# UNDERSTANDING THE WATER USE EFFICIENCY OF SUGAR BEET

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# Abstract

Climate change threatens crop production globally and in Europe hotter and drier summers are predicted which will result in a reduction in crop yields due to increasingly limited water availability. A sufficient supply of water is crucial to maximising crop yield because dry matter accumulation is directly proportional to water use in many environments, as solar radiation drives both photosynthesis and transpiration. Water use efficiency (WUE) has been studied in many of the major crop species including rice, wheat and maize, as they are grown in a wide range of climates, including those with poor water availability. From this research more WUE varieties of wheat have been developed to enable production in areas where water is limiting. This highlights that understanding crop WUE is a useful endeavour and that research should be undertaken in less studied crop species. One such species is sugar beet which is usually either irrigated or grown in climates with sufficient water availability. As water availability becomes increasingly limited it would be beneficial to understand how sugar beet uses water to inform crop management and identify traits for breeders linked to greater WUE to make the crop more resilient. This thesis explores WUE in sugar beet from the leaf to the crop level in controlled environment room and polytunnel experiments to understand how the plant uses water.

The three studies presented in this thesis have enabled sugar beet WUE to be characterised and have highlighted the anisohydric behaviour of the species. The first controlled environment study showed that sugar beet has fast stomatal responses compared to spinach which resulted in a similar level of intrinsic water use efficiency ($WUE_i$), despite reaching higher rates of stomatal conductance ($g_s$). The high rates of

$g_s$ could be linked to the transient wilting of the crop in the field and the concurrently high rates of $CO_2$ uptake and assimilation suggests wilting may not be detrimental to yield. Scaling up from the leaf level to the crop level, sugar beet were grown in large boxes in a polytunnel and exposed to four different irrigation regimes. Reduced water availability increased WUE (except in cases of extreme water limitation) and no acclimation (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to water deficit was evident as the plants responded similarly to repeated drought. Full recovery was observed after drought and the sugar beet showed an ability to continue to photosynthesise, even under severe water deficit, which is likely attributed to its anisohydric characteristics. The most significant finding of the study was that varietal differences in $WUE_i$ and associated traits were evident, including a lower stomatal density, which could possibly be linked to canopy architecture. Following on from these studies, diurnal $WUE_i$ in sugar beet was characterised to identify when the crop is most and least WUE during the day, and if water deficit affected this response. It was shown that $WUE_i$ declines later in the day as light, and therefore assimilation, declines faster than $g_s$. Water deficit increased $WUE_i$ and decreased the magnitude of the diurnal change in $WUE_{i,}$ as $g_s$ and A were reduced. The variety with greater $WUE_i$ in the second study also had higher $WUE_i$ in this study, but this did not lead to a greater dry matter WUE ($WUE_{DM}$) in any of the experiments which may be due to small biomass sample sizes. Diurnal fluctuations in leaf water content were also evident, although the plants did not wilt due to diurnal changes but did under water deficit, however the diurnal changes in temperature and VPD were not as great as those observed in the field due to limitations of the growth chamber.

Overall, the results show that sugar beet is a highly resilient crop species which is partly attributed to its anisohydric characteristics. Additionally, the difference in $WUE_i$ between commercial sugar beet varieties shows greater $WUE_i$ can be selected for without detriment to yield, as shown in the polytunnel experiment, and should be explored by breeders to develop varieties which will yield consistently well for growers as the climate changes.

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## List of abbreviations

| **Abbreviation** | **Definition** |
|---|---|
| $\delta^{13}C$ | Ratio of $^{13}C$ to $^{12}C$ |
| $\Delta g$ | Change in stomatal conductance |
| $\Phi PSII$ | Quantum yield of photosystem II |
| $\Delta t$ | Change in time |
| °C | degrees Celsius |
| A | Carbon dioxide assimilation |
| $a_1$ | Empirical coefficient - accounts for interactions of transpiration |
| ABA | Abscisic acid |
| $A_{max}$ | Maximum rate of assimilation |
| $A_{min}$ | Minimum rate of assimilation |
| $A_n$ | Net assimilation rate |
| ANOVA | Analysis of variance |
| BBRO | British Beet Research Organisation |
| C | Carbon |
| c. | circa |
| $C_a$ | $CO_2$ concertation in the atmosphere |
| $CEgs_{max}$ | Estimated maximum rate of stomatal conductance from log logistic 4 closing curve |
| $CEgs_{min}$ | Estimated minimum rate of stomatal conductance from log logistic 4 closing curve |
| CER | Controlled environment room |
| $Cg_s50$ | The point halfway between $CEg_{smax}$ and $CEg_{smin}$ |
| $C_i$ | Carbon dioxide concentration in the leaf |
| cm | centimetre |
| $CO_2$ | Carbon dioxide |
| $c_s$ | Carbon dioxide concentration at the leaf surface |
| cv | cultivar |

| | |
|---|---|
| $D_0$ | Empirical coefficient - accounts for interactions of carbon dioxide concentrations in the leaf |
| DAS | Days after sowing |
| DD | Double drought |
| DF | Degrees of freedom |
| DM | Dry matter |
| DRC | Dose response curve |
| DTI | Drought tolerance index |
| E | Transpiration |
| $E_s$ | Water lost through soil evaporation |
| ET | Evapotranspiration |
| Eq | Equation |
| Fig | Figure |
| Full | Fully irrigated |
| $F_v'/F_m'$ | Maximum PSII efficiency in the light |
| FW | Fresh weight |
| g | gram |
| $g_{(min,max)}$ | The maximum or minimum steady state level of stomatal conductance at full sun or shade |
| $g_0$ | Value of $g_s$ at the light compensation point |
| $G_c$ | Stomatal conductance to $CO_2$ |
| GC | Guard cell |
| $g_s$ | Stomatal conductance |
| $g_{smax}$ | Maximum rate of stomatal conductance |
| $g_{smin}$ | Minimum rate of stomatal conductance |
| $G_w$ | Stomatal conductance to water vapour |
| $H_2O$ | Water |
| HI | Harvest index |
| IRGA | Infrared gas analyser |
| Kg | kilogram |
| $K_{leaf}$ | Leaf hydraulic conductivity |

| | |
|---|---|
| KPa | kilopascals |
| L | litre |
| LAI | Leaf area index |
| LL.4 | Log logistic 4 curve |
| LSD | Least significant difference |
| Ltd | Continually water limited at approximately 50% field capacity |
| m | meter |
| min | minute |
| MJ | megajoule |
| mm | millimetres |
| mmol | millimole |
| mol | mole |
| MPa | megapascals |
| n | Sample size |
| N | Nitrogen |
| NaCl | Sodium chloride |
| NDWI | Normalised difference water index |
| nm | nanometre |
| $NPQ_t$ | Non-photochemical quenching without dark adaption |
| $OEgs_{max}$ | Estimated maximum rate of stomatal conductance from log logistic 4 opening curve |
| $OEgs_{min}$ | Estimated minimum rate of stomatal conductance from log logistic 4 opening curve |
| $Og_s50$ | The point halfway between $OEg_{smax}$ and $OEg_{smin}$ |
| PAR | Photosynthetically active radiation |
| PPFD | Photosynthetic photon flux density |
| PSG | Peristomatal groove |
| PSII | Photosystem II |
| $q_p$ | Photochemical quenching in the light |
| RH | Relative humidity |
| rpm | Revolutions per minute |

| | |
|---|---|
| Rubisco | Ribulose 1,5- bisphosphate carboxylase/oxygenase |
| RuBP | Ribulose 1,5- bisphosphate |
| RUE | Radiation use efficiency |
| RWC | Relative water content |
| sec | second |
| SD | Stomatal density |
| SD | Single drought |
| SE | Standard error |
| SLA | Specific leaf area |
| $Sl_{max}$ | Maximum rate of $g_s$ opening to an increase in PPFD |
| SLW | Specific leaf weight |
| SP | Stomatal pore |
| SPAD | Soil Plant Analysis Development |
| SS | Stomatal size |
| s.s | *sensu stricto* – ‘in the strict sense' |
| ssp | Subspecies |
| t | Time since step change in light |
| TLP | Turgor loss point |
| TW | Turgid weight |
| ug | microgram |
| UK | United Kingdom |
| USA | United States of America |
| VPD | Vapour pressure deficit |
| VWC | Volumetric water content |
| $W_a$ | Water vapour concertation in the atmosphere |
| $W_i$ | Water vapour concertation in the leaf |
| WS | White sugar yield |
| WUE | Water use efficiency |
| $WUE_{DM}$ | Dry matter biomass water use efficiency |
| $WUE_i$ | Intrinsic water use efficiency |
| $WUE_t$ | Instantaneous water use efficiency |

| | |
|---|---|
| $WUE_{WS}$ | White sugar yield water use efficiency |
| $\Gamma$ | Carbon dioxide compensation point |
| $\Delta^{13}C$ | Carbon isotope discrimination |
| $\delta_a$ | Atmospheric $^{13}C$ to $^{12}C$ ratio |
| $\delta_p$ | $^{13}C$ to $^{12}C$ ratio in plant tissue |
| µmol | micromole |
| $\tau$ | Time constant |
| $\Psi_{leaf}$ | Leaf water potential |

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## Joint authorship statement

The papers presented in this thesis were jointly authored and the breakdown for authorship is as follows:

**G. Barratt** - Lead author who carried out experimentation, data analysis and drafting of all manuscripts.

**L. McAusland** - Assisted in the experimental design and developing the protocol for assessing the speed of stomatal response in the study presented in Chapter two and provided feedback on the manuscript.

**B. Lomax** – Assisted with the carbon isotope discrimination protocol and data analysis in the study presented in Chapter three.

**E. Murchie** - Assisted with experimental design, interpreting chlorophyll fluorescence and gas exchange parameters, feedback on all manuscripts and is final author for chapter two.

**D. Sparkes** – Assisted with and oversaw all experimental design, practical work, data analysis, feedback on all manuscripts and is final author for Chapter three and Chapter four.

Additionally, anyone that provided practical assistance is listed in the acknowledgments in the relevant chapter.

## Copyright statement

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The remainder of this thesis has not yet been accepted for publication at the time of submission and is therefore the copyright of the authors.

# Chapter 1: General introduction and literature review

## 1.1 General introduction

Sugar beet is grown in temperate climates and accounts for 20% of white sugar produced worldwide (Punda and Prikhodko, 2009). It is a relatively new crop domesticated in the late 18th century (Fischer, 1989) and the area of sugar beet in Europe grew due to food security fears after the Second World War (Ward et al., 2008). Today sugar beet is appreciated as a profitable break crop, as it is spring sown, but falling world sugar prices threaten to reduce the growing area globally (OECD and FAO, 2019). Brazil can have a significant impact on the world white sugar price by adjusting supply, as Brazilian sugar cane factories are designed to switch between the production of white sugar and ethanol depending on demand from the world market (Koizumi, 2003). Worldwide the three biggest producers of sugar beet in 2019 were Russia (42.0MT), France (39.5MT) and the USA (30.0MT) (FAO, 2020).

The UK sugar beet market is a monopsony with British Sugar being the sole buyer of sugar beet grown under contract by farmers. Sugar beet is grown in the east of England to supply factories at Wissington and Cantley in Norfolk, Bury St Edmunds in Suffolk and Newark in Nottinghamshire. UK production averaged 7.7MT from 2008 to 2018 making it the 5th biggest producer of sugar beet in Europe behind France (36.6MT), Germany (26.9MT), Ukraine (14.4MT) and Poland (12.2MT) (FAO, 2020).

In Europe the crop has seen continual increases in annual yield of 1.5% in the past 10 years (Hoffmann and Kenter, 2018). However the loss of plant protection products, especially neonicotinoid seed treatments, has left the crop vulnerable to pests and diseases, especially beet virus yellows, spread by the aphid *Myzus persicae*, which can reduce yield by up to 47% (Clover et al., 1999). Fortunately breeders are working hard to develop resistant varieties (Stevanato et al., 2019).

In addition to the threat from pests and diseases there is also the challenge of climate change with wetter autumns and winters and hotter and drier summers that many countries, including the UK, will face in the future (David, 2017). It is imperative that the sugar beet crop continues to yield well as conditions become more challenging, and the hotter and drier summers are a particular threat to the crops yield. Drought tolerant traits of sugar beet varieties have been assessed (Ober and Luterbacher, 2002, Ober et al., 2005, Pidgeon et al., 2006), but this needs revisiting alongside work to understand and characterise the crop's water use efficiency (WUE) (Rytter, 2005, Bloch et al., 2006, Rajabi et al., 2008, Rajabi et al., 2009) to ensure that sugar beet remains a viable choice for growers as the climate changes.

## 1.2 Water use in crops

Dry matter accumulation in plants is directly proportional to water use, as solar radiation drives both photosynthesis and, through an increased vapour pressure deficit (VPD), transpiration (Tanner and Sinclair, 1983). This means that maximisation of yield in crop species requires adequate water supply, but this is often impossible, due to climate, or financially un-justifiable in agricultural systems. Additionally, water scarcity is an ever-increasing threat and phenotypes which enable yield to be maintained, or at least reduce losses, under water deficit are of growing importance.

In the UK, irrigation of the sugar beet (*Beta vulgaris,* ssp. *vulgaris*) crop is not usually economically viable (Hess et al., 2018) but periods of drought are common and can result in yield losses of up to 25% (Pidgeon and Jaggard, 1998). Characteristics which enable sugar beet to better withstand water deficits whilst maintaining yield are therefore of key interest and can be explored through the approach of water use efficiency (WUE). The following review will outline WUE at both the leaf and canopy level and will then assess specific behaviour and characteristics of sugar beet which influence WUE, including a focus on modelling stomatal responses and physiology. The behaviour of sugar beet under drought will also be outlined and traits related to drought tolerance which may affect WUE will be explored.

### 1.2.1 WUE at the leaf level

Water use efficiency (WUE) can be assessed at the leaf level and canopy level in crop species. At the leaf level WUE can be defined as 'the ratio of photosynthesis $CO_2$ assimilation (A) to transpiration of $H_2O$ (E) per unit leaf area' (Fageria et al., 2006). This is typically assessed through leaf gas exchange measurements and carbon isotope discrimination, which is discussed later. Utilising the main factors that define A/E it is possible to express them mathematically, with the following taken from Condon et al. (2004). Firstly, A is the product of stomatal conductance to $CO_2$ ($G_c$) and the concentration gradient of $CO_2$ between the atmosphere ($C_a$) and the inside ($C_i$) of the leaf:

$$A = G_c\ (C_a - C_i) \tag{1}$$

Secondly E is the product of stomatal conductance to water vapour ($G_w$) and the concentration gradient of water vapour between the inside ($W_i$) of the leaf and the atmosphere ($W_a$), which is the reverse of the $CO_2$ gradient:

$$E = G_w\,(W_i - W_a) \qquad (2)$$

Hence A/E can be expressed as:

$$\mathrm{A/E} = [G_c\,(C_a - C_i)]/[G_w\,(W_i - W_a)] \qquad (3)$$

This can then be further simplified through using the ratio of diffusivities of $CO_2$ and water vapour in air which equates to around 0.6:

$$\mathrm{A/E} \approx 0.6C_a\,(1 - C_a/\,C_i)]/(W_i - W_a) \qquad (4)$$

Eq.4 highlights that at the most basic level leaf WUE can be increased either through increasing the concentration gradient of $CO_2$ to increase uptake, or reducing the vapour pressure deficit (VPD), which is the difference between the vapour pressure inside the leaf compared to the vapour pressure of the air, to reduce transpirational water loss. Both of these gradients are heavily influenced by stomatal conductance ($g_s$) which is affected by a number of factors including; leaf water potential ($\Psi_{leaf}$), soil water content, humidity, internal resistance factors, plant and leaf age and solar radiation with increasing canopy temperature (Blad, 1983). $C_4$ plants are able to achieve a higher WUE than $C_3$ plants through increased rates of photosynthesis per unit leaf area coupled with lower stomatal conductance. Plant breeding has enabled the development of $C_3$ crop varieties with lower vernalisation and photoperiod requirements thus enabling earlier crop establishment which results in a reduction of E as $W_i$ is lower at cooler temperatures, reducing the gradient for water loss (Richards et al., 2002). Further maximisation of leaf level WUE could involve biochemical alterations such as increasing photosynthetic conversion efficiency (Sinclair et al., 1984). These are, however, difficult breeding objectives to achieve as these traits are hard to quantify, and inherent differences between varieties are rarely identified (Richards et al., 2002) although there is greater genetic variation in modern cultivars (Driever et al., 2014).

Assessing leaf level WUE usually requires measurement of leaf gas exchange which can produce values for instantaneous ($WUE_t$) and intrinsic ($WUE_i$) water use efficiency. $WUE_t$ is the ratio of net carbon assimilation (A) to transpiration (E) (Farquhar and Richards, 1984):

$$WUE_t = \frac{A}{E} \tag{5}$$

Whilst $WUE_i$ is the ratio of net carbon assimilation (A) to stomatal conductance ($g_s$) which is the rate $CO_2$ is entering the leaf (Farquhar et al., 1989):

$$WUE_i = \frac{A}{gs} \tag{6}$$

Both $WUE_t$ and $WUE_i$ can be measured using portable photosynthesis systems, which is the most common approach in current research. These open system instruments require the leaf to be inserted into a cuvette where the air is constantly circulated and VPD is constant. This means that there is a limitation to the results gathered from these measurements, as outside of cuvettes plants do not encounter such consistent air circulation (Condon et al., 2004). Portable photosynthesis systems can also be coupled with a multiphase flash fluorometer which enables chlorophyll fluorescence data to be collected. This is used to calculate parameters which assess the performance of photosystem II in different genotypes or extremes, such as drought, and is widely used in cereals (Sayed, 2003, Bartlett et al., 2012) but not in sugar beet and is an area which needs further exploration (Bloch et al., 2006, Choluj and Moliszewska, 2012). Two particularly useful publications are those of Maxwell and Johnson (2000) and Murchie and Lawson (2013) which provide a comprehensive introduction to chlorophyll fluorescence and best practice in its use, which is beyond the scope of this review.

Stomatal conductance is highly sensitive as it relies on stomatal responses which are determined by the $C_a : C_i$ gradient and $W_i : W_a$ ratio which in turn are influenced by a large range of factors. Environmental influences include; ambient $CO_2$ concentration, soil water status and evaporative demand as determined by irradiance, humidity and air temperature (Seibt et al., 2008). Plant characteristics which have an effect on these gradients include; Rubisco capacity, specific leaf area (leaf area /leaf dry weight) (SLA), leaf shape and chlorophyll content (Wilson, 1998). Medrano et al. (2015) examined WUE of grapevines using gas exchange measurements and highlighted how measurements from leaves located at different levels in the canopy could produce very different results as a consequence of the variation in microclimate within the canopy. This highlights the challenges encountered when measuring a process which is influenced by such a large range of factors making it highly variable. This also leads to difficulties relating leaf level WUE to canopy level WUE as these measurements are often not accurate enough to identify a significant relationship (Poni et al., 2009, Tomás et al., 2012). Medrano et al. (2015) identified increased leaf level WUE in grape vines under water deficit but the effect on canopy level WUE was variable both between years and genotypes. Additionally, these variations may be exacerbated by the fact that leaf gas exchange approaches often measure only one leaf in the canopy, despite variations in WUE between leaves (Medrano et al., 2012). Other factors such as dark respiration (Medrano et al., 2015), nocturnal conductance, which can vary based on how small an aperture the stomata can achieve (Snyder et al., 2003), and partitioning of carbon to non-photosynthetically active tissues in areas such as the roots (Boyer, 1996) are also unaccounted for in typical leaf gas exchange measurements.

Despite its many limitations measuring leaf level WUE is a useful tool for assessing crop WUE when its limitations are understood, and it is coupled with other approaches. A longer-term measurement of crop WUE is carbon isotope discrimination ($\Delta^{13}C$), which can support the results gathered from leaf gas exchange measurements.

### 1.2.2 Carbon isotope discrimination

Leaf gas exchange can be analysed through carbon isotope discrimination, where the ratio of the two naturally occurring stable carbon isotopes in the atmosphere, $^{13}C$: $^{12}C$ ($\delta^{13}C$), is analysed in plant tissues. $^{12}CO_2$ is lighter than $^{13}CO_2$ and diffuses into the leaf faster, where it is fixed more rapidly than $^{13}CO_2$ by Rubisco (Bierhuizen and Slatyer, 1965). As stomata close, the proportion of $^{13}CO_2$ in the stomatal cavity increases and it is more likely to be fixed (Boyer, 1996). This results in the plant accumulating relatively more $^{13}C$ than if stomata were always fully open. $\delta^{13}C$ is directly proportional to $WUE_i$ and can be expressed as the isotope discrimination value ($\Delta^{13}C$), which takes account of the atmospheric $^{12}C$ : $^{13}C$ ratio, and is inversely proportional to $WUE_i$ (Farquhar and Richards, 1984, Farquhar et al., 1989):

$$\Delta^{13}C = \frac{\delta_a - \delta_p}{1 + \delta_p} \qquad (7)$$

Where $\delta_p$ is the $\delta^{13}C$ calculated from the leaf tissue and $\delta_a$ is the atmospheric ratio of $^{12}C$ to $^{13}C$ which is either measured or taken to be -8‰. This value gives an integrated measure over time and the plant organ(s) sampled. The relative abundance of $^{12}C$ and $^{13}C$ in plant tissues compared to the atmosphere can be related to carbon uptake and stomatal control, and therefore WUE (Farquhar et al., 1982). Average values and trends in $\Delta^{13}C$ vary between species, with a typical $^{12}C$ : $^{13}C$ ratio in $C_3$ plants of 1.02 equating to a $\Delta^{13}C$ value of 20‰ (Condon et al., 2004). In $C_4$ species $\Delta^{13}C$ is less variable as PEP carboxylase, which fixes the carbon into the intermediary molecule oxaloacetate, does not discriminate between $^{12}C$ and $^{13}C$ as Rubisco does (Farquhar et al., 1989). In $C_3$ plants $\Delta^{13}C$ can vary greatly during the growing season as water deficits can induce stomatal closure. This means that it is often best to measure $\Delta^{13}C$ early in the growing season to assess inherent varietal WUE with plants being sufficiently watered to avoid stomatal closure (Richards et al., 2002), but this is not possible if the study is looking at WUE in response to drought. Due to the different hypotheses being addressed plant tissue samples for $\Delta^{13}C$ analysis are often taken at varying times which may lead to some of the differences identified between studies.

Traditionally $\Delta^{13}C$ is related to WUE using the linear model first proposed by Farquhar et al. (1982) which related $\Delta^{13}C$ to transpiration efficiency. Transpiration is proportional to A which is measured through $\Delta^{13}C$ and can thus be related to water loss. This model has been refined to take account of the effect of boundary layer and mesophyll conductance of transpiration and gas exchange respectively which in turn provides a more accurate the value for $WUE_i$ (Farquhar et al., 1989). Further understanding of the relationship between $\Delta^{13}C$ and plant WUE has highlighted limitations to the approach and that $\Delta^{13}C$ trends and comparisons can be better interpreted when accompanied by leaf gas exchange measurements, and information on environmental conditions (Seibt et al., 2008).

Sugar beet $\Delta^{13}C$ has not been as widely studied as it has in many other crop species, but variations under droughted and well-watered conditions are often evident. Rajabi et al. (2009) examined the genotype and environmental interaction of $\Delta^{13}C$ in sugar beet under water limited and irrigated conditions and highlighted the inverse relationship between $\Delta^{13}C$ and WUE. Significant genotypic differences in $\Delta^{13}C$ were identified between the sugar beet varieties examined. Additionally, it was shown that variation in $\Delta^{13}C$ was greater in conditions where water was not limiting, as opposed to the droughted treatments, with these results supported by previous research by Rytter (2005). Rytter (2005) did not, however, identify differences between genotypes as evidenced by Rajabi et al. (2009), but only compared two genotypes whilst Rajabi et al. (2009) used six. Despite conflicting evidence on differences in $\Delta^{13}C$ between sugar beet genotypes the differences between plants grown in well-watered and droughted conditions is often evident. Bloch et al. (2006) showed that the decline in $\Delta^{13}C$ for German sugar beet genotypes between well-watered and droughted treatments equated to a 24% increase in WUE. Similar to the study by that of Rytter (2005) only two genotypes were used and no differences in $\Delta^{13}C$ were found, this highlights that differences in $\Delta^{13}C$ are only present between some genotypes and more likely if they are not closely related. The study also reported that leaf $\Delta^{13}C$ in sugar beet is a better measure of WUE than root $\Delta^{13}C$ (Fig.1). Therefore, $\Delta^{13}C$ is a useful tool in understanding WUE in different environments, but its use in identifying genotypic differences needs to be explored further and may depend on the environment in which sugar beet is grown.

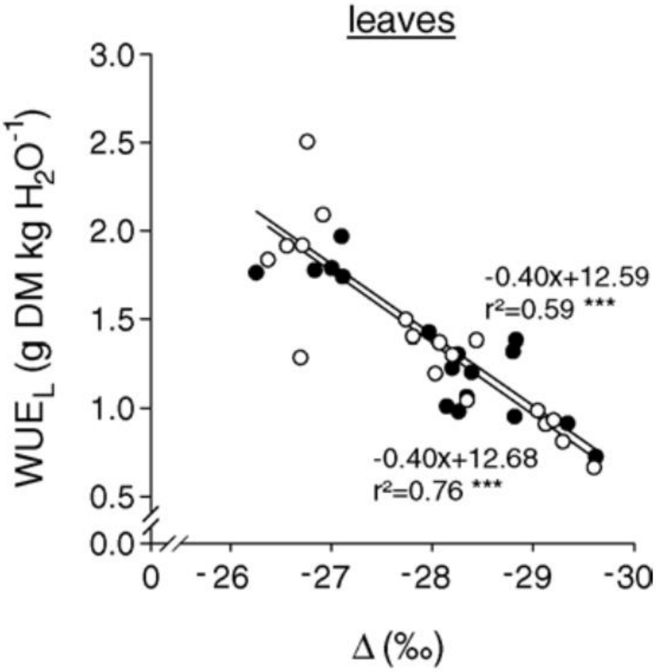

**Figure 1.** Relationship between WUE and $\Delta^{13}C$ of two sugar beet genotypes examined in pot grown sugar beet in a glasshouse. Genotype A represented by the white dots and genotype B by the black dots. The difference between the two varieties was ns. $r^2$ *** *P<0.001* (Bloch et al., 2006).

Additionally, research focusing on Australian wheat genotypes by Farquhar and Richards (1984) highlighted that the relationship between $\Delta^{13}C$ and yield is complex, and that conservative crop characteristics associated with low $\Delta^{13}C$ phenotypes may not be beneficial in all environments. In non-water limiting environments it has been shown that high $\Delta^{13}C$ wheat and barley phenotypes outperform low $\Delta^{13}C$ phenotypes. Low $\Delta^{13}C$ phenotypes in these environments are limited by reduced stomatal conductance and a lower photosynthetic capacity which reduces the total biomass accumulated by the plant, including harvestable product (Condon and Richards, 1993). High $\Delta^{13}C$ phenotypes may have an advantage in non-water limiting environments by achieving quicker canopy closure through more vigorous growth (Condon et al., 2004). In the UK sugar beet is sown in March and April, with the aim of achieving rapid canopy closure to maximum light interception and thus sugar formation and yield. It may therefore be the case that during this period high $\Delta^{13}C$ phenotypes may be beneficial, especially when the lower water loss gradient is also taken into account, but when water shortages occur later in the growing season low $\Delta^{13}C$ phenotypes may achieve a greater WUE, but this has not been examined. Overall, the relative advantage of low $\Delta^{13}C$ depends on the severity of the water stress.

### 1.2.3 WUE at the canopy level

WUE can be assessed at the canopy level where it can be defined most simply as the amount of dry weight (DM) accumulated per unit of water transpired by the crop (W) (Boyer, 1996):

$$WUE = \frac{DM}{W} \tag{8}$$

This can be manipulated further to take account of the percentage of this dry weight which is the desired product in the form of a harvest index (HI), as well as taking account of evapotranspiration (ET) which is the combined value for the water lost through soil evaporation ($E_s$), as well as that transpired (Fageria et al., 2006):

$$WUE = \frac{(DM \ x \ HI)}{ET} \tag{9}$$

ET is commonly calculated using the Penman-Monteith equation which requires values for daily mean temperature, wind speed, relative humidity and radiation interception to provide an estimate of ET, and this can be adjusted using a coefficient which is calculated for each crop species (Allen et al., 1998). Richards (1991) suggested looking at crop dry matter (DM) as a product of transpiration (E) and transpiration efficiency (TE), which is DM/ET:

$$WUE = \frac{TE}{1+(E_S/E)} \tag{10}$$

Richards (1991) highlighted, using this equation, two approaches to increasing canopy level WUE, firstly through increasing TE and secondly through increasing E relative to $E_s$. TE can be improved and $E_s$ reduced through improved crop management practices, such as reduced tilling to preserve soil moisture (Peterson et al., 1996) and breeding (Sinclair et al., 1984). The division between water transpired by the plant and that lost through soil evaporation changes as the growing season progresses, as canopy closure reduces losses from soil water evaporation, hence early canopy closure can help reduce $E_s$ (Richards et al., 2002). Early canopy closure is a key aspect of sugar beet agronomy enabling maximum light interception and thus sugar formation, which may help reduce $E_{s.}$ Additionally, the cooler early temperatures mean that $W_i$ is much lower relative to $W_a$ early in the season reducing E. TE is harder to address and relies more on breeding than management practices. A greater TE can be achieved in a number of ways including; maintenance of green leaf area, a greater HI and stomatal closure at night (Richards et al., 2002). Maintenance of sugar beet green leaf area through crop management practices is already promoted to help build sugar yield (BBRO, 2019), but an increased HI may rely more on breeding new varieties as management practices are already aimed at maximising sugar yields. The behaviour of sugar beet stomata at night has not been well documented, and this may be a useful trait to understand further.

In the temperate UK climate sugar beet yields are well correlated with intercepted radiation, providing that water is not limiting. In sunnier climates yield is most closely correlated with transpiration as light saturation results in plants achieving maximum assimilation rate rapidly whilst transpiration continues to increase (Werker and Jaggard, 1998). The main challenge for selection of varieties in the UK climate is the high variability in irradiance and rain. The most comprehensive study on sugar beet losses under drought was undertaken in 2006 and shows that between 1961-1990 there was a 64% chance of water deficit severe enough to lead to leaf senescence and yield loss each year, which highlights that water is not always limiting, but this is projected to increase up to 84% by 2050 (Richter et al., 2006). Werker and Jaggard (1998) identified correlations between light interception and ET to yield in UK sugar beet crops from 1980-1991. They showed both light interception and ET could be closely linked to sugar beet yield, and in years where irradiance was not limiting ET has an even closer correlation with yield. These results highlight the difficulty in selecting for phenotypes which will consistently perform across seasons. Much like the results from the $\Delta^{13}C$ analysis, a more conservative phenotype may perform well in the years where light or ET is limiting whilst a phenotype with less conservative traits may be preferable in other years.

Overall measuring and modelling plant WUE is challenging and must be tailored to the environment in which a plant is growing. Assessing WUE can, however, help to identify ways it may be improved, and provides a measure for the improvement of crop husbandry and breeding to better conserve water. As outlined above relationships between sugar beet growth and yield in relation to a variety of WUE measures shows it is a worthwhile approach to understanding sugar beet water use. It is therefore a useful tool and so long as its limitations are understood it provides an often easy and accessible way to monitor crop performance.

## 1.3 Wilting in sugar beet

The importance of $g_s$ with regards to WUE means it is of key interest when analysing the performance of plants in a range of environments, including the tendency of sugar beet to wilt when soil water is freely available. Wilting occurs when there is no longer sufficient turgor pressure, generated by the osmotic flow of water into the vacuole, on the cell wall to maintain turgidity (Tyree and Hammel, 1972). In severe cases this can result in plasmolysis, where the plasma membrane is pulled away from the cell wall (Lang et al., 2004). To maintain turgor pressure in the vacuole water must be taken up by the plant and this is driven by the plant water potential which decreases from the root to the shoot (Weatherley, 1970). If insufficient water is available in the soil for uptake by the roots then the water potential gradient can be reduced or lost. Environments where the external water potential is lowered, such as saline conditions, can also interfere with the plant water potential gradient (Zhao et al., 2020). However, plants can overcome such conditions if they are not prolonged or severe though by offsetting the loss in water potential with a change in osmotic potential which can be used to maintain leaf turgor (Turner, 2018). Stomata also play a key role in maintaining plant water status and leaf turgor through responding to changes in $\Psi_{leaf}$. $\Psi_{leaf}$ is dependent on leaf physiology which influences leaf hydraulic conductivity and hence the movement of water through the plant and into the leaf. Leaf hydraulic conductivity $K_{leaf}$ is defined by Sack and Holbrook (2006) as the 'ratio of water flow rate through the leaf to the driving force for flow, which is the Ψ across the leaf'. Stomatal conductance is increased when $K_{leaf}$ is able to compensate for losses through transpiration enabling $\Psi_{leaf}$ to be maintained. $K_{leaf}$ is maximised when the distance from the free flowing xylem to the leaf mesophyll is reduced and water reaches the stomatal pore for evaporation and subsequent transpiration easily (Sack and Holbrook, 2006). Temperature and irradiance also increase $K_{leaf}$ as the flux of water through the leaf is increased. The range of factors affecting $K_{leaf}$ means it is highly plastic trait over both time and plant age (Sack and Holbrook, 2006). Hydraulic conductivity and its interaction with $g_s$ means it is an important factor in plant wilting. Wilting reduces light interception as the canopy orientation is no longer optimal, reducing assimilation rates whilst transpiration continues at the same rate, thus reducing WUE so is an area of interest when assessing crop performance.

It is often observed that older leaves wilt faster than younger leaves when sugar beet is growing in the field. It was proposed by Lawlor and Milford (1975) that the wilting of older leaves is due to a greater hydraulic resistance, which reduces $K_{leaf}$. Under severe water deficits the mature sugar beet leaves wilt more readily despite stomata closing at a higher $\Psi_{leaf}$. Lawlor and Milford (1975) proposed that the ability of younger leaves to remain turgid during severe drought was due to their greater mechanical strength, the reduced sensitivity of stomata on younger leaves to $\Psi_{leaf}$ and the greater $k_{leaf}$, as the vascular network is less complex with this idea further supported by Fernandez and McCree (1991) through modelling water movement and resistances in sugar beet. It was also proposed by Milford and Lawlor (1975) that root size reduces hydraulic conductivity as it increases the distance that the water travels to reach the leaves. As the root size increases it becomes a greater percentage of the plant’s total biomass, as the canopy size is not increasing as quickly, so the forces driving water uptake are similar but resistance in the root is increasing. In particular the root to shoot interface has been identified as an area of high resistance which enlarges as the plant grows (Stieber and Beringer, 1984). Further evidence to support this is the behaviour of sugar beet in the glasshouse compared to the field. In the glasshouse $\Psi_{leaf}$ is observed to remain constant as VPD changes across a range of $\Psi_{soil}$, as plant water flux is moderated by internal resistance. In the field $\Psi_{leaf}$ is much more sensitive to VPD, even when soil water is not limiting, possibly as a consequence of increased hydraulic resistance at high water flux, greater than that achieved in glasshouse conditions, restricting water uptake to the leaves (Morillo-Velarde and Ober, 2008). These results also highlight the importance of field scale experiments if the research objectives are to enhance crop performance in practice. When sugar beet fail to supply water to the canopy the photosynthetic rate is reduced as stomata close due to the sudden decline in $\Psi_{leaf}$. This behaviour can result in the plant having an assimilation rate well below optimum given the sufficient availability of water and light and hence reduces the plant's WUE. Alternatively it has been observed in irrigated crops that the loss in leaf turgor does not reduce $\Psi_{leaf}$ sufficiently for stomata to close and therefore assimilation continued and yield losses were not as great as might have been expected (Kohl and Cary, 1969).

An additional influence on stomata is signalling from the root using the hormone abscisic acid (ABA) (Schulze, 1986). In some species, drying at the soil-root interface results in signals being sent to the leaves through the use of ABA in the xylem which can control stomatal aperture though the regulation of aquaporins in the guard cells (Davies et al., 2002). There is still debate as to how ABA initiates stomatal closure but there is evidence that ABA transported from the roots via the xylem acts as a chemical signal for stomatal closure whilst also decreasing leaf hydraulic conductance further driving this response (Pantin et al., 2013). Additionally there is evidence that the function of ABA can be altered by some of the products of photosynthesis which may have a small role in controlling stomatal aperture for optimal $WUE_i$ (Leymarie et al., 1998). There is also evidence that sensitivity to ABA may differ between young and old leaves with sensitivity to ABA declining with age (Willmer et al., 1988, Lee et al., 2011) which can lead to lower $WUE_i$ in older leaves (Raschke and Zeevaart, 1976).

As is evidenced there are a range of factors that affect the wilting characteristics of sugar beet, but the exact factors responsible for the differences in wilting between young and mature leaves, and the effect this behaviour has on yield, has not been proven in a range of environments and is an area which requires further study. The ability of sugar beet to osmotically adjust to address water deficits may also contribute to the differences in behaviour between young and mature leaves as part of its anisohydric behaviour which is explored in the next section.

## 1.4 Anisohydric behaviour in sugar beet

Plants have different mechanisms to respond to water deficits and these responses are usually adapted to their native environment. Crop species come from a range of environments and thus the way that they address water deficits and the effect it can have on yield reduction often varies, but $\Psi_{leaf}$ is often the dominant driver of stomatal control followed by VPD and these dominate over photosynthetic factors (Aasamaa and Sõber, 2011). Plants can broadly be grouped as either isohydric or anisohydric based primarily on their sensitivity to $\Psi_{leaf}$ (Tardieu and Simonneau, 1998). Isohydric plants 'maintain a constant $\Psi_{leaf}$ through control by stomatal conductance to limit transpiration' whilst anisohydric plants 'have a more variable $\Psi_{leaf}$ and keep stomata open and photosynthetic rates higher for longer, even with falling $\Psi_{leaf}$' often through osmotic adjustment (Sade et al., 2012). Although plants can be classed as isohydric or anisohydric there is in fact a continuum over which $\Psi_{leaf}$ controls stomatal closure, which can make it difficult to predict how some species respond to falling soil and therefore $\Psi_{leaf}$ (Meinzer et al., 2016, Meinzer et al., 2017). $\Psi_{leaf}$ and osmotic adjustment are strong indicators of where plants sit within the continuum with a more negative $\Psi_{leaf}$ at turgor loss point indicating anisohydry. It has been shown that steady state A and $g_s$ are higher in plants at the anisohydric end of the spectrum and they have a lower minimum $g_s$, faster stomatal response and photosynthetic activation, but at the cost of lower $WUE_i$ (Meinzer et al., 2017). The interactions between A, $g_s$ and $WUE_i$ mean that the benefit of the anisohydric response to water deficits is not always evident. It may be hypothesised that greater assimilation leads to greater crop yields in times of drought but equally the cost of osmotic adjustment may divert resources away from biomass accumulation (Munns, 1988). The ability of sugar beet to keep stomata open and gas exchange rates high during daily fluctuations in water deficits has been evidenced in America by Kohl and Cary (1969), with this study showing that through keeping stomata open sugar beet plants, under short term drought, avoided significant yield losses even when wilting was observed. It has also been shown that sugar beet does not adapt to drought and is able to recover to pre drought levels highlighting that the plant is not drought avoiding (Leufen et al., 2016), which may be linked to anisohydric response as plant is the resilient to low $\Psi_{leaf}$. The complex interactions which are present as part of the anisohydric response means species and climate specific research is necessary to understand crop responses in practice. Anisohydric behaviour is often observed in sugar beet, which osmotically adjust in times of drought or high levels of salinity to maintain leaf turgor. This behaviour affects the crop's conservation of water and may affect yield and is hence of key interest when looking to understand sugar beet WUE.

The mechanisms which enable sugar beet to maintain leaf turgor despite falling $\Psi_{leaf}$ as stomata remain open are still not fully understood. Vastarelli et al. (2013) monitored osmotic potential and relative water content (RWC) in sugar beet (*Beta vulgaris*, ssp. *vulgaris*) and its wild relative sea beet (*Beta vulgaris*, ssp. *maritima*) in pots under rain shelters. It was shown that sea beet has a greater capacity for osmotic adjustment but that both are able to adjust osmotically to water deficits. Leaf RWC remained stable in both treatments but on average leaf osmotic potential decreased from -0.1MPa to -2.1MPa in the droughted treatments whilst the well-watered plants showed no change. Osmotic adjustment was most effective when the onset of drought was slow, giving the plants sufficient time to adjust and prevent a significant reduction in biomass. A negative relationship between the concentration of compatible solutes for osmotic adjustment, in particular glycine betaine, and sucrose was highlighted, which may explain the lower levels of osmotic adjustment possible in sugar beet which has high concentrations of sucrose. Despite this being a small study, it highlights that the ability of sugar beet to osmotically adjust varies depending on both length and timing of drought and variety. It would be useful to analyse this behaviour in realistic conditions to analyse how resilient beet are *in situ*, as well as identifying if differences in the degree of osmotic adjustment between commercial varieties are evident as they are under high levels of salinity in sugar beet. A study of 46 sugar beet genotypes by Ober et al. (2005) also recognised varietal differences in osmotic adjustment under drought, but these differences did not correlate with yield so the relationship between osmotic adjustment and yield needs further exploration.

Varietal differences in the levels of osmotic adjustment are more widely studied with regards to salinity tolerance in sugar beet. Heuer and Plaut (1989) examined two commercial sugar beet cultivars and identified different responses to salinity. The salt sensitive variety had lower levels of $Na^+$ and $Cl^-$ and instead relied more readily on organic solutes to manage osmotic potential. It was suggested that these organic solutes interfere with the photosynthetic pathway or leak through the cytoplasm causing cell toxicity. Despite differing approaches in managing osmotic potential, and the associated effect on growth and $\Psi_{leaf}$, $g_s$ remained constant in both varieties, highlighting the high levels of osmotic adjustment possible in sugar beet. Katerji et al. (1997) also examined sugar beet responses to salinity at three levels, utilising a pressure volume curve, plotting $\Psi_{leaf}$ against RWC, to calculate the osmotic potential and turgor potential of the sugar beet. The results showed that through osmotic adjustment sugar beet were able to maintain leaf turgor and tolerate the salinity, showing only a slight reduction in yield compared to the plants under low salinity.

Further research by Ghoulam et al. (2002) identified varietal differences in response to increased salinity. Five cultivars were examined under four concentrations of NaCl and at the highest level of 200mmol differences in salinity tolerance were evident, much like those identified by Heuer and Plaut (1989) who tested at concentrations of 180mmol. A number of compatible solutes and inorganic ions were identified in osmotically adjusted leaves with glycine, betaine and proline being the most prevalent, although the role of proline in osmotic adjustment is still under debate it likely acts as an osmoprotectant (Ghoulam et al., 2002). Despite difficulty in its measurement, as it is measured *in vitro*, it is proposed that less tolerant varieties suffer greater electrolyte leakage which leads to cell toxicity and damage of the photosynthetic apparatus. In this study growth was found to be significantly reduced at the high NaCl concentrations in all varieties which is contrary to the results of Heuer and Plaut (1989) and Katerji et al. (1997), which both identified one variety where growth was not significantly affected by high salinity. This difference in salt tolerance can most likely be attributed to different breeding lines developed for specific environments and climates (Rozema et al., 2015). Therefore, differences in the levels of osmotic adjustment in sugar beet under drought may also be evident, especially between varieties from different breeding lines bred for different environments and climates, and this needs to be examined further.

McCree and Richardson (1987) tested the hypothesis that the anisohydric behaviour of sugar beet enables greater carbon gain through stomata remaining open for longer under water deficit, so that the rate of assimilation remains higher for longer compared to isohydric plants. Cowpea was chosen as a comparative isohydric species as it has similar rates of photosynthesis to sugar beet when water is not limiting. When examined under progressively drier conditions the results showed no benefit in anisohydric behaviour. It was highlighted that sugar beet was able to maintain leaf turgor until 8 days after drought, but water losses were much greater than the cowpea. Consequently, although carbon gain was greater in the sugar beet this performance could not be sustained for long, and the cowpea could maintain an erect canopy that continued carbon assimilation at a steady rate for longer before requiring further watering. This contrasting behaviour resulted in similar WUE for each species. Further studies have not been undertaken using this comparative approach as it is hard to derive accurate results from comparing two different species. Despite these challenges the results suggest the anisohydric behaviour of sugar beet may not be as beneficial as it first appears under long term drought. Anisohydric behaviour may, however, be beneficial under intermittent drought, when sugar beet is able to maintain leaf turgor, and this is an area which requires further study.

Overall, research is needed to understand how anisohydric behaviour in sugar beet affects WUE with regards to yield in typical field conditions, and whether this behaviour is beneficial. Examining commercial sugar beet varieties for different levels of osmotic adjustment under drought, which have been widely evidenced in sugar beet salinity studies, would aid understanding WUE in sugar beet. Varieties with different responses to drought could be used to examine WUE under different drought regimes to help identify those which would perform the best in the typical UK drought scenario. Differences are likely to be evident as Vastarelli et al. (2013) has shown between sea beet and sugar beet, in one of the few studies that focuses solely on anisohydric behaviour as it looks at osmotic adjustment in relation to drought rather than salinity.

## 1.5 Modelling stomatal responses

To understand how stomata will respond to the environment, including comparisons of the anisohydric and isohydric responses, there is a range of models that can be used which enable an estimation of $WUE_i$ to be made dependent on the effects of light, VPD, $CO_2$ concentration, $\Psi_{leaf}$ and other factors in isolation or in combination.

These models vary from steady state estimates to modelling dynamic responses to environmental variables. Two of the most widely used models to examine the effect of these variables on steady state $g_s$ are that of Jarvis et al. (1976) and Ball et al. (1987). The model of Jarvis predicts $g_s$ using functions to take account of the effect of stomatal conductance, photon flux density, air temperature, VPD, $\Psi_{leaf}$ and the ambient $CO_2$ concentration. It is limited by its failure to take account of any synergistic interactions, particularly the impact A has on the concentration of $CO_2$ around the leaf and thus the effect this has on zeaxanthin concentrations, which control guard cell turgor and therefore stomatal aperture and $g_s$ (Zeiger et al., 2002). The Ball-Berry model corrects this with a coupled steady state-model, making $g_s$ directly dependent on A, which was further modified by Leuning (1990) to account for the effect of the $CO_2$ compensation point on the concentration of $CO_2$ around the leaf:

$$g_s = g_0 + \frac{a_1 A_n}{(c_s - \Gamma)(1 + \frac{VPD}{D_0})} \tag{11}$$

Where $g_s$ is the stomatal conductance for $CO_2$ diffusion, $A_n$ is the net leaf $CO_2$ assimilation rate, $c_s$ is the $CO_2$ concentration at the leaf surface, $\Gamma$ is the $CO_2$ compensation point, $g_0$ is the value of $g_s$ at the light compensation point, and $a_1$ and $D_0$ are empirical coefficients which account for interactions of transpiration and $C_i$ concentrations respectively.

Although steady state $g_s$ is a useful measure in understanding what environmental variables drive stomatal control, and the maximum performance of a plant under these conditions it does not represent how most plants are responding in the field. In many understory species, steady state $g_s$ is rarely encountered and instead plants have to respond to transient periods of high light as sunflecks pass periodically though the canopy, with $g_s$ and A fluctuating in response. To assess dynamic responses studies have been undertaken exposing plants to changes in the environment, with light being the most widely studied due to it being a strong driver of stomatal responses (Lawson and Morison, 2004), and tracking the subsequent changes in $g_s$ and A. To model the response a number of approaches have been developed and adapted over time (Vialet-Chabrand et al., 2017). Firstly and most simply an exponential model can be fitted to the induction curves to estimate parameters of A and $g_s$, such as minimal and maximal values, the rate of change and the time taken to reach a given level of response such as 50% or 95% of the maximum. An example of how this can be useful is evidenced by McAusland et al. (2016) where 95% of the maximum rate of A provides the level up to which stomata are limiting A which is key to assessing the limitations of gs on A under fluctuating irradiance. McAusland et al. (2016) propose that this commonly used $Sl_{max}$ (maximum rate of $g_s$ opening to an increase in PPFD) is dependent on the amplitude of the response and that plants with smaller stomata express a greater change in $Sl_{max}$ despite the same change in stomatal aperture as plants with fewer, larger stomata. To overcome this a time constant describing time taken to achieve steady-state $g_s$ can be preferable. One of the most widely used approaches was developed by Kirschbaum et al. (1988) which separates the stomatal response into 3 phases as follows:

- Phase 1 - a biochemical signal in responses to changes in the environmental variables, which is sometimes known as the induction
- Phase 2 – the response in the guard cell ion channels and associated apparatus to increase or decrease osmotic potential in the guard cells, it is this stage that is the main reason for $g_s$ limitation to $A_{max}$
- Phase 3 -the change in osmotic potential causes an influx of water into or out of the guard cells which adjusts stomatal aperture, this stage is associated with $g_s$ overshooting the value needed for $A_{max}$

The latter two processes have higher time constants and result in the classic sigmoidal curve of stomatal response, and vary depending on the species being studied (Vico et al., 2011). This approach has been continuously improved to produce models of increased complexity which are accurate when compared to actual dynamic responses and take account of more factors, including the interaction with photosynthesis. For example Naumburg et al. (2001) added a photosynthetic module to their model to include Rubisco kinetics, ribulose 1,5- bisphosphate (RuBP) regeneration and electron transport to examine daily photosynthesis in understory species exposed to dynamic light regimes to represent sunflecks. Although models of increased complexity are necessary to study responses to dynamic factors from the biochemical to the whole plant level some hypotheses can be tackled using a more simplistic approach. An example of one of the most simplistic approaches is that of Assmann and Grantz (1990) who used blue light flashes to examine guard cell behaviour and its impact on the speed of stomatal response to VPD. The rate of stomatal opening was calculated as the change in $g_s$ divided by the change in time

($\Delta g/\Delta t$) between 10% and 90% of the magnitude of the blue light pulse whilst the rate of stomatal closure was calculated as ($-\Delta g/\Delta t$) between 90% and 10% (Fig.2).

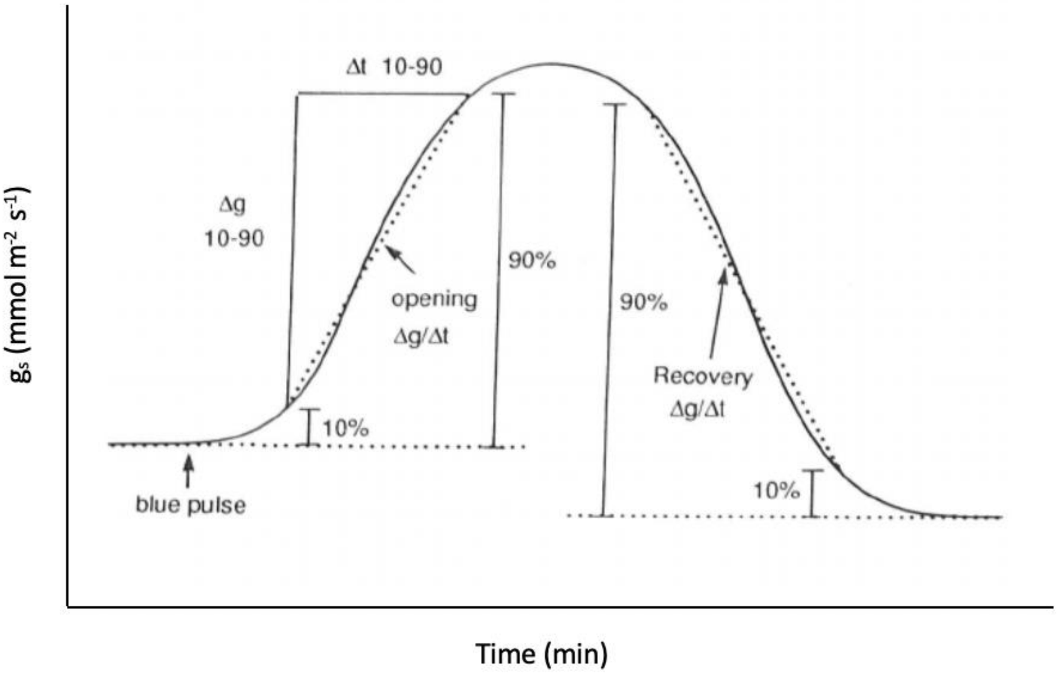

**Figure 2.** The ideal curve to calculate the speed of stomatal opening as the change in time ($\Delta g/\Delta t$) between 10% and 90% of the magnitude of the blue light pulse and closure (recovery) as ($-\Delta g/\Delta t$) between 90% and 10% (Assmann and Grantz, 1990).

The results showed that a higher VPD reduces pressure from surrounding epidermal cells on the guard cells due to increased transpiration enabling more rapid stomatal opening in response to blue light. Knapp (1993) used this method alongside a calculation to estimate $g_s$ over the entire response to fluctuating light to compare stomatal responses to light in $C_3$ and $C_4$ grasses using the following equation:

$$g = g_{(min,max)} \cdot [1 - \exp(-t/\tau)] \qquad (12)$$

Where $g_{(min,max)}$ is the maximum or minimum steady- state level of stomatal conductance at full sun or shade light, t is the time since the step change in light, and $\tau$ is a time constant signifying when 63% of the response has occurred. The use of

63% of the magnitude of the response was identified as a useful relative measure between species which differ significantly in their absolute response. To assess $g_s$ in the transition stage between each steady state level second order polynomial regressions were used as they best fitted the response between the steady state levels. The main highlight of the results was that $C_4$ grasses could respond more rapidly and better optimise A and $g_s$ under fluctuating light and were therefore better at conserving water. Another simplistic approach to assessing stomatal responses is that of Zipperlen and Press (1997), who assessed the speed of stomatal responses alongside photosynthetic induction of rainforest trees to compare carbon gain between species when encountering sunflecks. This model uses a sigmoidal function fitted to the light induction curves to give an estimate of either the rate of A or $g_s$ at either 50% or 90% of the maximum calculated value of A or $g_s$. To achieve this the model uses the asymptotic minimum A or $g_s$ (used to minimise noise from data points whilst the plant settles in the gas exchange cuvette for the first minute) the asymptotic maximum of A or $g_s$ during the light saturation period, the time period to achieve 50% or 90% of the maximum value, the inflection point of the curve for either A or $g_s$ and the slope parameters.

The benefit of using dynamic models and measurements can be shown by comparing how steady state $g_s$ values compare to those achieved under dynamic conditions. Steady state $g_s$ is easier to measure and if it is comparable to dynamic maximum $g_s$ it can be used without needing to take dynamic measurements. Studies have shown that $g_{smax}$ and $A_{max}$ measured under constant conditions is greater than that measured under dynamic conditions (Kaiser et al., 2018, Zipperlen and Press, 1997).

Conversely Naumburg and Ellsworth (2000) showed that in understory saplings grown under high $CO_2$ induction, loss was slower than those grown in ambient conditions resulting in higher carbon gain during sunflecks which was greater than that measured in steady state conditions, thus steady state values would underestimate daily carbon gain. This highlights that using dynamic responses to assess plant response is a more accurate reflection of how plants are behaving in the field and is a better approach for phenotyping than constant or steady state measurements alone. In addition to this, Lawson and Blatt (2014) argue that stomatal behaviour should be assessed under light levels and $CO_2$ concentrations encountered in the field. These more realistic conditions often result in different stomatal responses having no significant impact on carbon gain, as assimilation is not limited by stomatal aperture compared to studies where high levels of light are used.

## 1.6 Stomatal physiology

The importance of stomata in controlling transpiration and $CO_2$ through changes in stomatal conductance has so far been a focus of this review but stomatal physiology, such as stomatal size (SS) and stomatal density (SD), may also have an effect on the plant’s ability to control leaf gas exchange. Stomata vary in shape but there are two particularly notable types with most plants having two kidney shaped guard cells whilst grasses have two linear dumbbell shaped guard cells (Rudall et al., 2017). Dumbbell shaped guard cells require a smaller change in guard cell volume to control opening compared to kidney shaped guard cells which enables more rapid stomatal control leading to greater more rapid opening when exposed to light to maximise photosynthesis and rapid closing which increases $WUE_i$ (Grantz and Assmann, 1991). It has been suggested that grasses developed dumbbell shaped stomata as the evolved in the understory of tropical forests around 70 million years ago as greater stomatal control optimised plant performance to transient light (Hetherington and Woodward, 2003). It has also been shown using the fossil record that SD has changed according to the level of atmospheric $CO_2$ with a decrease in SD when $CO_2$ has increased (Royer, 2001). Such changes have also been evidenced in the medium term, with SD decreasing 67% in eight temperate tree species assessed as atmospheric $CO_2$ increased from 280 µmol $mol^{-}1$ to 340 µmol $mol^{-1}$ over a 200 year period covering 18th century industrialisation using herbarium records (Woodward, 1987). In addition to evolutionary studies of changes in stomatal morphology due to long and medium-term changes in atmospheric $CO_2$ there is also evidence that stomatal morphology can change in the short term in response to changing $CO_2$ and light intensity as leaves develop. Controlled experiments have mostly focused on changes in stomatal morphology in response to $CO_2$ and have shown that SD decreases and SS increases as $CO_2$ increases, much like the changes observed in the fossil record (Woodward et al., 2002). These changes result in a decrease in maximum $g_s$ whilst photosynthesis increases with greater levels of $CO_2$ and shows that changes in stomatal density can enhance $WUE_i$. However this change in SD has been shown to lead to reduced $WUE_i$ under lower levels of $CO_2$ (Woodward, 1987), highlighting that adaptions in stomatal morphology may only be beneficial if changes in the environment are prolonged. Light has also been shown to lead to altered SD in Tomato (*Solanum lycopersicum*) with leaves exposed to higher light having a greater

SD than those exposed to low light because light intensity drives the rate and duration of leaf expansion (Gay and Hurd, 1975). This higher SD led to a greater maximum rate of photosynthesis and concurrent increases in $g_s$ to maximise photosynthesis under the available light. There is limited research on the how environmental signals during leaf development are cascaded in the plant to control stomatal development this is an area of increasing interest. Not only does SS and SD influence maximum rates of $g_s$ and photosynthesis but also the speed of stomatal response. The leading theory of the relationship between stomatal size and density and the associated speed of responses is that SS an SD are inversely correlated, and that smaller stomata are faster to respond to the dynamic environment (Franks et al., 2009). With overall SD limited by the need for at least one epidermal cell of separation for stomata to function correctly (Franks and Beerling, 2009). This rapid response coupled with a high rate of A would be the ideotype for most agricultural crops to optimise carbon gain and minimise water loss through transpiration (Drake et al., 2013), as differences in $WUE_i$ are mostly driven by changes in $g_s$ rather than A (McAusland et al., 2013).

Increased stomatal speed limits excessive water loss as it reduces the disconnect between A and $g_s$. The discoordination between A and $g_s$ arises because stomatal responses to light are an order of magnitude slower than photosynthesis. This leads to stomatal limitation to A which varies significantly among species (McAusland et al., 2016). In addition to this, when plants reach $A_{max}$ $g_s$ continues to increase and a rapid decrease in $WUE_i$ results. Dumbbell shaped guard cells are less likely to reach this overshoot, and generally express greater stomatal control than kidney shaped guard cells resulting in a greater $WUE_i$, as less ion flux is needed to change guard cell turgor (Vico et al., 2011, McAusland et al., 2016). There is debate as to whether the increase in $WUE_i$ attributed to increased stomatal speed has a significant effect on daily WUE, which may be attributed to differences in the models used and is an area which requires further research. It has been evidenced that faster stomata result in up to 20% greater $WUE_i$ but when assessed over a day there was no significant difference compared to slower stomatal responses (Moualeu-Ngangue et al., 2016).

Although decreased SS and increased SD can lead to faster stomatal responses these characteristics can also lead to greater overall values of $g_s$ and A. Plants with the same cumulative pore size made up of smaller stomata rather than larger stomata generally have a greater $g_{smax}$ because of the 'edge effect' as there is a shorter diffusion pathway compared to larger stomata (Willmer and Fricker, 1996). However, in some species a greater maximum stomatal aperture can compensate for stomatal density to enable similar maximal rates of $g_s$ to be reached with increased SS and reduced SD (Büssis et al., 2006, Doheny-Adams et al., 2012, Monda et al., 2016). This can be dependent on other anatomical factors including guard cell size and shape, subsidiary cell number (Franks and Farquhar, 2007) and the distribution of stomata on the abaxial and adaxial leaf surface (Lu et al., 1993). Greater $g_s$ leads to lower $WUE_i$ as the relationship between A and $g_s$ is not linear as $g_s$ increases more than A under constant environmental conditions (Franks and Farquhar, 1999), but often results in increased biomass (Lawson and Blatt, 2014). This is supported by McAusland et al. (2016) who showed that high levels of WUE did not correlate with $A_{max,}$ as a reduction in $g_s$ often limits A. The ideal level of performance is the lowest $g_s$ that enables 95%

of $A_{max}$ to be reached as this is the optimal trade-off between water loss and assimilation without stomatal overshoot. In some cases a decrease in SS and increase in SD is evident during prolonged water deficit and can result in enhanced WUE, if $g_s$ is reduced whilst A is maintained, as has been evidenced in the perennial grass *Leymus chinensis* (Xu and Zhou, 2008) and potatoes (*Solanum tuberosum*) (Sun et al., 2014). However the relationship between $g_s$ and A means that this is not always evident, and can be dependent on the environmental conditions (Gaëlle et al., 2010).

In addition to responding to the dynamic environment there is evidence that there is a diurnal pattern influencing stomatal control. Matthews et al. (2018) showed in Arabidopsis that $g_s$ is affected by the intensity and the pattern of light, for example fluctuating sinusoidal vs non-fluctuating square wave, and the time point within the diurnal cycle. Plants grown under fluctuating light had notably faster stomatal responses at the start of the day, with greater $g_s$ values resulting in decreased $WUE_i$ with this difference in speed diminishing as the day progressed and $WUE_i$ increasing. This may have been an adaption in the fluctuating light plants, as although over the course of the day they received as much light as the non-fluctuating plants they received more at the start of the day. Although this diurnal response can be beneficial as it maximises A under high light it does in fact lead to a decrease in $WUE_i$ as $g_s$ reaches relatively higher levels than the non-fluctuating plants and drives an additional disconnect between $g_s$ and A.

The relationship between SS and SD, and the subsequent speed of stomatal response, has been focused on mainly as a way to expand our understanding of leaf gas exchange in dynamic environments, the cost of metabolic processes that drive guard cell turgor and as a way to assess understory species performance. Limited work has been undertaken to assess variation in SS and SD in crop species and how the speed of stomatal response varies and may be able to drive enhancements in carbon gain and WUE (Bertolino et al., 2019). Qu et al. (2016) used the high light to low light approach of Vico et al. (2011) to assess stomatal responses in rice to dynamic light to identify if these responses can increase drought tolerance. Varied responses between rice varieties in response to dynamic light were identified with those with faster closing stomata having a greater $WUE_i$, supported by $\Delta^{13}C$ results, leading to greater drought tolerance and higher biomass accumulation. Similarly, Faralli et al. (2019) assessed the speed of stomatal response in eight wheat cultivars to changes in light intensity, and also assessed the effect of different stages of development. Significant differences were identified in the speed of stomatal opening and closing between cultivars as well as the time taken to reach steady state A. The stage of development also influenced this with responses post anthesis slowing compared to booting. The effect of water deficit and elevated $CO_2$ was also assessed on one cultivar. Elevated $CO_2$ increased the speed of stomatal opening, which is contrary to the results of many studies but may be explained by the interaction with other environmental variables (Xu et al., 2016), which is complex and not within the scope of this review. Water deficit made the opening and closing response more asymmetric as it decreased the rapidity of stomatal opening and increased the speed of closure which agrees with the results of Barradas et al. (1994) and Haworth et al. (2018). However, research in *Nicotiana tabacum* showed an increase in the speed of the opening and closing phase under reduced water availability. *Nicotiana tabacum* has an asymmetrical stomatal response with the speed of closing being more rapid than opening even in the absence of water deficit. This highlights that an understanding of the asymmetric behaviour of each species may help to explain responses to the environment (Gerardin et al., 2018) and is mostly influenced by plant functional group and climate (Vico et al., 2011).

Despite the well supported theory that smaller stomata are faster to respond to the dynamic environment, there is evidence as to why this may not always be the case. Elliott-Kingston et al. (2016) have argued that plants that evolved under low atmospheric $CO_2$ concentrations generally close stomata faster than those that evolved under high atmospheric $CO_2$ concentrations. This enables sufficient $CO_2$ concentrations in the sub stomatal cavity and maximises $CO_2$ uptake and diffusion, as well as being faster to respond to dynamic environments to maximise WUE. They found that although smaller stomata are often faster within a single genus, this observation does not apply generally across plant taxa. Studies in Arabidopsis have also highlighted that stomatal size does not always correlate with the speed of stomatal response. Franks and Farquhar (2001) treated plants with ABA to increase stomatal density and reduce stomatal size as it has been evidenced that plants can manipulate stomata under drought. Within the ABA treated plants at a given guard cell turgor pressure, the stomatal aperture was half that of the control and when examined on an area scale $g_{smax}$ was lower in the ABA plants. The combination of reduced stomatal aperture, greater closure at zero turgor and a reduced $g_{smax}$ showed an increase in $WUE_i$ under drought conditions and explains why stomatal density may increase rather than decease under drought in some species. Increases in $WUE_i$ caused by increases in ABA can also be driven by reducing the sensitivity of guard cells to environmental stimuli such as $CO_2$ and light, although VPD has been shown to still have a significant effect (Haworth et al., 2018).

Research into the speed of stomatal responses in sugar beet has not been undertaken and is an area that provides an opportunity for study. Some studies have been undertaken on the stomatal physiology of sugar beet which has elliptical shaped guard cells and is amphistomatic (stomata on both the abaxial and adaxial leaf surface) with more commonly found on the abaxial surface (Burrows, 1969). A study by Luković et al. (2009) of 12 sugar beet genotypes identified a 40% difference in SD between the highest and lowest values observed under optimal water availability, with this difference evident on both the adaxial and abaxial surface. Such a significant difference in SD in the absence of water deficit suggests genotypic differences in SD of sugar beet could be explored as a breeding objective. This study was, however, carried out in optimum glasshouse conditions which means these significant observations may not translate to the field. In sugar beet, high SD has been shown to reduce WUE even when plants encounter low level water deficit as transpirational losses are great (Luković et al., 2009). Additionally, at higher SD the guard cells are smaller which enable stomata to remain open to some extent under water deficit and facilitate $CO_2$ uptake and assimilation, which is an anisohydric characteristic. If manipulation of stomatal number through breeding is viable in sugar beet then the SS and SD relationship of gas exchange of sugar beet stomata needs to be understood to identify if $WUE_i$ can be increased. Clearly there are complex interactions between SS and SD and the effect these traits have on $g_s$, A and $WUE_i$, which can be species and environment specific. It is therefore necessary to examine the diversity of stomatal physiology to understand the range of SS and SD in sugar beet varieties and if this is consistently evident. Changes in SS and SD in response to long term water deficits would also be beneficial to see if WUE increases under drought. In addition to this, the young and old leaves could be examined separately to see if there are significant differences in stomatal traits to identify whether this contributes to the witling response as discussed previously. Alongside these assessments of SS and SD it is necessary to characterise how these traits impact on the speed of stomatal responses in sugar beet to the dynamic environment, to assess WUE both instantaneously but also over time in more field like conditions. To do this it is clear that the right model to assess stomatal speed must be selected based on the hypotheses to be examined whilst respecting the biological limitations of the chosen model.

## 1.7 Sugar beet under drought - A useful approach to identifying differences in WUE related traits in sugar beet

In sugar beet, phenotypic differences related to drought tolerance which may be observed when water is freely available are not always as evident under drought. Experiments with both well-watered and droughted treatments are therefore utilised to identify traits which are consistent and enable plants to tolerate water deficits and help reduce yield losses. As sugar beet is derived from sea beet it retains some salinity and drought tolerance, such as the osmotic adjustment already discussed, but the extent to which these tolerances and their associated traits have been maintained in breeding lines and commercial sugar beet varieties varies. This variation has the potential to be significant as sugar beet is grown on four continents and has thus been bred to perform well in a multitude of environments, from temperate rain fed systems in Northern Europe to the irrigated systems in the Middle East (Morillo-Velarde and Ober, 2008). Understanding which traits enhance sugar beet drought tolerance through water conservation may help in the selection of traits to enhance WUE, but the complexity of the interactions between traits also means that they must be fully understood. Identifying if the diversity in these traits is evident through screening a range of sugar beet genotypes can also help in assessing whether they can be enhanced by breeders.

Ober et al. (2004) assessed the diversity of characteristics in sugar beet and their relation to yield to highlight the importance in identifying phenotype yield interactions. Breeders often fail to understand why a variety performs well under drought and understanding indirect traits, related to drought tolerance, may enable faster selection of plants when breeding (Richards et al., 2002). 46 sugar beet varieties were assessed over three years in the field. A drought tolerance index (DTI) was calculated to assess performance as the % of white sugar yield maintained under drought, normalised by the mean yield value for the trial. Significant differences between varieties in sugar yield, root yield and total DM under irrigated and droughted conditions were evident, such differences have also been evidenced under severe drought conditions in Iran (Sadeghian et al., 2000). One limitation of this study was the use of polythene rain shelters to impose drought. These can alter the field environment, affecting light intensity, light quality and humidity, so may not be directly comparable to field conditions. Further research by Tarkalson et al. (2014) however, highlighted such differences under less severe drought through the use of deficit irrigation compared to full irrigation, to avoid the limitations of rain shelters. In the study by Ober et al. (2004) DTI showed wide phenotypic variation to drought and some breeding lines had a greater DTI than the commercial varieties, highlighting that this is an area of opportunity for breeders. By comparing the varieties with high and low DTI values it also provided a basis for recognising the traits which enhance DTI, which may also help in identifying traits which can enhance WUE.

Ober et al. (2004) recognised that generally varieties which yielded well under irrigated conditions also yielded well under drought. Correlation between irrigated and droughted yields can be attributed to the fact that sugar beet has no stress sensitive developmental stage like legumes and cereals and thus water use is directly correlated with yield (Dunham, 1993). This is supported by the results of Brown et al. (1987) who showed that, regardless of drought timing, early or late, dry matter accumulation is always correlated with water use. There was, however, no significant correlation between potential yield, measured as yield under irrigation, and DTI and this trend was also evidenced in an earlier study by Ober and Luterbacher (2002). Ober et al. (2004) suggests this as evidence that the factors which influence DTI do not always greatly affect yield. The poor correlation between potential yield and DTI may mean that traits which reduce yield losses under drought may not always reduce yield potential. This would be highly beneficial in the UK climate where seasonal variation in drought length, timing and intensity means varieties need to perform well at varying soil moisture deficits. Sugar beet rooting traits leading to increased water uptake could be an area of physiology that may drive this behaviour by enhancing water availability under drought as more of the soil water is accessible to the plant and thus yield is maintained. Although no genotypic differences in the rooting depth of sugar beet have been observed there is evidence that water uptake differs with some sugar beet varieties better able to take up water from depth which results in a greater DTI (Ober et al., 2005). This could be associated with the observation that sugar beet roots have a delay between growth and maturity resulting in roots being present but no water uptake which could vary between genotypes (Fitters et al., 2017). Greater soil water access and up take maintains the water potential gradient through the plant helping to maintaining leaf turgor. This was evident in the work of Ober et al. (2005) as that plants which were able to access water from deeper in the soil profile had a reduced tendency to wilt and maintained a better canopy judged by greenness. This shows that rooting traits can affect plant water status, drought tolerance and the efficiency with which plants use the water available in the soil profile.

Despite the suspected influence of rooting traits on DTI, a number of indirect canopy traits were found to be significantly different between varieties and closely related to DTI and could therefore be used as selection criteria in breeding programmes. Firstly green canopy maintenance and a low wilting and senescence score, defined from 1-5 based on visual observations, was found to correlate well with DTI and showed phenotypic variation (Ober et al., 2005). The maintenance of green canopy under drought ensures light interception is maintained and thus assimilation and dry matter accumulation continue at a higher rate than in canopies which wilt or senesce faster. Sugar beet which have a larger more erect canopy architecture should maintain yield under drought, but these traits may not always increase WUE as the amount of water per unit of biomass accumulated does not necessarily differ. Water use may even increase as the larger canopy intercepts more radiation which raises leaf temperature and can increase transpiration rates (Blum, 2005).

Changes in leaf morphology were evident under drought and both specific leaf weight (DW/total sampled leaf area) and succulence index (FW-DW/total sampled leaf area) were negatively correlated with DTI. Ober et al. (2005) suggested that this was driven by the reduced leaf expansion rate evident in the genotypes susceptible to drought. Additionally, in the irrigated control succulence index was negatively correlated with both sugar yield and water use efficiency. There were no significant differences in RWC between the genotypes whilst in an earlier study by Shaw et al. (2002) RWC was found to be significantly lower in a drought susceptible genotype. RWC is an assessment of leaf water status and does not take account of leaf area whereas specific leaf weight and succulence index use leaf area and this may explain the differences in the observations. As plants drought and leaves become thicker this will be detected by specific leaf weight and succulence index but not necessarily by RWC. As leaf expansion rate declines and leaves become thicker this can aid water retention by reducing the surface to volume ratio for water loss through transpiration, although this may also reduce the rate of carbon uptake and assimilation (Farquhar et al., 1980). This could be why succulence index was negatively correlated with DTI as it may have led to lowered rates of photosynthesis and a reduction in yield. Additionally, increased succulence may result in stomata remaining open during drought as $\Psi_{leaf}$ is maintained, which ensures continued assimilation and dry matter accumulation. This will only result in greater WUE if the increase in water conservation is greater than the reduction in assimilation rate. The negative correlation between succulence index and WUE observed by Ober et al. (2005) suggests this is not evident in sugar beet. However, such trends in water use have been identified as the key driver of increased WUE under drought in temperate cereals, although it may come at a cost to yield potential (Richards et al., 2002). This reduction was evident in sugar beet with leaf succulence index being negatively correlated with sugar yield in the irrigated control Ober et al. (2005). These results show that traits which enable a greater DTI may enhance WUE, but as they focus on maintaining yield under drought through sustaining low rates of carbon uptake, assimilation and water use they may not result in a high yield potential under optimal conditions if the maximum rate of assimilation is limited. Focusing on drivers of WUE attributed to assimilation and dry matter accumulation, rather than just transpiration, may therefore also be useful if they can be identified.

Although not significantly correlated with DTI, differences in WUE between sugar beet varieties are evident, not because of differences in water use but rather dry matter accumulation (Ober et al., 2004, Ober et al., 2005). This may suggest that although all varieties use the same amount of water, the efficiency with which this is used to accumulate biomass differs. The most likely factor influencing dry matter accumulation is radiation use efficiency (RUE) which can be defined most simply as ‘the efficiency with which the plant converts intercepted radiation into dry matter’

(Sinclair and Muchow, 1999). RUE is determined by the photosynthetic performance of the plant and the ability of the plant to turn the carbohydrates produced from photosynthesis into biomass (Sinclair and Horie, 1989). For example, the average RUE of sugar beet is 1.4g DM per MJ solar radiation which is derived by plotting biomass against intercepted radiation and calculating the gradient of the line (Monteith et al., 1977). The ability of a plant or crop to intercept light is closely linked to canopy structure with the leaf area index (LAI, leaf area $m^2$/ground area $m^2$), leaf positioning, erectness and wilting all influencing how effectively the canopy intercepts radiation (Montieth, 1965). Sugar beet canopies with prostrate leaves achieve greater light interception and RUE with a relatively low LAI (<3), whilst canopies with more upright leaves require a LAI above 3.5 to maximise light interception and RUE with so that the light passes through to be intercepted by leaves deeper in the canopy (Duncan et al., 1967). The Brooms Barn sugar beet model highlights the importance of LAI on biomass accumulation as it reduces LAI based on the level of drought which in turn reduces the intercepted ration and therefore the yield potential of the crop (Qi et al., 2005). The potential difference in canopy architecture, RUE efficiency and the effect of these traits on WUE between sugar beet varieties needs to be explored further. These traits can then be compared to some of the traits which correlate with DTI, such as wilting to further understand how these traits interact to influence WUE and drought tolerance.

Overall, drought related traits may have a complex interaction with WUE and differences in WUE between varieties are evident under drought. Further research into the relationship between drought related traits and WUE is evidently needed but the complex interactions outlined here highlight the challenges in such an assessment.

## 1.8 Conclusion

Fundamentally WUE can be assessed at the leaf level through manipulating factors which affect carbon uptake, assimilation and transpiration. This can, in turn, help inform as to which traits may enhance sugar beet WUE.  At the canopy level more factors have an influence on WUE and this enables an even wider range of factors to be assessed which influence both plant and soil water losses. The assessment of WUE is clearly challenging due to the wide range of factors that influence it, some of which have been outlined here.

## 1.9 Research questions

In the case of sugar beet a number of areas with regards to WUE which require further exploration have been identified though this literature review. The areas that have been selected for further research are focused on understanding the stomatal physiology and responses of sugar beet to the environment, how WUE varies under water deficit and between varieties and whether varieties differ in traits associated with increased WUE. This has led to the development of the following research questions and hypotheses:

1. How quickly are sugar beet stomata able to respond to increasing light and is the speed of stomatal response related to anisohydric behaviour in sugar beet?

   *Hypothesis: Slow stomatal closure in sugar beet is attributed to a low stomatal density and large stomatal size which leads to a disconnect between* $g_s$ *and A and excessive water loss from transpiration.*
2. How is WUE affected by fluctuations in soil water availability in sugar beet?

   *Hypothesis: Sugar beet Intrinsic water use efficiency (*$WUE_i$*) and dry matter water use efficiency (*$WUE_{DM}$*) increase under water deficit.*
3. Does sugar beet acclimate (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to water deficit to increase WUE?

   *Hypothesis: Sugar beet shows no long-term physiological acclimation in response to water deficit due to its anisohydric behaviour.*

4. Are there differences in WUE between commercial sugar beet varieties with contrasting canopy architecture and does stomatal and leaf morphology vary between these varieties?

   *Hypothesis: Contrasting canopy architecture leads to differences in WUE and can be attributed to differences in stomatal and leaf morphology*

5. What is the diurnal change in $WUE_i$ in sugar beet and does this differ between varieties or under reduced water availability?

   *Hypothesis: WUE decreases as stomata open in response to increasing PPFD and decreases less in some varieties and in response to water deficit.*

6. What is the change in leaf water content in sugar beet diurnally, and could this explain why the crop wilts so freely compared to other crop species?

   *Hypothesis: Leaf water content declines as $g_s$ increases in response to increasing PPFD which leads to sugar beet wilting.*

## 1.10 Thesis overview

This thesis consists of three studies which have been written up as academic papers which have been, or will be, submitted to a relevant journal.

**Chapter 2: Anisohydric sugar beet rapidly responds to light to optimise leaf water use efficiency utilising numerous small stomata** – published in AoBPlants

This paper explores the speed of stomatal response in sugar beet and how this is related to stomatal morphology and the anisohydric response, as well as the subsequent effect on $WUE_i$. This chapter addresses the following research question and hypothesis:

1. How quickly are sugar beet stomata able to respond to increasing light and is the speed of stomatal response related to anisohydric behaviour in sugar beet?
   *Hypothesis: Slow stomatal closure in sugar beet is attributed to a low stomatal density and large stomatal size which leads to a disconnect between* $g_s$ *and A and excessive water loss from transpiration.*

**Chapter 3: Water use efficiency in contrasting commercial sugar beet varieties in response to fluctuating water availability** – to be submitted to Environmental and Experimental Botany once edited to reduce content**.**

This paper examines the effect of water availability on WUE in sugar beet and whether the plants undergo ang long term physiological acclimation to water deficit. Two varieties with contrasting upright and prostrate canopies were used to understand if differences in WUE between commercial sugar beet varieties with differing canopy architecture are present, and if the stomatal and leaf morphology differs between such varieties. This chapter addresses the following research questions and hypotheses:

2. How is WUE affected by fluctuations in soil water availability in sugar beet?

   *Hypothesis: Sugar beet Intrinsic water use efficiency ($WUE_i$) and dry matter water use efficiency ($WUE_{DM}$) increase under water deficit.*
3. Does sugar beet acclimate (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to water deficit to increase WUE?

   *Hypothesis: Sugar beet shows no long-term physiological acclimation in response to water deficit due to its anisohydric behaviour.*
4. Are there differences in WUE between commercial sugar beet varieties with contrasting canopy architecture and does stomatal and leaf morphology vary between these varieties?

   *Hypothesis: Contrasting canopy architecture leads to differences in WUE and can be attributed to differences in stomatal and leaf morphology.*

**Chapter 4: Diurnal responses of sugar beet and the effect on water use efficiency** – Further data needs to be collected before a paper can be submitted.

This paper focuses on five sugar beet varieties grown in a controlled environment room and exposed to a simulated diurnal light regime to identify how $WUE_i$ changes over the day and if this differs between varieties. Plants were also exposed to a water deficit to see if this altered diurnal $WUE_i$. Leaf water content was also assessed to identify if diurnal changes in leaf water content could help explain why sugar beet wilts freely in the field. This chapter addresses the following research questions and hypotheses:

5. What is the diurnal change in $WUE_i$ in sugar beet and does this differ between varieties or under reduced water availability?

   *Hypothesis: WUE decreases as stomata open in response to increasing PPFD and decreases less in some varieties and in response to water deficit.*
6. What is the change in leaf water content in sugar beet diurnally, and could this explain why the crop wilts so freely compared to other crop species?

   *Hypothesis: Leaf water content declines as $g_s$ increases in response to increasing PPFD which leads to sugar beet wilting.*

**Chapter 5: General discussion and conclusions**

The results of the three studies are summarised in this chapter and a general discussion of the work undertaken is presented.

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# Chapter 2: Anisohydric sugar beet rapidly responds to light to optimise leaf water use efficiency utilising numerous small stomata

The following chapter consists of a paper published in AoBPlants and explores the speed of stomatal response in sugar beet and how this is related to stomatal morphology and the anisohydric response, as well as the subsequent effect on $WUE_i$.

This chapter addresses the following research question and hypothesis:

1. How quickly are sugar beet stomata able to respond to increasing light and is the speed of stomatal response related to anisohydric behaviour in sugar beet?

   *Hypothesis: Slow stomatal closure in sugar beet is attributed to a low stomatal density and large stomatal size which leads to a disconnect between* $g_s$ *and* $A$ *and excessive water loss from transpiration.*

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STUDIES

# Anisohydric sugar beet rapidly responds to light to optimize leaf water use efficiency utilizing numerous small stomata

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## Abstract

Under conditions of high transpiration and low soil water availability, the demand for water can exceed supply causing a reduction in water potential and a loss of cell turgor (wilting). Regulation of stomatal aperture mediates the loss of water vapour ($g_s$), which in turn is dependent in part on the anatomical characteristics of stomatal density (SD) and stomatal size (SS). Anisohydric sugar beet (*Beta vulgaris*) is atypical, exhibiting wilting under high soil water availability. Spinach (*Spinacia oleracea*) belongs to the same family *Chenopodiaceae s.s.*, but demonstrates a more typical wilting response. To investigate the role of stomatal dynamics in such behaviours, sugar beet and spinach leaves were exposed to step-changes in photosynthetic photon flux density (PPFD) from 250 to 2500 μmol m$^{-2}$ s$^{-1}$. Using a four log-logistic function, the maximum rate of stomatal opening was estimated. Concurrent measurements of SD and SS were taken for both species. While sugar beet coupled faster opening with smaller, more numerous stomata, spinach showed the converse. After exposure to drought, maximum $g_s$ was reduced in sugar beet but still achieved a similar speed of opening. It is concluded that sugar beet stomata respond rapidly to changes in PPFD with a high rate and magnitude of opening under both non-droughted and droughted conditions. Such a response may contribute to wilting, even under high soil water availability, but enables photosynthesis to be better coupled with increasing PPFD.

**Keywords:** Anisohydric; speed of stomatal response; stomatal density; stomatal size; sugar beet; water use efficiency; wilting.

## Introduction

The largest areas of sugar beet (*Beta vulgaris* ssp. *vulgaris*) production are in Europe, Russia and North America, where it is grown for both sugar production and biofuel (Draycott 2006). Its wild ancestor is sea beet (*Beta vulgaris* ssp. *maritima*), which is thought to be the origin of the crop's salinity tolerance and suitability for the temperate climates in which sugar beet is grown (Ribeiro *et al.* 2016). Although sugar beet yields are increasing in the UK, losses of up to 25 % are evidenced in the driest years (Jaggard *et al.* 1998). Improving the resilience of the crop is important to maintain yields into the future as the world's climate changes and hotter, drier summers are predicted in the UK (David 2017). A number of studies have shown that drought tolerance varies between sugar beet genotypes and is associated with a range of traits from specific leaf weight to maintenance of canopy green area (Pidgeon and Jaggard 1998; Ober *et al.* 2004, 2005; Rajabi *et al.* 2009) but these studies did not assess how sugar beet regulate water use efficiency at the leaf level. Regulation of stomatal aperture mediates the rate of stomatal conductance ($g_s$) and assimilation (*A*) and it is the ratio of these two processes which gives a value for intrinsic water

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use efficiency ($WUE_i$); hence, the anatomical characteristics of stomatal density (SD) and stomatal size (SS) are important in determining these processes. Therefore, to understand $WUE_i$ in sugar beet, SD and SS and the effect these parameters have on the magnitude and speed of stomatal response must be understood.

A distinctive trait of the sugar beet crop is its tendency to wilt on bright and warm days, even when water is available in the soil profile. Research by Kohl and Cary (1969) demonstrated that light mist irrigation can reduce the prevalence of wilting. This suggests stomata are not closing as leaf water potential ($\Psi_L$) falls and that high levels of transpiration drive the wilting response. The reluctance of sugar beet stomata to close is attributed to reduced stomatal sensitivity to falling $\Psi_L$ and high levels of osmotic adjustment, rather than stomatal closure to reduce water losses through transpiration, which results in a rapid decline in $\Psi_L$ over the day (McCree and Richardson 1987). Plants that do not maintain a stable midday $\Psi_L$, including sugar beet, are described as an anisohydric, as opposed to isohydric plants which maintain midday $\Psi_L$ (Tardieu and Simonneau 1998). Despite wilting, the anisohydric response enables high photosynthetic rates to be maintained for longer periods than in isohydric plants, which close stomata sooner, and are suited to environments where water is abundant and droughts are short and of moderate severity (Sade *et al.* 2012). Key to the observation that sugar beet is anisohydric is the relationship between stomata and the environment and exploring this could identify if stomatal responses are a driver of wilting under high soil water availability.

Stomata respond to signals derived from the external and internal leaf environment to reduce water loss through transpiration and maximize $CO_2$ assimilation (Lawson *et al.* 2010). Declining plant water status (affected by factors such as vapour pressure deficit (VPD) (Nonami *et al.* 1991), soil water potential (Zhang and Davies 1990) and $\Psi_L$ (Brodribb and Holbrook 2003)), rising $CO_2$ concentrations in the intercellular air spaces (Xu *et al.* 2016) and low PPFD promote stomatal closure (Shimazaki *et al.* 2007), whilst the opposite conditions drive opening. For optimal $WUE_i$ stomata should open quickly in response to favourable conditions, to a magnitude which supports maximum *A*, without overshooting which would result in excessive $g_s$ and water loss (McAusland *et al.* 2016). There are a range of approaches to assess the impact of changing environmental variables on the speed and magnitude of stomatal response and most studies develop a model based on the sigmoidal response to step-changes in light (Kirschbaum *et al.* 1988; Assmann and Grantz 1990; Knapp 1993; Zipperlen and Press 1997; Vico *et al.* 2011; Drake *et al.* 2013). Step-changes in light are more representative of the field environment and facilitate plant responses more representative of those in the field compared to light curves in which light intensity changes gradually. This approach identifies the maximum and minimum rates of $g_s$ ($g_{smax}$, $g_{smin}$) and *A* ($A_{max}$, $A_{min}$) and the rate of change between the minimum and maximum giving a value for the speed of stomatal response in dynamic light (Kirschbaum *et al.* 1988). A popular approach is that of Knapp (1993) which uses a time constant to identify where 63 % of the magnitude of the change has occurred to give a measurement of the time taken to reach this point, whilst other studies derive values from different points such as 50 % (Drake *et al.* 2013) and 90 % of the maximum value for $g_s$ or *A* (Zipperlen and Press 1997). Alternatively, the change in stomatal response divided by the change in time between 10 and 90 % of the magnitude of the light pulse can be used as a more simplistic approach (Assmann and Grantz 1990). The model chosen depends on the hypothesis to be addressed and can be dependent on the asymmetry of opening and closing, which can be species- and environment-dependent (Vico *et al.* 2011).

The speed of stomatal response to dynamic conditions has a significant influence on $WUE_i$ and is related to the plant's SD and SS (Drake *et al.* 2013; Lawson and Vialet-Chabrand 2019), which have an inverse relationship in most species (Franks *et al.* 2009). A greater SD and reduced SS is typically associated with faster stomatal responses which increases the coordination between *A* and $g_s$ and increases $WUE_i$ (Lawson and Weyers 1999; Lawson *et al.* 2010; McAusland *et al.* 2016; Vialet-Chabrand *et al.* 2017), although this may not improve $WUE_i$ over a longer time scale (Moualeu-Ngangue *et al.* 2016). Given the different factors influencing stomatal dynamics, it is important to assess species individually and to understand the relationship between SD and SS, how this affects the speed of stomatal response and the impact this has on $g_s$ and *A*, and consequently $WUE_i$.

This study used dynamic light to assess the magnitude and speed of stomatal response and the relationship with SD and SS to enable an assessment of $g_s$, *A* and $WUE_i$ and identify if stomatal responses could be a driver of wilting in sugar beet. The hypothesis was that slow stomatal closure in sugar beet is attributed to a low SD and large SS which leads to a disconnect between $g_s$ and *A* and excessive water loss from transpiration. To address this hypothesis, spinach was selected as a comparison species as it also belongs to the family *Chenopodiaceae s.s.* but demonstrates a more typical wilting response. In addition to this it was hypothesized that water stress and wilting, which is often evident in the sugar beet crop, would alter the speed of stomatal response compared to well-watered plants to conserve water and increase $WUE_i$ at the expense of carbon fixation.

## Materials and Methods

### Plant material

Sugar beet (*Beta vulgaris* ssp. *vulgaris*) cv. Haydn and spinach (*Spinacia oleracea*) cv. Mikado were sown in 5-L pots containing a 1:1 mix of Kettering loam and sand and grown in a controlled environment room. Pots were placed on raised benches in a randomized block design, with eight replicates of each species, under fluorescent tubes (LUMILUX HO 54W/840 T5, Osram, Munich, Germany) which provided 12 h of light followed by 12 h of darkness, with an hour dawn and evening light adjustment. Three seeds were sown per pot and thinned to a single plant at 40 days after sowing (DAS) and hand-watered to prevent soil drying. Humidity was between 44 and 85 % with a daytime temperature of 22 ± 3 °C and night-time temperature of 6 ± 1 °C, monitored using a humidity and temperature data logger (TinyTag Ultra 2, Gemini Instruments, Chichester, UK). A split application totalling 1.05 g of ammonium nitrate was applied in solution with 15 mL applied at 35 DAS and 39 DAS.

### Drought treatment

Water was withdrawn from blocks 1 and 2 at 119 DAS and blocks 3 and 4 at 121 DAS for the drought treatment. The staggered water withdrawal ensured that the water deficits were comparable when measurements were taken, as each block took a day to measure. A capacitance soil moisture probe (ML 3 ThetaProbe, Delta T, Cambridge, UK) was used to monitor soil moisture content. The probe was inserted into the soil to 5 cm and percentage soil moisture recorded for each plant as gas exchange measurements were being taken. The spinach did not reach a water-stressed state as there was no wilting or decline in $A_{max}$ in the time constraints of the experiment whilst wilting was evident in the sugar beet. The drought responses are therefore focused on the results from the sugar beet observations.

## Gas exchange and chlorophyll fluorescence measurements

Leaves were dark-adapted for 30 min by wrapping in aluminium foil. The room was fully darkened when the leaves were unwrapped and placed into infrared gas analyser cuvette (LI-6800, LI-COR, Lincoln, NE, USA) with help of a green LED head torch (LUMii 10-465-200, LUMii, Coventry, UK) providing minimal light for the operator. Leaf 7–8 and 9–10 were used for the non-droughted and droughted measurements, respectively, and selected to ensure a uniform size, with spinach leaves of the same age as the beet leaves being selected for measurement.

Gas exchange measurements were taken using infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA). An auto log program within a control loop set PPFD in the gas exchange cuvette at 250 µmol m$^{-2}$ s$^{-1}$ for 15 min, 2500 µmol m$^{-2}$ s$^{-1}$ for 30 min and 250 µmol m$^{-2}$ s$^{-1}$ for 30 min. The maximum light intensity was identified following standard light–response curve procedures with 200 µmol m$^{-2}$ s$^{-1}$ PPFD step-increases in light intensity every 5 min and identifying the level at which *A* plateaued in both beet and spinach. The minimum light intensity was chosen as 10 % of this maximum light intensity. Gas exchange measurements of $g_s$, *A*, and leaf VPD and chlorophyll fluorescence parameters of $F_v'/F_m'$ (maximum photosystem II (PSII) efficiency in the light), ΦPSII (quantum efficiency of PSII electron transport in the light) and $q_p$ (photochemical quenching) were logged every minute of the 75-min program (15 at low light T1–T15, 30 at high light T16–T45 and a further 30 at low light T46–T75) using a multiphase flash fluorometer (LI-6800 multiphase flash fluorometer, LI-COR, Lincoln, NE, USA) (flash was 300 ms and 10 000 µmol m$^{-2}$ s$^{-1}$).

Standard settings were; flow 500 µmol s$^{-1}$, reference $CO_2$ 400 µmol, RH 50 % and leaf temperature 20 ± 3 °C, with matching at every measurement. The sugar beet and spinach measurements were taken at 90, 91, 92 and 96 DAS on blocks 1, 2, 3 and 4, respectively. The sugar beet non-droughted and droughted measurements were taken at 124, 125, 126 and 127 DAS on blocks 1, 2, 3 and 4, respectively. The VPD maintained in the LI-6800 chamber was between 1 and 1.2 KPa for the both the beet and the spinach [**see** Supporting Information—Fig. S1A], and for the non-droughted and droughted beet at the low light levels [**see** Supporting Information—Fig. S1B]. The spike at the onset of high light is due to the LI-6800 adjusting to maintain cuvette temperature and RH % as the stomata open and transpire. Once settled at high light VPD significantly ($P < 0.001$) increased to between 1.3 to 1.4 KPa for the beet and the spinach and between 1.1 to 1.3 KPa ($P = 0.009$) for the non-droughted and droughted beet. There was no significant difference in VPD between the sugar beet and spinach and the non-droughted and droughted beet.

## Modelling the light response

For the analysis of the speed of stomatal response, dose–response curves (DRCs) were calculated for each replicate using the $g_s$ data in the statistical programming and graphics package R (R Core Team 2019) using the freely accessible DRC package (Ritz *et al.* 2015). Model selection by comparison of different functions was utilized to identify which log-logistic function was most suited to the data set with log logistics 4 (LL.4) producing the best fit. Log-logistic curves require a stable start and end point to enable a realistic estimate of the upper and lower limit. For this reason, the 75 data points were split into a stomatal opening (switch from 250 to 2500 µmol m$^{-2}$ s$^{-1}$ PPFD) and a stomatal closing (switch from 2500 to 250 µmol m$^{-2}$ s$^{-1}$ PPFD) phase with 35 data points in each. The opening phase consisted of points T11–T45 (i.e. 11–45 min) (Fig. 1A), as $g_s$ was not consistently stable at T1–T10, and the closing phase T41–T75 (Fig. 1B). For opening, the first five data points (T11–T15) were therefore at low light to provide an estimate of the lower limit. The remaining 30 data points were then at high light (T16–T45) with stomatal conductance starting to plateau by the end of this period for estimation of the upper limit. For closing, the last 5 min of the high light period was used (T41–T45) to establish an upper limit followed by the 30 min of low light (T46–T75), with conductance starting to plateau at the end of this period for estimation of the lower limit. The estimated lower (OE$g_{smin}$—at opening, CE$g_{smin}$—at closing) and upper (OE$g_{smax}$—at opening, CE$g_{smax}$—at closing) limit to stomatal conductance ($g_s$) calculated using the LL.4 curve, could then be compared to the measured lower ($g_{smin}$) and upper ($g_{smax}$) $g_s$ values from the LI-COR. The point halfway between the estimated lower and upper limits of stomatal conductance (O$g_s$50—at opening, C$g_s$50—at closing) and the slope of the tangent of the line at the O$g_s$50 or C$g_s$50 provides an estimate of the speed of stomatal closure at that point for opening or closing, respectively. The mean curve parameters for the treatments were calculated using the LL.4 curves from each replicate and the mean LL.4 curves for each

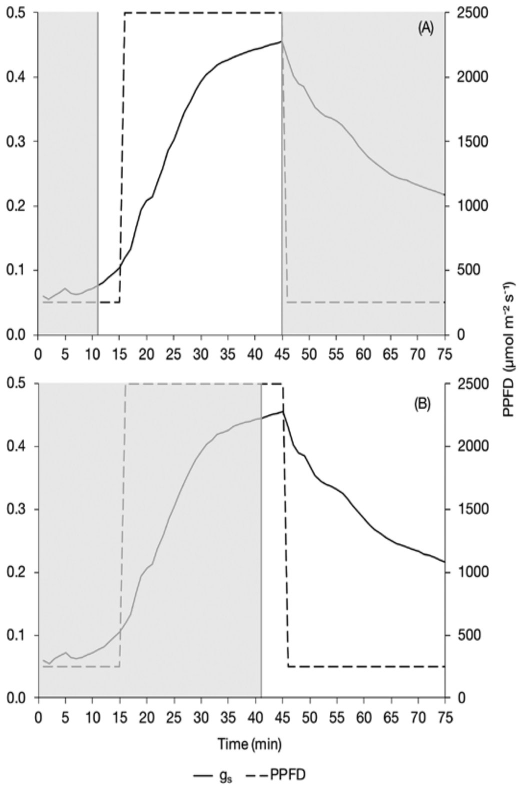

**Figure 1.** Stomatal conductance measured over a 75-min program (T1–T75) which was used to model stomatal opening and closing. Plants were exposed to 250 µmol m$^{-2}$ s$^{-1}$ for 15 min (T1–T15) followed by 2500 µmol m$^{-2}$ s$^{-1}$ for 30 min (T16–T45) and 250 µmol m$^{-2}$ s$^{-1}$ for another 30 min (T46–T75). To model stomatal opening an LL.4 function was used with the stomatal opening curve fitted using points T11–T45 (A) and the closing phase T41–T75 (B), which are located in the non-shaded regions of the figures.

treatment compared using two-way ANOVA in R to identify if treatments produced significantly different curves.

### Calculating intrinsic water use efficiency

Intrinsic water use efficiency was calculated using Equation (1) (Condon *et al.* 2002). The values for A and $g_s$ were collected using the infrared gas analyser as previously outlined.

$$\mathrm{WUE_i} = \frac{A}{g_s} \qquad (1)$$

### Stomatal anatomy

A stomatal impression of the abaxial and adaxial leaf surface of the gas exchange measurement leaf of each sugar beet and spinach replicate was taken after the non-droughted measurements at 97 DAS. Clear nail varnish was applied and left to dry for 20 min until no longer tacky. Clear tape was applied to the area and peeled to lift the dried varnish which was mounted on a microscope sample slide. Three images were taken from each sample slide using a microscope (Leica 5000B, Leica, Wetzlar, Germany) with a light source (Leica CTR5000, Leica, Wetzlar, Germany) at 100× magnification and cropped to 1 $\mathrm{mm^2}$ using the microscope scale for reference in Fiji (Schindelin *et al.* 2012). The stomata in the cropped images were manually counted using the Cell Counter plugin, with an average SD value of the abaxial and adaxial leaf surface calculated for each replicate from the three 1 $\mathrm{mm^2}$ areas counted.

Stomatal size was calculated by reducing the 1 $\mathrm{mm^2}$ image to 0.25 $\mathrm{mm^2}$ and randomly selecting 10 stomata to be measured. The stomatal pore (SP) length, peristomatal groove (PSG) length and guard cell (GC) width were measured and maximum theoretical conductance calculated for the adaxial and abaxial leaf surface using the method of Franks *et al.* (2009).

### Statistical analysis

Repeated-measures ANOVA was performed on the $g_s$, A, $F_v'/F_m'$, ΦPSII, $q_p$ and $\mathrm{WUE_i}$ data with time as the independent variable and a two-way ANOVA on the stomatal impressions data sets with species as the independent variable. Anomalous $\mathrm{WUE_i}$ values in excess of 200 at T17 were removed from the analysis as these were caused by the LI-COR automatically adjusting to the sudden increase in $g_s$ and A at the onset of high light to achieve the temperature and RH set points. GenStat 15th edition (VSN International Ltd, Hemel Hempstead, UK) was used for the statistical analyses except for the curve fitting which was performed in R as previously described.

## Results

### Sugar beet and spinach

*Speed of response to light in beet and spinach.* The sugar beet and spinach responded differently to the onset of high light (stomatal opening) and subsequent low light (stomatal closing) (Fig. 2). By fitting the LL.4 model and running a two-way ANOVA the two curves were identified as being significantly different ($P < 0.001$) (Fig. 3). The stomatal opening (Fig. 3A) of the sugar beet was faster with $\mathrm{O}g_s50$ estimated to be reached at 13.56 ± 0.60 min compared with 19.62 ± 4.87 min for the spinach (Table 1). At the estimated $\mathrm{O}g_s50$ the sugar beet stomata were still continuing to open rapidly and at a greater rate than the spinach with a slope of 2.91 ± 0.40 compared to 1.84 ± 0.52 (Table 1). The rapid opening of the sugar beet stomata was associated with a higher $\mathrm{OE}g_{smax}$ of 0.48 ± 0.02 mol $\mathrm{m^{-2}\ s^{-1}}$ (Table 1) which is close to the measured $g_{smax}$ of 0.46 ± 0.04 mol $\mathrm{m^{-2}\ s^{-1}}$ at T45, which is the last measurement taken during the 30-min high light period (Table 1). The $\mathrm{OE}g_{smax}$ of spinach was 0.45 ± 0.08 mol $\mathrm{m^{-2}\ s^{-1}}$ but the $g_{smax}$ reached at T45 was much lower at 0.36 ± 0.02 mol $\mathrm{m^{-2}}$ (Table 1), indicating that the spinach stomata were still in the process of opening at the end of the 45-min high light period. Both species had similar levels of $g_s$ prior to the onset of high light (Fig. 2) with the sugar beet $\mathrm{OE}g_{smin}$ of 0.08 ± 0.01 mol $\mathrm{m^{-2}\ s^{-1}}$ slightly less than the 0.10 ± 0.02 mol $\mathrm{m^{-2}\ s^{-1}}$ of the spinach, with both of these estimates close to the measured $g_{smin}$ at T11 (Table 1).

The stomatal closure LL.4 curves of the sugar beet and spinach (Fig. 3B) were also significantly different ($P < 0.001$). For both species, the rate of stomatal closure was slower than opening but the sugar beet was again faster than spinach, despite reaching a higher rate of $g_s$ in the high light period, with an estimated $\mathrm{C}g_s50$ of 16.81 ± 3.69 min and 22.41 ± 10.59 min, respectively (Table 1). At the estimated $\mathrm{C}g_s50$ the sugar beet had a slightly slower rate of closure with a slope of 1.90 ± 0.55 compared to 2.16 ± 1.17 in the spinach (Table 1). This may be attributed to the sugar beet having an initially rapid rate of closure which enabled it to reach a similar level of $g_s$ quickly (Fig. 3B), which had slowed by the $\mathrm{C}g_s50$. The $\mathrm{CE}g_{smax}$ of the sugar beet and the spinach was calculated to be 0.46 ± 0.02 mol $\mathrm{m^{-2}\ s^{-1}}$ and 0.35 ± 0.01 mol $\mathrm{m^{-2}}$, respectively, which is similar to the measured $g_{smax}$ at T45 of 0.46 ± 0.04 mol $\mathrm{m^{-2}\ s^{-1}}$ and 0.35 ± 0.02 mol $\mathrm{m^{-2}\ s^{-1}}$ (Table 1). The $\mathrm{CE}g_{smin}$ from the closing curves was the same for the beet and spinach, and close to the measured $\mathrm{C}g_{smin}$ at T75 for both species. The $g_s$ after the high light exposure (T46–T75) was higher than the pre-high light $g_s$ (T1–T15) (Table 1) because the plants did not return to the dark-adapted state in which they started.

*Assimilation and WUEi in sugar beet and spinach.* More open stomata facilitate greater $g_s$ and A; therefore, both $g_s$ ($P = 0.007$) (Fig. 2) and A ($P < 0.001$) (Fig. 4A) were significantly greater in the sugar beet than the spinach in the high light and subsequent low light period. The sugar beet reached an $A_{max}$ of 29.31 ± 1.04 μmol $\mathrm{m^{-2}\ s^{-1}}$ at T45 compared to 21.87 ± 0.86 μmol $\mathrm{m^{-2}\ s^{-1}}$ in the spinach ($P < 0.001$). The sugar beet also achieved significantly higher rates of A in the low light of 10.74 ± 0.13 μmol $\mathrm{m^{-2}\ s^{-1}}$ at T75 compared

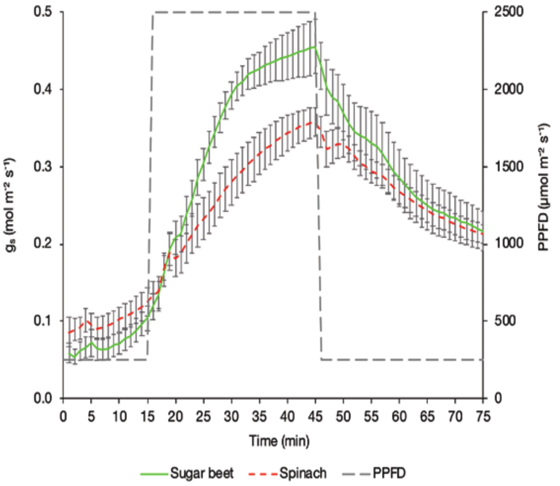

**Figure 2.** The stomatal conductance of sugar beet and spinach plants exposed to changing PPFD. Plants were exposed to a PPFD of 250 μmol $\mathrm{m^{-2}\ s^{-1}}$ for 15 min, 2500 μmol $\mathrm{m^{-2}\ s^{-1}}$ for 30 min and 250 μmol $\mathrm{m^{-2}\ s^{-1}}$ for 30 min, measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) with measurements logged every minute. These data were used to plot LL.4 curves and estimate stomatal speed. Error bars show SE ±, $n = 8$ sugar beet and 8 spinach.

to 9.21 ± 0.22 µmol $m^{-2}$ $s^{-1}$ in the spinach ($P < 0.001$). These values are greater than the post-dark adaptation values at T15, the end of the initial low light period, because the plants had by then undergone high light induction.

At the onset of low light the decoupling of $g_s$ and $A$ is evident in both sugar beet and spinach as $A$ declines almost instantly to a steady state due to the light requirement for photosynthesis (Fig. 4A) whilst $g_s$ declines more slowly (Fig. 2). When averaged over the whole response curve $g_s$ was not significantly different between the sugar beet and the spinach but $A$ was ($P = 0.002$). This is evident from T9 to T15 in the initial low light phase, at the onset of high light from T16 to T23, and at the end of the second low light phase from T68 onwards (Fig. 3B). The greater ratio of $A$ to $g_s$ (i.e. $WUE_i$) in the sugar beet over these time points therefore resulted in a trend of higher $WUE_i$ in the sugar beet during the initial low light phase, the start of the high light phase and then again later in the second low light phase ($P = 0.075$) (Fig. 4B).

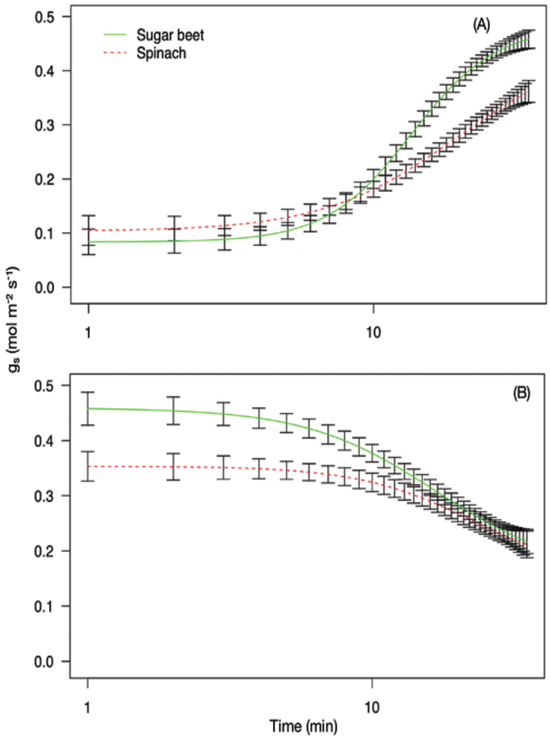

**Figure 3.** The LL.4 curves of stomatal conductance ($g_s$) of sugar beet and spinach. Stomatal conductance was measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) and fitted using plotted using the DRC package (Ritz *et al.* 2015) in the statistical programming and graphics package R (R Core Team 2019). The plants were exposed to a PPFD of 250 µmol $m^{-2}$ $s^{-1}$ for 15 min, 2500 µmol $m^{-2}$ $s^{-1}$ for 30 min and 250 µmol $m^{-2}$ $s^{-1}$ for 30 min. Curve (A) shows the curve fitted when using the measurements taken during the last 5 min of the initial low light period and the 30-min high light period. Curve (B) shows the curve fitted when using the measurements taken during the last 5 min of the high light period and the 30 min low light period. The curves were identified as being significantly different ($P < 0.001$) using a two-way ANOVA. Error bars show SE ± $n = 8$ sugar beet and 8 spinach.

## Chlorophyll fluorescence

Maximum PSII efficiency in the light ($F_v'/F_m'$) was not significantly different between species once the plants were stable at T10 (Fig. 5A). During the high light period differences in $F_v'/F_m'$ were evident between the beet and spinach with the beet maintaining a significantly higher ($P = 0.002$) ratio with values of 0.538 ± 0.006 compared to 0.476 ± 0.006 in the spinach at T45, indicating a lower value of non-photochemical quenching in the former, perhaps consistent with the higher value of $A$. Returning to low light, the sugar beet $F_v'/F_m'$ values remain significantly higher than the spinach with values at T75 of 0.737 ± 0.002 compared to 0.708 ± 0.004.

Sugar beet had a greater average PSII operating efficiency (ΦPSII) in the light ($P = 0.006$) consistent with the higher values of $A$ (Fig. 5B). ΦPSII was significantly greater ($P = 0.042$) at the end of the initial low light response (T7–T15) in the middle of the high light response (T26–T39, T42–T44) and consistently in the low light period (T46–T75) with a steady-state value at T75 of 0.708 ± 0.003 compared to 0.661 ± 0.006.

**Table 1.** Estimated $g_s$ parameters from LL.4 curves of sugar beet and spinach exposed to stepwise changes in light to induce stomatal opening (250 to 2500 µmol $m^{-2}$ $s^{-1}$ PPFD) and closing (2500 to 250 µmol $m^{-2}$ $s^{-1}$ PPFD), with measured $g_{smin}$ and $g_{smax}$ values for comparison. The average LL.4 curves of sugar beet and spinach, plotted from eight replicates each, were analysed using two-way ANOVA and shown to be significantly different ($P < 0.001$).

| | | Beet | | | Spinach | | |
|---|---|---|---|---|---|---|---|
| Parameter | Units | Output | SE | P-value | Output | SE | P-value |
| Opening | | | | | | | |
| OE$g_{smin}$ | mol $m^{-2}$ $s^{-1}$ | 0.08 | 0.01 | <0.001 | 0.10 | 0.02 | <0.001 |
| OE$g_{smax}$ | mol $m^{-2}$ $s^{-1}$ | 0.48 | 0.02 | <0.001 | 0.45 | 0.08 | <0.001 |
| O$g_s$50 | min | 13.56 | 0.60 | <0.001 | 19.62 | 4.87 | <0.001 |
| Slope | | 2.91 | 0.40 | <0.001 | 1.84 | 0.52 | <0.001 |
| T11 $g_{smin}$[a] | mol $m^{-2}$ $s^{-1}$ | 0.08 | 0.01 | – | 0.11 | 0.02 | – |
| T45 $g_{smax}$[b] | mol $m^{-2}$ $s^{-1}$ | 0.46 | 0.04 | – | 0.36 | 0.02 | – |
| Closing | | | | | | | |
| CE$g_{smin}$ | mol $m^{-2}$ $s^{-1}$ | 0.16 | 0.06 | <0.001 | 0.16 | 0.10 | ns |
| CE$g_{smax}$ | mol $m^{-2}$ $s^{-1}$ | 0.46 | 0.02 | <0.01 | 0.35 | 0.01 | <0.001 |
| C$g_s$50 | min | 16.81 | 3.69 | <0.001 | 22.41 | 10.59 | <0.01 |
| Slope | | 1.90 | 0.55 | <0.001 | 2.16 | 1.17 | ns |
| T75 $g_{smin}$[c] | mol $m^{-2}$ $s^{-1}$ | 0.22 | 0.03 | – | 0.21 | 0.01 | – |

[a] Measured $g_{smin}$ at T11 (pre-high light).

[b] Measured $g_{smax}$ at T45 (during high light).

[c] Measured $g_{smin}$ at T75 (post-high light).

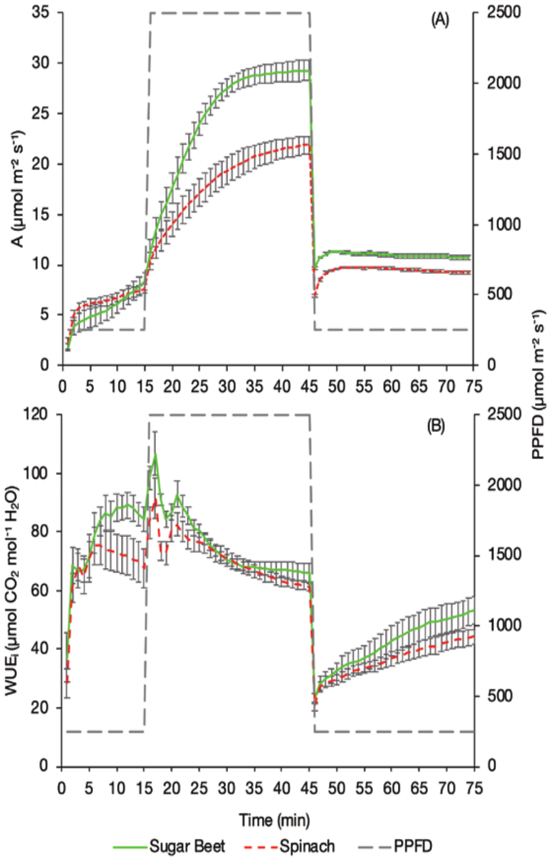

**Figure 4.** The assimilation (A) and WUE$_i$ (B) of sugar beet and spinach plants exposed to changing PPFD. Plants were exposed to a PPFD of 250 µmol m$^{-2}$ s$^{-1}$ for 15 min, 2500 µmol m$^{-2}$ s$^{-1}$ for 30 min and 250 µmol m$^{-2}$ s$^{-1}$ for 30 min, measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) with measurements logged every minute. Error bars show SE ± $n$ = 8 sugar beet and 8 spinach.

The level of photochemical quenching measured as $q_p$ (Fig. 5C) was not significantly different when averaged over the entire response cycle. There was a trend (P = 0.062) of greater $q_p$ in the sugar beet through all of the second low light period (T46–T75), with a steady-state value in this period of 0.960 ± 0.002 compared to 0.934 ± 0.004 in the spinach.

NPQ$_t$ was higher (P < 0.001) in the spinach than sugar beet at 2.5 compared to 1.9, respectively, averaged over all time points T1–T75, driven by differences under the high light and subsequent low light period (P < 0.001). Under high light NPQ$_t$ increased (P < 0.001) in both the sugar beet and the spinach and returned to levels comparable to pre the high light when the PPFD was decreased at T45 [see Supporting Information—Fig. S2A].

## Stomatal anatomy

Assessing SD and SS can provide an estimate of the maximum rate of $g_s$ a plant can attain and, in this case, can be compared to the estimated values from the modelled LL.4 curves. The sugar beet had significantly greater SD (P < 0.001) than the spinach on both the adaxial and abaxial leaf surface (Fig. 6A). Sugar beet had a smaller SS than spinach with all three parameters measured being significantly less, SP length (P < 0.001), PSG length (P < 0.001) and GC width (P = 0.003) (Fig. 6B). These parameters were then used to calculate the theoretical maximum stomatal conductance of the adaxial and abaxial leaf surface using the model of Franks and Beerling (2009) which were combined to produce an overall average. The theoretical maximum to $H_2O$ was 2.87 mol m$^{-2}$ s$^{-1}$ and 2.84 mol m$^{-2}$ s$^{-1}$ and to $CO_2$ which was 1.79 µmol m$^{-2}$ s$^{-1}$ and 1.78 µmol m$^{-2}$ s$^{-1}$ in beet and spinach, respectively. There was no significant difference between sugar beet and spinach in either parameter which supports the OE$g_{smax}$ value calculated from the LL.4 curves.

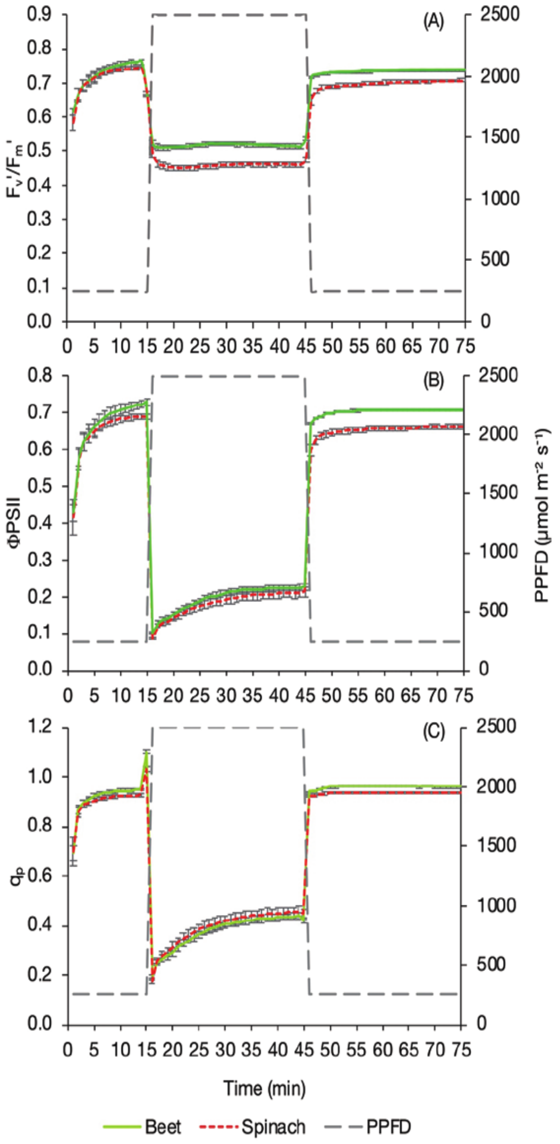

**Figure 5.** The F$_v$′/F$_m$′ (A), ΦPSII (B), $q_p$ (C) and of sugar beet and spinach plants exposed to changing PPFD. Plants were exposed to a PPFD of 250 µmol m$^{-2}$ s$^{-1}$ for 15 min, 2500 µmol m$^{-2}$ s$^{-1}$ for 30 min and 250 µmol m$^{-2}$ s$^{-1}$ for 30 min, measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) with measurements logged every minute. Error bars show SE ± $n$ = 8 sugar beet and 8 spinach.

## Light dynamic responses under drought

Sugar beet was selected for a focused analysis of dynamic responses to light under water deficit (drought) conditions. Droughted plants wilted and showed an altered stomatal response (Fig. 7). The fitted LL.4 curves of the non-droughted and droughted plants showed a significant difference (P < 0.001) in both stomatal opening (Fig. 8A) and closing phases (Fig. 8B). The droughted beet had a similar O$g_s$50 to the non-droughted beet

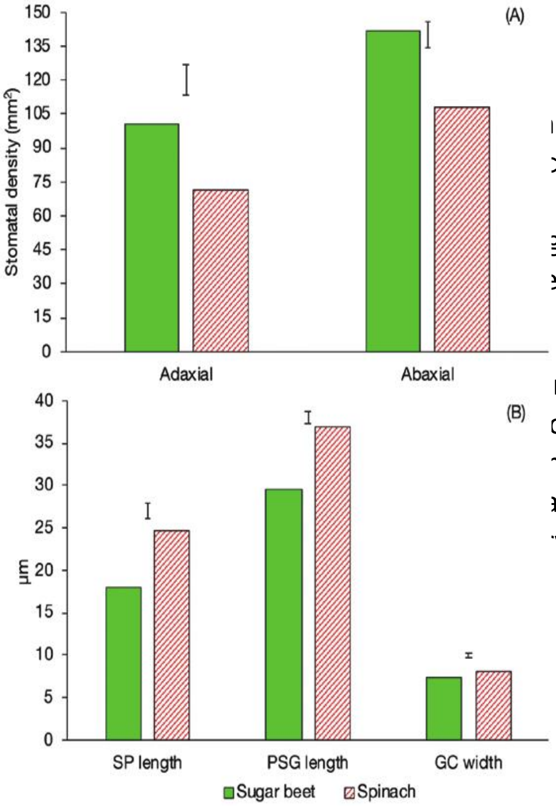

**Figure 6.** (A) The SD of the adaxial ($P < 0.001$, LSD = 6.90) and abaxial ($P < 0.001$, LSD = 5.90) leaf surface of spinach and sugar beet measured under optimal conditions. $n$ = 8 sugar beet and 8 spinach. (B) The SP length ($P < 0.001$, LSD = 0.864), PSG length ($P < 0.001$, LSD = 0.761) and GC width ($P = 0.003$, LSD = 0.217) of sugar beet and spinach measured under optimal conditions. $n$ = 8 sugar beet and 8 spinach.

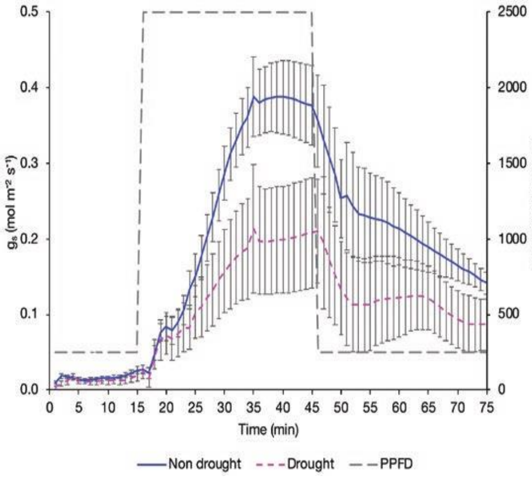

**Figure 7.** The stomatal conductance of non-drought and droughted sugar beet plants exposed to changing PPFD. Plants were exposed to a PPFD of 250 µmol m⁻² s⁻¹ for 15 min, 2500 µmol m⁻² s⁻¹ for 30 min and 250 µmol m⁻² s⁻¹ for 30 min, measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) with measurements logged every minute. These data were used to plot LL.4 curves and estimate stomatal speed. Error bars show SE ±, $n$ = 4 non-droughted and 4 droughted sugar beet.

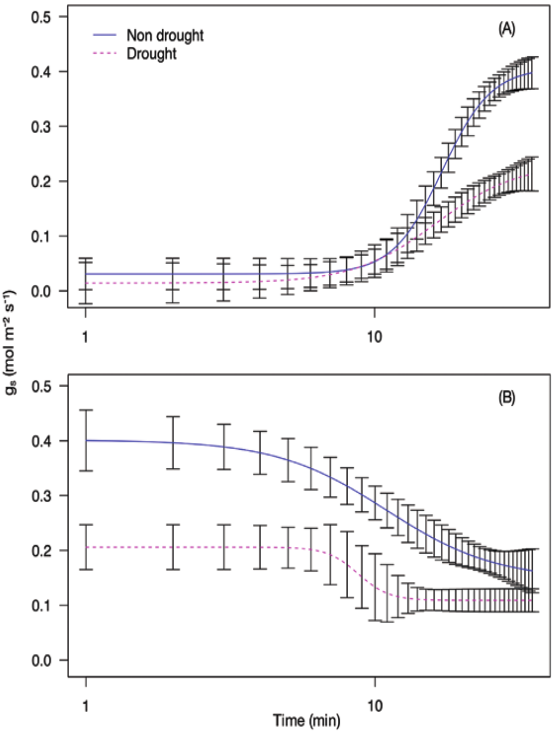

**Figure 8.** The LL.4 curves of stomatal conductance ($g_s$) of non-drought and droughted sugar beet. Stomatal conductance was measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) and fitted using plotted using the DRC package (Ritz *et al.* 2015) in the statistical programming and graphics package R (R Core Team 2019). The plants were exposed to a PPFD of 250 µmol m⁻² s⁻¹ for 15 min, 2500 µmol m⁻² s⁻¹ for 30 min and 250 µmol m⁻² s⁻¹ for 30 min. Curve (A) shows the curve fitted when using the measurements taken during the last 5 min of the initial low light period and the 30-min high light period. Curve (B) shows the curve fitted when using the measurements taken during the last 5 min of the high light period and the 30 min low light period. The curves were identified as being significantly different ($P < 0.001$) using a two-way ANOVA. Error bars show SE ±, $n$ = 4 non-droughted and 4 droughted sugar beet.

with an estimated time of 16.32 ± 2.47 min compared to 17.13 ± 0.71 min for the non-droughted, but with a slower rate of opening of 3.05 ± 1.39 compared to 5.11 ± 1.14 in the non-droughted (Table 2). This slower rate of response was associated with the reduced OE$g_{smax}$ of the sugar beet of 0.23 ± 0.04 mol m⁻² s⁻¹ compared to 0.41 ± 0.02 mol m⁻² s⁻¹ for the non-droughted, which were close to the measured $g_{smax}$ at T45 (Table 2). Returning to low light the droughted sugar beet reacted faster to close stomata with a C$g_s$50 of 8.73 ± 1.44 min and a rate of response of 8.12 ± 9.96 compared to 10.92 ± 2.57 min and 2.28 ± 1.17 for the non-droughted beet (Table 2). The OE$g_{smin}$ and CE$g_{smin}$ values were similar and close to the measured $g_{smin}$ at T11 and T75, respectively (Table 2), highlighting that $g_s$ values were not affected by water stress under low light, but were again estimated to be greater for the closing curve because the plants had acclimated to high light (Table 2).

## Assimilation and WUE$_i$ in droughted sugar beet

There was a trend of reduced $A$ ($P = 0.068$) in droughted sugar beet under high light (T16–T45) and averaged over the entire response curve $g_s$ was significantly lower ($P = 0.023$) in the droughted beet (Fig. 9A). This resulted in a lower average ratio of $g_s$ to $A$ and therefore a trend ($P = 0.083$) of higher WUE$_i$ in the droughted beet compared to the non-droughted beet from T26 onwards, meaning that the decline in $g_s$ was not proportional with the decline in $A$ (Fig. 9B).

There was no significant difference in the performance of PSII in the droughted sugar beet despite water stress, with no

**Table 2.** Estimated $g_s$ parameters from LL.4 curves of non-droughted and droughted sugar beet exposed to stepwise changes in light to induce stomatal opening (250 to 2500 µmol m$^{-2}$ s$^{-1}$ PPFD) and closing (2500 to 250 µmol m$^{-2}$ s$^{-1}$ PPFD), with measured $g_{smin}$ and $g_{smax}$ values for comparison. The average LL.4 curves of non-droughted and droughted sugar beet, plotted from four replicates each, were analysed using two-way ANOVA and shown to be significantly different ($P < 0.001$).

| | | Non-drought | | | Drought | | |
|---|---|---|---|---|---|---|---|
| Parameter | Units | Output | SE | *P*-value | Output | SE | *P*-value |
| Opening | | | | | | | |
| OE$g_{smin}$ | mol m$^{-2}$ s$^{-1}$ | 0.03 | 0.01 | <0.001 | 0.01 | 0.02 | ns |
| OE$g_{smax}$ | mol m$^{-2}$ s$^{-1}$ | 0.41 | 0.02 | <0.001 | 0.23 | 0.04 | <0.001 |
| O$g_s$50 | min | 17.13 | 0.71 | <0.001 | 16.32 | 2.47 | <0.001 |
| Slope | | 5.11 | 1.14 | <0.001 | 3.05 | 1.39 | <0.001 |
| T11 $g_{smin}$ [a] | mol m$^{-2}$ s$^{-1}$ | 0.02 | 0.00 | – | 0.01 | 0.00 | – |
| T45 $g_{smax}$ [b] | mol m$^{-2}$ s$^{-1}$ | 0.38 | 0.05 | – | 0.21 | 0.07 | – |
| Closing | | | | | | | |
| CE$g_{smin}$ | mol m$^{-2}$ s$^{-1}$ | 0.15 | 0.05 | <0.001 | 0.11 | 0.01 | <0.001 |
| CE$g_{smax}$ | mol m$^{-2}$ s$^{-1}$ | 0.40 | 0.03 | <0.001 | 0.21 | 0.02 | <0.001 |
| C$g_s$50 | min | 10.92 | 2.57 | <0.001 | 8.73 | 1.44 | <0.001 |
| Slope | | 2.28 | 1.17 | ns | 8.12 | 9.96 | ns |
| T75 $g_{smin}$ [c] | mol m$^{-2}$ s$^{-1}$ | 0.14 | 0.01 | – | 0.09 | 0.03 | – |

[a] Measured $g_{smin}$ at T11 (pre-high light).
[b] Measured $g_{smax}$ at T45 (during high light).
[c] Measured $g_{smin}$ at T75 (post-high light).

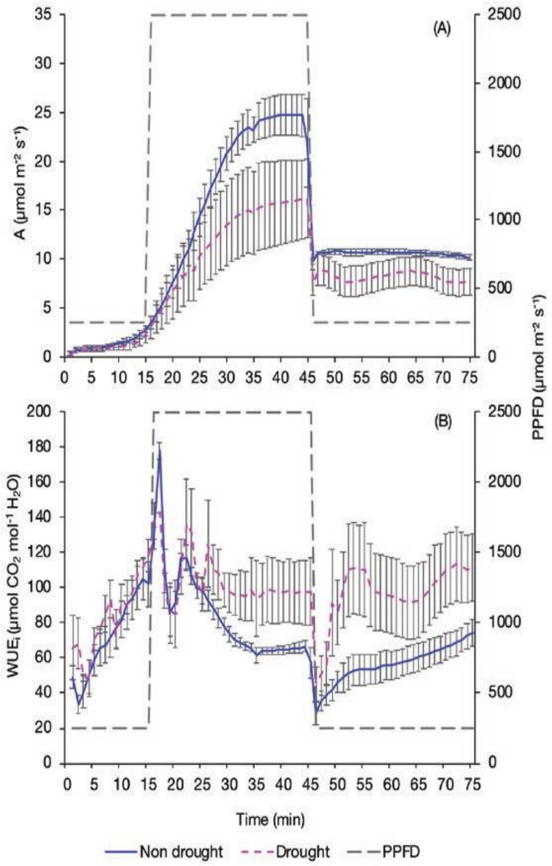

**Figure 9.** The assimilation (A) and WUE$_i$ (B) of non-drought and droughted sugar beet plants exposed to changing PPFD. Plants were exposed to a PPFD of 250 µmol m$^{-2}$ s$^{-1}$ for 15 min, 2500 µmol m$^{-2}$ s$^{-1}$ for 30 min and 250 µmol m$^{-2}$ s$^{-1}$ for 30 min, measured using an infrared gas analyser (LI-6800, LI-COR, Lincoln, NE, USA) with measurements logged every minute. Error bars show SE ±, *n* = 4 non-droughted and 4 droughted sugar beet.

significant differences in $F_v'/F_m'$, ΦPSII or $q_p$ ($P > 0.05$) between the non-droughted and droughted sugar beet.

No significant differences in NPQ$_t$ were evident between the non-droughted and droughted sugar beet but NPQ$_t$ did significantly increase ($P < 0.001$) under high light and decease under the subsequent low light [**see** Supporting Information—Fig. S2B] as was evident as in the beet and spinach comparison.

### The use of dose–response package to fit LL.4 curves to characterize stomatal opening

The fitting of an LL.4 curve using the dose–response package provided a quantifiable comparison between the sugar beet and spinach responses and is similar to the approach of Drake *et al.* (2013). The stability of the control of VPD and air temperature at the low light and high light levels prevented VPD being a factor in the stomatal response and ensures that light alone was the driver of stomatal control in the plants studied. There are little published data on spinach and beet $g_s$ but the OE$g_{smax}$ of 0.45 mol m$^{-2}$ s$^{-1}$ is identical to the control values produced by Downton *et al.* (1985) when assessing spinach responses to salinity. Whilst the OE$g_{smax}$ values for sugar beet are supported by the results of Katerji *et al.* (1997) who identified $g_{smax}$ at 0.46 mol m$^{-2}$ s$^{-1}$ which is close to the 0.48 mol m$^{-2}$ s$^{-1}$ estimated here. The OE$g_{smin}$ values are consistent with the values published for C$_3$ plants by (Flexas *et al.* 2002) and for spinach by the observations of Delfine *et al.* (1998). The use of these values as the upper and lower limit to estimate the speed of stomatal responses both with regards to opening and closing is therefore justified.

## Discussion

### The response of sugar beet and spinach to changes in light intensity

Sugar beet had a high SD and small SS which may have contributed to fast stomatal responses to changes in light intensity, enabling $g_{smax}$ and $A_{max}$ to be reached more rapidly than in spinach, and a reduced disconnect between $g_s$ and $A$. Therefore, the hypothesis that that sugar beet has slow stomatal responses attributed to a low SD and large SS is rejected.

As sugar beet stomata were faster to open in response to light compared to spinach, high levels of transpiration were quickly reached (Fig. 2). This coupled with the use of osmotic adjustment as $\Psi_L$ falls (McCree and Richardson 1987), as opposed to stomatal closure, may contribute to making the plant highly susceptible to wilting. The key role that transpiration plays in sugar beet wilting is supported by the findings of Kohl and Cary (1969) who observed that high light drives wilting in sugar beet and that wilting severity can be reduced by constant mist irrigation throughout the day. The high rate of transpiration is also likely to be coupled with other traits which prevent adequate water uptake to maintain leaf turgor, such as mesophyll thickness and leaf vein arrangement (Sack and Holbrook 2006), which also supports the observations of wilting in the field, even when water is freely available. Therefore, it is not large, slow stomata leading to excessive water loss during stomatal closure but small, fast-opening stomata, with a greater magnitude of response under transient light than the spinach, which enables high rates of transpiration and photosynthesis and is likely to be a driver of wilting in sugar beet. Additionally, as VPD was kept stable at high light but $g_s$ increased it is evident that light is a strong driver of stomatal responses in sugar beet, especially it is less responsive to reductions in $\Psi_L$ due to its anisohydric behaviour. This may be relevant to sugar beet's requirement for high rates of biomass production driven by high rates of photosynthesis. Under adequate water and high light, photosynthesis is often limited by the amount of photosynthetic components per unit leaf area, especially the enzyme Rubisco (Evans 1986). High stomatal conductance values are needed to drive these high assimilation rates, perhaps further increasing the likelihood of wilting.

The ability of sugar beet to reach $A_{max}$ and $g_{smax}$ faster than spinach alongside the increase in transpiration and the concurrent levels of high $WUE_i$ suggest that, even though sugar beet wilts under high light levels, the plant is maximizing its use of the available resources (Mrad *et al.* 2019). Anisohydric woody species have previously been shown to have fast stomatal responses to light but at a cost of reduced $WUE_i$ (Meinzer *et al.* 2017), but in this study the sugar beet $WUE_i$ was comparable to the isohydric spinach despite faster stomatal responses as the balance between $g_s$ and $A$ was maintained and excessive $g_s$ minimized. Plants that osmotically adjust have greater tolerance to water stress and this contributes to the ability of the plant to maintain photosynthetic performance, even when stomata remain open and $\Psi_L$ falls (Ludlow 1987). In addition to this, a high rate of transpiration leads to evaporative cooling which initially protects the plant's photosynthetic apparatus (Franks and Beerling 2009) before wilting. In comparison, the spinach is conserving water through a slower response but is not able to maximize the rate of $A$. Within the 30 min of high light intensity spinach only achieved 80 % of $g_{smax}$, while sugar beet achieved 96 %. In the field, light intensity can constantly fluctuate due to the movement of clouds and the sun's relative position throughout the day. The response of the beet may be optimal in these conditions as it is able to quickly open stomata to maximize $A$ whilst closing rapidly to reduce the disconnect between $A$ and $g_s$ (Lawson and Weyers 1999; Lawson *et al.* 2010; McAusland *et al.* 2016; Vialet-Chabrand *et al.* 2017). Conversely, the spinach would not respond fast enough to maximize its use of the higher light intensity in rapidly changing light conditions. On a consistently bright day, however, the spinach's more conservative response may be optimal to conserve water and reduce the likelihood of water stress throughout the day.

To ensure the anisohydric response and subsequent wilting is not detrimental to plant survival, sugar beet must maximize carbon fixation. The rise in $A$ in response to an increase in PPFD, termed photosynthetic induction, is the summation of a combination of processes, including (but not limited to) the rate of Rubisco activation and stomatal opening (Kaiser *et al.* 2016). Rapid induction requires efficient photosynthesis to optimize light capture and maximize carbon fixation. Sugar beet demonstrated significantly higher maximum ($F_v'/F_m'$) and operating (ΦPSII) PSII efficiency when compared to spinach at both high and low PPFD. This is also evident in the higher values of $q_p$ in sugar beet when recovering from the exposure to high light which demonstrates a greater proportion of open reaction centres in sugar beet, suggesting lower levels of NPQ investment (Murchie and Lawson 2013). Lower investment in NPQ means sugar beet is vulnerable to photoinhibition but avoids over protection of PSII, and is therefore capable of high photosynthesis rates and productivity (Kromdijk *et al.* 2016). This may be optimal for sugar beet as it is biennial, so needs to be highly productive for fast growth, and is adapted to latitudes away from the equator where PPFD is reduced and therefore photoinhibition rates are lower compared to latitudes closer to the equator.

There is a negative correlation between SS and SD across many species and conditions (Franks *et al.* 2009; Doheny-Adams *et al.* 2012) with more, smaller stomata enabling a greater rate of passage of $CO_2$ into the mesophyll for assimilation as the length of the diffusion pathway is reduced (Franks and Farquhar 2007). However, in this study the $g_{smax}$, from both the $OEg_{smax}$ from the LL.4 model and the theoretical $g_{smax}$ calculated from the stomatal anatomy is estimated to be only 0.03 mol $m^{-2}$ $s^{-1}$ higher in the sugar beet than the spinach. This suggests that it is a difference in the speed of stomatal opening, rather than the SD, that drives the difference observed between the beet and spinach in response to the changes in light intensity (Vialet-Chabrand *et al.* 2017). This is further supported by the sugar beet stomata being smaller, which enables them to react faster, as less ions and water movement is needed to drive changes in GC turgor (Hetherington and Woodward 2003; Drake *et al.* 2013). Additionally, when the SD and SS were used to calculate maximum conductance, using the Franks *et al.* (2009) model, there was no significant difference in the estimated maximum stomatal conductance between the sugar beet and spinach supporting the $Eg_{smax}$ and $Eg_{smin}$ from the LL.4 curves. The ability of spinach to reach a similar $g_{smax}$ could be explained by the greater SS leading to a slower stomatal response but larger maximum stomatal aperture, but this relationship is not present in all species (Büssis *et al.* 2006; Doheny-Adams *et al.* 2012; Monda *et al.* 2016).

## The effect of water stress on the response of sugar beet to changes in light

Water stress altered the speed of stomatal response with slower opening and faster closing, compared to the well-watered plants which increased $WUE_i$ at the expense of carbon fixation, as hypothesized. However, the magnitude of the stomatal response in the droughted sugar beet was greater than expected.

The reduction in $g_{smax}$ in the droughted beet shows that the maximum stomatal opening, or the stomatal conductance under any given PPFD, is lower in water-stressed plants. The reduction in $g_s$ also limits $A_{max}$ as the rate of $CO_2$ uptake is reduced as ribulose biphosphate synthesis can be inhibited (Tezara *et al.* 1999). The results of Ober *et al.* (2005) also show a reduction in the observed maximum assimilation rate $A_{max}$ under drought across genotypes, with evidence of reductions greater than 50 %, whilst in this study the average reduction in $A_{max}$ was 44 % under drought. The slower stomatal response under drought and relatively faster closing than opening has also been observed in French beans (*Phaseolus vulgaris*) and was driven by a greater sensitivity to plant $\Psi_L$ (Barradas *et al.* 1994) which was not assessed in this experiment and the driver in sugar beet may be different due to anisohydry and the reduced sensitivity to $\Psi_{L,}$ which could be explored further. As VPD was kept stable it is evident that water-stressed sugar beet reduce the magnitude of the stomatal response to changes in light compared to non-water-stressed beet. The observation that there was still a response from the droughted sugar beet to the high light shows that, even under severe water stress, where wilting was evident, the plant is still able to respond to environmental changes and effectively photosynthesize. The ability of the droughted plants to maintain a similar $F_v'/F_m'$, ΦPSII and $q_p$ to the non-droughted also shows that in sugar beet wilting is not necessarily detrimental to PSII and therefore the photosynthetic apparatus of the plant. This may be linked to sugar beet's anisohydric response, enabling photosynthesis to continue as $\Psi_L$ declines. The reduction in $g_s$ and *A* causes the ratio of the gradients for $CO_2$ uptake and $H_2O$ loss to increase which also leads to increases in $WUE_i$. Therefore, reducing stomatal aperture will lead to increases in $WUE_i$ which are beneficial under drought to make the most of any available water, and have been previously reported in sugar beet (Rytter 2005; Bloch *et al.* 2006; Topak *et al.* 2011).

In the UK, intermittent rather than terminal drought is common (Jaggard *et al.* 1998). The ability of sugar beet to respond to light, even when drought-stressed, is therefore beneficial as further water stress due to transpiration, as the stomata open for $CO_2$ uptake, is less risky in an intermittent drought than a terminal drought. The fact that drought stress is rarely terminal in the UK also suggests that the wilting response previously mentioned is not necessarily detrimental to the crop, as the temperate climate will enable rapid recovery, whilst the plant has maximized its use of the available light for carbon gain.

### Can we optimize the stomatal response of sugar beet?

Both the rapid response of sugar beet to high light and its ability to respond to light even when severely drought-stressed may be attributed to its ancestry. Sugar beet is descended from sea beet which is found across Europe. A study by Ribeiro *et al.* (2016), demonstrated the ability of some sea beet plants, found in differing environments in Portugal, to rapidly recover from severe drought and salinity stress. The greater level of allelic diversity in the sea beet suggests that the rapid response of commercial sugar beet, as shown in this study, could be changed through introgressing traits from the more conservative wild types. In addition to this, differences in drought tolerance and associated traits are evident, even within the current commercial sugar beet varieties (Ober *et al.* 2004, 2005; Luković *et al.* 2009; Rajabi *et al.* 2009; Schickling *et al.* 2010) and may provide another avenue to identify plants which have different levels of stomatal control. As discussed earlier, a more conservative sugar beet may be more productive in water-limited conditions, such as dry years in the UK where losses of up to 25 % (Jaggard *et al.* 1998) are evident and other areas of cultivation in Europe (Jones *et al.* 2003) and the USA (Cooley *et al.* 2015).

## Conclusions

Sugar beet responded more rapidly to increased light than spinach, likely due to smaller stomata. However, the lower SD and greater SS was not a limitation to the $OEg_{smax}$ of the spinach. The ability of sugar beet to react quickly compared to spinach enables $A_{max}$ and $g_{smax}$ to be reached rapidly but this may result in high levels of water loss through transpiration which, coupled with the anisohydric response, could drive wilting. Although this response may not be optimal when the weather is consistently dry, as soil water is used up rapidly, terminal drought is not usually of concern in most countries that cultivate sugar beet. The ability of sugar beet to maintain a low level of *A*, even when drought-stressed and without damage to the photosystems, also highlights its suitability to the short-term drought events common in many areas of cultivation. As the climate changes, and prolonged dry periods become more frequent, it may be necessary to utilize sea beet traits to breed more water conservative commercial sugar beet varieties.

## Supporting Information

The following additional information is available in the online version of this article—

**Figure S1.** The VPD of non-drought and droughted sugar beet plants (A) and droughted and non-droughted sugar beet (B) exposed to changing PAR of 250 μmol $m^{-2}$ $s^{-1}$ for 15min, 2500 μmol $m^{-2}$ $s^{-1}$ for 30min and 250 μmol $m^{-2}$ $s^{-1}$ for 30min, with measurements logged every minute and measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebaska, USA). (A) n = 8 Sugar beet and 8 spinach, (B) n = 4 non-droughted and 4 droughted sugar beet. Error bars show standard error.

**Figure S2.** The NPQt of non-drought and droughted sugar beet plants (a) and droughted and non-droughted sugar beet (b) exposed to changing PAR of 250 μmol $m^{-2}$ $s^{-1}$ for 15min, 2500 μmol $m^{-2}$ $s^{-1}$ for 30min and 250 μmol $m^{-2}$ $s^{-1}$ for 30min, with measurements logged every minute measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebaska, USA). Error bars show SE±, (a) n = 8 Sugar beet and 8 spinach, (b) n = 4 non-droughted and 4 droughted sugar beet.

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## Conflict of Interest

None declared.

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## Data Availability

The raw data are available as Supporting Information.

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## 2.12 Supporting information – S1

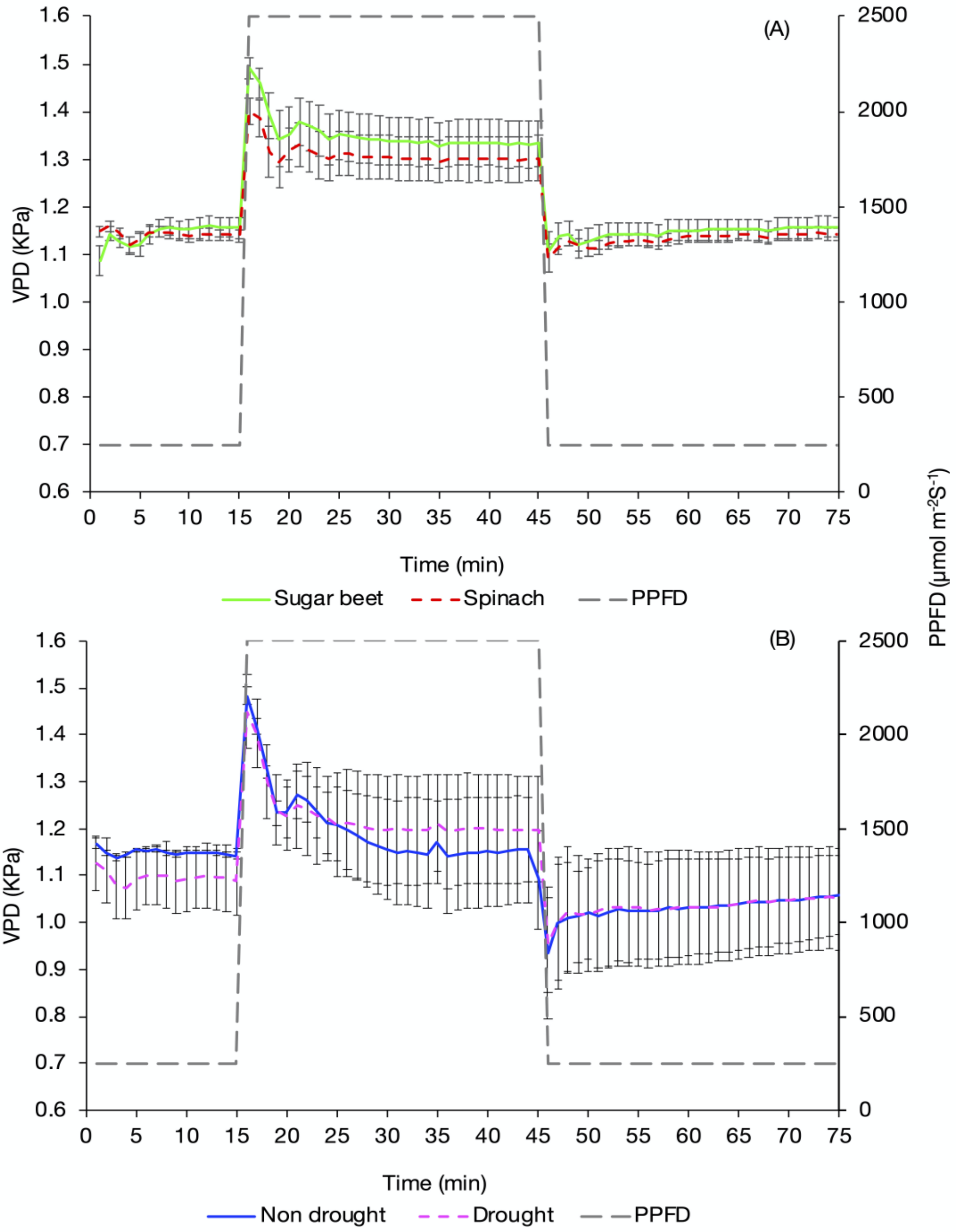

**Figure S1.** The VPD of non-drought and droughted sugar beet plants (A) and droughted and non-droughted sugar beet (B) exposed to changing PAR of 250 µmol $m^{-2}$ $s^{-1}$ for 15min, 2500 µmol $m^{-2}$ $s^{-1}$ for 30min and 250 µmol $m^{-2}$ $s^{-1}$ for 30min, with measurements logged every minute and measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebaska, USA). Error bars show SE±, (A) n= 8 Sugar beet and 8 spinach, (B) n= 4 non-droughted and 4 droughted sugar beet.

## 2.13 Supporting information – S2

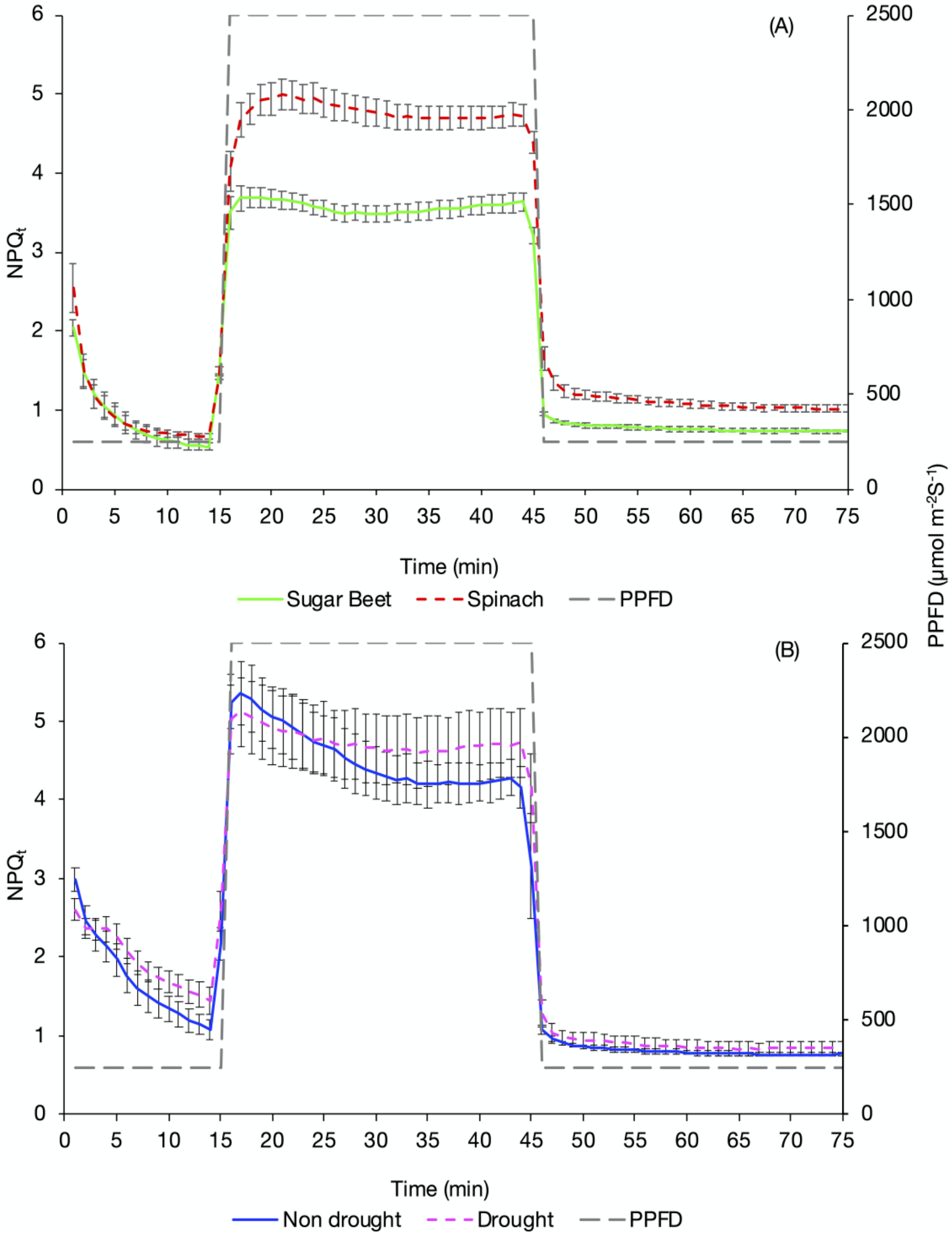

**Figure S2.** The NPQt of non-drought and droughted sugar beet plants (A) and droughted and non-droughted sugar beet (B) exposed to changing PAR of 250 µmol m⁻² s⁻¹ for 15min, 2500 µmol m⁻² s⁻¹ for 30min and 250 µmol m⁻² s⁻¹ for 30min, with measurements logged every minute measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebaska, USA). Error bars show SE±, (A) n= 8 Sugar beet and 8 spinach, (B) n= 4 non-droughted and 4 droughted sugar beet.

# Chapter 3: Water use efficiency in contrasting commercial sugar beet varieties in response to fluctuating water availability

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## 3.1 Abstract

Many areas of sugar beet production will face hotter and drier summers as the climate changes. Studies have examined drought tolerance in sugar beet but water use efficiency (WUE) has been less of a focus. An experiment was undertaken to examine the affect of fluctuating soil water deficits on WUE and identify if sugar beet undergoes any long-term physiological acclimation to water deficit to increase WUE. The study utilised two sugar beet varieties, one with an upright canopy and the other prostrate, to also examine if WUE differs between commercial varieties with contrasting canopy architecture. The sugar beet were grown under four different irrigation regimes (a fully irrigated, single drought, double drought and continually water limited) in large 610L boxes in an open ended polytunnel. Measurements of leaf gas exchange, chlorophyll fluorescence, relative water content (RWC) and SPAD were regularly undertaken and stomatal density, sugar and biomass yields and the associated WUE, specific leaf weight and $\Delta^{13}C$ were assessed. The results showed that water deficits generally increase $WUE_i$ and $WUE_{DM}$, but reduce yield. Sugar beet

recovered fully after severe water deficits, as assessed by the leaf gas exchange and chlorophyll fluorescence parameters and, except for reducing canopy size, showed no other permanent physiological changes due to drought, and therefore no changes in WUE or drought avoidance. The prostrate variety had a greater $WUE_{i,}$ measured using $\Delta^{13}C$, and traits associated with more water conservative phenotypes of a lower stomatal density and greater leaf RWC, whilst SPAD and leaf chlorophyll content changed inconsistently under water deficit and the relationship with WUE was unclear. The difference in $WUE_i$ between the two varieties suggests traits associated with greater $WUE_i$ may be linked to canopy architecture. However, the two varieties had a similar SLW, despite a previous observation that a higher SLW was associated with a lower $\Delta^{13}C$ and therefore higher $WUE_i$, suggesting that SLW is not a trait that can be used when selecting for sugar beet with a greater $WUE_i$.

## 3.2 Introduction

Climate change is causing hotter and drier summers in many areas of Europe (David, 2017) with crop yields increasingly limited by water availability (Angert et al., 2005). A sufficient supply of water is crucial to maximising plant yield because dry matter (DM) accumulation is directly proportional to water use in many environments, as solar radiation drives both photosynthesis and transpiration (Tanner and Sinclair, 1983). The relationship between photosynthesis and transpiration, known as water use efficiency (WUE) can be assessed at a range of scales from the leaf level, by assessing carbon uptake in relation to stomatal conductance ($g_s$), known as intrinsic water use efficiency ($WUE_i$) (Farquhar et al., 1989), to the crop level, by calculating the ratio of DM accumulated to water used by the crop ($WUE_{DM}$) (Boyer, 1996). In crops, WUE can be further defined to consider only the DM of the harvested product (Fageria et al., 2006). Increasing WUE can be achieved through manipulation of three key processes which operate from the leaf to the crop level, (i) reducing water loss, for example through soil evaporation and water passing beyond the root zone, (ii) reducing the rate of transpiration to carbon fixation and (iii) increasing the harvest index (Condon et al., 2002). A large number of traits are associated with these processes and those that influence photosynthesis and stomatal anatomy are of particular interest (Leakey et al., 2019). Environmental factors are also key to WUE with light, temperature and soil water availability affecting plant water use and carbon fixation (Hatfield and Dold, 2019). The relationship between carbon fixation and water use means that increasing crop WUE can be a trade-off between photosynthesis and transpiration (Blum, 2005). Despite this trade-off there has been success in breeding commercial wheat varieties which are more efficient in their use of water, without reducing yield potential under optimal conditions (Condon et al., 2002, Condon et al., 2004). This was achieved through identifying differences in carbon isotope discrimination ($\Delta^{13}C$) which is inversely related to $WUE_i$ (Farquhar and Richards, 1984) and allowed breeding for specific environments with low yield potential and water conservation is required. As breeders move away from focusing solely on maximising yield and look at traits which increase crop resilience, including those associated with WUE, it is important to understand if there are differences in such traits in sugar beet. Although there has been extensive work on drought tolerance in sugar beet (Ober and Luterbacher, 2002, Ober et al., 2004, Ober et al.,

2005) $WUE_i$ was not assessed and may be a useful trait when developing more WUE varieties for the regions in which climate change is leading to reduced water availability.

Sugar beet varieties are developed for a range of markets around the world, from the dry climates of the Middle East to the temperate climates of Europe and North America (Morillo-Velarde and Ober, 2008). Although there are many studies looking at the effects of irrigation on sugar beet in drier climates (Mohammadian et al., 2005, Topak et al., 2011, Li et al., 2019, Hassanli et al., 2010), in much of Europe, irrigation is not economically feasible (Řezbová et al., 2013) and sugar beet WUE must be increased to maximise the use of rainfall to reach the crop's full yield potential (Hoffmann and Kenter, 2018). Despite a maritime ancestry, which makes sugar beet more drought tolerant than many major crop species (Dunham, 1993), yield losses are still evident under drought with unirrigated losses in Europe ranging from 15-40% depending on the regional climate and soil type (Pidgeon et al., 2001). Significant variations in drought tolerance have been identified within the sugar beet germplasm and Beta genebank accessions driving further work to understand the level of drought tolerance in breeders' lines (Ober and Luterbacher, 2002). The work of Ober et al. (2004) compared sugar beet breeding lines and varieties grown for a range of climates to look at the drought tolerance index (DTI) (the fraction of irrigated yield maintained under drought, normalised by the mean yield across all genotypes in the trial) which was shown to be significantly different between genotypes. Genotype*environment interactions have been shown to significantly affect sugar beet yield (Hoffmann et al., 2009) and phenotypic differences related to drought tolerance, which may be observed when water is freely available, are not always as evident under drought (Ober et al., 2004). This highlights that traits must be tested under a range of conditions to fully understand how they may influence sugar beet WUE. However, the absence of any developmental stages which are more greatly affected by drought stress in sugar beet means that the timing of drought does not have an impact on final yield, and therefore the relationship between water use and yield is consistent regardless of drought timing (Dunham, 1993). Indirect canopy traits have a strong influence on DTI with greater green canopy maintenance, low wilting and senescence score, specific leaf weight (DW/total sampled leaf area) and succulence index (FW-DW/total sampled leaf area) all enhancing DTI (Ober et al., 2005). WUE assessed at the crop level shows significant differences between genotypes, which is driven by increased biomass accumulation rather than reduced water use (Ober et al., 2005). There was no assessment in these studies of $WUE_i$ and associated traits, such as $\Delta^{13}C$, and whether these could be correlated with DTI. $\Delta^{13}C$ is strongly correlated with $WUE_i$ (Farquhar and Richards, 1984) and provides an integrated measure of $WUE_i$ over time which is more reliable than direct leaf gas exchange measurements, which can be influenced by the environment at the time of measurement.

Few studies have considered $\Delta^{13}C$ in sugar beet but genotypic variations in $\Delta^{13}C$ have been evidenced and a relationship between SLW and $\Delta^{13}C$ identified, with a greater SLW equating to an increased $\Delta^{13}C$ and $WUE_i$ in breeding lines and hybrids (Rajabi et al., 2008). SLW was also correlated with DTI, suggesting that there may be a relationship between drought tolerance traits and WUE. Additionally, it has been shown that variation in $\Delta^{13}C$ is greater in conditions where water is not limiting as opposed to under drought (Rytter, 2005, Rajabi et al., 2009). This shows the importance of assessing $\Delta^{13}C$ under both irrigated and droughted conditions to ensure that the relationship with $WUE_i$ is understood. Sugar beet $\Delta^{13}C$ has also been used to show the increase of $WUE_i$ under drought as stomatal aperture is reduced. The decline in $\Delta^{13}C$ between well-watered and droughted treatments equated to a 24% increase in WUE, and the same study highlighted that leaf $\Delta^{13}C$ in sugar beet is a better measure of WUE than root $\Delta^{13}C$ (Bloch et al., 2006).

The diversity in sugar beet drought tolerance and $\Delta^{13}C$ in breeding lines and hybrids shows that sugar beet genotypes exist that are more efficient in their use of water. However, the relationship between $WUE_i$ and traits associated with drought tolerance has not been explored. The consistency of SLW in both increased DTI and $\Delta^{13}C$ suggest that canopy traits are closely associated with sugar beet $WUE_i$ and should be a key area of focus. In cereal crops it has been shown that canopy architecture can also affect $\Delta^{13}C$ with more erect leaves having a greater $\Delta^{13}C$ and achieving greater yields but the effect on WUE was not assessed (Araus et al., 1993). Sugar beet canopies can be classed as upright or prostrate and no research has been conducted on whether canopy architecture in sugar beet is related to WUE.

There is limited research on how leaf position within the canopy can affect stomatal morphology in crop species. However, there have been a number of studies examining the effect of canopy positioning on stomatal and leaf morphology in tree canopies. It has been shown that SD varies between sun and shade leaves and that this is driven by differences in cell differentiation as the leaf develops in Black alder (*Alnus glutinosa* L.) (Poole et al., 1996), opposed to differing rates of leaf expansion which can sometimes be attributed to differences in SD between sun and shade leaves (Boardman, 1977). There is evidence that leaf morphology can differ depending on the position of a leaf within the canopy (Van Wittenberghe et al., 2012) with comparison of 12 popular (Populus) genotypes showing the leaves higher in the canopy had a higher stomatal density and greater specific leaf area driven by a greater leaf thickness (Afas et al., 2007). Additionally, canopy structure can affect the environment experienced by the leaf (Baldocchi et al., 2002, Van Wittenberghe et al., 2012, Burgess et al., 2017), and differences in light intensity and $CO_2$ levels can also alter stomatal density and size. It has been observed in Tomato (*Solanum lycopersicum*) that leaves exposed to greater light intensities have a greater SD to enable greater maximum rates of photosynthesis (Gay and Hurd, 1975) and that SD decreases under increasing $CO_2$ in Arabidopsis (Woodward et al., 2002). These observations mean it could be feasible that differences in light intensity and $CO_2$ around leaves, driven by canopy architecture, could result in different stomatal morphology between sugar beet varieties with contrasting upright or prostrate canopies.

Previous work has been focused on the Beta genebank and breeders' lines rather than elite commercial varieties. So far, differences in traits associated with greater $WUE_i$ have not been detected in elite varieties which, if identified, would show that greater $WUE_i$ in sugar beet is a commercially viable trait for breeders to target. Although work is being undertaken to identify traits in wild sea beet populations, which may be introgressed into commercial varieties (Ribeiro et al., 2016), progress is slow as it relies on traditional breeding techniques. Therefore, it is useful to explore whether differences in $WUE_i$ and associated traits are evident in commercial varieties (Davis, 2006). If differences are identified, this would show that increased $WUE_i$ is already a viable trait in commercial sugar beet crops.

Therefore, in this study, two elite UK sugar beet varieties, with contrasting upright and prostrate canopies, have been selected to answer the research questions:

1. How is WUE affected by fluctuations in soil water availability in sugar beet?

   *Hypothesis: Sugar beet Intrinsic water use efficiency ($WUE_i$) and dry matter water use efficiency ($WUE_{DM}$) increase under water deficit.*

2. Does sugar beet acclimate (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to water deficit to increase WUE?

   *Hypothesis: Sugar beet shows no long-term physiological acclimation in response to water deficit due to its anisohydric behaviour.*

3. Are there differences in WUE between commercial sugar beet varieties with contrasting canopy architecture and does stomatal and leaf morphology vary between these varieties?

   *Hypothesis: Contrasting canopy architecture leads to differences in WUE and can be attributed to differences in stomatal and leaf morphology.*

## 3.3 Materials and methods

### 3.3.1 Box set up and plant materials

Two experiments were conducted in 2018 and 2019 using sugar beet varieties from different breeders, one with a prostrate canopy (cv. Cayman) (Prostrate) and the other with an upright canopy (cv. Sabatina) (Upright). To simulate a realistic canopy environment sugar beet was grown in plastic pallet boxes with a volume of 610 L, depth of 60 cm and surface area of 1.1 m$^2$. Boxes had drainage holes drilled in the bottom with membrane overlaid and filled with a sandy clay loam, (Landscape20, Topsoil, Peterborough, Cambridgeshire, UK). The boxes were filled in four stages and hand watered, for a fixed period of time in between each stage, to settle the soil. Boxes were filled to 15 cm, 45 cm then 60 cm and left for a minimum of 6 days before further filling, with a final top up to ensure soil was 60 cm in depth. Volumetric water sensors (ECH$_2$O EC-5, Meter group Inc, Pullman, Washington, USA) were buried 15cm from the bottom of the box in 2018 whilst in 2019 larger sensors (ECH$_2$O 10HS, Meter group Inc, Pullman, Washington, USA) were buried at 30 cm to get a reading over a larger soil volume of 1320 ml compared to 240 ml in 2018. Sensors were calibrated specifically to the soil used, as directed by the manufacturer’s protocol. Volumetric water content (VWC) was logged to a data logger every hour (Em5b, Meter group Inc, Pullman, Washington, USA). To enable full control of the water applied, the boxes were placed in an open ended polytunnel without environmental controls orientated East to West and covered in a diffuse polythene (SunMaster Diffused, XL Horticulture LTD, Ottery St Mary, Devon, UK) in 2018 and a clear polythene (SunMaster Clear, XL

Horticulture LTD, Ottery St Mary , Devon, UK) in 2019. In both years, the boxes were arranged in a split plot design, with watering regime on the main plot and variety on the sub plot. Measurements were taken on 32 boxes divided into four blocks of eight with discard boxes at the end of each row to ensure measurement boxes were part of a continuous canopy. A temperature and humidity sensor was suspended at canopy height and logged measurements every hour (TinyTag Ultra 2, Chichester, West Sussex, UK). In 2019, an additional sensor was suspended at the end of blocks 2 and 4 to identify if a temperature gradient was present but no differences were identified. Thermal time was higher in 2019 than 2018 with increased thermal time most evident in August and September (Supplementary Fig.S1).

### 3.3.2 Sowing and establishment

To ensure an optimal seedbed the boxes were raked to produce a fine tilth. Seeds were sown to represent, as closely as possible, the spacing of seeds in commercial sugar beet fields in the UK. A plywood board with holes drilled for correct seed spacing (Supplementary Fig.S2) was placed over the box and three seeds sown in each hole. Seeds were sown in three rows with 4 sowing locations per row with a row spacing between plants of 30 cm and to the edge of the box 15.5 cm and spacing within rows of 28 cm between plants and 13.5 cm to the edge of the box. Boxes were hand watered at regular intervals, and timed to ensure equal watering, to prevent soil drying and ensure good establishment. Plants were thinned once two true leaves were evident to give a total of 12 plants per box. Boxes were fertilised with ammonium nitrate in 2018 and ammonium sulphate in 2019, using a split application equating to 40 kg N $ha^{-1}$ followed by 80 kg N $ha^{-1}$. After each application of fertiliser, the boxes were watered equally. In 2018 and 2019 the seeds were sown on 9 April, in 2018 fertiliser was applied at 15 and 29 DAS with thinning at 32 DAS, whilst in 2019 fertiliser was applied at 16 and 29 DAS with thinning at 29 DAS.

### 3.3.3 Irrigation

An irrigation system consisting of drip irrigation pipe was installed after emergence with three lengths of pipe running between the sugar beet rows (Supplementary Fig.S2). Each subplot, consisting of two boxes, had a tap to switch irrigation on or off. Water was fed through a meter so the amount applied to the system could be measured. Four irrigation treatments were managed using this system; a fully irrigated control (Full), a single drought (SD), a double drought (DD) which had the SD treatment plus a second period of drought and a water limited treatment (Ltd) which was kept at approximately 50% field capacity. Irrigation was typically applied every 2-3 days in a 7-day period. The second water withdrawal for the DD started when the maximum rate of assimilation ($A_{max}$) returned to a level similar to the fully irrigated plants. Boxes were irrigated back to field capacity immediately at the end of a water withdrawal period. Timings were comparable between the two years, except for the DD which was later in 2018 than 2019. The first water withdrawal was at 65-96 DAS in 2018 and 73-92 DAS in 2019 and the second at 151-200 DAS in 2018 and 129-148 DAS in 2019. In 2018 the total amount of water applied per box was; Full – 110.9L, SD- 102.1L, DD-75.9L and Ltd- 41.3L and in 2019; Full – 102L, SD- 90.1L, DD-83.6L and Ltd- 28.9L. Soil moisture was monitored using the VWC sensors in 2018 and 2019 (Supplementary Fig.S3a and S3b) and irrigation adjusted accordingly.

### 3.3.4 Leaf gas exchange and chlorophyll fluorescence

Gas exchange measurements of maximum assimilation ($A_{max}$) and stomatal conductance ($g_s$) were taken using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA) and used to calculate $WUE_i$ (Condon et al., 2002):

$$WUE_i = \frac{A}{gs} \tag{1}$$

The two sugar beet located centrally in each box (6 and 7, Supplementary Fig.S2) were measured to ensure consistency. Settings were: flow 500 µmol $s^{-1}$, heat exchanger temperature 20°C (which gave a leaf temperature between 20°C and 28°C dependent on ambient conditions), RH 50%, light 1200 µmol $m^{-2}$ $s^{-1}$, and matched every 10 minutes. Chlorophyll fluorescence parameters of $F_v'/F_m'$ (maximum PSII efficiency in the light), ΦPSII (quantum efficiency of PSII electron transport in the light) and $q_p$ (photochemical quenching) were measured at the same time using a multiphase flash fluorometer (Li6800, LI-COR, Lincoln, Nebraska, USA) and a dark pulse (Murchie and Lawson, 2013). Data was logged once A, $g_s$, $CO_2$ sample and $H_2O$ sample were stable, which took between 5-10 minutes per leaf. Two leaves from each plant, totalling four leaves per box, were focused on in each year to cover each drought period 'measurement leaf 1' and 'measurement leaf 2'. Measurements started once the leaves were fully expanded with measurement leaf 1 used in 2018 throughout the period 77-133 DAS which includes the first drought from 65-96 DAS and in 2019 this leaf was used throughout 69-110 DAS which includes the drought at 73-92 DAS. Measurement leaf 2 was used in 2018 from 105-210 DAS, which overlaps with measurement leaf 1, to include the second drought at 151-200 DAS and in 2019 at 118-182 DAS to include the drought at 129-148 DAS. In 2019 measurements were also taken of ‘measurement leaf 3’ at 162-182 DAS to correlate with $\Delta^{13}C$, which will be outlined later. To reduce any effect of midday depression of photosynthesis, measurements were taken between 8:00 hr and 14:00 hr over 2 consecutive days with blocks one and two measured on the first day and blocks three and four on the second. For ease the DAS of the first day of measurements is used to denote the timing of the measurement. When gas exchange measurements were completed, thermal images of the canopy of each box were taken at a distance of 1 metre perpendicular to the edge of the box with a handheld camera (FLIR C2 thermal imaging camera, FLIR, Wilsonville, Oregon, USA) and analysed using thermal analysis and reporting software (FLIR Tools, FLIR, Wilsonville, Oregon, USA).

### 3.3.5 SPAD and chlorophyll extraction

SPAD values of the leaves used to measure gas exchange were recorded (SPAD-502, Minolta, Chioyda City, Tokyo, Japan) at regular intervals from 81-212 DAS in 2018 and 71-183 DAS in 2019 with three measurements taken from the leaf tip to give an average value.

In 2019, chlorophyll extraction was undertaken on leaf discs collected at 203 DAS from plants one, two and three (Supplementary Fig.S2). A SPAD measurement was taken and 1mm$^2$ leaf disc sample cut using a cork borer from the same area. Each leaf disc was wrapped in aluminium foil and placed in liquid nitrogen before storage in a -80°C freezer. At the same time three additional leaf discs were cut from each leaf and weighed together to determine FW and oven dried to record DW. This was then used as an indicator of the leaf water content to assess the relationship between leaf chlorophyll concentration SPAD and leaf water content:

$$Water\ content = FW - DW \qquad (2)$$

The frozen leaf discs were added to 2ml microcentrifuge tubes with 1.5ml 80% acetone and a ceramic bead before milling using a fast prep for two 20sec cycles. The extracted chlorophyll was then transferred to 15ml centrifuge tubes and topped up to 4ml using 3ml 80% acetone before centrifuging for 5min at 3000rpm. The spectrophotometer (Cary 50, Agilent, Santa Clara, California, USA) was zeroed using 2ml 80% acetone in the cuvette, which was repeated every four measurements. 2ml of the extracted chlorophyll was added to a cuvette and the absorption value measured at 646.6, 663.6 and 750nm. Chlorophyll *a* and *b* concentrations were then calculated using the equations of Porra (2002).

### 3.3.6 Relative water content

Relative water content (RWC) was measured at regular intervals from 66-213 DAS in 2018 and 74-177 DAS in 2019. Using a cork borer three 1cm diameter leaf discs were cut from a leaf on plants 2 and 3 representative of the new measurement leaf on plants 6 and 7 (Supplementary Fig.S2). The 6 leaf discs were weighed together to give an average fresh weight (FW) and submerged in reverse osmosis purified water for a minimum of 12 hours. Leaf discs were removed and patted dry using paper towel before weighing to record the turgid weight (TW). The 6 leaf discs were then wrapped in aluminium foil and oven dried at 80°C for a minimum of 48 hrs before removal from the foil and weighed to record the dry weight (DW). RWC was then calculated (Weatherley, 1950):

$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100 \qquad (3)$$

### 3.3.7 Stomatal impressions

Stomatal impressions of the abaxial and adaxial leaf surface of the final leaf selected for gas exchange measurements were taken at 219 DAS and 203 DAS in 2018 and 2019 respectively. Clear nail varnish was applied and left to dry for 20 minutes until no longer tacky, lifted using clear tape and mounted on a microscope sample slide. Three images were taken from each sample slide using a microscope (Leica 5000B, Wetzlar, Hesse, Germany) with a light source (Leica CTR5000 Wetzlar, Hesse, Germany) at 100x magnification and cropped to 1mm$^2$ using the microscope scale for reference in Fiji (Schindelin et al., 2012). The stomata in the cropped images were manually counted in Fiji using the Cell Counter plug in (Author: Kurt De Vos), with an average stomatal density (SD) for each sample calculated from the three 1mm$^2$ areas.

### 3.3.8 Harvest

Boxes were harvested at 226 DAS and 211 DAS in 2018 and 2019 respectively. The sugar beet were hand lifted with plants 6 and 7 taken for further analysis. The 10 remaining beet were topped and the leaves and roots weighed separately to determine FW. The canopy was then discarded, and the roots taken in woven polypropylene bags in an unrefrigerated van to the BBRO tare house at Wissington Sugar Beet factory, Norfolk, UK to determine sugar %. The leaves and roots of plants 6 and 7 were combined and weighed to determine FW before oven drying at 70°C and weighed to determine leaf and root dry matter (DM). The %DM of leaves and roots from plants 6 and 7 in each box was used to calculate the total DM from the total FW. The white sugar yield (WS) was calculated by multiplying the total FW by the sugar percentage. The total DM and WS for each box and the total amount of irrigation applied was then used to calculate the box level total dry matter water use efficiency ($WUE_{DM}$):

$$WUE_{DM} = \frac{Box\ total\ DM}{Total\ volume\ of\ water\ applied\ per\ box} \tag{4}$$

and WS water use efficiency ($WUE_{WS}$):

$$WUE_{WS} = \frac{Box\ total\ WS}{Total\ volume\ of\ water\ applied\ per\ box} \tag{5}$$

### 3.3.9 Carbon isotope discrimination

In 2019, measurement leaf 2 and 3 were removed at 209 DAS and freeze dried to determine the ratio of $^{12}C$ to $^{13}C$ (δ13C). Samples were milled (ZM200, RETSCH, Haan, North Rhine-Westphalia, Germany) to a fine homogenised powder and analysed at the British Geological Survey in Keyworth, Nottinghamshire, UK. Leaf $\delta^{13}C$ analyses were performed by combustion in an elemental analyser (ECS4010 4010 CHNSO, Costech, Milan, Lombardy, Italy) on-line to a triple trap and dual-inlet mass spectrometer (VG Optima, Isoprime Inc., Manchester, Greater Manchester, UK) with δ13C values calculated to the Vienna Pee Dee Belemnite (VPDB) scale using a within-run laboratory standards calibrated against NBS 18, NBS 19 and NBS 22. Replicate analysis of well-mixed samples indicated a precision of ±<0.1‰ (1 SD). $\delta^{13}C$ was used to calculate carbon isotope discrimination ($\Delta^{13}C$), which is inversely proportional to WUE (Farquhar et al., 1989):

$$\Delta^{13}C = \frac{\delta_a - \delta_p}{1 + \delta_p} \quad (6)$$

Where $\delta_p$ is the δ13C calculated from the leaf tissue and $\delta_a$ is the atmospheric ratio of $^{12}C$ to $^{13}C$ taken to be -8‰. $\Delta^{13}C$ (Farquhar et al., 1989).

$\Delta^{13}C$ was plotted against average $WUE_i$ on a per leaf basis, (calculated from the gas exchange values taken from measurement leaf 2 at 118, 140, 146, 153, 162, 169,174 and 182 DAS and measurement leaf 3 at 162, 169, 174 and 182 DAS), and DM WUE, WS WUE and SLW using averages calculated on a per box basis.

### 3.3.10 Specific leaf weight

Specific leaf weight (SLW) was calculated from measurement leaf 3, before processing to determine $\Delta^{13}C$, with the leaf passed through a leaf area meter (Li-3100, LI-COR, Lincoln, Nebraska, USA) to determine leaf area with DW calculated from the FW multiplied by the %DW derived from beet 6 and 7 at harvest:

$$SLW = \frac{DW}{Area} \quad (7)$$

### 3.3.11 Statistical analysis

Repeated measures ANOVA for a randomised block design with irrigation as a sub plot and variety and irrigation as factors was conducted on the $A_{max}$, $g_s$, $C_i/C_a$, $WUE_i$, $F_v/'F_m'$, ΦPSII, $q_p$, canopy temperature and RWC data. For the total leaf chlorophyll content, leaf water content, stomatal density, WS yield, Total DM, $WUE_{WS}$, $WUE_{DM}$, SLW and $\Delta^{13}C$ data a general ANOVA with the same parameters as the repeated measures ANOVA was used. All analysis was undertaken in GenStat 19th edition (VSN International Ltd., Hemel Hempstead, Hertfordshire, United Kingdom).

## 3.4 Results

### 3.4.1 Leaf gas exchange and $WUE_i$

To examine the effect of water availability on WUE the data was averaged across the two sugar beet varieties as there were no irrigation treatment*variety interaction. In 2018 measurement leaf 1 and 2 were measured at 105 – 133 DAS, covering the end of the first drought, whilst in 2019 measurement leaf 2 was not measured at the same time as measurement leaf 1, so did not cover the end of the first drought. $A_{max}$ and $g_s$ were significantly reduced in all treatments over time as leaves aged (Fig.1 and 2). Drought reduced $A_{max}$ and $g_s$ compared to the fully irrigated plants during the first drought in 2018 by 92 DAS, 23 days after water withdrawal, (*P<0.001*; Fig.1a and 2a) whilst in 2019 $A_{max}$ was reduced by 81 DAS, only 9 days after water withdrawal (*P<0.001*; Fig.1b) and $g_s$ was reduced, but not significantly, compared to the fully irrigated (Fig.2b). In 2018 there was a slight reduction in $A_{max}$ and $g_s$ in the DD treatment compared to the fully irrigated at 196 DAS but this was not significant (Fig.1c). The much later second drought in 2018 may be attributed to this as transpiration is reduced in cooler months and the thermal time was much lower during the second drought in 2018 than the second drought in 2019 (Supplementary Fig.S1). During the second drought in 2019, $A_{max}$ and $g_s$ were reduced in the DD treatment (*P<0.001*; Fig.2d) at 140 DAS, 11 days after water withdrawal. At 140 and 146 DAS the SD treatment had a higher $A_{max}$ and $g_s$ than the fully irrigated (*P<0.001*; Fig.1d and 2d). During this period temperatures exceeded 40°C from 136 – 140 DAS and the fully irrigated treatment saw a decline in VWC to levels similar to the water limited treatment before additional irrigation was applied at 141 and 142 DAS with this difference no longer evident at 153 DAS.

Recovery from the first drought, when $A_{max}$ was no longer significantly different to the control, in 2018 was at 133 DAS, 37 days after re watering (Fig.1a), and in 2019 was at 109 DAS, 18 days after re watering (Fig.1b). The less frequent measurements in 2018 mean that the speed of recovery was not as closely assessed as in 2019. Due to the lack of significant decline in A and $g_s$ under the second drought in 2018, recovery was only measured in 2019 and this was evidenced at 174 DAS, 46 days after re watering. The dip in $A_{max}$ in the fully irrigated at 174 DAS meant measurements were also taken at 182 DAS to ensure the results at 174 DAS were not anomalous (Fig.1d). The close relationship between $A_{max}$ and $g_s$ means that $g_s$ showed the same trends in recovery as $A_{max}$ (Fig.2). The decline and recovery of $A_{max}$ is further supported by the changes in chlorophyll fluorescence parameters (Supplementary Fig.S4, S5 and S6) of maximum photochemical efficiency in the light, $F_v$/'$F_m$' (Fig.S4), PSII operating efficiency, ΦPSII (Fig.S5), and photochemical quenching, $q_p$ (Fig.S6), which are largely consistent with those expected from a change in $A_{max}$ as a result of stomatal closure.

The continually water limited treatment had a reduced $A_{max}$ and $g_s$ comparable to the droughted treatments during water withdrawal, with the slower reduction in VWC in 2018 (Fig.1a and 2a) meaning the decline in $A_{max}$ and $g_s$ was not as rapid as it was in 2019 (Fig.1b and 2b). Once VWC was maintained at approximately 50% field capacity $A_{max}$ and $g_s$ were lower in the water limited treatment compared to the fully irrigated throughout the measurement periods in both years (*$P<0.001$*; Fig.1 and 2), except from 183 DAS onwards in 2018 (Fig.1c), and $g_s$ at 133 DAS as the fully irrigated leaf $g_s$ had also declined (Fig.2a).

$WUE_i$ was greater in the drought treatments of the first measurement leaf in 2018 with all treatments having a higher $WUE_i$ than the fully irrigated until 133 DAS (*$P=0.004$*; Fig.3a), which could be attributed to a lower relative decline of $A_{max}$ and $g_s$ in comparison as leaves age. The $C_i/C_a$ values show a significant reduction compared the fully irrigated until 133 DAS (*$P=0.006$*; Fig.4a) as stomatal aperture is reduced to conserve water which is also driving the increased $WUE_i$. The spike in $WUE_i$ and $C_i/C_a$ of the SD treatment at 98 DAS seems to be anomalous, with these differences not present at 105 DAS. In 2019 the difference in $WUE_i$ between treatments was not consistent and from 97-110 DAS the Ltd treatment had a lower $WUE_i$ than the fully irrigated, which is the only example of a water deficit treatment having a lower $WUE_i$ than the fully irrigated (*$P=0.003$*; Fig.3b). This was also reflected in the $C_i/C_a$ values (Fig.4b). In 2018 the Ltd treatment had a higher $WUE_i$ at 105 and 113 DAS in the second measurement leaf where it covers the first drought (*$P=0.004$*; Fig.3c). The $C_i/C_a$ also reflects this with the Ltd treatment being lower than the SD and DD treatment which were themselves lower than the fully irrigated (*$P=0.004$*; Fig.4c). At 196 DAS a higher $WUE_i$ was also evident in the Ltd treatment (*$P=0.004$*; Fig.3c) alongside a reduced $C_i/C_a$ (*$P=0.004$*; Fig.4c) compared to the other treatments. The DD treatment showed no increase in $WUE_i$ (Fig.3c) but $A_{max}$ and $g_s$ were not significantly reduced as previously outlined. In 2019, the second drought increased the $WUE_i$ of the DD treatment at 153 and 163 DAS with this difference no longer significant at 169 DAS (*P=0.022*; Fig.3d), just before recovery of $A_{max}$ and $g_s$ at 174 DAS. This also resulted in a decline in $C_i/C_a$ compared to the fully irrigated and SD to levels similar to the Ltd treatment (*P=0.027*; Fig.4d). The increase in $WUE_i$ (*P=0.022*; Fig.3d) and decrease in $C_i/C_a$ (*P=0.027*; Fig.4d) in the fully irrigated compared to the SD at 140 and 146 DAS can be attributed to the decline in VWC previously outlined.

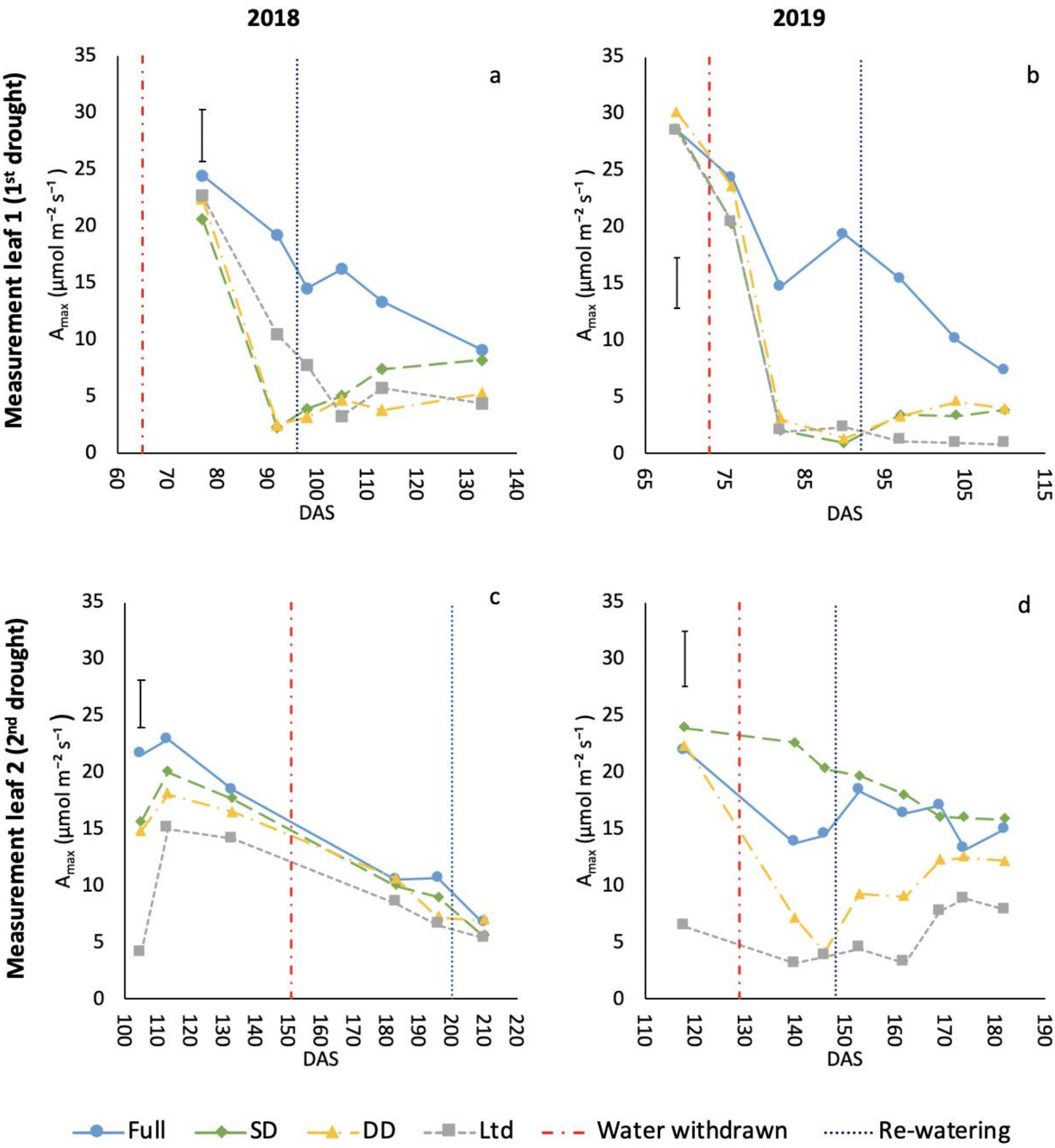

**Figure 1.** The $A_{max}$ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P<0.001* LSD=4.54 DF=190) and 2019 (b) (*P<0.001* LSD=4.45 DF=220) and measurement leaf 2 the second drought in 2018 (c) (*P=0.002* LSD=4.16 DF=191) and 2019 (d) (*P<0.001* LSD=4.75 DF=255). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

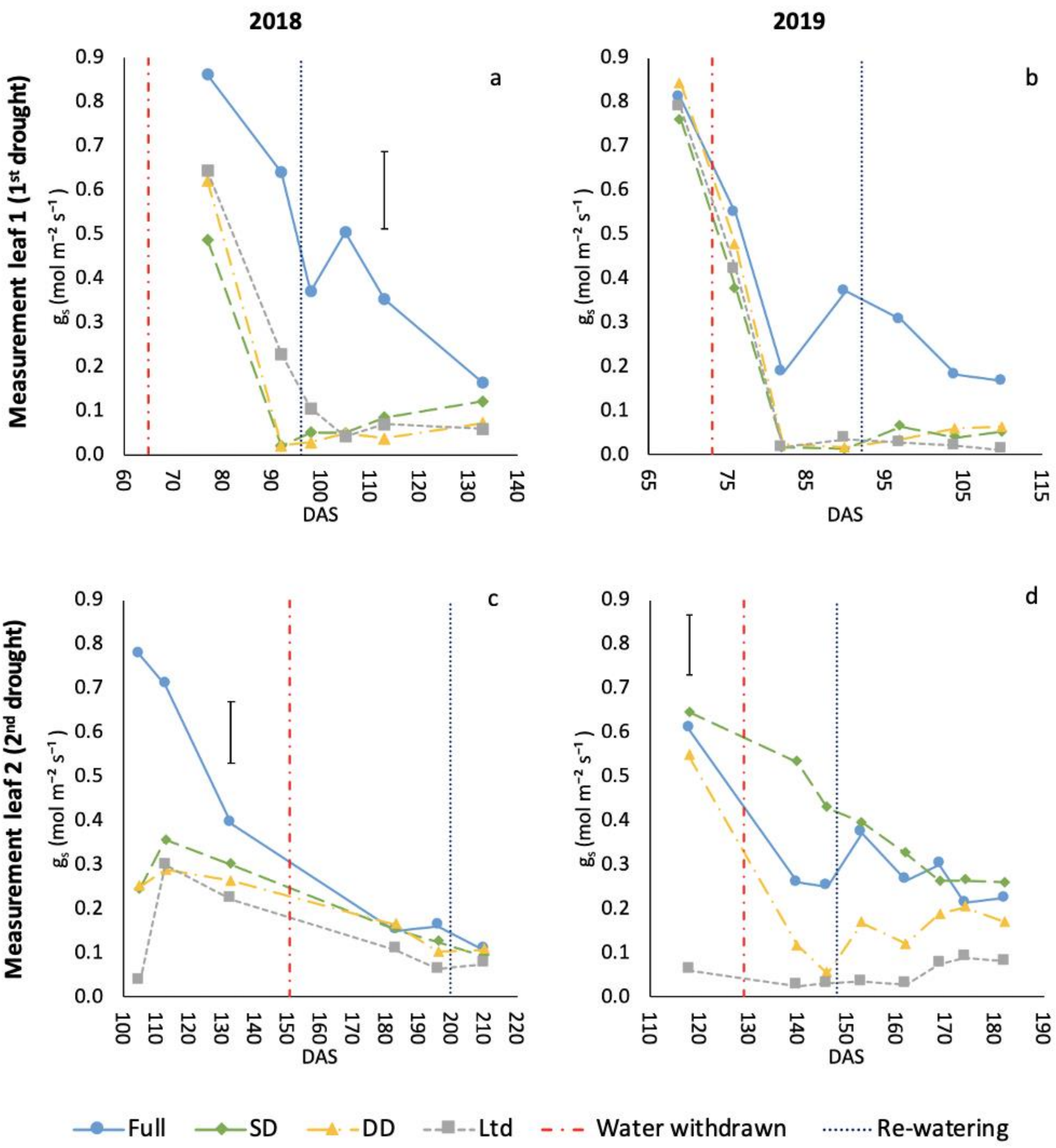

**Figure 2.** The $g_s$ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P=0.005* LSD=0.177 DF=190) and 2019 (b) (*P=0.133*) and measurement leaf 2 the second drought in 2018 (c) (*P=0.002* LSD=0.141 DF=191) and 2019 (d) (*P<0.001* LSD=0.136 DF=255). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

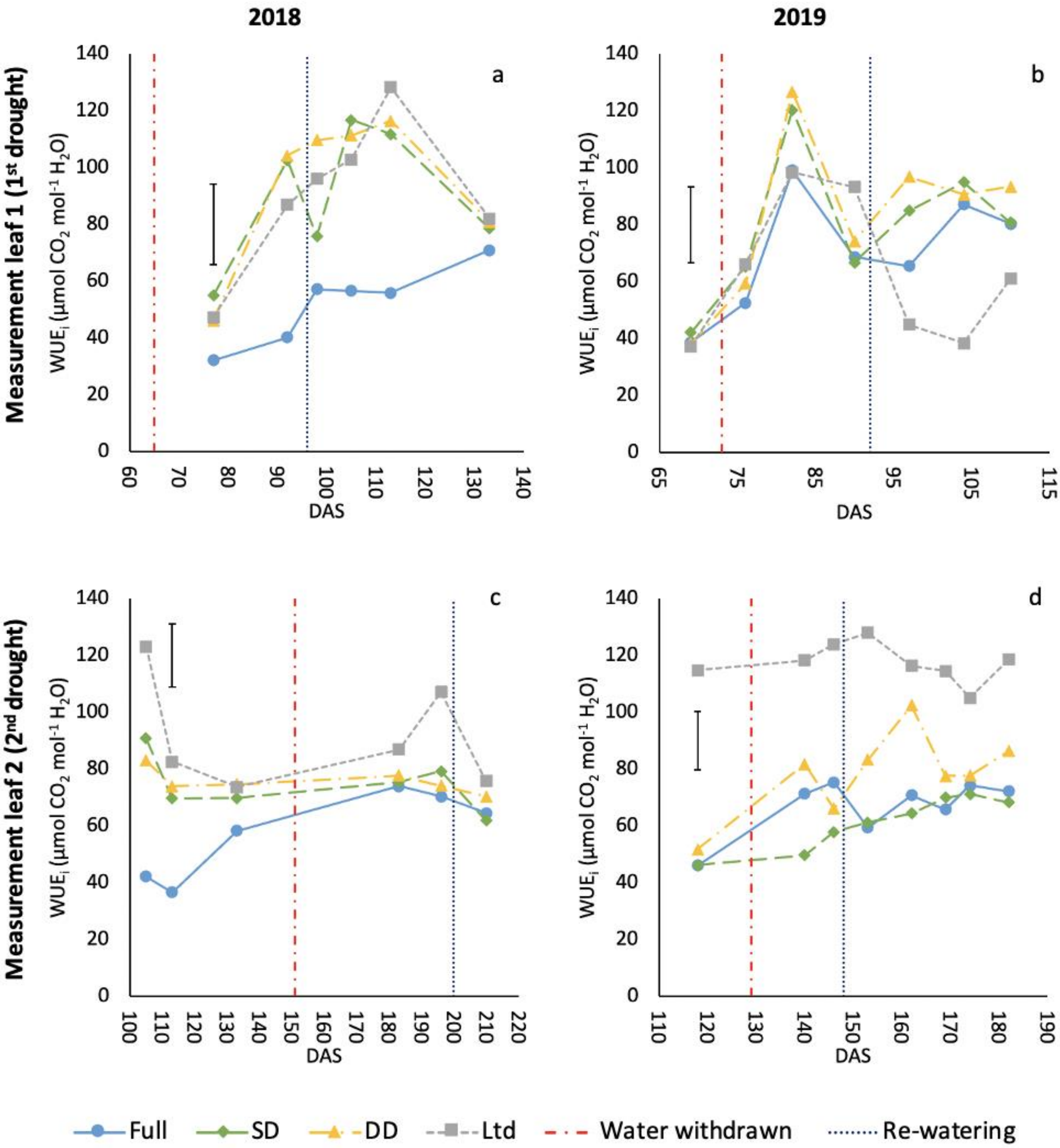

**Figure 3.** The WUE$_i$ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P=0.004* LSD=28.2 DF=190) and 2019 (b) (*P=0.003* LSD=26.5 DF=220) and measurement leaf 2 the second drought in 2018 (c) (*P=0.004* LSD=22.4 DF=191) and 2019 (d) (*P=0.022* LSD=20.4 DF=255). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

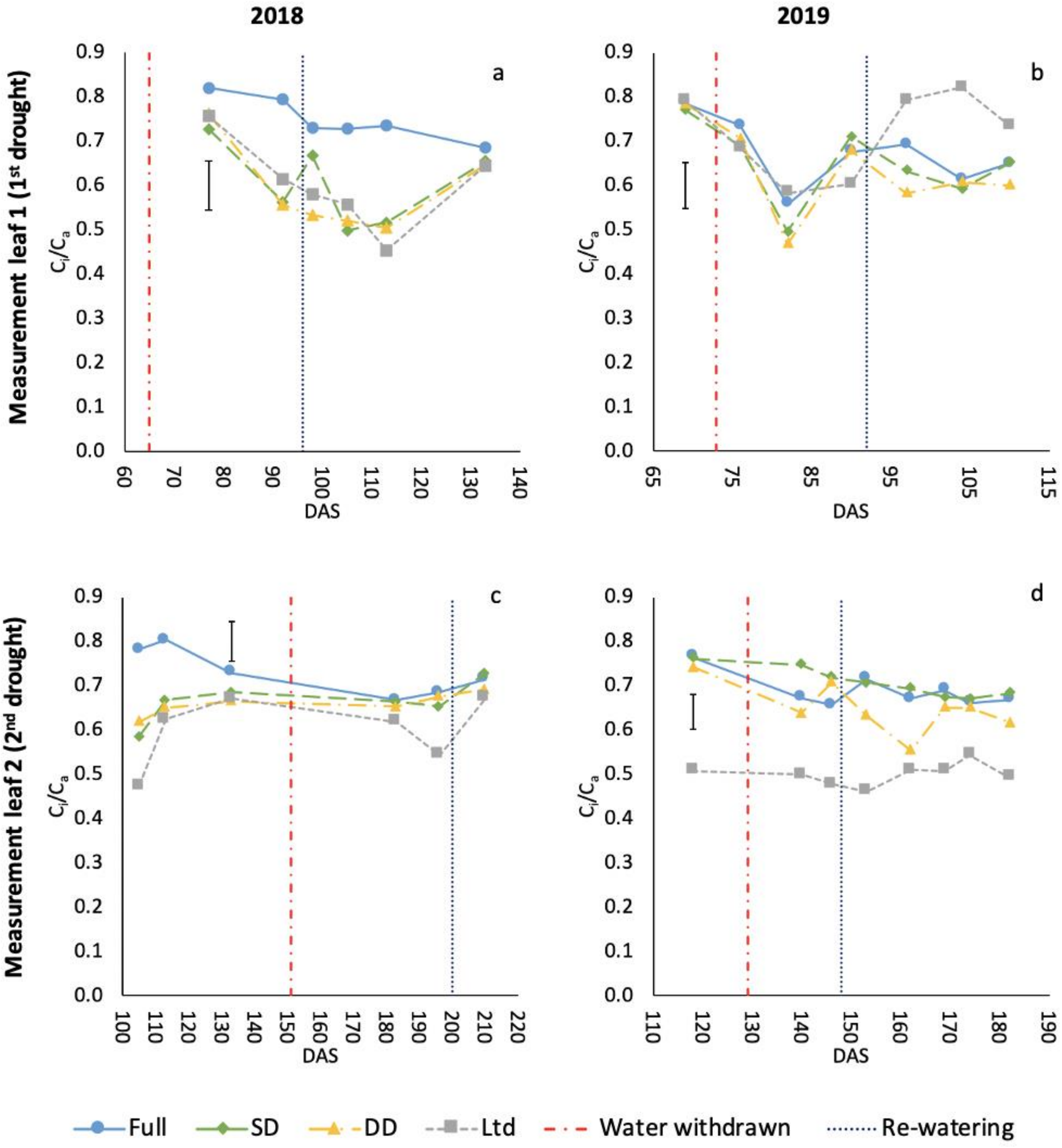

**Figure 4.** The $C_i/C_a$ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P=0.006* LSD=0.110 DF=190) and 2019 (b) (*P=0.003* LSD=0.104 DF=220) and measurement leaf 2 the second drought in 2018 (c) (*P=0.004* LSD=0.088 DF=191) and 2019 (d) (*P=0.027* LSD=0.079 DF=255). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

Averages for key measurements for each variety during the experiment are shown in figure 5. In 2018 there were no consistent differences in $A_{max}$, $g_s$ $WUE_i$ and $C_i/C_a$ between varieties. In 2019 there was no significant difference in $A_{max}$ between the two sugar beet varieties (Fig.5a), however $g_s$ was significantly higher in the upright variety of both measurement leaf 1 (*P=0.012*; Fig.5b) and 2 (*P=0.042*; Fig.5b). The non-significant difference in $A_{max}$ between varieties coupled with a significantly lower $g_s$ resulted in a trend (*P=0.072*) of greater $WUE_i$ in the prostrate variety in measurement leaf 1 (*P=0.072*; Fig.5c) and was significantly greater for measurement leaf 2 (*P=0.011*; Fig.5c). This greater $WUE_i$ was also associated with a lower $C_i/C_a$ for measurement leaf 2 of the prostrate variety (*P=0.012*; Fig.5d) and a similar trend for measurement leaf 1 (*P=0.069*; Fig.5d).

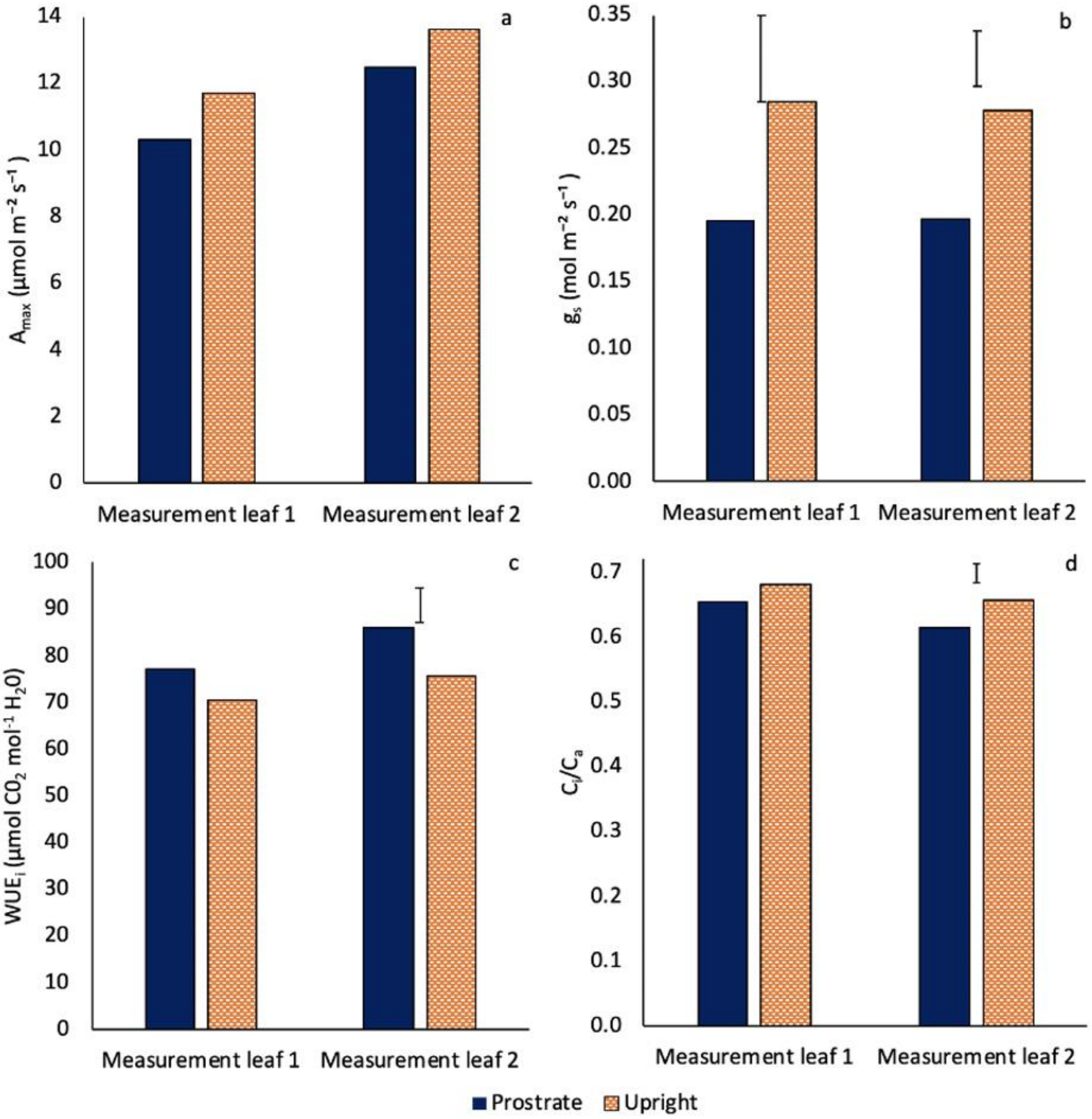

**Figure 5.** The average $A_{max}$ (ns), $g_s$ (Measurement leaf 1 *P=0.012* LSD=0.065, Measurement leaf 2 *P=0.001* DF=31 LSD=0.042), WUE$_i$ (Measurement leaf 1 *P=0.072*, Measurement leaf 2 *P=0.011* DF=31 LSD=7.32) and $C_i/C_a$ (Measurement leaf 1 *P=0.069*, Measurement leaf 2 *P=0.012* DF=31 LSD=0.03016) of two sugar beet leaves from varieties with a prostrate and upright canopy measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Leaf one was measured at 69, 72, 82, 90, 97, 104 and 110 DAS and leaf two at 118, 140, 146, 153, 162, 169, 174, 182 DAS.

### 3.4.2 Canopy temperature

Looking at the effect of water availability across the two varieties during the first drought an increase in absolute canopy temperature is evident. In 2018 at 78 DAS the temperature of the canopy was higher in the SD, DD and Ltd treatments than the fully irrigated and by 92 DAS this difference was significant ($P<0.001$; Fig.6a). The Ltd treatment had a lower canopy temperature than the SD and DD, but higher than the fully irrigated 13 days after watering ceased in the SD and DD treatments ($P<0.001$; Fig.6a). At 99 DAS the low air temperature of 21.2°C meant that no significant differences were evident but at 106 DAS the Ltd had a warmer canopy than the fully irrigated ($P<0.001$; Fig.6a). The SD and DD had canopy temperatures of 31.2°C and 30.8°C, with the SD significantly warmer than the fully irrigated, but both treatments cooler than the Ltd ($P<0.001$; Fig.6a). The same was evident at 113 DAS but with the SD no longer different to the fully irrigated. From 133 DAS onwards no significant differences were evident as the second drought was late in the season and temperatures were low (Fig.6a).

In 2019 the SD, DD and Ltd canopy temperatures were higher than the fully irrigated at 82 DAS, 9 days after watering ceased in the SD and DD treatments ($P<0.001$; Fig.6b). This difference was present until 111 DAS, 9 days after re watering from the first drought. At 140 DAS all treatments were heat stressed as they were close to or exceeding the ambient air temperature of 37.4°C, but with the DD, under second drought, and Ltd significantly warmer than the SD and fully irrigated ($P<0.001$; Fig.6b), which at this point had a lower VWC than the SD. At 154 DAS, 6 days after re watering, the DD had a similar canopy temperature to the fully irrigated and SD with these three treatments maintaining similar canopy temperatures for the reminder of the measurements. The Ltd canopy temperature remained significantly higher than the fully irrigated from 82 DAS onwards and at 104, 111, 140 and 163 DAS was warmer than the air temperature suggesting complete stomatal closure (Fig.6b).

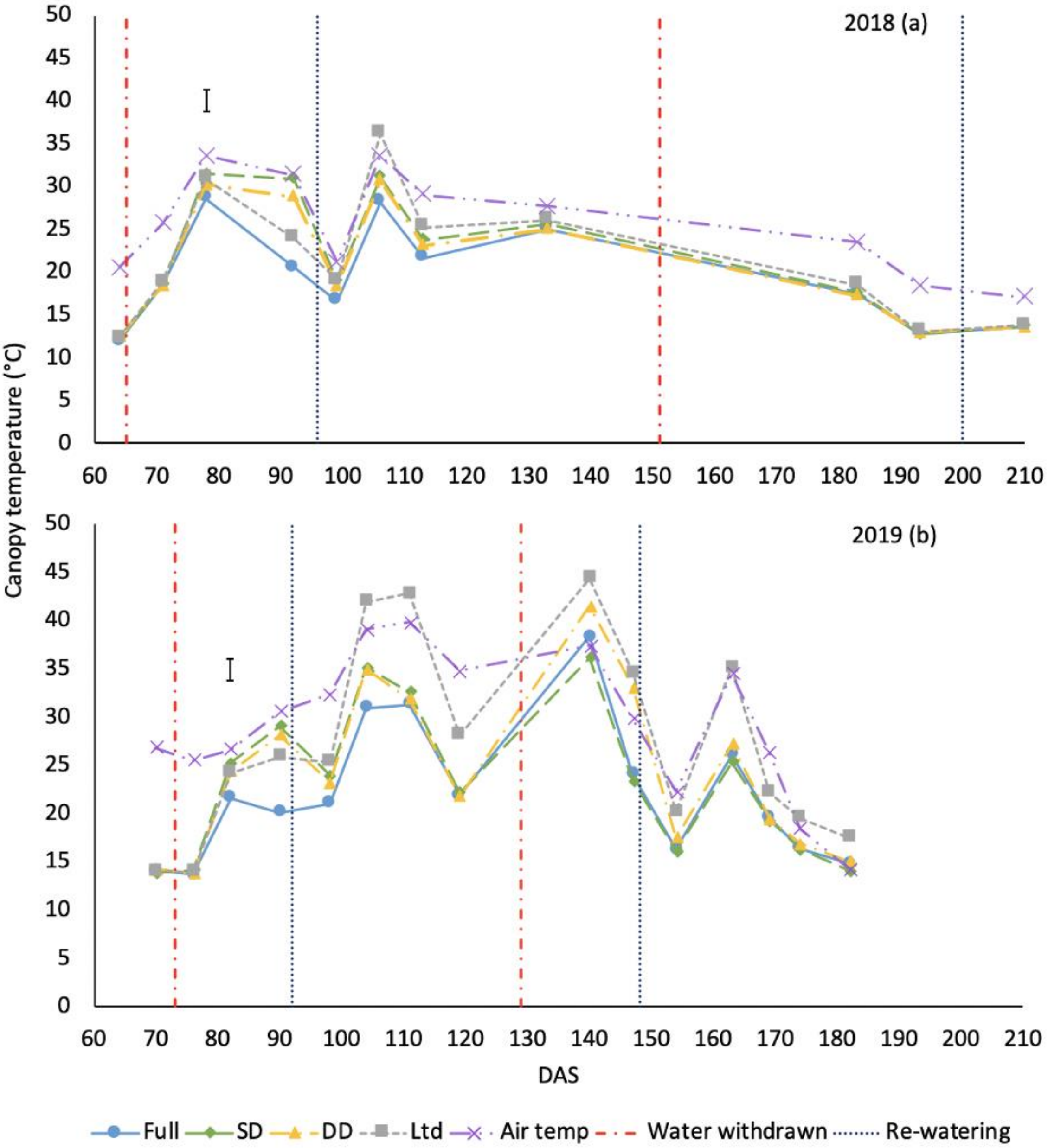

**Figure 6.** The canopy temperature of sugar beet grown under four different irrigation regimes in 2018 (a) (*P<0.001* LSD=2.56 DF=33) and 2019 (b) (*P<0.001* LSD=2.23 DF=42) and the air temperature at the time of measurement. Measured using a handheld thermal camera (C2, FLIR, Wilsonville, Oregon, USA) and temperature and humidity logger (TinyTag Ultra 2, Gemini data loggers, Chichester, West Sussex, UK). Error bars show irrigation*time interaction. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

In 2019 there was a variety*treatment*time interaction of canopy temperature. In the fully irrigated sugar beet the prostrate variety had a warmer canopy compared to the upright at 104, 111, 140, 147, 163, 174 and 182 DAS (*P=0.003*; Fig.7a). In the SD (Fig.7b) and DD (Fig.7c) treatments there were no significant differences between varieties during the first drought but during the second drought at 140 DAS the prostrate variety had a significantly warmer canopy than the upright in the DD treatment (*P=0.003*; Fig.7c), although both were above air temperature suggesting they had closed stomata. In the Ltd treatment the upright variety had a warmer canopy than the prostrate (*P=0.003*; Fig.7d), at 104, 111, 140 and 163 DAS, opposite to the observations in the fully irrigated treatment.

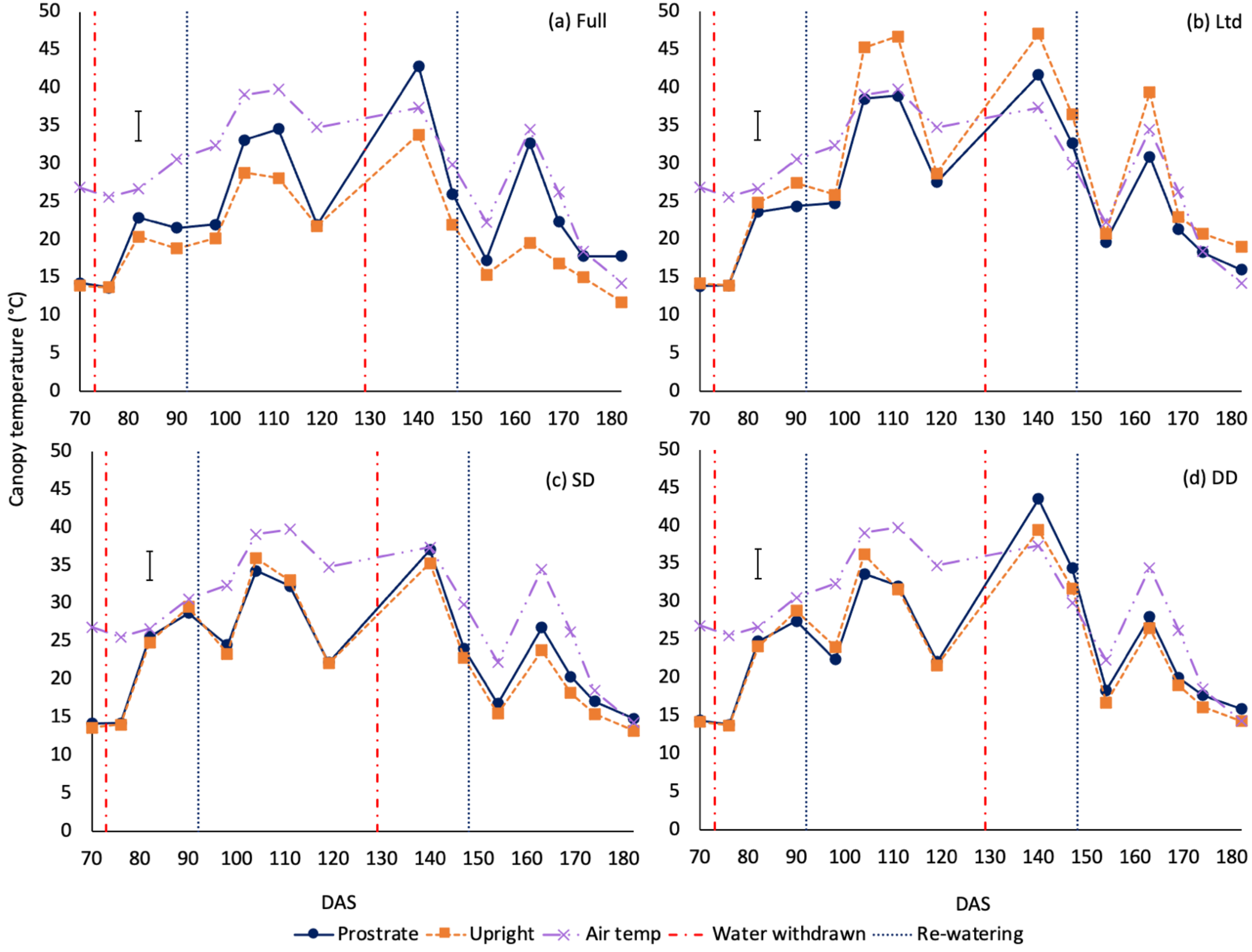

**Figure 7.** The canopy temperature of two sugar beet varieties with a prostrate and upright canopy grown under four different irrigation regimes and the air temperature at the time of measurement. Measured using a handheld thermal camera (C2, FLIR, Wilsonville, Oregon, USA) and temperature and humidity logger (TinyTag Ultra 2, Gemini data loggers, Chichester, West Sussex, UK). Error bars show variety*irrigation*time interaction (*P=0.003* LSD=3.88 DF=42). Irrigation regimes were a fully irrigated (Full) (a), continually water limited kept at approx. 50% field capacity (Ltd) (b), a single drought (SD) (c) 73 -92 DAS and a double drought (DD) (d) which was exposed to the single drought treatment plus an additional drought 118-182 DAS.

RWC declined in the sugar beet under drought as water availability reduced. This was evident during the first drought in 2018 at 93 - 101 DAS with recovery by 108 DAS except in the SD which was lower than the fully irrigated but similar to the DD and Ltd treatments (*$P<0.001$*; Fig.8a). In 2019, the first drought reduced RWC from 84 – 106 DAS with recovery by 121 DAS (*$P<0.001$*; Fig.8b). The second drought had the same effect in 2019 reducing RWC from 141-148 DAS with recovery by 154 DAS (*$P<0.001$*; Fig.8a). In 2018, the late drought did not significantly reduce RWC (Fig.8b). The Ltd treatment had a lower RWC compared to the fully irrigated once VWC had declined from 101-108 DAS in 2018 (*$P<0.001$*; Fig.8a) and 84-170 DAS 2019 (*$P<0.001$*; Fig.8b). Despite the decline in VWC in the fully irrigated at 140-146 DAS in 2019 and the concurrent reduction in $A_{max}$ and $g_s$ RWC did not significantly decline.

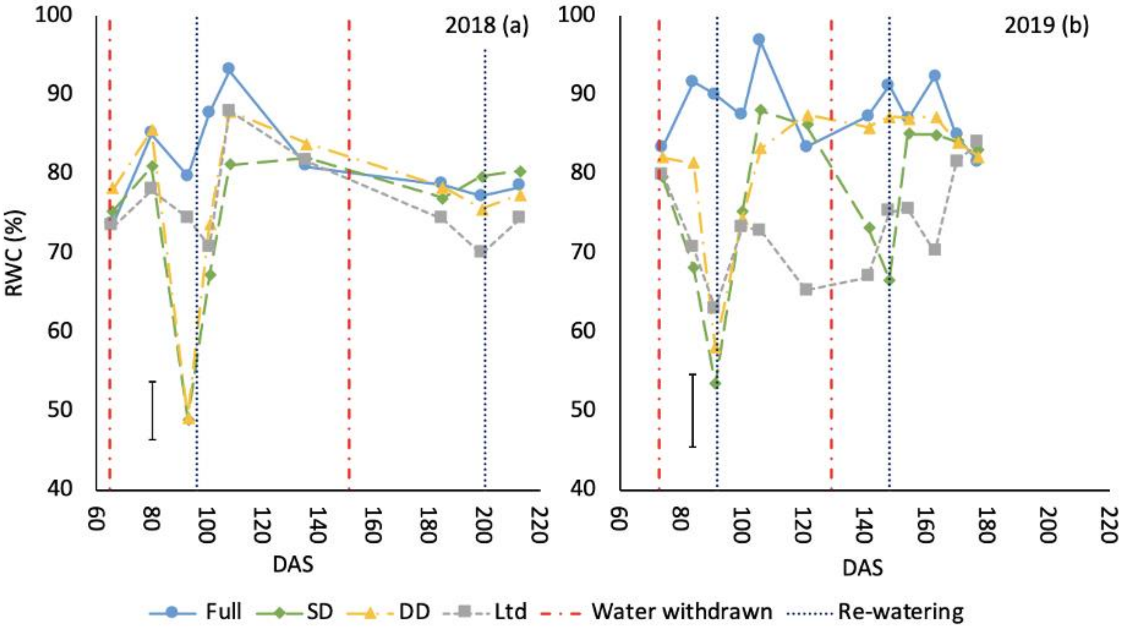

**Figure 8.** The RWC (%) of sugar beet grown under four different irrigation regimes in 2018 (a) (*P<0.001* LSD=7.34 DF=24) and 2019 (b) (*P<0.001* LSD=9.16 DF=33). Error bars shows irrigation*time LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS).

In both 2018 and 2019 the prostrate variety had a greater RWC than the upright variety when averaged across treatments (*P<0.001*; Fig.9).

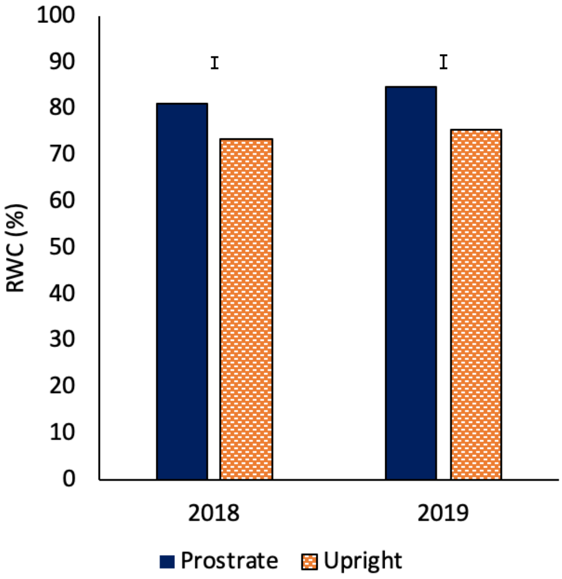

**Figure 9.** The RWC (%) of two sugar beet varieties with an upright and prostrate canopy in 2018 (*P<0.001* LSD=2.72 DF=1) and 2019 (*P<0.001* LSD=2.82 DF=1). Error bar shows variety LSD.

The average RWC, $WUE_i$, A and $g_s$ across all measurements and the SD of the adaxial and abaxial leaf surface were examined for year*irrigation*genotype interactions to identify if the response to water deficit was the same in 2018 and 2019. Only RWC showed differences between the two varieties in the response to the irrigation treatments between the two years. The prostrate and upright varieties showed a greater decline in RWC in the SD treatment in 2018 than 2019 (*P=0.014*). The upright variety also had lower RWC in the limited treatment but greater RWC in the well irrigated in 2019 compared to 2018 (*P=0.014*). These differences between years can likely be attributed to differences in the VWC and the apparent greater sensitivity of the upright variety's RWC to changes in soil moisture. In 2019 the VWC was higher on average than in 2018 which could explain the difference between years. In 2018 the period of water withdrawal was longer and a lower VWC was reached in the first drought which could have driven the difference evident in the SD treatments for both the upright and prostrate variety in 2018 vs 2019. Lastly, the lower RWC in the upright variety in the Ltd treatment could be attributed to the VWC be maintained at a lower level than in 2018. These observations highlight that the RWC of the upright variety may be more responsive to very low soil VWC.

### 3.4.4 Leaf chlorophyll and water content

The prostrate variety had greater total chlorophyll content on a per unit area basis in the Ltd treatment than all the other treatments, while in the upright variety, the fully irrigated and the DD had greater chlorophyll content than the SD and the Ltd (*P=0.008*; Fig.10a). The upright variety had greater chlorophyll content than the prostrate in the fully irrigated, SD and DD treatments whilst there was no significant difference in the Ltd treatment (*P=0.008*; Fig.10a). The ratio of chlorophyll *a* to *b* was not significantly different between irrigation treatments or variety. Leaf water content was also assessed to identify if this varied with chlorophyll content but the only difference was a greater water content in the prostrate variety compared to the upright in the full and Ltd, with the Ltd water content being significantly greater than that observed in any other treatment combination (*P=0.016*; Fig.10b).

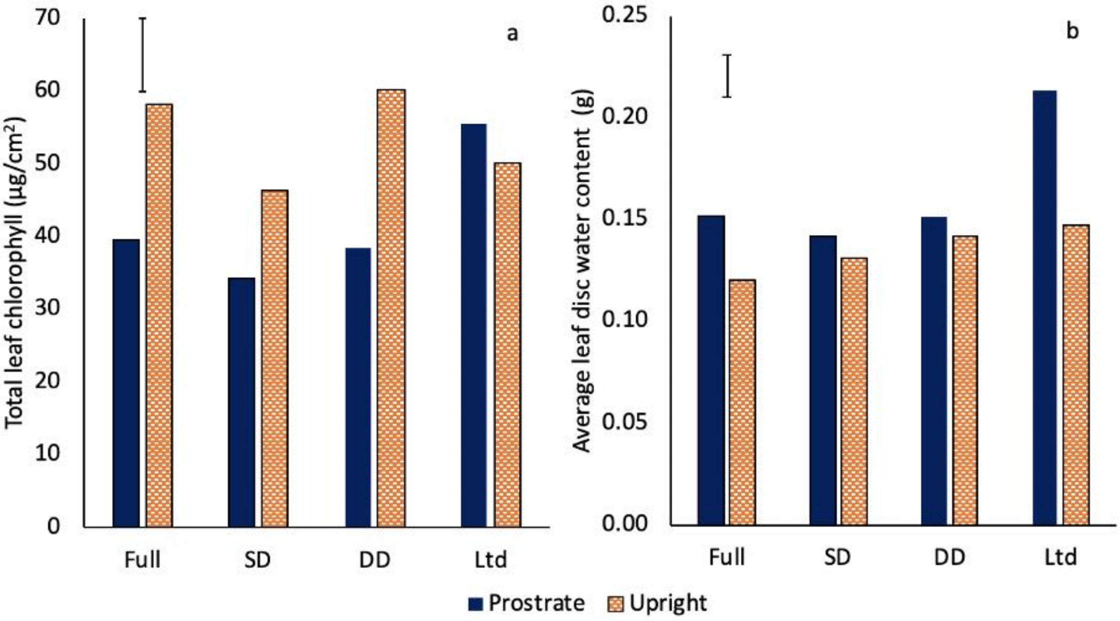

**Figure 10.** The total chlorophyll content (a) (*P=0.008* LSD=8.5 DF=31) and leaf water content (b) (*P=0.016* LSD=0.021 DF=31) of two sugar beet varieties with a prostrate and upright canopy grown under four different irrigation regimes, fully irrigated (Full), single drought (SD), double drought (DD) and continually water limited (Ltd). Error bar shows irrigation*variety LSD.

### 3.4.5 Stomatal density

The prostrate variety had a significantly lower stomatal density on both the adaxial and abaxial leaf surface in 2018 and 2019 (*P<0.001*; Fig.11). There was no consistent relationship between stomatal density and irrgation in either year.

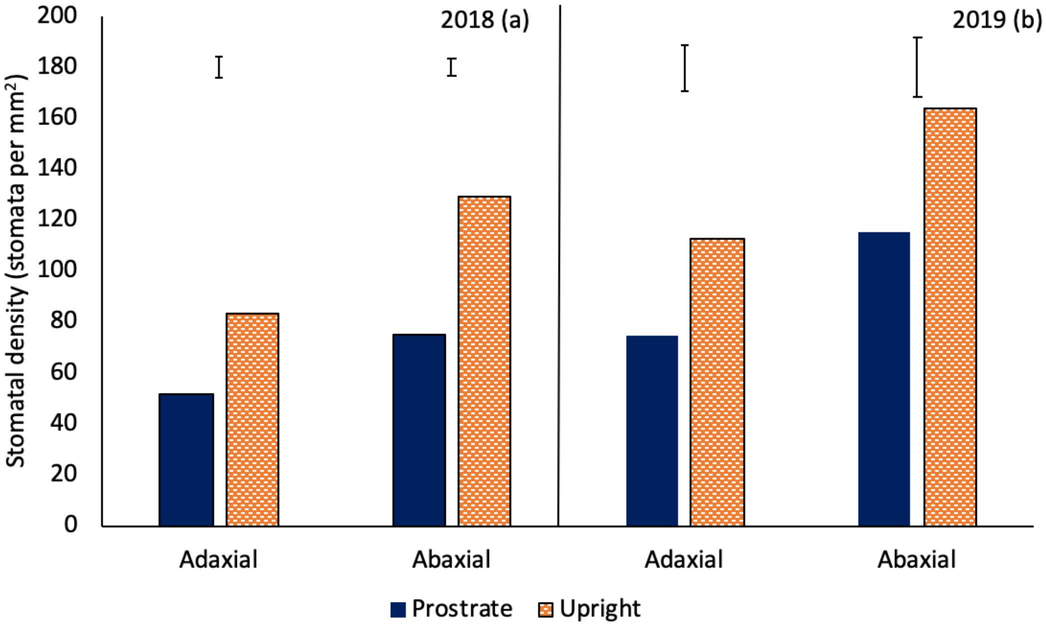

**Figure 11.** The stomatal density of the adaxial and abaxial leaves of two sugar beet varieties with a prostrate and upright canopy in 2018 (a) (adaxial *P<0.001* LSD=7.7 DF=31, abaxial *P<0.001* LSD=6.7 DF=31) and 2019 (b) (adaxial *P<0.001* LSD=18.1 DF=31, abaxial *P<0.001* LSD=23.1 DF=31).

### 3.4.6 Yield and WUE of dry matter and white sugar yield

There were no varietal differences in the total dry matter or white sugar yields and the $WUE_{DM}$ and $WUE_{WS}$ so these observations were averaged across the two varieties to focus on the affect of water availability. In 2018 and 2019 the same trends were evident in the total plant DM and WS with the fully irrigated having a greater total DM and WS in 2018 ($P<0.001$; Fig.12a) and 2019 ($P<0.001$; Fig.12b) than the other three treatments. In 2019, the Ltd treatment resulted in lower total DM and WS than the SD and DD ($P<0.001$; Fig.12b). In 2019 the sugar beet achieved a higher total DM and WS than 2018, except in the Ltd treatment, highlighting the differences in the crop's growth between years.

The $WUE_{DM}$ and $WUE_{WS}$ was higher in the Ltd treatment than the other three treatments and nearly double that of the fully irrigated and SD treatments in 2018 ($P<0.001$; Fig.12c). The DD had a higher $WUE_{DM}$ and $WUE_{WS}$ compared with the fully irrigated and SD in 2018 ($P<0.001$; Fig.12c), despite having a similar total DM and WS. In 2019, the $WUE_{DM}$ of the Ltd treatment was higher than the other three treatments ($P<0.001$; Fig.12d). The $WUE_{DM}$ of the fully irrigated, SD and DD were similar but the $WUE_{WS}$ in the SD and DD was lower than the fully irrigated ($P<0.001$; Fig.12d). Overall the extreme water deficit of the Ltd treatment increased WUE but the SD and DD treatments had inconsistent effects.

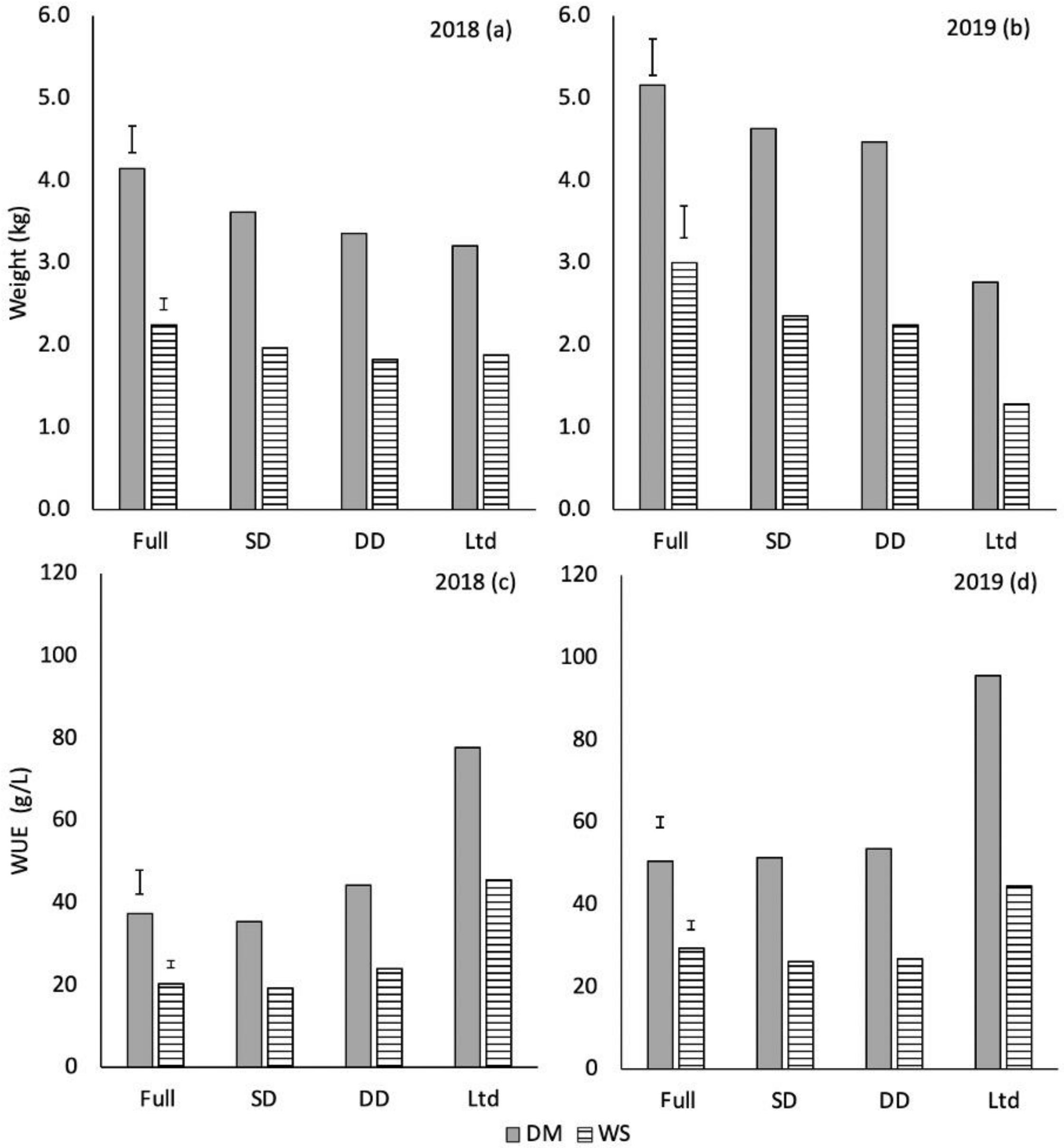

**Figure 12.** The amount of total dry matter (DM) and white sugar yield (WS) in 2018 (a) and 2019 (b) and $WUE_{DM}$ and $WUE_{WS}$ in 2018 (c) and 2019 (d) of sugar beet grown under four different irrigation regimes. (a) 2018 weights (DM $P<0.001$ LSD=0.322 DF=31 and WS $P<0.001$ LSD=0.142 DF=31). (b) 2019 weights (DM $P<0.001$ LSD=0.222 DF=31 and WS $P<0.001$ LSD=0.192 DF=31). (c) 2018 WUE ($WUE_{DM}$ $P<0.001$ LSD=5.76 DF=31 and $WUE_{WS}$ $P<0.001$ LSD=1.78 DF=31). (d) 2019 WUE ($WUE_{DM}$ $P<0.001$ LSD=2.52 DF=31, $WUE_{WS}$ $P<0.001$ LSD=2.09 DF=31). Error bars show irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 - 151-200 DAS and 2019 - 118-182 DAS).

### 3.4.7 Carbon 13 isotope discrimination

$\Delta^{13}C$ was negatively related to the average $WUE_i$ (*$P<0.001$*; Fig.13a), $WUE_{DM}$ (*$P=0.001$*; Fig.13b) and $WUE_{WS}$ (*$P=0.004$*; Fig.13c).

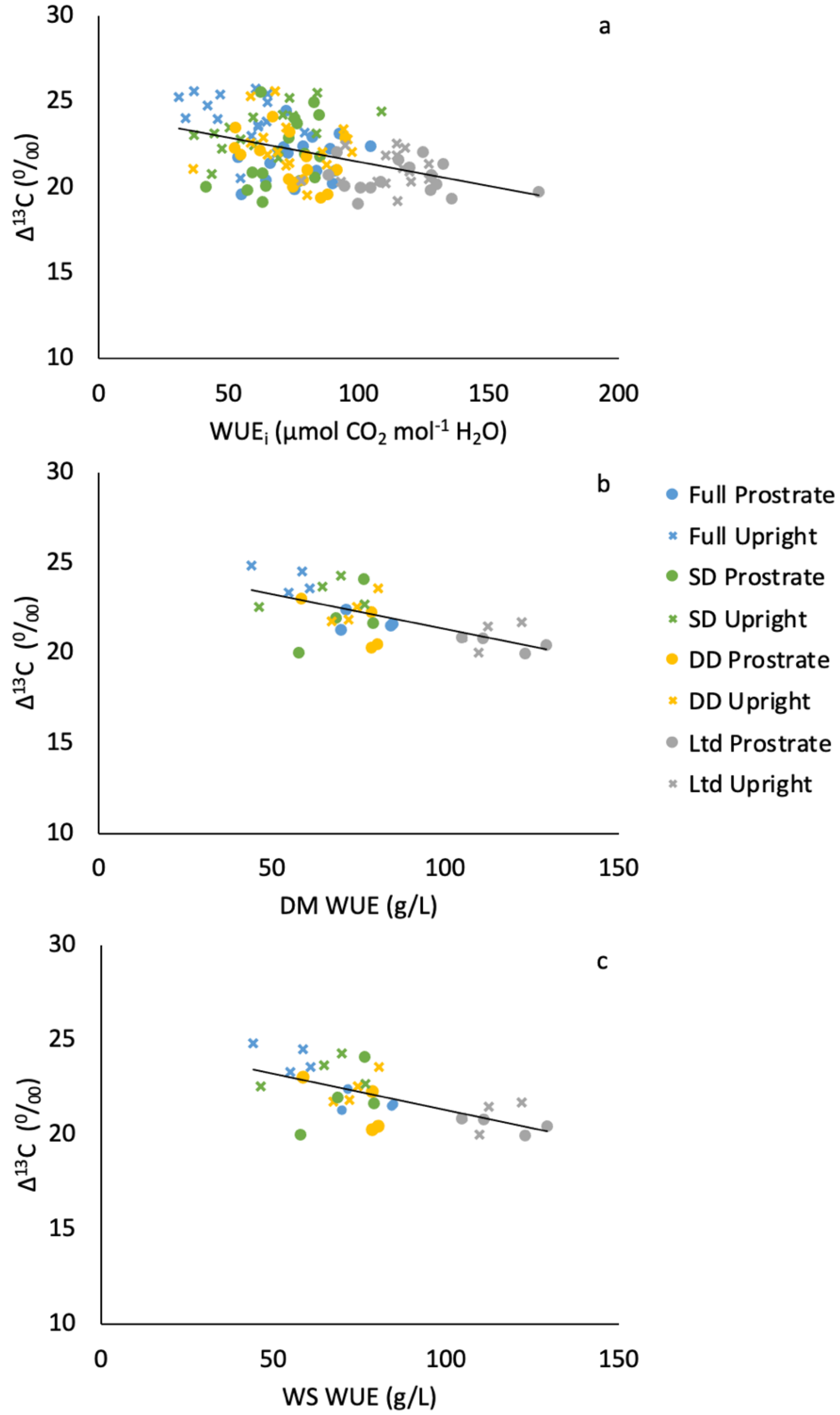

**Figure 13.** The relationship between $\Delta^{13}C$ and (a) $WUE_i$ (*$P<0.001$* $R^2=0.17$), (b) $WUE_{DM}$ (*$P=0.001$* $R^2=0.30$), (c) $WUE_{WS}$ (*$P=0.004$* $R^2=0.24$).

Water deficit reduced $\Delta^{13}C$ indicating increased $WUE_i$. The $\Delta^{13}C$ of measurement leaf 2, which was fully expanded at 118 DAS; before the second drought, was similar in the fully irrigated and SD treatments, with the DD lower but not significantly (Fig.14a). The Ltd treatment had a lower $\Delta^{13}C$ than the fully irrigated and SD but was not different to the DD treatment (*P=0.016*; Fig.14a).

Measurement leaf 3 was fully expanded at 162 DAS; after the second drought, with $\Delta^{13}C$ generally higher than in measurement leaf 2 (Fig.14a). This can be attributed to younger leaves being more active with higher transpiration, as shown by the gas exchange measurements, resulting in reduced $WUE_i$. The $\Delta^{13}C$ of measurement leaf 3 was lower in the DD than the fully irrigated and the difference was significant, unlike in measurement leaf 2, showing that water deficit was reducing $\Delta^{13}C$ and hence increasing $WUE_i$ at a greater magnitude in younger than older leaves (*P=0.016*; Fig.14a). The SD remained similar to the fully irrigated and the Ltd treatment $\Delta^{13}C$ was lower than the fully irrigated and SD but not the DD (*P=0.016*; Fig.14a).

Averaged across the watering regimes, the prostrate variety had a lower $\Delta^{13}C$ than the upright in both measurement leaf 2 (*P=0.001*) and measurement leaf 3 (*P=0.012*; Fig.14b).

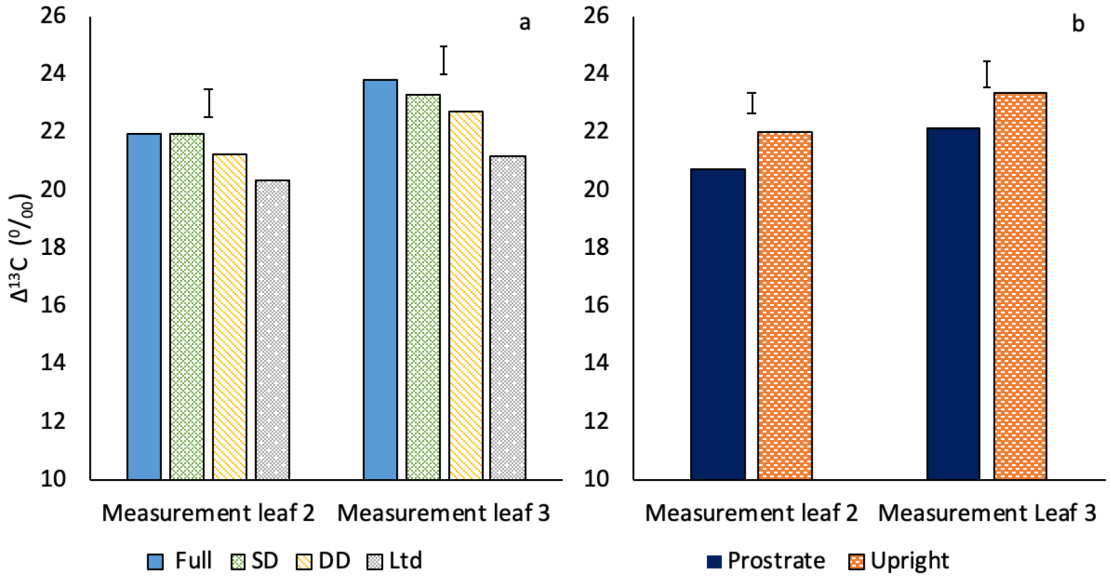

**Figure 14.** (a) The $\Delta^{13}$C of two measurement leaves of sugar beet grown under four different irrigation regimes, fully irrigated (Full), single drought (SD), double drought (DD) and continually water limited (Ltd). Error bar shows irrigation LSD of measurement leaf 2 (*P=0.016* LSD=0.975 DF=31) and measurement leaf 3 (*P=0.001* LSD=0.984 DF=31). (b) The $\Delta^{13}$C of two sugar beet varieties with a prostrate and upright canopy averaged across four watering regimes. Error bar shows variety LSD of measurement leaf 2 (*P=0.001* LSD=0.701 DF=31) and measurement leaf 3 (*P=0.012* LSD=0.909 DF=31).

To test the relationship between leaf traits associated with leaf water content and $WUE_i$ a regression was carried out between RWC and $\Delta^{13}C$. This highlighted the significantly lower (*$P<0.001$*) $\Delta^{13}C$ in the prostrate variety but also showed a significant positive correlation (*$P<0.001$*) between RWC and $\Delta^{13}C$ (Fig.15).

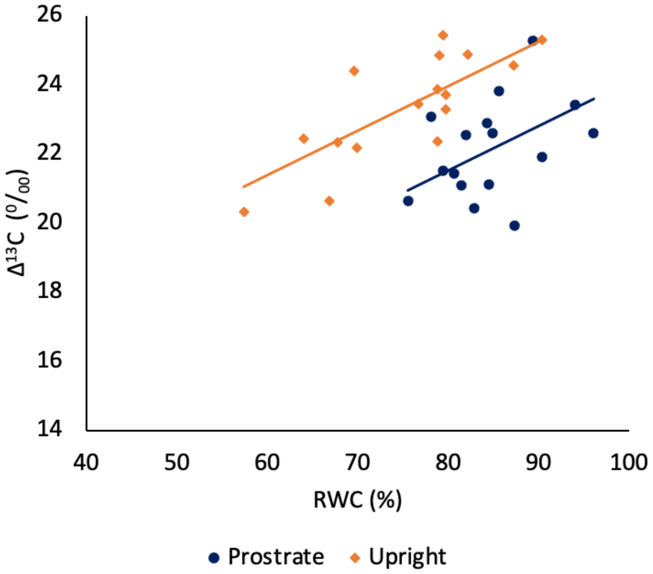

**Figure 15.** The relationship between $\Delta^{13}C$ and RWC of two sugar beet varieties with contrasting prostrate (*$P<0.001$*, $R_2$=0.15) and upright (*$P<0.001$*, $R_2$=0.64) canopies.

### 3.4.8 Specific leaf weight

$\Delta^{13}C$ was negatively correlated with SLW (*P=0.001*; Fig.16a). There was no significant difference in SLW between the varieties but high levels of water deficit increased SLW with the DD having a greater SLW than the fully irrigated and SD (*P=0.016*; Fig.16b). The SLW of the Ltd treatment was almost double the next nearest SLW value in the DD.

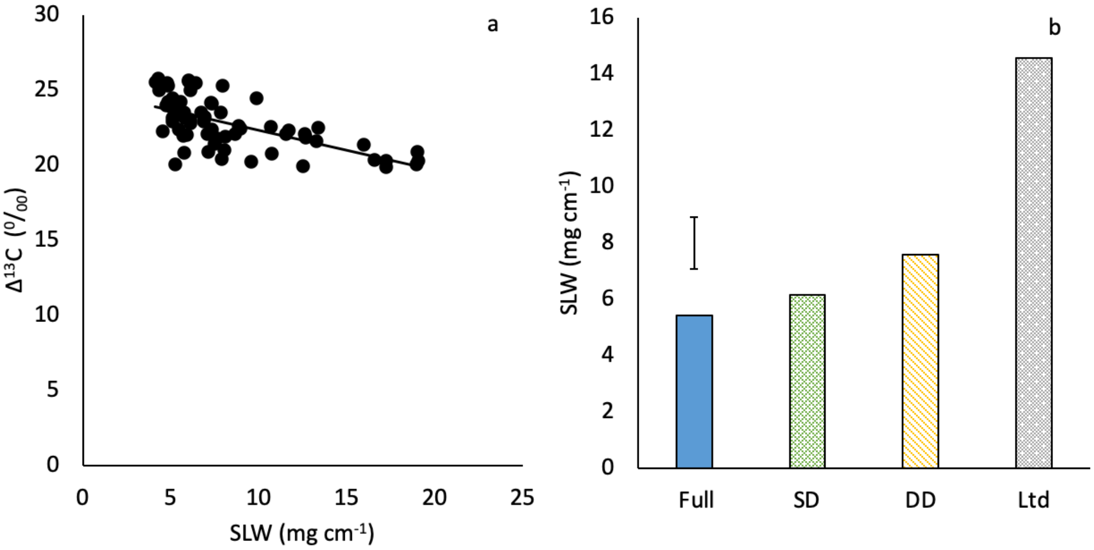

**Figure 16.** (a) The relationship between $\Delta^{13}C$ and SLW (*P<0.001* $R^2$=0.41) and (b) the SLW of sugar beet grown under four different irrigation regimes, fully irrigated (Full), single drought (SD), double drought (DD) and continually water limited (Ltd). Error bar shows irrigation LSD (*P=0.016* LSD=1.83 DF=31).

## 3.5 Discussion

The relationship between $\Delta^{13}C$, dry matter accumulation and $WUE_{DM}$ has been evidenced in sugar beet in response to drought (Bloch et al., 2006, Rytter, 2005) and the difference between the levels of $\Delta^{13}C$ in dry matter and soluble sugars (Monti et al., 2006) and leaf and root tissue (Bloch et al., 2006) compared. However, this is the first time a difference in $\Delta^{13}C$ has been identified in commercial sugar beet varieties and the relationship between WS and $\Delta^{13}C$ demonstrated. This shows that the objective of developing sugar beet varieties that are more efficient in their water use, without detriment to yield is viable, but these results are based only on two cultivars so should be interpreted with caution.

Increased $WUE_i$ under water deficit, as evident in the SD, DD and Ltd treatments, has been shown previously in sugar beet under drought (Bloch et al., 2006, Rinaldi and Vonella, 2006, Fitters et al., 2018). As the water deficit increased the stomata began to close to conserve water and $g_s$ declined with the reduction in transpiration causing an increased canopy temperature (Baker et al., 2007). The reduction in stomatal aperture also reduced $C_i/C_a$ which is associated with a greater $WUE_i$ and is related to a lower $\Delta^{13}C$ as the $CO_2$ in the sub stomatal cavity is not replenished as readily leading to greater a proportion of $^{13}C$ being fixed, thereby lowering the ratio of $^{13}C$ to $^{12}C$ (Seibt et al., 2008). There were two exceptions to this observation, firstly in 2019 the fully irrigated, SD and DD had similar $WUE_{DM}$ but the fully irrigated had a higher $WUE_{WS}$. This suggests that water deficit reduced the ratio of sugar accumulated to water used but did not alter the relationship between DM accumulation and water use which was comparable between the treatments. This could be attributed to different partitioning of sucrose in sugar beet as it has been identified that under drought phloem loading can be reduced, lowering the amount of sugar in the root but not in the plant overall (Mäck and Hoffmann, 2006). Secondly $WUE_i$ was reduced compared to the fully irrigated in the Ltd treatment of the first measurement leaf in 2019 whilst it was generally higher in all other observations. Across the same time period as the reduced $WUE_i$ the Ltd treatment also showed a greater $C_i/C_a$ ratio, whilst $A_{max}$ and $g_s$ were close to zero. An increase in $C_i$ has been observed in extreme cases of water deficit previously and is driven by an increase in non-stomatal limitations to photosynthesis with this point termed the $C_i$ inflection point which may explain this observation (Flexas and Medrano, 2002).

The measured gas exchange parameters recovered after drought but only partially due to the underlying leaf age related decline. It has been shown that leaves respond differently to heat stress depending on age with younger leaves showing responses of a greater magnitude compared to older leaves (Marias et al., 2017). However, when looking at the overlap of measurement leaf 1 and 2 from 105-133 DAS in 2018 it is evident that measurement leaf 1 reflected the trend of the response in measurement leaf 2 but not the magnitude. This means that, despite the overall decline in leaf activity with age, they still provided a reliable measure of the onset of drought and the subsequent recovery.

Sugar beet did not show any differences in WUE during the second drought compared to the first, suggesting the crop did not acclimate (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to avoid or better cope with water deficit. This has also been observed in glasshouse studies where the photosynthetic and biochemical responses in sugar beet were the same during three consecutive water deficit periods each separated by a recovery phase (Leufen et al., 2016). The maritime ancestry of sugar beet (Ribeiro et al., 2016) may be a driver of this with the plants showing drought tolerance (Dunham, 1993) and the photosynthetic apparatus being able to withstand severe water deficit and recover rapidly (Monti et al., 2006). This means there is no need to avoid a decline in leaf RWC, as the plant can continue to photosynthesise until the most severe levels of drought, and even then no long term damage occurs to the PSII, as shown by the recovery of the $F_v'/F_m'$, $\Phi$PSII and $q_p$ values which returned to levels similar to the fully irrigated once re watered. However, the recovery of maximum PSII efficiency in the light, and $A_{max}$, was not immediate and may have contributed to the reduced DM accumulation in the droughted treatments as evidenced in sugar beet by Bloch et al. (2006). The lack of any long-term physiological acclimation from the first drought was also reflected in the decline in $WUE_i$ and increase in $C_i/C_a$ as the sugar beet opened stomata and began to reach similar levels of $g_s$ and $A_{max}$ to the fully irrigated. The rapid recovery of leaf RWC, which recovered faster than PSII and leaf gas exchange, helps drive this recovery by ensuring the leaf has optimal conditions for photosynthesis (Lawlor, 2002), with the rapid recovery of RWC over daily cycles previously observed in sugar beet (Geiger et al., 1991). Not only was this recovery evident in these short-term measurements but also in the $\Delta^{13}C$ results where the SD leaves showed no difference compared to the fully irrigated treatment and therefore no long-term change in $WUE_i$. Water deficit has been shown to alter the stomatal density of leaves which develop under drought (Xu and Zhou, 2008, Sun et al., 2014) but there were no consistent changes in stomatal density highlighting further that sugar beet physiology changes little under water deficit. There is an exception to this which is the reduction in plant biomass which appears to have resulted in the crop having a reduced demand for water, likely due to a reduction in canopy size. Canopy size was not measured but water deficit has been shown to reduce leaf area in sugar beet (Fitters et al., 2017) which results in reduced radiation interception and DM accumulation (Brown et al., 1987). This was evidenced by the reduction in VWC in the fully irrigated treatment at 140 DAS which the SD did not encounter despite receiving the same amount of irrigation. This seems to have been beneficial under a slight water deficit but did not alter the response to the severe second drought. In the late second drought in 2018, the slight decline in $A_{max}$ and $g_s$ was not reflected in any other parameter and shows how late season drought can be hard to detect as the forces driving transpiration are reduced.

The difference in $\Delta^{13}C$ and associated traits between the two varieties supports the findings of Ober et al. (2004) and Ober et al. (2005), that there is variation in traits associated with drought tolerance and water use in sugar beet, despite suggestions that sugar beet varieties often lack the diverse traits due to being derived from a single population (Davis, 2006). The traits identified as being different between the varieties support the idea that the prostrate variety is more conservative in its use of water. Firstly, the greater RWC can be associated with more drought resistant phenotypes (Shaw et al., 2002) which this study has further shown to be linked to a greater $WUE_i$, although RWC has also been shown to have no association with drought tolerance although this may be driven by limitations in sampling for RWC in large scale field trials (Ober et al., 2005). RWC did not reduce the rate of carbon uptake and assimilation as has been observed in wheat (Farquhar et al., 1980) and a greater RWC can also enable plants to function for longer under water deficit (Xu et al., 2000). Secondly, the lower stomatal density in the prostrate variety may be associated with $WUE_i$ but complex interactions between stomatal density and size and the speed of stomatal response means that the relationship between SD and water use is debated. In potatoes a higher SD, which developed under drought, led to a greater $\Delta^{13}C$ and $WUE_i$ (Sun et al., 2014) and it has been suggested the smaller stomata are faster to close and reduce transpiration and increase $WUE_i$ (Drake et al., 2013). However, in Arabidopsis a lower stomatal density has been shown to be associated with reduced susceptibility to water deficit, which could explain that under the extreme water deficit of the Ltd treatment the prostrate variety had a cooler canopy than the upright (Doheny-Adams et al., 2012). Büssis et al. (2006) showed that increased stomatal aperture can compensate fewer stomata to enable similar levels of A and $g_s$ but that under high light intensities $g_s$ was reduced compared to the plants with a higher stomatal density. This may explain the higher canopy temperature in the prostrate variety in fully irrigated conditions as lower $g_s$ rate results in reduced transpiration cooling effect. This reduction in $g_s$ was also observed from the gas exchange measurements in 2019, leading to a greater $WUE_i$. This highlights how different stomatal densities may benefit different environments with a lower density making a plant more prone to heat stress under higher temperatures and irradiance, as maximum rates of transpiration and associated leaf cooling are reduced, (Lu et al., 1998) although such conditions are only present for short periods in much of the temperate sugar beet growing area. This also highlights the importance of understanding the genotype*environment interaction of different traits, as environmental conditions can significantly affect how traits drive sugar beet yield (Hoffmann et al., 2009). The lack of differences in the chlorophyll fluorescence parameters also suggest that the lower stomatal density would not limit photosynthetic capacity compared to the upright and is supported by the similarity in $A_{max}$ observed between the two varieties. All of these observations were supported by prostrate variety having a lower $\Delta^{13}C$ which equates to a greater $WUE_i$ (Farquhar et al., 1989). The similarity in final yield between the two varieties suggests that the lower stomata density was not detrimental to yield and may be a viable trait for increasing crop level WUE but must be considered alongside other associated traits such as stomatal size and leaf mesophyll conductance (Bertolino et al., 2019). The varieties had a similar SLW despite Rajabi et al. (2008) observing that a higher SLW was associated with a lower $\Delta^{13}C$ and greater $WUE_{DM}$, and this relationship did not vary depending on water deficit as had been previously observed (Rajabi et al., 2008). In this study SLW was correlated with $\Delta^{13}C$ but this was driven by difference in SLW due to water deficit, with plants exposed to a greater water deficit having a lower $\Delta^{13}C$ and $WUE_{DM}$. This suggests that the strong relationship between SLW, $\Delta^{13}C$ and $WUE_{DM}$ observed by Rajabi et al. (2008) may not always be evident on a varietal basis and other factors may be more strongly correlated with $WUE_{DM}$. Additionally Ober et al. (2005) observed that a greater SLW was associated with lower drought tolerance which could suggest that despite the differences in $WUE_i$ and the associated traits between the two varieties there DTI would not differ but this was not assessed in this study. Overall, the prostrate variety was more efficient in its use of water and had a reduced SD and greater RWC which are traits associated with increased WUE and drought tolerance, with the exception being a similar SLW to the upright. It is not known whether these traits are present in all sugar beet genotypes with prostate canopies, as only one was examined in this study, and this is an area for further research. Genetic variation in SD and canopy architecture may be dependent as leaf arrangement can lead to differences in the environmental conditions surrounding the leaf such as the size of the boundary layer and VPD, which may mean different SD are optimal to maximise $CO_2$ uptake while minimising transpiration which has been evidenced in trees (Warrit et al., 1980, Appleby and Davies, 1983).

Leaf chlorophyll content was greater in the upright variety except in the Ltd treatment, where the prostrate variety had a similar chlorophyll content and a greater leaf water content suggesting that under severe and prolonged water deficit the leaf morphology had changed, which was not evident in the upright variety. The increase in chlorophyll content was opposite to that typically observed under drought for many species such as tomato (Nankishore and Farrell, 2016), maize (Din et al., 2011) and rice (Pirdashti et al., 2009) and there was no change in the ratio of chlorophyll *a* and *b* which is also sometimes observed (Saeidi and Zabihi-e-Mahmoodabad, 2009). However, an increase in chlorophyll content has been observed in sugar beet exposed to drought previously (Hussein et al., 2008) and increased SPAD values (a proxy for chlorophyll content) were observed in sugar beet exposed to high level water deficits by Fitters et al. (2018), although these differences were no longer present after re watering and were attributed to a dilution effect. No such dilution effect is evident in this study as leaf water content was measured at the same time as collection of leaf discs for chlorophyll extraction. Additionally, the prostrate variety had a more stable chlorophyll content with an increase only evident in the Ltd treatment, whilst the upright variety was more variable, but this did not seem to affect the photosynthetic performance of the two varieties. It has been observed in barley that varieties with a more stable chlorophyll content when exposed to drought have greater drought tolerance (Li et al., 2006) but it has also been observed in wheat that a greater chlorophyll content confers greater drought tolerance (Talebi, 2011). The relationship between leaf chlorophyll content and WUE is therefore unclear in sugar beet and requires future study.

## 3.6 Conclusions

In conclusion, we have shown that water deficits tend to increase $WUE_i$ and $WUE_{DM}$ but reduce total yield. The recovery of sugar beet after drought has shown the crop's resilience and ability to recover from even the most severe drought and it is evident that the crop does not show any long-term physiological acclimation after these events to increase WUE or avoid future water deficits, except for reducing its canopy size. Despite only testing one variety of each contrasting canopy type, and the similarity in SLW, the greater $WUE_i$ of the prostrate variety and the difference in stomatal density and RWC compared to the upright suggests traits associated with greater $\mathrm{WUE}_i$ may be linked to canopy architecture.

## 3.7 Acknowledgments

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## 3.9 Supplementary

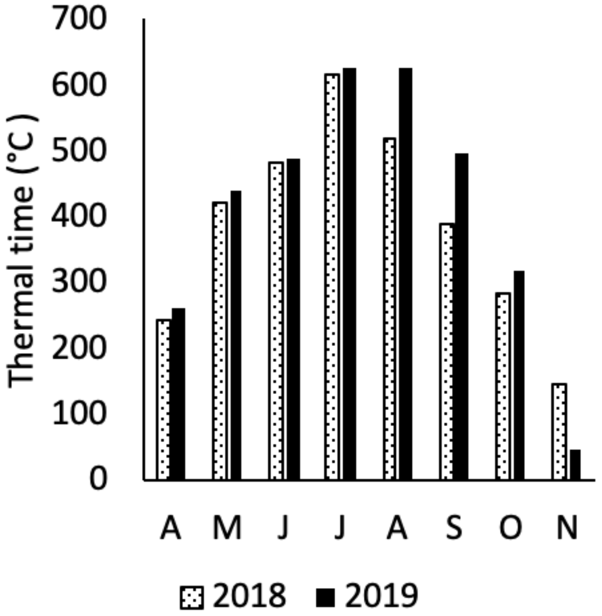

**Figure S1.** Cumulative thermal time for each month from April to November in 2018 and 2019. The thermal time in November is not comparable as harvest was later in 2018.

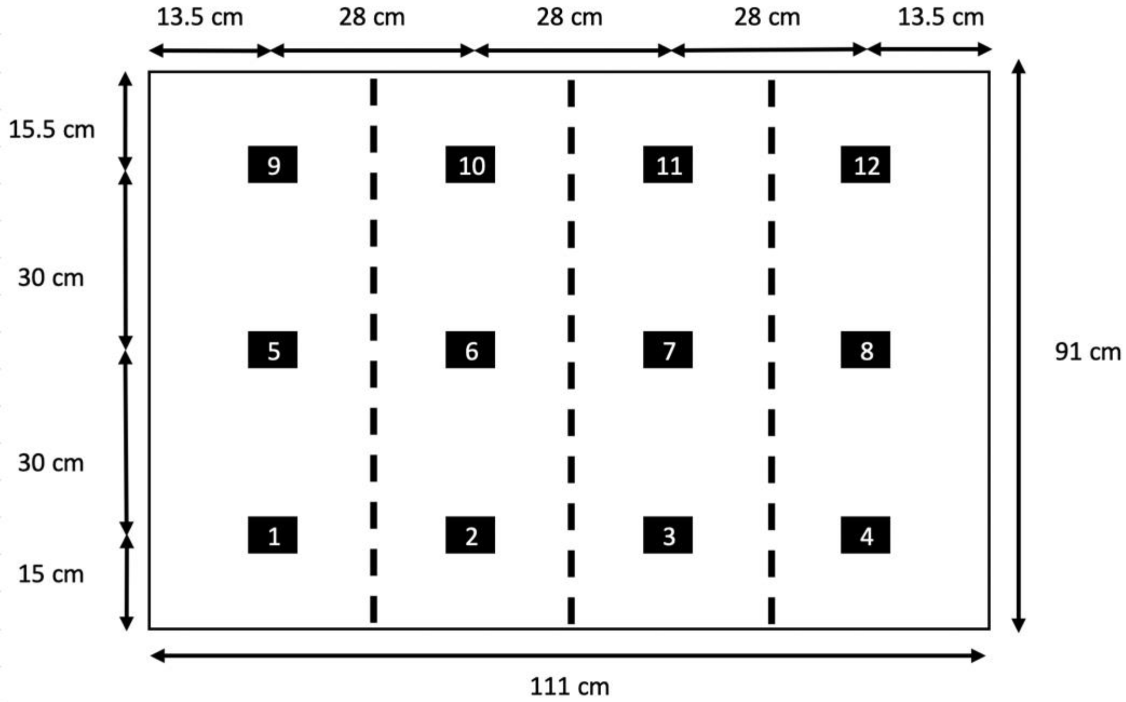

**Figure S2.** The seed spacing for the boxes used to grow sugar beet to examine WUE, each numbered box represents a sowing location and the dashed lines represent the location of the drip irrigation pipes.

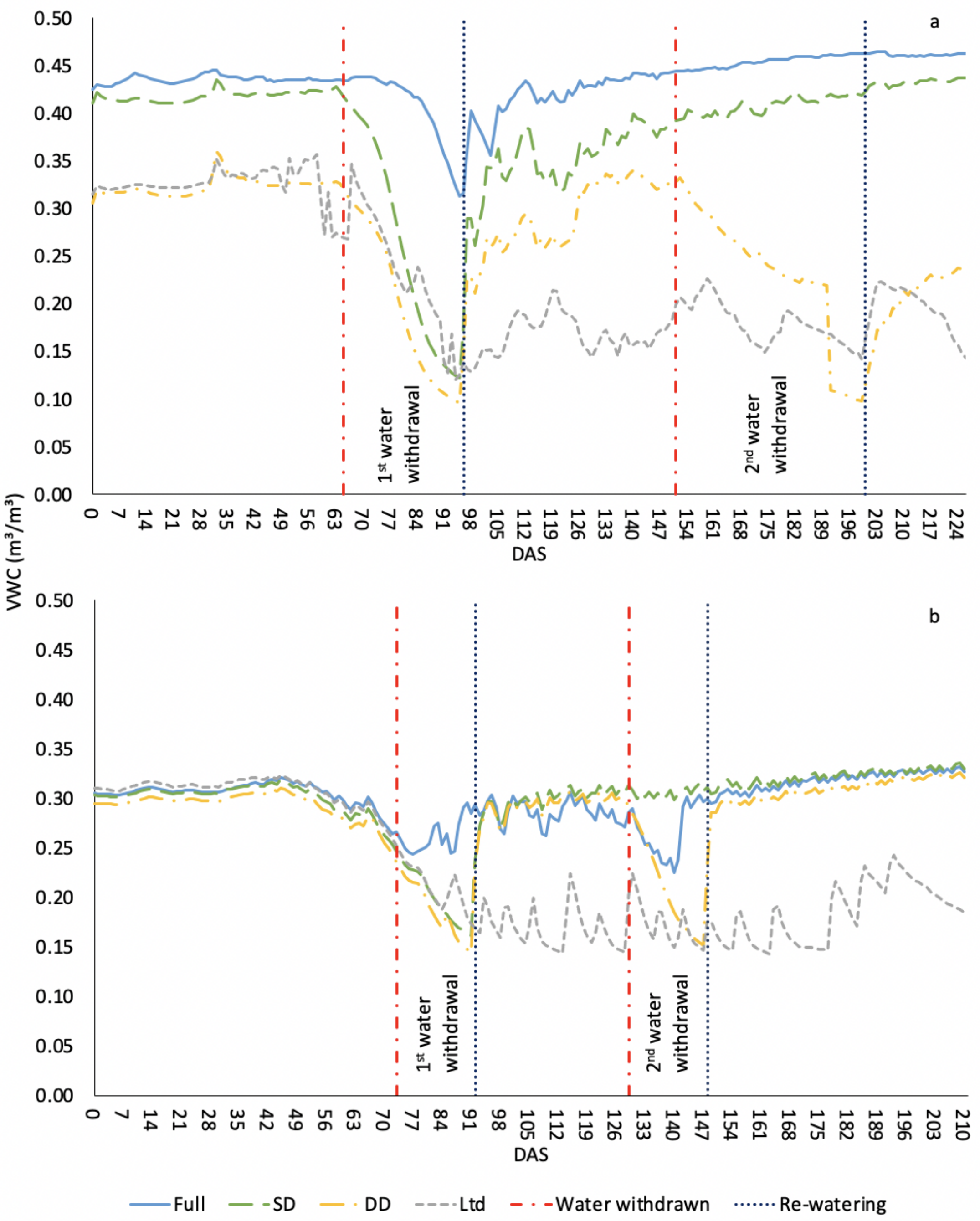

**Figure S3.** The average volumetric water content (VWC) of soil in 610L boxes containg 12 sugar beet plants grown under 4 different irrgation regimes in 2018 (a) and 2019 (b). In 2018 water was withdrawn from 65 DAS to 96 DAS for the single drought (SD), whilst the double drought (DD) was exposed to a second water withdrawal period from 151 DAS to 200 DAS. In 2019 water was withdrawn from 73 DAS to 92 DAS for the single drought (SD), whilst the double drought (DD) was exposed to a second water withdrawal period from 129 DAS to 148 DAS. No water was withdrawn from the fully irrigated (Full) or the water limited (Ltd) treatment boxes.

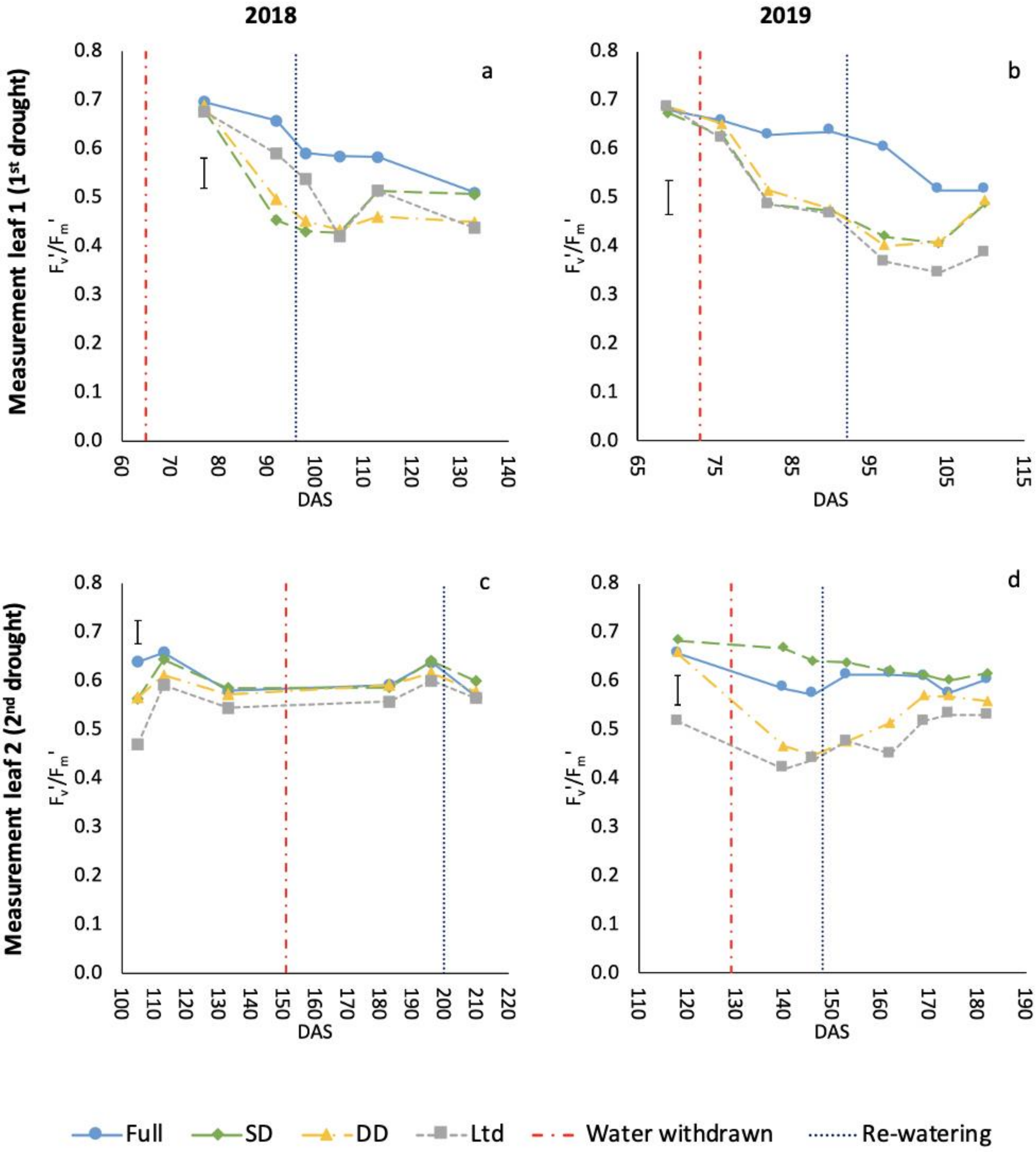

**Figure S4.** The $F_v$/’$F_m$’ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P<0.001* LSD=0.063 DF=190) and 2019 (b) (*P<0.001* LSD=0.071 DF=215) and measurement leaf 2 the second drought in 2018 (c) (*P=0.017* LSD=0.047 DF=191) and 2019 (d) (*P<0.001* LSD=0.061 DF=253). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS.

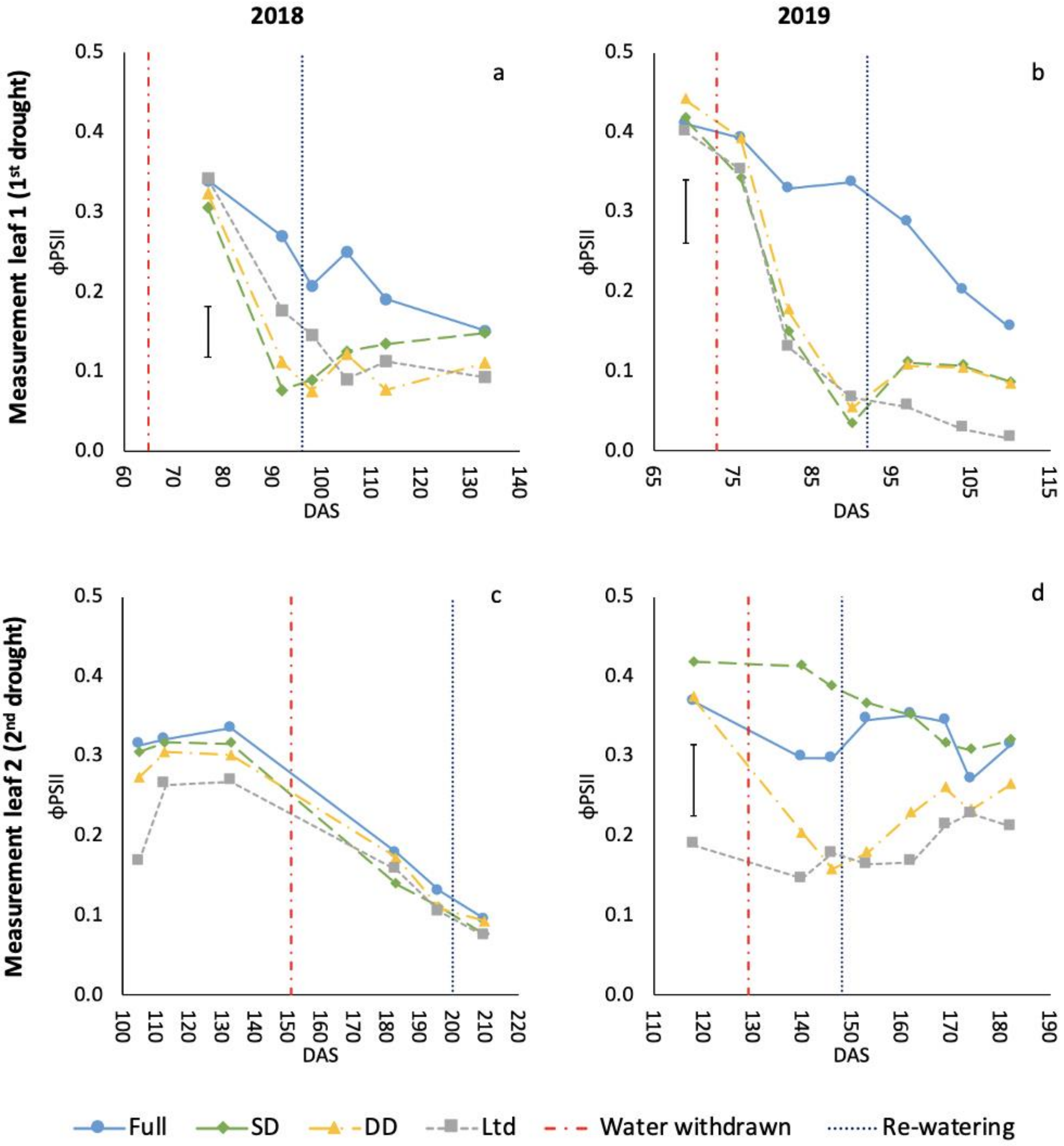

**Figure S5.** The ΦPSII of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P<0.001* LSD=0.064 DF=190) and 2019 (b) (*P<0.001* LSD=0.080 DF=207) and measurement leaf 2 the second drought in 2018 (c) (*P=0.144*) and 2019 (d) (*P<0.001* LSD=0.089 DF=253). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS.

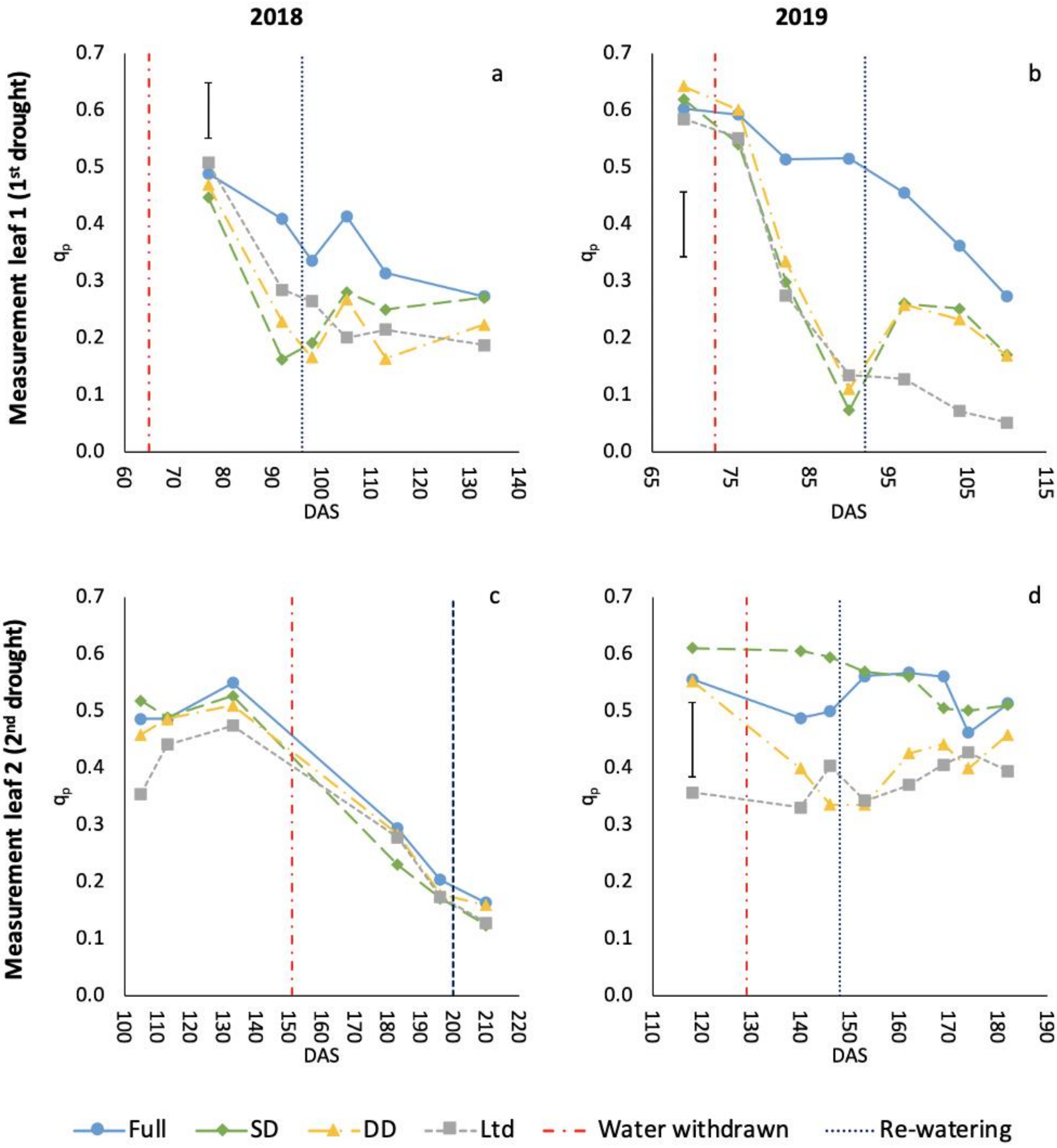

**Figure S6.** The $q_p$ of sugar beet grown under four irrigation regimes, measured using an infrared gas analyser (Li6800, LI-COR, Lincoln, Nebraska, USA). Measurement leaf 1 covers the first drought in 2018 (a) (*P=0.002* LSD=0.098 DF=190) and 2019 (b) (*P<0.001* LSD=0.115 DF=207) and measurement leaf 2 the second drought in 2018 (c) (*P=0.254*) and 2019 (d) (*P=0.011* LSD=0.130 DF=253). Error bars show time*irrigation LSD. Irrigation regimes were a fully irrigated (Full), a continually water limited kept at approx. 50% field capacity (Ltd), a single drought (SD) (2018 65-96 DAS and 2019 73 -92 DAS) and a double drought (DD) which was exposed to the single drought treatment plus an additional drought (2018 151-200 DAS and 2019 118-182 DAS.

# Chapter 4: Diurnal responses of sugar beet and the subsequent effect on water use efficiency

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## 4.1 Abstract

Intrinsic water use efficiency ($WUE_i$), calculated by dividing assimilation (A) by stomatal conductance ($g_s$), is often assessed at one timepoint during the day despite diurnal changes in the environment such as PPFD and temperature, which drive changes in $g_s$ and A. To fully characterise $WUE_i$ repeated measurements of leaf gas exchange over the day can provide a more representative assessment than singular measurements. This approach was used to assess how $WUE_i$ and leaf water content of sugar beet changes over the day, as the crop is observed to wilt even when water is freely available which could be linked to high rates of $g_s$. Additionally, five commercial UK varieties were used to examine if varietal differences in diurnal $WUE_i$ and leaf water content were evident in varieties bred for the same climate, and plants were exposed to a water deficit to identify the affect of reduced water availability on these parameters. There was also variation in the canopy architecture between the varieties with some having noticeably more upright or prostrate canopies. The five sugar beet varieties were grown in a controlled environment room (CER) and exposed to a diurnal light, temperature and humidity regime, with watering

withdrawn to induce a water deficit before rewatering. Gas exchange measurements of $g_s$ and A were used to calculate $WUE_i$, leaf temperature was assessed, and spectral reflectance measurements of leaves taken to calculate the normalised difference water index (NDWI) at four time points each day. The results showed that $WUE_i$ declined over the day as A and $g_s$ increased in response to increasing PPFD and temperature but recovered late in the day as these declined. Water deficit decreased A and $g_s$, which showed a reduced magnitude of response to increasing PPFD and temperature and resulted in a greater $WUE_i$ and increased leaf temperature. There was a trend of greater $WUE_i$ in Cayman than BTS1140 and Sabatina, suggesting there are differences in $WUE_i$ between commercial UK sugar beet varieties. NDWI showed a similar pattern to $g_s$ and the decline over the middle of the day could help to explain the transient wilting response often observed in sugar beet in the field.

## 4.2 Introduction

The efficiency with which crops use water has been a focus in many of the major crop species grown in regions where limited water availability is a threat due to climate change. Through the work of Farquhar and Richards (1984), which highlighted $^{13}C$ isotope discrimination ($\Delta^{13}C$) as a tool for selecting more water use efficient (WUE) plants, commercially viable wheat varieties with an increased WUE have been developed (Rebetzke et al., 2002, Condon et al., 2004). Crops which are predominantly grown in temperate climates have not received the same level of research interest as major crop species, such as wheat and maize, because water availability has not previously been a consistent threat to yield. As climate change now threatens to reduce water availability in some temperate regions, including parts of Europe (Maracchi et al., 2005) and North America (Motha and Baier, 2005) it is important to understand how crop species, which have not benefitted from a concerted research effort to deliver improvements in crop resilience, use water. One such crop is sugar beet, grown in many regions in the northern hemisphere, including Europe which accounts for approximately 70% of the world's sugar beet production by weight (FAO, 2020). Climate forecasts suggest that in the western regions of Europe, including the UK, this crop is at risk from increasingly drier summers (Jones et al., 2003) and therefore the efficiency with which it uses water requires further understanding. This will enable approaches to maximise the crop's water use efficiency to be identified and to reduce the threat from declining water availability.

Water use efficiency can be examined at the leaf or the crop level using a variety of methods. At the leaf level it can be calculated by dividing assimilation (A) by stomatal conductance to water vapour ($g_s$) to give intrinsic water use efficiency ($WUE_i$) or transpiration (E) to give instantaneous water use efficiency ($WUE_t$) (Hatfield and Dold, 2019). At the crop level, WUE can refer to the amount dry matter (DM) accumulated by the crop divided by the amount of water transpired ($WUE_{DM}$) (Boyer, 1996). The values used to calculated crop level WUE can be adjusted to take account of other factors for example the use of evapotranspiration, which takes account of water lost through soil evaporation in addition to transpiration (Sinclair et al., 1984), and the harvest index of the crop, as opposed to the total dry matter (Richards et al., 2002). A relationship between $WUE_i$ and $WUE_{DM}$ is not always evident and the reasons for this have been widely studied in grapevines (Poni et al., 2009, Tomás et al., 2012). Canopy structure can lead to large variations between leaves, meaning leaf measurements of $WUE_i$ are not representative of whole canopy $WUE_i$ (Medrano et al., 2012), and errors in measuring dark respiration can lead to carbon losses which are not accounted for in leaf gas exchange measurements (Medrano et al., 2015). Despite potential limitations in solely assessing $WUE_i$ the success in developing more WUE, wheat varieties through an understanding of $WUE_i$ suggests that it is worth assessing in other crops such as sugar beet.

Studies on WUE in sugar beet have been focused on identifying drought tolerant varieties. It has been shown that some varieties have an increased $WUE_{DM}$ which is driven by an increase in DM accumulation as opposed to a reduction in water use (Ober et al., 2005, Rajabi et al., 2009), although differences in $WUE_{DM}$ between varieties are not always evident (Rytter, 2005, Bloch et al., 2006). An increase in biomass as opposed to decreasing water use has also been evidenced as the driver of increased $WUE_{DM}$ in nematode tolerant and resistant sugar beet varieties (Hauer et al., 2015). Work has also been undertaken to understand how WUE in sugar beet changes under drought. WUE increases under drought as stomata begin to close and, as the forces driving transpiration are a magnitude greater than those driving $CO_2$ uptake, a greater reduction in transpiration than assimilation results (Zhang and Davies, 1990) and $WUE_i$ is increased (Meinzer et al., 1990). This response has been evidenced in sugar beet with both $WUE_{DM}$ (Rytter, 2005, Bloch et al., 2006, Ober et al., 2005) and $WUE_i$ (Bloch et al., 2006) increasing under drought. $WUE_{DM}$ and $WUE_i$ are therefore understood to some extent in sugar beet, with $WUE_{DM}$ being of greater focus despite the success in producing more WUE wheat by focusing on $WUE_i$. Many studies on $WUE_i$, including those on sugar beet, take measurements at one timepoint and under set conditions. Such approaches can be used to understand $WUE_i$ in crops (Anyia and Herzog, 2004, Liu et al., 2006, Kaminski et al., 2014) but multiple measurements across the day (Zur and Jones, 1984, Kumar et al., 2000, Medrano et al., 2012), or under dynamic conditions that plants would encounter in the field (McAusland et al., 2016, Vialet-Chabrand et al., 2016), enable a more comprehensive assessment of $WUE_i$. Alternatively $\Delta^{13}C$ provides an integrated measure of $WUE_i$ overtime which can give a more stable assessment of $WUE_i$ than gas exchange measurements but does not capture how the plant responds diurnally, and the effect this has on $WUE_i$, which can help identify if the plant is responding optimally to the environment to maximise $WUE_i$.

One study has been undertaken in sugar beet to understand how rapid onset artificial light regimes affect the regulation of photosynthesis compared to gradual onset light regimes, which are more representative of the natural environment (Geiger et al., 1991). The rapid light regime exposed the plant to the maximum irradiance of 800 $\mu mol\ m^{-2}\ s^{-1}$ for a 14-hour period whilst the gradual onset regime increased irradiance gradually to reach maximum irradiance halfway through this time period, before declining gradually back to darkness by the end of the 14 hours. Assimilation (A), $g_s$, leaf relative water content, ribulose 1,5-bisphosphate (RuBP) levels and Rubisco activation were measured. The interest in leaf water status arises from the tendency of sugar beet to wilt freely in the field, even when water is available, which may be linked to its anisohydric responses whereby stomata remain open as leaf water potential ($\Psi_{leaf}$) declines (Sade et al., 2012, Meinzer et al., 2017). The implication of this response on photosynthesis and crop yield is not fully understood. The study showed that two light regimes resulted in differences in the levels of RuBP and activation of Rubisco, but that this did not cause significant differences in the rate of photosynthesis. Additionally, under the rapid onset light regime, transpiration increased rapidly and led to wilting in some leaves, but an opportunity to assess $WUE_i$ was missed. This means that $WUE_i$ has not been assessed in sugar beet over the course of a day or under dynamic light.

Overall the difference in $WUE_{DM}$ between sugar beet varieties and the effect of drought is already an area of focus (Ober et al., 2005, Rytter, 2005, Rajabi et al., 2009, Bloch et al., 2006), and it is known how $WUE_i$ is affected by drought in sugar beet (Bloch et al., 2006). However, changes in $WUE_i$ over the course of the day and the effect of reduced water availability on $WUE_i$ over the day, or if varietal differences are evident, have not be assessed in sugar beet. Additionally, a reduction in RWC over the day has been evidenced, but whether this differs between varieties or is related to wilting has not been explored. Therefore, this study aims to address the following research questions and hypotheses:

1. What is the diurnal change in $WUE_i$ in sugar beet and does this differ between varieties or under reduced water availability?

   *Hypothesis: WUE decreases as stomata open in response to increasing PPFD and decreases less in some varieties and in response to water deficit.*

2. What is the change in leaf water content in sugar beet diurnally, and could this explain why the crop wilts so freely compared to other crop species?

   *Hypothesis: Leaf water content declines as $g_s$ increases in response to increasing PPFD which leads to sugar beet wilting.*

## 4.3 Materials and methods

### 4.3.1 Plant material

Sugar beet (*Beta vulgaris,* ssp. *vulgaris*) was grown in 5L pots in a two-thirds Kettering loam and one-third sand mix. Three seeds were sown per pot before thinning at 27 DAS. Five sugar beet cultivars from the British Beet Research Recommended list were grown (cv. Cayman, cv. Sabatina, cv. BTS1140, cv. Degas and cv. Haydn) with four replicates of each arranged in a randomised block design with these varieties representing a range of upright and prostrate canopy architecture (Cayman – Prostrate, Sabatina – Upright, BTS1140 - Upright, Degas – Prostrate and Haydn - Upright). As all of the varieties selected have been bred for the UK climate varietal differences could not be attributed to the breeding to suit different climates. Instead it could be identified if differences between varieties bred to succeed in the same climatic conditions were present. Pots were placed on benches in a controlled environment room (CER) (BDW80, Conviron, Winnepeg, Manitoba, Canada) and hand watered. Plants received 1.50g N in the form of ammonium nitrate fertiliser in a split application equal to 0.75g of N at 34 and 41 DAS. Light in the CER was provided by LEDs (BX - NS1 Spectrum, Valoya, Melkonkatu, Helsinki, Finland) which could produce a maximum PPFD of 1000 µmol $m^{-2}$ $s^{-1}$. From 0-108 DAS CER settings were as follows; maximum and minimum temperature 12°C ± 4°C, RH 65% ± 25%, 12-hour day length and a maximum PPFD of 600 µmol $m^{-2}$ $s^{-1}$. PPFD was ramped up for an hour after dawn, from 6:00, and down for an hour before dusk at 18:00. These settings were selected to prevent Aphanomyces root rot (*Aphanomyces cochlioides*) in the seedlings. Settings were gradually changed from 109 -117 DAS to reach a final maximum temperature of 26°C ± 4°C, RH 70% ± 25%, day length of 16 hours and maximum PPFD of 1000 µmol $m^{-2}$ $s^{-1}$. These settings were chosen to simulate a UK summer day in July, with an above average maximum temperature to ensure high rates of A and $g_s$. At 109 DAS the RH was increased to 70% ± 25% and the maximum PPFD to 1000 µmol $m^{-2}$ $s^{-1}$. From 109-112 DAS the day length was increased by 1 hour by moving dawn 30 mins earlier and dusk 30 mins later each day resulting in a final day length of 16 hours with dawn at 4:00 and dusk at 19:45. The LEDs in the CER could not produce a PPFD lower than 120 µmol $m^{-2}$ $s^{-1}$ and therefore light could not be ramped up or down below this value. For this reason from dawn until 7:30 PPFD was set at 120 µmol $m^{-2}$ $s^{-1}$, before increasing continuously to reach the maximum 1000 µmol $m^{-2}$ $s^{-1}$ at 12:00 when PPFD was then reduced continuously until 16:30 when PPFD was set to 120 µmol $m^{-2}$ $s^{-1}$ until dusk. The maximum temperature was increased by 2°C ± 4°C each day from 109-113 DAS and then 1°C ± 4°C each day from 114 -117 DAS to reach the final maximum of 26°C ± 4°C, which continued until the plants were harvested at 130 DAS. Each day the temperature continually increased from 4:15 to reach the maximum at 13:45 and then continually decreased to return to the minimum at 4:15. CER settings were monitored using the inbuilt temperature, humidity and light sensors and an additional temperature and humidity logger (TinyTag Ultra 2, Gemini data loggers, Chichester, West Sussex, UK).

### 4.3.2 Water withdrawal

Soil moisture was reduced by withholding watering from 120-125 DAS, with re watering at 126 DAS, after the day's measurements had been taken, and monitored using a capacitance soil moisture probe (ML 3 ThetaProbe, Delta T, Cambridge, Cambridgeshire, UK). Average soil moisture across all pots was; 17.4%, 13.3%, 10.5% and 23.1% at 122, 124, 126 and 129 DAS respectively.

### 4.3.3 Gas exchange, spectral reflectance indices and leaf temperature measurements

The newest fully expanded leaf on each plant was tagged at 115 DAS and used for the leaf temperature, leaf gas exchange and spectral reflectance measurements which were taken at four time points during the day at 10:00, 12:00, 14:00 and 15:30 with this repeated at 122, 124, 126 and 129 DAS. Leaf temperature was measured using a handheld thermal camera (C2, FLIR, Wilsonville, Oregon, USA) before gas exchange measurements were taken using an infrared gas analyser (IRGA) (Li6800, LI-COR, Lincoln, Nebaska, USA) with a clear chamber that had a 1x3cm aperture (6800-12A, LI-COR, Lincoln, Nebaska, USA) so that PPFD was provided by the CER LEDs. The PPFD at the level of the clear chamber was measured using the quantum sensor (Li190-R LI-COR, Lincoln, Nebaska, USA) attached to the IRGA and was lower at the level of the clear chamber than the CER PPFD setting because the plants were located and measured approximately 1 m below the LED light source. The average PPFD at the level of the clear chamber across the 35-minute measurement period was 515 µmol $m^{-2}$ $s^{-1}$, 770 µmol $m^{-2}$ $s^{-1}$, 499 µmol $m^{-2}$ $s^{-1}$ and 201 µmol $m^{-2}$ $s^{-1}$ at

10:00, 12:00, 14:00 and 15:30 respectively. Two infrared gas analysers were used and plants from blocks one and two were measured at the same time followed by blocks three and four. All measurements were completed within 35 minutes of the 10:00, 12:00, 14:00 or 15:30 start time. From the start to the end of each 35 min measurement period the change in the CER PPFD was <120 µmol $m^{-2}$ $s^{-1}$, temperature <1.5°C and RH <3%. The infrared gas analyser settings were; flow 500 µmol $s^{-1}$, reference $CO_2$ 400 µmol and RH 70%, with matching every 10 mins. The settings resulted in a VPD of 0.9 ± 0.15 in the IRGA chamber. Leaf temperature was set to match the CER settings as follows; 22°C at 10:00, 24°C at 12:00, 26°C at 14:00 and 24°C at 15:30. A comparison of the average CER and IRGA chamber environment at each measurement time point is available in Supplementary Table S1. Gas exchange data of assimilation (A) and stomatal conductance ($g_s$) was used to calculate intrinsic water use efficiency ($WUE_i$) (Condon et al., 2002):

$$WUE_i = \frac{A}{g_s} \tag{1}$$

Immediately after infrared gas analyser measurements were taken spectral reflectance of the same leaf was measured using a spectroradiometer with contact probe (ASD FieldSpec 4 Standard-Res Spectroradiometer, Malvern Panalytical, Malvern, Worcestershire, UK). The normalised difference water index (NDWI) was then calculated (Gao, 1996):

$$NDWI = \frac{(R_{860} - R_{1240})}{(R_{860} + R_{1240})} \tag{2}$$

### 4.3.4 Statistical analysis

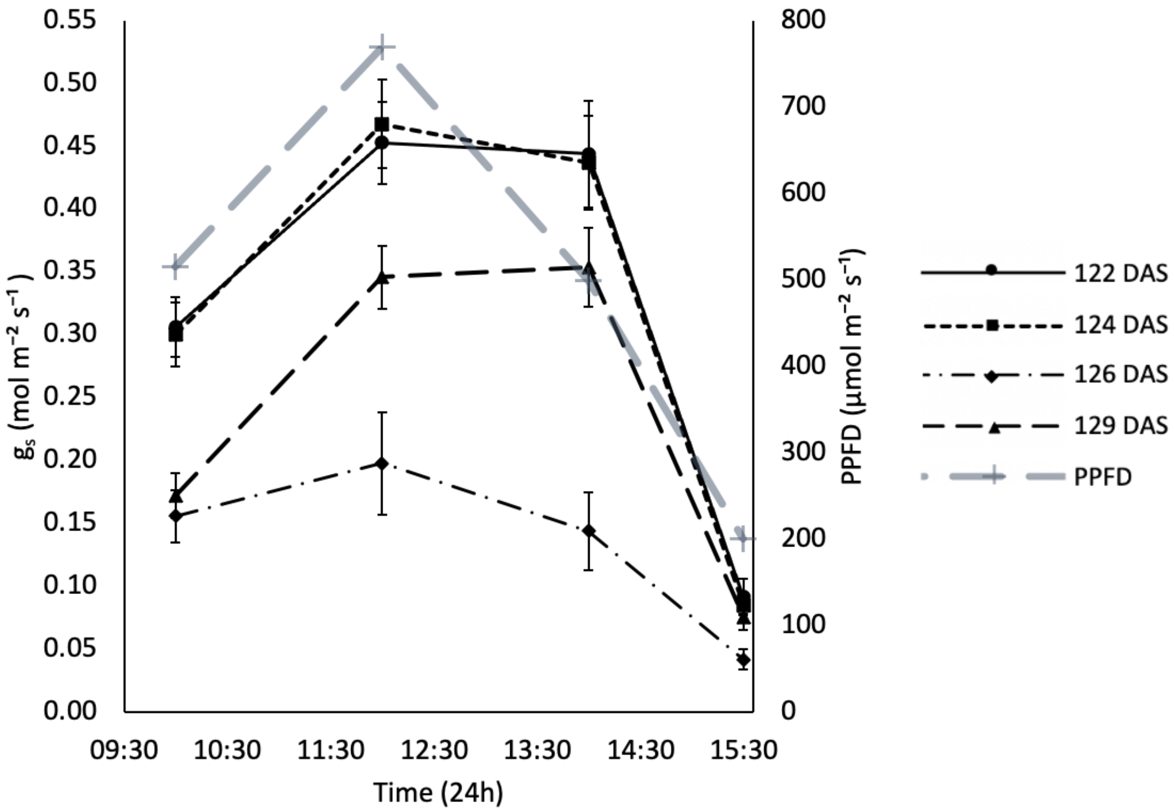
Repeated measures ANOVA for a randomised block design was undertaken on the A, $g_s$, $WUE_i$, leaf temperature and NDWI data with the DAS as a factor. All analysis was undertaken in GenStat 19th edition (VSN International Ltd., Hemel Hempstead, Hertfordshire, United Kingdom).

## 4.4 Results

### 4.4.1 Leaf gas exchange

To examine the gas exchange responses over time on each DAS the data from all the varieties was combined as there were no significant Variety*DAS interactions. This data is presented in a figure to show the trend over the day with an accompanying table showing both the time and DAS means and interaction. The exception to this is $g_s$ as there was a Variety*Time interaction (the $g_s$ of some varieties showed a different diurnal response but this did not differ between DAS) and this is presented separately.

Stomatal conductance increased in response to the increase in PPFD and temperature reaching a maximum between 12:00 and 14:00 ($P<0.001$) except under the highly reduced soil moisture content at 126 DAS. At 122 and 124 days a similar pattern was evident with $g_s$ increasing from c.0.3 at 10.00 to c.0.45 at 12.00, decreasing slightly by 14.00, then a large reduction to c.0.32 at 15.30 (Fig.1). At 126 DAS $g_s$ was less than half the values observed at 122 and 124 DAS and did not significantly change between 10:00 and 14:00 but reached a very low $g_s$ by 15:30 which is evident on all days as light declines and stomata close (Fig.1). Three days after re watering, at 129 DAS, $g_s$ started to recover and the significant increase ($P<0.001$) between 10:00 and 12:00 was again evident although $g_s$ was still significantly lower ($P<0.001$) than at 122 and 124 DAS (Fig.1).



| Time (24h) | 122 DAS | 124 DAS | 126 DAS | 129 DAS | Mean |
|---|---|---|---|---|---|
| **10:00** | 0.306 | 0.300 | 0.156 | 0.172 | 0.234 |
| **12:00** | 0.453 | 0.468 | 0.198 | 0.346 | 0.366 |
| **14:00** | 0.444 | 0.437 | 0.144 | 0.354 | 0.345 |
| **15:30** | 0.092 | 0.085 | 0.042 | 0.075 | 0.073 |
| Mean | 0.324 | 0.323 | 0.135 | 0.237 | 0.255 |

|  | **P** | **LSD** | **DF** |
|---|---|---|---|
| **DAS** | *<0.001* | 0.058 | 3 |
| **Time** | *<0.001* | 0.028 | 3 |
| **Time*DAS** | *<0.001* | 0.078 | 9 |

**Figure 1.** The stomatal conductance ($g_s$) of sugar beet at 10:00, 12:00, 14:00 and 15:30 at 122, 124, 126 and 129 DAS, averaged across varieties and the average PPFD at each time point. Measured using an infra-red gas analyser (Li800, LI-COR, Lincoln, Nebraska, USA) and with attached quantum sensor (Li190-R, LI-COR, Lincoln, Nebraska, USA). Error bars show ± SE and n=20. Table shows plotted means and means for each time point and day. P values, LSDs and DF from repeated measures ANOVA.

Three of the varieties had noticeably different $g_s$ responses across the day (Fig.2). At 12:00 BTS1140 and Sabatina had a greater $g_s$ than Cayman (*P=0.02*), whilst the other varieties had similar $g_s$. Sabatina $g_s$ continued to increase from 12:00 to 14:00 (*P=0.02*) whilst BTS1140 decreased in a similar pattern of response to the other varieties by 14:00 (Fig.2). At the lower light levels and temperatures at 10:00 and 15:30 no differences in $g_s$ were evident between the varieties (Fig.2).

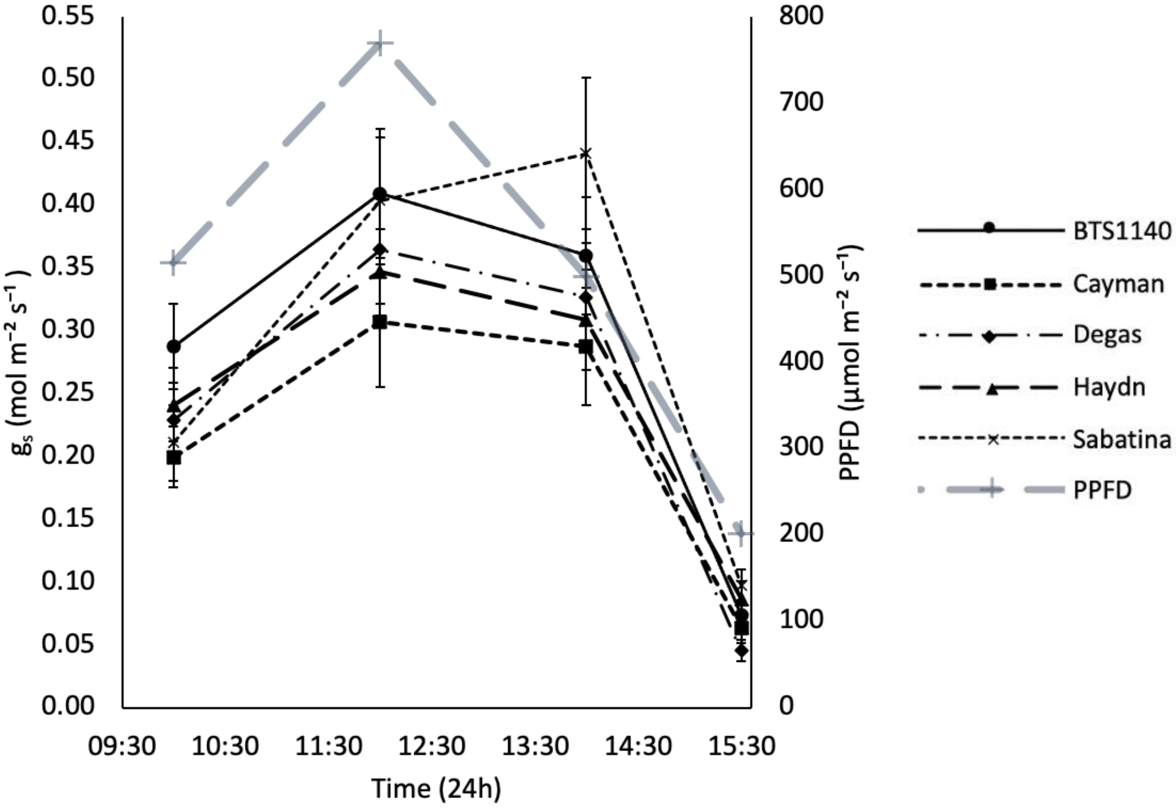
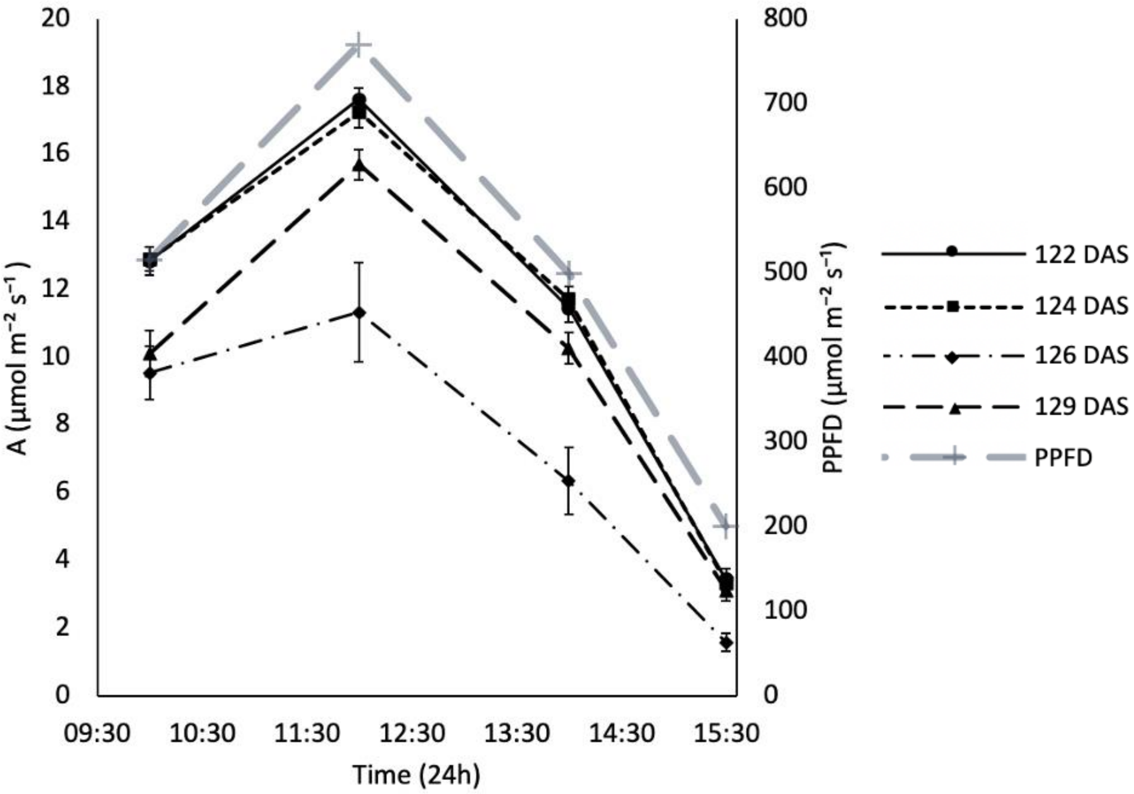


**Figure 2.** The stomatal conductance ($g_s$) of five sugar beet varieties measured at 10:00, 12:00, 14:00 and 15:30 averaged across 122, 124, 126 and 129 DAS and the average PPFD at each time point. Measured using an infra-red gas analyser (Li800, LI-COR, Lincoln, Nebraska, USA) and with attached quantum sensor (Li190-R, LI-COR, Lincoln, Nebraska, USA). Error bars show SE ± and n=4. Repeated measures ANOVA variety*time interaction *P=0.02* LSD=0.087 DF=12.

Assimilation increased with PPFD and temperature to reach a maximum at 12:00 at 122, 124 and 129 DAS but then declined (*P<0.001*) by 14:00 (Fig.3), unlike $g_s$ which remained at a similar level and only declined noticeably between 14:00 and 15:30 (*P<0.001*; Fig.1). The highly reduced soil moisture content at 126 DAS reduced A (*P<0.001*) and no increase was evident between 10:00 and 12:00, but the decline after 12:00 was similar to the other DAS, as PPFD decreased. Assimilation was lower at all-time points at 126 DAS compared to 122 and 124 DAS, when soil moisture content was still declining. After re watering, at 129 DAS, the recovery of A was similar to that of gs, with the pattern of response over the day similar to 122 and 124 DAS but A values lower at each time point (*P<0.001*). Assimilation was similar at 126 and 129 DAS at 10:00 but from 12:00 to 14:00 was greater at 129 DAS showing recovery after re watering, the same as $g_s$ (*P<0.001*).

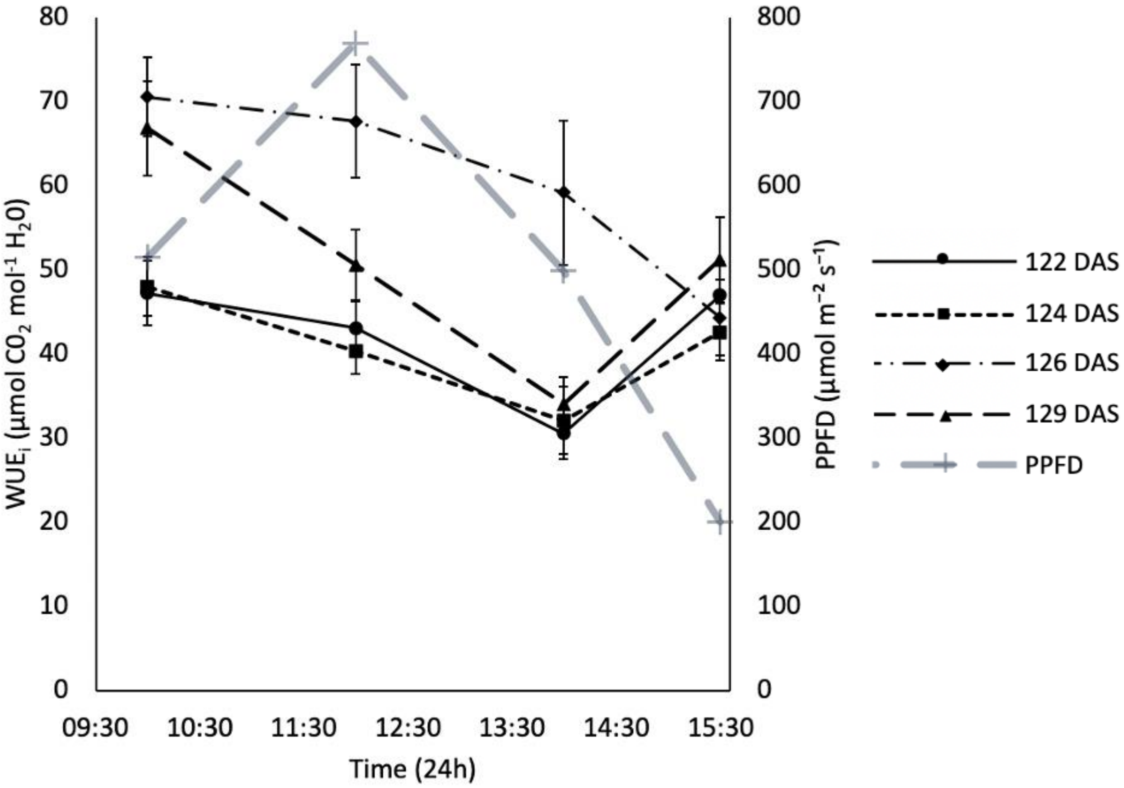


| Time (24h) | 122 DAS | 124 DAS | 126 DAS | 129 DAS | Mean |
|---|---|---|---|---|---|
| **10:00** | 12.85 | 12.91 | 9.54 | 10.13 | 11.36 |
| **12:00** | 17.64 | 17.25 | 11.34 | 15.70 | 15.48 |
| **14:00** | 11.46 | 11.72 | 6.35 | 10.28 | 9.95 |
| **15:30** | 3.45 | 3.33 | 1.58 | 3.13 | 2.87 |
| Mean | 11.30 | 11.35 | 7.20 | 9.81 | 9.92 |
| | | **P** | **LSD** | **DF** | |
| | **DAS** | *<0.001* | 1.35 | 3 | |
| | **Time** | *<0.001* | 0.64 | 3 | |
| | **Time*DAS** | *<0.001* | 1.77 | 9 | |

**Figure 3.** The assimilation (A) of sugar beet at 10:00, 12:00, 14:00 and 15:30 at 122, 124, 126 and 129 DAS and the average PPFD at each time point. Measured using an infra-red gas analyser (Li800, LI-COR, Lincoln, Nebraska, USA) and with attached quantum sensor (Li190-R, LI-COR, Lincoln, Nebraska, USA). Error bars show ±SE and n=20. Table shows plotted means and means for each time point and day. P values, LSDs and DF from repeated measures ANOVA.

$WUE_i$ increased during and after water the highly reduced soil moisture content at 126 DAS and 129 DAS respectively (*P<0.001*; Fig.4), with an overall greater $WUE_i$ at 126 DAS (*P<0.001*) driven by the differences in $g_s$ (Fig.1) and A (Fig.3) at 12:00 and 14:00. The higher $WUE_i$ at 129 DAS at 10:00, 12:00 and 15:30, although only significantly higher at 10:00 (*P<0.001*), resulted in a greater average $WUE_i$ on that day, when plants had been re watered, compared to 122 and 124 DAS (*P<0.001*), when soil moisture was still declining. On all measurement days $WUE_i$ declined throughout the day (*P<0.001*), driven by the greater decline in A than $g_s$ from 12:00 to 14:00 at 122, 124 and 129 DAS. At 126 DAS $WUE_i$ did not change significantly between 10:00 and 14:00 but was lower and similar to the other DAS by 15:30 (*P<0.001*; Fig.4), when $g_s$ had declined to similar levels as the other DAS, and A was lower than that observed at 122 and 124 DAS.

There was a trend (*P=0.058*) of lower $WUE_i$ in BTS1140 compared to Cayman, of 40.52 µmol $CO_2$ $mol^{-1}$ $H_2O$ compared to 54.6 µmol $CO_2$ $mol^{-1}$ $H_2O$, which could be attributed to the greater $g_s$ in BTS1140 (*P<0.001*) coupled with there being no difference in A between the varieties. Sabatina had a similar $WUE_i$ to both at 49.36 µmol $CO_2$ $mol^{-1}$ $H_2O$, despite also having a greater $g_s$ than Cayman (*P<0.001*), but the average A of Cayman was higher than BTS1140, although this was not significant.



| **Time (24h)** | **122 DAS** | **124 DAS** | **126 DAS** | **129 DAS** | **Mean** |
|---|---|---|---|---|---|
| **10:00** | 47.3 | 48.1 | 70.6 | 66.9 | 58.2 |
| **12:00** | 43.1 | 40.4 | 67.7 | 50.6 | 50.4 |
| **14:00** | 30.6 | 32.1 | 59.2 | 34.0 | 39.0 |
| **15:30** | 47.0 | 42.7 | 44.3 | 51.2 | 46.3 |
| Mean | 42.0 | 40.8 | 60.5 | 50.7 | 48.5 |

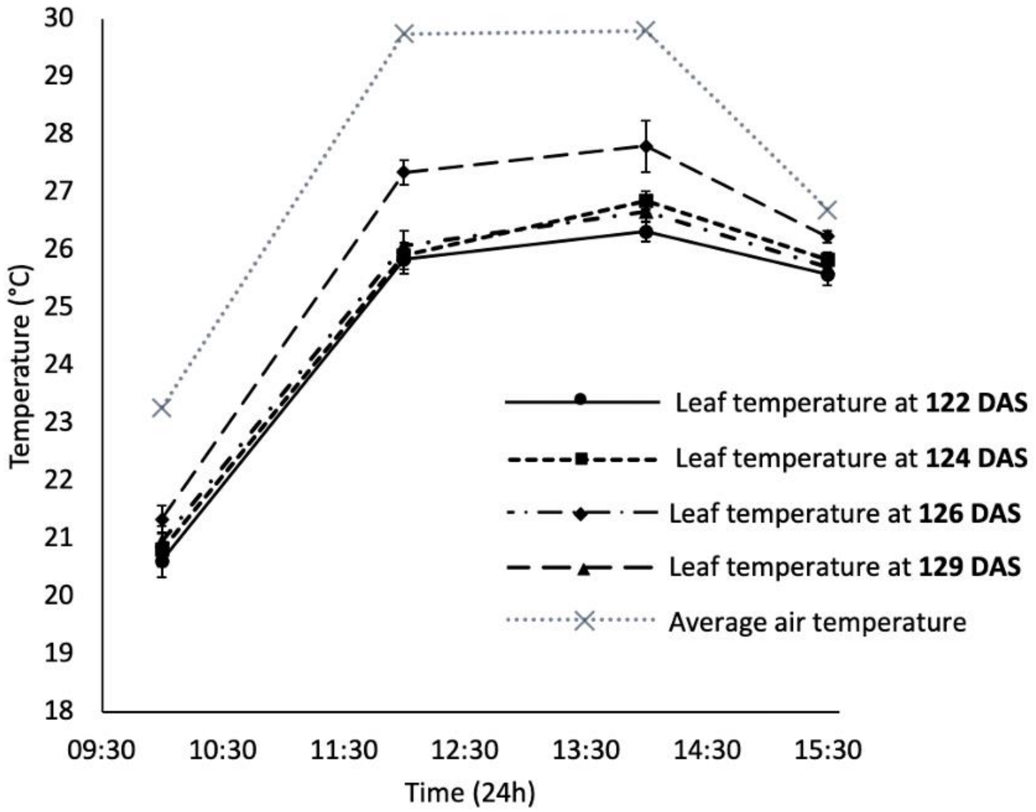
| | **P** | **LSD** | **DF** |
|---|---|---|---|
| **DAS** | *<0.001* | 8.26 | 3 |
| **Time** | *<0.001* | 5.33 | 3 |
| **Time*DAS** | *<0.001* | 12.51 | 9 |

**Figure 4.** The intrinsic water use efficiency (WUE$_i$) of sugar beet at 10:00, 12:00, 14:00 and 15:30 at 122, 124, 126 and 129 DAS and the average PPFD at each time point. Measured using an infra-red gas analyser (Li800, LI-COR, Lincoln, Nebraska, USA) and with attached quantum sensor (Li190-R, LI-COR, Lincoln, Nebraska, USA). Error bars show SE ± and n=20. Table shows plotted means and means for each time point and day. P values, LSDs and DF from repeated measures ANOVA.

### 4.4.2 Leaf temperature

On all DAS leaf temperature changed with the CER air temperature, with a significant increase until the maximum temperature at 14:00 followed by a decrease at 15:30 (*P<0.001*). Leaf temperature was higher under the highly reduced soil water content at 126 DAS (*P<0.001*; Fig.5), and a greater increase in canopy temperature was evident from 10:00 to 12:00 (*P=0.013*).

There was a significant difference in leaf temperature between the varieties (*P=0.008*) with Cayman and Degas having a higher leaf temperature than the other varieties.



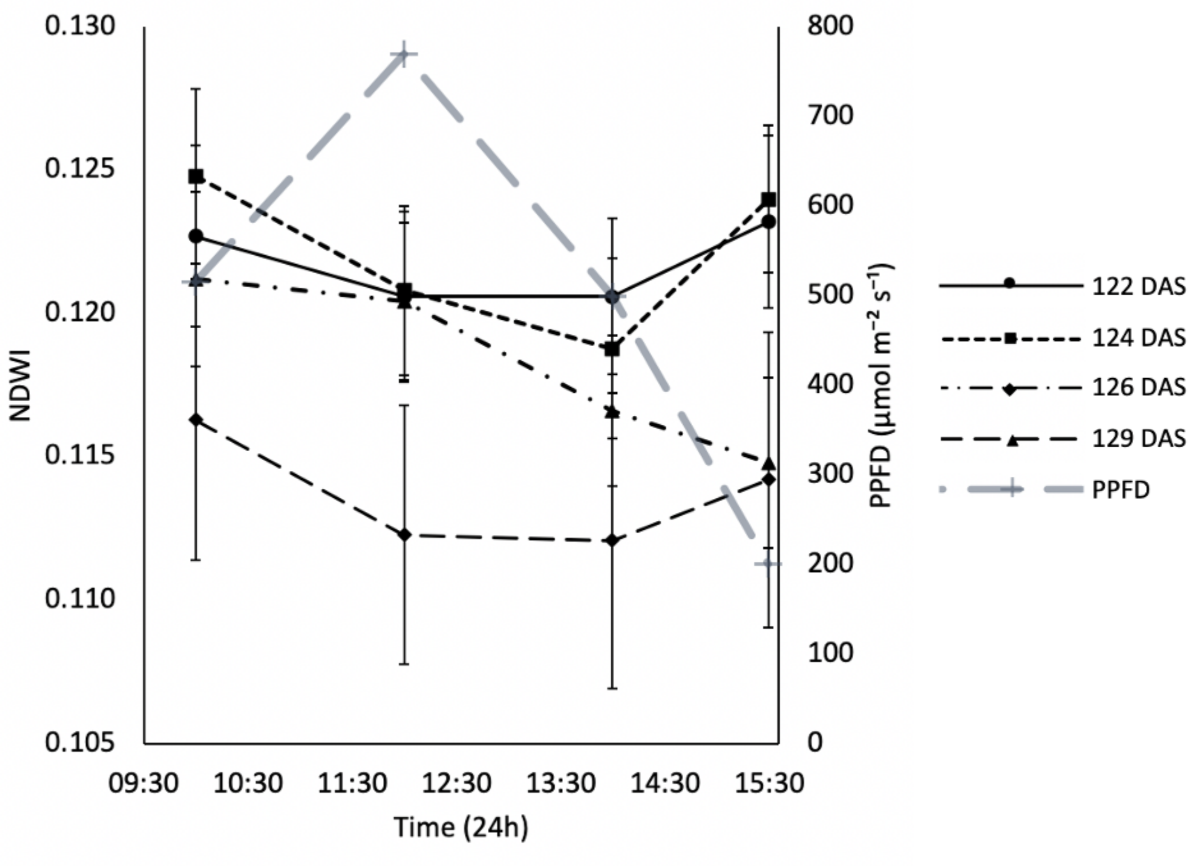
| **Time (24h)** | **122 DAS** | **124 DAS** | **126 DAS** | **129 DAS** | Mean |
|---|---|---|---|---|---|
| **10:00** | 20.61 | 20.81 | 21.34 | 20.97 | 20.93 |
| **12:00** | 25.84 | 25.91 | 27.35 | 26.08 | 26.29 |
| **14:00** | 26.32 | 26.86 | 27.80 | 26.67 | 26.91 |
| **15:30** | 25.58 | 25.84 | 26.24 | 25.71 | 25.84 |
| Mean | 24.59 | 24.85 | 25.68 | 24.85 | 24.99 |

| | **P** | **LSD** | **DF** |
|---|---|---|---|
| **DAS** | *<0.001* | 0.41 | 3 |
| **Time** | *<0.001* | 0.22 | 3 |
| **Time*DAS** | *0.013* | 0.56 | 9 |

**Figure 5.** The leaf temperature of sugar beet at 10:00, 12:00, 14:00 and 15:30 at 122, 124, 126 and 129 DAS and the average air temperature at each time point. Measured using a handheld thermal camera (C2, FLIR, Wilsonville, Oregon, USA) and temperature and humidity logger (TinyTag Ultra 2, Gemini data loggers, Chichester, West Sussex, UK). Error bars show SE ± and n=20. Table shows plotted means and means for each time point and day. P values, LSDs and DF from repeated measures ANOVA.

### 4.4.3 NDWI

NDWI is a non-destructive measurement of leaf water content, with a decrease in NDWI equating to a decrease in leaf water content. NDWI decreased as temperature increased from 10:00 to 14:00, and started to increase, to reach a similar value to that observed at 12:00, by 15:30 (*P<0.001*; Fig.6). A significantly lower NDWI was evident under the highly reduced soil moisture content at 126 DAS between 12:00 and 14:00 compared to 10:00 and 15:30 at 122 and 124 DAS (*P<0.001*). There were no significant differences in NDWI between 126 and 129 DAS, despite the average NDWI being higher from 10:00 to 12:00 at 129 DAS and close to the 122 and 126 DAS values, and the large decline in NDWI at 129 DAS from 12:00 to 15:30. Despite these changes in NDWI no diurnal pattern of wilting and recovery was observed but under water deficit some plants did wilt.



| Time (24h) | 122 DAS | 124 DAS | 126 DAS | 129 DAS | Mean |
|---|---|---|---|---|---|
| **10:00** | 0.123 | 0.125 | 0.116 | 0.121 | 0.121 |
| **12:00** | 0.121 | 0.121 | 0.112 | 0.120 | 0.119 |
| **14:00** | 0.121 | 0.119 | 0.112 | 0.117 | 0.117 |
| **15:30** | 0.123 | 0.124 | 0.114 | 0.115 | 0.119 |
| Mean | 0.122 | 0.122 | 0.114 | 0.118 | 0.119 |

| | P | LSD | DF |
|---|---|---|---|
| **DAS** | *ns* | - | - |
| **Time** | *<0.001* | 0.001 | 3 |
| **Time*DAS** | *<0.001* | 0.010 | 9 |

**Figure 6.** The NDWI of sugar beet at 10:00, 12:00, 14:00 and 15:30 at 122, 124, 126 and 129 DAS. Measured using a spectroradiometer (ASD FieldSpec 4 Standard-Res Spectroradiometer, Malvern Panalytical, Surrey, UK). Error bars show ±SE and n=20. Table shows plotted means and means for each time point and day. P values, LSDs and DF from repeated measures ANOVA.

## 4.5 Discussion

$WUE_i$ and leaf water content in sugar beet varied in response to changes in PPFD and temperature over the course of the day, with soil moisture content altering this response. The difference in $g_s$ between Cayman, BTS1140 and Sabatina coupled with similar levels of A resulted in the trend of higher $WUE_i$ in Cayman, which suggests that $WUE_i$ differs in commercial UK sugar beet varieties (which is consistent with the findings of Chapter three).

Measuring leaf gas exchange at four time points provided a more comprehensive data set to examine $WUE_i$ and highlighted how $WUE_i$ declines as $g_s$ and A increases and when declining PPFD drives a greater reduction in A than $g_s$. A decline in $WUE_i$ over the course of the day has been observed in other species (Zur and Jones, 1984, Kumar et al., 2000) and is driven by the reduction in PPFD leading to a rapid decline in A whilst the temperature and VPD decline is slower, causing transpiration and $g_s$ to be maintained at a higher rate for longer, therefore the ratio of $g_s$ to A is increased, as shown in this study. A similar relationship is observable under shorter term fluctuations in light, such as cloud passing over, where A declines rapidly, whilst $g_s$ shows little or no change (McAusland et al., 2016). Ideally stomata would react rapidly to prevent excessive water loss as light declines over the course of the day to optimise $WUE_i$. However, the processes that drive stomatal closure through changes in guard cell turgor result in a response that is a magnitude slower than A where the photosynthetic reaction can respond almost instantly (Vialet-Chabrand et al., 2016).

The overall difference in $WUE_i$ between the DAS would have been detected by measurements at only one time point per day at the high PPFD and temperature at 12:00 or 14:00. However, differences in the gas exchange parameters between the DAS and varieties were not always evident at 10:00 and 15:30 when PPFD and temperature was lower. This highlights the importance of measuring under high PPFD and temperatures when the force driving transpiration and photosynthesis are high enough to detect differences, but caution must be taken around midday as plants may show varying levels of midday depression (Hirasawa and Hsiao, 1999). This in itself could also be assessed and compared in sugar beet to see if varieties vary in midday depression and he subsequent effect on WUE which was not in the scope of this experiment. Measuring at multiple time points showed that the reduction, or absence, of the rise in $g_s$ and A to increasing PPFD and temperature under reduced soil moisture content was the driver of the differences in $WUE_i$ under reduced water availability. An increase in $WUE_i$ under reduced water availability has been observed in many crop species (Anyia and Herzog, 2004, Singh and Raja Reddy, 2011) including sugar beet (Bloch et al., 2006), as stomatal aperture is reduced and $g_s$ is reduced more than A as the forces driving transpiration are a magnitude greater. However, the reverse is sometimes evident and can be driven by differences in nutrient availability (Shangguan et al., 2000), developmental stage (Kumar et al., 2000) and genotype (Topbjerg et al., 2014). At the low light levels at 15:30, $WUE_i$ under reduced soil moisture content was the same as on the well-watered days. This response may be explained by the observation in grapevines that $WUE_i$ is much lower in shaded leaves in the canopy as the low light means A is low but stomata remain open and $g_s$ remains relatively high (Medrano et al., 2012). It could be that under low light the sugar beet leaves respond in the same way leading to the low $WUE_i$ at low light on all days. At 129 DAS $g_s$ and A increased to values greater than 126 DAS which led to a decline in $WUE_i$. However, $WUE_i$ remained higher than when the soil was drying at 122 and 124 DAS as $g_s$ and A were still lower, with the greatest differences evident at 10:00. At 10:00 the $g_s$ and A values at 129 DAS were similar those observed at 126 DAS but responded more to the increase in PPFD, so by 12:00 $g_s$ and A was greater at 129 DAS but remained lower than at 122 and 124 DAS, when soil moisture content was declining. This suggests that up to 10:00 the plants were slower to open stomata to conserve water than before the highly reduced soil moisture content at 126 DAS, but such adaptions are likely to be short lived as sugar beet have not been shown to adapt under drought as shown by the results in Chapter three and Leufen et al. (2016). Time constraints meant that measurements over an extended period, to see if these differences persisted, were not possible.

The high rates of $g_s$ led to a decline in leaf water content over the day as shown by the NDWI. NDWI is a well-established method of measuring water content in vegetation (Gao, 1996, Zhang and Guo, 2006, Rallo et al., 2014, Marusig et al., 2020). and provided a non-destructive way of measuring leaf water content which allowed repeated measurements of the same leaf. Sugar beet is anisohydric, so the stomata remain open as $\Psi_{leaf}$ falls (Sade et al., 2012, Meinzer et al., 2017), and therefore wilt often (Lawlor and Milford, 1975). This response can be beneficial under short term water deficit, including over the course of the day, as assimilation rates remain high even as leaves wilt (Kohl and Cary, 1969). In this study, the decline in NDWI across the day as $g_s$ increased showed that leaf water content declines as $g_s$ increases but recovers as $g_s$ declines. This has not been shown diurnally in sugar beet before but similar changes in RWC have been evidenced in other species (Rajagopal et al., 1977, Couderchet and Retzlaff, 1995, Chowdhury et al., 2015). These changes in RWC are likely associated with the wilting observed in the field on hot, sunny days when the VPD is high and subsequent recovery of leaf turgor by the following morning. Under the highly reduced soil water content at 126 DAS there was variation in the wilting of the plants, which was unrelated to variety, and may have been due to differences in the rate of drying of the soil between pots. This would not have been detected by the soil moisture probe as it only measured top 5 cm of soil and this may account for the greater SE observed. Despite this variation, an overall reduction in NDWI was evident at 126 DAS and more importantly there was still a decline in NDWI over the middle of the day as the plants still opened stomata, as shown by $g_s$ being above zero. This response shows that, even when wilted with low water availability, sugar beet are still opening stomata and photosynthesising but at a much-reduced rate than when water is freely available. This could be associated with the anisohydric response as the plant's photosynthetic apparatus are able to function under declining leaf water potential (Sade et al., 2012). This resilience is further shown by the recovery at 129 DAS when the plants were no longer wilted, as NDWI returned to the levels observed before the highly reduced soil moisture content at 126 DAS and $g_s$ and A had recovered to levels significantly greater than at 126 DAS. However, these values were lower than at 122 and 124 DAS, before the highly reduced soil water content, showing how A is reduced under low soil moisture content which when prolonged under drought reduces sugar beet yields (Monti et al., 2006). The late decline in NDWI at 129 DAS was not significant but suggests that sugar beet may be prone to wilting while the crop is recovering after leaf water content has severely declined due to a low soil moisture content, although no wilting was evident at 129 DAS.

Leaf temperature often reflects air temperature but is usually slightly lower as transpiration causes evaporative cooling from the sub stomatal cavity (Radin et al., 1994). This was reflected in the sugar beet which had a leaf temperature consistently lower than the air temperature, even under water deficit. Under reduced water availability an increase in leaf temperature was evident and can be attributed to the lower rates of $g_s$, which causes a reduction in the evaporative cooling from the sub stomatal cavity (Blum et al., 1989). It should be noted that the conditions of the IRGA are different to the ambient environment so the observed $g_s$ may be slightly different to the $g_s$ of the section of the leaf imaged with the thermal camera. However, the reduction under water deficit was so large that it is likely to have reduced the evaporative cooling of the leaf. Additionally, it has been evidenced that leaves orientated towards the sun at a horizontal angle are likely to be warmer than those in a more vertical position (He et al., 1996, Kalyar et al., 2013). This could explain the greater canopy temperature of Cayman and Degas as these varieties are from the same breeder and have more prostrate canopies than the other varieties whilst having similar $g_s$.

The lower $g_s$ of Cayman compared to BTS1140 and Sabatina, and consequent trend of greater $WUE_i$ in Cayman shows that varietal differences in $WUE_i$ are evident in commercial sugar beet varieties (and supports the Chapter three $\Delta^{13}C$ results). Differences have been shown in $WUE_{DM}$ but these studies have utilised breeders lines (Ober et al., 2005), varieties from contrasting climates (Rytter, 2005, Bloch et al., 2006) or specialist nematode varieties (Hauer et al., 2015). The trend of greater $WUE_i$ in Cayman compared to other varieties bred for the same climate shows that increased $WUE_i$ could be possible and commercially viable. Additionally, as A was maintained in Cayman despite the lower $g_s$ the greater $WUE_i$ is not reducing overall yield potential, as is sometimes evidenced (Blum, 2009) (which further supports the results of Chapter three, as no difference in $WUE_{DM}$ was evident between Cayman and Sabatina).

The lack of differences in $WUE_i$ and the fact leaf angle was not assessed means that the relationship between $WUE_i$ and leaf angle was not tested. However, leaf angle data is available for all four of the varieties from another unpublished experiment, with Haydn being the exception. This data shows that Sabatina has a consistently more upright canopy and Cayman a consistently more prostrate. Although BTS1140 is observed to have an upright canopy and Degas a prostrate, the data shows that these varieties can have leaf angles less extreme than Sabatina and Cayman respectively. In some of the canopy angle assessments from the unpublished experiments they are observed as having similar leaf angles. BTS1140 had a higher $g_s$ than Cayman, as did Sabatina, but none of the three varieties were different to

Degas. This suggests that differences in $WUE_i$ may only be evident when comparing varieties with canopies that are distinctly upright and prostrate.

Despite the benefits of measuring $WUE_i$ over the course of the day the approach is still limited by measuring one leaf within the canopy. Leaves respond differently throughout the day depending on where they are situated in the canopy, with this driven by differences in light interception and VPD. Therefore, leaf $WUE_i$ doesn't always correspond to plant $WUE_i$ (Poni et al., 2009, Medrano et al., 2012, Tomás et al., 2012). It has even been suggested that removing old and shaded leaves with low $WUE_i$ could increase overall plant WUE in grapevines (Medrano et al., 2012). To expand on the measurements taken at multiple times in the day it would be useful to also measure multiple leaves within the canopy to examine whole plant $WUE_i$ in sugar beet. This was not possible in this study as time in the CER was limited to prevent significant increases in $CO_2$. The suggestion of low $WUE_i$ is also of interest as older sugar beet leaves often wilt under reduced water availability and subsequently come in to contact with the hot soil surface where they are scorched and rapidly senesce. If these older leaves have a low $WUE_i$, as is evidenced in grapevines, whole plant WUE could be increased, and this is an area for further research. In addition to this the relationship between $WUE_i$ and $WUE_{DM}$ needs to be further assessed in sugar beet to ensure that a greater $WUE_i$ results in an overall more WUE crop. If this is the case then it will show breeders that greater $WUE_i$ is a viable selection trait in commercial beet varieties.

## 4.6 Conclusions

Overall, measuring leaf gas exchange, NDWI and leaf temperature throughout the day identified differences that would not have been detected by single measurements which enabled the aims of the study to be addressed. $WUE_i$ was shown to decrease over the day and there was a trend of greater $WUE_i$ in Cayman than BTS1140 and Sabatina. NDWI fluctuated over the day, which could help to explain the witling response often observed in sugar beet. The effect of reduced water availability was also shown, with the main driver of the daily reduction in $g_s$ and A, and the subsequent increase in $WUE_{i,}$ being the lack of response to increasing PPFD and temperature. Further studies are needed to assess if $WUE_i$ corresponds $WUE_{DM}$ in sugar beet. This research can then be used to encourage breeders to develop more WUE sugar beet varieties to secure sugar beet production in the many areas facing reduced water availability around the world.

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## 4.9 Supplementary

**Table S1.** The average PPFD, air temperature, leaf temperature, relative humidity (RH) and vapour pressure deficit (VPD) of a controlled environment room (CER) and IRGA chamber (IRGA) from a 35-minute measurement period starting at 10:00, 12:00, 14:00 and 15:30.

| **Time (24h)** | **PPFD[1] ($\mu$mol m$^{-2}$ s$^{-1}$)** | | **Air temp[2] (°C)** | | **Leaf temp[3] (°C)** | | **RH[2] (%)** | | **VPD[5] (kPa)** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *CER* | *IRGA* | *CER* | *IRGA* | *CER* | *IRGA* | *CER* | *IRGA* | *CER* | *IRGA* |
| **10:00** | 711 | 515 | 23.3 | 21.9 | 20.9 | 22.0 | 63.7 | 69.9 | 0.8 | 0.81 |
| **12:00** | 989 | 770 | 29.8 | 24.0 | 26.3 | 24.1 | 58.5 | 69.9 | 1.7 | 0.91 |
| **14:00** | 571 | 499 | 29.8 | 26.0 | 26.9 | 26.0 | 62.8 | 69.9 | 1.4 | 1.01 |
| **15:30** | 213 | 201 | 26.7 | 23.8 | 25.3 | 24.0 | 69.1 | 69.8 | 1.3 | 0.93 |

[1] *PPFD for the CER was taken from the LED settings, and for the IRGA chamber measured using the attached quantum sensor (Li190-R LI-COR, Lincoln, Nebaska, USA).*

[2] *Air temperature and relative humidity was measured in the CER using a temperature and humidity sensor logger (TinyTag Ultra 2, Gemini data loggers, Chichester, West Sussex, UK), and for the IRGA chamber using the inbuilt sensors (Li800, LI-COR, Lincoln, Nebraska, USA).*

[3] *Leaf temperature for the CER was measured using a thermal camera (C2, FLIR, Wilsonville, Oregon, USA), and for the IRGA chamber using the inbuilt thermocouple (Li800, LI-COR, Lincoln, Nebraska, USA).*

[4] *VPD was calculated from the thermal camera and temperature and humidity sensor data for the CER, and for the IRGA chamber using the inbuilt sensors (Li800, LI-COR, Lincoln, Nebraska, USA).*

# Chapter 5: General discussion and conclusions

## 5.1 Introduction

The aim of the work presented in this thesis was to gain a greater understanding of water use efficiency in sugar beet by addressing the six research questions and hypotheses highlighted by the literature review in Chapter one, and outlined again here under the chapter in which they are addressed:

**Chapter 2: Anisohydric sugar beet rapidly responds to light to optimise leaf water use efficiency utilising numerous small stomata**

1. How quickly are sugar beet stomata able to respond to increasing light and is the speed of stomatal response related to anisohydric behaviour in sugar beet?

   *Hypothesis: Slow stomatal closure in sugar beet is attributed to a low stomatal density and large stomatal size which leads to a disconnect between* $g_s$ *and A and excessive water loss from transpiration.*

**Chapter 3: Water use efficiency in contrasting commercial sugar beet varieties in response to fluctuating water availability**

2. How is WUE affected by fluctuations in soil water availability in sugar beet?

   *Hypothesis: Sugar beet Intrinsic water use efficiency (*$WUE_i$*) and dry matter water use efficiency (*$WUE_{DM}$*) increase under water deficit.*
3. Does sugar beet acclimate (defined as permanent changes in physiology opposed to short term changes such as reduced leaf gas exchange) to water deficit to increase WUE?

*Hypothesis: Sugar beet shows no long-term physiological acclimation in response to water deficit due to its anisohydric behaviour.*

4. Are there differences in WUE between commercial sugar beet varieties with contrasting canopy architecture and does stomatal and leaf morphology vary between these varieties?

   *Hypothesis: Contrasting canopy architecture leads to differences in WUE and can be attributed to differences in stomatal and leaf morphology*

**Chapter 4: Diurnal responses of sugar beet and the effect on water use efficiency**

5. What is the diurnal change in $WUE_i$ in sugar beet and does this differ between varieties or under reduced water availability?

   *Hypothesis: WUE decreases as stomata open in response to increasing PPFD and decreases less in some varieties and in response to water deficit.*

6. What is the change in leaf water content in sugar beet diurnally, and could this explain why the crop wilts so freely compared to other crop species?

   *Hypothesis: Leaf water content declines as $g_s$ increases in response to increasing PPFD which leads to sugar beet wilting.*

The overall findings will now be outlined and used to characterise water use efficiency and anisohydry in sugar beet. The challenge in understanding the impact of wilting in sugar beet will be explored as well as the lack of adaption under drought and the problems with stomatal physiology assessments. The complex relationships between $WUE_i$ and $WUE_{DM}$, and WUE and drought tolerance in sugar beet will also be examined. Implications of this work for breeders and growers will be outlined and future research suggestions arising from the work in this thesis presented.

## 5.2 Understanding water use efficiency and characterising anisohydry in sugar beet

Research on drought tolerance in sugar beet has helped to identify genotypic variation in $WUE_{DM}$ and potentially associated traits (Ober and Luterbacher, 2002, Ober et al., 2004, Ober et al., 2005, Rajabi et al., 2009) which can be used to breed varieties with greater drought tolerance and WUE. Studies on changes to WUE under drought have also been explored as part of this focus (Brown et al., 1987, Rytter, 2005, Bloch et al., 2006), and show how WUE increases under reduced water availability. The aforementioned work has assessed sugar beet $WUE_i$ but has focused on the effect of drought and has prevented a general characterisation of sugar beet $WUE_i$, which could help explain how the crop responds to the environment to manage water use and how anisohydric behaviour affects these responses. Examining sugar beet water use from $WUE_i$ at the leaf level through to the crop level $WUE_{DM}$, in the studies presented in this thesis, has enabled further characterisation of how sugar beet uses water and anisohydric behaviour in sugar beet.

The three studies presented in this thesis (Speed of stomatal response study- Chapter two, Water availability study – Chapter three and Diurnal study – Chapter four) show sugar beet to be a plant which can be described as risk taking but resilient. It has already been shown that sugar beet keep stomata open as leaf water potential ($\Psi_{leaf}$) falls (McCree and Richardson, 1987). The rapid stomatal responses identified in the speed of stomatal response study provide further evidence for this risk-taking response as A and $g_s$ increase rapidly in response to light, to maximise carbon gain, optimising the use of available light but this also leads to high rates of water loss through transpiration. High rates of A and $g_s$ and rapid stomatal responses have been evident in other anisohydric species (Alvarez et al., 2007, Meinzer et al., 2017) and suggest that on the continual spectrum of anisohydry to isohydry (Meinzer et al., 2016) sugar beet can be firmly classed as anisohydric. This risk-taking response has been identified as optimal in conditions where plants are able to recover from subsequent abiotic stress. Sugar beet is derived from sea beet (*Beta vulgaris,* ssp. *maritima*) in which anisohydry is likely beneficial because the marine environment in which it grows exposes the plant to osmotic potentials which reduce water availability. When exposed to this reduced water availability stomata which are less sensitive to fluctuations in $\Psi_{leaf}$ alongside osmotic adjustments to maintain leaf turgor enables stomata to remain open to facilitate photosynthesis in this environment (Vastarelli et al., 2013). This anisohydry can be beneficial under long term drought as low levels of $g_s$ and A are maintained which helps plant survival, and from a grower's perspective enables some level of continued biomass accumulation. The benefit of continued photosynthesis to plant survival under drought has been well studied in the Piñon-Juniper woodlands of the southwest USA, where mortality of the isohydric Piñon was 97% from 2000-2007 compared to 0.4% in the anisohydric Juniper, with this survival attributed to the ability to continue to photosynthesise under drought by maintaining greater hydraulic conductivity which ensures stomata remain open (McDowell et al., 2008). Juniper is however, prone to cavitation which can limit the speed of recovery post drought but the benefit of continued assimilation throughout the summer drought outweighs the inability to maximise use of late season rainfall (West et al., 2008). The $A_{max}$ of the sugar beet did fully recover in the water availability study, which is not always evident in other species (Kirschbaum, 1988, Gallé and Feller, 2007), but took 34 days on average which could be attributed to cavitation, but this was not assessed. The variation from 18-46 days to recover suggests there may be another factor involved which would likely be related environmental differences, most notably temperature. In the diurnal study A had recovered to 86% of the pre drought value, when averaged over all measurements taken over the day, 3 days after rewatering but measurements were stopped before it could be assessed if full recovery would be attained. The differences in the recovery speeds measured could be attributed to $A_{max}$ being measured in the water availability study under saturating light, whilst in the diurnal experiment A was measured under the ambient light of the growth room and at four time points. This shows that although $A_{max}$ may take time to recover after drought A, as would be observed in the field, recovers more rapidly as $A_{max}$ is pushing the plants' photosynthetic apparatus to the maximum under saturating light which would only be encountered during the brightest part of the day and by leaves at the top of the canopy (Murchie et al., 2003). This suggests that the anisohydric response in sugar beet is not detrimental to long term plant performance and therefore in temperate climates, where drought is intermittent, the anisohydric response appears beneficial, or at the very least not detrimental, to yield.

The impact of the anisohydric response on $WUE_i$ is complicated as it is affected by many factors including; soil moisture content, vapour pressure deficit (VPD) and atmospheric $CO_2$ (Condon et al., 2002). It has been observed that over a season $WUE_i$ is comparable between anisohydric and isohydric grapevines (Lovisolo et al., 2010) but that under drought isohydric varieties increase $WUE_i$ more rapidly, as $\Psi_{leaf}$ falls (Poni et al., 2007) and this greater $WUE_i$ is maintained at the start of re watering. However, the increased $WUE_i$ is due to reduced stomatal aperture restricting $g_s$ and A which limits the speed of recovery and therefore the anisohydric vines recovered faster, and this enabled better performance under moderate water stress (Pou et al., 2012). In the comparison with spinach, sugar beet had a comparable $WUE_i$ despite much greater A and $g_s$ so was maximising carbon gain under high light whilst maintaining a consistent level of $WUE_i$. This suggests that the rapid stomatal response is beneficial, but the plant was transpiring more water, so in environments where water availability may be poor this could be detrimental as leaf turgor is not recovered. Such a scenario was tested by McCree and Richardson (1987) who showed that initial high rates of $g_s$ and A in sugar beet led to similar ratio of carbon gain to water loss to the isohydric cowpea, which maintained leaf turgor for longer and had higher rates of A and $g_s$ after the sugar beet lost leaf turgor 8 days after the onset of drought. This increased water use of sugar beet was evident in the speed of stomatal response study as the spinach did not drought as quickly as the beet, as it was likely being more conservative in its use of water. These observations have also been evident when comparing Mediterranean shrubs with the anisohydric *Juniperus phoenicea.* Juniperus had higher $WUE_i$ as it was more responsive to the environment, but higher levels of total water use again made it more prone to drought than the isohydric species (Altieri et al., 2015). Therefore, the anisohydric response can make the crop prone to water stress despite similar $WUE_i$ which also highlights that $WUE_i$ alone does help in understanding how the crop uses water. It is also important to understand the stomatal dynamics and how the plant responds to the environment to fully characterise how and when the crop uses water.

## 5.3 Wilting - still challenging to assess

Although none of the three studies undertaken were able to identify the exact drivers of wilting in sugar beet, the high levels of $g_s$ attained by sugar beet and further evidence of anisohydric characteristics, could together explain the wilting seen in sugar beet in the field, even when water is available in the soil profile. The decline in NDWI as $g_s$ increased in the diurnal sugar beet study highlights the decline in leaf water content at high rates of $g_s$ but no wilting was observed, despite these diurnal fluctuations. However, the light levels in the controlled environment room (CER) were high but did not reach levels as high as would be observed in the field, and the LED light regime did not emit high levels of infrared light which increases the leaf temperature and can drive greater VPD between the leaf and the ambient air in the field. With both increased light and VPD, providing it is not so high as to induce stomatal closure, being drivers of increased $g_{s,}$ it could be that in the field environment $g_s$ may increase to rates above those observed in the CER, and this may result in the witling commonly observed in the field even when water is freely available. The decline in NDWI late in the day, even after re-watering, also suggests that the plant may be prone to wilting whilst recovering from a severe decline in leaf water content due to a low soil moisture content.

Prior to wilting sugar beet undergoes high levels of osmotic adjustment to offset the reduction in water potential in leaf cells and maintain $\Psi_{leaf}$ as water availability declines (Vastarelli et al., 2013). When osmotic adjustment is no longer sufficient to maintain $\Psi_{leaf}$ the leaf wilts and the $\Psi_{leaf}$ at this point known as the turgor loss point (TLP) (Bartlett et al., 2012). It has been shown that drought tolerant species that have a more negative TLP are shown to better resist drought with this mainly driven by osmotic adjustment (Bartlett et al., 2014). The level of compatible solutes, which are typically betaines, polyols and amino acids are not toxic to the cell organelles, including chloroplasts, in high concentrations, so differing levels of these should not affect the plants ability to photosynthesise (Chen and Jiang, 2010), although stomatal closure may already have had an effect on the rate of $CO_2$ uptake and photosynthesis. Of greater interest is the accumulation of inorganic ions such as $Na^+$ and $Cl^-$ which are usually accumulated under salt stress and at high concentrations can result in ionic toxicity (Munns, 2002). There is evidence that would suggest sugar beet is better able to avoid ionic toxicity because under salt stress there is an increase in the levels of $Na^+$ and $Cl^-$ but this does not reduce photosynthesis in the plant (Katerji et al., 1997). The reliance of sugar beet under salinity stress is attributed to the ability of sugar beet to accumulate glycine betaine which acts as a compatible solute (Shaw et al., 2002), a level low solute leakage from the vacuole (Ghoulam et al., 2002) where other saline resistant species such as spinach accumulate $Na^+$ and $Cl^-$ ions to protect the metabolically active parts of the leaf including the chloroplast (Robinson et al., 1983), and high levels of proline accumulation which acts as an antioxidant which can protect the photosynthetic apparatus enabling continued photosynthesis even as solute concentrations increase and leaf turgor is lost (Bohnert and Shen, 1998). Although drought stress does not expose the plants to the high levels of $Na^+$ and $Cl^-$ observed under salinity stress it suggests sugar beet is resilient to changes in solute concentrations which occur under drought which may explain the some of the observations in all three studies presented. Firstly, the ability of sugar beet stomata to open under high light, or in a diurnal cycle, to enable A, even when wilted, shows that the plant is still able to photosynthesise when leaf turgor is lost. Secondly, the resilience of PSII to reduced water availability and quick recovery from severe drought, as shown by the chlorophyll fluorescence measurements both in the speed of stomatal response and water availability study respectively. This resilience was also observed by Bloch et al. (2006) where only severe drought reduced the quantum yield of photosynthesis. The ability of PSII to resist declining plant water content in sugar beet shows it is well adapted to continue to photosynthesise even under water stress, albeit at reduced levels, and recover quickly, which again is likely attributed to its maritime ancestry. There is variation in the ability of sea beet and sugar beet to osmotically adjust (Vastarelli et al., 2013) and sea beet from different habitats has been shown to respond differently to abiotic stress with osmotic adjustment and biomass accumulation varying under drought (Ribeiro et al., 2016). This shows that there is variation in the resilience of sea beet under drought with these genotypic differences potentially linked to the variance in drought tolerance evident between sugar beet cultivars.

Although transient wilting appears not to be detrimental, wilting does pose a risk of leaves being damaged by coming into contact with the soil. Premature leaf senescence is often observed in sugar beet when wilted leaves touch the hot soil surface, with this evident in the water availability study although it was not measured or assessed. Losing leaves to scorching, when the leaves wilt and come in to contact with the hot soil surface and rapidly senesce, can reduce the area available to intercept light for photosynthesis (Earl and Davis, 2003). However, the loss of leaves may not be detrimental: older leaves wilt and are lost first (Lawlor and Milford, 1975) and these are much less active with lower $g_s$ and A than younger leaves as observed in the water availability study. Losing these leaves may therefore reduce the area from which water is lost through transpiration with these leaves contributing little to overall carbon gain as older leaves have lower rates of A. The loss of older leaves in grape vines has been shown to increase overall plant $WUE_i$ for exactly this reason (Medrano et al., 2012). Additionally, it has been shown that nutrient partitioning from older leaves lost under drought to younger leaves can help maintain plant function and improves drought tolerance (Munné-Bosch and Alegre, 2004). However, if the drought is prolonged and more leaves severely wilt and are lost through scorching on the hot soil surface then the loss of these younger and more active leaves is likely to reduce biomass accumulation, as observed in the limited water treatment in the water availability study. The plants had a much-reduced canopy size and it was observed that all but the most newly emerged leaf was prone to wilting and scorching, although again this was not recorded or measured.

Overall the resilience of PSII, as highlighted by the studies presented in this thesis, and the rapid response of sugar beet stomata to maximise carbon gain, which may result in wilting, is not detrimental in temperate climates where drought is moderate, and severe wilting and loss of younger leaves is uncommon. However, where drought is severe, this risk-taking strategy and long-term wilting is likely to be detrimental to the crop’s yield potential.

## 5.4 Adaption under drought and the problems with stomatal physiology assessments

The absence of any adaption to prevent a decline in PSII performance under drought had previously been observed by Leufen et al. (2016), in a study which showed that chlorophyll fluorescence parameters declined in a similar pattern in repeated drying cycles, despite differences in the level of osmotic adjustment and proline accumulation. This lack of adaptation is likely due to the resilience of the plant due to the anisohydric characteristics of sugar beet, as already outlined. The only consistent adaption observed was the reduction in canopy biomass which may help the plant reduce its total water use as overall transpiration is reduced, but this comes at the cost of reduced biomass accumulation, which is commonly observed in other crops (Sinclair et al., 1990, Jefferies and mackerron, 1993, Giunta et al., 1995). The key role stomata play in controlling water loss from the plant means that manipulation of stomatal size (SS) and stomatal density (SD) can be used to enhance drought tolerance and water use. Under low soil moisture, SS has been shown to decrease in *Arabidopsis thaliana* L. (Doheny-Adams et al., 2012) the grass *Leymus* *chinensis* L. (Xu and Zhou, 2008) and potatoes, *Solanum tuberosum* L. (Sun et al., 2014). Changes in SD are less consistent with the same studies identifying similar SD under drought as irrigated conditions (Doheny-Adams et al., 2012), an increase in SD under moderate drought but a decrease under severe drought (Xu and Zhou, 2008) and a decrease in SD under drought (Sun et al., 2014). No consistent changes in SD and SS due to drought were detected in the water availability study but there were challenges to accurately assess these traits, which may also explain why other studies have found contrasting results, although this could also be down to species differences. Firstly, the area assessed for SS and SD totalled 3mm$^2$ and even to assess this small area it took considerable amount of time to image and then manually count the number and assess the size of stomata. SD has been shown to vary significantly over the leaf surface and assessing such a small area may not be a true reflection of the SD over the whole leaf (Weyers and Lawson, 1997). There are many groups working to develop automated SD and SS tools (Fetter et al., 2019, Meeus et al., 2020) and these may help speed up the process and allow larger areas to be assessed to ensure an accurate assessment of SD and SS is attained. In addition to this challenge there is also the issue of wilting. Stomatal impressions were generally taken on leaves which were not wilted but to ensure leaves of a comparable age were compared, some of the leaves were wilted, which alters the SD as cells shrink and bring stomata closer together than on a well-watered leaf. The final problem is that of reduced leaf size under drought. The smaller leaves under drought may have a higher SD but this may be an artefact of the smaller leaf area i.e. the total number of stomata may be the same, but the SD is greater as the leaf area is reduced. This makes it hard to determine if there is an adaption in stomatal physiology under drought and may explain the inconsistent results observed in the water availability study. To overcome these issues the whole leaf needs to be examined and it would be better to assess leaves at full turgor, so droughted plants should be re watered to enable a consistent base line from which to assess SD and SS. This would then allow an accurate assessment of SD and SS and whether this changes under drought to aid water conservation. This, alongside our understanding of sugar beet stomatal kinetics from the speed of stomatal response study, would help in understanding if stomatal physiology changes under drought in sugar beet and if so, how these changes aid the plant under drought.

## 5.5 Assessing stomatal responses at the canopy level

The speed of stomatal response experiment showed that sugar beet stomata reacted rapidly to increased PPFD and this observation furthers the understanding of the impact anisohydry in sugar beet as discussed previously. Such studies are, however, limited in their ability to accurately represent stomatal responses as the detailed analysis of leaf gas exchange and chlorophyll fluorescence are only possible by inserting part of the leaf into an IRGA cuvette. Stomatal responses are highly complex and driven by a large number of processes, all influenced by the environment (Lawson et al., 2010). With regard to light there are two drivers of stomatal control termed the blue light response, which is photosynthesis independent and red light response which is photosynthesis dependent (Zeiger, 1983). The blue light response activates proton pumps to decrease water potential in the guard cell and hence drive water uptake into the guard cell to increase turgor thereby inducing stomatal opening (Iino et al., 1985). The red light response is more complex and is related to both a direct detection of red light but also the decline in $C_i$ as the plant photosynthesises (Sharkey and Raschke, 1981, Vavasseur and Raghavendra, 2005). When placed in the cuvette the leaf is exposed to an artificial light source which has a set red and blue light composition which can be altered but is kept consistent during measurements to allow statistical comparison. The composition of this light is therefore not representative of light from the sun which changes through the day and varies throughout the canopy (Robertson, 1966). This means that the measurements taken of the stomatal response characteristics may not be the same in the field when the light composition is different to that in the measurement cuvette. In addition to this, the cuvette removes the boundary layer and associated resistance which in a field environment would also be interacting with the light responses to drive stomatal control (Bunce, 1985). Therefore, the IRGA approach does not fully capture plant stomatal responses as they would occur in the field but does provide a way to assess stomatal control in a set environment.

To assess stomatal responses in a field setting, and to further understand if the observations in the IRGA translate to the field, it would be useful to assess whole canopy stomatal responses. The whole canopy approach also overcomes the issue of the differing activity of younger and older leaves and the variation across leaves previously mentioned. Rather than take individual IRGA measurements of leaves throughout the canopy, to account for differences in both leaf age and leaf position, chambers have been developed which allow whole canopy gas exchange to be measured (Burkart et al., 2007, Song et al., 2016, Jauregui et al., 2018). Whole canopy chambers still have limitations as the canopy is enclosed inside a large transparent container, which will alter the temperature, airflow and therefore VPD compared to the ambient conditions, but this is likely less severe than in the IRGA cuvette. An artificial light source could be used to change PPFD if needed but ideally measurements could be taken on a bright day using ambient light, for the reasons already outlined, with the plants shaded using a cover then exposed to high light so the increase in $g_s$ and A could be assessed and therefore the whole plant stomatal response examined. Such an approach would be similar to the measurements taken by McAusland et al. (2016) in the glasshouse to assess the impact of transient shading by clouds and the response of $g_s$ and A. Remote sensing of solar induced chlorophyll fluorescence (Meroni et al., 2009, Atherton et al., 2018) could also be undertaken. There is some debate as to the accuracy of SIF (Liu et al., 2016) and how best to interpret the data (He et al., 2020), so ground truthing would be needed to validate this approach but it has already shown to be reliable on the whole leaf scale in sugar beet (Raesch et al., 2014). Although not as accurate and detailed as those taken using a multiphase flash fluorometer (Murchie and Lawson, 2013), measurements of the responses of PSII to changes in light do not have to be sacrificed, which is one of the main attractions of using the IRGA with the multiphase flash fluorometer attachment. The use of a whole canopy gas exchange system would also be useful in studies such as the water availability study to capture the whole canopy activity, rather than a small area on old and new leaves of only two of the twelve plants, but such systems are cumbersome and may be impractical when many measurements need to be taken over the day which is why the handheld IRGA is still used. Hence, canopy chambers require considered experimental designs to allow their use and the benefits they infer, which could be coupled with handheld IRGA measurements for more rapid assessments. Through assessing gas exchange and associated physiology on individual leaves and whole canopies under varying environmental conditions it may also be possible to develop a model to predict canopy level WUE in sugar beet.

## 5.6 Limitations to assessing the relationship between $WUE_i$ and $WUE_{DM}$

The relationship between $WUE_i$ and $WUE_{DM}$ is complex, not least because water itself is not the driver of biomass accumulation but rather a function of the need to open stomata for $CO_2$ uptake, although it is important in facilitating the uptake of nutrients and transporting them around the plant. This disconnect was evident in the water availability study where varietal differences in $WUE_i$, from both IRGA and $\Delta^{13}C$ assessments did not equate to differences in root or shoot $WUE_{DM}$, but differences in drought treatment did. The lack of a variety*treatment interaction for the IRGA and $\Delta^{13}C$ observations suggest that this greater $WUE_i$ is due to an inherent difference between the varieties and not driven by water deficit, which is further supported by the greater $WUE_i$ observed in the prostrate variety (Cayman) in the diurnal response study. There are a number of reasons that an increased $WUE_i$ does not equate to a greater $WUE_{DM}$. Firstly, assessments of biomass are often focused only on above canopy biomass (French and Schultz, 1984, Roby et al., 2017, Weiler et al., 2019) and whilst in sugar beet research the storage root biomass is also commonly assessed

(Ober et al., 2005, Rytter, 2005, Bloch et al., 2006, Rajabi et al., 2009) the tip of the storage root is often lost when broken at harvest and the finer roots are not often extracted and assessed as this requires time intensive root washing. This leads to an inaccurate assessment of the plant's whole biomass which reduces the $WUE_{DM}$ value. A limitation in this area which was specific to the water availability study was the use of 610L boxes which may have led to the biomass samples being too small and variable to get an accurate dry matter value. Secondly, there are processes which are not captured in the IRGA measurement data, most notably nocturnal transpiration and dark respiration. Nocturnal transpiration has been shown in a range of species with rates typically between 5-15% of those observed in the daytime (Caird et al., 2007) and has shown to account for 10% of total daily transpiration in some species (Fuentes et al., 2014). However, a preliminary study undertaken as part of the diurnal response experiment showed that in a controlled environment room during the simulated night there was no transpiration from the sugar beet when measured using an IRGA. Although this was in a controlled environment, and therefore not fully representative of field conditions, it suggests that this may not be a source of major disparity between IRGA measurements of $WUE_i$ and $WUE_{DM}$ and the assessment of $\Delta^{13}C$ should account for nocturnal transpiration anyway. A more likely factor in the disparity between varietal $WUE_i$ and $WUE_{DM}$ is dark respiration in the roots. Dark respiration in the roots has been shown to be a key driver in the discrepancy between $WUE_i$ and $WUE_{DM}$ in grapevines but is still understudied (Medrano et al., 2015) and there is no published work on dark respiration on intact sugar beet storage roots, only on respiration post harvesting as this leads to sugar losses (Klotz et al., 2008).

Root $\Delta^{13}C$ is lower than leaf $\Delta^{13}C$, and dark respiration depletes $^{13}C$ in the roots, which is why leaf tissue is used to measure $\Delta^{13}C$ and correlates with $WUE_i$, therefore leaf $\Delta^{13}C$ does not take account of dark respiration in the roots either. Finally, as already discussed, there is the limitation that IRGA measurements are only taken from one leaf in the canopy under set conditions which is therefore unlikely to represent the whole plant water use and biomass accumulation. To overcome these challenges experimental design could be improved by growing sugar beet in the field with the use of a rain out shelter, to enable larger and more realistic biomass samples to be taken, the whole canopy approach to measuring $WUE_i$ used as discussed and the rate of dark respiration in the roots of the two varieties assessed to identify if this is the source of the discrepancy between varietal $WUE_i$ and $WUE_{DM}$.

## 5.7 Water use efficiency and drought tolerance in sugar beet

Just because a plant is more water use efficient it does not mean it is able to better maintain yield under drought, which is the typical definition of drought tolerance in crops (Ashraf, 2010). In the commonly cited review by Blum (2009) it is argued that effective use of water (EUW),rather than WUE should be the target when developing more drought tolerant crops. This is because greater WUE often comes at the cost of reduced photosynthesis which limits the plant’s ability to accumulate biomass, unless photosynthetic biochemistry can be altered. Blum (2009) even argues that the success with more WUE wheat in Australia can be attributed to the increased WUE leading to greater soil water conservation and therefore greater water availability during the critical flowering period, making the crop drought avoiding rather than drought tolerant. Therefore, it is argued that maximising soil moisture capture rather than conserving water through reduced transpiration should be a focus. In the water availability study, the prostrate variety (Cayman) had a greater $WUE_i$ and this did not appear to be at a cost to biomass accumulation, which may suggest that in sugar beet WUE can be increased without a cost to biomass accumulation, notwithstanding the challenges outlined in the previous section. However, the prostrate variety (Cayman) was identified to have a greater leaf relative water content than the upright variety (Sabatina), and in the comprehensive study by Ober et al. (2005) leaf traits associated with leaf succulence were negatively correlated with maintenance of green leaf foliage which was one of the key traits associated with a greater DTI. Additionally, the observation by Rajabi et al. (2008) that a greater SLW was correlated with an increased $\Delta^{13}C$ and drought tolerance was not evident in this study when comparing the varieties, further suggesting that leaf traits have an inconsistent association with WUE and drought tolerance. The discrepancy between leaf characteristics and WUE and the work on DTI could be due to the limited traits that any one study can look at whether this is due to time or financial constraints. In Ober's study stomatal size and density was not examined, and it is not known whether more succulent leaves always have a lower SD and greater SS or whether this is unique to the one prostrate variety (Cayman) examined in this study. The key importance of stomata on WUE and potentially the DTI of a variety was not examined in Ober's work and in the studies in this thesis only two varieties were examined. Therefore, it cannot be confidently concluded that leaf water characteristics alone are a driver of DTI as there may be a SS and SD influence which has not been assessed. This highlights the importance of assessing a range of traits in a drought tolerance study across a wide area of physiology. Ober et al. (2005) covered most areas from water uptake to photosynthesis to canopy and leaf traits but the lack of stomatal physiology measurements may have limited the study's ability to fully understand how leaf physiology affects DTI. Overall, it is important to understand the complex relationship between WUE and drought tolerance and that the two are not always related. The best approach is likely to be examining plants in the environment in which they will be grown so that the impact of increased WUE can be assessed alongside whether it confers greater drought tolerance in the given environment. This would reduce the risk of transferring a more WUE and drought tolerant genotype developed in one environment to another where the soil water availability and climate characteristics vary and the greater WUE no longer results in a greater drought tolerance.

## 5.8 Implications for breeders and growers

The impact of increased atmospheric $CO_{2,}$ from 400 ppm in 2020 to predicted levels of 720-1000 ppm by 2100 (IPCC, 2012), could increase crop WUE if no other changes in climate were observed (Bernacchi et al., 2007). However, the global increase in atmospheric $CO_2$ is predicted to increase global temperatures by an estimated 2.2C° on average (IPCC, 2012). This climate change and the already increasingly hotter and drier summers in much of the beet growing area (Maracchi et al., 2005, Motha and Baier, 2005) means that, even with an increase in atmospheric $CO_2$ and the potential for greater crop WUE, there is a concurrent risk of more frequent and severe droughts and the need for more WUE and drought tolerant crops is still present. The results of the three studies presented in this thesis show breeders that there are differences in $WUE_i$ between commercial sugar beet varieties. This is important as it shows that $WUE_i$ is independent of yield and can therefore be increased without a yield penalty that would make a variety unprofitable and undesirable to growers, which as outlined earlier is always a risk associated with greater $WUE_i$ as assimilation can also be reduced alongside $g_s$. The use of $\Delta^{13}C$ to identify more $WUE_i$ genotypes has also been highlighted and agrees with the work of Bloch et al. (2006). $\Delta^{13}C$ is the best trait for breeders to screen lines for greater $WUE_i$ as it is relatively high throughput. Although $\Delta^{13}C$ is not as rapid as remote sensing the traits related to $WUE_i$ such as stomatal density and RWC cannot currently be detected using such approaches. The inconsistency between $WUE_i$ and $WUE_{DM}$ does however, highlight the need to further understand the relationship between $WUE_i$ and $WUE_{DM}$ to ensure that the varieties selected for a greater $WUE_i$ using $\Delta^{13}C$ achieve a greater $WUE_{DM}$ as this is the most beneficial parameter for growers. In addition to this, if the greater $WUE_i$ and $WUE_{DM}$ is selected for to increase drought tolerance it is important that breeders develop new varieties with the consideration of the impact of soil type and climate for the reasons already outlined. Breeders also need to be aware of differences in canopy architecture between sugar beet varieties, of which the prostrate (Cayman) and upright (Sabatina) in the water availability study represented the extremes at both ends of a continuous spectrum. The lack of a consistent interaction between leaf morphology, stomatal characteristics and drought tolerance suggests that canopy architecture may be linked to WUE. Therefore, breeders should ensure that they continue to produce varieties with a range of canopy architectures and associated traits such as leaf water content, at least until the relationship between canopy traits WUE and drought tolerance is understood. It has been suggested that prostrate varieties in most crop species have a lower RUE which could reduce yield potential, as light interception is saturated at the top of the canopy (Duncan et al., 1967) which could result in this being an unfavourable trait with breeders. However, as breeders move to producing varieties based on yield stability and crop resilience, as well as yield potential, the conservation of more prostate varieties should be viable, especially if they have a greater WUE than upright varieties.

In summary the sugar beet ideotype for improved $WUE_i$ from the results of the three studies presented would be:

1. A stomatal density that is lower than in some sugar beet cultivars, but which still enables quick opening and closing.
2. A greater $\Delta^{13}C$ relative to other sugar beet cultivars.
3. A greater RWC relative to other sugar beet cultivars.
4. A prostrate canopy was associated with all of the above traits (expect of the speed of stomatal response) so this could be a desired trait for a $WUE_i$ ideotype but only one cultivar of each canopy type, upright and prostrate, was examined so this needs further exploration.

Growers across Europe are debating whether to continue growing sugar beet as the removal of neonicotinoid seed treatments has led to high rates of virus yellows infection, which is transmitted via aphids, and can reduce yield by up to 47% (Smith and Hallsworth, 1990). Clover et al. (1999) showed that there is no interaction between drought and virus yellows in terms of yield loss which suggests that more WUE varieties could reduce the lost yield potential in crops infected by virus yellows and exposed to drought. It may be that if virus yellows tolerant varieties are developed, and brought to the market before fully resistant varieties, they would also benefit from being more WUE to further reduce lost yield potential. For growers, the impact of more WUE varieties is also important to ensure crop resilience thereby increasing the likelihood of a good return on the crop. Even if some $WUE_i$ varieties were lower yielding in an average year, growers may accept a lower overall average yield if the variety is more stable under drought as this provides some insurance against increasingly unpredictable rainfall. This would be similar to the use of nematode resistant varieties, which have enabled growers to continue to grow sugar beet even in the presence of infestation (Kleine et al., 1998). Additionally, the contribution of the work presented here to further characterise anisohydry in sugar beet suggests that that wilting is not necessarily detrimental to yield potential and therefore growers should not be concerned about transient wilting, only longer-term wilting and leaf senescence. This observation should help growers to have greater confidence in the crop and suggests irrigation is not necessary when wilting is transient.

Overall, the work presented here should help breeders to better understand why WUE is important and that it is a viable breeding objective. This in turn will enable the development of more WUE and drought tolerant varieties that ensure sugar beet is a desirable crop for growers who face an increasingly uncertain future.

## 5.9 Future research

The studies presented in this thesis, and this discussion, have highlighted some areas which require further research. Firstly, it would be beneficial to undertake the canopy level assessments of sugar beet gas exchange and $WUE_i$ using canopy chambers for the reasons already outlined. This would also provide an opportunity to measure SS and SD under drought across the whole leaf using the stomatal counting software already mentioned to identify the relationship between SS, SD and leaf area to assess if SS and SD changes under drought, which could not be ascertained in the water availability study. Work also needs to be undertaken on a greater number of genotypes to identify if all prostrate varieties have a greater $WUE_i$, as identified in the water availability study, and whether there are consistent traits which can be attributed to this, especially SS and SD. If differences in SS and SD are identified, then these could be examined using the method in the speed of stomatal response study to assess the stomatal dynamics and how these may affect $WUE_i$. The assessment of traits and their consistency between prostrate and upright varieties would feed into the work also needed to understand the relationship between $WUE_i$ and drought tolerance as the traits identified could be tested under drought conditions to identify if they drive both greater $WUE_i$ and drought tolerance. Additionally, the absence of a relationship between $WUE_i$ and $WUE_{DM}$ in the varieties studied needs to be examined to see if this is because of the limited sample size or observable in the field, and if so the drivers of this explored so that the use of $WUE_i$ as a selection criteria for $WUE_{DM}$ is an option. One area that needs to be included in this work is rooting traits which are likely to help explain the relationship between

$WUE_i$ and $WUE_{DM}$. Finally, this work must be undertaken in diverse climates and soils to ensure the genotype and environment interaction is understood and that the findings are applicable to each sugar beet growing area.

## 5.10 Conclusions

The major conclusions from the studies presented in this thesis can be summarised as follows:

1. Sugar beet is a highly resilient anisohydric crop, and this is party driven by the maintenance of photosynthetic activity even under reduced water availability, which enables survival and full recovery of photosynthesis.
2. Transient wilting may not be detrimental to sugar beet crops as it could be linked to greater rates of $CO_2$ uptake and assimilation, driven by rapid stomatal opening, and turgor can be quickly recovered. However long-term drought stress and wilting will lead to reduced yield.
3. The presence of varietal differences in $WUE_i$ in commercial sugar beet varieties bred for the same climate shows that this is a realistic breeding objective as it does not come at the cost of reduced yield potential, as shown by differences in $WUE_i$ but not yield between the two elite cultivars studied.
4. Water stress increases $WUE_i$ and $WUE_{DM}$ in sugar beet unless it is severe and prolonged.
5. It is still not known which traits drive varietal differences in $WUE_i$ although the observation of varietal differences in stomatal size and stomatal density suggests that they could be associated with differences $WUE_i$. It is not known

if stomatal size and stomatal density help improve drought tolerance and this needs further research alongside work to identify other traits which drive both greater WUE and drought tolerance. If there are differences in SS and SD it may be the case that not all sugar beet exhibit the same rapid stomatal response.

6. The relationship between canopy structure and other traits such as stomatal density, stomatal size, SLW and leaf water content parameters such as RWC are not known. For this reason, breeders need to continue to produce varieties with varying canopy architecture, to avoid reducing the variability in traits which may be drivers of more WUE varieties.
7. Leaf level measurements have many limitations which need to be considered and the use of canopy chambers for canopy level gas exchange may enable a more accurate assessment of plant WUE in the field.
8. Assessing plant responses from short term fluctuations in light, diurnally and over the season enables a better understanding of how it responds to the environment and allows a more detailed understanding of how this could affect the plant's WUE.

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