Priming effect of leaf rust and salicylic acid in Russian wheat aphid resistance

By

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Dedication

I dedicate this dissertation to the beloved Holy Prophet Muhammad ﷺ (Peace Be Upon Him) and my beloved parents.

إِنَّ اللَّهَ فَالِقُ الْحَبِّ وَالنَّوَىٰ ۖ يُخْرِجُ الْحَيَّ مِنَ الْمَيِّتِ وَمُخْرِجُ الْمَيِّتِ مِنَ الْحَيِّ ۚ ذَٰلِكُمُ اللَّهُ فَأَنَّىٰ تُؤْفَكُونَ

Truly it is Allah Who causes the grain and the fruit-kernel to sprout. He brings forth the living from the dead and brings forth the dead from the living. Such is Allah. So whither are you tending in error?

(Ch. 6 Vs. 95)

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Declaration

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

…….28-11-2022……

Abbreviation

A

AA: Ascorbic acid

ABA: Abscisic acid

ANOVA: Analysis of variance

APX: Ascorbate peroxidase

ARC-SG: Agricultural Research Council-Small Grains

ASL: Acid-soluble lignin

AV: Avirulent

B

BTH: Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester

C

$CaCl_2$: Calcium Chloride

CAT: Catalase

CESA: Cellulose synthase catalytic subunit

cm^3: Cubic centimetre

CWPs: Cell wall proteins

ºC: Celsius degree

D

DAMPs: Damage-associated molecular patterns

Dn: *Duraphis noxia*

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E

eATP: Extracellular adenosine-5-triphosphate

EDTA: Ethylenediaminetetraacetic acid

F

FAD: Flavin adenine dinucleotide

F-C: Folin-Ciocalteu reagent

$Fe(OH)_2$: Ferrous hydroxide

Fe^{2+}: Ferrous ion

Fe^{3+}: Ferric ion

$FeCl_3$: Ferric Chloride

G

Ggt: *Gaeumannomyces graminis* var. *tritici*

GOPX: Guaiacol peroxidase

GPX: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione

GSSG: Oxidised glutathione

GST: Glutathione-S-transferase

H

H_2O_2: Hydrogen peroxide

HO_2^-: Hydroperoxyl ion

hpi: Hours post infestation

I

IV: Intermediate virulent

J

JA: Jasmonic acid

K

KCl: Potassium chloride

L

µl: Micro litre

LC-MS: Liquid chromatography-mass spectrometry

Lr: Leaf rust

LSD: Least significant difference

M

µM: Micromolar

M: Molar

MAMPs: microbe-associated molecular patterns

MAPK: Mitogen-activated protein kinase

MDHAR: Monodehydroascorbate reductase

mg ml^{-1}: milligram per millilitre

min: Minute

ml: millilitre

mM: Milimolar

MR: Moderate resistant

N

n: number of subsamples

NADPH: Nicotinamide adenine dinucleotide phosphate

NaHS: Sodium hydrosulphide

NBT: Nitro-blue tetrazolium

nm: Nanometer

NO: Nitric oxide

NPK: Nitrogen, Phosphorus and Potassium fertilizer

NPR: Non-expresser pathogenesis-related protein

O

$^1O_2$: Singlet oxygen

O_2^-: Superoxide anion

OH^-: Hydroxyl radical

P

PAL: Phenylalanine ammonia-lyase

PAMPs: Pathogen-associated molecular patterns

PGPB: Plant growth-promoting bacteria

PGPF: Plant growth-promoting fungi

PGPR: Plant growth-promoting rhizobacteria

Pgt: *Puccinia graminis* f. sp. *tritici*

pH: The negative logarithm of the hydrogen ion concentration

PME: Pectin methylesterase

POD: Peroxidase

PPO: Polyphenol oxidase

PR: Pathogenesis-related

Prxs: Peroxiredoxins

psi: Pounds per square inch

Pst: *Puccinia striiformis* f. sp. *tritici*

Pt: *Puccinia triticina*

PTEN: Phosphatase and tensin homolog

PTP: Protein tyrosine phosphatase

PVPP: Polyvinylpolypyrrolidone

R

R: Resistant

RCBD: Randomised complete block design

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPM: Revolution per minute

RuBP: Ribulose-1,5-bisphosphate

RWA: Russian wheat aphid

RWASA: Russian wheat aphid South Africa biotype S

S: Susceptible

SA: Salicylic acid

SAR: Systematic acquired resistance

SNP: Sodium nitroprusside

SOD: Superoxide dismutase

SRS: Sugar recovery standards

T

TaAGO5: Argonaute 5

TaGluD: β-1,3-glucanase gene

TaPME: PME gene family in bread wheat

TCA: Tricholoroacetic acid

U

UVPt: University of the Free State, *Puccinia triticina*

V

V: Virulent

v/v: Volume per volume

W

w/w: Weight per weight

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Table of contents

List of figures

List of tables

Chapter 1

1.1 Introduction

1.1.1 Background and motivation for the study

Wheat (*Triticum aestivum* L.) is one of the primary sources of carbohydrates for humans and livestock (Karakas et al., 2021). It is an essential cereal for the human diet and contributes to global food security. Almost 50% of calories for human consumption come from grains; out of this, about a quarter comes from wheat (González-Esteban, 2017).

Wheat grain is a rich source of carbohydrates, dietary fibre, vitamins (B-vitamins) and phytochemicals (Shewry and Hey, 2015). In addition to this, it has 13-17% bran, 2-3% germ and 80-85% mealy endosperm (Šramková et al., 2009). Wheat is a significant source of globulin, albumin, and amphiphilic protein content (Dubreil et al., 1998). Furthermore, wheat provides lipids and essential minerals like calcium, copper, iron, phosphorus, potassium, manganese, magnesium, and zinc (Rachon et al., 2015).

The current global wheat production is 642 million tons, and the future (2050) demand is about 840 million tons. This demand may be attained on limited resources (water, land) if new agronomic, physiological and genetic research strategies and practices are introduced (Sharma et al., 2015). Domestication of wheat occurred 10,000 years ago, and wheat spread worldwide as a major cereal crop. Its diverse adaptability to different environments makes it easy to domesticate. Genetic miscellany (ploidy level) of wheat and its progenitors reward novel diversity quickly in different climatic zones (Dubcovsky and Dvorak, 2007).

Commercial wheat cultivation started in South Africa in the early 1910s in Cape Town, with seeds introduced earlier by the Dutch traders (Nhemachena and Kirsten, 2017), and has become the second most crucial grain crop cultivated in South Africa after maize (Anonymous, 2021; Bester, 2014). Both tetraploid and hexaploid wheat cultivars are produced in approximately 90% of the available agro-climatic regions of South Africa (Lantican et al., 2005). The dominant wheat-producing areas are the Western Cape (winter rainfall, mainly dryland), Free State (summer rainfall, both dryland and irrigated), Northern Cape (irrigated) and North West (mainly irrigated) provinces. Even

though cultivation occurs in winter and summer rainfall regions, between 1983 and 2008, wheat was cultivated predominantly under dryland conditions where annual production averaged 1.5 to 3 million tonnes (2-2.5 tons/ha) (Nhemachena and Kirsten, 2017). However, about 30% of harvested wheat is produced under irrigation, where the yield potential varies between 6 to 12 tons/ha, with higher winter temperatures being the main limitation in the lower-yielding areas (Anonymous, 2021).

The major companies or institutions supplying improved wheat cultivars in South Africa are Sensako (now part of Syngenta), Pannar Seed (Corteva Agrisciences^{TM}) and the Agricultural Research Council-Small Grains (ARC-SG) (Nhemachena and Kirsten, 2017). The wheat varieties are constantly improved for high yield and tolerance or resistance to prevailing drought, salinity, heat, pests and diseases. In South Africa, the wheat industry contributes about USD 40 billion to the gross value of agricultural production (Jankielsohn, 2016) and 28 000 jobs (Bester, 2014).

Some pathogens (causing diseases like rust and powdery mildew) and pests similar to the Russian wheat aphid (RWA) significantly reduce yield and flour quality (Kazi et al., 2013). Russian wheat aphid infestations significantly challenge successful wheat production (Njom et al., 2017) because they reduce wheat yield and deteriorate flour quality (Girma et al., 1993). The emergence of RWA biotypes with increased virulence threatens wheat production and reduces the desired targets to meet the South African demand for high-quality wheat grain.

The RWA has been controlled by resistance breeding and pesticides. Even though these strategies effectively reduce the impact of RWA infestation, the aphid constantly evolves into virulent biotypes that overcome the resistance expressed in the cultivars. Efficient host resistance must be broad and durable to protect the plants (Jankielsohn, 2017). Furthermore, the rate of cultivar development should surpass that of biotype evolution. Unfortunately, resistance breeding often lags behind the appearance of new, virulent RWA biotypes, necessitating exploring alternative strategies to manage the aphids.

Aphicides have been used to curb aphid populations to maintain acceptable yields (Jankielsohn, 2021). Such chemicals are usually a mixture of contact and systemic pesticides, which are often expensive and less environmentally friendly, while

contact pesticides are less efficient because the aphids stay within rolled leaves and are unaffected by the chemicals.

Alternate, affordable, less detrimental to the environment, and equally effective at reducing aphid populations approaches that reduce plant damage and maintain yield are required. One potential approach uses priming agents like phytohormones and microbes as prophylactic (Llorens et al., 2020) measures before infestation. Ab Rahman et al. (2018) described priming as a powerful tactic to lower input costs in sustainable agriculture because it can potentially protect crops from pests and pathogens. Using priming agents such as hormones, mineral nutrients and biocontrol microorganisms to modulate plant defence responses can reduce the undesired toxic effects of chemicals. Some inorganic nutrients activate defence responses to insect infestation; for instance, phosphate and silicon-based fertilisers reduce insect infestation in monocots and dicots (Asiwe, 2009; Ab Rahman et al., 2018; Alhousari and Greger, 2018).

Similarly, exogenous application of plant hormones like jasmonic acid (JA) and salicylic acid (SA) improved plant vigour and tolerance to salinity stress in wheat (Yücel and Heybet, 2016). Furthermore, exogenous SA-activated defence responses reduced the number of aphids on the mustard plant (Mony et al., 2017) and reduced the disease index by tomato stem canker in the tomato-plant interaction (Esmailzadeh et al., 2008).

Besides chemicals and plant hormones, microbes like plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) were used as priming agents to promote plant growth and enhance defence responses to diseases in various crops (Singh et al., 2019). Seed treatment with these organisms, notably the PGPR, effectively reduced the aphid infestation effects on wheat (Naeem et al., 2018). Similarly, some fungi (ascomycetes, oomycetes and arbuscular mycorrhizal fungi) were used successfully to prime various crops, including cotton, for enhanced resistance to soil-borne diseases (Guo et al., 2014; Singh et al., 2019).

The understanding for exogenous SA application and inoculation of wheat with the leaf rust pathogen *Puccinia triticina* Eriks. (*Pt*) to prime plants for enhanced defence responses to RWA infestation is limited. Rust outbreaks can result in

extensive yield losses; therefore, the disease-causing pathogens have not been readily considered biocontrol agents against other pests in cereal crops. *Puccinia triticina* is a widely distributed and common wheat rust, with isolates known to be different in their virulence, and infections from *Pt* races are less damaging than stem rust and stripe rust (Huerta-Espino et al., 2011).

1.2 Problem statement

The rate at which aggressive RWA biotypes develop is alarming, and successful wheat production and grain quality maintenance are threatened. Various control strategies like resistance breeding and pesticide use are ineffective because of the rapid development of biotypes with increased virulence, and the regular application of pesticides is concerning from an environmental perspective and requires additional economic inputs. Furthermore, infestation by RWA deteriorates wheat grain quality. Therefore, efficient and environmentally friendly strategies are required to overcome challenges presented by aphids in wheat production and food sustainability.

1.3 Hypothesis

If *Pt* infection or exogenous application of SA sensitises wheat (*Triticum aestivum* L.) cultivars for increased resistance to RWA infestation, subsequent RWA infestation should reduce damage to wheat plants. The enhanced resistance responses in wheat cultivars by priming agents will be evident as reduced leaf damage, higher antioxidative cellular activity and cell wall reinforcement.

1.4 Objectives of the study

This study aimed to evaluate the efficacy of *Pt* isolates and SA in priming wheat for increased resistance to RWA infestation, which was achieved by pursuing the following objectives:

1. To determine the degree of RWA-induced leaf damage in wheat cultivars pre-treated with SA or pre-inoculated with *Pt* races at the seedling and flag leaf stages.
2. To elucidate the effect of SA or *Pt*-induced priming on the antioxidative responses of wheat to RWA (RWASA1) infestation at the seedling stage.
3. To investigate the effect of SA or *Pt* pre-treatment at the seedling stage on cell wall composition changes and their relationship with resistance to RWA (RWASA1) infestation.

1.5 The significance of the study

Wheat is a critical food component globally, and its production must meet the growing demands. On the other hand, the RWA is an economically devastating wheat pest that severely damages wheat and drastically reduces yield and quality. The aphid further evolves into resistance-breaking biotypes and can evolve pesticides-resistance. The study, therefore, explored the efficacy of an alternative strategy to protect wheat against RWA infestation. Efficient, equally sustainable, biodegradable, and affordable strategies could promise to curb the impact of aphids on wheat to enable efficient production and food security.

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Chapter 2

Host resistance mechanisms and Russian wheat aphid management in wheat production

2.1 Introduction

Wheat (*Triticum aestivum* L.) is the third largest source of carbohydrates after rice and maize for millions worldwide. Similarly, it is the second most important staple food in South Africa, with an annual production of 2.1 million tons (World-Grain.com). Successful wheat production depends on stable and high-yielding cultivars (Dube et al., 2019) and the resistance to biotic (pests and pathogens) and abiotic stress factors (including drought, salinity, and heat). One of the most economically devastating crop pests is the Russian wheat aphid [(RWA); *Diuraphis noxia,* Kurdjumov].

The RWA is an important pest of wheat, barley (*Hordeum vulgare* L.) and other grains (Mirik et al., 2007). The aphid severely damages wheat and reduces wheat production in South Africa and other wheat-producing regions (Jankielsohn et al., 2016). Aphid feeding can cause yield losses of up to 80% and deteriorate flour quality (Tolmay et al., 2007). The RWA populations and their devastating infestations have been controlled by pesticides (Jankielsohn, 2021) and resistance breeding (Tolmay et al., 2007). Improving host resistance through resistance breeding is considered more sustainable and reduces pesticide use, environmental hazards and toxicity (Jankielsohn, 2021).

Biological agents like parasitoids have also managed the RWA populations. However, this control strategy is challenging because the parasitoids require alternative hosts to multiply to sufficient levels, and the rate of RWA population increase is significantly higher than that of the parasitoids (Prinsloo, 2000). In all these, resistance breeding has been a better strategy for conferring host plant resistance. However, the successful identification and introduction of resistance genes in wheat cultivars with desired agronomic traits often lag behind the resistance-breaking RWA biotypes' rate of evolution.

Efficiently managing RWA infestations on wheat requires a concerted approach incorporating host resistance strategies, such as priming, which protects plants from biotic and abiotic stresses (Sibisi, 2014). Priming sensitises host plants for enhanced resistance to imminent biotic or abiotic stresses. Agents such as micronutrients, phytohormones, and microbes can prime plants for enhanced resistance responses to pests and pathogens (Naeem et al., 2018).

This review aims to collect and evaluate published information on plant defence mechanisms and the management strategies employed in host plant protection against pests, emphasising strategies that enhance host plant resistance, such as priming. The antioxidative responses and events associated with host cell wall modification will be emphasised as part of the inducible host plant protection strategies. The wheat-RWA system will be used as the primary example, and reference to other plant-pathogen/pest interactions will be used to provide further insight into mechanisms of host plant protection.

The information was gathered from databases such as Google Scholar, JASTOR, Science Direct and Web of Science, hosting peer-reviewed original research papers and review articles published in the last 25 years. The information generated identified critical elements in crop protection using host plant responses, forming the study's basis and future research objectives.

2.2 Russian wheat aphid

Although the Aphididae consists of about 50,000 species, fewer species; the pea aphid (*Acyrthosiphon pisum*), kissing bug (*Rhodnius prolixus*), green peach aphid (*Myzus persicae*) and RWA are economically significant (Burger and Botha, 2017). These aphid species are phloem-feeding agricultural pests. The RWA, Russian grain aphid or barley aphid (Kurdjumov, 1913), is an international insect pest of wheat (Jankielsohn et al., 2016).

2.2.1 Origin and distribution

The RWA was first recorded on the northern coast of the Black sea (former Union of Soviet Socialist Republic) in the early 1900s as a cereal crop pest (Yazdani et al., 2018). The RWA spread to major wheat-producing countries from Central Asia and became an international wheat pest (Jankielsohn, 2016). The Carpathian region has been classified as one of the model areas for RWA distribution and expansion from southeast to central European countries (Starý et al., 2003). Expansion of RWA to the Carpathian Basin resulted from natural expansion by one or two routes from Central Asia to Ukraine and adjacent countries and southwest to the Near East and Mediterranean (Basky, 1993; Starý, 1999). The adventive expansion route was another area from central-western Asia to the Mediterranean area (Starý, 1999).

The adult RWA spread by actively flying or using wind currents throughout the crop. However, long-distance dispersal occurs by hitchhiking on machinery, clothing or plant materials (Fact Sheet, Russian wheat aphid; https//www.planthealthaustralia.com.au).

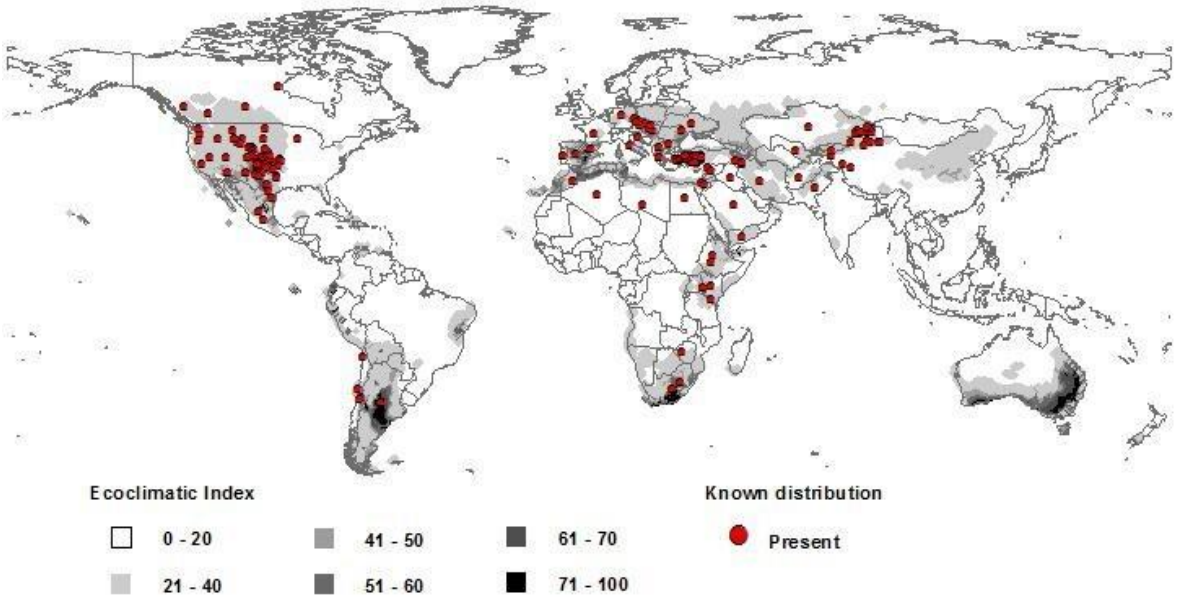

Fig. 2.1: Global distribution of the Russian wheat aphid. The eco-climatic indices' favourability ranges from 0 (do not persist) to 100 (optimum climate) (White et al., 2016).

In the African continent, RWA is present in Northern Africa (Morocco, Tunisia, Algeria), Eastern Africa (Ethiopia, Kenya) and Southern Africa (South Africa, Lesotho) (Fig. 2.1). Apart from Africa, RWA is also present in West Asia (Turkey, Yamen) (Chemeda,

2015). The RWA is also present in the United States of America (Ward et al., 2020) and Australia (Avila et al., 2019).

2.2.2 Russian wheat aphid distribution and diversity in South Africa

South Africa initially reported the RWA in 1978 in the dryland of Bethlehem, in the Eastern Free State, where winter and facultative wheat types are cultivated from June to December (Jankielsohn, 2011). Following the release of resistant cultivars, the RWA began to evolve into biotypes, and the second biotype, designated as RWA South African biotype 2 (RWASA2), was reported in 2005 (Tolmay et al., 2007), shortly followed by another biotype (RWASA3) reported in 2009 (Jankielsohn, 2011). The fourth biotype (RWASA4), restricted to the Eastern Free State, was recorded in 2011. The latest biotype, RWASA5, was also recorded in the Free State province in 2019 (Jankielsohn, 2019). Annual RWA surveys indicate that the biotypes are complex, diverse and dynamic in the Eastern Free State.

2.2.3 Russian wheat aphid feeding

Russian wheat aphid belongs to the Hemiptera, which secretes saliva and causes damage through physically drawing phloem sap or releasing toxins through saliva into plant cells. The RWA feeding occurs in four phases (Botha et al., 2005). The first phase (Phase 1) involves the pre-alighting behaviour, where the aphid randomly selects a plant as a potential feeding source. In the next phase (Phase 2), the aphid explores the plant surface to identify a suitable feeding area. In phases 3 and 4, the aphid probes the plant surface and searches for healthy tissues to draw nutrients.

The RWA preferentially feeds at the base of fresh leaves, which begin to roll inward as feeding progresses. In susceptible plants, aphids are protected in rolled leaves from contact pesticides. Matsiliza and Botha (2002) showed that *Sitobion yakini* (Eastop) preferred feeding on thin-walled phloem tubes because they are more functional in phloem loading and translocation. The phloem tubes consequently contain high content of sucrose and other desirable nutrients.

Russian wheat aphid feeding reduces shoot weight, plant height, spike number, and yield. Aphid feeding symptoms include changing leaf colour from green to yellowish, formation of whitish to purplish streaks, head distortion and trapping. Severe yield losses occur when the RWA infests the flag leaf, causing damage and rolling. Aphids move onto the newly emerged head and reduce grain quality if the head emerges. Severe infestation reduces plant vigour and often kills plants (Michaud and Sloderbeck, 2005). Up to 80% yield losses on susceptible South African wheat cultivars have been recorded (Tolmay et al., 2007). Furthermore, aphid feeding reduces host plant protein content and flour mixing quality (Girma et al., 1993).

Electrical penetration graph recordings of RWA infestation show that aphid stylet probing towards the phloem is less intense in the resistant than susceptible host plants (Khan et al., 2015). During probing and feeding, the aphids secrete saliva that paves the way to the phloem, protects their stylets and digests cell walls to access nutrients. The aphid saliva, mainly the watery saliva, contains various enzymes and proteins, including glucose oxidase, plant cell wall degrading enzymes and Ca^{2+}-binding proteins, which may function as effectors to suppress or induce plant defence responses (Hogenhout and Bos, 2011).

Aphid feeding on plants reduces cellular water content and subsequent loss of turgor pressure and plant growth. The RWA further reduces the biomass of all plant components. However, wheat plants distribute carbon components to decrease stem biomass and compensate for root and leaf growth under aphid infestation (Macedo et al., 2003).

2.2.4 Current Russian wheat aphid management practices in South Africa

The two main strategies used to control the RWA in South Africa are chemical control and breeding for resistant wheat cultivars (Jankielsohn, 2021). On a commercial scale, systemic pesticides control RWA infestation and prevent the populations from exceeding economic thresholds. However, these pesticides are toxic to humans and other insects, such as decomposers, pollinators, or predators. The chemicals can also contaminate the harvested grains (Fritschi et al., 2015). For example, Malathion and Dimethoate kill bees and RWAs but are carcinogenic to human beings. The chemicals

may also cause environmental pollution. With the intensive use of chemicals, there is also the possibility that RWA can develop tolerance to the active ingredients of insecticides (Marrone, 2019).

Biological control strategies such as introducing parasitoids have been used. However, a large parasitoid population that matches the pest population is required. Furthermore, insecticides that control other pests, like armyworms, may disrupt parasitoid activity (Umina et al., 2017). Parasitoids that control RWA populations require alternative parasitoid hosts. An alternative strategy, which can sometimes be complementary to biological control, is the development of resistant cultivars to reduce the impact of RWA infestation (Prinsloo, 2000). Developing resistant cultivars may reduce the ultimate cost of crop production, pest damage and environmental hazards. The resistant plant counters the toxic effects of aphids on leaves through the regeneration of ribulose-1,5-bisphosphate (RuBP), rubisco carboxylation (Gutsche et al., 2009) and upregulation of photosynthetic activities, while the susceptible plant cannot prevent the breakdown of photosynthetic machinery (Franzen et al., 2014).

Host-plant resistance is considered the most effective method of controlling RWA infestation (El Bouhssini et al., 2011). Wheat cultivars with resistance to the RWA have been developed elsewhere globally, and the first RWA-resistant cultivar in South Africa, Tugela *Dn1*, contained the *Duraphis noxia (Dn) 1* gene and was released for cultivation in 1992 (Dube, 2017). Different *Dn* gene series (*Dn1* to *Dn9*, *Dnx* and *Dny*) were introduced in different backgrounds to create resistant cultivars. However, the emerging biotypes have consistently overcome existing sources of resistance. The South African-RWA biotype 2 (RWASA2) recorded in South Africa in 2004 (Jankielsohn, 2011) has broken the resistance conferred by the *Dn1*, *Dn2*, *Dn3*, *Dn8* and *Dn9* genes, while genes *Dn4*, *Dn5*, *Dn6*, *Dn7*, *Dnx* and *Dnxy* remained effective in protecting plants from infestation (Tolmay and Booyse, 2017; Tolmay et al., 2020). Advanced host plant resistance incorporated various *Dn* genes in wheat germplasm to develop cultivars resistant to different South African biotypes. For instance, cultivars expressing *Dn5, Dn6, Dn7 and Dnx* expressed resistance to RWASA1, RWASA2 and RWAS3, and the gene *Dn2401* present in Cltr2401 conferred resistance to RWASA4 (Tolmay et al., 2020).

Since 1992, the release of resistant cultivars continued, and by 2006, cultivation of resistant cultivars covered 70-85% of the area planted for wheat in South Africa. These cultivars conferred resistance to the RWA, and fewer aphids developed on leaves on the main stem and tillers. However, Tolmay and Van Deventer (2005) reported that field trials showed variable resistance levels across the cultivars. Tolmay et al. (2007) further noted that specific cultivars hosted more RWAs, which were more damaging, during cultivar trials conducted at the Agricultural Research Council-Small Grain Institute (ARC-SG) in Bethlehem. In another study, Jankielsohn (2019) reported that out of the currently available wheat cultivars, 84% are resistant to RWASA1; 37% are resistant to RWASA2; 37% are resistant to RWASA3, and only 26% are resistant to RWASA4. Furthermore, in a recent study, only one genotype resisted the new, more virulent biotype, RWASA5 (Jankielsohn, 2021).

Studies on small RNAs that regulate genes for divulging resistance in wheat-plant against RWA infestation have predicted an *Argonaute 5* (*TaAGO5*) gene that confers resistance (Sibisi and Venter, 2020). They reported that a knockdown of *TaAGO*5 in resistant plants increased susceptibility to levels like RWA susceptible plants.

Even though resistance breeding is an effective control strategy against the RWA, the pace of resistance breeding is often relatively slow compared to the evolution of new resistance-breaking RWA biotypes. These biotypes are virulent to most of the RWASA1-resistant cultivars in South Africa. So, resistance breeding alone may not be sufficient to control RWA infestation. Additional strategies that employ host resistance are necessary to combat the RWA and may include the application of plant activators. Furthermore, biological control or priming agents can be used as host resistance activators. Alternative pest management strategies, such as priming by plant hormones or avirulent pathogens, can activate the host's defence responses. The activated responses could reduce the impact of all RWA biotypes. The priming strategy that controls pest populations is environmentally friendly and poses no health hazards to humans (Mony et al., 2017). A concerted effort that utilises resistance breeding to produce resistant cultivars and introduces priming agents to reduce pest pressure in wheat production to ensure crop protection may enhance food security.

2.2.5 Alternative Russian wheat aphid management practises: Priming

Priming refers to sensitising plants to respond fast and more vigorously to future biotic and abiotic stresses through treatment with non-pathogenic organisms, chemicals, or inorganic nutrients (Ameye et al., 2015; Balmer et al., 2015). It is an induced effect that activates host plant defence responses to increase the resistance to subsequent attacks.

The expression of resistance depends on the signalling pathways. Systemic acquired and induced resistance are examples of inducible forms of resistance. Using priming agents can reduce yield losses by about 20 to 85% (Walters et al., 2013). Examples of priming include seed treatment with salicylic acid (SA), which improves germination under drought stress and resistance to soil-borne diseases (Movaghatian and Khorsandi, 2013). A foliar application of SA also enhanced the resistance responses (Mony et al., 2017).

Information on wheat priming by microbes, especially rust fungi, is limited. Njom (2016) reported that wheat cultivars inoculated by *Puccinia triticina* (*Pt*) isolates showed priming effects to RWASA1 infestation based on proteomic results, while control plants did not show an enhanced defence response. The study did not relate the priming effect to phenotypic symptoms or describe the mechanisms of priming, which incorporates but is not limited to antioxidative responses and cell wall composition.

2.2.6 Priming agents: Inorganic nutrients and synthetic chemicals

Controlling pests and pathogens with synthetic pesticides is potentially harmful to the environment and considered less desirable. Consequently, plant activator application is an alternative approach to controlling pests and pathogens and improving plant health (Worrall et al., 2012).

Some micronutrients activate plant defence responses to insect populations. Specifically, high Silicon (Si) accumulation induces the Si-mediated defence pathways against pests (Alhousari and Greger, 2018). The phosphate fertiliser application also significantly lowers insect infestation (Asiwe, 2009), while increasing nitrogen application increases herbivore feeding. Zinc foliar application enhanced systemic

acquired resistance to tan-spot disease in durum wheat (Simoglou et al., 2006), while applying carbon nanotubes with or without nitrogen, phosphorus and potassium (NPK) fertilizers significantly improved plant growth, yield and antioxidants expression (Hasaneen and Omer, 2019).

A synthetic chemical like benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) has been used for induction of systemic acquired resistance (SAR) to protect plants against field diseases (Beckers and Conrath, 2007). The chemical is mobile in plants and sensitised the plants for enhanced resistance to subsequent infections. However, BTH was not readily adopted because farmers were interested in curative but not prophylactic applications.

2.2.7 Priming agents: microorganisms and bio-molecules

Different microorganisms can prime crop plants to enhance resistance to various stresses. Two different plant growth-promoting rhizobacterial (PGPR) strains (Gram-positive *Bacillus* sp. L81, isolated from *Pinus pinea* rhizosphere, and Gram-negative *Aeromonas* sp. AMG272 isolated from rice rhizosphere) primed rice plants for tolerance to abiotic stresses. These strains increased antioxidative enzymatic activities in rice plants, which scavenged the reactive oxygen species (ROS). The PGPR increased ascorbate peroxidase (APX) and pathogenesis-related (PR) protein chitinase in rice upon salt stress (García-Cristobal et al., 2015). *Pseudomonas fluorescens* has been used to prime Okra plants for drought stress. Infection increased the levels of various phenolic compounds, ascorbate and glutathione, and activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), APX, and guaiacol peroxidase (GOPX). The free amino acid and sugar contents were also elevated (Pravisya et al., 2019). The effects of the two PGPR strains (*Pseudomonas* sp. strain 6K and *Bacillus* sp. strain 6) on grain yield and defence responses against the RWA were tested in wheat cultivars. Evaluating the effect of the strains during RWA infestation, the results indicated increased phytoalexins and phenolic compounds, PR protein and transcript expression during RWA infestation (Naeem et al., 2018).

Fungi are the most important pathogens of cereal crops. The most common wheat rust disease is leaf rust (Lr), caused by Pt (Kolmer et al., 2013). Although the disease occurs more frequently in farmers' fields, the damage is often minor in comparison to stem rust (*Puccinia graminis* f. sp. *tritici*) (*Pgt*) and stripe rust (*Puccinia striiformis* f. sp. *tritici*) (*Pst*) (Terefe et al., 2009; Huerta-Espino et al., 2011). However, certain rust pre-infections increased resistance to stresses. For instance, Chen et al. (2015) reported that *Pst* infection enhanced wheat cultivars' resistance to biotic and abiotic stresses by maintaining the chlorophyll density and increasing antioxidant activities. Furthermore, Njom (2016) reported that prior infection by a leaf rust isolate (*Pt* race 3SA145) induced defence responses (antixenosis) in resistant (SST 347) and susceptible (SST 356) wheat cultivars to RWASA1 infestation.

Biotrophic fungi have remarkable variations in infection structures. The hyphae and appressoria invade the intracellular plant space with minor intercellular damage. Rust fungi, similarly, cause infection, and their extended presence within the cells activates defence responses for a long time compared to other priming agents, especially SA in the wheat plant (Mendgen and Hahn, 2002).

Applying leaf extracts like acetate (Z-3-hexenyl acetate, Z-3-HAC) to wheat primed it for enhanced defence responses to *Fusarium graminearum* (Ameye et al., 2015). Furthermore, purified plant or algal extracts containing damage-associated molecular patterns (DAMPs) have been used as potential plant vaccines to enhance resistance against herbivores in different crops (Quintana-Rodriguez et al., 2018). The DAMPs, including cell wall fragments, peptides, volatiles, extracellular Adenosine-5-triphosphate (eATP) and deoxyribonucleic acid (DNA) fragments, elicit resistant responses in crop species. In tobacco, eATP induced defence responses to Tobacco Mosaic Virus through PR gene expression (Chivasa et al., 2009). Similarly, plant extracts like the moringa leaf extract enhanced defence responses to pests and pathogens (Khan et al., 2020).

Phytohormones govern plant growth and responses to environmental factors. Ethylene, JA, and SA activate defence responses while abscisic acid (ABA), auxins, cytokinins, brassinosteroids and gibberellins promote plant growth (Denancé et al., 2013). The phytohormones can also activate resistance mechanisms against pests (Sytar et al., 2019).

Wheat plants resistant to *Fusarium graminearum* primarily initiate SA and JA-mediated defence pathways (Ameye et al., 2015). Wang et al. (2021) reported that the exogenous SA application induced callose deposition and closure of plasmodesmata in Arabidopsis. Furthermore, they reported that SA mediated cell-to-cell communication in innate immunity. Even though specific plant pathogen/pest effects occur, JA usually regulates defence responses during necrotrophic infection (Antico et al., 2012), while SA regulates defence responses during biotrophic invasion (Kou et al., 2021).

2.3 Resistance mechanisms

Plants cannot escape the environmental challenges that reduce growth and development. Potential pathogens such as bacteria, nematodes, fungi, and insects cause biotic stress (Moustafa-Farag et al., 2019), and plants activate different mechanisms to counter these stresses. In soybean, different varieties respond differently to aphid infestation; some use antibiosis and antixenosis defence responses (Baldin et al., 2018). Mafa et al. (2022) reported that wheat plant apoplastic β-1,3-glucanase and peroxidase (POD) activities were significantly higher in resistant (cv. Tugela *Dn5*) compared to susceptible wheat (cv. Tugela) to RWASA2 infestation. Furthermore, they observed the induction of holo-cellulose and crystallinity index to validate cell wall modification in cultivars resistant to RWASA2 infestation.

Plants use morphological and physiological responses to minimize and avoid biotic and abiotic stresses. In biotic stress, plants adopt different strategies like opening or closing stomata, leaf area and root length changes, and chlorophyll maintenance, regulated by different hormones. In the case of wheat, there are no specific morphological responses reported to resist RWA infestation. Wheat plants express physio-biochemical reactions to RWA infestation (Franzen et al., 2014).

Pathogens usually express virulence effects on host plants, increasing their access to nutrients and consequent ability to develop colonies. Plants, in response, mount defences to reduce pathogens' damaging effects and colonization. Plants employ primary defence responses for rapid de-localisation of pathogens, often species-

specific, and activate systemic defence responses that protect the entire plant (Hettenhausen et al., 2015).

Plants are primed by signalling molecules such as hydrogen peroxide (H_2O_2), nitric oxide (NO) donor sodium nitroprusside (SNP) and hydrogen sulphide (H_2S) donor sodium hydrosulphide (NaHS), melatonin and polyamines (Savvides et al., 2016). The exogenous application of H_2O_2 increases the cellular oxidative potential and requires efficient regulation by antioxidative species to prevent oxidative damage. Examples of antioxidative species include ascorbic acid (AA), glutathione and proline (Ellouzi et al., 2017).

2.3.1 Physiological and biochemical alterations

Microbial and hormonal treatment of wheat induces changes in plants' physiological and biochemical status. Treatment of plants with SA, JA and plant growth-promoting bacteria (PGPB) alters plants' biochemical status, increases disease tolerances, and enhances plant growth (Çakmakçı et al., 2017). The SA application enhances wheat's dry mass accumulation, water content, rubisco, carboxylic dismutase, and SOD activity (Sing and Usha, 2003). However, Franzen et al. (2014) studied photosynthetic capacity, chlorophyll and carbohydrate content in resistant and susceptible wheat cultivars and found no significant differences in chlorophyll contents among resistant and susceptible cultivars. They further showed that different responses in total protein and POD activities were related to specific pest and fungal infections (Franzen et al., 2014).

2.3.2 Cell wall modifications

The cytoskeleton is an intracellular framework (Henty-Ridilla et al., 2013) that consists of microtubules and actin filaments that mediate the intracellular transportation in tissues and respond to biotic and abiotic stresses. It performs innate immunity to oomycetes and fungi. Owing to pathogen and symbiotic microbe interactions, architectural changes in the cytoskeleton occur. Furthermore, plant cell and pathogen interactions rearrange cytoskeletal delivery as part of the defence expression (Schmidt

and Panstruga, 2007). During perturbation by effector proteins, two distinct changes in the cytoskeleton cause cell wall deposition (Kesten et al., 2017). One change is associated with pathogen-associated molecular patterns (PAMPs), where effector-triggered immunity is activated, and the other occurs during the interaction with microbe-associated molecular patterns (MAMPs), which activates the pattern-triggered immunity (Henty-Ridilla et al., 2013).

The plant cell responds to invasion during biological elicitation by expressing specific cell wall proteins (CWPs). These proteins reinforce the cell wall region, creating a physical barrier to invading pathogens. Cell wall proteins mediate the expression of downstream responses, such as the expression of the PR proteins (Rashid, 2016).

The plant cell wall is a highly organised and dynamic network that changes throughout cell life. It involves different components, including cellulose, hemicellulose and pectin polymers, aromatic substances, proteins, water and ions. It is a structural and functional barrier to pest and pathogen invasion and can be remodelled under stress stimuli (Houston et al., 2016). The plant cell wall consists of two layers, the primary and secondary cell walls. Lignin is a complex phenolic polymer and an essential component of the secondary cell wall. It strengthens plant cells and provides rigidity and hydrophobicity to allow cells to maintain turgor pressure, grow and transport water and nutrients. Cellulose, hemicellulose, and lignin in the ratio of 4:3:3 are the main components of the plant cell wall and form an essential component of lignocellulose (Chen, 2014).

Resistant and susceptible cotton plants showed increased lignin biosynthetic gene expression (3442 defence-responsive genes), phenylalanine ammonia-lyase (PAL), and POD against *Verticillium dahlia* strain V991 (Xu et al., 2011). Furthermore, these defence responses were faster and more intense in resistant than susceptible cotton plants and associated with lignification. Cellulose is a structural and functional main constituent of the cell wall that can reach up to 53% dry weight of the total fibre composition. The cell wall also contains wax, fatty substances, suberin and cutin (Abdel-Halim, 2014).

A difference in resistance and susceptibility to *Fusarium graminearum* was noted regarding the accumulation of methyl esterification and deoxynivalenol cell wall compounds in durum wheat (Giancaspro et al., 2018). Plant cell wall structural

modification occurs under the enzymatic activities of pectin methylesterase (PME) genes. Pectin methylesterase transfers signals from the apoplast to the cytoplasm. Furthermore, PME (*TaPME*) genes induce cell wall modification in wheat plants (Zega and D'Ovidio, 2016).

A high level of β-1,3-glucanase is present in many plant species. It performs vital roles in cell division, flower formation and seed maturity, material transportation through plasmodesmata, and stress responses (Balasubramanian et al., 2012). Furthermore, Balasubramanian et al. (2012) reported that the enzyme improves plant resistance against fungal pathogens. The activities of β-1,3-glucanase and chitinase were noted in resistant cultivars compared to susceptible ones in *Eruca sativa* and wheat (Balasubramanian et al., 2012; Mafa et al., 2022). Enzymatic activity increased throughout the expression period of up to 7 days of infection (Gupta et al., 2013). The β-1,3-glucanase gene (*TaGluD*) was identified as a candidate defence gene in wheat against a fungal pathogen (*Alternaria brassicicola*). Its transcripts were more than 60 times higher in resistant wheat cultivars, expressing *in vitro* antifungal activity against fungal species: *A*. *longipes* (30%), *Phytophthora capsici* (32%), *Rhizoctonia cerealis* (43%) *and R*. *solani* (42%) (Liu et al., 2009).

2.3.2.1 Cellulose

Cellulose is a tightly packed crystalline structure of the plant cell wall, arranged in linear chains of β-D-glucose. Non-covalent binding between β-D-glucose gives it high tensile strength equivalent to steel (Kozioł et al., 2017). Cellulose synthase catalytic subunit (CESA), present on the cytoplasmic side of the plasma membrane, is a crucial enzyme for cellulose synthesis (Endler and Persson, 2011). Modifications through CESA activity to the primary and secondary cell walls impart resistance to pathogens and abiotic stress (Bacete et al., 2018). Mutations in cellulose synthase genes and cell expansion regulating genes reduce cellulose concentration and activate lignin biosynthesis and expression of defence responses through jasmonate and ethylene-signalling pathways (Cano-Delgado et al., 2003).

Cell wall reinforcement enhances resistance to pathogens, including necrotrophic fungi (Bacete et al., 2018). Kesten et al. (2017) reported that cellulose-deficient mutant

plants are more sensitive to environmental stress than wild-type plants. Callose deposition between the cell wall and plasma membrane at the site of pathogen attack and the plasmodesmata causes a barrier to the pathogen's invasion (Wang et al., 2021). Furthermore, callose has a resilience capacity for the deformation of cellulose by pathogen attack. Cellulose, therefore, plays an essential role in plant-pathogen interactions. The cellulose synthase enzyme is responsible for cellulose production and strengthens the wheat plant cell wall during incompatible wheat-RWA interaction (Liu et al., 2011). The enzyme upregulates cellulose production in incompatible wheat interactions with either *D. noxia* or *Mayetiola destructor* (Liu et al., 2007).

2.3.2.2 Hemicellulose

Hemicellulose is a non-cellulosic compound present in both primary and secondary cell walls. Hemicellulose comprises glucose, xylose or mannose that form bridges between cellulose and pectin (Padayachee et al., 2017). Hemicellulose increases cell wall stiffening, enhancing the resistance response (Le Gall et al., 2015). Santiago et al. (2013) reported that leaves of a resistant maize inbred line to *Diatraea grandiosella* (Southwestern corn borer) and *Spodoptera frugiperda* (fall armyworm) showed a higher level of hemicellulose than the susceptible counterparts. Furthermore, cell wall polymers negatively correlated with fall armyworm feeding on resistant maize leaves (Hedin et al., 1996).

2.3.2.3 Lignin

Lignin is a phenolic compound that imparts the aqueous phase, impermeability and mechanical strength to the plant cell wall (Davin and Lewis, 2005). Predominantly, lignin deposition (lignification) at the secondary cell wall strengthens the plant cell wall (Vanholme et al., 2010). The rapid deposition of lignin and lignin-like phenolic compounds during biotic and abiotic stresses mediate multiple responses in the plant defence mechanism. Lignin is a physical barrier to pathogen invasion, and the phenylpropanoid pathway is responsible for lignin biosynthesis and defence responses (Lozovaya et al., 2007).

Lignin deposition occurred in response to *V. dahliae* in cotton and *Sclerotinia sclerotiorum* in *Camelina sativa* (Bellincampi et al., 2014). Lignin strengthens cell walls to resist the cell wall degrading enzymes and inhibits pathogen toxins from diffusing into the cytoplasm (Bellincampi et al., 2014), indicating that cell walls are reinforced through lignin deposition. Similarly, lignin joins cell wall polymers in wheat-RWA interaction and strengthens the cell wall to deter RWA infestation (Mafa et al., 2022).

2.3.3 Antioxidative responses

Insect and pathogen invasion triggers a change in calcium channels (Maffei et al., 2007) within host plants, sensed by calmodulin-like proteins and calcium-dependent protein kinases, which activate the accumulation of ROS and reactive nitrogen species (RNS) (Pan et al., 2019). Scavenging and compartmentalising ROS and RNS occur through multiple routes in a plant cell. A mitogen-activated protein kinase (MAPK) cascade is engaged upstream and downstream of ROS and nitric oxide (NO) production. Nitric oxide is a crucial signalling molecule regulated by *S*-nitrosylation. If these reactive species accumulate, they can be toxic to host cells, causing damage to DNA, proteins, lipids, and carbohydrates (Rowe et al., 2008), ultimately inducing oxidative stress, especially under abiotic stress conditions. The protective antioxidative defence machinery is upregulated to overcome oxidative stress and toxicity. Enzymatic (APX, CAT, glutathione reductase (GR), glutathione peroxidase (GPX), GOPX, glutathione-S-transferase (GST), monodehydroascorbate reductase (MDHAR), and SOD, and non-enzymatic (AA, glutathione, phenolic compounds, α-tocopherols, alkaloids and non-protein amino acids) activities control the cascades of oxidation reactions and protect plant cells (Gill and Tuteja, 2010).

2.3.3.1 Reactive oxygen species

Superoxide anion (O_2^-), H_2O_2, hydroxyl radical (OH^-), and singlet oxygen ($^1O_2$) are examples of ROS produced in plants under biotic and abiotic stress (Floriano-Sánchez et al., 2006). Reactive oxygen species play a significant role in plant defence against pests and pathogens. Rapid and prolonged accumulation of H_2O_2 occurs in wheat plants under stress conditions (Liu et al., 2010). In one study, H_2O_2 accumulated more

in resistant than susceptible wheat cultivars infected with leaf rust (Hafez et al., 2009). Russian wheat aphid infestation causes ROS accumulation, which under regulation, activates defence responses (Botha et al., 2005).

Reactive oxygen species are continuously produced in natural plant metabolism as a by-product of aerobic respiration, and cellular antioxidative reactions rapidly detoxify some ROS. The ROS are signalling molecules that control various processes, including activating the resistance response against pathogens, often associated with induced plant cell death (Apel and Hirt, 2004).

Reactive oxygen species, however, play a dual role in plant biology. They signal the activation of reactions necessary for cellular proliferation and differentiation at low concentrations, and elevated levels (oxidative burst) cause cell death (Mittler, 2017). The accumulation of ROS and programmed cell death correlate with the resistance of wheat during plant-pathogen interactions (Li et al., 2016). Virulent fungal strains induce the production of H_2O_2 in wheat, which is regulated by oxalate oxidase activity (Maksimov et al., 2009). A regulated increase in the production of H_2O_2 is part of the defence responses of resistant wheat to RWA infestation (Moloi and Van der Westhuizen, 2006). Furthermore, Kerchev (2011) observed H_2O_2 accumulation after three hours of RWA infestation in resistant wheat cultivars.

After perturbation by RWA, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase catalyses a differential production of H_2O_2 in resistant and susceptible plants (Moloi and Van der Westhuizen, 2006). Reactive oxygen species play a crucial role as secondary messengers under stress conditions (Xiong et al., 2002; Sharma et al., 2012). Hydrogen peroxide can oxidize critical cysteine thiol groups of phosphatases (PTEN, PTP1B, and MAPK) so that H_2O_2 is a signalling molecule (Veal et al., 2007). Reactive oxygen species also modify cell walls in wheat and rice-Hessian fly larval interaction (Lehmann et al., 2015). The hydrogen peroxide accumulating in response to biotic stress activates antioxidative systems and other defence pathways (Caverzan et al., 2016). Plant growth-promoting bacteria inoculation increase glutathione and ascorbic acid concentrations in specific plants and activate defence mechanisms in *Brassica napus* L. (Ren et al., 2019).

Reactive oxygen species are produced in resistant wheat plants after inoculation with *Pt* (Hafez et al., 2009). In a susceptible host-pathogen combination, less H_2O_2

accumulated than in the resistant plants, showing that ROS plays a central role in activating defence responses to infection.

2.3.3.2 Antioxidants

It is vital to regulate the amount of induced ROS by activating the accumulation of antioxidants in plant cells to minimise the potential damage. Enzymatic and non-enzymatic antioxidative activities are part of the detoxification mechanism of ROS.

2.3.3.3 Enzymatic antioxidative activities

Enzymatic antioxidative activities like APX, CAT, GR, GPX, GOPX, GST, MDHAR and SOD overcome the effects of ROS and protect plant cells from oxidative damage (Gill and Tuteja, 2010). Biotic factors like RWA infestation in wheat significantly induced the activity of SOD, GR, and APX in resistant cultivars (Moloi and Van der Westhuizen, 2008).

2.3.3.3.1 Superoxide dismutase (EC 4.6.1.2)

Superoxide dismutases are metal-containing enzymes that catalyse the dismutation of superoxide radicals to H_2O_2 and oxygen. The SODs occur in all aerobic organisms, essential in defence against oxidative stress. Superoxide dismutases are proposed as vital plant stress-tolerance enzymes. Plants have three types of enzymes that have metal cofactors: iron, manganese, and copper/zinc, that is, FeSOD, MnSOD, and Cu/ZnSOD (Zhang et al., 2015).

The inoculation of soil with *Trichoderma tomentosum* increased the activity of SOD, POD and PAL in wheat plants (Pittner et al., 2019). Debona et al. (2012) also reported that SOD, POD, APX and GST activity in a resistant wheat cultivar inoculated with the blast fungus (*Pyricularia oryzae*) significantly increased compared to the control. Similarly, SOD, GR and APX increased in resistant wheat cultivars infested with RWA (Moloi and Van der Westhuizen, 2008).

2.3.3.3.2 Peroxidase (EC 1.11.1.7)

Peroxidase is a vital enzyme present almost in all living organisms. It is a ROS-scavenging enzyme and contributes to lignification, cell wall elongation, wound healing, resistance to pathogen infection and suberization. Furthermore, POD is a remarkable environmental stress marker (Jouili et al., 2011). Soluble POD activity correlated with lignin deposition in the rose plant, indicating enhanced defence responses (Ekeke et al., 2019). Peroxidase oxidises guaiacol in the presence of H_2O_2 to form tetraguaiacol. Guaiacol is a reducing agent, changing its colour after losing protons to H_2O_2; water and oxygen molecules are the end products.

Peroxiredoxins (Prxs), localized in the cytosol, plastid, mitochondrion, and nucleus, are thiol peroxidases that destroy the peroxides as an antioxidative defence and part of the redox signalling network of the cell. Thiol-disulfide is a central part of the stress-sensing process and integrating information input system associated with redox regulation (Rouhier et al., 2004; Liebthal et al., 2017).

Singla et al. (2020) reported that *Pst* G-race inoculation increased the activity of CAT, POD and ascorbate-glutathione in the resistant barley cultivar RD2901, while glutathione/oxidised glutathione (GSH/GSSG) ratio remained stable in the susceptible cultivar. An increase in POD activity in wheat plants in response to RWA infestation is evidence of these defence responses (Moloi and Van der Westhuizen, 2008).

2.3.3.3.3 Ascorbate peroxidase (EC 1.11.1.11)

A primary H_2O_2 detoxifying system in plant cells is the ascorbate-glutathione cycle, in which APX plays a crucial role in catalysing the conversion of H_2O_2 into H_2O and O_2, using ascorbate as a specific electron donor (Huseynova et al., 2013). Ascorbate peroxidase is an H_2O_2-scavenging enzyme in plants and algae (Ishibashi et al., 2008). Higher APX activity indicates defence activation in resistant plants. Łukasik et al. (2012) reported that APX activity was higher in resistant triticale plants infested by bird cherry-oat aphid (*Rhopalosiphum padi* L.) and grain aphid (*S. avenae* F.), while ascorbate content was low in the susceptible plants. Similarly, resistant wheat cultivars significantly increased APX activities under RWA infestation (Moloi and Van der Westhuizen, 2008).

2.3.3.3.4 Catalase (EC 1.11.1.6)

Catalase is one of the earlier antioxidative enzymes induced to regulate ROS levels. It occurs in all H_2O_2-producing sites in the plant cell, such as the cytosol, chloroplast, mitochondria and peroxisomes (Sharma and Ahmed, 2014). Hydrogen peroxide scavenging by CAT isozymes is time and stress-specific within definite cells or cellular organelles. Regarding wheat-RWA interaction, RWA biotypes induced significantly higher defence-responsive enzymatic (CAT, *β*-glucanase and POD) activities (Lapitan et al., 2007). However, Yarullina et al. (2011) reported reduced CAT activity over time in *Septoria* leaf blotch-infected wheat plants, where SA inhibited CAT activity.

2.3.3.3.5 Glutathione reductase (EC 1.6.4.2)

Biotic and abiotic stresses activate GR (Bashir et al., 2007), a flavin-containing enzyme which reduces the GSSG into the reduced GSH form in the presence of an electron donor, NADPH (Farvardin et al., 2020). The GR plays a vital role in maintaining the cell ratio of GSH/GSSG and in metabolism (Elavarthi and Martin, 2010). Reduced glutathione converts to the GSSG form in the presence of ROS that donates electrons. In the presence of GR, the GSSG form converts to the GSH form (Couto et al., 2016).

2.3.3.4 Non-enzymatic antioxidants

2.3.3.4.1 Tocopherol

Tocopherol is a vital antioxidant known as vitamin E, commonly presented as α-tocopherol (Engin, 2009). Vitamin E scavenges active free radicals primarily by hydrogen atom transfer reaction to yield a nonradical product and vitamin E radical (Niki, 2014). The self-reaction of two molecules forms a dimer and trimer forms of α-tocopherol. The tocopherol radicals of γ- and δ-tocopherols prefer to react with each other to form a dimeric form that still reacts as an antioxidant (Yamauchi, 2009). Some evidence exists that α-tocopherol may affect the signalling pathway and redox reaction homeostasis by controlling the propagation of lipid peroxidation (Munné-Bosch, 2007).

2.3.3.4.2 Ascorbate and glutathione

A sharp increase in research on the signalling functions of ROS in conjunction with ascorbate and glutathione has been noticed in plants under biotic and abiotic stresses. Ascorbate and glutathione are part of the antioxidative system, regulating ROS levels. Reactive oxygen species directly oxidise ascorbate and quickly reduce glutathione. Due to this performance, ascorbate and glutathione are sacrificial nucleophiles (Kaur and Arora, 2013). Ascorbate and glutathione are different from other antioxidants because they associate with specific enzymes linking them to peroxide metabolism, exist as stable oxidised forms, and recycle to reduced forms through enzyme-catalysed reactions (Foyer and Noctor, 2011). Ascorbic acid contents decreased in triticale plants infested by cereal aphids, while POD activity increased throughout the infestation period (Łukasik et al., 2012).

Glutathione is an antioxidant in detoxification, redox homeostasis, and biosynthetic pathways. Glutathione can also interact with multiple proteins through thiol-disulfide links. Its interaction with ROS and cellular reductions confers a signalling function that activates defence responses (Noctor et al., 2012).

2.4 Conclusion

Currently, five RWA biotypes are present in South Africa and induce significant yield losses in wheat production. In South Africa, aphid-free wheat production can result in a medium to high grain yield potential. However, pest management through chemical pesticides is costly and potentially destructive to the environment, and the emergence of resistance-breaking biotypes challenges host resistance through breeding. An alternative strategy for durable aphid management that is less severe to the environment can be priming by plant activators and biotic agents. Research about wheat primed by fungi to RWA infestation has been reported based on proteomics. However, the role of fungal isolates and exogenous phytohormones, particularly SA, in antioxidative and cell wall-mediated defence responses has not been fully elucidated.

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Chapter 3

Efficacy of salicylic acid and *Puccinia triticina* isolates in reducing *Diuraphis noxia* damage in wheat

3.1 Introduction

Wheat is an important cereal crop that provides 20% of calories for humans worldwide (Muhammad et al., 2020). However, wheat production is at risk because of pest damage (Merrill and Peairs, 2013), including the Russian wheat aphid (RWA, *Diuraphis noxia*, Kurdjumov), a cosmopolitan pest that can reduce yield by up to 93% in different wheat-producing regions (Damte et al., 2018).

The RWA infestation in wheat production is primarily controlled by resistance breeding. Even though this is an effective strategy, the evolution of resistance-breaking biotypes weakens the durability of resistance against multiple RWA biotypes (Jankielsohn, 2017). Unfortunately, resistance breeding often lags behind the appearance of new, more virulent RWA biotypes, necessitating exploring alternative strategies to manage RWA. Producers use chemical aphicides to protect crops from devastating infestations and maintain acceptable yields. Aphicides have been used extensively in South Africa to curb aphid populations when only susceptible cultivars were available (Du Toit and Walters, 1984). Aphicides include contact and systemic pesticides. Systemic pesticides are usually more expensive and less environmentally friendly (Masinde et al., 2014), while contact pesticides are less efficient because the aphids take refuge within the rolled leaves.

Alternative affordable and environmentally friendly strategies equally effective at controlling aphid populations, reducing plant damage, and maintaining yields are required. One approach uses priming agents like phytohormones and microbes as prophylactic measures before infestation. Priming is a physiological state in which plants deploy intense and rapid defence responses to biotic and abiotic stresses (Beckers and Conrath, 2007; Balmer et al., 2015). Pre-treatment of plants with chemicals or avirulent microbes primes various plants, including wheat (*Triticum aestivum* L.), improving their tolerance to biotic stress (Blunk et al., 2019). Ab Rahman et al. (2018) further described priming as a powerful tactic to lower input

costs in sustainable agriculture because it can potentially protect crops from pests and pathogens. Using priming agents such as hormones, mineral nutrients, and biocontrol microorganisms to modulate plant defence responses can reduce the application of pesticides and their toxic effects.

Some inorganic nutrients activate defence responses to insect infestation; for instance, phosphate and silicon-based fertilisers reduce insect infestation in monocots and dicots (Asiwe, 2009; Ab Rahman et al., 2018; Alhousari and Greger, 2018). Similarly, exogenous application of plant hormones like salicylic acid (SA) and jasmonic acid (JA) improved plant vigour and tolerance to salinity stress in wheat (Yücel and Heybet, 2016). Exogenous application of SA activated the defence responses (Erdal et al., 2011) because it reduced the number of aphids, *Lipaphis erysimi*, on mustard (Mony et al., 2017; Feng et al., 2021) and lowered the disease index of tomato stem-canker in tomato plants (Esmailzadeh et al., 2008).

Besides chemicals and plant hormones, microbes like plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) have been used as priming agents to enhance defence responses to diseases in various crops and promote plant growth (Singh et al., 2019). Seed treatment with these organisms, notably the PGPR, effectively reduced aphid infestation effects on wheat (Naeem et al., 2018). Similarly, some fungi and fungus-like organisms (Ascomycetes, Oomycetes and arbuscular mycorrhizal fungi) have been successfully used to prime various crops, including cotton, for enhanced resistance to soil-borne diseases (Guo et al., 2014; Singh et al., 2019). Njom (2016) reported that wheat cultivars inoculated with *Puccinia triticina* Eriks. (*Pt*) isolates were primed for a resistance response to RWASA1. The primed condition was based on antixenosis and proteomic results, and to our knowledge, leaf rust pathogen *Puccinia triticina* Eriks. (*Pt*) inoculation of wheat to enhance the phenotypic defence responses (leaf damage) to RWA has not been tested.

This study's main objective was to investigate and compare SA and *Pt's* efficacy in reducing infestation damage by two South African RWA biotypes (RWASA1 and RWASA4) in various wheat cultivars. We used isolates of two *Pt* races with differential virulence and two concentrations of SA to prime wheat for increased

resistance to RWA infestation. The priming effect was evaluated at the seedling and flag leaf stages by assessing the degree of RWA-induced leaf damage in the different wheat cultivars.

3.2 Material and methods

We investigated the efficacy of SA and *Pt* isolates in priming various wheat cultivars for improved resistance to RWA infestation. Plants treated with SA or *Pt* isolates were artificially infested with the Russian wheat aphid biotypes (RWASA1 or RWASA4), and induced leaf damage in all the cultivars was measured. The following sections describe the wheat cultivars selected for this study, *Pt* isolates, and other procedures used to evaluate SA and *Pt* priming efficacy in reducing RWA-induced leaf damage.

3.2.1 Wheat cultivars

Wheat cultivars tested in this study originated from South Africa (PAN 3118, PAN 3161, PAN 3111, PAN 3133, PAN 3368, and SST 356) and Lesotho (Bolane, Tsholoha, Makalaote, and Mapili). These cultivars were selected based on their reaction to RWA biotypes and yield potential (ARC-Small grain production Guideline, 2020; https://www.arc.agric.za).

3.2.2 Wheat cultivation

Two different experiments were conducted on plants at the seedling and flag leaf stages under controlled conditions in Botany and Plant Pathology Greenhouses, the University of the Free State, Bloemfontein, South Africa (29° 6'27.08"S, 26°11'19.12"E). Since winter and spring types of wheat were used, all seeds were vernalised at 4°C for six weeks before planting. Plants were grown in a mixture of steam-sterilised red soil and peat (1:1) and were fertilised with a 0.2% (w/v) Multifeed-Classic water-soluble fertiliser [Effekto®, NPK Analysis 19:8:16 (43)] once per week for the duration of the trials. The fertiliser was dissolved in filtered

water at a rate of 5 g/l, and a 50 ml solution was applied to 20 plants per pot at the seedling stage and five plants per pot at the flag leaf stage. Growth conditions were according to Tolmay et al. (1999), where plants grew under natural light, and the temperature was maintained at 24±2 ºC (day: 13±1 hours) and 18±2 ºC (night 11±1 hours). All experiments were kept under nylon mesh cages (sieve size 315 µm) to inhibit aphid escape and biotype contamination.

3.2.3 Priming agents

Salicylic acid

Salicylic acid (SA, Sigma-Aldrich) was used as a priming agent to mobilise SA-mediated resistance pathways. Two SA solutions (1.5 mM and 3.0 mM) were prepared by initially dissolving a measured amount of SA in 4 ml absolute ethanol and then adjusting the dissolved SA to a total homogeneous solution of 400 ml in distilled water. Salicylic acid solutions were covered with aluminium foil.

We used a 100 ml atomiser spray glass bottle to apply SA solutions (foliar spray treatment) until runoff on plant leaves at the seedling and flag leaf stages. Two days later, the treated plants were infested with either RWASA1 or RWASA4. A mass of 20 clonal aphids (mature, apterous) per plant at the seedling stage and 150 clonal aphids at the flag leaf stage were scattered on wheat plants using a camel hairbrush to ensure equal distribution.

Leaf rust

Representative isolates of University of the Free State *Puccinia triticina* race 2 (UVPt2) (North American code CBBS), UVPt13 (SFDS), UVPt19 (SDDN), UVPt20 (CCPS), UVPt25 (CBPS) and UVPt26 (CFPS) (Long and Kolmer, 1989) were pre-screened for suitability in this study. The isolates of two races, virulent (UVPt26) and avirulent (UVPt13), to the wheat cultivars were selected for this study. The wheat variety Federation*4/Kavkaz was used to multiply UVPt13 spores and the cultivar SST 087 for UVPt26. Leaf rust urediniospores were stored at -80 ºC before use.

Urediniospores were heat-shocked at 46 ºC for 6 min in a water bath before inoculation. The spores were then dissolved in Soltrol® 130 isoparaffinic oil (Chevron Phillips Chemical Company LP) at 1 mg per 0.8 ml concentration. Plants were inoculated with the spore concentrate using a pressure pump (Vacuubrand® pump-model MZ2) set at 25 kPa, and attached to an inoculation nozzle (Pretorius et al., 2019). Inoculations were carried out in an enclosed booth and washed with a quick (60 s) rinse (spraying) with filtered water before and after inoculation. Inoculated plants were dried in a Conviron growth cabinet with fluorescent growth lights (200 µE/m^2/s light) set at 25 ºC for one hour. The plants were then transferred to a dew simulation chamber for overnight incubation at 19±1 ºC under >95% humidity, where urediniospores could germinate and cause seedlings or flag leaves infection. Forty-eight hours after inoculation, plants were infested with RWA as described above for the SA-treated plants.

Screening of leaf rust isolates

Isolates of *Pt* race UVPt2, UVPt13, UVPt19, UVPt21, UVPt25 and UVPt26 were used to determine the seedling responses of PAN 3118, PAN 3161, Bolane, and Tsholoha. The data were used to identify virulent and avirulent *Pt* races. The wheat varieties RL6078, carrying resistance gene *Lr*26 for UVPt13; RL6007 carrying resistance gene *Lr*26 for UVPt26, RL6042 carrying resistance gene *Lr*3bg for UVPt21 and Tobie, carrying resistance gene *Lr*32 for UVPt19 were used as standard checks to confirm the purity of the *Pt* isolates representative of the respective races.

3.2.4 Selection of RWA biotypes

Cultivar responses to four existing South African RWA biotypes (RWASA1-4) were determined in PAN 3118, PAN 3161, Bolane, and Tsholoha. Based on the results, RWASA1 and RWASA4 were selected to study wheat-RWA interactions under primed and non-primed conditions. The RWA biotypes were maintained on seedlings of the cultivar PAN 3118 enclosed in nylon mesh cages (315 µm). Wheat plants at the seedling or flag leaf stages were pre-inoculated with *Pt*

urediniospores or treated with SA before RWA infestation.

3.2.5 Treatments at seedling and flag leaf stages

The following treatments were applied to investigate SA and *Pt*'s efficacy in reducing RWA-induced damage on wheat leaves:

Table 3.1: Treatments at seedling and flag leaf stages to investigate the effect of salicylic acid and isolates of *Puccinia triticina* race UVPt13 and UVPt26 on modulating RWA-induced leaf damage

Seedling stage	Flag leaf stage
T_1 = control	T_1 = control
T_2 = RWASA1	T_2 = RWASA1
T_3 = 1.5 mM SA + RWASA1	T_3 = 1.5 mM SA + RWASA1
T_4 = 3.0 mM SA + RWASA1	T_4 = UVPt13 + RWASA1
T_5 = UVPt13 + RWASA1	T_5 = UVPt26 + RWASA1
T_6 = UVPt26 + RWASA1	T_6 = RWASA4
T_7 = RWASA4	T_7 = 1.5 mM SA + RWASA4
T_8 = 1.5 mM SA + RWASA4	T_8 = UVPt13 + RWASA4
T_9 = 3.0 mM SA + RWASA4	T_9 = UVPt26 + RWASA4
T_{10} = UVPt13 + RWASA4	
T_{11} = UVPt26 + RWASA4	

T = Treatment

Treatment 4 (3.0 mM SA + RWASA1) and T9 (3.0 mM SA + RWASA4) at the seedling stage were excluded at the flag leaf stage because a concentration of 1.5 mM and 3 mM SA did not induce significantly different responses in most cultivars at seedling stage (Table 3.7).

3.2.5.1 Wheat seedling growth stage

The experiment was conducted as a randomised complete block design (RCBD) with two replications. Each treatment was assigned randomly to 20 plants, and all experimental units were kept in separate gauze cages.

Venalised seeds were sown in plastic pots (1.8 l, 10×12×15 cm^3) filled with a mixture of red soil and peat (1:1). Seedlings were watered to saturation using reverse osmosis purified water every second day and fertilised (Multifeed-Classic) once per week. At the second leaf stage, 20 plants of each cultivar were sprayed with SA and two days later infested with the RWA (20 apterous adult aphids per plant). The leaves were evaluated for RWA-induced damage ten days later.

In *Pt* inoculation, a 0.012% w/v spore solution in isoparaffinic oil (1 mg *Pt* isolates per 0.8 ml Soltrol® 130 isoparaffinic oil) was prepared for a sample of 60 plants. Wheat plants were sprayed using a pressure pump (Vacuubrand® pump-model MZ2) attached to an inoculation nozzle at 25 kPa (Pretorius et al., 2019). Forty-eight hours after inoculation, each pot (Table 3.1) was infested with a mass of 20 apterous adult aphids per plant. A camel-hair brush spread the required mass of aphids on the plant. The aphids were allowed to settle and feed for ten days before evaluating infestation damage on the leaves.

3.2.5.2 Leaf damage rating

All plants were evaluated for RWA leaf damage using a leaf damage rating scale (Tolmay, 1995). The scale varied from no damage to plant death: 1 = no feeding symptoms; 2 = minor chlorosis; 3 = average chlorosis; 4 = major chlorosis; 5 = minor streaking; 6 = prominent streaking; 7 = minor leaf rolling; 8 = average leaf rolling; 9 = prominent leaf rolling; and 10 = plant death. Chlorosis varied from 1 to 3 depending on the density of chlorotic spots, while streaking varied from minor streaks to the whole leaf covered by streaks. Plants scored under the category of 1-3 were considered highly resistant (R), 4-7, moderately resistant (MR), and 8-10 susceptible (S).

3.2.5.3 Flag leaf growth stage

An experimental design and planting protocol like that used at the seedling stage was adopted. However, the vernalised and pre-germinated seeds were transplanted into 1.45 l plastic pots (11×12×11 cm^3), previously filled with a steam-

sterilised soil and peat mixture as described (3.2.2), and each pot contained five plants. Pots were watered daily using filtered water and fertilised once a week.

Eight weeks after transplanting, the plants were treated with SA or inoculated with *Pt* before RWA infestation. Two RWA biotypes (RWASA1 and RWASA4) were used for the infestation of the different wheat cultivars in 16 pots.

At this stage, entire plants were sprayed with a single SA concentration (1.5 mM) or inoculated with isolates of the two *Pt* races, UVPt13 or UVPt26. As described for the seedling stage, the same protocol was used to prepare SA and *Pt* spores.

Plants were partitioned according to their treatments (Table 3.1), primed with SA (1.5 mM), or inoculated with *Pt* isolates. Forty-eight hours after SA application or *Pt* inoculation, plants were infested with 150 aphids per plant. After fifteen days of infestation, plants were scored using the RWA damage rating scale (Tolmay et al., 1999; Jankielsohn et al., 2016).

3.2.6 Data analysis

The data were analysed using the general linear model procedure: PROC GLM SAS 9.4 (SA Institute, 1989). Following analysis of variance (ANOVA), the means were separated by the least significant difference (LSD) to investigate significant differences between treatments, cultivars, and cultivars' grouping according to treatment responses. Similar cultivars and treatments at $P \leq 0.05$ were grouped. Pearson's correlation analysis indicated the relationship between the seedling and flag leaf stage responses.

3.3 Results

Wheat cultivar responses were used to determine the virulence of the isolates of six *Pt* races and four RWA biotypes (Table 3.2). Two *Pt* races and two RWA biotypes were identified and used to explore the priming efficacy of leaf rust isolates and SA in reducing RWA damage.

Table 3.2 Effect of leaf rust (*Puccinia triticina, Pt*) races on specific wheat cultivars

Wheat cultivars/ control lines	***Pt* races**					
	UVPt2[1]	**UVPt13**	**UVPt19**	**UVPt21**	**UVPt25**	**UVPt26**
Bolane	3[2]	5	3	8	9	9
Tsholoha	7	5	5	8	9	9
PAN 3118	7	5	5	9	9	9
PAN 3161	8	8	8	4	8	8
RL6078	1	8	1	9	1	8
RL6007	3	2	1	2	8	8
RL6042	8	1	2	8	9	9
Tobie	2	2	9	2	2	2

[1]UVPt = University of the Free State *Puccinia triticina* (isolates of leaf rust races kept by the University of the Free State, South Africa)

[2]Conversion of rust ratings: Zero fleck = 1; fleck = 2; fleck 1 = 3; fleck 12 = 4; 2 (very small pustules) = 5; 2+ (small-sized pustules) = 6; 3- (medium size pustules) = 7; 3 (normal size of pustules) = 8; 3+ (large pustules) = 9 and 4 (fully developed pustules) = 10; 1-3 = avirulent; 4-6 = intermediate avirulent and 7-10 = virulent (modified screening scale by Terefe et al., 2014)

Analysis of variance indicated that *Pt* isolates induced significantly different reactions in the wheat cultivars treated. The isolate, UVPt13, caused necrotic flecks and small pustules on Bolane, Tsholoha and PAN 3118, indicating a resistant response. Therefore, this isolate was considered avirulent to three of the four wheat cultivars. The isolate of *Pt* race UVPt26 induced large pustules on the tested cultivars and was classified as virulent (Table 3.2). Responses to isolates UVPt2, UVPt19, and UVPt21 varied from avirulent to virulent among the four cultivars, whereas UVPt25 was virulent. The *Pt* isolates produced the expected responses on the wheat lines carrying specific *Lr* genes. Based on the results, the *Pt* isolate of race UVPt13 was selected as representative of the avirulent group and the isolate of UVPt26 as representative of the virulent group for use in subsequent experiments.

Russian wheat aphid virulence

The virulence of Russian wheat aphid biotypes 1-4 was tested on four wheat cultivars before their selection for further studies.

Table 3.3 Leaf damage rating scores in four wheat cultivar seedlings infested with four South African Russian wheat aphid (*Diuraphis noxia*) biotypes. Values are means ± SE (n=10).

Cultivars	RWA biotypes			
	RWASA1	**RWASA2**	**RWASA3**	**RWASA4**
Bolane	8.7 ± 0.10 S	8.3 ± 0.20 S	4.8 ± 0.09 MR	6.2 ± 0.45 MR
Tsholoha	8.0 ± 0.00 S	6.9 ± 0.55 S	6.3 ± 0.13 MR	6.7 ± 0.10 S
PAN 3118	9.9 ± 0.00 S	9.1 ± 0.10 S	8.8 ± 0.15 S	9.0 ± 0.05 S
PAN 3161	5.8 ± 0.05 MR	5.3 ± 0.15 MR	2.8 ± 0.05 R	5.0 ± 0.01 MR

R = resistant (1-3); MR = moderately resistant (4-6.5); S = susceptible (6.6-10); screening scale by Tolmay (1995).

The analysis of variance, summarised in Table 3.3, indicated that RWA biotypes induced varying damage symptoms on different cultivars. The RWA biotypes RWASA1 and RWASA2 induced similar mean damage rating scores on the wheat cultivars, slightly higher mean scores recorded for RWASA1. On the other hand, RWASA3 and RWASA4 induced variable effects on the wheat cultivars. Biotype 3 produced a wide range of responses, from susceptible to resistant, while RWASA4 caused moderately resistant responses, with only PAN 3118 and Tsholoha being susceptible (Table 3.3). Based on the results, RWASA1 and RWASA4 were selected for all the following experiments.

3.3.1 Salicylic acid and *Puccinia triticina* priming at the seedling stage

Two isolates of *Pt* races (UVPt13 and UVPt26) and two SA concentrations (1.5 mM and 3.0 mM) were used to prime wheat cultivars for enhanced resistance to RWASA1 and RWASA4 infestations, measured as the degree of leaf damage. A ten-point damage rating scale (Tolmay, 1995) was used to measure RWA-induced leaf damage after ten days of infestation.

Table 3.4 Analysis of variance in primed [*Puccinia triticina* race isolates (UVPt13 and UVPt26) and salicylic acid (1.5 mM and 3.0 mM)] wheat cultivars infested by Russian wheat aphid biotypes RWASA1 and RWASA4 at the seedling stage

SOV	Df	Mean Square	SOV	Df	Mean Square
Treatment	9	78.13**	Replication	1	$0.061^{N.S}$
Cultivar	9	48.022**	Treatment x Cultivar	99	2.98**

** = highly significant at P≤0.01; N.S = non-significant;

Coefficient of variation = 8.48%

Analysis of variance (Table 3.4) indicated that treatments, cultivars, and the treatment x cultivar interaction were highly significant, but replication was not significant. Russian wheat aphid-induced responses were significantly different in primed and non-primed plants. The analysis further indicated a significant interaction between the cultivars (genotype) and the applied treatments.

Table 3.5 Homogeneous grouping of cultivar responses to Russian wheat aphid infestation in the seedling stage after salicylic acid treatments and *Puccinia triticina* inoculation

Cultivar	Damage rating score	Group
Mapili Makalaote	7.78 7.74	A
Tsholoha	7.16	B
Bolane	6.69	C
PAN 3118	5.71	D
PAN 3111	5.30	E
SST 356	3.99	F
PAN 3161	3.35	G

n = 20, means grouping at P≤0.05

The homogenous grouping of wheat cultivars based on induced symptoms indicated their resistance or susceptibility. Infestation severely damaged the Lesotho landraces, Mapili and Makalaote, indicating their susceptibility to the aphid. The South African cultivars PAN 3111, PAN 3133 and PAN 3368 showed similar and moderately resistant responses to RWA infestation. The other cultivars (Tsholoha, Bolane, PAN3118, SST356 and PAN3161) ranged from resistant to susceptible (scores 3.35 to 7.16), with significantly different means.

Table 3.6 Treatments that incurred homogeneous Russian wheat aphid damage in different wheat cultivars at the seedling stage

Treatments	Damage rating score	Group
RWASA4	8.76	A
1.5 mM SA + RWASA4	7.97	B
3.0 mM SA + RWASA4	7.31	C
UVPt26 + RWASA4	6.37	D
RWASA1 UVPt13 + RWASA4	6.06 5.84	E
1.5 mM SA + RWASA1	4.93	F
3.0 mM SA + RWASA1	4.09	G
UVPt13 + RWASA1	3.35	H
UVPt26 + RWASA1	2.74	I

n = 20, means grouping at P≤0.05. R = resistant (1-3); MR = moderately resistant (4-6.5); S = susceptible (6.6-10); screening scale by Tolmay (1995).

The treatment effects (leaf damage) on wheat cultivars were grouped differently. Biotype 4 (RWASA4) induced the most severe leaf damage (mean damage score 8.76) and was regarded as more virulent than RWASA1 (mean damage score 6.06). However, RWASA4 infestation caused lesser leaf damage in cultivars primed by UVPt26 (mean damage score 6.37) and UVPt13 (mean damage score 5.84), indicating shifts to moderate resistance responses. Similarly, UVPt13 (mean damage score 3.35) and UVPt26 (mean damage score 2.74) primed wheat cultivars for enhanced resistance to RWASA1, shifting from moderately resistant to resistant. Wheat plants primed by SA showed a significant shift with improved levels of moderate resistance to RWASA1. Even though the resistance category did not change, SA induced a shift, reducing the damage to a lower end within the category. In contrast, all other treatments elicited significantly different responses. Overall, RWA induced minor damage in various wheat cultivars primed by *Pt*.

Phenotypic observation at the seedling stage indicated that RWASA1 caused streaking and leaf rolling in various wheat cultivars (Fig. 3.1, Image B). However, the RWASA1 infestation of SA-primed plants displayed only mild chlorosis and leaf streaking (Images C and D). Nevertheless, despite SA pre-treatment, wheat cultivars still expressed RWA feeding damage, exhibiting moderate resistance levels. In images E and F (Fig. 3.1), RWASA1 infestation of wheat cultivars primed by isolates of the two *Pt* races induced mild chlorosis in South African cultivars.

The Lesotho landraces showed streaking and minor leaf rolling, except Makalaote primed with UVPt13, which had leaf rolling.

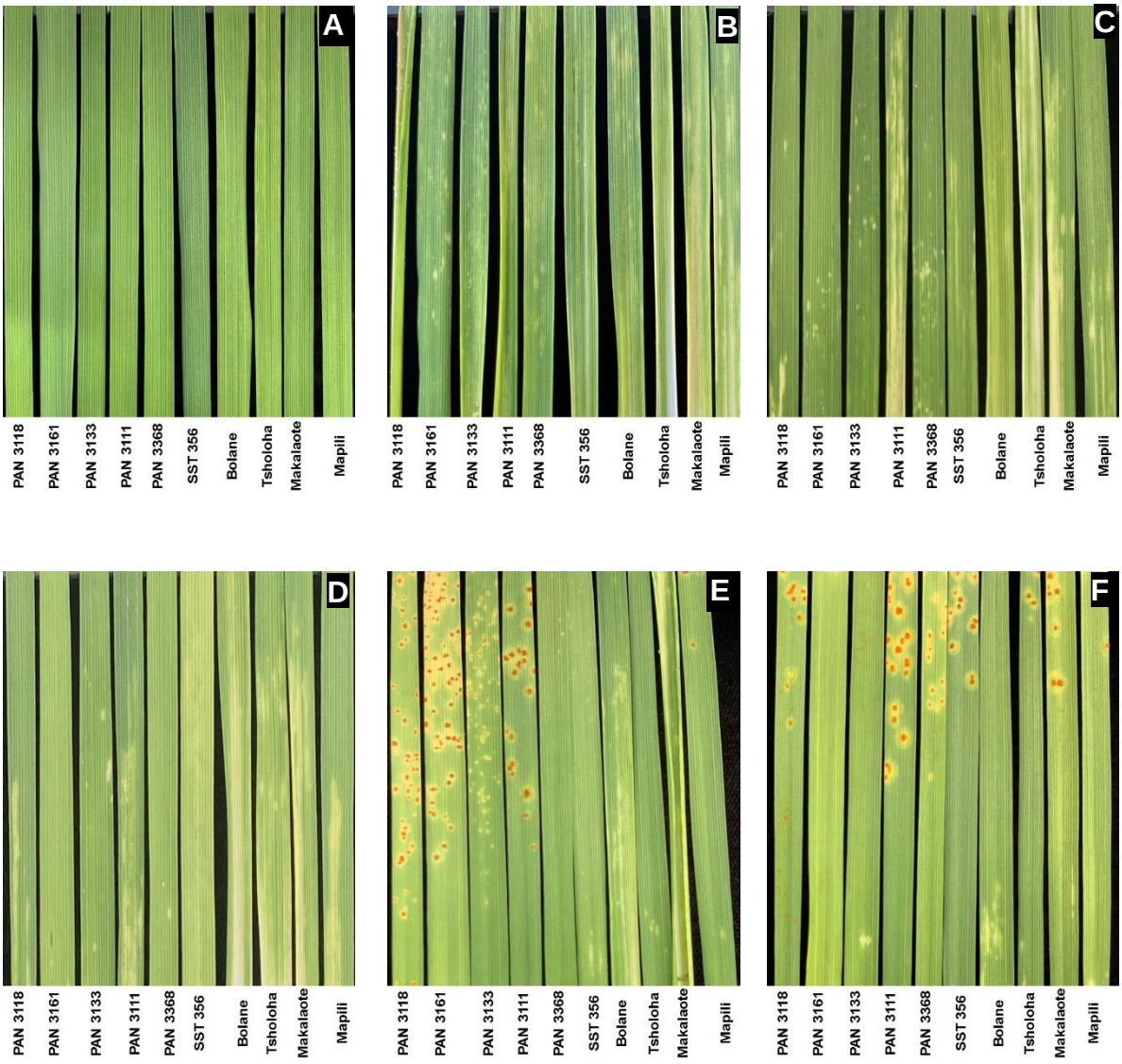

Fig. 3.1: Effect of Russian wheat aphid (RWASA1) infestation on leaf damage in wheat cultivars pre-treated with salicylic acid and isolates of *Puccinia triticina*. The images are from the second leaves of treated plants 10 days after aphid infestation. A: Control; B: RWASA1; C: 1.5 mM SA + RWASA1; D: 3.0 mM SA + RWASA1; E: UVPt13 + RWASA1; F: UVPt26 + RWASA1.

Table 3.7 Responses of wheat cultivars to Russian wheat aphid (RWASA1) infestation in salicylic acid and *Puccinia triticina* primed wheat cultivars at the seedling stage. Values are means±SE of leaf damage rating scores (n=2)

Cultivars	Treatments				
	RWASA1 (no priming)	**1.5 mM SA + RWASA1**	**3.0 mM SA + RWASA1**	**UVPt13 + RWASA1**	**UVPt26 + RWASA1**
PAN 3118	7.9 ± 0.55 S	6.5 ± 0.10 MR	6.2 ± 0.00 MR	2.1 ± 0.5. R	1.5 ± 0.25 R
PAN 3161	2.8 ± 0.50 R	1.4 ± 0.05 R	1.2 ± 0.00 R	1.0 ± 0.00 R	1.1 ± 0.10 R
PAN 3133	6.5 ± 0.50 MR	4.3 ± 0.20 MR	2.4 ± 0.60 R	2.7 ± 0.80 R	1.5 ± 0.10 R
PAN 3111	6.8 ± 0.00 S	6.4 ± 0.70 MR	5.4 ± 0.60 MR	1.3 ± 0.20 R	1.3 ± 0.00 R
PAN 3368	3.3 ± 0.15 R	3.3 ± 0.50 R	1.5 ± 0.15 R	1.3 ± 0.50 R	1.3 ± 0.20 R
SST 356	5.0 ± 0.30 MR	3.8 ± 0.25 R	2.3 ± 0.75 R	1.4 ± 0.05 R	2.0 ± 0.00 R
Bolane	5.7 ± 0.25 MR	4.1 ± 0.10 MR	4.2 ± 0.75 MR	5.4 ± 0.20 MR	5.1 ± 1.15 MR
Tsholoha	7.2 ± 0.45 S	6.1 ± 0.65 MR	4.6 ± 0.00 MR	4.7 ± 0.05 MR	4.7 ± 0.95 MR
Makalaote	8.2 ± 0.40 S	6.4 ± 0.15 MR	7.2 ± 0.15 S	8.0 ± 0.35 S	2.0 ± 0.70 R
Mapili	7.5 ± 0.60 S	7.2 ± 0.20 S	6.2 ± 0.10 MR	5.8 ± 0.70 MR	7.1 ± 0.30 S

Means grouping at P≤0.05. R = resistant (1-3); MR = moderately resistant (4-6.5); S = susceptible (6.6-10); screening scale by Tolmay (1995)

The cultivars PAN 3118, PAN 3111, Tsholoha, Makalaote, and Mapili were susceptible to RWASA1 infestation (Table 3.7). However, pre-treatment with SA (1.5 or 3.0 mM) improved their responses to moderate resistance. The only exception was Mapili at 1.5 mM SA pre-treatment, which remained susceptible. Inoculation with *Pt* races UVPt13 and UVPt26 before RWASA1 infestation increased resistance in PAN 3118 and PAN 3111, which scored resistant, while Tsholoha became moderately resistant. Makalaote, on the other hand, increased resistance when primed by UVPt26, and pre-inoculation of Mapili with UVPt13 improved RWASA1-induced response from susceptible to moderately resistant.

Without priming, cultivars PAN 3133, SST 356, and Bolane were moderately resistant to RWASA1. However, pre-treatment with SA (1.5 and 3.0 mM) improved their mean resistance responses. Leaf rust inoculation enhanced resistance to RWASA1 in PAN 3133 and SST 356 from moderately resistant to resistant, while in Bolane, although slightly lower mean values were recorded, it did not respond to SA or *Pt* pre-treatment. Even though PAN 3161 and PAN 3368 scored resistant to RWASA1 infestation with average chlorosis, SA and *Pt* pre-treatment improved their response from average to minor chlorosis.

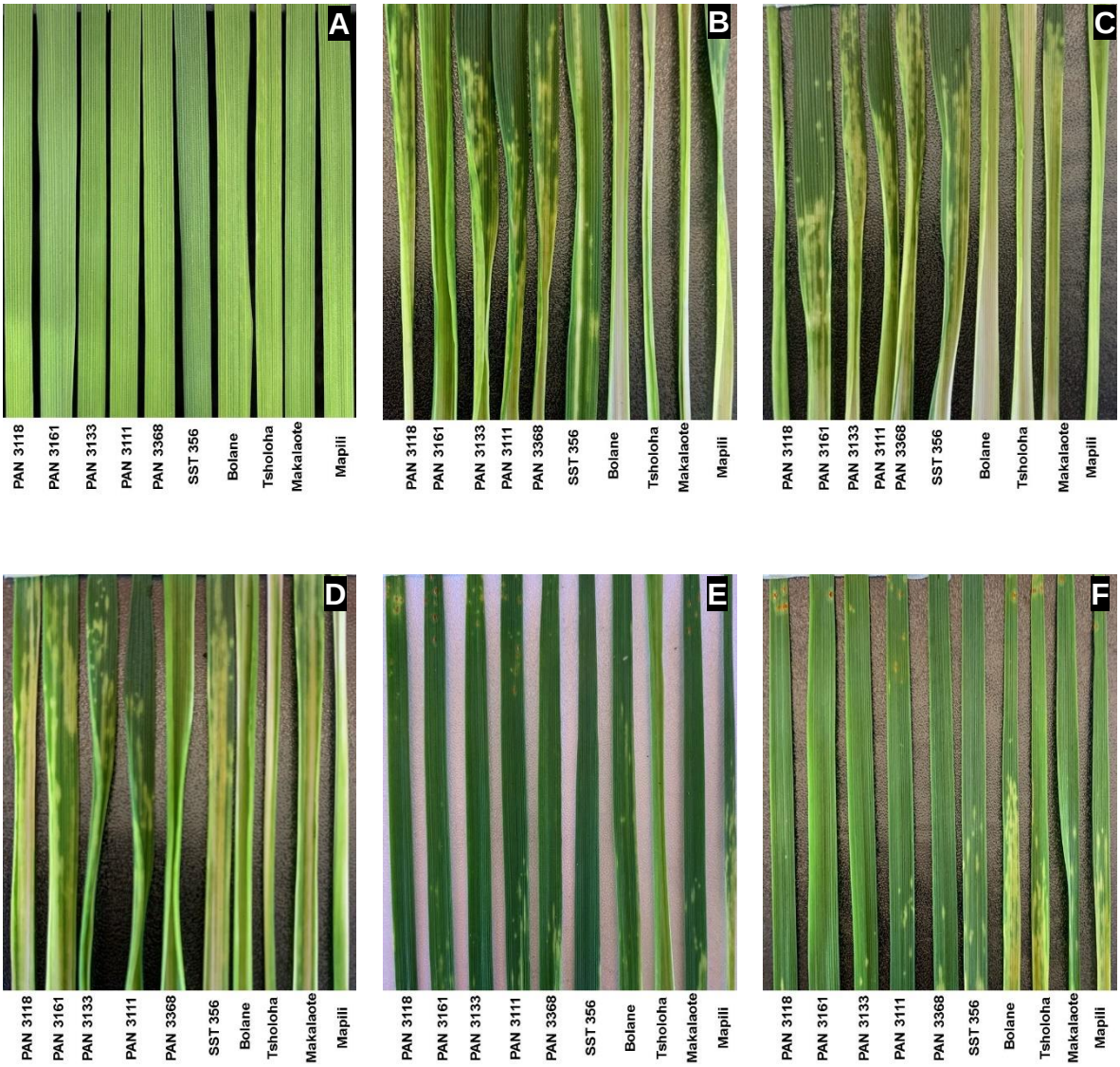

Fig. 3.2: Effect of Russian wheat aphid (RWASA4) infestation on leaf damage in wheat cultivars pre-treated with salicylic acid and isolates of Puccinia triticina. The images are from the second leaves of treated plants 10 days after aphid infestation. A: Control; B: RWSA4; C: 1.5 mM SA + RWASA4; D: 3.0 mM SA + RWASA4; E: UVPt13 + RWASA4; F: UVPt26 + RWASA4.

Almost all the wheat cultivars tested were susceptible to RWASA4 (Fig. 3.2, Image B). Salicylic acid (3.0 mM) reduced RWASA4-induced leaf damage in PAN 3118 and PAN 3111, and *Pt* races significantly reduced RWASA4-induced leaf damage in South African cultivars by reducing chlorosis and leaf rolling in some cultivars (Fig. 3.2, Images E and F).

Table 3.8 Responses of wheat cultivars to Russian wheat aphid (RWASA4) infestation in salicylic acid and *Puccinia triticina* primed wheat cultivars at the seedling stage. Values are means±SE of leaf damage rating scores (n=2)

Cultivars	Treatments				
	RWASA4 (no priming)	1.5 mM SA + RWASA4	3.0 mM SA + RWASA4	UVPt13 + RWASA4	UVPt26 + RWASA4
PAN 3118	9.2 ± 0.00 S	8.2 ± 0.10 S	6.4 ± 0.10 MR	4.0 ± 0.00 MR	5.2 ± 0.20 MR
PAN 3161	6.5 ± 0.00 MR	6.1 ± 0.45 MR	5.6 ± 0.25 MR	3.8 ± 0.25 MR	4.2 ± 0.20 MR
PAN 3133	9.6 ± 0.30 S	8.4 ± 0.05 S	7.8 ± 0.05 S	3.6 ± 0.40 MR	3.6 ± 0.10 MR
PAN 3111	9.2 ± 0.00 S	7.9 ± 0.15 S	6.3 ± 0.00 MR	4.2 ± 0.10 MR	4.0 ± 0.00 MR
PAN 3368	9.1 ± 0.10 S	8.1 ± 0.25 S	7.8 ± 0.10 S	5.1 ± 0.10 MR	6.8 ± 0.20 S
SST 356	6.5 ± 0.20 MR	5.1 ± 0.20 MR	5.2 ± 0.15 MR	4.3 ± 0.25 MR	4.5 ± 0.30 MR
Bolane	8.6 ± 0.15 S	9.2 ± 0.10 S	8.4 ± 0.50 S	8.2 ± 0.40 S	8.3 ± 0.15 S
Tsholoha	9.7 ± 0.05 S	9.0 ± 0.10 S	8.3 ± 0.30 S	8.4 ± 0.15 S	9.2 ± 005 S
Makalaote	9.8 ± 0.00 S	9.1 ± 0.15 S	8.7 ± 0.45 S	9.1 ± 0.05 S	9.2 ± 0.15 S
Mapili	9.6 ± 0.05 S	8.9 ± 0.05 S	8.9 ± 0.10 S	8.0 ± 0.05 S	8.4 ± 0.45 S

Means grouping at P≤0.05. R = resistant (1-3); MR = moderately resistant (4-6.5); S = susceptible (6.6 -10); screening scale by Tolmay (1995)

Almost all cultivars were susceptible to RWASA4, except PAN 3161 and SST 356, which were moderately resistant (Table 3.8). The Lesotho landraces, Bolane, Tsholoha, Makalaote, and Mapili, were susceptible to RWASA4 and pre-treatment with SA, or isolates of the two *Pt* races, did not improve their responses. However, *Pt* pre-inoculated cultivars PAN 3118, PAN 3133, PAN 3111 and PAN 3368 (Pt13) improved responses to RWASA4 infestation, shifting from susceptible to moderately resistant.

Salicylic acid (3.0 mM) pre-treatment improved the response to RWASA4 infestation from susceptible to moderately resistant in PAN 3118 and PAN 3111 (Table 3.8). However, even though lower mean damage scores were recorded, SA did not change the resistance responses of PAN 3133 and PAN 3368. In parallel, the isolates of *Pt* races UVPt13 and UVPt26 mediated decreases in the mean damage scores recorded, which in most instances resulted in changes from susceptible to moderate resistance in PAN 3118, PAN 3133, PAN 3368 and PAN 3111.

3.3.2 Salicylic acid and *Puccinia triticina* priming at flag leaf stage

All wheat cultivars at the flag leaf stage were pre-treated with either SA or isolates of *Pt,* and RWA-induced leaf damage was evaluated on the flag leaves. Priming with either SA or *Pt* caused a significant reduction in RWA-induced leaf damage in some wheat cultivars. Salicylic acid and *Pt* pre-treatment of different wheat cultivars induced different responses to the RWA biotypes. Responses were measured using the leaf damage rating scores, and data were analysed using linear model procedure SAS 9.4 (PROC GLM SAS9.4).

Analysis of variance (Table 3.9) indicated that treatments, cultivars, and the treatment x cultivar interaction were highly significant, but replications were not significant. According to the analysis, SA or *Pt* priming in the different wheat cultivars induced different responses to RWA infestation.

Table 3.9 Analysis of variance in primed [*Puccinia triticina* race isolates (UVPt13 and UVPt26) and salicylic acid (1.5mM)] wheat cultivars infested by Russian wheat aphid biotypes RWASA1 and RWASA4 at the flag leaf stage

SOV	DF	Mean Square	SOV	DF	Mean Square
Cultivar	9	40.18**	Replication	1	$0.016^{N.S}$
Treatment	7	13.50**	Treatment x Cultivar	63	3.83**

** = highly significant at P≤0.01; N.S = non-significant; Coefficient of variation = 4.47%

All wheat cultivars measured significantly different damage rating scores for both biotypes, except PAN 3111, Makalaote, PAN 3118, and Bolane, grouped as C (Table 3.10). Priming by both *Pt* race isolates mediated similar responses to RWASA1 infestation (Table 3.11). Biotype 4 (RWASA4) infestation of UVPt13 pre-inoculated wheat cultivars induced damage rating scores similar to RWASA1 infestation of unprimed wheat. RWA infestation induced significantly different damage scores in the different priming treatments.

Table 3.10 Homogeneous grouping of cultivar responses to Russian wheat aphid biotypes RWASA1 and RWASA4 infestation in the flag leaf stage after salicylic acid treatments and *Puccinia triticina* inoculation. Values are means±SE of leaf damage rating scores (n=16)

Cultivar	Damage rating score (Mean)	Group
Mapili	6.41	A
Tsholoha	5.73	B
PAN 3111	5.35	C
Makalaote	5.35	
PAN 3118	5.32	
Bolane	5.25	
SST 356	3.47	D
PAN 3368	3.07	E
PAN 3133	2.61	F
PAN 3161	1.57	G

Means grouping at P≤0.05

Biotype 4 (RWASA4) induced the severest leaf damage at the flag leaf stage. Correspondingly, the infestation of plants primed by SA and UVPt26 also caused high leaf damage scores. However, plants primed by UVPt13 incurred reduced leaf damage by RWASA4 feeding. Similarly, wheat cultivars primed by SA and the two *Pt* isolates sustained minor leaf damage to RWASA1 infestation (Table 3.11).

Table 3.11 Treatments that induced homogeneous Russian wheat aphid leaf damage in different wheat cultivars at the flag leaf stage. Values are means±SE of leaf damage rating scores (n=20)

Treatment	Damage score (Mean)	Group
RWASA4	5.53	A
1.5 mM SA + RWASA4	5.17	B
UVPt26 + RWASA4	4.95	C
RWASA1	4.63	D
UVPt13 + RWASA4	4.44	
1.5 mM SA + RWASA1	3.97	E
UVPt26 + RWASA1	3.32	F
UVPt13 + RWASA1	3.32	

Means grouping at P≤0.05

The wheat cultivar PAN 3111, which displayed susceptibility symptoms to RWASA1 without priming (Fig. 3.3, Image A), showed mild symptoms that

qualified it as resistant when pre-inoculated with UVPt13 (Fig. 3.3, Images A, C). *P. triticina* isolates pre-treatment also reduced damage severity in Makalaote, susceptible to RWASA1 before priming, but expressed moderate and strong resistance when primed by UVPt13 and UVPt26 (Fig. 3.3, Images A, C, D). SST 356, on the other hand, improved from moderately resistant to resistant when primed by SA or pre-inoculated with *Pt* (Fig 3.3).

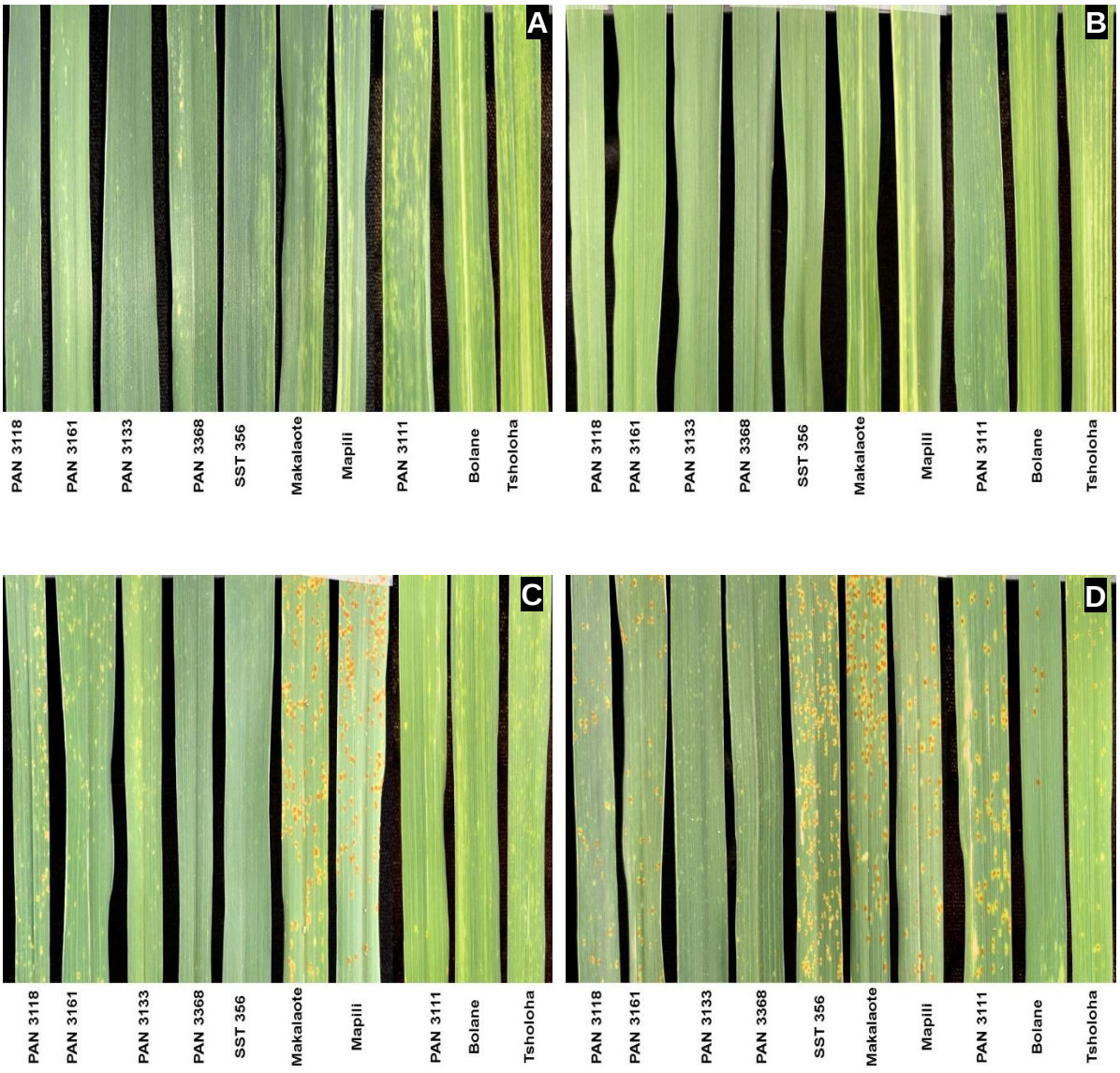

Fig. 3.3: Effect of Russian wheat aphid (RWASA1) infestation on leaf damage in wheat cultivars pre-treated with salicylic acid and isolates of *Puccinia triticina*. The images are from flag leaves 15 days after infestation. A: RWASA1; B: 1.5 mM SA + RWASA1; C: UVPt13 + RWASA1; D: UVPt26 + RWASA1.

Table 3.12 Responses of wheat cultivars to Russian wheat aphid (RWASA1) infestation in salicylic acid and *Puccinia triticina* primed wheat cultivars at the flag leaf stage. Values are means±SE of leaf damage rating scores (n=2)

Cultivars	Treatments			
	RWASA1 (no priming)	1.5 mM SA+ RWASA1	UVPt13 + RWASA1	UVPt26 + RWASA1
PAN 3118	3.1 ± 0.12 R	2.0 ± 0.12 R	2.7 ± 0.00 R	2.1 ± 0.12 R
PAN 3161	1.1 ± 0.12 R	1.3 ± 0.12 R	0.9 ± 0.12 R	1.0 ± 0.00 R
PAN 3133	1.2 ± 0.00 R	1.1 ± 0.12 R	0.5 ± 0.12 R	1.0 ± 0.00 R
PAN 3111	6.9 ± 0.12 S	5.5 ± 0.12 MR	3. 5 ± 0.12 R	6.8 ± 0.25 S
PAN 3368	2.2 ± 0.25 R	1.5 ± 0.12 R	2.1 ± 0.12 R	2.0 ± 0.00 R
SST 356	4.7 ± 0.12 MR	1.3 ± 0.12 R	1.7 ± 0.37 R	1.4 ± 0.00 R
Bolane	5.2 ± 0.00 MR	5.5 ± 0.12 MR	5.7 ± 0.12 MR	5.4 ± 0.00 MR
Tsholoha	7.3 ± 0.12 S	7.3 ± 0.12 S	5.4 ± 0.00 MR	5.4 ± 0.00 MR
Makalaote	6.7 ± 0.12 S	6.1 ± 0.37 MR	4.4 ± 0.25 MR	3.7 ± 0.37 R
Mapili	7.9 ± 0.12 S	8.1 ± 0.12 S	6.3 ± 0.12 MR	4.4 ± 0.25 MR

Means grouping at P≤0.05. R = resistant (1-3); MR = moderately resistant (4-6.5); S = susceptible (6.6-10); screening scale by Tolmay (1995)

Bolane and all the South African wheat cultivars, except PAN 3111, were resistant/moderately resistant to RWASA1 infestation at the flag leaf stage. Consequently, SA and *Pt* race UVPt13 pre-treatment reduced leaf damage severity in PAN 3111, and the response shifted to moderate resistance or resistance (Table 3.12). The cultivar remained susceptible when pre-inoculated with UVPt26. Priming treatments improved resistance levels in SST 356, which was moderately resistant before priming, but expressed resistance to RWASA1 after priming. Oddly, none of the priming treatments changed the reaction of Bolane to RWASA1 infestation (Table 3.12).

On the other hand, SA pre-treatment did not change the susceptibility to RWASA1 in Tsholoha and Mapili, but *Pt* inoculation improved the reactions, and the cultivars became moderately resistant. Salicylic acid and *Pt* also improved resistance to RWASA1 in Makalaote, which was susceptible before priming. The cultivar became moderately resistant (SA, UVPt13) and resistant (UVPt26) to RWASA1.

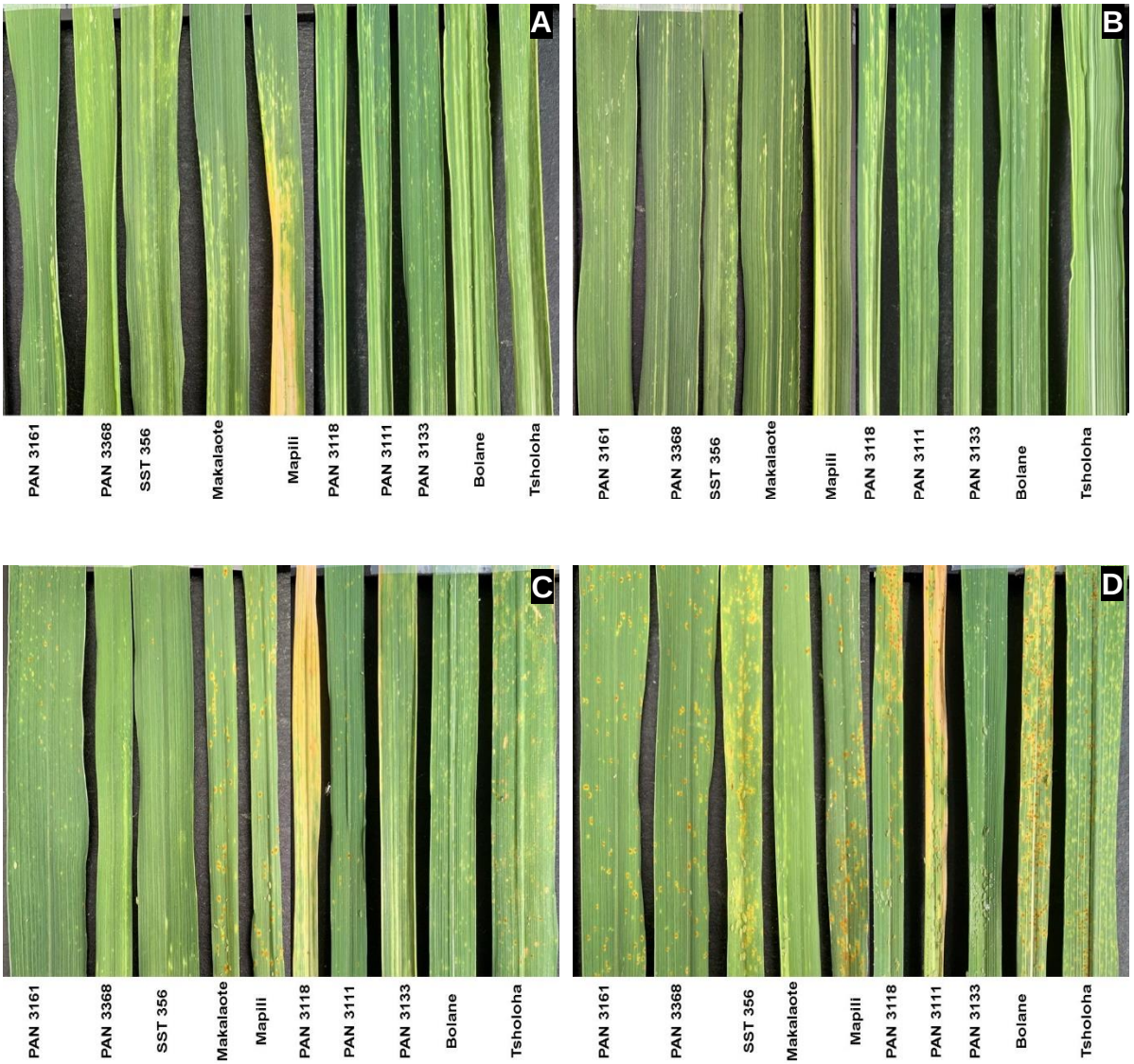

Fig. 3.4: Effect of Russian wheat aphid (RWASA4) infestation on leaf damage in wheat cultivars pre-treated with salicylic acid and isolates of *Puccinia triticina* inoculations. The images are from flag leaves 15 days after infestation. A: RWASA4; B: 1.5 mM SA + RWASA4; C: UVPt13 + RWASA4; D: UVPt26 + RWASA4.

Biotype 4 infestation induced variable chlorosis, striping and leaf rolling in wheat cultivars at the flag leaf stage. However, RWASA4 induced mild damage in leaves pre-inoculated with *Pt* isolates (Fig 3.4).

Table 3.13 Responses of wheat cultivars to Russian wheat aphid (RWASA4) infestation in salicylic acid and *Puccinia triticina* primed wheat cultivars at the flag leaf stage. Values are means±SE of leaf damage rating scores (n=2).

Cultivars	Treatments			
	RWASA4 (no priming)	1.5 mM SA + RWASA4	UVPt13 + RWASA4	UVPt26 + RWASA4
PAN 3118	8.7 ± 0.00 S	7.5 ± 0.10 S	8.5 ± 0.10 S	8.0 ± 0.20 S
PAN 3161	2.5 ± 0.00 R	2.3 ± 0.45 R	1.5 ± 0.25 R	2.0 ± 0.20 R
PAN 3133	4.5 ± 0.30 MR	3.4 ± 0.05 R	4.5 ± 0.05 MR	4.7 ± 0.10 MR
PAN 3111	5.7 ± 0.00 MR	5.0 ± 0.15 MR	3.7 ± 0.00 MR	5.7 ± 0.00 MR
PAN 3368	5.2 ± 0.10 MR	4.7 ± 0.25 MR	4.0 ± 0.10 MR	2.9 ± 0.20 R
SST 356	5.0 ± 0.20 MR	4.9 ± 0.20 MR	3.8 ± 0.15 MR	5.0 ± 0.30 MR
Bolane	5.2 ± 0.15 MR	5.0 ± 0.10 MR	4.7 ± 0.05 MR	5.3 ± 0.15 MR
Tsholoha	5.3 ± 0.05 MR	5.2 ± 0.10 MR	5.0 ± 0.30 MR	5.0 ± 0.05 MR
Makalaote	5.9 ± 0.00 MR	6.0 ± 0.15 MR	4.0 ± 0.45 MR	6.0 ± 0.15 MR
Mapili	7.3 ± 0.05 S	7.7 ± 0.05 S	4.7 ± 0.10 MR	5.0 ± 0.45 MR

Means grouping at P≤0.05. R = resistant (1-3), MR = moderately resistant (4-6.5), S = susceptible (6.6-10); screening scale by Tolmay (1995)

Most of the cultivars were moderately resistant or resistant (PAN 3161) to RWASA4 infestation, except PAN 3118 and Mapili, which were susceptible. Pre-treatment of PAN 3118, PAN 3161, SST 356, Bolane, Tsholoha and Makalaote did not affect the response to RWASA4 infestation. Some cultivars selectively responded to the priming agents; SA only shifted the reaction to resistant in PAN 3133, and UVPt26 mediated a resistant response in PAN 3368, while both isolates of the two *Pt* races induced a moderately resistant response in Mapili.

3.3.3 Correlation analysis of treatments at the seedling and flag leaf stages

The correlation analyses of wheat priming and induced reaction to RWA infestation at the seedling and flag leaf stages were evaluated to ascertain the relationship between the responses at different plant growth stages. The responses of wheat cultivars infested by RWASA1 and RWASA4 at the seedling and flag leaf stages showed a highly significant positive correlation (r = 0.57).

Table 3.14 Correlation coefficients (treatment comparison) between the mean leaf damage ratings at the seedling and the flag leaf stages

Treatment	R	Treatment	R
RWASA1	0.628**	RWASA4	0.548*
1.5mM SA + RWASA1	0.687**	1.5 mM SA + RWASA4	0.430
UVPt13 + RWASA1	0.735**	UVPt13 + RWASA4	0.060
UVPt26 + RWASA1	0.479*	UVPt26 + RWASA4	0.125

Pearson correlation t = 0.444 (0.05); r = 0.561 (0.01); * = P<0.05; ** = P<0.01

All treatments positively correlated at both seedling and flag leaf stages. The treatments produced relatively strong positive and significant correlations except for 1.5 mM SA + RWASA4 (0.430), UVPt13 + RWASA4 (0.06) and UVPt26 + RWASA4 (0.125). The treatment UVPt13 + RWASA1 induced the highest positive and highly significant correlation.

The correlation coefficient of the RWA biotype effect indicated that wheat cultivars showed a significantly positive relationship in the two stages, which was nonetheless weak. The induced leaf damage symptoms at the two growth stages showed that wheat cultivars expressed less damage severity at the flag leaf than at the seedling stage.

3.4 Discussion

Exogenous salicylic acid application

The RWA is an invasive and economically important pest of wheat and barley (Avila et al., 2019). It punctures phloem cells and sucks the sap, which diverts plant nutrients towards its development, reducing plant growth and yield. Different strategies exist to control aphid populations and reduce their feeding impact on plant growth to maintain crop productivity. Among them are resistance breeding and the production of resistant cultivars, which are weakened by the constant evolution of resistance-breaking biotypes (Venter et al., 2014). Alternative strategies that increase inherent plant defence responses, like priming, can protect crops from pests and pathogens and maintain plant productivity. This study evaluated the effect of exogenous SA application and *Pt* isolates infection in protecting wheat plants against RWA

infestation.

Salicylic acid is a signalling molecule that mediates defence response pathways during wheat-RWA interaction (Lu et al., 2016). Increased hormone content reduced aphid (*Lipaphis erysimi*) populations and infestation damage on mustard plants (Mony et al., 2017). Furthermore, previous studies have revealed that SA application induced resistance by reducing infestation intensity (aphid numbers) and ultimately increasing wheat yield (Mahmoud and Mahfouz, 2015). Higher hormone concentrations adversely affect pests, as Stella de Freitas et al. (2019) noticed that 16 mM SA foliar application disrupted nymphs' developmental stages.

In this study, SA priming through foliar application induced variable and minor responses dependent on the cultivars' biotype, resistance or susceptibility status, and growth stage. The priming protected susceptible seedlings from RWASA1, but not RWASA4, while the response of moderately resistant or resistant cultivars was not affected (Table 3.7, 3.8). Similar results were reported by Wang et al. (2021) that wheat seed priming by $CaCl_2$ reduces aphid (*Schizaphis graminum* Rondani) feeding efficiency, aphid population size and induced callose deposition at the seedling stage. At the flag leaf stage, 50% of susceptible or moderately resistant cultivars (Table 3.12) were protected from RWASA1, while only one change occurred in PAN3133, which induced a shift from moderate resistance to resistance against RWASA4 (Table 3.13). Similar results have been reported by Amin et al. (2008), where the foliar application of SA at the adult stage increased growth, carbohydrate content and yield of wheat plants and a key factor, SAR, to the pathogen (*F. graminearum*) infection (Sorahinobar et al., 2016).

Our study's results further prove that all wheat cultivars used in this greenhouse trial were more susceptible to RWASA4 than RWASA1, except PAN 3161 and SST 356 (MR) at the seedling stage. Additionally, a low concentration of SA (1.5 mM) did not prime defence responses to RWASA4 infestation, but a higher concentration (3.0 mM) mediated reduced leaf damage in only two cultivars (seedling stage: PAN3133 and Mapilili, flag leaf stage: PAN 3118 and PAN 3111), thus improving the resistance response to RWA (RWASA1 or RWASA4) in 20% of cultivars tested, either at seedling or flag leaf stage (Tables 3.7, 3.8, 3.12 and 3.13).

Priming by Puccinia triticina

Puccinia triticina is one of the most common wheat pathogens (Kolmer et al*.,* 2013) that often causes minor host damage compared to other wheat fungi (Terefe et al., 2009; Huerta-Espino et al., 2011). Rust outbreaks can result in extensive yield losses; therefore, the disease-causing pathogens have not been readily considered biological control agents against other pests in cereal crops. However, infections from *Pt* races are usually less damaging compared to stem rust (*P. graminis* f. sp. tritici; *Pgt*) and stripe rust (*P*. *striiformis* f. sp. *tritici*; *Pst*) (Huerta-Espino et al., 2011) and can sensitise host plants to react more robustly to subsequent damaging attacks.

This study used leaf rust (*Pt*) isolates to prime wheat cultivars at the seedling and flag leaf stages. Even though various indicators could measure the priming effect in plants, we used leaf damage rating scores to measure the effect of priming on RWA infestation in different wheat cultivars. Chen et al. (2015) measured chlorophyll concentration and antioxidative activities to evaluate the priming effect of stripe rust (*Pst*) infection on biotic and abiotic stresses. The use of pathogens in priming plant responses for enhanced resistance was also reported by Gholami et al. (2019). They observed an increase in resistance against take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) induced by prior inoculation with four different endophytic fungi. They concluded that *Coprinopsis urticicola* was a potential priming agent in improving wheat resistance to take-all disease. Overall, *Pt* race isolates reduced leaf damage in most susceptible seedlings; nonetheless, the isolates failed to enhance resistance against RWASA1 (Makalaote and Mapili) and RWASA4 (PAN3368 and the four Lesotho cultivars) in specific cultivars. At the flag leaf stage, UVPt13 enhanced the resistance of all the cultivars tested against RWASA1, while UVPt26 protected 90% of the cultivars tested. Most cultivars were moderately resistant to resistant against RWASA4, except PAN3118, which remained insensitive to priming and Mapili, which improved to moderately resistant. Our results, therefore, partially support the hypothesis that avirulent *Pt* race isolates colonising wheat seedlings could enhance the host resistance to RWA infestation, reducing leaf damage symptoms.

The SA and *Pt* priming correlation analysis indicated positive correlations at the seedling and flag leaf stages. The results show that wheat plants are amenable to priming at seedling or flag leaf stages to confer resistance to RWA infestation. Even though wheat cultivars were more resistant to RWA biotypes at the flag leaf than the seedling stages, we noted that the primed resistance depended on the initial resistance or susceptibility level to a particular biotype. Similar results were reported by Hawley et al. (2003) that RWA susceptible wheat (TAM 107) suffered severe leaf rolling at the seedling but not at the booting stage.

A comparison of SA and *Pt* as priming agents showed that *Pt* isolates were more efficacious than SA; they could be used as an alternative strategy for crop protection in plants instead of direct chemical pesticides. Since priming efficiency also depends on cultivars' resistance or susceptibility status, this study showed that wheat plants responded more effectively to priming agents at the flag leaf than at the seedling stages. However, the potential effect of the timing of the adult plant infestations and its potential impact on leaf-rolling and the possibility of a growth stage resistance cannot be confirmed unless field trials also provide supporting data. Some resistance to RWA biotypes in South African wheat cultivars enhanced their sensitivity to the priming agents more than the Lesotho landraces, which were entirely susceptible to RWA biotypes (Morojele, 2005).

3.5 Conclusion

The study demonstrated and compared SA and *Pt* isolates' efficacy in priming wheat for resistance to two RWA biotypes at the seedling and flag leaf stages. Wheat cultivars showed variant responses to infestation by the RWA biotypes, and the response to priming depended to some extent on the cultivar's resistance status and the growth stage. Priming by SA or *Pt* differentially reduced RWA damage in wheat cultivars. However, the *Pt* isolates were more efficacious priming agents than exogenously applied SA. Priming results at the flag leaf stage positively correlated to the seedling stage, indicating that wheat cultivars may respond similarly at both stages. Priming wheat with avirulent *Pt* isolates or defence-enhancing elicitors to induce pest resistance may present an alternative RWA management approach.

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Chapter 4

Antioxidant-mediated resistance to *Diuraphis noxia* in wheat primed by salicylic acid and *Puccinia triticina* isolates

4.1 Introduction

In nature, the wheat plant is exposed to biotic and abiotic stress factors that endanger its growth and productivity (Spanic et al., 2017). Biotic factors include pests such as the Russian wheat aphid (RWA), fungi, viruses, and bacterial infections. The RWA is a significant pest that draws phloem sap and causes nutrient drainage in cereals (De Wet and Botha, 2007), including wheat and barley.

Russian wheat aphid feeding causes turgor loss and reduces biomass and plant growth. Due to the compensation of carbon partitioning between the various sink tissues, there can be an ultimate reduction in grain yield of up to 93% (Burd and Burton, 1992; Damte et al., 2018). Heavy infestations can cause plant death as aphids shelter within the rolled leaves, where contact pesticides are ineffective (Turanli et al., 2012). Furthermore, controlling aphids with chemicals is environmentally less desirable and can contaminate food (Worrall et al., 2012). An alternative strategy to chemical control is enhancing host resistance through breeding programs or plant priming.

Plants accumulate reactive oxygen species (ROS) early during biotic and abiotic stress interactions, severely damaging plant cells and organelles (Caverzan et al., 2016). Even though ROS at high levels damage the cells and are toxic to pests and pathogens (Morkunas et al., 2011), lower concentrations are essential for signal transduction processes activating the defence responses. Therefore, plants need to establish an equilibrium between the production and detoxification of ROS, controlled by enzymatic and non-enzymatic antioxidative species.

Meanwhile, ROS interact with other signals, such as salicylic acid, and promote their accumulation. Reactive oxygen species accumulation and host resistance expression involve different mechanisms, including the hypersensitive response (Hargarten et al., 2017). Interactions of salicylic acid (SA) and jasmonic acid (JA) signalling pathways

with ROS in resistant cultivars coordinate the defence responses (Kachroo and Kachroo, 2007). Salicylic acid links to various essential components of plants to activate and enhance defence responses. For instance, SA interacts with the Non-Expresser of Pathogenesis Related Protein 1 (NPR1), the transcriptional regulator, to activate SA-mediated defence signalling pathways (Pokotylo et al., 2019).

Using elicitors or plant microbes to activate plant defence responses is an alternative approach to managing pest attacks (Worrall et al., 2012). Some microorganisms, phytohormones and inorganic nutrients have primed wheat plants to activate defence responses to subsequent challenges (Balmer et al., 2015). Exogenous application of hydro- and halo-priming agents activated antioxidative defence mechanisms and increased phenolics and proline levels in wheat plants under salt stress (Islam et al., 2015). In another study, exogenous application of Z-3-hexenyl acetate boosted JA-dependent defence signals against *Fusarium graminearum* (a causal agent of head blight of wheat) and reduced the necrotic lesions on spikelets (Ameye et al., 2015).

Pre-inoculation of wheat cultivars SST 347 (resistant to RWASA1) and SST 356 (susceptible to RWSA1) with *Pt* isolate 3SA145 before RWA infestation induced a resistant response (antixenosis) to RWASA1 infestation (Njom, 2016). A proteomic approach was used to evaluate resistance responses in wheat plants previously infected by *Pt* isolates before RWASA1 infestation. However, he did not measure the induced changes in the antioxidative capacity of primed plants during aphid infestation, which is unique to this study. The main objective of this study was to investigate the effect of RWA infestation on the antioxidative capacity of SA and *Pt* isolates primed wheat plants. We measured the various enzymatic and non-enzymatic antioxidative responses associated with Russian wheat aphid (*Diuraphis noxia*) resistance.

4.2 Materials and methods

We investigated the priming effect of SA and *Pt* isolates in wheat cultivars infested with RWASA1. The effect was measured as changes in enzymatic and non-enzymatic antioxidative activities. The following section describes the plant treatments and the assays to measure the antioxidative responses.

4.2.1 Wheat cultivation and sample collection

Wheat cultivars PAN 3118, PAN 3161 and PAN 3111 were selected based on their reaction to aphid (RWASA1) feeding as indicated by leaf damage scores on plants (Chapter 3, section 3.2.2). The wheat cultivar PAN 3161 is resistant to RWASA1, while PAN 3118 and PAN 3111 are susceptible but showed reduced leaf damage ratings when primed with SA and *Pt*.

The experiment was conducted as a randomised complete block design with three repeats in the greenhouse under natural light and controlled temperature (day: 22±2 ^{o}C, night: 18±2 ^{o}C). Wheat cultivars were planted in plastic pots (10×12×15 cm^3) filled with 1:1 red soil and peat. The plants were watered twice and fertilised once a week (Chapter 3, section. Leaf (second and third) samples were collected in liquid nitrogen at different hours post-infestation (hpi: 0, 6, 9, 12, 24, 48, 72 and 96). Samples were crushed in liquid nitrogen and homogenised in specific buffers to extract and measure the activities of specific enzymatic and non-enzymatic antioxidants.

4.2.2 Treatments applied to wheat plants to investigate priming effects

The wheat plants were treated as follows; control + RWASA1, 1.5 mM SA + RWASA1, and UVPt13 + RWASA1 (Chapter 3, Section 3.2.5).

4.2.3 Data analysis

The data for specific enzymatic activities and ascorbic acid content were analysed using the general linear model procedure: PROC GLMSAS 9.4 (SA Institute, 1989). Following analysis of variance (Two-Way ANOVA), the means were separated by the least significant difference (LSD) to indicate the significant differences between the treatments and responses. The cultivars and their responses to the treatments were further grouped according to their similarities.

4.2.4 Enzyme extraction (SOD, POD and GR)

A single protein extract from the leaf tissue was performed to measure the activities of superoxide dismutase (SOD), peroxidase (POD) and glutathione reductase (GR). Leaf tissue (1 g) was homogenised on ice using a mortar and pestle. The extraction buffer (4 ml) consisted of 50 mM potassium phosphate buffer, pH 7.0, containing 0.004 g polyvinylpolypyrrolidone (PVPP), 0.1% Triton X-100 (v/v), 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.04% (w/v) sodium-metabisulfite. The homogenised paste was centrifuged (Allegra X-30, Beckman Coulter) at 17 000 revolutions per min (rpm) for 15 min at 4 ^{o}C. The supernatant was separated into three aliquots and stored at -20 ^{o}C for enzyme assays.

4.2.5 Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase catalyses the scavenging of reactive oxygen radicals in a reaction that produces hydrogen peroxide (H_2O_2) (Getzoff et al., 1992). It is a three-dimensional enzyme-containing metallic co-factors (Mn, Cu or Fe). All three isozymes occur in cytosolic compartments, including the cytosol, chloroplast, mitochondria, and extracellular space (Bowler et al., 1994). The superoxide anions detoxification mechanism by SOD has been called a *ping-pong* due to the sequential reduction and oxidation of the metal co-factors and the associated oxidation and reduction of the superoxide radicals (Abreu and Cabelli, 2010). The following reaction is an example of the reaction of specific SODs. The Fe^{3+}-containing SOD detoxifies the superoxide radical and converts it to H_2O_2 (Abreu and Cabelli, 2010):

$$Fe^{3+}\ SOD + O_2^{\cdot -} \longrightarrow Fe^{2+}SOD + O_2$$

$$Fe^{2+}SOD + O_2^{\cdot -} (+2H^{+}) \longrightarrow Fe^{3+}SOD + H_2O_2$$

After detoxifying the superoxide radicals and producing H_2O_2, other enzymatic and non-enzymatic antioxidants like GR, POD, ascorbate peroxidase (APX), catalase (CAT) and tocopherol further work together with SOD to prevent any production of toxic species by both H_2O_2 and the superoxide radical.

Superoxide dismutase assay

To measure SOD activity, a reaction mixture of 50 mM potassium phosphate buffer, pH 7.8, containing 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 0.1 mM EDTA and 2 µM riboflavin was used. The assay was prepared in disposable polystyrene cuvettes, as indicated in Table 4.1. The reaction cuvettes were irradiated for 30 min by placing them under a fluorescent lamp in a box lined with aluminium foil. The absorbance of the reaction product was measured using a spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer) at 560 nm.

Table 4.1 Reaction mixture and irradiation to measure superoxide dismutase activity

	Reaction Mixture (µl)	**Extract (µl)**	**Irradiation**
Blank	970	30	No irradiation
Control	1000	0	Irradiated
Sample	970	30	Irradiated

The control was used to measure the maximum attainable absorbance at 560 nm. Superoxide dismutase activity was calculated using the following formula.

$$\text{SOD activity (\% inhibition of NBT)} = \frac{\text{Abs (Cont)} - \text{Abs (Samp)}}{\text{Cont Abs}} \times \frac{1}{\text{t (min)}} \times \frac{1}{[\text{Prot}]} \times \frac{\text{dil factor}}{1} \times 100$$

where,

$$\text{Dilution factor} = \frac{\text{Total volum in cuvette}}{\text{Volume of enzyme extract}}$$

Cont Abs: Absorbance of the control, Samp Abs: Absorbance of the sample, [Prot]: Protein concentration (mg ml^{-1}), dil factor: Dilution factor, t: time, min: minutes.

4.2.6 Peroxidase (EC 1.11.1.7)

Peroxidase is present in almost all living organisms. It acts in ROS scavenging and contributes to the lignification of plant cell walls, cell wall elongation, wound healing, resistance to infection and suberization.

Peroxidase oxidises guaiacol in the presence of H_2O_2 to form tetraguaiacol. Guaiacol is a reducing agent which changes colour after losing a proton to H_2O_2; the end product is water and oxygen.

$$4\,\text{Guaiacol} + 2H_2O_2 \xrightarrow[\text{Oxidation}]{\text{POD}} 8\,\text{tetraguaiacol (Brown Colour)} + 2H_2O$$

Enzyme assay

A modified method of Zieslin and Ben-Zaken (1991) was used. Briefly, the reaction mixture of 1 ml consisted of 840 µl 40 mM potassium phosphate buffer, pH 5.5, containing 0.2 mM EDTA, 100 µl 5 mM guaiacol, 10 µl enzyme extract and 50 µl 8.2 mM H_2O_2, which started the reaction. The change in absorbance was recorded using a spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer) at 470 nm for 180 s at 30 ^oC. Peroxidase activity was measured using the formula below:

$$\text{POD specific activity} = \frac{\text{dilution factor } \times \Delta\text{Abs}}{\sum \times [\text{Protein Content}]}\ \mu\text{mol tetraguaiacol ml}^{-1}\text{s}^{-1}(\text{U}) \,/\, \text{mg prot.}$$

$\sum_{Tetraguaiacol}$ = 26.6 mM^{-1} cm^{-1}, Dilution Factor = 1000/10, ΔAbs = Change in absorbance values.

4.2.7 Ascorbate Peroxidase (EC 1.11.1.11)

A significant H_2O_2 detoxifying system in plant cells is the ascorbate-glutathione cycle, in which APX plays a crucial role in catalysing the conversion of H_2O_2 to H_2O and O_2 using ascorbate as a specific electron donor (Huseynova et al., 2013). Ascorbate peroxidase is an H_2O_2-scavenging enzyme in plants and algae (Asada, 2006).

Enzyme extraction

The enzyme extract was prepared according to Moloi et al. (2016). Leaf tissue (0.5 g) was homogenised on ice using a mortar and pestle in a 5 ml solution of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1% (v/v) Triton X-100 and 1 mM ascorbate. The

leaf tissue paste was centrifuged (Allegra X-30, Beckman Coulter) at 15 000 rpm for 20 min at 4 ^{o}C. The supernatant was used as an enzyme extract.

Ascorbate peroxidase assay

The APX assay was performed according to a modified method by Pukacka and Ratajczak (2005). The reaction mixture (1 ml) consisted of 500 µl 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 200 µl 4 mM H_2O_2, 200 µl 0.68 mM ascorbate in phosphate buffer (100 mM pH 7.0) and 100 µl enzyme extract. A decrease in absorbance as a result of ascorbate oxidation was measured at 290 nm (Varian Cary-100 UV-VIS Spectrophotometer) for one min at 20 ^{o}C against a blank without the enzyme. An extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to calculate specific APX activity using the formula given below:

$$\text{APX specific activity} = \frac{\frac{V}{v} \times \frac{\Delta Abs}{\Delta t} \times \frac{1}{\varepsilon d}}{\text{Protein}} \text{ mM cm µg ml}^{-1}$$

V: volume in cuvette, v: enzyme extract volume, ∆Abs: change in absorbance,

∆t: reaction time, $\sum_{\text{ascorbic acid}}$: extinction coefficient (2.8 $mM^{-1}cm^{-1}$), d = path length (1 cm).

4.2.8 Catalase (EC: 1.11.1.6)

Catalase occurs in all H_2O_2 production sites in the cellular environment, such as the cytosol, chloroplast, mitochondria and peroxisomes (Sharma and Ahmed, 2014). The H_2O_2 scavenging by CAT isozymes within cells or cellular organelles is time, genotype, and stress-specific, affecting plants' defence signal processing.

Catalase works in peroxidatic or catalytic models. In the peroxidatic model, it reacts with hydrogen donors [ascorbic acid (AA), phenols, ethanol, formaldehyde] at a low concentration of H_2O_2. At a high concentration of H_2O_2, CAT works catalytically, where H_2O_2 acts as a donor and acceptor.

$$RH_2 + H_2O_2 \xrightarrow{\text{Catalase}} H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Firstly, the H_2O_2 oxidises the heme iron (Fe-OH) from an oxyferryl species to oxygen-rich iron-peroxide. In the second step, iron-peroxide reacts with H_2O_2 as a redundant and catalytically in the presence of ethanol. In this way, CAT reduces H_2O_2 into H_2O and O_2. Wheat plants without SA, *Pt* or infestation treatments were negative control. The treatments were RWASA1 infestation, 1.5 mM SA treatment, UVPt13 inoculation, 1.5 mM SA pre-treatment + RWASA1 infestation and UVPt13 pre-inoculation + RWASA1 infestation. The priming treatments were applied 48 hours before infestation, and leaf tissue was sampled at 0, 24 and 72 hpi.

Enzyme extraction and reaction assay

The enzyme was extracted using a protocol similar to SOD mentioned in section 4.2.5.

Catalase activity was determined by measuring the breakdown of H_2O_2 at 25 ^oC using a spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer) at 240 nm (Beers and Sizer, 1952). The reaction mixture of 1 ml contained 630 µl deionised water, 330 µl 59 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0, and 40 µl enzyme extract, which initiated the reaction.

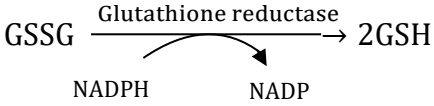
$$\text{CAT specific activity} = \frac{\text{Abs}}{\sum} \times \text{df} \times \frac{1}{[\text{Protein Content}]}$$

$\sum H_2O_2$ = 39.9 $M^{-1}cm^{-1}$, df: dilution factor = 1000/40, FW: Fresh weight, Abs: Absorbance at 240 nm.

4.2.9 Glutathione reductase (EC: 1.6.4.2)

Glutathione reductase is a critical ROS-scavenging enzyme activated by biotic and abiotic stresses (Bashir et al., 2007; Han et al., 2013). The enzyme has highly conserved domains, one binding flavin adenine dinucleotide (FAD) and NADPH, while the other forms an interface dimerisation domain. It accumulates in cellular regions with high electron flux, where ROS are produced. It recycles oxidised glutathione

(GSSG) back to reduced glutathione (GSH) in the presence of NADPH as an electron donor (Lascano et al., 2001). GR maintains the ratio of cell GSH/GSSG (Elavarthi and Martin, 2010).



The ROS donate electrons and convert the GSH to the oxidised form, GSSG. In the presence of GR, GSSG is reduced to GSH (Couto et al., 2016).

Glutathione reductase activity

Glutathione reductase activity was measured spectrophotometrically (Varian Cary-100 UV-VIS Spectrophotometer) by following a change in absorbance at 340 nm at 25 ^{o}C using a modified method (Smith et al.; 1988, Schaedle and Bassham, 1977). The reaction mixture of 1 ml contained 30 µl of 2 mM EDTA, 230 µl of 0.5 mM GSSG, 40 µl of enzyme extract, 470 µl 100 mM potassium phosphate buffer, pH 7.8, and 230 µl of 0.2 mM NADPH, which initiated the reaction.

Specific GR activity was calculated using the following formula:

$$\text{Glutathione reductase activity} = \frac{\Delta\text{Abs} \times \text{Dilution factor}}{\sum \times \text{Protein Concentration}}\ \mu\text{mol glutathione (U) / mg}$$

$\sum_{NADPH}$ = 6.22 mM^{-1} cm^{-1}, Dilution factor = 1000/40, ΔAbs = Change in absorbance

4.2.10 Non-enzymatic antioxidants: Ascorbic acid (EC 200-066-2)

Ascorbic acid (AA) regulates plant growth and scavenges excess ROS. The metabolite further controls several developmental processes like seed germination, cell division, cell wall elongation, epigenetic modification and transcription. It neutralises hydroperoxyl ($HO_2^{\cdot-}$) radical; the end product is H_2O_2. Hydrated AA reacts with peroxide radicals to produce monodehydroascorbate, H_2O_2, and water, the dehydrated ascorbic acid changes to AA in the presence of glutathione.

In studying AA content, an independent experiment was performed with additional treatments of priming agents used as a positive control (1.5 mM SA and UVPt13).

Total AA was extracted using a modified method of Law et al. (1983). Leaf tissue (1g) was homogenised in 10 ml of filtered 5% *m*-phosphoric acid in a pre-cooled pestle and mortar. Homogenised samples were centrifuged at 10 000 rpm for 30 min at 4 ^{o}C. The supernatant was transferred into an Eppendorf tube and used as the crude AA extract.

Ascorbic acid assay

The AA concentration was measured according to a modified protocol by Gillespie and Ainsworth (2007). A reaction mixture of 400 µl 50 mM potassium phosphate buffer, pH 7.4 with 5 mM EDTA, 200 µl supernatant, and 50 µl 10 mM dithiothreitol (DTT, to reduce the oxidised AA) was used. The reaction mixture was gently mixed and incubated at room temperature for 10 minutes. 50 µl 0.5 M *N*-ethylmaleimide (Sigma) was added (to remove excess DTT), gently mixed and incubated at room temperature for 2 minutes. Subsequently, 200 µl 10% TCA, 200 µl 43 % *o*-phosphoric acid and 200 µl 4% α,α-dipyridyl (Sigma) were added. Finally, 200 µl 3% $FeCl_3$ (passed through filter paper) was added to develop colour. Upon adding $FeCl_3$, the reaction mixture was immediately vortexed for 15-20 s to avoid precipitation. The reaction mixture was incubated in a water bath at 40 ^{o}C for 40 minutes. The absorbance was then read at 525 nm on a spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer). Deionised water was used as a blank.

The AA content of primed and non-primed plant samples was calculated using the equation below (Appendix Fig. 7.1).

$$\text{Ascorbic acid content} = \frac{\text{Abs} - 0.1549}{0.0166} \ \mu g\ ml^{-1}$$

Abs = Absorbance measured by the spectrophotometer

4.3 Results

The analyses of various antioxidative enzyme activities and the content of AA in primed and RWA-infested plants are reported in this section.

4.3.1 Superoxide dismutase (EC 1.15.1.1)

Table 4.2 Analysis of variance of superoxide dismutase activity in primed [*Puccinia triticina* (UVPt13) and salicylic acid (1.5mM)] and non-primed wheat cultivars infested by the South African Russian wheat aphid biotype 1 (RWASA1)

SOV	Df	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	3	21.25**	27.74*	90.79
Replication	2	3.92	17.23	18.91
Time (hpi)	7	43.39*	107.87**	439.96**
Treatment x Time	21	5.31	5.68	58.87

* = significant at P≤0.05; ** = highly significant at P≤0.01

The various treatments induced different responses (SOD activity) in PAN 3118 and PAN 3161, while responses were nonsignificant in PAN 3111 (Table 4.2). The replications and the interaction between priming and time were also nonsignificant for three wheat cultivars.

Table 4.3 Homogeneous grouping of the South African Russian wheat aphid (RWASA1) induced superoxide dismutase activity in salicylic acid-treated and *Puccinia triticina* inoculated wheat seedlings

PAN 3118			PAN 3161			PAN 3111		
Treatments	Means	Group	Treatments	Means	Group	Treatments	Means	Group
UVPt13 + RWASA1	5.91	A	UVPt13 + RWASA1	7.79	A	UVPt13 + RWASA1	16.50	A
RWASA1	4.50	B	RWASA1	6.42	BC	RWASA1	16.35	
SA + RWASA1	4.19		SA + RWASA1	6.34		SA + RWASA1	15.36	
Control	3.72		Control	5.16	D	Control	12.31	B

n = 24, means grouping at P≤0.05

Grouping of means indicated that treatments influenced SOD activity in wheat plants. UVPt13 pre-inoculation primed plants and induced the highest SOD activity in PAN 3118 and PAN 3161. However, priming did not induce activity that differed from RWASA1 infestation, as the treatments grouped in PAN 3111 (Table 4.3). However, PAN 3111 had constitutively higher SOD activity.

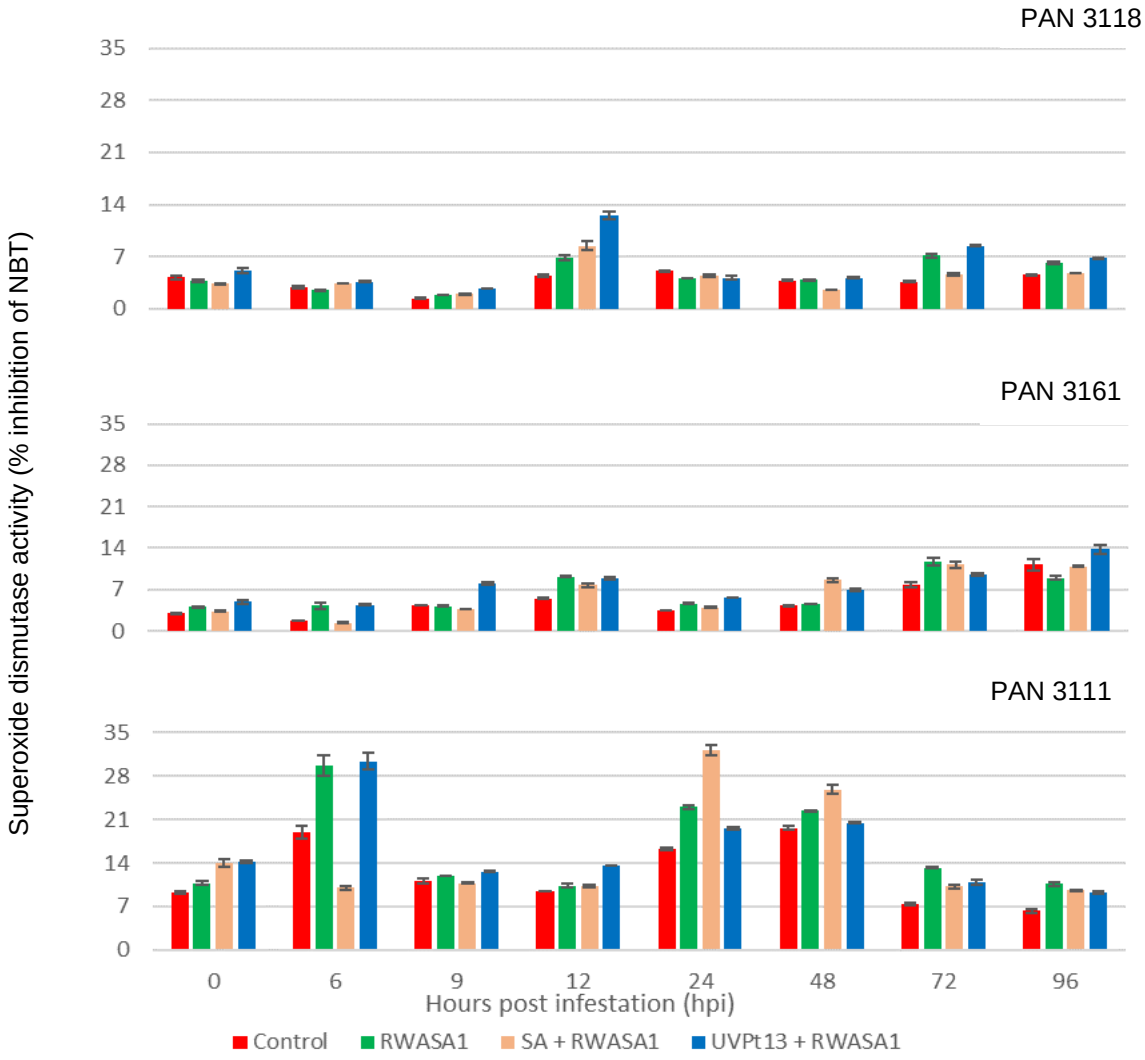


Fig. 4.1: Effect of South African Russian wheat aphid (RWASA1) infestation on SOD activity of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The bars represent means±SE from three biological repeats.

Wheat cultivars showed the highest level of SOD activity in plants pre-primed by UVPt13 isolate and the lowest in non-primed uninfested plants. PAN 3118 and PAN 3161 expressed significantly different and lowest SOD activity than the rest of the

treatments (Table 4.3, Fig. 4.1). In contrast, plants primed by SA showed a low non-significant difference in SOD activity than RWASA1 infested control. Wheat cultivars PAN 3118 and PAN 3111 showed variant SOD responses over time, while activity in PAN 3161 (resistant cultivars) gradually increased (Table 4.2, Fig. 4.1).

4.3.2 Peroxidase (EC 1.11.1.7)

Analysis of variance indicated that the various treatments induced significantly different POD activity in PAN 3118 and PAN 3161 but not PAN 3111. The effect of the duration of infestation (hours post infestation, hpi) was also significantly different in all the cultivars. However, the replications and interaction between the priming treatments and the duration of infestation were nonsignificant (Table 4.4).

Table 4.4 Analysis of variance of the South African Russian wheat aphid (RWASA1) induced peroxidase activity in primed [*Puccinia triticina* (UVPt13) and salicylic acid (1.5mM)] wheat cultivars

SOV	DF	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	3	0.265**	0.444**	0.902
Replication	2	0.008	0.264	4.37
Time (hpi)	7	0.429**	3.77**	63.28**
Treatment x Time	21	0.019	0.059	1.27

** = highly significant at P≤0.01

Infestation of primed and non-primed plants caused significantly different POD activity in PAN 3118 and PAN 3161 (Table 4.4). UVPt13 pre-inoculated cultivars showed higher POD activity than all other treatments, including the control. However, changes in POD activity were non-significant in PAN 3111, even though plants primed by UVPt13 showed relatively higher activity throughout the trial (Table 4.5, Fig. 4.2).

Grouping of means indicated that treatments influenced POD activity in wheat plants. Wheat cultivars pre-treated with SA or UVPt13 pre-inoculation induced a higher POD activity (Table 4.5). Wheat plants primed by the *Pt* isolate showed higher POD activity than those treated with SA, or infested controls (Table 4.5).

Table 4.5 Homogeneous grouping of South African Russian wheat aphid (RWASA1) induced peroxidase activity in salicylic acid and *Puccinia triticina* pre-inoculated wheat cultivars.

PAN 3118			PAN 3161			PAN 3111		
Treatments	**Means**	**Group**	**Treatments**	**Means**	**Group**	**Treatments**	**Means**	**Group**
UVPt13 + RWASA1	0.689	A	UVPt13 + RWASA1	0.829	A	UVPt13 + RWASA1	2.10	A
RWASA1	0.540	B	SA + RWASA1	0.642	B	SA + RWASA1	1.90	
SA + RWASA1	0.488	BCD	RWASA1	0.630		RWASA1	1.72	
Control	0.449	D	Control	0.498	C	Control	1.67	

n = 24, means grouping at P≤0.05

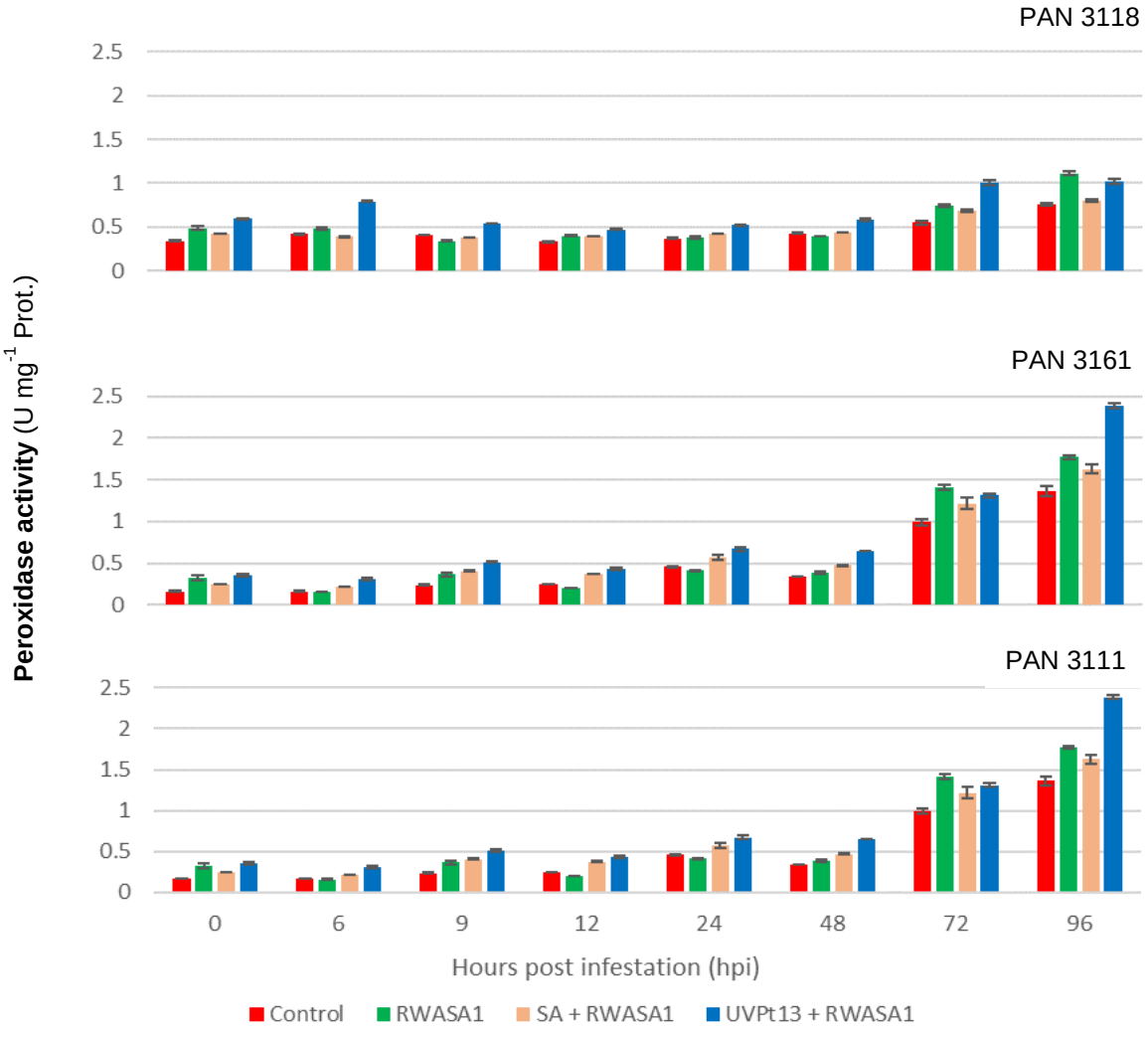


Fig. 4.2: Effect of South African Russian wheat aphid (RWASA1) infestation on POD activity of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The bars represent means±SE from three biological repeats.

Wheat cultivars showed a gradual increase in POD activity over time, especially in PAN 3111 and PAN 3161 (Fig. 4.2). Plants treated with SA or inoculated with UVPt13 before RWASA1 infestation showed higher POD activity than control and infested without prior treatment. In the later hours of infestation (72 hpi), RWASA1 infestation without prior treatment induced the highest activity in PAN 3161 and PAN 3111. However, this response was not sustained in the two cultivars, as 96 hpi, RWASA1 infestation of UVPt13 pre-inoculated plants showed the highest activity in the two cultivars (Fig.4.2).

4.3.3 Ascorbate peroxidase (EC 1.11.1.11)

Analysis of variance results revealed that APX activity was significantly different in primed and non-primed wheat cultivars, and the duration of infestation significantly affected the activity. On the other hand, replications and the interaction of priming treatments and time were nonsignificant (Table 4.6).

Table 4.6 Analysis of variance of South African Russian wheat aphid biotype (RWASA1) induced ascorbate peroxidase activity in primed [*Puccinia triticina* race isolate (UVPt13) and salicylic acid (1.5 mM)] wheat cultivars

SOV	DF	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	3	0.004**	0.008**	0.017**
Replication	2	0.0007	0.0001	0.0011
Time (hpi)	7	0.0009**	0.0014**	0.0165**
Treatment x Time	21	0.0002	0.0001	0.0010

** = highly significant at P≤0.01

Grouping of means indicated the lowest APX activity in the controls than plants primed by UVPt13 in all cultivars (Table 4.7). Infestation of SA-treated PAN 3161 induced APX activity that was significantly different from control plants, while in PAN 3118 and PAN 3111, the induced activity was not significantly different from that in controls (Table 4.7). UVPt13 priming, on the other hand, caused a significant increase in APX activity during RWASA1 infestation in all the cultivars (Table 4.7). Grouping of the means

indicates that APX activity significantly differed between control plants and plants primed by UVPt13.

Table 4.7 Homogeneous grouping of South African Russian wheat aphid (RWASA1) induced ascorbate peroxidase activity in salicylic acid-treated and *Puccinia triticina* inoculated wheat cultivars

PAN 3118			PAN 3161			PAN 3111		
Treatments	**Means**	**Group**	**Treatments**	**Means**	**Group**	**Treatments**	**Means**	**Group**
UVPt13 + RWASA1	0.051	A	UVPt13 + RWASA1	0.06	A	UVPt13 + RWASA1	0.116	A
RWASA1	0.033	BC	SA + RWASA1	0.030	BC	SA + RWASA1	0.067	B
SA + RWASA1	0.025	CD	RWASA1	0.024	CD	RWASA1	0.067	
Control	0.022	D	Control	0.018	D	Control	0.057	

n = 24, means grouping at P≤0.05

Russian wheat aphid infestation induced the highest biphasic APX activity in UVPt13 pre-inoculated wheat cultivars; first, it increased before 12 hpi and later increased at 24 hpi and beyond (Fig. 4.3). In contrast, in the susceptible cultivar, PAN 3118, the infestation of untreated and UVPt13 pre-inoculated induced similar levels of activity (Fig. 4.3). Infestation of UVPt13-inoculated plants induced a higher APX activity in the three tested cultivars than plants pre-treated with SA and the control (Table 4.7, Fig. 4.3).

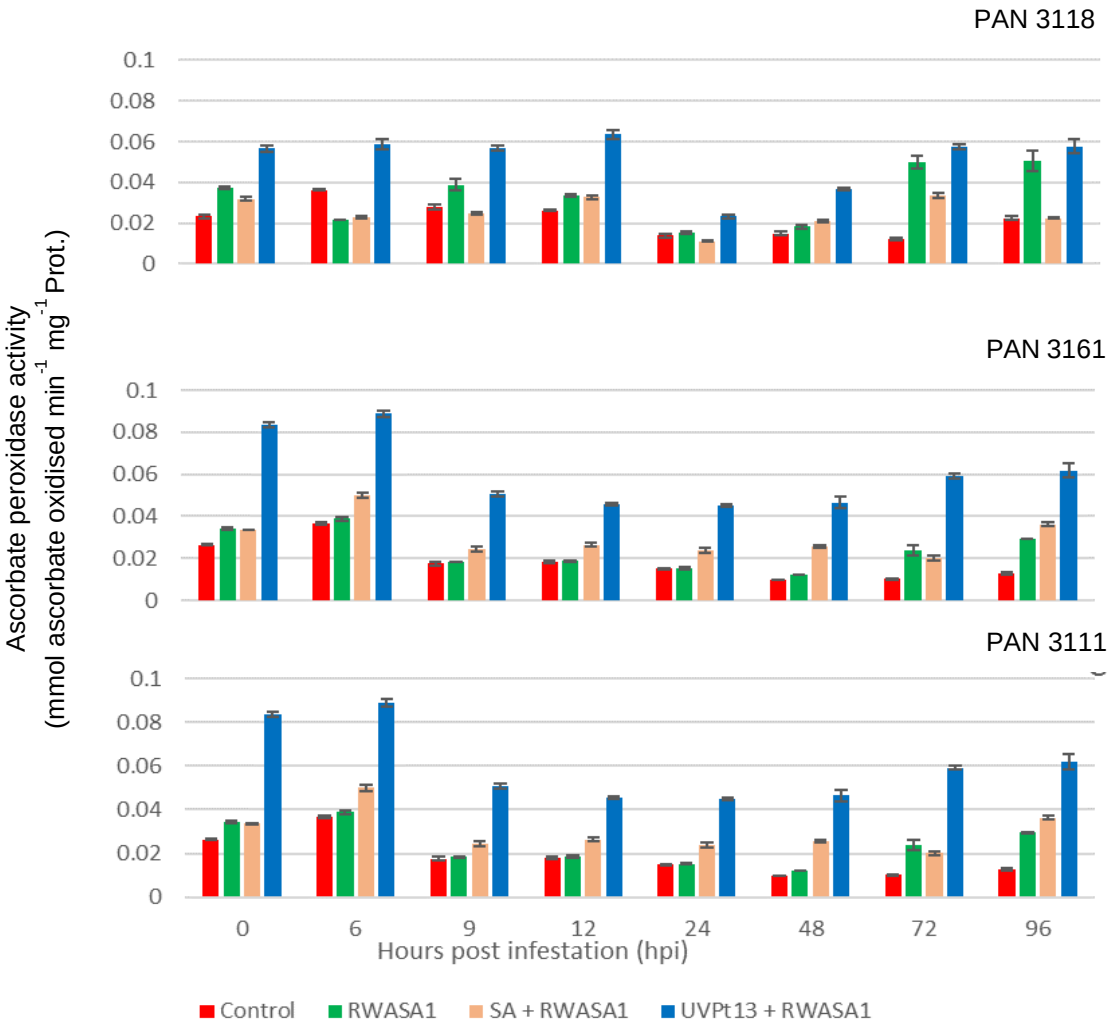


Fig. 4.3: Effect of South African Russian wheat aphid (RWASA1) infestation on ascorbate peroxidase activity of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The bars represent means±SE from three biological repeats.

4.3.4 Catalase (EC: 1.11.1.6)

Catalase activity was highly significant in all wheat cultivars under subjected treatments. However, induced activity was nonsignificant between replications except in PAN 3161 (Table 4.8). PAN 3118 showed similar activity throughout the sampling time from 0 to 96 hours post infestation, while PAN 3161 and PAN 3111 showed significantly different CAT activity throughout the sampling time (Table 4.8).

Table 4.8 Analysis of variance of catalase activity induced by South African Russian wheat aphid biotype 1 (RWASA1) infestation in primed [*Puccinia triticina* race isolate (UVPt13) and salicylic acid (1.5 mM)] and non-primed wheat cultivars

SOV	DF	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	3	0.0006**	0.0009**	0.0005**
Replication	2	0.00003	0.0001*	0.00002
Time (hpi)	7	0.00005	0.0001**	0.00014**
Treatment x Time	21	0.00004	0.00006*	0.00008*

* = significant at P≤0.05; ** = highly significant at P≤0.01

Grouping of means indicated that RWASA1 infestation of UVPt13-primed plants expressed the lowest CAT activity than the rest of the treatments (Table 4.9). Salicylic acid pre-treatment increased CAT activity only in PAN 3111 (Table 4.9).

Table 4.9 Homogeneous grouping of Russian wheat aphid (RWASA1) induced catalase activity of wheat cultivars pre-treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13

PAN 3118			PAN 3161			PAN 3111		
Treatments	Means	Group	Treatments	Means	Group	Treatments	Means	Group
Control	0.019	A	Control	0.017	A	SA + RWASA1	0.014	A
RWASA1	0.019		SA + RWASA1	0.015		Control	0.009	BC
SA + RWASA1	0.018		RWASA1	0.014		RWASA1	0.007	CD
UVPt13 + RWASA1	0.008	B	UVPt13 + RWASA1	0.003	B	UVPt13 + RWASA1	0.004	D

n = 24, means grouping at P≤0.05

In the early hours (6-12 hpi), RWASA1 infestation did not increase CAT activity in the three primed wheat cultivars (Fig. 4.4). Beyond 24 hpi, RWASA1 infestation of unprimed wheat cultivars induced higher activity in PAN 3118. In the other two cultivars, CAT activity of RWASA1-infested unprimed plants was similar to that of control and higher than that of primed plants, except at 96 hpi, where SA pre-treated plants expressed the highest activity, somewhat delayed for effective resistance to infestation (Fig. 4.4).

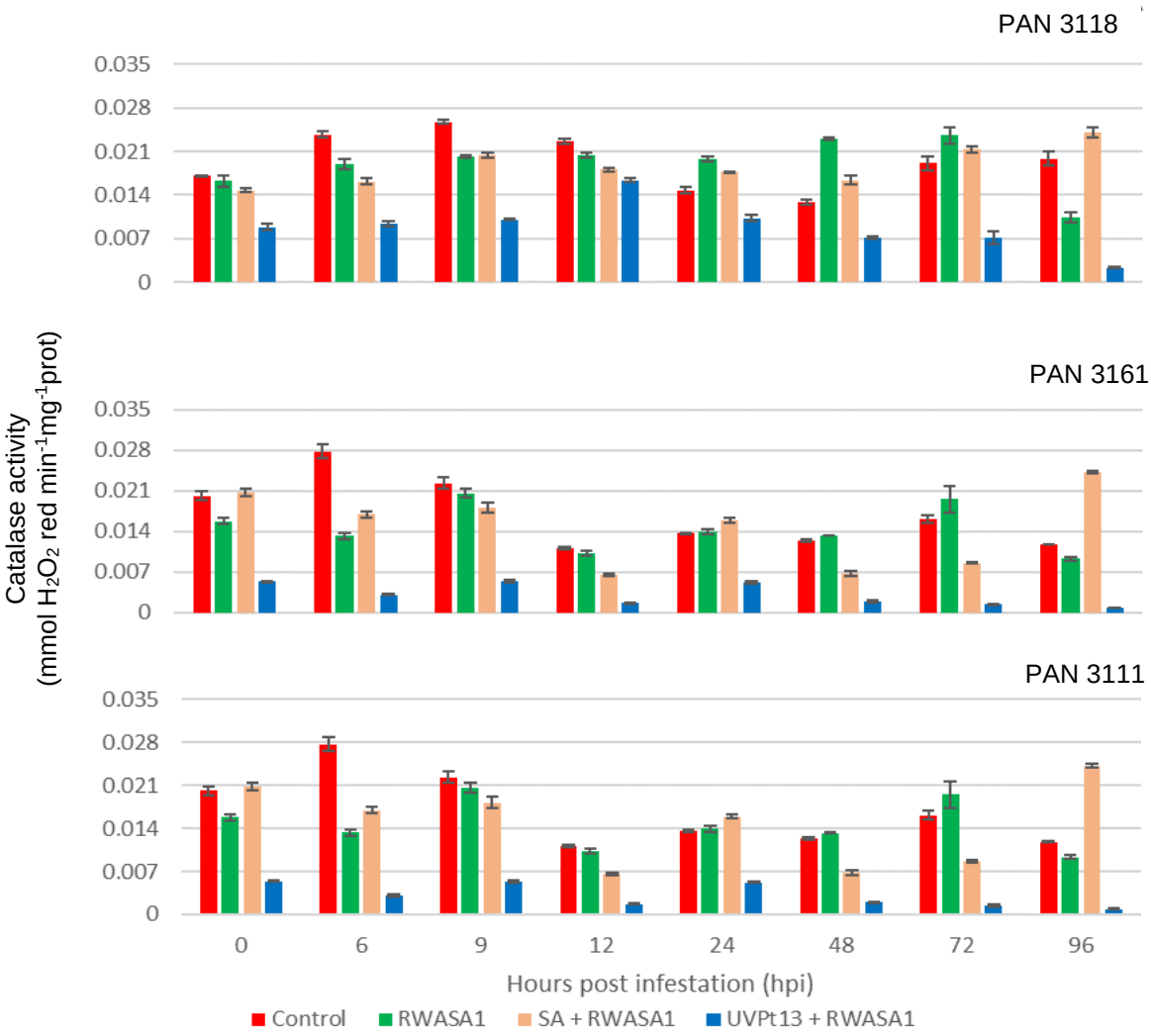


Fig.4.4: Effect of South African Russian wheat aphid biotype (RWASA1) infestation on catalase activity of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The The bars represent means±SE from three biological repeats.

4.3.5 Glutathione reductase (EC: 1.6.4.2)

The wheat cultivar PAN 3118 showed highly significant GR activity under different treatments, while PAN 3161 and PAN 3111 did not show any significant differences (Table 4.10). The interaction between time and treatments was also nonsignificant in all the cultivars. However, all cultivars showed highly significant activity at different sampling times (Table 4.10).

Table 4.10 Analysis of variance of glutathione reductase activity in primed [*Puccinia triticina* race isolate (UVPt13) and salicylic acid (1.5 mM)] wheat cultivars infested by Russian wheat aphid (RWASA1)

SOV	DF	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	3	2.381**	1.354	2.482
Replication	2	1.494	1.329	2.986*
Time (hpi)	7	4.992**	37.302**	14.112**
Treatment x Time	21	0.747	0.740	2.039

* = significant at P≤0.05; ** = highly significant at P≤0.01

Means grouping indicated that PAN 3118 responded similarly to all the treatments except the controls. The treatments did not induce significantly different responses in the other two cultivars, PAN 3161 and PAN 3111 (Table 4.11).

Table 4.11 Homogeneous grouping of glutathione reductase activity of South African Russian wheat aphid biotype 1 (RWASA1) infested wheat cultivars pre-treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13

PAN 3118			PAN 3161			PAN 3111		
Treatments	Means	Group	Treatments	Means	Group	Treatments	Means	Group
RWASA1	7.27	A	RWASA1	3.41	A	UVPt13 + RWASA1	5.84	A
UVPt13 + RWASA1	7.13		SA + RWASA1	3.05		RWASA1	5.53	
SA + RWASA1	7.07		UVPt13 + RWASA1	2.91		Control	5.12	
Control	6.55	B	Control	2.91		SA + RWASA1	5.00	

n = 24, means grouping at P≤0.05

Wheat cultivars did not show a significant variation in GR activity under different treatments. None of the treatmnets induced any variable responses in PAN 3161 and PAN 3111. Even though treatments induced a higher GR activity in PAN 3111 at 6 hpi, it was an isolated spike, and did not result in significantly different responses towards the treatments (Table 4. 11, Fig. 4.5).

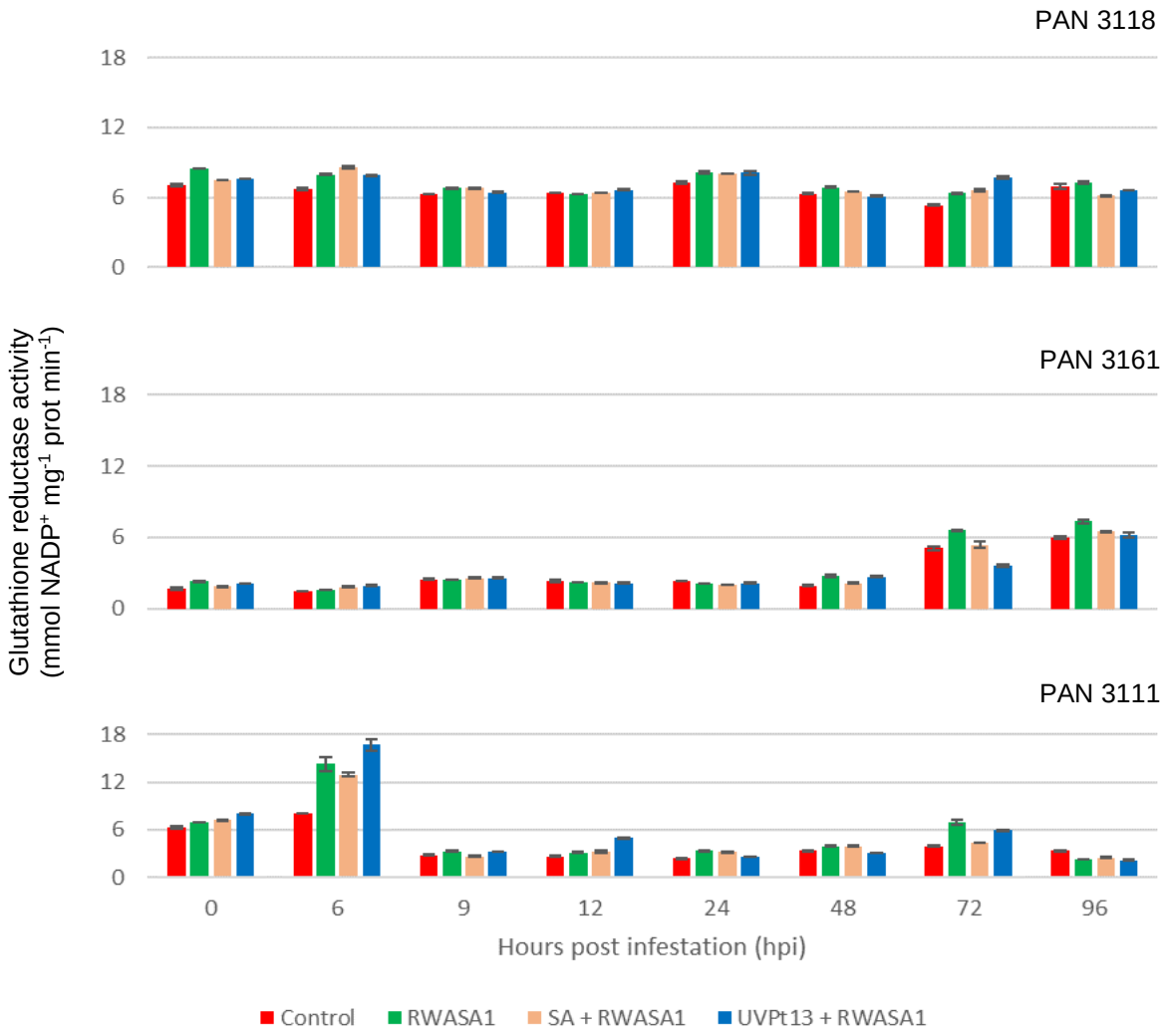


Fig. 4.5: Effect of South African Russian wheat aphid biotype 1 (RWASA1) infestation on glutathione reductase of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The bars represent means±SE from three biological repeats.

4.3.6 Ascorbic acid (EC 200-066-2)

Analysis of variance indicated that wheat cultivars PAN 3118, PAN 3161 and PAN 3111 showed highly significant AA contents in the three biological repeats. Sampling time showed significantly different AA contents in PAN 3161 and PAN 3111 (Table 4.12).

Table 4.12 Analysis of variance of ascorbic acid contents in primed [*Puccinia triticina* race isolate (UVPt13) and salicylic acid (1.5 mM)] wheat cultivars infested by Russian wheat aphid (RWASA1)

SOV	DF	Mean square		
		PAN 3118	PAN 3161	PAN 3111
Treatment	5	5.735	69.69**	15.92
Replication	2	313.34**	50.23**	496.92**
Time (hpi)	2	23.44	57.32**	69.53**
Treatment × Time	10	38.53**	11.22*	12.34*

* = significant at P≤0.05; ** = highly significant at P≤0.01

Even though treatments induced nonsignificant AA content, homogeneous grouping of treatments showed that wheat cultivars PAN 3118 and PAN 3161 expressed the highest AA contents in control plants while UVPt13 pre-inoculation in PAN 3111 induced activity higher than the rest of the treatments. Treatments did not influence AA content in PAN 3118, while in PAN 3161 and PAN 3111, the treatments induced changes in AA, which, nonetheless, did not increase the content upon RWASA1 infestation (Table 4.13).

Wheat cultivars without infestation and treatments showed the highest AA contents. Wheat cultivars inoculated with UVPt13 showed an increase in AA contents at 24 and 72 hpi, which was not higher than controls. Even though changes in AA content were measured in SA and UVPt13-treated PAN 3118 and PAN 3161, the values were not higher than in controls (Fig. 4.6).

Table 4.13 Homogeneous grouping of ascorbic acid contents of South African Russian wheat aphid biotype 1 (RWASA1) infested wheat cultivars pre-treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13

PAN 3118			PAN 3161			PAN 3111		
Treatments	**Means**	**Group**	**Treatments**	**Means**	**Group**	**Treatments**	**Means**	**Group**
Control	20.36	A	Control	23.38	A	UVPt13	19.01	AB
RWASA1	19.37		UVPt13	20.91	B	Control	18.28	BC
1.5mM SA + RWASA1	19.32		UVPt13 + RWASA1	19.40	BC	RWASA1	17.90	
UVPt13	19.08		RWASA1	17.54	CD	UVPt13 + RWASA1	16.21	
UVPt13 + RWASA1	18.70		1.5 mM SA + RWASA1	16.88	D	1.5 mM SA + RWASA1	16.07	
1.5 mM SA	17.95		1.5 mM SA	16.01		1.5 mM SA	15.96	CD

n = 9, means grouping at P≤0.05

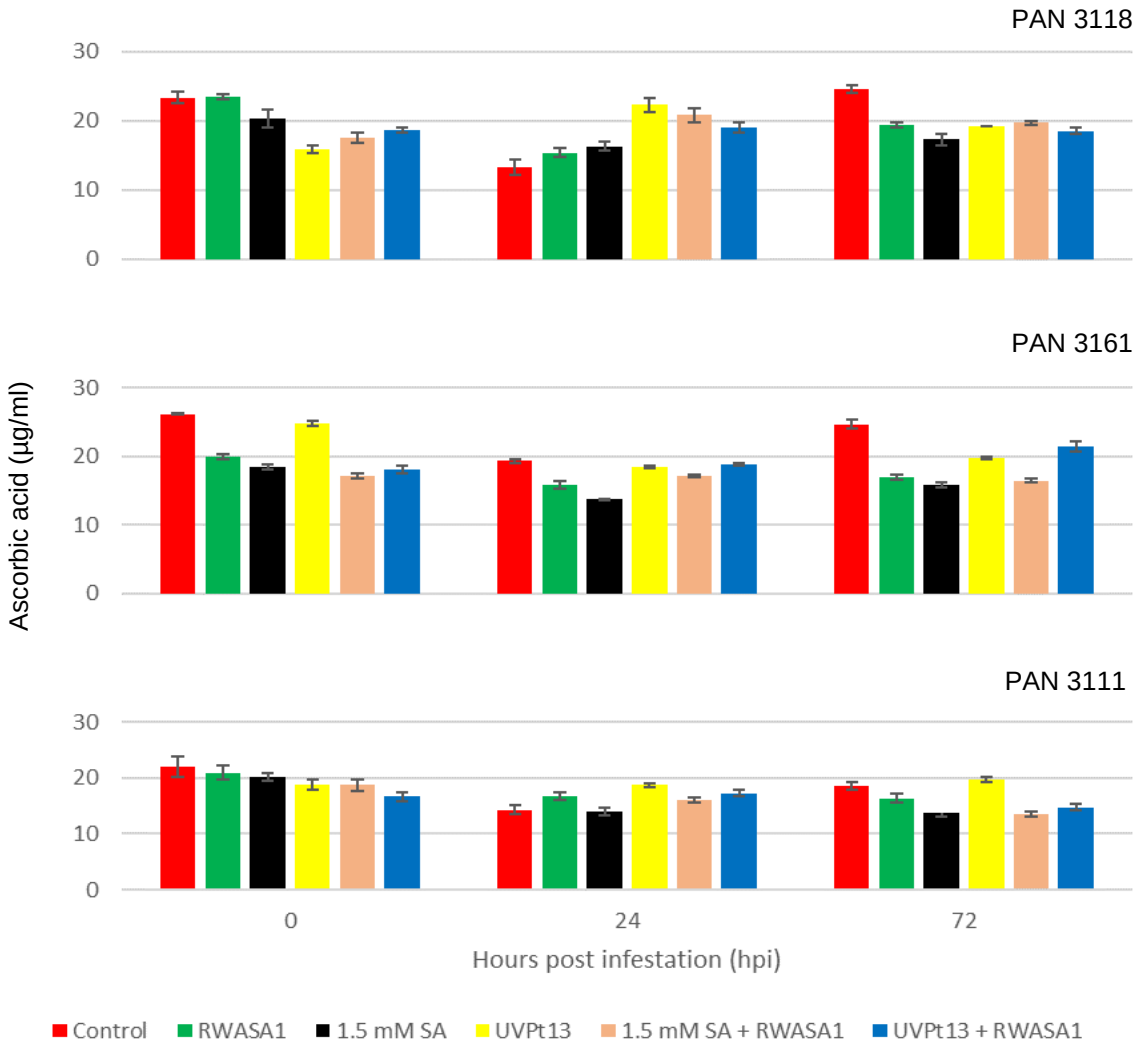


Fig. 4.6: Effect of South African Russian wheat aphid biotype 1 (RWASA1) infestation

on ascorbic acid content of UVPt13 pre-inoculated or SA pre-treated wheat cultivars. The bars represent means±SE from three biological repeats.

4.4 Discussion

Stressful conditions induce the accumulation of ROS in plants (Caverzan et al., 2016), which require scavenging by an equivalent amount of antioxidative species to prevent phytotoxicity (Ehsani-Moghaddam et al., 2006). A balance between the accumulation of ROS and antioxidants is an element of resistance.

Salicylic acid is a signalling phenolic compound that initiates defence responses against biotic and abiotic stresses (Kachroo and Kachroo, 2007) and mediates systemic acquired resistance (SAR). Exogenous application of SA induces SAR in plants and reduces induced symptoms by various bacterial, viral, and fungal pathogens (Wani et al., 2017; Wang and Xiang, 2020). *Puccinia triticina,* on the other hand, causes devastating effects on wheat growth and yield (Kaur et al., 2017). However, there are pathotypes such as UVPt13 that are avirulent to wheat. The study, therefore, investigated the effect of priming defence responses against RWASA1 using SA and an isolate of *Puccinia triticina* race UVPt13. We measured the antioxidative responses to RWA infestation in pre-treated wheat cultivars.

Wheat plants pre-inoculated with *Pt* isolates or pre-treated with SA enhanced the resistance response to RWA infestation by increasing the enzymatic antioxidants to higher levels than infested controls. However, wheat plants did not show significant differences in the content of a non-enzymatic antioxidant, AA.

Wheat cultivars pre-treated with SA or pre-inoculated with *Pt* isolate UVPt13 showed enhanced SOD, POD and APX activities. Superoxide dismutase scavenges superoxide radicals in plant cells (Ehsani-Moghaddam et al., 2006). Furthermore, the elevated activity of SOD is an indicator of resistance response to biotic and abiotic stresses (Lightfoot et al., 2017). The results agreed with Sorahinobar et al. (2021), who showed that SA-primed wheat seeds increased resistance to *F. graminearum* infection in resistant and susceptible wheat plants. The authors reported that primed plants increased the activity of POD, polyphenol oxidase and SOD and accumulated a high level of mRNA transcripts for PAL, β-1,3-glucanase, chitinase, cytochrome and

pleiotropic-drug resistance genes compared to control plants. Their findings agree with some of this study's results; POD and APX activities of PAN 3161 and PAN 3111 (Table 4.5, 4.7), and GR activities in PAN 3161 (Table 4.11) were higher in SA-treated plants than infested controls. Scavenging ROS protects plants against pest-induced oxidative stress (Moloi and van der Westhuizen 2008).

Previous research (Kiran et al., 2019) compared resistant and susceptible wheat genotypes to *Pt* isolates and reported that inoculated resistant genotypes showed increased enzymatic antioxidative activities of SOD and CAT, and PAL compared to non-inoculated plants. Furthermore, resistant cultivars showed high enzymatic activities compared to susceptible ones. Similarly, resistant wheat plants infected by *Puccinia striiformis* showed an increase in enzymatic antioxidants (APX, GR and GPX) compared to susceptible wheat plants (Chen et al., 2020). Therefore, incompatible interactions of wheat plants with *Pt* increase antioxidant activities and enhance defence mechanisms. This enhanced defence response can be utilised to counter RWA infestation. Similar results have been reported by Njom (2016) that *Pt* isolates 3SA145 enhanced defence responses in wheat cultivars to RWASA1 infestation through antixenosis and at proteomic levels.

Similarly, our findings indicate that wheat priming by an avirulent *Pt* isolate enhances wheat resistance by increasing the enzymatic antioxidative activities of SOD, POD and APX. The enzymes POD, SOD and CAT play critical roles in plant defence against different stresses, including insect herbivory. Additionally, antioxidants contribute to plant defence mechanisms against aphids (Zhao et al., 2016).

Even though CAT forms part of the resistance mechanism that detoxifies ROS, our results showed a significant reduction in CAT activity in plants primed by *Pt* before RWA infestation. Catalase activity was enhanced in *Vigna mungo* genotypes infested by whitefly (*Bemisia tabaci*) (Taggar et al., 2012) and exogenous SA application in wheat (Horváth et al., 2007). Debona et al. (2012) reported that wheat plants infected by *Pyricularia oryzae* increased the production of H_2O_2 compared to non-infected plants, and a reduction in CAT activity was associated with increased H_2O_2 production. The reduction in CAT activity could result from enhanced proteolysis caused by oxidative stress (Palma et al., 2002). Furthermore, they reported that most

peroxisomal proteins, such as CAT, glucose-6-phosphate dehydrogenase and glycolate oxidase, were endoproteolytically degraded.

The effect of priming on AA content was not apparent (Tables 4.12 and 13, Fig.4.6). The three biological replications were all significantly different ($P<0.05$), and the UVPt13 isolate increased AA content in PAN 3118 and PAN 3111 only at 24 hpi. The fact that responses were not repeatable makes it challenging to evaluate the effect of priming on AA content. Even though Yaman and Nalbantoğlu (2020) reported that exogenous application of SA increased AA content in herbicide-stressed wheat to levels of control, AA content occurs in minute concentrations in wheat leaves. Therefore, significant differences among treatments were difficult to determine.

Based on enzymatic antioxidative activities (SOD, POD and APX), the results revealed that exogenous application of SA and avirulent *Pt* infection could prime wheat plants and enhance defence responses to reduce RWASA1-induced damage on wheat. According to this study, the avirulent *Pt* isolate (UVPt13) proved an excellent priming agent compared to the exogenous application of SA (1.5 mM). Even though only one RWA biotype was used, priming plants with SA and avirulent *Pt* isolates may be an alternative strategy in RWA management. Further studies are required to establish the breadth of priming against various RWA biotypes. Investigation into the mechanisms of the primed responses, particularly in the cell wall region, which forms the first line of defence, could shed more light on the priming mechanisms.

4.5 Conclusion

Salicylic acid and the avirulent *Pt* isolate priming increased the activities of specific enzymatic antioxidants in the various wheat cultivars tested. The *Pt* isolate induced a higher antioxidant potential than SA priming. The results indicate that particular enzymatic antioxidants confer tolerance to RWASA1 infestation. Since the wheat cultivars showed different levels of responses, further studies to explore the apparent specificity in priming and mediating expression of antioxidant capacity in wheat are required. Nonetheless, the current findings contribute towards unravelling some mechanisms associated with priming-mediated tolerance to RWA infestation.

Additionally, the potential of *Pt* isolates priming needs further evaluation against various RWA biotypes and under field conditions.

4.6 References

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Chapter 5

Exogenous salicylic acid and *Puccinia triticina* pre-infection reduce cell wall modification in Russian wheat aphid infested wheat

5.1 Introduction

The plant cell wall is a complex matrix of polysaccharides that provides essential support and strength for plant survival. In addition, the matrix consists of a network of polymers, including proteins, aromatic substances, water and ions (Ochoa-Villarreal et al., 2012) that confer unique physical properties and structural architecture. Cell wall composition in vascular plants is approximately 35% pectin, 30% cellulose, and 30% hemicellulose; however, variation exists. Structural proteins contribute up to 15% to the cell wall components (Malinovsky et al., 2014).

The plant cuticle, hairs, waxes, antimicrobial enzymes and secondary metabolites protect plants from pests and pathogens (Malinovsky et al., 2014). Additionally, the plant cell wall forms a significant barrier that inhibits microbial and pest penetration, acting as a frontline barrier to stop their penetration and access to nutrients. Therefore, the plant cell wall is a physical and chemical barrier that inhibits successful infestation and infection (Malinovsky et al., 2014).

Structural properties of the plant cell wall are essential for its rigidity and resistance to biotic and abiotic stresses. Cellulose and hemicellulose provide cell wall rigidity, and pectin confers fluidity. Furthermore, cellulose and hemicellulose are implanted in the nebulous pectin polymers and are steadied by phenolic compounds and proteins. Hemicellulose binds the cellulose surface by preventing direct contact with microfibrils, while pectin links hemicelluloses for gel formation (Broxterman and Schols 2018).

Cellulose is a plant cell wall's main structural component and plays an essential role in plant growth and development. Three cellulose synthase enzymes in the plasma membrane catalyse cellulose synthesis (Somerville, 2006). Arabidopsis mutations in the cellulose synthase gene *CESA*3 reduced cellulose deposition in the primary cell

wall and increased lignin accumulation, making the plant resistant to powdery mildew (Caño-Delgado et al., 2003). Cellulose synthesis and deposition in the secondary cell wall are sensing mechanisms for cell wall integrity and enhance Arabidopsis's disease resistance to *Ralstonia solanacearum* and *Plectosphaerella cucumerina* (Hernández-Blanco et al., 2007). Furthermore, cellulose deficiency increased susceptibility in Arabidopsis infected with the vascular pathogen *Fusarium oxysporum* (Menna et al., 2021).

Hemicellulose is a polysaccharide matrix that holds cellulose microfibrils by hydrogen bonding. It is a linear or branched sugar molecule chain that varies between plant species and tissues. Galactans, xylans, glucomannans and mannans form part of the hemicellulose polysaccharides. Recently, Santiago et al. (2013) reported that leaves of resistant maize lines to *Diatraea grandiosella* (South Western Corn Borer) and *Spodoptera frugiperda* (fall armyworm) showed a higher level of hemicellulose than the susceptible counterparts. Furthermore, the cell wall polymers negatively correlated with fall armyworms feeding on resistant maize leaves (Hedin et al., 1996). They concluded that hemicellulose and proteins were associated with defence responses.

Lignin is an additional biopolymer of monolignols, primarily present in the secondary cell wall. It is a heterogeneous polymer made of three essential monolignols, p-hydroxyphenyl, syringyl and guaiacyl units (Xie et al., 2018). Lignin biosynthesis and deposition increase leaf toughness and play an essential role in plant defence against pest and pathogen invasion by physically blocking the cell wall region (Bagniewska-Zadworna et al., 2014). Furthermore, lignin deposition modifies the cell wall, making it more rigid. Menden et al. (2007) reported the induction of syringyl-rich lignin in wheat leaves during a non-compatible interaction with the fungus *Puccinia graminis*.

Ester bonds link phenolic compounds to other polymers (Gupta and De, 2017). Seven phenolic compounds in plant leaf tissue and eight in the root tissue have been identified. Cell wall-bound phenolic changes occur in response to stresses. Phenolic compounds like p-coumaric, ferulic, and sinapic acids and peroxidase (POD) activity increase under salt stress and contribute to cell wall stiffening (Haghighi et al., 2014).

Pests like the phloem-feeding RWA use their stylets to probe intercellularly towards the sieve tubes. All interactions of host plants with pests and pathogens trigger agitation of the host cell wall structure, reducing cell wall integrity and increasing infection success. In resistant interactions, cellular defence signals are transmitted, activating the cell structure's reinforcement. Therefore, we hypothesised that if the exogenous application of salicylic acid (SA) or infection by *Puccinia triticina* (*Pt*) isolates sensitised plants for increased resistance to subsequent attacks, associated cell wall reinforcements are involved. The study investigated the effect of RWASA1 infestation on the cell wall modifications of wheat plants pre-treated with SA or pre-inoculated with *Pt* (isolate UVPt13). The cell wall changes were determined by measuring and comparing the soluble lignin content, total phenolics, glucose, and xylose in primed and unprimed plants.

5.2 Materials and Methods

Three wheat cultivars, PAN 3118, PAN 3161 and PAN 3111, were selected based on their reactions to RWASA1 and pre-treatments with SA or *Pt* isolates, as shown in Chapter 3. At the second leaf stage, wheat seedlings were treated with 1.5 mM SA or inoculated with *Pt* isolate UVPt13 before RWASA1 infestation. The experiment was conducted in a greenhouse at the University of the Free State, Bloemfontein, South Africa (29.1074°S, 26.1873°E). The day temperature was maintained at 22 ºC with an 18 ºC setting applied at night. The plants grew under a natural photoperiod.

The experimental treatments were allocated using a randomised complete block design of two repeats. Wheat cultivars were assigned six treatments: control, 1.5 mM SA, UVPt13, RWASA1, 1.5 mM SA + RWASA1 and UVPt13 + RWASA1. Plant leaves were harvested by chilling in liquid nitrogen three times post infestation (hpi) 24, 48 and 96 hpi. Collected samples were crushed in liquid nitrogen and stored at -20 ºC before further analysis.

5.2.1 Sample preparation for the determination of glucose and xylose content

The samples were prepared according to a modified method by Sluiter et al. (2008). Oven-dried leaf samples (100 mg) were transferred to labelled test tubes. Sulfuric acid (H_2SO_4: 3.0 ml of 72%) was added to the leaf samples and mixed gently on a magnetic stirrer for 1 minute. The test tubes were then placed in a water bath set at 30 ºC for 60-min hydrolysis. During the incubation, samples were mixed with a stirring rod every ten min without removing them from the water bath.

After the 60-min hydrolysis, samples were removed from the water bath and diluted to a final concentration of 4% acid by adding the required volume of deionized water. Samples were mixed by inverting the tubes several times to blend the low and high acid phases. Samples were then autoclaved for one hour at 121 ºC and cooled at room temperature. The samples were further neutralised by adding 2 M $CaCO_3$ (BDH Chemicals, England) to a pH of 5 to 6 before filtration using Millex® syringe filters (0.45 µm) and transferring to Liquid chromatography-mass spectrometry (LC-MS) auto-sampler vials.

A set of sugar recovery standards (SRS) was taken throughout the analysis and used to correct losses due to the decomposition of sugars during dilute acid hydrolysis. The SRS included D-(+) glucose and D-(+) xylose, and their concentrations were chosen to closely resemble the glucose and xylose concentrations in the sample. The required amounts of glucose and xylose were weighed (0.1 mg) and dissolved in 10 ml of deionised water. The correct amount of 72% sulfuric acid (348 µl) was added to each SRS before autoclaving, pH neutralisation, filtering, and transferring to LC-MS autosamplers for running and calibration.

The samples were run on a Shimadzu UFLC with an LC20AB binary pump, SIL20A autosampler and column oven. Separation was performed by an XBridge Amide column (250 × 4.6 mm, 3.5 µm) (Waters) using a gradient elution program between 10% acetonitrile with 0.1% ammonium hydroxide as solvent A and 90% acetonitrile with 0.1% ammonium hydroxide as solvent B over a total run time of 25 min. Following injection at 100% A the eluent composition decreased to 70% A over 15 min, followed by a drop to 60% A at 16 min, an increase to 80% A at 19 min and a further increase to 100% A up to 21 min and then left to equilibrate up to 25 min before the next sample

injection. The column was kept at 40 °C for the duration of the analyses. Samples were analysed on a Sciex 4000QTRAP mass spectrometer in negative ionization multiple reaction monitoring (MRM) mode using three transitions per analyte. For glucose, the quantifying transition was 178.9 > 59.1 with qualifying transitions 178.9 > 89.1 and 178.9 > 119. The quantifying transition for xylose was 148.9 > 89.1 with qualifying transitions being 148.9 > 59.0 and 148.9 > 71.1. The source parameters were set at 15 psi curtain gas, collision gas at high, ionspray voltage at −4500 volts, heater temperature at 400 °C, nebulization gas at 50 psi and heater gas at 30 psi. Compound specific parameters were set as automatically optimized by the build in compound optimization wizard during continuous analyte infusion. A five-level serial dilution from 60 µg/ml to 0.006 µg/ml for each of the analytes was used as external calibrant with quantitation of the unknown sample levels from the linear range of the calibration curve. The instrument software Analyst 1.5 was used for acquisition and quantitation.

5.2.2 Total phenolic content determination using the Folin-Ciocalteu reagent

Total phenolic content was determined using the Folin-Ciocalteu (F-C) colourimetric method (Ainsworth and Gillespie, 2007). In this reaction, phenolic compounds transfer electrons to the phosphotungstic/phosphomolybdic acid complex and produce a green colour whose absorbance is read on a spectrophotometer at 765 nm.

Required reagents

Methanol: 95 % (v/v) methanol in distilled water, sodium carbonate: 700 mM Na_2CO_3 in distilled water, gallic acid: 10-250 µg/ml gallic acid prepared in 95% methanol and F-C reagent: 10% (v/v) F-C reagent.

Extraction of total phenolic compounds and Folin-Ciocalteu assay procedure

The leaf tissue extracts used for the cell wall carbohydrate study (section 5.2.1) were used to measure the total phenolic contents. The supernatant (100 µl) and F-C reagent (200 µl) were added into 2 ml Eppendorf tubes and vortexed gently. After the vortex, 800 µl solution of 700 mM Na_2CO_3 was added to the samples and incubated for an

hour at room temperature. Samples were then centrifuged for 5 min at 13 000 rpm. Then 1 ml samples and gallic acid standards were transferred into disposable polystyrene cuvettes, and absorbance was measured at 765 nm using a spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer).

Total phenolic content calculations

The standard curve of gallic acid was used to determine an equation used to calculate the total phenolic content in the samples (Appendix, Fig. 7.1):

$$Total\ phenolic\ content\ = \frac{Abs-0.17}{0.0067}\ \mu g/ml$$

5.2.3 Acid-soluble lignin

Hydrolysed sample aliquots (section 5.2.1) were used to measure acid-soluble lignin (ASL) using a UV-Visible spectrophotometer (Varian Cary-100 UV-VIS Spectrophotometer). Samples were read at 320 nm on the spectrophotometer, and the values were used to calculate the ASL content.

Acid soluble lignin calculation

$$\%\ \text{acid soluble lignin} = \left(\frac{\text{UVAbs}\ \times \text{Volum filtrate}\ \times\ \text{Dilution Factor}}{\sum\ \times \text{ODW sample}}\right) \times 100$$

ODW: Oven dry weight, Extinction coefficient (∑) = 30 L /g cm (Sluiter et al. 2008)

5.2.4 Data analysis

The data for glucose, xylose, total phenolic content and acid-soluble lignin was analysed using the general linear model procedure: PROC GLMSAS 9.4 (SA Institute, 1989). Following analysis of variance (Two-Way ANOVA), the means were separated by the least significant difference (LSD) to indicate the significant differences between the treatments and responses. If treatments failed to induce significant responses, the means (total phenolic contents, glucose, xylose, and acid-soluble lignin) were analysed using a T-test comparison. Treatment means were drawn over time to analyse the cell wall modification under primed and non-

primed conditions.

5.3 Results

Wheat cultivars' seedlings were treated with SA or inoculated with *Pt* isolates, and their responses were analysed to evaluate their cell wall content modification compared to control seedlings. Glucose (representing cellulose) and xylose (representing hemicellulose) were measured. Furthermore, ASL and total phenolic contents were also measured to study the cell wall modifications.

Analysis of variance did not show significant differences in the induced glucose, xylose and total phenolics, except acid-soluble lignin (Tab 5.1). Wheat cultivars showed highly significant differences in glucose, xylose and total phenolics. The sampling time, however, was significantly different except for acid-soluble lignin. T-tests were then performed to compare the treatment pairs (nonprimed and primed wheat cultivars).

Table 5.1 Analysis of variance of glucose, xylose, total phenolic content and acid-soluble lignin content induced by South African Russian wheat aphid biotype 1 (RWASA1) infestation in primed [*Puccinia triticina* race isolate (UVPt13) and salicylic acid (1.5 mM)] and non-primed wheat cultivars

SOV	DF	Mean Square			
		Glucose	Xylose	Total Phenolics	Acid-Soluble Lignin
Treatment	5	4586.12	21.38	552.80	0.35*
Time	2	667892.59**	870.39**	3490.89**	0.03
Cultivar	2	83990.89**	4.06	4214.84**	0.01
Replication	1	22823.14*	30.08	2710.01*	1.33**
Treatment*Time	10	923.97	26.28	918.58	0.21

* = significant at P≤0.05; ** = highly significant at P≤0.01

The LSD results for acid-soluble lignin indicate that wheat cultivars showed the highest ASL in SA-treated plants, followed by RWASA1 infestation. The lowest ASL was observed in UVPt13 pre-inoculated plants, followed by RWASA1 infestation of untreated plants (Table 5.2).

Table 5.2 Homogeneous grouping of acid-soluble lignin of South African Russian wheat aphid biotype 1 (RWASA1) infested wheat cultivars pre-treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13

Treatments	Means	Group
1.5 mM SA + RWASA1	24.27	A
RWASA1	24.22	AB
Control	24.22	AB
UVPt13	24.22	AB
1.5 mM SA	24.00	BC
UVPt13 + RWASA1	23.94	C

n = 18, means grouping at P≤0.05

5.3.1 Acid-soluble lignin

Lignin or lignin-like phenolic compounds are part of the plant defence responses against pests and pathogens (Bhuiyan et al., 2009a). Therefore, ASL was measured in primed and non-primed plants.

Table 5.3 Treatment comparison T-test for acid-soluble lignin in primed [1.5 mM salicylic acid (SA) and *Puccinia triticina* isolate UVPt13] and non-primed wheat seedlings from different cultivars (PAN 3118, PAN 3161 and PAN 3111)

	Control vs RWASA1	**RWASA1 vs 1.5 mM SA**	**RWASA1 vs UVPt13**	**RWASA1 vs 1.5 mM SA + RWASA1**	**RWASA1 vs UVPt13 + RWASA1**
T. Values	0.573	-1.225	-1.553	-0.670	0.298
P. Values	0.287	0.119	0.0699	0.256	0.384

T-test indicates that the ASL was non-significant in wheat plants under primed and non-primed treatments (Table 5.3).

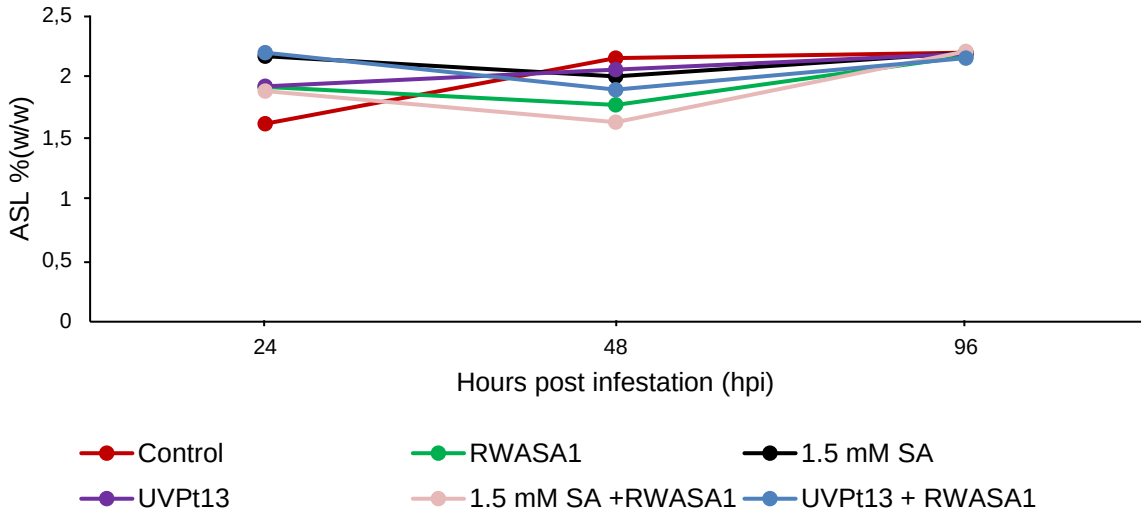

Fig. 5.1: Means of acid-soluble lignin content of wheat (PAN 3118) plants treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

At 24 hpi, the ASL content was not significantly different in all the treatments. Control plants of PAN 3118 and those pre-inoculated with UVPt13 increased ASL at 48 hpi, while all other treatments reduced ASL. However, primed plants recovered ASL contents at 96 hpi compared to 24 and 48 hpi (Fig. 5.1).

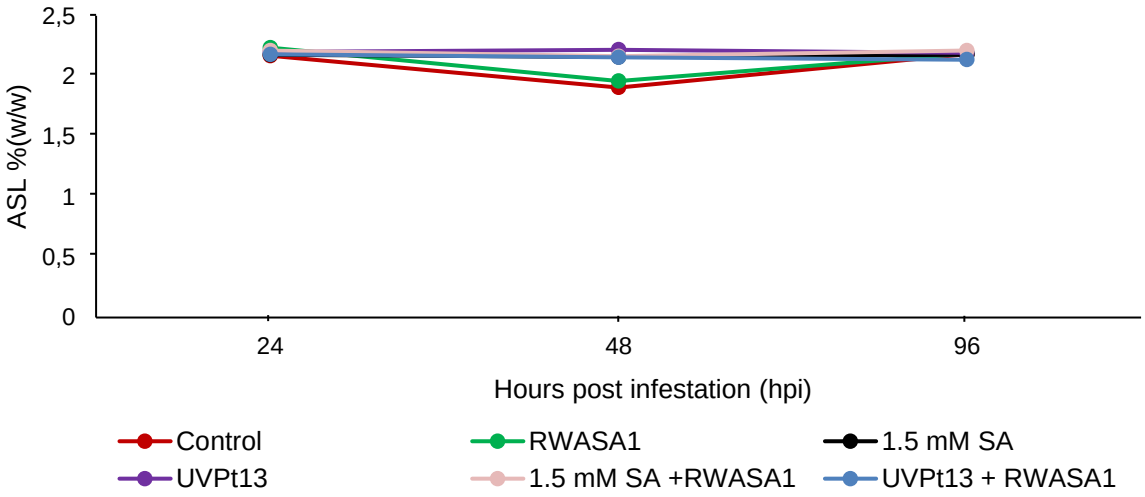

Fig. 5.2: Means of acid-soluble lignin content of wheat (PAN 3161) seedlings treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

All the treatments induced a similar level of ASL at 24 hpi, while at 48 hpi, there was a variation in seedlings under different treatments (Fig. 5.2). Control seedlings showed the lowest ASL at 48 hpi, while seedlings inoculated with *Pt* isolate UVPt13 showed the highest ASL levels. The control and RWASA1-infested plants recovered ASL at 96 hpi.

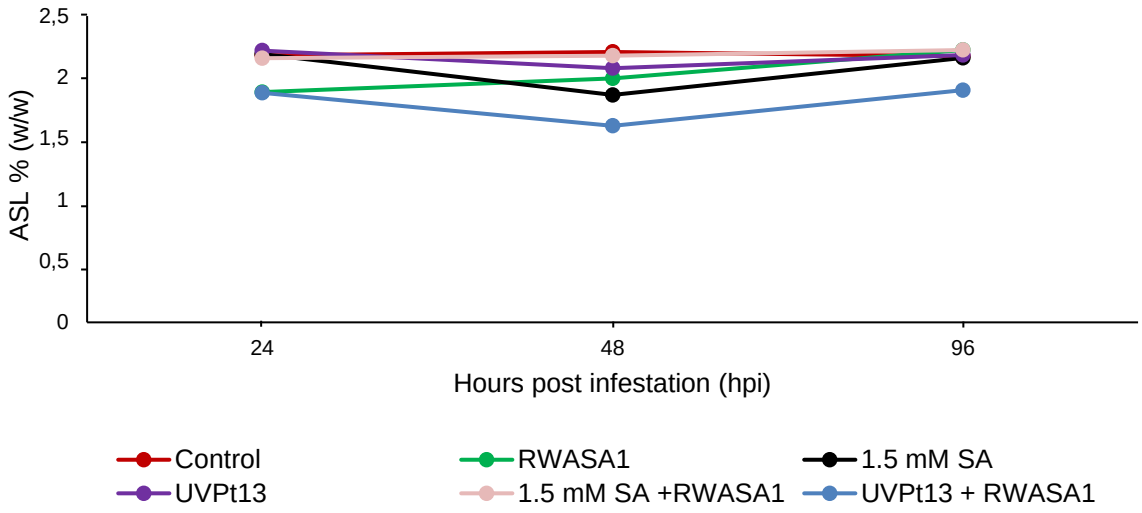

Fig 5.3: Means of acid-soluble lignin content of wheat (PAN 3111) seedlings treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

Wheat cultivar PAN 3111 showed consistent ASL levels in control, 1.5 mM SA + RWASA1 and UVPt13 treatments throughout the trial. At 24 hpi, wheat seedlings primed with SA before RWASA1 infestation showed higher ASL levels than the infested control (RWASA1) and UVPt13-primed plants (Fig. 5.3).

5.3.2 Total phenolic contents

Plant phenolic compounds are constituents of plant defence responses and protect plants against microbes and herbivores (War et al., 2012).

Table 5.4 Treatment comparison T-test for total phenolics in primed [1.5 mM SA and *Puccinia triticina* isolate UVPt13] and non-primed wheat seedlings from different cultivars (PAN 3118, PAN 3161 and PAN 3111)

	Control vs RWASA1	RWASA1 vs 1.5 mM SA	RWASA1 vs UVPt13	RWASA1 vs 1.5 mM SA + RWASA1	RWASA1 vs UVPt13 + RWASA1
T. Values	-0.185	0.436	-0.103	-0.190	-0.746
P. Values	0.427	0.334	0.459	0.425	0.233

T-test analysis showed that total phenolic contents were non-significant under different treatments in wheat cultivars. However, these treatments showed different probabilities of similarity. The total phenolic content in RWASA1 and UVPt13 pre-treated + RWASA1 infestation was not different (Table 5.4).

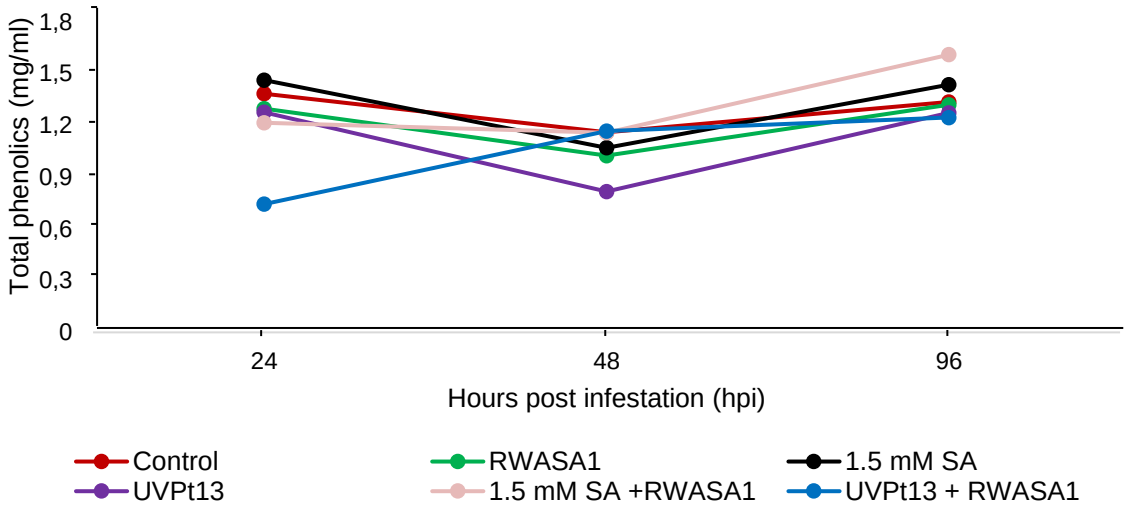

Fig. 5.4: Means of the total phenolic content of wheat (PAN 3118) seedlings treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

The treatments induced different levels of total phenolics at different times after infestation in PAN 3118 (Fig. 5.4). Seedlings infected by UVPt13 showed the lowest phenolic content at 48 hpi. However, UVPt13 infected, and UVPt13 and SA-primed seedlings measured higher phenolics content than the infested non-primed at 48 hpi.

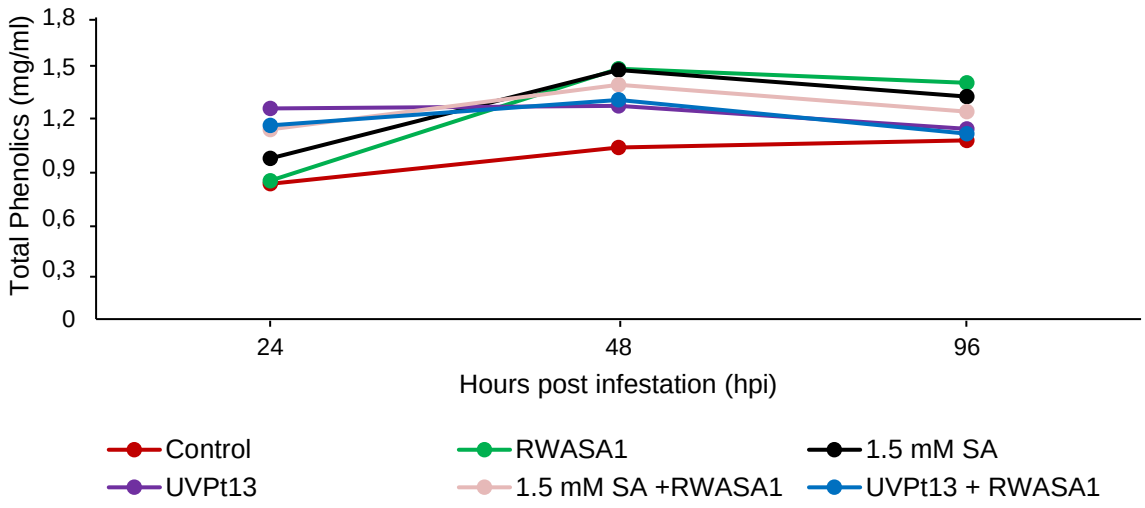

Fig. 5.5: Means of the total phenolic content of wheat (PAN 3161) seedlings treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

PAN 3161 is resistant to RWASA1; therefore, it consistently increased total phenolic compounds under primed and non-primed treatments (Fig. 5.5).

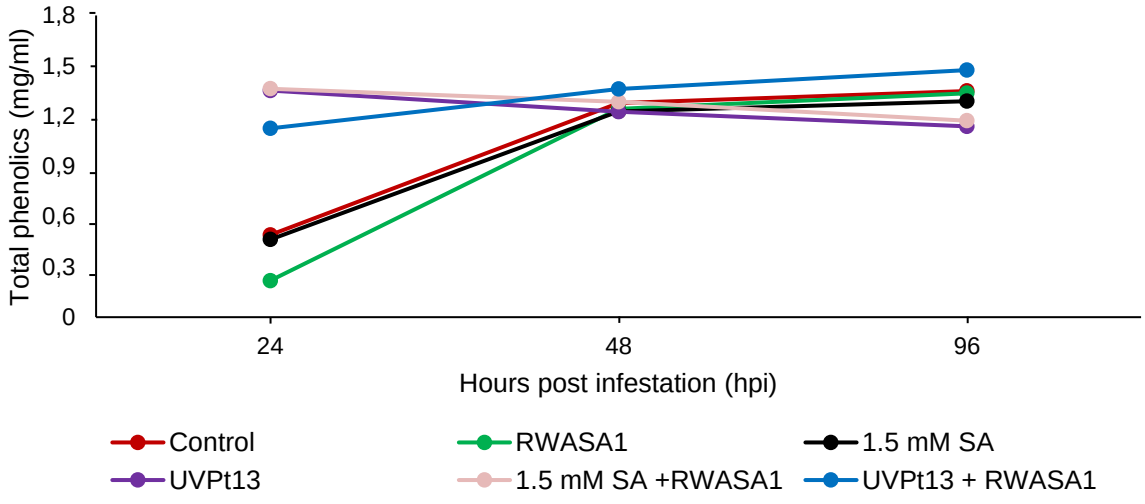

Fig. 5.6: Means of the total phenolic content of wheat (PAN 3111) seedlings treated with salicylic acid or inoculated with *Puccinia triticina* isolate UVPt13 before RWASA1 infestation.

The treatments induced different but non-significant total phenolic content in PAN 3111. Before and after priming (UVPt13 and 1.5 mM SA), Infestation induced a higher total phenolic content than at 24 hpi. Seedlings primed with UVPt13 before RWASA1 infestation showed increased total phenolic content throughout sampling times (Fig. 5.6).

5.3.3 Cellulose content in treated wheat cultivars

The treatment-induced cell wall modification was estimated by measuring the glucose content in the treated and control samples.

Table 5.5 Treatment comparison T-test for glucose in primed (1.5 mM SA and *Puccinia triticina* isolate UVPt13) and non-primed seedlings from different wheat cultivars (PAN 3118, PAN 3161 and PAN 3111).

	Control vs RWASA1	**RWASA1 vs 1.5 mM SA**	**RWASA1 vs UVPt13**	**RWASA1 vs 1.5 mM SA + RWASA1**	**RWASA1 vs UVPt13 + RWASA1**
T. Values	0.175	0.436	-0.103	-0.190	-0.746
P. Values	0.431	0.334	0.459	0.425	0.233

Wheat cultivars showed an almost similar level of glucose in all the treatments. Infestation or priming before infestation did not induce different glucose content in the cultivars (Table 5.5).

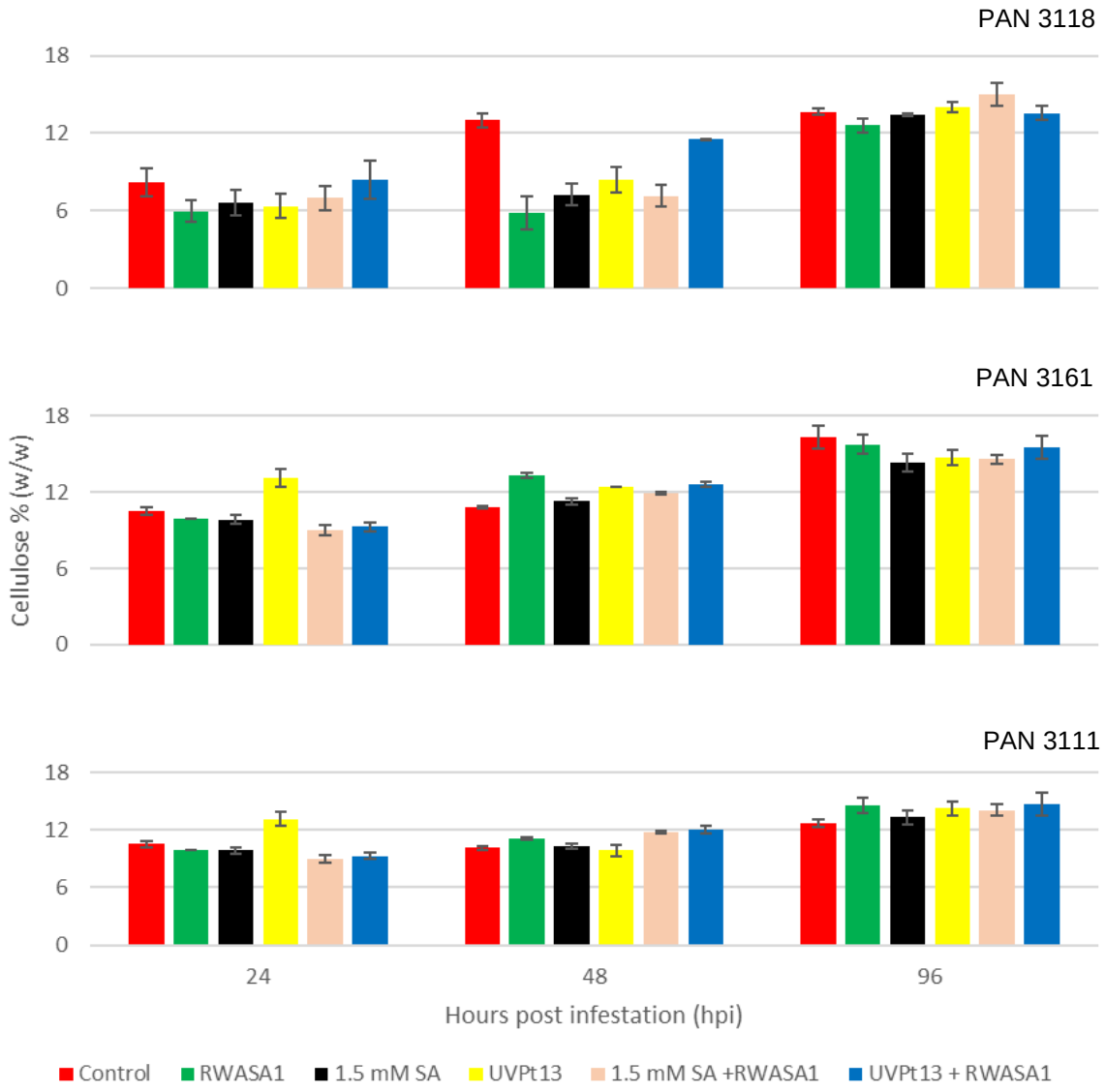

Fig. 5.7: Means±SE (n=6) cellulose (glucose) content (%) in cell walls of primed and non-primed seedlings of different wheat cultivars.

RWASA1 infestation reduced glucose content in seedlings (Fig. 5.7, PAN 3118) but was insignificant (Table 5.5). As the treatment duration increased, cellulose content recovered in primed (SA and *Pt* isolate) plants. The cultivar PAN 3161 is resistant to RWASA1, and under different treatments, increased cellulose content was measured, especially at 48 hpi (Fig. 5.7, PAN 3161). PAN 3111 showed no different responses under the different treatments (Fig.5.7 PAN 3111).

5.3.4 Hemicellulose contents in wheat cultivars

Like cellulose, lignin and pectin, hemicellulose protects plants against pests and pathogens. This study measured xylan content to evaluate the hemicellulose composition and cell wall modification during aphid infestation.

Table 5.6 Treatment comparison T-test of xylose in primed (1.5 mM SA, and *Puccinia triticina* isolate UVPt13) and non-primed seedlings from different wheat cultivars (PAN 3118, PAN 3161 and PAN 3111)

	Control vs RWASA1	**RWASA1 vs 1.5 mM SA**	**RWASA1 vs UVPt13**	**RWASA1 vs 1.5 mM SA + RWASA**	**RWASA1 vs UVPt13 + RWASA1**
T. Values	1.574	-1.229	-1.2181	-1.073	-1.968
P. Values	0.067	0.1183	0.120	0.149	0.0333*

* Significant at the probability of P<0.05

The treatments induced different levels of xylose. Furthermore, the T-test showed that seedlings infested by RWASA1 and UVPt13 + RWASA1 were significantly different (P<0.05; Table 5.6).

The treatments affected xylose content differently in PAN 3118 (Fig. 5.8 PAN 3181). At 24 hpi, all the treatments induced a lower xylose content except UVPt13 + RWASA1. However, as infestation proceeded, treatments increased xylose content, and at 48 hpi, content in control was about the same as in the UVPt13 + RWASA1. At 96 hpi, the xylose content of controls remained higher than other treatments, except 1.5 mM SA treatment. The treatment-induced increases were not sustained; at 96 hpi, control xylose was higher than all the treatments. In PAN 3111, the treatments 1.5 mM SA + RWASA1 and UVPt13 + RWASA1 induced xylose levels similar to those of the control (Fig. 5.8 PAN 3111). As treatment continued, 1.5 mM SA + RWASA1 treatment maintained the level of xylose to that of control (96 hpi). The cultivar showed a decline in xylose percentage in non-primed but RWASA1-infested plants compared to controls. Overall, results indicated that SA-induced xylose content in wheat cultivars enhances resistance to RWASA1 infestation.

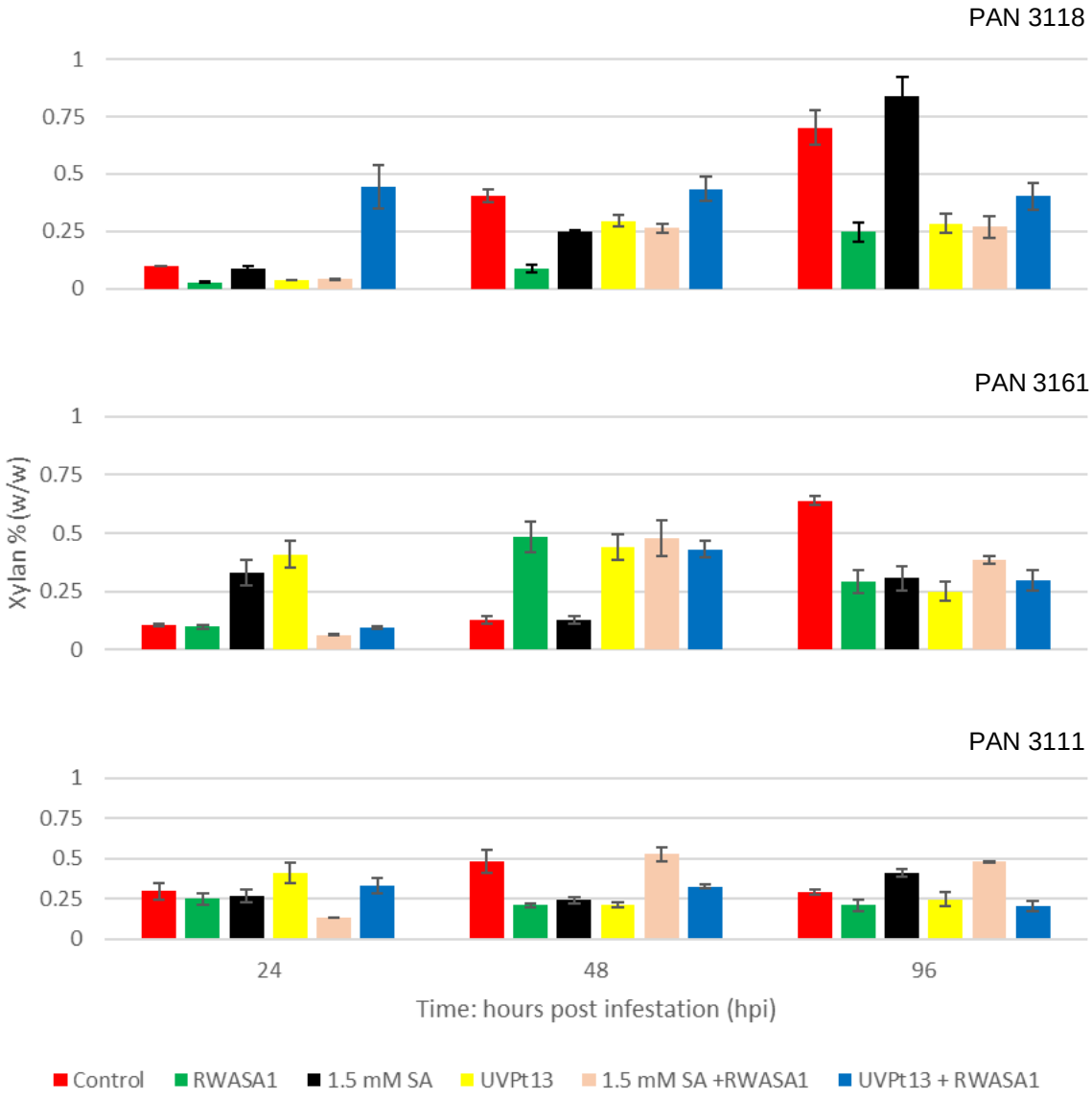

Fig. 5.8: Means±SE (n=6) xylose content (w/w %) in cell walls of primed and non-primed seedlings of different wheat cultivars.

5.4 Discussion

Total phenolic and acid-soluble lignin contents

Phenolic compounds are present in free and bound forms in the plant cell wall (Bonoli et al., 2004). Gupta and De (2017) reported that rice plants increased cell wall-bound phenolics under salinity stress in a salt-tolerant variety. In plant defence responses,

the phenylpropanoid pathway synthesises several secondary metabolites, protecting plants from abiotic and biotic stresses (Islam et al., 2019). Pathogen infection induces the synthesis of secondary metabolites (phenolics). In the current study, wheat PAN 3118 showed increased phenolic compounds when treated with UVPt13, UVPt13 + RWASA1, and 1.5 mM SA + RWASA1. Similarly, PAN 3111 showed higher total phenolics under UVPt13 + RWASA1 treatments, indicating that *Pt* isolates enhanced resistance to RWA infestation. This study indicated that primed wheat increased total phenolic, potentially protecting against RWA infestation.

Monolignol is critical in wheat plant defence responses to pathogens (Bhuiyan et al., 2009a). Syringyl lignin is one of the monolignols soluble in 72% sulfuric acid and can persist in 3% sulfuric acid (Yasuda et al., 2001). Wheat plants treated with a lignin synthesis inhibitor showed enhanced susceptibility to *P. graminis* (Kubalt, 2016). Similarly, gene silencing of monolignol synthesis enhanced wheat plants' susceptibility to powdery mildew infection (Bhuiyan et al., 2009b).

Lignification in infected plants may reduce infection or toxicity by pathogens. In our study, plants infected by avirulent *Pt* expressed a higher lignin content than controls. Furthermore, plants primed with SA also showed an increased lignin deposition. Wheat plants primed by either SA or avirulent *Pt* isolates enhanced defence responses to RWA infestation. Biotic and abiotic stresses enhanced phenylpropanoid pathways (Yadav et al., 2020) in plant tissues and increased enzymatic activities (Choudhary et al., 2020) and lignin deposition (Moura et al., 2010; Miedes et al., 2014).

Cellulose and hemicellulose content in primed and non-primed wheat plants

Cellulose is a crucial component of plant cell walls, which confers rigidity and causes a barrier to pest and pathogen invasion. Kesten et al. (2017) reported that cellulose-deficient Arabidopsis mutants were more sensitive to biotic stress than wild types. Infestation of the susceptible PAN 3118 with RWASA1 reduced cellulose content, while an infestation of primed PAN 3161 and PAN 3111 maintained cellulose content.

Xylan and xyloglucan are components of hemicellulose. Therefore, a change in xylan and xyloglucan content affects the defence responses. The Arabidopsis mutants with high xylose content enhanced resistance to a necrotic fungus, *P. cucumerina* (Wan et

al., 2021). Similarly, this study revealed that the RWASA1 infestation of wheat plants reduced xylose content and, consequently, the rigidity of the cell wall. However, UVPt13 (avirulent *Pt* isolate) pre-inoculation of PAN 3118 and PAN 3161 maintained the xylose content to that of controls, increasing the resistance.

Additionally, SA pre-treatment before RWASA1 infestation of PAN 3111 induced higher xylose content than the control. The results imply that priming maintained the rigidity of the cell wall and reduced aphid-induced degradation of the cell wall, thus increasing the resistance of treated plants. Zhao et al. (2021) reported that *P. striiformis* infection of barberry enhanced gene expression for pectin, hemicellulose and cellulose, while in wheat, a superficial level of gene expression was observed compared to control plants. Furthermore, they reported that lignin and pectin were significantly higher in wheat than in Barberry under *P. striiformis* infection. Additionally, Santiago et al. (2013) reported that resistant wheat cultivars demonstrated higher crude fibre and hemicellulose content during the Southwestern-Corn borer infestation.

5.5 Conclusion

Wheat cultivars primed by SA or inoculated with an avirulent *Pt* isolate showed different levels of reinforcement at the cell wall region. Results indicate that treatment with SA and infection with an avirulent *Pt* isolate can prime wheat plants to enhance their resistance against RWA infestation. The study shows that wheat cell wall rigidity is associated with RWA resistance. Future studies could investigate the role of cell wall composition and cell wall integrity during RWA infestations at different plant growth stages, on diverse germplasm and in natural environments.

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Chapter 6

6.1 General discussion

Successful wheat production faces abiotic (heat, drought and salinity) and biotic [fungal diseases and Russian wheat aphid (RWA) infestation] stresses in all global production regions. The RWA is an economically significant pest threat to successful wheat production globally, including in South Africa. It is an aggressive pest that punctures phloem cells and sucks cell sap, diverting host nutrients towards its development while reducing plant growth and yield.

Alternative to pesticides and resistance breeding, host plant defence enhancement using priming agents is an economically viable aphid management strategy. Priming agents like salicylic acid (SA) and microbes can reduce yield losses by up to 85% (Mony et al., 2017; Walters et al., 2013). In this study, the potential of SA and *Pt* race isolates in priming wheat cultivars to resist RWA infestation was evaluated.

Salicylic acid and avirulent *Pt* isolates significantly reduced RWASA1 and RWASA4-induced leaf damage in various wheat cultivars at the seedling and flag leaf stages. Furthermore, priming significantly increased enzymatic antioxidant activities and enhanced cell wall reinforcements by increasing cellulose, hemicellulose and soluble lignin contents. In priming the wheat cultivars for increased resistance to RWA, the avirulent *Pt* isolate (UVPt13) proved more efficacious than SA.

Salicylic acid (1.5 or 3.0 mM) priming through foliar application in the various wheat cultivars at the seedling and flag leaf stages improved reactions to RWA infestation (Chapter 3, Table 3.7-8, 3.12-13). However, a low concentration of SA (1.5 mM) did not prime defence responses to RWASA4 infestation; but a higher concentration (3.0 mM) mediated reduced leaf damage in PAN 3118 and PAN 3111, thus improving their resistance response.

Salicylic acid pre-treatment before RWASA1 infestation increased SOD, POD, APX, CAT and GR activities. However, RWASA1 infestation of primed plants induced variant effects on the non-enzymatic antioxidant (AA). In a different study, Sorahinobar et al. (2021) reported similar results where SA seed priming of wheat increased POD, polyphenol oxidase and SOD activities, which was evidence of enhanced defence

responses to *F. graminearum*. An increase in antioxidant activities in treated plants also showed enhanced defence responses to bacterial blight in rice (Samal et al., 2020). Their findings agree with some of this study's results; wheat cultivars showed higher levels of antioxidants in SA-treated plants than in infested controls.

Salicylic acid pre-treatment before RWA infestation induced higher xylose content than the control. The results imply that priming maintained the rigidity of the cell wall and reduced aphid-induced cell wall degradation.

The effect of *Pt* isolate as a priming agent to plant defence responses was measured by scoring induced leaf damage, antioxidant responses and cell wall composition. The avirulent *Pt* isolate UVPt13 reduced RWA (SA1 and SA4)-induced leaf damage at seedling and flag leaf stages. Reduced leaf damage indicated that *Pt*-priming enhanced defence responses in wheat cultivars. Furthermore, increased SOD, POD, APX and GR activities were also observed in pre-inoculated plants infested by RWASA1. This increase in enzymatic antioxidant SOD neutralises superoxide radicals in plant cells (Ehsani-Moghaddam et al., 2006) and produces H_2O_2 (Lightfoot et al., 2017).

A balance between the accumulation of ROS and antioxidants is an indication of defence responses (Malinovsky et al., 2014). However, a decline in CAT activity was noticed in wheat plants pre-inoculated by UVPt13 and infested by RWASA1. The reduced activity is a consequence of proteolysis by oxidative stress (Palma et al., 2002; Plaxton, 2019). The use of pathogens in priming plant responses for enhanced resistance was also reported by Gholami et al. (2019). They observed an increase in resistance against take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) induced by prior inoculation with four different endophytic fungi. They concluded that *Coprinopsis urticicola* was a potential priming agent that improved wheat resistance to take-all disease.

Puccinia triticina is one of the most common wheat pathogens (Kolmer et al., 2013) and causes minor damage than other rust fungi (Terefe et al., 2009; Huerta-Espino et al., 2011).

The results indicated that SA-treated or pre-inoculated plants showed wheat priming to RWA infestation. Similarly, successfully primed wheat plants infected by *Puccinia*

striiformis showed increased enzymatic antioxidants compared to Infested control plants. This enhanced host-defence response can be an alternative strategy to counter RWA infestation. Similar results have been reported by Njom (2016), where a *Pt* isolate (3SA145) enhanced antixenosis and other defence responses at the proteomic level against RWASA1 in wheat. However, he did not evaluate *Pt* isolates primed redox responses or any cell wall region changes. Our findings indicate that priming wheat plants with avirulent *Pt* isolate enhanced resistance by increasing enzymatic antioxidative activities.

Primed responses were further evaluated in the cell wall region. The UVPt13 + RWASA1 treatment in PAN 3111 showed a higher level of total phenolic contents than untreated RWA infestation. Therefore, an increase in the total phenolic compound indicates defence responses in wheat plants primed by SA and an avirulent *Pt* isolate to RWA infestation.

Wheat plants infected by avirulent *Pt* (UVPt13) expressed a higher lignin content than controls (Chapter 5: Fig 5.1,2,3). Similar results were reported by Southerton and Deverall (1990) that avirulent *Lr*20 strain infection on wheat leaves increases lignin content 75% greater than in control plants. Furthermore, plants primed with SA also showed increased lignin deposition (Chapter 5: Fig. 5.1,2,3). Wheat plants primed by either SA or the avirulent *Pt* isolate enhanced defence responses to RWA infestation. Increasing monolignols plays a critical role in wheat plant defence responses to pathogens (Bhuiyan et al., 2009a). Wheat plants treated with a lignin synthesis inhibitor showed enhanced susceptibility to *P. graminis* (Kubalt, 2016). Similarly, gene silencing of monolignol synthesis enhanced wheat susceptibility to powdery mildew infection (Bhuiyan et al., 2009b). Lignification in infected plants may reduce infection or toxicity by pathogens. Biotic and abiotic stresses enhanced phenylpropanoid pathways (Yadav et al., 2020) in plant tissues and increased enzymatic activities (Choudhary et al., 2020) and lignin deposition (Moura et al., 2010; Miedes et al., 2014).

The RWASA1 infestation reduced the cellulose contents of PAN 3118, while an infestation of SA and *Pt* isolate primed PAN 3161 and PAN 3111 maintained cellulose content. Cellulose is a crucial component of the plant cell wall, which confers rigidity and causes a barrier to pest and pathogen invasion. Kesten et al. (2017) reported that

cellulose-deficient Arabidopsis mutants were more sensitive to biotic stress than wild types.

A change in xylan and xyloglucan content affects disease defence responses. The Arabidopsis mutants with high xylose content enhanced resistance to a necrotic fungus, *P. cucumerina* (Wan et al., 2021). Similarly, this study revealed that the RWASA1 infestation of wheat plants reduced xylose content and, consequently, the rigidity of the cell wall. However, UVPt13 (avirulent *Pt* isolate) pre-inoculation of PAN 3118 and PAN 3161 maintained the xylose content to that of controls, increasing the resistance.

Based on leaf damage rating, enzymatic and non-enzymatic antioxidant activities and cell wall composition, the results revealed that exogenous application of SA or an avirulent *Pt* isolate (UVPt13) could prime plants and enhance defence responses to reduce RWASA1-induced damage on wheat. Since *Pt* isolates were efficacious priming agents compared to SA, they could be used as an alternative strategy for crop protection in plants instead of the direct use of chemical pesticides.

Wheat priming with SA and avirulent *Pt* isolates could be an environmentally friendly and durable strategy to reduce the impact of aphid infestation. However, the specificity demonstrated by SA and UVPt13 towards the Lesotho landraces and the biotype-specific reduction of leaf damage requires further investigation. In cases of successful priming, it could be interesting to evaluate the priming effect on yield potential and grain quality.

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Summary

Wheat is an important cereal crop that contributes carbohydrates and other nutrients to the human diet. However, successful wheat production faces abiotic and biotic stresses, reducing grain yield and nutritional quality. The Russian wheat aphid (RWA) is one of the significant and devastating wheat yield-reducing pests. This study aimed to investigate the efficacy of salicylic acid (SA) and *Puccinia triticina* (*Pt*) as priming agents in reducing RWA-induced damage in wheat cultivars. The objectives were to i) determine the impact of SA treatment or pre-inoculation with *Pt* isolates on RWA-induced leaf damage in wheat cultivars, ii) determine the activities of antioxidative systems during the primed resistance response, and iii) evaluate the role of cell wall composition during the infestation of primed plants.

Wheat cultivars were evaluated phenotypically by scoring induced aphid damage symptoms at the seedling and flag leaf stages. Salicylic acid differentially primed wheat cultivars and reduced the RWA biotypes induced leaf damage (increased resistance) at both growth stages. At the seedling stage, SA primed wheat cultivars non-specifically for enhanced resistance to RWASA1, while at the flag leaf stage, only PAN 3111, SST 356 and Makalaote showed increased protection. Priming towards RWASA4 resistance was particular; SA (3.0 mM) pre-treatment enhanced the resistance of only PAN 3118 and PAN 3111 at the seedling stage and PAN 3133 and Makalaote at the flag leaf stage. On the other hand, the *Pt* isolates effectively primed all the wheat cultivars for excellent resistance to RWASA1 except Mapili at the seedling and PAN 3111 at the flag leaf stages. However, pre-inoculation with the *Pt* isolates failed to enhance the resistance of the four Lesotho cultivars to RWASA4 at the seedling stage and PAN 3118 at the flag leaf stage. Lesotho landraces were less responsive to the priming agents, which requires further investigation. The induced responses at the seedling and flag leaf stages correlated positively in all the treatments.

The priming mechanisms on wheat cultivars were investigated by determining the induced biochemical changes during RWASA1 infestation. Three wheat cultivars (PAN 3118 and PAN 3111: susceptible to RWA biotypes, and PAN 3161, resistant to RWA, positive control) were used to study the antioxidant-related defence responses. Wheat plants pre-inoculated with *Pt* isolates or pre-treated with SA enhanced the

resistance response to RWA infestation by increasing the enzymatic antioxidants compared to infested controls.

Wheat cultivars pre-treated with SA or UVPt13 showed enhanced enzymatic antioxidant (SOD, POD and APX) activities, which are indicators of RWA resistance in wheat. Catalase activity was significantly higher in PAN 3111 in SA-treated than infested controls but declined in PAN 3161 pre-treated with SA. A significant reduction in CAT activity was noticed in plants primed with *Pt* before RWA infestation. The reduction in CAT activity could result from enhanced proteolysis caused by reported oxidative stress. The effect of priming on AA content was not apparent. The three biological replications were all significantly different ($P<0.05$). The fact that responses were not repeatable made it challenging to evaluate the effect of priming on AA content. Based on enzymatic antioxidative activities (SOD, POD and APX), the results revealed that exogenous application of SA and pre-inoculation with avirulent *Pt* isolates could prime wheat plants and enhance defence responses to reduce RWASA1-induced damage to wheat.

The composition of cell wall polymers, especially xylose, increased or maintained control levels in the *Pt* isolate (UVPt13) pre-inoculated cultivars, ensuring cell wall rigidity towards RWA penetration.

South African wheat cultivars were more responsive to priming agents than Lesotho landraces. Wheat cultivars showed similar responses at the flag leaf stage compared to the seedling stage. RWASA4 was more aggressive than RWASA1, possibly because of effector proteins released by RWASA4. Based on the responses, the avirulent *Pt* isolate (UVPt13) proved an excellent priming agent compared to SA (1.5 mM), suggesting that avirulent *Pt* isolates can be a worthy priming agent for improved resistance to RWA infestation. Thus priming plants with SA and avirulent *Pt* isolates could be an alternative strategy in RWA management.

Keywords: Antioxidants, peroxidase, cell wall sugars, induced resistance, leaf damage, lignin, peroxidase, Russian wheat aphid and total phenolics.

Chapter 7

Appendix

7.1 Standard curve derived from calculating ascorbic acid and total phenolic contents in plant tissue

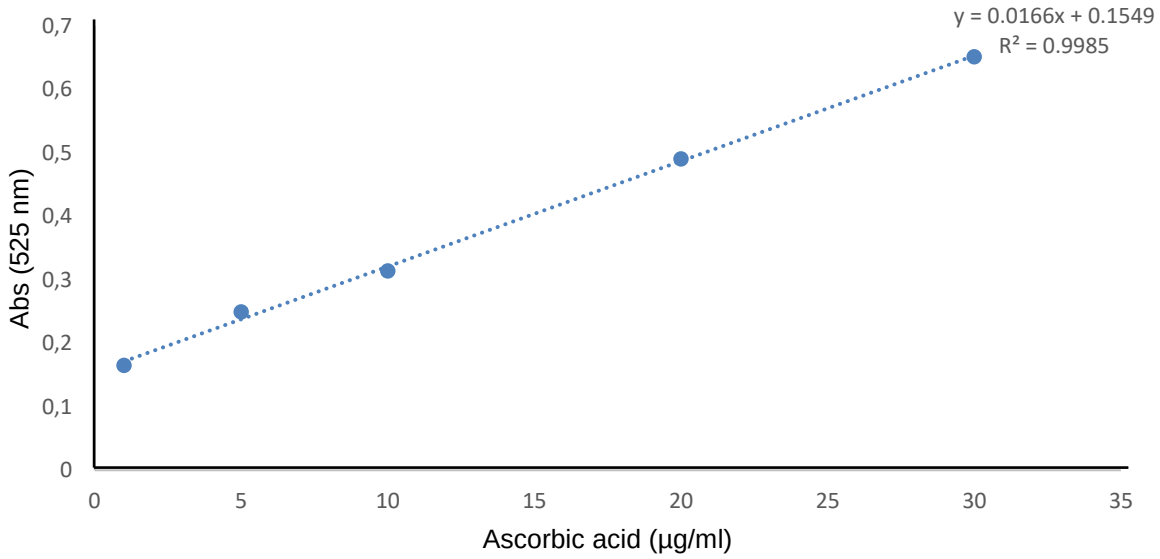

Fig. 7.1: Standard curve for ascorbic acid content.

The ascorbic acid content in the wheat plant samples was calculated using the equation given below. The equation was derived using the standard curve (Fig. 7.1).

$$\text{Ascorbic acid content} = \frac{\text{Abs} - 0.1549}{0.0166}\ \mu\text{g/ml}$$

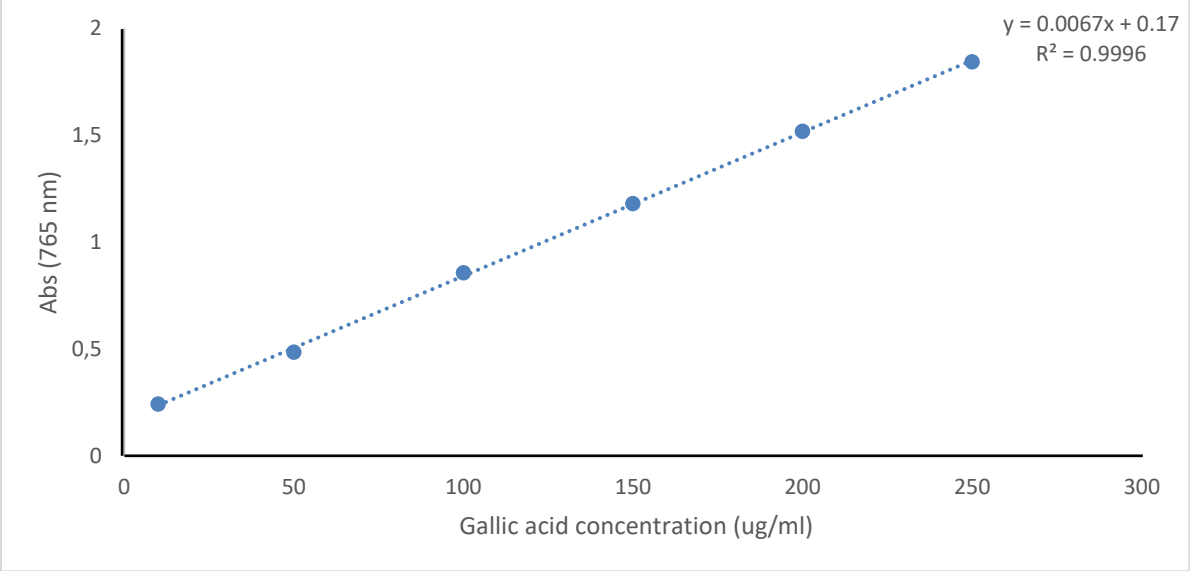

Fig. 7.2: Standard curve for gallic acid content.

Total phenolic content was calculated using the equation given below. The equation was derived using the standard curve (Fig. 7.2).

$$\text{Total phenolic content } = \frac{\text{Abs}-0.17}{0.0067}\ \mu\text{g/ml}$$