Factors affecting the resistance mechanisms of the Russian wheat aphid (*Diuraphis noxia*) on wheat.

BY

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DECLARATION

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

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Lieschen Bahlmann
June 2002

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To my brother Etienne: you are always in my thoughts, I will never forget you.

List of abbreviations

RWA	Russian wheat aphid
D. noxia	*Diuraphis noxia*
L:D	Light:Dark
SD	Standard deviation
PI	Plant Introduction
kDa	Kilo Dalton
ARC-SGI	Agricultural Research Council-Small Grain Institute
IWF	Intercellular washing fluid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T	Percentage of the total for acrylamide and bisacrylamide
C_{bis}	Concentration of bisacrylamide
MDH	Malate dehydrogenase
IEF	Isoelectric focusing
SEM	Scanning electron micrograph
TEMED	*N, N, N, 'N'*-tetramethyl-ethylenediamine
NADH	Nicotinamide adenine dinucleotide, reduced state
pI	Isoelectric point

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Chapter 1

Preface

The Russian wheat aphid (RWA) (*Diuraphis noxia*, Mordvilko) is a serious insect pest of wheat (*Triticum aestivum*) in South Africa. Damage from RWA infestations causes severe economic losses due to aphid feeding and the associated costs of insecticide application. This thesis addresses the insect-plant interactions as well as the factors affecting the resistance that the aphid encounters when feeding on resistant wheat.

In Chapter 2 a literature review is presented of the Russian wheat aphid focussing on aphid feeding. The sequence of events are discussed with the aphid initially finding a suitable host, location of the phloem, acceptance of the food source and ultimately with the withdrawal of the stylet. Also discussed is aphid feeding on resistant hosts.

Chapter 3 presents a comparison of Russian wheat aphid development when placed on susceptible ('Tugela') and resistant ('Tugela *Dn1*') wheat cultivars that are near-isogenic to each other. This study compares Russian wheat aphid fecundity, longevity and development to determine the influence of the resistant wheat cultivar.

In Chapter 4 an artificial diet was developed for the Russian wheat aphid. This is the first report of an artificial diet for the Russian wheat aphid. The diet developed was simple yet effective and enables potentially resistant compounds to be tested with their inclusion in an artificial diet to determine their effectiveness in inhibiting aphid fecundity and longevity.

Chapter 5 represents a study on the induction of proteins upon Russian wheat aphid infestation of a resistant wheat cultivar, 'Tugela *Dn1*'. Two-dimensional gel electrophoresis was used to confirm the presence of induced proteins and to identify these proteins.

Chapter 6 evaluates the influence of leaf epicuticular wax, leaf trichome length and density, of resistant and susceptible wheat cultivars. Their contribution to the resistance that the Russian wheat aphid encounters is discussed.

Chapter 2

Literature Review

2.1 Introduction

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), is a serious insect pest of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). The first published report of the aphid, as a pest on barley was by Kovalev *et al.* (1991). The aphid originated in southern Russia, countries bordering the Mediterranean Sea, Afghanistan and Iran (Walters *et al.*, 1980), but is considered a minor pest in these areas (Walters, 1984). It was first discovered in South Africa during 1978 on wheat in the northern Free State (Walters *et al.*, 1980), with severe outbreaks occurring during 1979 and 1980 (Du Toit & Walters, 1984).

Wheat is one of South Africa's major staple crops and constitutes 21% of the national arable land use together with other small grains (Marasas, 1999). The total wheat production in South Africa for the 1999/2000 season was 1.56 million tonnes (National Crops Estimates Committee, 2000). RWA infestation can result in plant yield losses of up to 90% under experimental field conditions. In yield loss trials, losses ranged from 20.6% to 91.2%, at an average of 56.8% (Hewitt, 1988). Unless preventative measures such as planting of resistant cultivars and chemical control are taken, the wheat yield loss will result in severe economic losses. The RWA caused over $1 billion in losses to the production of small grains in the western United States since its detection in 1986 to 2000 (Porter & Webster, 2000).

Other crops damaged to a lesser extent by the RWA include oats (*Avena sativa*), rye (*Secale cereale*) and triticale (x *Triticosale*). Many native and introduced grasses also serve as alternative

summer hosts to the RWA (Kindler & Springer, 1989). These allow the RWA to survive between wheat seasons.

2.2 Russian wheat aphid description and biology

The RWA is a small, light green aphid that is less than 2 mm in length. It can be distinguished from other aphids infesting wheat in South Africa by the short antennae and a projection above the caudal segment which gives it a "forked tail" appearance (Walters *et al.*, 1980) (Figure 2.1A).

RWA infested wheat typically has white, yellow and reddish-purple, to purple longitudinal streaks on the wheat leaves (Walters *et al.*, 1980). RWA feeding causes the double membrane of the chloroplast to be degraded after 10 days of feeding. This degradation of the chloroplast causes the characteristic streaking found on infested wheat (Fouché *et al.*, 1984). The aphids occur mainly on the new growth of the plant, in the axils of the leaves or within curled up leaves (Walters *et al.*, 1980). RWA feeding on wheat reduces growth and development of the plant, as well as a reduction in photosynthetic leaf area, which is also partly due to a combination of leaf stunting and new leaves not unfolding (Burd & Burton, 1992).

The rolling of leaves provides an optimum environment for RWA reproduction. This environment protects the RWA from contact insecticides and natural enemies that could be used as biological control agents (Miller *et al.*, 1994). Reed *et al.* (1992) showed that RWA parasitism by biological control agents was higher on plants that did not have leaf rolling. The RWA is

protected from the biological control agent by concealing itself in the enclosed leaf. Rolling of leaves during the heading stage can prevent the spike from extruding, which results in an obstruction of flowering and a consequent reduction in seed production (Miller *et al.*, 1994).

The RWA in South Africa reproduces parthenogenetically (i.e. reproduction takes place without fertilization) (Figure 2.1B). In South Africa, only winged (alate) or wingless (apterous) vivaparous females are found (Walters *et al.*, 1980). Winged females are only produced when the food source is depleted or under adverse environmental conditions (Robinson, 1992). In their native countries the RWA also undergoes sexual reproduction. Males and oviparae (sexually mature females) mate to produce eggs. These eggs then overwinter during the harsh winters experienced in these countries (Kiriac *et al.*, 1990).

No sexual forms have been reported from South Africa. However, in northwestern United States, where the RWA was also introduced, occasional oviparae have been reported (Kiriac *et al.*, 1990). The term gynocyclic was proposed to explain this phenomenon whereby only the occasional oviparae but no males are produced.

The immature RWA nymph reaches maturity in approximately 10 days (Aalbersberg *et al.*, 1987; Michels & Behle, 1988; Basky & Jordaan, 1997). This is dependent on several factors of which the most important is temperature. There is a significant decrease in nymphal developmental time with increases in temperature (Aalbersberg *et al.*, 1987; Girma *et al.*, 1990). Other factors influencing nymphal developmental time are the plant growth stage and plant quality. Aphids developed much faster when feeding on the jointing stage than on any other growth stage of wheat (Girma *et al.*, 1990). The influence of plant quality has also been observed in rape plants

(*Brassica napus*), where water stressed plants increased the rate of development of the cabbage aphid, *Brevicoryne brassicae* (Miles *et al.*, 1982). RWA life span is affected by the interaction of temperature and plant growth stage. There is an increased rate of mortality as temperature and plant age is increased (Michels & Behle, 1988; Girma *et al.*, 1990).

RWA reproduction (natality) is similarly affected by temperature and plant growth stage (Michels & Behle, 1988; Girma *et al.*, 1990). Natality increased with increasing temperatures (up to 20°C) and then decreased at higher temperatures. The highest number of progeny produced was observed when aphids fed on the jointing stage of wheat. The majority of RWAs develop through five nymphal stadia (5S) before the onset of reproduction. However, some nymphs start reproducing after four (4S) or six (6S) nymphal stadia (Girma *et al.*, 1990).

Aphid density also affects RWA development. RWA placed on wheat at densities of 10 and 40 aphids per plant revealed that the plants that were initially infested with 10 aphids showed larger populations after 15 days due to higher reproduction, lower mortality and less plant death (Quisenberry & Schotzko, 1994).

It has been reported that the RWA transmits several viruses to small grain crops. These include the barley yellow dwarf virus (BYDV), barley stripe mosaic virus (BSMV), brome mosaic virus (BMV) and *Rhopalosiphum padi* virus (RhPV) (Rybicki & Von Wechmer, 1984; Von Wechmer, 1984). Damsteegt *et al.* (1992) was, however, unable to duplicate these results and found that BSMV was not transmitted by the RWA, BMV was erratically transmitted (2.5%) and BYDV was occasionally transmitted (2.8%) to plants infested with RhPV.

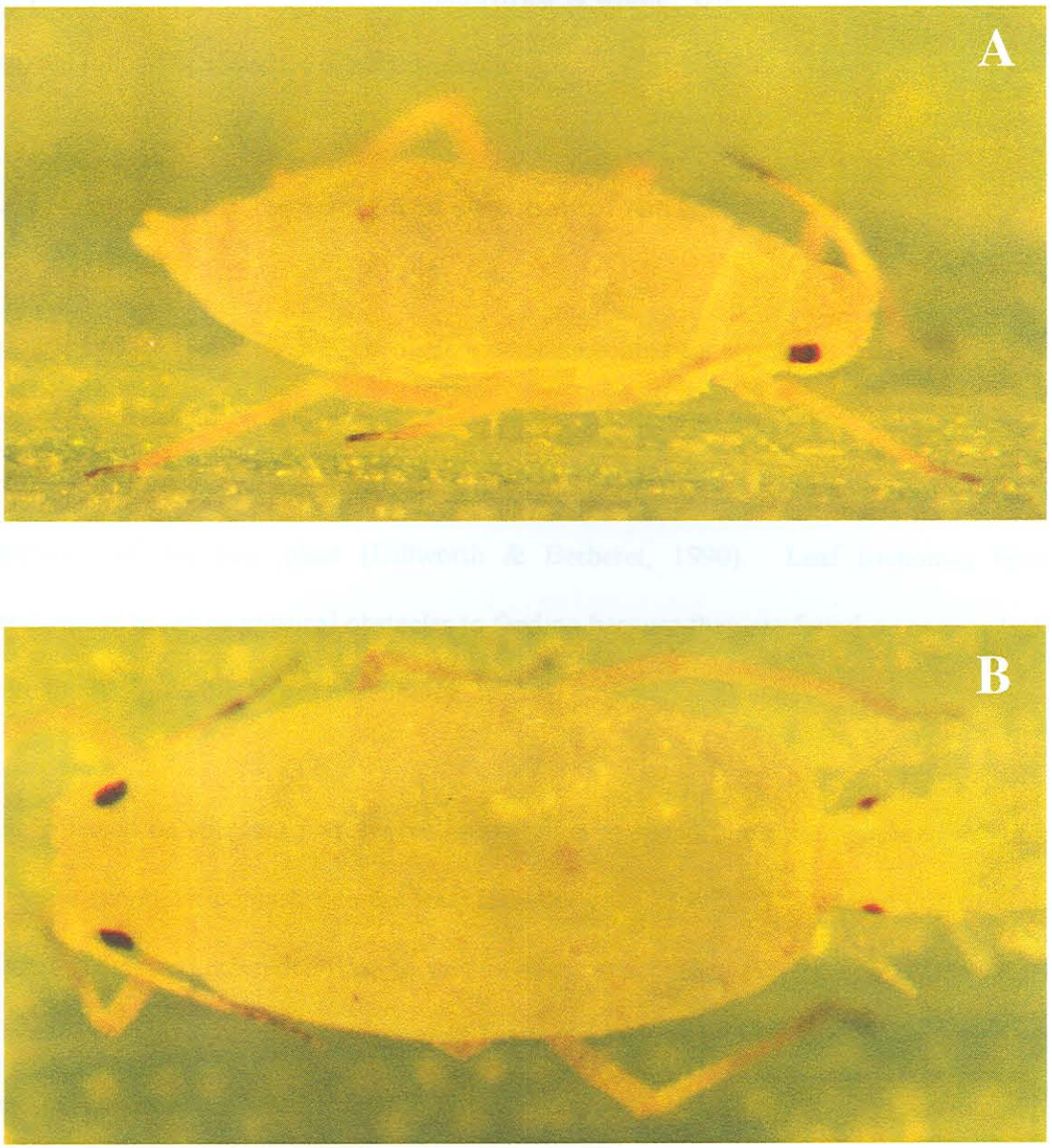

Figure 2.1. (A) The Russian wheat aphid. (B) A viviparous female Russian wheat aphid.

2.3 Feeding

The preferred host of the RWA in South Africa is wheat. Once the aphid has found a suitable host, the following sequence of action occurs:

2.3.1 Examination, probing and selection of the feeding site

The first surface that the RWA encounters when searching for a potential food source is the thin layer of lipids found on the surface known as the epicuticular wax. This covers the entire leaf surface. This wax covering is distinctive for each plant species and could play a role in RWA acceptance of the host plant (Dillworth & Berberet, 1990). Leaf trichomes have been hypothesized to act as physical obstacles to feeding because they are found on or near leaf veins, where the RWA feeds (Ni & Quisenberry, 1997b).

Aphids placed on resistant plants have been shown to spend more time engaged in nonfeeding behaviour (no stylet contact with the leaf) (Kindler *et al.*, 1992). This means that the aphids on resistant plants require more time to initiate feeding activities (Webster *et al.*, 1993). These indicate a physiological or morphological basis for the resistance observed. A RWA resistant wheat line, plant introduction (PI) 137739 was found to have the longest trichomes in comparison to two RWA susceptible wheat cultivars, 'Arapahoe' and 'Halt'. 'Arapahoe' and 'Halt' had the shortest trichomes but the highest trichome density. Trichomes are found on or near leaf veins and therefore long trichomes are likely to be an important physical obstacle as the phloem-feeding RWA probes close to leaf veins. The epicuticular wax ultrastructure was not found to

play a significant role on aphid-host preference (Ni & Quisenberry, 1997b). Similarly, Ni *et al.* (1998) found that the leaf epicuticular waxes of different cereal grains were similar and had little effect on the resistance towards the RWA.

Another factor that could influence an aphid's settling behaviour is when it encounters another aphid or a similar obstacle; a 'dummy' aphid. The black bean aphid, *Aphis fabae*, probed more often and remained longer near other aphids than apart from them (Ibbotson & Kennedy, 1951). Prado & Tjallingii (1997) showed that *A. fabae* had beneficial effects when they were placed on leaves that had previously been infested. This indicated the presence of phloem factors that enhances sieve element acceptance by the aphids, although mesophyll and non-vascular phloem tissue factors could not be excluded. This can be considered as host-plant acceptance. In contrast, the bird cherry-oat aphid, *Rhopalosiphum padi*, does not seem to benefit from gregarious living (De Barro, 1992), or from previous infestations (Prado & Tjallingii, 1997). *Rhopalosiphum padi* has been shown to display avoidance behaviour and increased mobility at high population densities due to a communicative odour stimulus (Pettersson *et al.*, 1995).

When the aphid finds a suitable probing site, the labium (which houses the stylets) are placed on the substrate (Miles, 1968). Sheath material is discharged from the tips of the stylets. Analysis of the sheath material of the milkwood bug, *Oncepeltus fasciatus*, showed that the proteinaceous stylet sheath consist of approximately 10% phospholipids (Miles, 1960; Miles, 1967). The sheath material gels rapidly as soon as it comes into contact with air or other aqueous media. The precursors of the sheath material occur together with companion molecules that prevent premature gelling. The companion molecules are free sulphydryl groups and free amino acids. Once the sheath material has been discharged, the amino acids diffuse away and the precursors

come into contact with oxygen, causing hydrogen and disulphide bonds to be formed respectively (Miles, 1964b). This causes the sheath material to gel.

The discharged sheath material gels between the stylets and the end of the labial groove (Miles, 1959b; Miles, 1999). This is known as a flange and forms a structure that is external to the substrate. The stylets are then forced into the plant. It has been hypothesized that the flange serves in securing the tip of the labium to the leaf surface during insertion of the stylets (Saxena, 1963) and also in preventing the stylets from slipping (Pollard, 1973). This could be due to the lipoprotein composition of the flange causing it to adhere to the leaf surface (Miles, 1964b).

Once the RWA stylets have penetrated the leaf surface, the stylets initiate the process of locating the phloem. The RWA feeds from the phloem sap (Fouché *et al.*, 1984).

2.3.2 Location of the food source

Once the aphid stylet has penetrated the epidermis, sheath material continues to be discharged and forms a lining around the stylet which continues along the chosen path. This is known as a stylet sheath. The first observation of a stylet sheath was by Prillieaux (Miles, 1968). It was originally thought that the stylet sheath was due to the reaction of the plant to insect penetration and not from the insect itself. However, an identical structure was found when aphids were allowed to feed on artificial media (Bennett, 1934).

The function of the stylet sheath is debatable. Tjallingii and Hogen Esch (1993) have shown that the stylet sometimes slides between the cell wall and the membrane and do not always follow an accurate intercellular path. During this process the stylet may pierce the plasmalemma and a small amount of watery saliva is discharged (Martin *et al.*, 1997). A small quantity of cell sap is sucked out and the stylet is then withdrawn. The cells that were punctured do not seem to partake in any hypersensitive response of the plant to the aphid. The sheath material has been shown to plug the gap in the cell wall and provide a surface so that repair of the membrane can occur (Tjallingii & Hogen Esch, 1993).

Aphids secrete another kind of saliva that is called the watery saliva. This saliva is water-soluble and does not gel. During the stylet path towards the phloem, the stylet often protrudes from the end of the sheath material during the formation of the stylet sheath (Miles, 1959b). The stylet discharges some watery saliva and this is then ingested together with any liquids surrounding the stylet tip (Campbell & Dreyer, 1990; Tjallingii & Hogen Esch, 1993; Martin *et al.*, 1997). Consequently, aphids can assess the chemical nature of the plant matrix polysaccharide and the cells that have been punctured, as well as determine the acceptability of the host plant by ingesting the discharged watery saliva (Campbell & Dreyer, 1990; Tjallingii & Hogen Esch, 1993).

The watery saliva contains a large number of amino acids that are probably unutilized products that have been absorbed from the phloem sap (Srivastava, 1989). The watery saliva shows strong reducing activity (Miles & Harrewijn, 1991). The pH of the watery saliva was found to be about eight (Miles, 1965).

Pectinases were found in the watery saliva (Adams & McAllan, 1956; 1958). Pectinases (pectinesterase and polygalacturonase) from the greenbug, *Schizaphis graminum*, have been shown to elicit responses in susceptible sorghum plants. These responses were chlorophyll loss and leaf discoloration, similar to that found when the greenbug feeds on susceptible sorghum plants (Ma *et al.*, 1998). It has been hypothesized that the released pectinases may digest the middle lamellar pectin. This facilitates stylet penetration (Dreyer & Campbell, 1987). Tjallingii and Hogen Esch (1993) found that the stylet does not follow a middle lamella path, contradicting Dreyer and Campbell (1987), but in fact follows the secondary walls. Also, stylet penetration seems to go faster than pectin degradation (Cherqui & Tjallingii, 2000). The action of pectinases on pectin also causes the release of pectic fragments, which may be biologically active and elicit plant responses (Ma *et al.*, 1998).

The enzyme amylase has been reported to occur but the validity of these claims is dubious (Miles, 1999). Oxidases are also found in the watery saliva (Miles, 1964a). During plant penetration by an aphid, polyphenols (like catechin) accumulate as a defense reaction to the aphid. Phenoloxidases catalyze toxic phenolics to non-toxic end products (Peng & Miles, 1988). Peroxidases are also reported to occur in the watery saliva of several aphids. This enzyme oxidizes a variety of defensive phytochemicals so that they are detoxified (Miles & Peng, 1989). Both the actions of phenoloxidases and peroxidases prevent the accumulation of products in the plant that will initiate a defense reaction to the aphid.

Several functions for the watery saliva have been proposed. Watery saliva can function as a lubricant to the stylets (Miles, 1999). The aphids can assess the chemical nature of the plant matrix polysaccharide and the cells that have been punctured, as well as determine the

acceptability of the host plant by ingesting the discharged watery saliva (Campbell & Dreyer, 1990; Tjallingii & Hogen Esch, 1993). Aphids have no Malpighian tubules and it has been postulated that the salivary glands assist with excretion (Miles, 1999). The composition of the saliva changes with diet and may even contain organic or inorganic radioisotopes (Lamb *et al.*, 1967; Forrest & Noordink, 1971). Other functions of the watery saliva are dependent on the salivary enzymes and have already been described.

The stylet follows an intercellular path until the phloem is reached (Fouché *et al.*, 1984). The next section will give an account of phloem ingestion.

2.3.3 Ingestion

Once the aphid stylet has entered the vascular bundle (phloem) no more sheath material is secreted. An electrical penetration graph (EPG) monitors aphid activities and the position of the stylet during probing. The EPG allows the probing behaviour of aphids as well as the aphid-plant relationship to be studied (Tjallingii, 1988). From the EPG data it was concluded that in general, aphids make three types of sieve element punctures (Tjallingii, 1990; Tjallingii & Hogen Esch, 1993). Firstly, a brief puncture into the phloem cells which is indistinguishable to punctures made into other cells. This seems to indicate ignorance or no recognition of the phloem cell by the aphid (Tjallingii & Hogen Esch, 1993). Secondly, a puncture into the phloem cell with the continuous discharge of watery saliva which is mixed with the phloem sap. This is known as the phloem salivation phase (E1) (Prado & Tjallingii, 1994). During E1 no saliva is ingested because the food canal within the stylets is filled with fluid (watery saliva). E1 indicates aphid

recognition but no ingestion of the phloem sap (Tjallingii & Hogen Esch, 1993). E1 is often but not always followed by E2. E2 is the phase in which saliva is discharged and mixed with the phloem sap. This watery saliva is then immediately ingested by the RWA and the saliva does not reach the plant tissue. This was proven using viruliferous *Rhopalosiphum padi* individuals. During E2 no virus was inoculated into the plant (Tjallingii, 1988; Prado & Tjallingii, 1994). This mixture of saliva and phloem sap is forced back into the stylets by phloem sap pressure. E2 indicates acceptance of the phloem sap with sap ingestion (Tjallingii & Hogen Esch, 1993). The phloem sap is not always accepted, in which case the aphid withdraws its stylet and probes again.

RWA feeding on resistant wheat and wheatgrass cultivars were found to spend significantly less time ingesting from the phloem compared to RWA on susceptible cultivars. RWA feeding on these resistant cultivars also spend more time in nonphloem ingestion. Kindler *et al.* (1992) suggested that the phloem sap of resistant plants do not provide the necessary structural and chemical cues for phloem acceptance by the RWA. The aphid subsequently turns to nonphloem feeding to survive. The same was observed when the RWA was allowed to feed on resistant and susceptible barley cultivars. Significantly less time was spent feeding on the phloem on the resistant cultivars when compared to the susceptible cultivars. Also, aphids on the resistant cultivars spend more time in nonphloem ingestion (Webster *et al.*, 1993).

RWA feeding on susceptible wheat significantly increased the concentrations of essential amino acids of the phloem, particularly tryptophan and leucine. Infested resistant wheat showed that levels of essential amino acids were slightly decreased (Telang *et al.*, 1999; Sandström *et al.*, 2000). The RWA has endosymbiotic bacteria (*Buchnera* sp.) which overproduce limiting amino acids which benefit their hosts (Douglas, 1988). These bacteria synthesize two essential amino

acids for the RWA, namely tryptophan (Lai *et al.*, 1996) and leucine (Thao *et al.*, 1998). There is a reduction in the number of gene copies of these two amino acids carried by the endosymbiotic bacteria in the RWA (Lai *et al.*, 1994; Thao *et al.*, 1998). Subsequently, a more efficient mechanism for utilizing nitrogen by the RWA has resulted in loss of copies of these genes by *Buchnera*. The RWA manipulates the nutritional quality of the phloem sap of susceptible wheat to improve the quality of its diet and reduces the need for provisioning by the endosymbiont.

2.3.4 Withdrawal of stylets

Once the aphid has completed feeding or has not accepted the phloem sap, the stylets are withdrawn. Withdrawal of the stylets may be completely through the stylet sheath or they may draw back the tips of the stylets for a short distance before the stylet is pushed through the side of the stylet sheath to produce a branch (Miles, 1972). Branched salivary sheaths have been shown to occur in both susceptible and resistant wheat cultivars (Ni & Quisenberry, 1997a). Complete withdrawal of stylets from the plants leaves the stylet sheath behind. The stylet sheath is sealed and filled up with a secretion so that no plant sap can enter (Kinsey & McLean, 1967). The secretion filling the stylet sheath upon withdrawal has been postulated to be watery saliva (Miles, 1959b; Kinsey & McLean, 1967).

RWA on resistant wheat cultivars were found to probe more and for a longer duration compared to aphids on susceptible wheat cultivars (Webster *et al.*, 1993). However, the number and duration of probes by the RWA on the susceptible and resistant wheat and wheatgrass cultivars did not differ significantly (Kindler *et al.*, 1992).

2.4 Methods of curbing RWA damage

Wheat is one of the major staple crops in South Africa (Marasas, 1999). The RWA can cause an average of 60% loss to yield (Hewitt, 1988). Four methods have been used to reduce the economic impact and the damage that the aphid causes:

2.4.1 Cultural practices

The RWA survives the summer season by living on alternate hosts, for example, rescue grasses (Kindler & Springer, 1989). Avoidance in planting these alternate hosts (*e.g.* barley) earlier than the planting of wheat will lead to a decrease in the numbers of the RWA. This also deprives the aphid of its refuge (Walters *et al.*, 1980). These refuges are the source of RWA infestations into wheat fields.

The choice of planting date should be reviewed. Walters *et al.* (1980) suggested that crops should not be planted before May. Also, wheat that is densely planted and well fertilized is not susceptible to heavy aphid infestations.

Cultural control of the RWA was largely practiced when the RWA was first discovered in South Africa. There were no RWA resistant wheat cultivars and little information was available on insecticide usage against this aphid (Du Toit & Walters, 1984).

2.4.2 Insecticides

RWA susceptible plants exhibit leaf rolling that provides an optimum environment that protects the RWA from contact insecticides (Miller *et al.*, 1994). RWA are also found in the axils of leaves and within the leaf whorl, which also restricts the action of contact insecticides (Walters *et al.*, 1980). Subsequently, systemic insecticides have proven more successful in controlling the RWA (Robinson, 1992).

The irresponsible use of insecticides is harmful to the environment and can destroy the aphid's natural enemies. The use of insecticides is also an expensive practice that increases production cost. Insecticide treated wheat may be unacceptable for export to Europe. Continued injudicial use of insecticides may accelerate the development of insecticide resistance by the aphid (Wolff *et al.*, 1994). More environmentally friendly methods of combating RWA infestations are desirable.

2.4.3 Biological control

The RWA is an introduced insect pest in South Africa, where its native natural enemies are absent. Consequently, the RWA has flourished on susceptible wheat.

Biological control agents of aphids can reduce the population levels of aphids. In South Africa, seven indigenous species of parasitic wasps and two species of predatory flies have been found to attack the RWA (Hayes, 1998). These natural enemies are, however, unable to prevent RWA

populations from causing severe damage on susceptible wheat (Aalbersberg *et al.*, 1988). Several natural enemies have been introduced into South Africa to combat the RWA. One of these is a parasitic wasp, *Aphelinus hordei*, introduced from the Ukraine in 1991 (Prinsloo, 1998). Female wasps lay their eggs inside the aphid and the larvae which hatch feed on the aphid. In field trials during 1996, parasitism of up to 72% was recorded (Hayes, 1998).

Other reports have shown that parasitism of the RWA also decreases aphid populations on susceptible wheatgrass (Reed *et al.*, 1992). However, susceptible wheatgrass that displayed leaf rolling had a decreased incidence of parasitism of the RWA because the RWA could hide from the parasitoid (Miller *et al.*, 1994). Farid *et al.* (1998) showed that *Diaretiella rapae* females (hymenopterous parasitoids) that emerged from RWAs that were maintained on resistant wheat, had a longer life span. RWAs fed on resistant wheat had no adverse effects on the parasitoid, indicating the compatibility of the resistant cultivar and the parasitoid.

The RWA lives and feeds in rolled leaves of susceptible plants and this provides some protection to the RWA against predators (Miller *et al.*, 1994). To combat this, the use of disease-causing fungi as a possible biological control agent against the RWA, have been attempted (Feng & Johnson, 1991). *Beauveria bassiana* has been shown to increase aphid mortality but has no significant effects on fecundity. Also, RWA nymphs may become infected when they come into contact with dead sporulating adults (Wang & Knudsen, 1993).

In South Africa, pathogenic fungi have been observed to attack the RWA, but only during the warm, moist summer months. The RWA is important only during the winter months when wheat is planted (Walters *et al.*, 1980). Six species of entomopathogenic fungi have been recorded in

South Africa. These include *Beauveria bassiana, Pandora neoaphidis, Conidiobolus obscurus, Conidiobolus thromboides* and *Entomophthora planchoniana* (Hayes, 1998; Hatting *et al.*, 2000). RWAs were shown to have higher susceptibility to *P. neoaphidis* (Hatting *et al.*, 2000).

Biological control agents are however, affected by environmental conditions. Introduced predators and parasitoids may not become active early enough to exert control over the RWA, as they do not have synchronized life cycles.

2.4.4 Planting of resistant cultivars

When the RWA was first discovered in South Africa on wheat during 1978 no wheat cultivars displayed any resistance (Walters *et al.*, 1980) and large scale insecticide applications were made annually. Emphasis was placed on identifying resistant *Triticum* genotypes from the RWA native countries (Du Toit, 1987). Attempts were then made to transfer this resistance to locally adapted cultivars by traditional breeding methods (Du Toit, 1989).

From the resistant wheat cultivars that were originally developed, 'Tugela *Dn1*' was the first resistant wheat cultivar to be released for commercial production in the world. 'Tugela', a local susceptible winter wheat cultivar, was crossed to PI 137739 (also known as SA 1684), a resistant wheat cultivar originating from Iran (Du Toit, 1987; 1989). The F_1 progeny were then backcrossed five times to 'Tugela'. The result was 'Tugela *Dn1*', a hard red, semi-dwarf bread wheat cultivar that can be cultivated under dryland conditions in the summer rainfall region (Small Grain Institute (SGI), Bethlehem). Resistance reactions showed that the resistance of

'Tugela *Dn1*' is controlled by a single dominant gene (*Dn1*) and that this gene is inherited independently to other RWA resistance genes (Du Toit, 1989).

The development and use of resistant cultivars may represent the most effective way to overcome RWA infestations of cereals. Resistant cultivars are environmentally safe and economical in comparison to the use of insecticides or biological control (Budak *et al.*, 1999).

Possible sources of resistance that have been described are antibiosis, antixenosis (nonpreference) and tolerance.

2.4.4.1 Antibiosis

Painter (1958) first coined the terms antibiosis, nonpreference and tolerance. The term antibiosis is defined as the ability of resistant plants to adversely affect the biology of an insect. Plants that express antibiotic resistance to insects are obviously less suitable hosts than susceptible cultivars (Mowry, 1994).

The most common category of resistance in wheat is antibiosis (Du Toit, 1987, 1989; Unger & Quisenberry, 1997). Aphids feeding on plants exhibiting antibiosis (resistant plants) experience decreased longevity, delayed development, decreased fecundity and an increase in restlessness (Painter, 1958; Scott *et al.*, 1991; Baker *et* al., 1992; Kindler *et al.*, 1992; Smith *et al.*, 1992). The most typical parameter that is used to assess antibiosis in wheat plants is RWA fecundity. This is assessed measuring the rate of nymphal production, the length of the nymphipositional

period (the period that nymphs are produced) and adult life span (Smith *et al.*, 1992; Unger & Quisenberry, 1997).

Very few wheat cultivars are found that display all three categories of resistance (Unger & Quisenberry, 1997). PI 137739 displays both antibiosis and antixenosis (Du Toit, 1987); low levels of tolerance to the RWA was found by Smith *et al.* (1992). Antibiosis causes selective pressure on the insect (Tolmay *et al.*, 1999). This can result in the insect developing resistance to the plant exhibiting antibiosis (Gould, 1998). Tolerance, which does not impose selection pressure on the insect, can be used in conjunction with other sources of resistance, to prevent or delay adaptation by the insect (Haile *et al.*, 1999).

Antibiotic resistance to the RWA seems to involve different and independent genes (Castro *et al.*, 2001). Subsequently, the genotypes carrying these genes need to be selected to broaden the genetic base from which to choose resistance genes in a breeding program.

2.4.4.2 Antixenosis (nonpreference)

The term antixenosis is defined as the nonpreference of plants for insect oviposition, shelter or food. This is primarily due to the lack of certain qualities of the plant (Painter, 1958).

Antixenosis is the inability of a plant to serve as a host for a pest. It is caused by physical or chemical plant factors that repel or deter insects from feeding and ovipositing on the plant (Tolmay *et al.*, 1999). Nicol *et al.* (1992) showed that there were significant correlations between

aphid nonpreference (plant resistance) and the concentration of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). DIMBOA is a hydroxamic acid that has deleterious effects on insects feeding on cereals (Mayoral *et al.*, 1996).

Antixenosis is measured by planting seeds of different cultivars randomly in a circle and releasing the RWA in the center. The distribution of the aphid is determined after a certain duration and the aphids preference is subsequently determined (Tolmay *et al.*, 1999). Some reports have indicated a great deal of variability in this approach (Webster *et al.*, 1987; 1991). Antixenosis seems to be the result of the action of multiple genes found on the group 7 chromosomes (Castro *et al.*, 2001).

As with antibiosis, antixenosis also causes selective pressure, but to a lesser degree than antibiosis. This can result in the aphid developing resistance to the plant exhibiting antixenosis. Subsequently, care should be taken when exposing the RWA to plants exhibiting only antixenosis for long periods of time.

2.4.4.3 Tolerance

Plants are tolerant if they survive under levels of infestation that would kill or severely injure susceptible plants (Painter, 1958). A resistant plant that displays tolerance has the ability to withstand infestation while still supporting insect populations that would severely damage susceptible plants (Scott *et al.*, 1991).

Known components of tolerance include plant vigour, compensatory growth, wound healing and changes in photosynthetic partitioning (Tolmay *et al.*, 1999). This mechanism of resistance is determined by measuring plant height, plant dry mass, plant fresh weight and the damage rating of the plants infested with the RWA (Du Toit, 1988; Unger & Quisenberry, 1997; Tolmay *et al.*, 1999).

The use of plant tolerance as a resistance mechanism against the RWA has been emphasized more than other systems (Haile *et al.*, 1999). Baker *et al.* (1992) found that moderate levels of tolerance measured in the greenhouse often correlates to a tolerance under field conditions. The genetic basis of the three resistance mechanisms (antibiosis, antixenosis and tolerance) show that multiple genes appear to govern resistance towards the RWA and that many of these resistant genes seem to be independent (Castro *et al.*, 2001). Pyramiding of genes enhances the genetic basis of resistance to the RWA and makes it more difficult for the RWA to overcome the resistance posed by the plant.

2.5 RWA induced protein alterations

The RWA feeds on phloem sap via a stylet that follows an intercellular path (Fouché *et al.*, 1984). Little information is available on the response of the plant to RWA feeding. Evidence suggests that plants react in a similar fashion to RWA infestation as defense reactions that are caused by pathogen attack, wounding or herbivorous insects (Givovich & Niemeyer, 1996; Botha

et al., 1998; Van der Westhuizen *et al.*, 1998). The objectives are to determine if RWA inducible responses are related to the resistance that plants possess.

RWA feeding on barley showed that on a susceptible 'Morex' line, a complex of proteins (≈ 22 to 24 kDa) disappeared from the protein profile although these had previously been present in the uninfested control. This same protein complex shifted its isoelectric point (toward the basic end) when the resistant barley line (PI 366450) was infested with the RWA (Porter, 1992). Using two-dimensional gel electrophoresis, the protein complex was found to be 23 kDa (Miller *et al.*, 1994). Porter & Webster (2000) found that in spring wheat there is a 24 kDa complex that was inhibited in a susceptible line ('Pavon') but this complex persisted in the resistant line (PI 140207) during RWA infestation.

PI 137739 (SA 1684) and other plant derivatives possessing the *Dn1* gene have been studied in terms of inducible changes as a result of RWA feeding. Rafi *et al.* (1996) showed an induction of three polypeptides (≈ 32, 33 and 35 kDa) upon RWA infestation in PI 137739 (RWA resistant) but not in a susceptible wheat cultivar ('Stephens'). It was suggested that these induced proteins may play a role in plant defense, involvement in the antibiosis displayed by PI 137739, interaction in the reproduction of the RWA or in senescence. Three proteins were also found to be altered in both the susceptible and resistant cultivars. These proteins showed a reduced expression and could be due to the RWA or to wounding by the aphid (Rafi *et al.*, 1996).

Protein profiles of the intercellular washing fluid (IWF) from 'Tugela' and 'Tugela *Dn1*' (these are near-isogenic lines) showed the induction of four complexes of proteins (28-33, 22-24, 18.5-

19.5 and 15.5-17 kDa) upon RWA infestation of the resistant cultivar. Western blots showed that these complexes consists of β-1,3-glucanase, chitinase and PR-S proteins (related to enzymes that possess antifungal activities). Not all proteins were identified (Van der Westhuizen & Pretorius, 1996). The RWA probes intercellularly and these induced proteins might play a role in the resistance that the RWA encounters when feeding on these resistant lines. The RWA has been shown to feed less from the phloem on resistant cultivars and turns to nonphloem feeding to survive (Kindler *et al.*, 1992). Here the RWA might encounter some of these induced proteins which possibly adversely affects the RWA.

When comparing 'Tugela' (RWA susceptible) and its near isogenic line 'Tugela *Dn1*' (RWA resistant), there was a reduction in the water soluble protein content of the third leaves after RWA infestation (Van der Westhuizen & Pretorius, 1995). RWA infestation of 'Tugela *Dn1*' caused an induction of the enzyme β-1,3-glucanase, which was not found for 'Tugela' (Van der Westhuizen *et al.*, 1998). Similar results were found for the enzyme chitinase (Botha *et al.*, 1998). The role of these enzymes in the defense of the plant to the RWA is not clear. One hypothesis is that their action releases oligosaccharides which triggers the defence reactions of the plant, similar to the reaction of the plant to hyphal penetration (Dreyer & Campbell, 1987).

Clearly, more investigations into the exact nature and mechanisms of the induced proteins are necessary to understand their role in the resistance encountered when the RWA feeds on a resistant plant.

2.6 Conclusion

Since the RWA was discovered in South Africa, it has become an important pest on wheat and numerous control methods have been investigated. The use of insecticides has not proven to be cost effective. Resistant plants have been developed and to date the RWA has not overcome this resistance. However, the possibility always exists that this could happen. Understanding the exact mechanism of resistance that these plants pose would help solve this problem if it does arise. The following chapters help in understanding the resistance that the plants possess and how the RWA responds to the resistance of the plants.

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CHAPTER 3

Comparison of Russian wheat aphid (*Diuraphis noxia*) developmental studies on resistant and susceptible near-isogenic wheat (*Triticum aestivum*) cultivars

3.1 Abstract

The Russian wheat aphid (*Diuraphis noxia*) is a serious insect pest of wheat in South Africa causing an average 60% losses in yield. Symptoms of infestation include leaf streaking, inward curling of leaves, leaf senescence and stunted growth. Russian wheat aphid feeding studies were conducted on two near-isogenic wheat lines, cvv. 'Tugela *Dn1*' (Tugela *5/SA 1684; Russian wheat aphid resistant) and 'Tugela' (Russian wheat aphid susceptible), and a third cultivar, 'Palmiet' (Russian wheat aphid susceptible). The development of first instar nymphs was monitored on wheat leaves of the three cultivars. Nymphs preferred the susceptible cultivars because more nymphs survived to reach reproductive maturity (80% for 'Palmiet' and 85% for 'Tugela') compared to the resistant cultivar (25% for 'Tugela *Dn1*'). However, those nymphs that survived to become reproductive adults showed no significant differences in their reproductive rates or life span on the three cultivars. The surviving aphids show no statistical differences when the reproduction and life span on resistant and susceptible wheat plants were compared. The resistance encountered by the RWA on the resistant 'Tugela *Dn1*' line showed that nymphs experienced difficulties in settling down and locating the phloem, causing 75% to die before becoming reproductively mature; indicating the antibiotic resistance of 'Tugela *Dn1*'.

3.2 Introduction

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), was first recognized as a serious insect pest on wheat (*Triticum aestivum*) in South Africa during 1978. Feeding damage by the RWA results in chlorotic lesions on the leaves (Walters *et al.*, 1980) and reduction in growth and development of susceptible plants (Burd & Burton, 1992) because of a reduction in photosynthetic leaf area as a result of leaf stunting and new leaves not unfolding.

The first RWA resistant wheat cultivar (cv. 'Tugela*5/SA 1684'; 'Tugela *Dn1*') was produced in South Africa and released for commercial production in 1989 (Du Toit, 1989). Almost normal growth occurs on resistant wheat plants and only small isolated chlorotic spots are found. There is also a lower RWA colonization on these plants (Quisenberry & Schotzko, 1994).

Most studies on the development of the RWA on wheat have focused on the effect of plant growth stage and temperature (Aalbersberg *et al.*, 1987; Michels & Behle, 1988, 1989; Girma *et al.*, 1990). There have also been investigations into the development of the RWA when reared on resistant and susceptible genotypes of wheat (Quisenberry & Schotzko, 1994; Zwer *et al.*, 1994; Rafi *et al.*, 1996). No developmental studies have been conducted comparing the near-isogenic wheat lines, cvv. 'Tugela' (susceptible to the RWA), 'Tugela *Dn1*' (resistant), as well as the cultivar 'Palmiet' (susceptible).

The diet of an aphid consists mostly of the phloem sap from the host plant. The phloem sap consists largely of sucrose and amino acids, where the sucrose acts as an attractant (Dreyer &

Campbell, 1987). When the RWA probes the plant, the stylet follows an intercellular path to the phloem (Fouché *et al.*, 1984). On resistant wheat lines, the RWA has difficulty in locating the phloem (Webster *et al.*, 1993). The probing time to reach the phloem was also found to be two times longer on the resistant lines (Kindler *et al.*, 1992; Webster *et al.*, 1993). This implies that the RWA encounters some resistance in the intercellular washing fluid (IWF), which is the path of the stylet. Aphid infestation of 'Tugela *Dn1*' results in the induction of four groups of proteins in the IWF (Van der Westhuizen & Pretorius, 1996). No induced proteins were found when the susceptible near-isogenic 'Tugela' cultivar was infested. Induced proteins were also found when a resistant barley line (Porter, 1992) and a resistant wheat line, PI 137739 (Rafi *et al.*, 1996) were infested with the RWA. These induced proteins in the infested resistant cultivar have been hypothesized to play a role in the resistance mechanism to the RWA. Studies comparing the RWA development and reproduction on susceptible and resistant wheat cultivars would help in understanding more about the resistance mechanisms of these plants.

The aim of this study was to infest the near-isogenic lines with the RWA and compare RWA development, longevity and fecundity. These lines were also compared to the susceptible 'Palmiet' cultivar which is not near-isogenic.

3.3 Materials and Methods

3.3.1 Aphids

Wheat (cv. 'Palmiet') was grown in a greenhouse at a temperature of 25±1°C. This cultivar is susceptible to the RWA. RWA obtained from the Agricultural Research Council – Small Grain Institute (ARC-SGI) in Bethlehem, South Africa, was maintained on this wheat.

3.3.2 Developmental studies

Adult apterous aphids were removed from RWA colonies and placed on wheat leaves cut from the 'Palmiet' cultivar. The second and third leaves were used. Three aphids were placed on each leaf. These leaves were placed individually in a petri dish (Michels *et al.*, 1987). The cut ends were placed between moistened paper towels to prevent desiccation of leaves. These leaves were replaced every three to four days (Michels & Behle, 1989), or when they showed signs of desiccation. The petri dishes were placed at 25±1°C with a photoperiod of 12:12 (L:D). A fine horse-hair paint brush was used to transfer aphids (Aalbersberg *et al.*, 1987).

Nymphs produced by the adults were removed daily and placed individually on a wheat leaf as described above. The age of these nymphs (first instar) was therefore known (Aalbersberg *et al.*, 1987). The wheat leaves used here were either one of three cultivars; two cultivars that were susceptible to the RWA ('Palmiet' and 'Tugela') and the other was resistant to the aphid ('Tugela

Dn1') (Tugela*5/SA 1684). The last two cultivars are near-isogenic lines. Each nymph/host-plant combination was replicated 20 times.

Individual nymphs were observed daily for moulting and survival. The presence of exuviae was used to establish that the aphid had moulted (Aalbersberg *et al.*, 1987). This indicated that the aphid had entered the next instar. When the aphids reached the adult stage (fifth instar), the number of progeny produced was also recorded.

3.3.3. *Statistical analyses*

Comparisons of the nymphal developmental period (before the onset of reproduction), progeny production and longevity was analyzed using a Student's t-Test (P = 0.05; Freund, 1976) with the Systat® 7.0.1 (1997) software.

3.4 Results

The development of RWA nymphs were observed on the three wheat cultivars ('Palmiet', 'Tugela' and 'Tugela *Dn1*'). Their ability to survive and become reproductively active is presented in Table 3.1. Nymphal survival to reproductive maturity differed markedly on the susceptible ('Palmiet' and 'Tugela') and resistant wheat cultivars ('Tugela *Dn1*'). RWA survival on the two susceptible cultivars exceeded that of the resistant cultivar .

Table 3.1. Russian wheat aphid survival and reproductive maturity. Twenty nymphs were placed on each of three wheat cultivars ('Palmiet', 'Tugela' and 'Tugela *Dn1*') at 25±1°C with a 12:12 (L:D) photoperiod.

Cultivar	Percentage becoming reproductive adults
'Palmiet'	80%
'Tugela'	85%
'Tugela *Dn1*'	25%

Nymphal developmental time. Developmental time of the nymphal life stages was observed on all three cultivars and the results are presented in Table 3.2. The duration in days spent by the aphids in the II to IV instar was not significantly different on the three cultivars. However, significantly less time was spent in the first instar by aphids on the resistant cultivar ('Tugela

Dn1'). The total duration spent in the developmental period was similar for the near-isogenic lines ('Tugela' and 'Tugela *Dn1*'), being 9.88 and 8.60 days respectively. Nymphs placed on 'Palmiet' remained in the developmental stage for a significantly shorter period (7.81 days) comparised to those on 'Tugela' (9.88 days).

Table 3.2. Duration of developmental stages in days for the Russian wheat aphid to reach the reproductive stage at 25±1°C with a 12:12 (L:D) photoperiod.

Cultivar	Developmental Stages (Instars) (Days) ± SD					Total
	1st*	2nd*	3rd*	4th*	5th*	
'Palmiet'	1.63±0.50^{a}	1.50±0.63^{a}	1.69±0.60^{a}	2.06±0.57^{a}	0.94±0.93^{a}	7.81±1.05^{a}
N	4	0	0	0	0	
'Tugela'	1.82±0.64^{a}	1.88±0.49^{a}	2.29±0.92^{a}	2.18±0.53^{a}	1.71±1.31^{b}	9.88±1.58^{b}
N	0	1	1	1	0	
'Tugela *Dn1*'	1.00±0.00^{b}	2.00±1.23^{a}	1.80±0.84^{a}	2.40±1.14^{a}	1.40±1.52^{ab}	8.60±1.52^{ab}
N	2	4	6	1	2	

*Means in the same column followed by the same letter are not significantly different (P>0.05).

N = the number of aphids out of the original 20 that died before reaching reproductive maturity.

Included in Table 3.2 is the instar life stage during which nonreproductively active nymphs died. On the susceptible cultivar ('Palmiet') all nymphal deaths occurred during the first instar. The

other susceptible cultivar ('Tugela') had one nymphal death in each of the instars from II to IV. The resistant cultivar ('Tugela *Dn1*') had more nymphal deaths in most of the instars.

Reproduction. RWA reproduction on the three cultivars is given in Table 3.3. Results only include those nymphs that survived to become reproductive. There are no significant differences for the average number of nymphs produced on all three wheat cultivars. Comparatively 1.36 nymphs were produced per day on 'Palmiet' and this was no different to those on 'Tugela' and 'Tugela *Dn1*' (1.29 and 1.23 nymphs, respectively).

Table 3.3. Average number of nymphs produced per day, average number of nymphs produced per adult and average life span of the Russian wheat aphid on three wheat cultivars. Aphids were placed at 25±1°C with a 12:12 (L:D) photoperiod. (Table only includes nymphs that survived to become reproductive).

Cultivar	Average Nymphs/ Day ± SD*	Average Nymphs/ Adult ± SD*	Average Life Span (Days) ± SD*
'Palmiet'	1.36±0.38[a]	11.81±6.99[a]	15.13±4.49[a]
'Tugela'	1.29±0.61[a]	13.82±11.52[a]	18.47±6.18[a]
'Tugela *Dn1*'	1.23±0.55[a]	10.20±8.35[a]	14.80±4.92[a]

*Means in the same column followed by the same letter are not significantly different (P>0.05).

Life span. Table 3.3 shows the average life span of the aphids on the three wheat cultivars tested. No significant differences are found amongst the three cultivars, suggesting that if a nymph survived to adulthood, the reproductive rate and average life span is not affected by the choice of the three host-plants used in this study.

3.5 Discussion

RWA nymphs were placed on three wheat cultivars: two were susceptible to the RWA ('Palmiet' and 'Tugela') and the other was resistant ('Tugela *Dn1*'), with the last two being the near-isogenic lines. Nymphs were shown to prefer the susceptible cultivars in that more nymphs survived to reach reproductive maturity when compared to the resistant cultivar. Michels & Behle (1988) found a 74% survival rate when the RWA was placed on a susceptible 'Scout 66' wheat cultivar. This survival rate is similar to that found in this study on the two susceptible cultivars.

The total time spent in the developmental period, the reproduction and life span showed that there were no significant differences between the near-isogenic lines 'Tugela' and 'Tugela *Dn1*'. However, less aphids survived to become reproductively active on the resistant 'Tugela *Dn1*' cultivar. Michels & Behle (1988) found that the RWA spent on average 8.5 days in the prereproductive period on a susceptible wheat cultivar. They found the RWA to produce an average of 1.30 nymphs per day. Both these findings do not seem to differ markedly from the findings of this study. However, 16.90 nymphs were produced per adult and the RWA had a life span of 22.60 days (Michels & Behle, 1988), which is more than that observed in this study.

The RWA resistant cultivar, 'Tugela *Dn1*', displays moderate levels of resistance (antibiosis). Antibiosis is defined as the manner in which resistant plants adversely affect the biology of the insect (Painter, 1958). This resistance is conferred by the *Dn1* gene from the plant introduction line (PI) 137739 (Du Toit, 1989). Painter (1958) described some effects resulting from the

feeding of insects on plants exhibiting antibiosis. These include death of the insect that often occurs during the first instar, abnormal life span, decreased fecundity and restlessness. When looking at the RWA on the resistant cultivar it was found that the aphid did not have a great likelihood of reaching adulthood (only 25%). However, death of the RWA only occurred 13% of the time in the first instar. The most deaths occurred in the third instar (40%). The life span and the fecundity were similar to those aphids on the susceptible cultivars. However, the aphids were more restless on the resistant cultivar. It has been shown that the probing time to reach the phloem is two times longer on resistant lines (Kindler *et al.*, 1992; Webster *et al.*, 1993). Aphids also made more brief probes on the resistant lines, although this was not found by Kindler *et al.* (1992). Therefore, aphids on the resistant lines had more difficulty in locating the phloem and less phloem sap was ingested. More time was spent on nonphloem feeding to survive.

In South Africa the RWA reproduces only by way of parthenogenesis (*i.e.* reproduction takes place without fertilization). Only winged (alate) or wingless (apterous) vivaparous females are found. Winged females are only produced on depletion of the food source or under adverse environmental conditions (Robinson, 1992). Countries where the RWA is indigenous have males and oviparae that mate to produce eggs. These eggs then overwinter during the harsh winters in these countries (Kiriac *et al.*, 1990). No sexual forms have been reported from South Africa.

All the RWA in South Africa are therefore asexual clones of each other. This then raises the question of how some individuals overcome resistance. The moderate levels of resistance (antibiosis) does not completely inhibit the aphid from feeding on the plant (Du Toit, 1987). One finds a lower colonization of aphids on infested resistant plants when compared to susceptible

plants (Quisenberry & Schotzko, 1994). Only small isolated chlorotic spots are found and growth is almost normal. Therefore, some aphids in a population do manage to survive on resistant wheat plants. This is in agreement with results found in this study. Twenty-five percent of aphids placed on the resistant cultivar manage to overcome the resistance posed by 'Tugela *Dn1*' and show reproduction and longevity similar to aphids placed on the susceptible 'Palmiet' and 'Tugela' cultivars.

This is the first reported case that the surviving aphids show no statistical differences when comparing reproduction and life span on resistant and susceptible wheat plants. Rafi *et al.* (1996) found that there was a higher reproductive rate of the RWA on the susceptible cultivar compared to that of the resistant cultivar. However, in their experiment, the aphids were placed in leaf cages at densities of 20, 40 or 80 aphids. No comparison can be drawn when comparing aphids on excised leaves to those on plants as the physiology of the plant tissue changes after leaf excision. Aphids often accept excised leaves more readily; they develop more quickly and are larger than those on whole plants (Blackman, 1988). However, the excised leaves of all three wheat cultivars in this study were subjected to the same environmental conditions and thus comparisons can be drawn about the aphid's life span and reproductive rates on the three cultivars tested.

This study has shown that when the RWA was placed on the near-isogenic lines ('Tugela' and 'Tugela *Dn1*') the aphid encountered some resistance in the resistant line. This resistance was encountered when the aphid started to probe the leaf initially (Kindler *et al.*, 1992; Webster *et al.*, 1993) and resulted in the death of the nymph before the aphid became reproductive. A small percentage of nymphs survived and displayed similar development, fecundity and life span to

those aphids on the susceptible cultivar. Aphids on resistant cultivars have difficulty in locating the phloem and then turn to nonphloem feeding to survive (Webster *et al.*, 1993). This does not have the same nutritional benefit as the phloem sap. Aphid infestation on 'Tugela *Dn1*' resulted in the induction of four groups of proteins in the intercellular washing fluid (Van der Westhuizen & Pretorius, 1996). These induced proteins have been hypothesized to play a role in the resistance of 'Tugela *Dn1*' to the RWA. On the resistant cultivar, the aphid will encounter these induced proteins when feeding on the nonphloem to survive. Further research would concentrate on identifying these induced proteins and determining if they play a role in the resistance that these plants pose to the RWA.

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Chapter 4

Development of an artificial diet for the Russian wheat aphid (*Diuraphis noxia*)

4.1 Abstract

The Russian wheat aphid, *Diuraphis noxia* (Mordvilko), has been a serious insect pest of wheat (*Triticum aestivum*) since its detection in South Africa during 1978. Insect damage has been curbed by the use of resistant wheat cultivars and chemical control, but little information is available on the exact mechanism of resistance that these cultivars possess. The development of an artificial diet would enable researchers to test possible resistant compounds for their deterrency to the Russian wheat aphid. Three sucrose concentrations (10, 20 and 30%) were tested to determine the optimal concentration required by the aphid. The aphid was found to prefer an optimal sucrose concentration of 20%. Further results showed that the Russian wheat aphid had dietary requirements for three essential amino acids (methionine, leucine and tryptophan). The addition of these amino acids to 20% sucrose resulted in an increase in the reproductive rate of the aphid. The addition of potassium phosphate and magnesium chloride was also found to be beneficial to the Russian wheat aphid. These two salts increased the life span of the aphid. In this study, an artificial diet was developed for the Russian wheat aphid that is simple, yet effective.

4.2 Introduction

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae), is a serious insect pest of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). It was first discovered in South Africa during 1978 on wheat in the northern Free State and has since become the most destructive pest on wheat in this country.

Feeding damage by the RWA results in chlorotic lesions on the leaves (Walters *et al.*, 1980) and a reduction in growth and development of susceptible plants (Burd & Burton, 1992). The aphids occur mainly on the new growth of plants, in the axils of the leaves or within curled leaves. RWA feeding on wheat causes a reduction in leaf area due to a combination of leaf stunting and new leaves not unfolding (Burd & Burton, 1992).

During nutritional requirement investigations of aphids in general, several chemically defined artificial diets have been developed. These artificial diets have been used as a feeding system in elucidating the nutritional requirements of aphids (Mittler, 1967), for studying their salivary proteins (Urbanska *et al.*, 1998; Cherquil & Tjallingii, 2000), as well as testing the deterrency of certain potentially resistant compounds (*e.g.* plant proteinase inhibitors) to aphids (Tran *et al.*, 1997).

Very little has been published on an artificial diet for the RWA. Damsteegt *et al.* (1992) exposed the RWA for 24 hours to a simple 20% sucrose solution containing *Rhopalosiphum padi* viruses (RhPV) to determine if the aphid transmitted viruses. Tran *et al.* (1997) reported on a more

complex diet than that of Damsteegt *et al.* (1992). However, this complex diet was developed specifically for the corn leaf aphid (*Rhopalosiphum maidis*) and was less useful for other cereal aphids (Kieckhefer & Derr, 1967). The RWA was only kept on this diet for 3 days to test the effectiveness of plant proteinase inhibitors in controlling the aphid on wheat. Thus, a simple yet effective artificial diet still needs to be developed for the RWA.

Investigations into the use of artificial diets for other aphids revealed that several constituents might be included in an artificial diet for the RWA. Methionine is strongly phagostimulatory to *Myzus persicae* (Mittler & Dadd, 1964), but not for *Aphis fabae* (Leckstein & Llewellyn, 1974); although methionine is essential for *A. fabae* to reach adulthood and to synthesize proteins. Furthermore, the RWA has endosymbiotic bacteria (*Buchnera* sp.) which overproduce limiting amino acids which benefit their hosts (Douglas, 1998). The bacteria synthesize two essential amino acids for the RWA, namely tryptophan (Lai *et al.*, 1996) and leucine (Thao *et al.*, 1998). There is a reduction in the number of gene copies of these two amino acids carried by the endosymbiotic bacteria in the RWA (Lai *et al.*, 1994; Thao *et al.*, 1998). Telang *et al.* (1999) found that the concentrations of both leucine and tryptophan increased significantly in infested susceptible wheat. Therefore a more efficient mechanism for utilizing nitrogen by the RWA has resulted in loss of copies of these genes by *Buchnera*. Conversely, increased levels of tryptophan and leucine in infested susceptible wheat may have reduced the need for provisioning by the endosymbiont. Retention of single copies of the genes has been selected for when the aphid encounters nutritionally deficient hosts. Methionine, tryptophan and leucine were therefore included in the artificial diet.

Omission of potassium phosphate and magnesium chloride from the diet of *M. persicae* resulted in a reduction in longevity and reproduction of these aphids (Dadd & Mittler, 1965). Similarly, survival and growth of the pea aphid, *Acyrthosiphon pisum*, was significantly increased by the presence of these two major salts (Auclair, 1965). Hence, potassium phosphate and magnesium chloride were included in the artificial diet.

The aim of this study was to develop an artificial diet for the RWA. An effective, simple artificial diet would enable RWA deterrence studies to be done to identify potential resistant compounds. These would be included in an artificial diet to determine their effectiveness in inhibiting aphid fecundity and longevity.

4.3. Materials and Methods

4.3.1 Aphids

Wheat (*Triticum aestivum* cv. 'Palmiet') was grown in a greenhouse at a temperature of 25±1°C. This cultivar is susceptible to the RWA. RWA obtained from the Agricultural Research Council – Small Grain Institute (ARC-SGI) in Bethlehem, South Africa, was maintained on this wheat.

4.3.2 Artificial diet

4.3.2.1 Testing optimal sucrose concentrations

Adult apterous aphids were removed from RWA colonies and placed on wheat leaves cut from the 'Palmiet' cultivar. Three adults were placed on each leaf. These leaves were placed individually in a petri dish (Michels *et al.*, 1987). The cut ends were placed between moistened paper towels to prevent desiccation of leaves. The leaves were replaced every three to four days (Michels & Behle, 1989), or when they showed signs of desiccation. The petri dishes were maintained at 25±1°C with a photoperiod of 12:12 (L:D). A fine horse-hair paint brush was used to transfer aphids to ensure that they were not harmed (Aalbersberg *et al.*, 1987).

Nymphs produced by the adults were removed daily and placed individually on a wheat leaf as described above. The age of these nymphs (first instar) was therefore known. Individual nymphs were observed daily for moulting and survival. The presence of exuviae was used to determine that the aphid had moulted (Aalbersberg *et al.*, 1987). This indicated that the aphid had entered the next instar. When the aphids reached the adult stage (fifth instar), each adult was individually

placed in a glass test-tube (135 mm in length), similar to that described by Mittler and Dadd (1964).

The open end of the test-tube was covered with a stretched Parafilm®M sachet containing 100 µl artificial diet (Kieckhefer & Derr, 1967). The first membrane was stretched to a tenth of its original thickness and placed over the top of the test-tube. The diet was then placed on the taut surface and covered with a second stretched membrane. Covering the sachet was a piece of yellow cellophane (Kieckhefer *et al.*, 1976; Tran *et al.*, 1997), which acted as an attractant to the aphid. The test-tube was placed upside down and gently tapped till the aphid fell onto the Parafilm®M sachet. The test-tubes were maintained at 25±1°C with a 12:12 (L:D) photoperiod.

Three diets were tested: a 10% sucrose, 20% sucrose, and 30% sucrose solution. These diet solutions were buffered to pH 7.0 with a 100 mM K_2HPO_4 solution (Bouwer, pers. comm[a].). The diets were filter sterilized (0.45 µm pore size) and stored at 4°C. Each adult/diet combination was replicated 20 times. Sachets were replaced every 24 hours to prevent bacterial contamination (Kieckhefer & Derr, 1967). Adults were observed daily for survival and production of nymphs.

4.3.2.2 Testing diet A and B

Adult aphids were placed on one of two artificial diets (Table 4.1). The optimal sucrose concentration used in Diet A and B was as determined in the previous section. Diet A contained all the components indicated in the table and Diet B was similar to Diet A except for the

[a] Bouwer, G. Department of Molecular and Cell Biology, University of the Witwatersrand, South Africa.

exclusion of magnesium chloride and potassium phosphate.

Newborn nymphs were placed on 'Palmiet' wheat leaves as discussed above. The adults that developed from these nymphs were placed on either Diet A or B. Twenty replicates were done on each diet. Adults were observed daily for survival and the production of nymphs.

4.3.3 Statistical analyses

Results were analyzed and compared for progeny production and survival of aphids when placed on the five artificial diets. Data was subjected to a Student's t-Test (P = 0.05; Freund, 1976) using the Systat® 7.0.1 (1997) software.

Table 4.1. Composition of Diet A and Diet B.

Components	A[a]		B[a]
L-amino acids			
Methionine	0.10g	Akey & Beck, 1971	0.10g
Leucine	0.20g		0.20g
Tryptophane	0.10g		0.10g
Sucrose	20.00g		20.00g
$MgCl_2.6H_2O$	0.20g	Dadd & Mittler, 1965	--
K_3PO_4	0.25g		--

[a]pH adjusted to 7.0 with 100 mM K_2HPO_4, and ddH_2O added to make 100 ml of diet.

4.4. Results

RWA adults were placed on diets of differing sucrose concentrations to determine the optimal sucrose concentration for the aphid. Three sucrose concentrations were tested, namely, 10%, 20% and 30%. The optimal sucrose concentration was then included in testing two further diets, Diet A and Diet B.

Testing optimal sucrose concentrations. RWA adults placed on the three sucrose diets (10%, 20% and 30% sucrose) showed that those on the 20% and 30% sucrose diets were more likely to overcome the adjustment from feeding on wheat leaves to feeding on the artificial diet and to become reproductively successful (Table 4.2). Only 45% of adults became reproductively successful on the 10% sucrose diet, as compared to 85% and 70% on the 20% and 30% sucrose diets, respectively.

Aphids that were reproductively successful on the three sucrose diets are presented in Table 4.3. Significantly more nymphs per adult were produced on the 20% than 10% sucrose diets. Aphids produced a comparative count of 5.82 nymphs on the 20% sucrose diet versus 2.56 nymphs on the 10% sucrose diet. Also, significantly more nymphs per day were produced on the 20% sucrose compared to the 30% sucrose diet (0.40 and 0.23 nymphs per day, respectively).

Table 4.2. Russian wheat aphid adults surviving to become reproductive. Twenty adults were placed on each of the five diets at 25±1°C and a 12:12 (L:D) photoperiod.

Diet	Adults survival and reproductive success
10% sucrose	45%
20% sucrose	85%
30% sucrose	70%
A	90%
B	80%

Table 4.3. Average number of nymphs produced per Russian wheat aphid, average number of nymphs produced per day and average life span on each of the five diets at 25±1°C at a 12:12 (L:D) photoperiod.

Diet	Average nymphs/ adult ± SD[y,z]	Average nymphs/ day ± SD[y,z]	Average life span (days) ± SD[y]
10% sucrose	2.56±2.01[a]	0.22±0.17[ab]	11.35±1.66[a]
20% sucrose	5.82±2.38[b]	0.40±0.19[a]	21.80±7.55[b]
30% sucrose	4.29±1.73[ab]	0.23±0.10[b]	20.00±7.75[b]
A	6.44±4.73[ab]	0.62±0.28[c]	19.60±9.53[b]
B	5.75±4.01[b]	0.78±0.35[c]	14.60±4.70[c]

[y] Means in the same column followed by the same letter are not significantly different (P>0.05) based on the Student's t-Test (Freund, 1976).

[z] The first and second columns only includes adults that survived to become reproductively active.

Longevity of the aphids on the three diets is presented in Table 4.3. The aphids had a significantly longer life span on the 20% and 30% sucrose diets compared to those on the 10% sucrose diet. Aphids had a longevity of 11.35 days on the 10% sucrose diet which was significantly less than the 21.80 and 20.00 days on the 20% and 30% sucrose diets, respectively.

Testing Diet A and B. The more complex Diets A and B showed similar results to the 20% and 30% sucrose diets when reproductive activity was compared (Table 4.2). Ninety percent of adults on Diet A successfully overcame the change from feeding on wheat leaves to an artificial diet as compared to that of diet B (80%).

RWA reproduction on Diet A and B is presented in Table 4.3. No significant differences were found for the number of nymphs per adult or number of nymphs per day on either of Diet A or B. However, significantly more nymphs per day were produced on Diets A and B compared to that of the three sucrose diets. The addition of three essential amino acids, methionine, leucine and tryptophan, showed a significant increase in the number of nymphs being produced each day.

RWA longevity studies showed that aphids survived significantly longer on Diet A than on Diet B (19.60 and 14.60 days, respectively) (Table 4.3). The omission of the two major salts in Diet B resulted in a significantly lower longevity when compared to Diet A.

4.5. Discussion

During the development of an artificial diet for the RWA (Diet A), it was found that aphids had a requirement for three important dietary sources. Firstly, a carbon source (sucrose) with an optimum concentration of 20%, that allowed a greater number of aphids to survive and become reproductively active when transferred to the artificial diets. This is probably because sucrose is a feeding stimulant (Dreyer & Campbell, 1987). The 20% sucrose concentration is in agreement with several other studies on aphids. *Myzus persicae* has an optimal sucrose range of 10% to 20% (Mittler & Dadd, 1963), while *Aphis gossypii* has an optimal sucrose concentration of 20% (Turner, 1971). However, not all aphids have similar sucrose requirements. *Acyrthosiphon pisum* required 35% sucrose to survive (Akey & Beck, 1971).

Secondly, the RWA showed a requirement for a nitrogen source (methionine, leucine and tryptophan). The addition of these three essential amino acids to 20% sucrose (Diet B) showed a significant increase in the average number of nymphs being produced each day as compared to that of the 20% sucrose solution. Thirdly, the addition of the two salts (Diet A) resulted in an increased life span. Similar results were found with *M. persicae* and *A. pisum* (Auclair, 1965; Dadd & Mittler, 1965).

Comparison of the life span of the RWA on wheat leaves to that on artificial diets showed similar results to that obtained on Diet A (Michels & Behle, 1988; Bahlmann, unpubl.). However, a reduction in the reproductive rate of the RWA on the experimental artificial diets was found. This could be due to a number of factors. The RWA feeds via a stylet that follows an

intercellular path until the phloem is reached. Feeding by the RWA results in ultrastructural damage with the chloroplast membrane being degraded. The subsequent release of nutrients from the chloroplast has been hypothesized to benefit the aphid as RWA feeding was shown to enhance the nutritional quality of the phloem (Fouché *et al.*, 1984; Telang *et al.*, 1999; Sandström *et al.*, 2000). The RWA also has an endosymbiont (*Buchnera*) that showed a reduction in the number of gene copies of leucine and tryptophan (Lai *et al.*, 1994; Thao *et al.*, 1998), the same amino acids that showed an increase in infested susceptible wheat (Telang *et al.*, 1999). The diet formulated in this study cannot match the natural phloem sap encountered by the aphid as well as the conditions encountered when the aphid is feeding on a susceptible wheat plant. The plant-insect-endosymbiont interaction therefore plays a complex role in the nutrition of the RWA.

In this study, a simple and effective diet was developed (Diet A) for the RWA. Although, it does not mimic the conditions encountered by the aphid in a wheat plant, it provides an easy and effective method of testing for the efficacy of potentially resistant compounds to the RWA.

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Chapter 5

Russian wheat aphid (*Diuraphis noxia*) induced protein alterations in wheat

5.1 Abstract

Several studies were undertaken to identify potential sources of resistance towards the Russian wheat aphid (*Diuraphis* noxia) since its detection in South Africa during 1978. Studies conducted on the intercellular washing fluid of wheat resistant to the Russian wheat aphid ('Tugela *Dn1*'), showed that proteins were induced within six days of infestation. These induced proteins were visible as five bands using SDS-PAGE analysis. Two proteins disappeared from the protein profile of 'Tugela *Dn1*' when the Russian wheat aphid was allowed to feed. Analysis of these proteins using two-dimensional gel electrophoresis indicated that these five induced protein bands corresponded to seven proteins. Subsequently, the ≈ 20 kDa band observed with SDS-PAGE analysis, was revealed as three proteins using two-dimensional gel electrophoresis with pI values of 5.0, 5.2 and 5.8. The induced proteins could not be sequenced because of their low concentrations in relation to the other proteins. Overexpression and underexpression of proteins were also visible after Russian wheat aphid infestation. Two of the induced proteins (≈ 36 and 26 kDa) are possibly β-1,3-glucanases. The other unique proteins in this study have not been identified.

5.2 Introduction

When a plant is exposed to adverse environmental stimuli, certain plant genes are activated that lead to an induction of proteins that partake in the defense response of the plant. The apoplast (intercellular space) plays an important role in the defense strategy of the plant with it being the site where signals originate and also were many defense-related proteins accumulate including the pathogenesis-related (PR) proteins (Bowles, 1990).

The RWA follows an intercellular path towards the phloem with its stylet (Fouché *et al.*, 1984). RWA feeding on resistant wheat, wheatgrass and barley culivars spend considerably less time feeding from the phloem when compared to RWA feeding on the susceptible cultivars. RWA on the resistant cultivars subsequently turned to nonphloem feeding to survive (Kindler *et al.*, 1992; Webster *et al.*, 1993). Van der Westhuizen & Pretorius (1996) found an induction of four groups of proteins in the intercellular fluids of a RWA infested resistant wheat cultivar, 'Tugela *Dn1*'. No induction of proteins was found in the susceptible wheat cultivar ('Tugela') during RWA infestation. Some of the induced proteins were serologically related to PR proteins and the hypothesis was made that these induced proteins are involved in the resistance the RWA encounters when feeding on resistant cultivars. The RWA subsequently encounters these induced proteins when searching for the phloem and, when engaged in nonphloem feeding on resistant cultivars, could possibly ingest these proteins.

Other studies have also shown the induction of proteins in resistant cultivars during RWA infestation (Porter, 1992; Miller *et al.*, 1994; Rafi *et al.*, 1996; Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998; Porter & Webster, 2000). Although some of these proteins have been serologically related to proteins like chitinase, β-1,3-glucanase and proteins that possess antifungal activity, many of the induced proteins still need to be identified. More investigations into the exact nature and mechanisms of the induced proteins are necessary to understand their role in the resistance encountered when the RWA feeds on a resistant plant.

In this study, the induction of proteins was examined in RWA infested and uninfested wheat, 'Tugela *Dn1*' ('Tugela*5/SA 1684', a RWA resistant cultivar). These induced proteins were analyzed using SDS-PAGE as well as two-dimensional gel electrophoresis so that these proteins could possibly be identified.

5.3 Materials and Methods

5.3.1 Plants

Wheat (*Triticum aestivum*) was grown in a greenhouse at a temperature of 25±1°C. RWA was maintained on the 'Palmiet' wheat cultivar, which is RWA susceptible. 'Tugela *Dn1*' was grown to the two leaf growth stage (stage 12; Trottman *et al.*, 1979). At this point the plants were infested with RWA. Aphids were collected with a fine horse-hair paint brush (Aalbersberg *et al.*, 1987) and gently tapped onto the wheat plants to be infested. Approximately five to six aphids were scattered onto each plant. 'Palmiet' plants that were infested with RWA were also placed next to the plants to be infested, so that the RWA could move off the 'Palmiet' plants onto 'Tugela *Dn1*' plants. Control plants were not infested with the RWA.

5.3.2 Intercellular washing fluid (IWF) extraction

IWF was extracted after six days of RWA infestation (Van der Westhuizen & Pretorius, 1996. IWF was extracted according to the method of Rohringer *et al.* (1983). The upper 8 cm of the wheat leaf was used. The cut ends were rinsed twice with distilled water. The leaves (1 g in fresh weight) were then placed with their cut ends first into a glass tube with buffer. The buffer consisted of 50 mM Tris-HCl (pH 7.8) containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and 5 mM mercaptoethanol (Van der Westhuizen & Pretorius, 1996). The glass tube was vacuum infiltrated for 30 minutes. The buffer was

infiltrated by slowly releasing the vacuum. The leaves were removed and quickly blotted dry on paper toweling. The leaves were then centrifuged at 500 *g* for five minutes at 4°C with their ends facing downwards. At the bottom of the centrifuge tube a perforated plastic disk was placed to provide space for the IWF extract. The IWF was recovered after centrifugation and stored at -20°C. The leaves were extracted three times for complete IWF protein collection. IWF was extracted from infested as well as uninfested 'Tugela *Dn1*'.

5.3.3 IWF contamination with cytoplasmic constituents

To establish whether the IWF had been contaminated with intracellular proteins, a malate dehydrogenase (MDH) assay was performed (Cooper, 1977). The assay was carried out at room temperature. Twenty µl of protein sample (corresponding to 5 µg protein) was added to 3.75 mM 1,4-dithiothreitol, 7 mM $MgCl_2$, 0.25 mM NADH, 2.3 mM oxalacetic acid and 80 mM phosphate buffer (pH 7.5) to make up a final volume of 1ml. The reaction was started by adding oxalacetic acid. The oxidation of NADH giving malate was followed spectrophotometrically at a wavelength of 340 nm.

Total protein was extracted from infested 'Tugela *Dn1*' using the method of Van der Westhuizen *et al.* (1998). Leaf tissue was frozen in liquid nitrogen and ground into a fine powder. Proteins were extracted with a 50 mM Tris-HCl buffer (pH 7.8) that contained 2 mM PMSF and 10 mM mercaptoethanol. After centrifugation at 10 000 *g* for 10 min the supernatant was removed and used for the MDH assay.

MDH activity from the IWF protein and total protein extraction was determined for 'Tugela *Dn1*' infested with the RWA and was replicated three times. MDH activity was expressed as µmol NADH.g^{-1} leaf fresh weight.min^{-1}. MDH contamination of less than 2% indicated little cytoplasmic contamination (Botha *et al.*, 1998).

5.3.4 Protein determination

The protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay reagent with gamma globulin as the standard.

5.3.5 SDS-PAGE analysis

Proteins were separated by SDS-PAGE (Laemmli, 1970) using a mini gel system containing 15 and 5% acrylamide (acrylamide: bisacrylamide, ratio of 37.5: 1) for the running and stacking gels, respectively (Van der Westhuizen & Pretorius, 1996). Electrophoresis was carried out at a constant voltage of 200 V.

IWF proteins were precipitated with 9 volumes of ice-cold acetone. This was placed at -70°C for 1 hr followed by centrifugation at 15 000 *g* for 20 min. The supernatant was removed and the pellet allowed to dry. The pellet was resuspended in 50 mM Tris (pH 7.8). Twelve µg of protein was loaded into the wells. Gels were then silver stained (Dunn, 1996). Samples loaded were IWF proteins of infested and uninfested 'Tugela *Dn1*'.

5.3.6 Two-dimensional gel electrophoresis

Isoelectric focusing was performed on the RWA infested and uninfested 'Tugela *Dn1*' IWF samples. The gel solution contained 5.5 g urea, 2.5 ml double distilled water, 1 ml acrylamide/bisacrylamide solution (40% T[1], 5% C_{bis}[2]), 2 ml of a 10% Nonidet P-40 solution, 0.4 ml ampholyte (pH 5-7), 0.1 ml ampholyte (pH 3-10), 7 µl *N, N, N, 'N'*-tetramethyl-ethylenediamine (TEMED) and 10 µl 10% ammonium persulphate. Gels were then cast in glass tubes that had a 1 mm diameter and were 15 cm long. They were allowed to polymerize for 1 hour at room temperature (Oosthuizen *et al.*, 2001).

The gel tubes were then placed in the upper chamber of a Hoefer SE600 gel electrophoresis unit. The anode solution consisted of 10 mM phosphoric acid and the cathode solution of 10 mM histidine. Five µl of sample overlay solution (9 M urea and 2% ampholytes) was added to the top of each gel (Oosthuizen *et al.*, 2001). Gels were then prerun as follows:

i) 200 V for 15 min

ii) 300 V for 30 min

iii) 400 V for 30 min

Protein samples (100 µg) were concentrated according to the method of Wessel & Flugge

[1] Percentage of the total for acrylamide and bisacrylamide
[2] Concentration of bisacrylamide

(1984) and resuspended to make a final volume of 20 µl. The protein samples were mixed with 5 µl sample buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholytes and 5% mercaptoethanol). This was then loaded into the pre-focused gels and overlayed with 5 µl sample overlay solution. The IEF gels were then run at 400 V for 16 h and then at 800 V for an additional hour (Oosthuizen *et al.*, 2001).

After IEF, the tube gels were squirted out with dH_2O and equilibrated in equilibration buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 5% mercaptoethanol) for 20 min (Oosthuizen *et al.*, 2001).

A uniform SDS-polyacrylamide separating gel (15% T, 2.7% C_{bis}) was prepared. The IEF gel was sandwiched onto the SDS-PAGE gel with an agarose sealing solution (0.5 M Tris-HCl pH 6.8, 1% SDS, 1% agarose). The SDS-PAGE gel was run at 5 W for 15 min followed by 10 W for 5 h 15 min. A constant temperature of 18°C was maintained during IEF and SDS-PAGE electrophoresis (Oosthuizen *et al.*, 2001). After electrophoresis, gels were silver-stained according to the method of Dunn (1996). Two-dimensional gel electrophoresis of the IWF protein samples of infested and uninfested 'Tugela *Dn1*' were replicated three times to ensure reproducibility of the protein profiles.

5.3.7 Analysis of protein profiles

The IWF sample of the uninfested 'Tugela *Dn1*' and the RWA infested 'Tugela *Dn1*' were replicated three times to ensure consistency of the protein profiles obtained. As the

number of spots obtained from the two-dimensional gel electrophoresis were few (due to only intercellular proteins being used), the spots were analyzed visually. The number of protein spots per gel was determined and the distinct differences between the two gels were marked.

5.4 Results

IWF was extracted from RWA uninfested and infested 'Tugela *Dn1*' to investigate the induction of proteins in the apoplast, which is the route that the RWA stylet takes to reach the phloem (Fouché *et al.*, 1984). A MDH assay was done to determine if there was any contamination from intracellular proteins.

MDH contamination. The percentage of MDH contamination is shown in Table 5.1. The percentage MDH contamination when extracting IWF was 0.14%. This is less than 2% which indicates minor cytoplasmic contamination (Botha *et al.*, 1998).

Table 5.1. Malate dehydrogenase assay (MDH) for IWF and total proteins from RWA infested 'Tugela *Dn1*'.

Protein sample	MDH μmol NADH. g^{-1} leaf fresh weight.min^{-1}	Percentage MDH contamination
IWF proteins from RWA infested 'Tugela *Dn1*'	0.28 ± 0.08*	0.14%
Total proteins from RWA infested 'Tugela *Dn1*'	204.60 ± 8.86*	

* The values given are for the average of three replications.

SDS-PAGE analysis. SDS-PAGE analysis of uninfested and infested 'Tugela *Dn1*' revealed that RWA infestation altered the protein composition of resistant wheat (Figure 5.1). Proteins were induced as well as differentially displayed when comparing the protein profiles. A total of five proteins were unique to the infested 'Tugela *Dn1*' profile (≈ 36, 26, 20 kDa and 2 proteins < 14.2 kDa). Two proteins were only displayed in uninfested 'Tugela *Dn1*' (≈ 29 and 24 kDa). Three proteins were differentially displayed in infested 'Tugela *Dn1*' in comparison to the protein profile obtained for uninfested 'Tugela *Dn1*'. Two proteins were overexpressed (≈ 45 and 22 kDa) and another was underexpressed (≈ 40 kDa) after RWA infestation.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis revealed that there were 48 spots on the uninfested 'Tugela *Dn1*' and 57 spots on the infested 'Tugela *Dn1*' gels (Figure 5.2). As with the SDS-PAGE protein profiles, proteins were induced as well as differentially displayed during RWA infestation (Figure 5.2). Seven protein spots were unique to 'Tugela *Dn1*' during RWA infestation (indicated by diamonds in Figure 6.2B). These unique spots confirmed the same five induced bands obtained with SDS-PAGE analysis (≈ 36 kDa, 26 kDa, three induced proteins all 20 kDa and two induced proteins < 14.2 kDa). As was expected, two-dimensional gel electrophoresis (separation according to protein charge and size) separated proteins of similar size that occurred in the same area on the SDS-PAGE gel.

Proteins whose expression is unique only to uninfested 'Tugela *Dn1*', are indicated by diamonds in Figure 5.2A. As with the SDS-PAGE protein profiles, two proteins were

found (≈ 29 and 24 kDa). RWA infestation also resulted in the up-regulation of a number of proteins, the most prominent being indicated by an oval (Figure 5.2B).

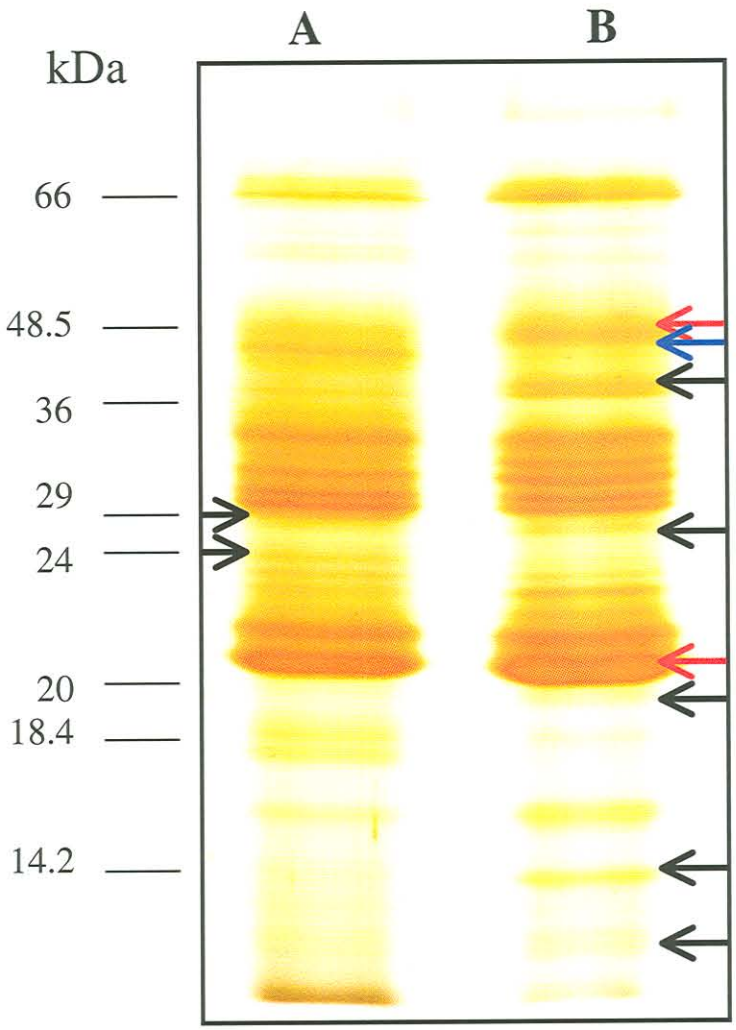

Figure 5.1. SDS-PAGE profiles of intercellular protein from (A) noninfested leaves of 'Tugela *Dn1*' and (B) RWA infested leaves of 'Tugela *Dn1*'. The vertical axis represents molecular masses (kDa). Black arrows indicate unique proteins in each profile. Red arrows indicate proteins that are overexpressed during RWA infestation. Blue arrows indicate proteins that are underexpressed during RWA infestation.

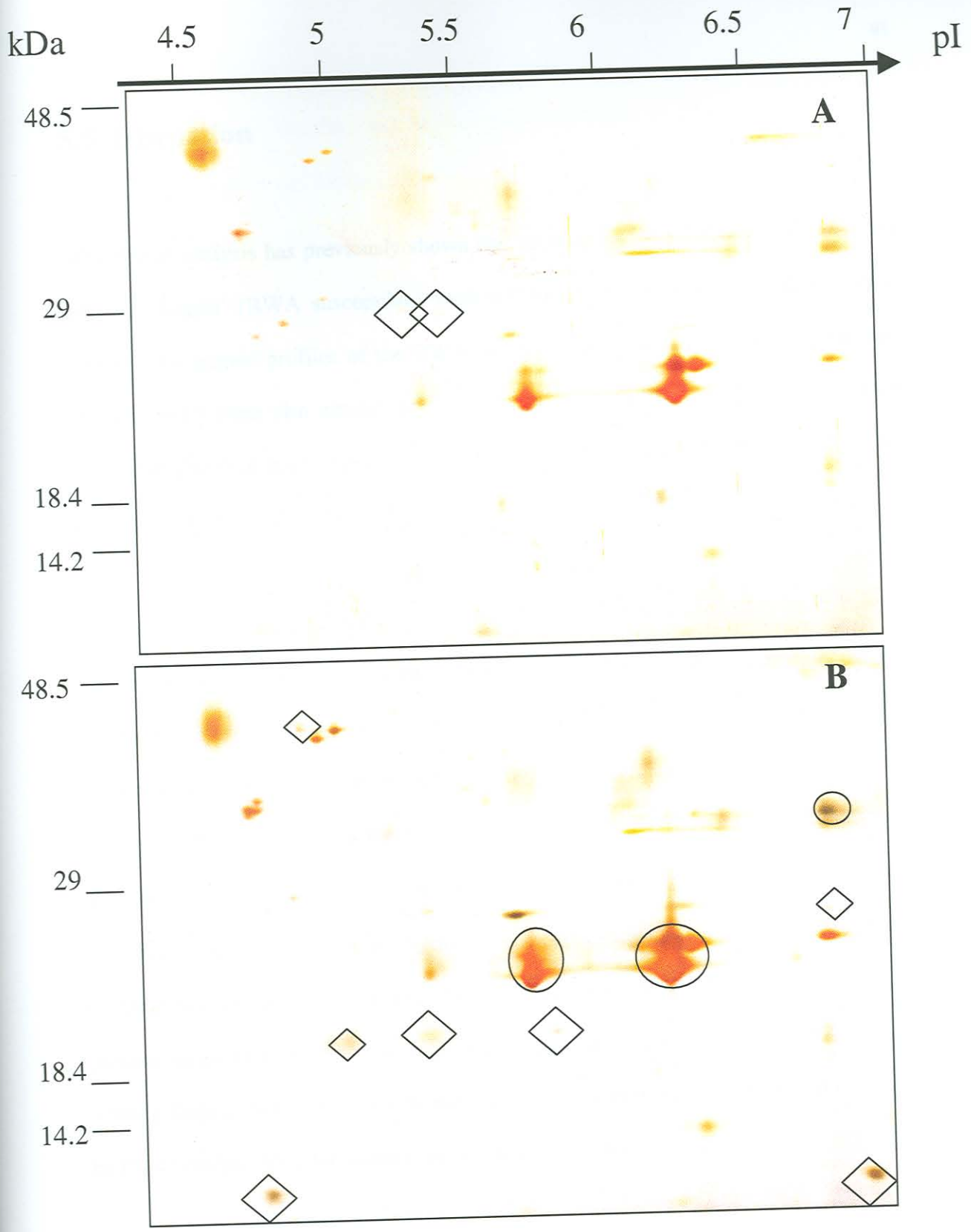

Figure 5.2. Protein profiles after two-dimensional gel electrophoresis of intercellular proteins from (A) noninfested 'Tugela *Dn1*' and (B) RWA infested 'Tugela *Dn1*'. The horizontal axes represents pI's of the isoelectric focusing gradients. The vertical axes represents molecular masses (kDa). Diamonds indicate unique proteins. Ovals indicate proteins that are overexpressed. The protein marker is shown on the left and the pI points to the top of each gel.

5.5 Discussion

SDS-PAGE analysis has previously shown that protein profiles of RWA uninfested and infested 'Tugela' (RWA susceptible) were similar (Van der Westhuizen & Pretorius, 1996). The protein profiles of the RWA uninfested near-isogenic lines ('Tugela' and 'Tugela *Dn1*') were also similar which indicates the lack of a constitutive resistance factor and also their near-isogenic nature (Van der Westhuizen & Botha, 1993; Van der Westhuizen & Pretorius, 1996).

Induction of proteins was confirmed during RWA infestation of the resistant cultivar ('Tugela *Dn1*'). The five induced proteins seen on the SDS-PAGE profile did not correspond to the induced proteins obtained by Van der Westhuizen & Pretorius (1996). They obtained induced proteins in the following molecular mass ranges: 28-33 (group 1); 22-24 (group 2); 18.5-19.5 (group 3) and 15.5-17 kDa (group 4). Group 1 proteins were serologically related to the β-1,3-glucanase and chitinase PR-proteins and have been implicated in defense against pathogens. The group 2 proteins were serologically related to chitinases and proteins that possess antifungal activity (PR-S proteins). Group 3 proteins appeared to represent a single protein that was overexpressed in RWA resistant wheat ('Tugela *Dn1*'). Group 4 proteins showed two induced bands that could possibly be PR-4 proteins (Van der Westhuizen & Pretorius, 1996).

This study shows an induction of five groups of proteins which corresponds to seven proteins. The sizes of these proteins are different to those found by Van der Westhuizen

& Pretorius (1996). The first and second induced proteins (≈ 36 and 26 kDa) could possibly be related to the PR-proteins, β-1,3-glucanases and chitinases. These enzymes have sizes that correspond to those of the induced proteins (Fink *et al.*, 1988; Van der Westhuizen *et al.*, 1998). β-1,3-glucanase were found to be induced during RWA infestation of 'Tugela *Dn1*' with the isozymes having pI values ranging from 9.3 to 3.6 (Van der Westhuizen *et al.*, 1998). RWA infestation also resulted in the expression of one chitinase isozyme with a p*I* of 5.5 (Botha *et al.*, 1998). The pI value of the first induced protein in this study was 4.9 and subsequently could be β-1,3-glucanase. The second induced protein has a pI value of 6.8 and is possibly also related to β-1,3-glucanase.

The second group of induced proteins observed by Van der Westhuizen *et al.* (1998), was serologically related to PR-S proteins possessing antifungal activity. There are similarities between a microbial attack on a plant and aphid infestation. β-1,3-glucanases digest cell wall constituents of plants, pathogenic fungi and bacteria and release elicitors that could activate the defense response of the plant. Chitinases hydrolyzes chitin of fungi and insects to protect the plant against attack (Botha *et al.*, 1998). The actual direct role of β-1,3-glucanase and chitinases in plant defense against the RWA is unknown. One hypothesis is that their action releases oligosaccharides which triggers the defence reactions of the plant, similar to the reaction of the plant to hyphal penetration (Dreyer & Campbell, 1987).

Group 3 proteins described by Van der Westhuizen *et al.* (1998) were not serologically related to any protein and their function is unknown. Occurring closely to this group of proteins was the three induced proteins observed in this study (all ≈ 20 kDa). The last two induced proteins in this study (< 14 kDa) are dissimilar to the group 4 proteins found by Van der Westhuizen *et al.* (1998). Also, the two proteins absent after RWA infestation could not be identified. Protein sequence analysis of induced proteins would help clarify the exact nature of these proteins. Two-dimensional gel electrophoresis has shown that bands obtained from SDS-PAGE analysis represent more than one protein.

Other studies have also indicated that changes in the protein profiles occur during RWA infestation. Miller *et al.* (1994) demonstrated the changes in proteins with two-dimensional gel electrophoresis of total protein from RWA infested 'Morex', a susceptible barley cultivar, and PI 366450, a resistant Afghanistan barley accession (plants were infested for six days). The PI 366450 cultivar showed a shift in the isoelectric point of a 23 kDa complex, with the complex being inhibited in 'Morex'. Porter & Webster (2000) showed an inhibition in a 24 kDa protein complex in a RWA infested susceptible wheat cultivar ('Pavon') that persisted in a RWA infested resistant cultivar (PI 140207). The protein profiles obtained were from total protein extracted from leaves that were infested for four days. Rafi *et al.* (1996) showed using SDS-PAGE analysis, enhanced and reduced expression as well as the appearance of unique proteins from total protein extracts. There was an appearance of a ≈ 53 kDa protein in RWA infested PI 137739 (RWA resistant wheat cultivar) at 11 days. This protein also appeared in the susceptible RWA cultivar ('Stephens') at day six.

RWA feeding does alter the expression of proteins upon infestation of plants. RWA feeding can cause the overexpression and underexpression of proteins as well as the appearance of novel proteins, as the plant attempts to defend itself against attack. The exact nature and role of these proteins is not known but they do seem to play a part in the defense of the plant against aphid feeding. RWA induced proteins are found in the apoplast (the route the stylet takes) and it has been shown that RWA on resistant plants feed less from the phloem and turn to nonphloem feeding to survive (Kindler *et al.*, 1992; Webster *et al.*, 1993). The induction of proteins (β-1,3-glucanase and chitinase) in the apoplast possibly aids the defense reaction of the plant to invasion (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998). Unidentified induced proteins could possibly act as a deterrent to the RWA when it turns to nonphloem feeding on resistant plants. Further research should identify these proteins and their roles tested in artificial diet experiments.

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CHAPTER 6

Leaf epicuticular wax ultrastructure and trichome effect on Russian wheat aphid (*Diuraphis noxia*) feeding

6.1 Abstract

The Russian wheat aphid (*Diuraphis noxia*) was first discovered on wheat in South Africa during 1978. It has since become a serious pest. The leaf epicuticular wax ultrastructure and leaf trichomes were examined on two Russian wheat aphid susceptible wheat cultivars ('Palmiet' and 'Tugela') and a Russian wheat aphid resistant wheat cultivar ('Tugela *Dn1*'). The lengths of the trichomes showed no significant differences in the three wheat cultivars examined. The resistant cultivar ('Tugela *Dn1*') had a significantly greater trichome density than the susceptible cultivars. Examination of the position of the trichomes revealed that there were differences for the adaxial and abaxial surfaces. Trichomes on all three wheat cultivars were found to occur mostly on the leaf veins of the adaxial surfaces, and on the leaf veins as well as between them on the abaxial surfaces. Leaf trichome density and position may act as physical obstacles to Russian wheat aphid feeding on leaf veins of adaxial leaf surfaces. The high trichome density on the leaf veins found in the resistant 'Tugela *Dn1*' cultivar could prevent the Russian wheat aphid from finding a suitable feeding site. Comparison of the scanning electron micrograph photos showed that the epicuticular wax structure was found to be similar for both the adaxial and abaxial surfaces amongst the three wheat cultivars. The wax structure was similar in the Russian wheat aphid resistant and susceptible cultivars and *does not seem to affect Russian wheat aphid feeding.*

6.2 Introduction

During its search for a potential food source, the Russian wheat aphid (*Diuraphis noxia*) (RWA) settles on a plant and comes into contact with the thin layer of lipids found on the surface known as the epicuticular wax. This covers the entire leaf surface. The chemical composition of these waxes are distinctive for each plant species and could play a role in RWA acceptance of the host plant (Dillworth & Berberet, 1990).

For the aphid *Sitobion avenae* feeding on wheat, cuticular waxes of leaves from wheat cultivars that were dark green and glossy (non-glaucous) were implicated in conferring resistance to this aphid. Conversely, glaucous (pale blue-green) plants were not found to be resistant to *S. avenae*. Fluorescent chromatography revealed that non-glaucous cultivars lacked diketones. Scanning electron microscopy revealed that non-glaucous leaves had a nearly smooth surface when compared to the glaucous leaves (Lowe *et al.*, 1985). They postulated that since diketones absorb ultraviolet light strongly, their absence in non-glaucous wheat would result in a visual deterrence to aphids. Also, the aphids may have difficulty clinging and probing the relatively smooth surface of the non-glaucous plants.

Ni & Quisenberry (1997) examined the epicuticular wax ultrastructure of two wheat cultivars, plant introduction (PI) 137739 (RWA resistant) and 'Arapahoe' (RWA susceptible). They found the wax ultrastructure to be similar in the two wheat cultivars

and not to play a significant role in host preference. Ni *et al.* (1998) also found that leaf epicuticular waxes of different cereal crops had little effect on the feeding of the RWA.

Leaf trichomes have also been implicated in the resistance of plants to aphids. Ni and Quisenberry (1997) postulated that leaf trichomes play a role as a physical obstacle to RWA feeding. RWA that fed on the resistant PI 137739 cultivar spent more time probing the leaves before penetration, than on the susceptible 'Arapahoe' cultivar. The RWA also probed less and the feeding duration was shorter on the resistant cultivar compared to the susceptible cultivar. The resistant cultivar was found to be the least preferred amongst the two cultivars. Examination of the leaf surface structure revealed that leaf trichomes of PI 137739 were more than six times longer than 'Arapahoe', although 'Arapahoe' had a higher trichome density (Ni & Quisenberry, 1997). Trichomes are found on or near leaf veins. Subsequently, long trichomes are likely to be an important physical obstacle to aphids that probe close to leaf veins during phloem feeding.

This study investigated the effect of the leaf epicuticular wax structure and trichomes on RWA feeding. Three wheat cultivars were examined; two were susceptible to the RWA ('Palmiet' and 'Tugela') and the other was resistant to the RWA ('Tugela *Dn1*'; 'Tugela*5/SA 1684'). 'Tugela' and 'Tugela *Dn1*' are near-isogenic lines, differing only by the dominant RWA resistant gene, *Dn1*. The cultivar SA 1684 is also known as PI 137739; PI 137739 and 'Tugela' are the parental lines of 'Tugela *Dn1*' (Du Toit, 1989).

6.3 Materials and Methods

6.3.1 Plants

Wheat (*Triticum aestivum*) was grown in a greenhouse at a temperature of 25±1°C. Three wheat cultivars were grown; two were susceptible to the RWA ('Palmiet' and 'Tugela'), while the third was resistant to the RWA ('Tugela *Dn1*').

6.3.2 Leaf trichome examination

The RWA feeds mostly on the adaxial surfaces of wheat leaves (Ni & Quisenberry, 1997), hence the adaxial surfaces of the second and third wheat leaves were examined for trichomes.

Trichomes were measured using a stereo microscope (5X magnification) that was attached to an AxioCam. Measurements were done using Axiovision 2.0.5.3 (1999). Twenty-one leaves were examined for each of the three cultivars. The length of four randomly selected trichomes was measured on each leaf.

The trichome density was recorded by counting the number of trichomes in a 3 x 2mm^2 area (Ni & Quisenberry, 1997). The area examined was in the center of the leaf. The trichomes were counted using a microscope at 10X magnification. Twenty-one leaves were examined for each of the three cultivars.

6.3.3 Leaf epicuticular wax ultrastructure and trichome position

The epicuticular wax ultrastructure of the second and third wheat leaves of the three cultivars were examined using a scanning electron microscope (SEM) (Jeol JSM-840 Scanning Microscope). Air-dried leaves were used (Gülz *et al.*, 1992) as the standard plant tissue fixation and dehydration process affects the leaf epicuticular wax structure by partially removing the wax (Ni & Quisenberry, 1997; Ni *et al.*, 1998). Ten leaves of each cultivar were placed in a sealed container containing silicon gel. The leaves were taped to the petri dish to prevent curling during drying. The leaves were allowed to air dry for seven days. Ten segments were taken from the center of each leaf for each of the adaxial and abaxial surfaces of each of the three cultivars. These were then mounted on aluminum stubs and sputter-coated with a gold-palladium alloy. The epicuticular wax ultrastructure was then examined on the SEM at 9 500X magnification and photographed.

The position of the trichomes on the adaxial and abaxial surfaces of the wheat leaves was also examined. Leaf segments prepared and used to examine the epicuticular wax ultrastructure (above) were examined at 60X magnification and photographed. Twenty photographs were taken for each wheat cultivar; ten for each of the adaxial and abaxial surfaces. Trichomes were counted between and on veins for both the adaxial and abaxial surfaces for the three wheat cultivars in a 460 x 550 μm^2 area that was randomly chosen.

6.3.4 Statistical analysis

Trichome length, trichome density data and the position of the trichomes were subjected to an Analysis of Variance (ANOVA) (P = 0.05) using the SYSTAT® 7.0.1 (1997) software.

6.4 Results

The leaf trichomes and the structure of the leaf epicuticular wax of three wheat cultivars were examined to determine if they could affect RWA food selection and feeding.

Leaf trichome examination. Leaf trichome data is given in Table 6.1. Trichome density was significantly different in the three cultivars ($F = 40.67$; $df = 2, 249$; $P < 0.05$). The resistant 'Tugela *Dn1*' had the most trichomes per mm^2 compared to the susceptible cultivars. The susceptible 'Tugela' cultivar had 1.7 times less trichomes per mm^2 in comparison to its near-isogenic line, 'Tugela *Dn1*'. 'Palmiet' was found to have 1.37 times less trichomes per mm^2 than 'Tugela *Dn1*'. Of all three wheat cultivars examined, 'Tugela' had the lowest trichome density, having an even lower trichome density than the other RWA susceptible cultivar ('Palmiet').

No significant differences were found for the length of the trichomes ($F = 2.38$; $df = 2, 60$; $P < 0.05$) in the three wheat cultivars examined. The average length of a trichome was 197.67 µm on the resistant 'Tugela *Dn1*' cultivar, which was comparable to the two susceptible cultivars (233.19 and 215.57 µm for 'Palmiet' and 'Tugela', respectively). High standard deviations were found due to the differences in sizes of the randomly selected trichomes.

Leaf trichome position. SEM examination at 60X magnification revealed that trichomes on the adaxial surfaces were mostly located on the leaf veins (Fig. 6.1). Conversely, the

trichomes on the abaxial surfaces were located on leaf veins as well as between leaf veins. This was statistically similar for all three wheat cultivars examined. For 'Palmiet', 'Tugela' and 'Tugela *Dn1*' the number of trichomes between the veins on the adaxial surfaces was significantly less to the number of trichomes between the veins on the abaxial surfaces for 'Palmiet' ($F = 26.55$; $df = 1, 18$; $P < 0.05$), 'Tugela' ($P = 22.46$; $df = 1, 18$; $P < 0.05$) and 'Tugela *Dn1*' ($F = 22.62$; $df=1, 18$; $P < 0.05$). The number of trichomes occurring on the veins of the adaxial and abaxial leaf surfaces were statistically similar for each of 'Palmiet' ($F = 0.86$; $df = 1, 18$; $P < 0.05$), 'Tugela' ($F = 0.01$; $df = 1, 18$; $P < 0.05$) and 'Tugela *Dn1*' ($F = 1.95$; $df = 1, 18$; $P < 0.05$).

Leaf epicuticular wax ultrastructure. Visual examination of the SEM photos (9 500X magnification) of the wheat leaves showed that the epicuticular wax ultrastructure was very similar amongst the three wheat cultivars on both the adaxial and abaxial surfaces examined (Fig. 6.2, 6.3, 6.4). Photos of the ultrastructure of the epicuticular waxes were examined and compared to existing data (Ni & Quisenberry, 1997; Ni *et al.*, 1998). The structure of the epicuticular waxes was found to occur as an irregular mixture that consisted mostly of curved rod-shaped waxes with few flakes. The density of the epicuticular wax was similar for both the adaxial and abaxial leaf surfaces, as well as for the three different cultivars.

Table 6.1. Trichome length and density in three wheat cultivars examined. Two cultivars ('Palmiet' and 'Tugela') are susceptible to the RWA and the third is resistant to the RWA ('Tugela *Dn1*').

Cultivar	Number of trichomes, mm^2		Trichome length, µm	
	Mean ± SD*	n	Mean ± SD*	n
'Palmiet'	20.24±7.54[a]	84	233.19±59.30[a]	21
'Tugela'	16.39±6.90[b]	84	215.57±42.94[a]	21
'Tugela *Dn1*'	27.73±11.49[c]	84	197.67±54.70[a]	21

*Means in the same column followed by the same letter are not significantly different ($P<0.05$).

n = the number of wheat leaves examined.

Table 6.2. Position of trichomes on the adaxial and abaxial surfaces of three wheat cultivars examined. Two cultivars ('Palmiet' and 'Tugela') are susceptible to the RWA and the third is resistant to the RWA ('Tugela *Dn1*').

Cultivar	Number of trichomes between veins, 253 μm^2			Number of trichomes on veins, 253 μm^2		
	Adaxial Mean±SD	Abaxial Mean±SD	n	Adaxial Mean±SD	Abaxial Mean±SD	n
'Palmiet'	0.40±0.52[a]	7.50±4.33[b]	10	6.90±3.18[a]	5.30±4.45[a]	10
'Tugela'	0.10±0.32[a]	6.60±4.33[b]	10	7.60±4.01[a]	7.80±3.99[a]	10
'Tugela *Dn1*'	0.40±0.52[a]	6.20±3.85[b]	10	9.70±3.59[a]	6.60±6.04[a]	10

*Means in the box followed by the same letter are not significantly different ($P<0.05$).

n = the number of wheat leaves examined.

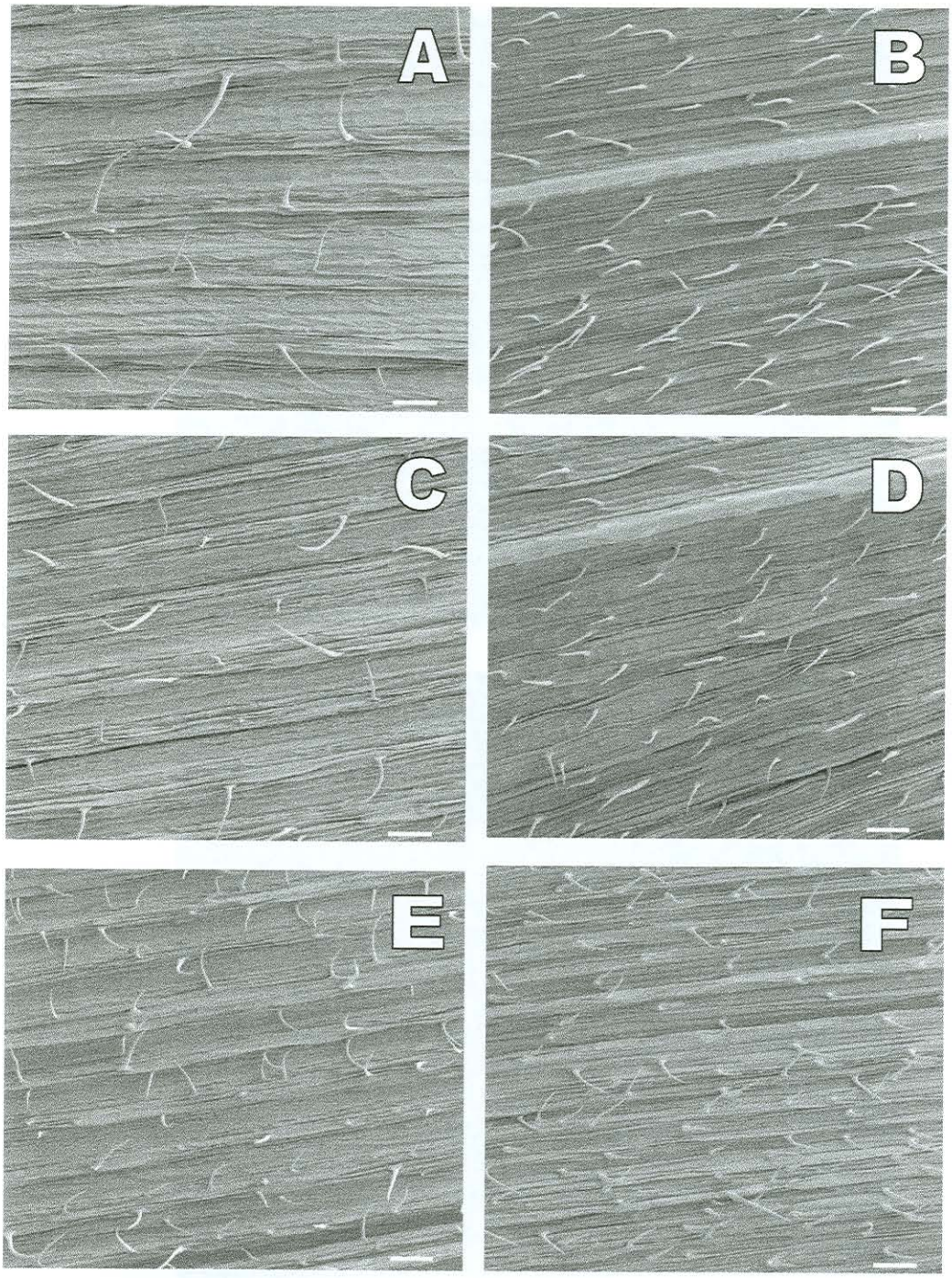

Figure 6.1. Scanning electron micrographs of the position of leaf trichomes on the adaxial and abaxial leaf surfaces of three wheat cultivars (60X; bars = 100 μm). (A) adaxial surface of 'Palmiet'. (B) abaxial surface of 'Palmiet'. (C) adaxial surface of 'Tugela'. (D) abaxial surface of 'Tugela'. (E) adaxial surface of 'Tugela *Dn1*'. (F) abaxial surface of 'Tugela *Dn1*'.

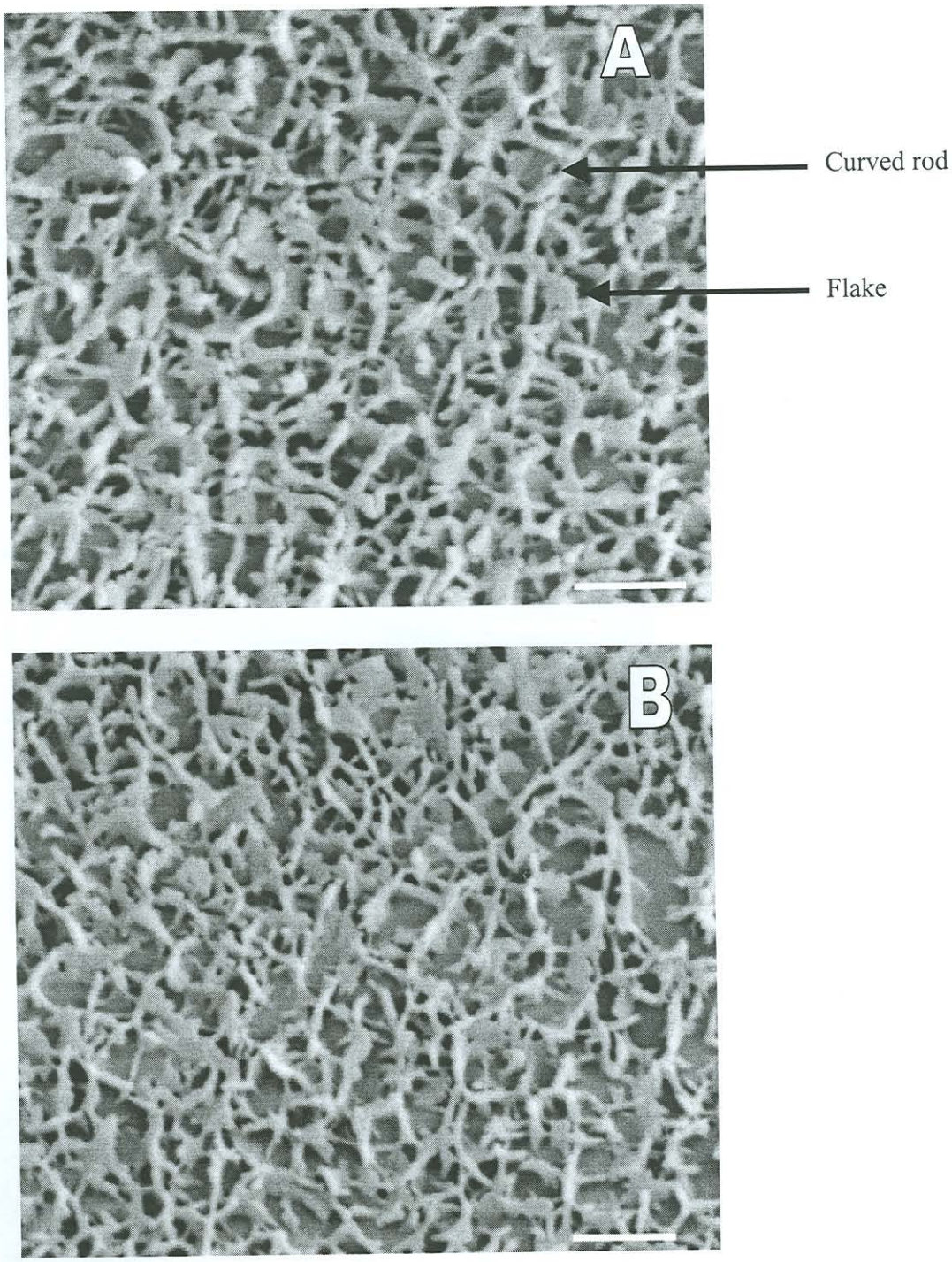

Figure 6.2. Scanning electron micrographs of epicuticular wax ultrastructure on the adaxial and abaxial surface of the 'Palmiet' wheat cultivar (9 500X; bars = 1μm). (A) adaxial surface. (B) abaxial surface.

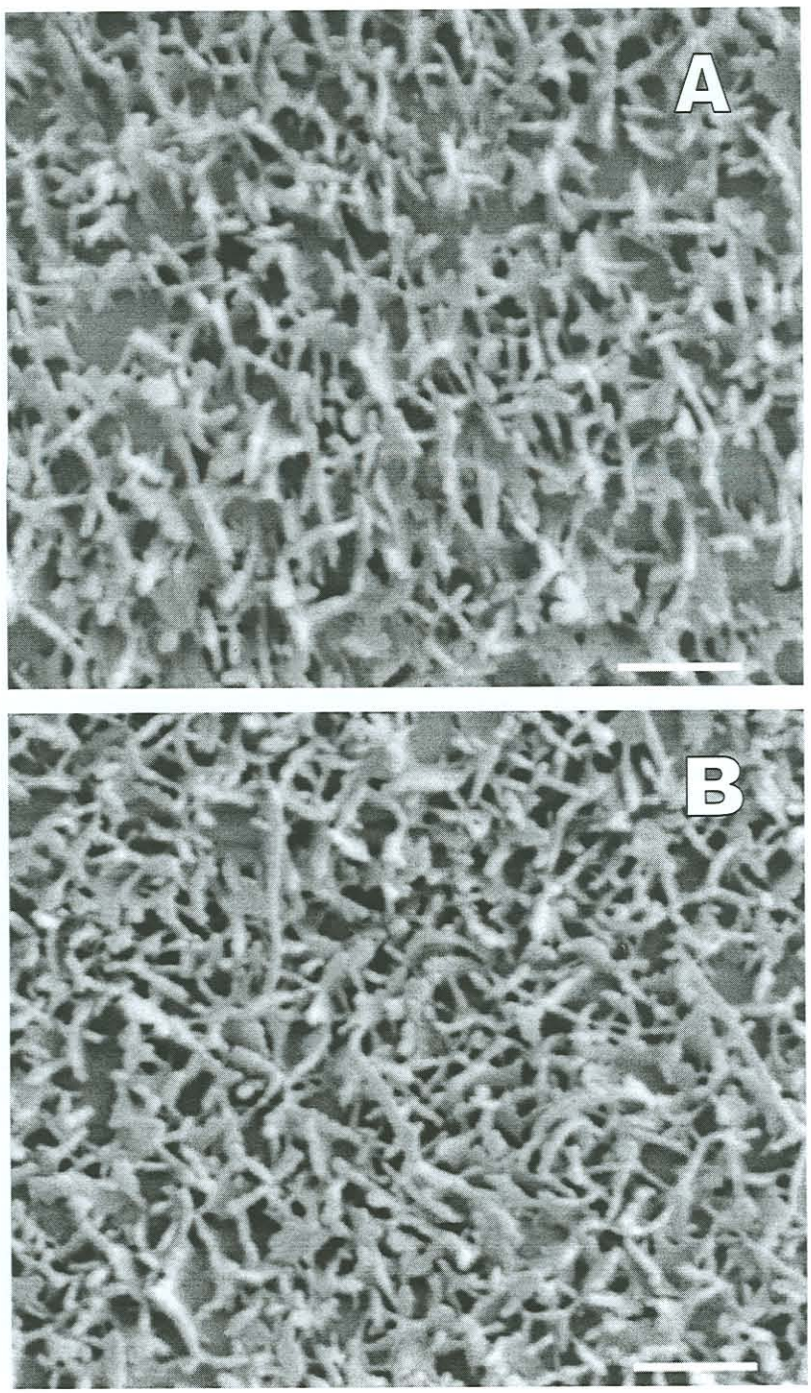

Figure 6.3. Scanning electron micrographs of epicuticular wax ultrastructure on the adaxial and abaxial surface of the 'Tugela' wheat cultivar (9 500X; bars = 1μm). (A) adaxial surface. (B) abaxial surface.

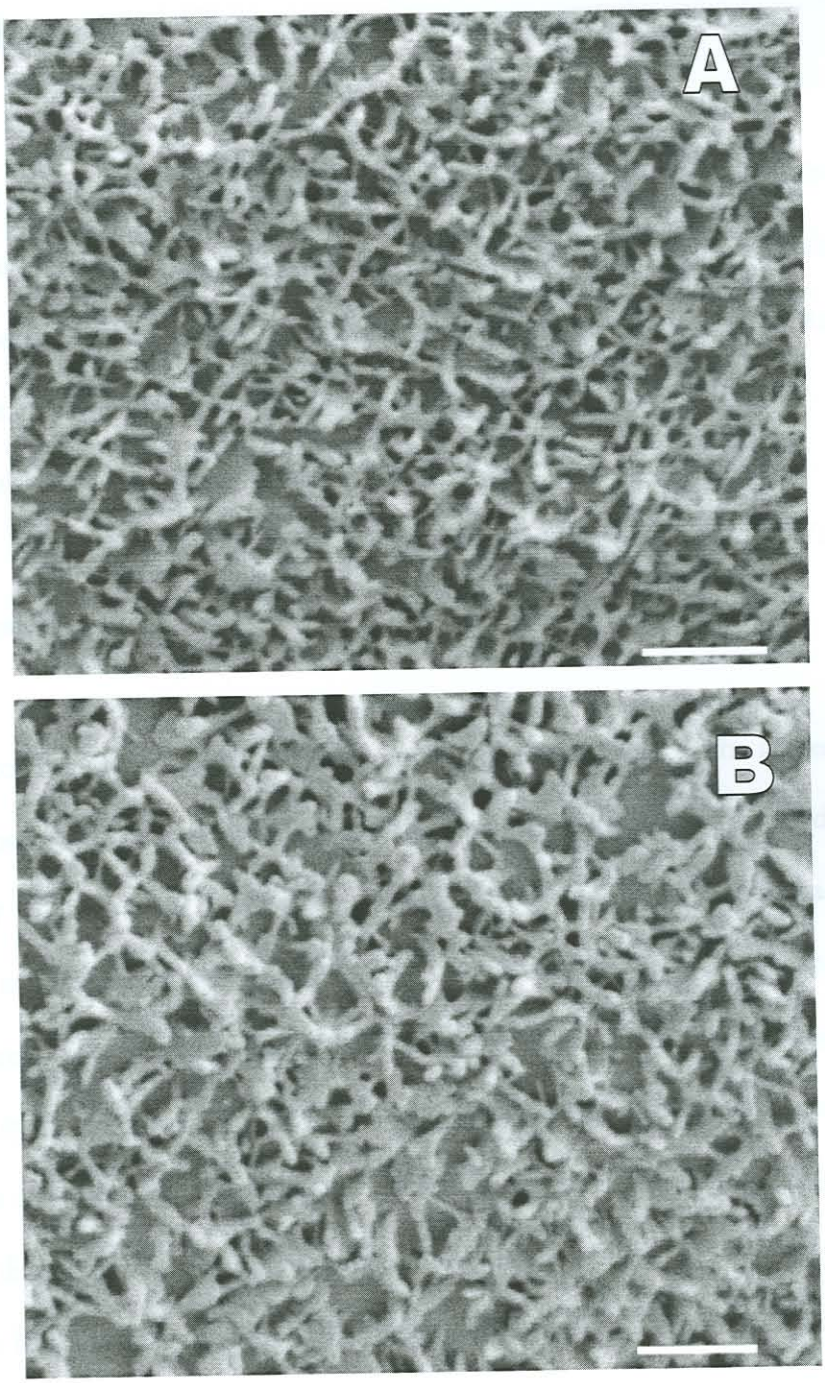

Figure 6.4. Scanning electron micrographs of epicuticular wax ultrastructure on the adaxial and abaxial surface of the 'Tugela *Dn1*' wheat cultivar (9 500X; bars = 1μm). (A) adaxial surface. (B) abaxial surface.

6.5 Discussion

The first surface that the RWA encounters when probing for a potential food source is the epicuticular wax covering the leaf surface. This wax covering is distinctive for each plant species and could play a role in RWA acceptance of the host plant (Dillworth & Berberet, 1990). Leaf trichomes could offer a physical obstacle to RWA feeding as these trichomes are found on or near leaf veins, where the RWA feeds (Ni & Quisenberry, 1997). The effects of these two leaf anatomical structures on RWA feeding were investigated.

Leaf trichome density was examined on the adaxial surfaces of the leaves and significant differences were found amongst the three wheat cultivars investigated. The resistant cultivar ('Tugela *Dn1*') had the highest trichome density. 'Tugela' had a significantly lower trichome density than 'Palmiet', when comparing the two susceptible wheat cultivars. There were no significant differences for the trichome lengths of the three cultivars investigated. Subsequently, only the density of the trichomes seems to play a role as an obstacle to feeding by the RWA. This is contrary to that found by Ni & Quisenberry (1997), that the antixenotic resistance of PI 137739 (SA 1684) was caused, in part if not totally, by long leaf trichomes and that trichome density did not contribute to the resistance of this cultivar when compared to a susceptible cultivar 'Arapahoe'. The cultivar PI 137739 had a lower trichome density compared to the susceptible wheat cultivar, 'Arapahoe' (Ni & Quisenberry, 1997).

The position of the trichomes showed that there were differences for the adaxial and abaxial surfaces. Trichomes on the three wheat cultivars were found to occur mostly on the leaf veins of the adaxial surfaces whereas they were found to occur on the leaf veins as well as between them on the abaxial surfaces. Similarly, Ni & Quisenberry (1997) found that trichomes were mainly found on the leaf veins on the adaxial side of the wheat leaves studied. The position and density of the trichomes could act as a physical impediment to the RWA gaining access to the leaf veins, where feeding occurs.

Cultivars PI 137739 and 'Tugela' are the parental lines of 'Tugela *Dn1*' (Du Toit, 1989). The trichome density of PI 137739 was approximately 14 trichomes per mm^2 (Ni & Quisenberry, 1997) which is similar to that of 'Tugela', but about half that of 'Tugela *Dn1*' (28 trichomes per mm^2). The trichome length of PI 137739 was much greater (473 µm) than that of 'Tugela' and 'Tugela *Dn1*' (216 and 198µm, respectively). PI 137739 displays high levels of antibiosis as well as some antixenotic resistance (Du Toit, 1989). Antixenosis is defined as the nonpreference of plants for insect oviposition, shelter or food (Painter, 1958). The nonpreference of 'Tugela *Dn1*' has manifested itself as a high trichome density. The RWA is less than 2mm in length (Walters *et al.*, 1980) and feeds preferentially on the adaxial surfaces of leaves (Ni & Quisenberry, 1997). As can be seen from Figure 6.4 (A) the high trichome density that occurrs mostly on the adaxial leaf veins (where the aphid feeds), would act as a physical impediment to RWA feeding. 'Tugela *Dn1*' would subsequently be nonpreferred by the RWA for feeding because of difficulties associated with reaching the leaf veins. RWA on other resistant plants have

been shown to be restless; they require more time to initiate feeding activities (Kindler *et al.*, 1992; Webster *et al.*, 1993).

A visual comparison of the SEM photos showed that the epicuticular wax structure was found to be similar for both the adaxial and abaxial surfaces amongst the three wheat cultivars. As the wax structure was similar on the RWA resistant and susceptible cultivars, the structure of the wax does not seem to play a role in RWA feeding. Lowe *et al.* (1985) found that on wheat cultivars that were resistant to *S. avenae*, the wax surface was relatively smooth and postulated that the insects had difficulty clinging to and probing these leaves. The findings of the leaf epicuticular wax ultrastructure agree with other studies on the influence of epicuticular wax on RWA feeding (Ni & Quisenberry, 1997; Ni *et al.*, 1998). Subsequently, leaf epicuticular wax ultrastructure does not appear to play important in RWA feeding.

The RWA feeds on leaf veins of the adaxial surfaces of leaves (Ni & Quisenberry, 1997). On the resistant wheat cultivar ('Tugela *Dn1*'), trichomes with a higher density than those of the susceptible cultivars ('Palmiet' and 'Tugela'), were found to occur mostly on the leaf veins of the adaxial surfaces. Subsequently, it was postulated that the density of the leaf trichomes plays a role in the antixenotic resistance that the RWA encounters when feeding on the resistant cultivar. The high density of trichomes acts as a physical impediment to the RWA reaching and feeding from the leaf veins on the adaxial leaf surfaces. The epicuticular wax structure and length of trichomes do not appear to be important in RWA feeding on the three wheat cultivars examined.

6.6 References

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Chapter 7

Summary

7.1 Summary

Diuraphis noxia (Russian wheat aphid; RWA) feeding on wheat is considered to be a great pest in South Africa. Due to wheat being a major staple crop in South Africa and with RWA potentially causing 60% loss in wheat yield, studies have been conducted on obtaining wheat that is RWA resistant. Recent studies have concentrated on identifying the resistance that these plants pose as the possibility remains that the RWA could overcome this resistance.

In this thesis, various aspects of the factors affecting the resistance encountered by the RWA on wheat were investigated. In Chapter 3 it was found that RWA nymphs were found to prefer the susceptible wheat cultivars ('Palmiet' and 'Tugela') in that more nymphs survived to reach reproductive maturity compared to nymphs on the resistant wheat cultivar ('Tugela *Dn1*'). The RWA nymphs that survived to adulthood on the resistant cultivar showed no significant differences to those surviving on the susceptible cultivars with regard to reproduction and longevity. This is the first reported case that the surviving aphids show no statistical differences when comparing reproduction and life span on resistant and susceptible wheat plants.

In Chapter 4 an artificial diet was developed for the RWA. It was found that the RWA had dietary requirements for three important sources. Firstly, a carbon source (sucrose) with an optimum concentration of 20%, that allowed a greater number of aphids to survive and become reproductively active when transferred to the artificial diet. Secondly, the RWA showed a requirement for a nitrogen source (methionine, leucine and tryptophan). The addition of these three essential amino acids to 20% sucrose showed a significant increase in the average number

of nymphs being produced each day as compared to that of only the 20% sucrose solution. Thirdly, the addition of the two salts (magnesium chloride and potassium phosphate) resulted in an increased life span. In this chapter, a simple and effective diet was developed (Diet A) for the RWA. Although, it does not mimic the conditions encountered by the aphid in a wheat plant, it provides an easy and effective method of testing for the efficacy of potentially resistant compounds to the RWA.

In an investigation into the influence of RWA feeding on the protein profiles of RWA resistant wheat ('Tugela *Dn1*'), it was shown that there is an induction of seven proteins. There are two proteins absent after RWA feeding on 'Tugela *Dn1*'. These proteins occurred in both SDS-PAGE analysis and two-dimensional gel electrophoresis, with proteins of the same size (occurring in the same band on the SDS-PAGE gel) being separated according to their charge. Subsequently, the ≈ 20 kDa band observed with SDS-PAGE analysis, was revealed as three proteins upon two-dimensional gel electrophoresis with pI values of 5.0, 5.2 and 5.8. These induced proteins could not be sequenced due to their low concentrations in relation to the other proteins. Overexpression and underexpression of proteins were also visible after RWA infestation. Two induced proteins (≈ 36 and 26 kDa) are possibly β-1,3-glucanases due to their molecular size and p*I* values corresponding to previous studies done. The ≈ 20 kDa induced protein occurs closely to the third group of proteins described by Van der Westhuizen *et al.* (1998). Their function is unknown. The other unique proteins in this study have not been identified.

In Chapter 6 the leaf epicuticular wax ultrastructure and leaf trichomes were examined on two RWA susceptible wheat cultivars ('Palmiet' and 'Tugela') and a RWA resistant wheat cultivar ('Tugela *Dn1*') to determine what effect these might have on the RWA resistance. The lengths of the trichomes gave no significant differences for the three wheat cultivars examined. Trichome density showed that the resistant cultivar ('Tugela *Dn1*') had a significantly greater trichome density than the susceptible cultivars. 'Tugela' had a significantly lower trichome density than 'Palmiet', when comparing the two susceptible wheat cultivars. When examining the position of the trichomes it was revealed that there were differences for the adaxial and abaxial surfaces. Trichomes of the three wheat cultivars were found to occur mostly on the leaf veins of the adaxial surfaces whereas they were found to occur on the leaf veins as well as between them on the abaxial surfaces. Leaf trichome density acts as a physical obstacle to RWA feeding, as these mostly occur on the adaxial leaf veins were the RWA feeds. Subsequently, only the density of the trichomes seems to play a role as an obstacle to feeding by the RWA. Comparison of the SEM photos showed that the epicuticular wax structure was found to be similar amongst the three wheat cultivars studied for both the adaxial and abaxial surfaces. As the wax structure was similar on the RWA resistant and susceptible cultivars, the structure of the wax does not seem to play a role in RWA feeding.

Knowledge obtained from this thesis will hopefully be used in providing a better understanding of the insect-plant interaction. The exact resistance encountered by the RWA when feeding on a resistant plant does, however require more investigation. Studies aimed at identifying the proteins induced upon RWA infestation would enable a better understanding of the insect-plant interaction.

7.2 Opsomming

Die Russiese koringluis (*Diuraphis noxia*) is een van die ernstigste peste van koring in Suid-Afrika. Die Russiese koringluis kan tot 60% skade in koringproduksie veroorsaak en daarom is studies gedoen om Russiese koringluis-weerstandbiedende kultivars te vind. Onlangse studies het gefokus op die identifisering van die weerstand want die moontlikheid bestaan dat die Russiese koringluis hierdie weerstand kan oorkom.

In hierdie tesis word verskeie aspekte van die faktore bespreek wat die Russiese koringluis tydens voeding teëkom. In Hoofstuk 3 is bevind dat die Russiese koringluisnimf die vatbare koring kultivars ('Palmiet' en 'Tugela') bo die weerstandbiedende kultivar ('Tugela *Dn1*') verkies. Meer nimfe oorleef om reproduktief te word op vatbare kultivars in vergelyking met nimfe op die weerstandbiedende kultivar. Die Russiese koringluisnimfe wat tot volwassenheid op 'Tugela *Dn1*' bereik, toon geen betekenisvolle verskille in vergelyking met die nimfe wat op die vatbare kultivars oorleef ten opsigte van reproduksie en lewensduur nie. Hierdie is die eerste verslag waar die oorlewende nimfe geen betekenisvolle verskille toon as na die lewensduur en reproduksie op vatbare en weerstandbiedende koringplante gekyk word nie.

In Hoofstuk 4 is 'n kunsmatige dieet vir die Russiese koringluis ontwikkel. Daar is gevind dat die luis drie belangrike voedingsbronne vereis. Eerstens, 'n koolstofbron (sukrose), teen 'n optimale konsentrasie van 20%. Hierdie konsenstrasie laat meer luise tot volwassenheid oorleef om reproduktief te word na oordrag op die kunsmatige dieet. Tweedens, die Russiese koringluis het 'n vereiste vir 'n stikstofbron (metionien, leusien en triptofaan). Byvoeging van hierdie drie

essentiële aminosure tot die 20% sukrose toon 'n betekenisvolle verhoging in die reproduktiwiteit (gemiddelde getalle nimfe per dag) wanneer dit met die suiwer 20% sukrose-oplossing vergelyk word. Derdens, byvoeging van twee soute (magnesiumchloried en kaliumfosfaat) gee 'n verhoogde lewensduur. In hierdie hoofstuk is 'n eenvoudige en effektiewe kunsmatige dieet ontwikkel (Dieet A). Hoewel dit nie die toestande wanneer die luis op 'n plant voed naboots nie, voorsien hierdie kunsmatige dieet 'n eenvoudige en effektiewe metode om die doeltreffendheid van potensiële weerstandbiedende verbindings te toets.

In 'n ondersoek om vas te stel wat die invloed van Russiese koringluisinfestering op die proteïenprofiele van weerstandbiedende koring ('Tugela *Dn1*') is, is bevind dat daar 'n induksie van sewe proteïne is (Hoofstuk 5). Daar is twee proteïene afwesig na Russiese koringluisinfestering. Hierdie sewe proteïene word aangetref wanneer proteïene in beide SDS-PAGE-jels, sowel as twee-dimensionele jel-elektroforese geskei word. Proteïene wat in dieselfde band op die SDS-PAGE-jel voorkom, word volgens lading met twee-dimensionele jel-elektroforese geskei. Vervolgens, wanneer die ≈ 20 kDa band wat waargeneem word met SDS-PAGE-analise, geskei word met behulp van twee-dimensionele jel-elektroforese, is die produk drie proteïene met pI-punte 5.0, 5.2 en 5.8, onderskeidelik. Die indentiteit van die geïnduseerde proteïene kon nie bepaal word nie, as gevolg van hul lae konsentrasies. Daar was ook proteïene wat meer of minder uitgedruk is na Russiese koringluisinfestering. Twee van die geïnduseerde proteïene (≈ 36 en 26 kDa) is moontlik β-1,3-glukanase, aangesien hul pI-punte en grootte ooreenstem met vorige studies op proteïene. Die ≈ 20kDa geïnduseerde proteïen lê naby aan die derde groep proteïene wat deur Van der Westuizen *et al.* (1998) beskryf is. Hulle funksie is

onbekend. Die funksies van die ander unieke proteïene wat waargeneem was in hierdie studie is ook nie bekend nie.

In Hoofstuk 6 is die blaarepikutikulêre wasultrastruktuur en blaartrigome in twee Russiese koringluis vatbare koringkultivars ('Palmiet' en 'Tugela') en in 'n weerstandbiedende koringkultivar ('Tugela *Dn1*') ondersoek om die effek daarvan op Russiese koringluisweerstand vas te stel. Die lengte van die trigome het geen betekenisvolle verskil tussen die drie koringkultivars wat ondersoek is, getoon nie. Wanneer trigoomdigtheid ondersoek is, is getoon dat die weerstandbiedende koringkultivar ('Tugela *Dn1*'), betekenisvol meer trigome besit as die twee vatbare kultivars. Wanneer die twee vatbare kultivars vergelyk word, het 'Tugela' 'n betekenisvolle laer trigoomdigtheid as 'Palmiet'. Blaartrigome kan 'n fisiese hindernis wees wanneer die Russiese koringluis voed, want die trigome word op die blaarare waar die luis voed, aangetref. Vervolgens speel net die trigoomdigtheid 'n rol as 'n fisiese hindernis vir die Russiese koringluis. 'n Ondersoek na die posisie van die trigome op die blaaroppervlak het getoon dat daar verskille is in die abaksiale en adaksiale oppervlakte. Trigome van die drie koringkultivars het meestal op die beaarde adaksiale oppervlakte op en tussen die blaarare voorgekom. Vergelyking tussen SEM-fotos van die blaarepikutikulêre wasultrastruktuur het aangetoon dat dit eenders was vir die drie kultivars vir beide die adaksiale en abaksiale oppervlaktes. Dus speel die wasultrastruktuur waarskynlik nie 'n rol in Russiese koringluisweerstand nie.

Kennis vauit hierdie tesis verkry kan gebruik word om 'n beter begrip te bekom oor insek-plant interaksies. Die presiese weerstand wat die Russiese koringluis teëkom as hy voed op 'n weerstandbiedende plant vereis nog navorsing. Studies gemik op die identifiseering van die

geïnduseerde proteïene sal wetenskaplikes in staat stel om 'n beter begrip te hê van die insek-plant interaksie.