Gallic acid modulates salt stress tolerance in soybean plants by regulating antioxidant capacity

Pateka Menzi

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Supervisor: Dr. Ashwil Klein

Co-supervisor: Dr. Marshall Keyster

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KEYWORDS

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Reactive oxygen species

Salt stress tolerance

ABSTRACT

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P. Menzi

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Soil salinity is one of the main limiting factors of plant growth and contributes to large portion of crop loss more especially in the semi-arid regions. Multiple studies have been done over the past few decades which have demonstrated how soil salinity hinders plant growth and development. Phenolic compounds have gained much importance due to their potential as antioxidants. Plant phenolics have been studied with the intention to identify compounds with protective roles against oxidative damage. Plants use these phenolic compounds for growth, reproduction, pigmentation and resistance to stresses.

Gallic acid (GA) is one of the phenolic compounds known to induce antioxidant activity and has been shown to enhance the activity of these enzymes under abiotic stress. However a large number of these studies are on human or animal cells. Only a few have explored the effect of GA in plants. The work presented in this study is two-fold. First, we analysed the influence of different concentrations (0.25 mM, 0.5 mM and 1 mM) of GA on the physiological and molecular response of soybean plants. This was done to determine the optimal concentration of GA, which promotes plant growth, and enhance biomass production while minimizing reactive oxygen species (ROS) accumulation. The second part of this study focused on using the determined concentration of GA to mitigate salt stress tolerance in soybean plants by analysing changes in various physiological and molecular parameters.

The results showed that exogenous GA differentially altered both physiological and molecular responses in soybean plants in the absence of salt stress. Low to moderate concentrations of GA (0.25 mM-0.5 mM) improved plant growth and biomass whereas the higher concentration (1mM) resulted in the complete opposite. Improved growth and biomass production observed in soybean plants treated with low to moderate GA are directly linked to the reduction in ROS accumulation and augmentation of antioxidant enzyme capacity. Based on these results we selected for lowest concentration of GA (0.25 mM) to regulate salt stress responses in soybean plants by monitoring changes in physiological parameters and various molecular responses.

Exogenous application of GA (0.25 mM) improved soybean growth and biomass prediction under salt stress (imposed by NaCl) conditions. Due to its antioxidative nature, GA restricted ROS accumulation at basal level (similar to control) and reversed the inhibitory effects caused by salinity stress. However the reduction in ROS accumulation in the combined treatment (GA + NaCl) could be attributed to the increase in the antioxidant activities of SOD and APX. These enzymes were able to minimise oxidative damage manifested by the increase in cell death. Taking in to consideration the increase in plant growth and antioxidant capacity coupled with the reduction in salt-induced ROS accumulation and oxidative damage under salt stress, it can be suggested that supplementation of GA notably improved soybean salt tolerance possibly via signals that regulate ROS accumulation during salinity stress.

DECLARATION

I declare that "**Gallic acid modulates salt stress tolerance in soybean plants by regulating antioxidant capacity** " 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.

Pateka Menzi December 2017

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APX	ascorbate peroxidase
BSA	bovine serum albumin
ca	circa
Cu/Zn – SOD	copper zinc superoxide dismutase
DAB	3,3'-Diaminobenzidine
DTT	dithiothreitol (Cleland's reagent)
EDTA	ethylenediaminetetraacetic acid
Fe – SOD	iron superoxide dismutase
GA	gallic acid
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
MDA	malondialdehyde
Mn – SOD	manganese superoxide dismutase
mM	milimolar
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitrotetrazolium blue chloride
PAGE	polyacrylamide gel electrophoresis
ppm	parts per million

POD	Peroxidase
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMED	N,N,N',N' - Tetramethylethylenediamine

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Soybean [*Glycine max* L (mer)] is one of the top commodity crops in the world including South Africa (de Beer and Prinsloo, 2013). These small yet important podded legumes are a great source of protein and are used in many forms. Soybeans have been incorporated in the human diet since the Shang dynasty era (ca 1500-1100 B.C) in Asia. Its many products include tofu, soy milk, miso and soy sauce which attracted many European travellers during the 1700's (du Toit 1942). Soybean was only introduced to South Africa in 1903 and since then the production of this crop has rapidly grown. The most prominent soybean products which are used as high protein meal and soybean oils. The high protein meal is a key ingredient for animal feed more especially in the pork and poultry industry (Dlamini *et al.,* 2014).

There is great potential in the market for soybean in South Africa however due the constraints in the soybean market such as government support, insufficient research and development (DTI, 2010), and environmental factors. These environmental factors include abiotic stresses (drought, salinity, heavy metal and UV radiation) which threaten the soybean industry. Salinity is amongst the most significant abiotic stressors which has been a limiting factor in plant production

mostly in arid and semi-arid climates (Hussain *et al.,* 2009; Acosta-Motos *et al.,* 2017). Soil salinity is estimated to affect ~800 million hectares of arable land (Munns and Tester, 2008).

Plants however have various mechanisms to regulate the accumulation of Reactive Oxygen Species (ROS) by scavenging these radicals through the use of ROS-scavenging antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1) a major scavenger of O2-, which results in the formation of H_2O_2 and O_2 (Dionisio-Sese and Tobita, 1998). Catalase (CAT; EC 1.11.1.6) dismutase's the H_2O_2 into H_20 and molecular O_2. Peroxidase (POD; EC 1.11.1.7), glutathione peroxidase (GPX; EC 1.11.1.9), ascorbate peroxidase (APX; EC 1.11.1.11) are also involved in the removal of H_2O_2 by producing water (Amirjani, 2010).

The defence mechanisms does not only include enzymatic antioxidants it also includes non-enzymatic active molecules that mediate stress. These active molecules such as phenolics can effectively supress the progression of oxidative damage. These plant phenolics such as GA (3,4,5-triphydroxyl-benzoic acid) is widely distributed in various plants and is a strong antioxidant (Niemetz and Gross, 2005). A number of studies have shown GA to have anti-tumoral, anti-mutagenic and antibacterial activity in various animal and human cells (Yeh and Yen, 2006). Furthermore GA pre-treatment has shown significant protective effects on the biochemical properties in induced myocardial infractions in Wistar rats (Priscilla and Prince, 2009). The pre-treatment of GA showed protective properties in the heart through the prevention of lipid peroxidation (Jadon *et al.,*

2007) and increased the levels of Glutathione (GSH), vitamin E and C in plasma and heart which shows the antioxidant potential of GA against injury caused by ROS (Priscilla and Prince, 2009). Recently studies on plants have been done in order to support the antioxidant effects of GA in plants. Exogenous application of GA has been found to enhance the activities of SOD, CAT, Peroxidase (POX) and APX in rice seedlings under salinity stress (Ozfidan-Konakci *et al.,* 2015).

The main focus of this review will be on the detoxification of ROS in plants under salinity stress using naturally occurring antioxidants using phenolic compound such as GA in order to mitigate salt stress or infer tolerance. In addition, it will demonstrate the importance of improving the growth of essential legumes such as soybean which are high commodity crops used for animal and human consumption. Furthermore, it will demonstrate improving the production of these food crops will in turn decrease food insecurity, undernourishment and increase economic growth.

1.2 Crop production

As a consequence of the growing population, human food consumption will be four times greater making the food availability be a relative crisis (Green *et al*., 2013). The food distribution worldwide has increased however, food production is at critical level as new challenges arise given the increase in demand for food . This has caused exigency in the production of processed foods and food supply, which results in producers being pressured into acquiring more land and resources (Campbell, 1991).

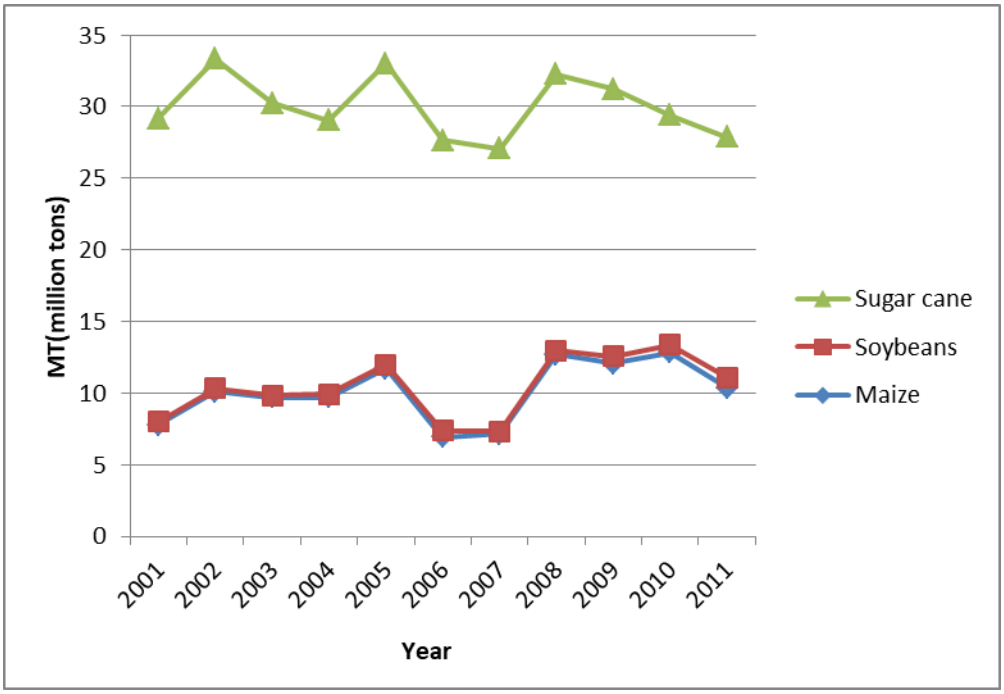


Figure 1.1: Production of the top 3 crops 2001-2011. A comparison of production of sugar cane, soybean and maize in South Africa. Maize being the least produced and sugar cane being the most produced in 2011 (FAOSTAT, 2013).

South Africa has 1.2 million square kilometres (km) of agricultural land, only 12% of that land is used for crop production (maize, wheat, sugar cane and sunflowers). South Africa is the main producer of maize in the Southern African Development Community (SADC) with more than 9000 commercial producers. The Southern African region consume maize their main source of carbohydrates (Brand SA, 2008). Of the three crops produced in South Africa (Figure 1.1) over period of 10 years, maize and soybean production was just over 10 MT in 2011. Furthermore, the production yield of cereal is 22.5% higher than pulses (Figure 1.2) which is worrisome as the nutritional value of legumes has been shown to be greater than cereals.

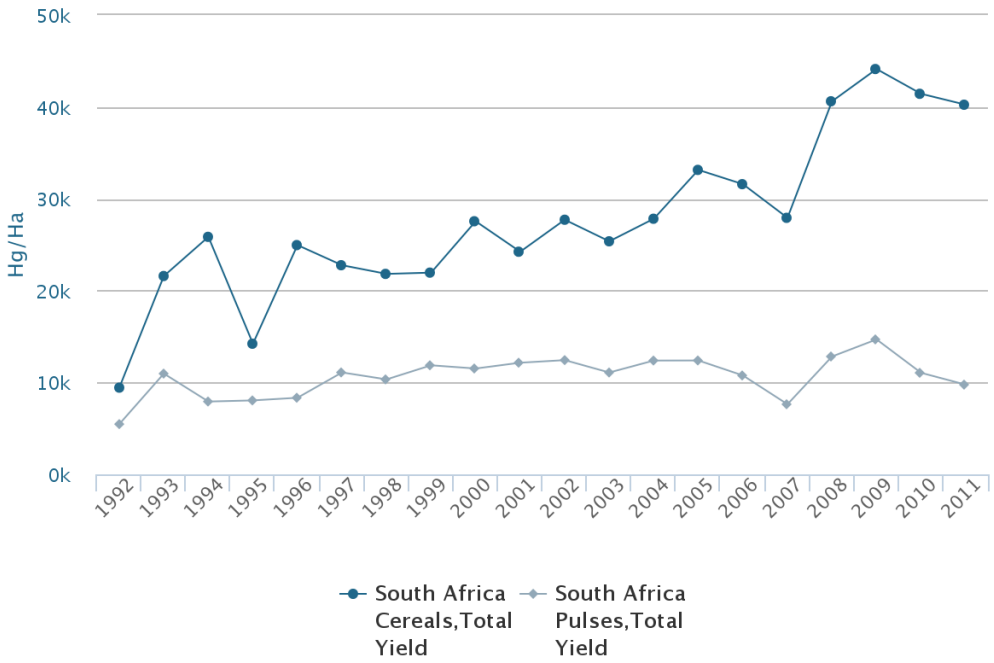


Figure 1.2: Total yield of cereals and pulses. Production of cereals has increased over the years whilst pulses have decreased. In 2011, the cereals were four times more than pulses. The units on the graph represents; Hectograms (100 grams) per hectare (Hg/Ha) (FAOSAT, 2013).

1.3 Legumes

Legumes are podded fruits which are cotyledons; they belong to the Leguminosae family and have been the main cultivated grain for centuries. The prominence of grain legumes globally is high as they are a significant source of nutrition in humans and animals. There are up to 19 000 species of legumes and these include the common bean (*Phaseolus vulgaris*), alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), lentil (*Lens culinaris* Med.), peanut (*Arachis hypogaea*) and soybean (*Glycine max* L. merr.) making them the third largest family of higher plants (Gepts *et al.,* 2005). Being the second most consumed crops, the role of this family is significantly disregarded, even though

they have shown to have higher nutritional value than most cereals. They are not only nutritious but also contribute to the ecosystem by providing nitrogen. The process is facilitated by nitrogen fixing bacteria (*Rhizobiaceae*, α-*Proteobacteria*) in the soil. This rhizobia bacterium interacts with the plant, by invading the roots which multiply and over a period of time root nodules form. Within the nodules inert N_2 is converted into NH_3 which is the biologically usable form of nitrogen (Zahran, 1999) this then reduces the need of for external inputs of nitrogen, by improving fertility and yield in nitrogen poor soils (Smykal *et al.,* 2012).

1.3.1 Soybean

1.3.1.1 History of soybean

Soybean (*Glycine max* L. Merr.) was initially domesticated during Zhou dynasty in the eastern half of northern China. The oldest available record however places soybean domestication during Shang dynasty (ca 1500-1100 B.C) (Hymowitz, 1970). The incorporation of soybean in the Asian diet with products such as tofu, soy milk, miso and soy sauce attracted many European travellers. This then lead to the pod being traded via the already established trade routes to the West. The oldest available record of soybean entering the United States was in 1756 (Hartman *et al.,* 2011). Soybeans were first introduced into South Africa in 1903 (Dlamini *et al.,* 2014), with little information of the farming of the field crop, farmers experienced side effects in production. This then led to the Department of Agriculture and Forestry worked extensively in order to minimise the difficulties faced by the farmers. The extensive research was aimed at minimising

reoccurring difficulties in production which could be eradicated by finding advanced production methods suitable in South Africa (du Toit, 1942). It was then recommended that the best place to produce the crop was in the KwaZulu Natal Province formerly known as Natal, when properly managed it would give optimal production (Hall 1930). Many stake holders were now involved in the improvement of the production of the crop such as the Feed Committee which wanted to improve production for animal feed. Over decades South Africa gradually increased its soybean production, however it was not until the late 1900 that momentum gained and output plummeted to 50 000 tonnes nationwide in 2010 (Opperman and Varia, 2011).

1.3.1.2 Soybean production and market value in South Africa

There is a great interest in South Africa in the use of soybean products due to the health benefits associated with these products. The consumption of soybean in South Africa is ~32% for oil and oil cakes (remaining solids after pressing soybean to extract the oil), 8% human consumption and 60% used for animal feed (DTI, 2010; DAFF, 2010). The protein content in soybean is a good source of protein for vegans and vegetarians (DAFF, 2010). The demand of soybean oil in the South Africa was ~ 1.3 MT in 2010, making it the top contender within SADC region. (Opperman and Varia, 2011). Other countries in the region (Malawi, Zimbabwe, Zambia and Mozambique) only had a demand of 0.2 tons in 2010 (Dlamini *et al.,* 2014). This can be attributed to the preference of soybean oil rather than

sunflower oil as it is more expensive in South Africa, therefore the dominance will always supersede those of fellow SADC countries.

The soybean industry in 2009 brought in~R1.1 billion and with production yields of up to 50 000 tons in 2010 which brought the production up by 5% (DAFF, 2013). The demand for soybean in South Africa is greater than the production yield; currently only 10% of the demand is produced locally and 90% of the protein meal is imported predominantly from Argentina and Brazil. The projection for demand in 2015 was estimated to be between 1 759 000 and 3 290 000 tons per annum (NAMC, 2011).

Since the study done by Hall (1930), the production of soybean has expanded to other provinces in the country. In his study, he suggested that soybean growth would be suitable in the Natal (KZN) region due to the rainfall in the region. However many studies have since been done in order to understand cultivation, production and processing of soybean in South Africa (du Toit 1942; Dlamini *et al.,* 2014). In Figure 1.3, a trend can be seen where the soybean production was greater in Mpumalanga (363000 tons) than any other province whilst the Western Cape and Eastern Cape have had the least amount of production with up to 1200 and 2100 tons respectively (DAFF, 2016). The production of soybean in South Africa fluctuates over time, on average the production ranges between 100 000 and 800 000 tons per annum with an average yield of 1.7 to 2 tons per hectare of land under dry conditions. One of the causes of the low production rate of soybean in the Eastern Cape is due to the assumption that the plant is

only suitable for commercial farmers, and the province is more saturated with subsistence farmers.

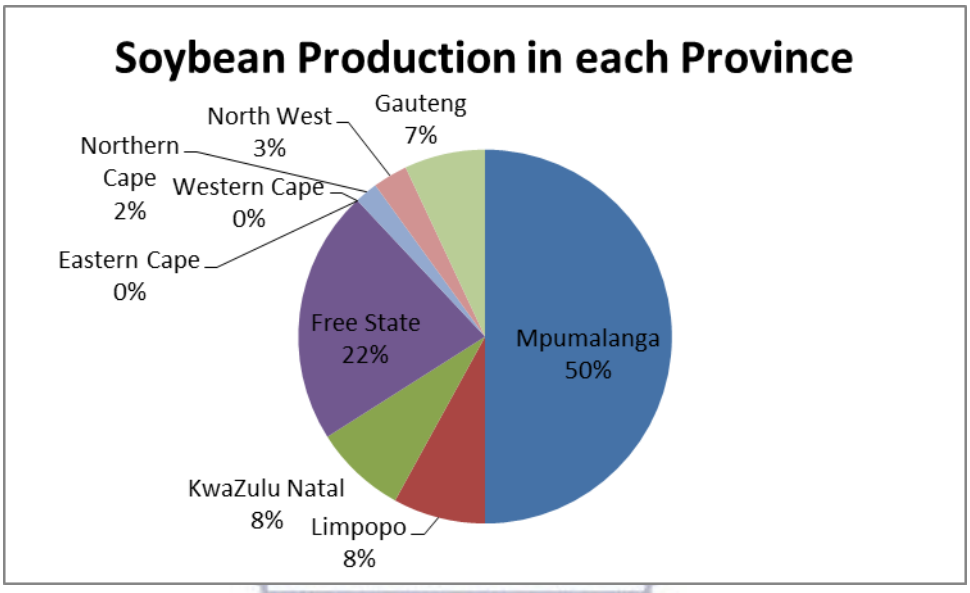


Figure 1.3: Production of Soybean per province in 2016. The data presented in the graph was retrieved from the National Department of Agriculture (DAFF, 2016).

The area used for the cultivation of soybean has increased gradually, in 1976 ~22 000 hectares was used for soybean, which gave an output of 17 900 tons (Dlamini *et al*., 2014). In 1993, the output increased to 63 100 tons even though the allocated land had decreased (Protein Research Foundation, 2013). The introduction of GMO in the soybean industry brought upon a surge of production and area allocated for the planting of soybean. The Compound Annual Growth Rate (CAGR) increased from 8.9% in 1976 up to 10.5% in 2012. When comparing the CAGR of output and area planted from 1997 to 2012, it is clear that the yields per hectare have drastically improved in the past 16 years (BFAP, 2013).

The demand for soybean is mainly from the processing or crushing industries, the increase for the meal and soybean oil is mainly as a result of rising income levels and improvement in the crushing capability. Domestically the rise in GDP has also contributed to a higher demand for livestock products, dairy products and consumption of vegetable oils. The majority of the seeds are sold to oil producers, animal feed and seed manufactures.

1.3.2.1 Nutritional composition and health benefits of soybean

Similar to other legumes soybeans are highly nutritious however, the macronutrient profile is more unique because soybeans have higher protein and fat content than other beans and are relatively low in carbohydrates; up to 36% of the calories contained in soybean are from proteins (Figure 1.4). These macronutrients include protein, fat, and fibre. Soy protein is a high quality protein. According to the guidelines adopted by the Food and Drug Administration and World Health Organisation it receives a rating of 1, which is the highest possible score (Messina, 2014). This in turn means that the quality of soy protein is equivalent to that of meat and milk derived proteins.

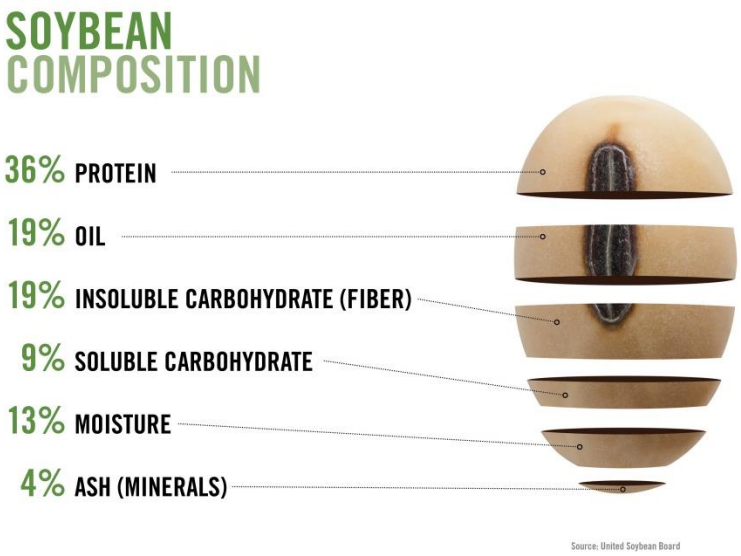


Figure 1.4: Nutritive composition of soybean seeds. Figure was adapted from Zurich Private Capital (2016).

The fat portion makes up 19% of the bean (Figure 1.4) and is extensively used both in food industry and directly by the consumers. Soybean oil is commonly used for cooking and commonly referred to as “vegetable oil”. The majority of the fat contained in soybean is unsaturated with 63% being polyunsaturated (linoleic acid), 23% monounsaturated (oleic acid) and 14% saturated (palmitic acid). The linoleic acid is an omega 3 fatty acid, and soybean is amongst the few plants which contain a substantial amount of omega 3 (USDA, 1979).

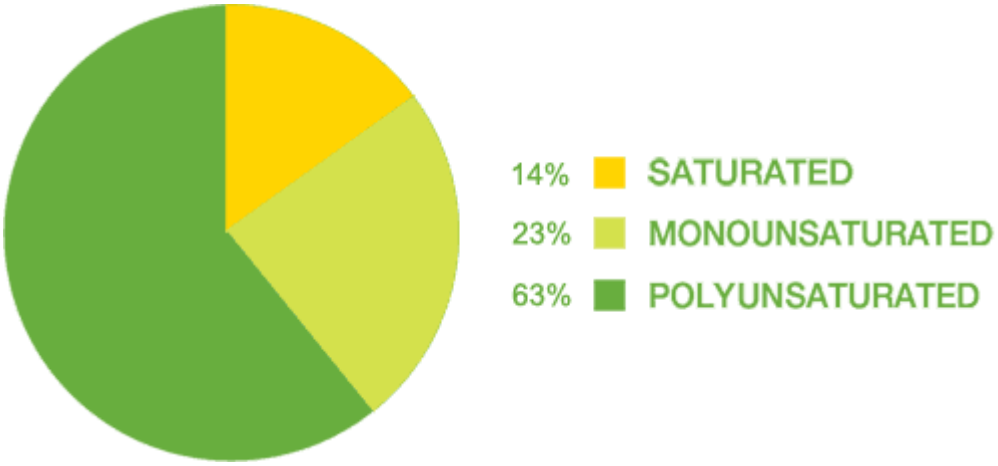


Figure 1.5: Fat composition of soybean. The data presented in this graph was adapted from Krishna (2015)**.**

The fibre content in soybeans makes up 19% (soluble) of the bean (Figure 1.4), and of that 9% is insoluble fibre. A single serving of the soybean provides ~ 8 grams (g) of dietary fibre; however some processing methods decrease the fibre content of the soy food significantly (Wang *et al*., 2009).

As soybean is mostly acknowledged for its source of plant proteins, apart from it being a good source of protein it also contains basic nutritive constituents; carbohydrates, dietary fibre, vitamins, minerals, flavonoids and peptides which have therapeutic attributes (Kim *et al.,*2006; Wang *et al.,* 2009). The low content of carbohydrates and fibre in soybean may contribute to the low glycaemic indexes which are beneficial for diabetic patients. In the same breath it reduces the risk of developing diabetes (Jenkins *et al.,* 1981). These dietary fibres have also been seen to have major protective effects against cardiovascular diseases (Anderson *et al.,* 1990; Van Horn, 1997). Epidemiological studies suggest that the

intake of complex carbohydrates and dietary fibre is inversely related to the development of coronary artery associated diseases (Trowell, 1972). A study by Gibson and Roberfroid (1995) shows how oligosaccharides are essential prebiotics which can favourably alter the micoflora of the colon. As a result of these findings in the study it has been proposed that due to its potential health benefit, the soy oligosaccharides can be attributed to the longevity of rural Japanese individuals. These individuals consume copious amounts of soy foods; furthermore soy oligosaccharides are used as a commercial sweetener in Japan (Hayakawa *et al.,* 1990; Anderson *et al.,* 1999)

The readily available minerals and vitamins in soybean have been attributed to it being able to reduce the risk of osteoporosis and hypertension (Dawson-Hughes *et al.,* 1990; Appel *et al.,* 1997). Osteoporosis affects ~ 20 million women in the United States and costs ~10 billion annually, the soy isoflavones have been proposed to preserve bone mineral density (Riggs *et al.,* 1990) human preliminary studies showed how soy isoflavones increased the bone mineral density in postmenstrual women (Brandi, 1992).

1.4 Abiotic stress

1.4.1 Salinity

Soil salinization is said to be the accumulation of soluble salts at the soil surface, and is characterised into primary and secondary salinization. Primary salinization is caused by natural processes; however secondary salinization occurs due to

human activities such as removal of deep rooted plants and improper irrigation (Farifteh, 2007).

Degraded agricultural land due to soil salinity has caused a drastic decline in productivity of plants and ultimately leading to the loss of agricultural yields (Patel *et al.,* 2009). Despite the well-documented cases of the severity of soil salinity being reported worldwide (Gao *et al.,* 2011; Mirlas, 2012; Mashimbye, 2013), there is still an increase in the soil salinity rather than a decrease. Nell (2009) found 5.1% of South African soils to be saline and 23% slightly saline. High concentrations of soil salinity can be detrimental to food crops such as soybean, maize, sorghum and wheat. Salt stress causes a decrease in osmotic potential, severe ion toxicity, nutrient imbalance, plant and cell death (Kumari *et al.,* 2016).

1.4.1.1 Salinity stress in soybean

Soybean is considered a moderately salt sensitive crop (Munns and Tester, 2008), salinity stress has significantly decreased soybean yields (Essa, 2002) through the inhibition of germination and post-germination growth (Saad-Allah, 2015). It also resulted in the reduction of the number and weight of the root nodules (Lauter *et al.,* 1981), shoot height and fresh and dry weights of the plant (Cicek and Cakirlar, 2008b). The chloride toxicity such as leaf chlorosis and reduced plant biomass. Saad-Allah (2015) performed a study in order to evaluate the effect of three sea salt stress levels on six varieties of soybean commonly cultivated in Egypt. Root and shoot development was stunted by the increasing

levels of salinity, furthermore the decline in hormones which stimulate growth and an increase in hormones which inhibit growth was observed.

The mechanism by which salt affects germination is still not clear, however osmotic and toxic effects are responsible for salt injury (Kumar and Sharma, 1990; Cramer *et al.,* 1994). When an ionic imbalance occurs, particularly of Ca^{2+} and K^{+} (Creda *et al.,* 1995; Essa, 2002) these ions have a detrimental effect on plant growth especially during salt stress.

Salinity stress causes an induction of excessive accumulation of reactive oxygen species (ROS) such as superoxide (O_2^{-}), hydroxyl radical (OH^{-}), hydrogen peroxide (H_2O_2) and peroxyl radical to name a few (Ahamad *et al.,* 2011). These ROS molecules can cause peroxidation of lipids, oxidation of proteins, DNA damage and inactivation of enzymes. Plants under salt stress have increased production of ROS and ROS-mediated membrane damage; which have been demonstrated to be major contributors to cellular toxicity (Sharma *et al.,* 2012).

1.5. ROS production

1.5.1 Basal level

Plant production worldwide has been adversely affected by abiotic stress these include drought, salinity, heavy metals, ultraviolet radiation and temperature (Carvalho, 2008). Abiotic stresses such as heavy metals disrupt cell redox homeostasis which results to an increase in the production of ROS and oxidative stress. The production of ROS incessantly produced as a by-product of metabolic

pathways which are confined in different cellular compartments like chloroplast, mitochondria and peroxisomes (Gill and Tuteja, 2010). However ROS is not only produced under oxidative stress, it is also produced at basal level as a signalling molecule to regulate biological and physiological processes (Schieber and Chandel, 2014). Under normal conditions ROS is involved in plant growth and development, response to abiotic and biotic stimuli and programmed cell death (Bailey-Serres and Mittler, 2006). There are four types of cellular ROS which include; singlet oxygen ($_1O^2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Winterbourn, 2008) that have intrinsic chemical characteristics that convene reactivity to diverse biological targets. The specificity in the production of ROS within its biological targets is an essential determinant of whether a redox signalling event or oxidative damage will occur (Dickinson and Chang, 2011). Incidentally when a signalling pathway reaction transpires, a H_2O_2-dependent oxidation of a particular protein will do so in close proximity to source of H_2O_2. For example, the production of H_2O_2 in close proximity of mitochondria, the mitochondria will move towards protein target so signalling pathway may activate (Rhee, 2006). Likewise the same effects can be seen in the production of O_2^- in the mitochondrial matrix; however the accumulation of O_2^- in cytosol results in a different outcome (Finkel, 2011). Thus proving that ROS production during signalling pathways is localised to specific target sites. The differentiation between redox signalling and oxidative stress is greatly determined by the type and concentration of the ROS (Schieber and Chandel, 2014).

1.5.2 ROS as a signalling molecule

The accumulation and production of ROS in signalling pathways is integrated with other signalling networks within the plant these include calcium signalling, cellular metabolic networks, redox responses and protein kinase network. In some instances ROS accumulation would pave way in activating these signalling networks or be a direct consequence of signalling through these networks (Mittler *et al.,* 2011). The integration of these ROS molecules can be seen with mitogen-activated protein kinases (MAPK) (Jammes *et al.,* 2009) which are involved during abiotic induced oxidative stress and in the production of nitric oxide (NO) which are involved in plant defences associated with resistance to pathogens (Asai *et al.,* 2008).

The ROS signal can be produced in a number of cells (mitochondria, chloroplast, and peroxisomes) in plant in response to pathogen, lesions or abiotic stress and be transmitted to the entire plant. Studies have identified that the first site of production of ROS is in the chloroplast. During photosynthesis, the photons from sunlight are seized into light-harvesting complexes (photosystem II and I) in the thylakoid membrane (Foyer *et al.,* 1994). This process is critical for plant survival; this exposes them to oxidative damage due to presence of polyunsatured fatty acids in the chloroplast envelope (Gill and Tuteja, 2010). In the electron transport chain oxygen is used as the final electron acceptor, which leads to the formation of ROS. Even though O_2 is non-reactive it is able to produce highly reactive $_1O^2$ via the triplet chlorophyll in reaction centre of photosystem II (Krieger-Liszkay,

2005). The reduction of a single electron of 0_2 then results in the formation of 0_2^- which is the primary ROS formed. When it accepts one electron and two protons, H_2O_2 is formed. In addition, these molecules interact with transitional metals such as copper and iron with which advanced interactions occur in the Haber-Weiss Mechanism or Fenton to give rise to OH (Gill and Tuteja, 2010).

1.6 Oxidative Damage to Biomolecules

1.6.1 Lipids

The production of these ROS molecules can be detrimental to many parts of the cell. Elevated levels of ROS as a result of abiotic stresses result in the damage of lipids, proteins and DNA (Sharma *et al.,* 2012). These can result in changes of intrinsic characteristics in the membrane such as fluidity, decreased enzyme function, inhibition of regulatory functions such as protein synthesis, DNA damage and eventually cell death. In lipids ROS overproduction augments lipid peroxidation, which exacerbates oxidative stress. The increase of lipid peroxidation finally results in the formation of malondialdehyde (MDA) (Tanou *et al.,* 2009), initially the O_2^- and OH molecules will interact with the methylene group found on a polyunsaturated fatty acids present in membranes which forms lipid peroxy radicals and hydroperoxide (Smirnoff, 1995). The production of MDA is a biochemical measure used to determine the extent of lipid peroxidation in the cell (Moller and Kristensen, 2004).

1.6.2 Proteins

Romero-Putertas *et al*. (2005) describes how ROS can attack proteins causing modifications of proteins which can directly affect the protein activity. Protein oxidation is detected by the increased concentrations of carbonylated proteins; however this is not the only modifications that result due to oxidative stresses. Other modifications include disulphide bond formation, nitrosylation, and glutathionylation (Yamauchi *et al.,* 2008), higher plants have been reported to exhibit protein modifications as a result of abiotic stresses. The oxidation of proteins is further demonstrated in pea plants under cadmium (Cd) stress, which resulted in cellular damage (Romero-Putertas *et al.,* 2005).

1.6.3 DNA

ROS is a major contributor of damage DNA, which is the cells genetic material oxidative damage in DNA can result in changes of polypeptide sequence, which results in the malfunctioning of proteins (Dizdaroglu, 1993). Oxidative attack on DNA bases generally encompasses the addition of OH group to double bonds. Purines, pyrimidine's and deoxyribose backbone react with OH groups (Imlay and Linn, 1988) to generate products which include hydroxylation of C-8 of the guanine forming 8-oxo-7,8 dehydro-2'-deoxygunosine and saturated products (Tsuboi, Kouda and Takeuchi, 1998). However other ROS molecules (H_2O_2 and O_2^-) do not interact with any of the bases and do not cause *in viro* strand damage (Halliwell and Aruoma, 1991). Ultimately even though the cell has mechanisms

which repair DNA damage, excessive damage to DNA by ROS lead to irreversible damage to the DNA which is detrimental to cell function (Dizdaroglu , 1993).

1.7 Antioxidants

1.7.1 Antioxidants found in plants

In order to balance the production of ROS under normal conditions, scavenging of these molecules are mediated by numerous antioxidant defence mechanisms. However even though the production of ROS must be at minimal levels, the cell cannot completely eliminate its production. These scavenging mechanisms involve both enzymatic and non-enzymatic antioxidants to detoxify the cell. The enzymatic enzymes include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST), guicol peroxidase (GPX), monohydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Gill *et al*., 2011; Miller *et al*., 2004; Singh *et al*., 2008). The non-enzymatic include ascorbic acid (ASH), glutathione (GSH), alkaloids, phenolic compounds, non-protein amino acids and α-tocophenols.

1.7.2 Enzymatic antioxidants

1.7.2.1 Superoxide dismutase

The major scavenging enzymes are SOD and CAT. SOD is the first contact enzyme as it the first to be produced, it catalyses the dismutation of O_2^- to oxygen and

H_2O_2 (Willekens *et al.,* 1997). It does so utilising transitional metal co-factors through the Haber-Weiss mechanism. The metal co-factors for SOD are classified into three types; manganese (Mn-SOD), copper/zinc (Cu/Zn-SOD) and iron (Fe-SOD) (Gill and Tuteja, 2010) which are found in diverse cellular compartments. SOD activity has been reported to increase in plants adversely affected by environmental stresses, such as heavy metals. Increased levels of SOD are predominantly associated with increased tolerance of plants against environmental stresses. Zaefyzadeh *et al*. (2009) has reported how increased levels of SOD can be used an indicator to screen for drought-resistance in plants and Gupta *et al*. (1993) has reported results which demonstrate how overproduction of SOD results in enhanced oxidative stress tolerance. A study done by Ginnakoula *et al*. (2010) on maize under aluminium stress demonstrated elevated levels of SOD where found to be associated with tolerance in the maize line, the level of membrane lipid peroxidation was similar to control plants.

1.7.2.2 Ascorbate peroxidase (APX)

The dismutation of H_2O_2 by APX requires a co-factor such as ascorbic acid (AsA) to reduce it to water and producing two molecules of MDHA. APX is an enzyme found in all ROS producing compartments, it is has an essential role in scavenging ROS in higher plants and regulates intracellular ROS levels (Noctor and Foyer, 1998). It scavenges H_2O_2 in a water-water and AsA-GSH cycle using ASH as an electron donor. There are at least five APX isoforms mainly thylakoid, glyoxisome membrane, cytosolic and chloroplast stromal soluble form (Carvalho, 2008).

Multiple studies done on hornworts, Chinese mustard (Wani *et al.,* 2012) and blackgram (Singh *et al.,* 2010) have displayed enhanced levels of APX under Cd stress. Significance in the increase of cystolic APX activity play a crucial role in providing protection against drought stress (Simonovicova *et al.,* 2004) similar effects were observed in transgenic plants exhibited increased tolerance of oxidative stress and pathogenic infection by *Phytophthora nicotianae* (Sarowar *et al.,* 2005).

1.7.2.3 Glutathione reductase (GR)

GR is also an enzyme which is involved in the AsA-GSH cycle, it's a NADPH reliant enzyme which catalyses the reduction of GSSG to GSH and maintain their ratio (Sharma *et al.,* 2012). A two-step catalytic reaction occurs the first being NADPH reducing the flavin moiety which is then oxidised, furthermore a redox active disulphide bridge is reduced to yield cysteine and thiolate anion. The second reaction involves the reduction of GSSG through thiol-disulfide altercation reaction (Ghisla and Massey, 1989). The activity of GR is essentially in photosynthetic tissues by chloroplastic isoforms (Li *et al*., 2005), in the chloroplast GSH and GR are implicated in the detoxification of H_2O_2 generated by the Mehler reaction (Biehler and Fock, 1996). Scavenging of ROS by GR can occur in two ways; directly by scavenges singlet oxygen, OH^- or O_2^-, or indirectly where it's a reducing agent which reutilises AsA from its oxidized form to reduced form by means of DHAR. Plants which exhibit increased levels of GR have been found to be stress tolerant. Pastori and co-author (1992) recognise the connection

between oxidative stress tolerance and GR activity, the oxidative stress caused by H_2O_2 may induce GR synthesis.

As the last enzyme which scavenges ROS in the Haliwell-Asasda pathway, there may still be some excess ROS which is still not detoxified. With the complexity involved in the detoxification of ROS, previous studies have shown that the combination of these enzymes in transgenic plants displays a synergistic effect on stress tolerance (Lim *et al.,* 2007). Multiple overexpressions of SOD, APX and DHAR in combination stresses have also proven to be beneficial in gaining tolerance (Lee *et al.,* 2009).

1.7.3 Non-Enzymatic antioxidants

1.7.3.1 Phenolic compounds

Non-enzymatic antioxidants are involved in oxidative defence system in plants; they interact with multiple cellular components and in addition have a crucial role in defence as enzyme co-factors (Gill *et al*., 2010) They are able to influence plant growth and development by controlling processes mitosis to cell elongation and ultimately cell death (Sharma *et al.,* 2012). There are vast number of these antioxidants one which plants utilise greatly are phenolics (phytophenolics), these are characterised by having at least one aromatic ring attached to one or more OH group (Crozier, Clifford and Ashihara, 2008). These phenoilcs are seen to be beneficial as they are involved in scavenging ROS such as O_2^-, H_2O_2, OH^- and $_1O^2$. Phytophenolics donate electrons to GPX-like enzymes which detoxify H_2O_2 (Sakihama *et al.,* 2002).

1.7.3.2 Gallic acid (GA)

Polyphenols such as GA (3,4,5-trihydroxybenzoic acid) (Habtemariam, 2011) are widely distributed in the plant kingdom. Incorporated in the human diet as antioxidants and are found in many fruits, vegetables and plant derived beverages such as tea, juices wine and coffee (Karamac *et al.,* 2006). GA is amongst one of the simplest polyphenols found in green plant tissues more specifically in tea leaves (Jayamani and Shanmugam, 2014). GA and its derivatives (Theaflavin-3-gallate, epigallocatechin-3-galate) represent a large family of plant secondary polyphenolic metabolites which are commonly known as antioxidants (Eslami *et al.,* 2010; Nabavi *et al.,* 2013).

Many complex polyphenols have been studied; GA has been reported to inhibit aggregation of the reduced and carboxymethylated form of milk. Furthermore, it reduces amyloid β (Aβ) levels in the mouse brain, and significantly improves the cognitive deterioration associated with Alzheimer's disease (Wang *et al.,* 2009). A large amount of scientific evidence shows that GA has antibacterial, antiviral, antifungal, anticancer and antioxidant activities (Li *et al.,* 2005). Nabavi and co-authors (2013) also determined that GA was able to inhibit nephrotoxicity and oxidative stress in renal tissues of rats.

1.7.3.3 Gallic acid biosynthesis

The biosynthesis of GA has been investigated since the late 60's by Dewick and Haslam (1969) and in the past six decades there have been three possible routes

which have been proposed. The first being through the β-oxidation of the side chain of the 3,4,5-trihydrocinnamic acid (Zenk, 1964, which (route A) implies that GA (4) can be produced from phenylalanine (1) via the same pathway as for caffeic acid and trihydrocinnamic acid (Figure 1.6). The second proposed route (B) is through the direct dehydrogenation of 5-dehydroshikimic acid (2) preserving oxygen functions from erythrose 4-phosphate (5) and phosphenol pyruvate (6) (Dewick and Haslam 1968, 1969). The last (route C) being the hydroxylation of protocatechuic acid (Kambourakis *et al.,* 2000).

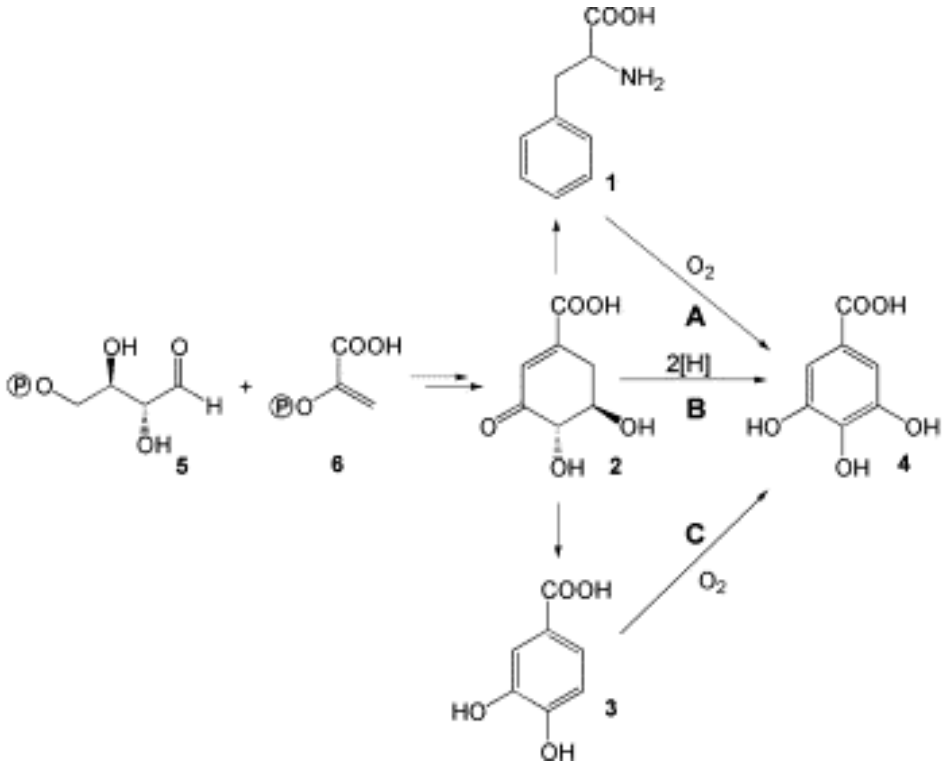


Figure 1.6: The biosynthetic pathway of gallic acid. Figure was adapted from Werner *et al.* (2004).

1.7.3.4 Antioxidant activity of Gallic acid

GA is also widely used in the pharmaceutical industry due to its biological and pharmacological activities, which include scavenging of free radicals (Badhani *et al.,* 2015), inhibition of squalene epoxidase (Abe *et al.,* 2000) and apoptosis of cancer cells (Liang *et al.,* 2012). GA does not only have antioxidant characteristics but also a pro-oxidant, it can either inhibit or endorse free radical production due to metal chelation. In the presence of iron GA promotes the hydroxyl radical production whereas in the absence of iron it is able to scavenge the radical (Habtemariam, 2011).

The efficiency of GA as an antioxidant was demonstrated by Yen *et al* (2002) where GA was able to scavenge up to 60% of H_2O_2 at a concentration of 4.17 mM GA. GA has strong scavenging activity on OH^- and H_2O_2,however GA shows no superoxide radical scavenging activities (Abdelwahed *et al.,* 2007). These phenolic compounds have been researched for prevention against diseases and interest in them has dramatically increased (Porat *et al.,* 2002). However, fewer studies have been done on the effect of GA on plant oxidative stress. Plants are a great source of energy for both plants and animals and increasing the production of these crops of paramount importance. The use of similar phenolic compounds has proven to be beneficial to the research community.

Ozfidan-Konakci *et al.* (2015) used GA on two rice cultivars in order to enhance tolerance against salt and osmotic stress induced by polyethylene glycol (PEG). They found that the exogenous application of GA (0.75 mM and 1.5 mM)

enhanced the activity of antioxidant enzymes such as SOD, CAT, POX and APX under salinity stress. Under PEG-induced osmotic stress, GA supressed the production of H_2O_2 and MDA and resulted in an up-regulation of the ROS-scavenging enzymes such as SOD and APX. Furthermore, the effects of GA (140 ppm) were also observed in pre-treated wheat seeds where the seedlings were exposed to water deficit conditions and showed that GA improved seedling growth (Bhardwaj *et al.,* 2015).

1.8 Project Aim and Objectives

The aim of this study is to investigate the effects of exogenously applied GA in modulating salt stress tolerance in soybean plants by exploring the following objectives:

- To monitor changes in plant growth parameters such as shoot and root fresh and dry weights
- Analyse the impact on photosynthetic metabolisms
- Measure changes in reactive oxygen species (O_2^-, H_2O_2) accumulation
- Evaluate the extend of oxidative damage manifested as cellular death
- Quantify antioxidant capacity by measuring changes in antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, peroxidase and glutathione reductase)

CHAPTER 2

METHODS AND MATERIALS

2.1. Chemical supplies

Table 2.1 List of chemical reagents and suppliers

Chemical	Supplier
Acetone	Merck Millipore
Acrylamide/Bis (40%)	BIO –RAD
Ammonium acetate ($C_2H_3O_2NH_4$)	Sigma Aldrich
Ammonium Persulfate (APS)	BIO –RAD
Ascorbic acid / Ascorbate	Sigma Aldrich
Bovine Serum Albumin (BSA)	Roche
Bradford Reagent (1X)	BIO –RA
5,5-Dithiobis(2-nitrobenzoic acid) (DTNB)	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Evans Blue	Sigma Aldrich
Gallic acid	Sigma Aldrich
Glacial acetic acid	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

Methionine	Sigma Aldrich
B-nicotinamide adenine dinucleotide (NADH)	Sigma Aldrich
Nitrotetrazolium blue chloride powder (NBT)	Sigma Aldrich
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
Promix	Windel Hydroponics
Propan-2-ol (isopropanol)	Merck Millipore
Riboflavin	Sigma Aldrich
Sodium hydroxide (NaOH)	Merck Millipore
Sodium molybdate (Na_2MoO_4)	Sigma Aldrich
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Thiobarbituric acid (TBA)	Sigma Aldrich
Trichloroacetic acid (TCA)	Merck Millipore
Tris(hydroxymethyl)-aminethane	BIO-RAD

2.2. Plant growth

Soybean (*Glycine max* L merr.) seeds (Natures choice, Highbury, Meyerton, South Africa) were surface sterilised with 0.35% of sodium hyperchlorite for 10 minutes, followed by five washes (in distilled water) of 5 minutes each. The seeds were then pre-soaked in distilled water for a further 30 minutes. Seeds were germinated on sterile moist filter paper in the dark for 48-72 hours. Germinated seeds were grown (two seeds per pot) in 1 L pots consisting of 3:1:1 ratio growth medium (silica sand, potting soil and organic promix) purchased from Windel Hydroponics, Durbanville, South Africa. The growth medium was kept moist by irrigation with distilled water during germination. Germinated seedlings (one plant per pot) were grown on a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2}.s^{-1} during the day phase.

2.3 Plant treatment

Treatment was performed at the VE (vegetative emerging) stage of growth. Control plants were supplied with 100 ml of distilled water every 48 hours for 14 days. For GA treatments, plants were supplied with different concentrations of GA (0.25 mM GA, 0.5 mM GA, 1 mM GA and 2 mM GA) dissolved in distilled water every 48 hours for 14 days. For NaCl treatments (over a period of 14 days); plants at the VE stage were supplied with distilled water that was supplemented

with 80 mM NaCl (NaCl, regarded as salt stress) every 48 hours for 14 days. The final concentration of 80 mM NaCl (to impose salt stress) was selected for this study based on work previously described by Klein (2012). For the combined treatments, plants were supplied with a combination of GA and NaCl (GA + NaCl) every 48 hours for 14 days.

2.4 Analysis of growth parameters

Following two weeks of treatment, plants were carefully removed from the growth medium to avoid any loss of plant material and rinsed with distilled. Five plants per 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).

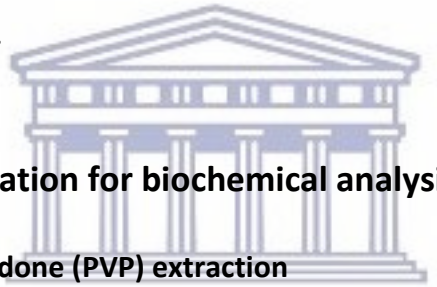
2.5 Cell viability

Cell viability was measured in the leaves of soybean plants using a modified method described by Sanevas *et al.* (2007). Fresh leaf tissue from three different plants per treatment (approximately 200 mg per treatment) were 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 from leaf tissue using 1% (w/v) SDS, after 1 hour incubation at 55°C.

Absorbance of the extracts from each treatment was measured at 600 nm to determine the level of Evans Blue up – take by the leaf tissue.

2.6 Determination of chlorophyll content

The chlorophyll content was calculated as described by Oancea *et al.* (2005), 100 mg of leaf sample was added to 1 ml of 80% (v/v) acetone. Each mixture was mixed by vortexing followed by centrifugation at 12 000 X g for 5 minutes. A 10X dilution of supernatant in acetone was done until samples were clear; samples were added on to 96 well microtitre plate. Absorbance readings were measured at 663nm and 645nm.



2.7 Sample preparation for biochemical analysis

2.7.1 Polyvinylpyrrolidone (PVP) extraction

Protein extraction from soybean plants was performed as described by Klein (2012). Leaf material (200 mg) were ground to a fine powder in liquid nitrogen and homogenized in 1 ml of extraction buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000] for determination/detection of antioxidant enzymatic activities (SOD, APX, POX, GR). The homogenates were centrifuged at 12 000 X *g* rpm for 15 minutes and the supernatants were stored at -20°C prior to use. Protein concentrations were determined according to Bradford, (1976), using bovine serum albumin (BSA) as a standard.

2.7.2 TCA extraction

Protein extracts were obtained from soybean leaf tissue (200 mg) that were ground to a fine powder in liquid nitrogen and homogenized in 1 ml of 10% trichloroacetic acid (TCA) for the detection of ROS biomarkers. The homogenates were vortexed and, centrifuged at 12 000 X *g* for 15 minutes and the supernatants were transferred to clean sterile Eppendorf tubes and stored at -20°C prior to use. Protein concentrations were determined according to Bradford, (1976), using bovine serum albumin (BSA) as a standard.

2.8 Quantification of ROS (superoxide and hydrogen peroxide) in soybean plants

For the detection of superoxide in soybean leaves, a modified method previously described by Russo *et al.* (2008) was used. Leaf material (100 mg) for each treatment were incubated in assay buffer (10 mM KCN, 10 mM H_2O_2, 2% SDS and 80 µM NBT dissolved in 50 mM KPO_4, pH 7.0) at room temperature for 20 minutes. Leaf material was homogenised using a mini plastic pestle. Homogenates were centrifuged at 13 000 X *g* for 5 minutes and absorbance's measured at 600 nm. Superoxide content was estimated using the extinction co-efficient of 12.8 $mM^{-1}.cm^{-1}$.

Hydrogen peroxide (H_2O_2) content in soybean leaves was measured using a modified a method described by Velikova *et al.* (2000). The reaction mixture consisted of 75 µl of the TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI.

Samples were incubated for 20 minutes at room temperature and absorbance's recorded at 390 nm. H_2O_2 content was calculated based on a standard curve constructed from the absorbance (A390 nm) of H_2O_2 standards.

2.9 Quantification of antioxidant enzyme activities in soybean leaves

2.9.1 Superoxide Dismutase

Soybean leaves were assayed for total SOD activity as described by Rao and Sresty (2000). The reaction mixture contained 50 mM KPO_4, pH 7.8, 13 mM methionine, 75 µM NBT, 0.1 mM EDTA and 2 µM riboflavin and 10 µl of extract. The reaction mixture was incubated for 20 minutes at 37 °C and absorbance readings were recorded at 560 nm. SOD activity was calculated based on the amount of enzyme that was required to cause a 50% reduction of NBT.

2.9.2 Ascorbate peroxidase

Total APX activity was measured in the leaves of soybean plants using a modified method by Asada (1984). Each reaction mixture contained 10 µl of PVP extract (extracts were supplemented with ascorbate to a final concentration of 2 mM) were added to the assay buffer (50 mM KPO_4, pH 7.0, 0.1 mM EDTA and 50 mM ascorbate). The reaction was initiated with 1.2 mM H_2O_2 in a final reaction volume of 200 µl and APX activity was calculated based on the change in absorbance at 290 nm using the extinction co-efficient of 2.8 $mM^{-1}cm^{-1}$.

2.9.3 Glutathione reductase

GR activity was measured using a modified method previously described by Smith *et al.* (1988) by following the rate of NADH oxidation at 340 nm. The assay mixture contained: 0.2 mM NADH, 0.5 mM GSSG, 1 mM EDTA in 100 mM KPO_4 pH 7.8 and 50 µl of enzyme extract in a 200 µl reaction. GR activity was calculated based on the oxidation of NADPH in the reaction, using the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

2.10 Detection of ROS scavenging antioxidant enzymes

2.10.1 Superoxide dismutase (SOD)

SOD activity was detected in the leaves of soybean plants using a modified method by Beauchamp and Fridovich (1971). Protein extracts (100 µg) from each treatment was separated on a 10% native polyacrylamide gel and specifically stained for SOD activity (as individual isoforms) using 2.5 mM nitroblue tetrazolium (NBT) and 0.5 mM riboflavin. SOD isoforms were identified by incubating the gels with 5 mM potassium cyanide (KCN), which inhibits Cu/ZnSODs or with 5 mM hydrogen peroxide (H_2O_2), which inhibits both Cu/ZnSODs and Fe-SODs (Archibald and Fridovic, 1982). MnSOD activity is resistant to both inhibitors.

2.10.2 Ascorbate peroxidase (APX)

Individual APX isoforms were detected in leaves extracts of soybean plants using a method described by Lee and Lee (2000). Prior to gel electrophoresis gels were equilibrated (20 minutes) in running buffer containing 2 mM ascorbate. Protein extracts (90 μg) from each treatment were separated on a 12% native polyacrylamide gel at 4˚C. Following electrophoresis, gels were incubated in 50 mM phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 minutes in the dark. The gel was then incubated in phosphate buffer (pH 7.0) containing 2 mM H_2O_2 and 4 mM ascorbate for 20 minutes in the dark. The gel was subsequently incubated in phosphate buffer (pH 7.8) containing 2.5 mM NBT and 2.8 mM N,N,N',N'-Tetramethylethylenediamine (TEMED) for 10 minutes with gentle agitation.

2.10.3 Peroxidase activity (POD)

Detection and visualisation of peroxidase activity was performed using a modified method by Hernandez *et al.* (2010). Leaf protein extracts (90 μg) from each treatment was separated on an 8% native polyacrylamide gel at 4°C. Following electrophoresis, the gels were briefly washed with 50 mM phosphate buffer (pH 7.0), followed by incubation in phosphate buffer containing 2 mM H_2O_2 and 0.04% DAB for 60 minutes. The stain was then discarded and gel was incubated in phosphate buffer containing 2 mM H_2O_2 for 10 minutes. The gel was washed with distilled water to terminate the reaction.

2.11 Densitometry analysis of antioxidant enzymes

Densitometry analysis was done on all the native PAGE gels for each antioxidant enzyme (SOD, APX and POD) after image acquisition, using the Spot Denso tool (AlphaEase FC imaging software V4, Alpha 88 Innotech Corporation). The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was scored as an average of the relative pixel intensities and expressed in arbitrary units from three independent gels. This was done by assigning control treatment pixel intensity to a value of 1 and expressing the rest relative to the control unless otherwise stated (Klein, 2012).

2.12 Statistical analysis

The data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at a 95% confidence interval, using GraphPad Prism 5.03 software.

CHAPTER 3

THE INFLUENCE OF EXOGENOUS GALLIC ACID ON THE PHYSIOLOGICAL AND MOLECULAR REPONSES OF SOYBEAN PLANTS

3.1 Introduction

Soybean (*Glycine max* L) represents one of the top produced food crops in the world, and is an excellent source of oil and protein for human consumption and feedstock. About 6% of the world's arable land is used for the cultivation of soybean (Murithi *et al.*, 2016). With the numerous bioactive factors and beneficial nutrients associated with soybean making it the top choice in improving food insecurity in developing countries. The global production of soybean since 1961 has risen by a factor of 10 from 27 million tons to 276 million tons in 2013 (FAOSTAT, 2013).

Currently the majority of the global soybeans are processed or crushed into meal and oil (Ali, 2010). Other products which are produced from soybean include; soymilk, natto, sprouts and tofu (Hartman *et al*., 2011). The southern Africa region has a total production of up to 861 000 tons as of 2010 (Tecgnoserve, 2011). The demand for soybean is 2 million tons in these regions is in the form of flavoured textured soy protein for human consumption. South Africa dominates both the demand and production of soybean in the region, which offers great

opportunities for both commercial and small holder farmers for increase in income.

Phenolic compounds have gained much importance due to their potential as antioxidants, plant phenolics have been studied with the intention to find compounds with protective roles against oxidative damage. Plants use these phenolic compounds for growth, reproduction, pigmentation and resistance to stresses (Hutzler *et al.,* 1998; Lattanzio *et al.,* 2006). Plant phenolics can be classified into two categories: (i) those synthesised during normal development and growth of plant tissue and (ii) those synthesised in response to infection, physical injury or stress. The induced phenolics may be produced at basal level under normal conditions; however under stress conditions (biotic or abiotic) synthesis of these phenolics may be enhanced (Lattanzio *et al.,* 2006).

The exogenous application of compounds has been a strategy used to either maintain or improve plant productivity under stress conditions. There are a great number of literatures which illustrates the benefits of using this method. The uses of compounds such as salicylic acid have been shown to have stimulatory effects on the plants physiological and morphological processes, growth, photosynthetic and metabolic processes (Kareem *et al.,* 2017). Abscisic acid is a phenolic compound, which was also used in grapes to improve the synthesis of anthocyanins thus improving the uniformity and quality of grapes (de Souza Leão *et al.,* 2014). Some of these studies have demonstrated how these phenolic compounds stimulate the activity of antioxidant enzymes.

Exogenous application of caffeic acid in soybean plants reduced cell viability, by enhancing ROS scavenging enzyme activity (SOD) under salinity stress (Klein *et al*., 2015). The use of GA in a study by Ozfidan-Konakci and co-authours (2014) on rice seedlings under osmotic and salinity indicates that GA improves the plants antioxidant capacity under stress conditions. Furthermore, the use of GA as a priming agent have been shown to enhance the enzymatic activity of SOD, CAT whilst increasing total phenolic content in wheat plants under drought stress (Bhardwaj *et al.,* 2017). This chapter explores the influence of exogenous GA (different concentration) on the physiological and biochemical responses of soybean plants.

3.2 Results

3.2.1 Gallic acid differentially alters growth parameters of soybean plants

The results shows that GA differentially altered shoot and root growth of soybean plants. Low concentrations of GA (0.25 mM) significantly enhanced shoot length (± 44%) when compared to the control plants (Figure 3.1 A). Although a significant increase in shoot growth (± 19%) was observed when plants were treated with 0.5 mM GA, this increase was not to the same level as observed for 0.25 mM GA. When soybean plants were treated with 1 mM GA, no significant changes in shoot growth was observed (Figure 3.1 A).

Low concentrations of GA (0.25 mM) significantly reduced root growth by ± 20% when compared to the control plants (Figure 3.1 B). When soybean plants was exposed to 0.5 mM and 1 mM GA, no significant changes in root growth was observed when compared to the control plants (Figure 3.1 B).

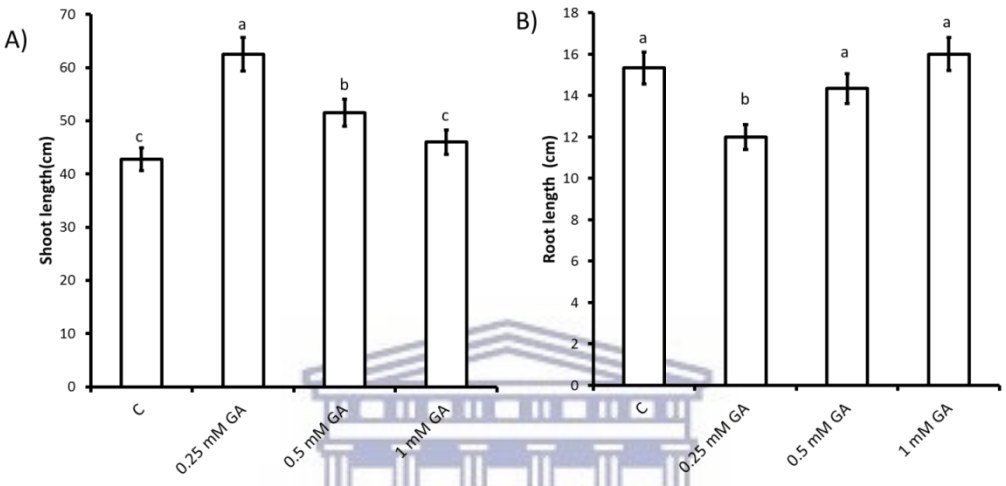


Figure 3.1 Gallic acid (GA) influences shoot and root growth in soybean plants. Plant shoot length (A) and root length (B) were measured after 14 days of exposure to different concentrations of GA. The error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

3.2.2 Gallic acid-induced changes in soybean biomass

Exogenous application of GA (different concentrations) influenced biomass production in soybean plants. The biomass of soybean plants (as seen for shoots and roots) was affected by GA (Figure 3.2 A-D) after 14 days of exposure. At low to moderate concentrations of GA (0.25 – 0.5 mM), shoot fresh weight was significantly reduced (± 20%) compared to control plants (Figure 3.2 A). However,

with higher concentrations (1 mM) of GA, shoot fresh weights significantly increased (± 20%). A similar trend was observed for shoot dry weights (Figure 3.2 B).

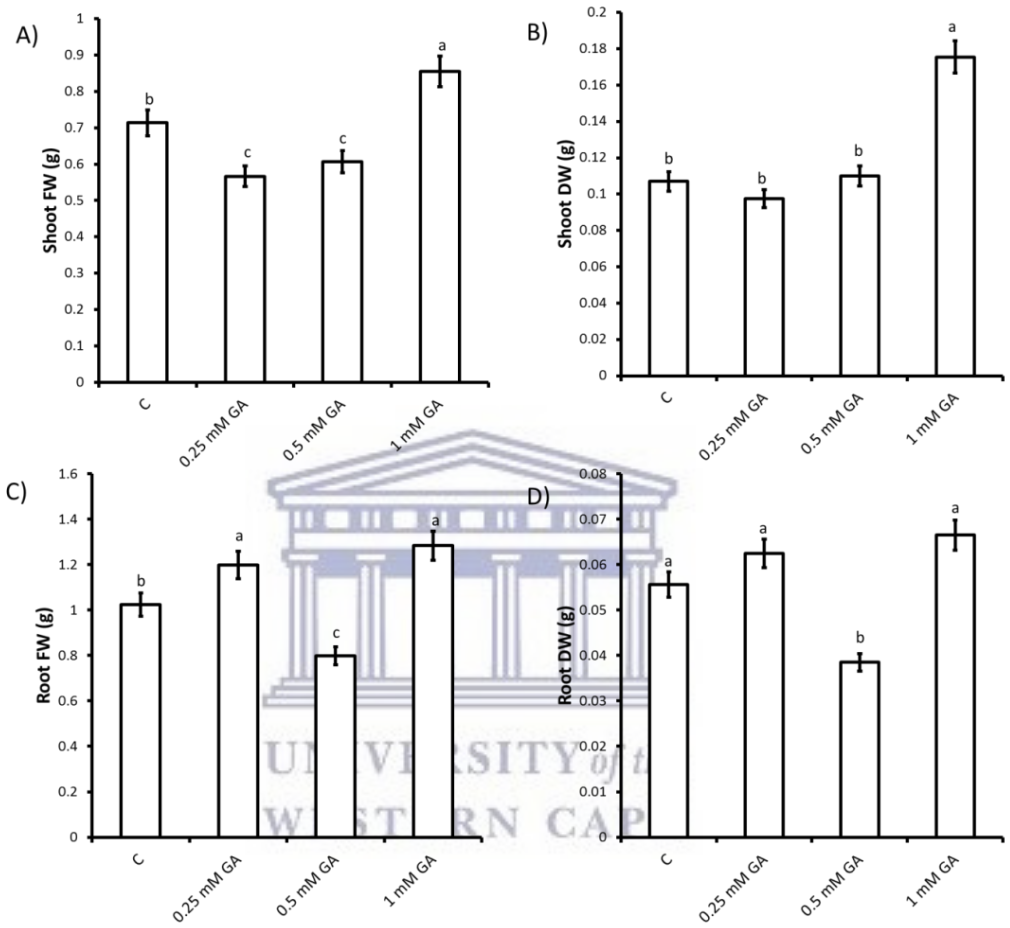


Figure 3.2 Biomass of soybean plants exposed to GA. The shoot fresh (A) and dry (B) weights, the root fresh (C) and dry (D) weight of each treatment. Each letter represents the means, which are significantly different from each other ($p < 0.05$). Error bars are means ± SE (n=3). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Root biomass was significantly altered in response to different GA concentrations (Figure 3.2 C-D). Root fresh weight was significantly enhanced in plants treated with 0.25 mM GA (± 16 %) and 1 mM GA (± 25%) respectively. However, 0.5 mM

GA significantly reduced (± 21%) root fresh weight when compared to the control plants (Figure 3.2 C). A similar trend was observed for root dry weights (Figure 3.2 D).

3.2.3 The effect of gallic acid on chlorophyll content in soybean plants

The photosynthetic pigments of soybean leaves were differentially influenced by GA. Increase in GA concentrations resulted in a significant decrease of green pigmentation (yellowing) (Figure 3.3 A) which ultimately lead to a decrease chlorophyll content in the leaves of soybean plants (Figure 3.3 B). Low GA concentration (0.25 mM) enhanced chlorophyll content by (± 8%) when compared to the control plants (Figure 3.3 B) and resulted in a more green leaf. The decrease in chlorophyll content due an increase in GA concentration ranged from ± 25 to ± 31%.

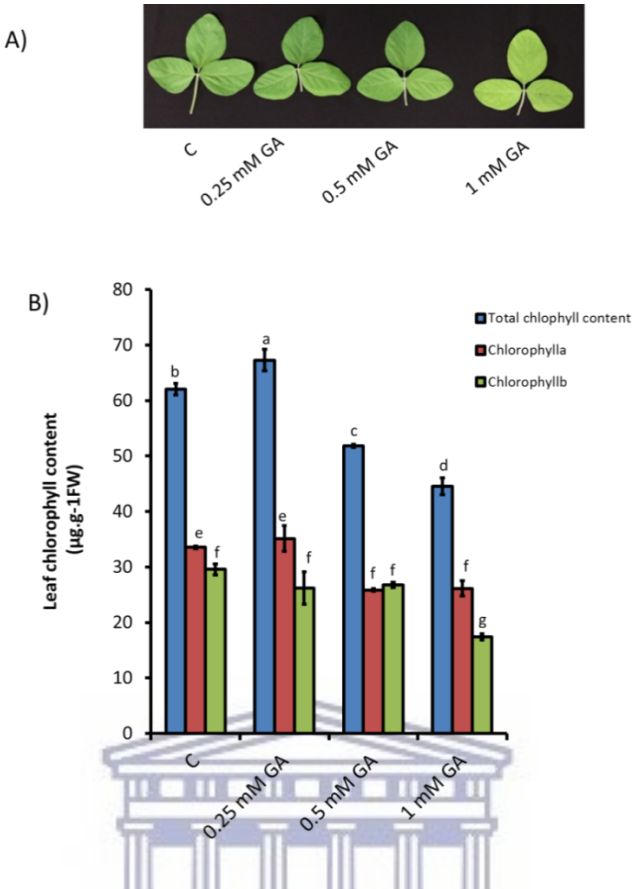


Figure 3.3 Changes in soybean leaf pigmentation and chlorophyll content. Image of the leaves (A) showing pigmentation and chlorophyll content (B) after 14 days of treatment. Error bars are representative of the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

3.2.4 Effect of gallic acid on ROS accumulation and oxidative damage

The influence of exogenous GA on ROS-induced oxidative damage was investigated. The results showed that 0.25 mM GA did not alter superoxide content (O_2^-) in soybean leaves when compared to the control plants (Figure 3.4 A). Plants treated with 0.5 mM GA showed approximately 5-fold increase in O_2^- content when compared to the control plants. Interestingly, when soybean

plants were treated with 1 mM GA, a significant reduction in O_2^- content was observed when compared to the control plants.

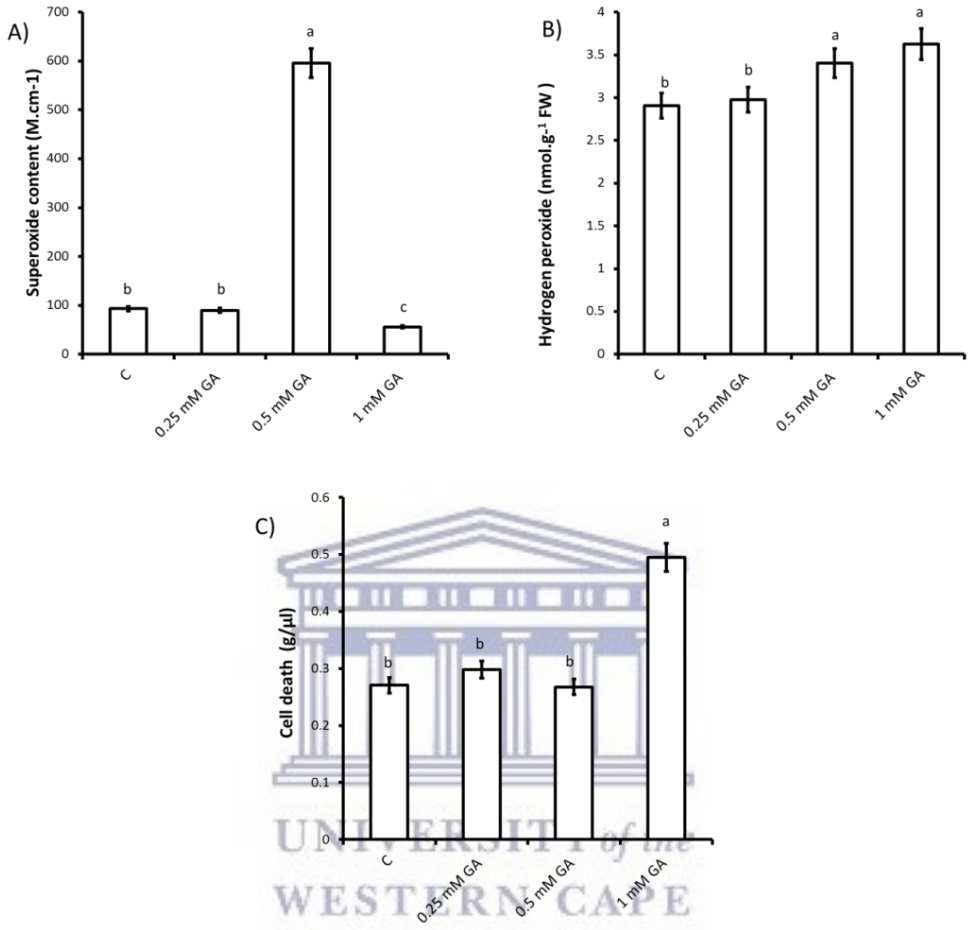


Figure 3.4 Influence of gallic acid on ROS-induced oxidative damage manifested as cellular death. Gallic acid-induced changes in superoxide (A), hydrogen peroxide (B) and cell viability (C) were analysed. Each letter represents the means which are significantly different from each other ($p < 0.05$). Error bars are means ± SE (n=3). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

The dismutation of O_2^- results in the production of hydrogen peroxide (H_2O_2) which would be further detoxified to either water or partially reduced OH (Liochev and Fridovich, 1999). Because of the detoxification of O_2- the level of H_2O_2 (Figure 3.4 B) increased with increasing concentrations of GA. The results illustrated that soybean plants treated with 0.25 mM GA showed no significant

changes in H_2O_2 levels compared to the control. However, in response to treatment with 0.5 mM to 1 mM GA, H_2O_2 content in the leaves of soybean plants was increased to levels more than observed for the control plants.

The increase in uncontrolled ROS usually results in enhanced oxidative stress, which leads to oxidative damage and ultimate cellular death. When analysing changes in death we observed that low GA concentration does not influence cell death. However, higher concentrations of GA (1 mM) significantly increased cell death (Figure 3.4 C) in the leaves of soybean plants, which could possibly be associated with an increase in lipid peroxidation (results not shown). In response to treatment with 1 mM, cell death in the leaves of soybean plants was increased by ± 83% (Figure 3.4 C).

3.2.5 Gallic acid differentially modulate antioxidant enzyme activity in soybean leaves

Total SOD activity was increased by an increase in GA concentrations (Figure 3.5 A). However the increase in the 0.5 mM treatment was not as pronounced as the increase observed for the other treatments. SOD activity was increased by ± 450% in the 0.25 mM treatment compared to the control. Treatment with 0.5 mM only increased SOD activity by ± 100% relative to the control. The highest increase in SOD activity was observed in the 1 mM GA treatment (± 600%) (Figure 3.5 A).

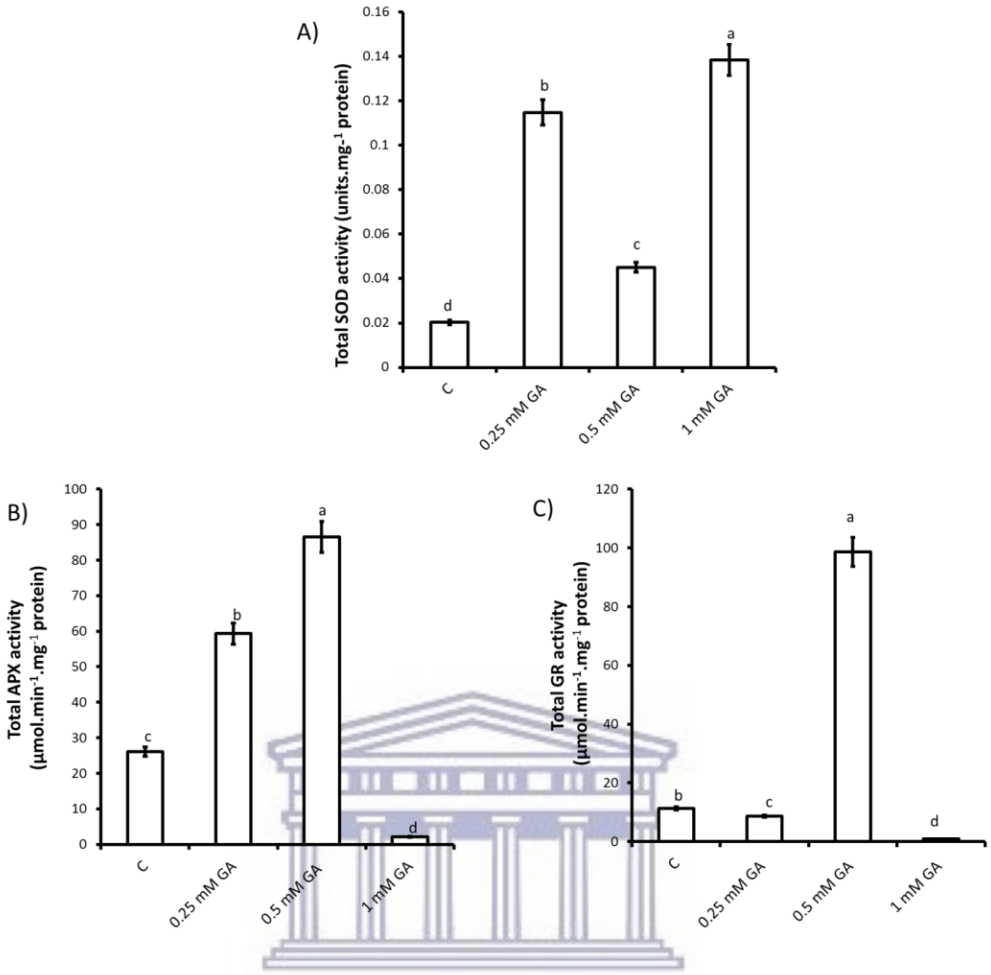


Figure 3.5 Gallic acid differentially regulate antioxidant enzyme activity in soybean leaf extracts. Measurement of the total SOD activity (A), APX activity (B) and GR activity (C) in leaves soybean leaves. Each letter represents the means which are significantly different from each other ($p < 0.05$). Error bars are means ± SE (n=3). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Total APX activity was differentially regulated by GA treatments (Figure 3.5 B). A significant increase in total APX activity in the leaves of soybean plants was observed in the 0.25 mM (± 128%) and 0.5 mM (± 232%) treatments (Figure 3.5 B). On the contrary, plants treated with 1 mM GA showed a marked reduction (± 92%) in total APX activity when compare to the control and other GA treatments (Figure 3.5 B).

Total GR activity in the leaves of soybean plants treated with GA were differentially regulated (Figure 3.5 C). A significant reduction in GR activity was observed in leaves of soybean plants treated with 0.25 mM (± 23%) and 1 mM (± 92%) GA.

3.3 Discussion

Phenolic compounds have important roles in the defence mechanisms in cells whether plant or animal cells. GA is classified as a polyphenols which are found in many different food sources. In this chapter, we analysed the influence of different GA concentrations on the physiological and molecular responses of soybean plants.

3.3.1 Gallic acid modulates changes in soybean plant growth and biomass production.

The stimulatory or inhibitory effects of exogenous application of phenolic compounds on the germination and early seedling growth have been reported (Reigosa *et al.,* 1999). The results presented here suggest that at low to moderate concentrations, GA will exhibit a positive effect on plant growth and development. The opposite was observed for root growth where the higher concentrations of GA promote root growth and lower concentration altered or suppressed root growth (Figure 3.1 B). A similar response was observed when 60 $\mu g.mL^{-1}$ of GA, was supplied to rice plants and showed significant improvement in shoot length (Singh *et al.,* 2017). Soybean plants treated with 0.25 mM GA

showed a significant increase in shoot length. This increase was reduced with higher GA concentrations albeit still higher than observed for the control plants.

Contrary to what was observed for shoot growth at low GA concentrations, root growth was significantly inhibited. The increase in shoot growth did not translate in increased biomass shown as fresh and dry weights. This phenomenon was also observed in a study by Klein *et al*. (2015) who demonstrated the inhibitory effect of low caffeic acid concentration in soybean biomass.

The root biomass as opposed to shoot biomass was remarkably increased in the 0.25 mM GA treatments (Figure 3.2 C). Roots are responsible for the intake of water and nutrients from the soil and support the above ground shoot growth yield which is highly dependent on the root biomass (Merill *et al.,* 2002). Evidently the shoot height (Figure 3.1 A) of the 0.25 mM GA significantly increased compared to the control. Parallel results were obtained by Gorni *et al.* (2016) where salicylic acid, a well investigated phenolic compound improved plant biomass of fennel at a much lower concentration. Biomass is influence by many factors such as soil humidity, air humidity, soil nutrient availability (Chatzistathis and Therios, 2013). In this study we have shown that GA is able to influence soybean biomass, the mechanism by which exogenous application of GA promotes both the shoot and root biomass is yet to be further investigated, by probing into the involvement of GA in nutrient uptake and cell wall lignification.

3.3.3 Impact of gallic acid on soybean photosynthetic capacity

Chlorophyll content is an imperative physiological measure which is directly related to the photosynthetic performance (Parashar *et al.,* 2014; Gorni and Pacheco, 2016). The apparatus of photosynthesis involves various components such as photosynthetic pigments and photosystems. Any damage to the plant that may reduce the overall photosynthetic capability of green plants. Changes in photosynthetic activity results in reduced levels of light harvesting chlorophyll proteins (Gill *et al.,* 2012; Malar *et al.,* 2014) which eventually leads to reduced plant growth. The data presented in the study suggests that exogenous application of GA at low concentrations (0.25 mM) can promote photosynthesis in soybean. A notable increase in chlorophyll *a* was observed (Figure 3.3) which contributed to the overall increase of chlorophyll content.

The application of higher concentrations of GA had deleterious effects on the photosynthetic capacity. However Yang *et al.* (2004) found that ferulic acid and p-coumaric acid reduced chlorophyll accumulation to levels to lower than untreated rice seedlings, these results were similar to those obtained in the study. Yildiztugay *et al.* (2017) showed contrary results as GAhad no notable effect on the photosynthetic capacity of soybean and similar results were also presented by Ozfidan-Konakci (2015) on two rice cultivars. Benzoic and cinnamic acids have been reported to reduce the chlorophyll content of soybean (Baziramakenga *et al.,* 1994; Yang *et al.,* 2004). Rice (1984) elucidated that the synthesis of porphyrin which is a precursor in the chlorophyll biosynthesis may

be inhibited by some phenolic compounds. The reduction in leaf chlorophyll could be the cause of reduction in shoot growth as observed (Figure 3.1 A). However this speculation would not be definite as the reduction of chlorophyll may occur after other physiological changes which are altered by phenolic compounds. The results suggest that exogenous application of GA at low concentration improves plant growth, biomass and photosynthetic metabolism (as seen for total chlorophyll content).

3.3.4 Effect of gallic acid on ROS accumulation and oxidative damage

Different types of active ROS like superoxide and hydrogen peroxide are the leading cause of cell deterioration (Bailey-Serres and Mittler, 2006). These molecules accumulate to excessive levels under stress conditions; however under normal conditions production of these molecules is required as signalling molecules. Plants are able to use enzymatic and non-enzymatic mechanisms in order to scavenge these molecules. The present results revealed that the accumulation of ROS such as O_2^- and H_2O_2 were unaffected by low concentrations of GA. However at higher concentrations GA significantly affected the production of O_2^- and H_2O_2. Yildiztugay *et al.* (2017) and Ozfidan-Konakci *et al.* (2015) observed contrary results to our study. At higher concentrations GA had no effect on the O_2- production or the H_2O_2 production on soybean and rice seedlings respectively. The reduction of ROS and free radicals O_2^- could have possibly triggered production of the antioxidant enzymes or directly due to

exogenous treatment of GA at low concentrations. The accumulation of H_2O_2 could be due to the scavenging of superoxide via SOD.

The additional accumulation and production of ROS molecules such as H_2O_2 are associated with lipid peroxidation (Foyer *et al.*, 1994) which can cause injury to the ultrastructure of cells, cause an imbalance in normal plant metabolism, and ultimately lead to cell death. Ksouri *et al.* (2007) postulated that phenolic acids' capacity to accumulate ROS differs according to diversity of the plant cultivars in stress tolerance. Phenolic acid such as GA inhibit lipid peroxidation by means of trapping the lipid alkoxyl radical. Our results (not shown) are in agreement with those obtained by Ozfidan-Konakci *et al.* (2015) where lipid peroxidation was reduced by 0.75 mM GA and 1.5 mM GA in two rice cultivars. Even with the reduction in lipid peroxidation, the level of H_2O_2 resulted in increased cell death of higher concentrations of gallic acid. Treatment with GA (0.25 mM) resulted in minimal accumulation of $O_2^{\cdot-}$, H_2O_2 and cell death, which suggests that the antioxidant capacity was induced at low concentration and diffusion of free radicals and restriction of peroxidative reactions was successful. Phenolic compounds are capable of modifying the peroxidation kinetics by altering the lipid packaging order (Arora *et al.,* 2000), in order to alleviate membrane fluidity and leakage (Michalak, 2006).

3.3.5 Gallic acid differentially alters antioxidant enzyme activity in soybean leaves

A common response when plants are exposed to stress is the accumulation of ROS molecules causing damage to lipids, carbohydrates, proteins, and DNA (Mittler, 2006). The antioxidant potential in plants can be enzymatic and non-enzymatic; the antioxidant enzymes evaluated such as SOD are of the first ROS scavenging enzymes activated. The accumulation of H_2O_2 is not only due to the dissemination of O_2- by SOD but also through β-oxidation, oxalate oxidase, glycollate oxidation during photorespiration or NADPH oxidase (Desikan *et al.,* 2003; Ozfidan-Konakci *et al.,* 2015). APX and GR are enzymes which detoxify H_2O_2 and the activity of these enzymes were also evaluated. Apart from GR exogenous application of GA generally enhanced the activities of both SOD and APX. The total SOD activity of the soybean leaves (Figure 3.5 A) in this study was increased in response to GA treatment, however 1 mM GA had remarkably higher levels of activity compared to other GA treatments. These results were contrary to those obtained by Ozfidan-Konakci *et al.* (2015) and Yildiztugay *et al.* (2017) who observed no changes to SOD activity in rice and soybean leaves respectively when compared to control treatment. Furthermore, our results are in partial agreement with those attained by Hassanein *et al.* (2015) who found that salicylic acid enhanced the activity of SOD by ± 28% in wheat leaves.

Total APX activity in soybean leaves was elevated by low to moderate concentration of GA, Yildiztugay *et al.* (2017) found similar results in soybean

treated with similar concentrations of GA. Higher concentrations of GA (1 mM) remarkably reduced APX activity and Ozfidan-Konakci *et al.* (2015) found similar results in the IR-28 rice cultivar. The increment of GR activity in 1 mM GA treatment could be activated as a result of the decline in APX activity as both enzymes are responsible for detoxifying H_2O_2.

The results presented here suggest that at low to moderate concentrations (0.25-0.5 mM), GA modulates antioxidant capacity in soybean plants to detoxify excessive ROS that would otherwise lead to oxidative damage and increased cellular death. Although high concentrations (1 mM) of GA slightly enhance plant root growth and biomass production it results in the increase in ROS molecules which cannot be scavenged by the counter antioxidant enzymes. The increase in root growth could be attributed to the plants trying to evade stress conditions due to the increase in GA concentrations. The increase in GA concentration significantly influence photosynthetic metabolism as can be seen for the yellowing of soybean leaves. Interestingly low concentration of GA (0.25 mM) inhibited root length but increased shoot length, root biomass, photosynthetic metabolism, reduced production of ROS and increased antioxidant capacity. With these attributes 0.25 mM deemed to be a fit concentration in order infer salt stress tolerance on soybean by improving antioxidant capacity and inhibiting the deleterious effects caused by salt stress.

CHAPTER 4

GALLIC ACID MODULATES SALT STRESS TOLERANCE IN SOYBEAN PLANTS BY REGULATING ANTIOXIDANT CAPACITY

4.1 Introduction

Salinity is one of the most significant abiotic stresses in the world which affects crop survival and yield. It has adverse effects on virtually all development stages during a plants life cycle (Zhu, 2016). Over 45 million hectares agricultural land worldwide has been impaired by salinity stress (Munns and Tester, 2008). Multiple studies have been done over the past few decades which have demonstrated how soil salinity content hinders plant growth and development through numerous pathways including osmotic stress, nutrient deprivation, ion toxicity, oxidative stress, membrane damage and reduction in cell growth and division (Zhu, 2016; Hanin *et al.,* 2016; Shu *et al.,* 2017).

Plants such as soybean are exposed to several abiotic stress conditions such as drought and salinity. Salt stress is a limiting factor which reduces the yield of many crops. Soybean is no exception to this and subsequently, development of improving levels of tolerance to salt has become an imperative matter for breeding innovative cultivars to withstand these harsh conditions. Soybean is the main source of high-quality oil and protein for human consumption. Consisting of ± 36% protein, soybean is one of the plants with the highest protein content.

With ± 19% oil content it's second only to groundnut amongst the leguminous family (Toorchi *et al.,* 2009). However, salt stress has significantly reduced soybean yield through inhibition of germination, decrease of nodulation, biomass and growth (Shu *et al.,* 2017).

Salinity stress leads to oxidative stress through an accumulation of ROS molecules such as O_2-, H_2O_2 and OH^- resulting in the imbalance of cellular redox state causing lipid peroxidation (Shi *et al.,* 2007). Plant cells possess antioxidant systems which are responsible for the balance of the redox state. The primary enzymatic defence system consist of multiple ROS scavenging enzymes such as SOD, APX, GR (Ahmad *et al.,* 2010) and POD (Assche and Clijsters, 1990) in order to maintain cellular integrity. However, the amplitude at which the ROS scavenging mechanism is activated is greatly dependent on a number of factors such as plant species, age, concentration and the time of exposure (Ortega-Villasante *et al.,* 2011).

In addition to enzymatic antioxidants non-enzymatic antioxidants also play a role in detoxifying ROS. These include phenolic acids which are utilised by plants to prevent the generation of toxic products by ROS. Phenolics provide protection in two ways by (i) regulating excess excitation of energy during photosynthesis metabolism during stress and (ii) enhancing the antioxidant capacity which, is dependent on the position and number of hydroxyl group in their structure (Sgherri *et al.,* 2003; Ozfidan-Konakci *et al.,* 2015).

GA (3, 4, 5-trihydroxybenzoic acid) is a key median molecule in the synthesis of plant hydrosable tannins (Muir *et al.,* 2011). GA prevents superoxide anion produced by xanthine oxidase, thus performing as ROS scavenger. As a result, GA with three hydroxyl groups seems to have a great potential in promoting the antioxidant response (Badhani *et al.,* 2015). The use of exogenous GA in mitigating abiotic stresses has recently been investigated in plants. Ozfidan-Konakci *et al*. (2015) found that GA restricts the damaging effects on rice exposed to salinity and osmotic stress. To date, no studies have investigated the influence of GA on the antioxidant defence system in plants exposed to salinity stress. Therefore, the aim of this chapter is to evaluate the effect of exogenously applied GA (0.25 Mm) on the physiological and responses of soybean plants exposed to long-term salt stress.

4.2 Results

4.2.1 Gallic acid and salt stress influences soybean growth parameters

Here we evaluated the influence of exogenous GA and salt stress on certain growth parameters of soybean plants. The results showed that GA significantly increased shoot length (± 44%) when compared to the control plants (Figure 4.1 A). Plants treated with 80 mM NaCl (to induce salt stress) significantly reduced shoot length (± 16%) when compared to the control plants. However, salt stress

plants supplemented with GA enhanced shoot growth to the same extent as observed in the GA treatment.

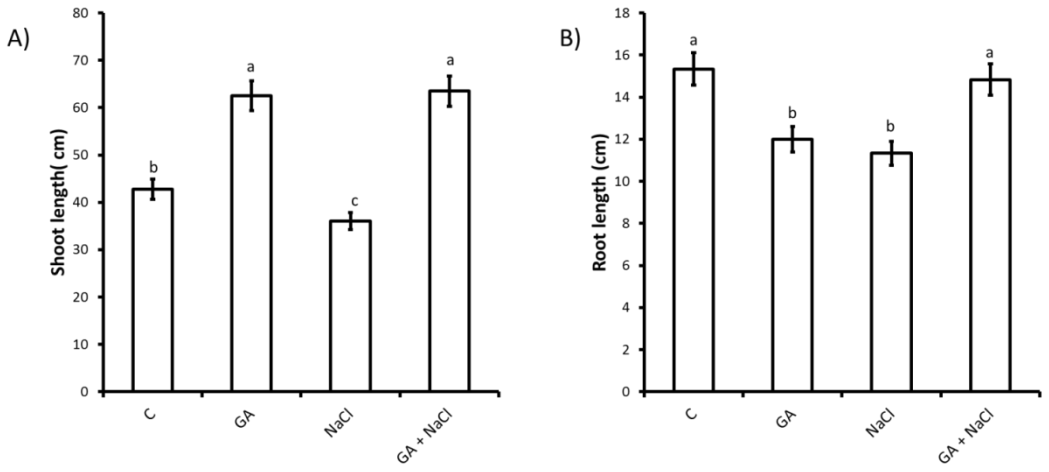


Figure 4.1 Influence of gallic acid on soybean exposed to salinity stress. Plant growth parameters were measured after 14 days of exposure to 0.25 mM GA, 80 mM NaCl and the combination of GA and NaCl. Shoot (A) and root (B) length. Each letter represents the means which are significantly different from each other ($p < 0.05$). Error bars are means ± SE (n=3). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

For root length, a significant reduction was observed in the GA treatment when compared to the control plants (Figure 4.1 B). A similar trend was observed for the salt stressed plants. However, when salt stress plants were supplemented with GA, root growth was restored to the level observed for the control plants (Figure 4.1 B).

4.2.2 Influence of salt stress and gallic acid on soybean biomass

Exogenous application of GA and salt differentially alter shoot and root fresh weight and dry weights. Salt stress significantly reduced shoot biomass (± 33%) when compared to control plants. A similar trend was observed in plants treated with GA although this decrease was not as severe as observed for salt stressed

plants (Figure 4.2 A). Interestingly, shoot fresh weight increased significantly (± 12%) in the combined treatment (GA + NaCl) when compared to the control plants. A similar trend was also observed for shoot dry weights (Figure 4.2 B).

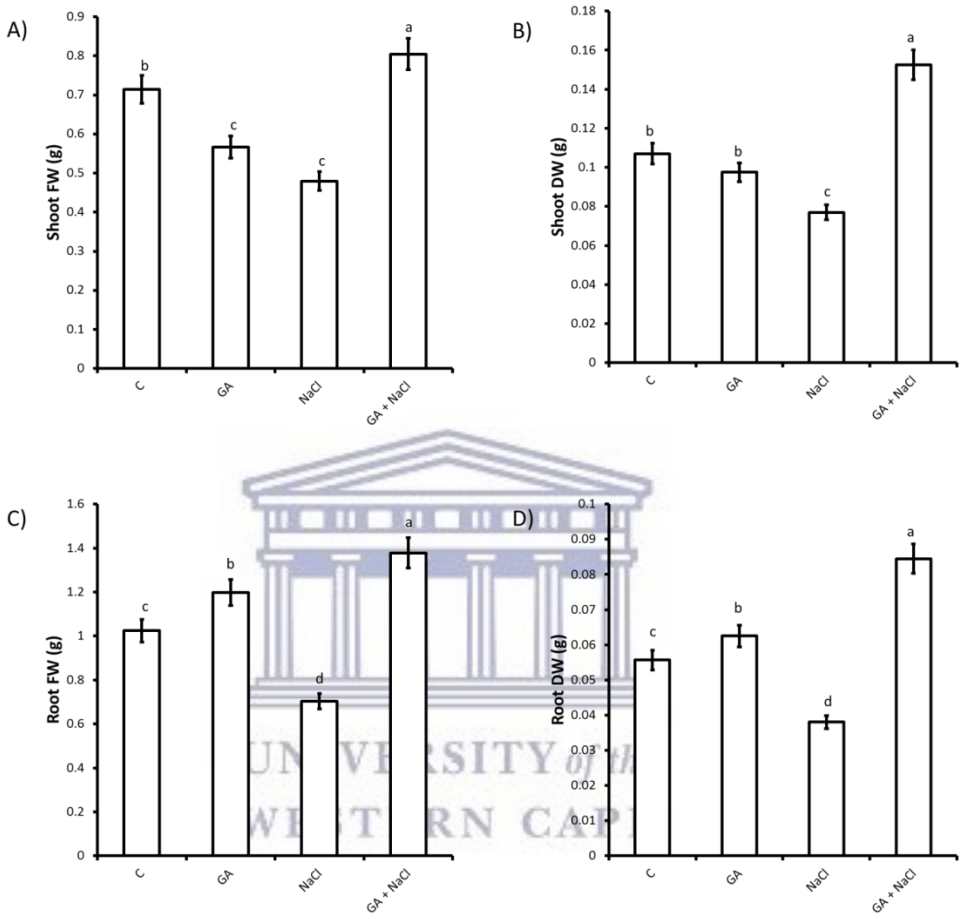


Figure 4.2 Biomass of soybean plants exposed to salt and gallic acid. Biomass was evaluated by measuring (A) leaf fresh weight (FW), (B) leaf dry weight (DW) coupled with (C) root fresh and (D) root dry weight at the end of the treatment period. The error bars are representative of the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Root fresh weight was significantly enhanced (± 16%) in the GA treatment whereas salt stress caused a significant reduction (± 31%) when compared to control plants (Figure 4.2 C). However, when salt stressed plants were supplemented with GA a marked increase (± 35%) in root fresh weight was observed even to a level exceeding that of the control plants. A similar trend in root dry weights was observed as seen for root fresh weight (Figure 4.2 D).

4.2.3 Effect of gallic acid and salt stress on chlorophyll pigments

Chlorophyll content was differentially influenced by exogenous GA and salt stress. A slight but significant increase (± 8%) in chlorophyll content was observed when soybean plants were treated with 0.25 mM GA. When soybean plants were exposed to long term salt stress, a significant reduction (± 36%) in chlorophyll content and yellowing of the leaves (Figure 4.3 A) was observed relative to the control plants (Figure 4.3 B).

Interestingly, when salt stress plants were supplemented with GA, the chlorophyll content was increased to levels slightly higher than those of the stress plant and an increase in green pigmentation was observed (Figure 4.3 A).

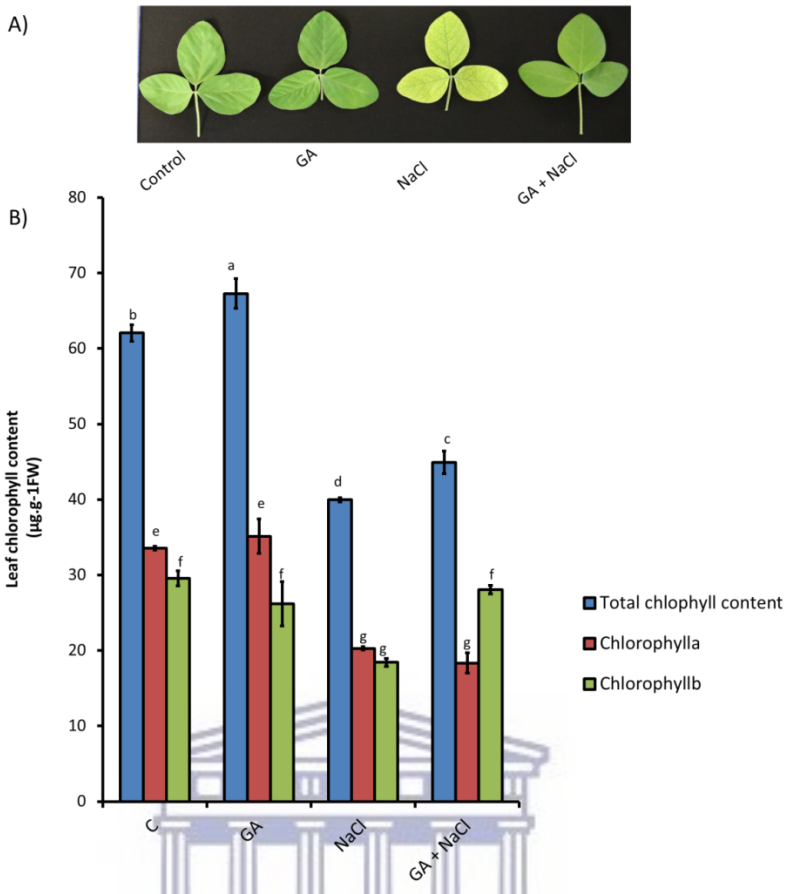


Figure 4.3 Leaf pigmentation and chlorophyll content of soybean leaves exposed to gallic acid and salt stress. Image represents the (A) second trifoliate leaves of various treatments and (B) total chlorophyll, chlorophyll *a* and *b* content were measured of soybean leaves following supplementation of 0.25 mM GA and 80 mM NaCl and 0.25 mM GA + 80 mM NaCl. Error bars are representative of the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

4.2.4 Effect of gallic acid and salt stress on ROS accumulation and oxidative damage in soybean plants

The effects of various treatments on ROS accumulation, oxidative damage and cell death were investigated because salinity stress enhances programmed cell death. It has been shown in literature that GA inhibits oxidative stress and is able to reverse the harmful effects caused by salt stress in rice, wheat and soybean grown in hydroponic conditions (Ozfidan-Konakci *et al.* 2015; Yildiztugay *et al.* 2017).

The results showed that exogenous application of GA did not alter superoxide content. However, salt stress significantly increased O_2^- content (± 530%) relative to the control (Figure 4.4 A). The combined treatment (0.25 mM GA + 80 mM NaCl) also increased O_2^- content (± 309%), relative to the control but was significantly lower than observed for the NaCl treatment.

For H_2O_2 accumulation, no significant change was observed in the GA and NaCl treatments relative to the control (Figure 4.4 B). However, a slight but significant increase was observed in the combined treatment (GA + NaCl).

Salt stress (induced by NaCl) increased in cell death (± 54%) relative compared to the control. The increase in cell death observed in the NaCl treatment was reversed (to the level observed for the control) when GA was supplemented to NaCl-treated plants (Figure 4.4 D).

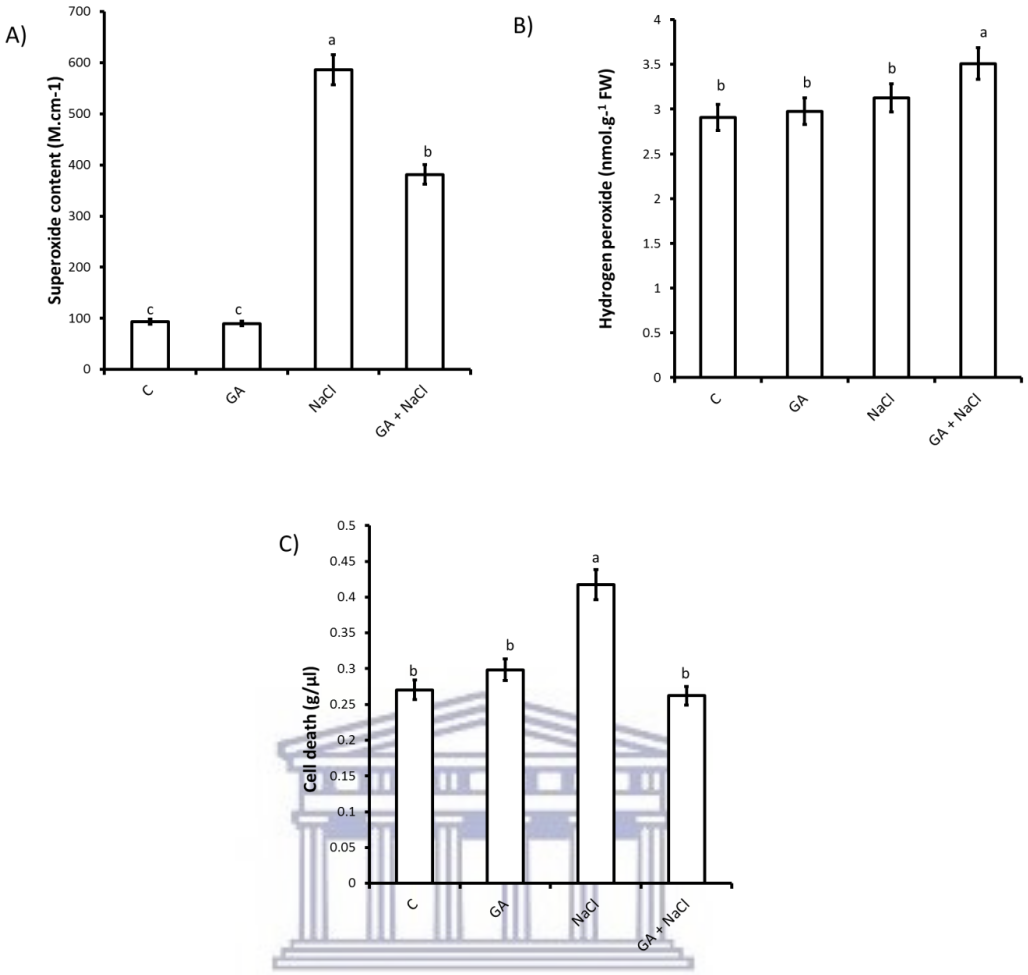


Figure 4.4 Gallic acid and salt stress influences ROS-induced oxidative damage and cell death. The superoxide (A), hydrogen peroxide (B), and cell viability (C) was evaluated in the leaves of soybean plants treated with 0.25 mM GA, 80 mM NaCl and 0.25 GA + 80 mM NaCl. Error bars are representative of the mean (±SE) of three independent experiments from three plants per treatment in each experiment. Means with different letters are significantly different from each other ($p < 0.05$). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

4.2.5 Gallic acid differentially alters antioxidant enzyme activity in soybean leaves under salt stress

Antioxidant enzymes activities in soybean leaves were differentially expressed in various treatments. Many studies have shown differences in enzymatic activities of antioxidant enzymes in various plant species (Gill and Tuteja, 2010). The results

showed that salt stress and GA differentially regulated total SOD activity in the soybean leaves after 14 days of treatment (Figure 4.5 A).

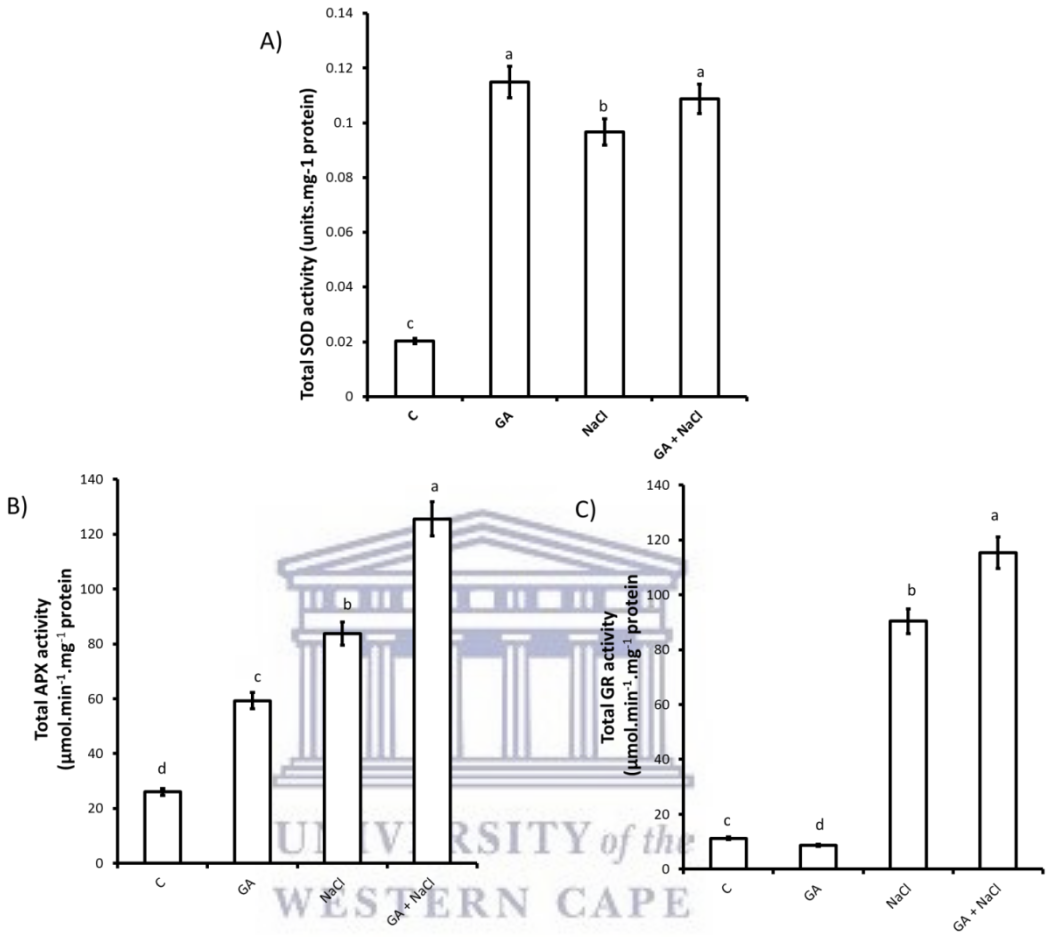


Figure 4.5 Gallic acid differentially modulate antioxidant capacity in soybean plants under salt stress. Measurement of the total SOD activity (A), APX activity (B) and GR activity (C) in the leaf extracts of soybean plants treated with 0.25 mM GA, 80 mM NaCl and 0.25 mM + 80 mM NaCl. Each letter represents the means which are significantly different from each other ($p < 0.05$). Error bars are means ± SE (n=3). The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Exogenous application of GA increase total SOD activity (± 450%) compared to the control (Figure 4.5 A). NaCl treatment also significantly increased SOD activity (± 385%) in contrast to control. Furthermore, the combination of GA and salt

stress (NaCl) increased SOD activity (± 440%) and this increase was augmented by GA. The increase in SOD activity observed in the combined treatment (GA + NaCl) was significantly higher than the control and slightly higher than observed in the NaCl treatment (Figure 4.5 A).

For total APX activity in the leaves of soybean plants, an exponential increase in activity was observed for all treatments with highest increased measured in the combined treatment (GA + NaCl). GA increased total APX activity by ± 128% with an even higher increase of ± 219% observed for the NaCl treatment (Figure 4.5 B). However, the highest increase in activity was observed in the combined treatment of ± 380% when compared to the control (Figure 4.5 B).

As expected, GR activity was significantly increased in response to treatment with NaCl. This increase was measured at ± 718% when compared to the control. Interestingly, exogenous GA reduced GR activity by ± 23% when compared to the control (Figure 4.5 C). However, when NaCl-treated plants were supplemented with GA, the enzymatic activity increased by ± 945% which is higher than the activity observed in the NaCl treatment and even higher than the control (Figure 4.5 C).

4.2.6 Detection of SOD activity in leaf extracts of soybean plants treated with gallic acid and salt stress

Superoxide dismutases (SOD) represent the first line of plant defence against ROS 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 shows the effect of exogenously applied GA and salt stress on the enzymatic activity of various SOD isoforms in the leaves of soybean plants. Leaf extracts from each treatment were separated on a 12% native polyacrylamide gel and stained for individual SOD isoforms. Seven SOD isoforms were detected using SOD specific staining and characterised based on their resistance/sensitivity to KCN and H_2O_2 (see section 2.10.1) After exposure to different SOD inhibitors (6 mM H_2O_2 and 6 mM KCN) the SOD isoform profile of soybean leaves included one manganese superoxide dismutase (MnSOD), one iron superoxide dismutase (FeSOD) and five copper/zinc superoxide dismutases (Cu/ZnSODs) (Figure 4.6). The densitometry analysis revealed that the activity of MnSOD was not significantly affected by GA treatment although a slight increase (± 6%) was observed in the NaCl treatment. The enzymatic activity of MnSOD was reduced (± 6%) in the NaCl treatment supplemented with GA (GA + NaCl) when compared to the control (Figure 4.6). The activity of FeSOD was only detected in the control and GA treatments with no significant changes observed. Interestingly, no activity for FeSOD was detected in the NaCl and combined treatments (GA + NaCl) (Figure 4.6).

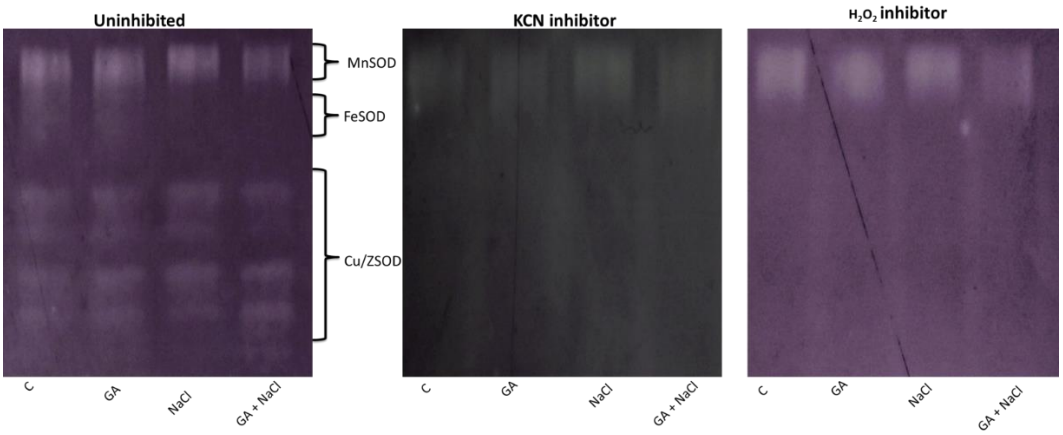


Figure 4.6 Differential expression of SOD activity. Assays were done on soybean plants treated with 0.25 mM GA, 80 mM NaCl, and 0.25 mM GA + 80 mM NaCl for a period of 14 days. The in-gels show the detection of SOD isoforms (A) with no inhibitors, (B) in the presence of 6 mM H_2O_2 and (C) in the presence of 5 mM KCN in response to the various treatments. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

The results suggest that the treatments differentially regulate Cu/ZnSOD isoform (Cu/ZnSOD 1, Cu/ZnSOD 2, Cu/ZnSOD 3, Cu/ZnSOD 4, Cu/ZnSOD 5) activity in leaf extracts of soybean plants (Figure 4.6 and Table 4.1). For Cu/ZnSOD 1, both GA and NaCl increase isoform activity by ± 5% whereas a higher decrease (± 11%) in activity was observed in the combined treatment. For Cu/ZnSOD 2, GA and NaCl decreased isoform activity relative to the control. Very low to no activity was detected for this isoform in the combined treatment. Statistically, no significant changes were observed for Cu/ZnSOD 3 in all the treatments. For Cu/ZnSOD 4, GA and the combined treatment (GA + NaCl) increased isoform activity as quantified using densitometry analysis. The increase observed in the GA treatment was ± 6% higher than the control, whereas the combined treatment increased activity by ± 8%. Contrary to what was observed in the GA treatments, NaCl reduced the activity of Cu/ZnSOD by ± 6% compared to the control. Except

for the combined treatment (GA + NaCl) no activity was in the control, GA and NaCl treatments. Therefore, there was no basis to estimate the increase and/or decrease in activity for Cu/ZnSOD 5.

Table 4.1: Densitometry analysis for SOD isoforms in soybean leaf extracts

Relative SOD Activity (Arbitrary Units)	SOD isoforms	Treatments			
		C	0.25 mM GA	80 mM NaCl	0.25 mM GA + 80 mM GA
	MnSOD	1 ± 0.050	0.969 ± 0.048	1.061 ± 0.053	0.936 ± 0.047
	FeSOD	1 ± 0.050	1.032 ± 0.052	n.d	n.d
	Cu/ZnSOD 1	1 ± 0.050	1.051 ± 0.053	1.039 ± 0.052	0.885 ± 0.044
	Cu/ZnSOD 2	1 ± 0.050	0.958 ± 0.048	0.936 ± 0.047	n.d
	Cu/ZnSOD 3	1 ± 0.050	1.023 ± 0.051	1.002 ± 0.050	1.005 ± 0.050
	Cu/ZnSOD 4	1 ± 0.050	1.066 ± 0.053	0.943 ± 0.047	1.081 ± 0.054
	Cu/ZnSOD 5	n.d	n.d	n.d	1 ± 0.05

Data presented in this table are the means standard error are means ± SE (n=3). All SOD isoforms were normalized using the control. The letters n.d in the table indicates that very low or no activity was detected. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

4.2.7 Gallic acid and salt stress differentially alters APX activity in soybean leaf extracts

Once SOD detoxifies O_2- to H_2O_2 other enzymes are activated in order to scavenge H_2O_2 such as APX. In this study, we annotate the influence of exogenously applied GA and salt stress on the enzymatic activity and expression of APX isoforms. For the detection of APX activity in soybean leaf extracts, 90 µg of leaf protein extract for each treatment was separated on a 12% native PAGE gel run at 4ºC. Three highly visible APX isoforms were detected in each of the treatments (Figure 4.7). The enzymatic activity of the APX isoforms detected in soybean leaf extracts is differentially regulated by exogenous GA and NaCl as indicated by densitometry analysis (Table 4.2). For APX 1, all treatments slightly reduced APX activity relative to the control with the highest reduction observed in the NaCl treatment. Salt stress (as imposed by NaCl) decreased APX 1 activity by ± 7% whereas the reduction in the GA and combined treatment was not as severe (Figure 4.7 and Table 4.2).

Apart from the combined treatment, no significant changes in APX 2 activity were observed in the GA and NaCl treatments (Figure 4.7 and Table 4.2).

For APX 3, GA increased the activity of the isoform by ± 9% when compared to the control. No significant changes were observed in APX 3 activity when treated with NaCl and the combination of GA and NaCl (Figure 4.7 and Table 4.2).

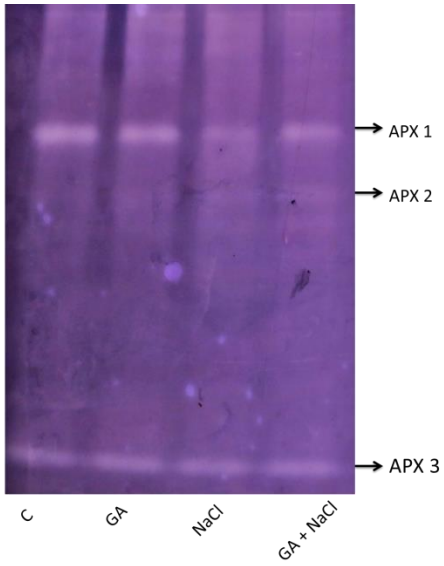


Figure 4.7 Detection of APX activity in soybean leaf extracts. In-gel activity assay were performed on leaf extract of soybean plants treated with 0.25 mM GA, 80 mM NaCl and 0.25 mM GA + 80 mM NaCl for a period of 14 days. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Table 4.2: Densitometry analysis of APX isoforms in soybean leaf extracts

Relative APX Activity (Arbitrary Units)	APX isoforms	Treatments			
		C	0.25 mM GA	80 mM NaCl	0.25 mM GA + 80 mM NaCl
	APX 1	1 ± 0.050	0.962 ± 0.048	0.924 ± 0.046	0.975 ± 0.049
	APX 2	1± 0.050	1.035 ± 0.052	1.038 ± 0.052	1.238 ± 0.062
	APX 3	1 ± 0.050	1.094 ± 0.055	1.033 ± 0.052	1.045 ± 0.052

Data presented in this table are the means standard error are means ± SE (n=3). All APX isoforms were normalized using the control. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

4.2.8 Peroxidase (POD) is differentially regulated by gallic acid and salt stress

In order to tolerate oxidative damage as a result of unfavourable conditions such as salinity stress plants have to possess a well-organised antioxidant defence system (Sairam *et al.,* 2002). Many studies have described that salt-tolerant plant species have elevate levels of their antioxidant enzyme activities in response to salt treatment (Cicek and Cakirlar, 2008a; Demiral and Turkan, 2005). The activity of POD was also evaluated with respect to anti-oxidative enzymes, various treatments present contrasting results. Here we analyse the influence of different treatments of POD activity as indicated for individual enzyme isoforms. The intensities (activity) of each isoform were quantified using densitometry analysis (Table 4.3). Five POD isoforms (POD 1, POD 2, POD 3, POD 4 and POD 5) were detected using antioxidant specific staining (section 2.10.3). All isoforms were differentially expressed by the different treatments (Figure 4.8).

The results showed that GA significantly increase POD 1 activity by ± 7% in leaf extracts of soybean plants when compared to the control (Figure 4.8 and Table 4.3). However, no activity for POD 1 was detected in the NaCl treatment. When NaCl-treated plants were supplemented with GA, POD activity was restored albeit not to the level observed for GA-treated or control plants (Figure 4.8 and Table 4.3). A similar trend was observed for POD 2 although the intensity of activity was not as pronounced as observed for POD 1.

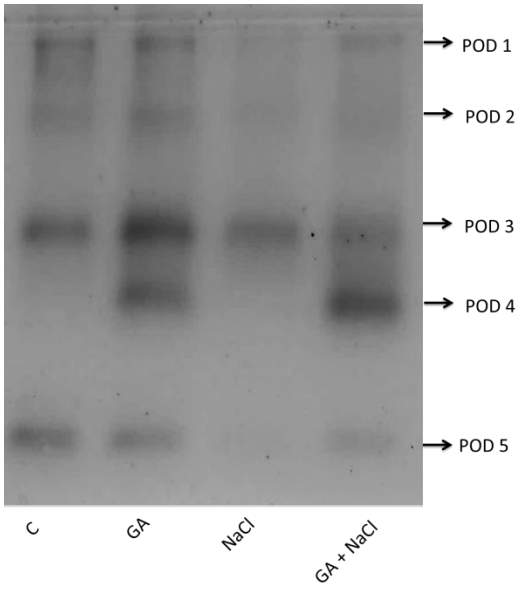


Figure 4.8 Gallic acid modulates POD activity in leaf extract of soybean plants under salt stress. POD activity was detected in leaf extracts of soybean plants using native PAGE in-gel activity assays. Soybean plants were treated with 0.25 mM GA, 80 mM NaCl and 0.25 mM GA + 80 mM NaCl for a period of 14 days. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

The enzymatic activity of POD 3 was increased by GA whereas a contrasting response was observed in the NaCl treatment (Figure 3.8 and Table 4.3).The increase in POD 3 activity observed in the GA treatment was severely restricted in the presence of NaCl (as seen for the combined treatment). The enzymatic activity of POD 4 is GA induced as no activity was detected in the control and NaCl treatment. Interestingly, the activity detected in the combined treatment (GA + NaCl) was more intense than the activity observed in the GA treatment.

Table 4.3: Quantification of POD isoforms in leaf extract of soybean plants

Relative POD Activity (% pixel intensity)	POD isoforms	Treatments			
		C	0.25 mM GA	80 mM NaCl	0.25 mM GA + 80 mM NaCl
	POD 1	1 ± 0.050	1.063 ± 0.053	n.d	0.948 ± 0.047
	POD 2	1 ± 0.050	1.024 ± 0.051	n.d	0.915 ± 0.046
	POD 3	1 ± 0.050	1.147 ± 0.057	0.960 ± 0.048	0.953 ± 0.048
	POD 4	n.d	1 ± 0.05	n.d	1.059 ± 0.053
	POD 5	1 ± 0.050	0.909 ± 0.045	0.740 ± 0.037	0.813 ± 0.040

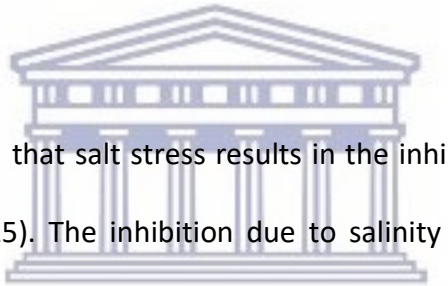
Data presented in this table are the means standard error are means ± SE (n=3). All POD isoforms were normalized using the control. The letters n.d in the table indicates that very low or no activity was detected. The capital letter C indicated in the labelling of treatments represents the 'control' used in the experiment.

Salt stress completely inhibited the enzymatic activity of POD 5 when compared to the other treatments. Although a decrease in activity of POD 5 was detected in the GA treatment it was not as severe as observed in the NaCl treatment. However, when NaCl treated plants was supplemented with GA, there was some recovery in POD 5 activity albeit not to the level of the control or even the GA treatment.

4.3 Discussion

In this part of the study we have investigated and analysed the influence of exogenously applied GA on long term salt stress more specifically on the physiological and molecular responses of soybean. The results obtained show the inhibitory effects of long term salt stress on plant growth and development. However when salt stressed soybean plants are supplemented with GA, salt-induced inhibition was reversed even though not to the level of the control.

4.3.1 Gallic acid improves soybean growth and biomass under long term salt stress



It is well documented that salt stress results in the inhibition of soybean growth (Hamayun *et al.,* 2015). The inhibition due to salinity in soybean results in an interruption of the whole life-cycle which ultimately leads to decrease in yields (Shu *et al.,* 2017). Similarly results annotated in this study showed that long term salt stress (induced by NaCl) resulted in the reduction in shoot and root growth (Figure 4.1). The roots are directly in contact with the saline soil and absorb nutrients and water which is translocated to the shoot. The behaviour of this tissue is crucial indicator for salt tolerance (Jamil *et al.,* 2004; Agarwal *et al.,* 2015). However salt stressed plants supplemented GA exhibited the reversed inhibitory effects caused by salt stress. Ozfidan-Konakci *et al.* (2015) showed that GA improved the growth rate of rice seedlings grown under salt stress in hydroponic conditions.

Reduction in plant biomass as a result of salt stress (80 mM NaCl) consequently resulted in the reduction in growth in the leaves by comparing to untreated control plants after 14 days. Salinity led to significant reduction in plant biomass (Figure 4.2), this coincided with similar data reported by Agarwal *et al*. (2015) and Queiroz *et al*. (2012). Conversely supplementation of GA to salt stressed soybean plants caused an improvement of both shoot and root dry weight to even higher levels than control. Taking into consideration these physiological parameters measured, GA showed rescuing effects on soybean. This is the first non-hydroponic study to report constructive effects of GA in plants especially soybean under salt stress.

4.3.2 Effect of gallic acid and salt stress on chlorophyll pigments

Salinity stress led to yellowing of the leaves (Figure 4.3 A) which in due course led to the damage of photosynthetic pigment composition. Similar data was reported by Al-Khanjari *et al.* (2002) in other legumes. Leaf chlorophyll content (Figure 4.3 B) was significantly reduced as a result of salt stress. The inhibitory effects of salt on chlorophyll have been proposed to be due to the enhancement of chlorophyllase activity (Jaiswal *et al.,* 2014). Reduction of photosynthetic pigments and biomass could also be due to the reduction of leaf size which was observed (Figure 4.3). Diminished leaf size could have arisen as a consequence of decline in water potential due to elevated levels of salt in the soil (Marcelis and Hooijdonk 1999; Queiroz *et al.,* 2012).

Previous studies have demonstrated how GA alleviates the reductions in photosynthetic efficiency of rice plants under salt stress (Ozfidan-Konakci *et al.,* 2015). Yildiztugay *et al.* (2017) also presented that GA alleviated soybean photosynthetic capacity as a result of cold stress. These results were contradictory to those obtained in both these studies. The reduction of chlorophyll content did however not affect plant growth and development and inhibited leaf chlorosis as it was experienced in the salt (alone) treatments.

4.2.3 Effect of gallic acid on ROS accumulation and oxidative damage of soybean plants exposed to salt stress

Salt stress induces inhibition of plant growth (Klein *et al.,* 2013) can be associated with excessive accumulation of O_2- and H_2O_2. Overproduction of ROS molecules such as superoxide and hydrogen peroxide are major factors responsible for lipid peroxidation (Tan *et al.,* 2008) and ultimately cause cell deterioration (Bailey-Serres and Mittler 2006) consequentially results in the reduction in plant biomass of plants exposed to salt stress. GA has been reported to restrict the production of H_2O_2 in soybean plants subjected to cold stress (Yildiztugay *et al.,* 2017). Results presented in this study suggest that GA restricts the production of O_2^- , however is unable to restrict the production of H_2O_2 in soybean plants subjected to salt stress. This then suggests that GA enhances antioxidant activity in order mitigate salt stress. As a result the cell death was brought to levels of control plant.

4.2.4 Gallic aid differentially alters antioxidant enzyme activity in soybean leaves subjected to salt stress

In order to minimise the oxidative damage as a consequence of salt stress, plants possess multiple mechanisms in order to eliminate or reduce the accumulation of ROS (Dalton *et al.,* 1986; Egbichi *et al.,* 2014). This involves the deployment of a number antioxidant enzyme which functions in detoxifying ROS. For the purpose of our study the activity of SOD which regulates the levels of O_2-, APX and GR which both detoxifies H_2O_2 were evaluated. Multiple studies have showcased the elevation of these enzymes in several plant species subjected to salt stress (Meloni *et al.,* 2003; Rasool *et al.,* 2013; Gengmao *et al.,* 2015).

In the present work, exogenous application of GA augmented SOD activity which accounted for reduction of O_2- content, a similar trend was also observed in salt stress plants supplemented with GA. Increase in SOD activity (Figure 4.4 A) was observed in the salt stressed (NaCl) plants, however this increase was insufficient in order to reduce O_2- production. This then suggests that GA is able to stimulate SOD activity, thus conferring efficient scavenging and to some degree tolerance. Both APX (Figure 4.4 B) and GR (Figure 4.4 C) activities were induced by salt stress relative to control plant. The combined treatment (GA + NaCl) had even higher levels of APX and GR, which would also suggest that total activity of these enzymes was solely enhanced as a result of supplementation of GA. Parallel data was also obtained by Yildiztugay *et al.,* (2017) in soybean under cold stress and Ozfidan-Konakci *et al.,* (2015) in rice under salt stress.

4.2.5 Gallic acid and salt differentially alters SOD activity/isoforms in soybean leaves

The data presented was in agreement with the notion that salt stress induces accumulation of ROS molecules such as O_2- (Jebara *et al.,* 2005). And as a result of that SOD activity was also enhanced, the activity of Cu/ZnSOD 4(Figure 4.5 and Table 4.1) was significantly increased by salt stress. However supplementation of GA on salt stress resulted in the increase of Cu/SOD 4. In addition GA exhibited similar effects, and it can be suggested that Cu/ZnSOD 4 activity is GA-induced. Cu/ZnSOD is localised in both the cytosol and chloroplast (Hernandez *et al*. 2000) and accounts for the increased total SOD (Figure 4.4 A) in both treatments. Sairam *et al* (2005) reported that enhancement in the activity of this SOD isoform resulted in the tolerance of a wheat genotype. Furthermore the activity of Cu/ZnSOD 5 was overexpressed in the combination treatment (GA + NaCl) which suggests that its activity contributes to the decrease

The activity of FeSOD was undetected in the salt (alone) and combination treatment (GA + NaCl). Induction of this isoform was seen to be greater at > 100 mM NaCl (Gomez *et al.,* 2004). Similar effects were also witnessed by Almansa *et al* (2004) and Gueta-Dahan *et al* (1997) and this was related to the low expression and activity of the enzyme in higher plants. MnSOD activity (Figure 4.5 A and Table 4.1) was differentially expressed in all treatments. Combination treatment (GA + NaCl) resulted in the reduction in activity however GA (alone) and NaCl resulted in the increase of the activity.

With the data presented it would then be suggested that exogenous application of GA did confer salt tolerance on soybean and this tolerance is arbitrated by the amplification of SOD activity that resulted in the scavenging of salinity stress-induced O_2- and consequently inhibiting cell death and increase in biomass. O_2-production also influences the activity of other ROS-scavenging enzyme pathways, APX and POD were investigated.

4.2.6 Gallic acid differentially alters APX activity/isoforms in soybean leaves under salt stress

One of the major ROS-scavenging enzymes such as APX is important in regulating intracellular H_2O_2 (Noctor and Foyer, 1998). APX exists in numerous isoforms localised in different compartments in plant cells and are differentially expressed and regulated under abiotic stress factors (El-Shabrawi *et al.,* 2010). APX isoforms are differentially expressed in all the subcellular compartments and salt stress tends to up-regulate their expression, based on plant species and salt sensitivity (Pandey *et al.,* 2015).

In our study total APX activity (Figure 4.4 B) was elevated in response to salt stress. An increase in APX 3 was clearly solely responsible for the increment of total APX activity. A clear reduction was however noted with APX 1 and APX 2 and this reduction was evidently caused by salt stress. Supplementation of GA in salt stressed soybean leaves resulted in the resulted in a significant increase in APX 2 and APX the activity of greatly influenced the increase in total APX activity

Taking into consideration the increase of GR activity (Figure 4.5.C) and APX, it can be hypothesised that the activity of these enzymes was inadequate in scavenging H_2O_2 (Figure 4.4.B) as a result of salt stress. In the event that antioxidant system is unable to adequately counteract the excessively high levels of H_2O_2 it accelerates cell death. However supplementation of GA to salt stressed soybeans resulted in the reduction of O_2- and cell death although not at levels of control plant.

4.2.7 Supplementation of gallic acid differentially alters POD activity/isoforms of soybean leaves under salt stress

PODs prevent oxidative damage to plasma membranes by scavenging peroxide radicals (Assche and Clijsters, 1990). PODs are found in either the bound state or free within the cell wall. Salt stress down-regulated POD activity and with inhibition of POD 1, POD 2 and POD 4 it could be hypothesised that this contributed to the increase in membrane leakage and subsequently cell death. Cicek and Cakirlar (2008b) presented similar data, as salt stress (NaCl) decreased the activity of POD in soybean plants in response to long term salt stress. However the activity of these POD isoforms was reinstated in the combined treatment (GA + NaCl) however at low levels. Supplementation of GA increased POD activity, with POD 4 being GA-induced it can be suggested that this resulted in the decrease in cell death. The increase of POD activity as a result of phenolic acid supplementation has been strongly associated with increase in lignin content and reduction in root growth (Devi and Prasad., 1996; Politycka, 1999).

Supplementation of ferulic acid in soybean also yielded an increase in cell-bound POD activity and lignin content (Dos Santos *et al.,* 2004). With the data presented this suggests that POD4 as a viable candidates for POD-mediated salt tolerance conferred by GA.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Soil salinity is one of the main limiting factors of plant growth and contributes to large portion of crop loss more especially in the semi-arid regions. The negative effect soil salinity has on agricultural land has contributed to the increase in the demand and rise of food crops and limited food resources. An average of 20% - 50% reduction in yield of essential food crops has been recorded as a result of salinity stress. Food security is therefore profoundly dependent on the development of food crops with improved resistance to abiotic stresses such as salinity. Soybean is an essential food crop as it is highly nutritious and forms an integral part of the human and animal diet.

An extensive range of adaptation and mitigation strategies are essential to improve crop production. These may include biotechnological strategies in order to confer tolerance through the use of naturally occurring antioxidants. Soybean is the main source of high-quality oil and protein for human consumption. However soybean is sensitive to salt stress and its production is not only essential for consumption but also contributes to the GDP of the country. South Africa being the main producer of soybean in the SADC region, increasing production of this crop is detrimental to our economy. In order to counter the delirious effects caused by long-term salt stress on soybean. This study explored the regulatory effects of exogenous GA on soybean in response to long-term salt stress.

Chapter three of the thesis explored the minimum concentration at which exogenous GA improved, plant growth, biomass, photosynthetic metabolism and antioxidant capacity. GA differentially altered both physiological and molecular responses in soybean plants. A low concentration of GA (0.25 mM) improved; shoot length, plant biomass and chlorophyll content. The study further analysed the role of GA on ROS accumulation and oxidative damage, and no significant changes compared to control were observed. The antioxidant capacity was enhanced by supplementation of GA as SOD and APX significantly increased. With these findings 0.25 mM presented to be a suitable concentration at which it improved plant growth and antioxidant capacity.

Chapter four described the regulatory effects of exogenous GA and salt stress on soybean growth, biomass, chlorophyll content, ROS accumulation and ROS scavenging enzyme activity. Salinity stress resulted in the inhibition on plant growth and increase in O_2^-, H_2O_2 and ultimately cell death. However GA supplementation on salt stressed soybeans improved; plant growth, biomass and the photosynthetic metabolism. Further analysis on resulted in a reduction of O_2^- and cell death.

Salt stress resulted in the increase of total SOD, APX and GR; however the increase of these enzymes was insufficient in scavenging ROS molecules. Supplementation of GA improved the antioxidant capacity and combination treatment (GA + NaCl) significantly increased the antioxidant capacity. The antioxidant activity was further analysed in non-denaturing polyacrylamide gels,

and two SOD isoforms were identified to be good candidates which contributed to the SOD-mediated salt tolerance. APX and POD also demonstrated similar effects. The role of GA in regulating salt stress in order to confer tolerance in soybean should not be limited to the alterations of ROS scavenging antioxidants but also extended to the analysis of genes and proteins expressed. This can be achieved by analysing deferentially expressed proteins and transcripts by GA via proteomics and transcriptomics.

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