ISOLATION AND ELICITING ACTIVITY OF THE RUSSIAN WHEAT APHID SALIVA IN THE RESISTANCE RESPONSE OF WHEAT

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“Every great advance in science has issued from a new audacity of imagination.”

-John Dewey

“A man’s mind plans his way, but the Lord directs his steps.”

-Proverbs 16:9

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DECLARATION

I declare that the Thesis herby handed in for the qualification *Magister Scientiae* at the University of the Free State, is my own work and that I have not previously submitted the same work for qualification at/in another University/Faculty.

Furthermore, I cede copyright to the Thesis in favour of the University of the Free State.

B TAIWE	**DATE**

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LIST OF ABBREVIATIONS

ARC-SGI	Agricultural Research Council-Small Grain Institute
ALP	Alkaline phosphatase
AOS	Allene oxide synthase
ATPase	Adenosine triphosphatase
Avr	Avirulence
Ca^{2+}	Calcium ions
CC	Companion cells
CEBiP	Chitin elicitor binding protein
D. noxia	*Diuraphis noxia*
dH_2O	Distilled water
Dn	*Diuraphis noxia*
EPG	Electrical penetration graph
ET	Ethylene
EF-Tu	Bacterial elongation factor
FACs	Fatty acid conjugates
Fig	Figure
GOX	Glucose oxidase
G-proteins	Guanine nucleotide-binding proteins
GTP	Guanosine triphosphate

H_2O_2	Hydrogen peroxide
HAMPs	Herbivore-associated molecular patterns
HIPVs	Herbivore induced plant volatiles
HPOD	Hydroperoxide
HR	Hypersensitive response
IPM	Integrated pest management
ISR	Induced systemic resistance
JA	Jasmonic acid
K^+	Potassium ions
LOX	Lipoxygenase
LRR	Leucine-rich repeat
LZ	Leucine zipper
MAMPs	Microbe-associated molecular patterns
MeJA	Methyl jasmonate
MeSa	Methyl salicylate
NBS	Nucleotide binding site
NBS-LRR	Nucleotide binding site-leucine rich repeat
NO	Nitric oxide
O_2^-	Superoxide anion
OH^-	Hydroxyl anion
OS	Oral secretions
PAMPs	Pathogen-associated molecular patterns

PFI	Phloem feeding insect
PGPR	Plant growth promoting rhizobacteria
POD	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related
PRR	Pattern recognition receptors
R-gene	Resistance gene
ROS	Reactive oxygen species
RWA	Russian wheat aphid
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEs	Sieve elements
Sp	Salivary proteins
SR	Systemic receptor
TLC	Thin-layer chromatography
TMV	Tobacco mosaic virus
UK	United Kingdom
USA	United States of America
USSR	Union of Soviet Socialist Republic

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INTRODUCTION

1.1 Wheat

Wheat is one of the most important cereal grain crops produced as staple food for mankind (Joshi *et al*., 2010). It is characterised as an annual or biennial grass with erect flower spikes and light brown grains, belonging to the Poaceae family. Wheat is a member of the genus *Triticum* and the main cultivated varieties include the bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum*), which both account for about 95% and 5% of world wheat, respectively (Kiplagat, 2005). Wheat can be cultivated either in spring (spring wheat) or autumn (winter wheat) and harvested in late summer (Curtis, 2002). The production of wheat has been reported to exceed all grain crops such as barley, oat, sorghum and even rice (FAOSTAT, 2006).

Wheat originated in the Fertile Crescent of the Middle East from where it spread to North Africa, Eurasia, Western Europe, America and the Southern hemisphere (Pagesse, 2000). Wheat cultivation owes its success to the fact that it can be grown in temperate regions such as Russia, and Western and Northern Europe. Countries responsible for almost 90% of wheat production in the world include China, EU countries, USA, India, Canada, Eastern European countries, Turkey, Australia and Argentina (Mitchell and Mielke, 2005). According to statistics the estimated average production for the annual year 2010/2011 is 648 million tons (IGC, 2011) and indications proclaim that by the year 2030 approximately 860 million tons will be produced by these countries (Maratheè and Gómez-MacPherson, 2000).

On the African continent, South Africa is the fourth largest wheat producer after Morocco, Egypt and Algeria (Latham, 2011). Wheat is cultivated throughout South Africa with the following provinces being the largest regions accountable for total

production: the Free State province (winter/spring-planted wheat in the summer rainfall region), and the Western Cape province (winter-planted wheat under dryland conditions) and spring wheat grown under irrigation in the summer rainfall region also in the Western Cape (Hatting *et al*., 2000). About 80% of South African wheat is produced under dryland conditions, 20% of the area planted to wheat is cultivated under irrigation and the industry is challenged by erratic rainfall patterns that may lead to wide fluctuations in yield (Hatting *et al*., 1999). In 2009, the production of wheat in South Africa was 1.958 million tons (FAOSTAT, 2009) against a domestic demand of about 3.1 million tons and according to the report by USDA Foreign Agricultural Service (2011), only about 1.5 million tons will be produced for the marketing year 2010/11. South Africa is therefore a net importer of wheat, largely from Argentina, Australia, France, UK and USA (Crop Estimates Committee, 2008). Biotic and abiotic stresses are dominant factors responsible for the declining wheat production in South Africa. Abiotic stresses include fluctuating climate conditions, acidic soils and preharvest sprouting after wet spells during wheat ripening. Biotic stress, for example, includes rust diseases in wheat such as stripe/yellow rust (*Puccinia* Westend *f. sp. striiformis* Eriks.) and leaf/brown rust (*Puccinia triticina* Eriks.) (Afzal *et al*., 2008). Insect pests such as the Russian wheat aphid are also regarded as crucial factors that account for high yield losses in wheat production.

The Russian wheat aphid (RWA) can cause about 21% to 92% yield losses on susceptible wheat cultivars in South Africa (Basky, 2003). The RWA is not only a pest to South Africa, but also to wheat producing countries such as the USA where yield losses and increased production costs, due to RWA infestations, have been estimated over $1 billion in the 10 years since the discovery of the aphid (Qureshi *et al*., 2005). The use of effective control measures against pests, particularly the RWA, will undoubtedly aid in preventing yield losses in wheat production.

1.2 RWA Control

1.2.1 Chemical control

RWA infestations can be controlled with the use of insecticides. However, leaf rolling caused by RWA feeding protects the aphid colonies, thus hampering the efficiency of, for instance, contact insecticides. In an attempt to overcome this, systemic insecticides such as disulfoton, dimethoate and demeton-s-methyl, as well as vapour action insecticides such as chlorpyriphos and parathion, and recently seed dressings such imidacloprid and thiametoxam have been applied (Nel *et al*., 2002). In South Africa, all the registered insecticides for the control of RWA are broad-spectrum systemic and contact organophosphates, except for imidacloprid and thiamethoxam (Nel *et al*., 1999).

The set back of these chemicals is their influence on natural enemies (ladybirds and wasps) that attack the RWA. However, some insecticides such as pirimicarb (active ingredient is organophosphate) which is registered as a control agent against aphids on wheat crops, have lower toxicity levels towards these natural enemies (Hatting, 2010). However, the use of insecticides can affect human health as well as the environment negatively. Moreover, aphids can develop resistance to these insecticides (Hatchett *et al.,* 1994, Isman, 1999). Due to these disadvantages alternative methods to control the RWA have to be developed.

1.2.1.1 Alternative chemical control

The use of semiochemicals such as methyl salicylate, menthol and 1,8-cineole obtained from essential plant oils for management of *D. noxia* infestations has been investigated (Prinsloo *et al*., 2007). In general, semiochemicals help natural enemies to locate and recognise their host or prey (Lewis and Martin, 1990). The olfactory signal originates from host plants and produces cues in response to herbivore attack (Vet and Dicke, 1992). Methyl salicylate is a volatile product of the phenylpropanoid

plant defence pathway and it is suggested that it may act as a signal for plant stress. A study by Prinsloo *et al.* (2007) showed that exposure of resistant plants to methyl salicylate significantly reduced RWA settling; whilst in susceptible cultivars there were increases in aphid populations even when treated with semiochemicals.

1.2.2 Biological control

Biological control of aphids on wheat is obtained by using predators and parasites (Iqbal *et al.*, 2008). Ladybird beetles and wasp parasites are natural enemies that play a role in increasing aphid mortality (Walters *et al.,* 1980; Nelson *et al*., 2004) and also in triggering avoidance behaviours that reduce infestation, feeding and reproduction. The disadvantage with using natural enemies of RWA is that natural enemies are polyphagous and are not species specific (Marasas, 1999). Other than being polyphagous, these natural enemies react only when there is a high RWA infestation and by then wheat plants are already under stress. Another drawback is that natural enemy populations develop much slower than those of RWA. The low population density of natural enemies has failed to reduce RWA populations to levels low enough to prevent economic damage to wheat (Basky, 2003).

1.2.3 Disease causing microbes

Entomopathogenic fungi with high epizootic potential have been used to reduce aphid populations. Species of fungi such as *Pandora neoaphidis*, *Conidiobolus obscurus* and *Entomophtora planchoniana* produce microscopic spores which germinate in contact with aphid skin (cuticle), penetrate the exoskeleton and may cause a fatal disease (Shah and Pell, 2003). Prevalence of infection may in some periods exceed 80%, indicating the possibility of utilising toxins from entomopathogenic fungi for microbial control of aphids (Nielsen and Wraight, 2009). Certain aphid species are particularly susceptible to toxins from fungi e.g. cotton aphids are frequently attacked by *Neozygites fresenii*, spotted alfalfa aphid by *Therioaphids trifolli* and *D.noxia* by *Pandora neoaphidis* (Hatting *et al*., 2000).

Kim *et al.* (2005) stated that parasitoid (*Aphidius colemani)* and fungus (*Verticillium lecanii)* may be used together for control of aphids but fungus application has to be timed to allow late-instar development of the parasitoid. Hatting *et al*. (2004) showed that *Beaveria bassiana* could control up to 60-65% of *Diuraphis noxia* in field conditions. However, a mycotoxin isolated from *Beauveria bassiana,* beauvericin, is a recognised toxic compound infecting maize, wheat and rice (Šrobárová *et al*., 2009). Even though beauvericin has the potential to decrease cell viability of wheat, the isolated toxin can be used as a biocontrol for RWA on wheat crops (Šrobárová *et al*., 2009). Entomopathogenic fungi require substantial humidity to be effective, thus are unlikely to cause death in arid regions where the RWA is most prevalent. In addition, toxins originating from entomopathogenic fungi are slow to kill and may be inhibited by low temperatures.

1.2.4 Host resistance

The most successful strategy for control of the RWA populations is breeding for resistance in cultivars. Since 1984 the use of host plant resistance has been a resourceful alternative over chemical control (Du Toit, 1988). Genetic resistance against the RWA was discovered in unimproved wheat germ plasm from central Asia and the Middle East (Harvey and Martin, 1990; Smith *et al.,* 1991). Because RWA populations all over the world interact differently with resistant cultivars (Puterka *et al.*, 1992), germ plasm of South African RWA populations had to be screened to ensure that correct genes were included in the breeding programme (Marasas, 1999). The single dominant resistance gene, *Dn1,* in wheat accession PI 137739, and *Dn2* in PI 262660 were the first in South Africa to be discovered in green house screening test at Agricultural Research Council-Small Grain Institute (Bethlehem) in 1986 (Du Toit, 1987). Cultivars containing *Dn1* resistance gene include ‘Tugela DN1’, ‘Molopo DN’, ‘Palmiet DN’ and ‘Betta DN’. Mostly, resistant plants consist mainly of these dominant single genes, *Dn1* or *Dn2*. Thereafter, efforts have been made to discover more sources of resistance to the aphid, and 10 genes conferring resistance to RWA have been identified from wheat and other cereals (Liu *et al*., 2002; Liu *et al*., 2005). These *D. noxia* (*Dn*) resistance genes include *Dn3* in *Triticum*

taushii accession (SQ 24), *Dn4* in PI 372129, *Dn5* in PI 294994, *Dn6* in PI 243781, *Dn7* from rye, *Dn8* and *Dn9* in PI 294994, and *Dnx* in PI 220127 (Nkongolo *et al.*, 1991; Liu *et al*., 2005). Wheat cultivars containing the *Dn7* resistance gene confer a high level of resistance to all currently existing US and South African biotypes (Lapitan *et al.*, 2007; Zaayman *et al.,* 2009). *Dn7* originated from a rye cultivar, Turkey 77, and was transferred to a wheat background via recombination between the 1RS telosome of Turkey 77 and 1BS/1BL translocation chromosome from a susceptible spring wheat “Veery” series cultivar, Gamtos (Lapitan *et al*., 2007).

The three known functional categories explaining host plant resistance include: (i) tolerance: plants can survive under levels of infestation that will kill or severely injure susceptible plants, (ii) antibiosis: resistant plants are able to affect the biology of the insect, and (iii) antixenosis: non-preference of plants for insect oviposition, shelter or food. For example, the effect of *Dn1* (PI 137739) represents antibiosis, *Dn2* (PI 262660) represents tolerance and antixenosis, and *Dn5* has been reported to exhibit antibiosis, antixenosis and tolerance (Du Toit, 1987; 1989).

About 70% of wheat farmers in the eastern parts of the Free State now plant resistant cultivars, and this has decreased the use of insecticides by approximately 35% between 1990 and 1996 (Marasas *et al.,* 1997). By the year 2006, 27 cultivars were released in South Africa including the commercial cultivar PAN 3144 known to contain resistance gene *Dn5*.

Although resistant wheat cultivars were developed and are presently the most effective strategy in keeping *D. noxia* infestations to the minimum (Basky, 2003), development of new virulent *D. noxia* biotypes overcoming resistance has been one of the most challenging crises. In South Africa, the presence of new resistance breaking biotypes was reported in 2005 (*RWASA2*, Tolmay, 2006) and 2009 (*RWASA3*, Jankielsohn 2011). The new biotypes have been reported as more

virulent than the initial RWA biotype. Under these circumstances, further research to develop new resistant cultivars has to continue and alternative methods to control RWA should be considered.

1.2.5 Alternative RWA control options

The use of elicitors of induced plant resistance has been proposed as an alternative Integrated Pest Management (IPM) strategy (Ozeretskovskaya and Vasyukova, 2002, Holopainen *et al*., 2009). Elicitors are natural or synthetic compounds that induce responses in plants similar to those induced by insect or pathogen attack (Karban and Kuć, 1999). Signalling pathways that are involved in inducing defence responses have led to the discovery and synthesis of elicitors. In a broad sense, elicitors are defined as chemicals from various sources that can trigger physiological and morphological responses in plants (Zhao *et al*., 2005). Generally, plants treated with a range of elicitors develop resistance to pathogens because multiple signalling pathways of intracellular defences are activated (Odjakova and Hadjiivanova, 2001; Garcia-Brugger *et al.*, 2006; Bent and Mackey, 2007; Holopainen *et al.*, 2009). The most common sources of elicitors are oral secretions and oviposition fluid from insects (Botha *et al*., 2005). Numerous biochemical compounds such as hydrolytic enzymes (cellulose, pectinases and glucose oxidase), structural proteins (glycoproteins), and other components such as volatiles (Miles, 1999; Eichenseer *et al*., 1999; Botha *et al*., 2005) in the insect regurgitant have been suggested as agents eliciting plant defence responses. It has been proposed that different virulence factors should be produced in saliva to result in the breakdown of resistance (Belefant-Miller *et al*., 1994). In the continuous search for elicitors, aphid regurgitants have been considered. De Vos and Jander (2009) demonstrated that salivary components of *Myzus persicae* (green peach aphid) contained a proteinaceous elicitor (3-10 kDa) which induced defence responses in *Arabidopsis thaliana*. Some of the insect-derived elicitors of plant defences that have been identified include; fatty acids and amino acid conjugates of oral secretions from *Spodoptera exigua* (Alborn *et al.,* 1997) and *Manduca sexta* (Halitschke *et al*., 2001),

and disulfooxy fatty acids in regurgitants of grasshopper *Schistocerca americana* (Alborn *et al*., 2007).

The application of elicitors to plants results in activation of defence responses and this seems to be a promising option for effective management of aphid infestations. RWA feeding on resistant cultivars activates signaling defence pathways that lead to expression of pathogenesis-related genes, thereby resulting in resistance in plants. However, the RWA tends to develop new virulent biotypes rapidly causing tremendous losses in wheat production (Tolmay, 2006; Tolmay *et al*., 2007). A better understanding of elicitors in RWA-wheat interactions could be a breakthrough in RWA management. Reports on RWA-wheat interactions include the identification of a glycoprotein elicitor in the intercellular wash fluids of Russian wheat aphid biotype 1 (*RWASA1*) infested resistant wheat (Mohase and van der Westhuizen, 2002a), and some eliciting activity in protein extracts of RWA [(RWASA1 and its mutant) van Zyl and Botha, 2008)]. This study was therefore initiated with the following objectives:

1. To investigate and compare the eliciting potential of aphid [*RWASA1* and Russian wheat aphid South African biotype 2 (*RWASA2*)] saliva, in the induction of defence responses in different wheat cultivars.
2. To isolate and characterize the elicitors in Russian wheat aphid saliva.

The findings from this study will aid in understanding the potential of aphid saliva in the induction of the resistance mechanism of wheat against the RWA. The results will further pave way for the identification and full characterisation of the elicitors, as well as the mechanism of their perception by different wheat cultivars.

LITERATURE REVIEW

2.1 The Russian wheat aphid

The Russian wheat aphid (RWA), *Diupharis noxia* (Kurdjumov) (Homoptera: Aphididae), is a small (1.6-2.1 mm long), spindle-shaped, soft-bodied, lime green insect. It has shortened antennae and reduced cornicles at the end of the abdomen (Hodgson and Karren, 2008). A distinguishing feature of the RWA from other cereal insects is the presence of an appendage (supracaudal process) above the caudal which gives the RWA the appearance of having two tails (Stoetzel, 1987).

2.1.1 Origin and distribution

The Russian wheat aphid (RWA), endemic to central Asia, southern Russia, Iran, Afghanistan and countries bordering the Mediterranean Sea (Ennahli *et al.*, 2009), has been a major pest of wheat and barley since 1912. The RWA was discovered in the southern and eastern parts of the African continent in the late 1970s (Torres, 1984). In South Africa, the RWA was reported as a pest of wheat in 1978 in the Eastern Free State and has since remained a serious pest of wheat crops (Walters, 1984). By 1980 the RWA was discovered in central Mexico and by 1986 infestations had reached the southern parts of Texas in the United States of America (Stoetzel, 1987). In Canada, RWA infestations were detected in 1988 (Jones *et al*., 1989); since then infestations have been reported in other countries. This pest is responsible for great losses in wheat especially in countries such as USA, South Africa and South America (Liu *et al.*, 2002). The sporadic outbreaks of RWA also occurred in the former Union of Soviet Socialist Republic (USSR), where losses of up to 75% were reported (Halbert and Stoetzel, 1998).

2.1.2 General morphology and reproduction

Diuraphis noxia has four nymphal instars and an adult stage. Aalbersberg *et al*. (1987) developed a simple strategy to diagnose the instars; by using morphology of the antennae, caudae and wing buds in conjunction with ratios between antennal segment lengths.

In South Africa the RWA reproduces asexually (Kiriac *et al*., 1990). Each female aphid gives birth to live daughters carrying embryonic granddaughters (parthenogenetic and viviparous) (Puterka *et al*., 1993). In other parts of the world, *D. noxia* reproduces sexually. Kiriac *et al*. (1990) reported an occurrence of sexual morphs (both oviparae and males, holocyclic) of RWA in several locations in the Soviet Union and Hungary (Basky, 1993).

According to Girma *et al*. (1990) reproduction of RWA nymphs is drastically delayed at low temperatures (10 ºC to 13 ºC); and reports indicate that temperatures above 25 ºC cause mortality (Michaud and Sloderbeck, 2005). During heavy infestations, increased proportions of immature aphids develop wings and migrate to healthier plants to begin new colonies (Michaud and Sloderberk, 2005). In the absence of suitable wheat hosts, winged females migrate to alternative crops (rescue grass, canary grass, false barley and wild oats) until wheat crops become available the following wheat season.

2.1.3 Host plants and volunteer wheat

The RWA is a severe pest of small grains including wheat (*Triticum aestivum* L., Poaceae) and barley (*Hordeum vulgare* L., Poaceae) (Halbert and Stoetzel, 1998). Survival of RWA is determined by different factors such as high temperatures and availability of suitable host plants. In South Africa, extremely cold weather conditions

induce RWA mortality. The absence of wheat during grain harvest in summer and the emergence of the next crop in late winter, forces RWA to migrate to alternative hosts (Jankielsohn, 2009). The RWA must therefore, locate alternative host plants such as rescue grass (*Bromus catharticus*), canary grass (*Phalaris minor*), false barley (*Hordeum murinum*) and wild oats (*Avena fatua*) to survive (Ni *et al*., 1998). These wild grasses have a major influence on the survival of the RWA. Perhaps attempts to control volunteer plants should be taken into account during the structuring of RWA control measures.

2.1.4 RWA biotypes

The presence of diversity within *D. noxia* populations has been noted in various parts of the world (Puterka *et al*., 1992). RWA biotypes are distinguished from each other based on their ability to overcome host resistance, their fecundity and the severity of damage they cause to plants (Jyoti *et al*., 2006; Burd *et al*., 2006; Weiland *et al*., 2008). A biotype is defined as a population (independent of geographic location) that is able to injure cultivated plants containing a specific gene(s) that was previously resistant to known aphid populations (Smith, 2005). Purteka *et al.* (1993) discovered strong similarities between RWA populations from USA and collections from South Africa, Mexico, France and Turkey; most variations were detected among populations collected from the Middle East and southern Russia during their reaction to resistant wheat lines in the USA. Basky (2003) described that biotypic variation does occur in *D. noxia* populations from Hungaria and South Africa. The Hungarian biotype caused damage to wheat cultivars resistant to the South African RWA populations. Moreover, differences between *D. noxia* from South Africa and Syria have also been reported (Black *et al*., 1992).

In 2003, a new biotype was identified in Colorado, USA, on the basis of its virulence to *Dn4*-based resistance in wheat (Haley *et al*., 2004; Qureshi *et al.*, 2005; Jyoti *et al.*, 2006). This biotype was designated as “biotype 2”, and had caused extensive yield losses as all cultivars that were marketed as resistant to the original RWA

during 2005 season were severely damaged by the new biotype (Collins *et al.*, 2005). Eight biotypes have so far been identified in the USA (Jyoti and Michaud, 2005; Burd *et al*., 2006; Weiland *et al*., 2008). South Africa underwent a similar situation, a new biotype was discovered in the Eastern Free State in December 2005 and designated as *RWASA2* due to its virulence towards existing resistant lines in South Africa (Boshoff and Du Toit, 2006; Tolmay, 2006; Tolmay *et al*., 2007). In 2009, a third RWA biotype, *RWASA3* that was also virulent to existing sources of resistance (*Dn1, Dn2, Dn3, Dn9* and *Dn4)* was discovered (Jankielsohn, 2011). The first biotype that emerged in 1978 was now designated as *RWASA1*.

2.1.5 Symptoms caused by RWA infestations

Damage symptoms due to RWA infestations are distinct in susceptible and resistant wheat cultivars. Visible symptoms at the RWA feeding site include chlorotic streaks and leaf rolling in susceptible plants and necrotic spots on resistant plants (Walters *et al*., 1980; Burd and Burton, 1992; Jimoh *et al*., 2011). The RWAs are mainly found on adaxial leaf surfaces, in the axils of young growing leaves or within rolled leaves. The rolled leaf shelters the RWA against climatic conditions (frost, rainfall or drought), natural enemies (ladybirds and parasitic wasps) and insecticides (contact insecticides) (Kindler *et al.*, 1991; Smith *et al.*, 1991). Chlorotic white spots are also visible symptoms indicating disruption of plant chloroplasts and cell membranes by salivary enzymes (Marasas, 1999). Other symptoms associated with aphid feeding include prostrate growth, and white, yellow and purple longitudinal streaks on leaf surfaces (Jyoti *et al*., 2006). Saheed *et al.* (2007) suggested that the injection of aphid saliva into xylem is the major cause of white and yellow streaks on leaves, as well as leaf rolling.

2.1.6 RWA feeding behaviour and saliva

Upon landing on plants, an aphid uses its mouthpart (stylet) to probe through the plant cells (with minimal tissue damage) before the phloem is reached. While probing, the aphid produces the gelling saliva (refer to 2.3.1.2) which acts as a protective sheath around the stylet and remains within the plant after stylet withdrawal (Tjallingii and Hogen-Esch, 1993; Giordanengo *et al*., 2010). As soon as the aphid stylet reaches phloem, it punctures and releases the watery saliva (refer to 2.3.1.3) before beginning to feed. It is prior and during feeding that this watery saliva is released and may contain multiple enzymes associated with defense responses (Ma *et al*., 2010).

2.1.6.1 RWA mouthparts

The RWA mouthparts are composed of a short triangular labrum. The labrum covers the base of the stylet bundle, the labium, which is a segmented and tubular organ with complex musculature that contracts and shortens during insertion of the stylet into plant tissue (Uzest *et al*., 2010). The stylet bundle (Fig 2.1) consists of two pairs of chitinous needle-like stylets, the inner pair of maxillary stylets, and the outer pair of mandibular stylet (moves independently when piecing leaf surface). The maxillary stylets are fixed together by interlocking grooves found on their inner surfaces where the grooves are opposed to form a food canal and a salivary canal in between (Dixon, 1973; Miyazaki, 1987).

Proboscis is a modified labium which consists of a sheath to hold stylet in a groove formed on its dorsal surface and five segments in which the terminal proboscis segment firmly grips the stylet and fixes the point of insertion (Uzest *et al*., 2010). The tactile receptor on the tip of the proboscis responds to leaf surface texture and enables aphids to detect the contours of veins, their preferred feeding site (Tjallingii, 1978). Probing is achieved by protraction of the mandibular followed by the maxillary stylet. Aphid mouthparts are remarkably adapted to their feeding habits, the thin and

flexible stylet bundles are able to pass intercellularly without damaging plant tissues (Tjallingii and Hogen-Esch, 1993).

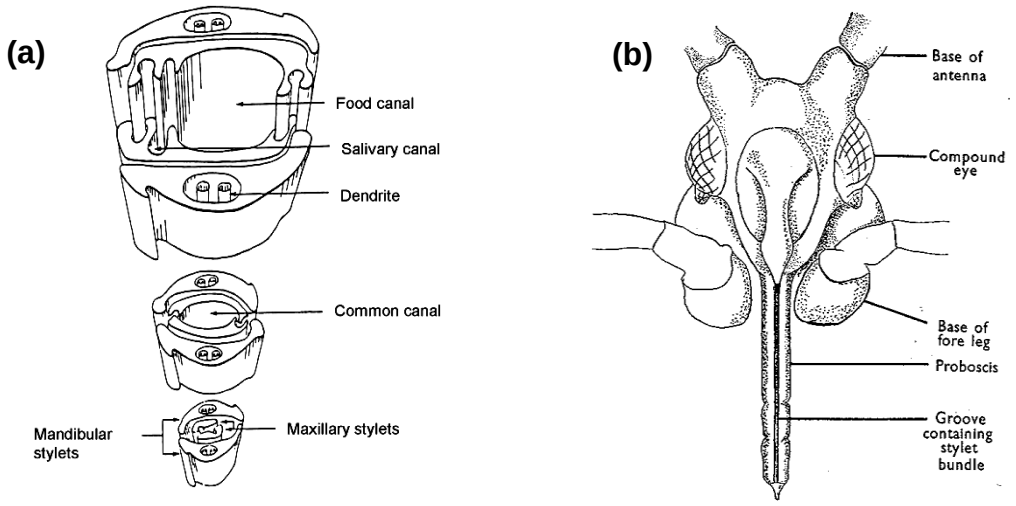

Figure 2.1: Schematic representation of the distal extremity of the stylet bundle of aphids; (a) two exterior mandibular stylets surround and protect two inner maxillary stylets (Uzest *et al*. 2010); (b) anterior view of the head and proboscis of an aphid to show the groove in the proboscis in which lies the stylet bundle (Dixon, 1973).

2.1.6.2 Sieve elements

The RWA feeds on the major veins of leaf tissue by intercellularly probing with its stylet through the cuticle, epidermis, and mesophyll until the phloem sieve elements (SEs) are reached (Walling, 2000). SEs are the enucleate conducting cells of the phloem, which contain a plethora of proteins and RNAs associated with long-distance signaling and defence responses (Fitzgibbon *et al*., 2010). When the SEs are punctured, the phloem sap is driven by turgor differences into the stylet food canal of RWA (Tjallingii and Cherqui, 1999; Will *et al.*, 2008; 2009). The phloem transports nutrients, defensive compounds, and informational signals throughout vascular plants (Turgeon and Wolf, 2009).

2.1.6.3 Phloem and sap composition

The majority of insects such as aphids, whiteflies, psyllids and plant hoppers, belonging to the suborder Homoptera are specialized to feed on phloem sap (Thompson and Goggin, 2006). These phloem-feeding insects provide additional challenges to plants as they deplete photosynthates, introduce vector viruses and chemical and/or protein effectors that alter plant defence signaling, induce infestation symptoms, and reduce plant growth and development (Kaloshian and Walling, 2005). The phloem plays an important role in plant nutrition and development, distributing a range of nutrients, water and signals from the source to sink cells (Dinant *et al*., 2010). The translocation in the sieve tubes occurs through mass flow driven by the pressure gradient between sources (high pressure) and sinks (low pressure) (Munch, 1930; Gould *et al*., 2005). It is due to this internal pressure that aphids can feed passively on phloem sap for prolonged periods.

The phloem sap is a mixture of carbohydrates (main component), proteins, amino acids, organic acids, and inorganic ions (Hall and Baker, 1972). The concentration of essential amino acids and other sources of nitrogen are low rendering the sap nutritionally insufficient (Sandström *et al*., 2000; Douglas, 2006). This deficiency is compensated by the primary endosymbiont bacteria (*Buchnera aphidicola*) of aphids; which synthesise and recycle the necessary essential amino acids (Douglas, 1998; Sandström and Moran, 1999). Severing of stylets to sample phloem exudates (stylectomy) has been used extensively to study the phloem contents (Dinant *et al*., 2010). There are speculations that aphids are capable of manipulating certain host plants' nutritional content. Evidence exists that aphid feeding can generate a nutritionally enhanced phloem diet (Telang *et al*., 1999). A study by Sandström *et al*. (2000) provided strong evidence that *D. noxia* and *Schizaphis graminum* alter the metabolism of their host plants to ingest increased concentrations of amino acids, especially essential amino acids. A common hypothesis suggests that aphids induce senescence-like changes and take advantage of the increased translocation resulting from breakdown of leaf proteins (Dorschner *et al*., 1987). Additional information provided by Telang *et al.* (1999) showed that during feeding *D. noxia* can

generate a nutritionally enhanced phloem diet. Stylet exudates of *D. noxia* and leaf exudates from wheat (*Triticum aestivum*) leaves of damaged and undamaged susceptible plants revealed changes in composition and levels of essential amino acids, which were not evident in the resistant plants, thus indicating nutritionally enhanced ingesta in susceptible plants fed on by *D. noxia*.

2.1.6.4 The electric penetration graph (EPG)

The EPG is an electronic system developed to study aphid salivary secretion and probing behaviour (McLean and Kinsey, 1964; Le Roux *et al*., 2008). The device can record signal waveforms that reflect different insect activities such as mechanical stylet work, saliva secretion and phloem sap ingestion (Reese *et al*., 2000; Will and van Bel, 2006; Will *et al*., 2008). The EPG has shown at least four phases of salivary secretion by aphids during plant penetration (Fig 2.2): (1) intercellular sheath secretion which envelopes the stylet, (2) intracellular potential drop salivation into cells along the stylet path as phloem sieve elements are punctured, (3) E1-initial phloem salivation (into SEs), and (4) E2-phloem feeding salivation (Cherqui and Tjallingii, 2000; Tjallingii, 2006; Harmel *et al*., 2008). The watery saliva excreted during probing is different from that secreted when the SEs are punctured because aphids have the ability to alter composition of salivary secretion in response to various chemical composition from the plant or their diet (Cooper *et al*., 2010; Moreno *et al*., 2011). According to Madhusudhan and Miles (1998), the watery saliva secreted by aphids during artificial feeding may not be similar to that released during direct feeding. Despite that the aphid salivary material recovered from artificial feeding medium (pure water, sucrose or amino acids) should at least indicate what aphids are capable of secreting into plants.

2.2 Defence responses

Plants lack a circulating adaptive immune system to protect themselves against pathogens or insects but are able to defend themselves by activating defence response mechanisms (Odjakova and Hadjiivanova, 2001; Maffei *et al*., 2007). The defence responses activated by aphid feeding are thought to be similar to those activated by bacterial, viral or fungal pathogens (Walling, 2000). Plants are not only exposed to pathogens and insects, but also environmental stresses such as heat, cold, water stress, mechanical and chemical stresses pose a threat to plants (Zhang *et al*., 1998). The interaction between plants and pathogens could either result in basic compatibility or basic incompatibility (Walling, 2000). In basic compatibility, the pathogens successfully colonize the plant and cause disease. However, plants also have a specific resistance mechanism called host incompatibility (Johal *et al*., 1995). Host incompatibility leads to activation of defence responses, therefore resulting in resistance (disease free) against pathogens.

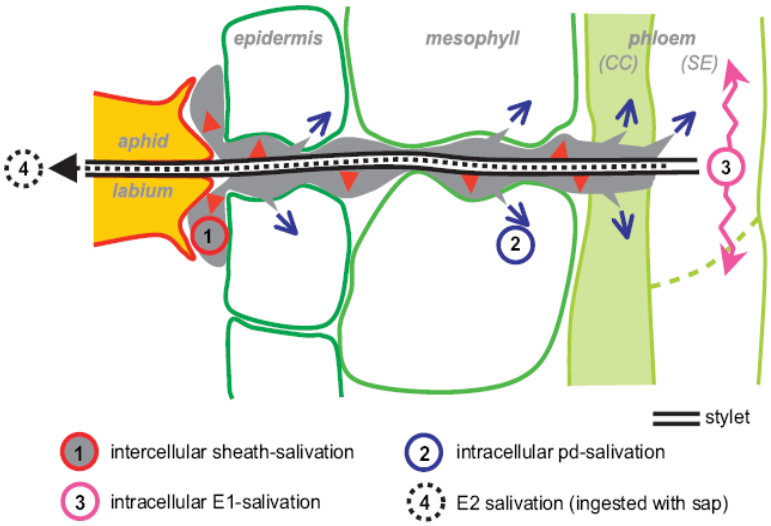

Figure 2.2: Feeding mechanism of the Russian wheat aphid. The numbers (1, 2, 3, and 4) represent salivation periods detected by the electrical penetration graph (EPG) technique (Tjallingii, 2006). CC: companion cell; SE: sieve element

2.2.1 General resistance

Plants protect themselves against pathogens by activating antimicrobial defence mechanisms that are either constitutive or inducible (Odjakova and Hadjiivanova, 2001). Constitutive (passive) plant defences include various structural and chemical cues that impede pathogen ingress, and/or deter colonization (antixenosis), growth, reproduction or survival (antibiosis) of the pathogen (Walling, 2000). These include morphological features such as thorns, trichomes and thickened cuticle as well as biochemical features such as accumulation of secondary metabolites (Hammond-Kosack and Jones, 1996; Walling, 2008). If the pathogen can overcome these preformed defences, the plant switches on the second line of defences (inducible). These defences involve the release of signaling compounds that are responsible for the induction of the defence responses (Ebel and Cosio, 1994; Nürnberger *et al*., 1994). The mounting of active defences is triggered by the recognition of structural or chemical features of pathogens or pests. This recognition is often mediated by pathogen associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides (Gómez-Gómez and Boller, 2002) or elicitors from plant pathogen or cell wall fragments (Garcia-Brugger *et al*., 2006). Elicitor recognition leads to initiation of signal transduction events that culminate in the activation of defence responses (Johal *et al*., 1995).

2.2.2 Host incompatibility

Host resistance is based on the recognition of specific elicitors that induce a specific response (Botha *et al*., 2005). In plant-aphid interactions, aphid derived elicitors [avirulence (*Avr)* gene products) are recognized by the *R* gene products in plants. This is followed by activation of aphid-specific resistance responses (Flor, 1971; Smith and Boyko, 2007). This is known as “gene-for-gene” (receptor-ligand) resistance. It has been postulated that *R* gene products act as receptors of *Avr* gene products, either directly or indirectly (Garcia-Brugger *et al*., 2006). The recognition between *Avr* and *R*-gene products initiates the generation of defence related signals that form part of the hypersensitive response, (HR) (Dangl and Jones, 2001), which

is triggered by a wide variety of pathogens and can occur within hours following recognition or pathogen contact (Morel and Dangl, 1997).

Even so the pathogen may try to avoid triggering these defence responses. The pathogen *Avr* genes can undergo mutation and enter without affecting the plant or being recognised. Therefore, the plant has to employ other strategies to defend itself for example through the “guard hypothesis”, in which the pathogenic effector is monitored by R-protein (guard), and changes caused by the effector activate the resistance when they interact with another plant protein (guardee) (Dangl and Jones, 2001; McDowell and Woffenden, 2003). It is presumed that insect saliva contains signals (avirulence effectors) that trigger the incompatible interaction using mechanisms proposed in guard hypothesis (Kaloshian and Walling, 2005).

2.2.3 Hypersensitive response

The hypersensitive response (HR) is characterized by the rapid formation of a localised cell and tissue death at the site of attempted pathogen ingress which correlates with exhibition of resistance (Mur *et al*., 2008). The expression of HR can occur in a single cell or can spread to numerous cells accompanying limited pathogen colonization (Hammond-Kosack and Jones, 1996). The HR is closely associated with defence responses such as the activation of calcium influxes, expression of the oxidative burst, induction of lipid peroxidation, as well as accumulation of signal molecules, for instance, nitric oxide, salicylic and jasmonic acids (Garcia-Brugger *et al*., 2006). Salicylic acid is responsible for triggering the expression of defence genes encoding certain pathogenesis related (PR) proteins. Also, antimicrobial phytoalexins accompany HR to further prevent infection by pathogens (Hahlbrook and Scheel, 1989).

2.2.4 Elicitor perception and signal transduction

Virulent pathogens present pathogen-associated molecular patterns (PAMPs), elicitors, and virulence factors that are perceived by the plant (Glazebrook, 2005). Signal perception in the plant cell relies on the presence of specific receptors for chemical signals of general recognition processes based on localized tissue injuries (Glazebrook, 2005). The pattern recognition receptors (PRRs) are members of the nucleotide-binding leucine-rich repeats (NB-LRR) family of proteins that activate the PAMP-triggered immunity. Corresponding PAMPs and PRRs that have been identified include β-glucan which corresponds with the extracellular glucan-binding protein (Mithöfer *et al*., 2000; Fliegmann *et al*., 2004); chitin which is perceived by the transmembrane LysM-containing receptor-like proteins, (CEBiP) (Kaku *et al*., 2006); and for flg22 and EF-Tu, the transmembrane Leucine-rich repeat receptor-like kinases FLS2 (Chinchilla *et al*., 2006) and EFR (Zipfel *et al*., 2006), respectively.

In race-specific interactions the recognition of the *Avr* gene products by *R*-gene products apparently occurs intracellularly, often involving leucine rich repeats (LRR)-containing proteins. However, not all R-proteins are intracellular, some occur as cell-surface receptors (Nürnberger, 1999; Bonas and Lahaye, 2002). Activation of plant defence responses requires timely perception of the aggressor, whether through recognition of race-specific elicitors or general elicitors (Mithöfer and Boland, 2008).

Following perception of elicitors by plant receptors effectors such as ion channels, GTP binding proteins (G-proteins), and protein kinases are activated. The activated effectors transfer elicitor signals to second messengers, which further amplify the elicitor signal to other downstream reactions (Ebel and Mithöfer, 1998; Blume *et al*., 2000). It should be noted that an elicitor signaling pathway may vary with perception of different elicitor signals or with target defence responses. The earliest reactions following elicitation is the activation of plasma membrane H^+-ATPase and opening of Ca^{2+} channels leading to rapid influx of Ca^{2+} and H^+ and efflux of K^+ and Cl^-. These

ion fluxes are prerequisites of MAP kinase activation and accumulation of reactive oxygen species (ROS, O^{2-}, H_2O_2 and OH^-) via the membrane-associated NADP(H) oxidase, peroxidases (apoplastic localized), oxalate oxidase and amine oxidase (Bolwell and Wojtaszek, 1997; Lamb and Dixon, 1997; Somssich and Hahlbrock, 1998; McDowell and Dangl, 2000). The H_2O_2 is an important ROS; it can cross membranes and directly induce cell signaling (Yin *et al*., 2010) of production of secondary metabolites such as phytoalexins, and more signaling molecules (salicylic acid, ethylene, jasmonic acid and nitric oxide). Furthermore, H_2O_2 produced at the plant cell surface drives rapid peroxidase-mediated oxidative cross-linking of structural proteins in the cell wall, reinforcing this physical barrier against pathogen ingression (Odjakova and Hadjiivanova, 2001).

2.2.5 Systemic acquired resistance

Systemic acquired resistance (SAR) is a type of induced resistance. When a plant becomes infected, it can develop resistance to a broad and distinctive spectrum of pathogens (Durrant and Dong, 2004). Systemic acquired resistance is mediated by SA produced or released from inactive conjugates during the HR (Johal *et al*., 1995). During SAR, plant resistance to pathogens is enhanced through systemic increases in SA levels and the expression of PR-proteins (Odjakova and Hadjivanova, 2001). Glucose oxidase from saliva of *Helicoverpa zea* feeding on soybean plants induced SAR against both the bacterial blight, caused by *Pseudomonas syringe* pv. *glycinea*, and the frogeye leafspot diseases caused by the fungus *Cerospora sojina* Hara (Felton and Eichenseer, 1999). Application of the salivary protein to mechanical wounds on soybean leaves triggered SAR against *Pseudomonas syringe* and *Cerospora sojina* that was comparable to insect feeding (Zhu-Salzman *et al*., 2005).

2.2.6 Induced systemic resistance

Induced systemic resistance (ISR) in plants is a defence mechanism that can be induced against a broad spectrum of pathogens (Hammerschmidt and Kuć, 1995). This type of induced resistance was named “rhizobacteria-mediated ISR” (van Loon *et al*., 1998; van Loon, 2007). Colonization of plant roots by strains of non-pathogenic plant growth-promoting rhizobacteria (PGPR) such as *Pseudomonas* (van Loon, 2007) and *Bacillus* (Kloepper *et al*., 2004), can induce a distinct broad-spectrum resistance response in both below and above ground parts of a plant (Conrath, 2009).

2.2.7 Signaling molecules

2.2.7.1 Salicylic acid

Salicylic acid (SA) is a phenolic acid crucial for HR responses (Smith and Boyko, 2007) that also promotes both local and systemic acquired resistance. Salicylic acid-dependant cascades use SA and its methyl salicylate (MeSA) to mediate SAR (Yin *et al*., 2010) and stimulate the expression of defence related genes such as the pathogenesis-related (PR) proteins. Aphid feeding induces expression of PR-genes and other transcripts associated with SA-mediated signaling in several plants including *Arabidopsis*, tomato, sorghum, wheat and tobacco (van der Westhuizen *et al*., 1998a,b; Moran and Thompson, 2001; Moran *et al*., 2002; Zhu-Salzman *et al*., 2004; Thompson and Goggin, 2006). In wheat, SA induction was observed in incompatibility, but not compatibility interaction with RWA (Mohase and van der Westhuizen, 2002b; Tsai, 2011).

2.2.7.2 Jasmonic acid

Jasmonic acid (JA) is a plant hormone and together with its methyl ester, methyl jasmonate (MeJa), are referred to as the jasmonates (Szczegielniak *et al*., 2005). Jasmonic acid and MeJa are transducers of elicitor signals for the production of plant secondary metabolites (Farmer *et al*., 2003), and induce resistance to chewing and cell-content feeding insects, as well as to certain fungal pathogens (Halitschke and Baldwin, 2004). When insects or pathogens attack plants, receptor recognition of elicitors initiates the octadecanoid-based pathway, where JA is formed from the C_{18} fatty acid linoleic acid (Blechert *et al*., 1995). Jasmonic acid and ethylene are both wound responsive and required for the elicitation of ISR by rhizobacteria (Pozo *et al*., 2004). Wounding and MeJA treatment induce the expression of lipoxygenase, allene oxide synthase and some stress-related genes (Creelman and Mullet, 1997). Jasmonate and ethylene co-operate to regulate the expression of genes and some jasmonate-inducible genes are not inducible in plants unable to produce or sense ethylene (Reymond and Farmer, 1998; Odjakova and Hadjiivanova, 2001). Microbe-associated molecular patterns (MAMPs) have been reported to stimulate JA and ethylene production, as well as up-regulate genes encoding proteins involved in the biosynthesis of JA and ET or pathogenesis-related proteins linked to SA-mediated responses (Denoux *et al*., 2008).

2.2.7.3 Ethylene

Ethylene (ET) is a phytohormone that regulates a wide range of plant processes, from growth and development to defence responses (Guo and Ecker, 2004; Zhao *et al*., 2005). Ethylene production can be induced by various stresses, such as wounding, ozone, microbial pathogens, insects, as well as elicitors (van Loon *et al*., 2006). While both JA and ET signaling pathways are essential for plant defence responses, ethylene is not a common signal for induction of plant secondary metabolites (Zhao *et al*., 2005).

2.2.7.4 Oxylipins

In plants, oxylipins are derived from C_{18} fatty acids like linolenic or linoleic acid via the lipoxygenase pathway (Vick, 1993). Oxylipins are classes of biologically active compounds that are generated by oxidative catabolism of polyunsaturated fatty acids (adding oxygen to the 9 or 13 position of C_{18} chain of linoleic and linolenic acids) by the coordinated action of lipase and lipoxygenase (Itoh *et al*., 2002). The biosynthesis pathway of JA-related cyclopentenone oxylipins via the octadecanoid pathway or non-enzymatic pathways is activated by elicitors caused by pathogens or insects (Zhao *et al*., 2005). Oxylipins act as signals to induce defence mechanisms in response to wounding, pathogen and insect attack (Shah, 2005).

Plant lipoxygenases (LOXs, EC 1.13.11; linoleate: oxygen oxidoreductase) constitute a large gene family of non-heme iron containing fatty acid dioxygenases, which are ubiquitous in plants and animals (Brash, 1999). Lipoxygenase catalyses the addition of molecular oxygen to fatty acids containing a *cis, cis,*-1,4-pentadiene system to give an unsaturated fatty acid hydroperoxide (Hamberg and Samuelson, 1967). Lipoxygenases are located in the cytoplasm and function in cell membrane lipid degradation and the production of plant defence signaling molecules such as jasmonic acid by action of allene oxide synthase (Smith and Boyko, 2007).

2.2.7.5 Nitric oxide

Nitric oxide (NO) is an important signaling molecule in plants. It is involved in the control of various physiological processes such as growth and flowering, regulation of stomatal aperture, xylem formation, stress and defence responses (Rodakowska *et al*., 2009). Nitric oxide activates the downstream defence signaling by the production of cGMP, which activates cyclic nucleotide-gated channels leading to Ca^{2+} and K^{+} influx, and downstream gene activation (Wendehenne *et al*., 2001). Reports indicate that *Avr* factors from pathogens stimulate NO production, which collaborates with reactive oxygen species (ROS) to promote disease resistance

(Delledonne *et al*., 1998). Wang and Wu (2004) stated that accumulation of ROS alone is not sufficient to mediate a strong disease resistance in plants, but it can act synergistically with NO to activate a stronger response. Reactive oxygen species and nitric oxide are therefore considered as primary defence signaling molecules (Bolwell, 1999). Accumulation of NO has been reported in RWA infested resistant wheat plants, thereby confirming the involvement of NO in RWA resistance response (Moloi, 2010). It was also discovered that NO acts as a signal for induction of pathogenesis-related (PR) proteins such as β-1,3-glucanase and peroxidase (Moloi, 2010).

2.2.8 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins have been defined as plant proteins that are induced by pathogens or related stress conditions (Cutt and Klessig, 1992) and are usually localised in the plant vacuole and the apoplast (Stintzi *et al*., 1993). Generally, PRs are low molecular weight proteins which can be extracted in acidic buffers. PRs were first detected in the early 1970s in tobacco leaves infected with *Tobacco mosaic virus* (TMV) (van Loon and van Kammen, 1970). Pathogenesis-related protein families (PR-1 to PR-17) from different plant species have been characterized and classified according to sequence similarities (van Loon *et al*., 1994; Fritig *et al*., 1998) (Table 1). A family could share similar biological activities, but differ in other properties such as substrate specificity, physiochemical properties or subcellular localization. The inducible pathogenesis-related proteins are mostly acidic proteins that are secreted into the intercellular space. In addition, basic pathogenesis-related proteins occur at relatively low levels in the vacuole (van Loon, 1997).

The PR families differentially play important roles in limiting activity, growth and spread of pathogens (van Loon *et al*., 2006). The β-1,3-glucanases (PR-2) and the endochitinases (PR-3, PR-4, PR-8 and PR-11) attack β-1,3-glucans and chitin which are components of the cell walls in most higher fungi (Honèe, 1999). Proteinase

inhibitors (PR-6) and chitinases have been reported to target nematodes and herbivorous insects. Germins and germin-like proteins (PR-15 and PR-16) have superoxide dismutase activity. The families of PR-12 (defensins) and PR-13 (thionins) both have broad antibacterial and antifungal activities; this has also been reported in PR-14 (lipid transfer proteins) (van Loon *et al*., 2006). Members of PR-1 and PR-5 families have been associated with activity against oomycetes (van Loon *et al.*, 2006).

The PR-5 proteins are thought to create transmembrane pores and have therefore been named permatins. The PR-1 proteins are markers of enhanced defensive state conferred by pathogen-induced SAR. The PR-6 (proteinase inhibitors) members act against herbivores and nematodes, and PR-7 members (endoproteinases) aid in microbial cell wall dissolution (van Loon *et al*., 2006). The PR-9 members have peroxidase activity which functions in cell reinforcements, thus limiting pathogen ingression. The PR-10 is the only family consisting of cytoplasmic proteins that might be specifically involved in antiviral activities; members have slight ribonucleases activity (Park *et al*., 2004). Lastly, PR-17 members have been discovered in infected tobacco, wheat and barley, and contain sequences that resemble the active site of zinc metalloproteinases (van Loon *et al*., 2006). The PR-17 family has not yet been characterized.

2.2.8.1 β-1,3-glucanases

β-1,3-Glucanases (EC 3.2.1.39) belong to the PR-2 family (Fritig *et al*., 1998). The endo β-1,3-glucanase produces oligomers of 2,6-glucose units from callose (β-1,3-glucan) (Stintzi *et al*., 1993) while the exo β-1,3-glucanase successively hydrolyses β-D-glucose units from non-reducing ends of β-1,3-D-glucan, releasing glucose units (Bielka *et al*., 1984). β-1,3-glucans are the major cell wall constituents of common fungal pathogens, bacteria and plants (Bowles, 1990). β-1,3-glucanases are responsible for the depolymerisation and mobilisation of the β-1,3-glucan and can therefore play a definite role in the mobilization of the glucan to the site of

infection and activation of the whole resistance mechanisms (Hinch and Clarke, 1980). In wheat, β-1,3-glucanases play a role in resistance against the RWA (van der Westhuizen *et al*., 1998a). It was reported that activities of β-1,3-glucanase in resistant cultivars resemble defence responses during pathogenesis and is part of HR (van der Westhuizen *et al*., 1998b).The accumulation of β-1,3-glucanase in infested plants may also be associated with expression of induced resistance to pathogens (Rahimi *et al*., 1996). Recently, a β-1,3-glucanase gene *TaGlu* has been isolated from *Puccinia striiformis* (*Pst*) infected wheat leaves and this gene was induced during both compatible and incompatible interations with *Pst*, but transcription level was much higher in the incompatible interaction (Liu *et al*., 2010).

2.2.8.2 Peroxidases

Peroxidases (EC 1.11.1.7) in plants can be induced by aphids (van der Westhuizen *et al*., 1998a) and pathogens (Reimers *et al*., 1992); wounding (Svalheim and Robertsen, 1990) and elicitors from fungal cell walls (Gotthardt and Grambow, 1992). These enzymes are involved in defence related events that occur in extracellular matrix (Bowles, 1990). Peroxidases play a major role in the production of H_2O_2 needed for lignin strengthening of cell walls and the formation of intermolecular cross links, suberin and the production of reactive oxygen species, which are associated with eliciting and signaling events as well as direct defence (Bowles, 1990; Mehdy, 1994). Peroxidases are broadly distributed in various tissues and cellular compartments in plants, and are responsible for plant physiological responses including auxin catabolism (Savitsky *et al*., 1999).

Table 1: Recognized families of pathogenesis-related proteins and their properties. [van Loon and van Strien (1999); van Loon *et al*. (2006)]

Family	Type member	Properties
PR1	Tobacco PR-1a	Antingal
PR2	Tobacco PR-2	β-1,3-glucanase
PR3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII
PR4	Tobacco 'R'	Chitinase type I, II
PR5	Tobacco S	Thaumatin-like
PR6	Tomato Inhibitor I	Proteinase-inhibitor
PR7	Tomato P_{69}	Endoproteinase
PR8	Cucumber chitinase	Chitinase type III
PR9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR10	Parsley "PR1"	Ribonuclease-like
PR11	Tobacco "class V" chitinase	Chitinase, type I
PR12	Radish Rs-AFP3	Defensin
PR13	*Arabidopsis* THI2.1	Thionin
PR14	Barley LTP4	Lipid-transfer protein
PR15	Barley OxOa (germin)	Oxalate oxidase
PR16	Barley OxOLP	Oxalate-oxidase-like
PR17	Tobacco PRp27	Unknown

2.3 Elicitors

Elicitors are low molecular weight compounds that can either originate from pathogen/insect (exogenous) or host (endogenous) and are able to induce defence responses in plants (Ebel and Cosio, 1994). They may either be abiotic, for example metal ions and inorganic compounds, or biotic elicitors originating from fungi, bacteria, virus, herbivores, plant cell wall components or chemicals released by plants upon pathogen or herbivore invasion (Zhao *et al*., 2005). Elicitors do not have a common chemical structure, but belong to a wide range of different classes of compounds including proteins, glycoproteins, glucans, lipids and synthetic molecules (Montesano *et al*., 2003). The distinct character of elicitors is that at low concentrations they are able to trigger plant defence reactions (Boller, 1995). This distinguishes elicitors from toxins, which may act only at higher concentrations and affect the plant destructively (Boller, 1995).

Elicitors can be divided into race-specific and non-race specific elicitors. The race-specific elicitors induce a response only in specific host cultivars (“gene-for-gene”, host incompatibility). Elicitors or avirulence determinants must be recognized by plant receptors or R-proteins, localized on the plasma membrane or the cytoplasm, before initiating signaling pathways (Zhao *et al*., 2005). Non-specific elicitors involve the induction of general defence mechanisms that lead to basic incompatibility (Montesano *et al*., 2003). Non-specific elicitors (general elicitors) induce defense responses in both host and non-host plants (Montesano *et al*., 2003).

2.3.1 Aphid saliva

The aphid saliva contains compounds that are capable of inducing defence responses in host plants. Miles (1972) reported that the watery saliva of most Homoptera is slightly alkaline, with pH of between 8 and 9. Miles (1999) also proposed that aphid saliva has the following functions: 1) to moisten food, 2) to pre-digest compounds in the food source (hydrolytic enzymes), 3) to facilitate the

“mechanical penetration” of cells during feeding, 4) to protect the stylet and prolong feeding time, and 5) to assist in dissolution of plant material during probing events. During feeding, the RWA secretes two types of saliva: the gelling saliva and the watery saliva. Composition of saliva secreted might vary due to the different diet compositions fed by aphids (Miles and Harrewijn, 1991; Urbanska *et al*., 1994; Cherqui and Tjallingii, 2000).

2.3.1.1 Salivary glands

Aphids have salivary glands that are paired, the right and left glands have two glandular units, a large principal gland and a smaller accessory gland (Tjallingii, 2006). The principal gland (innervated) plays a major role in sheath saliva production and contains eight secretory cells possibly responsible for secreting different components (Moreno *et al*., 2011, Roy and Dhandapani, 2011). On the other hand, the accessory gland does not appear to be innervated, and its cells do not show any differentiation (Moreno *et al*., 2011, Roy and Dhandapani, 2011). Accessory glands have been reported to transmit plant viruses by transferring the virus from haemolymph to salivary canal in the stylet and then into plants (Gray and Gildow, 2003). It has been inferred that the watery E1 saliva must originate from the accessory glands since E1 salivation (refer to 2.1.6.4, Fig 2.2) is responsible for inoculation of these viruses (Prado and Tjallingii, 1994). Protein C002 from salivary glands of the pea aphid whose sequence contains an N-terminal secretion signal was identified by Mutti *et al.* (2008). RNAi-based transcript knockdown of protein C002 resulted in severe reductions of pea aphid life span on fava bean (*Vicia faba*) leaves. Therefore this protein is essential for survival and/or feeding of pea aphid on its host plants (fava beans).

2.3.1.2 Gelling saliva

When the aphid settles on leaf tissue a small amount of gelling saliva, referred to as salivary sheath, is secreted before stylet insertion (E1, Fig 2.2) (Miles, 1999). The stylet secretes a viscous mixture of sheath precursors that gel rapidly when exposed to air. The access to oxygen allows oxidation of the sulphydryl groups to form disulphide bonds, which in turn promote formation of hydrogen bonds (Miles, 1999). The stylet can remain in contact with plant cells for hours to weeks (Walling, 2000). The origin of stylet sheath was first discovered by Hamilton (1935) in an artificial diet and was later accepted (Storey, 1939). This gelling saliva is secreted from the principal gland, forms a protective sheath around the stylet during probing, and prevents sap loss during intracellular penetration (Moreno *et al*., 2011). It seals the puncture site in the SE cell wall before and during the stylet tip piercing of SE plasma membrane (Tjallingii and Hogen-Esch, 1993). Thus, sheath saliva may reduce the influx of extracellular calcium (Ca^{2+}) into the SE lumen through the punctured site (Will and van Bel, 2006).

The gelling saliva consists of proteins, phospholipids, conjugated carbohydrates, and free amino acids (Kaloshian and Walling, 2005). The protein component [e.g. pectinase (Ma *et al*., 1990), cellulose and phenol oxidase (Miles, 1985) and peroxidase (Miles and Peng, 1989)] renders credence the involvement of sheath saliva in cell wall breakdown. Another function may be to shield the moving stylets from plant cells, and thus avoid triggering a defensive response by the plant that could include hypersensitive reactions or release of plant defensive chemicals (Resh and Cardé, 2009). In addition, the sheath's polyphenol oxidases may polymerize apoplastic phenolics (an induced defence) to prevent damage to plant cells (Walling, 2008). It is not clear whether unpolymerized sheath material acts as elicitors in plant-insect interactions (Walling, 2000; 2008).

2.3.1.3 The watery saliva

The watery saliva is a complex mixture of enzymes and other components responsible for eliciting plant defence signals (Baumann and Baumann, 1995, Urbanska *et al*., 1998). During probing the watery saliva which is secreted from accessory salivary glands is released into mesophyll cells (Martin *et al*., 1997) and then into the SEs. Ingestion of phloem sap occurs simultaneously with salivation, but at this stage the aphid saliva does not reach the plant. The phloem sap and aphid watery saliva mixture is forced into the food canal of the aphid by the hydrostatic pressure in the sieve elements (Tjallingii, 2006). Diffusion of the watery saliva surrounding the stylet into the plant tissue was first detected by using radiotracers (Madhusudhan and Miles, 1998). Moreover, the watery saliva plays important roles such as suppressing host defence responses, digesting external and ingested food material and secretion of certain metabolites (Carolan *et al*., 2009; Ma *et al*., 2010; Moreno *et al*., 2011). A wide array of compounds identified in the watery saliva include Ca^{2+}-binding proteins, pectinesterase, lipases, peroxidases, phenoloxidases, amylases, cellulose, sucrose, protease, and alkaline and acidic phosphatase (Miles, 1999; Funk, 2001; Will *et al*., 2007; Walling, 2009).

2.3.1.4 Enzymes in aphid saliva and salivary glands

In saliva and salivary glands of Hemiptera insects; 4 hydrolases (pectinesterase, polygalacturonase, cellulase, and amylase) and 5 oxido-reductases (catalase, peroxidase, catechol oxidase, superoxide dismutase, and ascorbate oxidase) have been detected (Madhusudhan and Miles, 1998). The role of hydrolases is to degrade polysaccharides in the cell wall, while the oxido-reductases interrupt redox balance of plants by affecting the generation and removal of hydrogen peroxide (Ni *et al*., 2000). These enzymes play key roles in feeding, digestion, metabolism, and self-detoxification processes of aphids (Miles and Peng, 1989; Miles and Oertli, 1993). Most of these enzymes may additionally act as elicitors of plant defence responses.

Oxido-reductase activities such as catalases, superoxide dismutases and ascorbate oxidases and not peroxidases, have been detected in salivary glands of *Diuraphis noxia* (Ni *et al*., 2000). A comparative study between *Diuraphis noxia* and *Rhopalosiphum padi* revealed that *D. noxia* salivary glands lacked peroxidase; but expressed catalase, whereas *R. padi* expressed peroxidase, but not catalase. These differences between aphid species may contribute to the differential types of damage on wheat plants (Ni *et al*., 2000). Peroxidases have also been found in salivary glands and stylet sheath of the rose aphid (Miles and Peng, 1989), and in the watery saliva of spotted alfalfa (Miles and Peng, 1989) and pea aphids (Madhusudhan and Miles, 1998).

The watery saliva and stylet sheaths of phytophagous Heteroptera contain polyphenol oxidase (catechol oxidase), which originates from the accessory glands of the salivary apparatus (Miles, 1999). The salivary oxidases in aphids are discharged in host plants and detoxify defensive phytochemicals responsible for defence responses (Miles, 1964; Peng and Miles, 1988; Urbanska *et al*., 1998). The salivary enzymes of aphids stimulate polyphenol oxidase (PPO) in plants, which acts as an anti-nutrient protein, causing bad digestion, or antifeedant activity, thus protecting plants from further damage (Urbanska *et al*., 1998). It is not clear whether the presence of PPO in wheat aphid saliva functions in triggering the defence gene expression during feeding in the host plant (Ma *et al*., 2010). Catechol oxidase and peroxidase from saliva of the rose aphid have been reported to oxidize phenolics and other allelochemicals in potato, Chinese cabbage and lettuce (Peng and Miles, 1991). Additionally, oxidases have also been reported to induce oxidative reactions that lead to chlorotic streaks in wheat leaves, and further investigations to prove this still need to be taken (Ma *et al*., 2010). The following enzymes have been detected in saliva and salivary glands of aphids:

2.3.1.4.1 Glucose oxidase

Glucose oxidase (GOX) is a salivary effector that suppresses wound signaling (Walling, 2000). It has been discovered in the hypopharyngeal glands of the worker honeybee (*Apis mellifera*) (Ohashi *et al*., 1999), saliva of *Myzus persicae* (Harmel *et al*., 2008) and also in the labial glands and salivary secretions of *Helicoverpa zea* (Eichenseer *et al*., 1999; Musser *et al*., 2005; Na and Chenzhu, 2004). It is needed to convert glucose into gluconic acid and H_2O_2, and might also serve as an elicitor in plants (Felton and Eichenseer, 1999). Glucose oxidase from the caterpillar, *Helicoverpa zea*, Boddie, was the first to be characterized as a salivary suppressor of nicotine, an inducible anti-herbivore defence molecule of tobacco (*Nicotiana tobacum*) (Musser *et al*., 2002; Na and Chenzhu, 2004). Furthermore, evidence was obtained that GOX from the caterpillar' s labial salivary gland extracts may be involved in the suppression of the pathogenicity of bacterial pathogens (Musser *et al*., 2005). Glucose oxidase-derived H_2O_2 is able to kill adapted or non-adapted plant microbes that enter the insect's feeding site, within the mid-gut GOX may serve as an effector by inhibiting ingested plant oxidative enzymes such as polyphenol oxidase, peroxidases and lipoxygenase that decrease the digestibility of plant proteins (Eichenseer *et al*., 1999).

2.3.1.4.2 Alkaline phosphatase

Alkaline phosphatase (ALP) is a ubiquitous enzyme in all organisms that is used to hydrolyze orthophosphate monoesters; their precise physiological function remains unclear, but they are considered to play a role in phosphate uptake and in secretory processes in epithelia of mammals (Yan *et al*., 2011). Alkaline phosphatase activity was discovered in saliva of whitefly (Bemisia spp., Homeoptera: Aleyrodidae) but not detected in aphid saliva from *Rhodobium porosum* Sanderson or *Aphis gossypii* Glover (Aphididae) (Funk, 2001). Cooper *et al.* (2010) were the first to discover this enzyme in *D. noxia* saliva. In Lepidoptera, ALP might be involved in absorption (Eguchi, 1995) and transport of nutrients across the gut or alkalinisation of the midgut lumen (Azuma *et al*., 1991). This enzyme has also been reported to play a

role in insect development, neural and renal function, and cuticle sclerotization (Chang *et al*., 1993; Yang *et al*., 2000; Yan *et al*., 2011). In whiteflies, higher secretions of ALP enhance feeding, thus ALP may play a role during whitefly feeding.

2.3.1.4.3 Lysozymes

Chewing insects such as caterpillars deliver mandibular and labial saliva and mid-gut regurgitants to the feeding site. Lysozymes, which hydrolyse the peptidoglycan of bacterial cell walls and induce defences against pathogens, are abundant in labial saliva, but absent in regurgitants (Liu *et al*., 2004). It is also possible that the salivary lysozyme would contact and hydrolyze the cell walls of the epiphytic bacterial microflora and pathogens that reside on plant foliage and release peptidoglycans which act as PAMPs (Gust *et al*., 2007).

2.3.1.5 Phloem-feeding insect derived elicitors

The phloem-feeding insect (PFI) saliva plays a major role in plant defence responses. In compatible interactions, PFI oral secretions are a potential source of *Avr* factors (Thompson and Goggin, 2006). In aphids no herbivore-associated molecular patterns (HAMPs) have been identified, however in some plant species there is good genetic evidence for *R*-gene mediated (gene-for-gene) resistance to phloem feeding insects (Walling, 2008). Given the significance of oral secretions in plant interactions with chewing insects, it is highly probable that insect secretions also mediate defence responses during interactions with PFIs. There are no *Avr* gene(s) which have been cloned from insects but Lapitan *et al*. (2007) identified a protein fraction isolated from the Russian wheat aphid (RWA) that could be a key in determining compatibility in plant-aphid interactions. When the fractions were injected into susceptible wheat cultivars, the protein induced the leaf-rolling symptom typical of RWA feeding in compatible interactions (Lapitan *et al*., 2007).

2.3.2 Insect-derived elicitors

Oral secretions (OS), a mixture of insect saliva plus regurgitants, from feeding insects often contain potent sources of elicitors, molecular patterns, and effectors that influence both direct and indirect defences (Mithöfer and Boland, 2008; Turlings and Ton, 2006; Felton and Tumlinson, 2008). Insect-derived elicitors identified in plant defence responses include volicitin, *N*-linolenoyl-l-glutamine, inceptin, caeliferin, bruchin and β-glucosidase (Howe and Jander, 2008). Others such as glucose oxidase (Eichenseer *et al*., 1999) and alkaline phosphatase (Funk, 2001) from larvae of the white cabbage butterfly (*Pieris brassicae*) have been identified.

2.3.3 Plant-derived elicitors

Elicitors are divided into two classes of regulatory oligosaccharides (oligosaccharins); those originating from the cell walls of microorganism (glucans, chitins, and chitosans) and those from the plant cell wall (Côtè *et al*., 1998). Four major classes of elicitor-active oligosaccharides have been identified: oligoglucan; oligochitin; oligochitosan and oligogalacturonides of plant origin (Ebel, 1998). Glucan elicitors are composed of 3-,6- and 3,6-linked β-glucosyl residues, and stimulate the production of phytoalexins, and newly synthesized antimicrobial low molecular weight compounds. These have been detected in culture filtrates of oomycets *Phytophthora sojae*, a pathogen of soy bean (Ebel, 1998). Glucan elicitors from fungal cell walls are released through hydrolytic enzymes (e.g. β-1,3-glucanases) released by infected plants. Reports indicate that they induce production of phytoalexins in numerous plants (Klarzynski *et al*., 2000).

A plant-derived elicitor, systemin, an 18-amino acid peptide, produced at wounded sites of tomato plants in response to attack by herbivorous insects, activates signaling pathways leading to defence responses (Scheer and Ryan, 2002). A

systemin receptor SR160, very similar to brassinosteroid receptor in *Arabidopsis* has been purified (Scheer and Ryan, 2002).

2.4 Herbivore-induced plant volatiles

In response to herbivory, plants emit specific blends of herbivore-induced plant volatiles (HIPVs). When plants are damaged by sucking arthropods (e.g. spider mites or aphids), antagonistic cross-talk of SA with JA seems to regulate the biosynthesis of HIPVs in the infested leaves (Ozawa *et al*., 2000; Leitner *et al*. 2005; Girling *et al*., 2008). Herbivory can be mimicked by adding crude insect regurgitants to a wound site eliciting volatile releases (Turlings *et al*., 1998). Several chemical classes of volatiles including terpenes, sulphides, nitriles, indole and others are released following herbivory (Paré and Tumlinson, 1999).

Volatiles induce plant systemic resistance in neighbouring plants (Zhao *et al*., 2009). herbivore-induced plant volatiles can prime plants to enhanced defence responses upon subsequent challenge (Turlings and Ton, 2006; Frost *et al*., 2008). Gene induction responses to aphid feeding in *Arabidopsis* are substantially different compared to those induced by artificial wounding. This indicates that there is specific plant recognition of some component, possibly oral elicitors of aphid-feeding (Moran and Thompson, 2001).

2.5 Calcium binding salivary proteins

The SEs regardless of plant origin (species and family), have the ability to clog (callose deposition) immediately after the slightest mechanical wounding (Will *et al*., 2007). This is done by rapidly sealing the plasma membrane lesion to prevent leakage of phloem sap into the apoplast (Will and van Bel, 2006). Wound-induced plugging of sieve plates (Donofrio and Delaney, 2001) involves synthesis of 1,3-β-glucan with a few 1,6 branches synthesized by a membrane-spanning 1,3-β-glucan synthase. β-1,3-Glucan is Ca^{2+} sensitive and can be degraded by

endo-1,3-β-glucanase (Will and van Bel, 2006). Dorschner (1990) postulated the presence of callose-hydrolysing β-1,3-glucanase in watery saliva that may serve as a weapon against sieve plate clogging.

The Ca^{2+} in SEs is available in low concentrations which increase upon wounding (van Bel and Gaupels, 2004). An increase in free Ca^{2+} or a change in redox state in the SEs initiates “clogging” of the sieve plates (Knoblauch *et al*., 2001; Will and van Bel, 2006). This “clogging” does not occur when the SEs are punctured during aphid feeding. Thus, aphids could be preventing the flow of Ca^{2+} into the sieve elements in two ways: 1) by closing off the wound caused by the stylet with gelling saliva to prevent Ca^{2+} inflow, and/or 2) preventing the plant from detecting changes in turgor pressure during feeding (Knoblanch and van Bell, 1998; Knoblauch *et al*., 2001).

Previously, research by Knoblauch and van Bell (1998) showed that when the phloem is punctured with a fluid filled microcapillary with the same diameter as an aphid stylet, deposition of “clogs” on sieve plates of broad bean, *Vicia faba*, were induced. Therefore, the secreted aphid saliva should possess some components preventing clogging of sieve plates. Furch *et al.* (2007) suggested that the watery saliva contains Ca^{2+} binding proteins that bind Ca^{2+} responsible for triggering activation of sieve-plate occlusion mechanisms. The researchers’ suggestions were supported by Will *et al*. (2007) who identified Ca^{2+} binding proteins in saliva of *Megoura vicaiae* (vetch aphid) feeding on *Vicia faba*. Evidently, aphid saliva does possess some anti-clogging properties (De Vos and Jander, 2009) and this provides a second tool that suppresses sieve plate plugging after sealing with sheath saliva.

Evidently, elicitors are an important aspect in plant-insect interactions and their ability to induce plant defence mechanisms creates a platform for better understanding of pathways involved in plant resistance. Enzymes from oral secretions and oviposition of insects have been identified as elicitors of plant defences, which may lead to the release of volatiles. These insect derived elicitors have been classified as HAMPs similar to MAMPs. Although no HAMPs from aphids

have been identified, aphid salivary secretion contains enzymes such as peroxidase, polyphenol oxidase and alkaline phosphatase.

The expression of induced defence responses such as the accumulation of PR-proteins associated with SA-mediated signaling (van der Westhuizen, 1998a,b; Mohase and van der Westhuizen, 2002a), and lipoxygenases associated with jasmonic acid (Berner, 2006), have been reported in RWA-wheat interactions. Even though RWA feeding induces slight wounding in plants, it elicits defences that involve the activation of both SA and JA-dependent signaling pathways. It is anticipated that RWA saliva comprises elicitors responsible for perception and signal transduction events involved in aphid resistance. Identification of such elicitors could be a breakthrough in understanding eliciting and signal transduction events associated with resistance to the RWA.

MATERIALS AND METHODS

The materials and methods described in this chapter were employed to investigate and compare the potential of aphid (*RWASA1* and *RWASA2*) saliva in the expression of defense responses in different wheat cultivars, and to elucidate some of the characteristics of the elicitor-active saliva fractions.

3.1 Plant material

Seeds of the wheat (*Triticum aestivum* L.) cultivar Tugela *Dn1* [*Dn1* resistance gene, PI 137739 (du Toit, 1989)] which is resistant to *RWASA1* and its near-isogenic cultivar Tugela, which is susceptible to all prevailing biotypes were used. A third cultivar, PAN 3144 [*Dn5* resistance gene, (Tolmay *et al*., 2007)] which is resistant to both *RWASA1* and *RWASA2* was also used. The RWA biotypes *RWASA1* and *RWASA2* [*Diuraphis noxia* (Kurdjumov)] were provided by the Agricultural Research Council-Small Grain Institute (ARC-SGI), Bethlehem, South Africa. Plants were grown under controlled conditions in the glass house at temperatures of 24 ºC (day) and 19 ºC (night), and kept in clean cages. The plants were watered daily, and fertilised (Multi-feed®) three times a week.

3.2 Aphid rearing

Mass rearing of aphids was maintained on susceptible wheat plants, *RWASA1* was multiplied on Tugela cultivar and *RWASA2* on Tugela *Dn1* cultivar in the glass house under prevailing day and night photoperiod.

3.3 Aphid saliva collection

A sterile glass container with diameter of 57 mm and 70 mm high was used as a feeding chamber for aphids (Fig 3.1). As described by Harmel *et al*. (2008), the top part was covered by double layer of Parafilm® (soaked in sterile distilled water overnight), and 2 ml of sterile distilled water (feeding medium) was dispensed between the two layers (Miles and Harrewijn, 1991). This feeding medium was filled such that it excluded any air and allowed fluid diet to flow into aphid stylets once feeding commenced. About 250 adult aphids (equivalent to 240 mg) of *RWASA1* or *RWASA2* were transferred from plants by carefully tapping onto a petri dish and gently transferring to the feeding chamber. The feeding chambers were placed in an incubator (24 ± 2 ºC), illuminated from above with constant yellow light (yellow filter). In order to concentrate the feeding medium with aphid saliva, another batch of weighed aphids replaced the feeding aphids after 6 hours and fed for another 14 hours. The watery saliva was collected after a total of 20 hours of feeding and stored at -20 ºC. The watery saliva was pooled in final volumes of 40 ml (10 000 aphids fed), and controls were subjected to the same procedure but without aphids.

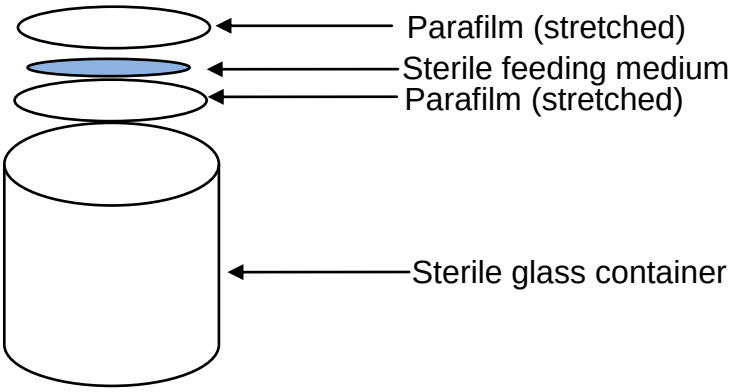

Figure 3.1: Diagram representing system used for collection of RWA saliva.

3.3.1 Concentration of the watery saliva

The collected watery saliva (40 ml) and the control feeding medium were freeze dried (Virtis Advantage) at -40 ºC for 24 hours. The dried residue was redissolved with 4 ml of distilled water (unless otherwise stated) and immediately injected into plants.

3.4 Determination of eliciting activity of elicitors in aphid saliva

3.4.1 Intercellular injection of elicitors from salivary material into plants

At the beginning of the third leaf growth stage, second leaves of different wheat plants (cultivars) were injected intercellularly with elicitors from the RWA salivary material using the Hagborg device (Hagborg, 1970) as shown in Fig 3.2. The leaves gently placed between the rubber stoppers were held firm by locking the forceps and elicitors from RWA salivary material (150 µl) were injected into the leaf with a sterile syringe (Mohase, 1998; Mohase and van der Westhuizen, 2002a). The control plants were intercellularly injected with control medium (refer to 3.3). Only the injected leaves were harvested 0, 3, 6, 9 or 48 hours after treatment. The leaves were quick frozen in liquid nitrogen and stored at -20 ºC.

Figure 3.2: Illustration of Hagborg device used for intercellularly injecting elicitors from RWA salivary material into wheat leaves (Hagborg, 1970).

3.4.2 Enzyme extractions

Leaf tissue (0.3 g) was ground in liquid nitrogen using a pre-cooled pestle and mortar. The powdered plant material was homogenised in 100 mM sodium acetate buffer (pH 5.5) at a ratio of 1:5 (plant material: buffer) (Mohase, 1998). The sodium acetate buffer contained 10 mM mercaptoethanol, 2 mM ethylene diamine tetra-acetic acid (EDTA) and 2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12000 xg for 20 minutes at 4 ºC and the supernatant stored as aliquots at -20 ºC was used as enzyme extract.

3.4.3 Protein determination

Protein concentrations were determined according to the Bradford (1976) method using Microplate Reader (Anthos, Zenyth 3100) at 595 nm. Bovine serum albumin (BSA, 0.5 µg μl^{-1}) was used as a standard.

3.4.4 Determination of enzyme activities

3.4.4.1 β-1,3-glucanase activity

A modified method of Fink *et al*. (1988) was used. The assay mixture containing 10 µl enzyme extract, 250 µl 2 mg ml^{-1} laminarin, and 240 µl 50 mM sodium acetate buffer (pH 4.5) was placed in a water bath at 37 ºC for 10 minutes. After the incubation, 500 µl of Somogyi reagent [0.2 g $CuSO_4$, 9 g Na_2SO_4, 1.2 g Na_2CO_3, 0.8 g $NaHCO_3$ and 0.6 g $C_4H_4KNaO_6.4H_2O$ in 50 ml distilled water (Somogyi, 1952)] was added and incubated at 100 ºC for 10 minutes. The reaction tubes were cooled under tap water, and 500 µl of Nelson's reagent [2.65 g $(NH_4)_6Mo_7O_{24}$, 2 ml 95-97% H_2SO_4, 0.32 g $Na_2HAsO_4.7H_2O$ in 50 ml distilled water (Nelson, 1944)] was added. The samples were vigorously shaken and absorbance was measured at 540 nm. β-1,3-Glucanase activity was estimated using a calibration curve generated with

different concentrations of glucose and specific activity was expressed as mg glucose mg^{-1} protein.

3.4.4.2 Peroxidase (POD) activity

A modified method of Zieslin and Ben-Zaken (1991) was used. The peroxidase assay consisted of 840 µl of 40 mM potassium phosphate buffer (pH 5.5) containing 2 mM EDTA, 100 µl of 5 mM guaiacol, 10 µl of enzyme extract and 50 µl of 8.2 mM H_2O_2. The change in absorbance was measured at 470 nm for 3 minutes at 30 ºC and peroxidase activity was determined using the molar extinction coefficient of guaiacol (2.66 $mM^{-1}cm^{-1}$) and specific activity was expressed as µmol tetraguaiacol mg^{-1} protein min^{-1}.

3.4.4.3 Lipoxygenase (LOX) activity

Linoleic acid substrate (2.5 mM linoleic acid in 0.15% (v/v) Tween 20) was prepared according to Ocampo *et al*. (1986). Linoleic acid (400 µl), Tween 20 (768 µl) and 40 ml methanol were added into a round bottomed flask and subjected to rotary evaporation at 60 ºC until dry. The residue was redissolved in 500 ml of 0.05 M sodium phosphate buffer (pH 9), divided into 5 ml aliquots and stored in air tight bottles at -20 ºC. Nitrogen gas was bubbled through the round bottom flask and air tight bottles during each aliquot transfer. During lipoxygenase assay the substrate was placed on ice and only used once.

Lipoxygenase activity was determined according to modified methods of Ocampo *et al*. (1986) and Grossmann & Zakut (1997). The reaction mixture consisted of 500 µl of 0.1 M sodium citrate phosphate buffer (pH 6.2), 25 µl of enzyme extract and 75 µl of 2.5 mM linoleic acid. The change in absorbance was measured at 234 nm for 10 minutes at 30 ºC. Lipoxygenase activity was calculated using the molar extinction

coefficient (9.6 x 10^{-7} $mM^{-1}cm^{-1}$) of hydroperoxide (HPOD) and specific activity was expressed as nmol HPOD mg^{-1} protein min^{-1}.

3.5 Fractionation of the salivary material

3.5.1 C18 Reverse phase chromatography

Forty millilitres of *RWASA1* or *RWASA2* salivary material was freeze dried (Virtis Advantage) for 24 hours and redissolved in 4 ml 70% (v/v) methanol. A portion of the mixture (2 ml) was fractionated using reverse phase chromatography on a pre-equilibrated (15 ml 70% methanol) stack of three C18 Sep Pak (Separations Waters) cartridges connected to a fraction collector (2112 Redirac, LKB Bromma). The column was eluted with 25 ml 70% (v/v) methanol at a flow rate of 0.25 ml min^{-1} (Minipuls 2, Gilson) and the eluate was collected as 0.5 ml fractions. The fractions were dried *in vacuo* at ambient temperatures, redissolved in 2 ml sterile distilled water and injected intercellularly into second leaves of plants at the beginning of the third leaf growth stage. The injected leaves were harvested 0, 3, 6, 9 or 48 hours after treatment. Enzyme activities of β-1,3-glucanase, peroxidase and lipoxygenase were determined as described previously (section 3.4.4).

3.5.2 Sephadex G-25 desalting

Salivary material of *RWASA1* or *RWASA2* was fractionated using PD-10 (Sephadex G-25, Separations) columns. Forty millilitres of *RWASA1* or *RWASA2* salivary material were freeze dried (Virtis Advantage) and redissolved in 2.5 ml of sterile distilled water. The samples were fractionated on a PD-10 column connected to a fraction collector (2112 Redirac, LKB Bromma) and eluted with 3.5 ml sterile distilled water at a flow rate of 0.25 ml min^{-1} (Minipuls 2, Gilson) and 0.5 ml fractions were collected. The fractions were immediately injected into second leaves of plants at the beginning of the third leaf growth stage. The leaves were sampled and induced enzyme activities determined as described in section 3.4.4.

3.6 Characterization of elicitor active fractions

3.6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using 11% separating gel and 6% stacking gel in a Mini-Protean II dual slab cell (Bio-Rad). Electrophoresis was carried out at a constant voltage of 200V for 45-60 minutes. The gels were silver stained according to Blum *et al.* (1987). Broad range molecular weight standards (Bio-Rad) were run alongside the protein samples.

3.6.2 Thin layer chromatography (TLC)

3.6.2.1 Detection of carbohydrates

Thin-layer chromatography (TLC) was performed on a 20 x 20 cm aluminium sheet pre-coated with Silica gel 60 F_{254} (Macherey-Nagel, Germany). The samples (equivalent to 2 µg protein) were loaded onto the plate at baseline in small portions with drying after each application. The TLC-plate was run in a solvent mixture of *n*-butanol: acetic acid: water (2:1:1) for 1 hour without any disturbances. The TLC-plate was then dried and sprayed with aniline reagent [Smith, 1960; (aniline (1 ml), diphenylamine (1 g), in 100 ml acetone (aniline reagent), and just before use 1 ml of 85% (v/v) Phosphoric acid was added to 10 ml of aniline reagent] and dried for 5 minutes (Baron and Economidis, 1963). After spraying and drying, the TLC-plate was developed in an oven at 120 ºC for 5 minutes (Baron and Economidis, 1963). Glucose (0.2 µg) was used as a standard.

3.6.2.2 Detection of amino acids

Similar procedure as in section 3.6.2.1 was used. Amino acids in samples (equivalent to 2 µg protein) were developed in solvent mixture of *n*-butanol: acetic acid: water (3:1:1) and detected by spraying with ninhydrin solution [0.25 g in 100ml

methanol, 20 ml glacial acetic acid and 4 ml 2,4,6-collidine]. The TLC-plate was heated at 105 ºC for 5 minutes (Baron and Economidis, 1963). β-Alanine (0.5 µg) was used as standard.

3.7 Partial purification of active fractions

Forty millilitres of *RWASA1* and *RWASA2* salivary material was freeze dried (Virtis Advantage) for 24 hours and redissolved in 4 ml 70% (v/v) methanol. A portion of the mixture (2 ml) was fractionated using reverse phase chromatography on a pre-equilibrated (15 ml 70% (v/v) methanol) stack of three C18 Sep Pak (Separations Waters) cartridges connected to a fraction collector (2112 Redirac, LKB Bromma). The column was eluted with 25 ml 70% (v/v) methanol at a flow rate of 0.25 ml min^{-1} (Minipuls 2, Gilson) and the eluate was collected as 0.5 ml fractions. Active fractions (12, 13, 17 and 18) were combined: fraction a (12 and 13); and fraction b (17 and 18) and dried *in vacuo* at ambient temperatures separately and each redissolved in 2.5 ml sterile distilled water. Fractions (a) and (b) were further fractionated on PD-10 columns separately. A portion (one half) of each fraction collected was also boiled for 30 minutes at 95 ºC. The collected fractions (unboiled and boiled) were injected into second leaves of plants at the beginning of the third leaf growth stage. The injected plants were harvested 0, 3, 6, 9 or 48 hours after treatment. Enzyme activities of β-1,3-glucanase and peroxidase were determined as described previously (section 3.4.4). Furthermore, the partially purified fractions were loaded on SDS-PAGE and TLC-plate.

3.8 Statistical analysis

For all assays, at least two sets of separate experiments were conducted, and within each experiment, assays were done in triplicate. The data was analyzed using Sigma Plot (version 7.0, 2001) of SYSTAT software followed by the *t*-test statements to ensure that mean values of two data columns [(resistant *Dn1* plants injected with elicitors from either *RWASA1* or *RWASA2* salivary material or sterile dH_2O);

(resistant (*Dn5*) plants injected with elicitors from *RWASA1* or *RWASA2* salivary) or (susceptible plants injected with elicitors of *RWASA1* or *RWASA2* salivary material and sterile dH_2O)] were significantly different (if P value is ≤ 0.05).

RESULTS

4.1 The effect of *RWASA1* and *RWASA2* salivary material on enzyme activities of peroxidase, β-1,3-glucanase and lipoxygenase.

The eliciting potential of aphid saliva was investigated in different wheat cultivars (Tugela, Tugela *Dn1* and PAN 3144) using enzyme activities of peroxidase (Fig 4.1), β-1,3-glucanase (Fig 4.2) and lipoxygenase (Fig 4.3) as indicators of defence responses (Mohase, 1998; Mohase and van der Westhuizen, 2002a; Berner, 2006). For results of independent replicate experiments, refer to Appendix (Figs 7.1, 7.2 and 7.3).

The following figures (Figs 4.1, 4.2 and 4.3) illustrate the effect of *RWASA1* and *RWASA2* crude salivary material on defence related enzyme activities in different wheat cultivars. Salivary material of *RWASA1* significantly induced an increase in peroxidase activity in Tugela *Dn1* (3.9-fold, $P = 0.0067$) and PAN 3144 (2.5-fold, $P = 0.000594$) cultivars (Fig 4.1). When comparing the effect of *RWASA1* salivary material on peroxidase activity in Tugela *Dn1* and PAN 3144 the induced activity was significantly ($P = 0.0239$) higher in Tugela *Dn1* than PAN 3144. *RWASA2* salivary material on the other hand induced a selective increase in peroxidase activity, only in PAN 3144 (2.4-fold, $P = 0.009$) cultivar. In contrast, the levels of peroxidase activity induced by *RWASA1* or *RWASA2* salivary material in PAN 3144 ($P = 0.683$) were not significantly different. Tugela cultivar did not show any induced activity of peroxidase after treatment with either salivary material of *RWASA1* or *RWASA2*.

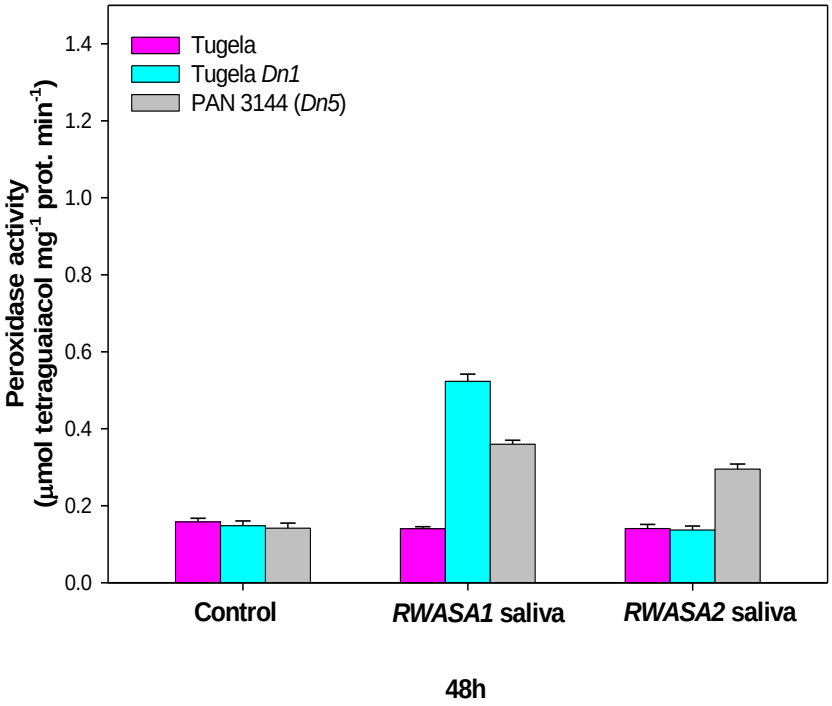

Figure 4.1: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on peroxidase activity of different wheat cultivars. Values are means ± SD (n=3).

Salivary (*RWASA1*) material, injected intercellularly into leaves, induced a differential increase in β-1,3-glucanase activity of different wheat cultivars (Fig 4.2). The highest increase in activity of β-1,3-glucanase was measured in Tugela *Dn1* (2.2-fold, *P* = 0.0058) and a slightly lesser increase was measured in PAN 3144 (1.9-fold, *P* = 0.00679). The levels of induced β-1,3-glucanase in Tugela *Dn1* and PAN 3144 were significantly (*P* = 0.0196) different. The salivary material of *RWASA2* on the other hand selectively induced an increase in β-1,3-glucanase activity only in PAN 3144 (1.7-fold, *P* = 0.0072). There was no significant difference (*P* = 0.0871) in the induced activity of β-1,3-glucanase in PAN 3144 when treated with either *RWASA1* or *RWASA2* salivary material. The salivary material of both *RWASA1* and *RWASA2* did not induce any increases in β-1,3-glucanase activity of Tugela cultivar.

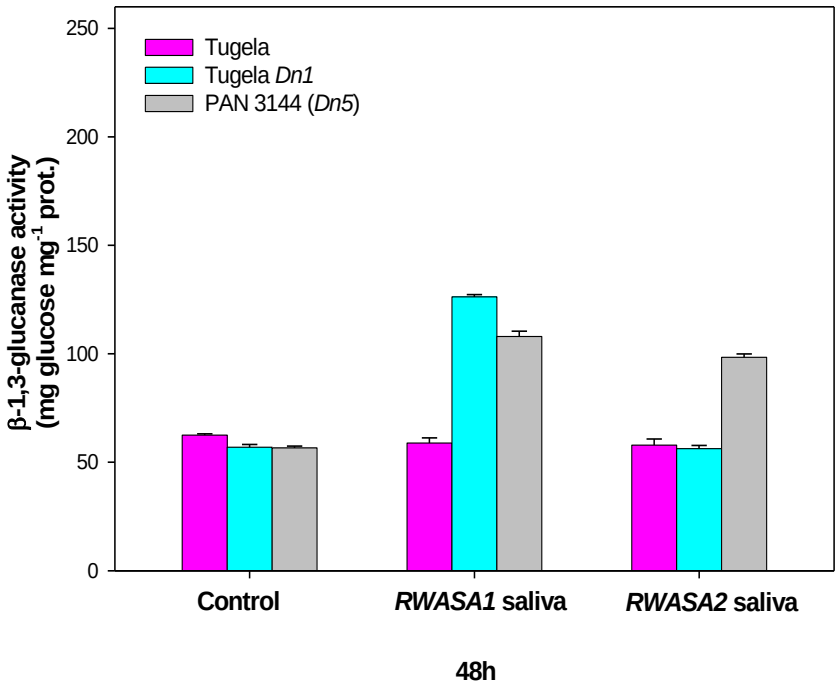

Figure 4.2: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on β-1,3-glucanase activity of different wheat cultivars. Values are means ± SD (n=3).

Aphid salivary material, intercellularly injected into the leaves, induced a differential increase in lipoxygenase activity of different wheat cultivars over a period of 48h (Fig 4.3). Elicitors in *RWASA1* salivary material slightly induced an increase in the activity of lipoxygenase as soon as 3h after treatment in Tugela *Dn1* (1.1-fold) and PAN 3144 (1.2-fold) cultivars. Elevated levels of lipoxygenase activity were further observed 6h and 9h after treatment in Tugela *Dn1* (1.5-fold; 1.8-fold) and PAN 3144 (1.3-fold; 1.7-fold), respectively. The highest level of increased lipoxygenase activity occurred 48h after treatment in Tugela *Dn1* (2.4-fold, $P = 0.000619$) and PAN 3144 (2-fold, $P = 0.00475$). Elicitors in *RWASA1* salivary material induced increases in lipoxygenase activity significantly ($P = 0.01359$) higher in Tugela *Dn1* than in PAN 3144 cultivar. Elicitors in salivary material of *RWASA2*, on the other hand were specific and only induced activity of lipoxygenase in PAN 3144 cultivar only. Increases in lipoxygenase activity also commenced as early as 3h (1.2-fold) after treatment. Similar to *RWASA1* salivary material, elicitors in *RWASA2* salivary

material induced the highest levels of lipoxygenase activity in PAN 3144 (1.8-fold, *P* = 0.00016) 48h after treatment. Nonetheless, elicitors in *RWASA1* and *RWASA2* salivary material induced similar (*P* = 0.0840) levels of lipoxygenase activity in PAN 3144.

The elicitors in salivary material of *RWASA2* appeared to be more specific, inducing higher defence related enzyme activities only in PAN 3144. Salivary material of *RWASA1* on the other hand contained elicitors that induced higher enzyme activities in both PAN 3144 and Tugela *Dn1* cultivars. However, the levels of enzyme activities were significantly higher in Tugela *Dn1* than PAN 3144 wheat in all the enzyme activities measured. It should be noted that elicitors in crude salivary material of both *RWASA1* and *RWASA2* induced increases in peroxidase, β-1,3-glucanse and lipoxygenase activities in PAN 3144, and there was no significant difference in the levels of activity induced by elicitors from saliva of both biotypes in all the enzyme activities in PAN 3144 cultivar (Figs 4.1, 4.2 and 4.3). Similar trends also occurred in a replicate experiment.

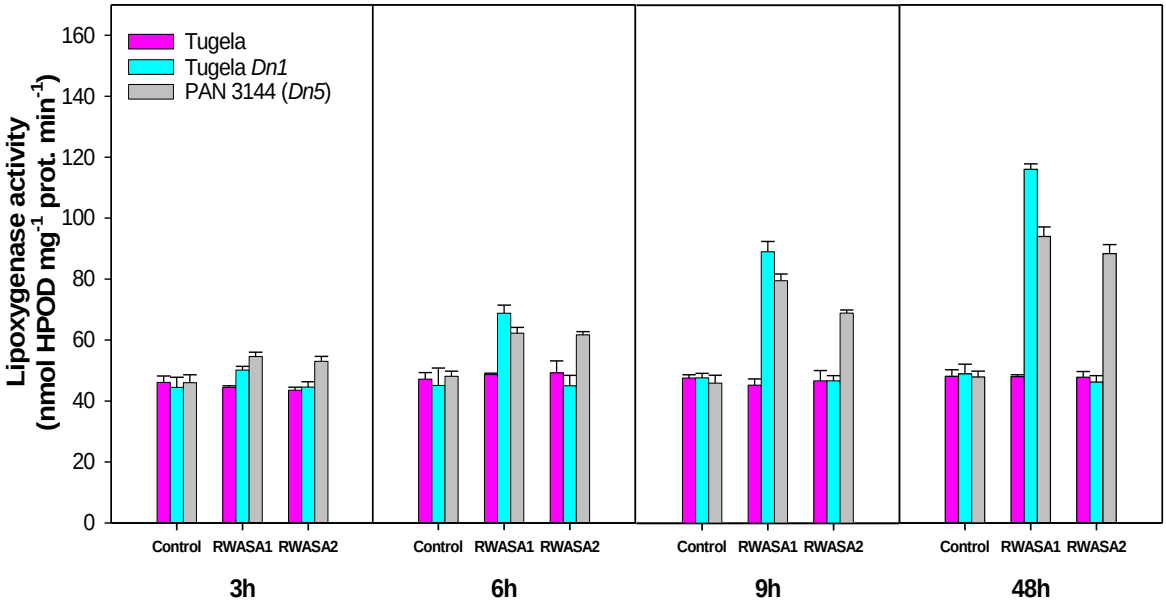

Figure 4.3: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

4.2 Fractionation of aphid saliva

4.2.1 The effect of C18 RP chromatography fractions on peroxidase, β-1,3-glucanase and lipoxygenase activities

In an attempt to isolate the potential elicitor, aphid saliva was fractionated using C18 reverse phase chromatography. Fifty fractions from *RWASA*1 or *RWASA2* saliva were collected, intercellularly injected into different wheat cultivars and defence responses were determined. Fractions 1 to 11 and the later fractions 19 to 50 did not induce any responses. Fractions 12, 13, 17 and 18 induced differential defense responses and only results of these inducing fractions will be shown. Peroxidase (Fig 4.4), β-1,3-glucanase (Fig 4.5) and lipoxygenase (Figs 4.6 and 4.7) activities were used to as indicators to quantify eliciting activity. For results of independent replicate experiments, refer to Appendix (Figs 7.4, 7.5, 7.6 and 7.7).

Salivary material (*RWASA1* and *RWASA2*) fractions 12, 13, 17 and 18 differentially induced increases of peroxidase activity in different wheat cultivars (Fig 4.4). Salivary fractions from *RWASA1* differentially induced a higher increase in Tugela *Dn1* (fractions 12: 5.9-fold; 13: 4.1-fold; 17: 4.8-fold; 18: 1.8-fold) than in PAN 3144 (fractions 12: 5.2-fold; 13: 3.4-fold; 17: 4-fold; 18: 1.6-fold) cultivar. A comparison of the effect of *RWASA1* fractions on induced activity in Tugela *Dn1* and PAN 3144 revealed that the fractions significantly (fractions 12: $P = 0.0282$, 13: $P = 0.0285$, 17: $P = 0.0113$, 18: $P = 0.01907$) induced higher peroxidase activity in Tugela *Dn1* than in PAN 3144 cultivar. Salivary fractions from *RWASA2* on the other hand induced a specific increase of peroxidase activity only in PAN 3144. All the fractions (12: 5-fold; 13: 3.4-fold; 17: 3.9-fold; and 18-1.5-fold) only induced an increase of peroxidase activity in PAN 3144. *RWASA1* and *RWASA2* saliva fractions did not induce any significant (fractions 12: $P = 0.09747$, 13: $P = 0.1854$, 17: $P = 0.0858$, 18: $P = 0.5201$) differences in the induced levels of peroxidase activity in PAN 3144. None of the fractions from either *RWASA1* or *RWASA2* saliva induced any increases in peroxidase activity in the Tugela cultivar.

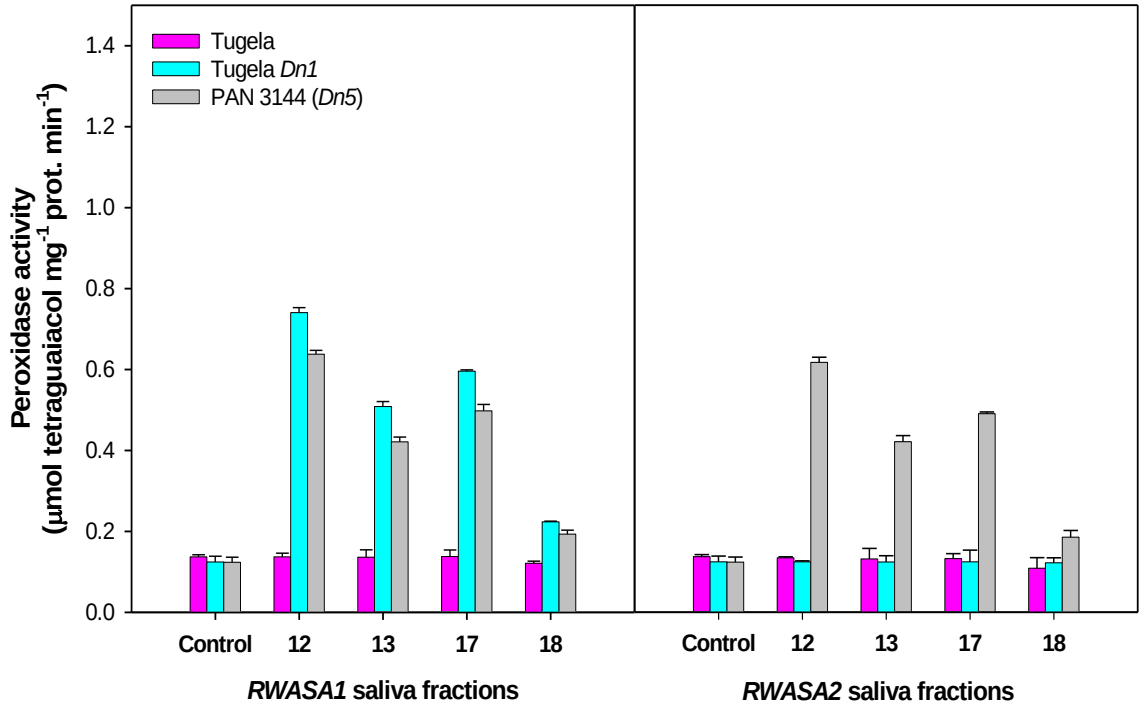

Figure 4.4: Effect of *RWASA1* and *RWASA2* salivary material fractions (C18 reverse phase chromatography) on peroxidase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

The fractions 12, 13, 17 and 18 from aphid saliva differentially induced increases of β-1,3-glucanase activity in different wheat cultivars (Fig 4.5). Salivary fractions from *RWASA1* differentially induced a higher increase in Tugela *Dn1* (fractions 12: 2.5-fold; 13: 1.7-fold; 17: 2.2-fold; 18: 1.4-fold) than in PAN 3144 (fractions 12: 2.2-fold; 13: 1.6-fold; 17: 1.8-fold; 18: 1.2-fold) cultivar. *RWASA1* saliva fractions induced activity of β-1,3-glucanase that was significantly (fractions 12: P = 0.03059, 13: P = 0.04468, 17: P = 0.01423, 18: P = 0.02867) higher in Tugela *Dn1* than in PAN 3144. Salivary fractions from *RWASA2* on the other hand induced a specific increase of β-1,3-glucanase activity. *RWASA2* fractions induced an increase of β-1,3-glucanase activity only in PAN 3144 (fractions 12: 1.9-fold; 13: 1.3-fold; 17: 1.7-fold; and 18: 1.2-fold). *RWASA1* and *RWASA2* saliva fractions did not induce any quantitatively significant (fractions 12: P = 0.3153, 13: P = 0.0818, 17: P = 0.1184, 18: P = 0.8907) differences in the β-1,3-glucanase activity of PAN 3144. There was no increased

activity measured in Tugela *Dn1* when treated with fractions of *RWASA2* saliva. None of the fractions from either *RWASA1* or *RWASA2* saliva induced any increases in β-1,3-glucanase activity in the Tugela cultivar.

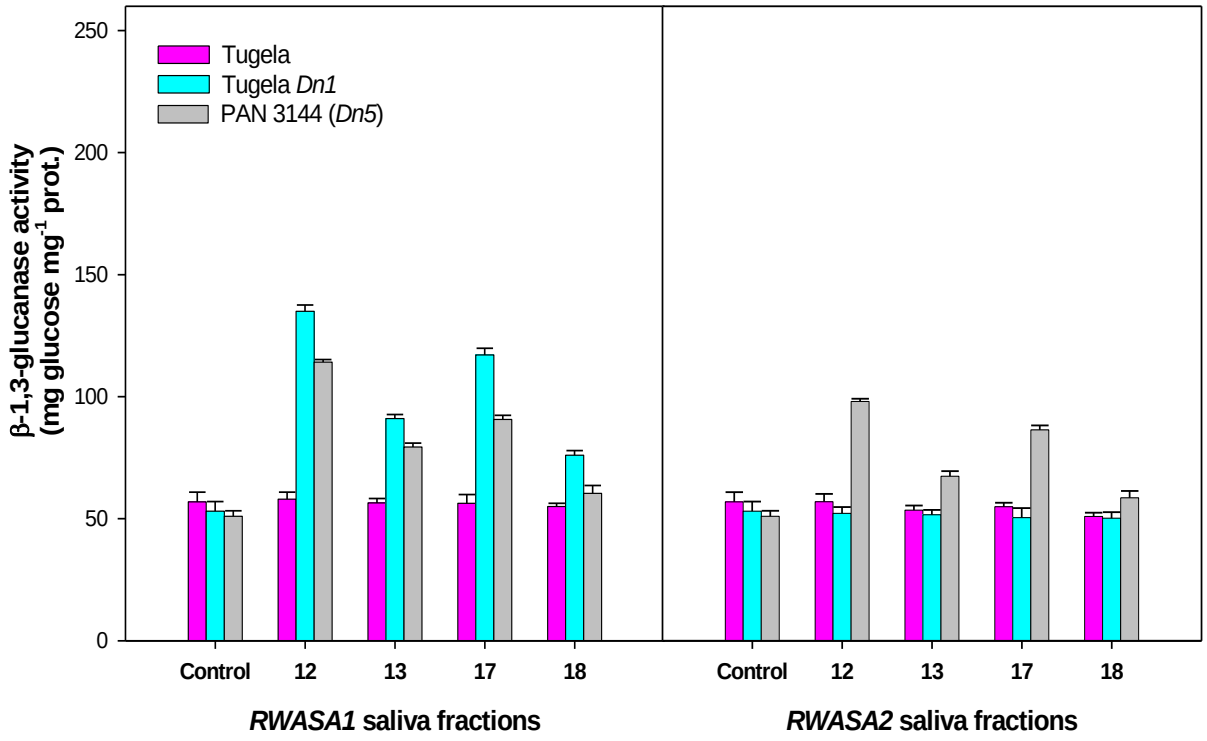

Figure 4.5: Effect of *RWASA1* and *RWASA2* salivary material fractions (C18 reverse phase chromatography) on β-1,3-glucanase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

RWASA1 salivary fractions differentially induced increased activity of lipoxygenase in Tugela *Dn1* and PAN 3144 cultivars (Fig 4.6). A slight induction occurred already as early as 3 hours after treatment in both Tugela *Dn1* (fractions 12: 1.2-fold; 13: 1.16-fold; 17: 1.2-fold; and 18: 1.16-fold) and PAN 3144 (fractions 12: 1.2-fold; 13: 1.11-fold; 17: 1.2-fold; and 18: 1.11-fold) cultivars. The degree of induction increased overtime; the highest induced activity occurred 48h after treatment in both Tugela *Dn1* and PAN 3144 cultivars. At this point the level of induced activity was significantly (fractions 12: $P = 0.00451$, 13: $P = 0.0397$, 17: $P = 0.0377$, 18: $P =$

0.0252) higher in Tugela *Dn1* (fractions 12: 2.6-fold; 13: 2.1-fold; 17: 2.2-fold; and 18: 1.8-fold) than in PAN 3144 (fractions 12: 2.3-fold; 13: 1.9-fold; 17: 2-fold; and 18: 1.6-fold). No increases in lipoxygenase activity in Tugela were measured following treatment with fractions of *RWASA1* saliva over the entire 48h treatment.

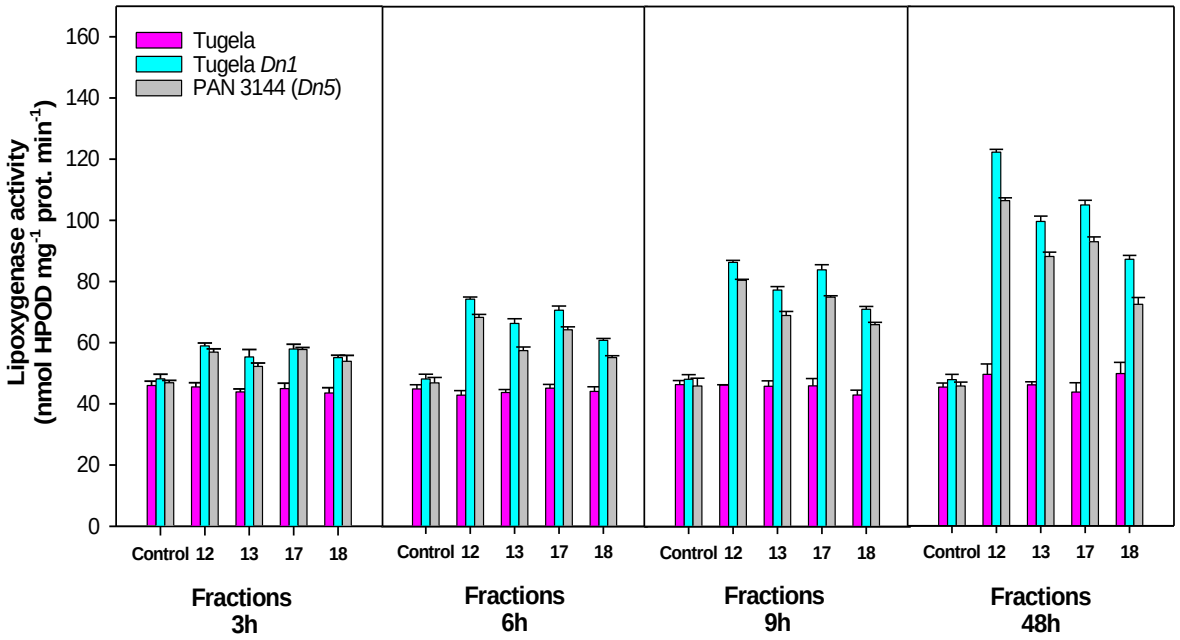

Figure 4.6: Effect of *RWASA1* salivary material fractions (C18 reverse phase chromatography) on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

C18 RP fractions (12, 13, 17 and 18) of *RWASA2* saliva specifically induced increases in lipoxygenase activity of PAN 3144 only (Fig 4.7). In PAN 3144 all *RWASA2* saliva fractions (12: 1.3-fold; 13: 1.1-fold; 17: 1.2-fold; and 18: 1.1-fold) induced an early increase in activity as soon as 3 hours after treatment. The induced activity increased overtime and 48h after treatment the highest levels were measured (fractions 12: 2.3-fold; 13: 1.7-fold; 17; 2.2-fold; and 18: 1.6-fold). No increases of lipoxygenase activity were measured in Tugela *Dn1* or Tugela cultivars over the entire 48 hours of treatment. *RWASA1* and *RWASA2* saliva fractions did not

induce any quantitatively significant (12: *P* = 0.6877, 13: *P* = 0.0722, 17: *P* = 0.8268, 18: *P* = 0.0737) differences in the activity of lipoxygenase in PAN 3144 wheat.

The tendency of fractions originating from *RWASA1* saliva inducing much stronger than those from *RWASA2* saliva was also observed with peroxidase (Fig 4.4) and β-1,3-glucanase (Fig 4.5) activities; where fractions 12 and 17 (*RWASA1* saliva) induced stronger enzyme activities in Tugela *Dn1* and PAN 3144. *RWASA2* saliva fractions were specific, inducing increased enzyme activities only in PAN 3144 wheat. *RWASA1* saliva fractions induced more pronounced increases in β-1,3-glucanase, peroxidase and lipoxygenase activities in Tugela *Dn1* than in PAN 3144 cultivar. Interestingly, when comparing induced levels of enzyme (peroxidase, β-1,3-glucanase and lipoxygenase) activities, C18 RP fractions seemed to induce much stronger than crude saliva. Results of independent replicate experiments also showed similar tendencies.

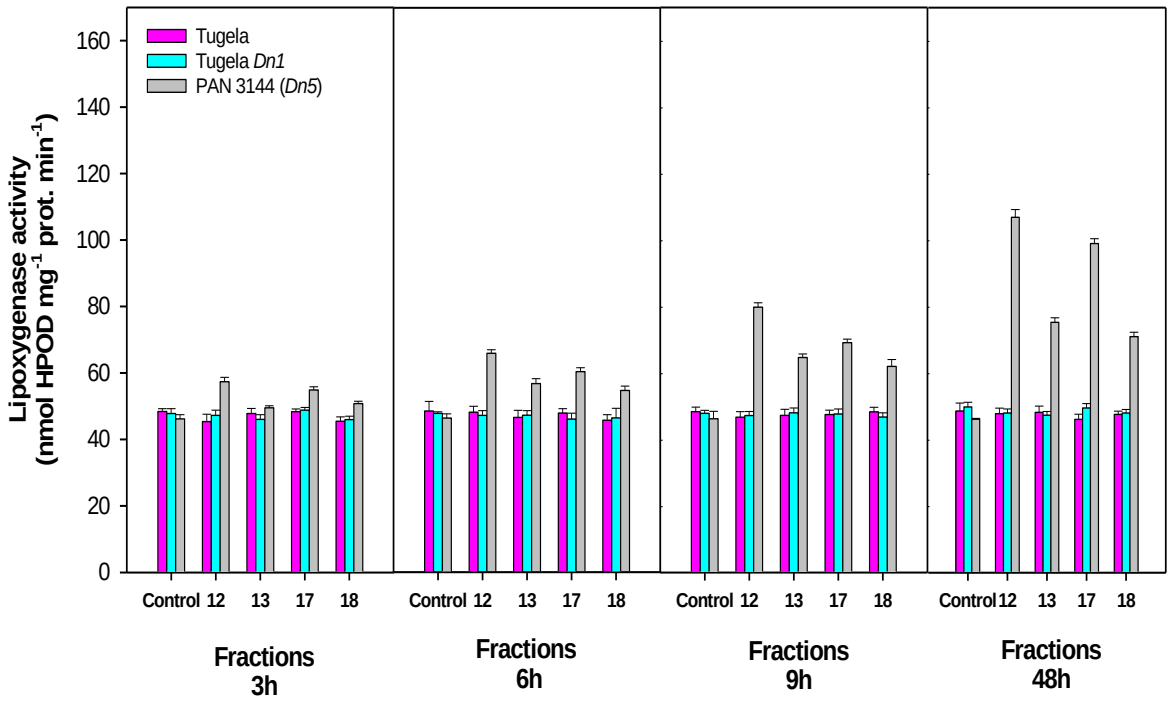

Figure 4.7: Effect of *RWASA2* salivary material fractions (C18 reverse phase chromatography) on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

4.2.2 Gel permeation

Further purification of aphid saliva was done by fractionating crude saliva using PD-10 (Sephadex G25 desalting) columns. The collected fractions were intercellularly injected into different wheat cultivars. Peroxidase (Fig 4.8) and β-1,3-glucanase (Fig 4.9) activities were used as markers of the eliciting activity. In an attempt to determine the nature of the potential elicitor in aphid saliva, the fractions were also heat treated. For results of independent replicate experiments, refer to Appendix (Figs 7.8 and 7.9).

Fig 4.8 illustrates the effect of salivary fractions on peroxidase activity in wheat. *RWASA1* saliva fractions differentially induced peroxidase activity in Tugela *Dn1* (Fig 4.8B, fractions 1: 2.4-fold; 2: 3.1-fold; and 3: 1.8-fold) and in PAN 3144 (Fig 4.8C, fractions 1: 2-fold; 2: 2.7-fold; and 3: 1.6-fold). All the fractions induced a slightly higher increase in peroxidase activity in Tugela *Dn1* than PAN 3144. Fraction 2 from *RWASA1* saliva induced the highest peroxidase activity in both Tugela *Dn1* (3.4-fold, $P = 0.00329$) and PAN 3144 (2.8-fold, $P = 0.00167$); the strongest induction occurred in Tugela *Dn1*. Fractions of *RWASA2* saliva selectively induced peroxidase activity only in PAN 3144 (fractions 1: 2.1-fold; 2: 2.4-fold; and 3: 1.3-fold). The levels of peroxidase activity in PAN 3144 induced by fractions from either *RWASA1* or *RWASA2* saliva were not significantly (fractions 1: $P = 0.0766$, 2: $P = 0.1369$, 3: $P = 0.1069$) different. The fractions from either *RWASA1* or *RWASA2* saliva did not induce any increases in activity of peroxidase in Tugela. Boiling of the fractions (*RWASA1* and *RWASA2* saliva) reduced peroxidase activity to almost control levels in all the wheat cultivars.

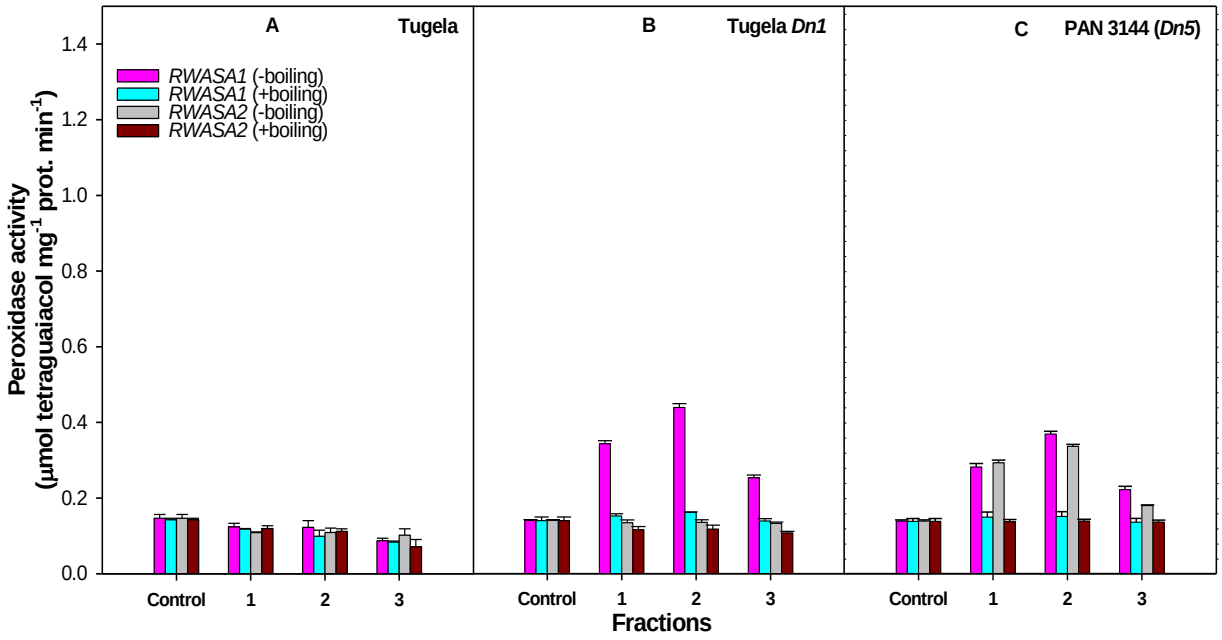

Figure 4.8: Effect of *RWASA1* and *RWASA2* salivary material fractions (PD-10) on peroxidase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

The fractions of *RWASA1* saliva induced differential increases of β-1,3-glucanase activity in Tugela *Dn1* (fractions 1: 2.8-fold; 2: 3.1-fold; and 3: 2.3-fold) and PAN 3144 (fraction 1: 2.7-fold; 2: 2.8-fold; and 3: 2.1-fold) cultivars (Fig 4.9). Fractions of *RWASA1* saliva significantly (1: P = 0.00062, 2: P = 0.00139, 3: P = 0.01423) induced higher activities of β-1,3-glucanase in Tugela *Dn1* compared to PAN 3144 cultivar. A specific induction of β-1,3-glucanase activity was observed in PAN 3144 after intercellularly injecting with fractions of *RWASA2* saliva [fractions 1 (2-fold), 2 (2.1-fold), and 3 (1.6-fold), Fig 4.9C]. Fractions of *RWASA2* saliva did not induce any β-1,3-glucanase activity in Tugela or Tugela *Dn1* cultivars. *RWASA1* and *RWASA2* saliva fractions did not induce any significant (1: P = 0.1248, 2: P = 0.1145, 3: P = 0.0853) quantitative differences in β-1,3-glucanase activity in PAN 3144. In addition, *RWASA1* saliva fractions also did not induce any increases in activity of β-1,3-glucanase in Tugela. All boiled fractions *(RWASA1* and *RWASA2* saliva) reduced β-1,3-glucanase activity to almost control levels in all the wheat cultivars.

Fraction 2 of both *RWASA1* and *RWASA2* saliva induced the highest activity of β-1,3-glucanase, this trend was also noted with peroxidase activity (Fig 4.8). A comparison of induced responses between C18 RP fractions and PD 10 fractions revealed that fractions from C18 RP chromatography induced higher levels in activity of the two enzymes. Similar trends also occurred in a replicate experiment.

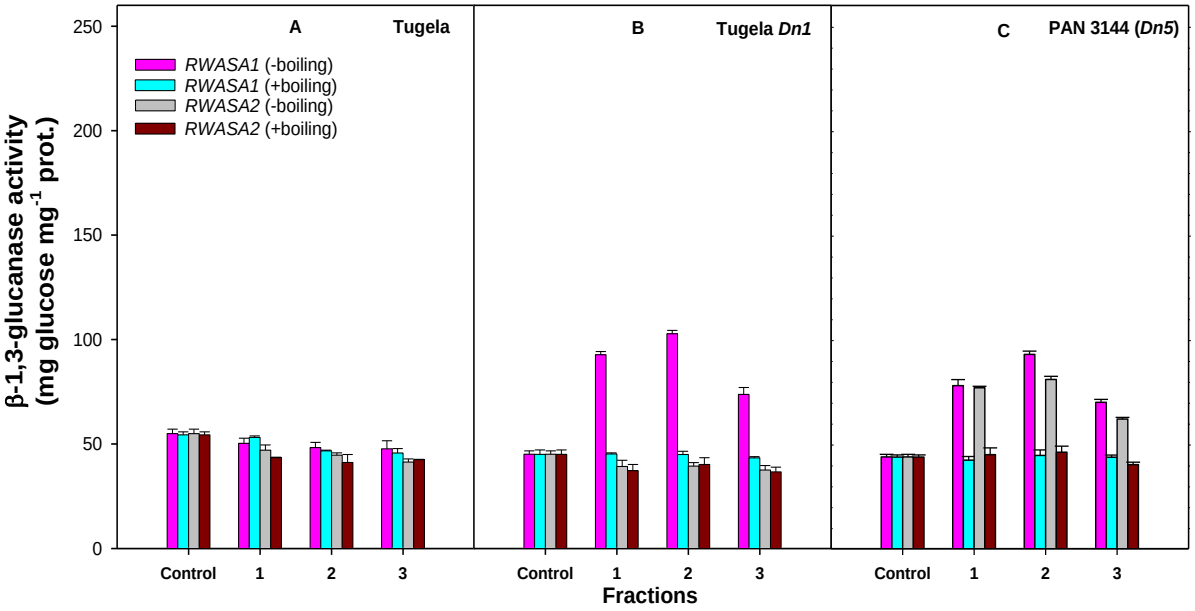

Figure 4.9: Effect of *RWASA1* and *RWASA2* salivary material fractions (PD-10) on β-1,3-glucanase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

4.2.3 Combined fractionation (C18 and PD 10)

The C18 RP chromatography fractions (12, 13, 17 and 18) of *RWASA1* and *RWASA2* saliva elicited increases in enzyme activities in different wheat cultivars. There is a high possibility that fractions 12 and 13, and 17 and 18, could contain the same active eliciting components inducing defence related enzymes. Further fractionation was done by firstly combining the C18 RP fractions 12 and 13 into one fraction (a) and 17 and 18 into another fraction (b). These fractions were individually passed through a pre-equilibrated PD-10 column. All the seven collected fractions were intercellularly injected into different wheat cultivars. Enzyme activities of peroxidase and β-1,3-glucanase were then used as indicators of the eliciting activity. The fractions were also boiled to determine the heat stability of the active molecules. For results of independent replicate experiments, refer to Appendix (Figs 7.10, 7.11, 7.12, 7.13, 7.14 and 7.15).

RWASA1 saliva fractions induced differential increases in activity of peroxidase in Tugela *Dn1* [(a_1: 2.1-fold; a_2: 3.3-fold; a_3: 1.6-fold; b_1: 2.2-fold; b_2: 2.8-fold; b_3: 1.4-fold), Fig 4.10A]. The highest increase in peroxidase activity was induced by fractions a_2 and b_2. *RWASA1* saliva fraction a_2 induced significantly (P = 0.00137) higher levels of peroxidase activity than *RWASA1* saliva fraction b_2 in Tugela *Dn1*. The *RWASA1* saliva fractions 4 to 7 did not induce any increase in peroxidase activity in Tugela *Dn1*. None of the *RWASA2* saliva fractions induced increased activity of peroxidase in Tugela *Dn1* (Fig 4.10B). All boiled fractions, either from *RWASA1* or *RWASA2* saliva, did not induce any increases in the activity of peroxidase in the Tugela *Dn1* cultivar.

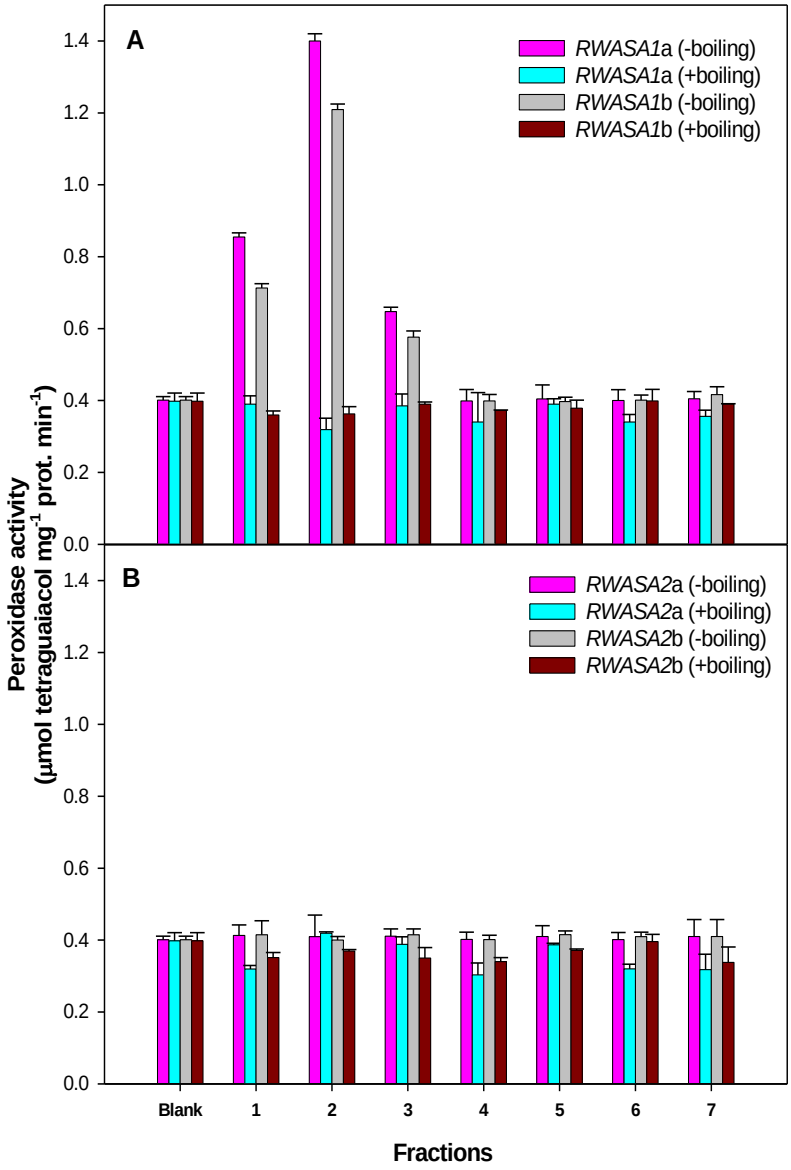

Figure 4.10: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in Tugela *Dn1* cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n = 3).

Partially purified fractions of *RWASA1* and *RWASA2* saliva differentially induced increases in peroxidase activity in PAN 3144 cultivar (Fig 4.11A, B). *RWASA1* saliva fractions (a_1: 2-fold; a_2: 2.8-fold; a_3: 1.4-fold; b_1: 1.9-fold; b_2: 2.4-fold; b_3: 1.4-fold) and *RWASA2* saliva fractions (a_1: 1.7-fold; b_1: 1.9-fold; a_2: 2.4-fold; b_2: 2.3-fold; a_3: 1.5-fold; b_3: 1.5-fold) induced differential increases in peroxidase activity of PAN 3144 wheat. Fraction 2 from either *RWASA1* saliva or *RWASA2* saliva significantly induced the highest increase in peroxidase activity. The difference in the degree of induction between *RWASA1* saliva fractions a_2 and b_2 was significant ($P = 0.03163$), as was noted between the *RWASA2* saliva fractions a_2 and b_2 ($P = 0.02404$). Although both *RWASA1* and *RWASA2* saliva fractions (a_2 and b_2) individually induced higher levels of peroxidase activity in PAN 3144; the levels of induced enzyme activity were not quantitatively different [fractions a_2 ($P = 0.1912$) and b_2 ($P = 1.115$) not significant for *RWASA1* and *RWASA2* saliva]. However, *RWASA1* saliva fractions significantly (a_2: $P = 0.0108$ and b_2: $P = 0.00441$) induced higher increases of peroxidase in Tugela *Dn1* than in PAN 3144. The fractions 4 to 7, originating from either *RWASA1* or *RWASA2* saliva did not induce any increase in peroxidase activity in PAN 3144 wheat. All boiled fractions did not induce any increases of peroxidase activity.

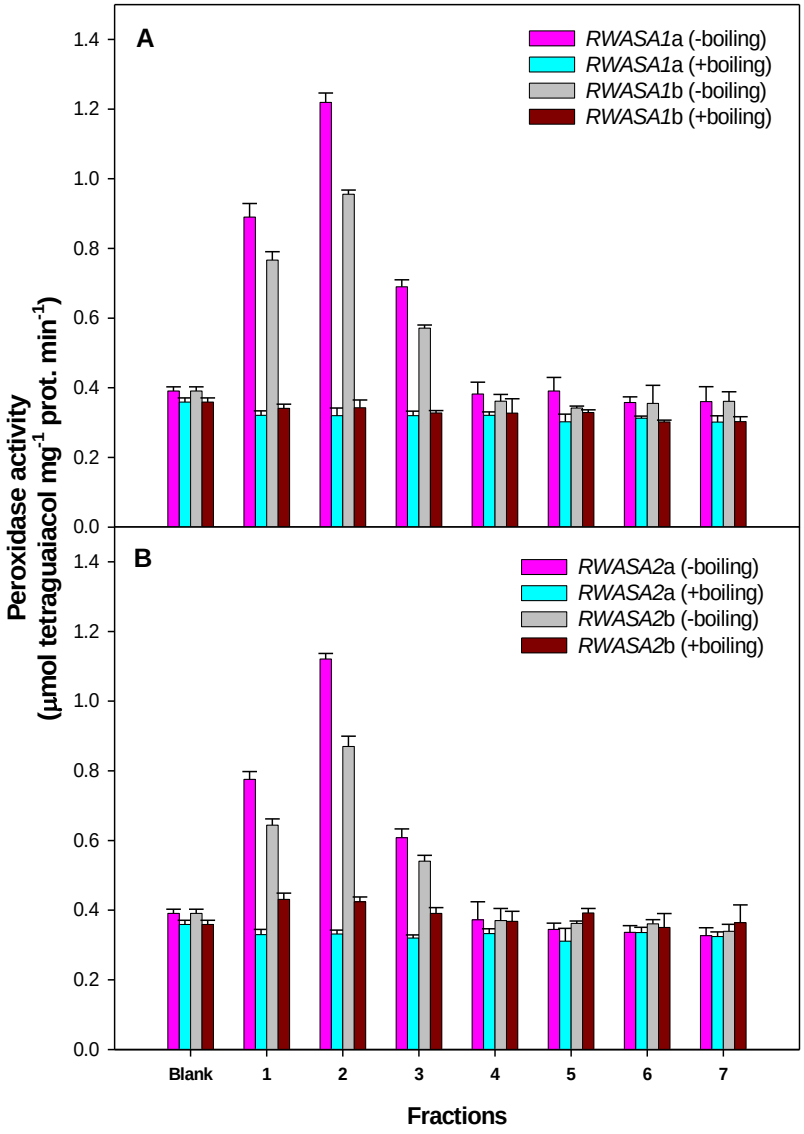

Figure 4.11: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in PAN 3144 cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n = 3).

RWASA1 and *RWASA2* saliva fractions did not induce any increased activity of peroxidase in Tugela cultivar (Fig 4.12A, B). All boiled fractions of either *RWASA1* or *RWASA2* saliva had any effect on peroxidase activity in Tugela.

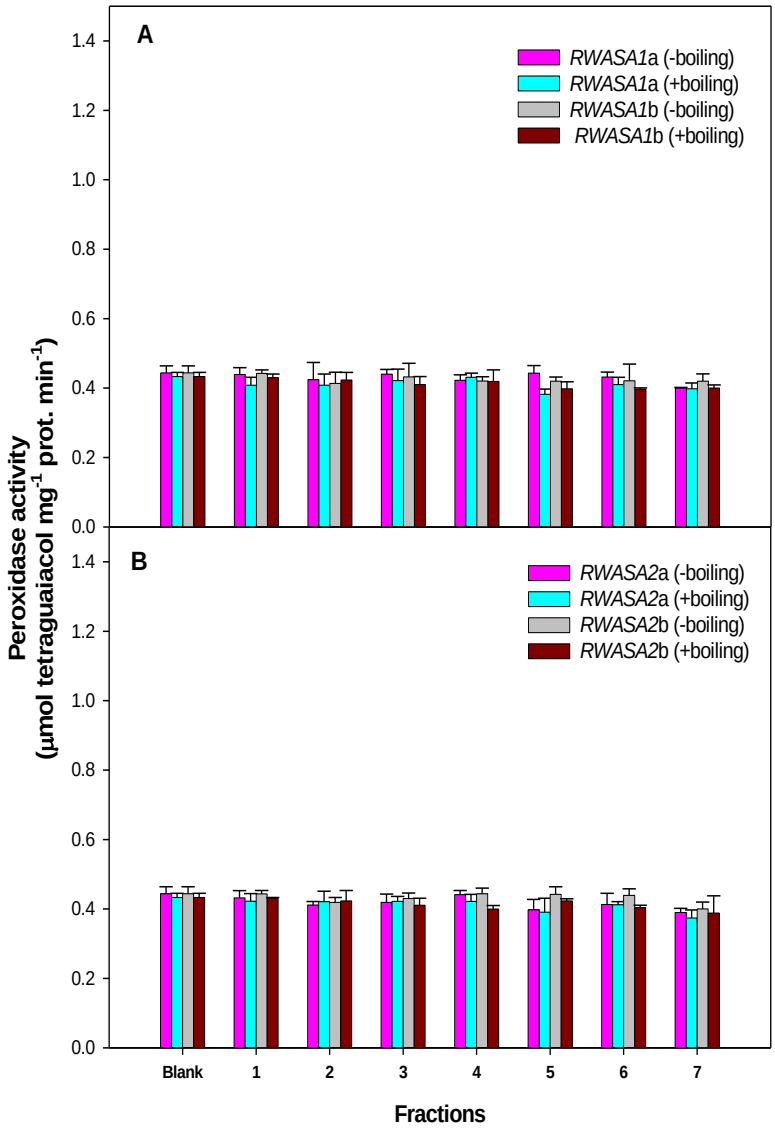

Figure 4.12: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in Tugela cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

The first three *RWASA1* saliva fractions differentially induced increases in activity of β-1,3-glucanase in Tugela *Dn1* [(a_1: 2.3-fold; b_1: 2.5-fold) and (a_3: 1.5-fold; b_3: 1.4-fold), Fig 4.13A]. The fractions a_2 (3.2-fold) and b_2 (2.5-fold) induced the highest

increase of β-1,3-glucanase activity. Fraction a_2 induced a significantly (P = 0.0381) higher β-1,3-glucanase activity than fraction b_2. Fractions 4 to 7 did not induce any increases in β-1,3-glucanase activity in Tugela *Dn1* cultivar. None of the fractions from *RWASA2* saliva induced any increases in activity of β-1,3-glucanase in Tugela *Dn1*. There were no changes in activity of β-1,3-glucanase in Tugela *Dn1* when plants were treated with boiled fractions of *RWASA1* or *RWASA2* saliva (Figs 4.13A, B).

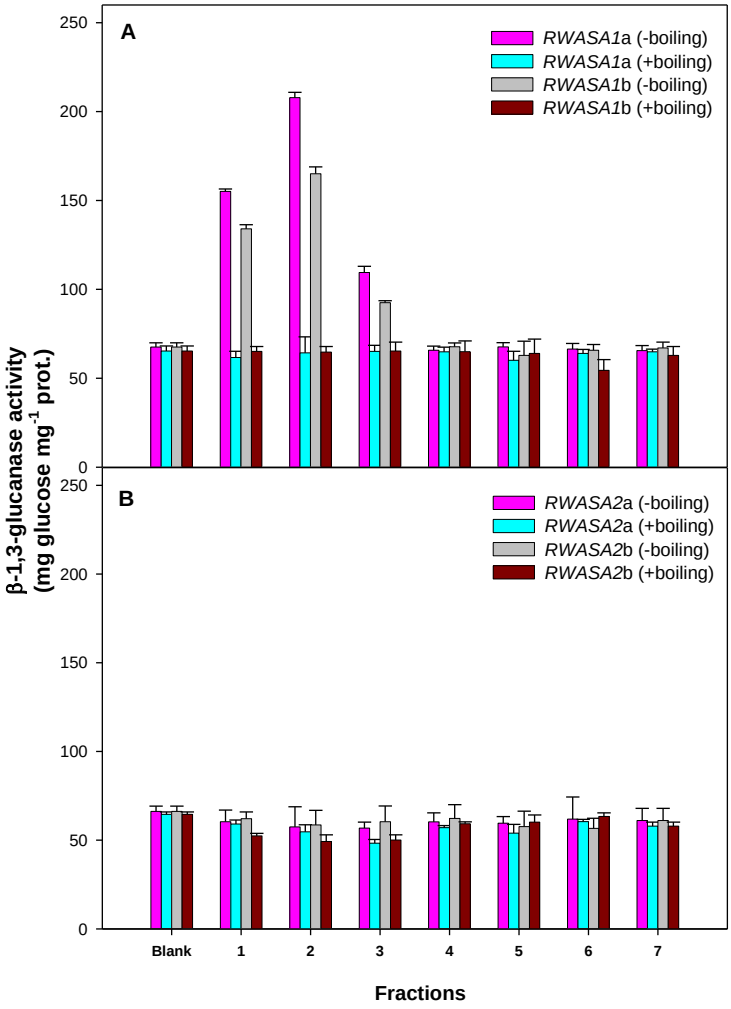

Figure 4.13: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanase activity in Tugela *Dn1* 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n= 3).

A similar pattern as in Fig 4.11 occurred for β-1,3-glucanase activity in PAN 3144 (Fig 4.14). Fraction 2 from either *RWASA1* saliva (a_2: 2.8-fold; b_2: 2.7-fold) or *RWASA2* saliva (a_2: 2.4-fold; b_2: 2.3-fold) induced the highest increase in β-1,3-glucanase activity. *RWASA1* saliva fractions a_2 and b_2 induced quantitatively different (P = 0.0010) enzyme activity levels. *RWASA2* saliva fractions a_2 and b_2 also induced different (P = 0.0293) levels of β-1,3-glucanase activity. Although both *RWASA1* and *RWASA2* saliva fractions (a_2 and b_2) induced higher levels of β-1,3-glucanase activity in PAN 3144; the overall induced levels were not quantitatively different (fractions a_2: P = 0.406 and b_2: P = 0.074). *RWASA1* saliva fractions (a_1: 1.9-fold; b_1: 1.9-fold a_3: 1.6-fold; and b_3: 1.4-fold) and *RWASA2* saliva fractions (a_1: 1.9-fold; b_1: 1.9-fold; a_3: 1.5-fold; b_3: 1.2-fold) also induced increased levels of β-1,3-glucanase activity in PAN 3144. The fractions 4 to 7, originating from either *RWASA1* or *RWASA2* saliva did not induce any increase in β-1,3-glucanase activity in PAN 3144 wheat. All boiled fractions did not induce any changes in β-1,3-glucanase activity.

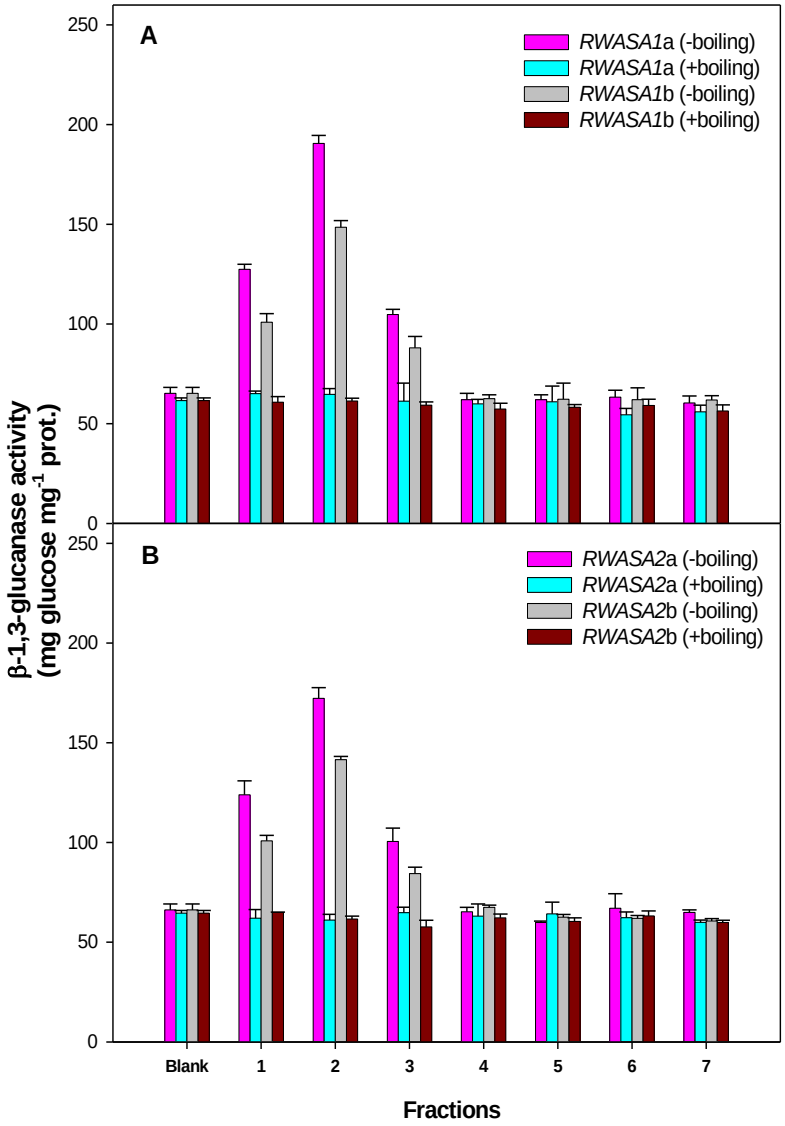

Figure 4.14: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanase activity in PAN 3144 cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

RWASA1 and *RWASA2* saliva fractions also did not induce any changes in activity of β-1,3-glucanase in Tugela (Fig 4.15A,B). All boiled fractions (*RWASA1* and *RWASA2*) did not have any effect on β-1,3-glucanase activity in Tugela. Similar trends also occurred in a replicate experiment.

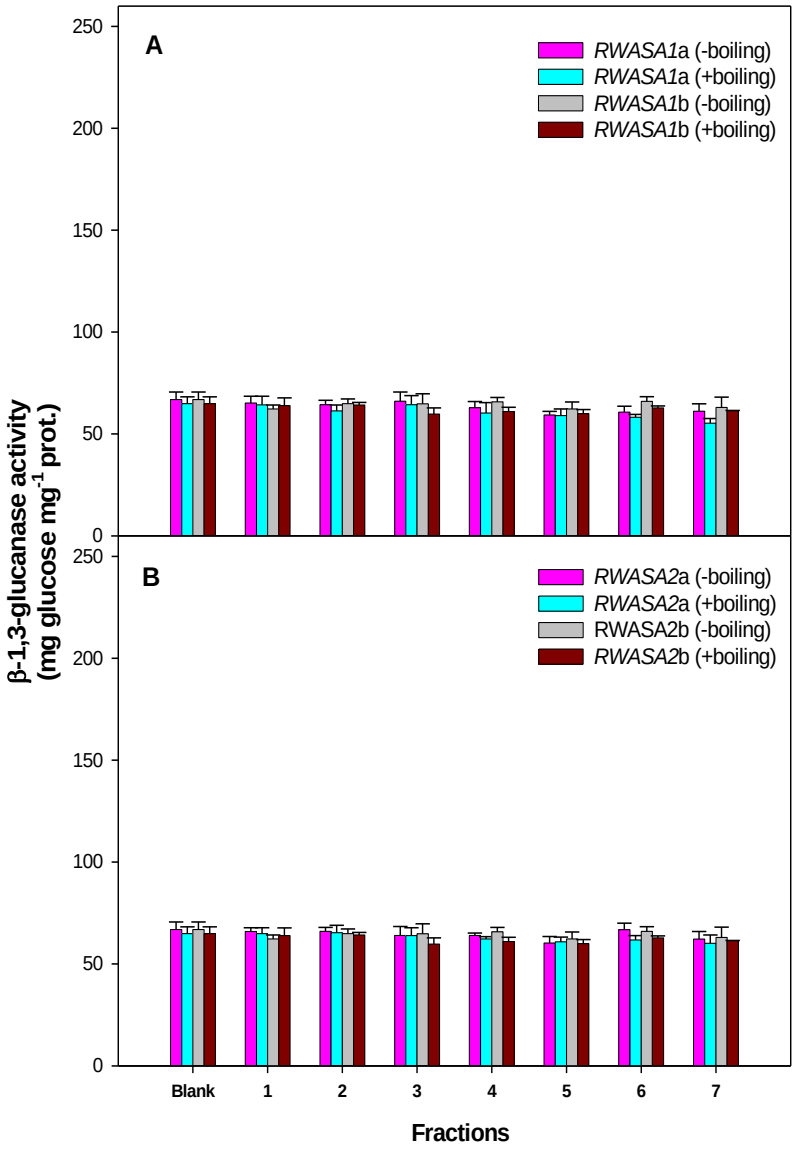

Figure 4.15: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanse activity in Tugela 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

4.3 Electrophoretic analysis of *RWASA1* and *RWASA2* salivary material and partially purified fractions

The electrophoretic separation of salivary proteins revealed about 23 polypeptide bands in crude saliva of *RWASA1* and approximately 21 polypeptide bands in crude saliva of *RWASA2*. The sizes of the polypeptides ranged between 232 kDa and 21 kDa (Fig 4.16). The polypeptide profiles of *RWASA1* and *RWASA2* crude salivary material showed more similarities than differences. Expression of proteins (denoted SP-salivary proteins) with the following sizes was similar in the salivary material of both biotypes: SP1 (232 kDa), SP2 (181 kDa), SP3 (133.5 kDa), SP4 (126.3 kDa), SP5 (109.7 kDa), SP7 (79 kDa), SP8 (73.7 kDa), SP9 (65.5 kDa), SP10 (64 kDa), SP11 (61.1 kDa), SP15 (45.2 kDa), SP19 (41 kDa), SP20 (39.3 kDa), SP22 (31.3 kDa), SP27 (23.1 kDa) and SP28 (21.3 kDa). However, differential expression as well as distinct expression of some proteins was evident in the salivary material of the two biotypes. Salivary proteins designated as SP4 with estimated molecular size of 126.3 kDa, SP7, SP8 and SP22 were more distinct in crude salivary material of *RWASA1* than *RWASA2*. In crude saliva of *RWASA2* SP2 and SP5 were expressed more than in *RWASA1* saliva. The expression of SP15 was similar in the crude salivary material of both *RWASA1* and *RWASA2*.

The expression of certain polypeptides was exclusive to either *RWASA1* or *RWASA2* crude salivary material. The following protein bands with sizes of 86.8 kDa (SP6), 56.1 kDa (SP13), 43.2 kDa (SP17), 34.4 kDa (SP21), 28.3 kDa (SP23), 27.3 kDa (SP24), and 24.9 kDa (SP26) were visual only in crude salivary material of *RWASA1*. The polypeptides denoted as SP12 (58.3 kDa), SP14 (47.2 kDa), SP16 (44.1 kDa), SP18 (42.9 kDa) and SP25 (26.6 kDa) were expressed only in *RWASA2* crude salivary material. There were no protein bands detected in control diets.

The polypeptide profile of the purified fractions (C18 RP followed by PD 10) of *RWASA1* and *RWASA2* saliva is represented in Figs 4.17 and 4.18. These fractions

differentially induced defence responses (peroxidase and β-1,3-glucanase) in resistant wheat cultivars (Figs 4.10, 4.11, 4.13 and 4.14). The most inducing fractions were fractions a_2 and b_2 for both biotypes (Figs 4.17 and 4.18). It is obvious that these fractions probably induced higher levels of activity because of their stronger protein expression. The expression of proteins in other fractions was not as intense as that of proteins in fractions a_2 and b_2. All the fractions had several polypeptide bands similar to crude saliva, thus indicating that there was purification. Further characterization of these inducing fractions is still required.

Polypeptides bands SP 1, 2, 4, 5, 7, 9, 10, 11, 13, 15, 17, 21 and 23 were present both in the *RWASA1* crude saliva and in the purified fractions, especially in fraction a_2 (Figs 4.16 and 4.17). The proteins SP 9, 10 and 11 were expressed more strongly in the purified (C18 followed by PD 10) fractions (a_2) than in crude saliva. In addition, SP13, 17, 21 and 23 were only expressed in fractions of *RWASA1*. It should be noted that these bands also appeared only in crude saliva of *RWASA1*. The polypeptide profile of purified inducing fractions and crude saliva originating from *RWASA2* showed expression of the following protein bands: SP1, 2, 3, 4, 5, 7, 9, 10, 11, 14, 15 and 18. The expression of the SP 9, 10 and 11 protein bands in the purified fractions (a_2) was also higher than in crude saliva (Figs 4.16 and 4.18). There were distinct bands (SP14 and SP18) evident from *RWASA2* saliva or its purified fractions that were not present in crude *RWASA1* saliva and its purified fractions.

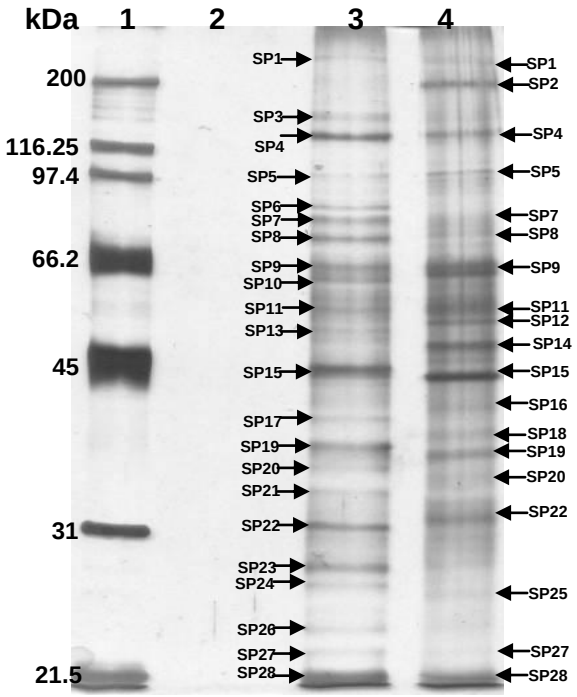

Figure 4.16: SDS-PAGE (11%) of saliva proteins from *RWASA1* and *RWASA2* crude salivary material. Ten microlitres of concentrated (x80, 1200 aphids per 10 ml sterile distilled water) salivary material was loaded per lane. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). **Lane 2**: Control diet (without aphids). **Lane 3**: *RWASA1* crude salivary material; **Lane 4**: *RWASA2* crude salivary material.

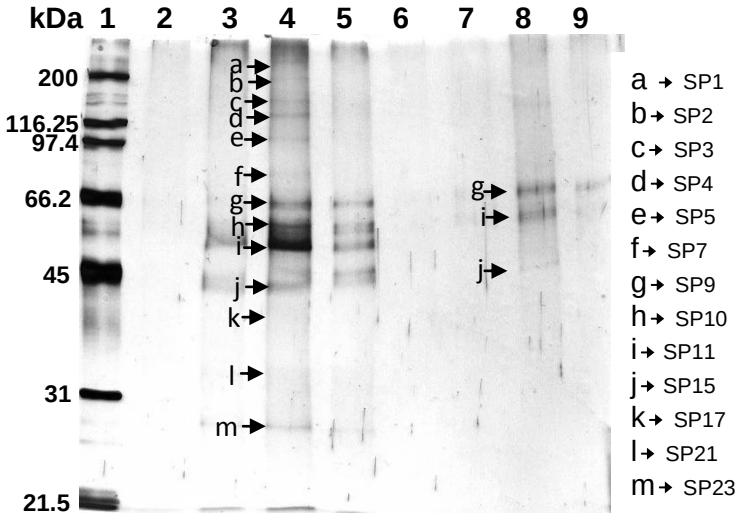

Figure 4.17: SDS-PAGE (11%) of purified fractions (C18 followed by PD 10) of *RWASA1* saliva. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). For all the fractions, 1 µg of protein was loaded; **Lane 3**: fraction a_1. **Lane 4**: fraction a_2, L**ane 5**: fraction a_3; **Lane 7**: fraction b_1; **Lane 8**: fraction b_2, and **Lane 9**: fraction b_3.

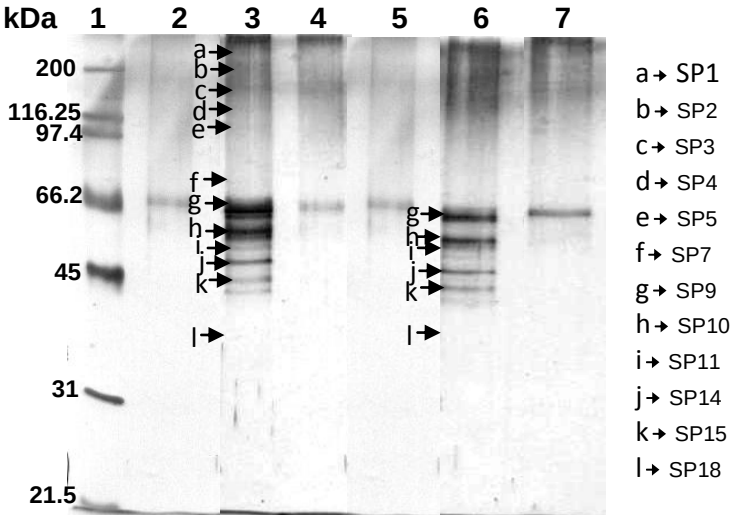

Figure 4.18: SDS-PAGE (11%) of purified fractions (C18 RP followed by PD 10) of *RWASA2* saliva. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). For all the fractions, 1 µg of protein was loaded; **Lane 2**: fraction a_1. **Lane 3**: fraction a_2, L**ane 4**: fraction a_3; **Lane 5**: fraction b_1; **Lane 6**: fraction b_2, and **Lane 7**: fraction b_3.

4.4 Qualitative thin layer chromatography (TLC) profile of elicitors in *RWASA1* and *RWASA2* saliva

Even though the TLC profiles of resistance inducing *RWASA1* and *RWASA2* crude salivary material showed major spots with approximately the same R_f values as that of glucose (Plate 4.1), certain spots with different R_f values were also present. The compounds with R_f values of 0.72, 0.85, 0.92, 1 and 1.15 were not detected in glucose, but in crude salivary material of both biotypes. A compound with R_f value of 1.7 was distinct in *RWASA1* saliva only, whilst a compound with R_f: 1.28 appeared only in *RWASA2* saliva. The TLC plate, when sprayed with aniline-diphenylamine reagent, *RWASA1* salivary material displayed an orange colour, whilst in *RWASA2* salivary material a dark brown colour appeared. The colour observed suggests that fructose or sucrose is one of the components in salivary material of *RWASA1* and *RWASA2*, thus carbohydrates are some of the constituents in RWA saliva.

The TLC profile of inducing fractions [(a_1; a_2; a_3; and b_1; b_2; b_3: C18 RP followed by PD 10)] from *RWASA1* and *RWASA2* saliva also indicated the presence of a compound with similar R_f value as glucose. A compound with an R_f value of 0.92 was detected in all salivary fractions of *RWASA1* and *RWASA2* (Plates 4.2 and 4.3). There were also faint spots with R_f values lesser than 0.92 in *RWASA1* and *RWASA2* fractions. The faint spots could correlate with spots located in crude saliva (R_f: 0.72 and 0.85). In addition to staining deeply on the TLC plate, the fractions a_2 and b_2 of *RWASA1* or *RWASA2* saliva also induced higher levels of enzyme activities than other fractions (see Figs 4.10; 4.11; 4.13 and 4.14).

The TLC profile of inducing fractions only showed two spots that also appeared in crude salivary material of *RWASA1* or *RWASA2* (Plates 4.2 and 4.3). There were no distinct bands in these fractions. During purification (C18 RP followed by PD 10 fractionation) simple sugars ran through the column due to their smaller size, proteins permeated the pores and were eluted during fractionation, also proven

during electrophoresis (Figs 4.17 and 4.18). However, the use of more sensitive techniques could lead to a thorough elucidation of resistance inducing components in aphid saliva.

Amino acids were also detected in saliva of *RWASA1* and *RWASA2* (Plates 4.4 and 4.5). Spraying of the plate with Ninhydrin solution resulted in the appearance of reddish spots. Different spots with R_f values of 0.34, 1.17 and 1.74 appeared in crude salivary material of *RWASA1*, whilst in crude salivary material of *RWASA2* spots with R_f values 0.43, 1 and 1.57 were detected (Plates 4.4 and 4.5). These spots were not detected in β-Alanine. Predominantly, reports indicate that aphid saliva contains a mixture of components including amino acids. The differential spots or amino acids could contribute to the content of elicitors in aphid saliva.

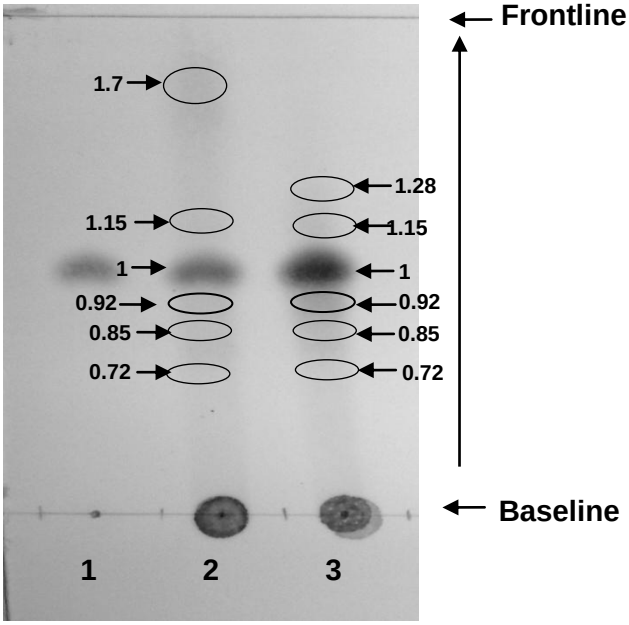

Plate 4.1: Qualitative TLC (sugars) profile of crude saliva from *RWASA1* and *RWASA2*. **Lane 1**: standard (glucose 0.2 µg). **Lane 2**: *RWASA1* crude saliva, 2 µg (protein equivalent). **Lane 3**: *RWASA2* crude saliva, 2 µg (protein equivalent).

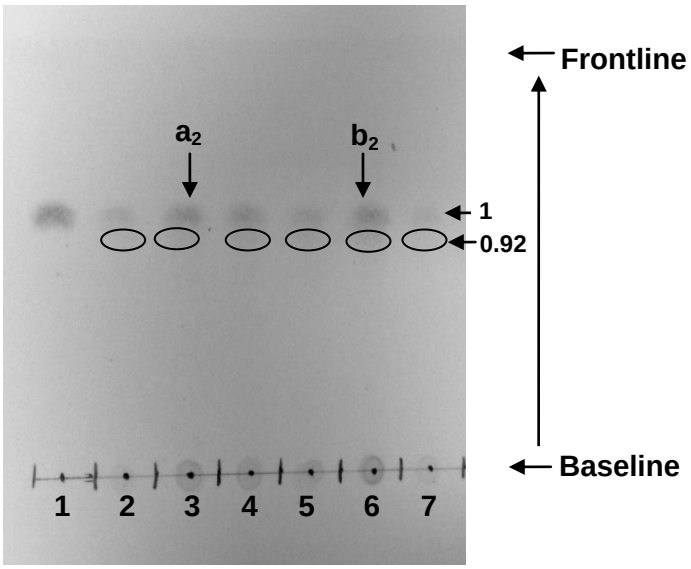

Plate 4.2: Qualitative TLC profile (sugars) of partially purified elicitors from *RWASA1* salivary material. The fractions were first fractionated with C18 reverse phase then active fractions (12 and 13= a; 17 and 18= b) passed through PD-10 column. Each lane was loaded with 4 µg protein equivalent of the fraction. **Lane 1**: standard (glucose 0.2 µg); **Lane 2**: a_1; **Lane 3**: a_2; **Lane 4**: a_3; **Lane 5**: b_1; **Lane 6**: b_2; and **Lane 7**: b_3.

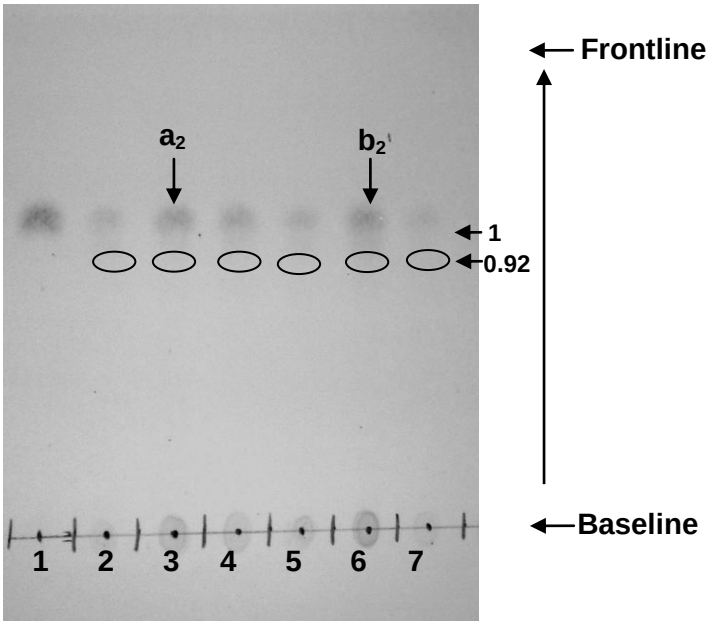

Plate 4.3: Qualitative TLC profile (sugars) of partially purified elicitors from *RWASA2* salivary material. The fractions were first fractionated with C18 reverse phase then active fractions (12 and 13=a; 17 and 18= b) passed through PD-10 column. Each lane was loaded with 4 µg protein equivalent of the fraction. **Lane 1**: standard (glucose 0.2 µg); **Lane 2**: a_1; **Lane 3**: a_2; **Lane 4**: a_3; **Lane 5**: b_1; **Lane 6**: b_2; and **Lane 7**: b_3.

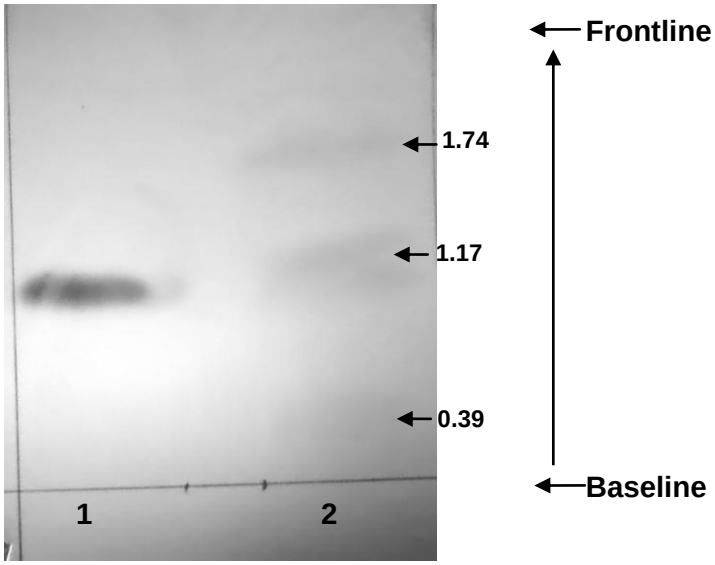

Plate 4.4: Qualitative TLC profile (amino acids) of *RWASA1* crude salivary material. **Lane 1**: β-alanine (0.5 µg). **Lane 2**: *RWASA1* crude salivary material, 2 µg of protein equivalent was loaded.

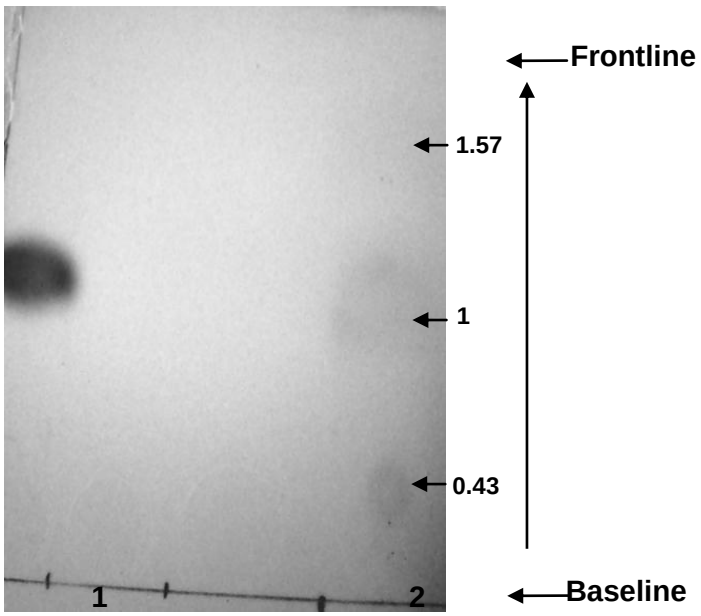

Plate 4.5: Qualitative TLC profile (amino acids) of *RWASA2* crude salivary material. **Lane 1**: β-alanine (0.5 µg). **Lane 2**: *RWASA2* crude salivary material, 2 µg of protein equivalent, was loaded.

DISCUSSION

The Russian wheat aphid is a destructive pest of wheat crops responsible for leading yield losses in South Africa and other wheat producing countries. Conquering RWA infestations has been challenging. Previously, chemical control was employed to curb infestations but this was regarded as expensive and harmful to the environment. The most promising strategy to manage RWA infestations remains as the creation of resistant cultivars through incorporating resistance genes in wheat cultivars. About 27 resistant wheat cultivars are now available in South Africa, even so, the rapid development of new RWA biotypes is still a challenge to plant breeders. Furthermore, the current resistance breaking biotypes have been reported to be more virulent than the old biotype (*RWASA1*) (Tolmay *et al.*, 2007; Jankielsohn, 2011). In these circumstances continuous efforts to introduce additional sources of resistance into commercially acceptable cultivars, and to improve overall strategies to control RWAs are certainly necessary. One approach could involve the identification and use of elicitors in aphid saliva to induce resistance mechanisms in wheat.

The aphid saliva has been proposed to contain various compounds, including amino acids, proteolytic enzymes (cellulose, estarases and lipases), peroxidases, phenoloxidase, sucrose and alkaline and acidic phosphatases (Miles, 1999; Funk, 2001; Will *et al.*, 2007; Walling, 2009; Ma *et al.*, 2010). Researchers working on plant-aphid interactions (van der Westhuizen *et al.*, 1998a,b; Berner and van der Westhuizen 2010a,b; Moloi and van der Westhuizen 2006, 2008) have indicated that RWA infestation induces various defence responses associated with the HR. In search for the source of elicitors in RWA-wheat interaction, Mohase and van der Westhuizen (2002a) indicated that treatment of plants with elicitors from the intercellular wash fluids of infested resistant plants induced responses similar to RWA infestation. Furthermore, protein extracts of the RWA induced different

defence responses in resistant and susceptible wheat cultivars (Lapitan *et al.*, 2007). Based on these reports, it was assumed that treatment of plants with aphid saliva could induce defence responses similar to those induced by direct aphid feeding in plants.

The RWA is a phloem feeding insect which inserts its stylet intercellularly until the phloem sieve elements are reached and feeding commences. During feeding, it regurgitates its soluble salivary contents into plant cells before ingesting phloem sap. It is assumed that the watery saliva contains a phytotoxin that elicits specific plant defence responses in resistant cultivars (Cherqui and Tjallingii, 2000). We collected RWA saliva by feeding both biotypes (*RWASA1* and *RWASA2*) sterile distilled water. This served to eliminate any contaminating compounds that could interfere with subsequent analysis of the feeding medium containing salivary secretions. Even though Madhusudhan and Miles (1998) pointed out that aphids probing through artificial membranes may not secrete saliva strictly similar to that injected into plants, biochemical compounds recovered from water into which aphids insert their stylets should at least provide an indication of what they are capable of secreting into plants (Madhusudhan and Miles, 1998).

Peroxidases, β-1,3-glucanases and lipoxygenases are well known as part of the resistance mechanism of wheat against the RWA (van der Westhuizen *et al.* 1998a,b; Mohase and van der Westhuizen 2002a; Berner 2006). These enzymes were therefore used as markers of the induced resistance response.

The injected *RWASA1* and *RWASA2* salivary material induced differential increases in activities of peroxidase, β-1,3-glucanase and lipoxygenase in different wheat cultivars. The induction of defence responses by elicitors in *RWASA1* saliva in cultivars containing resistance genes *Dn1* and *Dn5*, but not in the Tugela cultivar, which does not express any resistance genes is an indication that resistance genes mediate aphid resistance in wheat. The higher expression of

defence responses in Tugela *Dn1* compared to PAN 3144(*Dn5*) [Figs 4.1, 4.2 and 4.3] could relate to stronger recognition and perception of elicitors in *RWASA1* saliva by the R gene products in *Dn1* than *Dn5*-containing cultivars.

There are about 11 R genes (*Dn1*, *Dn2*, *Dn3*, *Dn4*, *Dn5*, *Dn6*, *Dn7*, *Dn8*, *Dn9*, *Dnx* and *Dny*) found in wheat and its relatives and mostly located on either the 1D or 7D chromosome in hexaploit wheat (Botha *et al.*, 2005). These *Dn* genes only confer resistance to RWA, but not to other aphids such as *Rhopalosiphum padi* (L) (Botha *et al.*, 2005). The *R*-mediated signaling events activate defence mechanisms that are shared by both specific and basal resistance pathways (Dangl and Jones, 2001). The differential expression of the wheat defence transcriptome was reported within one to two hours after infestation by RWA on resistant Tugela *Dn1* using cDNA AFLP analysis (Matsioloko and Botha, 2003). Wheat responds to RWA in a gene-for-gene manner resulting in either a compatible or an incompatible response. In resistant varieties, recognition of an aphid elicitor by a receptor or *R*-gene product is followed by activation of the signal transduction pathway resulting in expression of defence responses such as accumulation of PR proteins.

The effectiveness of the response of different wheat cultivars to dissimilar elicitors from saliva of RWA biotypes varies due to resistance genes expressed in the different cultivars (Botha *et al.*, 2005; van der Westhuizen *et al.*, 1998a). The relationship between the *Dn1* and *Dn5* genes and the elicitor(s) in *RWASA1* saliva is interesting. In direct aphid feeding, *RWASA1* infestation induced various defence related responses (Peroxidase, β-1,3-glucanase and lipoxygenase) in resistant *Dn1* (van der Westhuizen *et al.* 1998a;b; Berner 2006; Moloi and van der Westhuizen, 2006) and *Dn5* (Kgatisho *et al*., 2008; Tsai, 2011) containing cultivars than in the susceptible Tugela cultivar. Mohase and van der Westhuizen (2002a) also provided evidence that elicitors isolated from intercellular wash fluids of infested (*RWASA1*) resistant (*Dn1*) plants induced more defence responses in resistant (*Dn1*) than susceptible wheat cultivars. Evidence has been provided in this study that elicitor(s) originating from *RWASA1* saliva also trigger defence related

responses in both *Dn1* and *Dn5*, although at higher levels in *Dn1* containing cultivars. There is a possibility that the *Dn1* gene might express specific receptors that only recognise specific elicitors from *RWASA1* saliva. Usually such specificity in elicited responses is indicative of specific receptors that function in recognition and activation of the eliciting of the defence responses after attack (Botha *et al.*, 2005).

Phloem feeding insect secretions and enzymes in aphid saliva from potential sources of *Avr* factors that induce defence responses (Thompson and Goggin, 2006). Elicitor(s) in *RWASA2* saliva was very specific, inducing defence responses only in the cultivar with *Dn5* resistance gene (PAN 3144) which was released as RWA resistant cultivar before appearance of RWA biotype 2. Kgatisho *et al*. (2008) also noted this specificity where direct aphid (*RWASA2*) feeding selectively induced defence responses in cultivars expressing the *Dn5*, but not the *Dn1* resistance gene. The results of this study indeed confirm the fact that elicitor(s) in *RWASA2* saliva are different from those presented by *RWASA1* and have overcome the resistance conferred by *Dn1* resistance gene.

The *Dn5* resistance gene, in contrast to *Dn1*, confers resistance against elicitors from both the *RWASA1* and *RWASA2* saliva. This was evident from the results of this study as elicitor(s) from either *RWASA1* or *RWASA2* saliva induced defence responses in PAN 3144 (*Dn5*) wheat (Figs 4.1, 4.2 and 4.3). Tolmay *et al.* investigated the damage rating of the two RWA biotypes on different wheat cultivars and provided evidence that Tugela *Dn1* was still resistant to *RWASA1*, but susceptible to *RWASA2*, whereas PAN 3144 (*Dn5*) was resistant to both *RWASA1* and *RWASA2* (Tolmay *et al.*, 2007). Unexpectedly, elicitors from *RWASA1* and *RWASA2* saliva, which are presumed to be different, induced about the same levels of defence related enzyme activities in the cultivar PAN 3144. We therefore suggest that general receptors may be present in *Dn5* cultivars that recognise elicitors or avirulence determinants from both *RWASA1* and *RWASA2* saliva.

Induced activities of peroxidase in resistant wheat cultivars due to RWA infestations (van der Westhuizen *et al.*, 1998b) and the spotted alfalfa aphid in green lucerne leaves (Jiang and Miles, 1993) have been reported, as has been noted with elicitor(s) from aphid saliva in this study (Figs 4.1, 4.4, 4.8, 4.10 and 4.11). Similarly, elicitors from salivary material of other aphid species such as the green peach aphid (*Myzus persicae*) induced resistance responses in *Arabidopsis* (De Vos and Jander, 2009). In addition, Ma *et al.* (2010) provided evidence that polyphenol oxidase from saliva of *Sitobion avenae* and *Schizaphis graminum* acts as an elicitor triggering defence responses in wheat.

Peroxidases (PR9) are involved in defence-related events that occur in the extracellular matrix such as strengthening of cell walls by lignifications and formation of intermolecular crosslinks, suberin and the production of reactive oxygen species associated with eliciting and signaling of defence responses (Bowles, 1990; Mehdy, 1994; van der Westhuizen *et al.*, 1998b). The resultant rigidity of the cell wall could render aphid stylet penetration challenging. Van der Westhuizen *et al*. (1998a) reported that RWA infestations induced cell-wall thickening in mesophyll cells of resistant wheat which probably hinders aphid penetration.

β-1,3-Glucanase (PR2) is one of the enzymes induced during RWA infestations and is involved in aphid resistance in different wheat genotypes (van der Westhuizen *et al.*, 1998b). The increased levels of β-1,3-glucanase in resistant plants during RWA infestation confirms its involvement in the defence responses (van der Westhuizen *et al.*, 1998b). Higher levels of β-1,3-glucanase activity also occurred in resistant wheat cultivars treated with elicitor(s) from *RWASA1* or *RWASA2* saliva (Figs 4.2, 4.5, 4.9, 4.13 and 4.14). This suggests that β-1,3-glucanases are associated with resistance responses in wheat that are induced by elicitors in aphid saliva and are mediated by specific R-genes.

Glucanases found in plants are often endo-β-1,3-glucanases that produce oligomers of 2-6 glucose units from β-1,3-glucans (Stintzi *et al.*, 1993). In cell walls of fungal pathogens β-1,3-glucanases might act on glucans to release fragments (oligosaccharides or pectins) which may be perceived by plants as elicitors of defence responses (Klarzynski *et al.*, 2000; Wolski *et al.*, 2006). β-1,3-glucanase implicated in defence against pathogens has been reported for cereals including oat, barley and wheat (Bowles, 1990). The direct function of β-1,3-glucanase during RWA infestations is unknown, but reports indicate that this enzyme does accumulate in the apoplast of resistant wheat cultivars (van der Westhuizen *et al.*, 1998b). In addition, elicitors isolated from the apoplastic fluid of RWA infested resistant wheat plants also induced increases in β-1,3-glucanase activity (Mohase and van der Westhuizen, 2002a). The results of this study also indicate that β-1,3-glucanase is associated with the resistance response in wheat (Figs 4.2, 4.5, 4.9, 4.13 and 4.14)

Besides peroxidase and β-1,3-glucanase, lipoxygenase is also associated with the defence related responses in wheat during RWA infestation (Berner, 2006). The results of this study indicated that elicitors in aphid saliva induced differential increases in LOX activity in the different wheat cultivars treated (Figs 4.3, 4.6 and 4.7). The fact that LOX activity was induced only in cultivars containing resistance genes [Tugela *Dn1* and PAN 3144 (*Dn5*)], implicates LOX as part of the resistance mechanism towards RWA infestation. Similar tendencies of induced increases in LOX activity in infested resistant plants have also been reported [(*RWASA1-Dn1* and *RWASA1-Dn2*, Swart, 1999; Berner, 2006)]. The early induction of LOX activity (as soon as 3h after treatment with elicitors from saliva) indicated that this enzyme could be involved in the signaling response. In incompatible interactions, elicitors from *RWASA1* saliva induced much higher levels of LOX activity in Tugela *Dn1* than in PAN 3144 (Fig 4.3, 4.6 and 4.7). These findings provided additional evidence that the *Dn1* gene is highly effective against elicitors of *RWASA1* saliva. The results also confirm findings by Berner (2006) and Tsai (2011) on Tugela *Dn1* infested with *RWASA1*. Elicitors from *RWASA2* saliva were specific though, inducing increases in LOX activity only in the cultivar containing the *Dn5* gene (Fig

4.3). There were no increases in susceptible cultivars, Tugela and Tugela *Dn1*, when treated with elicitors of *RWASA2* saliva, thereby confirming that *Dn1* has lost resistance and/or *RWASA2* presents with new elicitor(s) not recognized by the receptors in *Dn1* containing cultivars.

Plant lipoxygenases have been considered as indicators of induced resistance in various plant insect interactions such as soybean against *Helicoverpa zea (*Felton *et al.*, 1994). As part of the resistance response, potato aphid (*Microsiphum euphorbiae*) and green aphid (*Myzus persicae*) feeding caused increases in lipoxygenase and *PR-1* RNAs in tomato leaves (Fidantsef *et al.*, 1999; Walling, 2000). The lipoxygenase pathway has been reported as essential for the success of defence response in wheat against the RWA (Berner, 2006). During insect attack, elicitors or signals are recognised and trigger the induction of lipase to convert phospholipids to linoleic acid and induce the lipoxygenase pathway. The lipoxygenase pathway products, oxylipins, serve as signal molecules that trigger the activation of defence related genes and as antimicrobial and antifungal compounds (Bleè, 1998; Holková *et al.*, 2010), or as intermediates in lipid peroxidation processes and defence responses (Murphy *et al*., 2006). The synthesis of oxylipins is catalysed by LOXs, which add molecular oxygen to polyunsaturated fatty acids (PUFAs) to yield the corresponding fatty acid hydroperoxides that are substrates for enzymes such as allene oxide synthase which is associated with synthesis of the jasmonates. These jasmonates are involved in signalling and regulation of plant defence gene expression (Shah, 2005). We speculate that the early increases in LOX activity following elicitor treatment (Figs 4.6 and 4.7) could initiate events leading to production of oxylipins that probably may be involved in signaling the activation of defence related genes.

It is interesting to note that elicitors from *RWASA1* saliva induce activity of defence related enzymes in both Tugela *Dn1* and Pan 3144 (*Dn5*) cultivars, whereas elicitors originating from *RWASA2* saliva induce defence responses only in PAN 3144 (*Dn5*) cultivar, again strongly confirming that elicitors in *RWASA2* saliva have

overcome the defences mediated by the resistance gene *Dn1* in wheat. It is therefore important to establish some of the properties of the elicitors isolated from *RWASA1* and *RWASA2* saliva that probably relate to their capacity to induce defence responses. In this regard SDS-PAGE was performed to establish the presence of and to separate the proteins in the salivary material of *RWASA1* and *RWASA2*.

Various researchers (Miles and Harrewijn, 1991; Madhusudhan and Miles 1998; Cooper *et al.*, 2010; 2011) have reported on the polypeptide profiles of aphid saliva of different aphid species collected using different feeding mediums such as pure water, sucrose and/or amino acids. Cooper and colleagues compared the polypeptide profiles of saliva from five aphid species [*Diuraphis noxia* (Kudjumov), *Diuraphis tritici* (Gillette), *Diuraphis mexicana* (Baker), *Schizaphis graminum* (Rondani), and *Acyrthosiphon pisum* (Harris)] and found that alkaline phosphatase (132kDa) was present in all the species (Cooper *et al.*, 2011). This alkaline phosphatase may have a universal role in aphid feeding.

The protein profiles of *RWASA1* and *RWASA2* salivary material revealed several polypeptide bands (Figs 4.16, 4.17 and 4.18). Common polypeptide bands shared by both biotypes, as well as unique bands were expressed (Fig 4.18). It is rather tempting to suggest that these unique polypeptide bands from either *RWASA1* or *RWASA2* salivary material are responsible for differential increases in induced enzyme activities in wheat plants (Figs 4.1; 4.2 and 4.3).

In our study, we suspect that the universal polypeptide band in RWA saliva may be SP4 (126.3 kDa). Although this SP4 polypeptide band occurred in both biotypes, and had a stronger expression in the saliva of *RWASA1,* it did not seem to be associated with RWA resistance response in wheat. Further characterization of the proteins in RWA saliva, that is beyond the scope of this study could elucidate general roles of the salivary proteins in aphid feeding and host resistance.

To further purify and characterize elicitors from aphid saliva various chromatographic techniques were used. Fractions from C18 reverse phase chromatography that eluted earlier (Figs 4.4, 4.5, 4.6 and 4.7) induced differential defence responses in various wheat cultivars. The earlier elution demonstrated the polar nature of the inducing fractions. The active fractions were further purified using PD-10 (Sephadex G25) columns. Instead of using group separation, individual fractions were collected from the columns. In this separation, elicitors in RWA saliva either entered the pores in the matrix at varying degrees or just passed through (larger molecules) and eluted first, thus elicitors were fractionated according to molecular size. The active fractions passed through the matrix and eluted earlier illustrating their relatively larger protein size. The partially purified fractions a_1, a_2, a_3 and b_1, b_2, and b_3 of *RWASA1* or *RWASA2* saliva differentially induced the activities of peroxidase and β-1,3-glucanase (Figs 4.10, 4.11, 4.14 and 4.15). The fractions (a_2 and b_2) originating from *RWASA1* saliva induced significantly higher levels of peroxidase and β-1,3-glucanase in Tugela *Dn1* than in PAN 3144 (*Dn5*). Elevated levels of activity induced by these fractions were much higher than those induced by crude salivary material, indicating a concentrating effect associated with fractionation.

The fact that these resistance eliciting fractions (*RWASA1* and *RWASA2* saliva) lost their inducing capacity after heat treatment (boiling) is an indication that elicitors in RWA saliva probably contain an active proteinaceous component that functions as an inducer of defence responses (Figs 4.10, 4.11, 4.14 and 4.15). This is in agreement with the report by Lapitan and colleagues (Lapitan *et al.*, 2007) who stated that the eliciting fractions of RWA salivary glands may have a proteinaceous character.

Furthermore, thin-layer chromatography (TLC) was employed to further establish the nature of elicitors in *RWASA1* and *RWASA2* saliva. We speculated that elicitors in RWA saliva might consist of amino acids and carbohydrates.

In addition to proteins being present in eliciting fractions of RWA saliva, qualitative TLC also revealed the presence of sugars (Plates 4.1, 4.2 and 4.3) and amino acids (Plates 4.4 and 4.5) in the inducing fractions. Differences in the spots indicating presence of carbohydrates in *RWASA1* and *RWASA2* salivary material were noted (Plate 1). The more spots detected in *RWASA2* than *RWASA1* salivary material could be an indication of the difference in the composition of elicitors in aphid saliva. The intense color of spots originating from purified fractions of *RWASA1* and *RWASA2* saliva could be an indication of the concentrating effect of the purification steps employed. These fractions also induced the highest defence responses in *Dn1* and *Dn5* cultivars. The differential expression of spots closely correlated to the differential expression of defence responses in *Dn1* and *Dn5* cultivars. Based on these results, it is obvious that carbohydrates might be one of the constituents of RWA saliva that contributes to the eliciting capacity that activates the defence responses in incompatible wheat-aphid interactions [(*RWASA1*-Tugela *Dn1*, *RWASA1*-PAN 3144 (*Dn5*) and *RWASA2*-PAN 3144 (*Dn5*)].

Elicitors consisting of proteins conjugated with sugars are not unique in plant defence responses. As mentioned in section 2.3, elicitors could be proteins, peptides, glycoproteins, lipids, or oligosaccharides (Nürnberger, 1999). Glycoproteins may be important elicitors that activate plant defense responses and promote plant disease resistance (Yang *et al*., 2009). Glycoproteins that induced defence responses in wheat have been reported in intercellular fluids of aphid infested resistant wheat cultivars (Mohase and van der Westhuizen, 2002a). The presence of glycoproteins in the inducing fractions of aphid saliva however still needs to be confirmed.

The results of this study provided evidence that elicitor(s) originating from aphid saliva increases some defence responses (peroxidase, β-1,3-glucanase and LOX) in resistant wheat plants, probably as part of an array of defence related reactions which collectively induce resistance against the RWA. In susceptible cultivars (Tugela and Tugela *Dn1*); the defence responses (activity of enzymes) were relatively low or insufficient for effective resistance. Our study confirms that RWA is

capable of secreting saliva in pure water. The polypeptide profiles of both biotypes had unique proteins bands, thus suggesting that protein components of aphid saliva might contribute to the differential responses induced in different wheat cultivars. The detection of carbohydrates and amino acids in RWA saliva evidently provided support that RWA saliva consists of a mixture of constituents, possibly glycoproteins. Continuation of this study focusing mainly on the complete characterization and possibly identification of the elicitors in aphid saliva, as well as the perception mechanisms by wheat plants, could shed more light on plant-aphid interactions. Such knowledge could undoubtedly be valuable in the continuous efforts to enhance aphid resistance in wheat.

CONCLUSION

The occurrence of the resistance breaking RWA biotypes is a great challenge to the wheat industry in South Africa. Resistance conferred by the *Dn1* gene which was abundantly used in breeding programmes against *RWASA1,* has been overcome by all the newly evolved SA biotypes including the *RWASA2* (Jankielsohn, 2011). Auspiciously, the *Dn5*-mediated resistance is still active against the *RWASA2*. The outcome of the interaction between the RWA and wheat depends entirely on elicitors originating either from RWA saliva or from the products of the interaction of the aphid with the plant. In addition to the presence of elicitors, detection by wheat receptors (gene products) is paramount to effective induction of the resistance response. This recognition may follow the gene-for-gene model, or employ the guard hypothesis, leading to resistance.

The *RWASA2* saliva obviously presents a modified elicitor(s) or a completely new elicitor(s) that eludes detection or is inefficiently detected by the receptors in *Dn1* containing cultivars. However since this elicitor(s) (from *RWASA2* saliva) is equally perceived by *Dn5*-containing cultivars as the elicitor(s) from *RWASA1* saliva, it is obvious that the receptors in *Dn5* containing cultivars are able to detect a broader range of elicitors than the highly specific receptors in *Dn1*-containing cultivars, and consequently the defence related pathways are activated.

The aim of this study was to isolate elicitors from saliva of two South African RWA biotypes, and determine and compare their eliciting activity in wheat cultivars containing different resistance genes. The isolated elicitor-active fractions were also partially characterized. It is worthwhile to note that according to various researchers artificial application of elicitors from insect salivary material to plants

induces similar defence responses as those induced by direct aphid feeding (Lapitan *et al*., 2007; De Vos and Jander, 2009). The results of this study will assist in providing clues in understanding the nature of elicitors presented by the *RWASA1* and *RWASA2* saliva, and the race specific (R-gene mediated) interactions with *Dn1* and *Dn5* containing cultivars, which could either be incompatible (resistance) or compatible (susceptibility).

Some attributes of the nature and eliciting activity of elicitors presented by aphid saliva in *Dn1* and *Dn5* containing cultivars include the following:

1. Intercellular injection of elicitors into different wheat cultivars resulted in either a resistant or a susceptible state.
    1.1 Elicitor(s) from RWASA1 saliva induced defence related responses (enzyme activities of peroxidase, β-1,3-glucanase and lipoxygenase) in both *Dn1* (Tugela DN) and *Dn5* (PAN 3144) containing cultivars (Figs 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.8 and 4.9). Obviously the resistance gene products of *Dn1* and *Dn5* are able to recognize elicitors in *RWASA1* saliva. Elicitors recognized could be either directly from saliva or the saliva could present enzymes that degrade cell wall components releasing fragments (oligosaccharides or pectins) that activate defence related responses.

    1.2 The elicitors present in *RWASA2* saliva on the other hand, induced defence responses in *Dn5* containing cultivars only, rendering recognition highly specific (Figs 4.1, 4.2, 4.3, 4.4, 4.5, 4.7, 4.8 and 4.9). The *Dn1*-containing cultivar (Tugela *Dn1*) could not confer defence responses when challenged with elicitors from *RWASA2* saliva. This newly evolved RWA biotype may possess a structure that completely eludes detection, or suppressors repressing activation of defence responses mediated by the *Dn1 gene*.

1.3 The *Dn5*-contaning cultivar detected elicitors from both *RWASA1* and *RWASA2* and activated the defence responses, implying that *Dn5* gene products recognize non-specific elicitors from differential sources.

2. Various chromatographic techniques were employed to isolate and characterize elicitors from *RWASA1* and *RWASA2* saliva.

2.1 Elicitor-active fractions (*RWASA1* and *RWASA2* saliva) emanating from C18 RP chromatography were polar in nature. Further purification of the active fractions revealed that elicitors in aphid saliva were larger proteins (24-232 kDa); this was also confirmed by SDS-PAGE. The purer fractions (C18 followed by PD 10) elicited higher increases in defence responses than the crude salivary material, C18RP fractions and PD 10 fractions (peroxidase and β-1,3-glucanase activity). The differential eliciting activity of these fractions was evident, elicitor-active fractions from *RWASA1* saliva induced higher defence responses in both *Dn1* and *Dn5* containing cultivars while the same fractions from *RWASA2* saliva induced activity in the *Dn5*-containing cultivar only. Loss of activity upon heating implied the proteinaceous character of these elicitor-active fractions.

2.2 SDS-PAGE revealed numerous polypeptide bands from these elicitor-active fractions. A positive test for carbohydrates and amino acids in the crude salivary material of both biotypes, and in the purer inducing fractions, probably suggests that elicitors from *RWASA1* and *RWASA2* saliva might contain a mixture of carbohydrates and amino acids (glycoproteins), and perhaps some other components responsible for eliciting defence responses in resistant wheat cultivars.

In conclusion, saliva of *RWASA1* and *RWASA2* contains elicitors or virulence factors perceived by wheat receptors leading to the resistant or susceptible state in wheat plants. Wheat cultivars containing *Dn1* and *Dn5* genes respond differently to elicitors in saliva of RWA biotypes, possibly because of the gene-for-gene interaction. *Dn1* gene products recognize specific elicitors from saliva of *RWASA1*, whilst *Dn5* gene products recognize distinct elicitors. Enzymes of defence responses such as peroxidase, β-1,3-glucanase and lipoxygenase that are involved in the overall resistance in wheat against RWA infestations are also elevated in resistant cultivars treated with elicitors from RWA saliva. Further research on characterization of elicitor active fractions, especially fractions a_2 and b_2 from both *RWASA1* and *RWASA2* saliva may lead to identification of elicitors that will provide a better tool in understanding elicitor perception by different wheat plants.

SUMMARY

The comparative effect of elicitors in Russian wheat aphid (*Diuraphis noxia*, Kurdjumov, RWA) saliva on the inducible defence related responses in different wheat (*Triticum aestivum* L.) cultivars was investigated. The elicitors emanated from the South African RWA biotype 1 (*RWASA1*) and biotype 2 (*RWASA2*) saliva. Elicitors from *RWASA1* and *RWASA2* salivary material were intercellularly injected into wheat cultivars, Tugela (lacks resistance genes), Tugela *Dn1* (*Dn1* resistance gene) and PAN 3144 (*Dn5* resistance gene) and the induced defence responses were measured. The accumulation of pathogenesis related (PR) proteins, peroxidase (PR9) and β-1,3-glucanase (PR2) involved in RWA resistance in wheat were used as indicators of the resistance response. Additionally, lipoxygenase (LOX) involved in the synthesis of lipid signals, jasmonates and other oxylipins, was also employed as a marker of resistance. Peroxidase, β-1,3-glucanase and LOX activities were determined spectrophometrically.

The elicitors in *RWASA1* saliva induced higher levels of peroxidase, β-1,3-glucanse and LOX activities in both *Dn1* and *Dn5* containing cultivars. However, the induced level of defence related enzyme activities was quantitatively higher in *Dn1* than *Dn5* containing cultivars. The purified elicitor active fractions from *RWASA1* saliva also induced enhanced defense responses that were significantly higher in *Dn1* than *Dn5* containing cultivars. This differential induction of defence responses suggests a stronger recognition of *RWASA1* elicitors by the *Dn1* gene products. In contrast, elicitors in *RWASA2* saliva interacted with the receptors in *Dn5*-containing wheat cultivars in a highly specific manner. The elicitors from crude saliva induced defence responses only in the *Dn5*, but not the *Dn1* containing cultivar. The purer eliciting fractions, collected from the various chromatographic separations, also induced selective defence responses that were evident only in PAN 3144 (*Dn5*)

Even though we suspect elicitors in the saliva of *RWASA1* and *RWASA2* to be distinct, they induced similar levels of defence related enzyme activities in the *Dn5* containing cultivar. The elicitor active fractions purified from *RWASA2* saliva induced similar responses in PAN 3144 (*Dn5*) as elicitors from crude saliva. This implies a strong capacity conferred by the *Dn5* mediated resistance, to recognize distinct elicitors from the saliva of the two aphid biotypes.

Polypeptide profiles of *RWASA1* and *RWASA2* crude and purified fractions of the salivary material, revealed expression of similar as well as distinct protein bands. These differences probably contributed to the disparity in the expression of defence related enzyme activities by saliva of *RWASA1* and *RWASA2* in wheat cultivars. Boiling of these fractions abolished the induced defence related activities, suggesting their proteinaceous character. Furthermore, the differential presence of carbohydrates and amino acids in elicitor active fractions originating from *RWASA1* and *RWASA2* saliva implies that in addition to proteins, carbohydrates or at least glycoproteins, could be one of active components in these inducing fractions.

The findings from this study confirm that the defence related enzymes (peroxidase, β-1,3-glucanase and lipoxygenase) associated with RWA resistance in wheat, are induced by elicitors in RWA saliva. The study has further indicated differential effects of elicitors in *RWASA1* and *RWASA2* saliva in wheat cultivars expressing different resistance genes. Elicitors in *RWASA2* saliva have overcome the resistance conferred by the *Dn1* resistance gene, but are avirulent to cultivars containing the *Dn5* gene. Furthermore, this *Dn5* resistance gene, not only confers resistance after treatment with elicitors in *RWASA2* saliva, but also in *RWASA1* saliva.

KEYWORDS: Russian wheat aphid, saliva, elicitors, wheat, resistance, induced defence responses, β-1,3-glucanase, peroxidase, lipoxygenase

OPSOMMING

Die effek van elisitore, teenwoordig in Russiese koringluisspeeksel (*Diuraphis noxia*, Kurdjumov, RKL), op geïnduseerde weerstandsreaksies in verskeie koring (*Triticum aestivum* L.) kultivars is vergelyk. Die elisitore kom voor in die speeksel van die Suid-Afrikaanse RKL-biotipe 1 (*RKLSA1*) en biotipe 2 (*RKLSA2*). Die *RKLSA1* en *RKLSA2* speekselmateriaal is intersellulêr ingespuit in die koring kultivars, Tugela (sonder weerstandsgene), Tugela *Dn1* (*Dn1* weerstandsgeen) en PAN3144 (*Dn5* weerstandsgeen) waarna die geïnduseerde weerstandsreaksies gemeet is. Die akkumulasie van patogenseverwante (PR) proteïene, peroksidase (PR9) en β-1,3-glukanase (PR2), betrokke in RKL weerstand in koring is gebruik as indikasie van die weerstandsrespons. Addissioneel is lipoksigenase (LOX) wat betrokke is in die sintese van lipiedseine, jasmonate en ander oksilipiene, ook as ‘n merker van weerstand gebruik. Peroksidase, β-1,3-glukanase en LOX-aktiwiteite is spektrofotometries bepaal.

Die elisitore in *RKLSA*-speeksel het hoër vlakke van peroksidase, β-1.3-glukanase en LOX aktiwiteite in beide *Dn1* en *Dn5* kultivars geïnduseer. Die geïnduseerde vlakke van die verdedigingsensiemaktiwiteite was kwantitatief hoër in *Dn1* as in *Dn5* kultivar. Die gesuiwerde elisitoraktiewe fraksies vanaf *RKLSA1* speeksel, het ook verhoogde verdedigingsresponse geïnduseer en dit was aansienlik hoër in Dn1 as in *Dn5* kultivars. Die differensiële induksie van ‘n verdedigingsrespons stel voor dat ‘n sterker herkenning van *RKLSA1*-elisitors voorkom in die *Dn1*-geenprodukte. Daarenteen, het die elisitors in *RKLSA2*-speeksel ‘n hoogs spesifieke interaksie met die koringkultivars aangetoon. Die elisitors vanaf die kruspeeksel, het ‘n verdedigingsrespons slegs in die *Dn5* geenbevattende kultivar getoon, maar nie in die *Dn1* geenbevattende kultivar nie. Die gesuiwerde elisitorfraksie, wat versamel is na verskeie chromatografiese skeidings, het ook geselekteerde verdedigingsresponse geïnduseer. Dit was slegs sigbaar in PAN 3144.

Al voorspel ons dat die elisitors in die speeksel van *RKLSA1* en *RKLSA2* verskillend is, het hulle eenderse vlakke van verdedigingsensiemaktiwiteite in die *Dn5* geenbevattende kultivar geïnduseer. Die gesuiwerde elisitoraktiewe fraksies vanaf *RKLSA2*-speeksel, het soortgelyke response in PAN 3144 as elisitore van kru-speeksel geïnduseer. Dit impliseer dat die *Dn5*-geen verdedigingsweg ‘n sterker

herkenningsvermoë het om spesifieke elisitore vanaf die twee koringluisbiotipes se speeksel te herken.

Polipeptiedprofiele van die *RKLSA1* en *RKLSA2* se kru- en gesuiwerde speekselfraksies het soortgelyke sowel as unieke proteïenbande getoon. Hierdie verskille het moontlik bygedra tot die diversiteit in die uitdrukking van verdedigings-verwante ensiemaktiwiteite deur die speeksel van *RKLSA1* en *RKLSA2* in die koring-kultivars. Verhitting van die fraksies bo 100°C het tot die staking in die geïnduseerde verdedigings verwante aktiwiteite gelei, wat op hul proteïnagtige eïenskappe dui. Verder het die differensiële teenwoordigheid van koolhidrate en aminosure in die *RKLSA1* en *RKLSA2* speekselelisitore, aangedui dat tesame met proteïene ook koolhidrate of glikoproteïene, een van die aktiewe verbindings in die induserende fraksies kan wees.

Die bevindinge van hierdie studie bevestig dat die verdedigingsverwante ensieme (peroksidase, β-1.3-glukanase en lipoksigenase), wat geassossieer word met RKL-weerstand in koring, deur elisitore in RKL speeksel geïnduseer word. Hierdie studie het verder aangedui dat die differensiële effek van elisitore in *RKLSA1* en *RKLSA2* speeksel verskillende weerstandsgene in koringkultivars tot uiting bring. Elisitore in *RKLSA2*-speeksel het die weerstand as gevolg van die *Dn1* weerstandsgene oorkom, maar is avirulent in kultivars wat die *Dn5* weerstandsgeen bevat. Verder het die *Dn5* weerstandsgeen weerstandbewerkstellig na behandeling met elisotore in beide *RKLSA1*-en *RKLSA2*-speeksel.

SLEUTELWOORDE: Russiese koringluis, speeksel, elisitore, koring, weerstand, geïnduseerde verdedigingsrespons, β-1.3-glukanase, peroksidase, lipoksigenase

APPENDIX

- Result of independent replicate experiments of crude saliva and fractions in activities of peroxidase, β-1,3-glucanase and lipoxygenase
- Results of independent duplicate experiments on SDS-PAGE and TLC

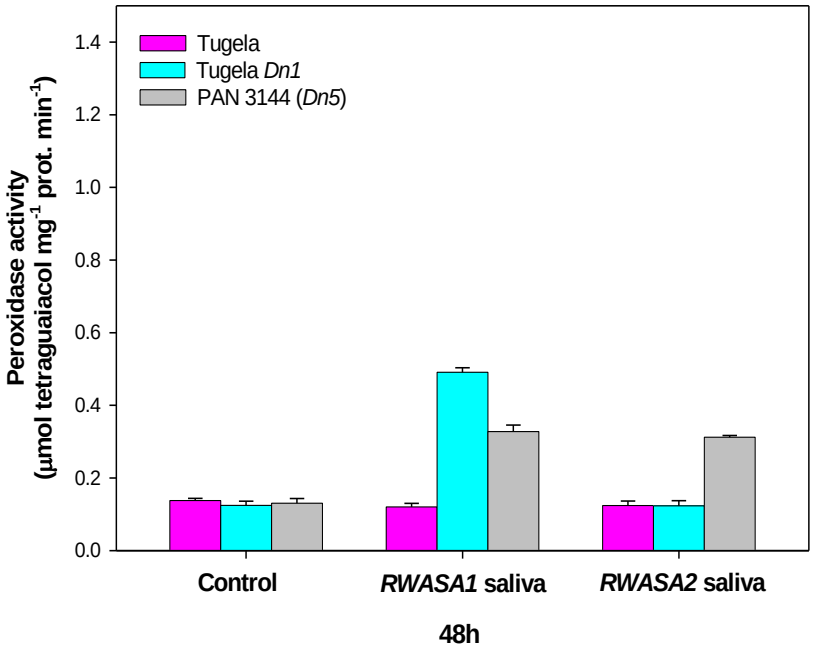

Figure 7.1: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on peroxidase activity of different wheat cultivars. Values are means ± SD (n=3).

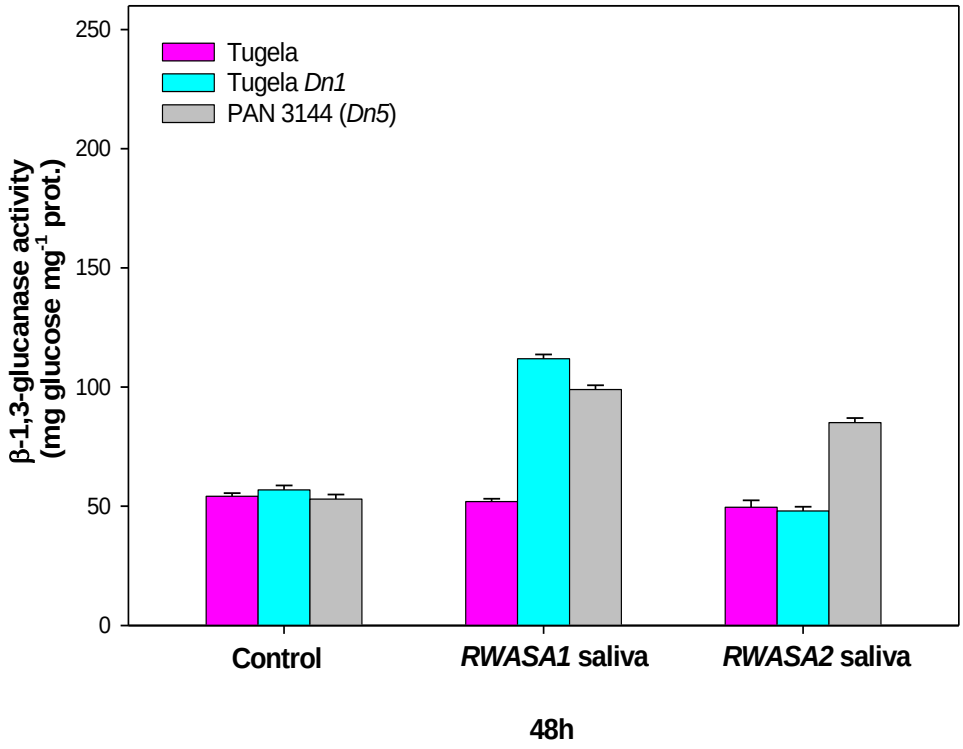

Figure 7.2: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on β-1,3-glucanase activity of different wheat cultivars. Values are means ± SD (n=3).

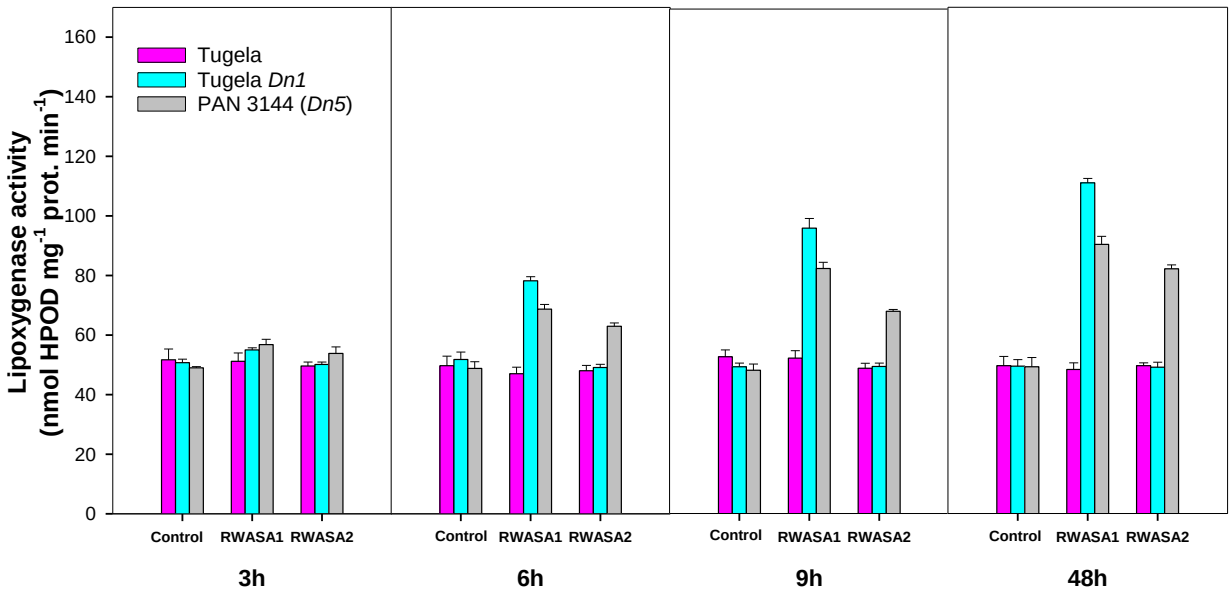

Figure 7.3: Effect of intercellularly injected aphid (*RWASA1* and *RWASA2*) salivary material on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

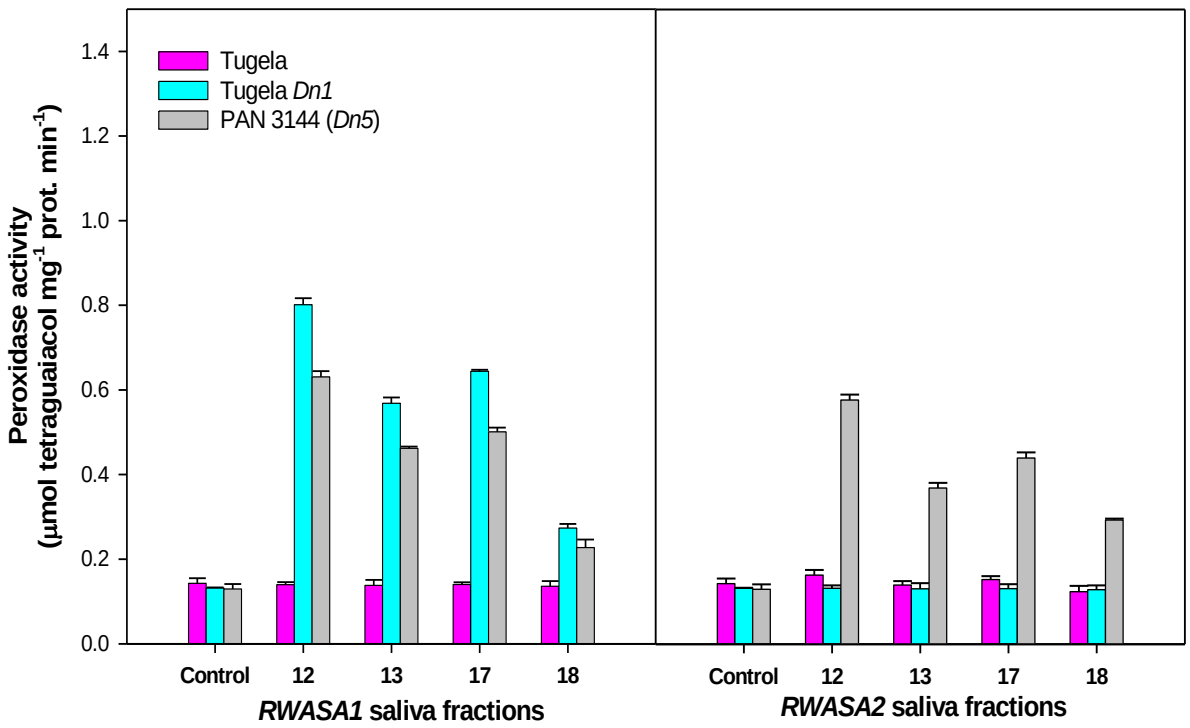

Figure 7.4: Effect of *RWASA1* and *RWASA2* salivary material fractions (C18 reverse phase chromatography) on peroxidase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

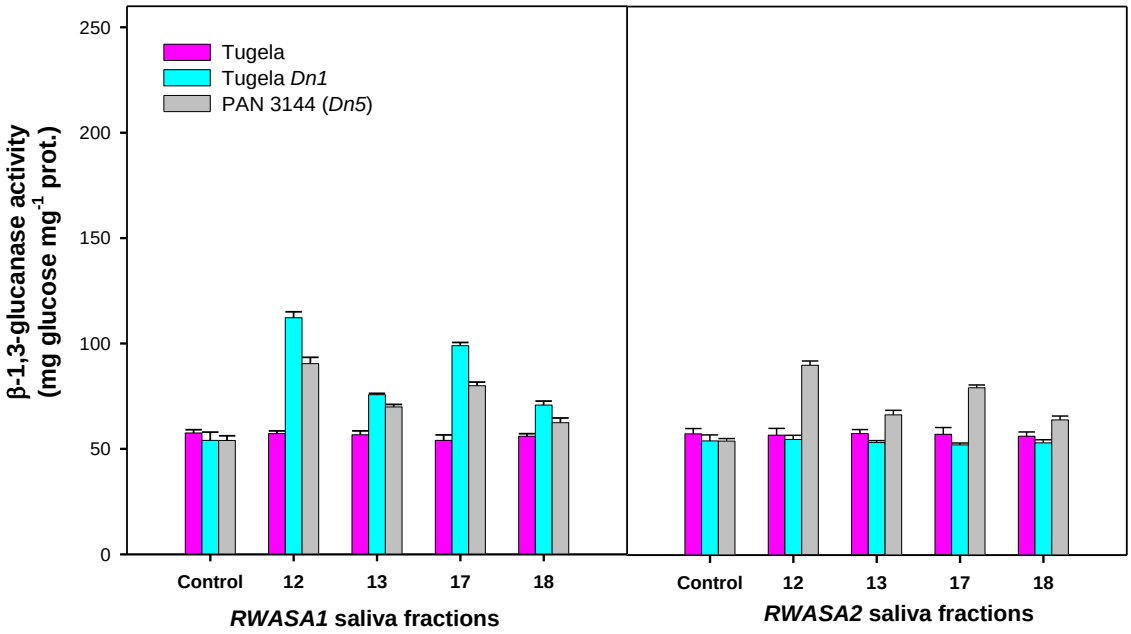

Figure 7.5: Effect of *RWASA1* and *RWASA2* salivary material fractions (C18 reverse phase chromatography) on β-1,3-glucanase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

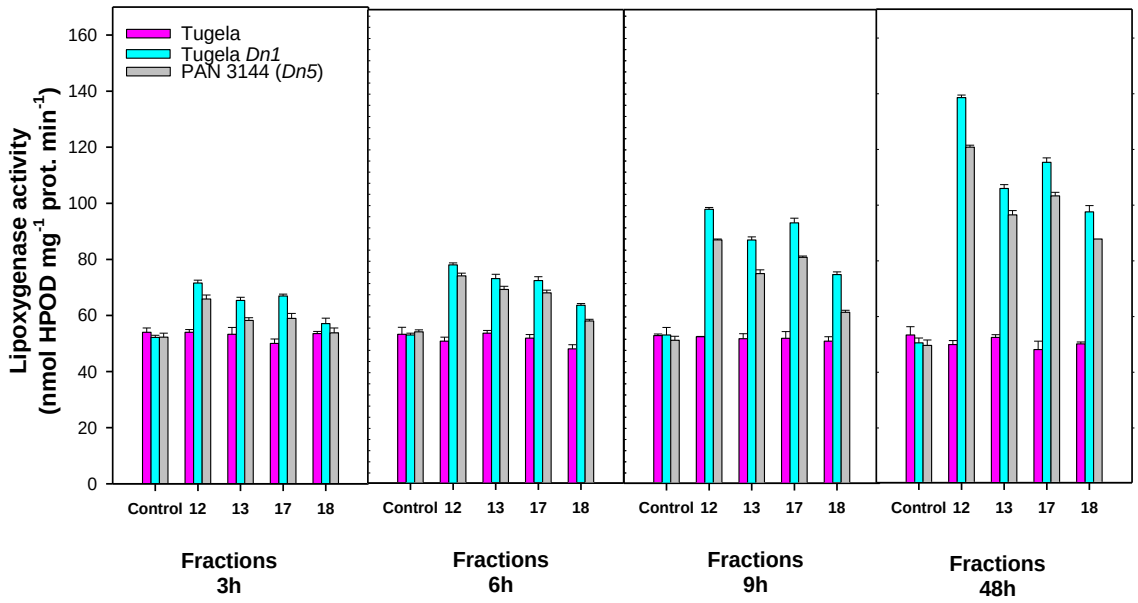

Figure 7.6: Effect of *RWASA1* salivary material fractions (C18 reverse phase chromatography) on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

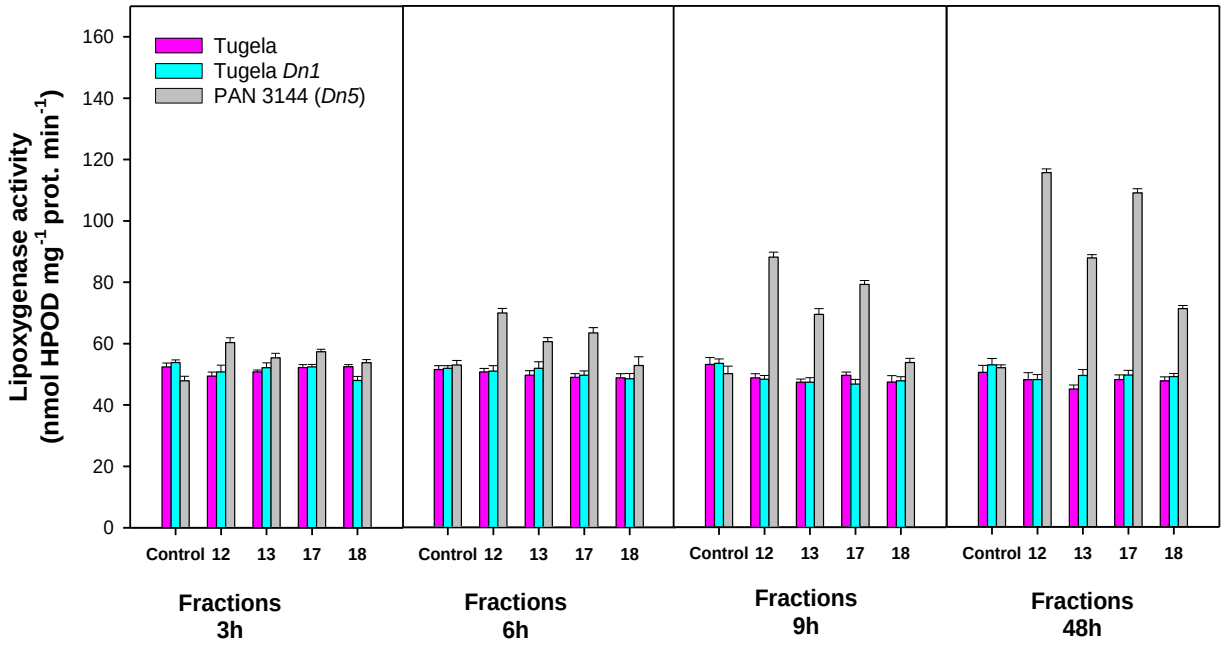

Figure 7.7: Effect of *RWASA2* salivary material fractions (C18 Reverse Phase chromatography) on lipoxygenase activity of different wheat cultivars. Values are means ± SD (n=3).

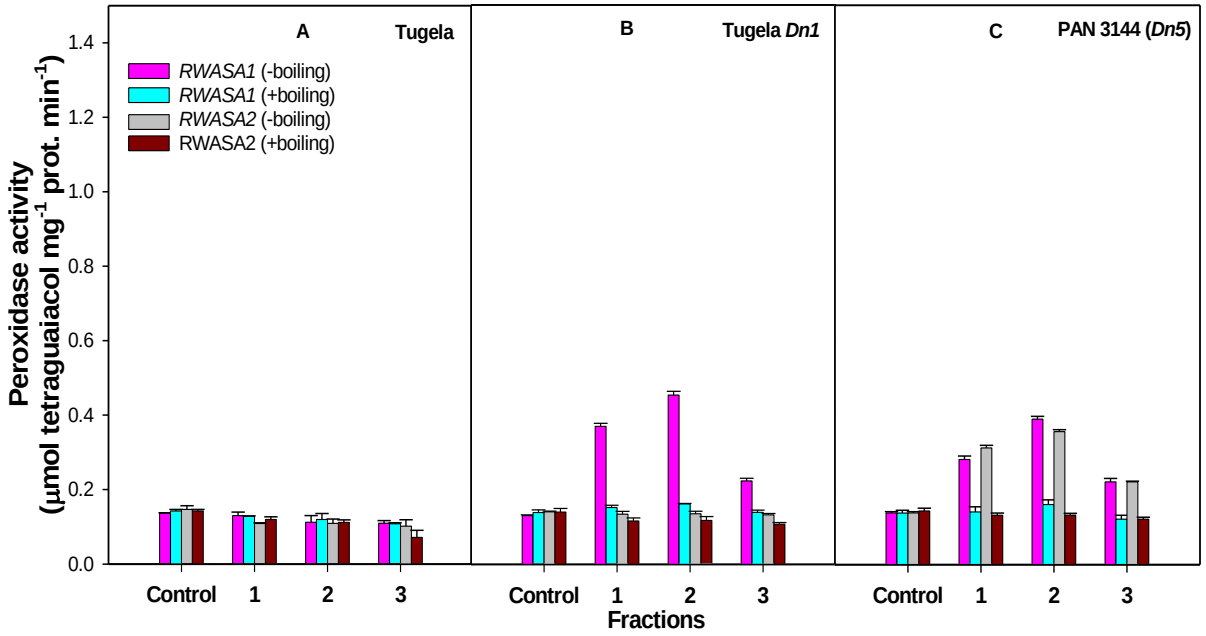

Figure 7.8: Effect of *RWASA1* and *RWASA2* salivary material fractions (PD-10) on peroxidase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

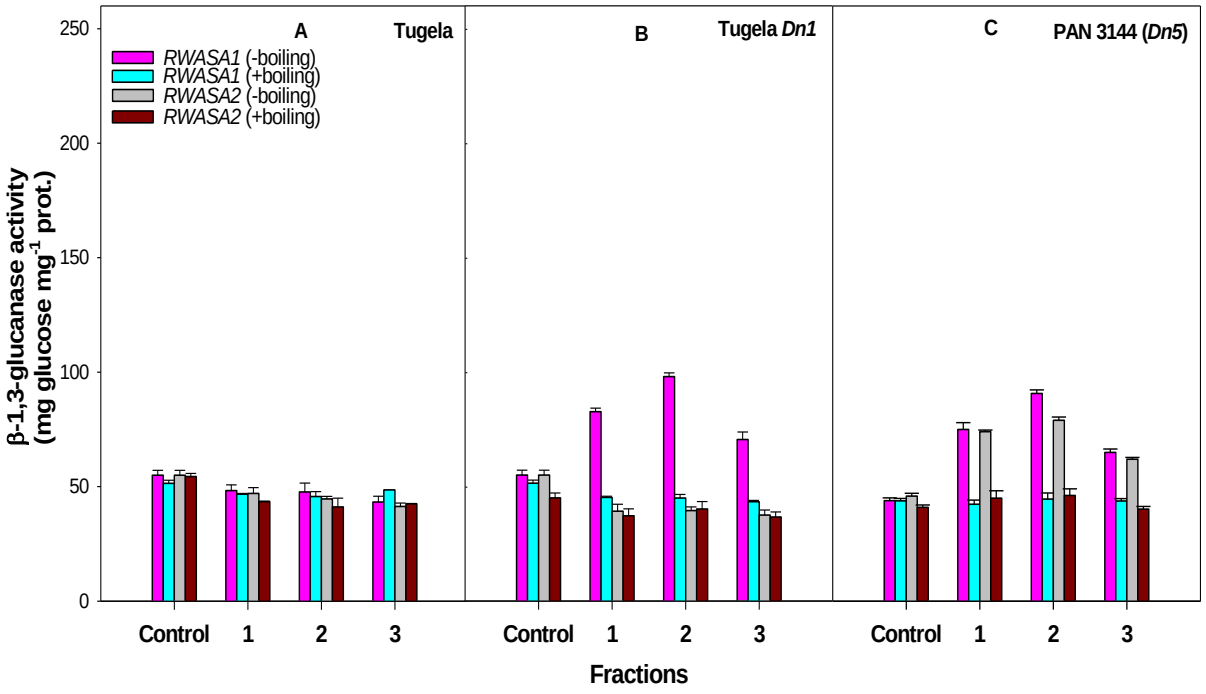

Figure 7.9: Effect of *RWASA1* and *RWASA2* salivary material fractions (PD-10) on β-1,3-glucanase activity of different wheat cultivars after 48h of treatment. Values are means ± SD (n=3).

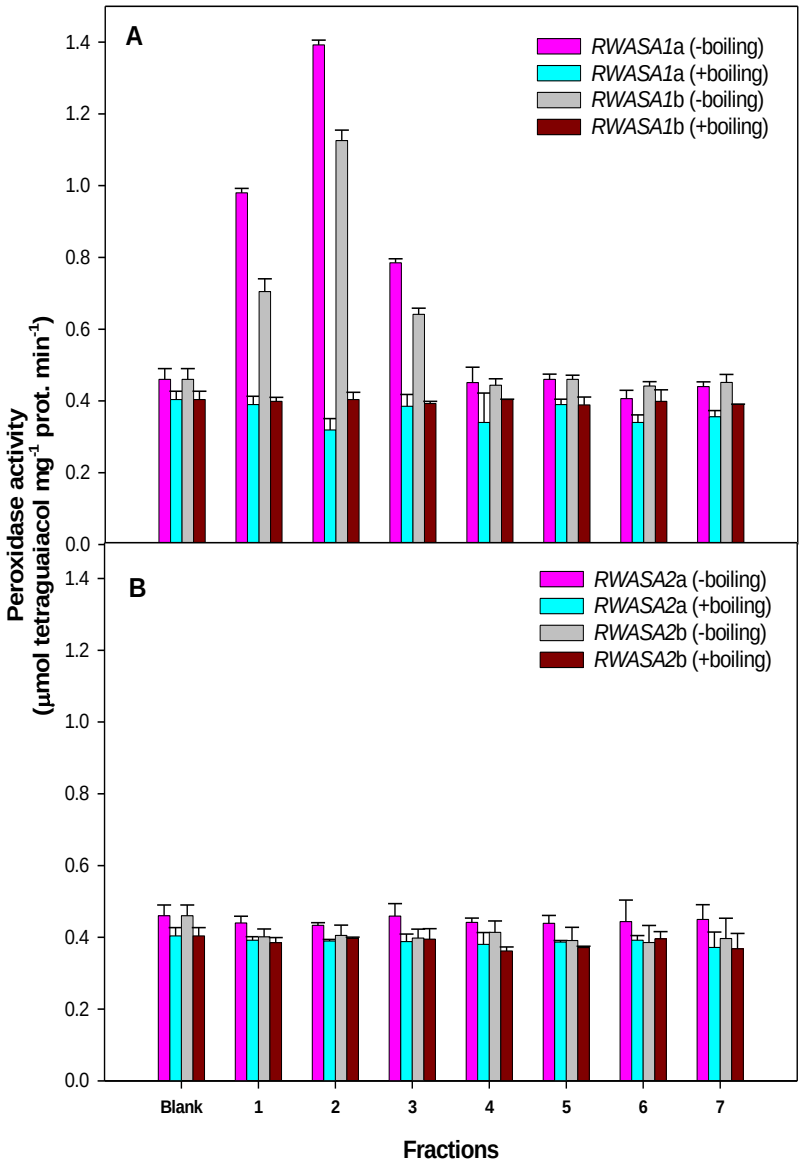

Figure 7.10: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in Tugela *Dn1* cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

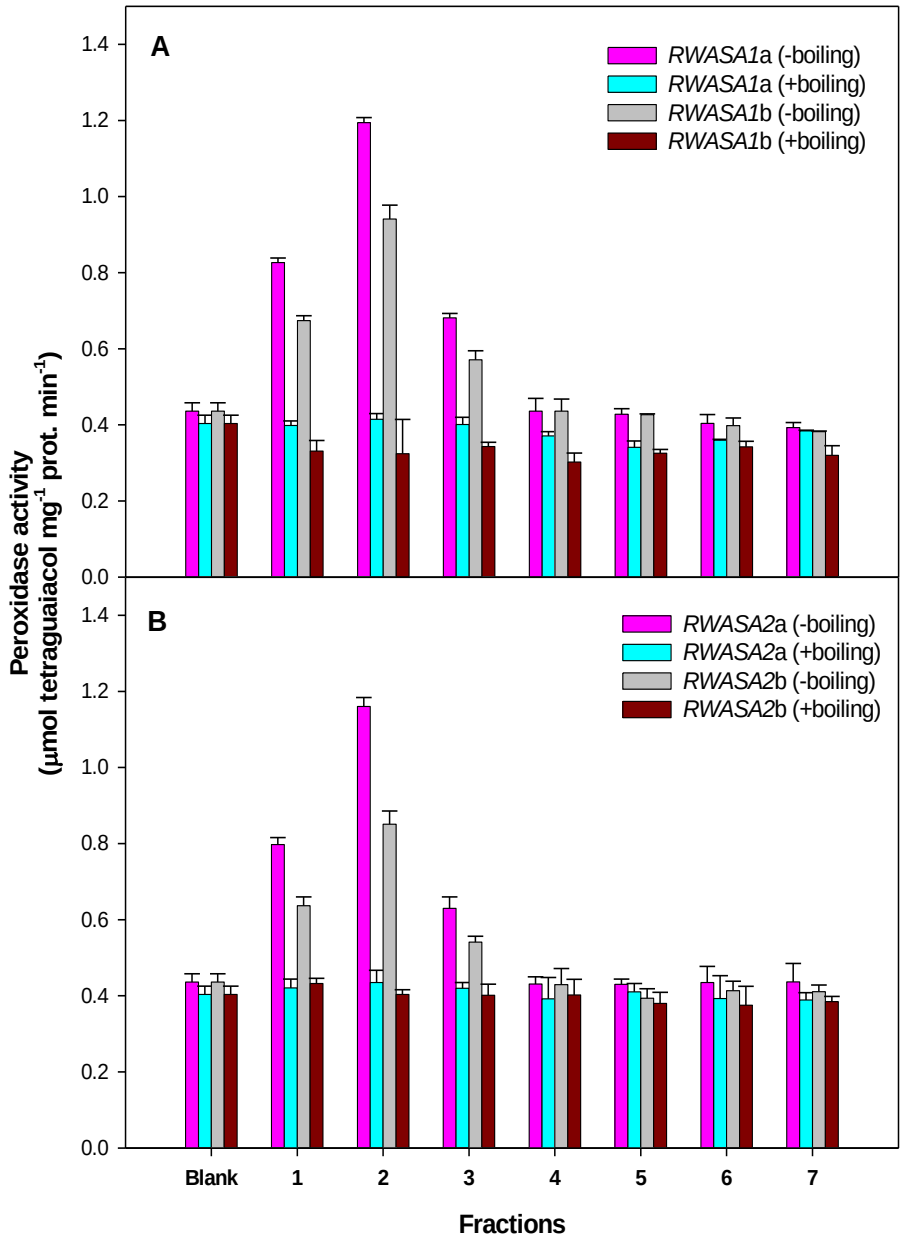

Figure 7.11: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in PAN 3144 cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

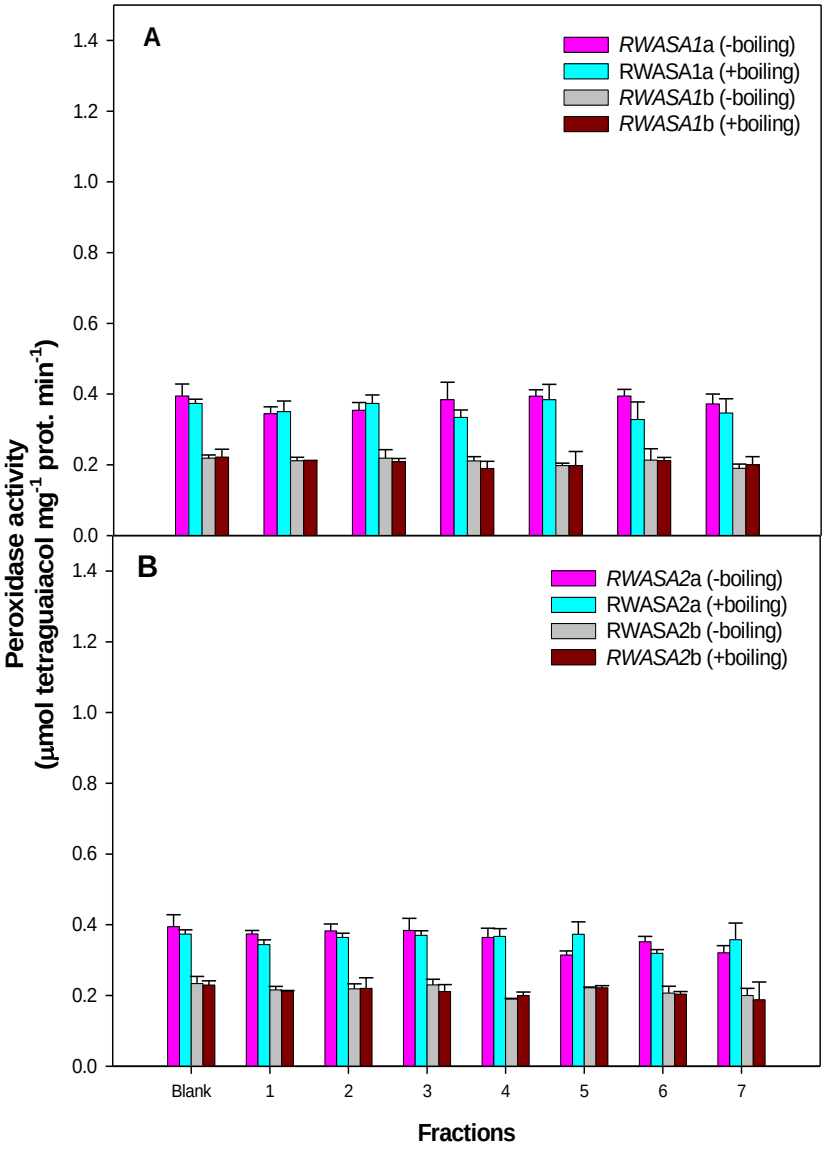

Figure 7.12: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on peroxidase activity in Tugela cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

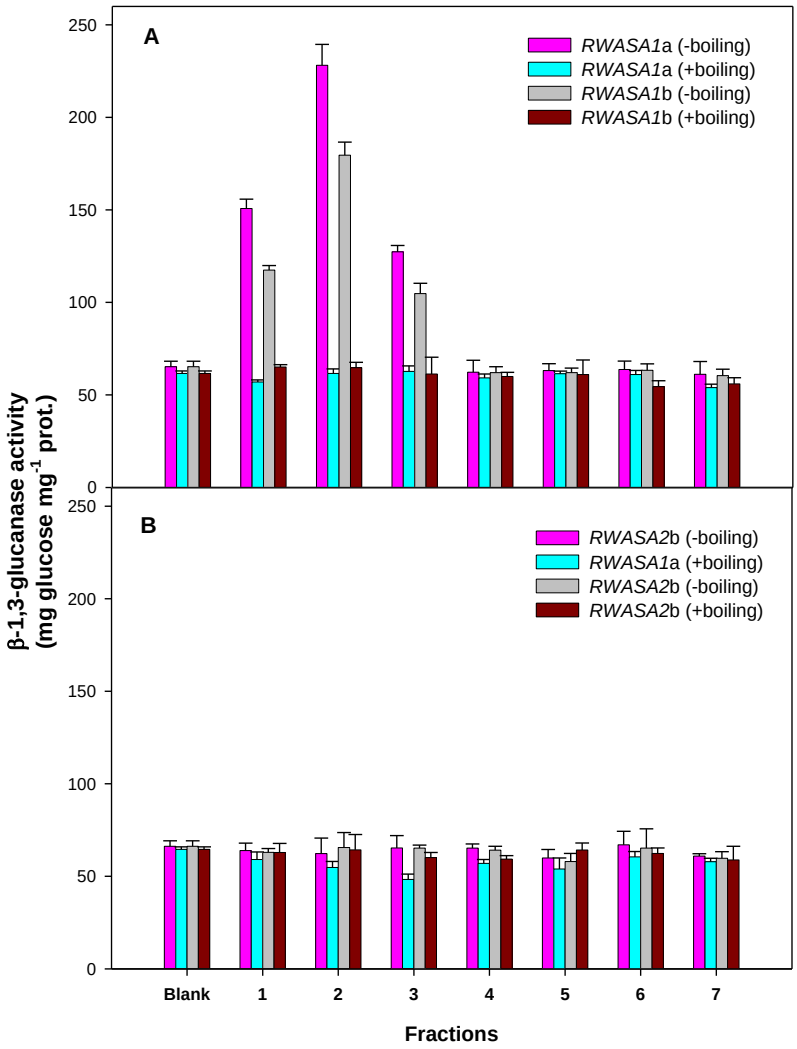

Figure 7.13: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanase activity in Tugela *Dn1* 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

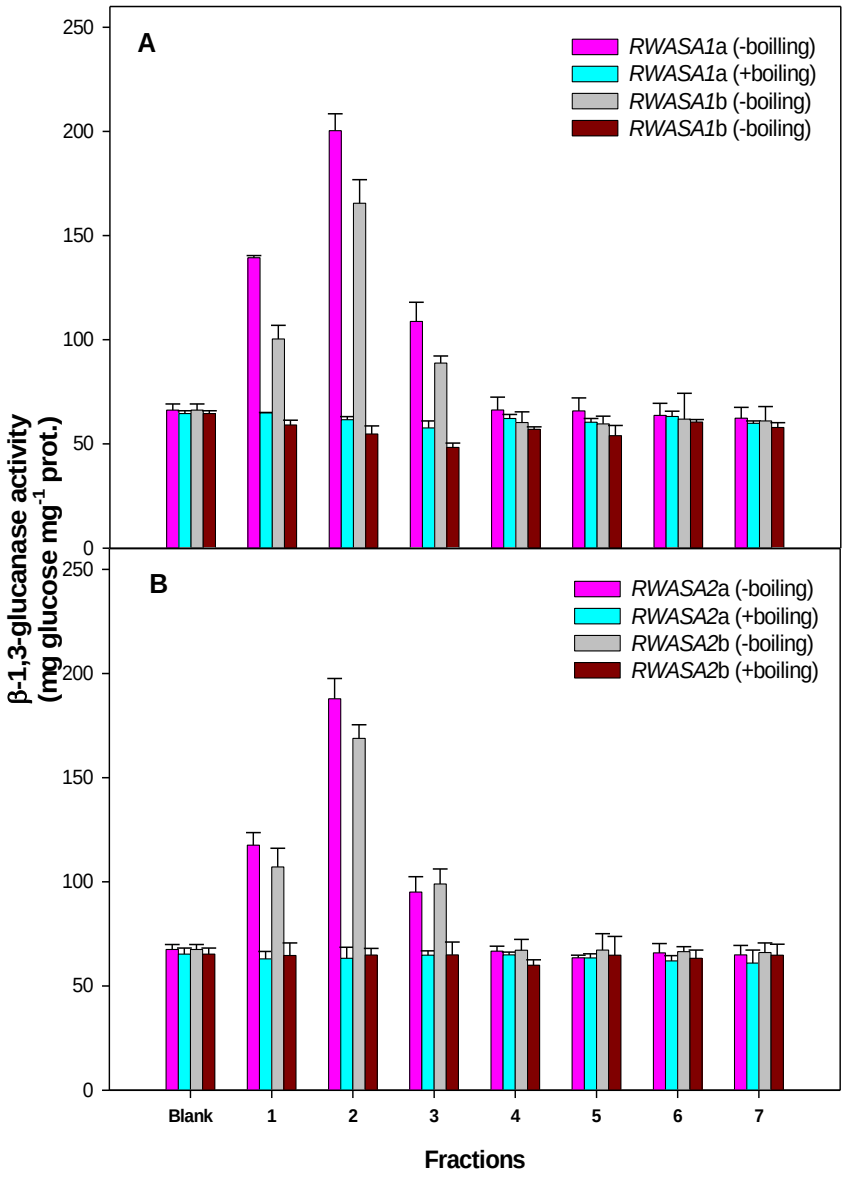

Figure 7.14: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanase activity in PAN 3144 cultivar 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

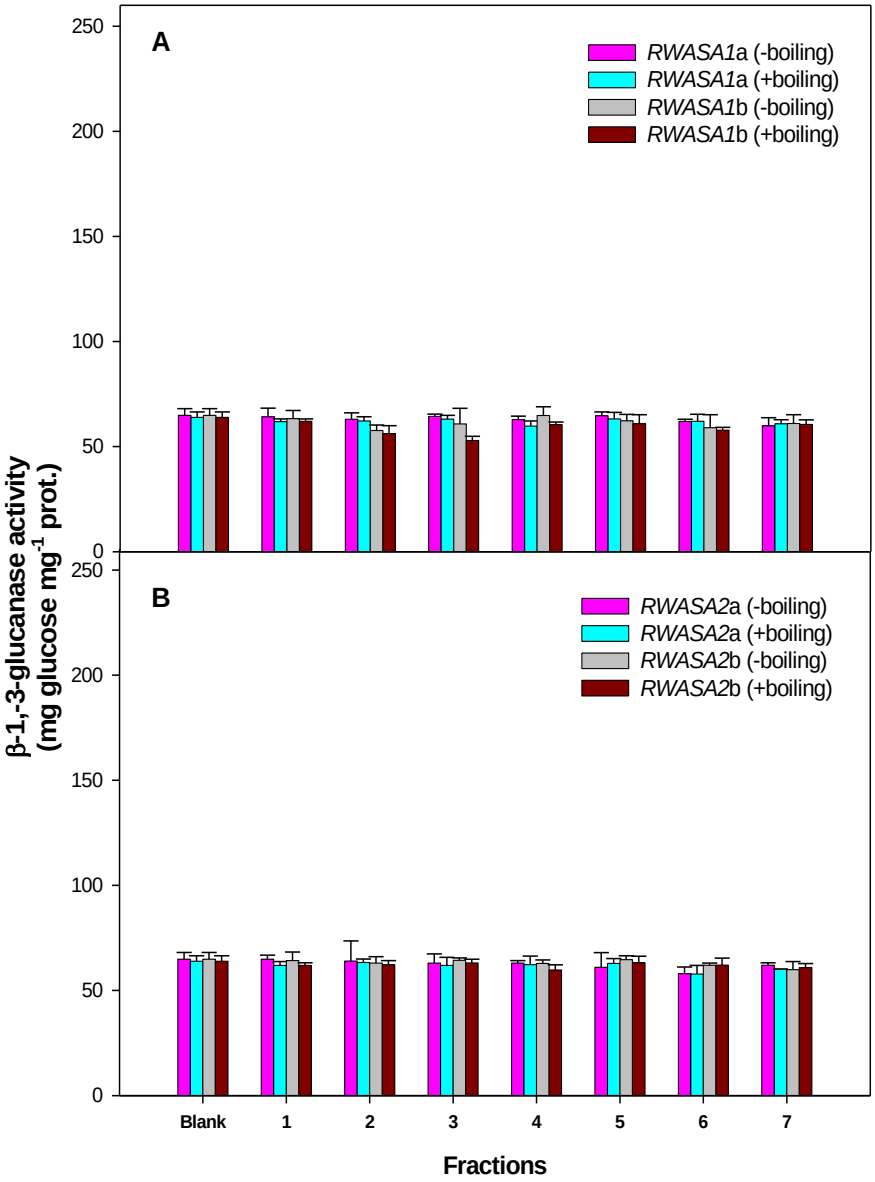

Figure 7.15: Effect of intercellularly injected *RWASA1* (A) and *RWASA2* (B) salivary material fractions on β-1,3-glucanase activity in Tugela 48h after treatment. *RWASA1* and *RWASA2* salivary material were first fractionated using C18 reverse phase chromatography, then active fractions (12 and 13= a; 17 and 18= b) passed through a PD 10 column. Blank is sterile water. Values are means ± SD (n=3).

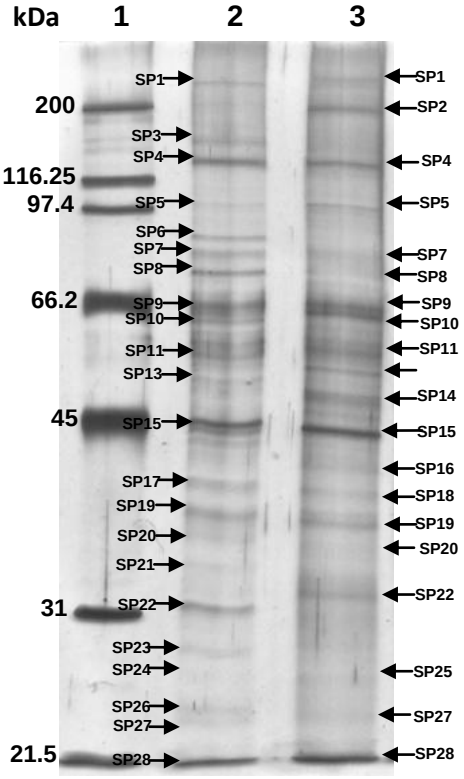

Figure 7.16: SDS-PAGE (11%) of saliva proteins from *RWASA1* and *RWASA2* salivary material. Ten microlitres of concentrated (x80, 1200 aphids per 10 ml sterile distilled water) salivary material was loaded per lane. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). **Lane 2**: (*RWASA1* salivary material); **Lane 3**: (*RWASA2* watery saliva)

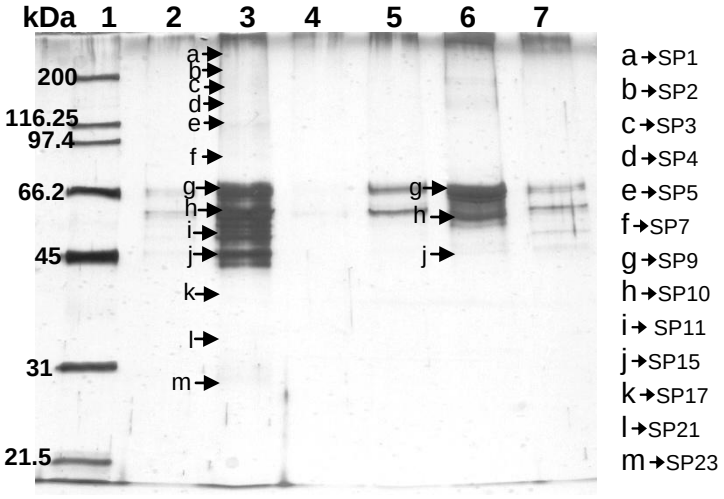

Figure 7.17: SDS-PAGE (11%) of purified fractions of *RWASA1* saliva. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). For all the fractions, 1 µg of protein was loaded; **Lane 2**: fraction a_1. **Lane 3**: fraction a_2, L**ane 4**: fraction a_3; **Lane 5**: fraction b_1; **Lane 6**: fraction b_2, and **Lane 7**: fraction b_3.

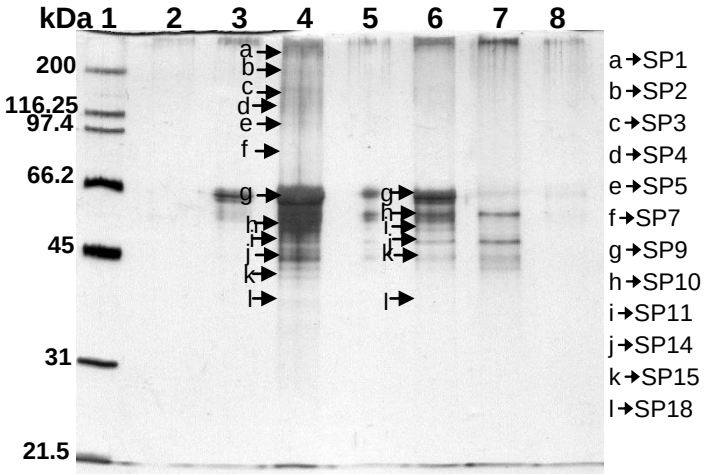

Figure 7.18: SDS-PAGE (11%) of purified fractions *RWASA2* saliva fractions. **Lane 1**: Broad range molecular marker proteins (Bio-Rad). For all the fractions, 1 µg of protein was loaded; **Lane 3**: fraction a_1. **Lane 4**: fraction a_2, L**ane 5**: fraction a_3; **Lane 6**: fraction b_2; **Lane 7**: fraction b_1, and **Lane 8**: fraction b_3.

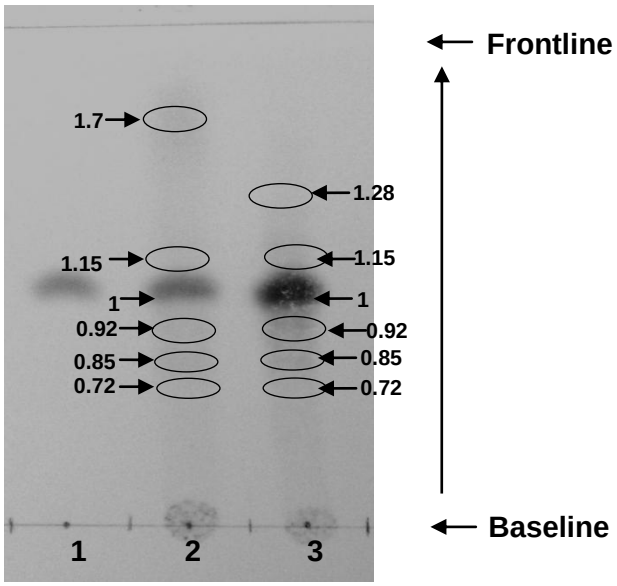

Plate 7.1: Qualitative TLC profile (sugars) of *RWASA1* and *RWASA2* crude salivary material. **Lane 1**: standard, glucose (0.2 µg). **Lane 2**: *RWASA1* crude salivary material, 2 µg was loaded. **Lane 3**: *RWASA2* crude salivary material, 2 µg was loaded.

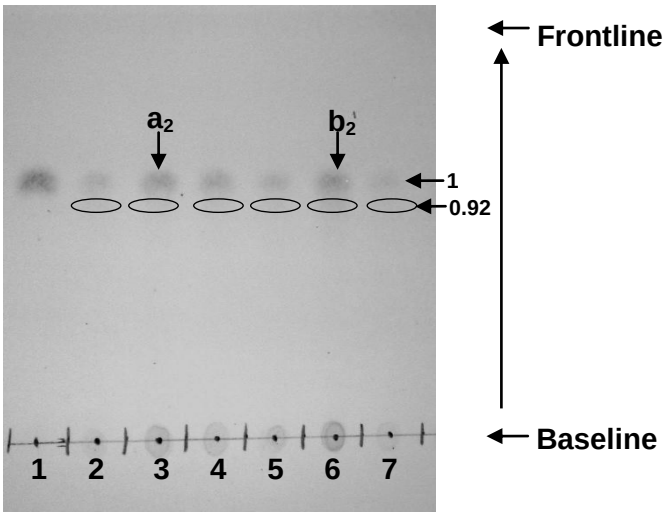

Plate 7.2: Qualitative TLC profile (sugars) of partially purified elicitors from *RWASA1* salivary material. The fractions were first fractionated with C18 reverse phase then active fractions (12 and 13=a; 17 and 18= b) passed through PD-10 column. Each lane was loaded with 4 µg protein of the fraction. **Lane 1**: standard, glucose (0.2 µg); **Lane 2**: a_1; **Lane 3**: a_2; **Lane 4**: a_3; **Lane 5**: b_1; **Lane 6**: b_2; and **Lane 7**: b_3.

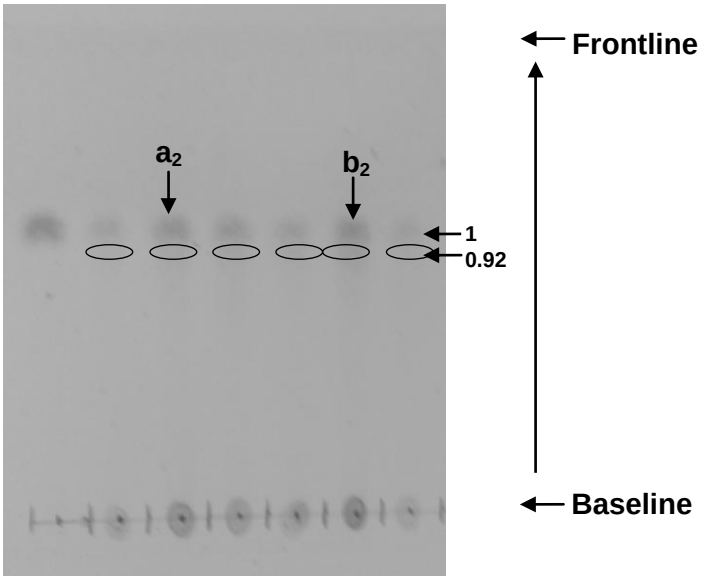

Plate 7.3: Qualitative TLC profile (sugars) of partially purified elicitors from *RWASA2* saliva. The fractions were first fractionated with C18 reverse phase then active fractions (12 and 13= a; 17 and 18= b) passed through PD-10 column. Each lane was loaded with 4 µg protein of the fraction. **Lane 1**: standard, glucose (0.2 µg); **Lane 2**: a_1; **Lane 3**: a_2; **Lane 4**: a3; **Lane 5**: b_1; **Lane 6**: b_2; and **Lane 7**: b_3.

1 2 3 4 5 6 7

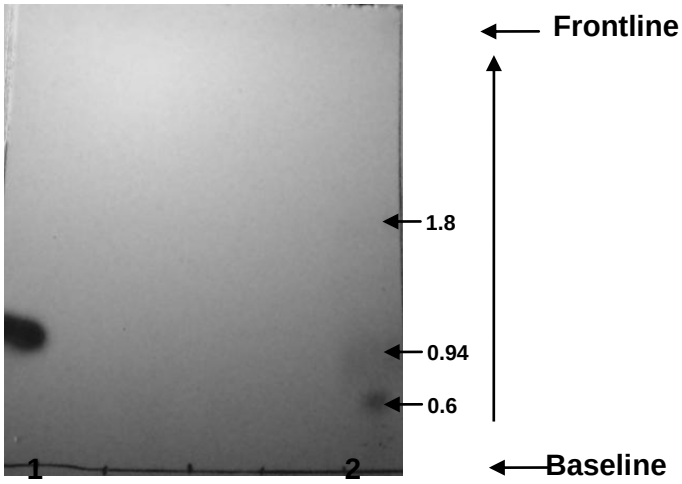

Plate 7.4: Qualitative TLC profile (amino acids) of *RWASA1* crude salivary material. **Lane 1**: β-alanine (0.5 µg). **Lane 2**: *RWASA2* crude salivary material, 2 µg of protein equivalent, was loaded.

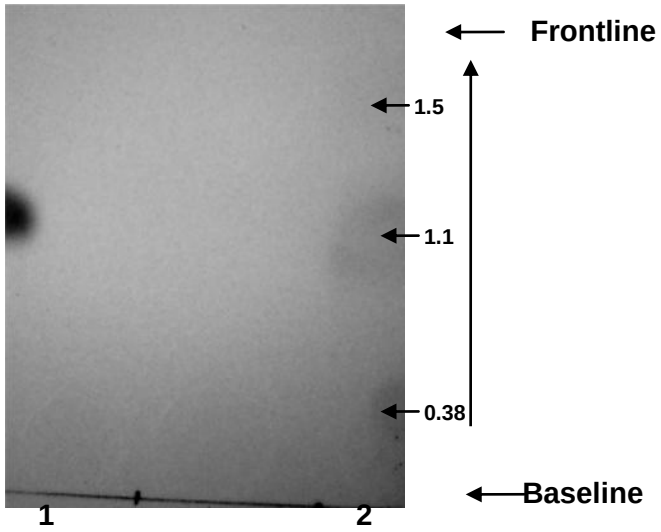

Plate 7.5: Qualitative TLC) profile (amino acids) of *RWASA2* crude salivary material. **Lane 1**: β-alanine (0.5 µg). **Lane 2**: *RWASA2* crude salivary material, 2 µg of protein equivalent, was loaded.

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