REGULATION OF PHOTOSYNTHESIS IN SORGHUM IN RESPONSE TO DROUGHT

A thesis submitted to the University of Manchester for the degree of PhD in the Faculty of Life Sciences

2014

CHUKWUMA COLLINS OGBAGA

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ABSTRACT

Changing climate in combination with growing world populations mean that there is growing need for plants to be grown on land that is currently considered marginal for agriculture. Sorghum is a C_4 plant that serves as an important food crop in Africa and India. It is also known to be highly drought tolerant but the mechanisms responsible for this tolerance are unclear. The overall aim of this study was to understand the drought tolerance mechanisms that enable the plant to maintain leaf function for a long time during water deficit.

In Chapter 2 of this thesis, I studied the underlying physiological mechanisms for tolerating drought in two sorghum varieties with differing degrees of drought tolerance compared to a closely related species, *Zea mays*. During progressive drought, the more tolerant sorghum variety Samsorg 17 maintained net CO_2 assimilation and photochemistry longest relative to the less tolerant Samsorg 40 and *Zea mays*. Differences were also seen in stomatal aperture, stomatal density, total chlorophyll content, chl a:b and A/Ci curve responses with maize more affected than the sorghum varieties.

In Chapter 3, I identified novel drought tolerance mechanisms in the sorghum varieties. The less tolerant Samsorg 40 lost PsbA (D1) and Rubisco proteins and reengineered its photosynthetic apparatus to accumulate amino acids and sugars in order to maximise survival under drought. Samsorg 17 maintained photosynthetic proteins notably PsbA (D1) and Rubisco and accumulated high constitutive sugar content allowing for the maintenance of transpiration and photosynthesis.

The two sorghum varieties had strikingly contrasting approaches of tolerating drought as demonstrated in Chapter 3. In Chapter 4, the aim was to characterise biochemical and metabolic changes that occur in response to drought. In particular, to identify sugars that are accumulated constitutively in Samsorg 17 and nitrogen sinks for lost N in Samsorg 40. My findings indicated a contrasting response in terms of sugar content in Samsorg 17 but support for amino acids as N sinks in Samsorg 40 as reported earlier. Sugars, sugar alcohols, lipids, organic acids, heat shock proteins and dehydrins were generally higher or more induced in Samsorg 17 relative to Samsorg 40. Samsorg 40 rather made amino acids. The implications of my findings and future work arising from this study were discussed in detail in the final chapter.

In conclusion, in this thesis, it was demonstrated that closely related plants can have mechanistically different physiological and biochemical mechanisms for responding to drought.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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ACKNOWLEDGEMENTS

I am sincerely grateful to everyone who helped me in one way or the other in the course of my PhD journey. In particular, I am immensely grateful to my supervisor Dr Giles N. Johnson for his supervision, assistance and patience especially in responding to some of my silly questions. I am also grateful to my advisor, Dr Thomas Nuhse, my external examiner, Dr Colin Osborne and internal examiner, Dr Jon Pittman for their comments, advice and support.

My sincere thanks to all members of our lab and visiting scientists who helped me with experimental suggestions/ideas, advice as well as with data analysis- Drs Mahmood Esna-Ashari, Habib-ur-Rehman Athar, Piotr Stepien, Milton Lima Neto, Beth Dyson, Mr Matthew Miller and Mr Stephen Chapman. Thank you to everyone in D.3513 and the members of plant science group for an excellent working environment and the good times we shared.

Many thanks to the priests at Manchester Catholic Chaplaincy - Fr. Ian Tomlinson, Fr. Tim Byron, Fr. William Pearsall and Fr. Edwin Thadeu: for their spiritual guidance and the entire members of the chaplaincy, for the happy moments and friendship.

Special thanks to my family and friends especially my dear parents for their love, moral and financial support and worthy of mention, for funding this research. I thank above all the holy Trinity- God the Father, Son and Holy Spirit for grace and direction.

ACRONYMNS

A	assimilation
ATP	adenosine triphosphate
CET	cyclic electron transport
Chl	chlorophyll
^{1}Chl	singlet chlorophyll
^{3}Chl*	triplet excited state of chlorophyll
Ci	internal CO_2 concentration
Cyt b_6f	cytochrome b_6f complex
DHN	dehydrin
DTT	dithiothreitol
E	transpiration
EDTA	ethylenediaminetetraacetic acid
Fd	ferredoxin
FNR	ferredoxin NADP reductase
FTIR	fourier transform infrared spectroscopy
Fv/Fm	maximum Photosystem II efficiency
G	stomatal conductance
GC-MS	gas chromatography mass spectrometry
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
LHC	Light Harvesting Complex
LHC I	Light Harvesting Complex I
LHC II	Light Harvesting Complex II
$MgCl_2$	magnesium chloride
NADP	nicotinamide adenine dinucleotide phosphate

NADPH	reduced nicotinamide adenine dinucleotide phosphate
NPQ	non-photochemical quenching
$^1O_2^*$	singlet oxygen
$^3O_2$	ground state oxygen
ΦPSII	efficiency of Photosystem II
PC	plastocyanin
PC-DF	principal component discriminant function
PEPC	phosphoenol pyruvate carboxylase
Pheo	pheophytin
PMSF	phenylmethanesulfonylfluoride
PPFD	photosynthetic Photon Flux Density
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
PSI ETR	rate of electron transport in electrons per PSI per second
PSII ETR	PSII Electron Transport Rate
P680	primary electron donor of PSII
$P680^+$	oxidised P680
P700	primary electron donor of PSI
$P700^+$	oxidised P700
Q_A	primary quinine electron acceptor of PSII
Q_B	secondary quinine acceptor of PSII
ROS	reactive oxygen species
Rubisco	ribulose 1,5 bisphosphate carboxylase oxygenase
RbcL	rubisco Large Subunit
RuBP	ribulose bisphosphate

RWC	relative water content
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOD	superoxide dismutase
SWC	soil water content
TBS-T	Tris-Buffered Saline Tween-20
Vpr	maximum PEP regeneration rate
WUE	water use efficiency

CHAPTER 1- GENERAL INTRODUCTION

1.1 Background of Project

The last twenty years have been marked by concerns over the changing climate (IPCC 1990, IPCC 2007, IPCC 2013). Climate change leads to extremes of weather conditions resulting from carbon dioxide (CO_2) emission and other greenhouse gases. It is associated with environmentally harsh conditions such as drought, floods and heat waves and affects plant morphology and physiology, in particular photosynthesis, by altering the ability of plants to capture light, CO_2, and water from the environment. The disruption caused by climate change might lead to food insecurity and poses a problem, as world population is predicted to reach 9 billion in 2050 (UN 2004).

One strategy, to tackle the growing demand for food, is to utilize marginal land (i.e. currently non-agricultural or uncultivable land) for the growth of agricultural crops. However, the problem with marginal land is the exposure to extremes of weather conditions such as drought and heat waves. Therefore, if marginal land is to be used for agricultural purposes, more stress tolerant crops should be developed. To do this, a better understanding of the effects of stresses such as drought on plants is needed. In this study, emphasis will be on drought and the overall aim is to examine the impact of drought on photosynthesis in the drought tolerant crop *Sorghum bicolor* with a view to identifying novel traits that are important in its drought tolerance and which might be transferred to less tolerant species.

1.2 Overview of drought

Drought is a major limitation on food production in Africa and Asia that occurs due to water deficit in the soil (Ngara et al. 2012). It can be caused by anthropogenic (human-induced) or

non-anthropogenic (natural) factors (IPCC 2007) . Anthropogenic causes include excessive use of land and deforestation, which reduces the ability of soil to retain water, while non-anthropogenic causes are natural climate variation (especially changes in ocean currents and increases in solar output) leading to reduced rainfall (IPCC 2007). Drought is generally grouped into three types: Hydrological, Agricultural and Meteorological (NOAA 2013). Hydrological drought occurs when water reserves available for use in lakes and reservoirs fall below normal. Agricultural drought is defined as a water deficit capable of limiting or reducing crop production. Meteorological drought on the other hand occurs when rainfall is less than average for a prolonged time (NOAA 2013). Agricultural and meteorological droughts are the most common types in Africa and Asia (Ngara et al. 2012). In the horn of Africa and South Asia, recurrent agricultural and meteorological droughts lead to desertification, massive food shortages and famine (Pantuliano and Pavanello 2009).

1.3 Plant Water Relations

1.3.1 Water Potential

Water potential can be defined as the potential energy of water per unit volume, compared to pure water (Braidwood et al. 2014). It determines the movement of water due to osmosis, gravity, pressure and even surface tension, and measures whether water is going to flow into or out of a cell (Spanner 1973, Steuter et al. 1981, Taiz and Zeiger 2002). It can be divided up into its contributing components such that:

$$\Psi = \Psi_S + \Psi_p \qquad \text{(Eqn 1.1)}$$

where Ψ_s is the solute potential and Ψ_p the pressure potential (Braidwood et al. 2014, Papendick and Camprell 1981). Water potential is a measure of Gibbs free energy and can be

described as Ψ= G/V, where G=Gibbs free energy and V= molar volume of water. This means that the lower the water potential, the lower the Gibbs free energy but the higher the potential energy (Braidwood et al. 2014, Papendick and Camprell 1981). The addition of solutes into pure water lowers water potential, which means lower Gibbs free energy. Pure water has a water potential of zero (0) bar i.e. 0 Pa, so physiological measurements of water potential are always negative. Stress conditions that can lower soil water potential include temperature, salinity and drought (Steuter et al. 1981, Taiz and Zeiger 2002).

Under normal circumstances, water flows from the soil, which has higher (less negative) water potential, through osmosis into the roots of the plants (which have lower, more negative, water potential) (Braidwood et al. 2014, Spanner 1973). Low water potentials in the root occur due to the presence of solutes, especially salts and sugars (Steuter et al. 1981). In plants, water potential becomes more negative from the roots to the leaves and then the atmosphere; that is, the atmosphere has the lowest water potential compared to leaves, stems and roots in that order (Spanner 1973, Steuter et al. 1981, Taiz and Zeiger 2002). Drought lowers soil water potential and in turn, reduces leaf water potential. When leaf water potential becomes greater than that of the soil (i.e. less negative relative to soil), water uptake becomes impossible. Plants close their stomata to avoid or delay extreme drops in leaf water potential from occurring (Ryan et al. 2014).

1.3.2 Stomata

Stomata are pores or openings that are found in the epidermis of the leaf and some other tissues and which allow control of gas exchange with the atmosphere (Swarthout and Hogan 2012, Xu and Zhou 2008). Specialised epidermal cells, referred to as guard cells, form the

boundary of the stomata and regulate stomatal openings. Carbon dioxide (CO_2), oxygen (O_2) and water are the main gases which show a physiologically relevant net flux into or out of the leaf (Swarthout and Hogan 2012, Xu and Zhou 2008). Entry of CO_2 is essential for photosynthesis. Loss of water, termed transpiration, is an unavoidable consequence of having open stomata and is influenced by changes in temperature, humidity and drought (Franks 2013, Xu and Zhou 2008). The link between stomatal control and CO_2 assimilation has been controversial (Jones 1998). What is clear is that stomatal aperture changes in response to availability of water, light, temperature and CO_2 and these changes are crucial for the adaptation of plants to their environment (Franks 2013, Haworth et al. 2011). Stomata are generally accepted by physiologists to play a major part in determining changes in CO_2 assimilation rates in plants. Other important processes determined by stomata control include: water use efficiency (balancing water lost in plant against carbon gained), leaf temperature and nutrient uptake (Haworth et al. 2011).

During drought, the stress hormone abscisic acid (ABA) is considered a major factor regulating stomatal aperture. ABA accumulates in the roots in response to low water potential and ABA is translocated to the leaf; where it induces the efflux of Ca^{2+} (in the cytoplasm) and K^+ (in the vacuole) through the guard cell's plasma membrane ion channels. Important channels are S-type anion, R-type anion and K^+ out efflux channels (Fig 1.1) (Kim et al. 2010). The efflux of the Ca^{2+} and K^+ leads to the depolarization of the plasma membrane and increases the osmotic potential of the guard cells causing water to leave the cell inducing shrinkage, which results in stomatal closure (Franks 2013, Li et al. 2000, Negi et al. 2008, Steuer et al. 1988, Vahisalu et al. 2008).

In contrast, during stomatal opening, water uptake occurs due to K^+ in channels inducing influx of K^+, Cl^- and NO_3^- inducing what is termed hyperpolarization of the guard cell plasma membrane (Kim et al. 2010). K^+ are transported into the vacuole via H^+/K^+ antiporter activities while the anions are transferred through both H^+/anion exchange mechanism and low affinity anion channels (nitrate transporter) (Fig. 1.1). Hyperpolarization is mediated by H^+- ATPase-dependent proton efflux with specific alleles involved identified as AHA1 and OST2 (Kim et al. 2010, Merlot et al. 2007). Hyperpolarization increases the guard cell turgor and induces stomatal opening (Kim et al. 2010).

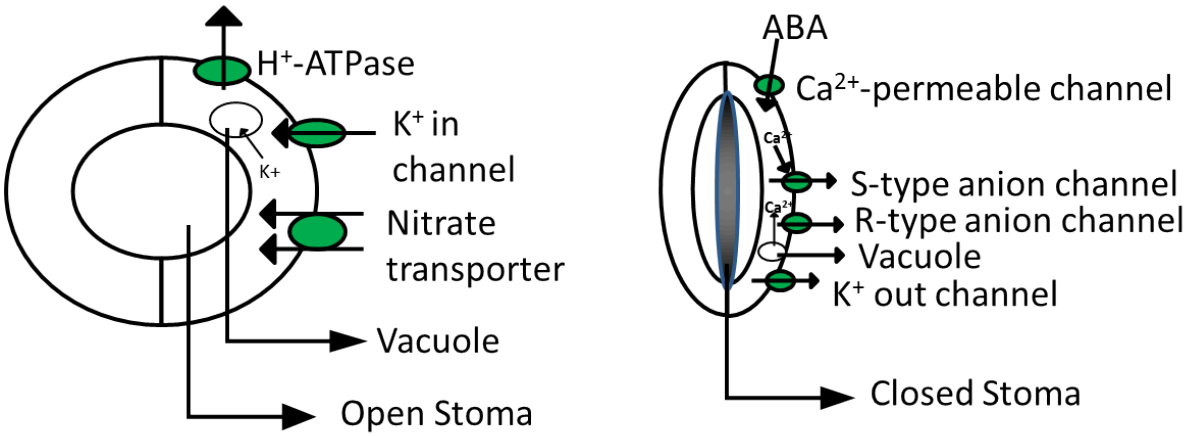


Fig.1.1. Regulation of guard cell opening. The stoma opens and retains its turgor due to the influx of K^+, Cl^- and NO_3^- through K^+ in channel and Nitrate transporter while it closes due to the efflux of cytoplasmic Ca^{2+} and vacuolar K^+ via S-type anion, R-type anion and K^+ out channels.

1.4 Photosynthesis

Photosynthesis in plants is the conversion of atmospheric CO_2 into organic carbon compounds driven by light. It occurs primarily in the chloroplast (Fig. 1.2), where light absorbed by chlorophyll is used to drive electron transport, producing ATP and reducing power that are used in the reactions of the Benson-Calvin cycle, to produce sugars. In the thylakoid membrane, the photosynthetic complexes are comprised of light harvesting complexes (LHCs) and reaction centres (RCs). LHCs consist of pigment binding proteins and are closely associated with the RCs. RCs are made up of pigments, numerous peptides and co-factors that drive electron transfer (Amerongen and Croce 2013, Hohmann-Marriott and Blankenship 2011, Zhu et al. 2010).

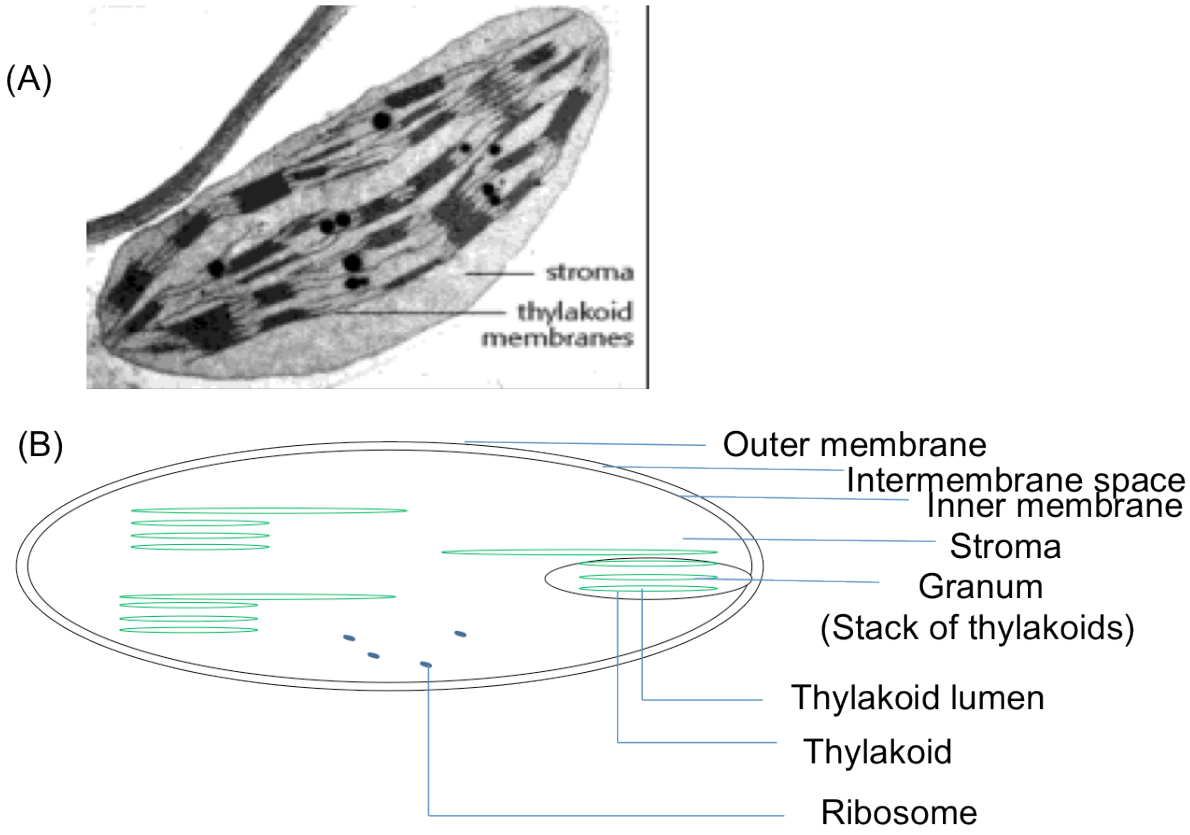


Fig. 1.2. Structure of the chloroplast. Structure shows electron micrograph (A) and diagrammatic representation (B) of a chloroplast. Photosynthetic components particularly stack of thylakoids, where light dependent reactions of photosynthesis take place, and stroma where the Benson-Calvin cycle reactions occur are shown. Electron micrograph of chloroplast was taken from Muller (2004).

1.4.1 Light Harvesting

Light is absorbed by light harvesting pigments, leading to the formation of an excited state. The absorbed energy, termed excitation energy, can be transferred by resonance transfer to the RCs to drive charge separation. This process is referred to as light harvesting. It is accomplished in green plants by the use of antennae, which ensure efficient energy transfer towards the RCs. The antennae also regulate light absorption to match fluctuating light levels,

demands of photosynthetic reactions and biochemical processes (Fleming et al. 2012, McConnell et al. 2010). The antennae of higher plants can be divided into a proximal antenna, which is part of or closely associated with the RC, and the light harvesting complexes (LHCI and LHCII), which are variable in size and composition (Dang et al. 2008, Keren et al. 2005, Picorel et al. 2004, Picorel et al. 2011). The pigments contained in the antennae are chlorophylls (chl a and chl b), xanthophylls and carotenoids (β-carotene) which are bound to various peptides (Li et al. 2004, Lince and Vermaas 1998). The LHCs act like a funnel in trapping the appropriate wavelength of light required for charge separation by the RCs (Demmig-Adams and Adams 1996, Zhu et al. 2010). The absorbed energy is transferred from one molecule to another by resonance energy transfer (Cheng 2006). The LHCs contain chlorophylls a and b and xanthophylls, whilst the proximal antennae bind chorophyll a and carotene only.

1.4.2 Photosynthetic electron transport chain

The higher plant photosynthetic electron transport chain is responsible for the movement of electrons from water to NADP accompanied by the net transfer of protons (H^+) across the thylakoid membrane from the chloroplast stroma to the thylakoid lumen. The proton electrochemical gradient formed drives adenosine triphosphate (ATP) generation by the chloroplast F_0F_1 ATP synthase (Andersson and Barber 1996, Barber and Tran 2013, Genty and Harbinson 1996a, Kramer and Crofts 1996, Owens 1996, Takahashi and Badger 2011).. The electron transport chain (ETC) in the chloroplast involves 2 reaction centres – Photosystem (PS) II and PSI – and a cytochrome b_6f (cyt b_6f) complex. These are connected via the mobile carriers plastoquinone and plastocyanin (Fig. 1.3).

PSII is the first protein complex in the ETC. It has the primary electron donor chlorophyll P680 in the RC and transfers energy from water to plastoquinone. The PSII core is made of a dimer of homologous peptides- D1 (PsbA) and D2 (PsbD)- and co-factors including pheophytin and plastoquinone. PSI has chlorophyll P700 as the primary electron donor in the RC and transfers light energy from plastocyanin to ferredoxin (Fd). Electron transfer in PSI involves chlorophylls (A_o, A_1) and FeS clusters F_A, F_B, F_x , Fd (Fromme and Mathis 2004, Vassiliev et al. 2001). The two photosystems have homologous structures. They each have a core, containing just chlorophyll a and β-carotene, and this is surrounded by LHCs (LHCII in PSII and LHCI in PSI) (Amerongen and Croce 2013, Blankenship 2002, Croce and Amerongen 2013, Demmig-Adams and Adams 1996, Gilmore and Govindjee 1999, Owens 1996, Romero et al. 2010, Rutherford and Faller 2003).

When charge separation occurs, $P680^+$ is produced, which is a strong oxidant that takes electrons originating from water, making oxygen, and passes them to pheophytin. The electrons are subsequently passed to plastoquinone, with the charge on P680 returning to a neutral state as a result of electrons gained from a manganese centre (Mn_4O_xCa). This is contained in the oxygen-evolving centre (OEC) on the lumenal side of the PSII RC (Kulik et al. 2007). The synthesis of oxygen from water, catalysed by Mn_4O_xCa, also results in the release of protons (H^+) into the thylakoid lumen (Fig. 1.3). H^+ release generates a pH gradient across the thylakoid membrane, which is ultimately used in the production of ATP (Barber and Tran 2013, Renger and Renger 2008, Takahashi and Badger 2011). The oxidation of water releases a total of four electrons and is the only known biological reaction where water is split into its component parts (Romero et al. 2010, Scott 2008).

The electrons passed to plastoquinone are further transferred to the cyt b_6f complex. This complex accepts electrons from plastoquinol. The accepted electrons are passed either through a linear or a cyclic (Q-cycle) electron transfer route. In the linear route, the electrons are transferred to the soluble electron acceptor plastocyanin in the thylakoid lumen, via the rieske Fe-S protein and cyt f (Fig 1.3). In the Q-cycle, electrons from plastoquinol (PQH_2) are used to reduce a second plastoquinone (PQ) transferred via two b cytochromes (cyt b_{563}). For each oxidised PQH_2, one electron passes through each of these two pathways, so that, on average, each electron passes twice through the cyt b_6f complex. For each oxidation of PQH_2, two protons are released into the thylakoid lumen. Reduction of PQ to PQH_2 in the Q-cycle results in the uptake of 2 protons from the chloroplast stroma. Thus, the cyt b_6f is responsible for a net movement of protons to the lumenal side of the membrane, contributing to the proton motive force (Andersson and Barber 1996, Barber and Tran 2013, Genty and Harbinson 1996a, Kramer and Crofts 1996, Owens 1996, Takahashi and Badger 2011).

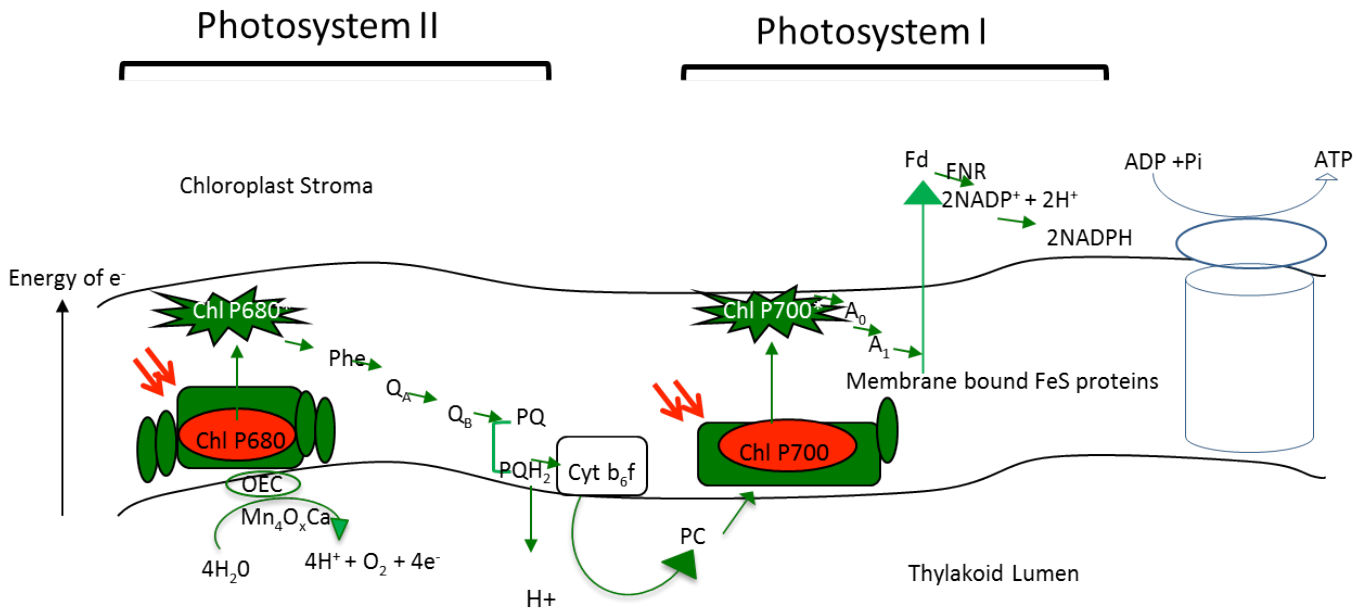


Fig. 1.3 The Z-Scheme of electron transport chain in Photosynthesis (adapted from Govindjee and Wilbert (2010)). Red arrows show light absorption by the photosystems. PSII= Photosystem II, OEC= Oxygen Evolving Complex, P680= PSII reaction centre chlorophyll, P680*=excited P680 after light absorption, Phe=Pheophytin, Q_A=Quinone A, Q_B=Quinone B, PQ= Plastoquinone, Cyt b_6f= Cytochrome b_6f, PC=Plastocyanin, PSI-Photosystem I, P700= PSI reaction centre chlorophyll, P700*=excited P700 after light absorption, A_0= special Chl a molecule (primary electron acceptor of PSI), A_1= phylloquinone (Vitamin K) molecule, Fd= Ferredoxin, FNR=Ferredoxin $NADP^+$ reductase.

PSI receives light activated electrons from plastocyanin and transfers them to Fd which is then oxidized by FNR to produce NADPH. Fd is an iron-sulphur protein. NADPH synthesized is used by the Benson-Calvin cycle (Foyer et al. 2012, Fromme and Mathis 2004). Reduced Fd can also reduce molecular oxygen to generate superoxide (O_2^-) instead of NADPH. While a small percentage of electrons passed to PSI (about 10%) is utilized in the production of O_2^-, most (about 90%) is used to drive NADPH production (Foyer et al. 1994, Foyer et al. 2012).

In addition to being passed into the Benson-Calvin cycle, electrons arriving at Fd can also be passed back into the electron transport chain in a cyclic pathway (Johnson 2011, Joliot and Johnson 2011). Cyclic electron transport (CET) involves light driven electron transfer involving only PSI, to generate the high **Δ**pH required to drive high energy state quenching (qE) and ATP synthesis. qE is a type of non-photochemical quenching (a protective mechanism) that occurs due to increased energization of the thylakoid (See Section 1.8.2) (Horton and Ruban 2005, Horton et al. 1996). CET is thought to occur when absorbed light exceeds utilization or when there is an imbalance in ATP:NADPH production (Hertle et al. 2013b, Johnson 2011). CET is important in maintaining effective photosynthesis in C_3 Plants (Clarke and Johnson 2001, Munekage et al. 2004). In C_4 plants however, CET is suggested to occur particularly in the bundle sheath cells, where PSI is thought to predominate (See Section 1.5). This conclusion is based on the observation that in C_4 plants, bundle sheath cells contain only trace amounts of PSII so are presumed to be unable to perform substantial linear electron transport. CET is difficult to estimate in C_4 plants however due to the different photosystem ratios in the different tissues, meaning that fluorescence and absorbance signals arise from different cell types (Heber and Walker 1992, Johnson 2011, Shikanai 2007).

At least three different pathways have been proposed for CET (Cruz et al. 2005). The first involves electron flux from Fd back to plastoquinone (PQ) with the electron transfer being catalysed by a putative ferredoxin plastoquinone reductase (FQR) (Bendall and Manasse 1995). The second involves electron flow from NADPH to PQ (Cruz et al. 2005, Johnson 2005, Kubicki et al. 1996, Livingston et al. 2010). This pathway is suggested to be mediated by an NADPH dehydrogenase (ndh) complex homologous to the mitochondrial NDH complex (Shikanai 2014, Yamamoto and Shikanai 2013). The third pathway is a route that involves the integration of FNR with Cyt b_6f to form a supercomplex (Zhang et al. 2001). FQR which catalyses electron flow from Fd back to PQ was recently suggested to be a complex involving the PGRL1 protein (Hertle et al. 2013b).

Various plants have been examined for CET under different conditions (Clarke and Johnson 2001, Edwards and Walker 1983, Golding and Johnson 2003, Heber and Walker 1992, Ivanov et al. 2001b, Johnson 2011, Munekage et al. 2002, Shikanai 2007). Edwards and Walker (1983) and Ivanov and co-workers (2001b) argued that cold stress could activate CET in winter Scots pine, a C_3 plant. Munekage and co-workers (2004) specifically investigated the effects of CET on two earlier proposed routes of CET (ndh and PGR5) on photosynthesis in Arabidopsis. They reported severe inhibition of growth in plants lacking both ndh complex and the PGR5 protein and suggested that CET is essential for photosynthesis in Arabidopsis (Munekage et al. 2004). Clarke and Johnson (2001) studied the effects of low temperature on CET while Golding and Johnson (2003) examined drought effects. CET was reportedly triggered in both stress conditions by those studies. However, there are other reports that found no evidence for CET under a range of non-stressful conditions (Genty and Harbinson 1996b, Genty et al. 1990).

1.4.3 Benson-Calvin Cycle

The Benson-Calvin cycle is a metabolic pathway present in the stroma of the chloroplast. Carbon enters the cycle as CO_2 and exits as triose phosphates (Glyceraldehyde-3-phosphate). The cycle can be divided into three stages- carbon fixation, reduction and regeneration (Fig 1.4) (Klekowski 1997). The ATP and NADPH needed to drive this cycle come from the light-dependent reactions of the ETC.

During Stage 1 (carbon fixation), CO_2 binds to a 5-carbon acceptor molecule, ribulose 1,5 bisphosphate (RuBP) to produce an unstable six-carbon compound, 3-keto-2-carboxyarabinitol-1,5-bisphosphate (3-Keto-CABP). This reaction is catalysed by ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) with the product splitting immediately after formation into two molecules of 3-phosphoglyceric acid (PGA) (Araújo et al. 2014, Bowyer and Leegood 1997).

$$CO_2 + RuBP \longrightarrow 2PGA$$

During Stage 2 (reduction), ATP and NADPH from the ETC convert PGA to glycerate-3-phosphate (G3P) (Bowyer and Leegood 1997, Klekowski 1997). The latter is needed as a precursor for sugar production. The third stage (regeneration), which involves the conversion of G3P back to RuBP using ATP, completes the cycle. For every molecule of G3P synthesised, three molecules of CO_2 enter the cycle and the latter completed six times to produce 0.5 molecule of six-carbon glucose ($C_6H_{12}O_6$). Hence the cycle utilises nine molecules of ATP and six molecules of NADPH for the net synthesis of one G3P and 0.5 molecule of $C_6H_{12}O_6$ (Fig 1.4) (Klekowski 1997).

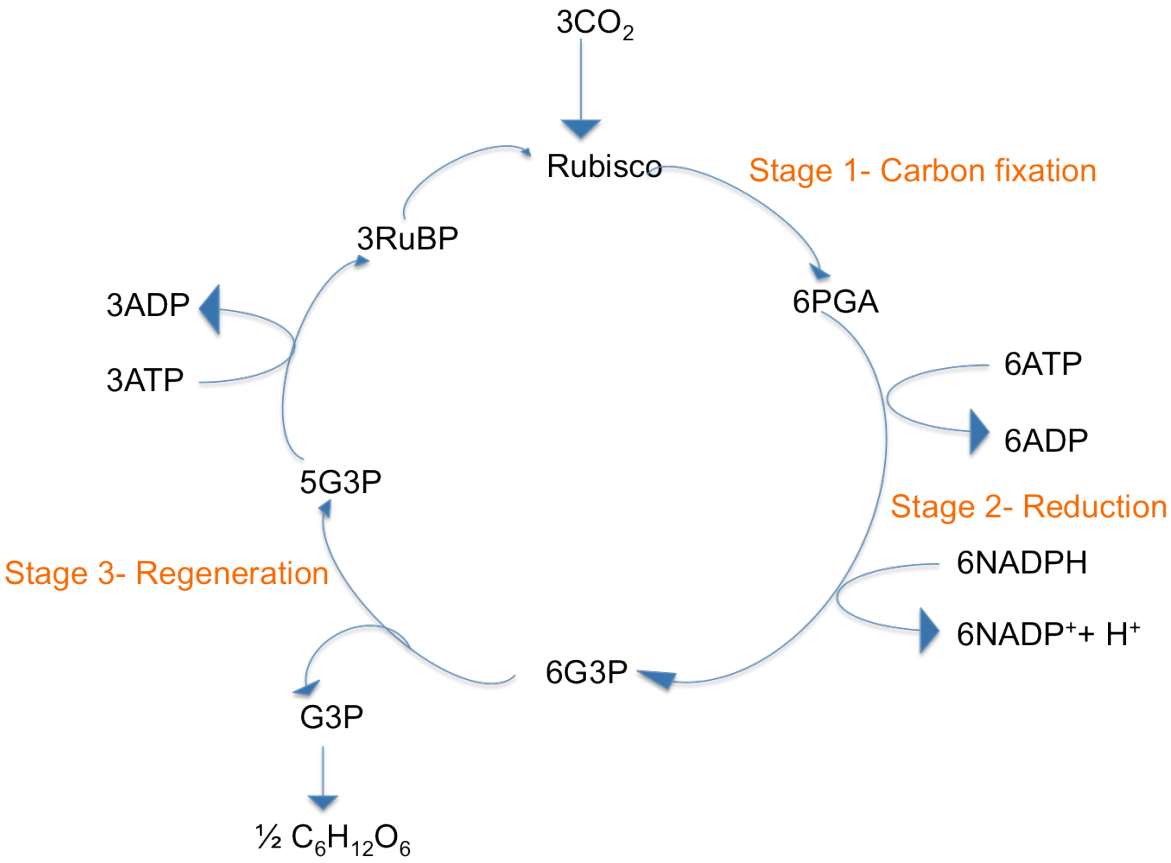


Fig 1.4 Benson-Calvin Cycle. In Stage 1 of the cycle, 3-phosphoglyceric acid (PGA) is formed. In Stage 2, ATP and NADPH convert PGA to glycerate-3-phosphate (G3P). G3P must be produced six times to make 0.5 molecule of six-carbon glucose. In Stage 3, G3P is converted back to RuBP using ATP for the completion of the cycle. RuBP, ribulose 1,5-bisphosphate, NADPH, reduced nicotinamide adenine dinucleotide phosphate, $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate.

The Benson-Calvin cycle is inactivated in the dark and needs to be activated at various steps most notably by the thioredoxin/ferredoxin activation system and by Rubisco activase. Thioredoxins are redox proteins that facilitate the reduction /oxidation of disulphide bridges in other proteins (Holmgren 1989). They have themselves catalytically active disulphide bonds, formed between two redox active cysteine residues, forming part of a characteristic motif (Trp-Cys-Gly-Pro-Cys) in the active site (Campbell et al. 2006). In plants, there are six different types of thioredoxins- thioredoxin f, m, x, y, h and o - that function in various cell compartments (Meng et al. 2010). However, only two (thioredoxin m and f) found in the chloroplast have clearly defined functions. All thioredoxin types contain a disulphide bond (-S-S-) in the oxidized form and two sulfhydryls (-SH-) in reduced form. The reduced thioredoxin-thioredoxin f activates several Benson-Calvin enzymes, including glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, fructose 1,6-bisphosphatase, sedopheptulose-1,7 –bisphosphatase and ribulose-5-phosphate kinase by reducing specific disulphides in these enzymes to thiols (Berg et al. 2002, Campbell et al. 2006, Michelet et al. 2013, Yano 2014).

Ferredoxin-thioredoxin reductase (FTR), a rieske protein (4Fe-4S) is crucial in the ferredoxin/thioredoxin regulatory chain. FTR uses reduced Fd to reduce thioredoxins, providing a link between the activity of the ETC and the activity of the Benson-Calvin cycle (Balsera et al. 2013, Foyer and Noctor 2000).

$$2Fd_{(red)} + \text{thioredoxin}_{(ox)} \xrightarrow{\text{FTR}} 2Fd_{(ox)} + \text{thioredoxin}_{(red)}$$

1.4.4 Rubisco

Rubisco is the most abundant protein on earth and is located in the chloroplast stroma (Feller 1998). Rubisco contains two subunits, the large (RbcL) and small (RbcS) subunits. In plants, these subunits form a holoenzyme consisting of 8 large and 8 small subunits, which have a combined molecular weight of ca 520kDa (Saschenbrecker et al. 2007, Spreitzer et al. 2005). The RbcL gene is present in the chloroplast of plants and the resulting polypeptide has a molecular weight of 55-kDa. RbcL forms the active site responsible for CO_2 fixation and has both catalytic and regulatory sites. In contrast, the RbcS gene is contained in the nucleus of plants and the protein product has a molecular weight of 13-kDa (Berg et al. 2002). RbcS enhances the catalytic activity and CO_2/O_2 specificity of the RbcL subunits (Berg et al. 2002, Genkov and Spreitzer 2009).

Rubisco catalyses either carboxylation or oxygenation reactions (Feller 1998). During carboxylation, PGA formed from the reaction of RuBP and CO_2 is used for either sugar production or regeneration of RuBP for the continuation of the Benson-Calvin cycle (Berg et al. 2002). On the other hand, during oxygenation, Rubisco reacts with molecular oxygen (O_2) instead of CO_2, to form one molecule of PGA and one of phosphoglycolate (PG). PGA re-enters the Benson-Calvin cycle while the carbon in PG has to be recovered through a series of reactions termed photorespiration (Fig. 1.5) (Berg et al. 2002, Bowyer and Leegood 1997). The latter involves enzymes and cytochromes located in the mitochondria and peroxisomes (Berg et al. 2002).

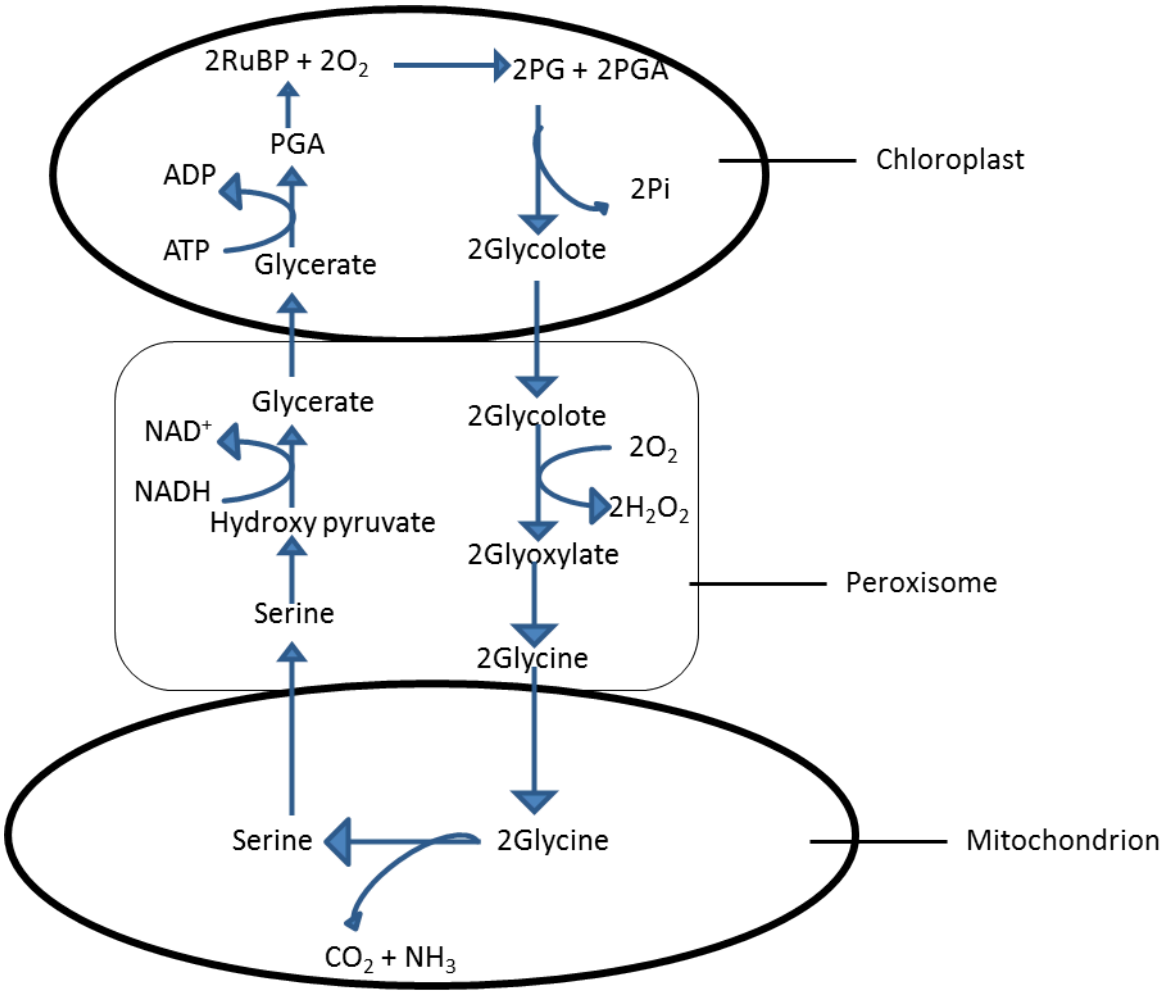


Fig.1.5 Photorespiration in plants. Oxygen (O_2) binds to ribulose 1,5 bisphosphate (RuBP) to make phosphoglycolate (PG). H_2O_2, hydrogen peroxide, CO_2, carbon dioxide, NH_3, ammonia, NAD^+, oxidized nicotinamide adenine dinucleotide, NADH, reduced nicotinamide adenine dinucleotide.

The role of Rubiso activase in activating the Benson-Calvin cycle cannot be overemphasized. Rubiso activase is a molecular chaperone that has the role of activating Rubisco and maintaining its activity (Zhang et al. 2002, Zhang and Portis 1999). The activase maintains activity by removing bound 2-Carboxy-D-arabitinol 1-phosphate (CA1P), a competitive inhibitor that occurs naturally in the chloroplast. CA1P can inhibit carboxylase or oxygenase reactions of Rubisco by binding tightly to the catalytic site of Rubisco. This happens at low light or in darkness (Khan et al. 1999). Upon transition to high light, Rubiso activase uses ATP from the ETC, together with light-activated CA1P-phosphatase, to remove CA1P. This renders CA1P non-inhibitory, thereby restoring full Rubisco activity (Khan et al. 1999, Scales et al. 2014, Zhang et al. 2002). Rubiso activase can also adjust the activation state of Rubisco to regulate the rate of CO_2 fixation (Carmo-Silva and Salvucci 2013). To do this, the activity of the activase is regulated by the ADP/ATP ratio of the chloroplast stroma, because ADP inhibits ATP hydrolysis by the activase.

There are two isoforms of the activase in plants- the large (46kDa) and small (43kDa) forms, which differ only at the carboxyl terminus (Carmo-Silva and Salvucci 2013, Zhang and Portis 1999). These isoforms are sometimes termed α and β- with the α-isoform being the longer polypeptide. The isoforms are the products of alternative splicing (a regulated process that occurs during gene expression which leads to a single gene capable of coding for multiple proteins) (Carmo-Silva and Salvucci 2013). Even though the two isoforms can activate Rubisco, most of Rubiso activase regulation occurs on the larger isoform (Zhang and Portis 1999). It is unclear why this is but might be related to the evolution of Rubisco.

Rubisco is reported to have evolved about 2.5 billion years ago in cyanobacteria. During that period the atmosphere had a high amount of CO_2 and limited O_2. Atmospheric CO_2 dropped in the late Miocene and into Pleistocene – (23-2 Mya) as a consequence of hot or warm climates often deficient in inorganic CO_2. This resulted in failure to distinguish between CO_2

and O_2 and adjustments in photosynthetic pathways as compensation. This resulted in the formation of the photorespiratory pathway, crassulacean acid metabolism (CAM) and C_4 photosynthesis (See Section 1.5) (Barsanti and Gualtieri 2006, Christin and Osborne 2013, Edwards et al. 2010, Sage et al. 2012). These mechanisms can concentrate CO_2 in cells where Rubisco is contained.

Attempts have been made to manipulate Rubisco to increase crop productivity with some problems reported. These problems include finding ways to specifically target Rubisco catalysis and an approach that can be used to produce a Rubisco activase capable of responding to changes in temperature (Parry et al. 2003, Parry et al. 2013).

1.5 Alternative Pathways for CO_2 Fixation

Plants use either C_3, CAM or C_4 mechanisms for photosynthesis. C_3 photosynthesis is used by majority of higher plants (approximately 85%) and simply involves the Benson-Calvin cycle as described (Section 1.4.3), while CAM and C_4 photosynthesis are modifications of this pathway utilised mainly by plants adapted to hot, arid or dry habitats. CAM and C_4 mechanisms produce better water use efficiency due to lack of photorespiration compared to C_3 and are used by about 10% and 5% of higher plants respectively (Yamori et al. 2014). C_3 photosynthesis originated about 2,800 Mya while CAM is thought to have evolved several times with the ability to endure periods of reduced precipitation (Forseth 2010). C_4 photosynthesis is the most recently evolved, and is assumed to have evolved about 10 Mya over 60 times independently due to increased aridity, high temperature and a reduction in atmospheric CO_2 from 400μmol mol^{-1} to 300 μmol mol^{-1}. At high temperature and low atmospheric CO_2, Rubisco fails to distinguish between CO_2/O_2. This favours C_4 plants which have a carbon concentrating mechanism enhancing their survival in warm climates (Christin

and Osborne 2013, Edwards et al. 2010, Forseth 2010, Griffiths et al. 2013, Osborne and Sack 2012, Sage et al. 2012). Plants utilising the various types of photosynthesis differ in their responses to stresses. For instance, C_4 plants generally have greater ability to tolerate high temperature than C_3 plants. C_4 plants also have reduced photorespiration compared to C_3 plants (Araújo et al. 2014, Whitmarsh and Govindjee 1999, Yamori et al. 2014).

C_3 photosynthesis occurs in most plants as the Benson-Calvin Cycle and is named as such because of the primary CO_2 fixing reaction involves the synthesis of the 3-carbon compound, PGA (Araújo et al. 2014). Examples of plants that use C_3 photosynthesis include grain cereals- such as rice, wheat, barley, vegetables-including tomatoes, spinach and trees such as-apple and peach (Whitmarsh and Govindjee 1999, Yamori et al. 2014).

CAM Photosynthesis is a photosynthetic pathway and mechanism in some plants adapted to arid conditions (Berg et al. 2002, Swarthout and Hogan 2012). Such plants, known as CAM plants, close their stomata during the day to avoid evapotranspiration but open them during the cooler more humid night, to accumulate CO_2 (Herrera 2009, Swarthout and Hogan 2012). Hence, CAM plants have high water use efficiency. Plants performing full CAM photosynthesis store the CO_2 that is accumulated at night in the vacuole as a 4-carbon organic acid, malic acid (Herrera 2009, Swarthout and Hogan 2012). Before storage in the vacuole, the CO_2 is fixed in the cytoplasm of the mesophyll by reaction with phosphoenolpyruvate (PEP) catalysed by the enzyme PEP carboxylase (Fig 1.6). The oxaloacetate (OAA) produced in this reaction is converted to malate by NAD^+ malate dehydrogenase. Malate is transported and stored in the vacuole as malic acid (Forseth 2010).

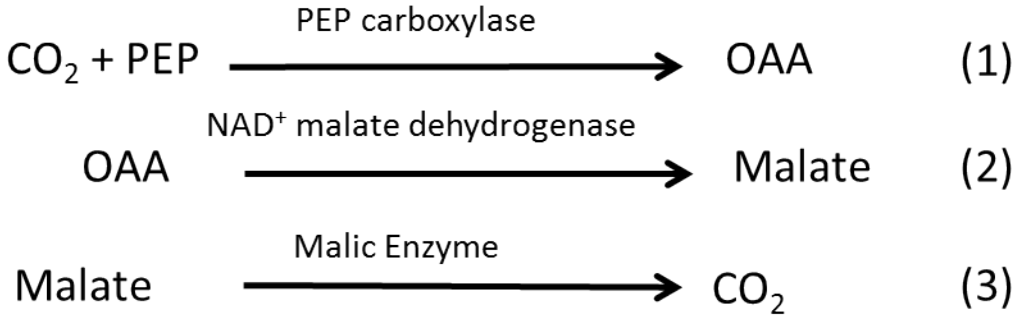


Fig. 1.6 Schematic representation of CAM photosynthesis. OAA is produced in the mesophyll cytoplasm by the reaction of PEP with CO_2. OAA is converted to malate and stored in the vacuole. The stored malate breaks down to CO_2 during the day.

During the day, the stored malate is transported to the chloroplast stroma where it breaks down to CO_2 that is concentrated around Rubisco for use by the Benson-Calvin cycle (Herrera 2009, Swarthout and Hogan 2012). In some CAM plants, during the day low levels of malic acid are formed from OAA in the cytosol. Such CAM plants are said to use phosphoenolpyruvate carboxykinase (PCK) biochemical pathway. There are two forms or subtypes of CAM plants- NADP malic enzyme (NADP-ME) and PCK, which differ, based on the daytime decarboxylation of malate (Dittrich et al. 1973, Yamori et al. 2014). Examples of plants that typically use CAM pathway are Pineapple, Agave and Cactus (Ritchie and Bunthawin 2010).

C_4 photosynthesis is sometimes called the Hatch-Slack pathway (Hatch and Slack 1966). C_4 plants have a unique anatomy (called Kranz anatomy). They have two different photosynthetic cell types-mesophyll and bundle sheath. Like CAM plants, C_4 plants form a 4-carbon molecule (OAA) as the first product of carbon fixation. The initial step of the pathway for C_4 plants involves stomatal diffusion of CO_2 into mesophyll cells. There, pyruvate is

converted to PEP by the enzyme pyruvate orthophosphate dikinase in the presence of inorganic phosphate (P_i) and ATP. This reaction produces a primary CO_2 acceptor, that is, a 3-carbon compound (C_3) PEP, adenosine monophosphate and inorganic pyrophosphate (PP_i) (Hatch and Slack 1966, Slack and Hatch 1997). PEP then binds to CO_2 to form OAA, catalysed by the enzyme PEP carboxylase. The reaction of PEP with CO_2 occurs even at low CO_2 concentrations due to the high affinity of PEP for CO_2. OAA is converted to malate in the mesophyll chloroplast (by NADP-malate dehydrogenase) and transported via plasmodesmata into the bundle sheath (Fig. 1.7) (Bowyer and Leegood 1997, Hatch and Slack 1966, Long and Spence 2013, Slack and Hatch 1997). In the bundle sheath, malate degrades to CO_2 and pyruvic acid. This decarboxylation leads to the generation of NADPH which is available for use in the Benson-Calvin cycle and an imbalance in the NADPH:ATP which is corrected through cyclic-driven ATP generation (Johnson 2011). CO_2 is used by the Benson-Calvin cycle while pyruvate is transported back to mesophyll (Hatch and Slack 1966, Slack and Hatch 1997). In the Benson-Calvin cycle, the CO_2 fixation is catalysed by Rubisco to produce sugars while pyruvate in the mesophyll is converted to PEP for the repetition of the cycle (Bowyer and Leegood 1997).

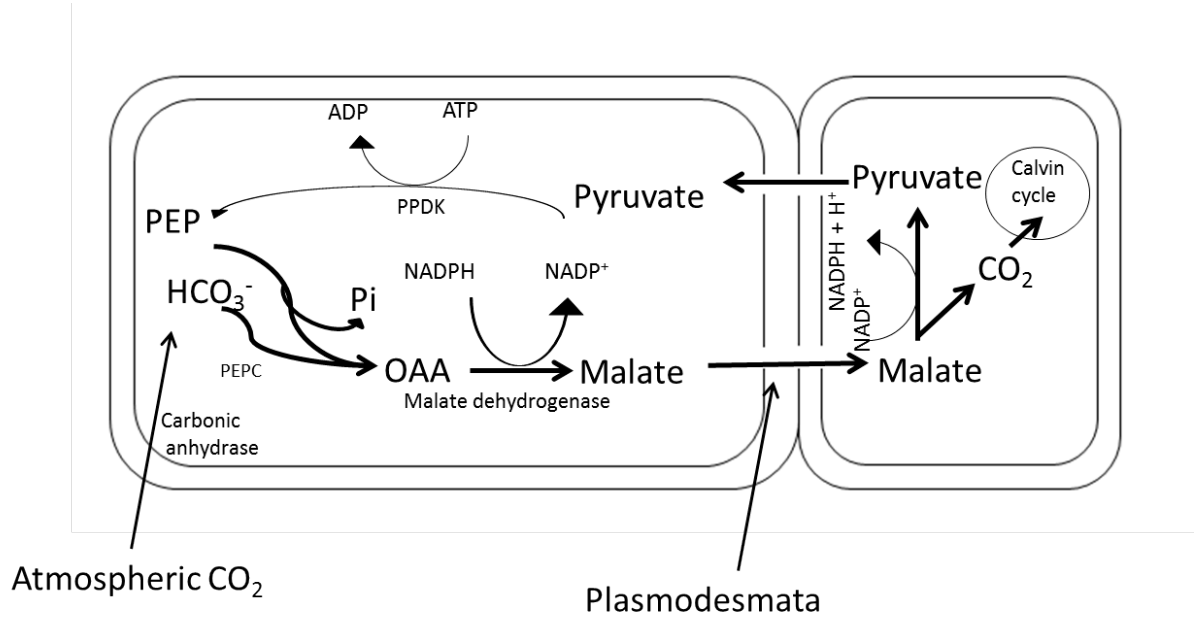


Fig. 1.7 Diagram of Hatch-Slack Pathway adapted from Koning (1994). Atmospheric CO_2 binds to PEP to form oxaloacetic acid, a 4-carbon compound in a reaction catalysed by the enzyme PEP carboxylase (PEPC). Malate is subsequently produced and transported into the bundlesheath cell. PEP, phosphoenolpyruvate, PEPC,phosphoenolpyruvate carboxylase, HCO_3^-, bicarbonate ion, $NADP^+$, nicotinamide adenine dinucleotide phosphate, NADPH, reduced nicotinamide adenine dinucleotide phosphate, PPDK, pyruvate phosphate dikinase.

C_4 plants that perform this type of photosynthesis are referred to as NADP-ME plants. Other types of C_4 plants are NAD-Malic Enzyme (NAD-ME) and PCK plants, with the differences being in the C_4 acid that is translocated into the bundle sheath, the location of the decarboxylating enzyme and the form of C_3 acid that is sent back to the mesophyll. For instance, in both NAD-ME and PCK forms, OAA produces aspartate in the cytosol instead of

malate. Aspartate is transported into the bundle sheath where it forms OAA (Fig. 1.8). In NAD-ME, OAA breaks down to malate and the latter is decarboxylated and pyruvate is synthesized. CO_2 released is transported into the bundle sheath chloroplast while pyruvate is converted to Alanine and sent to the mesophyll. In PCK, OAA is transported into the bundle sheath chloroplast where it is decarboxylated and PEP is formed (Long and Spence 2013, Sage and Pearcy 2000, Zhu et al. 2010). PEP is converted to Alanine and transported into the mesophyll (Fig. 1.8). Apart from being involved in photosynthesis, the biological role and significance of the different biochemical subtypes is unclear (Sage and Pearcy 2000, Wheeler et al. 2005). However, they have been linked to plant defence and stomatal control (Wheeler et al. 2005). The consensus at the moment is that CO_2 fixation occurs in the bundle sheath.

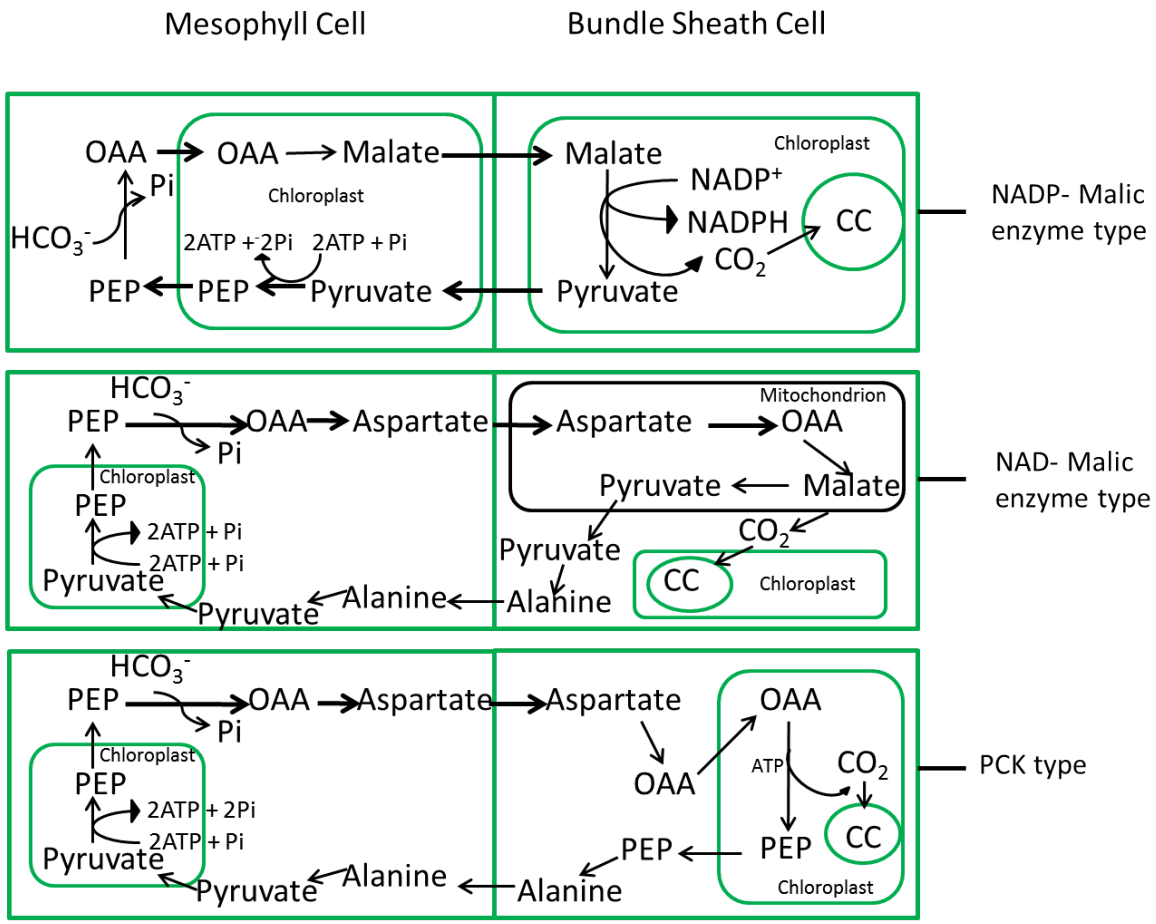


Fig 1.8. C_4 biochemical types. In NADP-Malic enzyme type (NADP-ME) and NAD-Malic enzyme (NAD-ME), the decarboxylating enzyme is malate. In the former, malate is decarboxylated in the bundle sheath chloroplast while in the latter its decarboxylation occurs in the mitochondrion before it is sent to the bundle sheath chloroplast. In phosphoenol pyruvate carboxykinase type (PCK), OAA is decarboxylated in the bundle sheath chloroplast. HCO_3^-, bicarbonate ion, OAA, oxaloacetate, CC, Benson-Calvin cycle.

C_4 plants, like CAM plants, have higher water use efficiency than C_3 plants, because they have a carbon concentrating mechanism, which involves PEP carboxylase (PEPC), which allows them to operate at lower stomatal conductance (Long and Spence 2013, Yamori et al. 2014). Unlike in C_3 plants, where a significant percentage of photorespiration occurs, due to CO_2 bound directly to RuBP in the presence of Rubisco, in C_4, PEPC has a higher affinity for CO_2 compared to Rubisco. PEPC increases the CO_2 accumulated by PEP in the chloroplast stroma thereby enhancing CO_2 fixation. This reduces the rate of photorespiration. However, during stress, for instance, high temperature, C_4 plants close their stomata. In such conditions, CO_2 available in the cytosol and stroma drops and photorespiration may still occur albeit at a much lower level compared to C_3 plants (Leegood 2007, Yamori et al. 2014). Examples of plants that use C_4 pathway include sugar cane, millet, maize and sorghum (Hatch 1999, Zhu et al. 2008).

1.6 Effects of drought on photosynthesis

Drought is known to have deleterious effects on plants. The most visible effect is wilting, which occurs as a result of a decrease in turgor pressure. Turgor pressure is the pressure exerted onto the cell walls due to the uptake of water into the cell down a concentration gradient (Campbell et al. 2008). This pressure pushes the plasma membrane against the cell wall of plants with plants described as turgid under such a state. During drought, turgor pressure decreases and the plant cell is said to be plasmolysed (Campbell et al. 2008). Drought effects may be seen at morphological, physiological or biochemical levels in plants (Ghannoum 2009, Medrano et al. 2002). During stresses such as high temperature and drought, morphological effects can prevent water uptake and distribution due to adjustments in rooting, shoots and leaves. In terms of rooting, comparison of the growth of seminal and

nodal roots has shown that, upon exposure to stress, the growth of the former is severely inhibited while the latter is unaffected (Cruz et al. 1992, Pardales et al. 1991, Salih et al. 1999)

It is well established that drought leads to inhibition of photosynthesis (Blum 2011, Chaves et al. 2003). However, the mechanism of this inhibition remains controversial (Chaves et al. 2009). There has been controversy as to whether the inhibition of photosynthesis induced by drought occurs due to stomatal or non-stomatal limitation (Chaves et al. 2009). Stomatal limitation involves a decrease in photosynthesis as a result of stomatal closure, induced by the accumulation of high concentrations of abscisic acid (ABA), thus preventing CO_2 entry. This is often regarded as the initial response of plants exposed to drought (Ghannoum 2009, Medrano et al. 2002). If limitation of photosynthesis is non-stomatal, then inhibition in photosynthesis is attributed to damage to the Benson-Calvin cycle, specifically reduced activity of photosynthetic enzymes. In this condition, biochemical factors, such as changes in the activities of PEP and Rubisco, are expected to limit photosynthesis (Ghannoum 2009, Medrano et al. 2002). Regardless of the limitation that induces drought, inhibition of photosynthesis results in leaf senescence/abscission and decrease in ATP concentration.

In the chloroplast, the thylakoid and stroma are susceptible to damage. It is now generally accepted that mild to severe drought damages photosystem II (PSII) (Cornic et al. 2004, Cousins et al. 2002, Golding and Johnson 2003) but not PSI (Golding and Johnson 2003, Shikanai 2007, Takahashi et al. 2009). Cornic and co-workers attributed severe damage to PSII during drought to a direct effect of the drop in net CO_2 uptake of the chloroplast caused by stomatal closure (Cornic et al. 2004). In the stroma, this drop in net CO_2 uptake causes a decline in the activities of the Benson-Calvin cycle enzymes, with the exception of ribulose 1,5 bisphosphate (RuBP), which is reportedly upregulated (Cornic et al. 2004, Cousins et al.

2002). Drought achieves this reduction by lowering intracellular CO_2 concentration (Ci) resulting in reduced CO_2 assimilation (Cousins et al. 2002). This leads to the accumulation of ATP and NADPH and in turn to accumulation of reduced plastoquinol, which promotes the formation of the reactive oxygen species- singlet excited oxygen ($^1O_2$*) in PSII.

1.7 Reactive Oxygen Species Production

Stress is any change in environmental factors capable of affecting plants morphologically, physiologically or biochemically, which can damage growth or even cause death. Changes in the environment may involve abiotic or biotic factors. Abiotic factors include extremes in temperature, humidity, drought etc. while biotic factors are damage inflicted on plants by living organisms such as insects, fungi, viruses, etc. (Wang et al. 2003).

During stress in plants, there is a tendency for absorbed light energy to be poorly utilised for photosynthesis. This can occur due to a disruption of CO_2 fixation, which leads to a blockage of electron transport and so results in an excess of absorbed light. In PSII, electron flow may be blocked at the rate-limiting step (Q_A) with electrons likely to return from the acceptor side to the donor side (charge recombination). Consequently, Chl P680 of PSII forms an excited triplet (^{3}Chl*) and in this state can react with ground state O_2 ($^3O_2$) to form singlet ground state chlorophyll (^{1}chl) and singlet excited oxygen $^1O_2$* (Foyer et al. 1994, Krieger-Liszkay 2005). The production of $^1O_2$* in PSII is capable of causing damage to the D1 protein – photoinhibition (Asada 2000, Krieger-Liszkay 2005). There are reports that the production of $^1O_2$* is favoured by increases in lifetime of excitation in PSII and absence of an alternative electron sink (Heyno et al. 2009, Krieger-Liszkay 2005). In contrast, there are other reports

suggesting that the synergistic maintenance of the D1 and oxygen-evolving complex can allow longer equitable electron dissipation during stress and prevent photoinhibition (Adams et al. 2013, Tikkanen et al. 2014). The lifetime of the $^1O_2$* produced is reduced by the presence of the PSII carotenoids (β-carotene and maybe accessory PSII chlorophyll, Chl Z) in the reaction centre, which return $^1O_2$* to $^3O_2$ or directly quench the excited ^{3}Chl*. However, if produced in excess, $^1O_2$* can damage the D1 protein of PSII (photoinhibition) and potentially cause more widespread damage (Foyer et al. 1994, Kitajima and Noguchi 2006, Krieger-Liszkay 2005).

The transportation of electrons to PSI from Cyt b_6f complex through plastocyanin is also affected during stress. The electrons already passed through membrane bound proteins to ferredoxin become blocked with electrons redirected to Mehler reaction, or water-water cycle, and fewer electrons to NADPH (Adams et al. 2013, Asada 1999b, Asada 2000). Molecular oxygen (O_2) is reduced to superoxide (O_2^-, Photoreduction) in the Mehler reaction and O_2^- is then degraded to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD) (Asada 2006, Foyer et al. 1994). H_2O_2 is further catalysed either enzymatically (in the presence of enzymes such as ascorbate peroxidase and glutathione peroxidase) to H_2O or non-enzymatically in the presence of Fe^{2+} and Mn^{2+} - the Fenton reaction to hydroxyl radicals ($OH^{\bullet}$) (Foyer and Noctor 2011, Ślesak 2007). The production of H_2O_2 and superoxide has been shown to accelerate the occurrence of photoinhibiton by inhibiting the repair of damaged PSII (Takahashi and Badger 2011, Takahashi et al. 2009).

1.8 Mechanisms of Stress Tolerance

Plants are often exposed to varying environmental conditions such as differing degrees of drought. Plants therefore need to adjust their photosynthetic apparatus in order to survive. Various protective mechanisms adopted in response to drought include non-photochemical quenching (NPQ), cyclic electron transport, compatible solute accumulation and stress-induced proteins (An and Liang 2013, Borovskii et al. 2002, Cruz de Carvalho et al. 2011, Demirevska et al. 2008, Griffin et al. 2004, Jiang and Huang 2002, Kavi Kishor and Sreenivasulu 2013, Mohammadkhani and Heidari 2008, Takahashi et al. 2009, Wood and Goldsbrough 1997, Yobi et al. 2012). This section reviews plants mechanisms for regulating photosynthesis and metabolism under stress, with particular reference to water deficit.

1.8.1 Non-Photochemical Quenching

Non Photochemical Quenching (NPQ) is a mechanism utilised by green plants and algae in protecting themselves from stress (e.g. drought), low irradiance or any other condition capable of causing death (Cousins et al. 2002, Horton and Ruban 2005). It may involve the dissipation of excess excitation energy as heat via molecular vibrations and arises mostly due to high energy quenching (qE) (Horton and Ruban 2005, Horton et al. 1996). Other types of NPQ that may be generated in response to adverse conditions are state transitions (qT) and photoinhibition (qI).

1.8.1.1 qE

qE is the dominant form of NPQ and occurs in the light harvesting complexes (LHCs). LHCs are able to sense pH and regulate excess absorbed light energy, which can lead to changes in thylakoid pH (ΔpH) during drought. In such conditions, electrons absorbed by LHCs for

linear electron transport (LET) may be down regulated to match the actual electron demand needed for LET (Ali and Komatsu 2006, Cousins et al. 2002, Zhou et al. 2007). This down regulation ensures that excess absorbed energy is harmlessly dissipated as heat without damaging the photosynthetic pigments. This quenching of excitation energy has been shown previously to correlate with the carotenoids of the xanthophyll cycle leading to changes in the kinetics of the cycle (Demmig-Adams and Adams 1996, Pinnola et al. 2013, Ruban and Horton 1999). At low thylakoid pH, which can occur during drought, the carotenoids violaxanthin can be converted to zeaxanthin in the presence of a catalyst-violaxanthin de-epoxidase (VDE) and the reaction takes place via an intermediate called antheraxanthin. Upon return to control conditions, the reverse of the reaction- zeaxanthin to violaxanthin - induced by the enzyme zeaxanthin epoxidase (ZE) occurs. Again antheraxanthin is produced as an intermediate (Baker 2004, Demmig-Adams and Adams 1996). The back and forth reaction, which has been shown to be typically activated in high and low light, is termed the xanthophyll cycle (Nayak et al. 2002, Niyogi et al. 2005). Changes in the cycle can be summarised to occur in response to induction or relaxation of ΔpH, with the former representing an energization of the thylakoid and leading to qE generation (Horton and Ruban 2005, Ruban and Horton 1999).

qE enables excess excitation energy in the thylakoid to be dissipated as heat. It occurs in LHCII in close association with the xanthophyll cycle carotenoids, with its activation influenced by the PSII proteins CP26, CP29 and PsBS (Livingston et al. 2010). However, it is not clear how qE and xanthophyll cycle are regulated (Ruban and Horton 1999, Ruban and Murchie 2012). What is clear is that qE induction can be enhanced by the xanthophyll pigments-antheraxanthin and zeaxanthin- and does not damage chlorophyll a/b proteins contained in the antenna complex of PSII and xanthophylls (Galle et al. 2010, Xu et al. 2011a)

1.8.1.2 qT

In low light, a different type of NPQ- qT may occur in response to over excitation of PSII relative to PSI. In this case LHCII protein complex is phosphorylated from State 1 (PSII) to State 2 (PSI). This occurs in the presence of a specific LHCII kinase, serine/threonine kinase (Stn7) (Bellafiore et al. 2005). When adequate LHCII proteins have been transported to PSI to match PSII, the kinase is turned off. This is referred to as state 1 to 2 transitions (Fig. 1.9). The switch from state 1 to state 2 is controlled by the redox state on plastoquinone and balances the excitation of the two photosystems. When PSII is underexcited relative to PSI, a transition from state 2 to state 1 mediated by LHCII phosphatase, a PP2C type protein phosphatase (TAP38 and PPH1) occurs (Allen 2003, Lunde et al. 2000, Puthiyaveetil et al. 2012, Wollman 2001).

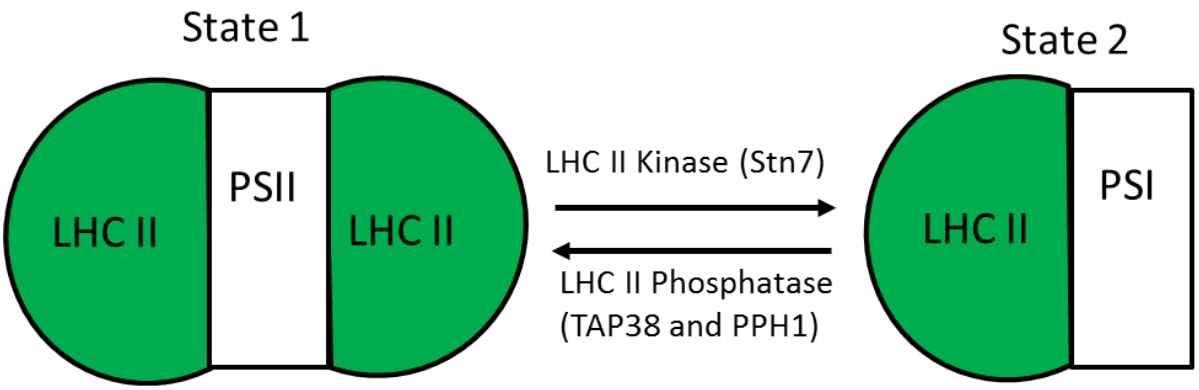


Fig.1.9. State transitions in green plants. Diagram shows two light harvesting complexes in photosystem II and one light-harvesting complex in photosystem I. LHII kinase is serine/threonine kinase (Stn7) while LHC II Phosphatase is regulated by PP2C type protein phosphatase (TAP38 and PPH1).

It is believed that qT allows plants to optimise their light capture at low light and prevents over excitation of one photosystem, which can lead to photoinhibition even at low light. Although it has been argued that qT can be activated in high light, experiments performed using barley and cyanobacteria revealed that qT is only important at low light intensities and that the absence of specific PSI-H subunit of PSI in plants prevents a transition from state 2 to 1 (Fujimori et al. 2005, Lunde et al. 2000, Mullineaux and Emlyn-Jones 2005, Walters and Horton 1991)

1.8.1.3 qI

qI which involves photo damage to the D1 protein of PSII reaction centre is a third type of NPQ and can occur during severe drought (Adams et al. 2013, Owens 1996). qI has two components- one which is protective and another which is damaging. The protective component may become activated upon sudden exposure to stress with excess energy dissipated, while the damaging component is activated upon prolonged exposure to stress. Unlike qE and qT, which have fast fluorescence relaxation kinetics (< 15 minutes), qI has a slow relaxation kinetic (> hour). Hence it is regarded as the most slowly relaxing component of NPQ (Walters and Horton 1991). qI-induced damage can be irreversible, especially after long term drought due to damage to PSII reaction centres (Walters 2005, Walters and Horton 1991).

1.8.2 Cyclic electron transport

CET involves a reduction in PQ by electrons originating from reactants after PSI, which leads to the translocation of protons from the stroma into the thylakoid lumen and generation of electrochemical gradient (ΔpH) or proton motive force (*pmf*) (Livingston et al. 2010). The

generation of ΔpH triggers the activation of photoprotective qE. During drought, LET may be disrupted and when that happens, CET functions to supply the reducing power needed by the Benson-Calvin cycle (Johnson 2011, Kohzuma et al. 2009). This results in enhanced ΔpH of the thylakoid and increased ATP synthase activity (Cruz et al. 2005). The latter has been explained as the reason for longer qE activity under water limiting conditions. Thus, there is strong evidence that ΔpH can increase both qE and net ATP production under stress conditions where CET is activated (Johnson 2011). Nevertheless, CET is activated during drought contributing to the balancing of the ATP/NADPH ratio together with the water-water cycle and malate shunt of the mitochondrion (Gao and Wang 2012, Huang et al. 2012, Kohzuma et al. 2009, Livingston et al. 2010, Zhang et al. 2011). Other factors that can affect ATP/NADPH ratio during drought are compatible solute production and stress-induced proteins (Hebbelmann et al. 2012, Sharma et al. 2011).

1.8.3 Compatible Solutes

Extreme conditions such as drought, salinity, heat waves, etc. lead to cell desiccation and water imbalance in plants (Chaves et al. 2003, Griffin et al. 2004, Kavi Kishor et al. 2005). These adverse effects result, without any form of regulation in the collapse of cells and eventually death of a plant. In order to survive such extreme conditions, mechanisms exist to minimise or prevent the occurrence of damage in response to desiccation.

Plants utilise primary and secondary metabolism in protecting themselves against extreme conditions. Primary metabolites are products of major metabolic process such as glycolysis (cytosol), Krebs cycle (mitochondrion) and Benson-Calvin cycle (chloroplast) while secondary metabolites include osmoprotectants or compatible solutes, alkaloids, carotenoids, cyanogenic glycosides etc. (Davey 2003, Kavi Kishor et al. 2005, Serraj and Sinclair 2002,

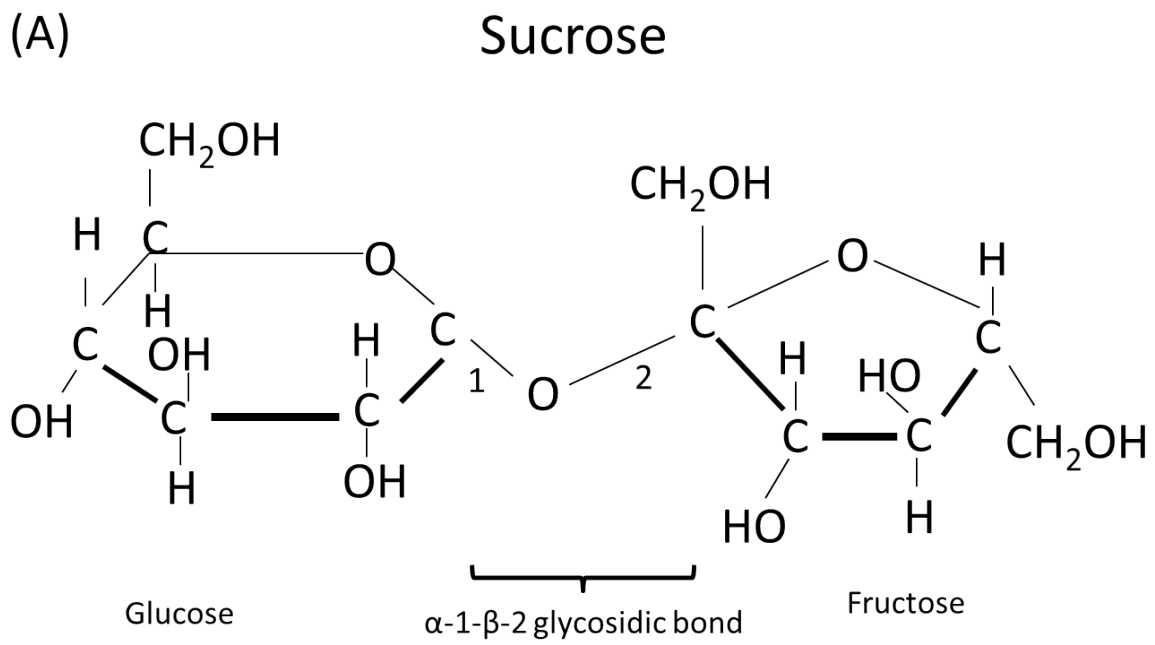
Yobi et al. 2012). Primary metabolites act as precursors for the synthesis of secondary metabolites (Kavi Kishor et al. 2005). Examples include glucose and sucrose (Adams et al. 2013, Delatte et al. 2011, Kavi Kishor et al. 2005, Nunes et al. 2013) while those of secondary metabolites are proline, glycine betaine (GB), sorbitol and trehalose (Ashraf and Foolad 2007, Davey 2003, Elbein et al. 2003, Garg et al. 2002). Secondary metabolites can accumulate in the cytoplasm of cells under low external water potential without inhibiting or damaging cytoplasmic components (Chaves et al. 2009, Kavi Kishor et al. 2005). They are thought to be involved in the protection of plants during stress conditions; for instance during drought, they lower the osmotic potential of the cytosol to prevent dehydration and loss of turgor (Chaves et al. 2009). The exogenous application of some secondary metabolites, for instance, GB, increases stress tolerance in plants (Chen and Murata 2008, Chen and Murata 2011); however the roles of hyperaccumulation during stress are unclear (Chaves et al. 2009, Kavi Kishor et al. 2005, Kavi Kishor and Sreenivasulu 2013). Hyperaccumulation of these small molecules has been reviewed as being a sign of stress, a protective mechanism or a coexisting mechanism (Chaves et al. 2009, Chen et al. 2007).

In the following subsections, compatible solutes will be discussed under two separate headings- carbohydrate based and nitrogen containing.

1.8.3.1 Carbohydrate-based

Carbohydrate-based compatible solutes are made up of monosaccharides, linked by glycosidic bonds, or sugar alcohols. They are highly soluble in water. Examples are the sugars- sucrose and trehalose and the sugar alcohols- sorbitol and mannitol (Garg et al. 2002, Hoffmann 2010, Ingram and Bartels 1996, Inman-Bamber et al. 2010, Nunes et al. 2013, Yobi et al. 2012).

Sucrose is a non-reducing disaccharide made up of the monosaccharides-glucose and fructose (Fig. 1.10A). The reducing ends of the two monosacharrides are linked together by an α-1β-2-glycosidic bond in such a way that binding to other saccharides is prevented (Beevers et al. 1952, Lemieux and Huber 1953). Trehalose is a disaccharide composed of two glucose subunits linked by an αα-(1-1)-glycosidic bond (Fig. 1.10B) and is also non-reducing (Elbein et al. 2003, Nunes et al. 2013). Sucrose and trehalose accumulation are believed to offer improved protection during drought (Garg et al. 2002, Hoffmann 2010, Ingram and Bartels 1996, Inman-Bamber et al. 2010, Nunes et al. 2013).



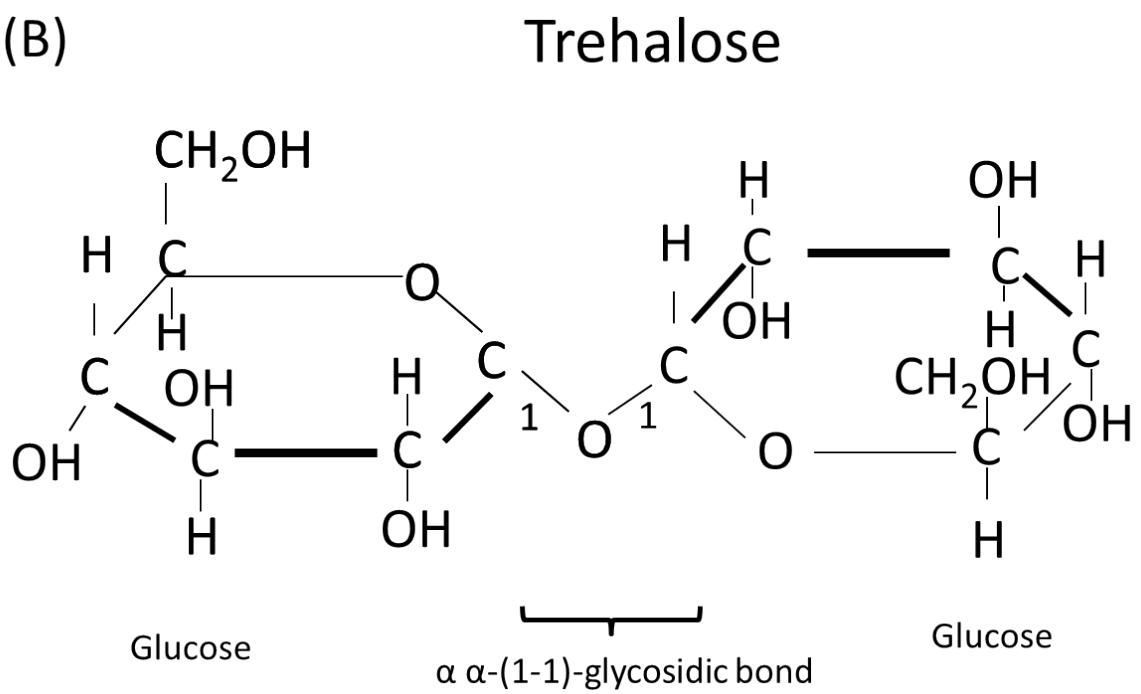


Fig.1.10. Examples of non-reducing disaccharides. Sucrose (A) and Trehalose (B). The reducing ends of the monosaccharides in Sucrose and Trehalose are linked by α-1β-2- and αα-(1-1) - glycosidic bonds respectively.

Sorbitol and mannitol are non-reducing sugar alcohols respectively derived from the reducing monosaccharides-glucose and mannose by the replacement of their aldehyde (-CHO) group with an alcohol ($–CH_2OH$) functional group (Fig. 1.11). They accumulate in response to low temperature and drought and play crucial roles in the regulation of osmotic potential. It is thought that they may scavenge free radicals generated during drought (Li et al. 2012, Yancey 2005).

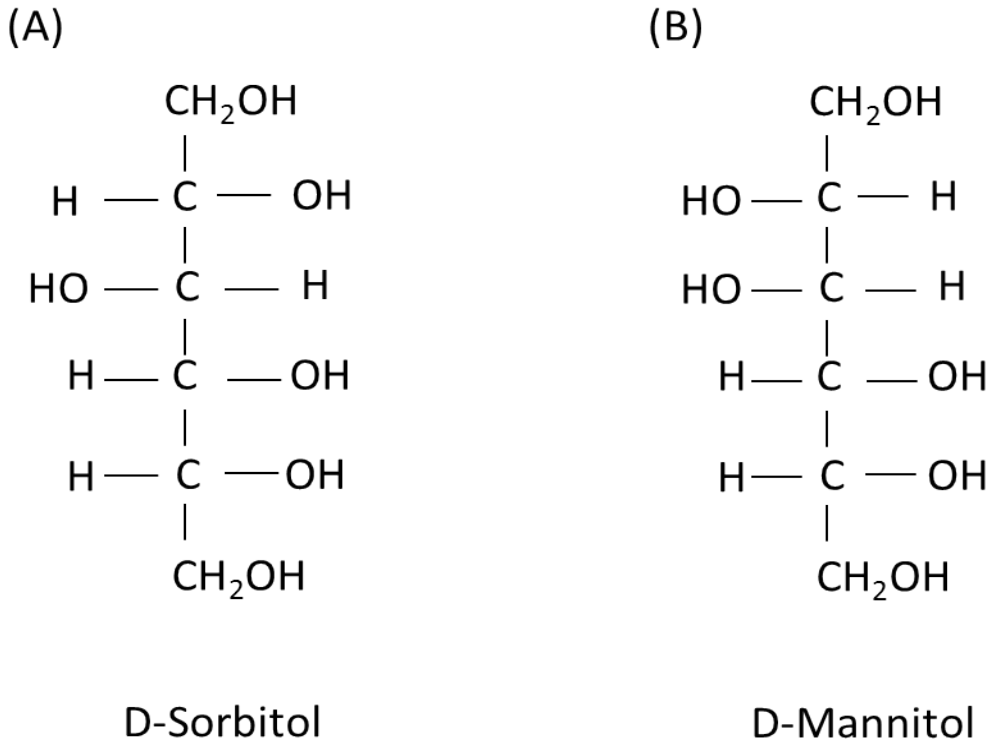


Fig. 1.11 Examples of non-reducing sugar alcohols. D-Sorbitol (A) and D-Mannitol (B). Structures derived from the monosccharides glucose and mannose by the replacement of their aldehyde (-CHO) group with an alcohol ($–CH_2OH$) functional group.

1.8.3.2 Nitrogen Containing

These types of compatible solutes are small molecules that contain nitrogen in their structure. Like carbohydrate-based compatible solutes, they are highly soluble in water. Examples are proline and glycine betaine (Delauney and Verma 1993, Kavi Kishor and Sreenivasulu 2013).

Proline is an amino acid that accumulates in plants and microorganisms during osmotic stress. Its accumulation is regarded as a mechanism to scavenge excess reactive oxygen species in order to protect against damage and ameliorate the effects of environmental stresses (Kavi

Kishor and Sreenivasulu 2013). The accumulation of proline depends on the species. For instance, barley and rice were reported to accumulate up to 3 or 4 fold of proline relative to control in response to osmotic and salt stresses respectively (Delauney and Verma 1993). The link between proline accumulation and stress adaptation in plants is however not clear.

The pathways of proline synthesis and degradation involve two major enzymatic processes. First, the rate limiting enzyme, crucial in the biosynthesis of proline in higher plants, D1-pyrroline-5-carboxylate synthetase (P5CS) converts glutamate (from cytoplasm or chloroplast) into pyrroline-5-carboxylate (P5C) (Fig. 1.12). P5C is then reduced to proline by a second enzyme D1-pyrroline-5-carboxylate-reductase (P5CR) (Kavi Kishor and Sreenivasulu 2013).

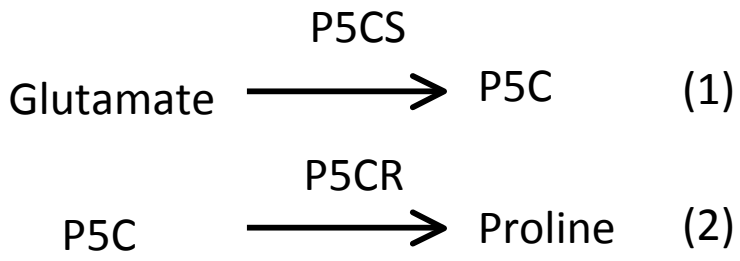


Fig. 1.12 Pathways of proline synthesis and degradation. Glutamate is converted into pyrroline-5-carboxylate (P5C) by D1-pyrroline-5-carboxylate synthetase (P5CS). P5C is subsequently reduced to proline by D1-pyrroline-5-carboxylate-reductase (P5CR).

GB is an amino acid betaine that is derived from glycine which occurs abundantly mainly in the chloroplast of plants, where it plays a vital role in osmotic adjustments (Ashraf and Foolad 2007). GB also occurs in bacteria, haemophilic archaebacteria, marine invertebrates and animals (Chen and Murata 2011). GB accumulates during osmotic stress and its

accumulation has been reported to contribute to drought tolerance (Serraj and Sinclair 2002, Yobi et al. 2012). There is evidence to show that transgenic plants overexpressing GB have improved tolerance to various abiotic stresses (Chen and Murata 2008, Chen and Murata 2011).

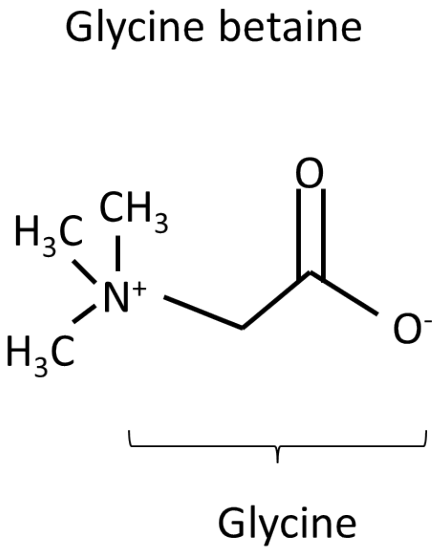


Fig. 1.13 Structure of glycine betaine. Glycine betaine is derived from glycine which is contained in the chloroplast.

In plants, GB is generated from two biosynthetic pathways. First, choline is oxidized to betaine aldehyde in a reaction catalysed by choline monooxygenase (CMO) (Fig. 1.14). Second, betaine aldehyde is oxidized to GB in the presence of the enzyme- NAD^+- dependent betaine aldehyde dehydrogenase (BADH) (Chen and Murata 2008, Chen and Murata 2011).

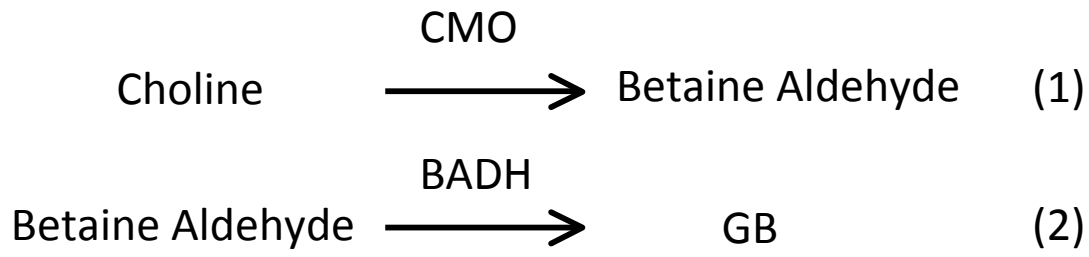


Fig. 1.14 Pathways of glycine betaine. Choline is first oxidized to betaine aldehyde in the presence of choline monooxygenase (CMO). Betaine aldehyde is then oxidized to GB in the presence of NAD^+- dependent betaine aldehyde dehydrogenase (BADH).

1.8.4 Stress - Induced Proteins

Exposure of plants to drought triggers the expression of stress-induced proteins. These proteins have been reviewed to either occur alone or together with other compatible solutes (Chaves et al. 2003, Griffin et al. 2004, Kavi Kishor et al. 2005, Wang et al. 2004, Xu et al. 2011b). These proteins include dehydrins and Heat Shock Proteins.

1.8.4.1 Dehydrins

Dehydrins (DHNs) are group II late embryogenesis abundant (LEA) drought proteins that accumulate in the cytosol, nucleus, chloroplast and mitochondria. They are known to have three-conserved segments- K, Y and S segments which affect their structures (Hanin et al. 2011). These segments are respectively named based on the presence of the amino acids- lysine, tyrosine and serine (Rorat 2006). They are expressed in response to stressful conditions in plant cells and are believed to protect cells from damage (Jagtap 1998, Mohammadkhani and Heidari 2008, Wood and Goldsbrough 1997).

1.8.4.2 Heat Shock Proteins

Heat shock proteins (HSPs) are stress-induced proteins. They are expressed mostly under temperature, heat and drought stresses (Jagtap 1998, Wang et al. 2004, Xu et al. 2011b). In plants, HSPs are classified according to their molecular weight into 5 major groups, namely, HSP 60, HSP 70, HSP 90, HSP 100 and the small HSPs, sHSP (Wang et al. 2004, Xu et al. 2011b). HSPs are involved in the maintenance of membrane protein stability and are expressed mostly in the cytosol and chloroplast. They are thought to have protective roles *in vivo* in replenishing stress damaged or degraded proteins thereby preventing misfolding (Wang et al. 2004, Xu et al. 2011b). However, their protective mechanisms during stress is unclear (Jiang and Huang 2002).

1.9 Sorghum

Sorghum (*Sorghum bicolor* L.), which is the fourth most important cereal worldwide, after rice, maize and wheat, originates from Sub Saharan Africa (Mutava et al. 2011). It is cultivated for different purposes. In Africa and many developing countries, it is cultivated primarily as a food crop while in developed countries, for instance, in the US, it is cultivated as a bioenergy and feedstock crop (Mutava et al. 2011). Sorghum can be separated into three types- grain, sweet and hybrid- with the difference being that the grain type grows taller and produces more biomass than the sweet type. Sweet sorghum instead accumulates sugars in the stem. The hybrid sorghum on the other hand combines the traits of both grain and sweet types, that is, produces both extensive biomass and has sweet stems (Gutjahr et al. 2013).

Sorghum is a C_4 drought tolerant cereal that does photosynthesis using NADP-ME biochemistry (See section 1.5) and grows in arid/semi-arid regions. It has been extensively

studied and its genome fully sequenced. This was done in order to identify physiological, molecular and biochemical mechanisms that can be used to improve the tolerance of other less tolerant cereals (Ngara et al. 2012, Paterson et al. 2009). In terms of the physiology of the plant, sorghum maintains photosynthesis, specifically PSII electron transport and net assimilation for a long time during water deficit (Kakani et al. 2011, Loreto et al. 1995, Zegada-Lizarazu et al. 2012). However, the mechanisms achieving this are unclear. Also, the recovery mechanism from water deficit has not been well reported in the literature. The molecular and biochemical mechanisms during drought are less studied but nevertheless have been reported (Kubicki et al. 1996, Mullet 2002, Paterson et al. 2009, Watling et al. 2000, Wood and Goldsbrough 1997). The genome of the plant was fully sequenced using whole-genome shotgun sequencing (Mace et al. 2013, Paterson et al. 2009) and the role of the ndh complex has been examined in the mesophyll and bundle sheath chloroplasts (See section 1.4.2). It was reported that three of the ndh subunits- H, J and K increased in the bundle sheath relative to mesophyll and so the increase was concluded to be responsible for ATP generation via CET (Kubicki et al. 1996). Although, there is growing evidence that CET ameliorates the effects of stress, literature reports on CET in C_4 plants are limited.
During drought, a 21KDa protein molecule was shown to be up-regulated and genes involved specifically in dehydration, high salinity and ABA differentially affected (Buchanan et al. 2005, Wood and Goldsbrough 1997). However, a complete characterisation of drought sensitive proteins- Heat Shock Proteins and Dehydrins has not been reported. Under high CO_2, the rate of photosynthesis in the plant is lowered but Rubisco content is unaffected (Watling et al. 2000). The maintenance of Rubisco under stress might have a metabolic cost or implications and this has not been examined.

1.10 Research aims and thesis outline

The principal aim of this thesis is to examine the mechanisms responsible for the maintenance of leaf function in sorghum for a relatively long time during drought.

Specific objectives were:

- To study the underlying physiological mechanisms behind longer maintenance of PSII efficiency and net assimilation under drought compared to other NADP-ME plants.

- To investigate acclimation to drought in sorghum cultivars with differing drought tolerance. This has been poorly reported, yet it is known that plants can adjust their metabolism during water deficit in order to limit the effects of the adverse conditions on growth, photosynthesis and crucial metabolic enzymes. This can lead to acclimation and was tested using gas exchange and chlorophyll fluorescence.

- To examine the regulation of PSII, PSI and Rubisco proteins as well as other biochemical processes, that may contribute to the maintenance of photosynthesis during water deficit. It was hypothesised that the maintenance of these proteins and the stress-induced proteins- heat shock proteins and dehydrins offer some degree of tolerance. This was tested using SDS-PAGE, western blots and biochemical assays.

- To study the metabolic changes that occur during drought in sorghum. There are very limited literature reports on such changes for C_4 plants. These were tested using metabolomics technologies- Fourier Transform Infra-Red spectroscopy and Gas Chromatography –Mass Spectrometry.

The thesis is presented in alternative format in accordance with the rules and regulations of the University of Manchester. The result chapters are presented in manuscript form and formatted to form a cohesive body of work. Detailed introductions, results sections (with specific hypotheses), discussions and conclusions are provided in the chapters of this thesis. Chapter 2 is a characterisation of the physiological mechanisms of drought response between sorghum and maize. Chapter 3 examines in more detail the contrasting responses and strategies adopted by two sorghum cultivars in response to drought (See Ogbaga et al. 2014). Chapter 4 is a chapter on biochemical analyses of droughted sorghum cultivars (Ogbaga et al *In preparation*). Finally, in Chapter 5, a comprehensive discussion of the findings is presented and future prospects outlined.

Chapter 2:

CHARACTERISATION OF THE RESPONSES OF SORGHUM AND MAIZE TO PROGRESSIVE DROUGHT

Authors:

Chukwuma C. Ogbaga, Giles N. Johnson

Address

The University of Manchester, Faculty of Life Sciences, Oxford Road, Manchester, M13 9PL, United Kingdom

Email Addresses

Chukwuma C. Ogbaga- chukwuma.ogbaga@manchester.ac.uk

Giles N. Johnson- giles.johnson@manchester.ac.uk

Author For Correspondence:

Giles N. Johnson

Tel: +44 161 275 5750

Fax: +44 161 275 5082

Email: giles.johnson@manchester.ac.uk

Key Words: Drought, Sorghum, Maize, Photosynthesis, Chlorophyll fluorescence, Photosystem I

Authors' contributions: CCO and GNJ designed the experiments. CCO performed all the experiments. CCO wrote the manuscript while GNJ provided comments. A part of figures 1 and 2 in this manuscript were shown in Chapter 3. Parts of these figures were shown in this chapter for comparison.

2.1 Abstract

Sorghum bicolor, a tropical C_4 plant, the first fully sequenced plant of African origin, is known to have marked drought tolerance. However little is known about mechanisms achieving this. The related species *Zea mays*, also a C_4 plant, is far less drought tolerant. Sorghum varieties with varying drought tolerance and maize were exposed for up to 12 days to drought. Gas exchange, Photosystem II and Photosystem I activities were measured using infrared gas analysis, chlorophyll fluorescence and NIR absorption spectroscopy respectively. Sorghum had a higher capacity to maintain photosynthesis than maize, with significant differences seen between the sorghum varieties, in terms of their ability to maintain photosynthesis under water deficit. Relative water content, photosynthesis, PSII electron flux (PSII ETR) and maximum PSII efficiency in the dark (Fv/Fm) were maintained up to Day 8 in the more drought tolerant Samsorg 17 whilst these parameters declined from Day 6 in the less tolerant Samsorg 40 and maize. In contrast to studies on C_3 plants, there was no increase in Photosystem I electron transport (PSI ETR) and NPQ during drought in either sorghum or maize. Based on this, it is suggested that, during progressive drought, there is no activation of cyclic electron flow in sorghum and maize, in contrast to reports on C_3 plants such as barley and Arabidopsis.

2.2 Introduction

Drought causes massive economic and agricultural losses and is forecast to be exacerbated in the future due to global climate change (Chen et al. 2012, IPCC 2013). Drought in arid regions is further compounded by competition for land between different economic purposes including industrial and agricultural uses to generate revenue and feed human population, respectively. Arable land available for agricultural use has, as a result, been in decline; hence there is need to breed crops that can produce maximum yield on limited land. In the tropics, particularly in Sub Saharan Africa, there have been continuous research efforts to produce water-use efficient crops that can thrive in marginal lands (Turyagyenda et al. 2013). There is also growing interest in understanding the drought tolerance mechanisms of widely cultivated staple drought tolerant crops such as Sorghum for the improvement of other crops. To do this, there is need to breed currently less tolerant staple crops to enable them to survive with limited water resources, to ensure food security.

Numerous morphological, physiological and biochemical changes occur in response to drought. Morphological effects are visible in the form of wilting in the shoots and leaves of green plants and adjustments in the rooting, water uptake and xylem structure can occur to offer some degree of drought tolerance (Carmo-Silva et al. 2009, Ghannoum 2009). In terms of physiology and biochemistry, photosynthesis becomes altered as a result of the disruption of the electron transport chain and the Benson-Calvin cycle (Flexas and Medrano 2002). Drought triggers a variety of specific physiological responses in plants, ranging from partial reduction in stomatal aperture (leading to a decrease in photosynthesis) to complete stomatal closure (Blum 1988, Blum 2011).

Stomatal closure is regarded as the earliest primary response of plants to drought stress, secondary responses being non-stomatal (biochemical), which only occurs during severe

stress (Chaves et al. 2003). The absence of stomatal effects in C_4 plants has been explained as being due to PEPC having a high affinity for CO_2 and hence being able to maintain CO_2 fixation for a relatively long time under drought (Carmo-Silva et al. 2012, Chaves et al. 2009, Ghannoum 2009). Plants can maintain open stomata using various mechanisms to acclimate or tolerate stressful conditions (Chaves et al. 2009, Chen et al. 2007, Yamori et al. 2014). During stress, loss of photosynthesis can occur to match the opening of the stomata. This occurs together with the down regulation of photosystem II and activation of cyclic electron flow to prevent the production of reactive oxygen species and for net ATP production (Takabayashi et al. 2005, Zegada-Lizarazu and Monti 2013). However in the absence of any form of regulation, water deficit alters linear electron transport ultimately leading to specific photosystem damage (photoinhibition) or production of reactive oxygen species (Asada 2006, Carmo-Silva et al. 2012, Medrano et al. 2002).

Sorghum bicolor is a tropical C_4 plant known to have marked drought tolerance and is the first fully sequenced plant of African origin (Paterson et al. 2009). Maize, also a C_4 plant, is however, not as drought tolerant as sorghum. In order to increase the drought tolerance of maize and other plants of the same biochemical subtype, it would be valuable to understand the mechanisms by which sorghum tolerates drought. Hence, in this present study, physiological responses of two sorghum cultivars − Samsorg 17 (a drought tolerant variety) and Samsorg 40 (less drought tolerant) − in response to progressive drought were examined. The responses of these sorghum plants with differing drought tolerance were compared with maize. Relative water content, gas exchange, chlorophyll fluorescence and Photosystem I photochemistry were studied in the two sorghum cultivars and maize. The results obtained are discussed in terms of identifying traits that contribute to drought tolerance.

2.3 Materials and Methods

Plant Material and Water deficit Induction

Seeds of Sorghum (*Sorghum bicolor* L. Cvs Samsorg 17- more drought tolerant and Samsorg 40-less drought tolerant) were provided by the Institute of Agricultural Research, Zaria, Nigeria. Maize seeds (Cv *Sundance*) were provided by Suttons, Paignton, UK. The seeds were grown in 3" pots filled with John Innes No.1 soil in a growth chamber with a 16h photoperiod and a day/night regime at 23°C/15°C and relative humidity (RH) of 40-50%. Photosynthetic photon flux density was 130 $\mu molm^{-2}$ s^{-1} (Light meter, SKYE instruments Ltd, UK provided by warm white LED lights, colour temperature =2800-3000 K). The plants were grown for 3 weeks before drought was imposed by withholding water for up to 12 days.

Soil Water Content and Leaf Relative Water Content

Soil Water Content (SWC) is expressed as a % maximum pot capacity i.e. % of pots containing water-saturated soils. Plant pots were then weighed every two days until day 12 and SWC (%) estimated relative to control.

Leaf relative water content (RWC) was calculated according to Barrs and Weatherley (1962). Leaf pieces were cut and weighed immediately to give fresh weight (Fw) of sample. Turgid weight (Tw) was obtained after floating leaf pieces on water for 4 hours. After recording fully turgid weight, leaves were dried at 70°C for 24h to determine the dry weight (Dw). RWC was estimated as:

$$(Fw-Dw)/(Tw-Dw) \times 100$$

Gas exchange measurement

Gas exchange was measured every 2 days until Day 12 using an Li-6400XT portable photosynthesis system (Licor, Lincoln, NE, USA) at a temperature of 25°C, PPFD 1000 $\mu molm^{-2}s^{-1}$, CO_2 concentration of 390 ppm and relative humidity (RH) of 50% ± 5. Fully expanded leaves were placed into LiCor extended reach chamber and allowed to equilibrate for 10 mins before gas exchange was measured. Water Use Efficiency (WUE) was calculated as net CO_2 assimilation (A) divided by transpiration (E) (Morison et al. 2008).

Stomatal density and Stomatal aperture determination

Stomatal density and stomatal aperture were determined using an impression approach on eight fully expanded leaves as described in Grant and Vatnick (2004). A thick patch of clear nail polish was applied to the abaxial leaf surface and allowed to dry. A clear tape was placed on the dried nail polish patch and peeled off to obtain a leaf impression. The leaf impression was trimmed with a pair of scissors, placed on a clean microscope slide and examined under a photomicroscope at 400X (Diameter-0.401mm, r-0.201, Area=0.127mm^2) to give stomatal density per unit area. Stomatal aperture, that is, the distance between the terminal ends of the guard cells of the stoma (Xu and Zhou 2008) was measured in micrometres (1 eye piece unit=2.5μm).

Chlorophyll estimation

Similar sized leaf pieces were cut and scanned using a flatbed Scanner (Canoscan LIDE 110). Leaf area was measured using ImageJ image analysis software (ImageJ 1.45s National Institutes of Health, USA). Leaf pieces were ground in a mortar and pigments extracted with 80% Acetone. Resultant extracts were assayed spectrophotometrically for total chlorophyll

content and chl a:b ratio in a glass cuvette using a USB4000 Spectrophotometer (Ocean Optics, Dunedin, USA) according to the method of Porra and Co-workers (1989) as shown below:

Chl a = 13.71.A (663.6-750) – 2.85.A (646.6-750)

Chl b = 22.39.A (646.6-750) – 5.42.A (663.6-750)

Chl a+b = 19.54.A (646.6-750) + 8.29.A (663.6-750)

Where A (X-750) is the difference in absorbance between X nm and 750nm

Photosystem II Efficiency and Photosystem I Electrons

Leaves were dark-adapted for 30 mins and used to estimate Photosystem II (PS II) efficiency and Photosystem I energy flux. For PSII Efficiency, measurements were made using WALZ PAM 101 fluorimeter and captured using laboratory written software (Labview, National Instrument). PPFD of actinic light [warm white LED (CREE MC-E; Colour temperature =2800-3000 K)] and saturation flashes were 1000 and 6000 μmol $mol^{-1}s^{-1}$ respectively. Maximum efficiency of Photosystem II, Fv/Fm and Non-Photochemical quenching, NPQ, were calculated as described in Maxwell and Johnson (2000) (see fluorescence induction curve below for more description):

Non-photochemical quenching (NPQ)- $(F_m^o - F_m')/ F_m'$

Efficiency of Photosystem II (ΦPSII)- $(F_m' - F_t)/ F_m'$

Maximum PSII efficiency in the light (F_v/F_m)- $(F_m^o - F_o)/ F_m^o$

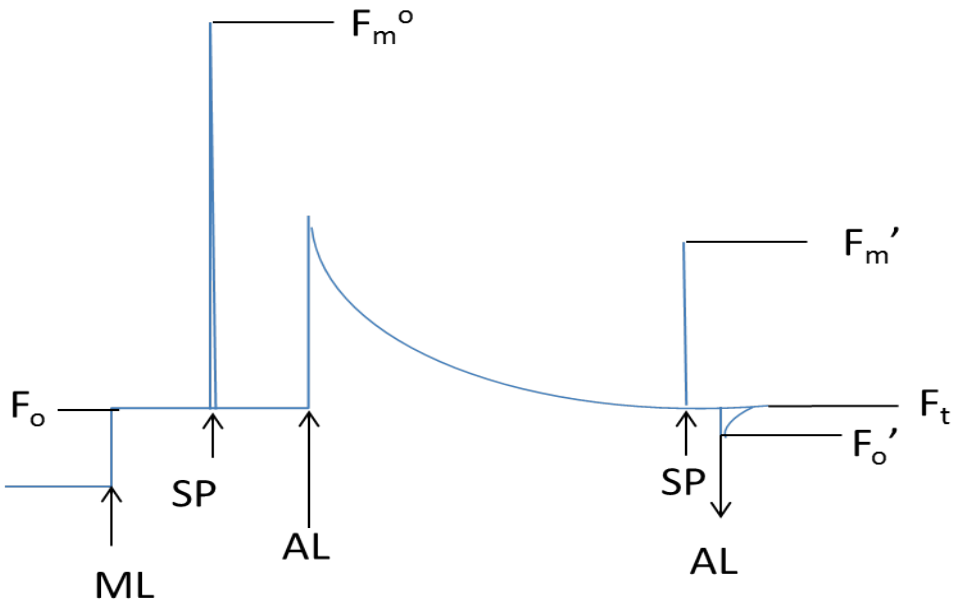


Fig. 2.1 Fluorescence Induction Curve. F_o, zero fluorescence level, ML, measuring light, SP, saturation pulse, F_m^o, maximum fluorescence level in the dark, AL, actinic light, F_m', maximum fluorescence in the light, F_t, steady state fluorescence, F_o', zero level fluorescence in the light [Adapted from Maxwell and Johnson (2000)].

Photosystem I redox state was measured using a PAM 101 fluorimeter with a P700ED dual wavelength emitter detector unit. Maximum P700 pool was estimated by illuminating a leaf with a far red light, provided by a Led Engin LZ4 far red LED (San Jose, CA, USA) (λ_{max} = 740 nm) to estimate the far red signal of the leaf. Leaves were then exposed to actinic light (1000 µmol $m^{-2}s^{-1}$) for 20 mins to attain a steady state. Actinic light was subsequently interrupted for periods of 100 ms to follow the light-dark decay of P700. Signals were normalised to the far-red signal to give P700 oxidation (PSI redox state). The decay of oxidized P700 from light to dark was fitted with a single exponential curve to estimate a first

order rate constant (k). PSI ETR was then calculated by multiplying the proportion of oxidized P700 by this rate constant (Proportion of oxidized P700 x k) to give a rate of electron transport in electrons per PSI per second (Clarke and Johnson 2001, Schreiber 2004).

A/Ci Curve

Measurement of A/Ci curve was performed using a portable photosynthesis system LI-COR 6400XT (Lincoln, NE, USA). Fully expanded leaves were clamped into a LiCor extended reach chamber under PPFD [warm white LED (CREE MC-E; Colour temperature =2800-3000 K)] of 1000 $\mu molm^{-2}s^{-1}$ at a temperature of 25°C. Reference CO_2 concentrations were supplied to the sample chamber with a CO_2 mixer across the series 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000 $\mu mol\ mol^{-1}$. The A/Ci curves were fitted according to Von Caemmerer model (von Caemmerer 2000) to estimate maximum carboxylation efficiency of Rubisco (Vcmax). Maximum PEP regeneration rate, Vpr, was calculated as the average of A values above the inflection of the curve, in this case, the last three A values from 1600 ppm to 2000 ppm.

Statistics

Statistical tests were performed using SPSS Statistics 20 statistical software (IBM United Kingdom Limited, Portsmouth, UK) to determine the significance of changes obtained at different pot capacities. Where significance is indicated, a two-way ANOVA with a Tukey post-hoc test was conducted, to compare the responses of the two sorghum lines to water deficit. Significantly different data points are labelled on figures with different letters.

2.4 Results

Pot water content, leaf water status and gas exchange response to progressive drought

Soil Water Content (SWC) dropped faster in maize pots relative to the sorghum varieties and tended to level off from Day 6. With water deficit, Samsorg 40 pots had lower SWC (%) compared with Samsorg 17 (Fig. 2.2). Relative water content (RWC) of leaves was measured to determine leaf water loss with water deficit (Barrs and Weatherley 1962). RWC was measured as pots dried up to day 12. On Day 6 when pot weights had fallen to 20-40% of their starting value (Fig. 2.2), RWC was maintained in both sorghum cultivars longer relative to maize (Fig. 2.2B). In maize, by Day 6, RWC had halved as it dried faster than in the two sorghum cultivars and was relatively maintained afterwards (Fig. 2.2). Loss of RWC occurred on Day 8 in the less tolerant Samsorg 40 and remained unaffected until Day 10 when severe drought had commenced with very dry plant pots in Samsorg 17.

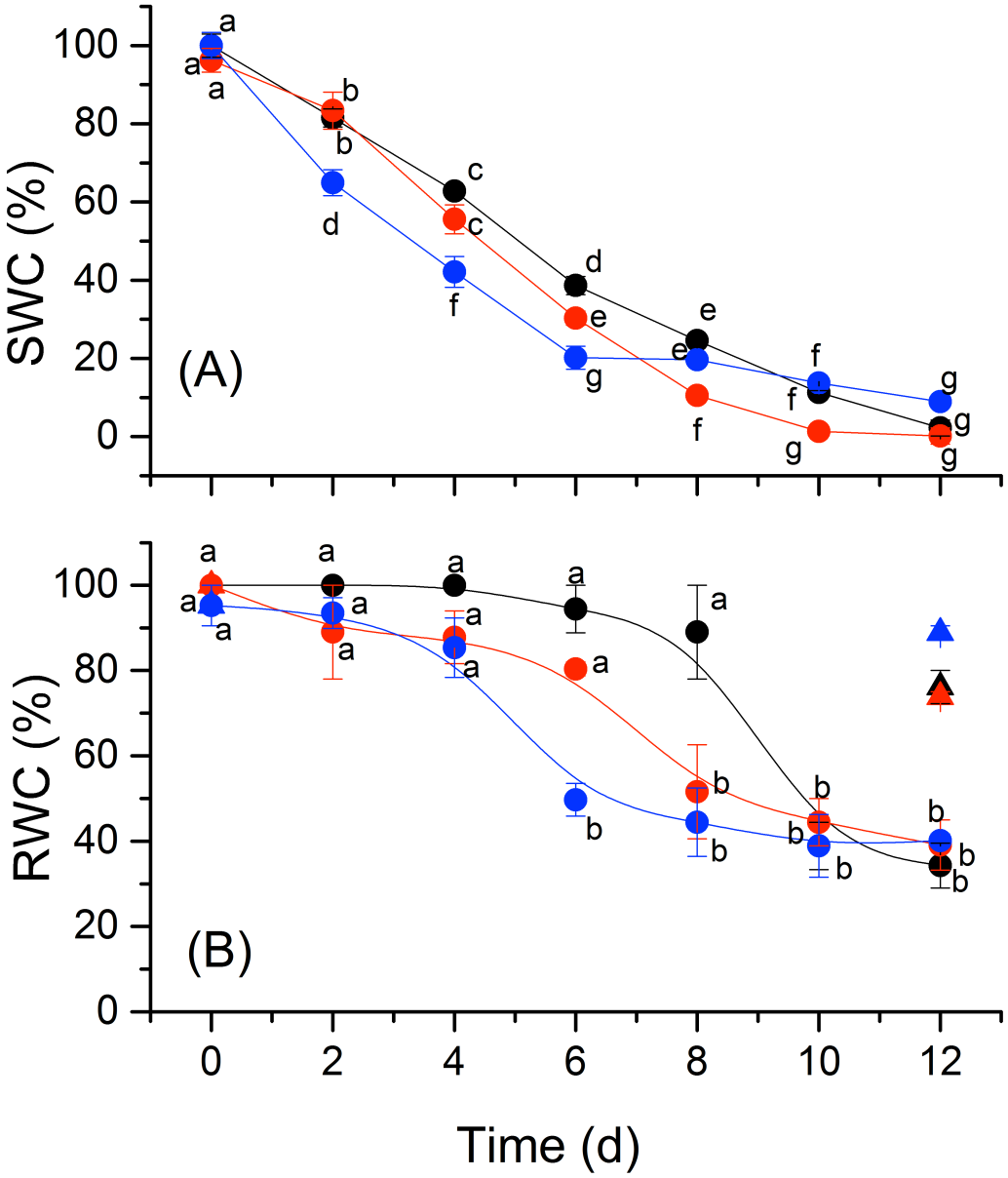


Fig. 2.2 The change in pot water content, relative to water saturation, in pots containing plants of Samsorg 17 (black circle), Samsorg 40 (red circle) and maize (blue circle) (A) and relative water content (B). Samsorg 17 control (black triangles), Samsorg 40 control (red triangles) and maize control (blue triangles). Control plants were not droughted and received adequate watering. SWC, soil water content, RWC, relative water content. All data represent the means ± SE of 4 replicates.

Gas exchange was measured for up to 12 days of drought in the sorghum cultivars and maize. Transpiration (E) measures the amount of water that a leaf is losing at a particular time i.e. the amount of water passing through the stomata. E was maintained until Day 6 in maize and Samsorg 40 dropping later, on Day 8, in Samsorg 17 (Fig. 2.3A). By Day 6, E had dropped to zero in maize and close to zero in Samsorg 40.

As indicated by measurements of stomatal conductance (G), maize closed its stomata by Day 6 whilst there was a drop in stomatal conductance in Samsorg 40 and Samsorg 17 by Day 6 and 10 respectively mirroring declines in transpiration (Fig. 2.3B).

In Samsorg 17, net CO_2 assimilation (A) was maintained up to Day 10 (Fig. 2.3C). This was in contrast to Samsorg 40 and maize, where net CO_2 assimilation reached close to zero by Day 6. Control plants were not droughted and received adequate watering.

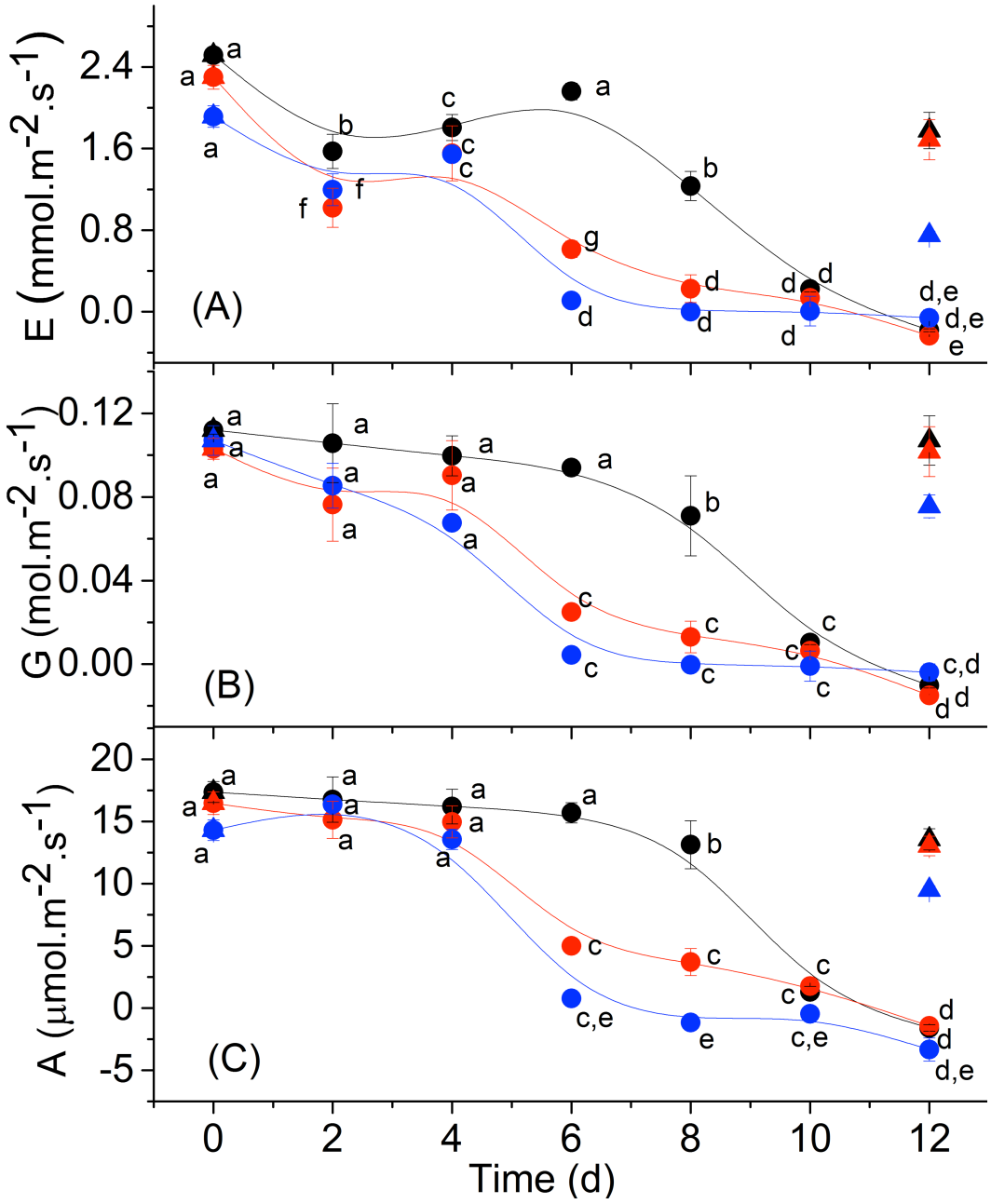


Fig. 2.3 Transpiration (A), Stomatal Conductance (B), Net CO_2 Assimilation (C) of sorghum plants- Samsorg 17 (black circle), Samsorg 40 (red circle) and maize (blue circle), Samsorg 17 control (black triangles), Samsorg 40 control (red triangles) and maize control (blue triangles). Control plants were not droughted and received adequate watering. Gas exchange

was measured on the third fully expanded leaves at ambient CO_2 concentration of 390 ppm, PPFD of 1000 μmol m^{-2} s^{-1} and a temperature of 25°C. All data represent the means ± SE of at least 3 replicates.

Water Use Efficiency and stomatal relations during water deficit

Maintenance of transpiration in Samsorg 17 relative to Samsorg 40 and maize suggests that water uptake continues the longest in Samsorg 17. It also suggests that Samsorg 17 plants retain open stomata for a long time relative to maize and Samsorg 40. Measurement of water use efficiency (WUE) in the plants showed similar patterns in both sorghum cultivars and maize. There was an early increase followed by a drop in WUE in both sorghum cultivars and maize. Estimation of WUE was not shown beyond Day 6, as average transpiration values in maize and Samsorg 40 were already zero (Fig. 2.4A).

Stomatal aperture and stomatal density were examined in the plants. In maize, there was a gradual decline in stomatal aperture through the experiment. In the two sorghum varieties, the stomata were smaller with less clear evidence of shrinkage with drought (Fig. 2.4B). However, stomata density tended to increase at the end of the experiment in the two sorghum varieties whilst unchanged in maize (Fig. 2.4C).

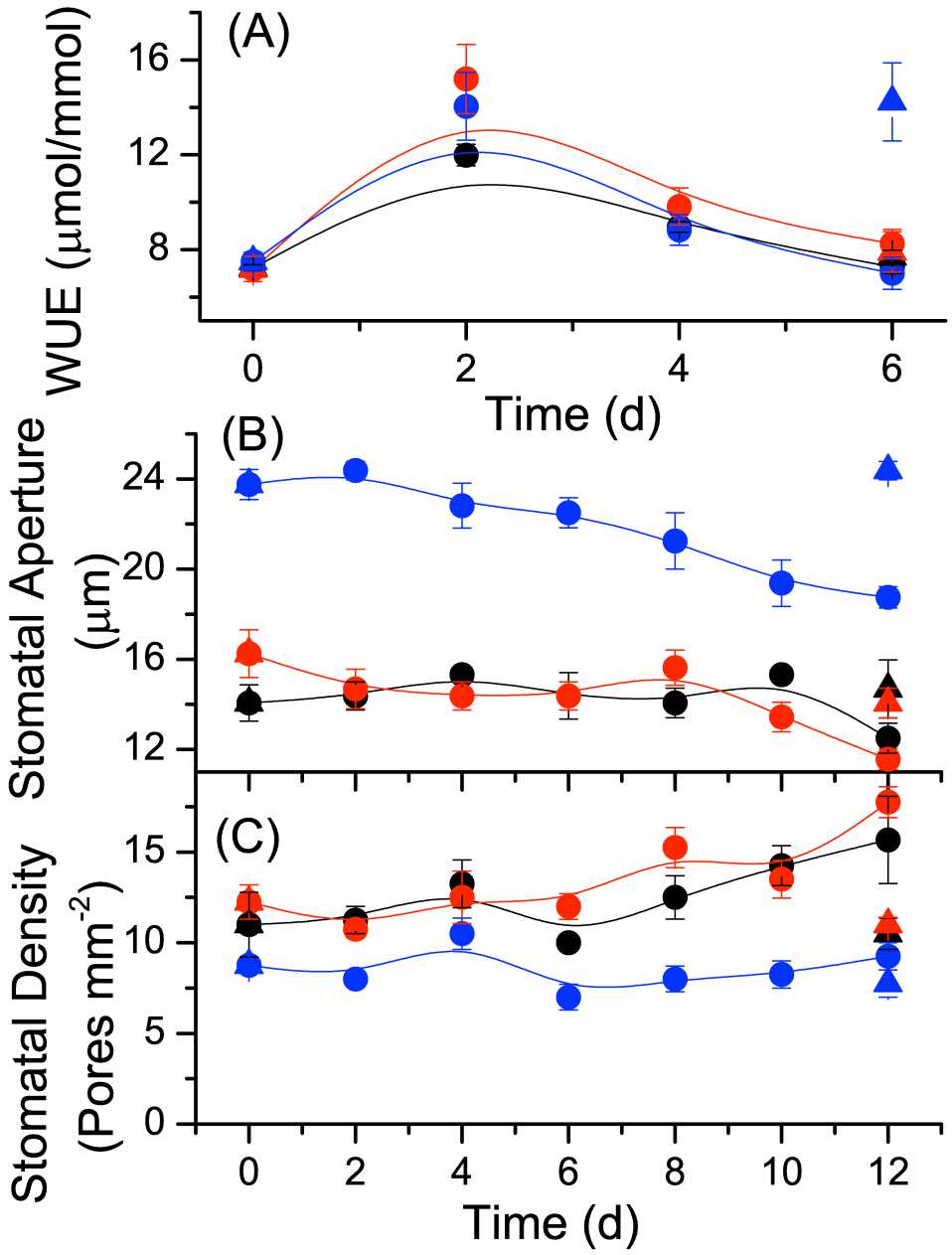


Fig. 2.4 Water Use Efficiency (A) Stomatal aperture (B) and stomatal density (C) to progressive droughting in Sorghum plants- Samsorg 17 (black circles), Samsorg 40 (red circles), Maize (blue circles), Samsorg 17 control (black triangles), Samsorg 40 control (red triangles) and maize control (blue triangles). Data represent the means ± SE of at least 8 impressions.

Changes in chlorophyll

Total chlorophyll (chl) and chlorophyll a:b (chl a:b) ratio were measured to characterise the stay-green trait morphologically observed in Samsorg 17 and Samsorg 40 relative to maize during drought. Chl per unit leaf area increased in Samsorg 40 and increased considerably more in Samsorg 17 in response to water deficit (Fig. 2.5A). Maize had a higher chl at the start of the experiment compared with the two sorghum varieties but this decreased with water deficit and more significantly when bleaching became noticeable by Day 6 (Fig. 2.5 A). Chl a:b was lower at the start of the experiment in maize relative to the two sorghum varieties. Chl a:b tended to increase with water deficit in maize whilst no substantial change was seen in the Samsorg 17 and Samsorg 40 (Fig. 2.5B).

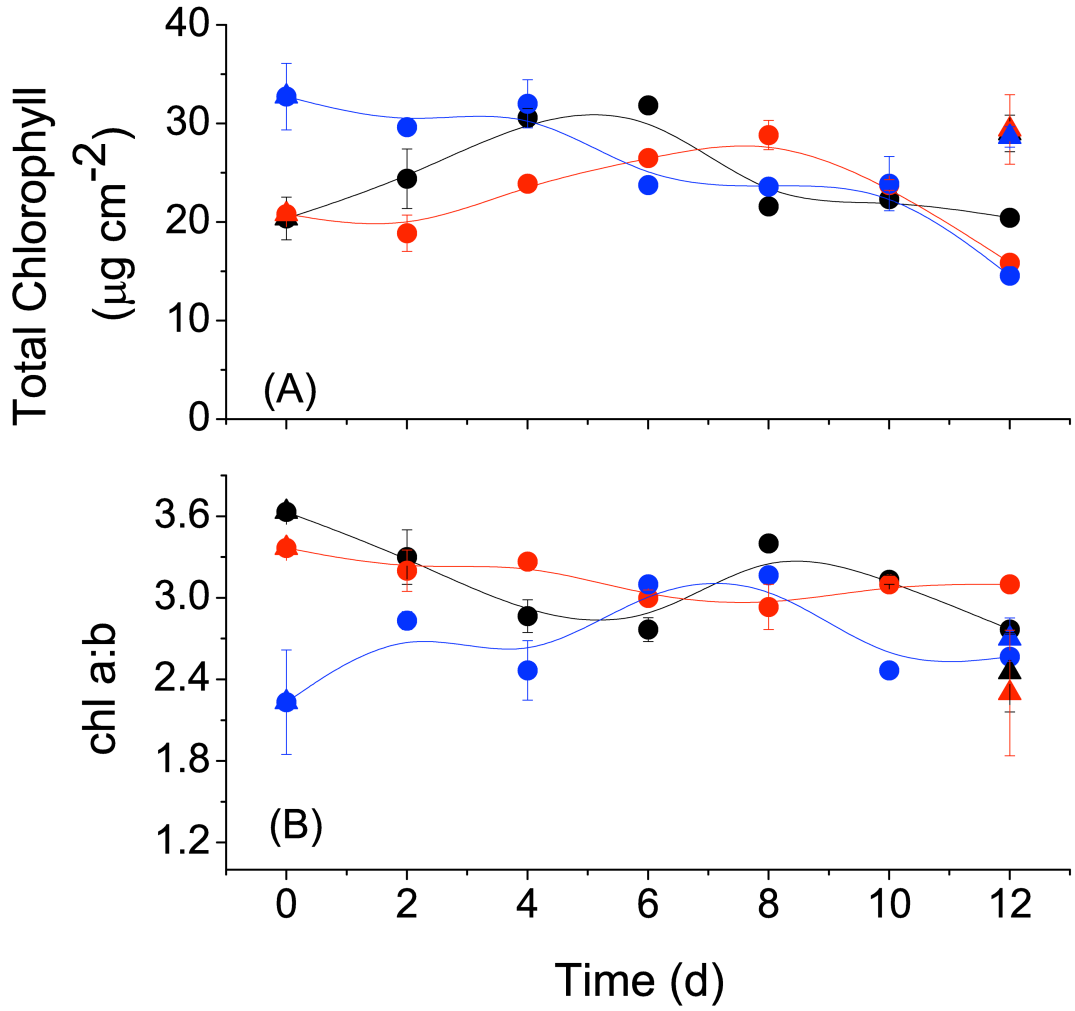


Fig. 2.5 Relationship between progressive drought and chlorophyll per unit area (A) and chlorophyll a:b ratio (B) for leaves of Samsorg 17 (black circles), Samsorg 40 (red circles), maize (blue circles). Samsorg 17 control (black triangles), Samsorg 40 control (red triangles) and maize control (blue triangles). All data represent means ± SE of at least 5 replicates.

Chlorophyll Fluorescence and Photosystem I electron transport

Chlorophyll fluorescence analysis can provide valuable information about the functioning of PSII. Using chlorophyll fluorescence analysis it is possible to estimate the efficiency of PSII (ΦPSII), i.e. the proportion of light absorbed by PSII that is used in photosynthesis under any given conditions (Genty et al. 1989). Multiplying ΦPSII by the irradiance gives an estimate of the relative rate of electron transport through PSII reaction centres (PSII ETR). PSII ETR in sorghum and maize in control conditions was ca 250 (Fig. 2.6A). As drought developed, PSII ETR dropped in parallel with the decline in CO_2 fixation. In maize, which is the least drought tolerant, and Samsorg 40, the less drought tolerant Sorghum variety, the decline in PSII ETR was significant from Day 6 and matched the drop in CO_2 Assimilation (Fig. 2.6A, 2.3C). Samsorg 17, the more drought tolerant Sorghum variety, lost its capacity to maintain PSII electron transport by Day 10 (Fig. 2.6A). A complete decline in PSII ETR occurred by Day 10 in all the plants examined.

When plants are subjected to high light and/or drought, they generate a pH gradient across the thylakoid resulting in the occurrence of non-photochemical quenching (NPQ) (Ruban and Murchie 2012). Subjecting the plants to PPFD of 1000 $\mu molm^{-2}s^{-1}$ and drought did not induce substantial increases in NPQ throughout the experimental period (Fig. 2.6B). However, in maize, by Day 8, when severe drought commenced, NPQ increased slightly although not significantly (ANOVA, $P > 0.05$) (Fig. 2.6B).

Decreases in the maximum quantum yield of PSII, Fv/Fm started by Day 8 in maize relative to Samsog 17. Fv/Fm in Samsorg 17 and Samsorg 40 were found to drop only during severe drought from Day 10 (Fig. 2.6C).

PSI ETR measures the total electron flux through PSI. In barley during drought, electron transport increased through PSI, relative to the control (Golding and Johnson 2003). The electron transport rate through PSI was measured throughout the experimental period in the sorghum cultivars and maize. PSI ETR was unaffected by drought in all plants (Fig. 2.6D). Comparison of PSII ETR and PSI ETR together with increases in NPQ has been used as evidence for cyclic electron flow in Arabidopsis and Thellungiella (Stepien and Johnson 2009). With no change occurring in NPQ, comparison of PSII ETR and PSI ETR of maize and sorghum suggests that no increase in cyclic electron flow was occurring (Fig. 2.6A, 2.6D). However, there was less correlation in the two parameters in Samsorg 40 compared with maize and Samsorg 17.

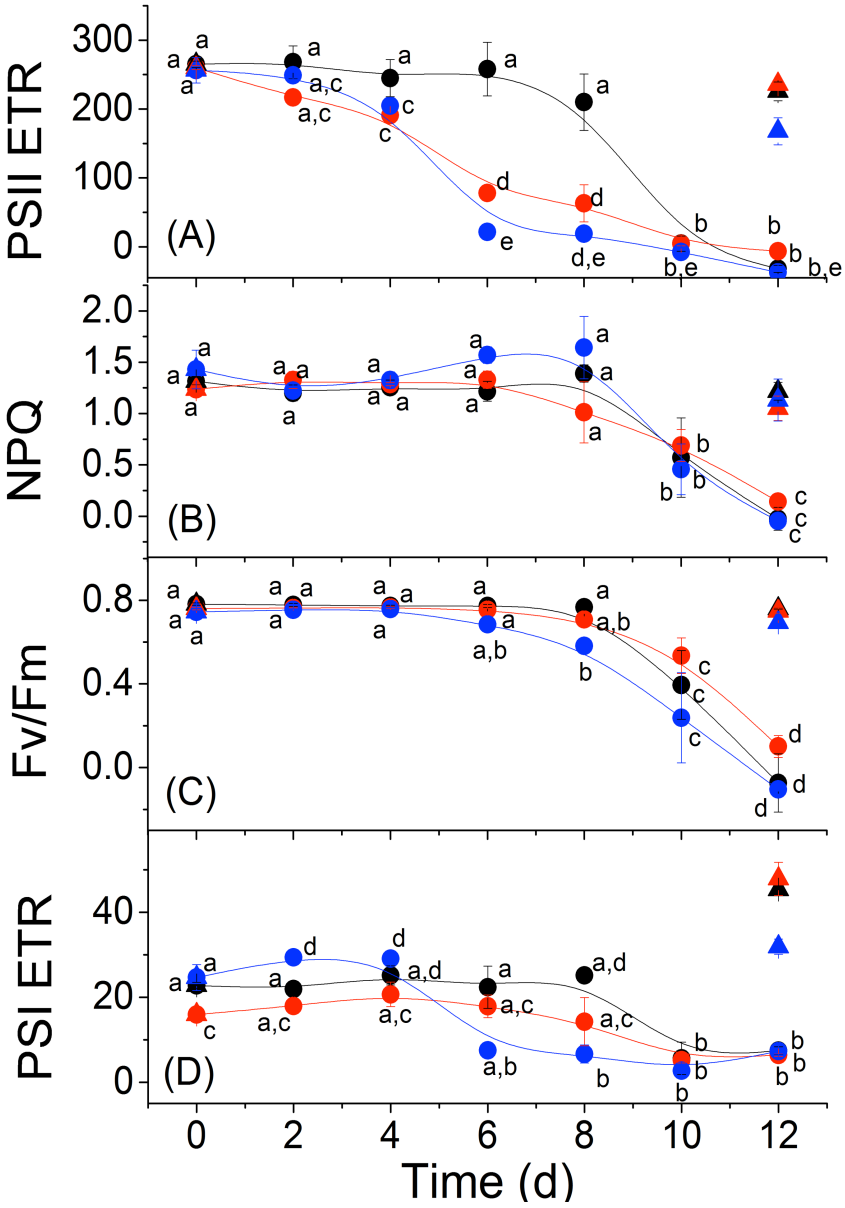


Fig. 2.6 PSII electron transport (A), Non photochemical quenching (B), Maximum efficiency of PSII (C) and PSI electron transport (D) of Samsorg 17 (black circles), Samsorg 40 (red circles), maize (blue circles) Samsorg 17 control (black triangles), Samsorg 40 (red triangles) and maize control (blue triangles). Measurements were taken at ambient CO_2 concentration of 390 ppm and PPFD of 1000 µmol$m^{-2}s^{-1}$, Saturating flash of 6000 µmol$m^{-2}s^{-1}$ and Temperature of 25°C. All data represent the means ± SE of at least 3 replicates. Apparent rise in NPQ in maize from day 6 to 8 was not significant (ANOVA, $P > 0.05$).

Biochemical limitation estimation in the two sorghum cultivars and maize

Early loss of PSII activity and Fv/Fm in maize suggests a possible occurrence of early non-stomatal or biochemical limitations in the plant. To investigate biochemical limitations under drought in the plants, A/Ci curves were measured. The shape of the A/Ci curve varied between plant varieties and with drought (Fig. 2.7). A/Ci curves were plotted up to Day 4, Day 6 and Day 8 in maize, Samsorg 40 and Samsorg 17 respectively, after which majority of the Ci values became unreliable. In Samsorg 40, there was a change in the relationship between A and Ci by Day 6 and by Day 8 in Samsorg 17 (Fig. 2.7). The A/Ci curve relationship was maintained in maize only up to Day 4.

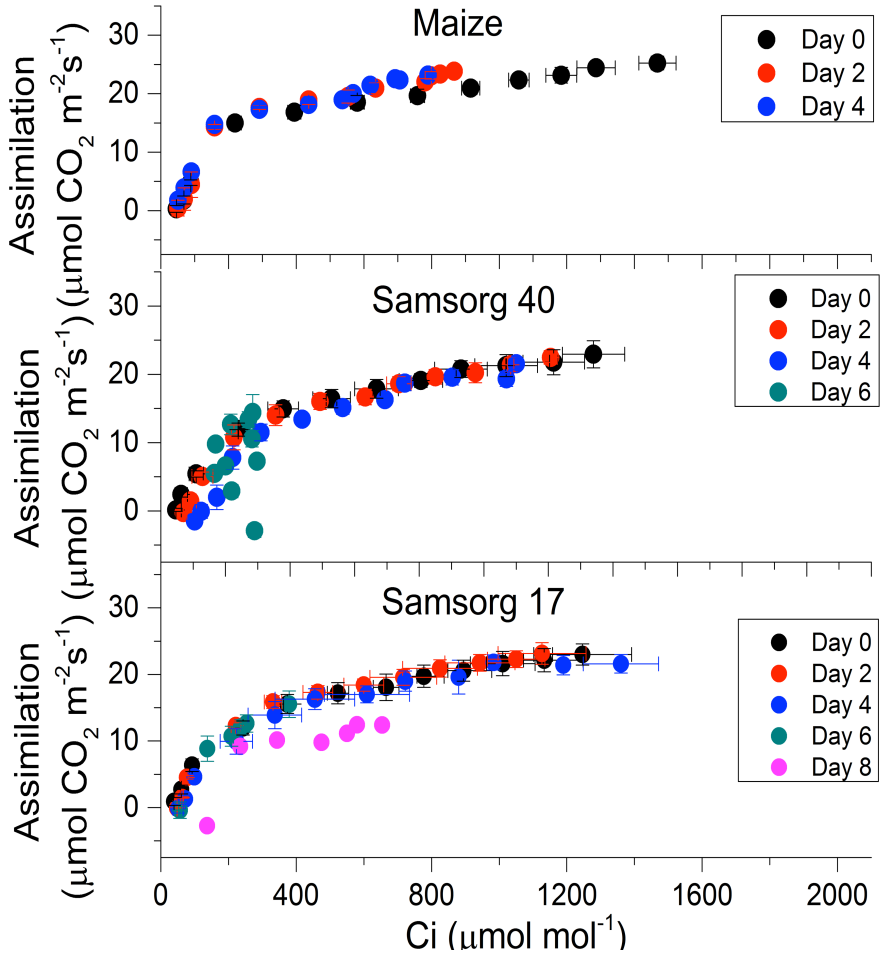


Fig. 2.7 The response of light-saturated carbon assimilation (A) to changes in intracellular carbon dioxide concentration (Ci) for Maize (A), Samsorg 40 (B) and Samsorg 17 (C) at different soil water contents. All data represent the means ± SE of at least 3 replicates.

The maximum regeneration efficiency of PEP (Vpr) was estimated from the A/Ci curves and it dropped markedly in all plants by Day 6 (Fig. 2.8). In maize, Vpr values for Day 6 were not shown as they became unreliable. Vpr also declined in the two sorghum cultivars by Day 6 more so in Samsorg 40 (Fig. 2.8).

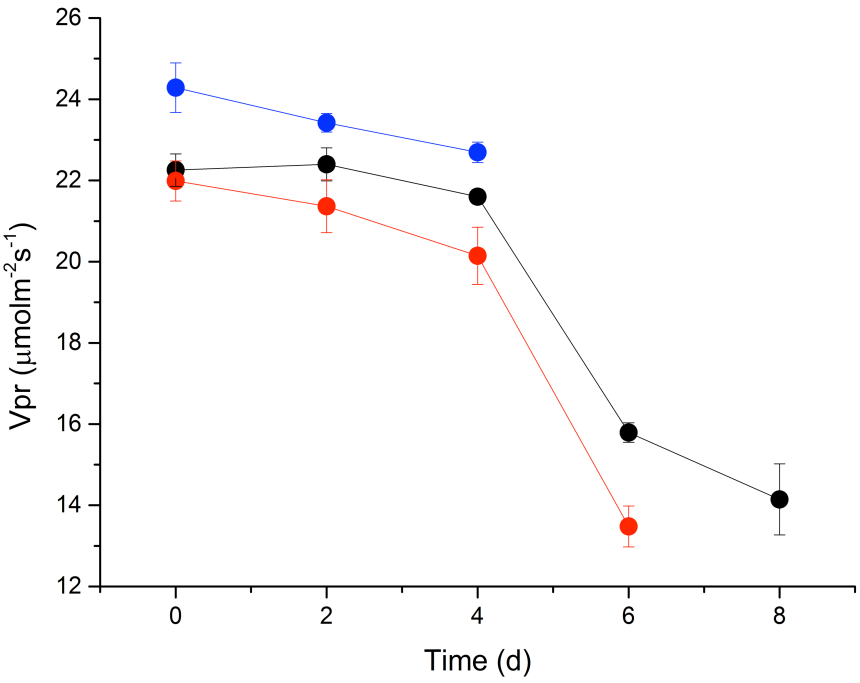


Fig. 2.8 Biochemical limitations to photosynthesis. Maximum regeneration efficiency of PEP (Vpr) for Samsorg 17 (black circles), Samsorg 40 (red circles), maize (blue circles). Vpr was estimated from the A/Ci curves of the plants. All data represent the means ± SE of at least 3 replicates.

The maximum carboxylation efficiency of Rubisco (Vcmax) also declined in all plants by Day 6 especially in maize where Vcmax values were already unreliable at that time point and were not shown (Fig. 2.9).

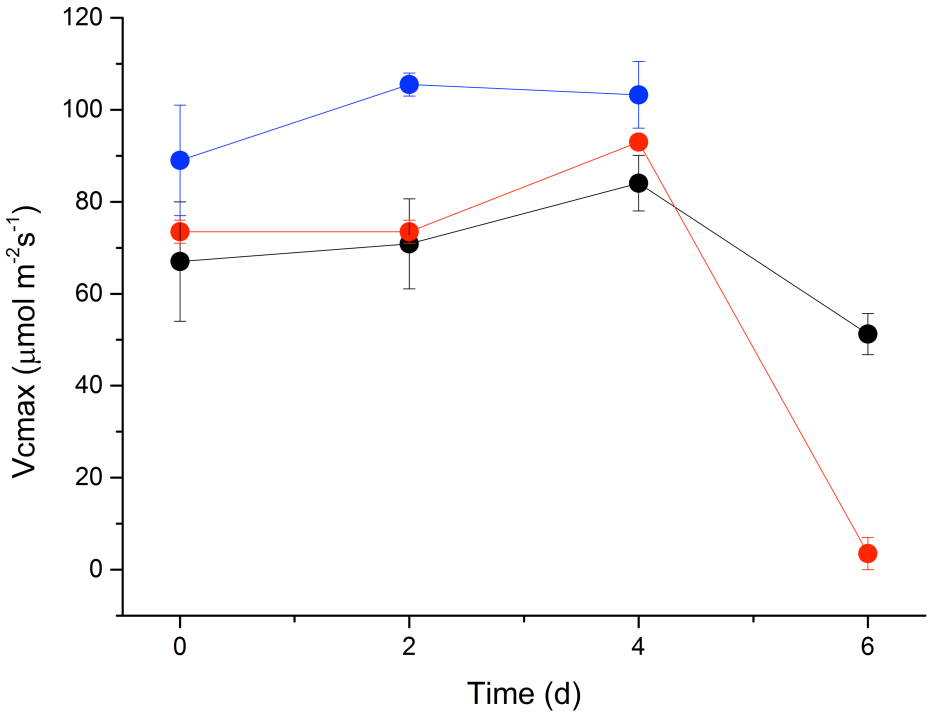


Fig. 2.9 Biochemical limitations to photosynthesis. The maximum carboxylation efficiency of Rubisco (Vcmax) for Samsorg 17 (black circles), Samsorg 40 (red circles), maize (blue circles). Vcmax was estimated from the A/Ci curves of the plants. All data represent the means ± SE of at least 3 replicates.

2.5 Discussion

Drought is an abiotic stress that affects plants differently depending on their sensitivity (Ghannoum 2009). Understanding the limitations of photosynthesis in sorghum, a naturally drought tolerant plant, could clarify drought tolerant mechanisms of the species and improve breeding of other important plants, such as maize. To better understand the physiological and biochemical mechanisms of drought tolerance, two sorghum cultivars- Samsorg 17 (more drought tolerant) and Samsorg 40 (less drought tolerant) were studied and compared with maize.

Samsorg 17 maintained relative water content up to 8 days whilst at the same time being extravagant in its water use, as reflected in high transpiration and longer open stomata (Fig. 2.2, 2.3). This was in contrast to maize and Samsorg 40 where stomatal conductance closed before relative water content dropped. Thus, whilst Samsorg 40 and maize avoid drought by closing their stomata earlier, in order to retain water longer, Samsorg 17 maintains photosynthesis longer, not by closing its stomata and reducing transpiration but, we deduce, by being able to better extract water from dry soil. Samsorg 17 lost RWC less as a result, relative to Samsorg 40 and maize. Higher pot weights were seen in maize under severe drought, but this difference could be attributed to the greater biomass present in maize pots rather than water being retained in the soil (Fig. 2.2).

The maize plants made larger but fewer stomata compared to the sorghum varieties. Larger stomata in the plants may have been responsible for maintaining similar stomatal conductance relative to the two sorghum varieties under watered conditions. However, having larger stomata does not mean better water use efficiency, as seen in Fig. 2.4A where all the plants had similar levels of water use.

Drought caused an early loss of chlorophyll in maize but an increase in chl a:b, reflecting a situation where there is loss of light harvesting complex relative to reaction centres. This suggests that a controlled down regulation of light capture occurred in the plant during drought. No substantial change in chl a:b of Samsorg 17 and Samsorg 40 suggests that there was no controlled down regulation of light capture in these plants, rather plants made more chlorophyll.

PSII and PSI ETR matched CO_2 assimilation in all the plants, albeit to a lesser degree in Samsorg 40 (Fig. 2.6). However, due to the presence of different tissues, mesophyll and bundle sheath cells, which contain mostly PSII and PSI respectively, a simple comparison of these parameters is dangerous. Hence, it is difficult to estimate actual electron fluxes in C_4 plants. Either way, the fact that there was a good PSII and PSI correlation in maize and Samsorg 17 is consistent with there being a lack of cyclic electron flow. In contrast in Samsorg 40, there was less correlation between the two parameters, suggesting that cyclic electron flow might increase per reaction centre in the plant during progressive drought. This result might be explained by the change in photosystem ratio shown later in Chapter 3. This represents a situation where Samsorg 40 is trying to protect itself from stress unlike maize and Samsorg 17. Maize and Samsorg 17 may have other strategies of dealing with stress but, as shown in this chapter, both plants down regulate their PSII and PSI to match the capacity of water splitting and the production of reducing power to the capacity for CO_2 fixation.

NPQ was not induced in all plants, probably because this parameter was already saturated at the high light measured (Fig. 2.6B). During severe drought of 10 days, there was a decline in Fv/Fm in all the plants i.e. the maximum proportion of the light energy arriving at an open (oxidised) PSII reaction centre that can be captured and converted to chemical energy by that centre (Fig. 2.6C). Fv/Fm data indicate that open PSII centres decreased by Day 8 in maize

and Day 10 in the two sorghum varieties, more so in Samsorg 40 (Fig. 2.6C). This decline may be as a result of slower turn-over due to reduced synthesis *de novo*, damage to the D1 protein of PSII, i.e. photoinhibition, or possibly from a protective down regulation of the PSII complex (Krieger-Liszkay 2005). With the knowledge that open PSII centres declined sooner in maize accompanied by early loss of PSII activity and total chlorophyll, the drop in Fv/Fm during severe drought presumably indicates photoinhibition.

It has been shown that spatial organization of photosynthetic capacity, that is, the organisation of the A/Ci curve, can be optimized for carbon uptake (Chen et al. 2008, Rayment et al. 2002). Alteration in the A/Ci curves can indicate changes in metabolites during drought and can occur together with disruption in energy dissipation. A/Ci curves were used to probe stomatal or biochemical changes occurring in the sorghum cultivars and maize. Both sorghum varieties and maize responded differently to drought. Decrease in Vpr occurred sooner in maize, relative to the two sorghum cultivars, and is consistent with a reduction in CO_2 fixation occurring earlier in maize (Fig. 2.7, 2.8). Reduction in Vpr occurred later in the two sorghum cultivars, but more significantly in Samsorg 40. By Day 10, changes in relationship of the A/Ci curves prevented further calculation of Vpr in all plants. One factor that has been suggested to affect change in relationship of A/Ci curves is acclimation and tolerance. Acclimation has been shown to prevent such changes in the relationship in response to nitrogen allocation and high temperature stress (Dreyer et al. 2001, Kattge and Knorr 2007). However, some studies did not find any evidence for acclimatory roles in maintaining the A/Ci relationship (Ainsworth et al. 2003). Maintenance of Vpr and better-organised A/Ci curves in Samsorg 17 would imply that this variety either acclimates or tolerates drought better than Samsorg 40 and maize. A better-organised A/Ci curve may have contributed to the enhanced carbon uptake seen in the more tolerant Samsorg 17. This was

reflected in the maximum carboxylation efficiency of Rubisco (Vcmax) which declined the least in the more tolerant Samsorg 17 (Fig. 2.9).

In conclusion, Samsorg 17 had a higher transpiration and maintained relative water content and net photosynthesis longer. Samsorg 17 therefore had a better photosynthetic machinery in contrast to the less drought tolerant Samsorg 40 and maize in response to water deficit. There was no clear evidence for the generation of non-photochemical quenching in either of the sorghum or maize plants and there was generally a good correlation between PSII and PSI in all plants. Based on this, it is concluded that cyclic electron flow is not activated during progressive drought in either sorghum or maize. This needs to be studied in detail as it has the potential of providing useful information regarding the best approach to improve drought tolerance in plants.

2.6 Acknowledgements

We would like to thank the Institute of Agricultural Research, Zaria, Nigeria for the provision of sorghum seeds used in this work.

Chapter 3:

SORGHUM (*SORGHUM BICOLOR*) VARIETIES ADOPT STRONGLY CONTRASTING STRATEGIES IN RESPONSE TO DROUGHT

Authors:

Chukwuma C. Ogbaga[1], Piotr Stepien[2], Giles N. Johnson[1]

Addresses

[1]The University of Manchester, Faculty of Life Sciences, Oxford Road, Manchester, M13 9PL, United Kingdom

[2] Wroclaw University of Life and Environmental Sciences, Department of Plant Nutrition, ul. Grunwaldzka 53, 50-357 Wroclaw, Poland

Email Addresses

Chukwuma C. Ogbaga- chukwuma.ogbaga@manchester.ac.uk

Piotr Stepien- piotr.stepien@up.wroc.pl

Giles N. Johnson- giles.johnson@manchester.ac.uk

Author For Correspondence:

Giles N. Johnson

Tel: +44 161 275 5750

Fax: +44 161 275 5082

Email: giles.johnson@manchester.ac.uk

(See: Ogbaga, CC., Stepien, P and Johnson, GN (2014) Sorghum (*Sorghum bicolor*) varieties adopt strongly contrasting strategies in response to drought. Physiologia Plantarum.)

Key Words: Drought, Sorghum, Compatible Solutes, Cyclic Electron Transport, Rubisco

Authors' contributions: CCO, PS and GNJ designed the experiments. CCO grew, treated, harvested and performed the vast majority of the work. PS assisted with the protein work and performed the total N and C:N experiments. CCO and GNJ wrote the manuscript.

3.1 Abstract

Sorghum is one of the most drought tolerant crops but surprisingly, little is known about the mechanisms achieving this. We have compared physiological and biochemical responses to drought in two sorghum cultivars with contrasting drought tolerance. These closely related cultivars have starkly contrasting responses to water deficit. In the less tolerant Samsorg 40, drought induced progressive loss of photosynthesis. The more drought tolerant Samsorg 17 maintained photosynthesis, transpiration and chlorophyll content until the most extreme conditions. In Samsorg 40, there was a highly specific down regulation of selected proteins, with loss of Photosystem II and Rubisco but maintenance of PSI and cytochrome b_6f, allowing plants to maintain ATP synthesis. The nitrogen released allows for accumulation of glycine betaine and proline. To the best of our knowledge, this is the first example of specific reengineering of the photosynthetic apparatus in response to drought. In contrast, in Samsorg 17 we detected no substantial change in the photosynthetic apparatus. Rather, plants showed constitutively high soluble sugar concentration, enabling them to maintain transpiration and photosynthesis, even in extremely dry conditions. The implications for these strikingly contrasted strategies are discussed in relation to agricultural and natural systems.

3.2 Introduction

The breeding of crop plants with enhanced drought tolerance is one of the great challenges for plant sciences, central to the maintenance of food security. Drought tolerance is a complex trait that is important at different growth stages and involves multiple adaptations. Fundamental to this is the ability to maximise the extraction of water from the soil whilst minimising loss from the leaves. Morphological adaptations include the development of deep roots, and alterations in leaf morphology and cuticle structure, while physiological adaptations involve changes in stomatal density to maximise water uptake and retention (Carmo-Silva et al. 2009, Cruz et al. 1992, Pardales et al. 1991, Salih et al. 1999). Biochemical adaptations include having water efficient photosynthetic pathways (CAM; C_4) and the accumulation of compatible solutes, including certain sugars and amino acids, to lower tissue water potential and enhance water uptake (Girma and Krieg 1992, Kavi Kishor et al. 2005, Kavi Kishor and Sreenivasulu 2013, Serraj and Sinclair 2002, Yobi et al. 2012). The latter are the focus of significant investment and research effort. In view of this, it is pertinent to look critically at the behaviour of naturally drought tolerant plants and to ask questions about which drought tolerance strategies are the most compatible with maintaining crop productivity.

Amongst widely grown crop species, sorghum has one of the greatest degrees of stress tolerance (Paterson et al. 2009). Like its close relative, maize, sorghum performs NADP-ME C_4 photosynthesis, however this alone does not explain its drought tolerance – sorghum is substantially more drought tolerant than maize (Kakani et al. 2011). In fact, in spite of various breeding efforts and widespread application of genetic analyses, surprisingly little is known about the physiological or biochemical traits giving rise to drought tolerance in this important species. However, it is thought that rooting depth, increased water use efficiency

and accumulation of solutes contribute to drought tolerance in this plant (Gutjahr et al. 2013, Zegada-Lizarazu and Monti 2013, Zegada-Lizarazu et al. 2012).

Compatible solute accumulation is a widespread response to various abiotic stresses, including cold, salinity and drought (Chen and Murata 2008). Accumulated solutes not only lower tissue osmotic potential, they are also thought to protect cells structurally and act as antioxidants (Chaves et al. 2009, Chen et al. 2007, Kavi Kishor et al. 2005, Kavi Kishor and Sreenivasulu 2013). Key features of compatible solutes are that they must be metabolically inert and able to accumulate to high concentrations. Notable examples include the sugars sucrose and trehalose, the amino acid proline and glycine betaine (GB) (Kavi Kishor et al. 2005, Serraj and Sinclair 2002, Yobi et al. 2012). The latter has received particular attention and efforts are being made to engineer plants to accumulate high concentrations of GB (Chen and Murata 2011). Plants overexpressing genes required for GB accumulation have been reported as having enhanced tolerance of various abiotic stresses, including drought, salt and extremes of temperature (Chen and Murata 2011). GB is suggested to specifically protect the photosynthetic apparatus from stress-induced damage (Chen and Murata 2008).

Accumulation of compatible solutes is expected to have a significant metabolic cost. In particular, accumulation of compounds containing nitrogen will result in a substantial diversion of N away from other processes, which may limit the supply of amino acids for protein synthesis (Feng et al. 2009, Gastal and Lemaire 2002, Lawlor 2002, Westhoff and Gowik 2010). Where GB is accumulating in response to stress, the plant will need to find N from other sources (Lawlor 2002).

In this paper we examine the physiological and biochemical responses to drought in two sorghum cultivars with differing degrees of drought tolerance. In particular, we have focused

on identifying biochemical features that give rise to drought tolerance. Strikingly, the plants show highly contrasting responses, in terms of changes in cellular processes.

3.3 Materials and Methods

Plant growth conditions and drought treatments

Seeds of Sorghum (*Sorghum bicolor* L) cv Samsorg 17 (more drought tolerant) and cv Samsorg 40 (less drought tolerant) were provided by the Institute of Agricultural Research, Zaria, Nigeria. The seeds were grown in 3" pots, one plant per pot, filled with John Innes No.1 soil in a growth chamber with a 16 h photoperiod and a day/night regime at 23°C/15°C. Photosynthetic photon flux density was 130 μmol m^{-2} s^{-1} (Light meter, SKYE instruments Ltd, UK, light provided by warm white LED lights, colour temperature 2800-3000 K) and relative humidity (RH) of 40-50%. The plants were grown for 3 weeks before drought was imposed using one of two methods. In progressive drought experiments, watering was completely withheld for up to 12 days. Water loss was estimated based on daily measurements of pot weight. In some experiments, plants were allowed to dry by withholding water until certain soil moisture content was reached and then that moisture content was maintained by rewatering on a daily basis with the water lost in the previous 24 hours. Soil water content (SWC) is expressed as a % maximum pot capacity. To estimate pot water capacity the soil from pots of fully water-saturated soil was weighed and then dried to constant weight at 105°C. The weight difference between water saturated and oven dried soil was taken as weight of water needed to bring pots to pot capacity and lower soil water contents (% pot capacity) were calculated accordingly.

Growth Analysis

Growth analysis was performed on Samsorg 17 and Samsorg 40 plants exposed to progressive drought for 12 days. Total leaf area and total above ground dry weight of tissues were measured at the start and end of the experiment. For leaf area measurement, leaf pieces were scanned using an Epson flatbed scanner and their area determined using ImageJ image analysis software (National Institutes of Health, USA). Tissues were subsequently dried in an oven at 70°C for 48 h to determine the total dry weight. Specific leaf area (SLA) was calculated as total plant leaf area divided by the leaf dry weight.

Leaf Relative Water Content

Leaf relative water content (RWC) was measured according to the method of Barrs and Weatherley (1962). The leaves were excised and weighed immediately to give actual fresh weight (Fw). Turgid weight (Tw) was determined after floating leaf segments on water for 4 hours and dry weight (Dw) measured after drying leaf pieces for 24h in an oven at 70°C. (RWC) was calculated as:

$$(Fw-Dw)/(Tw-Dw)* 100$$

Chlorophyll content / Chl a:b determination

Similar sized leaf pieces were cut and scanned using a Canon Scanner (Canoscan LIDE 110). Leaf area was measured using ImageJ image analysis software (National Institutes of Health, USA). Leaf pieces were ground in a mortar and pigments extracted with 80% Acetone. Resultant extracts were assayed spectrophotometrically for total chlorophyll content and chl

a:b ratio in a glass cuvette using USB4000 Spectrophotometer (Ocean Optics, Dunedin, USA) and calculated according to the method of Porra and co-workers (1989).

Measurement of Gas Exchange Parameters

Gas exchange parameters were determined using portable photosynthesis system LI-COR 6400XT (Lincoln, NE, USA) under a saturating PPFD of 1000 µmol m^{-2} s^{-1} provided by a warm white LED (CREE MC-E; Colour temperature =2800-3000 K) at temperature of 25°C. Fully expanded leaves were clamped into a LiCor extended reach leaf chamber at a CO_2 concentration of 390 ppm and relative humidity (RH) of 50%. Assimilation in the dark was recorded after allowing the leaf to equilibrate in the IRGA chamber for 10 minutes and assimilation in the light and transpiration were recorded at steady state, after 20 mins exposure to light.

Chlorophyll fluorescence and Photosystem I Measurements

Chlorophyll a fluorescence was measured using a Walz PAM 101 fluorometer connected to a computer with a National Instruments NI-6220 data acquisition card, running laboratory written software (Labview, National Instruments). Leaves adapted to darkness were exposed to an actinic light intensity of 1000 µmol m^{-2} s^{-1} and saturation flashes of PPFD of 6000 µmol m^{-2} s^{-1}. Non-photochemical quenching (NPQ), quantum efficiency of photosystem II (ΦPSII) and Fv/Fm were calculated as described by Maxwell and Johnson (2000).

Photosystem I redox state was measured using a PAM 101 fluorimeter with a P700ED dual wavelength emitter detector unit. Maximum P700 signal was estimated by illuminating a leaf with a far red light provided by a Led Engin LZ4 LED (λmax = 740nm; Led Engin, San Jose, CA, USA) with a saturating white flash being superimposed on the far red light at steady

state. The decay of P700 signal following a light-dark transition was fitted with a single exponential equation to yield a pseudo first order rate constant, k, for P700 reduction. PSI ETR was calculated by multiplying the proportion of oxidized P700 by this rate constant (proportion of oxidized P700 x k) to give a rate of electron transport in electrons per PSI per second (Clarke and Johnson 2001, Schreiber 2004).

Protein extraction and chloroplast isolation

1g whole-leaf or 250 mg whole-root soluble proteins were harvested at 8 hours into photoperiod and extracted in ice-cold 150 mM Tris-HCl buffer (pH 8.0), containing 20 mM EDTA, 15 mM $MgCl_2$, 2 mM PMSF, 0.1% [v/v] Triton X-100, 20 mM β-mercaptoethanol and 10% [v/v] glycerol) from tissue pre-ground in liquid nitrogen to a fine powder. The homogenates were briefly vortexed, subjected to freeze-thaw cycle and sonicated with VIBRA-CELL Ultrasonic Processor VC505 (VWR Jencons; 30 sec of 0.5 sec pulses) at 4^{o}C and finally centrifuged at 15 000g for 10 min. The supernatants containing leaf/root proteins were flash frozen and stored at –80^{o}C. Protein content was determined with Bradford protein assay (Sigma–Aldrich), using bovine serum albumin as a standard.

For determination of thylakoid proteins, chloroplasts were first isolated under dim light at 4^{o}C. Sorghum leaves were freshly harvested from plants transferred directly from growth light. The leaf material was chilled on ice and the leaves were cut into 5-mm pieces. The plant material was placed in a slush of isolation buffer (IB, frozen and semi-thawed) containing 50 mM HEPES-KOH (pH 7.8), 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM Na-ascorbate and 0.5% BSA, and ground in Polytron blender (Kinematica) with 5 bursts of 1s. The homogenate was filtered through a pre-moistened sandwich of 2 layers of 20-µm nylon mesh and one layer of absorbent cotton-wool. The chloroplasts were

quickly pelleted by centrifuging at 3000g for 5 min at 4ºC. The crude chloroplast pellet was resuspended in 3 ml of IB and carefully loaded onto a Percoll (Amersham) step gradient (10, 35 and 85% top to bottom). The gradients were centrifuged at 6500g in a swing-out rotor for 15 min. The broken chloroplasts of the upper band were removed; the intact chloroplasts (accumulated at the 35/85% interface) were collected and washed two times with 4 volumes of the wash buffer (WB, 50 mM HEPES-KOH (pH 7.8), 330 mM sorbitol, 2 mM EDTA, 2 mM $MgCl_2$), and the chloroplasts were collected by centrifugation at 2500g for 5 min. The final pellets were resuspended in small volume of WB, flash-frozen in liquid nitrogen and stored at –80^{o}C.

Total nitrogen content and C:N ratio determination

Samples from the two Sorghum varieties – Samsorg 17 and Samsorg 40 – subjected to 100, 40, 25 and 15 % of pot capacity water-regime, were collected at 8 hours into photoperiod. Harvested plant parts were separated into leaves and roots. Sub-samples were oven-dried at 105°C for 1 h and at 70°C for the next 48 h. They were subsequently weighed for determination of dry mass (DM) and finely ground with ball mill (Retsch, Germany). Ground sub-samples were loaded into tin capsules (50 mg sample), and dry-combusted in a CHN elemental analyzer in the presence of helium as a carrier gas (CE Instruments EA 1110, Thermo-Fisher, USA) to determine total N and C:N ratio.

SDS-PAGE and Immunoblot analysis

Protein samples for SDS-PAGE were incubated in 50 mM Tris-HCl (pH 6.8), containing 2% (w/v) SDS, 50 mM DTT, 1 mM PMSF, 5 mM EDTA, 10% (w/v) glycerol and 0.05% (w/v) bromophenol blue at 95^{o}C for 3 min. Total protein from Sorghum leaf/root (25µg) or chloroplast (15µg) were separated on 4-20% Precise Tris-Glycine SDS-PAGE (Thermo

Scientific) in Mini-PROTEAN 3 cell (Bio-Rad) at 35mA per gel. For immunoblot analyses, proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham) by electroblotting in western transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% [v/v] methanol) in Mini Trans-Blot cell (Bio-Rad; 60 min at 90 V). Blots were checked by Ponceau S staining to ensure even transfer. Membranes were blocked with 5% (w/v) BSA in TBS-T (20 mM Tris-HCl, pH 7.6, 125 mM NaCl and 0.15% [v/v] Tween-20) and then incubated overnight at 4^{o}C with primary antibodies against: photosynthetic components – Rubisco large subunit (RbcL), PSII (PsbA), chHSP60 (chCpn60; kindly provided by Prof. R. Galek, UP, Wroclaw, Poland), PSI (PsaD), cyt f (PetA; Agrisera). Binding of the primary antibody was detected using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:25000 polyclonal goat anti-rabbit IgG; Sigma) and ECL detection reagent (Amersham) according to the manufacturer's instructions.

Soluble Sugars Assay

Leaf samples were harvested at 8 hours into photoperiod, flash-frozen and freeze-dried for 48 hrs. Samples were homogenised and 50 mg weighed into 15 ml test tubes. 5ml of distilled water was added into the test tubes and boiled for 10 mins. Aqueous extract was filtered and total soluble sugars determined colorimetrically at wavelength of 490 nm according to DuBois and co-workers (1956) and Moreira Lobo and co-workers (2011).

Direct Injection Electrospray Ionisation Mass Spectrometry (DIMS)

30mg DW of tissue was extracted in aqueous solution. Extraction followed the methods of Fiehn and colleagues (2000b) and Allwood and colleagues (2006), with the modification that only aqueous extraction for polar compounds was adopted. 5µl of supernatants were pipetted into MS sample vials and loaded into 1200 series liquid chromatograph autosampler receiver

chamber connected to 6510 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, California, US). Direct flow injection was performed with 20% ACN, 0.1% Formic Acid, 0.3 ml/min, 350°C source and 6l/min drying gas. Spectra and Mass Spectrum were extracted using Agilent Mass Hunter Workstation Data Acquisition software for mass resolution of 4500. Spectra were recorded in positive (ES^+) mode using quadrupole time-of-flight (Q-TOF) mass analyser and relative quantification performed on positive ESI scans over m/z range of 100-1000 Th. Due to noise and non-zero m/z ion values obtained, data was binned to 0.0 Da using Agilent Mass Hunter Workstation Data Acquisition Software.

Statistics

Statistical tests were performed using SPSS Statistics 20 statistical software (IBM United Kingdom Limited, Portsmouth, UK) to determine the significance of changes obtained at different pot capacities. Where significance is indicated, a two-way ANOVA with a Tukey post-hoc test was conducted, to compare the responses of the two sorghum lines to water deficit. Significantly different data points are labelled on figures with different letters.

3.4 Results

Effects of drought induced by withholding water

When watering was completely withdrawn, the water content of the soil fell progressively, such that, after approx. 10-12 days, the soil was completely dry (Fig. 3.1A). There was a small but significant difference in the rate of water loss from pots depending on the Sorghum variety grown, with pots of Samsorg 40 losing water faster than Samsorg 17. Plants were destructively harvested at the end of the experiment. During the 12 day experiment, watered control plants showed a significant increase in leaf area (Fig. 3.1B). In droughted plants no increase in leaf biomass was apparent. Total above ground dry weight increased in both Sorghum lines, however this increase was similar in control and droughted plants (Fig. 3.1C). Specific leaf area was found to drop in both plant lines in response to drought (Fig. 3.1D).

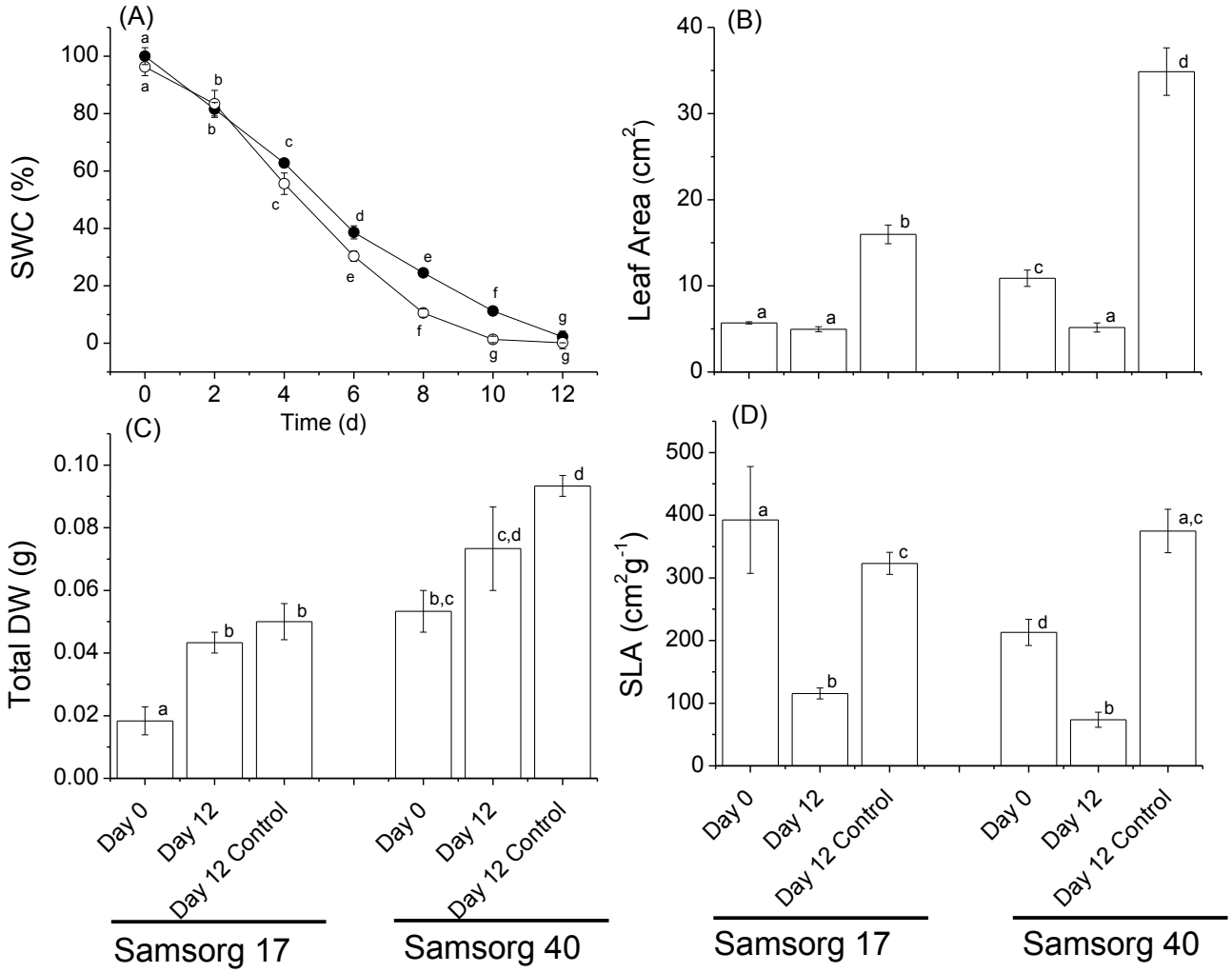


Fig. 3.1 The change in the pot water content, relative to water saturation (A), leaf area of plants (B), total above ground dry weight (C) and specific leaf area (D) of Samsorg 17 and Samsorg 40. All data represent the means ± SE of at least 3 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$). A part of this figure was shown in Chapter 2.

Relative water content (RWC) of leaves was measured to determine leaf water loss (Barrs and Weatherley 1962). During the first 6 days of drying, soil water content (SWC) fell to approx. 30-40% saturation. In the first 6 days of drying, leaf RWC differed in the two Sorghum lines (Fig. 3.2A). Samsorg 17 dried slower compared to Samsorg 40. From Day 8 onwards, a substantial drop in RWC was seen in Samsorg 40 plants (Fig. 3.2A). No significant change in the leaf RWC of Samsorg 17 was seen until Day 10 of treatment. By Day 12 of treatment, the RWC of both lines had fallen to approx. 35%.

Gas exchange was measured for up to 12 days in the two Sorghum cultivars. Stomatal conductance (G) differed in the two varieties (Fig. 3.2B). In Samsorg 40, withholding of water resulted in a closure of stomata by Day 6. Samsorg 17 did not drop significantly until Day 8, with the most substantial drop occurring by Day 10

Net Photosynthesis (A) in saturating light fell in response to drought in both Sorghum varieties, however the decline occurred earlier in Samsorg 40 than 17 (Fig. 3.2C). Although pot water had fallen substantially in both treatments, A was maintained in Samsorg 17 (Fig. 3.1A, 3.2C). The decline in A in Samsorg 40 preceded the decline in RWC, with A having fallen substantially on Day 6, whilst RWC was almost unchanged from control values. In Samsorg 17, there was a good correlation between A and RWC. No significant changes were seen in dark respiration rate across the experiment.

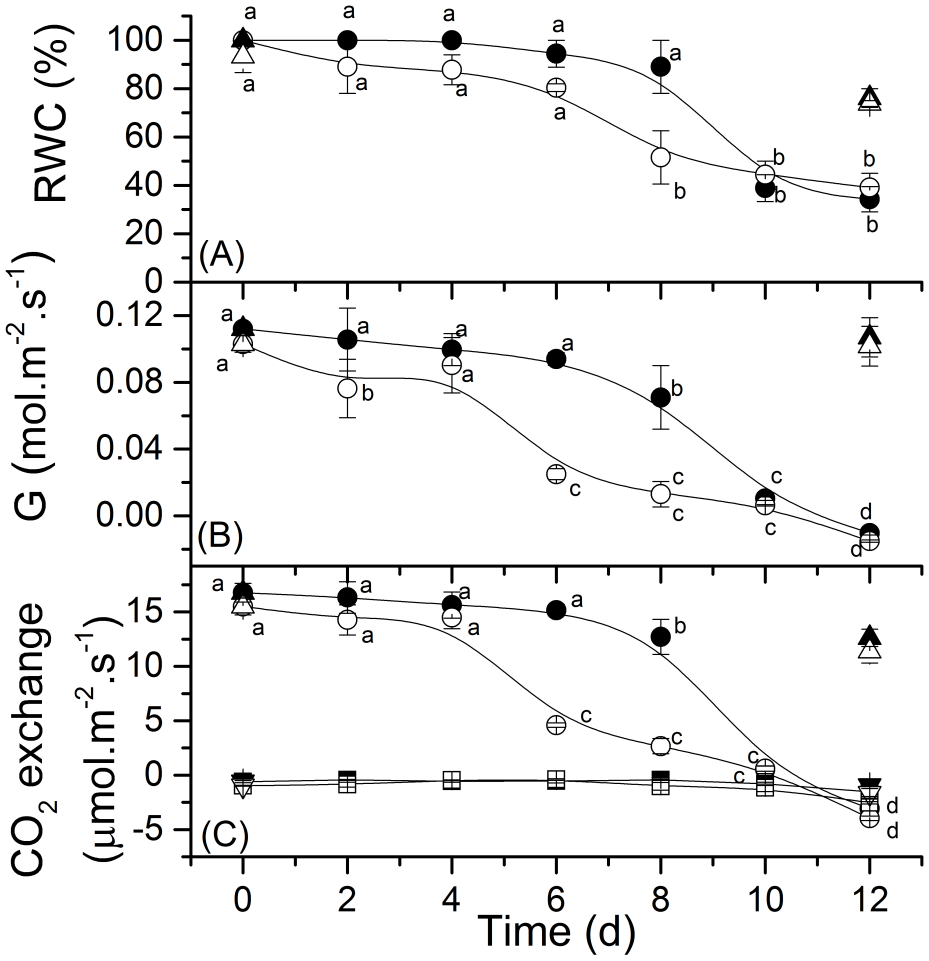


Fig. 3.2 Effect of progressive drought on water relations and photosynthesis in Sorghum. Plants were grown for 3 weeks under well-watered conditions and then watering was stopped. The change in leaf relative water content (A), stomatal conductance (B) and CO_2 exchange rate (C) of Samsorg 17 (closed circles) and Samsorg 40 (open circles) were measured every 2 days. Samsorg 17 control (closed triangles), Samsorg 40 control (open triangles), dark respiration in Samsorg 17 (closed squares) and dark respiration in Samsorg 40 (open squares). Measurements of gas exchange were performed at a CO_2 concentration of 390 ppm and an irradiance of 1000 $\mu molm^{-2}s^{-1}$. All data represent the means ± SE of at least 3 replicates.

Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$). A part of this figure was shown in Chapter 2.

Effects of prolonged drought on photosynthesis and water relations

Based on data presented in Fig 3.2, it can be seen that Samsorg 17 is able to maintain functionality for longer in response to progressive drought. In order to examine how the different Sorghum lines responded to more sustained drought, plants were allowed to dry to different degrees and then maintained for 5 days at close to those values, by watering appropriately on a daily basis. When pots were allowed to dry to 25% pot capacity, the RWC of plants of both Sorghum lines was maintained at close to 100% (Fig. 3.3A). Only when pots were dried to 15% of pot capacity was RWC markedly affected and then only in Samsorg 40. In both lines, RWC fell substantially when plants were maintained at 10% of pot capacity. The stomatal conductance (Fig. 3.3B) fell progressively in both Sorghum lines with decreasing water, with there being no significant difference between the two lines. The net photosynthesis was also affected differentially at 15% pot capacity, with Samsorg 17 being less repressed (Fig. 3.3C).

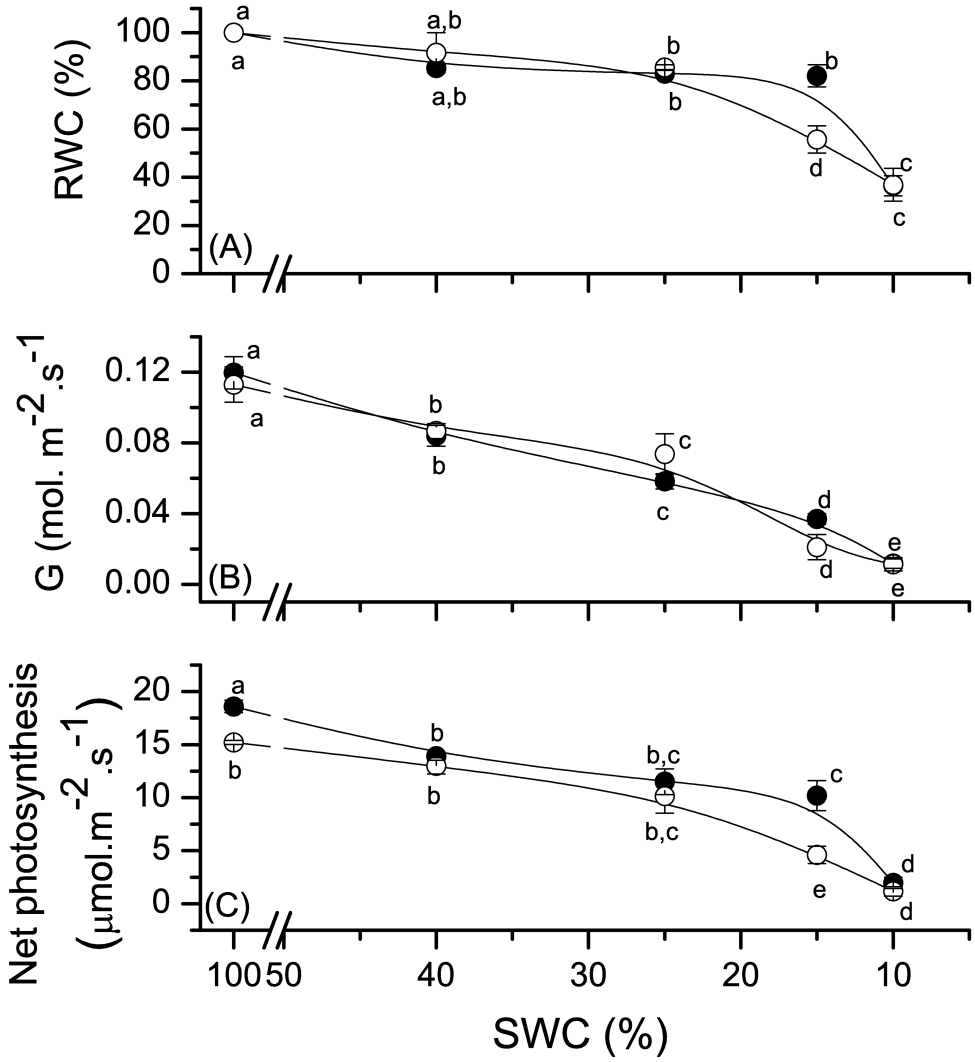


Fig. 3.3 Relative water content (A), Stomatal Conductance (B) and Photosynthesis (C) in Samsorg 17 (closed circles) and Samsorg 40 (open circles). Plants were maintained at different pot capacities for 5 days prior to measurement. Measurements were taken at an ambient CO_2 concentration of 390 ppm, PPFD of 1000 µmol m^{-2} s^{-1}, measuring temperature was 25°C. All data represent the means ± SE of at least 3 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$).

Measurements of chlorophyll fluorescence were performed to probe the responses of PSII to drought (Fig. 3.4). The maximum quantum yield of PSII, Fv/Fm, measured in dark-adapted leaves, is an indicator of damage occurring to the PSII reaction centre. In neither line was this significantly affected in plants exposed to drought down to 15% of pot water capacity. Only under the driest conditions, 10% of pot capacity was Fv/Fm affected, with this changing significantly in both lines (Fig. 3.4A). As drought increased, the relative PSII electron transport rate (PSII ETR) dropped in parallel with the decline in CO_2 fixation. In Samsorg 40, the less drought tolerant line, the decline in PSII ETR was significant at 15% pot capacity and matched the drop in CO_2 assimilation (Fig. 3.4B and 3.3C). At 10% pot capacity, PSII ETR fell to near zero in both lines.

Measurements of non-photochemical quenching in the two Sorghum lines, showed that this increased under drought in the tolerant line Samsorg 17, but less so in the more sensitive Samsorg 40 (Fig. 3.4C). Measurement of relaxation kinetics in the plants indicated that the contributions of protective high energy state quenching (qE) and photoinhibition (qI) did not differ significantly between the two lines (data not shown).

PSI electron flux, measured as PSI ETR, was maintained down to 15% pot capacity in Samsorg 17, while in Samsorg 40 PSI ETR was already affected at this level of drought (Fig. 3.4D). Overall the responses of PSI ETR and PSII ETR were similar (Fig. 3.4B, D).

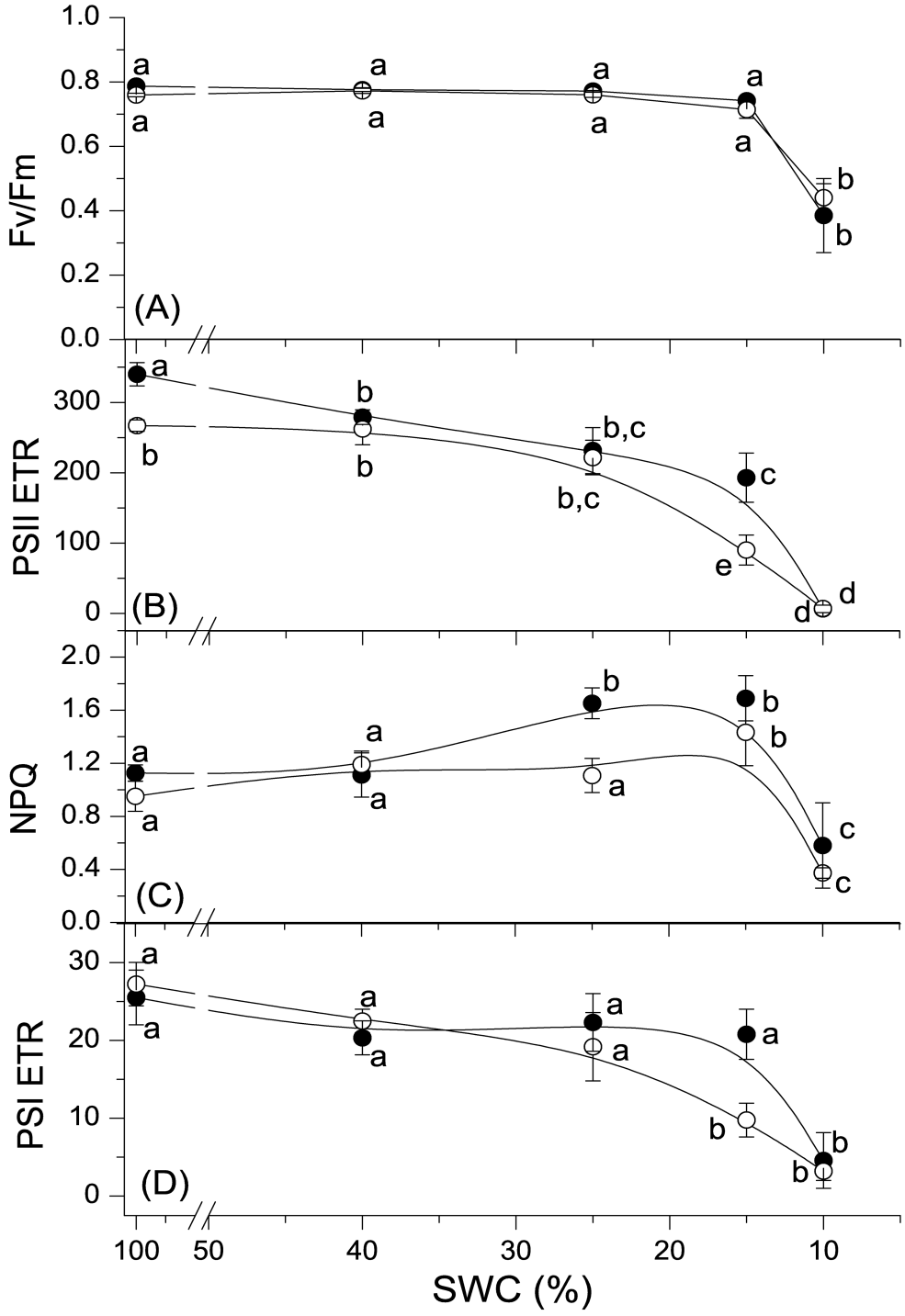


Fig. 3.4 Changes in chlorophyll fluorescence and PSI photochemistry measured at different pot capacities in Samsorg 17 (closed circles) and Samsorg 40 (open circles). Maximum quantum efficiency (A), Electron transport through PSII (B), NPQ (C) and Electron transport through PSI (D). Measurements were taken at ambient CO_2 concentration of 390 ppm, PPFD

of 1000 μmol m^{-2} s^{-1}, Saturating flash of 6000 μmol m^{-2} s^{-1} and temperature of 25°C. All data represent the means ± SE of at least 3 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$).

Changes in the composition of the photosynthetic apparatus in response to drought

When plants were maintained under different degrees of drought stress, there was a progressive loss of chlorophyll per unit leaf area in Samsorg 40 with increasing water stress (Fig. 3.5). At more moderate drought, this was accompanied by an increase in the chlorophyll a:b ratio, consistent with a specific loss of light harvesting complexes. However, at more severe levels of drought, chl a:b fell, indicating a preferential loss of reaction center complexes. In Samsorg 17, low level drought actually increased chlorophyll content per unit area, accompanied by an increase in chl a:b, implying an increase in reaction centres per unit area. Chlorophyll content only fell under the most extreme drought conditions, with no further changes in chl a:b being seen, implying a uniform loss of chlorophyll binding proteins.

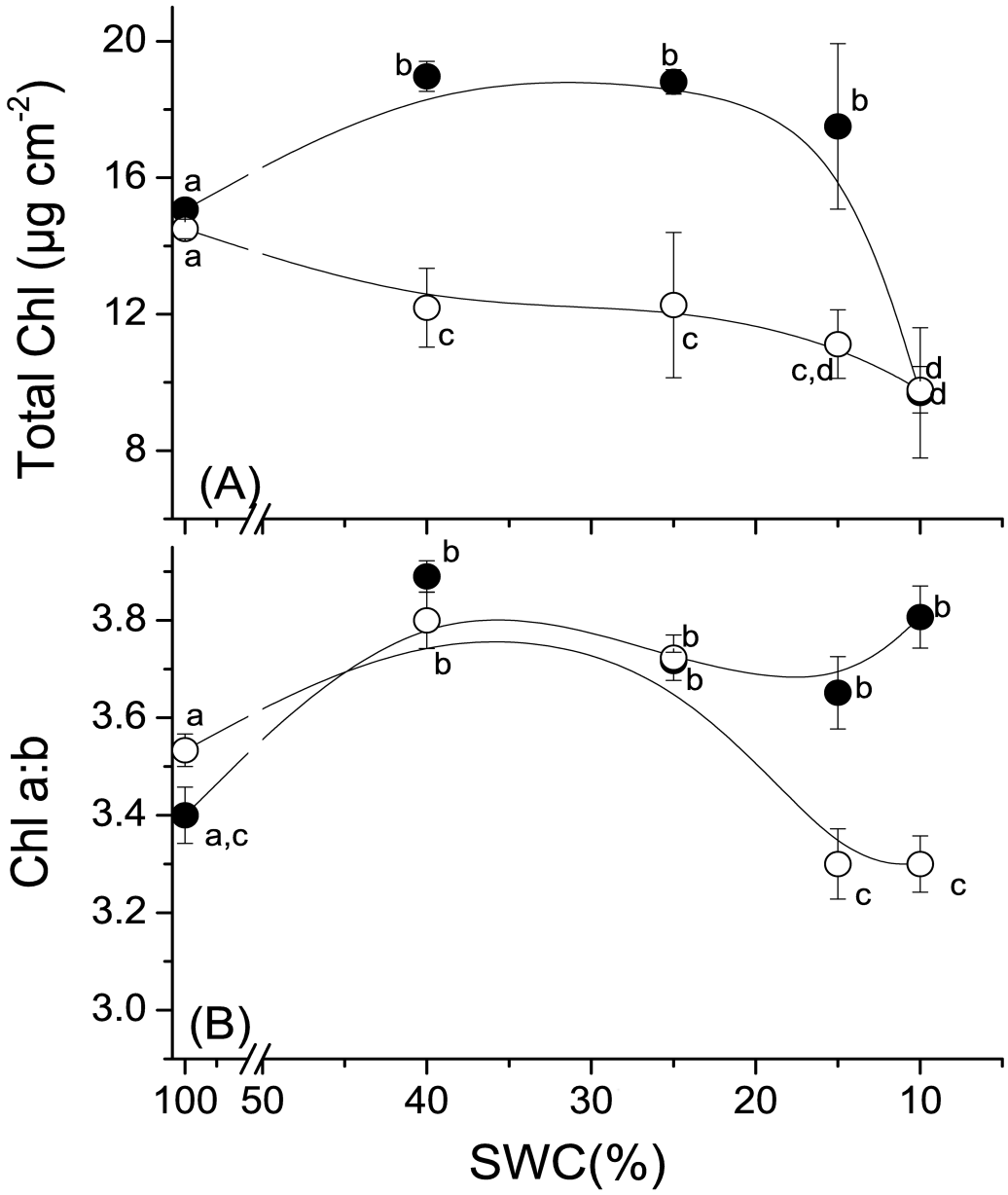


Fig. 3.5 Relationship between chlorophyll per unit area (A) and chl a:b (B) for leaves of Samsorg 17 (closed circles), Samsorg 40 (open circles) maintained for 5 days at different soil water contents. All data represent means ± SE of at least 5 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$).

Changes in chlorophyll content and composition imply a reengineering of the photosynthetic apparatus is taking place in response to drought. The photosynthetic apparatus represents a major proportion of total leaf protein. In Samsorg 40, total protein content of leaves declined significantly as drought progressed, with this effect already being apparent at the most moderate drought condition (Fig. 3.6A). At that level of drought, root protein was also lost, although more severe levels of drought resulted in no further decrease in root protein, per unit fresh weight (Fig. 3.6B). In Samsorg 17, no change in leaf chlorophyll was apparent at any level of drought, though there was a small loss of protein in roots.

Loss of protein in Samsorg 40 might represent a loss of total nitrogen (N) from the leaf or a diversion of N into other forms (e.g. accumulation of compatible solutes). Total nitrogen, expressed as a percentage of dry mass, in the leaf of Samsorg 17 increased in response to moderate drought while there was a decrease in leaf N in Samsorg 40 (Fig. 3.6C). Higher levels of drought gave rise to no further changes in leaf N in either cultivar, implying that N being lost from protein in Samsorg 40 was retained in the leaf. In the roots, total nitrogen, as a percentage dry mass, was not significantly affected in Samsorg 17 but decreased in Samsorg 40 with increasing drought (Fig. 3.6D). The leaf C:N ratio differed significantly between the two cultivars, already under control conditions, with the value in Samsorg 40 being lower. Drought induced opposite responses, with C:N increasing significantly in Samsorg 40 but tending to decrease in Samsorg 17, thus reversing the difference between the cultivars. The C:N ratio of roots under controlled conditions showed the same difference as in leaves, with Samsorg 40 having a lower ratio, which increase in response to mild drought. There was no significant change in root C:N with drought in Samsorg 17 (Fig. 3.6E, F).

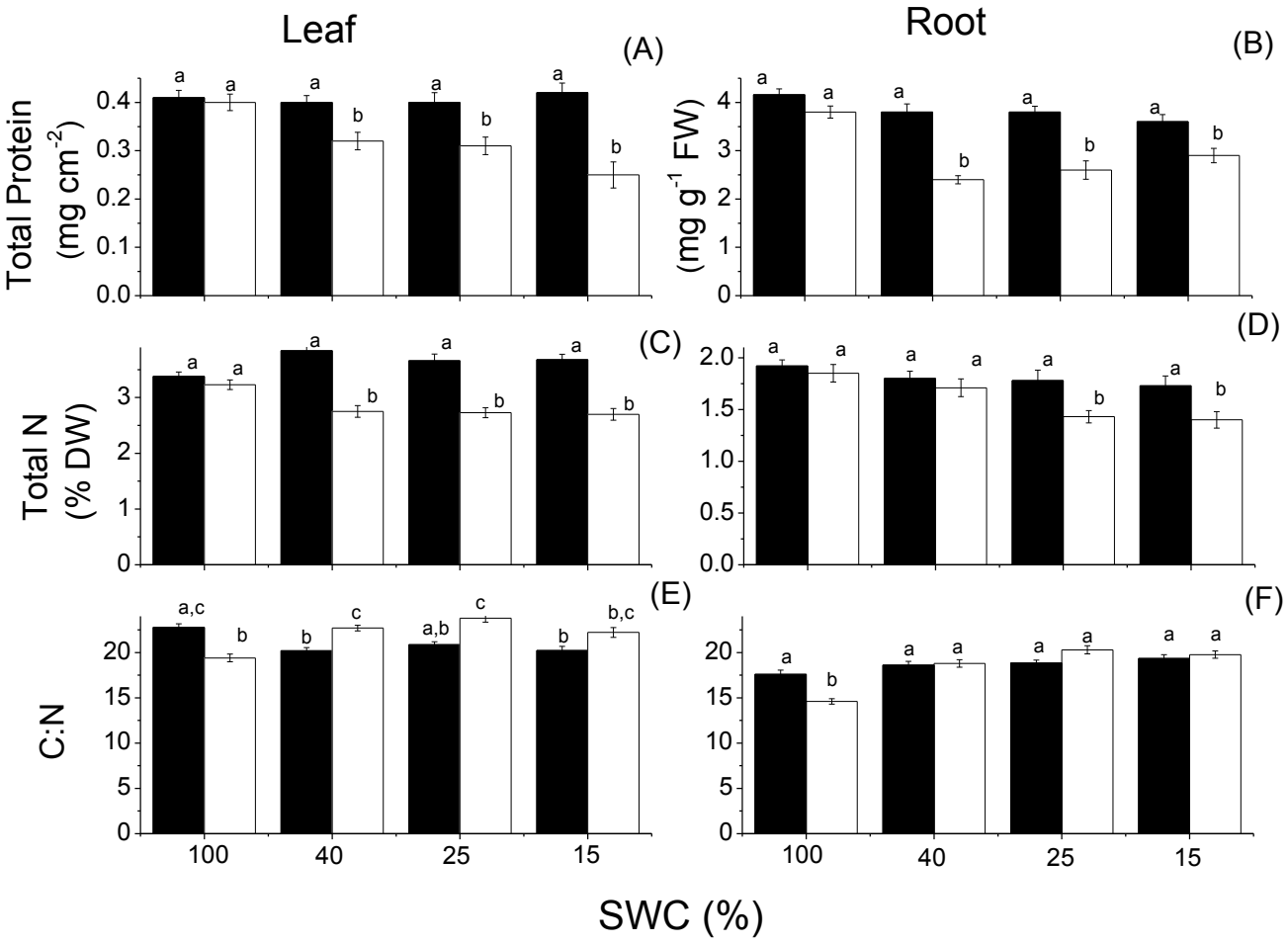


Fig. 3.6 Total leaf protein (A), total root protein (B), total leaf nitrogen (C), total root nitrogen (D), Leaf C:N ratio (E) Root C:N (F) of Samsorg 17 (black bars) and Samsorg 40 (open bars). Figures are from plants exposed to different degrees of drought. All data represent the means ± SE of at least 3 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$).

To better understand how the photosynthetic apparatus is reengineered under drought stress, Western blot analysis was performed for representative components of key photosynthetic complexes. In Samsorg 17, no changes in relative expression of any of the components of the photosynthetic apparatus measured were apparent (Fig. 3.7). In Samsorg 40, differential responses were observed in different proteins. Neither the PSI subunit (PsaD) nor cytochrome f (PetA) changed detectably, however, both Rubisco (RbcL) and PSII (PsbA) declined markedly as drought progressed (Fig. 3.7). Previous work has shown that the chloroplast localised heat shock protein, chCpn60, acts as a Rubisco chaperone (Demirevska-Kepova et al. 1999, Jagtap 1998, Wang et al. 2004, Xu et al. 2011b). In the leaf of Samsorg 40, chCpn60 declined in parallel with the RbcL down regulation (Fig. 3.6). Levels of this peptide were maintained in Samsorg 17 throughout the drought period.

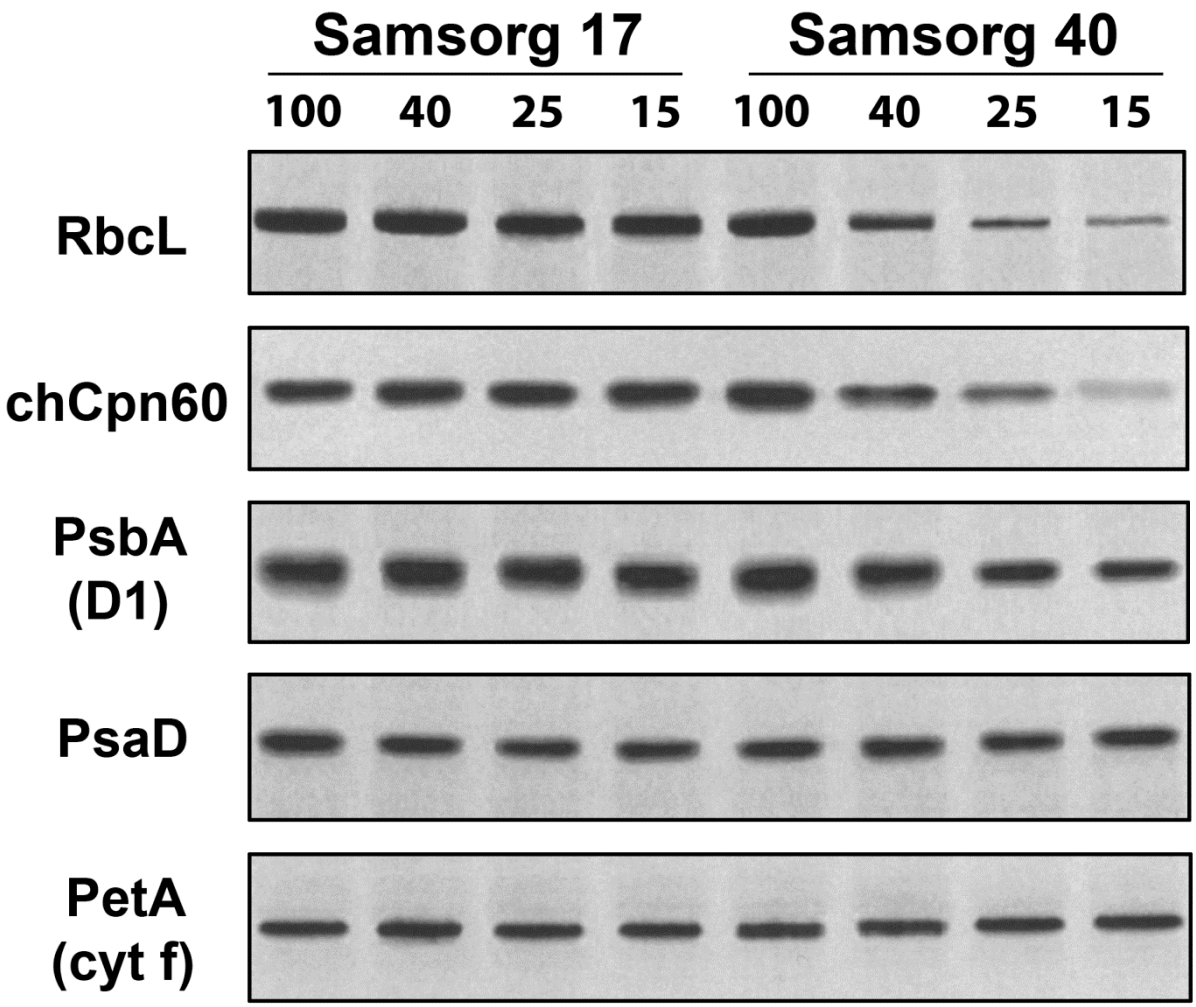


Fig. 3.7 Relative expression of components of the photosynthetic apparatus in Sorghum (var. Samsorg 17 and Samsorg 40) under drought treatment. Plants were allowed to dry and then maintained at different pot capacities (% SWC) for 5 days. Equal amounts of isolated chloroplast protein (15µg) were electrophoretically separated on glycine-SDS-PAGE (4-20%), electroblotted onto nitrocellulose membrane and probed with set of antibodies raised against RbcL, chCpn60, PSII, PSI and cyt f subunit peptides. Blots are representative of at least three independent experiments.

Changes in small molecules in response to drought

Rubisco and PSII together account for a significant proportion of total leaf protein, however, in Samsorg 40, leaf N is maintained below 40% soil water capacity, whilst substantial losses of protein, including Rubisco and PsbA, continue to occur. This implies that the N from protein degradation is being, to some extent at least, retained in the leaf. To identify possible sinks for this N, a non-targeted metabolomic approach was adopted. Amongst the metabolites identified using direct injection mass spectrometry (DIMS) the drought sensitive amino acids proline and glycine betaine both progressively increased in response to progressive drought with their being an earlier and greater appearance of glycine betaine. Colorimetric assay of total soluble sugars showed that these also increase in Samsorg 40 in response to water deficit (Fig. 3.8). The more drought tolerant Samsorg 17 maintained low levels of both proline and glycine betaine throughout the progressive drought treatment; however they contained a constitutively high sugar content (Fig. 3.8).

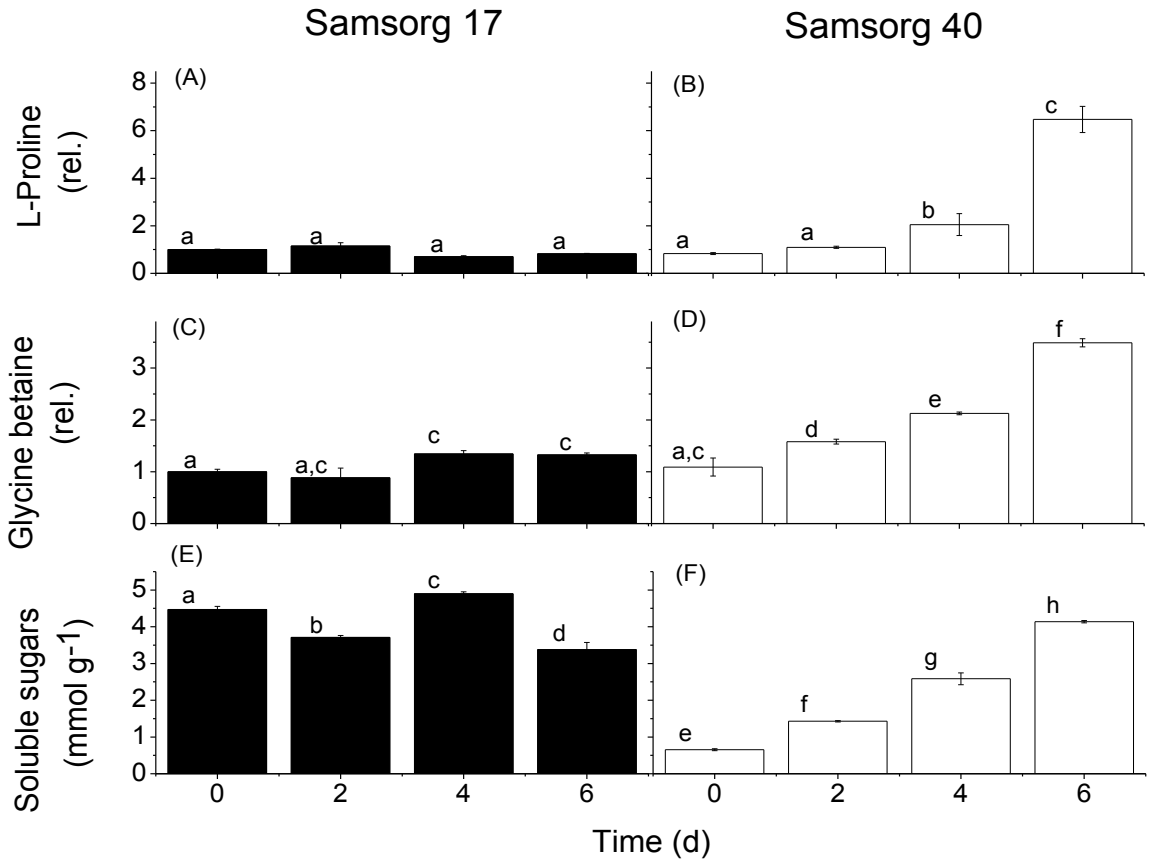


Fig. 3.8 Proline (A, B), glycine betaine (C,D) relative to Samsorg 17 control and soluble sugars (E, F) in Samsorg 17 (black bars) and Samsorg 40 (open bars) leaves exposed to progressive drought. Data are the means ± SE of at least 5 replicates. Significant differences are indicated by different letters on the figures (ANOVA, $P<0.05$).

3.5 Discussion

To increase the drought tolerance of crop species, we need to understand better the traits possessed by drought tolerant plants, so that these can be transferred into new varieties. In this study, two varieties of Sorghum – Samsorg 17, a highly drought tolerant cultivar and Samsorg 40, a less tolerant variety – were studied.

The two varieties differed in their growth under control conditions, with Samsorg 40 having a larger leaf area per plant and greater above ground dry weight, though with a lower specific leaf area (SLA). When exposed to progressive drought, both cultivars showed a loss of growth, in terms of leaf area but still increased in dry weight, resulting in a marked decrease in SLA in response to drought (Fig. 3.1). Samsorg 17 showed a more profligate use of scarce water resources, retained more open stomata longer, whilst being able to maintain leaf relative water content (RWC) even under very severe drought conditions, up to 8 days without water, while the RWC of Samsorg 40 had already declined significantly by same time point (Fig. 2A, B). At the same time, by maintaining transpiration, CO_2 assimilation was also maintained in Samsorg 17 (Fig. 3.2C, D). So, Samsorg 17 does not achieve better drought tolerance by conserving water, rather, this implies that it is better able to extract water from the dry soil than is Samsorg 40. When plants were maintained at particular levels of drought for 5 days, the difference between the two lines became less marked (Fig. 3.3), partly due to some recovery in Samsorg 40 and partly due to drought inducing responses in Samsorg 17 – e.g. there was some stomatal closure and loss of photosynthesis. Nevertheless, the point at which loss of function was occurring in Samsorg 17 was at a more severe level of drought than in Samsorg 40.

Inhibition of assimilation under drought conditions resulted in down regulation of Photosystem II electron transfer (PSII ETR). A significant decrease in PSII ETR was seen at 15% of Pot Capacity (PC) in Samsorg 40 and 10% PC in Samsorg 17 (Fig. 3.4B), with data correlating well with rates of assimilation. At the same time, responses of PSI ETR broadly mirrored those of PSII. These data need to be interpreted however in the context of changes occurring in protein composition and in light of their distribution in this C_4 plant. In Samsorg 17, there was no change in the relative content of different photosynthetic proteins detected (Fig. 3.7). Nevertheless, there was a decline in assimilation, a loss of PSII activity and an increase in NPQ (Fig. 3.3 and 3.4). The latter has widely been associated with cyclic electron flow, though electron transport to oxygen, the Mehler reaction, may also contribute to this (Asada 2000, Asada 2006, Golding and Johnson 2003, Stepien and Johnson 2009). Chlorophyll fluorescence in a Sorghum leaf will arise almost entirely from the mesophyll cells, whilst a substantial proportion of PSI activity can be presumed to be in the bundle sheath, where cyclic electron flow is thought to dominate (Heber and Walker 1992, Johnson 2011, Shikanai 2007). The rise in NPQ in Samsorg 17 is consistent with an increase in cyclic flow in the mesophyll (Heber and Walker 1992, Hertle et al. 2013b, Ivanov and Edwards 1997, Johnson 2011, Joliot and Johnson 2011, Munekage et al. 2002). Although there is no loss of PSI, relative to chloroplast proteins, in this cultivar, changes in chlorophyll are consistent with an increase in the total amount of reaction centres per unit area of leaf (Fig. 3.5). At the same time, we cannot exclude a redistribution of PSI between tissues.

The situation in Samsorg 40 is further complicated by the specific loss of PSII (Fig. 3.7). PSII ETR in this plant was maintained at all but the most severe levels of drought, however, this is estimated per PSII reaction centre, and, as these are decreasing in concentration the total flux through all PSII must also be falling (Fig. 3.4). PSI turnover tended to fall also;

however, we again cannot determine the contribution of different compartments to this flux. The fact that NPQ does not rise in this plant suggests that there is no increase in cyclic flow in the mesophyll. Additional NPQ is however not needed, as the excitation pressure on PSII is not increasing – PSII is being lost instead (Fig. 3.3 and 3.6). Measurements of Fv/Fm and the absence of degradation products of PsbA (D1 protein) breakdown imply that the loss of PSII is not related to photoinhibition but is, rather, a controlled process, whereby plants of Samsorg 40 are progressively decreasing PSII capacity (Adir et al. 2003, Long et al. 1994, Takahashi et al. 2009). This will have the effect of decreasing vulnerability to oxidative stress in the mesophyll as plants experience stress.

The progressive loss of PSII activity in the mesophyll reflects a reduction of primary CO_2 fixation, which will in turn result in a reduction in the extent to which Rubisco in the bundle sheath is supplied with CO_2 via the C_4 cycle (Carmo-Silva et al. 2012). The loss of Rubisco can be seen therefore as simply reflecting a decline in the demands for this enzymatic activity (Fig. 3.7). Loss of Rubisco might result from an increase in the rate of breakdown of the protein or a decrease in the rate of *de novo* synthesis. The loss of the chaperone protein chCpn60, which is associated with Rubisco synthesis, tends to support the latter (Wang et al. 2004, Xu et al. 2011b). Either way, the net loss of Rubisco protein, accompanied by the loss of PSII in the mesophyll and the overall loss of protein from the leaf, reflects a redistribution of resources, especially of nitrogen away from carbon fixation.

At the same time as PSII and Rubisco are being lost, maintenance of PSI and the cytochrome b_6f complex will allow cyclic electron flow to be relatively maintained. This is not required to protect PSII from damage through NPQ, but maybe important in maintaining the energy balance of the leaf. The components that are retained are those that are required for cyclic electron flow, which we suggest will be maintained in the bundle sheath and promoted in the

mesophyll. Cyclic flow will contribute ATP to support metabolic processes involved e.g. in synthesis of compatible solutes using breakdown products from degradation of proteins and other macromolecules (Chaves et al. 2009, Chen et al. 2007, Kavi Kishor et al. 2005, Kavi Kishor and Sreenivasulu 2013). The appearance in particular of glycine betaine and to a lesser extent proline and the accumulation of total soluble sugars under conditions where photosynthesis is decreasing are all consistent with the idea that a major reengineering of the leaf structure is occurring in this plant (Chaves et al. 2009). This represents a clear mechanism whereby Samsorg 40 is altering its tissues, both shoot and root, to maximise its survival under drought (Fig. 3.7).

This reengineering contrasts strongly with the response in Samsorg 17, which is considered to be the more drought tolerant plant (Ismaila et al. 2010, Jimoh and Abdullahi 2011, Ogbonna 2007, Simonyan et al. 2007). Samsorg 17 adopts a strategy of maintaining business as usual for as long as possible. It maintains a constitutively high pool of soluble sugars, reflected in its high C:N ratio under control conditions (Fig. 3.6 and 3.8). As the plant experiences drought, this allows it to maintain leaf relative water content, keep its stomata open longer and keep fixing carbon for longer. Nitrogen is not diverted from photosynthetic proteins (particularly from Rubisco which is a prominent cellular deposit of approx. 10% of total N in C_4 plants), so the nitrogen use efficiency of the plant is not compromised (Westhoff and Gowik 2010). The accumulation of large sugar concentrations will, of course, have a metabolic cost, however this maybe relatively small, if we assume that sugars can be redeployed at the point of grain filling. Indeed, they may represent a valuable store of fixed carbon and therefore not compromise overall plant productivity.

Given the contrasting responses of the two sorghum varieties to drought conditions, the question must be asked, which is the better? From an agronomic point of view, that of

Samsorg 17 is preferred – this variety has been bred as a drought tolerant cultivar. Farmers are interested in maintaining growth for as long as possible such that, provided drought is reversed soon enough, final yield is not compromised. It is possible however that the contrasted response of Samsorg 40 might, from an ecological point of view, actually be more successful. By having the flexibility to reengineer the leaf, this plant may increase its chances of survival, even if it is unable to maintain yield. Although Samsorg 17 is able to maintain business as usual longer, when exposed to sustained drought (up to 5 days in our experiments) its advantage is lost. At this point, the inability to reengineer leaf process may impede survival and recovery. Nevertheless, in terms of crop breeding strategies, we suggest that selecting for high tissue sugar content, rather than the ability to respond dynamically to drought, maybe the more successful approach.

3.6 Acknowledgements

We are grateful to the Institute of Agricultural Research, Zaria, Nigeria for kindly providing sorghum seeds used in this study. We also wish to express our gratitude to Reynard Spiess and Dr. Milton Lima Neto for help with Mass Spectrometry and soluble sugars assay respectively.

Chapter 4:

BIOCHEMICAL ANALYSES OF SORGHUM VARIETIES REVEAL DIFFERENTIAL MECHANISMS OF TOLERATING DROUGHT

Authors:

Chukwuma C. Ogbaga[1], Piotr Stepien[2], Beth C. Dyson[1], Giles N. Johnson[1]

Addresses

[1]The University of Manchester, Faculty of Life Sciences, Oxford Road, Manchester, M13 9PL, United Kingdom

[2] Wroclaw University of Life and Environmental Sciences, Department of Plant Nutrition, ul. Grunwaldzka 53, 50-357 Wroclaw, Poland

Author For Correspondence:

Giles N. Johnson

Tel: +44 161 275 5750

Fax: +44 161 275 5082

Email: giles.johnson@manchester.ac.uk

Ogbaga et al *In preparation* for submission to Plant Physiology

Key words: Sorghum, Drought, Photosynthesis, Compatible Solutes, Heat Shock Proteins, Dehydrins

Authors' Contributions

CCO, PS and GNJ designed the experiments. CCO grew treated and harvested plants and prepared the samples. CCO performed soluble sugar assays and analysed FTIR and GC-MS data. PS performed Western Blots. BCD ran the GC-MS samples. CCO wrote the manuscript.

4.1 Abstract

We have examined the biochemical responses of two sorghum cultivars of differing drought tolerance, Samsorg 17 (more drought tolerant) and Samsorg 40 (less drought tolerant), to sustained drought. Plants were dried to different degrees of drought and then maintained at that level of drought for 5 days. Responses were examined in terms of metabolic changes and the expression of drought induced proteins- Heat Shock Proteins and dehydrins. Changes in metabolites were studied using Fourier Transform Infra- Red (FTIR) Spectroscopy and non-targeted Gas Chromatography Mass Spectroscopy (GC-MS), while changes in protein expression were examined using Western blot analysis. Different responses of metabolites, Heat Shock Proteins and dehydrins were observed in the two cultivars. Metabolic changes involved changes in lipids, amino acids, polysaccharides and their derivatives. A total of 188 compounds with 142 known and 46 unknown compounds were detected in the two sorghum varieties. With water deficit, Samsorg 17 accumulated sugars, sugar alcohols and higher levels of lipids, while Samsorg 40 relied rather on making amino acids. It is concluded that the two Sorghum varieties adopt distinct approaches in response to drought, with Samsorg 17 being better able to maintain leaf function under severe drought conditions.

4.2 Introduction

Drought is one of the major limitations on food production in developing countries in Africa and Asia (Ngara et al. 2012). There is a growing need to produce crops that can survive extreme environmental conditions such as drought in order to maximise crop production. Detrimental effects of drought can be seen in the roots, shoots and leaves of green plants at morphological, physiological and biochemical levels. In this paper, the emphasis will be on the biochemical responses to drought, in terms of metabolism and selected proteins.

Exposure to drought triggers a variety of biochemical responses in plants. These responses include the accumulation of a variety of different compatible solutes and up-regulation of specific drought-induced proteins (e.g. heat shock proteins and dehydrins).

Compatible solutes are osmolytes that protect plants from osmotic stress, maintaining cytosolic osmotic balance during extreme conditions in plants (Kavi Kishor et al. 2005). Drought has been reported to induce the accumulation of compatible solutes in various herbaceous plants (Akashi et al. 2001, An and Liang 2013, Garg et al. 2002, Nakayama et al. 2000). Compatible solute accumulation has been reviewed as either being a sign of stress or induced as a protective mechanism which can minimise the effects of stress (Chaves et al. 2009, Chen et al. 2007, Kavi Kishor et al. 2005, Kavi Kishor and Sreenivasulu 2013) . On the one hand, their accumulation, enhanced by the up-regulation of abscisic acid (ABA), is seen as a plant's signalling mechanism that can potentially mitigate the production of reactive oxygen species (ROS) (Chaves et al. 2009). On the other hand, their accumulation is thought to contribute to the maintenance of the cytosolic energy balance of a cell (Kavi Kishor and

Sreenivasulu 2013). Examples of compatible solutes that have been identified as drought sensitive include nitrogen-containing compounds –e.g. glycine betaine, proline – and sugars – e.g. trehalose, glucose and sucrose (Kavi Kishor et al. 2005, Serraj and Sinclair 2002, Yobi et al. 2012). These osmolytes are metabolically expensive to produce and, in the case of amino acids, represent an important pool of nitrogen (Westhoff and Gowik 2010). Although compatible solutes are thought to maintain cellular homeostasis, there is no consensus yet regarding their role and mechanism of regulation *in vivo* in plants, especially during drought. What is known is that exogenous application of some compatible solutes, e.g. glycine betaine and proline, improves stress tolerance (Ashraf and Foolad 2007). It has been argued however that accumulation of solutes could be as a consequence of stress rather than an adaptive response (Ashraf and Foolad 2007, Chen and Murata 2011).

Heat shock proteins (HSPs) and dehydrins (DHNs) are broadly expressed in grasses under different stress conditions, such as high light, temperature, salinity, oxidative stress, heat, cold and drought and play crucial roles in maintaining cellular homeostasis, thereby protecting plants from stress (Jagtap 1998, Ngara et al. 2012, Wang et al. 2004, Xu et al. 2011b). HSPs help maintain protein stability and aid folding and refolding or are involved in the degradation of damaged proteins under stress conditions. HSPs are found throughout the cell (chloroplast and cytosol) and play a very important role in maintaining normal protein conformation (Wang et al. 2004). Although HSPs have been suggested to have protective roles (Wang et al. 2004, Xu et al. 2011b), especially in response to heat stress, their functions in other stress conditions, e.g. drought, is less clear (Jiang and Huang 2002). The levels of the chloroplast HSP60 were maintained in drought stressed *Sorghum bicolor* while those of cytosolic Heat Shock Protein HSC 70 were reportedly induced in *Festuca arundinacea* (Jiang

and Huang 2002, Ogbaga et al. 2014). However, the roles of other HSP families in relation to drought are unclear.

DHNs are Group II of late embryogenesis abundant (LEA) proteins that are hydrophilic, thermostable and accumulate in the cytosol, nucleus, chloroplast and mitochondria (Borovskii et al. 2002). They are characterised as having three conserved motifs, K, Y and S segments, named based on predominance of the amino acids – Lysine, Tyrosine and Serine respectively (Rorat 2006). Both classes of proteins – HSPs and DHNs – have been shown to be expressed in sorghum and maize in response to drought (Jagtap 1998, Mohammadkhani and Heidari 2008, Wood and Goldsbrough 1997) and have been suggested to have protective roles in both plants (Wang et al. 2004, Xu et al. 2011b).

Amongst widely cultivated crops, the C_4 grass *Sorghum bicolor* is one of the most drought tolerant species (Paterson et al. 2009). Having C_4 biochemistry allows it to maintain photosynthesis at low CO_2 concentrations, e.g. when stomata are significantly closed. However, this alone does not explain its drought tolerance – its close relative maize has similar C_4 metabolism but much lower drought tolerance. We showed previously contrasting responses in two sorghum varieties - Samsorg 17 and Samsorg 40 with differing degrees of drought tolerance, specifically in terms of high constitutive sugar content and loss of proteins respectively (Ogbaga et al. 2014). In this study, biochemical and metabolic responses to drought in these two sorghum cultivars were compared. In particular, we focussed on identifying the specific sugars accumulated by Samsorg 17 and the nitrogen sinks for lost proteins in Samsorg 40. The two cultivars are shown to differ substantially in their expression of HSPs and DHNs and accumulation of metabolites. The results are discussed in terms of identifying potential targets for developing better drought tolerance.

4.3 Materials and Methods

Plant material and growth conditions

Seeds of Sorghum (*Sorghum bicolor* L) cv Samsorg 17 (more drought tolerant) and cv Samsorg 40 (less drought tolerant) were provided by the Institute of Agricultural Research, Zaria, Nigeria. The seeds were grown in a growth room as described previously (Ogbaga et al. 2014). To estimate soil water content (SWC), % maximum pot capacity, fully saturated soil contained in 3'' plant pots was weighed and thereafter oven dried at 105°C to constant weight. The weight difference between the water-saturated and oven-dried soil was taken as the weight of water needed to bring pots to pot capacity and lower water levels (% pot capacity) estimated accordingly. Pots were then maintained at different water levels by re-watering daily with water lost in the previous 24 hours, for 5 days before samples were harvested.

Fourier Transform Infra-Red (FTIR) Spectroscopy

Fully expanded leaf and shoot tissues from different soil water contents were excised 8h into photoperiod with a pair of scissors, flash frozen in liquid nitrogen and lyophilised (freeze-dried) with ScanVac Coolsafe freeze dryer (Vacuubrand, Wertheim, Germany) for 48 hrs. Fourier Transform Infra-Red (FT-IR) spectroscopy was performed on 30 mg DW of tissues homogenised, transferred to 2ml microcentrifuge tubes and extracted in 600 µl of distilled water. 5µl of homogenate was loaded onto the wells of a silicon 96 well attenuated total reflection (ATR) target plate (Bruker, MA, USA) and dried at 60°C. The ATR plate was then placed in a Bruker Equinox-55 spectrometer and raw FTIR data recorded in the absorbance mode of wavenumber 4000 - 600 cm^{-1} with detector at $4cm^{-1}$. FTIR measures the infra-red spectrum of absorption or raman scattering of a material (Griffiths and de Haseth 2007). With this technique, absorbed light is re-emitted with lower intensity and changes in molecular

bonds (C-N bonds) in a sample under analysis detected. A mathematical algorithm is then used to convert raw IR data from intensity-vs-time into intensity-vs-frequency spectrum given in wavenumber cm^{-1}. Signals at particular frequencies are assigned to specific types of bonds and groups of molecules (Griffiths and de Haseth 2007). Principal components and loadings of the raw FTIR data were generated with the data analysis software, R (available at http://www.r-project.org/). These were plotted on Origin (OriginLab, Northampton, MA).

Principal component discriminant function analysis (PC-DFA), a supervised multivariate technique was performed on the principal components (PCs) of samples harvested at different soil water contents. This was done to determine the spectral regions of compounds that change with water deficit. For clarity, spectral regions of major compounds vary for lipids at ~ 3050-2800 cm^{-1}, proteins ~ 1750- 1250 cm^{-1} and carbohydrates ~ 1250- 900 cm^{-1} (Allwood et al. 2008, Correia et al. 2008). The conversion of the principal components of raw FTIR data into loadings allows for the differentiation of these spectral regions.

Gas Chromatography-Mass Spectrometry (GC-MS)

Fully expanded leaf and shoot tissues from plants exposed to different soil water contents were excised 8h into photoperiod with a pair of scissors, flash frozen in liquid nitrogen and lyophilised (freeze-dried) with ScanVac Coolsafe freeze dryer (Vacuubrand) for 48 hrs. 30 mg DW of tissues were extracted in methanol-chloroform-water mixture as described in Lisec et al. (2006) and Allwood et al. (2008). Polar phase extracts were dried using a speed vacuum concentration with 100 µL of an internal standard containing 200µg/ml of succinic-d4 acid, lysine-d4 and glycine-d5 (Sigma-Aldrich, St Louis, Missouri, USA). This was followed by a two- step derivatisation process according to Lisec et al. (2006) and Allwood et al. (2008), which involves first the addition of methoxyamine hydrochloride dissolved in

pure pyridine (Sigma-Aldrich) and second, treatment with a silylating compound *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA reagent; Macherey-Nagel, Duren, Germany). Samples were then run in a GC-MS ionisation source for ion formation and mass detection conducted using time-of-flight (TOF) mass analyser. GC-MS analyses were performed using a Leco Pegasus III (4D) GC x GC/MS in GC/MS mode (Leco Corp., St Joseph, MO) with a Gerstel MPS-2 autosampler (Gerstel, Baltimore, MD) and an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). Quality control samples were obtained by mixing equal volumes of the upper polar phase of all the sample extracts.

The retention time and/or index of metabolites identified were compared and matched with those from pure chemical standards using in-house libraries from the Manchester Metabolomics Database (Allwood et al. 2008). The metabolites were differentiated into polar and non-polar compounds (Allwood et al. 2006, Fiehn et al. 2000a).

SDS-PAGE and Immunoblot analysis

Protein extraction and chloroplast isolation were performed as described previously (Ogbaga et al. 2014).

Protein samples for SDS-PAGE were incubated in 50 mM Tris-HCl (pH 6.8), containing 2% (w/v) SDS, 50 mM DTT, 1 mM PMSF, 5 mM EDTA, 10% (w/v) glycerol and 0.05% (w/v) bromophenol blue at 95^{o}C for 3 min. Total protein from Sorghum leaf/root (25µg) or chloroplast (15µg) were separated on 4-20% Precise Tris-Glycine SDS-PAGE (Thermo Scientific, Waltham, MA, USA) in Mini-PROTEAN 3 cell (Bio-Rad, CA, USA) at 35mA per gel. For immunoblot analyses, proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham, Little Chalfront, UK) by electroblotting in western transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% [v/v] methanol) in Mini Trans-Blot cell

(Bio-Rad; 60 min at 90 V). Blots were checked by Ponceau S staining to ensure even transfer. Membranes were blocked with 5% (w/v) non-fat milk powder in TBS-T (20 mM Tris-HCl, pH 7.6, 125 mM NaCl and 0.5% [v/v] Tween) and then incubated overnight at 4^{o}C with primary antibodies against: Dehydrins (Abcam, Cambridge, UK); Heat Shock Proteins – HSC/HSP70 (Enzo, Exeter, UK), chHSP70, HSP90-2 and HSP101 (Agrisera, Vannas, Sweden). Dehydrins (DHNs) are Group II of Late Embryogenesis Abundant proteins with conserved motif in C-terminal amino acid sequence termed K-Segment. Anti-DHN antibody raised against this sequence recognises a number of polypeptides in the range 9-200 kDa. Binding of the primary antibody was detected using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000 polyclonal goat anti-rabbit IgG; Sigma) and ECL detection reagent (Amersham) according to the manufacturer's instructions.

Sucrose and Glucose Assays

100mg FW of sorghum samples were weighed and extracted in 1ml of distilled water. 0.1mL of sorghum extracts from different soil water contents (% pot capacity) were assayed for sucrose and glucose using Sucrose/D-Glucose assay kit (catalogue number K-SUCGL; Megazyme, Ltd., Bray, Ireland). Assays were performed according to the manufacturer's instructions. The principle of the kit involves the hydrolysis of sucrose by the enzyme β-fructosidase to D-glucose and D-fructose. Free D-glucose catalysed by glucose oxidase and peroxidase in the presence of *p*-hydroxybenzoic acid and 4-aminoantipyrine is then determined based on the concentration of a red coloured quinoneimine dye. FW results obtained were subsequently converted to DW using the formula: %DW= %FW / [1-(RWC/100)], where DW is dry weight, RWC is relative water content.

Statistics

A two-way ANOVA with a Tukey post-hoc test was conducted using SPSS Statistics 20 statistical software (IBM, Portsmouth, UK), to compare the responses of the two sorghum lines to water deficit. Different letters represent significant differences at $P<0.05$.

4.4 Results

Changes in metabolites during drought

Plants were allowed to dry to a range of different water contents and then maintained at those levels of dryness for 5 days, by re-watering pots daily with water lost in the previous 24 hrs. From the data obtained from the machine (in wavenumber cm^{-1}), clear differences were seen between the control plants e.g. in the lipid and amine regions (Fig 4.1).

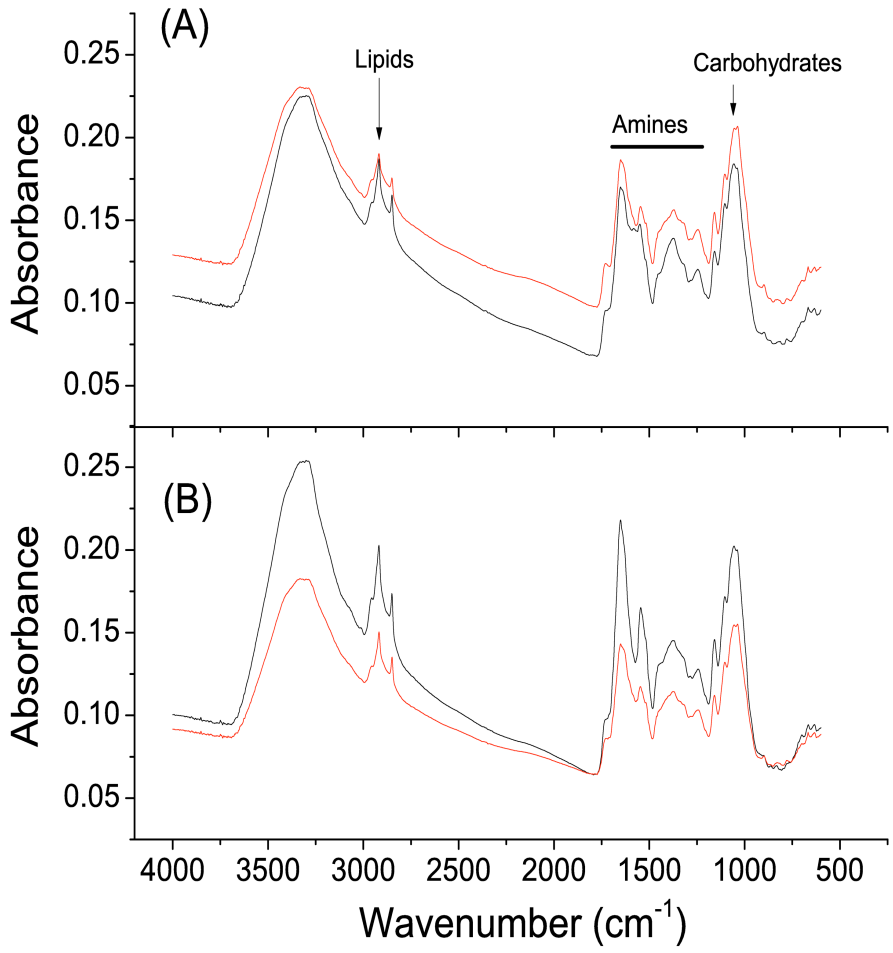


Fig. 4.1 Example FTIR Spectra of Samsorg 17 (A) and Samsorg 40 (B). Extracts from well watered plants (black lines) or plants exposed to 25% pot capacity (red lines) for up to 5 days. FTIR raw data were plotted using Origin (OriginLab, Northampton, MA).

Principal Component Discriminant Function (PC-DF) is a supervised multivariate analysis used to discriminate between the principal components of clusters of data. PC-DF of the FTIR spectra showed discrimination between plant lines under control conditions and with differing degrees of drought. In well-watered plants the two parent lines were separated.

With increasing water deficit, there is variation between the samples that is reflected mainly in PC-DF1 (Fig. 4.2A). Drying to 40% resulted in a substantial change in both plants, with this being most marked in Samsorg 40. This change could be described by both PC-DF2 and, to a lesser extent PC-DF1. Further drought resulted in a tendency for changes in PC-DF2 to reverse in Samsorg 40, but less so in Samsorg 17, with further changes being described by PC-DF1. Under the most extreme drought conditions, there was a tendency for the two lines to converge. Overall, majority of the changes were described by PC-DF1. Examination of the loadings indicated that changes in carbohydrates, amines and lipids all contributed to both PCDF1 and 2 suggesting that these are affected by drought (Fig. 4.2B).

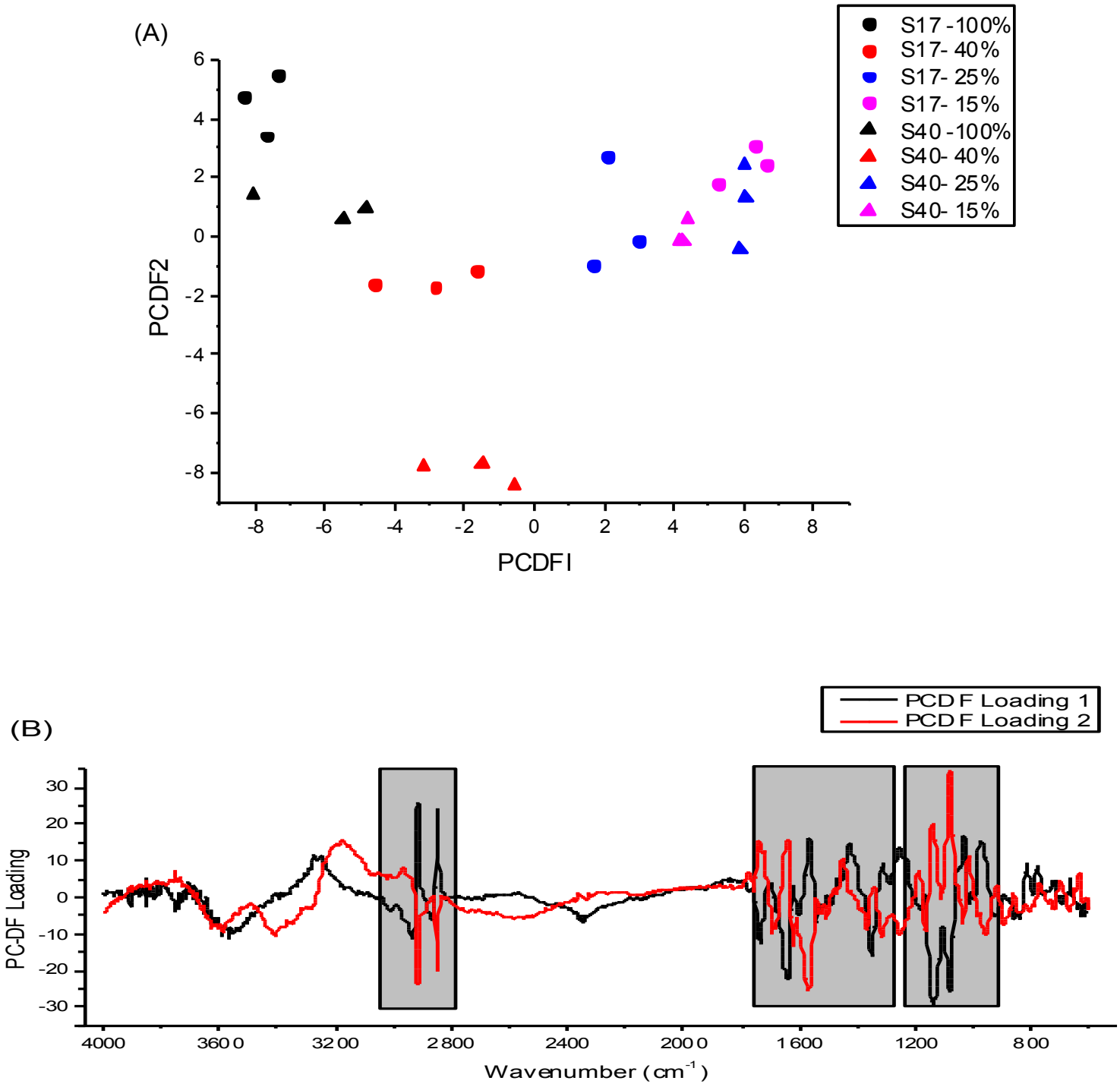


Fig. 4.2 Principal components discriminant function (A) and loadings (B) of Samsorg 17 and Samsorg 40 acquired using Fourier transform-Infrared spectroscopy. FTIR raw data were

acquired from machine and converted into principal components using the software R. Principal Component discriminant function (PC-DF) was performed on the principal components of the data. The generated discriminant functions were plotted using Origin (OriginLab, Northampton, MA). The shaded regions (from right to left) represent changes in carbohydrates, amines and lipids.

Changes in glucose and sucrose in response to drought

PC-DF analysis of FTIR data indicated the occurrence of changes in signals associated with carbohydrates in response to drought in both sorghum varieties. Soluble sugars were shown previously to be present at high concentrations constitutively in the tolerant Samsorg 17 whilst being induced in response to drought in Samsorg 40 (Ogbaga et al. 2014). Sorghum, especially sweet sorghum, is known to accumulate sucrose (Hoffmann-Thoma et al. 1996, Tarpley and Vietor 2007). Therefore, to determine whether the differences in sugar content between the two varieties studied here was due to sucrose, this was assayed using a linked enzyme assay, which also allowed us to estimate glucose content (Fig. 4.3).

When exposed to mild drought (40% pot capacity), there was a transient decrease in glucose content in both cultivars, with glucose content recovering at more severe drought levels. This effect was greater in Samsorg 17, where glucose content peaked at a concentration significantly above control levels (Fig. 4.3A). Both cultivars showed a further loss of glucose at the highest level of drought, compared to milder conditions. In Samsorg 17, there was a progressive accumulation of sucrose with increasing levels of drought. In Samsorg 40, there was no significant accumulation of sucrose. This contrasts to previous findings, where the former contained high concentrations of soluble sugars constitutively, whilst the latter accumulated sugars under drought conditions (Ogbaga et al. 2014). This suggests that sucrose

accumulation is not making a dominant contribution to the total sugar pool or that other sugars are changing in parallel with changes in sucrose and glucose. In an attempt therefore to identify further sugars accumulated under drought in the two varieties, plant material was analysed using non-targeted GC-MS analysis.

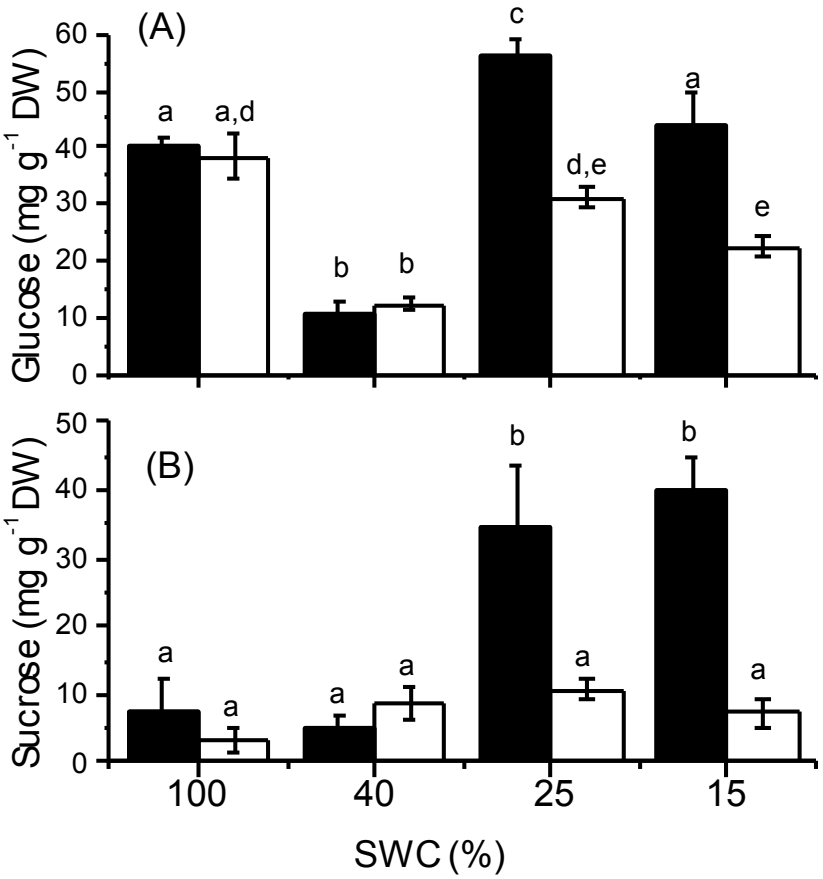


Fig. 4.3 Responses of the soluble sugars - glucose (A) and sucrose (B) to drought. Black bars are plants of Samsorg 17 while open bars are those of Samsorg 40. Data are mean of at least 3 replicates. Different letters represent significant difference, ANOVA, $P < 0.05$.

Metabolic differences between Samsorg 17 and Samsorg 40

A total of 188 compounds with 142 known and 46 unknown were detected in the two sorghum varieties using GC-MS. Table 4.1 below shows a list of major classes of compounds that were affected by drought (See Appendix I for complete list of compounds).

Table 4.1 Classes of compounds based on their functional groups (Larsen 2014). Compounds were detected with GC-MS. Significant proportion of the compounds identified are oxygen containing

Classes of Compounds	General Formula	Number detected
Alcohols	ROH	16
Aldehyde	RCHO	2
Carboxylic acids	RCO2H	30
Ethers	ROR'	3
Ketones	RR'C=O	3
Esters	RCO2R'	7
Amines	RNH2	14
Carbohydrates	ROH, RCHO, RR'C=O	25
Lipids	RCO2R'	2

GC-MS revealed that the drought tolerant Samsorg 17 contained, in addition to glucose and sucrose, significant amounts of fructose, galactose, lactose, cellobiose and sedoheptulose, relative to Samsorg 40 and to drought. The former also made more sorbose and a compound putatively identified as trehalose, but similar amounts of arabinose and 1,6-anhydroglucose compared with the latter (Fig. 4.3). The putative trehalose identification was as a result of the

disaccharide having a retention index that was greater than expected but closest to that of trehalose.

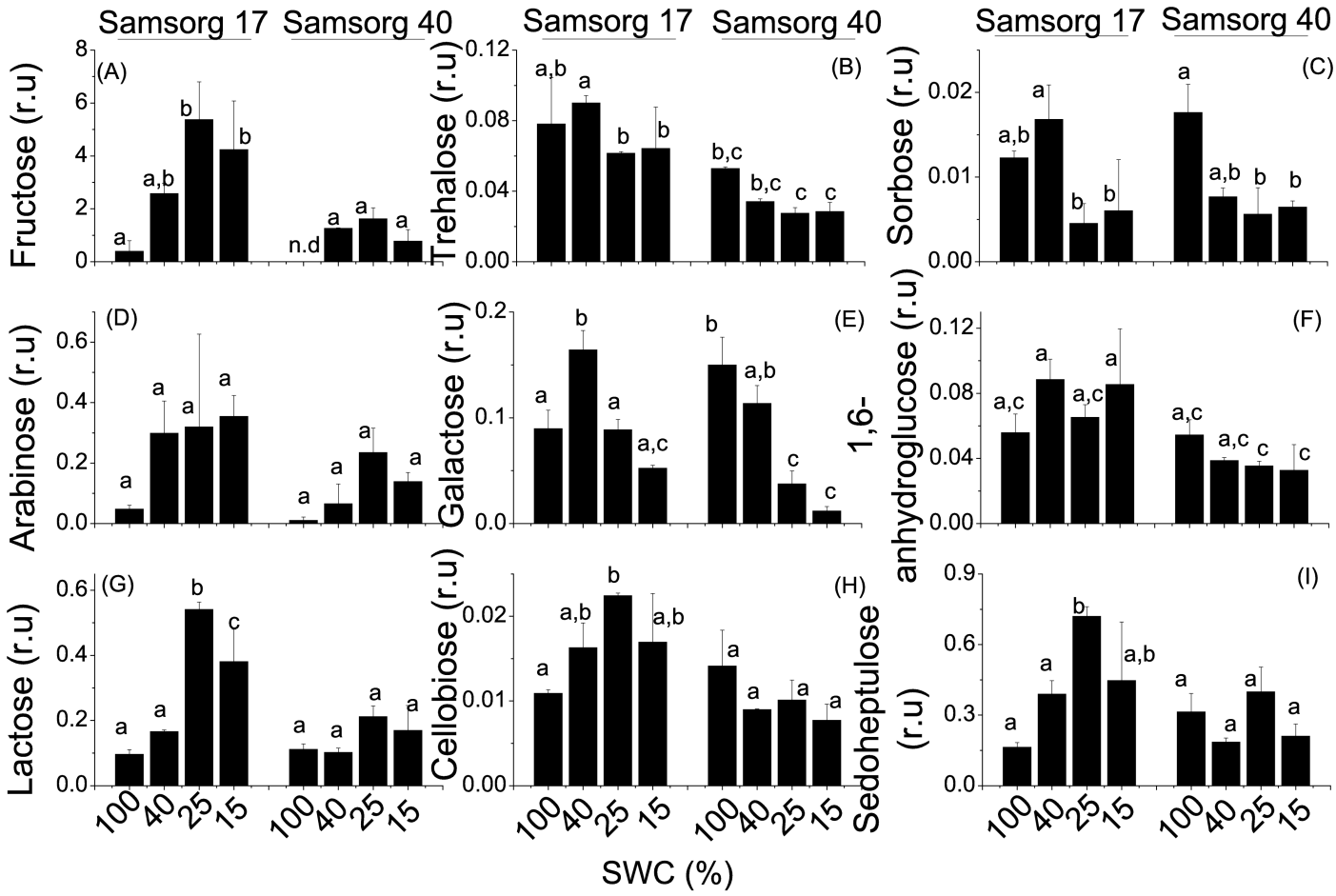


Fig. 4.4 Differences in sugars in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those of Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, n.d, not detected, ANOVA, $P < 0.05$.

Sugar alcohols have been suggested to play a role in drought tolerance, by acting as osmoprotectants, hence are thought to induce osmotic adjustments (dos Reis et al. 2012, Moing 2000). The sugar alcohols – ribitol, *myo*-inositol and xylitol – were significantly accumulated in Samsorg 17 but not in Samsorg 40 in response to drought (Fig. 4.5).

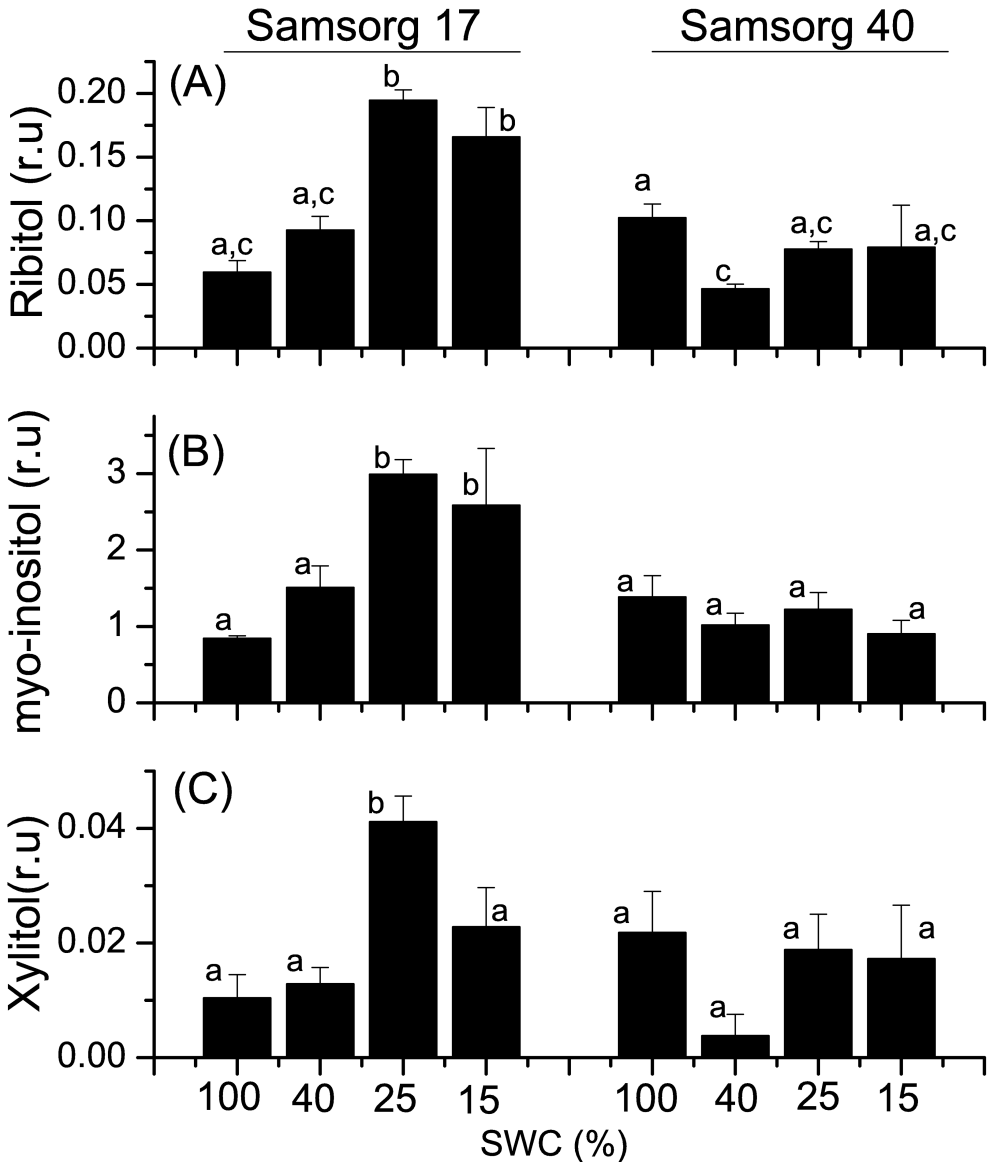


Fig. 4.5 Differences in sugar alcohols in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those of

Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, ANOVA, $P < 0.05$.

The osmoprotective role of amino acid accumulation during drought tolerance has been widely studied. It is thought that the accumulation of amino acids can remove reactive oxygen species formed in the cell, thereby improving tolerance (Good and Zaplachinski 1994, Montesinos-Pereira et al. 2014). The less drought tolerant Samsorg 40 accumulated the amino acids proline, threonine, phenylalanine, tryptophan, serine and asparagine in response to drought, relative to Samsorg 17 (Fig. 4.6). The only amino acid that was accumulated in higher proportions in Samsorg 17 was tyrosine.

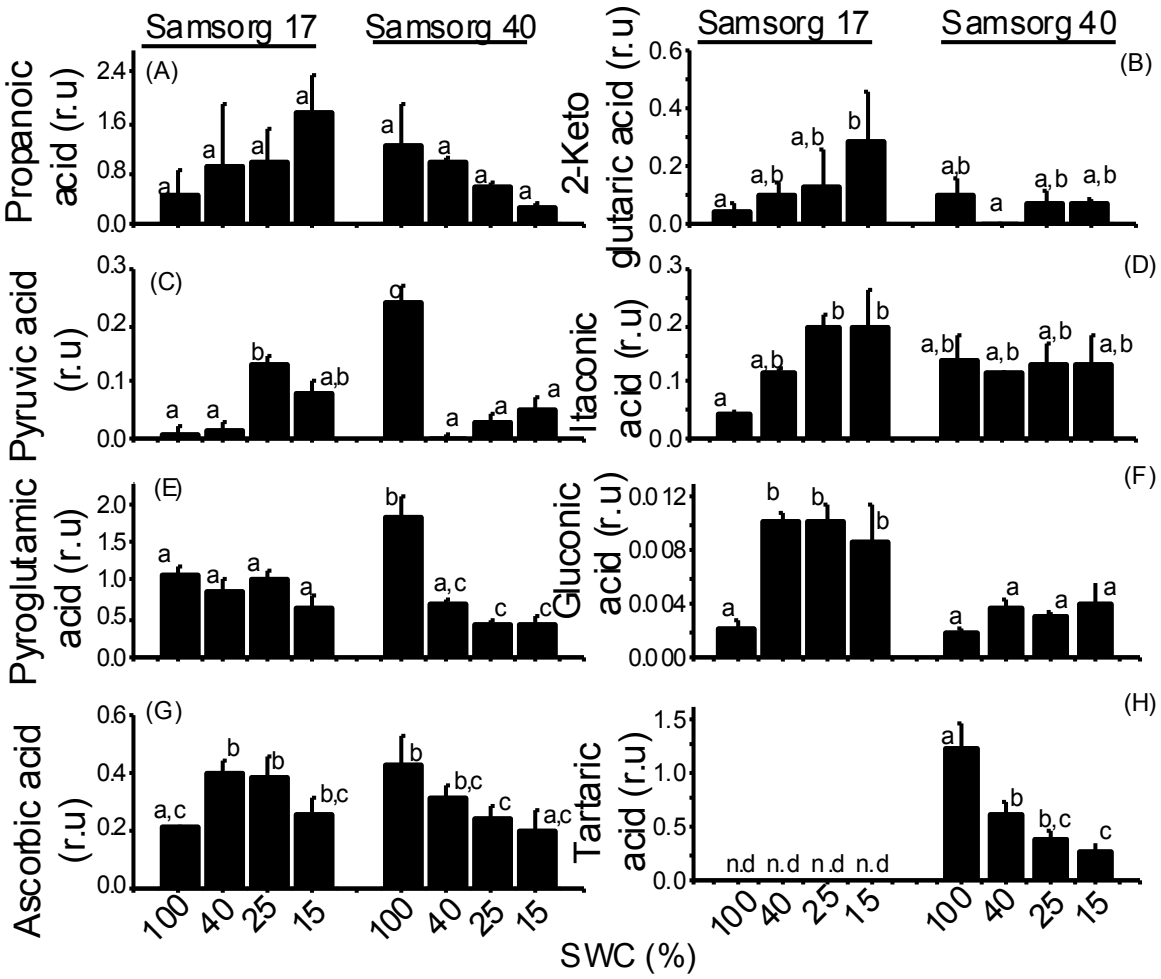


Fig.4.6 Differences in amino acids in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those of Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, n.d, not detected, ANOVA, $P < 0.05$.

Changes in lipid composition may help maintain cellular and membrane integrity during drought (Gigon et al. 2004). The fatty acid hexadecanoic acid and the fatty alcohol-isotridecanol were found constitutively in Samsorg 17 while in Samsorg 40; there was progressive loss of both. The lipid palmitin was not detected under well-watered conditions in Samsorg 17, unlike in Samsorg 40. The level of this lipid was higher in Samsorg 17 unlike in Samsorg 40 where progressive loss occurred.

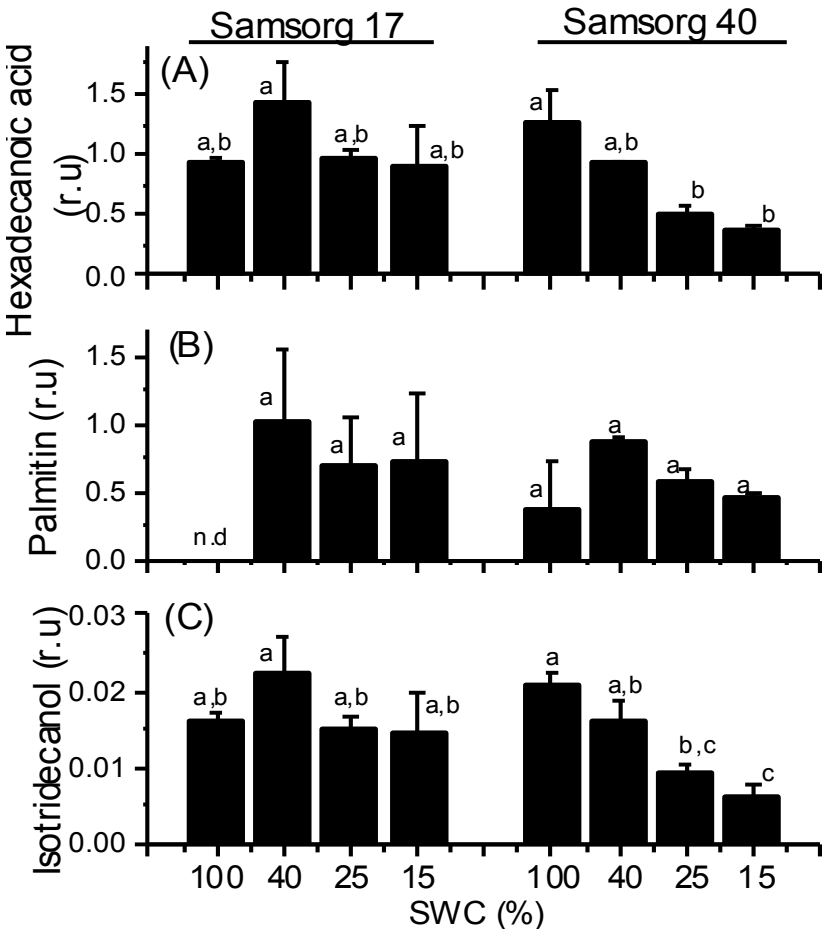


Fig. 4.7 Differences in lipids and fatty alcohol in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those

of Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, n.d, not detected, ANOVA, $P < 0.05$.

Various organic acids were detected in Sorghum leaves. Ascorbic acid, an important antioxidant, has widely been reported to be induced by stress and is believed to offer some degree of tolerance (Farooq et al. 2013, Shalata and Neumann 2001, Wang et al. 2010). There was some evidence for an accumulation of ascorbate under moderate drought in Samsorg 17, but in Samsorg 40 there was a progressive loss of this as drought progressed. Samsorg 17 accumulated more of the organic acids propanoic, 2-ketoglutaric, itaconic and gluconic acids. Levels of pyruvic and pyroglutamic acids were higher under well-watered condition in the less tolerant Samsorg 40 but with drought, dropped to the level seen in Samsorg 17. Tartaric acid was not detected in any treatments in Samsorg 17 but was present in significant amounts in Samsorg 40 dropping in concentration with drought (Fig. 4.8).

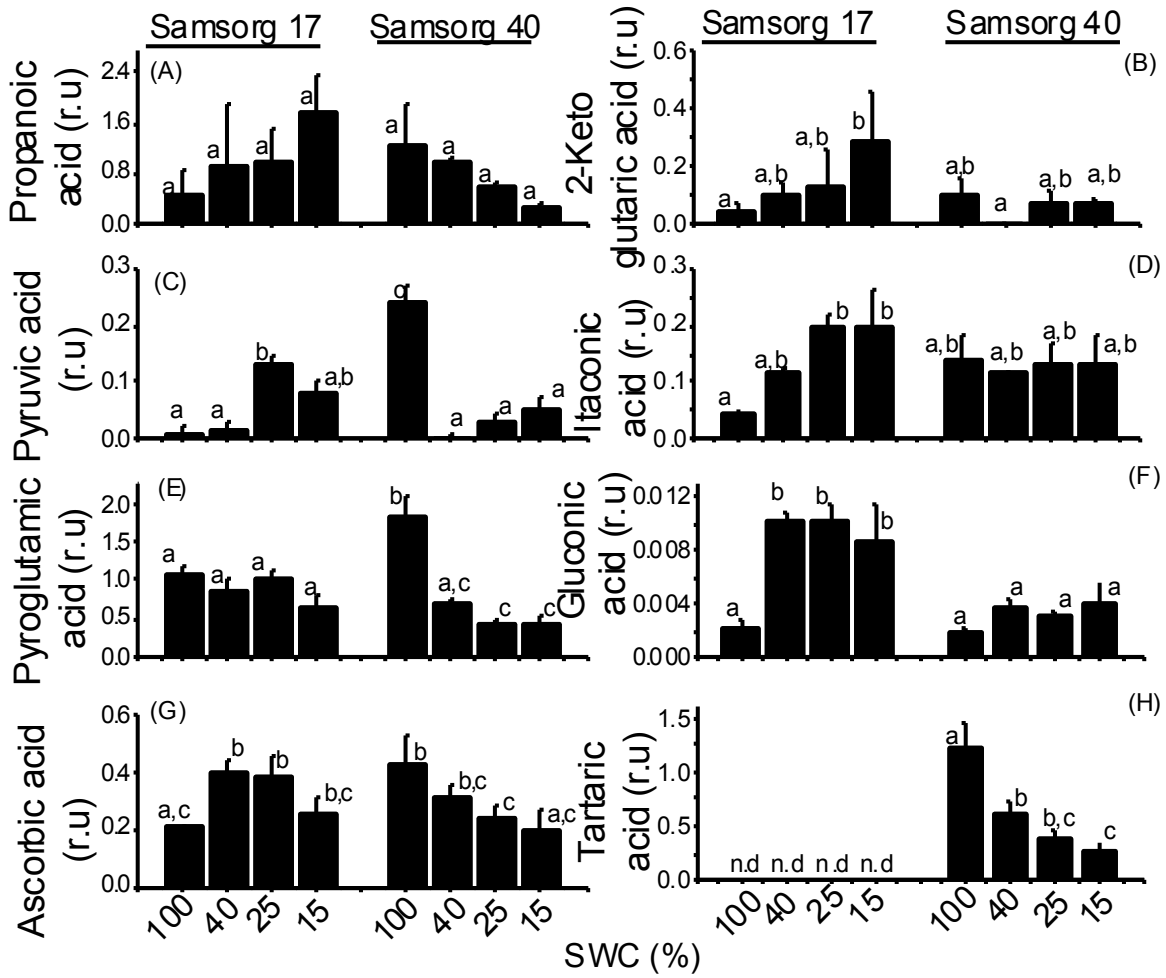


Fig. 4.8 Differences in other organic compounds in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those of Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, n.d, not detected, ANOVA, $P < 0.05$.

The levels of a number of unknown compounds varied between the two varieties. Upon exposure to drought, Samsorg 17 accumulated molecules of retention index (RI) 1464.9, 1673.9, 1846.8 and 1851 (Fig. 4.9). There were other unknown molecules that were differentially affected in response to drought with the least one having a RI of 1079 and the maximum an index of 3424.6 (See Appendix I for full list).

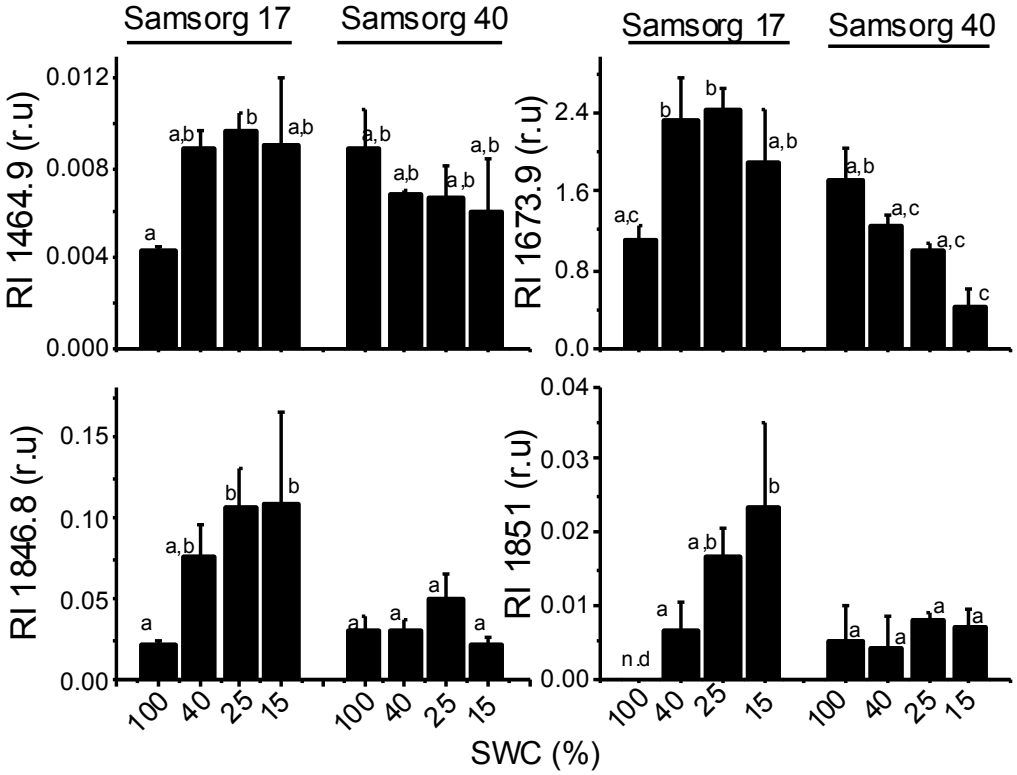


Fig. 4.9 Differences in major unknown compounds in the two sorghum varieties. For each panel, the responses of Samsorg 17 plants are shown first at four levels of drought followed by those of Samsorg 40. Data are mean of at least 3 replicates. Data were normalised to Glycine D5. Different letters represent significant difference, n.d, not detected, ANOVA, $P < 0.05$.

Changes in Heat Shock Proteins

Previous reports have shown that the chloroplast localised heat shock protein chCpn60 acts as a Rubisco chaperone (Demirevska-Kepova et al. 1999, Jagtap 1998, Wang et al. 2004, Xu et al. 2011b). In the leaves of Samsorg 40, chCpn60 (HSP60) declined in parallel with RbcL degradation (Ogbaga et al. 2014). Levels of this peptide were maintained in Samsorg 17 throughout the drought period (Ogbaga et al. 2014) . In order to determine whether the response of HSP60 was typical of other HSPs, we examined other HSP families - HSP70, HSP90 and HSP101.

Chloroplast localised heat shock protein chHSP70 is thought to be involved in protein import and translocation processes in chloroplasts. It also participates in the repair of damaged PSII proteins, by promoting either synthesis or assembly of new reaction center components (Cao et al. 2012, Medueno et al. 1993, Schroda et al. 1999). chHSP70 was significantly up-regulated from 40% PC in Samsorg 17. In Samsorg 40, a similar up-regulation was observed but this effect was less marked (Fig. 4.10). Chloroplast localised HSPs were not detected in root tissues (Fig. 4.10). The cytosolic protein, Heat Shock Cognate 70 (HSC70) ensures correct folding of polypeptide chains to proteins (Xu et al. 2011b). HSC70 was constitutively expressed in leaves of both Samsorg 17 and Samsorg 40; however the level of expression appeared stronger in the former than the latter (Fig.4. 10A). There was no clear indication that this expression was affected in response to drought in these tissues. In roots, there was some indication that HSC70 was induced by drought, especially in Samsorg 40 (Fig. 4.10B). Cytosolic HSP90, a molecular chaperone that only interacts with well-folded proteins was up-regulated in response to drought in leaves and roots of both cultivars; however Samsorg 17 appeared to show stronger expression than Samsorg 40. HSP 101 which belongs to a family of heat shock proteins named HSP 100s (or Clp proteins) and functions in protein

disaggregation and degradation accumulated in leaves during the late stages of drought in both lines, but considerably more so in Samsorg 40 (Fig.4. 10A). A similar pattern was observed in roots of Samsorg 40, whilst in Samsorg 17 a more even expression pattern was seen with differing degrees of drought (Fig. 4.10B).

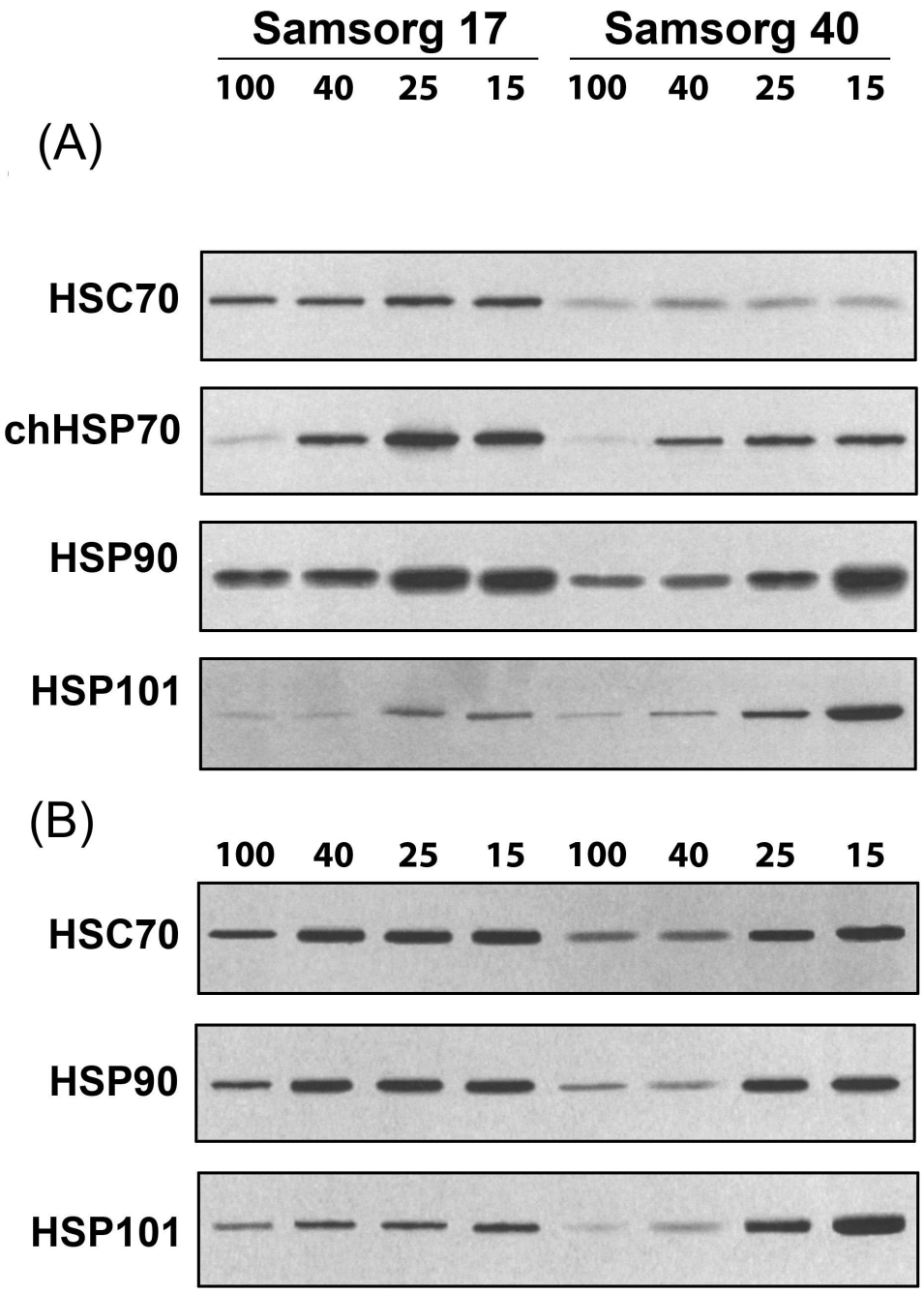


Fig. 4.10 Heat Shock Protein expression in the leaf (A) and root (B) of Samsorg 17 and Samsorg 40. Plants were maintained at four different water levels -100, 40, 25 and 15 % of pot capacity for 5 days. Equal amounts of whole leaf and root proteins (25µg) were

electrophoretically separated on Glycine-SDS-PAGE (4-20%), electroblotted onto nitrocellulose membrane and probed with sets of antibodies raised against HSP chaperone proteins. Blots are representative of at least three independent experiments.

Dehydrin/ Dehydrin-like protein expression

Dehydrins are Group II of Late Embryogenesis Abundant (LEA) proteins. They have a conserved lysine rich amino acid sequence and are thought to contribute to stress tolerance (Hanin et al. 2011, Rorat 2006). Samsorg 17 and Samsorg 40 showed contrasting DHN/ DHN-like protein expression when subjected to differing degrees of drought. In leaf tissue, peptides reacting to a DHN antibody ranged in size from 21 to 115 kDa. Amongst these, different expression patterns could be seen. Fewer bands were observed in roots, with bands at approx. 115, 37 and 21 kDa being absent (Fig. 4.11). The band at approx. 37kDa was constitutively expressed in the leaves of both cultivars; however bands appeared more intense in Samsorg 17 than Samsorg 40 (Fig.4.11A). Other bands showed more or less clear induction in response to drought. Bands in the range 52-69 kDa were observed in both cultivars and were induced in response to drought in both shoots and roots; however these tended to be induced sooner and/or more strongly in Samsorg 17 than in Samsorg 40. In contrast, the leaf only bands at 21 and 115 kDa showed earlier and stronger induction in Samsorg 40. The band at around 28 kDa showed similar expression patterns in roots and shoots of both cultivars, although expression appeared stronger in roots of Samsorg 17 (Fig. 4.11).

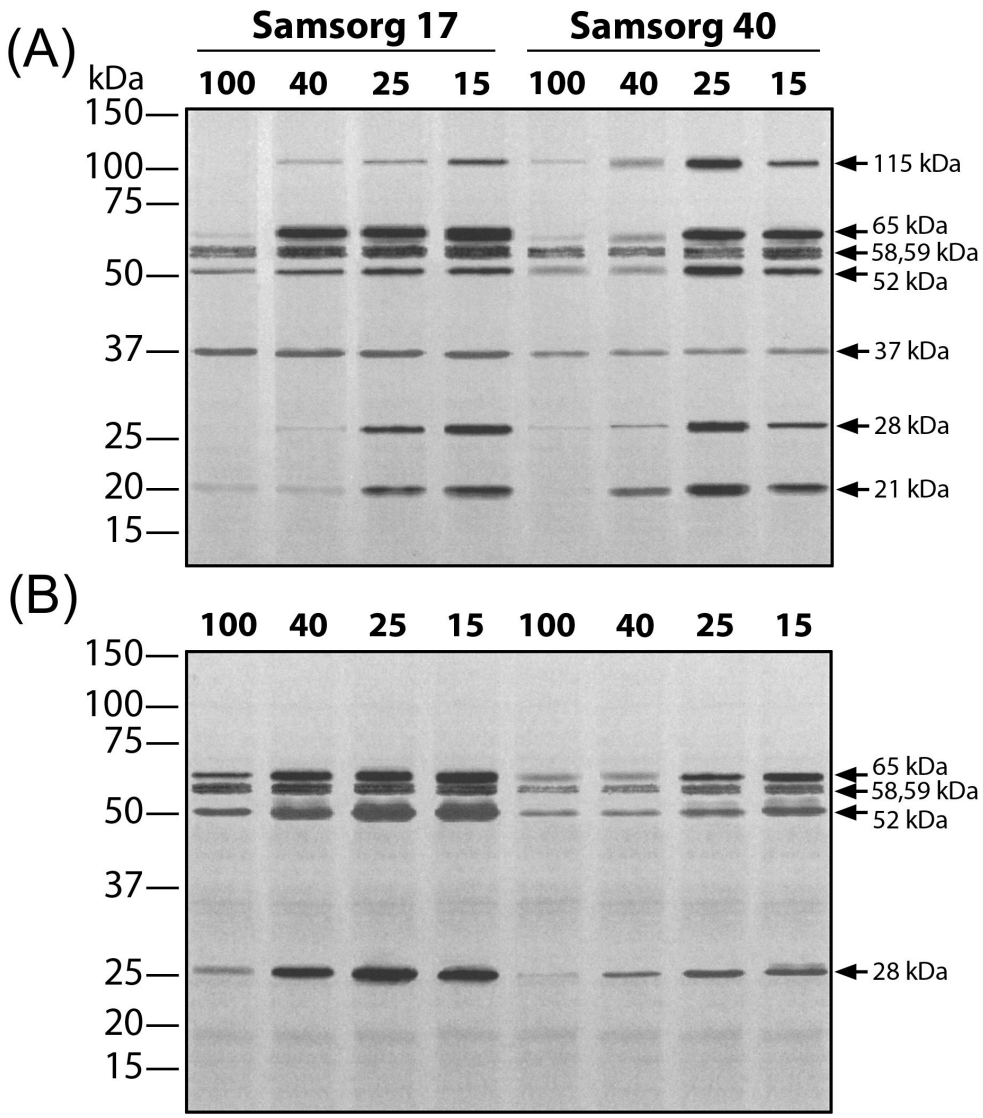


Fig. 4.11 DHN/ DHN-Like protein expressions in *Sorghum bicolor* L. (var. Samsorg 17 and Samsorg 40) under drought treatments-100 (Control), 40, 25 and 15% of pot capacity. Equal amounts of whole leaf (A) and root proteins (B) (25µg) were electrophoretically separated on Glycine-SDS-PAGE (4-20%), electroblotted onto nitrocellulose membrane and probed with set of antibodies raised against the K-segment of DHN. Blots are representative of at least three independent experiments.

4.5 Discussion

To increase the drought tolerance of crop species, we need to understand better the traits possessed by drought tolerant plants so that these can be transferred into new varieties. In this study, two varieties of Sorghum – Samsorg 17, a highly drought tolerant cultivar and Samsorg 40, a less drought tolerant one – were studied. Results obtained indicate that Samsorg 17 induced higher levels of heat shock proteins (HSPs) and dehydrins (DHNs) but differential accumulation of metabolites compared with Samsorg 40.

FTIR gives the infra-red spectrum of absorption or raman scattering of a material (Griffiths and de Haseth 2007). Together with PC-DF loadings, it is possible to determine what types of molecules are being affected by stress in plants. PC-DF performed showed clear discrimination or variation between the sorghum varieties. The sorghum samples were clearly different under well-watered and moderate drought conditions but converged at extreme drought. PC-DF loadings showed that sugars, amines and lipids are affected by drought in the two sorghum cultivars (Fig. 4.2) corroborating the changes identified on the raw spectra (Fig. 4.1)

It has been shown that sorghum accumulates sucrose (Hoffmann-Thoma et al. 1996, Tarpley and Vietor 2007). To determine if this contributed to the total sugar pool (Ogbaga et al. 2014), an enzyme linked assay was performed. The data indicate that Samsorg 17 is a better accumulator of sucrose. Both sorghum varieties contain high concentrations of glucose in response to drought. The data could not explain our earlier finding where sugars were found constitutively in Samsorg 17 but increased in Samsorg 40 (Ogbaga et al. 2014).

To identify sugars constitutively stored by Samsorg 17, mass spectroscopic analysis was conducted. GC-MS data revealed overall increases in the sugars- fructose, lactose, cellobiose and sedoheptulose in Samsorg 17 (Fig. 4.3, 4.4). There were also apparent higher levels of the monosaccharides– galactose, 1, 6-anhydroglucose, sorbose, arabinose, and a disaccharide putatively identified as trehalose – in Samsorg 17 (Fig. 4.4). This is consistent with sugar accumulation playing a crucial role in maintaining leaf function and photosynthesis during drought, as reported earlier (Garg et al. 2002, Massacci et al. 1996, Merewitz et al. 2011). However, the data contrast with our previous finding that Samsorg 17 stores high constitutive amounts of soluble sugars. In contrast, in Samsorg 40, excluding fructose and arabinose, there was little evidence for sugar accumulation. High level of fructose in both varieties, especially in Samsorg 17, suggests that fructose may contribute more to the total pool of soluble sugars than other sugars identified (See Appendix II). Overall, higher accumulation of soluble sugars in Samsorg 17 indicates that these play a role in lowering shoot and root water potential for longer water uptake. Differential amounts of sugars detected with the GC-MS contrasted with data obtained with colorimetric soluble sugars assay (Ogbaga et al. 2014). This questions the reliability of GC-MS in sugar estimation, especially considering the volatility of sugars. Other types of mass spectrometric approaches that can be used for sugar analysis include: Direct Injection Electrospray ionization, Liquid Chromatography Electrospray ionization and Liquid Chromatography using atmospheric pressure chemical ionization (Kumaguai 2001). Colorimetric results suggest that such assays might be better for sugar analysis. However, the trouble with the colorimetric assay used initially is the tendency to give cumulative estimation of both reducing and non-reducing sugars as well as sugar alcohols and structural polysaccharides (DuBois et al. 1956, Moreira Lobo et al. 2011). This makes it difficult to distinguish between actual soluble sugar contributions. Sugar alcohols

increased significantly in Samsorg 17 suggesting that these may have a role as osmoprotectants and may contribute to the stabilization of protein structure against degradation (Fig.4.5) (dos Reis et al. 2012, Moing 2000, Yobi et al. 2012).

The role of lipids in drought tolerance has been explained as being to maintain cellular integrity (Gigon et al. 2004). Higher fatty acids and the derivative isotridecanol, a fatty alcohol in Samsorg 17 support the view that lipids enhance plants survival during drought (Fig.4.7) (Zhang et al. 2005).

In Samsorg 17, there was up-regulation of the majority of the organic acids identified, unlike in Samsorg 40 (Fig. 4.8). One of such compounds up-regulated is ascorbic acid which has antioxidant properties and is thought to offer some degree of tolerance to plants. Ascorbic acid may protect membrane lipids from light-induced damage (Farooq et al. 2013, Shalata and Neumann 2001, Wang et al. 2010). The roles of the other organic acids detected are unclear. Organic acids differentially regulated between Samsorg 17 and Samsorg 40 plants are pyruvic, pyroglutamic, tartaric, itaconic, gluconic, propanoic and 2-keto glutaric acids. Pyruvic acid is a tricarboxylic acid (TCA) cycle intermediate, while pyroglutamic acid is an amino acid derivative (Roessner et al. 2000, Sanchez et al. 2008). Excluding the first three organic acids mentioned, apparent higher levels of organic acids in the more tolerant Samsorg 17 may suggest that they are protective.

Samsorg 40 plants responded to drought by accumulating amino acids, suggesting the utilisation of a different mechanism in protecting themselves from drought (Fig. 4.6). This supports our earlier conclusion that specific reengineering, involving the reallocation of nitrogen to amino acids, occurs in Samsorg 40 plants. Surprisingly Samsorg 17 accumulated more tyrosine. It is unclear why this was the case in Samsorg 17.

Metabolic alterations were accompanied by clear changes in the relative abundance of proteins present in the two sorghum lines (Ogbaga et al. 2014). Amongst these, changes were noted in the various HSPs and DHNs. These proteins have previously been characterised as being expressed in grasses in response to various stress conditions including light, temperature, salinity, oxidative stress, heat, cold and drought (Jagtap 1998, Ngara et al. 2012, Wang et al. 2004, Xu et al. 2011b). However little is known about the roles of these proteins in response to drought. What is known is that the maintenance of chloroplast localised heat shock protein chCpn60 and the cytosolic Heat Shock Protein (HSC 70) offer some level of drought tolerance (Jagtap 1998, Jiang and Huang 2002, Mohammadkhani and Heidari 2008, Ogbaga et al. 2014, Wood and Goldsbrough 1997).

Amongst the HSPs examined, chloroplast localised chHSP70, the constitutively expressed HSC70 and HSP90 all showed either higher apparent expression and/or induction at a lower level of drought in Samsorg 17 than Samsorg 40. chHSP70 functions in preventing aggregation and in assisting refolding of non-native proteins under both normal and stress conditions (Cao et al. 2012). It is involved in numerous protein import and translocation processes in the chloroplast (Wang et al. 2004, Xu et al. 2011b). A dual role of chHSP70 in the molecular protection of photosystem II has also been demonstrated. Evidence was found for an early role of chHSP70 during the reversible phase of PSII photoinactivation, suggesting a possible protective interaction with unfolding domains of PSII proteins. In addition, chHSP70 participates in the repair process by promoting either synthesis or assembly of new reaction center components (Schroda et al. 1999). Greater expression of chHSP70 in Samsorg 17 might be responsible, at least partially, for the maintenance of PSII protein complex and PSII electron transport under stress conditions (Fig.4.10) (Ogbaga et al. 2014). HSC70 is a house-keeping protein that is constitutively expressed in the cytosol

(Sung and Guy 2003). It prevents protein aggregation and hence ensures correct folding of polypeptide chains to proteins (Xu et al. 2011b). This probably contributed to the maintenance of protein structure in Samsorg 17, which improved the tolerance of the plant. HSP90 is a molecular chaperone that only interacts with well-folded proteins. HSP90 is distinct from many other molecular chaperones in that most of its known substrates are signal transduction proteins such as hormone receptors and signaling kinases. HSP90 only functions when large protein complexes involving co-chaperones such as chHSP70 have induced folding in the chloroplast (Wang et al. 2004, Xu et al. 2011b). It has also been demonstrated that HSP90 and HSC70 chaperones modulate specific and distinct signalling events important for fine-tuned regulation of stomatal aperture in response to various environmental conditions, seed germination, and transcriptional regulation of several abscisic acid (ABA) responsive genes. It was suggested that HSC70/HSP90 machinery may be implicated more generally in transcriptional responses to ABA for long-term adaptation to drought tolerance (Clément et al. 2011).The apparent up-regulation or earlier induction of these proteins in leaves and roots of Samsorg 17 is consistent with their role being to enhance the drought tolerance and continued survival of these plants under drought stress. In contrast HSP101 was the only HSP examined where apparent expression was higher in Samsorg 40, with only trace levels of this protein being detectable in leaves of Samsorg 17. HSP101 belongs to a family of heat shock proteins named HSP100s (or Clp proteins). Rather than the regular chaperone function of preventing protein aggregation and misfolding, this family functions in protein disaggregation and degradation (Wang et al. 2004, Xu et al. 2011b). The greater induction of this protein in Samsorg 40 is consistent with damage being induced and with damaged proteins having to be degraded. Curiously however, a comparatively higher level of

expression was seen in the roots of Samsorg 17, possibly reflecting an earlier reaction to drought in roots.

The functions of dehydrins (DHNs) are still under investigation, however the relationship between DHNs and drought has been examined (Lopez et al. 2003). It is believed they are induced by ABA during drought (Lopez et al. 2003, Schroeder et al. 2001). They are known to be thermostable and hence are thought, like HSPs, to have chaperone-like properties and roles in membrane structure stabilization (Borovskii et al. 2002, Demirevska et al. 2008). We found a generally higher induction of DHNs in Samsorg 17 than Samsorg 40 (Fig. 4.10). The higher expression levels and earlier induction of these proteins is again consistent with their playing a role in maintaining cell function for longer in response to drought.

4.6 Conclusions

In conclusion, in Samsorg 17, there was a general up-regulation of protective proteins- HSPs, DHNs- sugars, sugar alcohols and higher levels of lipids, organic acids and unknown compounds in contrast to Samsorg 40, the less tolerant sorghum cultivar. The up-regulation and higher levels of these molecules, in particular high amount of fructose in Samsorg 17, suggest that they play protective roles in maintaining leaf function and possible water content during water deficit. The mobilisation of N compounds in Samsorg 40, allows movement of N to young tissues for possible recovery. In the future, it would be useful to identify the unknown molecules that showed apparent higher levels in Samsorg 17 as they might provide information that can be utilised to improve drought tolerance in crops.

4.7 Acknowledgements

We would like to acknowledge Matthew Miller for help with FTIR data analysis.

CHAPTER 5- GENERAL DISCUSSION

Drought can occur to differing degrees, which can lead to a reduction in crop production. Understanding the drought tolerance mechanisms of plants would undoubtedly aid in the improvement of crops. In this thesis, the regulation of photosynthesis in response to drought was investigated in a drought tolerant C_4 plant, *Sorghum bicolor* and initially compared with less tolerant *Zea mays,* with the specific objective of identifying physiological differences between the two closely related species giving rise to differences in drought tolerance. The principal aim of the project was to examine the mechanisms responsible for the maintenance of leaf function in sorghum for a relatively long time during drought. Two sorghum cultivars − Samsorg 17 (more drought tolerant) and Samsorg 40 (less drought tolerant) were studied in detail.

In the first series of measurements presented in Chapter 2 of the thesis, drought was shown to affect photosynthesis substantially, with drought sensitive plants more susceptible. Sorghum, a naturally drought tolerant plant maintained Photosystem II (PSII) electron transport and net CO_2 assimilation for a longer time under drought conditions relative to maize (Blum 1996, Bosch and Alegre 2004, Chaves 1991, Zegada-Lizarazu et al. 2012). Based on the data from Chapter 2, it was shown that the more drought tolerant Samsorg 17 had an efficient mechanism of water uptake from dry soil compared to the less drought tolerant Samsorg 40 and maize. This implies that Samsorg 17 plants had a better organised rooting system and this enabled these plants tolerate drought better. This data adds to the knowledge that not only deep roots enhance water uptake but also rooting efficient in water uptake (Cruz et al. 1992, Pardales et al. 1991, Salih et al. 1999). Growth rate of Samsorg 17 was slower under control conditions compared with Samsorg 40 (Chapter 3, Fig. 1) but with drought attained similar rate seen in Samsorg 40. In Chapter 2, maize plants were shown to have made larger but fewer stomata relative to sorghum. The implications of fewer stomata are not yet clear, but it

could be an adaptive mechanism, hence the manipulations of stomatal traits still remain a target for future research (Lawson and Blatt 2014).

It was also shown that down regulation of PSII occurs at different rates in the two sorghum varieties and maize, probably preventing the formation of reactive oxygen species (ROS) during drought. This down regulation occurred in parallel with loss of net CO_2 assimilation to match charge separation. The occurrence of maximum charge separation and less CO_2 fixation would have meant a more reduced chloroplast. Without any form of regulation, this would have led to increases in reactive oxygen species (ROS) production. Feedback regulation of electron transport can decrease reducing power by limiting reaction centre turnover. The two sorghum varieties avoided the production of excess reducing power by matching charge separation with CO_2 fixation. Although ROS has not been directly measured, these data raised the question as to how long it takes for ROS with damaging capacity to be produced during drought in these plants. Work by Asada (Asada 1999a, Asada 2000, Asada 2006) investigated the roles of antioxidants and the Mehler reaction during stress conditions. Although there are controversies, it is thought on the one hand that the latter is not protective (Driever and Baker 2011). On the other hand, within the thylakoid, the Mehler reaction is also believed to act as an electron sink allowing longer linear electron flow up to a limited capacity (Adams et al. 2013, Asada 2006). This is a gap that should be studied in detail in the future. Enzymes such as glutathione and ascorbate that regulate ROS production are believed to remain unaffected in their activities except during leaf senescence (Noctor et al. 2014).

Various literature has examined PSII activity in response to drought in plants but there is limited information regarding the regulation of the entire photosynthetic electron transport (Carmo-Silva et al. 2012, Chaves et al. 2009).

The regulation of photosynthesis via non-photochemical quenching generation (NPQ) was studied (see Chapters 2 and 3). NPQ generation has been explained previously as a protective strategy adopted by plants under different conditions (Hertle et al. 2013a, Joliot and Johnson 2011, Munekage et al. 2002, Ruban and Murchie 2012). NPQ generation requires cyclic electron transport (CET) (Johnson 2011, Munekage et al. 2004). It is widely argued that CET occurs in C_4 plants, in particular in the bundle sheath cells, however it is difficult to measure CET in such plants due to different photosystem ratios in different cell types (Edwards et al. 2001, Ivanov et al. 2001a). Although there were clear differences between the two sorghum lines and maize, in none of the plants was there a significant increase in NPQ during progressive drought. This was interpreted as being due to the saturation of the thylakoid at the high light measured (Chapter 2). This result raised the question as to what stage or level of drought NPQ increases and by extension CET under high light. Data from Chapter 3 revealed that when plants were left longer at differing degrees of drought for up to 5 days, NPQ generation became apparent in the more drought tolerant Samsorg 17. Delayed increase in NPQ in Samsorg 17 plants indicates these plants utilise other mechanisms of adjustments to prevent early NPQ generation and increases in CET.

Regeneration efficiency of PEP (VPr) when estimated in the sorghum varieties and maize revealed that the more tolerant Samsorg 17 was the last to lose its Vpr when severe drought commenced by Day 6. This data suggests that CO_2 uptake by PEP occurs the longest in Samsorg 17. This was also reflected in the maximum carboxylation efficiency of Rubisco (Vcmax) of Samsorg 17 which was maintained the longest relative to Samsorg 40 and maize. This measurement was taken in high light and under such condition; it is difficult to specifically probe the bundle sheath where the actual CO_2 is produced after reactions with PEP and other mesophyll enzymes. Nevertheless, questions that should be asked are- how

does the bundle sheath differ in these plants? What are the roles of bundle sheath CO_2 leakiness in drought tolerance? Different responses may reflect slower development of drought or acclimation to drought. These questions have not been answered for sorghum and should be a target for future research. However, leakiness cannot be measured directly but can be inferred from a combination of gas exchange, PSII/PSI photochemistry and carbon isotope discrimination (Δ13 C). Δ13 C is based on comparing the theoretical models of Δ13 C with measured Δ13 C (Kromdijk et al. 2014). Leakiness has been inferred to increase at low light in maize and has been suggested to reduce carbon gain and crop production (Bellasio and Griffiths 2014, Kromdijk et al. 2008, Kromdijk et al. 2014, Yin et al. 2011).

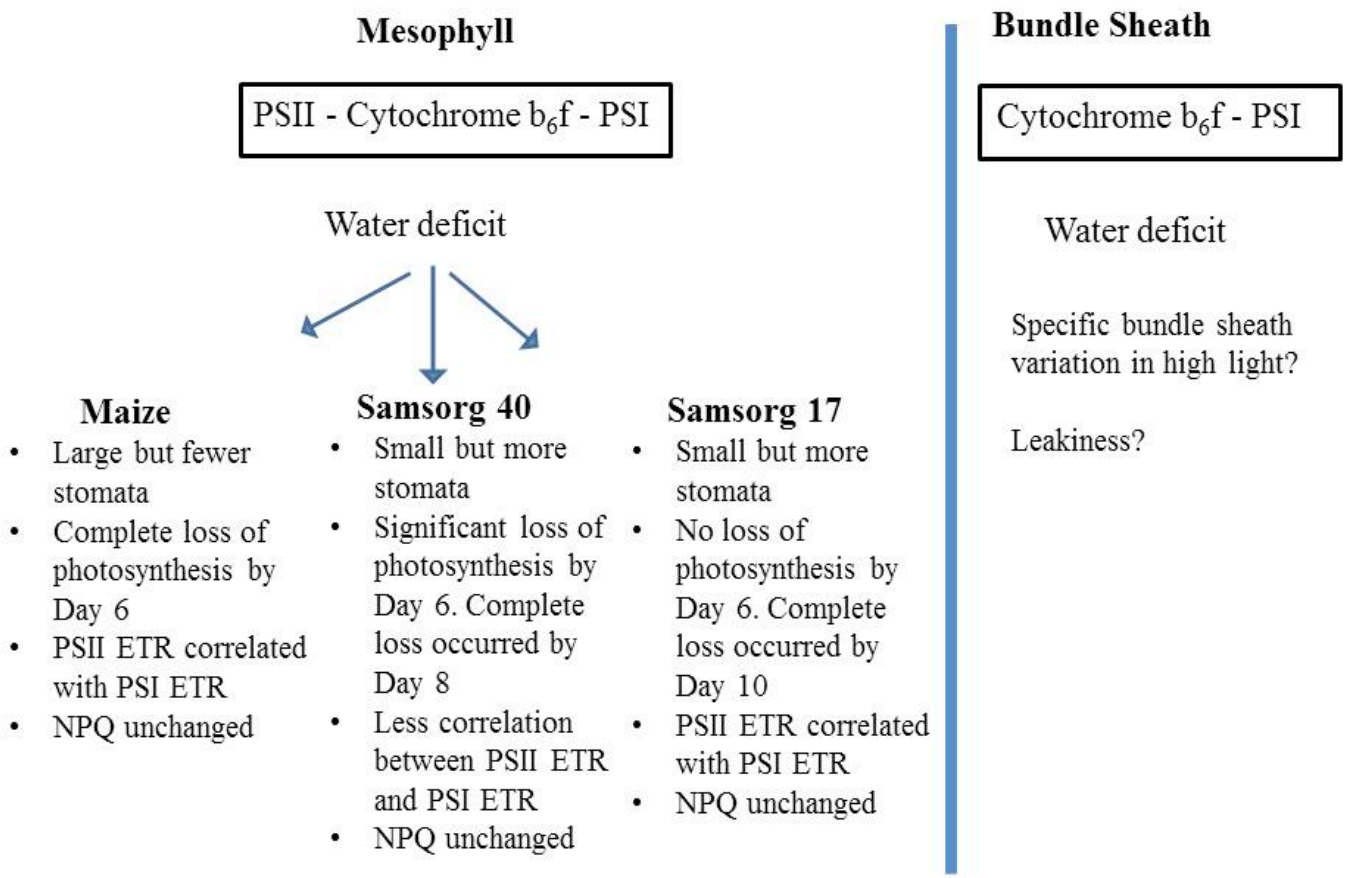


Fig. 5.1 Overview of the responses of the sorghum cultivars and maize to progressive droughting. The mesophyll has both photosystem II (PSII) and photosystem I (PSI) while the bundle sheath has mostly PSI.

Novel drought tolerance mechanisms were identified in this work. The mechanisms shown in Chapter 3 include maintenance of the Rubisco large subunit (RbcL) and high constitutive soluble sugar accumulation. Maintenance of RbcL in the more drought tolerant Samsorg 17 can be an indication of a more efficient CO_2 fixation during drought relative to Samsorg 40. High constitutive soluble sugars seen in Samsorg 17 offered protection during drought by lowering leaf, shoot and root water potential enabling better water uptake and longer leaf function in the plants. It was also shown in Chapter 3 that a specific reengineering of the photosynthetic apparatus occurred in the less tolerant Samsorg 40. Plants of Samsorg 40 lost proteins with the nitrogen released used for amino acid production and maintenance of ATP. The absence of such specific reengineering in Samsorg 17 means that the plant probably had a more active ATP resulting from a higher change in thylakoid pH gradient, which led to higher NPQ generation.

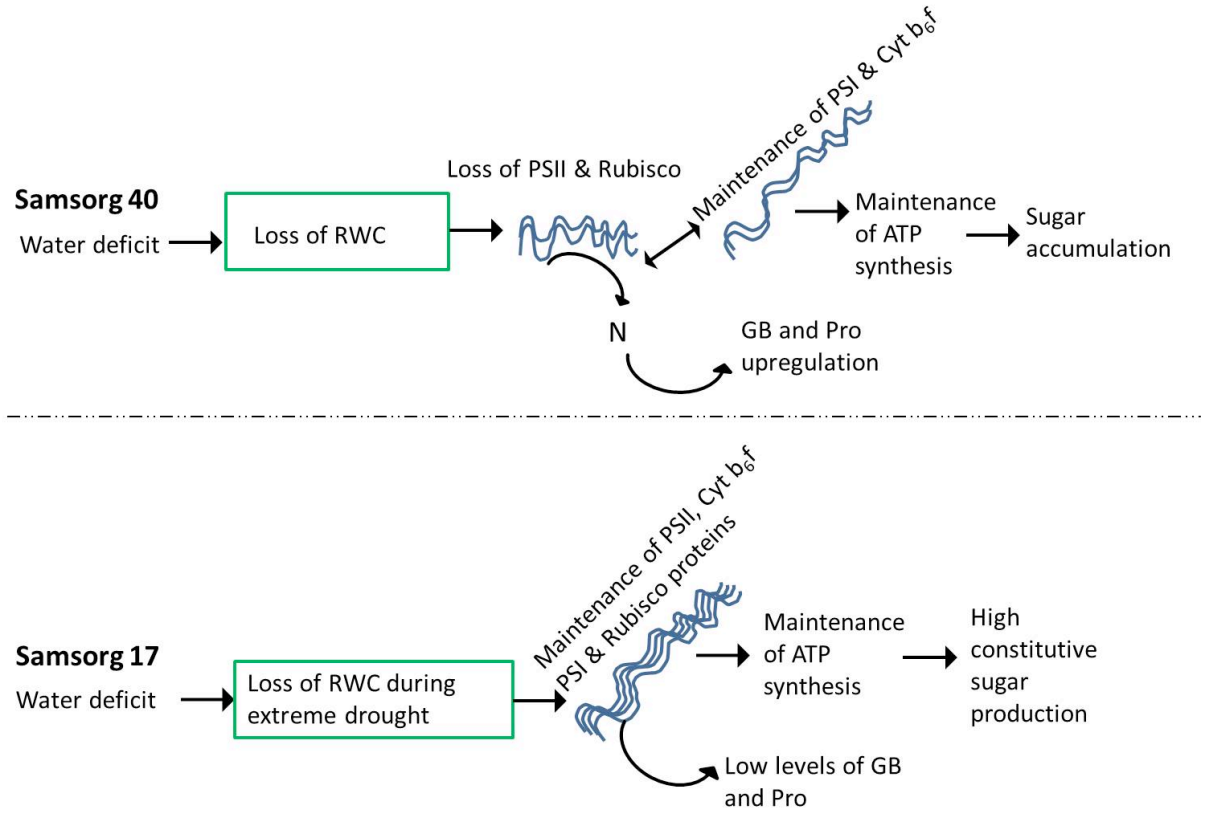


Fig. 5.2 Overview of the novel drought tolerance mechanisms in Samsorg 17 and Samsorg 40. Maintenance of photosynthetic proteins and high constitutive sugars were seen in Samsorg 17 in contrast to Samsorg 40 where loss of proteins and sugar accumulation occurred. RWC, relative water content, GB, glycine betaine, Pro, proline, N, nitrogen.

In Chapter 4, biochemical analyses of the tissues of Samsorg 17 and Samsorg 40 in terms of metabolism and selected proteins revealed that greater accumulation of sugars, sugar alcohols, lipids, organic acids, heat shock proteins and dehydrins are vital for drought tolerance. The tolerant Samsorg 17 accumulated these metabolites with greater protein induction or expression while the less tolerant Samsorg 40 relied on amino acid accumulation. These are

two different approaches of tolerating drought with the data indicating that that of Samsorg 17 is a better approach. The data from this chapter represent a situation where Samsorg 17 plants receive early signals and become equipped as quickly as possible to deal with drought while Samsorg 40 plants do not receive such signals and have to respond to the condition. Samsorg 17 may have a memory of activating and possibly remembering strategies which can enhance survival under drought (Niu et al. 2014). The metabolic data show that sorghum plants actually store nearly all the basic classes of nutrients or compounds contrary to the popular opinion in Africa that green plants are only rich in vitamins.

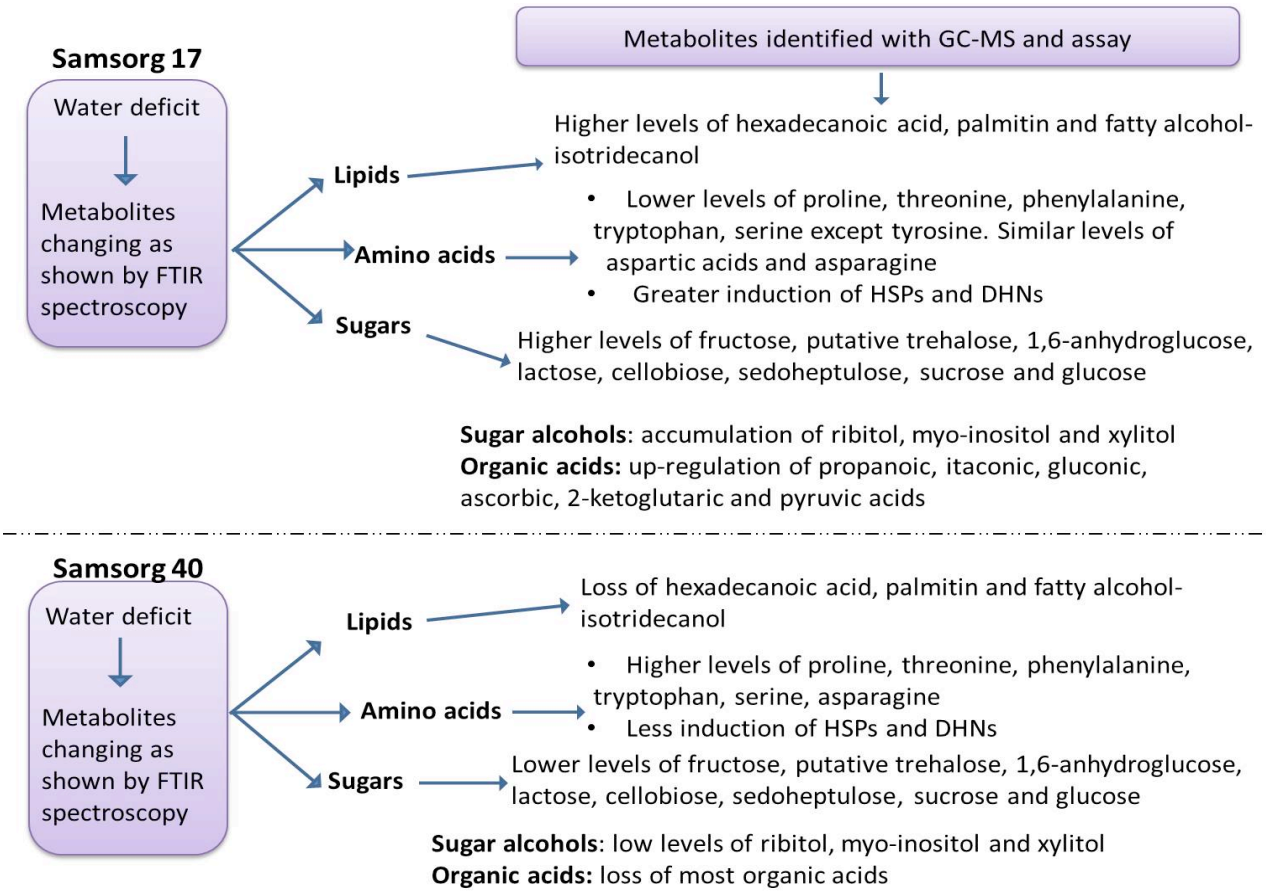


Fig. 5.3 Overview of the metabolites that change in response to drought in Samsorg 17 and Samsorg 40. The more drought tolerant Samsorg 17 makes higher levels of sugars, sugar alcohols, lipids, fatty alcohol and organic acids while the less drought tolerant Samsorg 40 rather relies on making amino acids.

5.1 Summary

In conclusion, it is believed that the data presented in this thesis have provided new insights into the regulation of photosynthesis during drought in sorghum. Particularly it has been shown in the thesis that NPQ generation, high constitutive soluble sugar accumulation, maintenance of specific photosynthetic proteins, greater induction of drought-induced proteins, low levels of compatible solutes and accumulation of various metabolites in sorghum are important for survival during drought. It is now proposed that the experiments should be repeated in a greenhouse and in the field to confirm reproducibility and that during breeding, the specific selection of high soluble sugars for instance and low levels of amino acids may be a good strategy to improve crop production during drought.

5.2 Future Research

Field experiments- Laboratory experiments use artificial conditions to study plants' responses to stresses. Results from such experiments are usually far from what could be obtained when similar experiments are performed in the field. Thus, field experiments in natural plant growth conditions would be useful to test reproducibility of the results reported in the thesis.

More synergistic studies- Repetition of the experiments presented in this thesis in the field may be followed up with further physiology on more sorghum varieties. Varieties with interesting traits can be studied further using a combination of approaches- phenomics (a high throughput phenotypic screening), genomics, transcriptomics, proteomics and metabolomics (Driever and Kromdijk 2013).

Modelling studies- The experimental designs and methods used in this project can be enhanced with modelling studies (Niu et al. 2014). For instance, the link between changes in net CO_2 assimilation and photosystem relations can be enhanced with such studies. These can be used to improve our understanding of the regulatory networks of drought on plants metabolism.

APPENDIX

APPENDIX I

Metabolites detected using GC-MS

ID	Retention Index
Silane, ethoxytrimethyl-	954.18
Asparagine_1807_2TMS	961.27
6-[3-Dimethylamino-1-hydroxypropyl]-2-phenyl benzothiazol	1019.5
2-Undecanethiol, 2-methyl-	1031.8
2-Undecanethiol, 2-methyl-:2	1038.8
Undecane, 4,7-dimethyl-	1044.2
Lactic acid_1058_2TMS	1053
Unknown 1	1079
l-Alanine, N-(trimethylsilyl)-, trimethylsilyl ester	1096.1
4-Hydroxy-3,4,6-trimethylhept-5-enoic acid lactone	1100.1
Pyridine, 3-trimethylsiloxy-	1120.8
Silane, trimethylphenoxy-	1132.6
Unknown 2	1150.8
Unknown 3	1151.9
Propanoic acid, 2-oxo-, methyl ester	1153
Valine_1169_1TMS	1169.6
Pyruvic acid_1175_1TMS	1170.4
Tridecane	1200.9
1-Pentamethyldisilyloxybutane	1222.5
Unknown 4	1231.2
Decane, 2,3,5,8-tetramethyl-	1237.9
Silanamine, 1,1,1-trimethyl-N-(trimethylsilyl)-N-[2-[(trimethylsilyl)oxy]ethyl]-	1240.4
Trimethylsilyl ether of glycerol	1258.9
Trimethylsilyl ether of glycerol:2	1259.9
Unknown 5	1260.8
Eicosane	1265
Isoleucine_1285_1TMS	1279.7
L-Leucine, N-(trimethylsilyl)-, trimethylsilyl ester	1291.8
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	1312.7
Unknown 6	1316.9
1-Butene-1,4-diol, diacetate	1317.7
Proline_1327_1TMS	1319
Glycine d5_1343_3TMS	1323.6
Unknown 7	1324.8
Unknown 8	1325.4
Isotridecanol-	1333.9
Serine_1361_2TMS	1346.6
Silanol, trimethyl-, phosphate (3:1)	1350.9

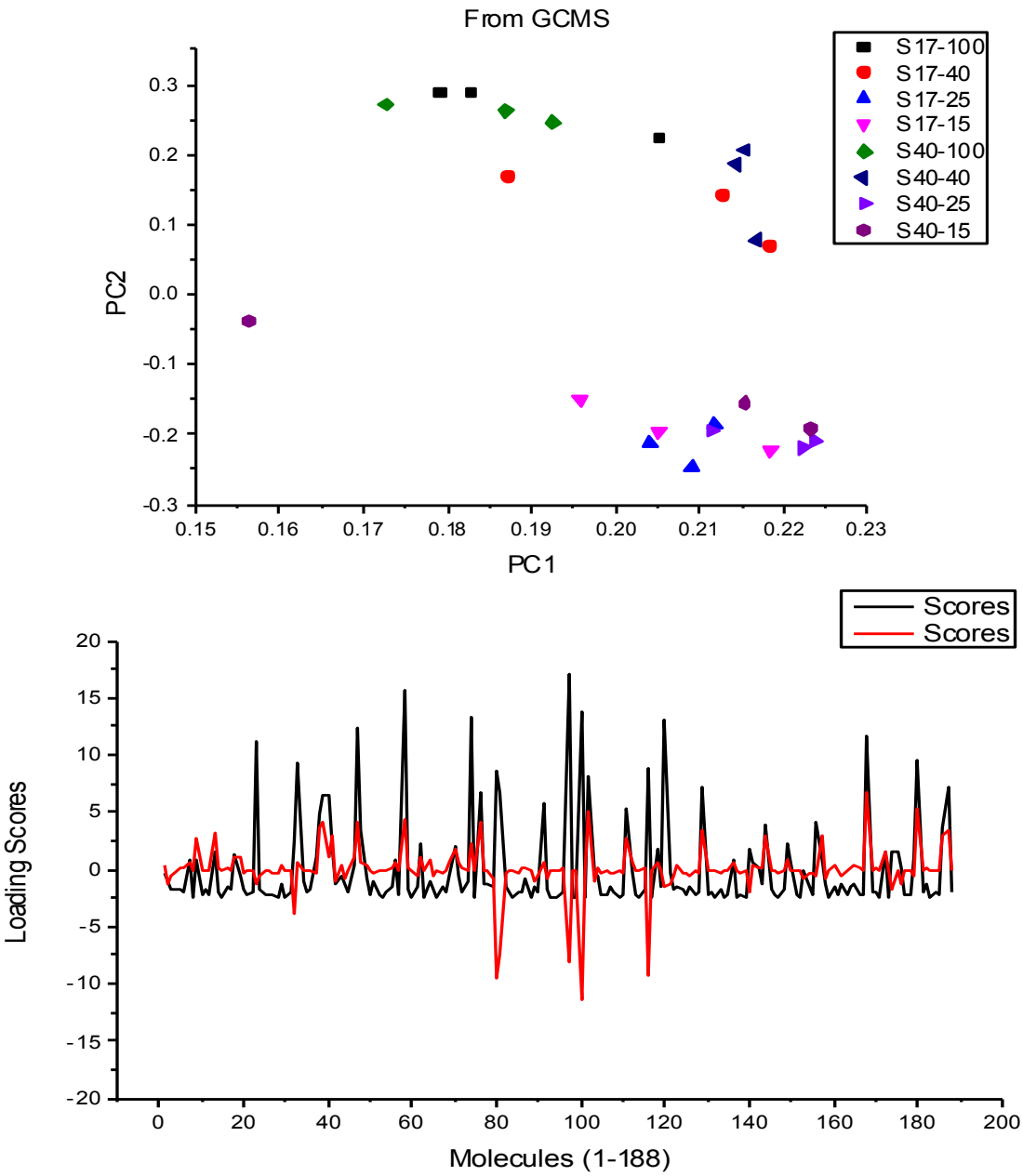
Phosphate_1372_4TMS	1351.5
Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	1359.1
Unknown 9	1360.5
1-Hexanamine,N-(2,2-dimethylhexyl)-2,2-dimethyl-	1361.3
Threonine_1384_2TMS	1367.7
Serine_1408_3TMS	1377.8
Threonine_1411_3TMS	1390.2
1-(2-Ethyl-[1,3]dithian-2-yl)-3-methyl-butan-1-ol	1407.9
Unknown 10	1409.1
Benzene, 1,1'-(1,1,10,10-tetramethyl-1,10-decanediyl)bis[3,4-dimethyl-	1410.1
Unknown 11	1411.9
1-Methyl-2-pyrrolidineethanol	1430.5
Itaconic acid_1461_2TMS	1447.5
Decane, 2,3,5,8-tetramethyl-:2	1449.8
Unknown 12	1464.9
Propanedioic acid, bis(trimethylsilyl) ester	1466.9
2(3H)-Furanone, dihydro-3,4-bis[(trimethylsilyl)oxy]-, trans-	1484.7
Heptadecane	1499.2
Isotridecanol-:2	1510.2
Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	1522.5
Aspartic acid_1553_2TMS	1535.5
Unknown 13	1538.5
Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	1539.5
2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.	1540.5
Unknown 14	1546.6
L-Aspartic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester	1555.9
Benzaldehyde, 4-[(trimethylsilyl)oxy]-	1572.7
Phenol, 4-[(5,6,7,8-tetrahydro-1,3-dioxolo[4,5-g]isoquinolin-5-yl)methyl]-, (R)-	1591.3
Ribitol_1660_5TMS	1623.4
Butanal, 2,3,4-tris[(trimethylsilyl)oxy]-, O-methyloxime, [R-(R*,R*)]-	1648.4
Unknown 15	1663.5
Glutamine, tris(trimethylsilyl)-	1664
1,3-Dioxolane	1664.4
Unknown 16	1666.3
2-Ethyl-acridone	1673
Unknown 17	1673.9
Unknown 18	1696.9
Pyroglutamic acid_1731_3TMS	1700.9
Unknown 19	1702.5
Unknown 20	1703.1
Fructose_1763_5TMS	1709.4
Fructose_1773_5TMS	1712.2
Fructose_1773_5TMS:2	1724.7
2-ketoglutaric acid_1739_2TMS	1727.7
Pipecolinic acid_1441_2TMS	1728.1

Eicosanoic acid, 2-ethyl-2-methyl-, methyl ester	1733.5
Phenylalanine_1768_2TMS	1738.1
Unknown 22	1744.4
Benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	1745.5
Unknown 23	1753.3
L-Proline, 1-(trimethylsilyl)-, trimethylsilyl ester	1758.2
Unknown 25	1761.4
Asparagine_1784_3TMS	1762.8
O,O,O'-Tris-trimethylsilylmalonate	1769.4
Galactofuranoside, methyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-, à-D-	1771.6
Unknown 27	1777.8
Unknown 28	1779.4
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	1784.3
Fructose_1831_5TMS	1789.1
Unknown 32	1794
Sorbose_1849_5TMS	1798.1
Fructose_1831_5TMS:2	1806
Unknown 34	1830.6
1-Propene-1,2,3-tricarboxylic acid, tris(trimethylsilyl) ester, (Z)-	1831.9
Arabinose_1669_4TMS	1836.2
1-Propene-1,2,3-tricarboxylic acid, tris(trimethylsilyl) ester, (Z)-:2	1838.1
Unknown 36	1839.8
Unknown 37	1840.9
1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester	1842.1
Unknown 38	1846.8
Unknown 39	1851
p-Trimethylsilyloxyphenyl-(trimethylsilyloxy)trimethylsilylacrylate	1862.7
Diethyl aminomalonate, trimethylsilyl deriv.	1870.4
Unknown 40	1871.2
L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester	1872.6
Gluconic acid_1942_6TMS	1877.2
Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester:2	1889.7
Glucopyranose, pentakis-O-trimethylsilyl-	1892.9
Unknown 42	1895.3
Heneicosane	1899.5
Pipecolinic acid_1441_2TMS:2	1923.7
myo-inositol_2014_5TMS	1950.6
Fructose_1763_5TMS:2	1956.9
Isoascorbic acid_2032_4TMS	1976.5
Galactose_1872_5TMS	1993.3
Tyrosine_2041_3TMS	2005.5
3-Hydroxymandelic acid, ethyl ester, di-TMS	2015.3
Fructose_1763_5TMS:3	2022.7
Unknown 43	2054
Tyramine_2093_2TMS	2068.3

Hexadecanoic acid_2101_1TMS	2091.4
à-L-Mannopyranose, 6-deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)-	2097.7
Cinnamic acid, p-(trimethylsiloxy)-, trimethylsilyl ester	2102.5
Unknown 44	2118.7
Adenine_2148_2TMS	2123.5
Unknown 45	2137.6
Silane, [(1-methoxy-1,3-propanediyl)bis(oxy)]bis[trimethyl-	2150
Ascorbic acid_2015_4TMS	2176.5
Ribitol_1662_5TMS	2180
Arabino-Hexos-2-ulose, 3,4,5,6-tetrakis-O-(trimethylsilyl)-, bis(dimethyl acetal)	2182.1
Erythritol_1477_4TMS	2183.5
Fructose_1763_5TMS:4	2187.2
Octadecane, 2-methyl-	2198.7
Trimethylsilyl 3,4-bis(trimethylsiloxy)cinnamate	2231.3
Unknown 46	2276.7
Octadecanoic acid_2292_1TMS	2277.4
1,6-anhydroglucose_1803_3TMS	2304.6
D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	2365.1
Tryptophan, bis(trimethylsilyl)-	2378.3
Fructose_1763_5TMS:5	2401.3
à-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-á-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	2417.2
à-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-á-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-:2	2435.5
Xylitol_1663_5TMS	2469.2
N,N',o-Tris-(trimethylsilyl)tryptophane	2487.2
Lactose_2567_8TMS	2490
Xylitol_1663_5TMS:2	2494.3
Silanamine, 1,1,1-trimethyl-N-(trimethylsilyl)-N-[2-[(trimethylsilyl)oxy]ethyl]-:2	2536.6
Palmitin_2606_2TMS	2546.3
Unknown 48	2547.6
Cellobiose_2579_?TMS	2575.1
Cellobiose_2579_?TMS:2	2636.5
Inosine_2818_4TMS	2644.8
Cellobiose_2674_8TMS	2652
D-Xylose, tetrakis(trimethylsilyl)-	2663
2-Monopalmitin trimethylsilyl ether	2666.3
Unknown 50	2680.8
Cellobiose_2674_8TMS:2	2682.6
1,6-anhydroglucose_1803_3TMS:2	2686.5
Unknown 51	2690.2
Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	2697.2
Trehalose_2614_8TMS	2698.6
(Methoxymethyl)trimethylsilane	2701.3
Unknown 53	2709.4
Tartaric acid_1700_4TMS	2723.2

1,1,3,3,5,5-Hexamethyl-2-thia-1,3,5-trisilacyclohexane	2730.4
Sucrose_2526_8TMS	2735.3
Sucrose_2526_8TMS:2	2741.5
Fructose_1763_5TMS:6	2771.5
Unknown 55	2782.5
threo-2,5-Hexodiulose, 1,3,4,6-tetrakis-O-(trimethylsilyl)-	2867.2
Sedoheptulose_2252_6TMS	2932.6
Unknown 56	2979.6
Propanoic acid, 2-methyl-2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	3034.5
Unknown 57	3068.7
Propanetriol, 2-methyl-, tris-O-(trimethylsilyl)-	3069.4
Propanedioic acid, bis[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	3100.5
Unknown 58	3182.5
Fructose_1763_5TMS:7	3406.5
Unknown 59	3424.6
á-l-Galactopyranoside, methyl 6-deoxy-2,3,4-tris-O-(trimethylsilyl)-	3491.3

APPENDIX II- Principal Components and loadings of compounds detected with GC-MS



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