Metabolic and proteomic responses of sorghum cell cultures to polyethylene glycol-induced osmotic stress

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DECLARATION

I, Dipuo Mosea, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification in Botany at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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Dipuo Mosea

DEDICATION

To my future daughter Zoe, my brother Mpho, mom Moleqoa and late dad Lekula.

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ABA	Abscisic acid
Ala	Alanine
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ARC	Agricultural Research Council
Arg	Arginine
Asp	Aspartic acid
BSA	Bovine serum albumin
CAT	Catalase
CBB	Coomassie brilliant blue
CHAPS	3- 3-Cholamidopropyl dimethylammonio]-1-propane sulfonate
DHAR	Dehydroascorbate reductase
DTT	Dithiothreitol Cleland's reagent
GBT	Glycine betaine
Gly	Glycine
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HILIC	Hydrophilic interaction chromatography
His	Histidine
iTRAQ	Isobaric tags for relative and absolute quantitation
K	Potassium
kDa	Kilo Dalton
LC/MS	Liquid chromatography-mass spectrometry
LEA	Late embryogenesis abundant

Leu	Leucine
Lys	Lysine
MDHAR	Enzymes monodehydroascorbate reductase
Met	Methionine
MW	Molecular weight
O_2^1	Singlet oxygen
OH^-	Hydroxyl radical
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
Phe	Phenylalanine
Pro	Proline
ROS	Reactive oxygen species
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
Ser	Serine
SOD	Superoxide dismutase
TEMED	N, N,N',N'-Tetramethylethylenediamine
TCA	Trichloroacetic acid
Thr	Threonine
Tre	Trehalose
Tyr	Tyrosine
Val	Valine
v/v	volume to volume
w/v	weight to volume

ABSTRACT

Climate change, population growth, and the emerging water crisis are negatively affecting agricultural productivity and, thus, food security. Climate change results in drought, which is a major osmotic stress. Osmotic stress triggers the overproduction and accumulation of reactive oxygen species, which result in oxidative stress. Plants also respond to drought stress by using a wide range of biochemical and molecular mechanisms. Examples include osmotic adjustment, antioxidant defense systems, the production of phytohormones, and changes in gene and protein expression patterns. Most of the major cereal crops are drought sensitive. However, sorghum (*Sorghum bicolor*) is well-adapted to survive under hot and dry conditions. Sorghum is thus a potentially good model system among cereals in drought stress response studies. The study aimed to evaluate sorghum cell cultures' metabolic and proteomic responses to polyethylene glycol (PEG)-6000- induced osmotic stress. The viability of the white sorghum cell cultures was monitored using the MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay after the cells were treated with 10 and 20% PEG osmotic stress. The levels of 16 metabolites and a sugar was analysed using hydrophilic interaction chromatography (HILIC) liquid chromatography-mass spectrometry (LC-MS) for control and 10 and 20% PEG-treated white sorghum cell suspension cultures. After 72 hours of 10 and 20% PEG-induced osmotic stress treatments, total soluble proteins (TSP) were extracted, and quantified using the Bradford assay and separated using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) to determine the quality of the extracts. An isobaric tag for relative and absolute quantitation (iTRAQ) analysis was conducted in the extracts followed by bioinformatics analysis of the positively identified osmotic stress-responsive proteins. The cell viability assays showed that both 10 and 20% PEG affected the metabolic activities of white sorghum cell cultures differently and, indeed, triggered osmotic stress, and the cell viability of the stressed samples declined relative to the control. In response

to the imposed osmotic stress levels, the metabolic profiles showed a dramatic decline in six amino acids namely leucine (Leu), methionine (Met), phenylalanine (Phe), serine (Ser) threonine (Thr), and valine (Val). A total of 177 and 229 white sorghum cell-cultured total soluble proteins were identified for the 10 and 20% PEG treatment experiments, respectively. Of these identified proteins, 28 and 48 were responsive to the 10 and 20% PEG treatments, respectively. Additionally, the study identified responsive proteins such as germins, peroxidases, and histones as proteins of interest because they were either uniquely responsive to severe stress or commonly responsive to the imposed osmotic stress. The results obtained added to the knowledge that can be used in breeding programmes for the improvement of cereal crops that are susceptible to drought stress.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The need for food and energy is expected to rise at an alarming rate in the next 30 years as the human population increases (Perez and Perez, 2022). The current 8 billion (Pison, 2022) global population is expected to increase by two to four billion by the year 2050 (United Nations, 2018). Furthermore, global water resources are increasingly becoming vulnerable to the pressures of climate change and population growth (Vörösmarty *et al*., 2000). A combination of climate change, population growth, and the emerging water crisis negatively affect agricultural productivity (Schmidhuber and Tubiello, 2008; Luz and Ferreira, 2018), and thus food security.

Among the abiotic stresses, drought has the most negative impact on agricultural crop productivity (Shanker *et al*., 2014). Extreme drought stress reduces plant survival rate, and biomass production, ultimately affecting crop yield (Tester and Bacic, 2005). In extreme cases, drought stress may result in plant death (Li *et al*., 2020). In peanuts (*Arachis hypogaea* L.), drought caused a reduction in seed number by up to 70% compared to well-watered plants (Vorasoot *et al*., 2003). With such devastating effects of water stress on crop yield, food security has become a serious concern.

Under the envisaged conditions of climate change, population growth, and the emerging water crisis, how possible is it to maintain adequate food provision? The world is now investing on developing crops that can still give maximum yield under harsh environmental conditions as a

potential solution. Therefore, our understanding of how plants adapt to abiotic stresses, including drought, has to improve (Boyer, 2010). Furthermore, plant breeding programmes depend on a better understanding of how agriculturally important crops respond to adverse environmental conditions. While most of the major cereal crops such as wheat (*Triticum aestivum*), maize (*Zea mays*), and rice (*Oryza sativa*) are drought-sensitive *(*Lesk *et al*., 2016), sorghum (*Sorghum bicolor*; Figure: 1.1) is well-adapted to hot and dry conditions (Rosenow *et al*., 1983). Sorghum is a good model system among cereals for drought stress response studies (Ngara and Ndimba, 2014).

Figure 1.1: An image showing sorghum (Louw, 2021).

1.2 Drought stress and its effects on plants

Worldwide, crop yields are negatively affected by environmental stresses such as drought, salinity, extreme temperatures, mineral deficiencies, and heavy metal toxicities (Mittler, 2006). In their natural environment, plants are simultaneously subjected to many of these different stresses (Alexieva *et al*., 2003). However, of all the environmental stresses, drought is regarded as the most detrimental to plant growth as it causes a continual reduction in their growth and development, more than any other stress (Lambers *et al*., 2008; Shanker *et al*., 2014).

Drought stress is a result of changes in rainfall patterns, high temperature and the greenhouse effect and they also compromise metabolic processes of plants (Xiong and Zhu, 2002). Agricultural drought is caused by a continuous shortage in rainfall combined with high surface temperatures that cause increased evapotranspiration in plants (Mishra and Cherkauer, 2010). The shortage of soil moisture content adversely affects plants at any growth stage. However, these effects vary depending on the duration and severity of the water stress, the growth stage of a plant, genotype, and species (Desclaux and Roumet, 1996; Reddy *et al*., 2004; Taiz and Zeiger, 2010). Furthermore, how the stress is imposed on a plant, whether it was gradual, abrupt, or the plant encountered multiple stress factors at the same time can also negatively affect the survival of that plant (Mittler, 2006). Overall, drought stress may cause a range of effects in plants as shown in Figure 1.2.

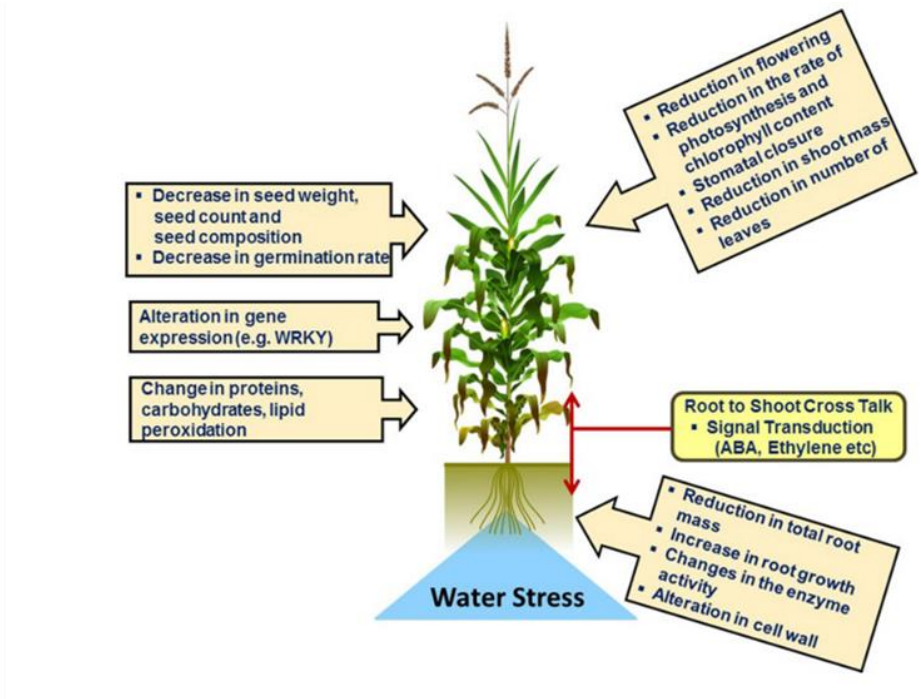

Figure 1.2: General effects of drought stress in plants (Ghatak *et al*., 2017).

1.2.1 Effects of drought on plant growth and yield

Drought stress delays seed germination due to reduced water uptake during imbibition, ultimately affecting plant growth (Okçu *et al*., 2005; Taiz and Zeiger, 2010). Plant growth is characterised by an irreversible increase in volume, size, and weight, including phases of cell division, elongation, and differentiation (Farooq *et al*., 2012). However, during periods of water stress, enzyme activities are impaired, turgor pressure is lost, and energy production decreases. These changes negatively affect plant growth processes (Taiz and Zeiger, 2010; Farooq *et al*., 2012). In sunflower (*Helianthus annulus L*.), drought causes loss of turgor pressure, inhibiting cell division and enlargement (Kiani *et al*., 2007; Hussain *et al*., 2009).

Dehydration also reduces the accumulation of dry matter in plants due to its effects on photosynthesis and the development of optimal leaf area (Asrar and Elhindi, 2011). In addition, limited water supply shortens the growth cycle of most plants as it triggers an early switch from

vegetative to reproductive stages (Desclaux and Roumet, 1996). While a substantial decrease in the economic yield of plants is caused by drought at vegetative stages, water deficit stress encountered during the reproductive stage is more detrimental to plant production (Çakir, 2004).

1.2.2 Effects of drought stress on mineral uptake and assimilation

Mineral nutrients absorbed by plants are used for growth, biomass production and require water for their solubilisation and translocation (Singh and Singh, 2004). However, under drought stress conditions, plants usually experience limited nutrient uptake (Tantawy *et al*., 2013). The nutrient uptake by summer maize (*Zea mays)* roots is greatly affected by drought, resulting in a decrease in the total amount of potassium (K) and phosphorus (P) found in the maize organs at different growth stages (Ge *et al*., 2012). The rate of nutrient diffusion in plants is also affected by limited soil water content (Singh and Singh, 2004).

Furthermore, during drought, root growth and function are affected, decreasing the uptake and transport of nutrients to the shoots (Hosseini *et al*., 2016). In addition, abscisic acid (ABA) signalling processes causes stomatal closure, resulting in reduced transpiration rates and limited nutrient uptake by roots and their transportation (Turner *et al*., 2001). Additionally, high oxidative stress under water deficits causes the leakage of cell membranes that further impairs nutrient uptake (Reddy *et al*., 2004), resulting in reduced mineral nutrition and plant growth (Ramakrishna and Ravishankar, 2011).

1.2.3 Effects of drought on photosynthesis

Plant growth and productivity depend on efficient CO_2 fixation during photosynthesis (Liang *et al*., 2018). However, under conditions of water stress, the photosynthetic capacity of most

crops is compromised due to the reduction in leaf surface area, stomatal closure, and impaired activities of photosynthetic enzymes, amongst others (Yamane *et al*., 2003). While stomatal closure is an important drought phenotypic trait aimed at reducing transpiration water loss, it also affects gaseous exchange during photosynthesis (Flexas and Medrano, 2002). Apart from stomatal closure, non-stomatal limitations of photosynthesis are equally important in reducing carbon fixation and biomass accumulation (Correia *et al*., 2006). For example, photosynthesis is disrupted by reduced activities of photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase, pyruvate phosphate dikinase, and malate dehydrogenase Reddy *et al*., 2004; Farooq *et al*., 2009).

1.3 Plant responses to drought stress

Plant responses towards drought stress can be at the whole plant, tissue, or cellular levels, ultimately resulting in morphological, biochemical, physiological, and molecular changes (Taiz and Zeiger, 2010). All these responsive changes determine the degree of a plant survival.

1.3.1 Morphological and physiological mechanisms against drought

Plants respond to stress through morphological and physiological mechanisms (Zhao *et al*., 2022). For example, plants escape drought by completing their life cycles before the dry season starts (Taiz and Zeiger, 2010). The desert ephemerals germinate, grow and flower during the rainy season and complete their life cycle before the dry season. Unfavourable seasons are also escaped by forming dormant seeds during a period of inadequate water supply (Taiz and Zeiger, 2010).

The effects of water deficit stress on plants can also be reduced by drought avoidance mechanisms (Raineri *et al*., 2015). Drought avoidance is defined as the ability of plants to

maintain high water status during periods of water deficits (Blum, 2005). For example, alfalfa (*Medicago sativa*) has deep roots that grow towards the water table during periods of insufficient water supply to avoid water limitation stress (Taiz and Zeiger, 2010). Biomass reduction is another common trait of plants found in drought-prone areas (Jamieson *et al*., 1995). Both the shoot and root dry weight in Asian red sage (*Salvia miltiorrhiza Bunge*) are reduced after an encounter with drought, but the shoots are more affected than the roots (Liu *et al*., 2011). The root length of sensitive wheat genotypes is mainly affected by drought due to a decrease in newly synthesized cell wall polysaccharides like pectin, hemicellulose, and cellulose (Piro *et al*., 2003). Genotypes with more root dry weight and root length density reaching deep water tables in soil layers are slightly affected by drought at pre-flowering in peanut (*Arachis hypogaea L.*) (Jongrungklang *et al*., 2011). In addition, most xerophytes have reduced leaf size to minimize any water loss through transpiration (Sinclair and Muchow, 2001; Blum, 2005).

1.3.2 Biochemical and molecular responses

Plants also respond to drought stress by using a wide range of biochemical and molecular mechanisms. Examples include osmotic adjustment, antioxidant defense systems, the production of phytohormones, and changes in gene and protein expression patterns (Mittler, 2006).

1.3.2.1 Osmotic adjustment

Drought reduces the water content of plant cells and tissue (Kiani *et al*., 2007; Hussain *et al*., 2009). In turn, the drought-stressed plants accumulate organic and inorganic solutes that help to maintain constant osmotic pressure in cells without decreasing the actual water status. This process is called osmotic adjustment or osmoregulation (Serraj and Sinclair, 2002). The solutes

do not disrupt the cell's enzymes, membranes, and organelles and are referred to as compatible solutes (Cechin *et al*., 2006). Examples of compatible solutes include soluble sugars, sugar alcohols, proline, glycine betaine, and organic acids. Apart from balancing the water status of plant cells, these solutes also protect cells from oxidative damage by reactive oxygen species (ROS) (Farooq *et al*., 2009).

1.3.2.2 Antioxidant defense responses

During drought periods, plants experience water deficiency, promoting oxidative stress that results from the over-accumulation of ROS. Plants respond to limited water supply by synthesising and accumulating the stress-signalling phytohormone ABA (Ackerson and Radin, 1983). ABA accumulation results in stomatal closure to reduce transpiration water loss (Cornic and Massacci, 2006). However, gaseous exchange is inhibited, ultimately resulting in oxidative stress and the reduction of photosynthesis (Baldocchi *et al*., 1985). The disruption of photosynthesis generates several ROS such as singlet oxygen (O_2^1), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Flexas and Medrano, 2002; Lawlor and Cornic, 2002). The over-accumulation of ROS interferes with metabolic processes of plants by causing the oxidative damage of lipids, proteins, nucleic acids and cell macromolecules (Lawlor and Cornic, 2002). To detoxify the reactive and damaging nature of ROS in cells, plants use enzymatic and non-enzymatic antioxidant defense systems (Simova-Stoilova *et al*., 2010). Rice seedlings (*Oryza sativa L.)* were subjected to 17 and 41.2% polyethylene glycol (PEG-6000) induced osmotic stress and high activities of the ascorbate regeneration enzymes monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Sharma and Dubey, 2005) were observed.

1.3.2.3 Molecular responses to drought

Water deficit stress triggers molecular responses such as the up- and down-regulation of genes, transcripts, proteins (Kavar *et al*., 2008) and metabolites (Da Fonseca-Pereira *et al*., 2019a). These response mechanisms involve ABA-dependent and ABA-independent pathways, as shown in Figure 1.3. Under conditions of limited water supply, genes involved in stress response are induced, and they trigger the accumulation of plant hormones such as ABA (Yamaguchi-Shinozaki and Shinozaki, 2006). The accumulated ABA in turn triggers stomatal closure and the expression of other stress-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 1997). A study on potatoes (*Solanum tuberosum. L*) under drought stress showed a high accumulation of the mRNA of a chloroplast drought-induced stress protein (CDSP) 32 (Broin *et al*., 2000). This protein reduces oxidative damage in the chloroplast (Broin *et al*., 2000).

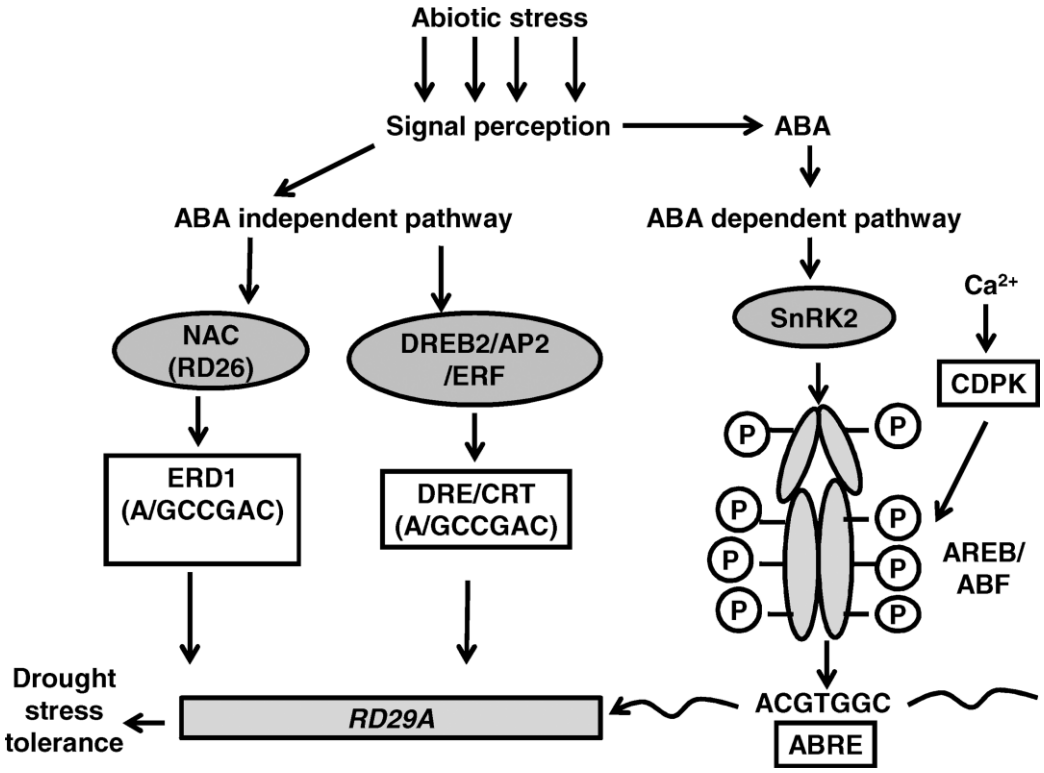

Figure 1.3: ABA-dependent and ABA-independent signalling in plants (Anami *et al*., 2009).

Plant cell culture experimental setups are widely used to study stress responses, and cells can be uniformly treated (Fehér, 2019). The cell culture study has large amounts of highly reproducible material, making suspension-cultured cells suitable for analyzing complex physiological functions at the cellular and molecular levels (Moscatiello *et al*., 2013) and can be used in osmotic stress studies (Ramulifho *et al*., 2019). On the other hand PEG-6000 is frequently used over other osmotica such as mannitol and sorbitol (Bartwal and Arora, 2017; Alqurashi *et al*., 2018) because of its high molecular weight solute that cannot penetrate the apoplastic space of cells (Alqurashi *et al*., 2018). Therefore, the effects of PEG-induced osmotic stress are not permanent, and cell wall damage is reversible (Hohl and Schopfer, 1991). PEG can trigger severe osmotic stress effects on cells (Hohl and Schopfer, 1991), which can be compared to those of air-drying plants (Hohl and Schopfer, 1991; Strauss and Agenbag, 1998). In contrast, the cell viability results of another study concluded that sorbitol as an osmoticum supports cell viability instead of compromising it (Ramulifho, 2017).

1.4 The aim and objectives of the study

The study aimed to evaluate the metabolic and proteomic responses of sorghum cell cultures to PEG-induced osmotic stress.

The objectives were to:

i) Evaluate the metabolic activity of sorghum cell suspension cultures in response to PEG-6000-induced osmotic stress,

ii) Identify PEG-induced osmotic stress-responsive proteins in sorghum cell suspension cultures,

iii) Perform bioinformatics analysis of the positively identified osmotic stress-responsive proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

White sorghum cell suspension cultures were used in this study. The cell cultures were initiated from callus generated in our research group (Ramulifho *et al*., 2019), using seed material obtained from Professor Bongani Ndimba, University of Western Cape/Agricultural Research Council (ARC), South Africa. The drought phenotypic trait of the white sorghum variety is, however, not known (Ngara *et al*., 2008; Ramulifho *et al*., 2019).

2.2 Plant tissue culture methods

2.2.1 Sub-culturing of white sorghum callus

White sorghum callus was sub-cultured on fresh sorghum callus medium [4.4 g/L MSMO medium; 3% (w/v) sucrose; 2.5 mg/L naphthalene acetic acid (NAA); 3 mg/L 2.4-dichlorophenolxyacetic acid 2,4-D, adjusted to pH 5.8 using 1 M NaOH, 0.8% agar] as described previously (Ngara *et al*., 2008). The plant hormones were prepared as follows; 3 mg of 2,4-D was dissolved in 100 µL of ethanol, and 2.5 mg of 1- NAA was dissolved in 100 µL of 1 M NaOH and independently made up to 1 mL with distilled water. For sub-culturing, six pea-sized callus masses were transferred onto the fresh medium in petri-dishes. Each sub-culturing cycle had six biological replicate plates. The plates were parafilm sealed and incubated under dark conditions in a Labcon growth chamber (Lab design Engineering, Maraisburg, South Africa) at 27°C for five weeks. Callus plates were monitored for growth during the incubation period.

2.2.2 Initiation and sub-culturing of white sorghum cell suspension cultures

White sorghum cell suspension culture was initiated using callus derived from three-day-old shoots (Ngara *et al*., 2008; Ramulifho *et al*., 2019). Fifty mililitres of sorghum cell suspension culture medium was poured into each of the four 250 mL Erlenmeyer flasks. The cell suspension culture medium had the same composition as the callus medium except for agar. A five-week-old actively growing friable callus lumps were placed into each of the flasks. The flasks were sealed with foil and incubated in a shaking incubator (Already Enterprise Inc., Taipei, Taiwan) at 27°C, under dark conditions, while shaking at 130 rpm. The flasks were topped up with sorghum cell suspension culture medium to 100 mL on the fourth day. The flasks were further incubated under the same conditions until the 10^{th} to the 12^{th} day.

Once the white sorghum cell suspension cultures were 10–12 days old, the cells were maintained in culture by transferring 30 mL of cell culture into a 250 mL flask containing 70 mL sorghum cell suspension medium. The flasks were sealed with foil and incubated at 27°C, under dark conditions, while shaking at 130 rpm. Cell cultures were sub-cultured into fresh medium every 10-12 days.

2.3 Polyethylene glycol-6000 induced osmotic stress treatment

In this study, osmotic stress was induced using polyethylene glycol (PEG)-6000 (CAT-NO; 81260, Sigma-Aldrich, St Lous, USA). Preliminary stress treatments were conducted using 10 and 20% PEG to establish the optimal concentration and duration of treatment to use. Treatment procedures were repeated on three independently established biological replicate cell suspension culture lines for each treatment. To reduce technical variation in the experiment, 8-day-old mother cultures were subdivided into three, 25 mL subcultures each for

the control, 10 and 20% PEG-induced osmotic stress treatments. The 10 and 20% PEG treatments were exerted using appropriate volumes of a 40% PEG-6000 stock solution. The control cell cultures were spiked with an equivalent volume of distilled water. Three biological replicates were prepared for each treatment group. Both the control and PEG-treated cell suspension cultures were incubated for a maximum of 72 hours under dark conditions at 27°C in a shaking incubator (Already Enterprise Inc.) at 130 rpm. Cell suspension cultures were sampled at 1, 6, 24, 48 and 72 hours for estimation of cell viability.

2.4 Assessment of cell viability using MTT assay

The MTT [3-(4, 5-dimethylthiazolyl2)-2, 5-diphenyltetrazolium bromide] assay (Ngara *et al*., 2008) was used to assess the viability of white sorghum cell suspension cultures following treatment with PEG-induced osmotic stress. During the stress treatment period, 150 µL of sorghum cell cultures of each treatment group, control, 10 and 20% PEG were sampled into 1.5 mL tubes. Three biological replicates, each with three technical replicate samples, were prepared. To each cell aliquot, 50 µL of a 5 mg/mL MTT stock solution was added. The samples were incubated and gently shaken at room temperature for 30 minutes and then allowed to settle on a flat bench-top for five minutes. After that, the samples were centrifuged at 9400 × *g* for five minutes. The resultant supernatant was discarded without disrupting the cell pellet. Then 1 mL of dimethyl sulphoxide (DMSO) was added to all the tubes before incubating for 10 minutes with gentle shaking. After incubation, the samples were allowed to settle on a flat bench-top and centrifuged for five minutes at 9400 × *g*. The supernatants were collected, and absorbance read at 490 nm using DMSO as a blank solution.

2.5 Protein extraction and quantification

2.5.1 Protein extraction from white sorghum cell culture

Eight days old white sorghum cell suspension cultures were treated with 10 and 20% PEG for 72 hours. Total soluble protein (TSP) was extracted from the cell suspension cultures as previously described (Ngara, 2009). Three biological replicates were used for control and the 10 and 20% PEG-treated cells. The ground cells of 0.5 *g* were transferred to 1.5 mL Eppendorf tubes and precipitated in 1 mL of 10% (w/v) trichloroacetic acid (TCA) on ice. The homogenate was centrifuged at 15 000 × *g* for 10 minutes at room temperature. Following centrifugation, the supernatant was discarded, and the pellet was washed three times with 1.5 mL of ice-cold 80% (v/v) acetone by centrifugation at 15 000 × *g* for 10 minutes per wash. The pellets were air-dried at room temperature for 10 minutes and re-suspended in 1 mL of urea extraction buffer [9 M urea, 2 M thioureas, and 4% 3-(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)] for each sample. The TSP was extracted in the urea extraction buffer overnight at room temperature with vigorous vortexing before centrifugation at 15 000 × g for 10 minutes. The TSP supernatant was then carefully collected and stored at -20°C pending protein quantification, gel electrophoresis and isobaric tags for relative and absolute quantification (iTRAQ) analysis.

2.5.2 Protein quantification

The TSP extracts were quantified using the Bradford assay (Bradford, 1976) with slight modification (Ngara, 2009). A stock solution of 5 mg/mL of bovine serum 35 albumin (BSA) was used to prepare the standard protein solutions, in 2 mL plastic cuvettes as shown in Appendix Table A1. The cell culture total soluble protein samples were also prepared in duplicate in plastic cuvettes by mixing 10 µL of each respective protein extract with 10 µL of 0.1 M HCl and 80 µL distilled water. The Protein Assay Dye Reagent Concentrate (BIO-RAD,

Hercules, California, USA) was diluted in a 1:4 ratio. A volume of 900 µL of the diluted Protein Assay Dye Reagent was added to all the BSA standards and sorghum protein samples, mixed and incubated for 5 minutes at room temperature. Absorbance values were read at 595 nm using a Jenway 7300 spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK). The 0 mg/mL BSA standard solution was used as a blank. A standard curve was plotted using BSA standards to estimate concentrations of the unknown protein samples.

2.6 One dimensional (1D) polyacrylamide gel electrophoresis (PAGE)

The quantified protein samples, and the Unstained Protein Ladder (New England Biolabs Inc, Massachusetts, USA), were separated on a 12% (v/v) 1D sodium dodecyl sulfate (SDS) polyacrylamide gel, as previously described (Laemmli, 1970). The gel was cast using 1 mm thick plates using a Mini PROTEAN Tetra Cell gel casting system (Bio-Rad) following the manufacturer's instructions. The mixture of equal volumes of protein samples plus sample buffer [100 mM Tris-HCL pH 6.8, 200 mM (w/v) DTT, 4% (w/v) SDS, 20% glycerol, a pinch of bromophenol blue] was pulse vortexed, centrifuged and denatured on a heat block for 3 minutes at 100 ºC mixer before they were loaded into the gel wells.

Gel electrophoresis was performed in an electrode running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS] in a Mini PROTEAN Tetra Cell tank (Bio-Rad) using a Basic PowerPac (Bio-Rad). Initially, the gels were run for 30 minutes at 100 V and then run at 150 V until the bromophenol blue dye reached the lowest level of the gel plates. After the gels were run, Coomassie Brilliant blue (CBB) R-250 staining solution [0.1% (w/v) 25 CBB R250, 40% (v/v) methanol, and 10% (v/v) acetic acid] was used to stain them overnight. The background of the gels was cleared using a destaining solution [40% (v/v) acetic acid and 10% (v/v)

glycerol]. Using the molecular imager Gel Doc™ XR+ with Image Lab™ Software version 5.2.1 (Bio-Rad), the gels were scanned, and the image was captured.

2. 7 Metabolites analyses

2.7.1 Sample preparation for metabolites

Metabolites of white sorghum cell suspension cultures were analysed following 72 hours of 10 and 20% PEG osmotic treatments. The cell suspension cultures were filtered using four layers of sterilized Miracloth (Merck, Darmstadt, Germany). To prepare for metabolite analysis, a total of 50 mg of cells was weighed directly into 1.5 mL Eppendorf tubes. A volume of 125 uL of 0.25 N HCl was added, and the sample pulse vortexed. The samples were heated at 60°C for 5 minutes on a heating block, followed by centrifugation at 9 400 × *g* for 5 minutes. The supernatant was transferred into new 1.5 mL Eppendorf tubes and stored at -20°C until shipment to Durham University, United Kingdom for metabolites analyses.

2.7.2 Amino acids and proline content analysis

Proline content was analysed using hydrophilic interaction chromatography (HILIC) liquid chromatography-mass spectrometry (LC-MS) on a Waters Acquity UPLC BEH Amide 1.7 µm 1.0x100 mm with Acquity BEH Amide guard column (Prinsen *et al*., 2016; Goche *et al*., 2020). Samples were prepared and 2 µL diluted (1:100) in solvent A (85% acetonitrile (MeCN), 10 mM ammonium formate and 0.15% formic acid (FA)), and column temperature was maintained at 40°C. Standards were also prepared in solvent A from 1 nM-20 µM. Optimal chromatographic separation was done using a gradient with both solvent A and B (85:15 H2O:ACN, 10 mM ammonium formate and 0.15% FA) at 0.2 mL/min flow rate. The initial concentration of solvent B was 0% and it was increased from 10-12 minutes to 100%. After

12.1 minutes the concentration was again decreased to 0%. The total run time was 18 minutes. The column was linked to mass spectrometry with ESI operated in positive ionisation mode and multiple reaction monitoring (MRM) mode transition (116:00>70.10) for proline and amino acids identification.

2.7.3 Glycine betaine content analysis

Glycine betaine content was also analysed using the HILIC LC-MS method. The chromatography separation was done using a Supelco Ascentis Express HILIC column (2.1 x 150 mm), which was maintained at 30°C. The sample volume was 2 µL diluted (1:50) in solvent A (85% MeCN + 10 mM ammonium formate and 0.15% FA). Standards were also prepared in solvent A from 10 nM-1000 nM. This chromatography separation was done using a gradient with solvent B (85:15 H2O: MeCN + 10 mM ammonium formate and 0.15% FA) at a flow rate of 0.4 mL/min. The gradient started at 0% for the first 7 minutes, increased after 7 minutes to 100% and maintained at 100% until 10 minutes elapsed. After 10 minutes, the concentration was then reduced to 0%, and the run time was 15 minutes. Glycine betaine analysis and identification was done using mass spectrometry with ESI operated in positive ionisation mode and MRM mode transition (118.1>58.10 and 118.1>59.2).

2.7.4 Abscisic acid analysis

Abscisic acid content was analysed using HILIC - LC-MS on a Waters Acquity UPLC BEH Amide 1.6 µm 2.1x100 mm with Acquity BEH Amide guard column (Prinsen *et al*., 2016; Goche *et al*., 2020). Samples were prepared and 2 µL diluted (1:100) in solvent A (H20 with 0.1% formic acid (FA)), and column temperature was maintained at 40°C. Optimal chromatographic separation was done using a gradient with both solvent A and B (95:5 H2O:

MeCN, with 0.1% formic acid (FA)) at 0.2 mL/min flow rate. The initial concentration of solvent B was 0% and it was increased from 10-12 minutes to 100%. After 12.0 minutes the concentration was again decreased to 0%. The total run time was 12.0 minutes. Standards were prepared from 0.2-100 nM: 2µl Column oven maintained at 40°C Autosampler at 4°C. The analysis was conducted by a mass spectrometer (AB Sciex) for abscisic acid analysis identification and quantification ESI operated in positive ionization mode. The transitions from MRF were 262.936.1>153.008.

2.8 iTRAQ analysis

2.8.1 Sample preparation for iTRAQ analysis

The white sorghum cell suspension culture TSP was acetone precipitated overnight at -20°C. The samples were centrifuged at 3 000 rpm for 10 minutes. The supernatants were discarded and the pellets washed 3 times with ice cold 80% (v/v) acetone. The sample mixture was spun at a maximum of 15000 × g for ten minutes, and the supernatant was gently discarded. Approximately 50 µL of ice-cold acetone was added to each Eppendorf tube, sealed with parafilm, and sent to Durham University iTRAQ analysis.

2.8.2 Sample labelling and analysis

The iTRAQ analysis of four biological replicates of control versus 10 and 20% PEG osmotic-stressed protein samples was conducted as follows. For each TSP sample, 12.5 µg of protein were used. The protein samples were acetone precipitated overnight at -20°C and centrifuged for 10 minutes at 15 000 × g. The samples were air-dried and re-solubilized using an iTRAQ Reagent-Multiplex Buffer Kit (AB Sciex) according to the manufacturer's instructions. For the cell suspension culture samples, 2.5 µl of the denaturant was added to each pellet and incubated

for 1 hour at 60°C. After that, 47.5 µl of dissolution buffer was added, and the samples vortexed for 20 minutes and centrifuged for 10 minutes at 15 000 × g. The supernatant was collected and mixed with 1 µl of reducing agent. The samples were incubated for 1 hour at 60°C, alkylated with 0.5 µl of cysteine blocking agent, vortexed, and set at room temperature for 10 minutes. All cell culture protein samples were separately digested with trypsin overnight at 37°C. The digested protein samples were vacuum-dried and resuspended in the ultrapure MilliQ water and the pH was adjusted to 7.5 using the dissolution buffer. Samples were all labelled with an 8-plex iTRAQ reagent kit following the manufacturer's instructions. The four control replicates were labelled using the tags with molecular weights with 113, 114, 115, and 116, and the 10 and 20% PEG stressed replicates were labelled 117, 118, 119, and 121, respectively. The 10 and 20% PEG-stressed samples were separately pooled to make one composite sample, which was vacuum dried and resuspended in 3 mL of buffer A (10 mM K_2HPO_4/25% acetonitrile, pH 2.8).

2.8.3 Sample clean-up for iTRAQ

Samples were cleaned-up using HILIC Solid phase extraction (SPE) cartridges (PolyLC Inc.), containing 300 mg of 12 µm polyhydroxyethyl-A, to remove the unincorporated label and buffer salts. The cartridges were equilibrated by sequentially adding 4 x 3 mL releasing solution (5% acetonitrile, 30 mM ammonium formate pH 3.0) and 4 x 3 mL binding solution (85% ACN, 30 mM ammonium formate pH 3.0). The dried iTRAQ-labelled peptide residue was dissolved in 75 µL of 3% acetonitrile (ACN), 0.1% formic acid (FA) followed by 150 µL of 0.3 M ammonium formate, pH 3. The pH of the mixtures were checked and adjusted to 3.0 using trifluoroacetic acid (TFA). The samples were centrifuged at 10000 × g for 10 minutes and then mixed with 1275 mL ACN. The resulting 1.5 mL samples were added to the SPE cartridges and the flow-through retained and passed through a second time. The columns were

then washed twice with 2 mL binding solution. Finally, the peptides were eluted with 2 x 1 mL releasing solution. The eluate was freeze-dried and re-suspended in 3% ACN, 0.1% formic acid for LC-MS/MS.

2.8.4 Liquid chromatography-mass spectrometry analysis

The mass spectrometry data was analyzed following Smith *et al*. (2015) with slight modifications. TSP proteins were identified and relatively quantified by processing the raw wiff data files against the relevant TrEMBL database sequences of *Sorghum bicolor* (downloaded in May 2018) using ProteinPilot™ 5.0.1 version 4895 software, incorporating the Paragon™ Algorithm 5.0.1.0.4874, (AB Sciex). An iTRAQ 8-plex peptide-labelled Paragon method for tryptic peptides with iodoacetamide cys-modification and data acquired on a TripleTOF 6600 spectrometer was used. Label bias correction was activated, the 'Thorough ID' and 'Run False Discovery Rate Analysis' alternatives were selected, and the Detected Protein Threshold setting was set at 0.05 (10%) Unused ProtScore (conf).

Peptide and protein tables were exported from ProteinPilot for further computing evaluation and filtering. The identified proteins with a single peptide were eliminated from the dataset. For quantitative evaluation, the abundance of every protein per sample consistent with the sample becomes acquired as a ratio to the 113-tagged sample. For the four biological replicates, the averages of the ratios were calculated for each protein in control and 10 or 20% PEG-osmotic-stressed samples. The average ratio of control to PEG osmotic-stressed sample average represented the fold-change in protein expression. Values above one signified an upregulation for the 10 and 20% PEG osmotic stress-responsive proteins, and values below

one denoted downregulation. The Student's t-test at 95% confidence level was used to analyse the average controls compared to the PEG-treated samples.

2.8.5 Bioinformatic analysis

The mass spectrometry-identified proteins were annotated using data retrieved from the UniProt database. The database predicted Gene Ontology annotation terms, biological processes, components, and molecular functions. All bioinformatics annotations and the accession number obtained from mass spectrometry data were used to retrieve data on family names, domains, and protein functions.

2. 9 Statistical analysis

Statistical analysis was carried out using a One-way analysis of variance for the cell viability assays and the metabolite analyses. Data was represented ± SE (n=3). Bars with different letters indicate statistical significance at $p \leq 0.05$ using the GraphPad Prism Version 5. The metabolites graph was uncluttered by not showing significant differences across time points. An asterisk was used to indicate a significant difference between the control and the 10 and 20% PEG treatments.

CHAPTER 3

EFFECTS OF PEG-INDUCED OSMOTIC STRESS ON THE GROWTH AND BIOCHEMICAL ACTIVITY OF SORGHUM CELL CULTURES

3.1 Introduction

Plant tissue culture is a combination of procedures that maintain the growth of plant tissues such as calli, organs, protoplasts, and cell suspension under aseptic conditions (Pierik, 1984). In the current study, white sorghum cell suspension cultures were used as experimental units to investigate the effect of polyethylene glycol (PEG)-6000-induced osmotic stress on the growth and biochemistry of plants.

Cell suspension cultures are derived from callus and maintained under suitable growth conditions with shaking in a liquid medium (Chawla, 2009). Currently, cell suspension cultures are widely used as experimental systems in several plant biology studies aimed at investigating a wide range of phenomena (Fehér, 2019). These cell cultures allow a uniform treatment of cells (Fehér, 2019). Researchers are provided with large amounts of highly reproducible material, making suspension-cultured cells suitable for analyzing complex physiological functions at the cellular and molecular levels (Moscatiello *et al*., 2013). Cell suspension cultures also allow immediate and uniform treatment of a population of cells (Moscatiello *et al*., 2013) and can be used in osmotic stress studies (Ramulifho *et al*., 2019).

Osmotic stress can be imposed on cell suspension cultures using different types of osmotica, such as mannitol, PEG, and sorbitol (Hohl and Schopfer, 1991). Of these compounds, PEG-6000 is frequently used (Bartwal and Arora, 2017; Alqurashi *et al*., 2018), because it is a high

molecular weight solute that cannot penetrate the apoplastic space of cells (Alqurashi *et al*., 2018). Therefore, the effects of PEG-induced osmotic stress are not permanent, and the damage caused to the cell walls is reversible (Hohl and Schopfer, 1991). Furthermore, unlike other chemicals, PEG triggers severe osmotic stress effects on cells (Hohl and Schopfer, 1991), which can be compared to those of air-drying plants (Strauss and Agenbag, 1998).

Earlier studies on tomato (*Lycopersicon esculentum*) cell suspension cultures reported increased free amino acid pools due to PEG-induced osmotic stress (Handa *et al*., 1983). These accumulating amino acids possibly function as osmolytes under water-stress conditions (Ghanbarzadeh *et al*., 2021). One of the earliest studies on sorghum callus cultures used PEG-8000 to investigate the difference in growth and free proline content between the control and PEG-treated cultures (Bhaskaran *et al*., 1985). The results showed no direct link between proline concentration and the PEG-induced osmotic stress (Bhaskaran *et al*., 1985).

The objective of this chapter was to evaluate the growth, metabolic activity, and metabolite profiles of white sorghum cell cultures in response to 10 and 20% PEG-induced osmotic stress.

3.2 Results

3.2.1 Maintenance of white sorghum cell suspension cultures

Callus is important for the generation of cell suspension cultures (Ngara *et al*., 2008). White sorghum cell suspension cultures were initiated from callus previously obtained from three-day-old shoots (Ramulifho, 2017; Ramulifho *et al*., 2019). Figure 3.1 shows white sorghum callus and the cell suspension cultures at different growth stages.

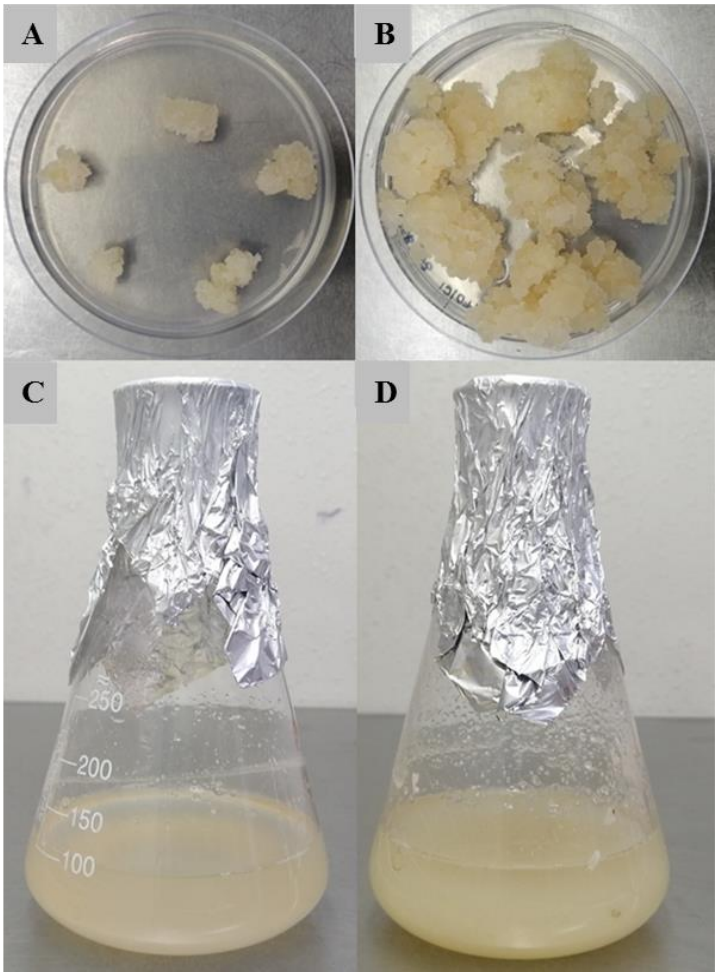

Figure 3.1: The different growth stages of white sorghum callus and cell suspension cultures. (**A)** and (**B)** show callus on day 1 and after three weeks on growth medium, respectively. (**C)** and (**D)** show 6 and 12-day-old cell suspension cultures, respectively.

3.2.2 The viability of PEG-treated white sorghum cell cultures

Preliminary osmotic stress assays were conducted to establish the concentration of PEG-6000 and the duration of treatment to use in the study. In the present study, PEG-6000 is referred to as PEG for brevity. Initially, white sorghum cell suspension cultures were exposed to 20% PEG, which is half the strength used for Arabidopsis (*Arabidopsis thaliana*) cell suspension cultures (Alqurashi *et al*., 2018). The 20% PEG concentration was chosen due to the difficulties experienced with working with a viscous 40% PEG solution.

Following the 20% PEG treatment, the viability of the white sorghum cell cultures was monitored at 0, 24, 48, and 72 hours using the MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. The 0-hour time-point denotes the sampling time immediately after applying the respective osmotic stress treatment. The MTT assay measures the metabolic activity of living cells (Mickisch *et al*., 1990). Absorbance readings are, therefore, proportional to the viability of the cell cultures (Mickisch *et al*., 1990). Figure 3.2A shows a graphical representation of the cell viability status of control and 20% PEG-treated white sorghum cell suspension cultures. The cell viability of the control and 20% PEG-treated sorghum cells was not statistically different at 0 hours. In contrast, at 24, 48, and 72 hours, the 20% PEG-treated cells had a drastic and significant decrease in viability. Based on the sharp decline in the viability of the 20% PEG-treated cells observed within 24 hours of treatment (Figure 3.2A), an additional treatment group using 10% PEG was included in the study.

When white sorghum cell suspension cultures were exposed to 10 and 20% PEG treatments in the same experiment, cell viability was monitored at 1, 6, 24, 48, and 72 hours. The results show that both 10 and 20% PEG significantly reduced the viability of sorghum cell cultures as early as 1 hour following stress treatments (Figure 3.2B). This low viability was observed

throughout the 72 hours of the experiment. However, the lowest cell viability readings were recorded at 6 hours for both PEG treatment groups (Figure 3.2B). Generally, the lowest cell viability was noted after 20% PEG-induced osmotic stress (Figure 3.2B).

A striking observation from the data was the increase in viability of the 10 and 20% PEG-treated cells at 24 hours relative to the control (Figure 3.2B). This showed that while the cells treated with both 10 and 20% PEG had compromised vitality after 6 hours of stress treatment, the cells were not completely dead. Therefore, the 72-hour time point was selected as an appropriate time point for evaluating osmotic stress-induced proteome changes of this sorghum cell culture line.

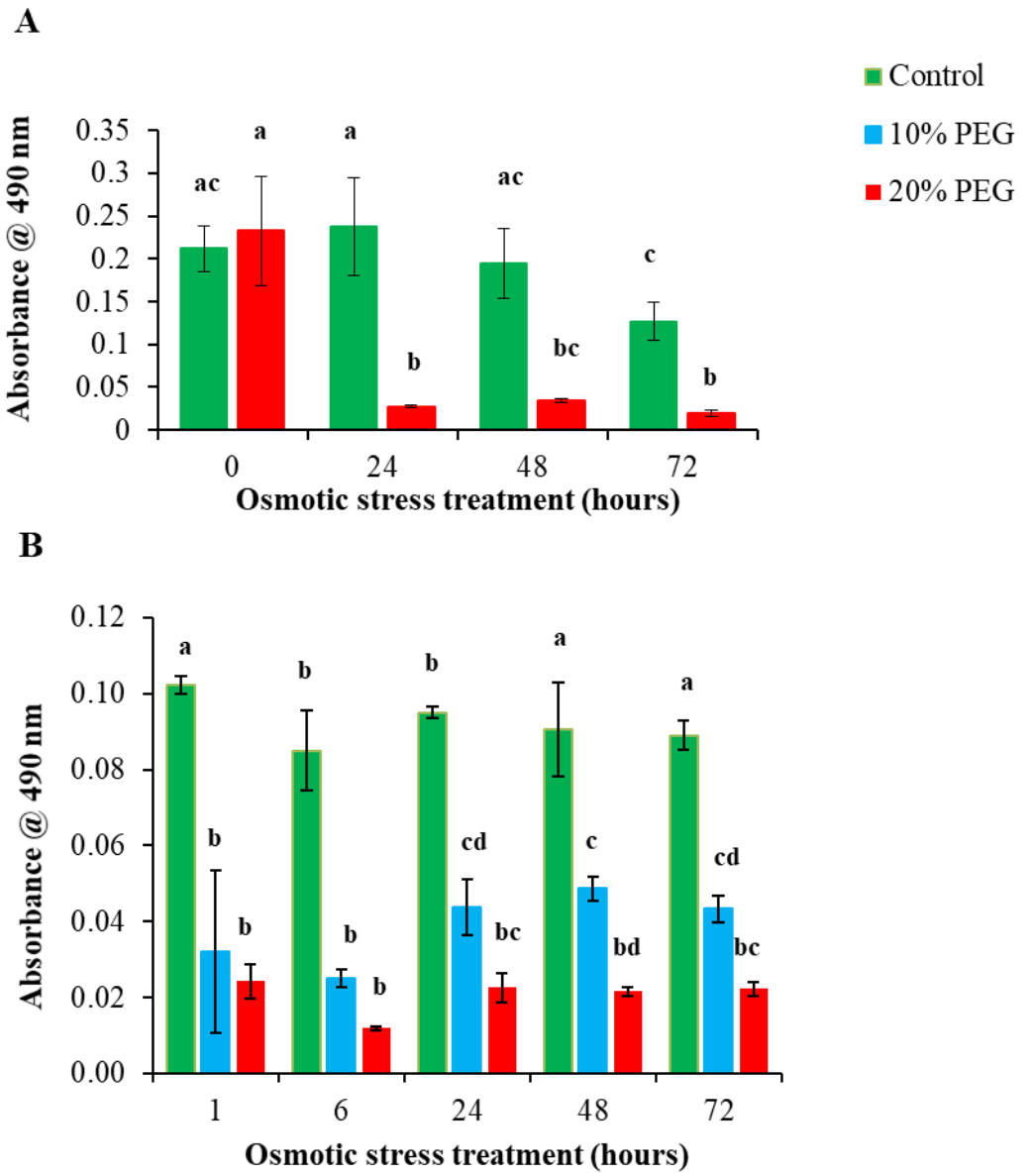

Figure 3.2: Effects of different concentrations of PEG on the viability of white sorghum cell suspension cultures. Sorghum cells were treated with 10 and 20% PEG over 72 hrs, and cell viability was estimated using the MTT assay at different time points. **(A)** MTT assay results of control and 20% PEG-treated cells sampled at 0 hours (immediately after stress induction), 24, 48 and 72 hrs. (**B)** MTT assay results of control, 10 and 20% PEG-treated cells taken at 1, 6, 24, 48 and 72 hours after stress treatment. Data represented ± SE (*n=3*) Bars with differing letters indicate statistical significance at $p \leq 0.05$ according to one-way ANOVA.

3.2.3 Molecular evaluation of the white sorghum cell suspension cultures

3.2.3.1 Metabolite profiling after PEG stress treatment

The levels of 16 metabolites and a sugar were analysed using Hydrophilic Interaction Chromatography (HILIC) Liquid Chromatography-Mass Spectrometry (LC-MS) for control, 10 and 20% PEG-treated white sorghum cell suspension cultures. The analyses were done in a time-course experiment at 1, 24, 48, and 72 hours. The 16 metabolites analysed included the phytohormone abscisic acid (ABA), osmolytes (proline (Pro) and glycine betaine), and amino acids such as alanine (Ala), arginine (Arg), aspartic acid (Asp), glycine (Gly), histidine (His), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser) threonine (Thr), tyrosine (Tyr) and valine (Val). For sugars, the content of trehalose was also analysed. Figure 3.3 shows the changes in the expression levels of the 16 metabolites for the control and 10 and 20% PEG treatments over 72 hours. Due to this experiment's nature, statistical analyses were done across treatments within a single time-point and not across time-points. Other researchers have used similar analyses in stress biology studies (Ramulifho *et al*., 2019; Lin *et al*., 2021).

In general, the expression patterns of the metabolites varied amongst the analytes (Figure 3.3). For example, ABA had insignificant differences for both the 10 and 20% PEG-treated cell cultures relative to the control, with the 20% PEG treatment having the lowest ABA content. Amongst the osmolytes, the levels of glycine betaine did not change much over the 72 hours of PEG treatment. However, Pro was significantly reduced at 1, 24, and 48 hours for a 10 and 20% PEG-induced osmotic stress treatment.

Amongst the other thirteen amino acids, two major trends were observed. Firstly, Leu, Met, Phe, Tyr and Val showed a drastic reduction in content for the 10 and 20% PEG treatments over the 72 hours relative to the control. Secondly, the rest of the amino acids showed a significant increase or decrease between the control at one point and another treatment at another time point, see Appendix Table A3.

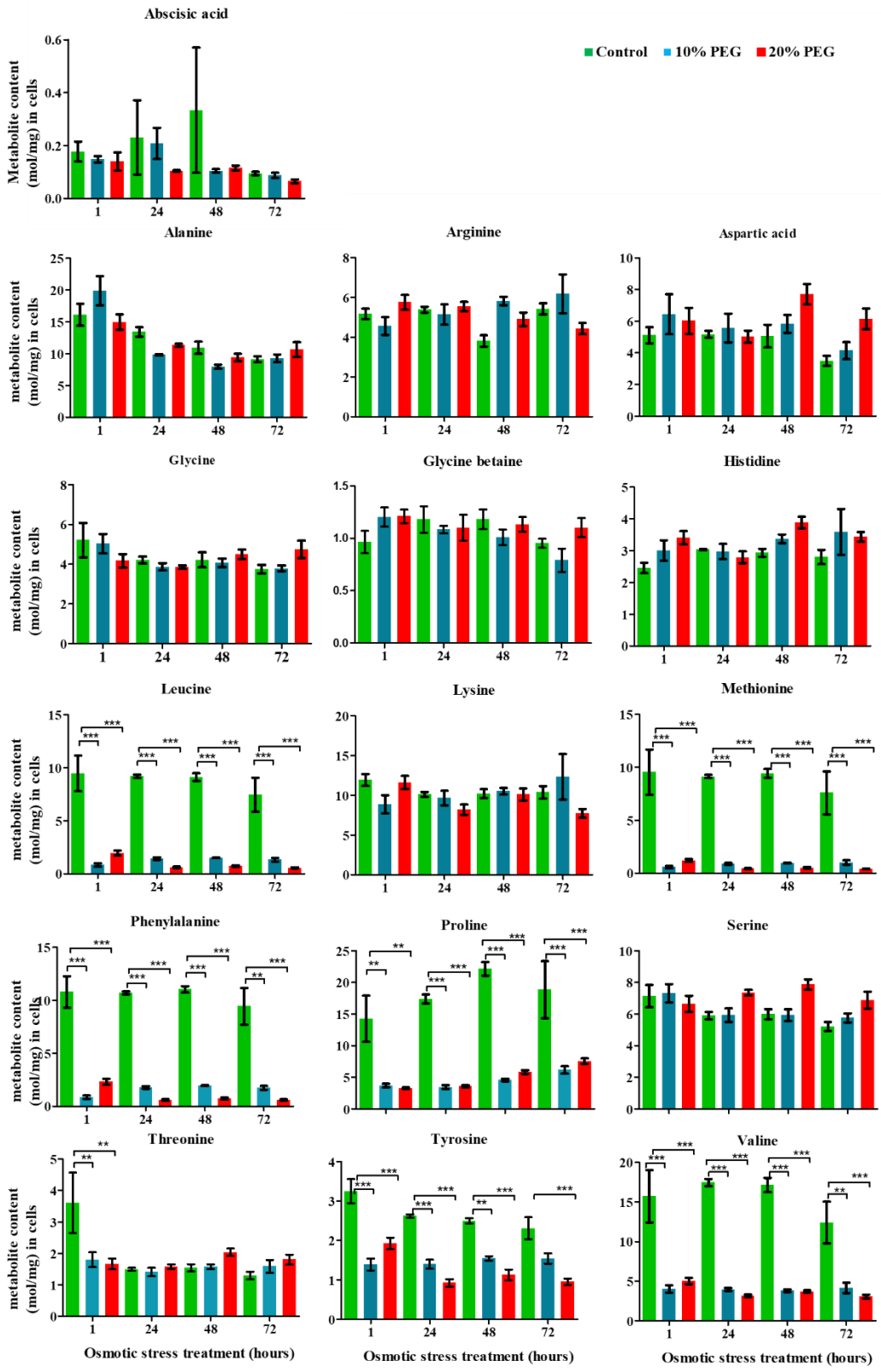

Figure 3.3: Metabolite profiling of white sorghum cell suspension cultures in response to PEG treatments. The levels of different metabolites were estimated at 1, 24, 48, and 72 hours after 10 and 20% PEG stress treatment using HILIC LC-MS. Bars represented ± SE (*n=3*) were *, **and *** indicated statistical significance at 5, 1, and 0.5% significance levels according to one-way ANOVA.

3.3 Discussion

Cell suspension cultures are used to study a range of biological phenomena, such as the differential expression of genes (Rustagi *et al*., 2015), proteins (Nouri and Komatsu, 2010), and metabolites (Da Fonseca-Pereira *et al*., 2019b) in response to environmental factors including salt, drought, and heat (Ngara *et al*., 2008; Ngara et al., 2018; Ngcala, 2020; Ramulifho *et al*., 2019). The current study focused on drought, also termed osmotic stress. Osmotic stress can be imposed on cell suspension culture systems using different types of solutes (Hohl and Schopfer, 1991; Ramakrishna and Ravishankar, 2011). The current study was designed to evaluate how the 10 and 20% PEG-induced osmotic stress would affect the growth, metabolic activity and metabolite accumulation of the white sorghum cell suspension cultures. The white sorghum suspension cell cultures (Figures 3.1C and D) were generated from callus (Figures 3.1A and B) prior to use in the study.

The study initially used 20% PEG to induce osmotic stress (Figure 3.2A). The results showed that 20% PEG drastically reduced the cell viability of the white sorghum cell suspension cultures. To avoid a study on completely dead cells after an early sharp decline in cell viability was noted within 24 hours (Figure 3.2A), a second level of osmotic stress treatment using 10% PEG was introduced (Figure 3.2B). This new experimental setup enabled a comparative study examining the impact of mild (10%) versus severe (20%) PEG-induced osmotic stress on the molecular responses of the sorghum cells. The results show that both 10 and 20% PEG significantly reduced the viability of sorghum cell cultures as early as 1 hour after stress treatments (Figure 3.2B).

Moreover, the experimental setup showed how mild (10% PEG) and severe (20% PEG) stress levels affected the cell viability of the sorghum cells differently. Ramulifho (2017), conducted

an MTT assay to estimate the cell viability of two, sorghum cell culture lines (ICSB 338 and the white sorghum) after exposure to sorbitol-induced osmotic stress. The cell viability results showed that sorbitol did not decrease viability relative to the control over 72 hours of treatment (Ramulifho, 2017). Therefore, PEG is a good osmoticum when compared to sorbitol in exerting osmotic stress. A study by Alqurashi *et al*. (2018), investigated severe water-deficit response by inducing a 40% PEG osmotic stress in Arabidopsis cell suspensions for 10 and 30 minutes. The results demonstrated that the cell viability of the Arabidopsis cells was compromised as evaluated by the fluorescein diacetate staining method. The study also suggested that future proteomic studies that focus on osmotic stress responses can sample cells as early as an hour after exposure to PEG treatments in cell cultures (Alqurashi *et al*., 2018). Overall, PEG is a good osmoticum to induce osmotic stress as early as 30 minutes to an hour on cell cultures for further cellular assays and evaluations.

In the current study, a striking observation from the data was the increase in viability of the 10 and 20% PEG-treated cells at 24 hours (Figure 3.2B). This showed that while the cells treated with both 10 and 20% PEG had decreased cell vitality after 6 hours of stress treatment, the cells were not completely dead. Therefore, the 72-hour time point was selected for evaluating osmotic stress-induced metabolic changes of this sorghum cell culture line. These results confirmed that cell viability is impacted differently depending on the type and intensity of the osmoticum used in experiments.

Plants synthesize many metabolites under abiotic stresses, including water deficits (Anjum *et al*., 2011; Valadez-Bustos *et al*., 2016). These metabolites range from phytohormones (Ramakrishna and Ravishankar, 2011), to amino acids, organic acids, betaines, and soluble carbohydrates (Anjum *et al*., 2011). The current study evaluated the accumulation of the

phytohormone ABA, osmolytes (glycine betaine and proline), amino acids (Ala, Arg, Gly, Asp, His, Lys, Leu, Met, Phe, Ser, Thr, Tyr and Val), and sugar (trehalose (Tre)) in response to PEG-induced osmotic stress. Both 10 and 20% PEG treatments were used to evaluate temporal variations of the metabolites over a 72-hour stress treatment period.

ABA is a phytohormone and a key stress signalling molecule in plant responses to stress (Raghavendra *et al*., 2010). The results from the current study showed no significant change in this phytohormone after 10 and 20% PEG osmotic stress relative to the control at 72 hours. The findings from this study could not support what is generally understood from the literature. A comparative study by Perales *et al*. (2005), used sorbitol to induce osmotic stress on the cell lines of rice embryos (*Oryza sativa L, cvs*. Bahia and Bomba). The tolerant line of rice embryos synthesized more ABA than the sensitive line in that study. However, the lack of significant reduction or increase of ABA in the current research could be a reflection of cellular changes which are tissue, species, cell line or even osmoticum related. Perales *et al*. (2005) used embryo-derived cell lines, and the sorghum cell culture was derived from a three-day-old white sorghum cell suspension shoot. The levels of ABA observed in this study may not necessarily reflect changes that could happen at the whole plant level.

Osmolytes accumulate in cells under stress to maintain certain physiological processes, such as photosynthesis and leaf expansion (Cechin *et al*., 2006). The two most evaluated osmolytes in response to stress are proline and glycine betaine. Proline is an osmoprotectant necessary in many metabolic pathways involved in osmotic balance after an encounter with drought (Di Martino *et al*., 2003). In this study, proline concentrations showed a significant decline in response to both 10 and 20% PEG osmotic stress relative to control at each time point (Figure 3.3). Other studies have observed an increase in proline content in maize (*Zea mays L.)*

seedlings and cell cultures (Ibarra-Caballero *et al*., 1988) after encountering drought stress. The increase in proline content is both tissue and age-dependent and that could be the reason there is no significant increase in this study. Some studies also argue that the non-correlation between proline accumulation and drought stress is a consequence of the mildness, severity, and duration of drought stress (Mohammadkhani and Heidari, 2008; Sofo *et al*., 2004). Furthermore, glycine betaine is abundant under osmotic stress in sorghum (Fracasso *et al*., 2016). However, the current study found no significant increase or decrease in glycine betaine contents (Figure 3.3).

Amino acids are some of the major biological molecules found in living organisms (Garrett and Grisham, 2016). They function as precursors and constituents of proteins (Garrett and Grisham, 2016). They also have a broader role as precursors for other compounds containing nitrogen, they affect membrane permeability, modulate ion transportation, and act as osmolytes (Rai, 2002). However, their precise role in plants depends on their nature, the developmental stage of the plant, and the prevailing environmental conditions (Rai, 2002). As such, the levels of amino acids in plant cells also change in response to abiotic stresses including water deficits (Rhodes *et al*., 1986). In the current study, two major trends were observed regarding the levels of amino acids in sorghum cells under 10 and 20% PEG treatment. For example, of the 13 amino acids analysed, only six amino acids showed a significant decrease in response to both the 10 and 20% PEG-induced osmotic stress relative to the control (Figure 3.3). These metabolites included Ser, Leu, Met, Phe, Val and Thr (Figure 3.3). These results are in contrast to those reported by Kovács *et al*. (2012) where the number of amino acids such as Glu, Gln, Asp, Thr, Ser, Ala, and Tyr had an accelerated transient increase when first observed after three days of 15% PEG treatment in wheat (*Triticum aestivum L*.). However, in another metabolomics study, osmotic stress induced in the roots of rice (*Oryza sativa L*.) with a -0.42

MPa PEG 6000 increased proteinogenic amino acids such as Pro, Ser, Glutamine (Gln), and asparagine (Asn) (Matsunami *et al*., 2020). Changes in the accumulation of amino acids have been reported in plants under stress (Rai, 2002). Lower molecular organic compounds such as these amino acids are crucial in understanding cellular functions under drought (Farooq *et al*., 2012).

Tre is a disaccharide of two glucose molecules joined by α, α-1,1-glycoside bond. Tre occurs naturally in most organisms, including plants, and its metabolism can be traced to ancient times (Avonce *et al*., 2006). In plants, Tre is in low concentration (Schluepmann and Paul, 2009), it is a signalling molecule that regulates carbon utilization for growth (Iturriaga *et al*., 2009; Schluepmann *et al*., 2012) and a molecular chaperone that contributes to osmotic stress tolerance (Avonce *et al*., 2006; Li *et al*., 2019). Tre was not detected in this study (refer to Appendix Table A2). Other researchers have reported that Tre metabolism can differ between sorghum genotypes (Li *et al*., 2019), possibly explaining why it was not detected in this study. Martínez-Barajas *et al*. (2011), proposed that the accumulation of trehalose 6-phosphate (Tre6P) in wheat (*Triticum aestivum*) is a good characteristic of grain development and improves crop yields. For better detection of Tre, future experiments could use the highly sensitive HILIC-ESI-MS (Hydrophilic-interaction chromatography) - (Electrospray ionization mass spectrometry) to quantify Tre (Toraño *et al*., 2012). The HILIC-ESI-MS method provides a high-throughput quantification platform for the undetectable Tre in conventional metabolomics platforms (Toraño *et al*., 2012). Furthermore, electrospray ionization (ESI)-MS is a selective detection method that offers an efficient means for the analysis of certain compounds with high polarity (Toraño *et al*., 2012).

CHAPTER 4

PEG-INDUCED OSMOTIC STRESS-RESPONSIVE PROTEINS OF WHITE SORGHUM CELL SUSPENSION CULTURES

4.1 Introduction

Drought resulting from low rainfall patterns and reduced soil moisture content is a threat to the growth and productivity of cereal crops (Orimoloye *et al*., 2022). Climate change also affects the water cycle, causing changes in the distribution patterns of crops (Lobell *et al*., 2011). During their growth, plants may experience varying extents of water deficit stress; and their subsequent responses may be specific to the levels of water deprivation, duration of exposure, the plant's growth stage, and/or species (Reddy *et al*., 2004). Some of the plant responses to water stress include the protection of cellular integrity and repairing the damage caused by the stress and its secondary effects (Oliver *et al*., 2000; Bravo *et al*., 2016). In addition, plants maintain the water status and physiological activities of cells by modulating gene expression, which results in changes in the synthesis and accumulation patterns of proteins and metabolites (Phukan *et al*., 2014; Akpinar and Cansev, 2022).

A plant's antioxidant system is an essential response mechanism for counteracting the negative effects of reactive oxygen species (ROS) during exposure to drought and other abiotic stresses (You and Chan, 2015). Although ROS act as signalling molecules at lower levels, their over-accumulation causes oxidative stress, and subsequent damage to DNA, lipids and proteins (Raja *et al*., 2017). ROS are maintained at optimum levels through redox reactions that are catalysed by enzymes such as superoxide dismutase (SOD), ascorbate peroxide (APX), glutathione reductase (GR), and catalase (CAT), non-enzymatic activities of glutathione, ascorbate, and alpha-tocopherol, among others (Mittler, 2017). Apart from the previously-

mentioned antioxidant enzymes, other drought-related proteins include molecular chaperones, late embryogenesis abundant (LEA) proteins, aquaporins, and dehydrins (Allagulova *et al*., 2003; Kosová *et al*., 2008; Gulen *et al*., 2009; Matthews, 2014).

Alterations in protein accumulation during drought response depend on the rates of transcription and translation, as well as their regulatory mechanisms, mRNA stability, and protein degradation (Greenbaum *et al*., 2003). These changes in protein expression may be studied through a range of gel-based and non-gel-based proteomics technologies (Monteoliva and Albar, 2004; Aslam *et al*., 2017). For example, the isobaric tag for relative and absolute quantitation (iTRAQ) coupled with mass spectrometry is one of the most widely used gel-free proteomic technologies for identifying and quantifying changes in stress-related proteins (Wiese *et al*., 2007; Hu *et al*., 2015; Ngara *et al*., 2018). Therefore, the chapter's objective was to perform a comparative iTRAQ and bioinformatic analysis of the 10 and 20% PEG-induced osmotic stress-responsive total soluble proteins of white sorghum cell suspension cultures.

4.2 Results

4.2.1 1D gel electrophoresis protein profiles after 10 and 20% PEG-induced osmotic stress

After 72 hours of 10 and 20% PEG-induced osmotic stress treatments, total soluble proteins (TSP) were extracted from white sorghum cell suspension cultures as described in Section 2.5. Four biological replicates were used for the extractions per treatment. The protein samples for the control, and 10 and 20% PEG-treated cells were quantified using the Bradford assay and separated on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) to determine the quality of the extracts. Figure 4.1 shows the TSP profiles of the control and PEG-induced osmotic-stressed samples. It was observed that the protein profiles

showed visible changes in the differential expression of the protein fractions in response to either the 10 or 20% PEG-induced osmotic stress relative to the control (Figure 4.1).

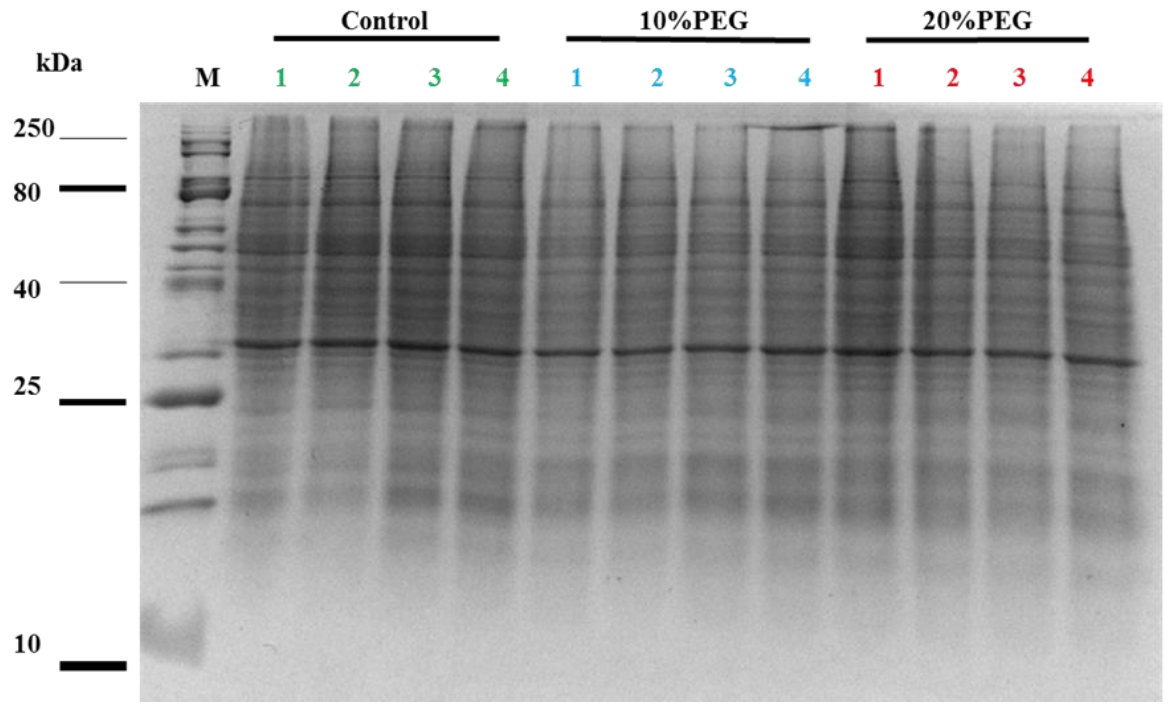

Figure 4.1: 1D SDS-PAGE analysis of white sorghum cell suspension cultures. Eight-day-old white sorghum cell suspension cultures were treated with 10 and 20% PEG to induce osmotic stress over 72 hours. Total soluble protein samples were separated on a 12% (v/v) SDS-PAGE gel. Approximately 10 µg of the protein extracts from each sample was loaded into each well. The gels were stained with Coomassie Brilliant Blue stain. The M-titled lane is loaded with the protein molecular weight marker measured in kilodaltons (kDa).

4.2.2 iTRAQ analysis of 10 and 20% PEG stress-responsive proteins

The extracted TSP samples of the control and the two PEG treatments were digested with trypsin and peptides labelled with iTRAQ tags and further analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In total, two separate sets of control total soluble protein samples were labelled with molecular weight tags 113, 114, 115, and 116

for each of the four biological replicates, respectively, while 117, 118, 119, and 120 tags were used to label independently the 10 and 20% PEG stressed samples. This yielded two iTRAQ experiments for this study. The first experiment consisted of the control and 10% PEG-treated samples, while the second experiment consisted of the control and 20% PEG-treated samples. The labelled peptides from each of the two iTRAQ experiments were separately analysed by LC-MS/MS and proteins identified based on 2 or more peptides with statistical confidence ≥ 95% were considered as positive identities. After the iTRAQ data clean-up process, a total of 177 and 229 white sorghum cell-cultured total soluble proteins were identified for the 10 and 20% PEG treatment experiments, respectively. Of these identified proteins, 28 and 48 proteins were responsive to the 10 and 20% PEG treatments, respectively. A summary of this data is presented in Table 4.1.

Table 4.1: A summary of the iTRAQ-identified proteins and induced changes in response to the PEG treatments.

PEG concentration	Identified proteins	PEG-responsive proteins	Protein regulation	
			Up	Down
10%	177	28	14 (50%)	14 (50%)
20%	229	48	19 (40%)	29 (60%)

4.2.3 Bioinformatic analyses of 10 and 20% PEG stress-responsive proteins

The stress-responsive proteins identified in both 10 and 20% PEG treatments (Table 4.1) were further annotated using data retrieved from the UNIPROT database. For all bioinformatics annotations, the UNIPROT protein accession numbers obtained from the mass spectrometry data were used as search terms to retrieve data on Gene Ontology (GO), protein family names,

domains, and protein function. This information is tabulated into two tables, with Table 4.2 focussing on 10% PEG and Table 4.3 on 20% PEG stress treatments.

For the 10% PEG treatment (Table 4.2), 28 osmotic stress-responsive proteins were involved in putative functions including metabolism (1), intracellular traffic (2), energy (3), transcription (7), protein synthesis (5), protein destination and storage (2), cell structure (1), disease/ defense (5), and unclassified (2). From this protein count, the transcription (25%), protein synthesis (18%), and disease/ defense (18%) functional groups had the greatest number of stress-responsive proteins as shown in Figure 4.2A. The data indicate that the sorghum cell suspension cultures responded to 10% PEG-induced stress by up- and down-regulating proteins in most functional groups. However, osmotic stress-responsive proteins involved in metabolism, and the protein destination and storage categories were all down-regulated (Table 4.2). On the other hand, transcription, protein synthesis, and disease/ defense functional groupings had more up-regulated proteins (Table 4.2).

White sorghum cell suspension cultures had 48 proteins responding to the 20% PEG-induced osmotic stress as illustrated in Table 4.3. These proteins were distributed amongst functional groups of metabolism (10), intracellular traffic (2), energy (1), transcription (9), protein synthesis (6), protein destination and storage (4), cell structure (3), disease/ defense (8), and unclassified (5). Functional groups with the highest number of 20% PEG-responsive proteins were metabolism (21%), transcription (19%), disease/defense (17%), and protein synthesis (13%), as shown in Figure 4.2B. The energy functional group contained only one protein, which was up-regulated. Furthermore, the transcription, protein synthesis, and disease/defence-related proteins were mostly up-regulated. In contrast, proteins grouped under metabolism, cell

structure, and the unclassified categories were all down-regulated after encountering the 20% PEG-induced osmotic stress (Table 4.3).

Table 4.2: List of 10% PEG-induced osmotic stress-responsive proteins identified in white sorghum cell suspension cultures.

Pro No:[a]	Accession[b]	Prot Name	Scor[c]	% Cov[d]	Seq Pep[e]	Ratio[h]	SD[g]	p-val[f]	GO Analysis[i]			Conserved domains and family name[j]
									p	f	c	
Metabolism												
45	A0A1B6QIM7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G123300	12.09	10.12	5	0.78	0.05	1.81E-02	carbohydrate metabolic process	beta-glucosidase activity	none	Domain not predicted; Glycoside hydrolase family 1
Intracellular traffic												
122	C5Z487	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3010G163600	5.79	11.98	3	1.10	0.05	2.08E-02	intracellular protein transport	GTPase activity	endomembrane system	Small GTP-binding protein domain; Small GTPase
136	A0A1W0VWX9	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G122200	4.87	8.14	2	0.74	0.02	2.12E-02	intracellular protein transport	GTPase activity	Golgi apparatus	Small GTP-binding protein domain; Small GTPase superfamily, ARF/SAR type
Energy												
11	C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G393900	27.85	38.59	16	0.89	0.05	9.49E-03	fructose 1,6-bisphosphate metabolic process	fructose-bisphosphate aldolase activity	cytosol	Domain not predicted; Fructose-bisphosphate aldolase, class-I
61	A0A1B6Q6S1	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G336000	10.48	8.59	6	1.16	0.09	3.62E-02	cellular carbohydrate metabolic process	2,3-bisphosphoglycerate-independent phosphoglycerate mutase activity	cytoplasm	Metalloenzyme; Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent
97	C5YW21	Malate dehydrogenase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G240700	7.52	12.35	4	0.73	0.15	2.77E-02	malate metabolic process	L-malate dehydrogenase activity	cytoplasm	Lactate/malate dehydrogenase, N-terminal; L-lactate/malate dehydrogenase
Transcription												
51	C5XDK0	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G210200	11.52	27.45	6	2.53	0.37	2.60E-04	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2B
62	A0A1B6P9Z6	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G213500	10.45	10.96	4	0.83	0.07	9.25E-03	nucleosome assembly	chromatin binding	chromatin	Domain not predicted; Nucleosome assembly protein (NAP)
79	C5XAT9	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G330000	8.54	28.89	5	0.41	0.03	5.97E-03	chromatin silencing	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
93	A0A1W0W662	Histone H4 OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G291066	8	23.66	4	2.44	0.57	8.56E-03	none	DNA binding	nucleosome	CENP-T/Histone H4, histone fold; HistoneH4
260	C5XAT8	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G329900	1.7	17.91	3	0.61	0.12	1.77E-02	chromatin silencing	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A

307	C5WPC5	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G417000	0.91	28.38	6	2.03	0.40	2.47E-03	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2B
383	C5YM38	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G149600	0.12	23.18	5	1.75	0.39	3.95E-02	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2B
Protein synthesis												
68	A0A1B6PQ87	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3005G050500	9.42	11.57	6	0.90	0.05	4.57E-02	ribosomal large subunit assembly	RNA binding	cytosolic large ribosomal subunit	Domain not predicted; Ribosomal protein L3
88	A0A1B6QFD0	Eukaryotic translation initiation factor 5A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G363100	8.13	26.25	5	1.71	0.11	1.06E-04	positive regulation of translational elongation	translation elongation factor activity	none	Translation elongation factor, IF5A C-terminal; Translation elongation factor IF5A-like
144	C5XD92	40S ribosomal protein S3a OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G364500	4.35	8.37	3	1.51	0.28	1.41E-02	translation	structural constituent of ribosome	cytosolic small ribosomal subunit	Domain not predicted; Ribosomal protein S3Ae
159	A0A1B6P9L7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G215200	3.56	10.96	2	1.79	0.39	3.49E-02	translation	structural constituent of ribosome	cytosolic large ribosomal subunit	Ribosomal L28e/Mak16; Ribosomal protein L28e
338	A0A1B6QMI3	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G334300	0.4	23.45	4	0.52	0.04	3.19E-02	translation	structural constituent of ribosome	cytosolic small ribosomal subunit	Domain not predicted; Ribosomal protein S19e
Protein destination and storage												
99	A0A1B6QA33	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G090500	7.33	8.08	4	0.67	0.06	4.93E-02	protein folding	unfolded protein binding	endoplasmic reticulum lumen	Domain not predicted; Calreticulin
168	C5Y675	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3005G064200 PE=3 SV=2	3.09	4.10	2	0.72	0.15	4.74E-02	proteolysis	peptidase activity	none	Xylanase inhibitor, C-terminal; Aspartic peptidase A1 family
Cell structure												
32	C5WT90	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G173300	14.47	17.86	8	0.88	0.02	1.93E-02	plant-type cell wall biogenesis	UDP-arabinopyranose mutase activity	cytosol	Domain not predicted; Reversibly glycosylated polypeptide
Disease/ defense												
27	Q4VQB2	Pathogenesis-related protein 10b OS=*Sorghum bicolor* OX=4558 GN=PR10	17.92	72.50	10	1.67	0.18	3.11E-03	abscisic acid-activated signalling pathway	abscisic acid binding	cytoplasm	Bet v I/Major latex protein; Bet v I type allergen
49	Q4VQB4	Pathogenesis-related protein 10c OS=*Sorghum bicolor* OX=4558 GN=PR10	11.71	85.53	6	1.16	0.07	4.51E-02	abscisic acid-activated signalling pathway	abscisic acid binding	cytoplasm	Bet v I type allergen; Glycoside hydrolase family 1

65	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G129700	10	26.67	5	2.41	0.62	3.93E-03	none	manganese ion binding	apoplast	Cupin 1; Germin
118	C5Z0N9	Peroxidase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G055300	6	11.29	3	1.38	0.19	1.89E-02	response to oxidative stress	peroxidase activity	plant-type cell wall	Haem peroxidase; Plant peroxidase
109	C5X6M3	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G138700	6.4	7.83	3	0.71	0.05	3.50E-02	defense response	none	integral component of membrane	C2 domain; Family not predicted
Unclassified												
16	C5XF10	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G104600	25.4	47.57	13	0.78	0.06	1.14E-03	none	none	none	NmrA-like domain; Family not predicted
29	C5YBP8	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3006G009000	16.38	20.04	9	1.19	0.08	7.44E-03	none	none	none	von Willebrand factor, type A; Family not predicted

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers acquired from the TrEMBL database incorporated within the UniProt database (http：//www.uniprot.org.

[c] Protein score developed by ProteinPilot software relating to the confidence of protein identification. A protein identification threshold of 1.3 was applied to the data, which only retains proteins identified with 95% confidence.

[d] Percentage coverage is defined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

[e] Sequenced peptides are the number of peptides that were sequenced and gave rise to protein identity. All proteins identified utilizing a single peptide were filtered out of the dataset.

[f] Probability value of the quantitative difference between the treatment and control protein abundance is due to chance alone.

[g] Standard deviation is calculated from the PEG osmotic stressed samples (n = 4) ratios.

[h] Ratio stands for the average fold-change (n = 4) induced by the 10% PEG treatment relative to the control. Values of one and above signify an upregulation.

[I] Gene ontology analysis as indicated on the UniProt database (http: //www.uniprot.org) P represents Biological Process, F for Molecular function, and C for Cellular Component.

[j] Conserved name as indicated in the UniProt database (http://www.uniprot.org).

Table 4.3: List of 20% PEG-induced osmotic stress-responsive proteins identified in white sorghum cell suspension cultures.

Pro No:[a]	Accession[b]	Prot Name	Scor[c]	% Cov[d]	Seq Pep[e]	Ratio[h]	SD[g]	p-val[f]	GO Analysis[i]			Conserved domains and family name[j]
									p	f	C	
Metabolism												
32	C5X183	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G094300	17.69	17.83	9	0.63	0.13	1.38E-02	sulfate assimilation	ATP binding	None	Sulphate adenylyltransferase; Family not predicted
33	A0A1B6QHY1	UDP-glucose 6-dehydrogenase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G084100	17.67	17.29	8	0.63	0.06	1.69E-02	glycosaminoglycan biosynthetic process	NAD binding	cytosol	UDP-glucose/GDP-mannose dehydrogenase, N-terminal; UDP-glucose/GDP-mannose dehydrogenase family
47	A0A1B6QIM7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G123300	13.92	11.51	6	0.59	0.13	1.75E-03	carbohydrate metabolic process	beta-glucosidase activity	None	Domain not predicted; Glycosyl hydrolase 1 family
54	C5WRH5	Nucleoside diphosphate kinase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G295200	12.73	32.21	6	0.75	0.09	4.05E-03	CTP biosynthetic process	ATP binding	None	Nucleoside diphosphate kinase-like domain; Nucleoside diphosphate kinase
71	A0A1B6PEZ5	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G014700	10.72	17.37	7	0.54	0.14	2.34E-02	fructose metabolic process	fructokinase activity	Cytosol	Carbohydrate kinase PfkB; Family not predicted
147	A0A1B6PBB3	Nucleoside diphosphate kinase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G259200	6.00	11.93	3	0.74	0.05	8.52E-04	CTP biosynthetic process	ATP binding	None	Nucleoside diphosphate kinase-like domain; Nucleoside diphosphate kinase
156	C5X972	Nucleoside diphosphate kinase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G306900	5.64	26.67	4	0.75	0.06	3.07E-02	CTP biosynthetic process	ATP binding	None	Nucleoside diphosphate kinase-like domain; Nucleoside diphosphate kinase
159	C5XIV5	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G149500	5.51	8.39	4	0.82	0.07	1.60E-02	D-xylose metabolic process	NAD+ binding	Cytoplasm	NAD(P)-binding domain; UDP-glucuronic acid decarboxylase
226	C5YIC1	Aconitate hydratase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G068100	2.86	2.61	3	0.58	0.09	1.24E-02	citrate metabolic process	metal ion binding	cytosol	Aconitase A/isopropylmalate dehydratase small subunit, swivel domain; Aconitase/Iron-responsive element-binding protein 2
332	C5XF87	Ketol-acid reductoisomerase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G239900	1.44	10.02	6	0.81	0.14	4.10E-02	isoleucine biosynthetic process	metal ion binding	chloroplast	Ketol-acid reductoisomerase, C-terminal; Ketol-acid reductoisomerase
Transcription												
60	C5XDK0	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G210200	11.62	33.99	7	2.29	0.41	1.26E-03	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2B
95	C5XAT9	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G330000	8.92	28.89	5	0.56	0.12	1.45E-02	chromatin silencing	DNA binding	Nucleosome	Histone H2A/H2B/H3; Histone H2B
115	A0A1W0VVX1	Histone H4 OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G057366	7.62	17.05	5	2.29	0.51	3.82E-03	nucleosome assembly	DNA binding	nucleosome	CENP-T/Histone H4, histone fold; Histone H4

121	C5XPA5	Histone H3 OS=*Sorghum bicolor* OX=4558 GN=SORBI_3004G170500	7.33	16.91	4	2.32	0.54	1.13E-02	none	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H3/CENP-A
235	C5XAT8	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G329900	2.66	17.91	3	0.61	0.08	1.63E-03	chromatin silencing	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
330	C5YP98	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3008G114500	1.46	11.76	2	0.50	0.08	1.88E-02	chromatin silencing	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
368	C5WPC4	Histone H2A OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G416900	1.11	10.00	2	1.83	0.19	4.32E-03	chromatin silencing	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
384	C5WPC5	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G417000	0.74	35.14	7	2.58	0.50	1.54E-03	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
471	C5XP45	Histone H2B OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G350100	0.12	32.85	6	1.87	0.14	3.34E-04	nucleosome assembly	DNA binding	nucleosome	Histone H2A/H2B/H3; Histone H2A
Protein synthesis												
65	C5YH46	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G178600	11.36	35.86	6	0.65	0.05	2.39E-02	ribosomal small subunit assembly	RNA binding	cytosolic small ribosomal subunit	Domain not predicted; Ribosomal protein S19e
155	A0A1B6P9L7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G215200	5.66	23.29	4	1.63	0.19	4.77E-02	translation	structural constituent of ribosome	cytosolic large ribosomal subunit	Ribosomal L28e/Mak16; Ribosomal protein L28e
164	C5YZ01	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G022400	5.28	10.19	4	1.99	0.49	8.07E-03	translation	mRNA binding	cytosolic large ribosomal subunit	Domain not predicted; Ribosomal protein L13, eukaryotic/archaeal
198	C5XAD5	Ribosomal protein L15 OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G047600	3.86	10.29	3	1.20	0.13	2.77E-02	translation	RNA binding	cytosolic large ribosomal subunit	Domain not predicted; Ribosomal protein L15e
201	A0A1B6QNT2	40S ribosomal protein S7 OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G403500	3.76	8.85	2	1.36	0.13	4.85E-02	translation	structural constituent of ribosome	cytosolic small ribosomal subunit	Domain not predicted; Ribosomal protein S7e
206	C5YMD1	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G019400	3.60	12.58	3	1.45	0.30	4.60E-02	translation	structural constituent of ribosome	cytosolic small ribosomal subunit	Ribosomal protein S13/S15, N-terminal; Ribosomal protein S15
Protein destination and storage												
17	C5YJ75	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3007G216300	26.17	16.91	13	1.47	0.25	1.96E-02	protein folding	Unfolded protein binding	cytosol	Histidine kinase/HSP90-like ATPase; Heat shock protein Hsp90 family

22	C5X0G5	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G236300	22.64	14.57	14	0.85	0.06	2.73E-02	ER-associated misfolded protein catabolic process	ATP binding	cytosol	AAA+ ATPase domain; AAA ATPase, CDC48 family
103	C5XQ74	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G208800	8.50	8.30	4	0.62	0.14	1.12E-02	proteolysis	aspartic-type endopeptidase activity	None	Xylanase inhibitor, C-terminal; Aspartic peptidase A1 family
134	A0A1B6QA33	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G090500	6.54	11.64	6	0.68	0.14	2.26E-02	protein folding	unfolded protein binding	endoplasmic reticulum lumen	Domain not predicted; Calreticulin
Cell structure												
24	C5WW94	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G197400	20.22	31.83	12	0.74	0.17	3.98E-02	none	ATP binding	Cytoskeleton	Domain not predicted; Actin family
43	C5WT90	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G173300	14.96	19.78	10	0.70	0.04	4.50E-05	plant-type cell wall biogenesis	UDP-arabinopyranose mutase activity	cytosol	Domain not predicted; Reversibly glycosylated polypeptide
76	A0A1B6QIB9	Tubulin alpha chain OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G107100	10.10	15.30	6	0.74	0.10	2.88E-02	microtubule cytoskeleton organization	GTPase activity	Microtubule	Tubulin/FtsZ, GTPase domain; Tubulin
Intracellular traffic												
88	C5XNL6	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G189000	9.25	22.66	5	1.16	0.10	3.25E-02	protein transport	GTPase activity	None	Small GTP-binding protein domain; Small GTPase
78	C5YW22	GTP-binding nuclear protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G240900	9.91	23.98	5	0.75	0.09	3.87E-02	protein import into nucleus	GTPase activity	nucleus	Small GTP-binding protein domain; Small GTPase
Energy												
258	C5YF55	Ubiquinol oxidase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3006G202500	2.12	4.53	2	1.45	0.29	4.61E-02	alternative respiration	metal ion binding	integral component of membrane	Alternative oxidase; Alternative oxidase
Disease/ defense												
20	C5Z529	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3010G173100	24.93	25.18	11	0.77	0.07	6.50E-03	glutathione metabolic process	glutathione transferase activity	None	AAA+ ATPase domain; Glutathione Transferase family
82	C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G129700	9.77	26.67	5	2.63	0.33	3.77E-04	none	manganese ion binding	apoplast	Cupin; Germin
89	C5X6M3	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G138700	9.22	15.36	5.00	0.78	0.08	4.99E-02	defense response	none	integral component of membrane	C2 domain; Family not predicted

117	C5Z0N9	Peroxidase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3009G055300	7.49	15.70	5	1.42	0.18	4.25E-03	response to oxidative stress	peroxidase activity	extracellular region	Haem peroxidase; Plant peroxidase
157	A0A1W0VY40	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G197200	5.62	8.72	3	0.63	0.10	1.86E-02	none	oxidoreductase activity	None	Non-haem dioxygenase N-terminal domain; Family not predicted
168	C5Y360	Peroxidase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3005G011300	5.07	9.15	3	2.73	0.78	5.06E-03	response to oxidative stress	peroxidase activity	extracellular region	Haem peroxidase; Plant peroxidase
216	A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3004G341200	3.27	3.03	2	1.55	0.33	3.84E-02	cell redox homeostasis	flavin adenine dinucleotide binding	Cytoplasm	FAD/NAD(P)-binding domain; Pyridine nucleotide-disulphide oxidoreductase, class I
279	C5XIY2	Peroxidase OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G152200	2.00	7.01	3	2.09	0.47	4.90E-03	response to oxidative stress	peroxidase activity	extracellular region	Haem peroxidase; Plant peroxidase
Unclassified												
18	C5XF10	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3003G104600	25.71	47.57	13	0.81	0.04	5.74E-03	none	none	None	NmrA-like domain; Family not predicted
25	C5X1K7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G247600	19.97	28.95	12	0.84	0.06	1.94E-02	none	NAD binding	None	NAD-dependent epimerase/dehydratase; Family not predicted
50	C5X1Q1	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G385900	13.46	21.94	8	0.84	0.04	9.42E-03	none	none	None	Domain not predicted; Ricin B-like lectin EULS3-like
97	C5WW09	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3001G042500	8.82	15.90	4	0.59	0.06	2.38E-05	none	none	None	Domain not predicted; Family not predicted
119	C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* OX=4558 GN=SORBI_3002G343600	7.35	16.52	5	0.72	0.11	1.67E-02	specification of floral organ number	enzyme inhibitor activity	None	Leucine-rich repeat-containing N-terminal, plant-type; Family not predicted

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers acquired from the TrEMBL database incorporated within the UniProt database (http://www.uniprot.org.

[c] Protein score developed by ProteinPilot software relating to the confidence of protein identification. A protein identification threshold of 1.3 was applied to the data, which only retains proteins identified with 95% confidence.

[d] Percentage coverage is defined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

[e] Sequenced peptides are the number of peptides that were sequenced and gave rise to protein identity. All proteins identified utilizing a single peptide were filtered out of the dataset.

[f] Probability value of the quantitative difference between the treatment and control protein abundance is due to chance alone.

[g] Standard deviation is calculated from the PEG osmotic stressed samples (n = 4) ratios.

[h] Ratio stands for the average fold-change (n = 4) induced by the 20% PEG treatment relative to the control. Values of one and above signify an upregulation.

[I] Gene ontology analysis as indicated on the UniProt database (http://www.uniprot.org) P represents Biological Process, F for Molecular function, and C for Cellular Component.

[j] Conserved name as indicated in the UniProt database (http://www.uniprot.org).

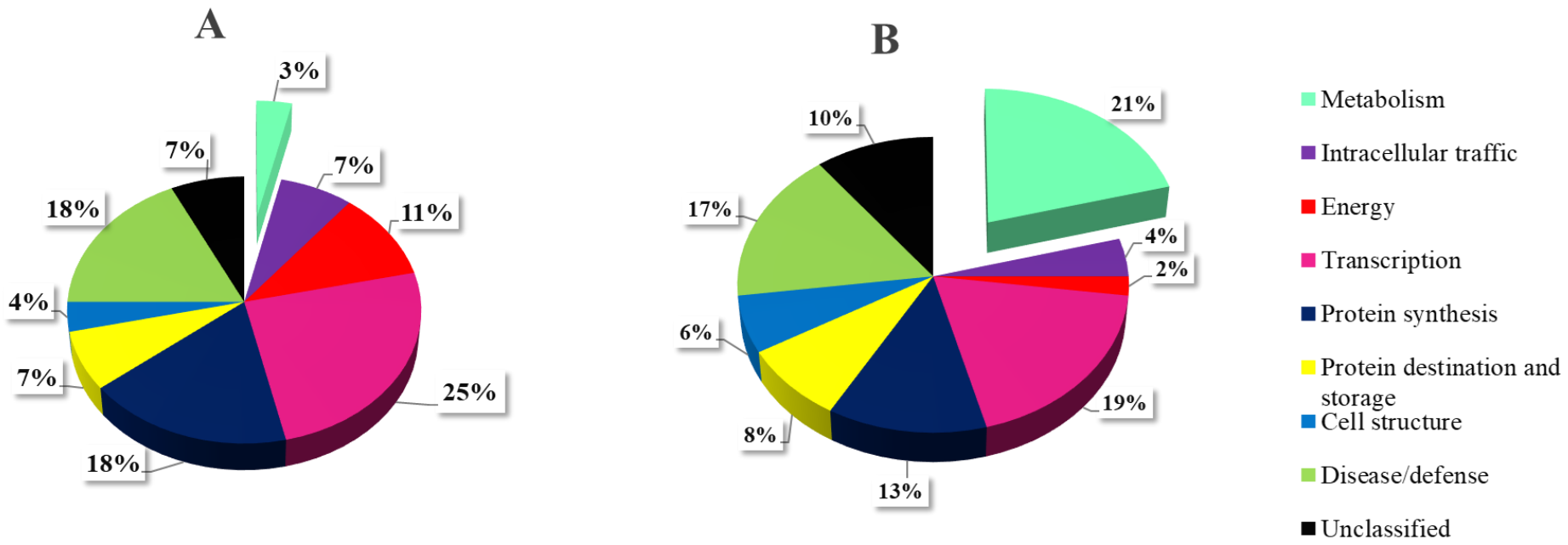

Figure 4.2: Functional categories of PEG-responsive proteins of white sorghum cell suspension cultures, after (**A**) 10% PEG and (**B**) 20% PEG osmotic stress.

4.2.4 Gene ontology annotation

Gene Ontology (GO) describes the molecular properties of genes and assists in the computational predictions of gene function (Zhao *et al*., 2020). The GO results for this study are presented in Tables 4.2 and 4.3 for the 10 and 20% PEG-responsive proteins, respectively. The results are further summarised in Figure 4.3.

It is apparent from Figure 4.3 that most of the differentially expressed proteins were down-regulated irrespective of the level of PEG concentration used to induce the osmotic stress. Figure 4.3 is also quite revealing in several ways concerning all three GO terms of biological process, molecular function and cellular location. A brief description of this data is given below. For example, 33 and 25% of the proteins were up-regulated within the molecular function after exposure to 10 and 20% PEG, respectively. Most of the up-regulated PEG-responsive proteins were involved in peroxidase activity and manganese ion binding regardless of the level of PEG-induced stress encountered. However, only proteins associated with the beta-glucosidase activity molecular functions were down-regulated in response to both PEG concentrations used.

Furthermore, Figure 4.3 illustrates that most of the up-regulated PEG-responsive proteins were in cellular components associated with the extracellular space such as the apoplast irrespective of the level of osmotic stress used. After 10% PEG-induced stress, most up-regulated proteins were in cellular components such as the plant-type cell wall and endomembrane system (Figure 4.3A). On the other hand, cellular components related to the integral component of the membrane and endoplasmic reticulum lumen had entirely down-regulated proteins irrespective of the PEG concentrations used. In contrast, Figure 4.3B shows that the increased accumulation

of proteins associated with the cytosolic large ribosomal subunit occurred as the stress was increased to 20% PEG. All the responsive proteins annotated within a specific cellular component showed an overall down-regulation of 42 and 39% for 10 and 20% of PEG-induced osmotic-responsive proteins, respectively. The integral component of the membrane and endoplasmic reticulum lumen had entirely down-regulated proteins after encountering 10 and 20% PEG-induced osmotic stress.

Figure 4.3 also shows the biological processes of the differentially expressed white sorghum cell suspension culture proteins. Following the 10% PEG treatment, the highly represented biological processes with all proteins up-regulated were the abscisic acid-activated signalling pathway, positive regulation of translational elongation, and responses to oxidative stress (Figure 4.3A). As the intensity of osmotic stress treatments increased to 20% PEG, additional biological processes with completely up-regulated proteins were cell redox homeostasis, alternative respiration, translation, and nucleosome assembly (Figure 4.3B). However, biological processes such as defense response, plant-type cell wall biogenesis, and proteolysis contained proteins that were down-regulated in both PEG stress treatments.

Figure 4.3: GO annotation for all the (**A**) 10 and (**B**) 20% PEG responsive proteins identified in sorghum cell cultures.

PGAM-i activity = 2,3-bisphosphoglycerate-independent phosphoglycerate mutase activity.

4.2.5 Bioinformatic analyses of the common and unique PEG stress-responsive proteins

The information collated in Tables 4.2 and 4.3 was further divided into three groups of common proteins, and those unique to each of the two PEG treatments. The results are presented in a Venn diagram (Figure 4.4) and show that 12 proteins were common to both treatment groups, while 16 and 36 were unique to 10 and 20% levels of stress, respectively.

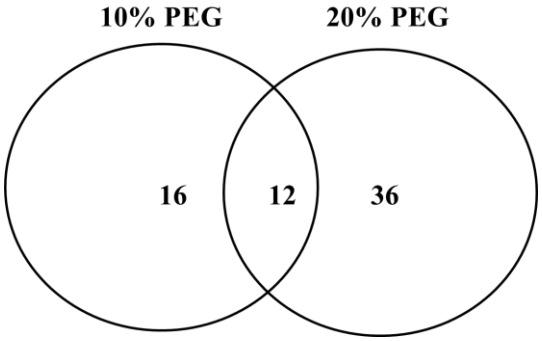

Figure 4.4: Venn diagram showing the number of stress-responsive proteins to 10 and 20% PEG.

The protein groups in Figure 4.4 are further presented in Table 4.4 for the common proteins and in Tables 4.5 and 4.6 for those unique to the 10 and 20% PEG treatments, respectively. In addition, a pictorial representation of the functional categories of these common and unique proteins identified in this study is shown in Figure 4.5. The 12 common proteins were involved in putative functions of metabolism (1), transcription (4), protein synthesis (1), protein destination and storage (1), cell structure (1), disease/ defense (3), and unclassified (1). Furthermore, each of the 12 proteins showed a similar expression pattern of either up or down-regulation in response to both PEG treatments. For example, an uncharacterised metabolism-related protein with accession number A0A1B6QIM7 was down-regulated in response to both

PEG concentrations, while a histone 2B protein (C5XDK0) was up-regulated. However, the asterically (*) marked three common proteins between the two stress treatments were statistically significant. The white sorghum cell cultures responded to both 10 and 20% PEG-induced osmotic stress by down-regulating all common proteins related to metabolism, protein destination and storage, cell structure, disease/ defense and unclassified, while the only protein synthesis-related common protein was up-regulated. However, in the transcription functional group, two proteins were up-regulated, while the other two were down-regulated (Table 4.4).

Table 4.4: List of osmotic stress-responsive proteins identified in white sorghum cell cultures that are common to both PEG treatments.

Accession[a]	Prot Name	10% PEG			20% PEG			10% vs 20% PEG	Family name[j]
		Ratio[b]	SD[c]	p-val[d]	Ratio[b]	SD[c]	p-val[d]	Ratio p-value[e]	
Metabolism									
A0A1B6QIM7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G123300	0.78	0.05	1.81E-02	0.59	0.13	1.75E-03	3.23E-02 *	Glycoside hydrolase family 1
Transcription									
C5XDK0	Histone H2B OS=*Sorghum bicolor* GN=SORBI_3002G210200	2.53	0.37	2.60E-04	2.29	0.41	1.26E-03	4.16E-01	Histone H2B
C5XAT9	Histone H2A OS=*Sorghum bicolor* GN=SORBI_3002G330000	0.41	0.03	5.97E-03	0.56	0.12	1.45E-02	5.47E-02 *	Histone H2A
C5XAT8	Histone H2A OS=*Sorghum bicolor* GN=SORBI_3002G329900	0.61	0.12	1.77E-02	0.61	0.08	1.63E-03	9.86E-01	Histone H2A
C5WPC5	Histone H2B OS=*Sorghum bicolor* GN=SORBI_3001G417000	2.03	0.40	2.47E-03	2.58	0.50	1.54E-03	1.38E-01	Histone H2B
Protein synthesis									
A0A1B6P9L7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G215200	1.79	0.39	3.49E-02	1.63	0.19	4.77E-02	4.93E-01	Ribosomal protein L28e
Protein destination and storage									
A0A1B6QA33	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G090500	0.67	0.06	4.93E-02	0.68	0.14	2.26E-02	9.26E-01	Calreticulin
Cell structure									
C5WT90	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G173300	0.88	0.02	1.93E-02	0.70	0.04	4.50E-05	1.93E-04 *	Reversibly glycosylated polypeptide
Disease/ defense									

C5WNY4	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G129700	2.41	0.62	3.93E-03	2.63	0.33	3.77E-04	5.49E-01	Germin
C5Z0N9	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3009G055300	1.38	0.19	1.89E-02	1.42	0.18	4.25E-03	7.76E-01	Plant peroxidase
C5X6M3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G138700	0.71	0.05	3.50E-02	0.78	0.08	4.99E-02	2.14E-01	Family not predicted
Unclassified									
C5XF10	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G104600	0.78	0.06	1.14E-03	0.81	0.04	5.74E-03	5.50E-01	Family not predicted

[a]Protein number assigned in ProteinPilot software.

[b]Protein accession numbers acquired from the TrEMBL database incorporated within the UniProt database (http://www.uniprot.org).

[c]Protein score developed by ProteinPilot software relating to the confidence of protein identification. A protein identification threshold of 1.3 was applied to the data, which only retains proteins identified with 95% confidence.

[d]Percentage coverage is defined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

[e]Sequenced peptides are the number of peptides that were sequenced and gave rise to protein identity. All proteins identified utilizing a single peptide were filtered out of the dataset.

[f]Probability value of the quantitative difference between the treatment and control protein abundance is due to chance alone.

[g]Standard deviation is calculated from the PEG osmotic stressed samples (n = 4) ratios.

[h]Ratio stands for the average fold-change (n = 4) induced by the 10 and 20% PEG treatment relative to the control. Values of one and above signify an upregulation.

[I]Gene ontology analysis as indicated on the UniProt database (http://www.uniprot.org) P represents Biological Process, F for Molecular function, and C for Cellular Component.

[j]Family name as indicated in the UniProt database (http://www.uniprot.org).

The data also showed that sorghum cell cultures responded to both PEG-treatments by altering the levels of proteins involved in metabolism, intracellular traffic, energy, transcription, protein synthesis, protein destination and storage cell structure, disease/ defense and unclassified functional groupings (Table 4.2), However, the metabolism and cell structure functional groups were absent amongst the unique 10% PEG treatment (Table 4.5).

As shown in Figure 4.5B, unique proteins identified in the 10% PEG treatment were, protein synthesis 4 (25%), energy 3 (19%), transcription 3 (19%), metabolism 2 (13%), disease/ defense 2 (13%) unclassified 1 (6%), protein destination and storage 1 (6%). For the 10% PEG-responsive proteins, categories including transcription, disease/ defense, and unclassified contributed to the up-regulated proteins. The proteins associated with energy (19%) were down-regulated, while those in the disease/ defense group were up-regulated (Table 4.5).

Table 4.5: List of osmotic stress-responsive proteins identified in white sorghum cell cultures that are unique to the 10% PEG treatment.

Accession[b]	Prot Name	Ratio[h]	SD[g]	p-val[f]	Family name[j]
Intracellular traffic					
C5Z487	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G163600	1.10	0.05	2.08E-02	Small GTPase
A0A1W0VWX9	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G122200	0.74	0.02	2.12E-02	Small GTPase superfamily, ARF/SAR type
Energy					
C5XFH6	Fructose-bisphosphate aldolase OS=*Sorghum bicolor* GN=SORBI_3003G393900	0.89	0.05	9.49E-03	Fructose-bisphosphate aldolase, class-I
A0A1B6Q6S1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G336000	1.16	0.09	3.62E-02	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent
C5YW21	Malate dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3009G240700	0.73	0.15	2.77E-02	L-lactate/malate dehydrogenase
Transcription					
A0A1B6P9Z6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G213500	0.83	0.07	9.25E-03	Nucleosome assembly protein (NAP)
A0A1W0W662	Histone H4 OS=*Sorghum bicolor* GN=SORBI_3002G291066	2.44	0.57	8.56E-03	HistoneH4
C5YM38	Histone H2B OS=*Sorghum bicolor* GN=SORBI_3007G149600	1.75	0.39	3.95E-02	Histone H2B
Protein synthesis					
A0A1B6PQ87	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G050500	0.90	0.05	4.57E-02	Ribosomal protein L3
A0A1B6QFD0	Eukaryotic translation initiation factor 5A OS=*Sorghum bicolor* GN=SORBI_3002G363100	1.71	0.11	1.06E-04	Translation elongation factor IF5A-like
C5XD92	40S ribosomal protein S3a OS=*Sorghum bicolor* GN=SORBI_3002G364500	1.51	0.28	1.41E-02	Ribosomal protein S3Ae
A0A1B6QMI3	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G334300	0.52	0.04	3.19E-02	Ribosomal protein S19e
Protein destination and storage					
C5Y675	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3005G064200	0.72	0.15	4.74E-02	Aspartic peptidase A1 family
Disease/ defense					
Q4VQB2	Pathogenesis-related protein 10b OS=*Sorghum bicolor* GN=PR10	1.67	0.18	3.11E-03	Bet v I type allergen
Q4VQB4	Pathogenesis-related protein 10c OS=*Sorghum bicolor* GN=PR10	1.16	0.07	4.51E-02	Glycoside hydrolase family 1

Unclassified						
C5YBP8	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3006G009000		1.19	0.08	7.44E-03	Family not predicted

[a]Protein number assigned in ProteinPilot software.

[b]Protein accession numbers acquired from the TrEMBL database incorporated within the UniProt database (http://www.uniprot.org).

[c]Protein score developed by ProteinPilot software relating to the confidence of protein identification. A protein identification threshold of 1.3 was applied to the data, which only retains proteins identified with 95% confidence.

[d]Percentage coverage is defined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

[e]Sequenced peptides are the number of peptides that were sequenced and gave rise to protein identity. All proteins identified utilizing a single peptide were filtered out of the dataset.

[f]Probability value of the quantitative difference between the treatment and control protein abundance is due to chance alone.

[g]Standard deviation is calculated from the PEG osmotic stressed samples (n = 4) ratios.

[h]Ratio stands for the average fold-change (n = 4) induced by the 10% PEG treatment relative to the control. Values of one and above signify an upregulation.

[I]Gene ontology analysis as indicated on the UniProt database (http://www.uniprot.org) P represents Biological Process, F for Molecular function, and C for Cellular Component.

[j]Family name as indicated in the UniProt database (http://www.uniprot.org.).

Table 4.6 show the proteins that are uniquely responsive to 20% PEG-induced osmotic stress. Overall, the 20% PEG treatment experiment induced a larger number of stress-responsive proteins than the 10% PEG treatment. Nine functional categories, namely, metabolism 9 (25%), protein synthesis 5 (14%), transcription 5 (14%), disease/ defense 5 (14%) unclassified 4 (11%), protein destination and storage 3 (8%), intracellular traffic 2 (6%), cell structure 2(6%) and energy 1 (3%) were uniquely identified in white sorghum cell suspension cultures in response to 20% PEG osmotic stress as shown in Figure 4.5C. Transcription, protein synthesis, energy, and disease/ defense categories had the most up-regulated proteins in response to the 20% PEG osmotic stress treatment shown in Table 4.6.

In response to 20% PEG, 39% of unique responsive proteins were up-regulated, and 61% were down-regulated (Table 4.6). Most responsive up-regulated proteins (Table 4.6) were theoretically categorized into transcription, protein synthesis, disease/ defense, and energy. However, most down-regulated responsive proteins were grouped under metabolism. Protein destination and storage, protein synthesis, and the unclassified-related protein were tremendously down-regulated towards the 20% PEG osmotic stress.

Table 4.6: List of osmotic stress-responsive proteins identified in white sorghum cell cultures that are unique to the 20% PEG treatment.

Accession[b]	Prot Name	Ratio[h]	SD[g]	p-val[f]	Family name[j]
Metabolism					
C5X183	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G094300	0.63	0.13	1.38E-02	Family not predicted
A0A1B6QHY1	UDP-glucose 6-dehydrogenase OS=*Sorghum bicolor* GN=SORBI_3001G084100	0.63	0.06	1.69E-02	UDP-glucose/GDP-mannose dehydrogenase family
C5WRH5	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3001G295200	0.75	0.09	4.05E-03	Nucleoside diphosphate kinase
A0A1B6PEZ5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G014700	0.54	0.14	2.34E-02	Family not predicted
A0A1B6PBB3	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3009G259200	0.74	0.05	8.52E-04	Nucleoside diphosphate kinase
C5X972	Nucleoside diphosphate kinase OS=*Sorghum bicolor* GN=SORBI_3002G306900	0.75	0.06	3.07E-02	Nucleoside diphosphate kinase
C5XIV5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G149500	0.82	0.07	1.60E-02	UDP-glucuronic acid decarboxylase
C5YIC1	Aconitate hydratase OS=*Sorghum bicolor* GN=SORBI_3007G068100	0.58	0.09	1.24E-02	Aconitase A/isopropylmalate Aconitase/Iron-responsive element-binding protein 2
C5XF87	Ketol-acid reductoisomerase OS=*Sorghum bicolor* GN=SORBI_3003G239900	0.81	0.14	4.10E-02	Ketol-acid reductoisomerase
Transcription					
A0A1W0VVX1	Histone H4 OS=*Sorghum bicolor* GN=SORBI_3003G057366	2.29	0.51	3.82E-03	Histone H4
C5XPA5	Histone H3 OS=*Sorghum bicolor* GN=SORBI_3004G170500	2.32	0.54	1.13E-02	Histone H3/CENP-A
C5YP98	Histone H2A OS=*Sorghum bicolor* GN=SORBI_3008G114500	0.50	0.08	1.88E-02	Histone H2A
C5WPC4	Histone H2A OS=*Sorghum bicolor* GN=SORBI_3001G416900	1.83	0.19	4.32E-03	Histone H2A
C5XP45	Histone H2B OS=*Sorghum bicolor* GN=SORBI_3003G350100	1.87	0.14	3.34E-04	Histone H2A
Protein synthesis					
C5YH46	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G178600	0.65	0.05	2.39E-02	Ribosomal protein S19e
C5YZ01	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3009G022400	1.99	0.49	8.07E-03	Eukaryotic/archaeal

C5XAD5	Ribosomal protein L15 OS=*Sorghum bicolor* GN=SORBI_3002G047600	1.20	0.13	2.77E-02	Ribosomal protein L15e
A0A1B6QNT2	40S ribosomal protein S7 OS=*Sorghum bicolor* GN=SORBI_3001G403500	1.36	0.13	4.85E-02	Ribosomal protein S7e
C5YMD1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G019400	1.45	0.30	4.60E-02	Ribosomal protein S15
Protein destination and storage					
C5YJ75	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3007G216300	1.47	0.25	1.96E-02	Heat shock protein Hsp90 family
C5X0G5	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G236300	0.85	0.06	2.73E-02	AAA ATPase, CDC48 family
C5XQ74	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G208800	0.62	0.14	1.12E-02	Aspartic peptidase A1 family
Cell structure					
C5WW94	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G197400	0.74	0.17	3.98E-02	Actin family
A0A1B6QIB9	Tubulin alpha chain OS=*Sorghum bicolor* GN=SORBI_3001G107100	0.74	0.10	2.88E-02	Tubulin
Intracellular traffic					
C5XNL6	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G189000	1.16	0.10	3.25E-02	Small GTPase
C5YW22	GTP-binding nuclear protein OS=*Sorghum bicolor* GN=SORBI_3009G240900	0.75	0.09	3.87E-02	Small GTPase
Energy					
C5YF55	Ubiquinol oxidase OS=*Sorghum bicolor* GN=SORBI_3006G202500	1.45	0.29	4.61E-02	Alternative oxidase
Disease/ defense					
C5Z529	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3010G173100	0.77	0.07	6.50E-03	Glutathione Transferase family
A0A1W0VY40	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3003G197200	0.63	0.10	1.86E-02	Family not predicted
C5Y360	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3005G011300	2.73	0.78	5.06E-03	Plant peroxidase
A0A194YU12	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3004G341200	1.55	0.33	3.84E-02	Pyridine nucleotide-disulphide oxidoreductase, class I
C5XIY2	Peroxidase OS=*Sorghum bicolor* GN=SORBI_3003G152200	2.09	0.47	4.90E-03	Plant peroxidase
Unclassified					
C5X1K7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G247600	0.84	0.06	1.94E-02	Family not predicted

C5X1Q1	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G385900	0.84	0.04	9.42E-03	Ricin B-like lectin EULS3-like
C5WW09	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3001G042500	0.59	0.06	2.38E-05	Family not predicted
C5XBP7	Uncharacterized protein OS=*Sorghum bicolor* GN=SORBI_3002G343600	0.72	0.11	1.67E-02	Family not predicted

[a]Protein number assigned in ProteinPilot software.

[b]Protein accession numbers acquired from the TrEMBL database incorporated within the UniProt database (http://www.uniprot.org).

[c]Protein score developed by ProteinPilot software relating to the confidence of protein identification. A protein identification threshold of 1.3 was applied to the data, which only retains proteins identified with 95% confidence.

[d]Percentage coverage is defined by the number of amino acids of sequenced peptides against the total length of the protein, with a threshold of at least 95% confidence.

[e]Sequenced peptides are the number of peptides that were sequenced and gave rise to protein identity. All proteins identified utilizing a single peptide were filtered out of the dataset.

[f]Probability value of the quantitative difference between the treatment and control protein abundance is due to chance alone.

[g]Standard deviation is calculated from the PEG osmotic stressed samples (n = 4) ratios.

[h]Ratio stands for the average fold-change (n = 4) induced by the 20% PEG treatment relative to the control. Values of one and above signify an upregulation.

[i]Gene ontology analysis as indicated on the UniProt database (http://www.uniprot.org) P represents Biological Process, F for Molecular function, and C for Cellular Component.

[j]Family name as indicated in the UniProt database (http://www.uniprot.org).

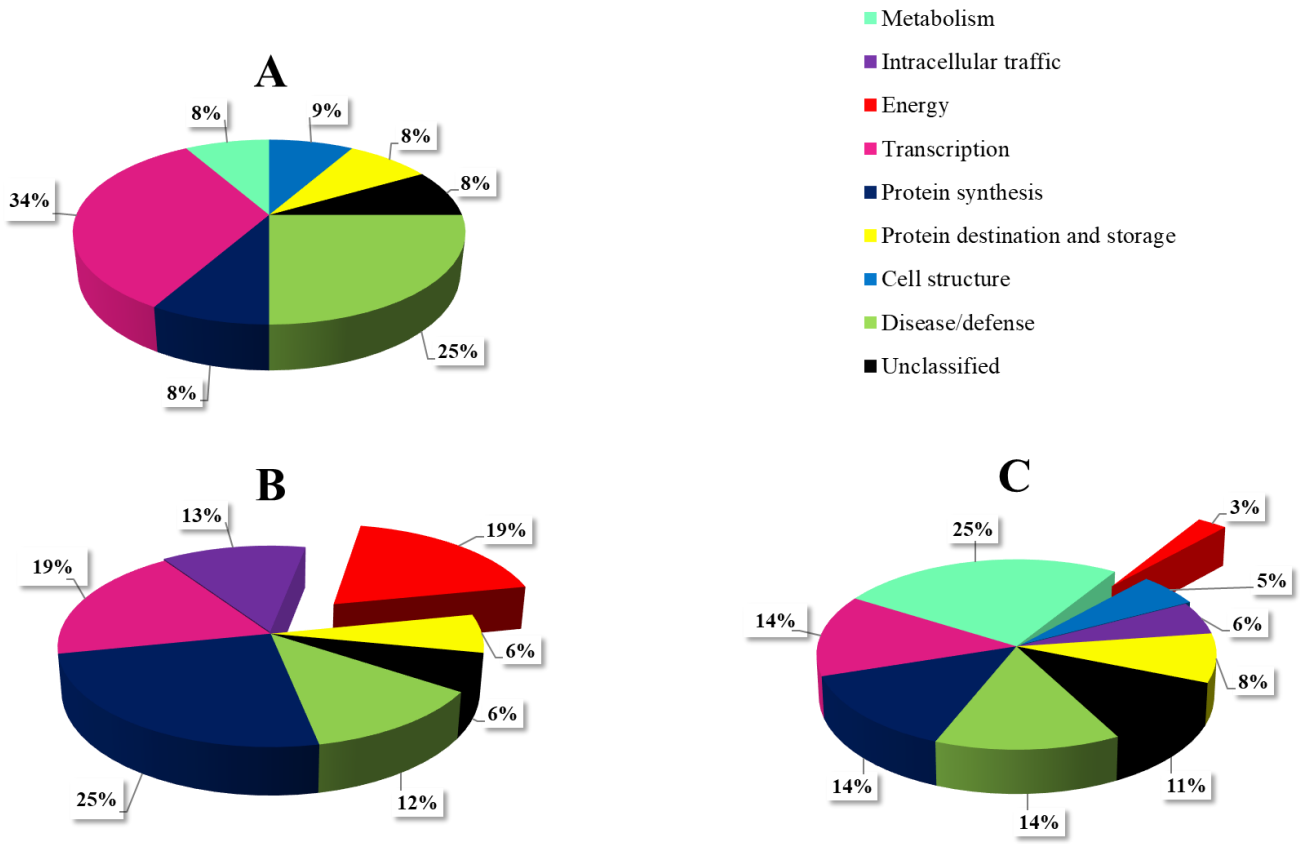

Figure 4.5: Functional categories of the common and unique PEG-responsive proteins of cell suspension cultures. The responsive proteins were classified into seven functional categories for (**A**) common to both 10 and 20% PEG and (**B**) unique to 10% PEG and (**C**) unique to 20% PEG osmotic stress treatments.

4.3 Discussion

This study used two different PEG concentrations to induce two levels of osmotic stress in white sorghum cell suspension cultures. The intensity of stress impacts plant proteomics depending on a plethora of factors; hence the current study assessed how white sorghum is affected by the two stress levels. Osmotic stress alters the relative abundance of responsive proteins, affects the nature of post-transcriptional and translational modification (PTMs), protein-to-protein interaction, and their biological functions (Kosová *et al*., 2018). The osmotic stress-responsive proteins were visually observed on a 1D gel (Figure 4.1). However, the visual display of the 1D gel did not show any up or down-regulation differences between the control and the PEG-treated samples. Ngara *et al*. (2008), reported that the 1D gel profiles of total soluble protein (TSP) are visually different from those of culture filtrate (CF) proteins. The TSP comprises of multiple, but less-abundant proteins, while CF exhibits few but relatively highly abundant proteins (Ngara *et al*., 2008). In the current study, the TSP expression profiles of the two osmotic stress treatments displayed numerous proteins but with similar expressions levels between all three treatment groups (Figure 4.1). The 1D gel did not show any visible changes possibly because of the type of proteins run, as many studies usually use culture filtrates on a 1D gel (Ngcala, 2018; Ramulifho, 2017). A plausible reason here is that 1D gels are not very sensitive is showing differential expression patterns of proteins found in low abundance.

The stress effects were monitored and quantified using the iTRAQ technique, a gel-free proteomic approach. Many researchers have used the iTRAQ method to study changes in protein expression levels in response to osmotic stress (Hu *et al*., 2015; Ngara *et al*., 2018). Proteomic data assist in understanding the functions of individual proteins and their broader role within complex cellular processes and locations (Gulcicek *et al*., 2005). The expression

level of a particular protein in response to stress can be correlated with the alteration in cellular processes during normal growth and under the influence of biotic and abiotic stimuli (Sánchez-Guerrero *et al*., 2019). Tables 4.2 and 4.3 and Figure 4.3 with Gene Ontology annotations reflected the differences in the biological processes, molecular functions, and cellular components of the PEG-responsive proteins identified in sorghum cells exposed to the two levels of PEG-induced osmotic stress. The abundances and uniqueness of responsive proteins highly depend on the stress level endured (Kosová *et al*., 2018); stress-tolerant genotypes differ in coping strategies and can also show opposite patterns in mild versus severe stress (Kosová *et al*., 2018). To show the correlation between the stress level and expressed proteins, the responsive proteins were tabulated into those that were common to both osmotic stress levels (Table 4.4) and the uniquely responsive proteins to each osmotic stress level (Table 4.5 and 4.6). These dynamic data provided by comparative iTRAQ data analysis bring a more in-depth understanding of osmotic stress response mechanisms in white sorghum cell suspension culture. Overall, the 20% PEG-induced osmotic stress had the highest number of responsive proteins compared to the 10% PEG-induced stress. In other studies, severe stress has been reported to induce the greatest number of responsive proteins compared to mild stress (Kosová *et al*., 2018).

The osmotic stress-responsive proteins unique to the 10 and 20% PEG treatments were grouped into seven and nine functional categories, respectively (Table 4.5 and 4.6). The molecular category with the most response proteins between the two stress treatments was metabolism (25%) after exposure to 20% PEG osmotic stress. Irrespective of the level of PEG-induced osmotic stress, sorghum cell cultures responded to the imposed stress by up-regulating the expression of the protein synthesis-related proteins while down-regulating those involved in metabolism, protein destination and storage, and cell structure (Table 4.5 and 4.6). Although

the 10 and 20% PEG osmotic stress induced a metabolic response, only 20% PEG gave a pronounced down-regulation of all the metabolism-related proteins (Table 4.6.), while 10% PEG generated no unique responsive proteins assigned the metabolism category.

Osmotic stress is known to affect cellular metabolism (Qamer *et al*., 2021), and stress-induced metabolic changes may result in the accumulation of metabolites and sugars (Matthews *et al*., 2013). Therefore, metabolism-related proteins may be considered important in osmotic stress. However, what precise role their down-regulation may play under osmotic stress needs further investigation. Nevertheless, white sorghum cell suspension cultures under PEG-induced osmotic stress down-regulated all metabolic-related proteins, possibly to conserve cellular energy towards signalling pathways and cellular disease/ defense machinery to regulate osmotic and oxidative stress (Goche *et al*., 2020) (Table 4.4 and 4.6). Therefore, these results possibly indicate that as cells encounter severe stress, cellular energy is re-directed where it is most required (Goche *et al*., 2020).

Osmotic stress causes oxidative stress due to the overproduction and accumulation of ROS (Ahmad *et al*., 2008). When plants encounter drought or osmotic stress, ROS act as signalling molecules, but cause oxidative stress at high concentrations (Hancock' *et al*., 2001). Oxidative stress negatively affects lipids, proteins, and nucleic acids (Miller *et al*., 2010). To detoxify the adverse effects of ROS generated by osmotic stress, cells employ enzymatic and non-enzymatic antioxidant-response mechanisms (Carvalho, 2008). Responsive proteins categorized in disease/ defense are crucial in the survival of plants after the harmful effects of osmotic stress (Nouri and Komatsu, 2010; Ngara *et al*., 2012). The current study shows an up-regulation of plant peroxidases, germin, Bev 1 type allergen, glycoside hydrolase family 1, and pyrine nucleotide-disulfide oxidoreductase families of proteins (Tables 4.4, 4.5, and 4.6).

Table 4.4 also shows that 67% of the common responsive proteins to both stress treatments were up-regulation classified in the disease/ defense category. A germin protein with accession number C5WNY4 was up-regulated in response to both PEG osmotic stress treatments within the disease/ defense functional category. Germin proteins are involved in signalling during seed germination (Patnaik & Khurana, 2001). These glycoproteins are also stable under extreme environmental conditions and are protease-resistant (Patnaik & Khurana, 2001). Table 4.4 indicates a cupin domain on the germin proteins, and they are associated with cell walls. Their increased accumulation in sorghum cell suspension culture possibly implicates them in osmotic stress response. These germin proteins were also immensely up-regulated in the sorghum secretome in response to high-temperature stress (Ngcala, 2018).

All the unique 10% PEG response proteins classified in disease/ defense were up-regulated, including a glycoside hydrolase protein (Table 4.5) The glycoside hydrolase protein family is a group of cellulose-degrading enzymes that modify cell walls (Mamo *et al*., 2014). These cellulose-degrading enzymes randomly attack the β-1,4-linkages within the polymer chain and release oligosaccharides (Moreira and Filho, 2016). Glycoside hydrolases are developmental- and stress-regulated enzymes rearranging the plant cell wall polysaccharides (Buckner *et al*., 2016). As cells under osmotic stress try to acclimate to stress, significant alterations are made at the cell wall, the plasma membrane, and the plant cell wall polysaccharides is rearranged by proteins such as the germins and glycoside hydrolases (Mamo *et al*., 2014).

An increase in the accumulation of disease/defense-related proteins is expected to be observed as the level of stress increases to 20% PEG to prevent oxidative damage caused by osmotic stress. However, in response to 20% PEG stress treatment, three out of five (60%) up-regulation

of most disease/ defense-related proteins is observed in this study (Table 4.6), even though after 10% PEG osmotic stress, white sorghum had a three out of three (100%) up-regulated all the responsive proteins assigned in disease/ defense category (Table 4.5). Most of the up-regulated PEG-responsive proteins were involved in peroxidase activity (Table 4.6). Lum *et al*, (2014), used rice (*Oryza sativa L*.) to evaluate the activity of antioxidant enzymes during PEG-induced osmotic stress, peroxidases were reduced in the drought-sensitive variety. A similar study showed peroxidases were up-regulated in the roots of rice (*Oryza sativa L. cv. IR64*) after withholding water to impose drought stress (Mehdi Mirzaei, 2012). The results further emphasize the important roles of peroxidases in response to water limitation.

When plants encounter stress, they control the gene expression patterns through complex transcriptional networks (Singh and Laxmi, 2015). All the H2B histone proteins were up-regulated in response to 10 and 20% PEG-induced osmotic stress (Table 4.4). The transcription category had the greatest number of responsive proteins that were up-regulated in response to both PEG stress treatments. These proteins were up-regulated possibly because histone proteins are the main structural components that assist in modifying chromatin structure and the main determinants of gene regulation (Trivedi *et al*., 2012). In response to stress, chromatin is modified, a process central to the transcriptional control of genes that respond to stress (Asensi-Fabado *et al*., 2017). However, a histone H2B protein was down-regulated in a Mediterranean shrub (*Cistus albidus*) after being subjected to a long-term summer drought (Brossa *et al*., 2015). In another study, the levels of histones were low in drought-sensitive maize (*Zea mays*) cultivar, while they increased in the drought-tolerant variety of a proteomic study under drought stress, implicating the role of histone proteins in stress-tolerance mechanism (Benešová *et al*., 2012). From the data obtained in the current study, it seems histone proteins are responsive to both 10 and 20% PEG-induced osmotic stress. These proteomic changes

possibly imply that histones are important for gene expressional changes necessary for white sorghum to survive the imposed stress.

Alternative oxidase functions in an energy-dissipating pathway that reduces ROS within the mitochondria (Pastore *et al*., 2001; Carvalho, 2008). This pathway is an alternative to the cytochrome pathway that produces water and reduces oxygen, by diverting electrons in the electron transport chain to produce water (Pastore *et al*., 2001). White sorghum cell cultures had only one protein belonging to the alternative oxidase family present and up-regulated in response to 20% PEG-induced osmotic stress (Table 4.6). This research throws many questions that still need further investigation regarding the distribution of energy-related proteins since at the most severe stress, only one protein is responsive whereas three proteins are responsive towards 10% PEG osmotic stress (Table 4.5).

Alternative oxidase also assists plants in recovering from extreme stress; lack of it will ultimately compromise the plant in recovery (Wang and Vanlerberghe, 2013). As stated above, it is part of alternative mitochondrial respiration, an additional cyanide-insensitive pathway for driving electrons to oxygen, a pathway that decreases the levels of ROS from osmotic stress (Wang and Vanlerberghe, 2013). Alternative oxidase is also induced in response to other stress, such as freezing in common wheat (Mizuno *et al*., 2008) and salinity in Arabidopsis (Smith *et al*., 2009).

CHAPTER 5

GENERAL CONCLUSIONS, AND RECOMMENDATIONS

Around the world, sorghum is the fifth most cultivated cereal crop (Food Security Department, 2019); it sustains human life with various industrial and economic applications related to feeding humans and animals, energy, biofuel, and fiber (Kimber *et al*., 2013). The natural drought-tolerant nature of sorghum makes it an important cereal for stress-related research (Kimber *et al*., 2013; Motlhaodi *et al*., 2017). It can survive in hot and dry environments where most cereal crops barely give maximum yield (Xin and Aiken, 2022).

Prior studies have discussed the importance of understanding and identifying plant molecular responses to drought stress to ensure food security by improving drought tolerance levels of crops (Kosová *et al*., 2018). Breeding and biotechnological applications improve crops (Matthews *et al*., 2013), and drought-responsive proteins further expand our understanding of adaptive responses that cereal crops deploy to survive environmental stresses (Ghatak *et al*., 2017). However, drought-responsive protein patterns may vary depending on crop species, genotype, and the intensity of the stress endured (Desclaux and Roumet, 1996; Reddy *et al*., 2004; Taiz and Zeiger, 2010).

In conclusion, the cell viability assays showed that both 10 and 20% polyethylene glycol (PEG) affected the metabolic activities of white sorghum cell cultures differently. Specifically, some research studies on osmolytes and some amino acids show that the responsive pattern of these cellular components are highly time-course dependent (Bianco-Trinchant and Le Page-Degivry, 1998). Therefore, there is a need to re-structure the experimental setup and review other extraction and analysis protocols for metabolites profiles.

There was a notable difference in the expression patterns of proteins induced by these two levels of PEG-induced osmotic stress. The quantitative proteomic method of isobaric tagging and mass spectrometry gave an insight into how sorghum regulates proteins in response to osmotic stress. To some extent, the results emphasized the theory that osmotic stress causes an overproduction of reactive oxygen species, whose adverse effects can be eliminated by the activity of antioxidant enzymes such as peroxidases and glycoside hydrolase, alterations in detoxification, metabolism, and protein synthesis related proteins under osmotic stress.

The study extrapolated the following recommendations:

1. The expression of target genes in response to PEG osmotic stress should be validated using quantitative real-time polymerase chain reaction,
2. The expression profiles of metabolites should be evaluated over an extended period of PEG osmotic stress treatment.
3. Different metabolite extraction protocols should be evaluated for other metabolites including trehalose and other sugars.

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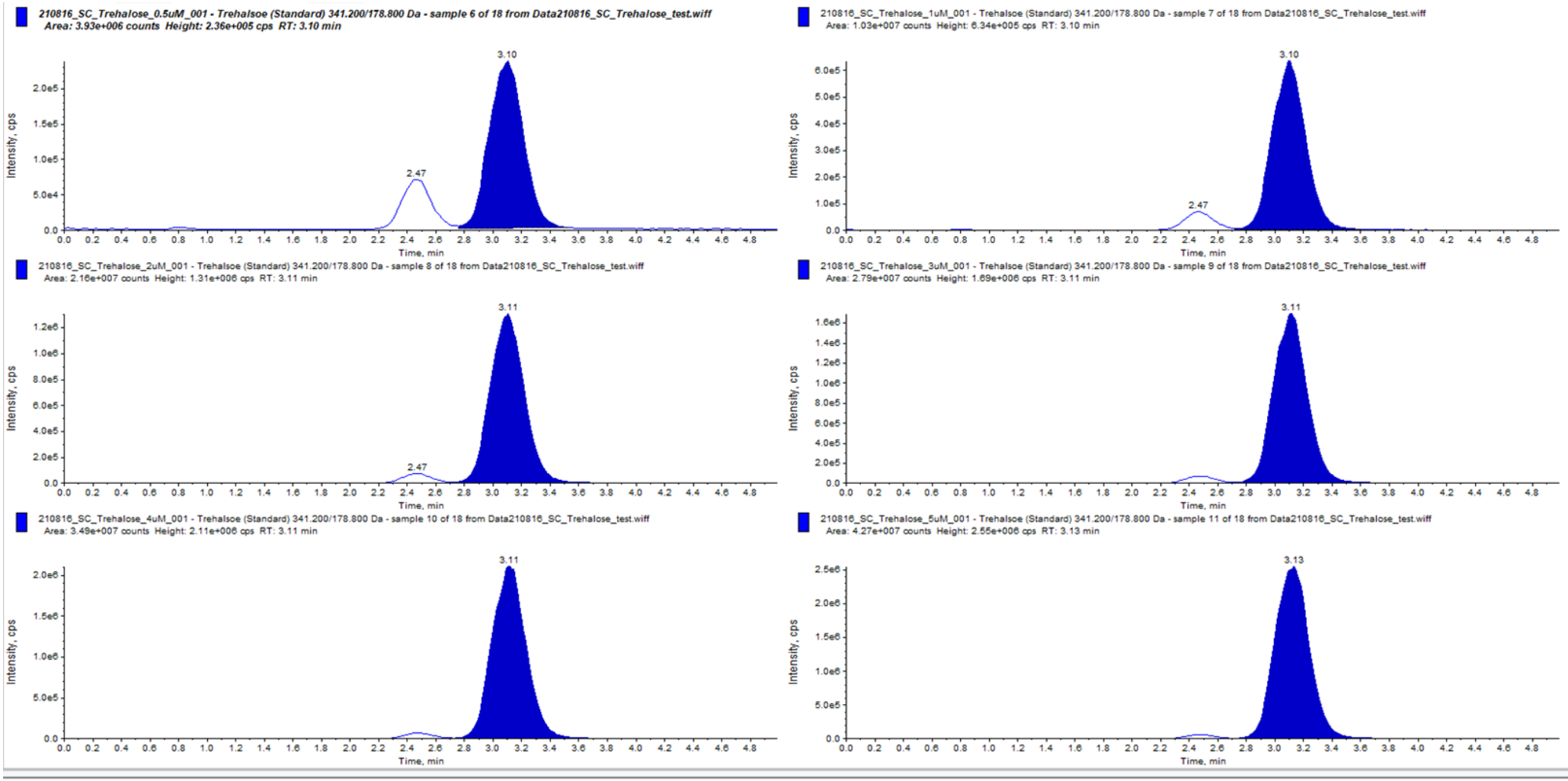
APPENDICES

Appendix: Bradford assay, trehalose standards and Gene Ontology annotations

Appendix Table A1: BSA standard solutions preparation used in protein quantification

BSAµg	BSA final concentration (mg/mL)	Urea extraction buffer (µl)	0.1 M HCl (µl)	Distilled water (µl)	Protein Assay Dye Reagent REAGENT
0	-	10	10	80	900
1	1	9	10	80	900
2	2	8	10	80	900
4	4	6	10	80	900
8	8	2	10	80	900
10	10	-	10	80	900

Appendix Table A2: Trehalose Standards 0.5-5µM



Appendix Table A3: Gene Ontology distribution profiles of 10% PEG responsive proteins

Gene Ontology Annotation	Protein regulation		Total number of proteins	Percent of protein regulation	
	Up	Down		Up	Down
Biological Process					
fructose 1,6-bisphosphate metabolic process	0	1	1	0	100
malate metabolic process	0	1	1	0	100
chromatin silencing	0	2	2	0	100
ribosomal large subunit assembly	0	1	1	0	100
protein folding	0	1	1	0	100
proteolysis	0	1	1	0	100
plant-type cell wall biogenesis	0	1	1	0	100
defense response	0	1	1	0	100
carbohydrate metabolic process	0	1	1	0	100
nucleosome assembly	1	3	4	25	75
intracellular protein transport	1	1	2	50	50
translation	2	1	3	67	33
cellular carbohydrate metabolic process	1	0	1	100	0
positive regulation of translational elongation	1	0	1	100	0
abscisic acid-activated signalling pathway	2	0	2	100	0
response to oxidative stress	1	0	1	100	0
not classified	1	2	3	33	67
Cellular component					
Golgi apparatus	3	1	1	0	100
cytosol	3	2	2	0	100
chromatin	3	1	1	0	100
endoplasmic reticulum lumen	3	1	1	0	100
integral component of membrane	3	1	1	0	100
cytosolic large ribosomal subunit	2	2	4	50	50
nucleosome	3	3	6	50	50
cytoplasm	3	1	4	75	25
endomembrane system	1	0	1	100	0
apoplast	1	0	1	100	0
plant-type cell wall	1	0	1	100	0
not classified	3	1	4	75	25
Molecular Function					
beta-glucosidase activity	0	1	1	0	100
fructose-bisphosphate aldolase activity	0	1	1	0	100
L-malate dehydrogenase activity	0	1	1	0	100
chromatin binding	0	1	1	0	100
RNA binding	0	1	1	0	100
unfolded protein binding	0	1	1	0	100
peptidase activity	0	1	1	0	100
UDP-arabinopyranose mutase activity	0	1	1	0	100
GTPase activity	1	1	2	50	50
DNA binding	4	2	6	67	33
structural constituent of ribosome	2	1	3	67	33
2,3-bisphosphoglycerate-independent phosphoglycerate mutase activity	1	0	1	100	0
translation elongation factor activity	1	0	1	100	0
abscisic acid binding	2	0	2	100	0
manganese ion binding	1	0	1	100	0
peroxidase activity	1	0	1	100	0
not classified	1	2	3	33	67

Appendix Table A4: Gene Ontology distribution profiles of 20% PEG responsive proteins

Gene Ontology Annotation	Protein regulation		Total number of proteins	Percent of protein regulation	
	Up	Down		Up	Down
Biological Process					
sulfate assimilation	0	1	1	0	100
glycosaminoglycan biosynthetic process	0	1	1	0	100
carbohydrate metabolic process	0	1	1	0	100
CTP biosynthetic process	0	3	3	0	100
fructose metabolic process	0	1	1	0	100
D-xylose metabolic process	0	1	1	0	100
citrate metabolic process	0	1	1	0	100
isoleucine biosynthetic process	0	1	1	0	100
ribosomal small subunit assembly	0	1	1	0	100
ER-associated misfolded protein catabolic process	0	1	1	0	100
proteolysis	0	1	1	0	100
plant-type cell wall biogenesis	0	1	1	0	100
microtubule cytoskeleton organization	0	1	1	0	100
protein import into nucleus	0	1	1	0	100
glutathione metabolic process	0	1	1	0	100
defense response	0	1	1	0	100
specification of floral organ number	0	1	1	0	100
chromatin silencing	1	3	4	25	75
protein folding	1	1	2	50	50
nucleosome assembly	4	0	4	100	0
translation	5	0	5	100	0
protein transport	1	0	1	100	0
alternative respiration	1	0	1	100	0
response to oxidative stress	3	0	3	100	0
cell redox homeostasis	1	0	1	100	0
not classified	2	6	8	25	75
Cellular component					
chloroplast	0	1	1	0	100
endoplasmic reticulum lumen	0	1	1	0	100
cytoskeleton	0	1	1	0	100
microtubule	0	1	1	0	100
nucleus	0	1	1	0	100
cytosol	1	5	6	17	83
integral component of membrane	1	1	2	50	50
cytoplasm	1	1	2	50	50
nucleosome	6	3	9	67	33
cytosolic small ribosomal subunit	5	1	6	83	17
apoplast	1	0	1	100	0
extracellular region	3	0	3	100	0
not classified	1	13	14	7	93
Molecular Function					
ATP binding	0	5	5	0	100
NAD binding	0	2	2	0	100
beta-glucosidase activity	0	1	1	0	100
fructokinase activity	0	1	1	0	100
aspartic-type endopeptidase activity	0	1	1	0	100

UDP-arabinopyranose mutase activity	0	1	1	0	100
glutathione transferase activity	0	1	1	0	100
oxidoreductase activity	0	1	1	0	100
enzyme inhibitor activity	0	1	1	0	100
GTPase activity	1	2	3	33	67
metal ion binding	1	2	3	33	67
RNA binding	1	1	2	50	50
Unfolded protein binding	1	1	2	50	50
DNA binding	6	3	9	67	33
structural constituent of ribosome	3	0	3	100	0
mRNA binding	1	0	1	100	0
manganese ion binding	1	0	1	100	0
peroxidase activity	3	0	3	100	0
flavin adenine dinucleotide binding	1	0	1	100	0
not classified	0	4	4	0	100