ALEXIN™-MEDIATED DEFENCE RESPONSES IN WHEAT DURING RUSSIAN WHEAT APHID (*DIURAPHIS NOXIA*) INFESTATION

by

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Dedication

I dedicate this thesis to my two daughters, Isabelle and Olivia Ferreira.

It doesn’t matter who you are, where you come from. The ability to triumph begins with you. Always.

- Oprah Winfrey

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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
APX	Ascorbate peroxidase
As-1	Activation sequence 1
ASM	Acibenzolar-S-methyl
ATP	Adenosine triphosphate
Avr	Avirulent
	B
BABA	β-aminobutyric acid
BTH	Benzo (1,2,3) thiadiazole-7-carbothionic acid *S*-methyl ester
	C
CAT	Catalase
Cu/ZnSOD	Copper/Zinc superoxide dismutase
	D
DHAR	Dehydroascorbate reductase
DTT	Dithiothreitol
	E
EDTA	Ethylenedinitrilotetraacetic acid
ETI	Effector-triggered immunity
ET	Ethylene
	F
FeSOD	Iron-superoxide dismutase
	G
GR	Glutathione reductase
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione-*S*-transferase
	H
H_2O_2	Hydrogen peroxide

HR	Hypersensitive response
	I
Inf	Infestation
INA	2,6-Dichloroisonicotinic acid/methyl ester
IS	Internal standard
ISR	Induced systemic resistance
IWF	Intercellular washing fluid
	J
JA	Jasmonic acid
	L
LAR	Localised acquired resistance
	M
MAPK	Mitogen-activated protein kinase
MeSA	Methyl salicylic acid
Mg	Magnesium
MnSOD	Manganese-superoxide dismutase
MR	Moderate resistance
MRM	Multiple reaction monitoring
MS	Moderately susceptible
	N
NBT	Nitro blue tetrazolium chloride
NLR	Nucleotide-binding leucine-rich repeat
NO	Nitric oxide
NPR	Non-expresser of pathogenesis-related
	O
O_2^-	Superoxide anion
	P
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PFI	Phloem-feeding insect
PGPR	Plant growth-promoting rhizobacteria

POX	Peroxidase
PRR	Pattern-recognition receptor
PR Proteins	Pathogenesis-related proteins
PTI	PAMP-triggered immunity
PVP	Polyvinylpyrrolidone

R

R	Resistance
R-gene	Resistance-gene
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonuclease
ROS	Reactive oxygen species
RWA	Russian wheat aphid

S

S	Susceptible
SA	Salicylic acid
SABP	Salicylic acid-binding protein
SAMT1	Salicylic acid methyltransferase 1
SAR	Systemic acquired resistance
SPE	Solid phase extraction
SOD	Superoxide dismutase

T

TDF	Transcript-derived fragment
Tris	Trishydroxymethyl aminomethane

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Chapter 1
Introduction

Wheat is the third largest crop produced on a worldwide basis and is a vital component of human nutrition. Over the last decade the use of fertilizers and pesticides has led to a global increase in wheat yields in many countries (Balkovic, Van der Velde, Skalsky, Xiong, Folberth, Khabarov, Smirnov, Mueller & Obersteiner, 2014). Yet, approximately 40-50% of the world's crop is still lost annually to pests and pathogens (US Department of Agriculture, 2015). Most pests are controlled by chemical pesticides because pesticides can kill a significant portion of the pest population quickly, thus preventing economic loss. However, pesticide pollution and residue on products are serious concerns in terms of the health and safety of the consumer, and also because of the effect they may have on the environment (Shi, Jiang & Chen, 2009). The search for more sustainable pest management methods has consequently become a priority in agriculture.

In South Africa, wheat is the second most important cereal crop produced in three distinct wheat producing areas, each with its own challenges (Agricultural Institute, 2017). The Free State province is one of the wheat producing areas. Wheat in this area is planted under dryland conditions in stored soil moisture accumulated during summer and autumn rains. However, a 50% decline in hectares planted with wheat has been observed during production seasons from 2004-2015 in this area (DAFF, 2018). Contributing factors are the prevailing drought and outbreaks of disease, as well as pests that render wheat planting uneconomical and no longer viable in this area.

The Russian wheat aphid (RWA, *Diuraphis noxia,* Kurdjumov) is the most harmful pest found in the wheat crop globally and in South Africa (DAFF, 2018). The occurrence of annual RWA outbreaks has been reported in the eastern Free

State, while other aphids occur only sporadically (DAFF, 2018). Resistance to pesticides is inescapable because of the rapid reproduction rate of aphids (Dogimont, Bendahmane, Chovelon & Boissot, 2010). The most sustainable method of protecting crops from the RWA is the use of resistant cultivars (El Bouhssini, Ogbonnaya, Ketata, Mossaad, Street, Amri, Kesser, Rajarams, Morgounov, Rihawi, Dabus & Smith, 2011) but new resistance-breaking biotypes are placing the industry under severe stress in the battle to constantly incorporate new resistant genes (Jankielsohn, 2013). Therefore, more sustainable methods to manage the RWA need to be explored.

Plants have developed the ability to identify elicitors and induce defence mechanisms by producing specialised morphological structures or secondary products that can be exploited in crop protection. Natural or synthetic compounds can induce systemically acquired resistance that is associated with the expression of priming, a state of defence readiness in plants. These compounds induce responses in plants similar to those triggered by phloem feeding insects or pathogen infection, including the RWA.

Alexin™ is a priming compound and can mediate the induction of defence mechanisms when plants are attacked by a pest or pathogen. Alexin™ is mostly registered on vegetables, fruit and tobacco, but its effect on cereals has not been described. Alexin™ application has shown success on horticultural plants such as tomatoes and potatoes that recovered after hail and frost bite. Celery pre-treated with Alexin™ effectively controlled septoria blight, compared to the other fungicides treatments used in the McDonald (2006) study. Carrots treated with Alexin™, salicylic acid and chitosan before inoculation with necrotrophic fungal pathogens showed reduced disease development (Jayaraj, Rahman, Wan & Punja, 2009).

OBJECTIVES OF THE STUDY

The aim of this study is to focus on the priming effect of Alexin™ and to investigate the potential of the product to mediate induced defence responses

in three resistant or susceptible wheat cultivars when challenged with the RWA. To achieve this, the objectives were:

1. To establish if Alexin™ will successfully prime different wheat cultivars to induce defence mechanisms when challenged with RWA infestation.
2. Determining what Alexin™ concentration mediates the most successful activation of defence responses when challenged with RWA infestation.
3. Identify what category (antibiosis, antixenosis or tolerance) of Alexin™-mediated response is induced during RWA-plant interaction.
4. Determine how defence responses such as accumulation of reactive oxygen species and induction of antioxidant enzymes are affected by Alexin™ treatment in various wheat cultivars.
5. Establish if there is an antagonistic response between hormones such as abscisic acid, jasmonic acid and salicylic acid when plants are treated with Alexin™.
6. Can Alexin™ successfully prime different wheat cultivars to control the RWA populations under field conditions.

Chapter 2
Literature review

2.1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is an essential food source worldwide: It also has a large genetic pool, which allows its cultivation in most regions of the world (Satorre & Slafer, 1999). As a very important cereal crop, it is also produced in South Africa in both summer and winter rainfall regions (Agricultural Institute, 2017). It is however susceptible to various pests including the Russian wheat aphid (RWA, *Diuraphis noxia*, Kurdjumov).

2.2 THE RUSSIAN WHEAT APHID

Distribution and description

On a global scale, aphids are an economically important group of insects with approximately one hundred different aphid species colonising crop plants (Blackman & Eastop, 2006). The RWA, for example, is one of the worst wheat pests, both globally and in South Africa. The RWA originates from the cold winter wheat production areas of eastern Europe and Russia (western Asia) (Annecke & Moran, 1982). Aphids can travel tremendous distances by means of air currents, with prevailing winds distributing them to all wheat-producing regions of the world. Until very recently, Australian wheat crops had not been colonised by the RWA; however, the agriculture department of Western Australia has now alerted farmers in South Australia, Victoria and New South Wales areas that the RWA has been detected in Australia (Government of Western Australia, 2016). Russian wheat aphids were introduced into South Africa in 1978, causing high yield loses in the dryland wheat areas of the Free State Province (Tolmay, Jankielsohn & Sydenham, 2013).

Although the RWA now occurs throughout South Africa, it continually reaches pest status in the dryland wheat areas (Prinsloo & Uys, 2015), while the brown wheat ear aphid (*Sitobion avenae*), oat aphid (*Rhopalosiphum padi*), rose grain aphid (*Metapolophium dirhodum*) and the wheat aphid (*Schizaphis graminum*), only occur sporadically (Agricultural institute, 2017). In South Africa, the rose grain aphid, oat aphid and brown wheat ear aphid occur in the Western Cape, preferring wetter conditions. The wheat aphid (*S. graminum*), causing damage to mature wheat, occurs in the eastern Free State dryland areas especially after the autumn rains (Annecke & Moran, 1982).

The RWA is a small (<2.0mm) insect that is spindle-shaped, pale yellow-green to grey-green in colour, with very short antennae (Prinsloo & Uys, 2015). This aphid is distinguished from other species by two posterior projections, giving the impression of a “double tail” (supracaudal process). These projections consist of the lower-positioned cauda, and a dorsally-positioned false cauda. The abdominal tubes or siphunculi are visible under the microscope (Annecke & Moran, 1982). There are two forms of the aphid in South Africa, namely the winged form called the alate, and the wingless form, known as the aptera. The head and thorax of the apterae are darker. The nymph is a replica of the adult wingless form, although much smaller (Photo 2.1).

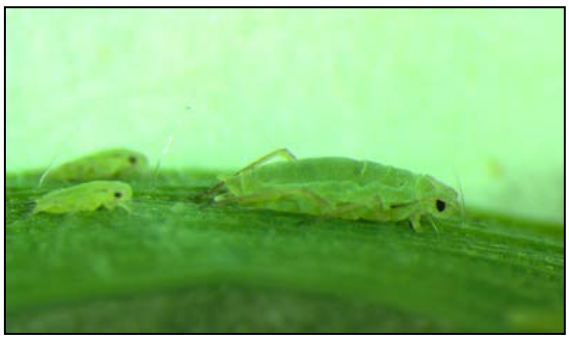

Photo 2.1: Russian wheat aphid, *Diuraphis noxia*. Nymphs and wingless adult female (photo taken by Dr Justin Hatting)

The RWA is a serious pest because females are parthenogenic, implying that the two sexes do not necessarily need to mate to reproduce (Annecke & Moran, 1982; Prinsloo & Uys, 2015). In South Africa the males are absent and females

give birth to live daughters (Annecke & Moran, 1982; Prinsloo & Uys, 2015). A single female can produce up to four nymphs per day that, under optimum conditions and depending on RWA biotype, host plant and environmental conditions (Prinsloo & Uys, 2015), reach adult stage very quickly. This can lead to many generations in a short period of time.

Russian wheat aphid feeding and host damage

The RWA feeds on small grain cereals such as barley, oats, rye, triticale and wheat. The aphid has a specialised piercing-sucking stylet to facilitate extracting sap from the vascular tissue (Prinsloo & Uys, 2015). It feeds mainly on the upper leaf surfaces of new growth, the axils of the leaves or within rolled leaves, secreting saliva into the plant and causing severe damage to plant cells (Prinsloo & Uys, 2015). During severe infestations, aphids cause leaf rolling, chlorosis, streaking and ultimate plant death (Annecke & Moran, 1982). Leaf rolling can cause ear trapping and malformations, which in turn lead to reduced or low quality seed, as well as severely reduced yield (Annecke & Moran, 1982; Prinsloo & Uys, 2015).

Infested resistant plants usually display small necrotic spots that appear on the leaf. Moderately resistant plants typically have white and pale yellow streaks which turn purple during cold conditions. Susceptible wheat plants have severe streaking and leaf rolling. Akhtar, Hussain, Iqbal, Amer and Tariq (2010) found that RWA infestation caused a significant loss in wheat yield. According to Karren (1989), each percentage point in the level of infestation will result in 0.5% yield loss of wheat at harvest. Mornhinweg, Brewer and Porter (2006) found that the effect of RWA feeding on grain yield and yield components varied with RWA resistance, with resistant lines showing increased grain yield or at least an increase in all three yield components.

Russian wheat aphid management

Although the RWA has a short life span, it can develop resistance to pesticides and, because reproduction is asexual, it can colonise plants very quickly, making the management of this aphid very difficult (Dogimont, *et al.*, 2010). The most economical and successful method of crop protection is the use of resistant cultivars (El Bouhssini, *et al.*, 2011). The advantage of resistant cultivars is that the resistance is already inbred and this protects the plant from seedling to adult stage. Aphids may be present on resistant hosts, but their numbers are more controlled throughout the season than is the case in susceptible plants. This reduces yield loss significantly in resistant wheat cultivars (Randolph, Peairs, Kroening, Armstrong, Hammon, Walker & Quick, 2003; Randolph, Peairs, Koch, Walker & Quick, 2005; Tolmay, Lindeque & Prinsloo, 2007).

There are different modes of resistance that can be explored and utilised in breeding plants that are resistant. The modes of resistance may be categorised in three functional groups: antibiosis, antixenosis and tolerance. Antibiosis is a measure of the plant's negative influence on the biology of the insect attempting to use that plant as a host (Norris, Caswell-Chen & Kogan, 2003). Antibiosis effect may reduce the body size and weight of the insect, prolong its development, reduce its fecundity, or induce failure to pupate or emerge. Antixenosis involves affecting pest behaviour through chemical or physical means to deter or reduce colonisation of the host plant (Norris, *et al.*, 2003). The pest will avoid feeding on the plant, or will be repelled by plant emissions and avoid ovipositing on the plant. Tolerance, on the other hand, is the ability of a plant to withstand pest feeding and reproduction damage (Norris, *et al.*, 2003). Various resistance genes can induce specific modes of resistance during RWA-wheat interaction; for example, the resistance gene *Dn1* induces antibiosis, *Dn2* confers tolerance, and *Dn5* combines all three modes of resistance – antibiosis, antixenosis and tolerance (Rafi, Zemetra & Qiusenberry, 1996).

The RWA is difficult to manage with pesticides or biological control strategies because it is protected within rolled leaves. However, in resistant cultivars the absence of leaf rolling exposes the insect to natural enemies and chemical applications, increasing compatibility with other management tactics (Hawley, Pears & Randolph, 2003; Tolmay, *et al.*, 2007). Although resistant wheat has kept aphid numbers at acceptable levels, new resistance genes must constantly be incorporated as pyramids into the wheat-breeding programmes to keep aphid numbers low (Jankielsohn, 2013).

Biological control methods such as fungi, wasps and predators may also be used together with other management strategies (Ennahli, El Bouhssini, Grando, Anathakrishnan, Niide, Starkus, Starkey & Smith, 2009). The aphid is very susceptible to entomophathogenic fungi, leading to fungal epizootics. The fungi, however, usually occur in the winter rainfall and irrigation regions (Prinsloo & Uys, 2015) where moist conditions prevail.

Indigenous South African parasitic wasps of the family Branconidae, as well as exotic species such as *Aphidius matricariae* (Haliday) and *Aphelinus hordei* (Kurdjumov), have been identified as natural enemies of aphids (Prinsloo & Uys, 2015), and Ladybird beetles are commonly found feeding on aphids. In the dryland wheat areas, however, these natural enemies are not successful in keeping aphid numbers low and pest outbreaks do occur.

Russian wheat aphid biotypes

While breeding of resistant cultivars reduces the impact of aphids on wheat, the aphids can counteract this by evolving into virulent biotypes. This evolutionary arms race between insects and plants is speeding up because, as much as artificial plant breeding processes favour the plant, RWA biotypes are also emerging more quickly (Jankielsohn, 2013). The discovery of new virulent RWA biotypes is a significant challenge to the wheat industry in South Africa.

The first RWA-resistant cultivar, Tugela*Dn1*, was released in South Africa in 1992 (Van Niekerk, 2001) and contained resistance gene *Dn1*. A new biotype *RWASA2*, virulent to *Dn1, Dn2, Dn3* and *Dn9,* was identified in 2005 (Jankielsohn, 2011). A third biotype, *RWASA3*, virulent to *Dn1, Dn2, Dn3, Dn4* and *Dn9* was reported in South Africa in 2009, and *RWASA4*, virulent to *Dn1, Dn2, Dn3, Dn4, Dn5* and *Dn9* was recorded in 2011 (Jankielsohn, 2011).

The cultivars used in this study were SST387, Elands and PAN3379. SST387 and PAN3379 are currently on the market and still protected by plant breeders' rights; therefore their pedigrees are unknown. Elands has been available on the market for many years. It is a dryland cultivar containing the *Dn1* gene and induces resistance to *RWASA1* (Hatting, Wraight & Miller, 2004). SST387 has a relatively high yield potential, medium resistance to *RWASA1*, yellow rust, stem rust and drought tolerance (Sensako, 2017). PAN3379 is a high yielding dryland wheat cultivar with resistance to all four known South African RWA biotypes (Pannar, 2017). Because of the continued emergence of new aphid biotypes, research must explore other control methods such as host defence mechanisms that may be inherent in plants.

Plants have evolved various strategies to defend themselves against pests and pathogens and by studying these protection mechanisms, more sustainable strategies to manage pests and pathogens might be devised.

2.3 PLANT IMMUNE SYSTEM

Defence or resistance mechanisms in plants include various components of the plants such as cuticles, needles, thorns, trichomes and waxes; these act as physical barriers to prevent invasion by potential attackers. In addition, plants can produce secondary metabolites as part of basal defence responses inhibiting pathogen growth or rendering the tissue less palatable to herbivores.

Elicitors

Basal defence responses are fast and effective if plants can recognise an invader and its associated elicitor repertoire. Elicitors are molecules produced by the pathogen, pest or the host plant when attacked; they induce physiological or biochemical responses linked to the expression of resistance (Desender, Andrivon & Val, 2007).

Elicitor recognition results in the activation of a series of host defence mechanisms, for instance cell wall reinforcement by deposition of callose and lignin, production of enzymes such as glucanases and peroxidases (Mohase & Van der Westhuizen, 2002a), biosynthesis of phytoalexins and pathogenesis-related (PR) proteins and expression of the hypersensitive response (HR) associated with programmed cell death (PCD) (Desender, *et al.*, 2007).

The structures of elicitors differ, depending on whether they emanate from pests, pathogens, or plant-pathogen/pest interactions. This implies that plant cells have different receptors which bind specific elicitors to trigger activation of defence-related genes in the nucleus (Darvill & Albersheim, 1984).

Induced defence responses in plants

Once the pathogen or pest has breached the outer layers of the plant cells, it should be recognised by receptors on plant cell membranes. Primarily the pattern-recognition receptors (PRR) located on the cell surface perceive the pathogen signatures (pathogen-associated molecular patterns, PAMPs) (Walter, 2011). This recognition can trigger the first level of immunity, known as PAMP-triggered immunity (PTI), which protects the plant from various pathogens. However, certain pathogens can release effectors to evade or suppress this line of resistance, rendering the plant susceptible and pathogens able to successfully invade the plant (Walter, 2011). To counter this invasion, plants have evolved receptor proteins that recognise the pathogen effectors. This leads to the activation of the second – more specific and robust – line of defence, the effector-triggered immunity (ETI) or resistant (R)-gene mediated

resistance (Pieterse & Dicke, 2007). Resistance genes are part of the plant's immunity, mediating various defence-related responses, including recognition of specific effectors, in order to express defence mechanisms. Resistance genes are part of multi-gene clusters and can occur as true alleles across naturally variant backgrounds. Resistance genes encode members of a diverse superfamily of intracellular nucleotide–binding leucine-rich repeat (NLR) receptors, which function intracellularly. Specific NLR proteins are activated by specific pathogen effectors (Dangl, Horvath & Staskawicz, 2013). The plant can therefore recognise a specific pathogen or pest and combat the threat of imminent disease with induced defence responses.

Over decades, the R genes of sexually compatible wild relatives have been identified and bred into cultivated crops, resulting in disease resistance. Effector proteins activate R genes and each pathogen can activate several different effectors; therefore, effectors are dependent on each other and each contributes to the activation of R genes. It is important to know the specific effectors that activate the R genes to control the specific pathogen strain, otherwise the R genes will not be activated (Dangl, *et al*., 2013). Resistant genes are very important in the case of RWA control. Resistant cultivars, functioning on a gene-for-gene basis, have been developed in South Africa. Evolving biotypes can however rapidly overcome the resistance they confer (Ricciardi, Tocho, Tacaliti, Gime, Paglione, Simmonds & Castro, 2010).

There are also overlaps in the components of PTI and ETI. These include cell wall fortification through callose and lignin synthesis, for example, production of secondary metabolites such as phytoalexins, and accumulation of PR proteins (Pieterse, Leon-Reys, Van der Ent & Van Wees, 2009). Phytoalexins are anti-microbial compounds synthesised and accumulated by plant cells after pathogen or pest attack (Walter, 2011). Pathogenesis-related proteins include anti-microbial β-1,3-glucanase and chitinase that degrade fungal walls. Anand, Zhou, Trick, Gill, Bockus and Muthukrishnan (2003) inserted two PR genes (chitinase and β-1,3-glucanase) into four transgenic wheat lines. The genes

were used singly or in different combinations to transform the susceptible wheat. Improved resistance in the glasshouse was accomplished with the expression of thaumatin-like proteins and a specific combination of chitinase and glucanase. No resistance was recorded under field conditions, and more studies are needed to determine why resistance was lost (Anand, *et al.*, 2003).

The recognition of pathogen-specific effectors is very effective, especially in regard to biotrophic pathogens and phloem-feeding insects like the RWA (Belefant-Miller, Porter, Pierce & Mort, 1994), because it leads to a burst of reactive oxygen species (ROS) that triggers a HR associated with PCD at the site of invasion. This keeps the pathogen isolated from the rest of the plant cells and stops further damage (Pieterse, *et al.*, 2009). Nutrients are also not easily accessible for the pathogen or RWA. Induced local resistance is usually transmitted to distant uninfected parts protecting the whole plant against the invader. The transmission of resistance to distal parts is often mediated by various signalling molecules including salicylic acid (SA).

2.4 SALICYLIC ACID

In plants SA is synthesised from a primary metabolite, chorismate, through two pathways: the phenylalanine ammonia-lyase (PAL) pathway via the enzyme PAL in the cytosol, and the chorismate via isochorismate pathway in the chloroplast (Vicente & Plasencia, 2011).

Salicylic acid is an important signalling hormone functioning as an activator of plant defence mechanisms in many different plant species. This very important physiological characteristic is part of the ETI or R-gene mediated resistance (Vlot, Dempsey & Klessig, 2009) leading to the expression of PR genes in localised and surrounding uninfected tissue (Ryals, Neuenschwander, Willits, Molina, Steinern & Hunt, 1996) and encoding proteins with antimicrobial or other defence responses during resistance (Walter, 2011). A very important part of activating plant defence mechanisms is the production and regulation

of ROS and the induction of the HR in gene-for-gene resistance (Ton, Pieterse & Van Loon, 2006).

As the levels of SA and ROS slowly accumulate in infected areas, the threshold to activate cell death is reached. The levels are also high enough to activate antioxidants and suppress cell death ensuring the survival of surrounding cells. The uninfected tissue is placed in a "primed state" with the different signals activating the R-gene-dependent specific defence mechanisms. In the infected cells SA and ROS levels accumulate fast, very quickly reaching the threshold, and cell death occurs (Alvarez, 2000). The manner in which SA is transported is not clear but the physical properties of SA show that it could be transported, metabolised and/or conjugated in plants; as SA is exogenously applied it is transported to other parts of the plant to activate a response (Raskin, 1992). The role of SA in the plant immune system and how it can help protect plants against many invaders is discussed below.

The role of salicylic acid in systemic acquired resistance

The induced resistance established in the area surrounding the infected area is called localised acquired resistance (LAR) (Hammerschmidt, 2009). Synthesis of signalling molecules that activate expression of defence-related genes in distal parts is also activated. In this respect, SA, jasmonic acid (JA) and ethylene (ET) are some of the key signalling molecules. These hormones have multiple roles in plants including mediation of defence responses against pests and pathogens. Their involvement is dependent on the host-pathogen interaction (Walter, 2011).

A total of six signalling pathways have been identified in plants responding to pathogen and pest attack (Walling, 2000). Four of the pathways are associated with responses to pathogen infection (Boughton, Hoover & Felton, 2006), while the SA pathway is also associated with phloem-feeding insects (PFI's) (Zarate, Kempema & Walling, 2007; Mohase & Van der Westhuizen, 2002b). These pathways are the ROS/nitric oxide (NO) pathway, the SA pathway, the JA/ET

sequential pathway and the JA/ET concomitant pathway (Boughton, *et al.*, 2006). The JA-dependent wound pathway and the JA-independent wound pathway are primarily associated with herbivores and are dependent on the feeding habit of the pest (Boughton, *et al.*, 2006). Following accumulation of a signalling hormone, a systemic defence response is usually established in distal plant parts, forming systemic acquired resistance (SAR) that protects undamaged tissues from subsequent pathogen and pest attacks (Hammerschmidt, 2009). Both local and systemic forms of resistance are frequently associated with expression of PR proteins.

The two important forms of resistance mechanisms induced are the induced systemic resistance (ISR) and SAR (Balmer, Pastor, Gamir, Flors & Mauch-Mani, 2015). The ISR, where JA and ET are key mediators, is triggered by beneficial organisms such as non-pathogenic plant growth-promoting rhizobacteria (PGPR) (Pieterse, Van Wees, Van Pelt, Knoester, Laan, Gerrits, Weisbeek & Van Loon, 1998). In contrast to SAR, ISR is not associated with PR gene expression or SA accumulation (Pieterse, Van Wees, Hoffland, Van Pelt & Van Loon, 1996).

For SAR to develop in systemic leaves, a signal generated in the inoculated leaf is transmitted via the phloem to the uninfected parts of the plant. The identity of the long distance signal that is responsible for activation of SAR is not clear (Champigny & Cameron, 2009). Some researchers (Shulaev, Leo & Raskin, 1995; Yalpani, Schulz, Daves & Balke, 1992) argue in favour of SA being the long-distance signal while others argue against it (Smith-Becker, Marois, Huguet, Midland, Sims & Keen, 1998; Vernooij, Friedrich, Morse, Reist, Kolditz-Jawhar, Ward, Uknes, Kessmann & Ryals, 1994). Studies in transgenic tobacco and *Arabidopsis thaliana* showed that the accumulation of SA is required in the distal tissue for expression of SAR (Delaney, Uknes, Vernooij, Friedrich, Weymann, Negrotto & Ryals, 1994; Vernooij, *et al.*, 1994). Although Rasmussen, Hammerschmidt and Zook (1991) showed that SA plays an important role in inducing resistance, the delay in its accumulation excludes it from being the primary systemic signal of induced resistance.

Seemingly, SA is transported in plants mostly as methyl salicylic acid (MeSA) (Vlot, Klessig & Park, 2008; Heil & Ton, 2008). Two enzymes control the balance between SA and MeSA. Salicylic acid-binding protein2 (SABP2) converts biologically inactive MeSA into active SA (Forouhar, Yang, Kumur, Chen, Fridman, Park, Chiang, Acton, Montelione, Pichersky, Klessig & Tong, 2005) and SA methyltransferase1 (SAMT1) catalyses the formation of MeSA from SA (Ross, Nam, D'Auria & Pickersky, 1999). Park, Kaimoyo, Kumar, Mosher and Klessig (2007) demonstrated that MeSA is crucial for long-distance SAR signalling in tobacco, but it still remains uncertain if this is the case in other plant species. Several signalling molecules such as lipid-derived JA (Truman, Bennett, Kubigsteltig, Turnbull & Grant, 2007), azelaic acid (Jung, Tschaplinski, Wang, Glazebrook & Greenberg, 2009) or peptides (Xia, Suzuki, Blount, Guo, Patel, Dixon & Lamb, 2004) and ROS (Alvarez, Pennell, Meijer, Ishikawa, Dixon & Lamb, 1998) have emerged as possible candidates. Nonetheless the most important argument is that SAR signalling is complex and may require a combination of several systemic signalling molecules (Conrath, 2009).

Non-expressor of pathogenesis-related gene 1 (NPR1) is the central regulator of SAR following SA perception, that mediates expression of PR genes (Cao Glazebrook, Clarke, Volko & Dong, 1997; Ryals, *et al.*, 1996). Non-expressor of pathogenesis-related gene 1 is an oligomer localised to the cytosol and its homeostasis is controlled by SA binding to NPR3/NPR4 in a concentration-dependent manner. At low SA concentrations, NPR1 exists in the oligomeric form which cannot induce defence genes. As the concentration of SA increases, SA binds to NPR3 and NPR4, ending its interaction with NPR1 (Moreau, Tian, Klessig, 2012). A change in the redox potential occurs (Mou, Fan & Dong, 2003) and the oligomeric form is reduced, causing the accumulation of NPR1 monomeric form in the cytoplasm (Dong, 2004). Both the monomerisation and oligomerisation of NPR1 involves *s*-nitrosoglutathione mediated *s*-nitrosylation (Feechan, Kwon, Yun, Wang, Pallas & Loake, 2005). The monomeric NPR1 form translocates to the nuclease where it functions as a transcriptional co-activator of defence responses that activate SAR. The change in redox potential allows

monomeric NPR1 to act as a co-factor for TGAs (part of the transcriptional factor family) to activate expression of defence related genes (Spoel & Loake, 2011). The TGA factors bind to As-1 (activation sequence 1), activating the expression of PR genes (Jakoby, Weisshaar, Droge-laser, Vicenta-Carbajosa, Tiedemann & Kroj, 2002).

Salicylic acid and reactive oxygen species (ROS) in the activation of defence responses

Pathogens and PFIs such as the RWA induce a broad spectrum of defence responses in plants. One of the first responses observed after a pathogen attack is an oxidative burst. A membrane-bound NADPH oxidase complex (Lamb & Dixon, 1997) mediates the rapid increase of ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in the apoplast (Belefant-Miller, *et al.*, 1994). The accumulation of ROS leads to the activation of SAR (Torres, 2010). The ROS therefore act as direct signals, inducing local and systemic responses but do not always involve PCD (Alvarez, Pennell, Meijer, Ishikawa, Dixon & Lamb, 1998). Cell death usually occurs in R-gene-dependent resistance but R-gene-resistance can also operate without inducing cell death (Alvarez, 2000). Therefore, cell death lesions might be a consequence of defence activation.

Reactive oxygen species signalling has been associated with hormones such as SA, JA, and ET in the regulation of defence responses towards pathogens and insects (Torres, 2010). Furthermore, H_2O_2 that accumulates in the chloroplasts and peroxisomes triggers SA biosynthesis, and activates certain defence responses such as transcriptional reprogramming, cell death and stomatal closure (Herrera-Vasquez, Salinas & Holuigue, 2015).

In most plants ROS initiate and establish plant defences and the hypersensitive response following successful pathogen recognition (Lamb & Dixon, 1997). Reactive oxygen species are indirectly responsible for the killing of pathogens because they mediate pH changes and ion fluxes that lead to the activation of specific proteases that can cause microbial death (Segal, 2008). Reactive

oxygen species also protect the plant directly by reinforcing the plant cell walls through lignification, driving oxidative cross-linking of the cell walls, while also inducing different cellular processes and regulation of defence genes (Lamb & Dixon, 1997).

The hypersensitive response (HR) is a defence mechanism that occurs after an infection or attack by most pathogens and PFIs (Moloi, 2002). This response kills cells around infected areas and limits the spread of the pathogen or feeding damage by the insect. Such response is activated after an avirulent (avr) gene product from the pathogen recognises and binds to the corresponding R-gene product from the plant (Morel & Dangl, 1997). A threshold of ROS and SA must be met to activate HR, even though transcriptional activation of defence responses can be activated below this threshold and HR is not always needed to induce resistance to a pathogen (Morel & Dangl, 1997).

Cell death is one of the most effective methods of depriving pathogens of nutrients, even lead to the death of the pathogens. Salicylic acid accumulates below the threshold level in the area surrounding the dead cells and plays a role in the anti-death functions to reduce the spread of cell death associated with HR (Alvarez, 2000).

Regulation of the accumulation of reactive oxygen species (ROS) and antioxidants by salicylic acid

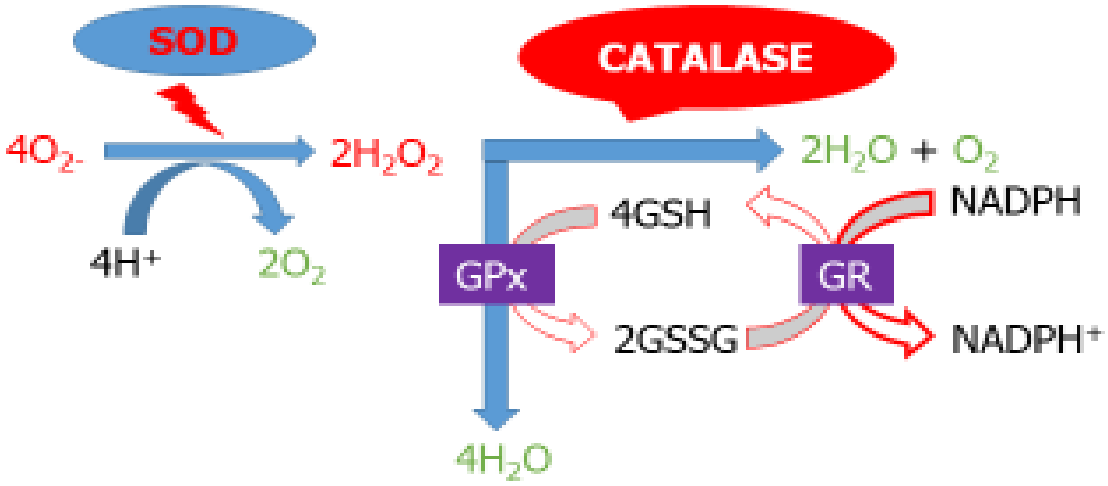

Figure 2.1: Regulation of ROS with antioxidant defence systems (illustration from Finosh & Jayabalan, 2013).

Although production of ROS is a very important defence response that also plays a role in the establishment of SAR, ROS-mediated signalling is controlled by a delicate balance between production and scavenging of ROS (Fig. 2.1). Reactive oxygen species scavenging enzymes, usually called antioxidant enzymes, include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). The level of SOD and APX or CAT activities in cells is crucial for determining the steady-state level of O_2^- and H_2O_2. Antioxidants can act at different stages of a cascade of oxidative stress and reduce ROS damage.

One of the very important ROS scavenging pathways includes SOD which converts O_2^- to H_2O_2 (Halliwell, 2006) in most intracellular and apoplastic compartments. Superoxide dismutase controls the over-accumulation of O_2^- in resistant wheat cultivars challenged with the RWA (Moloi & Van der Westhuizen, 2008). Superoxide dismutase has several isozymes, which can be classified by their location and catalytic metals. Manganese-superoxide dismutase (MnSOD) is confined to the mitochondria and iron-superoxide dismutase (FeSOD) to the chloroplast, while copper-zinc superoxide dismutase

(Cu/ZnSOD) exists in both the chloroplast and cytosol (Allen, 1995). Superoxide dismutase dismutates O_2^- to H_2O_2, then either CAT, APX or GPX can detoxify H_2O_2 to water and oxygen (Apel & Hirt, 2004) as part of the water-water cycle.

Salicylic acid-protein and protein-protein interactions occur downstream of SA signal transductions (Vlot, *et al.*, 2009). There are nine salicylic acid-binding proteins (SABPs) that are receptors for SA, with CAT being the first identified (Chen, Ricicliano & Klessig, 1993). Catalase is the main H_2O_2 scavenging enzyme in peroxisomes that converts H_2O_2 to H_2O and O_2 to control ROS (Hayes & McLellan, 1999; Hayes & Strange, 1995). Salicylic acid binds to CAT and APX, another SABP, inhibiting the activity of the two antioxidant enzymes and reducing the degradation of H_2O_2 (Durner & Klessig, 1995). Wang, Ma, Zang, Xu, Cao and Jiang (2015) showed that exogenously applied SA significantly reduced the activity of CAT and APX, while it enhanced SOD and peroxidase activities in the apricot fruit.

Salicylic acid interacts with glutathione (GSH), influencing pH levels (Foyer & Noctor, 2011); it also increases GSH reducing power, promoting ROS levels. As ROS levels increase, there is a decline in GSH, followed by a reductive phase associated with increasing GSH levels. As a result of this interplay between redox levels, regulated processes and defence responses are activated (Herrera-Vasquez, Salinas & Holuigue, 2015).

Glutathione peroxidase uses glutathione to reduce H_2O_2, while NAD(P)H regenerates reduced glutathione, a reaction catalysed by glutathione reductase (GR) (Hayat & Ahmad, 2007). The cytosol, with its ascorbate-glutathione cycle, and CAT in the peroxisomes may act as a buffer zone to control the overall level of ROS that reaches different cellular compartments during stress and also under normal metabolism conditions (Mitler, 2002).

Salicylic acid might also counteract oxidative damage leading to cell death (Alvarez, 2000). Pre-treatment of barley plants with SA increased the

antioxidant response by enhancing the activity of SOD, dehydroascorbate reductase (DHAR) and guaiacol peroxidase (POX) by 20%, 60% and 50% respectively, while the levels of APX and GR remained similar to those of the control (Ananieva, Christov & Popova, 2004). Treatment of maize with SA and low-temperature stress induced increased POX and GR levels, with no change in APX and SOD levels, and a decrease in CAT activity (Janda, Szalai, Tari & Paldi, 1999).

Exogenous SA application on Kentucky bluegrass increased SOD and CAT activities (He, Liu, Cao, Huai, Xu & Huang, 2005). In another study, heat-stressed SA-deficient transgenic plants were more prone to oxidative damage than were the non-transformed plants (Gaffney, Friendrich, Vernooij, Negrotto, Nye, Uknes, Ward, Kessmann & Ryals, 1993). Therefore, SA plays a signalling role in SAR that creates a feed-forward loop between H_2O_2 and SA synthesis and also as a source of ROS and a regulator of ROS scavenging.

Effect of various hormones on host defence responses

Plants respond differently to the feeding habits of insects. Chewing insects for instance induce the JA/Et pathway, while PFIs like the RWA induce the same defence responses as pathogens, including activating the SA-mediated pathway. These responses can also differ within insect species that have the same feeding habits. An experiment by Heidel and Baldwin (2004) tested the different signalling responses by different insect feeding guilds. They established that herbivorous caterpillars elevated JA levels and expression of JA-mediated genes, while other chewing-feeding insects caused an opposite response that resembled SA-mediated responses. The various responses could be a result of different inducing signals produced by different herbivores or mechanical damage. The aphid, a phloem-feeder, caused neither a JA-mediated response – as in the case of the caterpillars – nor an SA-mediated response as with the chewing-feeding insects (Heidel & Baldwin, 2004).

Nevertheless, some studies have shown that PFIs such as the silver leaf, whitefly and RWA induce SA-dependent responses (Zarate, *et al*., 2007; Mohase & Van der Westhuizen, 2002b). Li, Xie, Smith-Becker, Navarre and Kaloshian (2006) reported the importance of defence against aphids in tomato plant which had a neutral or negative effect in the reproduction of potato aphid (*Macrosiphum euphorbiae*) and whiteflies (Zarate, *et al*., 2007). The induction of SA-mediated defences increases SA levels within the phloem (Smith-Becker, et al. 1998). Aphids feed on phloem and thereby come into direct contact with SA. On the other hand, Donovan, Nabity and De Lucia (2013) observed a direct effect of SA on tobacco-adapted green peach aphids' (*Myzus persicae*) fecundity during artificial diet tests. Testing different concentrations of SA in artificial diet for aphids, Donovan, *et al*. (2013) reported decreased survival of the aphids, suggesting that SA itself may directly inhibit aphid growth.

When soybean plants were treated with β-aminobutyric acid (BABA) and challenged with soybean aphid (*Aphis glycines* Matsumura), the enzymatic activities, SA-signalling gene expression as well as ROS scavengers were primed with enhanced resistance against the aphid (Balmer, *et al*., 2015). Therefore, aphid stress increases SA signalling which is associated with increased SAR and PR gene expression (Vlot, *et al*., 2009).

Defining specific mechanisms of defence against aphids has been difficult because there is evidence of cross-talk between JA and SA, which leads to antagonist down-regulation of defence responses (Zarate, *et al*., 2007). During aphid feeding, JA levels are reduced as an effect of cross-talk between JA and SA, which down-regulates JA in response to increased SA production (Zarate, *et al*., 2007). In other studies, specific aphid-plant interactions induced both JA and SA-mediated defence responses (Heidel & Baldwin, 2004; Mohase & Van der Westhuizen, 2002b).

Transcriptomic and physiological evidence has revealed a variety of responses when cross-talk occurs, including negative outcomes in cases of antagonistic

interactions between two signalling pathways. This might occur between SA and JA signalling systems. Zarate, *et al*. (2007) showed that the activation of SA-mediated defences in mutants was associated with impaired JA defences and increased susceptibility to herbivorous insects and as expected SA levels were reduced when JA defences were activated, making plants more susceptible to pathogens.

De Vos, Van Zaanen, Koornneef, Korzelius, Dicke, Van Loon and Pieterse (2006) performed experiments in an attempt to understand the dynamics of SA-, JA- and ET-signalling in *Arabidopsis* after plants were stressed by pathogens and herbivorous insects with different modes of attack. The chewing caterpillar induced significant levels of SA-, ET- and JA-responsive genes. Although higher JA and ET levels were induced by the caterpillar feeding, the SA levels remained unaltered. In tests where herbivorous caterpillars were used on *Nicotiana attenuata,* JA levels were elevated during a 3-day attack. Interestingly, the SA concentrations also increased, although the transcriptional response showed that it was a JA-elicited response (Heidel & Baldwin, 2004). Therefore, eliciting SA-dependent defences does not always lead to suppression of SA- or JA-dependent defences (Ajlan & Potter, 1992) and can lead to increases or no change in JA and SA levels (Mohase & Van der Westhuizen, 2002b).

Abscisic acid (ABA) can induce defence mechanisms in some plant-pathogen interactions, while increasing susceptibility in others. Abscisic acid is an early defence response to halt pathogens by means of the activation of stomatal closure and callose deposits preventing the activation of SA and JA-dependent defences (Ton, Flors & Mauch-Mani, 2009). This could however lead to the suppression of PAL and SA as found by Ward, Cahill and Bhattacharyya (1989) in soybeans. The cross-talk between the signalling hormones SA, JA and ABA is complex, showing either synergistic or antagonistic effects, depending on the attacking organism. Such cross-talk may fine-tune the induced defence response, but further elaboration is still needed.

2.5 PRIMING

Chemicals can induce SAR that is associated with the expression of priming, a state of defence readiness in plants. Salicylic acid accumulates in the surrounding uninfected and distal plant parts, placing the plant in a “primed state”.

Salicylic acid has been demonstrated as one of the first compounds to induce resistance (White, 1979) even before it was described as an endogenous signal in SAR. Some examples of inorganic and organic compounds that prime plants include synthetic SA analogs: 2,6-Dichloroisonicotinic acid and its methyl ester (both are referred to as INA), and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH). Salicylic acid, INA, and BTH are all assumed to activate SAR through the same signalling pathway (Ryals, *et al*., 1996). The plant develops a “priming memory” and can quickly recognise the intruder or stress factor to rapidly induce the plant defence mechanisms to protect the plant. The primed state is also durable and can be maintained long after the encounter with the stressful event. The compounds that are able to prime plants and enhance resistance may be natural or synthetic, and are often called elicitors. They induce responses in plants similar to those triggered by herbivore feeding or pathogen infection. These compounds can, with moderate doses, directly induce and activate certain defence mechanisms and also prime cells to induce other defence genes when challenged (Goellner & Conrath, 2008).

When a plant is primed, the potential to induce defence responses is available but the defence cascade is not immediately activated. Only after the plant is exposed to the stress are the specific pathways triggered to activate the defence responses (Conrath, Pieterse & Mauch-Mani, 2002). The pathways thus induced are specific to the encountered challenges. This adaptability in priming, however, makes it difficult to trace the specific mechanism because an unrelated event may cause the same responses (Balmer, *et al*., 2015).

Plant priming can be separated into different stages (Fig. 2.2). The first stage of priming starts with the first exposure to the elicitor. During this stage levels of primary and secondary metabolites, enzymes and hormones are altered to place the plant in a state of readiness. Salicylic acid regulates the primed state during infection by activating expression of mitogen-activated protein kinases (MAPKs), production of ROS and increasing callose deposits (Balmer, *et al.*, 2015). The post-challenge primed stage is turned on when the plant is subsequently challenged by stress, ending the priming phase. The signals are expressed to fully activate defence responses to attack or counter the stress. This second stage quickly activates synthesis of phytoalexins, phenolics, callose, PRs, SA and JA (Balmer, *et al.*, 2015). The transgenerational primed state is a form of inherited resistance expressed in subsequent plant generations of primed parents (Balmer, *et al.*, 2015).

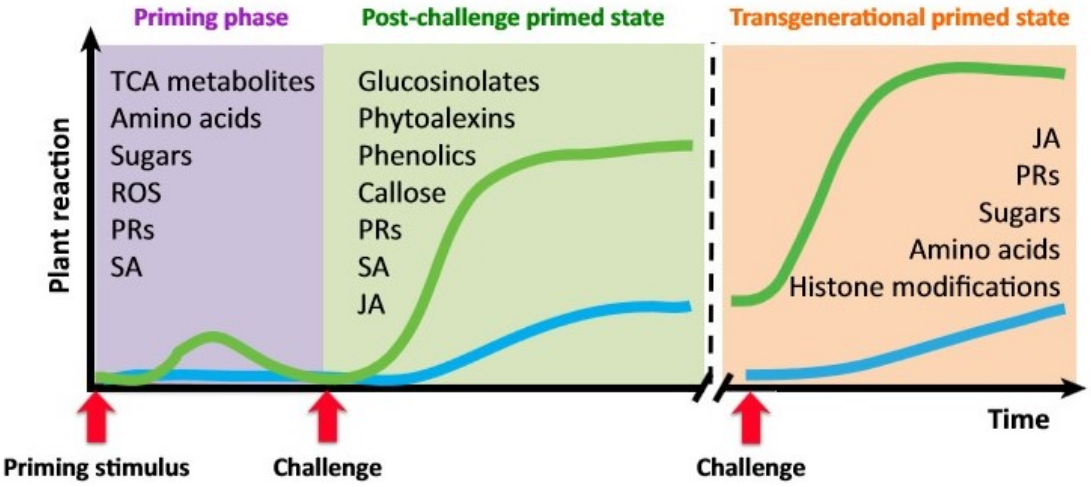

Figure 2.2: The various stages in priming (illustration from Balmer, *et al.*, 2015)

Despite the fact that priming can protect plants from a broad spectrum of pathogens and pests and can be maintained long after the initial stimulus, farmers favour fungicides because the pathogen is controlled immediately. Furthermore, priming is pro-active and must be sprayed before infestation, so, from the point of view of the farmer, if there are no pathogen/pest infestations in that particular season, money could have been spent unnecessarily.

Pathogens and pests can quickly develop resistance to pesticides, however, which can lead to the use of increased levels of chemicals to maintain production. The demand for safer and more sustainable methods is an urgent matter because of the environmental and health concerns associated with application of potentially toxic chemicals to arable land (Rapicavoli, 2015). In intensive production of arable crops there is a wide range of reliable and effective chemical and systemic products with which to protect plants.

Elicitors priming plants to induce defence responses have the potential for use in managing common pests and diseases and are especially important as part of an integrated approach to pest management. There are also commercial products used successfully as priming agents to manage pests and pathogens; for instance, Oryzemate®/probenazole (3-allyloxy-1,2-benzisothiazole-1,1-oxide) has been successfully used for 20 years in Asian rice production, especially against blast pathogen (*Magnaporthe grisea*) and remains one of the most important products for the protection of rice in Japan (Iwata, Umemura, & Midoh, 2004).

BION® and Actigard® are products containing ASM (acibenzolar-*S*-methyl) that are registered for a range of crops including bananas, lettuce, pears, tobacco, tomatoes and other leafy vegetables, cucurbits, and nuts against fungi (Leadbeater & Staub, 2007). In field studies, decreased disease severity brought about by ASM treatment was associated with a reduction in the number of race-change mutants and a suppression of disease caused by such mutants, which suggests that induced resistance agents may be useful for increasing the durability of genotype-specific resistance given by major R genes (Romero & Ritchie, 2004).

It should be borne in mind that the consumer demands conventional methods in crop production that minimise negative effects on the environment and also minimise pesticide residue on food. Therefore, the potential of priming agents in commercial farming still needs further studies to evaluate the effectiveness

of priming compounds against pests and pathogens, and their impact on plant growth.

Factors influencing the efficacy of salicylic acid priming

Plants possess an array of defence mechanisms to protect themselves from pests; however, plant defence is a costly business requiring energy and resources that could be used for growth and development. According to the 'growth-differentiation balance' hypothesis (Herms & Mattson, 1992) a metabolic competition for resources exists between plant growth and defence mechanisms. This hypothesis has also been studied in relation to SA synthesis and yield reductions (Cipollini, 2002), and the occurrence of SA-dependent responses associated with reduction in yield (Donovan, *et al.*, 2013). Induced resistance involves intense level of expression of defences causing the diversion of resources usually allocated for plant growth.

Most studies on the costs and benefits of induced resistance have focused on defences activated directly by the inducing agents (Walters & Fountaine, 2009), because induced resistance involves the intense expression of biochemical plant defences cost is an important factor to establish, as it could divert resources away from growth and yield. The possibility of a negative effect of induced resistance on yield and the variability in efficacy, represents a major obstacle to the implementation of induced resistance in agriculture (Walters & Heil, 2007). However, work by Van Hulten, Pelser, Van Loon, Pieterse and Tons (2006) has demonstrated benefits of priming in *Arabidopsis* and has shown that priming involves fewer costs than direct induction of defences and is beneficial in terms of the plant growth rate and fitness under disease pressure. In the case of priming, the plant is placed in a state of readiness and minimal resources are allocated for resistance expression when challenged with a compound, therefore, the plant does not incur additional costs unless it is challenged by pests or pathogens. The defence mechanisms are not turned on indefinitely, and therefore fewer resources are diverted from important growth processes until the plant is attacked and the need to induce full expression of

defence mechanisms exists. Therefore, plants must only be primed with low levels of eliciting compounds that do not activate the direct expression of defences causing negative effect on growth and fitness.

Induced resistance is a host response during pest, pathogen or abiotic stress. The expression of this response is affected by a range of factors, including host genotype, the environment and the extent to which plants have been induced in the field (Walters & Fountaine, 2009). This can be a risky form of defence in comparison with constitutive defence mechanisms. The defence response may be more specific, responding only to certain invaders or forms of abiotic stress (Heidel & Baldwin, 2004; Campbell, Fitzgerald & Ronald, 2002). For instance ASM (or BTH), a compound mimicking SA, enhances resistance to pathogens *Erysiphe graminus* and *Puccinia recondita*, by expressing only a few of the PR genes and it does not confer resistance to wheat head blight. This probably indicates that in wheat biotic stress chemical elicitors induce the expression of different gene sets suggesting that multiple defence pathways are followed (Campbell, *et al*., 2002).

Induced resistance can also lead to trade-offs causing negative cross-talk with other defence responses (Walters & Fountaine, 2009). According to Zarate, *et al*. (2007) the increase in SA levels can impair JA defences, making the host more susceptible to herbivorous insects, and the elevation of JA defences can suppress SA levels, exposing the plant to pathogens; therefore, the compound used might control a pest but cause a pathogen to infest the plant.

The expression of induced resistance can be influenced by the host genotype. Steiner, Oerke and Schonbeck (1988) determined more than thirty years ago that powdery mildew was controlled by *Bacillus subtilis,* but the level of control amongst the cultivars was different. This was again demonstrated in different lines of barley carrying different race-specific resistance genes to *B. graminis* F.sp. *hordei* (Martenelli, Brown & Wolfe, 1993). Hijwegen and Verhaar (1994) treated resistant cucumber with INA and showed increased resistance to the

powdery mildew fungus *Spaerotheca fuliginea*. The results were cultivar-dependent, with the highest levels of induced resistance in partially resistant cultivars and much lower levels of resistance in susceptible cultivars. On the other hand, Dann, Diers, Byrum and Hammerschmidt (1998) showed that induced resistance is not related to major resistance genes. They treated soybeans with INA and ASM where induced resistance to *Sclerotinia sclerotiorum* was greatest in susceptible cultivars.

Herman, Restrepo and Smart (2007) and Pasquer, Isidore, Zam and Keller (2005) studied gene expression under field conditions and also established that spring barley already expressed defence-related enzymes in untreated plants under field conditions. Heil and Ploss (2006) found that in wild plants there were already high levels of defence activity and following treatment with ASM, some of the species were capable of inducing higher levels of defence. These studies show that the environment influences plants and can influence induced resistance positively or negatively; therefore, field studies are needed to determine the factors that are important in influencing priming.

2.6 ALEXIN™

The product Alexin™ is a commercial product available for inducing resistance in plants. It is a liquid organic nutrient complex containing SA derivatives to boost the immune response of plants. As discussed, SA is an important defence-signalling hormone that induces accumulation of ROS as a first line of defence and mediates SAR. It is also a priming elicitor that can help a plant respond faster and more effectively against biotic and abiotic stress. Alexin™ also contains oligosaccharides, and nutrient elements such as calcium (Ca), magnesium (Mg), boron (B) and potassium (K).

Alexin™ application has shown success on horticultural plants and is mostly registered for vegetables, fruit and tobacco; its effect on cereals has not yet been described. Nulandis (a division of AECI limited) has done in-house trials

on the application of Alexin™ on tomatoes and potatoes, which showed remarkable protection and recovery from hail and frost bite. McDonald (2006) tested Alexin™ on the control of *septoria* blight of celery against calcium chloride and certain fungicides (Bravo 500™, Champ 2™, Quadris Cabrio™ and BAS 516) and found that Alexin™ was as effective as the fungicides in reducing pathogen impact. Treatments of carrots in the greenhouse with Alexin™, SA and Chitosan before inoculation with necrotrophic fungal pathogens showed reduced disease development over a 10-day period (Jayaraj, *et al.*, 2009). Chitosan was the most successful, followed by Alexin™ and SA. Alexin™ with its SA derivatives induced responses similar to that of SA, reducing the fungal colonisation. Hendricks, Hoffman and Lotze (2015) also found that Alexin™ reduced *Xanthomonas* infection and even though the efficiency varied between organs and seasons, significantly increased fruit size was induced.

Oligosaccharides present in Alexin™ are associated with plant defence responses that occur during plant-pathogen interactions (Larskaya & Gorshkova, 2015). Microorganisms induce hydrolytic reactions that release cell wall polysaccharide fragments, which serve as elicitors that trigger phytoalexin formation. One of the first reactions to oligosaccharides is the changing of the ionic flow and the flow of Ca^{2+} into the cell. Oligosaccharides also induce an oxidative burst (Larskaya & Gorshkova, 2015).

The macro nutrients in Alexin™ influence plant growth, yield and the success of activated defence mechanisms. Potassium is an important macro nutrient that triggers the activation of biochemical enzymes for the generation of Adenosine Triphosphate (ATP). The element is required for early growth, and plays a role in cellular osmoregulation (Wang, Zeng, Shen & Guo, 2013). The influence of K on soybean aphid (*Aphis glycines* Matsumura) population dynamics was examined in small plots or fields. Small plots with low K levels had high densities of aphid populations and improved aphid performance (Myers & Gratton, 2006). The results of the study provided evidence that K plays an important role in influencing soybean-aphid population dynamics.

Thus, proper potassium fertilisation may serve to benefit growth in soybean yields as well as reduce the probability of aphid outbreaks (Myers & Gratton, 2006). The role of potassium in crop resistance to disease was extensively reviewed by Perrenoud (1990), who showed that the incidence and rate of disease development may be reduced by adequate and balanced mineral nutrition in many crops and found that K fertility has been effective in reducing crop injury from diseases.

Alexin™ contains the essential macro nutrient Ca, which plays a role in cells by strengthening the extracellular matrix of the cell wall (Wu, Liu, Wang, Zhang & Xu, 2012). The movement of Ca ions is through either the symplastic or the apoplastic pathway (White, 2001). With Ca ions being a secondary messenger, the symplastic pathway dominates. The Ca ion fluxes are controlled and selective; essential for cell signalling (Wu, *et al*., 2012).). Changes in ion fluxes occur early in elicitor signal transduction, a rapid and temporary event (Conrath, Jeblick & Kauss, 1991). Vincent, Avramova, Canham, Higgins, Bilkey, Mugford, Pitino, Toyota, Gilroy, Miller, Hogenhout and Sanders (2017) observed a rise in Ca^{2+} around the feeding site of an *Arabidopsis* plant and linked this increase to plant resistance signalling during plant-aphid interaction. Salicylic acid-induced defence responses in plants require the presence of extracellular Ca^{2+}. Changes in transmembrane ion fluxes accompanying SA, pathogen and elicitor action are required for ROS generation (Hayat & Ahmad, 2007). It is especially important for the activation of plasma membrane NADPH oxidase. Calcium was required for the accumulation of SA leading to high levels of chitinase in tobacco leaves (Raz & Fluhr, 1992) and in the carrot suspension culture (Schneider-Müller, Kurosaki & Nishi, 1994). Calcium increases ammonium, potassium and phosphorous absorption and stimulates photosynthesis. Tests also showed that rice weight increased by 14% when extra Ca was applied at seed fill (Feagley & Fenn, 1998). There is evidence that Ca has no effect on insect performance (Hasemann, 1946; Salama, El-Sherif & Megahed, 1985) and according to Myers and Gratton (2006) it is unlikely that

Ca influences aphid population. Only Barker and Tauber (1951) showed that severe Ca deficiency can improve aphid performance.

Magnesium is an important macro nutrient involved in many enzyme activities. Magnesium is the central atom in chlorophyll and therefore very important for photosynthesis and can influence carbohydrate transport which, when not available at the right time, can limit yield. Under aluminium stress, Mg played a role in the structural stabilisation of tissues and homeostasis of ROS (Bose, Babourina, Shabala & Rengel, 2013).

Boron is a micro nutrient required for normal plant growth. The most important function of B is the role it plays in cell strengthening and development; it also stimulates and inhibits certain metabolic pathways. Boron is essential in cell division and the development of seed and fruit (Ahmad, Niaz, Kanwal, Rahmattullah & Rasheed, 2009). The involvement of B in ion fluxes such as Ca^{2+} and stimulation of NADPH oxidase in the cells of carrots has been reported by Barr, Bottger and Crane (1993). Interrelated functions that involve B also include nitrogen, phosphorous, potassium and calcium (Ahmad, *et al.*, 2009). According to field trials done with foliar B applications the final number of branches and pods on branches increased in soybeans (Schon & Blevins, 1990) and in four of the ten wheat cultivars treated with B foliar application significant yield increases were noticed (Korzeniowska, 2008).

2.7 Conclusion

Alexin™ has two very important qualities: firstly the SA derivatives prime the plant against pest and pathogens; and secondly the important nutrients contribute to plant defence mechanisms that keep the plant healthy. An approach where improved plant health and required systemic resistance can be combined with genetic resistance could provide the South African wheat industry with variable options for the safe and sustainable control of the RWA.

Chapter 3

Optimising Alexin™ concentration for induction of defence responses against Russian wheat aphid

3.1 INTRODUCTION

The Russian wheat aphid (RWA, *Diuraphis noxia,* Kurdjumov) is, on a global scale, a serious pest of wheat. In South Africa, regular RWA aphid outbreaks occur in the eastern Free State dryland wheat producing areas. The most successful aphid management strategy used in South Africa is cultivation of resistant wheat cultivars. These cultivars keep aphid populations low and reduce induced chlorophyll loss (Smith, Liu, Wang, Liu, Chen, Starkey & Bai, 2010). However, the existence of virulent aphid biotypes requires incorporation of new resistance genes into wheat, and priming might well be another effective defence mechanism that could be explored.

Priming enhances host defence mechanisms in relation to subsequent pest and pathogen attack. After an infestation, colonisation by beneficial microbes or treatment with various chemicals, a plant can acquire a primed condition – in other words, a condition in terms of which it recalls the previous attack or treatment. A primed plant can subsequently respond rapidly and effectively when exposed to biotic or abiotic stress (Goellner & Conrath, 2008).

Priming is a component of induced resistance and forms part of the systemic acquired resistance (SAR). Salicylic acid is one of the signalling molecules that is associated with SAR and the hypersensitive response (HR) (Ryals, *et al*., 1996). Mur, Naylor, Warner, Sugars, White and Draper (1996) were among the first researchers to introduce the idea of priming. They found that pre-application of SA in transgenic tobacco plants did not activate a strong

expression of defence genes, unless there was a subsequent pathogen infection.

Defence mechanisms such as expression of the HR that involve elevation of β-1,3-glucanase and peroxidase activities, and accumulation of phenolics, are associated with pathogen infection. The HR is also associated with resistance to infestation by phloem-feeding insects (PFIs) such as the RWA (Van der Westhuizen & Pretorius, 1996). Mohase and Van der Westhuizen (2002a) showed that elicitors released during RWA infestation induced high levels of β-1,3-glucanase and that inter-cellularly applied SA stimulated peroxidase activity (Mohase & Van der Westhuizen, 2002b).

β-1,3-glucanases occur as abundant proteins in seed plants; they display diverse physiological and developmental processes including cell division and seed germination (Cheong, Kim, Chun, Moon, Park, Kim, Lee, Han, Lee & Cho, 2000). This activity has been detected in cereal plants such as barley (Woodward & Fincher, 1982), maize (Jondle, Coors & Duke, 1989), oats (Yun, Martin, Gengenbach, Rines & Somers, 1993) and wheat (Jondle, *et al.*, 1989). These enzymes are also part of the defence mechanism induced by the RWA, SA and other chemical inducers (Ward, Payne, Moyer, Williams, Dincher, Sharkey, Beck, Taylor, Goy, Meins & Ryals, 1991; Malamy, Hennig & Klessig, 1992; Van der Westhuizen, Qian & Botha, 1998a). The substrate of β-1,3-glucanases, the β-1,3-glucan, is one of the major components of the cell walls of many fungi and this enzyme forms part of the defence mechanism as an antifungal protein that degrades fungal cell walls, thus debilitating proliferation of many potentially pathogenic fungi (Mauch & Staehelin, 1989; Cote, Cutt, Asselin & Klessig, 1991; Delp & Palva, 1999). Although it is doubtful that they have a direct effect on the RWA, these enzymes may play a role in the activation of defence genes (Van der Westhuizen, *et al.*, 1998a) and the release of glucan fragments which can act as elicitors (Boller, 1995).

Peroxidases are usually induced in plants where local lesions and chlorosis appear (Bates & Chant, 1970). These symptoms are also present during aphid feeding. Peroxidases are involved in defence-related events that occur in the extracellular matrix (Bowles, 1990). These include the strengthening of cell walls through lignification and formation of intermolecular crosslinks (Bowles, 1990). Van der Westhuizen, Qian & Botha (1998b) revealed that RWA infestation also induces cell wall thickening, a defence reaction associated with enhanced oxidative state, indicating that peroxidases are involved in the defence mechanism against RWA infestation in resistant cultivars.

Various experiments (Mohase & Van der Westhuizen, 2002b; Van der Westhuizen, *et al.*, 1998a & 1998b) have shown that peroxidases and β-1,3-glucanases can act as indicators of defence responses when plants are treated with activators such as SA prior to RWA infestation. The objective of this chapter was to identify the optimum Alexin™ concentration required to prime defence responses in various wheat cultivars during subsequent RWA infestation. The induced defences were measured as changes in β-1,3-glucanase and peroxidase activities.

3.2 MATERIALS AND METHODS

Plant material

Three South African dryland wheat cultivars, SST387, Elands and PAN3379, were chosen based on their market availability, yield potential and response (susceptible, resistant) to RWA infestation. SST387 and Elands are resistant to *RWASA1* but susceptible to the three other South African RWA biotypes (*RWASA2, RWASA3*, *RWASA4*), while PAN3379 is resistant to all four current South African RWA biotypes. SST387 is also drought tolerant. The seeds were obtained from the Agricultural Research Council – Small Grains (ARC-SG), South Africa.

Wheat plants were grown under greenhouse conditions at temperatures of 22 °C ± 4 °C. The plants were cultivated in trays and placed under cages covered with nets (315 micron) to enclose the aphids. Culture conditions and infestation procedures were as described by Du Toit (1988). For this study two of the four existing South African Russian wheat aphid biotypes, *RWASA1* and *RWASA2,* were used. In this experiment, aphids obtained from the ARC-SG were used to develop colonies. These colonies were maintained in glasshouses at the University of the Free State at a temperature of 24 °C ± 2 °C on the susceptible wheat cultivar, Tugela.

Treatments and infestation

Alexin™ (Reg no. B3835, Act no. 36 of 1947) is a registered trademark of AECI Limited (Nulandis® a division of AECI Limited). It is a liquid organic nutrient complex (containing salicylic acid derivatives, oligosaccharides, 4.5% potassium, 2.6% calcium, 0.8% magnesium and 0.2% boron). LI 700®, a product of Nulandis®, is an acidifier that increases the efficiency of pH sensitive agricultural remedies and improves the penetration properties of spray mixtures.

Two separate experiments were performed. In the first experiment plants were sprayed with different concentrations of Alexin™ before infestation with *RWASA1.* In the second experiment the Alexin™ concentration that yielded the most significant results was used to pre-treat plants which were subsequently infested with *RWASA2* to determine whether there is RWA biotype specificity when wheat is primed.

Treatment of plants with different Alexin™ concentrations

The optimal application rate of Alexin™ to protect wheat plants against different forms of biotic stress has not been established. The prescribed application rate of Alexin™ for tobacco is 0.5% [v/v (4L/ha)]. Alexin™ solutions containing LI 700® (0.06% v/v, adhesive) were prepared in sterile water. Different

Alexin™ concentrations [0.5%, 0.375% and 0.25% (v/v)] were used to establish the optimal concentration required for protection of wheat seedlings against RWA infestation. Alexin™ was applied as a foliar spray (fine mist) to seedlings at the beginning of the three-leaf stage.

Two days later aphids (10 apterous adults /plant) were placed on the plants. Alexin™ treated or untreated controls were not infested (Table 3.1). Each treatment consisted of 30 plants in three independent replicates.

Table 3.1: Treatments in glasshouse trial: Effect of RWA infestation on β-1,3-glucanase and peroxidase

Alexin (%v/v)	0	0.25	0.375	0.5
Uninfested RWASA1 RWASA2	Treatment 1 Treatment 5 Treatment 9	Treatment 2 Treatment 6	Treatment 3 Treatment 7 Treatment 10	Treatment 4 Treatment 8

Collection of the intercellular washing fluid (IWF)

The second and third leaves of 5 randomly selected plants at the three leaf stage were sampled at 0 and 48 h post infestation (hpi) and the IWF was extracted according to the method of Van der Westhuizen *et al*. (1998a). The leaves were cut into 7cm-long pieces and thoroughly rinsed in distilled water before 5 min vacuum-infiltration in extraction buffer (50 mM Tris-HCL, pH 7.8). The leaf pieces were then blotted dry with paper towel and placed in centrifuge tubes fitted with a perforated disc at the bottom. Leaves were centrifuged (*500 x g*) for 10 minutes at 4 °C. Subsequently, the IWF was collected at the bottom of the centrifuge tube, and the procedure was repeated with the same leaves. The combined IWF was frozen and stored at –20 °C until the assays for enzyme activities were performed.

Enzyme activity

Peroxidase and β-1,3-glucanase activities were measured as indicators of induced defence-related responses in Alexin™ treated and RWA infested plants.

Intercellular Peroxidase activity

A modified method of Zieslin and Ben-Zaken (1991) was used. The assay solution contained 840 µl potassium phosphate buffer (40 mM, pH 5.5), 100 µl guaiacol (5 mM), 50 µl H_2O_2 (8.2 mM) and 10 µl IWF (enzyme extract). The formation of tetraguaiacol was monitored at 470 nm using a Cary 100 UV-Visible spectrophotometer for 3 min at 30 °C. Peroxidase activity was expressed as mg tetraguaiacol mg^{-1} protein min^{-1}.

Intercellular β-1,3-glucanase activity

A modified method of Fink, Liefland and Mendgen (1990) was used. The assay solution contained 240 µl sodium acetate buffer (50 mM, pH 4.5), 250 µl laminarin (2 mg ml^{-1}) and 10 µl IWF (enzyme extract). The solution was incubated for 10 min at 37 °C, then 500 µl Somogyi reagent (Somogyi, 1952) was added. The mixture was heated at 100 °C for 10 min and then cooled. Arseno molybdate colour reagent (500 µl) of Nelson (1944) was added. The insoluble cuprous ions that formed as a result of the presence of glucose were completely dissolved to render a blue solution. The absorbance was read at 540 nm using a Cary 100 UV-visible spectrophotometer. A standard curve relating A_{540} to glucose concentration was used to calculate β-1,3-glucanase activity, which was expressed as mg glucose mg^{-1} protein min^{-1}.

Protein concentration

The protein concentration was determined according to the method of Bradford (1976) using Bio-Rad protein reagents and bovine [γ]-globulin as a standard. The absorbance of the coloured product was measured at 595 nm using a micro plate reader (Anthos Zenyth 3100).

Statistical analysis

The activities of peroxidase and β-1,3-glucanase were measured in triplicate within each of the three biological replicates. Outliers were assessed by boxplots and normality assessed by Normal Q-Q plots. Homogeneity of variances was assessed by Levene's test. Data were analysed using one-way analysis of variance (Anova, $p<0.05$, IBM SPSS statistics version 24) to determine enzyme activity responses between 0 hpi and 48 hpi. A two-way Anova was conducted if responses differed.

3.3 RESULTS

Effect of Alexin™ priming on *RWASA1*-induced enzyme activities

In SST387 there was no significant difference ($p=0.069$) in β-1,3-glucanse activity (Fig. 3.1 A) between untreated infested and Alexin™ pre-treated and infested plants at 48 hpi. The concentration of 0.5% (v/v) Alexin™ applied prior to infestation induced relatively higher β-1,3-glucanase activity at 48 hpi, which was nonetheless not significantly higher than in untreated infested plants.

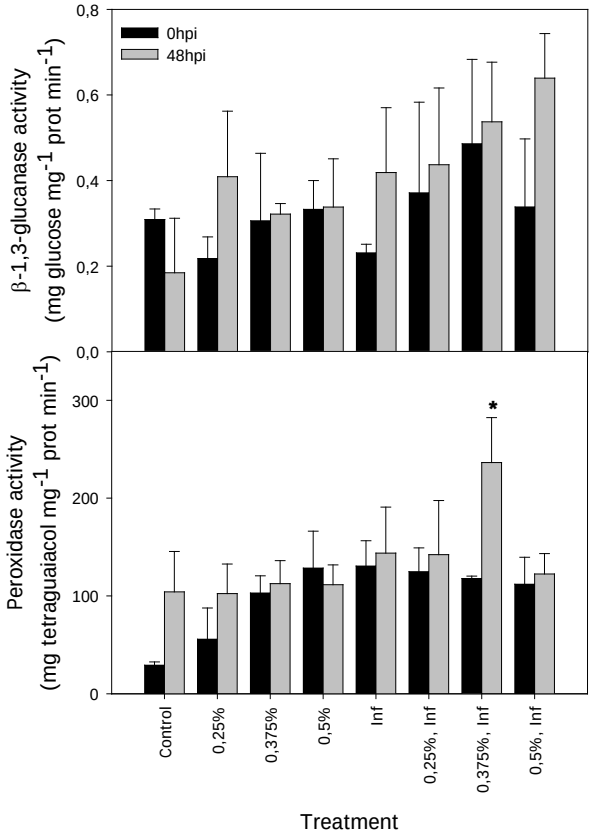

Figure 3.1: Effect of *RWASA1* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the resistant cultivar SST387. Alexin™ treatment in the absence of infestation: Control (0 Alexin™); 0.25% Alexin™; 0.375% Alexin™; 0.5% Alexin™. Alexin™ treatment followed by infestation (*RWASA1*) (Inf): 0 Alexin™ Inf; 0.25% Alexin™ Inf; 0.375% Alexin™ Inf; 0.5% Alexin™ Inf. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

A concentration of 0.375% (v/v) Alexin™ in SST387 induced higher peroxidase activity at 48 hpi than at 0 hpi in SST387 ($p=0.001$) (Fig. 3.1 B). However, this activity was not significantly ($p=0.142$) different from that in other infested treatments at 48 hpi.

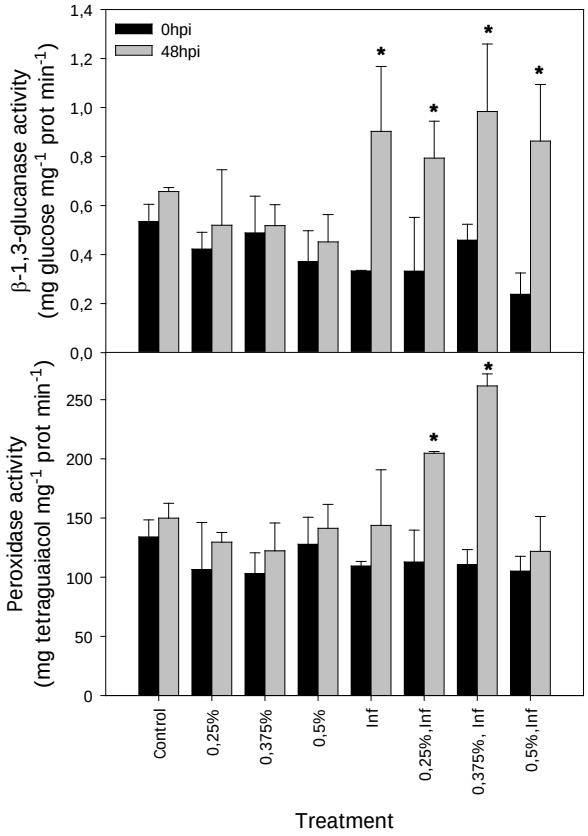

Figure 3.2: Effect of *RWASA1* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the resistant cultivar Elands. Alexin™ treatment in the absence of infestation: Control (0 Alexin™); 0.25% Alexin™; 0.375% Alexin™; 0.5% Alexin™. Alexin™ treatment followed by infestation (*RWASA1*) (Inf): 0 Alexin™ Inf; 0.25% Alexin™ Inf; 0.375% Alexin™ Inf; 0.5% Alexin™ Inf. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

There was no significant change in any of the uninfested Alexin™ treatments in Elands; neither the β-1,3-glucanase nor the peroxidase activities changed significantly (Fig. 3.2). As expected from a resistant cultivar, β-1,3-glucanase activity at 48 hpi was significantly higher in infested than in uninfested plants (Fig. 3.2 A: $p=0.000$), while the activity in Alexin™-treated infested plants did not differ significaty from the untreated infested plants ($p=0.224$) at 48 hpi.

A concentration of 0.25% or 0.375% (v/v) Alexin™ followed by infestation induced significantly higher peroxidase activity in Elands (Fig. 3.2 B: $p=0.001$). A concentration of 0.375% (v/v) induced the highest (2-fold) increase in activity at 48 hpi.

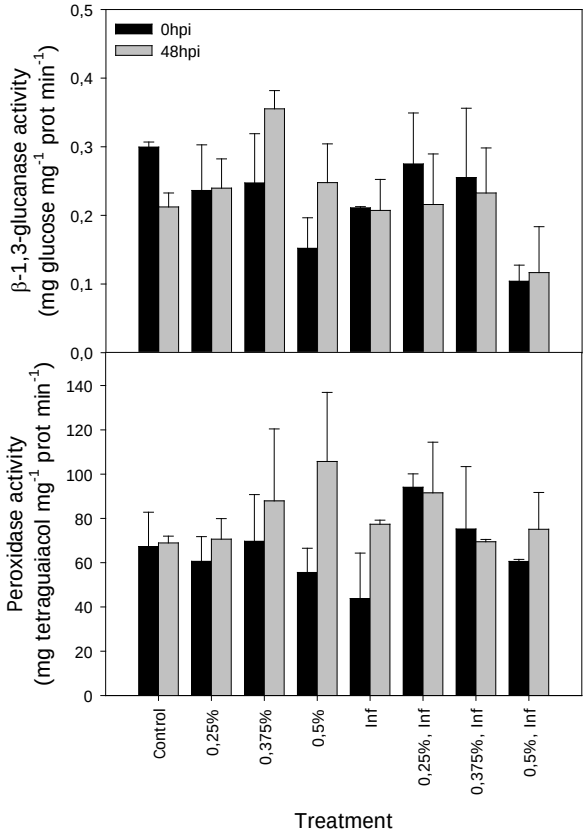

Figure 3.3: Effect of *RWASA1* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the resistant cultivar PAN3379. Alexin™ treatment in the absence of infestation: Control (0 Alexin™); 0.25% Alexin™; 0.375% Alexin™; 0.5% Alexin™. Alexin™ treatment followed by infestation (*RWASA1*) (Inf): 0 Alexin™ Inf; 0.25% Alexin™ Inf; 0.375% Alexin™ Inf; 0.5% Alexin™ Inf. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

Neither Alexin™ treatments alone nor those followed by infestation had any significant effects on β-1,3-glucanase (Fig. 3.3 A: $p=0.154$) or peroxidase (Fig. 3.3 B: $p=0.209$) activities in PAN3379.

Effect of Alexin™ (0.375%, v/v) on *RWASA2*-induced enzyme activities

There were increases though not significant in β-1,3-glucanase (Fig. 3.4 A: p=0.901) and peroxidase (Fig. 3.4 B: p=0.697) activities of 0.375% (v/v) Alexin™-treated infested and untreated infested SST387 at 48 hpi.

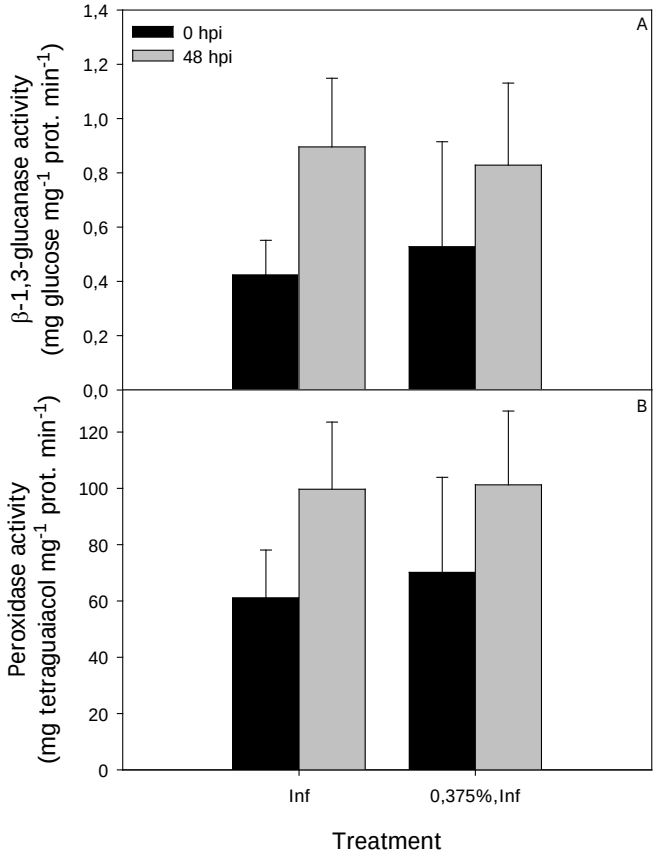

Figure 3.4: Effect of *RWASA2* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the susceptible cultivar SST387. Inf: Infestation of untreated plants; 0.375%, Inf: Infestation of Alexin™ (0.375% v/v) pre-treated plants. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

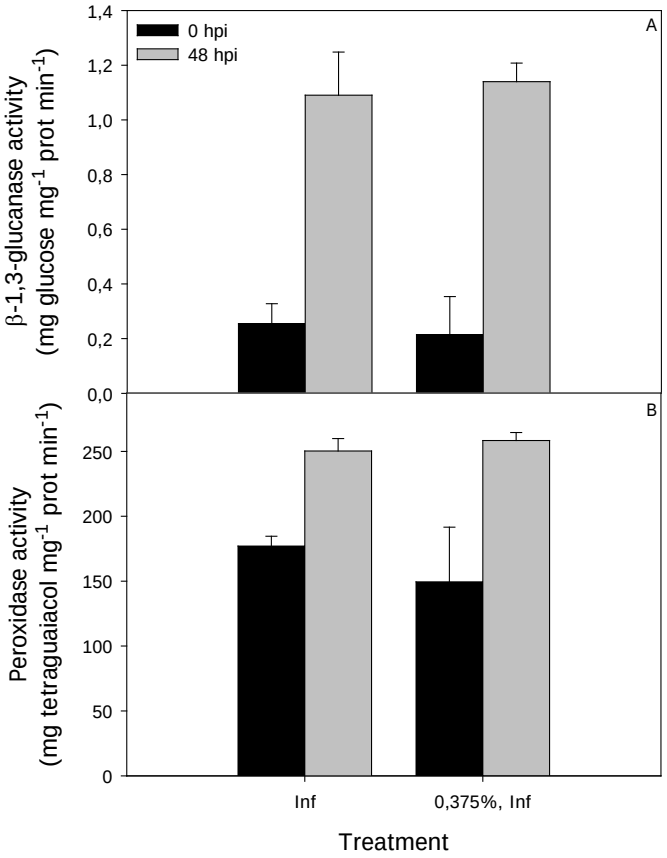

Figure 3.5: Effect of *RWASA2* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the susceptible cultivar Elands. Inf: Infestation of untreated plants; 0.375%, Inf: Infestation of Alexin™ (0.375% v/v) pre-treated plants. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

Even though Elands is susceptible to *RWASA2,* infestation alone and infestation of pre-treated Alexin™ induced increased activity of both enzymes. However, there was no significant difference in β-1,3-glucanase (Fig. 3.5 A: $p=0.47$) and peroxidase (Fig. 3.5 B: $p=0.77$) activities between the untreated infested and Alexin™-treated infested plants at 48 hpi.

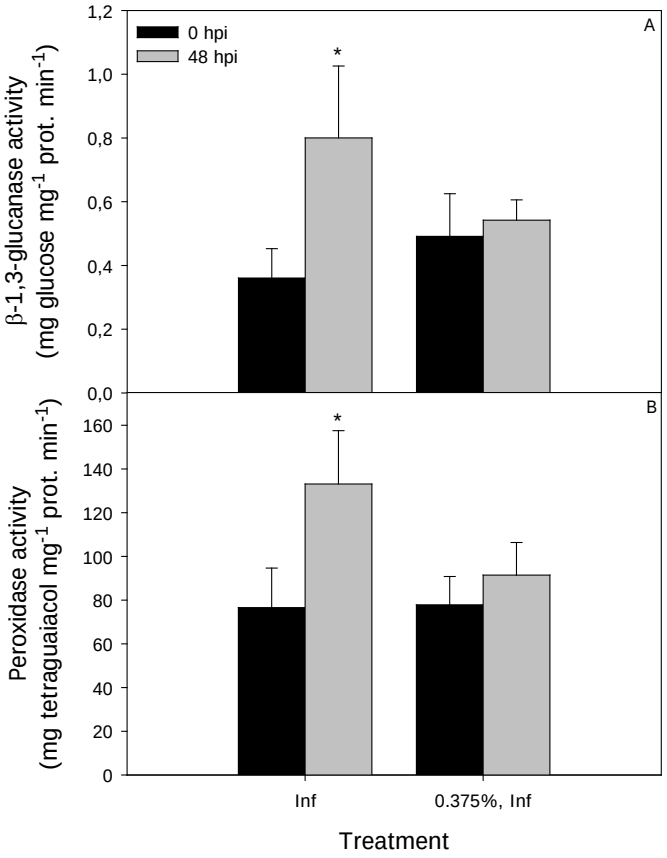

Figure 3.6: Effect of *RWASA2* infestation on β-1,3-glucanase (A) and peroxidase (B) activities in the resistant cultivar PAN3379. Inf: Infestation of untreated plants; 0.375%, Inf: Infestation of Alexin™ (0.375% v/v) pre-treated plants. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant difference ($p<0.05$).

The enzyme activities of infested untreated PAN3379 increased over time and were significantly higher at 48 hpi (Fig. 3.6A: p=0.035; B: p=0,009). This was expected because PAN3379 is resistant to *RWASA2.* In comparison to infestation of untreated and treated plants, Alexin™ pre-treatment tended to suppress β-1,3-glucanase and peroxidase activities within the 48 hpi of infestation.

3.4 DISCUSSION

Alexin™ is a plant activator which contains SA derivatives and other micronutrients, and which might be able to help the plant respond more rapidly to aphid attacks. In a primed state, the defence response is dormant, and the plant does not use excessive energy for protection until exposed to stress, when defence responses are aggressively activated (Van Hulten, *et al.*, 2006). This phenomenon has been observed in uninfested Alexin™ pre-treated cultivars: SST387 and Elands. The enzyme activities remained low since Alexin™ does not activate the full suite of defence response without exposure to stress.

Mohase and Van der Westhuizen (2002b) have demonstrated that peroxidase and β-1,3-glucanases are indicaters of plant defence activation when a signalling molecule such as SA is applied to plants prior to RWA infestation.

Wheat cultivars resistant to *RWASA1* such as *Tugela DN1* express relatively higher peroxidase and β-1,3-glucanase activities during infestation (Van der Westhuizen, *et al.*, 1998a & 1998b). It was therefore expected that SST387 ([1]Astrid Jankielsohn: personal communication), Elands (Sensako, 2017) and PAN3379 (Pannar, 2017), which are all resistant to *RWASA1,* would show increased enzyme activities when challenged with *RWASA1*. It was also hypothesised that if there was induction of the defence-related enzymes, then pre-treatment with Alexin™ would enhance this increase. Different concentrations of Alexin™ were applied to determine the amount that induced the highest response in defence-related enzyme activities during exposure to RWA infestations.

A concentration of 0.375% v/v Alexin™ induced a higher peroxidase activity during *RWASA1* infestation of SST387 and Elands (Fig. 3.1 B & Fig. 3.2 B). This was an indication that peroxidase may be involved in aphid resistance, perhaps

[1] Personal Communication: Dr A Jankielsohn, Agricultural Research Council – Small Grains (ARC-SG), South Africa.

with the strengthening of cell walls during aphid infestation. The increase in Alexin™ concentration corresponded with an increase of β-1,3-glucanase activity. Van der Westhuizen, *et al*. (1998a) also found an increase in β-1,3-glucanase activity in a resistant cultivar but not in the susceptible cultivar. They speculated that even though a direct role of β-1,3-glucanases could not be identified, the enzymes play a role in the RWA defence mechanisms. Alexin™ (0.375%, v/v) pre-treatment mediated induction of higher peroxidase activity in both SST387 and Elands and this concentration was identified as the optimal concentration capable of priming these wheat cultivars for protection against *RWASA1* and *RWASA2*.

Although increased enzyme activities were observed, in some experiments these responses were not significantly high. Such variations have also been reported in β-aminobutyric acid (BABA)-mediated responses (Van Hulten, *et al*., 2006). Induced callose depositions occurred only in cells that were in contact with the pathogen. The argument was that priming might give the plant some time to determine and activate the most competent defence mechanism; therefore, some defence responses might not be as prominent at the beginning of an attack as other defence responses.

The cultivar PAN3379 is resistant to all four known South African RWA biotypes (Pannar, 2017). Since the cultivar expresses such broad resistance, relatively higher enzyme activities in Alexin™ pre-treated plants were expected. However, Alexin™ pre-treatment suppressed RWA-induced enzyme activities. Despite β-1,3-glucanase and peroxidase activities being indicators of defence responses in resistant cultivars, they could not exclusively determine resistance or susceptibility of the wheat cultivars. The plant's defence system is complex and consists of several interacting factors: all these factors combined are what determines the susceptibility or resistance of a plant to a specific pest or pathogen.

Priming can produce varying results because induced resistance is a host genotype specific response during pest, pathogen or abiotic stress. Priming can potentiate responses though a particular pathway during exposure to a specific pest and can act through a different resistance mechanism to another pest. The expression of this response is affected by a range of factors, including host genotype, the pest or pathogen, the environment and the extent to which the plant has been already induced in the field with abiotic stress (Walters & Fountaine, 2009; Heidel & Baldwin, 2004; Campbell, *et al.*, 2002).

3.5 CONCLUSION

Alexin™ primed the cultivars Elands and SST387 to respond differently to *RWASA1*. This was mainly associated with peroxidase activity. However, Alexin™ pre-treatment supressed defence responses (peroxidase and β-1,3-glucanase) in the broad-resistance cultivar PAN3379. These results illustrate that even though Alexin™ can prime certain wheat cultivars for protection against the RWA, this priming effect is specific and depends at least on both host genotype and RWA biotype. Further tests are needed to elucidate the mechanisms of priming mediated though Alexin™.

Chapter 4

Screening for three different categories of Alexin^{TM}-mediated host plant resistance to Russian wheat aphid (*Diuraphis noxia*)

4.1 INTRODUCTION

Wheat is a very important food resource that is susceptible to various pests and pathogens that can cause economical damage. The Russian wheat aphid (RWA, *Diuraphis noxia*, Kurdjumov) is such a pest. This aphid reaches pest status regularly in the summer rainfall regions of South Africa, and more particularly, of the eastern Free State (Du Toit, 1987). Akhtar, *et al*. (2010) found that RWA infestation causes a significant loss to grain yield in wheat, and according to Karren (1989), each percentage of infestation level will result in 0.5% yield loss at harvest. Physiological and biological defence mechanisms in wheat can however affect aphid ecology, development and reproduction. Identifying these plant resistance nodes represents an effective alternative or supplementary pest management method.

The most successful management approach is host plant resistance (El Bouhssini, *et al*., 2011). In 1993, South Africa was the first country in the world to release RWA resistant wheat cultivars. Eight different cultivars were released and commercialised (Van Niekerk, 2001). The resistant cultivars, in combination with an integrated pest management programme, were successful in suppressing aphid numbers (Marasas, 1999). This control strategy seemingly contributed to the evolution of resistance-breaking biotypes, which created a significant challenge to the wheat industry in South Africa. It is therefore

important to constantly search and incorporate new resistance genes into wheat through dedicated breeding programmes (Jankielsohn, 2013).

Determining the resistance of a host plant is associated with three different nodes which can be categorised into antibiosis, antixenosis and tolerance (Painter, 1951). Antibiosis is a measure of the plant's negative influence on the biology of the insect. It is associated with reduction of body size, weight and fecundity, or failure to pupate or emerge, and with the prolonging of developmental stages (Norris, *et al.*, 2003). Antixenosis on the other hand can affect the pest behaviour by repelling or initiating the insect to avoid the plant as a food source or oviposition site. This effect is usually mediated by chemical or physical secretions and/or structures within the host plant (Norris, *et al.*, 2003). Tolerance, the last node of resistance, refers to the ability of a plant to withstand pest feeding and reproduction without significant damage (Norris, *et al.*, 2003). In such cases, yield is not significantly influenced by pest presence. Examples of resistance nodes have been observed in resistant wheat varieties: *Dn1* confers antibiosis, *Dn2* mediates tolerance, while *Dn5* affords a combination of antibiosis, antixenosis and tolerance towards the RWA (Rafi, *et al.*, 1996). These nodes of resistance, which may be enhanced by chemicals, resistance-inducing products, or plants expressing RWA resistance genes, are important in developing strategies to manage the aphid.

Combining resistance-breeding and resistance-inducing products may be an effective strategy and a long-term solution against resistance-breaking biotypes in wheat production. Alexin™ can be a very beneficial product which might boost inducible resistance responses in plants and thereby reduce the reliance on pesticides for crop protection. In this section we investigated the mode of Alexin™-mediated resistance (antibiosis, antixenosis or tolerance) in three different cultivars during RWA infestation.

4.2 MATERIALS AND METHODS

Plant material

Three dryland wheat cultivars were chosen based on their market availability, yield potential and response (susceptible, resistant) to RWA infestation. Cultivars SST387 and Elands are resistant to *RWASA1* but susceptible to the three other South African RWA biotypes (*RWASA2, RWASA3*, *RWASA4*). Additionally, SST387 is drought tolerant. PAN3379 is resistant to all four current South African RWA biotypes. The seeds used in these experiments were received from the Agricultural Research Council – Small Grains (ARC-SG) of Bethlehem, South Africa.

Wheat plants were grown under greenhouse conditions at temperatures of 22 °C ± 4 °C. The plants were cultivated in trays and placed in cages covered by nets (315 micron) to enclose the aphids. Culture conditions and infestation procedures were done as described by Du Toit (1988). For this study two of the four existing South African RWA biotypes, *RWASA1* and *RWASA2,* were used. Aphids used in this report were received from the ARC-SG and colonies were maintained on susceptible Tugela wheat. First-instar aphids for each biotype, which were obtained from reproducing adult aphids, were transferred and maintained on separate wheat plants until fifth instar (alatiform nymph) stage, and used in the following experiment.

Treatment and infestation

Alexin™ (Reg no. B3835, Act no. 36 of 1947) is a registered trademark of AECI Limited (Nulandis®, a division of AECI Limited). It is a liquid organic nutrient complex containing salicylic acid derivatives, oligosaccharides, 4.5% potassium, 2.6% calcium, 0.8% magnesium and 0.2% boron. LI 700®, a product of Nulandis®, is an acidifier that increases the efficiency of pH sensitive agricultural remedies and improves the penetration properties of spray mixtures.

An Alexin™ (0.375%, v/v) solution containing LI 700® (0.06% v/v, adhesive) in sterile water was prepared and applied as a foliar spray (fine mist) to wheat seedlings at the three-leaf stage. The outline of the treatments is shown in Table 4.1. Each experiment consisted of three independent replicates.

Table 4.1: Treatments in glasshouse trial: Screening for categories of resistance

Treatment	1	2	3	4
Alexin™ Infestation	0 (DH_2O) Infested with *RWASA1*	0.375% (v/v) Infested with *RWASA1*	0 (DH_2O) Infested with *RWASA2*	0.375% (v/v) Infested with *RWASA2*

Antixenosis

Two antixenosis tests were conducted using an olfactometer and the circle design choice test.

A Y-shaped olfactometer was used to determine attraction and/or repulsion of aphids towards control (untreated) or treated (0.375% v/v Alexin™) plants. A single pot per treatment was used, each containing three seedlings at the three-leaf stage. A pot with control plants was placed on the first arm of the Y and the pot with the treated plants on the second arm. Ten aphids were placed on a moist filter paper on the third arm. A gentle stream of air flowed from the two arms containing the potted plants towards the third arm where aphids were placed. The dispersal of aphids was determined after 24 hours.

The circle design choice test contained eight pre-germinated seeds of each cultivar planted in a circle in a pot. When seedlings reached the three-leaf stage, four seedlings of the same height and vigour were selected and kept growing while the other four were uprooted and discarded. These remaining seedlings were about 5 cm apart from each other in the circular form. Two days after Alexin™ treatment, 40 adult RWAs were placed on a filter paper in the middle of the pot. The aphids were allowed to distribute themselves within the pots towards their preferred seedlings over a period of 48 h. After 48 h the plants were cut off at soil level and the number of aphids counted on each

plant. A total of twenty pots of the same cultivar were prepared; seedlings in ten pots were treated with Alexin™ and seedlings in the other ten pots were left untreated as control. This was performed for all three cultivars at different times because of space constraints.

Antibiosis

Antibiosis as a form of host resistance was determined by calculating the intrinsic rate of increase (r_m) of the two South African biotypes – *RWASA1* and *RWASA2* – feeding on three different wheat cultivars. Twenty seeds of each cultivar were planted in rectangular trays (30 x 20 cm). At three-leaf stage, 10 seedlings of the same height were selected from each cultivar, while the rest of the seedlings were uprooted and discarded. One tray was left untreated as control and the other was treated with 0.375% (v/v) Alexin™. A single mature aphid was placed on a single leaf of each plant. The nymphs born to the aphid were counted each day for a period of fourteen days. The nymphs were immediately removed after the counting of each day.

The aphid population was calculated by determining the intrinsic rate of increase (r_m) using the following formula:

$$1 = \Sigma e^{-rm} l_x m_x$$

The age of aphids is represented by x (1 day). l_x is the probability of being alive on day x, m_x is the mean number of offspring on day x, and r is the intrinsic rate of increase (Birch, 1948).

Tolerance

Tolerance of the two RWA biotypes by the three different cultivars was determined using the same protocol as described above for determining antibiosis. Plants were treated with Alexin™ and two days after treatment a

single aphid was placed on a plant and allowed to reproduce for fourteen days. Plant height was measured at day one (before Alexin™ treatment) and the last measurement was taken 14 days after infestation. Plants in this experiment remained infested for a total of 14 days (Deol, Reese, Gill, Wilde & Campbell, 2001).

After plant height was measured, the damage rating on each plant was determined using a scale ranging from 1 to 10 (Tolmay, Van Der Westhuizen & Van Deventer, 1999). The scale places the cultivar in three different catagories, namely resistant (R), where chlorotic spots score between 1-4; moderately resistant (MR), where leaf striping has developed and scoring is between 5-6; and susceptible (S), where leaf rolling is prominent and scoring is between 7-10.

Photo 4.1: Description of RWA damage symptoms used for scroring. 1 – Small isolated chlorotic spots, 2 – Small chlorotic spots, 3 – Chlorotic spots in rows, 4 – Chlorotic splotches, 5 – Mild chlorotic streaks, 6 – Prominent chlorotic streaks, 7 – Severe streaks, leaves fold conduplicate, 8 – Severe streaks, leaves roll covulate, 9 – Severe streaks, leaves roll tightly, 10 – Plant dying (Tolmay, *et al.*, 1999).

To determine re-growth after aphid infestation, aphids were removed and the leaves were cut off at soil level. Leaves on the stumps were allowed to sprought

for a further 14 days and the extent of re-growth was measured by counting the number of new leaves emerging.

Data analysis

Outliers were assessed by boxplots and normality assessed by Normal Q-Q plots. Homogeneity of variances was assessed by Levene's test. Data were analysed using analysis of variance (Anova, IBM SPSS statistics version 24) to compare means of antixenosis, antibiosis and tolerance in wheat-RWA biotype interaction in relation to treatment with 0.375% (v/v).

4.3 RESULTS

Antixenosis

The olfactometer tests did not provide conclusive results and therefore the circle design choice test was used to determine antixenosis. The RWA biotypes did not show preference for any of the wheat cultivars either treated or not treated with Alexin™ (Fig. 4.1 A: *RWASA1*, B: *RWASA2*). There was no indication that either wheat genotype ($p=0.111$) or treatment ($p=0.11$) had any effect on aphid host preference.

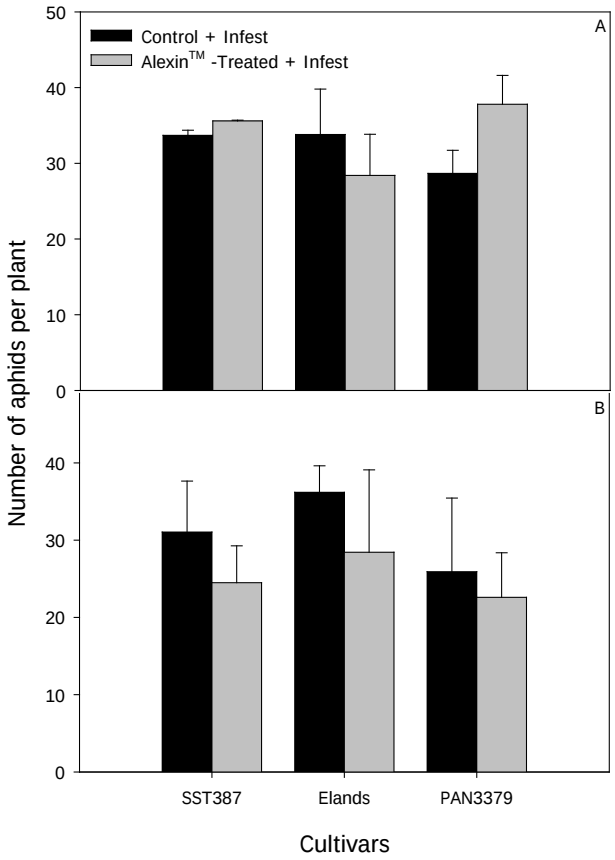

Figure 4.1: The mean number of aphids (A: *RWASA1*, B: *RWASA2*) on control or Alexin™-treated cultivars during the choice test. Error bars indicate standard deviation (n=3). There were no significant differences between the treatments ($p<0.05$).

Antibiosis

The intrinsic rate of increase (r_m) of *RWASA1* feeding on wheat was significantly lower in Alexin™-treated SST387 than in the untreated control (Fig. 4.2 A: $p=0.001$). The same could also be said about the intrinsic rate of increase (r_m) of *RWASA2* (Fig. 4.2 B: $p=0.008$) on SST387 treated plants.

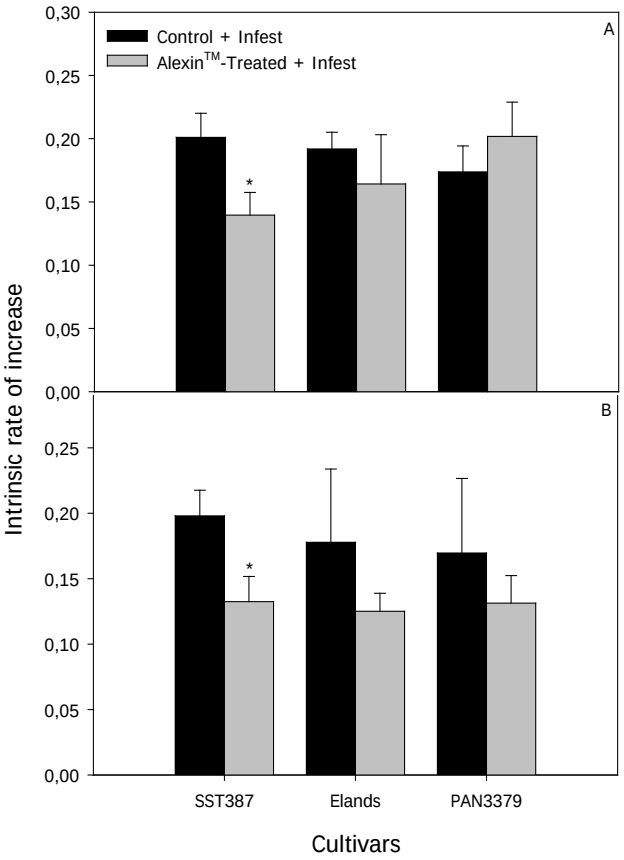

Figure 4.2: The intrinsic rate of increase of aphids (A: *RWASA1*; B: *RWASA2*) on control or Alexin™-treated cultivars. Error bars indicate standard deviation (n=3).
(*) indicates significant difference ($p<0.05$).

The *RWASA1* population was on average relatively lower in the treated cultivar than in the untreated control in Elands, but the values were not significantly different (Fig. 4.2 A: $p=0.171$). The same was found with the intrinsic rate of increase (r_m) of *RWASA2* (Fig. 4.2 B: $p=0.075$).

Alexin™ treatment in PAN3379 did not have any significant effect on the intrinsic rate of increase in the population of either *RWASA1* (Fig. 4.2 A: $p=0.102$) or *RWASA2* (Fig. 4.2 B: $p=0.193$).

Tolerance

Alexin™-mediated tolerance to aphid infestation (*RWASA1*) was evident in the cultivar SST387, where plant height was significantly (2.2-fold) taller than in the untreated plants (Fig. 4.3 A: p=0.014). Alexin™ treatment did not induce any significant differences in plant height in *RWASA1* infested Elands (Fig. 4.3 A: p=0.970) or PAN3379 (Fig. 4.3 A: p=0.932).

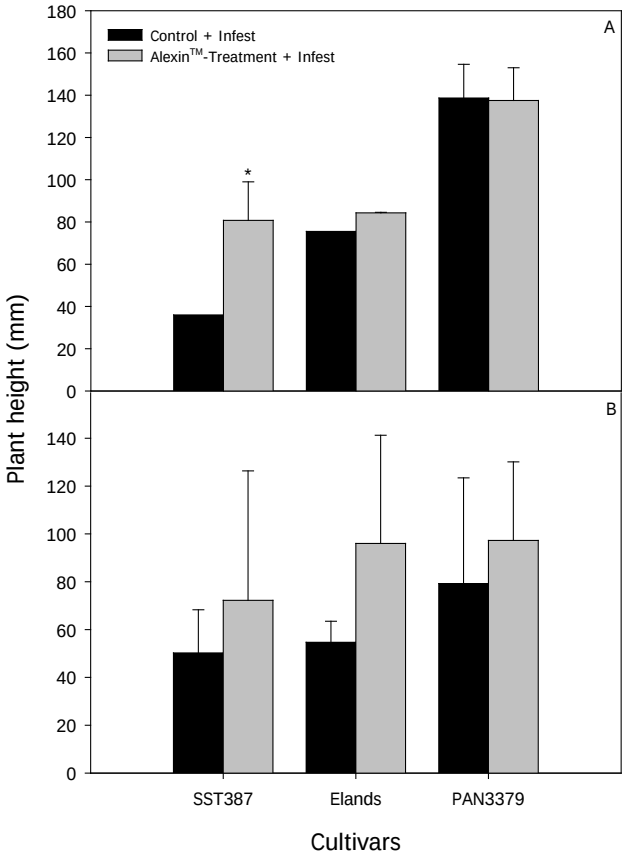

Figure 4.3: Plant growth rate (plant height) 14 d after infestation (A: *RWASA1*; B: *RWASA2*) in plants pre-treated or untreated (control) with Alexin™. Error bars indicate standard deviation (n=3). (*) indicates significant difference (p<0.05).

Growth was not significantly influenced in any of the cultivars pre-treated with Alexin™ and infested with *RWASA2* (Fig. 4.3 B: SST387 p=0.716; Elands p=0.915; PAN3379 p=0.955).

Table 4.2: Russian wheat aphid-induced damage rating scores (Tolmay, *et al.*, 1999). R: resistant; MR: moderately resistant; S: susceptible. Scores 1-3 = resistant; 4-6.5= moderately resistant; 6.5-10 = susceptible.

Cultivar	Treatment	Score and symptoms		Induced response	
		RWASA1	*RWASA2*	*RWASA1*	*RWASA2*
SST387	Control Infested	4, Chlorotic spots	8, Leaf rolling	MR	S
	0.375%, Infested	3.5, Chlorotic spots	5,Chlorotic spots & streaking	R	MR
Elands	Control Infested	4, Chlorotic spots	7.5, Streaking & leaf rolling	MR	S
	0.375%, Infested	4, Chlorotic spots	4.5, Chlorotic spots & streaking	MR	MR
PAN3379	Control Infested	2, Chlorotic spots	3, Chlorotic spots	R	R
	0.375%, Infested	2, Chlorotic spots	5, Chlorotic spots & streaking	R	MR

The Alexin™-treated SST387 showed tolerance to *RWASA1* because of the reduced symptoms and the shift from moderately resistant to resistant (Table 4.2). SST387 is susceptible to *RWASA2* and scored 8 but Alexin™ treatment shifted the damage rating score to moderate resistance of 5. Alexin™ pre-treatment and *RWASA1* infestation alone induced similar responses: moderate resistance (Elands) and resistance (PAN3379). However, Alexin™ treatment mediated the shift from susceptible to moderately resistant in Elands infested with *RWASA2*. Unexpectedly Alexin™ treatment suppressed defence responses in PAN3379 challenged with *RWASA2* from resistant to moderately resistant (Table 4.2).

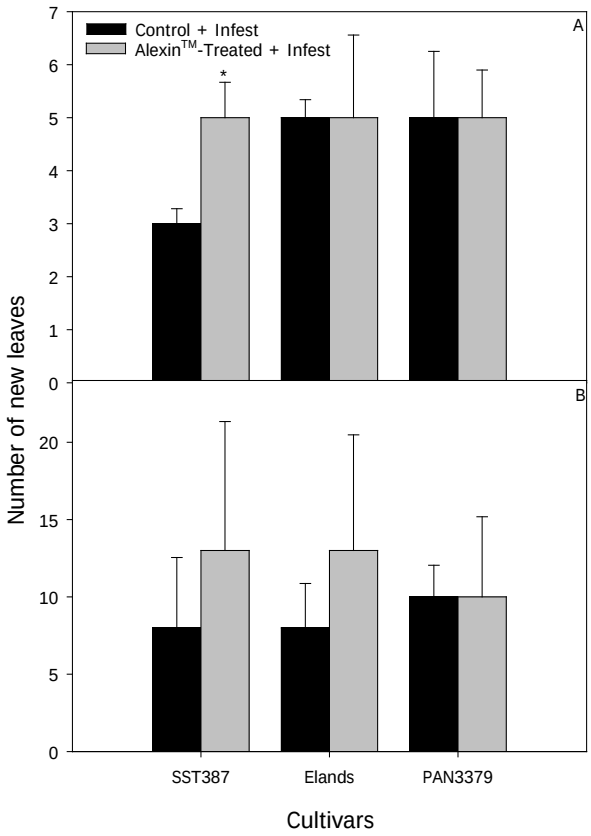

Figure 4.4: Mean number of new emerging leaves per plant (new leaves per plant) after 14 days following the infestation period (A: *RWASA1*; B: *RWASA2*) in plants pre-treated or untreated (control) with Alexin™. (*) indicates significant difference ($p<0.05$).

Alexin™ treatment induced significant recovery in terms of total number of leaves only in SST387 infested with *RWASA1* (Fig. 4.4 A: $p=0.003$) and not *RWASA2* (Fig. 4.4 B: $p=0.472$). In treated Elands (Fig. 4.4 A: $p=0.604$; B: $p=0.456$) and PAN3379 (Fig. 4.4 A: $p=0.670$; B: $p=0.937$) there was no significant number of emerging leaves recorded during infestations with either of the two RWA biotypes.

4.4 DISCUSSION

Chemicals or priming agents activating synthesis of certain secondary metabolites in plants that can act as insect repellents or feeding deterrents (Ohmart, Stewart & Thomas, 1985), but our antixenosis tests indicated that Alexin™ did not mediate any aphid attraction or repulsion behaviour in either

SST387 or Elands. The number of aphids feeding on PAN3379 control plants was not significantly lower than those feeding on treated plants.

Since it is often difficult to separate antixenosis and antibiosis because of their co-occurrence or co-inheritance (Wiseman, 1994), it was important to test both modes of resistance. Antibiosis is a defense mechanism that can influence the feeding behaviour of insects and consequently also their life cycle. Alexin™ treatment of the different wheat cultivars prior to infestation induced various effects on the population dynamics of the aphids. This treatment on SST387 influenced the RWA population negatively by suppressing the population growth rate of both *RWASA1* and *RWASA2*. This may suggest that Alexin™ treatment of SST387 induces broad resistance to both aphid biotypes.

The SA derivatives present in Alexin™ could be one of the elements that influence aphid fecundity. Donovan, *et al*. (2013) tested the effects of different concentrations of SA in an artificial diet and reported decreased survival of aphids, suggesting that SA may directly inhibit aphid growth. The aphid feeds on the phloem tissue and since SA is transported through the phloem, aphids could be directly exposed to SA derivatives. Furthermore, the micro-element boron present in Alexin™ also has an effect on cell wall formation (Ahmed, *et al*., 2009) and could increase cell wall rigidity, thus reducing the ease of cell penetration by aphid stylets, leading to reduced nutrient acquisition.

The cultivar Elands is susceptible to *RWASA2*, and PAN3379 is resistant to all biotypes. In these two cultivars, Alexin™ treatment supressed the *RWASA2* population, although not significantly. However, these cultivars are both resistant to *RWASA1*, and Alexin™ treatment did not significantly suppress the aphid population, especially in PAN3379, where there was no supression in comparison to the control.

Tolerance is entirely a plant response and no interaction is considered to occur between the plant and the insect (Wiseman, 1994). There are five physiological

plant responses associated with tolerance to insects: the increase of photosynthesis; high relative growth rates; increased tillering; high levels of carbon storage in roots; and the ability to reallocate carbon from roots to shoots after injury (Strauss & Agrawal, 1999). Hence, a plant is tolerant if pest infestation and wound healing do not compromise growth, general vigour or photosynthetic partitioning, and total yield is not reduced (Wiseman, 1994). When aphids start feeding on susceptible plants, symptoms such as chlorosis, leaf rolling and plant stunting develop and Burd, Burton and Webster (1993) illustrated that plant-growth measurements are a consistent indicator of plant stunting when compared to the control plant height. In this study Alexin™ mediated changes in plant height and the intensity of recovery indicates an expression of tolerance in treated SST387 towards *RWASA1*.

Several techniques are in use for assessing tolerance and antibiosis to aphids (Lazzari, Starkey, Reese, Ray-Chandler, Mccubrey & Smith, 2009). However, some fail to separate the two components, just as in this study it has been difficult to separate the two modes when SST387 had less aphid numbers and height increase. For this study it is presumed that tolerance might also be one of the resistance modes in SST387 because recovery occured in previously infested plants.

The Alexin™-mediated recovery (height and number of sprouted leaves) could be due to nutrients present in Alexin™. Potassium ions are for instance involved in synthesis of adenosine triphosphate (ATP), a high energy source in plants, with the electrical charge balance at the site of ATP production being maintained by potassium ions (Better Crops, 1998). The activation of enzymes associated with photosynthesis through regulation of stomatal movement and production of ATP are all influenced by potassium levels in a plant (Wang, *et al.*, 2013). Maintaining potassium nutrition is also important in plant resistance to biotic and abiotic stress. When potassium ions increase, the internal competition by pathogens for nutrient resources is decreased. This nutritional status can help the plant allocate more resources for plant defence and damage

repair by developing stronger cell walls, thus inhibiting pathogen or insect from obtaining nutrients (Wang, *et al*., 2013). Potassium regulates reactive oxygen species (ROS) and antioxidant accumulation that are important in defence mechanisms (Wang, *et al*., 2013). Nonetheless, Alexin™ treatment neither induced tolerance of Elands or PAN3379 to *RWASA1* nor SST387, Elands or PAN3379 to *RWASA2*.

Tolerance is an effective mechanism in resistance because selection pressure is less likely and the longevity of resistance under field conditions can be extended (Gullan, 1972). Antiobiosis and antixenosis on the other hand, might raise the evolution of new biotypes, leading to selection pressure (Gould, 1998). Even though Alexin™ mediated expression of tolerance to *RWASA1* in SST387, environmental factors usually affect tolerance more than any other type of resistance (Pedigo & Rice, 2006). Field experiments are therefore needed to verify tolerance resistance.

In susceptible cultivars, RWA infestation can induce chlorosis and leaf rolling. Leaf rolling has a negative effect on plant health and yield potential. The photosynthetic area is reduced and the flag leaf and ear can be trapped within the rolled leaf causing malformation of the ear and ultimately reducing the filling of the kernels. SST387 is susceptible to *RWASA2* (damage rating score: 8, Table 4.2) but Alexin™ treatment triggered the shift to moderately resistant (Table 4.2), where leaf rolling was reduced. The reduction in leaf rolling and striping could help plants maintain adequate rates of photosynthesis that support continued growth (Frazen, Gutshe, Heng-Moss, Higley, Gautam & Burd, 2007), thus overcoming the negative effects of aphid feeding. From the aphid perspective, it has been hypothesised that leaf rolling may be a primary factor for aphid survival and could limit the aphid host plant range (Burd, *et al*., 1993). Alexin™ is able to protect plants against disease, as reported by Jayaraj, *et al*. (2009), where pre-treated carrots were inoculated with *Altenaria radicina* and *Botrytis cinerea*. Hendricks, *et al*. (2015) also reported on Alexin™-mediated

protection of fruit trees from pathogens. This is the first report on Alexin™ increasing resistance to an insect pest in a cereal crop.

Alexin™-induced host damage on PAN3379 was interesting: Alexin™ suppressed defence responses from resistant to moderately resistant with *RWASA2* infestation. This was also evident in Chapter 3 with suppresion of glucanase and peroxidase activities. PAN3379 is resistant to the known RWA biotypes but the several complementary cellular pathways seem to be channelling defence mechanisms through a pathway different from SA and the application of Alexin™ seems to be influencing the plant defence mechanism negatively. The various outcomes of aphid-plant interactions may indicate that many novel genes and mechanisms involving plant perception and resistance to phloem feeding remain undiscovered.

4.5 CONCLUSION

Antibiosis is important in managing aphid numbers but could promote selection pressure. Plant tolerance is a very important category of resistance and because tolerance does not interfere with the insect's physiology or behavior, selection for virulent insect populations and the threat of emerging biotypes is presumed to be limited (Smith, 2005). Moreover, it may also help promote the effects of beneficial arthropods in agricultural settings (Smith, 2005). Alexin™ treatment elicited antibiosis and tolerance towards both *RWASA1* and *RWASA2* in SST387. The treatment induced a phenotypic shift towards resistance in Elands, with the response being categorised into antibiosis and tolerance. This is a very important shift because the plant did not roll the leaf to help enclose and protect the aphid; thus, aphids can still be exposed to predators and chemicals. By exploring the effect of Alexin™ on antibiosis, antixenosis and tolerance, it might be possible to determine induced intensity of selection pressure on RWA to evolve into biotypes. The shift in physiological symptoms could assist in indicating the potential of Alexin™ as part of an integrated pest management system to help manage aphids without overexploiting insecticides.

On the other hand, the treatment repressed aphid-induced tolerance and antibiosis in PAN3379 to a remarkable extent. Further investigations are needed because Alexin™ seems to regulate defence responses differently in various wheat genotypes, especially at biochemical and molecular level. It is important to decipher the effect of Alexin™ on specific defence components associated with induced resistance.

Chapter 5

Alexin™-mediated hormonal responses, redox reactions and gene expression in wheat challenged with the Russian wheat aphid

5.1 INTRODUCTION

Phloem-feeding insects are highly specific and in most cases induce defence responses similar to pathogenesis (Moran & Thompson, 2001). The Russian wheat aphid (RWA) on the other hand, causes minimal physical damage to host tissues during feeding. However, feeding does release saliva which contains effectors that induce physiological and biochemical changes leading to resistance or susceptibility; depending on the host genotype (Mohase & Taiwe, 2015).

The plant-aphid interactions result in the accumulation of reactive oxygen species (ROS), termed the oxidative burst (Moloi & Van der Westhuizen, 2006). The oxidative burst and antioxidant systems are two components that play an important role in the defence response (Moloi & Van der Westhuizen, 2008). The interplay between ROS and salicylic acid (SA) signalling was uncovered when application of Hydrogen peroxide (H_2O_2) and SA to tobacco and *Arabidopsis* plants induced accumulation of each other, suggesting their involvement in a self-amplifying feedback loop (Rao, Paliyath & Ormrod, 1996). After infection or attack, SA is synthesised and increases the initial H_2O_2 levels. As SA levels continue to increase, there is also a proportional accumulation of ROS, leading to a second oxidative burst, which is associated with cell death and expression of defence-related genes (Vlot, *et al.*, 2009).

The concentration of oxidant species requires regulation, otherwise they induce oxidative damage to the host cells. The antioxidative system can reduce ROS levels to prevent phytotoxicity. Salicylic acid can regulate levels of both oxidants and antioxidants. It can activate antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD) and it can inhibit catalase (CAT) and ascorbate peroxidase (APX) (Chen, Silva & Klessig, 1993). Russian wheat aphid infestation differentially induced activities of SOD, glutathione reductase (GR) and APX more in resistant than in susceptible wheat varieties (Moloi & Van der Westhuizen, 2008).

Salicylic acid is a signalling hormone that additionally interacts with other hormones such as jasmonic acid (JA) and abscisic acid (ABA) in the expression of defence responses that contribute to the resistance mechanism. However, hormone cross-talk can occur where the output may be antagonistic or synergistic (Pieterse, Van der Does, Zamioudis, Leon-Reyes & Van Wees, 2012). For instance, higher levels of SA antagonise JA-mediated defence responses as has been observed in *Arabidopsis*. Zarate, *et al*. (2007) showed that activation of SA-mediated defence responses in mutant lines that constitutively expressed SA defences (*cim10*) and impaired JA-mediated responses (*coi1*) induced herbivorous insect development. The opposite occurred when JA-mediated defence responses and lines with impaired SA synthesis (*npr1* and NahG) were activated, making the plant more susceptible to pathogens.

Salicylic acid, JA and ethylene cross-talk has been reviewed extensively (Pieterse, *et al*., 2009; Caarls, Pieterse & Van Wees, 2015). However, ABA and other hormones have received less attention. Abscisic acid has been associated with induced susceptibility of plants to fungal pathogens (Siciliano, Carneiro, Spadaro, Garibaldi & Gullino, 2015). Nonetheless the hormone can also induce defence responses by inducing stomatal closure and callose deposition when plants are attacked by pathogens (Ton, *et al*., 2009). Accumulation of ABA is an early defence reaction and it has been suggested that ABA is a starting signal that primes JA-dependent signalling (Balmer, *et al*., 2015), yet short term

treatment with ABA suppressed JA signalling (Yang, Liu, Dong, Cai, Tian & Wang, 2014). In soybean, ABA down-regulated β-1,3-glucanase activity (Rezonico, Flury, Meins & Beffa, 1998) and suppressed phenylalanine ammonia-lyase (PAL) activity, the latter being an important enzyme that generates SA (Ward, *et al.*, 1989). Therefore, depending on the biotic and abiotic stress, cross-talk between SA, JA and ABA is complex, showing either synergistic or antagonistic effects.

We hypothesised that since SA regulates the oxidant/antioxidant reactions during wheat-aphid interactions, exogenous application of formulations where SA is an active ingredient will accelerate the regulatory process and enhance host protection against RWA. This study therefore, investigates how Alexin™-priming affects levels of SA, JA and ABA, H_2O_2, antioxidant enzyme (SOD, CAT and GR) activities and the expression of specific genes associated with aphid resistance in susceptible and resistant genotypes.

5.2 MATERIALS AND METHODS

Plant material

Two dryland wheat cultivars were chosen based on their market availability, yield potential and response (susceptible, resistance) to RWA infestation. Cultivar SST387 is resistant to *RWASA1* but susceptible to the three other South African RWA biotypes (*RWASA2, RWASA3, RWASA4*) and is additionally drought tolerant. PAN3379 is resistant to all four current South African RWA biotypes. The seeds used in these experiments were received from the Agricultural Research Council-Small Grains (ARC-SG) in Bethlehem, South Africa.

Wheat plants were grown under greenhouse conditions at temperatures of 22 °C ± 4 °C. The plants were cultivated in trays and placed in cages covered with nets (315 micron) to enclose the aphids. Culture conditions and infestation procedures were as described by Du Toit (1988). For this study only *RWASA2*

was used. The aphids were received from the ARC-SG and colonies were maintained on susceptible wheat cultivar 'Tugela'.

Treatment and infestation

Alexin™ (Reg no. B3835, Act no. 36 of 1947) is a registered trademark of AECI Limited (Nulandis® a devision of AECI Limited). It is a liquid organic nutrient complex containing salicylic acid derivatives, oligosaccharides, 4.5% potassium, 2.6% calcium, 0.8% magnesium and 0.2% boron. LI 700®, a product of Nulandis®, is an acidifier that increases the efficiency of pH sensitive agricultural remedies and improves the penetration properties of spray mixtures.

Alexin™ (0.375%, v/v) solution containing LI 700® (0.06% v/v, adhesive) was prepared in sterile water and applied as a foliar spray (fine mist) to wheat seedlings at the three-leaf stage. Two days later (48h) aphids (10 aphids/plant; *RWASA2*, alatiform nymphs) were placed on all test plants (Alexin™-treated and untreated). Control plants were neither treated with Alexin™ nor infested. Table 5.1 indicates treatments applied to the plants. Sixty plants were used per treatment, and each treatment was performed in three independent biological repeats.

Table 5.1: Treatments in the glasshouse trial: Hormones, redox reactions and gene expression

Treatment	1	2	3
Alexin™ *RWASA2*	0 (DH_2O) No Infestation	0 (DH_2O) Infested	0.375% (v/v) Infested

Leaves were collected at different time intervals after aphid infestation [0, 3, 6, 12, 24 and 48 hours post infestation (hpi)]. During sampling, aphids were removed from randomly selected second and third leaves of the plants and the cut leaves immediately frozen in liquid nitrogen and stored at -20 °C or -80 °C for further assays.

Enzyme activity

Phenylalanine ammonia-lyase activity

Phenylalanine ammonia-lyase activity was extracted using the method described by Arz and Grambow (1995). Dowex was regenerated by subsequent washing in HCl, NaOH, NaCl and a final rinse in distilled water. Leaf tissue (300 mg) was ground on ice in a pre-cooled mortar and pestle containing 60 mg regenerated Dowex (1x4-200 ion exchange resin), 60 mg polyvinylpyrrolidone (PVP) and 60 mg washed sea-sand. The leaf tissue was homogenised in 0.1 M sodium borate buffer pH 8.8 (3 ml), containing 1 mM Ethylenedinitrilotetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). The homogenate was centrifuged at 15 000 rpm for 20 min at 4 °C and the supernatant was collected and used for the activity assay.

Sodium borate buffer pH 8.8, (0.1 M; 500 µl) containing 1 mM EDTA and 1 mM DTT, and 400 µl of enzyme extract were used for the assay. This reaction mixture was incubated for 2 min at 40 °C before addition of 100 µl L-phenylalanine (60 mM), which initiated the reaction. The blank consisted of 900 µl sodium borate buffer and 100 µl L-phenylalanine. The reaction proceeded for 10 min at 40 °C and change in absorbance was measured at 290 nm using a Cary 100 UV-Visible spectrophotometer. The enzyme activity was calculated using a calibration curve of cinnamic acid (CA 0.1 to 10 µg ml^{-1}). Enzyme activity was expressed as nmol CA mg^{-1} protein min^{-1}.

Hydrogen peroxide level

Hydrogen peroxide was extracted and quantified according to a modified method of Junglee, Urban, Sallanon and Lopez-Lauri (2014). Frozen leaf material (500 mg) was ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. Extraction buffer (3 ml) and PVP (10 mg) were added to the leaf powder. The extraction buffer consisted of 750 µl potassium phosphate buffer (10 mM, pH 5.8), 1,5 ml potassium iodide (1 M) and 750 µl trichloroacetic acid (0.01%). The homogenate was centrifuged at 15 000 rpm

for 10 min at 4 °C. The supernatant was used to determine H_2O_2 content. All steps were carried out on ice.

The reaction mixture contained 200 µl potassium phosphate buffer (10 mM, pH 5.8), 400 µl potassium iodide (KI, 1 M) and 200 µl enzyme extract. The reaction mixturewas incubated for 20 min at 25 °C in the dark. A blank consisted of potassium phosphate buffer and KI. Afterwards the absorbance of the samples was measured at 350 nm. Hydrogen peroxide content was expressed as nmol g^{-1} fresh weight.

Enzyme extraction (NADPH oxidase, SOD, GR and CAT)

Enzyme extractions were performed using a modified method of Milosevic and Slusarenko (1996). Frozen leaf material (1 g) was ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. The enzyme was extracted with 0.04 g insoluble PVP and 4 ml potassium phosphate buffer (50 mM, pH 7.0) containing 0.1% Triton X-100, 0.04% sodium meta-bisulfite and 10 mM EDTA. The homogenate was centrifuged at 17 000 rpm for 15 min at 4 °C. The supernatant was used to determine NADPH oxidase, SOD, GR and CAT activities. All steps were carried out on ice.

NADPH oxidase

A spectrophotometric method of Askerlund, Larsson, Wildell and Moller (1987) as modified by Rao, *et al*. (1996) was used to measure NADPH oxidase activity. The reaction mixture contained 540 µl potassium phosphate buffer (50 mM, pH 7.0), 300 µl NADPH, (150 µM) and 100 µl potassium cyanide (100 µM). The reaction was initiated by adding the enzyme extract (60 µl) and the decrease in absorbance was measured at 340 nm for 3 min at 25 °C using quartz cuvettes. Enzyme activity was expressed as ΔA_{340nm} mg^{-1} prot min^{-1}.

Superoxide dismutase

Superoxide dismutase activity was measured spectrophotometrically as described in a modified method of Milosevic and Slusarenko (1996). Enzyme extract (150 µl) was added to a reaction mixture consisting of potassium phosphate buffer (50 mM, pH 7.8), containing 13 mM methionine, 75 µM nitro blue tetrazolium chloride (NBT), 0.1 mM EDTA and 2 µM riboflavin. The sample was then placed 30 cm below two fluorescent lamps (2x 40 W) for 30 min in a box internally lined with aluminum foil. A reference cuvette, with enzyme and not irradiated, was used to measure the maximum absorbance at 560 nm, and a cuvette without the enzyme extract, that was irradiated, was used as a control. The SOD activity was expressed as % inhibition of NBT.

Glutathione reductase

The activity was determined by monitoring the GSSG-dependent (oxidised glutathione) oxidation of NADPH at 25 °C for 3 min at 340 nm, according to Foyer and Halliwell (1976). The reaction mixture (1 ml) contained 230 µl GSSG (0.5 mM), 30 µl EDTA (2 mM), 230 µl NADPH (0.2 mM), 450 µl potassium phosphate buffer (100 mM, pH 7.8) and 60 µl enzyme extract. The reaction was started by adding NADPH. The glutathione reductase activity was expressed as A_{340nm} mg^{-1} protein min^{-1}.

Catalase

The activity was determined by measuring the breakdown of H_2O_2 at 25 °C and the reaction was assayed for 1 min at 240 nm (Beers & Sizer, 1952). The reaction mixture (1 ml) contained 630 µl deionized water, 330 µl substrate (59 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0) and 40 µl enzyme extract, which initiated the reaction. Enzyme activity was expressed as µmol H_2O_2 red. mg^{-1} protein min^{-1}.

Extraction and analysis of hormones

The hormones (ABA, JA and SA) were extracted using a modified method of Forcat, Bennett and Mansfield, (2008) using the extraction mixture from

Segarra, Jáuregui, Casanova and Trillas (2006), with an additional use of solid phase extraction (SPE). The standards used were ABA, JA, SA and prednisolene at 0,01µl/mg suspended in MeOH. The hormones were expressed relative to the reference hormone.

Frozen (250 mg) leaf tissue was ground to a fine powder with liquid nitrogen and extracted in a 1.5 ml Eppendorf tube containing two silver beads (4.8 mm) and using 400 µl extraction solvent [MeOH/H_2O/acetic acid (10:89:1; v/v/v)]. The extraction mixture was vortexed for 2 min, incubated for 30 min on ice and then centrifuged at 13 000 rpm for 10 min. The extraction process was repeated twice and the supernatants were combined. For quantification normalisation purposes, an internal standard (IS, prednisolone) was added to each sample and three random samples were spiked with the hormonal standards (ABA, JA and SA).

The combined supernatant was extracted by solid phase extraction (SPE) cartridges. These cartridges (3 ml, 500 mg supelclean™ LC-SCX SPE tubes from Supelco) were conditioned with 6 ml MeOH and equilibrated with 12 ml MeOH/H_2O/acetic acid (10:89:1; v/v/v) before sample application at a slow rate of 2 drops per second. Cartridges were washed with 3 ml MeOH/H_2O/acetic acid (10:89:1; v/v/v), dried and the trapped analytes were eluted with MeOH/H_2O (80:20; v/v). The samples were dried in a Savant SC 210 SpeedVac Concentrator before reconstitution and analysis.

Sample analysis

Samples were analysed using an ABSCIEX 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu HPLC as a front end. All data acquisition and processing was performed using Analyst 1.5.2 (AB SCIEX) software.

Twenty microliters of each extracted sample were separated on a C18 (Restek Allure PFP propyl, 5µm, 50 x 2.1mm) column at a flow rate of 300 uL/min using a fast 1 min gradient from 1% to 100% mobile phase B (80% Acetonitrile containing 7.5 mM Ammonium formate); an additional 1 min at 100% B was included followed by column re-equilibration for a total 7 min analysis time in negative ionisation mode. Eluting analytes were ionised by electrospray in the TurboV ion source with a 550 °C heater temperature to evaporate excess solvent, 40 psi nebuliser gas, 40 psi heater gas and 25 psi curtain gas, with the ion spray voltage set at -4500 V.

To analyse the sample, a targeted Multiple Reaction Monitoring (MRM) workflow was followed on the instrument. During an MRM scan type the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass constitutes a transition. The instrument jumps between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting less than a second. If a transition is detected, the instrument's response is registered and this ion intensity value is plotted as a chromatogram.

The targeted analyses for the analytes consisted of 2 transitions each (jasmonic acid: 209.1>59.0, 209.1>165.2; abscisic acid: 263.1>153.1, 263.1>219.1; salicylic acid: 136.9>93.2, 136.9>75.2; prednisolone (IS): 359.2>329.1, 359.2>259.3). The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the second transition was used as the qualifier. The qualifier serves as an additional level of confirmation for the presence of the analyte. The retention time for these three transitions needs to be the same.

The integrated peak area of each quantifier was normalised to the integrated peak area of the IS (prednisolone). The normalised peak area values were used

in a relative quantitative fashion for comparing analyte levels in the different treatments.

Defence-related gene expression

Trizol Ribonuclease (RNA) extraction

Frozen leaf tissue (100 mg) was ground to a fine powder using liquid nitrogen. Total RNA was extracted using Trizol™ Reagent (Invitrogen™) (Chomczynski & Sacchi, 1987) and residual DNA was removed with 5 U DNasel (Fermentas) according to the manufacturer's instructions. The Nanodrop 2000 spectrophotometer (Thermoscientific, Waltham, Massachusetts) was used to determine total RNA concentration.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis was done according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin, Beaulieu, Huggett, Jaggi, Kibenge, Olsvik, Penning, & Toegel, 2010). RT-qPCR was performed using the Bio-Rad C1000 thermal cycler with CFX96 real-time attachment and the One-step KAPA™ SYBR® FAST qRT-PCR Universal (Kapa Biosystems). The optimum annealing temperature for all of the primer sets (Table 5.2) was determined using a temperature gradient from 58 to 70 °C, while the primer efficiency was determined with a 10 x serial dilution of RNA. Each 10 µl reaction contained 20 ng RNA template, 1x KAPA™ SYBR® FAST qPCR Master mix, 10 µM primer mix and 1x KAPA RT-mix. The amplification regime was 10 min at 50 °C and 3 min at 90 °C followed by 10 sec at 95 °C and 30 sec at the appropriate annealing temperature for 40 cycles. A melt curve was then done at the end of each cycle by increasing the temperature stepwise by 0.5 °C every 5 sec from 65 °C to 95 °C . The baseline and quantification cycles (Cq) were determined by the Bio-Rad CFX Software. RT-qPCR results were analysed using qBase ^{Plus} Software (Biogazelle). Each treatment was run in triplicate.

Reference genes had previously been validated (Paolacci, Tanzarella, Porceddu & Ciaffi, 2009; Castelyn, Appelgryn, Mafa, Pretorius & Visser, 2014) and used to normalise the expression of all experimental genes. Genes coding for phi-class glutathione-*S*-transferase F6 (*TaGSTF6*) and stress related-like protein and Inorganic pyrophosphate were validated (Table 5.2). Phenylalanine ammonia-lyase (PAL) primers were designed using Genbank accession number AY005474.1 (Primer3Plus **www.bioninformatics.nl**). The genes were expressed relative to the reference genes.

Table 5.2 Annealing temperature and amplification efficiency of primer pairs used for gene expression analysis.

Reference genes	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)	Efficiency values	R^2-value	Annealing temperature (°C)	Reference/ Accession number
GAPDH	TGTCCATGCCATGACTGCAA	CCAGTGCTGCTGCTTGGAATGATG	105	96.2%	0.971	59.5	Paolacci, *et al.*, 2009
18S rRNA	CCAGTGCTGCTTGGAATGATG	GTGACGGGTGACGGAGAATT	151	93.5%	0.992	59.5	Castelyn, *et al.*, 2014
Experimental genes	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**	**Amplicon size (bp)**	**Efficiency values**	**R^2-value**	**Annealing temperature (°C)**	**Reference/ Accession number**
Phenylalanine ammonia-lyase *(PAL)*	AAGCTGATGTTCGCGCAGTTCT	AAACCATAGTCCAAGCTGGGGT	103	107.8%	0.99	59.5	AY005474.1
Phi-Glutathione transferase F6 (*TaGSTF6*)	CTCCTGGGGTGGAGACAAT	GAAGGTGCTGGAGGTCTACG	95	98.8%	0.999	55.9	Schultz, 2014
Stress related-like protein indicator	CCTTGGTTGGTGACACATTC	CGTTCAGCCCATTTCTTTGC	137	99.8%	0.986	55.9	Botha, *et al.*, 2014
Inorganic Pyrophosphate	ACCGTCACTTCAGAGACAT	GCTGGGAGGAAATCATTCAC	119	98.5%	0.986	55.9	Botha, *et al.*, 2014

Data analysis

The enzyme activities, gene expression and hormone concentrations were measured in triplicate within each independent replicate. Outliers were assessed by boxplots and normality assessed by Normal Q-Q plots. Homogeneity of variances was assessed by Levene's test. Data were analysed using one-way analysis of variance (Anova, $p<0.05$, IBM SPSS statistics version 24) to compare means. A two-way Anova was conducted to determine whether the different treatments influence the stress reaction.

5.3 RESULTS

Phenylalanine ammonia-lyase plays a key role in the biosynthesis of SA and regulates the phenylpropanoid pathway (Chen, Silva & Klessig, 1993). Therefore, it is important to determine if and when PAL is activated because the upregulation of PAL increases SA and the induction of systemic acquired resistance (SAR).

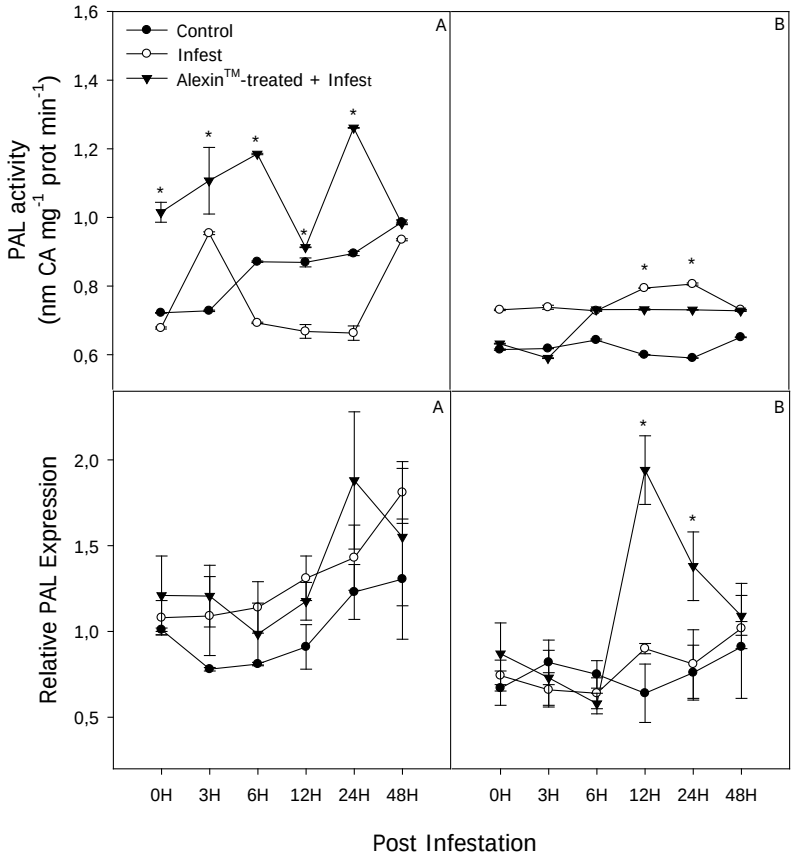

Figure 5.1: Effect of *RWASA2* infestation on PAL activity and expression in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Responses were measured at 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). (*) indicates significant differences ($p<0.05$).

Alexin™ treatment before infestation induced significantly higher PAL activity than *RWASA2* infestation alone in SST387 (Fig. 5.1 A: $p=0.00$). The activities were higher than in the control and the highest activity was recorded at 24 hpi. Phenylalanine ammonia lyase gene expression levels were not significantly ($p=0.63$) different between control, infestation and Alexin™ pre-treatment.

The activity of PAL was different in all the treatments in PAN3379 during the experimental period (Fig. 5.1 B: p=0.001). The differentially high activity was noted in infested plants without any prior treatment at 12 and 24 hpi. Alexin™ pre-treatment, on the other hand, increased activity from 6 hpi and the activity remained more or less constant and lower than those induced by infestation alone. Gene expression levels were significantly higher (p=0.000) at 12 hpi in Alexin™-treated plants compared to the control and infested untreated plants.

Although upregulation of PAL increases SA and the expression of SAR, H_2O_2 is also associated with plant defence signalling and the development of SAR (Chen, *et al.*, 1993). Hydrogen peroxide is generated via NADPH oxidase causing a rapid and intense oxidative burst. Therefore it was important to determine the activity levels of NADPH oxidase and H_2O_2.

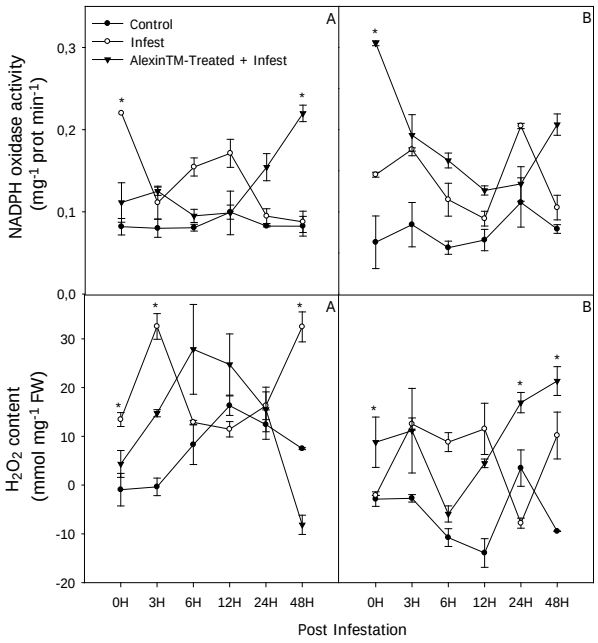

Figure 5.2: Effect of *RWASA2* infestation on NADPH oxidase activity and H_2O_2 concentration in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Activity levels were measured at 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). $^{(*)}$ indicates significant differences ($p<0.05$).

Infestation induced NADPH oxidase (Fig. 5.2 A: p=0.012) activity in SST387 Alexin™-treated SST387 plants from 12 hpi to 48 hpi. Interestingly, there was

already an increase in H_2O_2 (Fig. 5.2 A: p=0.00) level in Alexin™-treated plants. The level increased from just after infestation (0 hpi) and peaked at 6 hpi. Infestation of untreated plants induced increases in NADPH oxidase activity from 3 hpi, peaking at 12 hpi and declining thereafter. The H_2O_2 level spiked at 3hpi but was not sustained with further accumulation only after 24hpi.

A significant difference was also detected in NADPH oxidase (Fig. 5.2 B: p=0.009) and H_2O_2 (p=0.012) in PAN3379. Infestation of untreated plants induced the highest NADPH oxidase activity at 3 hpi and later at 24 hpi. The H_2O_2 content in these plants was high from 3 hpi and the level was sustained up to 12 hpi. This was expected because PAN3379 is resistant. The activity in Alexin™ pre-treated plants was at its highest at 0 hpi, and then infestation suppressed activity. The H_2O_2 content was also reduced by infestation, picking up only at 24hpi and 48 hpi.

Reactive oxygen species are important in protecting plants against intruders, but antioxidant enzymes need to keep the oxidative levels in check during stress episodes. This increase in antioxidant enzyme activities in either the chloroplast or other cytosolic compartments may improve protection against oxidative stress within plants (Foyer, Lelandias, & Kunert., 1994). Both SOD activity and ascorbate-glutathione cycle enzymes including GR are a part of regulatory components of plant cells under oxidative stress; coordination between these protective mechanisms is critical for plant survival (Ananieva, *et al.*, 2004).

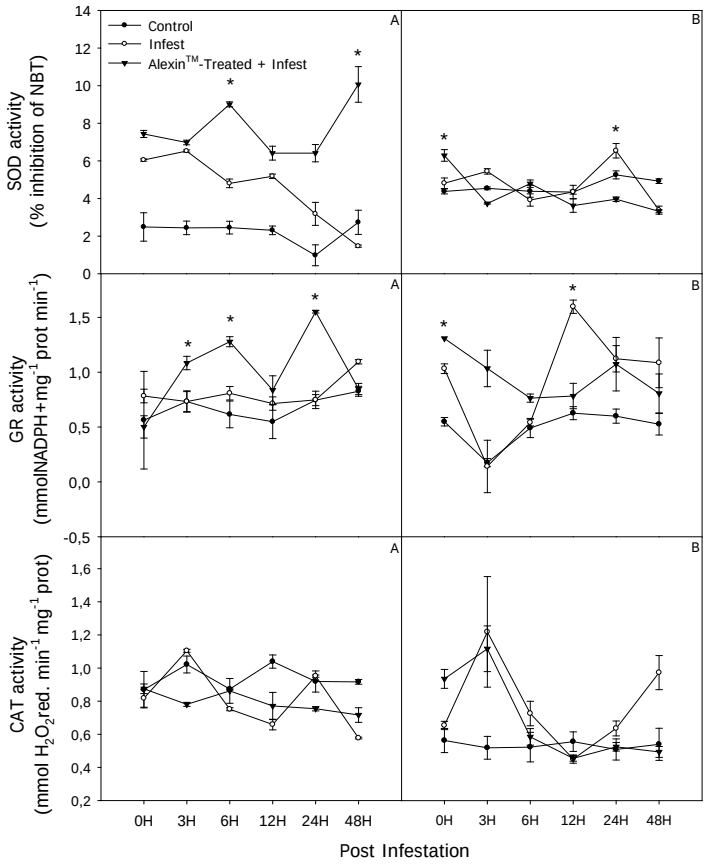

Figure 5.3: Effect of *RWASA2* infestation on SOD, GR and CAT activities in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Activity levels were measured 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). (*) indicates significant differences ($p<0.05$).

Alexin™ treatment mediated an increase in SOD activity of SST387 (Fig. 5.3 A: $p=0.000$) higher than in other treatments throughout the observation period. There was an earlier spike at 6 hpi and later at 48 hpi. In these plants, GR activity was significantly ($p=0.005$) higher than in all other treatments (untreated infested and control). Even though there was a general increase that was significantly higher from 3 hpi to 24 hpi (at which point activity began to decline), there was an isolated dip at 12 hpi. There were no significant ($p=0.946$) differences in CAT activity between the three treatments in SST387.

In the resistant cultivar PAN3379 (Fig. 5.3 B), infestation of untreated plants induced a significant SOD increase at 24 hpi ($p=0.029$). At the same time, Alexin™ treatment suppressed activity; SOD activity was highest at 0 hpi, before infestation. Infestation of untreated plants induced GR activity that

peaked at 12 hpi. As in SOD activity, Alexin™ suppressed GR activity throughout the 48 h study period. Once again the highest activity was at 0 hpi, and this declined gradually, as duration of infestation increased. Reminiscent of SST87, there were no significant differences (p=0.074) in CAT activity among all of the treatments.

The hormones associated with plant immune systems are small molecules that are important for the regulation of plant growth, development and reproduction. These hormones, ABA, JA and SA, are important in the complex network of communicating signalling pathways (Pieterse, *et al.*, 2009).

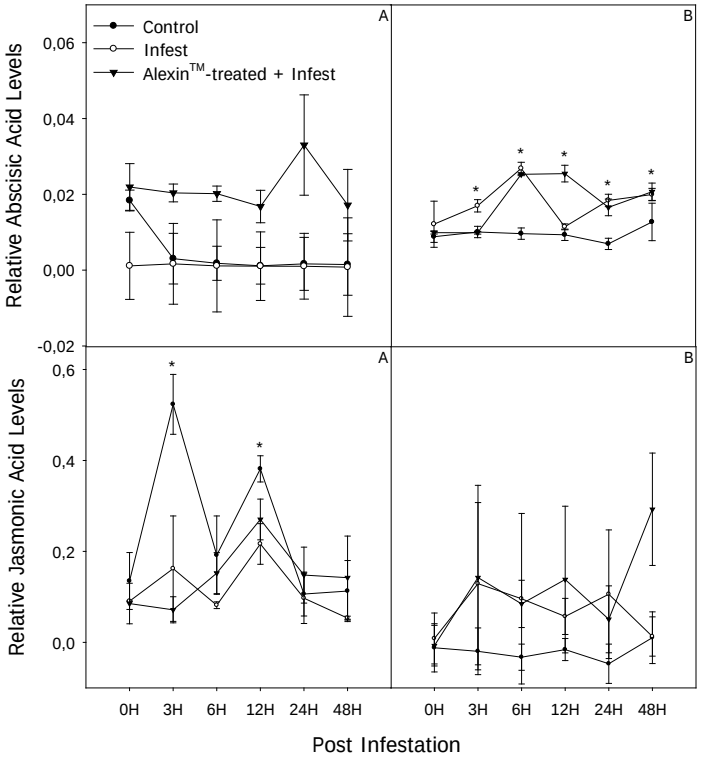

Figure 5.4: Effect of *RWASA2* infestation on ABA and JA in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Activity levels were measured at 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). (*) indicates significant differences (p<0.05).

There was an increase in the accumulation of ABA at 24 hpi in SST387, although there was no significant difference between treatments (Fig. 5.4 A: p=0.296). Accumulation of JA was significant (p=0.005), with the control being the highest at 3 hpi and JA levels were suppressed in infested and Alexin™-treated

plants. In PAN3379, ABA accumulation was significantly higher both in infested and infested Alexin™-pre-treated plants in comparison to the control (Fig. 5.4 B: p=0.026). In both treatments accumulation began as early as 3 hpi with a peak at 6 hpi. In Alexin™-pre-treated plants higher ABA levels were sustained up to 12 hpi, beyond which there was a gradual decline towards 48 hpi. The various treatments did not significantly affect the levels of JA in this cultivar (p=0.359).

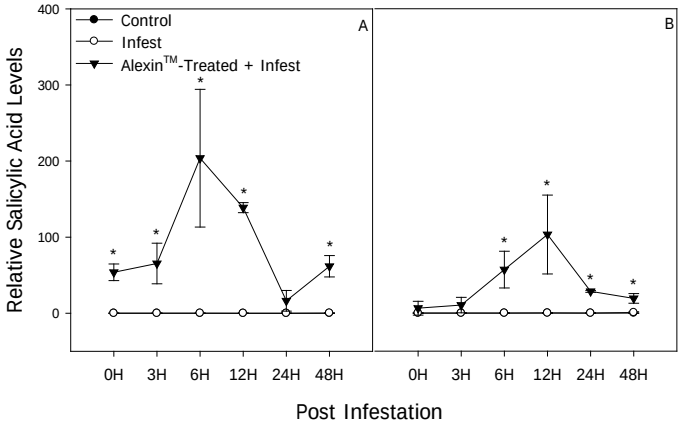

Figure 5.5: Effect of *RWASA2* infestation on SA in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Activity levels were measured at 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). (*) indicates significant differences (p<0.05).

Alexin™ treatment prior to infestation induced significantly (Fig. 5.5 A: p=0.00; B: p=0,002) higher SA content throughout the trial period in both SST387 and PAN3379. The differential increase occurred earlier and to quantitatively higher levels in SST387 than PAN3379. Infestation alone induced negligible amounts of SA. Salicylic acid accumulation was higher overall in both cultivars when compared to JA and ABA. The SA level in control and only infestation was extremely low and is not included in Fig. 5.5.

The following genes were identified as playing a role in RWA resistance and are associated with antibiosis and tolerance. Schultz (2014) established that the gene *TaGSTF6* is important for expression of antibiosis-type resistance during RWA infestation. Botha, Van Eck, Burger and Swanevelder (2014) identified

two transcript-derived fragment (TDF) interactors, namely inorganic pyrophosphatase and stress related-like protein interactor. The genes were classified into the stress and signal transduction category and are part of several TDFs that function as a component of photosynthesis.

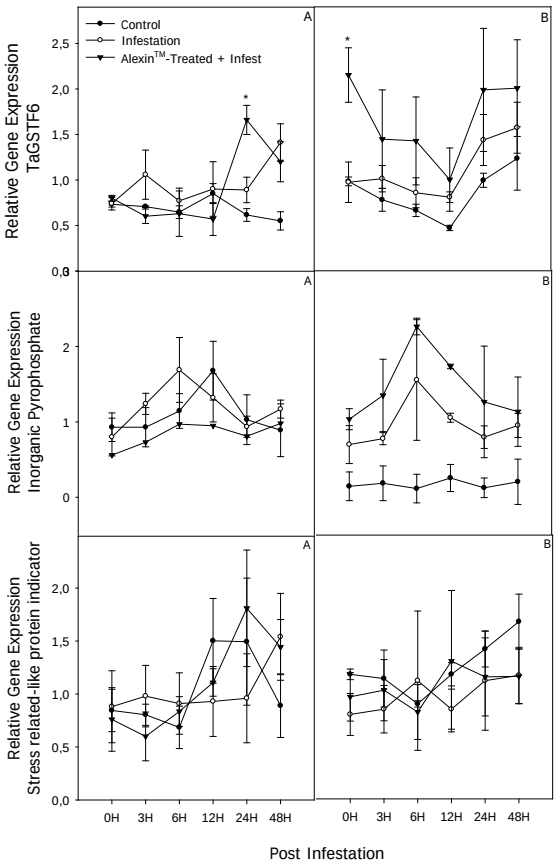

Figure 5.6: Effect of *RWASA2* infestation on the expression of *TaGSTF6*, inorganic pyrophosphate and stress related-like protein indicator gene levels in SST387 (A) and PAN3379 (B): control; infestation and 0.375% (v/v) infested. Activity levels were measured at 0, 3, 6, 12, 24 and 48 hpi. Error bars indicate standard deviation (n=3). (*) indicates significant differences ($p<0.05$).

Alexin™ treatment before infestation mediated a significant increase in expression of *TaGSTF6* gene levels, which occurred at 24 hpi in SST387 (Fig. 5.6 A: $p=0.001$). No significant differences were noted in the expression levels of either inorganic pyrophosphatase (Fig $p=0.479$) or stress related-like protein indicator ($p=0.568$).

In PAN3379 (Fig. 5.6 B) there was a significant difference between treatments at 0 hpi in *TaGSTF6* levels ($p=0.00$). Alexin™ treatment suppressed expression

of the gene. Infestation alone (p=0.00) and infestation of Alexin™-treated (p=0.001) plants induced significantly higher levels of the inorganic pyrophosphatase gene. But between the two treatments there were no significant differences (p=0.142). None of the treatments in any of the cultivars induced significant (p=0.302) differences in the expression of the stress related-like protein indicator.

5.4 DISCUSSION

This study focused on the effect of Alexin™-mediated defense responses associated with ROS and antioxidants coupled with an increase in SA and plant-aphid interactions. Many studies have shown that SA signaling usually activates these resistance responses and therefore by focusing on these responses we might be able to determine how the host responds to Alexin™ treatment on a biochemical and molecular level. Hormone accumulation particularly that of ABA, JA and SA were also investigated to determine whether antagonistic cross-talk could occur within the host plant when treated with Alexin™. The expression of defence-related genes associated with antibiosis and tolerance was determined since antibiosis and tolerance were expressed in Alexin™ treated SST387 (Chapter 4).

Previous studies performed with Alexin™ indicated disease control with accelerated defence responses (Jayaraj, *et al.*, 2009; McDonald, 2006). We therefore hypothesised that the resistant cultivar (PAN3379) would respond quicker to the infestation, activating expression of intense defence responses, while the susceptible cultivar (SST387) would also show increased resistance, although perhaps not as strongly as the resistant cultivar. However, in this study we determined that the defence responses such as PAL activity, H_2O_2, SOD and GR in the susceptible cultivar were significantly boosted by Alexin™, while responses were suppressed in the resistant cultivar.

This boost in defence responses of the susceptible cultivar was evident in the increase of PAL activity and SA accumulation. Reactive oxygen species are produced in plants during normal and stressed conditions, but when stressed, ROS production increases significantly to protect the plant (Ton, *et al.*, 2006). The increase in H_2O_2 level confirmed an oxidative burst protecting the plant against the RWA. Hydrogen peroxide is generated via NADPH oxidase but in this treatment NADPH oxidase activity only increased 24 hpi compared to the infestation treatment. Yet, there had already been an early increase of H_2O_2. Kadota, Sklenar, Derbyshire, Strasfeld, Asai, Ntoukakis, Jones, Shirasu, Menke, Jones and Zipfel (2014) recently suggested that NADPH oxidase can be primed after a series of rapid phosphorylation events, but does not trigger ROS production. Therefore it might be speculated that Alexin™ mediates the priming of NADPH oxidase in SST387, triggering a rapid and enhanced oxidative burst after infestation.

The high levels of oxidants also trigger antioxidant activity leading to the suppression of cell death and ensuring the survival of surrounding cells (Alscher, Erturk & Heath, 2002). This antioxidant response was also activated in the treated susceptible cultivar with the increase of SOD and GR activities. Superoxide dismutase is the first line of defence against ROS by catalysing the dismutation of O_2 to O_2^- and H_2O_2 (Trchounian, Petrosyan & Sahakyan, 2016). In treated SST387 there was an increase in SOD activity after an initial increase of H_2O_2. In findings of Moloi (2002), this response occurred in the resistant cultivar, agreeing with the hypothesis that the susceptible cultivar might increase RWA resistance responses when treated with Alexin™.

This significant increase of H_2O_2 also coincided with the increase in GR and corresponds with the findings of Pieterse, *et al*. (2009) in *Arabidopsis.* They demonstrated that glutathione is a major determinant of cellular redox homeostasis and that it coincided with the increase of SA genes (Pieterse, *et al.*, 2009). Therefore, changes in the cellular redox state play a role in the transduction of the SA signal. On the other hand, GR also causes glutathione

reduction and antioxidative processes in plants (Trchounian, *et al.*, 2016) that could be connected with the second increase in activity. Argandoña (1994) suggested that the second increase in GR after aphid (*Sitobion avenae*) infestation is to protect the plant against the physiological damage indirectly produced by the aphids. The second increase of GR activity in this study could have been a stimulant to protect the cells against oxidative damage, contributing to the hypothesis that Alexin™ might increase antioxidant activity to protect the plant.

The resistant cultivar did not respond as hypothesised and showed suppression with the Alexin™ treatment. The SOD and GR levels were high at 0hpi and suppressed the rest of the 48 hpi period, and when comparing NADPH oxidase and H_2O_2 levels with the antioxidants, it seems as if there was a delay in ROS accumulation. Therefore, in both cultivars it seems that Alexin™ treatment influences GR activity and it could be speculated that SA and GR work closely with each other, stimulating or even suppressing the induction of defence mechanisms.

The hormonal accumulation of ABA, JA and SA was also determined. Salicylic acid accumulation was significantly higher in both cultivars treated with Alexin™; therefore sufficient application was obtained and the plant did absorb the priming agent. Yet in both cultivars infestation alone did not significantly increase SA levels. This was expected in the susceptible cultivar but not in the resistant cultivar, which might have induced defence responses through other signalling pathways. Abscisic acid and JA are also associated with defence signalling during stress, yet both ABA and JA were demonstrated to be antagonistic to the onset of SA accumulation. In a comparison between the control, infested and treated plants of the susceptible cultivar, JA accumulation was the highest in control; the same antagonistic behaviour was observed with the increase of ABA at 24 hpi but a decrease in SA accumulation. According to Pieterse, *et al.* (2009), during activation of SAR the ABA genes were supressed,

indicating that ABA is an important regulator in abiotic and biotic stress responses.

Abscisic acid is an early defence response protecting the plant against pathogens by activating stomatal closure and callose deposits. This rapid defence response prevents the unnecessary activation of SA and JA-dependent defences and saves plant energy (Ton, *et al.*, 2009). However this could lead to the suppression of PAL and SA, as found by Audenaert, De Meyer and Höfte (2002) in tomato plants. This early response was noted in the resistant cultivar with infestation only, but was sustained in the treated plants. This could be the source of suppression in the Alexin™-treated PAN3379 defence mechanism. Therefore, cross-talk between the signalling hormones ABA, JA and SA is complex, showing either synergistic or antagonistic effects, depending on the host.

Genes associated with RWA-plant interactions were studied. The *TaGSTF6* gene is part of the glutathione-*S*-transferase (GST) group. The GST family is one of the larger groups found in plants and may play a role in limiting metabolic changes in the plant that increase during oxidative stress (Sappl, Oñate-Sánchez Singh & Millar, 2004). Schultz (2014) determined that H_2O_2 plays an important role in *Dn1*-mediated resistance and that H_2O_2 and *TaGSTF6* are necessary for an antibiosis resistance response. In this study there was an increased expression of *TaGSTF6* in the susceptible cultivar treated with Alexin™. These findings correlated with Chapter 4 findings regarding antibiosis-mediated responses in SST387. Schultz (2014) also determined this, even though with the silencing of *TaGSTF6* in BSMVGST and a down-regulation of H_2O_2, the plants still expressed moderate resistance. In this study, the gene was the highest at 0 hpi with Alexin™ treatment in PAN3379 but no increase was expressed in only infested plants; therefore, other resistance mechanisms could be playing a role in RWA-resistance in PAN3379.

Transcript-derived fragment genes tested in this study are part of a component associated with photosynthesis. Russian wheat aphid feeding can decrease chlorophyll in susceptible wheat (Wang, Chen & Li, 2004) which indicates damage to Photosystem Ⅰ（Botha, Lacock, Van Niekerk, Matsioloko, Du Preez, Loots, Venter, Kunert & Cullis, 2006). According to Botha, *et al*. (2014) the *Dn* gene was coupled with aphid recognition and is important in the eventual development of tolerance. In their study the inorganic pyrophosphatase increased at 2 hpi in the susceptible cultivar, while the resistant cultivars showed an increase from 0 hpi. In this study the gene was expressed in PAN3379 Alexin™ treatment and only infested treated plants and could indicate tolerance, thus confirming that other defence mechanisms were playing a role.

Another aspect to consider is how the plant receives the signal that induces priming. Gene-for-gene interaction is an important factor to consider when studying priming agents and the activation of resistance (Thakur & Sohal, 2013). Priming agents may be divided into two groups: “general” and “race specific”. While general priming agents are able to trigger defence in both host and non-host plants, race-specific agents induce defence responses leading to disease resistance only in specific host cultivars. Therefore, molecular mechanisms underlying priming are still poorly understood and future research is needed to fully understand the molecular basis of priming during interaction with the RWA.

5.5 CONCLUSION

Alexin™ enhanced SST387 resistance responses to RWA infestation, indicating that priming can play a very important part in pest management strategies to boost the defences of plants and to help advance the co-evolutionary arms race between plant and pest towards plant resistance. Nonetheless, chemical-host-aphid interactions are far more complex than it may seem, as proven by the PAN3379 and Alexin™ treatment, where SA accumulation was increased,

triggering suppression of the resistance response. This resistant cultivar did however show an oxidative burst and plant vigour in the absence of Alexin™, validating the premise that other defense mechanisms play a role in PAN3379-aphid interaction. Therefore, more collaborative efforts between wheat breeders and research in plant immunity are needed to ensure that both avenues are exploited to the advantage of plant health and wheat management systems.

Chapter 6

Screening for RWA-resistance in Alexin™-treated wheat cultivars under dryland field conditions

6.1 INTRODUCTION

Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are two of the most important cereal crops, together contributing about 41% of the world's cereal production (Fageria, Baligar & Jones, 2011). Wheat is a winter and spring cereal crop, with a growing season of 130 to 190 days. Winter wheat requires vernalisation for normal flower development. The ideal climate for spring wheat is warmer temperatures of between 22 and 34 °C; while for winter wheat, cooler temperatures, ranging from 3 to 25 °C, are more suitable (Fageria, *et al.*, 2011). The environment, particularly moisture or temperature conditions can strongly affect grain-filling (Garcia del Moral, Rharrabti, Villegas & Royo, 2003).

The Russian wheat aphid (RWA) is a very important economical pest that affects both wheat and barley. Sporadic outbreaks of the RWA occur in the eastern Free State where new virulent aphid biotypes often emerge. Each percentage of aphid infestation can lead to a 0.5% loss in yield (Karren, 1989). In South Africa yield losses of between 21% and 92% were recorded by Basky (2003).

Using resistant wheat crops is the most economically efficient method of reducing aphid growth and damage. However, with new RWA biotypes emerging and overcoming wheat resistance, alternative managing methods must be explored. One of these methods is priming.

Priming is a plant defence mechanism expressed following exposure to biotic and abiotic stress. In subsequent attack the plant can recall the previous stress and respond rapidly and efficiently (Conrath, 2009) because it has formed an immunological memory that potentiates rapid expression of resistance. The priming effect can also be induced by various chemicals (Walters, Walsh, Newton & Lyon, 2005). In this study Alexin™, a commercial plant activator that contains nutrients and salicylic acid derivatives, has been used as a priming agent, and we investigate its effect on the defence responses towards the Russian wheat aphid. Primed plants express a variety of defence responses when challenged by pests and pathogens. Jayaraj, *et al*. (2009) found that defence responses were induced in Alexin™-treated carrots when inoculated with *Alternaria radicula* and *Botrytis cinerea*. Since phloem-feeding insects (PFIs) can also stimulate the same response as plant pathogens (Mohase & Van der Westhuizen, 2002b; Van der Westhuizen, *et al*., 1998) it can be expected that Alexin™ might play a role in inducing rapid plant defence responses towards the RWA.

Salicylic acid is known as a plant growth regulator that increases bio-productivity. On horticultural species there is an increase in yield without affecting the quality of the fruits (Hayat & Ahmad, 2007). This was observed by Aristeo-Cortés (1998) where SA induced bigger tubers in carrots (60%), beet (16%) and radish (200%). Hayat, Fariduddin, Ali and Ahmad (2005) further found that growth traits of wheat were improved as a result of soaking the seeds in SA.

To determine wheat yield there are three important components: spike number, seeds per spike and kernel weight (Mornhinweg, *et al*., 2006). Mornhinweg, *et al*. (2006) tested RWA-infested barley and found that even though these yield components varied between resistant lines, these lines nonetheless had a higher yield than susceptible cultivars in the presence of RWA (Randolph, *et al*., 2005).

The scoring of RWA feeding symptoms on barley leaves is a good indication of RWA impact on yield as noted by Calhoun, Burnett, Robinson and Vivar (1991), who found that resistant barley with a low score of feeding symptoms produced higher yield. Therefore, it is important to determine if Alexin™ application under field conditions can induce tolerance and maintain or increase yield during RWA stress.

Induced resistance involves intense levels of expression of defences causing a diversion of resources usually allocated for plant growth. Most studies on the costs and benefits of induced resistance have focused on defences activated directly by the inducing agent; however, priming plants for resistance expression does not incur additional costs unless the plant is challenged by a pathogen or pest. Van Hulten, *et al*. (2006) demonstrated the benefits of priming in *Arabidopsis*, finding that induction of priming involved fewer costs than direct expression of defences, and that it was beneficial in terms of plant growth rate and fitness under disease pressure. In this study we assumed that Alexin™, a priming agent, would involve diversion of fewer resources from growth towards defence because it also contains important nutrients that might boost plant growth.

Although the glasshouse experiments showed tolerance and expression of defences in certain cultivars, it must be noted that even though screening for resistance under controlled glasshouse conditions is fast, it can be misleading. Conditions in the field can be more erratic and therefore can affect resistance. The objective of this field trial was therefore to determine whether Alexin™ could improve the resistance of wheat cultivars to RWA feeding under dryland field conditions and as a result improve yield. The three yield components and a field scoring system were used to measure yield and induced RWA feeding damage.

6.2 MATERIALS AND METHODS

Alexin™ (Reg no. B3835, Act no. 36 of 1947) is a registered trademark of AECI Limited (Nulandis® a division of AECI Limited). It is a liquid organic nutrient complex (containing salicylic acid derivatives, oligosaccharides, 4.5% potassium, 2.6% calcium, 0.8% magnesium and 0.2% boron). The optimum concentration of 0.375% (v/v) Alexin™ in sterile water containing a plant adhesive (LI700, 0.06%, v/v) was used. Alexin™ treatment was applied as a foliar spray to plants at three-leaf stage.

Six dryland winter wheat cultivars (Elands, Gariep, Senqu, PAN3118, PAN3379 and SST387) were cultivated in field trials for two wheat seasons (2014-2015 and 2015-2016) to determine yield and quality parameters after treatment with Alexin™. PAN3118 is susceptible to all the RWA biotypes; SST387, Elands, Gariep and Senqu are resistant to *RWASA1* and susceptible to *RWASA2*, *RWASA3* and *RWASA4*, while PAN3379 is resistant to *RWASA1*, *RWASA2*, *RWASA3* and *RWASA4*. Seeds were obtained from Agricultural Research Council-Small Grains (ARC-SG) in Bethlehem, South Africa.

The trials for both seasons were planted in a randomised complete block design with a plant density of 30 kg/ha and row spacing of 7 cm. A block consisted of 5 m X 5 row plots with four replications for each treatment and cultivar. A five row wheat hedge surrounded the randomized wheat blocks and the middle three wheat rows within the blocks were harvested for yield (kg/ha), hectolitre mass (kg/hl) and protein (12%) content to reduce contamination or drift between treatments. The cultivars were planted at ARC-SG in Bethlehem (S28.15663°E28.29116°).

Environmental conditions

Relative humidity (%), rain (mm), evaporation and temperature (ºC) were monitored by the Agricultural Research Council-Small Grains (ARC-SG), at

latitude: -28.16277; longitude: 28.29733; station name: Bethlehem: Small Grains stitute.

Field trial 2014-2015

The 2014-2015 field trial was planted on 11/08/2014. Plots were treated with Alexin™ on 14/10/2014 when the plants were at three-leaf stage and the second spray was on 11/11/2014 at tillering stage (Zadoks growth stage 10). The trial was evaluated for RWA damage on 05/12/2014 at stem extension (Zadoks growth stage 45) and harvested on 19/01/2015. The trial consisted of three treatments with four replicates.

Table 6.1 Treatments in first field study. Season 2014-2015.

Treatment	1	2	3
Alexin™ Application	0 (DH_2O) None	0.375% (v/v) One application	0.375% (v/v) Two applications

Field trial 2015-2016

The 2015-2016 trial was planted on 30/07/2015. Plots were treated with Alexin™ on 21/09/2015 when the wheat plants were at three-leaf stage. The trial was evaluated for RWA damage on 26/10/1015 at stem extension (Zadoks growth stage 45) and harvested on 07/01/2016. The trial consisted of two treatments with four replicates.

Table 6.2: Treatments in second field study. Season 2015-2016.

Treatment	1	2
Alexin™ Application	0 (DH_2O) None	0.375% (v/v) One application

Four-point damage rating scale

The cultivars were evaluated at adult stage using a 1-4-point scale where, 1 = no damage: Escape (E)/Resistant (R); 2 = chlorotic spots on leaves: Resistant (R); 3 = longitudinal streaks on leaves: Moderately susceptible (MS); 4 = rolling of leaves: Susceptible (S) (Photo 6.1.). Russian wheat aphid samples were

collected at each trial site and the biotype status of the aphids was determined through screening against a differential set with known resistance genes.

1. No visible damage: Escape(E)/ Resistance(R)	2. Chlorotic spots: Resistance(R)	3. Chlorotic streaks: Moderately Susceptible (MS)	4. Leaves rolled: Susceptible (S)

Photo 6.1: Four-point damage rating scale for Russian wheat aphid (*Diuraphis noxia)* resistance in adult wheat plants under field and natural RWA infestation (Jankielsohn, Masupha, & Mohase, 2016).

After harvest the yield (kg/ha), hectolitre mass (kg/hl) and protein (12%) content were determined for each plot.

Screening of RWA biotypes

Screening and identification of RWA biotypes were performed using the guidelines of Jankielsohn (2014). Each individual aphid sampled in the field was transferred to a wheat plant and placed in cages covered with nets (315 micron) to produce a clone colony for screening. The biotype of each RWA clone colony was determined by screening the feeding damage on eleven previously established plant resistance sources containing designated resistance genes *Dn1* to *Dn6* and *Dnx* as well as *Dny*.

Statistical analysis

The difference between treatments was analysed across all cultivars. Outliers were assessed by boxplots and normality assessed by Normal Q-Q plots. Homogeneity of variances was assessed by Levene's test. Data were analysed using one-way Anova (IBM SPSS statistics version 24, Least significant difference LSD, $p<0.05$). The means of host plant responses, RWA damage score, yield (kg/ha), hectolitre mass (kg/hl) and protein (12%) content in plants treated with Alexin™ in field trials were compared in six different wheat cultivars.

6.3 RESULTS

Field trial 2014-2015

Field trials were planted in the 2014-2015 wheat season to determine the effect of Alexin™ on six different wheat cultivars.

Table 6.3: Season 2014-2015. Comparison between resistance to Russian wheat aphid, RWA (damage rating score), yield (kg/ha), hectolitre mass (kg/hl) and protein content (12%) of six dryland wheat cultivars planted at ARC-SGI (Bethlehem; S28.15476°E28.28708°) and treated with Alexin™ application x1; x2. ^{abc} means without common letters differ significantly, ANOVA, LSD ($p<0.05$, SPSS).

Cultivar	Treatment	RWA damage	Yield (kg/ha)	Hectolitre mass (kg/hl)	Protein content (12%)
Elands	Control	1.75^{ad}	2.272^{ac}	78.3^{a}	14.8^{ad}
Elands	Alexin™x1	1.25^{ad}	1.9012^{abc}	74.6^{b}	14.3^{a}
Elands	Alexin™x2	1.5^{ad}	1.4812^{ab}	75.7^{a}	14.7^{ad}
Gariep	Control	3.75^{b}	1.4808^{ab}	72.6^{bc}	14.8^{a}
Gariep	Alexin™x1	2.75^{ab}	0.9436^{b}	71.1^{bc}	14.9^{ad}
Gariep	Alexin™x2	3.5^{b}	1.1396^{ab}	72.2^{bc}	14.9^{ad}
Senqu	Control	0.75^{acd}	1.4428^{ab}	75.0^{ab}	15.3^{ad}
Senqu	Alexin™x1	1.75^{ad}	0.9772^{b}	75.6^{a}	15.2^{ad}
Senqu	Alexin™x2	0.5^{acd}	0.688^{b}	74.8^{a}	15.9^{abd}
PAN3118	Control	3.25^{ab}	2.1484^{ac}	72.6^{bc}	14.9^{ad}
PAN3118	Alexin™x1	3.25^{ab}	1.9884^{abc}	72.8^{b}	15.0^{ad}
PAN3118	Alexin™x2	3.5^{ab}	2.044^{abc}	74.5^{b}	14.8^{ad}
PAN3379	Control	1.25^{ad}	1.2792^{ab}	71.5^{bc}	13.8^{a}
PAN3379	Alexin™x1	0.5^{acd}	1.524^{ab}	72.3^{bc}	13.9^{a}
PAN3379	Alexin™x2	0^{abcd}	2.1232^{ac}	72.5^{bc}	14.1^{a}
SST387	Control	3.5^{b}	2.2768^{ac}	68.95^{bcd}	14.5^{a}
SST387	Alexin™x1	0.5^{acd}	1.502^{ab}	67.075^{d}	14.7^{ad}
SST387	Alexin™x2	0^{abcd}	2.844^{c}	68.975^{bcd}	13.7^{ac}
	SE	±0.3226	±0.692	±6.843	±0.838
	LSD	0.3226	0.2316	0.7285	0.2549

The RWA damage was lower in Alexin™-treated single and double applications PAN3379 and SST387 (Table 6.3). There were significant differences in yield

(p=0.045), hectolitre mass (p=0.00) and RWA damage (p=0.00) between the controls and the Alexin™ treated cultivars, although there were no significant differences recorded in the protein content (p=0.117). SST387 had the highest yield, followed by PAN3118, Elands and PAN3379, while Senqu and Gariep had the lowest (Table 6.3). SST387 had the lowest hectolitre mass of all the cultivars, while Senqu had the highest protein content (Table 6.3). Nonetheless these values were not different from control.

Table 6.4: Season 2014-2015. Four-point damage rating scale for Russian Wheat Aphid (*Diuraphis noxia*) resistance in adult wheat under field and natural RWA infestation. [abc] means without common letters differ significantly, ANOVA, LSD (p<0.05, SPSS).

Cultivar	Control		Alexin™ x1		Alexin™ x2	
	Score	Damage rating	Score	Damage rating	Score	Damage rating
Elands	2[ad]	R	1[ad]	R	2[ad]	R
Gariep	4[b]	S	3[ab]	MS	4[b]	S
Senqu	1[acd]	R	2[ad]	R	1[acd]	R
PAN3118	3[ab]	MS	3[ab]	MS	4b	S
PAN3379	1[ad]	R	1[acd]	R	1[acd]	R
SST387	4[b]	S	1[acd]	R	1[acd]	R

1 = No visible symptoms (Resistant – R); 2 = Chlorotic spots (R); 3 = Chlorotic striping (Moderately Susceptible - MS); 4 = Leaves rolled (Susceptible – S)

The damage rating score on Gariep was lower in the 1x Alexin™ treatment and the damage rating on treated SST387 decreased significantly with Alexin™ treatment (Table 6.4). The significant difference for RWA damage rating score was p=0.00, SE ±0.3226 and the LSD 0.3226 (p<0.05).

Field trial 2015-2016

RWA damage was lower in the Alexin™-treated Elands, PAN3379 and SST387, but higher in the other Alexin™-treated cultivars (Table 6.5). PAN3118 and SST387 were stunted because of insufficient vernalisation. As a result, the hectolitre mass for these two cultivars could not be determined because the sample size was too small.

Table 6.5: Season 2015-2016. Comparison between resistance to Russian wheat aphid, RWA (damage rating score), yield (kg/ha), hectolitre mass (kg/hl) and protein content (12%) of six dryland wheat cultivars planted at ARC-SGI (Bethlehem; S28.15476°E28.28708°) and treated with Alexin™. [abc] means without common letters differ significantly, ANOVA, LSD ($p<0.05$, SPSS).

Cultivar	Treatment	RWA damage	Yield (kg/ha)	Hectolitre mass (kg/hl)	Protein content (12%)
Elands	Control	3,25[a]	1,0593[a]	75,1467[a]	16,8178[a]
Elands	Alexin™	2,25[b]	1,0013[a]	74,8600[a]	16,7862[a]
Gariep	Control	3[ab]	1,1743a	76,2200[a]	16,6739[a]
Gariep	Alexin™	3,5[a]	1,1243[a]	74,8333[a]	16,6680[a]
Senqu	Control	3[ab]	0,9593[ac]	75,5600[a]	16,7862a
Senqu	Alexin™	3,5[a]	0,9457[ac]	75,0200[a]	16,5244[a]
PAN3118	Control	0[abcd]	0,1053[b]	-	16,9991a
PAN3118	Alexin™	1,625[bc]	0,0670[b]	-	16,4340a
PAN3379	Control	3,75[a]	1,0317[a]	74,3700[a]	17,3338[a]
PAN3379	Alexin™	3[ab]	1,0143[a]	76,0000a	17,2941[a]
SST387	Control	0,75[abcd]	0,0190[b]	-	16,9102[a]
SST387	Alexin™	0a[bcd]	0,0250[b]	-	18,3280[ab]
	SE	±0.352	±0.13	±0.518	±0.910
	LSD	0.0294	0.0178	0.0357	0.0473

There were significant differences (Table 6.5) in yield ($p=0.0178$) between SST387 and PAN3118 that produced low yield, but differences between controls and the Alexin™ treatments in hectolitre mass ($p=0.217$) and protein content ($p=0.364$) were not significant. Gariep had the highest yield, followed by Elands, PAN3379 and Senqu (Table 6.5). Gariep had the lowest hectolitre mass of all the cultivars (Table 6.5). SST387 had the

highest protein content followed by PAN3379, Elands, Gariep, Senqu, while PAN3118 had the lowest protein content (Table 6.5).

Table 6.6: Season 2015-2016. Four-point damage rating scale for Russian wheat aphid (*Diuraphis noxia*) resistance in adult wheat under field and natural RWA infestation. [abc] means without common letters differ significantly, ANOVA, LSD ($p<0.05$, SPSS).

Cultivar	Control		Alexin™ x1	
	Score	Damage rating	Score	Damage rating
Elands	3[a]	MS	2[b]	R
Gariep	3[ab]	MS	4[a]	S
Senqu	3[ab]	MS	4[ab]	S
PAN3118	-	-	-	-
PAN3379	4[a]	S	3[ab]	MS
SST387	-	-	-	-

1 = No visible signs (Resistant – R); 2 = Chlorotic spots (R); 3 = Chlorotic striping (Moderately Susceptible - MS); 4 = Leaves rolled (Susceptible – S)

The damage rating score (Table 6.6) in Elands control indicated moderately susceptible, while the score rating on Alexin™ treated Elands showed a resistant response. PAN3379 showed a susceptible damage rating before treatment, while after Alexin™ treatment this cultivar showed a moderately susceptible rating. Because SST387 and PAN3118 were stunted, the scores for these two cultivars were excluded. The significant differences for RWA damage rating scores were $p=0.00$, SE ±0.352 and the LSD 0.00 ($p<0.05$), but these significant differences were primarily because of SST387 and PAN3118.

Environmental factors

During the 2015-2016 wheat season, South Africa experienced extreme drought conditions in the wheat producing areas (Fig. 6.1). The planted wheat trials were under severe drought stress. There was also a heavy RWA

infestation in the Bethlehem area and *RWASA3* was the dominant biotype (screening performed in glasshouse by Jankielsohn, 2015).

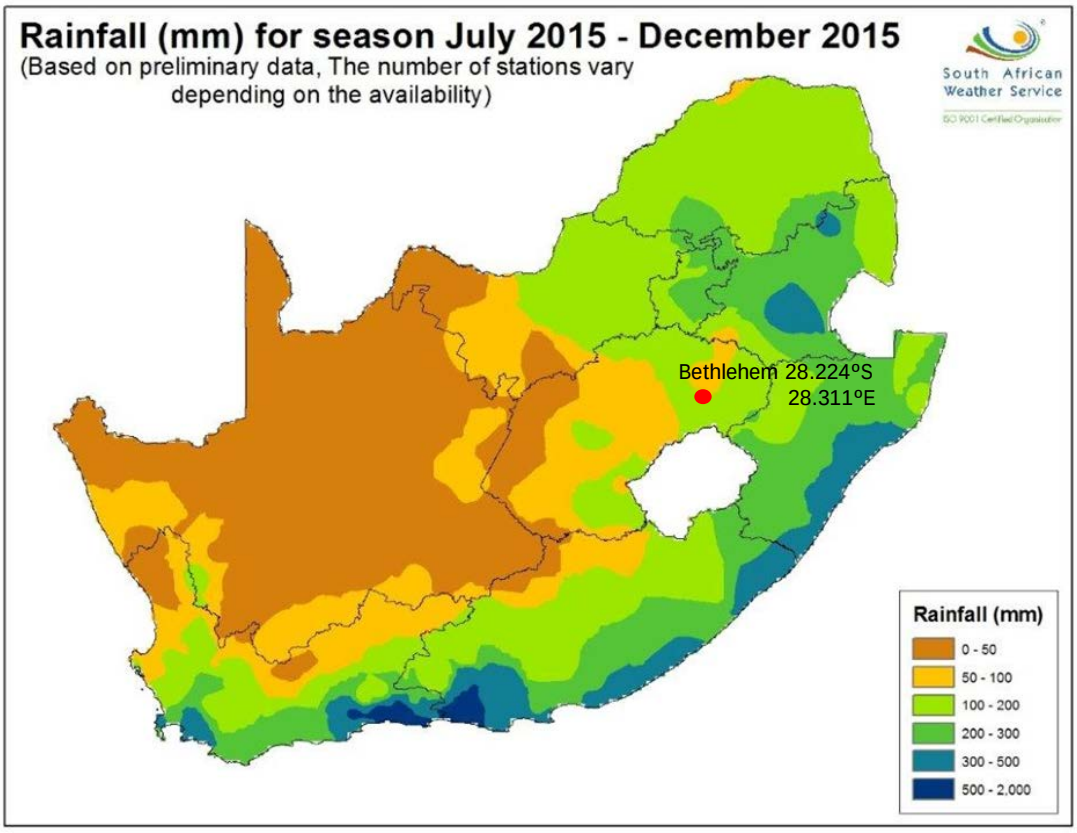

Figure 6.1: Rainfall pattern for July 2015 to December 2015 (South African weather service)

Table 6.7: Relative humidity, rainfall, evaporation and minimum temperature during the planted wheat season of 2014 and 2015 as recorded according to ARC, Bethlehem weather station.

Year	Month	Relative humidity (%)	Rain (mm)	Evaporation	Temperature Min (ºC)
2014	7	6,528	51,992	82,643	0
2014	8	10,265	52,851	81,335	0,343
2014	9	15,295	46,408	76,266	0,152
2014	10	14,278	46,995	74,106	6,271
2014	11	16,647	65,161	89,834	10,863
2014	12	19,870	64,787	89,695	14,04
2015	7	8,540	0,573	87,435	0,797
2015	8	12,695	0,000	78,539	2,663
2015	9	15,078	0,830	84,424	7,372
2015	10	19,114	1,040	81,188	10,819
2015	11	18,459	1,752	82,385	9,986
2015	12	22,774	1,269	85,666	14,755

During July, August and September 2015 almost no rainfall was recorded (Table 6.7) and the evaporation rate was also very high. The relative humidity was low, therefore the wheat did not grow in cool moist weather but rather under drought stress.

Table 6.8: Growth period and recommended planting dates according to the Small Grain Institute summer rainfall guidelines (2017) of the ARC-SG in comparison to the actual planting periods in 2014 and 2015 (Agricultural institute, 2017).

Cultivar	Growth period	Recommended planting dates	Actual plant dates 2014	Actual plant dates 2015
Elands	Medium	1 June–3 July	11/08/2014	30/07/2015
Gariep	Medium	1 June-3 July	11/08/2014	30/07/2015
Senqu	Medium	1 June-3 July	11/08/2014	30/07/2015
PAN 3118	Long	1 June-4 July	11/08/2014	30/07/2015
PAN3379	Short	3 June-1 Aug	11/08/2014	30/07/2015
SST387	Long	4 May-1 July	11/08/2014	30/07/2015

According to the guidelines, PAN3118 and SST387 need a long growth period that includes a period of exposure to low temperatures. A vernalisation period of at least one month at a minimum temperature below 5 ºC (Curtis, Rajaram & Macpherson, 2002) is required. Although the 2015 planting was much earlier, the minimum temperatures were higher during the 2015-2016 season, especially during the last two weeks of August. SST387 and PAN3118 therefore did not undergo the vernalisation period required for flowering and filling of kernels.

6.4 Discussion

Six winter wheat cultivars (Elands, Gariep, Senqu, PAN3118, PAN3379 and SST387) were cultivated in a field trial at ARC-Small Grains Institute, Bethlehem, in the 2014-2015 and 2015-2016 seasons, to determine yield and quality parameters as well as resistance towards RWA following Alexin™ treatment.

During the first field trial Alexin™ was applied twice, with an interval of a month, to determine whether there was a visible difference upon the second application. The glasshouse trials mentioned in Chapters 3 and 4 were at three-leaf stage, the first application in the field was also at three-leaf stage, and the second application took place during tillering (Zadoks growth stage 10). The damage rating score of SST387 was lower for treated plants (Table 6.3) and the four-point rating scale showed a shift from susceptible to resistant (Table 6.4), but there were no differences between one application and two applications for SST387. On the other hand, PAN3379 (Table 6.4) did not show a suppression in resistance as found in the glasshouse trials (Chapter 4). Therefore, a second application is not needed for the short growth season. Ross (1961) determined that systemic acquired resistance (SAR) can still persist 20 days after the initial pathogen inoculation. Field

tests conducted by Nulandis with Alexin™ on a peach orchard in Worcester in the Sandvliet region illustrated an effect on both infected fruit and general tree health. The trees were sprayed at an interval of 14 and 28 days to compare the efficacy of Alexin™ in controlling *Xanthomonas*. The longer spray interval produced better disease suppression, confirming that the induced resistance response in plants has a long lasting effect (Janse van Rensburg, 2013). Similar to our results, Stadnik and Buchenauer (1999) also found that reapplication of a priming agent did not increase the success of controlling the pathogen. They found that benzothiadiazole (BTH) was successful in controlling powdery mildew on wheat in the field, but not *Septoria* leaf blotch. An additional application showed no improvement in controlling *Septoria*. They also found no difference in yield in the treated plant compared to the control, as was observed in the present study during both seasons (Tables 6.3 and 6.4).

The second season, 2015-2016 (Fig. 6.1 and Table 6.7), was very dry and vernalisation did not occur in certain wheat cultivars. The harsh field conditions including drought, evaporation, and insect infestation showed that Alexin™ treatment did not activate efficient resistance and did not provide RWA control. Even though glasshouse trials (Chapters 3 and 4) showed that Alexin™ treatment successfully controlled the RWA population, results of the two field trials confirmed that there is a complex relationship between plants and their environment, which can only be revealed in field trials. Agents such as Elexa® and β-aminobutyric acid (BABA) also produced variable disease control results in field experiments (R). The lack of consistency and incomplete disease resistance control is not a surprise because induced resistance is a plant response and can be affected by many factors such as the abiotic environment, host genotype and the extent to which the plant in the field is already induced (Walters & Fountaine, 2009). Since effects of induced resistance agents could be

unpredictable because of environmental factors, such agents might be more successful in controlled production conditions such as in glasshouse vegetables.

Alternatively, Alexin™ could also be applied differently to plants to ensure induction of resistance and tolerance in field conditions. Alexin™ is a foliar spray but the possibility of manipulating the product to be used as a seed treatment or for root drenching to ensure good target delivery and coverage, might be worth investigating. Cotton seeds were treated with acibenzolar-S-methyl (ASM) prior to sowing in the field or being sprayed in the burrow on top of the seeds at sowing. These application procedures reduced the severity of black root rot (Mondal, Nehl & Allen, 2005). Application of BABA on wheat through root drenching inhibited the development of nematodes within roots (Oka & Cohen, 2001).

During the 2015-2016 season the most abundant biotype was *RWASA3*. The term biotype is used when an insect species has different populations and these populations vary in their virulence to a cultivar or cultivars (Pedigo & Rice, 2006). In South Africa four RWA biotypes have been documented. The glasshouse experiments were conducted with *RWASA1* and *RWASA2*, while *RWASA3* was prominent in the field study.

Insects have developed resistance to many control tactics and sustainable control methods must be explored. Induced resistance can be modulated by many factors and abnormal deviations of environmental factors can have extreme effects on the performance of resistant cultivars. Therefore, it is important to study various aspects of the induced defence mechanisms and target-specific facets which could be combined with other pest management tactics to reduce the “arms race” between pests and plants, but still keep aphid populations below the economic threshold.

6.5 CONCLUSION

Alexin™ had an effect on RWA damage and yield in the first season for SST387. The low RWA damage resulted in the highest yield in SST387. However, this trend could not be confirmed with the second season because of insufficient vernalization in SST387. Controlling aphids with Alexin™ seems to be genotype- and biotype-specific as described in previous chapters, as well as more dependent on growth and environmental conditions than with traditional controlling products, creating additional challenges. Priming and induction of resistance responses can be variable and inconsistent but may be an additional option for growers to protect their crops against RWA when integrated with cultivation of resistant cultivars in a more sustainable plant health management system. Although field studies were not consistent because of environmental factors such as temperature, drought and vernalisation, further studies must determine efficient uptake of priming products in the field and the potential of combining the products with other management strategies.

Chapter 7
General discussion

The Russian wheat aphid (RWA) is an economically important pest found in wheat and barley, globally and in South Africa. The most sustainable method to manage a pest population is to use resistant wheat cultivars. However, there is an “arms race” between the plant and the pest, which has proven to be a problem for the industry because large outbreaks of resistance-breaking biotypes have emerged, specifically in the dryland wheat areas of the eastern Free State. Therefore, other sustainable management methods need to be identified to help in managing RWA populations.

Plants are sessile and cannot escape pests, pathogens or unfavourable environmental conditions. For this reason, they have developed a broad range of induced responses to counteract such attacks and fluctuating environmental conditions. A pest, pathogen or beneficial microorganism attack can establish an important physiological state within a plant called the “primed” state (Conrath, 2009), which implies that if the plant is stressed again after an initial attack the plant defence responses will be enhanced to protect the plant. The primed state is durable and can be maintained long after the encounter with the stressful event (Goellner & Conrath, 2008). Priming only sensitises plants in anticipation of imminent attacks, and does not cause major fitness costs to the plant before the attack. Natural and synthetic chemicals can also prime a plant and induce defence responses when the plant is attacked. These induced defence responses have been studied extensively in cases where a plant is colonised by a pathogen. Mohase and Van der Westhuizen (2002b) identified that the

RWAs also induce the same defence responses as a pathogen when primed with salicylic acid (SA). Salicylic acid is one of the phenolics that accumulate in the surrounding uninfected and distal plant parts and can place the plant in a “primed state”.

Alexin™ has SA as its main active ingredient. The formulation also contains carbohydrates serving as sources of energy for the plant, as well as nutrient elements such as boron (B), calcium (Ca), magnesium (Mg) and potassium (K). During the current study, the role of Alexin™ as a possible priming agent on different wheat cultivars was investigated under control and RWA-infestation conditions. Three different cultivars were chosen: SST387 (resistant to *RWASA1*) with drought tolerance, Elands (resistant to *RWASA1*) and PAN3379 (resistant to all South African biotypes).

The question was posed as to whether Alexin™ could prime these cultivars and promote resistance to the RWA. Defence responses, such as expression of peroxidase and β-1,3-glucanase, were used to measure Alexin™-mediated defence responses within these cultivars. The cultivars SST387 and Elands showed increased resistance responses when challenged with *RWASA1*; however, the responses were not significantly higher when the plants were infested with *RWASA2.* This was, however, not the response in PAN3379. There were no increases in defence responses (peroxidase and β-1,3-glucanase) when challenged with *RWASA1,* and when challenged with *RWASA2* a suppression occured in the treated plants. Therefore, even though Alexin™ can prime certain wheat cultivars for protection against the RWA, the effect is highly specific, depending on the host genotype and biotypic status of the aphid.

The priming effect of Alexin™ was investigated further by determining the resistance nodes expressed when different cultivars were treated with Alexin™. The resistance was measured by identifying antixenosis, antibiosis

and tolerance. Also, RWA resistance was determined by phenotypic symptoms to recognise resistance, moderate resistance and suceptibility. SST387 responded positively to the treatment and showed antibiosis as well as tolerance responses during *RWASA1* infestation. Plant tolerance is a very important defence mechanism because it does not interfere with the insect's physiology or behavior and could decrease selection pressure for emerging biotypes (Smith, 2005).

Alexin™ mediated a shift from moderately resistant to resistant during *RWASA1* infestation in SST387. Eland and SST387 displayed a shift in the damage rating score from susceptible to moderate resistance when infested with *RWASA2*. This is important because moderate resistance is identified by streaking but excludes leaf rolling. Leaf rolling is a suscpetible response that helps RWAs to survive. Rolling encloses and protects the aphid within the leaf, so that chemicals and predators cannot reach the aphid. The rolled leaf also traps the ear of the plant, cutting off nutrients to the ear and leading to little or no seed formation. Therefore, Alexin™ increased resistance responses in susceptible cultivars SST387 and Elands but did not mediate any defence responses towards *RWASA2*.

The phenotypic damage rating score that shifted with Alexin™ treatment in SST387 from susceptible to moderately resistant and in PAN3379 from resistant to moderately resistant when challenged with *RWASA2* presented the opportunity to pose the following questions: What biochemical components of resistance and genes are induced in plants when primed with Alexin™ to enhance plant resistance to subsequent challenges and, on the other spectrum, to suppress plant resistance? Based on these questions we looked at reactive oxygen species (ROS), antioxidants, certain signalling hormones and some known RWA defence genes. The focus was mainly on

SST387 (susceptible to *RWASA2*) and PAN3379 (resistant to *RWASA2*) during infestation by *RWASA2*.

The resistance reponses in the susceptible cultivar were increased by Alexin™ treatment with an oxidative response and elevated antioxidant levels. Salicylic levels were also significantly higher, showing antagonistic responses towards JA. The gene (*TaGSTF6*) associated with antibiosis was upregulated and positively correlated with the findings in Chapter 4. The resistant cultivar expressed signs of suppression when treated with Alexin™, but also signs of an increase of SA concentration. It was nevertheless concluded that defence mechanisms might be induced by other defence signalling networks in PAN3379.

Even though glasshouse trials (Chapter 4) showed that the Alexin™ treatment successfully controlled RWA population in SST387, results of the two field trials (Chapter 6) confirmed that there is a complex relationship between plants and their environment, which can only be revealed in field trials. During the first field trial, Alexin™ was applied twice, but there were no obvious differences between the first and second applications, although Alexin™-mediated responses displayed a shift from susceptible to resistant in SST387 based on the four-point rating scale. Field tests with Alexin™ by Nulandis illustrated that a longer spray interval period produced better disease suppression, confirming that the induced resistance response in plants has a long lasting effect (Janse van Rensburg, 2013). A noteworthy response identified in the PAN3379 field study was that, based on the four-point rating scale, no suppression in resistance occurred as found in the glasshouse trials.

The second planting season was very dry and vernalisation did not occur in certain wheat cultivars. The harsh field conditions showed that Alexin™

treatment did not activate efficient resistance and did not provide RWA control. The Alexin™-mediated response seems to be genotype- and biotype-specific as previously mentioned, but the field studies highlighted the fact that priming is also dependent on growth and environmental conditions.

In conclusion, Alexin™ has a priming potential in wheat and can boost the resistance of a plant when challenged with RWA. Priming has the potential to help plants to activate resistance responses when needed, saving energy when not stressed. Further collaborated integrated pest management studies could create a platform to incorporate Alexin™ into the RWA management system. There is a great need for more field studies to focus specifically on when the product should be applied and to ensure that there is sufficient uptake of the product. These suggestions should be taken into account during future studies since it could be of importance in the marketing of Alexin™.

Chapter 8
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Summary

The Russian wheat aphid (RWA, *Diuraphis noxia* Kurdjumov) is a serious international pest occurring in wheat production areas in South Africa. The use of RWA-resistant cultivars is an effective pest management tactic, but the occurrence of resistance-breaking biotypes affects the durability of this strategy. We investigated the effect of Alexin™, a potential priming agent, on the defence responses of three different wheat cultivars challenged with two South African RWA biotypes-*RWASA1* and *RWASA2*. Alexin™ (0.375%, v/v) pre-treatment selectively mediated the expression of resistance responses in the wheat cultivars. The resistance response was measured in terms of enzyme activities of *β*-1,3-glucanase and peroxidase. To compliment these findings three host plant resistance nodes (antixenosis, antibiosis and tolerance) were used to screen for Alexin™-mediated resistance. Alexin™ treatment mediated tolerance and antibiosis to *RWASA1* and tolerance to *RWASA2* in the cultivar SST387. These changes were evident as reduced intrinsic rate of aphid population and phenotypic damage rating score from susceptible to moderately resistant. Alexin™ treatment however, compromised the defence responses towards *RWASA1* and *RWASA2* in PAN3379, a cultivar with reported resistance to all four known South African biotypes. To understand the mechanisms of priming in resistance to RWA, we measured the levels of reactive oxygen species (H_2O_2), antioxidant enzyme activities and certain defence hormones, as well as, expression of some stress related genes. Alexin™ mediated accumulation of H_2O_2 in the susceptible cultivar, increase in antioxidant enzyme activities (CAT, GR, SOD) and levels of Salicylic acid (SA) relative to Jasmonic acid (JA). On the other hand, the treatment suppressed defence responses in the resistant cultivar, except the relatively high level of induced SA. We concluded that defence mechanisms might be induced with other defence signalling networks. Even though field studies were not repeatable due to extreme seasonal variations in rainfall and temperature, the results indicate that Alexin™ has a potential to express systemic acquired

resistance in otherwise susceptible wheat cultivars, and as such can be incorporated into an integrated pest management system to improve aphid management in wheat.

Key words: Alexin™, Biotypes, Defence response, Priming, Russian wheat aphid, Salicylic acid, Tolerance