Proteomic Mapping of the *Sorghum bicolor* (L.) Moench Cell Suspension Culture Secretome and Identification of its Drought Stress Responsive Proteins

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Science, in the Faculty of Natural and Agricultural Sciences, Department of Plant Sciences, Qwaqwa Campus, University of the Free State.

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DECLARATION

I declare that **Proteomic mapping of *Sorghum bicolor* (L.) Moench cell suspension culture secretome and identification of its drought stress responsive proteins** is my original work, and has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Elelwani Ramulifho

January 2017

Signed_____

ABSTRACT

Drought (also known as osmotic stress), adversely effects crop productivity. With the projected increase in global surface temperatures, the frequency and intensity of drought is predicted to increase, worldwide. It is therefore important to develop crops that can withstand drought and thus alleviate food insecurity. However, the success of such breeding initiatives requires prior understanding of plant stress response mechanisms. Sorghum (*Sorghum bicolor*), a naturally drought tolerant cereal crop, is a potentially good model system for studying plant responses to drought stress. The objectives of this study were to establish a sorghum cell suspension culture system, map its secretome and identify the osmotic stress responsive proteins. In this study, seeds from eight sorghum genotypes, namely SA 1441, ICSV 210, ICSV 112, ICSV 213, ICSB 78, ICSB 338, Macia, and White sorghum, were used to establish callus and cell suspensions for use in secretome analysis. Murashige and Skoog Basal Salt with minimal organics medium supplemented with varying concentrations of plant growth hormones, 1-naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) were used for callus induction. ICSB 338 and White sorghum produced large friable callus masses on medium supplemented with 2.5 mg/L NAA and 3 mg/L 2,4-D. These callus masses were subsequently used to establish cell suspension cultures, which were further characterised in terms of cell growth and viability patterns following sorbitol-induced osmotic stress. The cell growth plots conformed to a typical sigmoidal growth curve with distinct lag, exponential, and stationary phases. Osmotic stress experiments were carried out on ICSB 338 and White sorghum cell cultures using 400 mM sorbitol for 72 hr. Cell viability and microscopic analysis indicated a change in metabolic activity and structural changes of cells following osmotic stress treatment. Culture filtrate proteins (referred to as secreted proteins in this study), were extracted from both cell cultures. Differential protein expressions of the secreted proteins of the two cultures were

observed on Coomassie Brilliant Blue-stained one-dimensional sodium dodecyl sulfate-polyacrylamide gels. The White sorghum secreted proteins after 48 hr of sorbitol treatment were further analysed by the isobaric tags for relative and absolute quantitation (iTRAQ) method. A total of 178 sorghum secreted proteins were positively identified, with some matching proteins from plant peroxidase, glycoside hydrolase, Expansin/Lol pl, germin, and peptidase C1A protein families. However, 78% of the 178 positively identified proteins were uncharacterised, possibly indicating novel sorghum proteins. SignalP 4.1 predicted signal peptides on 128 (72%) of the positively identified proteins, indicating that they are classically secreted into the extracellular matrix, while 50 (28%) were not. Out of the 178 positively identified secreted proteins, 152 were differentially expressed in response to osmotic stress with 148 (97%) and 4 (3%) being up-regulated and down-regulated, respectively. The osmotic stress responsive proteins were predicted to have putative functions in metabolism (33.5%), disease/defence (23%), protein destination and storage (13%), signal transduction (8%), energy (6.5%), cell growth/division (6%), cell structure (3%), intracellular traffic (1%), and secondary metabolism (1%); while 3% were unclassified and 2% unclear classifications, respectively. This study reports the first comprehensive sorghum cell suspension culture secretome map and its osmotic stress responsive proteins. The secretome mapping data reported in this study can be used as a reference for studies focussing on characterising sorghum secreted proteins in response to a wide range of biotic and abiotic stresses, thus further advancing existing knowledge on sorghum response networks.

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DEDICATIONS

I am dedicating this work to my parents, Mrs M. and Mr P. Ramulifho. You did not make it to secondary school, yet you understood how important is was for your children to be educated. Even when you did not have enough, with the little you had you supported us and prayed for us tirelessly. Today we are better because you loved us. Thank you mma na baba.

LIST OF ABBREVIATIONS

1D	One-dimensional
2,4-D	2,4-dichlorophenoxyacetic acid
2D	Two-dimensional
ABA	Abscisic acid
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBB	Coomassie Brilliant blue
CF	Culture filtrate
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CPS	Classical or conventional protein secretion pathway
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol Cleland's reagent
ECM	Extracellular matrix
ECS	Extracellular space
ESI	Electrospray ionization
EtOH	Ethanol
HCl	Hydrochloric acid
hr	Hour
Hsp	Heat shock protein
iTRAQ	Isobaric tags for relative and absolute quantitation
kDa	kilo Dalton
LC	Liquid chromatography
LEA	Late-embryogenesis abundant protein
LRR	Leucine-rich repeat
MALDI	Matrix assisted laser desorption/ionisation

MASCOT	Matrix Science
MES	2-(N-Morpholino)ethanesulfonic acid
min	Minutes
mRNA	Messenger Ribonucleic Acid
MS	Mass spectrometry
MS medium	Murashige and Skoog Basal medium
MSMO	Murashige and Skoog Basal Salt with minimal organics
MS/MS	Tandem mass spectrometry
mTRAQ	Mass differential tags for relative and absolute quantification
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MudPIT	Multidimensional protein identification technology
m/v	mass to volume
MW	Molecular weight
m/z	mass to charge ratio
NAA	1-naphthaleneacetic acid
NaOH	Sodium hydroxide
PAGE	Polyacrylamide gel electrophoresis
PCV	Packed cell volume
PGH	Plant growth hormone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-biphosphate carboxylase/oxygenase
SCV	Settled cell volume
SDS	Sodium dodecyl sulfate
SILAC	Stable isotope labelling by amino acids in cell culture
SP	Signal peptide
TCA	Trichloroacetic acid
TMT	Tandem mass tag

TOF	Time of flight
TSP	Total soluble protein
UPS	Unconventional protein secretion pathway
V	Volts
v/v	volume to volume
w/v	weight to volume

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OUTPUTS FROM THIS STUDY

1. Ramulifho, E., Tsilo, T. and Ngara, R. (2016). Secretome mapping of a *Sorghum bicolor* cell suspension culture system. Poster presented at the 2016 joint SAAB/SASSB conference, 10-13 January 2016, University of the Free State, Bloemfontein. *South African Journal of Botany*. DOI 10.1016/j.sajb.2016.02.160.

2. Results of Chapters 3 and 4 have been submitted for peer review as follows:
Ramulifho, E., Van As, J., Tsilo, T. and Ngara, R. Establishment of callus and cell suspension cultures of eight *Sorghum bicolor* (L.) Moench varieties. *Indian Journal of Plant Physiology*, Manuscript number: INPP-D-00023.

3. Ramulifho E, Tsilo T & Ngara R (2017). Establishment and characterisation of cell suspension cultures of two *Sorghum bicolor* varieties. Oral presentation presented at the 2017 SAAB conference, 8-11 January 2017, Lagoon Beach Hotel, Cape Town.

CHAPTER 1

LITERATURE REVIEW

1.1. Sorghum Production, Uses, and Potential Applications

Sorghum [*Sorghum bicolor* (L.) Moench; Figure 1.1] is a naturally drought tolerant crop (Rosenow *et al*., 1983) belonging to the family Poaceae. It is the fifth most produced cereal in the world after maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*; FAOSTAT, 2016). In addition, sorghum is one of the main staple foods in African and Asian countries, where it provides better household food security in drought prone areas (Henley *et al*., 2010). Apart from being used as a source of energy and micronutrients for humans, sorghum is also used as animal feed and a source of biofuel (Henley *et al*., 2010).

Figure 1.1. Cultivation of sorghum crop. Source: (Department of Agriculture and Food, Western Australia).

In the year 2014, approximately 68 million tons of sorghum were produced worldwide. Africa was the main producer, producing 43% of sorghum, followed by the Americas (39%), Asia (14%), and Oceania and Europe, both contributing 2% to the world's sorghum production (FAOSTAT, 2016). South Africa contributed about 151 000 tons of sorghum production in Africa, which approximate to about 0.5% of Africa's production.

Sorghum may potentially provide food in drought prone areas, because of its natural ability to withstand and grow under drought conditions, and thus address issues of food insecurity under the current global climatic change. The global mean surface temperature is estimated to rise in the range of 1.8°C to 4.0°C by the year 2100 (IPCC, 2007). With these projected increases in surface temperatures and the prevalence of drought episodes in Africa (Gan *et al*., 2016), food scarcity is imminent. It is estimated that by the year 2080, between 5 million and 170 million additional people worldwide will be at risk of hunger (Schmidhuber and Tubiello, 2007). As such, breeding for crops that are well-adapted to these unfavourable environmental conditions is becoming more urgent (IPCC, 2014).

However, the success of such breeding initiatives requires a prior understanding of plant stress response mechanisms. Sorghum can be used as a model plant system for studying mechanisms of drought tolerance in cereals (Ngara and Ndimba, 2014), because of its wide genetic diversity and its natural tolerance to drought. The information gained from such studies will then be implemented in breeding programmes aimed at producing more drought tolerant crops. Plant tissue culture systems are important experimental tools for studying plant stress response mechanisms.

1.2. Introduction to Plant Tissue Culture

Plant tissue culture involves a range of procedures used to maintain and grow plant tissues (calli, cells, and protoplasts) and organs (stems, roots, and embryos) in aseptic

or *in vitro* culture (Razdan, 1993). This technique is mostly used for the propagation, plant breeding, biomass production of biochemical secondary products, and scientific investigations of plants such as those focused on improving yield and quality. In the current study, the plant tissue culture technique was used to initiate and maintain sorghum callus and cell suspension cultures. Calli are unorganized masses of undifferentiated cells resulting from the uncoordinated and disorganized growth at the site of wounding in plants (George *et al*., 2008). Calli can either be friable or non-friable. Friable calli consist of loosely packed cells and show no apparent organ regeneration, while non-friable calli consist of densely packed cells, which are hard in texture (Evans *et al*., 2003). Some calli show organ regeneration to some extent and these are either called 'rooty' or 'shooty' callus depending on the type of organ regenerating (Frank *et al*., 2000).

In nature, calli are produced in response to different abiotic and biotic stimuli such as wounding and pathogenic attacks, respectively (Ikeuchi *et al*., 2013). In plants, wounding can be caused by strong winds, rain, snow and pathogen or insect attack (Lukaszuk and Ciereszko, 2012). These calli are thought to be a protective response by the plants against injury, infection and water loss, and often accumulate compounds such as phytoalexins and pathogen-related proteins involved in fighting the outside invader (Evans *et al*., 2003; Ikeuchi *et al*., 2013). Figure 1.2 A shows callus masses forming on a plant stem under natural conditions. In tissue culture (*in vitro*) conditions, callus formation (Figure 1.2 B) is induced by exogenously adding moderate to high levels of auxin and cytokinin plant growth hormones (PGHs), either individually or in combination. If either auxins or cytokinins are in excess, adventitious roots or shoots will form, respectively (George *et al*., 2008).

Figure 1.2. Callus masses produced in nature (A) and *in vitro* culture (B). Source: [Henderson State University (picture A) and Ramulifho Elelwani (picture B)].

In plant tissue culture, calli are important, mainly for plant regeneration and the establishment of cell suspension cultures (Evans *et al*., 2003). By definition, a cell suspension culture is a population of undifferentiated cells grown in liquid culture (Evans *et al*., 2003). Calli and cell suspension cultures are potentially useful experimental systems in the field of plant biology due to the high rates of cell multiplication, which provides a consistent supply of experimental units (Cai *et al*., 1987). Several research groups have used calli and/or cells in suspension as experimental systems in proteomics studies including the secretomics of different plant species and how they respond to an array of biotic and abiotic stress factors (Cho *et al*., 2009; Gupta *et al*., 2011; Ngara and Ndimba, 2011).

1.3. General Plant Responses to Abiotic Stress

The natural environment of plants is composed of a complex set of abiotic and biotic stress factors, which affect plant growth and development. Abiotic stress factors such as extremes in temperature, light, water supply and nutrient levels reduce the growth and

yield of plants below optimum levels (Cramer *et al*., 2011). These stress factors are a major concern in agriculture, as they reduce agricultural productivity, eventually resulting in severe economic losses and a reduction in food supply worldwide.

Plants are sessile and thus cannot move when their physical environment becomes unfavourable for normal growth and development. As a result, plants are constantly faced with the challenge of recognizing and responding to abiotic stress factors to avoid detrimental effects on their growth and development (Knight and Knight, 2001; Atkinson *et al*., 2015). In nature, plants encounter stress factors that occur concurrently, in contrast to short-term single stress factors, which are usually examined in laboratories (Knight and Knight, 2001). Plants respond to these abiotic stresses by activating cascades of molecular networks involved in stress perception, signal transduction and the expression of specific stress-related genes, proteins and metabolites (Figure 1.3; Vinocur and Altman, 2005). These responses may occur as a cross-talk, where components of one signal transduction pathway affects another pathway in the same or different tissue (Knight and Knight, 2001). Some of these responses include stress-related genes responsible for the production of proteins such as reactive oxygen species (ROS)-scavengers, antioxidants and chaperones (Wang *et al*., 2004; Vinocur and Altman, 2005).

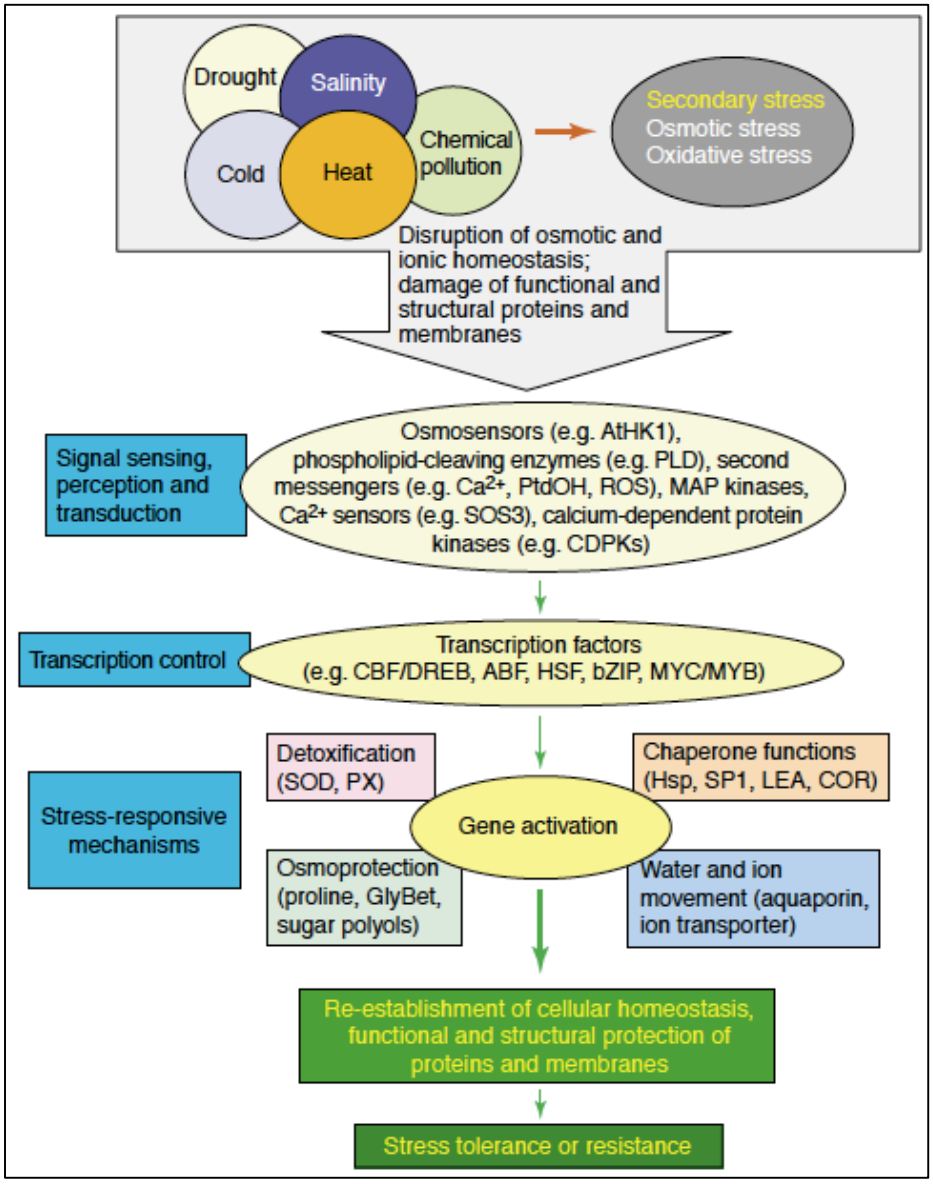

Figure 1.3. Plant response to abiotic stresses. Source: (Vinocur and Altman, 2005).

Reactive oxygen molecules are highly reactive and toxic, and cause damage to proteins, lipids, carbohydrates and nucleic acids, which may ultimately result in cell death. Stress-induced ROS accumulation is counteracted by the production of enzymatic antioxidants (superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and glutathione reductase) and non-enzymatic low molecular weight metabolites (cysteine, reduced

glutathione, and ascorbic acid; Gill and Tuteja, 2010). These ROS-scavengers detoxify ROS by catalysing reactions that convert toxic ROS molecules into molecules such as water and oxygen that are not harmful to plants. Chaperones such as heat shock proteins and late embryogenesis abundant (LEA) proteins are involved in protein folding, assembly, translocation, and stabilizing proteins and membranes. As a result, chaperones play an important role in maintaining cellular homeostasis by re-establishing normal protein conformation (Wang *et al*., 2004).

1.4. Drought Stress

Water is an important substance for plants to carry out biochemical processes such as photosynthesis (Heldt and Piechulla, 2004). Drought (also referred to as osmotic stress, water deficit, or dehydration) is experienced by the plant either when the roots are not absorbing enough water from the ground to transport to different parts of the plant or when the plant loses more water than normal via transpiration (Anjum *et al*., 2011). The sensitivity of the plants to drought differs depending on the duration and the severity of drought, plant species and their developmental stages, and on other abiotic stresses that may occur concurrently with drought stress (Demirevska *et al*., 2009).

Amongst the many abiotic stresses that plants are faced with, drought has the most adverse effects on plant growth and development and thus crop productivity (Anjum *et al*., 2011). As such, drought is a major threat to agriculture and food security worldwide. According to the South African Weather Services (http://www.weathersa.co.za), South Africa experienced the worst drought in the 2015/2016 growing season since 1906. This has negatively affected agricultural productivity as well as the country's economy.

1.4.1. Effects of Drought Stress on Plants

1.4.1.1. Growth and Yield

Plant growth is dependent on cell division, enlargement and differentiation, and involves genetic, physiological, ecological and morphological events and their interactions (Farooq *et al*., 2009). Drought stress impairs mitosis, obstructs cell elongation, and causes loss of turgor pressure, which in turn, results in reduced growth and yield (Farooq *et al*., 2009; Anjum *et al*., 2011). The first effect of drought on plants is impaired germination and poor crop stand. Germination and early seedling growth were impaired in five pea (*Pisum sativum*) cultivars following drought stress (Okçu *et al*., 2005), while plant growth and development during rice vegetative stage was also affected (Manickavelu *et al*., 2006).

Many yield determining physiological processes in plants respond to water stress. However, these processes are integrated in a complex way, making it difficult to pin point exactly how plants accumulate, combine and display these physiological changes over their entire life cycle (Anjum *et al*., 2011). Drought stress results in severe decline in yield traits of crops, which may be due to a disruption in leaf gas exchange (Farooq *et al*., 2009). Drought also results in reduced dry matter production (Nam *et al*., 2001) and causes infertility during flowering stages of some plants (Anjum *et al*., 2011).

1.4.1.2. Root Signalling Under Drought Stress

Apart from anchoring plants into the soil, root systems are important in water and mineral absorption, thus determining whether plants adapt to and survive water stress or wilt and die. Roots support growth throughout the plant's life-cycle and extract water from shallow soil layers that is otherwise easily lost by evaporation (Anjum *et al*., 2011). During water stress, abscisic acid (ABA) accumulates in the roots (Ollas *et al*., 2015). Together with cytokinins and ethylene, ABA triggers a signal cascade from roots to the shoots via xylem vessels, resulting in physiological changes, which determine the degree with

which a plant adapts to drought stress (Anjum *et al*., 2011). Abscisic acid in the roots is also believed to induce increased deep root growth, and thus continuously supplying water to the plant under drought stress (Blum, 1996). Furthermore, ABA causes an efflux of K^+ ions from guard cells, which results in loss of turgor pressure and ultimately stomatal closure (Anjum *et al*., 2011), and thus a reduction in water loss via transpiration. A 50-fold increase in ABA levels has been reported in pea plants under dehydration stress (Guerrero and Mullet, 1986) and ABA accumulation was also reported in *Arabidopsis thaliana* under water stress (Ollas *et al*., 2015).

1.4.1.3. Photosynthesis

Drought stress negatively affects the photosynthetic pathway, mainly due to the disruption of major photosynthetic components such as the thylakoid electron transport, the carbon reduction cycle, and the CO_2 supply (Farooq *et al*., 2009; Anjum *et al*., 2011). Photosynthetic rates are also reduced as a result of the reduction in leaf surface area. When the plant is under water stress, leaf expansion and area are greatly reduced due to leaf rolling and/or wilting, as this helps the plant retain water during drought stress (Blum, 1996). Consequently, less sunlight reaches the rolled or wilted leaves and CO_2 assimilation is also reduced due to small leaf area (Blum, 1996). Furthermore, as the plants close their stomatal openings during water stress, the rate and efficiency of gaseous exchange between the plant and the atmosphere is also impeded, resulting in reduced rates of photosynthesis. An imbalance between the production of ROS and the antioxidant defence systems also contribute to reduced photosynthetic rates in plants (Reddy *et al*., 2004), resulting in the accumulation of ROS, which cause oxidative damage to cellular constituents.

1.4.1.4. Respiration and Nutrient Relations

Plants spend a large quantity of energy in an attempt to cope with drought stress. Roots use carbon fixed in photosynthesis for their growth, maintenance and the production of

dry matter (Lambers *et al*., 1996). However, when plants are faced with drought stress, the rate of photosynthesis is reduced, meaning that only a limited quantity of carbon is fixed and reaches the roots. In wheat, more than 50% of the daily-accumulated photosynthates were transported to the roots and around 60% of this fraction was respired (Farooq *et al*., 2009). Reduced root respiration reduces the ability of the drought susceptible plant to carry out physiological activities and also normal growth (Farooq *et al*., 2009).

During drought stress, roots do not only lose their ability to respire maximally, but also negatively affects nutrient uptake and their transportation to different parts of the plant (Farooq *et al*., 2009). This further leads to a cascade of other negative responses such as reduced absorption of inorganic nutrients. Different plants respond differently to mineral uptake under water stress. However, most plants respond to water stress by increasing their nitrogen uptake, while decreasing phosphorus uptake (Farooq *et al*., 2009).

1.4.2. Mechanisms of Plants Responses to Drought Stress

Combinations of various morphological, biochemical, and physiological responses are important in determining whether the plant adapts to and survives or dies under drought stress. Plants have developed mechanisms such as drought escape, avoidance and tolerance in order to cope with drought stress as summarised in Figure 1.4.

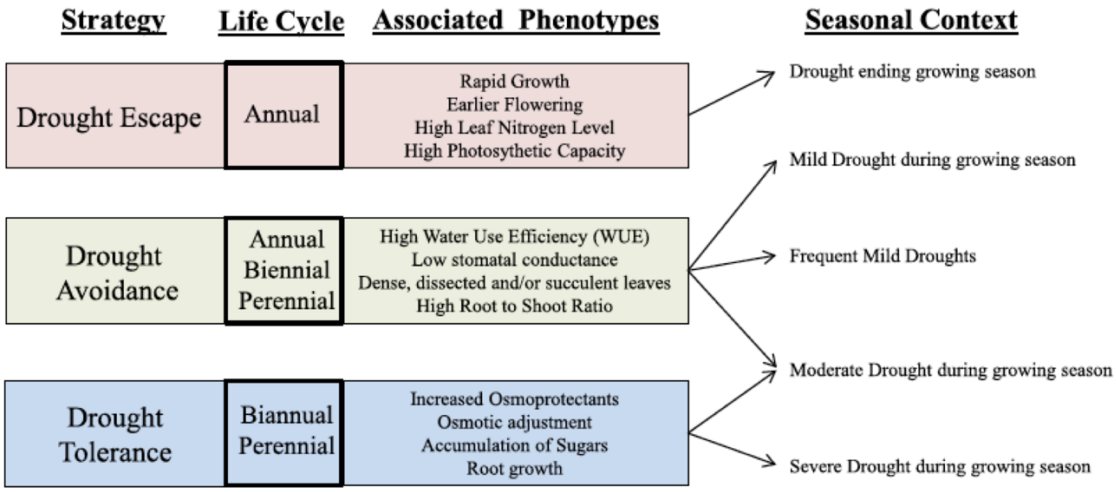

Figure 1.4. Three ways in which herbaceous plants respond to drought stress. Only representative phenotypes are listed here. Arrows indicate the environmental context that would most support each strategy. Source: (Kooyers, 2015).

1.4.2.1. Drought Escape

Drought escape involves rapid development to complete a life cycle before the onset of drought (Kooyers, 2015). This occurs when a physiological plant development is successfully matched with periods of soil moisture availability. Flowering time is the primary trait that is associated with drought escape and an early onset of flowering allows greater fitness in plants through higher seed set and/or greater seed mass (Farooq *et al*., 2009; Kooyers, 2015). This strategy has enabled the development of short-duration varieties in chickpea (*Cicer arietinum*), which in turn, helps in reducing yield loss due to terminal droughts (Kumar and Abbo, 2001). However, yield is largely correlated with the period that the crop survives under favourable growing conditions, meaning that plants still require more time to grow optimally to produce enough crop yield (Turner *et al*., 2001).

1.4.2.2. Drought Avoidance

Drought avoidance can be defined as mechanisms used by plants to conserve water at the whole plant level (Kooyers, 2015). Firstly, the plant reduces water loss to prevent dehydration by closing the stomata. Plant species such as rice (Islam *et al*., 2009) and tobacco (*Nicotiana tabacum*; Cameron *et al*., 2006) also increase the accumulation of wax on leaf surfaces to reduce water loss. Secondly, plants maintain water uptake through an extensive and prolific root system (Kooyers, 2015). Lastly, plants delay or rush between vegetative and flowering growth stages to avoid fruit abortion as a result of severe drought stress (Fang and Xiong, 2015). Traits that are often involved in drought avoidance include reduced leaf area, greater succulence, increased leaf reflectance, leaf rolling, and reduced stomatal size and density. Although these traits do not necessarily indicate drought avoidance at all times, drought avoiding plants generally have greater water use efficiency and may also lower or cease growth in response to drought (Farooq *et al*., 2009; Kooyers, 2015).

1.4.2.3. Drought Tolerance

Drought tolerance can be defined as the stability of plant performance as a result of different genetic traits that results in physiological, morphological, and biochemical changes that function to stabilise and protect cellular and metabolic integrity of plants during drought stress (Fang and Xiong, 2015). After the plant has perceived osmotic stress, a series of genes are induced and the products of these genes are divided into three categories. The first category involves proteins such as kinases and transcriptional factors that are involved in signalling cascades and are responsible for regulating other genes involved in drought response. The second category involves proteins such as aquaporins involved in the uptake and transport of water and ions. The last category involves proteins that are directly involved in protecting the plant against environmental stresses, such as the LEA proteins, osmotin, antioxidant enzymes, and proteins involved in the accumulation of compatible solutes (Fang and Xiong, 2015).

Compatible solutes also known as osmolytes or osmoprotectants are low molecular weight, usually non-toxic, and highly soluble organic compounds (Nahar *et al*., 2016). These improve the plant's tolerance to stress factors by osmotic adjustment, detoxifying ROS, mitigation of ionic toxicity, protection of photosynthetic and mitochondrial structure and metabolism, and stabilising membranes, enzymes and proteins (Nahar *et al*., 2016; Suprasanna *et al*., 2016). Osmolytes are categorised into different groups; amino acids (such as proline, glycine betaine, and a non-protein gamma-aminobutyric acid); sugars (such as trehalose, sucrose, and fructose); and sugar alcohols (such as mannitol, inositol, and sorbitol). A summary of these osmolytes and their putative protective roles under drought stress are listed in Table 1.1 below.

Table 1.1. Osmolytes and their mechanisms of protection during drought stress conditions.

Osmolytes Group	Mechanism of Protection/Role
Amino Acids (proline, glycine betaine)	ROS scavenging activity and singlet oxygen quenching ability, prevents membrane damage and ion toxicity, protect photosynthetic machinery, activates some stress-related genes, and maintains protein integrity.
Sugars (trehalose, sucrose, fructose)	Osmotic adjustment and stabilising membranes, reversible water absorption capacity, and increase thermostability.
Sugar alcohols (mannitol, inositol, sorbitol)	Facilitates osmotic adjustment, and act as signalling molecules.

Modified from Suprasanna *et al*., 2016.

As discussed in the above sections, plant response mechanisms to drought stress are complex. In order to fully understand these mechanisms, a large range of physiological, biochemical, genomics, transcriptomics, and proteomics techniques are routinely used to dissect the molecular responses.

1.5. Proteomics

1.5.1. How is Proteomics Defined?

Proteomics is defined as "the systematic analysis of a protein population in a tissue, cell or subcellular compartment" (van Wijk, 2001). It allows for both the qualitative and quantitative analysis of protein expressional changes during different developmental stages and in response to a range of abiotic and biotic factors (Eldakak *et al*., 2013).

1.5.2. Why Use Proteomics and not Other –Omics Technologies?

Cellular components are divided into sub-populations given an "–ome" suffix and their respective research focus with an "–omic" suffix (Soda *et al*., 2015). The principle of the central dogma is that genetic information in DNA (genome) is transcribed into an RNA copy (transcriptome), which in turn gets translated into protein (proteome).

The field of genomics focuses on complementing the genome sequence and assigning biological information to genes (Rai and Saito, 2016). Genomics studies have led to genome sequencing of Arabidopsis (The Arabidopsis Genome Initiative, 2000) and 85 other plant species (Rai and Saito, 2016), including sorghum (Paterson *et al*., 2009). Although genomics provides a global overview of the metabolic potential of organisms, it does not necessarily provide insight on how specific metabolic processes are regulated in different species under different environmental conditions (Rai and Saito, 2016).

On the other hand, transcriptomics focuses on the dynamics of RNA including different regulatory signals under different environmental factors (Soda *et al*., 2015; Rai and

Saito, 2016). Transcriptomics has increased our knowledge on how plants respond to different stress factors. However, some studies have reported that RNA levels do not necessarily correlate positively with protein abundance, mainly due to complex regulatory mechanisms involved in protein stability, abundance and post-translational modifications (Gygi *et al*., 1999).

Proteomic studies provide information on protein function, subcellular localization and enables the isolation of multi-subunit protein complexes whose individual subunit peptides cannot be determined from either the genomic or transcriptomic data (Rose *et al*., 2004). Furthermore, details on protein networks and metabolic functions under plant stress adaptive responses may also be generated (Soda *et al*., 2015). Other –omics (metabolomics, fluxomics and lipidomics) technologies are discussed by Rai and Saito (2016). Nonetheless, in order to obtain a comprehensive understanding of the genetic makeup of plants and their responses to different stress factors however, it is recommended to integrate different –omics technologies using a system biology approach (Soda *et al*., 2015).

1.5.3. Protein Extraction and Quantification

Protein extraction is an important step in preparing samples for proteomic analysis (Barkla *et al*., 2013) as it affects downstream processes in the proteomics workflow. Plant cells and tissues as compared to those from other organisms, have relatively low protein quantity, excess amount of proteases, and also contain compounds that interfere with proteome analysis (Rose *et al*., 2004; Chen and Harmon, 2006). Interfering compounds include secondary metabolites, lipids, and polysaccharides (Tsugita and Kamo, 1999). In addition, plant leaves consist of high levels of proteins such as Rubisco, which tends to dominate protein profiles, overshadowing other low abundant ones (Chen and Harmon, 2006).

Proteome quantitative data is important for gaining insight in the dynamics of proteins and their turnover rates. Quantification of low abundant proteins such as regulatory proteins may require that samples be fractionated or that sampling be focused onto specific cellular compartments or organelles within the plant cell (Barkla *et al*., 2013). For example, the extracellular matrix (Bhushan *et al*., 2006), cell wall (Jamet *et al*., 2008), and other subcellular proteomes (Millar and Taylor, 2015) have been analysed. Studying organelle proteomes helps in confirming the identities, functions, and location of proteins (Barkla *et al*., 2013).

Protocols that can extract total proteomes are important for detecting all proteins in a specific tissue, cell or cellular compartment. However, due to the protein complexity caused by a diverse range in molecular weight, charge, and post translational modifications amongst proteins, having a single extraction protocol that extracts all proteins with great efficiency is impossible (Rose *et al*., 2004; Chen and Harmon, 2006). However, the trichloroacetic acid (TCA)/acetone method remains the most commonly used protein extraction method, which helps in concentrating proteins as well as removing contaminants (Wu., Xiong *et al*., 2014).

1.5.4. Protein Separation and Identification

After the proteome has been extracted and quantified, the next set of experiments involves proteome separation and its identification. Different gel-based and non-gel based methods are available for separation and identification of proteins and some of these are discussed below.

1.5.4.1. Electrophoresis Protein Separation and Identification Techniques

Protein electrophoretic methods are used to separate complex protein mixtures, to determine subunit compositions and also verify homogeneity of protein samples (Gallagher, 2007). One dimensional (1D) sodium dodecyl sulphate (SDS) polyacrylamide

gel electrophoresis (PAGE) is one of the widely used gel-based methods, which fractionates proteins based on their molecular weights (Shi and Jackowski, 1998). However, 1D SDS-PAGE has limitations such as low resolution and that it denatures proteins; making it unsuitable for analysing native proteins whose biological activities need to be retained for further analysis (Shi and Jackowski, 1998).

On the other hand, two dimensional (2D) gel electrophoresis, an efficient method of separating and profiling proteins from biological systems is available and widely used (Cellulaire, 2002; Rabilloud, 2014). Like any other technology, 2D-gel electrophoresis has been criticized for its low resolution, under-sampling, and its inability to analyse transmembrane proteins (Cellulaire, 2002). Despite these limitations, some positive qualities unique to 2D-gel electrophoresis include reproducibility, due to the availability of immobilised pH gradient strips and the ability to analyse intact proteins as well as post-translational modifications (Rabilloud, 2014). For these reasons, the 2D-gel electrophoresis technique is still widely used in proteomics studies, even though some researchers prefer using the non-gel based methods.

1.5.4.2. Mass Spectrometry Protein Separation and Identification Techniques

Following separation of proteins using the gel-based techniques such as 1D and 2D gel electrophoresis, proteins of interest are identified by mass spectrometry (MS). Mass spectrometry is an analytical technique used to measure molecular weights of intact polypeptides and thus identify and characterise proteins (Chen, 2008). Generally, MS involves obtaining mass spectra based on the mass/size ratio (*m/z*) produced from fragmented peptides through a peptide precursor ion. In order to identify the proteins represented as spectra, the ion spectra are matched with other spectra that have been previously assigned to certain peptides in different databases. Alternatively, proteins can be identified by the use of sequence tags, which are a short string of complementary DNA sequence (Lin *et al*., 2003). In proteomics, MS has been used to catalog protein

expression data, define protein interactions and identify sites of protein modification (Han *et al*., 2008). Examples of mass spectrometry include matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) MS and electrospray ionization-mass spectrometry (ESI-MS; Lin *et al*., 2003).

Both MALDI-TOF MS and ESI-MS are still widely used in proteomics studies for the identification of gel-separated proteins. However, advances are now on shotgun proteomics methods, which involve a differential isotope labelling of proteins and peptides either metabolically, enzymatically or chemically using external tags (Cagney and Emili, 2002). Shotgun methods somewhat address limitations of gel-based methods such as their inability to analyse highly basic or hydrophobic proteins (Aggarwal and Yadav, 2016). In the current study, the isobaric tags for relative and absolute quantitation (iTRAQ) method, are used and discussed below.

1.5.4.2.1. iTRAQ

iTRAQ is a liquid chromatography (LC) based, stable isotope labelling technology that was first introduced in 2004 (Ross *et al*., 2004). Since its introduction, iTRAQ has been a useful tool, providing the proteomics community with an improved quantitative technique of high sensitivity for proteomes following different stress factors (Luo and Zhao, 2012). With its multiplexing ability, the proteome of multiple samples from different biological states can be simultaneously identified and quantified on one iTRAQ run.

iTRAQ labels consists of reagents, which allow for a 4-plex (114, 115, 116, and 117) or 8-plex (113, 118, 119, and 121) quantification (Ross *et al*., 2004). However, when the 4-plex and 8-plex methods were compared, higher protein identification rates were found when the 4-plex was used (Pichler *et al*., 2010). A summary of the iTRAQ experimental methodology is illustrated in Figure 1.5. Briefly, unlabelled experimental protein samples

are digested using trypsin, followed by an independent labelling with the different 4-plex or 8-plex isobaric tags (Luo and Zhao, 2012).

The tags attached to the balance group (Figure 1.5 A), react with the peptide at the N-terminus and the ε side chain of internal lysine residues, forming an amide linkage (Figure 1.5 B). Thereafter, labelled peptides are mixed and separated using LC. Peptides undergo fragmentation during MS/MS separation, generating a collection of spectra (Figures 1.5 C and D). The relative intensities of the resulting spectrum peaks (Figure 1.5 D) indicate the contribution of each sample to the total peptide intensity and can provide information on the relative abundance. Thereafter, search engines such as MASCOT (http://www.matrixscience.com) can be used to identify labelled peptides and subsequently, the corresponding protein based on peptide fragmentation data (Ross *et al*., 2004).

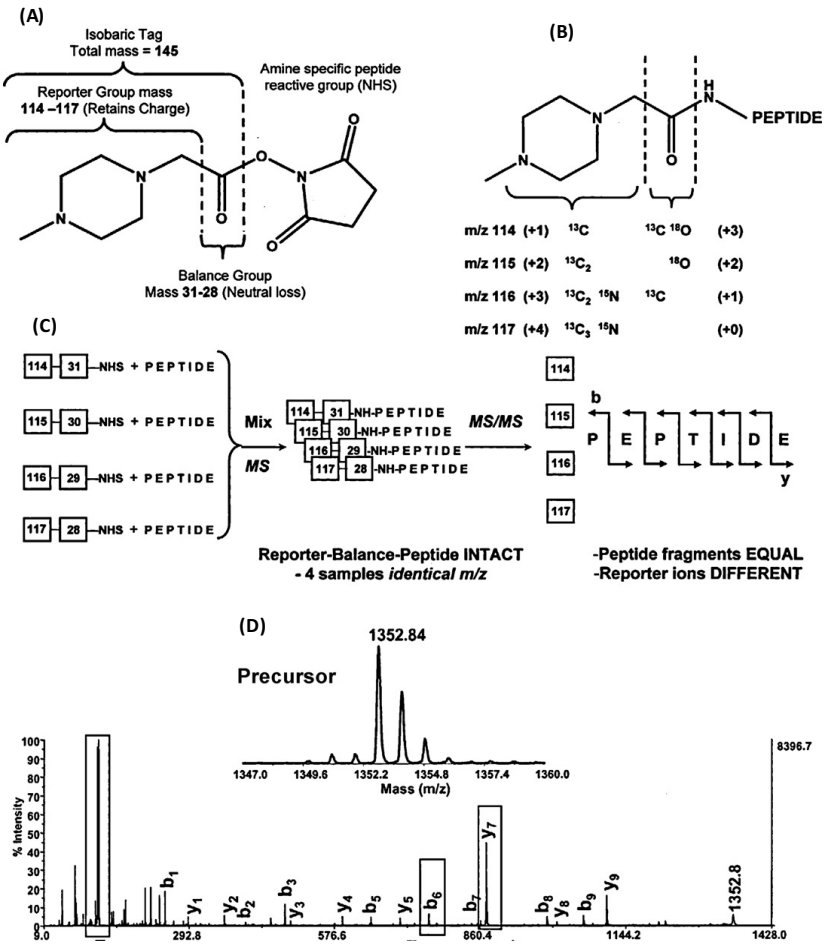

Figure 1.5. Schematic summary of the iTRAQ method. (A) Isobaric tags reacted with balance groups. (B) Tags react with the peptide N-terminus and the side chain of lysine residues to form an amide linkage. (C) Labelled peptides have the same mass on the MS scan. (D) MS/MS results and analysis for identifying proteins. Source: (adapted from Ross *et al*., 2004).

iTRAQ allows the simultaneous comparison of protein quantifications across multiple samples. In addition, it is easy to implement, allows sample replication, it is fast, and can be used to identify protein post translational modifications such as phosphorylation, methylation, acetylation, and fucosylation (Evans *et al*., 2012). However, iTRAQ is relatively expensive as compared to the other protein identification technologies. Recently, iTRAQ was used for the proteomic analysis of maize roots under heavy metal

stress (Li *et al*., 2016), and Arabidopsis extracellular matrix following fumonisin B1 treatment (Smith *et al*., 2015).

1.6. Plant Proteomics

When a stress response is induced, gene expression is altered, ultimately affecting both mRNA and protein expression in cells, which influence cellular biochemistry (Hu *et al*., 2015). Plant proteomics thus allows for the quantitative and qualitative analysis of proteins in plants under a range of physiological conditions (Chen and Harmon, 2006).

A number of proteomics studies aimed at understanding proteome responses in cereals under drought stress conditions have been conducted in maize (Benešová *et al*., 2012), sorghum (Jedmowski *et al*., 2014), sugarcane (*Saccharum officinarum*; Rahman *et al*., 2015), and wheat (Faghani *et al*., 2015), just to mention a few. In these studies, drought conditions were induced by either withholding water or addition of osmotica such as sorbitol, mannitol, polyethylene glycol, or sucrose in the plants' growth media. Following the stress treatments, the differentially expressed total soluble proteins of various plant tissues were then separated and identified using a combination of gel-based and/or non-gel based proteomics tools. Generally, the results indicated that proteins involved in carbohydrate, amino acid, nitrogen and energy metabolism were responsive to drought stress as they possibly function to restore metabolic homeostasis during stress conditions (Bohnert and Sheveleva, 1998).

Reactive oxygen species scavenging enzymes such as superoxide dismutase and catalases were also identified as being stress responsive in different crops (Jedmowski *et al*., 2014; Faghani *et al*., 2015; Rahman *et al*., 2015). These results are in line with the fact that during drought stress, oxidative stress increases resulting in excessive production of ROS (Farooq *et al*., 2009). Proteins involved in photosynthetic light-dependent reactions such as ATP synthase and coronatine-insensitive 1 protein, which

are also putatively involved in stomatal closure were both up-regulated (Jedmowski *et al*., 2014; Faghani *et al*., 2015). Other identified stress responsive proteins include chaperones, and proteins involved in defence, cytoskeleton stability, signal transduction, and protein metabolism (Rahman *et al*., 2015). Apart from using intact plant tissues for total soluble protein analysis, some proteomics studies focus on secreted proteins, their identities and also response patterns under drought stress.

1.7. The Plant Secretome

1.7.1. Definition of "Secretome"

The term "secretome" was first proposed in a genome-based study predicting both the secreted proteins as well as the secretion machinery in *Bacillus subtilis* (Tjalsma *et al*., 2000). Since then, the definition of "secretome" has became broader with advances in the field of proteomics. The "secretome" is defined as a set of proteins secreted into the extracellular matrix (ECM) either by a cell, tissue, or organism at any given time or under certain environmental conditions (Alexandersson *et al*., 2013; Krause *et al*., 2013). The secretome plays important roles in cell wall structure, cellular communication, and defence against different stress factors (Lum and Min, 2011).

1.7.2. Protein Secretion Pathways

Two secretory pathways, namely, the classical or conventional protein secretion (CPS) and the unconventional protein secretion (UPS), explain the different ways in which proteins are secreted out of cells into the ECM. In the CPS pathway, proteins are secreted via the endoplasmic reticulum (ER) - golgi - trans-golgi network (TGN) - plasma membrane (PM) or extracellular space (ECS) in the endomembrane (Labelled 1 in Figure 1.6; Drakakaki and Dandekar, 2013). This pathway is dependent on the N-terminally located signal peptides, which tag proteins for translocation into the ER lumen where the process of conventional protein secretion begins. This pathway is highly conserved in eukaryotes (Ding *et al*., 2014).

For a long period of time the CPS pathway was believed to be the only protein secretory pathway in plants. However, a few observations in some proteomic studies could not be explained using principles of this pathway (Alexandersson *et al*., 2013). Firstly, many signal peptide-lacking proteins (or leaderless proteins) were found on the outside of the PM in plant cells (Ding *et al*., 2014); and secondly, proteins, which contain signal peptides were found outside the PM, even though the proteins bypassed the Golgi apparatus (Ding *et al*., 2014). The secretion of these proteins can now therefore be explained by the UPS pathway (Drakakaki and Dandekar, 2013).

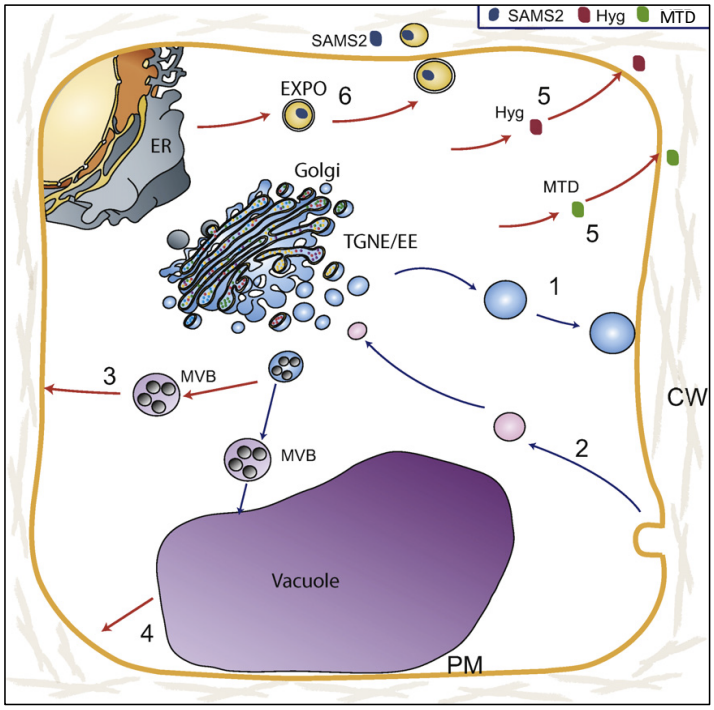

Figure 1.6. Protein secretion in plants. Shown are the CPS pathway (1), endocytosis (2), UPS through MVB (3), vacuole-PM fusion (4), Golgi-bypass pathway (5), EXPO double membrane organelle (6). Abbreviations: ER, endoplasmic reticulum; MTD, celery mannitol dehydrogenase; MVB, multivesicular body; Hyg, hygromycin phosphotransferase; PM, plasma membrane; TGN, trans-Golgi network; EE, early endosome; CW, cell wall; SAMS2, S-adenosylmethioninesynthetase 2; EXPO, exocyst-positive organelle. Source: (Drakakaki and Dandekar, 2013).

The UPS pathway has been extensively studied in mammals and yeast cells (Chua *et al*., 2012). It is believed that proteins are secreted via the UPS pathway if the presence of a protein in the ER/Golgi would disrupt ER functioning or if a protein has multiple functions, occurring in different cellular compartments (Ding *et al*., 2014). The pathway constitutes of the non-vesicular group, where cytoplasmic proteins have a direct path to the PM, and proteins, which need to be fused to a single membrane-bound structure or the protein, gets released from the PM (Drakakaki and Dandekar, 2013; Ding *et al*., 2014). In plants, evidence of this pathway (labelled 3 to 6 in Figure 1.6) is only starting to emerge and more than 50% of the plant secretome lacks signal peptides, thus supporting the UPS pathway (Robinson *et al*., 2016).

The above mentioned protein secretion pathways are responsible for secreting different functional proteins to required locations under different conditions. For example, when plants are exposed to stressful conditions, specific proteins are secreted to the ECM, where they function in plant stress response. It is for this reason that plant biologists study the secretome as a subset of the plant proteome.

1.7.3. How is the Plant Secretome Studied?

The *in vitro* and *in planta* systems are routinely used to study the composition of secreted proteins in plants (Alexandersson *et al*., 2013; Krause *et al*., 2013). In the *in vitro* plant system (Figure 1.7 A), secreted proteins are prepared from culture filtrate of cell suspension cultures (Krause *et al*., 2013). The proteins secreted into the liquid culture medium can be easily extracted using simple filtration and centrifugation steps (Alexandersson *et al*., 2013). The *in vitro* plant system has been widely used for secretome analyses because the cell suspension cultures can be easily maintained, and extraction procedures cause minimal cell damage (Alexandersson *et al*., 2013), while, the fraction of dead cells can also be estimated using the Evans blue dye (Agrawal *et al*., 2010). However, the main disadvantage of using the *in vitro* plant system in secretome

studies is that it does not provide a natural environment for cells and physiologically relevant treatments are difficult to apply (Agrawal *et al*., 2010). Nevertheless, this system has been used in secretome studies of rice (Cho *et al*., 2009), sorghum (Ngara and Ndimba, 2011), and chickpea (Gupta *et al*., 2011).

The *in planta* system involves an isolation of the apoplastic fluid from the extracellular space to prepare secreted proteins (Figure 1.7 B; Agrawal *et al*., 2010). The apoplast or ECS is the space outside the plasma membrane where plant cells exchange signals, water and solutes. The apoplast functions in defence against different stress factors, transport, osmotic homeostasis, cell adhesion, growth regulation and gas exchange, amongst others (Floerl *et al*., 2012). Secreted proteins are usually extracted from plant tissues using either the vacuum-infiltration-centrifugation (Agrawal *et al*., 2010) or the gravity-extraction method (Jung *et al*., 2008).

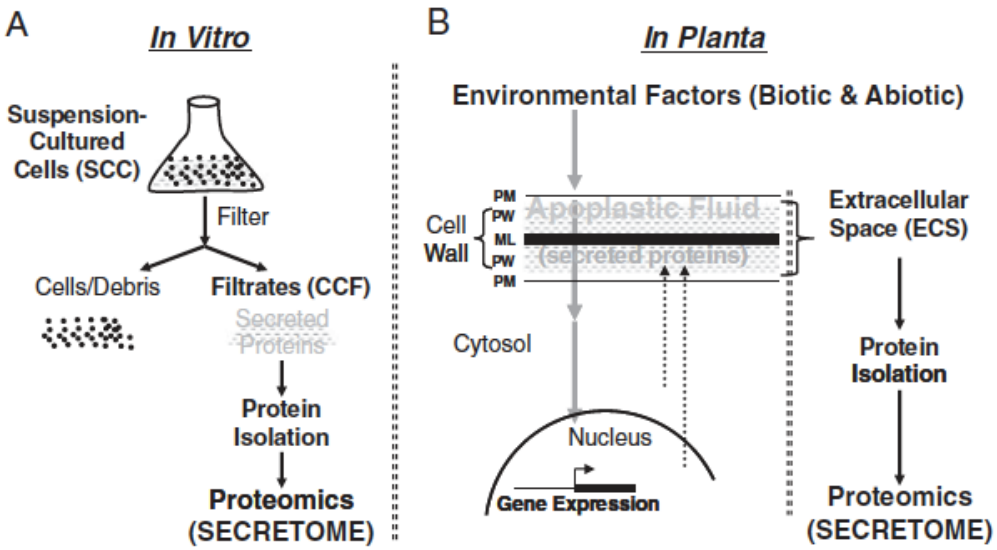

Figure 1.7. General overview of the *in vitro* (A) and the *in planta* (B) systems for secretome analysis. Source: (Agrawal *et al*., 2010).

The *in planta* system provides a natural environment for cells, where organ or developmental specific secretomes may be captured. However, it is difficult to extract the apoplastic secretome without damaging the cells (Agrawal *et al*., 2010). Therefore, intracellular contamination becomes a challenge and obtaining pure secreted protein fractions could be difficult (Pechanova *et al*., 2010). Nevertheless, the *in planta* system has been used in secretome studies of pea (Wen *et al*., 2007), tobacco (Delannoy *et al*., 2008), and rice (Jung *et al*., 2008).

1.7.4. Purity Assessment of Secreted Protein Fractions

In general, secreted protein fraction preparations of *in vitro* systems are contaminated with cytoplasmic proteins when cells lyse and/or die (Alexandersson *et al*., 2013); while the vacuum-infiltration-centrifugation method of extracting secreted proteins possibly damage cell walls and membranes, resulting in the release of cytoplasmic proteins into the ECS (Agrawal *et al*., 2010). The purity of secreted protein fractions may thus be assessed using enzymes activity assays, immunoblotting, and microscopy (Alexandersson *et al*., 2013; Krause *et al*., 2013).

Malate dehydrogenase has been used as a cytoplasmic marker and its enzyme activity to assess cytoplasmic contamination in plant secretome studies (Jung *et al*., 2008). However, soluble malate dehydrogenase has been shown to be present in the apoplastic fluid of some plant species such as barley (Li *et al*., 1989) and tobacco (Mäder and Schloss, 1979). Other cytosolic enzymes markers that have been used include catalase-peroxidase HPI (hydroperoxidase I; Dannel *et al*., 1995), glucose 6-phosphate dehydrogenase (Konozy *et al*., 2013), α-mannosidase (Delannoy *et al*., 2008), phosphoenolpyruvate carboxylase, and cytosolic aldolase (Tran and Plaxton, 2008).

In western blotting analysis, Rubisco (Gupta *et al*., 2015a), Hsp70 and actin (Cho *et al*., 2009), hexokinase (Cheng and Williamson, 2010) and α- (Jung *et al*., 2008) and β-

tubulin (Ngara and Ndimba, 2011) have been used as reference cytoplasmic proteins in assessing the levels of contaminants in the secreted protein fractions.

1.7.5. Plant Secretome Maps and Responses to Drought Stress

Proteins secreted into the ECM play important roles in cell wall structure, cell-cell interaction, and communication with the external environment, thus triggering defence responses against the different biotic and abiotic stimuli (Lum and Min, 2011). Following exposure to different abiotic and biotic stresses, proteins are continuously secreted into the ECM. However, some proteins may be classified as "housekeeping-proteins" because they are constitutively secreted irrespective of the prevailing environmental conditions. These proteins are mainly involved in the normal operation of the plant metabolic processes such as growth and development (Guerra-Guimarães *et al*., 2016).

Reported plant secretome studies involve a wide range of experimental designs (section 1.7.3) utilising different plant species and stress factors (reviewed by Alexandersson *et al*., 2013; Krause *et al*., 2013; Tanveer *et al*., 2014; Guerra-Guimarães *et* al., 2016). The sorghum secretome from non-treated cell suspension cultures has been mapped (Ngara and Ndimba, 2011). In that study, 2D gel electrophoresis and MALDI-TOF-TOF MS were used to separate and positively identify 14 secreted proteins. The identified proteins were categorised as peroxidases, germin proteins, oxalate oxidases, and α-galactosidases (Ngara and Ndimba, 2011).

Peroxidases help defend plants against oxidative stress by degrading ROS molecules generated during abiotic stress and/or pathogen infections, as well as stabilising metabolic homeostasis. Germin proteins and oxalate oxidases both function in plant defence against pathogenic attack, whereas α-galactosidases are involved in cell walls modifications (Guerra-Guimarães *et al*., 2016). Although sorghum leaf proteomics under salt stress (Swami *et al*., 2011; Ngara *et al*., 2012), cadmium stress (Roy *et al*., 2016),

and drought stress (Jedmowski *et al*., 2014) has been reported, there are still gaps in sorghum's secretome responses towards abiotic stress, including drought. However, drought responsive secreted proteins have been identified in other monocotyledonous crops such as rice.

Pandey and co-workers (2010) studied changes in secreted proteins of rice aerial tissues following 48 - 192 hr of water withdrawal. About 100 differentially expressed proteins were identified. Functions of these identified proteins include cell signalling (such as nucleoside diphosphate kinase, up-regulated 96 hr post stress induction), cell defence and rescue (such as L-ascorbate peroxidase, up-regulated throughout the stress period), cell wall modification (such as putative polygalacturonase, down-regulated at 96 hr), carbohydrate metabolism (such as phosphoglucomutase, up-regulated between 144 - 192 hr), secondary metabolism (such as chalcone isomerase, down-regulated at 48 and 96 hr), protein degradation (such as oligopeptidase, up-regulated between 48 - 192 hr), chaperones (such as chaperonin CPN60-1, up-regulated throughout the stress period), and protein biogenesis (such as putative elongation factor 2, up-regulated only at 144 hr; Pandey *et al*., 2010).

Plant cell suspension cultures are important experimental systems for studying protein expression including secretome changes in response to a range of biotic and abiotic stress factors. Little is known about sorghum secretome in response to either biotic or abiotic stress factors. The secretome expression map generated in this study can be used as a reference tool for other cereals in similar studies. Furthermore, the identified osmotic stress responsive proteins may lead to the identification of representative genes, which may be useful in different breeding programs aiming at producing drought stress tolerance crops in future.

1.8. Aim and Objectives of this Study

The aim of this study was to establish a sorghum *in vitro* culture system and to use proteomics tools to map its secretome and identify osmotic stress responsive secreted proteins.

The specific objectives were to:

(i) Initiate, propagate, maintain, and characterise sorghum callus and cell suspension cultures with contrasting phenotypes to drought stress tolerance.

(ii) Map the sorghum cell suspension culture secretome and identify drought stress responsive proteins using iTRAQ.

(iii) Perform bioinformatics analysis of the positively identified secreted proteins for subcellular localisation and putative secretory functions.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant Material

Seeds of eight sorghum (*S. bicolor*) genotypes were used in this study in order to select two genotypes which could readily induce large friable callus masses for use in subsequent experiments. Seven of these genotypes, namely SA 1441, ICSV 112, ICSV 210, ICSV 213, ICSB 338, ICSB 73, and Macia, were kindly donated by Dr Nemera Shargie, plant breeder at the Agricultural Research Council (ARC) - Grain Crops Institute, Potchefstroom, South Africa. The eighth genotype, White sorghum, previously used for callus induction by Ngara *et al*. (2008), was kindly donated by Prof Bongani Ndimba, University of Western Cape/ARC - Infruitec - Nietvoorbij, South Africa. The phenotypes of the genotypes were characterized as either drought tolerant, drought resistant, drought susceptible, or sweet grain, with the exception of White sorghum, whose drought response phenotype is unknown (Table 2.1). All genotypes used were white in colour.

Table 2.1. Sorghum genotypes used in this study.

No.	Genotype	Phenotype
1	SA 1441	Drought tolerant
2	ICSV 112	Drought resistant
3	ICSV 210	Drought resistant
4	ICSV 213	Drought resistant
5	ICSB 338	Drought susceptible
6	ICSB 73	Drought susceptible
7	Macia	Grain/Sweet
8	White sorghum	Unknown*

*Although phenotype is unknown, White sorghum is mainly used for pigeon and chicken feed (Agricol, 2016).

2.2. Sorghum Plant Tissue Culture

2.2.1. Seed Surface Decontamination

Seeds from each of the eight genotypes (Table 2.1) were surface decontaminated with 70% (v/v) EtOH for 1 min in a 50 mL Falcon tube. EtOH waste was discarded and the residual pipetted out. Seeds were then washed with commercial bleach [3.5% (m/v) sodium hypochlorite] containing 0.1% (v/v) Tween® 20 for 25 min with intermittent shaking. Thereafter, bleach solution was discarded and seeds were rinsed three times with sterile distilled water. Water waste was discarded and pipetted out in-between each wash. Seeds were then blotted and air-dried on sterile filter paper for 5 min before plating on sorghum seed germination medium.

2.2.2. Seed Germination and Explant Preparation

The surface decontaminated seeds (section 2.2.1) were plated onto plastic petri dishes containing sorghum seed germination medium [2.2 g/L Murashige and Skoog basal (MS) medium (Murashige and Skoog, 1962); 1% (w/v) sucrose; 5 mM 2-(N-Morpholino) ethanesulfonic acid (MES); pH 5.8, adjusted with 2M KOH; 0.8% (w/v) bacteriological agar] using sterile forceps. The petri dishes containing seeds were sealed with parafilm and incubated in a Labcon growth chamber (Labdesign Engineering, Maraisburg, South Africa), in the dark at 25°C for 4 days. After germination, the four-day old sorghum seedlings were removed from the petri dishes and placed on sterile filter paper. The shoots were cut into sections of about 5 mm and used as explants to induce callus formation.

2.2.3. Initiation and Maintenance of Sorghum Calli

Shoot explants (section 2.2.2) were placed onto plastic petri dishes containing sorghum callus initiation medium [4.4 g/L Murashige and Skoog Basal Salt with minimal organics (MSMO) medium; 3% (w/v) sucrose; pH 5.8, adjusted with 1 M NaOH; 0.8% (w/v) bacteriological agar] supplemented with plant growth hormones (PGHs) 1-naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) as represented in Table 2.2. Plant growth hormone NAA was prepared by dissolving 2.5 mg of NAA in 100 µL 1 M NaOH and topped up to 1 mL with distilled water. 2,4-D was prepared by dissolving 3 mg and 4 mg of 2,4-D, respectively, in 100 µL each of absolute EtOH and topped up to 1 mL with distilled water.

Table 2.2. A Latin square to test media with different concentrations of plant growth hormones, 2,4-D and NAA for optimal sorghum callus growth.

	2,4-D (mg/L)		
NAA (mg/L)	**0**	**3**	**4**
0	A	B	C
2.5	D	E	F

(A) represents sorghum callus induction control medium without any PGH; (B) only contains 3 mg/L of 2,4-D; (C) only contains 4 mg/L of 2,4-D; (D) only contains 2.5 mg/L of NAA; (E) contains 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) contains 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

Shoot explants from a single seedling were placed in one plate. This was done for at least six replicate plates. Plates were sealed with parafilm and incubated under the same conditions as described in section 2.2.2, with the exception that callus inductions were assessed over a 4 - 5 week period. Soft easily breakable (friable) calli were maintained in culture by sub-culturing 4 - 5 week old callus (broken into small pea sized pieces of about 0.5 g wet weight each) onto fresh sorghum callus induction media. Well established friable calli were maintained in culture.

2.2.4. Light Microscopic Analysis of Sorghum Calli

Four-week old friable calli were sent to the Laboratory for Microscopy, University of the Free State, Bloemfontein, South Africa for cell morphology evaluations. Calli microscopic evaluations were done using a Nikon E2000 Light microscope fitted with a Nikon DMX 1200 Digital camera (Tokyo, Japan) for imaging. Briefly, the specimen was placed on a drop of distilled water and lightly pressed between a microscope slide and cover slip. Edges of cover slip were sealed off with clear nail polish and allowed to dry before examination. This was repeated several times to obtain the least amount of damaged cells. Images were captured at different objective lens magnifications (40X and 100X) in order to get good representative images.

2.2.5. Initiation and Maintenance of Sorghum Cell Suspension Cultures

Friable calli (section 2.2.3) were used to initiate cell suspension cultures. Three large clumps of actively growing 4 - 5 week old friable calli (approximately 10 g total wet weight) were placed in a 250 mL Erlenmeyer flask containing 50 mL of sorghum cell suspension culture medium [4.4 g/L MSMO supplemented with 3% (w/v) sucrose, 3 mg/L 2,4-D, 2.5 mg/L NAA, pH 5.8]. Flasks were placed under dark conditions at 27°C on an orbital incubator shaker (Already Enterprise Inc., Taipei, Taiwan) with agitation at 130 rpm. After 4 days, the cell culture medium was topped up to a total volume of 100 mL with fresh medium and incubated until cultures reached the desired cell density. This was done for at least three independent biological replicates. To maintain the cultures, sub-cultures were done every 10 - 12 days by transferring 30 mL of culture to a 250 mL flask containing 70 mL of fresh medium.

2.2.6. Measurements of Cell Growth Parameters

2.2.6.1. Growth Curve Measurements

The growth curve of the suspension culture was monitored using the settled cell volume (SCV) method as described by Ngara and Ndimba (2011). Cell cultures were sub-cultured as described in section 2.2.5 and SCV readings were taken at two-day intervals (same time each day) starting from the day of sub-culturing. The readings were taken until the suspension cultures of three independently established cell cultures were too dense for consistent and reproducible sampling (Ngara and Ndimba, 2011). At each sampling time point, each flask from the three biological replicates was gently shaken and 1 mL aliquot of the cell culture was pipetted into a graduated 1.5 mL tube. Thereafter, the tube was left to stand for 10 min on a flat bench top and the volume of settled cells was estimated as presented in Equation 1 below:

Equation 1.

$$\text{SCV (\%)} = \frac{\text{Volume of settled cells}}{\text{Total volume of sample}} \times 100$$

2.2.6.2. Estimation of Cell Viability Using MTT Assay

The viability of cell suspension cultures was estimated using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Ngara (2009). Three independently established cell cultures were used as biological replicates. The cell cultures were sub-cultured into fresh medium as described in section 2.2.5. A volume of 150 μL of the cell culture was sampled into a 1.5 mL tube every second day starting from the day of sub-culturing (day 0) until the day cells reach the stationary growth phase.

The sampling was repeated for each biological replicate cell culture to give two technical replicates. To each 150 μL cell sample, 50 mL of a 5 mg/mL MTT (Sigma-Aldrich, St. Louis, USA) stock solution (prepared in distilled water) was added and incubated with an open lid for 30 min with gentle shaking in a fume hood. Cells were left to settle on a flat bench top for 5 min. Thereafter, the supernatant was carefully discarded without disrupting the cells. One millilitre of 100% (v/v) dimethyl sulfoxide (DMSO) was added to tubes containing only the cells, and incubated with gentle shaking for 10 min. Thereafter, the MTT treated cells were left to settle on a flat bench top for 5 min. The supernatant was then collected and its absorbance read on a spectrophotometer at 490 nm using DSMO as a blank solution.

2.3. Sorghum Protein Extraction

2.3.1. Protein Extraction from Culture Filtrate (CF) Fractions

2.3.1.1. Preliminary Extraction from Cell Culture Aliquots

Cell cultures were sub-cultured as described in section 2.2.5 and on day six after sub-culturing, flasks were placed in the laminar flow-hood to allow cells to settle. This was done for three independent biological replicate cell cultures. Once the cells had settled, 1 mL of culture filtrate (CF) was sampled into a 1.5 mL tube. This was done starting on the day that cells started growing exponentially (log phase) until the onset of the stationary phase on the cell growth curve. The collected CF was centrifuged at 13,400 *x g* using a Prism™ Microcentrifuge (Labnet International, Edison, USA) for 5 min. While avoiding disrupting the pellet, 800 μL of the CF was transferred into a new 1.5 mL tube. A volume of 200 μL of a 50% (w/v) trichloroacetic acid (TCA) stock solution (prepared in distilled water) was added to the tube to precipitate CF proteins.

Proteins were precipitated at 4°C overnight and also on ice for 30 min. Protein samples were centrifuged at 13,400 *x g* for 10 min and the supernatant was discarded. The pellet was washed once with 1 mL of 80% (v/v) ice cold acetone by centrifuging at 13,400 *x g* for 10 min. Thereafter, the pellet was air-dried at the bench top at room temperature for 4 min. The protein pellet was re-suspended in 20 μL of a 2X sodium dodecyl sulphate (SDS) gel loading buffer [100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% (w/v) SDS, 0.2% bromophenol blue, 20% (v/v) glycerol] by brief vortexing. The mixture was then boiled on an ACCUBLOCK™ Digital Dry bath (Labnet International) at 95°C for 5 min and loaded onto a 12% (v/v) 1D gel.

2.3.1.2. Large Scale Protein Extraction from Culture Filtrate

Cell cultures were maintained as previously described in section 2.2.5. On day 8 after sub-culturing, 30 mL of the cell cultures were filtered into a clean 50 mL Falcon tube

through 4 layers of sterile Miracloth (Merck kGaA, Dermstadt, Germany). The experiment was done for three independently established biological replicate cell cultures. The cells left on the Miracloth after filtering were rinsed three times with distilled water, crapped off, and immediately stored at -80°C until use in subsequent experiments.

The filtered culture medium was centrifuged at 2,500 *x g* for 10 min to collect a CF in the supernatant. One hundred percent acetone was added to the CF in a 1 : 4 (supernatant : acetone) ratio and proteins were precipitated overnight at -20°C. After protein precipitation, the samples were centrifuged at 15,700 *x g* for 10 min and the supernatant discarded. The pellet was washed three times with ice cold 80% (v/v) acetone by centrifuging at 15,700 *x g* for 10 min per wash. The pellet was air-dried at room temperature for 5 min and re-suspended in urea extraction buffer [7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS)] overnight with vigorous vortexing at room temperature. The solubilized protein mixture was centrifuged at 15,700 *x g* for 10 min. The supernatant containing CF soluble proteins was carefully collected and stored at -20°C, or until use in subsequent experiments.

2.3.2. Total Soluble Protein (TSP) Extraction from Cell Samples

Frozen cell samples (section 2.3.1.2) were finely ground using frozen and sterile mortar and pestle. Ground cells were transferred into a 15 mL Falcon tube and precipitated with 1 mL of 10% (w/v) TCA. Thereafter, cells were briefly mixed by vortexing and centrifuged at 13,400 *x g* for 10 min at room temperature. After centrifugation, the supernatant was discarded and the pellet was washed three times with 1.5 mL of ice cold 80% (v/v) acetone by centrifuging at 13,400 *x g* for 10 min. The pellet was air-dried at room temperature for 5 min and then re-suspended in urea extraction buffer. The TSP was extracted in urea extraction buffer at room temperature for at least 1 hr with vigorous vortexing at room temperature before centrifugation at 15,700 *x g* for 10 min. The

supernatant containing the TSP was carefully collected and stored at -20°C, or until use in subsequent experiments.

2.4. Osmotic Stress Treatments of Sorghum Cell Cultures

2.4.1. Time Course Stress Treatment

Cell suspension cultures of both ICSB 338 and White sorghum were sub-cultured as described in section 2.2.5. On day 8 after sub-culture, corresponding to the mid-log phase, each cell culture was sub-divided into two 50 mL sub-cultures for control and stress treatment. This was done for three biological replicate cultures. Osmotic stressed cells were treated by adding 4 mL of a 5 M sorbitol stock solution, making the final concentration of 400 mM sorbitol. The same volume of autoclaved distilled water (4 mL) was also added to all the control cultures. Immediately after treatment, the cultures were gently mixed by swirling and cells were sampled for the estimation of cell viability assays and protein extraction procedures. Cell sampling was repeated after 24, 48 and 72 hr of osmotic treatment.

2.4.2. Estimation of Cell Viability

2.4.2.1. The MTT Assay

The MTT assay was carried out on both control and sorbitol-treated cells sampled at 0, 24, 48, and 72 hr after treatment, and as is described in section 2.2.6.2.

2.4.2.2. The Evans Blue Assay

Cell viability was also estimated using the Evans blue assay as previously described by Ngara (2009), with minor modifications. Immediately after treatment (0 hr), each of the six flasks were gently shaken and two 1 mL aliquots of cell suspensions were sampled. Cells, at the 0 hr time point, in one cell suspension aliquot were deliberately killed by heating at 95°C on a heat block for 10 min. Cells in the other aliquot were left unheated. Both cell aliquots were left to settle on a flat bench top for 5 min and the medium was

discarded, leaving packed cells. Approximately 20 mg of the cells (wet weight) was weighed into a clean 1.5 mL tube. The cells were stained by adding 180 μL of distilled water and 20 μL of 0.5% (w/v) Evans blue solution prepared in distilled water, and incubated for 15 min with gentle shaking on a horizontal shaker at room temperature.

After incubation, stained cells were centrifuged at 2,300 *x g* for 5 min and thereafter, the supernatant discarded. The pellet was washed three times with 1 mL of distilled water through centrifuging at 2,300 *x g* for 5 min per wash. The supernatant was discarded in between each wash. After washing, 1.2 mL of 1% (w/v) SDS in 50% (v/v) methanol was added to the cells. The cells were ground using a plastic pestle and incubated at 50°C in an oven for 6 hr. After incubation, the homogenate was centrifuged at 2,300 *x g* for 5 min and 1 mL of the released Evans blue stain in the supernatant fraction was collected. The optical density of the released stain was measured spectrophotometrically at 600 nm using a 1% (w/v) SDS in 50% (v/v) methanol as a blank solution.

An assumption was made that at 0 hr post treatment, cells were 100% viable, with the possibility of a slight reduction in viability due to cell aging and nutrient depletion in the culture. Cell death using the Evans blue assay was estimated using the equation below (Equation 2). From this equation, cell viability was then considered as the difference from a 100% cell death.

Equation 2.

$$\text{Cell Death (\%)} = \frac{C_0 - X_t}{C_{B0} - C_0} \times 100$$

Where: C_0 is the absorbance of stain released from un-boiled cells at time 0 hr.
C_{B0} is the absorbance of stain released from boiled cells at time 0 hr.
X_t is the absorbance of stain released from boiled treated cells at different time point.

2.4.3. Light Microscopic Analysis of the Osmotic Stressed Cell Cultures

Microscopic analyses of control and sorbitol-treated cells were carried out in the Department of Zoology and Entomology, University of the Free State, Qwaqwa Campus, South Africa. Analyses were carried out on the ICSB 338 and White sorghum control and sorbitol-treated (400 mM) cell suspension cultures (section 2.4.1), at 48 hr and 72 hr post treatment. Cell culture evaluations were carried out using a Nikon Eclipse E200 Light microscope fitted with a DeltaPix Digital camera (Tokyo, Japan) for imaging. Briefly, a drop of the cell culture was placed on a microscope slide and lightly pressed with the cover slip. The cell cultures were then analysed on the microscope, with images being captured at different magnifications (10X and 40X) and in order to get good representative images.

2.4.4. Culture Filtrate (CF) Protein Extraction

Preliminary CF protein extraction was carried out for all the ICSB 338 and White sorghum cell cultures at 0 hr, 24, 48, and 72 hr, and as is described in section 2.3.1.1.

Preliminary protein extraction was done to see at which time point there was a difference in protein expression profile. Thereafter, a large scale CF protein extraction was done on all the ICSB 338 and White sorghum cell cultures at 48 and 72 hr post-treatment and as is described in section 2.3.1.2. For these extractions, 50 mL cell suspension cultures were sampled and the cells left on the Miracloth after filtering were discarded and not used any further. Extracted CF proteins were quantified and analysed on 1D SDS-PAGE.

2.5. Protein Quantification

The concentration of all protein extracts was estimated using a Bradford assay (Bradford, 1976) and as previously described by Ngara (2009). Bovine serum albumin (BSA) standards were prepared in duplicates from a 5 mg/mL stock solution (prepared in urea extraction buffer) in 2 mL plastic cuvettes as indicated in Table 2.3 below. Experimental protein samples were also prepared in duplicate in 2 mL plastic cuvettes by mixing 5 μL of each protein extract sample with 5 μL of urea extraction buffer, 10 μL of 0.1 M HCl, and 80 μL distilled water. The Protein Assay Dye Reagent Concentrate (Bradford reagent; Bio-Rad, Hercules, CA, USA) was diluted in a ratio of 1 : 4 (Bradford reagent : distilled water). Nine hundred microlitres of the diluted Bradford reagent was added to all the BSA standards and protein samples, mixed and incubated for 5 min at room temperature.

Table 2.3. Preparation of BSA standard solutions for protein quantification.

Concentration (μg)	BSA 5 mg/mL stock solution (μL)	Urea extraction buffer (μL)	0.1 M HCl (μL)	Distilled water (μL)
0*	0	10	10	80
5	1	9	10	80
10	2	8	10	80
20	4	6	10	80
40	8	2	10	80
50	10	0	10	80

*Blank solution

The absorbance was read at 595 nm on a Bioware II spectrophotometry (Biochrom, Cambridge, England) using a 0 mg/mL BSA standard as a blank solution. The standards were used to plot a standard curve from which concentrations of all the unknown protein extract samples were then determined.

2.6. Protein Gel Electrophoresis

2.6.1. One Dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein extract samples were separated on a 1D SDS-PAGE and as previously described (Laemmli, 1970). One dimensional gels were cast on 10.1 cm (width) x 8.3 cm (height) thick spacer glass plates (Bio-Rad) mounted with a 1 mm thick spacers using the Mini - PROTEAN® Tetra cell (Bio-Rad) gel casting system and according to the manufacturer's instructions. Resolving and stacking gels of 12% (v/v) and 5% (v/v) were prepared as indicated in Appendix 1.

Protein samples were prepared by mixing with 2X SDS gel loading buffer at a ratio 1 : 1 in a 1.5 mL tube. The mixture was heated on an ACCUBLOCK™ Digital Dry bath at 95°C

for 5 min. An appropriate volume of the denatured protein samples and 5 μL PageRuler™ unstained protein ladder (Thermo Fisher Scientific, Massachusetts, USA) were loaded onto the gel accordingly. Gel electrophoresis was carried out at 100V until the bromophenol blue tracking dye was about to run-off at the bottom of the gel. The gel was carefully removed from the gel cast and then stained.

2.6.2. Coomassie Brilliant Blue (CBB) Staining of SDS-PAGE Gels

Proteins separated on 1D SDS-PAGE gels (section 2.6.1) were visualised after staining with Coomassie Brilliant blue (CBB) R-250 stain [0.1% (w/v) CBB R-250 (Bio-Rad), 40% (v/v) methanol, 10% (v/v) acetic acid]. After electrophoresis, gels were dismantled from the gel cast and immersed in CBB stain overnight with shaking at room temperature. Thereafter, the gels were immersed in destaining solution [40% (v/v) methanol, 10% (v/v) acetic acid] with shaking at room temperature until protein bands were visible against a clear gel background. The gels were imaged using the molecular imager Gel Doc™ XR+ with Image Lab™ Software version 5.2.1 (Bio-Rad).

2.7. The iTRAQ Analysis

The iTRAQ analysis is an expensive procedure as the reagent kits are quite costly. Therefore, for this Masters dissertation, we decided to only analyse the White sorghum secretome. White sorghum friable callus plates were sent to the Department of Biosciences, Durham University, where cell suspension cultures were established as described in section 2.2.5. However, all iTRAQ data analysis including bioinformatics of the identified proteins was conducted at the University of the Free State-Qwaqwa Campus. Four biological replicates of cell culture were treated with 400 mM sorbitol for 48 hr. The culture filtrate (CF) proteins were extracted from both the controlled and sorbitol stressed cultures as described in sections 2.4.1 and 2.3.1.2, respectively, and subsequently used for the secretome mapping and differential protein expression using isobaric tags for relative and absolute quantitation (iTRAQ). For the secretome mapping

experiment, proteins were analysed by the multidimensional protein identification technology (MudPIT), which was part of an iTRAQ experiment, with only the iTRAQ control samples being used for mapping.

2.7.1. Sample Labelling and iTRAQ Analysis

Sample labelling and iTRAQ analysis was based on four biological replicates each of the controlled and sorbitol-treated CF protein extracts. Protein samples were prepared following the experimental procedure previously described by Smith *et al*. (2015) with minor modifications. Briefly, for each sample, 50 μg of protein were reduced with tris(2-carboxyethylphosphine) (TCEP) and alkylated with methyl-methane-thiol-sulfonate (MMTS). Thereafter, protein samples were digested using a 1:10 (w/v) trypsin to protein sample ratio. After digestion, samples were vacuum-dried, re-suspended in triethylammonium bicarbonate buffer (pH 8.5), and labelled with an 8-plex iTRAQ reagent kit (Applied Biosystems Sciex, Foster City, USA) and according to the manufacturer's instructions.

The four control replicates were labelled with tags of molecular weights 113, 114, 115, and 116, while sorbitol-treated replicates were labelled with 117, 118, 119, and 121 molecular weight tags. All eight samples were pooled to make one composite sample, which were then vacuum-dried and re-suspended in 3 mL of buffer A (10 mM K_2HPO_4/25% acetonitrile, pH 3.0). Thereafer, these were separated into 56 fractions on the Poly-LC strong cation exchange column (200 x 2.1 mm) at 300 nL/min on an Ettan LC (GE Healthcare, Pittsburgh, USA) high pressure liquid chromatography system. Peptide separation was performed using a biphasic gradient of: 0-150 mM KCl over 11.25 column volumes and 150–500 mM KCl in buffer A over 3.25 column volumes. A total of 56 fractions were collected over the gradient, and reduced to 30 by pooling those with low peptide concentration. The 30 fractions were dried down and re-suspended in 90 μL of 2% acetonitrile/0.1% formic acid. Aliquots of 20 μL from each fraction were

analysed by LC-MS/MS using a QStar Pulsar *i* mass spectrophotometer (Applied Biosystems).

2.7.2. Mass Spectra Data Analysis

Mass spectra data were analysed as described by Smith *et al*. (2015), with minor modifications. Briefly, a ProteinPilot software version 2.0.1 (Applied Biosystems) was used for data analysis against a TrEMBL database (downloaded in October 2013) sequences of the *Sorghum bicolor* only. As this is a secretome experiment, no bias correlation was applied to the quantitative data. A minimum threshold of 1.3 with a 95% confidence was set for peptide identification and a minimum score threshold of 2.0 with a 99% confidence for protein identification. All proteins identified on the basis of a single peptide were filtered out of the dataset. This gave rise to a total of 178 unique proteins, constituting the sorghum secretome mapping list of this study.

For quantitative analysis of the differentially expressed proteins, the abundance of each protein in all samples was calculated as a ratio to the 113-tagged control sample. Averages of the ratios for each protein across the four replicates each of the control and sorbitol-treated samples were calculated. The fold-change in protein expression as denoted by the ratio of control to sorbitol-treated samples, and as is shown in Equations 3 and 4 below. For the down-regulated proteins, the sorbitol-treated average was the numerator and the control was the denominator, with a negative sign denoting down-regulation. A Student's *t*-test at 95% confidence was used to compare the control averages to the sorbitol-treated averages.

Equation 3.

$$\textbf{Fold-change}_{\text{Up-regulated proteins}} = \frac{\text{Control average ratio}}{\text{Sorbitol-treated average ratio}}$$

Equation 4.

$$\textbf{Fold-change}_{\text{Down-regulated proteins}} = - \frac{\text{Sorbitol-treated average ratio}}{\text{Control average ratio}}$$

2.7.3. Bioinformatics Analysis

The UniProt database (http://www.uniprot.org; UniProt Consortium, 2014) was used to search for the theoretical molecular weights of the identified proteins, while the EMBL-EBI database (http://www.ebi.ac.uk/QuickGO/; Kulikova *et al*., 2004) was used for Gene Ontology analysis using three key terms (Biological Process, Biological Function, and Cellular Component). Conserved domains and family names of the identified proteins were searched against the InterPro database (http://www.ebi.ac.u/interpro/; Mitchell *et al*., 2014). Lastly, the presence of an N-terminal signal peptide on all identified proteins was predicted using a SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; Peterson *et al*., 2011).

CHAPTER 3

ESTABLISHMENT OF THE SORGHUM CALLUS AND CELL SUSPENSION CULTURES FOR USE IN SECRETOME STUDIES

3.1. Introduction

Sorghum is the fifth most important cereal crop after maize, rice, wheat and barley (FAOSTAT, 2016). It is mostly grown in the semi-arid regions of Africa and Asia because of its resilience to drought stress (Devnarain *et al*., 2016). Sorghum is considered to be the most recalcitrant crop for *in vitro* experiments (Pola, 2016). This is due to the accumulation of phenolics, low callus induction frequencies and a long callus generation period (Liu *et al*., 2015). To study and improve sorghum crop through biotechnology, requires amongst others, the use of callus and cell suspension culture systems. Therefore, efficient protocols for the production of these two plant systems are important (Pola, 2016).

Callus tissues are defined as masses of undifferentiated thin-walled parenchyma cells (Collin, 1998). In nature, when plants are wounded, calli are produced at the point of wounding as a protective mechanism to seal off the damaged tissue and thus preventing infection and water loss by the plant (Ikeuchi *et al*., 2013). *In vitro*, calli are induced by placing a plant tissue explant on a solid medium supplemented with plant growth hormones (PGHs), under aseptic conditions (Schaller *et al*., 2015). Plant explants differ between different tissue culture experiments and can be anything from seeds, roots, shoots, leaves, to inflorescence (Collin, 1998).

To avoid contamination of callus cultures, it is important that aseptic conditions are maintained at all times since the medium in which the calli are produced is also a good platform for microbial growth (Evans *et al*., 2003). Callus can be friable; consisting of

easily breakable cells and showing no apparent organ regeneration, or non-friable; consisting of densely packed, hard textured cells (Evans *et al*., 2003). Some calli show organ regeneration to some extent and these are either called ‘rooty’ or ‘shooty’ callus depending on the organ regenerating (Frank *et al*., 2000).

Friable calli are used to establish cell suspension cultures, which are defined as populations of rapidly growing undifferentiated cells grown in liquid medium (Evans *et al*., 2003). Cell suspension cultures are potentially useful experimental systems in the field of plant biology due to the high rates of cell multiplication, short lifecycle, and they can be easily maintained in culture by sub-culturing into fresh medium (Cai *et al*., 1987). The period of sub-culturing differs between plant species and also the inoculum cell density. For example, sorghum cell cultures were sub-cultured every two weeks (Ngara and Ndimba, 2011), while sugarcane cell cultures were either sub-cultured every 7 days (Calderan-Rodrigues *et al*., 2014) or every two weeks (Cesarino *et al*., 2013). On the other hand, Arabidopsis cell cultures were either sub-cultured every week (Oda and Fukuda, 2011) or every 15 days (Torregrosa *et al*., 2015), while tobacco had to be sub-cultured every week (Feng *et al*., 2015). The sub-culturing and maintenance of cell suspension cultures help in reducing cell aggregates from the first cell culture establishment experiment (Evans *et al*., 2003). With generations of sub-cultures, single cells slowly break off from cell clusters resulting in a finely suspended culture.

The main objective of this chapter was to initiate, propagate and maintain sorghum callus and cell suspension cultures to be further used in the secretome studies.

3.2. Sorghum Seed Germination

Due to the fact that White sorghum had previously been used in callus production procedures (Ngara *et al*., 2008), this sorghum genotype was excluded from all germination, contamination, and callus induction rate calculations in this study. Sorghum

genotypes (SA 1441, ICSV 112, ICSV 210, ICSV 213, ICSB 338, ICSB 73, and Macia; Table 2.1) were successfully germinated on sorghum seed germination medium as described in section 2.2.2. Germination was observed from as early as two-days post-sowing on most seeds from all genotypes. Figure 3.1 A below shows seeds that had germinated at day four post-sowing. Some seeds did not germinate as indicated in Figure 3.1 B, while other seeds had germinated but exhibited stunted seedling growth as indicated by the seedling with a black block around in Figure 3.1 C.

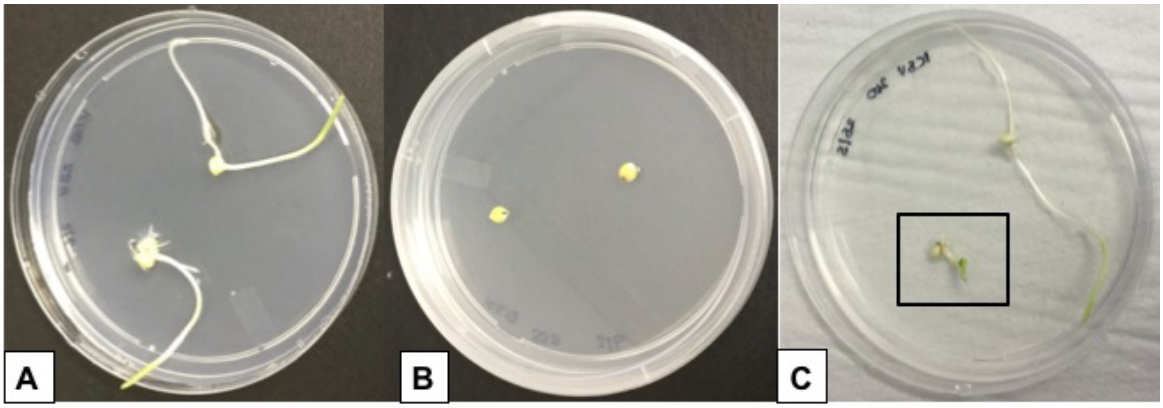

Figure 3.1. Sorghum seed germination results four-days post-sowing. (A) shows seeds, which successfully germinated. (B) shows seeds, which failed to germinate on sorghum seed germination medium. (C) highlights in the black rectangular block a seedling showing stunted growth.

The percentage germination and contamination rates of the seven sorghum genotypes were calculated based on the number of seeds that germinated or were contaminated per plate, respectively. In this study, a contaminated seed or seedling was characterized by being in close or direct contact with any microbial contaminants such as bacteria or fungi as illustrated in Figure 3.2 A below. On the other hand, a germinated seed was

characterized by the emergence of a shoot and/or root system four-days post-sowing as illustrated in Figure 3.2 B. Fifteen randomly selected plates containing two seeds each from each of the seven genotypes were selected for calculation of the percentage germination and contamination rates. The calculations were done using data obtained at day four post-sowing.

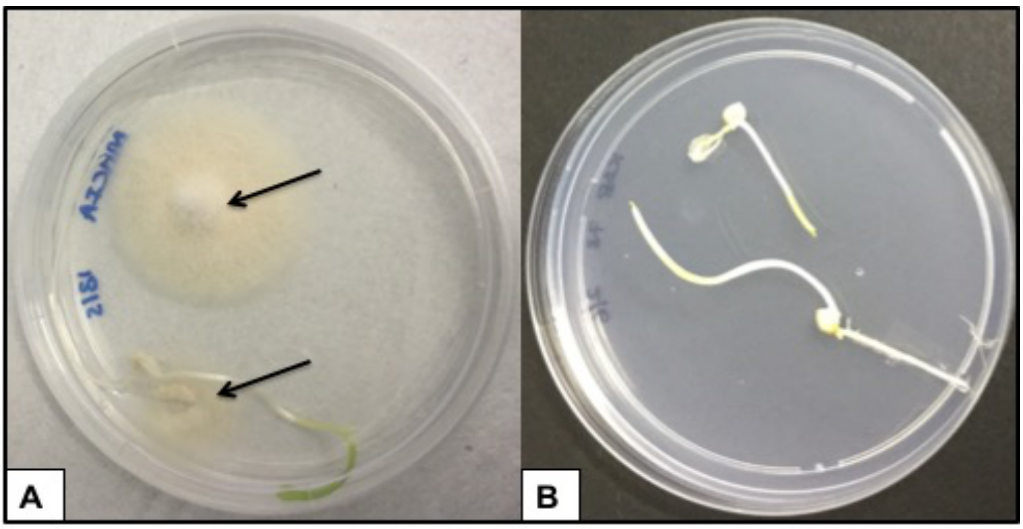

Figure 3.2. Plates showing the results of sorghum seed germination four-days post-sowing. (A) illustrates fungal contaminated seed and seedling (indicated by black arrows). (B) shows germinated seeds with no contamination.

Both SA 1441 and ICSB 338 showed the highest germination rates of 90% as compared to the other genotypes, whereas ICSV 213 had the lowest germination rate of 47% (Table 3.1). Overall, the percentage germination rates of all seven genotypes were statistically significant at 5% level of significance ($p < 0.05$) estimated using a One-way ANOVA. On the other hand, the overall percentage contamination rates of all seven genotypes were not statistically significant at 5% level of significance ($p < 0.05$). The

One-way ANOVA Tables of percentage germination and contamination rates are summarised in Appendix 2.

Table 3.1. Germination and contamination rates of different sorghum genotypes.

Sorghum Genotype	Percentage Germination rate*#	Percentage Contamination rate*
SA 1441	90 ± 0.280c	13 ± 0.229
Macia	83 ± 0.244c	17 ± 0.309
ICSV 210	77 ± 0.320bc	7 ± 0.176
ICSV 112	60 ± 0.338a	7 ± 0.176
ICSV 213	47 ± 0.297a	30 ± 0.254
ICSB 73	63 ± 0.297ab	17 ± 0.244
ICSB 338	90 ± 0.207c	27 ± 0.320

* Data presented as mean ± SD (n = 15).

Different letters indicate different means at 5% level of significance ($p < 0.05$).

3.3. Initiation and Maintenance of Sorghum Calli

Sorghum calli were successfully initiated from four-day old shoot explants cultured on sorghum callus induction medium supplemented with PGHs 2,4-D and NAA (Figure 3.3; section 2.2.3) for all genotypes. Figure 3.3 below shows a typical layout of the callus initiation experiment for all the seven genotypes.

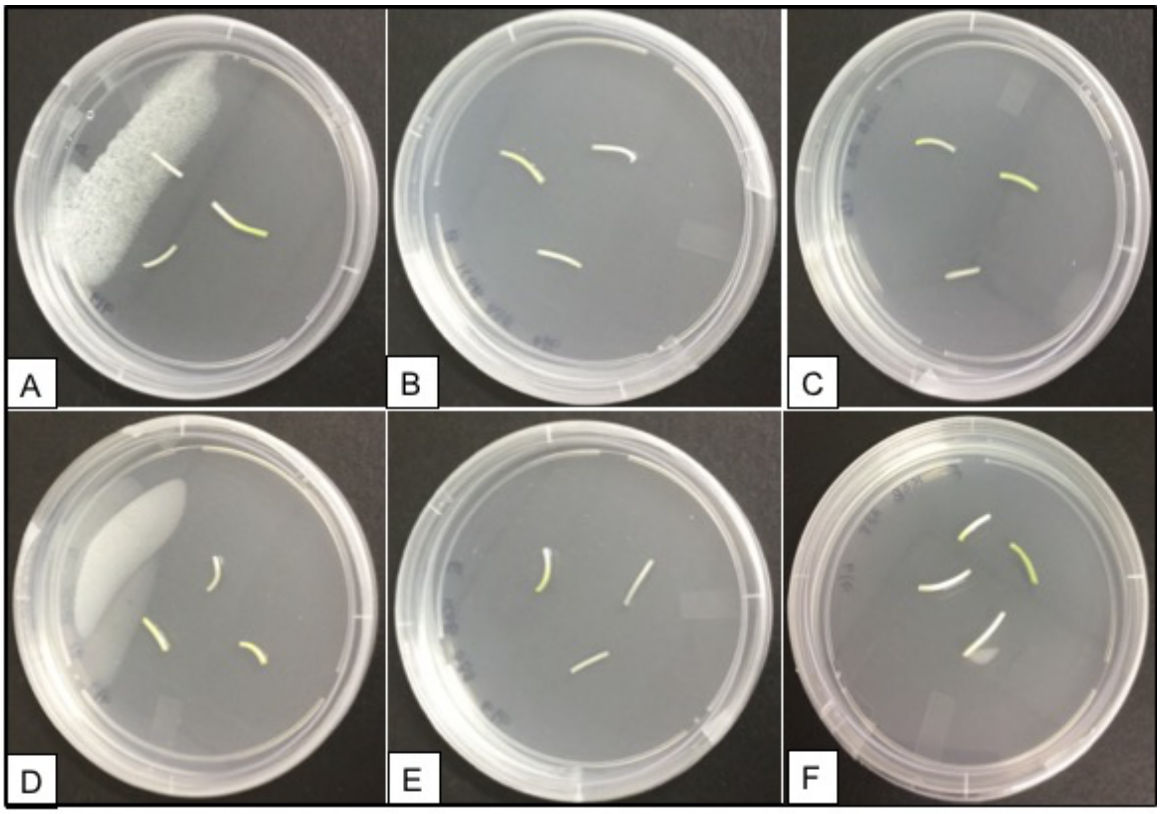

Figure 3.3. Petri-dish plates containing shoot explants at day 0 post-plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

Visual assessment of callus growth on different medium compositions was done to evaluate the influence of different PGH combinations on callus formation across the seven sorghum genotypes. Callus initiation results of three genotypes (SA 1441, ICSV 210 and ICSB 338) are presented in the following section. The results of the other four genotypes (ICSV 112, ICSV 213, ICSB 73 and Macia) are presented in Appendix 3.

3.3.1. SA 1441 Callus Induction

After five weeks of culturing on different medium types (A-F; Table 2.2; Figure 3.3), some shoot explants exuded a brown-purple pigment indicated by the black arrows in Figure 3.4. The intensity of the colour increased until the calli appeared black and eventually died. It is not clear if the pigment played any role in reducing the rate at which these calli proliferated. Medium A, with no PGH allowed the normal development of the seedling with roots and shoots and no callus was induced (Figure 3.4 A). Medium B supplemented with only 3 mg/L of 2,4-D allowed shoots to elongate with some formation of callus (Figure 3.4 B). Similar results were observed on medium C (4 mg/L of 2,4-D; Figure 3.4 C), medium E (3 mg/L 2,4-D and 2.5 mg/L NAA; Figure 3.4 E) and medium F (4 mg/L 2,4-D and 2.5 mg/L NAA; Figure 3.4 F). Medium D supplemented with 2.5 mg/L NAA mostly resulted in the development of roots, whereas few explants developed into shoots and callus (Figure 3.4 D).

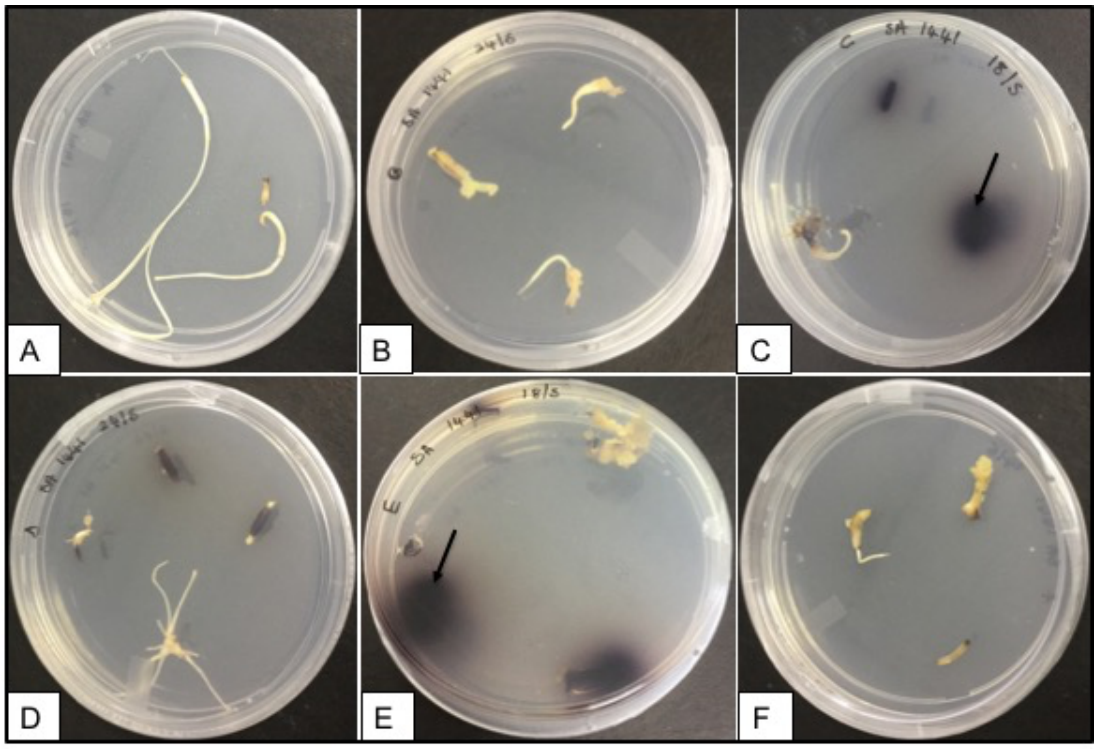

Figure 3.4. Petri-dish plates containing sorghum genotype SA 1441 shoot explants at 5-weeks post-plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

Despite the reduced number of calli induced (and correlating with pigment production), maintained calli proliferated and grew into large lumps after several generations of sub-culturing (Figure 3.5). The calli were cream-yellow in colour, had a globular shape, and not friable.

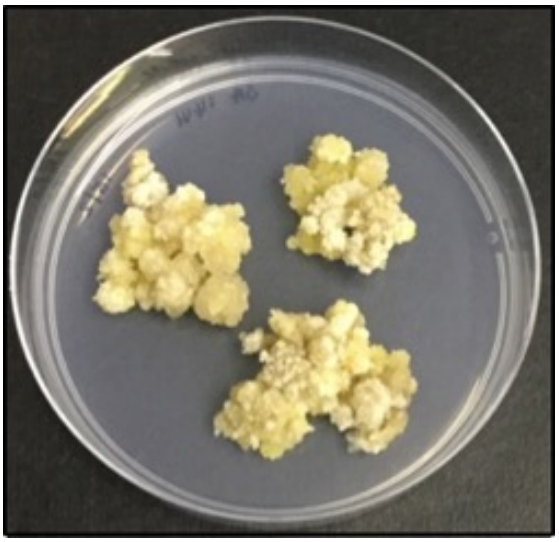

Figure 3.5. SA 1441 sorghum calli after several generations of sub-culturing.

3.3.2. ICSV 210 Callus Induction

ICSV 210 calli developed on media B, C, E, and F after five weeks of culture (Figure 3.6 B, C, E and F, respectively). Medium A showed normal development of seedlings, with roots and leaves, but no callus development as is seen in Figure 3.6 A. Shoot explants on medium D, mostly induced the formation of roots, few shoots and callus (Figure 3.6 D).

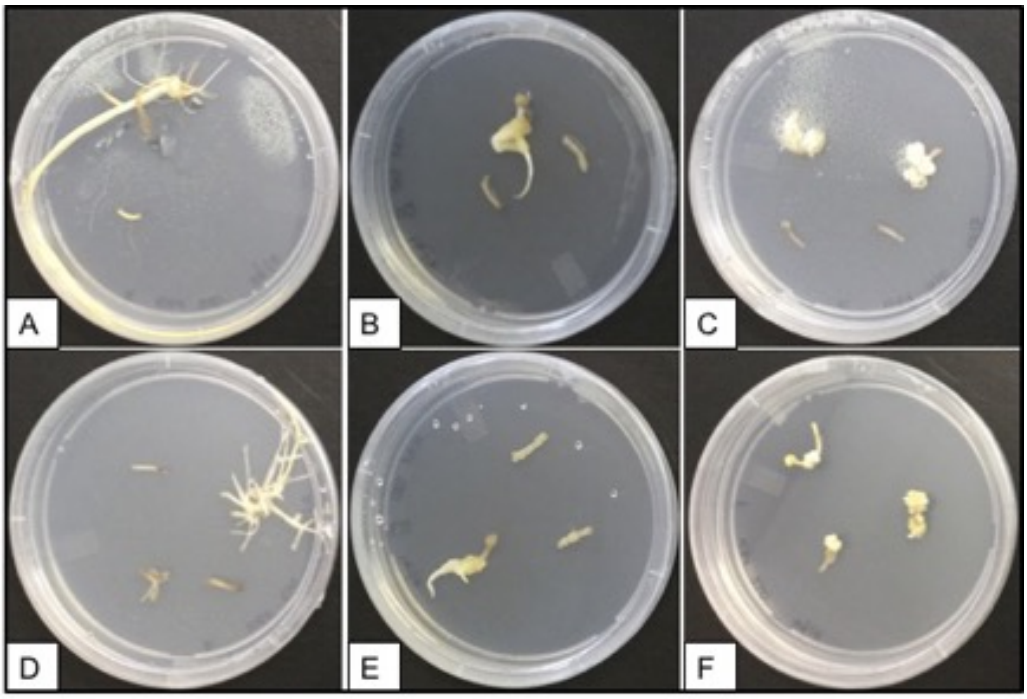

Figure 3.6. Petri-dish plates containing sorghum genotype ICSV 210 shoot explants at 5-weeks post-plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

After a few generations of sub-culturing, callus proliferated and grew into large masses, which were light cream in colour (Figure 3.7). Some calli from this genotype had root-like tissue structures (indicated by black arrow in Figure 3.7), which proliferated from individual cells across media B, C, E and F.

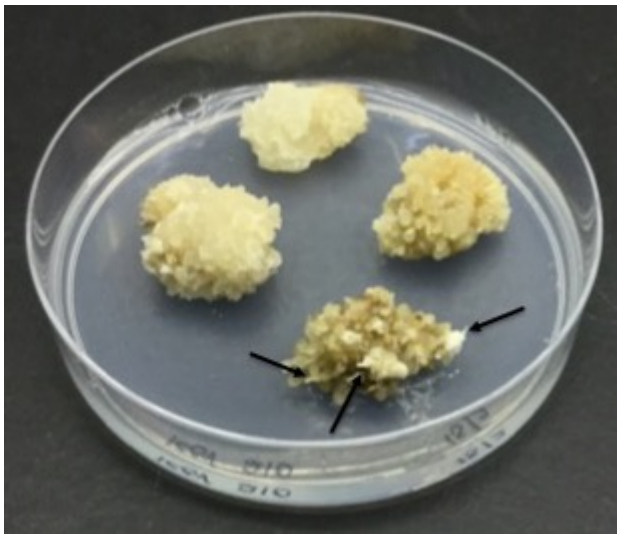

Figure 3.7. ICSV 210 calli after a few generations of sub-culturing. Black arrows indicate “rooty” tissues growing on calli.

3.3.3. ICSB 338 Callus Induction

Amongst all the seven genotypes used in this study, only the drought susceptible ICSB 338 type was able to induce callus on a range of media (Figure 3.8). Medium A gave similar results as was seen for the other genotypes. In media B, C, E, and F, shoots elongated to some extent and callus masses developed. Genotype ICSB 338 did not only produce roots on medium D as this was the case for genotypes ICSV 213, ICSB 73 and Macia, but it was also observed that calli were induced as well.

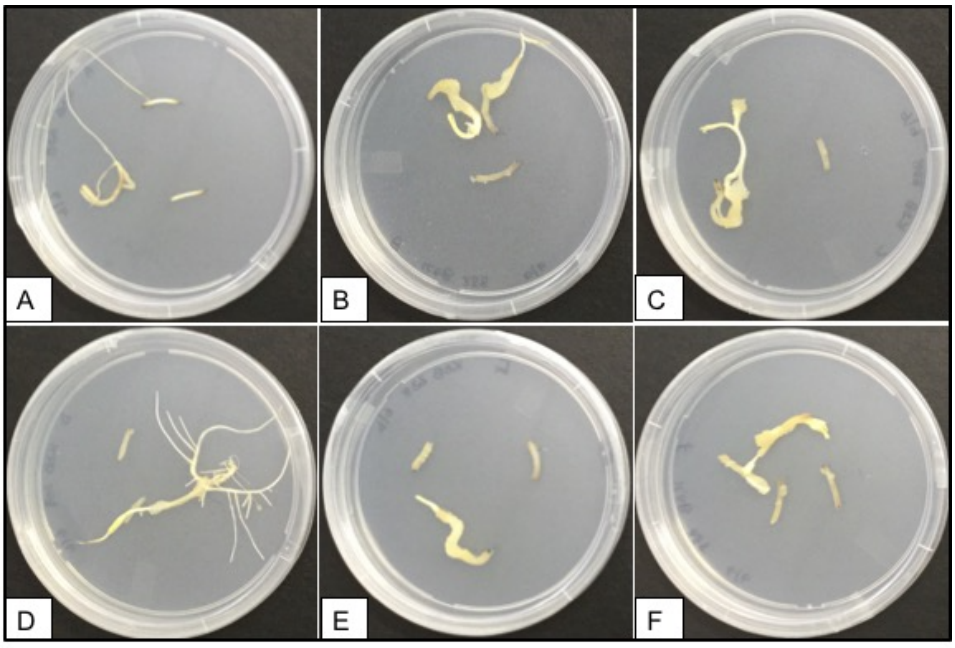

Figure 3.8. Petri-dish plates containing sorghum genotype ICSB 338 shoot explants at 5-weeks post-plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

Figure 3.9 A shows the sizes of the calli that were used for the purpose of sub-culturing and maintenance in culture on medium E. Figure 3.9 B shows the same callus plate after four weeks in culture. The callus grew into large cell masses, which were subsequently used to establish cell suspension cultures. The calli had a light cream colour and were highly friable.

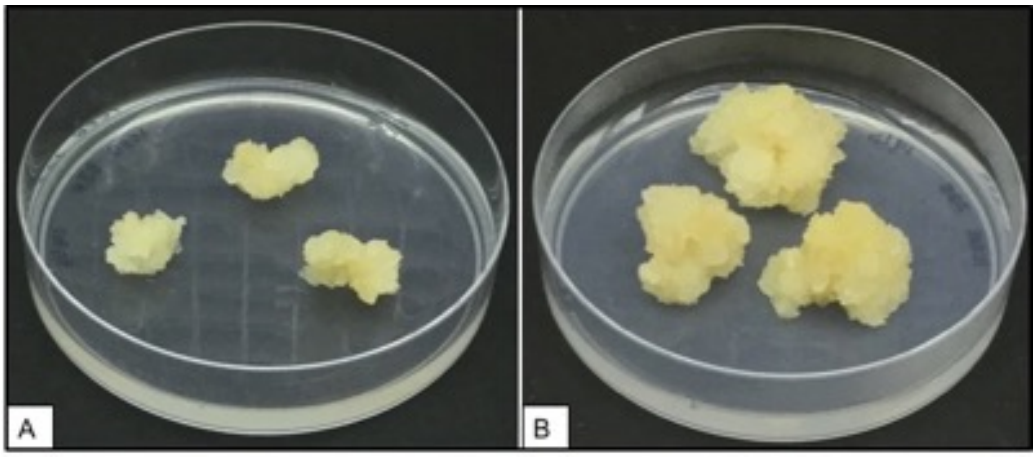

Figure 3.9. ICSB 338 calli on sorghum callus induction medium. (A) shows calli immediately after sub-culture. (B) shows calli four weeks post sub-culture.

It was also observed that different types of media (B, E and F) induced callus, which differed in both texture and morphology (Figure 3.10). Medium B produced clusters of globular-shaped calli, which were not friable. Calli produced on media E and F both had the same texture and morphology. These callus masses were made up of one cluster, had different shapes depending on direction of growth, and were highly friable.

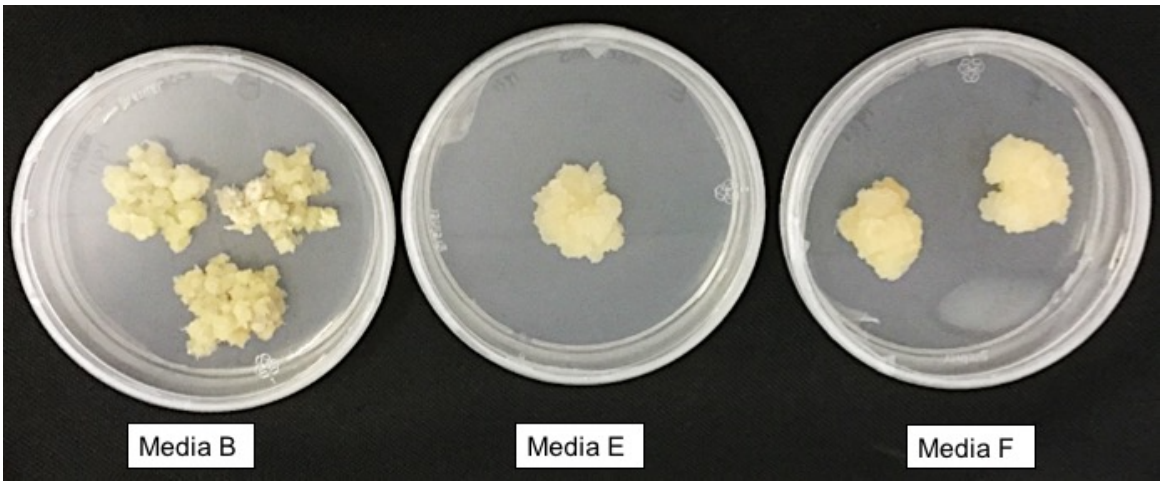

Figure 3.10. ICSB 338 sorghum calli on different sorghum callus induction media.

3.3.4. White Sorghum Callus Induction

White sorghum was previously used for callus induction by Ngara *et al*. (2008) and further regenerated in our Research Group at the University of the Free State, Qwaqwa Campus by an Honours student in 2015 (Lekekela, 2015). As a result, it was not included in germination, contamination and callus induction rate calculations. Previously, calli from shoot explants of this genotype were induced on medium E (3 mg/L 2,4-D and 2.5 mg/L NAA; Ngara *et al*., 2008). Subsequently, the same medium was used for this genotype in callus induction experiments and maintenance procedures in the current study. Figure 3.11 A shows the sizes of the calli that were used for the purpose of sub-culturing and maintenance in culture. Figure 3.11 B shows the same callus plate after four weeks in culture. The callus grew into large cell masses, which were subsequently used to establish cell suspension cultures.

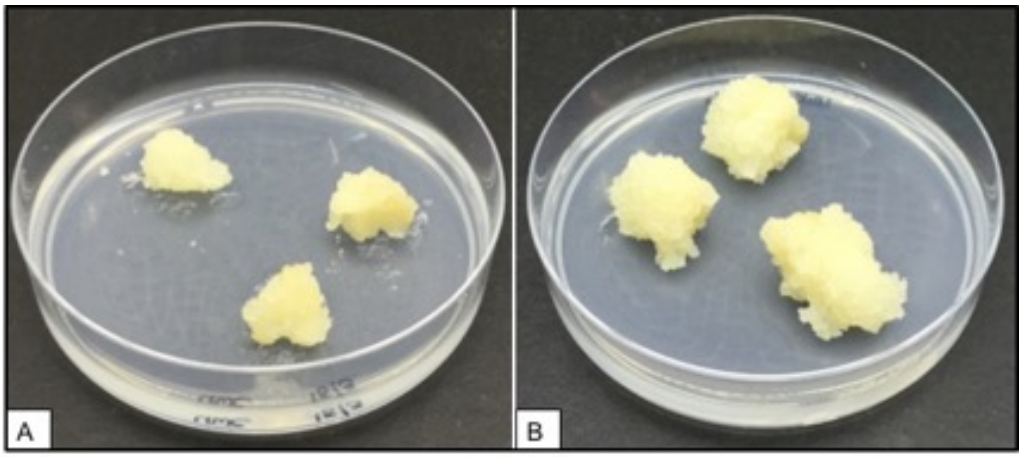

Figure 3.11. White sorghum calli on sorghum callus induction medium. (A) shows calli immediately after sub-culture. (B) shows calli four weeks post sub-culture.

3.3.5. Sorghum Calli Induction Rate and Induction Medium Selection

The rate of callus induction was calculated as a percentage based on the number of explants, which induced callus. For each medium type (Table 2.2; section 2.2.3), three explants per plate from two plates were assessed. The percentage callus induction rate ranged between 0% and 84% across different media and genotypes as presented in Figure 3.12. A Two-way ANOVA analysis showed that genotypes, different media, as well as their interaction had a statistically significant influence on callus induction rates, as indicated in Table 3.2 below. Medium A did not induce calli across all genotypes. Medium D did not induce callus for genotypes ICSV 213, ICSB 73, and Macia. Media B and E gave the highest callus induction rates for genotypes ICSV 210, ICSB 73, ICSB 338, and SA 1441 as compared to the other media types. Media B and F gave the highest callus induction rate for genotypes ICSV 213 and Macia. Lastly, high callus induction rates in genotype ICSV 112 were obtained from media B and C as compared to the other media types. In summary, based on a Two-way ANOVA analysis, genotypes ICSV 210 and ICSB 338, and media B and E had higher callus induction rates (Appendix 4).

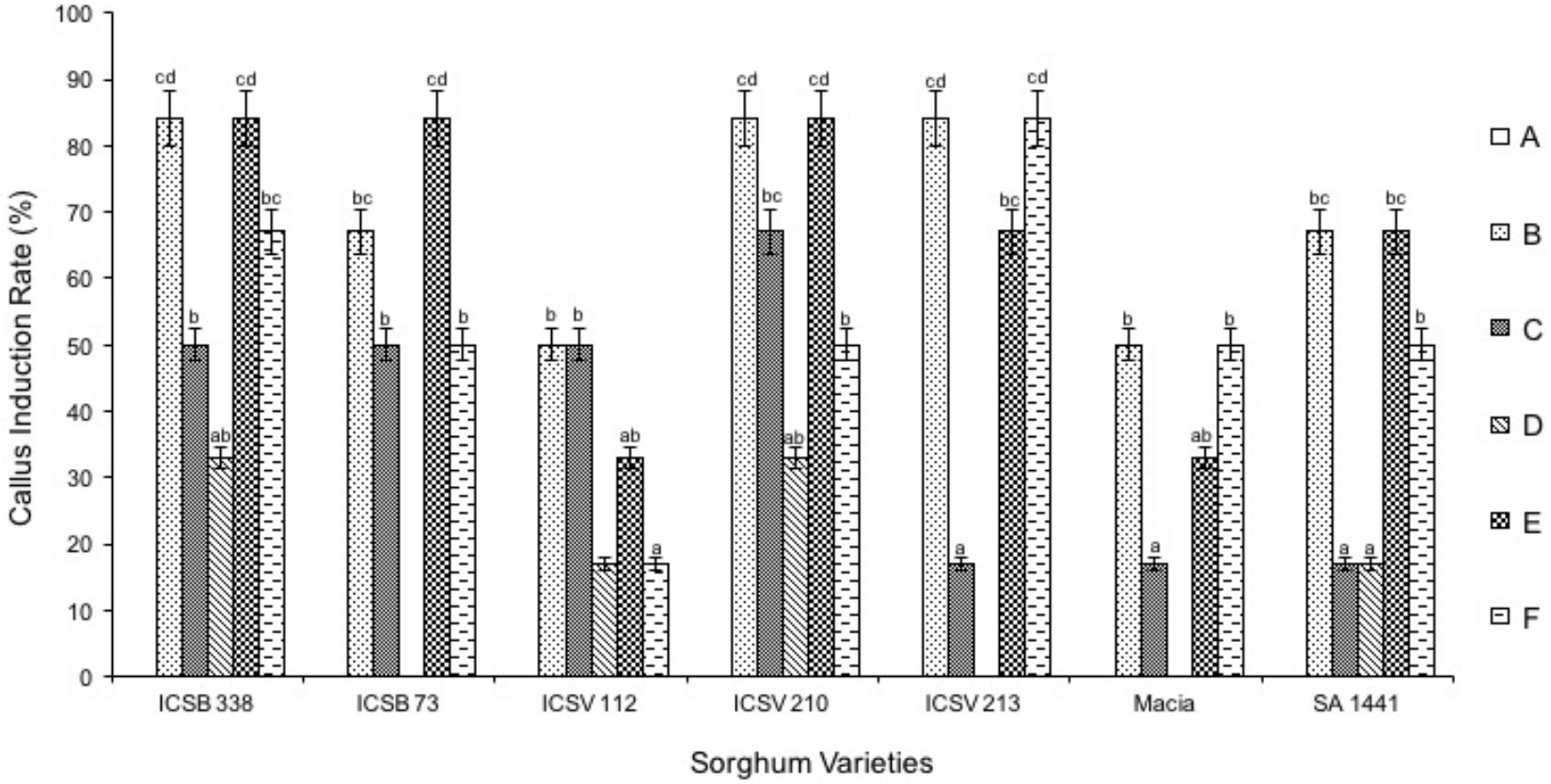

Figure 3.12. Callus induction results per sorghum genotypes. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA. Data presented as mean ± SD (n = 6). Different letters above error bars denote differences in mean difference at 5% level of significance ($p < 0.05$) analysed using Two-way ANOVA.

Table 3.2. A Two-way ANOVA analysis of sorghum percentage callus induction at 5% level of significance ($p < 0.05$).

Percentage Callus induction Rates					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Replicates	2	0.00508	0.00254	0.17	
Genotypes	6	1.31498	0.21916	14.72	<.001
Media	5	8.12934	1.62587	109.21	<.001
Genotypes x Media	30	1.96271	0.06542	4.39	<.001
Error	82	1.22074	0.01489		
Total	125	12.63284			

Results of callus induction media that produced large masses of friable calli between the six medium types are summarised in Table 3.3 below. Medium B produced large non-friable calli masses in genotypes SA 1441, ICSV 210, ICSV 112, ICSB 73, ICSB 338 and Macia. Medium C produced calli, which did not grow further during maintenance in all the genotypes except ICSV 112, which induced large non-friable calli. Medium D induced calli in SA 1441, ICSV 210, ICSV 112 and ICSB 338, however, the calli did not grow any further during maintenance. Medium E produced large friable calli for genotypes ICSB 338. Based on the observations made during these callus induction experiments, ICSB 338, which is characterised as drought susceptible (Table 2.1) produced friable callus more easily in comparison to the other genotypes. Although medium B induced more callus when compared to media A, C, D, and F, the masses were non-friable and thus not useful for the initiation of cell suspension cultures (Appendix 4). The same goes for genotype ICSV 210, which induced non-friable callus (Appendix 4). Based on these results, genotype ICSB 338 and medium E were selected for further use due to the production of large friable callus masses. Even though medium F seemed to have

produced large friable callus (Table 3.3) on ICSB 338, callus masses were smaller than the ones produced from medium E.

Table 3.3. A summary of callus initiation results of all seven sorghum genotypes used in this study.

Genotypes	Plant Hormonal Combinations*					
	A	B	C	D	E	F
SA 1441	-	++	+	+	++	+
ICSV 210	-	++	+	+	++	+
ICSV 213	-	+	+	-	++	++
ICSV 112	-	++	++	+	+	+
ICSB 73	-	++	+	-	++	+
ICSB 338	-	++	+	+	+++	+++
Macia	-	++	+	-	+	++

*Minus sign denotes lack of callus induction; (+) denotes induction of calli, which did not grow during calli maintenance. (++) denotes induction of large non-friable calli. (+++) denotes induction of large friable calli that were usable for establishing cell suspension cultures.

3.3.6. Light Microscopic Analysis of Sorghum Calli

Light microscopic analysis was done to observe the structure of sorghum callus cells derived from three sorghum genotypes, namely SA 1441, ICSB 338, and White sorghum. Figure 3.13 below shows the microscopic images of sorghum callus cells. Individual cells were observed in all the three genotypes (indicated by black arrows). These cells differed in shape, with cells from genotype SA 1441 and ICSB 338 having similar shapes and sizes. White sorghum calli had elongated rod-like shaped cells.

Clusters of cells were also observed (indicated by dotted black arrows) in SA 1441 and White sorghum.

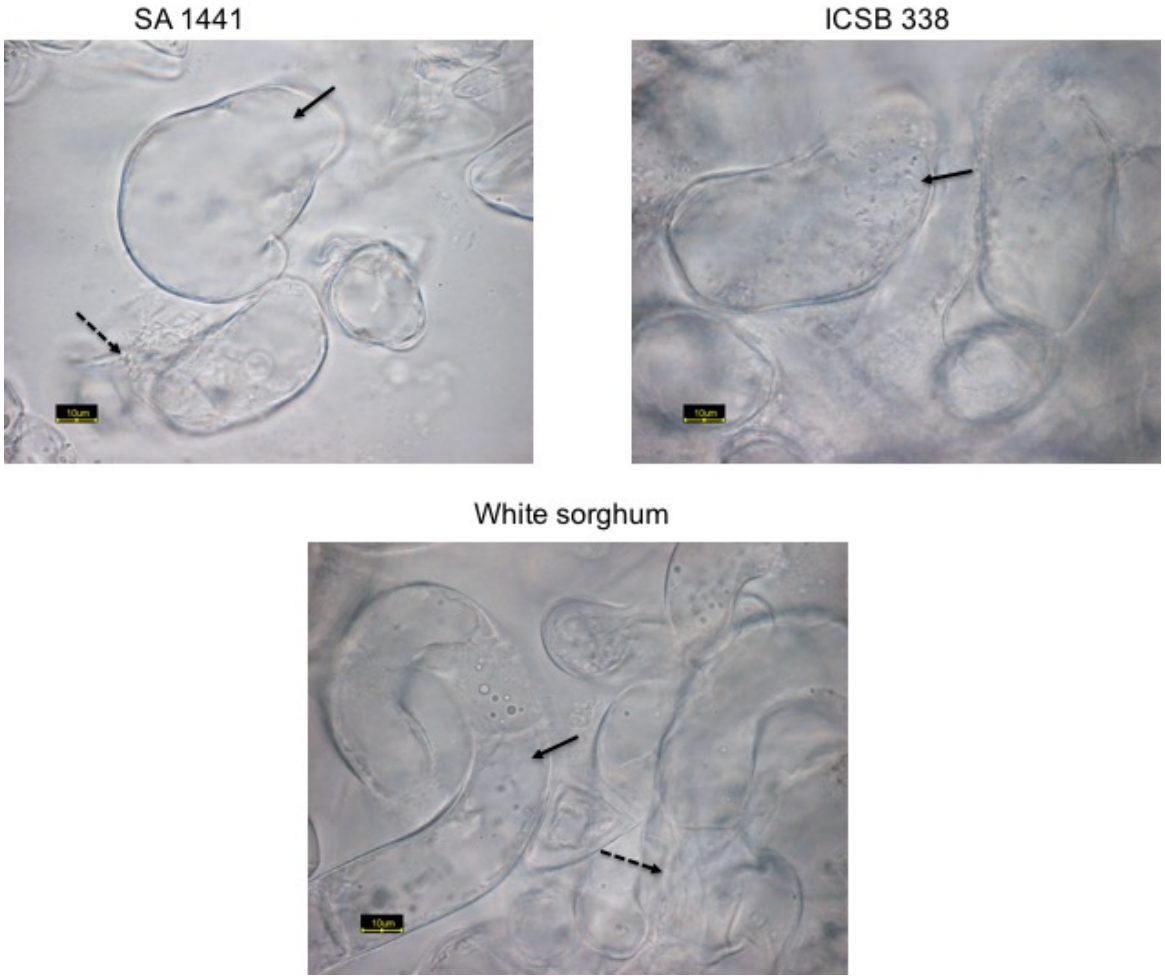

Figure 3.13. Light microscopic evaluation of different sorghum callus cultures. Individual cells are indicated by black arrows whereas cell clusters are indicated by black dotted arrows. The scale bar is 10 μm.

3.4. Initiation of Cell Suspension Cultures

From the seven sorghum genotypes that gave rise to callus, only one genotype was selected for use in establishing cell suspension cultures. The basis for selection was that the genotype must readily produce large amounts of friable callus masses upon induction in a reproducible manner across several experiments. Based on these selection criteria, ICSB 338 was ranked top of the list and thus selected for subsequent experiments. ICSB 338 was used in establishing cell suspension cultures together with White sorghum, which had previously been used for callus induction by Ngara *et al*. (2008) and replicated by Lekekela (2015).

Cell suspension cultures were initiated using four-week old actively growing friable calli (Figures 3.9 B and 3.11 B) as described in section 2.2.5. Figures 3.14 A and C below show liquid medium containing callus masses broken down into small pieces on the day of sub-culturing. During the period of incubation, these small pieces continued to break, releasing individual cells, which grew and multiplied in the liquid medium resulting in a dense cell suspension culture as is seen in Figures 3.14 B and D. During cell division and growth, the cells secreted proteins into the culture medium.

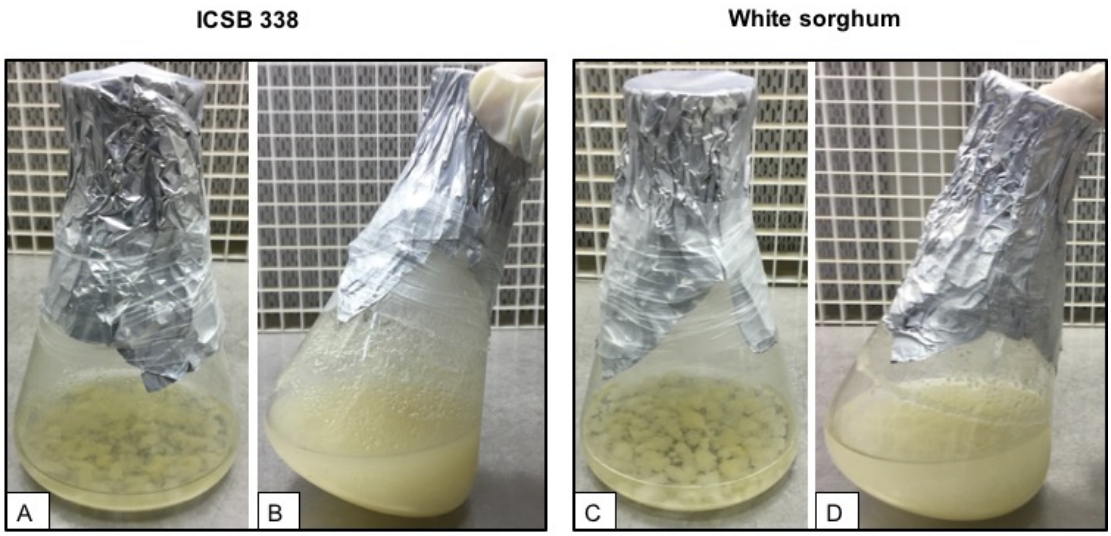

Figure 3.14. The establishment of ICSB 338 and White sorghum cell suspension cultures. ICSB 338 (Flask A) and White sorghum callus (Flask C) was broken into small pieces in liquid medium E (3 mg/L 2,4-D and 2.5 mg/L NAA) on the day of initiating cell suspension cultures. Flask B and D shows ICSB 338 and White sorghum cell culture lines after two generations of sub-culture, respectively.

3.5. Discussion

The plant tissue culture technique is very sensitive to contamination and requires that aseptic conditions are maintained throughout the experiments. One of the advantages of using tissue culture instead of an intact plant is the ability to control the physical and chemical environment in the laboratory to suit a particular experiment (Collin, 1998; George *et al*., 2008). Although the laboratory environment can be controlled, microorganisms still continue to be the major cause of culture contamination in plant tissue culture experiments (Cassells, 1991). Generally, nutrient medium is rich in organic compounds (such as sucrose), inorganic salts (micro- and macronutrients), carbon sources, vitamins, amino acids, and other compounds necessary for the growth (George *et al*., 2008). There is no way of selecting for only plant explants to grow on the medium.

For this reason, the nutrient medium provide a good growth platform for both fungal and bacterial microorganisms (Evans *et al*., 2003).

Microorganisms change the chemical environment by using the growth medium and the plant explant as food sources (George *et al*., 2008). In addition they also secrete metabolites into the medium, which greatly reduce the growth of plant explants and may ultimately lead to death of the plant tissues (Harabi *et al*., 2016). Percentage contamination rates in the seed germination experiment ranged between 7% and 30% across the seven sorghum genotypes evaluated in this study even though media, glassware and other laboratory instruments were sterilised by autoclaving. Contamination in tissue culture work is not a new observation; it has always been encountered even in the early ages of tissue culture (Langens-Gerrits *et al*., 1997).

To eliminate as much contaminants as possible, aseptic conditions should be enforced in tissue culture laboratories. For example, Langens-Gerrits *et al*. (1997) stated how hot water treated Lilium and Acer species had reduced contaminants in tissue culture. In sorghum tissue culture, however, 70% ethanol, commercial bleach, mercury chloride, and Teepol solutions amongst others, have been used to sterilise seed (Sai *et al*., 2006; Polumahanthi *et al*., 2014). The sources of these contaminants may be from the environment, where the plants were grown, human handling of the seeds, the plant, culture vessels, media, and also from the instruments used in tissue culture experiments (Evans *et al*., 2003). Some seeds contain seed-borne fungal and/or bacterial infections within the embryo, where they are not reached by the seed surface-sterilisation procedures used in plant tissue culture (Bishop *et al*., 1997).

In this study, germination was characterized by the emergence of shoot and/or root systems from the seeds cultured on sorghum seed germination medium, four-days post-sowing. *In vitro* seed germination is often hindered if the seeds were collected from an

open field and seeds are stored under unsuitable storage conditions (Bass and Stanwood, 1978). In the case of this project, limited supplies of field grown sorghum seeds were received. It is therefore important to have an efficient seed surface sterilisation protocol in order to end up with enough seeds, seedlings and thus explants for callus induction procedures. Different protocols for surface sterilisation exist, and these differ depending on the explants used and also on the species. Sorghum seeds have previously been surface sterilised using 70% (v/v) ethanol for 2 min and either sodium hypochlorite ranging from 2.5% - 50% (w/v; up to 30 min) or mercury chloride [0.1% - 1% (m/v), up to 4 min; Ngara *et al*., 2008; Sudhakar *et al*., 2008; Polumahanthi *et al*., 2014].

In the current study, 70% (v/v) ethanol was used to surface sterilise sorghum seeds for 1 min followed by a 3.5% (m/v) sodium hypochlorite. This method resulted in statistically non-significant contamination rates and statistically significant germination rates ranging between 47% and 90% (Appendix 2; Table 3.1). This method might not be the most effective method for surface sterilising sorghum seed since other studies used higher than 3.5% (m/v) concentrations of sodium hypochlorite solution. Ngara *et al*. (2008) used 12% (m/v) sodium hypochlorite from industrial bleach, but this could not be sourced during the duration of seed germination experiments. However, none of the studies mentioned above where sorghum seeds were surface sterilised showed records of the rate of seed contamination and germination. In all the studies mentioned above, the longest time in which 70% (v/v) ethanol was kept in contact with the seeds was 2 min. This might mean that prolonged stay of seeds in contact with 70% (v/v) ethanol might have adverse effects on seed germination.

In general, tissue culture work has many challenges, including contamination, reproducibility, and low callus formation efficiency (Karim *et al*., 2015; Liu *et al*., 2015). Problems that were encountered in this study include the production of potentially toxic

pigments possibly as a form of defence mechanism by the plant against wounding (Figure 3.4), low callus induction frequencies (Figure 3.12), and long periods of callus induction. These problems have been encountered in different sorghum cultivars, both sweet and grain sorghum (Cai *et al*., 1987; Sai *et al*., 2006; Liu *et al*., 2015). To produce enough callus masses for the purposes of establishing cell suspension cultures and microscopic analyses, it took between 5 - 9 months for genotypes ICSB 338, SA 1441 and White sorghum, whereas calli from the other genotypes (ICSV 112, ICSV 210, ICSV 213, ICSB 73 and Macia) used in this study died. In another study (Raghuwanshi and Birch, 2010), it took at least 12 - 16 weeks to produce large calli masses of sweet sorghum.

Different medium compositions have been used in different sorghum studies, looking for at least one medium type that will produce enough callus, be it for plant regeneration or molecular studies (Sai *et al*., 2006; Sudhakar *et al*., 2008; Liu *et al*., 2015). Sai *et al*. (2006) evaluated 24 diverse sorghum varieties for their callus induction responses with two types of media, MS and nutrient broth medium supplemented with 1 mg/L kinetin, 1 mg/L NAA and 1 mg/L 6-benzylaminopurine (NBKNB) using shoot tip explants. They reported a good response of callus induction for all the genotypes they used on both medium types. Liu *et al*. (2015) reported callus induction rates between 39% and 84% for all the eight media types used in the study, while Sudhakar *et al*. (2008) reported sorghum genotype IS3566 to have the highest callus induction rate than the other five genotypes used in the study. In the current study, sorghum genotype ICSB 338 and ICSV 210 showed higher callus induction rates, which were statistically significant according to a Two-way ANOVA (Figure 3.12; Appendix 4). However, during maintenance, ICSV 210 calli were not friable and showed reduced growth rates, and thus, could not be used to establish cell suspension culture. Differences in genotypic callus induction rates has also been recorded in other studies (Rao *et al*., 2000; Gupta *et al*., 2006).

In the current study, MSMO medium supplemented with different PGH combinations (section 2.2.3) was used for callus induction of all seven sorghum genotypes. The MSMO media B (3 mg/L 2,4-D) and E (3 mg/L 2,4-D and 2.5 mg/L NAA), produced high callus induction rates in all seven genotypes (Figure 3.12; Appendix 4). Although high induction rates were obtained, callus growth and reproducibility on these medium types was still low for some genotypes, such as ICSV 112 and Macia, and thus could not be used further in this study. However, media B and E efficiently gave statistically significant high callus induction rates for ICSV 210, and ICSB 338 (Figure 3.12; Appendix 4). But due to the inability of medium B to produce friable callus, only medium E, which produced friable callus could be used for maintaining callus in culture. These results are in agreement with Ngara *et al*. (2008). Most tissue culture studies combine two types of PGHs, auxin and cytokinins for callus induction (Sudhakar *et al*., 2008; Liu *et al*., 2015), but in the current study we combined two auxins PGHs (2,4-D and NAA). The callus induction procedure used in this study was adapted from a previous sorghum tissue culture experiment by Ngara *et al*. (2008). Low concentration of PGH 2,4-D is commonly used for callus induction in cereals (Chawla, 2009).

Light microscopic analyses were carried out to observe the structure of ICSB 338, SA 1441 and White sorghum callus cells. Single cells of different shapes and clustered cells were observed for all three genotypes. SA 1441 callus masses were made up of small globular callus, which were non-friable. There were fewer clustered cells viewed on SA 1441 field of view (Figure 3.13) and single cells could be visualised easily. However, this was hardly the case for ICSB 338 and White sorghum. Both the genotypes had friable calli made up of tightly packed cells, which made it difficult to observe single cells only on the field of view on the microscope. White sorghum cells were long and had a rod-like shape and for this reason, it was almost impossible to view single cells on high magnifications. However, single cells could be visualised on lower magnification (Figure 3.13).

Cell suspension cultures of ICSB 338 and White sorghum were successfully established from the friable callus masses (Figure 3.14) on medium E. These cells were maintained in culture by culturing 30 mL (containing 15 - 20% SCV) of cell culture and 70 mL fresh medium when sub-culturing the cell suspension cultures (section 2.2.5). The cell suspension cultures of both genotypes were finely suspended in medium E as indicated in Figures 3.14 B and D. These fine cultures were obtained after two generations of sub-culturing and were further characterised as discussed in Chapter 4.

CHAPTER 4

CHARACTERIZATION OF SORGHUM CELL SUSPENSION CULTURES

4.1. Introduction

Cell suspensions are defined as a population of rapidly growing undifferentiated cells that are grown in liquid medium (Evans *et al*., 2003). From these finely suspended cell cultures, different growth parameters such as cell growth and viability can then be assessed.

Cell growth can be assessed by monitoring the amount of settled cell volume (SCV) or packed cell volume (PCV) in culture, fresh and/or dry weight, and cell numbers (Evans *et al*., 2003). Cell growth measurements are then plotted against time, generating a growth curve, which shows the progression of cell growth over time. The typical sigmoidal growth curve consists of three distinct phases namely; the lag phase, the exponential phase and the stationary phase (George *et al*., 2008). During the lag phase, there is a very slow to no increase in cell density. The exponential phase, also known as the log phase represents a period where cells grow and multiply exponentially. Lastly, during the stationary phase, cell multiplication plateaus possibly due to nutrient exhaustion and, subsequently, cells start to die (George *et al*., 2008). The duration of each of these phases differs depending on the inoculum cell density, growth conditions and plant species (Gupta *et al*., 2011; Rahman *et al*., 2012).

Cell viability can be assessed across the growth curve using the MTT or Evans blue assays. The MTT-formazan assay is based on the principle that mitochondrial dehydrogenase of viable cells with active metabolism, converts the yellow tetrazole MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into a purple formazan, which is detected spectrophotometrically at 490 nm (Angius and Floris, 2015). The

Evans blue dye is effective in determining whether or not cells are alive in vascular plants (Crippen and Perrier, 1974). The dye penetrates through cells whose plasma membrane integrity is lost. The amount of dye absorbed by cells is then detected spectrophotometrically at 600 nm (Baker and Mock, 1994).

Cell suspension cultures are a potentially useful experimental system in plant biology, including for studies on the composition of secreted proteins (secretome) and their differential expression following both biotic and abiotic stress factors. As such, cell suspension cultures have been used to study plant responses to osmotic stress. In plants, limitations in water availability cause osmotic stress, also referred to as drought stress (Anjum *et al*., 2011). In cell cultures, osmotic stress can be induced using osmotica, such as sorbitol and polyethylene glycol. Sorbitol has been used to induce osmotic stress in sorghum (Ngara, 2009), sweet potato (*Ipomoea batatas*; Wang *et al*., 1999), and tobacco (Monetti *et al*., 2014) cell cultures. Polyethylene glycol has also been used in cell cultures of *Stevia rebaudiana* (Gupta *et al*., 2015b), and sugarcane (Patade *et al*., 2012).

The objective of this chapter was to characterise cell suspension cultures before and after exposure to sorbitol-induced osmotic stress.

4.2. Sorghum Cell Culture Growth Parameters

4.2.1. Measuring Cell Growth

The growth of ICSB 338 and White sorghum cell suspension cultures was assessed at two-day intervals using the SCV method as described in section 2.2.6.1. Cell growth measurements of three independently established cell suspension cultures per genotype were recorded and used to generate a growth curve over a period of 14 days. Figure 4.1 below shows the sigmoidal growth curves of ICSB 338 and White sorghum cell cultures. Both growth curves showed three distinct growth phases; the lag phase, the log phase and the stationary phase.

In the lag phase, there is no increase in SCV. This phase occurred from the day of sub-culturing until day 6 and day 4 for ICSB 338 and White sorghum, respectively. In the log phase, the SCV increased exponentially between day 6 - 10 and day 4 - 10 for ICSB 338 and White sorghum, respectively. Lastly, the cells went into the stationary phase, where cells continued to grow but at a slower rate. This occurred between day 10 - 14 for both ICSB 338 and White sorghum cell cultures. The SCV measurements were discontinued on day 14 because reproducibility and consistency became difficult when sampling 1 mL aliquots with a 1 mL pipette.

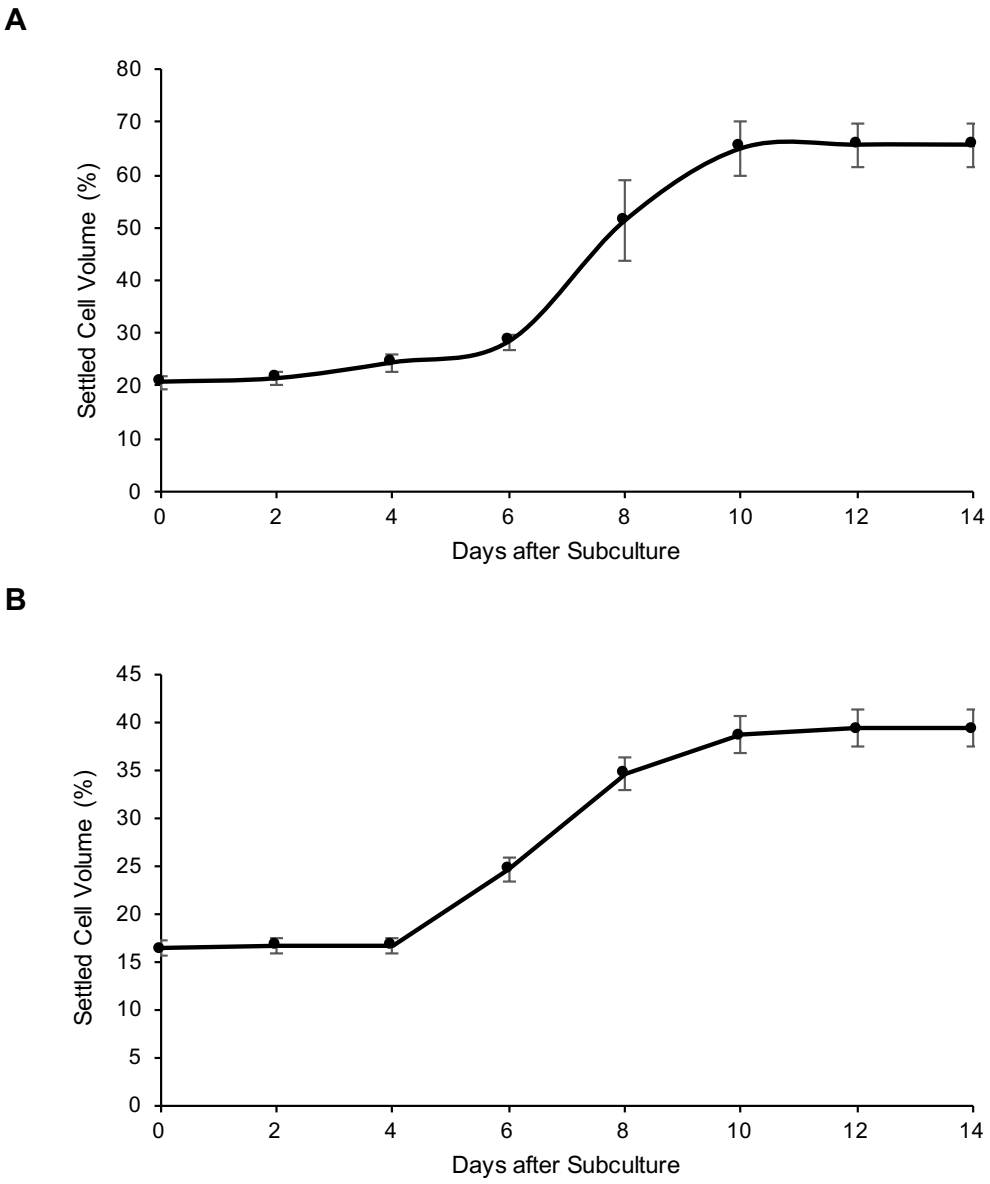

Figure 4.1. The growth curves of sorghum cell suspension cultures using the settled cell volume (SCV) method. (A) shows the ICSB 338 and (B) shows the White sorghum growth curve. The growth curve readings were done for three independent biological replicates for each cell culture. Data presented as mean ± se (*n*=3).

4.2.2. Measuring Cell Viability

The viability of cell suspension cultures of the two sorghum genotypes was estimated using the MTT assay as described in section 2.2.6.2. Figure 4.2 shows the cell viability of ICSB 338 and White sorghum cell suspension cultures from the day of sub-culturing (day 0) to day 14. At each time point, three independently established cell cultures were used as biological replicates. From each biological replicate per time point, sampling was carried out twice to give two technical replicates. Student's *t*-test statistical analysis was done at 5% level of significance. Generally, results indicate that cell viability was significantly low at days corresponding to the lag phases (day 0 - 4) of both cell cultures. Thereafter, a significant increase was observed between days 6 - 10, which corresponds to the exponential phases. A significant decrease in cell viability was observed during the stationary phase (day 10 - 14).

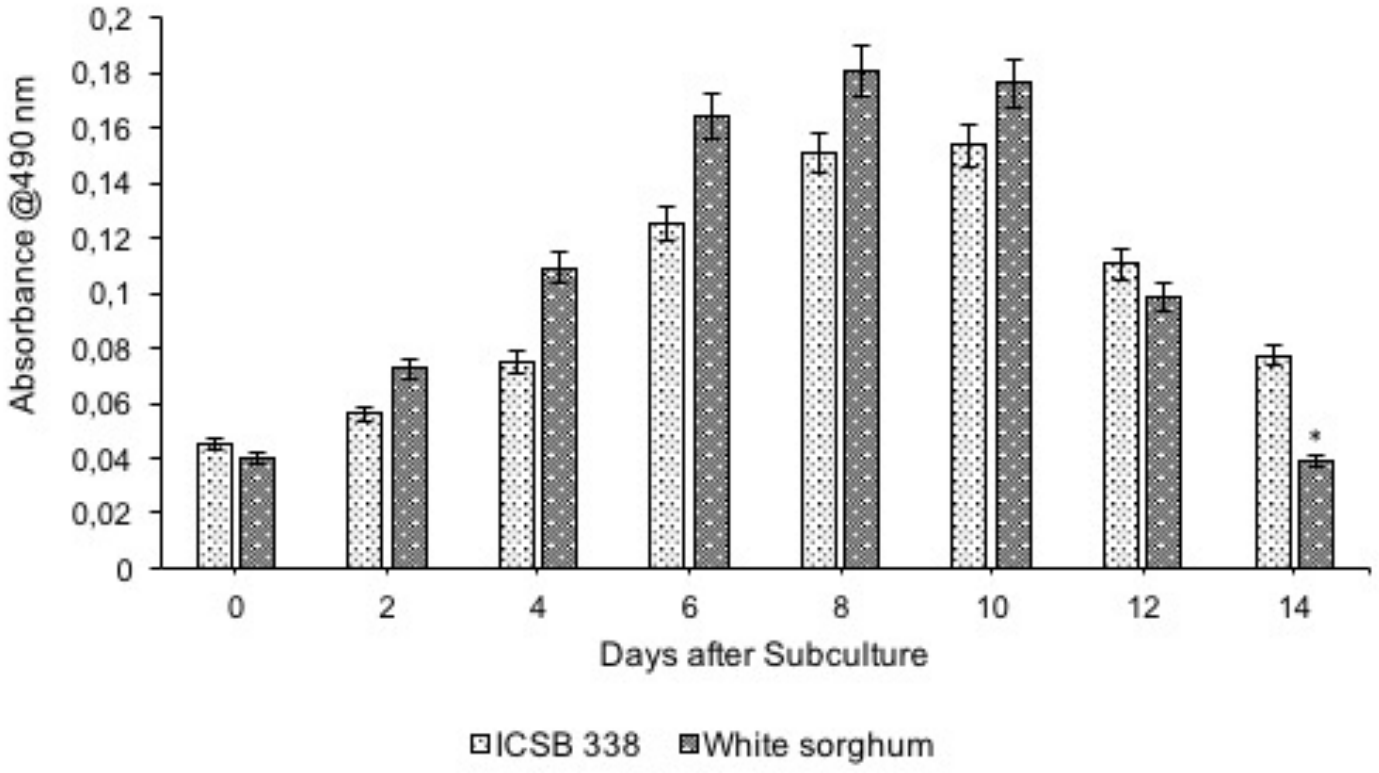

Figure 4.2. Cell viability of sorghum cell cultures using the MTT assay. (A) shows ICSB 338 and (B) shows White sorghum results. The cell viability readings at each time point is an average of two technical replicates from three independently established cell suspension cultures. Data presented as mean ± SD ($n = 3$). * indicates statistical significance at a 5% level of significance using Student's *t*-test ($p < 0.05$).

4.2.3. One-Dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Sorghum Cell Culture Proteomes

In order to study proteins secreted into the culture medium during the exponential growth phase (Figure 4.1), culture filtrate (CF) proteins were extracted every second day beginning from day 6 after sub-culturing. The protein extracts were resolved by 1D SDS-PAGE with CBB staining. The results indicated that from day 6 - 12, proteins were secreted into the culture medium at the same rate with the same expression pattern (results not shown). Based on these results, day 8 was selected for subsequent osmotic stress treatment experiments and protein extraction procedures.

Figure 4.3 shows the 1D SDS-PAGE analysis of sorghum CF and total soluble proteins (TSP) extracted on day 8 from three independently established sorghum cell suspension cultures of the two sorghum cell lines. From the gel, different CF and TSP protein expression profiles were observed. Culture filtrate proteins ranged between 10 - 100 kDa, whereas TSP ranged between from 10 - 200 kDa in extracts from both cell cultures. This confirms that the CF is enriched for secreted proteins and has a unique profile when compared to intracellular proteins. Due to difficulties in quantifying the CF and TSP proteins using the Bradford assay, volumes between 10 - 20 μL of the protein extracts were loaded in the gels. However, these particular protein extracts were not further used for any proteomic expression analysis work.

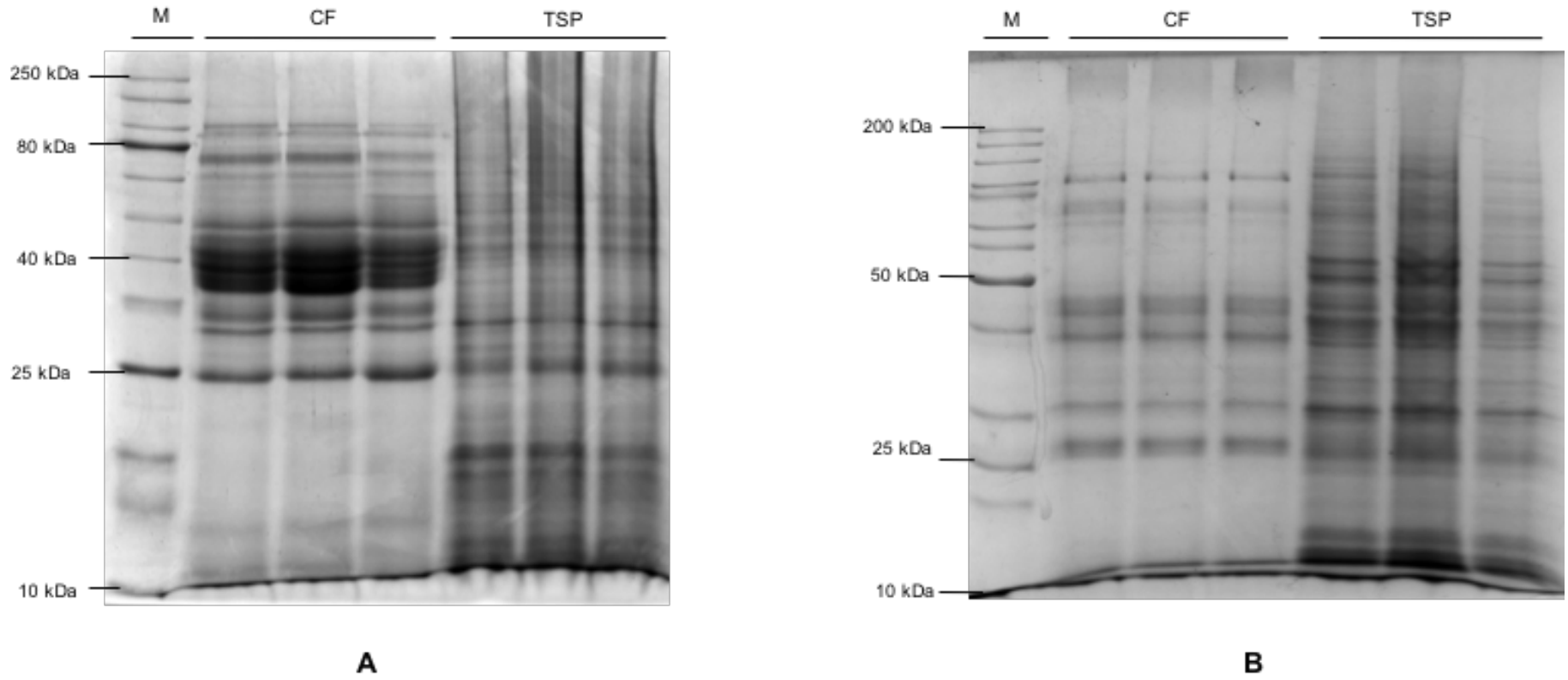

Figure 4.3. 1D SDS-PAGE analysis of sorghum cell suspension culture filtrate (CF) and total soluble protein (TSP) stained with CBB. (A) shows ICSB 338 and (B) shows White sorghum gel results. CF and TSP proteins were both extracted from cell cultures on day 8 post-sub-culture. Lane M represents the protein molecular weight marker measured in kDa.

4.3. Osmotic Stress Treatments of the Sorghum Cell Suspension Cultures

Osmotic stress treatments were carried out to assess the effect of stress on cell structure, viability, as well as protein expression profiles between control and treated samples. Treatments were carried out as described in section 2.4.1 using cell cultures at day 8 post-sub-culture. Osmotic stress in both the ICSB 338 and White sorghum cell cultures was induced by a final concentration of 400 mM sorbitol, while all control cultures were spiked with an equivalent volume of sterile distilled water. For each sorghum cell culture line, three biological replicates were used in the experiment.

Figure 4.4 below illustrates the appearance of the ICSB 338 (A) and White sorghum (B) controls and treated cell cultures, 72 hr post-treatment. Genotype ICSB 338 cell cultures were cream-yellow in colour (Figure 4.4 A) while the White sorghum cell cultures were cream in colour (Figure 4.4 B). Both control cell cultures had higher SCV compared to the sorbitol-treated cell cultures. The effect of osmotic stress on cell cultures was also assessed using cell viability assays (section 4.3.1 and 4.3.2), microscopic analysis of cell structure (section 4.3.3), and protein expression profiles (section 4.3.4).

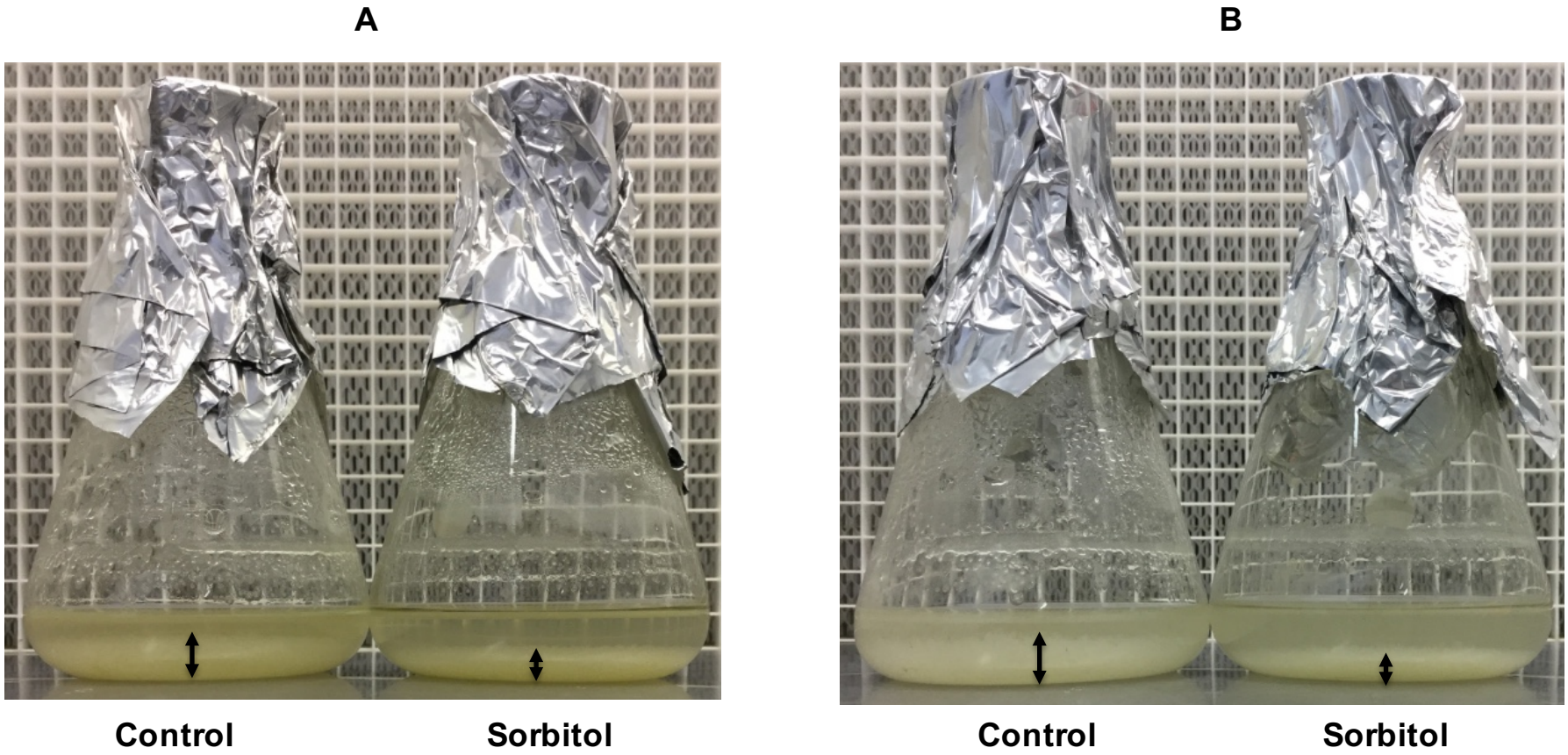

Figure 4.4. Sorghum cell cultures following osmotic stress treatment. (A) shows ICSB 338 cell cultures, while (B) shows White sorghum cell cultures. The settled cell volume in both control and treated cell cultures are indicated by black double arrows. All the cell cultures were treated for 72 hr starting from day 8 after sub-culturing.

4.3.1. The MTT Assay for Assessing Cell Viability

Cell viability results of controls and sorbitol-treated cells for the ICSB 338 and White sorghum cell cultures are shown below in Figures 4.5 A and B, respectively. Generally, the viability of the control cultures of both genotypes gradually decreased over the duration of the experiment (0 - 72 hr), although the decrease was not statistically significant. However, the sorbitol-treated cell fractions showed a statistically significant transient dip in cell viability 24 hr post treatment for both cultures (Figures 4.5 A and B). Thereafter, cells seemed to recover from the stress from 48 hr. A remarkable increase in viability was observed in White sorghum cells 72 hr post treatment. Statistical analysis was conducted using the Student's *t*-test at 5% level of significance.

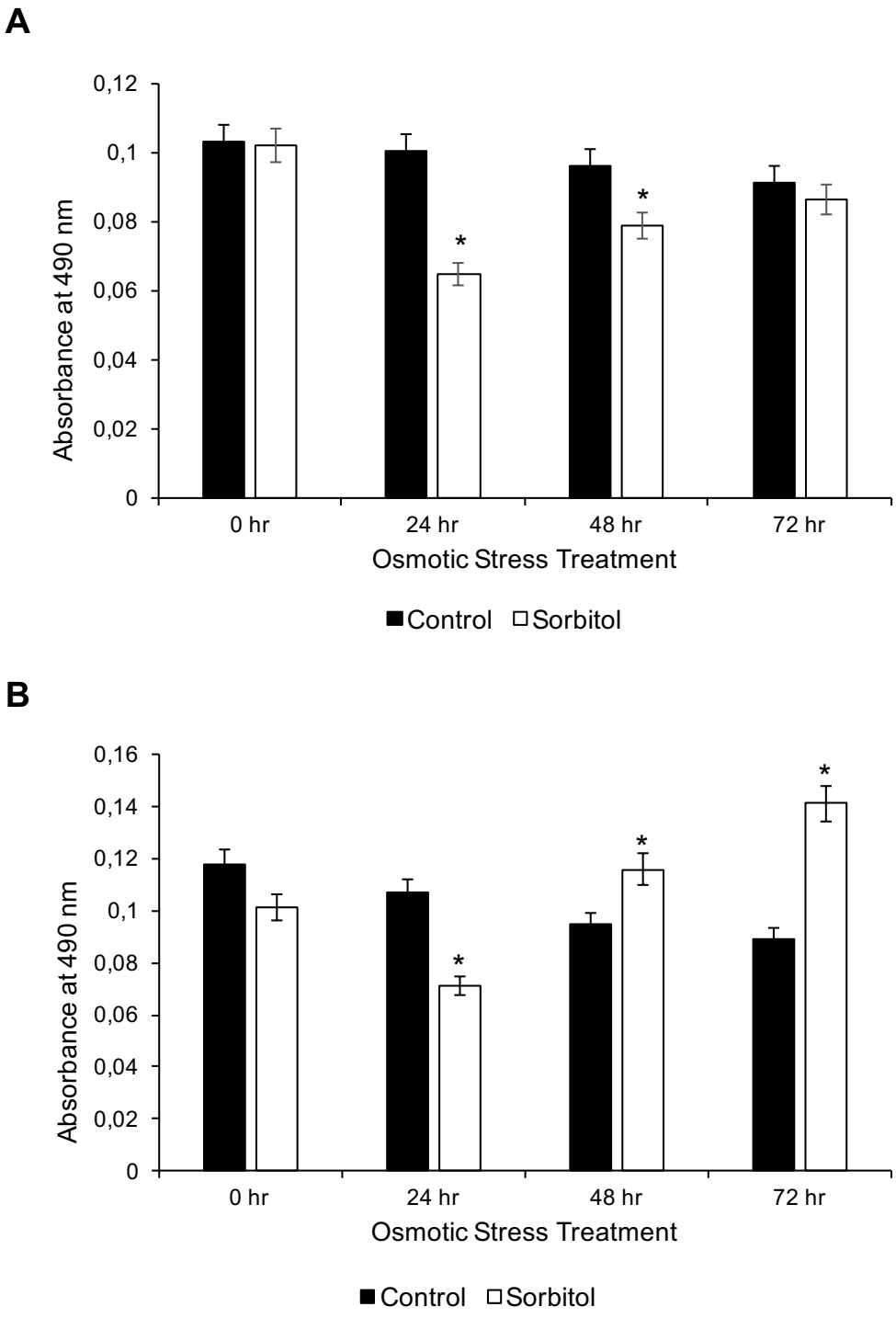

Figure 4.5. Cell viability of sorghum cell cultures following osmotic stress using the MTT assay. (A) shows ICSB 338 and (B) shows White sorghum results. The cell viability readings at each time point is an average of two technical replicates from three independently established cell suspension cultures. Data presented as mean ± SD (*n* = *3*). * indicates statistical significance at a 5% level of significance using Student's *t*-test ($p < 0.05$).

4.3.2. The Evans Blue Assay for Assessing Cell Viability

The Evans blue assay measures cell death, the inverse of which is cell viability. The Evans blue dye is absorbed by cells, which have a damaged plasma membrane (PM), thus making them permeable. This assay was used to estimate the cell viability of control and sorbitol-treated cells. Eight-day-old ICSB 338 and White sorghum cell cultures were used for osmotic stress treatments. However, cell viability measurements were only carried out starting from 24 hr post-treatment, with the assumption that the cell cultures used had 100% cell viability before treatment and immediately after treatment (0 hr). Statistical analysis was conducted using the Student's *t*-test at 5% level of significance.

Figures 4.6 A and B below show the cell viability results of the two cell cultures following 72 hr of osmotic treatment. There was an insignificant decrease in cell viability of the ICSB 338 controls between 24 (97%) and 48 hr (96%), followed by a statistically significant decrease at 72 hr (91%) post-treatment (Figure 4.6 A). The White sorghum control cells showed a statistically significant decrease in viability for the duration of the treatment (72 hr; Figure 4.6 B). For the sorbitol-treated cells, a significant decrease in cell viability was observed for the 72 hr of osmotic stress for both cell cultures. However, the ICSB 338 cells seemed to have been affected more by the 400 mM sorbitol (Figure 4.6 A), as compared to the White sorghum cells (Figure 4.6 B). This is explained by a steep decrease from 97% at 24 hr to 54% at 72 hr, whereas, for White sorghum, the decrease was from 95% at 24 hr to 72% at 72 hr post sorbitol treatment (Figures 4.6 A and B).

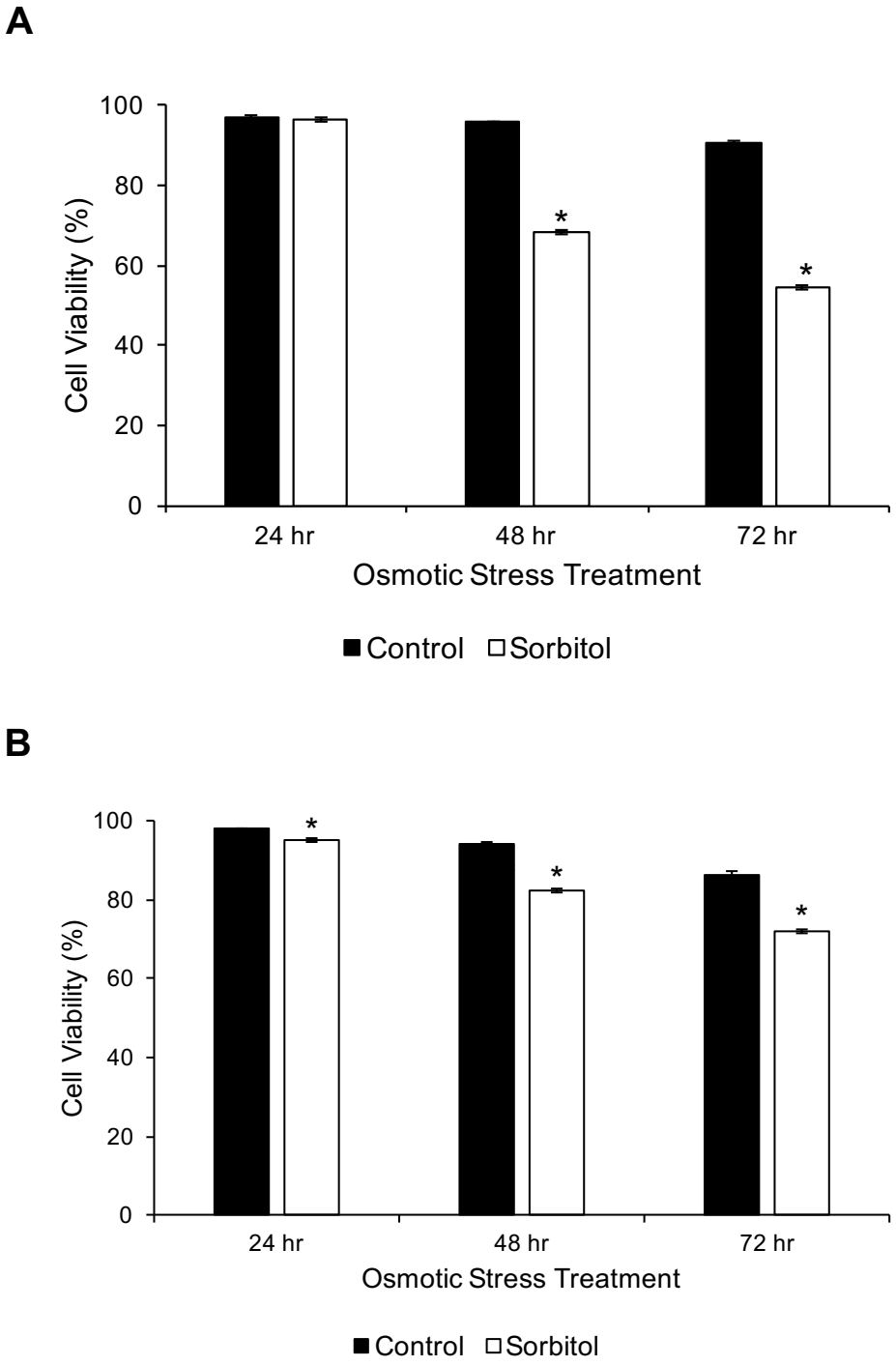

Figure 4.6. Cell viability of sorghum cell cultures following osmotic stress using Evans blue test. (A) shows ICSB 338 and (B) shows White sorghum results. The cell viability readings at each time point is an average of three independently established cell suspension cultures. Data presented as mean ± SD (*n* = *3*). * indicates statistical significance at a 5% level of significance using Student's *t*-test ($p < 0.05$).

4.3.3. Microscopic Analysis of Cell Structures

Day 8 cell suspension cultures of both the ICSB 338 and White sorghum were subjected to osmotic stress for 48 and 72 hr as described in section 2.4.1. Changes in cell structure of the ICSB 338 and White sorghum cells were assessed using a Nikon Eclipse E200 light microscope at a magnification of 40X. Figures 4.7 A and B illustrate the microscopic images of controls and sorbitol-treated cell cultures of the ICSB 338 and White sorghum, respectively. It is clear from Figure 4.6 that sorbitol treatment resulted in cell structural differences between the controls and treated cells. Both the ICSB 338 and White sorghum control cells were generally bigger in size as compared to the sorbitol-treated cells (Figures 4.7 A and B).

Following 48 and 72 hr of incubation, control cells of both the ICSB 338 and White sorghum consistently had evenly distributed cytoplasmic contents (CC). On the other hand, the sorbitol-treated cells seemed to have lost water to the external environment as a result of the osmotic stress. This observation is explained by plasmolysis of cells resulting in the cytoplasm detaching from the PM. Different levels of cell plasmolysis were observed between the ICSB 338 and White sorghum sorbitol-treated cells following 48 and 72 hr of treatment, with some cells that still had their cytoplasmic contents evenly distributed.

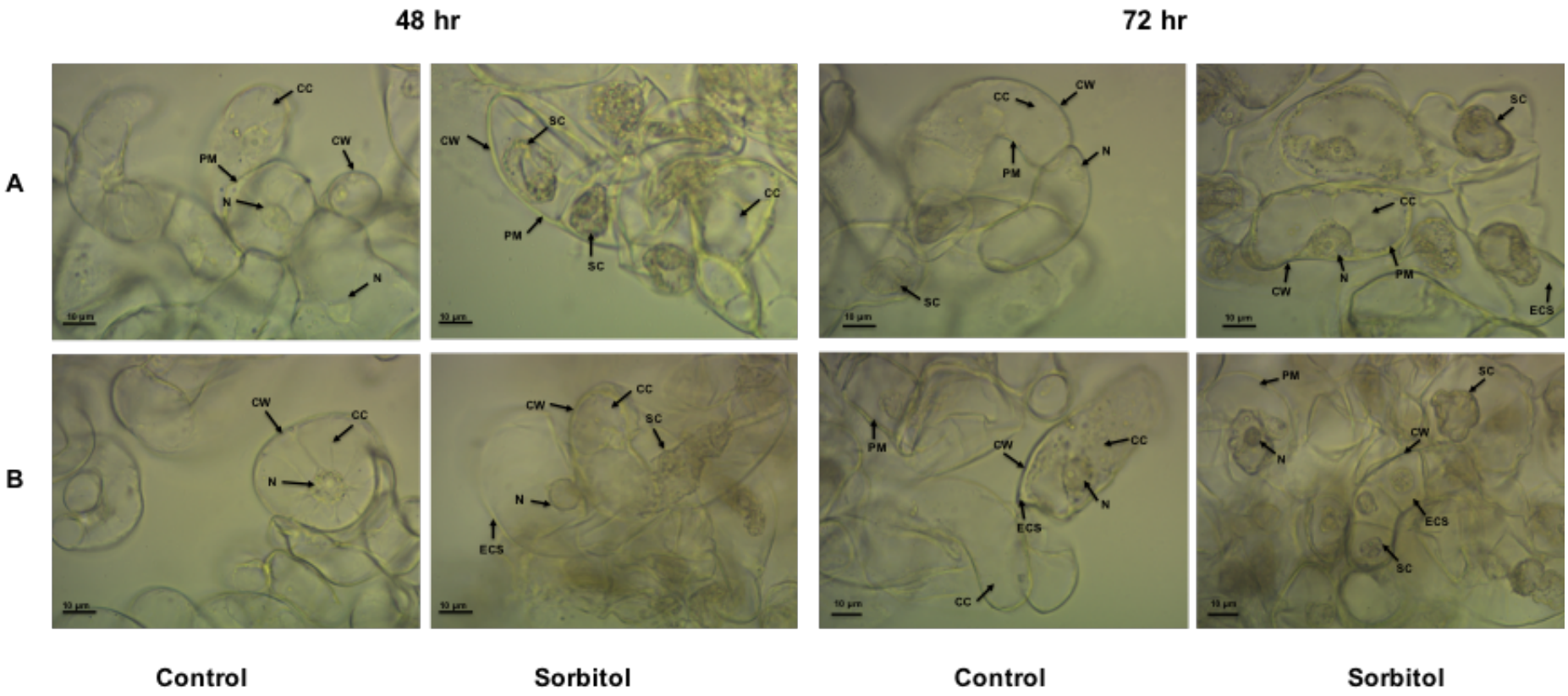

Figure 4.7. Light microscopic analysis of sorghum cell cultures following osmotic stress treatment. (A) shows ICSB 338 and (B) shows White sorghum cells following 48 and 72 hr osmotic stress. CW indicates cell wall; ECS, extracellular space; PM, plasma membrane; CC, cytoplasmic contents; N, nucleus; and SC, compressed cytoplasm. The scale bar is 10 μm.

4.3.4. Analysis of the Osmotic-Stressed Culture Filtrate on 1D SDS-PAGE

Culture filtrate (CF) proteins extracted from control and sorbitol-treated cell cultures at 48 and 72 hr, using the large-scale extraction method (described in section 2.3.1.2) were analysed on 1D SDS-PAGE. Protein expression profiling helps in deciding the time point in which proteins will be mapped. Figure 4.8 below illustrates a CBB stained 1D SDS-PAGE gel image taken using a Gel Doc™ XR+ molecular imager® (BioRad). There was a clear distinction in the ICSB 338 and White sorghum CF protein profiles at 48 and 72 hr. At the molecular range of 80 kDa, single bands of proteins were observed for ICSB 338, whereas, double bands were observed for the White sorghum around the same molecular range (indicated by a dark blue arrow).

Another distinction was observed between the controls and treated samples of the ICSB 338, where a single band of about 60 kDa was observed in the control sample and double bands for the treated samples at 48 hr. However, at 72 hr, the control samples expressed the double bands proteins while the treated sample expressed single band proteins (Figure 4.8). The ICSB 338 expressed small quantities (very faint protein bands) of proteins at about 25 kDa, whereas at the same molecular range, the White sorghum expressed more of these CF proteins of about 25 kDa at both time points (48 and 72 hr; red arrow; Figure 4.8). Although some protein expression patterns differed between the two cultures, some proteins were expressed in relatively similar intensities (indicated by light blue arrow).

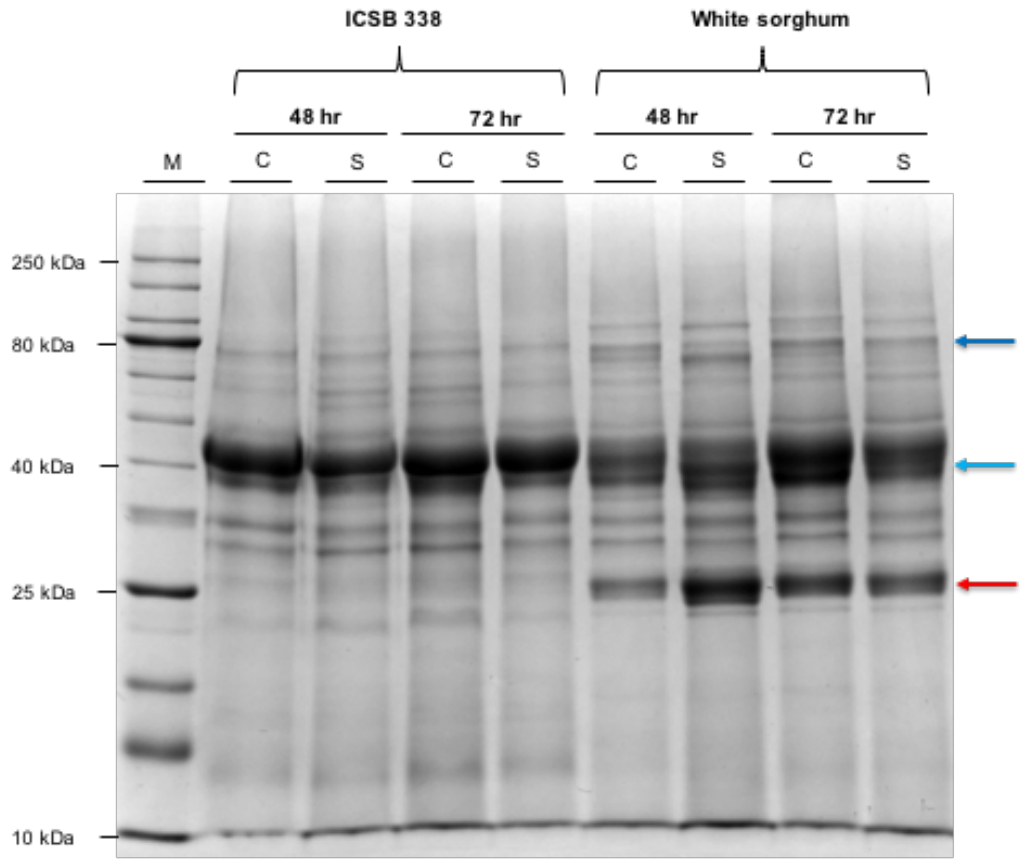

Figure 4.8. 1D SDS-PAGE analysis of sorghum culture filtrate (CF) proteins stained with CBB. The CF protein extracts from 48 and 72 hr osmotic stress treatments were analysed on this figure. Protein concentration of 10 μg from each sample was loaded in each respective well. Lane M represents the protein molecular weight marker measured in kDa. Lanes C represents controls CF, extracted from cell cultures treated with distilled water. Lanes S represents sorbitol treated CF, extracted from cell cultures treated with 400 mM sorbitol.

4.4. Discussion

Cell suspension cultures of the ICSB 338 and White sorghum were successfully established from the friable callus masses (Figure 3.14). The growth of the ICSB 338 and White sorghum cells was estimated using a SCV method over a 14-day period. Although both cell cultures had typical sigmoidal shaped growth curves (Figure 4.1), the duration of each of the lag and exponential phases differed between the cell cultures.

The duration of growth phases in plant cell suspension cultures are reported to differ depending on the inoculum cell density, growth conditions and plant species (Gupta *et al*., 2011; Ngara and Ndimba, 2011; Rahman *et al*., 2012). For example, the cell growth curve of White sorghum previously established by Ngara and Ndimba (2011), showed different lengths of growth phases from the current study. Although both studies used approximately the same inoculum density of between 15 - 16% SCV, the lag phase lasted for 8 days (Ngara and Ndimba, 2011) and four days in the current study (Figure 4.1). These distinct results could be attributed to differences in culture conditions and environments, and possibly technical differences in cell sampling.

Growth curve readings were terminated at day 14 for both cell cultures because of two reasons. Firstly, the colour of the cell cultures started changing from light yellow to brown, possibly indicating a reduced or change in cell metabolic activity. This observation can be correlated to low viability readings obtained when cells started browning (Figure 4.2). Secondly, the reproducibility and consistency in sampling became difficult even though cells were agitated before sampling. This is in agreement with observations reported by Ngara *et al*. (2008).

The viability of the ICSB 338 and White sorghum cell suspension cultures was estimated using the MTT assay over a 14-day period. It was observed that the results of the cell viability assays of both the ICSB 338 and White sorghum (Figure 4.2) were in agreement with the results from the growth curve (Figure 4.1). On the day of sub-culturing, the cells were fewer in number due to the culture dilution with fresh medium and cells adapting to a new environment (lag phase), thus low cell viability values. The cell viability estimate gradually increased with an increase in cell volume (log phase) and possibly metabolic activity. Thereafter, the cell viability decreased due to cells possibly stressing as a result of nutrient exhaustion (stationary phase; George *et al*., 2008).

Based on growth curve analysis and vital staining, the day onto which these two sorghum cell cultures must be sub-cultured for maintenance and keeping the cells alive can be determined. The cells should be sub-cultured every 10 - 12 days after the previous sub-culture for both the ICSB 338 and White sorghum. The results of the White sorghum growth curve previously reported by Ngara and Ndimba (2011), are in agreement with the results of the current study. The duration of maintaining cells in culture differs depending on the initial volume of either settled or packed cells, and also from species to species. *Abrus precatorius*, an important medicinal plant, requires that the cell suspension be sub-cultured every two weeks (Rahman *et al*., 2012), while *Jatropha curcas* and *J. gossypifolia* cell suspension require sub-cultures every 20 days (Ramos *et al*., 2013).

To assess if the total soluble proteins (TSP) were expressed in sorghum cells and/or secreted (CF) into the medium, a 1D SDS-PAGE analysis were done. Two proteomes; the TSP and CF from the cell suspension cultures of the ICSB 338 and White sorghum were extracted from cells and culture medium, respectively. Both the proteomes were quantified using the Bradford assay (Bradford, 1976) and analysed using the 1D SDS-PAGE. The Bradford assay has become the preferred method for protein quantification for many research groups (Bhushan *et al*., 2006; Oh and Komatsu, 2015). This assay relies on the binding of Coomassie Brilliant blue G250 dye to protein. The assay is simple, fast and sensitive (Kruger, 2009). However, the original Bradford assay has one major drawback; the binding specificity of the blue dye may lead to variation in response to different proteins (Kruger, 2009). To overcome this problem, several modifications were made to the original assay (Stoscheck, 1990). However, these modifications have resulted in a less robust assay that is more susceptible to chemical interferences (Kruger, 2009).

In the current study, challenges were faced during both the CF and TSP quantification. Low absorbance values were recorded at 595 nm. This in turn resulted in either negative or very low protein concentration values being obtained, and these extracts could not be used further than the 1D SDS-PAGE (Figure 4.3). These results could be due to two problems. Firstly, re-solubilizing the proteins in high volume of urea extraction buffer thereby resulting in too diluted proteins extracts. Secondly, it could be due to absorbance interference by chemical reagents used when re-solubilizing proteins or during protein quantification.

The ICSB 338 and White sorghum cell cultures were exposed to osmotic stress by treating with 400 mM of sorbitol, while control samples were treated with distilled water. Relative to the control samples, sorbitol-treated samples were assessed for any changes that could occur as a result of the introduced osmotic stress. Visual observations, cell viability and microscopic analysis were carried out for both the ICSB 338 and White sorghum sorbitol-treated cell cultures. After 72 hr post sorbitol treatment, a change in SCV between both the ICSB 338 and White sorghum control and treated cell cultures was observed (Figure 4.4). The SCV of the treated samples was lower than that of the control samples. This observation can be explained, possibly by the fact that when cells experience osmotic stress, they lose water to the outside environment with higher solute concentration via osmosis (Bray, 1997; Wang *et al*., 1999). As a result, cells tend to shrink and thus resulting in lower SCV. Conversely, control cells on the other hand do not lose water and remain turgid possibly because the solute concentration of the medium and that of the cell's cytoplasm are in equilibrium. This observation was also supported by the light microscopic analysis of the controls and sorbitol-treated samples, assessing structural changes of cells following osmotic stress. It was observed that the sorbitol-treated cells had compressed cytoplasm as compared to control cells, which generally had evenly distributed cytoplasmic contents (Figure 4.7). Similar cell shrinkage was observed in tobacco cells (Monetti *et al*., 2014) and sorghum (Ngara, 2009) after

treatment with 400 mM sorbitol and sweet potato after a 600 mM sorbitol treatment (Wang *et al*., 1999).

To investigate whether or not the introduction of sorbitol could cause cell death or change in metabolic activity, cell viability estimations were conducted using two assays, namely, the MTT and Evans blue. The two assays measure cell viability based on different principles. The MTT assay assesses cell viability by measuring cell active metabolic activity (Angius and Floris, 2015), whereas Evans blue measures the permeability of the plasma membrane of cells (Baker and Mock, 1994). Cell viability results revealed that the sorbitol did not cause cell death. Rather, it caused a transient decrease in cell viability 24 hr after treatment (Figure 4.5). However, at 48 hr following osmotic stress, cells appeared to have recovered from and adapted to the osmotic stress as explained by a significant increase in metabolic activity (for MTT assay; Figure 4.5). However, further studies need to be conducted to try and elucidate these observed increase in metabolic activity for the White sorghum in response to osmotic stress at 72 hr despite the decrease in viability as per Evans blue (Figure 4.6) results at the same time point. Techniques such as metabolic profiling could be done to investigate the underlying reasons for these changes.

Sorbitol treatment resulted in a decrease in cell viability, as shown by the Evans blue results (Figure 4.6). However, based on the observation made from the MTT assay, which measures metabolic activity, we propose that the cell viability measured by the Evans blue assay can be explained as observing a transient change in membrane permeability, which is not lethal. Elicitors such as chitosan have been found to cause similar transient changes in membrane permeability (or molecules leakage) when added to cell cultures (Ndimba *et al*., 2003). Such phenomenon happens when certain molecules are taken up by active transport or internalised via endocytosis. Sorbitol seemed to have the same effect in the current study as chitosan. Therefore, a possible

explanation of the increase in Evans blue uptake is perhaps a sorbitol-induced activation of endocytosis rather than plasma membrane damage leading to cell death. Thus, this study raises a caution to the use of this assay in cell death analysis.

In this study, sorbitol did not cause cell death within a time-frame of 72 hr (Figure 4.5). This is in agreement with a previous study by Ngara (2009) in which sorbitol did not reduce cell viability of sorghum cell cultures. However, in other species, sorbitol treatment activated cell death. For example, in tobacco BY-2 cell populations, a transient decrease in cell viability, which resulted in the activation of cell death, was observed from 2 hr until 24 hr post 400 mM sorbitol treatment (Monetti *et al*., 2014). In addition, Monetti and co-workers (2014) also observed an increase in ROS production following a 400 mM sorbitol treatment, suggesting its contribution to cell death.

The differences in protein expression between the two cell lines following the osmotic stress treatments were assessed using CBB stained 1D gels (Figure 4.8). There was a clear distinction between protein expression profiles of the ICSB 338 and White sorghum after 48 and 72 hr of treatment. Amongst other differences, the ICSB 338 showed a low expression (faint protein bands) of the 25 kDa proteins, whereas a much higher expression of these same molecular weight proteins was observed in White sorghum. The differences in protein expression between the two cell cultures may possibly explain their differences in cell growth patterns (Figure 4.1), cell viability of controlled cells (Figure 4.2) and osmotic stress treated cells (Figures 4.5 - 4.8) observed in the current study. However, no conclusions can be made on the above hypothesis without proper quantification and identification of the secreted proteins using advanced and highly sensitive gel-based and non-gel based proteomic tools.

CHAPTER 5

PROTEOMIC MAPPING OF THE WHITE SORGHUM CELL SUSPENSION CULTURE SECRETOME

5.1. Introduction

Under normal, non-stressful environmental conditions, plant cells continuously secrete proteins into the extracellular matrix (ECM). These proteins, called the secretome, play important functions in cell wall structure, cellular communication and plant defence (Lum and Min, 2011; Alexandersson *et al*., 2013). The secretome can either be studied from culture filtrate (CF) of cell suspension cultures (*in vitro*) or apoplastic fluids of whole plant organs (*in planta*; Krause *et al*., 2013) using different proteomics technologies (discussed in section 1.5.4). The composition of the cell culture secretome has been documented for crop species such as rice (Cho *et al*., 2009), chickpea (Gupta *et al*., 2011), and sorghum (Ngara and Ndimba, 2011).

In a previous sorghum study by Ngara and Ndimba (2011), secreted proteins were extracted from the CF of a White sorghum cell suspension culture system, separated by two dimensional (2D) gel electrophoresis and identified using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF-TOF) mass spectrometry (MS). A total of 14 proteins were positively identified as peroxidases, oxalate oxidases, α-galactosidases, and germin-like proteins (Ngara and Ndimba, 2011). These proteins have known functions in cell wall modifications, plant defence and signalling processes. Cho and co-workers (2009) identified 154 non-redundant secreted proteins from a rice cell suspension culture using multidimensional protein identification technology (MudPIT).

The identified proteins had functions in stress response (example, germin-like protein), metabolism (example, α-amylase), and protein modification (example, cysteine protease 1). In chickpea, 773 secreted proteins were identified from the cell suspension cultures using LC-MS/MS (Gupta *et al*., 2011). These proteins are involved in metabolism (example, α-glucosidase), signal transduction (example, lipase-lipoxygenase), transport (example, lipid transfer protein), and cell defence (example, pathogen-related protein).

Despite the improved advances in proteomic technologies, the importance of secreted proteins in normal plant cell development, and the fact that sorghum's genome has been sequenced (Paterson *et al*., 2009), sorghum secretome data is still limited. To date, only the 14 proteins identified by Ngara and Ndimba (2011) using gel-based proteomics are the only experimentally identified sorghum secreted proteins. Therefore, the current study explored the composition of the sorghum secretome using MudPIT, a non-gel based proteomics method.

The objectives of this chapter were to map the secretome of the White sorghum cell culture using MudPIT and to analyse the putative functions of the identified proteins in normal cell growth and developmental processes using bioinformatics tools.

5.2. MudPIT Analysis of the White Sorghum Secreted Proteins

White sorghum cell suspension cultures were established and maintained in culture as described in section 2.2.5. For the secretome mapping experiment, proteins were analysed by MudPIT, which was part of an iTRAQ experiment, with only the iTRAQ control samples being used for mapping. Secreted proteins used for iTRAQ analysis were precipitated from the culture filtrate using acetone, re-solubilised in urea buffer (section 2.3.1.2) and quantified using a modified Bradford assay (section 2.5).

For each of the four independent biological replicates used, 50 μg of the CF proteins were labelled with isobaric tags of molecular weights 113, 114, 115, and 116 and analysed by LC-MS/MS as described in section 2.7.1. The ProteinPilot software version 2.0.1 was used to analyse the resulting MS/MS data files against the TrEMBL database sequences of *Sorghum bicolor* only. All proteins that were identified on the basis of a single sequenced peptide were filtered out of the dataset to increase the confidence in protein identification. Table 5.1 below summarises the MudPIT results of a total of 178 positively identified proteins, constituting the sorghum-secreted proteins in this study. Of these proteins, 139 (78%) were identified as uncharacterised proteins (Table 5.1), meaning that their functional roles have not been experimentally verified.

Table 5.1. List of secreted proteins identified from the White sorghum culture filtrate (CF) using the iTRAQ and database searches.

Prot No.[a]	Accession[b]	Protein name	Scor[c]	% Cov[d]	Seq Pep[e]	Theor MW (kDa)[f]	SP[g]	GO analysis[h]			Conserved domains and family name[i]
								P	F	C	
1	C5Y1P4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G099000	79,54	72,76	133	34,38	Y	Chitin catabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase, catalytic domain; family not predicted
2	C5WSF9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301500	65,76	66,31	89	30,89	Y	Sexual reproduction	None predicted	Extracellular region	Expansin/allergen domain; Expansin/Lol pI family
3	C5WYQ4	Peroxidase OS=Sorghum bicolor GN=SORBI_001G360400	59,03	68,37	82	34,75	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
4	C5X5K6	Peroxidase OS=Sorghum bicolor GN=SORBI_002G416700	45,12	68,37	78	32,41	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
5	C5XYP5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G233700	42,88	34,82	32	84,26	Y	Carbohydrate metabolic process	Hydrolase activity, hydrolysing O-glycosyl compounds	Plant-type cell wall	Glycoside hydrolase, family 3 domain and family
6	A0A1B6QHZ6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089000	41,02	39,12	32	76,19	N	None predicted	None predicted	None predicted	Glycoside hydrolase domain; (Trans)glycosidases family
7	A0A1B6QG95	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416600	40,8	72,16	121	38,89	N	None predicted	None predicted	None predicted	Secretory-peroxidase domain; plant-peroxidase-like family
8	C5X532	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G123100	40,07	45,28	34	46,12	Y	Carbohydrate metabolic activity	Catalytic activity	Plant-type cell wall	Glycoside hydrolase superfamily domain, glycoside hydrolase family 27
9	C5XQV7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G085900	37,52	24,35	27	102,51	Y	Carbohydrate metabolic process	Catalytic activity	None predicted	Glycoside hydrolase superfamily domain; glycoside hydrolase 31
10	C5XI24	Peroxidase OS=Sorghum bicolor GN=SORBI_003G140700	37,11	53,01	38	38,09	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
11	A0A194YQ33	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G166700	36,45	40,79	26	64,68	Y	None predicted	None predicted	None predicted	Glycosyl hydrolases family 32 domain and family
12	C5WXC7	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_001G208100	35,39	55,87	37	46,96	Y	Carbohydrate metabolic process	Catalytic activity	Plant-type cell wall	Glycoside hydrolase superfamily domain; glycoside hydrolase family 27
13	C5Y360	Peroxidase OS=Sorghum bicolor GN=SORBI_005G011300	34,60	57,01	73	34,43	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family

14	A0A1B6PLA9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G104300	32,62	42,64	21	62,06	Y	None predicted	None predicted	None predicted	Domain not predicted; Gamma-glutamyl-transpeptidase family
15	C5XRC3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G374100	31,52	24,08	46	93,85	Y	Response to karrikin	Beta-galactosidase activity	Cell wall	Galactose-binding domain-like; glycoside hydrolase, family 35
16	C5Y8G7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G224500	31,05	45,68	38	37,53	Y	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic peptidase domain; aspartic peptidase A1 family
17	C5WSF0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300800	30,06	51,88	33	28,81	Y	Sexual reproduction	None predicted	Extracellular region	Expansin/pollen allergen domain; expansin/Lol pl family
18	C5X8J1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G301700	27,48	42,16	17	54,34	Y	None predicted	Amidase activity	None predicted	Amidase signature domain; amidase family
19	A0A194YMM6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G262500	26,92	44,81	15	36,53	N	None predicted	None predicted	None predicted	NAD(P)-binding domain; glyceraldehyde-3-phosphate dehydrogenase family
20	A0A1B6QMT3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G348900	26,82	33,33	27	50,13	Y	None predicted	None predicted	None predicted	None predicted
21	C5Z6U2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G210000	25,82	60,13	15	17,09	N	None predicted	Protein binding	None predicted	Ubiquitin domain and family
22	A0A1B6QI05	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089100	22,98	34,46	38	67,20	Y	None predicted	None predicted	None predicted	Glycoside hydrolase domain, (Trans)glycosidases family
23	C5Z240	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G003100	22,07	28,81	17	65,57	Y	Oxidation-reduction	Oxidoreductase activity	Plant-type cell wall	Cupredoxin domain; family not predicted
24	C5Z8N0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G118900	21,93	37,42	42	47,82	Y	None predicted	None predicted	None predicted	Fasciclin 1 domain; fasciclin-like arabinogalactan protein family
25	C5X3C1	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391300	21,66	40,65	32	39,09	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
26	C5XQ74	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G208800	21,06	41,51	20	53,53	N	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic peptidase domain; aspartic peptidase A1 family
27	C5XKE9	Endoglucanase OS=Sorghum bicolor GN=SORBI_003G015700	20,26	20,03	16	69,78	N	Polysaccharide catalytic	Cellulase activity	None predicted	Six-hairpin glycosidase domain; glycoside hydrolase family 9
28	C5Y397	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_005G132400	19,76	14,62	15	114,28	Y	Mannose metabolic	Alpha-mannosidase activity	None predicted	Glycosyl hydrolase family 38 domain and family
29	C5X8J4	Xyloglucan endotransglucosylase/hydrolase OS=Sorghum bicolor	19,12	44,90	16	35,07	Y	Xyloglucan metabolic	Xyloglucan: xyloglucosyl transferase activity	Cell wall	Xyloglucan endo-transglycosylase domain; xyloglucan endotransglucosylase/hydrol

		GN=SORBI_002G30200									ase family
30	C5WNY4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G129700	18,37	31,99	17	23,75	Y	Oxalate metabolic	Oxalate decarboxylase activity	Cell wall	Cupin 1 domain; germin family
31	C5YC92	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G018100	17,86	55,46	18	24,97	Y	Oxalate metabolic	Oxalate decarboxylase activity	Cell wall	Cupin 1 domain; germin family
32	C5Z864	Peroxidase OS=Sorghum bicolor GN=SORBI_010G232500	17,42	32,33	12	35,81	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
33	C5Z0P5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G055900	17,39	30,14	19	44,50	N	None predicted	None predicted	None predicted	Fasciclin 1 domain; fasciclin-like arabinogalactan protein family
34	C5Y1P1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G098700	17,19	49,35	64	33,72	Y	Chitin catabolic	Chitinase activity	Extracellular region	Glycoside hydrolase family 18 domain and family
35	C5XIY1	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152100	16,58	37,82	13	37,71	Y	Hydrogen peroxide catabolic	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
36	C5XB38	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055600	16,53	41,37	20	33,65	Y	Chitin catabolic	Chitinase activity	Extracellular region	Glycoside hydrolase family 18 domain and family
37	A0A1B6QIM7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G123300	15,84	21,83	9	56,50	Y	None predicted	None predicted	None predicted	Glycoside hydrolase domain, (Trans)glycosidases family
38	A0A1B6QN00	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G360500	15,74	33,53	12	35,97	Y	None predicted	None predicted	None predicted	Domain not predicted; Plant peroxidase family
39	C5WVG9	Cysteine proteinase inhibitor OS=Sorghum bicolor GN=SORBI_001G324800	15,59	40,74	11	14,38	Y	Negative regulation of cysteine-type endopeptidase activity	Cysteine-type endopeptidase inhibitor activity	None predicted	Cystatin domain and family
40	C5XWE5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G197600	15,43	11,95	10	81,75	Y	Lipid metabolic	Phosphoric diester hydrolase activity	None predicted	Glycerophosphodiester phosphodiesterase domain; glycerophosphoryl diester phosphodiesterase family
41	C6JSB7	Peroxidase OS=Sorghum bicolor GN=Sb0246s002010	15,19	75,31	73	33,76	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
42	C5XHF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G136200	14,53	21,46	13	23,04	Y	Plasmodesmata-mediated intercellular transport	Oxalate decarboxylase activity	Plasmodesma	Cupin 1 domain; germin family
43	A0A1B6PD28	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G113000	14,03	23,35	11	53,22	Y	None predicted	None predicted	None predicted	Metallophosphoesterase domain; purple acid phosphatase family
44	C5WRN5	Peroxidase OS=Sorghum bicolor GN=SORBI_001G444500	13,01	23,74	11	36,21	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
45	C5XIH4	Uncharacterized protein	12,68	29,06	13	57,68	Y	Phospholipid catabolic	Acid	None	Alkaline-phosphatase-like

		OS=Sorghum bicolor GN=SORBI_003G430100							phosphatase activity	predicted	domain
46	C5X1U2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G390300	12,12	44,29	9	16,83	N	None predicted	Calcium ion binding	None predicted	EF-hand domain; family not predicted
47	C5XPK9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G205600	12,01	8,646	11	65,66	Y	None predicted	None predicted	None predicted	Leucine-rich repeat domain; family not predicted
48	A0A1B6PNM7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G242000	11,82	16,22	11	49,18	Y	None predicted	None predicted	None predicted	Peptidase C1A domain and family
49	A0A194YGY2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G027000	11,76	20,63	7	48,05	N	None predicted	None predicted	None predicted	Domain not predicted; Enolase family
50	C5XX83	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G208700	11,67	28,52	11	28,43	Y	Amino sugar metabolic	Chitinase activity	Intracellular	Chitin-binding, type 1 domain; glycoside hydrolase, family 19
51	A0A1B6QGB6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416800	11,6	28,57	18	33,61	Y	None predicted	None predicted	None predicted	Domain not predicted; Plant peroxidase family
52	C5XIK1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G432700	11,5	17,93	12	48,75	Y	None predicted	None predicted	None predicted	Bulb-type lectin domain; family not predicted
53	C5Z6U1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G209900	11,48	14,92	7	57,79	Y	None predicted	Catalytic activity	None predicted	Phospholipase D-like domain; family not predicted
54	C5XYY5	Peroxidase OS=Sorghum bicolor GN=SORBI_004G105100	11,23	54,15	20	33,56	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
55	C5YVJ8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232200	11,07	19,78	14	28,71	Y	None predicted	None predicted	None predicted	Fasciclin 1 domain; family not predicted
56	C5YJ56	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G214700	10,79	28,63	10	28,06	Y	None predicted	None predicted	None predicted	Fasciclin 1 domain; family not predicted
57	C5Y8Y2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G235600	10,64	14,21	8	80,91	Y	Carbohydrate metabolic	Hydrolase activity, hydrolysing O-glycosyl compounds	Plant-type cell wall	Glycoside hydrolase family 3 domain and family
58	C5XHP8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419400	10,47	25,42	8	44,07	Y	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic peptidase domain; asparytic peptidase A1 family
59	C5WSE5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300400	10,42	28,28	12	31,85	Y	Sexual reproduction	None predicted	Extracellular region	Expansin/pollen allergen domain; expansin/Lol pI family
60	C5XHP7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419300	10,31	14,39	6	44,95	Y	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic peptidase domain; aspartic peptidase A1 family

61	C5YK12	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G100600	10,29	34	8	19,92	Y	None predicted	Electron carrier activity	Membrane	Photocyanin domain; family not predicted
62	C5WZU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G516000	10,24	21,79	16	27,75	Y	Amino sugar metabolic	Chitinase activity	Intracellular	Glycoside hydrolase domain; glycoside hydrolase family 19
63	C5XBP7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G343600	10,06	20,72	11	35,66	Y	None predicted	None predicted	None predicted	Leucine-rich repeat domain; family not predicted
65	A0A1B6PKE9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G056300	10	13,08	7	57,24	Y	None predicted	None predicted	None predicted	FAD linked oxidase domain; FAD-binding/transporter-associated domain-like family
66	C5XT36	Endoglucanase OS=Sorghum bicolor GN=Sb24P17cg_130	9,85	21,4	5	54,22	N	Polysaccharide catabolic	Hydrolase activity	None predicted	Six-hairpin glycosidase domain; glycoside hydrolase family 9
67	C5Y587	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G049800	9,85	24,52	8	23,51	N	Nuclear-transcribed mRNA catabolic, deadenylation-dependent decay	3'-5'-exoribonuclease activity	CCR4-NOT complex	Alginate lyase 2 domain; family not predicted
68	C5YBH7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G135500	9,3	14,74	7	60,81	Y	None predicted	None predicted	None predicted	Glyoxal oxidase, N-terminal domain; family not predicted
69	A0A1B6Q9F4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G057900	9,28	30,13	5	17,29	N	None predicted	None predicted	None predicted	Thioredoxin domain; thioredoxin-like family
70	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS=Sorghum bicolor GN=SORBI_004G205100	9,21	31,45	11	36,35	N	Glucose metabolic	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	None predicted	Glyceraldehyde 3-phosphate dehydrogenase, NAD(P) binding domain; glyceraldehyde/Erythrose phosphate dehydrogenase family
71	C5WN99	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G261600	8,49	11,05	11	57,33	Y	Carbohydrate metabolic	Hydrolase activity	Cytoplasm	Glycoside hydrolase, catalytic domain; family not predicted
72	C5X022	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G525000	8,38	7,991	7	49,22	Y	Carbohydrate metabolic	Hydrolase activity	Extracellular region	Pectin lyase fold domain; glycoside hydrolase family 28
73	C5YI15	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G066500	8,26	30,26	8	24,73	Y	Oxalate metabolic	Oxalate decarboxylase activity	Cell wall	RmlC-like cupid domain; germim family
74	A0A1B6Q9B0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G050900	8,15	16,17	14	40,79	Y	None predicted	None predicted	None predicted	Leucine-rich repeat, N-terminal domain; protein kinase-like family
75	C5Y5K2	Peroxidase OS=Sorghum bicolor GN=SORBI_005G051500	8	13,53	7	37,10	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
76	C5WXD7	Uncharacterized protein OS=Sorghum bicolor	7,71	32,44	14	24,42	Y	None predicted	None predicted	None predicted	Peptidase of plants and bacteria domain;

		GN=SORBI_001G209300									uncharacterised protein family, basic secretory protein
77	C5YY94	Peroxidase OS=Sorghum bicolor GN=SORBI_009G144800	7,64	23,31	5	35,06	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
78	C5Y1M2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G097400	7,48	14,93	4	40,57	Y	Metabolic process	Hydrolase activity	None predicted	Alpha/Beta hydrolase fold-3 domain; family not predicted
79	C5X502	Dirigent protein OS=Sorghum bicolor GN=SORBI_002G119900	7,48	17,39	6	19,12	Y	Phenylpropanoid biosynthetic process	Guiding stereospecific synthesis activity	Apoplast	Dirigent domain; plant disease resistance response protein family
80	A0A1B6Q7M0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G390700	7,42	14,26	4	53,82	N	None predicted	None predicted	None predicted	None predicted
81	A0A194YIA9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G044500	6,98	11,33	5	38,36	Y	None predicted	None predicted	None predicted	None predicted
82	A0A1B6QC86	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G189100	6,92	5,091	4	83,49	N	None predicted	None predicted	None predicted	Domain not predicted; glycoside hydrolase, family 81
83	C5XRX3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G294500	6,84	18,94	5	27,41	Y	Sexual reproduction	None predicted	Extracellular region	Expansin/pollen allergen domain; expansin/Lol pl family
84	C5XFX7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G247000	6,81	14,58	4	46,29	Y	Carbohydrate metabolic process	Hydrolase activity, hydrolysing O-glycosyl compounds	None predicted	Glycoside hydrolase domain; family not predicted
85	C5WT64	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G170700	6,7	9,243	4	79,37	Y	None predicted	None predicted	None predicted	Peptidase 58 & 553 domain; substilisin-like family
86	A0A1B6PTQ9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G204700	6,7	15,08	5	48,35	Y	None predicted	None predicted	None predicted	Pectin lyase fold/virulence factor domain; pectin lyase-like family
87	C5YVJ7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232100	6,59	27,35	4	24,90	Y	None predicted	None predicted	None predicted	Fasciclin 1 domain; family not predicted
88	C5XB39	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055700	6,57	24,1	10	33,60	Y	Chitin catabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase, catalytic domain; family not predicted
89	C5WSY5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G014700	6,49	15,53	4	51,21	Y	Carbohydrate metabolic process	Hydrolase activity	Anchored component of plasma membrane	Glycoside hydrolase, catalytic domain; glycoside hydrolase family 17
90	A0A1B6QEI0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G317600	6,46	22,35	4	18,69	N	None predicted	None predicted	None predicted	Chorismate mutase-like domain; chorismate mutase family
91	C5Y675	Uncharacterized protein	6,44	16,63	4	44,46	Y	None predicted	None predicted	None	Peptidase family A1 domain;

		OS=Sorghum bicolor GN=SORBI_005G064200								predicted	aspartic peptidase A1 family
92	C5XL59	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G024700	6,34	19,95	11	41,94	N	None predicted	None predicted	None predicted	Secretory peroxidase domain; plant peroxidase family
93	C5Z469	Peroxidase OS=Sorghum bicolor GN=SORBI_010G161600	6,26	8,28	14	33,21	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
94	C5WXN2	Carboxypeptidase OS=Sorghum bicolor GN=SORBI_001G348800	6,22	19,83	15	50,61	Y	Proteolysis	Serine-type carboxypeptidase activity	None predicted	Alpha/Beta hydrolase fold domain; peptidase S10, serine carboxypeptidase family
95	C5X5L7	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G417800	6,1	13,69	5	47,15	Y	Metabolic process	Hydrolase	Vacuole	Glycoside hydrolase superfamily domain; glycoside hydrolase family 27
96	A0A1B6Q6M7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G327700	6,09	18,8	4	14,89	Y	None predicted	None predicted	None predicted	None predicted
97	C5XIY0	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152000	6,05	14,84	7	37,65	N	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
98	C5YNA1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G172100	6,05	10,22	4	40,26	Y	Proteolysis involved in cellular protein catabolic process	Cysteine-type endopeptidase activity	Extracellular space	Peptidase C1A domain and family
99	C5XL56	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G023800	6,05	8,7333	3	61,96	Y	None predicted	None predicted	None predicted	Peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase A family
100	C5Y7Y7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G215500	6,03	16,07	14	11,69	Y	Negative regulation of endopeptidase activity	Serine-type endopeptidase inhibitor activity	Extracellular region	Proteinase inhibitor I12 domain
101	C5X578	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G128000	6,02	16,73	4	28,66	Y	None predicted	None predicted	None predicted	Carbohydrate esterases domain; family not predicted
102	A0A194YKZ5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G227400	6,01	6,122	3	70,38	N	None predicted	None predicted	None predicted	Glycoside hydrolase, catalytic core domain; (Trans)glycosidases family
103	C5YW21	Malate dehydrogenase OS=Sorghum bicolor GN=SORBI_009G240700	6	12,06	3	35,35	N	Malate metabolic process	Malate dehydrogenase activity	None predicted	Lactate/malate dehydrogenase domain; malate dehydrogenase, type 1 family
104	A0A194YU12	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G341200	6	8,889	5	53,04	N	None predicted	None predicted	None predicted	FAD-dependent pyridine nucleotide-disulphide oxidoreductase domain; FAD/NAD-linked reductases family
105	C5YWW2	Peroxidase OS=Sorghum bicolor GN=SORBI_009G113700	6	9,384	4	36,66	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
106	C5WT90	Uncharacterized protein	6	13,19	4	41,62	N	Cellulose biosynthetic	Intramolecular	Golgi	Nucleotide-disphospho-sugar

		OS=Sorghum bicolor GN=SORBI_001G173300						process	transferase activity	apparatus	transferases domain; reversibly glycosylated polypeptide family
107	C5YUD2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G072000	6	16,28	5	40,97	N	'*de novo*' IMP biosynthetic process	Phosphoribosylformylglycinamidine cyclo-ligase activity	Cytoplasm	AIR synthase-related protein domain; phosphoribosylformyglycinamidine cyclo-ligase family
108	A0A1B6QIX5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G142200	6	10,53	5	48,56	Y	None predicted	None predicted	None predicted	Glycoside hydrolase, catalytic core domain; (Trans)glycosidases family
109	A0A1B6PAU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G234800	6	6,275	6	54,37	Y	None predicted	None predicted	None predicted	Peptidase aspartic, catalytic domain; acid proteases family
110	A0A1B6QN96	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G371900	6	21,05	4	15,09	N	None predicted	None predicted	None predicted	Superoxide dismutase, copper/zinc binding domain; Cu, Zn superoxide dismutase-like family
111	A0A1B6Q818	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G416300	5,9	22,37	4	23,99	N	None predicted	None predicted	None predicted	Glutathione S-transferase (GST), C-terminal-like domain; GST C-terminal domain-like family
112	C5XCL8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G070200	5,72	17,62	3	20,94	Y	None predicted	None predicted	None predicted	Plant lipid transfer protein domain; family not predicted
113	C5XYB4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G229300	5,71	17,27	8	34,29	N	None predicted	None predicted	None predicted	Domain not predicted; phosphate-induced protein 1 family
114	C5XQJ2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G364300	5,67	18,48	5	31,68	Y	Chitin catabolic process	Chitinase activity	Extracellular region	Glycoside hydrolase family 18, catalytic domain and family
115	C5XQW7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G087300	5,27	18,06	10	31,60	Y	DNA catabolic process	Endonuclease activity	None predicted	Phospholipase C/P1 nuclease domain; S1/P1 nuclease family
116	C5WQH5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G149500	5,2	19,8	3	11,61	N	None predicted	None predicted	None predicted	None predicted
117	C5Y1P6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G099500	5,13	10,2	4	49,29	Y	None predicted	Hydrolase activity	None predicted	Domain not predicted; nucleoside phosphatase GDA1/CD39 family
118	C5X3C7	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391900	5	8,226	7	64,25	Y	Plant-type cell wall organisation	Peroxidases activity	Plant-type cell wall	Secretory peroxidase domain; plant peroxidase family
119	C5Y8K8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G228900	4,99	7,495	3	49,28	Y	Carbohydrate metabolic process	Hydrolase activity	Anchored component of plasma membrane	Glycoside hydrolase, catalytic domain; glycoside hydrolase family 17
120	C5YSB1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G048400	4,91	24,07	4	26,38	Y	None predicted	None predicted	None predicted	Alginate lyase 2 domain; family not predicted

121	A0A1B6QAK5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G113800	4,89	12,28	3	36,06	N	None predicted	None predicted	None predicted	Domain not predicted; spermine synthase family
122	A0A1B6PHE0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G120800	4,8	2,511	3	98,37	N	None predicted	None predicted	None predicted	Domain not predicted; peptidase M1 family
123	C5XFH6	Fructose-bisphosphate aldolase OS=Sorghum bicolor GN=SORBI_003G393900	4,76	18,03	4	38,56	N	Glycolytic process	Fructose-bisphosphate aldolase activity	None predicted	Aldolase-type TIM barrel domain; fructose-bisphosphate aldolase class-I family
124	A0A1B6QN59	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G366800	4,69	3,317	2	92,11	Y	None predicted	None predicted	None predicted	Coagulation factor 5/8 type, C-terminal domain; galactose-binding domain-like family
125	C5XHX2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G427700	4,51	12,95	5	23,24	Y	Oxalate metabolic process	Oxalate decarboxylase activity	Cell wall	Cupin 1 domain; germin family
126	C5Z475	Peroxidase OS=Sorghum bicolor GN=SORBI_010G162000	4,48	19,38	11	34,43	Y	Hydrogen peroxidase catabolic process	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
127	C5Z4E5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G044900	4,46	13,35	5	39,46	Y	None predicted	Hydrolase activity, acting on ester bonds	None predicted	SGNH hydrolase-type esterase domain; family not predicted
128	C5YZJ2	Peroxidase OS=Sorghum bicolor GN=SORBI_009G033400	4,37	14,79	7	36,06	N	Hydrogen peroxidase catabolic process	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
129	C5X6P7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G140400	4,37	31,4	5	17,60	Y	None predicted	Electron carrier activity	Anchored component of plasma membrane	Cupredoxin domain; family not predicted
130	C5XTG0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G166500	4,32	10,07	2	33,32	N	Nitrogen compound metabolic process	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	None predicted	Carbon-nitrogen hydrolase domain; N-carbamoylputrescine amidase family
131	C5WWQ2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G342600	4,31	4,795	3	64,78	N	Cell redox homeostasis	Thioredoxin-disulfide reductase activity	Cytoplasm	Thioredoxin domain; family not predicted
132	C5X9N2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G039000	4,26	17,11	3	16,37	Y	Intracellular sterol transport	None predicted	Intracellular	Immunoglobulin E-set domain; family not predicted
133	C5WP48	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_001G268700	4,24	6,157	3	111,61	Y	Mannose metabolic process	Alpha-mannosidase activity	None predicted	Glycoside hydrolase 38/57, N-terminal domain and family
134	C5YQ75	Peroxidase OS=Sorghum bicolor GN=SORBI_008G010500	4,21	40,55	31	37,73	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
135	A0A1B6PLT3	Uncharacterized protein OS=Sorghum bicolor	4,19	9,16	3	27,97	N	None predicted	None predicted	None predicted	Glycoside hydrolase, family 19, catalytic domain and

		GN=SORBI_006G132100									family
136	C5WQK1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G280000	4,18	7,879	3	54,17	Y	Proteolysis involved in cellular protein catabolic process	Serine-type carboxypeptidase activity	None predicted	Alpha/Beta hydrolase fold domain; peptidase S10, serine carboxypeptidase family
137	C5X780	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G007200	4,16	16,43	5	21,52	Y	None predicted	Electron carrier activity	Anchored component of plasma membrane	Cupredoxin domain; family not predicted
138	A0A1B6QEG2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G315800	4,1	8,451	5	38,92	Y	None predicted	None predicted	None predicted	Peptidase C1A domain and family
139	C5YRS3	Purple acid phosphatase OS=Sorghum bicolor GN=SORBI_008G037000	4,02	8,554	3	53,30	N	Dephosphorylation	Acid phosphatase activity	None predicted	Purpe acid phosphatase-like, N-terminal domain; family not predicted
140	C5YCY4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G160700	4,02	5,449	3	73,15	N	Carbohydrate metabolic activity	Hydrolase activity, hydrolysing O-glycosyl compounds	None predicted	Glycosyl hydrolase family 32, N-terminal domain; glycoside hydrolase, family 32
141	A0A1B6Q8G8	Uncharacterized protein (Fragment) OS=Sorghum bicolor GN=SORBI_003G440900	4,01	5,288	3	73,06	N	None predicted	None predicted	None predicted	Glycosyl hydrolase family 32, N-terminal domain; glycoside hydrolase, family 32
142	C5XHP9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419500	4	11,11	5	43,89	Y	Proteolysis	Aspartic-type endopeptidase activity	None predicted	Aspartic peptidase domain; aspartic peptidase A1 family
143	C5WPY8	Peroxidase OS=Sorghum bicolor GN=SORBI_001G277000	4	10,95	3	36,09	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
145	C5YBF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132700	4	12,5	4	28,77	Y	Amino sugar metabolic process	Chitinase activity	Intracellular	Chitin-binding, type 1 domain; glycoside hydrolase, family 19
146	A0A1B6QD45	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G237000	4	6,587	10	33,67	N	None predicted	None predicted	None predicted	Fasciclin 1 domain and family
147	C5Z998	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G255000	4	3,194	2	72,07	Y	Seed development	None predicted	None predicted	Leucine-rich repeat domain; protein WYRD family
148	C5WT45	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G168500	4	8,794	2	42,63	N	Negative regulation of endopeptidase activity	Serine-tyoe endopeptidase activity	Extracellular space	Serpin domain and family
149	C5Y7T1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G086000	4	8,261	2	49,85	Y	None predicted	Hydrolase activity	None predicted	Domain not predicted; Nucleoside phosphatase GDA1/CD39 family
150	C5X3W3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G246400	4	7,576	2	50,49	Y	Cell wall organisation	Hydrolase activity, acting on glycosyl bonds	Extracellular region	Pectin lyase fold/virulence factor domain; glycoside hydrolase, family 28
151	C5XC95	Uncharacterized protein	4	12,63	2	18,84	Y	None predicted	Electron carrier	Anchored	Cupredoxin domain; family

		OS=Sorghum bicolor GN=SORBI_002G345800							activity	component of plasma membrane	not predicted
152	C5XQ07	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G072300	4	7,51	3	26,97	N	Glycolytic process	Triose-phosphate isomerase actvity	Cytosol	Aldolase-type triosephosphate isomerase domain; triosephosphate isomerase family
153	C5Y2P0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G122300	4	14,79	2	15,40	Y	None predicted	None predicted	None predicted	Galactose-binding like domain; galactose-binding domain-like family
154	A0A1B6QFT1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G392000	3,66	5,277	2	40,53	N	None predicted	None predicted	None predicted	Domain not predicted; plant peroxidase family
155	A0A1B6QFT7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G392300	3,63	12,29	4	36,33	Y	None predicted	None predicted	None predicted	Domain not predicted; plant peroxidase family
156	C5WN51	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G119000	3,62	16,41	3	13,55	Y	None predicted	Electron carrier activity	Anchored component of plasma membrane	Cupredoxin domain; family not predicted
157	C5WPH2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G130400	3,51	14,2	3	17,49	Y	None predicted	None predicted	Vacuole	Domain not predicted; protein of unknown function DUF338 family
158	C5WSF3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301000	3,5	9,058	2	29,95	Y	Sexual reproduction	None predicted	Extracellular region	Expansin/pollen allergen domain; expansin/Lol pI family
159	A0A1B6P9F6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G190800	3,49	10,29	3	18,97	N	None predicted	None predicted	None predicted	Thioredocin domain; thioredoxin-like family
160	A0A1B6Q537	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G244600	3,48	6,509	6	35,27	N	None predicted	None predicted	None predicted	Glycoside hydrolase, catalytic core domain; (Trans)glycosidases
161	C5Z0N9	Peroxidase OS=Sorghum bicolor GN=SORBI_009G055300	3,46	7,438	3	38,28	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
162	C5X4N0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G255600	3,24	6,004	3	52,42	Y	Carbohydrate metabolic process	Hydrolase activity, hydrolysing O-glycosyl compounds	Anchored component of plasma membrane	Glycoside hydrolase,catalytic domain; glycoside hydrolase family 17
163	C5Z476	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G161900	3,23	23,05	9	34,86	N	Hydrogen peroxidase catabolic process	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
164	C5X455	Carboxypeptidase OS=Sorghum bicolor GN=SORBI_002G401200	3,17	5,513	2	56,62	Y	Proteolysis	Serine-type carboxypeptidase activity	None predicted	Alpha/Beta hydrolase fold domain; peptidase S10, serine carboxypeptidase family
165	A0A1B6PLT5	Uncharacterized protein OS=Sorghum bicolor	3,12	13,68	7	40,48	Y	None predicted	None predicted	None predicted	Galactose-binding like domain; galactose-binding

		GN=SORBI_006G133000									domain-like family
166	C5WRL4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G442200	3,11	10,79	2	38,57	Y	Dephosphorylation	Acid phosphatase activity	None predicted	Metallo-dependent phosphatase-like domain; acid phosphatase, type 5 family
167	C5XRU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G148100	3,02	12,44	5	23,32	Y	Oxalate metabolic process	Oxalate decarboxylase activity	Cell wall	RmlC-like jelly roll fold domain; germin family
168	C5XG88	Small ubiquitin-related modifier OS=Sorghum bicolor GN=SORBI_003G402600	2,93	51,52	3	10,84	N	Protein sumoylation	Protein tag	Nucleus	Ubiquitin domain; family not predicted
169	A0A1B6QJR7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G189000	2,91	7,98	2	43,29	N	None predicted	None predicted	None predicted	Domain not predicted; plant peroxidase family
170	C5WX83	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G058300	2,87	7,107	7	42,57	Y	None predicted	None predicted	None predicted	Leucine-rich repeat domain; family not predicted
172	C5X6H6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G431100	2,74	10,4	2	27,16	N	Response to oxidative stress	L-ascorbate peroxidase activity	Chloroplast	Plant peroxidase domain; plant ascorbate peroxidase family
173	C5XDR4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G217200	2,66	5,785	2	39,26	Y	Proteolysis involved in cellular protein catabolic process	Cysteine-type endopeptidase activity	Extracellular space	Peptidase C1A domain and family
174	C5YN91	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G171000	2,61	7,39	2	46,62	N	Cell redox homeostasis	Oxidoreductase activity	Cell	FAD/NAD(P)-binding domain; family not predicted
175	A0A194YLI0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G261000	2,57	15,35	2	20,55	Y	None predicted	None predicted	None predicted	Pectinesterase acyvity domain; plant invertase/pectin methylesterase inhibitor family
176	C5X3C2	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391400	2,45	20,82	12	39,13	Y	Response to oxidative stress	Peroxidase activity	Extracellular region	Secretory peroxidase domain; plant peroxidase family
178	C5Y171	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G142800	2,4	8,086	2	40,44	Y	Proteolysis involved in cellular protein catabolic process	Cysteine-type endopeptidase activity	Extracellular space	Peptidase C1A domain and family
179	A0A1B6QB11	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G133800	2,33	6,437	2	46,77	N	None predicted	None predicted	None predicted	FAD-dependent pyridine nucleotide-disulphide oxidoreductase domain; FAD/NAD-linked reductases family
181	A0A1B6PJF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G014400	2,27	7,231	2	51,66	N	None predicted	None predicted	None predicted	Pectate lyase domain; pectin lyase-like family
182	C5X8I3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G301600	2,21	33,73	14	54,13	Y	None predicted	None predicted	None predicted	Domain not predicted; amidase signature enzymes family

183	C5YBF3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132900	2,16	3,694	2	40,44	Y	None predicted	None predicted	None predicted	Galactose-binding domain-like domain; family not predicted

[a] Protein number assigned in ProteinPilot software.

[b] Protein accession numbers obtained from the TrEMBL database [incorporated within the UniProt database (http://www.uniprot.org)] searches against sequences of *Sorghum bicolor* only.

[c] Protein score generated 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 a 95% confidence.

[d] Percentage coverage is determined 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 peptide refers to the number of peptide that were sequenced and gave rise to protein identity. All proteins that were identified by means of a single peptide were filtered out of the dataset.

[f] Theoretical molecular weight (kDa) as annotated in the UniProt database (http://www.uniprot.org).

[g] Signal peptide predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). Y denotes the presence of a predicted signal peptide in the primary sequence of the protein, while N denotes the absence of a signal peptide.

[h] Gene ontology analysis as predicted by the EMBL-EBI database (http://www.ebi.ac.uk/QuickGO/). P denotes Biological Process, F denotes Functional Process, and C denotes Cellular Component.

[i] Conserved domains and family name as predicted by InterPro database (http://www.ebi.ac.uk/interpro/).

5.3. Bioinformatic Analyses on the Identified Sorghum Secreted Proteins

5.3.1. Prediction of Signal Peptides

Generally, secreted proteins have a cleavable N-terminal signal peptide required for protein translocation into the endoplasmic reticulum (ER; Drakakaki and Dandekar, 2013). In this study, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; Peterson *et al*., 2011) was used to identify the presence of signal peptides on all positively identified proteins. Out of the 178 positively identified sorghum secreted proteins (Table 5.1), 128 (72%) were predicted to be classical extracellular matrix proteins, with a signal peptide, whereas 50 (28%) were not (Figure 5.1). Most of the SP containing proteins were uncharacterised (77%). Other signal peptide containing proteins included peroxidases, carboxypeptidases, alpha-mannosidases, alpha-galactosidases, dirigent protein, and cysteine proteinase inhibitor (Table 5.1). Amongst proteins without a signal peptide were other peroxidases, endoglucanases, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, fructose-bisphosphate aldolase, purple acid phosphatase, and a small-ubiquitin-related modifier.

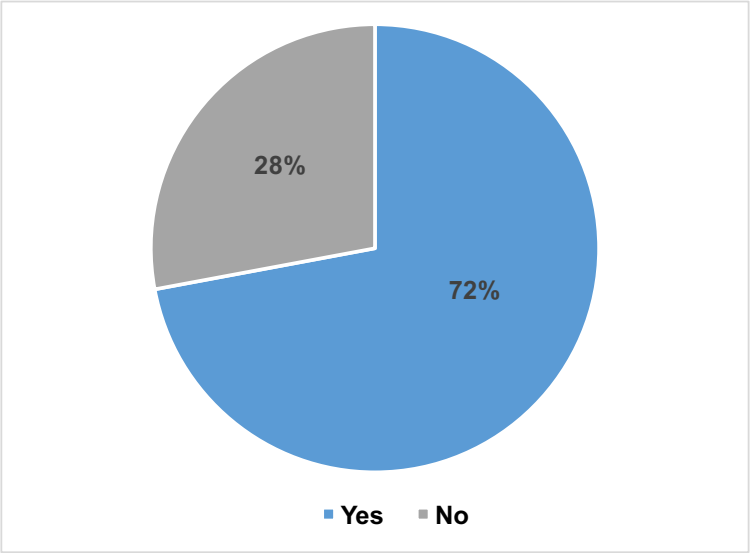

Figure 5.1. Signal peptide prediction on MudPIT identified sorghum culture filtrate proteins. Yes, indicates the presence of a predicted signal peptide in the primary sequence of the protein, and No indicates the absence of a predicted signal peptide.

5.3.2. Gene Ontology Analysis

All the 178 identified sorghum secreted proteins were submitted for Gene Ontology annotation for the determination of their cellular component, biological process, and functional process (Table 5.1). The annotations were done on EMBL-EBI database (http://www.ebi.ac.uk/QuickGO/) and results are shown in Figures 5.2 - 5.4 below. About 56% of the identified proteins did not have any predicted cellular component (Figure 5.2) and most of these proteins were uncharacterised (Table 5.1). Amongst the proteins with predicted cellular components, a larger proportion (20%) was predicted to be outside of the cell in the extracellular region (Figure 5.2) and dominated by peroxidases (Table 5.1). About 7% of the identified proteins were unexpectedly predicted to be in the intracellular part, which includes the cytoplasm, cytosol, nucleus, vacuole, golgi apparatus and the chloroplast (Table 5.1; Figure 5.2). Other important cellular components expected in secretome studies such as the apoplast (0.6%), plasmodesmata (0.6%), plasma

membrane (4%), cell wall (4%) and extracellular space (2%) were all predicted in this study (Table 5.1; Figure 5.2).

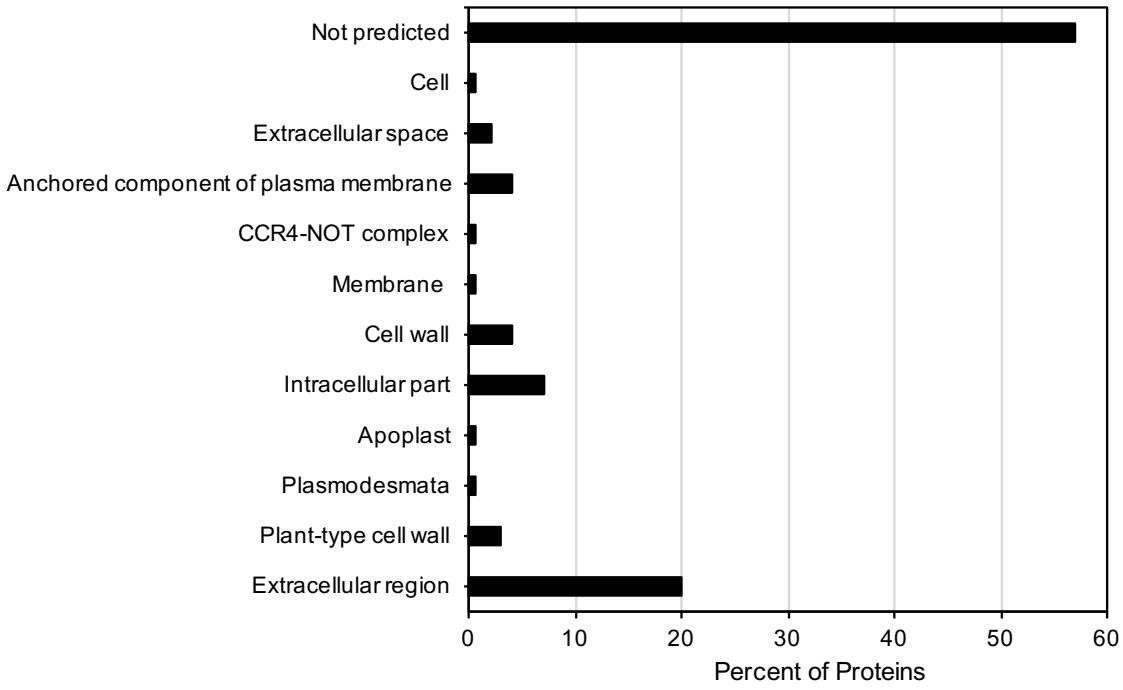

Figure 5.2. Cellular component predictions of the identified sorghum secretome based on GO annotation.

Most of the proteins amongst the 178 identified did not have any predicted biological processes. These proteins accounted for 45% of the identified proteins (Figure 5.3). The remaining 55% of proteins were distributed across several biological processes, with about 14% of these being involved in response to oxidative stress, carbohydrate metabolism (13%), catabolic process (7%) and proteolysis (6%; Figure 5.3). One protein, corresponding to 0.6% of identified proteins, was predicted to be involved in seed

development, an unusual biological process in secretome studies. This protein (protein no. 147, Accession number C5Z998, Table 5.1) is uncharacterised and does not have a predicted cellular component. However, it has a predicted signal peptide indicating that it is a classical secreted protein (Table 5.1).

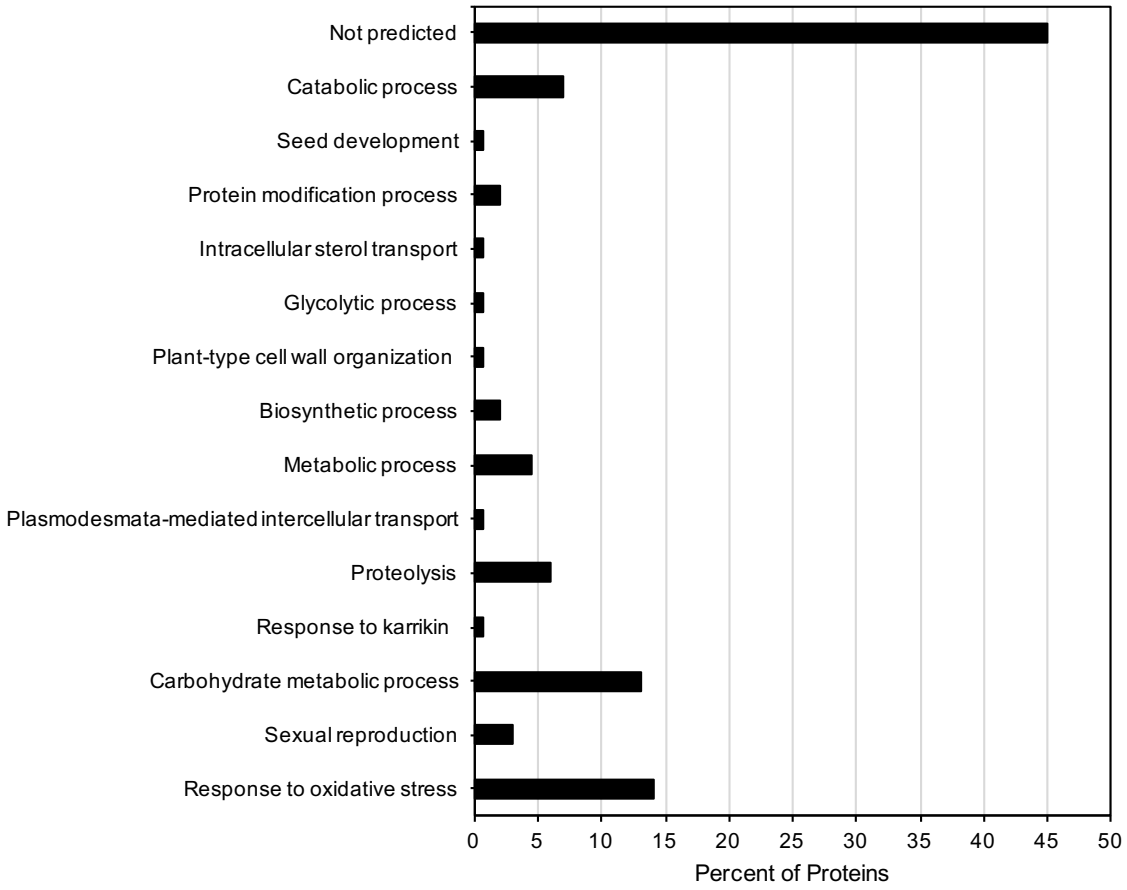

Figure 5.3. Biological process predictions of the identified sorghum secretome based on GO annotation.

The 178 positively identified proteins were annotated across a very broad range of molecular functions, including endopeptidase activity, peroxidase activity, chitinase, hydrolase activity, electron carrier, oxalate decarboxylase, and hydrolase activity, hydrolysing O-glycosyl compounds (Figure 5.4). Proteins with endopeptidase activity were annotated to be involved in proteolysis (Figure 5.3), and mostly localised in the extracellular space (Figure 5.2). These proteins were also predicted to have a signal peptide (Figure 5.1), indicating that they were secreted via the classical secretory pathway.

Proteins with peroxidase activity were annotated to be involved in response to oxidative stress (Figure 5.3). On the other hand, proteins with chitinase activity, hydrolase activity, oxalate decarboxylase activity, and hydrolase activity, hydrolysing O-glycosyl compounds were annotated as components of different metabolic processes. These processes included amino sugar, oxalate, and carbohydrate metabolism (Figure 5.3). However, proteins involved in electron carrier (Figure 5.4), constituting about 3% of the 178 identified proteins, did not have any predicted biological process (Table 5.1). These proteins were however annotated to be localised in the plasma membrane (Figure 5.2) and have predicted signal peptides on their primary sequences. A total of 43% of the 178 positively identified proteins had no predicted molecular functions (Figure 5.4).

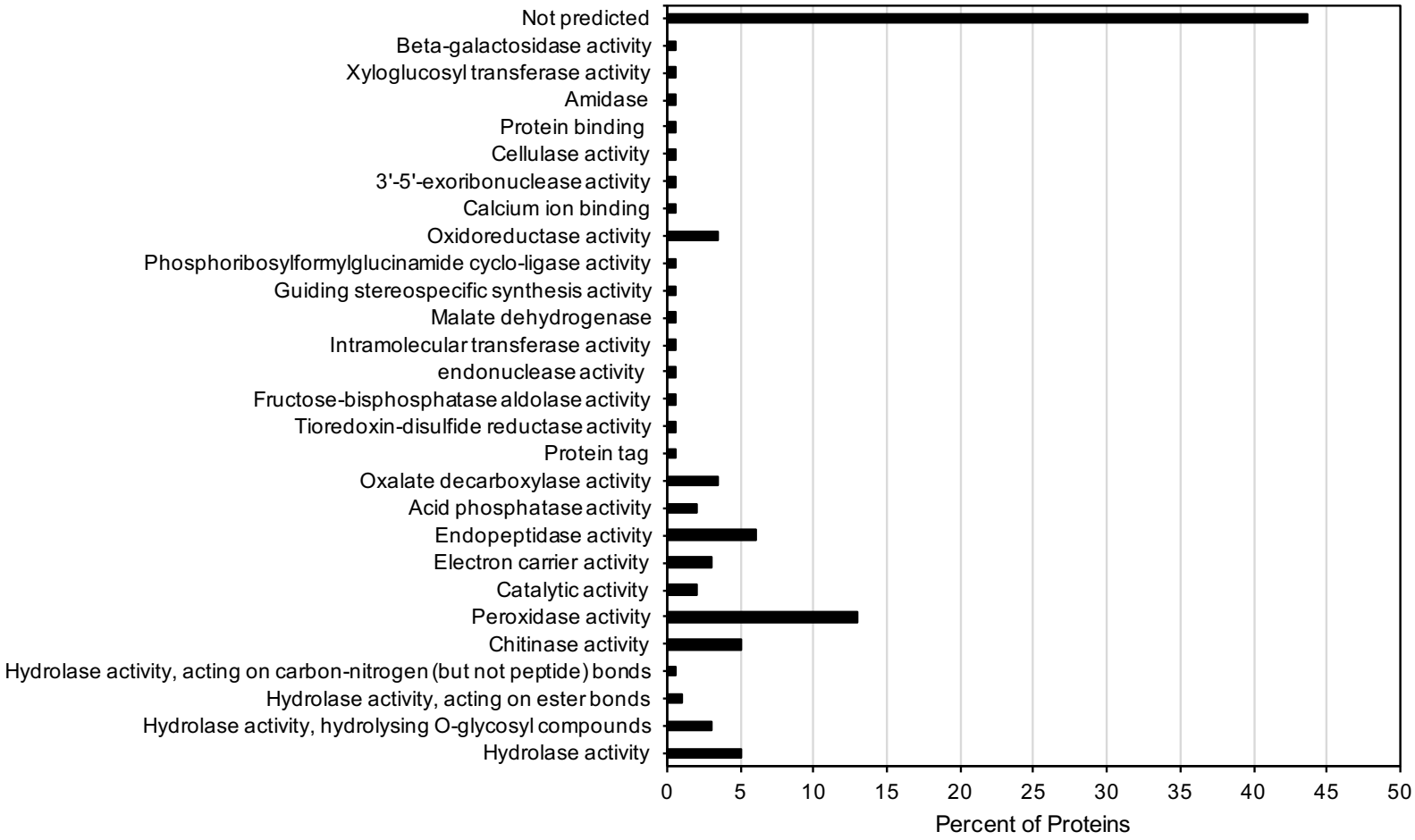

Figure 5.4. Molecular function predictions of the identified sorghum secretome based on GO annotation.

5.3.3. Identification of Conserved Domains and Family Names

Protein domains are distinct functional regions of a protein sequence that can exist and function independently of the rest of the protein chains (Marchler-Bauer *et al*., 2011). Protein families are a group of proteins sharing a common ancestor, and have similar protein structure, sequence, and function (Finn *et al*., 2013). Together with the protein families, protein domains assist in understanding a protein's function. The InterPro database (http://www.ebi.ac.uk/interpro/) was used to identify conserved domains and family names of all the 178 identified White sorghum secreted proteins. The first largest group of identified secreted proteins (46 proteins; 26%) had the glycoside hydrolase (GH) conserved domain and belonged to a wide range of glycoside hydrolase families including family 3, 9, 17, 18, 19, 27, 28, 31, 32, 35, and 81 (Table 5.1). The second largest group mostly consisted of peroxidases with the secretory peroxidase conserved domain (26 proteins; 15%) and belong to the plant peroxidase family (30 proteins; 17%).

Other conserved domains and families consisted of small groups of proteins ranging from 1 to 7. These domains include fasciclin 1 (proteins no. 24, 33, 55, 56, 87, and 146), cupredoxin (proteins no. 23, 129, 137, 151, and 156), cupin 1 (proteins no. 30, 31, 42, and 125), and leucine-rich repeat (LRR; proteins no. 47, 63, 74, 147, and 170). However, four of these proteins with the LRR domain (proteins no. 47, 63, 74, and 170) had no predicted cellular component, biological process, and molecular function (Table 5.1). The fifth protein (protein no. 147) was annotated to be part of seed development. All five LRR proteins had predicted signal peptides (Table 5.1). Although conserved domains and families may shed some light into a protein's function, there were a total of four positively identified uncharacterised proteins, two with signal peptides (proteins no. 81 and 96) and two without (proteins no. 80 and 116), that neither had a predicted cellular component, biological process, molecular function, conserved domain nor protein family name (Table 5.1).

5.4. Discussion

Proteins secreted into the ECM play important roles in cell wall structure, cellular communication, and defence against various stimuli (Lum and Min, 2011). Cell suspension cultures are a good experimental system for studying the secretome mainly due to the less invasive protein extraction methods used, which results in less cytoplasmic contaminants (Alexanderson *et al*., 2013). To date, there are only 14 positively identified sorghum secreted proteins that were identified from the White sorghum cell cultures using a combination of 2D gel electrophoresis and MALDI-TOF-TOF MS (Ngara and Ndimba, 2011). With an exponential increase in new technologies in the proteomics field, we saw a need to improve on the secretome map already reported by Ngara and Ndimba (2011); and thus assist in further characterising sorghum secreted proteins and work towards an understanding of their functions in growth and developmental processes.

The current study reports an improved sorghum secretome map using MudPIT. A total of 178 unique sorghum secreted proteins were positively identified, most of which are uncharacterised (78%). However, some identified proteins matched the identities of peroxidases, alpha-galactosidases, endoglucanases, dirigent protein, glyceraldehyde-3-phosphate dehydrogenase, carboxypeptidases, malate dehydrogenase, fructose-bisphosphate aldolase, and alpha-mannosidases (Table 5.1).

In the current study, 21 proteins were identified as peroxidases, and play important roles in response to oxidative stress (Table 5.1). These proteins have also been previously identified in secretome studies of sorghum (Ngara and Ndimba, 2011), rice (Jung *et al*., 2008; Cho *et al*., 2009) and chickpea (Gupta *et al*., 2011). Peroxidases have also been implicated in lignification, cross-linking of structural cell wall proteins, auxin catabolism (Hiraga *et al*., 2001) and possibly in programmed cell death regulation (Smith *et al*., 2015). Alpha-galactosidases, glyceraldehyde-3-phosphate dehydrogenases, malate

dehydrogenases, and fructose bisphosphate aldolases are considered house-keeping proteins because of their roles in growth and development (Jung *et al*., 2008; Guerra-Guimarães *et al*., 2016).

Endoglucanases are members of the GH family 9, consisting of cellulase activity, have roles in polysaccharide catabolism and are not classically secreted. Endoglucanases have not been identified in plant secretome studies. However, they are present in plants such as tomato (*Solanum lycopersicum*; Urbanowicz *et al*., 2007). Endoglucanases have however been identified in secretome studies of plant pathogenic oomycete *Phytophthora plurivora* (Severino *et al*., 2014) and a fungus, *Fusarium graminearum* (Brown *et al*., 2012) that infect important agricultural crops such as wheat, and both studies reported endoglucanases to be involved in plant cell wall degradation. The dirigent protein (protein no. 79) is a member of the plant disease resistance response protein family, functioning in lignin biosynthesis to repair or strengthen damaged cell walls (Jagadish *et al*., 2010). Lastly, carboxypeptidases, previously identified in chickpea (Gupta *et al*., 2011), Arabidopsis (Tran and Plaxton, 2008), and *Medicago trancatula* (Kusumawati *et al*., 2008) secreted proteins have been reported to function in protein turnover and mobilisation during seed germination (Kusumawati *et al*., 2008). These proteins do not have any predicted cellular component but they are classically secreted proteins (Table 5.1).

Out of the 178 identified proteins, 128 (72%) were predicted to have an N-terminal signal peptide, indicating that they are classical secreted proteins. Only 50 (28%) of the identified proteins did not have a predicted signal peptide using SignalP 4.1. As discussed in section 1.7.2, there have been reports of non-signal peptide containing proteins being secreted into the ECM via the non-classical secretory (UPS) pathway in plant cells (Drakakaki and Dandekar, 2013; Ding *et al*., 2014). However, the identification

of proteins without a signal peptide in this sorghum secretome study, does not necessarily suggest that they were secreted via the UPS pathway.

The 50 non-signal peptide containing proteins could have been in the ECM as contaminants from other cellular components or could have leaked out of the cell based on the molecular weight. For example, protein no. 168 (small ubiquitin-related modifier; SUMO), the smallest protein of the 178 identified proteins (with molecular weight of 10.84 kDa) is predicted to be localised in the nucleus functioning as a protein tag in the process of protein sumoylation (Table 5.1). This is in agreement with a report by Schwartz and Hochstrasser (2003), which suggests SUMO to be localised in the nucleus, functioning in protein modification, which results in protein's different molecular functions such as subcellular localisation or DNA binding, depending on the protein substrate (Hilgarth *et al*., 2004).

Most of the proteins without predicted signal peptides (proteins no. 6, 7, 19, 33, 49, 69, 80, 82, 90, 92, 102, 104, 110, 111, 113, 116, 121, 122, 135, 141, 146, 154, 159, 160, 169, 179, 181) were uncharacterised and also did not have any predicted cellular component, biological process, and molecular function; possibly indicating their novelty in this sorghum secretome study. However, some of these uncharacterised proteins were predicted to be part of plant peroxidase (protein no. 169), GH 32 (protein no. 141), and fasciclin (protein no. 146) protein families. These protein identities found in these families are in line with secretome studies; peroxidases lacking signal peptides from the plant peroxidase family and proteins from GH families have previously been identified in chickpea (Gupta *et al*., 2011), and both protein families function in cell wall formation and modifications (Lum and Min, 2011; Passardi *et al*., 2004). Fasciclin family proteins, functioning as cell adhesion molecules (Johnson *et al*., 2003) have previously been identified in Arabidopsis (Kaffarnik *et al*., 2009) and tobacco (Hafidh *et al*., 2016) secretome, but were reported to have predicted signal peptides.

A wide range of molecular functions were predicted in all 178 identified proteins, with peroxidase activity, endopeptidase activity, chitinase acttivity, hydrolase activity, and oxalate decarboxylase activity dominating the GO analysis, followed by the none predicted functions (Figure 5.4). Proteins with these molecular functions were mostly annotated as having roles in responses to oxidative stress, carbohydrate metabolism, catabolic processes, proteolysis, as well as other metabolic processes (Figure 5.3). Biological process and molecular function annotation data can be closely correlated to each other. For example, there was a high protein percentage with endopeptidase activity (Figure 5.4), which explains a higher protein percentage involved in proteolysis (Figure 5.3). Proteolysis is an important process where proteins are broken down into smaller polypeptides or amino acids; catalysed by proteases (Jung *et al*., 2008). The above-mentioned molecular functions are common in secretome studies (Jung *et al*., 2008; Cho *et al*., 2009; Gupta *et al*., 2011; Smith *et al*., 2015) and are thus found in the extracellular compartments.

A large number of the identified proteins (78%) are uncharacterised (Table 5.1). However, the InterPro database annotated 30 of these proteins to be members of the plant peroxidase family. Proteins belonging to this family are mainly responsive to oxidative stress (Figure 5.3) and have peroxidase activity (Figure 5.4). As discussed earlier in section 1.3, an excessive production of ROS in plant cells results in oxidative stress (Vinocur and Altman, 2005). In terms of cell suspension culture, stress could be in the form of continuous agitation of cells and also in terms of nutrients depletion in the medium. White sorghum cell suspensions used for CF proteins extraction were on day 10 of the growth curve (Figure 4.1 B). At this stage, cell growth is limited and cultures were in the stationary phase. As a result, the expression of a large number of peroxidases in this study (30 proteins; 17%) may have been triggered by any of the above-mentioned stresses.

A total of 43 proteins were classified as members of the different GH families such as 3, 9, 17, 18, 19, 27, 28, 31, 32, 35, 38, and 81, most of which are involved in polysaccharide hydrolysis (Henrissat and Davies, 1997). Proteins belonging to the GH 3 and 28 families are thought to be involved in restructuring the plant cell wall during expansion and differentiation (Minic *et al*., 2007). The substrates of these families mostly include cellulose, xylan, pectins, and xyloglucans (Jamet *et al*., 2008). In the current study, proteins targeting pectin degradation were identified, for example protein no. 150 (Table 5.1). Proteins belonging to the GH 17, 18, and 19 families, which include chitinases might also be involved in defence against pathogens (Jamet *et al*., 2008). In the current study, we found proteins of the GH 18 (protein no. 36) and 19 (protein no. 145) families to have chitinase activity. However, these proteins were annotated to have roles in amino sugar metabolism and chitin catabolism, respectively. Therefore, there are certainly other functions together with defence against pathogens that members of the GH 17, 18, and 19 are involved in. Proteins from different GH families have been identified across different plant and bacterial secretome studies; for example, in Arabidopsis (Smith *et al*., 2015), rice (Jung *et al*., 2008), chickpea (Gupta *et al*., 2011), and *Erwinia chrysanthemi* (centre rot; Kazemi-Pour *et al*., 2004). The above-mentioned studies agreed on the function of the proteins belonging to these GH families as associated with modifying and restructuring the cell wall during cell expansion and differentiation (Minic *et al*., 2007).

Although almost all identified proteins had some predicted conserved domain and/or family names to help predicting the function of the protein, five proteins had no predicted cellular component, biological process, molecular function, as well as domain and family. Three of these proteins (proteins no. 20, 81, and 96) have a signal peptide, which tells us that they are classically secreted proteins. However, this fact does not bring us any closer to predicting their functions. This is also true for the proteins that do not have predicted signal peptides (proteins no. 80 and 116). These five proteins and other

uncharacterised proteins in Table 5.1 have not been experimentally identified and verified and thus more studies such as gene knock-outs would need to be conducted to further characterise sorghum secreted proteins.

These results show that although the sorghum genome has been fully sequenced (Paterson *et al*., 2009), more studies still need to be done to annotate the expressed genes and proteins. Sorghum has intriguing genetics contributing to its tolerance towards drought. We should therefore take advantage of the newly developed molecular technologies and pay more attention in characterising these proteins. It is for this reason that this sorghum secretome map will be a foundation for further studies on characterising sorghum secreted proteins in response to drought (Chapter 6).

CHAPTER 6

PROTEOMIC IDENTIFICATION OF OSMOTIC STRESS RESPONSIVE SECRETED PROTEINS FROM WHITE SORGHUM SUSPENSION CULTURES

6.1. Introduction

Drought, also referred to as osmotic stress, adversely affects plant growth and development, and ultimately crop productivity. Africa is a relatively dry and hot continent mostly consisting of drylands and deserts, making it more prone to drought (Gan *et al*., 2016). According to the South African weather service reports (https://www.weathersa.co.za), the South African agricultural sector experienced massive crop losses of up to 100% in the 2015/2016 growing season due to drought. With the projected increase in global surface temperatures, the frequency and intensity of drought is projected to increase, worldwide. It is therefore important to develop crops that can withstand drought and thus alleviate food insecurity. However, the success of such breeding initiatives requires prior understanding of plant stress response mechanisms.

Sorghum, a naturally drought tolerant crop (Rosenow *et al*., 1983) is a potentially good model system for studying plant responses to drought stress (Ngara and Ndimba, 2014). However, for years, sorghum research has mainly focused on genomics and transcriptomics studies of this crop in responses to drought (Sanchez *et al*., 2002; Johnson *et al*., 2014). Although these studies have increased our understanding of sorghum's responses to drought stress, they only provide a global overview of response networks in plants. This is because the levels of genes and mRNA do not always correlate with that of proteins due to several post transcriptional and translational modifications that occur (Gygi *et al*., 1999). Since proteins drive many cellular functions

in living organisms, sorghum proteomics studies are thus important in understanding this crop's molecular responses to drought stress.

As discussed in sections 1.7 and 5.1, secreted proteins play important roles in plant growth and developmental processes as well as in response to internal and external stimuli. To date, only one study by Ngara (2009), reported on sorghum secretome analysis in response to drought stress. In that study, a combination of 2D gel electrophoresis and MALDI-TOF MS were used to separate and identify osmotic stress-responsive secreted proteins from the White sorghum suspension cultures. One protein, a putative wall-associated protein kinase, was differentially expressed in response to osmotic and salinity stress (Ngara, 2009). However, the secretome of other grasses such as rice in response to dehydration stress has been studied (Pandey *et al*., 2010). In that study, responsive proteins had functions in carbohydrate metabolism, cell defence and rescue, cell signalling processes, and cell wall modification, thus highlighting the affected metabolic functions under drought.

The current study explored sorghum secretome responses towards osmotic stress using isobaric tags for relative and absolute quantitation (iTRAQ). This highly sensitive liquid chromatography based technique uses different stable isotope tags to label one or more protein samples for simultaneous identification and quantification (Noirel *et al*., 2011). Recently, iTRAQ was used to identify and quantify *Arabidopsis* cell suspension culture secreted proteins following treatment with salicylic acid and exogenous ATP (Smith *et al*., 2015).

The objective of this chapter was to identify, quantify, and functionally classify the White sorghum suspension culture secreted proteins responding to sorbitol-induced osmotic stress.

6.2. iTRAQ Analysis of the White Sorghum Osmotic Stress Responsive Secreted Proteins

White sorghum cell suspension cultures were established and maintained in culture as described in section 2.2.5. At day 8 post-subculturing, the cell suspensions were treated with 400 mM sorbitol for 48 hr to induce osmotic stress as described in section 2.4.1. Secreted protein samples were processed for iTRAQ analysis as described in section 2.7.1. Due to the prohibitive costs of the iTRAQ reagents kits, we opted to only analyse the secretome at 48 hr and not at both time points. The four control replicates were labelled with tags of molecular weights 113, 114, 115, and 116, while sorbitol-treated replicate samples were labelled with 117, 118, 119, and 121 molecular weight tags. Quantitative analysis of the differentially expressed proteins was calculated as shown in Equations 3 and 4, with positive ratios (fold change values) denoting up-regulation, while negative values denote down regulation. The protein quantitation summary data obtained from the ProteinPilot is listed in Appendix 5.

Out of the 178 positively identified White sorghum secreted proteins (Table 5.1), 152 proteins were differentially expressed in response to osmotic stress as listed in Table 6.1. Of these 152 differentially expressed proteins, 148 (97%) were up-regulated, while 4 (3%) were down-regulated following stress treatment. Fold changes ranged from 1.21 (protein no. 56) to 38.7 (protein no. 155) and -1.42 (protein no. 175) to -2.40 (protein no. 92) for the up- and down-regulated proteins, respectively.

Table 6.1. List of the White sorghum secreted proteins that are differentially expressed in response to osmotic stress imposed by sorbitol.

Prot No.[a]	Accession[b]	Protein name[c]	Ratio[d]	SD[e]	p-value[f]
Metabolism					
5	C5XYP5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G233700	1,66	0,11	4,74E-03
6	A0A1B6QHZ6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089000	2,93	0,12	4,85E-06
8	C5X532	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G123100	2,05	0,05	7,96E-04
9	C5XQV7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G085900	1,46	0,07	1,30E-02
12	C5WXC7	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_001G208100	1,75	0,03	4,09E-03
15	C5XRC3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G374100	1,84	0,19	2,37E-03
22	A0A1B6QI05	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089100	2,19	0,08	1,79E-04
27	C5XKE9	Endoglucanase OS=Sorghum bicolor GN=SORBI_003G015700	2,84	0,35	2,25E-05
28	C5Y397	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_005G132400	4,24	0,28	1,10E-06
29	C5X8J4	Xyloglucan endotransglucosylase/hydrolase OS=Sorghum bicolor GN=SORBI_002G302000	3,58	0,24	1,42E-06
30	C5WNY4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G129700	2,07	0,18	1,57E-04
31	C5YC92	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G018100	2,18	0,30	3,17E-04
34	C5Y1P1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G098700	1,96	0,15	4,55E-04
36	C5XB38	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055600	2,33	0,16	7,22E-05
37	A0A1B6QIM7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G123300	1,68	0,07	3,13E-03
40	C5XWE5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G197600	2,52	0,16	3,59E-05
45	C5XIH4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G430100	1,55	0,15	9,16E-03
50	C5XX83	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G208700	1,56	0,11	7,48E-03
57	C5Y8Y2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G235600	1,52	0,12	1,67E-02
62	C5WZU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G516000	1,95	0,20	3,85E-05
66	C5XT36	Endoglucanase OS=Sorghum bicolor GN=Sb24P17cg_130	1,66	0,11	4,81E-04
67	C5Y587	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G049800	5,06	0,47	1,20E-06
71	C5WN99	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G261600	1,32	0,11	3,05E-02
72	C5X022	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G525000	2,00	0,11	1,51E-04
73	C5YI15	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G066500	1,95	0,13	1,69E-04
78	C5Y1M2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G097400	1,61	0,32	1,09E-02
82	A0A1B6QC86	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G189100	2,07	0,16	6,77E-04
84	C5XFX7	Uncharacterized protein OS=Sorghum bicolor	2,29	0,20	5,08E-05

		GN=SORBI_003G247000			
86	A0A1B6PTQ9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G204700	2,07	0,48	5,04E-03
88	C5XB39	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055700	2,35	0,11	1,15E-05
95	C5X5L7	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G417800	4,06	0,21	1,34E-06
99	C5XL56	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G023800	1,57	0,14	2,62E-03
101	C5X578	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G128000	1,52	0,10	9,00E-04
107	C5YUD2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G072000	1,49	0,25	7,69E-03
108	A0A1B6QIX5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G142200	1,47	0,09	3,67E-03
114	C5XQJ2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G364300	1,93	0,25	1,56E-03
115	C5XQW7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G087300	2,29	0,12	8,67E-05
119	C5Y8K8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G228900	-1,86	0,05	6,98E-03
124	A0A1B6QN59	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G366800	1,87	0,08	8,50E-04
125	C5XHX2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G427700	1,62	0,14	4,35E-03
133	C5WP48	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_001G268700	3,05	0,31	3,92E-04
135	A0A1B6PLT3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132100	1,51	0,21	1,62E-02
140	C5YCY4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G160700	2,42	0,42	2,17E-04
141	A0A1B6Q8G8	Uncharacterized protein (Fragment) OS=Sorghum bicolor GN=SORBI_003G440900	2,92	0,11	4,18E-05
145	C5YBF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132700	2,79	0,29	2,59E-05
160	A0A1B6Q537	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G244600	1,52	0,28	1,76E-02
162	C5X4N0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G255600	1,84	0,21	3,07E-04
165	A0A1B6PLT5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G133000	2,14	0,38	5,89E-04
167	C5XRU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G148100	3,01	0,42	8,74E-05
175	A0A194YLI0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G261000	-1,42	0,01	1,87E-02
181	A0A1B6PJF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G014400	2,20	0,32	8,41E-04
Energy					
19	A0A194YMM6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G262500	7,94	0,27	1,04E-08
49	A0A194YGY2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G027000	5,90	0,35	1,21E-07
61	C5YK12	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G100600	1,69	0,13	4,25E-04
70	C5XX52	Glyceraldehyde-3-phosphate dehydrogenase OS=Sorghum bicolor GN=SORBI_004G205100	4,49	0,29	5,19E-07
103	C5YW21	Malate dehydrogenase OS=Sorghum bicolor GN=SORBI_009G240700	5,43	0,89	2,77E-05
123	C5XFH6	Fructose-bisphosphate aldolase OS=Sorghum bicolor GN=SORBI_003G393900	4,01	0,53	2,75E-05
129	C5X6P7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G140400	2,34	0,14	1,53E-05
137	C5X780	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G007200	2,70	0,12	9,70E-06

151	C5XC95	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G345800	2,76	0,18	1,45E-05
152	C5XQ07	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G072300	5,32	1,29	1,52E-04
Cell growth/division					
2	C5WSF9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301500	3,18	0,19	3,58E-06
17	C5WSF0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300800	3,23	0,41	3,53E-05
33	C5Z0P5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G055900	2,95	0,23	3,77E-06
55	C5YVJ8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232200	1,54	0,05	7,35E-04
56	C5YJ56	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G214700	1,21	0,04	1,04E-02
59	C5WSE5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300400	3,14	0,23	7,20E-06
87	C5YVJ7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232100	2,36	0,11	1,38E-06
121	A0A1B6QAK5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G113800	2,97	0,29	3,91E-05
158	C5WSF3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301000	1,75	0,33	5,33E-03
Signal transduction					
43	A0A1B6PD28	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G113000	3,08	0,18	7,65E-06
46	C5X1U2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G390300	1,43	0,10	4,18E-03
47	C5XPK9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G205600	2,69	0,09	8,53E-07
53	C5Z6U1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G209900	2,96	0,16	5,80E-06
63	C5XBP7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G343600	1,34	0,03	2,08E-03
74	A0A1B6Q9B0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G050900	1,46	0,13	2,84E-02
117	C5Y1P6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G099500	2,44	0,15	3,59E-05
139	C5YRS3	Purple acid phosphatase OS=Sorghum bicolor GN=SORBI_008G037000	3,69	0,34	5,42E-06
147	C5Z998	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G255000	1,74	0,59	4,30E-02
149	C5Y7T1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G086000	1,78	0,21	1,03E-02
166	C5WRL4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G442200	1,75	0,31	2,12E-03
170	C5WX83	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G058300	1,57	0,12	3,61E-03
Intracellular traffic					
42	C5XHF1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G136200	1,99	0,16	2,65E-04
132	C5X9N2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G039000	4,15	0,38	4,03E-06
Protein destination & storage					
16	C5Y8G7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G224500	1,35	0,09	4,18E-02
21	C5Z6U2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G210000	2,82	0,36	5,01E-05
26	C5XQ74	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G208800	2,05	0,10	4,70E-04
48	A0A1B6PNM7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G242000	3,27	0,13	7,96E-07
58	C5XHP8	Uncharacterized protein OS=Sorghum bicolor	1,36	0,14	1,19E-02

		GN=SORBI_003G419400			
60	C5XHP7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419300	1,86	0,16	3,36E-04
85	C5WT64	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G170700	2,05	0,18	4,15E-04
91	C5Y675	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G064200	1,57	0,09	4,92E-03
94	C5WXN2	Carboxypeptidase OS=Sorghum bicolor GN=SORBI_001G348800	2,10	0,14	2,50E-04
98	C5YNA1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G172100	3,73	0,25	5,00E-06
109	A0A1B6PAU7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G234800	1,51	0,27	1,81E-02
112	C5XCL8	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G070200	1,97	0,19	1,11E-04
122	A0A1B6PHE0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G120800	3,13	0,41	8,07E-05
136	C5WQK1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G280000	2,77	0,28	3,61E-05
138	A0A1B6QEG2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G315800	4,42	0,25	3,79E-07
148	C5WT45	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G168500	3,55	0,33	6,16E-06
168	C5XG88	Small ubiquitin-related modifier OS=Sorghum bicolor GN=SORBI_003G402600	6,26	1,94	6,02E-04
173	C5XDR4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G217200	2,87	0,25	8,52E-06
178	C5Y171	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G142800	5,62	0,52	1,25E-06
Secondary Metabolism					
79	C5X502	Dirigent protein OS=Sorghum bicolor GN=SORBI_002G119900	3,01	0,31	2,02E-05
130	C5XTG0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G166500	6,10	0,45	5,54E-07
Cell structure					
39	C5WVG9	Cysteine proteinase inhibitor OS=Sorghum bicolor GN=SORBI_001G324800	1,95	0,16	2,29E-04
106	C5WT90	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G173300	2,48	0,28	2,43E-04
118	C5X3C7	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391900	-1,93	0,04	3,66E-03
150	C5X3W3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G246400	2,30	0,42	1,16E-03
Disease/Defence					
4	C5X5K6	Peroxidase OS=Sorghum bicolor GN=SORBI_002G416700	1,55	0,19	4,35E-03
7	A0A1B6QG95	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416600	2,08	0,03	6,75E-05
10	C5XI24	Peroxidase OS=Sorghum bicolor GN=SORBI_003G140700	1,36	0,09	3,67E-02
13	C5Y360	Peroxidase OS=Sorghum bicolor GN=SORBI_005G011300	2,73	0,31	4,80E-05
14	A0A1B6PLA9	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G104300	2,25	0,09	2,28E-05
23	C5Z240	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G003100	2,40	0,19	3,69E-05
25	C5X3C1	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391300	1,34	0,06	2,65E-02
32	C5Z864	Peroxidase OS=Sorghum bicolor GN=SORBI_010G232500	1,41	0,02	1,50E-02
35	C5XIY1	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152100	2,98	0,14	5,62E-06
38	A0A1B6QN00	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G360500	2,16	0,41	1,46E-03

41	C6JSB7	Peroxidase OS=Sorghum bicolor GN=Sb0246s002010	7,79	1,84	4,99E-05
44	C5WRN5	Peroxidase OS=Sorghum bicolor GN=SORBI_001G444500	1,73	0,06	3,15E-04
51	A0A1B6QGB6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416800	2,21	0,07	1,86E-05
52	C5XIK1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G432700	1,62	0,08	5,62E-03
68	C5YBH7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G135500	2,07	0,07	1,38E-04
69	A0A1B6Q9F4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G057900	5,63	0,26	6,39E-08
75	C5Y5K2	Peroxidase OS=Sorghum bicolor GN=SORBI_005G051500	1,53	0,15	6,09E-03
92	C5XL59	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G024700	-2,40	0,04	7,37E-04
97	C5XIY0	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152000	2,58	0,11	9,88E-06
104	A0A194YU12	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G341200	6,45	0,29	2,34E-08
110	A0A1B6QN96	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G371900	13,59	1,99	1,14E-05
111	A0A1B6Q818	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G416300	5,84	0,45	3,19E-07
120	C5YSB1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G048400	2,55	0,21	2,60E-05
126	C5Z475	Peroxidase OS=Sorghum bicolor GN=SORBI_010G162000	1,52	0,16	3,98E-03
128	C5YZJ2	Peroxidase OS=Sorghum bicolor GN=SORBI_009G033400	1,61	0,12	2,32E-03
131	C5WWQ2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G342600	8,47	2,43	6,43E-05
134	C5YQ75	Peroxidase OS=Sorghum bicolor GN=SORBI_008G010500	2,85	0,13	2,98E-06
143	C5WPY8	Peroxidase OS=Sorghum bicolor GN=SORBI_001G277000	1,90	0,11	5,44E-04
154	A0A1B6QFT1	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G392000	1,66	0,02	4,68E-04
155	A0A1B6QFT7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G392300	38,70	5,94	6,01E-06
159	A0A1B6P9F6	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G190800	4,28	0,35	1,95E-06
161	C5Z0N9	Peroxidase OS=Sorghum bicolor GN=SORBI_009G055300	2,76	0,11	9,30E-06
169	A0A1B6QJR7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G189000	2,10	0,39	2,17E-03
174	C5YN91	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G171000	3,42	2,19	8,12E-03
179	A0A1B6QB11	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G133800	5,62	0,92	1,42E-05
Unclear classification					
90	A0A1B6QEI0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G317600	4,45	0,12	9,13E-08
113	C5XYB4	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G229300	2,28	0,14	3,92E-05
157	C5WPH2	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G130400	1,94	0,37	2,85E-03
Unclassified					
20	A0A1B6QMT3	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G348900	3,09	0,24	6,02E-06
76	C5WXD7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G209300	2,67	0,13	2,62E-05
96	A0A1B6Q6M7	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G327700	1,62	0,07	3,01E-04

116	C5WQH5	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G149500	2,79	0,48	2,33E-04
153	C5Y2P0	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G122300	1,54	0,19	6,42E-03

[a] Protein number assigned in ProteinPilot software.

[b] Proteins accession obtained from the TrEMBL database [incorporated within the UniProt database (http://www.uniprot.org)] searches against sequences of *Sorghum bicolor* only.

[c] Proteins categorised based on functional categories according to Bevan *et al*. (1998). Percentage coverage, number of sequenced peptides, GO annotation, conserved domains and family names data are presented in Table 5.1.

[d] Ratio represents the average fold-change (n = 3) induced by treatment relative to control. Negative values indicate a down-regulation.

[e] Standard deviation

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

6.3. Functional Categories of the Differentially Expressed Secreted Proteins

The 152 differentially expressed proteins in response to osmotic stress were grouped into 11 functional categories according to Bevan *et al*. (1998), and results are shown in Table 6.1 and Figure 6.1. Due to the large number of uncharacterised proteins identified in the sorghum secretome map (Table 5.1) and the osmotic stress responsive proteins (Table 6.1), functional classification was conducted using data from conserved domains/protein families (Table 5.1), sequence similarity data on Uniprot as well as literature sources.

The most highly represented group of differentially expressed identified proteins is involved in metabolism (33.5%), followed by disease/defence (23%) and protein destination and storage (13%). Groups of proteins involved in signal transduction (8%); energy (6.5%); cell growth/division (6%); cell structure (3%); unclassified proteins (3%); unclear classification (2%); intracellular traffic (1%); and secondary metabolism (1%) had relatively lower numbers of proteins that were responsive to osmotic stress (Figure 6.1). A high percentage of differentially expressed proteins, functioning in metabolic

processes has also been observed in chickpea suspension culture secreted proteins in response to dehydration (Gupta *et al*., 2015). A brief description of some of the identities of the responsive proteins per category and their respective fold changes is given below.

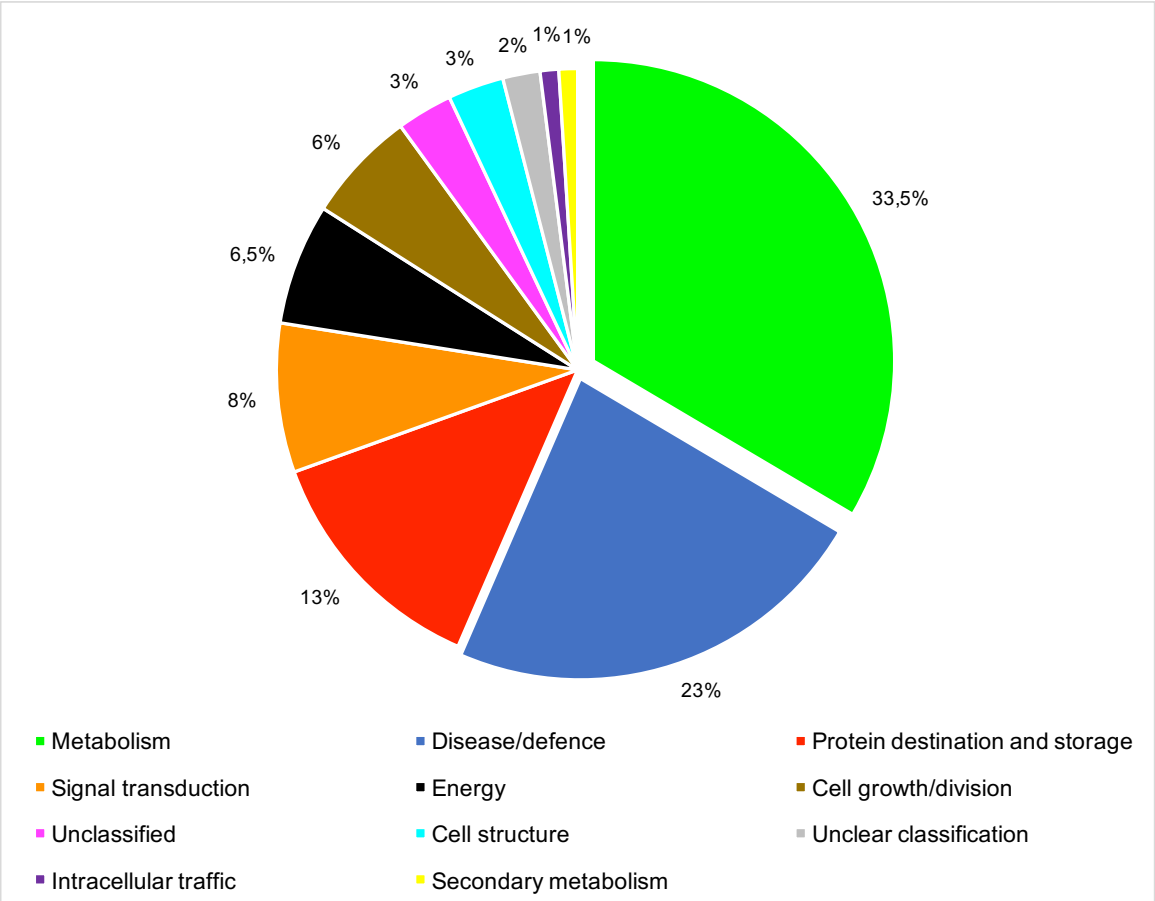

Figure 6.1. Functional characterisation of differentially expressed sorghum osmotic stress responsive secreted proteins.

6.3.1. Metabolism

Of the 152 differentially expressed sorghum secreted proteins (Appendix 5), a total of 51 (33.5%) proteins with putative functions in primary metabolism were responsive to osmotic stress (Table 6.1; Figure 6.1). Of these, a total of 16 proteins, namely, alpha-galactosidases (proteins no. 8, 12, and 95), endoglucanases (proteins no. 27 and 66), alpha-mannosidases (proteins no. 28 and 133), xyloglucan endotransglucosylase/hydrolase (protein no. 29) and uncharacterised proteins (proteins no. 5, 9, 57, 71, 72, 84, 119, 140 and 162), were involved in carbohydrate metabolism.

Table 6.1 shows an increase in abundance of alpha-galactosidases and eight uncharacterised proteins, except the uncharacterised protein number 119, which showed a decrease in abundance of -1.86. Alpha-galactosidases catalyse the hydrolysis of galactosyl residues from galacto-oligosaccharides and galacto-polysaccharides, which forms part in plant cell wall modifications (Fujimoto *et al*., 2003). Alpha-mannosidases (proteins no. 28 and 133) were significantly up-regulated in response to osmotic stress, with a fold change of 4.24 and 3.05, respectively (Table 6.1). Similar increase in abundance of this protein was observed in barley root and leaf proteomes in response to salt stress (Wu., Shen *et al*., 2014). Alpha-mannosidases catalyse the hydrolysis of terminal mannose from the N-glycan complex, which is important for the growth and development of plants (Strasser, 2015).

Other uncharacterised proteins in this functional group are involved in oxalate (proteins no. 30, 31, 73, 125, and 167), amino sugar (proteins no. 50, 62, 145), and glucose (protein no. 70) metabolic processes, whereas 16 uncharacterised proteins (proteins no. 6, 19, 22, 37, 82, 86, 99, 101, 108, 124, 135, 141, 160, 165, 175, and 181) did not have any predicted molecular functions and biological processes (Table 5.1). However, some of these uncharacterised proteins belong to the glycosyl hydrolase families, suggesting that they possibly function in cell wall modifications. Uncharacterised protein number 67

with an alginate lyase 2 domain and no predicted family had the highest fold change of 5.06 amongst all the other proteins in this functional group (Tables 5.1 and 6.1). According to the InterPro database (http://www.ebi.ac.uk/interpro/), alginate lyases catalyse the hydrolysis of the glycosidic linkage of the linear polysaccharide alignate through a beta-elimination, with proteins such as GH family 7, 16, and 32 listed as containing this domain in the database.

6.3.2. Disease/Defence

Figure 6.1 illustrates that 23% (35 proteins) of the 152 proteins that responded to osmotic stress have functions in disease and defence. This functional category mainly consists of proteins that matched the identity of peroxidases (proteins no. 4, 10, 13, 25, 32, 35, 41, 44, 75, 97, 126, 128, 134, 143, and 161; Table 6.1). All peroxidases had increased abundances in response to osmotic stress (Table 6.1), which is in agreement with results observed in potato (*Solanum tuberosum*) secretome following phosphite treatment (Burra *et al*., 2014) and in *A. thaliana* suspension culture secretome following nutritional phosphate deficiency (Tran and Plaxton, 2008). Furthermore, peroxidases were found to have increased abundance in soybean (*Glycine max*) root proteome under drought stress (Oh and Komatsu, 2015) and differentially expressed in shoot proteome of sensitive barley genotype under drought stress (Kausar *et al*., 2013). Peroxidases are part of multigene families that play important roles in the growth and development of plants, as well as in response to different biotic and abiotic stress factors. The functions of peroxidases are diverse and include lignification, suberisation, auxin catabolism, wound/healing, and defence against pathogens and oxidative stress (Hiraga *et al*., 2001).

Several other proteins in this functional category did not match any protein identities, possibly suggesting their novelty in stress adaptation. Most of these uncharacterised proteins, which had increased abundances (proteins no. 7, 23, 38, 51, 92, 131, 154, 155,

169, and 174) are involved in cell redox homeostasis and belong to the plant peroxidases family (Tables 5.1 and 6.1). One uncharacterised protein (protein no. 92), belonging to the plant peroxidase family had a decrease in abundance following osmotic stress (Tables 5.1 and 6.1). Although this is possibly a novel protein, similar decrease in abundance of a plant peroxidase family protein (peroxidase; accession number At5g64120) was observed in *A. thaliana* suspension culture secretome in response to phosphate deficiency (Tran and Plaxton, 2008). Two uncharacterised proteins, protein number 110 (member of the copper/zinc superoxide dismutase-like family) and 155 (member of the plant peroxidase family), had the highest fold changes of 13.59 and 38.70, respectively, when compared to other proteins involved in disease/defence (Table 6.1). Proteins in the two above-mentioned families function in restoring cell redox homeostasis by scavenging ROS (Hiraga *et al*., 2001).

6.3.3. Protein Destination and Storage

A total of 19 (13%) proteins were categorised to function in protein destination and storage as illustrated in Table 6.1 and Figure 6.1. All these proteins responded to osmotic stress with increased abundances (Table 6.1). However, a small ubiquitin-related modifier (protein no. 168) has the highest increase in abundance, with a fold change of 6.28 (Table 6.1). As discussed in section 5.4, this protein is involved in protein modifications that may direct a specific protein to a particular subcellular location (Hilgarth *et al*., 2004). A carboxypeptidase (protein no. 94) together with other uncharacterised proteins have similar proteolytic functions and belong to different peptidase families (Table 5.1). Peptidase family proteins are known to function in the removal of signal peptides from secretory proteins, protein shedding and turnover (Rawlings, 2013), and have previously been identified in *A. thaliana* suspension culture secretome subjected to phosphate deficiency (Tran and Plaxton, 2008). An increase in abundance of proteases was also reported in a 2-day drought treated soybean root proteome (Oh and Komatsu, 2015).

6.3.4. Signal Transduction

Figure 6.1 shows that 8% (12 proteins) of the differentially expressed proteins were involved in signal transduction. A purple acid phosphatase (protein no. 139) had a fold change of 3.69, higher than that of the other proteins (proteins no. 43, 46, 47, 53, 63, 74, 117, 147, 149, 166, and 170; Table 6.1), which all had increased abundances. Eleven out of 12 proteins did not match any protein identity, but some belong to purple acid phosphatase (proteins no. 43 and 166) and nucleoside phosphatase GDA1/CD39 (proteins no. 117 and 149) protein families (Table 5.1), while others did not have predicted families but had predicted conserved domains such as the EF-hand (protein 46), phospholipase D-like (protein no. 53), and LRR (proteins no. 47, 63, 74, 147, and 170) domains (Table 5.1).

Purple acid phosphatases are metalloenzymes that catalyse the hydrolysis of activated phosphoric acid esters and anhydrides such as ATP, under acidic conditions (Klabunde *et al*., 1996). Purple acid phosphatase isoforms were identified in *A. thaliana* secretome following phosphate deficiency (Tran and Plaxton, 2008). According to the InterPro protein domain and families predicting database (http://ebi.ac.uk/interpro/), proteins belonging to the nucleoside phosphatase GDA/CD39 family also have functions in ATP hydrolysis. Although this family has hardly been identified in secretome studies, it has been documented in potatoes (Handa and Guidotti, 1996). Leucine rich repeats play an important role in plant defence. They are involved in protein-protein interactions, which usually lead to the perception of a stress stimuli.

6.3.5. Energy

Energy is important for cells during defence as well as in normal growth processes. A total of 10 (6.5%) proteins were identified in this functional category, all with increased abundance (Table 6.1). Seven uncharacterised proteins were annotated to be members of the enolase (protein no. 49), glceraldehyde-3-phosphate dehydrogenase (GAPDH;

protein no. 19) and triosephosphate isomerase (protein no. 152) families and some having photocyanin (protein no. 61) and cupredoxin (proteins no. 129, 137, and 151) domains (Table 5.1). Amongst the uncharacterised proteins, proteins number 19, 49, together with malate dehydrogenase protein (protein no. 103) have significantly higher abundances of 7. 94, 5.90, and 5.43, respectively, compared to other proteins in this group (Table 6.1). Fructose bisphosphate aldolase (protein no. 123) and GAPDH (protein no. 19) are known glycolytic proteins and their increase in abundance has been observed in potato leaf proteome following treatment with different priming agents of plant resistance (Arasimowicz-Jelonek *et al*., 2013). Although these are glycolytic proteins, such proteins are believed to have other functions such as those related to plant defence in different subcellular locations (Muñoz-Bertomeu *et al*., 2010).

6.3.6. Cell Growth/Division

All 9 (6%) differentially expressed proteins involved in cell growth and division had increased abundance and could possibly be novel, since they did not match any protein identity (Table 6.1). Fold changes of proteins in this functional category ranged from 1.21 (protein no. 56) to 3.23 (protein no. 17). Four proteins (proteins no. 2, 17, 59, and 158) are members of the expansin/Lol pI family, while another four proteins (proteins no. 33, 55, 56, and 87) are members of the fasciclin family, and lastly, a protein (protein no. 121) from the spermine synthase family. Expansins are a large superfamily of proteins involved in key regulation of cell wall extension throughout a plant's growth and developmental stages (Cosgrove, 2005), and were found to be differentially expressed in rice suspension culture secreted proteins in response to different elicitors (Kim *et al*., 2009). A similar increase in abundance of ECM proteins from the fasciclin family has previously been observed in potato leaf secreted proteins responding to phosphite treatment (Burra *et al*., 2014).

6.3.7. Other Functional Groups

This group consists of functional categories that had relatively low number of differentially expressed proteins, namely, cell structure (4 proteins), secondary metabolism (2 proteins), intracellular traffic (2 proteins), unclear classification (3 proteins), and unclassified (5 proteins; Table 6.1). Of these proteins, one peroxidase (protein no. 118) functioning in cell structure had decreased abundance (Table 6.1). Peroxidases have multiple functions as discussed in section 5.4. All the other proteins in the different functional categories had increased abundances (Table 6.1).

Cysteine proteinase inhibitor (protein no. 39) involved in cell structure was amongst proteins with increased abundance as a result of osmotic stress (Table 6.1). This protein is listed amongst common dehydration responsive cell wall proteins in rice (Pandey *et al*., 2010). This protein degrades other proteins or peptides into smaller components (Martínez *et al*., 2012). Proteins that had significantly higher fold change in the above groups include the uncharacterised proteins number 90 (4.45 fold change), 130 (6.10 fold change), and 132 with fold change of 4.15 (Table 6.1). Five proteins under the unclassified category (proteins no. 20, 76, 96, 116 and 153) did not have any predicted functions, protein family or conserved domains (Table 6.1). These proteins could possibly be novel and need further characterisation.

6.4. Discussion

Plants continuously need to adapt to stress by activating cascades of molecular networks involved in stress perception, signal transduction and the expression of specific stress-related genes, proteins and metabolites (Figure 1.3; Vinocur and Altman, 2005). Groups of genes that are inducible by abiotic stresses, include those with direct involvement in protection against stress, such as those involved in the synthesis of osmoprotectants, detoxifying enzymes, and transporters. The second group include

genes that encode regulatory proteins such as transcription factors, protein kinases, and phosphatases (Vinocur and Altman, 2005).

In this study, the White sorghum cell cultures following a 48 hr exposure to 400 mM sorbitol-induced osmotic stress were used to investigate differential expression of the secretome using the iTRAQ method. A total of 152 White sorghum secreted proteins were identified as differentially expressed in response to osmotic stress (Appendix 5); making it the first study to provide an extensive sorghum secretome dataset in response to osmotic stress. The only other study, which reported sorghum secretome responses towards salt and osmotic stresses identified a single wall-associated protein kinase as being stress responsive (Ngara, 2009).

From a total of 152 differentially expressed secreted proteins, 148 (97%) and four (3%) were up-regulated and down-regulated, respectively; thus showing the effects of osmotic stress in the sorghum secretome. These results are in agreement with the results of the rice suspension culture secreted proteins in response to different elicitors (Kim *et al*., 2009). In addition, results the rice aerial tissue proteomes in response to dehydration (Pandey *et al*., 2010) showed that the proteins are involved in stress response. These observed changes in grass proteomes in response to different stimuli also indicate molecular changes that occur in plant cells under stress as discussed in section 1.3.

A large proportion (78%) of the iTRAQ identified White sorghum secreted proteins that responded to osmotic stress were uncharacterised, meaning that these proteins have not been verified experimentally. Therefore, the functions of these proteins remain speculative, until they are experimentally characterised (Ngara *et al*., 2012). As such, there is a high possibility that some of these proteins could be novel. Therefore, further characterisation of the structure and functions of these proteins through molecular cloning and activity assays is required. However, based on gene ontology classification,

and domain and protein family data of the identified proteins acquired from UniProt and InterPro databases, respectively, it was possible to infer some of the functions of these proteins (Table 5.1).

Differentially expressed proteins were functionally categorised according to Bevan *et al*. (1998), and this revealed that these proteins had functions in metabolism (33.5%), disease/defence (23%), protein destination and storage (13%), signal transduction (8%), energy (6.5%), cell growth/division (6%), cell structure (3%), intracellular traffic (1%), and secondary metabolism (1%; Table 6.1; Figure 6.1). Similar protein identities have been reported in sorghum leaf proteomes in response to drought (Jedmowski *et al*., 2014) and salt stress (Ngara *et al*., 2012). Proteins that did not have enough information in the two databases mentioned-above, were characterised as unclassified (3%) or unclear classification (1%; Table 6.1; Figure 6.1). In the current study, most of the proteins involved in primary metabolism were uncharacterised (Table 6.1), thus making it difficult to discuss their possible involvement in stress response. However, proteins identified in this group have hydrolytic functions of biomolecules such as polysaccharides, chitin, and phospholipids; which is consistent with some proteomics studies investigating stress responses (Benešová *et al*., 2012; Faghani *et al*., 2015).

Osmotic stress may result in membrane disorganization, protein activity loss, and/or denaturation, as well as the accumulation of ROS (Atkinson *et al*., 2015). Peroxidases dominated the disease/defence functional category (Table 6.1), with functions involving scavenging ROS molecules during oxidative stress (Table 5.1). It is expected that peroxidases are expressed with increased abundances in order to repair damage and prevent further oxidative damage when plants experience abiotic stress (Hiraga *et al*., 2001). Up-regulation of peroxidases has also been recorded in several secretome studies in response to a range of abiotic stresses such as in potato following phosphite treatment (Burra *et al*., 2014), barley under drought stress (Kausar *et al*., 2013), and

Arabidopsis following phosphate deficiency stress (Tran and Plaxton, 2008). There was one uncharacterised protein (Protein no. 92) belonging to the plant peroxidase family that was down-regulated in response to osmotic stress (Tables 5.1 and 6.1). Usually, when plants are experiencing oxidative stress, we do not expect peroxidases to be down-regulated. However, similar decrease in peroxidase levels have been observed in Arabidopsis secretome in response to phosphate deficiency (Tran and Plaxton, 2008).

The presence of proteins involved in protein destination and storage (Table 6.1; Figure 6.1) emphasises the movement of proteins and peptides between the intracellular and the ECM and also the role of secreted proteins in communication within and outside cells (Guerra-Guimareãs *et al*., 2016). It is important that plant cells communicate with the external environment for early perception of unfavourable conditions, subsequently resulting in early response and possibly survival of the plant. All positively identified proteins in this functional category were up-regulated, with small ubiquitin-related modifier (protein no. 168) having the highest fold change of 6.26 (Table 6.1). This intracellular protein, is responsible for protein modifications that may direct specific proteins in different subcellular localisation (Hilgarth *et al*., 2004). Other proteins in this category belong to peptidase families and have functions in removing signal peptides from secretory proteins (Rawlings, 2013), which is consistent with proteins identified in the extracellular compartment of cells.

Energy is required by the cells to perform different processes such as in signal transduction during defence as well as normal growth. In this study, proteins involved in energy production such as malate dehydrogenase (protein no. 103), fructose bisphosphate aldolase (protein no. 123), and several uncharacterised proteins were up-regulated proteins (Table 6.1). Although some glycolytic proteins (GAPDH and fructose bisphosphate aldolase) were identified in this study, it does not necessarily mean that they have roles in glycolysis in this study. This is because glycolysis is a known

intracellular pathway. However, some components of the glycolytic pathway are known to be secreted, and also enter the nucleus and assume different function from their primary metabolic roles. In potato proteome, they reported GAPDH binding to E3 ubiquitin ligase Siah1, which led to the nuclear translocation of these proteins, subsequently inducing cell death events (Arasimowicz-Jelonek *et al*., 2013). Except for the glycolytic proteins, it is highly expected that other secreted proteins involved in energy production are expressed in high abundances since more energy is required for plants to adapt to osmotic stress (Krasensky and Jonak, 2012).

Other proteins positively identified in this study include those involved in cell growth/division, cell structure, secondary metabolism, and intracellular traffic (Table 6.1; Figure 6.1). It was observed that most of the proteins that were differentially expressed in response to osmotic stress were uncharacterized, making it difficult to assign their functions in osmotic stress response. As such we recommend further characterization of these proteins, especially those that were greatly up-regulated (Table 6.1), since they might have important functions on how sorghum and other cereal crops respond and adapt to osmotic stress. The secretome analysis of this study revealed proteins involved in various mechanisms that may be enabling the White sorghum to be tolerant to osmotic stress.

Due to the highly sensitive nature of the iTRAQ technology in mapping and quantifying proteome changes even in small quantities, western blotting analysis was not conducted. It is possible that due to the compromised membrane integrity of the cells, there may be a free flow of cytosolic proteins in the ECM resulting in contamination of the secretome. However, the secretome results did not show any obvious collection of cytoplasmic leakage proteins. The leaderless proteins that were identified in this study have also been identified in other independent secretome studies (Jung *et al*., 2008; Gupta *et al*.,

2011), as discussed in Chapter 5. As such, these proteins might have dual locations and functions in cells (Drakakaki and Dandekar, 2013).

CHAPTER 7

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Sorghum (*Sorghum bicolor)* is a naturally drought tolerant crop with a completely sequenced genome, and thus a good experimental system for studying molecular responses to different abiotic and biotic stress factors. This study aimed to establish a sorghum cell suspension culture system, map its secretome and identify osmotic stress responsive extracellular proteins using proteomic tools. Of the seven sorghum genotypes used in the study, namely SA 1441, ICSV 210, ICSV 112, ICSV 213, ICSB 78, ICSB 338, and Macia, only ICSB 338, a drought susceptible variety readily produced friable callus masses for the establishment of cell suspension cultures. Further characterisation of cell cultures of ICSB 338 and White sorghum (used as a reference line in this study) using cell viability assays revealed that these two cell lines have different responses to osmotic stress.

Cell viability results showed that the ICSB 338 was more susceptible to osmotic stress compared to the White sorghum; and that the White sorghum cells recovered and/or adapted to osmotic stress more than the ICSB 338 cells. These results possibly indicated differences in stress response between the two cell cultures. Therefore, the ICSB 338 and White sorghum cell suspension cultures provide useful systems for use in comparative physiological, biochemical, and molecular studies in plants.

The proteomic analysis of the White sorghum secreted proteins using iTRAQ led to the identification of 178 proteins, constituting the first comprehensive sorghum secretome map, which will be an indispensable reference tool in plant secretome studies, worldwide. Out of the 178 positively identified sorghum-secreted proteins, 72% were predicted to have a signal peptide and thus localised in the extracellular matrix.

Although, the secretome map is now available, a large proportion (78%) of the 178 identified proteins were uncharacterised, meaning that they are still hypothetical proteins that have not been experimentally verified. As such, we recommend that more research be done to further characterise and elucidate the structure and function of these proteins as they could potentially be novel sorghum proteins with important functions in the physiology of plants.

When plants are faced with stressful conditions, cascades of molecular networks including genes, proteins, and metabolites, which assist in the perception of stress and response mechanisms toward the stress, are activated. These in turn determine whether or not the plant survives after experiencing the respective stress. The current study investigated the identities of the sorghum culture filtrate secreted proteins that responded to osmotic stress. Out of the 178 positively identified proteins, 152 responded to osmotic stress, with 148 proteins being up-regulated, while four were down-regulated. The osmotic stress-responsive proteins have functions in metabolism, disease/defence, protein destination and storage, signal transduction, energy, cell growth/division, cell structure, intracellular traffic, and secondary metabolism, while some could not be classified due to lack of protein information on different protein databases used. Overall, this study identified well-known osmotic stress responsive proteins and some novel, previously uncharacterised ones.

Some positively identified osmotic stress-responsive proteins were highly up-regulated. These include proteins number 19, 41 (peroxidase), 49, 67, 69, 103 (malate dehydrogenase), 104, 110, 111, 130, 131, 152, 155, 168 (small ubiquitin-related modifier), 178, and 179, which had fold changes ranging from 5.06 to 38.70. On the other hand, some positively identified proteins such as proteins number 92, 118 (peroxidase), 119, and 175 were down-regulated. As such, we recommend that these proteins be further characterised using molecular biology tools. For example, genes that

code for the above-mentioned proteins could be cloned and gene knockout experiments conducted for further characterisation of their roles in sorghum response to osmotic stress. Furthermore, other approaches such as metabolomics and systems biology could also be conducted on cell cultures to get a more comprehensive understanding of how sorghum responds to osmotic stress. The expression patterns on the above mentioned proteins and/or genes could also be evaluated in sorghum in response to other abiotic and/or biotic stress factors to understand their general roles in stress adaptation.

This Masters dissertation analysed the White sorghum proteome due to prohibitive costs of the iTRAQ reagents kits. However, further mapping and quantification of stress responsive secretome analysis of sorghum genotype ICSB 338 would need to be conducted in future studies. Furthermore, stress responsive genes of the White sorghum genotype will be further analysed using qPCR at the other time points, in order to evaluate temporal changes of these expressed genes during stress adaptive responses.

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APPENDICES

Appendix 1: One-Dimensional (1D) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Preparation.

Appendix 1-Table 1. Resolving and stacking gels preparation for 1D SDS-PAGE.

Reagents	**12% (v/v) Resolving gel**	**5% (v/v) Stacking gel**
Distilled water	4.3 mL	3.6 mL
40% (v/v) Acrylamide/ Bis solution	3 mL	0.625 mL
1.5 M Tris (pH 8.8)	2.5 mL	-
0.5 M Tris (pH 6.8)	-	0.63 mL
10% (w/v) SDS	0.1 mL	0.05 mL
10% (w/v) ammonium persulfate	0.1 mL	0.05 mL
N,N,N',N'-Tetramethyl-ethylenediamine	0.006 mL	0.005 mL
Total volume	**10 mL**	**5 mL**

Appendix 2: Percentage Germination and Contamination One-way ANOVA Tables

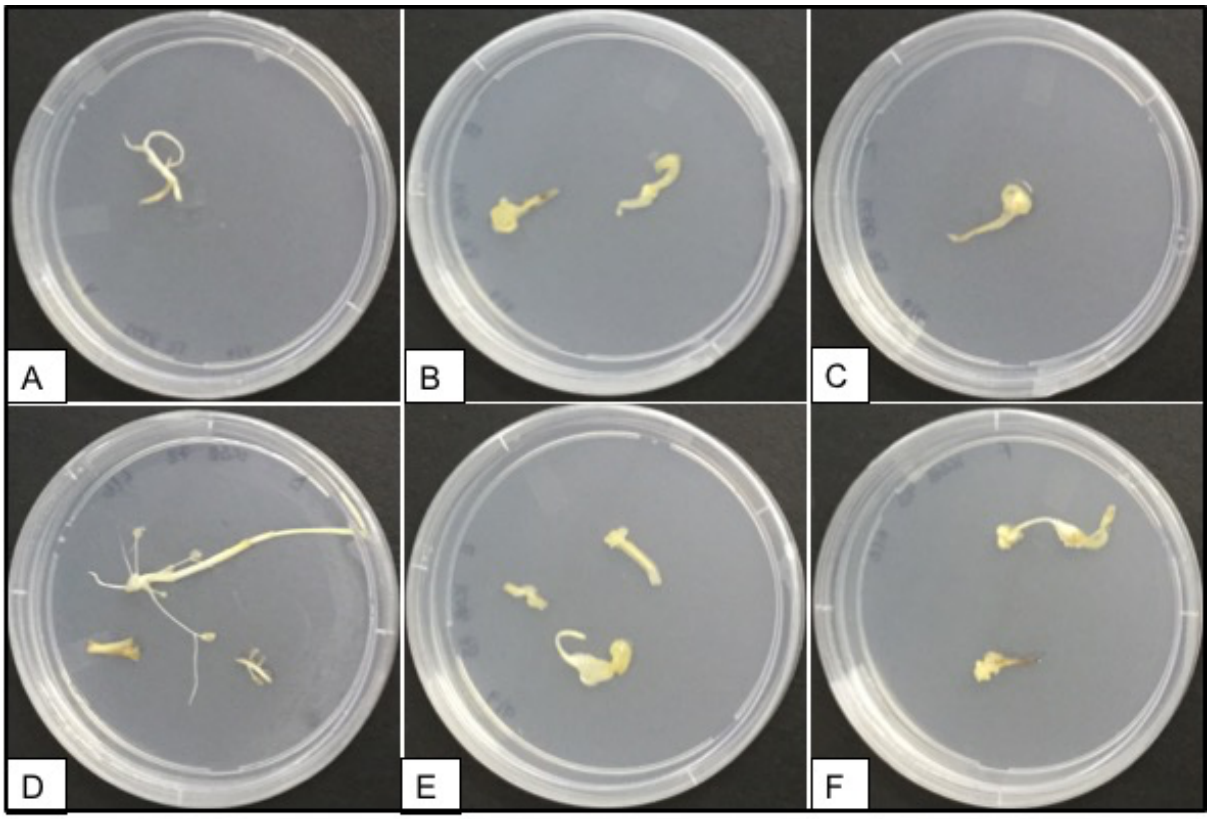
Appendix 2-Table 1. One-way ANOVA analysis of sorghum seeds percentage germination rates at 5% level of significance ($p < 0.05$).

Percentage Germination Rates					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Replicates	14	1.72857	0.12347	1.65	
Genotypes	6	2.48095	0.41349	5.51	<.001
Error	84	6.30476	0.07506		
Total	104	10.51429			

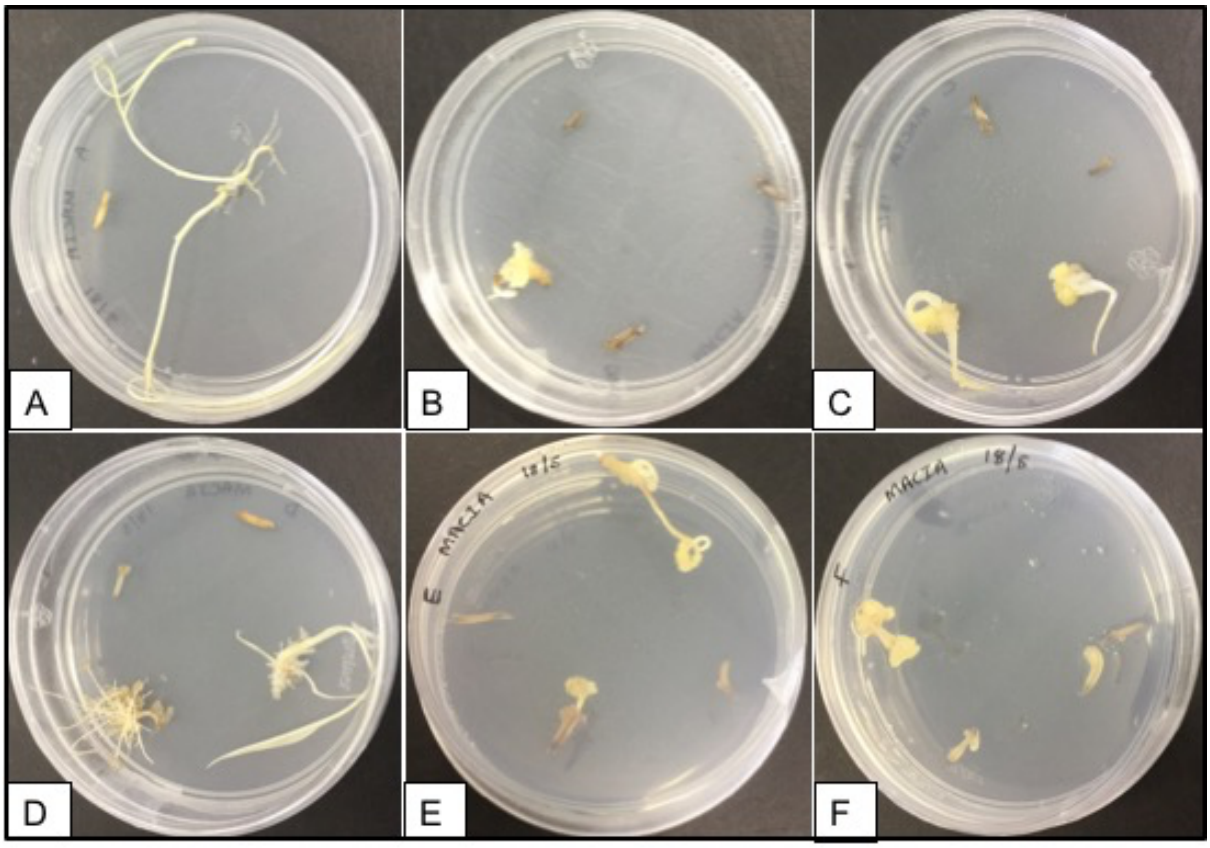
Appendix 2-Table 2. One-way ANOVA analysis of sorghum seeds percentage contamination rates at 5% level of significance ($p < 0.05$).

Percentage Contamination Rates					
Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Replicates	14	1.04762	0.07483	1.24	
Genotypes	6	0.73333	0.12222	2.03	0.070
Error	84	5.05238	0.06015		
Total	104	6.83333			

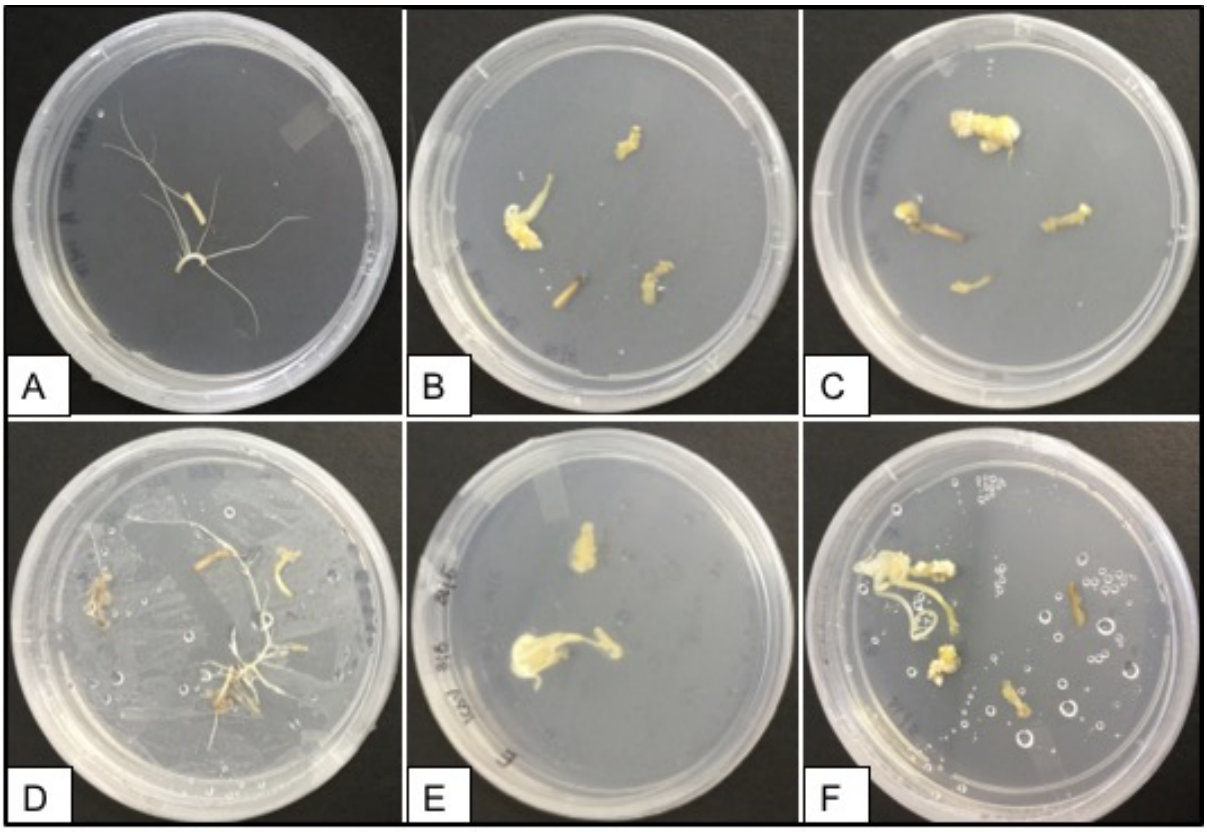
Appendix 3: Callus Induction Results



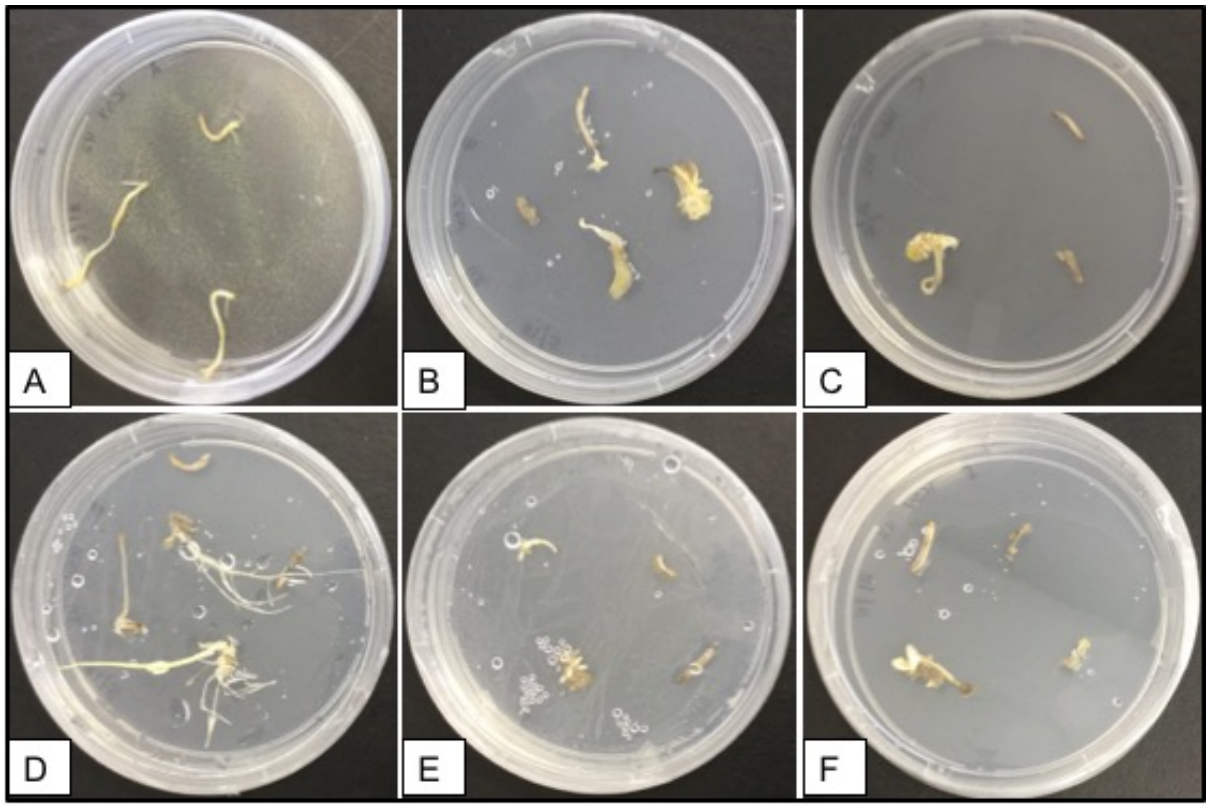
Appendix 3-Figure 1. Petri-dish plates containing sorghum genotype ICSB 73 shoot explants at 5-weeks post plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.



Appendix 3-Figure 2. Petri-dish plates containing sorghum genotype Macia shoot explants at 5-weeks post plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

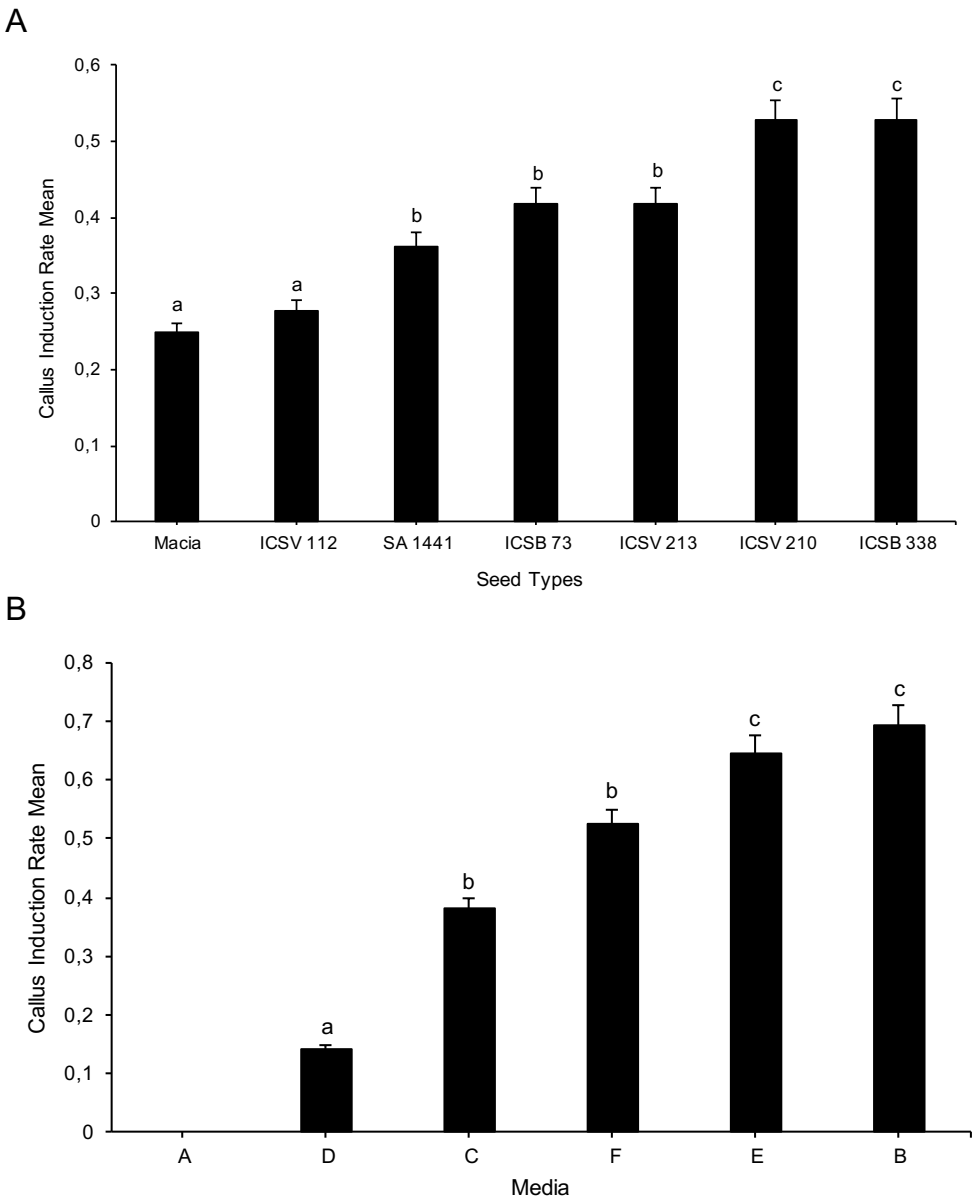


Appendix 3-Figure 3. Petri-dish plates containing sorghum genotype ICSV 213 shoot explants at 5-weeks post plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.



Appendix 3-Figure 4. Petri-dish plates containing sorghum genotype ICSV 112 shoot explants at 5-weeks post plating on different types of sorghum callus induction media. (A) represents control medium without any PGH; (B) Medium supplemented with 3 mg/L of 2,4-D; (C) Medium supplemented with 4 mg/L of 2,4-D; (D) Medium supplemented with 2.5 mg/L of NAA; (E) Medium supplemented 3 mg/L of 2,4-D and 2.5 mg/L of NAA and; (F) Medium supplemented with 4 mg/L of 2,4-D and 2.5 mg/L of NAA.

Appendix 4: The Influence of Different Genotypes and Media on Callus Induction



Appendix 4-Figure 1. The influence of different genotypes and media on callus induction based on their callus induction rate means. (A) shows genotypes plotted against their callus induction rate means, while (B) shows media plotted against their means. Means were generated using Two-way ANOVA at 5% level of significance ($p < 0.05$).

Appendix 5: The White Sorghum Culture Filtrate (CF) Secreted Proteins

Appendix 5-Table 1. List of the White sorghum culture filtrate secreted proteins.

Pro No.[a]	Scor[b]	% Cov[c]	Accession[d]	Name and Species	Seq Pep[e]	Ratios of control samples[f]				Mean[g]	SD[h]	Ratios of sorbitol-treated samples[i]				Mean[j]	SD[k]	Ratio[l]	p-value[m]
						113: 113	114: 113	115: 113	116: 113			117: 113	118: 113	119: 113	121: 113				
1	79,54	72,76	tr|C5Y1P4|C5Y1P4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G099000 PE=4 SV=1	133	1	1,04	1,26	1,74	1,26	0,34	1,18	1,12	1,43	1,14	1,21	0,15	0,96	0,82
2	65,76	66,31	tr|C5WSF9|C5WSF9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301500 PE=3 SV=1	89	1	1,00	1,52	1,39	1,23	0,27	3,80	3,81	3,82	4,20	3,91	0,19	3,18	3,58E-06
3	59,03	68,37	tr|C5WYQ4|C5WYQ4_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_001G360400 PE=3 SV=1	82	1	1,00	1,31	1,72	1,26	0,34	1,53	1,67	1,69	1,79	1,67	0,11	1,33	6,14E-02
4	45,12	68,37	tr|C5X5K6|C5X5K6_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_002G416700 PE=3 SV=1	78	1	0,98	1,27	1,43	1,17	0,22	1,68	1,69	1,81	2,09	1,82	0,19	1,55	4,35E-03
5	42,88	34,82	tr|C5XYP5|C5XYP5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G233700 PE=4 SV=1	32	1	1,04	1,41	1,82	1,32	0,38	2,07	2,21	2,33	2,16	2,19	0,11	1,66	4,74E-03
6	41,02	39,12	tr|A0A1B6QHZ6|A0A1B6QHZ6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089000 PE=4 SV=1	32	1	1,00	1,35	1,58	1,23	0,28	3,49	3,53	3,64	3,77	3,61	0,12	2,93	4,85E-06
7	40,80	72,16	tr|A0A1B6QG95|A0A1B6QG95_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416600 PE=4 SV=1	121	1	0,98	1,18	1,54	1,17	0,26	2,42	2,48	2,47	2,41	2,44	0,03	2,08	6,75E-05

8	40,07	45,28	tr|C5X532|C5X532_SORBI	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G123100 PE=3 SV=1	34	1	1,02	1,52	1,97	1,37	0,46	2,78	2,90	2,80	2,81	2,82	0,05	2,05	7,96E-03
9	37,52	24,35	tr|C5XQV7|C5XQV7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G085900 PE=3 SV=1	27	1	1,07	1,30	1,74	1,28	0,33	1,81	1,82	1,95	1,89	1,87	0,07	1,46	1,30E-02
10	37,11	53,01	tr|C5XI24|C5XI24_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_003G140700 PE=3 SV=1	38	1	0,98	1,21	1,66	1,21	0,31	1,69	1,71	1,52	1,67	1,65	0,09	1,36	3,67E-02
11	36,45	40,79	tr|A0A194YQ33|A0A194YQ33_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G166700 PE=4 SV=1	26	1	1,03	1,36	1,98	1,34	0,46	1,58	1,65	1,75	1,70	1,67	0,07	1,24	0,21
12	35,39	55,87	tr|C5WXC7|C5WXC7_SORBI	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_001G208100 PE=3 SV=1	37	1	1,10	1,41	2,02	1,38	0,46	2,44	2,41	2,45	2,39	2,42	0,03	1,75	4,09E-03
13	34,60	57,01	tr|C5Y360|C5Y360_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_005G011300 PE=3 SV=1	73	1	0,93	1,32	1,41	1,16	0,23	2,97	3,20	2,94	3,62	3,18	0,31	2,73	4,80E-05
14	32,62	42,64	tr|A0A1B6PLA9|A0A1B6PLA9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G104300 PE=4 SV=1	21	1	1,08	1,33	1,54	1,24	0,25	2,72	2,86	2,67	2,84	2,77	0,09	2,25	2,28E-05
15	31,52	24,08	tr|C5XRC3|C5XRC3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G374100 PE=3 SV=1	46	1	1,01	1,30	1,82	1,28	0,39	2,21	2,38	2,63	2,24	2,36	0,19	1,84	2,30E-03
16	31,05	45,68	tr|C5Y8G7|C5Y8G7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G224500 PE=3 SV=1	38	1	0,98	1,16	1,64	1,20	0,31	1,54	1,53	1,72	1,63	1,61	0,09	1,35	4,18E-02
17	30,06	51,88	tr|C5WSF0|C5WSF0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300800 PE=3 SV=1	33	1	0,93	1,16	1,35	1,11	0,19	3,45	3,85	3,06	3,96	3,58	0,41	3,23	3,53E-05

18	27,48	42,16	tr|C5X8J1|C5X8J1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G301700 PE=4 SV=1	17	1	1,01	1,30	1,82	1,28	0,38	1,61	1,68	1,64	1,57	1,62	0,05	1,27	0,12
19	26,92	44,81	tr|A0A194YMM6|A0A194YMM6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G262500 PE=4 SV=1	15	1	0,89	1,26	1,44	1,15	0,25	9,40	9,27	9,12	8,78	9,14	0,27	7,94	1,04E-08
20	26,82	33,33	tr|A0A1B6QMT3|A0A1B6QMT3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G348900 PE=4 SV=1	27	1	1,13	1,45	1,58	1,29	0,27	3,83	3,79	4,32	4,03	3,99	0,24	3,09	6,02E-06
21	25,82	60,13	tr|C5Z6U2|C5Z6U2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G210000 PE=4 SV=1	15	1	1,00	1,16	1,37	1,13	0,18	2,78	3,12	3,66	3,20	3,19	0,36	2,82	5,01E-05
22	22,98	34,46	tr|A0A1B6QI05|A0A1B6QI05_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G089100 PE=4 SV=1	38	1	1,12	1,62	1,83	1,39	0,40	3,05	3,07	3,15	2,95	3,05	0,08	2,19	1,79E-04
23	22,07	28,81	tr|C5Z240|C5Z240_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G003100 PE=4 SV=1	17	1	1,01	1,30	1,52	1,21	0,25	2,73	3,15	2,78	2,94	2,90	0,19	2,40	3,69E-05
24	21,93	37,42	tr|C5Z8N0|C5Z8N0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G118900 PE=4 SV=1	42	1	1,05	1,18	1,56	1,20	0,26	1,11	1,09	1,03	1,07	1,07	0,04	0,90	0,37
25	21,66	40,65	tr|C5X3C1|C5X3C1_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391300 PE=3 SV=1	32	1	1,00	1,19	1,57	1,19	0,27	1,51	1,58	1,65	1,62	1,59	0,06	1,34	2,65E-02
26	21,06	41,51	tr|C5XQ74|C5XQ74_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G208800 PE=3 SV=2	20	1	0,96	1,58	1,71	1,31	0,39	2,74	2,67	2,56	2,79	2,69	0,10	2,05	4,70E-04
27	20,26	20,03	tr|C5XKE9|C5XKE9_SORBI	Endoglucanase OS=Sorghum bicolor GN=SORBI_003G015	16	1	1,27	1,44	1,35	1,26	0,19	3,12	3,68	3,94	3,59	3,58	0,35	2,84	2,25E-05

				700 PE=3 SV=1															
28	19,76	14,62	tr|C5Y397|C5Y397_SORBI	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_005G132400 PE=3 SV=1	15	1	0,91	1,34	1,48	1,18	0,27	4,99	4,90	4,76	5,41	5,01	0,28	4,24	1,10E-06
29	19,12	44,90	tr|C5X8J4|C5X8J4_SORBI	Xyloglucan endotransglucosylase/hydrolase OS=Sorghum bicolor GN=SORBI_002G302000 PE=3 SV=1	16	1	1,06	1,44	1,42	1,23	0,23	4,21	4,70	4,53	4,20	4,41	0,24	3,58	1,42E-06
30	18,37	31,99	tr|C5WNY4|C5WNY4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G129700 PE=3 SV=1	17	1	1,05	1,22	1,56	1,21	0,25	2,36	2,48	2,75	2,41	2,50	0,18	2,07	1,57E-04
31	17,86	55,46	tr|C5YC92|C5YC92_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G018100 PE=3 SV=1	18	1	0,91	1,14	1,34	1,10	0,19	2,06	2,32	2,43	2,77	2,39	0,30	2,18	3,17E-04
32	17,42	32,33	tr|C5Z864|C5Z864_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_010G232500 PE=3 SV=1	12	1	0,98	1,14	1,58	1,17	0,28	1,65	1,63	1,68	1,64	1,65	0,02	1,41	1,50E-02
33	17,39	30,14	tr|C5Z0P5|C5Z0P5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G055900 PE=4 SV=1	19	1	1,06	1,18	1,35	1,15	0,15	3,63	3,36	3,08	3,47	3,38	0,23	2,95	3,77E-06
34	17,19	49,35	tr|C5Y1P1|C5Y1P1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G098700 PE=4 SV=1	64	1	1,13	1,35	1,77	1,31	0,34	2,54	2,79	2,48	2,50	2,58	0,15	1,96	4,55E-04
35	16,58	37,82	tr|C5XIY1|C5XIY1_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152100 PE=3 SV=1	13	1	0,95	1,18	1,56	1,17	0,28	3,37	3,40	3,54	3,67	3,50	0,14	2,98	5,62E-06
36	16,53	41,37	tr|C5XB38|C5XB38_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055600 PE=4 SV=1	20	1	1,11	1,30	1,72	1,28	0,32	2,75	3,10	3,05	3,03	2,98	0,16	2,33	7,22E-05
37	15,84	21,83	tr|A0A1B6QIM7|A0A1B6QIM7	Uncharacterized protein OS=Sorghum	9	1	0,95	1,23	1,70	1,22	0,34	2,02	2,10	2,11	1,96	2,05	0,07	1,68	3,13E-03

			_SORBI	bicolor GN=SORBI_001G123300 PE=4 SV=1															
38	15,74	33,53	tr|A0A1B6QN00|A0A1B6QN00_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G360500 PE=4 SV=1	12	1	1,08	1,26	1,75	1,27	0,34	2,80	2,59	2,32	3,29	2,75	0,41	2,16	1,46E-03
39	15,59	40,74	tr|C5WVG9|C5WVG9_SORBI	Cysteine proteinase inhibitor OS=Sorghum bicolor GN=SORBI_001G324800 PE=3 SV=1	11	1	1,04	1,26	1,54	1,21	0,25	2,12	2,39	2,46	2,42	2,35	0,16	1,95	2,29E-04
40	15,43	11,95	tr|C5XWE5|C5XWE5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G197600 PE=4 SV=1	10	1	1,05	1,41	1,69	1,29	0,33	3,12	3,31	3,44	3,12	3,25	0,16	2,52	3,59E-05
41	15,19	75,31	tr|C6JSB7|C6JSB7_SORBI	Peroxidase OS=Sorghum bicolor GN=Sb0246s002010 PE=3 SV=1	73	1	1,29	2,27	1,27	1,46	0,56	10,42	12,45	9,25	13,28	11,35	1,84	7,79	4,99E-05
42	14,53	21,46	tr|C5XHF1|C5XHF1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G136200 PE=3 SV=1	13	1	1,03	1,27	1,58	1,22	0,27	2,23	2,51	2,59	2,34	2,42	0,16	1,99	2,65E-04
43	14,03	23,35	tr|A0A1B6PD28|A0A1B6PD28_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G113000 PE=4 SV=1	11	1	1,11	1,45	1,77	1,33	0,35	3,94	4,12	4,03	4,35	4,11	0,18	3,08	7,65E-06
44	13,01	23,74	tr|C5WRN5|C5WRN5_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_001G444500 PE=3 SV=1	11	1	0,97	1,22	1,45	1,16	0,22	2,04	2,07	1,93	2,00	2,01	0,06	1,73	3,15E-04
45	12,68	29,06	tr|C5XIH4|C5XIH4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G430100 PE=4 SV=1	13	1	1,09	1,33	1,77	1,30	0,34	1,79	2,15	2,04	2,04	2,00	0,15	1,55	9,16E-03
46	12,12	44,29	tr|C5X1U2|C5X1U2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G390300 PE=4 SV=1	9	1	1,01	1,11	1,41	1,13	0,19	1,66	1,47	1,69	1,67	1,62	0,10	1,43	4,18E-03

47	12,01	8,65	tr|C5XPK9|C5XPK9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G205600 PE=4 SV=1	11	1	0,96	1,29	1,22	1,12	0,16	3,12	3,03	2,94	2,92	3,00	0,09	2,69	8,53E-07
48	11,82	16,22	tr|A0A1B6PNM7|A0A1B6PNM7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G242000 PE=4 SV=1	11	1	1,02	1,15	1,47	1,16	0,22	3,78	3,64	3,81	3,95	3,80	0,13	3,27	7,96E-07
49	11,76	20,63	tr|A0A194YGY2|A0A194YGY2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G027000 PE=4 SV=1	7	1	1,06	1,19	1,44	1,17	0,20	7,15	7,28	6,49	6,82	6,94	0,35	5,90	1,21E-07
50	11,67	28,52	tr|C5XX83|C5XX83_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G208700 PE=3 SV=1	11	1	1,04	1,17	1,71	1,23	0,33	1,78	1,89	2,02	1,98	1,92	0,11	1,56	7,48E-03
51	11,6	28,57	tr|A0A1B6QGB6|A0A1B6QGB6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G416800 PE=4 SV=1	18	1	0,90	1,19	1,38	1,12	0,21	2,41	2,50	2,41	2,55	2,47	0,07	2,21	1,86E-05
52	11,5	17,93	tr|C5XIK1|C5XIK1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G432700 PE=4 SV=1	12	1	1,05	1,41	1,82	1,32	0,38	2,17	2,02	2,19	2,17	2,14	0,08	1,62	5,62E-03
53	11,48	14,92	tr|C5Z6U1|C5Z6U1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G209900 PE=4 SV=1	7	1	1,18	1,33	1,72	1,31	0,31	3,77	4,07	3,72	3,92	3,87	0,16	2,96	5,80E-06
54	11,23	54,15	tr|C5XYY5|C5XYY5_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_004G105100 PE=3 SV=1	20	1	0,93	1,27	1,49	1,17	0,26	1,18	1,28	1,28	1,30	1,26	0,05	1,07	0,55
55	11,07	19,78	tr|C5YVJ8|C5YVJ8_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232200 PE=4 SV=1	14	1	1,17	1,34	1,48	1,25	0,21	1,92	1,91	1,99	1,88	1,93	0,05	1,54	7,35E-04
56	10,79	28,63	tr|C5YJ56|C5YJ56_SORBI	Uncharacterized protein OS=Sorghum bicolor	10	1	1,07	1,03	1,26	1,09	0,12	1,34	1,26	1,31	1,35	1,32	0,04	1,21	1,04E-02

				GN=SORBI_007G214 700 PE=4 SV=1															
57	10,64	14,21	tr|C5Y8Y2|C5Y 8Y2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G235 600 PE=4 SV=1	8	1	0,98	1,31	1,79	1,27	0,38	1,84	1,84	2,09	1,93	1,92	0,12	1,52	1,67E-02
58	10,47	25,42	tr|C5XHP8|C5 XHP8_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419 400 PE=3 SV=1	8	1	0,98	1,24	1,35	1,14	0,18	1,61	1,55	1,37	1,71	1,56	0,14	1,36	1,19E-02
59	10,42	28,28	tr|C5WSE5|C5 WSE5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G300 400 PE=3 SV=1	12	1	0,86	1,17	1,43	1,12	0,25	3,35	3,81	3,56	3,32	3,51	0,23	3,14	7,20E-06
60	10,31	14,39	tr|C5XHP7|C5 XHP7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419 300 PE=4 SV=1	6	1	1,01	1,40	1,44	1,21	0,24	2,29	2,03	2,39	2,33	2,26	0,16	1,86	3,36E-04
61	10,29	34,00	tr|C5YK12|C5Y K12_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G100 600 PE=4 SV=1	8	1	0,96	1,01	1,32	1,07	0,17	1,83	1,86	1,63	1,94	1,81	0,13	1,69	4,25E-04
62	10,24	21,79	tr|C5WZU7|C5 WZU7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G516 000 PE=3 SV=1	16	1	0,93	1,19	1,45	1,14	0,23	1,94	2,27	2,40	2,29	2,23	0,20	1,95	3,85E-04
63	10,06	20,72	tr|C5XBP7|C5X BP7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G343 600 PE=4 SV=1	11	1	0,96	0,93	1,22	1,02	0,13	1,39	1,40	1,33	1,39	1,38	0,03	1,34	2,08E-03
65	10	13,08	tr|A0A1B6PKE 9|A0A1B6PKE 9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G056 300 PE=4 SV=1	7	1	1,22	1,42	1,62	1,31	0,27	1,65	1,53	1,65	1,62	1,61	0,06	1,23	7,25E-02
66	9,85	21,40	tr|C5XT36|C5X T36_SORBI	Endoglucanase OS=Sorghum bicolor GN=Sb24P17cg_130 PE=3 SV=1	5	1	1,24	1,38	1,52	1,28	0,22	2,20	1,99	2,11	2,24	2,13	0,11	1,66	4,81E-04

67	9,85	24,52	tr|C5Y587|C5Y587_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G049800 PE=4 SV=2	8	1	1,12	1,39	1,39	1,22	0,20	6,04	6,74	6,35	5,63	6,19	0,47	5,06	1,20E-06
68	9,3	14,74	tr|C5YBH7|C5YBH7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G135500 PE=4 SV=1	7	1	1,15	1,70	1,53	1,34	0,33	2,81	2,68	2,78	2,83	2,78	0,07	2,07	1,38E-04
69	9,28	30,13	tr|A0A1B6Q9F4|A0A1B6Q9F4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G057900 PE=4 SV=1	5	1	0,86	1,03	1,19	1,02	0,14	5,97	5,79	5,38	5,89	5,76	0,26	5,63	6,39E-08
70	9,21	31,45	tr|C5XX52|C5XX52_SORBI	Glyceraldehyde-3-phosphate dehydrogenase OS=Sorghum bicolor GN=SORBI_004G205100 PE=3 SV=1	11	1	1,12	1,06	1,49	1,17	0,22	5,49	5,37	4,82	5,23	5,23	0,29	4,49	5,19E-07
71	8,49	11,05	tr|C5WN99|C5WN99_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G261600 PE=3 SV=1	11	1	1,14	1,14	1,59	1,22	0,26	1,59	1,76	1,62	1,49	1,61	0,11	1,32	3,05E-02
72	8,38	7,99	tr|C5X022|C5X022_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G525000 PE=3 SV=1	7	1	1,20	1,42	1,69	1,33	0,30	2,55	2,72	2,58	2,77	2,66	0,11	2,00	1,51E-04
73	8,26	30,26	tr|C5YI15|C5YI15_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G066500 PE=3 SV=1	8	1	0,98	1,12	1,46	1,14	0,22	2,15	2,27	2,08	2,39	2,22	0,13	1,95	1,69E-04
74	8,15	16,17	tr|A0A1B6Q9B0|A0A1B6Q9B0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G050900 PE=4 SV=1	14	1	0,89	1,01	1,63	1,13	0,34	1,56	1,56	1,83	1,65	1,65	0,13	1,46	2,84E-02
75	8	13,53	tr|C5Y5K2|C5Y5K2_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_005G051500 PE=3 SV=1	7	1	0,96	1,17	1,51	1,16	0,25	1,56	1,77	1,91	1,85	1,77	0,15	1,53	6,09E-03
76	7,71	32,44	tr|C5WXD7|C5WXD7_SORBI	Uncharacterized protein OS=Sorghum	14	1	1,29	1,30	1,90	1,37	0,38	3,55	3,58	3,79	3,76	3,67	0,13	2,67	2,62E-05

				bicolor GN=SORBI_001G209 300 PE=4 SV=1															
77	7,64	23,31	tr|C5YY94|C5Y Y94_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_009G144 800 PE=3 SV=1	5	1	0,88	1,20	1,33	1,10	0,20	1,24	1,17	1,15	1,14	1,17	0,04	1,07	0,51
78	7,48	14,93	tr|C5Y1M2|C5 Y1M2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G097 400 PE=4 SV=1	4	1	1,31	1,56	1,04	1,23	0,26	1,63	1,94	1,94	2,41	1,98	0,32	1,61	1,09E-02
79	7,48	17,39	tr|C5X502|C5X 502_SORBI	Dirigent protein OS=Sorghum bicolor GN=SORBI_002G119 900 PE=3 SV=1	6	1	0,98	1,08	1,45	1,13	0,22	2,97	3,51	3,70	3,39	3,39	0,31	3,01	2,01E-05
80	7,42	14,26	tr|A0A1B6Q7M 0|A0A1B6Q7M 0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G390 700 PE=4 SV=1	4	1	0,90	1,45	1,64	1,25	0,36	0,78	1,01	1,07	1,08	0,98	0,14	0,79	0,22
81	6,98	11,33	tr|A0A194YIA9| A0A194YIA9_S ORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G044 500 PE=4 SV=1	5	1	0,94	1,10	1,60	1,16	0,30	1,07	1,02	1,07	1,04	1,05	0,02	0,90	0,48
82	6,92	5,09	tr|A0A1B6QC8 6|A0A1B6QC8 6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G189 100 PE=4 SV=1	4	1	0,94	1,76	1,19	1,22	0,37	2,59	2,63	2,29	2,62	2,53	0,16	2,07	6,77E-04
83	6,84	18,94	tr|C5XRX3|C5 XRX3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G294 500 PE=3 SV=1	5	1	1,04	1,24	1,81	1,27	0,37	1,68	1,68	1,75	1,81	1,73	0,06	1,36	5,12E-02
84	6,81	14,58	tr|C5XFX7|C5X FX7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G247 000 PE=3 SV=1	4	1	0,92	1,10	1,37	1,10	0,20	2,67	2,28	2,69	2,44	2,52	0,20	2,29	5,08E-05
85	6,7	9,24	tr|C5WT64|C5 WT64_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G170 700 PE=4 SV=2	4	1	1,07	1,33	1,75	1,29	0,34	2,87	2,51	2,49	2,66	2,63	0,18	2,05	4,15E-04

86	6,7	15,08	tr|A0A1B6PTQ9|A0A1B6PTQ9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G204700 PE=4 SV=1	5	1	1,00	0,84	1,14	1,00	0,13	1,38	2,07	2,38	2,42	2,07	0,48	2,07	5,04E-03
87	6,59	27,35	tr|C5YVJ7|C5YVJ7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G232100 PE=4 SV=1	4	1	0,99	1,08	1,23	1,08	0,11	2,56	2,45	2,48	2,68	2,54	0,11	2,36	1,38E-06
88	6,57	24,10	tr|C5XB39|C5XB39_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G055700 PE=4 SV=1	10	1	1,10	1,49	1,89	1,37	0,40	3,08	3,20	3,30	3,30	3,22	0,11	2,35	1,15E-04
89	6,49	15,53	tr|C5WSY5|C5WSY5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G014700 PE=3 SV=1	4	1	1,22	1,50	2,18	1,47	0,51	1,89	1,91	1,68	2,18	1,92	0,20	1,30	0,16
90	6,46	22,35	tr|A0A1B6QEI0|A0A1B6QEI0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G317600 PE=4 SV=1	4	1	0,88	0,90	1,32	1,03	0,20	4,64	4,49	4,68	4,44	4,56	0,12	4,45	9,13E-08
91	6,44	16,63	tr|C5Y675|C5Y675_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G064200 PE=4 SV=2	4	1	0,96	1,09	1,59	1,16	0,29	1,78	1,85	1,93	1,72	1,82	0,09	1,57	4,92E-03
92	6,34	19,95	tr|C5XL59|C5XL59_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G024700 PE=4 SV=2	11	1	1,07	1,24	1,49	1,20	0,22	0,51	0,49	0,45	0,54	0,50	0,04	0,42	7,37E-04
93	6,26	8,28	tr|C5Z469|C5Z469_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_010G161600 PE=3 SV=1	14	1	1,21	2,08	1,46	1,44	0,47	2,00	1,62	2,05	2,68	2,09	0,44	1,45	8,82E-02
94	6,22	19,83	tr|C5WXN2|C5WXN2_SORBI	Carboxypeptidase OS=Sorghum bicolor GN=SORBI_001G348800 PE=3 SV=1	15	1	0,97	1,06	1,60	1,16	0,30	2,58	2,33	2,53	2,29	2,43	0,14	2,10	2,50E-04
95	6,1	13,69	tr|C5X5L7|C5X5L7_SORBI	Alpha-galactosidase OS=Sorghum bicolor GN=SORBI_002G417800 PE=3 SV=1	5	1	0,92	1,30	1,64	1,22	0,33	4,91	4,65	5,06	5,13	4,94	0,21	4,06	1,34E-06

96	6,09	18,80	tr|A0A1B6Q6M7|A0A1B6Q6M7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G327700 PE=4 SV=1	4	1	1,06	1,11	1,40	1,14	0,18	1,77	1,83	1,93	1,89	1,85	0,07	1,62	3,01E-04
97	6,05	14,84	tr|C5XIY0|C5XIY0_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_003G152000 PE=3 SV=1	7	1	1,04	1,02	1,50	1,14	0,24	2,94	2,80	3,05	3,01	2,95	0,11	2,58	9,88E-06
98	6,05	10,22	tr|C5YNA1|C5YNA1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G172100 PE=3 SV=1	4	1	0,93	1,25	1,73	1,23	0,36	4,83	4,66	4,59	4,24	4,58	0,25	3,73	5,00E-06
99	6,05	8,73	tr|C5XL56|C5XL56_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G023800 PE=4 SV=1	3	1	1,11	1,56	1,38	1,26	0,25	1,82	1,97	2,17	1,95	1,98	0,14	1,57	2,62E-03
100	6,03	16,07	tr|C5Y7Y7|C5Y7Y7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G215500 PE=4 SV=1	14	1	1,09	1,29	1,16	1,13	0,12	1,17	1,28	1,50	1,67	1,41	0,23	1,24	7,76E-02
101	6,02	16,73	tr|C5X578|C5X578_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G128000 PE=4 SV=1	4	1	0,86	1,20	0,99	1,01	0,14	1,58	1,56	1,39	1,63	1,54	0,10	1,52	9,00E-04
102	6,01	6,12	tr|A0A194YKZ5|A0A194YKZ5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G227400 PE=4 SV=1	3	1	0,70	0,91	0,61	0,80	0,18	1,01	0,85	1,44	1,22	1,13	0,25	1,40	8,36E-02
103	6	12,06	tr|C5YW21|C5YW21_SORBI	Malate dehydrogenase OS=Sorghum bicolor GN=SORBI_009G240700 PE=3 SV=1	3	1	0,94	1,27	1,57	1,20	0,29	7,05	6,66	5,19	7,04	6,49	0,89	5,43	2,77E-05
104	6	8,89	tr|A0A194YU12|A0A194YU12_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G341200 PE=4 SV=1	5	1	1,02	1,24	1,29	1,14	0,15	7,58	7,08	7,11	7,63	7,35	0,29	6,45	2,34E-08
105	6	9,38	tr|C5YWW2|C5YWW2_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_009G113700 PE=3 SV=1	4	1	1,05	1,90	1,68	1,41	0,45	0,97	0,78	1,33	1,53	1,15	0,34	0,82	0,40

106	6	13,19	tr|C5WT90|C5WT90_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G173300 PE=4 SV=1	4	1	0,95	1,02	1,67	1,16	0,34	3,04	2,53	2,78	3,17	2,88	0,28	2,48	2,43E-04
107	6	16,28	tr|C5YUD2|C5YUD2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G072000 PE=3 SV=1	5	1	1,29	1,33	1,45	1,27	0,19	1,70	1,65	2,14	2,08	1,89	0,25	1,49	7,69E-03
108	6	10,53	tr|A0A1B6QIX5|A0A1B6QIX5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G142200 PE=4 SV=1	5	1	1,18	1,16	1,54	1,22	0,23	1,78	1,91	1,81	1,68	1,79	0,09	1,47	3,67E-03
109	6	6,27	tr|A0A1B6PAU7|A0A1B6PAU7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G234800 PE=4 SV=1	6	1	1,05	0,96	1,48	1,12	0,24	1,88	1,31	1,78	1,84	1,70	0,27	1,51	1,81E-02
110	6	21,05	tr|A0A1B6QN96|A0A1B6QN96_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G371900 PE=4 SV=1	4	1	0,90	1,16	1,15	1,05	0,12	12,51	15,96	12,60	16,06	14,28	1,99	13,59	1,14E-05
111	5,9	22,37	tr|A0A1B6Q818|A0A1B6Q818_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G416300 PE=4 SV=1	4	1	1,23	1,16	1,38	1,19	0,16	6,91	6,40	7,07	7,49	6,97	0,45	5,84	3,19E-07
112	5,72	17,62	tr|C5XCL8|C5XCL8_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G070200 PE=4 SV=1	3	1	1,03	1,11	1,32	1,12	0,15	2,47	2,20	2,02	2,11	2,20	0,19	1,97	1,11E-04
113	5,71	17,27	tr|C5XYB4|C5XYB4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G229300 PE=4 SV=1	8	1	1,01	1,40	1,49	1,23	0,26	2,82	2,67	2,70	2,97	2,79	0,14	2,28	3,92E-05
114	5,67	18,48	tr|C5XQJ2|C5XQJ2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G364300 PE=3 SV=1	5	1	0,96	1,12	1,63	1,18	0,31	2,05	2,22	2,64	2,19	2,27	0,25	1,93	1,56E-03
115	5,27	18,06	tr|C5XQW7|C5XQW7_SORBI	Uncharacterized protein OS=Sorghum	10	1	0,95	1,09	1,60	1,16	0,30	2,55	2,56	2,72	2,80	2,66	0,12	2,29	8,67E-05

				bicolor GN=SORBI_003G087 300 PE=4 SV=1															
116	5,2	19,80	tr|C5WQH5|C5WQH5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G149 500 PE=4 SV=1	3	1	1,12	1,00	1,45	1,14	0,21	2,80	3,05	3,89	3,02	3,19	0,48	2,79	2,33E-04
117	5,13	10,20	tr|C5Y1P6|C5Y1P6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G099 500 PE=3 SV=1	4	1	0,86	1,20	1,47	1,13	0,26	2,78	2,97	2,64	2,68	2,77	0,15	2,44	3,59E-05
118	5	8,23	tr|C5X3C7|C5X3C7_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391 900 PE=3 SV=1	7	1	0,98	1,34	1,48	1,20	0,25	0,63	0,61	0,67	0,58	0,62	0,04	0,52	3,66E-03
119	4,99	7,50	tr|C5Y8K8|C5Y8K8_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_005G228 900 PE=3 SV=1	3	1	0,95	1,06	1,50	1,13	0,25	0,55	0,57	0,64	0,66	0,61	0,05	0,54	6,98E-03
120	4,91	24,07	tr|C5YSB1|C5YSB1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_008G048 400 PE=4 SV=1	4	1	0,98	1,04	1,43	1,11	0,21	2,54	2,81	2,94	3,04	2,83	0,21	2,55	2,60E-05
121	4,89	12,28	tr|A0A1B6QAK5|A0A1B6QAK5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G113 800 PE=4 SV=1	3	1	1,05	1,73	1,11	1,22	0,34	3,88	3,75	3,69	3,22	3,64	0,29	2,97	3,91E-05
122	4,8	2,51	tr|A0A1B6PHE0|A0A1B6PHE0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G120 800 PE=4 SV=1	3	1	0,84	1,53	1,12	1,12	0,30	3,20	3,31	3,42	4,11	3,51	0,41	3,13	8,07E-05
123	4,76	18,03	tr|C5XFH6|C5XFH6_SORBI	Fructose-bisphosphate aldolase OS=Sorghum bicolor GN=SORBI_003G393 900 PE=3 SV=1	4	1	0,84	1,25	1,51	1,15	0,29	4,74	4,80	5,04	3,83	4,60	0,53	4,01	2,75E-05
124	4,69	3,37	tr|A0A1B6QN59|A0A1B6QN59_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G366 800 PE=4 SV=1	2	1	0,89	1,35	1,62	1,22	0,34	2,19	2,30	2,25	2,38	2,28	0,08	1,87	8,50E-04

125	4,51	12,95	tr|C5XHX2|C5XHX2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G427700 PE=3 SV=1	5	1	1,15	1,23	1,77	1,29	0,33	2,13	2,24	2,06	1,92	2,09	0,14	1,62	4,35E-03
126	4,48	19,38	tr|C5Z475|C5Z475_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_010G162000 PE=3 SV=1	11	1	1,17	1,45	1,55	1,29	0,25	1,74	2,02	2,13	2,00	1,97	0,16	1,52	3,98E-03
127	4,46	13,35	tr|C5Z4E5|C5Z4E5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G044900 PE=4 SV=1	5	1	1,05	1,44	2,05	1,39	0,48	1,11	1,16	1,12	1,24	1,16	0,06	0,84	0,38
128	4,37	14,79	tr|C5YZJ2|C5YZJ2_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_009G033400 PE=3 SV=1	7	1	1,12	1,21	1,63	1,24	0,27	1,85	2,05	1,96	2,14	2,00	0,12	1,61	2,32E-03
129	4,37	31,40	tr|C5X6P7|C5X6P7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G140400 PE=4 SV=1	5	1	0,94	1,02	1,34	1,08	0,18	2,33	2,53	2,60	2,65	2,52	0,14	2,34	1,53E-05
130	4,32	10,07	tr|C5XTG0|C5XTG0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G166500 PE=4 SV=1	2	1	1,25	1,47	0,87	1,15	0,27	7,35	7,33	6,38	6,93	7,00	0,45	6,10	5,54E-07
131	4,31	4,79	tr|C5WWQ2|C5WWQ2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G342600 PE=4 SV=1	3	1	1,31	2,15	2,11	1,64	0,57	13,66	12,05	12,48	17,39	13,90	2,43	8,47	6,43E-05
132	4,26	17,11	tr|C5X9N2|C5X9N2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G039000 PE=4 SV=1	3	1	0,82	1,15	1,26	1,06	0,19	4,17	4,04	4,88	4,51	4,40	0,38	4,15	4,03E-06
133	4,24	6,16	tr|C5WP48|C5WP48_SORBI	Alpha-mannosidase OS=Sorghum bicolor GN=SORBI_001G268700 PE=3 SV=1	3	1	0,52	0,93	1,77	1,05	0,52	3,05	3,03	3,10	3,67	3,21	0,31	3,05	3,92E-04
134	4,21	40,55	tr|C5YQ75|C5YQ75_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_008G010500 PE=3 SV=1	31	1	0,96	1,29	1,41	1,16	0,22	3,18	3,42	3,23	3,45	3,32	0,13	2,85	2,98E-06

135	4,19	9,16	tr|A0A1B6PLT3|A0A1B6PLT3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132100 PE=4 SV=1	3	1	1,12	1,26	1,78	1,29	0,34	2,12	2,13	1,85	1,71	1,95	0,21	1,51	1,62E-02
136	4,18	7,88	tr|C5WQK1|C5WQK1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G280000 PE=3 SV=1	3	1	1,13	1,61	1,08	1,20	0,27	3,10	3,19	3,30	3,73	3,33	0,28	2,77	3,61E-05
137	4,16	16,43	tr|C5X780|C5X780_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G007200 PE=4 SV=1	5	1	0,98	1,20	1,56	1,18	0,27	3,16	3,21	3,35	3,07	3,20	0,12	2,70	9,70E-06
138	4,1	8,45	tr|A0A1B6QEG2|A0A1B6QEG2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G315800 PE=4 SV=1	5	1	1,07	1,25	1,56	1,22	0,25	5,42	5,22	5,75	5,22	5,40	0,25	4,42	3,79E-07
139	4,02	8,55	tr|C5YRS3|C5YRS3_SORBI	Purple acid phosphatase OS=Sorghum bicolor GN=SORBI_008G037000 PE=3 SV=1	3	1	1,36	1,86	1,90	1,53	0,43	5,46	5,41	6,14	5,57	5,64	0,34	3,69	5,42E-06
140	4,02	5,45	tr|C5YCY4|C5YCY4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G160700 PE=3 SV=1	3	1	1,66	1,56	1,72	1,48	0,33	3,22	3,52	3,43	4,19	3,59	0,42	2,42	2,17E-04
141	4,01	5,29	tr|A0A1B6Q8G8|A0A1B6Q8G8_SORBI	Uncharacterized protein (Fragment) OS=Sorghum bicolor GN=SORBI_003G440900 PE=4 SV=1	3	1	0,94	1,16	1,89	1,25	0,44	3,58	3,79	3,67	3,53	3,64	0,11	2,92	4,18E-05
142	4	11,11	tr|C5XHP9|C5XHP9_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G419500 PE=4 SV=1	5	1	1,19	1,27	2,04	1,38	0,46	0,97	1,07	0,73	0,79	0,89	0,16	0,65	9,24E-02
143	4	10,95	tr|C5WPY8|C5WPY8_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_001G277000 PE=3 SV=1	3	1	0,83	1,05	1,47	1,09	0,27	1,93	2,04	2,21	2,11	2,07	0,11	1,90	5,44E-04
145	4	12,5	tr|C5YBF1|C5YBF1_SORBI	Uncharacterized protein OS=Sorghum bicolor	4	1	1,34	1,68	1,72	1,43	0,34	3,67	4,27	4,21	3,83	4,00	0,29	2,79	2,59E-05

146	4	6,59	tr|A0A1B6QD45|A0A1B6QD45_SORBI	GN=SORBI_006G132700 PE=3 SV=1 Uncharacterized protein OS=Sorghum bicolor	10	1	3,69	2,90	3,39	2,75	1,21	3,00	3,66	4,49	2,66	3,45	0,81	1,26	0,37
147	4	3,19	tr|C5Z998|C5Z998_SORBI	GN=SORBI_002G237000 PE=4 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	0,77	1,50	1,50	1,19	0,36	1,75	1,70	1,92	2,95	2,08	0,59	1,74	4,30E-02
148	4	8,79	tr|C5WT45|C5WT45_SORBI	GN=SORBI_010G255000 PE=4 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	1,11	1,08	1,55	1,18	0,25	3,77	4,47	4,44	4,15	4,21	0,33	3,55	6,16E-06
149	4	8,26	tr|C5Y7T1|C5Y7T1_SORBI	GN=SORBI_001G168500 PE=3 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	1,13	2,24	2,18	1,64	0,66	2,91	2,72	2,83	3,20	2,91	0,21	1,78	1,03E-02
150	4	7,58	tr|C5X3W3|C5X3W3_SORBI	GN=SORBI_005G086000 PE=3 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	1,26	1,60	0,87	1,18	0,32	3,11	2,65	2,16	2,96	2,72	0,42	2,30	1,16E-03
151	4	12,63	tr|C5XC95|C5XC95_SORBI	GN=SORBI_002G246400 PE=3 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	1,22	1,18	1,72	1,28	0,31	3,49	3,80	3,48	3,39	3,54	0,18	2,76	1,45E-05
152	4	7,51	tr|C5XQ07|C5XQ07_SORBI	GN=SORBI_002G345800 PE=4 SV=1 Uncharacterized protein OS=Sorghum bicolor	3	1	1,12	1,48	1,55	1,29	0,27	5,05	8,14	7,10	7,10	6,85	1,29	5,32	1,52E-04
153	4	14,79	tr|C5Y2P0|C5Y2P0_SORBI	GN=SORBI_003G072300 PE=3 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	1,43	1,77	1,32	1,38	0,32	1,97	2,18	2,37	2,00	2,13	0,19	1,54	6,42E-03
154	3,66	5,28	tr|A0A1B6QFT1|A0A1B6QFT1_SORBI	GN=SORBI_005G122300 PE=4 SV=1 Uncharacterized protein OS=Sorghum bicolor	2	1	0,96	1,12	1,44	1,13	0,22	1,87	1,85	1,88	1,89	1,87	0,02	1,66	4,68E-04
				GN=SORBI_002G392000 PE=4 SV=1															

155	3,63	12,29	tr|A0A1B6QFT7|A0A1B6QFT7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G392300 PE=4 SV=1	4	1	1,47	2,43	1,20	1,52	0,63	65,73	56,49	22,34	54,64	58,96	5,94	38,70	6,01E-06
156	3,62	16,41	tr|C5WN51|C5WN51_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G119000 PE=4 SV=1	3	1	0,93	1,13	1,44	1,13	0,23	0,91	0,98	1,13	1,28	1,08	0,16	0,96	0,74
157	3,51	14,20	tr|C5WPH2|C5WPH2_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G130400 PE=4 SV=1	3	1	0,92	1,07	1,41	1,10	0,22	1,97	2,01	2,68	1,89	2,14	0,37	1,94	2,85E-03
158	3,5	9,06	tr|C5WSF3|C5WSF3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G301000 PE=3 SV=1	2	1	0,86	0,94	1,10	0,97	0,10	1,36	2,15	1,64	1,66	1,71	0,33	1,75	5,33E-03
159	3,49	10,29	tr|A0A1B6P9F6|A0A1B6P9F6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_009G190800 PE=4 SV=1	3	1	0,92	1,27	1,41	1,15	0,23	5,09	4,49	5,30	4,81	4,92	0,35	4,28	1,95E-06
160	3,48	6,51	tr|A0A1B6Q537|A0A1B6Q537_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_003G244600 PE=4 SV=1	6	1	1,15	1,21	1,68	1,26	0,29	1,66	1,80	2,31	1,91	1,92	0,28	1,52	1,76E-02
161	3,46	7,44	tr|C5Z0N9|C5Z0N9_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_009G055300 PE=3 SV=1	3	1	1,21	1,46	1,77	1,36	0,33	3,75	3,76	3,88	3,62	3,75	0,11	2,76	9,30E-06
162	3,24	6,00	tr|C5X4N0|C5X4N0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G255600 PE=3 SV=1	3	1	1,22	1,19	1,44	1,21	0,18	2,19	1,95	2,43	2,33	2,23	0,21	1,84	3,07E-04
163	3,23	23,05	tr|C5Z476|C5Z476_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G161900 PE=4 SV=2	9	1	1,01	1,49	1,72	1,31	0,36	1,38	1,56	1,69	1,63	1,57	0,13	1,20	0,22
164	3,17	5,51	tr|C5X455|C5X455_SORBI	Carboxypeptidase OS=Sorghum bicolor GN=SORBI_002G401	2	1	1,20	1,19	1,78	1,29	0,34	1,56	1,45	2,16	1,74	1,73	0,31	1,33	0,11

				200 PE=3 SV=1															
165	3,12	13,68	tr|A0A1B6PLT5|A0A1B6PLT5_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G133000 PE=4 SV=1	7	1	1,10	1,21	1,18	1,12	0,09	2,15	2,63	2,02	2,80	2,40	0,38	2,14	5,89E-04
166	3,11	10,79	tr|C5WRL4|C5WRL4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G442200 PE=4 SV=1	2	1	1,33	1,42	1,51	1,32	0,22	1,85	2,33	2,53	2,50	2,30	0,31	1,75	2,12E-03
167	3,02	12,44	tr|C5XRU7|C5XRU7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G148100 PE=3 SV=1	5	1	0,86	1,06	1,32	1,06	0,19	3,14	3,72	2,71	3,18	3,19	0,42	3,01	8,74E-05
168	2,93	51,52	tr|C5XG88|C5XG88_SORBI	Small ubiquitin-related modifier OS=Sorghum bicolor GN=SORBI_003G402600 PE=3 SV=1	3	1	1,15	1,35	1,35	1,21	0,17	6,59	9,86	8,40	5,49	7,58	1,94	6,26	6,02E-04
169	2,91	7,98	tr|A0A1B6QJR7|A0A1B6QJR7_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G189000 PE=4 SV=1	2	1	0,98	1,20	1,75	1,23	0,36	2,24	3,03	2,80	2,29	2,59	0,39	2,10	2,17E-03
170	2,87	7,11	tr|C5WX83|C5WX83_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_001G058300 PE=4 SV=1	7	1	0,99	1,02	1,50	1,13	0,25	1,67	1,81	1,67	1,92	1,77	0,12	1,57	3,61E-03
172	2,74	10,40	tr|C5X6H6|C5X6H6_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G431100 PE=3 SV=1	2	1	0,63	0,67	0,99	0,82	0,20	0,61	0,67	1,25	0,64	0,79	0,31	0,96	0,88
173	2,66	5,78	tr|C5XDR4|C5XDR4_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G217200 PE=3 SV=1	2	1	0,95	1,16	1,32	1,11	0,17	3,27	3,06	3,47	2,91	3,18	0,25	2,87	8,52E-06
174	2,61	7,39	tr|C5YN91|C5YN91_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_007G171000 PE=4 SV=1	2	1	1,5	2,46	2,47	1,86	0,73	5,47	9,61	5,45	4,86	6,35	2,19	3,42	8,14E-03

175	2,57	15,35	tr|A0A194YLI0|A0A194YLI0_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_010G261000 PE=4 SV=1	2	1	0,95	1,18	1,41	1,14	0,21	0,79	0,80	0,80	0,81	0,80	0,01	0,70	1,87E-02
176	2,45	20,82	tr|C5X3C2|C5X3C2_SORBI	Peroxidase OS=Sorghum bicolor GN=SORBI_002G391400 PE=3 SV=1	12	1	1,16	1,24	1,75	1,29	0,33	1,44	1,43	1,37	1,39	1,41	0,04	1,10	0,48
178	2,4	8,09	tr|C5Y171|C5Y171_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_004G142800 PE=3 SV=1	2	1	1,07	1,35	1,71	1,28	0,32	7,97	6,85	7,16	6,89	7,22	0,52	5,62	1,25E-06
179	2,33	6,44	tr|A0A1B6QB11|A0A1B6QB11_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G133800 PE=4 SV=1	2	1	1,29	1,33	1,72	1,33	0,30	7,19	7,22	6,74	8,83	7,50	0,92	5,62	1,42E-05
181	2,27	7,23	tr|A0A1B6PJF1|A0A1B6PJF1_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G014400 PE=4 SV=1	2	1	0,95	1,57	1,76	1,32	0,40	3,28	3,01	2,55	2,76	2,90	0,32	2,20	8,41E-04
182	2,21	33,73	tr|C5X8I3|C5X8I3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_002G301600 PE=4 SV=2	14	1	0,88	1,07	1,32	1,07	0,18	1,40	1,21	1,26	1,30	1,29	0,08	1,21	6,82E-02
183	2,16	3,69	tr|C5YBF3|C5YBF3_SORBI	Uncharacterized protein OS=Sorghum bicolor GN=SORBI_006G132900 PE=4 SV=1	2	1	1,31	1,94	1,77	1,51	0,43	1,90	2,03	1,60	2,21	1,93	0,26	1,28	0,14

[a] Protein number assigned in ProteinPilot software.

[b] Protein score generated 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 a 95% confidence.

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

[d] Proteins accession obtained from the TrEMBL database [incorporated within the UniProt database (http://www.uniprot.org)] searches against sequences of *Sorghum bicolor* only.

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

[f] Values indicate the abundance of each protein from the four replicate control samples presented as a ratio to the 113-tagged sample

[g] Mean of ratios of each protein from the control samples (n=4).

[h] Standard deviation of the ratios of control samples (n=4).

[i] Values indicate the abundance of each protein from the four replicate sorbitol-treated samples presented as a ratio to the 113-tagged sample..

[j] Mean of ratios of each protein from the sorbitol-treated samples (n=4)..

[k] Standard deviation of the ratios of sorbitol-treated samples (n=4).

[l] Ratio represents the average fold-change (n = 4) induced by treatment relative to control. Positive values indicate an up-regulation.

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