The impact of delayed nodule senescence by tissue-specific cysteine protease inhibitor expression in soybean [*Glycine max* (L.) Merr.] development and response to abiotic stress

By

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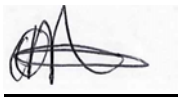
Declaration

I, **Elizabeth Ofentse Mathibela**, declare that this thesis, which I hereby submit for the degree MAGISTER SCIENTIAE (Plant Science) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution**.**

Signature: 

Date: 2021-07-27

Dedication

To the passionate, humble, and exceptional leader Dr. Matome Eugene Makgopa. Although you are gone, your teachings will echo in the lives you have touched, especially mine. You saw potential in me and regardless of your past student record and my below-average marks, you allowed me to study towards my postgraduate degree. You went far beyond your job description and ensured I found a mentor in you. Thank you for all the lollipops after every failed experiment, for pushing me to step out of my comfort zone, and for exposing me to every opportunity that groomed me to not only know how to follow protocol but to invent and test new protocols. We were never ready to lose you, but we are confident that you have equipped us to fully live, love, laugh, and have a celebratory braai for every milestone. Heaven laboratory is having a blast with you around. Till we meet again, a re tlo ba hemisa. A ro ba fa chance ebile ro lwa till dilo stofong!

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Summary

Environmental sustainability and food security are among the major global challenges. Excess use of fertilizers and urbanization significantly reduces sustainability of suitable environments for agricultural purposes. It is therefore important to develop solutions that can potentially improve food security and maintain adequate yield with the available agricultural resources without further damaging the environment. Soybeans are a multifaceted crop used as feed, food, and other industries. Apart from their nutritive value, these plants can fix nitrogen through specialized organs in their roots due to association with *Rhizobia* known as nodules. Nitrogen is an important and limiting element in crop production and nitrogen fixing crops can fix atmospheric nitrogen to a form used by plants reducing the use of nitrogen fertilizers.

Soybean yields are negatively affected by both natural and stress induced senescence of nodules since it limits nitrogen supply to the plants resulting in nitrogen deficiency. Senescence is due to accelerated and unregulated activity of proteases, specifically cysteine proteases. In this study, transgenic soybean plants expressing a cysteine protease inhibitor (*OC-I*) specifically in root nodules were characterized under natural and stress-induced tolerance. The hypothesis being that *OC-I* will delay nodule senescence resulting in prolonged nitrogen supply which will delay leaf degradation for nutrient remobilization thus leading to improved growth. Unlike ectopic expression in previous studies, tissue-specific expression did not lead to reduced growth of the transgenic lines as compared to wild type under normal conditions. Transgenic lines maintained significantly higher photosynthetic machinery (chlorophyll) as well reduced oxidative damage (hydrogen peroxide and malondialdehyde) under natural and stress induced-conditions senescence. The reduced oxidative damage was due to improved antioxidant pool (GST, POD, CAT), osmoprotectant and protective pigment accumulation (proline, anthocyanin, carotene). Ureide content accumulation in root nodules indicated significant nitrogen fixation in transgenic lines as compared to wild type. Metabolite accumulation was also investigated to understand partitioning patterns of the plants. Accumulated data suggest the involvement of *OC-I* in senescence tolerance.

Dissertation composition

Chapter 1 of this MSc dissertation is a literature review focused on the importance soybean in multiple industries as well as the challenges faced in soybean production. Information on biological nitrogen fixation as well as nodule development and senescence are highlighted. Drought stress was the focus as the major stress-induced senescence discussed and how the proteolysis pathway changes in response to this stress. The problem statement, aims, objectives and hypothesis of the study are outlined at the end of this chapter. **Chapter 2** characterizes the transgenic *OC-I* lines in natural senescing conditions to investigate if tissue-specific expression of *OC-I* delays nodule senescence and how above ground organs might respond to the delay. **Chapter 3** further characterizes the transgenic lines under drought conditions to understand how these transgenic plants respond to stress-induced senescence. In this chapter, root nodules are the focus since there is limited data available on how root nodules respond to drought stress. Both chapter 2 and chapter 3 have phenotypical, physiological, and biochemical changes observed in either root nodules or leaves in respond to the conditions the plants are in. **Chapter 4** discusses the general conclusions reached in the study as well as prospects. The end of each chapter has a **list of all cited literature** in that specific chapter.

Contents

List of figures

Abbreviations and symbols

Abs	Absorbency
Ala	Alanine
ANOVA	Analysis of Variance
APX	Ascorbate Peroxidase
Asn	Asparagine
CAT	Catalase
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
Cys	Cysteine
Gln	Glutamine
Gly	Glycine
GR	Glutathione reductase
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
His	Histidine
Lb	Leghemoglobin
Leu	Leucine
LPO	Lipid Peroxidation
MDA	Malondialdehyde
Nod	Nodulation
OC-I	*Oryzacystatin I*
OD	Optical density
PCR	Polymerase chain reaction
Phe	Phenylalanine

PIs	Protease Inhibitors
POD	Peroxidases
Pro	Proline
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Trp	Tryptophan
Val	Valine
WT	Wild type

Chapter 1

Literature review

1.1. Introduction

1.1.1. The importance of soybean

Soybean (*Glycine max* (L.) Merr.) is an important legume plant originating from East Asia in countries including China, Korea and Japan (Lee et al., 2011, Sedivy et al., 2017). The fields and roadsides of many Asian countries are dominated by *Glycine max*. This specie belongs to the subgenus Soja, which also includes *Glycine Soja* and *Glycine gracilis*. Molecular, morphological, and cytological data indicate that *G. Soja* is the ancestor of both *G.max* and *G. gracilis*. *G. gracilis* is considered the weedy or semi-wild form of *G. max* (Singh et al., 2007). Soybean was introduced to Sub-Saharan Africa by Chinese traders due to its potential for being a commercial crop as food, animal feed and source of industrial raw material (Khojely et al., 2018). Soybean is used as a cheap source of protein and is one of the most widely researched economically important crops worldwide (Dwevedi and Kayastha, 2011), making it an excellent candidate for genetic modification to improve its nutritious value and increase tolerance to both biotic and abiotic stress.

The dry soybean seed is composed of 36% protein, 19% oil, 19% insoluble carbohydrates, 9% soluble carbohydrates, 13% moisture and 4% ash. Soybean is an agriculturally and economically important crop due to its vast uses as food, feed and other industrial applications including paint, plastics and cleaning agents (Hershman et al., 2011). Argentina, the United States of America and Brazil dominate the global soybean production, and made up 80% of the total production in 2018 (Prisăcaru and Șevciuc, 2020). In Sub-Saharan Africa, Nigeria and South Africa are the leading Soybean producers, contributing 1.32 and 0.73 million tons of soybean grain in 2019 (Hartman and Murithi, 2019). There is great potential for Africa to become one of the major global soybean producers due to its suitable climates, and available land (Foyer et al., 2019). Soybean is a good source of plant based protein and with the world shifting towards a healthier lifestyles (Zhang et al., 2017) more research has been focused on improving the growth and development of this crop. Soybean oil consists of 22.8% total monosaturated fatty acids, 15.6% total saturated fatty acids, and 57.7% total polyunsaturated fatty acids making it the second largest vegetable oil produced globally (Vagadia et al., 2017).

Apart from its economic importance, soybean plants provide agricultural land sustainability due to their ability to form root nodules and fix nitrogen. Soybean root nodules are formed due to a symbiotic relationship between the soil borne microbe *Bradyrhizobium japonicum* and the roots of the soybean plant (Puppo et al., 2005). The relationship is referred to as symbiotic

because the plants gain nitrogen due to microbial action and the microbes gain access to nutrients from the plant (Desbrosses and Stougaard, 2011). Due to this nitrogen fixing ability, the introduction of legumes to degraded soil can accelerate soil reclamation and enable pioneer natural succession of native vegetation (Pérez-Fernández et al., 2016).

1.1.2. The development and functioning of root nodules

Nodulation involves infection with a *Rhizobia* species and then formation of a symbiotic organ known as a nodule (Suzaki et al., 2015). The initiation of nodule formation involves the activation of the root epidermis's defense signaling cascade by the rhizobium derived lipochitin oligosaccharides, known as nodulation (Nod) factors (Suzaki and Kawaguchi, 2014). These Nod factors are encoded for by Nod genes which are divided into two groups: Early Nod genes and Late Nod genes. Early Nod genes are expressed early in nodule formation prior to nitrogen fixation and late Nod genes are expressed when the complete structure of the nodule is already formed and nitrogen fixation will commence (van de Wiel et al., 1990). Early Nod gene signal induces oscillation of calcium and other, yet to, be identified signaling molecules to the cortex inducing reverse epigenetic reprogramming that leads to the loss of cell specialization of the cortical cells in infected root hairs (Oldroyd, 2013). These dedifferentiated cortical cells are then reprogram to from nodules (Szczyglowski et al., 1998) as shown in Fig. 1.1.

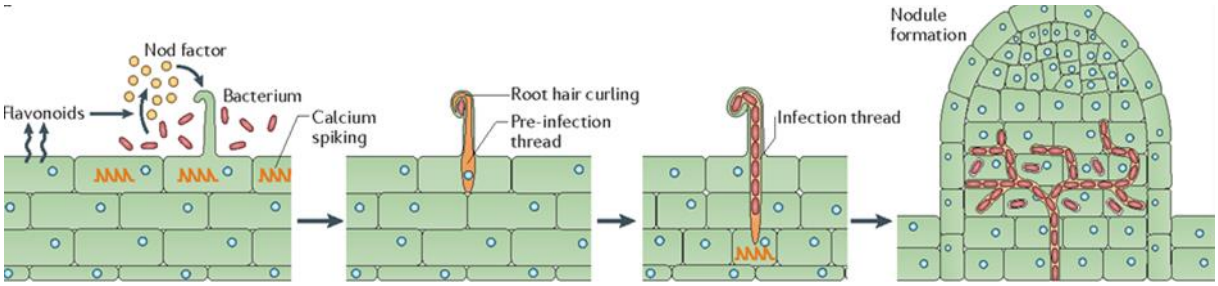

Figure 1.1: Rhizobial nodule formation as depicted by Oldroyd, (2013). Cortical cells release flavonoids that attract *Rhizobia* to the roots. Nod factors in the roots facilitate curling of the root hairs providing a suitable environment for bacterial infection. Following the formation of an infection thread, a canker like organ develops in the root, known as a nodule allowing for nitrogen fixation.

Legumes have two types of nodule morphology, determinate and indeterminate as depicted in Fig. 1.2 (Kazmierczak et al., 2020). Determinate root nodules stop growing after approximately 2-3 weeks post inoculation due to the termination of meristem division in the cortex (Newcomb, 1981). Soybean (*Glycine*) and bean (*Phaseolus*) species have determinate root

nodules. Indeterminate root nodules continuously grow as a result of the addition of meristem cells at the apex (Newcomb, 1981). Alfalfa (*Medicago*) and pea (*Pisum*) species develop indeterminate root nodules. Soybean nodules are further classified as either crown or lateral nodules depending on where they develop. Crown nodules develop early on the main tap root while lateral nodules develop on lateral roots to compensate for the decomposition of crown nodules as plant growth progresses (Vorster et al., 2013).

Microbes that can fix nitrogen require 16 moles of adenosine triphosphate (ATP) to reduce one mole of nitrogen (Figueiredo et al., 2013). In legumes, the ATP is provided by the host plant through photosynthesis and the nitrogenase is from the bacterial symbiont (Sahabi, 2016). Root nodules can fix atmospheric nitrogen due to the presence of Nitrogenase enzyme in these organs. Nitrogenase has Fe-S, Fe-Mo, Fe-V cofactors at its core for efficient nitrogen fixation by the binding of nitrogen at its core (Jangir et al., 2020). The ideal functioning conditions of nitrogenase include; (1) oxygen diffusion resistant outer cell layer of root nodules, (2) binding and transport of oxygen by leghemoglobins in infected cells of the nodule interior and (3) restriction of diffusion of oxygen into bacteria by external mucilage (Mus et al., 2016). Nitrogenase catalyzes the breakdown of one of the bonds between the two nitrogen molecules and add a hydrogen atom to produce the nitrogen needed for plant growth (Postgate, 1982).

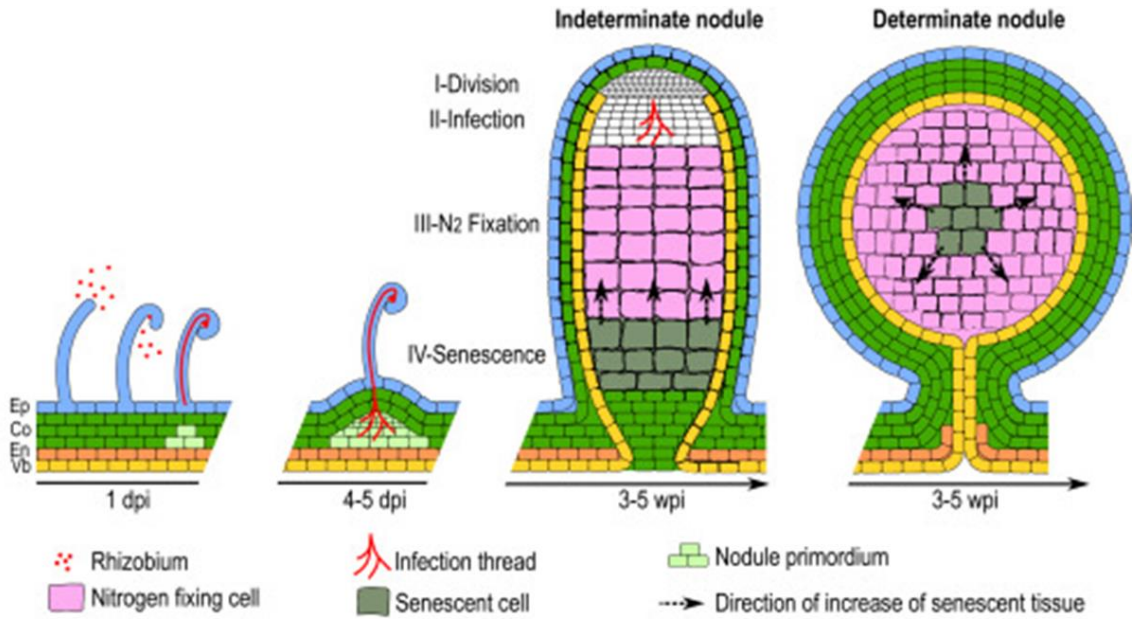

Figure 1.2: Depiction of nodule formation days post inoculation (dpi) and weeks post inoculation (wpi) and development of interderminate and determinate root nodules. Image extracted from Kazmierczak et al., (2020).

1.1.3. Nodule senescence

Nodule lifespan is dependent on legume plant species, for example, leguminous perennials (such as alska clover) have longer-lived nodules as compared to fast-growing legume annuals, such as soybean (Puppo et al., 2005). The average life-span of fast-growing legume nodules is 10-12 weeks after inoculation in the absence of stress (De Beer, 2012). Determinate nodule senescence initiates at the center of the nodule spreading to the edges and indeterminate nodule senescence starts at the point where the nodule is closest to the root and progresses to the apex of the nodule (Puppo et al., 2005, Guerra et al., 2010). The decrease in nitrogenase activity is directly proportional to progression of nitrogen senescence and can be visibly analyzed by loss of pink color in active root nodules to a non-active greenish color as a result of the degradation of the heme-group of leghemoglobin (Van de Velde et al., 2010).

Leghemoglobin (Lb) is a phytoglobin that facilitates the diffusion of oxygen in nitrogen fixing root nodules (Sudhakar et al., 2016). Leghemoglobin is made of a heme kinship and a single polypeptide (globin) whose amino acid sequence differs depending on the legume species (Singh and Varma, 2017). The function of Lb involves supplying oxygen to the nitrogen fixing bacteria and protecting nitrogenase from being inactivated by exposure to oxygen (Wittenberg, 2012). For efficient oxygen transport Lb has to be converted to its ferric form through the action of ferric leghemoglobin reductase resulting in the formation of oxyleghemoglobin (Becana Ausejo et al., 1995, Urarte et al., 2008).

Senescing root nodules are at risk of oxidative damage due to the autooxidation of oxyleghemoglobin to the ferric form associated with production of O_2^- which degrades to form hydrogen peroxide (Puppo et al., 1981). Reactive oxygen species (ROS) can also result from the strong reducing conditions required for the fixation of nitrogen, or degradation of nodule proteins such as hydrogenase and uricase (Dalton et al., 1991). The increase in ROS concentrations in root nodules accelerates nodule senescence by provoking nodule membrane lipid peroxidation as well as bacteroid membrane peroxidation (Mathieu et al., 1998, Puppo et al., 1991).

Soybean suffers from nitrogen deficiency in field conditions specifically at the flowering stage since this is when nodule senescence is initiated (Quain et al., 2015). Planting in the absence of an inoculant or in degraded soils, which prevents successful nodulation also promotes nitrogen deficiency in the crop. External application of nitrogen fertilizers as well as inoculating with bacterial strains that can survive in adverse conditions, such as soil acidity

offers a solution, but it is not always cost effective (Merbach and Schilling, 1980, Nwadinigwe and Onyeidu, 2012). However, even with the use of these solutions, premature nodule senescence can occur because of abiotic stresses like drought, defoliation, chilling stress, salt stress and exposure to heavy metals (Gordon et al., 1990, Balestrasse et al., 2003, Dupont et al., 2012, Kunert et al., 2016).

1.1.4. Drought stress and response

Water for agriculture is a scarce commodity, which is of great concern since large amounts of water are needed for food production to feed the increasing population (Motoshita et al., 2018). South Africa is ranked among the top thirty driest countries worldwide with most of its soils sandy, poorly buffered and acidic (Mabhaudhi et al., 2019, Twomlow et al., 2006). Climate change plays a major role in increasing water demands, with agriculture being heavily affected as compared to other sectors especially is southern Africa where temperatures are expected to increase at 1.5-2 times the global rate of temperature increases (Wang et al., 2016, Dedekind et al., 2016). Under these predicted weather conditions southern and tropical parts of Africa are predisposed to the occurrence of droughts and floods (Dedekind et al., 2016).

Drought, defined as prolonged precipitation deficiency from what is expected leading to reduced anthropogenic and environmental productivity (Hayes et al., 1999), is one of the major consequences of climate change. It is one of the crucial limiting factors in grain crop production due to its ability to negatively affect germination potential, early seedling growth, root and shoot dry weight and vegetative growth (Daryanto et al., 2015, Okçu et al., 2005, Manickavelu et al., 2006). In order to improve crop yield and maintain the yield for food security reasons, plants with traits that ensure survival under both well-watered and drought conditions are essential (Basu et al., 2016).

Plants have developed several strategies to deal with drought stress, which include drought escape, tolerance or avoidance (Chaves et al., 2003). Plants can use more than one of these strategies to deal with water deficit (Shavrukov et al., 2017). Drought escape refers to the plant's ability to complete its life cycle prior to any physiological changes caused by water deficit (Kooyers, 2015). Escape strategies rely on rapid reproduction under severe conditions. Successful reproduction under unfavorable conditions is dependent on the plant's ability to effectively partition assimilates to developing fruit (Chaves et al., 2003). Drought tolerance is the ability of a plant to endure water deficit environments (Basu et al., 2016). Tolerance strategies involve maintaining high turgor pressure in cells through osmotic adjustment

(Morgan, 1984). Drought avoidance involves the prevention of tissue dehydration by minimizing water loss while maximizing water uptake (Ehleringer and Cooper, 1992, Fang and Xiong, 2015).

Drought results in increased solute concentration in the soil as compared to the internal environment of roots leading to reverse osmosis which leads to molecular, physiological and biochemical changes in plants (Kaur and Asthir, 2017, Waraich et al., 2011). Molecular changes in response to drought include increased abscisic acid biosynthesis gene to regulate growth and upregulation of stress related protein genes such as late embryonic abundant proteins (LEA), dehydrins, heat shock proteins, drought response element binding (DREB) and N-acetylcysteine (Brodribb and McAdam, 2013, Yoshida et al., 2010, Macková et al., 2013, Liu et al., 2014). Biochemical responses to drought involve increased antioxidative enzymes, reduced ROS and increased accumulation of stress metabolites such as proline and polyamines (Hochberg et al., 2013, Yang et al., 2017, Chakraborty et al., 2015). Physiological traits related to drought stress include reduced leaf growth as a function of lower turgor pressure, reduced photosynthesis as a result of limited carbon dioxide due to closed stomata and under severe drought lack of RuBisCO regeneration and reduction of photosynthetic pigments (Shivakrishna et al., 2018, Farooq et al., 2010, Aranjuelo et al., 2011). Drought accelerates leaf senescence as a mechanism to reduce canopy cover and optimization of water and nutrient remobilization to organs crucial for future survival of the plant (Griffiths et al., 2014).

The effect of drought on photosynthesis, and the resulting senescence, prompt biochemical responses in plants to confer adaptation to the stress signal. Plants increase the activity of certain proteases, such as cysteine proteases to induce early tissue senescence (Chaves et al., 2003) as a survival mechanism. Senescing tissues lead to the production of ROS such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), singlet oxygen (O_2) and hydroxyl radicals (OH) which are metabolic by-products, but if not carefully regulated can result in reduced growth and development (Nxele et al., 2017). The accumulation of ROS can prompt stress related response in plants (Lee et al., 2012) and if the plant fails to deploy effective detoxification, high ROS levels can result in oxidative damage to cells and increased proteolysis, which can trigger programmed cell death.

At early drought stages plants prioritize phenotypical changes to absorb water deep in the soil through improved root systems and managing water loss by partially closing their stomata (Hu and Xiong, 2014). As the severity of the stress increases plants employ biochemical changes

involving enzymatic and non-enzymatic antioxidants to avoid oxidative damage and osmoprotectant to maintain osmotic potential (Lee et al., 2012). This is because drought resistance can be linked to effective detoxification of ROS resulting in reduced oxidative damage. The osmoprotectant and non-enzymatic antioxidants include accumulation of soluble sugars, betaine, spermines, proline and glutathione (Seki et al., 2007 and Lee et al., 2012) to maintain cell turgor pressure. The enzymatic antioxidants include increased activity of catalase (CAT), superoxide dismutase (SOD), glutathione-s-transferase (GST), glutathione reductase (GR), and ascorbate peroxidase (APX) as an attempt to detoxify cells (Seki et al., 2007).

1.1.5. Cysteine proteases and their inhibitors

Proteolysis is a process of protein breakdown. Although proteolysis plays an important role in many aspects of plant physiology and development (Vierstra, 1996), it can have detrimental effects on the plant if not properly controlled. Proteolysis is essential for various cellular housekeeping and stress responses, which include removal of misfolded/damaged proteins; supplying amino acids needed to make new protein synthesis; assisting in the maturation of zymogens and peptide hormones by limited cleavages; homeosis and development by reducing the abundance of key enzymes or regulatory proteins; and programmed cell death (Davies, 1982, Ellis et al., 1991, Gatenby and Viitanen, 1994). The rate of proteolysis is tightly regulated by the interaction between proteolytic enzymes known as proteases and protease inhibitors. The indication that proteases and their inhibitors are specifically involved in the response of plants to drought stress originated from observations that drought conditions often bring about senescence of plant tissue and that senescence was closely interconnected with increased proteolysis that involves several proteases (Kidrič et al., 2014).

1.1.5.1. Plant cysteine protease classification

Proteases catalyze the breaking of peptide bonds between amino acids resulting in protein degradation (Radisky, 2017). Proteases are classified into different groups according to their ability to either partially or completely degrade a specific protein, and the identity of their active site (Fan and Guo-Jiang, 2005, González-Rábade et al., 2011). The action can either be a limited form of proteolysis, where the protease cleaves a limited number of peptide bonds of the target protein leading to activation or maturation of the protein. The other group is referred

to as, or unlimited proteolysis, which could occur, where when proteins are degraded to single amino acids (Fan and Guo-Jiang, 2005) as represented in the schematic depicted on Fig. 1.3.

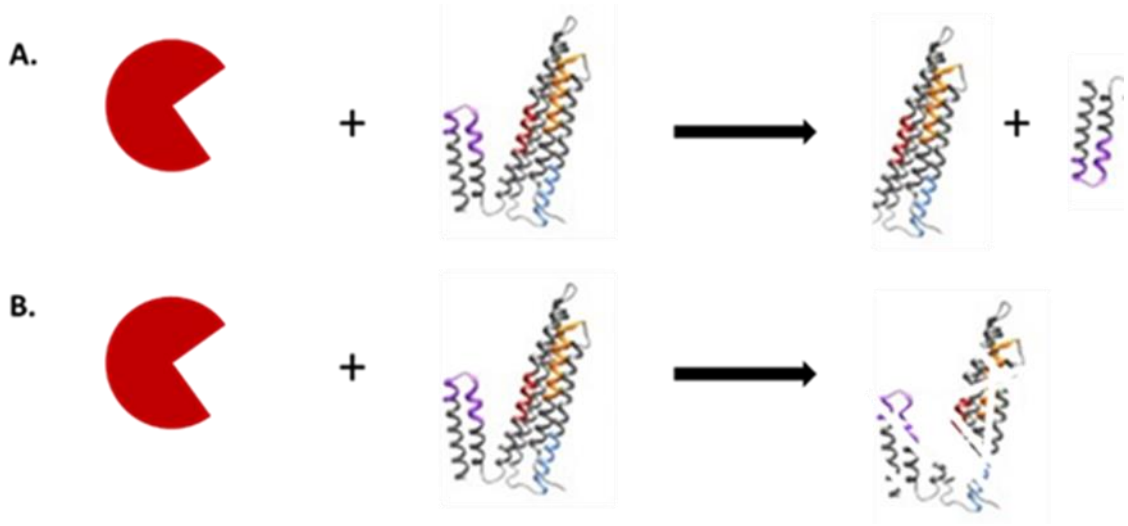

Figure 1.3: The action of proteases. (A) limited proteolysis where only a few peptides are cleaved, (B) unlimited proteases where the entire protein is degraded. Adopted from García-Lorenzo, (2007).

Plant cysteine proteases are well characterized proteolytic enzymes, which play vital roles in senescence, seed germination and defenses (Martínez et al., 2012). These enzymes have strong proteolytic activity against a broad range of protein substrates and function at various pH's and temperatures (González-Rábade et al., 2011). The MEROPS database classified CPs into ten clans (CA, CD, CE, CF, CH, CL, CM,CN, CO and C-) with most enzymes belonging to the papain family, clan CA (Rawlings et al., 2010). Cysteine proteases play crucial roles in drought susceptibility. Drought tolerant wheat cultivars have shown to have suppressed cysteine protease gene expression while susceptible cultivars showed augmented expression suggesting involvement of these proteases in drought susceptibility (Simova-Stoilova et al., 2010). In soybean root nodules, drought conditions have shown to upregulate the expression of cysteine proteases resulting in premature nodule senescence (Marquez-Garcia et al., 2015, Cilliers et al., 2018).

1.1.5.2. Papain proteases

Plant papain proteases are a large group of proteolytic enzymes belonging to clan CA and cysteine protease class (Rawlings et al., 2010). Their functions have related to plant/pathogen interactions, immunity, seed germination, senescence, and stress response like drought (Grudkowska and Zagdańska, 2004, Kempema et al., 2007, Chen et al., 2010, Misas-Villamil

et al., 2016). These enzymes are synthesized in an inactive form containing a signal peptide responsible for protein degradation and auto-inhibitory peptide to prevent unplanned protein degradation (Coulombe et al., 1996). The active domain has Cys-His-Asn chain for catalytic activity and is activated by a pH dependent cleavage of the conserved salt bridges (prodomain) within the enzymes (Hasilik et al., 2009, Schröder et al., 2010, Liu et al., 2018).

In soybean, chinese milkvetch, pea and berrelclover, papain protease genes play important roles in nodule initiation, development, and senescence (Vincent and Brewin, 2000, Pierre et al., 2014, Mergaert et al., 2020). Soybean papain proteases genes *CYSP1* (*GmCYSP1*) and *GmPLCP* (*Glyma.04g190700*), negatively affect nodule development and enhance senescence (Alesandrini et al., 2003, Yuan et al., 2020). Transcriptomic profile data from soybean nodules showed upregulation of 14 papain cysteine proteases at the onset of senescence suggesting their involvement in accelerated nodule senescence (Van Wyk et al., 2014). If the activity of these proteases goes unregulated, premature nodule senescence because of biotic/abiotic stress can cause yield losses.

1.1.5.3. Plant cysteine protease inhibitors

Protease inhibitors limit the action of proteases by tightly binding to them thus inhibiting their peptide hydrolysis activity (Van der Hoorn, 2008). Protease inhibitors are classified according to which catalytic group of proteases they inhibit as well as the mode of action of their activity (Habib and Fazili, 2007). Following cellular production of protease inhibitors, the basic mode of action of protease inhibitors involves using their substrate-like region (active site), on the surface, to bind to the active site of the specific protease (Habib and Fazili, 2007). Fig. 1.4 describes the basic mode of action of protease inhibitors to prevent proteolysis using a cysteine protease inhibitor (phytocystatin). Upon interaction with a cysteine protease, phytocystatins form an irreversible bond known as cysteine protease-cystatin complex thus preventing protein degradation (Kunert et al., 2015). This irreversible interaction is made possible by the Gln-Xaa-Val-Xaa-Gly motif in the core of the peptide, Xaa can be any amino acid depending on plant species, a Pro-Trp or Leu-Trp dipeptide in the C-terminal and a conserved glycine residue in the N-terminal region of the phytocystatin (Benchabane et al., 2010).

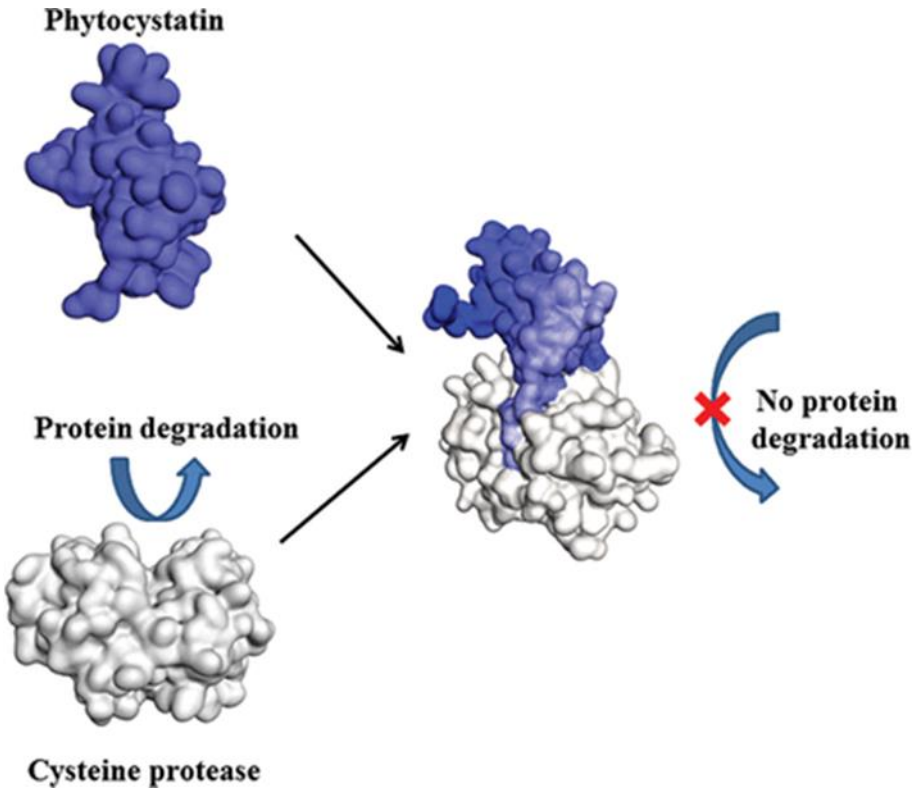

Figure 1.4: Basic mode of action of protease inhibitors. Protease inhibitors bind to the active site of a specific protease and alter its functioning. Adopted from Kunert et al., (2015).

Cysteine protease inhibitors family is characterized by a group of endogenous cysteine protease inhibitors (PIs) containing multiple cystatin-like motifs (Rai et al., 2017). Cysteine protease inhibitors are divided into four super families (Oliveira et al., 2003). Family one comprises of stefins, family two is known as cystatins, family three is referred to as kinogens and family four are known as phytocystatins (Habib and Fazili, 2007). Most cysteine protease inhibitors are small in size and lack putative glycosylation sites (Martínez et al., 2012).

In soybean plants cysteine protease inhibitors regulate internal processes in the plant contributing to abiotic stress tolerance (Mangena, 2020). However, the quantity of cystatins in mature soybean plants is low due to the decomposition of the transcribed mRNA during seed germination allowing for cysteine protease synthesis to facilitate germination (Arai et al., 2002). This decrease in phytocystatins makes soybean susceptible to cysteine protease senescence when exposed to stressed conditions. The identity and functioning of most of the endogenous cysteine protease inhibitors in soybean, just like most leguminous crops, remain unknown (Mangena, 2020). Although there is evidence of increased cystatins in root nodules as a response to drought the increase does not sufficiently compensate for the rapid degradation thus not significantly improving drought tolerance.

1.1.5.4. *Oryzacystatin I* (*OC-I*), a papain protease inhibitor

Oryzacystatin I (*OC-I*) is a cysteine protease inhibitor naturally occurring in rice seeds and is the first well defined cysteine protease inhibitor of plant origin (Kondo et al., 1990). Abe et al., (1987) was the first study to successfully clone *OC-I* and establish homology with animal-based cysteine protease inhibitors. In their study, conserved regions of animal cysteine protease inhibitors Phe-Ala-Val, Pro-Trp-Met and Gln-Val-Val-Ala-Gly were used to screen and isolate *OC-I* from a rice cDNA library. It was only in 2000 that the first 3d structure of *OC-I* was determined using nuclear magnetic resonance spectroscopy to confirm the homology relationship suggested by Abe et al., (1987) (Nagata et al., 2000). Being the first plant-based cystatin to be isolated, *OC-I's* involvement in plant-plant, plant-pathogen and plant-environment interaction become of great interest.

In rice seeds, *OC-I* plays vital role in the inhibition of papain-protease activity thus promoting storage protein accumulation during flowering (Kondo et al., 1990). Biotechnological applications have associated overexpression of *OC-I* with tolerance to drought, heat, light stress, stem nematode infection and Colorado potato beetle larval development (Demirevska et al., 2010, Gao et al., 2011, Cingel et al., 2017). Demirevska et al., (2010) observed protection of antioxidant enzymes in *OC-I* transformed tobacco plants under drought, heat and chilling stress; and non-stress conditions. In biotic stress conditions, Cingel et al., (2017) reported stunted growth of the colorado potato beetle by the inhibition of the pathogen cysteine proteases through *OC-I* and *OC-II* interaction in transgenic potato plants.

Plant cysteine protease inhibitors act as defense proteins (Martínez et al., 2012). Their defense role is the most analyzed and has been theorized from their ability to impede the activity of digestive proteases from insects and acari in experiments using artificial diets and bioassays on plants overexpressing specific cysteine protease inhibitor (Atkinson et al., 2004, Álvarez-Alfageme et al., 2007, Carrillo et al., 2011). The expression of *OC-I* in different plant species has shown both biotic and abiotic stress tolerance.

1.2. Problem Statement

Climate change, food security and limited arable land are among the major global challenges (Dawson et al., 2016). It is of great importance that these challenges be addressed to ensure sustainable food production for the future. Climate change has a direct effect on sustainable agricultural land and food security. It is difficult to prevent climate change yet vital to combat its consequences since more than 900 million people are suffering from chronic hunger as

indicated by the food and agricultural organization (World Health Organization, 2019). Africa and Asia potentially being at the highest risk of chronic food insecurity. To secure global food production there must be major re-focusing of production strategies. Instead of combating challenges individually it is pivotal to employ strategies that combine global challenges and yields a sustainable solution.

Nodulation gives legume species a superior advantage in degraded soils (Chaer et al., 2011). This is based on the nitrogen fixing capabilities of legumes resulting in ability to remove toxic ions while improving soil fertility by increasing soil nitrogen (Abiala et al., 2018). The role of legumes in increasing soil fertility is through promotion of nutrient cycling, increasing carbon incorporation in the soil, the restoration of soil nutrients and minimization of erosion (Moura et al., 2016). Legumes can deal with several consequences of soil degradation. *Hedysarum carnosum*, a pastoral legume, had increased detoxification of Na+ by accumulating the ions in leaves and maintained high nitrogen fixation in salt degraded soil (Kouas et al., 2010). The latter is a characteristic of legume's ability to absorb heavy metals/toxins in root and safely translocate them to leaves where the substances accumulate (Pajuelo et al., 2011). The observation on *Hedysarum carnosum* suggest that these plant species can potentially improve soil fertility by increasing available nitrogen in the soil and detoxifying harmful ions in degraded soils. Although other crops, such as maize, have been reported to have been reported to reclaim soils degraded by salt in China (Luo et al., 2018), legumes, such as soybean, have been reported to be more tolerant to soil degradation consequences such as high salinity (Butcher et al., 2016). Soybean's superiority over maize under degraded soils was reported in different tillage treatment of heavily degraded Chinese soils. The report indicates significant increase in soybean yields under no tillage degraded soils and significantly decreased yield in maize under the same treatment (Chen et al., 2011).

Most studies characterizing the effect of drought on soybean are focused on above ground traits rather than root nodules (Kunert et al., 2016). Nodules are important organelles in soybean since they are a result of *Rhizobia* association with plant roots leading to nitrogen fixation (Kunert et al., 2016). Drought causes early nodule senescence leading to reduced nitrogen supply to the plant and ultimately plant death (Cilliers et al., 2018). It is vital to characterize tissue specific expression of *OC-I* in soybean since constitutive expression showed reduced growth due to cost of gene expression in normal conditions (Makgopa, 2014). The improvement of soybean is important since this crop can potentially improve food security and land fertility for sustainable food production.

Symptoms of nitrogen deficiency due to the onset of nodule senescence and cost of constitutive expression of *OC-I* in soybean lead to the hypothesis that tissue specific *OC-I* overexpression in root nodules will result in delayed nodule senescence. Delayed nodule senescence will then be followed by prolonged nitrogen supply resulting in reduced nitrogen deficiency. And since the transgene will not be expressed constitutively, the cost of reduced growth is expected to be mitigated significantly in transgenic lines as compared to the wild type.

1.3. Aim and objectives

The aim of this study was to characterize transgenic soybean (*Glycine max.* (L) Merr.) William 82 lines expressing *OC-I* in root nodules. These lines (L21, L22 and L23) were generated by Makgopa, (2014) and since the lines were independently transformed using agrobacterium infiltration, different responses to age induced as well as drought induced senescence were expected depending on where the transgene *(OC-I)* integrated in the specific line's genome. The aim was realized by analyzing phenotypical changes (nodule and above ground organs growth parameters), physiological alterations (accumulation of photosynthetic and protective pigments, changes in osmoprotectant production), biochemical differences (antioxidant enzymes activity, ROS production and cysteine protease activity analysis) and metabolite production (glucose, starch, and total saccharides). Transgene expression through RT-qPCR was conducted to verify differential gene expression due to different transgene incorporation.

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Chapter 2

Effects of tissue specific expression of a cysteine protease inhibitor (*Oryzacystatin I*) on age-induced senescence of Soybean (*Glycine max* (L.) Merr.) plants.

Abstract

Root nodules are specialized organs developed in mainly leguminous plant species and are a result of a symbiotic relationship with a diazotrophic bacterium, which fix nitrogen. Due to this symbiotic relationship legumes can survive in nitrogen deficient soils and rehabilitate the soils thus reducing the use of nitrogen rich fertilizers. These organs are vital in growth and development of legumes. Soybean plants suffer from nitrogen deficiency when the root nodules begin to senesce. Natural senescence of plants is defined as the degradation of cells exposed to optimal conditions as a result of aging and it is the final stage of every plant organ. Proteases play a vital role in proteolysis-associated senescence. Studies have shown an increase in cysteine protease activity in naturally senescing tissues of soybean, Arabidopsis and tobacco. The study aims to investigate the delay of nodule senescence by inhibiting the activity of cysteine proteases using a cysteine protease inhibitor (*Oryzacystatin I*). The hypothesis being that delayed senescence will alleviate nitrogen deficiency thus promote improved growth throughout the growing season. *OC-I* transgenic lines showed slightly higher growth as compared to the wild type. This was associated with the significantly high chlorophyll content and photosynthate assimilates at different levels of natural senescence progression (top, middle and bottom leaves). The transgenic lines did not have the same need as wild type plants to degrade old leaves and remobilize nutrients to reproductive growth due to increased nodule number, weight and ureide content, which indicate delayed nodule senescence. The nodules and leaves had improved antioxidant enzymes, likely to insure effective detoxification. The results suggest the involvement of *OC-I* in delaying senescence and thus possibly reducing the use of nitrogen fertilizers in crops carrying this transgene.

2.1. Introduction

Proteases play a vital role in proteolysis-associated senescence since they are responsible for protein degradation. Cysteine proteases (such as papain) are predominantly expressed in both natural and stress induced senescence. Cilliers et al., (2018) reported increased cysteine protease activity in root nodules of soybean exposed to drought. The severity of the stress resulted in more than 2-fold increase in cysteine protease activity in soybean root nodules (Marquez-Garcia et al., 2015, Cilliers et al., 2018). Naturally senescing tissues of soybean, Arabidopsis and tobacco have shown increased cysteine protease activity (Otegui et al., 2005, Martínez et al., 2008). This suggests involvement of cysteine proteases in senescence and the hypothesis that inhibitions of these proteases by their inhibitors, like *oryzacystatin I* (*OC-I*) can potentially delay senescence. *Oryzacystatin I* (*OC-I*) is a well characterized rice endosperm protease inhibitor whose activity has been associated with abiotic stress tolerance (Makgopa, 2014, Marquez-Garcia et al., 2015, Quain et al., 2015).

Root nodules are specialized organs developed in mainly leguminous plant species and are a results of a symbiotic relationship with a diazotrophic bacterium (Yoneyama and Natsume, 2010). The purpose of this relationship is to fix atmospheric nitrogen to reduce nitrogen deficiency in the host and supply symbiont bacteria with nutrients from the host (Ratcliff et al., 2008, Luo et al., 2018). Due to this symbiotic relationship legumes can survive in nitrogen deficient soils and rehabilitate the soils thus reducing the use of nitrogen rich fertilizers. Root nodules are important for most legume growth and development; however, they are highly sensitive to biotic and abiotic stress. Several studies have highlighted the response of root nodules to biotic and abiotic stress which include increasing defense, development, and repair related proteins such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione reductase (de Zélicourt et al., 2012, Baig et al., 2018, Xu et al., 2012).

Soybean (*Glycine max* (L.) Merr.) is an important legume plant originating from Asia (Sedivy et al., 2017). The crop has been domesticated due to its excellent protein content, numerous vitamins and minerals, fiber and high levels of fatty acids (Dwevedi and Kayastha, 2011). It is one of the most widely researched health-promoting and affordable food (Dwevedi and Kayastha, 2011), making it a great candidate for genetic modification to improve its nutritious value and increase tolerance to both biotic and abiotic stress. The aim of this study was to characterize the response of soybean plants expressing a cysteine protease inhibitor in root

nodules under natural/age-induced senescence. The hypothesis is that *OC-I* will delay age-induced senescence leading to prolonged nitrogen supply thus improving yield.

2.2. Materials and methods

2.2.1 Plant growth

Transgenic *Glycine max* (L.) Merr. Williams 82 seeds, transformed as described by Makgopa (2014), were surface sterilized in 100% bleach (5 minutes) and rinsed 5 times with distilled water followed by imbibition in distilled water for 3 hours. The seeds were planted 5 cm deep in wetted vermiculite contained in sandscape pots (width 300 mm, height 285 mm and thickness 2 mm). The seeds were covered with 5 g rhizobium strain WB74 (Soygro Ltd, Potchefstroom) to induce nodulation. The plants were grown in a controlled environment chamber at day and night temperatures of 28°C/20°C respectively and irradiance of 400 $mol^{-2}s^{-1}$ at 12 hours day and 12 hours night cycle. Plants were watered with distilled water every second day. Nitrogen free Hoagland solution (500 ml) was supplied to the plants once a week (Hoagland and Arnon, 1950). The solution comprised of macronutrients 1 M $MgSO_4.7H_2O$, 1 M KH_2PO_4, 1 M FeDTA, 1 M $CaCl_2.H_2O$, and 50 mM KCL. The macronutrient solution was added to a micronutrient solution consisting of 46 µM H_3BO_3, 3.9 µM $MnSO.H_2O$, 3.9 µM $ZnSO_4.7H_2O$, $CuSO_4.2H_2O$ and 0.1 µM $Na_2MoO_4.2H_2O$. The Hoagland's solution was made up to the desired volume and the pH was adjusted to 6.8 with 1M NaOH.

2.2.2 Growth Parameters

Physiological analysis was conducted at harvest (ten weeks after germination when flowering had initiated) as follows: Leaf number was recorded before the leaf samples were collected for fresh and dried weight measurements. Leaves for dry weight were stored at 75°C for two days before determining dry weights. Leaf water content was measured as the difference between the fresh and dry weight of the leaves as described by Quain et al., (2015). Nodule number and weight of crown nodules (initial nodules formed on the main root of the plant. Since these nodules are the first nodules produced, as compared to lateral nodules, they are expected to be affected by age-induced senescence earlier than lateral nodules) was recorded. Leaves from the top, middle and bottom, representing different leaf ages/developmental stage, of each plant were used for biochemical analysis.

2.2.3 Hydrogen peroxide content

Hydrogen peroxide (H_2O_2) content measurements were performed as described by Velikova et al., (2000) with minor modifications. Nodules/ leaves (100 mg) were crushed in liquid nitrogen and then mixed with five volumes of 6% (w/v) trichloroacetic acid (TCA). The samples were homogenized using a vortex and then centrifuged at 10 000 x.g for 10 min to pellet the plant material. A volume of 200 µl of the supernatant was transferred to a new microfuge tube 300 µl of 0.5% (w/v) thiobarbituric acid (TBA) was added. Standards ranging between 0 mM and 100 mM of 30% H_2O_2 (Sigma Aldrich, SA) were prepared in deionized water. In a 96 well microplate, 50 µl crude enzyme extract and the prepared standards were mixed with 1.25 mM dipotassium hydrogen phosphate (K_2HPO_4) and 250 mM potassium iodide (KI) and incubated for 20 minutes at room temperature (25°C). The plate was then read at 390 nm on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). A standard curve using results from the standards was generated and the equation of the curve was used to determine H_2O_2 content in the samples.

2.2.4 Protein content

Root nodule/leaf tissue (100 mg) was homogenized in 0.05 M Tris-HCL (pH 8). The Tris-HCL improves solubility of proteins preventing them from denaturing and being damaged. Total protein content was then analyzed using Bradford's assay because it is the most accurate procedure for determining protein concentration in a solution. Bovine Serum Albumin (BSA, Sigma-Aldrich, SA) was used as a standard (Bradford, 1976). Optical density at 595nm (OD_{595}) was measured with a BMG FLUOstar Optima microplate reader (BMG Labtech, Germany) to determine protein concentration. The equation of the standard curve was used to quantify the results from the microplate reader.

2.2.5 Papain-like protease activity

Papain-like protease activity was measured as described by Carrillo et al., (2011). Protein (5 mg) from nodule extracts were used for measuring papain-like protease activity in extracts. Reactions were individually loaded in triplicates into a black, flat-bottom 96 well plates (Nunc, AEC Amersham). Each well had 10 µl of protein sample, 82 µl of 50 mM sodium phosphate buffer (pH 6.0) containing 10 mM L-cysteine (Sigma-Aldrich, Germany) and 8 µl of 200 µg/µl substrate (Z-Phe-Arg-MCA). The substrate was added last and fluorescence development was measured with a BMG FLUOstar Optima microplate reader (BMG Labtech, Germany) at 37°C with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Reactions were

monitored over a 10 min period. The negative control reaction contained reaction buffer, substrate, and protein extraction buffer.

2.2.6 Catalase activity

Catalase (CAT) activity was determined as described by Sekmen et al., (2014). A reaction mixture containing 100 mM Tris-Cl buffer and 30% H_2O_2 was prepared in each well of a 96-well microplate. Ten microgram of extracted protein was used to start the reaction. Immediately after the addition of the protein extract, the activity of catalase was quantified with spectrophotometry on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski) at 240nm collecting readings every minute for 5 minutes. Catalase activity was then calculated as the change of absorbance at 240nm every minute. One unit of CAT was defined as the amount of enzyme that decreases 0.1 of absorbance at 240 nm per minute as described by Herzog, (1973). Fifty microlitres of Tris-Cl buffer was used as reference.

2.2.7 Glutathione-S- transferase activity

Glutathione-S-transferase was performed as described by Mannervik et al., (1985). A reaction mixture containing 1 mM glutathione (GSH, Sigma Aldrich, SA), 1 mM 1-Chloro-2,4-dinitrobenzene (CDNB) and 10 µM crude enzyme were loaded in 96 well micro plate and incubated for 3 minutes at laboratory room temperature (25°C). Following incubation absorbance was read at 340 nm on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski) to determine kinetic rate of glutathione-s-transferase. The reaction mixture containing 100 mM Tris-Cl served as a negative control. The extinction coefficient (ε) of 0.0096 $\mu M^{-1}.cm^{-1}$ was then used to calculate GST activity as indicated by the equation below:

$$GST\ activity = \frac{A_{sample\ at\ 3\ mins} - A_{sample\ at\ 0\ mins}}{\varepsilon} \ldots\ldots\ldots Eq.\ 1$$

2.2.8 Peroxidase activity

Peroxidase activity was performed as described by Miao et al., (2010). A reaction solution containing 0.2% Guaiacol (Sigma Aldrich, SA), 100 mM Tris-Cl, 30% H_2O_2 and 10µg crude enzyme was placed in a 96 well microplate. In the presence of peroxidase, the conversion of H_2O_2 to water and oxygen will result in a brown rusty compound floating in the sample as a result of guaiacol decomposition. Guaiacol acts as a positive control for the presence of POD and samples without crude enzymes were used as negative control. Tris-Cl was used as

reference. The plate was read at 470 nm. One unit of POD was defined as the amount of enzyme that increases 0.01 of absorbance at 470 nm per minute on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski).

2.2.9 Ureide assay

Root nodules (100 mg) or leaves (100 mg) were homogenized in liquid nitrogen and the powder was dissolved in 0.2 M NaOH. Allantoin standards were prepared in 0.2 M NaOH. The reaction mixture and standards were boiled at 95°C for 20 minutes to convert allontoin to allantoic acid. The presence of allantoic acid was observed by the red coloration of the samples after boiling. The negative control, consisting of all reagents except sample tissue and allantoin remained colorless after boiling. The samples were then cooled and centrifuged at 10 000 x.g to pellet plant tissue. Following centrifugation the supernatant was transferred to a clean eppendorf tubes and diluted for further analysis according to Young and Conway (1942). The diluted samples and standards were boiled with 0.5 M NaOH for another 10 minutes and thereafter combined with 0.33% phenylhydrazine (Sigma Aldrich, SA) and 0.65 M HCl in a 1:1 ratio and boiled for 2 minutes. The reaction mixes were analyzed using UV spectrophotometry measured at an absorbance of 525 nm on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). A standard curve was set up with 1, 2, 4, 6, and 8 µg of allantoin (Sigma Aldrich, SA) and the equation derived from the curve was used to calculate the ureide content.

2.2.10 Chlorophyll and carotenoid content

Pigment was extracted from soybean leaves in 100% ethanol as described by Meloni et al., (2003). Soybean leaves (100 mg) were ground in liquid nitrogen and resuspended in 10 ml 100% ethanol to keep pigment concentrated. The samples were stored at -4°C for a week under dark conditions to prevent chlorophyll degradation. Following the incubation chlorophyll content was determined by spectrophotometry on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski) as described by Arnon, 1949. Chlorophyll A, B and total chlorophyll were calculated as described in Eq.2,3 and 4 respectively. Carotenoid content was calculated as described by Zavřel et al., (2015) using Eq.5. Chl stands for chlorophyll, Ab is abbreviation for absorbance and Car stands for carotenoids. To convert the data from µg/ml to µg/ g of plant material, resultant values were divided by the mass of tissue used in pigment extraction. Absolute ethanol (100%) was used as reference to eliminate the influence of ethanol on spectrophotometry readings.

$$Chla\ (\mu g/ml) = 13{,}36\ (\text{Ab } 664\text{nm}) - 5{,}19\ (\text{Ab } 648\text{nm}) \ldots\ldots\ldots Eq.2$$
$$Chlb\ (\mu g/ml) = 27{,}43\ (\text{Ab } 648\text{nm}) - 8{,}12\ (\text{Ab } 664\text{nm}) \ldots\ldots\ldots Eq.3$$
$$Chltotal\ (\mu g/ml) = ChlAa + Chlb \ldots\ldots\ldots Eq.4$$
$$Car\ (\mu g/ml) = [1(Ab\ 470 - Ab\ 720)] - [2.86\left(\frac{Chla}{221}\right)] \ldots\ldots\ldots Eq.5$$

2.2.11 Starch and glucose determination

Starch was determined using the modified protocol of Zavřel et al. (2018). Pigments were extracted as described for chlorophyll and carotenoid content analysis (section 2.9). Samples were centrifuged at 10 000 x.g at 4°C for 20 minutes to pellet the material. The pellet was dissolved in 30% potassium hydroxide (KOH) and incubated at 95°C for 90 minutes to remove alkali-sensitive saccharides. Samples were then cooled at room temperature for 10 minutes and combined with pre-cooled (4°C) 100% ethanol. The mixture was incubated overnight at -20°C to continue the removal of alkali-sensitive saccharides. Following the overnight incubation, samples were centrifuged at 20 000 x.g at 4°C for an hour. The supernatant was discarded, and the pellet was allowed to dry under vacuum at 60°C for 1 hour. The pellet was re-suspended in 1 N hydrochloric acid (HCl). The samples were boiled at 95°C for 30 minutes to completely dissolve the pellet and hydrolyze starch. Following boiling, the samples were allowed to cool at 25°C for 10 minutes. To neutralize 1 N HCl in samples, 1 N sodium hydroxide (NaOH) was added, and samples were briefly vortexed. Samples were then centrifuged at 15 000 x.g at room temperature for 10 minutes and supernatant transferred to a clean tube and pellet discarded. In a fume hood 5% phenol was added to the samples, followed by incubation at room temperature for 15 minutes. After incubation, 96% sulfuric acid was added to the samples to hydrolyze the glucose. The samples were loaded on 96 well microtitre plate for analysis and read at 490 nm on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). A standard curve of 25 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 300 µg/ml and 500 µg/ml of D-Glucose was used to determine glucose concentration. Sulfuric acid was used as reference. For starch concentration the resultant glucose concentration was divided by the factor of 0.9 because the ratio molar mass of starch ($C_6H_{10}O_5$) and glucose ($C_6H_{12}O_6$) is 0.9.

2.2.12 Total saccharide determination

Saccharides were determined using a modified method of Zavřel et al., (2018). Pigments were extracted as described in 2.9. Samples were centrifuged at 10 000 x.g and pellet collected. The pellet was dissolved in sterile distilled water and combined with 5% phenol. The samples were further incubated at room temperature for 15 minutes. Following incubation, 200 µl 96%

sulfuric acid was added to completely hydrolyze carbohydrates and the samples were transferred to 96 well microtitre plate and read at 490 nm at PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). D-glucose, D-Fructose and D-galactose standard curves were prepared, since glucose, fructose and galactose are the most abundant saccharides in plants, as described in section 2.11 and used to analyze total saccharides in prepared samples.

2.2.13 Anthocyanin content

Anthocyanin content was determined as described by Nakata and Ohme-Takagi (2014). Five milligrams of leaf samples were measured and ground with liquid nitrogen. Five volumes (1 ml per mg) of extraction buffer (45% methanol and 5% acetic acid) were added to the ground material and mixed thoroughly. After mixing, samples were centrifuged for 5 minutes at 12 000 x.g and supernatant collected in a new tube. The supernatant was centrifuged at 12 000 x.g for a further 5 minutes to remove any plant residue. The resulting supernatant was transferred to a 96 well microtitre plate and read at 530 nm and 637 nm at PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). Anthocyanin absorbs optimum light at 530 nm and no light 637 nm, therefore the measurements were corrected from scattered light using values observed at 637 nm (Lange et al., 1971).

2.2.14 Proline content

Proline content was measured as described by Chen and Zhang, (2016). Reaction solutions of 3% sulphosalicylic, acetic acid and 2.5% acid-ninhydrin were prepared, followed by an addition of 5 µg crude protein. The solutions were then boiled at in a water bath (75°C) for 15 minutes. Following boiling, the solutions were cooled on ice for 5 minutes. The cooled solutions were poured in 96 well microtitre plate and read at an absorbance of 520 nm using a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). L-Proline standards were prepared as described in section 2.2.11 and used to estimate proline content in the prepared solutions.

2.2.15 Statistical analysis

Results are reported as the mean ± standard error (SE) values of at least three replicates. Two-way and one-way analysis of variance (ANOVA) in GraphPad Prism 6 were used to evaluate the effects of drought stress on wild type and transgenic lines. Means were compared using Tukey's test. P-values less or equal to 0.05 were considered statistically significant.

2.3. Results

2.3.1 *OC-I* expression does not confer growth constraints

Ten week old plants that have reached flowering stage were analyzed to compare if there are any growth differences between wild type and transgenic lines. There were minor visible differences, such as transgenic lines having slightly longer stems than wild type, between the wild type and transgenic lines as shown in Fig. 2.1A, and this is supported by no difference in leaf number (Fig. 2.1B). There was a trend of increased fresh and dry weight in two of the transgenic but no significant difference (Fig. 2.1C-D) and L21 and L22 show higher pigmentation (dark green leaves) as compared to the wild type and L23 (Fig. 2.1E). When looking at leaf water content, there were no statistically significant difference between wild type and transgenic lines.

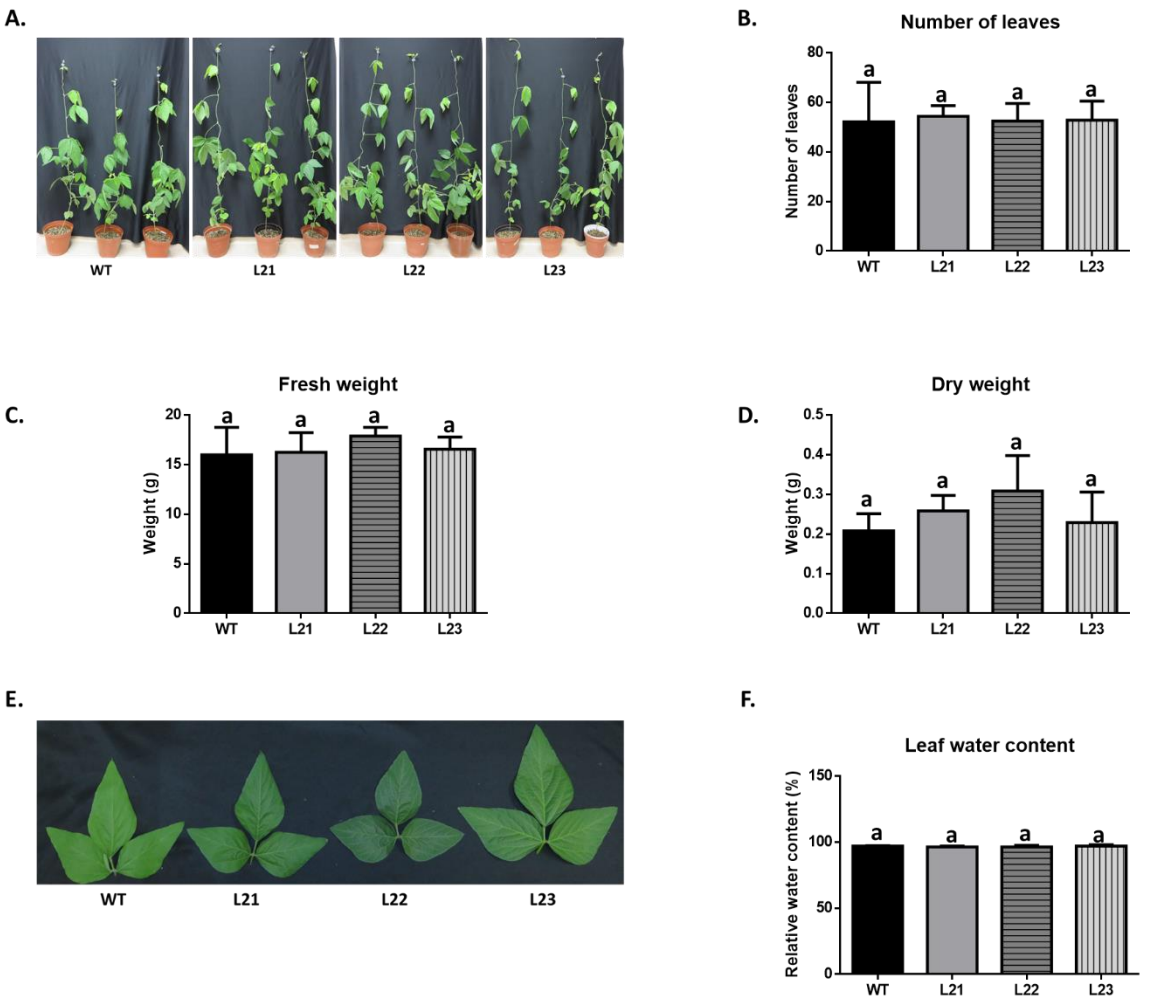

Figure 2.1: Images to compare overall plant development of different lines and wild type in the same vegetative growth stage (A). (B-D) Number of leaves were counted and fresh weight as well as dry weight of at least 6 biological replicates were measured, respectively. Leaf morphology was observed, followed by measurement of leaf water content (E-F). Error bars represent standard error (n=6). One-way ANOVA was used to test for significance at $P<0.05$.

2.3.2 Antioxidant enzyme levels in leaves change as senescence progresses

Biochemical assays were conducted on leaves at different growth stages (top, middle, and bottom) to check how the plants are responding to age induced senescence. As the leaves age catalase activity decreases more than 80% from top to bottom leaves in both wild type and transgenic lines, with L23 having the largest decreases (Fig. 2.2A). On the other hand, the opposite is observed with peroxidase activity (Fig. 2B). Transgenic lines, except L23, have the highest increase in POD activity (Fig. 2.2B) and there is a general increase in GST activity as

age induced senescence progresses in the leaves of transgenics (Fig. 2.2C), however there was not significant difference in the wild type, GST levels across leaves. Despite the transgenic lines showing high antioxidant activity in all growth stages, there is no significant difference when compared to the wild type (Fig. 2.2A-C). No statistical significance is further observed in hydrogen peroxide activity (Fig. 2.2D).

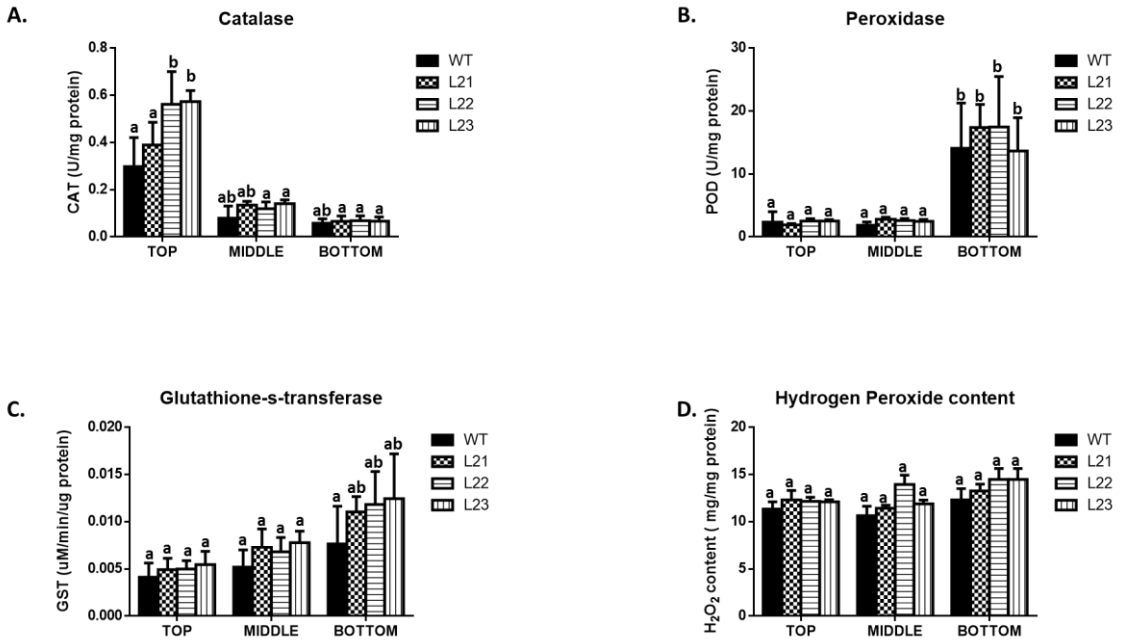

Figure 2.2: Biochemical assays were conducted to test for the antioxidant pool of leaves. A-B, Catalase and Peroxidase activity was determined to test hydrogen peroxide detoxification as observed in D. Glutathione-s- transferase was studied to quantify the detoxification of reduced glutathione. Bars with different letters are significantly different at $P<0.05$ from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 10.

2.3.3 Transgenic lines show increased Pigment and osmoprotectant production in leaves

Pigment and osmoprotectant production in leaves was analyzed to test how senescence affects leaves at different senescing stages. In all senescing stages, wild type maintained statistically significantly higher chlorophyll A content in comparison with transgenic lines, however the opposite is observed with chlorophyll B content (Fig. 2.3A-B). Despite the wild type maintaining increased chlorophyll A content, there was no statistical change in chlorophyll

content for both wild type and transgenic lines throughout senescing stages (Fig. 3A). Transgenic lines have significantly higher total chlorophyll content when compared to the wild type (Fig. 2.3C) with no significant increases in total chlorophyll content throughout different senescing stages. Throughout all the different senescing stages transgenic lines produce significantly higher protective pigment, carotene, as compared to the wild type (Fig. 2.3D). Although not statistically significant, transgenic line L21 maintained high levels of the osmoprotectant proline (Fig. 2.3E).

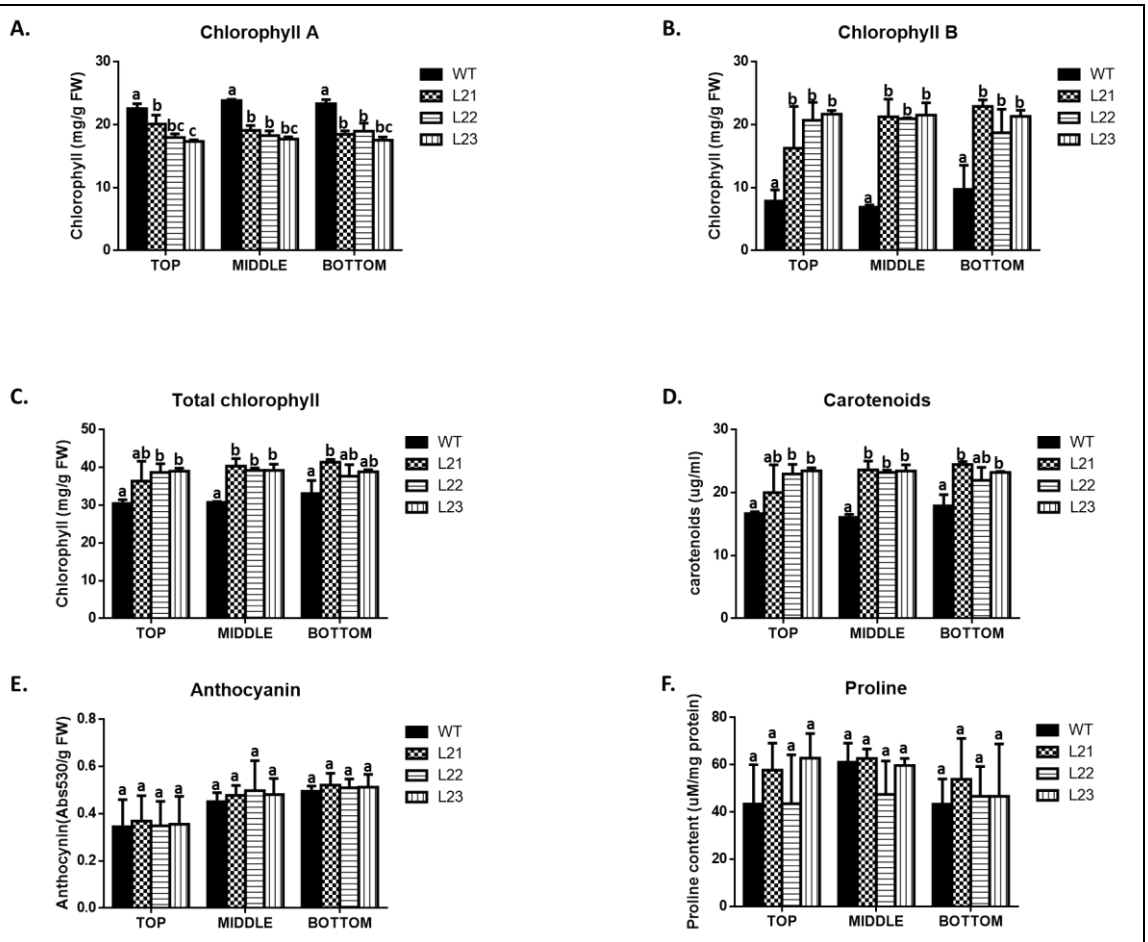

Figure 2.3: Pigment (A-E) and osmoprotectant (F) were analyzed to check how the plants respond to age induced senescence. Bars with different letters are significantly different at $P<0.05$ from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 10.

2.3.4 Improved metabolite production in leaves

Total soluble protein decreases in leaves experiencing different levels of age induced senescence with the highest protein content observed in the top leaves (Fig. 2.4A). Transgenic lines showed the highest decrease in soluble protein content (L21 with 92%, L22 with 88% and L23 with 86%) as compared to the wild type (74%) from top to bottom leaves. When analyzing starch, glucose, and carbohydrate content in leaves transgenic lines L21 and L22 behave in a similar manner while wild type and L23 show similar responses to age induced senescence.

Transgenic lines L21 and L22 had the lowest stored starch content in all growth stages (top, middle, and bottom) as compared to wild type and transgenic line L23 (Fig. 2.4B). A similar trend is observed with photosynthate production (Fig. 2.4C), however L21 and L22 show highest usable carbohydrates in all growth stages, as compared with wild type and L23 (Fig. 2.4D). Apart from top leaves of L23, transgenic lines maintain lowest ureide content in all growth stages as compared to wild type (Fig. 2.4E). Despite the statistical insignificance, the wild type showed a trend of lowest protease activity in all growth stages as compared to all transgenic lines (Fig. 2.4F).

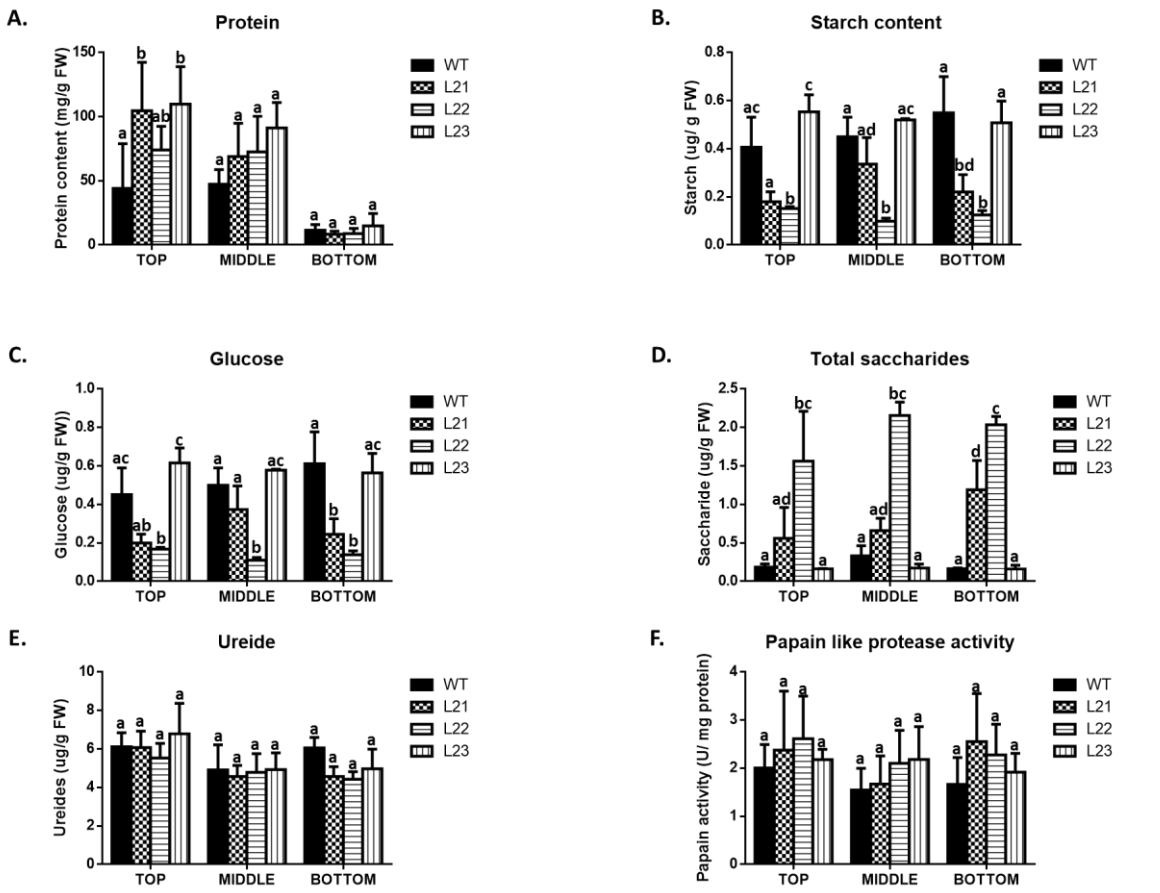

Figure 2.4: Protein content (A), stored starch (B), photosynthetic products (C), working saccharides (D), nitrogen fixation capabilities (E) and papain-like protease activity (F) was investigated to observe how these parameters are affected by aging. Bars with different letters are significantly different at P<0.05 from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 10.

2.3.5 Physiological and biochemical analysis of root nodules

Transgenic lines, except L23, have significantly higher number of nodules as compared to wild type which corresponds to increased nodule weight (Fig. 2.5A-B). Despite the low nodule number, transgenic line L23 had significantly higher nodule weight as compared to wild type nodules (Fig. 2.5B). The transgenic lines showed a trend of statistically non-significant increase in soluble protein content which correlates to the statistically significant decrease in papain-like protease activity as compared to wild type (Fig. 2.5C-D). Due to decreased protease activity transgenic line's antioxidant pool improved (Fig. 2.5E-H). Transgenic lines had increased catalase, glutathione-s-transferase (GST) and ascorbate peroxidase (APX) activity (Fig. 2.5E-G). Increased antioxidant pool provides effective detoxification which allowed

transgenic lines to exhibit a trend of statistically no significant increase in ureide content production as compared to wild type (Fig. 2.5H).

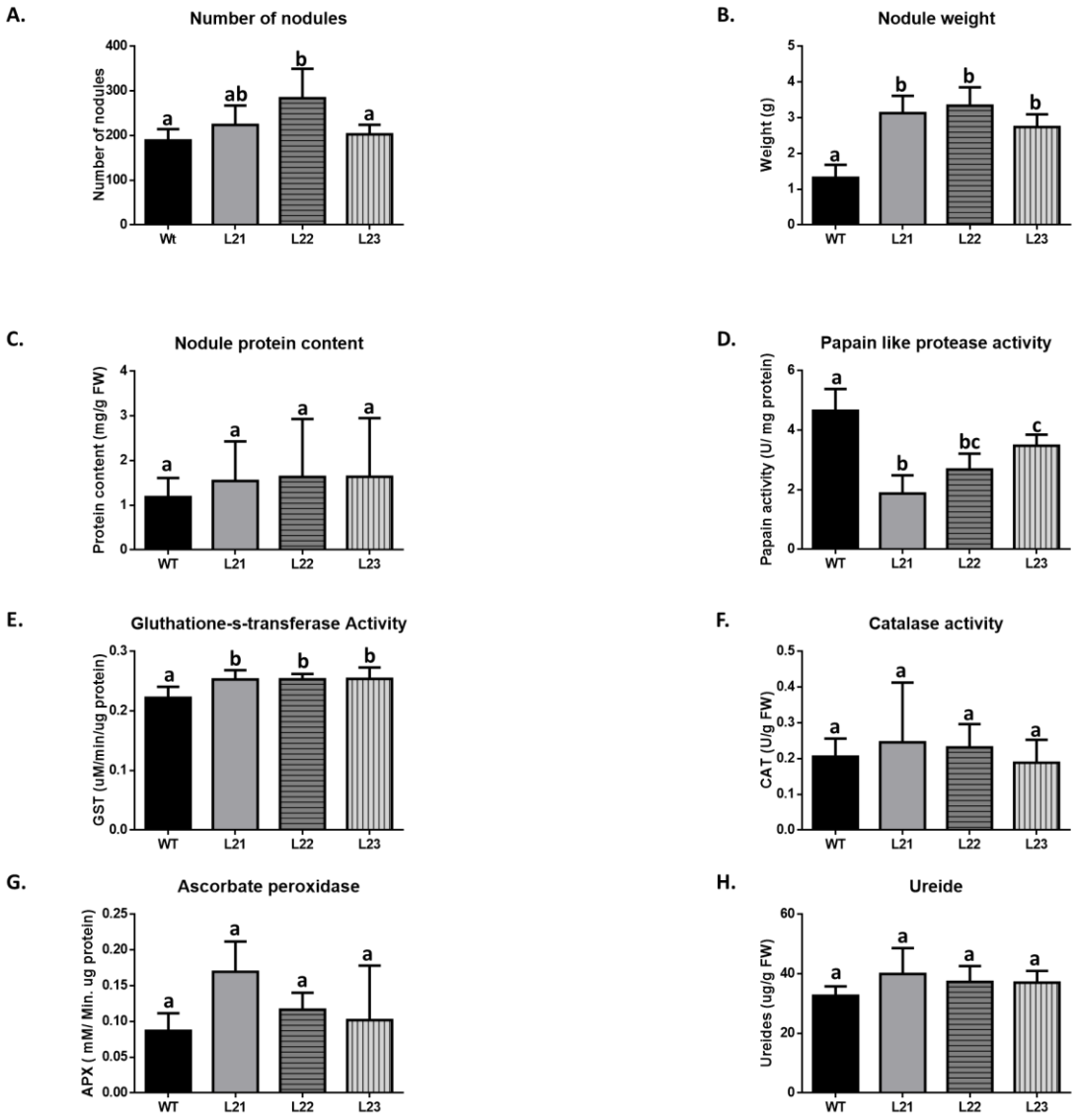

Figure 2.5: Physiological (A-B) and biochemical (C-G) analysis including nitrogen fixation abilities (H) on nodules was investigated to observe how these parameters respond to age induced nodule senescence. Bars with different letters are significantly different at $P<0.05$ from one-way ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 10.

2.4. Discussion

During natural ageing senescence there is a sequence of morphological, physiological, and biochemical processes that occur. Initial phenotypic symptoms of senescence is a loss of green color ending in yellowing under severe senescence due to loss of chlorophyll (Zhao et al., 2018). The sampled plants were in the same vegetative stage and were expected to show similar onset of age induced senescence. However, although whole plant phenotypic observations showed minimal phenotypic differences (Fig. 2.1A) closer visual inspection of the leaves revealed a lighter green color in wild type leaves as compared to transgenic line leaves (Fig. 2.1E). Yellowing of senescing leaves is correlated to total number of leaves indicating a source-sink relationship supporting degradation of old leaves to supply nutrients for the development of new leaves (Diaz et al., 2005).

Senescence can reduce the photosynthetic capacity of plants due to the degradation of proteins, pigments and membranes (Falqueto et al., 2010). Both chlorophyll A and B work as photosynthetic receptors (Khaleghi et al., 2012). The main difference is that chlorophyll A is the principal pigment associated with photosynthesis while chlorophyll B is an accessory pigment responsible for collecting energy and passing it on to chlorophyll A (Ishikita et al., 2006). Due to its primary function in photosynthesis, plants with high chlorophyll A content are expected to have improved light harvesting capability thus increased photosynthetic capacity. Transgenic lines showed significantly decreased chlorophyll A as compared to the wild type plants (Fig. 2.3A). Kura-Hotta et al., (1987) reported that decreased chlorophyll A content in rice leaves is due to senescence, which further reiterates the benefits of increased chlorophyll A content as observed in wild type plants.

However increased light harvesting capabilities is not the only requirement for successful photosynthesis. Chlorophyll B plays an important role in assembly of antenna complexes of thylakoid membrane, mobility and diffusion of membrane molecules, thermal energy dissipation and grana repair (Voitsekhovskaja and Tyutereva, 2015). The latter suggests chlorophyll B can possibly improve light usage as well as chloroplast repair during senescence. Since transgenic lines exhibit significantly higher chlorophyll B content as compared to wild type plants (Fig. 2.3B) and our findings suggest that they will show improved repair during senescence which may lead to improved photosynthesis in each growth stage. Since transgenic lines also maintained significantly increased total chlorophyll in each growth stage in relation to wild type (Fig. 2.3C) it shows balanced synthesis and repair of both chlorophyll A and B,

which can potentially increase photosynthesis. The maintenance of equilibrium in thylakoid repair to accommodate light harvesting can be related to the hypothesized nutrient source allowing transgenic lines to maintain production with delayed senescence.

Carotenoids, anthocyanins, and proline are protective piments, hormones and osmoprotectant respectively, produced by plants in response to stress. In all growth stages transgenic lines maintained the highest levels of these compounds (Fig. 2.3D-F). Carotenoids play important roles in increasing light absorption spectrum for photosynthesis and are involved in phytohormone biosynthesis (Rodrigo-Baños et al., 2015). These pigments absorb light in a spectral region (blue-green) which the sun irradiates maximally, regulate the flow of energy within the photosynthetic apparatus to protect them from photoinduced damage thus enhancing overall efficiency of the photosynthetic light reactions (Polívka and Frank, 2010, Hashimoto et al., 2016). The significant increase of carotenoids in transgenic lines suggest improved protection as well as light harvesting abilities of the generated transgenic lines (Fig. 2.3D) throughout all stages of senescence.

Anthocyanins play an important role in plant-environment interactions by delaying senescence in unfavorable conditions, protecting tissues against severe UV exposure, metal chelating and acting as an antioxidant (Nishio, 2000, Buchweitz et al., 2012, Park et al., 2012, Bi et al., 2014, Tattini et al., 2014). Ghaffari et al., (2019) reported that foliar application with proline induced anthocyanin and carotenoid accumulation. The former is due to proline's capability in safeguarding cells and their functions as well as shielding plant tissue from damages caused by ROS (Ghaffari et al., 2019). Our study supports Ghaffari et al., (2019) by showing how endogenous accumulation of proline probably helps with maintaining high levels of photosynthetic pigments (Fig. 2.3A-C), protective pigments (Fig. 2.3D-E), and antioxidative enzyme activity (Fig. 2.2A-C) in aging leaves.

Increased protective protein levels in the transgenic lines suggest an increase in soluble protein content. This suggestion was proven correct by high protein content observed in top and middle leaves of transgenic lines as compared to the wild type (Fig. 2.4A). However, this observation is different in bottom leaves. This may be due to high rate of total chlorophyll degradation in old leaves as compared to young leaves (Fig. 2.3C), because chlorophyll proteins make up more than 70% of leaf proteins (Lin and Wu, 2004). Cysteine proteases, especially papain-like proteases, are some of the most abundant proteins during leaf senescence (Carrión et al., 2013, Poret et al., 2016). This is as a result of protein remobilization. In all growth stages transgenic

lines show increased papain-like protease activity (Fig. 2.4F) suggesting increased protein remobilization to protective enzymes as well as growth and development.

Mishra et al., (2006) reported decreased fresh and dry weight in senescing tissues due to water loss in Catharanthus roseus. An opposite observation was made in transgenic Arabidopsis plants indicating that delayed leaf senescence leads to increased biomass content and constant maintenance of leaf water content (Mahmood et al., 2016). Due to the trend of increased dry biomass content and no visible water loss in transgenic lines when compared to wild type (Fig. 2.1C, D, F) it is likely an indication of delayed senescence which may be related to a possible alternate nutrient source exhibited by the generated transgenic lines. Delayed leaf senescence contributed to significant yield increases in maize since the leaves maintained a longer growth phase thus accumulating increased nutrients for partitioning to the grain (Fehr, 1984). The observation of delayed senescence in the transgenic lines suggests a possible improvement in yield due to improved partitioning of nutrients because of the longer growth phase of the leaves. However, yield would need to be tested in a separate study.

Glucose is the primary product of photosynthesis and can be incorporated in carbohydrates for transport to developing tissues, or stored as starch, which can be degraded to soluble carbohydrates during dormancy or at seed stage to maintain respiration and other cellular functioning (Regier et al., 2010). When observing these metabolites, wild type and transgenic line L23 behave in a similar manner by significantly increasing photosynthate production (glucose) and storing the glucose in a form of starch (Fig. 2.4 B-C). On the other hand, transgenic lines L21 and L22 invest in significantly higher incorporation of glucose in saccharides, which can be transported to developing tissues (Fig. 2.4D). This is a beneficial investment since the plants were harvested in flower initiation. This means the wild type and transgenic line L23 show signs of delayed flowering due to investment of metabolites in storage, while L21 and L21 have begun to invest in flowering thus preparing for pod filling.

Even though senescence is vital for the redistribution of nutrients it is a process responsible for the over production of active reactive oxygen species (Panda and Sarkar, 2013). To ensure growth, catalase and the ascorbate-glutathione cycle enzyme play an important role in the detoxification of the reactive oxygen species (Scandalios, 1997). Hydrogen peroxide (H_2O_2) and oxide (O_2^-) are among the most notorious reactive oxygen species (ROS) involved in accelerated senescence, which in turn up regulate production of antioxidative enzymes such as CAT and POD (Zhang et al., 2011).

The transgenic lines showed increased H_2O_2 in all growth stages because of partitioning to flowers (Fig. 2.2D) and this increase results in increased CAT levels (Fig. 2.2A), which was a similar observation made by Zhang et al., (2011). However, there was a significant increase in CAT activity on top leaves of transgenic lines as compared to wild type, while POD showed a trend of increased rates the bottom leaves of transgenic lines as compared to wild type (Fig. 2.2A, B). This observation is related to the affinity of CAT and POD for H_2O_2. Catalase has a lower affinity for H_2O_2 while peroxidases have a higher affinity (Anjum et al., 2016, Heck et al., 2010). This may explain why at low H_2O_2, in the top leaves we observed high CAT activity, while at high H_2O_2 in the bottom leaves, there was high POD activity (Fig. 2.2A, B).

Glutathione-s-transferases (GSTs) are a group of enzymes that play major role in detoxification of xenobiotics (foreign chemicals) as well as reactive oxygen species (ROS) and are induced by several environmental factors including senescence (Kunieda et al., 2005). This group of enzymes quench the effect of ROS by conjugation with glutathione (Kumar and Trivedi, 2018). There was a statistically significant increase in GST activity in leaves from top to bottom (Fig. 2.2C). The transgenic lines maintained a trend of high GST levels in all growth stages (Fig. 2.2C), which is correlated to tolerance to natural and stress related senescence (Dixon and Edwards, 2010, Liu et al., 2013, Csiszár et al., 2014) since GSTs reduce accumulation of ROS thus promoting delayed senescence.

Prolonged nitrogen fixation/supply is dependent on the ability of nodules to survive age related senescence. Ureides play a vital role in nitrogen utilization in legumes (Takagi et al., 2018) and an increase in ureide content is related to an increase in nitrogen utilization. Since the wild type and transgenic line L23 maintained a clear trend of high ureide content in all senescing stages of their leaves (Fig. 2.4E), it is implied that these plants have ae higher nitrogen utilization. This supports the hypothesis suggesting that the wild type and transgenic L23 senesces to help remobilize nutrients to actively growing tissues. Increased ureide content in leaves has been reported to cause negative feedback to root nodules resulting in decreased nitrogen fixation, which can further cause limited nitrogen supply to the whole plant (Serraj and Sinclair, 2003, Todd et al., 2006, Marquez-Garcia et al., 2015). Transgenic lines show a trend of increased ureide content, with L21 and L22 showing the highest levels in root nodules, indicating limited negative feedback from the leaves (Fig. 2.5H). Transgenic line L23, similar to wild type plants, show decreased ureide content in nodules as compared to the other transgenic lines supporting the feedback inhibition associated with accumulation of ureides in leaves (Fig. 2.4E,2.5H).

Decreased feedback inhibition allows prolonged nitrogen supply to leaves, thus eliminating the need for the plants to senesce to remobilize nutrients to actively growing tissues.

Another factor directly correlated to nitrogen fixation is nodule weight. Nodules with greater weights offer bigger surface areas for efficient nitrogen fixation (King and Purcell, 2001). Transgenic lines are able to support increased nitrogen fixation as compared to wild type due to greater nodule weight as well increased number of high weighing nodules (Fig. 2.5A-B). This prolonged nitrogen supply, in a form of ureides, in transgenic lines is associated with delayed senescence observed by physiological and biochemical attributes studied in the leaves.

Root nodules are a site of intense biochemical activity thus making them susceptible to damage from ROS as well as reactive nitrogen species (RNS) (Becana et al., 2010). This high capacity of ROS and RNS requires high antioxidant defenses such as antioxidant enzymes GST, APX and CAT (Dalton et al., 2009). Transgenic lines show increased GST activity with relation to the wild type indicating effective detoxification (Fig. 2.5E). Apart from H_2O_2 detoxification, catalase activity is positively correlated to nitrogen fixation in root nodules (Ali et al., 2016, Francis and Alexander, 1972). Transgenic lines L21 and L22 show a trend of the highest catalase activity as compared to wild type and transgenic line L23 (Fig. 2.5F) indicating possible efficient nitrogen fixation. Catalase activity in transgenic line L23 and wild type reaffirms the effect of feedback inhibition of nitrogen between root nodules and shoots.

Ascorbate peroxidases (APX) play a key role in the ascorbate-glutathione cycle for the detoxification of H_2O_2 (Caverzan et al., 2012). Transgenic lines exhibit a trend of increased APX activity as compared to the wild type suggesting efficient ROS removal (Fig. 2.5G). With regards to the antioxidant enzymes studied, transgenic lines exhibit increased antioxidant pool as compared to the wild type, which can be related to the incline in increased protein content (Fig. 2.5C) as observed in leaves. Transgenic line's nodules are able to detoxify oxidative species and delay senescence due to the inhibition of cysteine proteases, specifically papain-like proteases (Fig. 2.5D). Papain-like proteases play key roles in nodule senescence by decreasing bacterial symbiont infection process as well as lifespan of nodules (Van Wyk et al., 2014, Yuan et al., 2016, Yuan et al., 2017). This delay in nodule senescence leads to prolonged nitrogen supply to shoots thus delaying proteolysis-associated senescence of leaves for nutrient remobilization.

The generated transgenic lines show different levels of senescence delay, with L23 showing most physiological and biochemical changes similar to the wild type. This observation is due

to independent transformation of the transgenic lines which allows for different incorporation of the gene within the plant genome. Depending on where the transgene is incorporated, it will be differentially expressed in different transgenic lines. Chapter 3 will explore *OCI* expression levels in the root nodules of the transgenic lines to investigate how differentially expression affects response to senescence.

2.5. Conclusion

Tissue specific expression of *Oryzacystatin I* delays nodule senescence and prolongs nitrogen fixation in nodules and nitrogen supply to shoot, which can lead to improved yield even under normal environmental conditions. The mobilization of nitrogen is likely achieved by the inhibition of papain-like proteases in the nodules resulting in decreased protein degradation as a consequence of age-related senescence. This leads to nodules showing decreased ROS (H_2O_2) production and an improved antioxidant enzyme (GST), which results in improved efficiency in nitrogen fixation measured as ureide content. The delay in nodule senescence then prolongs nitrogen supply to shoots reducing the requirement of leaves to degrade to remobilize nutrients to actively growing tissues. This then delays leaf senescence by increasing antioxidation (GST) resulting in improved photosynthetic capacity (total chlorophyll content) which can maintain increased nitrogen supply while maintaining active growth and partitioning to reproductive growth.

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Chapter 3

Effects of expressing a cysteine protease inhibitor (*Oryzacystatin I*) in root nodules on drought tolerance of (*Glycine max* (L.) Merr.)

Abstract

Environmental sustainability and food security are among the major global challenges. Excess use of fertilizers and urbanization significantly reduces suitable environments for agricultural purposes. Biotic and abiotic stress accounts to more than 50% yield losses worldwide. Drought being one of the major limiting factors in yield due to its ability to increase protease activity. This poses a threat to food security especially in water poor countries such as South Africa. This research aims to assess the genetic improvement conferred by delaying nodule senescence and its effects on growth and development of a high protein and environmentally friendly crop, soybean, under drought conditions by delaying nodule senescence. The working hypothesis was that the expression of *oryzacystatin I* (*OC-I*) in root nodules will decrease papain-like protease activity leading to delayed nodule senescence and increased nitrogen supply to shoots under drought conditions. Physiological and biochemical parameters were analyzed to test this hypothesis. Transgenic lines maintained a higher biomass, showed reduced tissue damage, and improved nodule mass when compared to the wild type under drought conditions. The transgenic lines also had significantly higher antioxidant pools and reduced reactive oxygen species indicating effective detoxification under both normal and drought conditions. The root nodules of the transgenic lines continued to display significantly higher ureide content than the wild type under drought conditions. Taken together, these results suggest that *OC-I* plays an important role in conferring drought tolerance in soybean by delaying drought-induced nodule senescence.

3.1. Introduction

Biological plant stress is the alteration of morphology, physiology, biochemistry and genetics in plants as a response to unfavorable environments (Lichtenthaler, 1996). Plant growth and productivity is severely affected by biotic and abiotic stress (Jaleel et al., 2009). Drought is one of the major limiting stressors in plant production (Farooq et al., 2012). Drought is a meteorological phenomenon defined by limited rainfall resulting in below average productivity of the affected area (Bullock et al., 2017). Drought stress in plants is considered as moderate water loss as a result of transpiration, which further leads to stomatal closure and decreased gaseous exchange (Jaleel et al., 2009, Farooq et al., 2012). Underground plant organs such as roots and root nodules are the first sensors for drought stress and thus play a vital role in studying drought stress sensitivity/tolerance (Kunert et al., 2016).

Biological nitrogen fixation is the conversion of atmospheric nitrogen (N) to ammonia (NH_3) which can be used by plants for assimilation into amino acids (Lam et al., 1996). This biological process is predominantly found in bacterial species. There are bacterial species such as *Rhizobia* that can form symbiotic relationships with leguminous plants to fix nitrogen (Mus et al., 2016). This process is important in plant production and reduces the costs associated with applying nitrogen fertilizers. Drought stress affects this symbiotic nitrogen fixation by decreasing the concentration of *Rhizobia* in the soil. The decrease in bacterial concentration results in limited nodulation, dissociation of nodules, which causes a decrease in nitrogen supply to shoots and forces senescence of leaves for nutrient remobilization (Fenta et al., 2014, Cilliers et al., 2018). Premature nodule degradation as a result of drought has been associated with an increase in cysteine proteases (Cilliers et al., 2018).

Cysteine proteases are proteolytic enzymes involved in the hydrolyses of peptide bonds of the substrate by cysteine residue on their active site (Corvo et al., 2018). Plant cysteine protease activity of these enzymes has been associated with seed germination, senescence and defense (Martínez et al., 2012). Papain-like proteases are the most abundant cysteine proteases and specifically upregulated when senescence is initiated (Liu et al., 2018, Yuan et al., 2020). The inhibition of papain-like proteases can potentially delay nodule senescence. Cysteine proteases are the natural inhibitors of papain-like proteases (Martínez et al., 2012). The well characterized rice cysteine protease inhibitor, *Oryzacystatin I* (*OC-I*), has shown effective activity against papain-like protease across plant species (Masoud et al., 1993, Premachandran et al., 2021). Ectopic expression of *OC-I* has been shown to improve drought tolerance in

soybean and tobacco plants, however reduced growth under normal conditions was also observed in the transgenic lines (Demirevska et al., 2010, Quain et al., 2015).

The aim of this study was to characterize transgenic soybean (*Glycine max* (L.) Merr. Williams 82) *oryzacystatin I* (*OC-I*) lines under the control of a soybean nodule specific promoter (leghemoglobin promoter) under well-watered and drought conditions. The nodule specific promoter (leghemoglobin promoter) has been previously characterized to be expressed specifically in root nodules (Carvalho et al., 2003). The objectives included physiological and biochemical analysis of transgenic lines as compared to the wild type under the two conditions. The hypothesis was that *OC-I* will prevent drought induced premature nodule senescence leading to prolonged nitrogen supply in unfavorable conditions.

3.2. Materials and methods

3.2.1. Plant growth and analysis of growth parameters

Plants were grown as described in section 2.2.1. For drought treatment, the pots were covered with clear plastic bags to ensure that water is only lost through transpiration. Drought treatment was started at 4 weeks because that is when nodulation is initiated. The plants were subjected to drought conditions for 20 days when the soil had reached 50% drought and plants were showing wilting phenotype. Growth parameters were measured as described in section 2.2.2.

3.2.2. Cell viability

Cell viability was performed as described by Vijayaraghavareddy et al., (2017). For microscopic analysis soybean leaves were cut with into 10 discs with borer (1 cm diameter) on opposite sides of the main vein. The discs were placed in petri plates and submerged in Evans blue dye and left to shake in an orbital shaker (Hybaid shaker model HB-SHK 1, ThermoFisher Scientific South Africa) at 75 revolutions per minute for 20 minutes at room temperature. This allows the dye to penetrate the leaf discs equally. Following shaking, the leaf discs were washed with distilled water five times until unbound dye washed out. The discs were then analysed by light microscope (Zeiss AXIO Imager.M2). Whole leaf tissue damage was assessed by staining as described for leaf discs and to quantify staining at the whole leaf level Evans blue dye was extracted from the leaves by grinding 100 mg of stained leaf tissue in 1% Sodium dodecyl sulfate (SDS). SDS acts as a lysis buffer to break down cell membranes allowing the dye to be exposed. Samples were then centrifuged at 10000 x.g for five minutes. The supernatant was transferred into a 96 well microtitre plate and read on a

PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski) at 600 nm. Evans blue standards ranging from 0 ng to 100 µg were used to determine the concentration of dye present in samples. A standard curve using results from the standards was generated in excel and the equation of the curve was used to determine the extent of which the dye penetrated the samples.

3.2.3. Determination of lipid peroxidation

Lipid peroxidation was determined as previously described by Zhang et al., (2007). Soybean leaves (100 mg) were ground in liquid nitrogen and transferred to Eppendorf tubes. Five volumes of 6% (w/v) Trichloroacetic acid (TCA) as added to the Eppendorf tubes. The samples were homogenized using a vortex and then centrifuged at 10 000 x.g for 10 minutes to pellet the plant material. The supernatant (200 µl) was transferred to new Eppendorf tubes followed by the addition of 300 µl of 0,5% (w/v) Thiobarbituric acid (TBA). The samples were mixed by brief vortexing. A small hole was punched on the Eppendorf lids to prevent the lids from opening under high temperature incubation. The sample tubes were placed in a heating block and was allowed to incubate at 90 °C for 20 minutes. Following the 20 minutes incubation, the samples were incubated on ice for 10 minutes. Samples were then centrifuged at 10 000 x.g for five minutes to pellet plant material. All extracted samples were then loaded in triplicates (technical replicates) on a 96-well microtitre plate. The plate was then read at 532 nm and 600 nm on a PowerWave™ microplate spectrophotometer (BioTek® instruments, INC, Winooski). The absorbance at 600 nm was subtracted from the absorbance at 532 nm to correct for the non-specific turbidity. Lipid peroxidation was determined by measuring activity of malondialdehyde (MDA) using spectrophotometry applying the extinction coefficient of 155 $Mm.cm^{-1}$.

3.2.4. Antioxidant assays

Hydrogen peroxide content, Catalase, Glutathione-S-trasferase and Peroxidase were analyzed as described in sections 2.2.4, 2.2.6, 2.2.7, 2.2.8 respectively.

3.2.5. Protein, pigment and ureide content

Protein content, ureide assay, chlorophyll content and proline content were examined as described in sections 2.2.4, 2.2.9, 2.2.10 and 2.2.14 respectively.

3.2.6. RNA extraction and cDNA synthesis

Root nodules (100 mg) were ground in liquid nitrogen and RNA extracted following manufacturer's instructions using the Plant RNA Mini Kit (Zymo Research, USA). The RNA quantity and quality were determined using nanodrop (Nanodrop ™ Thermo Scientific,

USA). Pure RNA was then used for first strand cDNA synthesis using hexamer primers (Thermo Scientific). To detect *OC-I* expression, 2X SYBR green (Bio-Rad, UK) with solution concentrations as suggested by manufacturer's instructions. The 40s ribosomal S8 gene was used as a reference gene: Forward primer 5' GCC AGC CTG CTA ACA CTA AG 3' and Reverse primer 5' AAG AGT CTG AGT ACG CAC AAG 3' (GenBank accession TC100533). Reactions were carried out in a real time thermo cycler (Bio-Rad, UK) with *OC-I* gene specific primers Forward primer 5' TCA CCC AGC AAC AAG AAG 3' and Reverse primer 5'GCA TCG ACA GGC TTG AAC T 3' set to conditions 95°C for 5 min followed by 29 cycles: 95°C for 30 s; 60°C for 30 s; 72°C for 30 s and an addition 5 min at 72°C in a CFX96 Touch™ Real-Time PCR Detection System using a 96-well Clear Multiplate PCR Plates (Bio-Rad, California). The relative expression of *OC-I* was determined by comparing Cq values of target gene (*OC-I*) to that of a reference gene (40S) as described by Livak & Schmittgen, (2001). Quantifications were based on the fold-change in gene expression normalized to an endogenous reference gene and relative to the untreated control. Calculations were carried out with the $2^{-\Delta\Delta Cq}$ method using three independent replicates (Livak and Schmittgen, 2001).

3.2.7. Papain-like protease activity

Papain-like protease activity was measured as described in section 2.2.5.

3.2.8. Statistical analysis

Results are reported as the mean ± standard error (SE) values of at least three biological replicates. Two-way analysis of variance (ANOVA) in GraphPad Prism 6 was used to evaluate the effects of drought stress on wild type and transgenic lines. Means were compared using Tukey's test. P-values less than or equal to 0.05 were considered statistically significant.

3.3. Results

3.3.1. Delayed nodule senescence improves growth of soybean leaves and *OC-I* expression decreases papain-like protease activity in root nodules

To assess the effect of nodule expression of *OC-I*, data on plant phenology, leaf number, fresh weight, relative water content and chlorophyll content was collected. Fig. 3.1A shows phenotypical development of the wild-type and transgenic lines at six weeks of well-watered and 20 days of drought conditions. There was visible stunted growth in plants exposed to drought conditions as compared to those under well-watered conditions, with minimal differences between wild type and transgenic lines. There was however significantly decreased papain-like protease activity in wild-type plants as compared to transgenic plants

under drought conditions and no significant differences under well-watered conditions (Fig. 3.1B). Under drought conditions, the wild type had an average of 50% higher papain-like protease activity as compared to the transgenic lines which is 86% higher than all plants assessed in well-watered conditions. The increase in papain-like protease activity in the wild type is related to significantly lower protease inhibitor expression in these plants as compared to transgenic lines (Fig. 3.1C). Transgenic lines L21 and L22 showed a significant increase of 2-fold in *O-CI* expression under drought conditions, which likely resulted in lower papain-like protease activity in these plants. Differences in expression levels of *OC-I* in transgenic lines is likely due to where the gene was inserted in the plant's genome.

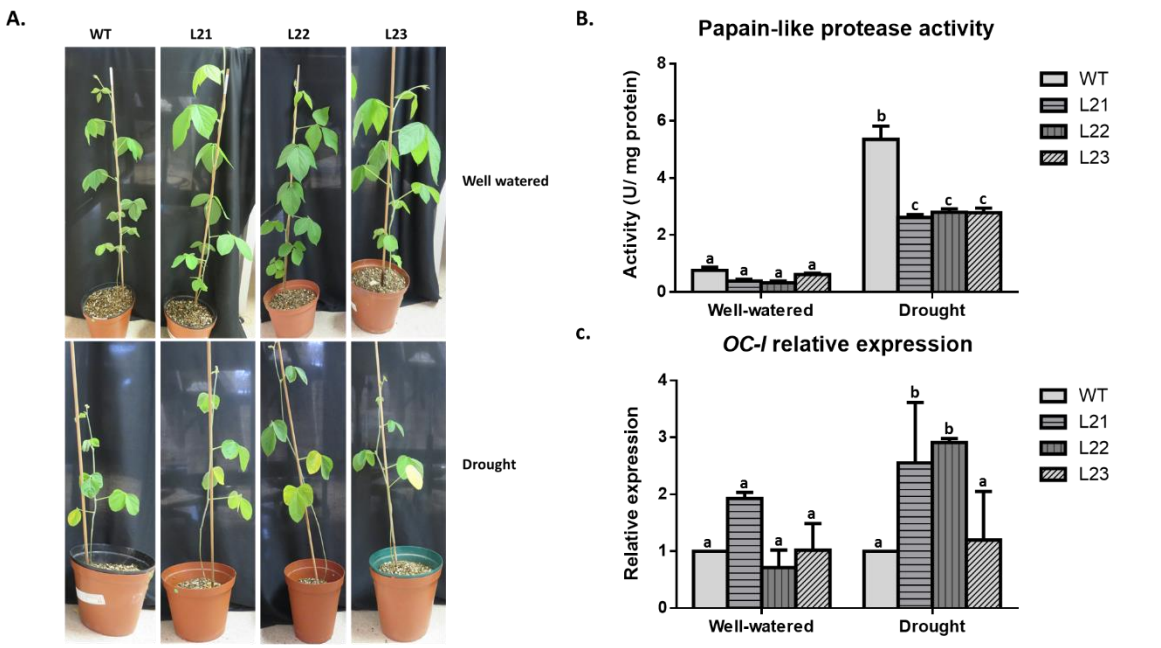

Figure 3.1: (A) Phenological assessment of six weeks old soybean plants and (B) papain-like protease activity observed and (C) relative expression of *OC-I* in root nodules of the plants subjected to well-watered and drought conditions for 20 days. Bars with different letters are significantly different at $P<0.05$ from two-way ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 12.

Transgenic lines L21, L22 and L23 had no significant differences in leaf number as compared to the wild type under well-watered conditions (Fig. 3.2A). However, under drought conditions, both the transgenic lines and the wild type showed a decrease in leaf number with wild type having the largest decrease (60%) when compared to L21 (25%), L22 (41%) and L23 (27%) decreases showed by transgenic lines (Fig. 3.2A). All transgenic lines except L22, had a

significantly higher number of leaves under drought conditions than wild type plants (Fig. 3.2A). Between the transgenic lines, there was no significant difference in the number of leaves under drought conditions (Fig. 3.2A).

In well-watered conditions transgenic lines L22 and L23 maintained a significantly higher fresh weight as compared to wild type plants and L21 plants (Fig. 3.2B). Although L22 had a larger decrease of fresh weight (88%) under drought conditions compared to the well-watered plants in relation to 85%, 80% and 86% of wild type, L21 and L23 respectively, there is no significant differences. There was a trend of all transgenic lines maintaining a slightly higher fresh weight as compared to the wild type under drought conditions (Fig. 3.2B). There was no significant difference in relative water content between transgenic lines and wild type under well-watered conditions (Fig. 3.2C). Under drought conditions the transgenic lines maintained a significantly higher level of relative water content (30%, 23 % and 23% of L21, L22 and L23 respectively) when compared to wild type plants (Fig. 3.2C). Wild type had the largest decrease in relative water content when comparing well-watered and drought conditions (40%) and in comparison, to L21 (18%0, L22 (26%) and L23 (24%) under drought conditions.

Transgenic lines had significantly higher level of chlorophyll content when compared to wild type under well-watered and drought conditions (Fig. 3.2D). Under drought conditions transgenic lines had significantly higher chlorophyll content (between 40 and 80 µg/g) as compared to wild type at approximately 20 µg/g. When comparing between well-watered and drought conditions, the wild type had the largest decrease in chlorophyll content (49%) as compared to L21 (38%), L22 (35%) and L23 (6%) decreases shown in the transgenic lines (Fig. 3.2D). This further supports observation that transgenic lines maintain physiological functions under drought conditions that are like the physiological functions of these plants under well-watered conditions.

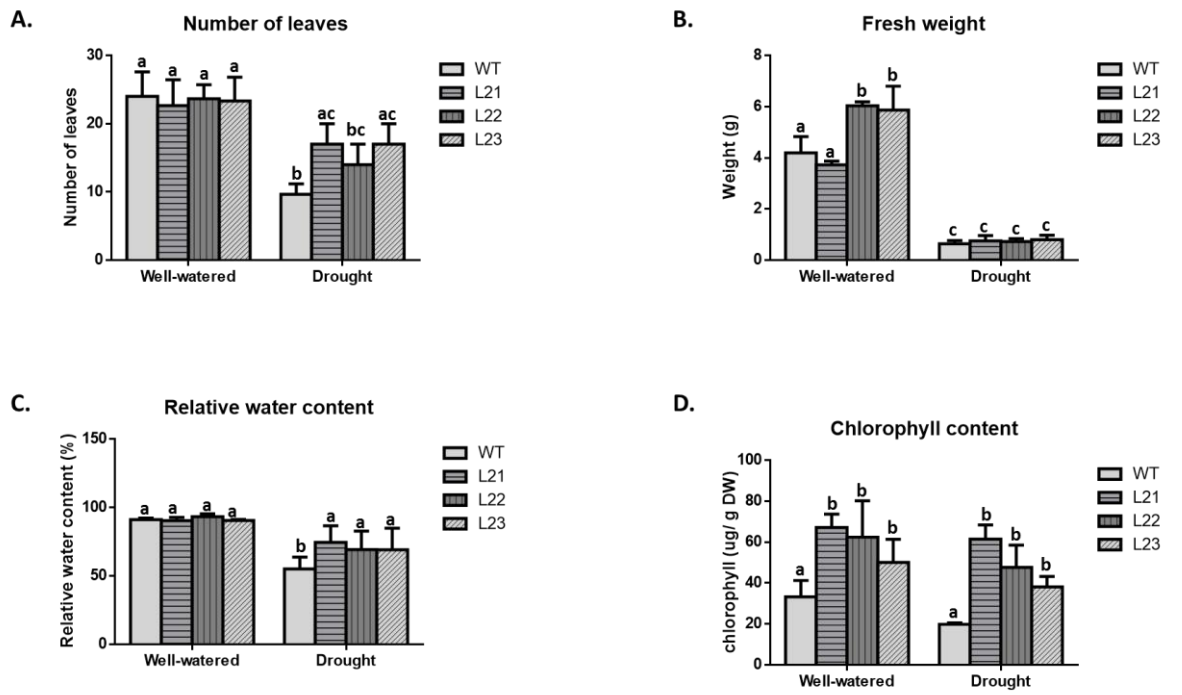

Figure 3.2: A. Number of leaves for 6 weeks old plants exposed to well-watered and drought conditions, B. Fresh weight of harvested leaf material, C. Relative water content estimated from the fresh and dry biomass and D. Chlorophyll content of soybean leaves under well-watered and drought conditions. Bars with different letters are significantly different at $P<0.05$ from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 12.

3.3.2. Expression of *OC-I* in root nodules improves leaf cell viability under drought conditions

Microscopic images indicated minimum staining in wild type and transgenic lines under well-watered conditions (Fig. 3.3A). Excessive staining was observed in the leaf discs of wild type plants under drought conditions, while transgenic lines showed limited tissue damage and staining (Fig. 3.3A). Evans blue spectrophotometric quantification indicate no significant difference in staining under well-watered conditions with a trend of transgenic lines having slightly less stain when compared to wild type (Fig. 3.3B). Under drought conditions transgenic lines have significantly less staining, an average of 50%, as compared to wild type (Fig. 3.3B). The wild type plants showed more damaged tissue with 81% of the tissue penetrated by Evans blue solution. Penetration of Evans blue solution was significantly lower in transgenics at 77%

for L21, 79% for L22, and 76% for L23 when comparing well-watered and drought conditions (Fig. 3.3B).

Under well-watered conditions there is no significant difference of MDA (malondialdehyde) concentration between wild type and transgenic lines, however, there does appear to be a slight trend towards lower MDA concentrations in the transgenic plants (Fig. 3.3C). The difference in MDA concentrations is more pronounced under drought conditions with transgenic lines L22 and L23 maintaining a significantly lower concentration when compared to wild type (Fig. 3.3C).

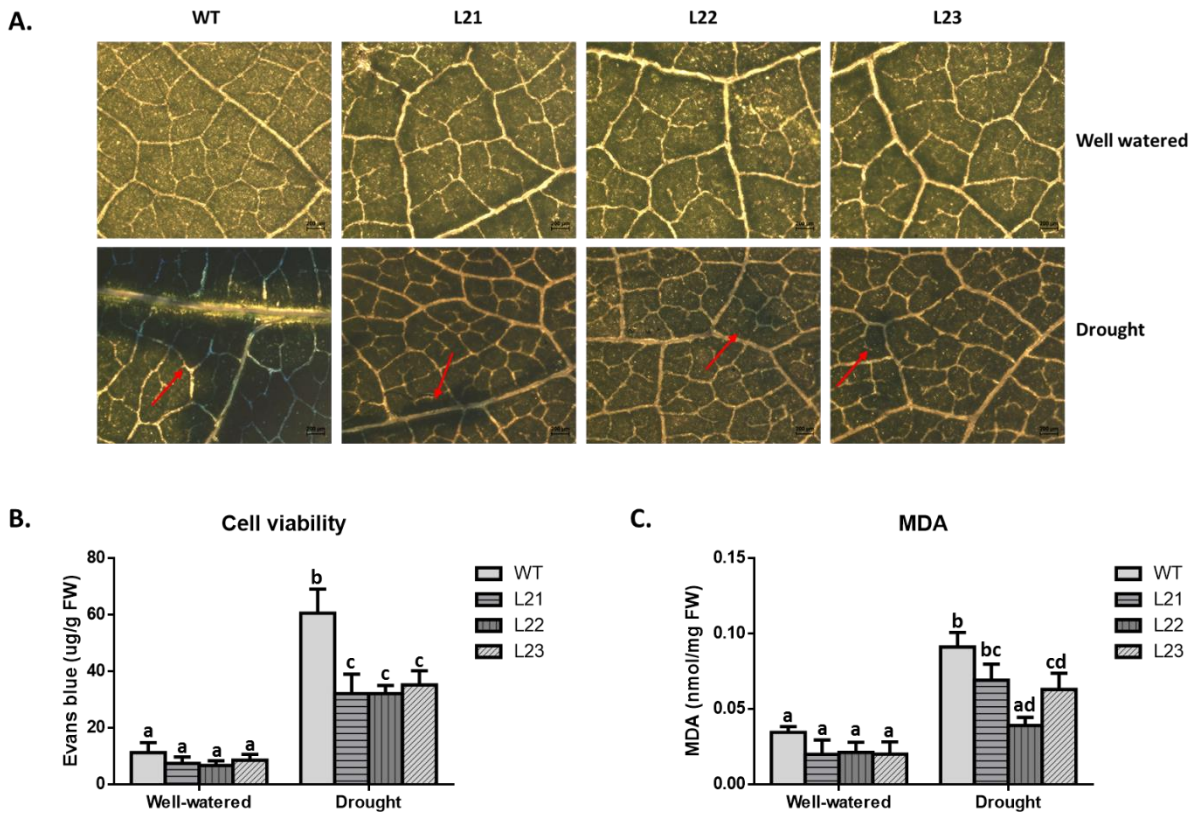

Figure 3.3: A. Light microscope images of Evans blue stained leaf disks. B. Spectrophotometric quantification of Evans blue stain of whole leaves. Arrows on the bottom panel of A point to the stain indicating tissue damage. D. Malondialdehyde (MDA) measurement in leaves. Bars with different letters are significantly different at P<0.05 from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 12.

3.3.3. Physiological and nitrogen fixing properties of root nodules under drought conditions were improved

Physiology of the root nodules was investigated to determine whether *OC-I* has any physiological effects on the nodules and if nodule specific increased gene expression could improve nitrogen fixation. Transgenic lines had higher nodule number when compared to wild type under well-watered conditions and L21 and L23 maintained the highest nodule number under drought conditions (Fig. 3.4A). Transgenic lines have significantly higher nodule biomass under well-watered conditions and there were no significant differences in nodule biomass under drought conditions (Fig. 3.4B).

Under well-watered conditions all nodules exhibit the characteristic pink color indicating nitrogen fixation and wild type losses the characteristic pink color under drought, whereas the transgenic lines maintain the color even through the drought treatment (Fig. 3.4C). Transgenic lines L21 and L22 have significantly higher levels of ureides under drought conditions indicating higher nitrogen fixation and transport as compared to wild type (Fig. 3.4D). The ureide content results correlate with the pink color observed under drought conditions. Transgenic lines L21 and L22 showed a darker pink color as compared to wild type and L23 indicating significantly higher nitrogen fixation under drought conditions.

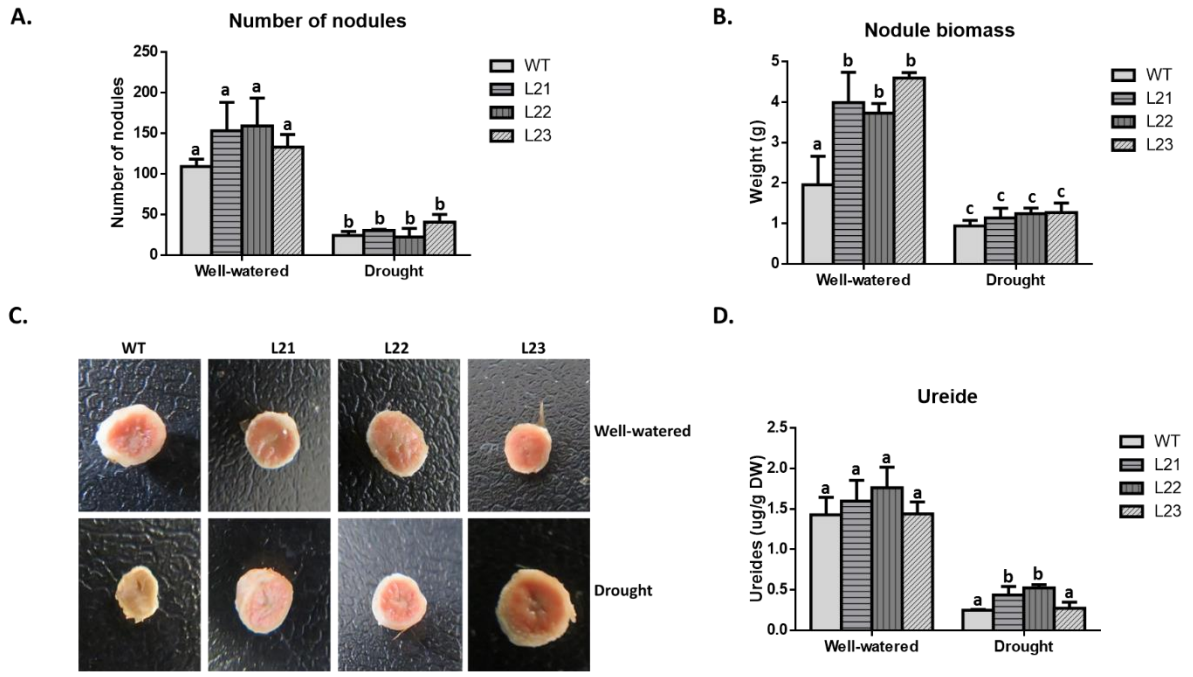

Figure 3.4: A. Number of root nodules counted from each harvested plant, B. Overall biomass of nodules per plant harvested, C. Vertically dissected nodules to observe the characteristic pink color and D. Ureide content from harvested nodules. Bars with different letters are significantly different at $P<0.05$ from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 12.

3.3.4. Improved antioxidant pool in root nodules

The transgene (*OC-I*) was expressed under the control of a root nodule-specific promoter (Leghemoglobin promoter) therefore it was important investigate the effect of increased *OC-I* expression in nodules, by investigating enzyme functions that are related to the drought stress response. Under well-watered and drought conditions transgenic lines appear to have a slightly

lower level of total protein content, which was more pronounced under drought conditions, with a significant difference observed between transgenic and wild type plants (Fig. 3.5A). When comparing each line to their well-watered counterpart, transgenic line 22 had the highest increase of protein content (88%) as compared to the observed 87% (Wild type), 86% (L21) and 81% (L23) observed (Fig. 3.5A).

Testing catalase, peroxidase, glutathione-S-transferase and proline provide us with insight into the antioxidative and osmotic responses of the transgenic lines and wild type to drought, with reference to well-watered conditions. Under well-watered conditions transgenic lines appear to have a decreased (but not significant) hydrogen peroxide content as compared to the wild type (Fig. 3.5B). Drought significantly increased hydrogen peroxide content in wild type plants as compared to the transgenic lines (Fig. 3.5B). Although not statistically significant, transgenic lines also displayed elevated peroxidase activity as compared to the wild type under well-watered conditions and this trend was also observed under drought conditions. Transgenic line L23 showed significantly higher peroxidase under drought conditions as compared to wild type (Fig. 3.5C). When comparing each line to their well-watered counterpart, wild type has the highest (92%) increase in peroxidase activity as compared to the observed 81%, 81% and 89% increases observed in L21, L22 and L23, respectively, indicating increased stress resulting in increased antioxidant response.

There was no significant difference in catalase activity between the transgenic lines and wild type plants when tested under well-watered conditions lines (Fig. 3.5D). The transgenic lines displayed a slightly lower catalase activity as compared to wild type under drought conditions (Fig. 3.5D). Wild type plants had the highest (89%) increase of catalase activity as compared to 87% (L21), 79% (L22) and 82% (L23) observed between the tested treatments. Under well-watered conditions glutathione-S-transferase activity of wild type was not significantly lower than that of the transgenic lines, but L22 and L23 suggest a trend toward higher GST activity (Fig. 3.5E). Transgenic lines showed a lower GST activity under drought conditions than wild type plants. The wildtype plants had the highest increase (33%) when compared to 30%, 15% and 19% increases exhibited by L21, L22 and L23, respectively between treatments in related lines. Increased proline content results in increased osmoprotection in the plants. Wild type has lowest proline content as compared to the transgenic lines under both well-watered and drought conditions, with L21 and L22 having significantly higher proline content under drought conditions as compared to wild type and L23 indicating improved osmoprotection (Fig. 3.5F).

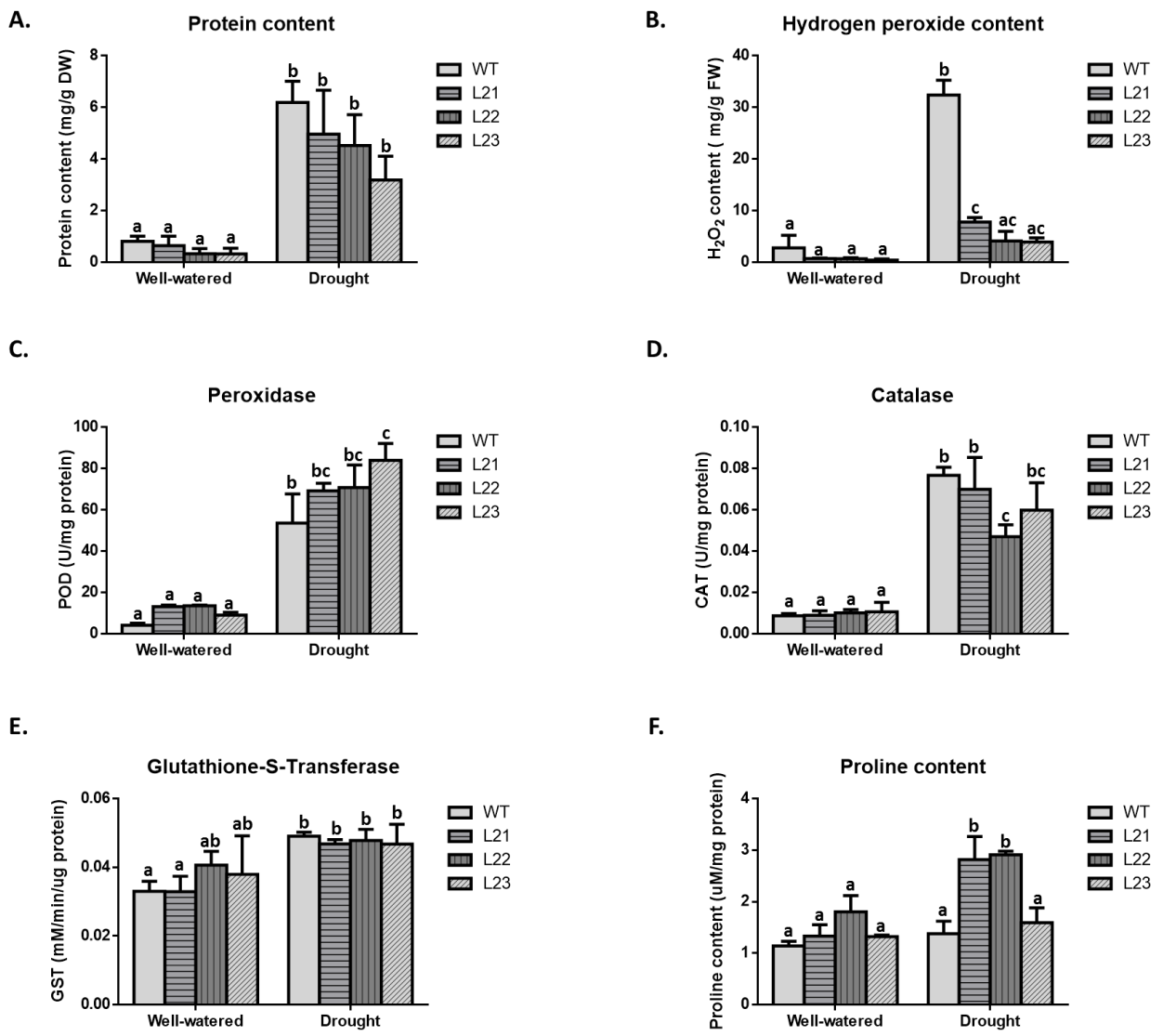

Figure 3.5: A. Protein content, B. Hydrogen peroxide content, C. Peroxidase activity, C. Catalase activity, Glutathione-S-transferase activity and F. Proline content extracted from harvested nodules. Bars with different letters are significantly different at P<0.05 from two-way (between and within treatments and lines) ANOVA. Four columns in a block (WT and three transgenic lines) were analyzed for significant differences. Error bars represent standard error n= 12.

3.4. Discussion

Oryzacystatin I (*OC-I*) is a rice protease inhibitor responsible for the inhibition of cysteine proteases (Cingel et al., 2017). Increased cysteine protease expression is known to be induced by drought conditions resulting in senescence (Cilliers et al., 2018, Gomez-Sanchez et al., 2019). To delay senescence and prolong nitrogen supply, by the inhibition of senescence associated with cysteine proteases, transgenic soybean plants expressing *OC-I* in root nodules

were generated by Makgopa, (2014) and further characterized in this study. No significant phenotypical differences were observed in the transgenic plants under both well-watered and drought conditions suggesting no visible tolerance to drought stress (Fig. 3.1A). In all the studied phenological, physiological and biochemical processes the transgenic lines behave differently between each other and the wild type. This is likely due to the agrobacterium based independent transformation with the transgene used to generate each line. Independent transformation leads to the different lines incorporating the transgene in different regions of the genome, which may be euchromatin, where the gene is constitutively expressed, or heterochromatin, where gene expression is limited (Babu and Verma, 1987). As indicated in Fig. 3.1C, transgenic lines have different levels of gene expression indicating different locus integration of the transgene in the different lines. Since transgenic lines L21 and L22 have the highest *OC-I* expression as well as improved physiological and biochemical responses to drought, it appears likely that the gene was inserted in a suitable region for gene expression as compared to the location the gene was inserted in L23.

There were significant expression differences in the cysteine protease (Fig. 3.1B), papain-like protease, between wild type and transgenic plants especially under drought conditions. This suggests that the increased biochemical functioning of *OC-I* in the nodules may result in delayed senescence and prolonged nitrogen supply ultimately leading to drought tolerance. Further phenotypic, physiological, and biochemical analysis were conducted to investigate to what extent increased *OC-I* levels in root nodules might be influencing drought tolerance in soybean.

When plants experience a water deficit, the first physiological process to indicate stress is leaf development (Farooq et al., 2010). As a response to drought, plants acquire adaptations such as waxy leaves or reduced number of leaves as a mechanism to reduce water loss under water deficit conditions. Cultivars with reduced leaf sizes/number are considered drought tolerant, but this has a negative impact on yield under non-stress conditions (Sinclair and Muchow, 2001, Farooq et al., 2010). Under drought conditions the wild type plants had significantly fewer leaves, which likely is an adaptation to reduce water loss and promote drought survival (Bangar et al., 2019).Transgenic lines on the other hand, except L22, did not exhibit a significant reduction of leaf number from well-watered to drought stress conditions (Fig. 3.2A), suggesting better drought tolerance and these plants may likely also maintain improved yields even under drought conditions as observed by Farooq et al., (2010).

Under drought conditions Fenta et al., (2012) reported a shift of shoot/root ratio of 1-2 indicating resource partitioning from shoots to roots. This can be a disadvantage to shoot growth, which may result in decreased yield. However, in the current study the transgenic lines maintained a higher biomass under drought conditions as compared to the wildtype (Fig. 3.2B). This indicates that transgenic lines are likely partitioning more resources to shoot growth more efficiently compared to wild type under drought conditions. Under well-watered conditions transgenic lines, except L21, maintain significantly higher shoot biomass as compared to wild type indicating improved partitioning therefore potentially increased yield.

Physiological adaptation to drought is the ability to regulate water loss under water deficit conditions (Basu et al., 2016) and protection of photosynthetic machinery during these periods (Fenta et al., 2012). Transgenic lines maintained a high relative water content under drought conditions as compared to the wild type plants (Fig. 3.2C). This could be an indication of more efficient water use and reduced transpiration (Fang and Xiong, 2015). Alternatively, it could indicate improved osmoregulation as a result of increased proline content in root nodules (Fig. 3.5F), which may lead to drought tolerance. The ability of the transgenic lines to maintain relatively similar water content under well-watered conditions and a higher water content under drought as compared to the wild type (Fig. 3.2C), suggests that transgenic lines have improved growth and development regardless of the conditions. Similar results were observed by Le Roux et al., (2019). These phenological and physiological observations are likely to be related to the prolonged nitrogen supply conferred by higher levels of OC-I protein protecting root nodules from drought induced senescence. It has previously been established that increased nitrogen supply improves plants growth and yield and is a likely explanation for our observations (Sanz et al., 2011).

Li et al., (2006) and Huseynova, (2012) reported a significant decrease in chlorophyll content in drought sensitive barley and wheat cultivars as compared to tolerant cultivars. This suggests that relative chlorophyll content can be an indicator for drought sensitivity/tolerance. In this study, the transgenic lines, maintained a significantly higher chlorophyll content than the wild type plants under well-watered and drought conditions suggesting a possible increase in photosynthetic output (Fig. 3.2D). These findings point towards two hypotheses: (1) transgenic lines are drought tolerant and (2) transgenic lines can potentially have improved yield under both well-watered and drought conditions. Prolonged nitrogen supply to shoots results in increased photosynthetic capabilities since proteins of Calvin cycle and thylakoids represent the majority of nitrogen in leaves (Evans, 1989) thus may explain the increased chlorophyll

content in the transgenic lines. The improved phenological features (increased leaf number, fresh weight, and leaf water content) allows for the transgenic lines to maintain increased photosynthetic capabilities (as indicated by increased chlorophyll content in transgenic lines) related to increased nitrogen supply.

Tissue damage is an indicator of oxidative damage due to ROS production in response to water-stress (Akcay et al., 2010). No visible tissue damage was observed under drought conditions (Fig. 3.1A), so to test for physiological and biochemical tissue damage two approaches were taken. The physiological approach assessed the extent and quantity of Evans blue staining penetrating the leaves (Fig. 3.3A and 3.3B), and the biochemical approach quantified the production of MDA (Fig. 3.3C). Microscopic images from the physiological approach indicated no staining of any of the leaves under well-watered conditions (Fig. 3.3A). Under drought conditions wild type showed increased dye penetration, indicating a higher level of tissue damage as compared to the transgenic lines (Fig. 3.3A). Vijayaraghavareddy et al., (2017) reported similar results, where their plants were subjected to stress and showed increased staining compared to the control plants, which indicated extensive tissue damage due to stress conditions.

Since these results were obtained from leaf discs, they do not represent the level of tissue damage in whole leaves but rather near the main vein it was important to confirm results at whole leaf level to provide an overview of how the leaves are responding to stress. Whole leaf spectrophotometric quantification was performed to give clear indication of Fig. 3.3A at whole leaf level. The results observed in Fig. 3.3B support what was observed in Fig. 3.3A. Under both well-watered and drought conditions transgenic lines maintained a strong trend of minimal tissue damage as compared to wild type. With the transgenic lines having significantly less dye penetration under drought conditions indicating significantly low tissue damage as compared to the wild type (Fig. 3.2B). Phillips, (2012) also observed increased dye absorbance in 3 weeks drought stressed soybean plants suggesting increased tissue damage. Although the physiological approach to determine tissue damage produced reliable results, it was important to understand if the tissue damage was only mechanical wounding or if there were some level of physiological/biochemical effects. Lipid peroxidation (LPO) is defined as the removal of electrons from lipids and membranes by ROS resulting in damaged cells (Jambunathan, 2010). The primary products of LPO are lipid hydroperoxidases and the most studied secondary products are MDA, hexanal, propanal and 4-hydroxynonenal (Ayala-Astorga and Alcaraz-Meléndez, 2010). MDA has been widely used as LPO biomarker (Khoubnasabjafari et al.,

2015) and proved to be an effective marker in this study as well. Transgenic lines had significantly lower MDA under both well-watered and drought conditions indicating reduced LPO thus less tissue damage (Fig. 3.3C), which supported the results of the Evans staining (Fig. 3.2B and 3.3A) and indicated the transgenic plants were better protected against drought conditions.

The main causes of tissue damage in leaves is as a result of the disturbance in the equilibrium of ROS production by metabolic process such as photosynthesis (Hasanuzzaman et al., 2020). Since chlorophylls are the powerhouse of photosynthesis they are regarded as one of the major ROS producers in leaves (Foyer and Noctor, 2003, Møller and Sweetlove, 2010, Wu et al., 2015). Increased internal ROS in chlorophylls results in these organs being the first to be degraded as a result of stress conditions. Transgenic lines showed significantly high levels of chlorophyll compared to wild type yet maintain reduced tissue damage (Fig. 3.2D and 3.3A-C) indicating a more efficient ROS production equilibrium related to increased nitrogen supply from nodules delaying chlorophyll degradation to escape drought.

The physiological and biochemical approach of studying cell damage suggests that transgenic lines maintain intact tissue under both normal and unfavorable conditions as compared to the wild type. All physiological and biochemical analysis on leaves suggest drought tolerance of the transgenic lines since the plants maintain high leaf content, fresh biomass, and potential effective water use efficiency under both well-watered and drought conditions in relation to the wild type. The transgenic lines maintain lower tissue damage and high chlorophyll content, which are indicators of drought tolerance and potentially improved yield. Leaf phenology and biochemistry indicate improved growth and development of transgenic lines relative to wild type plants. This partially supports the hypothesis of the study that protecting root nodules can improve plant growth and development by prolonging nitrogen supply thus protecting photosynthetic organs and promoting efficient partitioning of photosynthates. To test the protective abilities of increased OC-I protein levels, physiological and biochemical analysis of nodules was performed.

The process of nodulation is important in legumes since nodules provide a suitable environment for biological nitrogen fixation (Ferguson et al., 2019). Under drought conditions increased nodulation has previously been observed in drought tolerant plants (Talukdar, 2013, Furlan et al., 2017). In the current study, the transgenic lines maintained high nodulation, represented as a trend of increased nodule number, under well-watered and drought condition (Fig. 3.3A).

These findings suggest that a certain level of drought tolerance in transgenic plants can be expected.

Nodule drought tolerance has been associated with the plant's ability to transport photosynthetic products to nodules and increased nodule biomass (King and Purcell, 2001). This is because nodule biomass is positively correlated to nitrogen fixation (Hwang et al., 2014). Since transgenic lines have significantly higher chlorophyll content under drought conditions, we assume increased photosynthesis and photosynthate transport. This assumption is supported by a trend of increased nodule biomass under drought conditions in transgenic lines as compared to wild type (Fig. 3.4B). Taking into consideration King and Purcell, 2001's association of nodule drought tolerance between efficient photosynthate transport and nodule weight, transgenic lines have improved drought tolerance and improved photosynthate transport under well-watered conditions due to increased nodule biomass (Fig. 3.4B). For the above reasons transgenic lines are expected to have increased nitrogen fixation.

Ureides are a primarily long-distance transport form of nitrogen, which is transported from nodules to shoots for nitrogen supply (Tajima et al., 2004). Soybean transgenic lines expressing common bean ureide permease (*UPS1*) transporter in nodules resulted in increases ureide synthesis (Carter and Tegeder, 2016). These results suggest that an increase in ureide transport results in increased biosynthesis of ureides. This suggestion was supported by increased ureides in transgenic soybean nodules as reported by Carter and Tegeder, (2016). The *OC-I* transgenic lines L21 and L22 maintained a trend of higher ureide content as compared to wild type under well-watered conditions (Fig. 3.4D), indicating potentially increased ureide transport as reported by Carter and Tegeder, (2016). It is a widely known fact that nitrogen fixation decreases in response to drought. Drought tolerant soybean cultivars have previously been shown to possess a higher ureide accumulation in nodules when compared to susceptible cultivars (Cerezini et al., 2017). The *OC-I* transgenic lines L21 and L22 showed significantly increased ureide content in nodules (Fig. 3.4D), indicating drought tolerance and possible improved transportation to the shoots. These results support a positive correlation between nodule weight and nitrogen fixation, as previously suggested by Cerezini et al., (2017).

Active nitrogen fixing nodules show the characteristic pink color indicating the presence of Leghemoglobin (Pommeresche and Hansen, 2017). Fig. 3.4C shows the characteristic pink color indicating nitrogen fixation in root nodules. Under well-watered conditions, transgenic lines and wild type maintain the pink color, while the wild type loses the color under drought

conditions indicating reduced nitrogen fixation. Under both well-watered and drought conditions transgenic lines show superior nodule physiology indicating improved nitrogen fixation under both conditions. These results support the second part of the study hypothesis, prolonged and improved nitrogen fixation under drought conditions. Nodule phenology and nitrogen fixation studies showed that higher levels of OC-I protein confer drought protection to root nodules. The remaining question to be addressed was how this drought protection might work at biochemical level.

Water stress results in reduced protein content due to its ability to induce ROS production, which leads to inhibition of protein synthesis or cause protein degradation or both (Senthil et al., 2018). Different authors have contradictory results on the effect of environmental changes on protein content. But it has been reported that in order for the plant to survive, new proteins have to be synthesized in order to try and deal with the stress (Fazeli et al., 2007). The wild type maintains a clear trend of high levels of protein content under both well-watered and drought conditions (Fig. 3.5A). Protein content increased in both transgenic lines and wild type in response to drought stress, which suggests the synthesis of protective proteins. Under drought conditions the transgenic lines had a strong trend of lower levels of protein when compared to wild type plants (Fig. 3.5A), which indicates that the plants are under less stress thus no need for significant increase in production of survival proteins. Drought induces the transcription of genes expressing proteins associated with drought tolerance (Kavar et al., 2008). The transgenic lines in this study showed the highest increases in protein content, indicating more effective production of vital proteins compared to the wild type plants and can likely be attributed to the inhibition cysteine protease activity. This suggestion is supported by the ability of transgenic lines to outperform wild type as indicated by the physiological traits presented in this study. Transgenic lines increase protein content in a target specific manner, targeted for protection, while wild type increase protein content for repair as well as protection. Which is why wild type plants have high protein content yet still grow poorly in drought condition as compared to the transgenic lines.

Nodules harbor a wide range of biochemical processes making them highly susceptible to oxidative damage (Becana et al., 2010). It is therefore important that the generation of ROS are regulated to prevent premature senescence. Hydrogen peroxide was the chosen ROS to study because Furlan et al., (2012) established the relationship between stress related oxidative damage to enhanced H_2O_2. Drought causes an increase in H_2O_2 in leaves and nodules, which is associated with increased MDA levels (Furlan et al., 2012). Under well-watered conditions,

transgenic lines and wild type have low H_2O_2 as compared to the increase observed under drought conditions (Fig. 3.5B). Transgenic lines maintain significantly lower H_2O_2 content as compared to wild type under drought conditions (Fig. 3.5B). This suggests that transgenic lines have established either enzymatic or non-enzymatic biochemical process to protect nodules from oxidative damage under drought. The establishment of these biochemical process is highly dependent on the ability of OC-I protein to inhibit the action of cysteine proteases targeted to protective enzymes. In soybean cells, oxidative stress (like high hydrogen peroxide content) induces cysteine protease activity, which leads to the degradation of protective proteins thus programmed cell-death in plants (Solomon et al., 1999). The inhibition of cysteine proteases, such as observed in this study, allows the nodules to effectively detoxify the ROS due to limited expression of cysteine proteases.

Enzymatic antioxidant and osmoprotectant profile of transgenic lines and wild type was studied to further understand how the transgenic lines are preventing accumulation of ROS. Peroxidases (POD) play important role in the detoxification of H_2O_2 to water (Rácz et al., 2018). Since H_2O2 increases due to drought stress POD are expected to increase in order to detoxify the cells. Under well-watered conditions transgenic lines have high POD activity as compared to the wild type (Fig. 3.5C), which correlates to the trend of lower H_2O_2 in transgenic lines as compared to wild type under well-watered conditions (Fig. 3.5B). A similar trend is observed under drought conditions, where transgenic lines maintained a trend of higher POD activity indicating effective detoxification as indicated by significantly lower H_2O_2 in transgenic lines as compared to wild type in Fig. 3.5B. Similar results were observed in drought tolerant wheat cultivar where POD activity increased with a decrease in osmotic potential (Esfahani et al., 2010). Catalase, just like POD, plays a role in detoxification of H_2O_2. The difference between catalase and POD lies in the dual, hyperoxidase and peroxidase, activity of catalase (Mafakheri, 2011). As with POD, an increase of H_2O_2 is expected to be accompanied with increased catalase activity to for effective detoxification. Under well-watered conditions transgenic lines and wild type have almost similar catalase activity but under drought conditions transgenic lines maintain low catalase activity as compared to the wild type.

Glutathione-S-transferases (GST) play a major role in the detoxification of xenobiotics and oxidative stress metabolism (Liu et al., 2013). These enzymes are abundant in root nodules and are likely to provide antioxidant defenses important for nitrogen fixation support (Dalton et al., 2009). The over expression of GST has led to biotic and abiotic stress tolerance in Arabidopsis (Kao et al., 2016). In this study two of the transgenic lines (except L21) maintain a trend of

higher GST levels under well-watered conditions when compared to the wild type plants (Fig. 3.5E). These results suggest that L22 and L23 are prepared to deal with unfavorable conditions. Malefo et al., (2020) reported similar results in transgenic Arabidopsis indicating the ability of protease inhibitors to improve the antioxidant pool of plants regardless of the environmental conditions.

Proline is one of the most common osmolytes that accumulate in response to environmental changes (Szabados and Savoure, 2010, López-Gómez et al., 2014). Apart from being an excellent osmolyte, proline paly vital roles such as activation of antioxidant pathways (Nounjan et al., 2012, Osman, 2015 and Ghaffari et al., 2019). Accumulation of proline content induces the activity of superoxide dismutase and peroxidases, specifically ascorbate peroxidase (Soshinkova et al., 2013).Transgenic lines L21 and L22 maintain a higher proline content as compared to wildtype in drought conditions (Fig. 3.4D), indicating effective osmoprotection of nodules under both conditions. These results suggest a mechanism of drought induced nodule senescence resulting in ROS production, followed by proline accumulation. Proline accumulation then leads to the induction of POD, which is protected from protease degradation by OC-I protein and leads to effective detoxification in transgenic lines as compared to the wild type. The characteristic of increased proline content has also previously been observed in drought tolerant cultivars (Mafakheri et al., 2010 and Furlan et al., 2017).

3.5. Conclusion

The physiological data compiled in this study suggests that tissue-specific expression of *OC-I* in soybean plants leads to improved biomass under well-watered and drought conditions. The transgenic lines maintain effective detoxification of ROS in root nodules due to the inhibition of drought-induced cysteine proteases, leading to improved nodule life under unfavorable conditions. Improved nodule life is beneficial since it results in prolonged nitrogen supply to the plant, which contributes to improved growth and yield. This enhanced nitrogen supply plays an important role in the assembly of amino acids which are vital protein building blocks in plant cells. *OC-I* expression significantly improves nitrogen fixation indicated by ureide content under both well-watered and drought conditions, which will decrease a plant's dependence on nitrogen-based fertilizers. Therefore, the transgenic lines can potentially have improved growth in nutrient poor soils.

The proposed mode of action of *OC-I* involves the inhibition of proteases responsible for premature nodule senescence, improving plant's antioxidant and osmoprotectant pool under both well-watered and drought conditions. This study suggests the involvement of *OC-I* in drought stress tolerance by improving not only nodule life, but also growth and development of the plant by protecting photosynthetic machinery. The outcome of this study provides more information on how manipulating proteolysis in plants can improve crops for survival in the changing climates. The improvement of grain legumes is important to provide better nutrition to the population with the limited agricultural resources available.

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Chapter 4

General conclusions and future recommendations

The aim of the study was to characterize transgenic soybean plants expressing a cysteine protease inhibitor, *Oryzacystatin* (*OC-I*) in root nodules under drought conditions and age-induced senescence. The hypothesis was that tissue-specific inhibition of cysteine proteases will delay nodule senescence and thus prolong nitrogen supply to the shoots while eliminating the disadvantage of dwarf plants caused by constitutive expression. At least six replicates per transgenic line and wild type were studied under well-watered, drought and pre-flowering conditions to assess the effect of tissue-specific expression of *OC-I*.

Leaves were harvested at different growth levels (top, middle, bottom) of pre-flowering plants to study age induced senescence. The results indicated a delay of senescence in transgenic lines while still sustaining reproductive growth. This was because of delayed nodule senescence and decreased ureide feedback inhibition between nodules and leaves. Due to this delay in senescence, transgenic lines L21 and L22 appear to invest more in partitioning photosynthates to flowers than storage. These results support the hypothesis of the study with minor exceptions for transgenic line L23. Since L23 showed some level of ureide feedback inhibition, nitrogen supply was limited thus leading to these plants initiating leaf senescence earlier than other transgenic lines to maintain reproductive growth.

Under well-watered conditions transgenic lines show similar shoot growth when compared to the wild type plants indicating that no shoot growth constraints were conferred by expressing *OC-I*. Despite similar shoot growth, transgenic lines showed increased total chlorophyll content suggesting improved light harvesting capabilities, which might be translated to increased photosynthesis. This was due to the trend of higher nodule number and weight supporting more efficient nitrogen fixation in transgenic lines as compared to the wild type. All observed findings are likely due to decreased papain-like protease activity, which results in delayed nodule senescence irrespective of which growth conditions the plants were exposed to.

Throughout the study transgenic lines L21 and L22 showed improved performance in all studied conditions while transgenic line L23 shared a few similar responses with the wild type. The former is attributed to the independent transformation of the transgenic lines. This means that the transgene (*OC-I*) was incorporated in different places of each transgenic line and depending on where the gene is located, it was expressed differently. Transgenic line L21 and L22 show, on average, the highest *OC-I* relative expression. This suggests that high *OC-I* expression allows for higher papain-like protease inhibition, thus delayed nodule senescence

and improved nitrogen supply to shoots.

To prevent random transgene integration promoting uniform expression in all transformed lines, genome editing nucleases such as Zinc-finger nucleases and clustered regularly interspaced short palindromic repeat (CRISPR)–Cas-associated nucleases can be used. These nucleases introduce the gene of interest in specific targeted sites of the genome by inducing double stranded breaks at the target site and inserting the gene of interest and allowing the cellular machinery to repair the break (Li et al., 2020). The presented study show that high expression levels of *OC-I* delay nodule senescence while low expression levels show variable results, therefore the location of the transgene in high *OC-I* expressing lines (L21 and L22) and used for more precise gene editing. The integration site of *OC-I* in transgenic lines L21 and L22 can be observed by nodule genome sequencing and searching the genome for *OC-I* sequence.

Although the study provides evidence on improved growth and development of transgenic plants expressing *OC-I*, it does not offer any solid proof on increased yield and seed quality. Growing the plants till seed production could have been a good indication on whether prolonged nitrogen supply improves yield or not. Yield studies can be conducted by terminating the experiment when the soybean plants have reached seed maturity (R8) followed by measurements of pod and seed number, as well as seed weight per plant and per transgenic line (including wild type) to indicate yield (Alsajri et al., 2020, Chen et al., 2020). Since soybean is grown primarily for the highly nutritious seeds, seed quality studies would give this study an overall conclusive answer. The seed quality studies could include seed morphology and metabolomics to test how increased nitrogen supply affects seed production and quality. Seed metabolites such as protein, stachyose, oil, raffinose, linoleic acid, oleic acid and palmitic acid can be quantified as indicators of soybean seed quality (Alsajri et al., 2020).

The major limitation of the study was planting space restrictions. Due to small planting chambers, only a small number of plants could be planted per trial to assure statistical accuracy. This led to age-induced senescence to be studied only as it progresses per plant and not at different vegetative/reproductive growth stages (Nodulation, pre-flowering, pod-filling, and seed maturity). Molecular, physiological, and biochemical results from these different growth stages would have provided a more well-rounded view on how *OC-I* expression changes through the growth season and how nitrogen fixation and shoot growth is

affected by the transgene expressional differences. If enough space was provided per growth season, drought at different growth stages would have been conducted to determine how each stage respond drought-induced senescence and which growth stage is more susceptible to the senescence. The other limitation of the study is the fact that it was conducted in well-controlled environments which means the observed results can be different if the plants are exposed to field conditions. To overcome these limitations, a bigger growth chamber could be prioritized which accommodates the harvest of statistically accurate biological replicates per growth stage. For verification of the observed results, field trials could be conducted to study how the transgenic lines respond to drought/ age-induced senescence in conditions that are not closely controlled. Field trials on the generated lines would offer a broader view of stress tolerance and some insight to the optimal cultivation conditions of these transgenic lines in the future.

The human population is exponentially increasing, and we seem to be losing in the race of providing sustainable food with limited resources. Soybeans are high in protein and due to their ability to nodulate, they can potentially rehabilitate degraded soils. This offers food security while improving soil fertility to allow for rotational farming with other major crops. Naturally, soybean plants can potentially mitigate two global challenges, food insecurity and degraded soils. The tolerance to abiotic can be archived by understanding mechanisms by which the stress affects plants. Drought induces senescence by increasing protease, especially cysteine proteases, activity. This study has shown that inhibition of cysteine proteases by their natural inhibitors delays nodule senescence, resulting in prolonged nitrogen supply to shoots leading to drought stress tolerance as well as potential increased yield under normal conditions. This contributes to the research for improving sustainable food production and ensuring zero world hunger in 2050.

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