Molecular and physiological characterization of transgenic soybean (*Glycine max*) over-expressing oryzacystatin-I (OCI) in their root nodules

By

Mogale Clinton Hlokwe

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Supervisor: Dr M.E. Makgopa

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DECLARATION

I, Mogale Clinton Hlokwe, declare that the dissertation, which I hereby submit for the degree, Magister Scientia, 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.

Date:……..

Signature:…….

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ABSTRACT

Pre-mature nodule senescence is a naturally occurring process that often has a negative impact on plant growth and development. Nodule senescence is regulated by a class of enzymes known as cysteine proteases. In this study, transgenic soybean (*Glycine max*) were investigated in order to determine whether nodule senescence can be prolonged by reducing the activity of cysteine proteases. Transgenic lines over-expressing a cysteine protease inhibitor (oryzacystatin-I) in root nodules were characterized under different growth conditions. Differential transgene expression occurred in transgenic lines. The presence of OCI was detected by determining cysteine protease activity using a fluoremetric assay which utilizes a fluorogenic cysteine protease substrate. Under well-watered conditions, OCI did lower cysteine protease activity in transgenic plants and also improved the growth rate of trifoliate leaves. The most potent effects of OCI were observed when plants were less than 9 weeks old. This was maybe due to a spike of OCI expression in nodules. Although cysteine protease activity was also lowered under drought conditions, detectable effects of OCI on plant biology were difficult to characterize in drought-treated transgenic lines. Overall, the results of this study demonstrated that organ-specific over-expression of a cysteine protease inhibitor can alter certain plant functionalities that are related to the activity of those cysteine proteases. Future work will focus on determining OCI sensitive proteases and the respectable pathways which they are involved in.

COMPOSITION OF DISSERTATION

Chapter 1 consists of a literature review on the importance of legumes and how protease-protease inhibitor complexes have an influence on plant biological functions. It also outlines what is plant stress and how it impacts plant development or other cellular processes. The rationale, aim and objectives of this study are provided at the end of this chapter. **Chapter 2** reports the identification of transgenic lines expressing OCI in their root nodules. This chapter focuses on determining the appropriate week for characterizing the effects of OCI. It also deals with determining the impact of OCI on plant biology or phenotype during normal growth conditions. **Chapter 3** reveals whether OCI inhibition can alter plant biology under drought conditions and whether these morphological or phenotypic changes correspond to enhanced growth. **Chapter 4** outlines the concluding arguments for the relevant information found in this MSc study and also provides insights on future works. The last section has a list of all literature cited in this dissertation.

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ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	Micromolar
µmol	Micromoles
APX	Ascorbate peroxidase
BNF	Biological nitrogen fixation
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CDNB	2, 4-Dinitrochlorobenzene
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
E-64	Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FU	Fluorescence unit
Fv/Fm	PSII maximum quantum yield
FW	Fresh weight
g	Grams
GST	Glutathione S-transferase
hrs	Hours
dH_2O	Distilled water
IWUE	Instantaneous water-use efficiency

kDa	Kilo-Dalton
MDA	Malondialdehyde
ml	Millilitre
mg	Milligram
min	Minute
mM	Millimolar
mol	Moles
N	Normality
N_2	Nitrogen
NC	Negative control
nm	Nanometer
Nod	Nodulation
NH_3	Ammonia
O/N	Overnight
OCI	Oryzacystatin-I
PAR	Photosynthetically active radiation
PBS	Phosphate buffer saline
pH	Log hydrogen ion concentration
pLeg	Leg-haemoglobin promoter
PC	Positive control
PCD	Programmed cell death
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PLCP	Papain-like cysteine protease
PI	Protease inhibitor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
s	Seconds

SE	Standard error
SMC	Soil moisture content
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
U	Units
UK	United Kingdom
USA	United States of America
V	Voltage
v/v	Volume per volume
VPE	Vacuolar processing enzymes
WC	Water content
w/v	Weight per volume
WT	Wild-type
x	Any amino acid
xg	Times the force of gravity
ZA	South Africa
Z-Phe-Arg-MCA	Z-phenylalanine-arginine-7-amido-4-methylcoumarin
%	Percentage
°C	Degree Celsius

Chapter 1

Introduction

1.1 Legumes

Leguminous plants are valued in modern day agriculture due to their various contributions to our society. Nearly 30% of the world's primary crops harvested in the world are from grain and forage legumes [Kaufman *et al.,* 1997]. These plants produce protein rich seeds and fruit along with nutritious fodder [Puppo *et al.,* 2005]. This makes them an important source of dietary protein intake for both humans and animals [Keyser *et al.,* 1992; Quain *et al.,* 2015;]. Legume trees are now been looked at as a future renewable energy resource for making biofuels [Cordovilla *et al.,* 1999]. In recent years, interest has fallen upon legumes of the bean family, Fabaceae, due to the isoflavone secondary metabolites they can produce. These isoflavone secondary metabolites, for example genistein and daidzein, are compounds known to help with the prevention and treatment of cancer [Kaufman *et al.,* 1997].

1.1.1 Soybean and nitrogen fixation

An important economical legume belonging to the Fabaceae family is the soybean plant, *Glycine max* [Keyser *et al.,* 1992; Quain *et al.,* 2015]. Over the decades more than 35% of processed vegetable oils have been made from soybean seeds. These processed oils are, in turn, used for various industrial food products such as bread and margarine [Mello-Farias and Chaves, 2008; Vorster *et al.,* 2013]. Other reported benefits of soybean are reducing blood cholesterol levels, prevention of diabetes and obesity, and increasing health of the bowel and kidney [Friedman *et al.,* 2001].

Like other members of its family, soybean can enrich soil nitrogen content by forming an endosymbiotic relationship with the soil bacteria, collectively, known as rhizobia [Cordovilla *et al.,* 1999; Puppo *et al.,* 2005]. The bacteria grow in

specialised *de novo* formed plant organs called root nodules [Martinez *et al.,* 2008; Sulieman *et al.,* 2014]. The plant provides the bacteria with nutrients and energy and in turn the bacteria fixes atmospheric nitrogen into ammonia [Puppo *et al.,* 2005; Sulieman *et al.,* 2014]. The ammonia is then incorporated into organic molecules to act as a source of nitrogen for the plant. The fixation of atmospheric nitrogen (N_2) into ammonia (NH_3) is called biological nitrogen fixation (BNF) [Keyser and Li, 1992]. This conversion reaction is regulated by a complex of enzymes which are collectively called nitrogenase. The reaction catalysed by nitrogenase is very energy consuming and sensitive to oxygen, hence needs to occur in an environment-controlled organ [Keyser and Li 1992; Martinez *et al.,* 2008; Sulieman *et al.,* 2014]. Biological nitrogen fixation (BNF) not only improves soil fertility but it also promotes sustainable agriculture. This process provides farmers of developing countries with an alternative to using expensive chemical fertilizers [Puppo *et al.,* 2005; Martinez *et al*., 2008].

1.1.2 Nodule development

During the normal growth of soybean roots, chemical compounds known as flavonoids; are secreted at the tips of each emerging root hairs. These compounds attract rhizobium bacteria to root tips and also stimulate the expression of *Nod* genes [Stougaard *et al.,* 2000]. *Nod* genes found in rhizobium bacteria result in Nod factors which promote the growth of an infection thread inside root hairs (Figure 1.1). As invagination of the cell membrane enables the infection thread to penetrate the interior structure of the root, another structure called bacteroids forms in the root cortex [Schultze and Kondorosi, 1998].

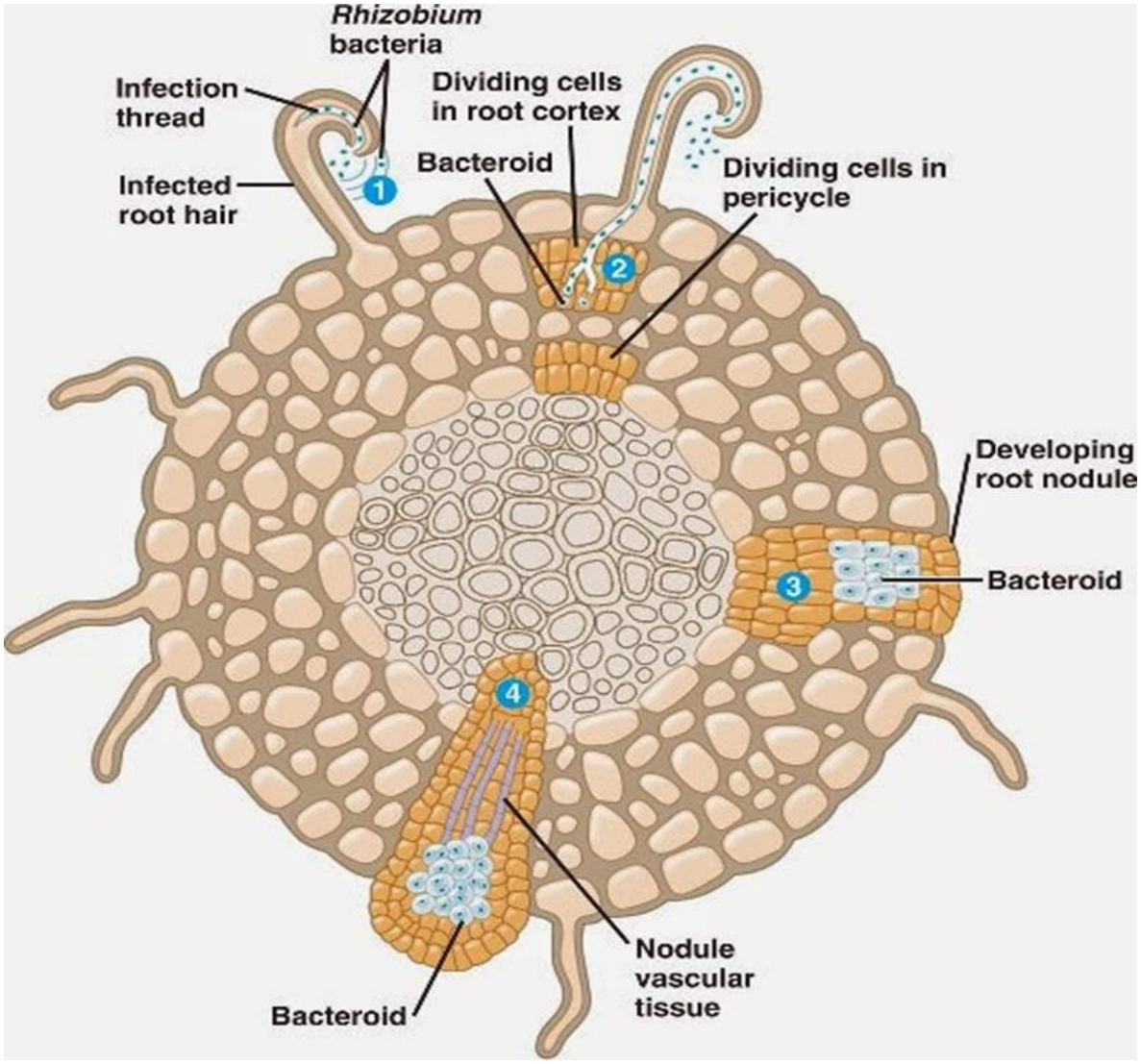


Figure 1.1: Development of a root nodule in a determinate legume. 1) Elongation of root hair and establishment of an infection thread, 2) Infection thread penetrates root cortex while stimulating root cells to divide, 3) Root cells fuse around vesicle holding bacteria (bacteroid); 4) Vascular tissue develops in nitrogen-fixing nodule [Campell *et al.,* 2011].

Bacteroids are specialised vesicles that house rhizobium bacteria in root cells. Infection thread moves deeper into root structure stimulating multiplication of cortex and pericycle cells [Stougaard *et al.,* 2000; Puppo *et al.,* 2005]. Growth of rhizobium bacteria throughout the infection thread produce a nitrogen-fixing bacteroid that is surrounded by masses of cortex and pericycle cells [Schultze and Kondorosi, 1998]. These cells eventually combine to form the specialised plant organ called root nodule. The maturing nodule begins to develop vascular and sclerenchyma tissue while its diameter increases as time progresses. Vascular tissue enable metabolites to be exchanged between the plant and bacteria, while sclerenchyma cells limit the amount of oxygen present in nodules [Colebatch *et al.,* 2004].

There are two types of nodules found in legumes which can differentiated by the manner in which they develop. Legumes, such as soybean, have determinate nodules which are characterized by their spherical shape (Figure 1.2a). The development of these nodules begins in meristem cells of the outer cortex and cellular division usually yields after ten days of infection [Hirsch *et al.,* 2000]. Matured determinate nodules have non-active meristem cells and senescence initiates from the centre spreading towards the exterior of the nodule [Puppo *et al.,* 2005].

In contrast, indeterminate nodules have a cylindrical or branched structural shape (Figure 1.2b). These type of nodules are found in legumes such as alfalfa or peas and consist of five distinctive zones [Timmers *et al.,* 2000]. Meristem cells that are constantly replicating even in the absence of rhizobium bacteria comprise the first (I) zone. The second (II) zone has an infection thread that will later develop into a bacteroid. In the third (III) zone, a growing bacteroid begins nitrogen fixation. [Hirsch *et al.,* 2000]. The fourth (IV) zone appears later on during development as

it is the area where senescence occurs. In the fifth (V) zone, rhizobium bacteria are free-living and have no symbiotic benefits to the plant [Timmers *et al.,* 2000].

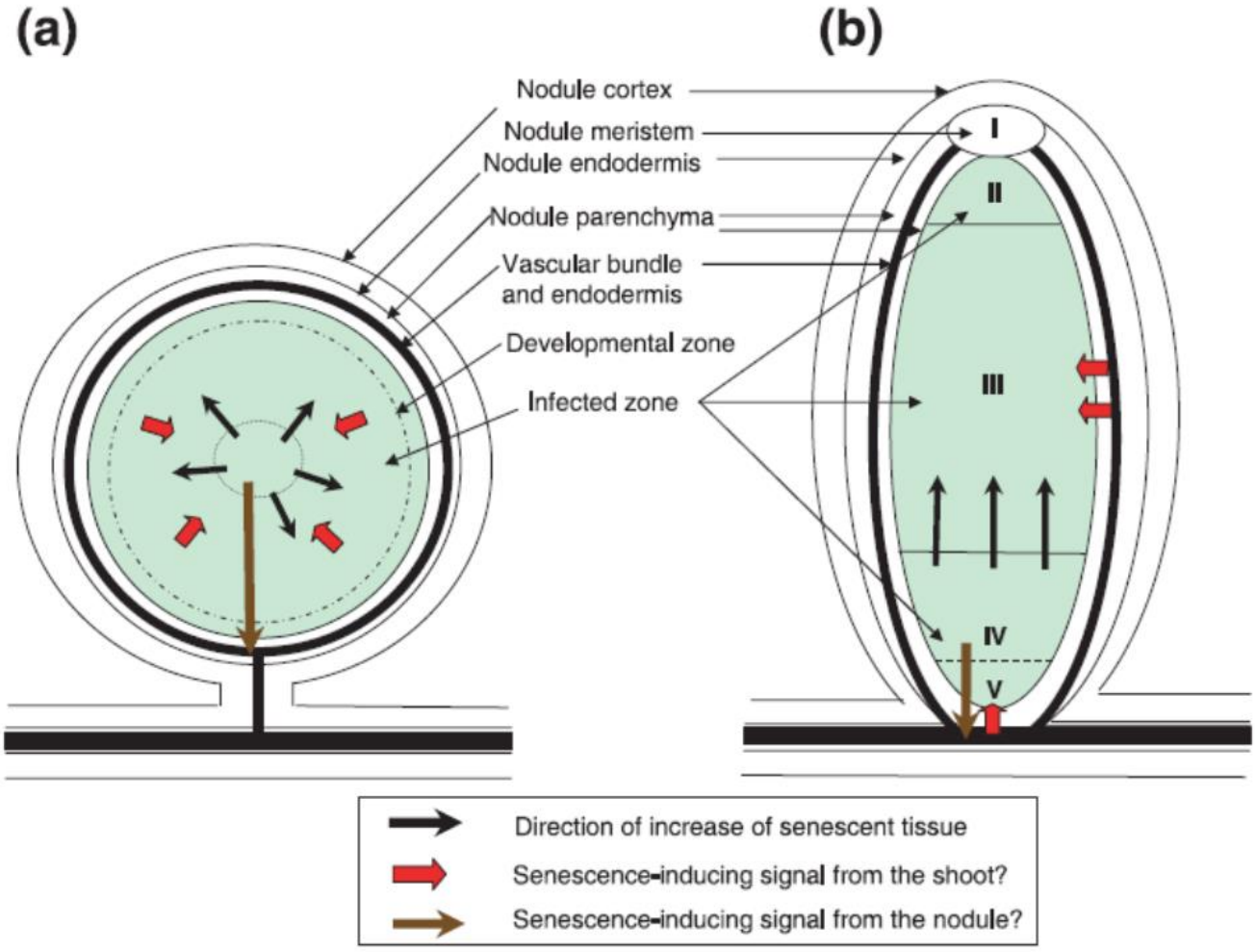


Figure 1.2: Illustration of the structural and developmental differences between **a)** determinate and **b)** indeterminate nodules [Puppo *et al.,* 2005].

1.1.3 Nodule senescence

One of the setbacks with the relationship between soybean and rhizobia is that it is affected by numerous factors such as temperature, lack of nutrients, acidic soil or salt and osmotic stress [Demirevska *et al.,* 2008]. Under such unfavourable conditions, premature nodule senescence occurs and the symbiotic relationship is terminated resulting in little to no nitrogen fixation [Cordovilla *et al.,* 1999; Demirevska *et al.,* 2008]. Nodule senescence can be seen as part of the final stages of nodule development [Puppo *et al*., 2005]. During this stage, the nodules systematically breakdown and the bacteria are removed. In fast growing herbaceous plants, nodule senescence can occur naturally after ten to twelve weeks [Puppo *et al.,* 2005; van de Velde *et al.,* 2006].

However, as mentioned above, external factors can cause senescence to occur prematurely. In the recent years, it was discovered that premature nodule senescence is mostly due to the high sensitivity of BNF to external changes [Arrese-Igor *et al.,* 2011]. Nodule senescence can be characterised genetically, biochemically and physiologically. Visibly, we know that the nodule tissue goes from red to green and that this decolourization correlates to the decrease of active leghaemoglobin present [van de Velde *et al.,* 2006]. Another hallmark of nodule senescence, besides structural changes of organelles, is the high proteolytic degradations that result in a decrease amount of total soluble protein. This degradation of protein is regulated by a group of enzymatic peptides called proteases [Pfeiffer *et al.,* 1983; Grudkowska and Zagdanska, 2004; van de Velde *et al.,* 2006].

1.2 Proteases: the proteolytic proteins

Proteases are hydrolytic enzymes required in regulating a multitude of biological processes in living organisms [Nagase *et al.,* 1999]. In plants, proteases help regulate processes such as flower senescence and remobilization of nutrients in seed during germination [Quain *et al.,* 2014]. This group of enzymes all catalyse cleavage reactions where water is added to an amide and ester bonds which involves electron pairing on the side chain carbon of the peptide bond to be cleaved [Vanaman *et al.,* 1999]. Proteases usually have a very high affinity for diverse substrates which in turn means that they can be specialized for a specific functionality. It is for this very reason why protease have become important for modern day industry such as the food biotechnology processing and detergent industry [Nagase *et al.,* 1999; Tavano *et al.,* 2013]. The specificity of these enzymes arises from their ability to recognize NH_2 or COOH end of a polypeptide along with the reactive chains around its amino acid sequences either on the amino- or carboxyl- terminal of the bond to be cut [Nagase *et al.,* 1999; Vanaman *et al.,* 1999].

Over the decades researchers have discovered that proteases can be defined in terms of where they cleave a peptide bond. Aminopeptidases are able to cleave amino acids that have bonds at the amino terminus of a peptide, while carboxypeptidases function by cleaving bonds of amino acids found at the carboxyl terminus [Barrett et al., 1986]. Collectively, these peptidases are called exopeptidases. Endopeptidases are the most common proteases and cleave bonds of amino acid residues occurring in the middle of the peptide. These protease are regularly classified as possessing a serine residue in their reactive site and usually found in vacuoles [Boller *et al.,* 1986; Huffaker *et al.,* 1990]. Endopeptidases can further be sorted into different classes based on prominent functional groups found at their active sites [Grudkowska and Zagdanska, 2004; Fan and Wu, 2005]. From this we can classify proteases into seven catalytic

classes namely: asparagine, aspartic, cysteine, glutamate, metallo, serine and threonine protease [Rawlings *et al.,* 2011; Deu *et al.,* 2012]. For the purpose of this research, only cysteine proteases and their inhibitors will be discussed in detail.

1.2.1 Cysteine proteases

Cysteine proteases (CPs) are generally produced as 30–50 kDa pre-enzymes, which are translocated to acidic organelles such as lysosomes or vacuoles [Dolenc *et al.,* 1995]. Once in these organelles, the CPs will function in the hydrolysis of other polypeptides [Rawlings *et al.,* 1999]. Cysteine proteases are usually synthesized with a signal peptide that determines which organelle they will be located to. The removal of signal peptides after translocation results in CPs having a molecular mass that ranges between 20 to 35 kDa [Dolenc *et al.,* 1995]. The functionality of cysteine proteases differs from dismantling of organelles (senescence) to precursor protein activation (including pre-enzymes and pre-hormones) [Rawlings *et al.,* 1993; Beers *et al.,* 2000]. Other cysteine proteases (caspases) can induce a kind of programmed cell death (PCD) called apoptosis [Wex *et al.,* 2002].

In recent years, researchers have seen that the mechanisms of apoptosis have many similar characteristics to that of plant hypersensitive response. Plant hypersensitive response is a protection mechanism that also includes PCD [Dangl *et al.,* 2001]. Cysteine proteases are also required for a multitude of other plant processes like fruit ripening, development of embryo and seedling, signalling of growth factors, sorting protein reserves during seed germination or regulating post-translational modification of peptides during seed development [Gruis *et al.,* 2002; Fan *et al.,* 2005; Salas *et al .,* 2008]. The expression of cysteine proteases

was furthermore found in the inflorescence of daylilies and in the rotting leaves of maize and tomato [Smart *et al.,* 1995; Drake *et al.,* 1996].

Cysteine proteases were first discovered in 1879 from the fruit of the plant, *Carica papaya*, and thus was named papain [Barret *et al.,* 1994]. Interestingly, papain was also the first cysteine protease to have its three dimensional structure determined. The term 'papain-like' cysteine protease (PLCP) was derived from sequences that were similar to papain [Barrett *et al.,* 2001; Wiederanders *et al.,* 2003]. These and other proteases are usually grouped into the same families or clans based on the other various traits they share as such as common three dimensional structures, similarity between nucleotide sequences or biochemical affinity to certain ligands [Barrett *et al.,* 1994; Barrett *et al.,* 2001]. An updated and detailed classification of cysteine proteases is available on the MEROPS database (http://merops.sanger.ac.uk). Since most cysteine proteases found in plants belong to either the papain (C1) or the legumain (C13) family, only these two will be discussed in detail.

1.2.1.1 Papain-like cysteine proteases (PLCPs)

Papain-like cysteine proteases are members of the CA clan and fall under the C1A subfamily. Clan CA proteases are characterised by their sensitivity to the general cysteine protease inhibitor, E64 [Barrett *et al.,* 2001]. The active enzyme conformation of these proteases is maintained by a conserved sequence of amino acids and usually have disulphide bridges in their reactive sites [Wiederanders and Kaulman, 2003]. Plant PLCPs are generally produced in an inactive form or precursor in order to prevent degradation of peptides in the cytoplasm. They are activated when certain amino acid residues are cleaved either by the precursor itself or another proteolytic enzyme [Wiederanders *et al.,* 2003; Wiederanders and

Kaulman, 2003]. The amino acid sequence removed represents an inhibitory propeptide, which when cleaved causes conformational change in PLCP's functional structure [Wiederanders *et al.,* 2003].

Papain-like cysteine proteases are usually sorted based on their similarity to mammalian CPs such as cathepsin B-, F-, H- and L-like. These similarity are according to parameters such as phylogenetic relations or DNA structures [Martinez and Diaz, 2008]. There are structural and sequence variances between these various cathepsins, which correlates to their functionality [Musil *et al.,* 1991]. The Cathepsin B and L subfamily usually have some similarities in the amino acid sequence of cysteine residues required in the formation of disulphide bonds. Both of these cathepsins have endopeptidase functionality but cathepsin B also has carboxypeptidase activity [Musil *et al.,* 1991; Martinez and Diaz, 2008]. Structurally, cathepsin B proteases have an occluding loop in their active site that is not present in cathepsin L proteases. The peptide loop is responsible for carboxypeptidase functionality [Musil *et al.,* 1991]. Typically, cathepsin L proteases have a conserved motif (E-R-F-N-I-N) in the interior of their reactive site which is absent in cathepsin B [Martinez and Diaz, 2008].

1.2.1.2 Legumain-like cysteine protease

These cysteine protease are often called 'legumain-like' because their first template protease was initially extracted and characterised from the legume, *Canavalia ensiformis* [Müntz and Shutov, 2002]. Legumains require an acidic pH environment in order to function, hence they are typically found in the cell walls or vacuole. Legumains are also known for their distinct affinity for aspartic residues [Müntz and Shutov, 2002]. These cysteine proteases have the ability to cleave any peptide bond that is flanked by an asparagine residues [Ishii *et al.,*

1994]. Legumains that reside and function in vacuoles are often referred to as vacuolar processing enzymes (VPEs). Interestingly, VPEs lack any nucleotide sequences that are similar to other CPs [Ishii *et al.,* 1994]. However, they do have a reaction site that contains the same residues, namely histidine and cysteine, as other cysteine proteases. Legumains also differ from PLCPs in that they are insensitive to E64 inhibition [Ishii *et al.,* 1994; Müntz and Shutov, 2002].

1.3 Protease inhibitors

The activities of proteases are usually regulated at the transcriptional and/or post-translational stages. In the latter, they can either have their side chain residues modified or regulated by an inhibitor [Martinez and Diaz, 2008]. Protease inhibitors (PIs) are naturally occurring proteins that can down regulate the functionality of proteases [Habib and Fazili, 2007]. They can also be defined as low molecular mass peptides that function in various defence mechanisms of plants [Lievens *et al.,* 2004]. Most fungi and herbivorous animals use proteases to breakdown or digest plant material as they fed. Plants use PIs to reduce the activity of hydrolytic enzymes found in these pests [Johnson *et al.,* 1989]. Inhibition occurs when the active-site of the substrate region of a protease binds to the corresponding reactive site on the surface of the inhibitor thus blocking the site for the actual substrate. This bonding between PI and protease is reversible [Habib and Fazil, 2007; Margis-Pinheiro *et al.,* 2008]. There are PIs for each class of proteases however; we will only be looking at cysteine PIs in detail.

1.3.1 Cysteine protease inhibitors

Inhibitors of CPs are usually divided into categories according to parameters such as similarity in primary sequence, number of disulphide bridges or peptide

molecular size [Grudkowska and Zagdanska, 2004; Habib and Fazili, 2007]. These categories are namely cystatins, kininogens, stefin and phytocystatins. Cystatins are defined by conserved sequences at the N- and C- terminus of their polypeptides and has two disulphide bonds [Turk *et al.,* 1991]. These protease inhibitors have a typical molecular mass of 13-14 kDa. Kininogens are much larger than all the other cysteine PIs as their molecular size ranges from 88-144 kDa. These inhibitors consist of a peptide with a conserved amino acid sequence, Q-V-V-A-G [Turk *et al.,* 1991].

Stefin inhibitors are single-chained peptides that synthesized without a disulphide bond or signal sequence [Oliveira *et al.,* 2003]. They also do not undergo glycosylation after translation. The smallest cysteine PIs are found in the stefins and phytocystatins category where peptides are about 11 kDa in size [Turk *et al*., 1991]. However, there are some exceptions as some phytocystatins found in potato tubers can contain multi-cystatin domains [Walsh and Strickland, 1993]. Phytocystatins are inhibitors exclusively found in plants and will be discussed in more detail.

1.3.1.1 Phytocystatins

Phytocystatins is another name given to protease inhibitors that limit the activity of cysteine proteases belonging to the C1A (papain) family [Martinez *et al.,* 2007]. Interestingly, there are phytocystatins with extensions on their carboxyl terminal which enables them to inhibit some proteins of the C13 family. Most phytocystatins are synthesized as low molecular mass peptides (12–16 kDa) that lack disulphide bonds or glycosylation modifications [Ishi *et al.,* 1994; Margis-Pinheiro *et al.,* 2008]. Their tertiary structure is composed of regions with conserved P-W and Q-V-V-A-G motifs along with an alpha helix. The alpha helix

has a {L-V-I-A-G-T-R-K-E-F-Y-A-S-V-I}-x-{E-D-Q-V-H-Y-F-Q-N} consensus sequence [Habib *et al.,* 2007; Oliveira *et al.,* 2003]. Phytocystatins function by binding at the active site of a protease, thereby blocking access of a possible substrate (Figure 1.3). The binding forms a reversible complex that deactivates the protease [Chu *et al.,* 2011]. These interactions between inhibitor and CP usually involves different amino acids located at various regions of the protein. Typically, phytocystatins are translated with a signal sequence indicating that they are not located in the cytosol [Oliveira *et al.,* 2003].

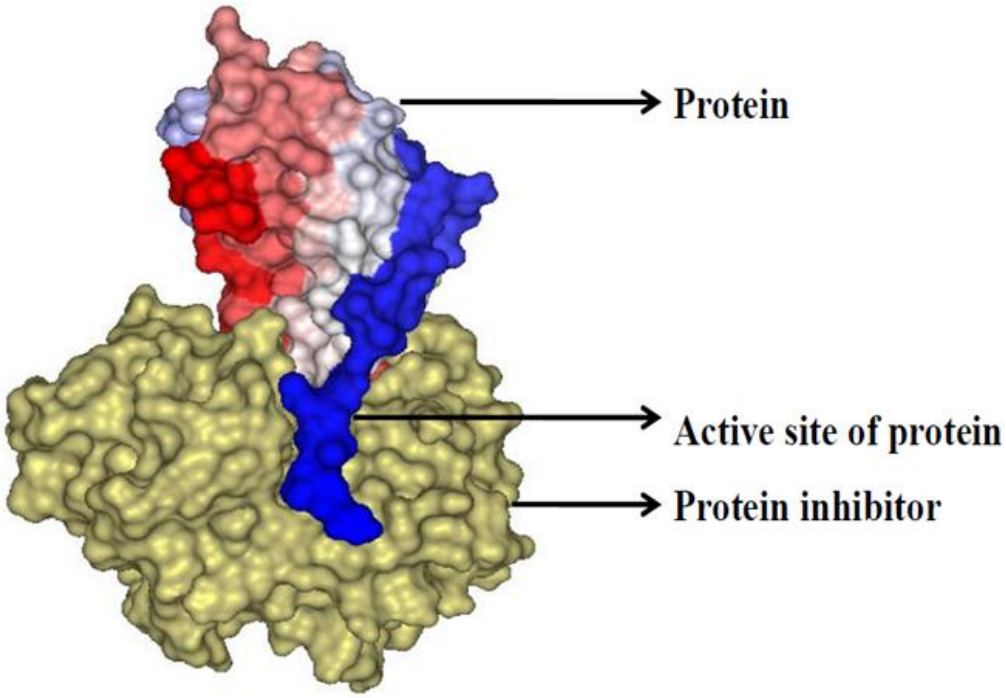


Figure 1.3: An illustration of a protease inhibitor binding to active site of target protease [M Du Plessis, 2013 (UP)].

These PIs are often described as peptides mainly involved in two cellular processes, namely regulating the turnover of polypeptide and acing as defence peptides [Martinez *et al.,* 2009; Benchabane *et al.,* 2010]. In the latter,

phytocystatins are responsible for plants' natural defence system against pests by inhibiting digestive proteases of phytophagous insects which impairs overall digestion [Ryan *et al.,* 1990]. Moreover, moulting and non-digestive polypeptides regulation can also be affected by PI activity [Faktor *et al.,* 1997]. In another study, they showed that transgenic potato plants were more resistant to nematodes such as the potato cyst nematode *Globodera pallida* through over-expression of a cysteine protease inhibitor [Urwin *et al.,* 2001]. Furthermore, artificial diets of the oryzacystatin-I (OCI) and chicken egg white cystatin significantly reduced the survival and growth of the peach potato aphid *Myzus persicae* [Cowgill *et al.,* 2002].

However, the most important functions of phytocystatins has to do with regulating the activity of endogenous cysteine proteases. The lack of these inhibitors would result in the degradation of other polypeptides that is essential for various physiological processes [Solomon *et al.,* 1999; Martinez *et al.,* 2009]. Phytocystatins are also involved in other levels of growth and development such as distribution of metabolites in tubers or seedlings or apoptosis [Solomon *et al.,* 1999; Weed *et al.,* 2009]. Another important function associated with phytocystatins is their ability to increase tolerance to some abiotic stresses. An example is the over-expression of *AtCYSa* and *AtCYSb* in transgenic Arabidopsis which lead to an improved tolerance to low temperatures, drought and high salinity [Zhang *et al.,* 2008]. Similarly, van der Vyver *et al.,* 2003 reported that constitutive expression of OCI in transgenic tobacco reduced the adverse effects of chilling stress on photosynthesis.

1.4 Plant stress

Plants are living systems that require energy and water in order to grow and reproduce [Puppo *et al.,* 2005]. As a living organism, plants encounter factors that negatively impact their growth and development. These factors that hinders a plant's well-being or ability to function normally can be perceived as plant stresses [Mahajan and Tuteja, 2005]. Generally, there are two types of stressors namely abiotic and biotic stressors. Biotic stressors are typical stresses that are caused by other living organisms such as bacteria or nematodes [Chaves *et al.,* 2003]. Abiotic stressors refers to stresses that are linked to growth conditions in an environment such as soil salinity, nutrients or availability of water. Stresses in plants can initiate a multitude of biochemical, molecular, morphological and physiological responses [Chaves *et al.,* 2003].

Although various stressors lead to different responses, most defence mechanisms in plants are initiated by similar signalling pathways [Wang *et al.,* 2003]. Generally, external stimuli activate receptors at the surface of plant cells which in turn leads to the activation of a cascade of pathways (Figure 1.4). These pathways often involve a flux in intracellular Ca^{2+} concentration which either activates or deactivates Ca^{2+} sensitive proteins [Huang *et al.,* 2012]. An incline in intracellular Ca^{2+} concentrations enables Ca^{2+} sensors to bind with phosphatases/kinases resulting in the expression of genes involved in plant defence [Yang *et al.,* 2010].

Typically, a biotic stressor would activate genes that are required to synthesize toxic proteins [Dang *et al.,* 2015]. An example are the thionins produced in barley which have a pesticide effect on *Rosellinia necatrix* or *Manduca sexta* larvae. After repair or prevention of infection, homeostasis returns in the cell [Berrocal-Lobo *et al.,* 2009; Molina *et al.,* 1993]. While, abiotic stressors usually activate genes for chaperones, heat shock factors, late embryogenesis abundant

proteins or antioxidants. These gene products function by maintaining the integrity of plant cellular structures [Mahajan and Tuteja, 2005].

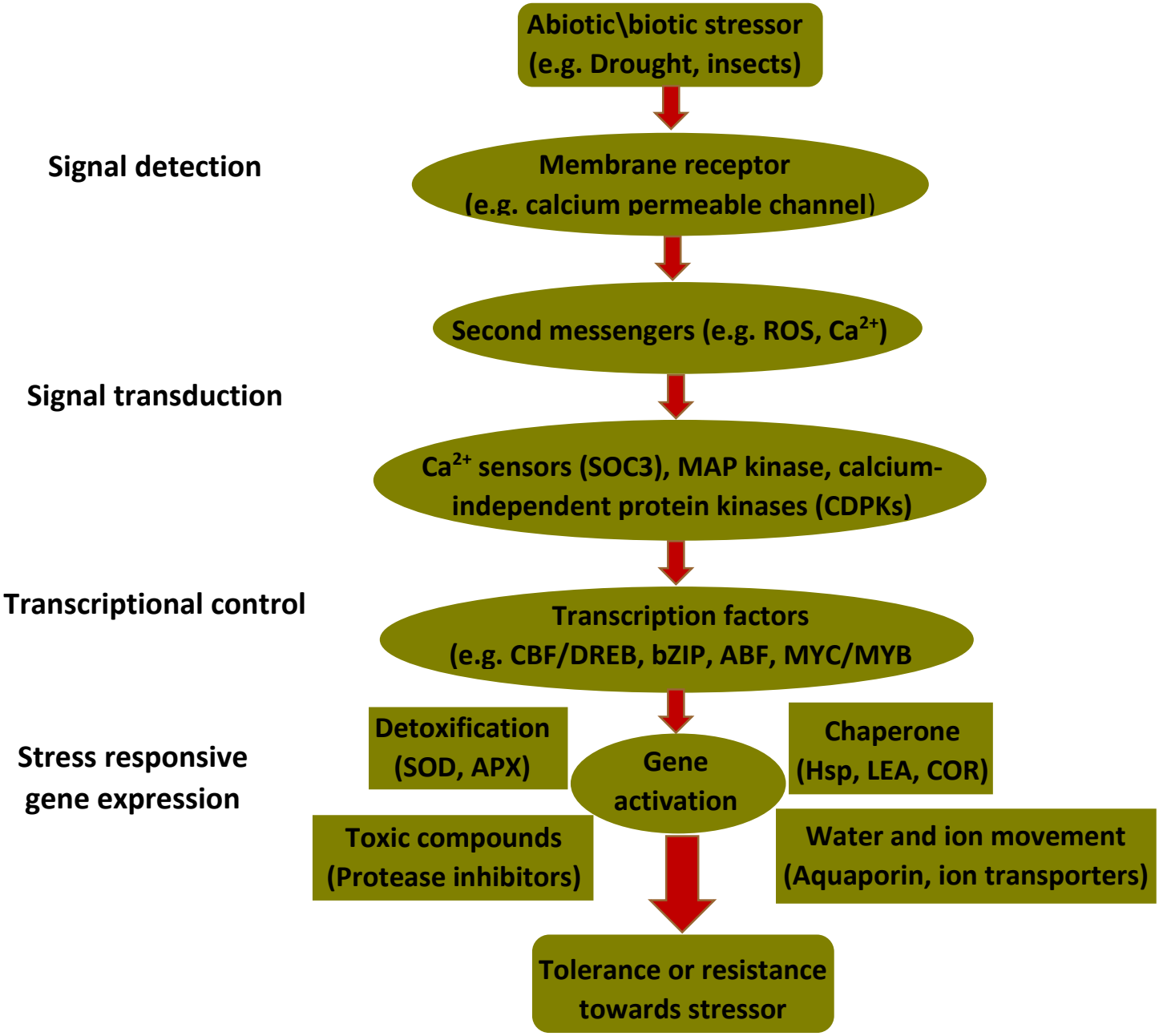


Figure 1.4: Schematic representation of generic pathways initiated when a plant is exposed to various stressors. Extracellular disturbance is recognized by membrane-bound receptors which promotes the activation of large and complex intra-cellular signalling cascade including the generation of secondary signal molecules. The overall process leads to the expression of a multitude of genes involved in stress response [Modified from Makgopa, 2014].

1.4.1 Drought in plants

Drought is one of the major abiotic stressor limiting crop yield today [de Pavia Rolla *et al.,* 2013]. This is especially true for legumes since drought can lead to drought-induced nodule senescence. The process of nodule senescence has been correlated with a marked decline in the major activities involved in removal of reactive oxygen species (ROS) [Evans *et al.,* 1991; Becana *et al.,* 2000] This is important because oxidative damage to biomolecules has been proposed as one of the most important mechanisms triggering nodule senescence in stressed nodules [Gogorcena *et al.,* 1995; Escuredo *et al.,* 1996; Gogorcena *et al.,* 1997]. In *Medicago truncatula*, drought-induced senescence was characterized by the increase of proteolytic activity and free Fe^{2+}/Fe^{3+} levels [Dhanushkodi *et al.,* 2018]. As drought is predicted to elevate over the next decades due to the global changes in climate, it is important for researcher to better under drought responses in plants [Jury *et al.,* 2007].

1.4.1.1 Physiological and morphological responses to drought stress

Drought stress in plant is defined as the rate of transpiration in leaves exceeding the overall water intake [Lawlor and Cornic, 2002]. Drought responses in plants can be attributed to a variety of factors such as leaf architecture, plant species genome, duration of drought and developmental stage of plant [Bray *et al.,* 1997]. Water deficit can lead to a variety of responses in plants. During drought conditions, photosynthesis rates are known to decrease. This is mainly due to the stomata closing to avoid severe water loss via transpiration [Yordanov *et al.,* 2003]. Limited opening of stomata, in turn, also restricts the absorption of carbon dioxide, a crucial molecules required for carbon assimilation in plant cells [Zlatev

and Lindon, 2012]. Stomatal closure is initiated when plants produce the hormone, abscisic acid, in response to drought stress [Xu *et al.,* 2010].

Researchers have known for quite some time that drought stress usually leads to an increase in biomass ratio of root/shoot [Wu *et al.,* 2008]. This can be observed in Asian Scot pine plants, were drought stress results in seedlings having larger roots while the shoot was smaller. Such traits are important for drought survival as they suggest that these plants have better water-use efficiency or higher water uptake capacity [Cregg and Zhang, 2001; Achten *et al.,* 2010]. In soybean, exposure to drought causes a decrease in nitrogen fixation and seed yield [Serrai *et al.,* 1999; Clement *et al.,* 2008]. The former is due to premature nodule senescence which occurs during drought conditions.

1.4.1.2 Mechanisms of drought adaptation

Collectively, the various types of responses initiated by drought stress eventually lead to a mechanism for handling this stress. Plants usually have three different mechanisms involved in drought adaptation, namely: drought tolerance, drought avoidance and drought escape [Chaves *et al.,* 2003]. These drought adaptation mechanisms can also be used in combination during different development stages, resulting in what is typically referred to as drought resistance (Figure 1.5). Drought escape is a mechanism commonly used by plants with a short life span. These plants usually mature early quickly so that by the time drought occurs their reproductive stage is done [Manavalan *et al.,* 2009]. Researchers have since developed transgenic plants with similar traits. An example is the Flowering Locus (FT) gene, *MdFT1*, which was isolated from apple and over-expressed in *Arabidopsis thaliana* [Tränkner *et al.,* 2010]. *MdFT1* over-expression resulted in transgenic lines that developed flowers sooner when compared to wild-type.

Discovery of such FT genes can be useful in engineering crop plants that can escape drought via early flowering.

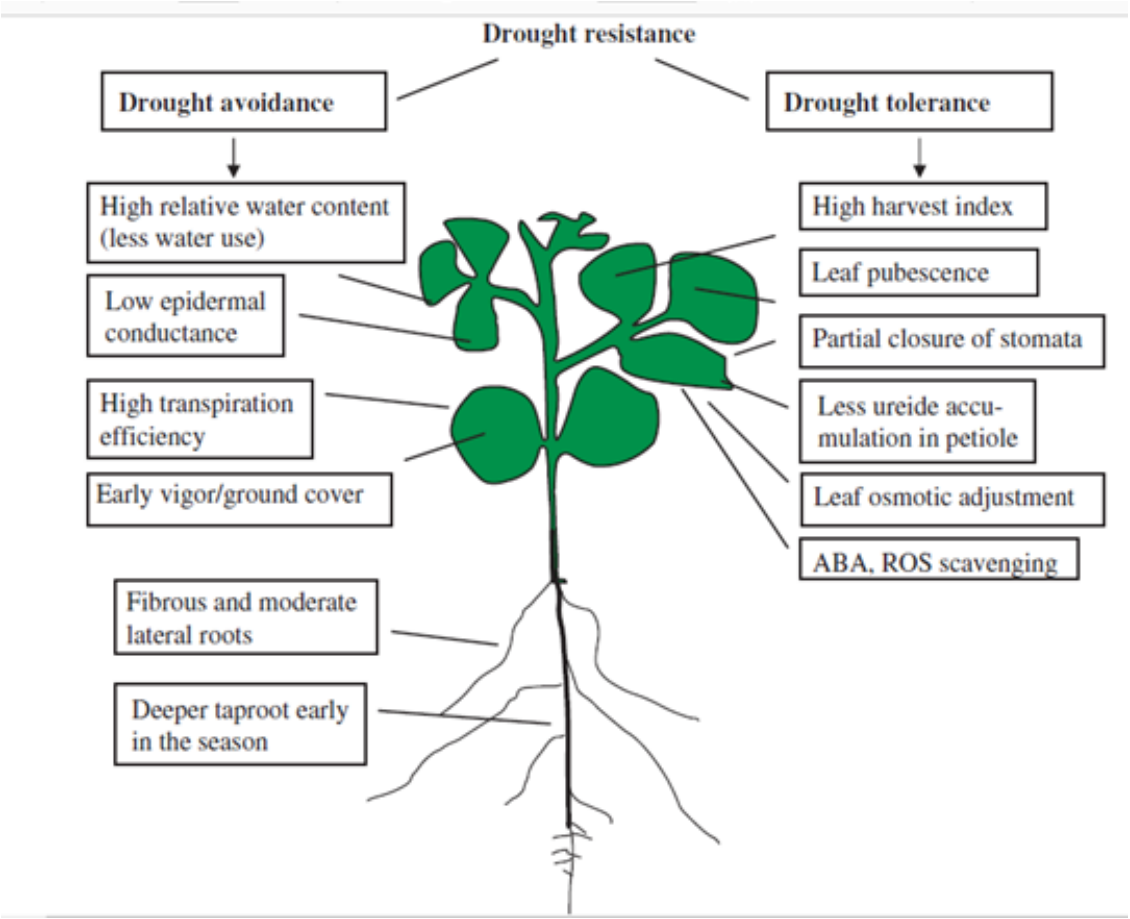


Figure 1.5: Illustration of how drought resistance in a soybean plant is comprised of drought avoidance and tolerance [Manavalan *et al.,* 2009].

Drought avoidance is a mechanism that functions by preventing the occurrence of drought stress in the plants [Chaves *et al.,* 2003]. This can be achieved by a number of ways. One way is reducing water loss by closing stomata which in turn limits the rate of transpiration in leaves [Manavalan *et al.,* 2009]. Another can involve the development of a larger root system in order to increase surface area for water absorption (Figure 1.5). The establishment of an early ground cover or a deep well-structured root system soon after germination is also common in this kind of drought adaption [Manavalan *et al.,* 2009]. Mechanisms of drought avoidance can also be used to improve plant growth and development during such unfavourable growth conditions. Researchers have shown that over-expressing a water channel protein (aquaporin) gene, *RWC3*, in rice can lead to an improvement in leaf water potential and water absorption in roots [Lian *et al.,* 2004].

The final mechanism for coping with water deficit is drought tolerance. Drought tolerance refers to the plants ability to withstand drought conditions as it continues to grow and development [Passioura, 1997]. This mechanism is linked to a variety of strategies which include modification to biochemical and physiological processes (Figure 1.5) [Taji *et al.,* 2002; Bartels and Sunkar, 2005]. In recent years, most researchers have focused on how the accumulation of secondary metabolites can lead to the development of drought tolerance in some plants [Singh *et al.,* 2015]. These metabolites can result in osmotic changes that improve water retention in shoot systems [LeRudelier and Boullard, 1983; Bohnert and Jensen, 1996]. Some metabolites can also function as ROS scavengers, which are produced in abundance during drought stress [Smirnoff and Cumbes, 1989; Smirnoff *et al.,* 1993]. Studies have showed that introducing a myo-inositol O-methyltransferase gene, *IMT1*, in *Nicotiana tabacum* cultivar SR1 can lead to the accumulation of specific solutes, which helps improve the plant's protection

against drought and salt stress [Sheveleva *et al.,* 1997]. Furthermore, it was also shown that introducing a *Datura stramonium* polyamine biosynthesis gene*, adc*, in rice can lead to an increase in polyamine levels, which in turn enhances drought tolerance [Capell *et al.,* 2004].

1.4.2 Phytocystatins involved in abiotic stress tolerance

Programmed cell death (PCD) is one of the biological processes that occur during plant response to stress [Solomon *et al.,* 1999]. As mentioned earlier, PCD is linked to high protease activity and the activity of these proteases can be regulated by inhibitors. Hence, damage to plant cells by stress leads to the synthesis of PI, including cystatins [Zhang *et al.,* 2008]. Previous investigations have demonstrated that the expression of certain phytocystatins are associated with response to several abiotic stresses [Masoud *et al.,* 1993; Habib and Fazili, 2007; Zhang *et al.,* 2008]. In tomatoes (*Lycopersicon esculentum*), salt stress can lead to the accumulation of various cystatins and other genes linked to damage functions [Dombrowski, 2003]. In another study, they discovered that cystatin expression increased in the roots and leaves of chestnut plants during salt or cold stress [Pernas *et al.,* 2000].

Furthermore, cold stress leads to an increase of a cold-response associated cystatin (Hv-CP1) in the vegetative tissue of barley plants [Gaddour *et al.,* 2001]. In cowpea (*Vigna unguiculata*), levels of accumulated cystatins in leaves correlated to the degree of cultivar susceptibility towards drought stress. The most drought-tolerant cowpea cultivars had the highest amount of cystatins accumulated in their leaves [Diop *et al.,* 2004]. These discoveries of how endogenous phytocystatins can influence plant development during abiotic stress has led to the development of transgenic plants carrying such cystatins. In the

last decade, researchers have produced numerous transgenic plants that have demonstrated the ability of phytocystatins to enhance abiotic stress tolerance [Van der Vyver *et al.,* 2003; Zhang *et al.,* 2008; Demirevska *et al.,* 2010].

1.5 Problem statement

Soybean is one of the world's most important crops and its growth is usually in pair with rhizobium bacteria [Puppo *et al.,* 2005]. There is a symbiotic relationship that naturally exists between soybean and rhizobia bacteria [Cordovilla *et al.,* 1999]. In this partnership, the plant provides nutrients and the bacteria supply a cost effective source of nitrogen via nitrogen fixation [Keyser and Li, 1992; Puppo *et al.,* 2005]. When soybean is under unfavourable abiotic conditions such as insufficient water or salinity. Under such unfavourable conditions early nodule senescence occurs and the symbiotic relationship is terminated resulting in no nitrogen fixation [Demirevska *et al.,* 2010]. This has a negative impact on plant growth and development especially if the soil is low in nitrogen.

It has been shown in previous studies that nodule senescence is regulated by a class of proteases called the cysteine proteases. Furthermore, it was also shown that the activity of these cysteine protease can be regulated by protease inhibitors like cystatin [Demirevska *et al.,* 2010]. This research project will focused on the molecular, biochemical and physiological characterisation of transgenic soybean plants that are expressing the cysteine protease inhibitor (OCI) from rice in their nodules. The rationale for this is that the OCI will protect proteins that are degraded by the papain-like cysteine proteases thus increasing the longevity of the symbiotic relationship between the plant and bacteria. In turn, the plants should have an improved growth rate and better tolerance to abiotic stresses in particular drought stress. Our working hypothesis is that transgenic soybean plant

over-expressing OCI will lead to improved plant growth and its ability to withstand drought. The knowledge obtained from this study can be used to generate drought tolerant cultivars of other leguminous crops that of are great importance to agriculture, especially in Africa, where water and source of nitrogen are major limiting agricultural factors.

1.6 Aim and objectives

The main aim of this research project was to investigate whether ectopic OCI expression in root nodules can limit the occurrence of nodule senescence. In turn, enhancing overall plant growth and development.

The following objectives were established in order to achieve our set aim:

- Molecular characterization of the OCI expressing transgenic soybean plants to confirm gene expressions so as to facilitate selection of transgenic plants expressing a protease inhibitor in order to perform phenotypic analysis
- Morphological and physiological analysis of transgenic soybean lines to determine a possible growth promoting effect of OCI expression on plant performance under well-watered conditions
- Phenotypic analysis of drought-treated and well-watered transgenic soybean to determine a possible effect of OCI expression on plant performance under different growth conditions.

Chapter 2

Identification and characterization of transgenic soybean lines grown under well-watered conditions

2.1 Introduction

Genetic engineering or transformation refers to the incorporation of foreign DNA sequences into an organism's genome where the coding sequence is translated [Stitt and Sonnewald, 1995]. Over the decades, genetic engineering or transformation has emerged as the latest technology to be used in agriculture for crop improvement [Sinclair *et al.,* 2004]. Genetic transformation is favoured over traditional methods of crop improvement, such as plant breeding, because it enables crops to be transformed with genetic material from a multitude of different type of biological organisms [Gasser, 1989; Somers *et al.,* 2003; Sinclair *et al.,* 2004]. Transformation can lead to alterations in plant biochemistry and physiology which in turn affect plant traits such as photosynthesis rates, capacity to produce harvestable yield or growth rates [Sinclair *et al.,* 2004]. Transformed soybean lines were not produced in this study, rather seeds transformed with OCI were obtained from a previous research study [Quain *et al.,* 2015].

All soybean transformations were produced using an *Agrobacterium*-mediated transformation. This method of transformation was favoured because it is inexpensive, simple and has a high transformation efficiency [Gelvin *et al.,* 2003, Mello-Farias and Chaves, 2008]. These facilities that provided soybean transformation service followed the Paz *et al*., 2006 method. As part of their investigations, these previous researchers designed various cassettes carrying protease inhibitor genes under the control of different promoter sequences. These cassettes were designed so that transgenes are expressed in different organs or cellular compartments. Cellular compartment expression was achieved by including a signalling peptide sequence in some cassettes.

Our seeds of interest were transformed with a cassette carrying the cysteine protease inhibitor, OCI. This cassette had OCI under the regulation of a leg-haemoglobin (pLeg) promoter. The cassette was incorporated into a pTF101.1

plasmid to enable amplification in bacteria and also for herbicide resistance selection capabilities. In root nodules, the pLeg promoter regulates the expression of leg-haemoglobin [Sulieman and Tran, 2014]. Leg-haemoglobins primarily function in nodules to control the levels of oxygen, which can inhibit nitrogen fixation [Keyser and Li, 1992; Sulieman and Tran, 2014]. The desired construct was thus designed so that OCI will only be expressed in root nodules and not in other plant tissues or organelles

In this chapter, we used PCR to identify which transformed soybean lines have OCI present in their genome. We also used qPCR to determining whether transgenic plants were actively expressing OCI in their root nodule. Since ectopic expression often results in low protein yield [Benchabane *et al.,* 2008], another aim of this chapter was to investigate in which weeks is the highest levels of OCI expression occurred. The most effects of OCI inhibition were observed at those weeks where OCI expression was relatively high. This ensured that characterization was done at a time period when OCI is still active in plant cells. Thereafter, the T_2 generation were further characterized to determine their morphological parameters and biochemical trends.

2.2 Material and Methods

2.2.1 Plant material and growth

Commercial wild-type soybean seeds (*Glycine max,* Williams 82) were obtained from Pannar Seed (Greytown, South Africa). Transgenic seeds were obtained as mentioned above. Seeds of the wild-type and the T1 generation of transformed were sown with *Bradyrhizobium japonicum* (strain WB 74-1) powder (Soygro bio-fertilizer Limited, South Africa). Seedlings were grown in pots [17.5 cm x 20 cm diameter (top) and 13.1 cm] in medium-grade vermiculite (Mandoval PC, South

Africa). Plants were grown under artificial lights up to a 13 hrs photoperiod at 600 mM $mol^{-2}s^{-1}$ photosynthetically active radiation (PAR) and 60% relative humidity in an environmentally controlled growth chamber (phytotron) at 25°C day/ 16°C night temperature. Plants were watered twice a week with distilled water and three times a week with a nitrogen-free Hoagland solution to obtain nodule formation. Pots were re-arranged periodically to allow uniform exposure to light in the growth chamber.

2.2.2 Harvesting of plant material

For sample collection, three plants (for each time point) were harvested after 6, 8, 9, 13 and 18 weeks. Root nodules or trifoliate leaves were collected over these periods and immediately stored after each harvest in a -80°C freezer for further analysis.

2.2.3 Morphological parameters

For each experiment morphological parameters, such as overall: leaf number, stem height, shoot length, fresh and dry weights of upper plant organs, were compared in six to eight weeks old soybean plants from each line. Leaf number was measured by counting the number of trifoliate leaves from base to tip throughout plant development until the eighth week. Shoot length was determined by measuring the shoot from the base to the tip of the shoot. Chlorophyll a and b concentrations in leaves were determined as described by Lichtenaler and Wellburn, 1983. Briefly, one leaflet from each trifoliate leaves was grounded in liquid nitrogen and 10 ml of ice-cold 95% ethanol was added. The mixture was incubated at -20˚C for at least one week before centrifuging at 12 000 xg for 5 min. The supernatant was collected and absorbance determined at 648 nm and

664 nm using a spectrophotometer. Concentrations were determined using extinction coefficients for chlorophyll a and b when extracted in 95% ethanol. The fresh biomass, for both shoots and roots, was determined at the end of week 18 by weighing plant material. Dry biomass was weighed after plant materials were dried in an oven at 70°C for three days. Water contents were calculated as the difference between the first and second measurements as: WC (%) = [(fresh weight - dry weight)/fresh weight)] x 100.

2.2.4 Primer design

The nucleotide sequence of OCI (table 2.1B) was obtained from the NCBI database [http://www.ncbi.nlm.nih.gov/tools] and Primer3 software (online) [http://frodo.wi.mit.edu/primer3] was used to generate primers for both PCR and qPCR. The primers were synthesised at Inqaba Biotec (ZA). The sequences for OCI and its primers are provided in table 2.1A. A 40S ribosomal protein S8 gene from *Medicago sativa* (accession no. TC100533) [Van de Velde *et al.,* 2006] was used as a reference or housekeeping gene. Primers sequences are provided in table 2.1A.

Table 2.1: (A) Primer sequences for OCI and reference gene (40S) used during PCR; **(B)** coding DNA sequence of OCI gene [accession no. M29259.1].

A

Primers	**Sequence (5' to 3')**
OCI PCR forward primer	ATG TCG AGC GAC GGA GGG CC
OCI PCR reverse primer	GAT GGG CCT TAG GCA TTT GC
OCI qPCR forward primer	TCA CCC AGC AAC AAG AAG
OCI qPCR reverse primer	GCA TCG ACA GGC TTG AAC T
40S forward primer	GCC AGC CTG CTA ACA CTA AG
40S reverse primer	AAG AGT CTG AGT ACG CAC AAG

B

OCI Sequence (5’ to 3’)
ATGTCGAGCGACGGAGGGCCGGTGCTTGGCGGCGTCGAGCCGGTGGGGAACGAG AACGACCTCCACCTCGTCGACCTCGCCCGCTTCGCCGTCACCGAGCACAACAAGA AGGCCAATTCTCTGCTGGAGTTCGAGAAGCTTGTGAGTGTGAAGCAGCAAGTTGTC GCTGGCACTTTGTACTATTTCACAATTGAGGTGAAGGAAGGGGATGCCAAGAAGCT CTATGAAGCTAAGGTCTGGGAGAAACCATGGATGGACTTCAAGGAGCTCCAGGAG TTCAAGCCTGTCGATGCCAGTGCAAATGCCTAAGGCCCATC

2.2.5 DNA extraction and PCR

DNA was extracted from the root nodules of six week old plants as described by Edwards *et al.,* 1991. A mass of 100 mg fresh nodules were ground in liquid nitrogen then 400 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) was added to the powdered material. The mixture was centrifuged at 13 000 xg for 1 min. After the supernatant was collected, equal amounts of isopropanol were added and centrifuged at 13 000 xg for a further 5 min. The pellet was washed with 250 µl of 70% ethanol and centrifuged at the same speed for 2 min. The resulting pellet was resuspended in 50 µl of distilled water. Thermo Scientific Master Mix (2x) was used to setup a 10 µl PCR reactions which consists of 5 µl of Mater Mix, 1 µl of DNA template (80 ng/µl), 0.5 µl of each (forward and reverse) 10 µM primer and 3 µl of nuclease-free water. The reaction was carried out in an AU/Palm Cycler (Corbett Life Science cycler, Australia) as indicated: 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.

2.2.6 RNA extraction and cDNA synthesis

Pre-stored nodules (100 mg) were ground in liquid nitrogen and used for total RNA extraction with a Plant RNA Mini Kit (ZymoResearch, USA) according to the manufacturer’s instructions. The RNA quantity and purity was determined with a

spectrophotometer (Nanodrop® Thermo Scientific, USA). Any impurities were removed with a MiniPrep RNA concentrator kit (ZymoResearch, USA) according to manufacturer's instructions. Extracted RNA was then used to synthesize cDNA with an ImProm II reverse transcriptase kit (Promega, USA) according to manufacturer's instructions. First stand cDNA synthesis reactions were performed with random hexamer primers (Thermo Scientific, USA). Real-time PCR reactions were conducted using SYBR Green 2x (Bio-Rad, UK). The reaction mixture was 10 µl with the following composition: 5 µl of SYBR Green, 1 µl of DNA template (80 ng/µl), 0.5 µl of each (forward & reverse) 10 µM primer and 3 µl distilled water. Reactions were conducted in a real-time thermal cycler (Bio-Rad, UK) using the same parameters as the PCRs in section 2.2.5. A melting curve analysis was also included between 75°C and 95°C temperature intervals. The relative expression of OCI was determined by comparing Cq values of target gene (OCI) 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 whereby $\Delta\Delta Cq = (Cq^{untransformed} - Cq^{reference\ gene}) - (Cq^{transformed} - Cq^{reference\ gene})$.

2.2.7 Gel electrophoresis

All PCR products were visualised on a 2% agarose gel ran for 40 min at a constant voltage of 80V in TAE buffer. Samples were prepared by mixing 5 µl of PCR product and 1 µl 6x DNA loading dye (Fermentas, Canada), which contains gelred (Biotium, USA) for easy visualization of samples during the electrophoresis process. Gel images were visualized using an ultraviolet (UV) transilluminator and gel imaging software (Bio-Rad, UK).

2.2.8 Protein extraction

Pre-stored root nodules of 100 mg in mass were placed in liquid nitrogen and ground into a powder. Nodule protein was extracted by adding 500 µl of protein extraction buffer (50 mM Tris-HCl, pH 7 and 5 mM EDTA) to the powdered plant material. The mixture was centrifuged at 13 000 xg for 40 min at 4°C and the supernatant containing the protein was stored at -80°C. Before storing, an aliquot of the protein samples was taken for further analysis. Total soluble protein content was determined using the Bradford (Bio-Rad, UK) assay reagent [Bradford *et al.,* 1976] and bovine serum albumin (BSA) (Sigma-Aldrich, USA) as a standard. The absorbance was determined at 595 nm with a spectrophotometer. Five concentrations of the BSA (1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml) were prepared through a serial dilution in order to obtain a protein standard curve. In each standard & protein sample, 5 µl was loaded in triplicates onto a clear 96 well plate (Nunc, AEC Amersham) and 200 µl of the Bradford reagent was added. The reactions were placed in the dark and incubated at room temperature for 20-30 min. The absorbance was determined using a micro-plate reader (BMG FluoroStar Omega, Germany) with protein extraction buffer, mixed in Bradford reagent, as the blank.

2.2.9 Papain-like cysteine protease (PLCP) activity assay

Total soluble protein (~ 5 mg/ml) from nodule extracts were used for measuring PLCP activity or cathepsin L-like activity in extracts. Reactions were individually loaded in triplicates into a black, flat-bottom polysorp 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 fluorescence spectrophotometer (BMG FluoStar Galaxy) at 37°C with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Reactions were monitored over a 10 min period time period. The control or blank reaction contained reaction buffer, substrate and protein extraction buffer.

2.2.10 Ureide extraction

The rate of nitrogen fixation was measured indirectly by determining ureide content in root nodules. This was done by grinding 100 mg of nodules in liquid nitrogen. A volume of (1000 µl) 2 M NaOH was added to the powdered material and boiled for 20 min before cooling on ice. This was followed by centrifugation at 10 000 xg for 10 min then the collected supernatant was diluted eight times with distilled water. Allantoin (Sigma-Aldrich, Germany) was used as the standard for preparing serial dilution in order to obtain a ureide standard curve. These standards had to be first treated in order to convert all allantoin into allantoic acid, which is the actual compound being measured. A volume of (80 µl) 5 M NaOH was added before boiling the samples for 10 min and chilled on ice. Followed by 160 µl of phenylhydrazine/HCl and boiling again for 2 min. The mixture was chilled for 5 min prior to adding solution 400 µl of HCl/KFeCn and incubating at room temperature for 10 min. This treatment was also done to the protein samples before measurements. The phenylhydrazine/HCl solution was prepared by mixing equal parts of 0.33% phenylhydrazine (v/v) (Sigma-Aldrich, Germany) with 0.65 N HCl (Sigma-Aldrich, Germany) and incubated at -20°C before use. While, the HCl/KeFeCN solution was prepared by adding 5 ml of 1.67% KFeCN (w/v) (Sigma-Aldrich, Germany) to 20 ml of 0.65 N HCl (Sigma-Aldrich, Germany). Prepared standards and samples were left at room temperature for 10 min before readings at an absorbance of 525 nm with a spectrophotometer were taken. This

section was based on how ureide content was determined as described by Young & Conway, 1942.

2.2.11 Enzymatic anti-oxidant assays

Another biochemical analysis of interest was the effect of ectopic OCI expression on enzymes involved in anti-oxidant response. The aim here was to determine whether OCI had an influence on anti-oxidant defence as they respond to oxidative stress, which can occur as a result of normal growth or other factors like drought.

2.2.11.1 Glutathione-S-transferase (GST) activity assay

Glutathione-s-transferase assays were carried out according to the method of Mannervik *et al.,* 1985. A volume of 20 µl extracted protein (section 2.2.8) from the respective samples was aliquoted into different plastic cuvettes (Whitehead Scientific, SA). To each of the protein samples, a volume of 980 µl reaction mixture containing 1 mM 2, 4-dinitrochlorobenzene (CDNB), 1mM glutathione made up in phosphate buffer saline (PBS) buffer (pH 6.5) were added. The absorbance was measured at 340 nm after 3 min to determine the kinetic rate. Using the extinction coefficient of 0.0096 $\mu M^{-1}.cm^{-1}$, the GST activity was calculated.

2.2.11.2 Ascorbate peroxidase (APX) activity assay

A modified method of Singh *et al.,* 2007 was used to determine total ascorbate peroxidase activity. Assays were prepared in triplicates onto a clear 96 well plate (Nunc, AEC Amersham) with each well loaded with 10 µl extracted protein (section 2.2.8), 188 µl of ascorbate buffer (71.43 mM K_2HPO_4,0.36 mM ascorbate and 0.71 mM H_2O_2) and 5 µl of 5 mM ascorbic acid. The absorbance was

measured at 290 nm after 10 min to determine the kinetic rate. Using the extinction coefficient of 2.8 $mM^{-1}.cm^{-1}$, the APX activity was calculated.

2.2.12 Malondialdehyde (MDA) extraction

A modified method by Zhang *et al.* 2007 was followed for the lipid peroxidation assay. A mass of 100 mg nodules was ground into a powder in liquid nitrogen and 5x volumes of 6% (w/v) trichloroacetic acid (Sigma-Aldrich, Germany) was added. The samples were homogenised using a vortex and then centrifuged at 1000 xg for 10 min to pellet the plant material. A volume of the supernatants (200 µl) was transferred into new tubes, to these tubes 300 µl of 0.5% (w/v) thiobarbituric acid (Sigma-Aldrich, Germany) was added. The samples were briefly mixed once again using a vortex. Parafilm was used to wrap around the lids of the tubes to ensure that it remained sealed during the high temperature incubation. The sample tubes were placed in a heating block and incubated at 95°C for 20 min. Following the incubation at high temperature, the samples were incubated on ice for 10 min. Once the incubation on ice was completed, the samples were centrifuged at 13 000 xg for 5 min. All extracted samples were then loaded in triplicate on a clear 96 well microtitre plate (Nunc, AEC Amersham). Absorbance readings were taken at 532 nm and 600 nm using a spectrophotometer. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to correct for the non-specific turbidity. The extinction co-efficient of 155 $mM.cm^{-1}$ was used to determine MDA concentrations.

2.2.13 Statistical analysis

Data for all experimental parameters were analysed using Student's t-test at stringency level of $p < 0.05$ comparing wild-type soybean plants directly to

transformed soybean plants. In all experimental at least three biological replicates were used. One-way ANOVA was further employed to confirm significant differences found in statistical data using the Tukey method as a post-hoc test at stringency level of $p < 0.05$ on a GraphPad Prism windows interface.

2.3 Results

2.3.1 Confirming presence of OCI in root nodules

The seeds of five independent soybean lines transformed with OCI were grown up to six weeks after germination. Genomic DNA extracted from root nodules were amplified with PCR using the OCI primers mentioned in sector 2.2.4. The appearance of the 300 bp band on the gel electrophoresis image indicated presence of our desired gene (Figure 2.1A). A control gene (*40S*) was also used to ensure no errors occurred during PCR. From the gel it can be seen that four of the five lines had OCI transgene in their root nodules. Interestingly, the same primer sets, either for *OCI* or *40S* gene, appear to generate amplicons with different sizes in the independent soybean lines. After confirming integration of OCI in these lines, qPCR analysis was performed to determine which lines are actively expressing OCI in their root nodules. Initial qPCR results showed that three of the four lines were expressing the transgene namely: L21, L22 and L23.

Furthermore, OCI expression was determined from week six to week eight to better characterize between the transgenic lines (Figure 2.1B). In figure 2.1B for all the transgenic lines, the least level expression of OCI was observed in week 6. In each independent transgenic line there were different levels of expression with L21 showing the highest expression, at weeks six and eight, when compared to other transgenic lines. These two lines (L22 and L23) both showed the highest

expression at week 7 and were marked by a decrease in expression once they are 8 week old.

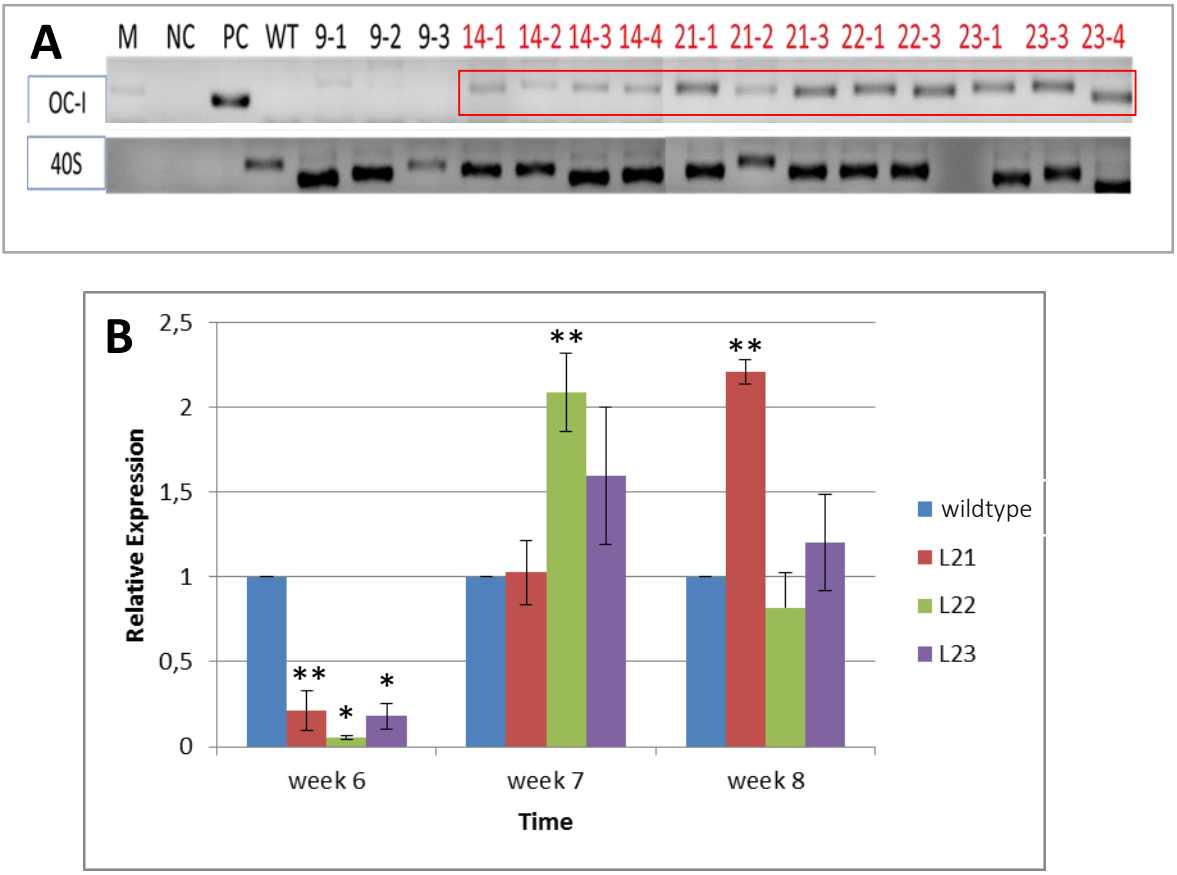


Figure 2.1: Molecular detection of OCI in root nodules of transgenic lines 21, 22, 23 and wild-type soybean **(A)** PCR products after amplification with OCI and 40S primers. Numbers in red represent transgenic lines with OCI and the red box indicates 300 bps band. **(B)** Relative expression of OCI in soybean root nodules at various weeks. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± standard error (SE) of three individual plants (* $p<0.01$, ** $p<0.001$).

2.3.2 Morphological characterization

Following the determination of presence and expression of OCI in root nodules, the next step was to determine whether OCI had an effect on plant growth and development. Transgenic lines (L21 & L23) grew more trifoliate leaves by the end of week 8 than wild-type with L21 having more significant trifoliate leaves (Figure 2.2A), however there was no significant difference in the chlorophyll concentration of their leaves (Figure 2.2B). Furthermore, chlorophyll concentrations of transgenic and wild-type soybean plants seemed to share no particular trend. Visually, wild-type plants had more yellow leaves than transgenic lines (Figure 2.3).

The overlapping of slopes in figure 2.2C suggested that there was a similar growth rate in the shoots between transgenic lines and wild-type. However, transgenic lines (L21 & L22) had a significantly lower wet (fresh) mass at week 18 compared to wild-type plants (Figure 2.2D). This difference in fresh shoot mass could also be visualised in the images of plants. An interesting observation with the plant shoots was that both dry mass and shoot water content showed no significant difference between transgenic lines and wild-type plants. The root biomass of transgenic lines, similar to shoot dry mass and water content, showed no significant difference compared to wild-type (Figure 2.2E).

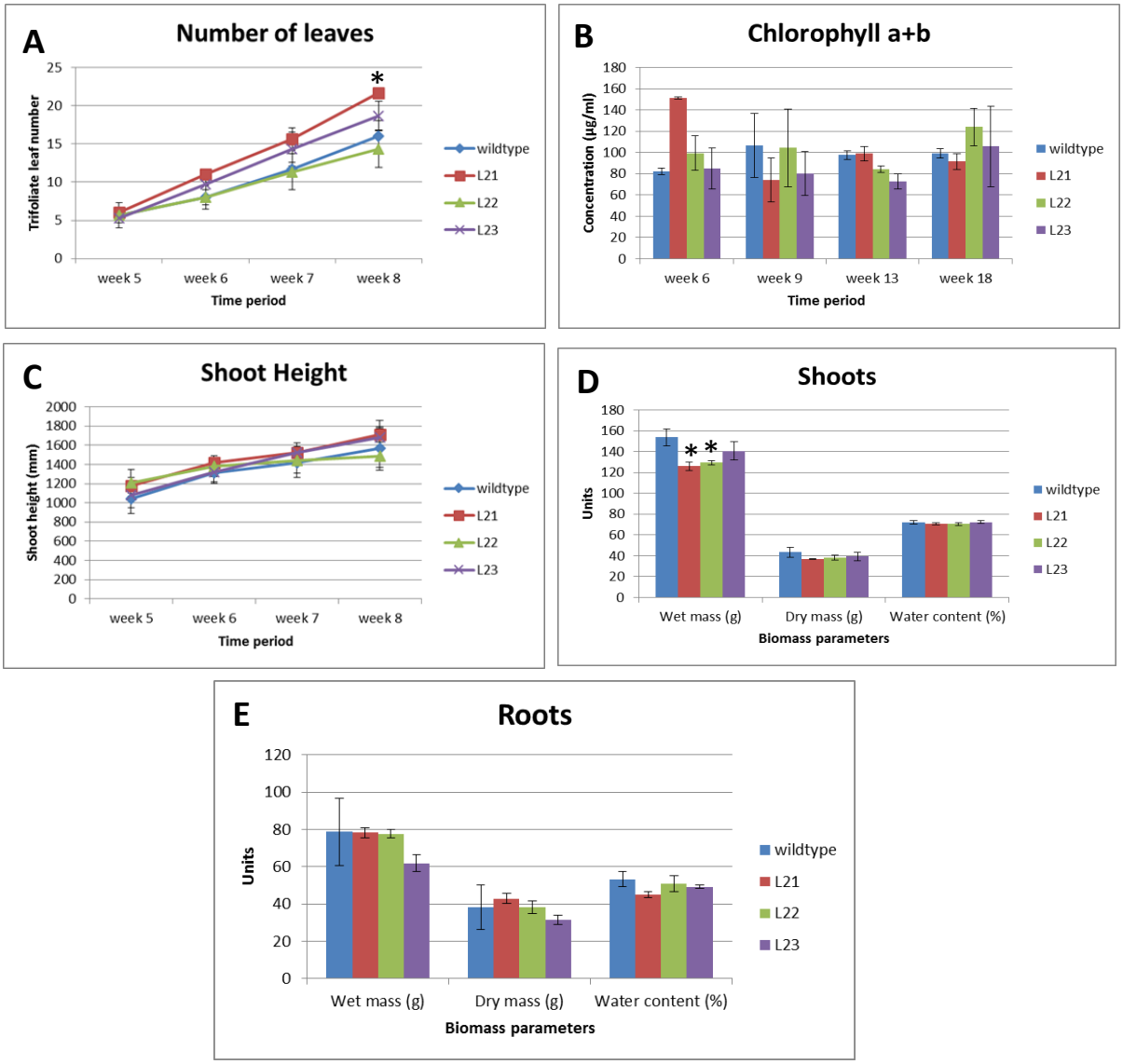


Figure 2.2: The effects of ectopic OCI expression on morphological traits of transgenic lines 21, 22, 23 and wild-type soybean **(A)** number of trifoliate leaves, **(B)** chlorophyll a+b content at week 18, **(C)** height of shoot, **(D)** biomass & water content of shoots at week 18 and **(E)** biomass & water content of roots at week 18. . Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05).

Figure 2.3: Images of transgenic lines 21, 22, 23 and wild-type soybean at the end of week 18.

2.3.3 Total soluble protein content

The content of total soluble protein was measured so that we could determine whether OCI is active in root nodules when expressed by transgenic lines. A trend was observed with the soluble protein content where the transgenic lines had more proteins than wild-type (Figure 2.4A). Although in week 9, it was only L21 that followed this trend, week 9 and week 13 showed the trend being continued through all transgenic lines. Another trend was seen with the protein content throughout all soybean plants, where protein concentration increased from week 9 to week 13 then followed by a decline from week 13 to week 18. The incline from week 9 to week 13 was much higher in transgenic lines than wild-type.

2.3.4 PLCP activity

Papain-like protease activity was determined in wild-type and transgenic plants to determine whether OCI is active in nodules. In all soybean lines, PLCP activity decreased from week 9 to week 13 then increased from week 13 to week 18 (Figure 2.4B). Transgenic lines (L22 & L23) showed a higher decline in activity from week 9 to week 13 when compared to wild-type, while the wild-type had a higher incline than transgenic lines at week 13 to week 18. An inverse correlation exists between protein content and PLCP activity, where a decrease in PLCP activity (week 9 to week 13) marked an increase in protein during the same period and vice versa. This indicated that PLCP activity has an influence on total soluble protein content.

2.3.5 Ureide content

Ureide content was measured to conclusively determine whether OCI is acting on proteases by affecting nitrogen fixation. The rate of nitrogen fixation changes

throughout all soybean plants similarly to how protein content shifted in between week 9, week 13 and week 18 (Figure 2.4C). Nitrogen fixation rates increased from week 9 to week 13 then declined from week 13 to week 18. The transgenic lines (L21 & L22) had the least incline from week 9 to week 13. No significant difference was observed in ureide content between transgenic lines and wild-type which indicated that all soybean lines have similar nitrogen fixation rates.

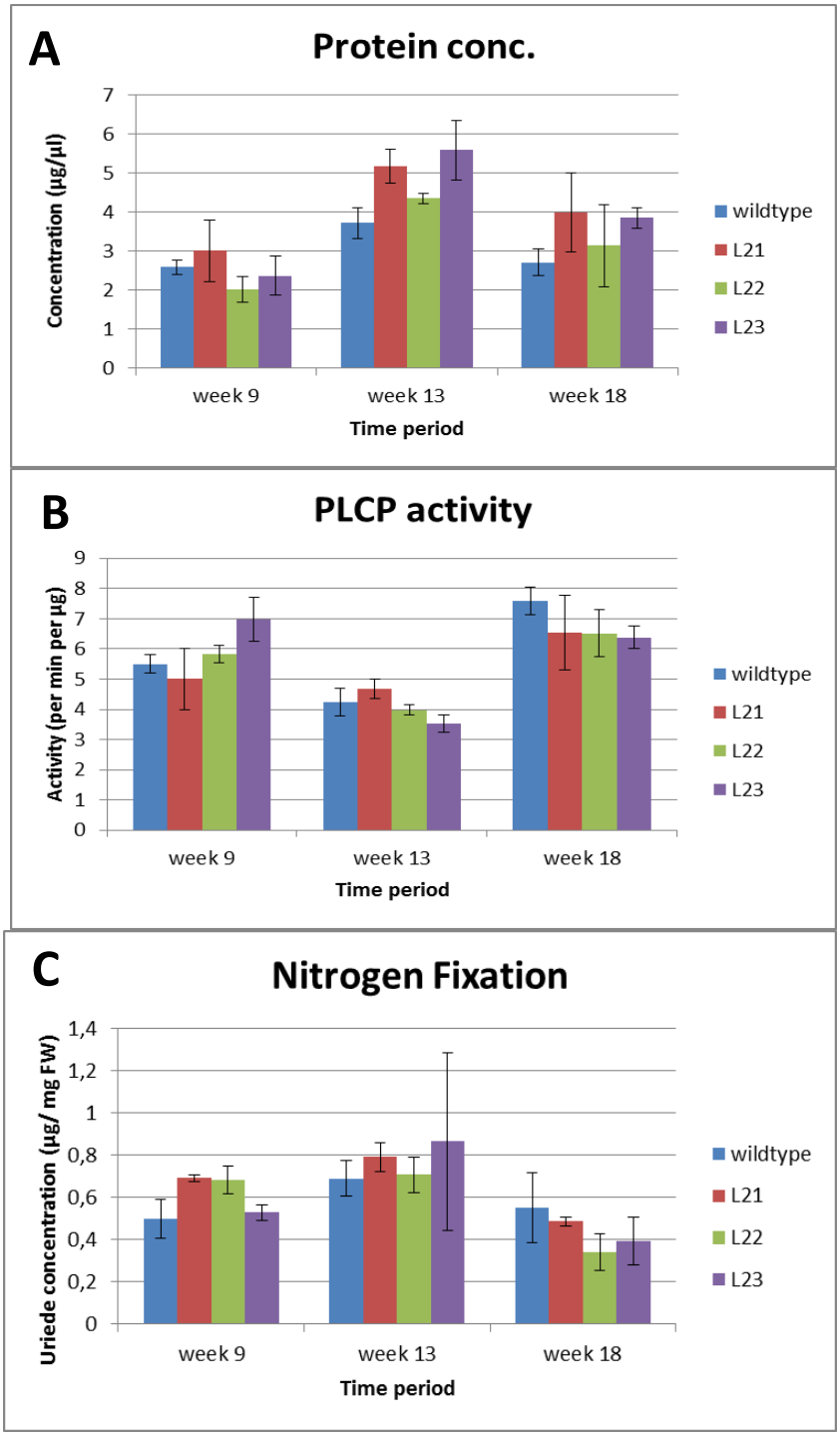


Figure 2.4: The effect of ectopic OCI expression on **(A)** total soluble protein content, **(B)** PLCP activity and **(C)** nitrogen fixation rate in root nodules of transgenic lines 21, 22, 23 and wild-type soybean at different weeks after germination. Data represent the mean ± SE of three individual.

2.3.6 Lipid peroxidation

Lipid peroxidation was measured to serve as a general indicator of the levels of cellular oxidation occurring in root nodules. The concentration of MDA in wild-type was gradually decreasing throughout week 9 to week 13 (Figure 2.5A), while transgenic lines had an increase in concentration from week 9 to week 13 and declined from week 13 to week 18. For transgenic lines, the highest incline was observed from week 9 to week 13 in L21 and L22 showed the highest decline in week 13 to week 18. Line 22 also had a MDA concentration significantly higher than wild-type at week 13, which suggests that wild-type has less oxidative activity.

2.3.7 Enzymatic anti-oxidant activity

The response to oxidation in root nodules is mediated by a variety of mechanisms. Some of these mechanisms involve the activity of anti-oxidant enzymes. Among these enzymes are glutathione-S-transferase and ascorbate peroxidase. The GST activity results (Figure 2.5B) were interesting because they had a trend similar to the PLCP activity data (Figure 2.4B). There was decrease in GST activity from week 9 to week 13 and an incline between weeks 13 and 18. This trend was observed in all soybean lines. Transgenic lines and wild-type soybean plants seemed to share no particular trend in their APX activity (Figure 2.5C). A noteworthy observation about this APX dataset is that at week 9 transgenic line 22 had a significantly higher APX activity than wild-type.

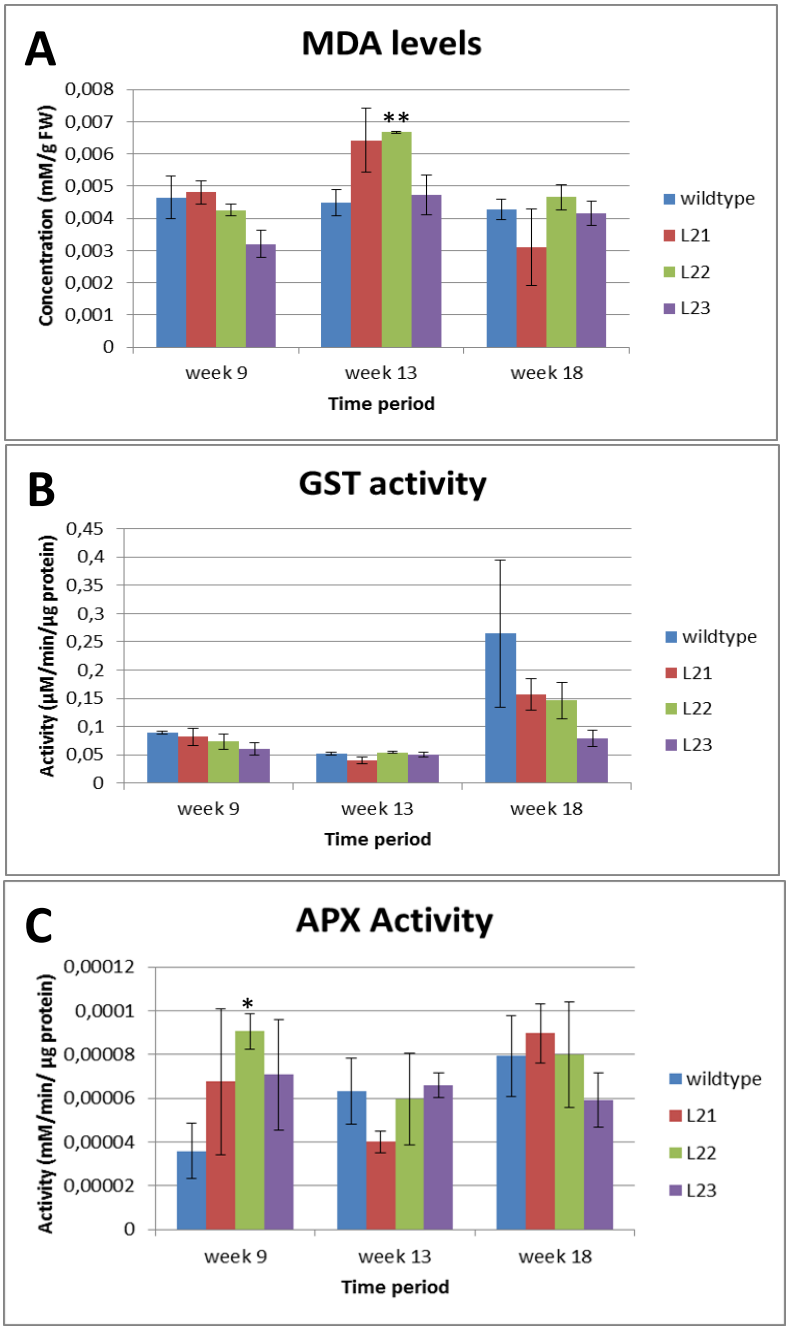


Figure 2.5: The effects of ectopic OCI expression on **(A)** lipid peroxidation and anti-oxidative activity of **(B)** GST and **(C)** anti-oxidation activity of APX in root nodules of transgenic lines 21, 22, 23 and wild-type soybean at different weeks after germination. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01).

2.4 Discussion

In this chapter, the main focus was to characterize soybean plants over-expressing OCI in their roots. Independent soybean lines transformed with OCI were identified. Furthermore, expression of the OCI gene was confirmed in some of these lines. The appearance of amplicons with different sizes can be attributed to impurities during DNA preparation. The presence of impurities can have an influence on DNA mobility during gel electrophoresis [Fan *et al.,* 1991]. These impurities were a consequence of the type of DNA preparation method used, which is cost effective but yields a crude nucleic acid mixture [Edward *et al.,* 1991]. Relative expression of OCI was characterized from weeks 6 to 8 for all transgenic soybean lines (L21, L22 and L23). Oryzacystatin-I expression of weeks 9, 13 & 18 were difficult to quantify due to low concentration of nucleotides obtained during RNA extraction (data not shown). A possible reason why this occurred is that nodule senescence has already began. Programmed cell death (PCD), like senescence, is a natural process and occurs as early as 5 weeks after nodulation in some soybean plants [Alesandrini *et al.,* 2003]. During PCD, there is a marked increase in protease activity along with high levels of reactive oxygen species (ROS). Programmed cell death is viewed as an onset of senescence processes and often marked by a decline in nitrogenase activity [Levine *et al.,* 1996; Desikan *et al.,* 1998*;* Alesandrini *et al.,* 2003]. The occurrence of PCD so early on during plant growth suggests that nodule senescence is initiated before plants are 9 weeks old. Although in some instances, nodule senescence has been shown to occur after ten to twelve weeks [Puppo *et al.,* 2005; van de Velde *et al.,* 2006].

The similar trend shared by transgenic lines and wild-type for both protein content and PLCP suggested that the effects of OCI are minimal by the time transgenic lines reach 9 weeks old. Overall, PLCP and protein content results indicate that

OCI does have some effect on protease activity in root nodules. Similar results were reported by other researchers. Kim *et al.,* 2007 showed that the activity of an endogenous protease, in a transgenic plant, can be reduced by an exogenous protease inhibitor. Moreover, other previous results reported by Van der Vyver *et al.,* 2003 and Prins *et al.,* 2008 proved that OCI inhibition can lead to an increase in soluble protein content.

Legumes obtain their nitrogen supply from two sources namely minerals found in the soil or via nitrogen fixed in root nodules [Keyser and Li, 1992; Tajima *et al.,* 2004]. During this MSc study, soybean plants were watered with nitrogen-free Hoagland's solution and distilled water. This was done to ensure that nodulation occurred and that the plants had one source of nitrogen supply. The presence of no significant difference in ureide content at the different weeks, together with the similar trend in concentrations between transgenic lines and wild-type suggested that ectopic OCI expression had no detectable effect on nitrogen fixation. It was suspected that this was due to the low levels of OCI occurring during these weeks.

Lipid peroxidation occurs when ROS produced in cells react with the interior of the plasma membrane. This process does occur naturally in all plants and is often used as an indicator of the overall oxidative state in the cell [Zhang *et al.,* 2007]. Plants are known to actively produce ROS which may control many different physiological processes such as biotic and abiotic stress-response, pathogen defence and systemic signalling [Dalton *et al.,* 1999]. An excess accumulation of ROS in the cell results in oxidative stress [Sharma *et al.,* 2012]. Transgenic lines and wild-type showed variations in cell oxidative states and response to oxidation. Usually, ROS can function as secondary messengers, protective or harmful biomolecules [Gratao *et al.,* 2005]. The functionality of ROS molecules are determined by the equilibrium between ROS production and scavenging in plant cells [Foyer and Noctor, 2005; Gratao *et al.,* 2005]. The variations in MDA levels

and activity of anti-oxidant enzymes in this study suggested that there are different states of equilibrium between the production and the scavenging of ROS in all soybean lines. Furthermore, OCI inhibition did not have any detectable influence on antioxidant functionality. Similar results were reported in studies conducted by Li *et al.,* 2015. In their research, they showed that ectopic expression of a cystatin, from *Jatropha curcas*, into tobacco plants did not reduce oxidation. In contrast to our findings, Belenghi *et al.,* 2003 showed that over-expressing phytocystatin (AtCYS1) can reverse the effects caused by oxidative stress, in turn preventing plant PCD.

Oryzacystatin-I (OCI) inhibition had minimal effect on shoot development as indicated by the lack of significant differences in the phenotypic analyses between transgenic lines and wild-type. Moreover, our initial results suggested that OCI inhibition does not lead to major improvements in the growth rate of shoot or root systems. Interestingly, this MSc results contradicted with the findings by Gutiérrez-Campos *et al.,* 2001 and Zhang *et al.,* 2008. Gutiérrez-Campos et al., 2001 illustrated that constitutive expression of OCI in tobacco plants can lead to an increase in shoot height, dry weight and fruit-bearing capabilities. OCI inhibition accelerated growth rate in these transgenic tobacco but also reduced their life cycle. Similarly, Zhang *et al.,* 2008 reported that over-expressing a cysteine PI in transgenic *Arabidopsis* increased fresh weight during both normal and stress growth conditions. These differences in this MSc results to theirs suggested that the effect of OCI inhibition on phenotype differs based on plant type or organelle location, where transgenic soybean lines expressing OCI in leaves tend to have more chlorophyll [Quain *et al.,* 2014]. While OCI expression in root nodules does not influence leaf chlorophyll content but does increase trifoliate leaf number (Figure 2.2A and B).

The observation of these minimal/non-detectable effects of OCI expression could be explained by the low concentrations of the inhibitor at different weeks. This study showed that OCI is expressed at high levels at week 8 for all transgenic lines. Thus, it is possible that OCI expression declines after week 8, resulting in minimal differences between transgenic lines and wild-type. Another observation worth noting was the variation in OCI expression in each independent transgenic line. These differences in OCI expression could have aroused as a result of a number of factors such as transgene location in genome, copy number of transgene or DNA modification (methylation) [Muskens *et al.,* 2000; Lechtenberg *et al.,* 2003; Tang *et al.,* 2007]. Similar variations in expression were also reported by Marra *et al.,* 2009 in their studies with transgenic soybean lines, where a cysteine protease propeptide (PROHGCP) from *Heterodera glycines* was expressed in roots. Differential expression in these transgenic lines correlated with the different amounts of PROHGCP detected during Western blot analysis.

In the next chapter, transgenic soybean lines over-expressing OCI in their root nodules were further characterised. Soybean plants grown under drought conditions were investigated in order to determine whether ectopic OCI expression, in root nodules, improves drought tolerance.

Chapter 3

Characterization of transgenic soybean lines

grown under drought conditions

3.1 Introduction

Drought is one of the major abiotic stresses that have very severe damage on commercial crops. It often results in worldwide reduction of crop yield, which in turn has a major impact on food security [Bray *et al.,* 2000]. The frequency and severity of droughts are predicted to increase as a result of climate change together with increases in the land areas experiencing drought [Jury and Vaux, 2007]. Soybean is one of the crops that are severely affected by drought [Clement *et al.,* 2008]. Drought leads to a decrease in nitrogen fixation which decreases seed yield [Serraj *et al.,* 1999]. In particular, the symbiotic relationship between the plant and the bacteria is very susceptible to environmental stresses. It was already known for 40 years that drought stress negatively affects nitrogen fixation as well as nitrogenase activity and that biological nitrogen fixation and plant biomass decline under water stress [Zahran, 1999; Pimratch *et al.,* 2008].

Drought-induced senescence is marked by the degradation of symbiosis partner proteins, such as leg-haemoglobin. When leg-haemoglobin is broken-down there is a release of free iron, which in turn react with hydrogen peroxide generating an excess of ROS [Becana *et al.,* 1998; Puppo et al., 2005; DeLaat *et al.,* 2014]. Accumulation of ROS activates oxidative stress signalling pathways which function in collaboration with other hormonal responses [Noctor *et al.,* 2014]. There are many studies that have investigated the impact of drought on different aspects of biological nitrogen fixation. Their findings showed that drought reduces specific activity in root nodules, limits the formation of new nodules and nodules shrink in biomass [Serraj *et al.,* 1999; King and Purcell 2001; Streeter 2003]. In this chapter, transgenic soybean plants were grown under drought conditions.

The main aim was to determine whether ectopic OCI expression can reduce the effects of drought-induced senescence. Since, the results in chapter 2 indicated

that OCI expression is highest at week 8 after germination we decided to investigate the effects of OCI during drought conditions prior to week 9. The objective of this chapter was to characterize any biochemical, molecular, morphological and physiological changes that affect plant growth and development.

3.2 Material & methods

3.2.1 Plant material and growth

Commercial soybean and transgenic seed were sown with *Bradyrhizobium japonicum* (strain WB 74-1) powder. Seedlings were grown in pots [17.5 cm x 20 cm diameter (top) and 13.1 cm] in fine-grade vermiculite. Plants were grown under natural light conditions which were extended with artificial lights up to a 13 hrs photoperiod at 600 mM $mol^{-2}s^{-1}$ photosynthetically active radiation (PAR) and 60% relative humidity in an environmentally controlled green-house (phytotron) at 25°C day / 16°C night temperature. Plants were watered twice a week with de-ionised water and three-times a week with a nitrogen-free Hoagland solution to obtain nodule formation. Pots were re-arranged periodically to allow uniform exposure to light in the growth chamber.

3.2.2 Drought treatment

Drought trial plants were grown as described in section 3.2.1 until they reached 7 weeks old. Afterwards, water was withheld for the next 7 days to allow soil moisture content (SMC) to reach 30%, referred to as drought conditions. At the end of week 8 root nodules were harvested along with those of well-watered (control).

3.2.3 Harvesting of Plant Material

For sample collection, three plants were harvested after 8 weeks. Root nodules (or trifoliate leaves) were collected over this period and immediately stored after each harvest in a -80°C freezer until further analysis.

3.2.4 Morphological parameters

Morphological parameters were determined at described in section 2.2.3 for both growth conditions. Chlorophyll a and b concentrations in leaves were determined as described by Lichtenaler and Wellburn, 1983 in section 2.2.3. Nodule mass were determined by weighing fresh nodules after harvesting and nodule number was obtained by counting nodules as they were harvested.

3.2.5 RNA extraction and cDNA synthesis

Pre-stored root nodules were ground in liquid nitrogen and used for total RNA extraction with a Plant RNA Mini Kit according to the manufacturer's instructions. Extracted RNA was then used to synthesize cDNA with an ImProm II reverse transcriptase kit used according to the manufacturer's instructions. The same qPCR primer sets were used for OCI and reference (40S) gene (refer to table 2.1). The reaction mixture was 10 µl with the following composition: 5 µl of SYBR Green, 1 µl of DNA template (100 ng/µl), 0.5 µl of each (forward & reverse) 10 µM primer and 3 µl distilled water. Reactions were initiated by placing in a real-time thermal cycler and ran with same parameters as in section 2.2.5 with a melting curve analysis added at between 75°C and 95°C. For a detailed description refer to section 2.2.6.

3.2.6 Protein extraction

Pre-stored root nodules ground into a powder while in liquid nitrogen. Total proteins was extracted by adding a protein extraction buffer to the powdered plant material. The mixture was centrifuged at 13 000 xg for 40 min at 4°C and the supernatant containing the protein was stored at -80°C. Total soluble protein content was determined using the Bradford assay reagent and BSA as a standard. In each standard and protein sample, 5 µl was loaded in triplicates onto a clear 96 well plate and 250 µl of the Bradford reagent was added. The reaction placed in the dark and incubated at room temperature for 10 min. The absorbance was determined using a micro-plate reader with protein extraction buffer as the blank. For a detailed description refer to section 2.2.8.

3.2.7 PLCP activity assay

Total soluble protein (~ 5 mg/ml) from nodule extracts were used for measuring PLCP activity. Reactions were individually loaded into a black, flat-bottom polysorp 96 well plates. Each well had 10 µl of protein sample, 82 µl of sodium phosphate buffer (pH 6.0) containing 10 mM L-cysteine and 8 µl of 200 µg/µl substrate (Z-Phe-Arg-MCA). Fluorescence development was measured with a fluorescence spectrophotometer at 37°C with excitation and emission wavelengths of 360 nm and 450 nm, respectively. Reactions were monitored over a 10 min period time period. All reactions were carried out in triplicate. As a control, the reaction contained the buffer and substrate but no protein extract. For detailed description refer to section 2.2.9.

3.2.8 Ureide extraction

Root nodules were grounded in liquid nitrogen and NaOH was added to the powdered material before boiling for 20 min and cooling on ice. This was followed by centrifugation at 10 000 xg for 10 min then the collected supernatant was diluted eight times with distilled water. NaOH was added before boiling the samples for 10 min and chilled on ice. Followed by adding of phenylhydrazine/HCl and boiling again for 2 min. The mixture was chilled for 5 min prior to adding solution of HCl/KFeCn and incubating at room temperature for 10 min. Prepared standards and samples were left at room temperature for 10 min before reading at an absorbance of 525 nm with a spectrophotometer. For detailed description refer to section 2.2.10.

3.2.9 Enzymatic anti-oxidant assays

3.2.9.1 GST activity assay

Extracted protein (section 3.2.6) from the respective samples were aliquoted into different plastic cuvettes. To each of the protein samples, a reaction mixture containing 1 mM CDNB, 1 mM glutathione made up in PBS buffer (pH 6.5) were added. The absorbance was measured at 340 nm after 3 min to determine the kinetic rate. For detailed description refer to section 2.2.11.1.

3.2.9.2 APX activity assay

Assays were prepared in triplicates onto a clear 96 well plate with each well loaded with 10 µl extracted protein (section 3.2.6), 188 µl of ascorbate buffer and 5 µl of 5 mM ascorbic acid. The absorbance was measured at 290 nm after 10 min to determine the kinetic rate. For detailed description refer to section 2.2.11.2.

3.2.10 MDA extraction

Root nodules were ground into a powder in liquid nitrogen and 6% (w/v) trichloroacetic acid was added. The sample was homogenised using a vortex and then centrifuged at 1000 xg for 10 min to pellet the plant material. The supernatants was transferred into a new tubes, to these tubes 0.5% (w/v) thiobarbituric acid was added. The samples were briefly mixed once again using a vortex. The sample tubes were placed in a heating block and was allowed to incubate at 90°C for 20 min. Following the incubation at high temperature the samples were incubated on ice for 10 min. Once the incubation on ice was completed the samples were centrifuged at 13 000 xg for 5 min. All extracted samples were then loaded in triplicate on a 96 well microtitre plate. The plate was then read at 532 nm and 600 nm on a spectrophotometer. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to correct for the non-specific turbidity. For detailed description refer to section 2.2.12.

3.2.11 Physiological parameters

In this study, we focused on photosynthetic CO_2 assimilation, stomatal conductance, and transpiration rates as physiological parameters. These parameters were determined on the central leaflet of the central leaflet in each case of attached first trifoliate (bottom), fourth trifoliate (middle) and seventh trifoliate (top) leaves between 4 and 6 hrs after the start of the photoperiod using the LI-6400XT portable photosynthesis system (LI-COR, USA). Instantaneous water-use efficiency (IWUE) values were calculated as the ratio between CO_2 assimilation rates and stomatal conductance values as described previously by Soares-Cordeiro *et al.,* 2009.

3.2.12 Statistical analysis

Data for all experimental parameters were analysed using Student's t-test at stringency level of $p < 0.05$ comparing wild-type soybean plants directly to transformed soybean plants. In all experimental at least three biological replicates were used. One-way ANOVA was further employed to confirm significant differences found in statistical data using the Tukey method as a post-hoc test at stringency level of $p < 0.05$ on a GraphPad Prism windows interface.

3.3 Results

3.3.1 Relative OCI expression

The relative expression of OCI in the root nodules of soybean plants grown under well-watered and drought conditions are shown below (Figure 3.1). In this figure, it could be seen that OCI expression was high under both well-watered and drought conditions. OCI expression was highest when plants were well-watered and showed a decline in expression when plants were under drought conditions. Line 21 had the highest OCI expression of the transgenic lines under both growth conditions with expression being significantly high when plants were well watered. Similar expression levels were seen in well-watered L21 plants during initial characterizations in chapter 2 (Figure 2.1B). The least levels of OCI expression were observed in L23 under both growth conditions with expression being significantly low when plants were under drought.

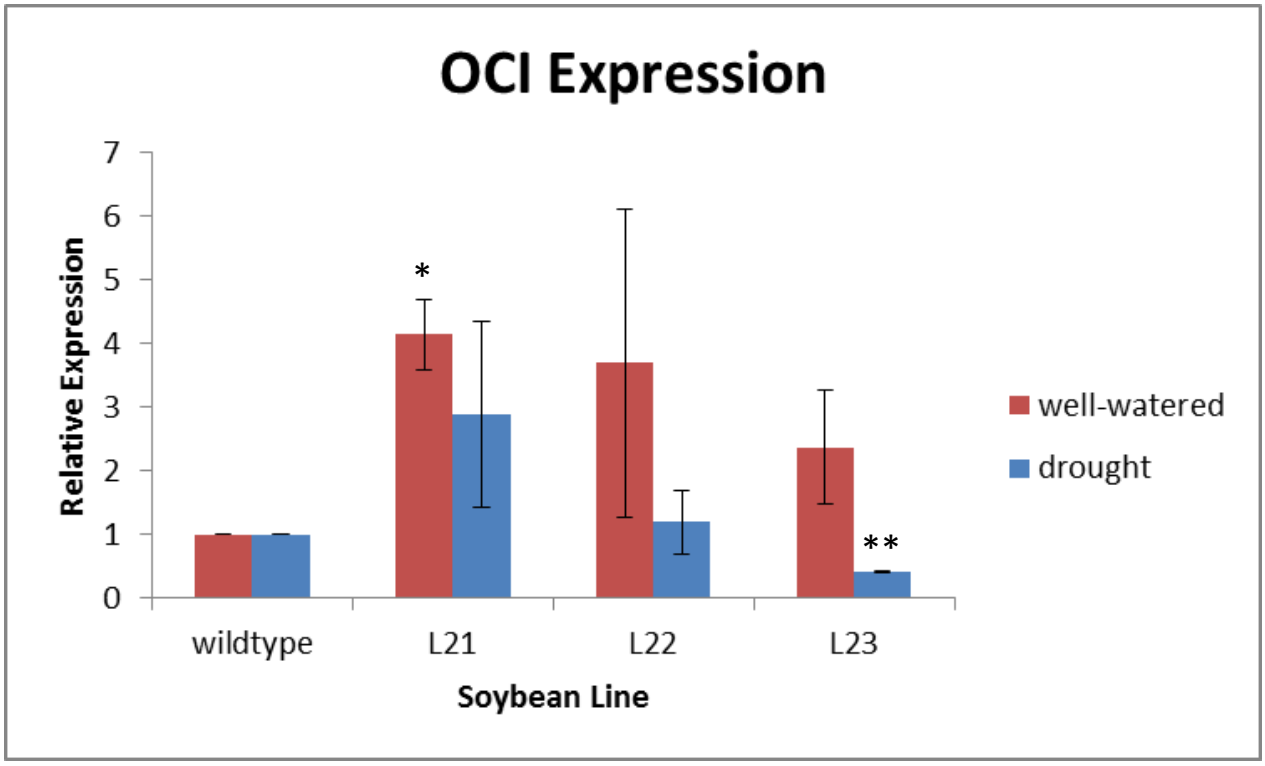


Figure 3.1: Relative OCI expression in root nodules of transgenic lines 21, 22, 23 and wild-type soybean during well-watered and drought conditions (30% SMC). Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants. (*p<0.01, **p<0.001).

3.3.2 Morphological characterization

The biomass (fresh and dry) of roots and shoots were both marked by a decrease in weight when plants were under drought conditions, except for dry root mass which had an increased for almost all drought-treated plants (Figure 3.2A-D). No significant difference was observed between fresh root weight of transgenic lines and wild-type during both growth conditions (Figure 3.2A). However, the dry root weight of well-watered transgenic lines (L21 and L23) was significantly higher than wild-type (Figure 3.2C). Interestingly, drought-treated L22 was the only soybean line that showed a decrease in dry root mass. Shoot fresh weight of well-watered transgenic lines were higher than wild-type, with L21 being the only transgenic line significantly higher than wild-type (Figure 3.2B and D). This trend was not seen in shoot biomass under drought conditions, where biomass was similar in all soybean lines. Water content in roots of well-watered transgenic lines (L22 and L23) were significantly less than wild-type (Figure 3.3A). There was no difference in root water content under drought conditions and all soybean lines showed a similar decline in water content. Shoot water content (Figure 3.3B) was less when compared to root water content. This was indicated by the slight decline of shoot water content during drought.

Although shoot water content was similar for all soybean lines under both growth conditions, well-watered L21 had a shoot water content significantly lower than wild-type. Well-watered transgenic lines had nodules that weighted less than well-watered wild-type, with L23 significantly lower than wild-type (Figure 3.3C). There was a decline in nodule mass for all soybean lines under drought conditions. However, no significant difference was seen between nodule mass of transgenic lines and wild-type under drought conditions. Interestingly, drought treated wild-type showed the highest decline in nodule mass when compared to transgenic lines. Similar to other morphological parameters, nodule number decreased in all

soybean lines during drought conditions (Figure 3.3D). Although no significant difference was found between nodule number of transgenic lines and wild-type under both growth conditions, there was a trend observed in which transgenic lines seemed to have more nodules than wild-type during both growth conditions. Visually, the wild-type looked similar to transgenic L23, with both plants having a more “bush-ish” shoot compared to other transgenic lines (L21 and L22) (Figure 3.4). Transgenic line 21 showed the least amount of leaves. Bottom (old) trifoliate leaves of all soybean lines had yellowed and senescing while the top (young) trifoliate leaves were still green. Overall, it seemed that a 7 day drought did not have such an adverse influence on the physical appearance of both transgenic lines and wild-type plants.

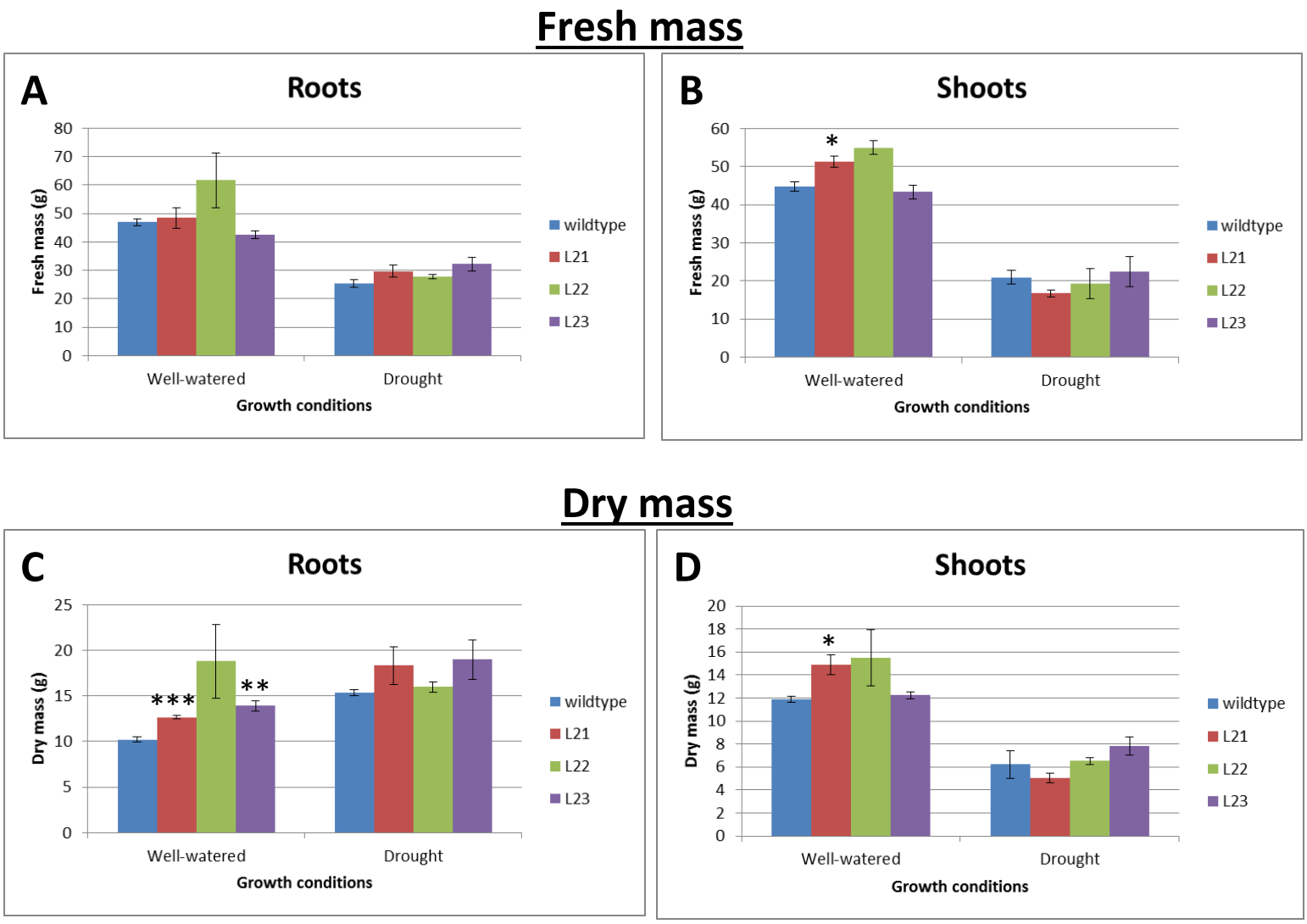
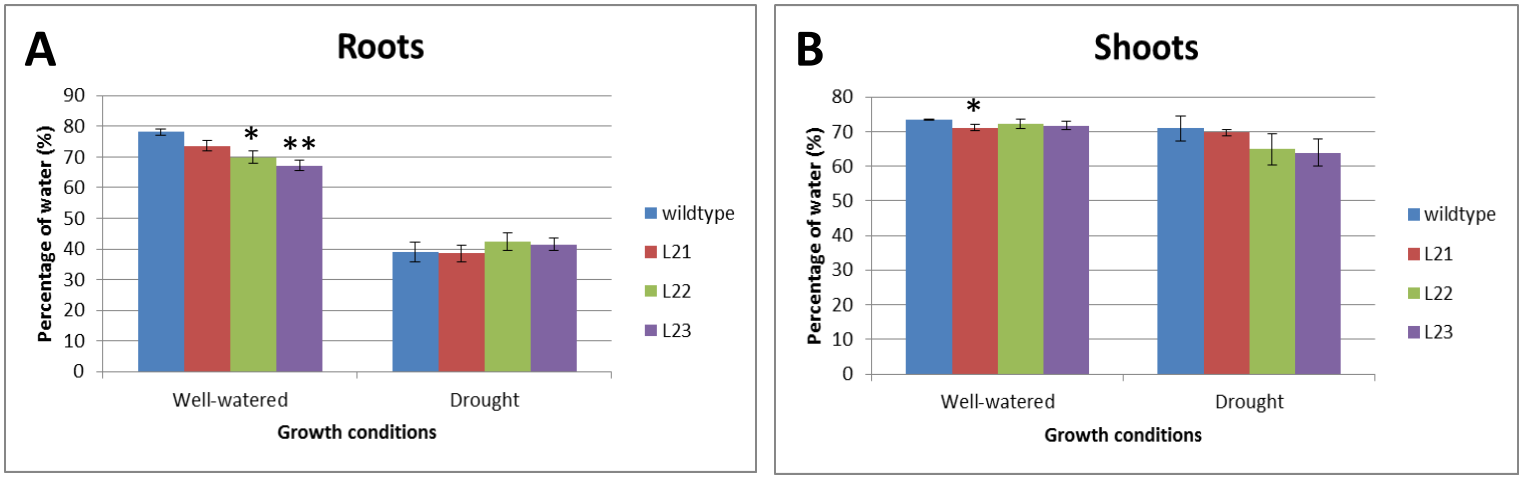


Figure 3.2: Effects of drought on plant biomass of the transgenic lines 21 22, 23 and wild-type soybean as represented by **(A)** fresh root weight, **(B)** fresh shoot weight, **(C)** dry root weight and **(D)** dry shoot weight. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01, *** p<0.001).

Water content



Nodules

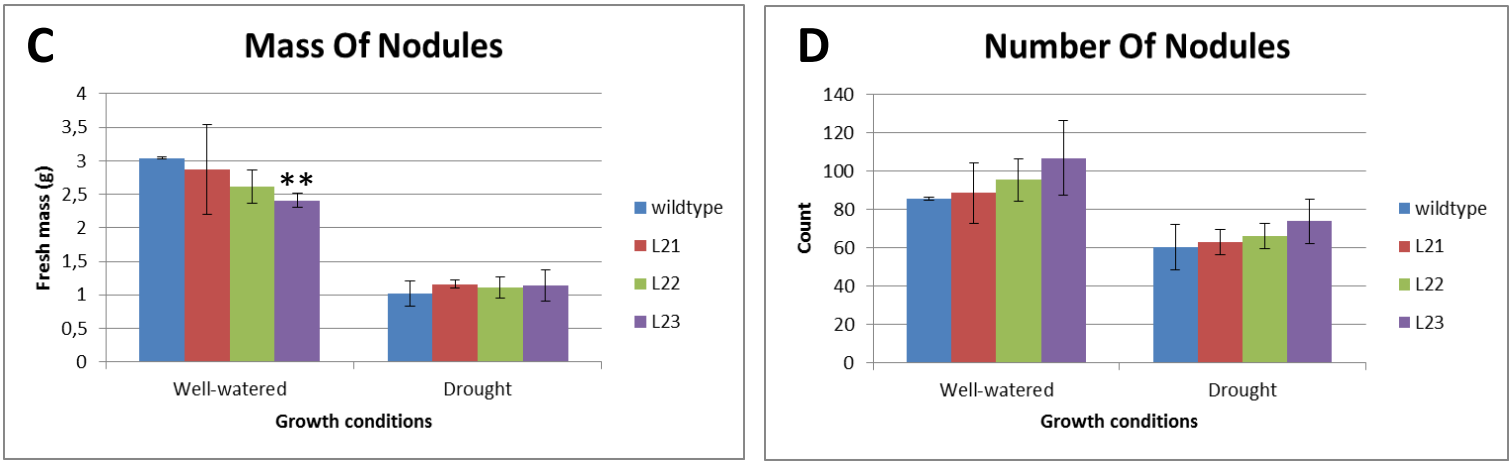


Figure 3.3: Effects of drought on transgenic lines 21, 22, 23 and wild-type soybean as indicated by **(A)** roots water content, **(B)** shoots water content, **(C)** mass of nodules and **(D)** number of nodules week 8. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01).

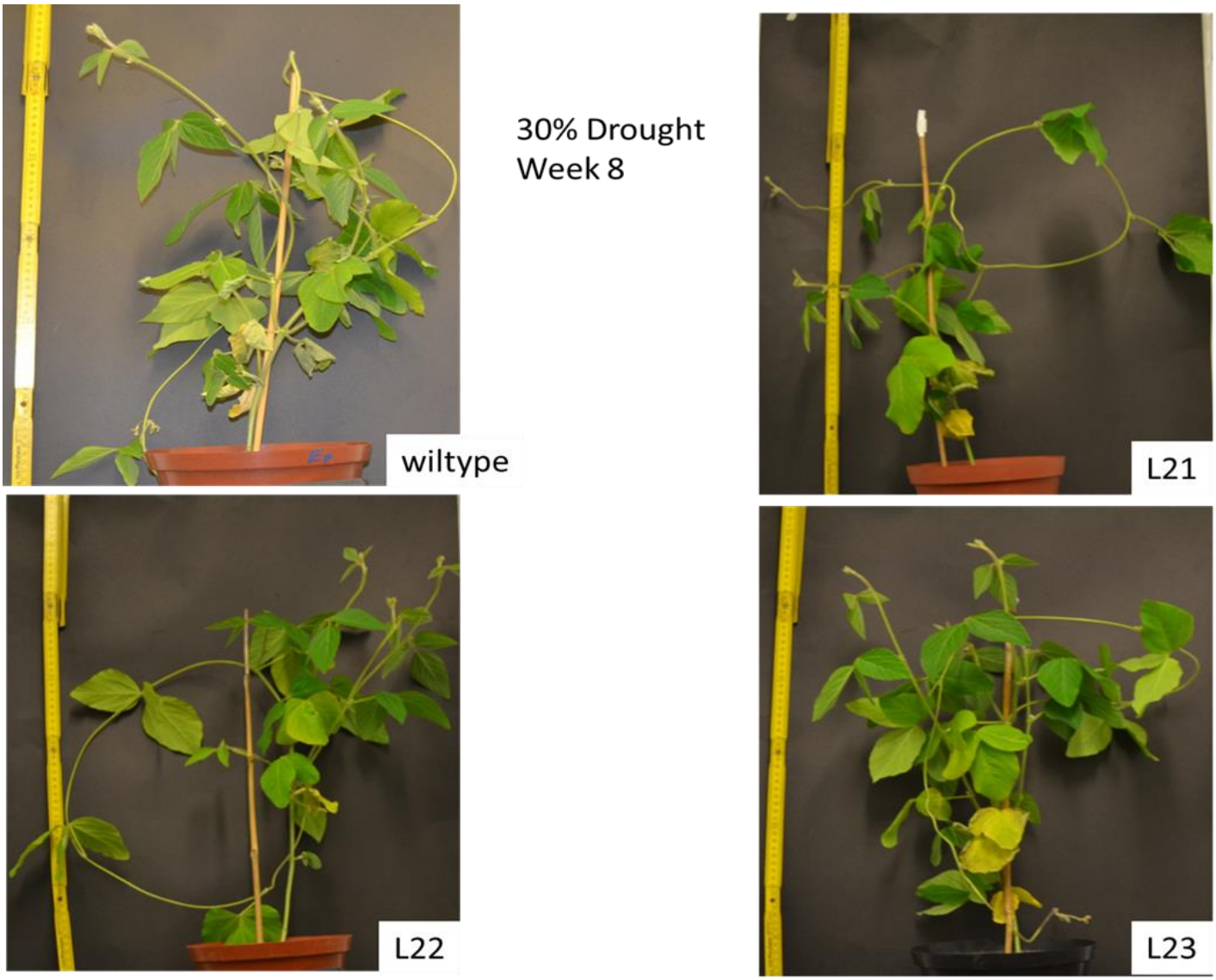


Figure 3.4: Images of transgenic lines 21, 22, 23 and wild-type soybean after exposure to drought conditions (30% SMC) for seven days.

3.3.3 Total soluble protein content

The concentration of soluble proteins was similar for all soybean lines under well-watered conditions (Figure 3.5A). Similar to OCI expression, protein content decreased when plants were under drought. Interestingly, transgenic line L23 was the only soybean plant to show an increase in protein content under drought when compared to well-watered L23 plants. Drought-treated transgenic lines had a significantly higher protein content when compared to wild-type.

3.3.4 PLCP activity

The incline in PLCP activity when plants are under drought correlated to the decline in protein content under similar growth conditions (Figure 3.5). This same event was observed with soybean plants characterized in chapter 2. The wild-type showed a higher incline in PLCP activity when plants were under drought compared to transgenic lines (Figure 3.5B). The activity of PLCP was significantly lower in transgenic lines (L21 and L22) for both growth conditions when compared to wild-type. This indicated that OCI is active in root nodules after its expression.

3.3.5 Ureide content

There seemed to be no uniform trend with the rate of nitrogen fixation (Figure 3.5C). During well-watered conditions, no significant differences were observed between transgenic lines and wild-type. Under drought stress, L22 was the only transgenic line with a nitrogen fixation rate that was significantly lower than wild-type. Nitrogen fixation increased in all soybean lines when plants were under drought conditions with L21 being the only expectation, which showed a decrease in nitrogen fixation when plants were under drought. However, no significant difference was seen in the rate of nitrogen fixation between transgenic lines and wild-type during drought conditions.

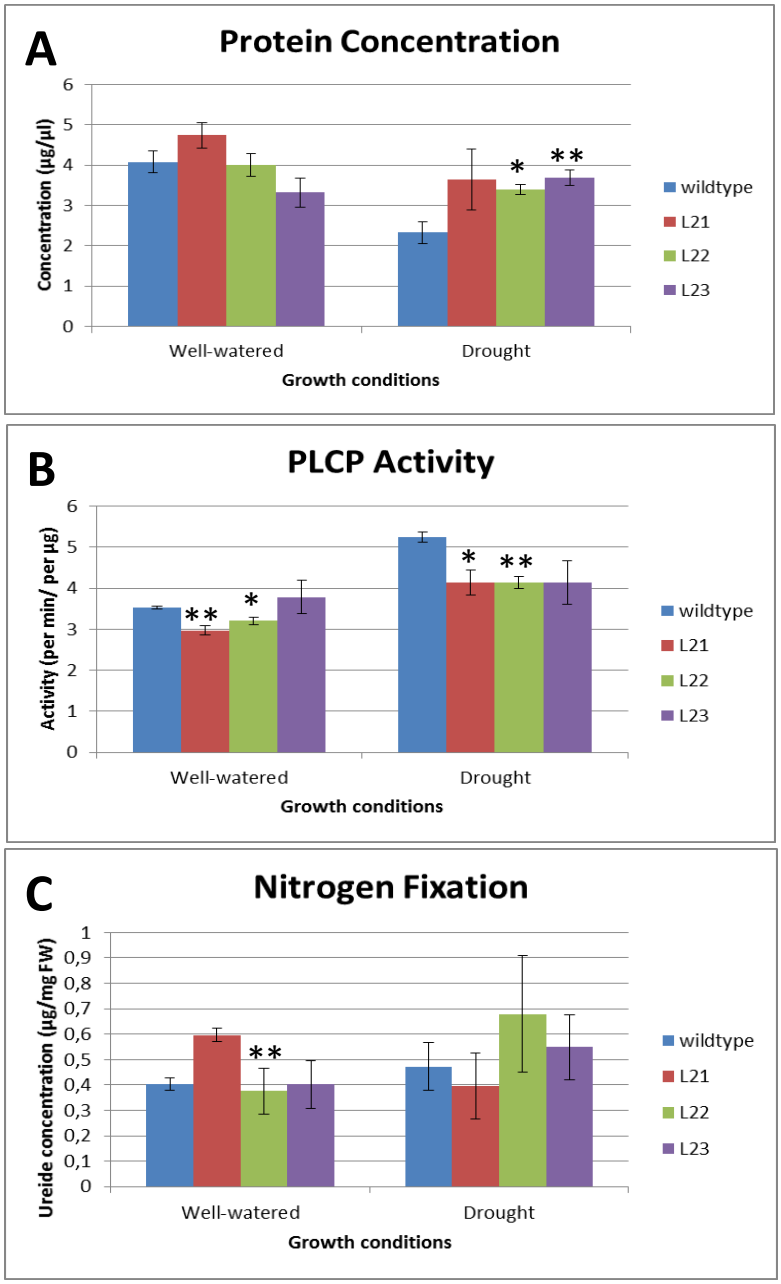


Figure 3.5: Effects of drought on root nodules of transgenic lines 21, 22, 23 and wild-type soybean as represented by **(A)** total soluble protein content, **(B)** PLCP activity and **(C)** nitrogen fixation. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01).

3.3.6 Lipid peroxidation

The concentration of MDA increased in all soybean lines when plants were not watered, but had no significant difference between transgenic lines and wild-type (Figure 3.6A). Drought-treated wild-type showed a higher incline in MDA level when compared to drought-treated transgenic lines, while the incline in all independent transgenic lines was similar. During well-watered conditions, transgenic lines (L21 and L23) had significantly higher levels of MDA than wild-type.

3.3.7 Enzymatic anti-oxidant activity

Similar to MDA concentration, GST activity increased during drought in all lines expect for L23, which had a decrease in activity (Figure 3.6B). Drought-treated transgenic lines (L21 and L22) had GST activity significantly lower than wild-type. Wild-type also had the highest incline in GST activity under drought conditions. All soybean lines had similar levels of GST activity during well-watered conditions. Although there was no significant difference in APX activity under drought conditions, APX activity of all drought soybean lines increased (Figure 3.6C). The highest incline in APX activity was observed in L22, while the least incline was seen in L23. There was no significant difference between the overall APX activity of transgenic lines and wild-type during drought conditions. Well-watered transgenic lines (L22 and L23) had an APX activity significantly lower than wild-type with L23 showing the least APX activity of all soybean lines.

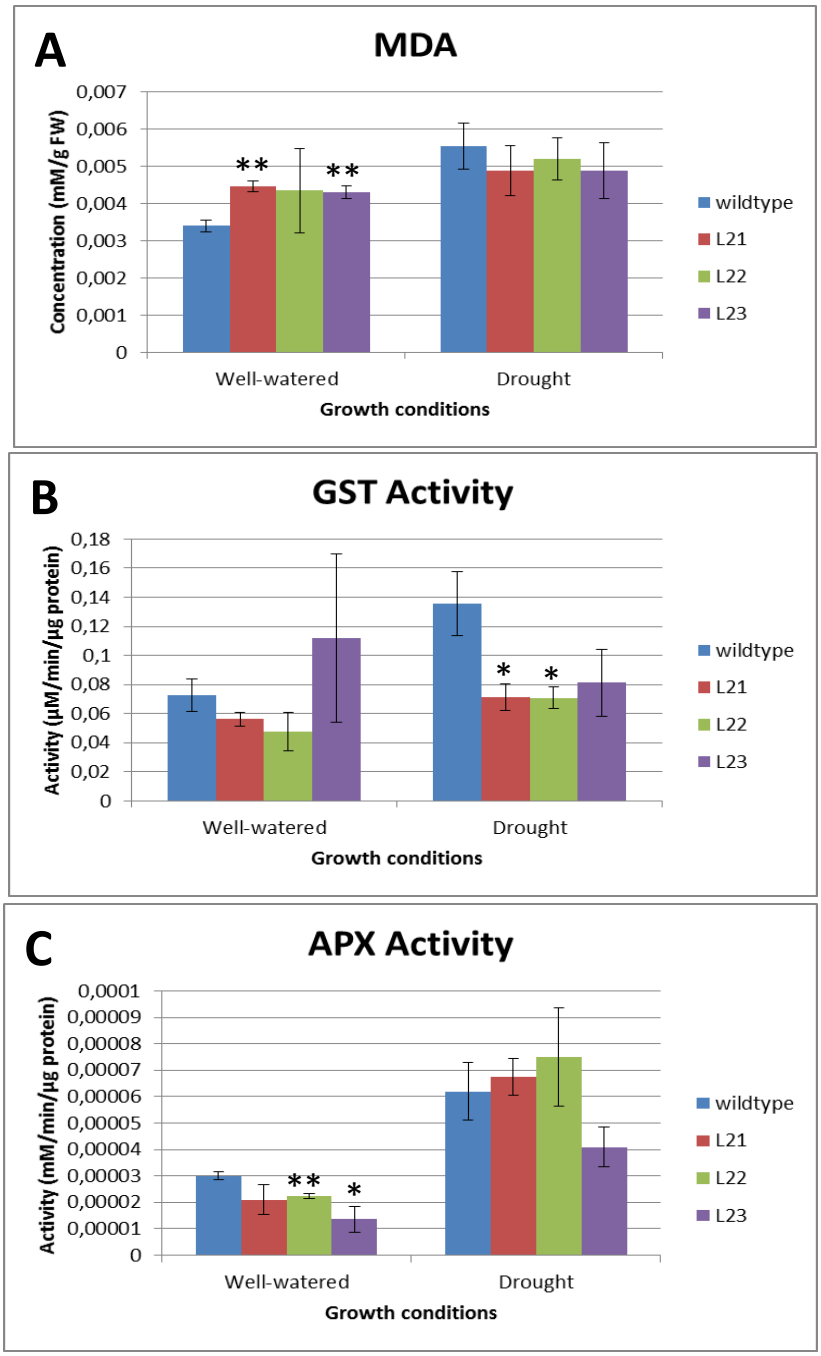


Figure 3.6: Effects of drought on transgenic lines 21, 22, 23 and wild-type soybean as represented by **(A)** MDA levels, and anti-oxidant activity of **(B)** GST activity and **(C)** APX activity. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01).

3.3.8 Physiological characterization

Various physiological parameters were determined in this section in order to investigate whether OCI inhibition in root nodules can influence development in shoots. Leaflets at different ages were measured using a LI-COR photosynthesis system machine. The concentration of chlorophyll was significantly lower in well-watered transgenic lines than well-watered wild-type (Figure 3.7). Well-watered wild-type had similar chlorophyll content between its young and older leaflets, while watered transgenic lines had varying chlorophyll content between its young and older leaflets. Although, there was no significant difference in the chlorophyll content between transgenic lines and wild-type under drought conditions. Drought-treated transgenic lines had an incline in chlorophyll content at all leaflets when compared to wild-type.

There was no significant difference between photosynthesis rates of transgenic lines and wild-type under both growth conditions (Figure 3.8). This suggested that photosynthesis rates are similar in all soybean lines even when plants are stressed. As expected there was a decrease in photosynthesis in drought-treated plants. Similarly to photosynthesis rates, there was no significant difference between stomatal conductance of transgenic lines and wild-type (Figure 3.9). All soybean lines showed a decrease in stomatal conductance when under drought conditions.

Transpiration rate of well-watered transgenic line L22 was significantly lower in intermediate aged (middle) leaves then well-watered wild-type (Figure 3.10). No other significant differences were observed between transpiration rates of transgenic lines and wild-type under well-watered conditions. Transpiration rates decreased drastically in all soybean lines when plants were grown under drought conditions. However, no significant differences were seen between transpiration rates of drought-treated transgenic lines and wild-type. The exact same trend for

stomata conductance is observed as CO_2 assimilation and transpiration. These findings suggested that photosynthesis was affected by transpiration and stomatal conductance. Transgenic lines had similar IWUE values under both growth conditions, while wild-type showed a decrease in IWUE under drought conditions (Figure 3.11). This suggested that the availability/usage of water in the leaves of transgenic lines was less affected by drought than those of wild-type. Similar to IWUE, no significant difference was observed between intracellular CO_2 concentration (Ci) of transgenic lines and wild-type under both growth conditions (Figure 3.12). Furthermore, Ci levels remained at similar levels in all soybean lines under both growth conditions.

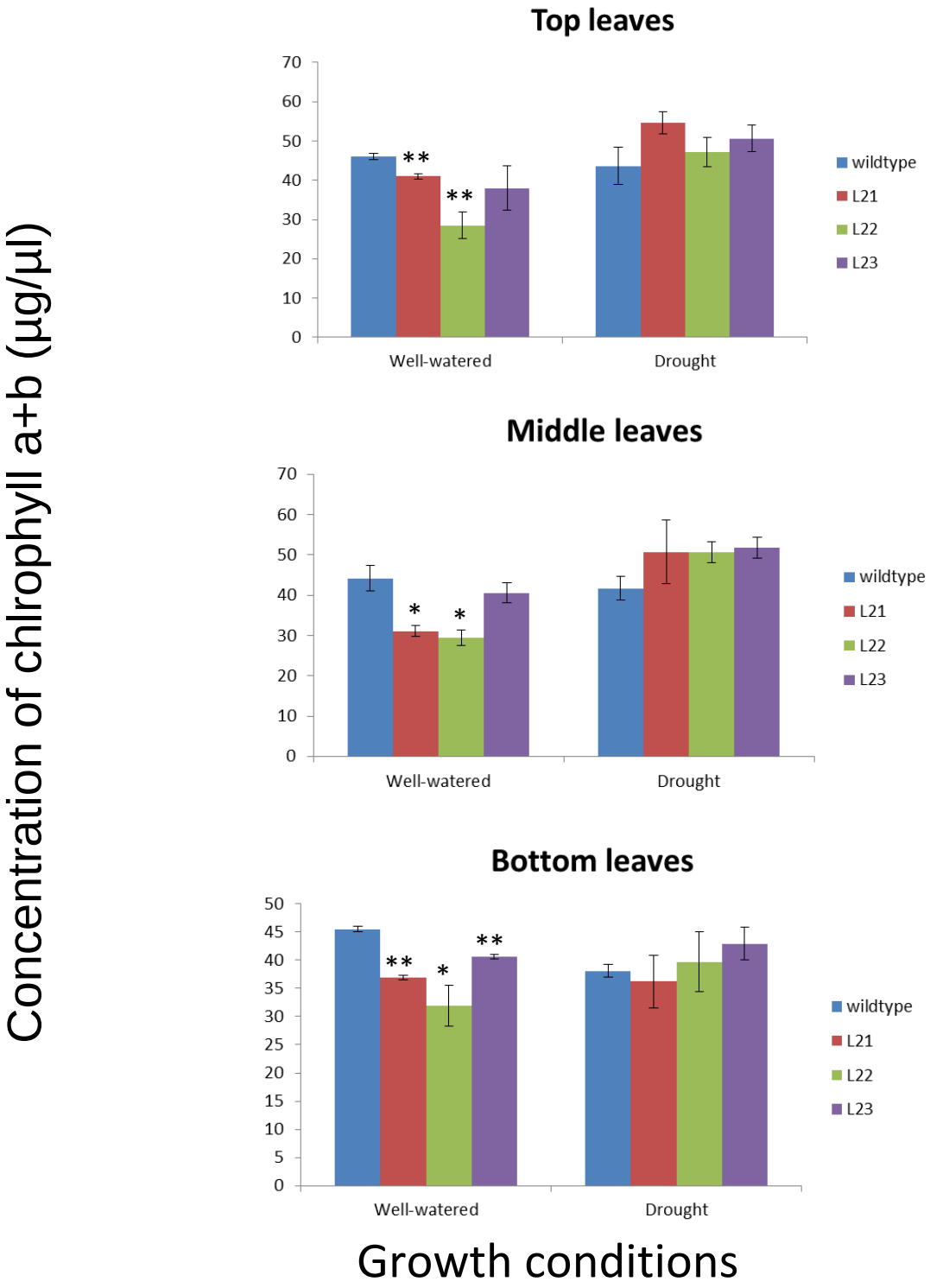


Figure 3.7: The effects of OCI inhibition on leaf chlorophyll content after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of three individual plants (*p<0.05, **p<0.01).

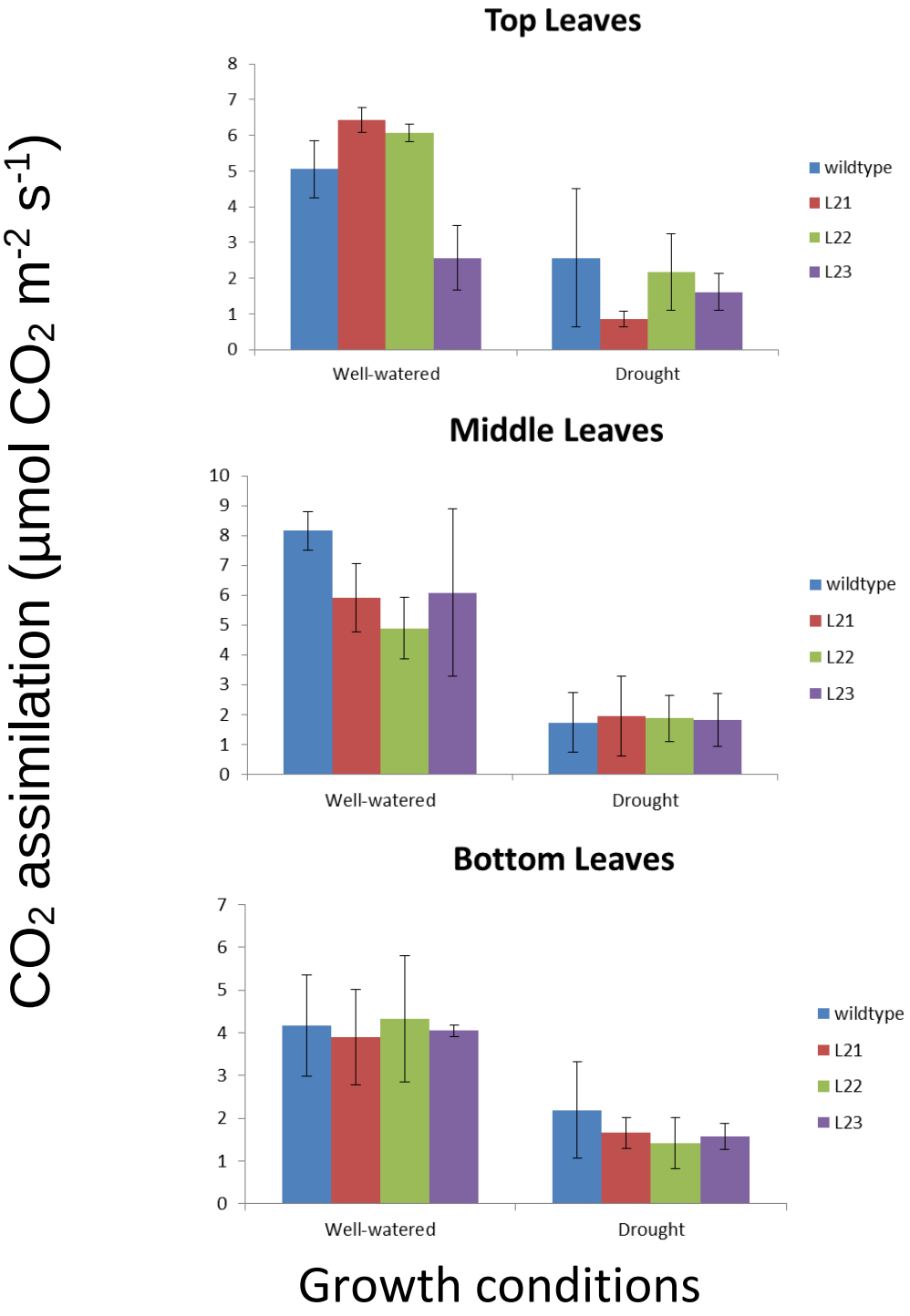


Figure 3.8: The effects of OCI inhibition on photosynthetic carbon assimilation after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Data represent the mean ± SE of three individual plants.

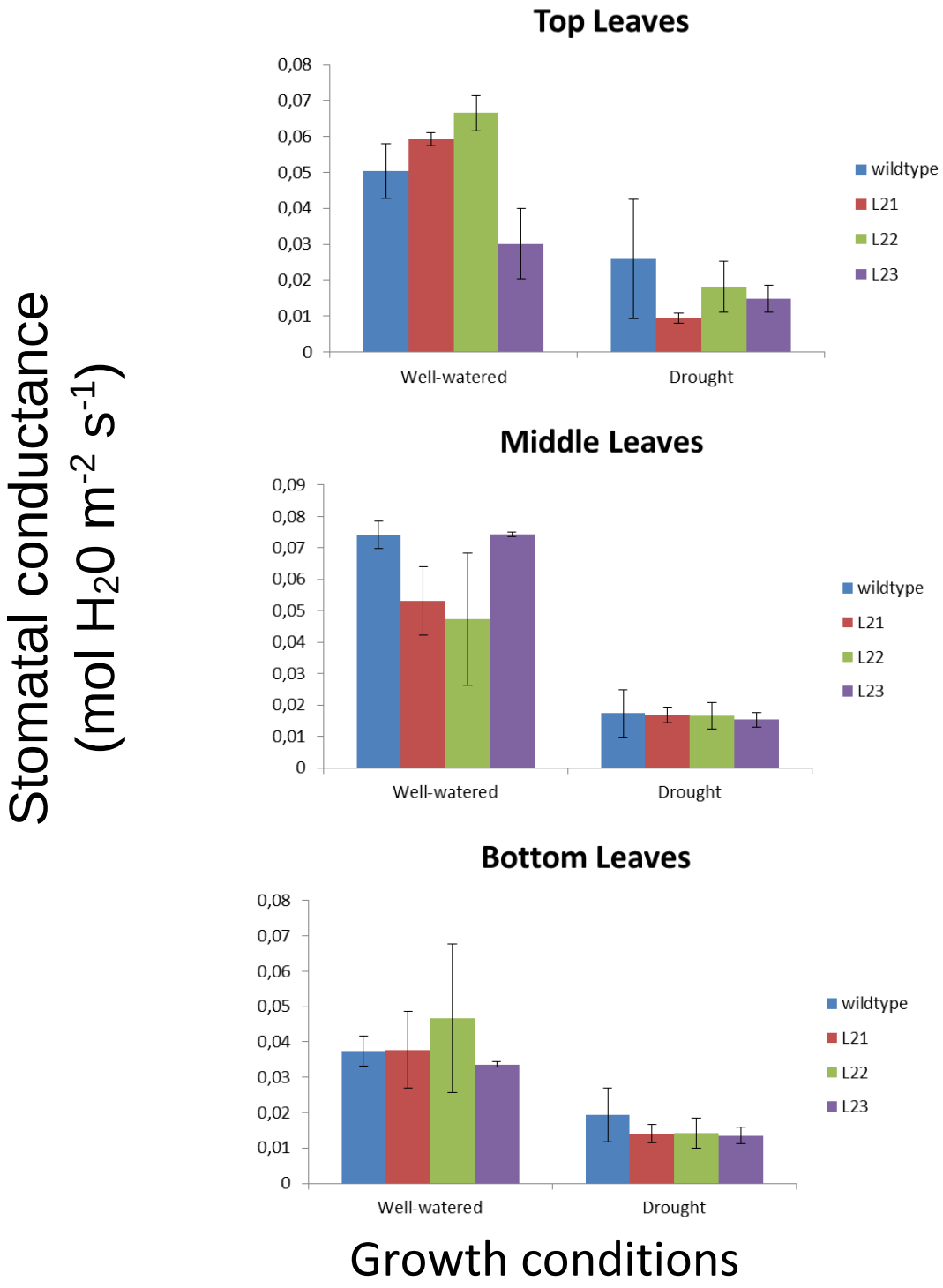


Figure 3.9: The effects of OCI inhibition on stomatal conductance after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Data represent the mean ± SE of three individual plants.

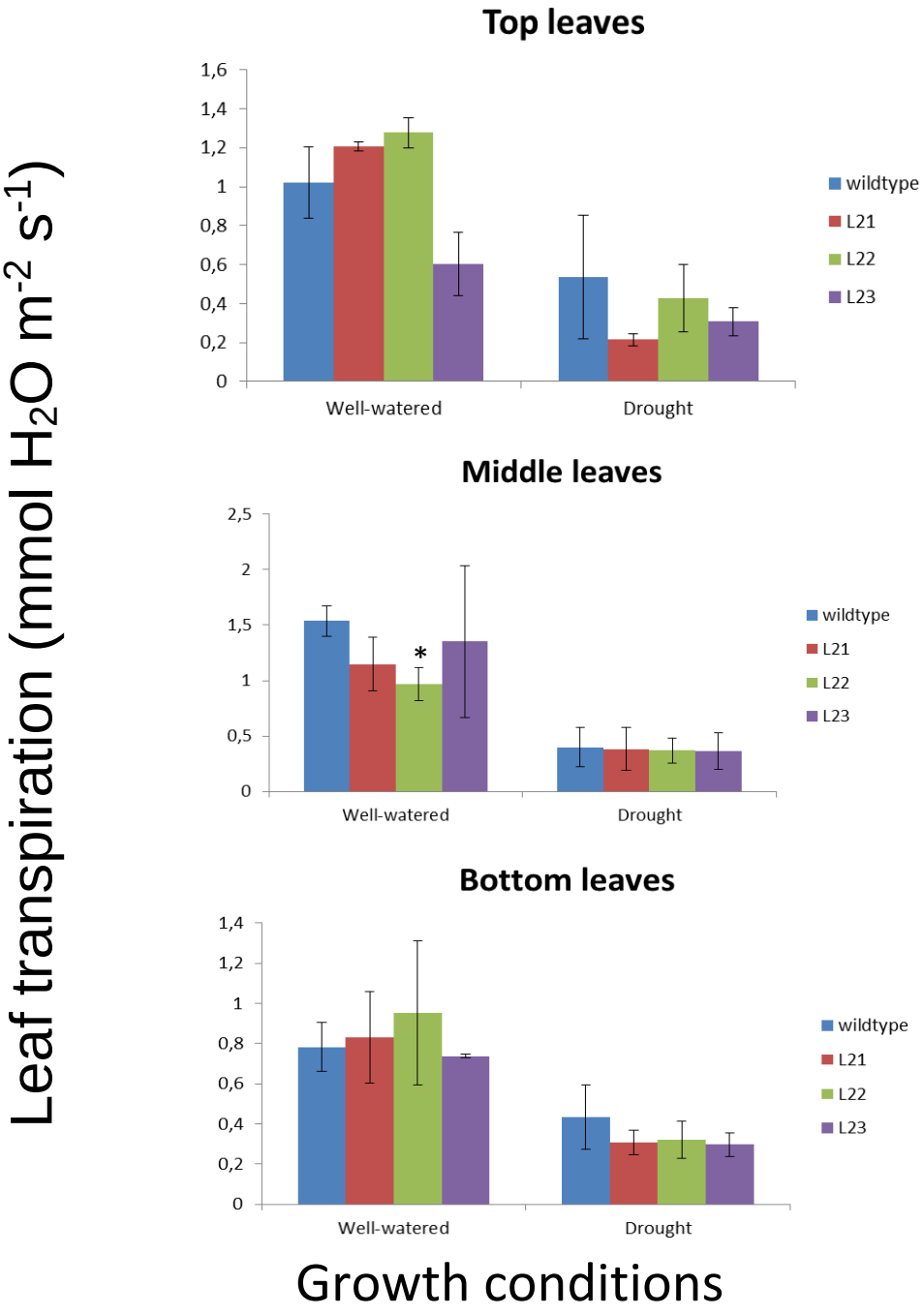


Figure 3.10: The effects of OCI inhibition on transpiration after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Significant differences were determined via the Student's t-test and are indicated by asterisks. Data represent the mean ± SE of 3 individual plants (*p<0.05).

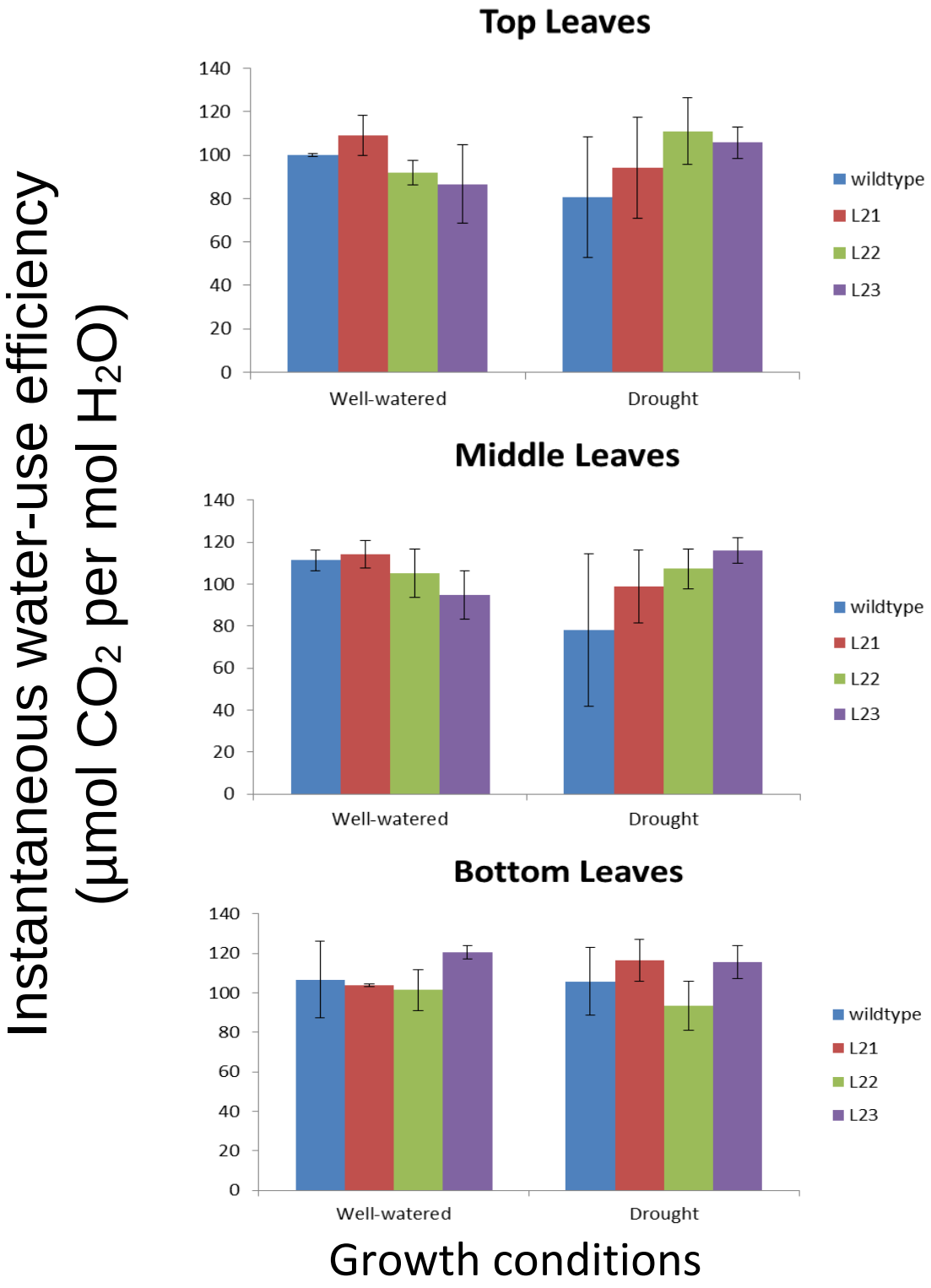


Figure 3.11: The effects of OCI inhibition on instantaneous water-use efficiency after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Data represent the mean ± SE of three individual plants.

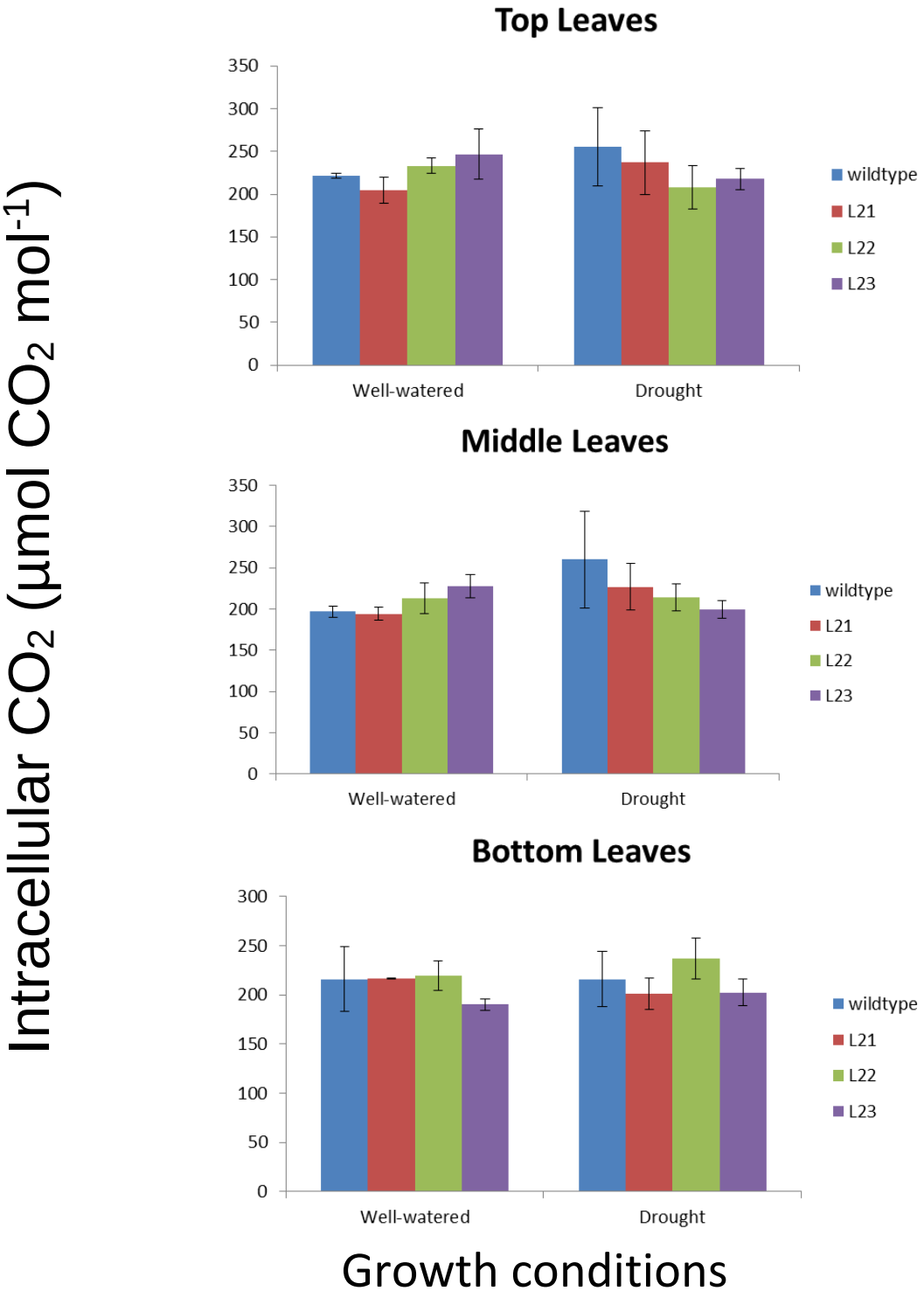


Figure 3.12: The effects of OCI inhibition on intracellular CO_2 concentration after SMC reached 30% (drought conditions). Measurements were taken in three leaf ranks (top, middle and bottom) for transgenic lines 21, 22, 23 and wild-type soybean. Data represent the mean ± SE of three individual plants.

3.4 Discussion

Biological nitrogen fixation (BNF) is an important process to agriculture due to its various benefits on production cost, environment and crop yield [Easterling *et al.,* 2007; Salon *et al.,* 2011]. Moreover, these benefits associated with BNF also promote local agricultural sustainability [Peoples *et al.,* 1995; Jensen and Hauggaard-Nielsen, 2003]. The absence of nodulation or premature senescence can have adverse effects on plant development and crop yield [Arrese-Igor *et al.,* 2011]. Since both premature and natural root nodule senescence are regulated by the activity of cysteine proteases, our interest was in trying to reduce the functionality of these proteases. Cysteine proteases play a role in a number of plant processes, such as seed germination, fruit ripening and flowering, and are usually negatively regulated by phytocystatins, which are plant cysteine proteases inhibitors [Abe and Arai, 1991; Alonso and Granell, 1995].

Phytocystatin are known to regulate cysteine proteases involved in movement of storage peptide, programmed cell death, senescence and seed development [Arai *et al.,* 2002; Sugawara *et al.,* 2002; Szewińska *et al.,* 2013]. Oryzacystatin-I is an example of such a phytocystatin and inhibits α, β and γ cysteine proteases that are produced during rice seed germination [Watanabe *et al.,* 1991]. Our earlier results (in chapter 2) showed that OCI can influence the activity of PLCP when it's expressed in relatively high levels. This was further supported by the OCI expression data of this chapter. Interestingly, the expression of OCI was reduced in all transgenic lines during drought growth conditions. This was in contrast with findings by Zhang *et al.,* 2008, where transgenic *Arabidopsis* had an incline in OCI expression during drought stress. Similarly, Rui *et al.,* 2015 reported that abiotic stress increases phytocystatin (JcCPI) expression in transgenic plants during drought.

The decline in OCI expression during drought conditions could possibly be due to the regulatory function of the pLeg promoter. During drought, leg-haemoglobin is one of the major nodule proteins degraded as drought-induced senescence occurs [Becana *et al.,* 1998; DeLaat *et al.,* 2014]. As such, leg-haemoglobin expression would also be expected to decrease in respond to drought-induced senescence. Since OCI expression was regulated by the same promoter, it is suspected that its expression would also be affected by drought in a similar manner. Usually, endogenous cystatins show an incline in expression during drought conditions [Wang *et al.,* 2015]. In a previous study, Jangpromma *et al.,* 2014 reported that a 5 day drought can increase the expression of endogenous phytocystatins in different sugarcane cultivars. Perhaps, expression of these cystatins is regulated by a drought-response promoter in those plants.

Elevated MDA concentrations indicated that plant cells were experiencing high levels of oxidative stress, which is frequent during drought conditions [Saneoka *et al.,* 2004, Farooq *et al.,* 2009]. The production of ROS is also a common occurrence in plants during drought [Farooq *et al.,* 2009]. This accumulation of ROS, along with increased vacuolar protein levels, leads to premature nodule senescence and elevated protease activity [Kinoshita *et al.,* 1999; Vandenabeele *et al.,* 2003]. The data in this MSc study confirmed these prior findings, where drought resulted in increased PLCP activity and accumulation of ROS, as suggested by the inclination of enzymatic anti-oxidant activity. Hence, OCI expression had a minimal effect on preventing drought-induced nodule senescence. Interestingly, our results contradicted findings reported in a previous study. In that study transgenic *Arabidopsis* over-expressing OCI had a reduction in PLCP activity and increased soluble protein content under drought conditions [Makgopa, 2014].

The increase in ureide content during drought conditions correlated with findings by previous researchers, which showed that ureides (allantoin and allantoic acid) accumulated in root nodules of plants experiencing drought stress [Vadez *et al.,* 2000; Ladrera *et al.,* 2007]. This accumulation of ureides suggested that even if nitrogenase activity was reduced or inhibited by drought, the extent of inhibition experienced under these conditions was not sufficient to prevent ureide accumulation in the nodules. Furthermore, this data implies that OCI expression does not lead to improved nitrogen fixation during drought conditions.

The occurrence of high nodules numbers along with less nodule mass in transgenic lines indicated that individual root nodules were overall smaller in these lines. This also demonstrated that, in some part, the process of nodulation is sensitive to OCI inhibition. Similar findings were obtained in a previous studies, showed that transgenic soybean lines over-expressing OCI also resulted in increased nodule numbers [Quain *et al.,* 2015]. The high dry mass and low water content of roots in transgenic lines together with the larger dry and fresh shoot mass suggested that OCI inhibition had a positive impact on the functionality of the shoot and root systems during well-watered conditions.

However, this MSc data also indicated that OCI inhibition leads to a decline in shoot growth during drought conditions. Interestingly, similar findings were reported by Makgopa, 2014, indicating that drought-treated transgenic soybean had a decrease in shoot height. Previous studies by Fenta *et al.,* 2011 and Cilliers *et al.,* 2018 demonstrated that drought does lead to enhanced root growth and reduce shoot development in wild-type soybean plants. This was unexpected as another researcher reported that ectopic OCI expression can lead to altered root architecture in transgenic Arabidopsis [Quain *et al.,* 2014]. The morphological data in this chapter implies that OCI expression in root nodules has minimal/no detectable effect on root and shoot development under drought. Thus, drought

can lead to an increase in root biomass (dry mass) but OCI inhibition limits drought's negative influence on nodule formation and activity.

Previous findings of this research group have shown that OCI inhibition can have an influence on leaf chlorophyll concentration. The results in their research demonstrated that OCI inhibition has a positive effect on chlorophyll content during well-watered conditions [Quain *et al.,* 2014]. Similarly, Tan *et al.,* 2016 reported that over-expressing a phytocystatin, MpCYS5, in transgenic Arabidopsis increases leaf chlorophyll content during abiotic stress. However, the expression of OCI was not organ-specific in those transgenic plants. In contrast to these earlier studies, this MSc study investigated transgenic soybean plants expressing OCI only in their root nodules. Transgenic lines in this study had less leaf chlorophyll when compared to wild-type and also showed a decline in chlorophyll content during drought.

Physiological parameters of drought-treated soybean lines (including wild-type) correlated with other findings by previous researchers, where drought conditions lead to a decrease in stomatal conductance, photosynthetic rate and leaf transpiration in non-transgenic plants [Ohashi *et al.,* 2006; Warren and Adams, 2006]. CO_2 assimilation data indicated that OCI inhibition does not have an influence on photosynthesis under both growth conditions. Furthermore, this data implied that OCI expression did not prevent reduction in photosynthetic rates at all trifoliate leaf hierarchy during drought conditions. This was in contrast with the findings by Van der Vyver *et al.,* 2003 and Demirevska *et al.,* 2010, which reported that over-expressing OCI in transgenic tobacco plants can protect photosynthetic rates during abiotic stress.

In this MSc study, there was a lack of significant differences in physiological parameters between transgenic and wild-type soybean lines, under both growth conditions, which implied that OCI inhibition has minimal effect on leaf functionality when it is over-expressed in root nodules. However, previous researchers have demonstrated that actively expressing OCI in leaves of transgenic plants can enhance certain physiological traits during various abiotic stresses [Demirevska *et al.,* 2010; Quain *et al.,* 2014; Quain *et al.,* 2015].

In the next final chapter, there is a general conclusion on the findings generated in this research and also a discussion on future recommendations for further studies.

Chapter 4

General conclusions & Future recommendations

The underlining aim for this MSc project was to determine whether over-expressing a protease inhibitor can affect plant growth and development, in particular nodule senescence. The hypothesis is that nodule-specific expression of an exogenous protease inhibitor can lead to improved phenotypic traits. Previous studies done in the same research group as this MSc proved that ectopic expression of a protease inhibitor can alter certain plant phenotypes during stress. Protease inhibitors usually function by reducing the activity of a specific class of proteases. Thus, over-expressing protease inhibitors can be a useful biotechnology tool in discovering the function of cysteine proteases under different growth environments. The sole focus of this MSc study was on transgenic soybean lines over-expressing OCI in a specific plant tissue, namely root nodules.

The first outcome of this MSc research project was the successfully detection of active OCI expression in root nodules of transgenic soybean lines. Expression of OCI was further confirmed by measuring the activity of cysteine proteases in root nodules. This addressed the set objective of selecting transgenic plants expressing a cysteine protease inhibitor prior to performing phenotypic analysis. The second outcome was achieved by analysing the effect of OCI inhibition on morphological parameters and activities of antioxidant enzymes when plants are grown under normal conditions. The final outcome of this MSc study involved an extensive phenotypic characterization to explore the exact function of cysteine proteases in soybean plant development and performance under drought stress.

The data in this MSc soybean study demonstrated that tissue/organ-specific ectopic expressing of OCI can alter certain plant functionalities that are related to cysteine protease activity. Oryzacystatin-I (OCI) inhibition was most effective when expressed at high levels, which occurs before the plants reach 9 weeks old. In future studies, it is recommended that the OCI expression be compared to leg-

haemoglobin expression. This will enable researchers to determine whether the decline in OCI expression, after week 9, is due to the Leg promoter. The expression of OCI should also be characterised beyond week 9 using both semi-quantitative and quantitative PCR.

Originally, transgenic soybean lines over-expressing OCI, under the regulation of different promoter sequences, were produced to investigate whether OCI inhibition can improve plant growth. In these studies, the focus was on transformed soybean plants carrying OCI under the cauliflower mosaic virus promoter (CaMV), p35S. The CaMV promoter is strong and constitutive but often does not limit expression to a specific plant tissue or organ [Odell *et al.,* 1985; Benfey and Chua 1989]. This method of expression has some setbacks such as it reduces the rate of plant growth, which is a result of the plant exhausting too much energy expressing the transgene [Olhoft *et al.,* 2003; Paz *et al.,* 2006; Podevin *et al.,* 2012].

Another setback is that we cannot discriminate the independent effects of OCI inhibition on shoot and root systems. Since the CaMV promoter enables the expression of a transgene in both shoot and root systems, it is expected to result in more influence on plant growth and development. Hence, the difference in phenotypic observation between previous transgenic soybean lines and transgenic lines studied in this MSc. For future works, it is suggested that researchers generate transgenic soybean lines where OCI expression is regulated by a stress-inducible promoter. This will cause an elevation in OCI expression during stress, which in turn contributes towards better understanding of phytocystatin functionality under these conditions.

Transgenic lines of this MSc study showed no significant improvement in both morphological and physiological parameters during drought conditions. In some instances, the presence of OCI did result in plants having improved growth.

Unfortunately, the effects of OCI inhibition were not uniform throughout all transformed lines, which made it difficult to investigate the exact pathways it affects. In future studies, it would be recommended to include a soybean line that went through the transformation process but do not have the OCI gene. This will enable us to determine whether the alterations we observed were caused by OCI inhibition or the transformation process, itself. Another recommendation is to include other morphological parameters such as germination period, number of flowers and number of seeds produced per plant can provide more useful details during characterization.

The data of this research study also showed that OCI inhibition was most potent when plants were well-watered and about 8 weeks of age. After 8 weeks or under drought conditions, transgenic soybean lines behaved similar to the wild-type and it was suspected that this behaviour might be a consequences of the decline in OCI expression during these periods. In future works, it is recommended to increase the number of biological replicates for each soybean line. The higher amount of samples might enable the future researchers to successfully profile OCI relative expression past week 8. In addition, Western blot analysis can be useful in determining whether relative expression past week 8 correlates to the amount of OCI present in nodules. Unfortunately, Western blotting and photographic evidence of nodule senescence were overlooked during this study due to research project timeline and limited stock of nodule samples. In hindsight, such parameters would have enabled better understanding of some data sets such as ureide concentrations and relative OCI expression.

No previous research endeavours have focused on investigating nodule-specific expression of a phytocystatin in transgenic soybean plants. This means that there is limited literature to which this MSc data can be correlated to. Overall, this MSc study has proved that OCI inhibition can lead to pleiotropic effects in transgenic

plants even when expressed exclusive in nodules. Similarly, other researchers have discovered that constitutive expression of a phytocystatin leads to similar pleiotropic effects in transgenic plants [Gutiérrez-Campos *et al.,* 2001; Munger *et al.,* 2012]. Thus, it can be concluded that the extent of this pleiotropic effect on a transgenic plant depends on factors such as growth stage of plant, regulatory sequences upstream of transgene and plant species.

For other future works, the acetylene reduction methodology should also be included in determining the influence of OCI on BNF. This method uses gaseous exchange for real-time detection of nitrogen fixation rate in nodules and will give a more accurate analysis on this process [Ligero *et al.,* 1986]. It is also recommended that more focus be aimed at discovering OCI sensitive proteases and the respectable pathways which they are involved in. The discovery of such proteases and pathways is key in obtaining a better understanding of plant protease/protease inhibitor systems and how they impact plant growth, development and performance. In turn, this knowledge can be used to generate commercial legume crops that are more tolerant towards unfavourable growth conditions.

REFERENCES

Abe, M. and Arai, S. 1991. Some properties of a cysteine proteinase inhibitor from corn endosperm. Agricultural and Biological Chemistry, Vol. 55, pp: 2417-2418

Alesandrini, F., Mathis, R., van de Sype, G., Herouart, D. and Puppo, A. 2003. Possible role of a cysteine protease and hydrogen perioxde in soybean nodule development and senescence. New Phytologist Vol. 158, pp: 131-138

Alonso, J.M. and Granell, A. 1995. A putative vacuolar processing protease is regulated by ethylene and also during fruit ripening in Citrus fruit. Plant Physiology, Vol. 109, pp: 541-547

Arai, S., Matsumoto, I., Emori. Y. and Abe, K. 2002. Plant seed cystatins and their target enzymes of endogenous and exogenous origin. Journal of Agriculture, Food Chemistry, Vol. 50, pp: 6612–6617

Arrese-Igor, C., Gonzlez, E.M., Marino, D., Ladrera, R., Larrainzar, E. and Gil-Quintana, E. 2011. Physiological responses of legume nodules to drought. Plant Stress, Vol. 5(1), pp: 24-31

Barrett, A.J. 1986. The classes of proteolytic enzymes. Edited by Dalling, M.J. Plant Proteolytic Enzymes: 1-16. CRC Press, Boca Raton

Barrett, A.J. 1994. Classification of peptidases. Methods Enzymology, Vol. 244, pp: 1–15

Barrett, A.J., Rawlings, N.D. 2001. Evolutionary lines of cysteine peptidases. Biological Chemistry, Vol. 382, pp: 727

Bartels, D. and Sunkar, R. 2005. Drought and salt tolerance in plants. Critic Review Plant Science, Vol. 24, pp: 23-58

Becana M, Moran JF, Iturbe-Ormaetxe I. 1998. Iron-dependent oxygen free radical generation in plants subjected to environmental stress: Toxicity and antioxidant protection. Plant and Soil, Vol. 201: 137–147

Becana, M., Dalton, D.A., Moran, J.F., Iturbe-Ormaetxe, I., Matamoros, M.A. and Rubio, M.C. 2000. Reactive oxygen species and antioxidants in legume nodule. Physiolgia Plantaru, Vol. 109, pp. 372-281

Beers, E.P., Woffenden, B.J. and Zhao, C. 2000. Plant proteolytic enzymes: possible roles during programmed cell death. Plant Molecular Biology, Vol. 44, pp: 399-415

Belenghi, B., Acconcia, F., Trovato, M., Perazzolli, M., Bocedi, A., Polticelli, F., Ascenzi, P. and Delledonne, M. 2003. AtCYS1, a cystatin from Arabidopsis thaliana, suppresses hypersensitive cell death. FEBS Letter, Vol. 270, pp: 2593–2604

Benchabane, M., Goulet, C., Rivard, D., Faye, L., Gomord, V., and Michaud, D. 2008. Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnology Journal, Vol. 6, pp: 633-648

Benchabane, M., Schluter, U., Vorster, J., Goulet, M.C. and Michaud, D. 2010. Plant cystatins. Biochimie, Vol. 92, pp: 1657-1666

Benfey, P.N. and Chua, N-H. 1989. Regulated genes in transgenic plants. Science, Vol. 244, pp: 147-181

Berrocal-Lobo, M., Molina, A., Rodríguez-Palenzuela, P., García-Olmedo, F., Rivas, L., 2009. Leishmania donovani: Thionins, plant antimicrobial peptides with leishmanicidal activity. Experimental Parasitol, Vol.122, pp: 247–249

Bohnert, H.J., Jensen, R.G. 1996. Strategies for engineering water stress tolerance in plants. TIBTECH, Vol.14, pp. 89-97

Boller, T. and Wiemken, A. 1986. Dynamics of vacuolar compartmentation. Annual Review of Plant Physiology. Vol. 37, pp: 137-164

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, Vol. 72, pp: 248-254

Bray, E.A., Bailey-Serres, J.E. and Weretilnyk, E. 2000. Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R (eds) Biochemistry and molecular biology of plants. American Society of Plant Biologists, Rockville, MD, pp: 158–1249

Bray, E.A. 1997. Plant responses to water deficit. Trends in Plant Science, Vol. 2, pp: 48-54

Campbell, N.A., Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P.V. and Jackson, R.B., 2011. Biology, Editor Wilbur, B., Eighth edition (International edition), Pearson Benjamin Cummings, San Franciso, USA, pp: 795

Chaves, M. M., Pereira, J. S. and Maroco, J. P. 2003. Understanding plant responses to drought-from genes to the whole plant. Functional Plant Biology, Vol. 30, pp: 239-264

Chu MH, Liu KL, Wu HY, Yeh KW, Cheng YS. 2011. Crystal structure of tarocystatin-papain complex: Implications for the inhibition property of group-2 phytocystatins. Planta, Vol. 234, pp, 243–254

Clement, M., Lambert, A., Herouart, D. and Boncompagni, E. 2008. Identification of new up-regulated genes under drought stress in soybean nodules. Gene, Vol. 426, pp: 15-22

Cilliers, M., van Wyk, S.G., van Heerden, P.D.R., Kunert, K.J. and Vorster, B.J. 2018. Identification and changes of the drought-induced cysteine protease transcriptome in soybean (*Glycine max*) root nodules. Environmental and Experimental Botany, Vol. 148, pp: 59–69

Colebatch, G., Desbrosses, G., Ott, T., Krusell, L., Montanari, O., Kloska, S., Kopka, J. and Udvardi, M.K. 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. The Plant Journal, Vol. 39, pp: 487-512

Cordovilla, M., Liger, F. and Lluch, C. 1999. Effect of salinity on growth, nodulation and nitrogen assimilation in nodules of faba bean (*Vicia faba* L.) Applied Soil Ecology, Vol. 11, pp 1-7

Cowgill, S. E., Wright, C. and Atkinson, H. J. 2002. Transgenic potatoes with enhanced levels of nematode resistance do not have altered susceptibility to non-target aphids. Molecular Ecology, Vol. 11, pp: 821-827

Dalton, T.P., Shertzer, H.G. and Puga, A. 1999. Regulation of gene expression by reactive oxygen, Annual Review Pharmacological Toxicology, Vol. 39, pp. 67-101

Dang, L., Van Damme, E.J.M., 2015. Toxic proteins in plants. Phytochemistry, Vol. 117, pp: 51–64

Dangl, J.L. and Jones, J.D.G. 2001 Plant pathogens and integrated defence responses to infection. Nature, Vol. 411, pp: 826-833

de Paiva Rolla, A., Carvalho, J.C., Fuganti-Pagliarini, R. Engels, C., do Rio, A. Marin, S.R.R., Neves de Oliveira, M.C., Beneventi, M.A., Marcelino-Guimara˜es, F.C., Bouc¸as Farias, J.R., Neumaier, N., Nakashima, K., Yamaguchi-Shinozaki, K and Nepomuceno A.L. 2013. Phenotyping soybean plants transformed with rd29A:AtDREB1A for drought tolerance in the greenhouse and field. Transgenic Resarch, Vol 23(1), doi:10.1007/s11248-013-9723-6

DeLaat DM, Colombo CA, Chiorato AF, Carbonell SAM. 2014. Induction of ferritin synthesis by water deficit and iron excess in common bean (*Phaseolus vulgaris* L.). Molecular Biology Reports, Vol. 41, pp: 1427–1435

Demirevska, K., Simova-Stoilova, L., Vassileva, V., Vaseva, I., Grigorova, B. and Feller, U. 2008. Drought-induced leaf protein alterations in sensitive and tolerant wheat varieties. General Applied Plant Physiology, Vol. 34, pp: 79-102

Demirevska, K., Simova-stoilova, L., Fedina, I., Georgieva, K. and Kunert, K. 2010. Response of oryzacystatin I transformed tobacco plants to drought, heat and light stress. Journal of Agronomy and Crop Science, Vol. 196, pp: 90-99

Devienne-Barret, F., Justes, E., Machet, J. and Mary, B. (2000). Integrated control of nitrate uptake by crop growth rate and soil nitrate availability under field conditions. Annals of Botany, Vol. 86, pp: 995-1005

Desikan, R., Reynolds, A., hancock, J.T and Neill, S.J. 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defense gene expression in Arabidopsis suspension cultures. Biochemical Journal, Vol. 330, pp. 115-120

Deu, E., Verdoes, M. and Bogyo, M. 2012. New approaches for dissecting protease functions to improve probe development and drug discovery. Nature Structural & Molecular Biology, Vol. 19, pp: 9-16

Dhanushkodi, R., Matthew, C., McManus, M.T. and Dijkwel, P.P. 2018. Drought-induced senescence of *Medicago truncatula* nodules involves serpin and ferritin to control proteolytic activity and iron levels. New Phytologist, Vol. 220 (1), pp: 196-208

Diop, N.N., Kidric, M., Repellin, A., Gareil, M., d'Arcy-Lameta, A., Pham Thi, A.T. and Zuily-Fodil, Y. 2004. A multicystatin is induced by drought-stress in cowpea (*Vigna unguiculata* (L.) Walp) leaves. FEBS Letters, Vol. 577, pp: 545-550

Dolenc I, Turk B, Pungercic G, Ritonja A, Turk V. 1995. Oligomeric structure and substrate induced inhibiton of human cathepsin C. Journal Biological Chemistry, Vol. 270, pp: 21626–31

Dombrowski, J.E. 2003. Salt stress activation of wound-related genes in tomato plants. Plant Physiology, Vol. 132, pp: 2098-2107

Drake, R., John, I., Farrell, A., Cooper, W., Schuch, W. and Grierson, D. 1996. Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. Plant Molecular Biology. Vol. 30, pp: 755-767

Du Plessis, M. 2013. Cysteine proteases activity and gene expression studies in soybean nodules during development and drought stress. MSc dissertation, University Of Pretoria, http://repository.up.ac.za

Dubey, V. K., Pande, M., Singh, B. K. and Jagannadham, M. V. 2007. Papain-like proteases: Applications of their inhibitors. African Journal of Biotechnology, Vol. 6, pp: 1077-1086

Easterling, W.E., Aggarwal, P.K., Batima, P., Brander, K.M., Erda, L., Howden, S.M., Kirilenko, A., Morton, J., Soussana, J.F., Schmidhuber, J. and Tubiello, F.N. 2007. Food, fibre and forest products. Climate change 2007: impacts, adaptation and vulnerability. In: Parry, M.L., Canziani, O.F., Palutikof, J.P., van der Linden, P.J. and Hanson, C.E. (eds) Contribution of working group II to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, pp 273–313

Edwards, K., Johnstone, C. and Thompson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research, Vol. 19, pp: 1349

Escuredo, P.R., Minchin, F.R., Gogorcena, Y., Iturbe-Ormaetxe, I., Klucas R.V. and Becana, M. 1996. Involement of activated oxygen in nitrate-induced senescene of pea root nodules. Plant Physiology, Vol: 110, pp. 1185-1195

Evans, P.J., Gallesi, D., Matheu, C.H., Hernandez, M.J., De Felipe, M.R., Halliwell, B. and Puppo, A. 1991. Oxidative stress occurs during soybean nodule senescence. Planta, Vol. 2088, pp. 73-79

Faktor, O. and Raviv, M. 1997. Inhibition of molt in Spodoptera littoralis larvae treated with soybean Bowman–Birk protease inhibitor. Entomologia Experimentalis et Applicata, Vol. 82, pp: 109-113

Fan, J-B., Korman, S.H., Cantor, C.R. and Smith, C.L. 1991. *Giardia lamblia*: haploid genome size determined by pulsed field gel electrophoresis is less than 12 Mb. Nucleic Acids Research, Vol. 19(8), pp: 1905-1908Fan, S. and Wu, G. 2005. Characteristics of plant proteinase inhibitors and their applications in combating phytophagous insects. Botanical Bulletin of Academia Sinica, Vol. 46, pp: 273-292

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D. and Basra, S.M.A. 2009. Plant drought stress: effects, mechanisms and management. Agronomy Sustainable Development, Vol. 29, pp: 185-212

Fenta, B.A., Driscoll, S.P., Kunert, K.J. and Foyer, C.H., 2011. Characterization of drought tolerance traits in nodulated soybeans. Journal of Agronomy Crop Science, Vol. 198, pp: 92–103

Foyer, C.H. and Noctor, G. 2005. Redox homeostasis and antioxidant signalling: A metabolic interface between stress perception and physiological responses, Plant Cell 17, pp: 1866-1875

Friedman, M. and Brandon, D. L. 2001. Nutritional and health benefits of soy proteins. Journal of Agricultural and Food Chemistry, Vol 49, pp: 1069-1086

Gaddour, K., Vicente-Carbajosa, J., Lara, P., Isabel-Lamoneda, I., Diaz, I. and Carbonero, P. 2001. A constitutive cystatin-encoding gene from barley (I*cy*) responds differentially to abiotic stimuli. Plant Molecular Biology, Vol. 45, pp: 599-608

Gasser, C.S. and Fraley, R.T. 1989. Genetically engineering plants for crop improvement, Science, Vol. 244 (4910), pp. 1293-1299

Gelvin, S.B. 2003. *Agrobacterium*-mediated plant transformation: The biology behind the “gene-jockeying” tool. Microbiology and Molecular Biology Reviews, Vol. 67, pp: 16-37

Gogorcena, Y., Itube-Ormaetxe, I., Escuredo, P.R. and Becan, M. 1995. Antioxidant defense against activated oxygen in pea nodules subjected to water stree. Plant Physiology, Vol.108, p: 753-759

Gogorcena, Y.,Gordon, A.J., Escuredo, P.R., Minchin, F.R., Witty, J.F., Moran, J.F. and Becana, M. 1997. N2 Fixation, carbon metabolism and oxidativedamage in nodules of dark-stressed common bean plants. Plant Physiology, Vol. 113, pp: 1193-1201

Gratao, P.L., Polle, A., Lea, P.J. and Azevedo, R.A. 2005. Making the life of heavy metal stressed plants a little easier. Function Plant Biology, Vol. 32, pp: 481-494.

Grudkowska, M. and zagdanska, B. 2004. Multifunctional role of plant cysteine proteinases. Acta Biochimica Polonica, Vol. 51(3), pp: 609-624

Gruis, D.F., Selinger, D.A., Curran, J.M. and Jung, R. 2002. Redundant proteolytic mechanisms process seed storage proteins in the absence of seed-type members of the vacuolar processing enzyme family of cysteine proteases. The Plant Cell, Vol. 14, pp: 2863-2882

Gutiérrez-Campos, R., Torres-Acosta, J.A., Pérez-Martínez, J. and Gómez-Lim, M.A., 2001. Pleiotropic effects in transgenic tobacco plants expressing the Oryzacystatin I gene. Hortscience, Vol. 36 (1), pp: 118–119

Habib, H. and Fazili, K. M. 2007. Plant protease inhibitors: A defense strategy in plants. Biotechnology and Molecular Biology Review, Vol. 2, pp: 68-85

Harper, J.E. 1987. Nitrogen metabolism. In Soybeans: Improvement Production and Uses. 2nd edition. Ed. J RWilcox. Am. Society Agronomy Madison. WI, pp: 497-533

Hirsch, A.M. 1992. Tansley Review, 40, Developmental biology of legume nodulation. New Phytologist, Vol. 122, pp: 211-237

Huang, G.T., MA, S.L., Bai, L.P., Zhang, L., MA, H., Jia, P., Liu, J., Zhong, M. & uo, Z.F. 2012. Signal transduction during cold, salt, and drought stresses in plants. Molecular Biology Reports, Vol. 39, pp: 969-987

Huffaker, R. 1990. Proteolytic activity during senescence of plants. New Phytologist, Vol. 116, pp: 199-231

Jangpromma, N., Saito, A., Araki, T., Jaisil, P., Songsri, P., Sakda Daduang, S., Kawaguchi, Y., Dhiravisit, A. and Thammasirirak, S. 2014. Molecular cloning and characterization in eukaryotic expression systems of a sugarcane cysteine protease inhibitor gene involved in drought tolerance. Turk Journal Botany, Vol. 38, pp: 724-736

Jensen, E.S. and Hauggaard-Nielsen, H. 2003. How can increased use of biological N2 fixation in agriculture benefit the environment? Plant Soil, Vol. 252, pp: 177–186. doi: 10.1023/A:1024189029226

Johnson, R., Narvaez, J., An, G. and Ryan, C. 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defence against Manduca sexta larvae. Proceedings of the National Academy of Sciences (USA), Vol. 86, pp: 9871-9875

Jury WA, Vaux HJ. 2007. The emerging global water crisis: managing scarcity and conflict between water users. Advances in Agronomy, Vol. 95, pp: 1–76

Ishii, S.I. 1994. Legumain: Asparaginyl endopeptidase. Methods in Enzymology, Vol. 244, pp: 604-615

Kaufman, P.B., Cuke, J.A., Brielmann, H., Boik,J. and Hoyt, J.E. 1997. A Comparative Survey of Leguminous Plants as Sources of the Isoflavones, Genistein and daidzein: Implication for Human Nutrition and Health. Journal of Alternative and Complementary Medicine, Vol. 3(1), pp: 7-12

Keyser, H.H. and Li, F. 1992. Potential for increasing biological nitrogen fixation in soybean. Plant and Soil, Vol. 141, pp: 119-135

Kim, T. G., Kim, H. M., Lee, H. J., Shin, Y. J., Kwon, T. H., Lee, N. J., Jang, Y. S. and Yang, M. S. 2007. Reduced protease activity in transformed rice cell suspension cultures expressing a proteinase inhibitor. Protein Expression and Purification, Vol. 53, pp: 270-274

King, C.A. and Purcell, L.C. 2001. Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Science, Vol. 41, pp: 1099–1107

Kinoshita T, Yamada K, Hiraiwa N, Kondo M, Nishimura M, Hara-NishimuraI. 1999. Vacuolar processing enzyme is up-regulated in the lytic vacuoles of

vegetative tissues during senescence and under various stressed conditions. Plant Journal, Vol. 19, pp: 43–53

Ladrera R, Marino D, Larrainzar E, Gonzalez EM, Arrese-Igor C. 2007. Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. Plant Physiology, Vol. 145, pp: 539–546

Lawlor, D. and Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, Cell & Environment, Vol. 25, pp: 275-294

Lechtenberg, B., Schubert, D., Forsbach, A., Gils, M. and Schmidt, R. 2003. Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing. The Plant Journal, Vol. 34, pp: 507-517

Levine, A., Pennell, R., Alvarez, M., Plamer, R and Lamb, CJ. 1996. Calcium-mediated apoptosis in plant hypersensitive disease resistance response. Current Biology, Vol. 6, pp. 427-437

LeRudulier, D., Bouillard, L. 1983. Glycine betaine, an osmotic effector in Klebsiella pneumoniae and other members of the Enterobacteriaceae. Applied Environmental Microbiology, Vol. 46, pp: 152-159

Lian, H.L., Yu, X., YE, Q., Ding, X.S., Kitagawa, Y., Kwak, S.S., Su, W.A. and Tang, Z.C. 2004. The role of aquaporin RWC3 in drought avoidance in rice. Plant and Cell Physiology, Vol. 45, pp: 481-489

Lievens, S., Goormachtig, S. and Holsters, M. (2004). Nodule-enhanced protease inhibitor gene: emerging patterns of gene expression in nodule development on *Sesbania rostrata*. Journal of Experimental Botany, Vol. 55, pp: 89-97

Lichtenaler, H.K. and Wellburn, A.R. 1983. Determination of total carontenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemistry Society Trans, Vol 11, pp: 591- 592

Ligero F., Lluch C. and Olivares J. 1986. Evolution of ethylene from roots of Medicago sativa plants inoculated with Rhizobium meiiloti. Journal of Plant Physiology, Vol. 125, pp: 361-365

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, Vol 25, pp: 402-408

Mahajan, S. and Tuteja, N. 2005. Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics, Vol. 444, pp: 139-158

Makgopa, M.E., 2014. Production and characterization of transgenic *Arabidopsis* and soybean (*Glycine max* (L.) Merrill.) plants over-expressing oryzacystatin I (OC-I). PhD thesis, University Of Pretoria, http://repository.up.ac.za

Manavalan, L.P., Guttikonda, S.K., Phan Tran, L.-S. and Nguyen, H.T. 2009. Physiological and molecular approaches to improve drought resistance in soybean. Plant and Cell Physiology, Vol. 50, pp: 1260-1276

Mannervik, B. 1985. The isoenzyme of glutathione transferase. Advances in Enzymology and Related Areas of Molecular Biology, Vol. 57, pp: 357-417

Margis-Pinheiro, M., Carina, A., Zolet, A.C., Loss, G., Pasquali, G. and Margis, R. 2008. Molecular evolution and diversification of plant cysteine proteinase inhibitors: new insights after the poplar genome. Molecular Phylogenetic Evolution, Vol. 49, pp: 349–355

Marra, B.M., Souza, D.S.L., Aguiar, J.N., Firmino, A.A.P, Sarto, R.P.D., Silva, F.B., Almeida, C.D.S., Cares, J.E., Continho, M.V., Martins de Sa, C., Franco,

O.L. and Gross de Sa, M.F. 2009. Protective effects of a cysteine proteinase propeptide expressed in transgenic soybean roots. Peptides, Vol: 30(5), pp: 825-831

Martinez, M., Diaz-Mendoza, M., Carrillo, L. and Diaz, I. 2007. Carboxyl terminal extended phytocystatins are bifunctional inhibitors of papain and legumain cysteine proteinases. FEBS Letters, Vol. 581, pp: 2914–2918

Martinez, M. and Diaz, I. 2008. The origin and evolution of plant cystatins and their target cysteine proteinases indicate a complex functional relationship. BMC Evolutionary Biology, Vol. 8, pp: 198-210

Martinez, M., Cambra, I., Carrillo, L., Diaz-Mendoza, M. and Diaz, I. 2009. Characterization of the entire cystatin gene family in barley and their target cathepsin L-like cysteine-proteases, partners in the hordein mobilization during seed germination. Plant Physiology, Vol. 151, pp: 1531–1545

Masoud, S.A., Johnson, L.B., White, F.F. and Reeck, G.R. 1993. Expression of a cysteine proteinase inhibitor (Oryzacystatin-I) in transgenic tobacco plants. Plant Molecular Biology, Vol. 21, pp: 655–663

Mello-Farias, P.C. and Chaves, A.L.S. 2008. Advances in *Agrobacterium*-mediated plant transformation with enphasys on soybean. Scientia Agricola, Vol. 65, pp: 95-106

Mengel, D.B., Segars, W. and Rehm, G.W. 1987. Soil fertility and liming. In Soybeans: Improvement. Production and Uses. Second Edition. Ed. J R Wilcox. American Society of Agronomy. Madison. WI, pp: 461-496

Molina, A., Goy, P.A., Fraile, A., Sánchez-Monge, R. and García-Olmedo, F., 1993. Inhibition of bacterial and fungal plant pathogens by thionins of types I and II. Plant Science, Vol. 92, pp: 169–177

Munger, A., Coenen, K., Cantin, L., Goulet, C., Vaillancourt, L.-P., Goulet, M.-C., Tweddell, R., Sainsbury, F. and Michaud, D. 2012. Beneficial 'unintended effects' of a cereal cystatin in transgenic lines of potato, *Solanum tuberosum*. BMC Plant Biology, Vol. 12, pp: 1-12

Müntz, K. and Shutov, A.D. 2002. Legumains and their functions in plants. Trends in Plant Science, Vol. 7, pp: 340-344

Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T. and Katunuma, N. 1991. The refined 2.15 A X-ray crystal structure of human liver cathepsin B: The structural basis for its specificity. EMBO Journal, Vol. 10(9) pp: 2321–30

Muskens, M. M., Vissers, A. A., Mol, J. M. and Kooter, J. 2000. Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Molecular Biology, Vol. 43, pp: 243-260

Nagase, H. and Woessner, J.F., Jr. 1999. Matrix metalloproteases. Journal of Biology. Chemistry, Vol. 274, pp: 21491-21494

Neill, S., Desikan, R. and Hancock, J., 2002. Hydrogen peroxide signalling. Current opinion in Plant Biology, Vol: 5, pp: 388-393

Noctor, G., Mhamdi, A and Foyer, C.H. 2014. The roles of reactive oxygen metabolism in drought: Not so cut and dried. Plant Physiology, Vol.164, pp: 1636–1648

Odell, J.T., Nagy, F. and Chua, N-H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35s promoter. Nature, Vol. 313, pp: 810-812

Ohashi, Y., Nakayama, N., Saneoka, H. and Fujita, K. 2006. Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants, Biology Plant, Vol. 50, pp: 138–141

Olhoft, P. M., Flagel, L. E., Donovan, C. M. and Somers, D. A. 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta, Vol. 216, pp: 723-735

Passioura, J. 1997. Drought and drought tolerance. Drought tolerance in higher plants: Genetical, physiological and molecular biological analysis. Netherlands: Springer, pp: 1-5

Paz, M., Martinez, J., Kalvig, A., Fonger, T. and Wang, K. 2006. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. Plant Cell Reports, Vol. 25, pp: 206-213

Peoples, M.B., Herridge, D.F. and Ladha, J.K. 1995. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Plant Soil, Vol: 174, pp: 3–28. doi: 10.1007/BF00032239

Pernas, M., Sánchez-Monge, R. and Salcedo, G. 2000. Biotic and abiotic stress can induce cystatin expression in chestnut. FEBS Letters, Vol. 467, pp: 206-210

Pfeiffer, N.E., Torres, C.M., and Wagner, F.W. 1983. Proteolytic activity in soybean roots nodules. Plant Physiology, Vol. 71, pp: 797-802

Pimratch, S., Jogloy, S., Vorasoot, N., Toomsan, B., Patanothai, A. and Holbrook, C. 2008. Relationship between biomass production and nitrogen fixation under drought-stress conditions in Peanut genotypes with different levels of drought resistance. Journal of Agronomy and Crop Science, Vol. 194, pp: 15-25

Podevin, N. and DU Jardin, P. 2012. Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants. GM Crops and Food: Biotechnology in Agriculture and the Food Chain, Vol. 3, pp: 296-300

Prins, A., Van der Heerden, P.D.R., Olmos, E., Kunert, K.J. and Foyer, C.H. 2008. Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a mdel for dynamic interactions with ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. Journal of Experimental Botany, Vol. 59, pp: 1935-1950

Puppo, A., Groten, K., Bastian, F., Carzaniga, R., Soussi, M., Lucas, M.M., De Felipe, M.R., Harrison, J., Vanacker, H. and Foyer, C.H. 2005. Legume nodule senescence: Roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytologist, Vol. 165, pp: 683-701

Quain, M.D., Makgopa, M., Marquez, B., Comadira, G., Fernandez, G., Olmos, E., Schnaubelt, d., Kunert, K.J. and Foyer, C. 2014. Ectopic phytocystatin expression leads to enhanced drought stress tolerance in soybean (*Glycine max*) and *Arabidopsis thaliana* through effects on strigolactone pathways and can also result in improved seed traits. Plant Biotechnology Journal, 2014, pp: 1-11

Quain, M.D., Makgopa, M.E., Cooper, J.W., Kunert, K.J. and Foyer, C.H. 2015. Ectopic phytocystatin expression increases nodule numbers and influences the responses of soybean (*Glycine max*) to nitrogen deficiency. Phytochemistry, Vol. 112, pp: 170-187

Rawlings, N.D. and Barrett, A.J. Evolutionary families of peptidases. 1993. Biochemistry Journal, Vol. 290 (1), pp: 205–18

Rawlings, N. D., Barrett, A. J. & Bateman, A. 2011. Asparagine peptide lyases: A seventh catalytic type of proteolytic enzymes. Journal of Biological Chemistry, Vol. 286, pp: 38321-38328

Rui, L., Wang, W., Wang, W., Li, F., Wang, Q., Xua, Y. and Wang, S. 2015. Overexpression of a cysteine proteinase inhibitor gene from *Jatropha curcas* confers enhanced tolerance to salinity stress. Electronic Journal of Biotechnology, Vol. 18, pp: 368–375

Ryan, C.A.1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annual Review of Phytopathology, Vol. 28, pp: 425–449

Salas, C.E., Gomes, M.T.R., Hernandez, M. and Lopes, M.T.P. 2008. Plant cysteine proteinases: Evaluation of the pharmacological activity. Phytochemistry, Vol. 69, pp: 2263-2269

Salon, C., Avice, J.C., Larmure, A., Ourry, A., Prudent, M. and Voisin, A.S. 2011. Plant N fluxes and modulation by nitrogen, heat and water stresses: A review based on comparison of legumes and non-legume plants. In Abiotic Stress in Plants: Mechanisms and Adaptations Eds. A Shanker and B Venkateswarlu. Intech. doi: 10.5772/23474

Saneoka, H., Moghaieb, R.E.A., Gnanasiri S. Premachandra, G.S. and Fujita, K. 2004. Nitrogen nutrition and water stress effects on cell membrane stability and leaf water relations in *Agrostis palustris* Huds. Environmental and Experimental Botany, Vol. 52, pp: 131-138

Schaller, A. 2004. A cut above the rest: the regulatory function of plant proteases. Planta, Vol. 220, pp: 183-197

Schultze, M. and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. Annual Review of Genetics, Vol. 32, pp: 33-57

Serraj, R., Sinclair, T.R. and Purcell, L.C. 1999. Symbiotic N2 fixation response to drought. Journal of Experimental Botany, Vol. 50, pp: 143-155

Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany. 2012, pp: 1-12

Sheveleva, E., Chmara, W., Bohnert, H.J. and Jensen, R.C. 1997. lncreased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* SR1. Plant Physiology, Vol. 115, pp: 1211-1219

Sinclair, T.R., Purcell, L.C. and Sneller, C.H. 2004. Crop transformation and the challenge to increase yield potential. Trends in Plant Science, Vol.9 (2), pp: 70-75

Singh, H.P., Batish, D.R., Kohli, R.K. and Arora, K. 2007. Arsenic-induced root growth inhibition in mung bean (*Phaseolus aureus* Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regulation, Vol. 53 (1), pp: 65-73

Singh, M., Kumar, J., Singh, S., Singh, V.P. and Prasad, S.M. 2015. Roles of osmoprotectants in improving salinity and drought tolerance in plants: A review. Review Environment Science and Biotechnology, DOI 10.1007/s11157-015-9372-8

Smirnoff, N. and Cumbes, Q.J. 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry, Vol. 28, pp: 1057-1060

Smirnoff, N. 1993. Tansley review No 52: the role of active oxygen in the response of plants to water deficit and desiccation. New Phytologist, Vol. 125, pp: 27-58

Soares-cordeiro, A. S., Driscoll, S. P., Pellny, T. K., Olmos, E., Arrabaça, M. C. & foyer, C. H. 2009. Variations in the dorso-ventral organization of leaf structure and Kranz anatomy coordinate the control of photosynthesis and associated signalling at the whole leaf level in monocotyledonous species. Plant, Cell and Environment, Vol. 32, pp: 1833-1844

Solomon M, Belenghi B, Delledonne M, Levine A. 1999. The involvement of cysteine proteases and protease inhibitor genes in programmed cell death in plants. Plant Cell, Vol. 11, pp: 431–444

Somers, D.A, Samac, D.A. and Olhoft, P.M. 2003. Recent advances in legume transformation. Plant Physiology, Vol. 131, pp: 892-899

Smart, C.M., Hosken, S.E., Thomas, H., Greaves, J.A., Blair, B.G. and Schuch, W. 1995. The timing of maize leaf senescence and characterisation of senescence-related cDNAs. Physiologia Plantarum, Vol. 93, pp: 673-682

Stitt, M. and Sonnewald, U. 1995. Regulation of metabolism in transgenic plants. Annual Review of Plant Physiology and Plant Molecular Biology, Vol. 46, pp: 341-368.

Stougaard, J. 2000. Regulators and regulation of legume root nodule development. Plant Physiology, Vol. 124, pp: 531-540

Streeter, J.G. 2003. Effects of drought on nitrogen fixation in soybean root nodules. Plant Cell Environment, Vol. 26, pp: 1199–1204

Sugawara, H., Shibuya, K., Yoshioka, T., Hashiba, T. and Satoh, S. 2002. Is a cysteine proteinase inhibitor involved in the regulation of petal wilting in senescing carnation (*Dianthus caryophyllus* L.) flowers? Journal of Experimental Botany, Vol. 53, pp: 407–413

Sulieman, S and Tran, L.T. 2014. Symbiotic nitrogen fixation in legume nodules: Metabolism and regulatory mechanisms. International Journal Of Molecular Sciences Vol. 15, pp: 19389-19393

Szewińska, J., Prabucka, B., Krawczyk, M., Mielecki, M. and Bielawski, W. 2013. The participation of phytocystatin TrcC-4 in the activity regulation of EP8, themain prolamin degrading cysteine endopeptidase in triticale seeds. Plant Growth Regulation, Vol. 69, pp: 131–137

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana.* Plant Journal, Vol. 29, pp: 417-426

Tajima, S., Nomura, M. and Hiroshi Kouchi, H. 2004. Ureide biosynthesis in legume nodules. Frontiers in Bioscience, Vol. 9, pp: 1374-1381

Tan, Y., Wei, X., Wang, P., Sun, X., Li, M. and Ma, F. 2016. A Phytocystatin Gene from Malus prunifolia (Willd.) Borkh., MpCYS5, confers salt stress tolerance and functions in endoplasmic reticulum stress response in Arabidopsis. Plant Molecular Biology Rep, Vol. 34, pp: 62–75

Tang, W., Newton, R. J. and Weidner, D. A. 2007. Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine. Journal of Experimental Botany, Vol. 58, pp: 545-554

Tränkner, C., Lehmann, S., Hoenicka, H., Hanke, M.-V., Fladung, M., Lenhardt, D., Dunemann, F., Gau, A., Schlangen, K., Malnoy, M. and Flachowsky, H. 2010. Over-expression of an FT-homologous gene of apple induces early flowering in annual and perennial plants. Planta, Vol. 232, pp: 1309-1324

Tavano, O.L., 2013. Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, Vol. 90, pp: 1-11

Teresa Capell, T., Bassie, L. and Christou, P., 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. Plant Biology, Vol. 101 (26), pp: 9909–9914

Timmers, A.C.J., Soupène, E., Auriac, M.C., de Billy, F., Vasse, J., Boistard, P. and Truchet, G. 2000. Saprophytic intracellular rhizobia in alfalfa nodules. Molecular Plant-Microbe Interactions, Vol. 13, pp: 1204-1213

Turk, V. and Bode, W. 1991. The cystatins: protein inhibitors of cysteine proteinases. Federation of European Biochemical Societies Letters, Vol. 285, pp: 213-219.

Urwin, P. E., Troth, K. M., Zubko, E. I. and Atkinson, H. J. 2001. Effective transgenic resistance to *Globodera pallida* in potato field trials. Molecular Breeding, Vol. 8, pp: 95-101

Vanaman, T.C. and Bradshaw. 1999. Proteases in cellular regulation Minireview Series. The Journal Of Biological Chemistry, Vol. 274(29), pp: 20047

van de Velde, W., perez Guerra, J.C., De Keyser, A., De Rycke, R., Rombauts, S., Maunoury, N., Mergaert, P., Kindorosi, E., holsters, M. and Goormachtif, S. 2006. Aging in Legume Symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. Plant Physiology, Vol.141, pp; 711-720

Van der Vyver, C., Schneidereit, J., Driscoll, S., Turner, J., Kunert K.J. and Foyyer, C.H. 2003. Oryzacystatin-I expression in transformed tobacco produces a conditional growth phenotype and enhance chilling tolerance. Plant Biotechnology Journal, Vol. 1, pp: 101-112

Vadez V, Sinclair TR, Serraj R. 2000. Asparagine and ureide accumulation in nodules and shoots as feedback inhibitors of N2 fixation in soybean. Physiologia Plantarum, Vol. 110, pp: 215-223

Vandenabeele S, Van Der Kelen K, Dat J, Gadjev I, Boonefaes T, Morsa S, Rottiers P, Slooten L, Montagu MV, Zabeau M et al. 2003. A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. Proceedings of the National Academy of Sciences (USA), Vol. 100, pp: 16113–16118

Vorster, BJ., Schluter, U., Du Plessis, M., van Wyk, S., Makgopa, M.E., Ncube, I., Quain, M.D., Kunert, K. and Foyer, C.H. 2013. The cysteine protease Inhibitor system explored in soybean nodules development. Agronomy, Vol 3, pp: 50-570

Walsh, T.A. and Strickland, J.A. 1993. Proteolysis of the 85-kilodalton crystalline cysteine proteinase inhibitor from potato releases functional cystatin domains. Plant Physiology, Vol. 103, pp: 1227-1234

Warren, C.R. and Adams, M.A. 2006. Internal conductance does not scale with photosynthetic economics of water and nitrogen use in photosynthesis, Plant Cell Environment, Vol. 29, pp: 192–201

Watanabe, H., Abe, K., Emori, Y., Hosoyama, H. and Arai, S. 1991. Molecular cloning and gibberellin-induced expression of multiple cysteine proteinases of rice seeds (oryzains). Journal of Biological Chemistry, Vol. 266, pp: 16897-16902

Wang, W., Vinocur, B. and Altman, A. 2003. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta, Vol. 218, pp: 1-14

Wang, W., Zhao, P., Xue-mei Zhou, X., Xiong, H. & Sun, M. 2015. Genome-wide identification and characterization of cystatin family genes in rice (*Oryza sativa* L.). Journal of Plant Cell Report, Vol. 34, pp: 1579–1592

Weeda, A.M., Kumar, G.N.M. and Knowles, N.R. 2009 Developmentally linked changes in proteases and protease inhibitors suggest a role for potato multicystatin in regulating protein content of potato tubers. Planta, Vol. 230 (1), pp: 73-84

Wex, T., Buhling, F. and Wex, H. 2001. Human cathepsin W, a cysteine protease predominantly expressed in NK cells, is mainly localized in the endoplasmic reticulum. Journal of Immunology, Vol. 167, pp: 2172– 8

Wiederanders, B., Kaulman, G. and Schilling K 2003. Functions of propeptide parts in cysteine proteinases. Current Protein Peptide Science, Vol. 4, pp: 309–326

Wiederanders, B. 2003. Structure–function relationship in class CA1 cysteine peptides propeptides. Acta Biochemia Polonica, Vol. 50, pp: 691–713

Xu, Z., Zhou, G. and Shimizu, H. 2010. Plant responses to drought and rewatering. Plant Signaling and Behavior, Vol. 5, pp: 649-654

Yang, T., Ali, G.S., Yang, L., Du, L., Reddy, A.S.N. and Poovaiah, B.W. 2010. Clacium/calmodulin-regulated recptor-like kinase CRLK1 interacts with MEKK1 in plant. Plant Signaling and Behavior, Vol. 5, pp: 991-994

Yordanov, I., Velikova, V. and Tsonev, T. 2003. Plant responses to drought and stress tolerance. Bulgarian Journal of Plant Physiology, pp. 187-206

Young, G.E., Conway, C.F. 1942. On the estimation of allantoin by the riminischryver reaction. Journal of Biological Chemistry, Vol. 142, pp: 839–853

Zahran, H.H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews, Vol. 63, pp: 968-989

Zhang, X., Liu, S. and Takano, T. 2008. Two cysteine proteinase inhibitors from *Arabidopsis thaliana*, AtCYSa and AtCYSb, increasing the salt, drought, oxidation and cold tolerance. Plant Molecular Biology, Vol. 68, pp: 131–143

Zhang, F.Q., Yang, Y.S., Lou, Z.P. and Dong, J.D. 2007. Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plants seedlings (*Kandelia candel* and *Bruguira gymnorrhiza*). Chemosphere, Vol. 67 (1), pp: 44-50

Zlatev, Z. and Lindon, F. C. 2012. An overview on drought induced changes in plant growth, water relations and photosynthesis. Emirates Journal of Food and Agriculture, Vol. 24, pp: 57-72