Initiation of defence responses by plant extracts and their insecticidal role against the Russian wheat aphid in wheat.

by

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DECLARATION

I, the undersigned, hereby declare that this dissertation, prepared for the degree of Magister Scientiae in Botany which was submitted by me to the University of the Free State, is my original work and has not been submitted previously to any other University/Faculty. All sources of materials and financial assistances used for the study have been duly acknowledged. I further cede copyright of the dissertation in favour of the University of the Free State.

Lubabalo Saba 16/11/2015

DEDICATION

To my family, words can never be enough to show my gratitude towards your unconditional love and support throughout these years. I have come to know 4 “L’s” of life, love and lessons learnt through hard work and dedication. I have been fortunate enough to have been blessed with friends who have wished me well through life many trials and tribulations and I dedicate this to my family and friends.

“To live is to suffer, to survive is to find some meaning in that suffering.” – Friedrich Nietzche

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LIST OF ABBREVIATIONS AND SYMBOLS

Aa	*Artemisia afra*
Ag	*Agave attenuata*
ANOVA	Analysis of variance
ARC-SGI	Agricultural Research Council Small Grain Institute
BABA	β-aminobutyric acid
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
BTH	Benzothiadiazole
Ca	Circa
Cv	Confidence interval
DCM	Cultivar
DDT	Dichloromethane
DEET	Dichlorodiphenyltrichloroethane
DF	N, N-diethyl-3-methylbenzamide
DMSO	Degrees of freedom
DOA	Dimethyl sulphoxide
DPPH	Department of Agriculture
EDTA	Diphenylpicrylhydrazyl
ETI	Ethylenediaminetetraacetic acid
ETS	Effector-triggered immunity
F	Effector-triggered susceptibility
FAOSTAT	F-statistic
FID	Food and Agriculture Organisation of the United Nations Statistics
FL	Flame ionization detector
GC-MS	Fiducial limit
GLV	Gas chromatography Coupled with Mass Spectroscopy
HPI	Green leaf volatile
HR	Hours post infestation
	Hypersensitive response

ISR	Induced systemic resistance
IWF	Intercellular wash fluid
JA	Jasmonic acid
LC_{50}	Lethal concentration killing 50% population
LRR	Leucine-rich repeats
MAMP	Microbial-associated molecular pattern
MeJA	Methyl jasmonate
NB	Nucleotide binding
NIST	National Institute of Standard and Technology
ORN	Olfactory receptor neurons
P	Probability
PAL	Phase alternative line
PAMP	Pathogen-associated molecular pattern
PR	Pathogenesis related
R_f	Distance travelled by a given compound divided by the distance travelled by the solvent front
ROS	Reactive oxygen species
RWA	Russian wheat aphid
RWASA1	Russian wheat aphid South African biotype 1
SA	Salicylic acid
SAR	Systemic acquired resistance
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
VOC	Volatile organic compound
Zc	*Zanthoxylum capense*

LIST OF SI UNITS

%	Percentage
°C	Degrees centigrade
cm	Centimetre(s)
g	Gram(s)
h	Hour(s)
h.p.i	Hour post infestation
M	Molar(s)
mg	Milligram
min	Minute(s)
mL	Millilitre(s)
mM	Millimolar(s)
pH	Power of hydrogen
s	Second(s)
U	Unit(s)
µg	Microgram(s)
µl	Microlitre(s)
w/v	Weight per volume
v/w	Volume per weight

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Chapter 1
General introduction and Rationale

Introduction and rationale

Wheat (*Triticum* spp.) is an important grain crop cultivated throughout the world primarily due to its adaptability to many different environments including areas considered inhabitable such as the Arctic Circle (Briggle and Curtis, 1987). This adaptability is largely due to the complex nature of the plant's genome. The different varieties of wheat include the common wheat (*Triticum aestivum* L.) or bread wheat comprising approximately 95% of wheat found in the world, durum wheat (*Triticum durum*) used in the making of macaroni, spaghetti and pasta products and einkorn wheat (*Triticum monococcum*), considered to be the oldest form of domesticated wheat due to no evidence of hybridization with other grasses or grains (Takumi *et al.,* 1993; Elias, 1995; Kiplagat, 2005).

Crop intensification has resulted in a threefold increase of global crop production in the past 50 years (FAOSTAT, 2013). The intensification process revolves around the input of fertilizers, innovative cropping systems and use of pesticides to enhance production yield (Hobbs and Morris, 1996; Tscharntke *et al.,* 2005; Stoop, 2011). The production of wheat worldwide per capita surpasses all other crops and its stability is a major concern for many nations (FAOSTAT, 2013). The production stability is often shaken when the cereal crop is exposed to adverse abiotic and biotic stresses.

The health and survival of a plant is often determined by its ability to survive attack from pests and pathogens. A plant's ability to defend itself from a pest or pathogen is largely dependent on its capability to activate a defence response that may either be passive or active. Passive responses are innate and can be attributed to structural barriers or the positioning of deterrent compounds at strategic sites that prevent further colonisation of tissue (Almagro *et al.,* 2009). Active responses, also known as induced responses are the result of an invader's presence signalling a cascade of defence responses, which includes the hypersensitive response, production of pathogenesis-related (PR) proteins, production of reactive oxygen species (ROS), cell wall lignification and the reinforcement of the cell wall through cross-linkages, all in an effort to minimize damage or disease caused by the invader (Czaninski *et al.,* 1993; Durrant and Dong, 2004).

The induced defence response aids in the plant's natural ability to cope with an advancement of a pest or pathogen in its environment. However, a plant's ability to survive constant attack

from a pest or pathogen may require more than just its natural ability but perhaps introducing strategies to enhance resistance may prove beneficial.

One beneficial approach is the use of plant activators that work in the manipulation of chemical signals plants receive from damaged neighbouring plants and allow for the induction of a robust defence response in anticipation of a pest or pathogen attack (Goellner and Conrath, 2008). This is known as a priming response and chemicals such as salicylic acid, jasmonic acid, acibenzolar-S-methyl (BTH) and isonicotinic acid are known to induce a defence response in plants (White, 1979; Cohen *et al.,* 1993; Görlach *et al.,* 1996).
Plants also have the ability to recognise general chemical structures associated with microbes through receptor sites and once these structures bind to a receptor, they elicit a downstream defence response resulting in either mechanical or biochemical defence mechanisms (Nürnberger and Brunner, 2002). These chemical structures are known as elicitors.

With agricultural pests, synthetic pesticide usage over the past few decades has proven to be very effective in controlling and managing pests that would otherwise have had a damaging effect on crop yields and production. However, the continual use of these chemical pesticides has seen a breakout in resistance from pests, detrimental effects to non-target organisms and an overall degradation to the environment (Bocquené and Franco, 2005; Coat *et al.,* 2006).
The breakaway from modern pesticides use towards naturally derived products has become a common trend for many developed countries. The use of biopesticides has gained renewed interest by many European and North American governments as an alternative for pest management due to heightened awareness of environmental, health and pollution impact posed by synthetic pesticides (Hynes and Boyetchko, 2006; Thakore, 2006).

It was once widely considered that biopesticides fall under a niche market of agrochemical products with a low possibility of new developments due to their application not fitting into the traditional "fast killing" mode of action (Waage, 1997; Greaves, 2009). However, now with major global stake holders and policy makers having taken a keen interest in robust sustainable development practices, market trends into the entire global pesticide market have shown an increase in biopesticide trade and a steady decline in the synthetic chemical pesticide markets (Thakore, 2006). This shows greater farmer acceptance of these natural products having efficacy in safer pest control and conventional crop management methods (Thakore, 2006). Greater farmer acceptance of biopesticide use is also apparent with current trends towards

"organically" produced food growing at rapid rates in developed countries. Within the European Union, the area of organic cultivation spans 7.6 million hectares with an increase of 7.4 % a year (European commission Directorate General, 2013).

Plants possess the ability to produce active compounds (secondary metabolites) that are of keen interest in numerous industries based on their properties. More than 100 000 chemical types have been reported which indicate a large diversity (Hadacek, 2002). These compounds include a vast array of phytochemicals that may function in the defence mechanisms of a plant by either switching on defence signals or by deterring pests and pathogens with their toxic capabilities (Edreva *et al.,* 2008; Bartwal *et al.,* 2012; Bednarek, 2012).
In relation to deterrence of pests, repellents are of keen interest as they may act locally or at a distance in achieving a deterrence effect on an arthropod by limiting its ability to act on a surface. Natural repellents have been favoured over the past few decades as a new source of eco-friendly tactics in deterring arthropods possibly due to the low toxicity levels, good efficacy and consumer awareness of the dangers of synthetic products (Katz *et al.,* 2008).

In South Africa, the Russian wheat aphid (*Diuraphis noxia* Kurdjumov, Order Hemiptera; Family Aphididae) has long been regarded as a troublesome pest of cereal grains and this is possibly due to its lack of natural enemies in the region (Aalbersberg *et al.,* 1989).
The *Diuraphis* sp. has been documented for its ability to survive on alternate hosts such as wild grasses making them adaptable to periods of change in seasonality (Weiland *et al.,* 2009; Jankielsohn, 2013). Studies on biological control using generalist predators e.g. carabids (*Pterostichus cupreus* L.) of cereal aphids in the early growing season showed a significant decrease in aphid densities. However, later in the growing season where conditions are optimal for aphids, the same application proved to be insufficient as aphid density peaks were still high enough to have damaging effects on crops (Brewer *et al.,* 2013).

Moreover, the application of synthetic insecticides has shown disadvantages for many farmers as it, firstly is too costly and secondly, over time, insects develop resistance towards the insecticides (Thomas and Waage, 1996). A partially successful management strategy thus far for aphids has been resistant breeding of wheat that work in tandem with host plant resistance bred into bread quality wheat. The resistant genes *Dn*1, *Dn*2 and *Dn*5 (*Dn* denotes *Diuraphis noxia* resistance) were ear-marked and used as potential resistance sources in breeding of wheat lines that were resistant towards aphid biotypes (Tolmay *et al.,* 2007). The main issue of

concern with breeding for resistance is the aphid's ability to break resistant lines and rearing susceptibility with the emergence of a new aphid biotype (Stoner, 1996). This has been well documented in *Schizapus graminum* on wheat (Thomas and Waage, 1996). Resistant breaking biotypes are a common trend in aphid populations and four biotypes have developed since the first documented Russian wheat aphid (RWA) biotype discovery in South Africa in 1978 (Walters *et al.,* 1980; Jankielsohn, 2014). Control of this pest is of great concern as it can cause yield losses of 21% to 92% (Kiplagat, 2005).

This study was undertaken with the aim of finding more environmental friendly ways to protect wheat plants from attacks by the RWA and a better understanding of the mechanisms of natural plant substances as botanical insecticides, repellent products and 'plant activators' in the resistance response of wheat in general. *Artemisia afra*, *Agave attenuata* and *Zanthoxylum capense* were the plants chosen to carry out our objectives. These plant species have been well documented in literature as potential sources of bioactive compounds that range from being antimicrobial, larvicidal and antimycobacterial (Graven *et al.,* 1992; Brackenbury and Appelton, 1997; Masoko and Nxumalo, 2013). Plant-derived substances have shown to be biologically effective in either repelling or killing insects with their application (Rattan, 2010). It is worth considering that plant extract applications may provide a possible solution in controlling the RWA by enhancing resistance in wheat plants that will deter the insect. Therefore, the aim of this study was to measure the effect of plant extracts on wheat plants and their possible role in enhancing resistance through repellency, insecticidal or through the induction of a defence response.

Objectives:

1. General identification of polar and non-polar compounds of *Agave attenuata*, *Zanthoxylum capense* and *Artemisia afra* using thin layer chromatography with different detection sprays and gas chromatography coupled with mass spectroscopy.
2. Insecticidal properties of these extracts.
3. Screening for aphid responses to the odours of the extracts and essential oil, using a four-arm olfactometer.
4. Screening for aphid / plant acceptance with a no-choice aphid-settling test on plants sprayed with the polar, non-polar and essential oil extracts under glasshouse conditions.
5. Assessing the phenotypic damage on infested wheat plans after treatment with extracts and essential oil.

6. The *in vitro* activities of β-1,3-glucanase and peroxidase enzymes isolated from intercellular wash fluid from infested and non-infested susceptible and resistant wheat, treated with *Ar. afra* and *A. attenuata* extracts and essential oil under glasshouse conditions.

For all experimental purposes, the Russian wheat aphid South African Biotype 1 (RWASA1) was used.

Chapter 2
Literature review

2.1 Origin and distribution of the RWA

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphidadae) is native to southern Russia, Afghanistan, Iran and other countries bordering on the Mediterranean. From its first detection in 1901 in Crimea, the aphid has spread to other parts of the world (Fig. 2.1) and has manifested its role as a troublesome pest in parts of Asia, Southern Africa, North America, South America, Northern and Eastern Africa and central Europe (Walters *et al.*, 1980; Gilchrist *et al.*, 1984; Lukasova *et al.*, 1999; El-Bouhssini *et al.*, 2011; Ngenya *et al.*, 2014).

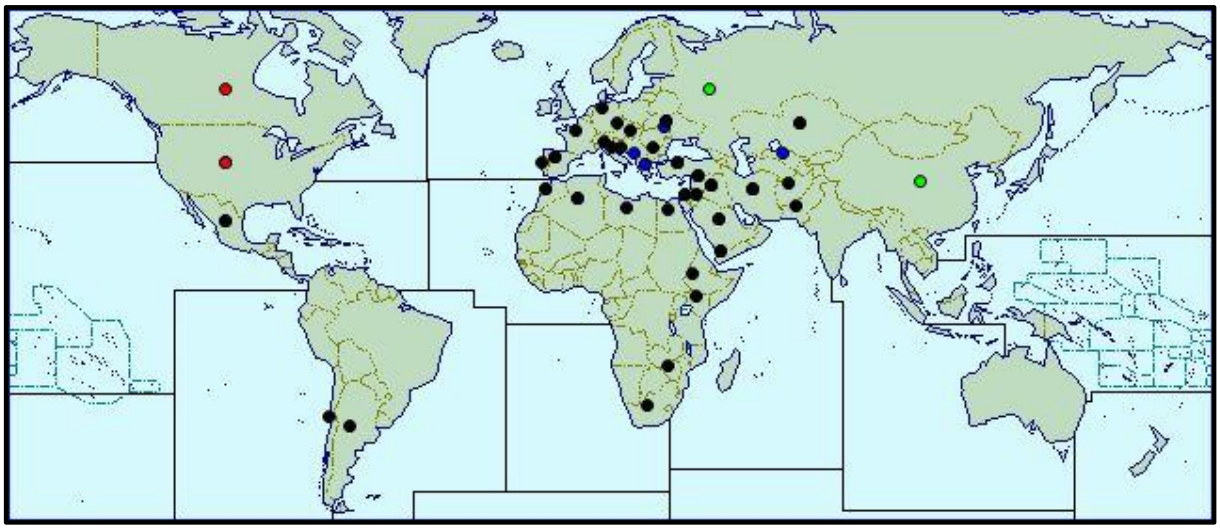

Figure 2.1: Distribution of RWAs in different parts of the world (http://www.cabi.org/isc/datasheet/9887).

In South Africa, from its first detection in 1978 in the Eastern Free State region, the RWA has managed to acclimatise itself in other parts of the country such as the Western Cape where wheat is also abundant (Walters *et al.*, 1980; Jankielsohn, 2014). Four RWA biotypes exist in South Africa and are scattered throughout the different regions (Fig. 2.2).

Dispersal of aphids and their successful distribution in an environment can be attributed to a sum of key factors including a short development time, polyphenism and clonal reproduction (Lombaert *et al.*, 2006). Polyphenism is a form of phenotypic plasticity that allows for two distinct phenotype variations with no intermediate form from the same genotype (Nijhout, 1999). In aphid populations this is observed with the apterous and winged forms existing within the same clonal population (Lombaert *et al.*, 2006). Polyphenism in aphids is largely driven by

environmental conditions that dictate the availability of food from particular farming styles and the large scale movements of winged aphids increases the dispersal capacity to find new and profitable environments (Miller and Pike, 2002).

Crops only provide adequate resources for a limited period of the year and phytophagous insects such as the RWA make use of alternate hosts during periods where ideal hosts (crop plants) are not present (Kindler and Springer, 1989; Weiland *et al.*, 2009). The alternate hosts are mainly grass species of which RWA makes use of during periods of over summering and when fewer resources are available (Weiland *et al.*, 2009). This in turn make RWA survivorship a little more robust compared to other crop insects.

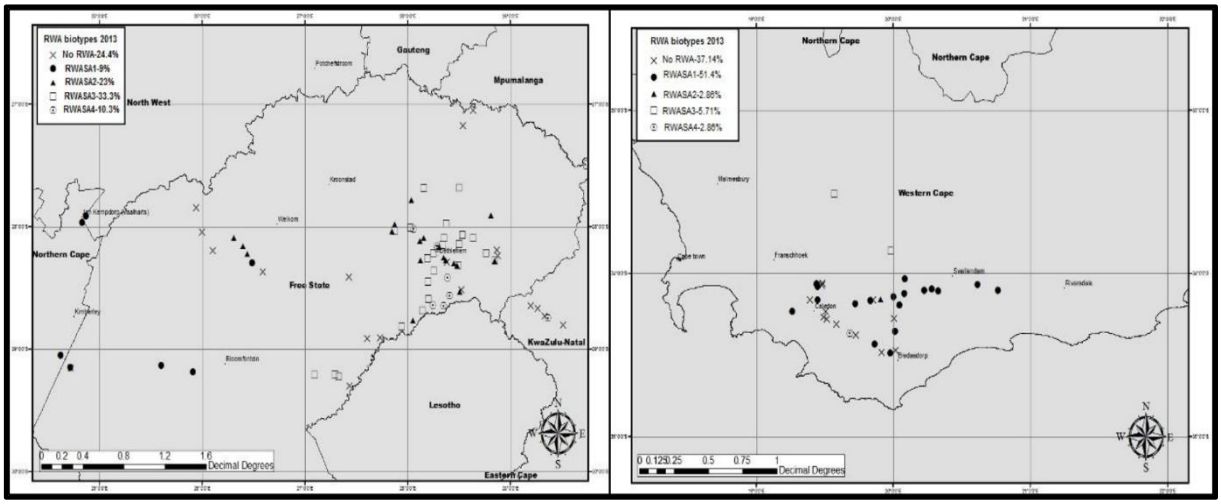

Figure 2.2: Distribution of the different South African RWA biotypes in South Africa (Jankielsohn, 2014).

2.2 Environment and suitability

The RWA thrives in dry hot conditions but has been recorded to survive in conditions of below -20°C (Butts, 1992). The production of nymphs from the females has shown to increase in a typical lifecycle when an increase in temperature between 5 and 20°C occurs (Michels and Behle, 1988). The aphid has also been recorded to survive heavy rain periods (Araya, 1991).

2.3 Mode of feeding

Aphids typically feed at the base of newly formed leaves and on the inflorescence of barley (*Hordeum vulgare* L.) and wheat (Kindler and Springer, 1989). Unlike grazing insects that remove large portions of plant tissue, the aphids inflict minimal plant damage as they possess a flexible stylet that penetrates into the leaf area, finding its way to the phloem sieves via an intercellular route where the withdrawal of assimilates occurs (Miles, 1999).

When the stylet penetrates into the host tissue, the aphid secretes two types of saliva, a proteinacious gel saliva and a watery saliva (Giordanengo *et al.,* 2010). The secretion of both types of saliva does not occur simultaneously and is timed by the movement of the stylet entry into the host tissue and the start of the ingestion phase (Tjallingii and Hogenesch, 1993; Prado and Tjallingii, 2007). The secretion of the gelling saliva forms a supportive sheath around the stylet and seals off surface sites where the stylet has punctured reducing further damage to host tissue and disarming the plants defence (Will *et al.,* 2007). Once the stylet reaches and penetrates the sieve cell, the watery saliva is released (Prado and Tjallingii, 2007). The watery saliva may contain enzymes such as cellulase, polyphenoloxidase, glucose-oxidase and pectinase that allow for breakdown of cell walls, change in cellular redox levels and sequestering toxicity of defensive secondary metabolites such as phenolic compounds and induction of systemic responses (Peng and Miles, 1991; De Bruxelles and Roberts, 2001; Divol *et al.,* 2005).

The piercing of sieve elements for the assimilation of food substances at the source-sink site results in a general response of phloem occlusions (Knoblauch and van Bel, 1998). Occlusions are blockages that prevent loss of valuable material. Occlusions in phloem makes use of two mechanisms; callose depositions in sieve pores and phloem protein plug formation in sieve plates (van Bel, 2006). Both mechanisms are reversible if the damage to the sieve element is not detrimental (Knoblauch *et al.,* 2001; van Bel, 2006; Furch *et al.,* 2007). Occlusions are a plant's general response to prevent sap loss and both mechanisms are associated with an influx of Ca^{2+} ions in the cell (Furch *et al.,* 2007).

The Ca^{2+} channels are mechano-sensitive and the puncturing effect of the stylet into the sieve cells activates this influx of Ca^{2+} ions (Will and van Bel, 2006; Will *et al.,* 2007). Calcium ions are an important component in many signal transduction pathways and aphids may counteract

this by always injecting watery saliva into sieve elements immediately after penetration (Prado and Tjallingii, 1994; Eckardt, 2001). The saliva (watery) components (proteins) may compete for free Ca^{2+} and may in turn prevents occlusions and allow aphids to feed from one sieve element for a longer period (Eckardt, 2001; Knobluach *et al.*, 2001; Will *et al.*, 2007).

2.4 Description and damage caused on host plants

Diuraphis noxia is a pale green, spindle shaped arthropod with a length of approximately 2 mm. It has a characteristic supracaudal process, short antennae that distinguishes it from other aphids and has a double tail feature (Walters *et al.*, 1980). When the RWA feeds on a suitable host appropriating susceptibility, it releases a toxin that causes symptoms such as longitudinal leaf rolling caused by the inhibition of chlorophyll production, yellowing at leaf tips and white streaks (hot weather) or purple streaks (cool weather) running across the leaf surface (Walters *et al.*, 1980; Kazemi *et al.*, 2001).

Heavily infested plants are flattened and their growth is stunted. The damaging effects of infestation are more prominent in the seedling stages, however if infestation occurs later in a mature plant, one can expect the flag leaf to curl and the head of the wheat plant never fully emerges causing poor grain maturation (Peairs, 1998; Akhtar *et al.*, 2010). The presence of aphids on wheat plants affects the overall quality which leads to a lowered grain yield.

2.5 Host plant resistance

According to Thomas and Waage (1996), there are three known functional categories explaining host plant resistance which include:

1. Tolerance: plants can survive under levels of infestation that will kill or severely injure susceptible plants.
2. Antibiosis: plants produce secondary compounds with defensive capabilities that protect them from herbivores. These compounds may reduce growth, alter physiology, delay maturation or induce various physical or behavioural abnormalities in herbivores. With insects, resistant plants are able to affect the biology of the insect.
3. Antixenosis: plants possess physical or chemical properties making them unpalatable to herbivores, this usually involve feeding repellents. This is a non-preference type of

resistance and may involve physical traits such as waxes or tough epidermis that does not provide the pest with a desirable substrate.

All three strategies play a role in making the plant highly resistant to the RWA (Webster *et al.,* 1987; Miller *et al.,* 2003).

2.5.1 Categories of plant defence

Plants possess innate immunity and therefore rely on defence response mechanisms for their protection (Odjakova and Hadjiivanova, 2001). The activation of the defence response is similar for all invaders including pathogens, bacteria and herbivore attacks (Walling, 2000). Plants have the ability to detect the presence of microorganisms and herbivores through an intricate surveillance system that recognises either microbial-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), mechanical damage caused by abiotic or biotic wounding or the presence of elicitors secreted by phytophagous insects into host cells (Dangl and Jones, 2001; Brunissen *et al.,* 2009). The perception of a pathogen or insect threat is a prerequisite for the activation of a defence response by a host plant.

2.5.1.1 Basal defences

Basal defences occur in both resistant and susceptible host plants and may include traits like cell wall modifications and emission of plant volatiles that attract or repel aphids (Goggin, 2007). Basal defences can be viewed in both a qualitative and quantitative manner. In a quantitative manner, basal defences allow a plant to overcome any form of attack by a pest or pathogen that can cause heavy infestation or spread of infection (Dangl and Jones, 2001). In a qualitative manner, basal defences refer to the inability of a pathogen to overcome a plant's defence mechanism due to its unadaptability to a host plant (Heath, 1997; Lipka *et al.*, 2005). Basal defences ultimately lead to casade of signalling molecules that give the required defence response.

2.5.1.2 Non-host resistance

Non-host resistance is regarded as the most durable form of resistance a plant can employ against an invader (Nürnberger and Lipka, 2005). This particular type of resistance is thought to rely on a multiple protective layer that makes use of constitutive barriers and inducible reactions (Mysore and Ryu, 2004). Structural barriers or preformed barriers are the first obstacle a pest or pathogen has to overcome before it can invade a host plant. In the case of grazing insects, structural defence is an important component employed. Structural defence by definition is avoidance or tolerance strategies employed by plants that deter herbivore feeding (Hanley *et al.,* 2007). Morphological characteristics like spinescences (thorns, prickles and spines) deter herbivore feeding through physical contact and in some instances, their removal result in greater feeding by the herbivore on the plant of choice (Wilson and Kerley, 2003). In the case of fungal penetration into a host plant cell, the cytoskeleton plays an important role in the integrity of resistance towards fungi and when affected, non-host resistance is greatly compromised (Yun *et al.,* 2003). Constitutive barriers such as the cell wall not only provide mechanical defence to microorganisms (fungi) but also play an important role in the synthesis of active compounds that activate defensive genes (Narváez-Vásquez *et al.,* 2005).

The inducible reactions include the synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, PR-proteins as well as the strengthening of the preformed barriers like the cell wall (Nürnberger and Lipka, 2005). Two types of non-host resistance exist and a plant may utilize either one in defending itself. Type 1 non-host resistance is symptomless (no hypersensitive reaction; HR) and a pathogen is unable to overcome the first obstacle being the preformed barriers e.g. cell wall, secondary metabolites and antimicrobial products (Mysore and Ryu, 2004). The non-host pathogen may still however possess general elicitors that result in *PR* gene expression in a host plant through systemic acquired resistance (SAR) leading to a defence response (Lu *et al.,* 2001). Type 2 non-host resistance shows a HR response symptom and is the most widely considered type of non-host resistance. In Type-2 non host resistance, a pathogen overcomes the preformed barriers described above and penetrates into the host cell and specific elicitors are recognized by the plants surveillance system and a defence reaction leading to HR is employed (Mysore and Ryu, 2004). Cell death is a common feature with this type of non-host resistance (Mysore and Ryu, 2004). Signalling pathways are triggered by the ability of a plant to detect the invader (pathogen or pest) by elicitors through the plant-pathogen

interaction. Elicitors are molecules that have the ability to induce a defence related response (Ebel and Cosio, 1994).

2.5.1.3 *R*-gene mediated resistance

Resistant-gene mediated defence responses rely on the genotype of a plant to recognise a particular pathogen that has overcome non-host resistance. The genes are characterised by a nucleotide binding leucine-rich repeat (NB-LRR) motif that allows for disease resistance by the recognition of specific pathogen effector proteins (Chisholm *et al.,* 2006). The interaction of the *R*-gene and avirulent gene products from plant and pathogen is highly specific and this leads to disease resistance (Jones and Dangl, 2006). A co-evolution exists between plant and pathogen interaction and disease resistance is governed by the plant's ability to evade pathogen success over time. The effector-triggered immunity (ETI) results from this interaction and leads to an increased defence response, if not, effector-triggered susceptibility is employed (Fig 2.3) (Jones and Dangl, 2006). Cloning of *R*-genes into plants has led to the understanding that plants with specific *R*-genes may play a defensive role to unrelated pathogens that share a common motif (Hammond-Kosack and Jones, 1997). The binding of this effector to the *R*-gene product results in the signalling of a cascade network that results in defence response that would be associated with basal defences in susceptible genotypes (Goggin *et al.,* 2001). *R*-gene mediated defences are often associated with the HR response (Jones and Dangl, 2006). This particular type of defence has allowed for the cloning of specific genes in susceptible genotype that would now be able to confer resistance to both pathogen and insects in the case of the *Mi-1.2* gene in tomatoes (Rossi *et al.,* 1998).

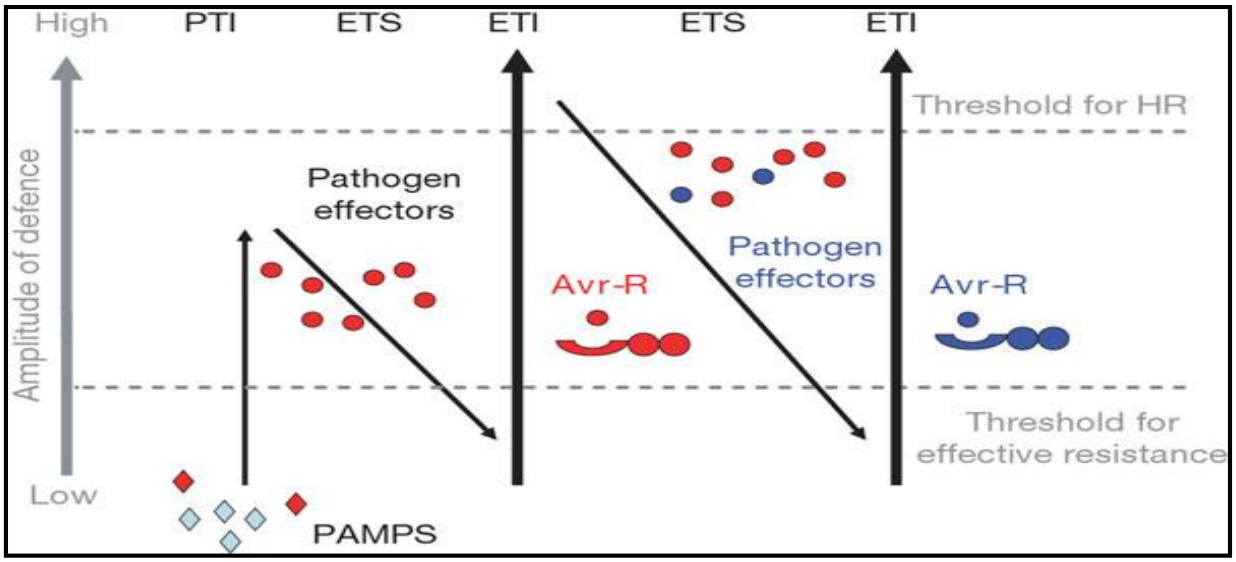

Figure 2.3: Zigzag model of plant pathogen interactions (adapted from Jones and Dangl, 2006). Avr-R – Avirulence resistance-resistance-protein interaction, PTI-Pattern-triggered immunity, ETI-effector-triggered immunity, ETS-effector-triggered susceptibility, HR – hypersensitive response, PAMPs – Pathogen-associated-molecur-patterns. Model describes interaction of two levels of plant immunity (PTI and ETI).

2.5.1.4 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins are low molecular weight water soluble proteins that play an indicative role in the defence response in plants against both biotic and abiotic stresses (Van Loon *et al.,* 1998). These particular proteins are localised in vacuoles, cell walls and chloroplasts (Payne *et al.,* 1990). According to Van Loon *et al.* (1998), there are approximately 19 families of PR-proteins that exist in the plant kingdom. Pathogenesis-related proteins may be coded by host plants as a response to local attack but they can also be formed following any kind of infection (Scherer *et al.,* 2005).

Glucanases belong to the PR-2 family and their expression can result from different circumstances such as fungicide treatment, pathogen invasion or insect infestation (Siefert *et al.,* 1996; Van der Westhuizen *et al.,* 1998b; Gupta *et al.,* 2012). Most of the glucanases in plants are endo-glucanase which are responsible for the production of glucan oligomers from callose (Stintzi *et al.,* 1993). Callose is produced by transmembrane proteins and its accumulation is the result of disruptions within the cell wall components. Glucanase is responsible for its removal (Jacob and Northcote, 1985; Delmer, 1987). Glucanase plays a major role in many of the plant's physiological processes including fruit maturation, cereal

germination and flower development (Hinton and Pressey, 1980; Stuart *et al.*, 1986; Hoj *et al.*, 1989; Neale *et al.,* 1990; Ori *et al.*, 1990). In the defence response, β-1,3-glucanase can turn glucan oligomers into soluble forms that can elicit a defence response (Darvill and Albersheim, 1984). Pathogenesis-related proteins can therefore either generate signal molecules or actively play a mechanical role in the defence response. Chitinases (PR-3) and glucanases (PR-2) have been shown to possess antifungal activity (Mauch *et al.,* 1988).

Peroxidases are a well known class of PR-proteins belonging to the PR-9 sub-family and have been documented to play a role in the downstream defence responses (Van Loon *et al.,* 2006). Plant peroxidases are glycoproteins that are located in vacuoles and cell walls (Passardi *et al.,* 2005). The locality of peroxidases at the cell wall is advantageous as this PR protein is highly associated with cell wall lignification and suberization in response to wounding or attack from a pathogen through its involvement in the production of H_2O_2 (Almagro *et al.,* 2009). The production of H_2O_2 creates a highly toxic environment that prevents further spread of infection (Passardi *et al.,* 2005). The reinforcement of cell walls through lignification and suberization protect above and below ground structures respectively (Ros-Barcelò, 1997; Bernards *et al.,* 2004). In addition to its defensive role, peroxidases play other crucial physiological roles due to their different enzymatic isoforms and versatility (Passardi *et al.,* 2005).

2.5.1.5 Hypersensitive response

When the pre-existing physical and chemical barriers of a host plant are no longer efficient in a plant's defence efforts against an invader, a plant will employ a natural inducible defence response against that invader, leading to intricate signalling pathways that will restrict further damage and allow for the plant to survive (Morel and Dangl, 1997; Mur *et al.,* 2008). This is known as the hypersensitive response (HR). The HR is characterized by the rapid formation of 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 the 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 signalling molecules, for instance, nitric oxide, salicylic acid (SA) and jasmonic acid (JA) (Garcia-Brugger *et al.,* 2006). After HR activation, unaffected distal parts of the plant may develop resistance through systemic acquired resistance (SAR) through

signalling molecules that lead to defence reactions that include the production of reactive oxygen species, biosynthesis of phytoalexins and PR-proteins, lignification and strengthening of the cell wall (Hammond-Kosack and Jones, 1996; Thakur and Sohal, 2012). Salicylic acid is responsible for triggering the expression of defence genes encoding certain PR-proteins. Also, antimicrobial phytoalexins accompany HR to further prevent infection by pathogens (Hahlbrock and Scheel, 1989).

The first response to pathogen invasion is an oxidative burst that results in the accumulation of reactive oxygen species in a cell that leads to the HR (May *et al.,* 1996). These attack the invading pathogen and are extremely toxic to cells. Plants possess radical detoxifying enzymes (superoxide dismutase, catalase and peroxidases) and non-enzymatic antioxidants (ascorbate, glutathione, tocopherol and phenolic compounds) that protects the plant from oxidative damage at the sites of ROS generation (Ahmad *et al*., 2008; Thakur and Sohal, 2012). Hypersensitivity is the localization of a pathogen by the death of a limited number of host cells that restricts the pathogen from invading any further to other cells (Passardi *et al.,* 2005).

In plant-insect interactions, HR-based resistance has been reported for piercing and sucking insects (Walling, 2000). Gall-inducing insects such as *Dasineura marginemtorquens* appear to have an HR type response on host plants by showing rapid cell death, accumulation of phenolics, induction of ROS compounds and SA accumulation (Dangl *et al.,* 1996; Ollerstam *et al.,* 2002; Ollerstam and Larrson, 2003). A gene-for-gene interaction has been identified as a possible mediator for the hypersensitive response with the bluegreen aphid (*Acyrthosiphon kondoi* Shinji) in *Medicago truncatula* (Klinger *et al.,* 2009). Insects that deposit their eggs on host plants induce a HR response similar to that of pathogens (Fatouros *et al.,* 2014). *Pieris rapae* egg deposition showed the expression of *PR-1* gene and a HR necrosis symptom was visible on *Brassica nigra* plants (Fatouros *et al.,* 2014). Resistant plants have shown a HR response as in the case of barley towards RWA (Belefantmiller *et al.,* 1994). The RWA resistance response conveyed by the incorporated *Dn* resistance genes has shown not to be a wounding response, but a HR response.

2.5.1.6 Signalling and priming effect

Within a plant, a cross network of communication exists and once a plant has activated its defence response locally, systemic resistance is the next step in enhancing its resistance by spreading of the defence efforts to uninfected parts of the plant (Kunkel and Brooks, 2002; Jung *et al.,* 2009; Thakur and Sohal, 2012). Early events in the perception of invaders by a plant generate many events that either directly or indirectly interconnect different signalling pathways that will lead to metabolic changes through a specific physiological response (Zhou *et al.,* 2005). The type of interaction between a plant and its invader plays a major role in influencing the response and which pathway will be activated. Signalling molecules include jasmonic acid, salicylic acid and ethylene (ET) (Kessler and Baldwin, 2002; Durrant and Dong, 2004).

Two types of systemic resistance responses occur, namely systemic acquired resistance and induced systemic resistance. Both types regulate resistance responses to distal parts of the plant, however they employ different mechanisms to achieve the required response. Systemic acquired resistance (SAR) makes use of SA and is associated with *PR* gene expression (Van Loon *et al.,* 2006). Induced systemic resistance shows no connection to SA or *PR* gene expression however it relies more on JA and ET for its response (Pieterse *et al.*, 1998).
Jasmonic acid and its ester, methyl jasmonate form part of the jasmonates. Jasmonic acid is a plant hormone that may be responsible for the production of secondary metabolites in response to insect feeding (Farmer *et al.,* 2003; Halitschke and Baldwin, 2005). Pathogen-associated molecular patterns and wounding have the ability to induce JA signal transduction towards distal parts of the plants for the required defence response (Turner *et al.,* 2002; Atkinson and Urwin, 2012). The jasmonic acid and ethylene defensive pathways seem to be activated by necrotrophic interactions whilst salicylic acid pathways are activated by biotrophic interactions (Lecourieux-Ouaked *et al.,* 2000; Spoel *et al.,* 2003). Signalling can be induced by phloem-feeding insects such as the RWA and this was documented with ROS accumulation in infested resistant wheat lines (Moloi and van der Westhuizen, 2006).

Priming can be described as an enhanced and augmented defence response that results in a faster induced plant defence response prior to an attack (Goellner and Conrath, 2008). Plants that are primed thus exhibit a stronger activation of an inducible defence response and can be induced either biologically or chemically. Biological priming results from localised attack by a

biological organism (pathogen) that can elicit SAR to distal parts of a plant that are uninfected (Durrant and Dong, 2004). Plant tissue that is affected by the pathogen produce a systemic signal that is transported to unaffected parts for SA-dependent defences (Jung *et al.,* 2009). Priming of an induced systemic response can also occur in plants through the presence of a non-pathogenic organisms such as *Pseudomonas fluorescens* that is dependent on the NPR1 pathway (Pieterse *et al.,* 1998). Plants exposed to beneficial microorganisms respond more efficiently and quicker to an attack as opposed to those that were not exposed. This has been well documented in tomato plants colonized by mychorhizal fungi that showed significantly more PR-protein accumulation after an attack by *Phytophthora parasitica* compared to those tomato plants that were not colonized by mychorhizal fungi (Cordier *et al.,* 1998). This state of enhanced resistance is not only limited to exposure to beneficial microorganisms but treatments with plant activators in low doses can also prime a plant (van Hulten *et al.,* 2006).

Plant activators are chemical inducers that systemically induce a defence response and can be categorised into two types, biological and chemical inducers. Their application allows for a broad-spectrum disease resistance response as SAR is employed (Yoshida *et al.,* 2010). Signalling molecules such as SA and JA can be considered as natural plant activators as they have the ability to induce a defence response by limiting disease symptoms through the activation of defence-related genes (Potlakayala *et al.,* 2007; Lahlali *et al.,* 2014). Chemically induced priming is caused by synthetic analogs that can mimic biological forms of priming through exogenous application of the synthetic compounds. Treatment of plants with different chemical stimuli plays a positive role that can either initiate ROS build up, cause callose depositions or induce expression of SA and JA-inducible genes (Zimmerli *et al.,* 2000; Sauerborn *et al.,* 2002).

In *Arabidopsis*, treatment with β-aminobutyric acid (BABA) resulted in callose depositions and SAR priming expression (Zimmerli *et al.,* 2000). Beta-aminobutyric acid is active at low concentrations and can help a plant to defend itself against a vast number of diseases (Jakab *et al.,* 2001). *In vitro* application of BABA, inhibited spore germination of *Penicillium italicum* in *Citrus senensis* (Tavallali *et al.,* 2008). Benzothiadiazole (BTH) is another chemical stimulant that leads to numerous defence related responses, including an increase in phenolic content in *Brassica juncea*, synthesis of phytoalexins and PR-proteins which prevented infestation of a parasitic weed *Orobanche cumana* in *Helianthus annuus* (Sauerborn *et al.,* 2002; Guleria and Kumar, 2006). Benzothiadiazole has also been used on wheat to treat

powdery mildew infection which resulted in the induction of wheat genes that encode for a lipoxygenase protein (Görlach *et al.,* 1996). Probenazole applied to rice induces the accumulation of salicylic acid conferring resistance to *Magnaporthe grisea* (Iwai *et al.,* 2007).

The application of plant activators in crop management strategies has numerous advantages that can improve a plant's health in an environment by lowering disease outbreaks, attraction of natural predators and reduced insecticide use and environmental hazards due to their low toxicicty (Freeman and Beattie, 2008). The initiation of defence responses by activators is another step in promoting natural-based immunity (von Rad *et al.,* 2005). When SAR is initiated, higher levels of PR-proteins accumulate within the plant and the hypersensitive response is employed (Odjakova and Hadjivanova, 2001).

Herbivore attack can also result in a priming response through the release of volatile organic compounds that may attract natural enemies of the herbivore (Kishimoto *et al.,* 2005). Volatile organic compounds (VOCs) at high concentrations also have the ability to induce systemic responses in plants and is associated with JA (Ton *et al.,* 2007; Frost *et al.,* 2008a). A subset of VOCs known as herbivore-induced plant volatiles are released in the presence of herbivore attack and the signals are known to have short-transmission distances between intact plants or neighbouring intact-plants (Kim and Felton, 2013). Volatiles produced in response to herbivore and insect feeding, which also include green leaf volatiles (GLV) are capable of priming defences that may include accumulation of secondary metabolites and JA (Frost *et al.,* 2008b; Hirao *et al.,* 2012). The priming response by GLVs also works through indirect defence responses by attracting enemies of herbivores and this enhances plant resistance (Schuman *et al.,* 2012).

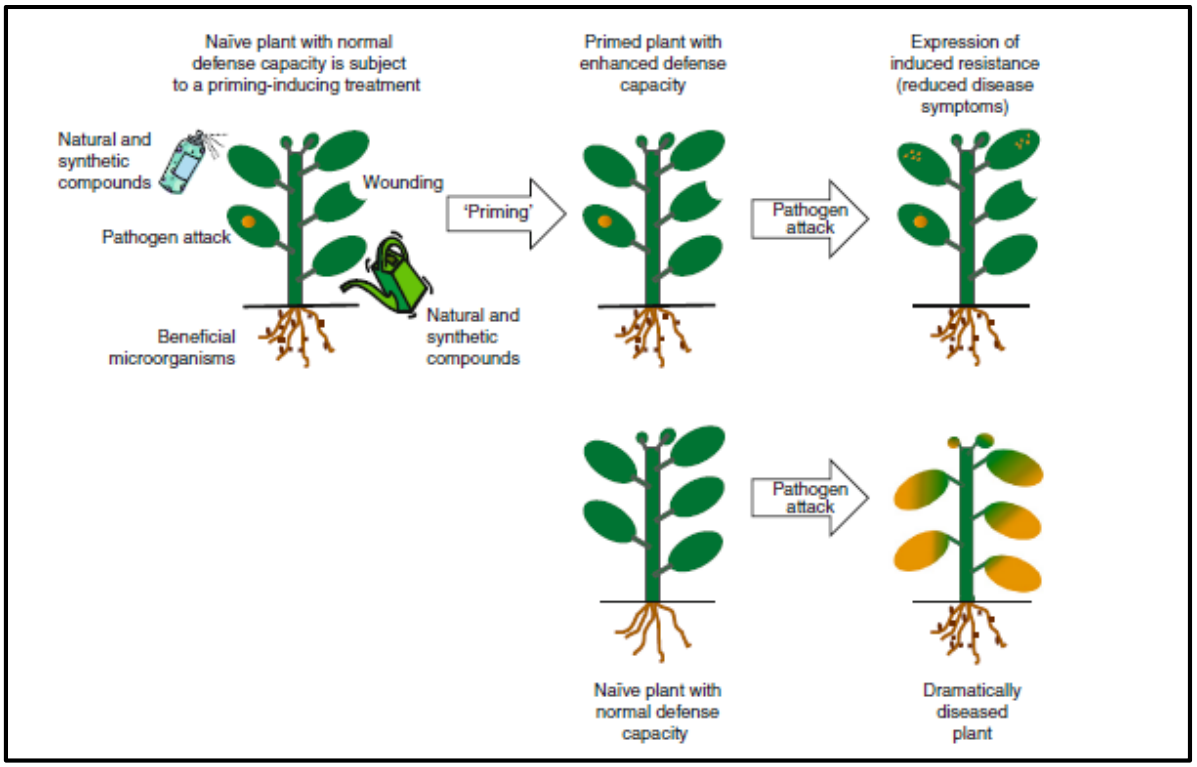

Figure 2.4: A plant's potential response to priming effect (Conrath *et al.*, 2006).

2.6 Control of pests

2.6.1 Biological control of aphids

The biological control of pests in agriculture relies largely on predation, parasitism or the release of pathogens (van Rijn and Sabelis, 2005). The predation of aphids by ladybugs alone is a partially effective measure to control population sizes, however when used in combination with some form of chemical, genetic or cultural method in an integrated pest management system, it proved to be more effective (Du Toit *et al.*, 1987). The parasitism employed by *Diaeretiella rapae* and entopathenomogenic fungi are also examples of biological control used to control aphids (Vu *et al.*, 2007; Silva *et al.*, 2011). Biological control with the natural enemies of the RWA proved not to be successful in susceptible cultivars (Aalbersberg *et al.*, 1989). An understanding of insect-insect interactions requires much more investigation and further research on how it can be properly managed by farmers (Richter, 2010).

2.6.2 Breeding for resistance

Breeding for resistance requires the identification of closely linked markers to resistant genes that can be validated in wheat backgrounds and introgressed into the host plant. Most work done on RWA resistance breeding has made use of microsattelite markers that locate the position of genes of interest. Currently there are 11 resistance genes (*Dn*1-9, *Dn*x and *Dn*y) used to confer resistance towards the RWA (Puterka *et al.,* 2014). A gene for gene interaction has been hypothesised to induce resistance against the RWA and breeding programs make use of these genes by introducing resistant genes in combinations for cultivar development (Boyko *et al.,* 2006).

Because RWA biotypes all over the world interact differently to host plants, germplasm of RWASA biotypes has to be screened accordingly for the possible resistance genes available. In South Africa, the first commercial resistant cultivar released was Tugela *DN* (containing the *Dn*1 gene) that proved successful to the biotype (RWASA1) found at that time (van Niekerk, 2001). The outbreak of new biotypes (RWASA2 and RWASA3) in 2005 and 2011 were virulent to four genes (*Dn*1, *Dn*2, *Dn*3, *Dn*9) and five genes (*Dn*1, *Dn*2, *Dn*3, *Dn*4, *Dn*9 respectively) (Jankielsohn, 2011). A fourth biotype was found in 2011 in South Africa and was reported to be virulent to *Dn*5 (Jankielsohn 2014).

The first North American resistant cultivar released was Halt (containing *Dn*4 gene) which exhibited antibiosis, antixenosis and tolerance towards the RWA (Quick *et al.,* 1996; Hawley *et al.,* 2003; Miller *et al.,* 2003). Some sources for resistance genes include rye and closely related ancestors of wheat (Anderson *et al.,* 2003). The majority of the known genes conferring resistance to RWA's are located on the D genome of wheat and one on the 1RS/1BL translocation (Liu *et al.,* 2001; Anderson *et al.,* 2003). The *Dn*7 gene was introduced in wheat through translocation from a rye chromosome to a wheat chromosome and this particular gene exhibits resistance to many biotypes of RWA's in North America and Africa (Lapitan *et al.,* 2007).

Breakthrough in modern biotechnology and breeding programmes has been a sought after feature as this has provided the best strategy in dealing with the RWA (Boyko *et al.,* 2006). In South Africa, germplasms have provided accession lines to resistant wheat varieties for commercial farmers to combat RWA outbreaks. Germplasms with novel genes provide

different sources of resistance to RWA's and ensures the transfer of genes to local wheat cultivars (El-Bouhsinni *et al.,* 2011). Wild relatives of wheat plants provide to germplasm development and international collaborations provide different accession lines that aid in breeding programmes (El-Bouhsinni and Nachit, 2000). The utilisation of germplasms may target the manipulation of secondary metabolites in crops that provide resistance towards the RWA (Niemeyer *et al.,* 1992). Breeding for resistance has beneficial long term effects and by planting resistant wheat plants, the application of insecticides is avoided. The main problem with breeding for resistance is the aphid's ability to break resistant lines and rearing susceptibility with the emergence of new aphid biotypes (Stoner, 1996).

2.6.3 Chemical control

2.6.3.1 Insecticides and their slow downfall in modern agriculture

Insecticides have long been considered as an alternative strategy in managing pests throughout the world. The application at some stage was only thought to enhance crop yields without having any deleterious effects on the environment, however research in modern agriculture has shown that phosphorous containing products can have an effect on non-target organisms (Riemens *et al.,* 2008). Modern agriculture is faced with ever increasing challenges of food shortage and consumer awareness associated with applications of insecticides. Organophosphate insecticides are the most widely used insecticides in the world (Mansour *et al.,* 2009). The toxic nature and exposure of insecticides on humans has been reported to have many health risks (Clem *et al.,* 1993; Briassoulis *et al.,* 2001; Fenske *et al.,* 2005).

Nicotine alkaloid synthetic derivatives are still used to control pests such as aphids in greenhouses, however their effectiveness is questionable due to their low insecticidal activity and high mammalian toxicity (Nauen *et al.,* 2001). In the case of the RWA, the option of chemical control is greatly limited due to the aphid's feeding habit and positioning itself on the inside of rolled leaves making it difficult for contact with the insecticide (Michaud and Sloderbeck, 2005). For effectiveness, systemic insecticides would be required to be sprayed over the entire plant and this would require larger volumes making it undesirable for farmers. Sytemic insecticides can also negatively affect beneficial nectar feeding insects causing another pitfall of insecticide use. (Stapel *et al.,* 2000). Continual use of insecticides to control pests generally results in pesticide resistance from insect populations over time (Brealey *et al.,* 1984).

The above mentioned, coupled with sustainability practices enforced by many developed states denounces the careless application of broad-spectrum insecticides and increase in the integration of cultural methods and biological control in pest management has been adopted (Wijnands, 1997). Furthermore, the rise in the amount of land currently utilised for organic farming has cut a big portion of the insecticide market share and many statutory bodies have slowly phased out many products (Thakore, 2006). Current trends in research are shifting towards natural product development.

2.7 Biopesticides

The breakaway from traditional pesticides use towards naturally derived products has become a common trend for many developed countries as the goals towards sustainable practices have intensified. During the past decade, a lot of emphasis has been based on creating these natural products and market trends have shown that farmers have adopted the use of these conventional products as alternatives (Thakore, 2006; O'Brien *et al.,* 2009).
This has led to the establishment and research towards biopesticide formulation. Biopesticides are pesticides derived from natural products (Copping and Menn, 2000). They are further characterised as biochemical pesticides and microbial pesticides based on their active ingredient/s (O'Brien *et al.,* 2009).

Microbial pesticides are the most widely used group of biopesticides based on the success of soil-borne bacterium *Bacillus thuringiensis* (Bt) being a very effective biocontrol agent against lepidopteran insects (Srinivasan, 2012). A particular successful story is the effectiveness of microbial pesticides to the destructive diamond blackmoth in Asia and Africa (Iqbal *et al.,* 1996). *Bacillus thuringiensis* has also been incorportated into maize and has proved successful for nearly two decades since commercialization in 1996 (Hutchison *et al.,* 2010). Maize with Bt has shown a decrease in pesticide usage, improved pest management and an increase in yield production worldwide (Christou *et al.,* 2006; James, 2012).

Entomopathogenic viruses have also been commercialized into biopesticides and are effective to various insects on vegatables (Kumari and Singh, 2009). Entomopathogenic fungi have been reported to play a role in controlling insects in tropical environments and may possess ovicidal and larvicidal effects (Ekesi *et al.,* 2002).

Biochemical pesticides include insect pheromones, plant extracts and oils, insect growth regulators and plant growth regulators (O'Brien *et al.,* 2009). Biochemical pesticides differ from conventional pesticides by their mode of action being non-toxic towards non-target organisms (Gupta and Dikshit, 2010). They include substances that interfere with growth or mating or substances that attract or repel pests (Gupta and Dikshit, 2010).

The shift of focus to botanical insecticides is largely due to health and ecological issues posed by their synthetic counterparts. A few factors worth considering with the application of botanical pesticides are the environment, stability of the botanical insecticide, resistance build up towards botanical insecticides, the role of secondary metabolites and the formulation of a natural product (Pavela, 2009). Pests generally build up resistance over time to synthetic insecticides due to their continual use and exposure to the same treatment. Synthetic pesticides target a particular site of a pest and make use of one mode of action unlike phytochemicals. Phytochemicals generally target more than one biological system depending on the insect (Rattan, 2010; Mann and Kaufman, 2012).

Plants produce numerous active compounds in their arsenal of defence that are deterent to pests through fumigation or are toxic substances that may effect normal growth and development of an insect or result in death (Mann and Kaufman, 2012). Botanical insecticides are by definition plant derived insecticides and their role has been under investigation in controlling pest populations (Isman, 2000; Isman and Grieneisen, 2014).

Plants have evolved in such a way that the most successful plant species survive primarily by synthesizing moderately or highly toxic compounds that target specific sites of other biological organisms e.g. insects (Rattan, 2010). These sites include receptor sites, membranes, ion-channels and other cellular components that affect insect physiology (Harborne, 1993; Rattan, 2010). A pest found in its suitable environment usually does not struggle for resources whilst the application of a new botanical insecticide faces a challenge of its long lasting effect in the field. Botanical insecticides are reported to be unstable after some time and they lose their efficacy in the field (Khan *et al.,* 2012). Pyrethins from *Chrysanthemum* sp. have long been used as botanical insecticides and have also been reported to have limited stability in the field due to their sensitivity to light and heat (Koul and Walia, 2009). They are generaly formulated with synergists such as piperonyl butoxide to improve their efficacy (Koul and Walia, 2009).

Since the environment plays a huge role in determining what the efficacy of a botanical insecticide will be, formulation is equally important as to determine the longevity and release of the active ingredients into a particular environment. Micro-encapsulations, blending of compound mixtures, dust formulation and film-forming formulation are a few delivery methods used (Pavela, 2009; Masuda, 2011; Mann and Kaufman, 2012).

One possible advantage natural pesticides offer as opposed to their synthetic counterparts is their broad spectrum ability to play a dual role of being both a repellent or antimicrobial agent and insecticide (Rattan, 2010). Limonene oil has been reported to possess such activities (Lindgren *et al.,* 1996; Liuk *et al.,* 1999). It has been established that novel secondary metabolites have the ability to reduce the biological fitness of insects by targeting different biological systems through their functioning (Isman, 2006).

2.7.1 Repellent products and their mechanism

Repellent products can act locally or at long distances by affecting an arthropod's olfactory receptor neurons (ORN) or their gustatory receptor neurons through contact action (Dickens and Bohbot, 2013). The mechanism employed on ORN can either block sites that serve as attractants for example the application of N, N-diethyl-3-methylbenzamide (DEET) on human skin that changes the volatiles being released in repelling mosquitoes (Syed and Leal., 2008). The effects on gustatory receptor neurons work as feeding deterrents that require direct contact with a surface and mediates avoidance behaviour by an arthropod (Schoonhoven and van Loon, 2002). Essential oils and terpenes from different plant species function as repellents to different arthropod's (Nerio *et al.,* 2010). Plant-derived volatiles may act as repellents by stimulating neurons in arthropods that detect non-host semio-chemicals (Guerrero *et al.,* 1997). This feature can also be induced by herbivore attack where plants change their volatile emission profiles to mimic non-host plants and avoid further insect colonization on that particular plant (Pickett *et al.,* 2003). Through contact interaction with a plant, repellency is achieved by antifeedant mechanisms e.g. in the case of the bean aphid, *Megoura crassicauda* which can distinguish between its host and non-host plant by detecting specific chemicals that are highly abundant and mask feeding stimulants (Ohta *et al.,* 2006). Antifeedants such as alkanoic acids are effective against the pine weevil, *Hylobius abietis* (Månnson *et al.,* 2006).

2.8 Wheat in South Africa

Wheat is an important cereal grain primarily used for human consumption whilst the poorer quality grain is used as animal feed (Breitenbach and Fenyes, 2000). In South Africa, wheat is the second largest grain produced after maize, however due to various socio-economic factors, the most prominent contributing factor being population size increase, the demand for wheat in South Africa has increased and in meeting such demands, South Africa has now become a net importer of wheat (Breitenbach and Fenyes, 2000; Baiyegunhi and Sikhosana, 2012). Wheat belongs to the family *Poaceae* and the main cultivated type is bread wheat which can either be cultivated in the autumn months or spring months (Kiplagat, 2005). In South Africa, wheat is cultivated throughout the major provinces; however the major regions of cultivation are the Western Cape and Free State (Fig. 2.5) which bear the summer rainfall patterns producing spring wheat.

2.8.1 Factors in declining wheat production

Abiotic stress factors such as cold, drought, salinity and nutrient stress have a significant effect on world agriculture and it has been proposed that they result in yield loss of more than 50% of beneficial crops (Wang *et al.,* 2003). Drought is a polygenic environmental stress that plays a significant role in wheat development and has been identified as a key factor resulting in grain yield loss and underdeveloped plants (Killic and Yagbasanlar, 2010). Many factors can affects a plant's tolerance to water stress including the duration of stress, the plant's growth stage and the severity of drought (Chaves *et al.,* 2014).

Salinity has been reported to affect the production of wheat in many of the major growing wheat regions of the world (Turki *et al.,* 2012). A major problem associated with salinity includes its affect on soil fertility and the certain characterisics of wheat such as yield and quality are affected by this (Asgari *et al.,* 2012; Turki *et al.,* 2012). Increase in salt stress affects early seedling growth in wheat and this in turn affects the yield (Rahman *et al.,* 2011).

With biotic stress, disease prevalence and yield losses are the two main problems associated with wheat production. Exposure to organisms such as viruses, obligate parasites, insects, soilborne pathogens, residue borne and semi-biotrophic pathogens can have a devastating effect on a plant's health (Duveiller *et al.,* 2007). Viruses in wheat can be transmitted through numerous vectors that can include insects (*Rhopalosiphum padi*) and obligate parisites

(*Polymixa graminis, Puccinia graminis*) (Henry and Plumb, 2002). It has been noted that wheat is susceptible to at least 50 viruses (Brunt *et al.,* 1996).

Soilborne pathogens include the cereal nematodes (cereal cyst nematode [*Heterodera sp.*]), root lesion nematode *Pratylenchus thornei* and the dryland root rot caused by *Cochliobolus sativus* (Verma and Spurr, 1987; Duveiller *et al.,* 2007). These pathogens do not cause significant damage under optimum conditions, however in dryland conditions they will have detrimental effects on the yield (Nicol and Ortiz-Monasterio, 2004; Duveiller *et al.,* 2007).

In addition to abiotic and biotic pressure, socio-economic factors such as access to machinery (mechanisation), labour, irrigation, seed availability, grain pricing and marketing, level of credit towards agricultural sector and population densities play a definitive role in the production potential of wheat in most developing countries (Kosina *et al.,* 2007). Mechanisation refers to the availability of power sources and tools in a production system. In wheat, the loss of machinery usually results in more intensive manual labour that lowers the production output capabilities (Rahman *et al.,* 2011).

The level of education of farmers has shown that literate farmers produce more wheat as opposed to illiterate farmers (Iqbal *et al.,* 2014). In South Africa, education and shortage of management skills has been identified as a problem in our society and improvement is needed to not only scale up production but also improve commercialization in agriculture (Jordaan and Grobler, 2011). Strategies have however been put in place through agricultural and education training being provided by government to educate and improve the level of agricultural acumen of rural communities (Department of Agriculture of South Africa, 2005). Coupled with education, the basic knowledge of science in agricultural practices and access to the best possible resources is not limited to only commercial farmers.

Agricultural policies by government linked to the above-mentioned factors also play a role in wheat production. Land reform policies implemented in African states such as Zimbabwe has also resulted in the loss of production potential in farming communities and a steady decline was observed with maize production (World Bank, 2007). It was also established that farming communities struggled to provide enough food for themselves (Richardson, 2005).

When one looks at all the above factors, it is clear that wheat production can be affected adversely not only by abiotic or biotic stresses but also poor infrastructure and socio-economic factors that result from it.

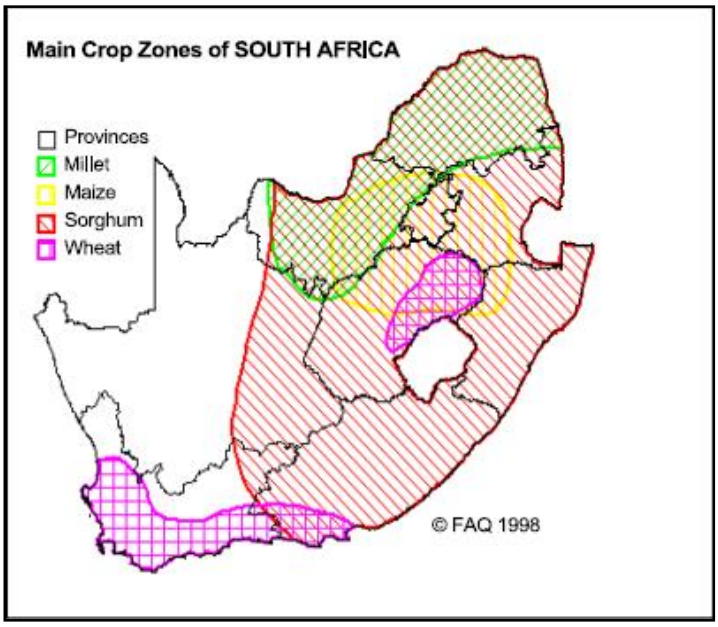

Figure 2.5: Main crop zones of South Africa (FAO/GIEWS 2001)

2.9. Bioactive compounds found in plant species

In addition to the constitutive barriers, plants also have the ability to constitutively produce secondary metabolites during the defence response that may act directly or indirectly. The different classes of secondary metabolites includes terpenes, phenolics, nitrogen-and-sulphur containing compounds and oxylipins, all of which play a definitive role in some way during the defence response. Terpenes have been reported to possess antimicrobrial properties and volatile signalling ability that attract insect predators (Paré and Tumlinson, 1999). Phenolic compounds provide a diverse array of defence related responses including deterrence capabilities and growth inhibitory towards insects (Rattan, 2010). Nitrogen-and-sulphur containing compounds play a role in plant-pest interactions by being anti-feedant or toxic substances towards pests (Fürstenberg-Hägg *et al.*, 2013). Pyrrolizidine alkaloids have been documented to possess toxic properties towards insects (Hartmann, 1999). Oxylipins are secondary metabolites generated from the oxidation of fatty acids and are best known for their role in signalling pathways (Blée, 2002). The oxylipins are utilized in pathways to generate signalling molecules such as JA, which plays an important role against insects (Farmer and Ryan, 1992).

2.9.1 *Artemisia afra*

Artemisia is a large genus of plants that belong to the *Asteraceae* family. The genus in particular comprises about 300 species of well known aromatic and medicinal plants distributed throughout the world (Burits *et al.,* 2001). The common name *of Ar. afra* is the African wormwood plant and it is indigenous to South Africa and can be described as a highly aromatic bushy shrub that can grow up to 1.5 m in height and is deciduous in areas with cold winters (Hilliard, 1977; Burits *et al.,* 2001). *Artemisia afra* is planted in temperate and sub-tropic regions. Actively growing in the summer months, it is able to withstand quite low temperatures during the winter months. *Artemisia afra* is distributed in all provinces of South Africa except Northern Cape (Hilliard, 1977).

The essential oil is known to possess numerous bioactive compounds such as thujone, camphor, alpha-pinene and 1,8-cineole of which all have characteristic bioactivities. It is used mainly in traditional medicines for the treatment of ailments like influenza, fever, diabetes, gastro-intestinal disorders and intestinal worms (Hilliard, 1977).

2.9.2 *Zanthoxylum capense*

Zanthoxylum capense is indigenous to South Africa and belongs to the *Rutaceae* family. It is a multi-branched tree that can range between 5 to12 m in height (Schmelzer and Gurib-Fakim, 2013). The tree has for a long time been associated with having medicinal properties and has been used to treat ailments such as flatulent colic, stomach ache and fever (Van Wyk *et al.,* 1997). The leaves of the plant can also be used for the treatment of sores. Apart from medicinal uses, the plants biological activity is related to that of pellitorine (Steyn *et al.,* 1998). Pellitorine is known to have ovicidal properties against the potato beetle (Ginesta *et al.,* 1994). The plant is also rich in alkaloids that have antimycobacterial activity (Luo *et al.,* 2013).

2.9.3 *Agave attenuata*

Agave attenuata belongs to the family *Agavaceae* and is native to Tropical America. The plant is an evergreen, succulent species that lacks spines. In South Africa, it is regarded as a non-invasive species and mainly distributed as an ornamental plant grown in gardens (Brackenbury and Appleton, 1997). Previous literature reports showed the presence in the plant of steroidal saponins (Mendes *et al.,* 2004). Some species of this genus are used in traditional medicine in alleviated high blood pressure in humans (Hackman *et al.,* 2006). The plant is also known to possess compounds that have anti-inflammatory properties (Mendes *et al.,* 2004). It is highly toxic to the snail *Bulinis africanus* which is an intermediatory host of *Schistosoma haematobium* (Brackenbury and Appelton, 1997).

Owing the nature of the bioactive compounds present in the above mentioned plant species, it is worthwhile considering that the application of crude extracts of these plants' may enhance resistance of wheat plants against the RWA through repellency, insecticidal or the induction of defence-related responses.

Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Wheat seedlings

Seeds of near-isogenic wheat (*Triticum aestivum* L.) lines, resistant cultivar (cv.) Tugela *DN*, containing the *Dn*1 (PI 137739) resistance gene (Du Toit, 1989) and susceptible Tugela to the RWA, were obtained from the Agricultural Research Council – Small Grain Institute (ARC-SGI), Bethlehem, South Africa. Twenty seedlings per pot were cultivated in a sterilized soil/peat (3:1) mixture at day and night temperatures of 22ºC and 16ºC. The plants were watered daily and fertilized with 50 ml of Multifeed classic® (2.5 g L^{-1}, w/v) per pot, twice per week.

3.1.2 Plant material

Leaf material of plant species were collected at various locations in South Africa and identities were confirmed by a qualified botanist, Professor Johan du Preez at the University of the Free State. *Artemisia afra* material was obtained from Bethlehem (28°14’ S, 28°18’ E) in the eastern part of the Free State. *Zanthoxylum capense* material was collected in the Lowveld Botanical gardens in Nelspruit (25°44’ S, 30°96’ E), Mpumalanga and *Agave attenuata* material was collected from Bloemfontein (29°07’ S; 26°11’ N).

3.1.3 Experimental insects

Russian wheat aphid (RWA) *Diuraphis noxia* (Kurdjumov) biotype RWASA1 was originally supplied by the ARC-SGI, Bethlehem and was continuously multiplied on susceptible wheat plants (Tugela cv.) under greenhouse conditions. Colonies were maintained in cages.

3.1.4 Other materials

All the chemicals used, i.e. methanol, dichloromethane (DCM), ethyl acetate, dimethyl sulphoxide (DMSO) acetic acid, sulphuric acid and ethanol were of the purest grade available and purchased from Merck (Germany). Aluminium thin-layer chromatography plates (Silica gel 60 F_{254}; 20 x 20 cm^2) were also purchased from Merck (Germany).

3.2 Methods

3.2.1 Preparation of crude plant extracts and essential oil

All plant material was dried in an oven at 40°C. Dried macerated leaf material (20 g) was successively extracted using water, methanol and dichloromethane as solvents [1:20 (w/v)]. Water extracts were boiled for 5 min then left at 35°C in a water bath for 1 h. Methanol and DCM extracts were thoroughly mixed and extracted in a waterbath at 35°C for 1 h, thereafter all the extracts were filtered using Wattman® No 1 filter paper. The water extract was lyophilized, while the methanol and dichloromethane extracts were vacuum dried using a Büchi® Rotavapor. The dried extracts were kept at 4°C. Essential oils were isolated from fresh leaf material by conventional hydro-distillation in a Clevenger-type apparatus for 4 h. A round bottomed flask was packed with fresh leaf material [circa (ca) 300 g] and ca 200 ml of distilled water was added. The flask was sealed, the cooling system was set up and the apparatus was heated using an electric heating mantle. The oil was collected and stored at 4°C in the dark until further use.

3.2.2 Treatment of wheat and infestation procedure

The different plant extracts were applied as a foliar spray to 17-day-old (three-leaf growth stage) susceptible and resistant wheat plants at a concentration of 1 g L^{-1} as described by Cawood *et al.* (2010). Either the extract or the essential oil were dissolved separately in 30 ml 1% DMSO and applied to the wheat seedlings by means of a hand spray until runoff. One set of pots was sprayed with 1% DMSO to serve as negative control. Culture conditions (3.1.3) with minor modifications and infestation procedures were done as described by Du Toit (1988). After plants were sprayed with plant extracts and essential oil, they were all left to dry and were then infested by scattering Russian wheat aphids (RWAs) onto the leaves, according to each bio-assay experiment. Another set of plants (resistant and susceptible) were sprayed with 1% DMSO solution and left as uninfested and infested controls.

3.2.3 Chromatographic techniques

3.2.3.1 Thin layer chromatography

Thin layer chromatography (TLC) of the plants aqueous, methanol and DCM extracts and essential oil were performed in order to visualize any compounds present. Extracts were dissolved in their appropriate extraction solvents at 10 mg mL^{-1} and 10 -15 µL applied to the TLC. In this study for the essential oil and DCM extracts, the mobile phase in which the plates were developed comprised of toluene : ethyl acetate (93:7). The mobile phase for development of the aqueous and methanol extracts was chloroform: methanol: water: acetic acid (65: 35: 5: 1). Compounds resolved on the plate were visualized using either general or specific methods. Ultraviolet (UV) light indicates fluorescent compounds. They were examined at 365 nm (long) and at 254 nm (short) wavelength UV light (Evans, 2002). Alternatively, colourless compounds required a chemical reaction in order to visualize their location (Smith and Seakins, 1976). Spray reagents to produce coloured derivatives were used, namely *ρ*-anisaldehyde-acetic acid, vanillin-sulphuric acid, 5% ferric chloride and dragendorf reagents, prepared according to the standard methods described by Wagner and Bladt (1996). A 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent was used to illustrate compounds with antioxidant activity (Glavind and Holmer,1967).

3.2.3.2 Gas chromatography and mass spectrometry

Analytical gas chromatography was carried out using a Perkin Elmer Clarus 500 gas chromatography system fitted with Zebron Zb-5ms fused silica capillary column (30 m x 0.25 mm, 0.25 µm film thickness). Carrier gas was helium at a flow rate of 1.5 mL min^{-1}. Column temperature was initially kept at 60°C for 5 min, then gradually increased to 280°C at a rate of 2°C min^{-1}, and held for 10 min. Essential oil and DCM samples (1 µL, appropriately diluted in hexane) were injected at 280°C with split mode (1:50). The concentration of the samples was 10 mg mL^{-1}. The flame ionization detector (FID) was set at 280°C. A Perkin Elmer Clarus 560 S mass spectrometer was used to identify components seperated by GC according to their retention indices and GC-MS fragment patterns. The identification of components was done with the use of the National Institute of Standard and Technology (NIST) version 3.0 database.

3.2.4 Insecticidal bioassay

A laboratory assay of the different plant extracts and essential oil were carried out on RWASA1 to assess the bio-efficacy of the extracts as described by Singh *et al.* (2010). The spray solutions (0.1, 0.5, 1 and 10 mg mL^{-1}) were prepared in 1% DMSO. Twenty adult aphids from the colony were transferred to a clean open petri dish and were subjected to direct spraying of the extract solution at room temperature. Experiments were run daily from 12:00 to 16:00 p.m. The spray bottle was held 30 cm away from the petri dish for even distribution of the extract mist. To prevent aphids from escaping, filter paper was used to cover the petri dish. After one hour, the aphids that were still alive were counted and transferred to a new petri dish with fresh wheat leaf material as a food source. These petri dishes were closed and placed in a growth cabinet with diurnal light with day and night temperatures of 24°C and 18°C. Mortality of the aphids was recorded at 1 h and 24 h post treatment. Each experiment was repeated 20 times for each plant extract. From each plant species, the best extract was chosen for further analysis to determine LC_{50} values (Finney, 1971). Commercial product Raid® for crawling insects and 1% DMSO in distilled water were used as positive and negative controls respectively.

3.2.5 Repellency bioassay

3.2.5.1 Olfactometer

A four-arm olfactometer (Pettersson, 1970; Pettersson, 1994) was used to test the response of aphids to the different extracts and essential oil (Fig. 3.1). It consists of an enclosed Perspex chamber divided into a central area (A) and four arm zones (12 cm diameter). The floor of the chamber was fully covered with white filter paper. Air was drawn from the centre of the olfactometer at 400-500 ml min^{-1} where discreet air currents were established in the four arms. Odor source vials (B) were connected to each of the four arm zones. Each odor source vial contained a piece of Wattman no.1 filter paper onto which 10 µl of the extract (10 mg mL^{-1}), or the control solution, was deposited. An odor field was established by introducing the extract into two neighboring arm zones, while the opposite two arms contained the respective solvent in which the extract was dissolved and served as control. Activated charcoal filters (C) were fitted at the end of each glass vial to ensure clean air entering the odor sources.

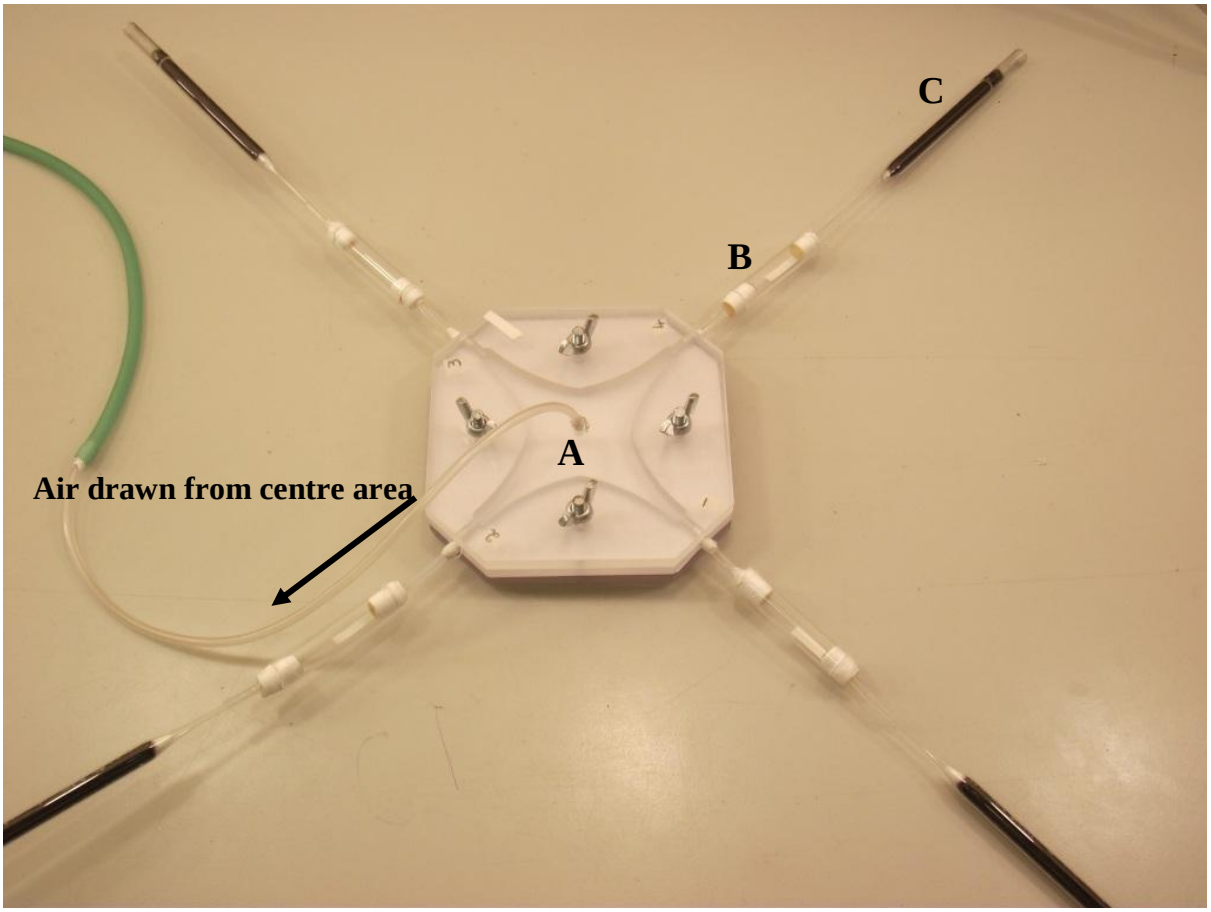

Figure 3.1: A four arm olfactometer, indicating the centre area A, the odour vial B, and charcoal filter C.

A single aphid was introduced into the olfactometer and observed for 10 min. The time spend and number of entries made into either treated or untreated zones by the aphid in the olfactometer was done by using EthoVision XT (Noldus technologies, Netherlands) software package. The tests were performed in a windowless room with a constant light source. Live tracking motion of the insect during the 10 min run was possible with the use of a stage camera (PAL definition: 786 x 576). The temperature in the room was kept constant at 25°C. Experiments were conducted daily between 08:00am and 16:00pm. Each treatment was repeated with 20 individuals. Olfactometers and odor sources with charcoal filters were changed after every five replications. Data for both control arm zones and both treated arm zones were pooled to give single figures for the time spent and the number of entries into these zones. The mean duration is described as time spent per visit by the aphid in the olfactometer chambers and calculated by dividing the time spent through the number of visits by the aphids.

3.2.5.2 Leaf settling

Resistant and susceptible wheat were treated with the different plant extracts and essential oil at 3 leaf stage as described in 3.2.2. A no-choice aphid settling test (Ninkovic *et al.,* 2002; Glinwood *et al.,* 2004) was used to assess aphid settling on treated plants. A 50 ml falcon tube was placed over the youngest fully developed leaf, which was always the second leaf. The upper end of the tube was covered with a sponge and the lower end with a sponge plug with a slit for the leaf. The tube was supported with a skewer to minimize mechanical damage to the test plant. Ten aphids were placed in the tube. After 2 h and 72 h the number of live aphids that settled (not walking) on the leaf, was recorded. According to Prado and Tjallingi, (1997) two hours is sufficient time for aphids to settle and penetrate the phloem. Five plants were randomly selected for each treatment. Control plants were sprayed with 1% DMSO solution (v/v).

3.2.6 Symptom analysis

Plants used for the leaf settling experiment (3.2.5.2) were left for 7 days whereafter symptoms on the plants and number of live aphids were recorded. Symptom development on plants was analyzed according to an adapted method described by Tolmay *et al.* (1999) by using a 1-10 damage rating scale where: 1 = small isolated chlorotic spots; 2 = small chlorotic spots; 3 = chlorotic spots in rows; 4 = chlorotic splotches; 5 = mild chlorotic streaks; 6 = prominent chlorotic streaks; 7 = severe streaks, leaves fold conduplicate; 8 = severe streaks, leaves fold convolute; 9 = severe streaks, leaves roll tightly; 10 = dying plant. A mean score of $1 \leq 2.5$ represented a highly resistant plant (no leaf curling), $2.6 \leq 6.5$ represented a medium resistant plant, $6.6 \leq 10$ represented a susceptible plant (curled leaves and severe chlorosis).

3.3 Induction of defence response

Wheat seedlings cv. Tugela and Tugela *DN* were grown as described in 3.1.1. At the third leaf stage, wheat plants were sprayed with the different extracts and essential oil and were left for 24 hours. After 24 hours, plants were infested with RWASA1 in a caged setup where each plant had an average of 20 aphids per plant. Leaves were harvested from 0 to 144 h time intervals for assay purposes.

3.3.1 Collection of intercellular wash fluid

Leaves from both the resistant and susceptible plants were cut in 10 cm long pieces, thoroughly rinsed in distilled water, and then vacuum infiltrated with 50 mM Tris buffer (pH 7.8) for 5 minutes (Jung *et al.*, 1993). The leaves were dried on a blotting paper, inserted vertically in a centrifuge tube with a perforated disc at the bottom, and centrifuged (5000 x *g*) at 4°C for 5 minutes. After centrifugation, the intercellular wash fluid (IWF) was collected from the bottom of the centrifuge tube, and the procedure was repeated using the same leaves. The combined IWF was stored at –20°C for the assay of the intercellular β-1,3-glucanase and peroxidase activities.

3.3.2 Protein determination

The protein content of the enzyme extracts was determined according to a modified method of Bradford (1976), where BioRad (Bio-Rad laboratories GmbH) reagent was used to determine protein concentrations. The assay mixture consisted of 160 µL distilled water, 40 µL Biorad, and 10 µL enzyme extract or standard (0.5 mg mL^{-1}). The absorbance was measured at 595 nm using the Biorad microplate reader. Bovine serum albumin (BSA) was used as a standard.

3.3.3 Peroxidase (EC 1.11.1.7) activity assay

A modified method of Zieslin and Ben-Zaken (1991) was used. The assay mixture (1 mL) contained 10 µL IWF, 50 µL 8.2 mM H_2O_2, 100 µL 50 mM guaiacol, 340 µL double distilled water and 500 µL 80 mM potassium phosphate buffer (pH 5.5). The absorbance increase was measured at 470 nm for 3 minutes at 30 °C against a blank containing all the reagents except for the IWF, which was replaced by 50 mM Tris buffer (pH 7.8). The increase in absorbance (tetraguaiacol formed) represented the rate of H_2O_2 reduction by peroxidase with guaiacol as a hydrogen donor. The enzyme activity was expressed as mM tetraguaiacol mg^{-1} prot. min^{-1} (using guaiacol extinction coefficient of 26.6 mM^{-1} cm^{-1}).

3.3.4 β -1,3-Glucanase (EC 3.2.1.39) activity assay

A modified method of Fink *et al.* (1988) was used. The assay mixture contained 10 µL IWF, 250 µL of a 2 mg mL^{-1} laminarin solution and 240 µL 50 mM sodium acetate buffer (pH 4.5).

After incubation at 37°C for 10 minutes, 500 µL of Somogyi reagent [0.2 g $CuSO_4$; 9 g Na_2SO_4; 1.2 g $NaCO_3$; 0.8 g $NaHCO_3$ and 0.6 g potassium tartrate in 50 mL double distilled water, (Somogyi, 1952)] was added and incubated at 100°C for 10 minutes. After cooling under tap water, 500 µL of Nelson's reagent [2.65 g $(NH_4)_6$ Mo_7O_{24}; 2 mL 95-97 % H_2SO_4; 0.32 g $Na_2HAsO_4.7H_2O$ in 50 mL double distilled water, (Nelson, 1944)] was added. The absorbance (which represented the amount of glucose formed) was measured at 540 nm. The blank and the glucose standards used to prepare a standard curve were subjected to the same procedure. The quantity of glucose produced from laminarin was determined from the glucose standard curve (which was subjected to similar experimental conditions) and β-1,3-glucanase activity was expressed as mg glucose mg^{-1} prot. min^{-1}.

3.4 Statistical analysis of data

The statistical analysis of experimental data was performed by Professor Robert Schall, Statistical Consultation Unit, Department of Mathematical Statistics and Actuarial Science, University of the Free State. The SAS Institute Inc (2009) statistical software package was used.

Olfactometer; number of visits: For each pair of visit counts (that is, given a certain plant species, extract and replicate) the number of visits in the treatment arms of the olfactometer follows a binomial distribution Bin (p,n), where p is the probability that the aphid visits the treatment arms of the olfactometer, and n is the total number of visits (treatment and control) observed. Based on this data [number of visits in the treatment arms of the olfactometer over the total number of visits (treatment and control)] a certain combination of plant species and extracts could be considered effective if the average proportion is less than 0.5; similarly, a certain combination of plant species and extract could be considered more effective than another such combination if the average proportion for the former is smaller than the average proportion for the latter.

In order to explore the efficacy of plant species, extracts, and of the various combinations of plant species and extract the number of visits in the treatment arm of the olfactometer was analysed using a generalized linear model (GLIM) with the following specifications:

1. Distribution: Number of visits in the treatment arm has a binomial distribution Bin(p,n), where p is the probability that the aphid visits the treatment arms of the olfactometer, and n is the total number of visits (treatment and control) observed

2. Link function: Logistic
3. Linear predictor: Two-way layout fitting the factors plant species, extract, and the plant species by extract interaction.

The model allowed for extra-binomial variation, and test statistics, standard errors and CIs were scaled using the ratio of the Pearson chi-square statistic over its degrees of freedom.

Olfactometer; time spent: For each pair of time measurements (that is, paired with respect to plant species, extract and replicate) the difference was calculated between the time spent in the treatment and in the control arms of the olfactometer. Based on this data, a certain combination of plant species and extract could be considered effective if the mean value in the time difference is negative; similarly, a certain combination of plant species and extract could be considered more effective than another such combination if the mean time difference for the former is smaller than mean time difference for the latter.

In order to explore the efficacy of plant species, extracts, and of the various combinations of plant species and extract the time difference was analysed using a two-way analysis of variance (ANOVA) fitting the factors plant species, extract, and the plant species by extract interaction. From this ANOVA, F-statistics and associated P-values were obtained for the effects in the model.

Insecticidal properties: The difference in mortality rate between each treatment and the control was assessed using a Pearson chi-square test. The difference was statistically significant in all cases, with a P-value < 0.0001 ($P < 0.0001$) in all cases (data not shown in the Results Section). Furthermore, the mortality data for the top performing extract for each plant species was further analysed using Probit analysis (SAS procedure PROBIT). The explanatory variable was $\log_{10}$ (concentration). Because of extra-binomial variation, all standard errors and test statistics were scaled by the ratio of the Pearson chi-square goodness-of-fit statistics and its degrees of freedom.

For each of the three treatments, the estimated Probit model slope is reported, as well as the estimated LC_{50} value together with 95% Fiducial Limits for the LC_{50} value (these Fiducial Limits can be interpreted as confidence limits).

Leaf settling experiment: A generalized linear model specifying a Poisson distribution for the number of aphids settling at 2 h and 72 h, respectively, was used; the linear predictor was that for a one-way layout (treatment). Treatments were copared to the control by estimating the rate ratio of the number of aphids settled; a P-value associated with the null hypothesis that the ratio is 1 is reported.

Symptom analysis: For each cultivar, the disease rating data was analysed using a one-way analysis of variance fitting the effect "treatment" (10 species / extract combinations and control). From this ANOVA, pairwise differences between the mean disease ratings for the 11 treatments were calculated, together with 95% confidence intervals (CIs) for the mean difference, and a P-value associated with the null hypothesis that the mean difference is zero.
For each cultivar, the average infestation counts were analysed using a one-way analysis of variance fitting the effect "treatment" (10 species/extract combinations and control). From this ANOVA, pairwise differences between the mean infestation counts for the 11 treatments were calculated, together with 95% confidence intervals (CIs) for the mean difference, and a P-value associated with the null hypothesis that the mean difference is zero. This mean number was expressed as the percentage compared to the controls.

Induction of defence: The enzyme data was analysed separately for each time point using a three-way ANOVA fitting the factors treatment, cultivar, and condition (uninfested and infested), and all interaction terms. The t-test statistics and probability-values were calculated associated with the comparison of the treatments.

Chapter 4
Results and Discussion

4.1 Extraction of plant material

The percentage dried plant material recovered after drying was 46.31%, 13% and 43.90% for *Ar. afra, A. attenuata* and *Z. capense* respectively, while the percentage essential oil yield (v/w) from *Ar. afra* was 1.7%. The percentage essential oil extracted is efficiently comparable to studies done by Burits *et al.* (2001). Only the essential oil of *Ar. afra* was extracted as *A. attenuata* and *Z. capense* did not yield any oil from extraction through hydro-distillation. Studies by Kothari and Seshadri (2010) on seed extracts showed that a higher yield extraction was directly proportional to anti-bacterial activity of higher magnitude. The extraction process can thus be regarded as crucial step in any plant formulation before running bioassays. Many solvents are used to extract phytochemical compounds from plant material. An important factor governing the choice of solvents used in an extraction is the type of phytochemical groups that are targeted for extraction if they are known (Houghton and Raman, 1998). In this study, no particular phytochemical group was targeted for extraction as it was not established which compounds could have bioactivity against the RWA. The solvents chosen for extraction purposes had varying polarities with different chemical properties which allow for the extraction of different compounds from the plant material used. The different solvents therefore provide a broad spectrum of possible phytochemicals that can be extracted from the different plant species. The mass that each solvent extracted from 1 g dried leaf material was determined, calculated as percentage (g/g) extracted and recorded in Table 4.1.

Table 4.1: Plant extract recovery.

Plant name	**Percentage mass residue extracted**			
	Solvent used for extraction			
	Water	**Methanol**	**DCM**	**Average %**
Ar. afra	25.00	9.80	9.20	**14.67**
A. attenuata	33.80	5.70	2.32	**13.94**
Z. capense	17.00	4.00	4.61	**8.54**
Average %	**25.27**	**6.50**	**5.38**	

The average yield was obtained for crude extracts of *Ar. afra* (14.67%) followed by *A. attenuata* (13.94%) while *Z. capense* yielded the least (8.54%). Water was the most efficient extractant with an average of 25.27% dried plant material extracted, followed by methanol (6.5%) and

DCM (5.38%). A few factors may attribute for the water showing a higher extraction yield compared to the other two solevents namely, the efficiency of the extraction technique and the nature of available extractable compounds found in a plant species (Ahmad, 2009; Sultana *et al.*, 2009).

Water as an extractant also has many advantages including its non-toxic nature, its high availability and its ability to extract numerous compounds including terpenoids, phenolics, sugars, amino acids, anthocyanins, saponins and polypeptides (Houghton and Raman, 1998; Bart, 2011). The high percentage mass recovery resulting from the extraction with water was primarily due to the fact that compounds present in the three plant species being hydrophilic in nature. *Ar. afra* was found to contain hydrophilic compounds (Liu *et al.*, 2009). Methanol used as a solvent for extraction can extract similar compounds to water due to their polarities being very close, however it may also extract compounds like polyphenols and quassinoids (Houghton and Raman, 1998). Dichloromethane is a non-polar solvent that can extract compounds such as non-polar phenolics, terpenoids and alkaloids (Guillén and Manzanos, 1998). In comparison to other studies, the efficiency of extraction for *Ar. afra* and *Z. capense* compares well for the water and dichloromethane extracts (Ntutela *et al.*, 2009; Masoko and Nxumalo, 2013).

4.2 Chromatographic techniques

4.2.1 Thin layer chromatography

Thin layer chromatography studies were carried out as an initial screening process in order to determine and visualise the locality of the various compounds by using R_f values. Figure 4.1 and 4.2 illustrate the TLC results for water, methanol and DCM extracts of *Ar. afra*, *A attenuata* and *Z. capense*. Different compounds were visible under UV light and when detection spray agents were applied.

In all the extracts, a number of compounds were observed with similarity in the chemical composition between water and methanol extracts (Fig. 4.1). Under UV-256 nm light, blue spots appear in the polar extracts for *Ar. afra* only. A yellow spot (R_f = 0.86) appears for *Z. capense* under the same light in the methanol extract (Fig. 4.1). However, in the non-polar DCM extracts of *Ar. afra*, *A. attenuata* and *Z. capense* blue spots were visible under UV-256 nm (Fig.

4.2). Dark green spots were also visible in *A. attenuata* and *Z. capense* under this illumination. On the baseline, there are compounds with a green colour that did not migrate on the plate (Fig. 4.2).

In the polar extracts, under 365 nm, the highly fluorescent compounds at R_f = 0.47 and 0.92 (blue in colour) found in *Ar. afra* were also visible (Fig. 4.1). Except for the compounds on the baseline that did not migrate, no other spots appeared under this light for the polar extracts of the other two plant species. A blue spot occured in the DCM extract of *Ar. afra* under UV-365 nm light and many red spots in both *A. attenuata* and *Z. capense* (Fig. 4.2). Fluorescence detection is an effective tool in identifying compounds in plants and thin layer chromatography provides a quick, cost effective way in the identification process. The separated compounds on a TLC plate were identified based on their R_f values, developing system used for separation and the colour reaction of spots when detection spray reagents were applied. Under UV light, the bright blue fluorescence represent compounds with conjugated double bonds (Wagner and Bladt, 1996). Such compounds may represent alkaloids, flavonoids or triterpenes (Barbetti *et al.*, 1986; Wagner and Bladt, 1996). The bright red (UV-365nm) spots are representative of photosynthetic pigments (Subramoniam *et al.*, 2012).

With the detection reagents, the presence of phenolic compounds was determined using ferric chloride solution and the blue-grey colour spots represented compounds with a phenol ring (Jork *et al.*, 1990). It is proposed that the use of ferric chloride with occasional heating provides the colour change and in visible light the colour spot on the plate is made possible due to the d-d transitions in the octahedral complex between Fe^{3+} ion and hydroxyl groups of phenols (Rungsimakan, 2011). From the different plant extracts, *Ar. afra* methanol extract showed prominent blue-grey spots at R_f = 0.16, 0.69, 0.74 and 0.92 and *Z. capense* methanol extract at R_f = 0.18 and 0.32, indicating the presence of phenolic compounds.

After spraying the TLC's with the general detection reagents *p*-anisaldehyde-acetic acid or vanillin-sulphuric acid, many different compounds could be observed (Fig. 4.1 and 4.2). With *p*-anisaldehyde, the identification of sugars (green), phenolic compounds (green), sapogenins (yellow), terpenes (pink) and steroids (blue) were possible (Jork *et al.*, 1990). In the polar extracts of *Ar. afra*, pink spots were visible (Fig 4.1), indicating the presence of terpenes and according to studies by Liu *et al.* (2009), *Ar. afra* is rich in terpenes. Numerous green spots were visible in the polar extracts of *A. attenuata* (Fig. 4.1). Although the green spots may

represent sugar molecules, it has been seen that saponins may attach to sugar molecules (Adel *et al.,* 2000). This plant is known to possess saponins (Mendes *et al.,* 2004) and the green compounds may indicate the presence of saponins. Prominent blue spots were visible in the polar and non-polar extracts of *Z. capense* (Fig. 4.1). According to literature, flavonoids, sterols and terpenes, among others, have been identified from *Z. capense* (Waterman and Grundon, 1983; Adesina, 2005).

Vannilin-sulphuric acid reagent spray revealed a complex mixture of compounds present in the chromatograms (Fig. 4.1 and 4.2). Flavonoids (yellow colour) and terpenes (violet) were detected (Wagner and Bladt, 1996). The yellow colour appeared in the polar extracts of *Ar. afra* and *Z. capense* (Fig. 4.1) and quantification studies for *Ar. afra* by Avula *et al.* (2009) and *Z. capense* by Amabeoku and Kinyua (2010) revealed the presence of flavonoids.

Dragendorf reagent spray was used for the detection of alkaloids, which are precursors of amino acids that may have allelopathic functioning (Wink, 1999). Bright orange spots reveal the presence of alkaloids. No compounds were detected on the TLC plates for the presence of alkaloids (Fig. 4.1 and 4.2), however some *Zanthoxylum spp.* have been reported to contain alkaloids in their structures (Negi *et al.,* 2011). The phytochemical constituents of a plant can be affected by numerous factors including seasonality, biotic and abiotic factors thus some compounds may be absent in a particular extract and TLC may help monitor this absence (Moure *et al.,* 2001).

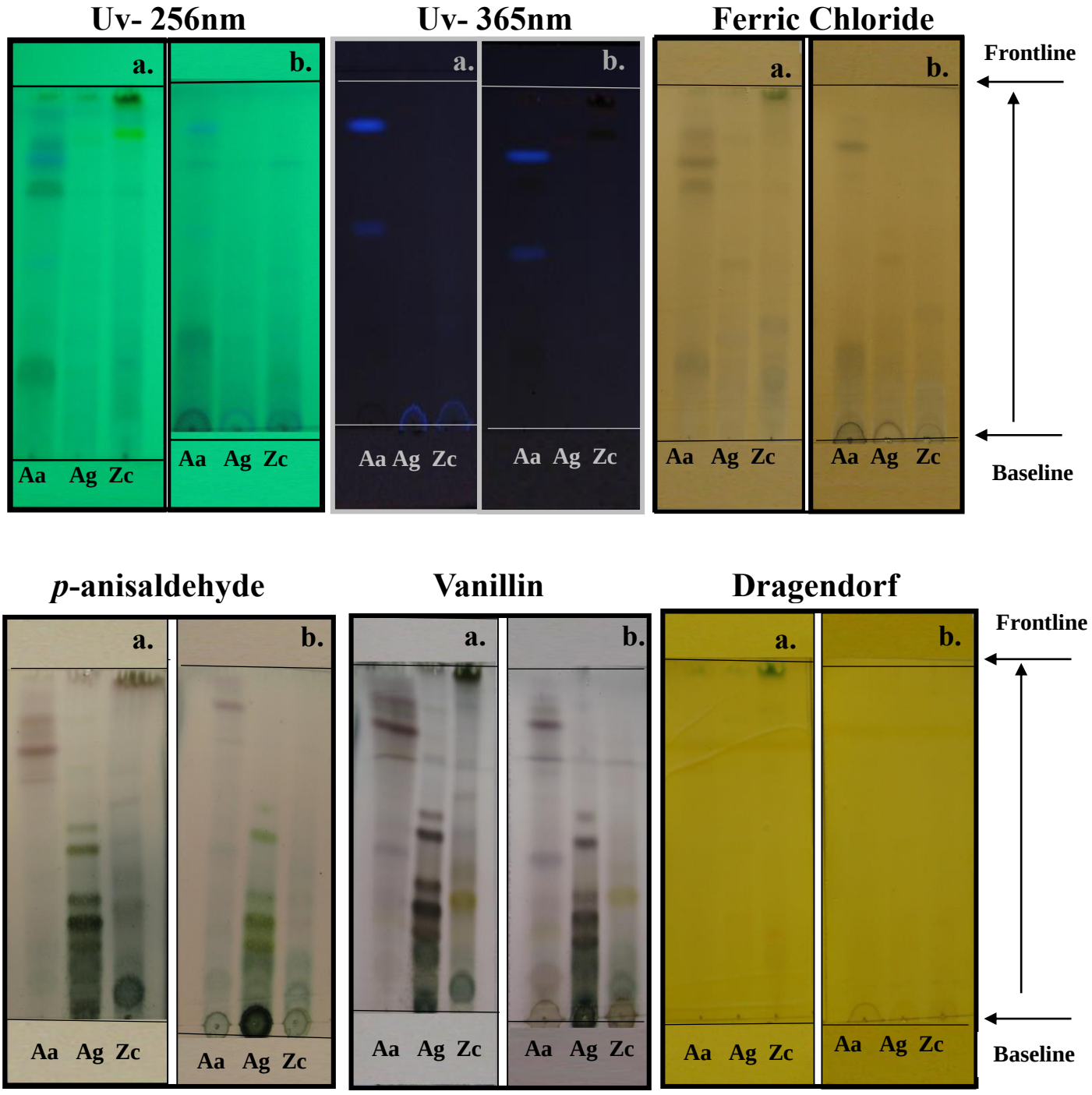

Figure 4.1: Qualitative TLC profile of polar water and methanol extracts. a: Methanol extract, b: Water extract. Lane Aa= *Ar. afra*, Lane Ag = *A. attenuata*, Zc = *Z. capense* Ar: 150 µg, Ag:150 µg , Zc:100 µg extract equivalent. Plates developed in $CHCl_3$:MeOH:H_2O:acetic acid [65:35:5:1%]

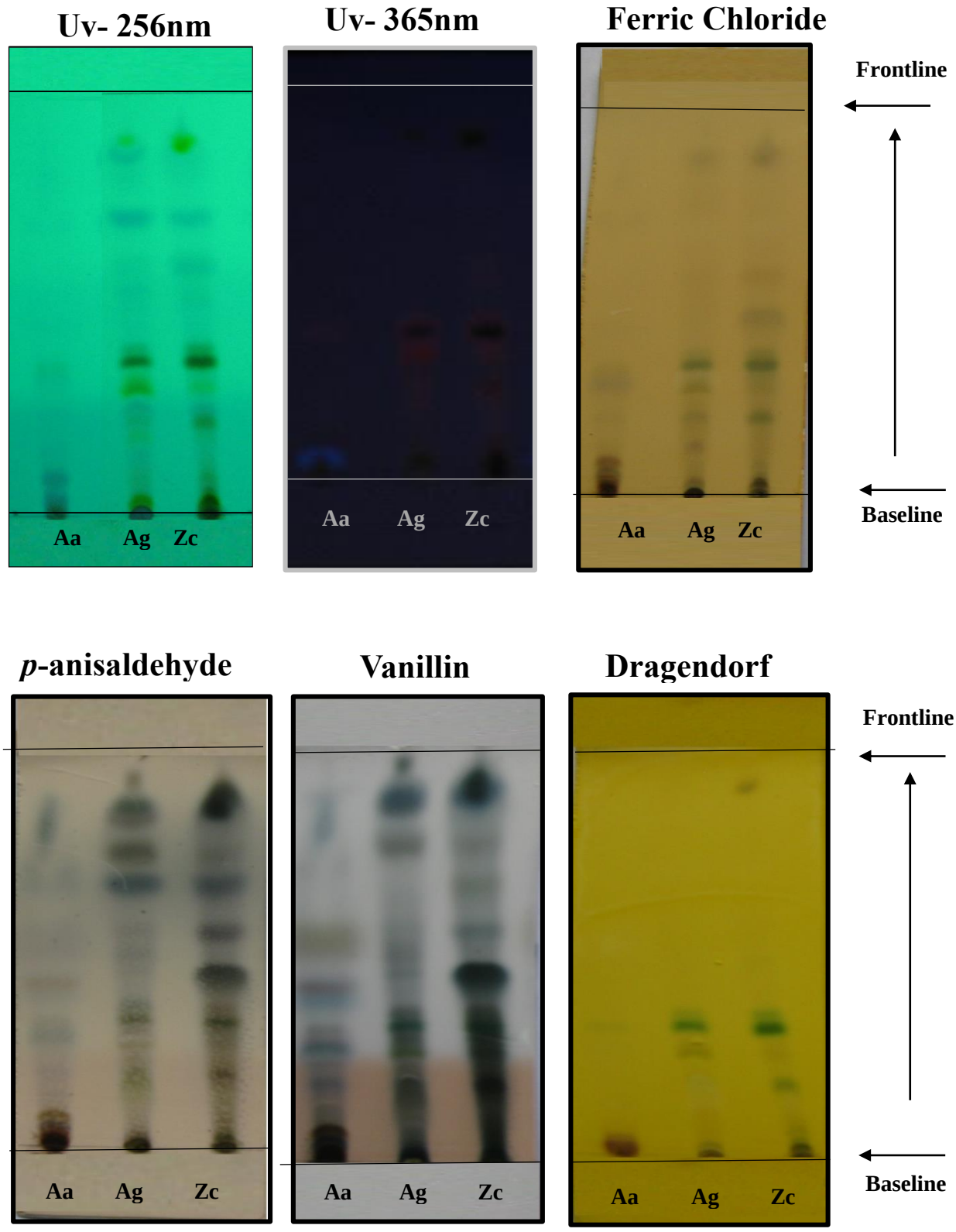

Figure 4.2: Qualitative TLC profile of non-polar DCM extracts. Lane Aa= *Ar. afra*, Lane Ag = *A. attenuata*, Zc = *Z. capense* Aa: 150 µg , Ag:150 µg , Zc:100 µg extract equivalent. Plates developed in Toluene: Ethyl acetate [93:7].

4.2.1.1 DPPH antioxidant activity

The use of DPPH reagent in a qualitative manner to determine which compounds possess antioxidant properties, proved positive for all the extracts of the three plant species (Fig. 4.3). DPPH measures the electron donating activity of the compound by its ability to scavenge off radicals (Lü *et al.*, 2010). The yellow spots present on the TLC plate on a mauve background represent the antioxidant compounds (Bondet *et al.*, 1997).

DPPH* + H—A → DPPH—H + A*

(Purple) Yellow

The compounds possessing antioxidant activity appeared to be the more polar extracts of *Ar. afra* and *Z. capense* ($R_f \leq 0.5$; Fig 4.3) that were identified earlier as phenolic compounds (Fig. 4.1). Antioxidant compounds protect a plant from any damage caused by oxidative stress (Gupta *et al.*, 2006).

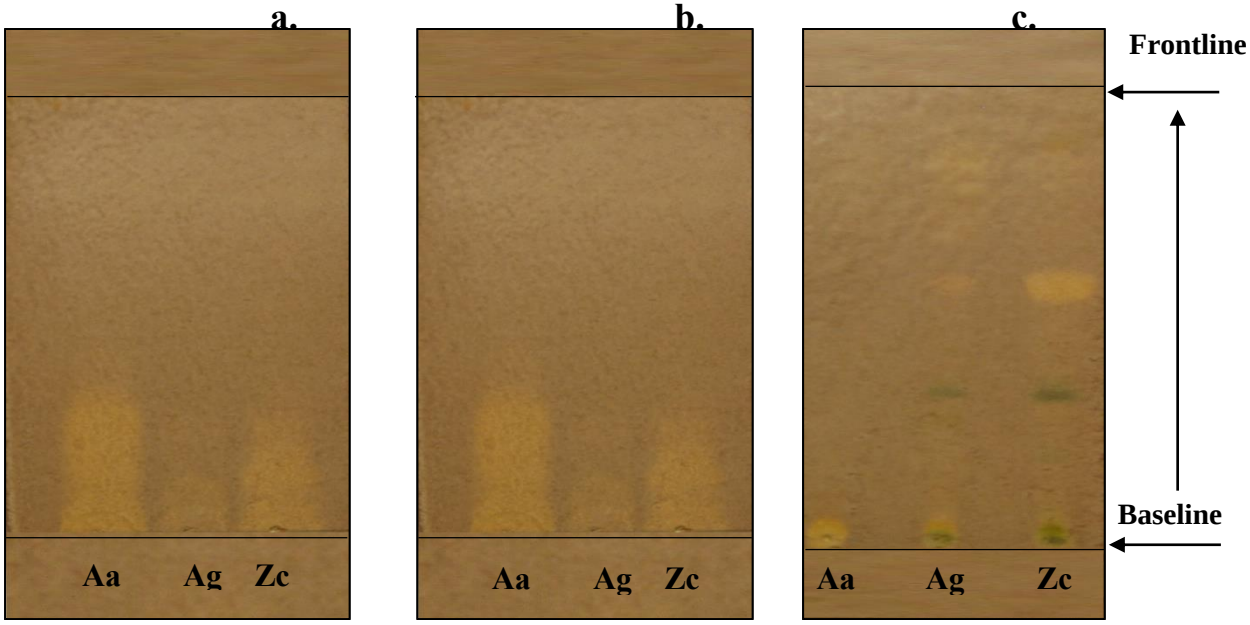

Figure 4.3: Qualitative TLC profile of antioxidant activity displayed by polar and non-polar polar extracts. **a:** Methanol extracts **b:** Water extracts **c:** DCM extracts. Lane Aa = *Ar. afra*, Lane Ag = *A. attenuata*, Zc = *Z. capense.* **Plate a,b:** developed in $CHCl_3$:MeOH:H_2O:acetic acid [65:35:5:1%] **c** developed in Toluene: Ethyl acetate [93:7].

4.2.2 GC-MS analysis

4.2.2.1 Composition of essential oil of *Ar. afra*

Thirteen compounds were identified in the essential oil of *Ar. afra,* making up a total composition of 92.24% (Table 4.2). The major components present were eucalyptol (12.87%), camphor (15%), α-thujone (29%), β-thujone (12%) and 1,8-cineole (7.42%). The oil is characterised by many of the major compounds been released within the the first 25 min of the run (Fig. 4.4).

Table 4.2: GC-MS results of essential oil of *Ar. afra*

Retention time (min)	Compound name	Area (%)
2.01	3-Hexanol	5.45
3.45	α-Terpinene	3.17
5.23	α-Pinene oxide	0.99
6.52	Thuja-2,4(10)-diene	3.74
9.34	Eucalyptol	12.87
10.39	1,8-Cineole	7.42
14.12	α-Thujone	29
15.22	ß-Thujone	12
16.17	Artemisia triene	0.67
17.01	Camphor	15
18.32	Artemesia triene	1.43
21.33	Hexadecane	0.43
25.22	Octanol	0.07
Total		**92.24**

Area (%) of compound = height of peak x width of peak at ½height x Total area^{-1}

Essential oils are a complex mixture characterised by high number of hydrocarbons (terpenes) and oxygenated compounds which gives their characteristic odour properties (Nerio *et al.,* 2010). The composition of the essential oil of *Ar. afra* revealed numerous compounds through GC-MS (Table 4.3) that were comparable to work done previously by Graven *et al.*(1992),

Chagonda *et al.* (1999); Viljoen *et al.* (2006); van Wyk (2008). Thujone, cineole and eucalyptol are compounds highly associated with *Artemisia spp*. and the difference in their percentage composition is a factor for differentiation between the species (Ghorbani-Ghouzhdi *et al.,* 2008). Genetic differences and environment contribute to the variation in oil compositions (Novak *et al.,* 2006; Viljoen *et al.,* 2006).

The properties of many oils is their repellency effect and insecticidal role against many insects due to their volatility and toxicity (Nerio *et al.,* 2010). Some monoterpenes found in the essential oil such as, α-pinene and cineole have already been described as providing repellency to mosquitoes (Park *et al.,* 2005). Alpha-thujone is a biological active compound and is useful as an insecticide and an anthelminthic agent for the treatment of parasitic worms (Hold *et al.,* 2000). Eucalyptol (1,8-cineole) and camphor are also found in the essential oils of *A. vulgaris* and according to studies by Wang *et al.* (2006); the essential oil showed good fumigant activity against *Tribolium castaneum* at varying concentrations.

Therefore it is expected that in this study, the essential oil, based on its chemical composition and previous reports in literature, may play a role in the management of the Russian wheat aphid. No studies to my knowledge have tested the oil in possible insecticidal effects against the Russian wheat aphid. Repellency work done by Richter (2010) reported the oil's role as a possible natural repellent, however the integration to its direct contact and physiological effects on wheat has not been done before.

4.2.2.2 Composition of DCM extracts

All the DCM extracts were dissolved in hexane for analysis on GC-MS. The hexane solvent eluted just after 1.2 min (Fig. 4.5 and 4.6) and was not taken into consideration when percentage area calculations were performed. Good separation of the different constituents of DCM plant extracts were obtained during gas chromatography (GC; Fig. 4.4; 4.5 and 4.6)

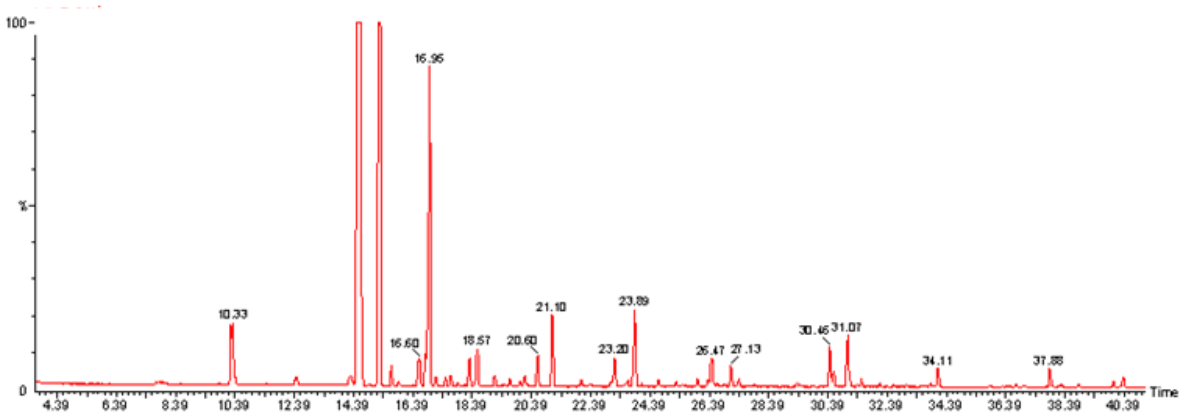

Figure 4.4: GC-chromatogram of DCM extract of *Ar. afra* .

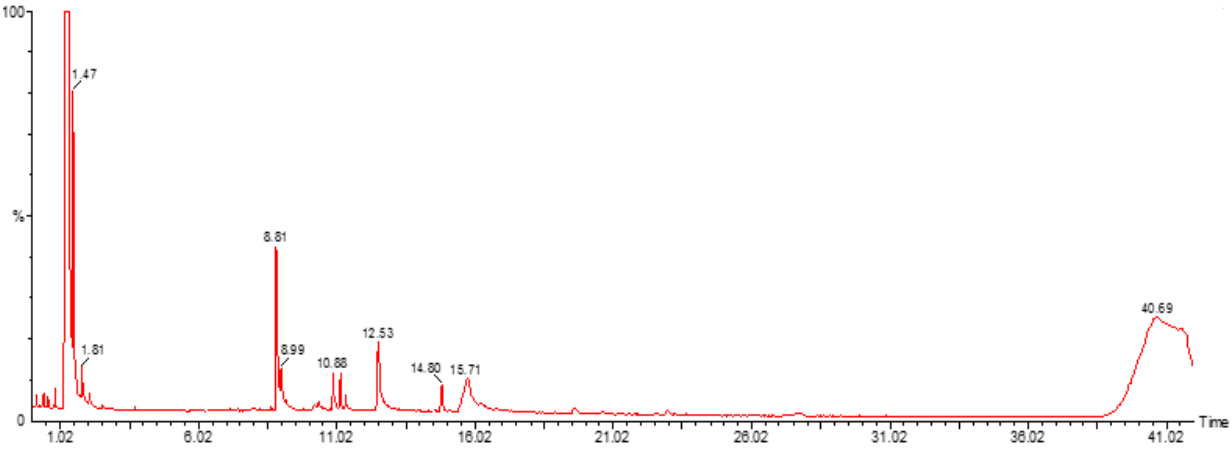

Figure 4.5: GC-chromatogram of DCM extract of *A. attenuata*.

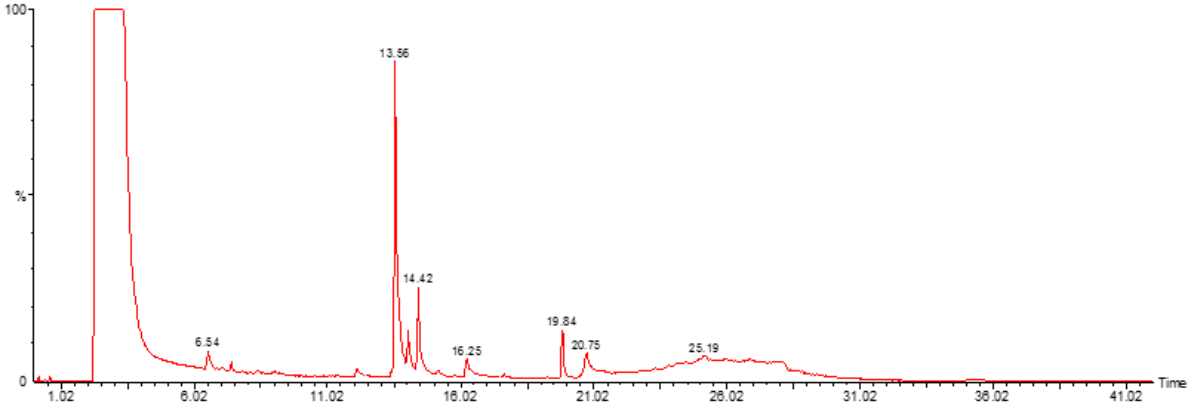

Figure 4.6: GC-chromatogram of DCM extract of *Z. capense.*

In comparison to the essential oil of *Ar. afra*, the DCM extract contained 15 compounds of which only four were also detected in the oil. The major constituents present in the DCM extract were α- and ß-thujone and consists of 66.86 % of the total area (Table 4.3).

Table 4.3: Chemical constituents and their recorded biological activities of an *Ar. afra* DCM extract.

Retention time (min)	Compound name	Area (%)	Nature of compound	**Activity
10.33	Eucalyptol	2	Monoterpenoid	Antihelmintic
14.39	α –Thujone	29.47	Monoterpene	Antibacterial,larvicidal
15.28	β –Thujone	37.36	Monoterpene	Antibacterial,larvicidal
16.96	Camphor	1.6	Terpenoid	Insecticidal
18.57	Borneol	2.08	Terpene	Antiacetylcholinesterase
20.6	7-Methyloct-3-en-2-one	1.07	Ketone	No activity reported
21.1	Piperitol	2.91	Alcohol	No activity reported
23.2	4-Hexen-1-ol, acetate	0.6	Alcohol	No activity reported
23.89	E-10-Pentadecenol	4.6	Alcohol	No activity reported
26.47	2-Ethenyl-1,3,3-trimethylcyclohexene	1.9	Aromatic ether	Carcinogenic*
27.13	2-Acetyl-4,6-octadienoic acid	3.72	Ester	Anti-tumour
30.46	1-Octyne	0.9	Alkyne	No activity reported*
31.07	2-Methyl-6-methylene-1,7-	0.4	Alkene	No activity reported
34.11	α-Methyl-1-adamantanemethylami	0.41	Isoquinoline	No activity reported
37.88	Fluoroacetylene	0.38		Carcinogenic
Total		**89.4**		

Area (%) of compound = height of peak x width of peak at ½height x Total area^{-1}

** *Dr. Duke's* Phytochemical and *Ethnobotanical* Databases.

*Pubchem Project NCBI

Six compounds were identified from the DCM extract of *A. attenuata* (Table 4.4). The GC-MS analysis of *A. attenuata* also confirmed the presence of hexadecanoic acid, which was reported by Rizwan *et al.* (2012) as part of the hexane fraction of a methanol extract of *A. attenuata*. Methyl jasmonate was also found in this particular fraction and is known to be an inducer of defence genes (Farmer and Ryan, 1990).

Table 4.4: Chemical constituents and their recorded biological activities of an *A. attenuata* DCM extract.

Retention time (min)	Compound name	Area (%)	Nature of compound	**Activity
1.47	2,5-Dimethyl-3,4-hexanediol	23	Alcohol	Anti-inflammatory*
8.81	Methyl jasmonate	6.4	Ester*	Plant defensive-gene* inducer
10.88	Tetradecanoic acid	3.86	Fatty acid	Anti-tumour*
12.53	n-Hexadecanoic acid	4.91	Fatty acid	Larvicidal*
14.8	1,10-Decanediol	1.38	Alcohol	No activity reported
15.71	1,4-Cyclohexanedimethanol	4.04	Alcohol	No activity reported
Total		**43.58**		

Area (%) of compound = height of peak x width of peak at ½height x Total area^{-1}
** *Dr. Duke's* Phytochemical and *Ethnobotanical* Databases.
*Pubchem Project NCBI

Six compounds were identified in the DCM extract of *Z. capense* (Table 4.5). Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol) is known to have insect repellent abilities and n-hexadecanoic acid possesses larvicidal activity (Singh *et al.,* 2012). It is possible that the extract might have the ability to be insecticidal in our study. (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene (E–(β)-farnesene), a sesquiterpene, functions as alarm pheromones in some aphids alerting them of danger (Cui *et al.,* 2013). The presence of terpenes was also confirmed previously by TLC (Fig. 4.2).

Table 4.5: Chemical constituents and recorded biological activities of a *Z. capense* DCM extract.

Retention time (min)	Compound name	Area (%)	Nature of compound	**Activity
6.54	(E)-7, 11-Dimethyl-3-methylene-1,6,10-dodecatriene	2.41	Sesquiterpene	Insect repellent*
13.56	2,4-Dimethyl-hex-1-ene	25.98	Alkene	No activity reported*
14.42	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3.80	Terpene	Insect repellent *
16.25	n-Hexadecanoic acid	2.64	Fatty acid	No activity reported
19.84	3-Tetradecyne	2.92	Terpene	No activity reported*
20.75	Undec-10-ynoic acid	3.21	Fatty acid	No activity reported*
Total		**40.96**		

Area (%) of compound = height of peak x width of peak at ½height x Total area^{-1}
** *Dr. Duke's* Phytochemical and *Ethnobotanical* Databases.
*Pubchem Project NCBI

The DCM extracts of all plant species analysed by GC-MS revealed numerous bioactive compounds found within the non-polar extracts (Table 4.3 - 4.5). The presence of terpenes, fatty acids, alcohols reveal the complex nature of compounds found in the different extracts. To corroborate the value of these plant extracts in a study of this nature, a few important aspects have to be considered in their usage such as the possible repellent, insecticidal and plant defence inducing capabilities of the extracts against RWA in wheat. Singling out compounds from the different extracts as a possible reason for a desired response would be false, however literature supports that some of these compounds do possess bioactivities as shown in Tables 4.3 - 4.5 that have not been explored in agricultural practices against the RWA as control agents.

4.3 Insecticidal activity

The commercial insecticide Raid® for crawling insects and 1% DMSO in water (v/v) were used as positive and negative controls, respectively. All plant extracts and the essential oil treatment (10 mg mL^{-1}) caused significant mortality of RWA's at both 1 h and 24 h post treatment compared to the negative 1% DMSO control (Table 4.6; $P < 0.05$; Pearson chi-square test).

Treatments with *Ar. afra* lead to 50 - 54% mortality after 1 h, 63.5 – 99% with *A. attenuata* and 58.5 - 63% with *Z. capense* treatments. After 24 h mortality increased for all treatments (Table 4.6) which indicates that, within the first hour mortality is primarily caused by contact administration, followed by a rise in mortality where the lethal effect is probably caused through ingestion. Importantly, since adult aphids were used, the fitness of the aphids was compromised as under no conditions did multiplication occur after exposure to the different extracts. In general, all the plant extracts showed high efficacy after exposure to only a single treatment by causing mortality of at least 70% at the end of 24 hours (Table 4.6). For *Ar. afra* the most effective treatment were the essential oil with increased mortality from 50 to 89.50% in 24 h, DCM extract of *Z. capense* (65.75 to 98.75%) and all the *A. attenuata* extracts killed more than 96% of aphids after 24 h.

The positive control insecticide used (Raid®), caused 100% mortality after 1 h and according to the product information sheet this product "kills on contact" due to its two active ingredients imiprothrin and cypermethrin (Deluise, 2012), which are synthetic pyrethroids. Imiprothin is highly toxic to sodium ion channels of insects (very low toxicity to mammalian sodium channels) that ultimately leads to paralysis (Davies *et al.*, 2007), while cypermethrin is extremely hydrophobic and causes membrane ion channels to stay open much longer, showing great potency as a neurotoxin (Cox, 1996).

The evaluation of plant extracts causing mortality at both 1 h and 24 h post treatment indicates the presence of active toxic compounds and the value of botanical insecticides as possible control agents towards RWA's.

The top performing extracts from each plant species were further analysed using probit analysis to obtain the LC_{50} for mortality at 24 h. For each of the three treatments, the estimated probit model slope was reported, as well as the estimated LC_{50} value together with 95% fiducial limits for the LC_{50} value (Table 4.7).

Table 4.6: Mortality of RWAs 1 h and 24 h post treatment with different plant extracts.

Treatment	Extract	Mortality			
		1 h post treatment		24 h post treatment	
		%	N (out of 400)	%	N (out of 400)
Water (-)	1% DMSO	0	0	0	0
Commercial product (+)	Raid®	100*	400	100*	400
Artemisia afra	Water	50*	200	74*	296
	Methanol	54*	216	70.75*	283
	Dichloromethane	53.75*	215	75.25*	301
	Essential oil	50*	200	89.50*	358
Agave attenuata	Water	71.50*	286	98.75*	395
	Methanol	58.50*	234	97.50*	390
	Dichloromethane	63.50*	254	96.50*	386
Zanthoxylum capense	Water	68*	272	93.25*	373
	Methanol	65*	260	81*	324
	Dichloromethane	65.75*	263	98.75*	395

(+) = positive control; (-) = negative control; h = hour(s). N = number of dead aphids. Pearson chi-square test $P < 0.05$. * = significant.

Table 4.7: Toxicity (LC_{50}) of plant extracts 24 h post treatment.

Plant species	Extract	Total no. of insects	Slope	LC_{50} (mg mL^{-1})	95% FL	Chi^2	P-value
Ar. afra	Essential oil	1229	0.9277	0.396	[0.24;0.57]	86.39	$P < 0.05$
A. attenuata	Water	1148	1.0393	0.838	[0.41;1.49]	30.47	$P < 0.05$
Z. capense	DCM	1022	1.4938	0.555	[0.433;0.693]	148.23	$P < 0.05$

FL = fiducial limits; P = Probability (P-value associated with null-hypothesis of zero slope).

The cytotoxic effect of the plant extracts towards RWA was statistically significant for the three treatments, and the LC_{50} values showed the essential oil of *Ar. afra* to be the most toxic (LC_{50}

= 0.396 mg mL^{-1}; FL = [0.24;0.57]) followed by DCM extract of *Z. capense* (LC_{50}= 0.555 mg mL^{-1}; FL = [0.41;1.49]) and water extract of *A. attenuata* (LC_{50} value = 0.893 mg mL^{-1}) ; FL = [0.43;0.69] being the least toxic.

Secondary metabolites are known to possess insecticidal properties and their ability to kill or repel insects have been reported (Broussalis *et al.,* 1999). From TLC profiles of the different plant extracts, it was seen that different phytochemicals were present (including terpenes, phenolics and saponins) and the presence of these compounds may provide key roles in intergetrated pest management strategies (both repelling and insecticidal). Terpenes have been reported to have a longer lasting effect through synergising with other compounds (toxins) and act as solvents to facilitate their diffusion across membranes (Hummelbrunner and Isman, 2001; Rattan, 2010). Although durational effects was not part of the study, evidence of the essential oil of *Ar. afra* having a longer lasting effect can be seen in Table 4.6 (158 killed from 1 h to 24 h). At sublethal concentrations, the different plant extracts managed to reduce the pest population at both 1 h and 24 h.

Considering the possible constituents of the water extract of *A. attenuata* and its fast mode of killing, one need to recall the possible effect of saponins found in the extract. The detergent properties of saponins may allow for permeation into cell membranes, releasing substances into cells quicker that result in the fast mode action within the first hour. Saponins are molecules made up of a water-soluble glucidic chain attached to a liposoluble structure known as an aglycone (Thakur *et al.,* 2011). Saponins are found in numerous plant species and some insects (*Platyphora* spp.) sequester saponins from host plants for their own defence (Termonia *et al.,* 2002). The biological role of saponins in plant defence against insects, raised renewed interest as it has been established that they provide feeding deterrents (Shinoda *et al.,* 2002) and in this study the entomotoxicity from the direct spraying bioassay may be considered. In entomotoxicity, the application of saponins has been reported to have possible insecticidal effects on insects when exposed by the cleaving off of an active aglycone molecule whilst feeding (Tava and Avato, 2006). The actual targeted biological pathway causing saponins to be insecticidal has not yet been described (De Geyter *et al.,* 2007). The permeability theory of saponins cannot be taken into consideration as firstly no reports have shown a saponin being able to penetrate the insect's cuticle and even when applied with an abrasive insecticide in order to damage the cuticle, no significant increase in insecticidal ability was observed (Chaieb, 2010).

The toxicity of oils to insects' works via contact action and this can affect biological systems that results in feeding dormancy of a pest (Matsumura and Beemen, 1982). The essential oil has numerous chemical properties within its arsenal and is comprised mostly of monoterpenes and its derivatives. The oil's properties range from deterring a specific pest by repellency and interference with the growth and development of the insect (Mann and Kaufman, 2012), but can have no effect on possible predators of the pest, as they may not be harmed by the residue effects of the oil, unlike with conventional insecticides.

Compounds identified in the DCM extract of *Z. capense* include terpenes, phenolics and flavonoids. Terpenes can be toxic to a variety of insects and their combined effect with other compounds allow for their activity (Rattan, 2010). In the case of terpenes, they may allow for the passage of other compounds through membranes that may be cytotoxic to a pest (Gershenzon and Dudareva, 2007). The availability of terpenes is important when it comes to a mixture of compounds as it allows for better activity through either volatile communication (deterrence or an attractant) and enhanced toxicity by permeating itself and other compounds to a target area (Akiyama *et al.*, 2005; Kunert *et al.*, 2005; Pemmaraju *et al.*, 2013). Two of the major constituents of *Z. capense* DCM include phytol and (E)-β-farnesene of which both are regarded as insect repellents. Phytol is also a major constituent of different flora reported to have good repellent and insecticidal properties (Cruz-Ezstrada, 2013).

4.4 Repellency

4.4.1 Olfactometric response of RWAs

The results derived from the no choice test using the olfactometer indicate that aphids are repelled by the majority of the plant extracts over a 10 min interval. The two variables chosen to measure the efficacy of the extracts in repelling the aphids were the number of visits (Table 4.8) and time spent (Table 4.9) in the arms of the olfactometer. The mean duration was calculated as a factor indicating the strength of repellency of a particular treatment.

Number of visits

The analysis of the visits data by a generalized linear model (GLIM) showed that singularly, the effect of plant species, solvent extracts (water, DCM and methanol) and crude plant extract treatments fitted the model (Table 4.8). This analysis showed that, overall, there were no

statistically significant differences between plant species, solvent extracts and the crude plant extracts regarding their effectiveness in number of visits.

Table 4.8: Number of visits: Likelihood ratio statistics for model effects.

Effect	DF	F-Value	P-value
Plant species	2	0.57	0.5672
Extract (Solvent)	3	0.99	0.4005
Crude plant extracts	4	1.85	0.1202

DF= degrees of freedom; F= F- statistics; P = probability.

However, the results presented in Table 4.8 do not imply that the plant extracts (treatments) were not effective (since Table 4.8 only suggests plant treatments do no differ in efficacy), nor do the overall test results rule out the possibility that particular plant extracts differed significantly in their efficacy. A more detailed analysis of these aspects presented below showed that the efficacy of a particular plant extract can be assessed by comparing the number of visits into the treated arms with the total number of visits (treatment and control): a particular plant extract can be considered effective if the probability of visits in the treated arms is less than 0.5. For all plant extract treatments, the estimate of the probability of a visit to the treatment arms of the olfactometer, together with a 95% confidence interval (CI) for that probability as derived from the fit of the above mentioned GLIM (Table 4.9). With the exception of *Ar. afra* water extract, all plant extracts proved to be effective treatments as suggested by the fact that the estimated probabilities of a visit in the treatment arms was below 0.5. The most effective extracts from *Ar. afra*, *A. attenuata* and *Z. capense* were DCM, water and methanol respectively. These extracts were also significantly more effective than their control treatments ($P < 0.05$), indicated by the fact that the upper limit of the 95% CI for the probability of visits to the treatment arms were less than 0.5 (Table 4.9).

Table 4.9: Probability of RWA visits occurring in treated arms of the olfactometer during a 10 min interval.

Plant species	N	Extract	Estimate	95% CI	P-value
Artemisia afra	20	Dichloromethane	0.3092	0.1812 to 0.4753	0.0254*
	20	Methanol	0.4234	0.3100 to 0.5454	0.2174
	20	Water	0.5570	0.4405 to 0.6676	0.3375
	20	Essential oil	0.4839	0.3296 to 0.6413	0.8446
Agave attenuata	20	Dichloromethane	0.3981	0.2719 to 0.5394	0.1562
	20	Methanol	0.4122	0.2940 to 0.5415	0.1832
	20	Water	0.3782	0.2757 to 0.4929	0.0377*
Zanthoxylum capense	20	Dichloromethane	0.4252	0.3153 to 0.5429	0.2121
	20	Methanol	0.2817	0.1754 to 0.4196	0.0027*
	20	Water	0.4051	0.2858 to 0.5367	0.1562

CI = confidence interval; N = number of replicate(s); P = probability; * = significant.

To determine the most effective treatment, a pairwise comparison (Table 4.10) was done and the relative efficacy of those treatments was characterized by the odds ratio (OR) namely the odds of a visit occurring in the treatment arms for treatment A over the odds of a visit occurring in the treatment arm for treatment B. An odds ratio < 1 indicates that a particular treatment is more effective than another for a visit to occur. Results confirmed that, the *Z. capense* methanol extract was the most effective treatment for the number of visits of all plant extracts followed by the DCM extract of *Ar. afra*.

Table 4.10: Pairwise odds ratio[1] between treatment combinations.

Treatment A		Treatment B				
Plant species	**Extract**	**Plant species**	**Extract**	**Odds Ratio**	**95% CI**	**P-value**
Artemisia afra	Dichloromethane	*Artemisia afra*	Methanol	0.6097	0.2583 to 1.4393	0.2589
			Water	0.3559	0.1527 to 0.8296	0.0167
			Essential oil	0.4775	0.1836 to 1.2416	0.1295
		Agave attenuata	Dichloromethane	0.6769	0.2732 to 1.6773	0.3993
			Methanol	0.6382	0.2656 to 1.5333	0.3153
			Water	0.7359	0.3157 to 1.7157	0.4777
		Zanthoxylum capense	Dichloromethane	0.6052	0.2589 to 1.4148	0.2464
			Methanol	1.1414	0.4489 to 2.9022	0.7811
			Water	0.6574	0.2720 to 1.5892	0.3517

[1] Ratio Treatment A / Treatment B of odds for visits occurring in the treatment arms of olfactometer; CI= confidence interval; P = probability.

Time spent

For each pair of time measurements taken under a particular treatment (plant extract), the difference between the times spent in the treatment and control arms of the olfactometer was calculated. The resulting differences were analysed using a two-way analysis of variance (ANOVA). The main effect of plant species and extract (solvent) proved to be not statistically significant, but the plant extracts interaction was statistically significant (Table 4.11).

Table 4.11: Time spent: Two-way ANOVA fitting effects of the model.

Effect	Num DF	Den DF	F-Value	P-value
Plant species	2	190	1.95	0.1447
Extract (Solvent)	3	190	0.84	0.4734
Crude plant extract	4	190	3.17	0.0151*

DF = degrees of freedom; P = probability; * = significant.

The efficacy of a particular plant extract can be assessed by estimating the mean difference in time spent in the treatment arms versus the control arms of the olfactometer; a mean difference

below zero would indicate an efficacious treatment. The estimate of the mean difference of time spent in the treatment arms versus the control arms for all plant extracts, together with a 95% CI, as derived from the two-way ANOVA are presented in Table 4.12. All plant extracts except *Ar. afra* water have a repellent effect (mean < 0) relative to their control treatments. In terms of ranking, *A. attenuata* water extract and DCM extracts of *Ar. afra* and *Z. capense* had the lowest mean difference (highest efficacy) among the extracts (Table 4.12).

Table 4.12: Mean time spent in treatment arms compared to controls during 10 min interval.

Plant species	Extract	Mean (s)	95% CI	P-value
Artemisia afra	Dichloromethane	-216.58	-350 to -74.46	0.0028*
	Methanol	-0.1620	-138.38 to 138.05	0.9982
	Water	115.10	-23.11 to 253.32	0.1021
	Essential oil	-43.05	-181.27 to 95.16	0.5397
Agave attenuata	Dichloromethane	-72.87	-211.09 to 65.33	0.2996
	Methanol	-120.14	-258.35 to 18.08	0.0881
	Water	-243.29	-381.51 to 105.08	0.0006*
Zanthoxylum capense	Dichloromethane	-143.78	-281.99 to -5.56	0.0415*
	Methanol	-77.09	-215.31 to 61.12	0.2726
	Water	-62.70	-200.88 to 75.44	0.3722

CI = confidence interval; P = probability; s = seconds; * = significant.

The pairwise differences indicated the efficacy of the plant extracts against one another and a particular treatment A could be considered more effective than Treatment B if the mean difference (MD) is < 0 (Table 4.13). The two most effective extracts were the DCM extract of *Ar. afra* and the water extract of *A. attenuata*. In terms of effectiveness, the plant extracts showed variation for time spent in treatment arms for both polar (methanol and water) and non-polar extracts (DCM and oil) further justifying the model effects represented by the Two-way ANOVA (Table 4.11).

Table 4.13: Pairwise differences in mean time spent between treatment arms of the olfactometer in 10 min interval.

Treatment A		**Treatment B**				
Plant species	**Extract**	**Plant species**	**Extract**	**Mean difference**	**95% CI**	**P-value**
Artemisia afra	Dichloromethane	*Artemisia afra*	Methanol	-212.42	-364.99 to 25.93	0.0333*
			Water	-327.69	-407.89 to -16.95	0.0011*
			Essential oil	-169.53	-523.15 to 132.22	0.0887
		Agave attenuata	Dichloromethane	-139.71	-335.17 to 55.76	0.1602
			Methanol	-92.44	-287.91 to 103.02	0.3521
			Water	30.71	-164.76 to 226.17	0.7570
		Zanthoxylum capense	Dichloromethane	-68.80	-264.27 to 126.66	0.4883
			Methanol	-135.49	-330.96 to 59.97	0.1731
			Water	-149.91	-345.38 to 45.55	0.1320

CI = confidence interval; P = probability.

Mean duration

The mean duration effect of the plant extracts represents the summation of events (time spent and visits) used to quantify the RWA's behaviour towards the different plant extracts. The repelling ability of a particular plant extract is considered effective if the mean duration is low. A longer mean duration value indicated that a particular plant extract is less efficacious as the aphid is likely to spend more time per visit in the treatment arms. All treatments proved significant ($P = 0.0378$) using a one-way ANOVA Tukey-test ($P < 0.05$). In terms of ranking of extracts for the plant species, the essential oil of *Ar. afra* proved to be the most effective for *Ar. Afra* extracts, water extract for *A. attenuata* extracts and the DCM extract for *Z. capense* extracts. Overall, the water extract of *A. attenuata* showed the lowest mean duration for all treatments (Fig. 4.7).

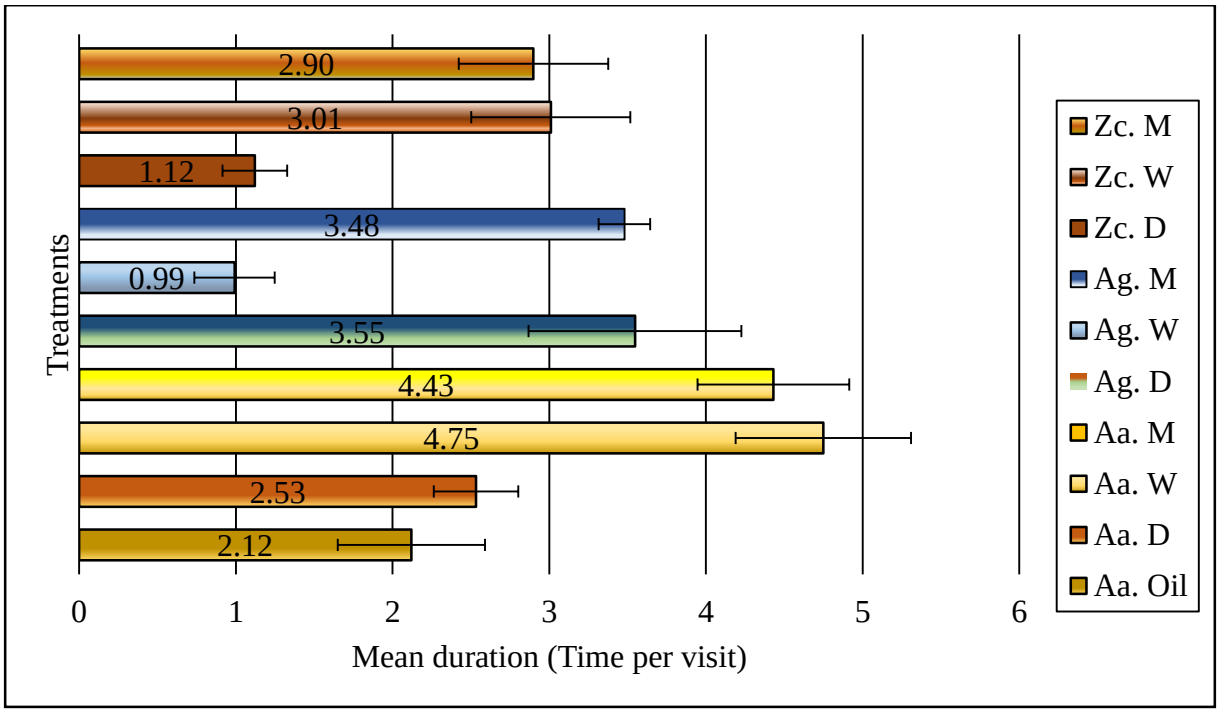

Figure 4.7: Mean duration ± S.E of RWA in different treatment arms of olfactometer during a 10 min interval. Tukey's test ($P < 0.05$). Aa = *Ar. afra*, Ag. = *A. attenuata* , Zc = *Z. capense*; W = water extract, D = DCM extract and M = methanol extract.

The delivery system of the four arm olfactometer provided an efficient measure to study RWA behaviour towards the different volatiles presented in a closed chamber. Significant responses were obtained and in some instances, the insect chose to spend less time in the treatment arms, showing that the RWA could perceive and discriminate the odours presented to it. The initial screening for repellency using the olfactometer reveals that the essential oil and certain plant extracts have the ability to repel aphids. It is the excitation of receptors in an insect's olfactory system that brings about their behavioural response (Webster *et al.*, 2012). The compounds present in the different plant extracts may act singularly or in a combined synergistic manner to bring about repellency (Webster *et al.*, 2012).

From extracts of *Ar. afra*, the essential oil and DCM proved most efficient in repelling the RWA. It has been found that volatile compounds such as camphor, eucalyptol and thujone have the ability to act as fumigant and neurotoxic substances towards insects (Gillij *et al.*, 2008; Rattan, 2010). Both extracts possessed similar volatile compounds e.g. eucalyptol and camphor as well as α- and ß- thujone that might act as repellents towards the aphids. Menthol and 1,8-cineole have been reported to act as a repellent towards RWA (Prinsloo *et al.*, 2007). Essential oils are volatile oils that are made up mostly of terpenoids and their repellent properties have

been investigated against numerous insects. The monoterpene constituents of some oils have been reported to repel *Culex* species of mosquitoes (Choi *et al.*, 2002). Compounds such as turmerone and dehydroturmerone from turmeric oil have shown to be strong repellents to grain pests such as the red flour beetle, *Tribolium casteneum* (Herbst.) (Chahal *et al.*, 2005). Oils from *Ocimum* spp. have generally been used as repellents (Tawatsin *et al.*, 2001; Kiplang'at and Mwangi, 2012) against mosquitoes. The duration effect of oils varies for repellency with some oils showing efficacy from 2 hours up to 4 weeks (Ansari and Razdan, 1994; Tawatsin *et al.*, 2001; Oyedela *et al.*, 2002).

The DCM extract of *Z. capense* had the lowest mean duration between the extracts of *Z. capense*. It was established through GC-MS that phytol and E–(β)-farnesene were found in its constituents. Phytol is a highly repellent diterpene that has been reported to be repellent against mosquitoes (Odalo *et al.*, 2005). E–(β)-farnesene is a volatile sesquiterpene found in various plants and animals with a primary function of chemical communication and is well reported as an alarm pheromone (Vandermoten *et al.*, 2012). The release of an alarm pheromone usually agitates aphids, removing them from their host plants. Alate (wingless) aphids are recognized as being more sensitive to the alarm pheromone release and often don't colonize a host plant emitting the volatile (Vandermoten *et al.*, 2012). The presence of two repellent volatiles in the extract may work synergistically in repelling the RWA. Combination of volatiles tend to affect the behavioural responses of insects better than a singular compound and studies have reported this with black bean aphid, *Aphis fabae* and maize weevil, *Sitophilus zeamais* (Webster *et al.*, 2008; Ukeh *et al.*, 2012).

Most repellent compounds reported in literature are non-polar and it is expected that they would be the active compounds in repelling RWAs, however from what can be observed from the behavioural responses in the olfactometer where the water extract of *A. attenuata* showed the lowest mean duration for all treatments against RWA, insects can also be sensitive to polar compounds. The exact nature of repellency towards *A. attenuata* water extract cannot be ascribed to anything else except an excitation in the antennal complex of the RWA, as polar compounds are known to excite olfactory neurons in the antennae of insects (Meijerink and van Loon, 1999). Although not a great deal of work has been done on agricultural pests, many reports show mosquitoes are repelled by polar substances such as lactic acid (Shirai *et al.*, 2001). In a closed environment such as an olfactometer, the extract's ability to cause repellency

was well studied as the experimental setup firstly removes all other odours present, focusing primarily on the presence of odours provided by treatments.

4.4.2 Leaf settling bioassay

Susceptible and resistant wheat plants were treated with the essential oil and plant extracts prior to infestation as described in chapter 3 (3.2.5.2). A no-choice aphid-settling test (Ninkovic *et al*. 2002) was used to assess the aphid's acceptance of plants exposed to the essential oil and plant extracts. Data was analysed for the distribution of aphids that settled on treated wheat plants at 2 and 72 hpi compared to control plants and a P-value was assigned for the ratio of active treatments against the control. The effect of the treatments was considered effective if $P < 0.05$.

During the first 2 hpi, the essential oil, water and methanol extracts of *Ar. Afra*, as well as the DCM extract of *A. attenuata* significantly reduced aphid settling by 53.12, 43.75, 46.88 and 40.63% respectively on the susceptible cultivar (Table 4.14), while the same treatments only somewhat limited the settling ability in resistant wheat when compared to the controls (Table 4.15). The *Z. capense* methanol extract, although not significant, managed to lower aphid settling by 34.38% in the susceptible cultivar and in the resistant, the DCM extract lowered settling by 26.09%. At 72 hpi however, susceptible plants treated with the water extract of *Ar. afra* reduced aphid settling by 60% ($P = 0.0116$) and the DCM extract of *A. attenuata* had 44 % less aphids that settled on them. In the resistant wheat, it was only extracts of *A. attenuata* and the methanol extract *of Z. capense* that lowered aphid settling to a certain degree (37 to 20%) when compared to the control.

In general, the mean number of aphids that settled on both wheat varieties increased over time, however some treatments performed better at managing the increase in number. The essential oil and DCM treatments of *Ar. afra* proved not to be successful in managing aphid settling on both susceptible and resistant wheat cultivars over a 72 h period.

Table 4.14: Mean number of RWAs that settled on a **susceptible** wheat leaf at 2 and 72 hpi.

Treatments		Mean no. aphids[1] ± S.D settling per leaf (n = 5)			
		2 h	P-value	72 h	P-value
Ar. afra	Essential oil	3.0 ± 1.58	0.0032*	8.4 ± 1.30	0.5440
	Dichloromethane	5.8 ± 1.10	0.6406	13.4 ± 6.58	0.2540
	Water	3.6 ± 1.14	0.0176*	4.0 ± 2.00	0.0116*
	Methanol	3.4 ± 1.48	0.0104*	8.2 ± 3.90	0.4927
A. attenuata	Dichloromethane	3.8 ± 1.58	0.0286*	5.6 ± 2.00	0.0736
	Water	4.4 ± 2.27	0.1000	10 ± 3.39	1.000
	Methanol	4.4 ± 2.30	0.1000	9.80 ± 5.30	0.9416
Z. capense	Dichloromethane	4.8 ± 0.41	0.1952	8.2 ± 3.91	0.4927
	Water	4.4 ± 1.14	0.1000	9.2 ± 1.64	0.7662
	Methanol	4.2 ± 0.45	0.0682	8.4 ± 4.04	0.5440
Control	1 % DMSO	6.4 ± 2.07	-	10 ± 3.00	-

[1]Ten aphids were used in each settling test. All extracts were dissolved in 1% DMSO (1 g L^{-1}). P = probability. * = significant. ($P < 0.05$).

Table 4.15: Mean number of RWAs that settled on a treated **resistant** wheat leaf at 2 and 72 hpi.

Treatments		Mean no. aphids[1] ± S.D settling per leaf (n = 5)			
		2 h	P-value	72 h	P-value
Ar. afra	Essential oil	3.6 ± 1.34	0.3651	7.6 ± 3.58	0.7842
	Dichloromethane	4.8 ± 3.11	0.8653	7.2 ± 4.09	0.6441
	Water	3.6 ± 1.48	0.3651	8.8 ± 2.39	0.7918
	Methanol	3.8 ± 1.48	0.4736	7.2 ± 3.96	0.6441
A. attenuata	Dichloromethane	3.6 ± 0.55	0.3651	5.2 ± 1.48	0.1404
	Water	5.4 ± 1.34	0.5112	6.4 ± 2.81	0.3939
	Methanol	5.0 ± 1.01	0.7372	5.8 ± 1.40	0.2469
Z. capense	Dichloromethane	3.4 ± 1.67	0.2718	7.2 ± 3.58	0.7842
	Water	3.6 ± 1.82	0.3651	8.8 ± 2.31	0.7918
	Methanol	5.6 ± 1.52	0.4163	6.6 ± 1.37	0.4514
Control	1 % DMSO	4.6 ± 2.30	-	8.2 ± 3.36	-

[1]Ten aphids were used in each settling test. All extracts were dissolved in 1% DMSO (1 g L^{-1}). P = probability. * = significant. ($P < 0.05$).

4.4.3 Symptom analysis

It was demonstrated that the settling ability of the aphids over 72 h was compromised by the application of some plant extracts prior to infestation. The treated plants were left for 7 days and analysed for phenotypic damage caused by the aphids (Fig. 4.8). A one-way ANOVA was used for the analysis of the mean damage score, with significance measured at $P < 0.05$; similarly, a one-way ANOVA was used for the analysis of the average number of live aphids after 7 days on treated plants.

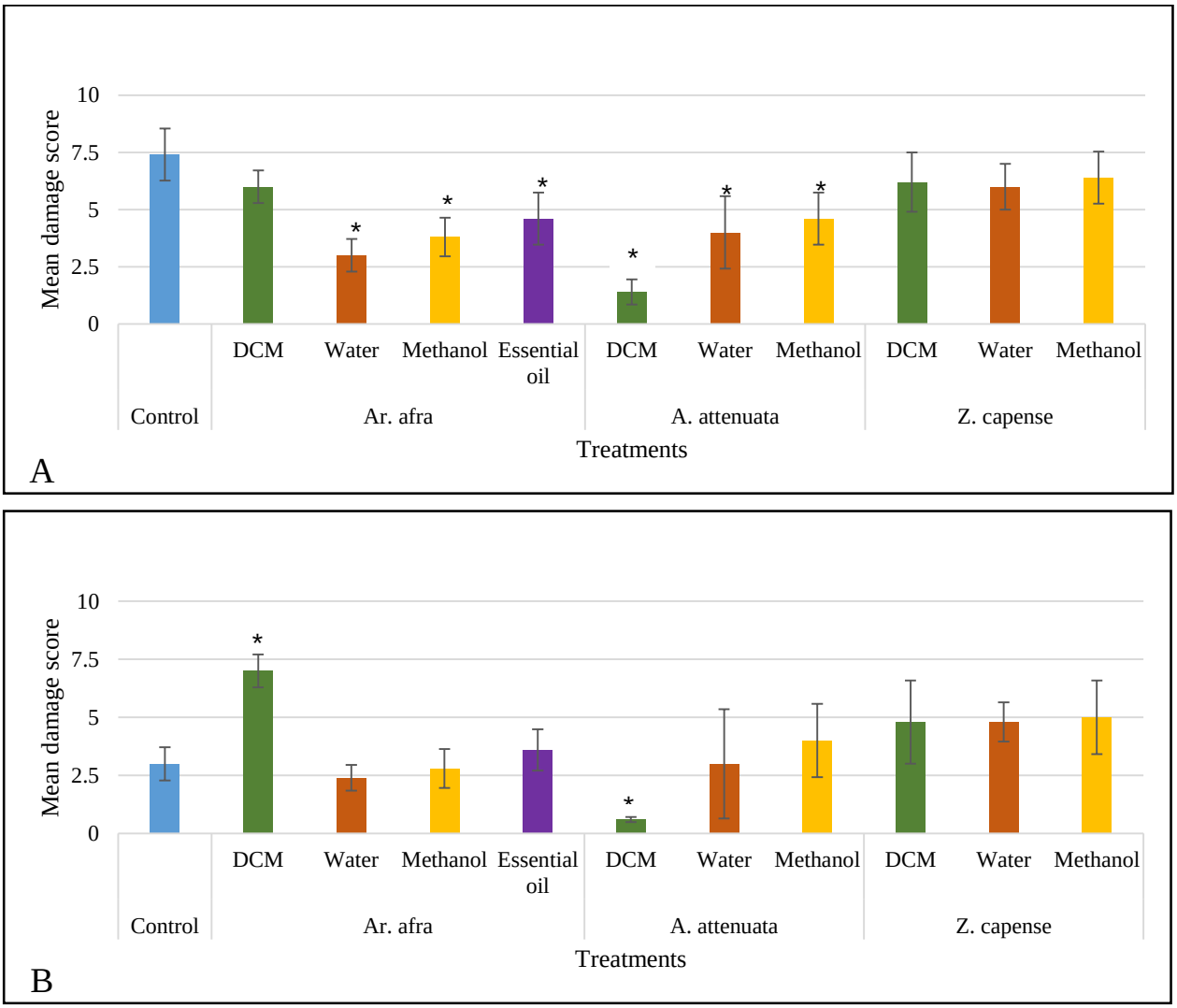

Figure 4.8: Mean damage rating scores ± SE of treated Tugela (A) and Tugela *DN* (B) wheat plants after 7 days of infestation with RWASA1. Scores: $1 \leq 2.5$ represented a highly resistant plant (no leaf curling), $2.6 \leq 6.5$ represented a medium resistant plant, $6.6 \leq 10$ represented a susceptible plant (curled leaves and severe chlorosis). * = Significant ($P < 0.05$).

Repellency of RWA from the direct application of extracts on wheat plants as opposed to the closed chambers of an olfactometer (Table 4.10- 4.13) showed that some of the weaker extracts (Aa. water, Ag. DCM) showed lower damage ratings as opposed to the more repellent extracts (Aa. oil, Aa. DCM, Zc. DCM) from the olfactometer bioassay. The water extract of *Ag. attenuata* was consistent in being repellent in both the olfactometer bioassay (Fig. 4.7) and leaf setlling assay in controlling disease symptoms (Fig. 4.8 A and B). Thus the effectiveness of these extracts against the RWA cannot be attributed to odour responses alone. The different plant extract treatments showed to be able to control disease symptoms by reducing the severity caused by infestation when comparing to the control. In particular, the DCM extract treatment of *A. attenuata* and water extract *Ar. afra* managed to lower disease symptoms significantly in both susceptible and resistant wheat cultivars (Fig. 4.8).

Table 4.16: Mean number of live aphids after 7 days as a percentage of the control.

Treatment		**Tugela**	**Tugela *DN***
		% of Control	% of Control
	Control	100	100
Ar. afra	Essential Oil	112	78.3
	Dichloromethane	109	129
	Water	51	73
	Methanol	51	96
A. attenuata	Dichloromethane	32	51.8
	Water	72.4	81.1
	Methanol	75.7	92.9
Z. capense	Dichloromethane	86	78.5
	Water	87.8	114
	Methanol	86.6	113
F-statistic		13.51	1.13
P-value		P < 0.05	0.358

P = probability; Tukeys test P < 0.05.

The application of most foliar plant extract treatments lowered disease symptoms in infested wheat plants. In general, treatments that limited the aphid's settling ability on susceptible wheat plants (DCM extract of *A. attenuata* and water extract of *Ar. afra*) during the 72 h period, also lowered the prevalence of disease symptoms associated with RWA infestation significantly ($P_{Ag.\ DCM} < 0.0001$; $P_{Aa.\ water} < 0.0001$). Considering the average number of live aphids found on the treated plants after 7 days (Table 4.16), there seems to be a relationship between the number of live aphids and the damage scores (Fig. 4.8). The DCM extract treatment of *A. attenuata* showed 68 and 48.2% lower number of live aphids when compared to the control in susceptible and resistant wheat plants respectively and also the lowest damage scores in both wheat varieties. Although symptom analysis revealed that resistance was achieved in susceptible plants after seven days against the RWA through the application of these DCM extracts (Fig. 4.8 A), the essential oil and methanol of *Ar. afra* as well as the water and methanol extracts of *A. attenuata* were also effective in lowering disease symptoms in the susceptible wheat, although the number of live aphids that settled after 72 were not significantly less (Table 4.15). The resistant wheat cultivar had the advantage of possessing the *Dn*1 gene which confers resistance to RWASA1. In general it was found that lower aphid numbers on wheat plants, presented less damage on plants. It was clear that almost no symptoms were present on the resistant plants treated with the DCM extract of *A. attenuata* (Fig. 4.5 B).

Various factors influence an aphid's performance on settling on host plants, including the plant genotype and the developmental stage (Reinink *et al.,* 1989). The developmental stage is a crucial part as aphids are known to leave older plants in search of younger plants (Harrington and Taylor, 1990). This may be attributed to nutritional quality or preference for softer tissue as older foliage is harder and tougher, making it difficult to penetrate. The plant genotype plays a crucial role in establishing what the mechanism of resistance is used against the aphid and whether antibiosis, antixenosis or tolerance strategies are employed (Thomas and Waage, 1996).

Aphids feed on a plant only when they accept it as food source and the number of aphids settling on treated plant leaves is a good indicator of their preference. From the different treatments the two extracts that proved to be effective in both wheat cultivars were *Ar. afra* water extract and the DCM extract of *A. attenuata*. Plants treated with these two extracts seem to exhibit antibiosis as the number of aphids present after 7 days was lower than the control plants. Plants

that exhibit antibiosis affect biological development of an insect and in turn limit the level of infestation (Goggin, 2007).

Plants have the ability to produce many volatiles that influence the behaviour of insects to utilise or not utilise the plants resources for feeding and oviposition (Van den Berg *et al.*, 2008). Volatiles present host cues for phytophagous insects to distinguish between host and non-host plants (Sole *et al.*, 2010). Olfaction plays an important role in colonization of host plants by insects such as RWA (Powell and Hardie, 2001). The ability for insects to differentiate between host and non-host plants lies largely to what volatiles the plants emit and phytophagous insects make use of this mechanism (Webster *et al.*, 2012). Phytophagous insects are attracted to plants by a blend of volatile compounds (Bruce *et al.*, 2005; Webster *et al.*, 2012).

Artemisia afra polar extracts showed to be weak repellents in the olfactometer bioassay (Fig. 4.7), thus its ability to lower aphid settling may be associated with other factors, probably the actual defence mechanism of wheat plants or other compounds present. A study on plant semiochemical applications on RWA populations influencing their settling ability by Prinsloo *et al.* (2007) indicated that no significant effects could be gathered in field trials although repellent products showed positive results in olfactometric studies. The *A. attenuata* DCM extract however, showed repellent ability from the olfactometer bioassay and also good insecticidal properties. Methyl jasmonate was also found to be part of its constituents in its GC-MS profile (Table 4.5). Methyl jasmonate (MeJA) is a volatile organic compound derived from jasmonic acid and may act as inducer of plant defence (Walters *et al.*, 2012). In wheat, cis-jasmone was reported to increase the production of phenolic compounds and hydroxamic acids (Moraes *et al.*, 2008). Hydroxamic acids are known to confer resistance in wheat against aphids (Niemeyer *et al.*, 1992). The hydroxamic acids are phagorepellents in wheat that affect aphid feeding and delay aphid feeding during probing (Giovich and Niemeyer, 1991). Methyl jasmonate can also induce a plant to produce multiple different types of defense chemicals such as phytoalexins (Xu *et al.*, 2003), nicotine or proteinase inhibitors (Farmer and Ryan, 1990). The proteinase inhibitors interfere with the insect's digestive process and discourage the insect from eating the plant again (Xu *et al.*, 2003). Although MeJA was not the only compound found in the *A. attenuata* DCM extract, its presence cannot be ruled out as it may be one of the causes for reduced aphid settling.

The exact nature of how the plant extracts caused reduced aphid settling and minimised damage is unknown. It has been established however that these extracts do possess insecticidal and repellent properties and may have caused an avoidance strategy through numerous channels such as contact irritancy caused by the treatments or a change in volatile mixture that is not desirable for host location or a mediated defence response by the host plants that deters the aphids from settling on host plants. Contact irritancy occurs when an insect makes physical contact with a chemical residue on a surface that usually disrupts normal behaviour (Potikasikorn *et al.,* 2005). Insecticides such as dichlorodiphenyltrichloroethane (DDT) have shown to change behavioural response of mosquitoes (*Anopheles minimus*) through contact irritancy (Chareonviriyaphap *et al.,* 2001).

4.5 Initiation of defence responses by crude plant extracts.

From the results obtained so far, the largest impact on repellency, leaf settling, aphid population and disease symptoms on wheat was caused by *Ar. afra* and *A. attenuata* extracts and essential oil. Therefore the decision was made to investigate only the effect of these two plant extracts on the *in vitro* activities of two PR-proteins, ß-1,3-glucanase and peroxidase. Data was analysed separately for each time point using a three-way ANOVA fitting the factors treatment, cultivar, and condition (uninfested and infested), and all interaction terms; significance was measured at $P < 0.05$. The t-test statistics and P-values associated with the comparison of the treatments were calucated. The application of the crude plant extract treatments prior to infestation had a significant effect on β-1,3-glucanase (Table 4.17) and peroxidase enzyme activities (Table 4.18) under conditions of infestation and non-infestation for both resistant and susceptible wheat cultivars over a 144 h period.

Table 4.17: Analysis of variance fitting the effects of plant extract treatments on wheat plants for β-1,3-glucanase enzyme activity under different conditions at different time intervals.

Condition	Time interval (h)	NUM DF	DF	F-Value	P-Value
IR	24	5	48	703.63	< 0.0001
IS	24	5	48	481.68	< 0.0001
R	24	5	48	399.06	< 0.0001
S	24	5	48	153.74	< 0.0001
IR	48	5	48	281.03	< 0.0001
IS	48	5	48	152.11	< 0.0001
R	48	5	48	168.65	< 0.0001
S	48	5	48	97.59	< 0.0001
IR	72	5	48	384.99	< 0.0001
IS	72	5	48	320.41	< 0.0001
R	72	5	48	47.82	< 0.0001
S	72	5	48	217.53	< 0.0001
IR	144	5	48	1508.86	< 0.0001
IS	144	5	48	143.59	< 0.0001
R	144	5	48	56.28	< 0.0001
S	144	5	48	52.03	< 0.0001

IR = infested resistant; R = uninfested resistant; IS = infested susceptible; S = uninfested susceptible; DF = degrees of freedom; F = F statistic for treatment (comparing 6 treatments); P = probability.

Table 4.18: Analysis of variance fitting the effects of plant extract treatments on wheat plants for peroxidase enzyme activity under different conditions at different time intervals.

Condition	Time interval (h)	NUM DF	DF	F-Value	P-Value
IR	24	5	48	26.62	< 0.0001
IS	24	5	48	20.19	< 0.0001
R	24	5	48	46.15	< 0.0001
S	24	5	48	11.66	< 0.0001
IR	48	5	48	15.93	< 0.0001
IS	48	5	48	6.75	< 0.0001
R	48	5	48	25.16	< 0.0001
S	48	5	48	16.46	< 0.0001
IR	72	5	48	22.69	< 0.0001
IS	72	5	48	44.64	< 0.0001
R	72	5	48	16.24	< 0.0001
S	72	5	48	25.85	< 0.0001
IR	144	5	48	108.29	< 0.0001
IS	144	5	48	19.19	< 0.0001
R	144	5	48	81.24	< 0.0001
S	144	5	48	13.07	< 0.0001

IR = infested resistant; R = uninfested resistant; IS = infested susceptible; S = uninfested susceptible DF = degrees of freedom; F= F statistic for treatment (comparing 6 treatments); P = probability.

4.5.1 The effect of *Ar. afra* extracts on the *in vitro* enzyme activities of β-1,3-glucanase and peroxidase in wheat.

The application of crude *Ar. afra* plant extract treatments affected both β-1,3-glucanase and peroxidase activities in susceptible and resistant wheat plants under conditions of infestation and non-infestation with RWA's (Fig. 4.9 and 4.10). The water extract is mainly responsible for the significant increase ($P < 0.0001$) in β-1,3-glucanase activity in uninfested susceptible and resistant wheat plants (Fig. 4.6 A, C). In the infested plants treated with the essential oil, β-1,3-glucanase enzyme activity only increased after 48 h and was significantly the highest at 144 h in the resistant plants ($t = -13.23$; $P < 0.0001$) compared to the control (Fig. 4.6 D).

Peroxidase activity also increased significantly through application of the essential oil ($P < 0.0001$) and water extracts ($P < 0.0001$) for both uninfested susceptible and resistant plants (Fig. 4.7 A and C). In the infested resistant plants, peroxidase enzyme activity was 1.75 fold higher at 144 h ($t = -12.41$; $P < 0.0001$) than in the control when treated with the essential oil (Fig. 4.7 D). This increase in peroxidase activity corresponded with increased β-1,3-glucanase enzyme activity (Fig. 4.6 D). If we consider the repellent nature of the essential oil from previous bioassays, it can be speculated that during the first 24 h, aphids were repelled by the essential oil and therefore the plant did not respond in inducing a defence response. However after 48 h, aphids were no longer repelled and the plants respond to aphid infestation ensuing increased β-1,3-glucanase and peroxidase activities.

Susceptible **Resistant**

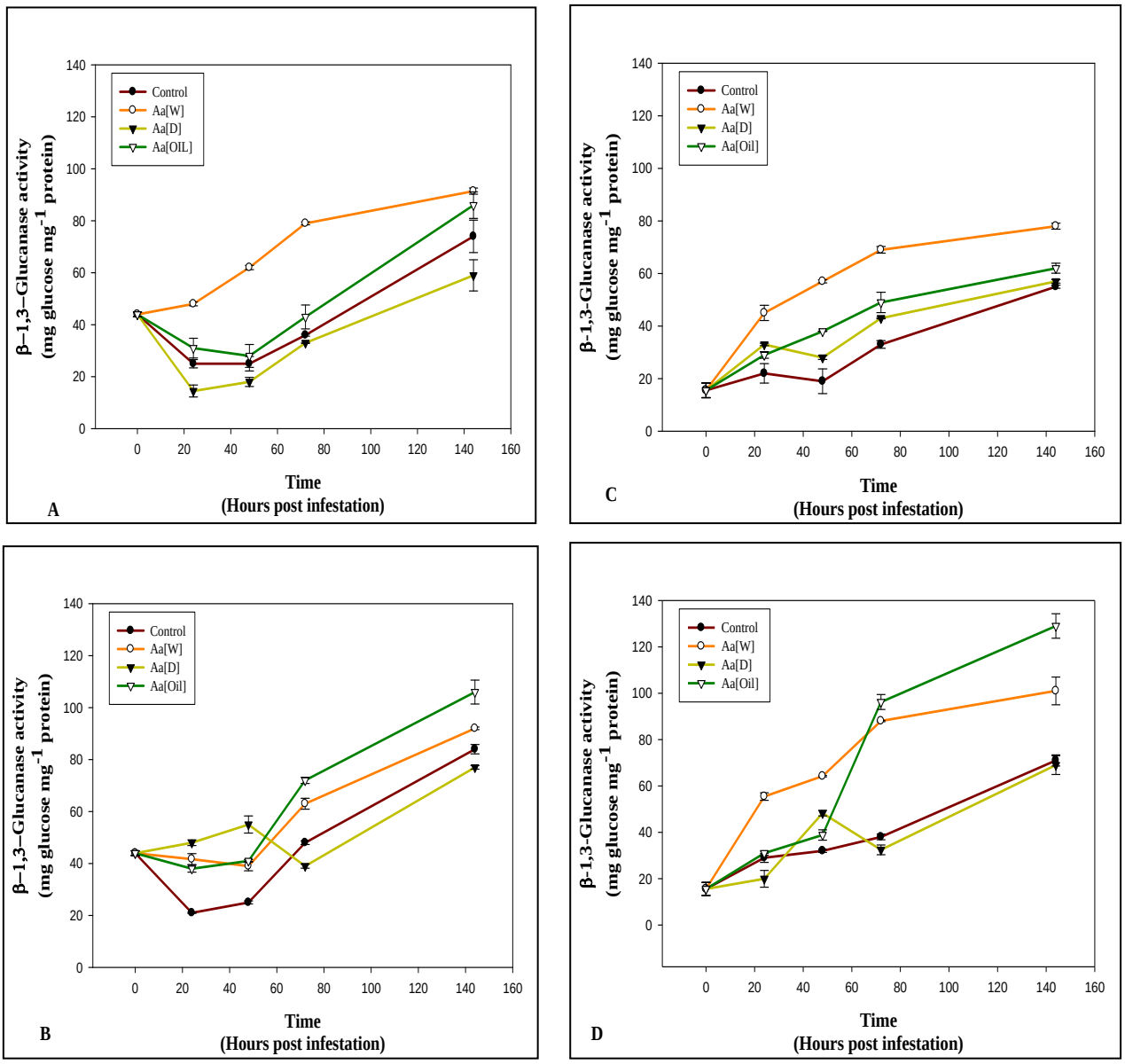

Figure 4.9: Effect of *Ar. afra* crude plant extracts on β-1,3-glucanase activity in wheat plants infested with RWASA1 from 0 to 144 h after foliar treatment. Values are means ± SD (n = 3). Aa [W] = *Ar. afra* water extract, Aa [D] = *Ar. afra* DCM extract, Aa [OIL] = *Ar. afra* essential oil. A, C = Uninfested; B, D = Infested.

Susceptible **Resistant**

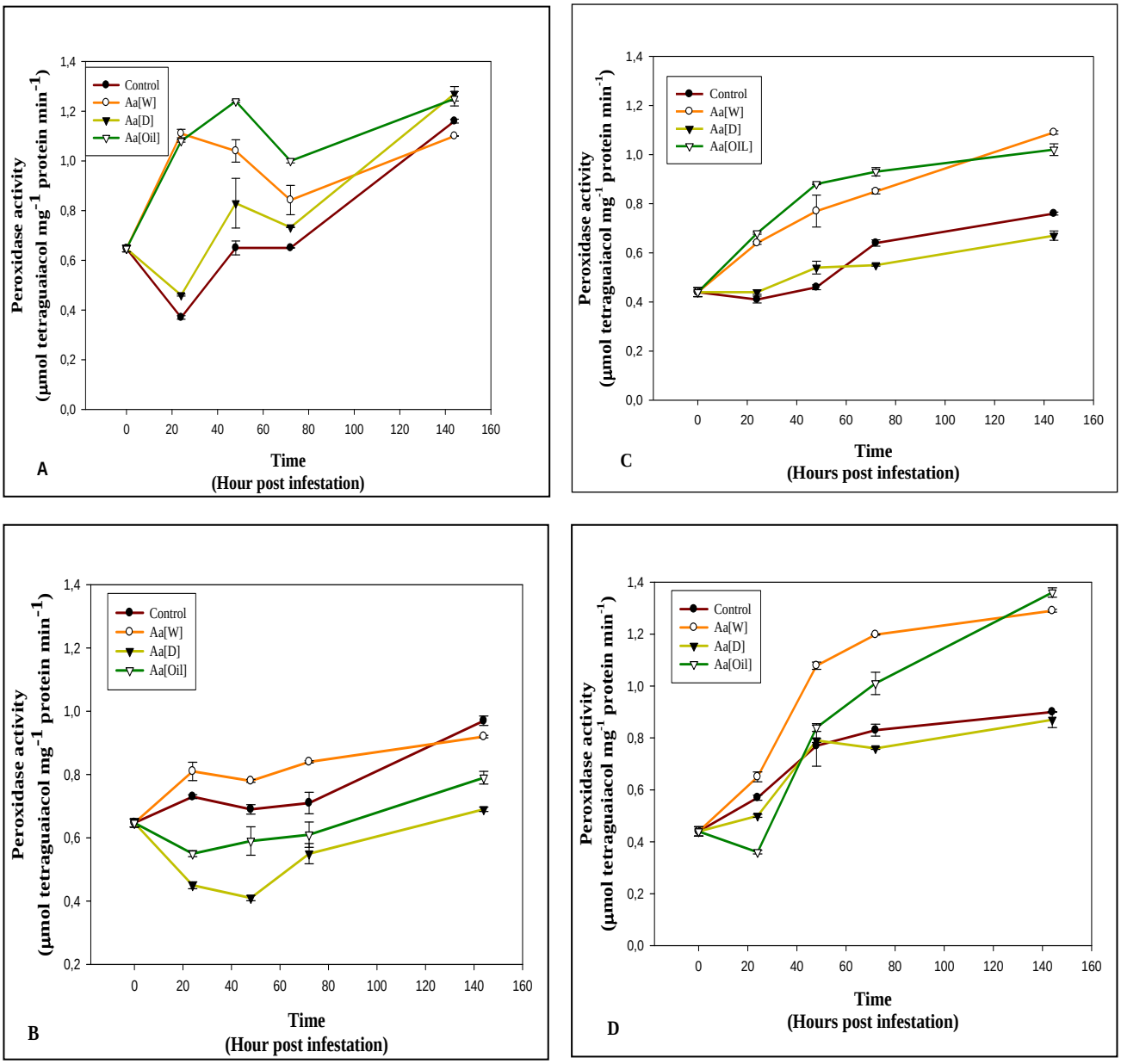

Figure 4.10: Effect of *Ar. afra* crude plant extracts on peroxidase activity in wheat infested with RWASA1 from 0 to 144h after foliar treatment. Values are means ± SD (n = 3). Aa [W] = *Ar. afra* water extract, Aa [D] = *Ar. afra* DCM extract, Aa [OIL] = *Ar. afra* essential oil. A, C = Uninfested; B, D = Infested.

4.5.2 The effect of *A. attenuata* extracts on the *in vitro* enzyme activities of β-1,3-glucanase and peroxidase in wheat.

Treatment with the crude *A. attenuata* extracts affected both β-1,3-glucanase and peroxidase activities significantly ($P < 0.05$) in wheat plants under conditions of infestation and non-infestation with RWA (Fig. 4.8 and 4.9). In comparison to the controls, uninfested susceptible and resistant wheat plants treated with *A. attenuata* water extract resulted in higher β-1,3-glucanase activities throughout the 144 h period (Fig 4.8, A and C). From 24 h, enzyme activity increased significantly by 1.73 fold ($t = -8.51$; $P < 0.0001$) and peaked at 144 h with 1.81 fold higher activity ($t = -5.94$; $P < 0.0001$) in uninfested susceptible wheat plants, treated with *A. attenuata* water extract measured against the control (Fig. 4.8 A). The DCM extract of *A. attenuata* treatment showed no significant increase in β-1,3-glucanase activity for the uninfested susceptible wheat plants ($P > 0.05$).

In the uninfested resistant wheat plants, significant higher β-1,3-glucanase enzyme activities were recorded with both water ($P < 0.05$) and DCM ($P < 0.05$) extracts of *A. attenuata* from 48 h to 144 h when compared to the control (Fig. 4.11 C) . No significant increase in β-1,3-glucanase activity could be detected in the infested plants (Fig 4.11 B and D).

For peroxidase activity, the water extract treatment inhibited enzyme activity under most conditions during 144 h period with the DCM extract treatment increasing enzyme activity at later periods for both susceptible and resistant wheat plants (Fig. 4.12). In the uninfested susceptible wheat plants treated with DCM extract, higher peroxidase enzyme activity levels at 72 h and 144 h were noticed (Fig. 4.12 A). In the uninfested resistant plants, higher enzyme activity was recorded at 72 h ($t = -3.76$; $P = 0.0005$) and 144 h ($t = -8.31$; $P < 0.0001$) for plants treated with DCM extract of *A. attenuata* (Fig. 4.12 B). Infested resistant wheat plants treated with the DCM extract treatment showed significantly higher enzyme activity ($P < 0.0001$) throughout the 144 h period (Fig. 4.12 D).

The invovlment of β-1,3-glucanase in the defence response has been shown by the induction of β-1,3-glucanase activity during a 144 h infestation period. van der Westhuizen *et al.* (1998b) found that high levels of β-1,3-glucanase were induced during aphid infestation on wheat. It has also been proven that β-1,3-glucanase activity levels rise with the infection of a pathogen in most plants as seen with *Penicillium digitatum* on grapefruit increasing levels of enzyme

activity in different tissues (McCollum *et al.,* 1995). Beta-1,3-glucanase and chitinase have the ability to hydrolyze chitin and β-1,3-glucans which are major constituents of fungal cell walls thus higher levels of enzyme activity are associated with the inhibition of fungal growth and plant defence (Cawood *et al.,* 2010). In barley, the induction of β-1,3-glucanase in response to fungal infection was reported (Jutidamrongphan *et al.,* 1991) In wheat, it can be considered that increases in β-1,3-glucanase activitiy forms part of the general resistance response against the RWA.

Peroxidases take part in various plant defence related strategies including the production of toxic ROS that play a major role in signalling during plant-pathogen interactions, strengthening of cell wall and suberin formation that are associated with the resistance response (Bowles, 1990; Mehdy, 1994; Wojtaszek, 1997). The induced response is often characterized with an increase in peroxidase activity (Rameshsundar *et al.,* 2001). In wheat, susceptible plants infested with RWA initially showed low levels of peroxidase activity with a differential induction over time, however resistance cultivars exhibited higher levels of peroxidase activity throughout (van der Westhuizen *et al.,* 1998a).

Enhanced levels of apoplastic β-1,3-glucanase and peroxidase activities in treated infested wheat leaves may be involved in reactions that reinforce the cell walls to resist penetration by the aphids ensuing that aphid feeding is restricted. The enhanced levels in the treated non-infested wheat plants may be linked to an elicitor response priming the plant and expressed systemically.

Plant extracts are known to be repellent and/or insecticidal, besides, the application of the extracts used in this study have shown to have both properties and may perhaps work directly against the aphid. The induction of defence responses prior to infestation or in uninfested wheat plants remains difficult to explain. If we consider the water extract of *Ar. afra* being a weak repellent through an olfactometer bioassay and moderately insecticidal when compared to the other extracts, its prominent ability to induce a defence response and lowering disease symptoms in wheat may be ascribed to compounds that act as elicitors in inducing resistance responses in wheat. Agricultural crops treated with plant extracts have been reported to show induced systemic resistance responses and a decrease in disease symptoms (Ramamoorthy *et al.,* 2002; Cawood *et al.,* 2010).

Oils are generally known for their repellent properties and deterrence effect but research on alternative measures in controlling bacterial spot showed the Indian clover oil increased PR-protein levels when applied to tomatoes (Lucas *et al.*, 2012). Secondary metabolites have been reported to increase PR-proteins, and phenolic compounds like oxalic acid application has been shown to induce both β-1,3-glucanase and peroxidase activities in rice towards *Rhizoctonia solani* (Jayaraj *et al.,* 2010). Interestingly, the water extract of *Ar. afra* has shown to possess phenolic compounds and also induced both enzymes in this study. The actual mechanism of disease resistance by the application of plant extracts suggests that their active compounds either act directly or indirectly with the RWA or induce a systemic response, resulting in lowered disease symptoms or damage.

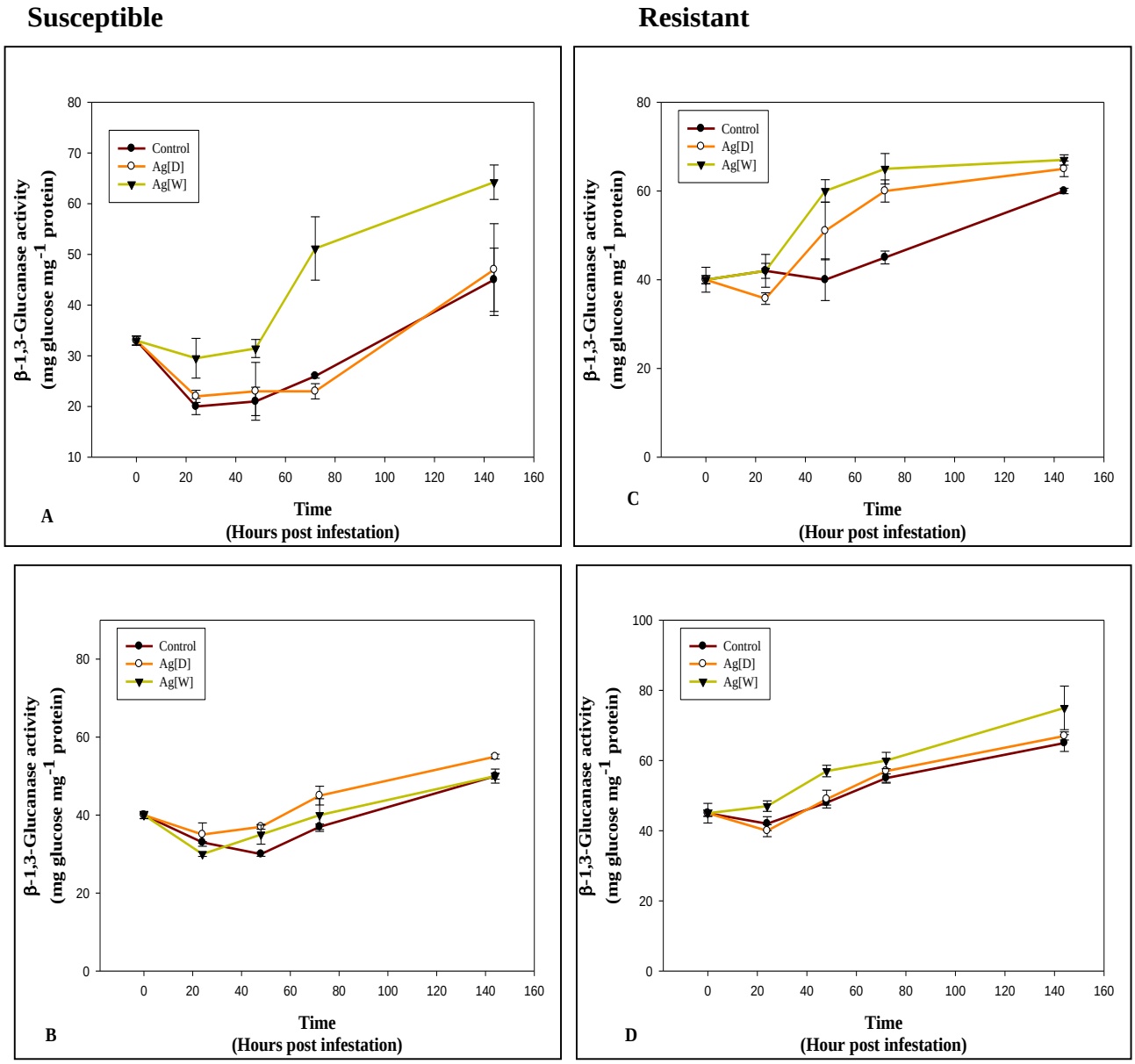

Figure 4.11: Effect of *A. attenuata* crude plant extracts on β-1,3-glucanase activity in wheat plants infested with RWASA1 from 0 to 144 h after foliar treatment. Values are means ± SD (n = 3). Ag[W] = *A. attenuata* water extract, Ag[D] = *A. attenuata* DCM extract. A, C = Uninfested; B, D = Infested.

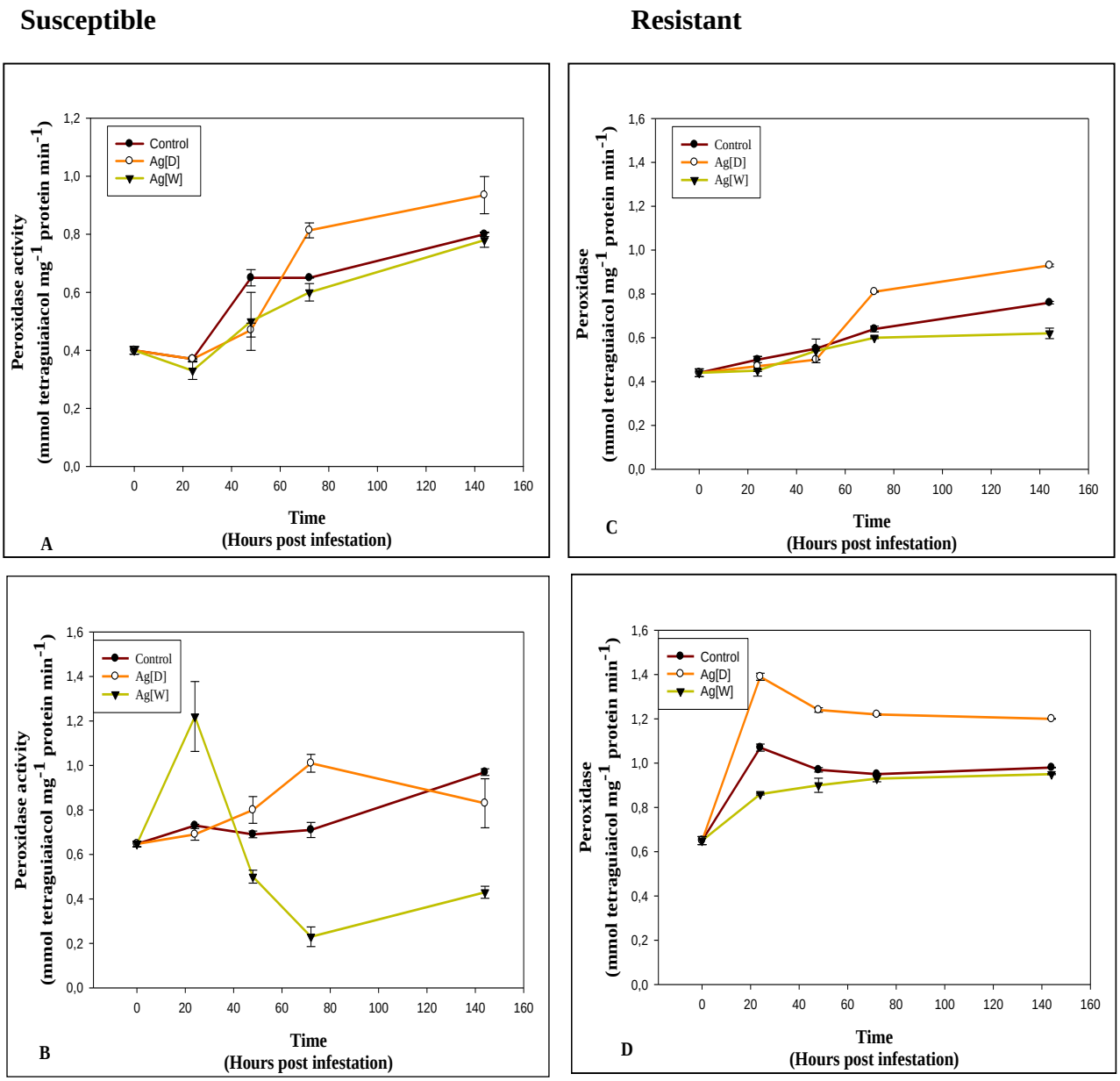

Figure 4.12: Effect of *A. attenuata* crude plant extracts on peroxidase activity in wheat plants infested with RWASA1 from 0 to 144 h after foliar treatment. Values are means ± SD (n = 3). Ag[W] = *A. attenuata* water extract, Ag[D] = *A.attenuata* DCM extract. A, C = Uninfested; B, D = Infested.

Chapter 5
Conclusion

Conclusion

The initial aim and objectives stated at the beginning of the project were met, with the plant extracts having been shown to provide protection in some way through their application against the RWA.

By means of chromatographic techniques, numerous compounds were identified in the different plant species under scrutiny. With the aid of thin layer chromatography, terpenes, phenolic compounds and flavonoids were identified in the extracts of *Ar. afra*. Saponins were only identified in the polar extracts of *A. attenuata*. *Zanthoxylum capense* extracts possessed flavonoids and terpenes. Additionally, the polar extracts of *Ar. afra, A. attenuata* and the non-polar extract of *Z. capense* displayed antioxidant activities. The GC-MS analysis of the essential oil and DCM extracts revealed the presence of unique compounds with various activities.

The insecticidal activities of the extracts were confirmed in terms of their ability to reduce biological fitness and numbers through direct spraying in laboratory tests. The water and DCM extracts of *A. attenuata*, essential oil of *Ar. afra* and the DCM extract of *Z. capense* proved to be extremely potent in killing up to 98% of aphids after 24 h.

The initial test for repellent properties of the extracts was confirmed in the olfactometer bioassay and some of the extracts proved to repel the RWA in closed chambers. The DCM extract and essential oil of *Ar. afra*, water extract of *A. attenuata* and DCM extract of *Z. capense* were highly effective in repelling aphids as shown by their low mean durations in olfactometer arms.

The application of mainly the polar extracts and essential oil of *Ar. afra* and DCM extract of *A. attenuata* on wheat plants enhanced resistance, for instance, disease symptoms were lowered after seven days and a lesser amount of aphids were observed to settle on wheat plants within the first 72 hours, compared to the controls. Interestingly, the water extract of *Ar. afra* which proved to be poorly repellent in the olfactometer bioassay, lowered aphid settling and reduced disease symptoms significantly after seven days in susceptible wheat seedlings. The DCM extract of *A. attenuata* treatment on wheat plants enhanced resistance significantly in both wheat varieties through a drastic reduction in disease symptoms and aphid numbers after seven days.

In addition, foliar application of the water extract and essential oil of *Ar. afra* significantly increased *in vitro* activities of ß-1,3-glucanase and peroxidase PR-proteins extracted from the intercellular wash fluid from uninfested and infested wheat plants. The water extract of *A. attenuata* only caused increases in β-1,3-glucanase activity in uninfested wheat, while the DCM extract of *A. attenuata* treatment was responsible for an increase in peroxidase activity in all the experiments.

The exact mechanism of how these plant extracts provide resistance through repellency, insecticidal and induction of defence is unknown and may be caused by a particular compound or one can suggest that the compounds found in the extracts act synergistically. However further research needs to be done through fractionation and isolation of individual compounds from these extracts and through bioassays to reveal the active compound/s that provide resistance against the RWA in wheat.

Summary

The Russian wheat aphid (*Diuraphis noxia*: Kurdjumov) is considered a harmful pest in South Africa and many other parts of world. The application of insecticides to host plants poses two unique problems as firstly it is too costly and the health risks associated with synthetic applications is usually met with consumer resistance. Plant species like *Artemisia afra*, *Agave attenuata* and *Zanthoxlum capense* are rich in bioactive compounds which are widely used in different industries. The bioactive compounds in the plant species and their possible role in providing protection against aphids served as the rationale for the basis of this study. In this study, the essential oil of *Ar. afra* and polar and non-polar extracts from the three plant species mentioned, were investigated for their repellent and insecticidal abilities towards the Russian wheat aphid (RWA) and their ability to induce a defence response in wheat.

Water, methanol and dichloromethane (DCM) crude extracts were prepared from each plant using dried ground leaf material. The essential oil of *Ar. afra* was obtained through hydro-distillation from fresh leaf material.

The identification of compounds present in the different plants through thin layer chromatography revealed the presence of terpenes and phenolics in *Ar. afra* extracts. In *A. attenuata*, steroids and saponins were highlighted as being present, while *Z. capense* extracts showed the presence of steroids, flavonoids and other phenolic compounds.

Essential oil of *Ar. afra* and DCM extracts of the three plant species were analysed using gas chromatography coupled to mass spectrometry. In the essential oil, compounds found in high abundance were eucalyptol (12.87%), camphor (15%), α–thujone (29%), β-thujone (12%) and 1,8-cineole (7.42%). Alpha-thujone (29.47%) and β-thujone (37.36%) were identified as the major constituents in the DCM extract of *Ar. afra*. Compounds present in *A. attenuata* DCM extract revealed the presence of methyl jasmonate (6.4%) and 2,5-dimethyl-3,4-hexanediol (23%). The DCM extract of *Z. capense* consist of, among others, 2,4-dimethylhex-1-ene (25.98%) and E – (β)-farnesene (2.41%) .

Laboratory bioassays were carried out to evaluate the insecticidal activity of the essential oil and other crude extracts against the RWA by spraying the insects directly with a 10 mg mL^{-1} solution. Mortality was recorded at 1 h and 24 h after treatment. All the plant extracts and

essential oil caused significant mortality of 50% or more after 1 h and at least 70% at the end of 24 hours. *Ar. afra* essential oil proved to be the most toxic with a LC_{50} – value of 0.396 mg mL^{-1}.

Initial screening for repellency of the essential oil and extracts was performed using a four arm olfactometer. Both polar and non-polar extracts repelled the RWA. However, the mean duration recorded in repeated 10 min cycles showed the The DCM extract and essential oil of *Ar. afra,* water extract of *A. attenuata* and DCM extract of *Z. capense* were highly repellent.

The repellent properties and abilities of the essential oil and plant extracts to limit RWAs settling on susceptible and resistant wheat leaves, ensuing lowered disease symptoms after 7 days, were exploited in a leaf settling bioassay under glasshouse conditions. At 2 h.p.i, the essential oil and polar extracts of *Ar. afra* and DCM extract of *A. attenuata* reduced aphid settling significantly when compared to the control in the susceptible cultivar. The water extract treatment of *Ar. afra,* DCM extract treatment of *A. attenuata* maintained lower number of aphid settling on wheat leaves even after 72 h.p.i in the susceptible cultivar. In the resistant cultivar, no significant effects were observed, however the DCM extract treatment of *A. attenuata* still maintained to lower aphid settling after 72 h.p.i. The symptom analysis revealed that the extracts mentioned above also lowered the prevalence of disease in wheat after 7 days and the number of live aphids were less.

The intiation of defence responses by the extracts of *Ar. afra* and *A. attenuata* was comparable to what was observed in the leaf settling bioassay. β-1,3-Glucanase and peroxidase activity increased with the application of the water and essential oil extracts of *Ar. afra* in both uninfested and infested plants during a 144 h period. Furthermore, an increase in peroxidase activity was observed when wheat was treated with the DCM extract of *A. attenuata*.

The results obtained in this study, indicate that the plant extracts contain bioactive compounds that possess the ability to aid in the control of the RWA through various mechanisms and their integration in pest management strategies may prove to be beneficial.

Keywords: insecticidal activity, repellency, PR-proteins, olfactometer, leaf settling, Russian wheat aphid, wheat, chromatography.

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