Proteomic analysis and physiological response of wheat treated with a triglyceride purified from *Lupinus albus* L. seed

by

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

Marie Curie

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Declaration

I declare that the dissertation submitted by me for the degree Philosophiae Doctor at the University of the Free State, South Africa is my own independent work and has not previously been submitted by me to another University. I furthermore concede copyright of the dissertation in favour of the University of the Free State.

Signed in Bloemfontein, Free State, South Africa.

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Abbreviations

A

ABA	abscisic acid
AdoHcyase	adenosylhomocysteinase
ADP	adenosine-5’-diphosphate
ALDP	fructose-bisphosphate aldolase
ANOVA	analysis of variance
ARF	ADP-ribosylation factor
AS	acid saturation
ASM	acibenzolar-*S*-methyl
ATP	adenosine-5’-triphosphate
Avr	avirulence

B

BRs	brassinosteroids
BTH	benzo (1, 2, 3) thiadiazole-7-carbotioic acid S-methyl ester

C

C	carbon
c	concentration

CAB	Chlorophyll *a-b* binding protein
CAs	carbonic anhydrases
CC	ComCat®
CDC	cell division control protein
cDNA	Complementary deoxyribonucleic acid
CDSP32	chloroplast drought-induced stress protein of 32 kD
CEC	cation exchange capacity
CEF	cyclic electron flow
CER	CO_2 exchange rate
CKs	cytokinins
CO_2	carbon dioxide
Cq	quantification cycle
C_r	CO_2 concentration of reference
C_s	CO_2 concentration of sample
CtpA	carboxyl-terminal peptidase
CV	coefficient of variation
Cyt b_6f	cytochrome b_6f complex

D

d	light path
DH-JA	dihydrojasmonic acid

DM	dry mass
DTT	dithiothreitol

E

E	reaction efficiency
EA	extractable acid
EDTA	ethylenediaminetetraacetic acid
EDU	ethylene diurea
EF-2	elongation factor 2
ESPACE	european stress physiology and climate experiment
ET	ethylene
EtBr	ethidium bromide
ETI	effector-triggered immunity
ϵ	extinction coefficient of NADPH at 340 nm

F

F	air flow rate
F-6-P	fructose-6-phosphate
FAO	food and agriculture organization of the united nations
FBPase	fructose-1,6-bisphosphatase

FHB	fusarium head blight
FNR	ferredoxin NADP(+) reductase
FTR	ferredoxin-thioredoxin oxidoreductase
FtsH	filamentation temperature-sensitive H
F_v/F_m	maximum quantum efficiency of PSII
ΦPSII	quantum efficiency of PSII

G

G-6-P	D-glucose-6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
GR	glutathione reductase
GRP-94	glucose-regulated protein 94
GS2	glutamine synthetase leaf isozyme

H

H_2O_2	hydrogen peroxide
HBL	homobrassinolide
HCF136	PSII stability/assembly factor

HK	hexokinase
Hpi	hours post infestation
Hpt	hours post treatment
HR	hypersensitive response/ reaction
HSPs	heat shock proteins

I

IAA	indole-3-acetic acid
ICS	isochorismate synthase
IPCC	intergovernmental panel on climate change
ISR	induced systemic resistance
IWF	intercellular wash fluid

J

JA	jasmonic acid

K

K	potassium
KIN	kinetin

L

LC/MS/MS	liquid chromatography tandem mass spectrometry
LHCBs	light-harvesting chlorophyll *a*/*b*-binding proteins
LHCII	light harvesting complex II

M

M	average expression stability
MAP	mitogen activated protein
ME	β-mercaptoethanol
MeJA	methyl jasmonate
MeSA	methyl salicylate
MIQE	minimum information for publication of quantitative real-time PCR experiments
MOPS	3-(N-morpholino)-propanesulfonic acid
MS	mass spectrometry
MSE	MeSA esterase
MW	molecular weight

N

N	nitrogen

NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotineamide adenine dinucleotide phosphate-oxidase
NAR	net assimilation rate
N-ATCA	N-acetyl thiazolidine carboxylic acid
NO	nitric oxide
NPQ	non-photochemical quenching
NPR1	non-expresser of *PR1*

O

$^1O_2$	singlet oxygen
O_3	ozone
OEC	oxygen evolving complex
OEE1	oxygen evolving enhancer protein 1
OEE2	oxygen evolving enhancer protein 2
OEE3	oxygen evolving enhancer protein 3
OsGER5	*Oryza sativa* germin-like protein 5
OsPR-10	*Oryza sativa* pathogen-related protein class 10
OsSGT1	*Oryza sativa* UDP glucose: SA glucosyltranferase

P

P	phosphorous
PAMP	pathogen associated molecular pattern
PBZ1	probenazole-inducible protein
pD1	precursor D1
PGI	phosphor-glucoisomerase
PGK	Phosphoglycerate kinase
PGM	phosphoglucomutase
PGPR	plant growth-promoting rhizobacteria
Ppb	parts per billion
PPIase	peptidyl-prolyl cis-trans isomerise
PQH_2	dihydroplastoquinone
PR	pathogen related
PRKase	phosphoribulokinase
Probenazole	3-prop-2-enoxy-1,2-benzothiazole1,1-dioxide
PRRs	pattern recognition receptors
Prx	peroxiredoxin
PSI	photosystem I
PSII	photosystem II
PTI	PAMP-triggered immunity

Q

qPCR	quantitative polymerase chain reaction

R

RLI	RNase L inhibitor-like protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
Rubisco	ribulose-1, 5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1, 5-bisphosphate
RWA	russian wheat aphid
RWASA2	RWA biotype 2

S

S	leaf area
SA	salicylic acid
SABP2	salicylic acid binding protein 2
SalT	salt-induced proteins
SAR	systemic acquired resistance

SBPase	sedoheptulose-1,7-bisphosphatase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphide-polyacrylamide gel electrophoresis
SS	*Lupinus albus* L. cv. Betsuhana White seed suspension
SWC	seaweed extract

T

TMV	tobacco mosaic virus
Tris-HCl	tris-(hydroxymethyl) aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
TRX	thioredoxin
TRX-M	thioredoxin M-type
Tm	tunicamycin
Triton-X 100	octyl phenol ethoxylate
TK	transketolase
TIM	triosephosphate isomerise
TSP	tea seed powder
Tef 1-α	translation elongation factor 1 alpha subunit

V

v	sample volume
V	final volume

W

WAK 1	wall-associated kinase 1
W_r	transpiration rate of reference
W_s	transpiration rate of sample

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Introduction

CHAPTER 1

Wheat is known as one of the 'big three' crops with a worldwide annual harvest of approximately 600 million tonnes (Ehteramian *et al*., 2012). It is grown at various altitudes, from sea level to 4500 m above sea level, indicating range of cultivation (http://www.eolss.net). About 95% of cultivated wheat is hexaploid bread wheat while tetraploid durum wheat makes up the majority of the remaining 5% (Shewry, 2009).

With an ever-increasing world population, the production of cereal crops must increase to keep up with demand. Agriculture is currently unable to provide adequate food as is evident in the 178 million children suffering from malnutrition in large parts of South Asia and Africa (Vermeulen *et al*., 2012). Relative water scarcity and the low potential of arable lands available to subsistence farmers, makes them especially vulnerable to malnutrition (Ortmann and Machethe, 2003). Subsistence farmers therefore employ more intensive practises in order to strive towards household food security (Aliber and Hart, 2009). This is an ever-increasing problem even in high-income countries where a large part of the population is food insecure. Agriculture is further threatened by changes in the global climate (Ziska *et al*., 2012). Farmers therefore need to implement proactive measures to increase food production which, instead of being a regional incentive, should be implemented globally (Ziska *et al*., 2012).

The yield of cereal crops has risen exponentially since the beginning of the Green Revolution. According to Food and Agriculture Organization of the United Nations (FAO) (2010) this increase is due to improvements in agricultural techniques and control of cultivars through the use of chemical fertilisers and pesticides. Hole *et al*. (2005) predicted pesticide use will double as a result of the growing demand for food. Some of these chemicals however have a long-lasting negative effect on the surrounding environment and species diversity. It is transported over great distances and finally ends up in aquatic systems (Van Dyk and Pletschke, 2011). The use of pesticides containing DDT is especially harmful, since it has a half-life of 3 to 20 years and accumulates within the food chain (Lintelmann *et al*., 2003). Dasgupta *et al*. (2005) noted that the health effects of Bangladeshi farmers were directly related to the amount of pesticides they used in crop cultivation. Many of these farmers were still using banned pesticides, including Dursban, Diazinon and Megaphos (Rahman and Hossain, 2003). Geiger *et al*. (2010) showed that, compared to farms where organic

principles are practised, the use of pesticides decreased the surrounding plant and bird species diversity.

Agriculture is therefore turning to more environmentally friendly practices to maximize yield increases. One of these practices is the use of bio-stimulants that have a lower impact on the environment since they mainly consist of natural molecules like plant hormones, enzymes, vitamins and various other biologically active compounds (García-Martínez *et al*., 2010). Application of bio-stimulants in small quantities stimulates the synthesis of natural hormones, enhances nutrient uptake, stimulates root growth and increases the resistance response when plants are exposed to stress conditions (Grabowska *et al*., 2012).

Treatment of crops with bio-stimulants has shown significant yield increases. Rathore *et al*. (2009) indicated that foliar application of a *Kappaphycus alvarezii* seaweed extract increased growth parameters and yield in soybean (*Glycine max* L.) and enhanced nutrient uptake. Application of Fantac, a mixture of 5% N-Acetyl thiazolidine carboxylic acid (N-ATCA) and 0.1% folic acid, significantly increased rice grain yield between 11.3% and 16.2% above the control (Srivastava *et al*., 2010). The application of an organic root bio-stimulant on *Amaranthus cruentus* seedlings resulted in a significant increase in vegetative growth and root development when combined with a mineral fertiliser (Akande, 2006). The search for novel compounds that could have a significant effect on crop yield is therefore ongoing.

Even though bio-stimulants are successfully used on horticultural and agricultural crops, the end results depend on the species and cultivar, as well as the time of application and concentration of the bio-stimulant. It was shown that when Aminoplant was applied to spinach, it had no effect on yield parameters and no significant yield increase was found (Kunicki *et al*., 2010).

During a recent study, Van der Watt and Pretorius (2013) utilised various bio-assays to analyse the *in vitro* and *in vivo* effect of a *Lupinus albus* L. cv. Betsuhana White seed

suspension (SS) on grain crops. Results indicated that a single application of SS to maize and wheat, increased yield compared to control untreated plants. Initially SS did not significantly increase hypocotyl growth of Cress seedlings, but instead root growth was enhanced (Van der Watt and Pretorius, 2013). SS, however, enhanced root and coleoptile growth in wheat and maize seedlings. This supplied the rationale for the isolation and identification of the active bio-stimulatory compound(s) within SS.

Following standard activity directed chromatographic and bio-assay procedures, the active bio-stimulatory compound was identified as glyceryl trilinoleate (SS a.i.) (Van der Watt and Pretorius, 2013). The glyceryl trilinoleate (SS a.i.) significantly enhanced the respiration rate of monoculture yeast cells and increased root growth in maize and wheat, thereby indicating the bio-activity of this compound.

The aim of this study was to determine whether a 60% purified *Lupinus albus* L. seed suspension called SS, increased yield in wheat and if this compound can successfully be used in agricultural practises. Even though it was previously postulated that photosynthesis is targeted by SS application (Janse van Rensburg, 2009), the exact mechanism of SS function is still unknown. The role and effect of SS will be assessed during a glass house trail and in response to stress conditions.

Literature Review

CHAPTER 2

2. Introduction

Globally, between 1.2 and 1.5 billion hectares are used for crop cultivation while another 3.5 billion are used for grazing (Howden *et al*., 2007). Even though agriculture is one of the largest land users across the globe, agricultural production will have to increase significantly to meet the projected growth in human population and per capita food demand (Howden *et al*., 2007). With a projected world population of 9 billion by 2050 (Roberts, 2011), the sustainable production of crops is of utmost importance.

Global food security is challenged by various factors, including water shortage, high energy cost and climate change (Simelton *et al*., 2012). Climate change can be in the form of higher temperatures and a lack of water causing longer periods of drought. Countries relying on agriculture as a source of income and food will be more vulnerable to climate change and climate variability (Bryan *et al*., 2009). According to the Intergovernmental Panel on Climate Change (IPCC) (2007), warming will be greater in sub-Saharan African countries in comparison to the global average which pose a significant threat to food and water security within this region.

2.1 Environmental changes and their effect on crops

Grain crops provide approximately two-thirds of the total annual human and animal protein intake (Tubiello *et al*., 2007). The management of natural resources is therefore of cardinal value in order to provide food for a growing population while still conserving soil and water resources (Cassman *et al*., 2003). The success of agriculture depends largely on the climate because heat, light and water are the main driving forces of crop growth. Changes in these three factors could have a significant effect on food production. Developing countries are more susceptible to the impacts of drought and heat as they do not always have the financial resources to cope with fluctuations in climate.

Certain environmental changes can however be beneficial to agriculture. Increased carbon dioxide (CO_2) levels account for approximately one third of the observed 1% increase in global wheat production and according to Tester and Langridge (2010) higher temperatures could also benefit plants. At high latitudes and altitudes growth inhibition occurs due to low temperatures. This phenomenon could be alleviated should the mean global temperature increase. Offsetting this aspect is the fact that drought patterns could be altered and the development of novel pests and diseases could occur.

2.1.1 Effect of higher CO_2 levels

Atmospheric CO_2 levels are currently 40% higher in comapirson to that at the dawn of the industrial revolution (Lindroth, 2010). According to the IPCC (2007) the projected atmospheric CO_2 concentration will increase from 280 $mmol.mol^{-1}$ on ground level to between 500 to 1000 $mmol.mol^{-1}$ by the end of the century. This will be accompanied by a subsequent increase in global temperatures of between 1.8 and 5.8°C.

Carbon (C) and nitrogen (N) constitute 45% and 5% of plant dry matter respectively and are two crucial structural elements. The maintenance of the C/N ratio is of utmost importance in maintaining plant growth development (Wingler *et al*., 2006; Zhang *et al*., 2007). Increased photosynthetic rate is the initial response of C_3 plants to elevated CO_2 concentrations (Taub, 2010). The stimulation is however not always maintained at the maximum level. This might be due to reduced stomatal conductance whereby intercellular CO_2 is depleted and less is available for use by photosynthetic machinery (Kant *et al*., 2012). Decreased photosynthetic rate in CO_2 rich environments could also be a result of a decrease in electron transport to Ribulose-1-5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation, ultimately changing the N metabolism of the plant. The latter is accompanied by changes in growth, development and yield (Kant *et al*., 2012).

The increase of CO_2 levels as a result of the use of fossil fuels however has a 'fertilizing' effect on most plants (http://www.co2science.org/about/position/globalwarming.php). It was shown that the increase in atmospheric CO_2 had a positive effect on plant growth (Alberton *et al*., 2010). C_4 crops including maize, sorghum and millet are not as sensitive to fluctuations in CO_2 concentrations as others (Leakey *et al*., 2006). C_4 photosynthesis should theoretically not be affected by elevated CO_2 concentration as it is usually saturated. Leakey *et al*. (2006) showed that in the absence of drought, elevated CO_2 levels had no significant effect on key photosynthetic enzymes in maize. Sun *et al*. (2012) indicated that when strawberries were grown at elevated CO_2 levels at low temperatures, yield and quality increased. When they were grown at elevated CO_2 levels at high temperatures, strawberry production decreased. They stated that research must focus on the combined effects of high temperature and elevated CO_2 levels in order to obtain adequate crop yield.

In a study by Vogan and Sage (2012) the authors illustrated the effects of low CO_2 concentrations and increased temperatures on C_3, C_3-C_4 and C_4 crops. C_3-C_4 crops originated during the intermediate stage of the C_4 evolution and include crops where the photo-respiratory cycle occurs between the bundle sheath and mesophyll cells (Vogan and Sage, 2012). Seedlings were grown in a growth chamber and chlorophyll content determined once each gas exchange response was completed. Plants grown at normal CO_2 concentrations did not have any effect on leaf N, leaf C/N ratio or specific leaf mass. Results did however indicate that, when grown at low and elevated CO_2 concentrations, 180 and 380 $\mu mol.mol^{-1}$ respectively, Rubisco content in leaves were 60% lower while the C/N ratio was 25% higher in C_4 species form the genera *Flaveria*, *Heliotropium*, and *Alternanthera* in comparison to other functional types. When the temperature was however increased to 37°C, a 50% increase in net CO_2 assimilation was observed in C_3-C_4 species compared to C_3 species. This illustrated the advantage of the C_3-C_4 pathway at low atmospheric CO_2 concentrations in warm climates.

2.1.2 Effect of higher ozone levels

Ozone (O_3) concentrations have risen within the last century reaching a global mean of approximately 50 parts per billion (ppb) on the surface (Fiscus *et al*., 2005). Even though substantial progress has been made in the past decade to understand the effect of rising O_3 concentration on crops, 25% of the Earth's surface could be exposed to O_3 concentrations in excess of 60 ppb. The projected increase in surface O_3 is said to be between 20 and 25% by 2050 (IPCC, 2007). This could have significant effects on agricultural yield since ozone pollution causes yield reductions at concentrations as low as 20 ppb (Ashmore, 2002).

Increased ozone levels interact with leaf content to form reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hydroxyl radicals (Fiscus *et al*., 2005). Unscavenged ROS induces necrosis, early senescence and abscission. The result is a transient decrease in stomatal conductance (Kollist *et al*., 2007; Vahisalu *et al*., 2010). A photosynthetic decrease is however not thought to be associated with decreased conductance as recovery occurs within 30 to 40 min of O_3 treatment (Kollist *et al*., 2007).

According to Long *et al*. (2005) a 20% increase in O_3 will decrease rice and maize yields by 4% and 5% respectively, while wheat yield will decrease by 9% and soya with 12%. By 2050, yield reductions could be as high as 8.9%, 9% and 17.5% for barley, wheat and rice respectively (Feng and Kobayashi, 2009). Even though predicted yield changes are variable within species, all point to reduced yield. Yield increases as a result of increasing atmospheric CO_2 in C_3 crops will be eliminated by 2050 due to increasing O_3 levels while yield in C_4 species will decrease by 5% (Jaggard *et al*., 2010).

A compound that could possibly protect plants from O_3 is ethylene diurea (EDU). Singh and Agrawal (2010) found that EDU had to be applied to wheat crops at all developmental stages in order to prevent O_3-induced yield losses. The effect of O_3 on wheat yield was thus more cumulative.

2.1.3 Effect of variable temperatures

Increased temperatures as a result of climate change will have a significant effect on current commercial crops like wheat (Shewry, 2009). In order to obtain maximum yield, wheat must be grown between 15 and 20°C. These temperatures increase the duration of grain filling and yield a higher starch accumulation per kernel (Dupont and Altenbach, 2003). Depending on the variety, rate of grain fill can either increase or decrease with increased temperatures. In some varieties, the rate of grain fill is constant while kernel weight decreases (Dupont and Altenbach, 2003). Guedira and Paulsen (2002) noted significant decreases in kernel weights when plants were subjected to a temperature of 30°C during grain fill. Dough strength seemed to increase with increasing temperatures up to 30°C (Randall and Moss, 1990), but above this threshold dough strength weakened with reduced quality (Blumenthal *et al*. 1991). Schlenker and Roberts (2009) also indicated yield increases in maize at 29°C, soybeans at 30°C and cotton at 32°C. These temperatures were seen as the threshold for crops since incubation of maize at 40°C for 24 h caused a decrease in yield of 7% with similar results being obtained for the other crops.

According to Peng *et al*. (2004) decreases in the rice yield can be directly attributed to the increase in night-time temperatures as a result of global warming. They indicated a significant decrease in grain-filling and yield when rice was grown at a constant day temperature of 33°C but increasing night temperatures from 25 to 33°C. Zhang *et al*. (2013) analysed the effect of low and high night temperatures on rice yield. They conducted field experiments from 2009 to 2011 and observed an increase of 4.8°C, 4.0°C, 3.9°C and 3.8°C in night temperatures over the 4 seasons. Rice plants exposed to high night temperatures showed a decrease in yield of between 8% and 16.7% compared to plants exposed to low night temperatures. Low biomass and harvest index attributed to the negative effect of high night temperatures on rice yield. Similar results were obtained by Cheng *et al*. (2009) when they observed a significant decrease in brown rice yield following high night temperature exposure.

As temperatures increase, the periodic plant life cycle is accelerated thereby shortening the growth period. A reduction of grain yield under irrigated, well watered wheat crops were associated with an increase in mean temperatures during the growing season (Lobell and Ortiz-Monasterio, 2007). A reduction in grain production of up to 50% was observed following increased temperatures of 2°C during the Australian growing season (Asseng *et al*., 2011). The authors noted that most of these reductions were as a result of leaf senescence due to temperatures above 34°C.

During high temperatures the sexual reproductive phase is especially vulnerable and could have significant consequences on agricultural crops (Thuzar, 2010). Prasad *et al*. (1999) showed that in groundnut, there was a significant negative correlation between pollen viability and production and temperatures above 34°C. At these temperatures the degeneration of the tapetal layer of pollen is associated with pollen sterility and reduced yield (Porch and Jahn, 2001). In other grain crops, increased heat caused sterility of the pollen with no reproduction being possible. Endo *et al*. (2009) indicated that even though rice plants exposed to increased temperatures showed normal pollen grain formation, the seed had reduced germination rates. High temperatures increase anther indehiscence which ulimatley cause floret sterility (Matsui *et al*., 2001).

Low temperatures also affect many economically important crops. The response however differs between species. Airaki *et al*. (2012) showed that pepper plants exposed to low temperatures for a maximum of three days showed oxidative stress during the first 24 hours. There after plants recovered by regulating changes in the antioxidant metabolism. Similar results were obtained by Cui *et al*. (2005) when rice seedlings were exposed to temperatures ranging from 5 to 15°C. Proteomic analysis indicated that many of the up-regulated proteins were involved in antioxidant reactions and 43.9% of the identified proteins were chloroplast associated. Yan *et al*. (2006) indicated that when rice seedlings were exposed to a temperature of 6°C for 6 or 24 h with a subsequent 24 h recovery at 28°C, photosynthesis was the functional group that was most affected. Chilling temperatures also caused leaf rolling and increased relative electrolyte leakage indicating the effect of decreased temperatures on membrane permeability.

2.1.4 Effect of drought

The availability of water directly affects plant growth and crop production. In Africa, there is not enough water even under normal conditions to produce high crop yields. In Sub-Saharan Africa, 95% of cereal production is dependent on rainfall (Wani *et al*., 2009). During periods of drought millions of people face starvation. In 2009 poor rainfall caused an increase of 53-million food-insecure people in Sub-Saharan Africa (FAO, 2008). Excessive rainfall on the other hand has severe effects on young seedlings and plant roots as waterlogged soil causes roots to rot. Crops that rely on growing-season rainfall lose more water through evaporation from the soil, making them more vulnerable to excessive rainfall (Sadras, 2003) while crops relying on water stored within the soil lose less water through evaporation while growing (Hatfield *et al*., 2001).

As temperatures increase, so does the water holding capacity of air. Even if no precipitation changes occur, evapo-transpiration rates will increase as a result of increasing temperatures, thereby enhancing drought incidence. Global crop yield is thus dependent on water availability. According to the IPCC (2007), water resources could decline by as much as 30% by the middle of the century therefore also increasing the land area affected by drought stress. Due to the low heritability of tolerance, it has been difficult to increase yield of drought affected crops (Neumann, 2008).

Guóth *et al.* (2009) recently analysed the effect of drought stress on two drought tolerant and two drought sensitive wheat cultivars. Results indicated a more pronounced decrease in water potential in the sensitive cultivars while no significant effect was observed on chl *a* and chl *b* levels in all cultivars. Non photochemical quenching (NPQ) levels increased significantly in drought sensitive cultivars, while the number of kernels per spike, the kernel weight per spike and the 1000-kernel weight decreased significantly in the sensitive cultivars. Yield was also decreased in one of the tolerant cultivars.

The changes in environmental conditions as a result of climate change will thus have a dire effect on the worldwide crop production. It may however increase production in, for example, very dry areas. Nevertheless, agriculture will be affected by climate changes. One of the key aspects of plant growth affected by these environmental conditions is photosynthesis.

2.2 The effect of environmental changes on photosynthesis

The process of photosynthesis that fuels life on Earth is localised to the highly compartmentalized chloroplasts, consisting of various light-dependent complexes, including photosystem II (PSII), cytochrome b_6f complex (Cyt b_6f) and photosystem I (PSI).

The plant thylakoid membrane consists of approximately 80% galactolipids (Shimojima and Ohta, 2011). In comparison to other biological membranes, it is a relatively fluid structure which is important for photosynthetic transmembrane diffusion. PSII is a membrane bound protein complex that harvests light energy and consists of chlorophyll and carotenoid pigments (Hamdani *et al*., 2011). PSII also contain the PSII outer antenna proteins and the light-harvesting chlorophyll *a*/*b*-binding proteins (LHCBs), which are the most abundant membrane associated proteins (Xu *et al*., 2012). Many intrinsic and extrinsic proteins are involved in PSII of which the extrinsic polypeptides on the luminal side are the most important. Together three polypeptides, the oxygen evolving enhancer protein 1 (OEE1), the oxygen evolving enhancer protein 2 (OEE2) and the oxygen evolving enhancer protein 3 (OEE3) form the oxygen evolving complex (OEC) that maintains the manganese cluster (Debus, 1992). This is the site of water oxidation as well as the formation of PSII core proteins D1 and D2. The PSI complex is also situated in the thylakoid membrane and mediates light-driven electron transport to the ferredoxin- nicotinamide adenine dinucleotide phosphate (NADP) complex.

The Calvin-Benson cycle (Calvin cycle) is associated with carbon fixation during which CO_2 is incorporated via the reaction between Ribulose-1-5-bisphosphate (RuBP) and Rubisco

(Hohmann-Marriott and Blankenship, 2012). Rubisco is, however, an inefficient enzyme that catalyses two competing reactions namely carboxylation and oxygenation (Andersson *et al*., 1989). Environmental changes like temperature and drought can increase the oxygenase reaction. If Rubisco oxygenase activity can be reduced, carbon assimilation and photosynthesis would increase significantly.

Photosynthesis can also serve as a sensing system to detect various environmental changes (Pfannschmidt and Yang, 2012) with light, temperature and water being the most prominent abiotic factors that affect photosynthesis. The photosynthetic light reaction is mainly temperature-independent while the Calvin cycle is light-independent but extremely temperature and substrate sensitive. Biotic and abiotic factors can thus disturb the balance between the two photosynthetic reactions that will ultimately affect photosynthetic efficiency.

2.2.1 Sensing effect of light stress

Illumination of plants can be experienced in one of two ways: (i) optimal light and (ii) excess light. When a plant experiences optimal light, photo-damage and repair are in balance since the absorbed light quanta is utilized during various biochemical reactions. During excess light conditions the quanta exceed utilization (Allahverdiyeva and Aro, 2012). This causes photo-oxidative damage to the photosynthetic apparatus causing photo-inhibition. PSII photo-damage occurs at all light intensities and, depending on the environmental conditions, can even occur at low light intensities.

CAM plants are able to survive under fluctuating light conditions while some are very resistant to high light stress (Cela and Munné-Bosch, 2012). This is unlike C_3 and C_4 plants. At low light intensities, C_4 plants have a lower quantum yield for CO_2 uptake (Krall and Pearcy, 1993) and lack the capacity for maintaining a high state of photosynthetic induction (Sage and McKown, 2006). Since photo-respiration is almost completely repressed, C_4 plants are also more sensitive to photo-inhibition (Niyogi, 2000).

Photo-inhibition occurs as a result of exposure to high irradiance thereby hindering PSII activity, which is independent of the change of pigment concentration (Goh *et al*., 2012). The first reaction centre to lose activity is the OEC (Tyystjärvi, 2008) indicating that photo-inhibition occurs as a result of net photo-damage to PSII (Fig. 2.1). PSI is not as sensitive to light-induced damage compared to PSII but Takahashi and Murata (2008) reported that following ten photoperiods of high light intensity, inhibition of PSI also occurred. The cyclic electron flow (CEF) cycle surrounding PSI protects this photosystem and avoids photo-inhibition of PSII. The latter occurs when the rate of photo-damage to PSII exceeds the rate of PSII repair (Takahashi *et al*., 2009).

Damage to PSII can also be demonstrated by the solar action spectrum of PSII damage. This model suggests that under incident sunlight, the photo-damage to PSII is associated with UV wavelengths as well as yellow light wavelengths (Takahashi and Badger, 2011).

2.2.1.1 Proteases involved in degrading damaged PSII subunits

PSII consists of more than 20 subunits with the D1 and D2 proteins forming the core of the complex (Kato and Sakamoto, 2009). Yamamoto (2001) illustrated that the D1 protein undergoes irreversible oxidative damage by ROS following strong illumination. The D2, PsbH and Cyt b559 proteins can also be damaged after light exposure but this occurs less frequently (Rokka *et al*., 2005).

The process of D1 degradation has been studied for the past 20 years, but the proteases involved in degradation have only recently been characterized. Two important protease families are involved in D1 degradation. The first is the Deg-family of proteases. They are present in bacteria, higher plants and mammals. Deg proteases are nuclear-encoded serine proteases (Kato and Sakamoto, 2009). In *Arabidopsis*, four Deg proteases are found in the thylakoid membrane, three in the thylakoid lumen (Deg1, 5, 8) while Deg2 is associated with the stromal side of the thylakoid lumen (Schubert *et al*., 2002).

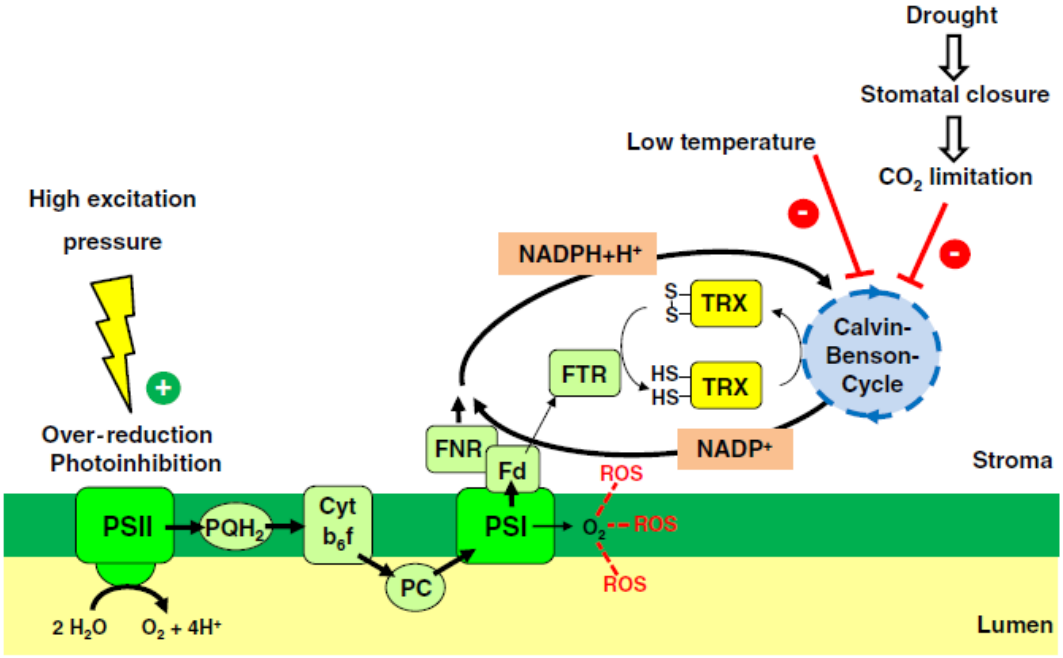


Fig. 2.1. The interacting effects of light, temperature and drought on photosynthesis. The scheme represents the photosynthetic electron transport chain and the dark reaction (Pfannschmidt and Yang, 2012). They are connected via the $NADPH+H^+/NADP^+$ redox reaction and the FTR/thioredoxin system. Red arrows represent the repression of the Calvin cycle while black arrows represent electron flow (Pfannschmidt and Yang, 2012). Cyt b_6f - cytochrome b_6f complex; FNR – ferredoxin NADP(+) reductase; FTR – ferredoxin-thioredoxin oxidoreductase; PQH_2 – dihydroplastoquinone; TRX – thioredoxin.

It was initially thought that Deg2 is involved in D1 protein degradation *in vitro* (Hauβühl *et al*., 2001). Recent studies indicated the role of Deg1 (Kapri-Pardes *et al*., 2007), Deg5 and Deg8 (Kato *et al*., 2012) in D1 protein degradation. Deg1 assists the stroma-exposed FtsH and Deg2 proteases in D1 degradation through cleavage of the lumen-exposed regions of the protein. Since Deg5 and Deg8 is also located on the luminal side of the thylakoid membrane, Kato *et al*. (2012) postulated that these proteases cleave D1 at the luminal loop thereby connecting transmembrane helices C and D. The cleaved D1 products then form a substrate for FtsH.

The FtsH-family of proteases plays the most significant role in D1 degradation. FtsH proteases belong to the ATPase family that are ATP-dependent membrane-bound zinc metalloproteases (Tomoyasu *et al*., 1993). In higher plants FtsH is thylakoid bound (Lindahl *et al*., 1996). The functional domains of the bacterial enzyme are conserved in the plant FtsH (Lindahl *et al*., 2000). FtsH proteases are located between the grana and stroma–exposed region of the thylakoid membrane (Fristedt *et al*., 2009) and catalyse the degradation of soluble and membrane-bound proteins (Koppen and Langer, 2007).

Arabidopsis contains 12 FtsH homologs of which nine are chloroplast bound (Sakamoto *et al*., 2003). The four major isoforms of this complex, located in the thylakoid membrane, is FtsH1, FtsH2, FtsH5 and FtsH8 (Rodriguez *et al*., 2011). Mutants that lack the FtsH2 (yellow variegated 2) and FtsH5 (yellow variegated 1) homologs have shown a leaf-variegated phenotype (Sakamoto *et al*., 2002). FtsH2 is involved in D1 protein degradation (Kato and Sakamoto, 2012) and the removal of D1 following heat damage (Kamata *et al*., 2005) and UV-B irradiation (Cheregi *et al*., 2007). FtsH2 also degrades unassembled PSII subunits within the thylakoid membrane (Komenda *et al*., 2006) and regulates the levels of glucosyl-glycerol phosphate synthase involved in osmoprotection (Stirnberg *et al*., 2007).

2.2.1.2 Repair of PSII

The PSII repair cycle involves several steps. Firstly, monomerization of the PSII complex occurs after detachment of the light harvesting complex II (LHCII) antenna complex. The PSII core then moves from the grana to the stromal lamellae (Kirchhoff *et al*., 2008). Secondly, OEC proteins dissociate from PSII and disassembly of the core monomer takes place (Aro *et al*., 2005; Allahverdiyeva and Aro, 2012). Thirdly the D1 protein is degraded by catalysis by the FtsH protease and the *de novo* synthesis of the precursor D1 (pD1) protein takes place (Bailey *et al*., 2002). The pD1 protein matures through cleavage of C-terminus amino acids by carboxyl-terminal peptidase (CtpA) (Kato and Sakamoto, 2009). The PSII reaction centres and the OEC extrinsic proteins reassemble and photo-activation of the PSII complex takes place (Rokka *et al*., 2005).

2.2.2 Sensing effect of temperature stress

Temperature stress is one of the most important abiotic factors that limit crop production. Both elevated and low temperatures have a significant effect on photosynthesis and other cellular processes.

2.2.2.1 Low temperature

The cell membrane is one of the first structures to be affected by low temperatures. During low temperatures, the increase in relative and absolute polyunsaturated fatty acid content is of utmost importance as this aid in maintaining cellular integrity and chloroplast function (Campos *et al*., 2003). When tobacco leaves and roots were frozen at -3°C, electrolyte leakage increased to 80 and 75%, respectively, indicating the destruction of plant membranes exposed to chilling temperatures (Popov *et al*., 2012). Following root and leaf hardening, lipid content in tobacco leaves increased by approximately 20%.

A study done by Leonardos *et al*. (2003) indicated that wheat plants exposed to 5°C showed higher photosynthetic rates in the morning when light intensity and CO_2 concentration was high. Their data is consistent with the fact that, during the day, a feedback inhibition of photosynthesis occurred. They also showed that the decrease in photosynthetic rate between 08:00 and 16:00 could be associated with an increase in photo-oxidative damage during cold stress.

Similar results were obtained when two maize (*Zea mays* L.) cultivars, one tolerant and one susceptible, were exposed to cold stress at 5°C (Aroca *et al*., 2003). In temperature-sensitive maize that was pre-treated with drought, the effect of chilling was decreased. One day after recovery from chilling, the tolerant and drought pre-treated sensitive cultivar showed higher net photosynthetic rates in comparison to non-drought pre-treated plants. The increase in net photosynthesis decreased the probability of generating ROS even though following drought pre-treatment, hydrogen peroxide (H_2O_2) content increased. The authors postulate a signalling role for drought pre-treatment in enhancing cold tolerance in maize.

Hajiboland and Habibi (2011) investigated the effect of chilling stress, with or without acclimation, on winter and spring wheat. As expected, maximum quantum efficiency of PSII (F_v/F_m) and CO_2 assimilation rates decreased in the spring cultivar but not in the winter cultivar, whereas increased NPQ levels was observed only in the winter cultivar. Both peroxidase activity and H_2O_2 levels were higher in the spring cultivar compared to the winter cultivar. The reduction in photosynthetic capacity in spring wheat could be assigned to non-stomatal limitations of photosynthesis. These limitations include the decrease in F_v/F_m causing damage to the photosynthetic apparatus (Allen and Ort, 2001) followed by impaired CO_2 fixation (Liang *et al.,* 2007) that could ultimately lead to the generation of ROS and photo-oxidative damage to PSII.

2.2.2.2 Elevated temperature

The Calvin cycle enzymes are sensitive to elevated temperatures that explain the inhibition of carbon assimilation at moderate temperatures (Sharkey, 2005). The decrease in Rubisco activity is due to the heat-sensitive enzyme Rubisco activase (Law and Crafts-Brandner, 1999). Light saturated CO_2 exchange rates (CER) decreased when leaf temperatures of cotton (*Gossypium hirsutum*) and wheat (*Triticum aestivum*) increased. The decline in CER and F_v/F_m levels both correlated to decreased Rubisco activity.

Elevated temperatures also cause membranes to become permeable and alter the macroscopic structure of chloroplasts (Tóth *et al*., 2005). Heat stress therefore has a significant effect on thylakoid membrane integrity as shown by Mathur *et al*. (2011a). They indicated that when spinach leaves were exposed to elevated temperatures, thylakoid destacking increased. Similar results were obtained by Semenova (2004).

The PSII reaction centre consists of three components: PSII α, PSII β and PSII γ (Hsu and Lee, 1991). Mathur *et al*. (2011b) observed that in wheat seedlings exposed to temperatures ranging from 25 to 45°C, the proportion of α centres decreased while β and γ centres increased. They postulated that the active PSII α centres were converted to inactive PSII β and PSII γ reaction centres. Their results also indicated that antenna size heterogeneity could recover at temperatures of up to 40°C, but irreversible damage occurred at 45°C.

Heat-induced cross linking of D1 protein with D2 protein leads to a decrease in PSII activity (Ohira and Yamamoto, 2001). One of the most important extrinsic proteins of the oxygen evolving complex is coded by the *PsbO* gene. This gene is also referred to as the Mn-stabilizing protein (Heredia and De las Rivas, 2003). During high temperature conditions, two of the four Mn atoms are released from the OEC thereby inactivating this complex. Almeselmani *et al*. (2012) recently analysed the effect of heat on a tolerant (C306) and susceptible (PBW343) wheat cultivar. Results indicated that the expression of both *PsbO* and

PsbA increased in the tolerant cultivar but was similar to control treatment following prolonged heat stress. A smaller reduction in total chlorophyll content and chlorophyll *b* attributes to the tolerance in C306 which in turn maintains the expression of *rbcL* and *rbcS* and Rubisco activity (Almeselmani *et al*., 2012). In a study done by Velikova *et al*. (2012), it was shown that in isoprene-emitting transgenic *Arabidopsis* plants exposed to heat stress the production of ROS and reactive nitrogen species (RNS) were lower in comparison to wild-type plants. The authors thereby postulate that in heat stressed plants, isoprene quenches ROS and has a stabilizing effect on the thylakoids.

2.2.3 Sensing effect of water stress

Drought is the natural phenomenon where the level of water availability is significantly lower compared to normal over a specific period of time (Vicente-Serrano *et al*., 2013). During drought conditions, shoot growth decreases while the plant still tries to maintain root growth (Sharp, 2002) resulting in an increased root to shoot ratio (Pinheiro and Chaves, 2011). This in turn alters carbon and nitrogen metabolism in various organs. Antonio *et al*. (2008) illustrated that when *Lupinus albus* plants were placed under water stress the stem components, stele and cortex, were less affected compared to leaves. The stem components showed a 15% reduction in water capacity in comparison to leaves that showed a decrease of 50%, thereby illustrating the water retention ability of stem components. Upon rehydration, plant water status was fully restored.

Drought also negatively affects photosynthesis, usually resulting in stomatal closure and limitations in gas exchange (Fig. 2.1) (Jaleel *et al*., 2009). When decreased stomatal conductance is combined with high irradiance, the rate of reducing power production overcomes the rate of use in the Calvin cycle. This is a well known mechanism in C_3 plants whereby photosynthesis is down-regulated to produce photo-inhibition (Adams *et al*., 1988). In severe cases, drought leads to an imbalance in metabolism ultimately causing plant death (Jaleel *et al*., 2008).

Drought-induced stomatal closure is triggered by abscisic acid (ABA) and decreased CO_2 availability to the photosynthetic apparatus (Michelozzi *et al*., 2011). These conditions cause a reduction in photosynthetic electron transport thereby inducing oxidative stress (Borsani *et al*., 2001). Huang *et al*. (2012) found that when the resurrection plant *Paraboea rufescens* was exposed to mild drought stress, CEF was significantly affected and leaves indicated a decrease in the ability to utilise linear electron flow products. Increased CEF and NPQ levels protected PSII from photo-inhibition while alleviating the over-reduction of PSI.

Two grass genotypes, one drought resistant (Midnight) and the other drought sensitive (Brilliant), were exposed to drought stress and then re-watered. Hu *et al*. (2010) indicated decreased net photosynthetic rates, stomatal conductance and transpiration. Photochemical efficiency, the carboxylation rate of Rubisco and RuBP regeneration significantly decreased after drought stress. Relative water content was restored to 90% of that of the control following re-watering. Net photosynthetic rates, stomatal conductance and the maximum quantum efficiency of PSII partially recovered after rehydration.

2.3 Agrochemicals used in agriculture

Novel approaches are required to safeguard crops against biotic and abiotic stress factors while still ensuring optimal yield. These include various agricultural techniques, like crop rotations and conservation tillage (http://www.sustainabletable.org), that have been successfully implemented (Majathoub *et al*., 2004). Agrochemicals called plant activators are compounds that are able to activate the plant defence response in the form of systemic acquired resistance (SAR) (Noutoshi *et al*., 2012) thereby conferring enhanced disease resistance to crops. In order to be classified as a plant defence activator, a compound must be able to induce an identical resistance response in the plant in comparison to a spectrum of different pathogens. An advantage of plant activators is the fact that no resistant microbe has emerged as a result of the treatment (Noutoshi *et al*., 2012).

2.3.1 The plant defence response

Crop plants must be able to defend themselves against a variety of pathogens which includes fungi, viruses and bacteria. Even though pesticides, including microbicides, fungicides and insecticides are widely used, there is still an estimated 14% crop loss due to disease (Noutoshi *et al*., 2012).

Plants are constantly subjected to various pathogenic attacks and have therefore evolved both passive and inducible defence mechanisms. The passive or innate defence response is ever-present but when pathogens overcome it, plants rely on an inducible active defence response for protection. Genetic traits in both the host and pathogen determine the ability of a plant to respond to pathogen infection through either of the defence pathways.

2.3.1.1 Innate immune response

The innate or basal defence response includes the recognition of pathogen associated molecular patterns (PAMPs) (Truman *et al*., 2006). PAMPs are small pathogen derived molecules that are non-specific. PAMPs act as general elicitors of the defence response and function in vertebrates and non-vertebrates that include Gram-negative bacteria, Gram-positive bacteria and methylated bacterial DNA fragments (Medzhitov and Janeway, 2002). Even thought various PAMPs are known, PAMP receptors have only been identified for flagellin, chitin and the translation elongation factor EF-Tu (Zipfel, 2008). PAMP recognition occurs via pattern recognition receptors (PRRs) located in the cell wall (Pitzschke *et al*., 2009). Once activated, PRRs initiates an intracellular signalling pathway resulting in PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). The activation of PTI is mediated via the mitogen activated protein (MAP) kinase cascade that relays the signal from the PRRs to the downstream components (Pitzschke *et al*., 2009).

2.3.1.2 Induced defence mechanisms

The inducible defence response usually involves the hypersensitive response (HR), SAR and the induced systemic resistance (ISR).

2.3.1.2.1 Hypersensitive response

The gene-for-gene defence response in plants is well defined (Martin *et al*., 2003). In order to overcome pathogen effectors, plants have developed the ability to directly or indirectly recognise a pathogenic avirulence (avr) protein or effector resulting in effector-triggered immunity (ETI) (Chisholm *et al*., 2006; Jones and Dangl 2006). This recognition response results in the activation of the HR in surrounding plant cells. In contrast, when no recognition occur virulence factors alter the host virulence targets causing disease susceptibility (Truman *et al*., 2006).

Two important signalling molecules that contribute to the activation of the HR in plants following fungal pathogen infection (Yoda *et al*., 2006) are H_2O_2 and nitric oxide (NO) (Delledonne *et al*., 2001). The production of ROS is initiated by the reduction of molecular oxygen and/or by the transfer of excess excitation energy to O_2 causing the formation of a singlet oxygen ($^1O_2$) (Alscher *et al*., 2002). ROS accumulation is detrimental to plant health since it damages various macromolecules including DNA, proteins, chlorophyll and membranes (Blokhina *et al*., 2003).

2.3.1.2.2 Systemic acquired resistance

A second type of inducible long-lasting, host resistance is termed systemic acquired resistance (Ryals *et al*., 1996). SAR provides a broad, non-specific resistance that is

expressed throughout the plant. In order for SAR to be initiated in systemic tissue, inoculated tissue must generate a phloem transported signal (Shah, 2009).

The development of SAR is associated with salicylic acid (SA) biosynthesis and pathogenesis related (*PR*) gene expression (Hunt *et al*., 1996). Plants that are unable to accumulate SA, failed to activate SAR. Park *et al*. (2007) indicated that in *NahG* transgenic tobacco that failed to accumulate SA, the SA-derivative methyl salicylate (MeSA) accumulated and acted as a SAR signal. The subsequent hydrolysis of MeSA back to SA by SA-binding protein 2 (SABP2) then triggers SAR. Since MeSA is unable to induce disease resistance and a subsequent defence response in *NahG* transgenic tobacco, it is rendered as biologically inactive and unable to induce *PR*-gene expression (Koo *et al*., 2007).

Dempsey and Klessig (2012) recently reported on various phloem-mobile SAR signals that have been identified (Fig. 2.2). The proposed model for SAR activation is as follows: the pathogen-induced NtSAMT1/AtBSMT1 activity in leaves converts some of the accumulated SA to MeSA. The elevated levels of SA then inhibits MeSA esterase (MSE) activity of SABP2/specific members of the AtMES family. MeSA accumulates and is transported to the systemic leaves via the phloem. Once it reaches the systemic leaves, MeSA is converted back to SA through the SABP2/AtMES MSE activity. The release of SA will then trigger SAR and a defence response will be initiated accordingly.

2.3.1.2.3 Induced systemic resistance

Induced systemic resistance in plants is induced through plant growth-promoting rhizobacteria (PGPR) (Vallad and Goodman, 2004) of which *Pseudomonas* species are the best known (Raaijmakers *et al*., 2010). ISR is phenotypically similar to SAR (Desoignies *et al*., 2013) but does not involve the induced expression of *PR*-genes or accumulation of SA (Pieterse *et al*., 1996). Yan *et al*. (2002) illustrated that ISR does however rely on jasmonate and ethylene regulating pathways.

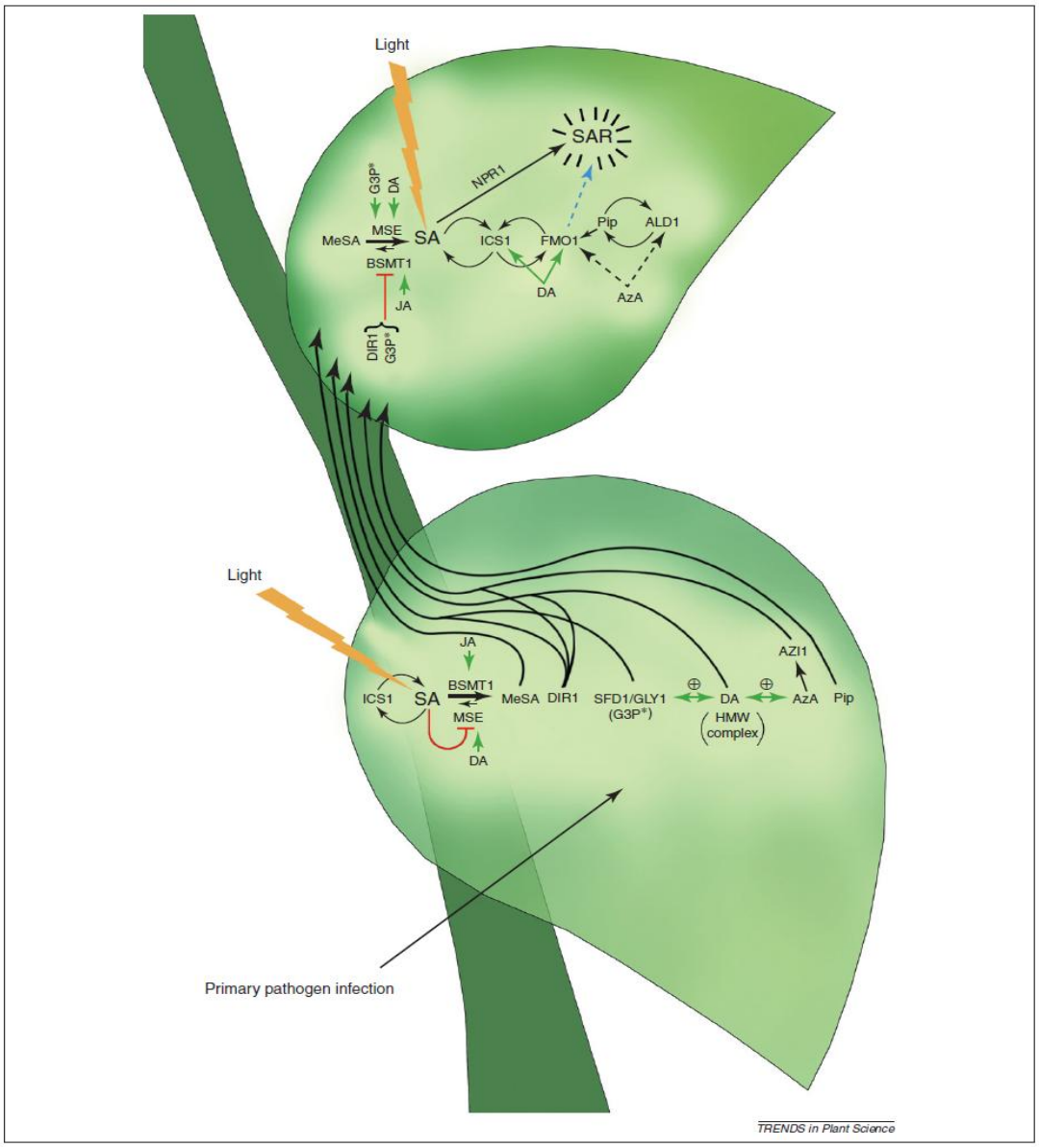


Fig. 2.2 Working model of putative SAR long-distance signalling. The various compounds involved in SAR signalling are as indicated (Dempsey and Klessig, 2012).

2.3.2 Plant activators and the plant defence response

Defence priming is thought to be induced through treatment with a natural or synthetic compound. Priming in plants entails the rapid activation of a defence response against various stress factors (Conrath, 2011). The defence response could either be in the form of SAR (Beckers *et al*., 2009; Jung *et al*., 2009), ISR (Conrath *et al*., 2002) or wound-induced resistance (Chassot *et al*., 2008) to name a few. One hypothesis on priming is that it involves cellular proteins important in signalling. These signalling proteins are activated once biotic or abiotic stress exposure occurs and a rapid, robust activation of immunity, defence and stress tolerance is established (Conrath *et al*., 2006).

2.3.2.1 Salicylic acid

SA is important in PTI- and ETI-mediated defence responses as SA levels increase in pathogen infected plants. This in turn activates various defence genes including the *PR1* gene, also known as a molecular marker for SA signalling (Tsuda *et al*., 2008).

Li *et al*. (2012) analysed the effect of SA in the protection of rice against infection with *Magnaporthe oryzae*. Proteomic analysis identified 36 proteins that were differentially expressed following SA treatment. The identified proteins were involved in defence, antioxidant activity and signal transduction. The influence of SA application to salt tolerant wheat was also investigated (Kang *et al*., 2012) following exposure to various salt concentrations. Global expression profiling identified up-regulated proteins involved in various cellular and metabolic processes, including signal transduction, stress defence, energy metabolism and photosynthesis. Application of SA to oilseed rape (*Brassica napus* L.) increased resistance to *Sclerotinia sclerotiorum* thereby confirming the positive role of SA in the establishment of a defence response (Wang *et al*., 2012).

2.3.2.2 Jasmonic acid

Jasmonic acid (JA) is an important defence hormone whose signalling pathway has been well studied in various crops. JA and its methyl ester, methyl jasmonate (MeJA), are two signalling molecules collectively called jasmonates. Biotic and abiotic stress induced responses are regulated by jasmonates. Once induced, JA is conjugated to Ile by means of JA conjugate synthase JAR1 activity (Staswick and Tiryaki, 2004) leading to the formation of JA-Ile (Fonseca *et al*., 2009).

JA-induced signalling is negatively controlled by SA (Leon-Reyes *et al*., 2010). Studies indicated that glutathione and JA also interacts as JA activates expression of the glutathione synthetase and glutathione reductase (GR) encoding genes (Xiang and Oliver, 1998). Recently Han *et al*. (2013) analysed the effect of an oxidative stress signalling mutant, *cat2*-triggered glutathione accumulation, on JA signalling. Results indicated that glutathione accumulation is required for the induction of the JA pathway and that by blocking this accumulation, the expression of JA-linked genes induced by H_2O_2 is terminated.

JA-dependent pathways affect several defence responses. Various studies have shown that *PR*-genes are induced following JA exposure (Rakwal *et al*., 2001). Kim *et al*. (2003) indicated that JA application increased the levels of a PR10-class protein, PBZ1. They indicated that in suspension cultured rice cells, JA is able to induce the expression of defence related genes. Peng *et al*. (2012) recently analysed the pathogen-responsive gene, *WRKY30*, in rice. Transcript levels of this gene increased rapidly following treatment with JA. Resistance to rice sheath blight fungus, *Rhizoctonia solani*, and blast fungus, *Magnaporthe grisea*, increased in rice overexpressing the *WRKY30* gene. These results together with the expression of JA synthesis-related genes *LOX*, *AOS2* and *PR3* and *PR10*, indicated the importance JA plays in the mediation of a defence response (Peng *et al*., 2012) thus indicating its role as a plant activator.

2.3.2.3 Ethylene

The gaseous plant hormone ethylene (ET) plays a crucial role in signal transduction during plant-pathogen interactions (Harrach *et al*., 2008). Ethylene production is induced by fungal toxins, pathogen invasion and endogenous elicitors amongst other things (Díaz *et al*., 2002). Ethylene activates various defence related processes in plants, including phytoalexin production, PR-protein synthesis and cell wall alterations. Depending on the plant-pathogen interaction, exogenous ET application can either induce a defence response or have no effect (Li and Yen, 2008).

More recently endogenous ET was genetically manipulated in rice to enhance resistance to rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) (Helliwell *et al*., 2013). The ET biosynthetic gene, *OsACS2*, was placed under control of a pathogen-inducible promoter, *PBZ1*. Molecular, physiological and pathological analyses indicated that the overexpression of *OsACS2* in transgenic rice led to the production of ET and *PR*-gene expression. Increased resistance was obtained against rice blast and sheath blight.

2.3.2.4 Probenazole

The most successful plant activator to date is Probenazole (3-prop-2-enoxy-1,2-benzothiazole1,1-dioxide, Oryzemate®). It was discovered almost 36 years ago through a fungicide screening (Watanabe *et al*., 1977). Probenazole can protect rice against blast fungus as well as bacterial blight. This reduces the use of common pesticides while ultimately decreasing yield loss (Noutoshi *et al*., 2012). Probenazole is not a SA analogue but might function through priming a defence response in plants. The effect of probenazole was seen in *Arabidopsis* (Yoshioka *et al*., 2001) and rice (Iwai *et al*., 2007; Umemura *et al*., 2009) plants.

It was however shown that Probenazole regulates the accumulation of SA once induced through pathogen infection and act upstream of SA. Pre-treatment of rice with Probenazole increased SA accumulation and PR protein levels. Iwai *et al*. (2007) also observed that Probenazole-induced resistance in rice could be attributed to the regulation of SA accumulation. Cloning of the Probenazole-responsive gene indicated that it codes for *Oryza sativa* UDP-glucose:SA glucosyltransferase (*OsSGT1*) and contributes to the SA signalling mechanism in rice (Umemura *et al*., 2009). The exact mode of action is, however still unclear (Noutoshi *et al*., 2012).

2.3.2.5 Actigard®

Another successful plant activator is acibenzolar-*S*-methyl (ASM). ASM is distributed by Syngenta Crop Protection and is called Actigard® in the USA and Bion® in Europe (Vallad and Goodman, 2004). The active ingredient in Actigard® is benzo (1, 2, 3) thiadiazole-7-carbotioic acid S-methyl ester (BTH) and its role as a plant activator has been elucidated in various studies (Pasquer *et al*., 2005; Van Hulten *et al*., 2006).

Foliar application of Actigard® on salmonberries significantly reduced disease severity of three *Phytophthora ramorum* isolates (Bailey *et al*., 2012). Actigard® is able to protect tomato plants against bacterial speck (*Pseudomonas syringae*) and spot (*Xanthomonas campestris*) by activating the plant's natural defence responses through the activation of SAR (http://www.syngentacropprotection.com).

2.3.3 Bio-stimulants in agriculture

Plant growth can be improved through the use of various naturally occurring and artificially derived compounds containing bio-stimulatory properties (Mora *et al*., 2010). They can either act as elicitors or provide microelements, organic compounds or nutrients that can be utilised

by plants. Various compounds have been registered as fertilisers while others are known as bio-stimulants that aid in nutritional absorption and transport of macro- and microelements. Several studies reported on the beneficial effects of bio-stimulants and illustrated increased root and shoot growth, water uptake and resistance to stress (Maini, 2006; Nagy *et al*., 2010). Bio-stimulants improve crop yield while simultaneously reducing the use of fertilisers. Most bio-stimulants contain plant hormones, amino acids and sea kelp extracts (Maini, 2006; Nagy *et al*., 2010).

2.3.3.1 Abscisic acid

Abscisic acid is a phytohormone that is produced during various climatic conditions and plays an important role in seed formation and germination (Rodríguez-Gazio *et al*., 2009). Chibani *et al*. (2006) analysed the effect of ABA treatment on seed dormancy in *Arabidopsis*. Application of ABA to non-dormant seeds inhibited seed germination and decreased the accumulation of proteins involved in energy and protein metabolism.

The ABA and biotic stress signalling responses share various similarities. Both rapidly leads to the formation of ROS (Guan *et al*., 2000) and generates the same NADPH-dependent respiratory burst oxidase homologs (Torres *et al*., 2002). When ABA is perceived by guard cell receptors following water stress, signalling pathways are activated that initiate stomatal closure (Desikan *et al*., 2004). Downstream of ABA, reversible protein phosphorylation and the activation of signalling compounds, such as G-proteins, leads to the closing of the stomata (Schroeder *et al*., 2001).

Exogenous application of ABA to soybean plants significantly increased both chlorophyll content as well as dry weight. Photosynthetic capacity also increased following ABA treatment of soybean seedlings (Travaglia *et al*., 2009). Studies also indicated that foliar treatment of ABA significantly increased yield and alleviated water stress in wheat (Travaglia *et al*., 2010).

2.3.3.2 Kelpak®

Kelpak® (Kelp Products, Simon's Town, South Africa) is a seaweed concentrate (SWC) developed from *Ecklonia maxima* (Osbeck) Papenfuss (Papenfus *et al*., 2012). Application of SWCs to plants increased root growth (Jones and Van Staden, 1997), vegetative growth (Steveni *et al*., 1992) as well as fruit quality and quantity (Rathore *et al*., 2009).

Beckett *et al*. (1994) analysed the effect of Kelpak® on tepary bean (*Phaseolus acutifolius* L.) yield. They showed a significant increase in bean weight and nitrogen concentrations. Results indicated that Kelpak® acted as a bio-stimulant and not just a fertilizer. Researchers have identified cytokinins and auxins (Stirk *et al*., 2004), as well as polyamines (Papenfus *et al*., 2012) in Kelpak®. Papenfus *et al*. (2013) recently reported on the significant effect Kelpak® had in relieving nutrient deficiency in N-deficient okra seedlings (*Abelmoschus esculentus* (L.) Moench). They postulated that this effect might be due to the fact that Kelpak® acts in synergy with plant growth regulators.

2.3.3.3 ComCat®

ComCat® (CC), derived from *Lychnis viscaria* L. seeds (Roth *et al*., 2000) is a natural bio-stimulant containing brassinosteroids (BRs) as the active substance (Van der Watt and Pretorius, 2011). Exogenous application of ComCat® to tomato plants increased fruit quality during storage (Melkamu *et al*., 2008). ComCat® also induced activity of PR-proteins (http://comcat.info/descript.html) and significantly increased yield in crop plants (Melkamu *et al*., 2008) and vegetables (Schnabl *et al*., 2001; Workneh, 2002).

2.3.3.4 *Lupinus albus* L. cv. Betsuhana White seed suspension

Lupins are legumes that belong to the family Fabaceae and consist of 450 species (Sanz *et al*., 2010). They are usually consumed as appetizers and are grown to improve soil quality (Sanz *et al*., 2010; Kordan *et al*., 2012). Van der Watt and Pretorius (2011) screened a *Lupinus albus* L. cv. Betsuhana White seed suspension (SS) to elucidate its bio-stimulatory properties. Results indicated that foliar application of SS increased yield in carrots, lettuce and beetroot. The active bio-stimulatory compound in SS is glycerol trilinoleate, a triglyceride consisting of three fatty acid moieties.

Effect of SS on yield and associated parameters in wheat

CHAPTER 3

3.1 Introduction

The global demand for food, feed and fuel has a direct effect on agricultural crop production (Naylor *et al*., 2007). In order to achieve the desired yield, farmers are faced with two options. The first is to increase the agricultural area under production, but this could increase the disruption of ecosystems. While the arable land area per capita decreased significantly since the 1960s, the potential for increase exists predominantly in South America and Africa (FAO, 2011). The second, more acceptable option to increase yield is to increase productivity on existing farmland (Edgerton, 2009).

During the last half of the twentieth century, the yield of arable crops has increased significantly in both developed and developing countries. Wheat yield in the United Kingdom rose from three to eight tons per hectare and from 1.08 to 2.7 tons per hectare worldwide (Jaggard *et al*., 2010). The increased yield could be attributed to farmers using nitrogen-rich fertilizers, chemicals for crop protection and improved crop varieties (Jaggard *et al*., 2010).

To supply food for all, various options have to be explored in order to increase crop yield. The use of bio-stimulants in agricultural practices has received much attention in recent years. One of the most attractive characteristics of bio-stimulants is that these molecules are natural (García-Martínez *et al*., 2010). One very useful bio-stimulant is seaweed extract (Zodape, 2009). According to the FAO (2006), more than 15 million metric tons of seaweed products are produced annually. These products are used successfully as bio-stimulants on agricultural and horticultural crops (Stirk *et al*., 2004). Chemical analysis of seaweed extracts indicated that most of these products contain a wide variety of plant growth regulators that include cytokinin, auxin and ethylene (Zhang and Ervin, 2004).

Auxins are phytohormones that mainly control plant shape (Tanaka *et al*., 2006) and play an important role in growth regulation in response to stimuli (Vanneste and Friml, 2009). The main naturally occurring auxin in plants is indole-3-acetic acid (IAA) (Zhao, 2010). Ahmed

et al. (2012) indicated that exogenous application of IAA to date palms significantly increased fruit quality parameters, including fruit weight and length and yield. The positive effect of IAA on root and shoot growth of crop plants was shown under various stress conditions (Chaudhry and Rasheed, 2003; Sheng and Xia, 2006). When sunflowers were grown in soil that was either moderately of highly contaminated with heavy metals from sewage sludge, the addition of IAA increased root and stem biomass of plants grown in the moderately contaminated soil. Since IAA did not have a significant effect on growth of plants in the highly contaminated soils, it indicated that the effect of IAA had an upper limit of effectiveness (Liphadzi *et al*., 2006). Agami and Mohamed (2013) observed substantial decreases in growth, relative water and pigment content in wheat plants exposed to cadmium. However, when the seeds were first soaked in IAA prior to planting, increases were observed in all three these parameters.

Cytokinins (CKs) belong to a group of phytohormones that regulate cell division and morphogenesis and are involved in flower and seed development, seed germination and nutrient uptake (Zalabák *et al*., 2013). Javid *et al*. (2011) recently reported on the effect of kinetin (KIN) on rice under salt stress. Foliar application of KIN to both salt-stress susceptible and resistant rice cultivars increased yield as well as the 1000-grain weight. The observed increase was associated with increased starch, glucose, fructose and sucrose concentrations. Exogenous application of cytokinin to *Alyssum murale* plants significantly increased plant biomass and transpiration rates (Cassina *et al*., 2011).

The gaseous hormone ET regulates various plant growth and developmental processes including germination, fruit ripening and leaf senescence (Bleecker and Kende, 2000). The post-harvest loss in agricultural crops is largely due to the increase in ET production during senescence and ripening (Goren *et al*., 2011). Antagonists of ET are therefore important in agricultural practices as they are able to protect plants from exogenous and endogenous ET (Sisler *et al*., 2003). The application of ET antagonists blocks ET receptors to extend the harvest season and improve the quality and shelf-life of various crops, fruits and vegetables.

The BRs occurring ubiquitously within the plant kingdom form a family of hormones (Roth *et al*., 2000; Schnabl *et al*., 2001) that functions in various plant organs including flower buds, fruits, seeds, roots, stems and leaves (Bajguz and Hayat, 2009). Exogenous application of BRs increases growth and yield of various economically important crops. BRs increase the number of ears as well as the number and weight of kernels per ear of various cereal crops (Rao *et al*., 2002; Ali *et al*., 2008). BRs also increase the number of pods per plant and total seed yield in various leguminous crops (Vardhini and Rao, 1998; Rao *et al*., 2002; Hayat and Ahmed, 2003). Even though BRs usually have an enhancing effect on growth and yield, many studies could not indicate a positive correlation between application and ultimate yield. It was found that the time of application (Khripach *et al*., 2000; Sasse, 2003), length of BR exposure (Sasse, 2003) and the mode of application (Fariduddin *et al*., 2008) play pivotal roles in the establishment of increased yield. Various studies also found that the plant species and genotype also determine the effectiveness of BRs (Kang *et al*., 2007; Ali *et al*., 2008).

Commercial bio-stimulants are produced to increase yield of agricultural and horticultural crops (Khan *et al*., 2009). Majathoub *et al*. (2004) analysed the effect of four bio-stimulant applications on wheat. Foliar application of commercially available Vigro, Biomin, Humiplus and Humacare improved various growth parameters including the number of tillers, grain number per spike, grain size and yield. Even though increased growth rates were observed, only Vigro indicated statistically significant changes in yield compared to the control treatment. This was probably due to the fact that Vigro also increased root mass thereby increasing water and nutrient uptake.

ComCat® is one of a handful of bio-stimulants that have been successfully commercialised. It is produced from the seeds of European plants (Agraforum AG, Germany). This activator contains a mixture of 12 different wild plants extracts including BRs, auxins and gibberellins (Schnabl *et al*., 2001) of which BRs form the active ingredients. CC has been shown to enhance root development in vegetable crops (Van der Watt and Pretorius, 2011) and increase yield in tomato plants (Melkamu *et al*., 2008). Treatment of wheat and maize plants with ComCat® ROW markedly increased dry kernel yield while application of ComCat® VEG increased yield of cabbage and carrots (Hüster, 2011).

The bio-stimulatory ability of a *Lupinus albus* L. cv. Betsuhana White seed suspension, referred to as SS, was evaluated when the crude extract was applied to plants (Van der Watt, 2005). SS increased the respiration rate of monoculture yeast cells, as well as coleoptile length and root growth in cabbage (Van der Watt and Pretorius, 2011). SS also significantly increased yield of vegetable crops (Van der Watt and Pretorius, 2011) and the PAN 3377 wheat cultivar (Van der Watt, 2005). Further purification of the crude SS extract identified the active component as a triglyceride, glycerol trilinoleate. Subsequent research used the 60% purified SS preparation for treatment of the Thatcher+*Lr34* wheat cultivar (Janse van Rensburg, 2009).

The aim of this study was to determine whether the 60% purified SS preparation also increased yield in the Thatcher+*Lr34* wheat cultivar and if it did, through which mechanism does it occur within the plant.

3.2 Materials and methods

3.2.1 Materials

Seed from the *Puccinia triticina* (*Erikss. & Henn*) resistant wheat (*Triticum aestivum* L.) cultivar Thatcher+*Lr34* was previously used (Van der Merwe, 2008; Janse van Rensburg, 2009) to identify differentially expressed genes following SS treatment. Seed was provided by the Department of Plant Sciences at the University of the Free State.

A pot trial was conducted in the glass house at a ±25°C day/18°C night cycle at the University of the Free State during the 2012 growth season. Statistical trials were laid out in a randomised block design including three blocks with five replicates for each treatment.

3.2.2 Preparation of soil and fertiliser treatment

Prior to the start of the study, soil was sent to the ARC Small Grain Institute (Bethlehem) for analysis. The results are summarised in Table 3.1, which indicates the measured parameters and the associated values compared to the recommended norms. Fertiliser treatments were based on the soil analysis and the recommended withdrawal norms for an expected yield potential of 7 ton ha^{-1}. Soil analysis indicated that potassium (K) content was within the norm and therefore no additional K was added. Both the N and phosphorous (P) content was below the norm and was supplemented with KAN/LAN (28%) and super-phosphate (10.5%) respectively. Fertiliser levels were calculated based on pots with a diameter and depth of 35 cm. The initial fertiliser treatment was applied at a concentration of 50 kg ha^{-1} N and 20 kg ha^{-1} P. Soil was added to pots until they were 75% full. The N and P fertilisers were mixed with 20% of the remaining soil and added to the pots where after seeds were sown. The pots were then filled with the remaining 5% soil. For subsequent treatments, the fertiliser was dissolved in 50 ml distilled water and applied directly to pots.

Table 3.1. Analysis of soil samples for the SS pot trial. AS – acid saturation; CEC – cation exchange capacity; () – results as a % of CEC; EA - extractable acid.

	Norms	**2012**
Sand (%)		94
Clay (%)		4
Leem (%)		2
CEC ($cmol_c kg^{-1}$)		4.07
pH (KCl)		7.0
P ($mg\ kg^{-1}$)	Medium-high (25-35)	32.2
K ($mg\ kg^{-1}$)	High (>120) (6-12)	313.2 (20)
Ca ($mg\ kg^{-1}$)	Medium (200-400) (55-75)	391 (48)
Mg ($mg\ kg^{-1}$)	High (>120) (20-30)	157.2 (32)
Na ($mg\ kg^{-1}$)	Low (<10) (<5)	5.2 (1)
EA (%)		0.00
AS (%)		0.00
Ca/Mg ratio		1.52
(Ca^+ Mg)/K ratio		4.05

The second fertilizer application was two weeks post germination and consisted of 50 kg ha^{-1} N and 20 kg ha^{-1} P. The third and fourth fertiliser treatments of 40 kg ha^{-1} N were administered at growth stages 8 or 9 and 14 (Meier, 1997).

Seedlings infested with aphids were treated with 7.5 ml l^{-1} Pyrinex® while red spider mites were controlled with 2 ml l^{-1} Red Spidercide® using a knapsack sprayer.

3.2.3 Preparation of SS and treatment of seedlings

SS was suspended in distilled water containing 0.5 ml l^{-1} polyoxyethylenesorbitan monolaurate (Tween 20) to a final concentration of 0.5 g l^{-1}. The positive control, ComCat® ROW, was prepared as a 50 mg l^{-1} solution in water containing 0.5 ml l^{-1} Tween 20, while water containing 0.5 ml l^{-1} Tween 20 was used as a negative control. Foliar treatments were applied until runoff on seedlings at the 3-4 leaf stage corresponding to growth stage 13-14 (Meier, 1997). Seedlings used for analysis were harvested at 0, 2, 4 and 6 weeks post treatment. One seedling per time interval was harvested for each treatment and replicate and used for all subsequent analyses.

3.2.4 Physiological analyses

3.2.4.1 Quantification of chlorophyll content

A total of 0.3 g wheat leaf tissue was homogenized in 5 ml 100% acetone. Samples were centrifuged at 17 000 *g* for 5 min at 25°C. One millilitre of the chlorophyll extract was used to determine the absorbance spectra at 470, 644.8 and 661.6 nm respectively. Acetone was used as the blank. Analysis was done using the UV-probe 2.42 program and the Shimadzu UV-2450 UV-visible spectrophotometer. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b* and carotenoid content were determined using the following Excel-based formula:

Chlorophyll *a* content = (11.24*661.6 nm value)-(2.04*644.8 nm value)

Chlorophyll *b* content = (20.13*644.8 nm value)-(4.19*661.6 nm value)

Chlorophyll *a*+*b* content = (7.05*661.6 nm value)+(18.09*644.8 nm value)

Carotenoid content = (1000*470 nm value)-(1.9*chlorophyll *a* content)-(63.14*chlorophyll *b*)/214

3.2.4.2 Extraction and determination of sucrose, D-glucose and D-fructose content in wheat leaves

Wheat leaf samples (0.3 g) were suspended in 80% (v/v) ethanol and incubated at 80°C for 15 min to stop all enzyme activity. Tissue was homogenized using a Retsch Mixer Mill MM400 (Cole-Parmer) at 10 Hz for 15 sec and centrifuged at 17 000 *g* for 5 min at 25°C. One millilitre supernatant was transferred to an Eppendorf vial and incubated at 70°C until all ethanol evaporated. After leaving samples overnight at room temperature, the sugar pellet was dissolved in 1 ml water and stored at -20°C. Sugar content was determined using the

Enzytec^{TM} D-Glucose/D-Fructose/Sucrose kit (R-Biopharm) according to the manufacturer's instructions.

The principle of the R-Biopharm enzymatic technique for determining sucrose, D-glucose and D-fructose content in solid tissue is as follows. The D-glucose concentration is determined before and after the enzymatic hydrolysis of sucrose, while the D-fructose content is determined subsequent to the determination of the D-glucose content. At pH 7.6, hexokinase (HK) catalyses the phosphorylation of D-glucose by adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) [1].

$$\textbf{D-Glucose + ATP} \xrightarrow{\textbf{HK}} \textbf{Glucose-6-phosphate + ADP} \qquad \textbf{[1]}$$

In the presence of glucose-6-phosphate dehydrogenase (G-6-PDH), the formed D-glucose-6-phosphate (G-6-P) is specifically oxidized by NADP to gluconate-6-phosphate with the formation of reduced nicotinamide adenine dinucleotide phosphate-oxidase (NADPH $+H^+$) [2].

$$\textbf{G-6-P + NADP}^+ \xrightarrow{\textbf{G-6-PDH}} \textbf{gluconate-6-phosphate + NADPH + H}^+ \qquad \textbf{[2]}$$

The NADPH formed in this reaction is stoichiometric to the amount of D-glucose and is measured by means of its absorbance at 340 nm. Hexokinase also catalyses the phosphorylation of D-fructose to fructose-6-phosphate (F-6-P) in the presence of ATP [3].

$$\textbf{D-Fructose + ATP} \xrightarrow{\textbf{HK}} \textbf{Fructose-6-phosphate + ADP} \qquad \textbf{[3]}$$

On completion of reaction [3], F-6-P is converted by phosphor-glucoisomerase (PGI) to G-6-P [4].

$$\textbf{Fructose-6-phosphate} \xrightarrow{\textbf{PGI}} \textbf{Glucose-6-phosphate} \qquad \textbf{[4]}$$

G-6-P reacts again with NADP to form gluconate-6-phosphate and NADPH [2]. The amount of NADPH formed is now stoichiometric with the amount of D-fructose. At pH 4.6, sucrose is hydrolyzed by β–fructosidase (invertase) to D-glucose and D-fructose [5].

$$\textbf{Sucrose} + H_2O \xrightarrow{\beta\text{–fructosidase}} \textbf{D-glucose} + \textbf{D-fructose} \qquad [5]$$

The D-glucose determination after inversion (total D-glucose) was carried out according to the principle outlined above. The sucrose content was calculated from the difference of the D-glucose concentration before and after enzymatic inversion.

The sugar content was calculated by means of the following equation and expressed as µmol g^{-1} dry mass (DM):

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \ g\ l^{-1}$$

where c = concentration

V = final volume (ml)

v = sample volume (ml)

MW = molecular weight (g mol^{-1})

d = light path (cm)

ϵ = extinction coefficient of NADPH at 340 nm (= 6.3)

It follows for sucrose,

$$c = \frac{3.02 \times 342.3}{\epsilon \times 1.0 \times 0.1 \times 1000} \times \Delta A_{sucrose} = \frac{10.34}{\epsilon} \times \Delta A_{sucrose}\ g\ l^{-1}$$

for D-glucose

$$c = \frac{3.02 \times 180.16}{\epsilon \times 1.0 \times 0.1 \times 1000} \times \Delta A_{D\text{-glucose}} = \frac{5.441}{\epsilon} \times \Delta A_{D\text{-glucose}} \text{ g } l^{-1}$$

and for D-fructose.

$$c = \frac{3.04 \times 180.16}{\epsilon \times 1.0 \times 0.1 \times 1000} \times \Delta A_{fructose} = \frac{5.477}{\epsilon} \times \Delta A_{fructose} \text{ g } l^{-1}$$

3.2.4.3 Measurement of photosynthesis and transpiration rates

Photosynthesis and transpiration rates were measured at the specified time intervals using a Portable Photosynthetic LI-6400XT System (Li-Cor Biosciences). Net photosynthesis was measured using this system by means of the following formula:

$A = F(C_r – C_s (1000 – W_r/1000 - W_s))/100S$

where F = air flow rate

C_r = CO_2 concentration of reference

C_s = CO_2 concentration of sample

W_r = transpiration rate of reference

W_s = transpiration rate of sample

S = leaf area

Transpiration rate was measured using the formula:

$E = F(W_s - W_r)/100S(1000 - W_s)$

where F = air flow rate

W_r = transpiration rate of reference

W_s = transpiration rate of sample

S = leaf area

3.2.5 Measuring plant height as representative of vegetative growth

In order to analyse the effect of SS and CC on growth, plant length was measured at 0, 2, 4 and 6 weeks post treatment. A one meter ruler was used and height measured in cm from the soil surface to the tip of the single longest top leaf.

3.2.6 Determination of leaf fresh:dry mass ratio

The fresh mass of harvested leaf material was measured at specified time intervals and, subsequently, dried at 70°C for one week. The dry mass was then determined and the ratio of fresh mass:dry mass calculated.

3.2.7 Quantification of grain yield

Once the drying cycle of the mature wheat was completed and the ears harvested, three wheat plants per replicate for each treatment (15 plants in total) were used to determine yield potential. The number of ears per plant was counted and weighed. Subsequently, the grain kernels were removed from the ears, counted and weighed. Final yield was represented as ton ha^{-1} by extrapolating pot area to hectares.

3.2.8 Statistical analysis of data

The Agrobase Generation II version 34 software program (Agronomix Software, Winnipeg, MB, Canada) was used to calculate the analysis of variance (ANOVA) for all data sets. Statistical analysis was done using GenStat, 2012 (Introduction to GenStat for Windows, 16th ed., VSN International, Hemel Hemstead, Hertfordshire HPI. IES, UK). Treatments that differed significantly were indicated using the (*) symbol.

3.3 Results

To quantify the effect of SS on the growth and yield of Thatcher+*Lr34* wheat, various parameters were used. Besides grain yield, these included plant height as a growth parameter and physiological parameters such as sugar content, chlorophyll and carotenoid content and photosynthesis rate.

No significant differences between treatments in terms of chlorophyll *a* (Fig. 3.1a), chlorophyll *b* (Fig. 3.1b), chlorophyll *a*+*b* (Fig. 3.1c) or carotenoid (Fig. 3.1d) content were observed. However, a significant difference ($P<0.01$) was found for both chlorophyll *b* content and chlorophyll *a*+*b* content over the four measured time intervals. No significant differences were observed for the interaction between treatment and time intervals for all measured parameters.

The photosynthesis rate (Fig. 3.2a) decreased markedly two weeks after treatment for all treatments where after it remained constant for the duration of the study. No significant differences were found between treatments, but a highly significant difference ($P<0.01$) was observed over the four time intervals for all treatments. On the other hand, transpiration rates (Fig. 3.2b) seemed to increase gradually to reach a maximum four weeks after treatment. This increase was significant ($P<0.01$) over time, but not between treatments.

Glucose content (Fig. 3.3a) was not significantly affected by any of the treatments, but there appeared to be a marked increase following SS treatment after two weeks. Thereafter a decrease in glucose content was observed for all treatments over the next four weeks. The fluctuations that occurred in fructose content (Fig. 3.3c) were similar to those observed for glucose content. A marked increase was once again observed two weeks post SS treatment but this increase was not significant between treatments. A significant difference ($P<0.01$) was obtained between time intervals regarding glucose and fructose content. No

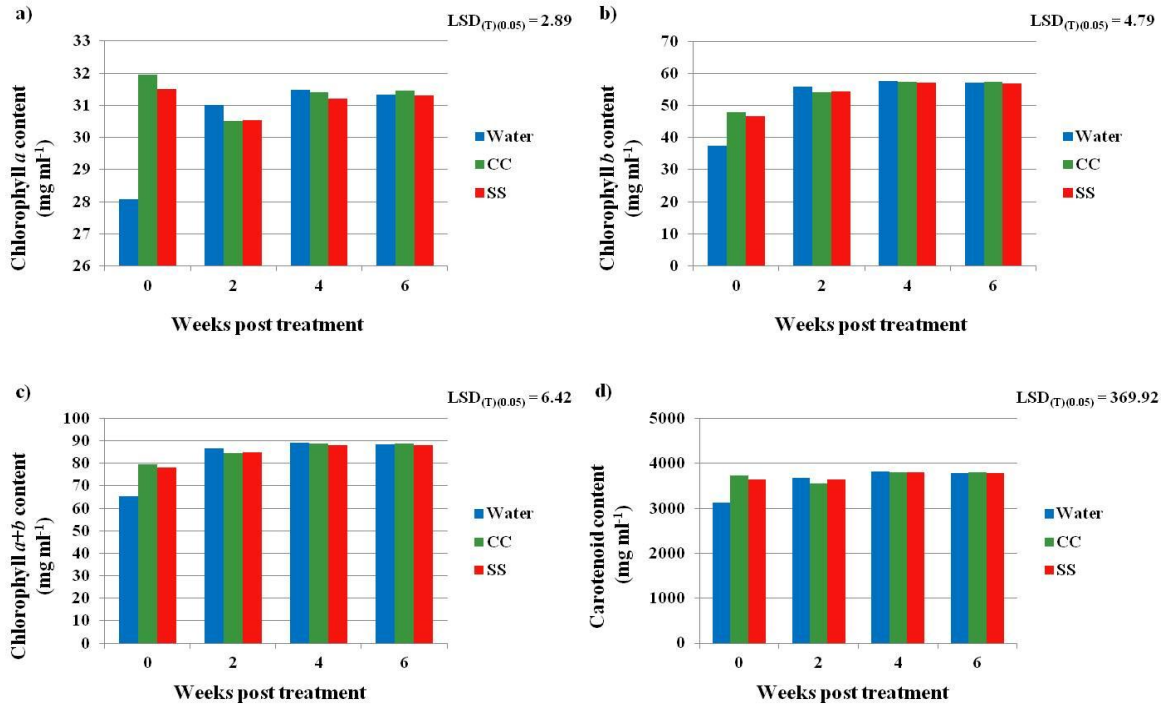


Figure 3.1. The response of wheat cv. Thatcher+*Lr34* chlorophyll content to treatment with CC and SS. In a) chlorophyll *a*, b) chlorophyll *b*, c) chlorophyll *a*+*b* and d) carotenoid content is given where n=15.

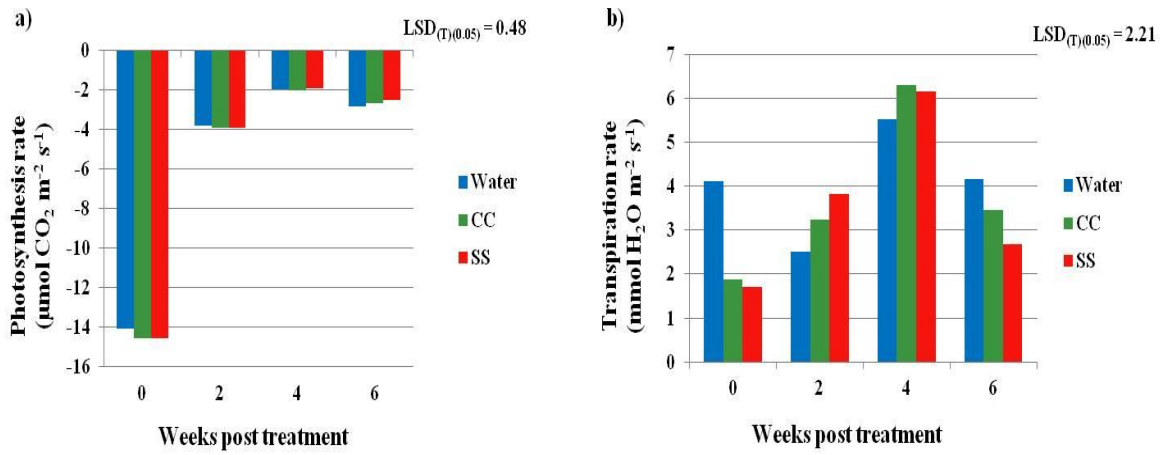


Figure 3.2. The response of wheat, cv. Thatcher+*Lr34*, to treatment with water, CC and SS in terms of a) photosynthesis and b) transpiration rates (n=15).

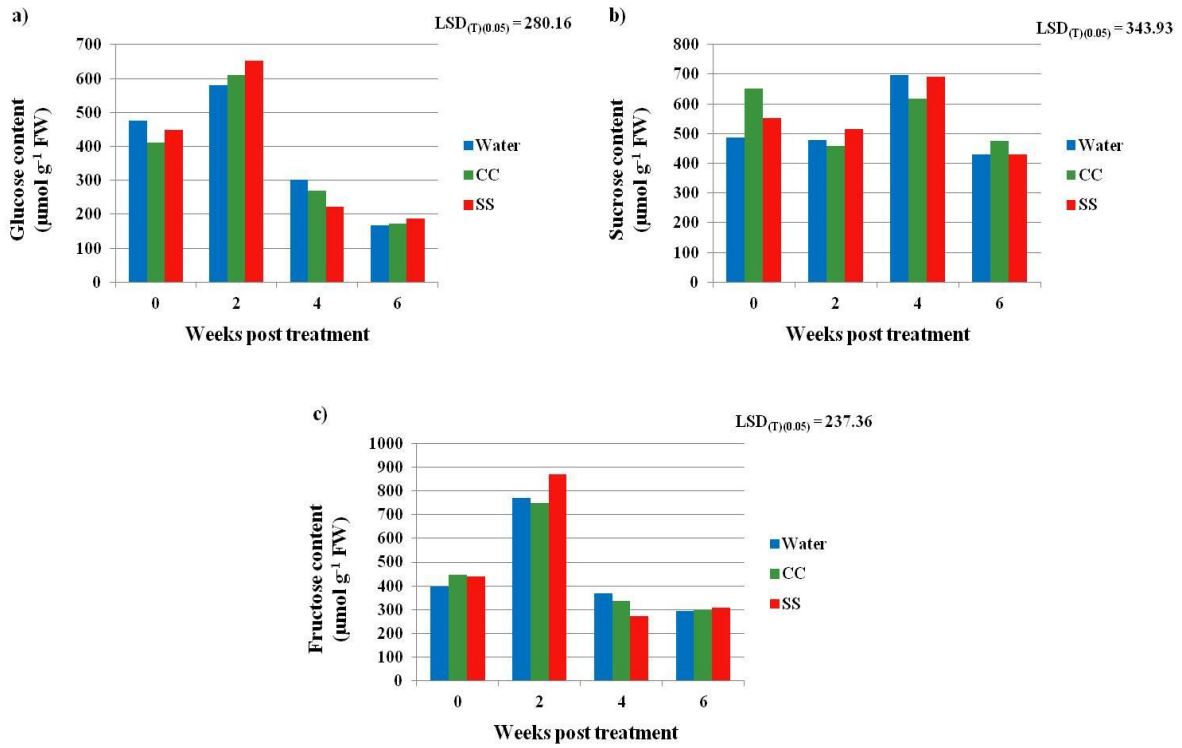


Figure 3.3. The response of wheat, cv. Thatcher+*Lr34*, to treatment with water, CC and SS in terms of a) glucose, b) sucrose and c) fructose content in leaves (n=15).

significant difference was observed in sucrose content (Fig. 3.3b) between treatments or over the four time intervals.

As could be expected, significant differences ($P<0.01$) were observed in plant height (Fig. 3.4) over the four measured time intervals. A marked increase in plant length was observed at two weeks post treatment, but this increase was not significant between treatments. No significant interaction was found between the treatments and time intervals.

The fresh mass:dry mass ratio (Fig. 3.5) decreased over time for all treatments. This decrease was significant between time intervals ($P<0.01$) but not between treatments.

No significant yield increases were observed between treatments (Fig. 3.6). Foliar application of CC and SS markedly, but not significantly, decreased yield by 200 kg ha^{-1} and 700 kg ha^{-1} respectively, compared to the water treatment.

Linear correlations were also calculated (Table 3.2) in order to determine the degree to which two variables were related. Correlation analysis indicated highly significant ($P<0.01$) correlations between fresh mass:dry mass ratio and chlorophyll content and plant height and photosynthesis. Surprisingly, there were no significant correlations between chlorophyll content and photosynthesis parameters, or sugar content and photosynthesis parameters. A significant correlation ($P<0.05$) was, however, observed between plant height and transpiration, while photosynthesis rate and plant height also correlated positively ($P<0.01$).

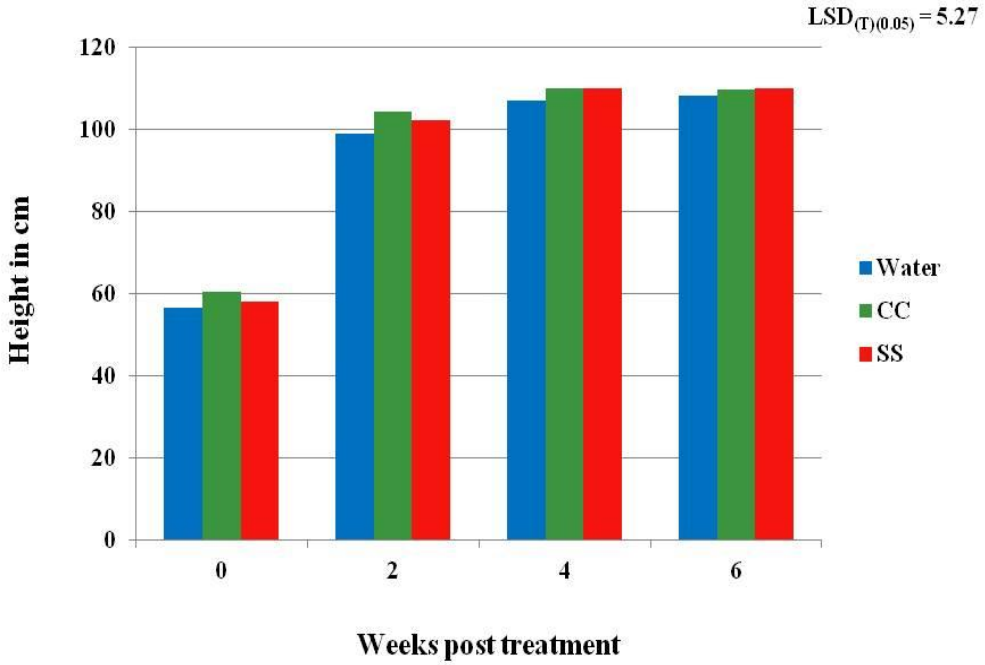


Figure 3.4. Growth response of wheat, cv. Thatcher+*Lr34*, to treatment with water, CC and SS in terms of plant height where n=15.

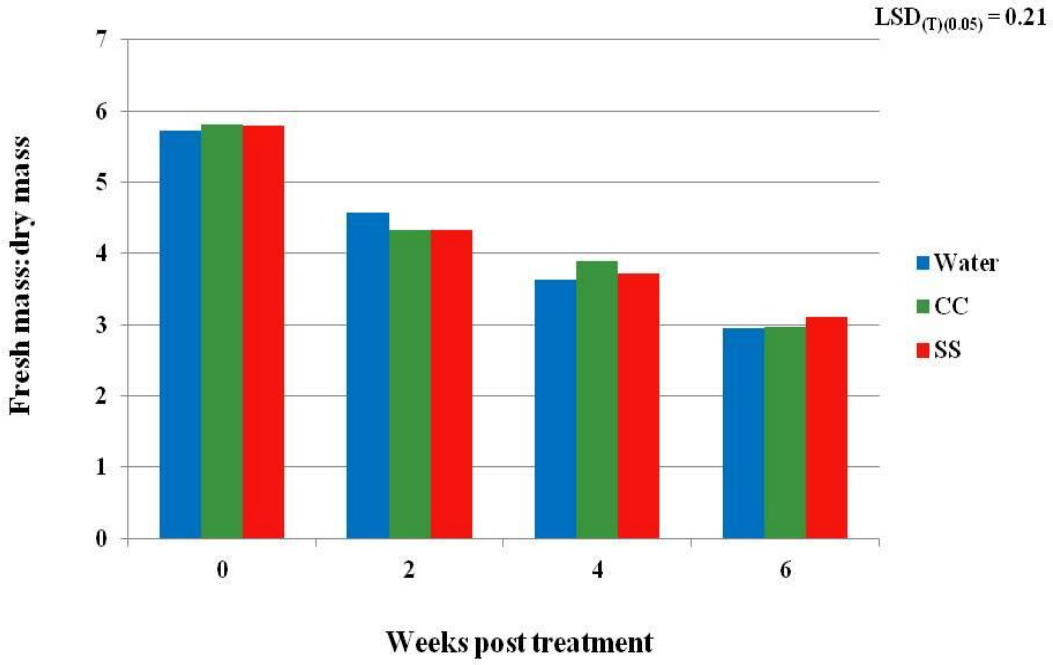


Figure 3.5. Effect of CC and SS treatment on the fresh mass:dry mass ratio in Thatcher+*Lr34* wheat compared to the water control (n=15).

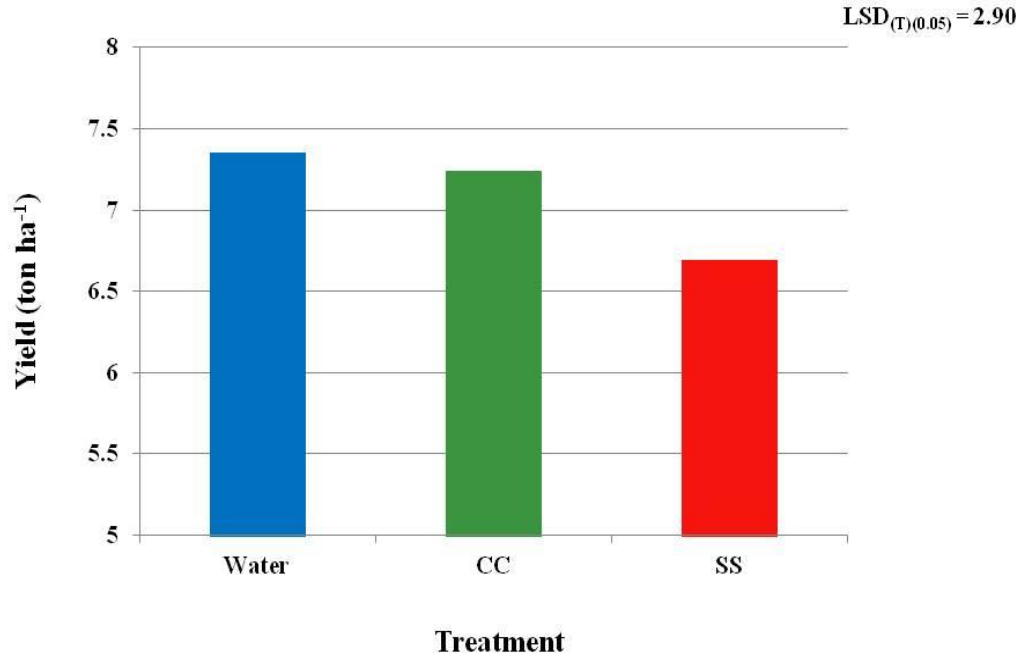


Figure 3.6. The yield response of wheat, cv. Thatcher+*Lr34*, to treatment with water, CC and SS when applied at growth stage 13 – 14 (n=15).

Table 3.2. Significant correlations between measured characteristics following treatment with CC and SS. Significance is indicated as ** for P<0.01 and * for P<0.05.

	Fresh mass:dry mass	**Chlorophyll *a* content**	**Chlorophyll *b* content**	**Chlorophyll *a*+*b* content**	**Length**	**Transpiration**	**Photosynthesis rate**	**Glucose**
Chlorophyll *b*	-0.7414**	0.4121*						
Chlorophyll *a*+*b*	-0.6747**	0.6193**	0.9706**					
Length	-0.9050**		0.8256**	0.7539**				
Transpiration					0.4232*			
Photosynthesis rate	-0.8933**		0.8248**	0.7439**	0.9768**	0.3789*		
Glucose	0.4661**	-0.5507**		-0.3445*		-0.4569**		
Sucrose		-0.3388*						
Fructose						-0.3852*		0.9119**
Carotenoids	-0.3626*	0.9399**	0.6694**	0.8252**	0.3837*		0.3631*	-0.5462**

3.4 Discussion

The application of bio-stimulants to agricultural crops in order to either induce resistance towards abiotic and biotic stress conditions or increase yield has gained interest lately. Popular commercialised bio-stimulants used worldwide *inter alia* include Kelpak® and ComCat®. These products are applied as a foliar treatment or directly to roots, but always additional to fertilizer and never on its own (Ferrini and Nicese, 2002). Kelpak®, a seaweed concentrate, contains a mixture of cytokinins, auxins (Stirk *et al*., 2004) and polyamines (Papenfus *et al*., 2012) while ComCat® contains brassinosteroids as an active ingredient (Khripach *et al*., 2000). It is accepted that different products have different mechanisms of action and it is, therefore, acknowledged that they have different effects on different crops. The selection of an appropriate bio-stimulant is dependent on the objective.

Ferreira and Lourens (2002) reported on the yield improving effect of Kelpak® in canola (*Brassica napus*). Foliar application at 2 l ha^{-1} at the 4-leaf growth stage (cultivars Monty and Dunkeld) significantly increased the yield. The natural bio-stimulant, ComCat®, was developed and commercialized in Germany. The active substances of this natural product were identified as 24-episescasterone, 24-epi-castasterone and epi-brassinolide. All three belong to the brassinosteroid phytohormone group and are certified as plant growth promoters (Khripach *et al*., 2000). BR has an effect on plant development, in particular reproduction, maturation, senescence and seed as well as fruit yield. An important feature of BRs is their ability to also increase the quality of crops. In rice, optimal growth and yield was observed following foliar treatment of 24-epibrassinolide at a concentration of 100 µM (Krishnan *et al*., 1999). In wheat however, growth was promoted at the much lower concentration of 0.104 µM and 0.052 µM 24-epibrassinolide under normal and saline conditions, respectively (Ali *et al*., 2008). In this study ComCat® was used as positive control at the optimum concentration of 200 g ha^{-1} recommended by the manufacturers (Agraforum, Germany). ComCat® instead of the seaweed extract Kelpek® was used as the product was developed from seed extracts of land plants that compared more closely to the seed suspension of *Lupinus albus* tested in this monograph.

During the current study, various parameters were used to determine the effect of the 60% purified SS preparation, a triglyceride called glycerol trilinoleate, on wheat, cv. Thatcher+*Lr34*. These comprised a single growth parameter, plant height and yield parameters including ear number, ear mass and kernel mass as well as physiological parameters related to yield capacity including pigment content, photosynthesis rate and sugar production. No significant differences ($P<0.01$) in plant height were observed between treatments at the four measured time intervals. As yield increase is the principle objective for applying bio-stimulants from an agricultural perspective, emphasis was placed on this aspect. As it was previously shown that the crude SS extract significantly increased the yield of lettuce and carrots by 20 ton ha^{-1} and 22.2 ton ha^{-1} respectively under field conditions (Van der Watt and Pretorius, 2011), photosynthesis parameters were used to obtain information on its possible mechanism of action. A further rationale was that the crude SS extract also markedly increased dry kernel yield in the PAN 3377 wheat cultivar (Van der Watt, 2005).

Foliar application of the 60% purified SS preparation to Thatcher+*Lr34* wheat seedlings did not improve grain yield. Instead it had no significant effect on yield. The question that arose from this observation was whether the purified form of SS had the opposite effect on the physiology of wheat in real terms. Photosynthesis being the main primary metabolic process accountable for producing sugars and eventually determining yield, therefore, became the main focus of this study in order to establish the uncharacteristic response of wheat to the purified form of SS.

Foliar treatment of wheat, cv. Thatcher+*Lr34*, with the 60% purified SS preparation had no significant effect on chlorophyll *a*, *b*, total chlorophyll or carotenoid content compared to both the negative (water) and positive controls. Although significant differences ($P<0.01$) in chlorophyll content were observed between time intervals, not at any stage were the differences between treatments significant. Surprisingly, despite the fact that pigment content was not affected by any of the treatments, the photosynthesis rate in Thatcher+*Lr34* leaves decreased drastically two weeks following treatment. However, what was surprising was that the photosynthesis rate remained substantially low for the following four weeks in all treated plants including the water control. This was confirmed by the insignificant differences

between treatments at all four time intervals in terms of glucose, fructose and sucrose content. However, a marked increase in glucose and fructose content two weeks post treatment and the dramatic decrease during the following four weeks were in concert with the decrease in photosynthesis rate over the same time period. It seemed, therefore, that a simple answer to the decrease in yield was not to be found in pigment content only, but it was rather a result of interference with another facet of the photosynthesis process.

An initial literature survey revealed that very little research has been done in the past on the response of plants to treatment with fatty acids. However, results contrasting to the findings in this study were found in the few reports available. For example, fatty acids were shown to be involved in defence mechanisms of plants and to promote chlorophyll synthesis (Wink, 1999). This is the opposite of findings in this study, namely that chlorophyll levels were unaffected. Closest to the current findings is the work done by Finger (2000) on wine grapes. In field experiments conducted in northern Virginia during 1998, oils reduced photosynthesis, fruit maturity, and crop yield in Chardonnay and Cabernet Sauvignon wine grapes. Vines were treated with JMS Stylet oil (1.5%) at 1870 l ha^{-1} applied to the canopy. The net assimilation rate (NAR) and soluble solid content were significantly decreased by the oil treatment compared to the untreated control.

Fairly recently Seigler (1998) and Edqvist and Farbos (2003) reported on the plant growth regulatory activity of plant sterols and fatty acids. Despite these reports, very little information on the growth regulating action mechanism of either lipids or fatty acids was found in the literature and must therefore be regarded as highly speculative. Van der Watt (2005) formulated two postulates in an attempt to explain the possible mechanism of action of the triglyceride contained in SS in promoting seedling growth as well as increasing yields in the field crops tested.

The first postulate of van der Watt (2005) is based on the assumption proposed by Seigler (1998) that exogenously applied fatty acids might slightly increase the level of available fatty acids to be oxidized via α- and β-oxidation (Seigler, 1998). Most fatty acids occur in nature

as esters of glycerol (commonly referred to as triglycerides or tri-acylglycerols) that either plays a role in energy supply or is stored as components of phospholipids, glycolipids and waxes. Some are converted to alcohols, aldehydes, olefins, hydrocarbons, acetylenic compounds and other secondary metabolites (Seigler, 1998). When SS, containing trilinoleate as active substance, is applied to crop plants as a foliar spray, it is assumed that this increases the level of available fatty acids after being taken up by the leaves and its stimulatory effect might be via normal metabolism. As photosynthesis forms an extremely important part of anabolic metabolism in plant cells and is directly related to the production of assimilates translocated to harvestable parts of crops during the grain filling or fruit development stage, the possibility exists that available fatty acids might affect the process. Anderson and Beardall (1991) reported a marked change in fatty acid composition, including an increase in the amount of linolenic or linoleic acid, during chloroplast development. Fatty acids have been shown to be crucial in the protection of the photosynthetic machinery under cold conditions (Sewon *et al*., 1997). Linolenic acid levels decreased while linoleic acid levels increased as a response to cold treatment leading to a hardened state characterized by the accumulation of chlorophylls. It is postulated that exogenously applied trilinoleate (SS) might evoke the same increase in chlorophyll production in crop plants as was reported under cold stress conditions (Sewon *et al*., 1997).

The second postulate (van der Watt, 2005) is based on the possible action of fatty acids as second messengers. In oil rich seeds, such as lupine, the process of second messenger production is characterized by the mobilization of storage lipids that serve as a major carbon source during the growth of seedlings (Park *et al*., 2002). However, it is postulated that in seeds with low lipid content, exogenously applied trilinoleate might supply either a metabolic or a growth stimulus or both via second messengers as an action mechanism normally not in contention in these seeds. The question is how this stimulus could possibly be activated?

There are two known pathways for metabolizing the hydroperoxides of linoleic and linolenic acid. The first pathway leads to the synthesis of JA while the other to that of dihydrojasmonic acid (DH-JA) (Claeys *et al*., 1986). Jasmonic acid is probably the best characterized of the potential chemical messengers derived from the lipoxygenase-dependant oxidation of fatty

acids. Perhaps one of the greatest surprises in recent years is the wide range of physiological activities induced by JA in plants (Seigler, 1998). One of the first physiological activities demonstrated for JA by Aldridge *et al*. (1971), after its isolation from culture filtrates of the fungus *Lasiodiplodia theobromae*, was that it inhibited plant growth, but only at high or toxic levels. The phytohormone effects of JA are similar to those of ABA, an established phytohormone. As a result Sembdner and Parthier (1993) argued that JA should be recognized as a representative of a unique class of phytohormones. In this regard JA shares both chemical and biological similarities with the plant growth regulator ABA as both compounds inhibit growth and promote the senescence of detached leaves at high levels while low levels can have a stimulatory effect. This confirms its typical hormone-like action.

The results obtained during this initial study with Thatcher+*Lr34* wheat did not demonstrate the potential of SS in its purified form as a promising bio-stimulant for manipulating this crop. However, both the 60% purified SS extract and CC (positive control) decreased grain yield following foliar treatment. These results were not anticipated since prior studies indicated the positive effect of these compounds on various yield associated factors. This caused us to speculate on possible reasons.

The first possibility is that, since CC and SS contain plant hormones and triglycerides, respectively, application could induce an antagonistic effect of these compounds within Thatcher+*Lr34*. The antagonistic effect of BRs dates back to 1992. In a study done by Clouse *et al*. (1992) it was apparent that BRs stimulated elongation of auxin-depleted soybean epicotyls. Exogenous application of ABA to rice plants decreased expression of various protein encoding genes involved in cytokinin synthesis and degradation (Tsai *et al*., 2012). The antagonistic effect of cytokinins and auxins has also been shown to be important in controlling root development (Ioio *et al*., 2008). Ioio *et al*. (2007) indicated that there is a balance between the antagonistic effects of cytokinins and auxins. This balance is necessary for the establishment of the *Arabidopsis* root meristem size. The balance between cell differentiation (cytokinins) and cell division (auxins) therefore controls root growth in *Arabidopsis*.

The time of application of plant hormones is also of importance (Van Loon *et al*., 2006). When ET is applied prior to pathogen inoculation, it can either reduce disease development or have no effect. However, treatment with ET after pathogen inoculation increased disease development. The time of bio-stimulant application plays a pivotal role in the plants' response to treatment (Khripach *et al*., 2000; Amzallag, 2002). Under normal conditions, BRs usually have a positive effect on plant growth and yield. Holá *et al*. (2010) indicated increased yield in maize plants following treatment with 24-epibrassinolide, a naturally occurring BR, and the synthetic androstane, an analogue of castasterone. These results were obtained under field conditions when plants were treated at leaf stage 3-4 or leaf stage 6-7. When BRs were applied during leaf stage 3-4, the length of leaves 7 to 10 increased significantly. The authors, however, indicated the opposite effect when application of BRs occurred at leaf stage 6-7. According to Hüster (2011), since CC contains a range of natural phytohormones, some crops might be sensitive to changes in CC concentration and application at various growth stages.

Foliar treatment of SS to a drought-sensitive wheat cultivar and one drought-resistant cultivar indicated opposing effects of this bio-stimulant during analysis (Van der Watt, personal communication). Further, SS in its purified form as was used in this study might lack the synergistic effect of other compounds contained in the crude SS form as was applied by Van der Watt (2005). Based on previous work by Van der Watt and Pretorius (2013), it is unlikely that the active ingredient was lost during SS preparation and that inhibitors were co-purified. Future work should however include the purified glycerol trilinoleate as a positive control.

In conclusion, no significant results were obtained during this glass house study but it became apparent that the role of the 60% purified SS extract did not affect growth parameters in Thatcher+*Lr34* wheat. The exact functioning following SS treatment, needs to be elucidated if this product is to become an important agricultural commodity. In order to try and understand the functionality of SS, the role thereof will be investigated in more detail in the following chapters.

Proteomic analysis of wheat treated with SS

CHAPTER 4

4.1 Introduction

With an ever increasing world population, the demand for food increases annually by 2% (www.csustan.edu/agstudies/documents/.../WorldPopandFood.ppt). Wheat provides 20% of the daily human protein and calorie intake, making it the second most important food crop after rice (www.wheatinitiative.org). Therefore, the cultivation of wheat and the ultimate protection of yield are of cardinal value. Farmers and researchers continuously investigate new approaches to increase crop yield with one such an approach being the use of plant activators/bio-stimulants.

Plant activators are agrochemicals that could for instance stimulate a plant's defence response through the induction of SAR thereby providing enhanced resistance against a broad range of diseases (Von Rad *et al*., 2005). SA, JA and ET are natural plant activators whose roles within plants have been extensively studied.

The role of SA was first described by White (1979) when he illustrated that the exogenous application of SA reduced disease symptoms caused by Tobacco Mosaic Virus (TMV). Anand *et al*. (2008) indicated that SA inhibits the induction of the *repABC* operon and virulence genes in *Agrobacterium tumefaciens*, while silencing of the *ICS* (isochorismate synthase), *NPR1* (non-expresser of *PR1*) and *SABP2* genes enhanced *Agrobacterium* infection in *Nicotiana benthamiana*. Treatment of wheat with SA prevented the accumulation of mycotoxins and reduced the effect of Fusarium head blight (FHB) (Makandar *et al*., 2012). In wheat expressing the *NahG* gene where SA is actively metabolised, the effects of FHB were more severe.

The synthetic plant activator BTH increases resistance against powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) and leaf spot (*Septoria* spp.) in wheat (Görlach *et al*., 1996). While BTH is not effective against all pathogens, the induced defence response is much stronger than that triggered by SA or JA (Pasquer *et al*., 2005).

Plant activators are not only effective against biotic stress factors, but also against abiotic factors. In a recent study, the exogenous application of JA improved the tolerance of wheat to UV-B exposure by alleviating decreased PSII function due to UV-B (Liu *et al*., 2012). Pre-treatment with JA also significantly increased chlorophyll (chl α and chl β) levels within the plants.

Only a few plant activators or bio-stimulants have been commercialised. ComCat® is one of only a few bio-stimulants that contain BRs as active compounds (Van der Watt and Pretorius, 2011). Brassinosteroids are phytohormones that increase crop yield (Schnabl *et al*., 2001) as well as resistance against biotic (Nakashita *et al*., 2003) and abiotic factors (Kagale *et al*., 2007). The three active compounds in CC are epibrassinolide, 24-episescasterone and 24-epicastasterone (Khripach *et al*., 2000). CC improves root development and increases the photosynthetic rate of treated plants (Van der Watt, 2005). Pre-harvest treatment of tomatoes with CC also increased yield as well as fruit quality during storage (Melkamu *et al*., 2008).

In a search for new plant activators, Van der Watt (2005) analysed native South African plants for bio-stimulatory activity. A *Lupinus albus* L. cv. Betsuhana White seed suspension called SS had significant bio-stimulatory activity. SS improved yield in lettuce, beetroot and carrots and caused a significant increase in root length (Van der Watt and Pretorius, 2011). The active bio-stimulatory compound in SS is glycerol trilinoleate, a triglyceride.

Proteomics present valuable tools to analyse the effect of plant activators or bio-stimulants on plants. Mass spectrometry (MS) analysis of protein samples not only identifies multiple proteins within a specific sample (Adachi *et al*., 2006), but also measure protein abundance (Steen and Pandey, 2002). The importance of proteomic analysis is rising due to the fact that proteins can be directly linked to gene function (Pandey and Mann, 2000).

The two quantification methods that are used during MS analysis include label-free quantification and stable isotope labelling quantification (Nesvizhskii *et al*., 2007). The latter

enables the direct comparison of relative peptide abundance, thereby evading problems that could occur due to ion-suppressive effects of coeluting peptides (Tang *et al*., 2004). The disadvantages of isotopic labelling include the cost and the constraint of pairwise comparisons between samples which in turn causes difficulties regarding large scale studies and retrospective comparisons (Old *et al*., 2005).

One of the most well known label-free methods of MS is termed spectral counting whereby the number of MS/MS spectra, assigned to each protein, is compared (Old *et al*., 2005). Advantages of spectral counting include the measurement of relative protein abundances and the fact that they are theoretically and computationally simple (Little *et al*., 2010). The use of label-free methods is usually more cost effective as well. Label-free quantitative methods do, however, have several shortcomings. The first is as a result of false-positive discriminative peptides. This occurs due to chromatographic variability between different LC-MS experiments. The second limitation is that peptide identification is necessary for quantification since the MS/MS spectra use databases such as SEQUEST and MASCOT (Little *et al*., 2010). It is thus possible to lose a number of important peptides during a database search causing a certain number of spectra to be assigned to an incorrect class of peptides for various reasons, including sequence variations and post-translational modifications (Lee *et al*., 2011).

A proteomic approach was used to analyse the effect of BRs on *Arabidopsis* (Deng *et al*., 2007). Cellular processes targeted by BRs include signalling, vesicle transport and hormone biosynthesis. Brassinosteroids also induced JA biosynthesis that could aid in the activation of a defence response. When Kang *et al*. (2012) investigated the effect of SA on salt tolerance in wheat, proteomic results indicated an increase of proteins involved in signal transduction and the defence response, thereby confirming SA's positive role in salt adaptation.

Kim *et al*. (2003) analysed the effect of JA, SA and H_2O_2 on protein expression in suspension-cultured rice cells with proteomics. The authors showed that the *Oryza sativa* pathogen-related protein class 10 (OsPR-10), probenazole-inducible protein (PBZ1) and salt-

induced proteins (SalT) from incompatible reactions were reduced earlier in comparison to compatible reactions. They concluded that proteomic analysis could differentiate changes in the timing and amount of protein expression that had been induced by JA, SA and elicitor application during incompatible and compatible plant-pathogen interactions.

Since no significant differences were obtained between the 60% purified SS and water treatments during the glass house study (Chapter 3), it was decided to analyse the wheat proteome following SS treatment in order to give possible reasons for the non-significant data. Using a shotgun proteomic approach, it was proposed that by identifying differentially expressed polypeptides in the treated wheat seedlings, possible cellular mechanisms targeted by SS application could be identified.

4.2 Materials and methods

4.2.1 Cultivation and treatment of wheat seedlings

Wheat (*Triticum aestivum* L.) seedlings (cv. Thatcher+*Lr34*) were germinated and grown in a soil:compost (1:1) mixture in a glass house at ±23°C. Plants were watered daily and 1 $g.l^{-1}$ Multifeed P® (5:2:4) fertilizer was applied twice a week. Plants were transferred to a growth chamber at ±23°C with a 12 h day/night cycle with a light intensity of 1500 micro-einsteins.$m^{-2}.s^{-2}$ once they reached the third leave stage.

The SS plant activator was prepared by resuspending the 60% pure SS molecule (Van der Watt, 2005) in water to a final concentration of 0.5 g l^{-1}. Tween 20 was added to a final concentration of 0.5 ml l^{-1} to ensure optimal SS uptake by the leaves. Water containing 0.5 ml l^{-1} Tween 20 only was used as control. The solutions were applied as a fine mist to three leaf seedlings until runoff. Leaf material was harvested at 0 and 24 hours post treatment (hpt) and stored at -80°C until further use. Three independent biological repeats were done.

4.2.2 Extraction and quantification of total protein

Frozen leaf tissue was ground to a fine powder in liquid nitrogen. Protein extraction and quantification was done according to Donnelly *et al*. (2005). In short, 500 mg tissue was suspended in 1 ml chilled 10% (v/v) trichloroacetic acid in acetone containing 0.07% (v/v) β-mercaptoethanol (ME). The solution was vortexed and incubated at -20°C for 1 h. After centrifugation for 1 h at 45 000 *g* (Beckman L8-M Ultracentrifuge), the resulting pellet was washed three times with chilled acetone/0.07% (v/v) ME with centrifugation for 30 min at 45 000 *g* between washes. The final wash solution was removed and the pellet air-dried.

Protein pellets were solubilized in 8 M urea, 2% (v/v) octyl phenol ethoxylate (Triton-X 100), 60 mM dithiothreitol (DTT) (30:900 w/v) for 1 h at 37°C and vortexed every 15 min. After ultrasonication with a microtip (Branson Sonifier B12) at 35% for 2 min, the solution was incubated for another hour at room temperature. The mixture was finally centrifuged at 45 000 *g* for 20 min and the supernatant collected. Protein concentration was determined using Bradford reagent with a few modifications (Ramagli and Rodriguez, 1985). The Bradford Protein Assay Dye (Bio-Rad) was diluted three-fold with water and both the samples and standard (10 µl) mixed with 10 µl water and 180 µl diluted Bio-Rad before the absorbance was read at 595 nm.

4.2.3 SDS-PAGE separation of total protein

Sodium dodecyl sulphide-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was done according to Laemmli (1970). A total of 100 µg protein was dissolved in loading buffer [1% (w/v) sodium dodecyl sulphide (SDS), 4% (v/v) glycerol, 50 mM DTT, 13 mM Tris-(hydroxymethyl) aminomethane (Tris-HCl) (pH 6.8), 0.04% (w/v) bromophenol blue]. Proteins were denatured at 94°C for 5 min, chilled on ice before separation for 40 min at 10 $V.cm^{-1}$ on a 12% (w/v) polyacrylamide running gel with a 6% (w/v) stacking gel using 0.1 M Tris-HCl (pH 8.3), 0.15 M glycine, 0.1% (w/v) SDS as running buffer. After separation, the gel was stained with Coomassie blue [0.2% (w/v) Coomassie Blue R250, 0.1% (w/v) Coomassie Blue G250, 7% (v/v) acetic acid, 43% (v/v) methanol] for 15 min at room temperature and then destained with 7% (v/v) acetic acid, 43% (v/v) methanol.

4.2.4 In-gel trypsin digestion of resolved polypeptides

After washing the gel twice for 15 min each with chromatography grade water, each gel lane was cut into five segments and each segment into 1x1 mm pieces. The gel pieces were first washed with water, then twice with 50% (v/v) acetonitrile and finally with 100% acetonitrile. The acetonitrile was replaced with 10 mM DTT, 0.1 M NH_4HCO_3 and the gel pieces

incubated at 56°C for 45 min. After removing the supernatant, the gel pieces were incubated in the dark for 30 min at room temperature in 55 mM iodoacetamide in 0.1 M NH_4HCO_3. After washing the gel pieces twice as previously described, they were dehydrated in 100% acetonitrile and air-dried in a fume hood for 5 min. After rehydration on ice in 50 mM NH_4HCO_3 containing 5 ng/µl trypsin, the tubes were incubated overnight at 37°C.

After the digest solution was transferred to new tubes, the remaining gel pieces were covered with 5% (v/v) formic acid, vortexed and incubated at room temperature for 15 min. The formic acid was replaced with 100% acetonitrile, the samples vortexed, incubated for 15 min at room temperature and centrifuged for 5 min at 12 000 *g*. The supernatant was transferred to the tubes containing the digest solution. These steps were repeated where after the digest solution was dried in a Speed Vac and resuspended in 20 µl 5% (v/v) formic acid.

4.2.5 Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis

Nano LC/MS/MS analysis was performed on the recovered peptides when 5 µl of each digest was injected and concentrated on a C18 reverse phase trapping column. The digest was eluted and separated on a custom packed C18 reverse phase column (150 mm x 0.75 µm, Nucleosil 120-3 C18, Macherey-Nagel GmbH and Co). The peptides were separated off the column using a 10 to 25% (v/v) acetonitrile in 0.1% (v/v) formic acid gradient over 50 min for a total run time of 2 h including re-equilibration steps at a flow rate of 350 nL/min on an Agilent 1200 series nanoLC. Peptides were analysed on an AB SCIEX 4000QTRAP hybrid triple quadrupole ion trap mass spectrometer fitted with a nanospray source. A survey scan of between 400 and 1200 Da was performed to scan for eluting peptides. An enhanced resolution scan was performed on peptides to determine the charge state of each peptide before fragmenting the peptides in the collision cell, producing enhanced product ions from which sequence information was extruded on an in-house Mascot 2.4 server using the latest Swissprot database. The protein identification data from Mascot was further processed using ProteoIQ 2.4 (Nusep) for easy comparison of relative protein quantitation using spectral counting across biological groups. This gave an indication of proteins that were up- or down-

regulated in comparison to the control treatment. Functional analysis of the identified polypeptides was done with the UniProt Databasis using Gene Ontology (GO) software.

4.2.6 Quantitative PCR analysis of selected genes

Quantitative polymerase chain reaction (qPCR) analysis was done according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin *et al*., 2010). Total RNA was extracted using Trizol reagent (Invitrogen™) and residual DNA removed with 5 U DNaseI (Fermentas) according to the manufacturer's instructions. The concentration of extracted RNA was determined spectrophotometrically (Sambrook and Russell, 2000). RNA quality was determined by separating 500 ng total RNA on a 1% (w/v) denaturing agarose gel containing 0.41 M formaldehyde. RNA was resuspended in RNA buffer [0.5 M 3-(N-morpholino)-propanesulfonic acid (MOPS), 50% (v/v) formamide, 6.5% (v/v) formaldehyde, 50 $\mu g.ml^{-1}$ ethidium bromide (EtBr)] where after samples were denatured at 65°C for 15 min. Prior to separation, RNA loading buffer [50% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.25% (w/v) bromophenol blue] was added (Sambrook and Russell, 2000). Samples were separated for 45 min at 10 $V.cm^{-1}$ in a 1x MOPS (5 mM sodium acetate, 50 mM EDTA, 20 mM MOPS pH 8) running buffer and results photographed using the Bio-Rad gel documentation system. Complementary deoxyribonucleic acid (cDNA) was synthesised using the ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's guidelines. qPCR analysis was performed using the Bio-Rad C1000 thermal cycler with CFX96 real-time attachment and the KAPA™ SYBR® FAST qPCR kit (Lasec).

All qPCR reactions contained 2 µl diluted cDNA template, 1x KAPA™ SYBR® Fast qPCR Master mix and 10 pmol of each primer. The amplification regime was as follows: denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and 30 sec at the specific annealing temperature (Table 4.1). A melt curve was included from 65-95°C with 0.5°C increments every 5 sec to confirm the absence of non-specific amplification products. A temperature gradient was first used to determine the optimal annealing temperature of each

primer pair. Thereafter a 1:4 cDNA dilution series was used to determine the amplification efficiency of each primer set.

Prior to gene expression analysis, reference gene validation was done. Nine candidate reference genes (Table 4.1) were tested in order to obtain the most stable reference gene combination. The quantification cycle (Cq)-values were imported into the GeNorm-Plus2 software (Biogazelle) to obtain the average expression stability (M) values. qPCR analysis of three randomly selected experimental genes was done using three biological repeats, three technical repeats at two different time intervals. Replicate variability was set to 0.5 and outliers were excluded from analysis. Expression results were analysed using qBase^{Plus} software (Biogazelle).

Table 4.1. Nucleotide sequences of primers used for qPCR analysis of selected genes.

Reference genes							
Gene name	**Reference**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**	**Reaction efficiency (E)**	**R^2-value**
ADP-ribosylation factor (*ARF*)	Paolacci *et al*., 2009	GCTCTCCAACAACATTGCCAAC	GCTTCTGCCTGTCACATACGC	165	56	95.2%	0.953
RNase L inhibitor-like protein (*RLI*)	Paolacci *et al*., 2009	CGATTCAGAGCAGCGTATTGTTG	AGTTGGTCGGGTCTCTTCTAAATG	242	60	109.5%	0.957
Cell division control protein (*CDC*)	Paolacci *et al*., 2009	CAAATACGCCATCAGGGAGAACATC	CGCTGCCGAAACCACGAGAC	227	56	98%	1.000
GAPDH	Jarošová and Kundu, 2010	TGTCCATGCCATGACTGCAA	CCAGTGCTGCTTGGAATGATG	105	60	88.4%	0.997
18S rRNA	Jarošová and Kundu, 2010	GTGACGGGTGACGGAGAATT	GACACTAATGCGCCCGGTAT	151	60	87.6%	0.996
β-tubulin	Jarošová and Kundu, 2010	CAAGGAGGTGGACGAGCAGATG	GACTTGACGTTGTTGGGGATCCA	84	60	70.1%	0.989
Histone H3	Paolacci *et al*., 2009	GTCACCATCATGCCCAAG	CAACACATTCCACTTCCG	109	59.5	101.3%	0.985
Translation elongation factor 1 alpha-subunit (Tef 1-α)	Giménez *et al*., 2011	GCCCTCCTTGCTTTCACTCT	AACGCGCCTTTGAGTACTTG	91	59.5	102.6%	0.988
α-tubulin	Clarke and Rahman, 2005	GGCTTGTGTCTCAGGTTATCTCATC	CATGGAGGATGGCTCGAAGG	216	59.5	99.4%	0.934

Table 4.1. Continued

Experimental genes							
Name	**Accession number**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**	**Reaction efficiency (E)**	**R^2-value**
Carbonic anhydrase	P40880	GTGGAATCAAGGCTCTGCTC	GACGAACGGGTAGGTCAAGA	203	59.5	95.9%	0.977
Endoplasmin homolog	P36183	ATGGGAGTCCAAAGCTGATG	ACATCAACCTCCTTGGTTGC	204	59.5	100.9%	0.986
ATP-dependent zinc metalloprotease FtsH2	Q655S1	GTGTCGATGAAGCAAAGCAA	TCCGACAAACATCTCCACAA	215	55.9	107.4%	0.994

4.3 Results

A shotgun proteomic approach was used to analyse the effect of the 60% purified SS treatment on the wheat proteome. A visual comparison of the polypeptide profiles of 24 hpt SS and water treated wheat did not indicate any significant differences (Fig. 4.1). LC/MS/MS analysis was used to identify differentially expressed polypeptides. Expression levels were determined by expressing the spectral values of the SS treatment at 24 hpt relative to that of the water treatment at the same time interval. Polypeptides with a positive spectral count were considered to be up-regulated following SS treatment while those with a negative spectral count were considered to be down-regulated.

A total of 366 polypeptides were initially identified with a 75% accuracy using LC/MS/MS of which 72 were identified with 95% accuracy (Table 4.2). In all cases, where one polypeptide was represented by 2 or more homologs, the homologs were excluded and only one representative polypeptide was used for analysis. Of the 72 identified polypeptides, 22 showed increased spectral counts in SS treated plants at 24 hpt while 39 showed a decrease with 4 showing no change (Fig. 4.2).

In order to only analyse polypeptides that were truly affected by SS treatment, only polypeptides that were up-regulated above 0.4 and down-regulated below -0.4 spectral counts were used for further analysis. These values were obtained by subtracting the normalised spectral count value of water from that of SS. The above mentioned parameters were randomly chosen. The up-regulated polypeptides included the PSII stability/assembly factor HCF136, elongation factor 2, carbonic anhydrase, endoplasmin homolog, catalase 1, oxygen-evolving enhancer protein 2, ATP-dependent zinc metalloprotease FtsH2, Rubisco activase B, PSI reaction centre subunit II, adenosylhomocysteinase, actin-1 and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. Down-regulated polypeptides included triosephosphate isomerase, ATP synthase subunit beta, Rubisco activase A, cytosolic phosphoglycerate kinase, phosphoglucomutase, PSI reaction centre subunit III, elongation factor Tu, glyceraldehyde-3-

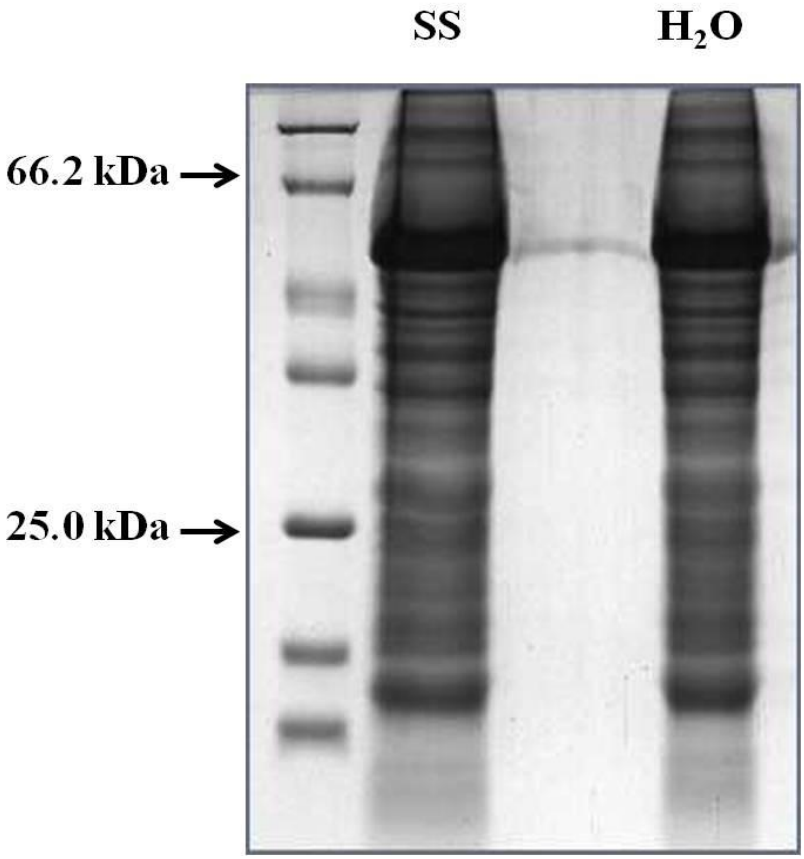


Figure 4.1. SDS-PAGE separation of total polypeptides isolated from Thatcher+*Lr34* wheat treated with SS and water respectively. A total of 100 µg protein was separated on a 12% (w/v) SDS-PAGE gel. Molecular size fragments are as indicated.

Table 4.2. Proteomic identification of polypeptides following SS treatment of wheat seedlings.

Polypeptide identification	**Accession number**	**EC-number**	**Functional category**
50S ribosomal protein L5, chloroplastic	Q9ZST0		Translation
50S ribosomal protein L9, chloroplastic (CL9)	Q8L803		Translation
60S ribosomal protein L9	P49210		Translation
Actin-1	P53504		Binding
Adenosylhomocysteinase (AdoHcyase)	P32112	EC 3.3.1.1	Other
ATP synthase subunit beta, chloroplastic	P20858	EC 3.6.3.14	Photosynthesis
ATP-dependent zinc metalloprotease FtsH2, chloroplastic	Q655S1	EC 3.4.24.-	Photosynthesis
Beta-amylase	P93594	EC 3.2.1.2	Catalytic mechanisms
Carbonic anhydrase, chloroplastic	P40880	EC 4.2.1.1	Photosynthesis
Catalase-1	Q43206	EC 1.11.1.6	Photosynthesis
Chlorophyll *a-b* binding protein 1B-21, chloroplastic (LHCI type I CAB-1B-21) (LHCI-730 chlorophyll *a/b* binding protein) (Light-harvesting complex I 21 kDa protein)	Q9SDM1		Photosynthesis
Chlorophyll *a-b* binding protein of LHCII type III, chloroplastic (CAB)	P27523		Photosynthesis
Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	Q7X9A6	EC 1.10.99.1	Photosynthesis
Elongation factor 2 (EF-2)	O23755		Protein synthesis
Elongation factor Tu, chloroplastic	Q43467		Protein synthesis
Endoplasmin homolog (Glucose-regulated protein 94 homolog) (GRP-94 homolog)	P36183		Protein synthesis
Ferredoxin-dependent glutamate synthase	Q08258	EC 1.4.7.1	Photorespiration
Ferredoxin-NADP reductase, chloroplastic (FNR)	P41343	EC 1.18.1.2	Electron transport
Fructose-1,6-bisphosphatase, chloroplastic (FBPase)	P09195	EC 3.1.3.11	Gluconeogenesis
Fructose-bisphosphate aldolase, chloroplastic (ALDP)	Q40677	EC 4.1.2.13	Gluconeogenesis
Germin-like protein 8-14 (Germin-like protein 1) (Germin-like protein 5) (OsGER5)	Q6ZBZ2		Binding

Glutamine synthetase leaf isozyme, chloroplastic (GS2) (Glutamate--ammonia ligase)	P13564	EC 6.3.1.2	Nitrogen metabolism
Glutamine synthetase, chloroplastic	P14655	EC 6.3.1.2	Nitrogen metabolism
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) A, chloroplastic	P19866	EC 1.2.1.13	Glycolysis
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	P08477	EC 1.2.1.12	Glycolysis
Glycine dehydrogenase [decarboxylating], mitochondrial	P26969	EC 1.4.4.2	Photorespiration
NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Q8LK61	EC 1.2.1.9	Calvin cycle
Nucleoside diphosphate kinase 1	O81372	EC 2.7.4.6	ATP binding
Oxygen-evolving enhancer protein 1, chloroplastic (OEE1) (33 kDa subunit of oxygen evolving system of PSII) (33 kDa thylakoid membrane protein) (OEC 33 kDa subunit)	P27665		Photosynthesis
Oxygen-evolving enhancer protein 2, chloroplastic (OEE2) (23 kDa subunit of oxygen evolving system of PSII) (23 kDa thylakoid membrane protein) (OEC 23 kDa subunit)	Q00434		Photosynthesis
Oxygen-evolving enhancer protein 3-2, chloroplastic (OEE3) (16 kDa subunit of oxygen evolving system of PSII) (Ferredoxin-NADP reductase-binding protein) (BP) (OEC 16 kDa subunit)	Q41806		Photosynthesis
Peptidyl-prolyl cis-trans isomerase (PPIase)	P21569	EC 5.2.1.8	Protein folding
Peptidyl-prolyl cis-trans isomerase CYP20-2, chloroplastic (PPIase CYP20-2)	Q9ASS6	EC 5.2.1.8	Protein folding
Peroxiredoxin-2E, chloroplastic	Q949U7	EC 1.11.1.15	Photosynthesis
Peroxiredoxin-2E-2, chloroplastic	Q7F8S5	EC 1.11.1.15	Photosynthesis
Phosphoglucomutase, cytoplasmic (PGM)	Q9SNX2	EC 5.4.2.2	Carbohydrate metabolism
Phosphoglycerate kinase (PGK), chloroplastic	P12782	EC 2.7.2.3	Calvin cycle
Phosphoglycerate kinase, cytosolic	P12783	EC 2.7.2.3	Glycolysis
Phosphoribulokinase, chloroplastic (PRKase)	P26302	EC 2.7.1.19	Calvin cycle
Photosystem I reaction centre subunit II, chloroplastic (PSI 20 kDa subunit)	P36213		Photosynthesis
Photosystem I reaction centre subunit III, chloroplastic (Light-harvesting complex I 17 kDa protein)	P13192		Photosynthesis
Photosystem I reaction centre subunit IV, chloroplastic (PSI 10.8 kDa polypeptide)	P13194		Photosynthesis

Photosystem II stability/assembly factor (HCF136), chloroplastic	Q5Z5A8		Photosynthesis
Probable fructose-bisphosphate aldolase 1, chloroplastic	Q9SJU4	EC 4.1.2.13	Glycolysis
Probable galacturonosyltransferase-like 8	O48684	EC 2.4.1.-	Structural
Protein FD (bZIP transcription factor 14)	Q84JK2		Flower development
Rubisco large subunit	Q31886	EC 4.1.1.39	Photosynthesis
Rubisco large subunit	Q05985	EC 4.1.1.39	Photosynthesis
Rubisco small subunit	Q40004	EC 4.1.1.39	Photosynthesis
Rubisco activase A	Q40073		Photosynthesis
Rubisco activase B	Q42450		Photosynthesis
Rubisco large subunit-binding protein subunit alpha, chloroplastic (60 kDa chaperonin subunit alpha) (CPN-60 alpha) (Fragment)	P08823		Photosynthesis
Rubisco large subunit-binding protein subunit beta, chloroplastic (60 kDa chaperonin subunit beta) (CPN-60 beta) (Fragment)	Q43831		Photosynthesis
Stromal 70 kDa heat shock-related protein, chloroplastic (Fragment)	Q08080		Stress response
Thioredoxin M-type, chloroplastic (Trx-M)	Q9ZP21		Electron transport
Transketolase, chloroplastic (TK)	Q7SIC9	EC 2.2.1.1	Calvin cycle
Triosephosphate isomerase, chloroplastic (TIM) (Triosephosphate isomerase)	P46225	EC 5.3.1.1	Glycolysis
Uncharacterized 341.7 kDa protein in psbD-psbC intergenic region	Q32065		Photosynthesis
UTP-glucose-1-phosphate uridylyltransferase	Q43772	EC 2.7.7.9	Carbohydrate metabolism

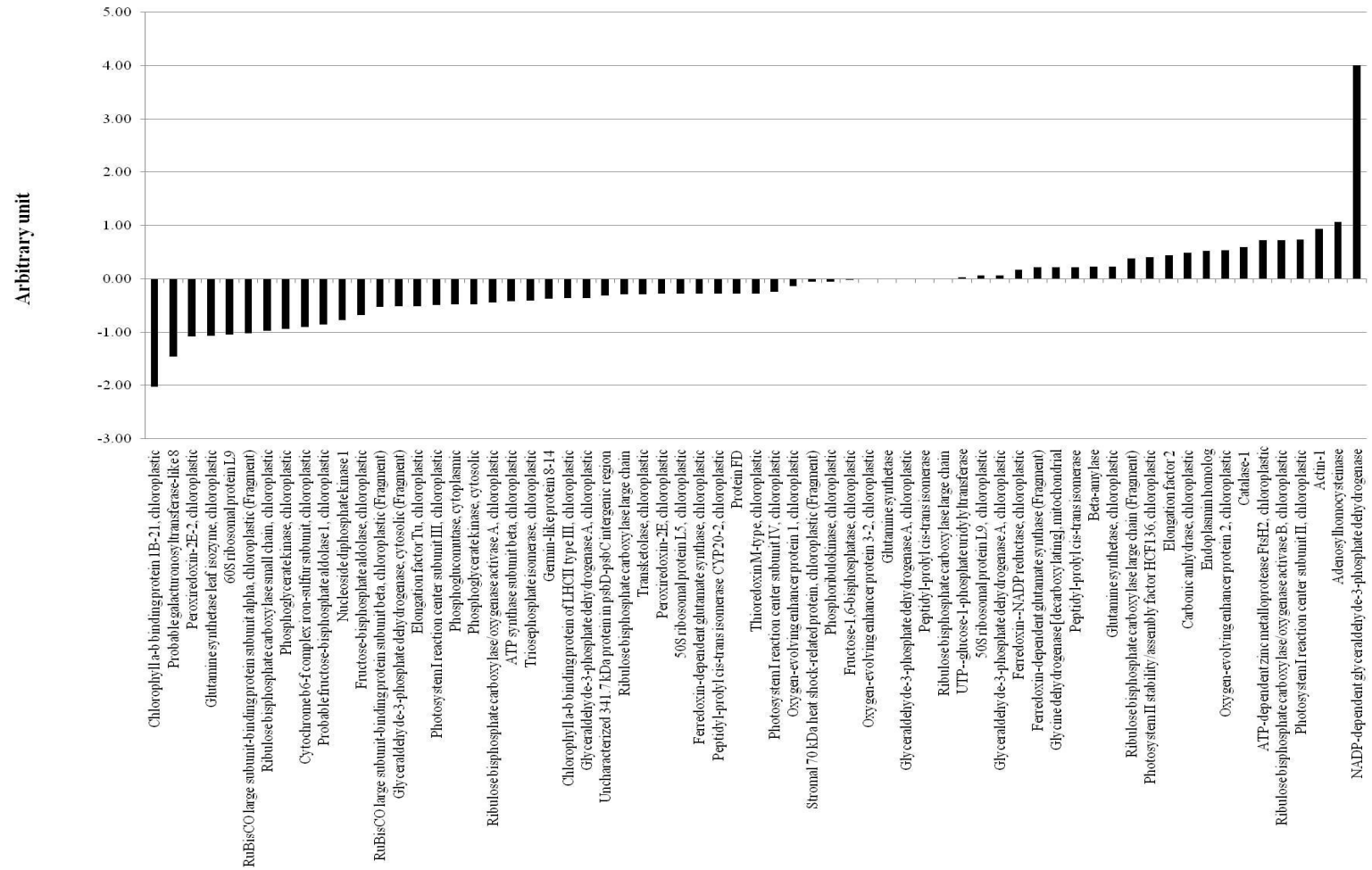


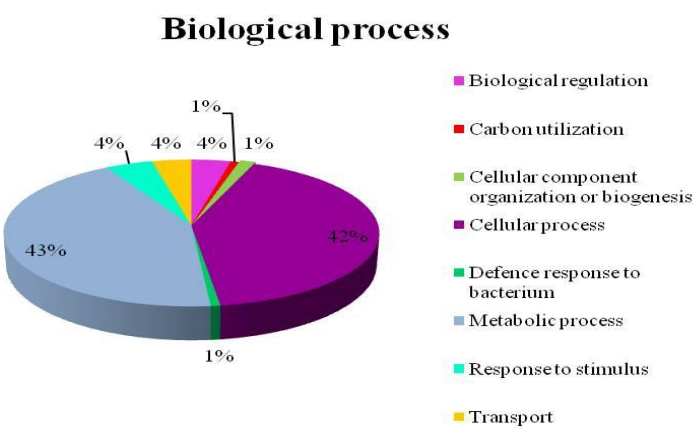
Figure 4.2. Relative differential expression levels of identified polypeptides in SS treated wheat seedlings. Only polypeptides down-regulated below the -0.4 level and up-regulated above the 0.4 level are shown.

phosphate dehydrogenase, Rubisco large subunit-binding protein subunit beta, fructose-bisphosphate aldolase, nucleoside diphosphate kinase 1, probable fructose-bisphosphate aldolase 1, cytochrome b6-f complex iron-sulfur subunit, phosphoglycerate kinase (chloroplastic), Rubisco small subunit, Rubisco large subunit-binding protein subunit alpha, 60S ribosomal protein L9, glutamine synthetase leaf isozyme, peroxiredoxin-2E-2, probable galacturonosyltransferase-like 8 and chlorophyll *a-b* binding protein 1B-21.

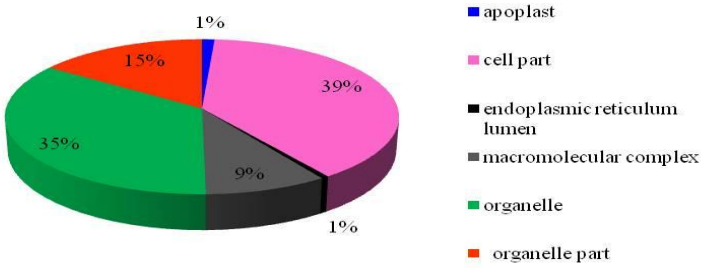
Functional analysis of all 72 identified polypeptides was done with the UniProt Databasis using GO software. The GO analysis divided the polypeptides according to their Biological process, Cellular component and Molecular functions respectively (Ashburner *et al*., 2000). The Biological process represents the series of processes a cell or organism uses to detect the reduction of primary carbon sources. The Cellular component represents the part of the cell or extracellular environment in which a polypeptide is located, while the Molecular function represents the activity of the polypeptide on a molecular level.

When classified according to their Biological function (Fig. 4.3a), the largest functional categories consisted of polypeptides involved in metabolic (43%) and cellular processes (42%) followed by polypeptides involved in transport (4%), response to stimuli (4%) and carbon utilization (1%). The largest part (39%) of the Cellular component graph (Fig. 4.3b) constituted of polypeptides associated with the cellular part. These included polypeptides associated with the mitochondrial and chloroplast thylakoid membranes. Another 35% consisted of polypeptides associated with various organelles while 15% represented organelle parts. Polypeptides involved in the macromolecular complexes, endoplasmic reticulum lumen and apoplast accounted for 9%, 1% and 1%, respectively. In terms of Molecular function (Fig. 4.3c), the largest portion (47%) of polypeptides was involved in binding that included cofactor-, metal ion-, nucleotide-, peptide- and RNA binding. Polypeptides involved in catalytic activity (43%) formed the second largest group that included those involved in oxidoreductase and UTP-glucose 1- phosphate uridylyltransferase activity. The catalase group (2%) included polypeptides involved in photosynthesis.

a)



b)



c)

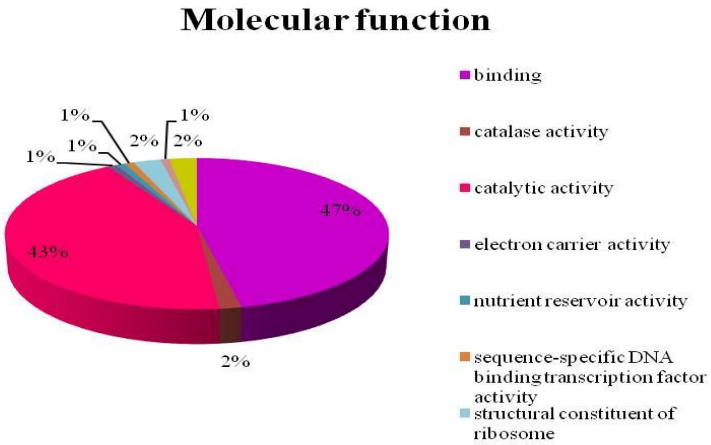


Figure 4.3. Gene ontology analysis of 72 differentially regulated polypeptides following SS treatment. Protein classes as given by Gene Ontology: (a) Biological process, (b) Cellular component and (c) Molecular function. The identification of a group of polypeptides is given as a proportion of the total amount of identified polypeptides.

The proteomic data indicated that the main target of SS treatment is photosynthesis. The polypeptides involved in photosynthesis were thus further classified under the three major aspects of photosynthesis, namely electron transport, carbon fixation and repair / protection (Table 4.3). To support the proteomic data, the induced expression of three genes whose encoded polypeptides showed increased spectral counts, was confirmed using qPCR. These genes were representative of each of the three groups of photosynthesis related polypeptides (Table 4.3).

Preceding qPCR analysis, total RNA was separated on a 1% (w/v) denaturing agarose gel in order to confirm its quality and quantity (Fig. 4.4). The expression stability of nine candidate reference genes was evaluated using qBase software (Table 4.1). This was done in order to obtain the most stable reference gene or gene combination for normalization of the expression data. The coefficient of variation (CV) and M values were calculated using GeNorm (Table 4.4). The two most stable reference genes identified were those encoding the RNase L inhibitor-like and Histone H3 proteins. The combined CV-value for the two genes was below the required value of 0.5. The expression levels of the three experimental genes were thus expressed relative to this combination of reference genes.

The expression of all three chosen genes was induced in SS treated wheat at 24 hpt (Fig. 4.5). The expression of the cell division protease FtsH homolog 2 (Fig. 4.5a) and endoplasmin homolog (Fig. 4.5c) encoding genes showed only minor increases in expression. While the expression of the carbonic anhydrase encoding gene (Fig. 4.5b) differed between water and SS treated plants at 0 hpt, a significant difference was observed at 24 hpt with a two and a half-fold increase in expression.

Table 4.3. The three main aspects of photosynthesis affected by SS treatment. Differentially expressed polypeptides that play a role in each aspect, are listed.

Electron transport	**Calvin cycle / carbon fixation**	**Repair / protection**
Up-regulated		
Photosystem I reaction centre subunit II	Carbonic anhydrase	ATP-dependent zinc metalloprotease FtsH2
	NADP dependent glyceraldehyde-3-phosphate dehydrogenase	Catalase-1
	Rubisco activase B	Endoplasmin homolog
		Oxygen-evolving enhancer protein 2
		Photosystem II stability/assembly factor HCF136
Down-regulated		
	Fructose bisphosphate aldolase	Peroxiredoxin
	Phosphoglycerate kinase	
ATP synthase subunit beta	Rubisco activase A	
Chlorophyll *a-b* binding protein 1B-21	Rubisco small subunit	
Cytochrome b6-f complex iron-sulfur subunit	Rubisco large subunit binding protein subunit alpha	
Photosystem I reaction centre subunit III	Rubisco large subunit binding protein subunit beta	
	Transketolase	
	Triosephosphate isomerase	
Unchanged		
	FBPase	
	GAPDH	
	Phosphoribulokinase	
	Rubisco large subunit	

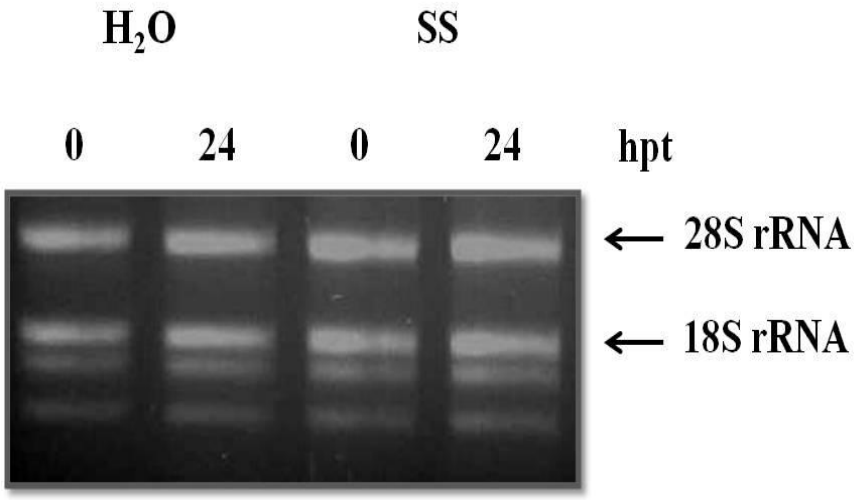


Figure 4.4. Total RNA extracted from wheat seedlings treated with water and SS respectively. Time intervals are as indicated.

Table 4.4. Analysis of reference gene stability. The corresponding M and CV values as determined by GeNorm, are listed. The combination of the most stable reference genes are indicated with an asterisk (*).

Gene name	**M value**	**CV value**
ADP-ribosylation factor	1.064	0.667
*RNase L inhibitor-like protein	0.898	0.4
Cell division control protein	0.888	0.359
GAPDH	1.058	0.549
18S rRNA	0.981	0.413
β-tubulin	1.114	0.585
*Histone H3	0.863	0.341
Translation elongation factor 1 alpha-subunit	0.937	0.335
α-tubulin	1.519	0.81
Average	1.036	0.495

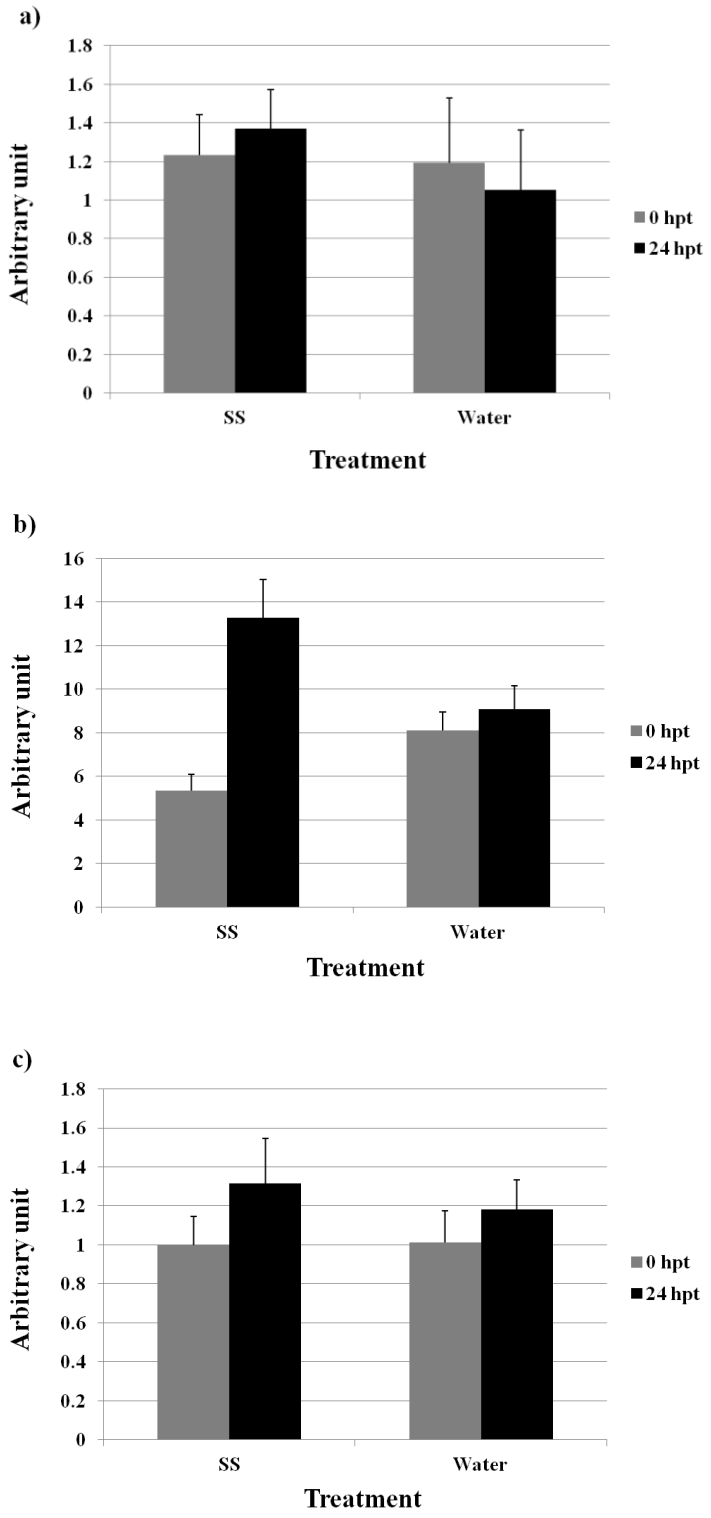


Figure 4.5. Validation of proteomic data using qPCR analysis. The expression of genes encoding a) ATP-dependent zinc metalloprotease FtsH2, b) carbonic anhydrase and c) endoplasmin homolog are indicated. Time intervals and treatments are as indicated.

4.4 Discussion

During a previous study, the application of the crude SS extract to the wheat cultivar PAN 3377 increased yield (Van der Watt, 2005). This indicated that SS could be exploited as a possible bio-stimulant that could increase yield in crops. However, as indicated in Chapter 3, a glass house trial of Thatcher+*Lr34* wheat treated with the 60% purified SS product did not indicate any significant differences in yield or any of the other measured characteristics between the SS and water treatments. Proteomic analysis was therefore used to identify cellular processes targeted by the purified SS product so as to explain the glass house results.

Despite identifying 72 differentially expressed polypeptides, it was decided to mainly focus on polypeptides that were up-regulated above an arbitrary unit of 0.4 or higher or down-regulated below an arbitrary unit of -0.4 or lower, except in the cases of key regulatory enzymes. The rationale for choosing these parameters was that some polypeptides that showed both up- and down-regulation fell between these limits. Examples are the glyceraldehyde-3-phosphate dehydrogenase A (chloroplastic), peptidyl-prolyl cis-trans isomerase and Rubisco large subunit polypeptides (Fig. 4.2).

The proteomic results were validated with qPCR expression analyses (Fig 4.5). Out of the three genes tested, only the gene encoding carbonic anhydrase showed significant induced expression. Since the proteomic analysis of the current study was done at 24 hpt, it is however possible that the *FtsH2* and endoplasmin genes could have been induced and subsequently repressed within the 24 h period. Since noteworthy induction levels were however indicated for carbonic anhydrase, the proteomic results were considered valid.

The identification and characterisation of chloroplast proteins is of great importance in order to understand photosynthesis and its associated processes. Based on their localization and function, chloroplast proteins are divided into three broad groups namely electron transport, the Calvin cycle / carbon fixation and repair of the photosynthetic apparatus (Van Wijk,

2012). The obtained proteomic results of the current study suggested that the effect of the 60% pure SS on wheat could mainly be explained through its influence on these three photosynthetic protein groups within the chloroplast.

Chloroplast proteins include those associated with the light reactions or the electron transport chain. They are located in the thylakoid membrane bilayer and are thus thylakoid-bound (Van Wijk, 2012). Of the five identified polypeptides associated with the electron transport chain, only the PSI reaction centre subunit II was up-regulated while the subunit III was down-regulated (Table 4.3). Based on their identities and functions, the down-regulation of the other three polypeptides implied a decrease in the harvesting of light (chlorophyll *a-b* binding protein), electron transport (cytochrome b6-f complex iron-sulfur subunit) and ATP synthesis (ATP synthase subunit beta) following SS treatment or an overall down-regulation of electron transport process.

One up-regulated electron transport associated polypeptide that was identified was FNR that showed a small increase (arbitrary unit of 0.28) in polypeptide levels (Table 4.3). FNR catalyzes the final step of the electron transport chain whereby electrons are transferred to $NADP^+$ from reduced ferredoxin (Hajirezaei *et al*., 2002). The authors showed that over-expression of FNR increased photosynthetic ability thereby increasing biomass production and indicating the regulatory role of FNR in photosynthesis in tobacco. In contrast, transgenic tobacco plants expressing the antisense *FNR* gene had increased susceptibility to photo-oxidative damage due to reduced electron transport (Palatnik *et al*., 2003).

Chloroplast enzymes also include enzymes involved in the Calvin cycle which interact with thylakoid membranes (Van Wijk, 2012). In order to supply food for a growing population, improvements to the photosynthetic efficiency could hold the key to increased yield (Rosenthal *et al*., 2011). It is especially carbon fixation during the Calvin cycle that could play a pivotal role (Bar-Even *et al*., 2010).

For that reason, researchers have previously identified which enzymes within the Calvin cycle hold the best promise for manipulation to improve photosynthetic capacity. This was done by using transgenic plants that expressed an antisense copy of a specific gene. It was found that the majority of the Calvin cycle enzymes were present in excess (Raines, 2003). These included Rubisco (Stitt *et al.*, 1991), glyceraldehyde 3-phosphate dehydrogenase (Price *et al*., 1995), fructose 1,6-bisphosphatase (Koβmann *et al*., 1994), phosphoribulokinase (Paul *et al*., 1995) and aldolase (Haake *et al*., 1998). It was only when the enzyme levels decreased more than 50% that a decrease in photosynthetic activity was evident.

There were only two enzymes within the Calvin cycle that held true potential for manipulation, namely sedoheptulose-1,7-bisphosphatase (SBPase) (Harrison *et al*., 1998) and transketolase (Henkes *et al*., 2001). Even small reductions (20%) in the levels of these two enzymes severely reduced photosynthetic capacity, indicating that their *in vivo* concentrations were just enough to drive photosynthesis.

The current proteomic data showed that the levels of the majority of the Calvin cycle enzymes were either reduced or remained unchanged (Fig. 4.2, Table 4.3). The first of the three stage Calvin cycle (Raines *et al*., 1999) is the carboxylation of ribulose-1,5-bisphosphate when Rubisco binds CO_2 to it to form 3-phosphoglycerate. Proteomic analysis implied a decrease in Rubisco activity due to reduced expression of the small subunit of Rubisco, of both the large subunit Rubisco binding proteins and of Rubisco activase A, even though Rubisco activase B was up-regulated (Table 4.3).

Carbonic anhydrases (CAs) are Zn-containing metalloenzymes whose activity and expression are decreased by Zn deficiency (Sasaki *et al*., 1998). The main function of CA is catalysing the reversible hydration of CO_2 (Fabre *et al*., 2007). Yusuf *et al*. (2012) indicated that the treatment of Indian mustard (*Brassica juncea*) with SA increased the tolerance of plants to nickel and saline stress through elevated CA activity. The fact that CA is up-regulated (Table 4.3; Fig. 5b), indicated that even though Rubisco levels and by implication activity were

decreased, the continued efficient fixation of CO_2 by Rubisco was ensured by enhanced CA activity through the increased availability of CO_2.

During the second reductive stage of the Calvin cycle, 3-phosphoglycerate is reduced to form glyceraldehyde 3-phosphate through the use of ATP and NADPH (Habenicht *et al*., 1997) The two enzymes involved in the phosphorylation (phosphoglycerate kinase) and reduction (NADP glyceraldehyde 3-phosphate dehydrogenase) reactions were repressed and induced respectively.

The last stage of the Calvin cycle entails the regeneration of the CO_2 acceptor molecule, rubilose 1,5 bisphosphate via triosephosphates by using 10 different enzymes (Rao and Terry, 1989). Of these ten enzymes, triosephosphate isomerase, fructose 1,6 bisphosphate aldolase and transketolase were down-regulated while the levels of FBPase and phosphoribulokinase remained unchanged. The key enzyme SBPase was down-regulated, but was not included in table 4.4 since its identification fell below the 95% identification cut-off.

Sedoheptulose-1,7-bisphosphatase is crucial in maintaining the balance between carbon leaving the Calvin cycle and carbon needed for maintaining RubP levels. Lefebvre *et al*. (2005) indicated that the over-expression of SBPase in tobacco brought about increased photosynthetic rates and a 30% increase in biomass yield. Similar results were obtained when Rosenthal *et al*. (2011) found that greater carbon assimilation and electron transport rates were found in tobacco over-expressing SBPase grown at elevated CO_2 concentrations in comparison to wild type plants. They also indicated an increase in electron transport rates in the tobacco mutants.

Combined, these results indicated that the application of the 60% pure SS repressed the photosynthetic process, or at the very least did not in any way increase photosynthetic capacity. This could explain the results of chapter 3 where the application did not lead to any increases in yield, growth or sugar content above the water control. The exact mechanism

how SS application led to a repressed electron transport chain and Calvin cycle is at this stage not clear.

The last category of chloroplast associated proteins is involved in the assembly and disassembly of the photosynthetic machinery, repair of damaged photosystem components and protection of the general photosynthetic process. This is the largest and most diverse category consisting of protein kinases (O'Malley *et al*., 2008), phosphatases and thioredoxins (Van Wijk, 2012), isomerases (e.g. TPL40), proteases (e.g. FtsH, DegP and Clp) (Wagner *et al*., 2012) and assembly factors (e.g. HCF136 involved in PSII assembly) (Kang *et al*., 2012).

Electron transfer from reduced ferredoxin to thioredoxin coordinates the activities of the light and dark photosynthetic reactions (Pfannschmidt and Yang, 2012). The observed down-regulation of thioredoxin can be attributed to the down-regulation of the Calvin cycle proteins Rubisco, SBPase and phosphoribulose kinase. The down-regulation of thioredoxin can also be associated with the down-regulation of peroxiredoxin (Brion *et al*., 2002). Research identified a novel plant thioredoxin was identified in potato namely chloroplast drought-induced stress protein of 32 kD (CDSP32) (Broin *et al*., 2002). They considered whether the eubacterial 2-Cys peroxiredoxin related plastidic protein, BAS1, is targeted by CDSP32. Affinity chromatography indicated that CDSP32 does indeed target BAS1 in the chloroplasts. *In vitro* analysis indicated that CDSP32 reduced BAS1 and that this plastidic protein displayed a CDSP32-dependent peroxidase activity. Plants lacking CDSP32 indicated decreased F_v/F_m values when exposed to photo-oxidative stress while chlorophyll content also decreased indicating photosynthetic membrane damage. The thioredoxin, CDSP32, plays an important role in oxidative damage which can be associated with its role as an electron donor to BAS1 peroxiredoxin.

Catalase and peroxiredoxin (Prx) is expressed in cells as hydrogen peroxide-decomposing enzymes to yield H_2O (Dietz *et al*., 2006). All Prx have a more or less similar structure which includes a thioredoxin fold and functions in peroxide detoxification during photosynthesis.

The implicated decrease in both electron transport and carbon fixation by the SS treatment would lead to an increase in photo-inhibition by the excess excitation energy. This over-excitation results from the accumulation of NADPH that cannot be sufficiently used because of down-regulated carbon fixation. The induced expression of catalase-1 following SS treatment indicated the role of catalase in the SS treated plant as hydrogen peroxide-decomposing enzymes and could possibly illustrate the sensitivity of plants, treated with SS, to O^{-2} and H_2O_2 inactivation (Dietz *et al*., 2006). It is suggested that SS application did not directly induce the catalase expression genes, but rather that it was a consequence of the increased ROS levels.

SS application had a significant effect on the OEC. Even though a down-regulation was seen in the oxygen evolving enhancer proteins 1 and 3, SS application increased the levels of OEE2 (Fig. 4.2). Verica *et al*. (2004) indicated that in cacao leaves both OEE2 and OEE3-2 protein encoding genes were induced following MeJA and BTH treatment, respectively. OEE2 is involved in the regulation of PSII where it interacts with a wall-associated kinase 1 (Wak 1) (Yang *et al*., 2003). This interaction possibly alters the formation of ROS leading to the induction of defence-related genes and regulation of the hypersensitive response.

Increased crop yield not only depends on enhanced photosynthetic rates but also on photosynthetic repair (Zhu *et al*., 2010). PSII is more vulnerable to photo-oxidative damage compared to PSI and requires an excellent repair mechanism (Lindahl *et al*., 2000). Light-induced damage to the OEC of PSII is repaired via synthesis of the D1 protein of the PSII core. Once D1 light-induced damage occurs, the PSII complex migrates from the grana thylakoid to the stroma thylakoid where it partly disassembles. Two proteases, Filamentation temperature-sensitive H (FtsH) and Deg, a serine protease, degrades the damaged D1 protein and a new D1 is synthesized and inserted into the stroma thylakoid ultimately forming a fully functional PSII complex again (Kato and Sakamoto, 2009). Proteomic analysis indicated that the ATP-dependent zinc metalloprotease, FtsH2, was up-regulated following SS application (Fig. 4.2). Application of exogenous SA to sorghum also induced the chloroplast FtsH protease (Salzman *et al*., 2005). Similar results were obtained by Chen *et al*. (2011) when

they treated *Arabidopsis* plants with MeJA and found increased FtsH2, -5 and -8 protein levels.

Another polypeptide involved in the repair of PSII is Ptr ToxA binding protein (Manning *et al*., 2009). It was previously found that within 2 h, SS treatment did induce the expression of the gene significantly (Viljoen *et al*., unpublished data). The fact that the polypeptide was not detected during the proteomic analysis, could be due to it being present at lower levels within the plant. The up-regulation of FtsH2, Ptr ToxA binding protein and the PSII stability factor (HCF136), as well as catalase, clearly implicated that following SS treatment, ROS was produced due to the excess of energy not utilized. This repair and detoxification system was therefore induced to ensure continued optimal photosynthesis.

Various heat shock proteins (HSPs) were also up-regulated following treatment. They included a putative HSP (HSP90) (data not shown as this falls outside the chosen parameters) and an endoplasmin homolog that belongs to the HSP90 family (Fig. 4.2). Gene expression analysis indicated a significant increase in the endoplasmin homolog expression (Fig. 4.5c). Endoplasmin is a HSP90 chaperone that is located in the endoplasmic reticulum (Klein *et al*., 2006). It is also known as a GRP94 and was discovered when glucose was withheld from cell cultures (Shiu *et al*., 1977). Microarray studies indicated that when *Arabidopsis* plants were treated with tunicamycin (Tm), an inhibitor of N-glycosylation and inducer of the unfolded protein response, endoplasmin levels increased more than seven-fold (Martínez and Chrispeels, 2003), thus indicating the role of endoplasmin in protein folding. Its activity is poorly understood in plants, however. The expression of this gene could possibly assist in the repair of the damaged D1 protein since an increase in protein folding could increase the rate of D1 repair. This is an aspect that needs further investigation.

In conclusion, proteomic analysis indicated that SS affected wheat by mainly targeting the photosynthetic machinery. Since various Calvin cycle enzymes are down-regulated, this could have resulted in a decrease in electron transport. This increased catalase activity in order to remove ROS and the consequent oxidative damage. To further protect the plant, a

repair mechanism of the PSII is activated to ensure functional photosynthetic apparatus. These results therefore supplied a feasible explanation for the non-significant yield associated data that was obtained during chapter 3.

Effect of SS application on membrane stability in stressed wheat seedlings

CHAPTER 5

5.1 Introduction

Plants could be exposed to various abiotic and biotic stress factors on a daily basis. These include pathogens and pests, strong light, high temperatures and drought, to name a few. Their survival depends on how plants are able to cope with these factors. According to the IPCC (2007), these stress factors will increase in future as a result of global climate changes.

Within the agricultural sector, bio-stimulants and plant activators can be used to increase stress tolerance of crops in order to maximise yield even under stressful conditions (García *et al*., 2012). Plant activators are compounds that activate the plant's own defence responses in the form of SAR (Noutoshi *et al*., 2012). Acibenzolar-*S*-methyl was the first compound to be marketed as an inducer of SAR as it induces resistance in various crops including cereals, tobacco and vegetables (Walters *et al*., 2005).

One of the major economic pests that threaten wheat and barley (*Hordeum vulgare* L.) crops worldwide, is the Russian Wheat Aphid (RWA) *Diuraphis noxia* (Mordvilko) (Srinivas *et al*., 2012). More than seven RWA biotypes are known, but RWA biotype 2 (RWASA2) remains the largest threat to wheat and barley crops and is the most virulent (Randolph *et al*., 2008). RWAs are phloem feeders that feed on photo-assimilates within the host plant (Valdez, 2010). They feed by inserting a stylet into the intercellular sieve elements of host plants but cellular penetration could also occur causing cell wall disruptions. Damage to the plants is caused by injected phytotoxins that break down the chloroplast and cellular membranes (Will and Van Bel, 2006; Lapitan *et al*., 2007). Insect feeding has a negative effect on thylakoid membrane stacking thus decreasing LHCII functioning. Botha *et al*. (2006) postulated that in resistant cultivars, thylakoids remain intact allowing maximum photosynthetic activity. In susceptible plants, thylakoid membranes are disrupted, leaf chlorosis occurs and plants die as a result of energy shortage.

Heat is one of the best studied abiotic factors affecting crop production. Plants experience heat stress as either mild (30°C) or extreme (40°C and above). Mild heat stress causes reversible membrane alterations, while extreme heat leads to irreversible membrane damage (Kumar *et al*., 2012).

Elevated temperatures increase membrane permeability and inhibit electron transport (Zhang *et al*., 2009a). The effect of moderate heat stress (37°C) on sensitive and tolerant rice genotypes was analysed using ultra-structural characteristics (Zhang *et al*., 2009b). The authors indicated increased membrane permeability in both genotypes. Mesophyll cells in the flag leaves of the tolerant rice cultivar were tightly arranged while some stomata were closed. Analysis of the sensitive genotype indicated open stomata and unstructured mesophyll cells. High temperatures also caused a loose arrangement of the grana thylakoid layer and the chloroplast envelope was blurred. The nucleus was disintegrated and increased osmiophilic granules were visible. Enami *et al*. (1994) also indicated that upon heat treatment of spinach leaves, PSII becomes disorganised while water splitting and the OEC is disrupted.

Heat-exposed *Jatropha curcas* L. plants also indicated a 28% increase in electrolyte leakage following exposure to 43°C heat stress (Silva *et al*., 2010). Lipid peroxidation, as well as ROS scavenging, increased. Increased levels of NPQ indicated the excess energy is not used to drive photosynthesis but instead is dissipated as excess heat. This indicated a photo-protective effect against photochemical damage.

Exposure of *Cicer arietinum* L. plants to heat stress of 46°C for 2 h induced significant membrane injury (Chakraborty and Tongden, 2005). Exogenous application of SA reduced membrane injury and induced the activity of the stress associated enzymes peroxidase and ascorbate peroxidase. This indicated a positive role for SA in protecting plants from oxidative damage as a result of increased temperatures.

In some cases the application of an activator or bio-stimulant could also have a negative effect on membrane structure and function.When *Vigna radiata* L. Wilczek cv. T-44 plants were treated with 28-homobrassinolide (HBL) and exposed to elevated temperatures of 40°C, a decrease in growth and photosynthetic parameters were found (Hayat *et al*., 2010). This was accompanied by increased electrolyte leakage with decreasing membrane stability. Control plants treated only with HBL did not indicate any changes in electrolyte leakage but did show an increase in the membrane stability index.

Prior to this study, it was believed that SS application mainly targets the photosynthetic machinery and that a possible increase in photosynthetic output could explain the increase in yield obtained in previous studies (Van der Watt and Pretorius, 2011). In chapter 3, results indicated the opposite when the application of the 60% purified SS preparation to Thatcher+*Lr34* wheat actually decreased the yield. This result was confirmed when most of the photosynthesis associated polypeptides identified in SS treated wheat in chapter 4 were down-regulated after foliar application. SS treatment did however induce the up-regulation of various polypeptides putatively involved in membrane stability and PSII repair.

With this in mind, the aim of this study was to determine the effect of pre-treatment of wheat seedlings with the 60% purified SS preparation on wheat chloroplast structure and membrane stability after heat treatment and RWA infestation respectively. The hypothesis is that SS could protect plants from abiotic and biotic stress factors through stabilising membranes by activating various repair mechanisms.

5.2 Materials and methods

5.2.1 Plant propagation and SS treatment

The RWA susceptible wheat (*Triticum aestivum* L.) cultivar Tugela was used during this study. Seed was planted in pots containing a 1:1 soil and compost mixture. Seedlings were watered daily and 1 g l^{-1} Multifeed P® (5:2:4) was applied twice a week. The 60% pure SS preparation was dissolved to a final concentration of 0.5 g l^{-1} in water containing 0.5 ml l^{-1} Tween 20. Wheat seedlings were sprayed with the SS solution until runoff. Control plants were sprayed with water containing 0.5 ml l^{-1} Tween 20 only.

5.2.1.1 Heat stress exposure

Seedlings were grown in growth cabinets at 1500 micro-einsteins m^{-2} s^{-2} illumination with a 12 h day (24°C)/12 h night (18°C) cycle. When seedlings reached the third leaf stage, they were treated with SS and water respectively. Half the seedlings were then moved to a second growth cabinet at an extreme heat temperature of 42°C (Kumar *et al*., 2012) with a 12 h light/12 h dark cycle with the same light specifications. Seedlings were harvested at 0, 6, 12, 24, 48 and 96 hpt and frozen at -80°C. For the measurement of electrolyte leakage, four seedlings were harvested at each time interval for each of the four treatments. Three independent biological repeats were done.

5.2.1.2 Russian wheat aphid infestation

Tugela seedlings used for RWA infestation were grown in a glass house at 23°C. Once seedlings reached the third leaf stage, they were treated with SS and water respectively. Half the plants of each treatment were then infested with RWA while the other half remained

uninfested. RWASA2 infestation was done by gently brushing approximately 15 aphids on each plant. Prior to harvesting, the aphids were gently removed from the plants. Seedlings were harvested at 0, 6, 12, 24, 48 and 96 hours post infestation (hpi) and stored at -80°C. Four seedlings were harvested for each treatment at each time interval to measure electrolyte leakage. Three independent biological repeats were done.

5.2.2 Transmission electron microscopy analysis

Seedlings harvested during both the biotic and abiotic stress treatments were cut into several 5 x 2 mm pieces. Leaf samples were fixed overnight in 3% (v/v) glutardialdehyde, 0.1 M sodium phosphate buffer (pH 7.0) and then rinsed for 15 min in fresh buffer solution the following morning. The buffer solution was replaced with 1% (v/v) OsO_4 in 0.1 M sodium phosphate (pH 7.0) buffer for 1 h where after the material was rinsed twice with the fixing buffer for 15 min each. Samples were dehydrated in a series of acetone dilutions [50%, 70% and 95% (v/v)] for 20 min each with two final dehydration steps in 100% acetone for one hour each.

The dehydrated samples were embedded in Spurr epoxy (Spurr, 1969) in order to make thin sections for microscopic examination. The acetone was replaced by epoxy in the following stages: an initial 1:1 acetone:epoxy embedding for 1.5 h followed by a 1:2 acetone:epoxy embedding for 1.5 h and a final impregnation step of 100% epoxy for 8 h. The last step was repeated overnight in a vacuum desiccator. Lastly, the samples were embedded in the epoxy at 70°C for 8 h in a special mould.

The embedded leaves were sectioned with a Leica ultramicrotome EM UC7 (Vienna, Austria) using a newly prepared glass knife for each sample. Sixty nanometer sections of approximately 0.1 x 0.2 mm were cut and stained with 6% (w/v) uranyl acetate for 20 min and lead citrate for 10 min (Reynolds, 1963). The CM100 Philips transmission electron microscope (FEI, The Netherlands) was used for ultrastructure analysis. Images were

captured with a Megaview II digital camera (Soft Imaging System, Münster, Germany) at 1376 x 1032 x 8BPP resolution.

5.2.3 Electrolyte leakage analysis

Intercellular wash fluid (IWF) was collected from both the RWA and heat treated seedlings according to Cawood *et al*. (2010). In short, harvested leaves were cut into 7 cm long pieces, rinsed in distilled water and dried on blotting paper. The leaves were vacuum infiltrated with 50 mM Tris-HCl pH 7.8 where after they were dried on Whatman filter paper and placed in a centrifuge tube with a perforated disc. After centrifugation at 2000 *g* at 4°C for 10 min, the IWF was collected. The procedure was repeated and the combined IWF freeze-dried. The residue was dissolved in 10 ml deionised water and the conductivity measured using an electrical conductivity meter (HI 98129 pH/conductivity /TDS tester, Hanna Instruments). Electrolyte leakage for each time interval was calculated using the following formula:

$L_{(SS)}/L_{(Water)}$

A decreasing value would indicate decreased leakage and *visa versa*.

5.2.4 Gene expression analysis

Frozen wheat seedlings were ground to a fine powder in liquid nitrogen. Total RNA was extracted from leaf tissue (ca. 200 mg) using Trizol® Reagent (Invitrogen™) according to the manufacturer's instructions. RNA was treated with 5 U DNaseI (Fermentas) according to the manufacturer's instructions to remove possible DNA contamination. RNA concentration was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) (Sambrook and Russell, 2000). A total of 500 ng RNA was separated on a 1% (w/v) denaturing agarose gel containing 0.41 M formaldehyde to confirm RNA quality (4.2.6).

Expression analysis was done using the KAPA™ SYBR® Fast One-Step qRT-PCR Universal kit (KAPA Biosystems) and the Bio-Rad C1000 thermal cycler with CFX96 real-time attachment. Primer optimisation was done by running a gradient reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) reaction and a standard curve for each primer pair. The temperature at which no primer dimers occurred was chosen as the optimal annealing temperature (Table 5.1). Since reference gene validation was previously done (4.2.6), the *RLI* and *Histone H3* reference genes were used for expression analysis.

The expression levels of three experimental genes were analysed during this study: an oxygen evolving enhancer protein 2 (*OOE2*), a metalloprotease (*FtsH2*) and a PSII stability factor (*HCF136*) encoding gene. Each reaction contained 50 ng RNA template, 1x KAPA™ SYBR® Fast qPCR Master mix, 1x KAPA RT mix and 10 pmol of each primer. The RT-qPCR amplification regime consisted of an initial cDNA synthesis step at 42°C for 5 min followed by denaturation at 95°C for 5 min and 40 cycles of 95°C for 3 sec and 30 sec at the specific annealing temperature. A melt curve was included from 65°C to 95°C increasing every 5 sec by 0.5°C. Three biological repeats with two technical repeats were analysed for each RT-qPCR reaction. Cq-values were imported into GeNorm-Plus2 software (Biogazelle) and outliers excluded from analysis. RT-qPCR results were analysed with qBase^{Plus} software (Biogazelle).

Table 5.1 Oligonucleotides used during this study.

Name	**Reference /Accession number**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**	**Reaction efficiency (E)**	**R^2-value**
RNase L inhibitor-like protein	Paolacci *et al*., 2009	CGATTCAGAGCAGCGTATTGTTG	AGTTGGTCGGGTCTCTTCTAAATG	242	60	102.3%	0.974
Histone H3	Paolacci *et al*., 2009	GTCACCATCATGCCCAAG	CAACACATTCCACTTCCG	109	59.5	103.9%	0.898
Oxygen evolving enhancer protein 2	Q00434	CAGAAGAATGACGAGGCTGC	CATCAGTTTGAACCCCTCGC	201	55.9	111%	0.931
ATP-dependent zinc metalloprotease FtsH2	Q655S1	GTGTCGATGAAGCAAAGCAA	TCCGACAAACATCTCCACAA	215	55.9	91%	0.901
PSII stability/assembly factor (HCF136)	Q5Z5A8	CGTCCTGCTGAAAACAACCA	CAGCAGCCTTTCACATGTGT	234	59.5	105.7%	0.986

5.3 Results

In chapters 3 and 4, the effect of SS was analysed under non-stressed conditions. To help formulate a possible role for the 60% purified SS preparation in wheat, it was decided to expose seedlings to two different stress conditions that influence membrane stability within the plants.

The initial studies were done to analyse the possible effect of SS application on chloroplast ultrastructure. Analysis of chloroplast ultrastructure indicated no significant differences between water and SS plants under normal non-stressed conditions at 96 hpt (Fig. 5.1). The tonoplast was intact and thylakoids were clearly visible within the chloroplasts. Plasmodesmata were visible which indicated efficient cellular activity. Part of a nucleus is visible in each photo.

Incubation of wheat seedlings at 42°C affected chloroplast ultrastructure significantly (Fig. 5.2). Once the plants were stressed at 42°C for 96 h (Fig. 5.2c and d), the chloroplasts started moving away from the cell wall, which is a clear indication of the experienced stress. Within the chloroplasts, thylakoids were disorganised and not intact. A larger magnification of chloroplast ultrastructure indicated visible differences between the water and SS treatments (Fig. 5.3). Chloroplasts of SS treated plants were structured and the plasmalemma was intact with well developed plasmodesmata thus indicating cell activity. In the control plants, the grana and thylakoids showed degeneration since inter-thylakoidal swelling was clearly visible, whereas a less apparent effect was observed in SS treated plants.

Analysis of electrolyte leakage showed that treatment with SS resulted in a decrease in electrolyte leakage measured at 24°C at 6 hpt (Fig. 5.4) in comparison to 0 hpt. A similar yet not signification decrease was observed in the heat stressed plants (Fig. 5.4). There after leakage increased for both treatments, even though less leakage occurred at 48 hpt compared to 0 hpt.

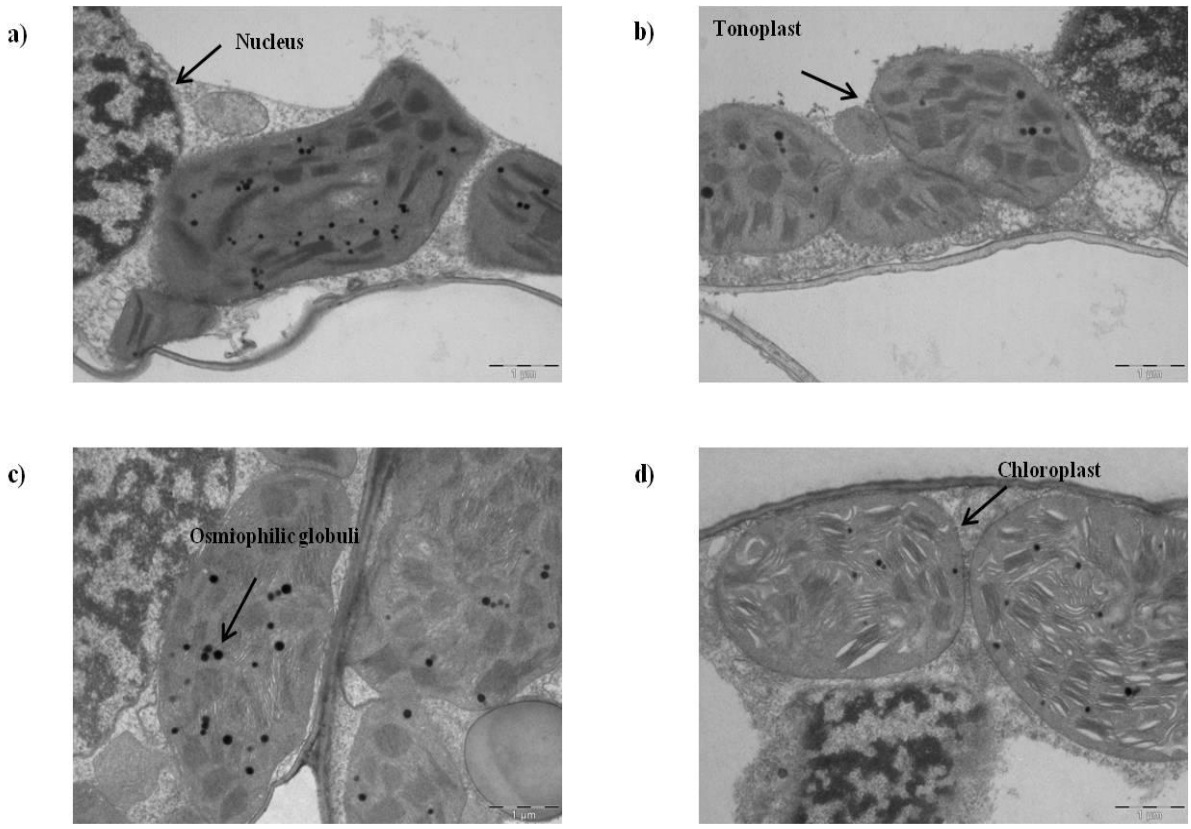


Figure 5.1. Chloroplast ultrastructure of wheat seedlings grown at 24°C following treatment with water and SS respectively. Indicated are leaves treated with (a) water and (b) SS at 0 hpt and (c and d) 96 hpt, respectively. Scale bar = 1 µm.

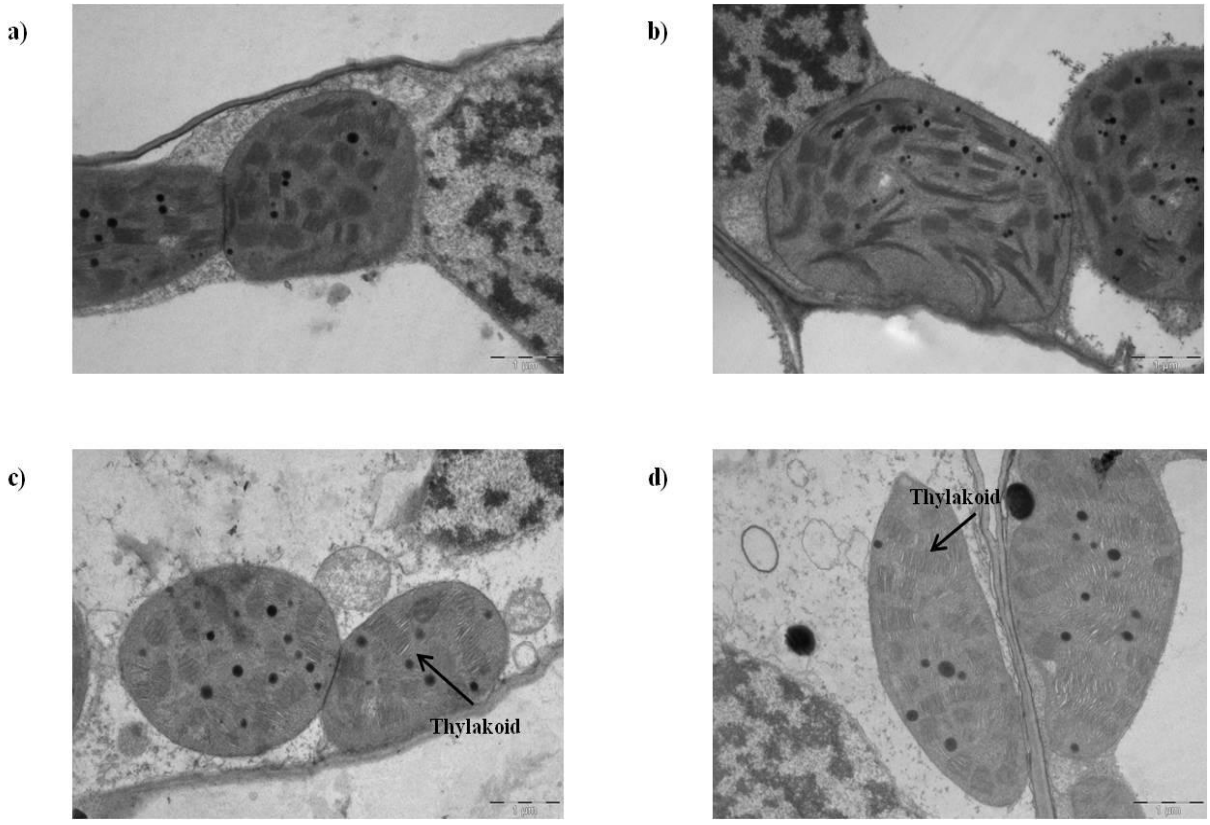


Figure 5.2. Ultrastructure of wheat chloroplasts following treatment with water and SS and incubation at 42°C. Indicated are leaves treated with (a) water and (b) SS at 0 hpt and (c and d) 96 hpt, respectively. Scale bars = 1 µm.

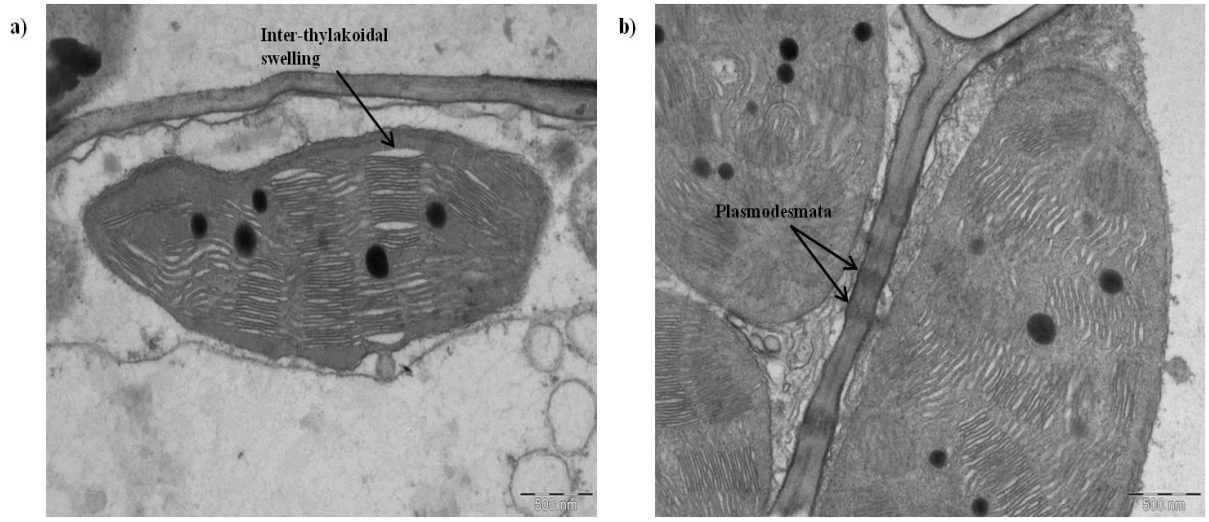


Figure 5.3. Chloroplast ultrastructure of water and SS treated wheat at 96 hpt after incubation at 42°C. Indicated are the treatments with (a) water and (b) SS respectively at 42°C. Scale bars = 500 nm.

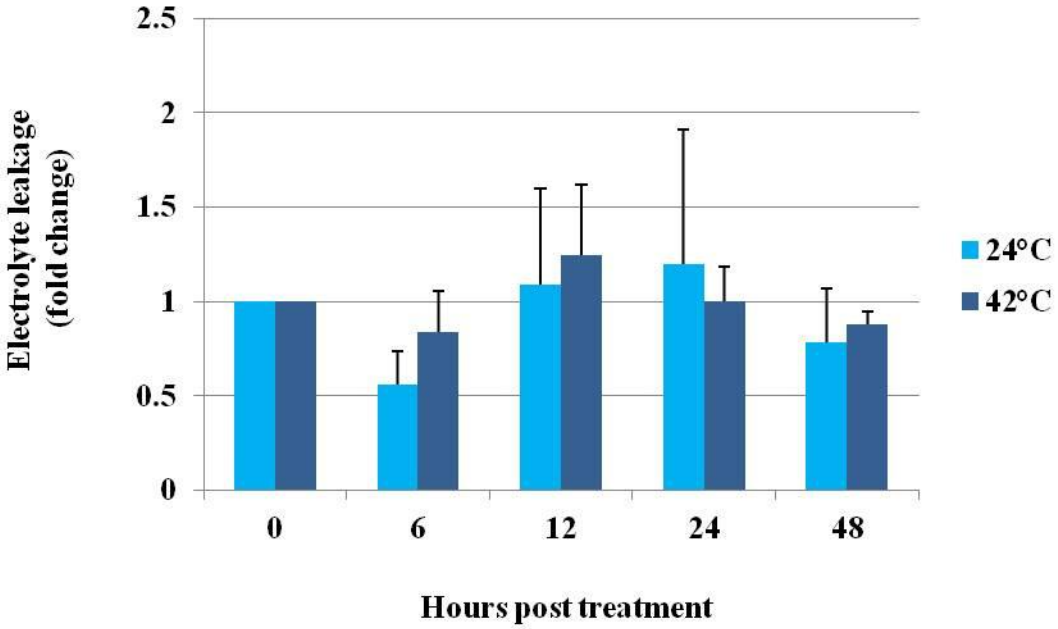


Figure 5.4. Electrolyte leakage of water and SS treated wheat grown at 24°C and 42°C. Time intervals are as indicated and electrolyte leakage is given as a fold change.

Overall, the effect of SS treatment was deemed not significant as indicated by the overlapping standard deviations.

There were no clear differences between SS and water treatment in the expression of *FtsH2* (Fig. 5.5a) and *OEE2* (Fig. 5.5c) from 0 to 24 hpt. Expression analysis of *HCF136* (Fig. 5.5b) showed a 10-fold increase in expression following treatment with SS when incubated at 24°C. The control treatment also indicated an increase in gene expression but was significantly different at 24 hpt from SS treatment.

Following prolonged exposure at 42°C, no induction was observed in *FtsH2* (Fig. 5.6a) or *OEE2* (Fig. 5.6c) gene expression. A clear increase was observed in the expression of *HCF136* (Fig. 5.6b) for both water and SS treated plants at 24 hpt. The increased gene expression was also significantly different between the SS and water treatments at 24 hpt.

The role of SS on membrane structure as part of a biotic stress condition in the form of RWA infestation was also investigated. Once again analysis of chloroplast ultrastructure did not indicate clear differences between water and SS treatments in uninfested wheat seedlings at 0 and 96 hpt (Fig. 5.7). Chloroplasts contained intact thylakoids that were attached to the tonoplast. Plasmodesmata are visible in Fig. 5.7a.

At 96 hpi, a difference was found between the water (Fig. 5.8c) and SS (Fig. 5.8d) treated RWA infested wheat seedlings. Chloroplast ultrastructure was negatively affected in RWA infested plants following SS treatment. The chloroplasts of SS treated wheat were separated from the cell wall while the thylakoids were not as organised as seen in water treated RWA infested wheat (Fig. 5.8c).

Electrolyte leakage again did not indicate any significant differences following SS treatment of uninfested wheat seedlings (Fig. 5.9). While electrolyte leakage in the SS treated RWA

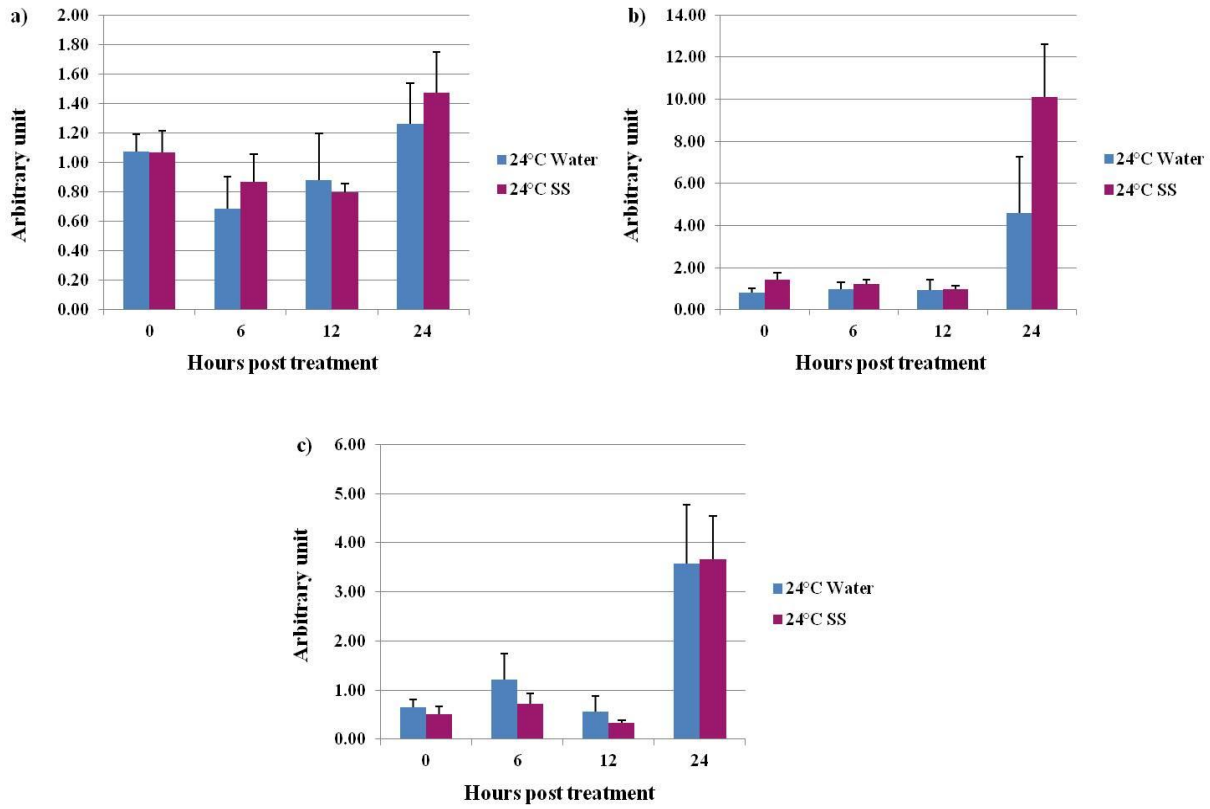


Figure 5.5. Gene expression analysis of wheat treated with water and SS incubated and grown at 24°C. Expression analysis of (a) *FtsH2*, (b) *HCF136* and (c) *OEE2* is shown. Time intervals are as indicated for each treatment.

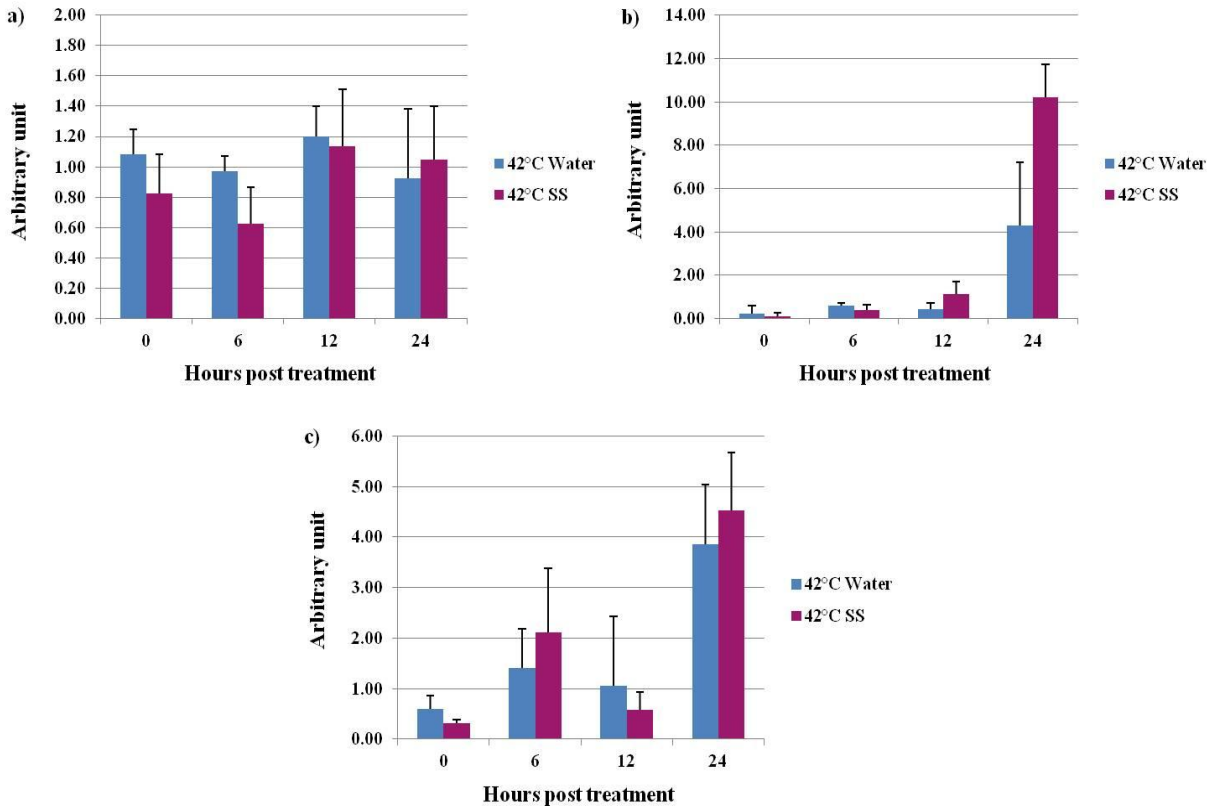


Figure 5.6. Gene expression analysis of wheat incubated at 42°C following treatment with water and SS, respectively. Expression analysis of (a) *FtsH2*, (b) *HCF136* and (c) *OEE2* is shown. Time intervals are as indicated for each treatment.

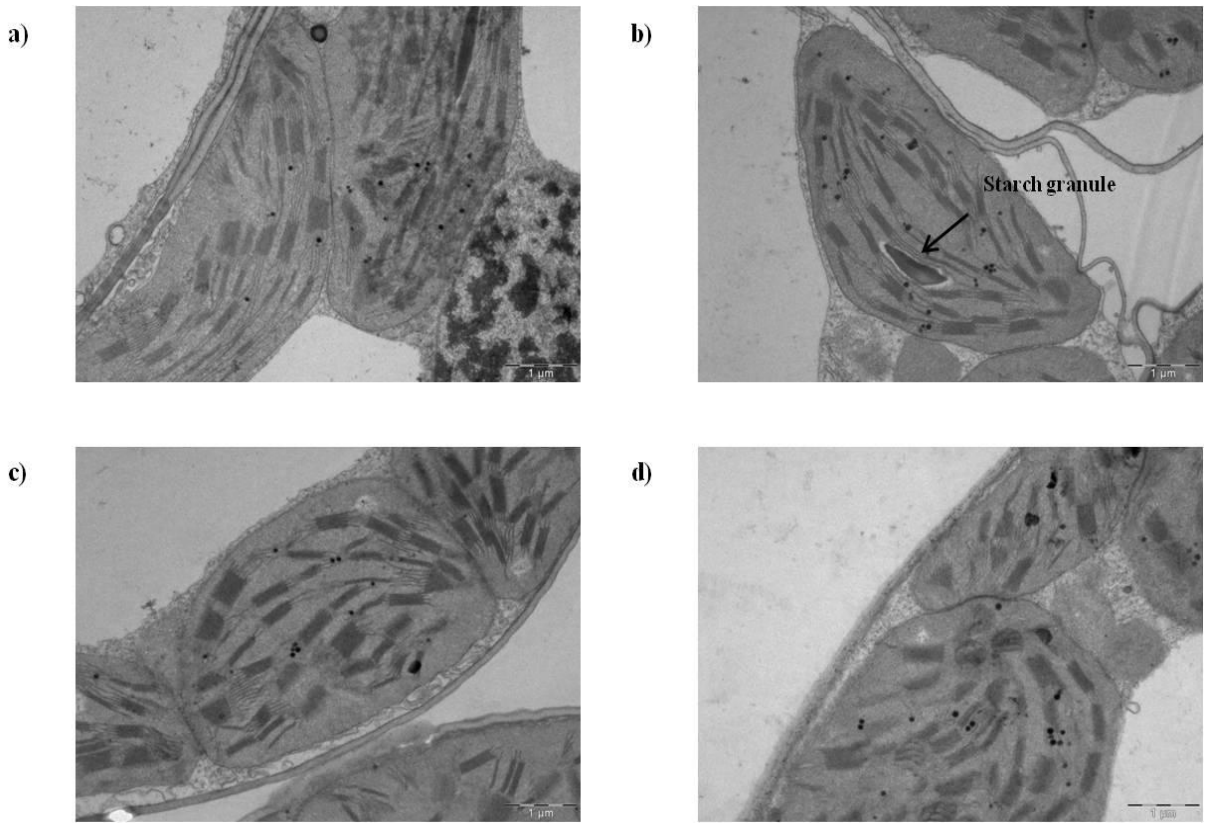


Figure 5.7. Analysis of chloroplast ultrastructure following treatment with water and SS of uninfested seedlings. In (a) water and (b) SS treated leaves grown at 0 hpt is shown, while in (c) water and (d) SS treated uninfested seedlings are illustrated at 96 hpt. Scale bars = 1 µm.

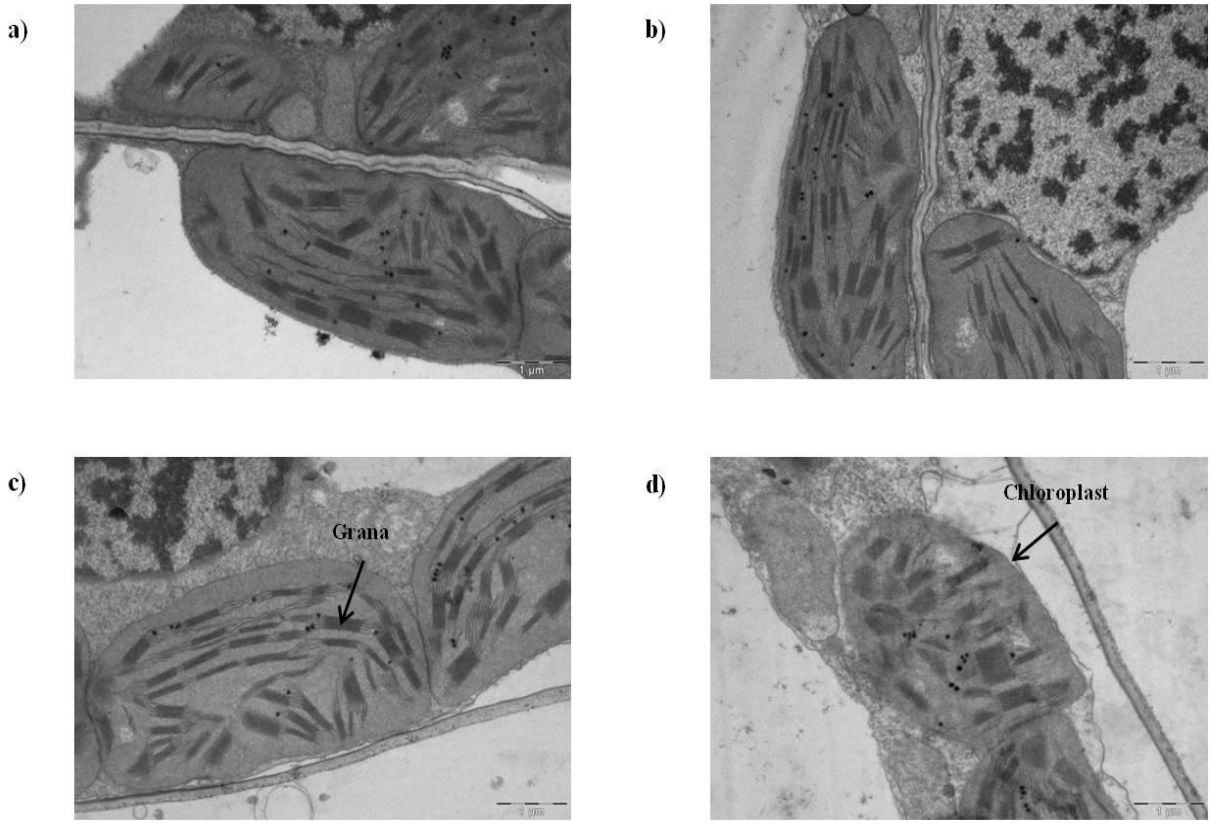


Figure 5.8. Analysis of chloroplast ultrastructure following treatment with water and SS of RWA infested seedlings. In (a) water and (b) SS treated chloroplast ultrastructure is shown at 0 hpi, while in (c) water and (d) SS treated infested seedlings are illustrated, respectively at 96 hpi. Scale bars = 1 µm.

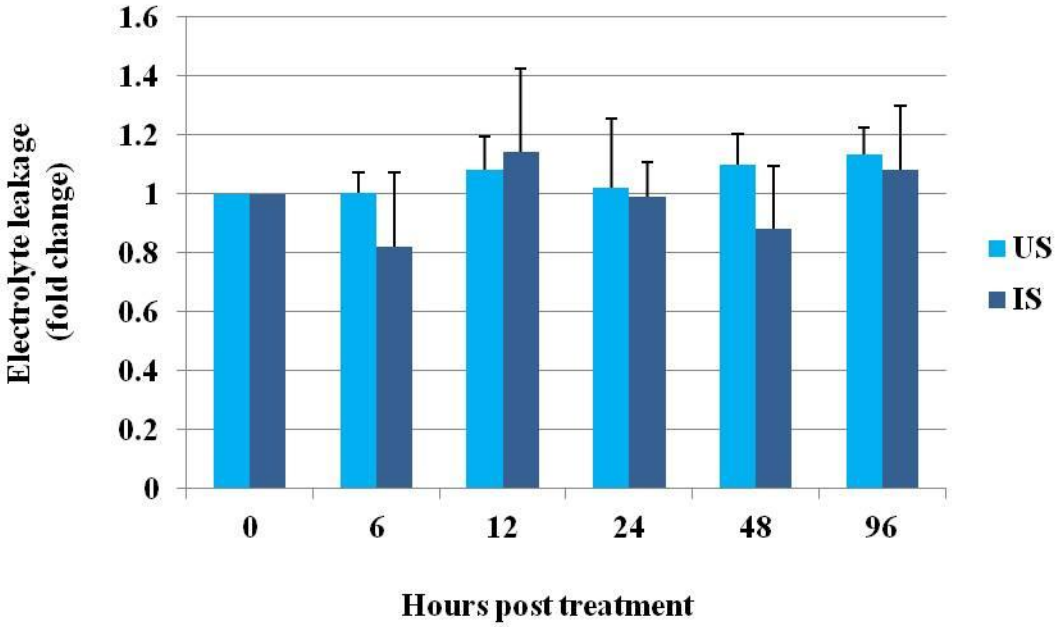


Figure 5.9. Electrolyte leakage of SS treated infested and uninfested wheat. Time intervals are as indicated and electrolyte leakage is given as a fold change.

infested seedlings decreased at 6 hpi with a subsequent increase later on, the changes were not significant due to overlapping standard deviations.

Treatment of uninfested Tugela wheat with SS did not result in any changes in gene expression of *FtsH2* (Fig. 5.10a) or *HCF136* (Fig. 5.10b). A marked increase was observed in *OEE2* (Fig. 5.10c) gene expression at 24 hpt for both water and SS treatments but no significant difference was found between the two treatments.

A two and a half-fold increase was observed in the expression of *HCF136* (Fig. 5.11b) 24 hpi following SS treatment and RWA infestation. SS treatment also increased the expression of *OEE2* (Fig. 5.11c) at 24 hpi more than 16-fold compared to 0 hpi. Even though the expression of *OEE2* also increased following water treatment and RWA infestation, this increase was significantly different from SS treatment at 24 hpi.

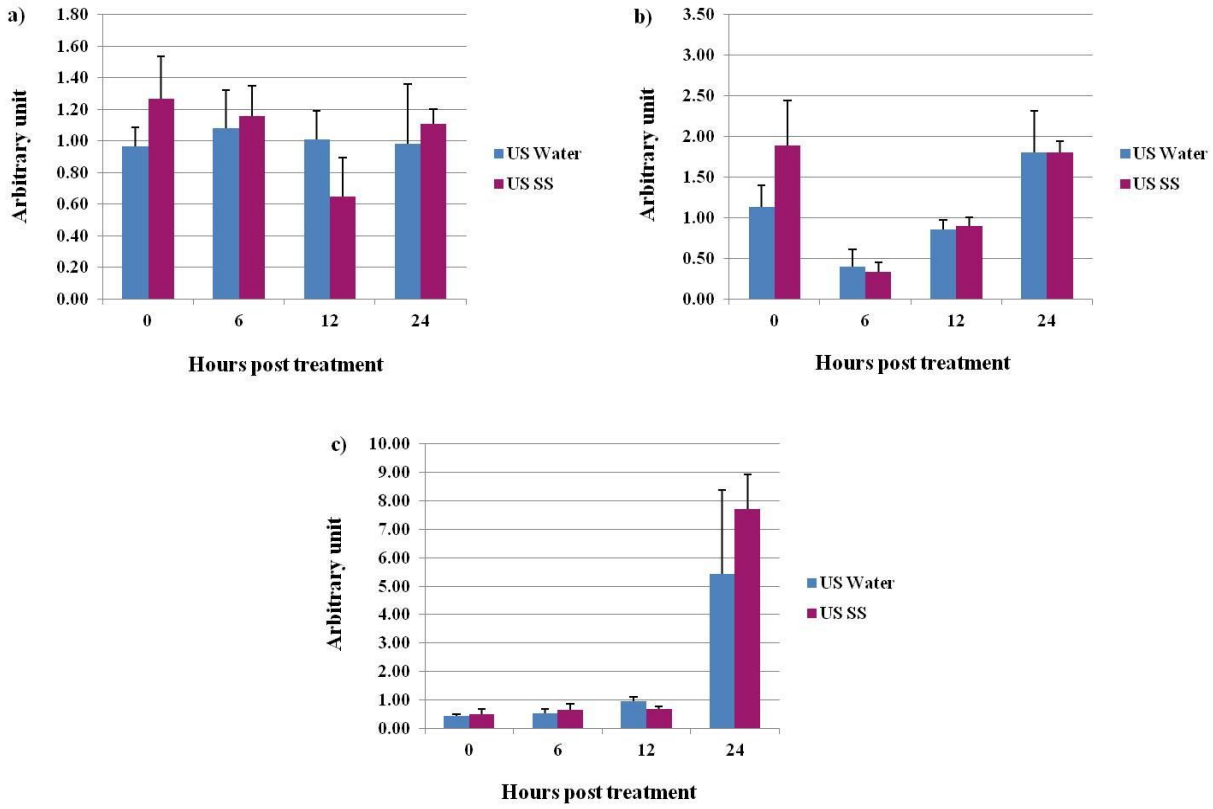


Figure 5.10. Gene expression analysis of uninfested Tugela seedlings after treatment with SS and water. Expression analysis of (a) *FtsH2*, (b) *HCF136* and (c) *OEE2* is illustrated. Time intervals are as indicated for each treatment.

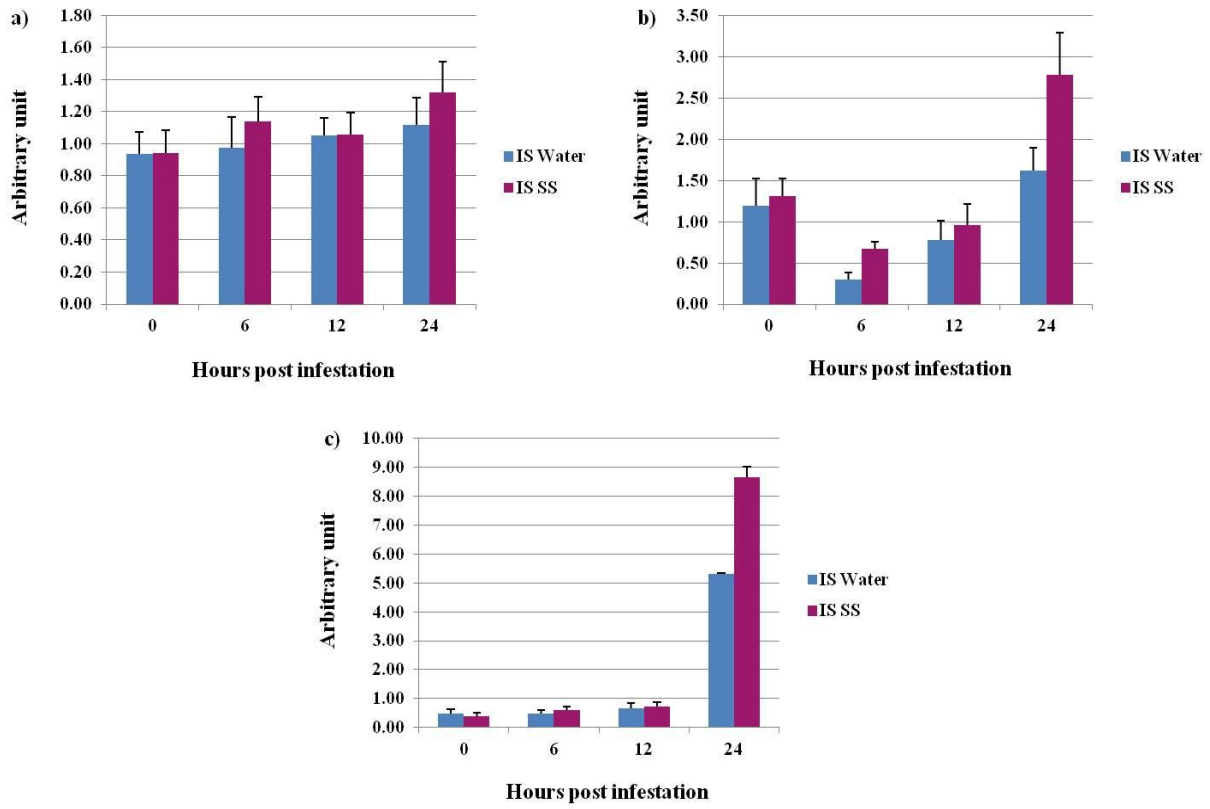


Figure 5.11. Gene expression analysis of RWA infested Tugela seedlings after treatment with SS and water. Expression analysis of (a) *FtsH2*, (b) *HCF136* and (c) *OEE2* is illustrated. Time intervals are as indicated for each treatment.

5.4 Discussion

The effectiveness of bio-stimulants differs between plants and under different conditions. The selection of the most suitable product that could increase photosynthesis and yield in various crops is therefore crucial. The effects of most compounds however only become evident once plants are exposed to some sort of stress factor (Hamza and Suggars, 2001).

One of the marked changes that occur as a result of biotic or abiotic stresses is changes in membrane integrity. When plants are exposed to heat stress, membrane permeability is altered (Tóth *et al*., 2005) together with the macroscopic chloroplast structure (Kreslavski *et al*., 2008). This also includes decreased integrity of the thylakoid membranes. Temperatures in excess of 35°C cause a decrease in membrane stacking leading to the disorganisation of thylakoid membranes which is accompanied by increased ion leakage and changes in energy distribution between PSI and PSII (Wahid and Shabbir, 2005). At temperatures above 42°C, state transition is inhibited even though various PSII reaction centres still remain active.

Salem-Fnayou *et al*. (2011) analysed the effect of heat stress on grapevine and observed several structural alterations. Chloroplasts were more globular and thylakoid membrane orientation was disrupted. They also observed a reduction in granal stacking thickness. When *Cucumis sativus* L. seedlings were exposed to elevated temperatures, chloroplasts became abnormal and swollen (Gao *et al*., 2011). The authors observed non-parallel, disorganised lamellae compared to control plants. Pre-treatment of cucumber seedlings with paraquat, however, increased thylakoid organisation and decreased abnormal shaped chloroplasts following heat stress. Luo *et al*. (2010) indicated similar results when they observed that heat stressed wheat showed damage to the chloroplast envelope with a subsequent increase in membrane fluidity. When heat-stressed wheat was however pre-treated with trehalose, the extent of damage to the grana lamellae and chloroplast envelope decreased.

Since chloroplast ultrastructure and electrolyte leakage are good indicators of the effect of stress conditions on membrane integrity (Kim *et al*., 2012), it was decided to use them to determine whether the 60% purified SS preparation benefits wheat during exposure to heat and RWA infestation. Initial analysis indicted no significant differences between treatment with SS and water under control conditions. However, both the biotic and abiotic stress conditions had an influence on chloroplast ultrastructure. The results indicated a positive effect of SS on chloroplast ultrastructure following heat exposure with less damage being evident. In contrast, SS application had a negative or no effect on chloroplast ultrastructure when wheat was infested with RWASA2. In both cases, this was however not associated with changes in electrolyte leakage since no significant differences were observed, even though decreased leakage was implied at 6 and 48 hpt (Fig. 5.4). By increasing the number of replicates, the statistical analysis could possibly indicate a marked decrease in electrolyte leakage following treatment.

Kim *et al*. (2012) recently transferred the rice cDNA clone, *Oshsp26*, to Tall fescue (*Festuca arudinacea* Schreb.) plants through *Agrobacterium*-mediated transformation. *Oshsp26* is a chloroplast-localised small heat shock protein encoding gene that protects plants from heat stress by playing a protective role within the chloroplast. When grown under normal control conditions, no differences were observed in electrolyte leakage between transgenic and non-transgenic plants. When heat stressed at 42°C, less leakage was observed in the *Oshsp26* transgenic line compared to the non-transgenic control plants. These results were supported by higher F_v/F_m levels in the transgenic plants compared to control plants. They concluded that *Oshsp26* containing transgenic plants showed improved heat stress tolerance that could possibly be associated with the protection of PSII.

This led to expression analysis of three genes that are proposed to be influenced by chloroplast membrane stability since they function within the repair mechanism of PSII. During extreme heat stress, the D1 protein of PSII is most sensitive to damage (Ohira and Yamamoto, 2001). Exposure of spinach to moderate heat stress resulted in the cleavage of the D1 protein while FtsH protease, a membrane-bound ATP-dependent zinc metalloprotease,

directed the degradation of the phosphorylated D1 (Mohanty *et al*., 2012). The major FtsH complex is located within the chloroplast thylakoids (Sakamoto *et al*., 2003).

Yue *et al*. (2010) isolated two FtsH2 encoding full-length cDNAs from maize (*Zea mays* L.). When the expression of these genes were analysed in response to water deficit, low temperatures, high salt and ABA treatment, opposing results were obtained. The expression of *ZmFtsH2B* was markedly increased upon water deficit and ABA treatment, while *ZmFtsH2A* expression was constitutive in all tested conditions. Transgenic tobacco overexpressing *ZmFtsH2A* and *ZmFtsH2B* did not improve drought tolerance. These results indicated that the two specific genes may not necessarily be directly involved in all stress conditions. The current results showed constitutive expression for the *FtsH2* gene following heat (Fig. 5.6a) and RWA stress (Fig. 5.11a). It is thus proposed that the FtsH2 protein identified through the proteomic study could feature more prominent during other stress conditions such as for instance high-light stress. This is an aspect that should be investigated in future.

The OEE2 protein is involved in the regulation of PSII stabilisation (Ifuku *et al*., 2005). The down-regulation of this protein, through RNA interference, caused drastic decreases in plant growth as well as quantum yield of PSII (Ifuku *et al*., 2005). The up-regulation of the *OEE* genes is associated with photo-inhibition (Wang *et al*., 2012) and high light stress (Mahong *et al*., 2012). Yang *et al*. (2003) observed that this protein interacts with wall-associated kinase 1 (WAK1) and that when *Arabidopsis* was infected with *P. syringae*, OEE2 phosphorylation is enhanced. Their data suggests that OEE2 could also play a role in defence signalling. This suggestion was supported by a study that showed that *Pinus nigra* (Arnold) inoculated with *Diplodia scrobiculata* and *Sphaeropsis sapinea* contained significantly more OEE1 and OEE2 polypeptides (Wang *et al*., 2006).

The expression of the OEE2 encoding gene was significantly up-regulated in the SS treated plants following RWA infestation (Fig. 5.11c) but not during the heat stress treatment. The induced expression of *OEE2* was probably not necessary in the heat stressed plants where SS

treatment facilitated the protection of chloroplast membrane structure and per implication PSII reaction centres. It is however well known that RWA infestation causes damage to chloroplast ultrastructure and by implication PSII reaction centres (Botha *et al*., 2006). When tobacco plants were infected with Pepper mild mottle tobamovirus, OEE2 polypeptide levels decreased significantly (Pérez-Bueno *et al*., 2004) illustrating the vulnerability of the OEC and thylakoid membranes to biotic stress factors.

It is thus possible that the up-regulation of the OEE2 encoding gene could be a result of the activation of a reconstruction process of PSII centres that were destroyed following infestation. The application of the 60% pure SS preparation therefore do not seem to have a direct effect on the protection of chloroplast membranes during RWA feeding but it did initiate the expression of a gene involved in maintaining PSII stability and assembly. A secondary role in defence signalling as was postulated by Van der Watt (2005) can however not be excluded.

The last membrane stabilising gene that was analysed following SS treatment was the PSII stability / assembly factor HCF136 encoding gene. *HCF136* codes for a hydrophilic protein that is localised in the lumen of thylakoids. Inactivation of the *Arabidopsis* HCF136 encoding gene through mutation caused a PSII-lacking phenotype with no stable accumulation of photosynthetic core complex proteins (D1, D2, CP47 and CP43) (Meurer *et al*., 1998). Since HCF136 is required for PSII reaction centre assembly and stability (Plücken *et al*., 2002), the significant induced expression of *HCF136* during both stress conditions (Fig. 5.6b and Fig. 5.11b) illustrated that SS application could benefit stressed plants to repair PSII reaction centres that were damaged.

Combined, the data suggests that the 60% pure SS preparation could possibly play a more significant role in the assembly and repair of PSII complexes than in membrane stability, as was originally postulated. This supports a previous study where treatment of heat-stressed wheat seedlings with the 60% pure SS preparation improved both the F_v/F_m and quantum efficiency of PSII (ΦPSII) levels compared to the untreated controls (Janse van Rensburg,

2009). However, the moleculae did have a positive effect on chloroplast membrane stability. Taking this into account, the current study indicates that SS does indeed have a positive effect on plants experiencing abiotic stress in the form of heat, but the precise mechanism involved, requires further investigation.

General discussion

CHAPTER 6

Agriculture uses a substantial amount of the Earth's resources (Cassman *et al*., 2003). Crop production, pasture and livestock grazing use 38% of the total land area (Wood *et al*., 2000), while irrigation of crops account for 80% of all freshwater consumption. The application of N-rich fertilisers accounts for more than 50% of the total N used for human activities (Smil, 1999). The use of these resources is thus important but may ultimately have a negative effect on the environment. The conversion of land from natural forests and wetlands to agro-ecosystems reduces biodiversity and decreases the habitat of wildlife and plant communities (Cassman *et al*., 2003). The withdrawal from water bodies reduces the amount of water available to sustain wildlife and plant populations while the use of excess nitrogen fertilisers causes contamination within water bodies thereby increasing nitrous oxide emissions that has the potential to cause more damage than CO_2 (Waldhoff *et al*., 2011).

The demand for wheat has increased significantly in various countries including Africa. This demand is projected to increase with 2.6% annually until 2020 (Shiferaw *et al*., 2013) while it is projected to increase with 60% in the developing world by 2050 (Nelson *et al*., 2010). Even though several improvements have been made over the last two decades, it was realised that breeding efficiency must increase in order to supply in the increasing demand for food while still protecting natural resources for future generations.

Farmers, however, do not have the luxury of time in order to wait for the genetic identification of complex traits affecting yield and are thus turning to more conventional methods. This includes the use of crude extracts from wild plants that possess bio-stimulatory properties (Arthur *et al*., 2003). These extracts are usually environmentally friendly and do not pose a risk of damage to fauna. Natural products could possibly increase crop yield on available arable land and aid in food security (Van der Watt and Pretorius, 2013).

In a recent study the growth regulatory effect of Tea Seed Powder (TSP) from *Camellia* spp. was analysed (Andresen and Cedergreen, 2010). TSP is believed to contain saponins as the active ingredients (Sparg *et al*., 2004). Saponins are plant secondary metabolites that are able to induce the plant's natural defence response (Wu *et al*., 2005). Beet, oat and barley crops

were soil-treated with various concentrations of TSP and significant increases in biomass ranging from 27% to 41% were observed. As a foliar spray, TSP brought about a 14% to 26% increase in biomass. During the first season, an increase of 38% in strawberry yield was observed, while during the second season, no significant increase was found, indicating the varying effects TSP, and by implication other bio-stimulants, have. This posed the question as to what effect other bio-stimulants could have and how they could be used to facilitate yield increases.

During the current study, the role of SS as a possible bio-stimulant was investigated under controlled and stress conditions. As a crude extract of a *Lupinus albus* L. seed suspension, the bio-stimulatory properties of SS were established when significant crop yield increases were found (Van der Watt and Pretorius, 2011). The active ingredient of the crude SS extract was determined to be glycerol trilinoleate, a triglyceride with three unsaturated linoleic acid fatty acids attached to a glycerol moiety (Van der Watt, 2005).

Throughout the current study, the bio-stimulatory activity of a 60% purified SS preparation and not that of the crude extract, was tested. The rationale for this was four-fold. Firstly, if the crude SS preparation was used, it would have been difficult to know with certainty whether the induced gene expression or associated yield increase was the result of the active ingredient, glycerol trilinoleate or of any other compounds within the extract. Secondly, previous work (Van der Merwe, 2008; Janse van Rensburg, 2009) indicated that the 60% pure SS preparation mainly targets the photosynthetic machinery which indicated the potential to improve yield.

Thirdly, any secondary responses caused by other compounds within the crude extract would be eliminated. Chowdhury *et al*. (2007) treated mango plants with a crude garlic extract. Even though a yield increase was found, treatment with the extract also increased the number of diseased fruit compared to other tested extracts. This indicated that the crude garlic extract triggered a complex array of pathways and/or mechanisms within the plant. By using the 60% purified SS preparation a similar situation would be avoided. Finally, the commercialization

and patenting of a single compound as a bio-stimulant would be much easier, since the effect of its application would be more defined.

To our surprise, no significant increases in yield or any of the other tested yield parameters were found for both the 60% pure SS or the positive CC control (Chapter 3). In fact, both treatments led to a marked, but not significant decrease in yield. This was despite the fact that previous work showed that both bio-stimulants significantly increase yield. For SS, this was seen in vegetable crops and the wheat cultivar PAN 3377 after the foliar application of the crude SS extract (Van der Watt and Pretorius, 2011).

The enhancing effect of CC on yield was also shown in various studies (Melkamu *et al*., 2008; Hüster, 2011). However, other studies indicated no yield increases following CC application (Van der Watt and Pretorius, 2011), possibly explaining the glass house results. During a previous study, Sekoli (2009) could not indicate an increase in yield when CC was applied to carrots. Comcat® contains BRs as active ingredients that occur in low concentrations within the plant. When the effect of the BR analogue BB-16 was analysed, results showed that when applied consecutively to passion fruit (*Passiflora edulis* f. *flavicarpa*, Degener), yield significantly increased above the control (Gomes *et al*., 2006). When applied there after, no significant yield increase was observed. The study indicated the importance of the time of bio-stimulant application at various growth stages. According to Hüster (2011) some field crops might be sensitive to CC treatment and initiate an antagonistic effect within the plant or have no significant effect at all.

Since SS application did not indicate any significant difference in yield, it was postulated that during the preparation of the 60% pure SS extract, the compound/s responsible for increased yield could have been excluded, leaving only the glycerol trilinoleate as active ingredient which was unable to increase yield. To test this hypothesis and investigate which aspects of plant growth were targeted by the pure SS preparation, proteomic analysis of treated wheat seedlings was done (Chapter 4).

One of the most important factors associated with yield is photosynthesis. Various studies suggest that the only way to increase crop yield, is to increase the efficiency of photosynthesis (Long *et al*., 2006; Mitchell and Sheehny, 2006). The European Stress Physiology and Climate Experiment (ESPACE) project conducted a study where they analysed the effect of ambient and elevated CO_2 concentrations on spring wheat during 3 consecutive growing seasons. They indicated that photosynthesis increased by 50% under elevated CO_2 levels resulting in a 35% increase in grain yield (Bender *et al*., 1999; Mitchell *et al*., 1999). These results support the notion that a sustained increase in photosynthesis correlates to increased crop yield (Long *et al*., 2006). Sinclair *et al*. (2004) however indicated lower yields when they found that an increase of 33% in leaf photosynthesis translated to an 18% increase in biomass but only a 5% increase in grain yield.

The proteomic results supported the glass house trial results with several key polypeptides involved in the photosynthetic processes of the Calvin cycle and electron transport being significantly down-regulated after treatment with purified SS (Chapter 4). This included polypeptides that code for key enzymes that regulate the whole photosynthetic process, namely SBPase and transketolase (Harrison *et al*., 1998). The implied decrease in photosynthesis could therefore explain the non-significant effect of SS on yield parameters that were observed. The exact mechanism whereby the 60% pure SS preparation affected the decrease, is however unknown.

Since photosynthesis was mainly down-regulated following SS treatment, this would cause an increase in excess energy that should have been utilised during photosynthesis. This excess energy could significantly increase oxidative damage associated with increased ROS production. The observed increased in catalase enzyme levels would however be able to counteract the ROS damage to cellular compartments by directly converting H_2O_2 to water and molecular oxygen (Liu *et al*., 2010). The question remains how glycerol trilinoleate could facilitate these changes in the wheat cell proteome?

During chloroplast development, an increase in linolenic (18:3) and linoleic acid (18:2) is observed (Anderson and Beardall, 1991). Together with other unsaturated fatty acids like oleic acid and arachidonic acid, they influence the stability of cellular membranes (Falcone *et al*., 2004). Wada *et al*. (1994) postulated that the degree of unsaturated fatty acids could affect both the thylakoid membranes and membrane bound proteins thereby affecting electron transport.

According to Upchurch (2008), the modification of membrane fluidity is associated with changes in the levels of unsaturated fatty acids which are regulated by fatty acid desaturases. When exposed to high temperatures, long term adaptations include the desaturation of fatty acids within the thylakoid membranes thereby decreasing the fluidity of the membranes (Harwood *et al*., 1994; Zheng *et al*., 2011). This causes changes in lipid-protein interactions leading to protein degradation. However, it was shown that the levels of linoleic acid also increase during periods of high temperature stress, most likely due to the fact that it might fulfil a specific role during heat stress (Falcone *et al*., 2004). This accumulation takes about 60 h after temperature increase before the changes are detected.

The application of SS in the form of glycerol trilineolate could thus possibly aid in the adaptation to heat stress through linoleic acid accumulation in the thylakoid membranes, possibly giving the heat stressed plants a head start above the controls (Chapter 5). This was supported by previous work when SS treated wheat seedlings showed higher F_v/F_m levels compared to water treated seedlings under heat stress conditions (Janse van Rensburg, 2009).

Elevated temperatures also induce the expression of heat shock protein encoding genes (Sung *et al*., 2003) through the detection of changes in membrane fluidity (Horvath *et al*., 1998). Even in the absence of heat stress, SS application induced the accumulation of heat shock proteins such as endoplasmin (Chapter 4) that could assist in the PSII repair mechanism. Endoplasmin is a HSP90 chaperone located in the endoplasmic reticulum (Klein *et al*., 2006) which controls proper protein folding ensuring a faster rate of PSII repair. Even though adjustments to the fatty acid saturation levels of the thylakoid membranes prevent decreased

photosynthetic efficiency through damaged photosystems, the PSII reaction centre is sensitive to temperature changes. This is due to the D1 protein within the PSII complex being very susceptible to damage caused by heat (Berry and Björkman, 1980).

FtsH proteases play an important role in the degradation of the damaged D1 complex where after a newly synthesised D1 protein is inserted back into the thylakoid stroma forming a functional PSII complex once again (Kato and Sakamoto, 2009). It was shown that the unsaturated level of the membrane influences this repair of PSII (Moon *et al*., 1995). Even though the proteomic results implied increased FtsH2 levels after SS treatment (Chapter 4), *FtsH2* and *OEE2* expression were not induced in heat stressed plants treated with SS. This was most probably due to the protective effect of the 60% pure SS / linoleic acid treatment on the chloroplast membranes (Chapter 5).

The most noteworthy induction of gene expression was seen for *HCF136* (Chapter 5). HCF136 is a thylakoid protein that is associated with PSII assembly and stability (Plücken *et al*., 2002). Ingle *et al*. (2007) indicated that HCF136 abundance decreased following water stress. The authors proposed that the reduction could serve as a component of photosynthetic shutdown since membrane stability was now compromised. The upregulation of this gene during both heat and biotic stress by SS confirms the overall protective effect of linoleic acid application to the thylakoid membranes.

Finally, yet another protein that influences thylakoid membrane stability is Ptr ToxA Binding Protein (Manning *et al*., 2007), a homolog of the *Arabidopsis* Thf1 protein that plays a role in the formation of thylakoids (Wang *et al*., 2004). Though not identified during the current study, the expression of this gene was found to be significantly up-regulated after SS treatment under normal conditions (Janse van Rensburg, 2009).

The expression of *OEE2* was up-regulated by SS application following RWA infestation (Chapter 5). When *Pisum sativum* L. seedlings were inoculated with *Uromyces pisi*,

proteomic analysis indicated the up-regulation of OEE2 (Barillia *et al*., 2012). Despite being part of PSII and playing a role in photosynthesis, OEE also functions during signalling initiated by Wak1, a plasma and cell wall bound receptor protein (Yang *et al*., 2003). It is proposed that in RWA infested Tugela, OEE2 could possibly play a role in the activation of a defence response when damage to PSII in the form of the D1 protein would activate defence signalling mediated by Wak1, leading to the induction of an appropriate response.

In conclusion these observations suggest that, even though foliar application of SS was not able to induce increased yield, this unsaturated fatty acid had two important effects on chloroplast thylakoids. The first was to induce the expression of several genes involved in the alleviation of damaged PSII through its enhanced repair following damage. Secondly, it provided heat stressed plants with the advantage of rapid changes to the fatty acid saturation levels of membranes during heat stress. This would ensure the optimal protection of the photosynthetic apparatus during elevated temperatures. It is postulated that since polypeptides coding for photosynthetic related genes is decreased following SS application, membrane stability is increased in order for the plant to still maintain adequate levels of photosynthetic functioning to keep alive during heat stress.

Therefore based on this study, the 60% pure SS preparation on its own has no significant use as a bio-stimulant in agriculture. However, when applied in combination with another product which was proven to increase yield in various agricultural crops, the 60% pure preparation will be of value. This is especially true considering the proposed increase in global temperature as a result of global warming. It is also important to remember that these results are based on a glasshouse trial and a single tested concentration. There are, however clear differences between glasshouse and field trials at various concentrations. These suggestions should be taken into account during future studies since it could be of importance for the commercialisation of this novel bio-stimulant.

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CHAPTER 7

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Summary

The agricultural use of bio-stimulants to increase crop yield is increasing as a result of the ever growing human population. Since bio-stimulants do not have the negative environmental effects usually associated with fungicides and pesticides, the identification and testing of new bio-stimulants are a priority. It was previously shown that treatment of wheat cultivar PAN 3377 with a crude *Lupinus albus* L. seed suspension extract increased the yield compared to an untreated control.

The agricultural use of bio-stimulants to increase crop yield is increasing as a result of the ever growing human population. Since bio-stimulants do not have the negative environmental effects usually associated with fungicides and pesticides, the identification and testing of new bio-stimulants are a priority. It was previously shown that treatment of wheat cultivar PAN 3377 with a crude *Lupinus albus* L. seed suspension extract increased the yield compared to an untreated control.

During the current study, the bio-stimulatory effect of a 60% purified extract called SS was investigated. The initial aim was to analyse the effect of the purified SS extract on yield of the wheat cultivar Thatcher+*Lr34*. No significant increases in yield or any of the associated parameters were obtained in response to SS treatment. This indicated that, while the crude and complex SS extract increased yield in wheat, the purified molecule was unable to do it.

To validate the glass house results, proteomic analysis of SS treated wheat cultivar Thatcher+*Lr34* was done. Results indicated that the purified SS extract mainly targets the photosynthetic machinery of the plant by altering three main pathways. While the levels of polypeptides active in both the Calvin cycle and electron transport chain were generally down-regulated following treatment, a few polypeptides involved in the PSII and general repair mechanisms, were up-regulated. These results were validated by qPCR analysis of three randomly selected genes from each of the mentioned pathways.

When wheat cultivar Tugela was treated with the 60% pure SS extract and exposed to heat stress, a stabilising effect on chloroplast ultrastructure was observed. Thylakoid membranes were intact and no induction of the membrane stabilising gene *OEE2* was observed. However, when infected with RWA, the 60% pure SS extract was unable to facilitate chloroplast membrane stabilisation. This was supported by the induced expression of both the *OEE2* and *HCF136* genes.

This study concludes that the 60% pure SS extract, of which glycerol trilinoleate is the active ingredient, cannot be used as a bio-stimulant independently. It is proposed that during the purification of the crude SS extract, additional components responsible for increasing yield in wheat, was lost and thereby its ability to increase yield. This product could however be of value when used in combination with other compounds that is able to facilitate yield increases.

Keywords: *Lupinus albus* L. seed suspension, bio-stimulant, yield, photosynthesis, membrane stabilisation.

Opsomming

Die gebruik van bio-stimulante om in die landboubedryf opbrengs te verhoog, word al hoe belangriker as gevolg van die groeiende wêreldpopulasie. Aangesien bio-stimulante nie soos fungisiedes en pestisiedes ‘n negatiewe invloed op die omgewing het nie, is die ontwikkeling van nuwe bio-stimulante ‘n prioriteit. Dit is reeds voorheen bewys dat die behandeling van die PAN 3377 koringkultivar met ‘n *Lupinus albus* L. saadsuspensie ekstrak opbrengs verhoog in vergelyking met die onbehandelde kontrole.

Tydens die huidige studie is die vermoë van ‘n 60% suiwer SS ekstrak om opbrengs te verhoog, getoets. Aanvanklike was bepaal of die ekstrak die opbrengs van die koringkultivar Thatcher+*Lr34* kan verhoog. Geen statisties betekenisvolle verhoging in opbrengs of enige van die geassosieerde parameters, was gekry nie. Dit was in direkte teenstelling met die SS ru-ekstrak.

Om die glashuisresultate te bevestig, is ‘n proteonomiese ontleding van SS behandelde Thatcher+*Lr34* koringsaailinge gedoen. Die gesuiwerde SS ekstrak het hoofsaaklik die fotosintese proses geteiken met vlakke van polipeptiede betrokke by beide die Calvinsiklus en elektronoordragsisteem wat afgereguleerd was. In teenstelling was ‘n aantal polipeptiede betrokke by PSII se herstelmeganisme opgereguleer. Die resultate is met die qPCR analise van drie lukraak geselekteerde gene bevestig.

Behandeling van die Tugela koringkultivar met die 60% suiwer SS ekstrak tydens die blootstelling van die plante aan hittestres, het ‘n beskermende effek op die chloroplast ultrastruktuur gehad. Tilakoïedmembrane was intak en geen induksie van die membraan stabiliserende geen *OEE2* is waargeneem nie. Na infestasie met die Russiese koringluis, was SS egter nie in staat om membraanstabiliteit te fasiliteer nie. Dit het gelei tot die geïnduseerde uiting van beide die *OEE2* en *HCF136* gene.

Ten slotte het hierdie studie getoon dat die 60% suiwer SS ekstrak wat gliserol trilinoleaat as aktiewe bestanddeel bevat, nie selfstandig as ‘n bio-stimulant gebruik kan word nie. Dit word

voorgestel dat addisionele komponente van SS wat veranwoordelik is vir verhoogde opbrengs, tydens die suiweringsproses verlore gegaan het. Dit het daartoe gelei dat die gesuiwerde SS sy vermoë om opbrengs te verhoog, verloor het. Hierdie produk kan egter van waarde wees indien dit in kombinasie met ander bio-stimulante wat wel opbrengs verhoog, gebruik word.

Sleutelwoorde: *Lupinus albus* L. saadsuspensie, bio-stimulant, opbrengs, fotosintese, membraanstabiliteit.

Appendix

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