# Exogenous caffeic acid alters molecular responses in *Salvia hispanica* L.

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**A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the department of Biotechnology, University of the Western Cape**



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December 2016

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# KEYWORDS

2D SDS – PAGE
Antioxidant enzymes
Caffeic acid (CA)
CA – induced
CA – recovered
Carbohydrate metabolism
Chia (*Salvia hispanica* L.)
Defence proteins
Detoxifying enzymes
Lipid peroxidation
MALDI – TOF MS
Nitrogen metabolism
Photosynthesis
Pigmentation
Plant proteomics
Protein identification
Protein synthesis
Proton transport
Pseudocereals
Reactive oxygen species
Salt – induced
Salt stress tolerance

# ABSTRACT

**Exogenous caffeic acid alters molecular responses in *Salvia hispanica* L.**

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Masters Thesis, Department of Biotechnology, University of the Western Cape

Salt stress is one of the most important abiotic stresses, resulting in the accumulation of ROS, which amount to great agricultural losses by influencing plant yield and in turn threaten sustainable agriculture and food security worldwide. ROS accumulates to levels that can become toxic to plants and is dependent on the scavenging ability of the antioxidant system to maintain redox homeostasis. Caffeic acid (CA) is a known antioxidant that have been shown to reduce the formation/production of ROS in legume plants exposed to salt stress. However, its' effect on pseudocereal plants such as chia have not been elucidated. This study investigated the influence of exogenous caffeic acid (at a final concentration of 100 µM) on the physiological and biochemical responses of chia plants under salt stress (100 mM). Furthermore, this study also investigated how caffeic acid and salt stress influenced protein changes in chia leaves using gel based proteomic analysis coupled with mass spectrometry.

The results showed that salt stress significantly reduces plant growth, biomass, relative water content, and chlorophyll metabolism. Contrary to what was observed for salt stress, caffeic acid improved plant growth, biomass and chlorophyll content. When salt stressed plants was supplemented with caffeic acid, the negative effects observed in the salt treatment was reversed albeit not to the level of the untreated

control. A similar trend was observed for ROS accumulation as denoted by hydrogen peroxide content, superoxide levels and the extent of lipid peroxidation. Caffeic acid and salt stress differentially altered antioxidant enzyme activity to control ROS metabolism. Although a significant increase in antioxidant enzyme activity was observed in salt stressed plants, this increase was not sufficient to counter the deleterious effects caused by salt stress. However, exogenous supplementation of caffeic acid to salt stressed plants notably reversed the negative effect caused by salt stress although not to the level of the control plants.

A total of 21 leaf based proteins with different degrees of abundance (across all treatments) was identified using gel based proteomic analysis. These proteins were functionally characterised into six broad categories. These categories include carbohydrate metabolism (43 %), proton transport (14 %), nitrogen metabolism (9 %), protein synthesis (10 %), detoxification (19 %) and disease/defence (5 %). Interestingly, most of the proteins identified was induced by caffeic acid (76 %), whereas only one protein was upregulated in response to salt stress and the remaining proteins were induced in the combined treatment (19 %). Most of the positively identified proteins was localised to the chloroplast with some found in the thylakoid membrane.

Based on the results obtained in this study we suggest that caffeic acid could serve as a regulatory signalling molecule in modulating salt stress tolerance in chia plants. This is evident by improved plant growth and biomass coupled with reduced ROS production. Moreover, the caffeic acid induced proteins identified in this study could also serve as potential biomarkers to enhance salt stress tolerance in chia plants.

# DECLARATION

I declare that "Exogenous caffeic acid alters molecular responses in *Salvia hispanica* L.**"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Shelby Ann Jones December 2016

Signed ……  …….

# ACKNOWLEDGEMENTS

Firstly, I would like thank the **Father in Heaven** for the strength and guidance bestowed upon me throughout this research project.

I thank my supervisor, **Dr. Ashwil J Klein**, for his commitment and dedication toward my completion of this degree. I am grateful for the endless time he spent educating me. Thank you for keeping an open door policy. I certainly would not have reached this far without your supervision.

I extend my appreciation to all individuals who contributed to my understanding of plant science including **Dr. Lizex H.H. Hüsselmann** for his continuous and immediate assistance. I would like to thank my co – supervisor, **Dr. Marshall Keyster**, along with the entire **Plant Omics Laboratory** for your intellectual conversations, constructive criticism and valuable advice contributing to this degree.



A special thanks to my parents, **Gillian Ann Jones** and **Wayne Jones**, for their continuous support (emotionally and financially). Thank you for being my pillars of strength and constantly pushing me, not only to do my best, but also to be the best person I can be. To my mother, THANK YOU for enduring all my stressful episodes. I know it wasn't easy. To my father, know that I am proud of you. I love you both.

To all my siblings across both families, **Chloë Hannah Thomas**, **Skye – Leigh Jones** and **Connor Meinking**, for acting as motivation to be the perfect role

model. Most importantly, I am highly thankful for my youngest brother, **Troy Wayne Jones**, for mending broken families through his birth.

To **Adria Michelle Burrows**, words cannot express how grateful I am for your friendship. Thank you for always being there and not allowing my moods to push you away. Thank you for your safe and reliable transport to and from the University of the Western Cape. May this only be the beginning.

I acknowledge the **National Research Foundation (NRF)** for funding the proposed research as well as the **Proteomics Research and Service Unit** along with the other **Biotechnology laboratories** at the University of the Western Cape for providing their facilities.

I would like to extend immense appreciation to the Regional Secretory, **Ms. Nomonde Mgumane** of the National Bargaining Council for the Clothing Manufacturing Industry (Western Cape Chamber). The Fees Must Fall Movement imposed severe stress upon myself during a crucial write – up period. Thank you to Ms. Mgumane for providing me with a space to concentrate in order to complete this degree. Furthermore, thank you to **Mr. Barry Galant** and **Mr. Ricardo de Bruin**, within the IT department, for providing me with computer facilities and unlimited internet access to conduct the research for this degree. Words cannot express the gratitude I feel for the **National Bargaining Council for the Clothing Manufacturing Industry (Western Cape Chamber)**.

Finally, to **Riyaad Addinall**, thank you for stepping into my life when you did. The continuous support and upliftment in my darkest periods has definitely

contributed to the completion of this degree. Thank you for always being nothing but a desk away and always making sure I am emotionally kept for the following day. You have been nothing but a pillar attached to my side and I am nothing but appreciative for your benevolent actions. Thank you for assisting me in the laboratory but most importantly, thank you for opening my eyes to greater things. Shukraan for all that you do and may Allah grant your kind heart a successful career ahead.

**December 2016**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| APX | ascorbate peroxidase |
| AsA | ascorbic acid |
| BSA | bovine serum albumin |
| CA | caffeic acid |
| CAT | catalase |
| CBB | coomassie brilliant blue |
| Cu/Zn – SOD | copper zinc superoxide dismutase |
| DHAsA | dehydroascorbate |
| DHAR | dehydroascorbate reductase |
| DTT | dithiothreitol (Cleland's reagent) |
| EDTA | ethylenediaminetetraacetic acid |
| EST | expressed sequence tag |
| Fe – SOD | iron superoxide dismutase |
| GR | glutathione reductase |
| GSH | reduced glutathione |
| GSSG | oxidised glutathione |
| IEF | isoelectric focusing |
| pI | isoelectric point |
| IOA | iodoacetamide |
| IPG | immobilised pH gradient |
| MALDI – TOF | matrix assisted laser desorption/ionisation-time of flight |
| MDA | malondialdehyde |
| Mn – SOD | manganese superoxide dismutase |
| MS | mass spectrometry |
| MS/MS | tandem mass spectrometry |
| MW | molecular weight |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NBT | nitrotetrazolium blue chloride |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| PMF | peptide mass fingerprinting |
| PVP | polyvinylpyrrolidone |
| ROS | reactive oxygen species |
| SDS | sodium dodecyl sulphate |
| SOD | superoxide dismutase |
| TBA | thiobarbituric acid |
| TCA | trichloroacetic acid |
| TEMED | N,N,N′,N′ - Tetramethylethylenediamine |
| TFA | trifluoroacetic acid |
| TOF | time of flight |
| XTT | 3 – bis(2 – methoxy – 4 – nitro – 5 – sulfophenyl) – 2H – tetrazolium – 5 – carboxyanilide |
| 1D | one – dimensional |
| 2D | two – dimensional |
| 2DE | two – dimensional gel electrophoresis |
| 2D SDS – PAGE | two – dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis |

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1. Introduction

Food security, a globally used term, is defined as the accessibility to healthy and high quality nutritional food sources, throughout a particular region, at all times (Drimie and McLachlan 2013; Labadarios *et al*. 2011). Furthermore, food systems are explained as the interaction between (and within) the biogeophysical and human environments which leads to the production, processing, distribution, preparation and finally, food consumption (Gregory *et al*. 2005). Therefore, functioning foods systems, include three components: (i) food availability, (ii) food access, as well as (iii) food utilisation (Figure 1.1). Hence, these food systems are recognised as the underpin for food security (Gregory *et al*. 2005).



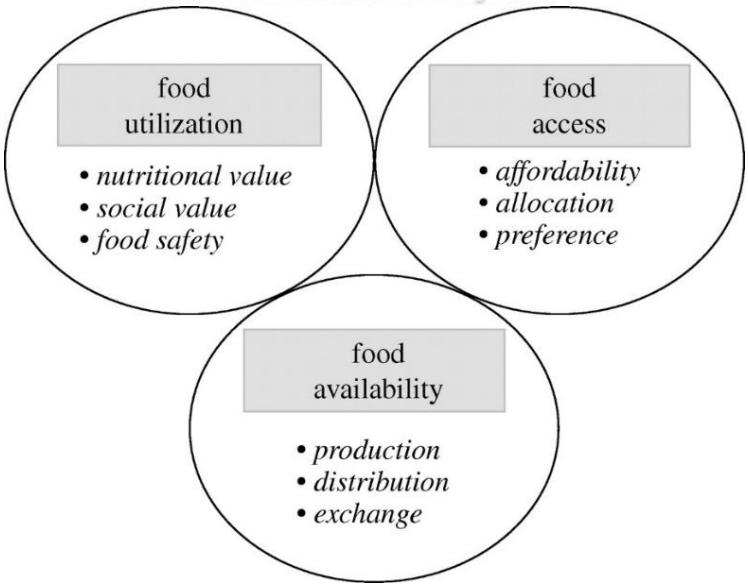



**Figure 1.1. Food systems with their respective functions.** The diagram was adapted from Gregory *et al.* 2005.

The food security status within a particular domain is highly influenced by plant – based food sources, such as cereals and pseudocereals. The importance of these crops are expressed through its valuable nutritional and medicinal properties. These properties were discovered in numerous cereals, including sorghum (*Sorghum Bicolor* L. Moench) and maize (*Zea mays* L.), as well as pseudocereals such as buckwheat (*Fagopyrum esculentum* Moench), quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus cruentus* L.) (Goncalves *et al*. 2016; Ramatoulaye *et al*. 2016; Rouf Shah *et al*. 2016; Tang *et al*. 2016). These crops have been exploited for various food sources categorised into fats and oils, carbohydrates, vitamins and salts, proteins, beverages, spices, as well as drugs for the prevention and curing of various infections and diseases (Muhammad and Amusa 2005). Therefore, food crops can serve as a valuable contributing factor to improve the food security status within food insecure domains.

Food insecurity has become alarmingly worrisome within the African continent (Figure 1.2). However, according to Talebpour *et al*. (2015), South Africa is only at medium risk which was further explained by Drimie and McLachlan (2013). These authors amended the original food security definition to accommodate the South African population since various divisions of South Africa are declared food secure, whilst few areas remain food insecure. Therefore, suggesting that not every region within South Africa has access to foods that are essential for healthy living (Drimie and McLachlan 2013; Labadarios *et al*. 2011) and thus forming the basis of food crop research in aid of improving the food insecure domains of South Africa.

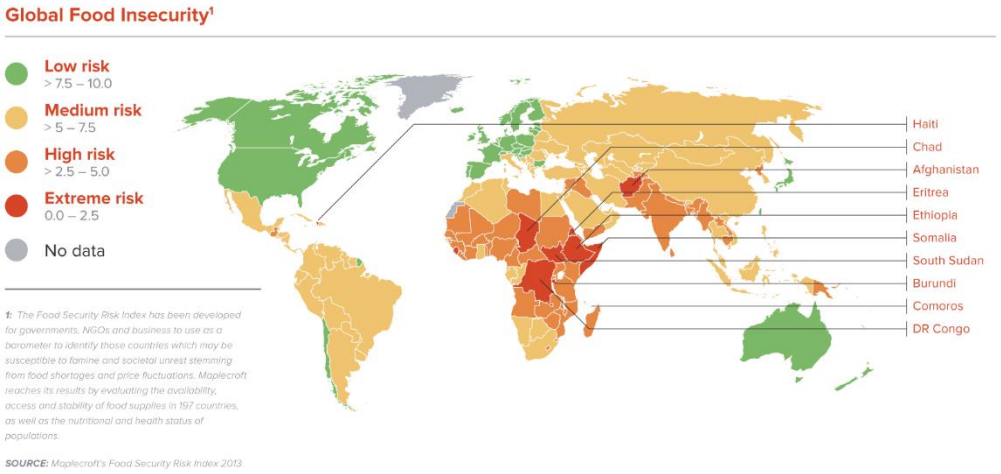



**Figure 1.2. The distribution of global food insecurity in 2012 – 2013.** The map was adapted from Talebpour *et al*. 2015.

South Africa remains to be one of the fastest growing populations (Larkins *et al*. 2008) and therefore, despite the advances witnessed in the South African economy since the Apartheid era (political and economical), the country remains overwhelmed with poverty, unemployment and food price volatility (Altman *et al*. 2009; Drimie and McLachlan 2013; Labadarios *et al*. 2011). In addition to these economic impacts, South Africa is highly affected by climate change. This could lead to significant changes in rainfall patterns which could ultimately result in drought and high saline environments (Slingo *et al*. 2005). Therefore, as a consequence, the country suffers a persistence of hunger and malnutrition (Drimie and McLachlan 2013), contributing 204 million undernourished individuals (sub – Saharan including South Africa) to the 814 million within developing countries (Labadarios *et al*. 2011).

Therefore, a wealth of research has shown that climate variability has a direct link to food security as various weather conditions impact certain valuable crops. Thus, climate change halts food production and ultimately negatively impacting the food

security status in South Africa (Gregory *et al*. 2005; Lobell *et al*. 2008; Slingo *et al*. 2005). Hence, given these "short wave shocks" such as food price volatility and "long wave shocks" which refers to climate change (Drimie and McLachlan 2013), ordinary South African citizens continue to experience the severe pressure through these adverse conditions, enforcing a greater struggle to meet their basic household needs (Labadarios *et al*. 2011).

Although these wave shocks collectively reduces the strength of the food security status in South Africa, those fortunate of accessing healthy food sources consume maize, wheat and rice, which contribute to 60% of the world's food supply (Pimentel *et al*. 1997). In addition to these crops, advances in plant research has led to the discovery of superfood crops that have shown to medicinally and nutritionally contribute to human health. Many of these superfood crops, such as buckwheat, quinoa and amaranth are characterised as pseudocereals. However, research in the pseudocereal, *Salvia hispanica* (chia), remain in its infancy.



Therefore, in order to improve food security, this research addresses climate change and the highly nutritional pseudocereal, *Salvia hispanica*, to improve food production and reduce food insecurity not only in South Africa, but worldwide.

## 1.2. The importance of pseudocereals

Pseudocereals are defined as crops that are evolutionary distant from cereals, yet remain to produce grains, and are characterised by an excellent nutrient profile (Alvarez – Jubete *et al*. 2010; Berti *et al.* 2005). The nutritive value of pseudocereals are associated with their protein content which provides a substantial group of bio –

macromolecules required for various physiological functions (Mota *et al*. 2016), in addition to their polyphenol and high dietary fibre content (Tang *et al*. 2016).

However, extensive research focused on cereal crops instead, given its 60% contribution to the world's food supply (Pimentel *et al*. 1997). Thereafter, upon the discovery of gluten – based diseases such as celiac disease (CD), plant researchers diverted their focus toward gluten – free sources. Alvarez – Jubete *et al*. (2010) explains CD to be an autoimmune enteropathy which is triggered via the ingestion of gluten containing grains in genetically susceptible individuals. This understanding has driven research towards the discovery and knowledge of gluten – free grains, such as amaranth (*Amaranthus retroflexus*), quinoa (*Chenopodium quinoa*), buckwheat (*Fagopyrum esculentum*) and chia (*Salvia hispanica*), all classified as pseudocereals (Galova *et al*. 2015; Tang *et al*. 2016). Furthermore, given its low allergenicity, pseudocereals have also been recommended for babies as a replacement for rice (Mota *et al*. 2016). Therefore, these pseudocereals have been implemented into, not only gluten sensitive individuals diets, but also healthy diets (Gorinstein *et al*. 2007).

## 1.3. *Salvia hispanica* as an alternative food source

*Salvia hispanica*, a pseudocereal commercially known as chia (Figure 1.3), was first documented in the codices of the 16$^{th}$ century in Mexico, whereby a volume of ethnobotanical information was provided in addition to describing the large agricultural areas that were solely devoted to chia cultivation. Therefore, chia can be described as an ancient crop as they were first discovered and cultivated by pre – Columbian communities in Mexico and Guatemala (Ayerza and Coates 1999; Ixtaina

*et al*. 2010; Sandoval – Oliveros and Paredes – Lopez 2012). However, although chia served as a staple food source for these pre – Columbian communities, the "chia rage" had become lost in history when the Spanish invaded the Aztecs as it resulted in a reduction in chia cultivation. This was due to the Spanish banishing chia cultivation as a means of control, and consequently limiting chia survival (Joseph 2004). It wasn't until the 1990's when Dr. Wayne Coates, the founder and writer of chia research, stimulated chia production for nutritional composition studies. This was achieved through the initiation of a project by Agropecuaria El Valle S.A., an Argentinian company, in order to determine the feasibility of commercialising chia production in Argentina and Columbia (Coates 1996).

Although this pseudocereal originated in central Mexico and Guatemala, it is now grown in various countries with Australia currently noted as the largest producer of these chia seeds (Crawford *et al*. 2012; Timilsena *et al*. 2016). In addition to Australia, chia is also produced in Argentina (Ayerza 2016), Brazil (de Freitas *et al*. 2016), Italy (Bochicchio *et al*. 2015) and various other countries contributing to a worldwide annual production of 30 000 tons (Daniells 2013).

Chia, classified within the mint (*Labiatae*) family, has been described as a crop that is capable of growing in arid and semiarid conditions (Ixtaina *et al*. 2008; Mohd Ali *et al*. 2012; Reyes – Caudillo *et al*. 2008). A total of 900 species exist within the *Salvia* genus which has all been explored for various chemical studies relating to the isolation of polyphenols, diterpenoids and tanshinones from various plant tissues. Chia was known to be one of the major crops of the 16$^{th}$ century (Reyes – Caudillo *et al*. 2008) and although this plant served as a staple food source for the pre –

Columbian communities, modern age communities primarily recognises chia plants for their oil applications (Ixtaina *et al*. 2008; Ixtaina *et al*. 2010).

**Figure 1.3. Matured *Salvia hispanica* (chia) plants grown in the field.** Chia can grow up to 2 m tall.

### 1.3.1. Botanical characteristics

Chia oil, which is known to consist of highly nutritional components, is located within the seed. Mohd Ali and authors (2012) had described chia seeds as oval – shaped with a diameter of 1 – 2 mm (Figure 1.4). These authors further describe the chia plant's to grow as tall as 2 m with opposite arranged leaves in its' natural and accommodating environment. As viewed in Figure 1.3, upon maturation, chia plants exhibit purple colour flowers which have been described by Mohd Ali *et al*. (2012) to range between 3 – 4 mm, with fused corollas.

**Figure 1.4. Seeds of *Salvia hispanica* (chia).** Chia seeds are oval shaped and are 1 – 2 mm in diameter (Daniells 2013).

### 1.3.2. Nutritional value

The seeds that are produced from this species are prized for their nutritional and medicinal properties since the Mayans and Aztecs exploited this seed as a food supplement for energy, endurance and strength (Sandoval – Olivero and Paredes – Lopez 2012). Hence, the interest in chia lies within its oil content, protein composition, antioxidant activity and dietary fibre content (Ixtaina *et al.* 2008). Due to its high dietary fibre content, the chia seed is considered highly nutritional given the volumes of published research proving that the consumption of dietary fibre reduces cholesterolaemia, modifies the glycemic and insulinaemic responses as well as alter the intestinal functions and antioxidant activities (Reyes – Caudillo *et al.* 2008). According to Sandoval – Olivero and Paredes – Lopez (2012), researchers are interested in the high levels of natural antioxidants (phenolic compounds) within the

chia seed. These phenolic compounds, specific to chia, include caffeic and chlorogenic acid, kaempferol, quercetin and myricetin (Mohd Ali *et al.* 2012; Sandoval – Olivero and Paredes – Lopez 2012).

### 1.3.3. Chia oil – Medicinal value

Chia seeds comprises 25 – 40 % oil (Mohd Ali *et al*. 2012) that consists of linoleic (17 – 26 %) and α – linolenic (50 – 57 %) acids (Ayerza 1995). The α – linolenic acid, also referred to as omega 3 (ω – 3) polyunsaturated fatty acid, appears to be advantageously dominant thus covering 60 % of the oil content. Therefore, given that ω – 3 is essential for growth and development, chia oil has become medicinally important across the globe (Ixtaina *et al*. 2012). The ingestion of high levels of ω – 3 has been proven to play an important role in the treatment as well as the prevention of diabetes, arthritis, hypertension, cancer, coronary heart disease (CHD) and other inflammatory and autoimmune disorders (Ixtaina *et al*. 2012). Studies in the United States have shown that CHD is regarded as one of the main causes of death in Americans. However, it has also been proven that a change in diet (increasing ω – 3 fatty acid consumption) could result in reducing the chances of contracting the disease (Ayerza and Coates 2009).

## 1.4. The influence of abiotic stress on plants

Like other plants, chia is also exposed to several abiotic stresses, including salt stress, which is a consequence of acid rain. Saline soil, unfortunately contributes to many agricultural losses due to the presence of high levels of sodium within the soil, thus

diminishing accommodating environments, as well as inducing plant water deprivation by decreasing the osmotic potential of the soil. In addition to these consequences, high levels of salt could also cause nutrient imbalances and shortages (Sairam and Tyagi 2004). Therefore, maintaining the homeostasis of the ion concentrations within the plant is of high importance as it contributes to the physiology of the living cells. Therefore, proper regulation is required to minimise toxic ions and accumulate essential ions (Zhu 2003). Under normal conditions, the plant experiences high levels of potassium ($K^+$) and low levels of sodium ($Na^+$; Figure 1.5) which is a homeostasis that is essential for many cytosolic enzymatic activities and maintaining membrane potential. Therefore, when the plant is exposed to salt stress, the $Na^+$ interrupts the plants' successful uptake of $K^+$ and thus when the plant accumulates $Na^+$ within the plant, it becomes toxic to the naturally occurring enzymes (Zhu 2003). In addition to $Na^+$ becoming toxic to the plant, because the plant experiences low water availability, it initiates its protective mechanism by closing its stomata in order to reduce transpiration. However, as a consequence to the closure of the stomata, it prevents the entering of carbon dioxide which is essential for photosynthesis to occur and thus, limiting the successful survival of the plant (Amjad *et al*. 2014).

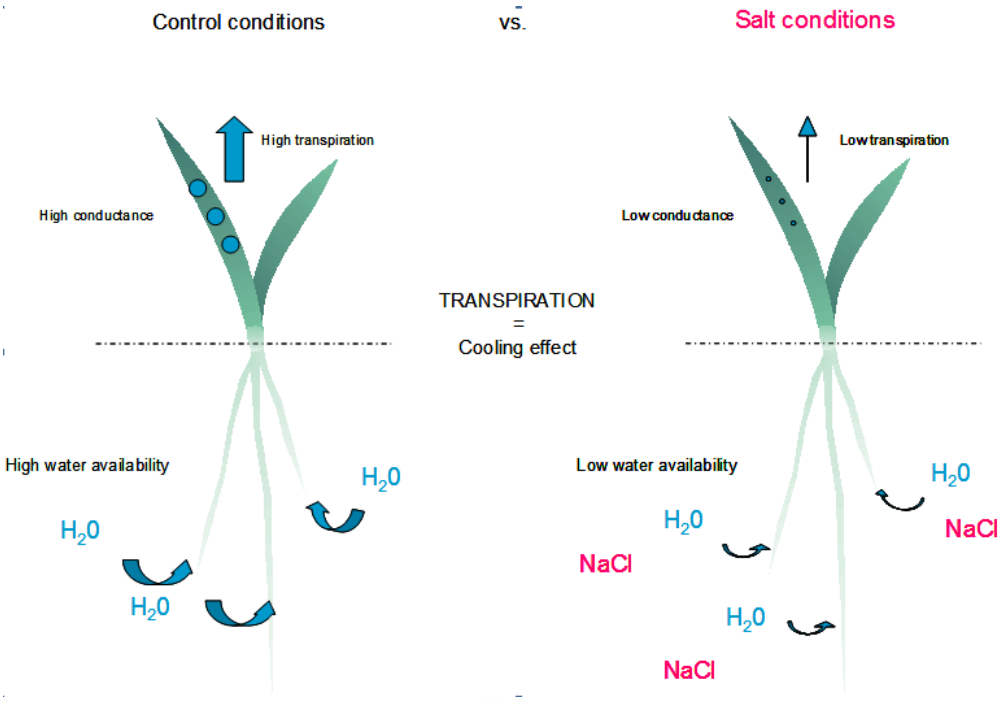



**Figure 1.5. Diagram illustrating the effects of salt stress on plant growth.** Salt stress causes water reduction and water stress within the plant as the sodium chloride prevents successful uptake of sufficient water (Berry and Sirault 2010).



When the plant is exposed to abiotic stresses, or other stresses, it imposes oxidative strain on the plant. Stress exposure initiates the accumulation of toxic molecules known as Reactive Oxygen Species (ROS; Mittler 2002). Therefore, oxidative stress is explained as the generation of an imbalanced state of excessive reactive oxygen overcoming the plants' antioxidant capacity, thereby leading to the oxidation of biomacromolecules (lipids, proteins, DNA and enzymes; Dai and Mumper 2010). Hence, ROS molecules are easily detected by the plant and thus serve as a signalling molecule for various biosynthetic pathways (Mittler 2002).

## 1.5. Stress induced ROS accumulation

ROS are intermediates that are reduced forms (more toxic) of atmospheric oxygen including $H_2O_2$ (hydrogen peroxide), $O_2^-$ (superoxide radicals) and $HO^-$ (hydroxyl radicals). Under normal conditions, the production of ROS is low, not detrimental to the plant and serves as signalling molecules in various metabolite pathways (Mittler 2002). However, when the plant is exposed to various stress conditions (including salt stress), it could result in the increased production of ROS. This accumulation is believed to be a by – product of stress metabolism (Miller *et al.* 2008) which could lead to lipid peroxidation, disruption of DNA strands and the inactivation of various essential enzymes (Bandeoglu *et al*. 2004; Cheng and Song 2006).

There are two substances that can be measured to determine the plants' strength capabilities in response to a specific stress. This begins with the extent of ROS accumulation which can be measured via $H_2O_2$ content, followed by the extent of lipid peroxidation, due to ROS accumulation, which can be elucidated via malondialdehyde (MDA) content. Given that $H_2O_2$ is a ROS classified molecule and MDA is known to be a naturally occurring by – product of lipid peroxidation, the increase in ROS results in the increase in cell death (as a consequence of lipid peroxidation). Therefore, the higher the levels of $H_2O_2$ and MDA, the more stressed the plant (Wang *et al*. 2013).

Furthermore, because ROS plays an important role in the plant in terms of signalling molecules and mediating stress tolerance, but at the same time serve as a cytotoxic compound (Lee and Lee 2000), its' concentrations are regulated by a large, complex network of genes referred to as the "ROS gene network" (Chaudhary *et al.* 2009;

Miller *et al.* 2008). Thus, to regulate the levels of ROS within the plant, it initiates scavenging mechanisms that help keep ROS concentrations at controllable and safe levels (Miller *et al.* 2008).

### 1.5.1. Mechanisms of ROS scavenging in plants

Since the accumulation of ROS is a result of the impairment of the transportation of electrons within the chloroplast and mitochondria, which causes the production of superoxide radicals (Bandeoglu *et al.* 2004), there are various mechanisms of ROS scavenging that exists within a plant to avoid the over – production of these toxic molecules. These mechanisms involve enzymatic and non – enzymatic antioxidants (Figure 1.6; Mittler 2002). Antioxidants are compounds that sole function is to either delay, inhibit or prevent the effects of oxidative stress. Therefore, antioxidants prevent the reduction or oxidation of oxidizable material through free radical scavenging as well as weakening the effects of oxidative stress (Dai and Mumper 2010). The activation of these enzymes are essential for the determination of safe levels of superoxide radicals (Mittler 2002). One of the pathways involved in ROS scavenging is known as the Halliwell – Asada pathway, or more commonly referred to as the ascorbate – glutathione pathway (Inze and Van Montagu 1995) (Figure 1.6).

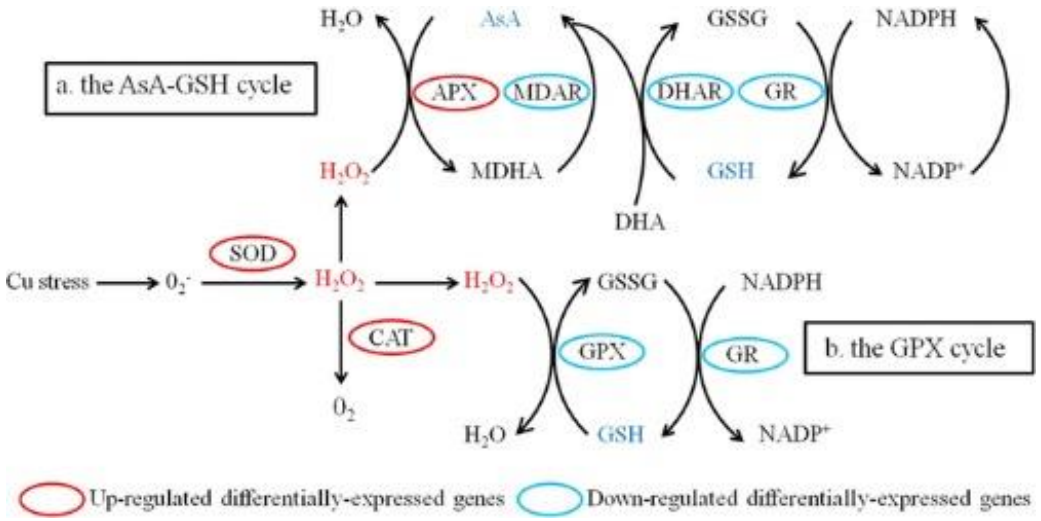



**Figure 1.6. The redox cycling of ascorbate as described by the Halliwell – Asada pathway.** The pathway involves using enzymatic and non – enzymatic antioxidants to assist in the scavenging of ROS molecules which accumulates in response to stress (Inze and Van Montagu 1995).



### 1.5.1.1.Enzymatic mechanisms

The Halliwell – Asada pathway (Figure 1.6), also referred to as the ascorbate – glutathione pathway, involves five enzymatic ROS scavenging antioxidant enzymes. These include superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR: EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) (Inze and Van Montagu 1995).

### Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme that is abundant in aerobic organisms and its sole purpose is to reduce toxic superoxide radicals ($O_2^-$) to less toxic hydrogen peroxide ($H_2O_2$). SOD, being the first enzyme in the ROS scavenging system, is a

gene that is expressed once the organism is exposed to stress which is indicated by increased levels of $O_2^-$ (Cheng and Song 2006; Lee and Lee 2000).

$$O_2^- \xrightarrow{\text{SOD}} H_2O_2 + H_2O$$

**Ascorbate peroxidase**

The SOD enzyme reduces high levels of $O_2^-$ into $H_2O_2$, which in turn activates the Halliwell – Asada $H_2O_2$ scavenging enzyme, known as ascorbate peroxidase (APX). Hydrogen peroxide is a form of ROS and therefore, even though it is not as detrimental as $O_2^-$, it remains toxic in accumulative forms. APX enzymes function in reducing $H_2O_2$, using ascorbate (AsA) (Figure 1.6), to release monodehydroascorbate (MDHA) (Asada 1992).

$$\text{Ascorbate} + H_2O_2 \xrightarrow{\text{APX}} \text{MDHA} + H_2O$$

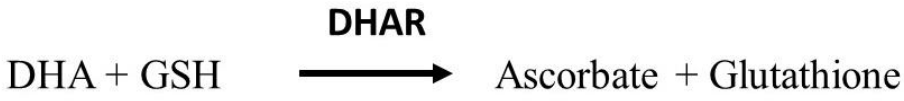


**Monodehydroascorbate reductase**

Although MDHA has been declared safe for plant survival (Asada 1992), the generation of MDHA activates the enzyme, monodehydroascorbate reductase (MDHAR) to allow for the regeneration of ascorbate (AsA). Therefore, MDHAR reduces MDHA, using NADPH as electron donors, yielding ascorbate molecules as end products. However, MDHA, theoretically, has a short half – life and thus majority of MDHA is converted into dehydroascorbate (DHA). Hence, since the reduction of MDHA yields ascorbate as an end product (Figure 1.6), the MDHAR serves as a key role within the ROS scavenging system (Gill and Tuteja 2010).


**Dehydroascorbate reductase**

Dehydroascorbate reductase (DHAR), like MDHAR, functions in the regeneration of ascorbate. However, DHAR yields oxidised glutathione (GSSG) from reduced glutathione (GSH). This enzyme is only activated when excess MDHA is converted into DHA (Gill and Tuteja 2010).




**Glutathione reductase**

Glutathione reductase (GR) functions as the final enzyme within the Halliwell – Asada pathway (Figure 1.6) in order to reduce toxic species and to yield ascorbate and glutathione (Mittler *et al*. 2004). According to my understanding of the Halliwell – Asada pathway, GR reduces the GSSG into a reduced form of glutathione with the help of an electron donor (NADPH) as GSH is required for DHAR to convert DHA back into ascorbate and GSSG (Mittler *et al.* 2004). Therefore, GR functions as a catalyst in the regeneration of ascorbate (Lee and Lee 2000).


SOD, APX and GR all form part of the Halliwell - Asada scavenging pathway as they work together to neutralize the harmful effects caused by the over production of ROS. Although there are other forms ROS scavenging, it is believed that the enzymes in

the Halliwell - Asada pathway has a greater affinity for $O_2^-$ and $H_2O_2$ and is more effective as it covers a greater surface area within the plant (Lee and Lee 2000; Mittler 2002).

### 1.5.1.2. Non – enzymatic mechanisms

Upon the activation of the enzymatic antioxidants, the complete ascorbate – glutathione (Halliwell – Asada) pathway oxidises and generates ascorbate and glutathione (Lee and Lee 2000). These non – enzymatic mechanisms serve as redox buffers that could possibly influence the expression of various genes that are associated with abiotic (as well as biotic) stresses (Miller *et al.* 2008).



**Ascorbic acid/Ascorbate**

Ascorbic acid, also known as ascorbate, but commercially known as vitamin C, serve many functions within plants considering it is a ubiquitous molecule found within most eukaryotic organisms. One of its functions is serving as an antioxidant and participates in a wide range of processes within the plant that are required for its' survival, including the resistance to various environmental stresses (Smirnoff and Wheeler 2000). It is considered one of the most powerful ROS scavenger (directly scavenging $O_2^-$ and $OH^-$) as it donates numerous electrons in enzymatic and non – enzymatic reactions (Gill and Tuteja 2010). Ascorbate is well synthesised and used in the Halliwell – Asada pathway as it is required as a substrate for the scavenging of $H_2O_2$ by APX to yield MDHA which in turn is reduced to ascorbate in the presence of an electron donor (NADPH; Figure 1.6).

**Glutathione**

Glutathione is a molecule that is of high importance within the plant systems by serving essential roles in the plant. These roles include functioning as an antioxidant as well as serve as a cofactor of various enzymatic processes among other roles (El – Enany 1997). This metabolite can occur in two forms: reduced glutathione (GSH) and oxidised glutathione (GSSG). To generate GSSG from GSH, the cys thiol group is oxidised. However, the reverse reaction is catalysed by GR using NADPH as electron donors (Yannarelli *et al.* 2007). In terms of the Hallwell – Asada pathway, glutathione serves as a substrate. However, its antioxidant capacity allows it to scavenge $O_2^-$, $H_2O_2$ and $OH^-$, thus assisting in the reduction of toxic ROS molecules (Gill and Tuteja 2010).



## 1.6. Plant polyphenols

Plants naturally produce phenolic or polyphenolic compounds as secondary metabolites that has now been referred to as plant polyphenols (Duthie *et al*. 2003; Pandey and Rizvi 2009). They are largely distributed throughout most plant tissues (Martin and Appel 2010) and are involved in diverse functions that contribute to the health and survival of the plant (Duthie *et al*. 2003).

### 1.6.1. Importance of plant polyphenols

Plant polyphenols exhibit vital roles in physiology and morphology within plants. They contribute to growth, reproduction, pigmentation, pathogen resistance, structure and lignification, predator resistance, pollination and allelopathy (Balasundram *et al*.

2006; Duthie *et al*. 2003; Martin and Appel 2010). Specifically to food crops, these compounds may contribute to bitterness, colour, flavour, stringency and odour (Pandey and Rizvi 2009).

Furthermore, these polyphenols are essential components of the human diet given its antioxidant properties, free radical scavenging capabilities and their ability to reduce the effects of oxidative stress – induced tissue damage relating to chronic diseases. Hence, some polyphenols function as antibiotics and enforce anticancer, anti – inflammatory, antimutagenic, antiulcer, anti – carcinogenic and antidiarrheal effects (Balasundram *et al*. 2006; Karakaya 2004; Martin and Appel 2010).



### 1.6.2. Biosynthesis of plant polyphenols

Phenolics are derived from the pentose phosphate, shikimate and phenylpropanoid pathways (PPP) within plants (Figure 1.7; Balasundram *et al*. 2006; Duthie *et al*. 2003; Randhir *et al*. 2004). These pathways function simultaneously in order to synthesise these polyphenols. The generation of phenolic compounds begin with the oxidative PPP providing a precursor, known as erythrose – 4 – phosphate, for the shikimate pathway. As a response, the shikimate pathway converts these sugar phosphates into aromatic amino acids such as phenylalanine, which in turn becomes the precursor for the phenylpropanoid pathway and ultimately producing various phenolic compounds (Lin *et al*. 2016).

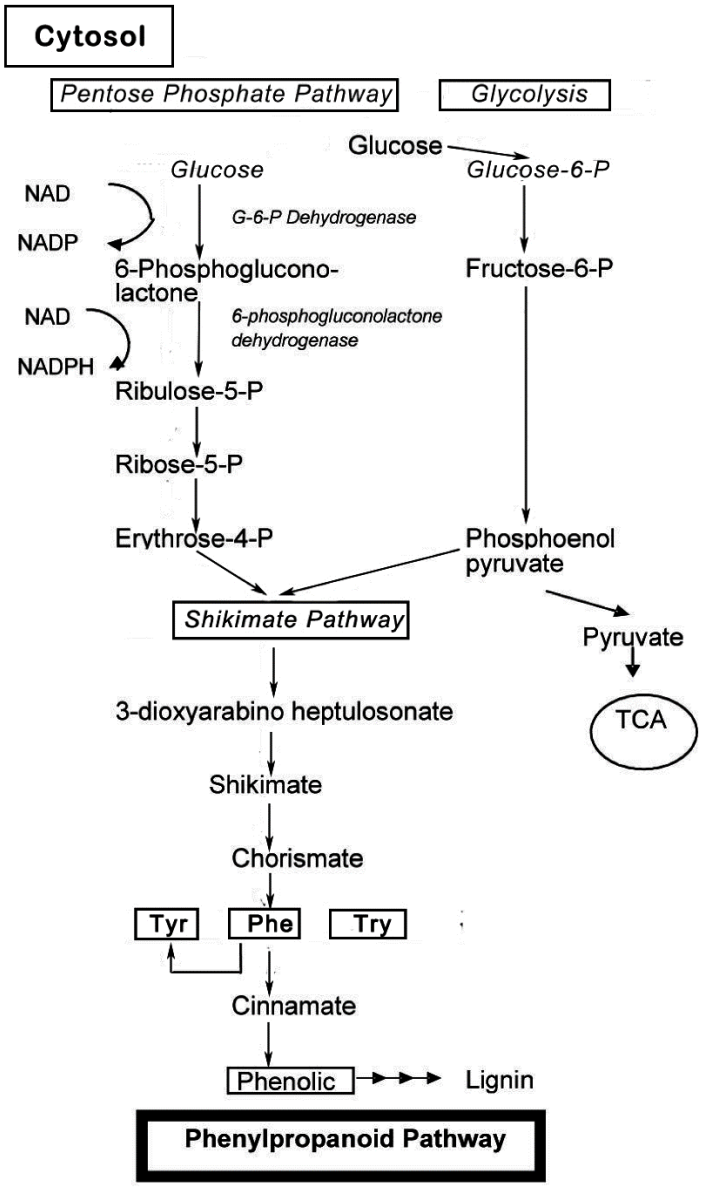



**Figure 1.7. Synthesis of phenolic compounds via the pentose phosphate pathway (PPP).** Oxidative PPP provides a precursor erythrose – 4 – phosphate for the shikimate pathway. As a response, the shikimate pathway converts these sugar phosphates into aromatic amino acids such as phenylalanine (Phe), which in turn becomes the precursor for the phenylpropanoid pathway (Lin *et al*. 2016).

### 1.6.3. Structure and classes of plant polyphenols

The capabilities of plant polyphenols' antioxidant power are dependent on its chemical structure (Figure 1.8). They possess the ideal chemical structure for free radical scavenging activities as they retain phenolic hydroxyl groups that are prone

to donate an electron or a hydrogen atom to a free radical. They also exhibit an extended conjugated aromatic system that delocalizes an unpaired electron (Balasundram *et al*. 2006; Dai and Mumper 2010).

Plant polyphenols, or phenolic compounds, include phenolic acids, flavonoids, tannins, stilbenes and lignans (Dai and Mumper 2010). All these phenolic compounds have two common characteristics: the presence of at least one aromatic ring hydroxyl – substituted; and the fact that they are bound to sugars and/or proteins (Giada 2013).

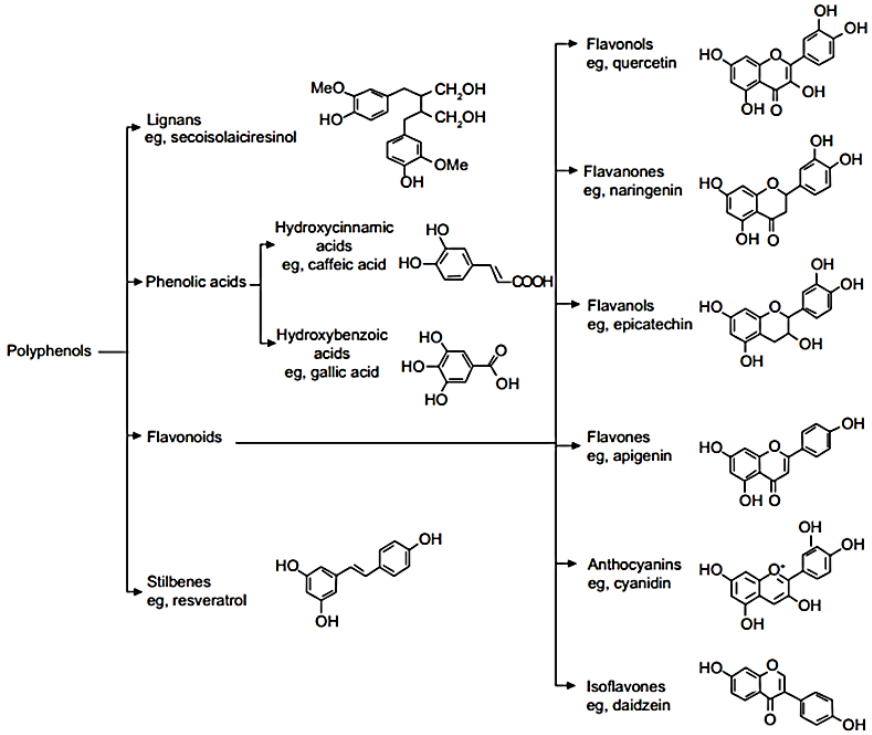



**Figure 1.8. Classification and chemical structures of plant polyphenols.** The diagram was adapted from Martin and Appel (2010).

More than 8 000 phenolic structures have been identified (Dai and Mumper 2010; Martin and Appel 2010; Pandey and Rizvi 2009) and of these structures, flavonoids, including phenolic acids, are known to be most abundant, covering over half of the known phenolic compounds (Balasundram *et al*. 2006). Flavonoids can be further

divided into six subgroups: flavonols, flavanones, flavanols, flavones, anthocyanins, isoflavones, based on the oxidation state of the central C ring (Dai and Mumper 2010; Karakaya 2004; Martin and Appel 2010).

On the other hand, phenolic acids are divided into two classes: hydroxybenzoic acids (seven carbon atoms) and hydroxycinnamic acids (nine carbon atoms). Benzoic acid derivatives include gallic acid, vanillic and syringic acids whereas the cinnamic acids include ferulic acid, sinapic acid and most importantly (to this study), caffeic acid (Balasundram *et al*. 2006; Dai and Mumper 2010).

### 1.6.4. Biosynthesis and function of phenolic acids in plants

Phenolic acids, famous for their pharmacological characteristics, are known to be one of the most abundant secondary metabolites and vital bioactive compounds within the plant system. They are also appreciated for their contribution to unique taste, flavour and health – promoting properties found in fruits and vegetables. Hence, there is a direct link between phenolic content and crop quality (Ghasemzadeh and Ghasemzadeh 2011)



Recently, the importance of phenolic compounds and its role in food crops have been excessively studied given phenolic acids featuring in agricultural, chemical, biological as well as medicinal studies. Therefore, given the extensive phenolic research, the biosynthetic pathway of these compounds are well known (Figure 1.9; Ewane *et al*. 2012; Dixon and Paiva 1995; Sakihama *et al*. 2002) and are described to derive from the shikimate pathway from simple sugars resulting from primary metabolism.

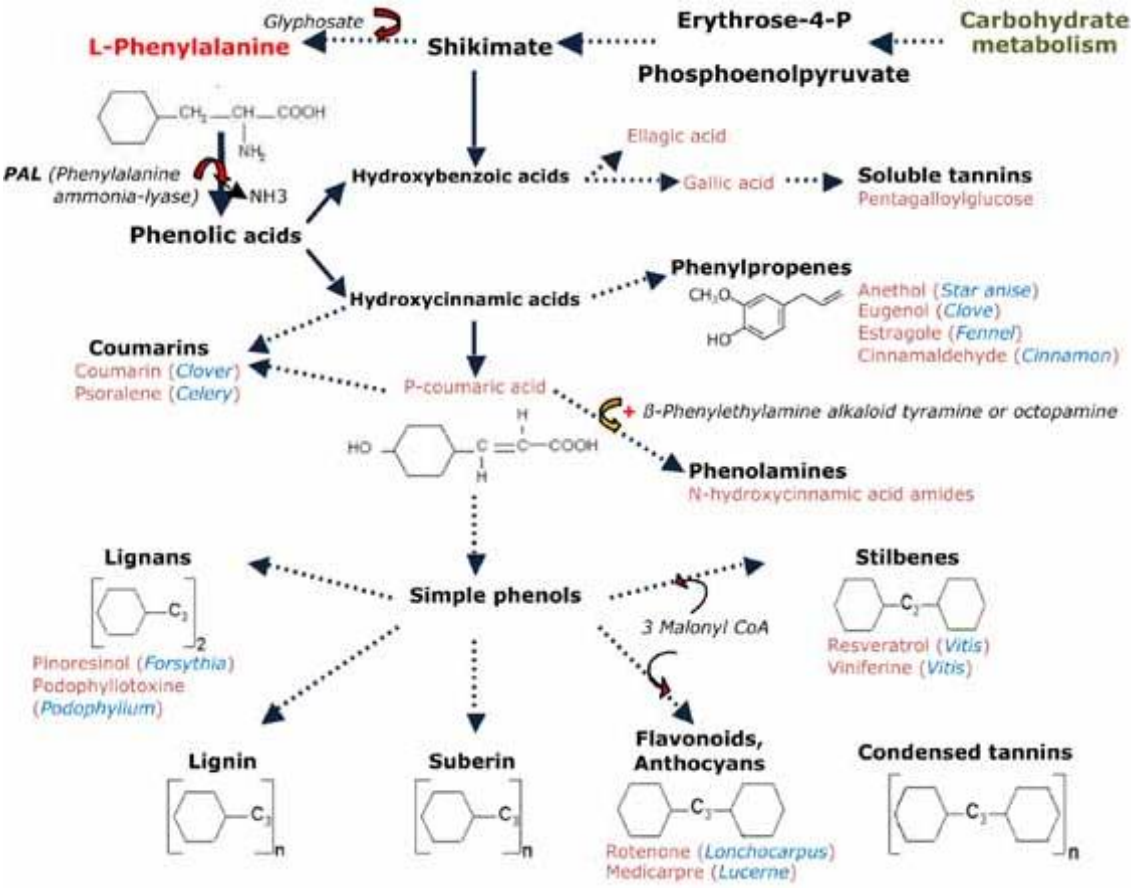



**Figure 1.9. Biosynthesis of phenolic compounds.** The diagram was adapted from Ewane *et al.* (2012).



The diagram illustrates the synthesis of phenylalanine via carbohydrate metabolism. These carbohydrates transform into erythrose – 4 – phosphate (pentose phosphate pathway) and phosphoenolpyruvate (glycolysis). The transformed carbohydrates then enter the shikimate metabolic pathway. Thereafter, phenylalanine deamination occurs via phenylalanine ammonia – lyase (PAL), inducing the first crucial stage in the biosynthesis of phenolic compounds. The diagram also indicates the division of phenolic acids, hence, these acids are widely distributed within the plant as hydroxycinnamic and hydroxybenzoic acids (Ewane *et al*. 2012).

### 1.6.5. Biosynthesis of hydroxycinnamic acids

The biosynthesis of hydroxycinnamic acids (caffeic, ferulic, 5 – hydroxy ferulic and sinapic acids) uses phosphoenolpyruvate and erythrose – 4 – phosphate as its starting material (Figure 1.10). Thereafter, the shikimate pathway leads to the synthesis of phenylalanine and tyrosine, initiating phenyl deamination via PAL (phenylalanine ammonia – lyase; Krause *et al.* 2003).

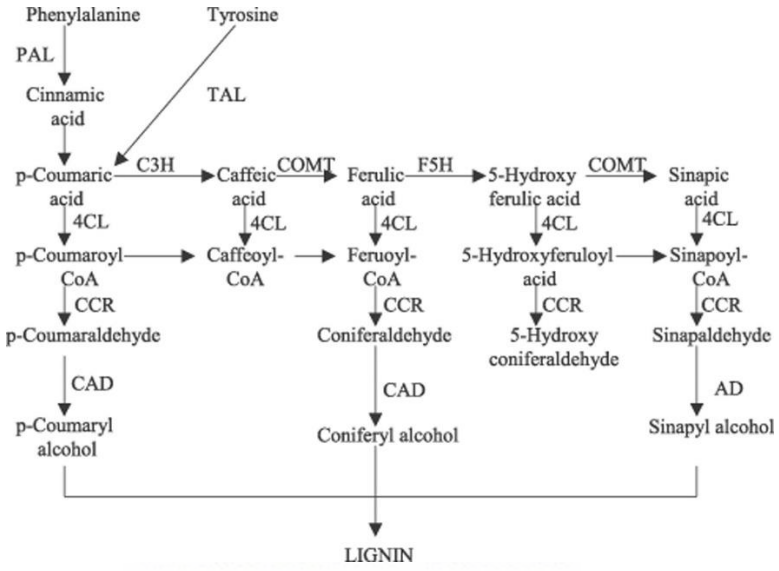



**Figure 1.10. Biosynthesis of hydroxycinnamic acids.** The diagram was adapted from Krause *et al.* (2003).

In plants, tyrosine ammonia – lyase (TAL) converts tyrosine into 4 – hydroxycinnamic acid (*p* – coumaric acid), which can later be transformed into either caffeic, ferulic or sinapic acid. This pathway is responsible for the production of numerous secondary metabolites as well as lignin and lignin precursors known as CoA derivatives (Krause *et al.* 2003).

These CoA derivatives are known to possess multiple purposes. They are believed to be the chemical source to cell bound hydroxycinnamic acids. In addition, the thioester linkage formation between the CoA and cinnamate is known to activate the carbonyl group on the hydroxycinnamic acid, inducing various condensation and conjugation reactions that initiates the production of flavonoids and stilbenoids (El – Seedi *et al*. 2012). However, the first hydroxycinnamic acid derived from *p* – coumaric acid, as well as the most abundant in fruit sources, is caffeic acid.

### 1.6.6. Caffeic acid as a phenolic compound with antioxidant properties

The hydroxycinnamic compound, caffeic acid (CA), has been described as the most abundant phenolic acid in many agricultural products including vegetables, cereals, legumes and fruit sources (Belay *et al*. 2016). They are often esterified with chlorogenic acid, a major phenolic compound found in coffee (Figure 1.11; Dai and Mumper 2010; Karakaya 2004). Caffeic acid (3,4 – dihydroxycinnamic acid) has been shown to serve as an α – tocopherol protectant in LDL (low – density lipoprotein). In addition, this phenolic acid, along with its conjugates (chlorogenic and caftaric acids) have been shown to possess powerful antioxidant properties in various systems (Gulcin 2006).

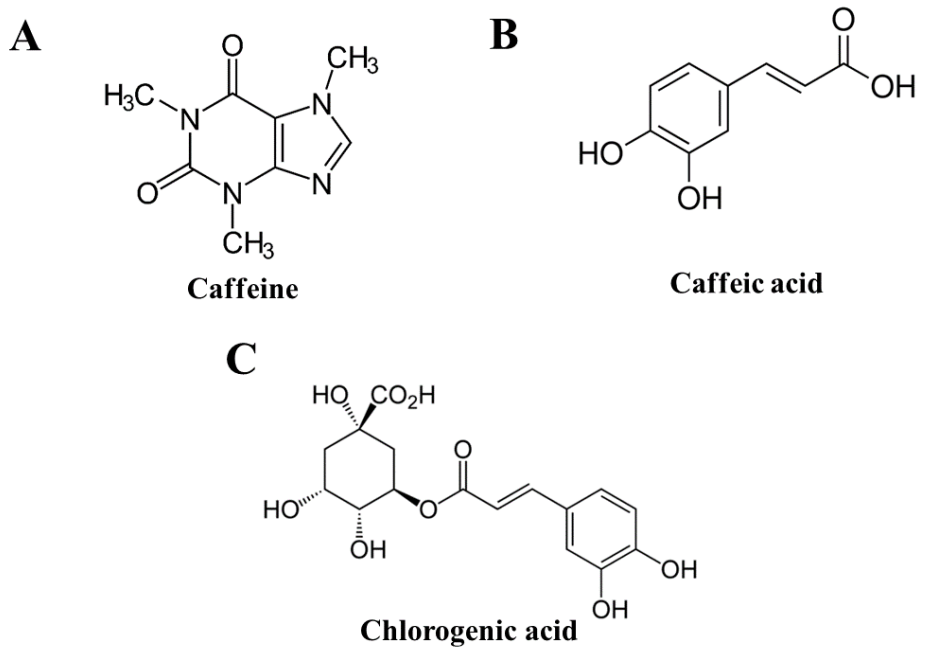



**Figure 1.11. Molecular structure of caffeine (a), caffeic acid (b) and chlorogenic acid (c).** The diagram was adapted from Belay *et al*. (2016).



As previously described, antioxidant compounds exist solely to prevent the effects of oxidative stress (Dai and Mumper 2010). Caffeic acid (CA) has been recognised as one of those antioxidants given its involvement in anti – inflammatory, antiviral, immunomodulatory functions, anticarcinogenic and antioxidant activities (Klein 2012). Aside from this compound being a part of these numerous essential activities, they function in inhibiting lipid peroxidation in plant cells, reduce lipoxygenase activity and completely block ROS accumulation (Sud'ina *et al.* 1993).

Phenolic compounds, generally, scavenge free radicals through a series of coupled reactions involving enzymatic and non – enzymatic antioxidants. However, caffeic acid, in comparison to ferulic and *p* – coumaric acids, has been recognised as a superior antioxidant in inhibiting LDL oxidation whilst scavenging free radicals and

singlet oxygen (Gulcin 2006). Hence, given its powerful status within recent studies, caffeic acid has been deemed to serve as a potent antioxidant within various systems.

### 1.6.6.1. Caffeic acid in plants

Caffeic acid is a secondary metabolite that can be isolated from a variety of plant species (Table 1.1). They serve as effective substrates of polyphenol oxidases and if conditions allow, they may undergo oxidation in plant tissues or in various plant – based products (Gulcin 2006).

**Table 1.1.** Detection of caffeic acid in different plant species. The table was adapted from Klein (2012).

| Plant species | Organ | [] as caffeic acid (mg/kg), fresh weight | Hydrolysis |
|---|---|---|---|
| | Vegetables | | |
| **Bean** | Hulls, unripe fruit | 12-14; <0.5-9 | Enzymatic |
| **Cabbage** | Outer leaves | 11-44 | Enzymatic |
| **Carrot** | Whole vegetable | 18-96 | Enzymatic |
| **Pea** | Unripe seeds | <0.5-1 | Enzymatic |
| **Potato** | Peel | 63-280 | Enzymatic |
| | Fruits | | |
| **Grape fruit** | Fruit/peel | 11-40 / 14-51 | Enzymatic |
| **Lemon** | Fruit/peel | 13-27 / 16-35 | Enzymatic |
| **Orange** | Fruit/peel | 19-50 / 12-36 | Enzymatic |
| **Strawberry** | Fruit | <0.5-14 | Enzymatic |
| **Watermelon** | Fruit/peel | <0.5 | Enzymatic |

Although caffeic acid forms one of the most common cinnamic acids isolated from crops plants (Rice 1995), research has also shown its role in plant growth inhibition in response to various concentrations. Batish *et al*. (2008) has shown caffeic acid

concentrations ranging between 0 mM to 10000 mM had significantly supressed the root growth as well as impair adventitious root formation (ARF) in mung bean (*Vigna radiate*). In addition, this allelopathic effect was also witnessed in soybean (*Glycine max*) seedlings whereby caffeic acid had shown to impair soybean root biomass (Bubna *et al*. 2011). However, in 2013, Klein *et al*. further explains, through soybean studies, that along with the reduction in root growth, also witnessed in their study, they also observed the antioxidant effect of caffeic acid by means of reduced ROS accumulation. Furthermore, these authors justify stunted growth by explaining that caffeic acid causes premature lignification of plant cell walls (Klein *et al.* 2013). Therefore, although various studies have shown that caffeic acid could cause plant growth inhibition, other studies also show the inhibition effect parallel to the antioxidant effect of caffeic acid.



## 1.7. Proteomics

The term "Proteomics" derived from "proteome" expression which was first used in 1995 to describe the protein complement to the genome (Blackstock and Weir 1999; Chen and Harmon 2006). Proteomics has since been defined as the study and determination of various protein properties. The technique studies the protein expression levels, interactions as well as post – translational modifications (PTM) on a large scale to generate a global, integrate understanding of cellular processes as well as networks at a protein level (Aebersold and Mann 2003; Blackstock and Weir 1999).

### 1.7.1. Significance of proteomic research

The development of proteomic studies stems from researchers realising that although the extensive DNA information availability, which was usually obtained via various high – throughput technologies, including transcript imaging, differential display, SAGE (serial analysis of gene expression) and DNA microarrays (Anderson and Seilhamer 1997; Lockhart and Winzeler 2000), one still cannot elucidate biological function (Pandey and Mann 2000). This was due to the above – mentioned techniques not guaranteeing information based on the quality and the quantity of the resulting gene products (proteins; Ngara 2009; Zivy and de Vienne 2000), hence the introduction of proteomic studies.

In addition, proteins tend to undergo post – translational modifications (PTM) such as glycosylation, proteolytic processing or phosphorylation, consequently giving rise to the production of various isoforms from a single gene product (Abbott 1999; Ngara 2009). Therefore, PTM proteins play a vital role in subcellular localization of proteins within a cell, as well as regulating the function and maintaining protein stability (Kersten *et al*. 2009; van Wijk 2001; Zivy and de Vienne 2000). Hence, given that PTM proteins show variation in response to various physiological states, only the investigation of protein expression itself would contribute to a better understanding of gene functions in response to various environmental conditions or physiological states (Dubey and Grover 2001).

Although DNA information could provide answers to various genomic queries, proteins are believed to be to the effectors of biological function. Therefore, the expression levels of all these proteins could provide more accurate data when characterising a biological system (Cox and Mann 2007). Thus, since there is a direct

relationship between genes (genomics) and proteins (proteomics), researchers begin to embark on plant proteomics to contribute to the available genomic data to allow for a better understanding of the biological networks within various plant species.

### 1.7.2. Plant proteomics

Although proteomics has been used extensively in many areas since the 1970's when researchers began building protein databases (Pandey and Mann 2000), plant proteomics was still in it's infancy in the early twenty first century (Chen and Harmon 2006; van Wijk 2001). However, the successful completion of the *Arabidopsis* genome sequence (model organism) had initiated the application of high – throughput technologies in plant – based studies, hence providing greater knowledge and understanding of complex biological networks (Kersten *et al*. 2002). Therefore, there is a direct link between proteomic studies and genomic data given that genome sequences are important resource tools for protein identification. However, where there is a lack of fully annotated sequences, protein identification can be determined via similarity searches of homologous proteins in closely related species (Carpentier *et al*. 2008).

The genome sequence availability for *Arabidopsis* has evoked further research including rice (*Oryza sativa*) and poplar (*Populus*) as well as introducing expressed sequence tag (EST) databases and gene indices for numerous plant species giving rise to the advances in plant proteomics (Chen and Harmon 2006).

### 1.7.2.1. Applications of proteomics in plant sciences

Once the *Arabidopsis* genome was published, many researchers embarked on plant proteomic research. In 2015, Jorrin – Novo and authors compiled a review on the advances on plant proteomics whereby stating all the crops that appeared in the Proteomics journal. However, not one article was dedicated to a pseudocereal. Thus, research regarding pseudocereal proteomic analysis remains limited. To date only a few documented cases can be accessed in the public domain. Galova *et al*. (2015) comparatively analysed the leaf proteomes of cereals (wheat) and pseudocereals (amaranth), respectively. This study identified distinct differences (using 2D PAGE analysis) in protein expression profiles between cereals and pseudocereals within the 40 to 200 000 molecular weight (MW) range and isoelectric point (pI) region of 9 – 11. Proteins detected in this molecular weight range were only present in the cereal proteome and absent in the pseudocereal proteome. The key nutritional difference between a cereal and a pseudocereal crop is gluten – based (Tang *et al*. 2016). Hence, it was no surprise for Galova *et al*. (2015) to identify those absent proteins as gluten – associated proteins involved in celiac disease (CD). This in turn supports the evidence that pseudocereals are gluten free as explained in the section 1.2. In addition, the proteome profile of amaranth in response to salt stress have also been described by Huerta – Ocampo *et al*. in 2014, thus creating a basis for the research proposed in this study.

# Project aims and objectives

This research aims to determine the effect of exogenously applied caffeic acid on chia (*Salvia hispanica* L.) plants under salt stress. The effect of salt stress on cereal crops are well documented, however, limited information in the public domain focuses on how pseudocereals respond to saline environments. Furthermore, to our knowledge, the effect of exogenously applied caffeic acid on pseudocereal crops have not been elucidated. Therefore, this study aims to achieve the effect of caffeic acid under salt stress on the physiological responses of chia plants by analysing various growth parameters. These parameters include shoot and root length and biomass, relative water content and photosynthetic pigments. In addition, this study targets the biochemical responses of chia plants (to exogenous caffeic acid and salt stress) by investigating the overproduction of ROS molecules ($O_2^-$ and $H_2O_2$), lipid peroxidation, cell death and various enzymatic antioxidant activities (SOD, APX and GR). Moreover, this report extends to gel based proteomic studies, coupled with mass spectrometry, in order to elucidate the effect of exogenously applied caffeic acid, under salt stress, on the chia leaf proteome. Hence, since the relationship between caffeic acid and salt stress, as well as its combined effect on pseudocereals, have not been reported, it provides a knowledge gap for which this research will contribute.

# CHAPTER 2

# METHODS AND MATERIALS

## 2.1. General chemicals and suppliers

**Table 2.1.** List of chemicals and reagents used in this study

| Chemical / Reagent | Supplier |
|---|---|
| Acetone | Merck Millipore |
| Acrylamide/Bis (40 %) | BIO – RAD |
| Acetonitrile (ACN) | Merck Millipore |
| Agarose D – 1 LE | White Scientific |
| Ammonium acetate ($C_2H_3O_2NH_4$) | Sigma Aldrich |
| Ammonium Bicarbonate (AmBic) | Merck Millipore |
| Ammonium nitrate ($NH_4NO_3$) | Sigma Aldrich |
| Ammonium Persulfate (APS) | BIO – RAD |
| Ascorbic acid / Ascorbate | Sigma Aldrich |
| Bio – Lyte 3/10 Ampholyte (100 X) | BIO – RAD |
| Borric acid ($H_3BO_3$) | Sigma Aldrich |
| Bovine Serum Albumin (BSA) Fraction V | Roche |
| Bradford Reagent (1X) | BIO – RAD |
| Bromophenol blue | Sigma Aldrich |
| Caffeic acid | Sigma Aldrich |
| Calcium chloride ($CaCl_2$) | Sigma Aldrich |
| 3-[(3-Cholamidopropyl)dimethylammonio]1-Propanesulfonate CHAPS | Sigma Aldrich |
| Cobalt (II) chloride ($CoSO_4$) | Sigma Aldrich |
| Coomassie® brilliant blue (CBB) R-250 | BIO – RAD |
| Copper (II) sulfate ($CuSO_4$) | Sigma Aldrich |
| 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) | Sigma Aldrich |
| Dithiothreitol (DTT) Cleland's reagent | Fermentas |

| | |
|---|---|
| Electrode wicks (gel-side down) | BIO – RAD |
| Ethanol 99.9% | Kimix |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma Aldrich |
| Ethylenediaminetetraacetic acid ferric sodium salt (Fe-Na EDTA) | Sigma Aldrich |
| Evans Blue | Sigma Aldrich |
| Glacial acetic acid | Merck Millipore |
| Glucose | Merck Millipore |
| Glutathione disulfide (GSSG) | Sigma Aldrich |
| Glycerol | Merck Millipore |
| Glycine | BIO – RAD |
| Hydrochloric acid (HCl) | Merck Millipore |
| Hydrogen peroxide ($H_2O_2$) | Merck Millipore |
| Iodoacetamide (IOA) | BIO – RAD |
| Magnesium sulfate ($MgSO_4$) | Sigma Aldrich |
| Manganese (II) sulfate ($MnSO_4$) | Sigma Aldrich |
| 2-(N-Morpholino)ethanesulfonic acid (MES) hydrate | Sigma Aldrich |
| β-mercaptoethanol | Amresco |
| Methanol | Merck Millipore |
| Methionine | Sigma Aldrich |
| Methylthiazolyldiphenyl-tetrazolium bromide (MTT) | Sigma Aldrich |
| Mineral oil (PlusOne DryStrip Cover Fluid) | GE Healthcare |
| B-nicotinamide adenine dinucleotide (NADH) | Sigma Aldrich |
| Nitrotetrazolium blue chloride powder (NBT) | Sigma Aldrich |
| PageRuler™ unstained protein ladder | Fermentas |
| Phenazine methosulfate (PMF) | Sigma Aldrich |
| Phenylmethylsulfonyl fluoride (PMSF) | Amresco |
| Polyvinylpyrrolidone (PVP) MW: 40 000 | Sigma Aldrich |
| Potassium cyanide (KCN) | Sigma Aldrich |
| Potassium hydroxide pellets | Merck Millipore |
| Potassium iodide (KI) | Sigma Aldrich |
| Potassium nitrate ($KNO_3$) | Sigma Aldrich |

| | |
|---|---|
| Potassium phosphate monobasic ($KH_2PO_4$) | Sigma Aldrich |
| Potassium phosphate dibasic ($K_2HPO_4$) | Sigma Aldrich |
| Potassium sulfate ($K_2SO_4$) | Sigma Aldrich |
| Promix Organic | Cypress House Trading |
| Propan-2-ol (isopropanol) | Merck Millipore |
| Ready strip$^{TM}$ IPG strips | BIO – RAD |
| Riboflavin | Sigma Aldrich |
| Sodium chloride (NaCl) | Merck Millipore |
| Sodium dodecyl sulfate (SDS) | BIO – RAD |
| Sodium hydroxide (NaOH) | Merck Millipore |
| Sodium molybdate ($Na_2MoO_4$) | Sigma Aldrich |
| Sucrose | Merck Millipore |
| N,N,N',N'-Tetramethylethylenediamine (TEMED) | BIO – RAD |
| Thiobarbituric acid (TBA) | Sigma Aldrich |
| Thiourea | Sigma Aldrich |
| Trichloroacetic acid (TCA) | Merck Millipore |
| Trifluoroacetic acid (TFA) | Merck Millipore |
| Tris(hydroxymethyl)-aminethane | BIO – RAD |
| Trypsin | Promega |
| Urea | Sigma Aldrich |
| 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) | Sigma Aldrich |
| Zinc sulfate monohydrate ($ZnSO_4$) | Sigma Aldrich |



## 2.2. Stock solutions and buffers

**Table 2.2.** List of stock solutions and buffers prepared for this study

| Stock solution / Buffer | Composition |
|---|---|
| **Agarose sealing solution (0.5 %)** | 0.5 % (w/v) agarose prepared in 1 X SDS-PAGE running buffer with a tint of bromophenol blue. |

| | |
|---|---|
| **Acetone (80 %)** | 80 % (v/v) acetone in distilled water. |
| **APS (10 %)** | 10 % (w/v) APS in distilled water. The solution was freshly prepared before use. |
| **APX spectrophotometer buffer** | 50 mM $K_2HPO_4$ at pH 7.0; 0.2 mM EDTA; 0.25 mM ascorbic acid in d.$H_2O$. |
| **BSA stock solution I (5 mg/ml)** | 5 mg/ml BSA in PVP extraction buffer |
| **BSA stock solution II (5 mg/ml)** | 5 mg/ml BSA in IEF buffer |
| **Caffeic acid (5 mM) stock solution** | 5 mM caffeic acid dissolved in 6 % ethanol and made up with d.$H_2O$. |
| **CBB stock solution (1.25 %)** | 1.25 % (w/v) CBB R-250 in d.$H_2O$. |
| **CBB staining solution I** | 50 ml of 1.25 % (w/v) CBB stock solution, 10 % (v/v) glacial acetic acid and 25 % (v/v) propan-2-ol in d.$H_2O$. |
| **CBB staining solution II** | 6.25 ml of 1.25 % (w/v) CBB stock solution, 10 % (v/v) glacial acetic acid and 10 % (v/v) propan-2-ol in d.$H_2O$. |
| **CBB staining solution III** | 6.25 ml of 1.25 % (w/v) CBB stock solution and 10 % (v/v) glacial acetic acid in d.$H_2O$. |
| **Destaining solution** | 10 % (v/v) acetic acid and 1 % (v/v) glycerol in d.$H_2O$. |
| **Ethanol (70 %)** | 70% (v/v) ethanol in d.$H_2O$. |
| **Equilibration buffer** | 6 M urea; 2 % (w/v) SDS, 50 mM Tris – HCl, pH 8.8 and 20 % (v/v) glycerol in d.$H_2O$. |

| | |
|---|---|
| **Evans blue stock solution (0.5 %)** | 0.5% (w/v) Evans blue in d.$H_2O$. |
| **GR spectrophotometer buffer** | 100 mM $K_2HPO_4$ at pH 7.8; 0.5 mM GSSG; 1 mM EDTA; 0.2 mM NADH in d.$H_2O$. |
| **HCl (1 M) for pH** | 1 M HCl in d.$H_2O$. |
| **IEF buffer** | 7 M Urea; 2 M thiourea; 4 % (w/v) CHAPS; 20 mM DTT; 1 % (w/v) bromophenol blue in d.$H_2O$. |
| **$KH_2PO_4$ (1M) stock solution** | 1 M $KH_2PO_4$ in d.$H_2O$. |
| **$K_2HPO_4$ (1M) stock solution** | 1 M $K_2HPO_4$ in d.$H_2O$. |
| **KI (0.5 M) stock solution** | 0.5 M KI in d.$H_2O$. |
| **KOH (5 M) for nutrient solution pH** | 5 M KOH in d.$H_2O$. |
| **Native gel running buffer stock solution (5 X)** | 25 mM Tris-base; 192 mM glycine in d.$H_2O$. |
| **Native gel loading dye (6 X)** | 375 mM Tris-HCl at pH 6.8; 50 % (v/v) glycerol; 0.02 % (w/v) bromophenol blue in d.$H_2O$. |
| **Nutrient solution (1 X)** | 0.5 mM $K_2SO_4$; 0.5 mM $MgSO_4$; 1 mM $CaCl_2$; 10 mM $KNO_3$; 2 mM $NH_4NO_3$; 8 mM $KH_2PO_4$ buffer at pH 6.4; 30 µM $H_3BO_3$; 10 µM $MnSO_4$; 0.7 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 1 µM $Na_2MoO_4$; 0.2 µM $CoSO_4$; 50 µM Fe – Na EDTA and 10 mM MES at pH 6.4 in d.$H_2O$. |

| | |
|---|---|
| **PVP extraction buffer** | 40 mM $K_2HPO_4$ at pH 7.4; 1 mM EDTA; 5 % PVP MW = 40 000; 5 % glycerol in d.$H_2O$. |
| **SDS buffer** | 0.1 M Tris-HCl, pH 8.0; 2 % (w/v) SDS; 5 % (v/v) β-mercaptoethanol; 30 % (w/v) sucrose and 1 mM PMSF in d.$H_2O$. |
| **SDS gel loading dye** | 100 mM Tris-HCl at pH 6.8; 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 200 mM DTT in d.$H_2O$. |
| **SDS running buffer stock solution (5 X)** | 25 mM Tris-base; 192 mM glycine; 0.1 % (w/v) SDS in d.$H_2O$. |
| **SDS (10 %) stock solution** | 10 % (w/v) SDS in d.$H_2O$. |
| **SOD spectrophotometer buffer** | 50 mM $KPO_4$ at pH 7.4; 13 mM methionine; 75 µM NBT; 0.1 mM EDTA; 2 µM riboflavin in d.$H_2O$. |
| **TCA (6 %) extraction buffer** | 6 % (w/v) TCA in d.$H_2O$. |
| **TCA/Acetone (10 %)** | 10% (w/v) TCA in acetone. |
| **TCA (20 %) / TBA (0.5 %)** | 0.5 % (w/v) TBA in 20 % (v/v) TCA stock solution |
| **Tris-HCl (0.1 M), pH 7.9** | 0.1 M Tris in d.$H_2O$ adjusted to pH 7.9 with concentrated HCl. |
| **Tris-HCl (0.5 M), pH 6.8** | 0.5 M Tris in d.$H_2O$ adjusted to pH 6.8 with concentrated HCl. |
| **Tris-HCl (1.5 M), pH 8.8** | 1.5 M Tris in d.$H_2O$ adjusted to pH 8.8 with concentrated HCl. |

## 2.3. Plant growth and treatment

Chia (*Salvia hispanica*) seeds (purchased from Faithful to Nature, Cape Town, South Africa) were germinated on wet filter paper in a dark environment for 2 – 3 days. The germinated seedlings were transplanted (1 per pot) in a moist (distilled water) promix growth medium (Stodels Garden Centre, Brackenfell, South Africa) and were allowed to grow in a growth room on a 16 hour light/8 hour dark cycle at 25°C until the first leaves were fully expanded. Seedlings were supplemented with 50 ml of 1 X nutrient solution (see section 2.2) at 2 – day intervals until the collar of the second true leaves were visible.

At this stage, control plants were supplied with 1 X nutrient solution every second day. For treatments, the nutrient solution was supplemented with the following final concentrations: 100 µM caffeic acid, 100 mM NaCl and a combination of 100 µM caffeic acid with 100 mM NaCl. Treatments or nutrient solution (40 ml per pot) were applied to each plant directly at the base of the stem of the plant in the pot every second day for 21 days.



## 2.4. Analysis of plant growth

After 21 days of treatment, plants were removed from the growth medium, being careful to avoid any loss of shoots and roots during the up – rooting of the plants. Plants from each treatment were scored for shoot length, root length, shoot and root fresh weight (FW) and shoot and root dry weight (DW). The DW was determined by heating the shoot and root samples in an oven at 55°C for 48 hours as described by Valentovic *et al*. (2006).

## 2.5. Measuring relative water content

Relative water content (RWC) was measured as described by Mohammadkhani and Heidari (2007) using the following formula:

$$RWC = \frac{Fresh\ weight\ (FW) - Dry\ weight\ (DW)}{Turgid\ weight\ (TW) - Dry\ weight\ (DW)}\ x\ 100$$

## 2.6. Measurement of leaf chlorophyll and carotenoid content

Chlorophyll and carotenoid content was estimated using a modified method of Lichtenthaler and Wellburn (1983). Leaf tissue (100 mg) was submerged in 1 ml of 80 % acetone. Leaf extracts were vortexed and centrifuged at 10 000 $X\ g$ for 10 minutes. This process was repeated until a clear pellet was observed. The absorbance of different fractions (200 µl) was recorded at 470 nm, 663 nm, and 646 nm. Chlorophyll and carotenoid content was calculated using the following formulas:



$$Chlorphyll\ \underline{\boldsymbol{a}}\ (\mu g/ml) = 12.21\ (A_{663}) - 2.81\ (A_{646})$$

$$Chlorphyll\ \underline{\boldsymbol{b}}\ (\mu g/ml) = 20.13\ (A_{663}) - 5.03\ (A_{646})$$

$$Carotenoids\ (\mu g/ml) = (1000A_{470} - 3.27[Chl\ a] - 104[Chl\ b]) / 227$$

## 2.7. Measurement of cell viability

Cell viability was measured in the leaves of chia plants using a modified method described by Sanevas *et al*. (2007). Fresh leaf tissue was harvested from three different plants per treatment (approximately 100 mg per treatment) and stained with 0.25 % (w/v) Evan's Blue for 60 minutes at room temperature. The leaves were washed with distilled water for 90 minutes at room temperature to remove surface –

bound dye. This process was followed by the extraction of the Evans Blue stain (taken up by dead cells) from leaf tissue using 1 % (w/v) SDS, after 1 hour incubation at 55°C. Absorbance of the extracts was measured at 600 nm to determine the level of Evans Blue up – take by the leaf tissue

## 2.8. Protein extraction for biochemical analysis

Leaf tissue from all treatments were harvested and ground into a fine powder using liquid nitrogen. Leaf material (200 mg) was homogenized in 1 ml of 6 % (w/v) trichloroacetic acid (TCA) for analysis of $H_2O_2$ content and lipid peroxidation or in 1 ml of homogenizing buffer (PVP) (see section 2.2) for the measurement of antioxidant enzymatic activities. Protein concentrations for all assays were measured in extracts as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA).



## 2.9. Assays for ROS accumulation

For the detection of $O_2^-$ in chia leaves, a modified method by Able *et al*. (1998) was used. Leaf material (100 mg) for each treatment were homogenized in 500 µl of 50 mM potassium phosphate buffer (pH 8.2) which contained 0.12 mM 3 – bis(2 – methoxy – 4 – nitro – 5 – sulfophenyl) – 2H – tet – trazolium – 5 – carboxyanilide (XTT). The homogenate was incubated in the dark at room temperature for 20 minutes and centrifuged at 12 000 *X g* for 5 minutes to obtain the $O_2^-$ extract. The $O_2^-$ extracts were then measured as described by Sutherland and Learmonth (1997).

For $H_2O_2$ content analysis in chia leaves, a method previously described by Velikova *et al*. (2000) was used. The reaction mixture contained 50 µl of TCA protein extract, 5 mM $K_2HPO_4$ (pH 5.0) and 0.5 M KI. The sample mixture was incubated at room temperature for 20 minutes and the absorbance readings were recorded at 390 nm. Therefore, the $H_2O_2$ content was calculated based on the standard curve constructed from the absorbance ($A_{390}$ nm) of $H_2O_2$ standards.

## 2.10. Measurement of lipid peroxidation

The extent of lipid peroxidation (malondialdehyde; MDA) in chia leaves were monitored as described by Buege and Aust (1978) with slight modifications. TCA protein extracts (200 µl) from each sample was mixed with 400 µl of 0.5 % TBA (dissolved in 20 % TCA) and boiled at 95°C for 30 minutes, followed by an ice incubation for 10 minutes. The sample mixture was then centrifuged at 12 000 *X g* for 5 minutes and the absorbance of the supernatant was measured at 532 nm and 600 nm respectively. The concentration of MDA was calculated using a molar extinction coefficient 155 $mM^{-1}$ $cm^{-1}$.



## 2.11. Quantification of the antioxidant enzyme activity within chia leaves

For all the antioxidant enzyme assays, sample extracts were prepared using PVP homogenizing buffer as described in section 2.2.

Total SOD activity was measured as described by Beyer and Fridovich (1987). The sample reaction mixture consisted of 10 µl PVP protein extract and 190 µl of SOD spectrophotometer buffer (see section 2.2) in a final volume of 200 µl. The reaction was initiated when the sample mixture was exposed to light for 15 minutes or until a colour change was observed. The absorbance was measured at 560 nm and SOD activity was calculated based on the amount of enzyme that was required to reduce 50 % of NBT to blue formazan.

For APX activity, a modified method was adapted from Asada (1984) where each reaction contained 10 µl PVP protein extract and 180 µl of APX spectrophotometer buffer (see section 2.2) in a final volume of 190 µl. The reaction was initiated through the addition of 10 µl $H_2O_2$ (90 µM), and the absorbance measured at 290 nm. APX activity was calculated using the extinction coefficient 2.8 $mM^{-1}$ $cm^{-1}$.

GR activity was determined at 340 nm by following the rate of NADPH oxidation as described by Esterbauer and Grill (1978). The reaction mixture consisted of 0.2 mM NADPH, 0.5 mM glutathione disulfide (GSSG), 1 mM EDTA, 100 mM $K_2HPO_4$ at pH 7.8, and 50 µg of enzyme extract in a 200 µl reaction. The GR activity was calculated based on the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}$ $cm^{-1}$.

## 2.12. Detection of ROS scavenging antioxidant isoforms in response to exogenous caffeic acid and salt stress

### 2.12.1. Superoxide dismutase

Native gels were electophoretically separated on a 12 % polyacrylamide gel according to Laemmli (1970). SOD activity was then detected using a modified method of Beauchamp and Fridovich (1971) using 100 µg of total protein extract from each sample. For the detection of SOD activity and identification of individual SOD isoforms, specific inhibitors were used. These inhibitors included potassium cyanide (KCN; inhibits the copper/zinc – containing Cu/ZnSOD) or $H_2O_2$ (inhibits both Cu/ZnSOD and the iron – containing FeSOD). However, the identification of manganese – SOD's (MnSOD) are evident via its resistance to both KCN and $H_2O_2$.



### 2.12.2. Ascorbate peroxidase

Individual APX isoforms were detected as described by Mittler and Zilanskas (1993) using 60 µg of total protein extract. This was achieved by incubating the native gel (described in section 2.12.1) in 50 mM phosphate buffer ($KPO_4$; pH 7.8) containing 4 mM ascorbate and 2 mM $H_2O_2$. The gel was then washed with 50 mM $KPO_4$ (pH 7.8) for 1 minute prior to a final incubation (staining solution) containing 50 mM $KPO_4$ (pH 7.8) with 209 µl TEMED and 15 mg NBT. The native gel remained in solution until visible APX isoforms were detected.

### 2.12.3. Glutathione reductase

GR activity was detected as described by Lee and Lee (2000) with slight modifications. GR activity (as individual isoforms) was visualized in the native gel

by an incubation in 50 mM Tris – HCl buffer (pH 7.9) containing 4.0 mM glutathione disulfide (GSSG), 1.5 mM NADPH, and 2 mM 5,5' – dithiobis(2 – nitrobenzoic acid) (DTNB) for 10 minutes. The GR activity was negatively stained in the dark with a solution containing 0.6 mM 3 - (4,5 – dimethylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide (MTT) and 0.8 mM phenazine methosulfate (PMS) for 5 – 10 minutes at 30°C.

## 2.13. Densitometry analysis of ROS scavenging enzymes

The in – gel images for SOD, APX and GR enzymatic activities were analyzed by densitometry analysis using the Spot Denso tool of AlphaEase FC imaging software (Alpha Innotech Corporation). The individual enzymatic gels were measured and represented as arbitrary values (relative enzymatic activity) of three independent gels according to Klein (2012). The enzymatic activity (for SOD, APX and GR) of each isoform in the treatments were measured as an average of the relative pixel intensities and expressed in arbitrary units. This was achieved by assigning the isoform with the lowest pixel intensity to a value of 1 and expressing the rest of the pixel intensities for that particular enzymatic isoform in the other treatments relative to this isoform.



## 2.14. Profiling the leaf proteome of *Salvia hispanica*

### 2.14.1. Sample preparation for proteomic analysis

Protein extraction was performed following an optimized method adapted from Ngara 2009. A total of 200 mg of ground up leaf material from each treatment were

homogenized in 10 % (w/v) TCA/Acetone through vortexing and a 13 000 *X g* centrifugation step for 6 minutes. The supernatants were discarded and the resultant pellets were initially washed with an 80 % (v/v) methanol plus 0.1 M ammonium acetate (met – $NH_4Ac$) solution, followed by an 80 % (v/v) acetone wash. Thereafter, the pellets were air dried at room temperature for an hour, followed by resuspending the pellets in a 1:1 phenol and SDS buffer (see section 2.2) ratio. Samples were then incubated on ice for 6 minutes followed by a 13 000 *X g* centrifugation for another 6 minutes. The upper phenol phase was then transferred to a sterile tube which was later filled with the 80 % (v/v) methanol plus 0.1 M ammonium acetate (met – $NH_4Ac$) solution to allow for protein precipitation at – 20ºC overnight. The precipitates were subjected to a 13 000 *X g* spin for 6 minutes after which the supernatants were discarded revealing a white pellet. The pellets underwent two washes (pellet resuspension) with 100 % (v/v) methanol followed by an 80 % (v/v) acetone wash. After the final wash step, the pellets were allowed to air dry at room temperature for an hour. Thereafter, the pellets were dissolved in IEF buffer (see section 2.2) and stored at – 20ºC. Protein concentrations were determined according to Bradford in 1976.



### 2.14.2. One dimensional polyacrylamide gel electrophoresis (1D PAGE)

Protein samples, prepared in a 1:1 ratio with 6 X SDS gel loading dye (see section 2.2), were boiled at 95ºC for 5 minutes, and subjected to a 1D PAGE. A 1D PAGE analysis was used to resolve proteins according to their molecular weight with the aid of the Mini – Protean III® Cell gel casting system (Bio – Rad). Resolving 12 % gels (40 % Acrylamide/Bis stock solution (37:5:1); 1.5 M Tris – HCl, pH 8.8;

0.5 M Tris – HCl, pH 6.8; 10 % SDS; 10 % APS, 16 mM TEMED) were gently poured between two glass plates according the manufacturer's manual (Bio – Rad). The liquid gel was overlaid with isopropanol and allowed to polymerize for about 30 minutes. When this time had elapsed, the isopropanol was decanted and the plates were rinsed with $dH_2O$. A 5 % stacking gel (40 % Acrylamide/Bis stock solution (37:5:1); 0.5 M Tris – HCl, pH 6.8; 10 % SDS; 10 % APS, 16 mM TEMED) was prepared and poured on top of the resolving gel and a 10 – well comb was inserted to form wells necessary to load the samples. The gel was allowed to polymerize for about 15 minutes. Gels were then placed into the buffer tank according to the manufacturer's guide (Bio – Rad). Upon polymerization, the combs were removed, the tank was filled up with 1 X SDS running buffer (see section 2.2) and the samples were loaded. Gels were electrophoresed at 120 V until the dye reached the bottom of the gel for approximately 90 minutes.



### 2.14.3. Two dimensional polyacrylamide gel electrophoresis (2D PAGE)

Protein samples were separated according to their isoelectric points (pI's) and molecular weights (MW), using immobilized pH gradient (IPG) strips (pH 4 – 7) in the first dimension coupled with SDS – PAGE analysis in the second dimension.

#### 2.14.3.1. Rehydration of immobilized pH gradient (IPG) strip

Protein samples (100 µg) from each treatment were premixed with 10 X ampholytes (1.25 µl), 0.2 % (w/v) DTT and made up to a final volume of 125 µl with DeStreak solution. Samples were loaded onto a balanced re – swelling tray. The IPG strips

(7cm long, pH range 4 – 7) were gently positioned on top of each sample, gel side facing downward, avoiding the formation of air bubbles. Strips were overlaid with mineral oil and allowed to passively rehydrate for 16 hours at room temperature.

### 2.14.3.2. Isoelectric focusing (IEF)

After rehydration, the IPG strips were rinsed with $dH_2O$, blotted on tissue paper to remove excess water and each placed on a focusing platform, with the gel side facing downwards. Pre – wet electrode pads (wicks) were positioned at each end of the strips. The strips were overlaid with mineral oil and focused with the IEF program as described in Table 2.3.

**Table 2.3.** Isoelectric focusing (IEF; BIO – RAD) parameters for 7 cm IPG strips

| Phase | Volts (V) | Hours (Hrs) / Volt Hours (Vhrs) |
|---|---|---|
| **Phase 1** | 250 | 0h15 minutes |
| **Phase 2** | 4 000 | 1h00 |
| **Phase 3** | 4 000 | 12 000 Vhrs |



### 2.14.3.3. Equilibration of IPG strips

After IEF, IPG strips were equilibrated in SDS – containing buffers (equilibration buffer) to solubilize focused proteins and allow SDS binding prior to second dimension SDS – PAGE. The focused IPG strips were incubated gel side up in re – swelling tray channels containing 2.5 ml equilibration buffer (see section 2.2), firstly containing 2 % (w/v) DTT for 15 minutes followed by 2.5 % (w/v) iodoacetamide (IOA) for another 15 minutes with gentle agitation at room

temperature. After equilibration, the isoelectric focused proteins were ready for separation on second dimension SDS-PAGE as described below (section 2.14.3.4).

#### 2.14.3.4. Second dimension by SDS PAGE analysis

The SDS gels were prepared as outlined in section 2.14.2, with the omission of the stacking gel. Each IPG strip, was positioned directly above the resolving gel and sealed with melted agarose gel. Electrophoresis was performed as described in section 2.14.2.

## 2.15. Coomassie Brilliant Blue (CBB) staining

For both the 1D and the 2D SDS – PAGE gels, proteins were visualized using the coomassie brilliant blue (CBB) R – 250 staining protocol, involving three consecutive steps. On completion of electrophoresis, the gels were carefully removed from the glass plates and stained with CBB I (see section 2.2). This was followed by two 30 minute staining steps with CBB II (see section 2.2) and CBB III (see section 2.2), respectively. After the staining steps, the gels were submerged in a destaining solution (see section 2.2) at room temperature with gentle agitation until protein versus background ratio is appropriate for visualization was obtained. The gels were imaged using the Molecular Imager PharosFX Plus System (BIO – RAD).

## 2.16. Comparative analysis of 2D SDS – PAGE gels

The 2D SDS – PAGE analysis was done using PDQuest™ Advanced 2D analysis software version 8.0.1 Build 055 (BIO – RAD). The 2D gels were imaged using the Molecular Imager PharosFX Plus System (BIO – RAD) and analysed according to the PDQuest™ Advanced 2D Analysis Software user manual (BIO – RAD). All analyses in experiments were made using three biological replicates per treatment group. The gels were normalised with the aid of the local regression model compensating for gel to gel differences in spot quantities due to non – expression related variations. Before differential protein expression was done, spots were manually edited using the consensus tool to obtain spot expression consensus across all biological replicates in treatment groups. A protein spot was considered differentially expressed between samples when it had a p – value of less than 0.05 and a fold change of more than 1.5. Three biological replications were used for the analysis. Protein spots of interest were manually picked using sterile pipette tips for identification by mass spectrometry analysis.



## 2.17. In – gel trypsin digestion and peptide extraction

Briefly, the differential protein spots were manually excised from 2D gels and washed twice in distilled water for 10 minutes. The gel pieces were destained (50 % acetonitrile and 25 mM ammonium bicarbonate) and sonicated for 3 – 5 minutes. The gel pieces were dehydrated by washing twice in 50 % acetonitrile (ACN) for 10 minutes. After dehydration the gel pieces were digested overnight in 20 ng of sequencing grade trypsin (Promega) at 37°C according to the manufacturer's guide.

Peptides were extracted with a 10 µl solution of 30 % ACN and 0.1 % trifluoroacetic acid (TFA) (Sigma) for 30 minutes at room temperature and stored at 4°C for further analysis.

## 2.18. Protein identification by MALDI – TOF MS/MS

Differential expressed proteins were identified using the ultrafleXtreme MALDI TOF system (Bruker Daltonics, Germany) with instrument control through Flex control 3.4. A small fraction (1 µl) of peptide extract produced by the in – gel digestion was placed on an MALDI anchor chip and allowed to air – dry at room temperature. Each sample on the anchor chip was covered with 1 µl solution of 0.4 mg/ml α – cyano – 4 – hydroxycinnamic acid in a mixture of acetonitrile (ACN) and 0.1 % trifluoroacetic acid (TFA) (70:30) and then air dried. The mass spectra were acquired on an ultrafleXtreme TOF mass spectrometer (Bruker Daltonics, German). Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics, Germany). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Data captured by MALDI – TOF MS/MS were a result coupled with Mascot v2.2.03 (http://www.matrixscience.com) against NCBI [Taxonomy: Viridiplantae (Green Plants)] and SwissProt using the following parameters: 0.2 Da mass tolerance, one missed cleavage, carbamidomethylation of cysteines as fixed modifications and oxidation of methionine as variable modifications.

## 2.19. Functional classification of positively identified proteins

Proteins were grouped into functional categories using data available on the UniProt database (www.uniprot.org) as well as literature sources.

## 2.20. Bioinformatic analysis

All experiments described were performed three times independently, with measurements taken from eight (plant growth measurements) or three (for all other experiments) different plants for each treatment in each of the three independent experiments (100 µM caffeic acid, 100 mM NaCl and a combination of 100 µM caffeic acid with 100 mM NaCl). For statistical analysis, one – way analysis of variance (ANOVA) test was used for all data and means (for three independent experiments) were compared according to the Tukey – Kramer test at 5 % level of significance, using GraphPad Prism 5.03 software.

# CHAPTER 3

# EXOGENOUSLY APPLIED CAFFEIC ACID REGULATES THE PHYSIOLOGICAL RESPONSES OF *SALVIA HISPANICA* UNDER SALT STRESS

## 3.1. Introduction

Plant physiological studies are described as the application of modern physics and chemistry to ultimately understand plants and what accounts for their survival (Salisbury and Ross 1992). The physiological appearance of plants, one component of physiological studies, are the first indicators as to whether the plant had undergone some sort of stress represented mainly by leaf discolouration and stunted shoot length.

Plant growth is dependent on multiple factors or processes for optimal development. These activities involve complex interrelationships between the processes within the meristems (involving a supply of metabolites) and the effect of internal and external factors on these specific processes (Moorby 1981). Hence, in plant systems, environmental changes serves as the key external factor, thus consequently altering internal environments by means of ROS accumulation (Choudhury *et al.* 2016). This ripple effect ultimately affects the supply of essential metabolites and therefore, negatively impacting the growth of the plant.

Salt stress have been shown to negatively influence various physiological parameters by limiting water uptake, by means of stomatal closure (section 1.4), thus influencing

the physiological indicators mentioned above (Aroca *et al*. 2012; Roy *et al*. 2014). The effect of long term salt stress have been studied in rice (Mishra *et al.* 2013), soybean (Klein *et al*. 2013) and various other cereal crops. However, limited information in the public domain focuses on the effect of salt on pseudocereals.

Based on reports published on soybean (section 1.6.6.1; Klein *et al.* 2013; Bubna *et al*. 2011) the work in this report focuses on using caffeic acid (CA) in order to alleviate the effects of salt stress. Although Bubna *et al*. (2011) concluded negative impacts of caffeic acid on soybean plants, Klein *et al*. (in 2013 and 2015) reports positive effects of caffeic acid within soybean plants in response to saline conditions. However, there is limited information available on the effects of salt stress on pseudocereals and the relationship between salt and caffeic acid on the pseudocereal, chia, have not been shown. Therefore, this part of the thesis focuses on the effect of caffeic acid in chia plants, under salt stress, by monitoring various growth parameters and photosynthetic pigment studies.



## 3.2. Results

### 3.2.1. Caffeic acid improves plant growth under salt stress

Chia plants were grown and treated as described in section 2.3. This section describes the influence of exogenous caffeic acid and salt stress on chia growth and development. The results show that caffeic acid and salt stress differentially influences plant growth (Figure 3.1). Caffeic acid significantly improved plant growth, whereas the opposite was observed in response to salt stress when compared to the untreated control. However, plant growth was significantly improved in salt

stressed plants supplemented with caffeic acid albeit not to level of the control plants (Figure 3.1).

The shoot length in the caffeic acid treatment was ± 16 % higher compared to the control, whereas in the salt treatment, the shoot length was significantly inhibited by ± 36 % in comparison to the control (Figure 3.1 B). However, the shoot length in the combined treatment (CA + NaCl) was augmented by ± 24 % compared to the salt treatment, albeit not to the level of the control or the caffeic acid treated plants.

Apart from the caffeic acid treatment, the root length was not significantly altered in the other treatments (Figure 3.1 A and C). The caffeic acid treated plants exhibited a ± 15 % increase in root length compared to the control plants. Interesting to note was that root volume (not measured) was significantly influenced by the exogenous combined caffeic acid and salt treatment. Caffeic acid significantly increased root volume in whereby the opposite was observed in the salt treated plants in comparison to the control plants. However, root volume in the salt treated plants supplemented with caffeic acid was increased to a level higher than that observed for the salt treatment, albeit not to the level of the control and caffeic acid treated plants. This suggests that exogenous caffeic acid could improve chia plant growth under salt stress.

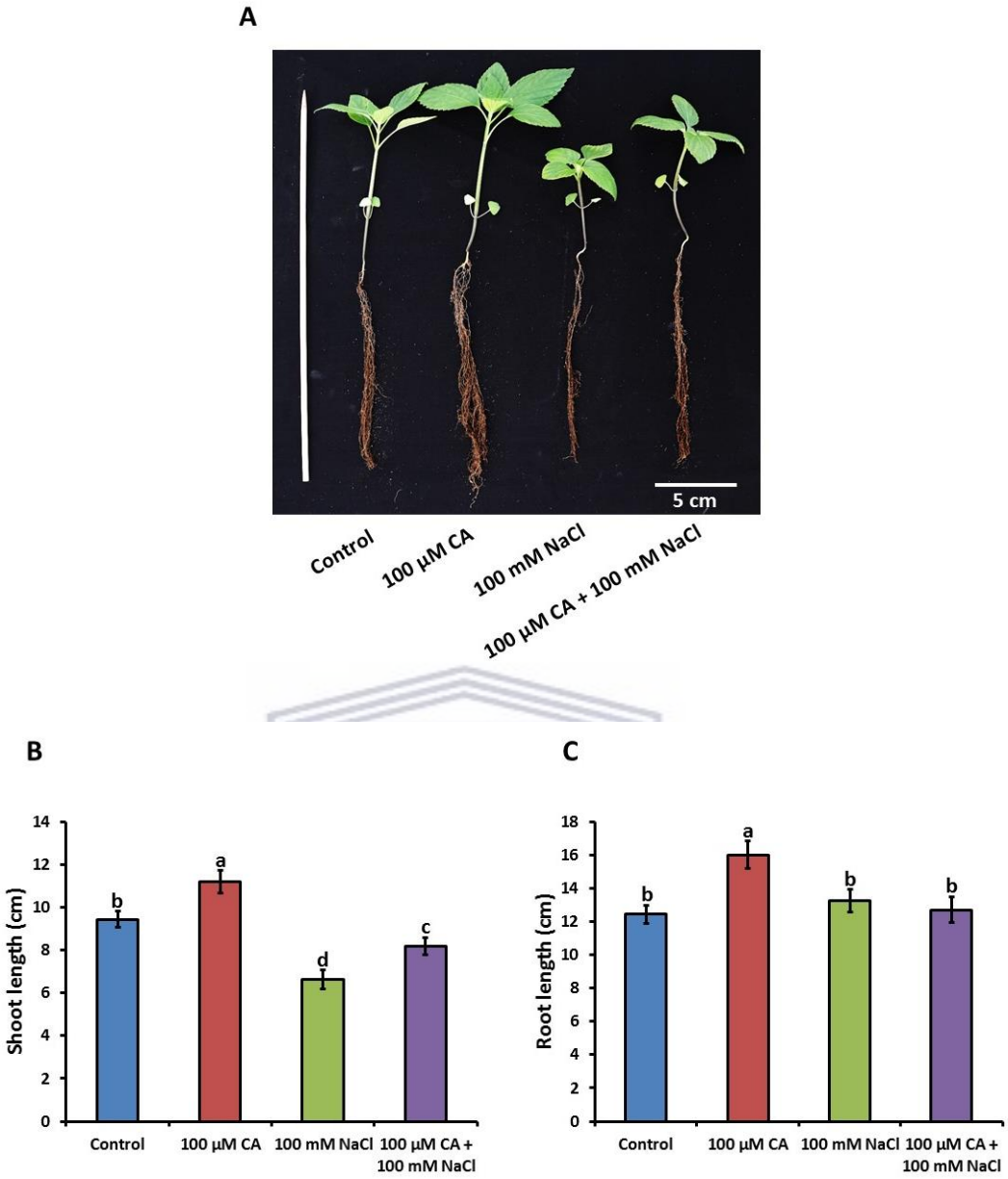



**Figure 3.1. The influence of caffeic acid and salt stress on chia plant growth.** Plant growth parameters include individual representatives of each treatment (A), shoot length (B) and root length (C). Error bars are representative of the mean (± SE) of three independent experiments from 8 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

### 3.2.2. The effect of caffeic acid and salt stress on chia biomass

Exogenous caffeic acid and salt stress differentially influences chia biomass as observed for shoot and root fresh and dry weights (Figure 3.2). Figure 3.2 shows that

plants exposed to caffeic acid (100 µM CA) promotes shoot and root development and subsequent plant survival.

Exogenous application of caffeic acid increased shoot fresh weight by ± 17 %, whereas a significant reduction of ± 40 % was observed in the salt treatment when compared to the untreated control (Figure 3.2 A). The reduction in shoot fresh weight observed in the salt treatment was reversed (albeit not to the level of the untreated control) when salt stress plants were supplemented with caffeic acid. Interestingly, the trend observed for the shoot fresh weight in response to the different treatments was also witnessed for the root fresh weight (Figure 3.2 B), whereby a significant reduction (± 45 %) in root fresh weight was observed in response to 100 mM NaCl in comparison to the control plants. However, plants treated with caffeic acid showed to promote plant root formation by ± 26 % (100 µM CA) as well as exhibit a ± 40 % increase in response to 100 µM CA + 100 mM NaCl in comparison to 100 mM NaCl.

This phenomenon was also observed for the shoot and root dry weights (Figure 3.2 C and D). The shoot dry weight was improved by ± 40 % compared to the control in response to caffeic acid whereas a significant reduction (± 20 %) was observed as a consequence of salt stress. However, shoot dry weight in the salt treatment supplemented with caffeic acid was increased by ± 30 % in comparison to the salt treated plants, although not to the level observed for the control (Figure 3.2 C). In addition, root dry weights expressed a similar trend presenting a ± 34 % increase in response to caffeic acid and a subsequent decrease (± 23 %) in response to salt. The effect of the combined treatments (caffeic acid + salt stress), once again, show to reverse the effects of salt (± 31 % increase compared to the salt treatment) albeit not to the level of the untreated control (Figure 3.2 D).

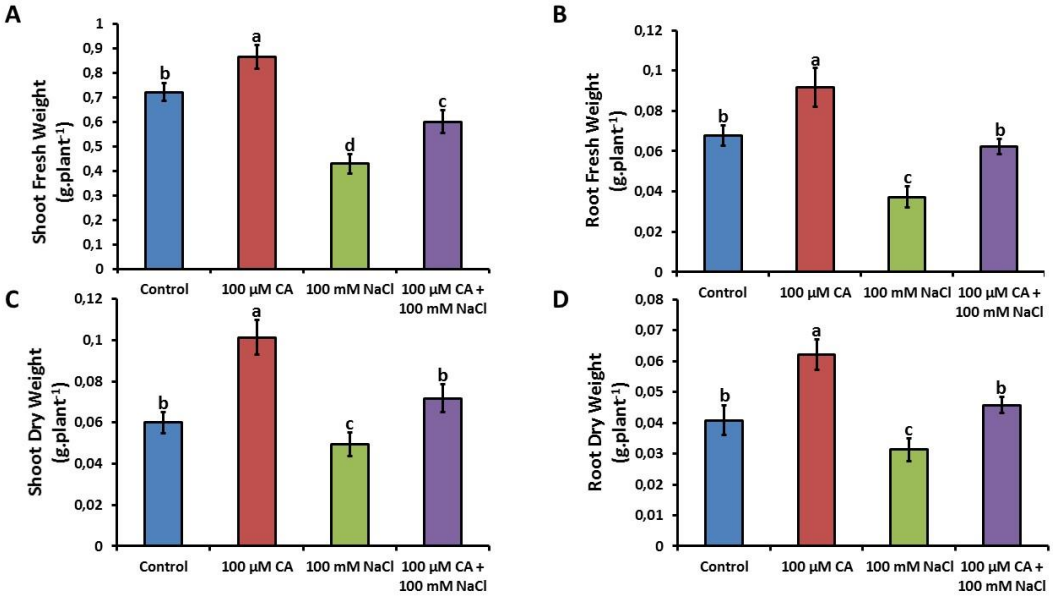



**Figure 3.2. The effect of caffeic acid on chia plant biomass under salt stress.** Plant biomass is represented by shoot (including the leaf; A) and root (B) fresh weights as well as shoot (C) and root (D) dry weights. Error bars are representative of the mean (± SE) of three independent experiments from 8 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

### 3.2.3. Caffeic acid improves water retention in salt stressed plants

The survival of plants in nature depends on its ability to retain water. Here we measured water retention of chia plants exposed to caffeic acid and salt stress. Exogenous caffeic acid did influence water retention as seen in Figure 3.3. However, salt stress reduced water retention in chia leaves by ± 30 % compared to the control. Interestingly, when salt stressed plants were supplemented with caffeic acid, water retention (as seen for RWC) was significantly improved to levels observed for the control (Figure 3.3). This suggests that caffeic acid not only reverses the effects of salt stress by improving water retention but also promotes plant survival.

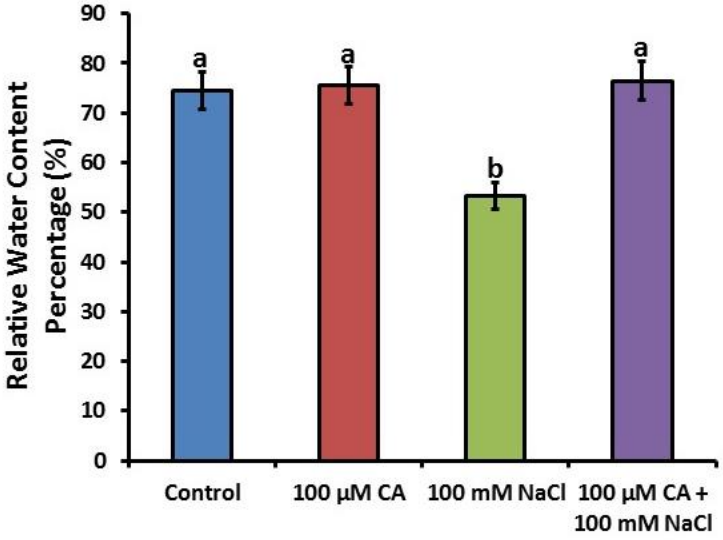



**Figure 3.3. The effect of exogenous 100 µM CA, 100 mM NaCl and the combination of the two on the relative water content in chia leaves.** Error bars are representative of the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

### 3.2.4. The effect of caffeic acid and salt stress on chlorophyll pigments and carotenoid content

Caffeic acid and salt stress differentially influences photosynthetic metabolism and carotenoid biosynthesis in chia leaves (Figure 3.4). A significant increase in leaf chlorophyll content (Figure 3.4 A) was observed in response to caffeic acid. This increase was approximately ± 16 % higher compared to the control. Contrary to what was observed in response to caffeic acid, salt stress reduced leaf chlorophyll content by ± 50 %. On the other hand, supplementation of the salt treatment with caffeic acid alleviated the salt – induced reduction in chlorophyll content as the chlorophyll content in the combined treatment (caffeic acid + salt stress) was statistically similar to that observed from control the plants.

For carotenoid content, no significant difference was observed in the caffeic acid treatment when compared to the control (Figure 3.4 B). Salt stress reduced carotenoid content by ± 28 % compared to the control. Interestingly, the combined treatment significantly enhanced the carotenoid content to levels even higher than seen for the control. This increase was ± 19 % higher than control and ± 42 % higher than the salt treatment (Figure 3.4 B).

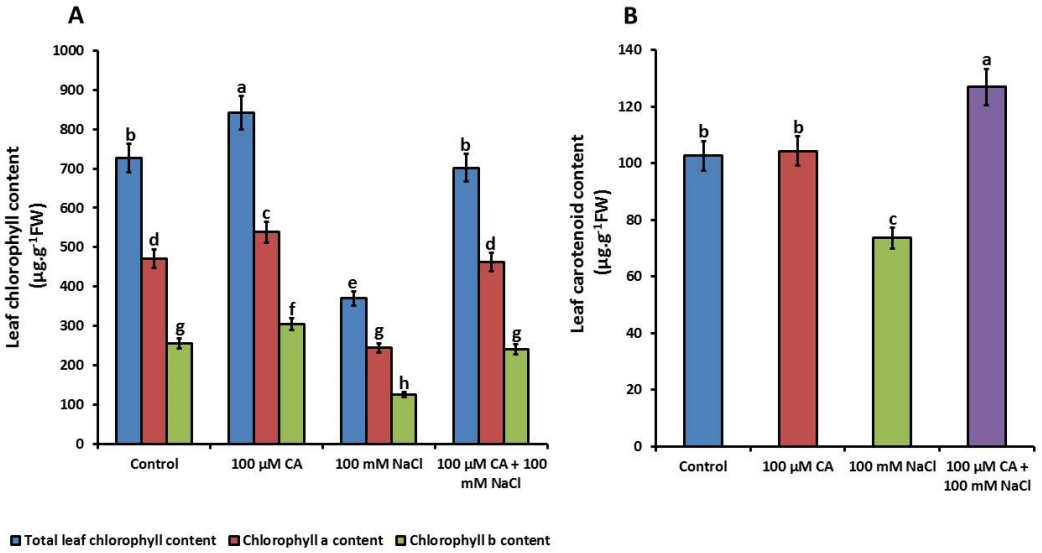



**Figure 3.4. Caffeic acid and salt stress differentially alters photosynthetic pigments (A) and carotenoid content (B) in chia leaves.** Error bars are representative of the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

## 3.3. Discussion

Here, we have analysed the influences of exogenously applied caffeic acid and long term salt stress on the physiological responses of *Salvia hispanica* L. The results show that salt stress inhibits plant growth and development whereas caffeic acid

show to exhibit the opposite effect. In addition, when salt stressed plants are supplemented with caffeic acid, the salt – induced inhibition is reversed although not to the level observed for the control.

### 3.3.1. Caffeic acid improves chia growth and biomass under salt stress

Contrary to what has been reported in literature, we have demonstrated that exogenously applied caffeic acid improves plant growth and biomass under salt stress in chia plants (Figure 3.1; Figure 3.2). Previous studies have shown that caffeic acid inhibits root elongation in leafy spurge (*Euphorbia esula*) (Barkosky *et al*. 2000). However, in this study, we have shown that caffeic acid increases root length and root volume (based on visual observation) relative to the control (Figure 3.1 A). The inhibitory effect of salt stress on plant growth and development have been well documented including this study (Al Hassan *et al*. 2015; Bor *et al*. 2003; Keyster *et al*. 2013). However, the combined treatment of salt stress and caffeic acid did not improve root length in comparison to the salt stress plants alone (Figure 3.1 A and C). On the other hand, root volume was significantly more in the combined treatment when compared to the salt treated chia plants but still less than the control and the caffeic acid treatment (Figure 3.1 A). It is evident across Figures 3.1 to 3.2 that not only does caffeic acid improve shoot elongation, root elongation as well as shoot and root biomass, but also show signs of rescue under saline conditions across all the fresh weight experiments. It is clear from these initial studies that caffeic acid appears to have a positive effect on this particular pseudocereal. Thus, since caffeic acid studies are not well documented, this is the first study to report the positive effects of caffeic acid on root volume under salt stress in pseudocereal plants, such as chia.

### 3.3.2. Exogenous caffeic acid improves water retention under salt stress conditions

To analyse the beneficial effects of caffeic acid under salt stress, it is important to consider the role of caffeic acid in plant water retention, since the initial reduction in plant growth, after salt treatment, is a consequence of the osmotic effects caused by salts (Munns and Tester 2008). The ability to retain water under salt conditions (as seen in this study; Figure 3.3) can improve salt tolerance by mitigating an excessive ion concentration by a dilution effect (Romero – Aranda *et al*. 2006). In this study, exogenously applied caffeic acid could maintain a higher water content under salt stress conditions (Figure 3.3). Based on correlation analysis in this study, a direct relationship exists between RWC (Figure 3.3) and plant dry weight (Figure 3.2). This suggests that enhanced salt tolerance of chia plants could be partially attributed to higher RWC.



### 3.3.3. Photosynthetic metabolism is differentially altered by exogenous caffeic acid and salt stress

Photosynthetic pigment composition, such as chlorophylls and carotenoids, are directly linked to the physiological status of plant leaves (Gitelson *et al*. 2006). This statement is supported by the fact that various electron transfers occurring during photosynthesis within the chloroplasts depend on the above – mentioned plant pigments (Fassnacht *et al*. 2015). Chlorophylls – a and – b (green pigments) function in the absorption of solar light energy in order to transfer it into the photosynthetic apparatus (Gitelson *et al*. 2006; Kira *et al*. 2015). The results presented in this study showed a significant decrease in the chlorophyll – a and chlorophyll – b content, leading to a significant overall decrease in the total chlorophyll content observed in

salt treated chia plants (Figure 3.4 A). The adverse effect of photosynthesis under salt stressed conditions was previously described in different crop species such as sultana vines (Fisarakis *et al*. 2001), cowpea (Taffouo *et al*. 2009), cotton (Meloni *et al*. 2003) and wheat (Raza *et al*. 2006). However, there are many reports showing little or no changes or even the stimulation in the photosynthesis capacity of plants under low salt stress (Hawkins and Lewis 1993). In fact, the effect of salt stress on photosynthesis depends on the salt concentration in addition to the plant species or genotypes. Chia plants treated with 100 mM NaCl experienced a significant reduction in chlorophyll content (Figure 3.4 A) as salt inhibits the synthesis of chlorophyll in addition to activating the chlorophyllase enzyme which is responsible chlorophyll degradation. Furthermore, salt stress (imposed by 100 mM NaCl) is also responsible for the inhibition of Rubisco and PEP carboxylase which is essential for photosynthesis (Al Hassan *et al*. 2015; Soussi *et al*. 1998). Therefore, the reduction in chlorophyll content by treatment with 100 mM NaCl, represents a physiologically stressed sample. On the other hand, exogenous application of caffeic acid to salt stressed plants improved photosynthetic capability (Figure 3.4 A) by abolishing salt – induced leaf chlorosis as previously described for soybean plants (Klein *et al*. 2015).

On the other hand, carotenoids (yellow pigments), in addition to light – harvesting, could serve as photoprotective molecules by means of the xanthophyll cycle. The role of carotenoids within this cycle allows for excess energy to dissipate, thus reducing the level of damage within the photosynthetic system (Fassnacht *et al*. 2015; Kira *et al*. 2015; Safafar *et al*. 2015). Therefore, alterations in plant pigments, especially the chlorophylls and carotenoids, have been directly associated with stress phenology.

This statement is supported by the fact that the reduction of chlorophyll content often implies stress and its ratio with upregulated carotenoids comfortably allows for the analysis of the physiological state of the plant (Fassnacht *et al*. 2015; Kira *et al*. 2015). However, the results presented in this study is in contradiction to how salt stress influences carotenoid biosynthesis in plants as we have shown that salt stress significantly reduces carotenoid content in chia leaves (Figure 3.4 B). However, Marti *et al*. (2016) further explains that the content of carotenoids are greatly dependent on the species and the environmental conditions. Therefore, chia leaves correspond to the research performed on tomato leaves (not fruits) whereby no correlation exists between $Na^+$ and carotenoid content (Al Hassan *et al*. 2015; Juan *et al*. 2005; Tuna 2014) thereby reducing the level of carotenoids within a salt – stressed sample. In addition, although exogenous caffeic acid does not influence carotenoid production under physiological conditions, a significant increase in carotenoid content was observed in the salt treatment supplied with caffeic acid (Figure 3.4 B). This increase was even higher than observed in the control plants. This suggests that caffeic acid improves photosynthetic metabolism and carotenoid biosynthesis under salt stress and thus enhancing salt stress tolerance in chia plants.

# CHAPTER 4

# CAFFEIC ACID – INDUCED ROS SCAVENGING IN CHIA PLANTS

## 4.1. Introduction

Salt stress is one of the major abiotic stress factors that adversely affects crop productivity. High concentrations of salts in soils account for large decreases in the yield of a wide variety of crops (Tester and Davenport 2003). It is expected that increased salinisation of arable lands will have devastating global effects, rendering useless for crop production around 30 % of agricultural lands within the next 25 years, and up to 50 % by the year 2050 (Wang *et al*. 2003). Elevated levels of salt ions in soil solution, surrounding plant roots, induce an imbalance in water potential between plant root cells and ambient soil solution, resulting in cellular dehydration. Exposure of plants to salt and other abiotic stress factors activates various physiological and developmental changes. These alterations are regulated by the expression of different genes and the accumulation of their translated proteins, activating diverse physiological, metabolic, and defence systems to survive (Valliyodan and Nguyen 2006).

Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Bor *et al*. 2003; Candan and Tarhan 2003). ROS production is one common feature of all aerobic organisms during their normal metabolic activities. Oxidative damage of lipids, proteins and nucleic acids, through

the alteration of normal cellular metabolism, is an important indicator of ROS (Imlay 2003). Abiotic stress conditions, such as drought and salt stress, increase ROS production in plants, and depending on their natural and genetic capacity, they have developed enzymatic and non – enzymatic defence strategies against ROS. It is well documented that salt stress promotes oxidative damage and plants with constitutive and induced antioxidant levels have better resistance to damage (Parida and Das 2005).

Antioxidant enzymes play an important role in scavenging ROS through a series of complex reactions. These reactions include the dismutation of superoxide ($O_2^-$) to hydrogen peroxide ($H_2O_2$) by superoxide dismutase (SOD). The $H_2O_2$ is then detoxified by various enzymes like ascorbate peroxidase (APX) and glutathione reductase (GR) (Noctor and Foyer 1998). Various researchers have reported that an increase in the activities of these enzymes are closely related to salt stress tolerance in many plant species.



Caffeic acid (CA) has emerged as an inhibitor of root growth in plants, exerting its growth – inhibiting effects by modulating the generation of ROS and increasing lignification (Bubna *et al*. 2011; Singh *et al*. 2009). The role of caffeic acid in alleviating salt stress has been associated with enhanced scavenging of $O_2^-$ via the augmentation of SOD activity (Klein *et al*. 2013). Salt stress tolerance induced by exogenous caffeic acid is proposed to be resulting from the reduction of the extent of cell death caused by $O_2^-$ accumulation during salt stress (Klein *et al*. 2013). Given the role of caffeic acid as an antioxidant with the capacity to inhibit ROS production (Jayanthi and Subash 2010), as well as the link between caffeic acid and plant salt stress tolerance (Klein *et al*. 2013), this study investigates the role of caffeic acid in

controlling ROS scavenging in chia plants under salt stress. Although a similar study have been performed on legume plants, to our knowledge this is the first study that describes how caffeic acid controls ROS production and antioxidant enzyme activities in pseudocereal (chia) plants under salt stress.

## 4.2. Results

### 4.2.1. The effect of exogenous caffeic acid and salt stress on ROS biomarkers

Chia plants were grown and treated as described in section 2.3. The effect of the exogenous caffeic acid and salt stress on ROS production ($O_2^-$ and $H_2O_2$) in the leaf tissue of chia plants was investigated given that salt stress is known to cause excessive ROS accumulation. A few lines of research have shown that caffeic acid inhibits oxidative stress and should therefore reverse the negative effects caused by salt stress if it were to enhance salt tolerance in chia plants. Exogenously applied caffeic acid have significantly reduced $O_2^-$ content by ± 30 % relative to the untreated control (Figure 4.1 A). Plants treated with 100 mM NaCl (to impose salt stress) enhanced $O_2^-$ content by ± 50 % compared to the untreated plants (Figure 4.1 A). However, when salt stressed plants were supplemented with caffeic acid, $O_2^-$ content was significantly reduced compared to the levels observed in the salt treatment. This reduction was ± 42 % lower compared to the salt treatment albeit still higher (± 15 %) than observed for the untreated control (Figure 4.1 A).

For $H_2O_2$ content, a similar profile to $O_2^-$ content was observed (Figure 4.1 B). Plants treated with caffeic acid (100 µM CA) reduced $H_2O_2$ content by ± 30 % relative to the untreated control, whereas those plants that were subjected to 100 mM NaCl

significantly increased $H_2O_2$ content by ± 48 % compared to the untreated controls (Figure 4.1 B). For salt treated plants supplemented with caffeic acid, $H_2O_2$ content was reduced by ± 34 % in comparison to the salt treatment although not to the levels observed for the control plants (Figure 4.1 B). The results obtained here demonstrates that exogenously applied caffeic acid can reduce salt – induced oxidative damage by controlling ROS production under salt stressed conditions in chia plants.

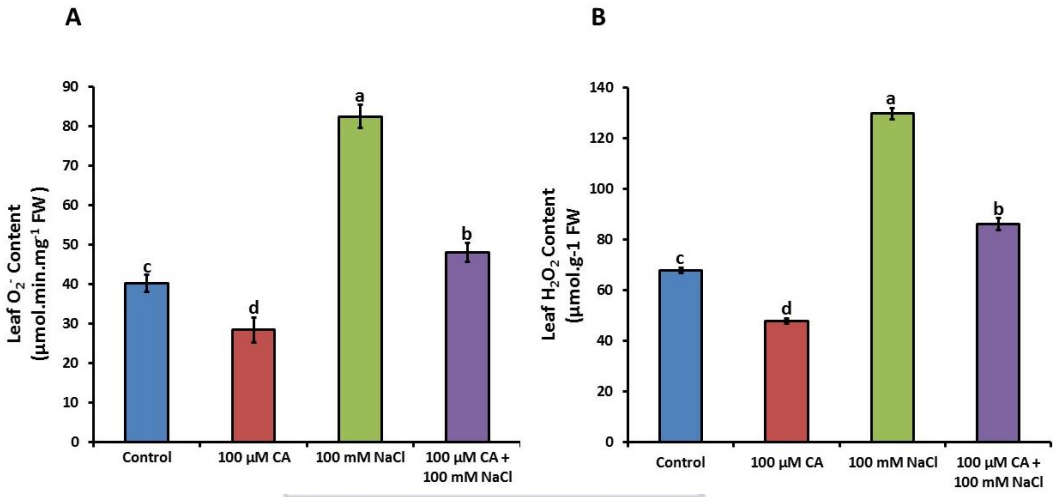



**Figure 4.1. The effect of exogenous caffeic acid (100 µM CA) and salt stress (100 mM NaCl) on superoxide content (A) and hydrogen peroxide levels (B) in the leaf tissue of chia plants.** Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).



### 4.2.2. Exogenous caffeic acid restricts salt – induced cell death by inhibiting oxidative damage

The extent of malondialdehyde (MDA) accumulation, which is indicative of lipid peroxidation, in the various treatments, was measured as an estimate of oxidative damage. The MDA content in chia plants treated with 100 µM caffeic acid was ± 13 % lower compared to the untreated control plants. In response to treatment with 100 mM NaCl, MDA content in chia leaves was increased by ± 50 % compared to the

untreated controls (Figure 4.2 A). However, when salt stressed plants were supplemented with caffeic acid, MDA content was reduced by ± 38 % in comparison to the salt treatment although not to the level observed for the untreated control (Figure 4.2 A).

The increase in MDA content in response to salt stress was manifested as an increase in cell death (as shown for Evans blue uptake) (Figure 4.2 B). A similar trend for cell death was observed as seen for the MDA analysis (Figure 4.2 A). A significant reduction of ± 22 % in Evans blue uptake (manifested as cell death) was observed in response to caffeic acid when compared to the untreated control (Figure 4.2 B). Cell death in the leaves of chia plants exposed to salt stress was significantly increased by ± 55 % in comparison to the untreated control. However, this increase was significantly reduced in salt stressed plants that were supplemented with exogenous caffeic acid, although not the level observed for the untreated controls (Figure 4.2 B).

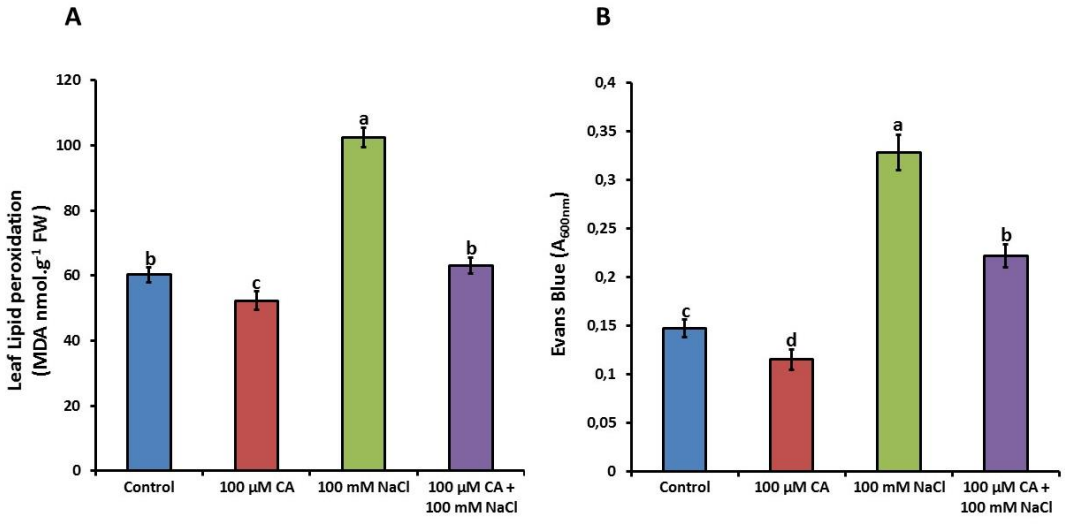



**Figure 4.2. The influence of caffeic acid and salt stress on lipid peroxidation (A) and the extent of cell death (B).** Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

### 4.2.3. Caffeic acid differentially regulated antioxidant enzyme activity under salt stress

Differential regulation of antioxidant enzyme activity in various plant species to salt stress have been well documented (Abogadallah 2010; Gill and Tuteja 2010). In addition, there is evidence that such response is modulated by exogenously applied caffeic acid (Klein 2012; Klein *et al*. 2013).

Caffeic acid and salt stress differentially regulated total SOD activity in the leaves of chia plants. Caffeic acid reduced SOD activity by ± 26 % whereas a significant increase of ± 62 % was observed for salt stress when compared to the untreated controls (Figure 4.3 A). A significant reduction in SOD activity (± 35 %) was observed in the salt treatment supplemented with caffeic acid when compared to the salt treatment although not to the level of the untreated control (Figure 4.3 A).

For leaf APX activity, a similar trend to SOD activity was observed. Caffeic acid inhibited APX activity by ± 31 % when compared to the untreated control (Figure 4.3 B). Salt stress augmented APX activity by ± 58 %. However, for salt stressed plants supplemented with caffeic acid, APX activity was reduced to levels complementing that of the untreated control plants although still higher to what was observed in the caffeic acid treatment (Figure 4.3 B).

Contrary to what was observed for SOD and APX activity, GR activity was significantly increased in both caffeic acid and salt stress treatments (Figure 4.3 C). However, in the salt treatment supplemented with caffeic acid, GR activity was reduced to levels lower than observed for the caffeic acid and salt treatment albeit higher than the untreated control (Figure 4.3 C).

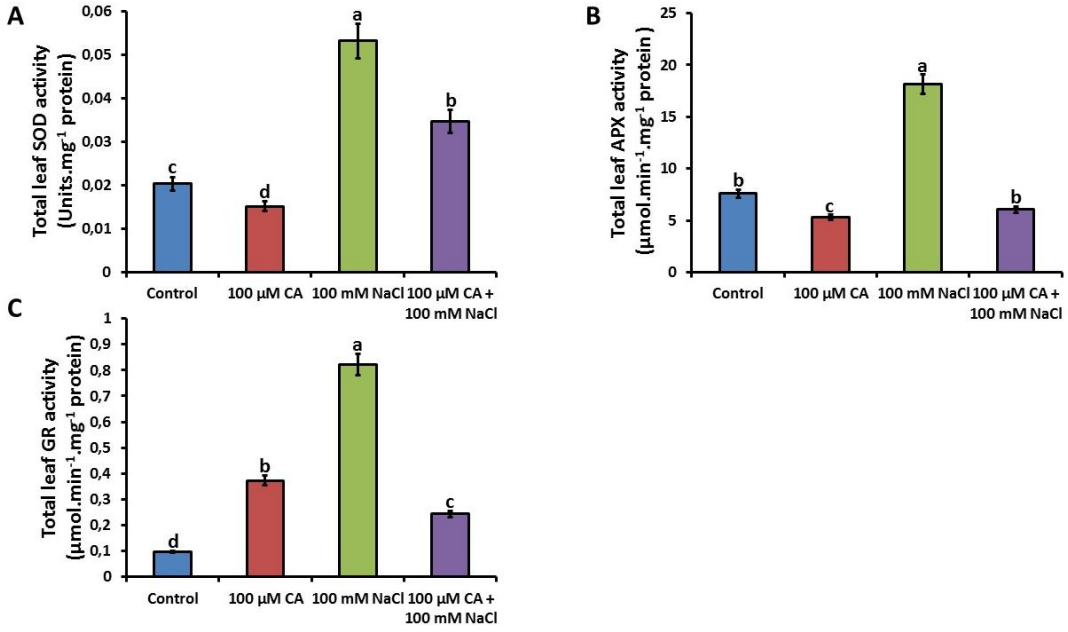



**Figure 4.3. Caffeic acid and salt stress differentially alters antioxidant activity in the leaves of chia plants.** Total SOD (A), APX (B) and GR (C) enzymatic activity were measured spectrophotometrically in the leaves of chia plants. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).



### 4.2.4. The effect of exogenous caffeic acid and salt stress on the activity of individual SOD isoforms

Superoxide dismutases (SOD, EC 1.15.1.1) represent the first line of plant defence against ROS in the array of enzymes that function to protect the plant cells against oxidative stress. Therefore, SOD is classified as a chain – breaking group of enzymes since they scavenge superoxide and yield another form of ROS; hydrogen peroxide ($H_2O_2$). This study demonstrates the effect of exogenous caffeic acid and salt stress on the enzymatic activity of various SOD isoforms in chia leaves. Leaf extracts from each treatment were separated on a 12 % native polyacrylamide gel and stained for individual SOD isoforms. A total of four SOD isoforms was detected in the uninhibited control gel (Figure 4.4 A). The activity of each SOD isoform was differentially regulated in response to caffeic acid and salt stress (Figure 4.4). The

characterisation of SOD isoforms in chia leaves was determined using KCN and $H_2O_2$ as described in section 2.12.1. Analyses of these results, based on resistance and sensitivity to KCN and $H_2O_2$, suggests the existence of two manganese SOD's (MnSOD1 and MnSOD2), one iron SOD (FeSOD) and one copper – zinc SOD (Cu/ZnSOD) (Figure 4.4). Based on densitometry analysis (Table 4.1), caffeic acid and salt stress differentially alter the activity of each SOD isoform. MnSOD1 was only detected in the caffeic acid treatments and absent (or very low in abundance) in the control and salt stress treatments. The activity of MnSOD2 was significantly reduced by ± 55 % in the caffeic acid treatment when compared to the untreated control (Figure 4.4; Table 4.1). In response to salt stress, the activity of MnSOD2 was augmented by ± 38 % compared to the untreated control. Furthermore, when salt stressed plants were supplemented with caffeic acid, MnSOD2 activity was significantly reduced compared to the salt treatment. The activity observed was like that of the untreated control (Figure 4.4; Table 4.1). For FeSOD, the activity detected in response to caffeic acid was ± 12 % higher compared to the untreated control. The salt treatment reduced FeSOD activity by ± 23 %, whereas salt treated plants supplemented with caffeic acid improved FeSOD activity to a levels slightly beyond that of the untreated control (Figure 4.4; Table 4.1). A similar trend in activity to FeSOD was observed for Cu/ZnSOD.

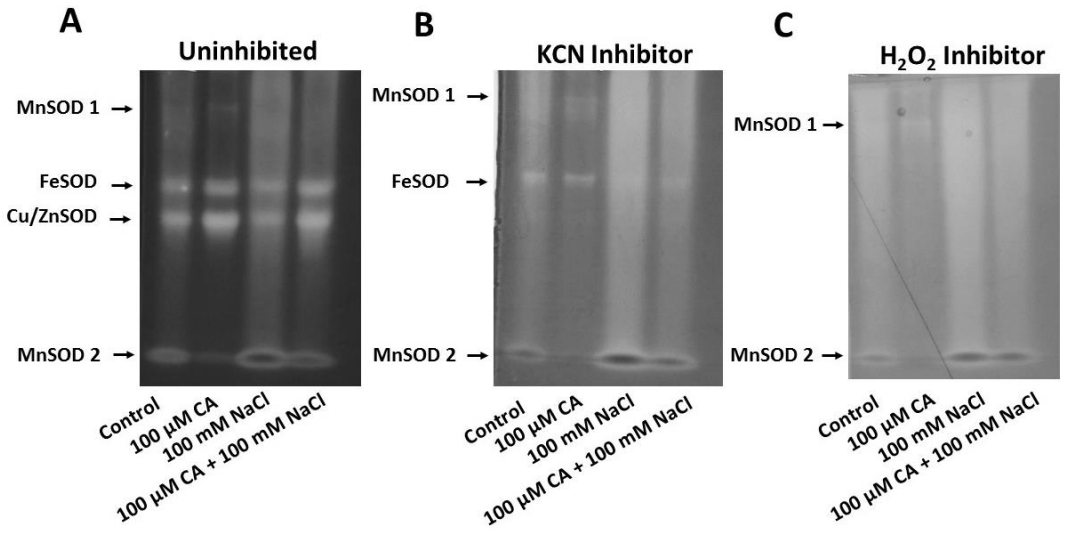



**Figure 4.4. Caffeic acid and salt stress differentially regulate SOD isoform activity.** In – gel activity assays were performed on chia leaves treated for 21 days. The native polyacrylamide gels shows the detection of SOD isoforms with no inhibitors (A), in the presence of 5 mM KCN (B) and in the presence of 6 mM $H_2O_2$ (C) in response to the various treatments.

**Table 4.1.** Tabular representation of relative SOD isoforms in chia leaves.

| Relative SOD Activity (Arbitrary Units) | Chia leaf SOD isoforms | Treatments | | | |
|---|---|---|---|---|---|
| | | Control | 100 µM CA | 100 mM NaCl | 100 µM CA + 100 mM NaCl |
| | MnSOD1 | $0.00 \pm 0.00^{c}$ | $1.49 \pm 0.07^{a}$ | $0.00 \pm 0.00^{c}$ | $1.00 \pm 0.05^{b}$ |
| | MnSOD2 | $2.23 \pm 0.11^{b}$ | $1.00 \pm 0.05^{c}$ | $3.59 \pm 0.18^{a}$ | $2.12 \pm 0.11^{b}$ |
| | FeSOD | $1.27 \pm 0.06^{b}$ | $1.44 \pm 0.07^{a}$ | $1.00 \pm 0.05^{c}$ | $1.52 \pm 0.08^{a}$ |
| | Cu/ZnSOD | $1.25 \pm 0.06^{b}$ | $1.48 \pm 0.07^{a}$ | $1.00 \pm 0.05^{c}$ | $1.53 \pm 0.08^{a}$ |

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

### 4.2.5. Caffeic acid and salt stress alters APX activity

While SOD is responsible for scavenging $O_2^-$, yielding high levels of $H_2O_2$, additional enzymes are activated for $H_2O_2$ scavenging such as ascorbate peroxidase (APX). In relation to the Halliwell – Asada pathway, APX is the primary enzyme responsible for scavenging $H_2O_2$. Here, we describe the influence of exogenous caffeic acid and

salt stress on the enzymatic activities of individual APX isoforms. The results show that only two APX isoforms (as labelled from the top of the gel) was identified (Figure 4.5 A). For APX1, no significant changes in enzymatic activity was observed for the different treatments (Figure 4.5 A and B).

For APX2, exogenous application of caffeic acid significantly reduced enzymatic activity by ± 60 %, compared to the untreated control Figure 4.5 C). On the contrary, salt stress significantly enhanced enzymatic activity by ± 50 % compared to the untreated control. However, the increase in activity observed the salt treatment was reversed when salt stressed plants were supplemented with caffeic acid albeit not to the level of the untreated control (Figure 4.5).

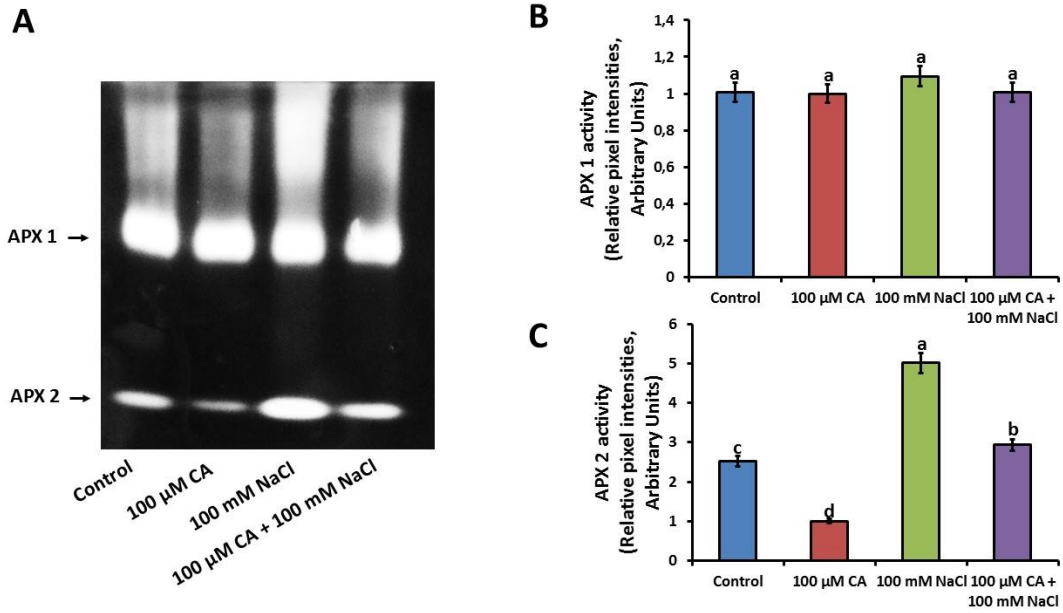



**Figure 4.5. The effect of caffeic acid and salt stress on APX activity.** In – gel activity assays were performed on chia leaves treated for 21 days. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

### 4.2.6. The influence of caffeic acid and salt stress on GR activity

Although APX plays an important role in the conversion of $H_2O_2$ to water, GR is also an essential catalyser in the conversion of $H_2O_2$ to maintain the redox state of ascorbate and glutathione, since it converts GSSG to GSH that is used by DHAR to regenerate ascorbate (section 1.5.1.1; Hernandez *et al*. 1999). GR is responsible for recycling GSSG to GSH and controls the redox status in plant cells. Here we describe the influence of exogenous application of caffeic acid and salt stress on GR activity in chia leaves. The results show that two GR isoforms (GR1 and GR2) was detected after activity specific staining as described in section 2.12.3 (Figure 4.6). The enzymatic activity observed for both GR isoforms were differentially regulated by caffeic acid and salt stress (Figure 4.6). Based on densitometry analysis (Figure 4.6 B and C), GR1 activity was not altered in response to caffeic acid, whereas a significant increase of ± 38 % was observed in the salt treatment compared to the untreated control. When the salt treatment was supplemented with caffeic acid, GR1 activity was reduced to a level below that of the control (Figure 4.6 B)



For GR2, no or very low enzymatic activity was detected in the control sample. Interestingly, GR2 was only detected in caffeic acid treatments (Figure 4.6 A and C). A reduction in GR2 activity was observed in the combined treatment when compared to the caffeic acid treatment.

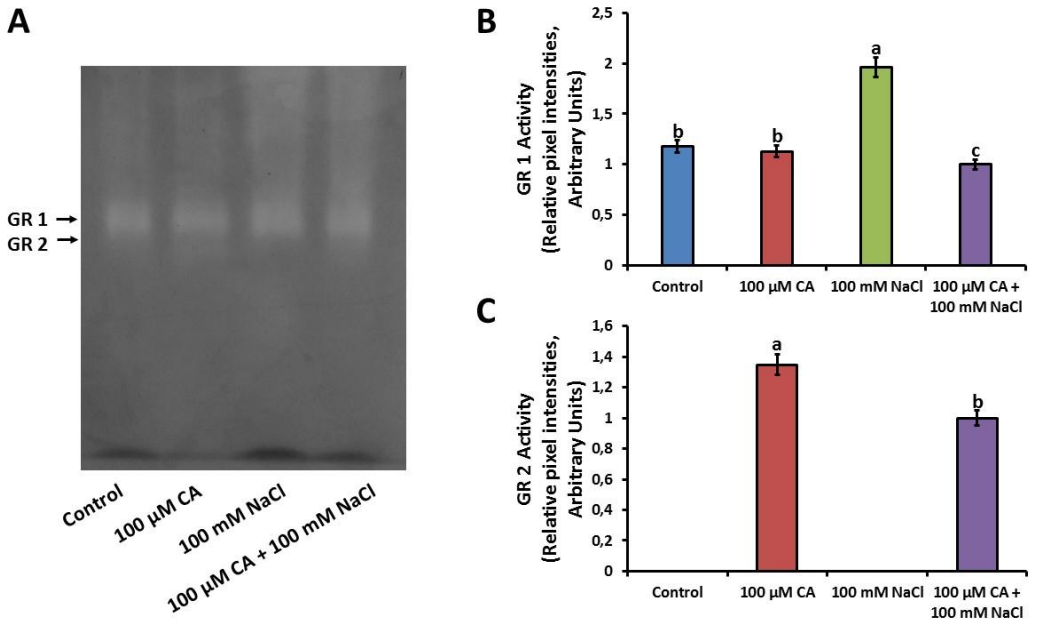



**Figure 4.6. The effect of caffeic acid and salt stress on GR activity in chia leaves.** In – gel activity assay was performed on chia leaves treated for 21 days. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$).

## 4.3. Discussion

### 4.3.1. Exogenous caffeic acid modulates ROS accumulation by restricting oxidative damage

Salt stress has a reputation of negatively affecting plant growth (Keyster *et al*. 2013; Parida and Das 2005) and is known to be associated with ROS accumulation (Miller *et al*. 2010). The deleterious effects of ROS production in response to abiotic stresses and the modulatory role of various enzymatic and non – enzymatic antioxidants in controlling these molecules have been observed in various crops species including liquorice (*Glycyrrhiza uralensis Fisch*), sorghum (*Sorghum bicolor* L. Moench), cucumber (*Cucumis sativus* L.), alfalfa (*Medicago sativa* L.) and many other valuable

cereals (Boldaji *et al*. 2012; Lee and Lee 2000; O'Donnell *et al*. 2013; Pan *et al*. 2006).

The role of caffeic acid in scavenging ROS have been previously described in Wan *et al*. (2014). However, in a recent study by Klein *et al*. (2015), the authors demonstrated that exogenous caffeic acid does restrict oxidative damage by inhibiting ROS production under salt stressed conditions in soybean root nodules. These results are in support of our findings that caffeic acid protects chia plants under salt stressed conditions by inhibiting ROS production (Figure 4.1) and thus restricting ROS – induced oxidative damage and limiting cellular death (Figure 4.2). Therefore, the level of plant cells that are able to remain viable in response to salt stress supplemented with caffeic acid in comparison to salt stressed conditions, instigated enzymatic research in order to determine the effect of an exogenous antioxidant on various endogenous antioxidants.



### 4.3.2. Salt stress tolerance in chia plants is mediated by caffeic acid – induced antioxidant capacity

The involvement of ROS scavenging in long – term salt stress tolerance in chia plants mediated by caffeic acid is supported by the enhancement of antioxidant enzymatic activity in the various treatments. Apart from GR, the enzymatic activities of SOD and APX were differentially regulated in response to exogenously applied caffeic acid and/or salt stress compared to the untreated control. However, the salt stress – induced increase in these enzymatic activities was significantly higher than the increase in the enzymatic activities seen where salt stress treatment was supplemented with caffeic acid. This result could suggest that the supplementation of exogenous

caffeic acid to chia plants could reduce the demand for enhanced enzymatic activity under salt stress since plant experiences reduced signs of stress in the presence of caffeic acid (Figures 4.1 and 4.2).

#### 4.3.2.1. SOD activity is differentially expressed by caffeic acid and salt stress in chia leaves

Superoxide dismutase (SOD) represents the first line of defence when the plant undergoes stress given its high affinity for superoxide radicals (section 1.5.1.1). They are considered chain – breaking enzymes (Alscher *et al*. 2002; Gill and Tuteja 2010). In that case, the total SOD activity was determined spectrophotometrically (Figure 4.3 A) from which a significant increase in total SOD activity within the 100 mM NaCl treated sample was witnessed, indicating the immediate requirement for survival. Interestingly, the SOD levels in the combined treatments showed a significant decrease in comparison to the salt, presenting the first implication of caffeic acid limiting the need for excessive antioxidant activity under salt stress. Subsequently, chia leaves were then subjected to native PAGE analysis in order to identify the individual isoforms contributing to total SOD activity (Figure 4.4) from which, generally, three isoforms were identified. However, intriguingly, a fourth SOD isoform was identified in those samples that were subjected to exogenous caffeic acid which was eventually recognised as a MnSOD. However, the contribution of each isoform, including the CA – induced SOD, toward superoxide radical scavenging varied across all treatments.

The analysis of individual SOD isoforms (FeSOD, MnSOD and Cu/ZnSOD) would require the knowledge of the origin of ROS accumulation which Miller *et al*. (2010)

describes to be within the chloroplast, mitochondria as well as the peroxisome. These authors also emphasise that the main cellular compartment housing ROS accumulation is the chloroplast which is a result of the limitation of $CO_2$ fixation along with the over – reduction of the electron transport chain. In addition to the chloroplast, the mitochondria is also responsible for high levels of ROS as a result of over – reduction of the electron transport chain (Miller *et al*. 2010).

Theoretically speaking, different SOD isoforms are localised within various subcellular compartments whereby FeSOD's (Iron – SOD) are found within the chloroplast, MnSOD's (Manganese – SOD) within mitochondrial and peroxisomal cells and finally Cu/ZnSOD's (Copper Zinc – SOD) are found in chloroplasts as well as the cytosol (Alscher *et al*. 2002; Gupta *et al*. 1993). Given that $O_2^-$ radicals can be generated at any location that contains an electron transport chain, it would not be surprising to witness SOD activity within all those subcellular locations.



However, various environmental conditions could affect the availability of metals and thus diminish the effectiveness of the enzyme. FeSOD's suffer from this environmental change condition as the increase in $O_2$ within the environment results in the reduction of Fe (II) availability which causes a shift to the more available metal, Mn (III). MnSOD's effectively catalyses $O_2^-$ by attracting the negatively charged $O_2^-$ molecule to the positively charged amino acid present at the active site of SOD. Mn (III) then donates an electron directly to $O_2^-$ resulting in a reduction of one $O_2^-$ molecule and thus forms $H_2O_2$ through proton reactions (Alscher *et al*. 2002). Given that MnSOD's derived from FeSOD's, they have similar electrical properties and thus the transition from using iron to manganese did not require an immense change in SOD protein structure.

In addition, Alscher *et al*. (2002) explains that the various SOD isoforms respond differently based on their location as well as the location as to where the oxidative stress affects most. These authors further explained that although MnSOD's are mitochondrial – localized, they can also be targeted to the chloroplast, thereby increasing their protective abilities.

Based on the research conducted in this report, involving chia plants, referring to the SOD native gel (Figure 4.4), the fact that the effectiveness of various SOD isoforms depends on environmental conditions as well as the location of the SOD, and the stress, could provide answers as to why the MnSOD's show greater expression differences across all treatments in comparison to the FeSOD and Cu/ZnSOD. Furthermore, the fact that the level of MnSOD2 is significantly upregulated in response to salt stress, as well as the presence/absence of MnSOD1 across the treatments, could be a consequence of the readily available Mn (III) metal as well as the fact that MnSOD's can be targeted to areas apart from the mitochondria. However, although the information availability on SOD activity and its response to stress is in abundance, given that the proteome and transcriptome of chia plants have not been well studied and thus there is no data relating to the plants proteomic and transcriptomic behaviour, one can only suggest the contributing SOD isoforms.

The public domain provides volumes of information on the effects of salt stress on SOD activity in crop plants (Hernandez *et al*. 1999; Klein 2012), which directly supports the result in this study. However, besides Klein *et al*. (2013) and Klein (2012) (performed on soybean), there is no information available on the relationship between salt and caffeic acid and its combined effect on pseudocereals such as chia. The results in this report show reduced SOD activity in the caffeic acid treated plants

(Figure 4.3 A) in comparison to the control. In addition, the level of SOD activity is also diminished in the salt stressed plants that were supplemented with caffeic acid (Figure 4.3 A) compared to the 100 mM NaCl. Since there are reduced levels of $O_2^-$ in caffeic acid treated plants as well as salt stressed plants supplemented with caffeic acid, the reduction of SOD activity was expected and thus suggesting that exogenous caffeic acid could reduce the necessity for enhanced antioxidant enzyme activity.

#### 4.3.2.2. APX activity is differentially expressed by caffeic acid and salt stress in chia leaves

According to Caverzan *et al*. (2012) and Pandey *et al*. (2015), ascorbate peroxidase (APX) is labelled as the key enzyme within the ascorbate – glutathione (Halliwell – Asada) pathway by functioning to reducing $H_2O_2$ to $H_2O$ and $O_2$, using ascorbate (AsA) as an electron donor (section 1.5.1.1). APX is valued for its ability to be functional within the chloroplast, mitochondria, peroxisomes as well as in the cytosol (Mittler *et al*. 2004; Shigeoka *et al*. 2002). Caverzan *et al*. (2012) continues to explain that generally, APX activity, along with other enzymatic antioxidants, increases in response to various environmental stress which is clearly observed in the 100 mM NaCl treated sample within the spectrophotometer analysis (Figure 4.3 B) as well as the native PAGE data (Figure 4.5). However, as with SOD isoforms, various APX isoforms are usually classified according to their subcellular localization known as mitochondrial-, chloroplastic-, cytosolic - and peroxisomal/glyoxysomal – APX isoenzymes which can be identified using the Peroxidase database (Caverzan *et al*. 2012; Dabrowska *et al*. 2007; Pandey *et al*. 2015).

Though, unlike SOD isoforms, Pandey *et al*. (2015) further explains that APX isoforms across all subcellular compartments are upregulated in response to salt stress given its great sensitivity to abiotic stresses. Therefore, in the case of this report, the two APX isoforms identified in chia leaves were labelled as APX1 and APX2 and was not further classified given the limited sequence information availability within the *Salvia hispanica* species.

However, although no significant enzymatic changes was observed for APX1, APX2 showed distinct alterations in expression levels across all treatments (Figure 4.5 C). As previously stated, chia leaves experienced a distinct increase in APX activity in response salt. However, the application of exogenous caffeic acid significantly reduced total APX activity (Figure 4.3 B) as well as APX2 expression levels (Figure 4.5 C) in response to 100 µM CA in comparison to the control. This finding could suggest that exogenous caffeic acid limits the demand for active enzymatic antioxidants given the reduction in ROS molecules present within the plant system when exposed to caffeic acid (Figure 4.1).

The relationship between caffeic acid and salt are not well documented and thus the effect of these exogenous compounds (combined) on pseudocereals antioxidant systems have been done. The results in this report show that salt stress supplemented with caffeic acid reduces total APX activity (Figure 4.3 B). However, since the exogenous application of caffeic acid to salt stressed chia plants reduced $H_2O_2$ production molecule formation (Figure 4.1 B) as well as lipid peroxidation and cell death (Figure 4.2), the demand for excessive APX activity is reduced in comparison to salt stressed chia plants.

#### 4.3.2.3. GR activity is differentially expressed by caffeic acid and salt stress in chia leaves

The function of the Halliwell – Asada pathway is to utilize its' enzymatic and non – enzymatic antioxidants, in parallel, to assist in reducing ROS molecules to levels less toxic to the plant (Pandey *et al*. 2015). The final active enzyme in the pathway is known as glutathione reductase (GR) and have been observed in bean (*Phaseolus vulgaris* L.; Cakmak and Marschner 1992) and maize plants (Yannarelli *et al*. 2007). GR maintains a cellular redox state by means of catalysing the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) through the oxidation of NADPH (section 1.5.1.1), resulting in maintaining a balance between reduced GSH and AsA pools (Hossain *et al*. 2012). As expected, increased GR enzymatic activity within the salt stressed plant was observed (Figure 4.3 C; Figure 4.6). However, different from the $O_2^-$ and $H_2O_2$ scavenging enzymes (SOD and APX), the level of GR activity in the caffeic acid treated plants showed a significant increase in comparison to the control (Figure 4.3 C). It is possible that because the expression levels of SOD and APX activity could be directly linked to the concentrations of ROS molecules present within the plant, the expression of these enzymes in the caffeic acid treated plants are lower than the control. However, since GR's main function in the Halliwell – Asada pathway is to serve as a catalyst in the regeneration of ascorbate (Lee and Lee 2000), its upregulated expression in response to caffeic acid could serve as a protective mechanism.

On the other hand, a second GR isoform was identified that was unique to those plants subjected to caffeic acid and hence referred to as CA – induced (Figure 4.6). The presence of an additional GR isoform in response to caffeic acid could also explain

the upregulation of total GR activity (Figure 4.3 C) in chia plants in response to 100 µM CA compared to the untreated control plants.

However, salt stressed chia plants supplemented with caffeic acid reduced GR activity to a level below that of the control which is in contradiction to what was observed for SOD and APX. Since the effect of caffeic acid on the entire Halliwell – Asada pathway within pseudocereal crops had not been elucidated, we suggest that there could be a direct link between caffeic acid biosynthesis and GR induced ROS scavenging to promote salt stress tolerance in chia plants.

# CHAPTER 5

# EXOGENOUS CAFFEIC ACID AND SALT STRESS ALTERS THE LEAF PROTEOME OF CHIA PLANTS

## 5.1. Introduction

Plant polyphenols are naturally occurring polyphenolic compounds within plant systems (Pandey and Rizvi 2009). They are involved in diverse functions that contribute to the health and survival of the plant (Duthie *et al*. 2003). In response to salt stress, polyphenolic compounds are known to reduce reactive oxygen and thus participates in the defence against ROS accumulation (Ksouri *et al*. 2007). The most abundant phenolic compound described in food crops is known as caffeic acid (CA) (Belay *et al*. 2016).



The exogenous application of caffeic acid on food crops have been shown to enhance salt tolerance in soybean root nodules through the reduction of superoxide radicals (Klein *et al*. 2013). These authors focused on the physiological and biochemical responses to caffeic acid and salt stress, thereby showing that the exogenous supplementation of caffeic acid under salt stress enhanced antioxidant activity and thus improves salt tolerance in soybean plants.

However, the effect of caffeic acid on plant proteomes have not been described, thus making this study a first of its kind. Given the distinct physiological and biochemical changes observed in chia plants in response to salt and caffeic acid, including the combined treatment (Chapter 3 and 4), changes in protein abundance should be

observed. Therefore, this study investigated the changes in leaf protein abundance under salt stress in the presence of caffeic acid.

## 5.2. Results

### 5.2.1. Separation and visualisation of chia leaf samples on 1D SDS – PAGE

Chia leaf samples were subjected to 1D SDS – PAGE to evaluate protein quality and abundances prior to 2D SDS – PAGE analysis. A total of 10 µg of total protein extract (section 2.14.1) of all chia samples were separated and visualised on a CBB stained 12 % polyacrylamide gel (Figure 5.1). The protein expression across all treatments showed high similarities which suggested uniform protein loading across all treatments. It was also evident that the separated proteins were of high quality with no visible streaking or protein degradation. The results show that protein extracts from all treatments covered the molecular weight (MW) range between 14.4 and 116 kDa in which some bands were more expressed than others. Distinct differences in the chia leaf protein profile in response to the various treatments were observed as indicated by the different arrows (Figure 5.1). Exogenously applied caffeic acid increased protein abundance/expression as shown for the band intensities compared to the control sample. However, the opposite was observed in the salt stress treatments where some of the proteins identified were downregulated when compared to the untreated control. Interestingly, the reduction in band intensity observed in the salt treatment was reversed when salt stressed plants were supplemented with exogenous caffeic acid. The increase in protein abundance/expression (as seen for increased band intensities) could be attributed to more than one protein separating as a single band, whereas the opposite can be suggested for the proteins with low

expression/abundance. This in turn illustrates a limitation associated with 1D SDS – PAGE analysis. Therefore, the separation of proteins in the second dimension could aid in protein identification as all proteins would separate as individual spots.

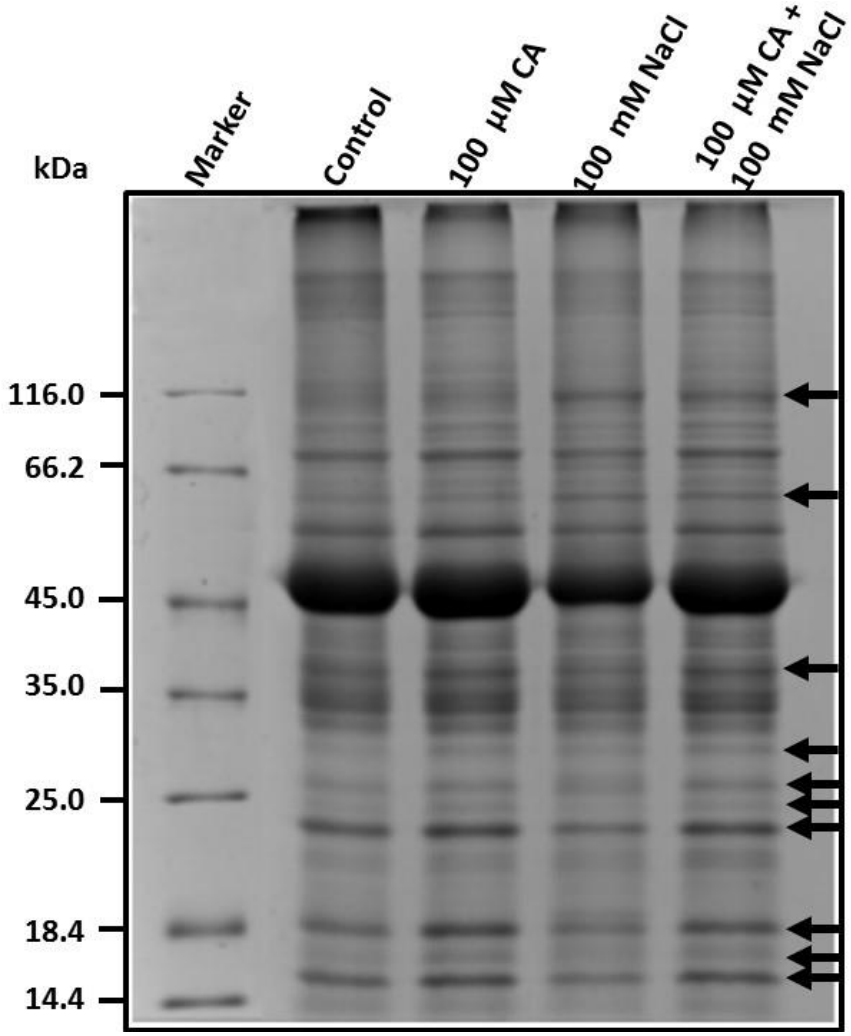



**Figure 5.1. One – dimensional leaf proteome profile of chia in response to caffeic acid and salt stress.** Protein extracts from different treatments were size fractionated on a 12 % denaturing 1D SDS polyacrylamide gel.

### 5.2.2. Detection of differential expressed proteins in chia leaves exposed to caffeic acid and salt stress

Two – dimensional gel electrophoresis was used for the detection and identification of differential expressed proteins in chia leaves in response to exogenously applied

caffeic acid and salt stress. Using 1D SDS – PAGE analysis, proteins were separated based on their MW whereas 2D SDS – PAGE resolved proteins based on their MW as well as their isoelectric point (p*I*), allowing for precise comparison between protein samples. A fraction of chia leaf protein extract (100 µg) from three independent biological and technical replicates of each treatment were resolved on 7 cm IPG strips of pH range 4 – 7 and separated on a 12 % (v/v) 2D SDS – PAGE gels (section 2.14.3). The well resolved CBB stained protein spots were visualised and imaged as described in section 2.15 and 2.16. The result show that most protein spots are confined within the 25 – 116 kDa range with an experimental IEF pH restriction of 4 – 7 (Figure 5.2).

**Table 5.1.** Tabular representation of various categories identified in this study based on differentially expressed proteins across the two – dimensional gels

| Class | Regulation | Spot Numbers | Total |
|---|---|---|---|
| **I** | CA ↑; S ↓; CA + NaCl ↑ | 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 12; 13; 14; 15; 16; 17 | 16 |
| **II** | S ↓; CA + NaCl ↑ | 18; 19; 20; 21 | 4 |
| **III** | CA ↓; S ↑; CA + NaCl ↓ | 11 | 1 |

** ↑ Up-regulated ↓ Down-regulated

The results in Table 5.1 show that caffeic acid and salt stress (imposed by 100 mM NaCl) significantly alters the leaf proteome of chia plants (Table 5.1; Figure 5.2). Table 5.1 is divided into three distinct classes. Class I represents protein spots that are upregulated in response to caffeic acid (even the combined treatment) and downregulated by salt stress. Class II represents protein spots that are downregulated salt stress and recovered by caffeic acid. However, showed no difference in expression levels in response to caffeic acid in comparison to the untreated control.

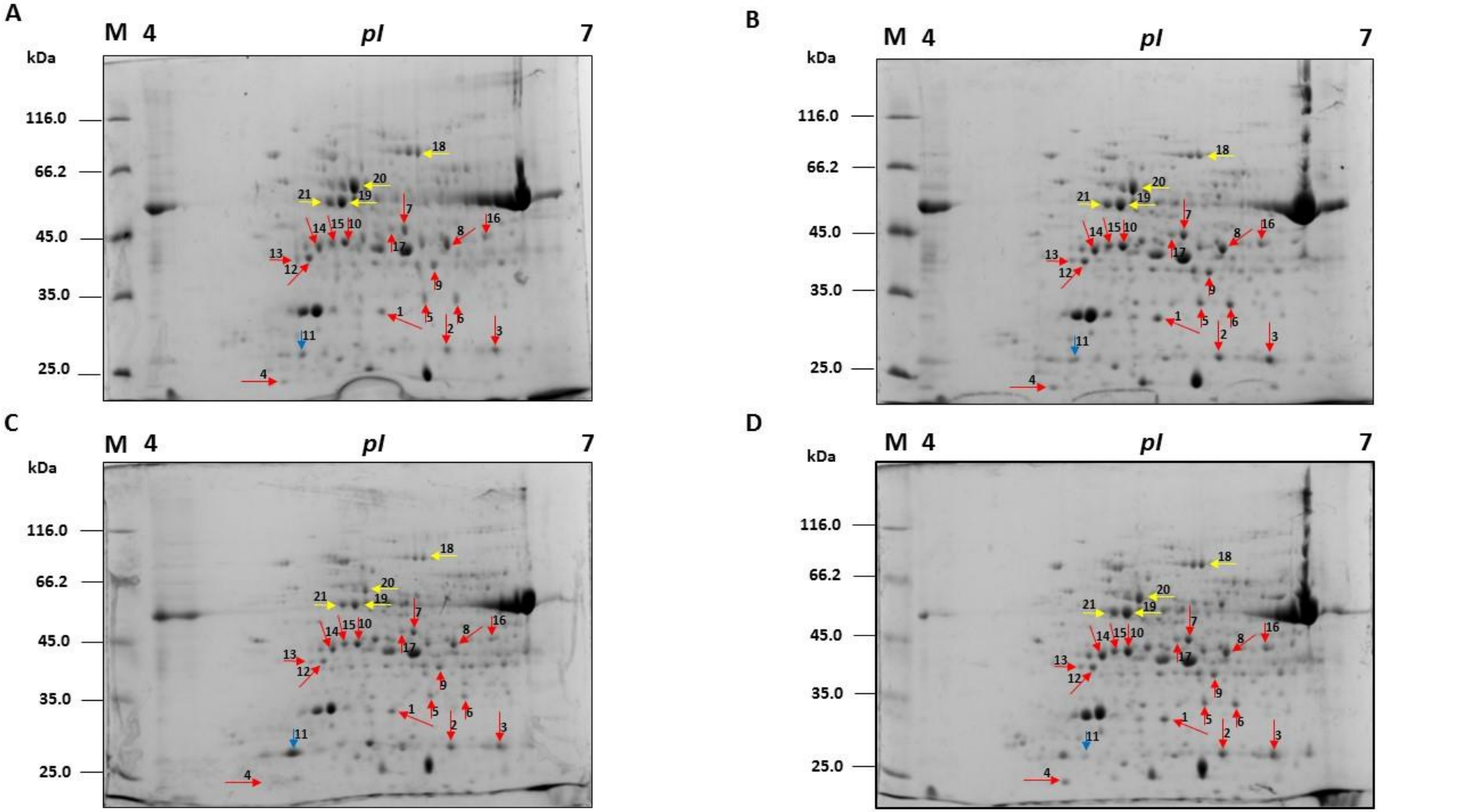



**Figure 5.2. Representative CBB stained two – dimensional SDS PAGE gels of chia leaves in response to different treatments.** Chia leaf samples (control (A), 100 µM CA (B), 100 mM NaCl (C) and 100 µM CA + 100 mM NaCl (D)) were separated on a 12 % denaturing 2D SDS polyacrylamide gel. Red arrows represent CA – induced spots; yellow arrows represent CA – recovered spots and blue arrows representing salt – induced spots.

Class III represents protein spots that are upregulated by salt stress but inhibited by caffeic acid. The protein spots represented in each class was plotted as a pie chart (Figure 5.3). The result shows that 76 % of the protein spots detected in this study were induced by caffeic acid whereas 19 % of the spots was recovered by caffeic acid when suppressed by salt stress. Only 5 % of detected protein spots was induced by salt stress (Figure 5.3).

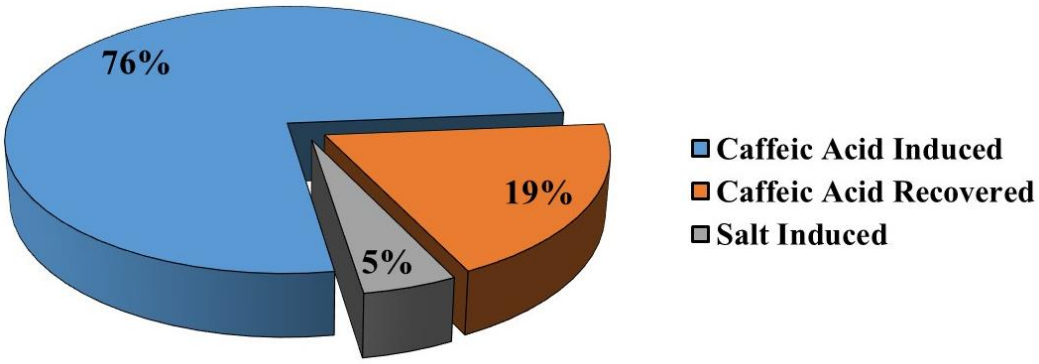



**Figure 5.3. Graphical representation of the influence of caffeic acid and salt stress on protein expression in chia leaves**



### 5.2.3. Identification and functional characterisation of differentially expressed chia leaf proteins

A total of 21 spots were well resolved, reproducible, abundant spots that were excised and identified using MALDI M/S (Table 5.2) as described in section 2.18. The results obtained from mass spectrometry were tabulated showing spot numbers, protein ID's, accession numbers, molecular weight search (MOWSE) scores, molecular weights, isoelectric points, location of the identified protein as well as its expression. Proteins were identified by means of a newly developed *Salvia hispanica* transcriptomic database converted to a peptide database using TRINITY version Trinityrnaseq_r2013-02-25 (Haas *et al*. 2013) against the NCBI protein datasets of *Sesamum indicum* and *Erythranthe guttata* as primary related species.

**Table 5.2.** Protein identification of differentially expressed chia leaf proteins spots using MALDI – TOF MS/MS

| Spot No.[a)] | Protein Name | Species | gi\|[b)] | Accession No.[c)] | MOWSE score[d)] | Matching peptides | Exp. MW(kDa)/ pI[e)] | Location[f)] | Expression [g)] |
|---|---|---|---|---|---|---|---|---|---|
| | **Carbohydrate Metabolism** | | | | | | | | |
| **5** | Ferredoxin-NADP reductase, leaf type isozyme, chloroplastic | *Erythranthe guttata* | 604319636 | XP_012345049.1 | 383.84 | 9 | 35.90/ 6.20 | Chloroplast stoma | CA-induced |
| **6** | Ferredoxin-NADP reductase, leaf type isozyme, chloroplastic | *Erythranthe guttata* | 604319636 | XP_012345049.1 | 504.14 | 7 | 35.90/ 6.20 | Chloroplast stoma | CA-induced |
| **8** | Phosphoglycerate kinase chloroplastic | *Sesamum indicum* | 747078374 | XP_011086346.1 | 476.60 | 9 | 64.50/9.83 | Chloroplast | CA-induced |
| **9** | Fructose-bisphosphate aldolase 1 chloroplastic | *Erythranthe guttata* | 747082340 | XP_012837114.1 | 354.53 | 6 | 42.00/5.75 | Plastoglobule | CA-induced |
| **12** | Sedoheptulose-1,7-bisphosphatase, chloroplastic | *Sesamum indicum* | 604342107 | XP_011084948.1 | 375.28 | 10 | 49.90/8.64 | Chloroplast | CA-induced |
| **13** | Sedoheptulose-1,7-bisphosphatase, chloroplastic | *Sesamum indicum* | 604342107 | XP_011084948.1 | 236.08 | 6 | 49.90/8.64 | Chloroplast | CA-induced |
| **14** | Phosphoribulokinase, chloroplastic-like (Thioredoxin F1 chloroplastic) | *Erythranthe guttata* | 604316997 | XP_012847146.1 | 259.13 | 5 | 54.40/5.98 | Chloroplast stroma | CA-induced |
| **16** | Glyceraldehyde-3-phosphate Dehydrogenase B chloroplastic | *Sesamum indicum* | 747055657 | XP_011074072.1 | 94.74 | 2 | 46.40/9.58 | Chloroplast stroma | CA-induced |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **18** | Transketolase chloroplastic | *Sesamum indicum* | 747044732 | XP_011090550.1 | 170.94 | 4 | 92.70/6.23 | Chloroplast thylakoid membrane | CA-recovered |
| | **Proton Transport** | | | | | | | | |
| **19** | ATP synthase CF1 beta subunit (chloroplast) | *Sesamum indicum* | 335059307 | YP_004935673.1 | 438.18 | 8 | 55.40/5.17 | Chloroplast thylakoid membrane | CA-recovered |
| **20** | ATP synthase CF1 alpha subunit (chloroplast) | *Sesamum indicum* | 496538586 | YP_004935651.1 | 176.51 | 3 | 57.60/5.20 | Chloroplast thylakoid membrane | CA-recovered |
| **21** | ATP synthase CF1 beta subunit (chloroplast) | *Sesamum indicum* | 335059307 | YP_004935673.1 | 490.03 | 8 | 55.40/5.17 | Chloroplast thylakoid membrane | CA-recovered |
| | **Nitrogen Metabolism** | | | | | | | | |
| **10** | Glutamine synthetase leaf isozyme chloroplastic like | *Sesamum indicum* | 747086629 | XP_011071523.1 | 278.71 | 6 | 45.20/5.12 | Chloroplast | CA-induced |
| **15** | Glutamine synthetase leaf isozyme chloroplastic like | *Sesamum indicum* | 747086629 | XP_011071523.1 | 152.87 | 4 | 45.20/5.12 | Chloroplast | CA-induced |
| | **Protein synthesis** | | | | | | | | |
| **7** | Elongation factor TuB, chloroplastic | *Sesamum indicum* | 1024013872 | XP_011101284.1 | 279.38 | 5 | 45.00/6.09 | Chloroplast | CA-induced |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **17** | Elongation factor TuB, chloroplastic | *Sesamum indicum* | 1024013872 | XP_011101284.1 | 232.54 | 7 | 45.00/6.09 | Chloroplast | CA-induced |
| | **Detoxifying enzymes** | | | | | | | | |
| **1** | Carbonic anhydrase chloroplastic-like isoform X1 | *Sesamum indicum* | 747100747 | XP_011098473.1 | 415.61 | 9 | 35.70/ 6.30 | Cytoplasm | CA-induced |
| **2** | Carbonic anhydrase chloroplastic-like isoform X2 | *Sesamum indicum* | 747100747 | XP_011098474.1 | 348.74 | 8 | 27.60/6.02 | Cytoplasm | CA-induced |
| **3** | Carbonic anhydrase chloroplastic-like isoform X2 | *Sesamum indicum* | 747100747 | XP_011098474.1 | 458.10 | 9 | 27.60/6.02 | Cytoplasm | CA-induced |
| **4** | 2-Cys peroxiredoxin BAS1 chloroplastic-like | *Sesamum indicum* | 747092875 | XP_011094222.1 | 88.86 | 2 | 28.50/4.72 | Chloroplast | CA-induced |
| | **Disease/Defence** | | | | | | | | |
| **11** | Thaumatin-like protein | *Sesamum indicum* | 604334283 | XP_011072290.1 | 123.24 | 3 | 25.50/4.81 | Secreted | Salt-induced |

**a) Spot number as indicated on 2D gel images (Figure 5.2)
**b) gi| numbers obtained from the newly developed *Salvia hispanica* transcriptomic database generated using TRINITY version and searched against the NCBI protein database
**c) Accession number in the National Center for Biotechnology Information (NCBI) database
**d) MOWSE score for MALDI-TOF
**e) Experimental MW and pI were estimated from the 2D gels shown in Figure 5.2
**f) Subcellular location of the proteins as predicted by UniProt (http://www.uniprot.org/)
**g) Describe the expression levels of each treatment relative to the untreated control as shown in Figure 5.2

Positively identified proteins were functionally characterised based on their involvement in various cellular processes using a combination of similarity searches with Universal Protein Sequence database (http://www.uniprot.org/) and various other literature sources (The EU Arabidopsis Genome Project 1998; Ngara 2009). These categories include carbohydrate metabolism, proton transport, nitrogen metabolism, protein synthesis, detoxifying enzymes and disease/defence. (Table 5.2; Figure 5.4).

Most of the proteins identified in this study were associated with carbohydrate metabolism (43 %) followed by detoxification (19 %). In addition, positively identified proteins are also shown to be involved in proton transport (14 %), protein synthesis (10 %), nitrogen metabolism (9 %) and disease/defence (5 %) (Figure 5.4).

Interestingly, all the positively identified spots involved in carbohydrate metabolism were caffeic acid induced, apart from spot 18 that was instead recovered by caffeic acid under salt stress. Protein spots involved in nitrogen metabolism, protein synthesis and detoxification are also caffeic acid induced whereas all proteins involved in proton transport, along with spot 18, were classified as class II CA – recovered proteins (Table 5.1; Table 5.2; Figure 5.3). In addition, spot 14 represented the only spot to be successfully identified as a defence protein in response to salt stress in chia leaves.

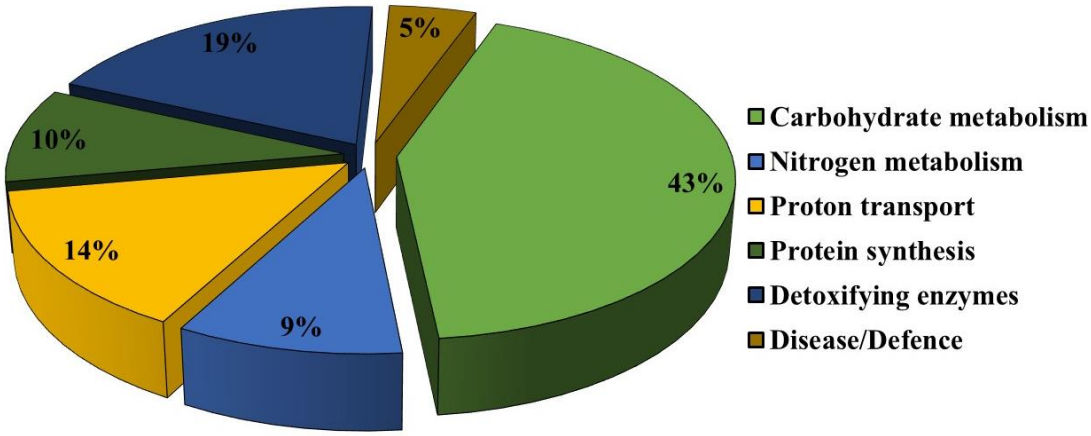



**Figure 5.4. Functional characterisation of identified proteins in chia leaves across all treatments.**

### 5.2.4. Subcellular localisation of positively identified proteins

Protein function is dependent on subcellular localisation (van Wijk 2001). Localisations of the positively identified chia leaf proteins were predicted using a combination of similarity searches with Universal Protein Sequence database (http://www.uniprot.org/), Reactome: A Curated Pathway Database (http://www.reactome.org/) and various literature sources whereby majority of the positively identified proteins were localised to the chloroplast (67 %). Some proteins were found in the cytoplasm (25 %) and the rest were identified as secreted proteins (8 %) (Figure 5.5). Proteins localised to the chloroplast were further sub localised in the general chloroplast (40 %), chloroplast stroma (27 %), chloroplast thylakoid membrane (27 %) and plastoglobuli (6 %) (Figure 5.5).

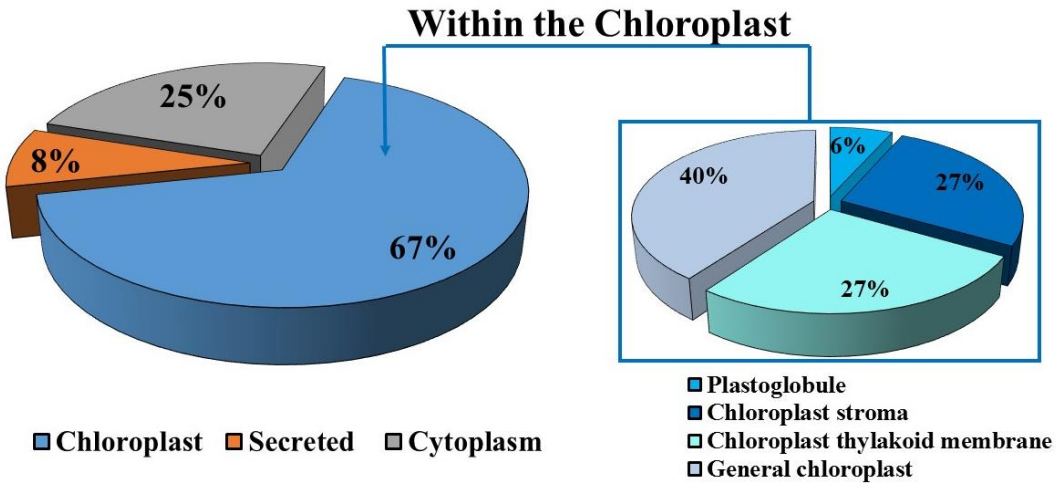



**Figure 5.5. Subcellular localisation of positively identified proteins expressed in chia leaves.**

### 5.2.5. Proteins observed in multiple spots

A total of six classes of proteins were observed in multiple spots in the 2D gels (Figure 5.2; Figure 5.6). These classes include chloroplastic ATP synthases (spots 19, 20 and 21), chloroplastic carbonic anhydrases (spots 1, 2 and 3), elongation factors (spots 7 and 17), ferredoxin – NADP reductases (spots 5 and 6), glutamine synthetases (spots 10 and 15) and sedoheptulose – 1,7 – bisphosphate (spots 12 and 13). The multiple protein – spotting pattern in this chia leaf proteome can be subdivided into two groups. Group 1 represent proteins that have the same NCBI accession number and molecular weight, with different pI values. Group 2 include proteins with the same protein ID with different accession numbers, molecular weight and pI values. Proteins found in group 1 are elongation factors (spots 7 and 17), ferredoxin – NADP reductases (spots 5 and 6), glutamine synthetase leaf isozymes (spots 10 and 15) and sedoheptulose – 1,7 – bisphosphate (spots 12 and 13). On the other hand proteins found in group 2 are the ATP synthase proteins (spots 19, 20 and 21) and the carbonic anhydrase (CCA) proteins (spots 1, 2 and 3).

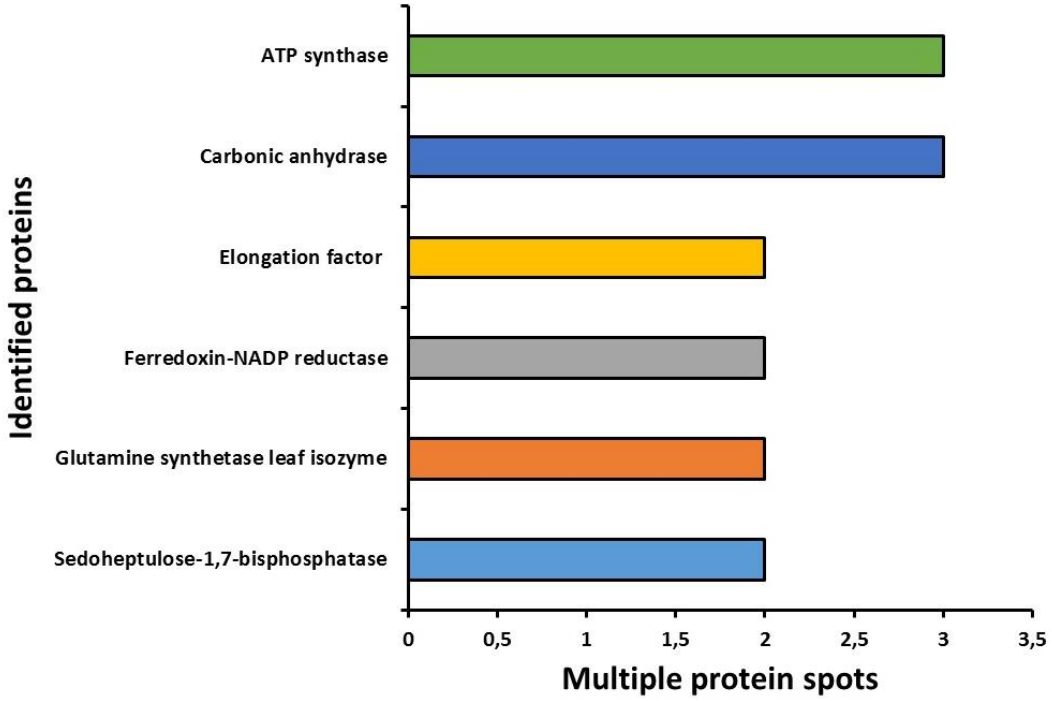



**Figure 5.6. Protein classes identified in multiple spots.**



## 5.3. Discussion

Here we describe the influence of exogenous caffeic acid and salt stress on the leaf proteome of chia plants. Two – dimensional gel electrophoresis coupled with mass spectrometry was used to identify and functionally characterise differentially expressed proteins in the chia leaves. Although the influence of salt stress on leaf proteomes of important cereal crops have been well documented, no evidence exists in the public domain that describe the effect of caffeic acid on protein expression and its role in modulating protein changes under salt stress. To our knowledge, this study is a fist of its kind. The results presented here demonstrates that exogenous caffeic acid does influence protein abundance/changes in chia leaves exposed to salt stress (Figure 5.2). Most of the differentially expressed protein spots visualised, detected and identified were CA – induced and were involved in various metabolic

processes (Table 5.2). Positively identified proteins (21) were grouped into six distinct functional categories based on their putative functions and subcellular localisation. These categories include carbohydrate metabolism, proton transport, nitrogen metabolism, protein synthesis, detoxifying enzymes and disease/defence. The roles of each of these categories are described below:

### 5.3.1. Functional characterisation of proteins identified in chia leaves

**Carbohydrate metabolism**

From the 21 proteins positively identified in this study, 9 are associated with carbohydrate metabolism. These proteins were identified from spots 5, 6, 8, 9, 12, 13, 14, 16 and 18 (Table 5.2). The proteins in this category represent most proteins identified in this study (43 %; Figure 5.4). Proteins involved in carbohydrate metabolism participates in various biological processes and metabolic pathways such as photosynthesis and glycolysis. All proteins (except spot 18) identified in this functional category were upregulated in response to caffeic acid and down regulated by salt stress (Class I; Table 5.1). However, the expression levels of these proteins were significantly upregulated in the salt stress treatment supplemented with caffeic acid compared to the salt stressed plants (Figure 5.2). On the other hand, spot 18 was categorised as a Class II protein (Table 5.1). Apart from the current study, no evidence exists describing the influence of caffeic acid on protein expression, whereas the effect of salt stress is well documented. The reduction of photosynthesis – related proteins in response to salt is consistent with rice, wheat, soybean and potato (Nouri *et al*. 2015) and could be due to non – specific DNA damage (Pessarakli 2016).

In addition, a reduction in photosynthesis could also stem from the accumulative effects of ROS which include ROS molecules reacting with various proteins and lipids immediately associated with photosynthesis (Lawlor and Tezara 2009).

**Proton transport**

Proton transport (14 %; Figure 5.4) represents the third major functional category of the proteins identified in this study. These proteins, identified as ATP synthases (spots 19, 20 and 21), are involved in transporting protons across the chloroplast thylakoid membrane (Table 5.2; Figure 5.5). ATP synthases are known to serve as the general energy currency of the cell (Engelbrecht and Junge 1997) by converting ADP to ATP in the presence of a proton gradient across the thylakoid membrane (McCarty 1992; von Ballmoos and Dimroth 2007). This, in turn, drives a wide variety of energy consuming cellular processes during plant cell growth and development. Therefore, given that various cellular processes are dependent on ATP production, the inhibition of $K^+$ ions in the presence of salt (section 1.4) reduces the photosynthetic rate as a result of limited $K^+$ ions required for the charge balance at the site of ATP production (Shabala and Pottosin 2014). Therefore, all processes dependent on ATP, including carbohydrate metabolism, are inhibited which is clearly represented in the 100 mM NaCl treated sample given the reduction in the expression of majority of the proteins (Figure 5.1; Figure 5.2). On the other hand, the ATP synthase (spots 19, 20 and 21) expression levels were not altered in response to caffeic acid. However, in the salt stress treatment supplemented with caffeic acid, ATP synthase expression was enhanced to a level significantly higher than observed

for the salt treatment (Class II; Table 5.1). This could suggest that caffeic acid modulates salt stress tolerance by controlling ATP synthase activity under salt stress.

**Nitrogen metabolism**

Nitrogen (N) contributes significantly to plant biomass and serves as an essential component of most biomolecules. Inhibition in nitrogen metabolism significantly alters phenotypic characteristics, thus reducing plant growth and yield in various crop species (Beatty *et al*. 2016). Glutamine synthetase (spots 10 and 15; Table 5.2) contributes significantly to nitrogen metabolism by catalysing the first step in ammonia assimilation (Miflin and Habash 2002). This protein separated as two distinct isoforms (spot 10 and 15; Figure 5.2) contributing to 9 % of the 21 positively identified proteins in this study (Figure 5.4). Exogenous caffeic acid enhanced glutamine synthetase abundance/expression relative to the untreated control whereas the opposite was observed for salt stress (Class I; Table 5.1; Figure 5.2). This could be a result of stomatal closure in response to salt stress, thereby reducing various nitrogen source uptake (Hu *et al*. 2014). A reduction in nitrogen source uptake inhibits nitrite and nitrate reductase functioning to yield ammonia which in turn inhibits the effect of glutamine synthetase (spots 10 and 15) under salt stress. However, the expression of glutamine synthetase was significantly higher in the combined treatment when compared to the salt treatment. This suggest a regulatory role of caffeic acid in nitrogen metabolism under salt stress conditions.

**Protein synthesis**

Protein synthesis plays a vital role in providing the cells with the necessary proteins and enzymes which participate in various biological processes within the cell (Ngara 2009). This category constitutes 10 % of the positively identified proteins (spots 7 and 17) within this study (Figure 5.4). These proteins are chloroplast elongation factors Tu (EF – Tu) (Figure 5.2; Table 5.2), which migrated as two isoforms and have been shown to play an important role in protein synthesis in tobacco plants (Murayama *et al*. 1993; Sugita *et al*. 1994). Caffeic acid and salt stress differentially alters EF – Tu expression relative to the untreated control. Caffeic acid increased protein abundance of EF – Tu whereas salt stress reduced EF – Tu abundance which was in contrast to what was observed in Ndimba *et al*. (2005). However, the reduction in EF – Tu abundance observed in the salt treatment was reversed when caffeic acid was supplied to the salt treatment (Class I; Table 5.1; Figure 5.2).



**Detoxifying enzymes**

The 2 – Cys peroxiredoxins BAS1 (2 – Cys PRX BAS1) enzyme was successfully identified as a detoxifying enzyme within the chia leaf proteome (spot 4; Table 5.2). These enzymes form part of a large family of peroxidases in which Baier and Dietz (1996 a and b) have studied and introduced into the public domain. These authors have justified that the two cysteine residues conserved in all 2 – Cys peroxiredoxins allow them to function in the detoxification of peroxides (alkyl hydroperoxides and $H_2O_2$). In addition to 2 – Cys PRX BAS1, various carbonic anhydrase isoforms (spots 1, 2 and 3) have been identified as detoxifying enzymes (Table 5.2). These proteins are known to play a vital role in various physiological functions, including the

reduction of $O_2^-$ and $H_2O_2$ as well as contribute to the carboxylation/decarboxylation reactions, involving both photosynthesis and respiration (Ellis 2006; Moroney *et al*. 2001). The detoxifying enzymes represent the second major functional category of proteins identified in this study (19 %; Figure 5.4). However, although salt stress imposes severe stress on chia plants, the expression of these detoxifying enzymes under salt stress are reduced in comparison to the control and thus increasing the level of stress given the inactivity of these detoxifying enzymes. This result corresponds to the expression level of 2 – Cys PRX BAS1 in *Arabidopsis* plants under abiotic stress (Seki *et al*. 2002). On the other hand, since the effect of exogenous caffeic acid under salt stress have not been documented, it is interesting to note the upregulation of these detoxifying enzymes in response to caffeic acid in comparison to the control (Figure 5.2). In addition, the supplementation of exogenous caffeic acid under salt stress increased 2 – Cys PRX BAS1 and carbonic anhydrase expression levels beyond that of the salt stressed plant (Class I; Table 5.1). Therefore, suggesting that exogenous caffeic acid promotes the detoxification of peroxides as well as reactive oxygen.



**Disease/Defence**

Although majority of the salt exposed chia protein profile was downregulated, one highly upregulated protein, in response to salt stress, was successfully identified as a thaumatin – like protein (TLP) (spot 11; Figure 5.2; Table 5.2). Plant TLP's are known to play a vital role in plant defence and stress responses (Zhao *et al*. 2010). The distinct upregulation of this protein under salt stress corresponds to a study performed on *Halogeton glomeratus* (Halogeton; within the amaranth family) whereby TLP spots were significantly abundant after salt treatment, expressing the

role of TLP in secondary cell wall development in plants (Wang *et al*. 2015). On the other hand, the application of exogenous caffeic acid showed to have no effect on this particular enzyme, however, the supplementation of caffeic acid under salt stress significantly reduced the expression level of the TLP enzyme in comparison to the salt stress treatment (Class III; Table 5.1). This result suggests that the exogenous application of caffeic acid reduced the demand for enhanced defence activities.

### 5.3.2. Subcellular localisation of proteins identified in chia leaves

Most proteins involved in carbohydrate metabolism (section 5.3.1) are located within the chloroplast or the chloroplast stroma given its involvement in photosynthesis and glycolysis (Table 5.2; Figure 5.5). However, fructose – 1,6 – bisphosphate aldolase 1, based on UniProt searches, has been localised within in the plastoglobule. Plastoglobules are globular compartments found within plastids and were usually viewed as lipid and carotenoid storage particles (Brehelin *et al*. 2007). However, more detailed studies had shown a possibility of plastoglobules functioning within the chloroplast. Using mass spectrometry methods, Vidi *et al*. (2006) identified chloroplastic metabolic enzymes within the *Arabidopsis* plastoglobule proteome, one of them being fructose – 1,6 – bisphosphate aldolase 1. Vidi and co – workers (2006) thus concluded that plastoglobules are not just lipid storages and that the presence of these enzymes suggest its involvement in metabolic pathways. However, Lundquist *et al*. (2012) studied fructose – 1,6 – bisphosphate aldolase 1 further and kindly excluded its involvement in the plastoglobule proteome explaining that they are not enriched in the plastoglobule fraction and that their abundance remains within the

stroma and the thylakoid membrane. On the other hand, Nacir and Brehelin continued to argue this statement in 2015, expressing that fructose – 1,6 – bisphosphate aldolase 1 enzyme could partition between plastoglobules and other compartments of the chloroplast and thus should not be completely excluded from the plastoglobule proteome, but rather be considered as enzymes that exhibit functional roles within the plastoglobules as well as in other plastid compartments (Nacir and Brehelin 2015).

In addition, transketolase (spot 18), functioning in carbohydrate metabolism, as well as all ATP synthase proteins (spots 19, 20 and 21) are all localised within the thylakoid membrane (Table 5.2; Figure 5.5). The thylakoid membrane functions in the fluidity of various lipids, membrane proteins, ions, pigments and various biological substances. However, any form of environmental stress can alter the fluidity and the composition of the stressed membrane (Tian *et al*. 2016) which was evident in the changes observed in the expression levels of these particular proteins in response to different environments (caffeic acid and salt).



Furthermore, thaumatin was the only protein to be involved in the secretome (Figure 5.5), which is described as proteins that are released into the extracellular space at any given time and under certain conditions through various secretory mechanisms (Agrawal *et al.* 2010).

### 5.3.3. Multiple proteins identified in chia leaves

Given the existence of multiple proteins (Figure 5.6), it is possible that chia leaf proteins could have undergone post – translational modifications (PTM) (Pejaver *et al*. 2014). In addition, the identification of multiple proteins could be a result of a

combination of PTM, transcription, translation and protein turnover as a response to environmental change (Abdallah *et al*. 2012). Furthermore, proteins encoded by multigene families could also aid in multiple protein identification (Degand *et al*. 2009) (Table 5.2; Figure 5.6). The identification of multiple proteins were also observed in proteomic studies involving maize leaves (Porubleva *et al*. 2001) and sorghum leaves (Ngara 2012).

# CONCLUDING REMARKS AND FUTURE PROSPECTS

The consequences of salt stress on food crop production have been extensively reviewed. Salt stress have detrimental downstream effects on economically important food crops due their sensitivity to abiotic stress conditions and therefore contribute significantly to food insecurity within various regions. Chia is regarded as a potential alternative food source due to its nutritional and medicinal characteristics. Interestingly, chia is deemed to be naturally drought tolerant although this hypothesis has not been scientifically tested. This study described the regulatory effects of caffeic acid (small signalling molecule) to long term salt stress by monitoring the physiological and molecular responses of chia plants. The results presented in this thesis is divided into three research chapters.



Chapter three described the effect of exogenous caffeic acid and salt stress on chia growth, biomass, relative water content and photosynthetic metabolism. Caffeic acid and salt stress differentially alters plant growth, total chlorophyll, and beta carotenoid content. Caffeic acid improved plant growth (Figure 3.1) and biomass (Figure 3.2) whereas salt stress showed an inhibitory effect. Interesting to note is that in the combined treatment (caffeic acid + salt stress), caffeic acid reversed the negative effects caused by salt stress by improving plant growth, biomass, and photosynthetic ability.

Chapter four described the regulatory role of caffeic acid by enhancing salt stress tolerance through differentially modulating ROS metabolism and antioxidant

capacity in chia plants. Salt stress significantly enhanced ROS biomarkers, which is a common phenomenon previously described in literature. However, caffeic acid (under salt tress conditions) controls the increase in ROS molecules to levels that are no longer toxic to plants. This in turn restricted the extent of lipid peroxidation and ultimate cellular death (Figure 4.2). The exogenous application of caffeic acid reduced antioxidant enzymatic activity. This result, in correlation with reduced ROS levels, suggests that caffeic acid reduces the demand for enhanced antioxidant enzyme activity.

Furthermore, chapter five comparatively analysed changes in the leaf proteome of chia plants in response to exogenous caffeic acid and salt stress. Although the influence of salt stress on plant proteomes are well documented, this study is the first to describe the effect of caffeic acid on plants proteomes and how caffeic acid regulates changes in the leaf proteome under salt stress. Using gel based proteomic analysis (2D PAGE coupled with mass spectrometry), we have positively identified 21 differentially expressed proteins, some of which could serve as potential biomarkers to improve salt stress tolerance in chia and other cereal and pseudocereal food crops. Some of these markers include ferredoxin – NADP reductase, phosphoglycerate kinase, fructose – bisphosphate aldolase, sedoheptulose – 1,7 – bisphosphatase, phosphoribulokinase, glyceraldehyde – 3 – phosphate among other caffeic acid induced or recovered proteins identified in Table 5.2.

The identification of energy and photosynthesis related proteins coupled with improved plant growth and biomass (as observed in chapter 3) suggest that caffeic

acid could serve as a plant growth promoting agent (alone) or as a regulatory signalling molecule under salt stress conditions. However, more in depth research is required to support this hypothesis. The role of caffeic acid in modulating salt stress tolerance should thus not be limited to the modification of the ROS scavenging antioxidant system and changes in protein abundance but extended to analysing the transcriptome and metabolome changes. This would provide great insight as well as pave the way for genetic engineering to enhance salt tolerance to sensitive crops.

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