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Elucidation of possible virulence factors present in Russian wheat aphid *(Diuraphis noxia)* biotypes' saliva

BY

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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Magister Scientiae* to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other University.

RA van Zyl

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ELUCIDATION OF POSSIBLE VIRULENCE FACTORS PRESENT IN RUSSIAN WHEAT APHID *(DIURAPHIS NOXIA)* BIOTYPES' SALIVA 37

PREFACE

The Russian wheat aphid (RWA) is a pest of cereals, such as wheat and barley. It feeds on these hosts by injecting saliva into the plants' phloem tissue and consuming the mixture of saliva and photoassimilates. It has been proposed that the insect's saliva contains elicitors or virulence factors, which cause the symptoms typically observed in susceptible wheat cultivars. These are leaf rolling, chlorotic streaking, a decrease in yield and death in cases of heavy infestation. In contrast, resistant plants display symptoms typical of defence responses, such as the formation of necrotic lesions and an increase in the expression of pathogenesis related proteins. But, most importantly, RWA feeding on these hosts does not result in their subsequent death.

The objectives of the present study are thus to elucidate any putative virulence factors, present in insect saliva, that can result in the breakdown of resistance of cultivars and thus, lack of recognition and/or delayed onset of the plants' defence responses.

Thus, this thesis investigates the RWA on protein level to determine which components of these insects induce the different changes observed in the resistant and susceptible plants. Also, it examines whether or not the biotypes uniquely altered their elicitors in response to selective pressure.

In **Chapter 1** a brief introduction is presented on the Russian wheat aphid, its distribution and the effects of its feeding on resistant and susceptible wheat cultivars.

In **Chapter 2** a literature review provides insight on how the Russian wheat aphids feed and survive on wheat. It also outlines the control mechanisms which plants could employ to withstand attack from pests and pathogens.

In **Chapter 3** proteins were extracted from different parts of two Russian wheat aphid biotypes and separated on high pressure liquid chromatography (HPLC). Two biotypes were selected for the study to provide comparative information on the development of biotypes and/or their virulent elicitors. The presence of the potential elicitors was determined by examining the extent of leaf rolling chlorotic streaking/spots on injected plants' leaves, determining the activity of defence related enzymes of the injected plants and visualizing the proteins extracted from these plants on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. This was done in order to examine the plants on phenotypic, enzymatic and proteomic levels, which could confirm the results obtained on three different levels. It was found that resistant cultivars react similarly to the two biotypes, but that the RWA biotypes differ significantly on a protein level. Potential motivations for these variations are discussed.

Results presented in this dissertation represent the outcomes of a study conducted from March 2005 to December 2006 in the Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, under the supervision of Prof. A.-M. Botha-Oberholster. Chapter 3 is being prepared to be submitted for review in Insect Biochemistry and Molecular Biology.

The following conference paper and poster presentations were generated from results presented in this dissertation.

1. Van Zyl, R. A., Mathabe, P., Hlongwane, C., Bahlmann, L. & Botha, A.-M. 2005. Proteins expressed in wheat in response to Russian wheat aphid *(Diuraphis noxia)* infestation. *Abstracts / Comparative Biochemistry and Physiology* **Part A 141** p234.
2. Van Zyl, R. A. & Botha, A.-M. 2006. Wheat *(Triticum aestivum)* responses to injection by Russian wheat aphid *(Diuraphis noxia)* salival proteins. *South African Genetics Society 19th Congress: Bloemfontein, South Africa, 2nd-4th April 2006.* Programme and Abstracts, p30.
3. Van Zyl, R. A. & Botha, A.-M. 2007. Proteins are eliciting defence responses in wheat to *Diuraphis noxia*. *13th International congress on Molecular Plant-Microbe Interactions: Sorrento, Italy, 21st -27th July 2007. In press*.

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ABBREVIATIONS

ANOVA:	Analysis of Variance
APS:	Ammonium persulfate
ATP:	adenotrinucleotidephosphate
Avr:	Avirulence
β:	Beta
C teminal:	Carboxyl-terminal
CC:	Coiled-coil
CD:	Co-dominance
cm:	Centimeter
CRP:	Conservation Reserve Program
º:	Degrees
ºC:	Degrees Celsius
DN	*Diuraphis noxia*
DI:	Dominant independent
eLRR:	Exoplasmic Leucine-rich repeats
FAOSTAT	Food and Agriculture Organization
GlcNAc:	N-acetylglucosamine
IGC	International Grains Council
h p. inj.:	Hours post injection
HPLC:	High pressure liquid chromatography
HR:	Hypersensitive response
kb:	Kilo basepairs
kDa:	Kilo Dalton
JA:	Jasmonic acid
LRR:	Leucine-rich repeats
µg:	Microgram
µl:	Microlitre
ml:	Millilitre
MYA:	Million years ago
min:	Minute

m/v:	Mass per volume
M:	Molar
N terminal:	Amino terminal
$(NH_4)_2SO_4$:	Ammonium sulphate
nm:	Nanometer
NBS:	Nucleotide binding site
p. inj.:	Post injection
PCD:	Programmed cell death
PEST:	Pro-Glu-Ser-Thr
P.F.:	Purification Factor
PI:	Plant introduction
PK:	Protein kinases
PR:	Pathogenesis Related
R:	Recessive
R-gene:	*Resistance*-gene
RLK:	Receptor-like kinases
ROI:	Reactive oxygen intermediates
ROS:	Reactive oxygen species
rpm:	Rotations per minute
RWA:	Russian wheat aphid
SA:	Salicylic acid
SA1:	South African1 RWA biotype
SAM:	South African Mutant Biotype
SAR:	Systemic acquired resistance
SD:	Single dominance
SD:	Standard deviation
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser/Thr:	Serine/Threonine
SSH:	Suppression subtraction hybidization
TEMED:	N,'N,'N,'N,'-Tetramethylethylenediamine
TIR:	Toll-Interleukin-like repeat
Tris:	Tris (hydroxymethy) – aminomethane

TYA:	Thousand years ago
V:	Volt
v/v:	Volume per volume
w/v:	Weight per volume

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CHAPTER 1

INTRODUCTION

Wheat *(Triticum aestivum)* is a very important crop. Present and future requirements dictate that its production must exceed all other grains', making it the most cultivated crop in the world, with production in tons exceeding even that of rice (Food and Agriculture Organization (FAOSTAT), 2006). This wide cultivation practice is probably owing to the high versatility of this cereal: not only is it used in the production of dough (to make various breads, pastries and pastas), it is also used to make alcoholic beverages and fed to animals as fodder. A potential function of wheat might be the generation of biofuels in a world which is running out of natural energy resources. In addition to its being versatile, wheat is one of the few crops which can be cultivated successfully in temperate regions, such as Russia and Western and Northern Europe. The International Grains Council (IGC) forecasts that 607 million tons of wheat will be consumed during 2007. This is correlated to the prediction of a 3.5% increase of wheat cultivated land areas (IGC, 2007). Thus, in order for the required amount of wheat to be cultivated, factors which affect yield negatively should be limited.

The Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko) is such a factor. The RWA is a parthenogenic pest, which can wreak havoc on wheat and barley. This feeding causes chlorotic streaking and leaf rolling, and most importantly, reduced yield, in plants susceptible to attack (Walters et al., 1980). This preference for wheat causes great economic and social damage when

one considers the immense agronomic importance of this crop. It follows that the presence of the insect in most wheat producing countries has led to quite serious economic losses world wide. For example, losses of about $800 million were reported in the USA (Morrison & Peairs, 1998).

Different methods have been employed to control this insect in the field. Unfortunately, chemical control is rendered useless by the fact that the leaf rolling symptom provides the insects with protection (Burd & Burton, 1992) from any insecticides which might have been sprayed on the growing crops (Smith et al., 1992). Secondly, no biological controls have been identified, which might have an adverse affect on the insect's distribution on crops (Farid et al., 1998; Prinsloo et al., 1998). Thus, the most successful strategy to date has been the development and cultivation of crops displaying resistance to the infestation of the pest.

Seven resistant near-isogenic lines were developed and released in South Africa over the past 16 years (Tolmay et al., 2006). This resistance against the insects is mediated through three different mechanisms: antibiosis, antixenosis and tolerance (Smith et al., 1992). Flor (1955) first proposed that hosts recognize components of its pathogens, and that this recognition leads to the activation of defence responses against attack of this pathogen. Thus, in the case of wheat-RWA interaction, when one of the Resistance genes (*R*-genes) in the host plants is capable of recognizing a specific, complementary Avirulence (*Avr*) gene, it triggers a hypersensitive response (HR) in the plant (Botha et al., 2006). This first step in defence or recognition signaling is characterized by the release of reactive oxygen species (ROS) to produce peroxide, superoxide and nitric oxide, resulting in cell death in the region of infection to prevent further spread of the pathogen (Staskawicz et al., 1995). The HR in turn mediates the activation of systemic acquired response (SAR) (Ryals et al., 1996), which is associated with the activation of the Pathogen-Related (*PR*) genes, such as glucanases, chitinases and defensins (Bowles, 1990; Lawton & Lamb, 1987)).

Thus, as a result of their success in combating RWA attack, resistant cultivars are cultivated worldwide. Regrettably, this has forced the aphids to overcome this obstacle via the development of new biotypes. These are individuals of a species which might appear similar to the other members of the populations, but some aspect of their feeding behaviour infers virulence to host cultivars, which usually exhibit resistance to the insects (Basky, 2003; Puterka et al., 1993). These changes may be brought about through environmental changes, such as changes in climate or food source.

In addition to eight biotypes, which were described in the USA (Botha et al., 2006; Haley et al., 2004; Webster et al., 1987), the development of a new South African biotype was reported to occur in the Eastern Free State (Boshoff & Du Toit, 2006). Biotypes are described by their feeding behaviour and consequent effect on their hosts' responses (Smith et al., 1992). It was proposed or hypothesized that the different virulent factors should be produced in saliva to result in these observations in breakdown of resistance (Belefant-Miller et al., 1994).

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Wheat is one of the few crops, which can be cultivated in temperate regions; because of this, it is one of the most widely cultivated crops in the world. However, it is not infallible to biotic, such as pests, and abiotic stresses, for example drought. An example of a biotic stress is the Russian wheat aphid. This insect feeds on the wheat plants in a manner which damages the membranes of cells and chloroplasts of the wheat hosts (Fouché et al., 1984): this damage results in an overall reduction in the energy production of the plant as the normal functioning of cells are impaired (Botha et al., 2005). A consequence of this is a reduction in yield, or even death (Jones et al., 1989; Walters et al., 1980). The Russian wheat aphid has caused great economic losses world-wide: and since its discovery in South Africa, losses in yield of between 21 to 90% have been experienced (Basky, 2003), while in the USA, losses of about $800 million were due to the aphid's presence (Morrison & Peairs, 1998). However, in regions endemic to the aphid, the cultivars seem to have developed resistance mechanisms to withstand attack from the RWA. Thus, in understanding these mechanisms, the knowledge could be used in breeding resistant or tolerant crops to combat the damage inflicted on crops by this pest. Not only can this knowledge be applied to wheat, but to other crops as well, seeing that the association of wheat with the aphid (and *vice versa*) could provide a model for host-pest interactions.

2.2. Wheat

Wheat was the first crop to be domesticated. This occurred in the Fertile Crescent approximately 152 000 years ago (Figure 2.1). Owing to this, that region is often referred to as the "Cradle of Civilization", because domestication of crops changed the present lifestyle of those humans from hunter-gatherers to farmers.

Figure 2.1. The Fertile Crescent (http://www.accuracyingenesis.com/ararat.html).

By the contribution of the diploid *Aegilops speltoides* genome (BB) *circa* 3 million years ago (Figure 2.2), this initially cultivated wheat species, einkorn (*Triticum urartu)* (AA), later evolved into the tetraploid emmer wheat (AABB), which was then cultivated throughout the Middle East. These above-mentioned species aren't grown on a wide scale anymore: they have been replaced by the tetra- and hexaploid wheat species, *T. turgidum* (durum wheat) and *T. aestivum* (bread wheat) (Kellogg, 2001). These two species are cultivated wide-spread to meet the ever increasing demands of the world's population (Atwell, 2001).

3 MYA	*T. urartu*	+	*Ae. speltoides*	=	*T. turgidum*
	AA		BB		AABB
8 TYA	*T. turgidum*	+	*Ae. tauschii*	=	*T. aestivum*
	AABB		DD		AABBDD

Figure 2.2. Evolution of *Triticum aestivum* (**MYA:** million years ago; **TYA:** thousand years ago) (Kellogg, 2001)

The hexaploid form of wheat, bread wheat *(Triticum aestivum)*, is the result of a relatively recent evolutionary cross between a wild type (*Aegilops tauschii*) and durum wheat (*Triticum turgidum*). In *T. aestivum*, the quadroploid AABB portion was provided by *T. turgidum* and the diploid DD portion by *Ae. tauschii* [(Johnson & Bhave, 2004; Martín et al., 1999) Figure 2.2]. Each basic set represents seven chromosomes and the genome consists of six sets of these seven chromosomes (labelled numerically). The enhanced fitness associated with polyploidy was (and is) probably the driving force behind bread wheat being cultivated in most of the of the world's temperate regions (Martín et al., 1999). At present, it is the staple diet of 40% of the world's population and it represents 20% of the total calories consumed (Gill et al., 2004). Thus, it is of interest to find methods to prevent the loss of yield as a result of the effects of abiotic and biotic stresses.

2.3. Russian wheat aphid

The Russian wheat aphid *(Diuraphis noxia* Mordvilko*)* (Homoptera: Aphididae) is a pest, which causes the destruction of small grain crops (Smith et al., 1992).

Figure 2.3. Examples of some of the maturity stages of *Diuraphis noxia* (Mordvilko) (Homoptera: Aphididae). A nymph (A), an adult (B) and a winged adult (C). (Photograph courtesy of Leon van Eck).

2.3.1. Morphology and physiology

The RWA is a red-eyed greenish aphid, approximately 2 mm in length. It varies in colour from pale yellow to green to grey-green. Its body is spindle-shaped and covered in a powdery, mealy wax. It can be easily distinguished from other aphids by its characteristic very short antennae, condensed cornicles, absent siphunculi and a supracaudal process, the latter of which gives it a "forked-tail" appearance (Dürr, 1983; Robinson, 1992; Stoetzel, 1987; Walters et al., 1980).

2.3.2. Life cycles

It can occur in a winged (alatae) or unwinged (apterae) form (Figure 2.3). In general, the apterae dominate the population, whereas the alatae only occur when the present feeding site has become

unsuitable and the population needs to migrate to "greener pastures". The apterae reach maturity after two weeks and can give birth to a maximum of four live nymphs daily [(Dürr, 1983; Robinson, 1992; Walters et al., 1980) Figure 2.3]. The exclusive presence of apterae and alatae females represents the anholocyclic reproducing populations: they only reproduce parthenogenetically throughout the year (Puterka et al., 1993). Sexual morphs do occur, but do so rarely (Kiriaç *et al*., 1990). When they do, holocyclic reproduction transpires, where parthenogenesis occurs predominantly, except when a sexual generation emerges in the autumn. This is done to ensure the survival of the population as eggs in adverse conditions, such as harsh winters (Puterka et al., 1993). The male form does not occur in South Africa (Walters et al., 1980).

2.3.3. Host plants and volunteer wheat

The RWA prefers to feed on wheat, barley and the triticale; although, the damage is most severe on wheat and barley. This means that it can survive on "volunteer wheat", such as the *Bromus*, *Arena, Agrotricum, Hordeum* spp. and other wild grass species, in periods when its preferred hosts aren't being cultivated (Kindler & Springer, 1989; Ni et al., 1998; Prinsloo et al., 1997; Walters et al., 1980). Owing to the Conservation Reserve Program (CRP) programs in the USA, to promote the planting of these natural grasses in an attempt to preserve natural biodiversity of these grasses, it could in fact be causing the Russian wheat aphid presence to be ever-present and at a wider range, because it increases the availability of hosts on which the pest can occur successfully throughout the year (Kindler & Springer, 1989). The *Agropyron* spp. (wheat grass) is the most susceptible of the volunteer wheats (Jones et al., 1989).

Rainfall seems to affect the number of the aphid population negatively; they are probably washed off the leaves and fall onto the soil. Interestingly, the aphid number then increases dramatically once they have managed to climb back onto the plants. This is probably owing to the fact that the

plant's health status improves in the respite gained when the aphids are on the soil, and can sustain more aphids than previously (Kriel et al., 1986). In general, it seems to prefer a less humid habitat (Stáry et al., 2003).

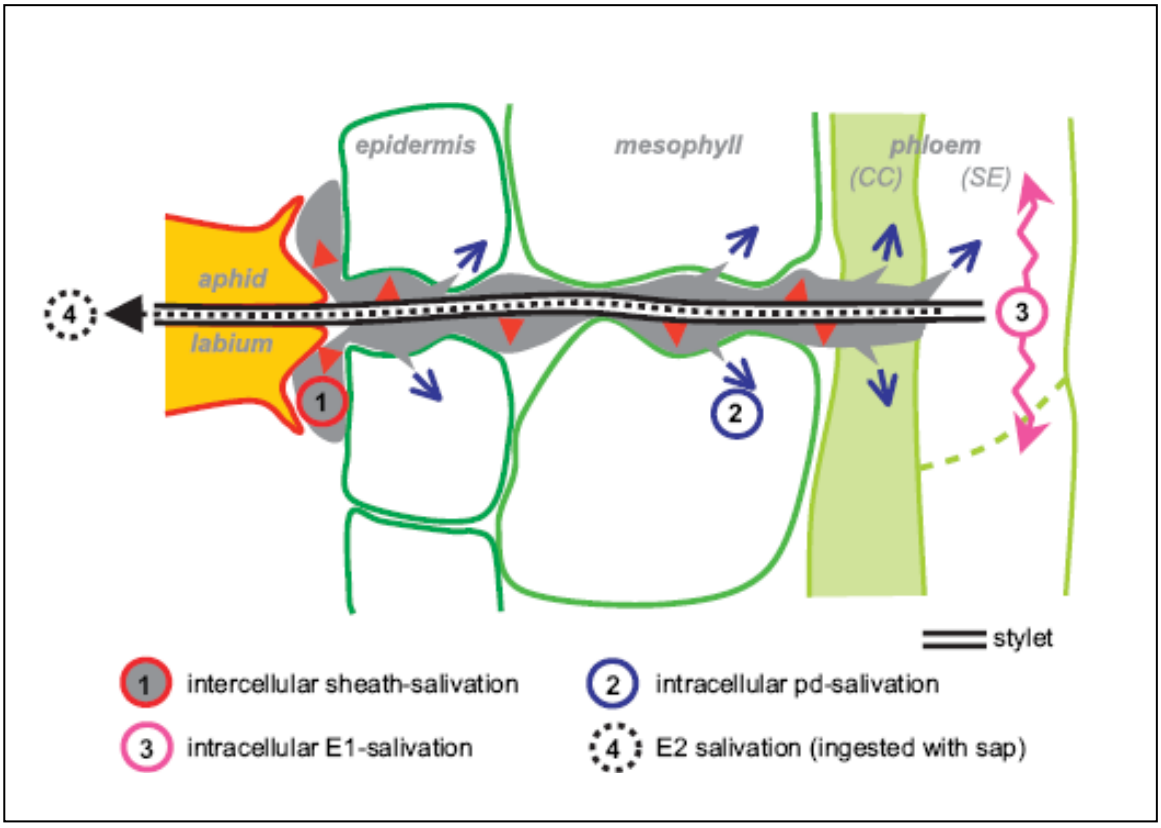

Figure 2.4. Feeding mechanism of the Russian wheat aphid. Numbers indicate the different periods of salivation as detected by Electrical Penetration Graph (EPG) readings (**CC:** companion cell; **SE:** sieve element) (Tjallingii, 2006)

2.3.4. Feeding mechanism and behaviour of RWA and resulting effects on host plants

2.3.4.1. Feeding mechanism

They are usually found on the first three cm of the basal regions of leaves 1 and 2, and only move further up the leaf in extreme cases of overcrowding (Kriel et al., 1986). When feeding on its preferred host, the aphid will remain feeding on the individual plant until an increased aphid

population depletes its host of all its available nutrients. When this occurs, the aphids move to a new plant (Knudsen & Schotzko, 1999). The aphid uses pierce and suck mechanism to feed on the phloem of its host plants: according to Pollard (1973), it repeatedly uses its stylet to stab between the mesophyll cells in the leaf tissue, to discover the location of the phloem in the vascular bundle, from where it obtains its nutrients (Figure 2.4). While stabbing into the tissue, it produces a sheath to protect the probing stylet.

After probing the leaf at various locations with its stylet, it will settle at the most favourable site and continue feeding from the phloem. It does so by injecting its watery saliva into the plant and sucking this saliva-phloem sap mixture into its gut, where the photoassimilates, generated during the host plant's photosynthetic processes, are digested (Tjallingii, 2006). It is proposed that this saliva might contain a phytotoxin, which is the instigator of the susceptible plants' destruction (Belefant-Miller et al., 1994; Heng-Moss et al., 2003; Wang et al., 2004).

McLean and Kinsey (1964, 1967) developed an electronic system to scrutinize insect behaviour. They later found that the waveforms produced during salivation and ingestion-related processes differed from each other. When Girma et al. (1992) applied this method to study the RWA's feeding behaviour, 3 waveforms were observed instead; it seemed to represent salivation, a multiple X-wave section (comprising of multiple Volt peaks) and ingestion. Further investigation led to the resolution of these waveforms into 6 steps of RWA feeding; namely non-probing, penetration, salivation, X-wave, phloem-ingestion and non-phloem ingestion.

As can be expected, aphids feeding on sorghum, which is less infallible to RWA feeding, took longer to probe and salivate and in contrast, spent a much shorter time ingesting nutrients not within the phloem. Using the same technology, Ni and Quisenberry (1997) found that the RWA make

more probes diurnally than nocturnally, although the time spent probing was shorter in the day in comparison to that in the night. Strangely enough, when monitoring feeding patterns on PI 137739, a resistant cultivar, the RWA made more nocturnal probes than in the day and spent more time probing and less time feeding.

2.3.4.2. Aphid saliva

According to Miles (1972) saliva in aphids have the following functions: 1) it moistens food, 2) it probably contains hydrolytic compounds which are required for pre-digestion of compounds in the food source, 3) it facilitates the "mechanical penetration" of cells during feeding, 4) it contains solidifying components that produce the sheath that protects the stylet and prolongs feeding time, and 5) assists in dissolution of plant material during probing events. The aphids contain two types of salivary glands. The primary gland, which probably produces the sheath saliva that protects the stylet during probing and feeding, and then the accessory glands, that produce the watery saliva used in the detection of the phloem and the ingestion of its contents. The products of the two gland types are able to mix with each other without the formation of a solid "sheath-like" compound; thus, whenever the glands are removed, both types of saliva are removed in combination (Miles 1967). The sheath also seals the "damaged" cells (Miles, 1999).

The primary gland is much more differentiated than the accessory gland. Tjallingii (2006) proposed that there are two types of salivation, E1 and E2. E1 salivation seems to occur when the insects are probing into the phloem's sieve elements and E2 salivation once the phloem has been detected and the aphid has settled down to feed. This saliva probably contains amino acids, phenolics, oligosaccharides, amylases, cellulases, esterase or lipases, phosphorylase, acid phosphatase, phenolase or peroxidase, pectinases and other proteolytic enzymes (Fouché et al., 1984; Miles, 1972; Ni & Quisenberry, 2003; Robinson, 1992) and compounds involved in normal feeding. The

elicitor is probably a necessary component of normal aphid function. Chen et al. (2004) found that about half of the mRNAs expressed in salivary glands of Hessian fly contained putative secretion signals at their N-terminals. Most variation occurred in these N terminal regions, making one assume that they are ideal candidates for elicitors. In addition to this belief, these genes clustered close to other known *Avrs* on chromosome 2A of the insect. They are predicted to be small proteins (8.5 to 10 kDa) and have pI values between 9.92 and 10.90.

2.3.4.3. Symptoms

Symptoms of feeding (which can be even induced by the presence of a lone aphid) are longitudinal chlorotic streaking and leaf rolling in susceptible and necrotic spots in the resistant cultivars (Walters et al., 1980). In cold weather, this streaking is reddish-purple owing to an anthocyanin pigment; otherwise, it is white or yellow (Jones et al., 1989; Walters et al., 1980). In extreme cases, feeding on the flag leaf causes the bending of ears, which turn white, and results in a decrease in yield (Jones et al., 1989; Walters et al., 1980).

These are probably a result of the interfering effect on photosynthesis following the destruction of the chloroplasts. Loss of chloroplast arrangement and degradation of their membranes were observed in leaf pieces four hours after treatment with extracts from whole RWAs. This disruption of the thylakoid membranes results in the release of chlorophyll into the cells (Fouché et al., 1984). It seems that resistant cultivars manage to overcome this detrimental effect by the collapse of affected cells in the region of feeding, thus preventing the release of cell content and subsequent destruction of surrounding tissue (Belefant-Miller et al., 1994). These collapsed cells usually occur around the many-branched sheaths, which are observed when aphids feed on resistant cultivars (Belefant-Miller et al., 1994). Chlorophyll content decreases in all plants until four days after infestation. Then the levels stabilize in the resistant plants while it decreases in susceptible plants to

such low concentrations, that the plants die (Heng-Moss et al., 2003; Van der Westhuizen & Pretorius, 1995; Wang et al., 2004). This is probably caused by the interruption of normal photosystem II electron transport (Burd & Elliott, 1996). This is confirmed by the fact that RWA infestation causes less damage to susceptible plants in the absence of light (Macedo et al., 2003): thus disruption of normal chloroplast activities and the aftereffect thereof are probably the basis for plant death. It is now believed that the preservation of the chloroplasts, which ensures their normal function and energy production in cells, is the method employed by resistant wheat plants to survive RWA virulence (Botha et al., 2005).

2.3.5. Distribution and habitat

2.3.5.1. Distribution

The RWA was first labelled as a serious wheat pest in South Africa in 1978. At the beginning of 1979 it occurred only in the Eastern Free State; however, by September of the same year, it was found in most parts of the Western Free State, Lesotho and isolated spots in Transvaal and Natal (Walters et al., 1980). Since its discovery in 1978, the aphid is now found in all areas of the world where wheat is cultivated, except Australia. It has been proposed that the aphid is endemic to the former USSR, the Balkans, Iran, Turkey and regions in the Middle East (Anderson et al., 2003). The first resistant lines of barley and wheat were collected from these endemic regions; they probably exist because of sympatric evolution with the aphid, where selective pressure resulted in the emergence of these lines (Webster et al., 1991).

2.3.5.2. Biotypes

A biotype is defined as “[a population] within an insect species that [has] the ability to damage plant entries normally resistant to that insect” (Smith et al, 1992). As can be expected, selective pressure has forced the emergence of different RWA biotypes, which can overcome the resistance

presented by the resistant cultivars. These biotypes are similar in morphology, but differ in the severity of their attack. Moreover, crops, which are resistant to one biotype, might be susceptible to another and *vice versa*. For example, the Hungarian biotype can attack cultivars, which are resistant to the South African biotype (Basky, 2003; Puterka *et al*., 1993). The different biotypes are characterized by the plant's response to their feeding. The biotype classification was initiated by Puterka et al. (1992), who used a series of wheat, barley and rye differentials to characterize a world collection of RWA populations. Biotype 2 was first identified by its virulence to the *Dn4* based resistant winter wheat cultivars (Haley et al., 2004). Now it displays resistance to *Dny* as well (Jyoti et al., 2006). It was proposed that seven biotypes occurred world wide by the early nineties (Puterka et al., 1992). North American biotype screening to date has been based on virulence to wheat lines containing the *Dn4* or *Dn7*, which is adequate for identifying biotypes other than 1 and 2. Based on these differentials, at least six potentially new biotypes have been identified from Colorado and surrounding states (Botha et al., 2006). A biotype from Chile is resistant to *Dn4*, while one from the Czech republic is to *Dn4* and *Dnx* and another from Ethiopia is to *Dn4* and *Dnx* (Smith et al., 2004).

Phylogenetic studies indicate that the most diversity occurs in the Eastern and Northeastern regions of the Mediterranean. This is to be expected, seeing as the RWA is thought to be endemic to this area. These populations follow a holocyclic life cycle. The existence of biotypes is probably the result of a fairly recent step in RWA evolution, considering that many biotypes cluster in the same allomorph group. It would seem as though the RWA populations in France, USA, Mexico and South Africa were all originally from Turkey. This suggests that they were spread to their current location by commerce (Puterka et al., 1993).

2.3.6. RWA in the South African context

The RWA is the most aggressive of the aphids infecting wheat grown in South Africa – it can reduce the yield by up to 90% (Ahern & Brewer, 2002). Owing to the fact that the RWA can withstand temperatures as low as 0.5°C, it could not be combated using naturally occurring bio-enemies (Hatting et al., 1999). The region in the Free State, where wheat is cultivated, has a dry, temperate climate, which suits the aphid perfectly because it originated in arid regions (Basky, 2003). Unfortunately, this renders the use of insecticides inefficient. This is further enhanced by the fact that aphid feeding causes the leaves to curl up and conceal the aphids from insecticides (Basky, 2003; Ni & Quisenberry, 2003). Thus, it would make sense to rather breed resistant wheat cultivars, which could withstand aphid attack (Basky, 2003).

2.3.7. Control

2.3.7.1. Cultural practices

In regions where the norm is to plant crops more densely, it would seem as though aphid attack is less severe (Walters et al., 1980). However, this might be an effect of an overabundance of plant material, masking the effect that an aphid population could have on a more sparse harvest; considering that the amount of nymphs born is a limiting factor in population expansion. Also, the cultivars planted in these regions might be more resistant than those grown elsewhere.

The greatest economic loss is associated with infestation of the wheat during the flag leaf and second leaf stage (Kriel et al., 1986). If the aphid could be controlled to prevent damage at this stage of the plant's development, it could prevent the large-scale loss considerably.

The aphids do not seem to cause as much damage to rye, oats or maize. If all other measures fail, it might be suggested to change the type of crop cultivated to one, which might stand a better chance of survival to RWA attack.

The volunteer wheats create an environment where the aphids can survive over winter or summer, depending on the crop of choice. Perhaps, by replacing these in the surrounding fields, where wheat or barley is cultivated, with a less preferred Triticale, such as oats or rye, could prevent the emergence of the aphid in full force the next planting season (Walters et al., 1980).

2.3.7.2. Biological control

Diaeretiella rapae was evaluated as a potential biological control agent of RWA and appeared to be able to survive well in the presence of the aphids (Farid et al., 1998). *Aphelinus varipes* was an unsuitable organism for biological control, because it is only active after the middle of December, and by that time the aphid infestation would have raged unchecked and its damage would be in full swing (Prinsloo, 1998).

2.3.7.3. Chemical Control

Owing to the destructive nature of this pest's feeding behaviour on susceptible crops and the ineptness of using insecticides (owing to the protection offered to the insects by the rolling of leaves) and biological control measures to stem the spread of this pest, it has become generally accepted that the best measure of control is via the breeding and cultivation of tolerant or resistant wheat cultivars. In order to do this most successfully, the best strategy would be to pyramid the different resistance genes, because this will hopefully prevent the emergence of resistant aphid strains as a result of selective pressure.

2.3.8. Genes associated with Russian wheat aphid resistance

2.3.8.1. Resistance mechanisms associated with RWA in wheat

It is accepted that three mechanisms of resistance occur as proposed by Smith et al. (1992), which are antibiosis, antixenosis and tolerance. Antibiosis is resolved by the effects feeding has on aphid fecundity, nymphipositional period, daily amount of nymphs produced, number of days that aphids are produced and adult longevity. Antixenosis depends on the number of nymphs that can survive on a plant and the effect their feeding has on the height of the plant, its wet weight (WW), dry weight (DW) and leaf number. Tolerance is indicated as a relative "degree in reduction" of the plant's height, WW and DW in relation to an uninifested plant. Tolerance is often associated with an increase in plant growth, despite the presence of feeding aphids (Castro et al., 2001). It is believed that these mechanisms are a result of a "gene-for-gene" interaction (Flor, 1955) between components in the host plant and those in the invading agent (Botha et al., 2006).

It would appear as though the resistance mechanisms against the greenbug (*Schitzaphpis graminum*) and the RWA are independent from each other and probably regulated by different genes. These different relevant genes then regulate antibiosis, antixenosis and tolerance in an independent manner. This is advantageous, because it supplies us with many sources to combine and to pyramid, for the prevention of the emergence of resistant RWA biotypes.

2.3.8.2. Tracking down the genes and their locations

In 1989, Du Toit announced that the resistance in line PI 137739, was caused by a single dominant gene, called *Dn1*. Examples of cultivars proposed to contain this gene are 'TugelaDN', 'MolopoDN', 'PalmietDN' and 'BettaDN'. Since then, eleven putative genes for resistance have been identified (Table 2.1), annotated *Dn1* to *Dny* (Botha et al., 2006). *Dn1, Dn2*, *Dn5* and *Dnx* are all proposed to be located on chromosome 7D (Liu et al., 2001) Thus, the resistance is probably

conferred by the contribution of *Ae. tauschii's* genome to *T. aestivum*. Smith et al. (1992) proposed that the resistance conferred by *Dn1*, *Dn2* and PI 294994 was owing to low levels of antibiosis and not antixenosis. Du Toit (1987) suggested that *Dn1* conferred antibiotic resistance. Formusoh et al. (1994) also confirmed this. Porter and Webster (2000) propose that the *Dn1* gene also confers resistance in the accession line PI1140207, despite having a phenotype, which differs from PI 137739.

Table 2.1. Summary of the genes associated with RWA resistance, their accession lines in which they occur and the mode of inheritance.

Gene	Wheat Accession *(T. aestivum)*	Mode of inheritance	Reference
Dn1	PI 137739	SD	Du Toit, 1989
Dn2	PI 262660	SD	Du Toit, 1989
dn3	SQ24 *(Ae. tauschii)*	R	Nkongolo, 1991a; Nkongolo et al., 1991b
Dn4	PI 372129	SD	Nkongolo, 1991a
Dn5	PI 292994	SD	Marais & Du Toit, 1993
Dn6	PI 243781	SD	Dong & Quick, 1995
Dn7	1B/1R Translocation from Rye	SD	Marais et al., 1994
Dn8	PI 294994	SD	Liu et al., 2001
Dn9	PI 294994	SD	Liu et al., 2001
Dnx	PI 220127	SD	Liu et al., 2001

SD: single dominant; **CD**: co-dominant; **DI**: dominant independent; **R**: recessive

2.3.8.3. Previous studies on infestation-induced protein expression in wheat

Various studies have indicated that within 48 hours, the protein profile of cereal plants begin to change when they are infested with the RWA. When comparing the two Near-Isogenic Lines (NILS), 'Tugela' (susceptible) and 'TugelaDN', it was seen that RWA infestation resulted in an

increase in the number of proteins in the resistant cultivar and the reverse in the susceptible; i.e. a decrease in protein numbers. Although the aphids prefer to feed at the bases of the leaves, the profiles of all the different parts of the resistant leaf are similar, indicating a global expression of "protective components". A 56 kDa organel encoded protein was absent in the susceptible plants, whereas a 100 kDa nuclear encoded protein was highly induced in the resistant plants (Van der Westhuizen & Botha 1993).

It was found that the expression of proteins of four molecular weight ranges (28-33, 22-24, 18.5-19.5 and 15.5-17.0 kDa) was induced in the apoplasm of resistant lines of wheat (Van der Westhuizen & Pretorius, 1996). A corresponding group, 22-24 kDa, was induced in a resistant line of barley from Iran (PI 366450) after RWA infestation when compared to the susceptible cultivar 'Morex' (Webster et al., 1991). A similar study comparing this same 'Morex' to a resistant accession line from Afghanistan (PI 366450), resolved that a 23 kDa protein complex was lost in the susceptible line, while it had a pI shift in the resistant. This was also accompanied by a considerable loss of chlorophyll in 'Morex' after infestation (Miller et al., 1994). Likewise, a 24 kDa protein complex was inhibited as a result of RWA infestation in susceptible wheat cultivar 'Pavon' in comparison to resistant PI 140207 (Porter & Webster; 2000). Serological studies imply that these proteins are related to the Pathogen-Related proteins tobacco (PR-2) and barley (32 kDa) and tobacco (PR-Q and PR-5) chitinases (Van der Westhuizen & Pretorius 1996).

Immunogold labelling indicated that in resistant plants high concentrations of β-1,3-glucanase accumulate mostly in the cells in the vascular bundles in response to RWA infestation; especially in these cells' walls and chloroplasts (Van der Westhuizen et al., 2002). This could explain the Belefant-Miller et al. (1994) study, in which it was proposed that the resistant plants manage to survive by the collapse of the cells closest to aphid probing and feeding and also by the

maintenance of higher chlorophyll levels (Belefant-Miller et al. 1994). Also, seven isoforms of β-1,3-glucanases have been observed in the apoplast (Van der Westhuizen et al., 1998a). Perhaps, in conjunction, the endo- and exocellular forms manage to attack the pathogen before it has entered the cell and then protect the membranes in the cells if it has, thus preventing the alteration of optimal cellular conditions, which might result from leakage of organel content into the cell.

Three forms of chitinase isoforms are present in wheat and are expressed in response to different triggers. The first group is associated with RWA feeding and had a pI value of 5.1, while the second group is expressed after ethylene induction: two bands with pI values of 4.1 and 6.8 were observed. Lastly, mechanical wounding also triggered the presence of a single band with a pI of 4.1 (Botha et al., 1998). Thus, one can see that the resistance response associated with RWA infestation is unique to the pest and not a result of the probing or general Systemic Acquired Resistance (SAR).

It has also been observed that Salicylic acid (SA) is upregulated to a greater extent in resistant plants about 48 hours post infestation (pi). Similarly, peroxidase expression is also increased within 48 hours pi and accumulates in the apoplast (Mohase & Van der Westhuizen, 2002; Van der Westhuizen et al., 1998b). It is proposed that salicylic acid is involved in the signalling, which results in the activation of the SAR. Upregulation of peroxidases results in the thickening of cell walls and the release of reactive oxygen species (ROS).

In contrast, catalase activity is inhibited as SA content increases: intercellular administration of SA has the same effect. Normally, catalase is an anti-oxidant, which converts hydrogen peroxide into water and oxygen. Hydrogen peroxide is an example of a ROS and its concentration will increase when catalase is inhibited (Mohase & Van der Westhuizen, 2002). The accumulation of ROS is an indication that the hypersensitive response (HR) has been activated.

Seeing as the saliva of the RWA contains a phytotoxin, it can be expected that its presence will elicit a "detoxification" response. It has been observed that the expression of esterase and superoxide dismutase is increased in resistant and susceptible wheat, barley and oats plants after RWA infestation (Ni & Quisenberry, 2003). This could be expected seeing as superoxide dismutase is involved in the process of rendering superoxide radicals harmless. However, this was not observed after infestation with *Rhopalosiphum padi* (Hemiptera: Aphididae), another cereal pest, which does not induce the chlorosis observed after RWA feeding (Ni & Quisenberry, 2003). This once again confirms a unique resistance mechanism associated with the RWA and cereals.

2.4. Resistance Mechanisms in Plants

2.4.1. Introduction

Plants have evolved in a manner to deter organisms from exploiting them of nutrients. Firstly, they had to develop means in which it could detect unwanted invasions and deal with the problem at hand. Secondly, it had to have mechanisms in place to deter or even kill potential pathogens and pests from settling or feeding (McDowell & Dangl, 2000).

After an attack has been detected, it results in the localized programmed cell death of infected cells, cell wall fortification in the region of infection and the release of anti-microbial compounds (Hammond-Kossack & Jones, 1996). This first step is known as the Hypersensitive response (HR) and is characterised by the occurrence of brown, dead tissue (Stakman, 1915). It is further characterized by the deposition of callose on the cytosolic face of cell walls; cellular ion influxes leading to an influx of calcium into the cytosol and an oxidative burst of Reactive oxygen intermediates (ROI), such as nitric oxide, superoxide and peroxide; the accumulation of phenolics and other antimicrobial compounds; and the production of autofluorescent compounds (Dangl & Jones, 2001; Harris et al., 2003).

Secondly, a broad-based Systemic acquired resistance (SAR) occurs, which affords an extended period of resistance against a wider range of pathogens (Ryals et al., 1996). Of interest, is that the biotrophic pathogens, which obtain nutrients from live tissue, activate defense pathways dependant on SA and necrotrophs, which attain nutrients from dead tissue, have the corresponding response on JA and ET (Thomma et al., 2001). This mediates the differential expression of a range of SAR proteins, such as the *Pathogenesis related* (*PR*) proteins, of which chitinase or β-1,3-glucanase are examples (Ward et al., 1991). The temporal and spatial expression of the *PR*-genes differs in plant tissues of a single plant. For example, when wheat plants were treated with SA, JA or *Fusarium culmorum*, *PR4* was instantly induced in coleoptiles, whereas it only appeared in the roots 30 days after infection. *PR1* and *PR5* were expressed constantly in control and infected plants. Wounding produced changes of all the *PR*-genes in only the mature leaves (Bertini et al., 2003). Once again, this suggests that defence related activities are strictly regulated in plants.

2.4.1.1. Components of *R*-gene mediated resistance

2.4.1.1.1. Classes of *R*-genes

It is generally believed that resistance is mediated by a direct interaction between an Avirulence factor and its corresponding *R*-gene product, for example *Pi-ta* in rice and *Avr-Pi-ta*, from *Magnaporthe grisea*, which causes rice blast disease (Bryan et al., 2000). In general, four protein domains are associated with the *R*-genes: Nucleotide binding sites (NBS), Receptor-like kinases (RLK), Protein kinases (PK) and Leucine Rich Repeats (LRR). The most abundant class of *R*-genes is the group of NBS-LRR proteins, followed by the Ser/Thr protein kinases and then the exoplasmic LRRs (eLRR), which are transmembrane proteins with either, an internal C terminal or a kinase domain [(Dangl & Jones, 2001; Hammond-Kosack & Parker, 2003) Figure 2.5]. It was proposed that *Avr-Pi-ta* is a protease which could potentially activate the *Pi-ta* gene product by cleaving it.

(Orbach et al., 2000). However, it would appear as though this simple type of binding is in fact the exception (see later: Guard hypothesis in Section 2.4.2.1.2).

2.4.1.1.1.1. Nucleotide binding site-Leucine rich repeats (NBS-LRR)

The NBS-LRR genes consist of an N terminal NBS domain and a C terminal LRR domain. In addition, most contain either a coiled-coil (CC) or, a Toll-Interleukin-like repeat (TIR) domain in addition to the NBS. The monocots only contain CC-NBS-LRR, whereas the dicots contain both, indicating that the TIR domain is more ancient than the CC domain. It also follows that NBS regions were associated with TIRs before the divergence of angio- and gymnosperms. Thus, NBS-LRR mediated defense must be an old mechanism of plant resistance (Meyers et al., 1999). The CC-NBS-LRR probably consist of many subfamilies which differ in respect to their CC regions (Dangl & Jones, 2001). There are approximately 600 NBS-LRR in rice (Goff et al., 2002) and 149 *R*-genes in *Arabidopsis*, of which the latter is distributed into 60% TIR-NBS-LRR and 30% CC-NBS-LRR. They found many duplication and deletion events which could explain the clustered arrangements of this class of *R*-genes in the genome (Meyers et al., 2003).

The most famous NBS-LRR is *Pi-ta* (as mentioned above). Another is *RRS1* from *Arabidopsis*, which confers *Ralstonia solanacearum* resistance (See Figure 2.5 E). It contains a WRKY motif at its C terminal. This motif also occurs in Zinc-finger transcription factors. Thus, it probably functions by binding to *cis*-acting components of resistance, because binding of RRS1 to PopP2 results in this complex being localized to the nuclear membrane (Deslandes et al., 2003). *Mi-1.2* in tomato renders resistance against potato aphids *(Macrosiphum euphorbiae)* and root-knot nematodes *(Meloidegogyne)*. Both these pathogens feed intracellularly, on the leaves and roots, respectively, thus perhaps the resistance mechanism to deter this feeding is similar, even though the

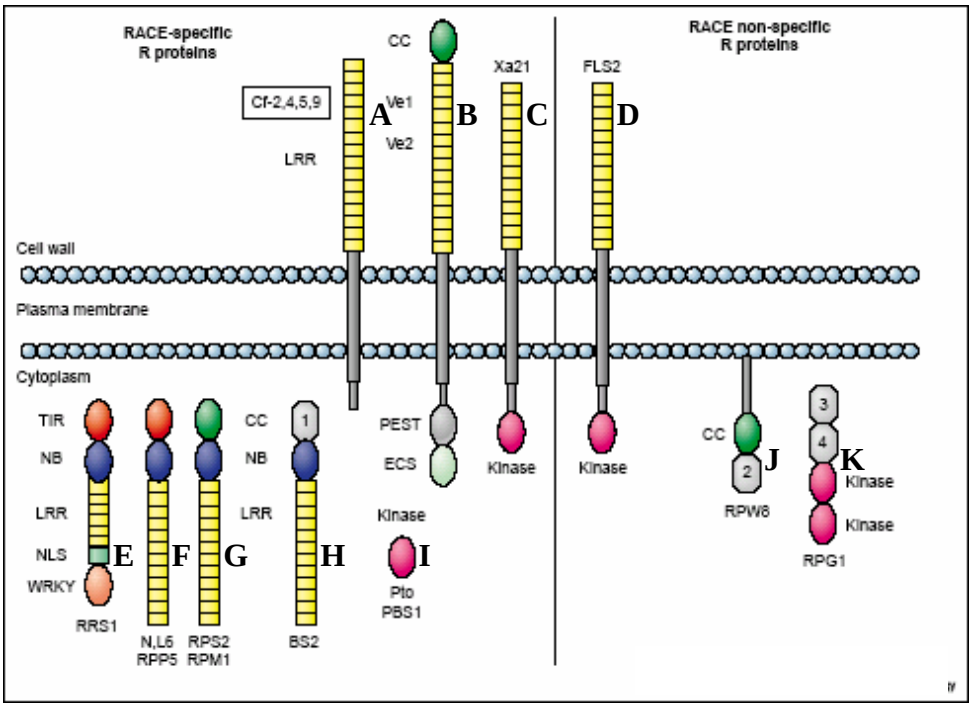

Figure 2.5. Schematic representation of the different classes of *R*-genes in plants. (**Ve1:** *Verticillium alboatrum* 1 resistance in tomato (lacks the PEST domain) (B); **Ve2:** *V. alboatrum* 2 resistance in tomato (lacks the CC domain) (B); **1,2,3 and 4:** proteins lacking in homology to any known sequences; **BS2:** bacterial speck resistance 2 (H); **Cl-2,3,5,9:** *Cladosporium fulvum* resistance to the races 2, 3, 5 and 9 (A); **CC:** Coiled-Coil; **ECS:** endocytosis signal; **L6:** flax rust resistance 6; **LRR:** Leucine-rich repeat; **NB:** Nucleotide Binding site; **NLS:** nuclear localization sequence; **PEST:** Pro-Glu-Ser-Thr-like sequence; **PBS1:** resistance to *Pseuomonas* bacterial speck expressing avrPphB (I); **Pto:** *P. syringae* pv. Tomato resistance (I); **RPG1:** resistance to *Puccinia graminis* f. sp. *tritici* (K); **RPM1:** resistance to *P. syringae* pv. *maculicola* expressing avrRPM1 or AvrB (G); **RPP5:** resistance to *Peronospora parasitica* (F); **RPW8:** resistance to powdery mildew (J); **RRS1:** resistance to *Ralstonia solanacearum* (E); **TIR:** Toll-Interleukin-like repeat **Xa21:** resistance to *Xanthomonas oryzae* p. v. *oryzae* (C) (From: Hammond-Kosack & Parker, 2003).

organisms bear very little resemblance to each other (Rossi et al., 1998). *Mi* is a CC-NBS-LRR *R*-gene, which probably occurs in the cytoplasm. The CC region of the N terminal region 1 is required for the successful recognition by the functional *Mi-1.2*, from which defence is then mediated through signaling in the LRR (Hwang et al., 2000; Hwang & Williamson, 2003). The potato *Rx* gene acts against the coat protein (CP) of Potato virus X (PVX) and it is a CC-NBS-LRR. When

different combinations of the gene are co-expressed, the gene is still functional (Moffett et al., 2002). Another example of this class is *Prf*, required for *Pto*-induced resistance against *Pseudomonas syringae* p.v. tomato (*Pst*), which causes bacterial speck in tomatoes. *Prf* consists of an NBS, LRR and a Leucine-zipper motif [(Salmeron et al., 1996) See later in 2.4.2.1.2)].

2.4.1.1.1.2. Nucleotide binding site-Leucine rich repeats (NBS-LRR) and Receptor-like kinases (RLK)/Protein kinases (PK) (LRR and RLK/PK)

Examples of this class are *XA21* (Figure 2.5 C) and *XA26* in rice, which confers resistance against *Xanthomonas oryzae* p.v. *oryzae (Xoo)*. They contain a LRR, a transmembrane region and a cytoplasmic C terminal Serine/Threonine protein kinase (Song et al., 1995; Sun et al., 2004). The two genes are expressed constitutively during the plant's entire lifespan (Sun et al, 2004).

2.4.1.1.1.3. LRR and a short cytoplasmic portion

Similarly to the above, the *Cf* genes in tomato, which confers resistance to *Cladosporium fulvum*, contain a transmembrane region and a LRR, but lack the cytoplasmic regions (Figure 2.5 A). Tomato encodes for four *R*-genes annotated *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*. Each of these mediate resistance by recognizing their corresponding *Avr* genes, *Avr2*, *Avr4*, *Avr5* and *Avr9*. The *Cf* genes share 90% homology and appear to have arisen by duplication events seeing as they are arranged as tandem repeats (Thomas et al., 1998).

2.4.1.1.1.4. Only PK

As stated previously in 2.4.2.1.1.1, *Pto* is required for tomato's resistance against *P. syringae* (Figure 2.5 I). It encodes a cytoplasmic Serine/Threonine protein kinase (Martin et al., 1993). This gene is physically linked to the NBS-LRR *Prf*, which is essential for successful resistance. Seeing as *Pto* has kinase activity, it could potentially act via phosporylation events in the cell's cytoplasm,

such as cleaving the NBS region of *Prf* (Salmeron et al., 1996). Similarly Barley *Rpg1* (Figure 2.5 K) encodes a cytoplasmic protein kinase, which is necessary for resistance against stem rust (*Puccinia graminis* f. sp. *tritici)* (Brueggeman et al., 2002). When barley was transformed with *Rpg1*, the F1 progeny segregated in a ratio of 3:1, indicating single dominant inheritance of the gene (Horvath et al, 2003). However, it contains two of these kinase domains located in tandem to each other. Perhaps its action is similar to that of *Pto*, because it is also located close to many putative NBS-LRRs (Ayliffe & Lagudah, 2004).

2.4.1.1.1.5. LRR and conserved domains

An example of this class, are the *Ve* genes in tomato (Figure 2.5 B). The *Ve* genes confer resistance to *Verticillium alboatrum*. *Ve1* contains a LRR and a cytoplasmic CC domain, whereas *Ve2* has a Pro-Glu-Ser-Thr (PEST) domain instead of the latter. The cytoplasmic domains contain sequences homologous to those in mammalian cells associated with receptors controlling endocytosis and cell degradation, for instance the erythropoietin cytokine receptor. The authors propose that the external domains might induce a direct signal via their internal domains when an *Avr* or other stimulus is recognized extracellularly (Kawchuk et al., 2001).

2.4.1.1.2. Guard hypothesis

As an alternative to the classic model that the direct interaction of the *Avr* and its specific *R*-gene mediates the defence response, the guard hypothesis was suggested in an attempt to explain the following interactions. For example, it is proposed that the *Pto-AvrPto* interaction might be required for virulence of the pathogen and that the *Prf* protein recognizes this interaction by "guarding the *Pto*" and alerts the cell of its invasion by pathogens (Figure 2.6). The *Avr* enters the cell when the bacteria *P. syringae* comes in contact with the cell's surface. The *Avr* interacts with *Pto*. This interaction is recognized by *Prf*, which is "guarding" *Pto* and leads to the release of oxygen

intermediates and calcium influxes. The *AvrPto* interaction also causes the phosphorylation of *Pti1* by *Pto* which is also a serine protease. *Pto* also binds to the transcription factors *Pti4*, *Pti5* and *Pti6*,

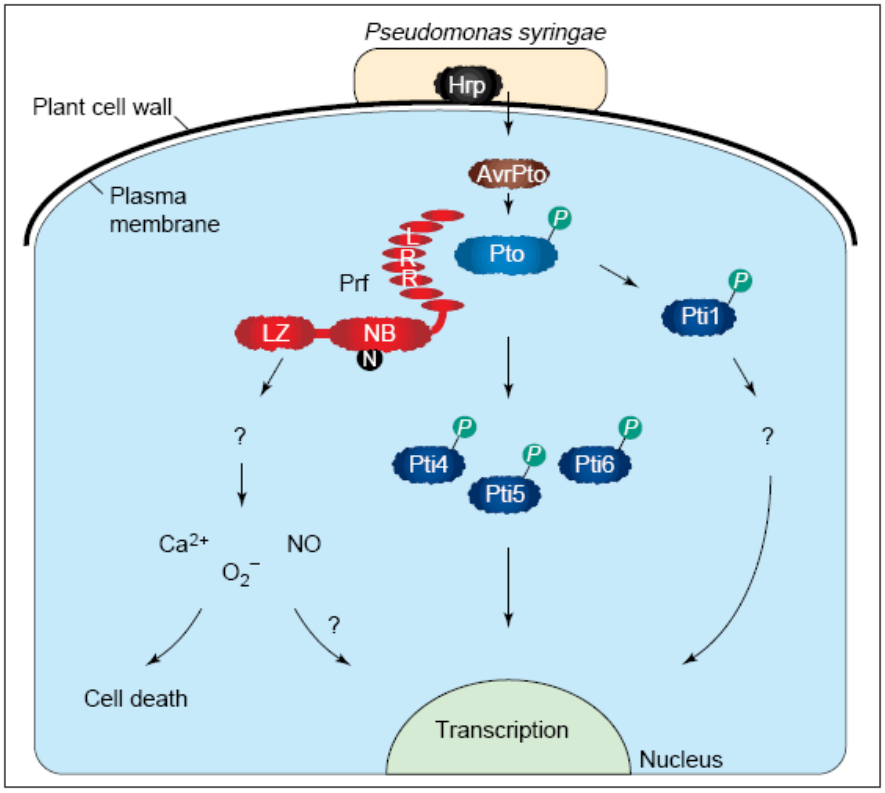

Figure 2.6. A schematic representation of the Guard Hypothesis as proposed by Van der Biezen (1998), using the interaction between Pto and Prf as an example (**Hrp:** contact-dependent bacterial secretion system; **LRR:** Leucine-rich repeat domain; **LZ:** leucine-zipper motif; **N, NTP, NB:** nucleotide-binding site; **NO:** nitic oxide; *P*: Phosphate). (From: Van der Biezen & Jones (1998).

which are associated with defence responses. This probably leads to a signal of attack being relayed through the cytoplasm to the nucleus and the infected cell dies to prevent the spread of infection (Dangl & Jones, 2001; Van der Biezen & Jones, 1998). Thus, in the absence of the required *R*-gene,

the *Avr* factors can interact and hinder the regulators of plant defense or support the action of suppressors of plant defense.

As with *Pto* and *Prf*, *RPM1* in *Arabidopsis* (Figure 2.5 G), which acts against *P. syringae,* also requires an additional gene, *RIN4*, for the activation of the HR. However, in uninfected cells, *RIN4* is expressed and regulates basal defence negatively. It seems as though the *Avr* gene product targets RIN4 protein with the aim to phosphorylyze it and thus enhance its negative effect on defence. However, when the Avr interacts with RIN4 while RPM1 is "guarding" it, the cell activates its Hypersensitive response (Mackey et al., 2002).

2.5. Conclusion

Thus, it can be seen that, as in most host-pathogen interactions, that resistant wheat plants and the Russian wheat aphid relate to each other in a very specific manner. Although the precise mechanism for detection of the insects' elicitors is unknown, it can be assumed that the wheat's *R*-gene or genes is a member of one of the classes described above. The RWA has means of overcoming resistance, which these *R*-genes grant to their plants, and this characteristic provides the possibility that the insects might be able to threaten all resistant cultivars in the future. Thus, by identifying the nature of the elicitors will reveal how the insects manage to attain this and facilitate the breeding of crops with the appropriate genes to combat this phenomenon.

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CHAPTER 3

ELUCIDIATION OF POSSIBLE VIRULENCE FACTORS PRESENT IN RUSSIAN WHEAT APHID *(DIURAPHIS NOXIA)* BIOTYPES' SALIVA

3.1. Abstract

The Russian wheat aphid (RWA) [*Diuraphis noxia* (Mordvilko) (Homoptera: Aphididae)] feeds with a pierce-and-suck mechanism: its saliva is mixed with the photoassimilates in the phloem of its host and sucked up. It is proposed that the saliva contains certain elicitors, which although necessary for normal feeding, also renders the susceptible plants unable to withstand "attack" from the insects, and results in these plants' damage or death. Resistant plants probably contain mechanisms to detect these elicitors and launch defence strategies. The objective was to elucidate which proteins in saliva elicit these symptoms and/or defence mechanisms. Guts were extracted from SAM and SA1 mutants: the former is a mutant biotype, which emerged after being fed exclusively on resistant wheat for five years. The guts were purified via size exclusion high performance liquid chromatography and injected into resistant and susceptible plants, 'Tugela' and 'TugelaDN'. SDS-PAGE analysis indicated that the biotypes differed in regards to each other's total protein complement with a single protein of differing size. Plants only displayed the normal phenotypic symptom of leaf rolling when injected with pure guts. However, peroxidase and chitinase activity of plants increased within seven hours when injected with fractions containing the putative elicitors. Proteins were differentially expressed in plants in response to these injections. It was found that the two putative elicitors identified in the HPLC fractions differ significantly from

each other in size. The SAM elicitor probably developed by means of a duplication event of its counterpart present in the SA1 biotype.

3.2. Introduction

The Russian wheat aphid (*Diuraphis noxia* Mordvilko) has been considered a pest of wheat (*Triticum aestivum*) in South Africa since its local discovery in 1978 (Walters et al. 1980). At present, it occurs worldwide (with the exception of Australia) in wheat producing countries and causes great economic losses in South Africa (Basky, 2003) and the USA (Botha et al., 2006). The RWA feeding has effects on the susceptible plants: these are leaf rolling, the development of chlorotic streaking, a reduction of normal growth, which leads to a decrease in yield and even death, in the case of extreme infestation (Walters et al., 1980).

It is now commonly believed that these symptoms are a result of interference with normal chloroplast functioning (Botha et al., 2005, 2006). It has been reported that RWA feeding leads to the destruction of cell and chloroplast membranes (Fouché et al., 1984). Also that it results in a decrease in chlorophyll content in plants (Botha et al., 2005; Fouché et al., 1984; Heng-Moss et al., 2003; Wang et al., 2004). This would lead to a loss of energy and also a disruption of the cells' standard homeostasis. Thus, it would follow that the resistant plants have some manner of maintaining normal chloroplast function and energy production, which enables them to function despite RWA infestation.

The first South African commercial cultivar displaying this resistance was 'TugelaDN'. This was created by introducing the *Dn1* gene from the accession line PI 137739 into the susceptible line 'Tugela' (Du Toit, 1989). It was heralded as being an ideal crop as fewer RWAs infested these plants in comparison to susceptible plants (Quisenberry & Schotzko, 1994). Unfortunately, the

existence and cultivation of this and other resistant cultivars has resulted in the global development of different RWA biotypes by means of selective pressure. These different biotypes might all appear similar in regards to their morphology, but some of them differ in their feeding behaviour and render previously reported resistant cultivars susceptible (Basky, 2003; Puterka et al., 1993). At present, two aphid biotypes have been reported as such in South Africa: the wild type biotypes annotated South African 1 (SA1) and South African 2 (SA2). A third biotype, namely South African Mutant (SAM), was developed under selective pressure in the laboratory and displays resistance and feeding preference to the resistant cultivar 'TugelaDN'. Another example is the emergence of six RWA biotypes in the USA (Botha et al., 2006). These were all identified on the alleged resistant cultivar, 'Gamtoos R', which contains the *Dn7* gene (Anderson et al., 2003).

It is believed that the insects inject a phytotoxin into their hosts' phloem as part of their pierce-and-suck feeding process, and that this compound is responsible for the symptoms observed in the plants (Belefant-Miller et al., 1994). Its recognition by the resistant wheat plant elicits a defence response against RWA pathogenesis. It is believed that this recognition mechanism is a result of a "gene-for-gene" interaction between components in the host plant and those in the invading agent (Botha et al., 2005, 2006). The first step in this response is the release of reactive oxygen species (ROS), such hydrogen peroxide, into the cells. Thus, the presence of ROS indicates that the hypersensitive response (HR) has been activated (Lamb & Dixon, 1997).

The cells either defend themselves via cell wall thickening by up to 12% (Van der Westhuizen et al., 1998a) or by experiencing programmed cell death. The latter is observed as necrotic lesions (Fouché et al., 1984). Subsequent to these activities, the systemic acquired resistance (SAR) commences through cascades mediated by salicylic acid and jasmonic acid, that lead to the expression of *Pathogeneisis related* (*PR*)-genes, such as chitinase (Van der Westhuizen et al.,

1998a), and genes necessary for chloroplast maintenance, such as ATP synthase (Botha et al., 2005).

Thus, understanding how this defence response is triggered provides the potential to screen wheat or other cereal populations for other potential genes with resistance against RWAs. Also, the very specific interaction between the RWA and wheat, make this an ideal model for studying insect-host interactions with the aim of applying the knowledge obtained thereof on other organisms' defence mechanisms. However, in the case of wheat, studies such as these are impeded by the fact that its genome is 16 000 Mb (megabases) (Gill et al., 2004). Even with more and more sequence data becoming available, examining the masses of DNA (and its subsequent large proteome), sets a time limiting factor on obtaining relevant information. However, the RWA genome is considerable less bulky. It has been reported that aphid genomes are probably between 200 and 800 Mb in size (www.genomesize.com; Gregory, 2002). This will be much easier to examine and the results obtained thereof can then be utilized to search for specific wheat genes. It follows that the first objective of this project was to shed light on the nature of the alleged elicitors present in the gut of the RWA. Secondly, it was to determine whether the gut composition, in its entirety or in fractions collected from high pressure liquid chromatography (HPLC), would elicit the same defence responses in plants as observed during normal RWA infestation.

3.3. Materials and Methods

3.3.1. Plant material

Wheat plants were grown in the greenhouse at a constant temperature of 20°C: two near-isogenic lines 'Tugela' (RWA susceptible) and 'TugelaDN' (Tugela/*5 SA 1684) (RWA resistant). SA 1684 contains the *Dn1* gene and was obtained from the accession line PI 168988 from Iran (Du Toit, 1987). This gene confers resistance to RWA in the form of antibiosis (Du Toit, 1987).

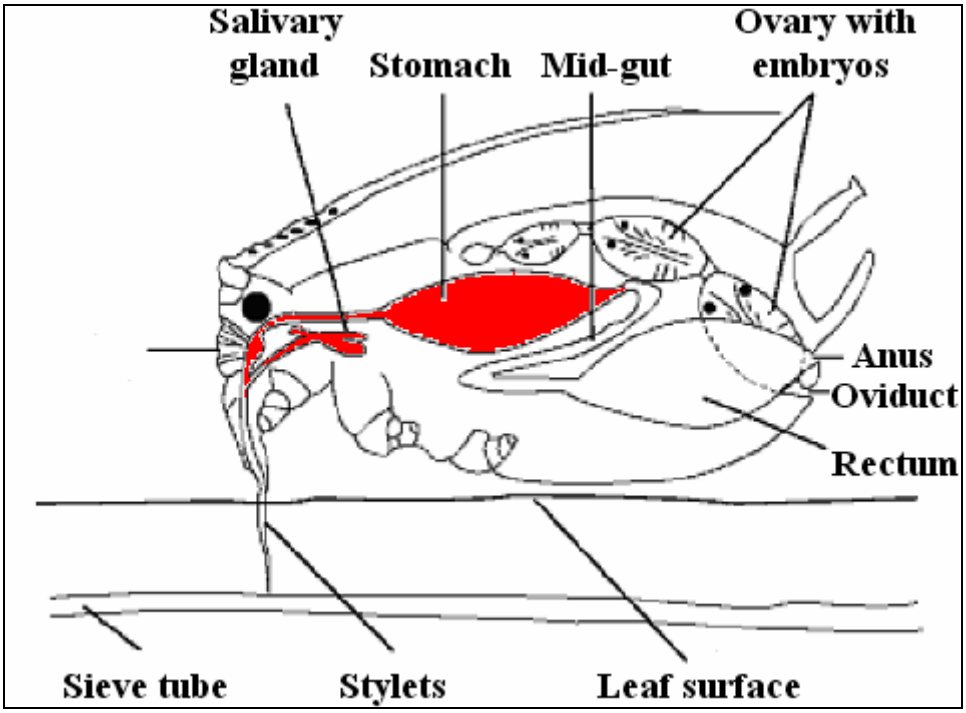

Figure 3.1. Schematic representation of RWA to indicate (in red) which portion of the insects were excised as gut (i.e., gut = stylet, salivary glands and stomach) (www.insected.arizona.edu).

3.3.2. Aphid biotypes

All experiments were performed on material obtained from two biotypes of the Russian wheat aphid (RWA) *Diuraphis noxia* Mordvilko, which are annotated as South African Biotype 1 (SA1) and South African Mutant (SAM). The latter was obtained using selective pressure over a period of five years. The wild type SA biotype was force fed on resistant 'TugelaDN' plants. These plants are known to contain the *Dn1* resistance gene (see above) (Du Toit, 1987). If one assumes that RWA reproduce once three-weekly (Walters et al., 1980), then it follows that the mutant biotype developed over approximately 87 generations under this selection pressure. The RWA population underwent two genetic bottlenecks; namely, shortly after transfer from the susceptible 'Tugela' to its resistant NIL 'TugelaDN'; and then again in 2004. At present, the two biotypes can be discerned on genetic level using AFLP profiling (Walters & Botha, unpublished results) and mitochondrial OC-1 gene sequence (Li et al., unpublished results). These aphid populations were maintained in

growth chambers at a constant temperature of 20°C on the wheat cultivars, 'Scheepers' (RWA susceptible) and 'TugelaDN', respectively.

3.3.3. Extraction of proteins

3.3.3.1. RWA proteins

3.3.3.1.1. Extraction of total RWA proteins

Proteins were extracted from the SAM and SA1 RWA biotypes. Whole aphids were ground in liquid nitrogen and extracted with 1 ml ice-cold extraction buffer (0.25 M Potassium phosphate buffer, pH 7.5). The extract was centrifuged at 8 000 rpm for 15 minutes at 4°C. The supernatant was collected and stored at -20°C.

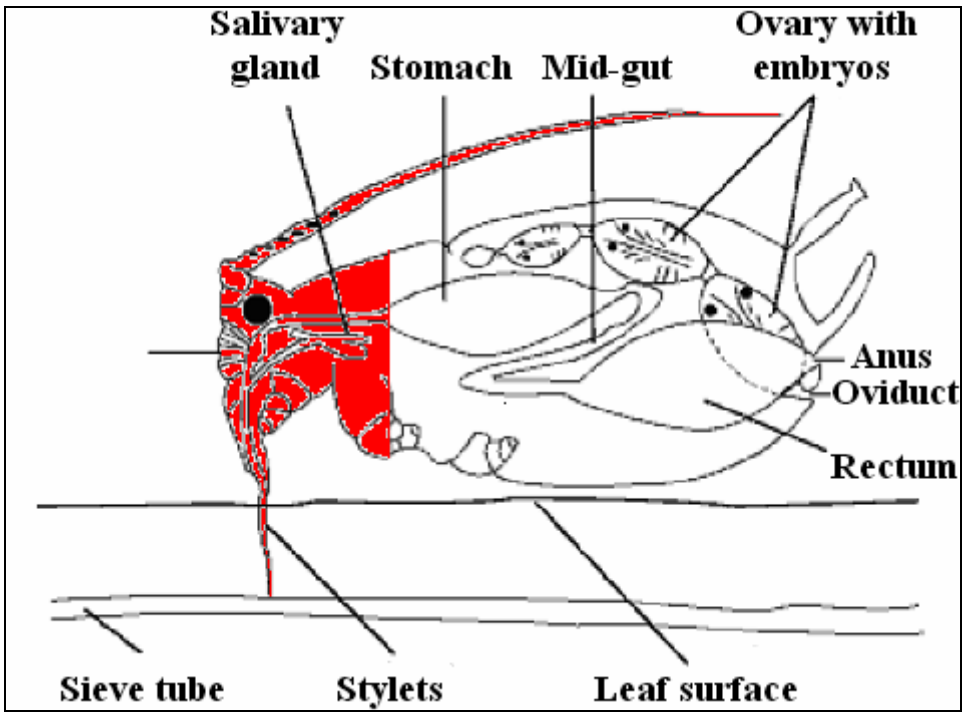

Figure 3.2. Schematic representation of RWA to indicate (in red) which portion of the insects was excised as heads (i.e., head = stylet and salivary glands) (www.insected.arizona.edu).

3.3.3.1.2. Extraction of RWA guts

Five hundred guts were extracted from each aphid biotype, resulting in a total of 1 000 glands. This was done under a stereomicroscope (Stemi SV 6, Zeiss, Germany) set at magnification of 1 250x. Aphids were pinned to the surface of a glass petri dish with a blunt dissection needle, before the insects were longitudinally slit open behind the head. The guts, silvery in appearance, were then scooped or pulled up and placed in ice-cold extraction buffer (0.25 M potassium phosphate buffer, pH 7.5). The extracted portion representing the gut portion is highlighted in Figure 3.1.

3.3.3.1.3. Extraction of RWA heads

Three hundred heads were extracted from each aphid biotype. This was done under a stereomicroscope (Stemi SV 6, Zeiss, Germany) set at magnification of 1 250x. Aphids were pinned to the surface of a glass petri dish with a blunt dissection needle and the heads were cut off just below the first pair of legs, using a sharp dissection blade and placed in ice-cold extraction buffer (0.25 M potassium phosphate buffer, pH 7.5). The extracted portions of the insects are highlighted in red in Figure 3.2.

3.3.3.1.4. Protein concentration of extracted RWA proteins

Protein concentrations were determined using a Bradford protein assay dye reagent (Bio-Rad, USA; Bradford, 1976). A curve using gamma-globulin as standard, is presented in Figure 3.3. The absorbance was determined at a wavelength of 595 nm using a Multiskan *Ascent V1.24* plate reader (Thermo Electron Corporation, USA).

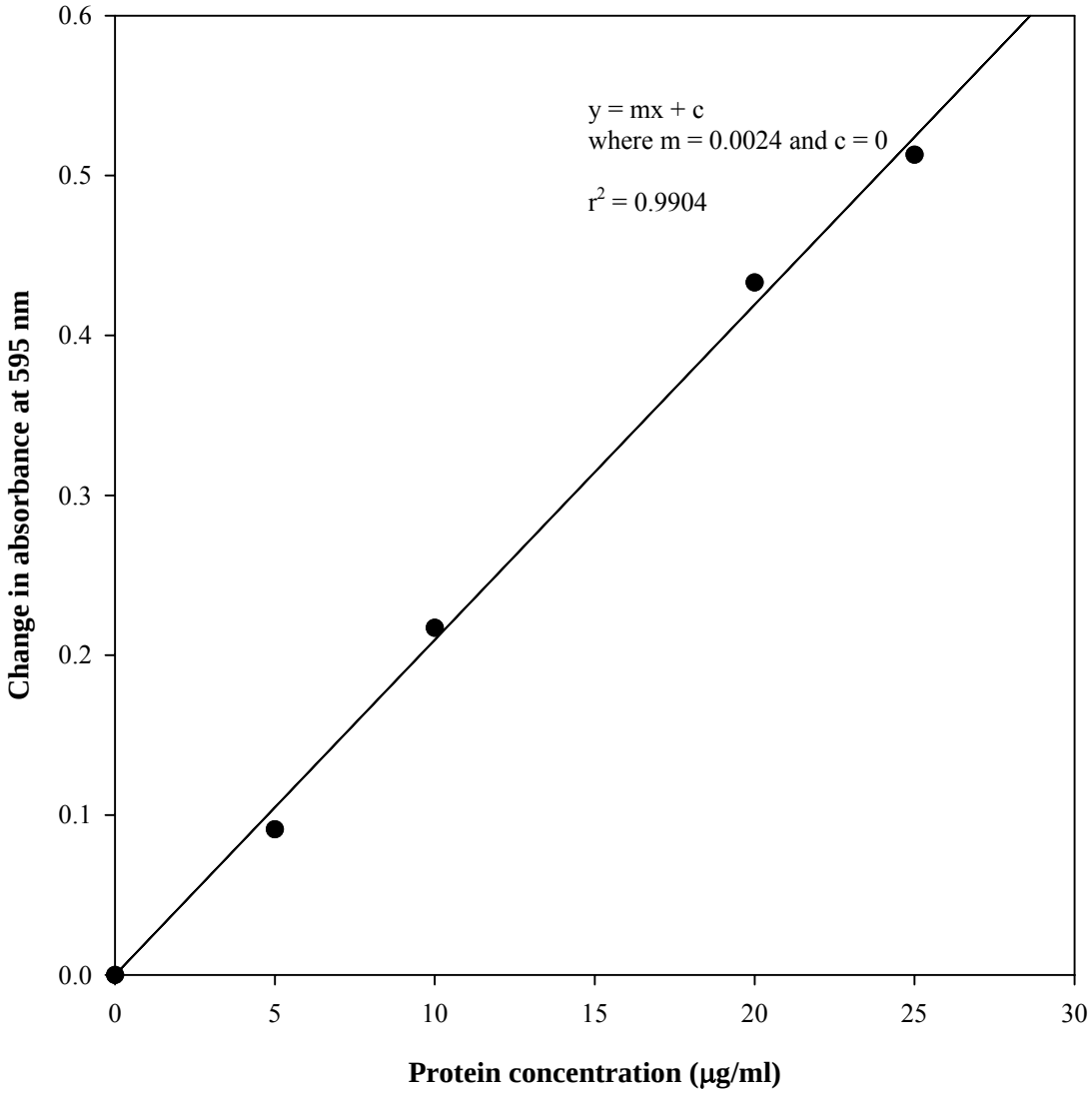

Figure 3.3. Gamma-globulin standard curve for the determination of concentration of proteins extracted from both biotypes of aphids before and after their purification with ammonium sulfate precipitation and HPLC.

3.3.3.1.5. Purification of RWA protein extracts

3.3.3.1.5.1. Ammonium sulfate precipitation

Solid ammonium sulfate, $(NH_4)_2SO_4$, was ground to powder using a mortar and pestle. The extract was made up to a volume of 5 ml with cold 0.025 M potassium phosphate buffer, pH 6.8. During

constant stirring at 4°C, it was brought to 65% saturation by gradual addition and dissolution of $(NH_4)_2SO_4$. After further stirring for an hour, the solution was centrifuged at 15 000 g for 15 minutes at 4°C (Sorvall RC-5B PLUS Superspeed Refrigerated Centrifuge, Thermo Electron Corporation, USA). The collected supernatant contained the non-proteinaceous fraction (metabolite and carbohydrate components), while the proteinaceous pellet was resuspended in 1 ml cold 0.025 M potassium phosphate buffer, pH 6.8.

3.3.3.1.5.2. Dialysis

Dialysis was performed overnight to remove salt impurities from the collected fractions. The dialysis tubing was pre-treated by soaking it overnight in potassium phosphate buffer (0.025 M, pH 6.8) containing 0.1% sodium azide. The samples were placed in the dialysis tubing (14 mm in diameter containing a pore radius of 24Å; Cope, London, U.K.), sealed with two clips and placed in 4 litres of the same phosphate buffer, at constant stirring at 4°C for 16 to 24 hours. The dialyzed samples were removed from the tubing and stored at 4°C.

3.3.3.2. Extraction of wheat proteins for enzyme activity assays and SDS-PAGE

3.3.3.2.1. Extraction of wheat proteins

The leaf tissue was ground in liquid nitrogen and 2 ml extraction buffer was added (100 mM sodium acetate buffer, pH 5.5, 10 mM mercapto-ethanol, 2 mM EDTA, 2 mM PMSF) (Mohase & Van der Westhuizen, 2002). The crude extract was centrifuged at 12 000 g for 20 minutes at 4°C. The supernatant was decanted and kept on ice until the assays were performed (catalase and peroxidase activity) or stored at -20°C.

3.3.3.2.2. Protein concentration of wheat sample extracts

Protein concentrations were determined using a Bradford protein assay dye reagent (Bio-Rad, USA; Bradford, 1976). A curve using gamma-globulin as standard, is presented in Figure 3.4. The absorbance was determined at a wavelength of 595 nm using a Multiskan *Ascent V1.24* platereader.

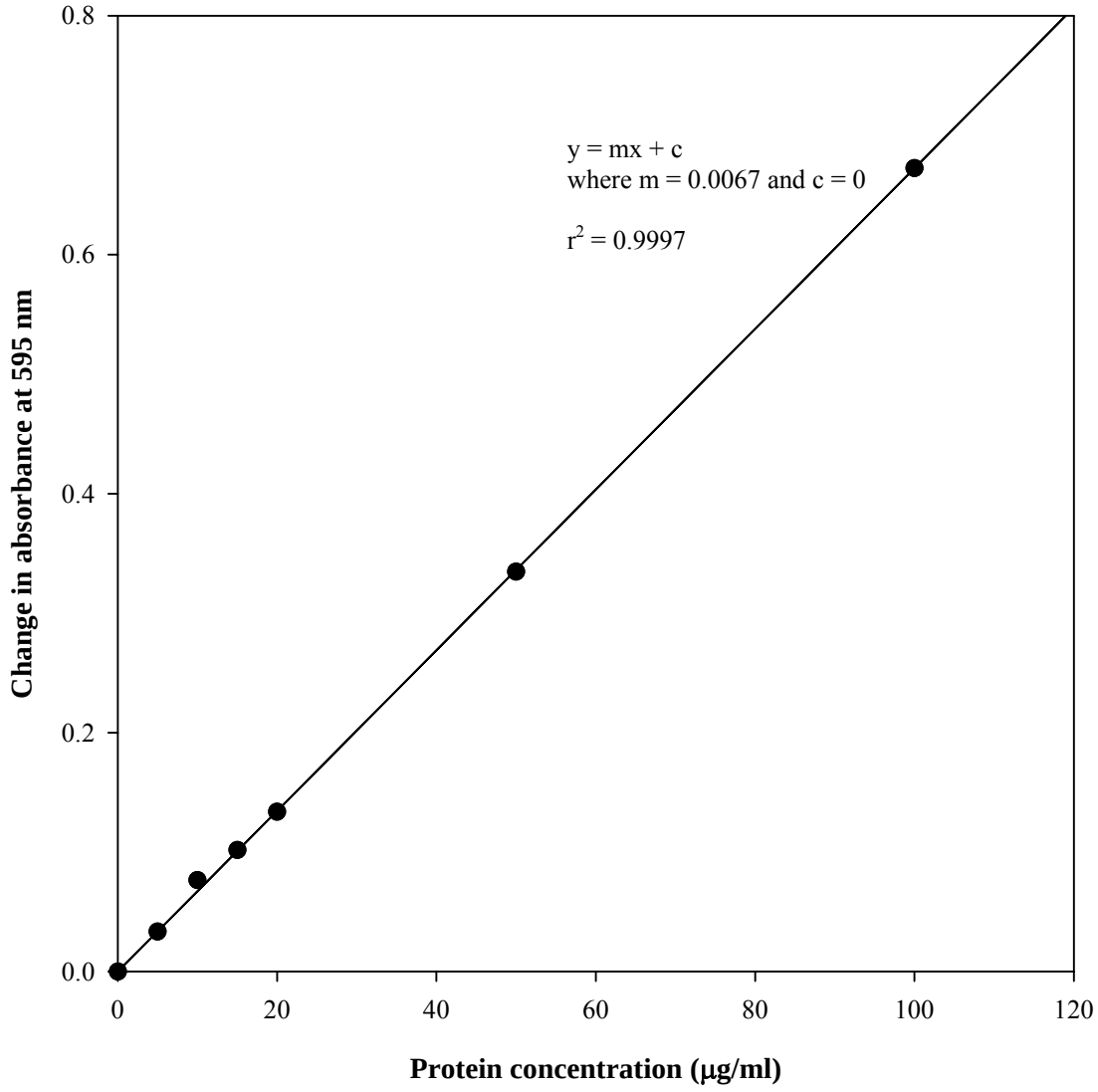

Figure 3.4. Gamma-globulin standard curve for the determination of concentration of proteins extracted wheat samples.

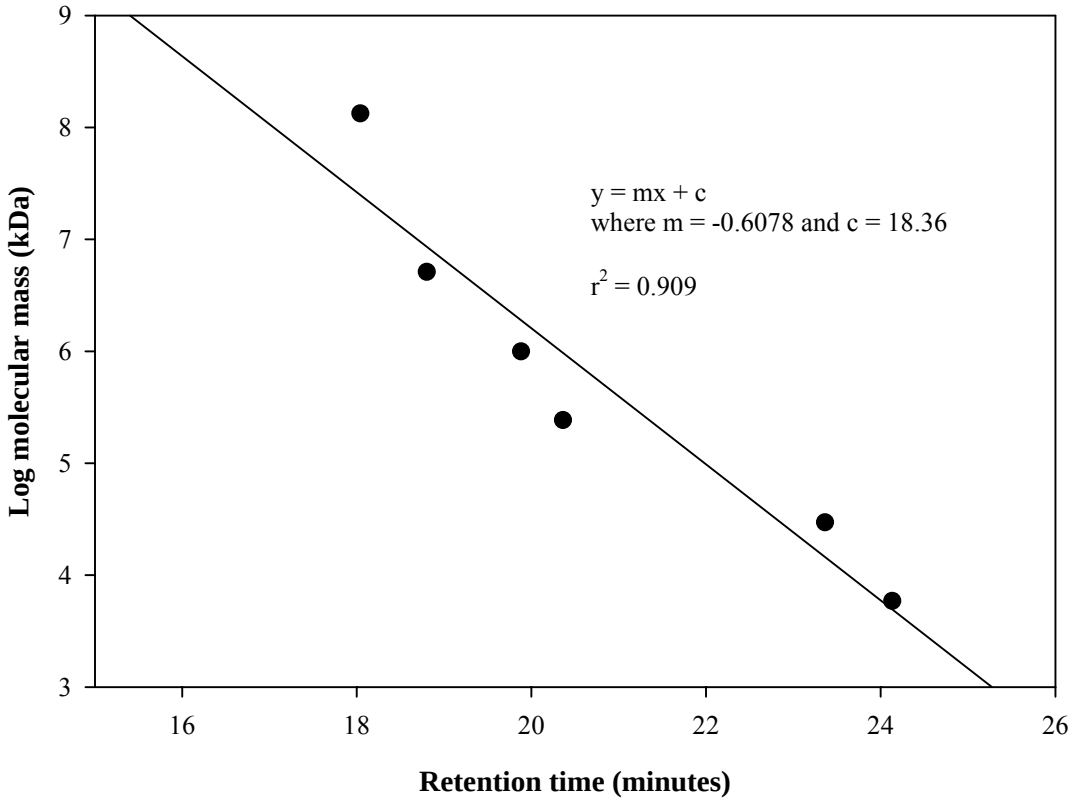

Figure 3.5. Standard curve for the determination of protein sizes in kDa.

3.3.4. High pressure liquid chromatography (HPLC) of RWA protein extracts

According to the test injections, we could confirm that the protein fraction of the saliva was responsible for the physiological defence response observed in the plants. Thus, test runs of HPLC were performed on the total RWA and salivary gland protein extracts. This was done by running samples on a Phenomenex column (Biosep-SEC-S 3000, USA), for 65 minutes at a flow rate of 0.5 ml min^{-1} in 0.025 M phosphate buffer, pH 6.8, using a Waters 2C HPLC. A 100 µg of protein was loaded for total protein extract, while 30 µg was used for head proteins and 10 µg for gut proteins. Absorbance was measured at 280 nm. Fractions were collected at one-minute intervals during the runs. Thereafter 10 µl of each collected fraction was injected into plants either as pooled samples (*x* x 10 µl) or individual (1 x 10 µl) fractions (where *x* is the amount of fractions in a pooled sample).

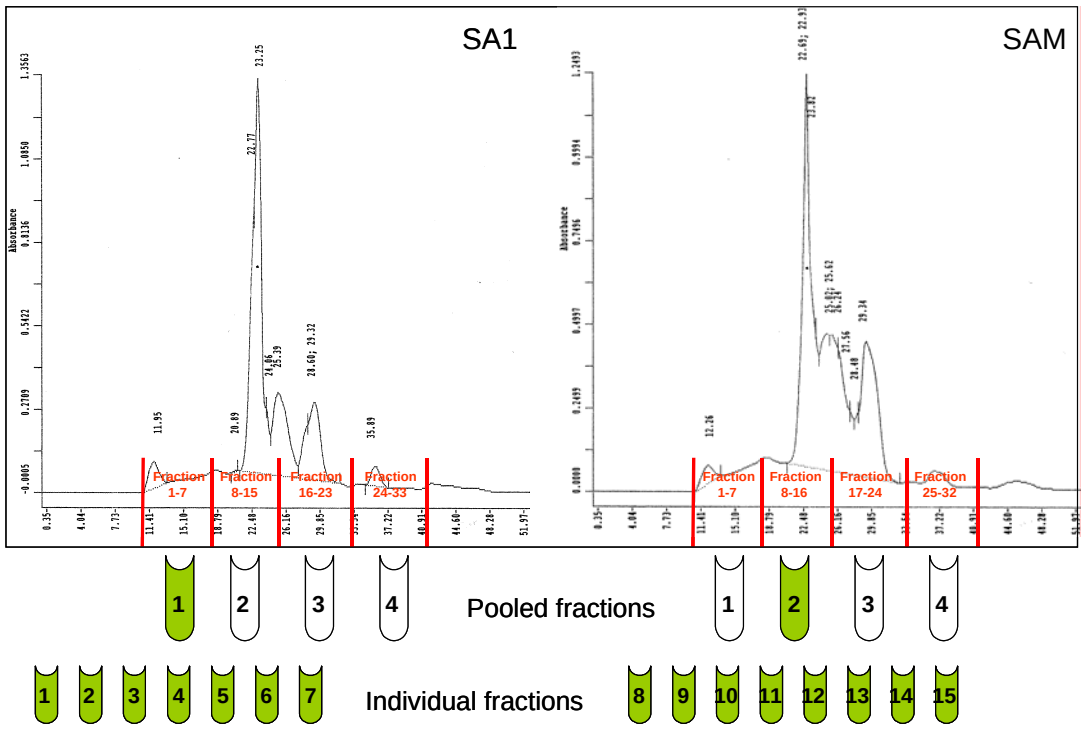

Figure 3.6. Collection and pooling of fractions after separation on HPLC. Pooled factions = 5 individual fractions pooled together prior to injection; Individual fractions = separate individually collected fraction injected.

(Figure 3.6). After 7 hours, total protein was extracted from the leaves of these plants for use in enzyme activity assays. A standard curve is presented in Figure 3.5 as constructed from Dalton Mark VI protein marker (Sigma, USA).

3.3.5. Measurements and determination of the plants' responses to injections of gut fractions from both biotypes of RWAs

Injections of the gut protein extracts from both the RWA biotypes, were performed on 'Tugela' and 'TugelaDN' plants. A total volume of 50 µl of the gut fractions were injected into each plant.

Extracts were injected into the veins of the plants using insulin needles. After 48 hours, the plants were photographed and evaluated phenotypically.

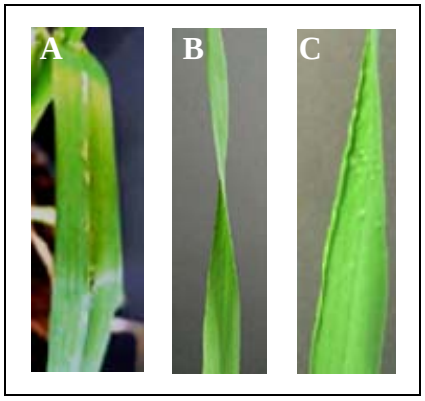

Figure 3.7. Phenotypic changes observed in leaves in response to RWA gut injections (**A:** site of injection at time=0, where the leaf is displaying no leaf rolling; **B:** 180º leaf rolling; **C:** puckering of the flag leaf).

3.3.6. Phenotypic evaluation and leaf rolling

The rate of leaf rolling was determined in RWA injected 'Tugela' and 'TugelaDN' plants, by observing leaf rolling (Figure 3.7 B) [measured as degrees of turning in relation to the leaves' initial rolling at time = 0 (leaf rolling $_{\text{time=7hours}}$/leaf rolling $_{\text{time=0 hours}}$)] in response to the RWA proteins. In extreme cases, the appearance of puckering or rippling was observed in the flag leaves of injected plants (See Figure 3.7 C): 'puckering' was defined as the crumpling of a small area of leaf tissue, giving it an uneven, corrugated appearance.

3.3.7. Activity assays of defence-related enzymes

The activity assays were performed on proteins extracted from plants, which had either been injected with pure gut or with fractions of proteins obtained after HPLC analysis.

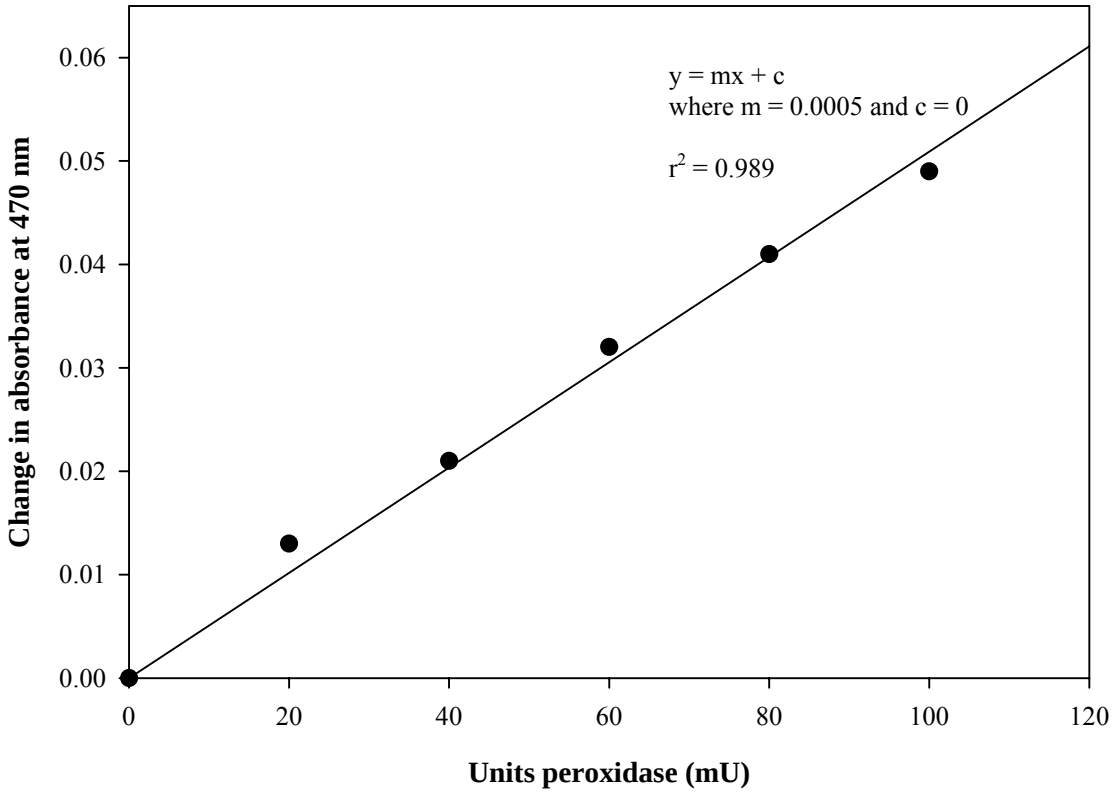

Figure 3.8. Standard curve for the determination of peroxidase activity assays of proteins extracted from wheat samples.

3.3.7.1. Peroxidase activity (E.C. 1.11.1.7)

For peroxidase activity determination, 1 ml of reaction mixture consisted of 100 mM potassium phosphate buffer, pH 5.0, 3 mM peroxide, 3 mM guaiacol and 1% (v/v) enzyme extract (as cited by Van der Westhuizen et al., 1998b). The reaction was initiated by the addition of the enzyme extract. The assays were performed at 470 nm for a period of 30 seconds on a *SpectroPlus*^{TM} spectrophotometer (Bio-Rad, USA). Enzyme activity was expressed as µmol tetraguaiacol. $min^{-1}.mg^{-1}$ protein. A standard curve is presented in Figure 3.8.

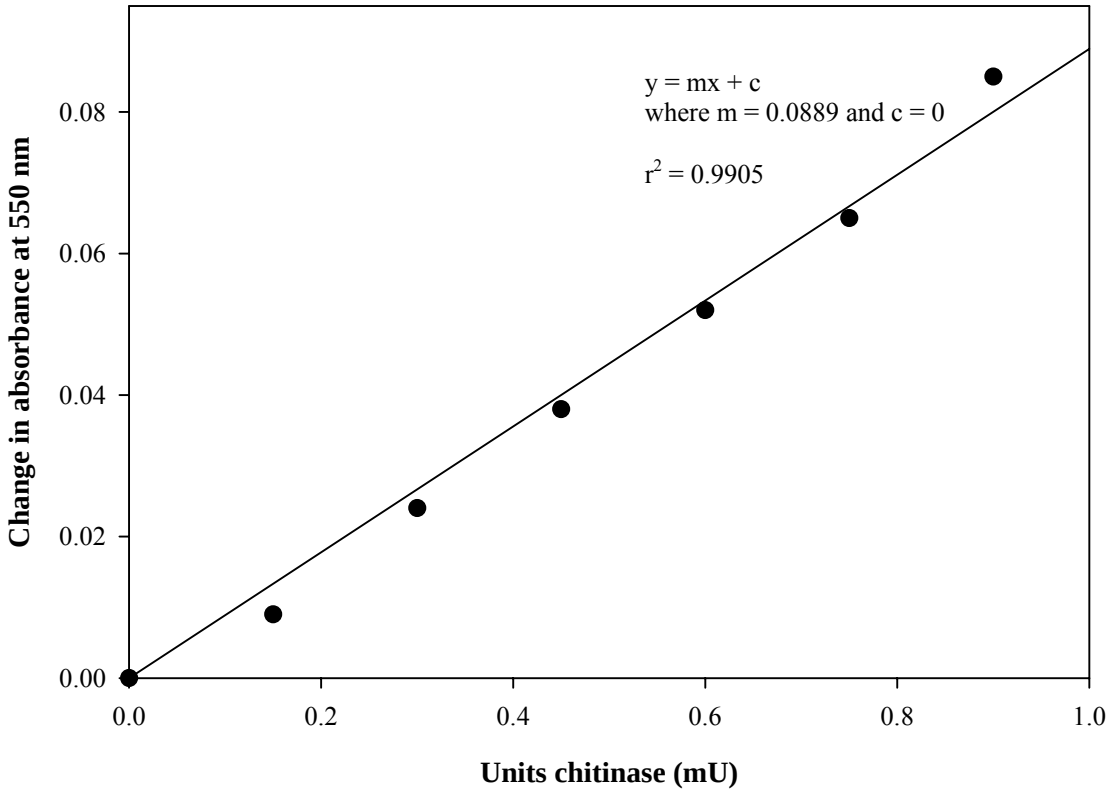

Figure 3.9. Standard curve for the determination of chitinase activity assays of proteins extracted from wheat samples.

3.3.7.2. Chitinase activity (E.C. 3.2.1.14)

For chitinase activity determination, a modified method as cited by Van der Westhuizen et al. (1998b) was used. The reaction was as follows: 0.5 ml of reaction mixture consisted of 28 mM sodium acetate buffer, pH 6.5, 0.6 mM sodium azide, 1 mg chitin colloidal (Sigma, USA) and 4% (v/v) enzyme extract. The mixture was incubated at 37ºC for two hours. After centrifugation (1000g, 2 min) 0.3 ml of the supernatant was used in a second reaction mixture containing 20 µl 1.5% (m/v) desalted snail gut enzyme (cytohelicase) and 30 µl of 1 M phosphate buffer (pH 7.1).

Chitin oligomers formed in the first reaction were hydrolysed at 37°C for 30 min after which the resulting GluNAc was determined. A mixture of 0.25 ml secondary reaction solution and 50 µl 0.8 M sodium borate buffer (pH 9.1) was heated in a boiling water bath for 3 min. After cooling and the addition of 1.5 ml 1 % (m/v) 4-dimethyl-aminobenzaldehyde, the mixture was incubated at 37°C for 30 min and subsequently cooled. The change in absorbance at 550 nm was measured on a *SpectroPlus*™ spectrophotometer (Bio-Rad, USA) to determine chitinase activity. Enzyme activity was related to the formation of N-acetylglucosamine (GlcNAc) equivalents (µmol GlcNAc. $h^{-1}.mg^{-1}$ protein). A standard curve is presented in Figure 3.9.

3.3.7.3. Catalase activity (E.C. 1.11.1.6)

For catalase activity determination, 1 ml of reaction mixture consisted of 50 mM potassium phosphate buffer, pH 6.5, 15 mM hydrogen peroxide, and 5% (v/v) enzyme extract (Li et al., in press). The reaction was initiated by the addition of the enzyme extract. After scanning the reaction between 200 and 250 nm, it was found that the most catalase activity was recorded at 247 nm on a *SpectroPlus*™ spectrophotometer (Bio-Rad, USA). Thus, all other assays were performed at this wavelength for a period of 90 seconds. Enzyme activity was related to the degradation of hydrogen peroxide (µmol H_2O_2 $min^{-1}.mg^{-1}$ protein). A standard curve is presented in Figure 3.10.

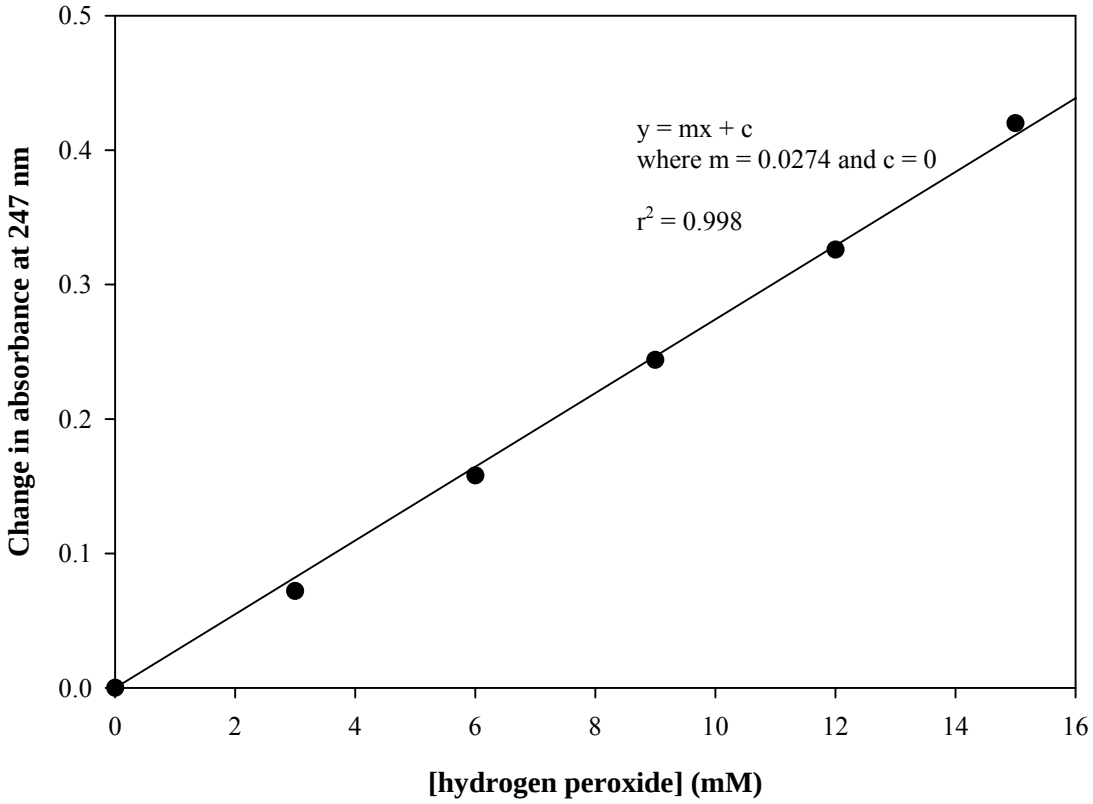

Figure 3.10. Standard curve for the determination of catalase activity assays of proteins extracted from wheat samples.

3.3.8. Statistical analysis

Analysis of variance (ANOVA) was calculated between the groups using the *ezANOVA* program (www.sph.sc.edu/omd/rorden/ezanova/home.html#between). H_o was the hypothesis that all the values are the same and was not rejected in the event of $0.01<P<0.05$.

3.3.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% running and 5% stacking gel, ratio 37.5:1, acrylamide: bisacrylamide; 10% APS and 10% TEMED) were cast and run according to the protocol of Laemmli (1970). Gels were run on a *Hoeffer* gel system (Amersham, U.K.) at 130V. Gels were visualized with 2% (w/v) Coomassie staining. Wheat

samples loaded contained 3 µg protein and the RWA samples varied in protein concentrations over a range from 1 to 4 µg. Protein band sizes were determined by comparing them to the marker Dalton Mark VI (Sigma, USA).

3.4. Results

3.4.1. Protein extraction

Proteins were extracted from total RWAs, their heads or their guts (refer to Figures 3.1 and 3.2). This was done to determine the factor of protein exclusion by extracting only the heads or guts in comparison to total protein. After extraction, 441.7 µg ml^{-1} protein was obtained for the total SAM RWAs, while 589 µg ml^{-1} protein was obtained for the SA1 RWAs. When extractions were made of the guts, 58 µg ml^{-1} and 66 µg ml^{-1} protein was obtained for the SAM and SA1 aphids, respectively, and 83.33 µg ml^{-1} and 72.75 µg ml^{-1} for their head protein extractions (Table 3.1).

Table 3.1. Concentrations of proteins extracted from the RWAs.

Sample		Protein concentration (µg ml^{-1})
SAM [a]	:Total protein	441.70
SAM	:Heads	83.33
SAM	:Guts	57.95
SA1 [b]	:Total protein	589.43
SA1	:Heads	72.75
SA1	:Guts	66.91

a: South African Mutant Biotype **b:** South African Biotype 1

3.4.2. Purification of protein extracts

In addition to the selective exclusion of protein via the extraction of the heads and guts, these aforementioned proteins were then further resolved with $(NH_4)_2SO_4$ precipitation followed by dialysis and HPLC. The protein concentrations of these samples and their resulting factor of purification for each step are presented in Table 3.2. By extracting heads instead of total RWA, a 5.3 (SAM) and 8.1 (SA1) fold reduction in protein were measured. When guts were extracted, SAM RWAs produced 7.6 times less protein and the SA1 RWAs 8.8 times less. Purification by $(NH_4)_2SO_4$ precipitation and dialysis yielded SAM protein samples with a concentration of 24.68 µg ml^{-1} and a purification factor of 17.9 and SA1 samples with 42.27 µg ml^{-1}, purified by factor

13.9. HPLC then purified the samples even more by a factor of 102.7 yielding 4.30 µg ml^{-1} proteins for the SAM sample and by a factor of 121.3 for the SA1 yielding 4.86 µg ml^{-1} protein.

Table 3.2. Reduction in the amount of protein through exclusion and purification of the proteins**.**

RWA Biotype	Purification Procedure									
	Total protein		Head protein		Gut protein		$(NH_4)_2SO_4$ precipitation & dialysis		HPLC	
	[Protein] (µg ml^{-1})	P. F. [c]	[Protein] (µg ml^{-1})	R. [d]	[Protein] (µg ml^{-1})	R.	[Protein] (µg ml^{-1})	P. F.	[Protein] (µg ml^{-1})	P. F.
SAM [a]	441.70	1.0	83.33	5.3	57.95	7.6	24.68	17.9	4.30	102.7
SA1 [b]	589.43	1.0	72.75	8.1	66.91	8.8	42.27	13.9	4.86	121.3

a: South African Mutant Biotype, **b:** South African Biotype 1, **c:** Purification Factor, d: Reduction in the amount of protein through exclusion.

3.4.3. High Pressure Liquid Chromatography (HPLC) of RWA protein extracts

HPLC was performed on the RWA protein extracts to resolve the proteins in the samples according to size. The profiles obtained from the total protein fractions were comparable in the following instances: a small peak after a retention time (rt) of 12.26 (Figure 3.11 A) and 11.95 (Figure 3.11 B), a smaller peak at 18.32 rt (Figure 3.11 A) and 18.79 rt (Figure 3.11 B), a large peak at 23.25 rt (Figure 3.11 A) and 22.93 rt (Figure 3.11 B), a moderate peak at 29.32 rt (Figure 3.11 A) and 29.34 rt, at another small peak at 35.89 rt (Figure 3.11 A) and 36.3 rt (Figure 3.11 B) and an even smaller, broad peak approximately 43 rt (Figure 3.11 A) and 45 rt (See Figure 3.11 B).

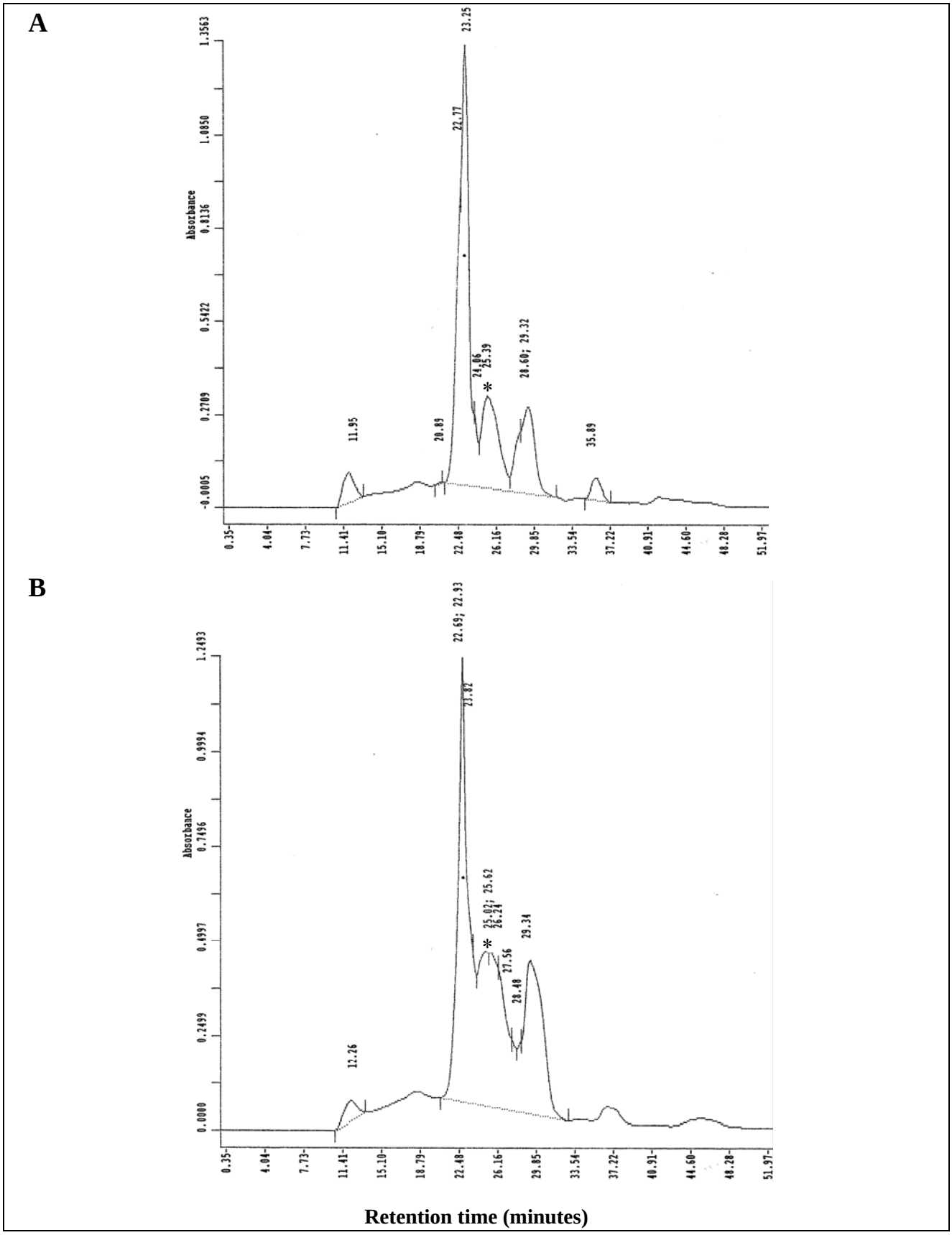

Figure 3.11. HPLC profiles of total RWA protein extracted from SA1 biotype (A) and SAM biotype (B). Absorbance = 280 nm. Asterisks indicate differences between different biotype's profiles at the same retention time.

The most significant difference is observed after the retention time of 25: a single peak occurs in the SA1 profile at 25.39 (Figure 3.11 A), while a double peak occurs in that of the SAM profile at 25.62 and 26.24 (Figure 3.11 B).

The profiles obtained from the gut proteins (Figure 3.12) showed more variation than the total RWA protein presented in Figure 3.11. But the following peaks occur in both profiles: a peak at retention times 13.87 and 12.025, 3 large peaks escalating in absorbance at retention times 18.79, 21.74 and 23.4 (Figure 3.12 A) and 22.04, 22.72 and 23.26 (Figure 3.12 B) respectively, a moderate peak at retention times 27.39 and 27.08, a peak at 39.07 rt and 38.696 rt, a peak at 43.37 rt, a very small peak at 21.97 rt and an even smaller peak at 60.58 rt. The peaks at retention times 13.87 and 39.065 are relatively larger in the SA1 profile with absorbances of 0.0123 and 0.00475 (Figure 3.12 A) in comparison to 0.0017 and 0.0042 (Figure 3.12 B), while the peak at 43.37 rt is larger for the SAM profile at an absorbance of 0.0079 (Figure 3.12 B) in contrast to 0.0001 in Figure 3.12 A. An additional very small peak occurs in Figure 3.11 B at retention time 35.75.

The profiles of the head samples (Figure 3.13) showed less variation than that of the gut proteins (Figure 3.12). The same peaks occur in both profiles at the following retention times: a medium sized peak at 12.025 rt, a large peak at 20.262 rt, a peak at 30.586 rt and a small peak at 37.59 rt. As in Figure 3.11, the most significant difference occurs around 25 minutes in Figure 3.13 A. A medium sized peak occurs on the side of the large peak at 22.47 rt and a smaller peak at 25.24 rt, but in Figure 3.13 B a single peak occurs at 25.24 rt. The absorbance of the peak at retention time 30.59 is 0.0128 (Figure 3.13 A) and larger than its equivalent in Figure 3.13 B, which has an absorbance of 0.0039. The large peak at 20.262 rt corresponds to the small peaks at the same time in Figure 3.11.

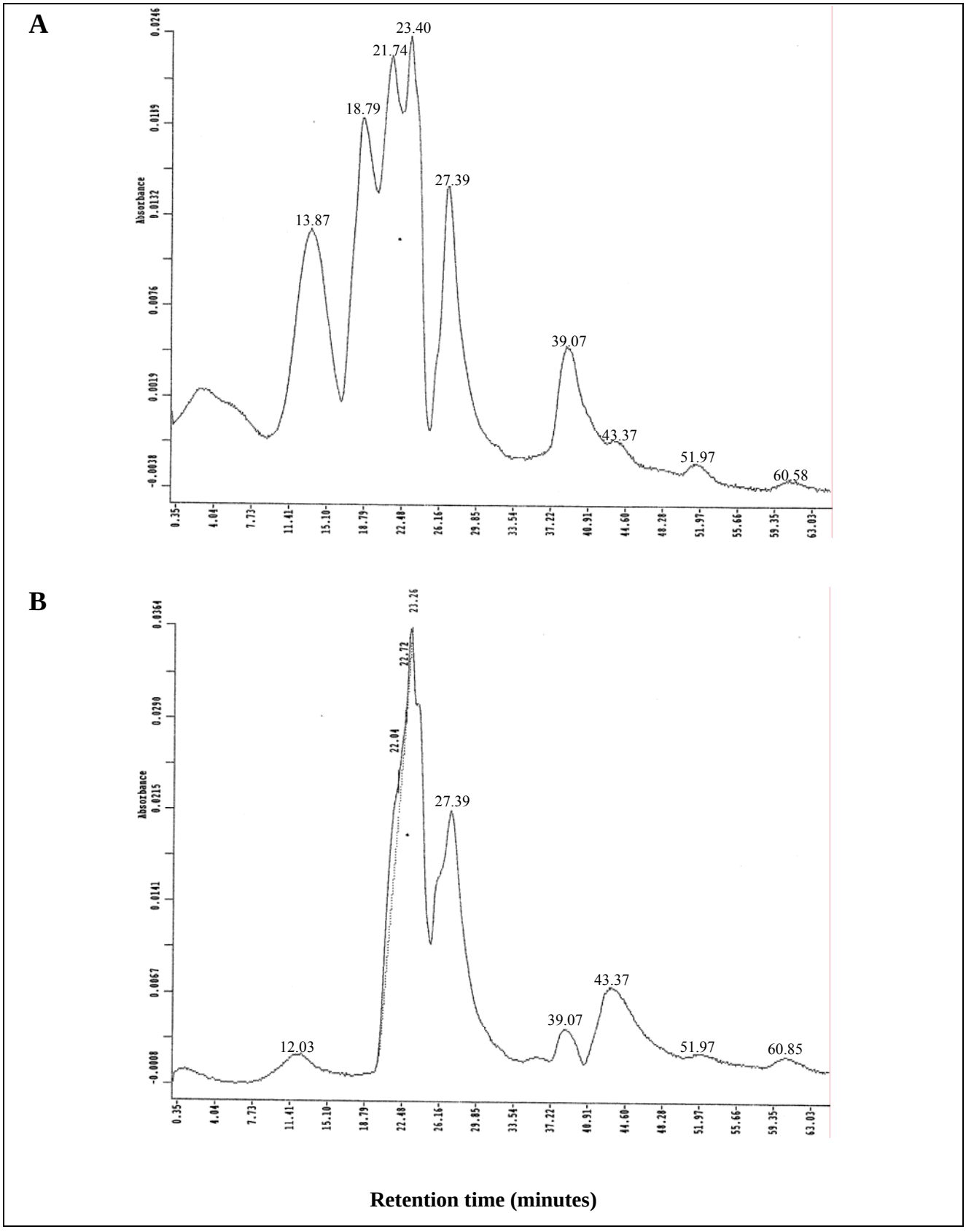

Figure 3.12. HPLC profiles of RWA protein extracted from the guts of the SA1 biotype (A) and the SAM biotype (B). Absorbance = 280 nm.

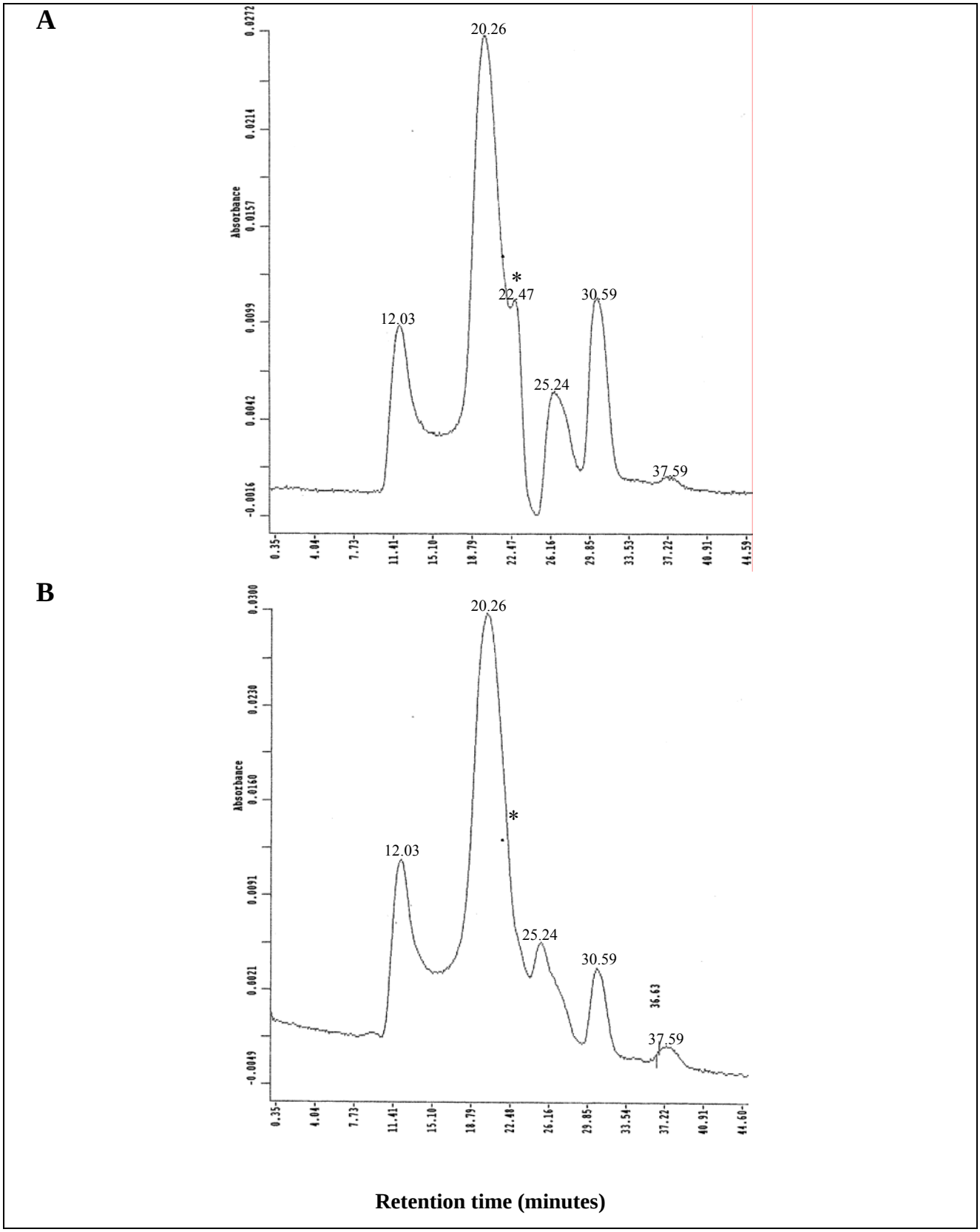

Figure 3.13. HPLC profiles of head RWA protein extracted from the SA1 (A) and the SAM biotype (B). Absorbance = 280 nm. Asterisks indicate differences between different biotype's profiles at the same retention time.

3.4.4. Measurements and determination of the plants' responses to injections of purified protein fractions from both biotypes of RWAs

The non-proteinaceous and proteinaceous phases were injected into plants to determine which phase could possibly contain the elicitors which cause the symptoms of normal RWA feeding. The responses of the plants to the biotypes' gut injections were evaluated phenotypically and by measuring the amount of leaf roll responses.

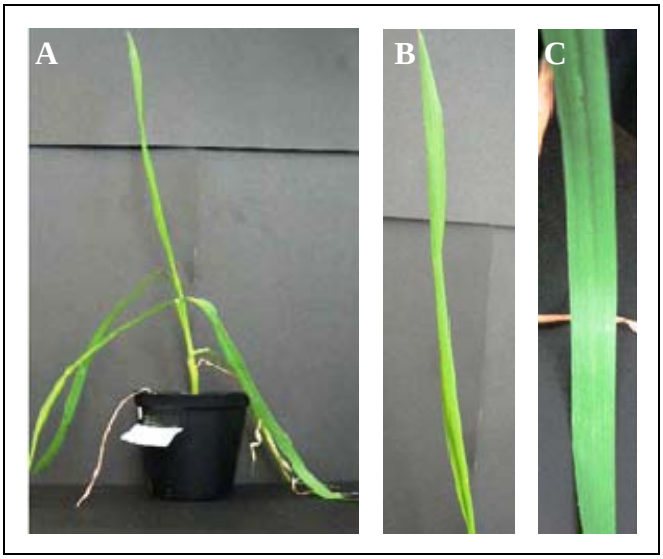

Figure 3.14. 'TugelaDN' 48 hours post injection with 50 µl phosphate buffer (**A:** Whole plant; **B:** Flag leaf; **C:** Basal leaf).

Only plants injected with protein (Figures 3.14 and 3.15) displayed any significant symptoms when compared to plants injected with buffer (Figure 3.14). All the leaves had started rolling, mimicking the symptoms of aphid feeding on susceptible wheat plants (Figures 3.14 and 3.15 A, B and C). Within 24 hours, the flag leaf was puckered (Figure 3.15 D and Figure 3.16.D) and necrotic lesions had appeared on the basal leaves (Figure 3.15 E and Figure 3.16E).

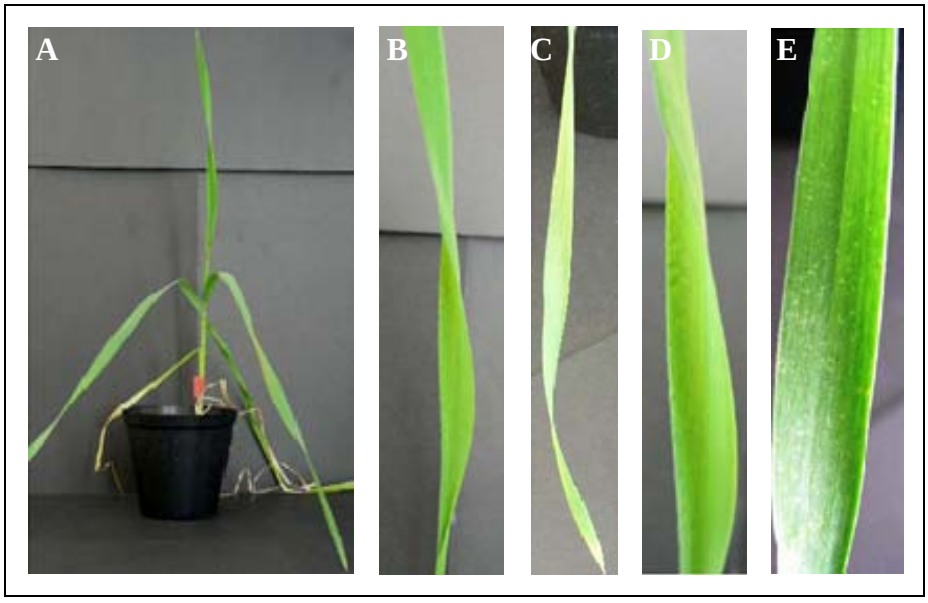

Figure 3.15. 'TugelaDN' 48 hours post injection with 50 µl SAM gut extracts (**A:** Whole plant; **B:** Flag leaf; **C:** Basal leaf **D:** puckering on the flag leaf; **E:** necrotic lesions on the basal leaf).

In all cases, the symptoms on plants injected with SA1 type saliva were the most severe (Figure 3.16).

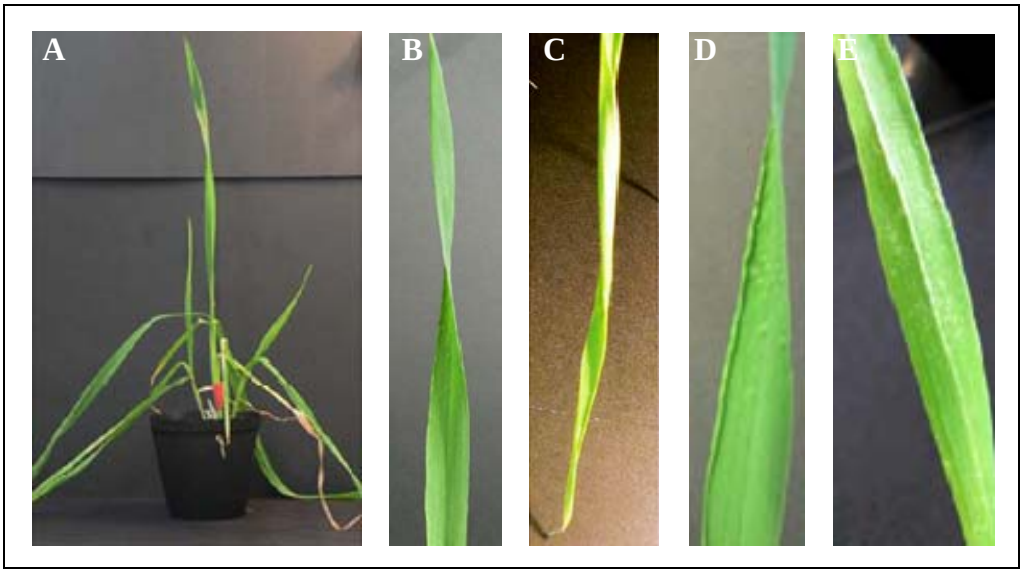

Figure 3.16. 'TugelaDN' 48 hours post injection with 50 µl SA1 RWA gut extracts (**A:** Whole plant; **B:** Flag leaf; **C:** Basal leaf; **D:** puckering on the flag leaf; **E:** necrotic lesions on the basal leaf).

3.4.5. Leaf rolling

3.4.5.1. Plants injected with salivary extract

Leaf rolling is a characteristic feature of RWA feeding on susceptible plants. It was initially anticipated that this could be used as a measure of eliciting action of each gut extract phase or HPLC fraction. The degree of leaf rolling of the flag and basal leaves was measured in each plant before injection and just before protein extraction of each sample. It was then converted to a fold value in comparison to that of the non-injected plants in the sample batch. The first leaf rolling experiment was performed with SA1 RWA gut extracts on 'Tugela' plants to determine a rough guide to which we could compare all other data (See Figure 3.17 and Table 3.3).

Table 3.3. Degree of leaf rolling recorded for 300 minutes in the flag and basal leaves of 'Tugela' plants post injection with SA1 RWA gut extracts.

Treatment	Degree of leaf roll (Flag leaf)	Std error	Degree of leaf roll (Basal leaf)	Std error
Not injected	1.000	0.000	1.000	0.000
Buffer	1.000	0.000	0.806	0.100
30 min. p. inj.[a]	2.250	0.000	1.667	0.272
60 min. p. inj.	2.250	0.000	4.000	0.000
90 min. p. inj.	2.750	0.204	4.000	0.000
120 min. p. inj.	2.875	0.102	6.000	0.000
150 min. p. inj.	2.875	0.102	6.000	0.000
180 min. p. inj.	2.875	0.102	6.000	0.000
210 min. p. inj.	2.875	0.102	6.000	0.000
240 min. p. inj.	2.875	0.102	6.000	0.000
270 min. p. inj.	2.875	0.102	6.000	0.000
300 min. p. inj.	2.875	0.102	6.000	0.000

a: minutes post injection

After 30 minutes, the degree of leaf rolling in the flag and basal leaves in relation to the non-injected plants was at 2.250±0.000 and 1.667±0.272 respectively. By 60 minutes, the flag leaves

were similarly rolled as at time interval 30, but the basal leaves had doubled in intensity and were 4 times as rolled as the control plants. At 90 minutes, the basal leaves were unchanged, but the flag leaves had increased in leaf rolling to 2.75±0.204 times that of the control. By 120 minutes the degree of rolling was fixed for both the leaf types: 2.875±0.102 times for the flag leaves and 6.000±0.000 times for the basal leaves.

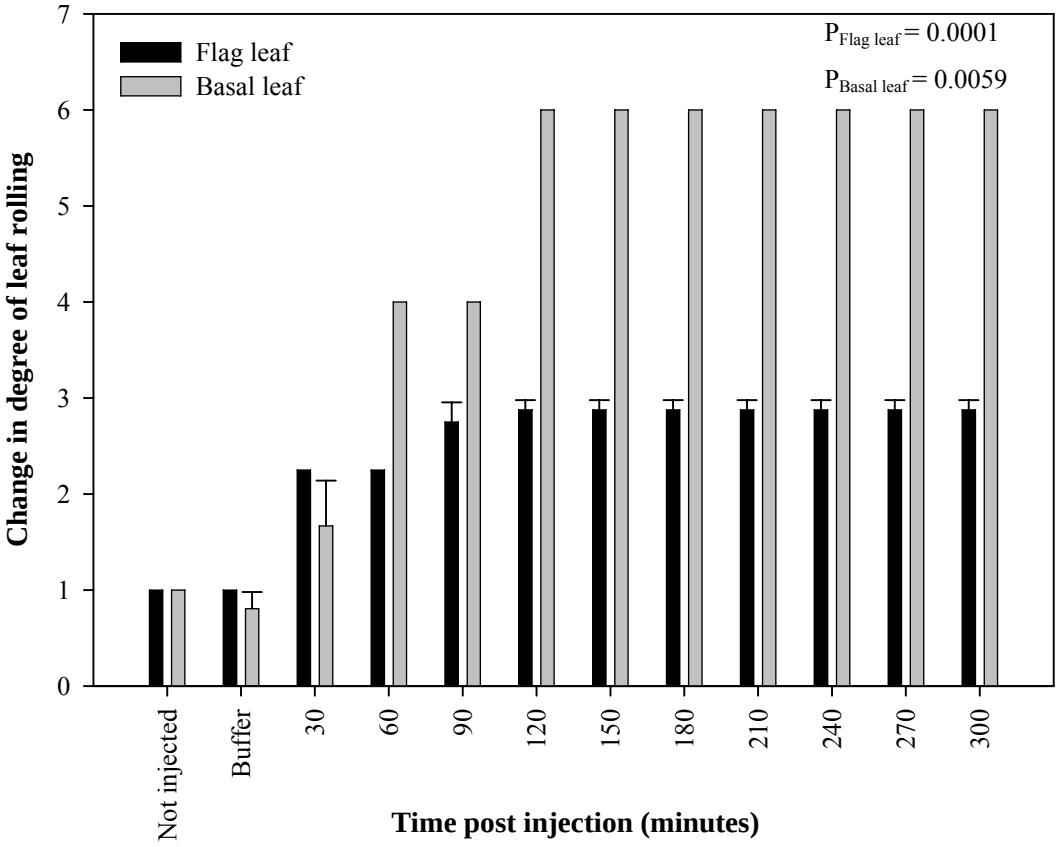

Figure 3.17. Degree of leaf rolling in 'Tugela' plants post injection with RWA gut extracts ($P_{Flag\ leaf} = 0.0001$ and $P_{Basal\ leaf} = 0.0059$,where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

The second set of leaf rolling data was collected during the time trial experiment on 'TugelaDN' to determine the time interval post injection displaying the highest peroxidase activity (Table 3.4. and Figure 3.18). Most of the plants' flag leaves had increased values in regards to their leaf rolling: the

non-injected plants by 1.111±0.000 times, 5 hour post injection by 1.778±0.619, 6 h. p. inj. by 1.089±0.048 and 7 h. p. inj. by 1.655±0.362. Three of the plants hadn't undergone any change in leaf rolling: 1, 2 and 9 h. p. inj. The buffer injected plants, 3 h. p. inj., 4 h. p. inj. and 8 h. p. inj. had decreased in leaf rolling, by 0.550±0.041, 0.847±0.211, 0.960±0.195 and 0.788±0.099 respectively.

Table 3.4. Degree of leaf rolling recorded for 9 hours in the flag and basal leaves of 'TugelaDN' plants post injection with RWA gut extracts.

Treatment	Degree of leaf roll (Flag leaf)	Std error	Degree of leaf roll (Basal leaf)	Std error
Not injected	1.111	0.000	1.000	0.000
Buffer	0.550	0.041	1.333	0.136
1 h. p. inj.[a]	1.000	0.000	1.000	0.000
2 h. p. inj.	1.000	0.000	1.500	0.000
3 h. p. inj.	0.847	0.211	0.565	0.225
4 h. p. inj.	0.960	0.195	0.761	0.327
5 h. p. inj.	1.778	0.619	1.233	0.570
6 h. p. inj.	1.089	0.048	0.944	0.182
7 h. p. inj.	1.655	0.362	0.939	0.233
8 h. p. inj.	0.788	0.099	1.329	0.275
9 h. p. inj.	1.000	0.000	1.214	0.175

a: hours post injection

Similar to the flag leaves, the basal leaves also had no pattern in regards to their leaf rolling. The non-injected and 1 h. p. inj. plants remained unchanged; the buffer injected, 2 h. p. inj., 5 h. p. inj., 8 h. p. inj. and 9 h. p. inj. had increased by 1.333±0.136, 1.500±0.000, 1.233±0.570, 1.329±0.275 and 1.214±0.175 respectively; whereas, 3 h. p. inj., 4 h. p. inj., 6 h. p. inj. and 7 h. p. inj. had decreased by 0.565±0.225, 0.761±0.327, 0.944±0.182 and 0.939±0.233 respectively.

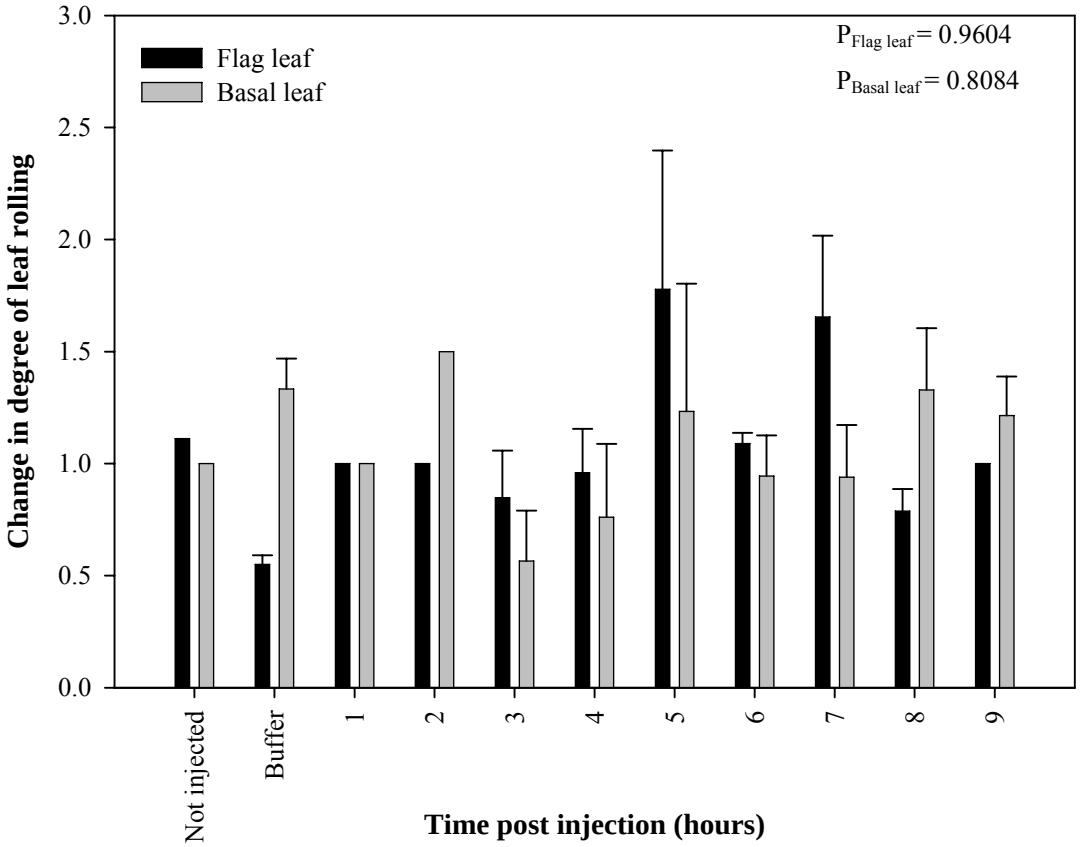

Figure 3.18. Degree of leaf rolling recorded for 9 hours in the flag and basal leaves of 'TugelaDN' plants post injection with RWA gut extracts ($P_{Flag\ leaf}$ = 0.9604 and $P_{Basal\ leaf}$ = 0.8084,where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

3.4.5.1.1. Plants injected with HPLC fractions

The leaf rolling patterns observed on the 'TugelaDN' plants injected with the pooled HPLC samples (Figure 3.19 and Table 3.5) were as sporadic as those from the time trial plants. Most of the flag leaves were more rolled at 7 h. p. inj. than they were at the initiation of the experiment: the non-injected, SAM.3, SAM.4, SA1.1, SA1.2 and SA1.4 injected plants by 1.111±0.000, 1.511±0.248, 1.111±0.111, 1.571±0.297, 1.155±0.200 and 1.051±0.151 respectively. The plants injected with buffer, SAM.1, SAM.2 and SA1.3 showed decreased rolling by 0.550±0.041, 0.906±0.107 and 0.767±0.051 respectively.

Table 3.5. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'TugelaDN' plants post injection with pooled HPLC fractions of RWA gut extracts.

Treatment	Degree of leaf roll (Flag leaf)	Std error	Degree of leaf roll (Basal leaf)	Std error
Not injected	1.111	0.000	1.000	0.000
Buffer	0.550	0.041	1.333	0.136
SAM[a].1	0.906	0.107	0.750	0.144
SAM.2	1.063	0.141	0.458	0.253
SAM.3	1.511	0.248	0.965	0.099
SAM.4	1.111	0.111	0.917	0.083
SA1[b].1	1.571	0.297	0.867	0.133
SA1.2	1.155	0.200	0.933	0.218
SA1.3	0.767	0.051	0.644	0.099
SA1.4	1.051	0.151	1.044	0.289

a: HPLC fractions collected after injection of South African Mutant; **b:** HPLC fractions collected after injection of South African Biotype 1

Most of the basal leaves had reduced leaf rolling: SAM.1, SAM.2, SAM.3, SAM.4, SA1.1, SA1.2 and SA1.3 injected plants' leaf rolling was reduced by 0.750±0.144, 0.458±0.253, 0.965±0.099, 0.917±0.083, 0.867±0.133, 0.933±0.218 and 0.644±0.099 respectively. The buffer and SA1.4 injected plants had increased levels of leaf rolling by 1.333±0.136 and 1.044±0.289. Only the non-injected plants' leaf rolling had remained unchanged.

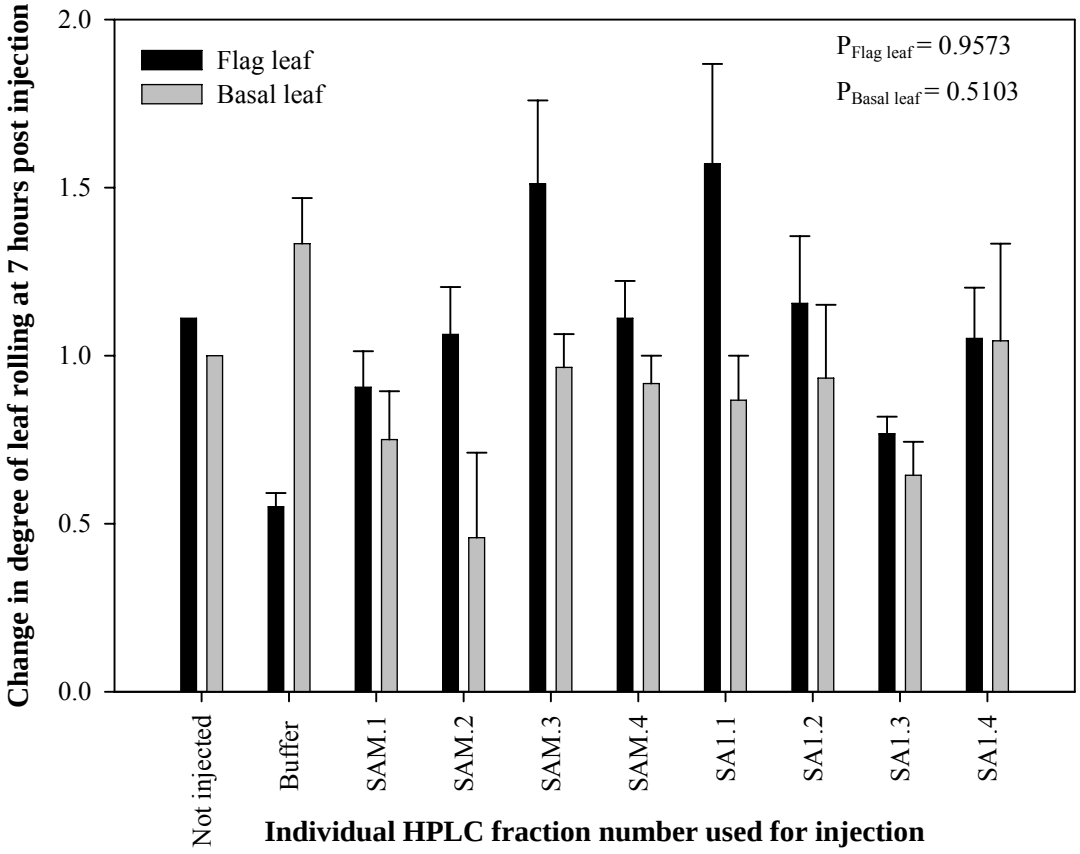

Figure 3.19. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'TugelaDN' plants post injection with pooled HPLC fractions of RWA gut extracts (**SAM:** South African Mutant Biotype, **SA1:** South African Biotype 1) ($P_{Flag\ leaf} = 0.9573$ and $P_{Basal\ leaf} = 0.5103$,where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$)

Leaf rolling was also determined for 'TugelaDN' plants injected with the individual HPLC fractions (Figure 3.20 and Table 3.6). Most of the flag leaves had decreased leaf rolling: buffer, SAM.1, SAM.2, SAM.6, SA1.10, SA1.11, SA1.13, SA1.13 and SA1.14 injected by 0.550±0.041, 0.762±0.078, 0.800±0.163, 0.750±0.204, 0.833±0.136, 0.250±0.204, 0.750±0.204 and 0.750±0.204 respectively. Five plants' leaf rolling had increased: non-injected, SAM.3, SAM.5, SA1.12 and SA1.15 by 1.111±0.000, 1.143±0.117, 1.125±0.306, 1.250±0.204 and 1.500±0.408 respectively. SAM.4, SAM.7, SA1.8 and SA1.9 had remained the same.

Table 3.6. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'TugelaDN' plants post injection with the individual HPLC fractions of RWA gut extracts.

Treatment	Degree of leaf roll (Flag leaf)	Std error	Degree of leaf roll (Basal leaf)	Std error
Not injected	1.111	0.000	1.000	0.000
Buffer	0.550	0.041	1.333	0.136
SAM.1	0.762	0.078	0.900	0.081
SAM.2	0.800	0.163	0.667	0.000
SAM.3	1.143	0.117	1.000	0.000
SAM.4	1.000	0.000	0.900	0.000
SAM.5	1.125	0.306	0.900	0.081
SAM.6	0.750	0.204	0.825	0.061
SAM.7	1.000	0.000	1.200	0.000
SA1.8	1.000	0.000	1.000	0.000
SA1.9	1.000	0.074	1.100	0.081
SA1.10	0.833	0.136	1.000	0.000
SA1.11	0.250	0.204	0.500	0.408
SA1.12	1.250	0.204	0.833	0.046
SA1.13	0.750	0.204	1.000	0.000
SA1.14	0.750	0.204	0.789	0.009
SA1.15	1.500	0.408	1.000	0.000

a: HPLC fractions collected after injection of South African Mutant; **b:** HPLC fractions collected after injection of South African Biotype 1

As in the case of the flag leaves, most of the basal leaves also had decreases in leaf rolling: SAM.1 by 0.900±0.000., SAM.2 by 0.667±0.000, SAM.4 by 0.900±0.000, SAM.5 by 0.900±0.081, SAM.6 by 0.825±0.061, SA1.11 by 0.500±0.408, SA1.12 by 0.833±0.046 and SA1.14 by 0.789±0.009. The non-injected, SAM.3, SA1.8, SA1.10, SA1.13 and SA1.15 injected plants remained the same. Leaf rolling in buffer, SAM.3 and SA1.9 injected plants were increased by 1.333±0.136, 1.200±0.000 and 1.100±0.081 respectively.

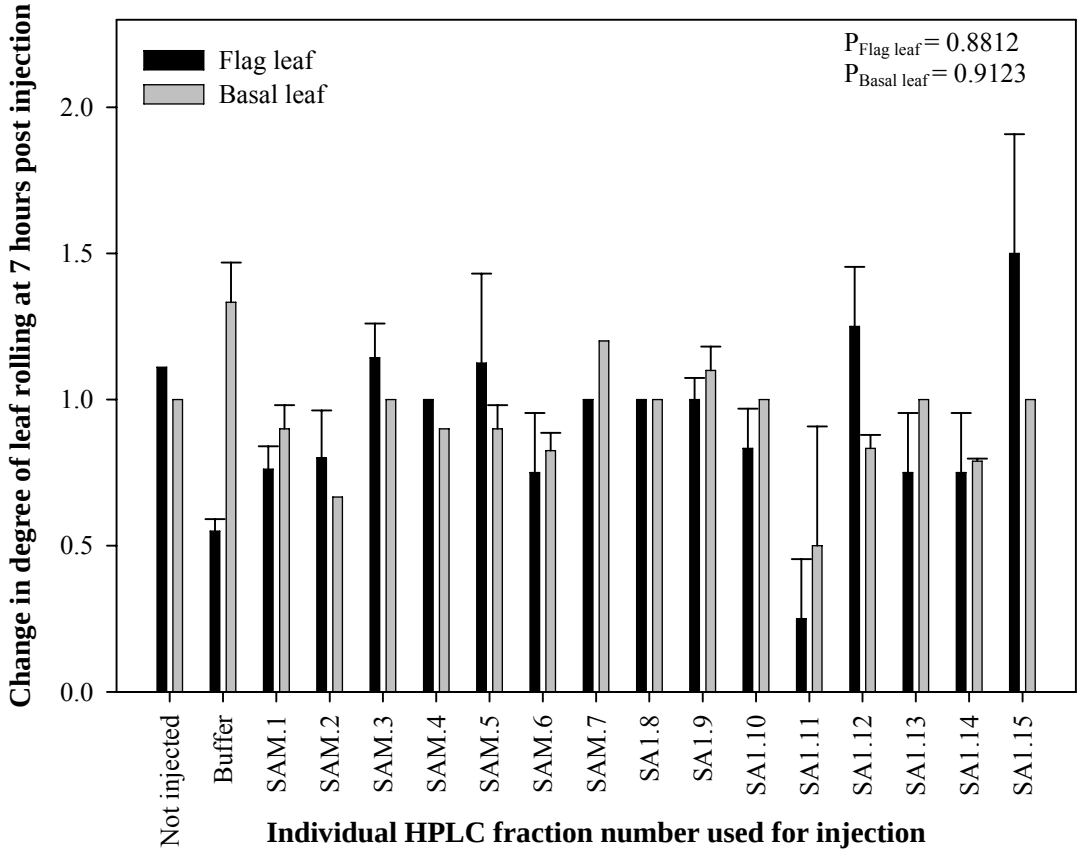

Figure 3.20. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'TugelaDN' plants post injection with the individual HPLC fractions of RWA gut extracts (**SAM:** South African Mutant Biotype, **SA1:** South African Biotype 1) ($P_{Flag\ leaf}$ = 0.8812 and $P_{Basal\ leaf}$ = 0.9123,where $H_0 \neq H_A$ when P<0.01 or P>0.05)

Lastly, the leaf rolling was determined for the 'Tugela' plants injected with the individual HPLC fractions (Table 3.7 and Figure 3.21). Most of the flag leaves had increased levels of leaf rolling: SAM.1, SAM.2, SAM.3, SAM.4, SAM.5, SAM.6, SA1.10, SA1.11 and SA1.12 injected by 1.063±0.141, 1.296±0.353, 1.233±0.145, 1.167±0.167, 1.167±0.167 1.133±0.333, 1.500±0.255 and 1.250±0.144 respectively. The buffer, SA1.8, SA1.12, SA1.13 and SA1.15 injected plants remained the same. The non-injected, SAM.7, SAM.9 and SA1.14 injected plants had decreased rolling by 0.900±0.000, 0.952±0.047, 0.963±0.037 and 0.833±0.167 respectively.

Table 3.7. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'Tugela' plants post injection with the individual HPLC fractions of RWA gut extracts.

Treatment	Degree of leaf roll (Flag leaf)	Std error	Degree of leaf roll (Basal leaf)	Std error
Not injected	0.900	0.000	1.000	0.000
Buffer	1.000	0.000	0.806	0.100
[a]SAM.1	1.063	0.141	1.267	0.371
SAM.2	1.296	0.353	0.794	0.120
SAM.3	1.233	0.145	1.111	0.111
SAM.4	1.167	0.167	0.925	0.042
SAM.5	1.167	0.167	1.278	0.147
SAM.6	1.333	0.333	1.167	0.167
SAM.7	0.952	0.047	0.806	0.100
[b]SA1.8	1.000	0.000	1.167	0.167
SA1.9	0.963	0.037	0.948	0.052
SA1.10	1.500	0.255	1.056	0.242
SA1.11	1.250	0.144	1.349	0.206
SA1.12	1.000	0.000	1.262	0.145
SA1.13	1.000	0.000	1.000	0.000
SA1.14	0.833	0.167	0.952	0.047
SA1.15	1.000	0.000	1.333	0.333

a: HPLC fractions collected after injection of South African Mutant; **b:** HPLC fractions collected after injection of South African Biotype 1

The basal leaves were mostly rolled: SAM.1, SAM.3, SAM.5, SAM.6, SAM.8, SA1.10, SA1.11, SA1.12 and SA1.15 injected plants had increased levels of leaf rolling by 1.267±0.371, 1.111±0.111, 1.278±0.147, 1.167±0.167, 1.167±0.167, 1.056±0.242, 1.349±0.206, 1.262±0.145 and 1.333±0.333 respectively. Buffer, SAM.2, SAM.4, SAM.7, SA1.9 and SA1.14 injected plants were less rolled by 0.806±0.100, 0.794±0.120, 0.925±0.042, 0.806±0.100, 0.948±0.052 and 0.925±0.047 respectively. Only the non-injected and SA1.13 injected plants were unchanged.

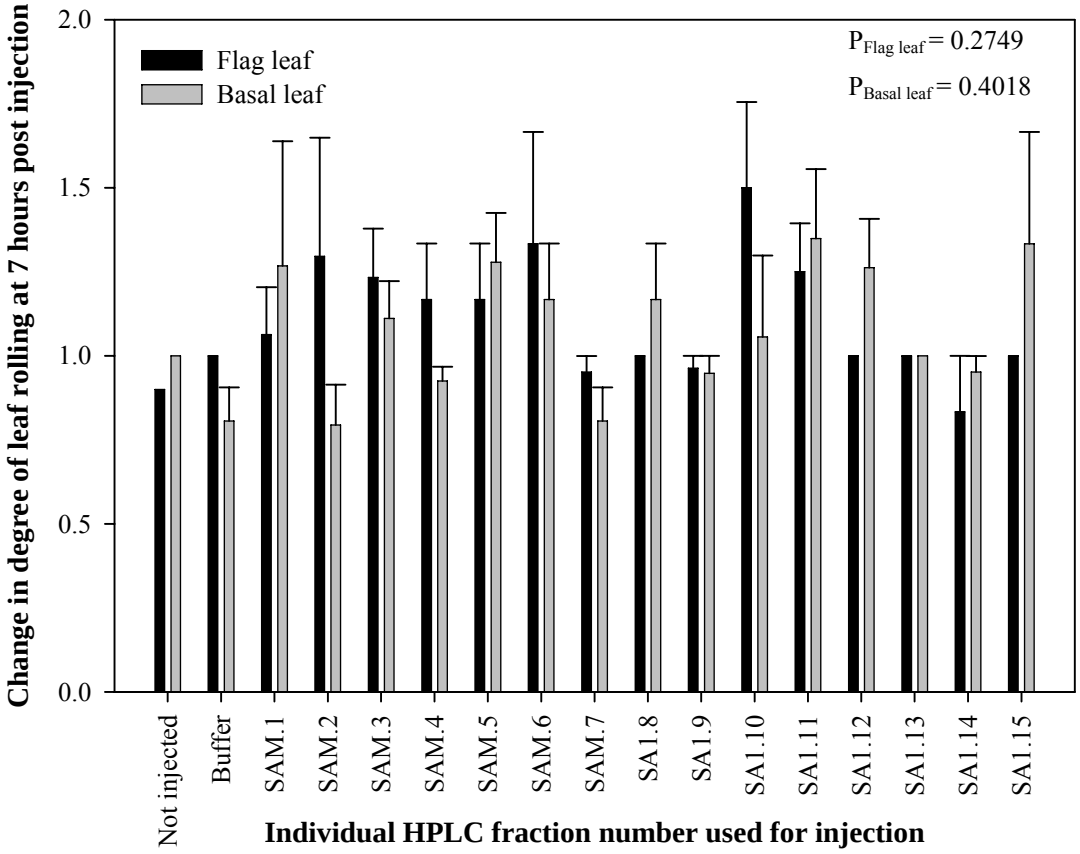

Figure 3.21. Degree of leaf rolling after 7 hours in the flag and basal leaves of 'Tugela' plants post injection with the individual HPLC fractions of RWA gut extracts (**SAM:** South African Mutant Biotype, **SA1:** South African Biotype 1) ($P_{Flag\ leaf}$ = 0.2749 and $P_{Basal\ leaf}$ = 0.4018,where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

3.4.6. Activity assays of defence-related enzymes

Enzyme activity assays were performed on injected plants to determine the effect of the injected fractions on these plants. The enzymes assayed were chosen based on their proposed function in the cells' defence responses and normal functioning.

Table 3.8. Peroxidase activity assays performed on proteins extracted at different time intervals from 'TugelaDN' wheat plants after injection with 20 µl SA1 RWA gut extract.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Peroxidase (µmol tetraguaiacol. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[b]
0 h. p. inj.[a]	1.050	0.100	23.633	0.000	1.00
1 h. p. inj	0.873	0.100	26.219	0.000	1.11
2 h. p. inj.	1.153	0.130	22.886	0.000	0.97
3 h. p. inj.	1.861	0.160	26.725	0.000	1.13
4 h. p. inj.	1.621	0.180	24.961	0.000	1.06
5 h. p. inj	1.454	0.115	27.748	1.963	1.17
6 h. p. inj	1.338	0.080	28.363	0.031	1.20
7 h. p. inj	1.284	0.110	38.303	6.991	1.62
8 h. p. inj	1.304	0.110	29.393	0.000	1.24
9 h. p. inj	1.146	0.125	26.194	12.089	1.11

a: hours post injection; **b:** activity calculated relative to that of non-injected plants, where the non-injected plants' activity = 1.00

3.4.6.1. Peroxidase Assay

Peroxidase is an indicator that the defence response has been activated. It is directly involved in producing and regulating the compounds of the reactive oxygen species (ROS) burst, which is initiated after an "attack" by a pathogen or pest has been launched (Lamb & Dixon, 1997). It is

expected that 'TugelaDN' will have higher expression after injection (Van der Westhuizen et al., 1998a).

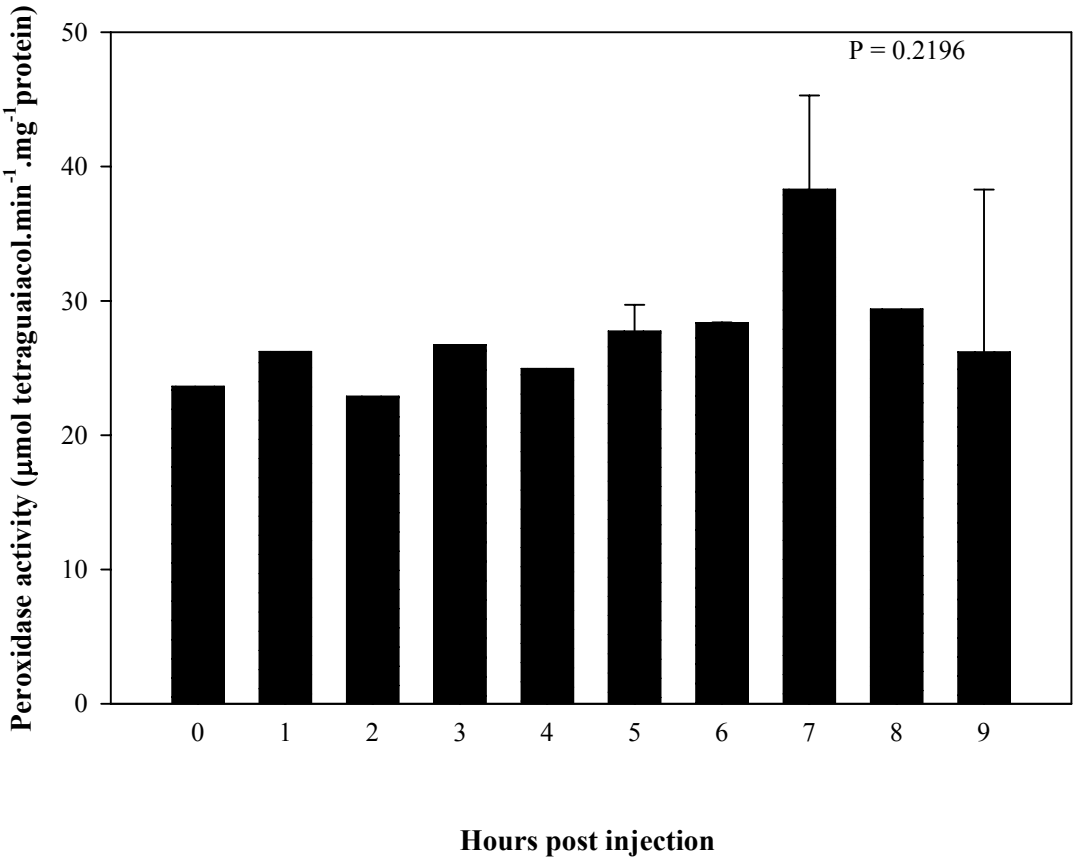

Figure 3.22. Peroxidase activity assays performed on proteins extracted at different time intervals from 'TugelaDN' wheat plants after injection with 20 µl SA1 RWA gut (P=0.2196 where $H_0 \neq H_A$ when P<0.01 or P>0.05).

3.4.6.1.1. Plants injected with gut proteins

To determine the time interval at which this defence response was the most active, plants were injected with purified gut extract at different time intervals for a period of nine hours and sampled at hourly intervals. Peroxidase activities were assayed in proteins extracted from these plants (Table

3.8 and Figure 3.22). The activity of plants 0 h. p. inj. was 23.633±0.000, 1 h. p. inj. was 26.219±0.000, 2 h. p. inj. was 22.886±0.000, 3 h. p. inj. was 26.725±0.000, 4 h. p. inj. was 24.961±0.000, 5 h. p. inj. was 27.748±1.963, 6 h. p. inj. was 28.363±0.031, 7 h. p. inj. was 38.303±6.991, 8 h. p. inj. was 29.393±0.000 and 9 h. p. inj was 26.194±12.089.

It was found that the most significant difference to that of the non-injected plants, was that of time interval 7 hours after expression started peaking at 5 hours and 6 hours. After that time interval, the activities decreased again. All subsequent experiments were performed for the duration of 7 hours.

Table 3.9. Peroxidase activities of 'TugelaDN' plants after injection with the pooled gut HPLC fractions.

Treatment	**Protein concentration of sample [mg ml^{-1}]**	**Fresh mass of sample (g)**	**Activity of Peroxidase (µmol tetraguaiacol. min^{-1}.mg^{-1} protein)**	**Std error**	**Relative Activity[c]**
Not injected	0.799	0.060	16.000	0.000	1.00
Buffer	0.924	0.080	19.800	3.103	1.24
SAM[a].1	0.890	0.110	26.963	0.673	1.69
SAM.2	1.074	0.110	19.219	0.124	1.20
SAM.3	0.962	0.107	20.413	1.656	1.28
SAM.4	0.938	0.097	19.470	1.047	1.22
SA1[b].1	0.940	0.117	20.797	1.359	1.30
SA1.2	0.971	0.123	27.164	0.500	1.70
SA1.3	0.999	0.120	17.608	0.260	1.10
SA1.4	1.133	0.123	16.866	1.276	1.05

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

3.4.6.1.2. Plants injected with HPLC fractions

To determine the most relevant HPLC batches, the plants' peroxidase activities were determined 7 hours post injection. The batches consisted of fractions collected during HPLC, which were subsequently pooled into batches (refer to Figure 3.6). Subsequently, the fractions in the batches, which caused significant enzyme activity in injected plants, were further investigated on enzymatic level. Henceforth pooled fractions are referred to as "pooled fractions" and the fractions in these samples are referred to as "individual fractions".

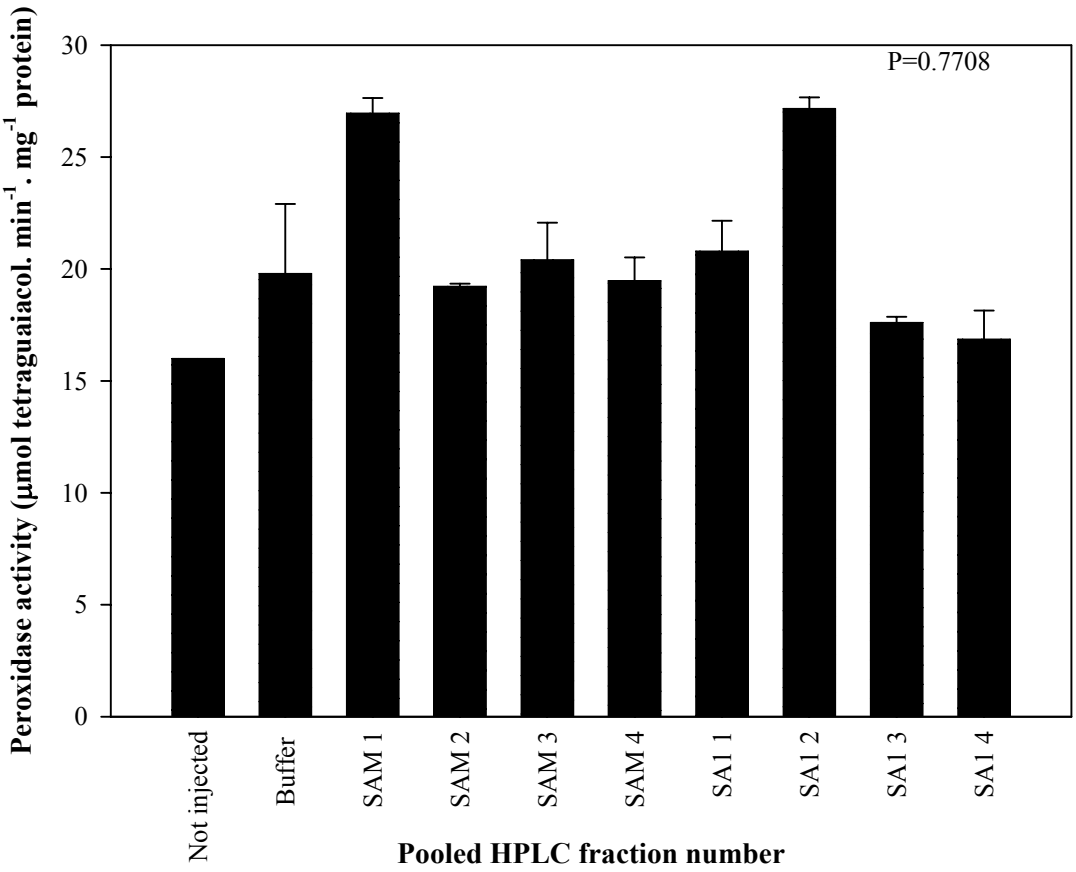

Figure 3.23. Peroxidase activity assays performed on proteins extracted from 'TugelaDN' wheat plants 7 hours after injection with the pooled gut HPLC fractions (P=0.7708 where $H_0 \neq H_A$ when P<0.01 or P>0.05).

All activities were greater than that of the non-injected plant, 16.000±0.000, and most were similar to that of the buffer-injected plants, 19.8±3.103, which was approximately 1.24 times more active than the non-injected (Table 3.9 and Figure 3.23). Plants injected with SAM.2, SAM.3, SAM.4, SA1.1, SA1.3 and SA1.4 had activities of 19.219±0.124, 20.413±1.656, 19.470±1.047, 20.797±1.359, 17.608±0.260 and 16.866±1.276, respectively.

Table 3.10. Peroxidase activities of 'TugelaDN' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Peroxidase (µmol tetraguaiacol. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.587	0.110	16.019	0.000	1.00
Buffer	0.533	0.080	18.863	0.000	1.18
SAM[a].1	0.749	0.110	12.627	0.287	0.79
SAM.2	0.666	0.100	11.573	0.123	0.72
SAM.3	0.762	0.120	12.755	0.229	0.80
SAM.4	0.856	0.085	19.532	0.380	1.22
SAM.5	0.333	0.080	43.089	3.518	2.69
SAM.6	0.788	0.115	13.553	0.227	0.85
SAM.7	0.635	0.120	18.384	0.078	1.15
SA1[b].8	0.644	0.095	16.989	0.990	1.06
SA1.9	0.727	0.120	33.991	2.734	2.12
SA1.10	0.524	0.085	38.014	3.344	2.37
SA1.11	0.520	0.120	19.897	0.817	1.24
SA1.12	0.911	0.130	11.392	0.013	0.71
SA1.13	0.824	0.100	7.019	0.189	0.44
SA1.14	0.560	0.105	21.529	2.021	1.34
SA1.15	0.641	0.105	15.068	1.465	0.94

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

However, the activities of plants injected with fractions SAM.1, 26.963±0.673, and SA1.2, 27.164±0.500, were approximately 1.7 times higher than that of the non-injected plants, prompting us to further examine the HPLC fractions this batch consisted of.

Table 3.11. Peroxidase activities of 'Tugela' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Peroxidase (µmol tetraguaiacol. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.566	0.103	5.531	0.225	1.00
Buffer	0.518	0.090	6.107	0.274	1.10
SAM[a].1	0.737	0.107	8.539	0.633	1.54
SAM.2	0.776	0.110	6.762	0.413	1.22
SAM.3	0.767	0.130	6.375	0.088	1.15
SAM.4	0.813	0.113	7.455	1.116	1.35
SAM.5	0.690	0.093	7.255	0.447	1.31
SAM.6	0.536	0.093	7.073	0.468	1.28
SAM.7	0.621	0.110	6.242	0.000	1.13
SA1[b].8	0.746	0.123	7.742	0.311	1.40
SA1.9	0.624	0.097	7.158	0.828	1.29
SA1.10	0.746	0.103	6.835	0.138	1.24
SA1.11	0.824	0.103	5.688	0.042	1.03
SA1.12	0.821	0.103	8.333	0.014	1.51
SA1.13	0.855	0.107	9.388	0.154	1.70
SA1.14	0.741	0.103	6.924	0.386	1.25
SA1.15	0.797	0.093	7.560	0.117	1.37

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

When the fractions of the two significant HPLC batches were injected into 'TugelaDN' plants, the activities of the plants differed more from each other in regards to their peroxidase activities (Table

3.10 and Figure 3.24). The activity of the buffer plants, relative to that of the non-injected plants, was 1.18 times higher. This was similar to the relative activity observed in the non-injected and buffer injected plants in Table 3.9 and Figure 3.23.

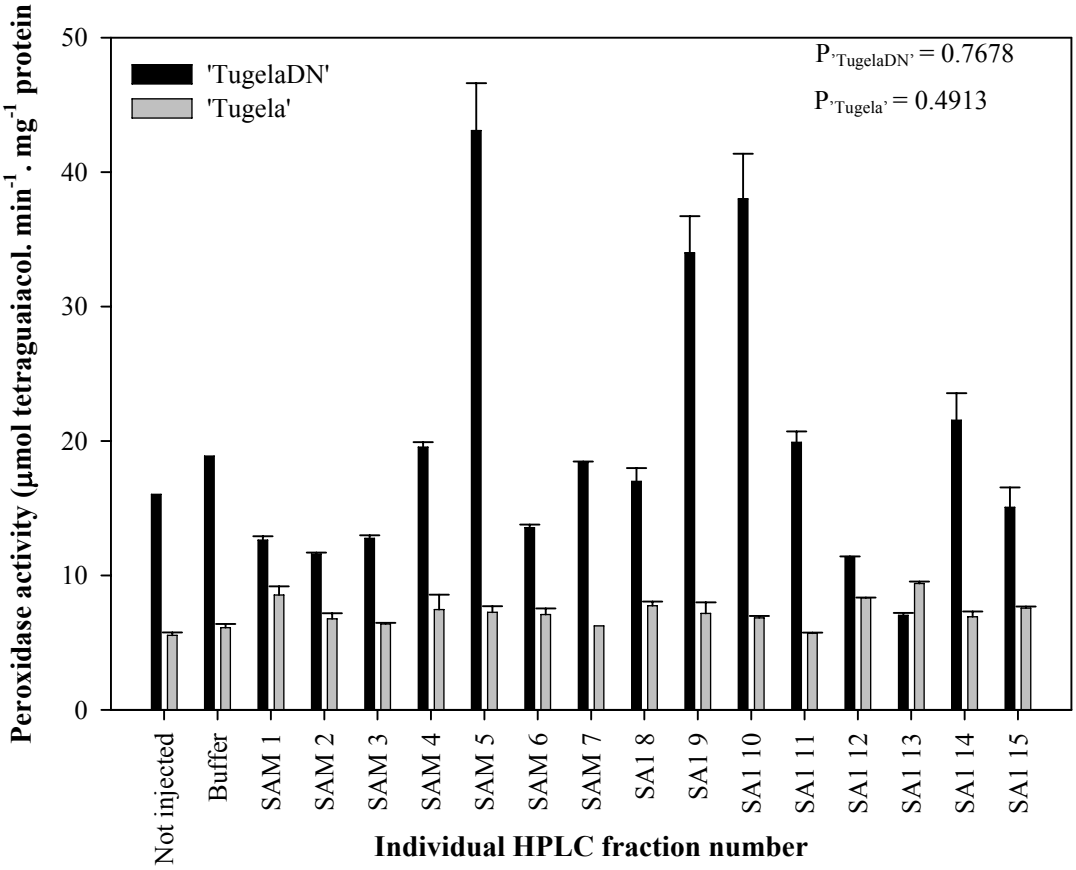

Figure 3.24. Peroxidase activity assays performed on proteins extracted from 'Tugela' (grey bars) and 'TugelaDN' (solid bars) wheat plants after injection with the individual gut HPLC fractions ($P_{'TugelaDN'}$ = 0.7678 and $P_{'Tugela'}$ = 0.4913, where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

However, although some of the plants' activity was similar to that of the buffer-injected plant, 18.863±0.000, namely SAM.4 at 19.532±0.380, SAM.7 at 18.384±0.078, SA1.11 at 19.897±1.817,

most of the expression was lower than that of the negative control at 12.627±0.287 for SAM.1, 11.573±0.123 for SAM.2, 12.755±0.229 for SAM.3, 11.392±0.013 for SA1.11, 7.019±0.189 for SA1.12 and 15.068±1.465 for SA1.15. The SA1.14 injected plants had higher activity than the controls at 21.529±2.021 (Table 3.10).

In contrast, fractions SAM.5 and SA1.9 and 10 caused a significant increase in peroxidase activity at 43.089±3.518, 33.991±2.734 and 38.014±3.344 respectively. These were 2.69, 2.12 and 2.37 fold higher than that of the non-injected plants.

When these same fractions were injected into 'Tugela' plants, a different pattern emerged (Table 3.11 and Figure 3.24). All the plants had lower activities than their 'TugelaDN' counterparts. The plants injected with buffer, at 6.107±0.274, were only 1.1 times more active than the non-injected plants at 5.531±0.225. Also, most of the activities were comparable to that of the buffer-injected plants. The activity of SAM.2 was 6.762±0.413, SAM.3 was 6.375±0.088, SAM.4 was 7.455±1.116, SAM.5 was 7.255±0.447, SAM.6 was 7.073±0.468, SAM.7 was 6.242±0.000, SA1.8 was 7.742±0.331, SA1.9 was 7.158±0.828, SA1.10 was 6.835±0.138, SA1.11 was 5.688±0.042, SA1.14 was 6.924±0.386 and SA1.15 was 7.560±0.117. The biggest differences were observed in SAM.1 at 8.539±0.633, SA1.12 at 8.333±0.014 and SA1.13 at 9.388±0.154, which were 1.54, 1.51 and 1.7 times more active than the negative control.

3.4.6.2. Chitinase Assay

Chitinase was chosen as an indicator of the plants' systemic acquired resistance (SAR) because it is an unbiased indicator of defence induced phenotypic traits (Heil & Ploss, 2006).

3.4.6.2.1. Plants injected with HPLC fractions

As with the peroxidase assays, chitinase activities were determined at 7 hours post injection.

Table 3.12 . Chitinase activities of 'TugelaDN' plants after injection with the pooled gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Chitinase (µmol GlcNac. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	1.050	0.100	5.358	0.000	1.000
Buffer	0.560	0.080	6.613	1.489	1.234
SAM[a].1	0.923	0.120	18.628	0.426	3.477
SAM.2	0.960	0.100	1.171	0.000	0.219
SAM.3	0.972	0.107	1.890	1.335	0.353
SAM.4	0.975	0.102	1.503	0.701	0.280
SA1[b].1	0.936	0.110	3.003	0.000	0.561
SA1.2	0.944	0.125	18.164	0.042	3.390
SA1.3	0.965	0.110	11.655	0.020	2.175
SA1.4	1.133	0.120	14.397	0.044	2.687

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

The buffer injected plants, at 6.613±1.489, were 1.234 times more active than the non-injected, at 5.358±0.000 (Table 3.12 and Figure 3.25). Most of the plants showed lower activity in comparison to the non-injected: SAM.2 at 1.171±0.000, SAM.3 at 1.890±1.335, SAM.4 at 1.503±0.701 and SA1.1 at 3.003±0.000. Plants injected with SA1.3 (11.655±0.020) and SA1.4 (14.397±0.044) had higher chitinase activity, whereas the activities of plants injected with fractions SAM.1, 18.628±0.426, and SA1.2, 18.164±0.042, were 3.477 and 3.390 times higher than that of the non-injected plants.

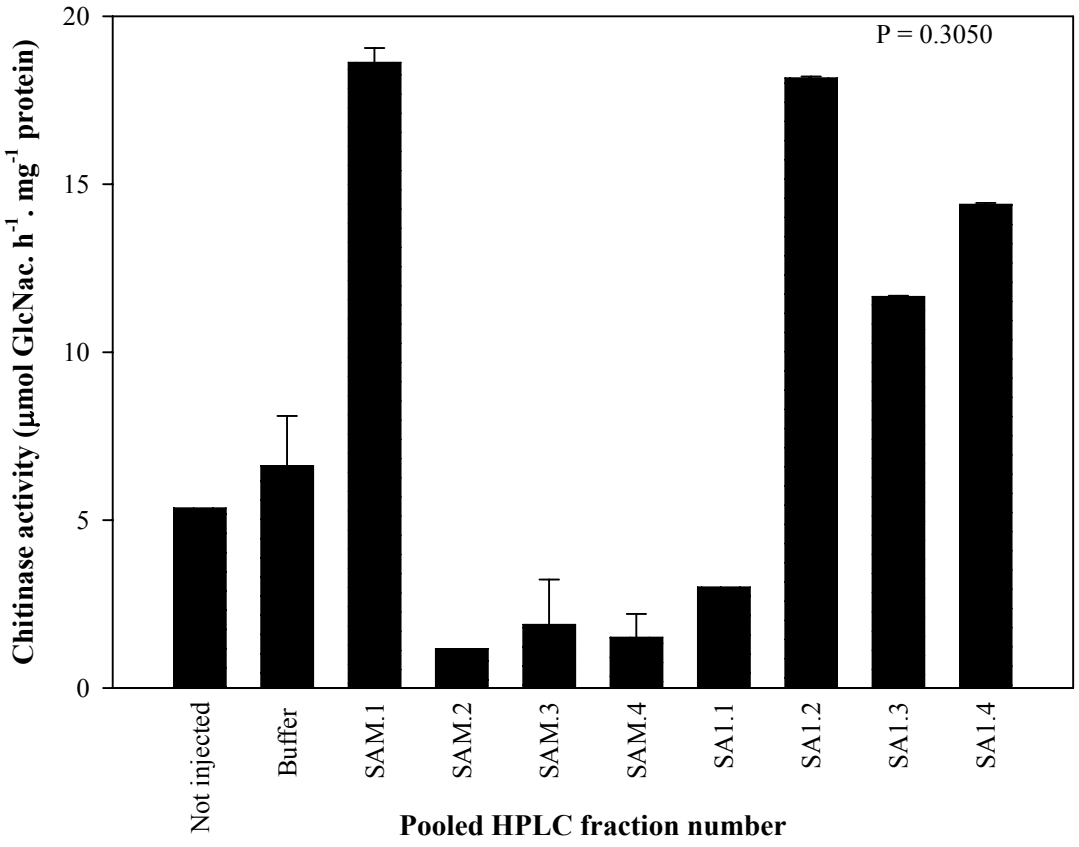

Figure 3.25. Chitinase activity assays performed on proteins extracted from 'TugelaDN' wheat plants after injection with the pooled gut HPLC fractions . (P = 0.7678, where $H_0 \neq H_A$ when P<0.01 or P>0.05).

The injection of 'TugelaDN' plants with fractions from batches SAM.1 and SA1.2 gave comparable patterns to that of the peroxidase activity assays (Table 3.13 and Figure 3.26).

Table 3.13. Chitinase activities of 'TugelaDN' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Chitinase (µmol GlcNac. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.924	0.080	7.002	0.030	1.000
Buffer	0.560	0.080	17.538	0.543	2.505
SAM[a].1	0.749	0.110	14.287	0.430	2.040
SAM.2	0.666	0.100	11.862	0.093	1.694
SAM.3	0.762	0.120	5.541	0.065	0.791
SAM.4	0.856	0.120	5.258	0.000	0.751
SAM.5	0.333	0.080	22.681	1.812	3.239
SAM.6	0.788	0.115	9.280	0.005	1.325
SAM.7	0.635	0.090	3.786	0.787	0.541
SA1[b].8	0.644	0.095	17.703	1.053	2.528
SA1.9	0.494	0.100	15.954	0.000	2.279
SA1.10	0.524	0.085	23.176	4.314	3.310
SA1.11	0.520	0.120	9.236	0.169	1.319
SA1.12	0.911	0.130	9.916	0.530	1.416
SA1.13	0.824	0.100	7.837	0.112	1.119
SA1.14	0.560	0.105	8.635	0.342	1.233
SA1.15	0.733	0.130	12.271	0.000	1.753

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

The chitinase activity of buffer-injected plants (17.538±0.543) were 2.505 times more active than the non-injected plants, 7.002±0.030. Although three plants had less activity than the non-injected plants, SAM.3 (5.541±0.065), SAM.4 (5.258±0.000) and SAM.7 (3.786±0.787), most of them had lower activity when compared to the buffer-injected plants. Plants injected with SAM.2, SAM.6,

SA1.9, SA1.11, SA1.12, SA1.13, SA1.14 and SA1.15 had activities of 11.862±0.093, 9.280±0.005, 15.954±0.000, 9.236±0.169, 9.916±0.530, 7.837±0.112, 8.635±0.342 and 12.271±0.000. The activity of SA1.8 was higher at 17.703±1.053. However, two fractions had much higher levels of activity in comparison to the buffer-injected plants: SAM.5, at 22.681±1.812, and SA1.10 at 23.176±4.314. These were 3.239 and 3.310 times more active than the non-injected plants.

Table 3.14. Chitinase activities of 'Tugela' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Chitinase (µmol GlcNac. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.527	0.105	12.393	0.565	1.000
Buffer	0.562	0.100	4.020	0.221	0.324
SAM[a].1	0.746	0.095	9.169	0.854	0.740
SAM.2	0.795	0.110	8.106	0.399	0.654
SAM.3	0.763	0.130	2.966	0.179	0.239
SAM.4	0.750	0.105	8.237	0.430	0.665
SAM.5	0.766	0.100	9.920	0.926	0.800
SAM.6	0.501	0.100	3.966	0.172	0.320
SAM.7	0.605	0.105	8.129	1.066	0.656
SA1[b].8	0.807	0.125	8.428	0.602	0.680
SA1.9	0.622	0.105	6.293	0.389	0.508
SA1.10	0.815	0.110	8.286	0.157	0.669
SA1.11	0.816	0.100	5.834	0.115	0.471
SA1.12	0.842	0.095	5.257	0.540	0.424
SA1.13	0.936	0.110	9.311	0.013	0.751
SA1.14	0.723	0.100	2.363	0.218	0.191
SA1.15	0.851	0.095	1.636	0.100	0.132

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

When the 'Tugela' plants were injected with the same fractions, none of the plants had higher activity than the non-injected sample (12.393±0.565) (Table 3.14 and Figure 3.26). Even the buffer-injected plants (4.020±0.221) had lower activity. The fractions with the highest activity relative to the non-injected plants were SAM.1, at 9.169±0.854, SAM.5 at 9.920±0.926 and SA1.13 at 9.311±0.013. Plants injected with SAM.2, SAM.3, SAM.4, SAM.6, SAM.7, SA1.8, SA1.9, SA1.10, SA1.11, SA1.12, SA1.14 and SA1.15 had activities of 8.106±0.399, 2.966±0.179, 8.237±0.430, 3.966±0.172, 8.129±1.066, 8.428±0.602, 6.293±0.389, 8.286±0.157, 5.834±0.115, 5.257±0.540, 2.636±0.218 and 1.636±0.100 respectively.

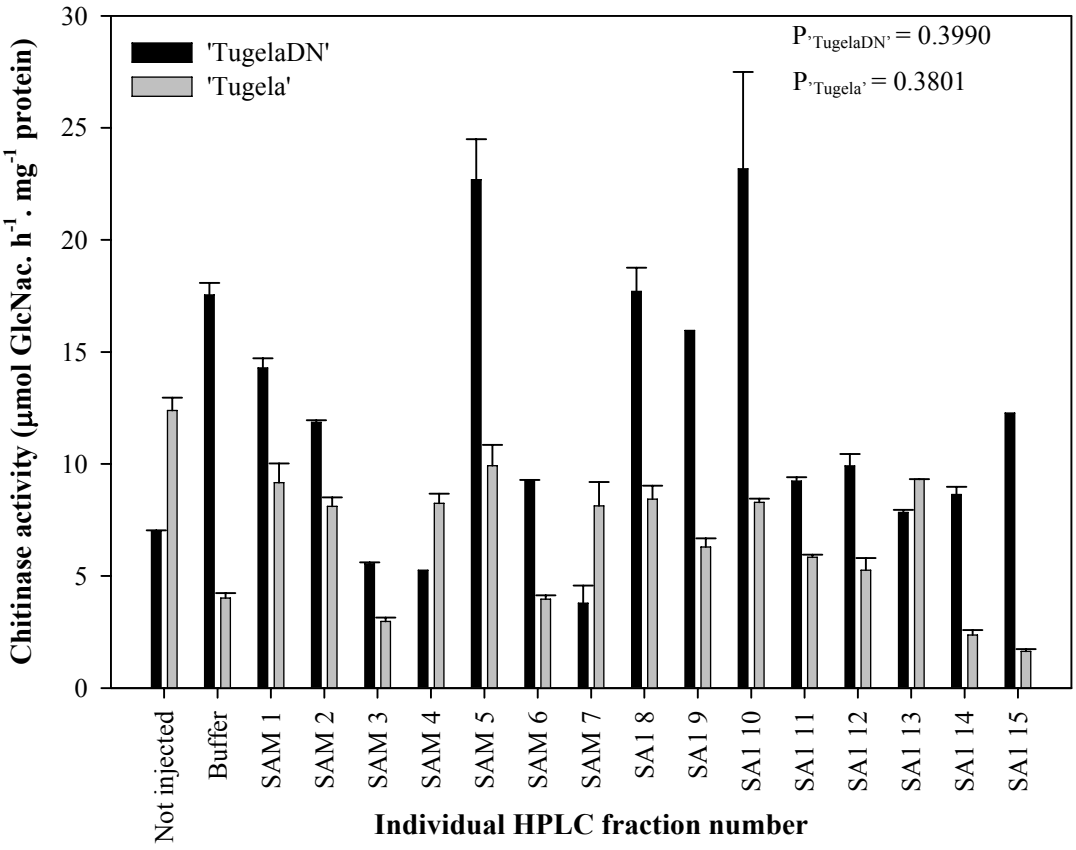

Figure 3.26. Chitinase activity assays performed on proteins extracted from 'Tugela' (grey bars) and 'TugelaDN' (solid bars) wheat plants after injection with the individual gut HPLC fractions. ($P_{'TugelaDN'}$ = 0.3990 and $P_{'Tugela'}$ = 0.3801, where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

3.4.6.3. Catalase assay

Catalase, in conjunction with peroxidase, has a function in maintaining H_2O_2 at stable levels in normal cells (Blokhina et al., 2003). Thus, in the event of higher levels of this reactive oxygen species, one would expect the enzyme to increase in concentration too.

3.4.6.3.1. Plants injected with HPLC fractions

Catalase was first assayed in 'TugelaDN' plants, which had been injected with the pooled HPLC fractions (Table 3.15 and Figure 3.27). The non-injected and buffer-injected plants had the same activity at 0.667±0.000. SAM.1, SAM.2, SAM.3, SA1.1 and SA1.3 injected plants had lower activities at 0.439±0.020, 0.659±0.148, 0.544±0.032, 0.651±0.015 and 0.285±0.065.

Table 3.15 . Catalase activities of 'TugelaDN' plants after injection with the pooled gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Catalase (µmol H_2O_2 degraded. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.799	0.060	0.667	0.000	1.000
Buffer	1.050	0.100	0.667	0.000	0.999
SAM[a].1	0.890	0.110	0.439	0.020	0.658
SAM.2	1.074	0.110	0.659	0.148	0.987
SAM.3	0.962	0.107	0.544	0.032	0.815
SAM.4	0.938	0.097	0.692	0.015	1.036
SA1[b].1	0.940	0.117	0.651	0.035	0.976
SA1.2	0.971	0.123	0.701	0.040	1.050
SA1.3	0.999	0.120	0.285	0.065	0.428
SA1.4	1.133	0.123	0.677	0.103	1.015

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

Plants injected with SAM.4 (0.692±0.015), SA1.2 (0.701±0.040) and SA1.4 (0.677±0.103) had higher activities than those of the negative control.

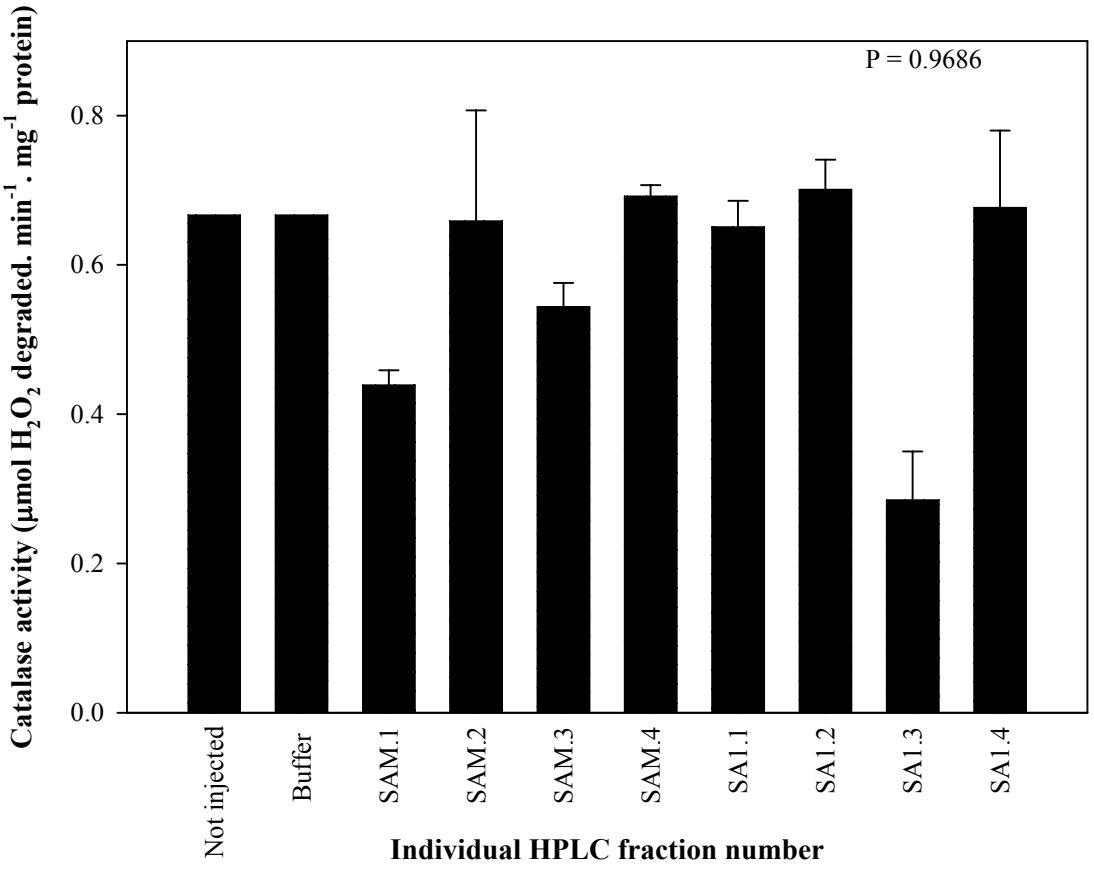

Figure 3.27. Catalase activity assays performed on proteins extracted from 'TugelaDN' wheat plants after injection with the pooled gut HPLC fractions (P = 0.9686, where $H_0 \neq H_A$ when P<0.01 or P>0.05).

In the plants injected with the individual HPLC fractions, the catalase activities of most of the 'TugelaDN' plants were higher than that of the negative controls: 1.125±0.022 for the non-injected and 1.040±0.064 for the buffer-injected (Tables 3.16 and Figure 3.28). Plants injected with SAM.1, SAM.2, SAM.3, SAM.4, SA1.8, SA1.9, SA1.10, SA1.12 and SA1.13 had activities of 1.474±0.021,

1.299±0.108, 1.262±0.020, 1.233±0.101, 1.212±0.101, 1.438±0.083, 1.321±0.035, 1.189±0.070 and 1.149±0.112. Activity from injection with SAM.5 was 1.025±0.057, SAM.6 was 1.051±0.054, SAM.7 was 0.975±0.070, SA1.11 was 1.116±0.131, SA1.14 was 1.123±0.063 and SA1.15 was 1.104±0.084 and these activities were all lower than that of the negative controls.

Table 3.16. Catalase activities of 'TugelaDN' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Catalase (µmol . H_2O_2 degraded. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.566	0.103	1.125	0.022	1.000
Buffer	0.518	0.090	1.040	0.064	0.925
SAM[a].1	0.737	0.107	1.474	0.021	1.311
SAM.2	0.776	0.110	1.299	0.108	1.155
SAM.3	0.767	0.130	1.262	0.020	1.122
SAM.4	0.813	0.113	1.233	0.101	1.096
SAM.5	0.690	0.093	1.025	0.057	0.911
SAM.6	0.536	0.093	1.051	0.054	0.935
SAM.7	0.621	0.110	0.975	0.070	0.866
SA1[b].8	0.746	0.123	1.212	0.101	1.078
SA1.9	0.624	0.097	1.438	0.083	1.279
SA1.10	0.746	0.103	1.321	0.035	1.175
SA1.11	0.824	0.103	1.116	0.131	0.992
SA1.12	0.821	0.103	1.189	0.070	1.058
SA1.13	0.855	0.107	1.149	0.112	1.022
SA1.14	0.741	0.103	1.123	0.063	0.998
SA1.15	0.797	0.093	1.104	0.084	0.982

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

The 'Tugela' non- and buffer injected plants had activities of 0.426±0.041 and 0.443±0.056 respectively (Table 3.17 and Figure 3.28). Most of the plants injected with the HPLC fractions had lower activities than these negative controls: SAM.2, SAM.3, SAM.4, SAM.6, SAM.7, SA1.8, SA1.9, SA1.10, SA1.11, SA1.12, SA1.13, SA1.14 and SA1.15 had values of 0.422±0.061, 0.396±0.026, 0.409±0.047, 0.302±0.022, 0.399±0.019, 0.317±0.039, 0.290±0.039, 0.285±0.003, 0.280±0.033, 0.331±0.018, 0.269±0.041, 0.336±0.024 and 0.319±0.038 respectively.

Table 3.17. Catalase activities of 'Tugela' plants after injection with the individual gut HPLC fractions.

Treatment	Protein concentration of sample [mg ml^{-1}]	Fresh mass of sample (g)	Activity of Catalase (µmol H_2O_2 degraded. min^{-1}.mg^{-1} protein)	Std error	Relative Activity[c]
Not injected	0.924	0.080	0.426	0.041	1.000
Buffer	0.560	0.080	0.443	0.056	1.040
SAM[a].1	0.749	0.110	0.447	0.050	1.050
SAM.2	0.666	0.100	0.422	0.061	0.992
SAM.3	0.762	0.120	0.396	0.026	0.930
SAM.4	0.856	0.085	0.409	0.047	0.959
SAM.5	0.333	0.080	0.449	0.005	1.054
SAM.6	0.788	0.115	0.302	0.022	0.710
SAM.7	0.635	0.120	0.399	0.019	0.938
SA1[b].8	0.644	0.095	0.317	0.039	0.745
SA1.9	0.727	0.120	0.290	0.039	0.682
SA1.10	0.524	0.085	0.285	0.003	0.670
SA1.11	0.520	0.120	0.280	0.033	0.659
SA1.12	0.911	0.130	0.331	0.018	0.776
SA1.13	0.824	0.100	0.269	0.041	0.631
SA1.14	0.560	0.105	0.336	0.024	0.789
SA1.15	0.641	0.105	0.319	0.038	0.748

a: South African Mutant; **b:** South African Biotype 1; **c:** activity calculated relative to that of non-injected plants, where the non-injected plants'activity = 1.00

Only plants injected with SAM1 and SAM.5 had comparable activities to the control plants' at 0.447±0.050 and 0.449±0.005.

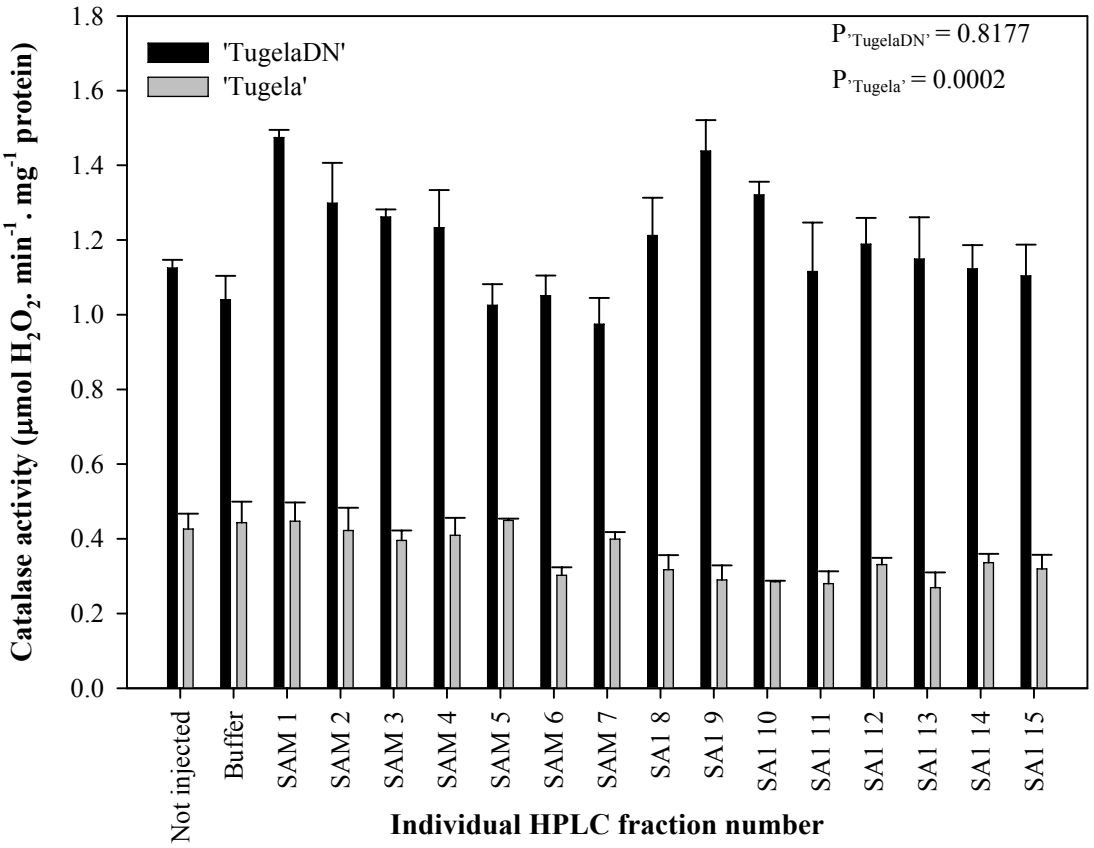

Figure 3.28. Catalase activity assays performed on proteins extracted from 'Tugela' (grey bars) and 'TugelaDN' (solid bars) wheat plants after injection with the individual gut HPLC fractions ($P_{'TugelaDN'}$ = 0.8177 and $P_{'Tugela'}$ = 0.0002, where $H_0 \neq H_A$ when $P<0.01$ or $P>0.05$).

3.4.7. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.4.7.1. Wheat protein profiles in response to injection with RWA proteins

SDS-PAGE was performed using the proteins extracted from the injected wheat samples to determine if any changes occur within the proteomes after injection and also to compare if different changes occurred between the two wheat cultivars.

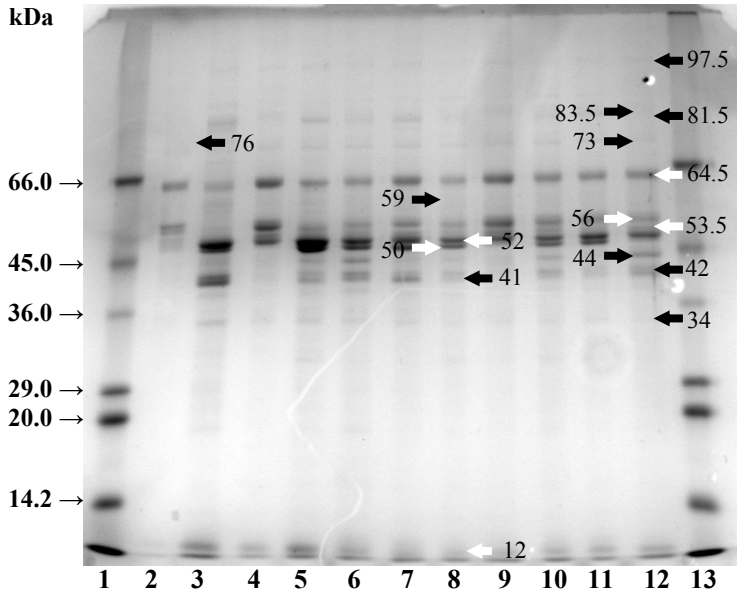

Figure 3.29. SDS-PAGE analysis of 'TugelaDN' proteins (3µg per lane) extracted at different time intervals after injection with 20 µl SA1 RWA gut extracts (**1. & 13.** Dalton MVI; **2.** Not injected; **3.** Buffer; **4.** 1 h. p. inj.; **5.** 2 h. p. inj.; **6.** 3 h. p. inj.; **7.** 4 h. p. inj.; **8.** 5 h. p. inj.; **9.** 6 h. p. inj.; **10.** 7 h. p. inj.; **11.** 8 h. p. inj.; **12.** 9 h. p. inj.). {White arrows indicate bands expressed in all lanes and black arrows indicate differentially expressed bands}.

In the time trial experiment done on 'TugelaDN' plants, it can be seen that changes occur in the protein profile of these plants at the different time intervals (See Figure 3.29). Protein profiles of the non-injected plants' proteins seem totally different in comparison to the injected plants (Figure 3.29, lane 2). Protein bands of 64.5 kDa, 56 kDa, 53.5 kDa, 52 kDa, 50 kDa and 12 kDa in size

were present in all the treatments. A band of 76 kDa only occurs in the non-injected plants (lane 2). In the other lanes (3-12), differentially expressed bands of the following sizes appear in response to injection: 97.5 kDa, a double band of 83.5 and 81.5 kDa, 73 kDa, 59 kDa, three bands of 44 kDa, 42 kDa and 41 kDa, and 34 kDa. The three bands are expressed at a lower intensity from time intervals 5 to 8 hours post injection, but seem to recover by time 9 hours.

The profiles of the susceptible cultivar, 'Tugela' are presented in Figure 3.30. Bands of the following sizes occur throughout: 97.5 kDa, 83.5 kDa, 81.5 kDa, 73 kDa, 64.5 kDa, 53.5 kDa, 52 kDa, 50 kDa, 44 kDa, 34 kDa and 12 kDa. Although the polypeptide profiles of plants injected with the SA1 fractions seem similar to each other and the non-injected plants, it appears as though there were less of bands 52 kDa, 50 kDa, 44 kDa and 34 kDa (Figure 3.30 A, lanes 6-10) in plants injected with SAM.3, 4, 5, 6 and 7.

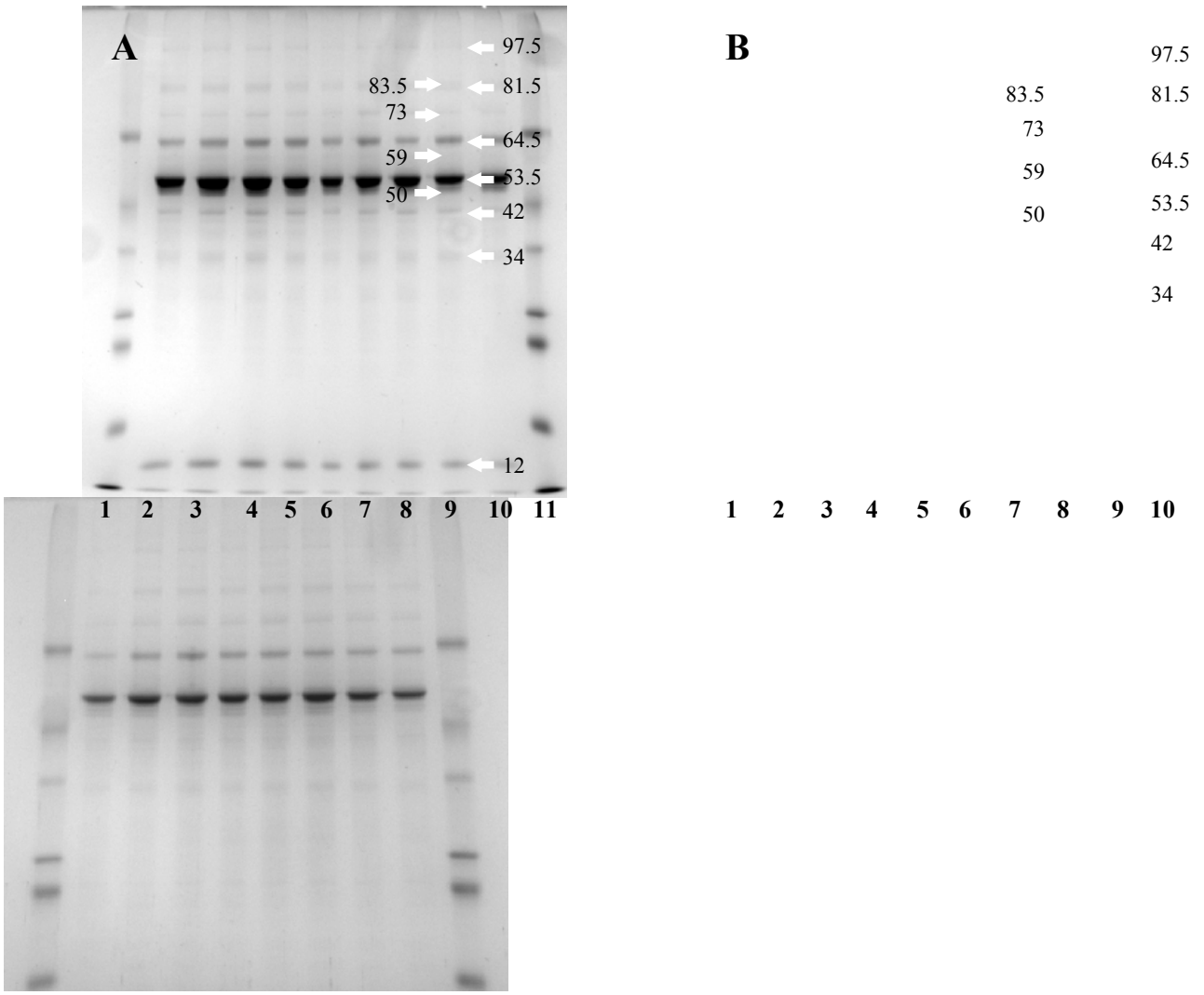

Figure 3.30. SDS-PAGE analysis of proteins (3µg per lane) extracted from 'Tugela' injected with the individual SAM (A) and SA1 (B) HPLC fractions. (**A: 1. & 11.** Dalton MVI; **2.** Not injected; **3.** Buffer; **4.** SAM.1; **5.** SAM.2; **6.** SAM.3; **7.** SAM.4; **8.** SAM.5; **9.** SAM.6; **10.** SAM.7. **B: 1. & 10.** Dalton MVI; **2.** SA1.8; **3.** SA1.9; **4.** SA1.10; **5.** SA1.11; **6.** SA1.12; **7.** SA1.13; **8.** SA1.14; **9.** SA1.15). {White arrows indicate bands expressed in all lanes}.

In contrast, the 'TugelaDN' profiles differ much from their 'Tugela' counterparts. As observed in the time trial gel (Figure 3.31), the non-injected plants' proteins are different in comparison to the injected plants (Figure 3.31, lane 2). Protein bands of the following sizes were shared: 97.5 kDa, a double band of 83.5 and 81.5 kDa, 73 kDa, 59 kDa, three bands of 44 kDa, 42 kDa and 41 kDa, and 34 kDa. The three proteins of 44 kDa, 42 kDa and 41 kDa, which lessen in intensity in the time trial gel (Figure 3.29), are also expressed at lower concentration in the plants injected with fractions SAM.5 and SA1.10 (Figure 3.31 A., lane 8 and Figure 3.31 B., lane 6). (These are the final HPLC

fractions which caused the most significant enzyme activity differences in Section 3.4.6). Unlike the time trial gel, the band of 64.5 kDa, is expressed less in these gels in response to injection. Thus only bands of 56 kDa, 53.5 kDa, 52 kDa and 12 kDa are expressed in all the injected plants to the same level.

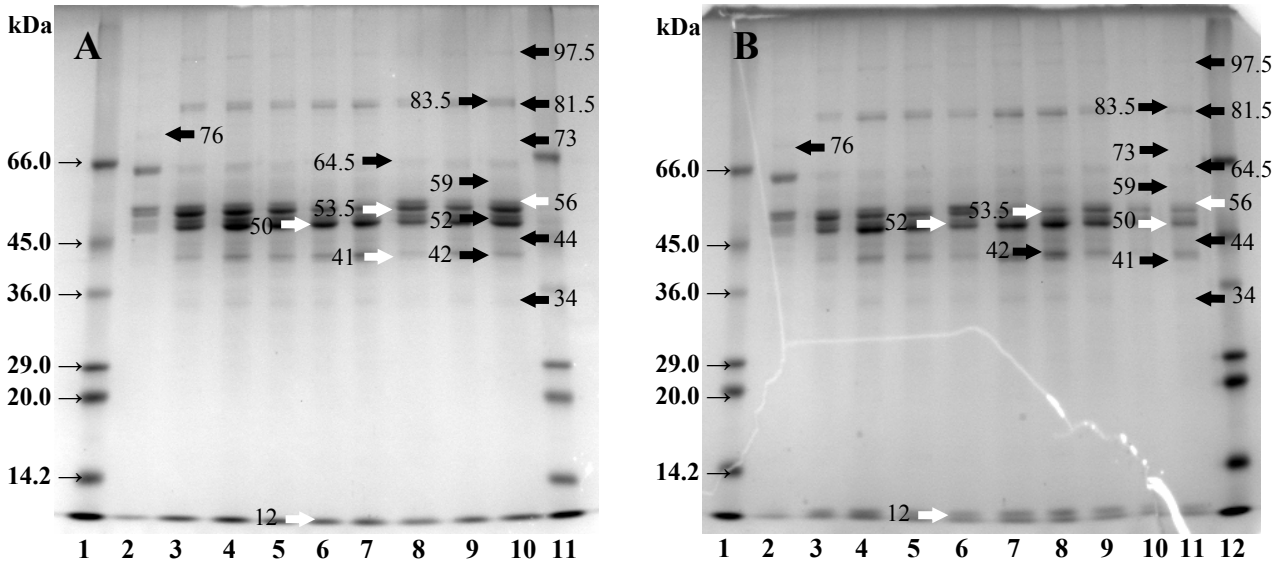

Figure 3.31. SDS-PAGE analysis proteins (3µg per lane) extracted from 'TugelaDN' injected with the individual SAM (A) and SA1 (B) HPLC fractions (**A: 1. & 11.** Dalton MVI; **2.** Not injected; **3.** Buffer; **4.** SAM.1; **5.** SAM.2; **6.** SAM.3; **7.** SAM.4; **8.** SAM.5; **9.** SAM.6; **10.** SAM.7. **B: 1. & 12.** Dalton MVI; **2.** Not injected; **3.** Buffer; **4.** SA1.8; **5.** SA1.9; **6.** SA1.10; **7.** SA1.11; **8.** SA1.12; **9.** SA1.13; **10.** SA1.14; **11.** SA1.15.). {White arrows indicate bands expressed in all lanes and black arrows indicate differentially expressed bands}.

3.4.7.2. RWA protein profiles

The RWA proteins were run at different protein concentrations to be able to visualize all the bands (Figure 3.32). Nineteen protein bands were observed for both of the biotypes' proteomes with only two distinct differences observed between the two. The first difference is the presence of an additional band of 29.4 kDa in the SA1 biotype (Figure 3.32, lanes 7-10) which is absent from the SAM biotype's profile (Figure 3.32, lanes 2-5). Likewise, the SAM profile has a band of about 56.7 kDa, which does not occur in the SA1 profile. In addition to these, the following bands were present

for all the lanes: 103 kDa, 100 kDa, 82.7 kDa, 79.7 kDa, 78 kDa, 73.7 kDa, 69.3 kDa, 66.9 kDa, 63.4 kDa, 58.7 kDa, 54.2 kDa, 48 kDa, 44.3 kDa, 41.4 kDa, 38.3 kDa, 30.4 kDa, 23.1 kDa and 12.7 kDa.

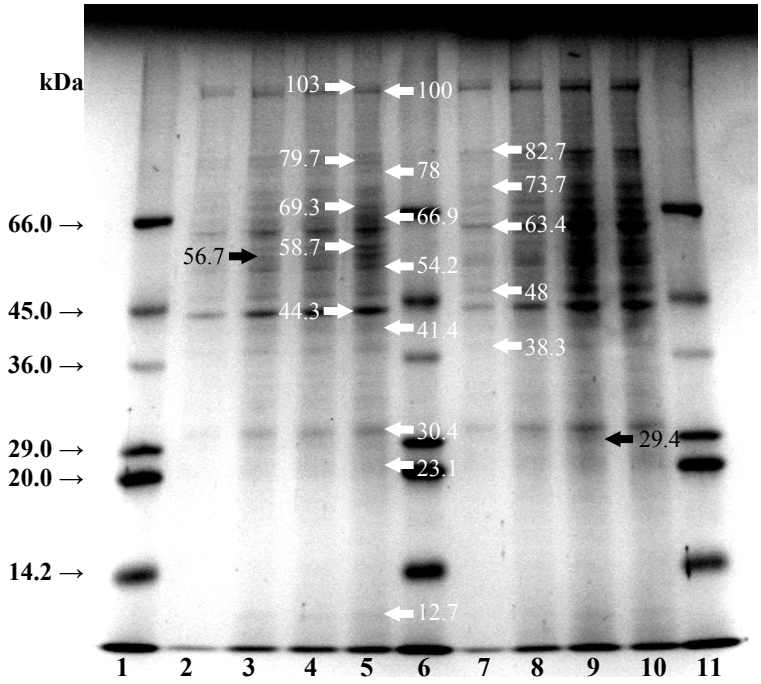

Figure 3.32. SDS-PAGE of 2 dilution series of proteins extracted from the two RWA biotypes. (**1. & 6. & 11.** Dalton M VI; **2.-6. SAM Biotype: 2.** 1 µg; **3.** 2 µg; **4.** 3 µg; **5.** 4 µg. **7.-10. SA1 Biotype: 7.** 1 µg; **8.** 2 µg; **9.** 3 µg; **10.** 4 µg.) {White arrows indicate bands expressed in all lanes and black arrows indicate differentially expressed bands }.

3.5. Discussion

The Russian wheat aphid is an insect that feeds with a pierce and suck feeding mechanism. It does so by injecting saliva into the phloem, and ingesting the saliva-photoassimilate mixture into its gut. It is proposed that the insect's saliva contains a toxin (Belefant-Miller et al., 1994), which elicits a specific plant defence response in resistant cultivars and causes the symptoms associated with RWA feeding in susceptible ones.

A model has been suggested to explain the mode of defense action in resistant plants in response to RWA feeding (Botha et al., 2006): As the aphid feeds, its elicitor is recognized by receptors of *Resistance* (*R*)-genes in the plants' cell walls. This interaction results in the activation of the hypersensitive response (HR) and a subsequent cascade of defence events: the release of reactive oxygen species, such as peroxidase and superoxide; the thickening of cell walls; and the activation of other defence responses. Secondly, salicylic acid (SA) and jasmonic Acid (JA) mediated pathways result in the expression of genes involved in programmed cell death (PCD) and maintenance of chloroplast integrity and photosynthetic processes (such as ATP synthase and thioredoxin), and *Pathogenesis-Related* (*PR*)-genes (i.e. chitinases and glucanases). Affected cells undergo HR-mediated PCD and this is observed as necrotic lesions on the leaves (Botha et al., 2005). The rest of the plant's cells experience Systemic acquired resistance (SAR). In contrast, the susceptible plants' leaves start rolling (Walters et al., 1980) and later suffer from chlorosis as the plants' chloroplasts are damaged by their disrupted redox potential (Burd & Elliott, 1996). The latter is observed as longitudinal streaking in extreme cases of infestation (Walters et al, 1980).

It has been proposed that the saliva probably contains amino acids, phenolics, proteolytic enzymes (such as cellulases, esterases and lipases) and compounds involved in the feeding mechanism (Fouché et al., 1984; Miles, 1972, 1999; Ni & Quisenberry, 2003). It follows that the elicitor is probably one of the above.

Support for the proteinaceous character of the elicitor was obtained during a study by Li et al. (in press). They injected 'Gamtoos S' and 'Gamtoos R' plants with total US1 or US2 RWA extract, which had been separated into protein and non-proteinaceous phase. Resistant plants ('Gamtoos R') had increased levels of peroxidase, catalase and glucanase. Leaf rolling was observed almost immediately in the susceptible 'Gamtoos S' plants.

Based on these results, we decided to further purify the RWA protein extracts and inject those into plants. In order to do so, we extracted guts, because our prior knowledge of RWA feeding suggests that the elicitor present in the saliva would be present in the guts. Our study also differed in respect to the RWA biotypes used for protein extraction and the plants injected: we determined the effect of SAM and SA1 gut extractions on 'Tugela' and 'TugelaDN' plants. The effects were monitored by observing leaf rolling in the leaves and also by determining the activities of peroxidase, chitinase and catalase. These proteins were selected because they would acts as markers for reactive oxygen species (ROS) (Lamb & Dixon, 1997), SAR (Ward et al., 1991) and normal chloroplast function (Ni & Quisenberry, 2003), respectively.

The South African 1 (SA1) biotype is maintained on the susceptible wheat cultivar 'Scheepers'. The other population, South African Mutant (SAM) is maintained on the resistant cultivar 'TugelaDN'. The latter population was developed by forcing the insects to consume the resistant cultivar instead. Over a period of five years the population is now so altered from the original wild type population in respect to its feeding preference on the resistant cultivar, that it can be considered a biotype in its own right.

There was no specific pattern observed in regards to the amount of protein extracted between the two RWA biotypes. Head proteins were less than the gut proteins (Table 3.1). This is surprising since the gut extracts originating from guts of 500 individuals and the heads of 300. One can then assume that the heads had a much higher protein density than the guts. This is probably not too unexpected when one considers that the heads contain many different features, such as the eyes, mouth pieces, glands, antennae and two legs. In contrast, the gut protein extracts should contain the lining of the organ, proteins associated with the digestion of photoassimilates and the proteins present in the ingested phloem (Figures 3.1 and 3.2).

Different profiles were obtained for the different types of extracts of RWA proteins (Figures 3.10, 3.11 and 3.12). As expected, the total protein extracts yielded the most intricate profiles. This is probably owing to the fact that the whole aphids' protein complement was resolved, whether it was feeler or antennae protein, in addition to gut or saliva. The most significant difference between these two biotypes' profiles, was a single peak in the SAM's and a double peak in the SA1's at the retention times of 25 and 26 minutes. Similarly, the head extract profiles also contain a single difference: a peak is present at the retention time of 22.47 of the SA1 profile, whereas it is absent in the SAM profile. The gut profiles were similar in regards to the amounts of peaks and their locations; however, most of the peaks differed in their intensities. The large peak at retention time 23 minutes, which is present in all the total and gut HPLC profiles, is absent in the head profiles. This indicates that the large peak is a protein present in the guts of the insects. Perhaps the differences observed in all the profiles are a reflection of changes that ensued because of selection pressure that was placed on the SAM biotype when it was forced to adapt to feeding on the resistant cultivar 'TugelaDN' (Habibi et al., 2001).

The head proteins were extracted in the hope of obtaining samples containing the salivary glands (Figure 3.2), the source of the alleged elicitors present in the saliva (Belefant-Miller et al., 1994). However, only the single small peak at retention time 22.47 in the SA1 profile distinguishes the two. Perhaps the concentration of glands in relation to total head was too low to be detected. It is probable that the head contains mostly conserved proteins with specific function and thus little variation is expected to occur between the biotypes. This might apply to the glands' structure: they are probably similar, but only differ in regards to the composition of the saliva they produce.

Perhaps differences occur in the mouth pieces of the two insects to facilitate different feeding mechanism styles: Girma et al. (1992) reported that RWA switched to feeding only in the mesophyll instead of the phloem of the resistant 'CI17882' plants. It is possible that the SAM mutants could have adapted the manner of feeding on a mechanical as well as physiological or biochemical level. Seeing as the two biotypes were reared on two different wheat cultivars, this could account for some of the differences. For example, perhaps the surface structures of the 'Scheepers' plants are different from that of the 'TugelaDN' plants. The excessive amounts of trichomes present on the 'TugelaDN', but not 'Tugela' plants, possibly affect normal RWA feeding (Bahlmann et al., 2003). This, in combination with differing composition of the cell walls, might lead to the aphid's stylet increasing in length to assist in probing. Or, the composition of the phloem might differ in the two cultivars leading to the insect ingesting (Mittler & Dadd, 1965) and digesting the obtained nutrients in diverse ways, and requiring a different combination and intricate balance of digestive enzymes to achieve this (Miles, 1999). This might clarify why the gut proteins seem to be composed of a greater variety of proteins than the heads when one compares the number of peaks. It might also explain why the corresponding peaks in the two gut profiles usually differed in absorbance from each other.

In the SDS-PAGE performed on the RWA, only two differences were observed between our two biotypes. A larger band of 56.7 kDa was differentially expressed in the SAM RWAs and a smaller band of 29.4 kDa was present only in the SA1 proteome (Figure 3.32). If only a single protein difference does occur between the two biotypes, then it is possible that these two proteins have the potential of being the *Avrs*. It indicates that all other proteins are conserved in the two biotypes, but that the differing proteins had to alter in order for the insects to be able to function normally without suffering detection during feeding. Each of the biotypes had a total of nineteen protein bands which

could be visualized. This implies that the insects consist of very few proteins to perform all the creatures' basal functions.

To date, SDS-PAGE protein profiles of the Homopteran species have not been reported previously. However, it has been indicated that by exposing Hemipteran species to feeding on different substrates, can alter these aphids' salivary protein profiles (Habibi et al., 2001). The different insects in that study either ingested mesophyll or phloem cells' content. They hypothesized that the insects are able to change their salivary protein content in response to the recognition of different diets, and perhaps also do so to evade detection by some plant compounds. It could simply be a mechanism to "stabilize their biochemical milieu" (Habibi et al., 2001). The non-phytophagous Hemipteran did not change its salivary profile when feeding on other substrates (Habibi et al., 2001). They propose that this was observed because the saliva of the entomophagous insects is not in contact with any of the plants' tissues.

Thus, perhaps the potential to adapt the components of saliva in response to feeding is also a characteristic of the Homopteran pierce-and-suck insects. This is probably the case, because RWA change their feeding habits to ingest the content of mesophyll cells instead of phloem when feeding on resistant wheat (Girma et al., 1992). It will be informative to monitor whether or not SA1 RWA modify their protein profiles in response to feeding on 'TugelaDN' and also if SAM RWA respond differently to exposure to susceptible cultivars, such as 'Tugela' and 'Scheepers'.

When gut protein extract from the different RWA biotypes was injected into 'TugelaDN' plants, leaf rolling, puckering of the flag leaves and the appearance of necrotic lesions were observed (Figures 3.13, 3.14 and 3.15). Necrotic lesions are a characteristic of programmed cell death (Becker et al., 1993). The presence of necrotic lesions indicated that the hypersensitive response

had been activated in the resistance plants following injection by the gut extracts. This was to be expected, seeing as plants containing the *Dn1* gene should have defence mechanisms in place to deal with RWA infestation (Du Toit, 1987).

Perhaps all these symptoms were observed in the injected 'TugelaDN' plants because the gut proteins were so concentrated: 20 guts were extracted in 10 µl phosphate buffer, and 50 µl of extract was injected into plant, which is equal to 100 guts. Thus, the plants were subjected to an equivalent of heavy aphid infestation. Normal infestation experiments are conducted by placing six RWA on a leaf (Van der Westhuizen et al., 1998b). Thus, injected gut extract was the equivalent of approximately 20 fold the specified requirement to successfully monitor plant defence responses post infestation. It was presumed that leaf rolling experiments would be a good indicator of RWA infestation and would be useful as a tool to identify the elicitors in the HPLC fractions. In order to quantify these observations, 'Tugela' plants were also injected with RWA guts and the degree of leaf rolling determined over time (Figure 3.16). By 90 minutes, the leaf rolling was constant in the flag leaves and by 120 minutes in the basal leaves. Li et al. (in press) observed that susceptible plants experienced leaf rolling almost immediately subsequent to injection. However, when the leaf rolling was measured in all subsequent experiments where plants were injected with HPLC fractions, no specific pattern of leaf rolling was observed.

Epinasty or hyponasty is the upward or downward curling of leaves, respectively (Van Volkenburgh (1999). This is mediated in the Venus Fly trap *(Dionaea muscipola)* by the influx of ions and water (and subsequent increase in turgor and growth) to the cells in the opposite direction of the leaf curling event. Thus, upper cells shrink as lower cells increase in size and the leaf curls up (Williams & Bennett, 1982). It was proposed that the leaf rolling in wheat in response to RWA infestation is caused by a decline in turgor in the leaf cells, which inhibits normal cell growth processes (Burd & Burton et al., 1992). The RWA feed on the upper side of the leaf: stylets pierce these cells and

affect their turgor negatively. As the turgor increases in the non-penetrated abaxial cells, they might swell rapidly in conjunction to the upper cells shrinking and/or collapsing. This causes the leaves to start rolling (Botha et al., 2006).

Peroxidase, chitinase and catalase activities were measured since these enzymes have been shown to be indicators of plant defence (Mohase & Van der Westhuizen, 2002; Van der Westhuizen et al., 1998b). When 'TugelaDN' plants were injected with the pooled gut HPLC fractions (Table 3.9 and Figure 3.23), all the injected plants had higher peroxidase activity than the non-injected plants. This may be in response to the wounding effect that the injections caused. Wounding induces the expression of an isoform of the SAR enzyme, chitinase (Botha et al., 1998). Since an increase in peroxidase expression precedes the activation of SAR (Lamb & Dixon, 1997), this increase in expression post wounding might also apply to peroxidase. The plants injected with the SAM.1 and SA1.2 batches' activities were both 1.7 times higher than the non-injected plants. It was assumed that these batches contained the fractions which contain the elicitors of the two biotypes because it had been previously reported that peroxidase is upregulated in response to RWA infestation (Mohase & Van der Westhuizen, 2002; Van der Westhuizen et al., 1998b). These pooled samples were then selected for further examination on individual fraction level.

When individual fractions were injected into 'Tugela' and 'TugelaDN' plants (Tables 3.10 and 3.11 and Figure 3.24), the 'Tugela' plants had peroxidase activities similar to those of its buffer-injected plants, which was very similar to the non-injected plants. The highest peroxidase activities were obtained for the fractions SAM.5 and SA1.10. These plants had 2.69 and 2.37 times more activity than the non-injected plants. Although the peroxidase activities were three times lower in the non-injected 'Tugela' plants in comparison to the 'TugelaDN' non-injected, activity is still present in the susceptible plants. This is probably because all cells contain catalase and peroxidase to regulate the

balance of reactive oxygen species in the cells (Lamb & Dixon, 1997). However, the 'TugelaDN' plants contain higher levels of peroxidase than the 'Tugela' plants (Figure 3.24). Perhaps this provides the resistant plants with a head-start to counter attack from the aphid and this infers the *Dn1* containing plants' antibiotic (Du Toit, 1987) activity.

Chitinase increases in expression as a response to RWA infestation (Van der Westhuizen and Pretorius, 1996; Van der Westhuizen et al., 1998b). When 'TugelaDN' plants were injected with the same batches of HPLC fractions (as used in the peroxidase activity assays), it was found that plants injected with the SAM.1 and SA1.2 batches had the highest chitinase activities (Table 3.12 and Figure 3.25). These activities were 3.477 and 3.390 times higher than the non-injected plants.

The non-injected 'Tugela' plants had a higher chitinase activity than the corresponding 'TugelaDN' plants (Tables 3.13 and 3.14 and Figure 3.26). In fact, all the injected 'Tugela' plants had lower activities than the non-injected. Chitinase is expressed at low levels in susceptible plants but increases at delayed rates that make it unable to elicit a response against RWA feeding (Van der Westhuizen et al., 1998b). Perhaps wounding responses result in a decrease of chitinase activity in the event of no pest being recognized by the plants' defence responses. However, the 'TugelaDN' plants, injected with SAM.5 and SA1.10, were 3.239 and 3.310 times higher than the non-injected plants.

A final assay was performed to confirm our results. However, most of the plants appeared to not have any change in catalase activity in comparison to the non-injected plants. The biggest changes were observed for the SAM.1 and SA1.3 batches which were 0.658 and 0.428 times lower in regards to catalase activity than the non-injected plants (Table 3.15 and Figure 3.27). When the

HPLC fractions were injected into the plants, the SAM.5 and SA1.10 plants were 0.911 and 1.175 times more active than non-injected plants (Table 3.16 and 3.17 and Figure 3.28).

This was in contrast to the results of Li et al. (in press). They stated that the activity of catalase was increased when 'Gamtoos R' plants were injected with RWA protein extracts. This was not found in the 'TugelaDN' plants. On the other hand, Mohase and Van der Westhuizen (2002) reported that the activity of catalase decreased in the event of RWA feeding. They stated that SA concentration was inversely correlated to catalase expression. Considering that catalase is a molecule which detoxifies peroxide, this would make sense since an increase in hydrogen peroxide concentration is a consequence of the HR (Blokhina et al., 2003). This could explain why the SAM.1 batch caused a lower catalase activity. However, neither of the previous studies can clarify why plants injected with SA1.2 (our putative batch containing the elicitor of SA1) had 0.976 times lower activity than the non-injected plants. Perhaps batch SA1.3 contains another component which is recognized by the plants and results in a defence response via the decrease in catalase expression.

It is quite possible that the genetic backgrounds and genes conferring resistance cause a different defence response elicited by RWA infestation. 'Gamtoos R' resistance is caused by the *Dn7* gene (which is a result of a 1B/1R translocation event in rye (Marais et al., 1994)) whereas 'TugelaDN' contains the *Dn1* gene instead (Du Toit, 1987). *Dn1* is supposed to act through antibiosis (Wang et al., 2004) and *Dn7* through antixenosis (Anderson et al., 2003). Seeing as antibiosis has adverse effects on the feeding insects and antixenosis causes non-preference (Smith et al., 1992), the mechanisms to obtain these outcomes are probably different.

Matsioloko & Botha (2003) reported that defence responses of resistant wheat plants are activated within two hours after RWA infestation. Perhaps by seven hours post injection, the plants had

achieved all they could via the HR and the environment of the plants' cells was returning to normal. By seven hours the SAR was already defending the entire plant against any subsequent RWA attack: this is indicated by the increased levels of chitinase by this time (Figures 3.24 and 3.25). It is more than probable that the injection of plants results in a much quicker defence of plants and that a quicker detoxification method (and subsequent normalization of catalase levels) will follow.

The proteins extracted from the 'TugelaDN' plants, used in the time-trial gel, were visualized on SDS-PAGE gels to monitor if any changes would occur on a proteomic level over time (Figure 3.29). The non-injected plants were the most different of all the samples (Figure 3.29). Bahlmann (2002) found that proteins were differentially expressed in infested 'TugelaDN' plants: e.g. a band of about 36 kDa was newly expressed, 45 kDa was overexpressesed and 40 kDa was downregulated. These bands correspond with the band patterns observed in Figure 3.29. A band was observed of about 34 kDa, which corresponds to the 36 kDa band. This could possibly be a protein related to barley chitinase T (Van der Westhuizen & Pretorius, 1996).

Three bands (41, 42 and 44 kDa) were upregulated in response to injection. The 41 and 44 kDa bands probably correspond to the bands of 45 and 40 kDa (Bahlmann, 2002). However, these bands are expressed at lower intensities in the profiles of plants between 5 to 8 hours post infestation and appear again at 9 hours. The 'TugelaDN' plants injected with the SAM.5 and SAM.10 HPLC fractions (Figure 3.31) also display this phenomenon of the three bands of 41, 42 and 44 kDa. A band of 45 kDa was reported to be expressed at lower intensity in infested 'Tugela' and TugelaDN' plants (Van der Westhuizen & Pretorius, 1996). It was suggested that RWA could deliberately hinder normal protein expression in barley (Porter, 1992). This implies that the lower expression of 45 kDa in this study is probably caused by RWA feeding by means of the plants' exposure to the elicitors in saliva, whereas higher expression is an indicator of wounding.

Reports by Van der Westhuizen & Botha (1993) indicated that the resistant plants expressed a band of 56 kDa and 100 kDa in reponse to RWA infestation. In this study a 56 kDa band was observed, that was constitutively expressed in all 'TugelaDN' plants, while being absent in the 'Tugela' plants (Figures 3.28, 3.29 and 3.30). Furthermore a band of 97.5 kDa was detected in all the 'Tugela' plants, and it was induced in the injected 'TugelaDN' plants.

When comparing the SDS-PAGE polypeptide profiles of the two cultivars, the profiles of the two cultivars' non-injected plants differ from each other. However, the two cultivars are near-isogenic lines: 'Tugela' plants were crossed with the accession line SA1684, which contained the *Dn1* gene. The F1 population was subsequently backcrossed and selected until the plants displayed RWA resistance conferred by the *Dn1* gene, while still exhibiting a phenotype similar to 'Tugela' (Du Toit, 1987, 1989). It is easy to assume that the plants are mostly similar when considering the genetic history of the two cultivars; however, biochemically one cannot predict how the presence of a single gene might affect the expression of other genes and their subsequent proteins. Thus, it would follow that these differences in the 'TugelaDN' plants might occur constitutively and thus the plant is most probably always "geared up and ready" for attack. This would imply that the relevant *R*-gene, responsible for launching the defence response, will be expressed in uninfested as well as infested resistant plants. If more than one gene is required for resistance, then it is quite possible that the susceptible cultivar could contain most of the necessary components of the defence mechanism, while lacking a single gene required for activation of that response.

The results obtained from the enzyme activity assays and the SDS-PAGE polypeptide profiles of the extracted plant proteins led us to believe that the relevant fractions containing the elicitors are SAM.5 (collected between retention times 13 and 14 minutes) and SA1.10 (collected between

retention times 18 and 19 minutes), for the SAM and SA1 biotypes, respectively. There is a 5 minutes difference between the proteins' retention times, indicating a relative difference in protein size. Thus, the elicitor in SAM increased its size. According to the standard curve, the proteins should have approximate molecular weights of 55.065 kDa and 103.138 kDa for SA1.10 and SAM.5, respectively.

It is possible that the different mechanism of virulence between the two biotypes can be contributed to a single protein change. This can be supported by the fact that the SDS-PAGE gels of the RWA extracts indicate that the biotypes each contain a single protein differentially expressed in the other. Interestingly, the SAM biotype's protein band of 56.7 kDa is approximately twice as big as the differentially expressed band in the SA1 biotype (29.4 kDa). If these differentially expressed bands are in fact the virulence factors, then this might imply that the evolutionarily younger biotype, SAM, exists as a result of a duplication event of the original virulent factor of the older SA1 population. A nymph takes three weeks to attain maturity (Walters et al., 1980). Thus, the new biotype is (5 years*52 weeks)/(3 weeks/RWA generation) ≈ 87 RWA generations younger.

In the HPLC profiles, the SAM proteins eluted earlier at an shorter retention time. This is to be expected because the SEC-S 3000 column separates proteins according size, with the bigger proteins emerging first (www.phenomenex.com). However, in conjunction with the information obtained from the SDS-PAGE, these results indicate that the SAM elicitor is in fact larger than the 56.7 kDa band: it is double that at a size of 103 kDa. The same applies for the SA1 aphids: instead of the elicitor being the dimer at 29.4 kDa, it is also double the size, at 55 kDa. Thus, this indicates that the SAM elicitor is double that of the SA1. This implies that the SAM resistance was developed via the duplication of SA1's. Insect antibiotic resistance is either as a result of gene amplification or transcription factors acting in *trans* on a gene conferring resistance (Hemingway,

2000). An example of the former is the duplication of gene portions of esterase encoding genes in peach-potato aphids (*Myzus persicae* Sulz.). Resistance is directly correlated to the amount of amplification of these gene portions (Field et al., 1988).

However, the band of 103 kDa occurs in both the biotypes SDS-PAGE images, unlike the two differentially expressed bands, which are half (SAM) and quarter (SA1) the size of it. Perhaps even though the protein is present in all RWA biotypes, its translational modification differs in each. This could be a mechanism to avoid detection by the plant. It would also provide a great source of variation for the elicitor. If the elicitor consists of more than one subunit, then the modification of each and the rearrangement of their construction would provide more than one mechanism to circumvent recognition by the plant. Alternatively, perhaps all the different duplications of the same gene occur next to each other in tandem on the same chromosome, but their expression differs between the two insects.

The size of the elicitors was in contrast to what was expected: in Hessian Fly (*Mayetiola destructor*), it is proposed that a multiple *Avr* gene family occurs on chromosome 2A. However, most of these *Avrs'* gene products were predicted to have sizes of between 8 and 10 kDa (Chen et al., 2004). The midguts and salivary glands of these insects contained five groups of putative proteinase inhibitors, which contained proteins smaller than 15 kDa (Maddur et al., 2006). These are much smaller than the proposed elicitor sizes of 103 and 55 kDa. However, it is still possible that the RWA elicitors occur as a multiple gene family on a single chromosome: in the event of the proteins being duplication events, they will occur as duplicated genes on a single chromosome. The Hessian Fly *Avrs* contain putative signaling domains at their N-terminals, displaying high homology to serine proteases (Chen et al., 2004). The groups of proteinase inhibitors are probably members of a serine proteinase inhibitor family, which contains the same location of cysteine residues in the

members' amino acid sequences (Maddur et al., 2006). Thus they all belong to a gene family, which encode for gene products with similar function. Similarly, if the RWA elicitors are required for normal feeding mechanisms, which cannot be lost owing to selective constraints, then it would make sense that the new elicitors would have the same or similar function to the original one.

In the case of the US biotypes, the reactive protein extract from USA1 eluted much later from the HPLC column than that of the US2 biotype, which evolved at a later stage. The latter protein also emerged at retention time of 14 minutes while US1's elicitor occurs at 28 minutes (approximately 7 kDa) (Lapitan, Van Zyl & Botha, unpublished data). However, that would imply that the US2 protein is 14 times bigger than US1's. Thus, the US2 elicitor might also a result of a series of duplication events. If duplication is the normal mechanism of overcoming plant resistance, then perhaps the SA biotypes are younger than US1 and SAM is of a similar age to US2. This is probably true for the SAM and US2 biotypes seeing as US2 was distinguished from US1 in 2003 (Haley et al., 2004) and SAM was forced to start developing resistance in 2002. However, the SA1 biotype was identified South Africa in 1978 (Walters et al., 1980) whereas the US1 biotype was only discovered in the USA in 1986 (Stoetzel, 1987; Webster et al., 1987). This would imply that the US1 biotype is younger than the SA1 biotype. However, the RWA was already present in Mexico in 1980 (Gilchrest, 1984, as cited by Stoetzel, 1987). The US1 biotype is probably part of this population when one considers the proximity of Mexico to the USA and would then be of a corresponding age to the SA1 biotype.

At present, the biotypes need to be further investigated in order to obtain conclusive results regarding their ages and relatedness. The pattern of biotype development can be further verified by the examination of other new biotypes and their corresponding old biotypes. It would be the most ideal scenario if the original aphids were to be collected from their endemic habitats. Vicki Tolmay

reported that a new SA biotype has emerged in the Eastern Free-State (Boshoff & Du Toit, 2006). It will be interesting to observe how this RWA has changed in comparison to the other SA biotypes.

Ongoing studies aim to obtain amino acid sequence data of these elicitors and consequently generate proteins from these to confirm the elicitor and/or develop probes for Western Blots, which can be utilised to identify individuals of the different RWA biotypes. Also, analysis of the sequence data might lead to the elucidation of these elicitors' structure and possibly even putative function. This could shed light on whether or not these proteins are part of a family or are novel events occurring at random. Understanding how the aphids evolve, will enable breeders to impede this biotype development via the creation of crops displaying resistance to specific biotypes.

However, one should not forget that the potential of RWA biotypes to develop is probably not restricted to the mutant insects. If it can occur in a laboratory environment by restricting the insects' food source, then the same would apply by the exclusive planting of a single resistant cultivar in the field. Potentially, this could lead to global emergence of super virulent biotypes. This has once again reiterated the importance of developing tolerant, rather than antixenotic or antibiotic lines.

3.6. Literature cited

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CHAPTER 4

SUMMARY

The Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko) is a serious pest of small grains, such as wheat and barley. Considering the fact that wheat is the second most consumed crop in the world, ways to undermine the effects of RWA feeding are of extreme importance. This has driven the search for obtaining novel germplasm displaying resistance against the pest. Unfortunately, the RWA has means of overcoming resistance in cultivated lines which contain resistance genes, and by doing so, manages to inflict the same damage as it would on susceptible plants. In comparison to the original insects, these new "biotypes" are exactly the same in all aspects except in those regarding their feeding behaviour on these resistant crops.

This dissertation concentrated on determining the ways the biotypes might have developed to obtain its new virulence. The RWA feeds with a pierce-and-suck mechanism, which implies that the saliva of the insects is in constant contact with the plants' phloem during feeding. It was decided to take a closer look at the protein complement of the saliva from the South African biotypes, SA1 and SAM. Proteins were extracted from their total bodies, guts and heads. The proteins in these extracts managed to elicit symptoms in resistant 'TugelaDN' plants typical of RWA feeding: leaf rolling and necrotic spotting. The HPLC profiles obtained from these extracts indicated that differences occurred between the biotypes' in regards to peak number and/or intensity. These differences could either reflect mechanical barriers or physiological differences or even be a manifestation of the

differing phloem proteins of the cultivars the insects were maintained on. SDS-PAGE of the biotype total proteins indicated that the insects contained 19 protein bands and one of these was differentially expressed in each of the biotypes.

Even though leaf rolling was observed after injections of total gut extracts, the same was not observed for any later injections of HPLC fractions of this same extract. Perhaps the elicitors were too dilute after purification and could not elicit the same phenotypic response. However, this was not applicable to the enzymatic activity of injected plants. Resistant plants reacted with an increase in peroxidase and chitinase activity and an unchanged catalase activity within seven hours post injection with fractions containing the putative eliciting agents.

The SDS-PAGE of the wheat proteins extracted after injections showed no variation in proteins from susceptible plants, but variation in the resistant cultivars did occur. Eight proteins were expressed differentially in the injected plants and one in the non-injected, whereas four were expressed constitutively in both. Three bands had lower intensities in plants which were injected with the fraction containing the putative elicitors. In conjunction with the enzyme activity assays, the putative SAM elicitor eluted at an approximate retention time of 14 minutes and that of SA1 at 19 minutes. Correlation to the SDS-PAGE data confirmed that these proteins were approximately 103 kDa and 56 kDa in size, respectively. These fractions were not located in peaks on the HPLC profiles, indicating that they are expressed in low abundance. This suggests that the differences observed in regards to peaks probably reflect changes in the insect not linked to virulence.

The results indicate that the biotypic virulence is caused by a single protein change. It is possible that these changes were brought about by duplication events because the SAM elicitor is approximately double in size of SA1’s elicitor. However, a band of 103 kDa also appears in the

SA1 SDS-PAGE profile. Perhaps the larger band occurs in all biotypes and the virulence is a result of posttranslational modifications of the protein. However, sequencing of these proteins will reveal a lot about the nature of these elicitors.

Presently, sequence analysis is in progress on these proteins, and if enough amino acid sequence data is obtained from the N and C terminals, then degenerate primers might be deduced for these regions. This will enable for the amplification of the gene from the aphid genome. If this is successful, the amplified DNA can be sequenced and its homology determined to other known DNA sequences. The complete gene product can be inserted into a plasmid construct and its protein expressed in transformed insect cells. If purified protein is expressed at a regular interval and it elicits the same response as extracts from RWAs, then the functional role of the protein will be confirmed. This will pave the way of future experiments by facilitating the collection of RWA fractions and thus, decreasing the time expenditure of discovering the way that host plants defend themselves against these pathogens.

Furthermore, an added advantage is that the elicitors will be stabile for the entire study: thus, it won't change its nature as it did in the SA1 mutant in response to feeding on the resistant cultivar. Alternatively, the amino acid sequence obtained can be used to construct a synthetic protein from new. The proteins obtained from either of these techniques can then be used to produce antibodies to use as markers during Western blot analysis to identify potential elicitors in other biotypes. Additionally, ELISA plates could be designed, utilizing these antibodies to provide farmers with a diagnostic tool to assign biotypic identity to RWA as they are discovered in their fields. Knowing which biotypes are present will facilitate and improve the choice of management practices when combating a specific RWA infestation.

At present, only sequencing of the pea aphid genome has been commenced. Thus, DNA sequence data is limited in regards to any pierce-and-sucking insects and their relevant genes involved in feeding. Thus, most sequence data obtained from the RWA will be novel and enhance the database of already known aphid genes. It might also provide information on the nature of putative aphid *Avrs* (such as organization in the genome, amount or mutation rate), but also shed light on the mode of defence mechanisms in the resistant host plants. Nevertheless, not all the data will be novel: conserved protein domains present in the sequences could contain homology to other organisms' protein structures, such as signaling domains or structural features, and provide information about the basic nature and function of these elicitors.

This study indicated that the mutant lines can evolve rather rapidly: to give an approximate time span, it took five years to obtain this. Therefore, the potential exists to create novel mutant lines by forcing them to feed on other resistant lines. Perhaps it would be a better option to use the SA1 aphids, which have already displayed the potential to change in response to the different hosts. It would also provide a reference population to compare any new biotypes to. If the new biotypes display similar changes in their elicitors, similar patterns could evolve in aphid populations globally. If this is the case, evolving biotypes could be predicted and new wheat cultivars could be developed based on mechanisms to pre-empt changes in different RWA biotypes.

It has been indicated by the emergence of the SAM biotype that selective pressure can force the development of new biotypes. This is a mini enactment of what can happen in the field in the case of only resistant wheat lines being cultivated, and thus the need to diversify in terms of resistance genes placed in the fields should be emphasized in integrated management practices. Lastly, the information obtained from our sequence data will enable the development of new cultivars

displaying tolerance, rather than antixenosis and antibiosis, to feeding aphids and thus, stem the materialization of novel biotypes exhibiting severe virulence.