PLANT APHID INTERACTIONS – EFFECTS OF *DIURAPHIS NOXIA* AND *RHOPALOSIPHUM PADI* ON THE STRUCTURE AND FUNCTION OF THE TRANSPORT SYSTEMS OF LEAVES OF WHEAT AND BARLEY

THESIS

Submitted in fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

of

RHODES UNIVERSITY

by

SEFIU ADEKILEKUN SAHEED

Department of Botany

December, 2007

Abstract

The infestation of the cultivated grain crops by phloem feeding aphids has generated a great deal of interest over the years, due to the serious damage they cause to the crops and yield losses that result. The mechanism of the interaction between aphids and host plants remains largely unknown in spite of efforts to understand the basis of aphid feeding on grain crops. Greater efforts are required to explain the mechanism(s) of this interaction in order to achieve sustainable agriculture. This thesis focused on an investigation of the mechanism of feeding by the Russian wheat aphid, *Diuraphis noxia* Mordvilko (RWA) and the bird cherry-oat aphid, *Rhopalosiphum padi* L. (BCA) on barley and wheat cultivars. These two aphids co-occur naturally, but they inflict very different feeding effects on host plants. Structural and functional approaches were employed to investigate their feeding habits and these were then related to the observed differences in their host plants. Transmission electron microscopy (TEM) techniques were used to study the ultrastructural damage, while fluorescence microscopy techniques – using aniline blue fluorochrome (a specific stain for callose) and 5, 6-CFDA (a phloem-mobile fluorophore) – were employed to investigate the functional response to damage via wound callose formation and phloem transport capacity respectively. RT-PCR and quantitative real-time RT-PCR techniques were used to investigate the regulation of the genes involved in callose synthesis and degradation at the transcriptional level.

Morphological observation of the damage caused by the aphids show that infestation by RWA results in extensive leaf chlorosis, necrosis and rolling, while infestation by BCA does not lead to any observable symptoms within the same period. Interestingly, the population study shows that BCA breeds faster than RWA within the two-week experimental period. The ultrastructural study of feeding damage caused by the two aphids on the vascular bundles of susceptible barley cv Clipper, shows a different patterns of damage. Probing the vascular bundles results in the puncturing of vascular parenchyma by both aphids, but severe damage occurs in sieve tubes-companion cell complex during sustained feeding by RWA. In contrast, less damage occurs when BCA feeds on the phloem. Drinking from the xylem by RWA results in deposition of a large quantity of electron-dense watery saliva, which apparently seals the xylem vessels completely, by blocking all the pit membrane fields between the xylem vessels and associated parenchyma cells. In contrast, drinking from xylem by BCA results in deposition of a dense, granular saliva into the xylem vessels only, which does not appear to totally occlude the pit membrane fields. This is the first known report in which ultrastructural evidence of aphids' drinking in xylem is provided. The comparative effects of RWA feeding on a susceptible Betta and resistant Betta-*Dn1* wheat cultivars showed that after two weeks, the Betta cultivar expressed damage symptoms such as chlorosis, necrosis and leaf roll, while few chlorotic patches and necrotic spots occur in resistant Betta-*Dn1* cultivars.

An ultrastructural investigation of the feeding damage caused to all leaf tissues revealed, for the first time, that RWA is capable of both intra- and inter-cellular probing within mesophyll cells. Probing in the mesophyll cells induces a more severe damage in susceptible Betta than in the resistant Betta-*Dn1* counterpart. Similar differences in damage occurred during feeding in the thin-walled sieve tubes of the phloem, with the sieve tubes of the Betta showing more damage than that of the resistant Betta-*Dn1*. However, drinking from xylem resulted in the characteristic occlusion of metaxylem vessels by copious deposition of saliva by RWA in both Betta and Betta-*Dn1* cultivars. In all cases of probing, feeding, and drinking by RWA in both cultivars, all probed cells with evidence of salivary material deposit and those cells adjacent to salivary material deposit, exhibit significant damage in susceptible Betta cultivar, whereas similar cells in Betta-*Dn1* cultivars do not show as damage as severe.

Investigation of the functional response of the plants to feeding by aphids through the deposition of wound-induced callose shows that formation and deposition of wound callose occurs in both longitudinal and cross veins within 24h of feeding by RWA. This deposition increases through short-term feeding (72h) and prolonged feeding (14d). This is in sharp contrast to the observations with BCA feeding, where little or no callose formation occurs within the same time frame. Callose formation and deposition occurs only when a higher population of BCA feeds on barley leaves. This is the first report of aphid-induced wound callose by BCA. In all cases of

callose deposition, aphid stylet tracks were associated with callose and the deposition of callose appears to be a permanent feature, because wound callose remained in the leaf tissues even after 120h of the aphids' removal. Wound callose signals (defence and anti-defence) are discovered to be transported in the phloem tissues and are dependent on the direction of assimilate flow. Examination of the possible regulation of wound callose genes at the transcriptional level shows that the two expressed glucan synthase gene sequences (*GSL* – genes involved in callose formation) analysed did not show any significant increase or regulation upon aphid infestation. Contrary to expectation, all three aphid-induced β-1, 3-glucanases (genes which are thought to be involved in callose degradation) showed higher expression in RWA-infested tissue than in BCA-infested tissue. The results of the feeding damage on the transport capacity of the phloem shows that BCA infestation does not lead to a significant reduction in the phloem transport capacity during short-term feeding (72h), while RWA-infested leaves showed considerable reduction in the transport capacity of the phloem within the same period. However, prolonged feeding (14d) by BCA induces a considerable reduction on the transport capacity of the phloem on the infested tissues. In contrast, a marked reduction in the transport capacity of the phloem occurs in RWA-infested leaves and in most cases, complete cessation of transport ensues.

In conclusion, these data collectively suggest that RWA is a serious and most destructive phloem feeder in comparison to the BCA. RWA causes

severe damage to all cellular tissues of the host plants, which result in apoplasmic and symplasmic isolation of xylem and phloem tissues, while BCA infestation does not result in such isolation within the same time and population levels. Resistance genes appear to function by conferring resistance to cell damage on the resistant cultivars during aphid feeding. Responses by plants to aphid infestation via wound callose deposition are again shown to be species-specific. A quick response results when RWA feeds, even at a very low population level, while a response occurs only at a higher infestation level by BCA, and this response was shown as not regulated at the transcriptional level. Differences in the damage to leaf tissues and wound callose deposition eventually lead to varying degrees of damage to the transport capacity of the phloem. These differences in the damage signatures are hereby suggested to be the cause of the diversity in the observed damage symptoms and the yield losses upon infestation by the two aphid species.

Acknowledgements

To the Glory of Allmighty Allah (SWT), and the memories of my late father Imam Saheed Atanda Quadri Adekilekun and my great uncle, Khaleefah Jamiu Ayinla Quadri Adekilekun, I dedicated this thesis.

My profound gratitude goes to my principal supervisor, Prof. CEJ Botha for the opportunity giving to me, to work under his supervision and tap from his wealth of experience. I am eternally grateful to him for his encouragements, supports and for his concerns about my academic and personal life. My sincere appreciation goes to my co-supervisor, Prof. LMV Jonsson of the Södertörn University College, Huddinge, Sweden, for her guidance, encouragements and supports.

I wish to thank the entire members of CEJB research group esspecially, Dr Lin Liu, for his expert advice and assistance with TEM techniques and Dr Elizabeth Ade-Ademilua, for her support during the beginning of this study. I thank members of LJO research group in Sweden especially, Thérèse Gradin, Kristina Larsson, Izabela Cierlik and Dr Gabriele Delp, for their supports during my visits to the group and for painstakingly putting me through the molecular biology techniques. Prof. Graeme Bradley is appreciated for his contribution to the callose studies. I thank Shirley Pinchuck and Marvin Randall of the Rhodes University EM unit for their support with the use of TEM facilities. Special thanks to the

entire staff of the department of Botany especially, David Forsdyke and Jay Narsai for their constant help throughout the study period. My friend, Shola Adeyemi is appreciated for his help with statistical analysis.

I appreciate the National Research Foundation (NRF), Pretoria, South Africa for the two years Grant-holder's student bursary, the Dean of Research office, Rhodes University, for student support in 2005 and Mellon Mentor Funds given in 2007. I thank The Swedish Foundation for International Cooperation in Research and higher Learning (STINT) for student support given during all my visits to Sweden. I also appreciate the study leave granted me by the Obafemi Awolowo University (OAU), Ile-Ife, Nigeria.

I must not forget to appreciate the support of my brother Dr Jeleel Adekunle Jimoh, for his financial support throught the entire period of this study. Special thanks to all my friends in Grahamstown, other parts of South Africa, Nigeria and Sweden. Mention must be made of friends like Drs Olajide Ibrahim (Jyde), Olanrewaju Shaibu (Larry) and Elijah Oyeyemi; Messrs Tony Odiwe, Wole Olowoyo, Kunle Adebowale, Olumide Alebiosu (Olumba), Sheriff Salisu, Rasheed Adeleke, Humphrey Atebe, Pumeza Magadla, Kedi Maseko, Nomtandazo Mini, Zanele Godola, Zimkhita Qengwa, Phila Gaga, Gbade Osunsoko, Mr & Mrs

Oladepo, Saheed Adebayo, Sofia Magarioteli and Susan Aghahowa. Your supports in various ways which are too numerous to mention helped a lot.

I thank my mentors at the department of Botany, OAU, Nigeria, Drs A.A. Adelusi, S.O. Oke and H.C. Illoh for their support and for making my sojourn into the academia world a reality. Members of the UNIFEMGA, Ile-Ife branch and Ede Crystal Club are appreciated for taking care of my home fronts while I was away.

I will like to thank my mother, Alhaja Sarat Saheed Adekilekun and my siblings, Badirat, Kabirat, Kudirat, Mudathir and Umar (Deji) for their inspirations, prayers, supports and understanding. I appreciate the entire members of Adekunle Adegbite family (my in-laws) for their financial supports and prayers especially during the tough period of actualising this dream. The Adekilekun family of Edeland home and abroad are appreciated for their supports, prayers and concerns.

Finally, special thanks to my dearest wife, Adenike Zainab Saheed-Adekilekun for her love, care, support and sacrifices while I was away for this study. My hearty gratitude goes to my boys, Abdul-Rafii and Abdul-Quadri for being peaceful and for understanding that I needed to be away from them. I love you lots.

Table of contents

List of figures

List of tables

1 Chapter 1: General introduction

1.1 Plant and aphid interactions – the background

In their natural environment, plants encounter various biotic and abiotic challenges, which usually lead to interactions between them, such as those that occur between plants and aphids. The interactions between plants and their predators are very complex and these affect crop protection, selection and cultivation. Studies of plant-insect interactions have generated keen interest among scientists over the years and research into this area has incorporated many fields of study including ecology, evolution, behaviour, physiology and biochemistry. These disciplines form the foundation for the development of phytocentric, or plant-based hypotheses that are used to explain the diverse and complex form of herbivory (Moran et al., 2002 and references cited). Plants are known to undergo various chemical and morphological changes in their response to herbivores or pathogens attack (Karban and Baldwin, 1997). These responses could be either constitutive or induced and may serve as a defence mechanism by deterring subsequent herbivory. However, in some cases, prior infestation can improve the performance of later-arriving herbivores (Gange and Brown, 1989; Underwood, 1998; Agrawal and Sheriffs, 2001). Increasing evidence suggests that induced responses and their consequences are highly species-specific (Walling, 2000). Plants have been reported to show some ecologically induced response to aphid attack. For example, Chamberlain et al., (2001) reported

that volatiles released by the plants in response to aphids' attack might elicit a response from the aphid predators, which may then attack the aphids, thereby resulting in an advantage for the plants.

1.2 The aphids

'Aphids' is a broad term referring to any members of the Aphidoidea, though there are some uncertainties about the families to be included (Ilharco and van Harten, 1987). However, most work that has involved the study of aphid feeding has been concerned with the species in the order Homoptera and the family aphididae; this is because feeding strategies vary significantly between these families (Miles, 1999). The aphids in this family produce no gall structures. They feed from the sieve elements of the phloem and they produce large amount of honeydew as excreta. In addition, they lead a fully mobile life (Miles, 1999). Most aphid species that are within the group of Aphidoidea according to Miles, (1999), do little perceptible damage to plants, which serve as a source of food to them, providing they exist in small numbers and do not carry plant viruses. There are exceptions though, which include the greenbug *Schizaphis graminum*, and the Russian wheat aphid, *Diuraphis noxia*. Miles, (1999) suggests that some components of the aphid saliva might be responsible for the toxicoses caused by some species.

1.2.1 The Russian wheat aphid (RWA – *Diuraphis noxia* Mordvilko)

1.2.1.1 History and distribution

The Russian wheat aphid (RWA) is indigenous to Southern Russia and some countries bordering the Mediterranean Sea, Iran and Afghanistan. RWAs are serious pests to wheat and barley in Russia since 1900, as well as minor pests to other small grains in countries in the Middle East and the Mediterranean littoral of North Africa and Europe. It was first recorded as a pest to wheat in South Africa during 1978 (Walters et al., 1980), and by the end of 1979 it had becomes a serious pest, spreading throughout the wheat producing areas of eastern and western Orange Free State of South Africa as well as Lesotho (Walters et al., 1980). The RWA was first discovered in USA in 1986 (Webster et al., 1987) but it has now spread throughout North and South America (Peairs et al., 1989; Ortego and Delfino, 1994) causing extensive damage to barley, bread wheat as well as pasta wheat (Castro et al., 1996). Damage evaluation runs into billions of US dollars annually with costs arising from yield losses and insecticide inputs (Morrison and Peairs, 1998; Webster et al., 2000). Because of the stated destructive effects, the RWA has been described as a major pest of small grains in South Africa, United States, Canada and many countries of the world (Walters, 1984; Hein et al., 1990).

1.2.1.2 Morphology and life cycle

The RWA is a relatively small aphid. It is less than 2mm in length, green to grey coloured and possesses an elongated and spindle-shaped body. It has

extremely short antennae, reduced cornicles sometimes called tailpipes, a characteristic projection above the cauda (tail) of a forked tail or "double tail" and the absence of prominent siphunculi, which is common in other aphid species. These clearly distinguish the RWA from the rest of Southern African wheat aphids (Walters et al., 1980). The RWA lives in one of two forms, the winged (alate) and the wingless (apterous) females. Male forms are not known to occur locally normally, as they reproduce only parthenogenetically without fertilization (Walters et al., 1980; Schotzko and Smith, 1991). The winged females mostly develop when the host plant conditions become unfavourable either by being under stress or when the host plant has reached a certain growth stage when it can no longer provide a favourable habitat, most especially with the respect to source of food for the aphid. The winged form then relocates to a more favourable habitat, spreading to nearby fields or further afield. They do not ordinarily have the capacity to fly long distances on their own, but the RWA can migrate to distant fields many kilometres away, with the use of convection currents and prevailing winds (Walters et al., 1980; Kriel et al., 1984; Schotzko and Smith, 1991). The winged females settle on the new suitable host and began to feed immediately, producing nymphs which grow into wingless females, which start reproducing new nymphs within two weeks, provided the conditions remain favourable (Walters et al., 1980). These new individuals live for up to 60 to 80 days and they are capable of producing about 80 offspring in their lifetime, producing up to four nymphs per day (Walters et al., 1980; Peairs, 1998). The new

offspring then infest the host plant's new leaves as they emerge, forming dense colonies within the rolled leaves, which are prevented from unrolling, a strategy that protect the aphids from their predators, parasites as well as sprayed insecticides (Webster et al., 1987). Once the host plants' ears appear, the aphid population begins to decline and they develop into winged forms, which then migrate in search of new favourable host (Walters et al., 1980).

The RWA tolerates extremely low temperatures, explaining its winter survival in the southern Russia where it originates, and particularly the northern part of United States (Peairs, 1998; CAPS Program, 1994). The aphid can survive a temperature as low as -25^{o}C; however, its lifespan and reproductive rate reduce when the temperatures fall below -10^{o}C, while higher temperatures and rainfall lead to high mortality and reductions in their numbers (Walters et al., 1980). The ability to survive the oversummering period between small grain harvest and autum planting on *Bromus* spp and volunteer wheat plants is a critical advantage to the survival of the aphid. The fact that the infested *Bromus* plants were found during the summer period when no infested wheat plant were found in wheat growing regions of the Free State in South Africa indicates the importance of this grass on the seasonal cycle of the aphid (Aalbersberg et al., 1988). It should be noted that, irrespective of the ability of the RWA to either oversummer or overwinter, its ability to disperse rapidly and reproduce quickly gives it an advantage to re-infest any areas where it may have been eliminated by the lack of oversummering hosts, severe winter

and any possible unfavourable environmental factors. These are factors known to contribute to its spread throughout the western United States (Peairs, 1998). The rapid infestation of wheat fields by the RWA can be from 20% to 80% within two weeks when the conditions are favourable (Walters et al., 1980).

1.2.1.3 Symptoms of infestation

The infestation of plants by RWA has been shown to be accompanied by extensive chlorosis and necrosis as well as other visible damage symptoms. These symptoms include distinct white, yellow, purple, or at times reddish-purple longitudinal streaks, with severe leaf roll in fully expanded leaves and the prevention of unrolling of new developing leaves (Walters et al., 1980; Hewitt et al., 1984; Riedell, 1989; Saheed et al., 2007a). A single aphid may cause distinct symptoms, but heavy infestation of the host plants often result in a flattened appearance as young tillers may lie almost parallel to the ground. The flag leaf, is most often infested if the host plant is at the later growth stage. This results in the leaf ear becoming bent and turning white, an indication that poor yields will result (Walters et al., 1980). Walters (1984), Rybicki and Von Wechmar, (1984) have reported that RWA is equally capable of transmitting viral diseases like other grain aphids. Damsteegt et al., (1992) later reported a clone of the RWA that has the ability to transmit some plant pathogenic viruses, such as barley mosaic virus (BMV), barley yellow dwarf virus (BYDV) as well as sugarcane mosaic virus (SMV). The feeding and associated symptoms have been reported globally to lead to significant quality and yield losses in the infested plant (Du Toit and Walters, 1984; Pike

and Allison, 1991), with yield losses ranging between 30% and 60% in the infested plants recorded in South Africa (Du Toit and Walters, 1984).

1.2.2 The bird cherry-oat aphid (BCA – *Rhopalosiphum padi* L.)

1.2.2.1 History and distribution

BCA is also a serious pest of many cereal crops. It could be a direct pest of the crops or an important vector of viruses. The aphid is described as one of the most serious pests of spring-sown cereals in Sweden (Wiktelius et al., 1990). However, the aphid does not cause serious direct damage to cereals in most part of continental Europe: its principal effect on crops is the transmission of barley yellow dwarf virus (BYDV) along with others – up to 15 have been reported (Chan et al., 1991). This BYDV virus has been reported as the cause of one of the more serious diseases of grain crops globally resulting in about 87% yield losses (Canning et al., 1995). BCA has been reported as an infrequent problem in Ontario (Agronomy guide, 2002), but is known to be a pest of many grain crops, such as wheat, barley, oats, rye, and triticale in the United States (UCIPM, 2002), and sometimes it may attack sorghum and corn (Stoetzel and Miller, 2001).

1.2.2.2 Morphology and life cycle

Like RWA, BCA is also a small aphid about 2 mm long or less. The body is bulb-shaped, dull olive-green in colour, and has patches of red-orange near the rear of the abdomen between a pair of tubes called cornicles. The cornicles

and legs are pale green, and the antennae are long and black. The young aphids are usually light green in colour. Like the RWA, the BCA also consist of two forms, winged – alate; and the wingless – apterous (Agronomy Guide, 2002). The aphids normally cluster on the upper sides of the leaves near the base of young plants; they later climb to the top up to the leaf whorls. In Sweden, where the BCA was regarded as a serious pest of cereal crops, it is holocyclic, exhibiting a complete cycle of reproducing both parthenogenetically and sexually, but the aphid is anholocyclic in the rest of continental Europe, where it is permanently parthenogenic (Dixon, 1971; Wiktelius et al., 1990). The BCA is monophagous on its primary host, which is *Prunus padus* (Bird Cherry) in Sweden and the rest of Europe (Wiktelius et al., 1990), but it is *Prunus virginiana* in United States (Stoetzel and Miller, 2001). The aphid overwinters as an egg on its primary hosts. However, the aphid is oligophagous (broad hosts) on its secondary hosts during summer. These hosts consist of most cereals such as barley, wheat, oats, rye, and triticale (Wiktelius et al., 1990; UCIPM, 2002). The name, bird cherry-oat aphid, was a coinage from its primary and one of its secondary hosts (bird cherry and oat).

1.2.2.3 Symptoms of infestation

The infestation of cereal crops by the BCA do not result in severe symptoms and damage to the infested plants. Heavy populations have, however, been reported to cause a golden yellow streaking on the leaves, which may appear to have a large bronze patch on the field (Agronomy Guide, 2002; UCIPM,

2002). The infestation by the BCA does lead to substantial loss in terms of yield (Leather et al., 1989; Riedell et al., 1999). This could be about 21% due to the aphid feeding in wheat alone, but a yield loss of about 58% could result when the combination of the effects of BYDV with aphid feeding is considered (Riedell et al., 1999). However, Riedell et al., (1999) reported that experiments conducted under field environments generally show larger yield losses due to BYDV damage than those seen in greenhouse experiments reported.

1.3 The host plants

The aphids have a variety of secondary hosts; in this case, most of the cultivated cereals that are grain crops serve as their host, including barley, wheat, rye, oats, triticale, maize and at times sorghum. The focus of this research is on two of the most widely cultivated cereal crops, barley and wheat; they both belong to the same family graminae – Poaceae.

1.3.1 The secondary host – barley (*Hordeum vulgare* L.)

Barley is an ancient domesticated crop and one of the first crops in old world agriculture. This crop was first discovered growing wild as a grass in Asia many thousands of years ago. The Chinese cultivated barley, thus making it one of their first commercially grown crops (Washington Barley Commission, 2007). The Egyptians and Greeks consumed barley for medicinal purposes in ancient times and it serves as source of nourishing food. However, the crop is thought to have found its way to North America with Christopher Columbus

during the journey to the New World (Washington Barley Commission, 2007). Badr et al., (2000) suggested that the Israel-Jordan area in the southern part of the Fertile Crescent is the region where the crop was brought into culture and that the Himalayas are the region of domesticated barley diversification. Irrespective of the place of origin or center of domestication, barley is one of the most cultivated grain crops of the world today, providing a good source of nutrition for people and livestock.

Barley grain consists of mostly starch and protein. There are three common uses of barley: the grain is primarily used as animal feed, such as in feeding cattle, swine, and poultry animals. In most cases, barley kernel may be rolled, ground, or flaked, prior to being fed to the animals. The second most important use of barley is in the making of malt, which is used in liquor such as beer, also in malted milk and as flavourings of variety of foods. The third and the least common use of barley is as food for humans. It may be purchased in several forms like pearl barley, where the outer hull and part of the bran layer has been removed, and this is sold in many supermarkets. Barley flour, flakes, and grits are other barley foods, available in health food and specialty stores. Barley may be used commercially as ingredients or additives to many of the prepared foods, such as breakfast cereals, breads, cookies and snack bars (Washington Barley Commission, 2007).

1.3.2 The secondary host – wheat (*Triticum aestivum* L.)

Wheat is known to originate from southwestern Asia, where earliest remains of the crop was found in Syria, Jordan, and Turkey (Gibson and Benson, 2002). Present-day wheat has been linked to primitive relatives discovered in some of the oldest excavations of the world in eastern Iraq, and this dates back to about 9 000 years (Gibson and Benson, 2002). Areas such as the Nile Valley, India, China and even England have revealed archaeological findings that show that bread wheat was grown in about 5000 B.C. However, wheat was reported to be first grown in the United States in 1602 on an island off the Massachusetts coast (Gibson and Benson, 2002). Since that time, human beings have depended on wheat as a source of nutritious food for themselves and their animals.

Wheat is one of the leading cereal crops that have been produced, consumed and marketed in the world today. The crop provides about 35% staple food and about 20% of the calories for the world's populations (FAO, 1998; Gibson and Benson, 2002). World wheat production during the year 2000 was more than 570 million metric tones (Gibson and Benson, 2002). This was grown on approximately 520 million acres. Cultivated wheat crops are mainly used as food for humans. These consist of two types: bread wheat (*T. aestivum*), this is also known as common wheat, and pasta wheat (*T. durum*), which is used in making pastas. The common wheat, unlike other plant-derived food, contains gluten protein, which makes leavened dough rise by

forming minute gas cells that hold carbon dioxide during fermentation, an attribute which favoured its use in making bread and biscuits.

1.3.3 The anatomy of the host plants

Understanding the anatomy of the leaves of the host plant is very important, as this allows a more precise investigation of the activity of the aphids and the mechanisms involved in aphid feeding on its host plant and in the primary site of feeding – the vascular bundles. The anatomy of barley leaf is typical of any of the grasses in the *Poaceae* (Blackman, 1971; Ellis, 1987; Evert et al., 1996). Therefore, the knowledge of the anatomy of the barley leaf blade can be extended to other members of the family – like wheat, in this case. Anatomical descriptions of mature barley leaves undertaken in the past years have been few, prior to the work of Dannenhoffer et al., (1990). Many articles before it only mentioned the vasculature, or described the anatomy of barley leaf superficially, or only as it relates to the aspects of their immediate interest (Rovenska and Natr, 1981; Cherney and Marten, 1982).

The mature barley leaf, typical of grasses, is an elongated structure that divides morphologically into leaf blade and sheath. The leaf contains a system of longitudinal vascular strands that are interconnected by numerous transverse veins, which are widely separated by loosely arranged mesophyll cells arranged in more or less three distinct layers (Dannenhoffer et al., 1990; Evert et al., 1996). In a cross-section of the mature leaf, three orders of longitudinal vascular bundles can be recognised; large, intermediate and small

bundles, based on their size, the composition of their xylem and phloem and the nature of their contiguous tissues (Dannenhoffer et al., 1990; Evert et al., 1996). In barley, most strands intergrade from one bundle type into another as they descend the leaf, beginning with small bundle anatomy at their distal ends (Dannenhoffer et al., 1990, 1994). As in other grasses, large and intermediate bundles in the barley leaf are associated with girders or strands of hypodermal sclerenchyma (Botha et al., 1982; Dannenhoffer et al., 1990; Evert et al., 1996). The longitudinal strands in mature leaves are surrounded for the most part or at times, partly, by two bundle sheaths, consisting of an outer parenchymatous and inner mestome sheath cells (Evert et al., 1996). At maturity, the third leaf of barley has about 21-28 vascular strands in the widest portion of the leaf blade (Dannenhoffer et al., 1990).

However, the anatomy of the leaf sheath is rather different from that of the blade: while the leaf blade has longitudinal vascular bundles located in ridges, the sheath has the bundles in abaxial furrows embedded in ground tissues and ridges that contain large lacunae (Dannenhoffer et al., 1990) separate them from one another. In addition to the mestome sheath, the vascular bundles in the leaf sheath contain parenchymatous bundle sheaths which were poorly differentiated. They intergrade with the surrounding ground tissue in appearance, including the colourless cells above and the chlorenchymatous cells below. Only two types of longitudinal bundles occur in the sheath of barley leaf: large and intermediate. However, they are the continuations of

corresponding strands from the leaf blade. In addition, the sheath of the flag leaf is only different from that of the third leaf primarily in the degree of the chlorophyllous tissue development (Dannenhoffer et al., 1990).

1.3.4 The vascular bundles – primary site of aphid feeding

In a cross-section of the vascular bundles in barley, large bundles are characterized by the presence of two large metaxylem vessels and a protoxylem lacuna; the xylem vessels are distributed one on each side of the lacuna and they have a distinct metaphloem. The intermediate bundles on the other hand, lack protoxylem and conspicuous large metaxylem vessels, but protophloem is often present, though it could be obliterated in mature bundles. Metaphloem is always present. The bundles are usually surrounded by mestome sheath (Botha et al., 1982; Dannenhoffer et al., 1990; Evert et al., 1996). The small bundles, however, are generally smaller than the intermediate bundles. They do contain metaxylem vessels and the largest of the small bundle may contain protoxylem. Bundles may be surrounded by a mestome sheath, which may be disrupted on the xylem side. For most of the length of the leaf blade, the marginal strands are small bundles (Dannenhoffer et al., 1990).

Metaphloem typically consist of two distinct sieve tubes – thin and thick-walled – though thick-walled sieve tubes may be absent in the smallest of the small bundles (Dannenhoffer et al., 1990). The thin-walled sieve tubes are so called because their cell wall is thin. They are associated with companion cells

and, are usually described as the first to mature or the first-formed metaphloem (Evert et al., 1978; Botha et al., 1982; Botha and Evert 1988; Dannenhoffer et al., 1990). The thick-wall sieve tubes, on the other hand, are described as the late-formed metaphloem. They usually consist of one or sometimes two sieve tubes that occur in close proximity to metaxylem. Thick-wall sieve tubes lack companion cells and they have thickened cell walls (Evert et al., 1978; Dannenhoffer et al., 1990; Evert et al., 1996). A number of studies have shown that barley and other members of Poaceae, which include wheat, sugarcane, maize and the southern African grasses like *Themeda triandra,* contain both thin- and thick-walled sieve tubes at least in the intermediate and small bundles (Botha et al., 1982; Colbert and Evert, 1982; Dannenhoffer et al., 1990).

Studies have shown that the different orders of bundle in the leaf blades of both C_3 and C_4 grasses have largely different functions (Lush, 1976; Altus and Canny, 1982; Fritz, et al., 1989). The small and intermediate bundles primarily function as loading bundles, while large bundles are primarily involved in longitudinal transport (Altus and Canny, 1982; Evert et al., 1996). The structure and as well as the functions of the sieve tube elements (both thin- and thick-walled) in the members of the Poaceae family have also been under critical review. Fritz et al., (1983) reported that thick-walled sieve tubes might be involved in the photosynthates' retrieval from the transpiration stream and/ or from the apoplast, but the thin-wall sieve tubes have been

demonstrated to be most functional in terms of transport and phloem loading than the thick-wall counterpart (Matsiliza and Botha, 2002). In addition, the studies conducted by Evert et al., (1996), Botha and Cross, (1997) in the small and intermediate bundles of barley source leaves revealed that the common wall between the thick-wall sieve tubes and other cell types contain very few plasmodesmata. They therefore concluded that both sieve tube-companion cell complexes and thick wall sieve tubes might be virtually symplastically isolated from the rest of the leaf. In contrast, the report of the investigation by Haupt et al., (2001) shows that both the intermediate and large bundles of sink leaves in barley contain relatively high frequencies of plasmodesmata between cells in the vascular bundles and that, connections between thick-walled sieve tubes and adjoining cells were abundant unlike source leaves where this is not the case. This suggests that apoplastic solute loading mostly occurs in source leaves and symplastic loading occurs in sink leaves.

1.4 Aphid feeding on host plants

1.4.1 Antibiosis, antixenosis and tolerance

Feeding on host plants by the aphids has generated many issues and concerns for decades. Scientists have used different approaches to obtain a better understanding of the mechanisms involved in this interaction in an attempt to proffer explanations for these complex interactions and to eventually find solutions to the problems generated by aphid feeding. In spite of these

enormous efforts, these mechanisms are not yet fully understood. There have been many attempts to study and understand the mechanism underlining the plant-aphid interactions: Leszczynski et al., (1989) concisely described the three different resistant mechanisms of plants against aphids, which were antixenosis, antibiosis and tolerance. Leszczynski et al., (1989) described antixenosis as the mechanism that occurs when resistant plants deter the aphids from settling on and colonizing them. Antibiosis occurs when the plant restricts the rate of the aphid's increase, while tolerance occurs when the plant is able to tolerate the nutrient drain caused by the aphids. Plant responses to aphid feeding have been reported to be largely species-specific; the signals which elucidate these plant responses have been suggested to be aphid derived, particularly from the aphid saliva secreted during stylet penetration, probing and subsequent feeding activities (Gill and Metcalfe, 1977). This position was established during their study of antibiosis as an effective means of resistance in barley to corn leaf aphid (*Rhopalosiphum maidis*). Antibiosis has been reported to play a crucial role in the interaction with this aphid, as mortality levels are high, but only for the corn leaf aphid, as the resistant strain of barley used in their study was found not be resistant to other aphid species (Gill and Metcalfe, 1977). This report gives an insight into different behavioural mechanisms during infestation of a host plant by the aphid, a critical focus of this thesis.

1.4.2 Physiological effects of aphid feeding on host plants

The physiological effects of feeding by aphids on host plants vary greatly. When aphids feed, redirection of assimilates into the aphid gut occurs instead of these been transported through the veins. This redirection to the gut results in the formation of secondary sinks by the feeding aphids (Botha and Matsiliza, 2004). The drain of assimilates has been demonstrated to be very strong and localized, as plants react to such redirection to the aphid's gut in some respects as if it were a bud (Hill, 1962). The aphids in this case are competing with other parts of the plant, which are as a result of the aphid feeding, denied assimilates, eventually resulting in observable deficiency symptoms in the infested plants. There is evidence that plant height, shoot weight and number of spike are significantly reduced when the RWA feeds in wheat (Girma et al., 1993) and these are in addition to the earlier reported visible symptoms characteristic of the RWA infestation. It is important to state here that devastating yield losses result when drought and aphid infestation occur simultaneously. Aphid infestation causes drought-stress symptoms in leaves of infested barley, even in the presence of sufficient root moisture (Cabrera et al., 1994). Cabrera et al., (1994) eventually conclude that some of the metabolic changes that occur in barley cv Aramir under aphid infestation could be due to water stress induced by the aphid. RWA infestations have equally been shown, to decrease the water potential in barley leaves and increase proline and glycine-betaine accumulation in leaves (Riedell, 1989). In the same vein, the efficiency of light capturing in barley

has been reported to be decreased by *Sitobion avenae* (Blanco et al., 1992), while the greenbug aphid, *Schizaphis graminum,* has been shown to decrease the photosynthesis during the infestation of barley leaves (Gerloff and Ortmann, 1971).

1.4.3 Damage control through callose formation

Plants have been shown to respond to the disruption of their cell structure by herbivore probing and feeding, by the formation of callose. Callose production and deposition in plant tissues has been shown to be a very important and effective plant response to wounding. Callose is β-1, 3-glucan. It was first discovered in the mid-1800s (see Currier 1957; Evert and Derr, 1964) and was described as a soft carbohydrate blockage found in intercellular connections (Currier, 1957). Callose synthesis is initiated when β-1, 3-glucan synthase is activated subsequent to the disruption of plasma membrane integrity (Kauss, 1985). Callose formation occurs quite rapidly, mostly within a minute of wound initiation (Radford et al., 1998, Nakashima et al., 2003). It is deposited between the plasma membrane and the cell wall of the plant. Callose is generally electron-lucent when viewed in electron micrographs (Stone and Clarke, 1992) and it fluoresces under UV light when stained with the aniline blue fluorochrome (Stone et al., 1985). Wounding of plant cells and the initiation of wound callose formation seems to be as a result of elevated calcium ion levels in a mechanism involving calmodulin (Botha and Cross, 2001 and references cited). Callose has been used in several studies to identify phloem elements that are active in sap transport (Evert and Derr, 1964; Botha

et al., 2000). They (callose) are largely produced in the sieve elements of the phloem to restrict the flow of sap through cells (Currier, 1957) and thus seal the pores in damaged phloem to prevent assimilate loss (Sjölund, 1997). Callose synthesis and deposition in plant-microbe interaction have also been demonstrated (Donofrio and Delaney, 2001; Jacobs et al., 2003), during which plant cells respond to attack by synthesizing and subsequently depositing callose, rapidly, as plugs or plates which are deposited close to the site of pathogen invasion (see Ryals et al., 1996; Donofrio and Delaney, 2001 and references cited). These callosic deposits seem to function as a physical barrier against microbial penetration (Stone and Clarke, 1992) and this could be achieved by either immobilizing or slowing the invading microorganisms.

1.4.4 Ultrastructure reveals functional damage

Structural and functional damage of cereal crops under aphid infestation have been poorly investigated. While several papers partly address the damage to host plant ultrastructure during aphid feeding, they do not focus in detail on structural damage or more importantly on the functional aspect of probing-related damage. For example, Fouché (1983) and Fouché et al., (1984) were concerned with the changes which occurred to mesophyll cell organization, more especially to the chloroplasts. The study was conducted by treating leaves with whole-body extracts of the RWA. No attempt was made to show structural details or the pattern of the damage with respect to feeding aphids or possible responses by the host plants. The work by Al-Mousawi et al., (1983) on the ultrastructural damage caused by greenbug aphid (*Schizaphis*

graminum Rondani), in resistant and susceptible wheat cultivars shows different damage in the mesophyll cells of the host plant by the probing aphids. However, damage to the vascular bundles was not shown by this study. It is pertinent to note that conclusions relating to the effects of feeding aphids on the plant may not therefore be justified, unless they include studies based on probing and feeding activities, as these should illustrate feeding effects. Apart from the studies mentioned above, other structural and ultrastructural studies conducted in the past rarely explain plant and aphid interactions, or more importantly, plant reactions or defences to aphid attack based on changes or damage to cell structure. Several of these studies, such as those by Botha and Mabindisa 1977, Botha et al., 1977, Botha and Evert 1978, Matsiliza and Botha 2002, focused primarily on using feeding aphids to examine the probing pathways and preferential feeding sites. This thesis represents one of the first studies of cell damage, in which ultrastructural assessment of the damage has been attempted.

1.4.5 Rationale behind the use of the RWA and BCA on wheat and barley

The RWA and BCA are, as stated elsewhere, serious cereal crops pests, especially of wheat and barley. These aphids have diverse effects on host plants. RWA is known to cause observable, severe symptoms such as longitudinal streaking of the leaves, rolling of fully expanded leaves and prevention of unrolling of newly formed leaves. The RWA also causes extensive chlorosis and necrosis in infested plants. Single aphids have been

reported to be capable of causing distinct symptoms (Walters et al., 1980). In contrast, small colonies of the BCA do not generally cause any observable symptoms, except for some evidence of a golden yellow streaking, reported to occur upon infestation of the field by heavy populations (Agronomy Guide, 2002; UCIPM, 2002). RWA and BCA have different ecological strategies. RWA escapes periods when the host cereals (winter-grown) are not available by oversummering on grasses like *Bromus* spp and BCA overwinters on *Prunus* spp when the host cereals (spring-sown) are not accessible. In addition, BCA also prefers to feed on the lower parts of the plant and forms smaller aggregates than does RWA (Wiktelius et al., 1990; Pettersson, 1994), whereas RWA feeds in dense aggregates, preferentially in rolled leaves (Walters et al., 1980; Burd and Burton, 1992).

Despite the differences in their ecology and also in the elicited symptoms in host plants, the two aphid species are known to cause substantial yield loss upon infestation and have been reported to naturally co-occur on the same host plant (Feng et al., 1992; Wraight et al., 1993). Substantial efforts are being made globally to curb the huge losses due to the infestation by establishing efficient control methods. The methods that were used in the past, and which are still partly used now, involved the use of insecticides (contact or more expensive systemic insecticides), along with the cultural practice of delayed planting, growing of non-host crops, eradication of oversummering or overwintering hosts (Walters, 1984; Pike, 1988; Du Toit, 1989a). The most

effective, economical and environmentally sound methods of control obtainable today involve the use of resistant cultivars. Many resistant cereal cultivars are now available (Du Toit, 1987; Liu et al., 2001), although there is a disturbing evolution of new, more virulent biotypes of these aphids with the ability to overcome the existing resistant cultivars (Quick et al., 2001; Haley et al., 2004; Tolmay et al., 2007). There is, therefore, an increasing need for more profound knowledge of the mechanism and effects of aphid feeding on these cereal crops. The differences with respect to damage, as outlined above between the RWA and BCA, provide us with an opportunity to effectively explore their mechanisms of feeding on cereals, thereby enabling us to attempt to relate the effects on the host plants in terms of the observable symptoms, growth and feeding pattern to their structural damage. The result of a structure-function study such as this one, should be of some use to breeders in determining what strategies to employ to totally or partially, curtail the damage caused to vegetative barley and wheat plants. Examination of structural damage to transport systems could possibly enable or serve as a basis for the inclusion of more targeted resistance which would result in plants with greater survivability.

1.5 Research objectives

Given the background above, the focus of this work was to gain additional primary knowledge about the structural and functional relationships of the damage caused by both RWA and BCA using non-resistant and resistant cultivars. It was felt that this will help to give a deeper insight into the mechanisms deployed by these aphids during their infestation of the plants, as well into the responses initiated by the plants to survive these attacks. It was obvious that in order to understand the mechanism involved in plant-aphid interactions, it would be essential to have detailed knowledge of damage at the ultrastructural level to vascular bundles and associated tissues in the leaves where the aphids feed. The susceptible barley cultivar cv Clipper used in this study (except in Chapter Four, where wheat cultivars are used as a comparison) provides us the opportunity of investigating the effects of these feeding aphids on the plants with little or no interference from any resistance genes. In the same light, the use of susceptible wheat Betta and resistant Betta *Dn1,* provides the opportunity to evaluate the effect of the resistant gene *Dn1* as contained in this resistant cultivar over the near-isogenic susceptible counterpart Betta. These two cultivars were selected to provide baseline information on the mechanisms involved in resistant cultivars that could be extended to other cultivars containing other genes. Given the differences between the aphids (BCA and RWA), as well as the non-resistant and resistant barley and wheat cultivars discussed here, it was felt that an in-depth study of subcellular and cellular damage was necessary in order to gain more

information regarding damage caused by these aphid pests. In part, the damage, or damage limitation, is under molecular control, thus experiments were conducted to investigate regulation at the transcriptional level of the callose genes to see how much correspondence could be obtained between what could be seen at the wide field fluorescence microscopic level, as compared to molecular approach.

This study therefore involved evaluating structural and functional damage caused by these aphids by:

1. The evaluation of the damage to the cell and tissue ultrastructure of barley by RWA and BCA, using transmission electron microscope (TEM) techniques, with a view to gain clear understanding of the differences in the patterns of ultrastructural damage. This is related to observed differences in the visible symptoms of their host plants (Chapter Three).
2. The investigation of the ultrastructural damage caused by RWA in the susceptible and resistant wheat cultivars selected for RWA. This was aimed at gaining more knowledge about the mechanism of resistance, by comparing the observed differences in the damage to cell ultrastructure in the more resistant Betta-*Dn1* to the susceptible near-isogenic counterpart, Betta cultivar (Chapter Four).
3. The investigation of the functional response of the plant to the feeding by the aphids, through the formation of aphid-induced wound callose.

Here I examined the wound callose formation resultant from aphid probing, report on the up- and downstream movement of wound callose signals, as well as investigate the sustainability or otherwise of the deposited callose in plant tissues as caused by the two aphid species. Fluorescence microscope techniques are used in these experiments to examine the patterns of the plant's response to the aphid feeding and relate it to its functions in the observed differences in damage symptoms (Chapter Five).

4. A molecular investigation of the expression of wound callose genes (callose synthase and β, 1, 3 glucanase) was carried out to establish the differences in up- or down-regulation of the genes responsible for wound callose formation. These comparative studies add new information to our understanding of the expression and regulation of specific genes induced after aphid infestation (Chapter Six).
5. The functional effects of the feeding damage to the transport of assimilate via the phloem in the plants' vascular bundles was investigated using fluorescence microscope techniques. This provides additional information about the comparative damage to the export capacities of the phloem. This is to gain further knowledge about the mechanism of different patterns of feeding by these two aphid species and the effects this has on the transport of assimilates by the infested plants (Chapter Seven).

In summary, this thesis represents my effort to gain a clear understanding of the differences and similarities of the effect of RWA and BCA feeding on cereals. The study brings together a structural, functional and molecular approach, providing some unique new insight into aphid feeding, causes and the effects of the feeding.

1.6 Hypotheses

The hypotheses upon which this study is based were:

1. The RWA would cause more severe structural and functional damage to the infested leaf, because it is highly aggressive and will elicit distinct symptoms on the host plant, than BCA, which is recognised as a less aggressive and non-symptomatic aphid.
2. There would be more extensive structural damage to vascular bundles and other leaf tissues in the susceptible cultivars than in the resistant cultivars.

2 Chapter 2: Materials and methods

2.1 Host plant material

Barley seeds (*Hordeum vulgare* L. cv Clipper) and the seeds of the susceptible and resistant wheat cultivars (*Triticum aestivum* L. cv Betta and Betta-*Dn1* respectively) were obtained from the Agricultural Research Council-Small Grain Institute, Bethlehem, South Africa. The seeds were pre-germinated in Petri dishes and sown in potting soil (60:40, peat: vermiculite mixture – Greenfingers, Port Elizabeth, South Africa) in plastic pots. The plants were watered twice a week with Long-Aston nutrient solution (Hewitt, 1966), and grown under controlled environment (Conviron S10H Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 24^{o}C, 66% RH (day) and 22^{o}C, 60% RH (night) with a 14h photoperiod. Light sources were a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, USA) and frosted incandescent 60W bulbs (Philips, Eindhoven, The Netherlands) and irradiance levels were 250µmol m^{-2} s^{-1} at 20 cm above soil level.

2.2 Aphid colony maintenance

The Russian wheat aphids (*Diuraphis noxia* Mordvilko SA-1) and Bird cherry-oat aphids (*Rhopalosiphum padi* L.) were also obtained from The ARC – Small Grain Institute, Bethlehem, South Africa. Colonies of the two aphids were maintained separately on young susceptible barley (for barley-based experiments) and susceptible wheat (for wheat-based experiments) plants. The colonies were kept in insect cages in separate growth cabinets. These aphid

colonies were raised on feeder plants for at least three generations to avoid the effects of previous hosts (Shufran et al., 1992). New, young feeder plants were introduced to the colonies every two weeks to ensure that healthy host plants were available for the aphid colonies to feed from constantly. The growth cabinets used in the aphid experiments were set at 18^{o}C, 66% RH (day) and 15.5^{o}C, 66% RH (night) and 14h photoperiods. A small, single-leaf aphid cage was used to keep the aphids on leaves of interest during the transmission electron microscopic experiments (Chapters Three and Four), while Clipcage of 2cm in diameter, as described by Noble (1958), were used in fluorescence microscopy and molecular biology experiments (Chapters Five to Seven).

2.3 Transmission electron microscopy procedures

2.3.1 Experimental set-up and aphid infestation

In this thesis, two experiments were conducted, which involved using transmission electron microscope procedures. The first experiment is reported in Chapter Three, and it deals with ultrastructural differences caused when BCA and RWA feed on a susceptible barley cultivar, Clipper. The second experiment is reported in Chapter Four, and this deals with ultrastructural investigation of feeding damage caused by RWA when it feeds on susceptible (Betta) what cultivars and resistant (Betta-*Dn1*) wheat cultivars. These two experiments have similar experimental set-ups. Five aphids each (unless otherwise stated) were carefully transferred to either the second or the third leaf above the coleoptiles with a camel hairbrush. After the aphids were

allowed to settle on the leaves, a small, single-leaf aphid cage was used to cover the aphids and keep them on the leaves of of their choice. Ten replicate plants were established for each treatment. The aphids were allowed to feed and reproduce for two weeks, after which the visible damage symptoms were noted, before the infested leaves and those of the control were selected for the transmission electron microscopic study of feeding-related damage. In the case of barley cv Clipper experiments (see Chapter Three), aphid population increases from the original five aphids during the two-week course of the experiments were determined.

2.3.2 Leaf material treatment

Leaf material was cut into strips from leaves of control as well as infested plants. The strips were then carefully trimmed and diced into smaller pieces (approximately 2 x 2mm) into cold 6% parafomaldehyde-glutaraldehyde in 0.05M sodium cacodylate buffer, using a sharp, clean, single-edge razor blade. The leaf segments were placed in small vials, and subjected to a very slight vacuum (17000 kg/m sec^2) for 1h before being placed in the refrigerator at 4^oC in a fresh change of fixative overnight. The segments were then washed in three changes of cold 0.05M sodium cacodylate buffer pH 7, after which they were immediately transferred to cold 2% osmium tetroxide in 0.05M sodium cacodylate buffer and kept in the refrigerator overnight. The leaf segments were then washed in cold buffer and dehydrated in a cold graded ethanol series, followed by two changes in propylene oxide. Spurr's (1969) epoxy resin was used in embedment of the leaf tissues. Ulthrathin sections

(silver to gold) were cut using a diamond knife (Drukker, Netherlands) and the sections were collected on 300 mesh copper grids (SPI suppliers, Philadelphia, USA). The sections were stained with uranyl acetate followed by lead citrate, and they were viewed and photographed at 80KV using a JEOL JEM 1210 transmission electron microscope (JEOL, Tokyo, Japan).

2.4 Fluorescence microscopy – Aniline blue treatments

2.4.1 Experimental set-up and aphid infestation

Unless otherwise stated, five adult aphids were confined in clipcage to the mid-region of a fully expanded leaf for each of the aphid feeding treatments. In addition, investigation of the effect of feeding by a large BCA population feeding on barley leaves involved introducing 50 adult aphids to clipcage. An empty cage served as control. Ten replicate plants were set up per treatment. A mature leaf (second leaf above coleoptile) and newly expanded leaf (fourth or fifth leaves above coleoptiles) were selected as source and sink leaf respectively during the short-term feeding experiments, while the second or third leaf above coleoptiles was selected for long-term feeding experiments.

For the wound callose formation and signal transport experiments, the enclosed aphids were allowed to feed for 24, 48 and 72 hours (short-term feeding responses), and 7 and 14 days (long-term feeding responses). After this the leaves were sacrificed for the study of feeding-related wound callose

formation using the aniline blue fluorochrome treatments and fluorescence microscope to visualize the wound callose.

For the wound callose formation pattern resulting from high BCA population feeding experiments, two procedures were adopted. The first treatment extended the long-term experiments (14d) for an additional one week (21d). On average, the aphid population under the extended conditions was about 70 aphids (see Table 3.1). In the second treatment, 50 adult aphids were allowed to feed for 72h, after which the leaves were selected for study of wound callose formation.

For the wound callose persistence experiments, only RWA was used in this study. The aphids were allowed to feed for 96h (4 days) after which all aphids were gently and carefully removed from the leaves and the clipcages replaced. Leaves were then randomly selected for the study of wound callose presence 0, 24, 48, 72, 96 and 120 h after removal of the aphids from the leaves.

2.4.2 Preparation of aniline blue fluorochrome

Aniline blue fluorochrome (4'4-[carbonyl bis (benzene 4,1-diyl) bis (imino)] bis benzensulphonic acid (Biosupplies Australia Pty Ltd) was made up as follows: 0.1 mg of the compound was diluted in 1 ml of distilled water to make the stock solution that was foil wrapped and stored at 4^{o}C. The stock solution was diluted in a ratio of 1:3 with distilled water to produce the

working stock (427 μM in distilled water), and was kept foil-wrapped at 4^{o}C until needed.

2.4.3 Leaf material treatment

In the aniline blue fluorochrome experiments, whole leaves were cut from the plant, after which the clipcage was gently removed and the area where the aphids were feeding was marked for the aphid infestation treatment. The area covered by the clipcage was marked in the control treatment. The leaves were transferred immediately into Ca^{+} free buffer (10mM MES (2-[morpholino] ethanesulfonic acid), 0.5 mM $MgCl_2$, 0.5 mM KCl and 125 mM mannitol, adjusted to pH 7.2). The abaxial surface of the leaves was gently scraped under the buffer on a glass plate using a single-edge razor blade in order to remove the cuticle and the underlying epidermal tissue in order to expose "windows" into the underlying mesophyll and vascular tissues. Working solution of the aniline blue fluorochrome was applied dropwise to the leaf strips on glass slide and covered with 20 x 50mm coverslips. The tissue was incubated with the fluorochrome solution for 30 minutes at 20^{o}C, and then washed with a fresh Ca^{+} free MES buffer. Examination of callose fluorescence was carried out under UV light, using an Olympus BX61 wide-field fluorescence Digital Imaging Microscope (Olympus Tokyo Japan, supplied by Wirsam Scientific Johannesburg, South Africa), fitted with aniline blue filter cube (with an excitation of 425-440 nm and emission of 475 nm). Images were saved in a database using the program analySIS (Soft Imaging System

GmHb, Germany), and imported as bitmaps to Corel Draw 12 (Corel 2003) for presentation.

2.5 Fluorescence microscopy – 5, 6-CFDA treatments

2.5.1 Experimental set – up and aphid infestation

The experiments where the xenobiotic 5, 6-CFDA was used to monitor phloem transport (reported in Chapter Seven) involved infestation of barley leaves with five adult aphids each of BCA and RWA. The aphids were confined using clipcage to the mid-region of a fully-expanded leaf for each of the aphid feeding treatments after the aphids have settled on the leaves while the control leaves carry empty cage. The aphids were allowed to feed for 72h (short-term) and 14d (long-term) before the leaves were further subjected to 5, 6-CFDA treatment in order to investigate the phloem transport capacity of the leaves in each treatment. Source and sink leaves were used during short-term feeding experiments, while only source leaves were used for long-term experiments, with 20 replicate plants established for each treatments.

2.5.2 Preparation of 5, 6-CFDA (5, 6-carboxyfluorescein diacetate)

The stock solution of 5, 6-CFDA (C-195, Molecular Probes, Eugen, Oregon USA) was first made by adding 1ml of 0.2% dimethylsulphuroxide (DMSO) to 100mg units of 5,6CFDA. This was foil wrapped and stored at -5^{o}C until needed. A working solution was then made by adding 1μL aliquots of the

stock solution to 1ml of distilled water, foil wrapped and stored in -5^{o}C until needed. The working solution was added directly to the abraded leaf surface and once loaded, the acetate moiety cleaved and the resultant 5,6CF eventually transported within the phloem.

2.5.3 Leaf material treatment

The experiments involving the use of 5, 6-CFDA were carried out using intact plants. The leaves were gently abraded, to allow access of the fluorophore, on the abaxial surface with a sterilized needle. Source leaves were abraded on the part above the clipcage, while sink leaves were abraded on the part below the clipcage. This is taking into consideration the classical pattern of assimilates movement, which is known to be basipetal (lamina tip to base) in source leaves and acropetal (lamina base to tip) in sink leaves (Turgeon, 1989). The abraded portion was then rinsed with distilled water before 100µL of working strength 5, 6-CFDA was added and covered with transparent polythene film (Housebrand, Brackenfell, South Africa) to prevent evaporation. The 5, 6-CFDA was allowed to be taken up through the abrasion and transported for 3h. At the end of the loading and transportation time, the leaves were detached, placed on a glass slide and covered with silicone oil, to prevent 5, 6-CFDA from leaking from the leaf. The fluorescence front, the distribution and the distance transported from the point of application of 5, 6-CFDA in control, as well as aphid infested, leaves were observed under UV light. The Olympus BX61 wide-field fluorescence microscope described above was used with a U-YFP filter set (10C/Topaz 41028, Chroma technologies, Battlebro USA)

with excitation of 513 nm and an emission of 527 nm. The images were also saved in a database and presented as described in the aniline blue treatment above.

2.6 Callose genes expression

2.6.1 Experimental set-up and aphid infestation

Ten-day-old plants were infested with aphids, BCA and RWA, by placing the clipcages around the second leaf above coleoptiles and placing 20 adult aphids per plant within the clipcages. 24 replicate plants were set up for each treatment. At time zero (same day as putting aphid cages on) plant tissues equivalent to the tissue caged-in were harvested into 15ml sterilized falcon tubes, frozen immediately in liquid nitrogen and stored at –80ºC. After 24, 48 and 72h, aphids were brushed off the infested leaves and the plant tissue within the clipcages was harvested as described above and stored at –80ºC until further use. Controls were treated the same way, except for the presence of aphids. Eight plants per treatment were pooled together in a 15ml falcon tube in triplicate, immediately frozen in liquid nitrogen, and then kept at –80ºC. Total RNA was extracted from the plant materials thus harvested and subjected to RT-PCR as well as real-time RT-PCR procedures.

2.6.2 RNA extraction

Plant material from barley cv. Clipper was harvested in 2 biological replicates with 8 individual plants in each. Total RNA was isolated from 100mg frozen plant powder using Total RNA Purification from Plant (Macherey-Nagel,

USA), according to the kit protocol. DNA was digested during the purification and purified RNA was eluted in RNase-free water.

2.6.3 RT-PCR

RT-PCR was performed by using SuperScript™ One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, USA). Thirty ng of total RNA was used as template. For callose synthases, specific primers were designed for all three sequences potentially coding for callose synthase that are present on the Barley1 GeneChip (Affymetrix). For β-1, 3-glucanases, specific primers were designed for four of the sequences potentially coding for β-1, 3-glucanases that are present on the Barley1 GeneChip. The sequences were chosen among all sequences on the chip with similarity to β-1, 3-glucanases because they were expressed in barley leaves with aphids in a microarray experiments (Delp G, Gradin T, Åhman I, Jonsson LMV, unpublished). Primer sequences are given in Table 2.1. Cycling conditions on PTC-100™ Programmable Thermal Controller, MJ Research, Inc. were: 45ºC for 30 min. and 94ºC for 2 min., 35 cycles (actin, all callose synthases and glucanase 10477) or 32 cycles (glucanases 1636, 1637_s and 1639) of 94ºC for 30 sec., 57ºC for 30 sec. and 72ºC for 1 min., and finally 72ºC for 5 min. Different cycle numbers were chosen to avoid over-amplification. Products were run on 2% agarose gels run in 1 x TBE (Sambrook et al., 1989) and visualized by ethidium bromide.

Table 2.1 Sequences of primers used for RT-PCR

	Contig no.	Primer sequences
Callose synthases	4949	F1 5'-CCACCATCGTGATCCTTATCGTGAT-3' R1 5' CATGATCGCCGGCTTCAGTGCCTGA-3'
	8428	F1 5′-GTGGAAAACAGTGCGCTCGTTGGCT-3′ R1 5′-GCCTGGTTGAACAGTAGCCGTGTCT-3′
	13152	F1 5′-AGTGCACTAGCATTCTTAGCAACTG-3′ R1 5′-CAGCGTCGTACATCCGAGAGAT-3′
β-1,3-glucanases	1636	F1 5′-GTTCCTGAGGCCCATCCTTAACTT-3′ R1 5′-CGTCGAAGAGGTTGGTGTATGTCAA-3
	1637_s	F1 5′-GAGAGGAGCTTCGGGCTCTTCAA-3′ R1 5′-AGTTAACGCATGCTTGGTTGCACTC-3′
	1639	F2 5'-ACGCGCAGGCGTACAACCAGGGATT-3' R2 5'-ACGACGCAGCTTATTCGACCACGAA-3'
	10477	F1 5'-TCCGGCTCCTACTGAGCACTGAAAG-3' R1 5'-ATGCACTACGCCTGTACAGAGCTGC-3'

2.6.4 Real-time RT-PCR

Real-time RT-PCR was performed using iScript™ One-Step RT-PCR Kit with SYBR® Green (BIO-RAD, USA) according to kit protocol. Thirty ng of total RNA was used as template. Only one replicate (with 8 plant individuals) was analyzed for the basic β-1, 3-glucanase AJ271367 (1637_s) since real-time data was going to serve only as a complement to the RT-PCR data. For contig1639, potentially coding for an acidic β-1, 3-glucanase, two replicates was analyzed and the mean for both calculated. All reactions were prepared as duplicates. Actin was used as a reference gene to ensure normalization of expression levels (primers; Actin F 5′9-TTCTCGACTCTGGTGATGGTGT-3′ and ActinR 5′-CAAGCTTCTCCTTGATGTCCCT-3′). Also, a no template control was run. Cycling conditions on MyIQ™ Single-Color Real-Time PCR Detection System (BIO-RAD) were 50ºC for 10 min., 95ºC for 5 min., and 45 cycles of 95ºC for 10 sec. and 59ºC for 30 sec. A melting curve was run after the PCR, starting at 95ºC for 1 min., 55ºC for 1 min. and then 80 cycles, increasing each cycle by 0.5ºC, starting at 55ºC for 10 sec. For calculating the relative transcription ratio between sample and control compared to reference gene, a formula by Pfaffl (2001) was used (ratio = $(E_{target})^{\Delta CPtarget\ (control\text{-}sample)}$ / $(E_{ref})^{\Delta CPref\ (control\text{-}sample)}$). Results were correlated with primer efficiency by using LinRegPCR software (Ramakers et al., 2003).

3 Chapter 3: Ultrastructural damage caused by RWA and BCA in a susceptible barley cultivar

Preamble

The data presented in this Chapter are in part, as published in Saheed et al., 2007a (Appendix A).

3.1 Introduction

Aphids are phloem-feeding insects, so tend to cause little serious perceptible damage to the plants that serve as their source of food. As previously discussed in Chapter One, BCA appears to cause no visible host plant damage when feeding, although its infestation is known to result in substantial yield loss (Leather et al., 1989 Riedell et al., 1999). In contrast, the RWA is known to cause an extensive chlorosis and necrosis upon infestation (Walters et al., 1980; Riedell, 1989). Investigations have shown that RWA and BCA do not only elicit different effects on their host plants, but also that they have different ecologies (Walters et al., 1980; Wiktelius et al., 1990). An example of this differing ecology is that BCA prefers to feed on the lower parts of the plant and in smaller aggregates (Wiktelius et al., 1990; Pettersson, 1994), whereas RWA feeds in dense aggregates and prefers to remain in rolled leaves (Walters et al., 1980; Burd and Burton, 1992).

Studies that involve investigations at the enzyme, protein and transcriptional level have demonstrated that both aphid species induce responses in their host plants. The RWA and the BCA have both been shown to induce pathogenesis-related proteins, such as β-glucanases and chitinases, and it has been reported that the induced responses are stronger and at higher levels in resistant cultivars than in susceptible cultivars (Van der Westhuizen et al., 1998a, b; Forslund et al., 2000). There are also marked differences in these induced responses. An example is the amino acid composition of the phloem sap of wheat, which was found to change upon RWA infestation, whereas BCA infestation had no such effect in the same wheat cultivars (Sandström et al., 2000). There are also differences in oxidative responses between the two aphid species. For example, RWA have been reported to induce peroxidase activity in infested plants (Van der Westhuizen et al., 1998a, Ni et al., 2001), but this is not the case in BCA-infested plants (Forslund et al., 2000; Ni et al., 2001). Peroxidase results lend support to the earlier observations of the formation of chlorotic yellow spots, which appear after RWA infestation, and the absence of these regions after BCA infestation. There is at the time of this investigation no explanation to the curling of the leaves and the yellow streaking in the RWA-infested susceptible plant.

Miles (1999) suggests that aphid saliva might be responsible at least in part for some of the observed damage. Saliva is ejected during probing as well as during feeding activities (Prado and Tjallingii, 1994; Martin et al., 1997;

Matsiliza and Botha, 2002). Aphid saliva is known to be of two types. The first forms the solid and supportive stylet sheath, which is left behind during probing, and the second is described as non-gelling watery saliva (Miles, 1999). The salivary sheath apparently contains proteins with active sulphydryl groups and exhibits enzyme activity, including polyphenol oxidase, peroxidase as well as glucanase. In addition, oxidases and pectinases have been reported present in the watery saliva (Miles, 1999).

It is clear that two distinct sources of damage must exist: the first one is phloem-related and the second is xylem-related damage. There is evidence that phloem probing causes substantial damage to the capacity of the phloem to transport assimilate. However, the question remains – how much damage is caused to the phloem? A second and equally important aspect is the evidence of 'drinking' from xylem by aphids. The principal question addressed in this chapter is what level of damage the leaves sustain – especially the vascular system - during aphid probing and feeding. The investigation of feeding effects at the TEM level were considered to be important for several reasons, chief of which is that little if any detailed information relating to cell and tissue damage exists in the literature. Past structural and ultrastructural studies were primarily used to examine the pathway of aphid stylets in the plant tissue during feeding activities. Some of these studies used the aphids to examine the potential functionality of specific phloem structures (Evert et al., 1968; 1973;

Botha and Mabindisa, 1977; Botha et al., 1977; Botha and Evert, 1978; Matsiliza and Botha 2002; Botha and Matsiliza 2004).

In this chapter, the investigations were based on the hypothesis that leaf curling, necrosis and chlorosis were caused by changes to the transport system, as a result of aphid feeding and subsequent saliva deposition. This is because, while aphid-induced phloem damage causes reduction of assimilate transport, which the plant may well be able to compensate for. Additionally, drinking water from functional xylem elements causes severe damage, possibly blockage within the vessels themselves, and precluding exit of water to surrounding parenchymatous elements that may preclude movements and / or recycling of nutrients. Little attention has been given to the damage occurring at the cellular level as a result of aphid feeding which could explain aphid-feeding symptoms such as leaf curling and yellow streaking. In this chapter, the cell damage inflicted by RWA was examined in a susceptible barley cultivar (cv Clipper) and this was compared with the apparently less damaging BCA. The aim was to use the novel tool of leaf ultrastructures to gain a better understanding of diverse mechanisms of aphid feeding. The use of the susceptible barley cultivar (cv Clipper) in the experiments reported here could hopefully serve as a reliable basis for future ultrastructural studies on other less susceptible cultivars.

3.2 Experimental overview

It is pertinent to recap briefly the materials and methods used in these experiments. Unless otherwise stated, second or third leaves above the coleoptiles were infested with five juveniles each of RWA or BCA, single-leaf aphid cage was used to cover and keep the aphids on leaves of interest. The aphids were allowed to feed and reproduce for two weeks, after which the leaves were selected and processed for the electron microscopy investigation of feeding-related damage. Aphid population increase from the original (five aphids) during the two-week time course was determined from ten replicate plants.

3.3 Results

3.3.1 Visible symptoms of infestation

The experiments confirm that there are differences in the visible symptoms of infestation exhibited by the two aphids. These include the distinct chlorosis and necrosis, as well as rolling of leaves that were observed in RWA-infested leaves (Fig. 3.1A). These symptoms did not develop in BCA-infested leaves throughout the two-week experimental period (Figure 3.1B).

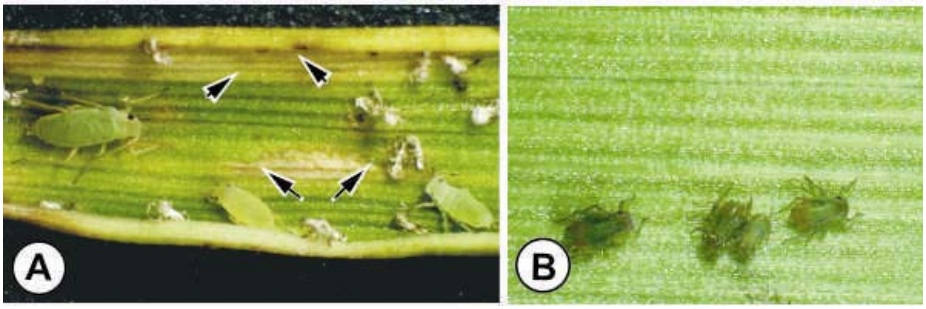

Fig. 3.1 Feeding aphids and their associated symptoms after two weeks

Figure 3.1 shows differences in symptoms of infestation by the aphids.
Fig. A is a RWA-infested leaf in which leaf rolling, chlorosis (arrowheads) and necrotic regions (arrows) are evident.
Fig. B shows a BCA infested leaf with no observable symptoms.

3.3.2 Population counts

An interesting observation was the differences in the rate of increase in population by the two aphids from the starting number of five aphids within the two-week period. The BCA population increased more rapidly than that of RWA (Table 3.1).

Table 3.1 Russian wheat aphid (RWA) and Bird cherry-oat aphid (BCA) populations per leaf, two weeks post-infestation with five aphids per leaf from ten replicates

Aphid type	Mean populations
RWA	34.9 ± 2.6
BCA	69.6 ± 3.7

p-values < 0.05 (<.0001), CI for difference of means = [-44.3, -25.1], ± SE.

3.3.3 Ultrastructural investigation

The details of the ultrastructural damage to vascular tissue from barley leaf blades are presented in this chapter.

3.3.3.1 Vascular system of control tissue

In the control leaf tissue; as expected, all the vascular structures appear intact, normal and are assumed functional (Fig. 3.2). Figs 3.2 A-C shows details of part of an intermediate vascular bundle where two thick walled sieve tubes (solid circles) as well as a thin-walled sieve tube (open circle) are visible. In Figs. 3.2 D and E, pore-plasmodesmal fields (pore-pd, PD) between a companion cell and sieve tubes are unoccluded.

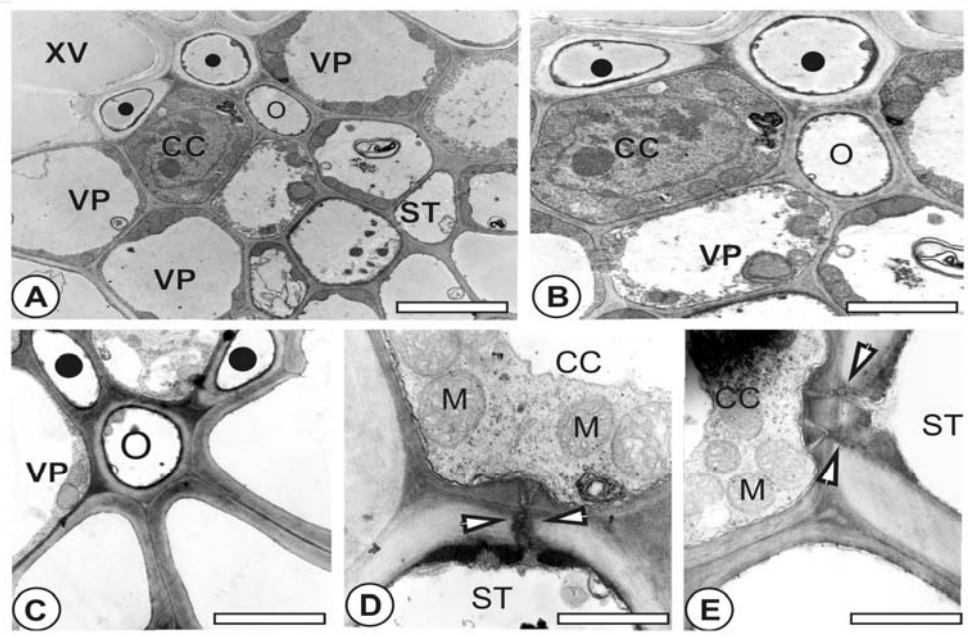

Fig. 3.2 Transmission electron micrographs of vasculature from control barley leaves

In **Fig. 3.2 (A-E)**, TEM images of control barley leaves, intermediate vascular bundles are illustrated.

Figs. A-B show a close spatial relationship between the thick-walled sieve tubes (solid circles) and the xylem vessels (XV), as well as to a thin-walled sieve tube (open circle). Vascular parenchyma cells (VP), and companion cells (CC) are associated with the thin-walled sieve tubes.

Fig. C illustrates a thin-wall sieve tube (open circle) and thick-walled sieve tubes (solid circles)

Figs. D-E Unoccluded, presumably functional, characteristically-branched pore plasmodesmal connections between thin-walled sieve tubes (ST) and companion cell (CC), the mitochondria are intact.

Scale bars; A-C=5 μm; D-E = 1 μm.

3.3.3.2 Damage to vascular system during RWA feeding

The characteristic damage caused by RWA to the vasculature in barley leaves is illustrated in Figure 3.3. The probing of the xylem vessels (XV) during feeding results in the vessels being sealed with electron-dense material (Fig. 3.3A), which is characteristic of RWA-probed vessels. The vascular parenchyma cells exhibit signs of severe plasmolysis upon probing by the aphids and possibly tasting from their contents. The plasmamembrane and cytoplasm are withdrawn from the cell wall in most cases (Fig. 3.3B). Accretion of salivary material (SS) often obliterated the xylem parenchyma cells adjacent to punctured tracheary elements. Along with this, adjacent mesophyll cells were often damaged during the probes of xylem (Fig. 3.3C). The sieve tubes showed various degrees of damage; for example, in Fig. 3.3D, thin-walled sieve tubes (ST) show evidence of having been punctured. The endoplasmic reticulum in the middle sieve tube, however, remains parietally localized (open dart), whilst in the uppermost sieve tube, the ER appears interspersed with deposits of electron-dense material; and amorphous callose (solid dart). The sieve tubes also suffer from plasmolysis, from mild to severe, in which the plasmamembrane and the underlying cytoplasm was disrupted and pulled away from the cell walls (Figs. 3.3 M-N). The extensive formation of callose (C) was mostly associated with punctured and disrupted sieve tubes, which occurs in associated companion cell where encapsulation of the plasmodesmatal is evident (Figs. 3.3K, L). In many instances, plasmodesmatal

and pore plasmodesmatal connections were occluded with electron-dense and electron lucent material (Figs. 3.3I, J).

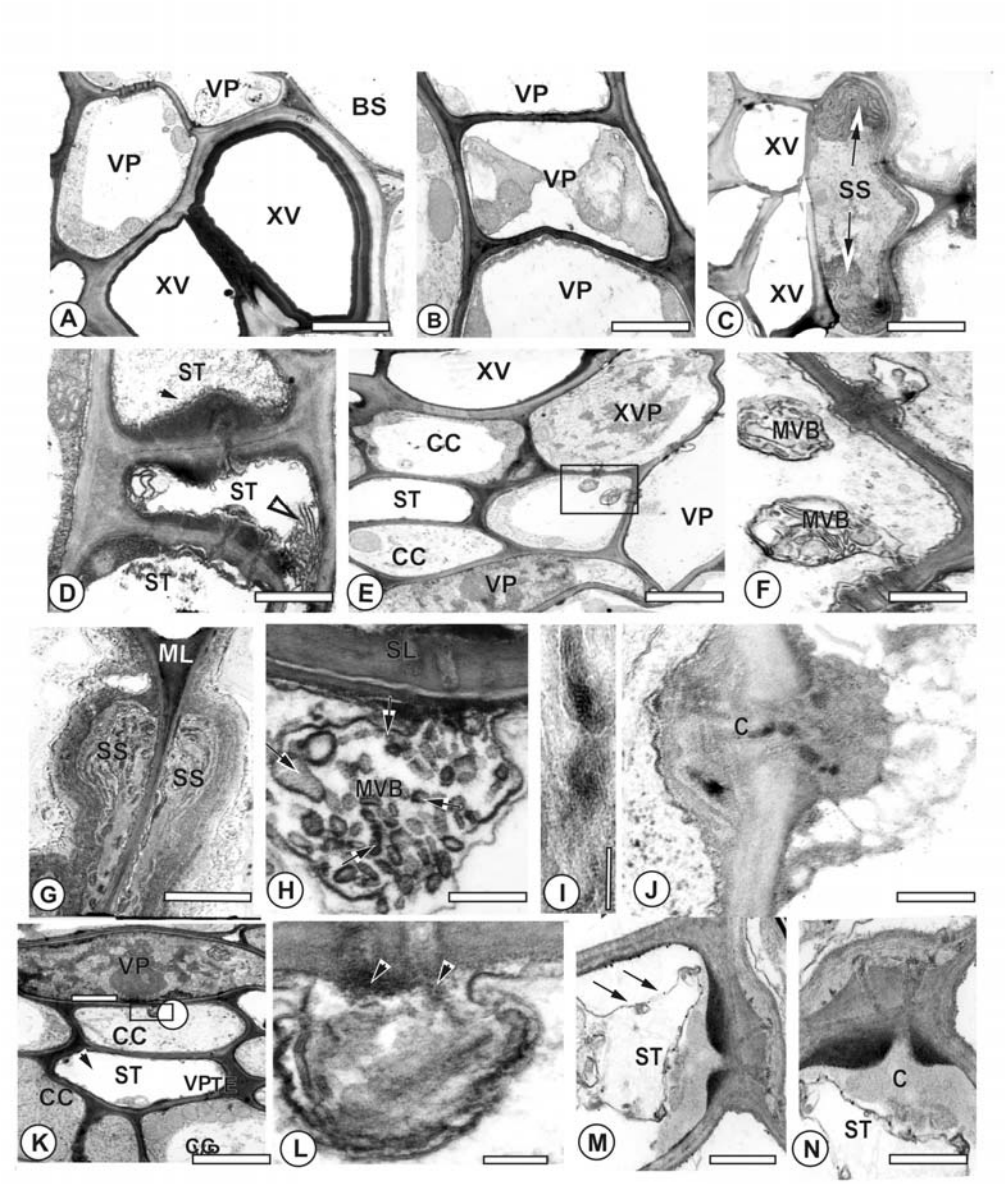

Fig. 3.3 Transmission electron micrographs from RWA-infested barley leaves

Figure 3.3 (A-N) reveals ultrastructural damage caused by RWA feeding:
Fig. A. illustrates typical punctured xylem vessels, with electron-dense salivary ejecta encasing the walls of the vessels, occluding the pit membrane between the vessels. Surrounding parenchymatous cells appear unaffected. Part of an undamaged bundle sheath cell (BS) appears unaffected.
Fig. B. shows vascular parenchyma (VP) in which the central cell is severely plasmolysed, presumably after aphid puncture.
Fig. C shows part of an intermediate vascular bundle in which salivary sheath material (SS) has completely obliterated the xylem parenchyma cells adjacent to xylem vessels. Mesophyll cells to the right have also been punctured and contain salivary material.
Fig. D shows three thin-walled sieve tubes from a small, intermediate vascular bundle. Damage is extensive, but endoplasmic reticulum remains parietally localized (open dart). In the uppermost sieve tube, the ER is interspersed with extensive electron-dense deposits. Pore connections to the upper sieve tube are obliterated by electron-dense material. Amorphous callose is deposited at the neck of pores in contrast to open pores between sieve elements in control tissue (Figs. 3.2 D-E).
Fig. E shows part of an intermediate vascular bundle. Multivesicular bodies (box and detailed in Fig. F) are present in the companion cell.
Fig. F shows that all plasmodesmata appear closed and are associated with distorted plasma membranes.
Fig. G reveals a multilayered salivary material deposit on either side of the common wall between two vascular parenchyma cells. (ML = middle lamella.)
Fig. H shows a multivesicular body adjacent to plasmodesmata between a mestome sheath cell (above) and a vascular parenchyma cell (below). The suberin lamella (SL) forms a compound middle lamella between these cell layers. Plasmodesmata are occluded by electron-dense material.
Fig. I: high resolution of a single plasmodesma from Fig. J.
Fig. J: plasmodesmata between parenchymatous elements appear blocked by electron lucent and electron dense material. Plasmodesmal apertures are associated with callose (C).
Fig. K shows thin-wall sieve tube, companion cell and associated vascular parenchyma. There is no plasmolysis or obvious damage to sieve tube but there is callose encapsulation in companion cell (box).
Fig. L shows a higher magnification (from Fig. K) of plasmodesmata between parenchyma and companion cell with heavy encapsulation by callose and salivary constituents.
Fig. M shows part of a probed thin-walled sieve tube where the plasma membrane and cytoplasm have pulled away from the cell wall (arrows).
Fig. N is a part of the Fig. M. showing electron-dense material, as well as callose (C) that is intermixed with extensive salivary deposits associated with the plugged pore-plasmodesma.
Scale bars: A – C = 5 µm; D = 3 µm; E = 5 µm; F = 2 µm; G = 2µm; H = 100 nm; I = 100 nm; J = 250 nm; K = 5 µm; L = 100 nm; M = 3 µm; N = 1 µm.

Saliva ejected by probing or feeding aphids often appeared layered or polylamellate (Fig. 3.3G) where the common wall between the adjacent parenchymatous cells have been punctured. This results in the deposition of salivary material on both sides of the wall. Feeding by RWA results in formation of multivesicular bodies (MVB, details in Fig. 3.3H), which usually develop as a result of sudden pressure loss often associated with the vascular parenchyma linked to damaged sieve tubes. (See box in Fig. 3.3E and at higher magnification in Fig. 3.3F.)

3.3.3.3 Damage to vascular system during BCA feeding

The ultrastructural damage associated with BCA feeding is as illustrated in Figure 3.4 (A-K). Comparison of the data presented in Fig. 3.4 with the damage caused by RWA (Fig. 3.3) leaves little doubt that BCA causes or induces less structural damage to the vascular system in barley leaves. Damage caused by BCA is mostly partial plasmolysis, which was often evident, as was obliteration of xylem (Figs. 3.4A-C). In Fig. 3.4B, the extent of the damage to the xylem vessels is evident. The xylem probed by BCA often contained granular salivary material, which occupies much of the lumen of the vessel in this case (Figs. 3.4A-C). The damage that occurs here appears less severe than is the case in RWA-probed xylem. It should be noted that the adjacent thick-wall sieve tube (TST, Fig. 3.4B) is apparently partially plasmolysed, similar plasmolysis occurs in companion cells (Figs. 3.4D, K).

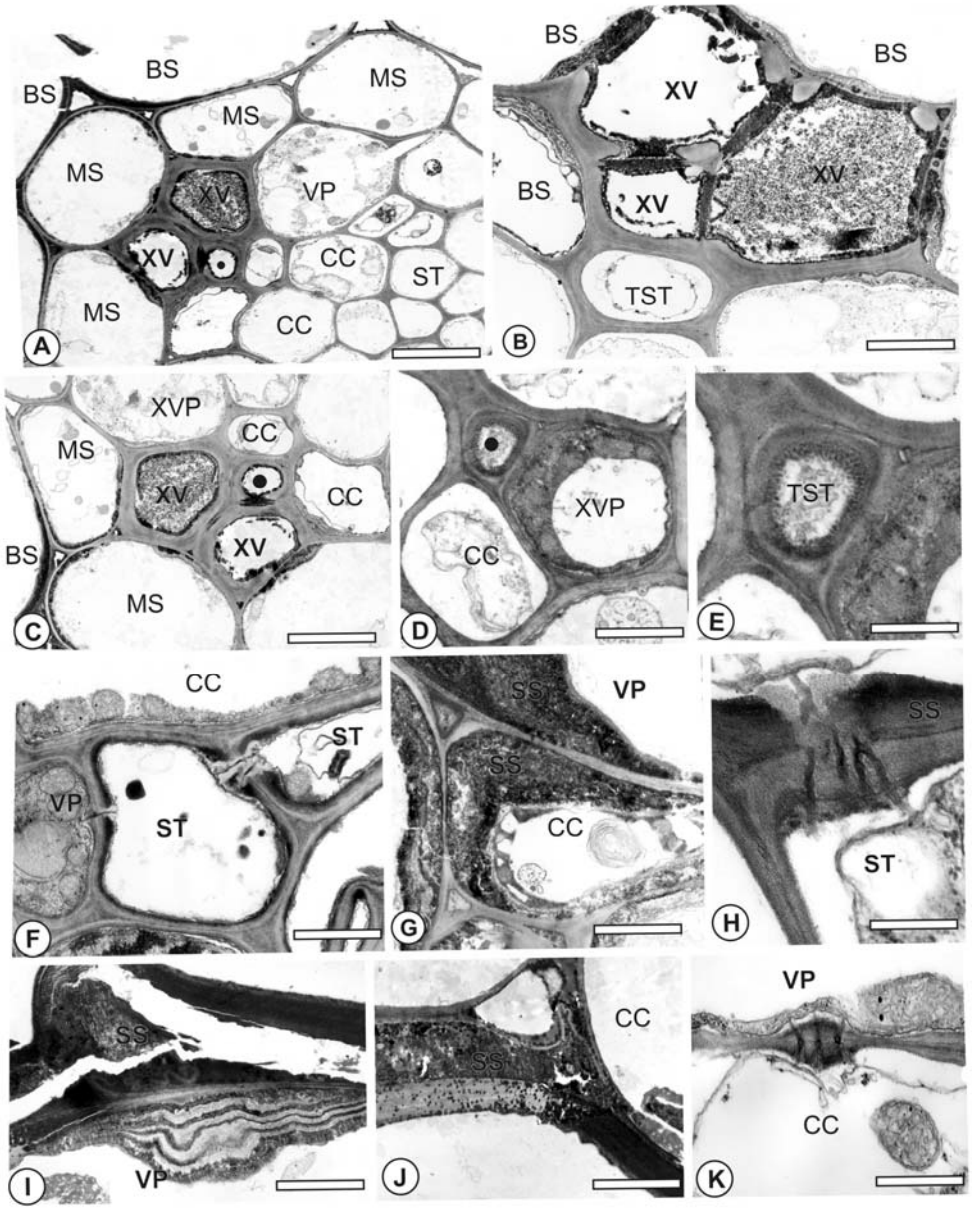

Fig. 3.4 Transmission electron micrographs from BCA-infested barley leaves

Figure 3.4 (A-K) illustrates aspects of damage caused by the BCA feeding:
Fig. A. shows details of part of a small vascular bundle. Cells within this vascular bundle show limited aphid-associated damage. Xylem vessels (XV) have been probed for water. Salivary deposits are loosely granular and electron-dense, but do not encapsulate cell walls, as is the case with RWA xylem probes (see Fig.3.3A for comparison).
Fig. B shows that vessels are not totally occluded by the loosely granular salivary deposit. The thick-walled sieve tube (TST) is partially plasmolysed.
Fig. C shows a detail of the vascular bundle with granular salivary deposit in the xylem and partial plasmolysis of adjacent cells.
Fig. D shows details showing xylem vascular parenchyma (XVP) adjacent to a companion cell (CC) with cells variously-plasmolysed.
Fig. E shows a higher magnification (from Fig. D) of a thick-wall sieve tube and adjacent xylem vascular parenchyma cell with salivary deposits.
Fig. F show two aphid-probed thin-walled sieve tubes (ST), the sieve tube to the right shows more evidence of plasmamembrane disruption and lateral sieve areas between these cells are plugged with callose. Plasmodesmata between the sieve tube at the centre and its associated vascular parenchyma cell appear unoccluded.
Fig. G shows salivary sheath material (SS) deposited on either side of the vascular parenchyma-companion cell wall interface.
Fig. H Pore-plasmodesmata between a thin-walled sieve tube (below) and a companion cell (above) are occluded. Pore plasmodesmata are partially occluded.
Fig. I shows deposition of salivary constituents splitting the wall of the vascular parenchyma and its adjacent cell. Note the disruption of the plasma membrane from the cell wall.
Fig. J shows electron-dense salivary material deposited along cell wall and middle lamella (ML) of companion cells.
Fig. K shows plasmodesmata partly occluded between a vascular parenchyma cell (above) and a companion cell (below).
Scale bars: A = 20 µm; B = 10 µm; C = 15µm; D =5µm; E = 2µm; F = 5µm; G = 2µm; H-K = 1µm.

The probing and eventual feeding by BCA in the thin-walled sieve tubes (Figs. 3.4F, H) resulted in the sieve area pores that connect the two sieve tubes to be partially occluded by callose; this is far less than observed in RWA-probed phloem. However, damage to vascular parenchyma and companion cells was often more extensive (Figs. 3.4E, G, I-J). Note that salivary sheath material (SS) is often deposited along the walls of the cells separating the cytoplasm from the wall (Figs. 3.4I-J) and also the deposition of saliva constituents as well as the disruption of organelles within the companion cell (Fig. 3.4G) and xylem vascular parenchyma (Fig. 3.4E).

3.4 Discussion

It was of interest that the population growth study of the two aphid species showed that BCA reproduced more quickly than RWA did over the two-week period (Table 3.1). However, these results are consistent with the observation by Messina et al., (2002) where BCA population growth was shown to increase faster than that of RWA after infestation on wheat. The logical conclusion would be that more feeding associated damage would be observed with BCA due to the larger number of feeding aphids. This is because damage to plants is assumed to be caused by the puncturing of cells by the aphid stylet, feeding in the phloem and subsequent salivary constituents' deposition (Miles, 1999). However, this was not the case. Leaves fed on by the larger BCA population remained visibly healthy (Fig. 3.1B). In contrast, in leaves infested by smaller populations of RWA, marked symptoms of chlorosis, necrosis, as well as leaf roll occurred (Fig. 3.1A).

The experiments conducted by Prado and Tjallingii (1994) as well as Martin et al., (1997) have both demonstrated that puncturing of parenchyma cells by aphids during their probing activities before they eventually reach sieve tubes (the primary feeding site), involves the discharge of watery saliva. Penetration of plant leaves by aphid stylets has been reported to vary from an apparently highly precise process, to one which, at times, appears haphazard (Matsiliza and Botha, 2002 and references cited therein). The studies conducted by Fouché, 1983 and Fouché et al., (1984) have demonstrated that changes

occurred in the cell organization of wheat tissue when wheat leaves were treated with extracts from whole body of RWA. Their results cannot, however, be compared directly to the present study, which focuses on the damage caused by stylet penetration during probing and subsequent aphid feeding. Aphids have been reported to drink from xylem (Tjallingii, 1994) during electrical penetration graph (EPG) recordings. The results presented in this chapter might be the first ultrastructural evidence for a confirmation of drinking from xylem elements. Puncture and drinking from xylem elements by the RWA and BCA resulted in markedly different damage signatures. RWA seem to eject a large quantity of electron-dense, fluid ('watery') saliva. This, in all instances, appears to completely seal the xylem vessel (see Fig. 3.3A), and in particular the saliva blocks all membrane pit fields between xylem vessels and other associated parenchyma cells. In contrast, BCA appear to deposit dense granular saliva in the xylem vessels (see Figs. 3.4 A-C). This saliva matrix does not appear to occlude the membrane of xylem vessels – such as xylem parenchyma pit fields. It is tempting therefore, to speculate that the less aggregated deposits resulting from BCA drinking induces less physiological degrading of the apoplasmic transport system than would seem to be the case with RWA water drinking processes. In the case of RWA-feeding, the xylem elements are ensheathed by an amorphous, electron-dense layer, which seems to completely isolate these elements from surrounding xylem parenchyma, thereby precluding the exchange of vital water and mineral nutrients with adjacent cells. As a result of blocking parts

of the xylem system and thus preventing solute exchange in these regions, leaf roll and chlorosis / necrosis could develop.

There are differences in the pattern of damage produced by probing, feeding and water-drinking aphids. In all cases, RWA elicits more visible structural damage than does BCA, when probes of comparative tissues are examined (compare Fig 3.3 to Fig. 3.4). Cell wall probing produces similar results, with a polylamellate salivary deposit commonly found associated with inter- as well as intracellular probes. In both cases, RWA and BCA can separate cells along the middle lamella. The results presented here also confirm that both RWA and BCA puncture parenchyma cells during their search for functional sieve tubes within the phloem tissue and that the cells probed by RWA (Figs. 3.3 B, C, E and F) appear to be more affected by probing and may be rendered non-functional. In contrast, similar vascular parenchyma and associated phloem probes by BCA do not seem to be as marked or severe (Figs 3.4 A-G).

Clearing (blowing) of stylets (evidenced by deposition of salivary material observed here both inter- as well as intracellularly) suggests that the aphids might analyse the substrate contained in the cells that they penetrate (see also Klingauf, 1987; Tjallingii, 1995; Miles, 1999 among others). Probing and puncturing of cells along the pathway may help direct the aphids towards their food source (phloem tissue) and water source (xylem vessels). When the aphid reaches the sieve tubes, salivary sheath production stops, but feeding is

preceded by ejection of watery saliva. The aphids then start to ingest cell sap and maintain a sustained feeding pattern (Tjallingii, 1995; Prado and Tjallingii, 1997; Miles, 1999). This present study shows that the sustained feeding by RWA from sieve elements results in complete and apparently irreversible damage (Figs. 3.3 D, M and N) whereas BCA does not appear to cause such irreversible damage to the sieve elements from where they feed (Figs. 3.4 F and H).

The evidence reported in this chapter provides useful and novel information relating to the damage inflicted by the aphids on the transport tissues of barley leaves. In the absence of positive identification of virus populations, the data presented demonstrate that the physical puncturing of the cells by the aphid stylets and the subsequent deposition of saliva during the feeding process elicit differing response. The results reported here demonstrate that much of the damage inflicted by RWA in comparison to BCA could be due to complete destruction of the cells. Resultant rapid pressure loss can precede irreversible physiological damage, in addition to reported effects associated with saliva deposition itself. The sealing of xylem vessels by blocking the pit field membranes by watery saliva material and the extensive damage to sieve tube-companion cells complex of the phloem cannot be ignored.

Considering the above evidence, I therefore suggest that leaf rolling and streaking symptoms shown by plants infested with RWA might, in part, be

due to the apoplasmic and symplasmic isolation of both xylem and phloem respectively, leading to limitation in nutrient exchange from these cells into adjacent cells. It is further suggested that chlorosis and necrosis might appear during probing, where puncturing of cells could induce oxidative stress while the sealing of xylem and phloem can generate leaf rolling and streaking symptoms.

4 Chapter 4: Ultrastructural damage caused by RWA in susceptible and resistant wheat cultivars

Preamble

This chapter explores the use of wheat cultivars that were susceptible or resistant to RWA and investigates ultrastructural damage during RWA feeding. This is because there is no commercial cultivar of barley available in South Africa that is resistant to RWA and that could be used for this study. Hence, the use of the two near-isogenic wheat cultivars, since wheat and barley has similar ultrastructures. However, the data presented in this chapter are in part, as published in Saheed et al., 2007b (Appendix B).

4.1 Introduction

The Russian wheat aphid became a significant problem in South Africa in 1978 (Walters et al., 1980) and still causes major economic losses not only in South Africa, but also in North and South America. The RWA has been described as one of the most destructive pests of small grains (Kovalev et al., 1991), due to the extensive chlorosis, necrosis and leaf roll it caused upon infestation, apart from the substantial yield loss. Because of these serious effects, a series of efforts has been engineered into breeding for resistance to this aphid. To date, about 10 genes conferring resistance to RWA have been identified from wheat and other related cereals (Liu et al., 2002, 2005). Many of the resistant accessions and their near-isogenic counterparts carrying

resistance genes have been used in various studies with the aim of unravelling the mechanisms for RWA-induced plant damage and that of resistance mechanisms.

The resistance gene *Dn1* is one of the resistance genes that have been incorporated into many cultivars of wheat. It was first identified in South Africa in the common wheat accession PI 137739 from Iran (Du Toit, 1987, 1988, 1989a, b). *Dn1* has been introduced into different wheat cultivars such as Betta, Tugela and Molopo, creating near-isogenic lines (Du Toit, 1989a). The effect of the *Dn1* gene has been reported to be mostly antibiotic. This operates by causing a reduction in the growth rate of the feeding aphid's population, as well as a reduction in fecundity and aphid biomass (Du Toit, 1987, 1989b; Budak et al., 1999; Heng-Moss et al., 2003). These are the effects that could be observed in the resistant Betta- *Dn1* wheat as compared to susceptible Betta cultivar. The reduction in aphid biomass was also reported in Tugela-*Dn1* as compared to Tugela near-isogenic line (Wang et al., 2004). In addition, apart from antibiosis effects, Du Toit (1987) reported some levels of antixenosis in Betta-Dn1, but this was not confirmed in the experiments conducted by Budak et al., (1999). However, tolerance effects were found to be absent in the wheat lines containing *Dn1* genes (Du Toit, 1989b; Budak et al., 1999). Lines carrying the *Dn1* gene have been reported to exhibit symptoms such as chlorotic spots and leaf streaks, but the symptom rating is

lower than on the corresponding susceptible lines (Du Toit, 1988; Wang et al., 2004; Botha et al., 2006).

Extensive studies of the biochemical effects of RWA infestation have been carried out using many near-isogenic lines of wheat as well as barley cultivars. Some hydrolytic enzymes that belong to the pathogenesis-related proteins group, such as β-1, 3-glucanase (Van der Westhuizen et al., 1998a), peroxidase as well as chitinase (Van der Westhuizen et al., 1998b) have been found to be induced by RWA infestation in the resistant *Dn1* lines. In contrast, no induction, a very late induction, or a lower degree of induction can occur in the corresponding susceptible lines. This suggests that RWA induces a hypersensitivity response in resistant lines, an idea that was further supported by the early accumulation of hydrogen peroxide and increased levels of NADPH oxidase activity in RWA-infested resistant Tugela-*Dn1* lines (Moloi and Van der Westhuizen, 2006). Other studies have focused on the chlorosis and changes in ultrastructure of the chloroplasts (Fouché et al., 1984) as well as the levels of chlorophyll and carotenoids in susceptible and resistant lines upon RWA infestation (Heng-Moss et al., 2003; Wang et al., 2004; Botha et al., 2006). RWA often co-occurs with the non-symptomatic BCA, but RWA-resistant *Dn1* lines do not show any antibiosis effects against this aphid (Messina and Bloxham, 2004), indicating that the resistance mechanism is not activated by the bird cherry-oat aphid or that it acts specifically against RWA. In Chapter Three (Saheed et al., 2007a), the differences in damage at the

ultrastructural level caused by the RWA and BCA, when they both feed on vascular bundles of the susceptible barley cultivar cv Clipper, were clearly demonstrated. The damage to conducting elements may partly explain the severe symptoms caused by RWA infestation and the absence of such symptoms upon infestation by the BCA.

In this chapter, the investigation focuses on further ultrastructural effects of RWA infestation on susceptible wheat cultivar Betta and the resistant Betta-*Dn1*. This is with a view to gain further understanding of:

1. The mechanisms of the RWA feeding which leads to the severe visible symptoms observed in susceptible plants.
2. The resistant mechanism that prevents RWA proliferation (antibiosis) as well as reduces or possibly, delays such symptoms in the resistance cultivar.

The hypothesis behind this investigation is that less structural damage will be visible in the resistant than in the susceptible cultivars and that the ejection of saliva into the xylem, as previously reported in Chapter Three, is a causal factor in the visible symptoms during infestation. This is because copious salivary deposition would effectively limit if not totally block the transfer of water and essential nutrients that occurs during the transpirationally driven ion exchange and recycling process. In addition to the known hypersensitivity induced by the *Dn1* gene, leaf roll is slower to appear, and other effects

associated with RWA feeding are delayed in *Dn1,* due to lower aphid population growth rates than in the non-resistant Betta.

4.2 Experimental overview

In the experiments reported in this chapter, the second or third fully expanded leaf of the susceptible (Betta) and resistant (Betta-*Dn1*) wheat cultivars were infested with five adult aphids, and single-leaf aphid cages were placed over the infested leaves. Empty aphid cages were placed on the control leaves and 10 replicate plants were established for each treatment. The aphids were allowed to feed and reproduce for two weeks, after which visible damage symptoms were noted before the infested leaves and those of the control were selected for the transmission electron microscopic study of feeding-related damage.

4.3 Results

4.3.1 Symptoms of infestation

The probing and feeding activities of RWA resulted in noticeable damage to susceptible Betta, but was less evident in resistant Betta-*Dn1* cultivar. Symptoms such as chlorosis, necrosis and leaf roll were observed in the susceptible cultivar Betta within the two-week experimental period, while the resistant cultivar Betta-*Dn1* exhibited few chlorotic patches and necrotic spots within the same time frame.

4.3.2 Ultrastructural damage

4.3.2.1 Control tissue

The details of ultrastructure from the control susceptible Betta and the near isogenic resistant Betta-*Dn1* cultivars are not different and they are as illustrated in Figure 4.1 (A-E), the cells structures are intact and are apparently functional. Figures 4.1 A-B shows details of part of an intermediate vein, which includes thin-walled sieve tube elements, associated vascular parenchyma and companion cells that appear normal and undisturbed. Vascular parenchyma and companion cells associated with thin-walled sieve tube elements at higher magnification (Figs. 4.1 C-D) show undamaged mitochondria, intact endoplasmic reticulum and middle lamella. Figure 4.1E illustrates a thick-walled sieve tube with a plasma membrane that is parietal and intact.

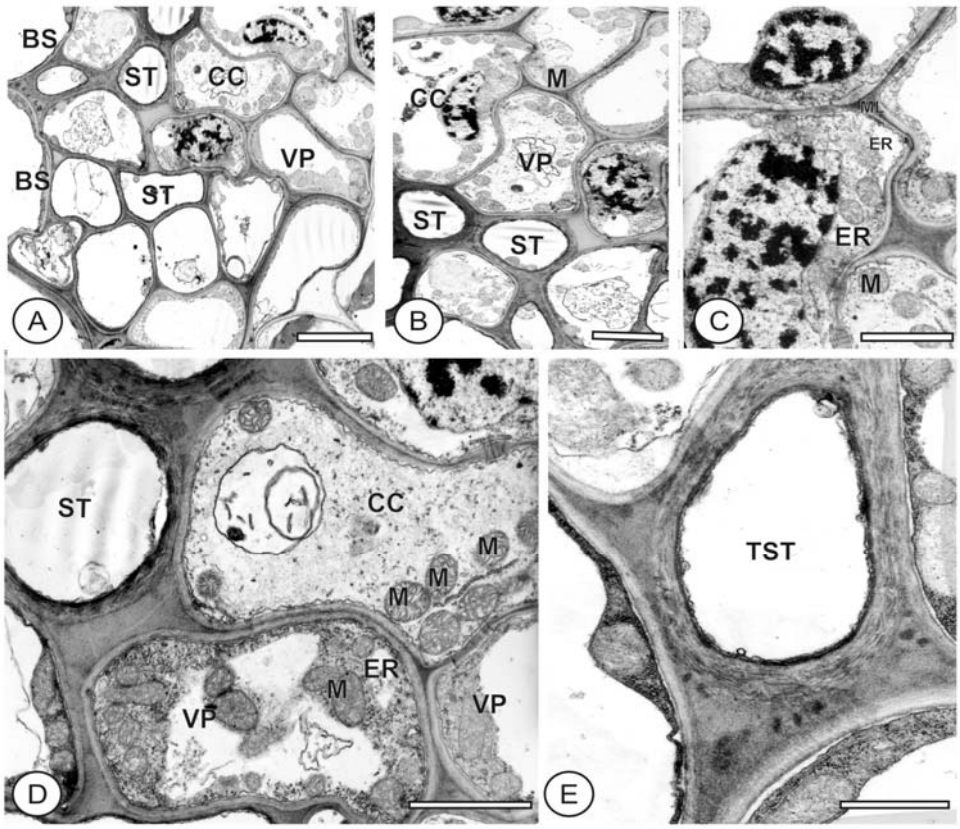

Fig 4.1 Transmission electron micrographs illustrating vasculature from control wheat leaves

Figure 4.1 (A-E) show details of control, non-infested wheat cv Betta and Betta-*Dn1*.

Figs. A and B: part of an intermediate vein including thin-walled sieve tube element (ST), associated vascular parenchyma (VP), companion cells (CC) and bundle sheath cells (BS). Note that the thin-walled sieve tube elements and other cells are intact.

Figs. C and D: details of vascular parenchyma (VP) and companion cells (CC) associated with thin-walled sieve tube elements (ST). Mitochondria (M), endoplasmic reticulum (ER) and middle lamella (ML) are intact.

Fig. E: thick-walled sieve tube element (TST) showing plasma membrane that is parietal and intact.

Scale bars A-E = 5 μm.

4.3.2.2 Probing in mesophyll cells

Probing of mesophyll cell by RWA during its pathway to the vascular bundle results in severe cell damage to the cells probed and punctured. Figure 4.2 (A-D) illustrates the damage caused by RWA in the susceptible Betta cultivar, while Fig. 4.2 D shows similar probing in resistant Betta-*Dn1*. The mesophyll tissue penetrated or punctured in the susceptible cultivar (Figs. 4.2 A, C) shows evidence of damage to cell walls, disruption of cell cytoplasm and destruction of constituent and organelles. Probing by RWA usually results in mesophyll cells being split apart and the cleavage of the plasmodesmatal fields ensues, while the saliva sheath that is usually left behind occupies the middle lamella region (Figs. 4.2 B-E). In addition, probed mesophyll cells in susceptible Betta have the walls of adjacent cells as well as the cytoplasm destroyed while plasmolysis is often evident (Figs. 4.2 B-D). In few instances, organelles like chloroplast can appear intact (Fig 4.2 D). In contrast, mesophyll cell damage was not as severe in *Betta-Dn1* leaf blade (Fig. 4.2 E).

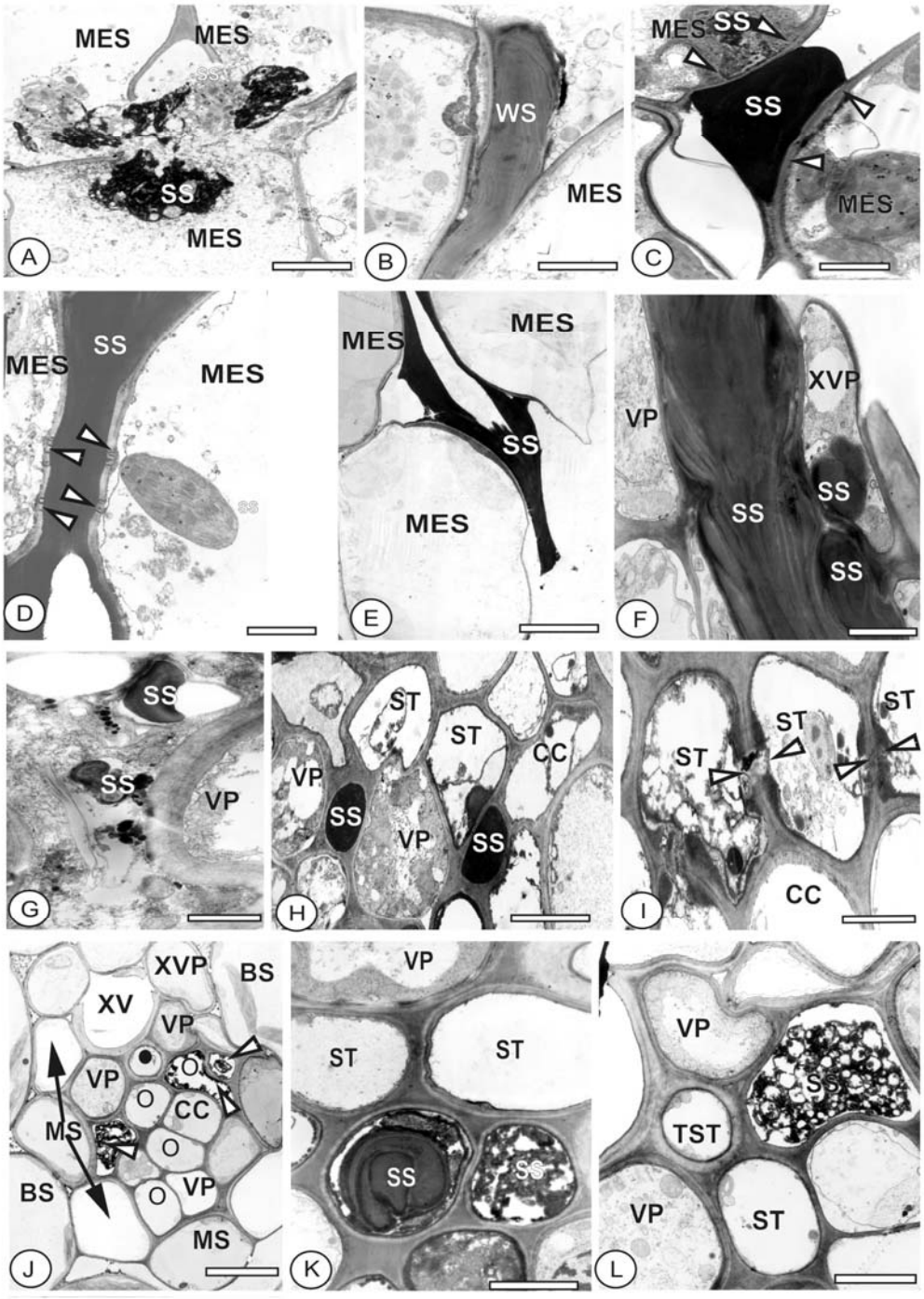

Fig. 4.2 Ultrastructural damage in mesophyll cells and phloem systems in wheat cultivars

Figure 4.2 (A-L) illustrates aspects of RWA probing and feeding damage to mesophyll and phloem systems, in Betta and Betta-*Dn1*. **Fig. A** shows junction between probed mesophyll cells (MES) in Betta cultivar. Cell walls were destroyed and cell contents disrupted and intermixed with salivary material (SS). **Fig. B** shows part of a stylet sheath between two mesophyll cells in the leaf blade of Betta cultivar. Note that the cell walls have been punctured and saliva is present within the mesophyll cell to the left (SS). **Fig. C:** a stylet sheath is in the intercellular space between mesophyll cells (MES) of a Betta cultivar. Both walls in contact with the sheath show damage at the contact zone (arrows) and saliva (SS) has leaked into the cells. **Fig. D** shows a probe between two mesophyll cells of a Betta cultivar leaf in which the aphid has split the cells (presumably along, or in the middle lamella region) separating plasmodesmata in the process (arrowheads). The mesophyll cells show signs of disruption, yet the chloroplast in the cell to the right appears normal. **Fig. E** shows probing in Betta-*Dn1* leaf blade. Stylet sheath (SS) deposition occurs between mesophyll cells, which apparently are neither plasmolysed nor otherwise damaged.
Fig. F shows detail of a probe near a large metaxylem vessel (XV) of a large vascular bundle in a Betta-*Dn1* leaf blade. Copious saliva (SS) was deposited, as the aphid appeared to puncture a number of cells sequentially while probing for phloem. Despite the presence of saliva, the punctured xylem parenchyma cell to the right (XVP) does not appear to be damaged or plasmolysed.
Fig. G shows completely disrupted vascular parenchyma in an intermediate vascular bundle of a Betta cultivar.
Fig. H shows details of obliteration of the phloem tissue due to extensive feeding in a vascular bundle of a Betta cultivar. Two thin-walled sieve tubes (SS) were completely obscured by saliva and plasmolysis was evident in other sieve tubes. Companion cells and phloem parenchyma were extensively damaged in this large intermediate vascular bundle.
Fig. I shows three adjacent thin-walled sieve tubes (ST) from an intermediate bundle of a Betta cultivar with cytoplasmic damage and callose deposition (arrowheads) in lateral sieve area pores.
Fig. J shows part of a small intermediate vascular bundle in Betta-*Dn1* leaf blade. A metaxylem vessel (XV), several xylem parenchyma cells (XVP), one thick-walled sieve tube (solid dot), several thin-walled sieve tubes (open circles) and associated companion cells (CC) and vascular parenchyma (VP). Mestome sheath (MS) cells are visible inside the bundle sheath (BS); damage is limited to only cells with saliva deposits (arrowheads).
Fig. K shows thin-walled sieve tubes in a large bundle of Betta-*Dn1*, which are undamaged, in contrast to adjacent elements, which contain saliva.
Fig. L shows details of undamaged thick- and thin-walled sieve tubes in an intermediate vascular bundle of Betta-*Dn1* after being probed. Salivary complex (SS) is vesiculated. Surrounding cells do not appear to have been damaged.
Scale bars: A = 2 μm; B = 5 μm; C = 5 μm; D = 3 μm; E = 5 μm; F = 5 μm; G = 3 μm; H = 10 μm; I = 5 μm; J = 15μm; K = 3 μm; L = 5 μm.

4.3.2.3 Probing and puncturing of vascular parenchyma

Vascular parenchyma are often probed and obliterated during penetration by RWA on the way to the phloem and xylem tissues. These probes resulted in deposition of a great deal of salivary sheath material, often in the region of the middle lamella. This often resulted in the separation of the plasmodesma of adjacent cells. At times, salivary ejecta are often deposited inside the punctured cell. In the susceptible Betta cultivar, punctured vascular parenchyma cells (Fig. 4.2 G) show severe damage to the cytoplasm and organelles. The common observation in this cultivar is that cells adjacent to salivary deposit show severe damage to cell walls, including the cytoplasm as well as organelles. Damage was to all intents, similar to that in the mesophyll cells (see Fig. 4.2 C). In contrast, inter- as well as intra-cellular probes of intermediate and small bundles in Betta-*Dn1* cultivar do not appear to result in severe damage to vascular parenchyma (Fig. 4.2 F). In this case, the vascular parenchyma (right) adjacent to the salivary deposit (left) does not appear to show signs of damage in this image as compared to what was observed in susceptible cultivar. It appeared as if cell disruption was an isolated event in some instances, with individual, rather than whole groups of cells, destroyed during feeding (see also Figs. 4.2 J, L for similar examples in vascular bundle).

4.3.2.4 Phloem feeding – thin-walled sieve tubes

The primary site of aphid feeding is the sieve tubes of the phloem. In the susceptible and resistant cultivars used in this study, the RWA probed and fed

from the thin-walled sieve tubes extensively. In Betta cultivar, this feeding resulted in severe damage to the phloem, as shown in Fig. 4.2 H, in this example, four sieve tubes, a companion cell as well as phloem parenchyma have been punctured. These sieve tubes show severe plasmolysis, as their plasma membranes have ruptured and torn away from the cell walls in places. In addition, two thin-walled sieve tubes are occluded with salivary sheath material, while the remaining parenchyma cells are variously plasmolysed. Callose deposition in the lateral sieve area pores of the punctured, plasmolysed sieve tubes (Fig. 4.2 I) was very common. In sharp contrast, thin-walled sieve tubes in the vascular bundle of Betta-*Dn1* leaf blade usually show little or no evidence of plasmolysis (Figs. 4.2 J-L), as they appeared undamaged, including those adjacent to salivary material deposit. However, obliterated thin-walled sieve tubes were occasionally encountered.

4.3.2.5 Phloem feeding – thick-walled sieve tubes

Thick-walled sieve tubes do not appear to be favoured site of feeding by aphids. It seemed that they were probed through, while searching for the thin-walled sieve tubes. However, probing in the thick-walled sieve tubes of the susceptible Betta cultivar sometimes resulted in the plasmolysis of the sieve tube. Figure 4.3 B shows the thick-walled sieve tube (solid circle), which was plasmolysed during what is interpreted as an extensive probe. In contrast, plasmolysis of thick-walled sieve tubes was not often seen in Betta-*Dn1* leaf blade bundles, even when stylet probes (Figs. 4.2 J, L and 4.3A) obliterated vascular parenchyma cells adjacent to the thick-walled sieve tubes.

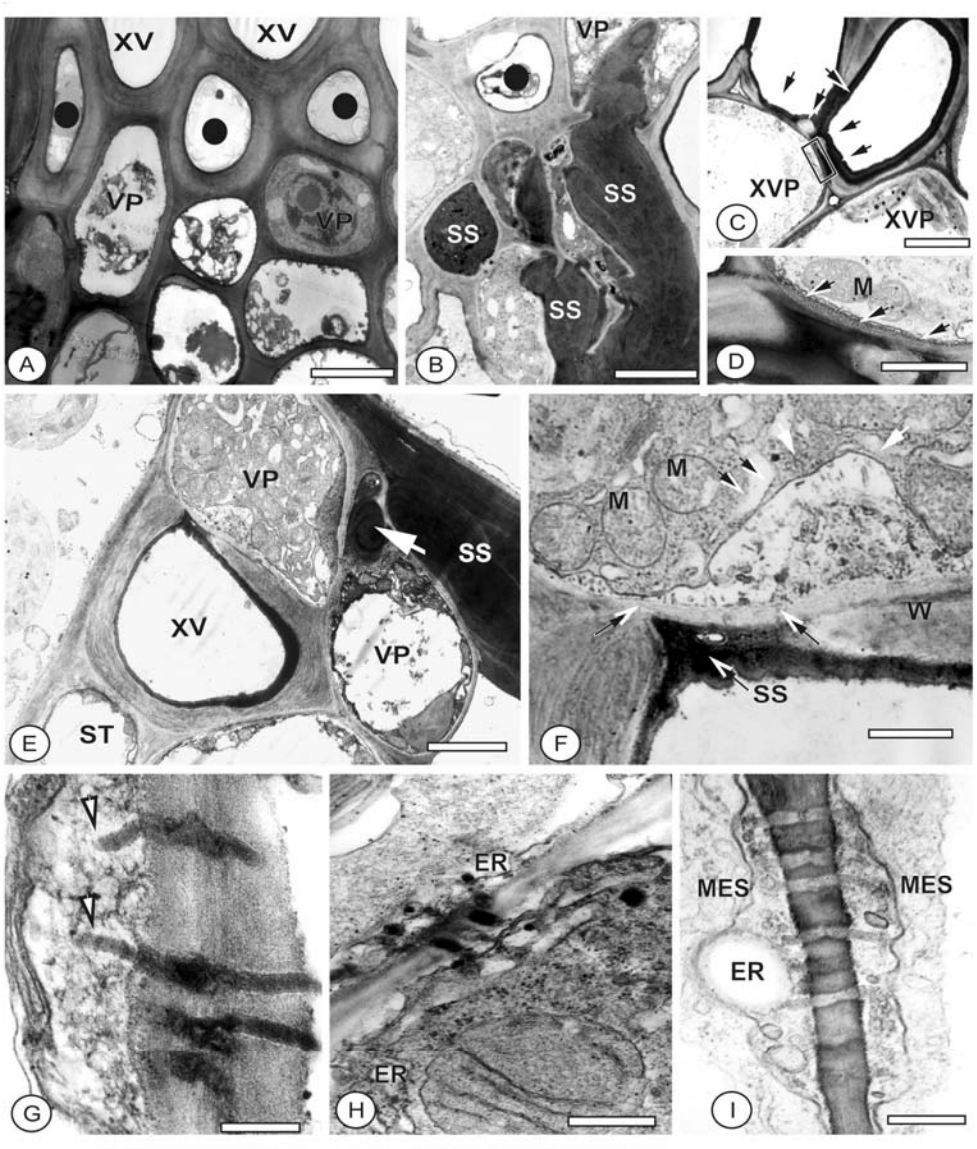

Fig. 4.3 Ultrastructural damage in part of phloem, xylem and plasmodesmatal in wheat cultivars

Figure 4.3 (A-I) shows aspects of damage caused by RWA probing in thick-wall sieve tubes and drinking from xylem in Betta and Betta-*Dn1*.
Fig. A shows detail of undamaged thick-walled sieve tubes (solid dots) in a Betta-*Dn1* leaf blade which is adjacent to thin-walled sieve tubes in which the content has been disrupted and plasmolysed.
Fig. B shows an extensive probing of the phloem tissue in an intermediate vein of a Betta leaf blade. Both thin- and thick-walled sieve tubes were probed. Saliva (SS) has obliterated several cells.
Fig. C shows part of a group of metaxylem vessels from an intermediate bundle of a Betta cultivar. Note that saliva completely encases the inner walls of the xylem elements and occludes pit membranes between the vessels (arrowheads) as well as the pit membrane between the lowermost vessel and its associated vascular parenchyma cell (paired arrowheads).
Fig. D shows detail of pit membrane in rectangle in Fig. C. Note that the saliva has not crossed the fenestrated wall structure associated with the xylem parenchyma, and that saliva completely blocks the pit membrane on the metaxylem vessel side of the wall.
Fig. E shows details of a transverse vein in a Betta leaf blade, which was probed, leaving a stylet sheath (SS). The solitary xylem vessel is ensheathed by saliva, as is the pit membrane between this cell and its associated vascular parenchyma cell (VP). The right-most vascular parenchyma cell was punctured – saliva occupies part of the cell (arrowhead) and the cytoplasm has been disrupted.
Fig. F shows detail of the pit membrane from Fig. E. Saliva (SS) has occluded the pit membrane, and ejecta have crossed the membrane, exuding into the parenchyma cell. The plasma membrane has separated from the cell wall (arrowheads point to plasma membrane). The space between the cell wall and the plasma membrane is occupied by saliva and granular, electron-dense material. W = cell wall.
Fig. G shows detail of plasmodesmatal field between a bundle sheath (right) and vascular parenchyma cell (left) in an intermediate bundle in Betta leaf blade. Ectodesmata (arrowheads) have formed, possibly due to plasmolysis caused by probing.
Fig. H a plasmodesmatal field between two vascular parenchyma in the leaf blade Betta cultivars, the cells shows occlusion of plasmodesmata.
Fig. I shows plasmodesmatal field between two mesophyll cells in a Betta-*Dn1* leaf blade. Plasmolysis has resulted in extrusion of the plasmodesmata (forming ectodesmata); some vesciculation of the cytoplasm has occurred.
Scale bars: A = 5 μm; B = 5 μm; C = 5 μm; D = 500 nm; E = 5 μm; F = 2 μm; G = 500 nm; H = 800 nm; I = 250 nm.

4.3.2.6 Drinking from xylem

It has been argued in Chapters One, Three, and some previously cited references that aphids are capable of drinking from the xylem and feeding from the phloem. In the experiments reported in this chapter, there was no apparent difference when RWA probed the xylem in either Betta or Betta-*Dn1*. The xylem vessel walls in both cultivars were lined with an amorphous, electron-dense layer of saliva. This, as earlier reported in Chapter Three, is characteristic of RWA, and these saliva deposits appear to seal the xylem vessels and occlude pit membrane connections between the vessels and their surrounding xylem parenchyma (Figs. 4.3 C-F). The pit fields between metaxylem vessels, as well as those between the xylem and associated xylem parenchyma, were occluded and plugged with these salivary deposits (Figs. 4.3 C-D).

4.3.2.7 Plasmodesmal fields

Plasmodesmata suffer severe disruption and damage during probing and feeding by the aphids. Figures 4.3 G-I illustrate aspects of disruptions between plasmodesmal fields in both Betta and Betta-*Dn1* cultivars. In the Betta cultivar, plasmodesmata were often blocked and/or occluded with callose (Fig. 4.3 H), and formation of ectodesmata is very common (Fig. 4.3 G). This is possibly as a result of the rapid plasmolysis of the cells in question. Partial disruption of plasmodesma are the common observations in Betta-*Dn1* cultivar, while ectodesmal formation is rare. Fig. 4.3 I shows the

plasmodesmata between two mesophyll cells of a Betta-*Dn1* that are partially plugged and some plasmolysis of cells can be seen.

4.4 Discussion

4.4.1 Stylet-related damage

RWA subsists primarily on the fluid contents of the plant cells. Its principal feeding site is the sieve element, which can be reached via either stomata or puncturing of the epidermal or mesophyll cells. The penetration of the mesophyll cell of wheat during RWA probing has been proposed to be entirely intercellular (Fouché, 1983; Fouché et al., 1984). In the work reported here and based upon TEM investigation, there is strong evidence of intercellular (Figs. 4.2 B-E) as well as intracellular (Figs. 4.2 A, C) probing of mesophyll tissue.

In the susceptible Betta cultivar, both inter- and intracellular cellular probing resulted in severe cellular damage to the mesophyll cells. The mesophyll cells that were adjacent to salivary material deposits were equally damaged, as plasmolysis, disruption of the cytoplasm and its organelles, as well as cell wall damage ensue (Figs. 4.2 B-D). The punctured cells containing salivary deposits displayed severe damage to the cell cytoplasm and its organelles (Fig. 4.2 A). Interestingly, this did not appear to be the case and was not obvious in the resistant Betta-*Dn1* cultivar, where mesophyll cells that were probed

intracellularly, or those adjacent to salivary sheath deposits (Fig. 4.2 E), or even those which had been punctured, do not show signs of damage to cytoplasm or constituent organelles.

4.4.2 Phloem damage

After probing the mesophyll, the aphids reach the vascular bundles, where penetration appears to be entirely intracellular. The sequence of probing begins with the bundle sheath, followed by the vascular parenchyma, xylem elements and sieve element-companion cell (SE-CC) complex (Evert et al., 1973; Fouché et al., 1984; Matsiliza and Botha, 2002).

In Chapter Three it was suggested that the sealing of xylem by RWA during the process of blowing or clearing of their stylets, as well as severe phloem damage they caused during feeding, could lead to isolation of xylem and phloem conducting elements, and that this could induce leaf roll and streaking symptoms (Saheed et al., 2007a). These observations are further confirmed in the results reported in this chapter, where severe plasmolysis, damage to the plasma membrane, cytoplasmic contents, cell walls and organelles in intracellularly-probed cells, as well as to cells adjacent to salivary material deposition and to those containing saliva, was more evident in the susceptible cultivar (Figs. 4.2G-I) than in the resistant cultivar, which showed reduced damage to cell walls, cytoplasm and organelles (Figs. 4.2 F, J-L).

Damage to thin-walled sieve tubes of the phloem due to multiple probing and feeding was severe in susceptible Betta cultivar (Figs. 4.2 H-I). This is not surprising, because thin-walled sieve tubes and their associated companion cells in Poaceae family are the prime target for feeding by aphids (Botha and Matsiliza, 2004). In contrast, this damage appears to be not as severe in resistant Betta-*Dn1* (Figs. 4.2 J-L) where the phloem shows little or no damage. However, damage to thick-walled sieve tubes appears to be minimal in both susceptible and resistant cultivars. This is also not unexpected, as thick-walled sieve tubes are generally symplasmically isolated from the thin-walled sieve tube-companion cell complexes in many grasses (Botha, 2005) and they are not the preferred site of feeding by the aphids (Matsiliza and Botha, 2002). Some thick-walled sieve tubes show plasmolysis in susceptible Betta cultivar (Fig. 4.3 B), but not so in the resistant Betta-*Dn1* (Figs. 4.2 J, L and 4.3 A).

4.4.3 Saliva damage

Copious saliva was found in the entire probing and feeding pathway, including sieve elements in both the susceptible and resistant cultivars. However, all cells probed with salivary material deposit, or adjacent to salivary deposit, show significant damage in susceptible Betta cultivar, whereas similar cells in Betta-*Dn1* cultivars do not show as severe damage as seen in Betta. It appeared as if cell disruption was in a resistant cultivar, an isolated event in many instances, with individual cells, rather than whole groups of cells, destroyed during probing and feeding.

The results presented in this chapter suggest that reduced, cell and organelle damage (resistance to cell damage) occurs in the resistant Betta -*Dn1* plants. This resistance to damage might be because of the accumulation of higher levels of PR-proteins, peroxidase and / or defence-related products reportedly found in the cell apoplast of some resistant wheat cultivars, including Betta-*Dn1* (Van der Westhuizen and Pretorius, 1996; Van der Westhuizen et al., 1998a). Peroxidase has been shown to be involved in direct defence, such as lignification, formation of intermolecular crosslinking and suberin formation, as well as the production of reactive oxygen species associated with eliciting, and signalling events inducing defence mechanism (Bowles, 1990; Mehdy, 1994). Therefore, by limiting penetration of salivary components, as part of hypersensitive response (HR) (Moloi and van der Westhuizen, 2006), peroxidase may confer some protection to probed cells in the resistant cultivar, thereby preventing subsequent damage to the cells and their organelles.

4.4.4 Xylem-related damage

Aphids in general have been previously reported to drink periodically from the xylem (Tjallingii, 1994) while they feed from the phloem. In Chapter Three (Saheed et al., 2007a), RWA and Bird cherry-oat aphid (BCA) have been shown tap the xylem in barley in order to drink water, and in the process that they eject salivary material. RWA have been shown to deposit watery, smooth saliva that, in all cases, completely coats the inner face of the walls of

xylem vessels. The extensive coating supports the argument for "watery" saliva. This is not the case when BCA taps the xylem. Here, the aphids eject a more granular salivary matrix, which does not appear to occlude the pit membrane or pit apertures between metaxylem vessels and adjacent xylem parenchyma. The occlusion through deposition of copious saliva, a characteristic of RWA, was observed within the metaxylem vessels of all vein classes within Betta and Betta-*Dn1* plants. It is unlikely that gelling saliva would spread as evenly or effectively to seal up the pit membranes, as is the case when RWA taps the xylem for water. Evidence for complete blocking of pit membranes by saliva (see Figs. 4.3 D, F) lends overwhelming support for the suggestion that streaking and wilting in Betta and Betta-*Dn1* may be caused through the prevention of water and nutrient flow to parenchymatous elements from the xylem when RWA feeds on leaves of the plant. Symplasmic transport of assimilates could also be disrupted due to occlusion of plasmodesmata and callose deposition within sieve tubes. Ectodesmata formation is usually associated with plasmolysis (Fig. 4.3 G) and it is generally taken as an indicator of severe damage to plasmodesmata. This often occurred in Betta cultivars, but not in Betta-*Dn1*. Limited plasmodesmatal disruptions (Fig. 4.3 L), by partial or no occlusion by callose, were commonly observed in resistant cultivar.

The apparent lack of occlusion of plasmodesmata in the Betta-*Dn1* cultivar could perhaps be attributed to β-1, 3-glucanase (an enzyme that degrades

callose), which has been shown to accumulate to a greater extent in the resistant cultivars (Van der Westhuizen et al., 1998a). Despite the evidence presented here, some assimilates could still be symplasmically trafficked in adjacent cells despite significant redirection to the aphid's gut in resistant Betta-*Dn1* cultivar, but that reduction of phloem related transport could be greater in the non-resistant Betta cultivar.

In conclusion, the extensive cellular and subcellular damage caused by RWA to mesophyll and vascular tissues of Betta and to a lesser extent in Betta-*Dn1* leaves is confirmed by the observed morphological symptoms. The disruption of the phloem and xylem transport systems by aggressive feeding and probing of the RWA suggests a marked loss in the physiological function of these transport systems in the susceptible Betta, in comparison to the resistant Betta-*Dn1,* where this appears reduced. This reduction in observed damage in resistant Betta-*Dn1* could perhaps be a combination of a reduced number of feeding aphids (antibiosis) and enhanced cell wall strengthening effects due to the activity of PR-proteins such as peroxidase.

Tapping the xylem for water, as a result of the aphid's habit of egesting or regurgitating watery saliva has an additional, fatal effect. The consequence of xylem vessels sealing is loss of functionality, which will result in the cessation of water offloading to vascular parenchyma cells and as a result reduction in the available water supply well to the phloem, where loading will be adversely

affected. The decrease in water availability, associated nutrient recycling and phloem loading leads to water and nutrient stress in the plant and decreases assimilate flow. The visible result is leaf streaking, necrosis and leaf curling. These are manifestations of severe stress that do not preclude any potential hypersensitivity response.

5 Chapter 5: Wound callose formation in response to RWA and BCA feeding in barley leaves

Preamble

The data presented in this chapter are in most part, published as part of data presented in Saheed et al., 2007c; submitted to Physiologia Plantarum.

5.1 Introduction

Wound callose formation and deposition have been reported to be a rapid response, which may occur within a minute of cell wounding (Gunning and Steer, 1996; Radford et al., 1998; Nakashima et al., 2003). Callose is deposited between the cell wall and the plasmamembrane and it is associated with sieve plates and pore-plasmodesma between the companion cells and associated sieve tubes. In addition, locally deposited callose in sieve pores and plasmodesmata could occur either as a response to wounding or some other physiological stress, and it could be deposited as plugs or plates (normally referred to as papillae) in response to pathogen infection (Stone and Clarke, 1992; Donofrio and Delaney, 2001). The formation of callose during wounding has been shown to lead to a reduced rate of transport in phloem tissues of wheat leaves (Botha and Matsiliza, 2004). The rapid formation of callose in sieve pores of the phloem has for years been considered to be an effective means by which plants respond to wounding. Callose, therefore,

helps to seal the pores in damaged phloem tissue thereby preventing the loss of assimilates (Sjölund, 1997).

Recent studies have shown that some aphid species cause wound callose formation (Botha and Matsiliza, 2004; De Wet and Botha, 2007). These aphids include RWA and the grass aphid – *Sitobion yakini* (Eastop). In addition, these aphid species have been reported to cause wound callose formation in wheat cultivars known to be susceptible to RWA. However, no evidence of wound callose formation was observed in a resistant cultivar which contains resistance gene against RWA when been fed upon by *S. yakini* (de Wet and Botha, 2007). Aphids, which damage the phloem tissue during penetration and subsequent feeding, can feed from sieve tubes of the phloem for prolonged periods. It has been suggested that aphids can prevent wounding reactions, such as callose formation and protein plugging, by sealing the stylet puncture site with sheath saliva and that the watery saliva injected into the sieve elements may interact with sap constituents in some unexplained way (Will and Van Bel, 2006; Will et al., 2007).

Wound callose formation suggests that its formation may at least partly explain the severe symptoms caused by RWA infestation. This notion finds support from the results of the ultrastructural study (Chapter 3), where the effects of feeding by RWA and BCA were compared and in Saheed et al., (2007a). BCA can co-occur with RWA on wheat and other cereals, but on its

own does not appear to cause any visible damage symptoms (Messina et al., 2002), especially at low levels of infestation. This is unlike RWA, which is capable of causing distinct symptoms even as a result of a single aphid feeding (Walters et al., 1980; Saheed et al., 2007a). In Chapter 3, it was shown that the differences in the salivary egestion into xylem elements by RWA and BCA, together with effects on phloem tissue, cause distinct differences. RWA causes formation of callose in sieve pores and also in plasmodesmata and pore plasmodesmata between companion cells and sieve tubes, whereas in BCA-infested tissue, only sieve pores appeared to contain callose (Chapter 3; and in Saheed et al., 2007a). However, a study has shown that β-1, 3 glucanase (an enzyme that is suggested to digest callose) accumulates in higher levels in resistant cultivars when compared with the susceptible type during RWA infestation of wheat (Van der Westhuizen et al., 2002), and during BCA infestation of barley cultivars (Forslund et al., 2000).

In this chapter, I report on my experiments to further elucidate the underlining mechanism of aphid feeding and the response by plants to the damage caused. Understanding the synthesis and distribution pattern of wound callose formation (the response to wounding) by these two aphid species (RWA and BCA) was considered important. Using a susceptible barley cultivar (Clipper) in this study presented a unique opportunity to evaluate wound callose induction with little or no masking and/or interference due to induced β-1, 3 glucanase activity. In this chapter, the functional response of plant to aphid

feeding through formation of wound callose was investigated and the results of these investigations are presented here. The aim was to explore the pattern of wound callose formation during short- and long-term feeding by the two aphid species using a callose-specific fluorochrome. In addition, the investigation of the persistence, or possible removal of the wound callose after it has been deposited were carried out; and lastly, the examination of the movement of the wound callose signals, whether up- or down-stream of the assimilate flow was carried out, and the results presented here.

5.2 Experimental overview

Given that the formation and distribution patterns of callose in aphid-fed leaves would indicate the level of wounding and its spread as a result of signal transmission, it was felt that callose distribution could best be investigated in the following ways:

(a) Wound callose formation and distribution during short- as well as long-term feeding by RWA and BCA, using a callose-specific fluorochrome (aniline blue fluorochrome; details of this fluorochrome are recorded in the 'Materials and Methods' section).

(b) Wound callose formation by higher populations of BCA feeding in short-term as well as long-term experiments.

(c) Persistence of deposited wound callose after aphid removal. Here, experiments involved allowing feeding for a pre-determined time and after the removal of the aphids, leaf recovery was examined at fixed time intervals.

(d) Signal transport in the phloem needed to be investigated downstream and upstream of aphid infestation in mature source leaves, and in immature sink leaves.

5.3 Results

5.3.1 Distribution of wound callose in control leaf tissues

The development of wound callose associated with the damage caused by scraping of the leaves was effectively minimised by scraping the leaf surface under Ca^{+} free MES buffer at pH 7.2. Figs. 5.1 A-D show part of the leaf blade from control, uninfested leaf tissue after 72h, which is the time used for short-term response. Similar response results were observed after 24 and 48h (data not shown). Fig. 5.1 A and C illustrate part of the leaf blade occupied by the clipcage in source leaves, while Figs. 5.1 B and D show similar parts in sink leaves. There is little or no callose-associated fluorescence, except that which was associated with sieve plates and pore-plasmodesmal units (PPUs) in small (SV), intermediate (IV) vascular bundles and in cross veins (XV). As expected, callose-associated fluorescence did not show any marked increase (data not shown), even in the 14d control leaves.

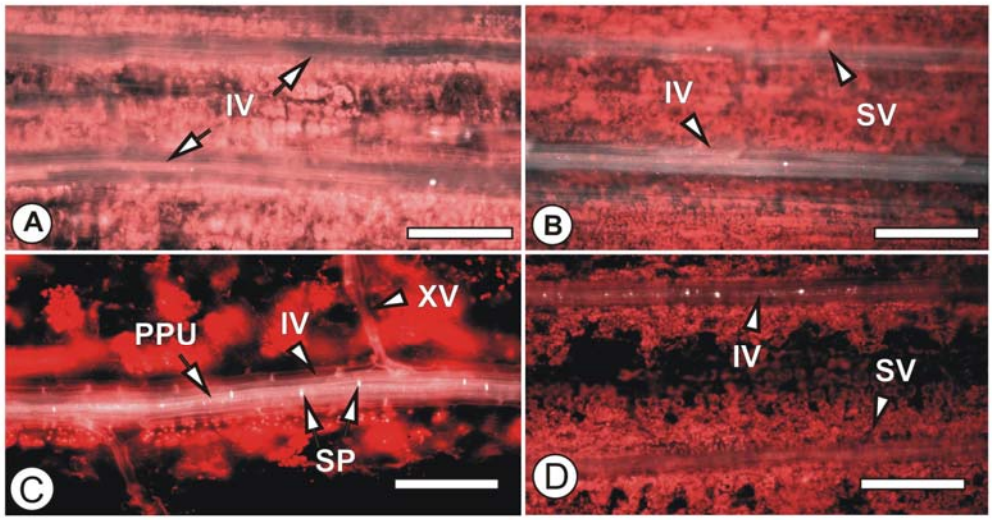

Fig. 5.1 Callose formation in control barley leaves

Figure 5.1 (A-D) shows distribution of callose formed as a result of scraping in control barley leaves. The portions occupied by the aphid cage in source leaves are shown in **Figs. A and C**, while similar portions in sink leaves are shown in **Figs. B and D.** Note that there is no evidence of wound callose formation in intermediate vein (IV), small vein (SV) as well as cross vein (XV) in these leaves, except callose associated with sieve plates (SP) and pore plasmodesma units (PPUs).
Scale bars: A = 200 µm; B = 200 µm; C = 100 µm; D = 200 µm.

5.3.2 Formation of wound callose in BCA-infested leaves

Interestingly, infestation of sink as well as source leaves by BCA for short-term (24 - 72h) does not result in any noticeable formation of wound callose in all classes of longitudinal vein orders or in cross veins. Limited wound callose formation was, however, observed along longitudinal veins after long-term (14d) feeding, but little evidence of formation was found in cross veins. Figure 5.2 A-D show parts of the leaves where the aphids were feeding in source (Figs. 5.2 A and C) and sink (Figs. 5.2 B and D) leaves. No wound callose-associated fluorescence was observed in all the small (SV), intermediate (IV) and cross (XV) veins. Sieve plates were, however, associated with callose as well as PPUs. These results were similar to those observed in control, uninfested leaves (Fig. 5.1). Little or no formation of wound callose was observed even after 7d (data not shown) of BCA feeding. However, after 14d (long-term) of continuous feeding by BCA, some wound callose was observed. It should be noted here that sink leaves would have transit into source leaves within 14d of growth. Figs. 5.2 E-H illustrate aspects of wound callose-associated fluorescence developed in source leaves, but examined after 14d (long-term) of BCA feeding. The distribution of wound callose in longitudinal veins where the aphids were allowed to feed shows that the formation of wound callose in these tissues is limited. Smaller deposits, or punctate spots, mark callose deposition associated with PPUs and plasmodesmatal fields.

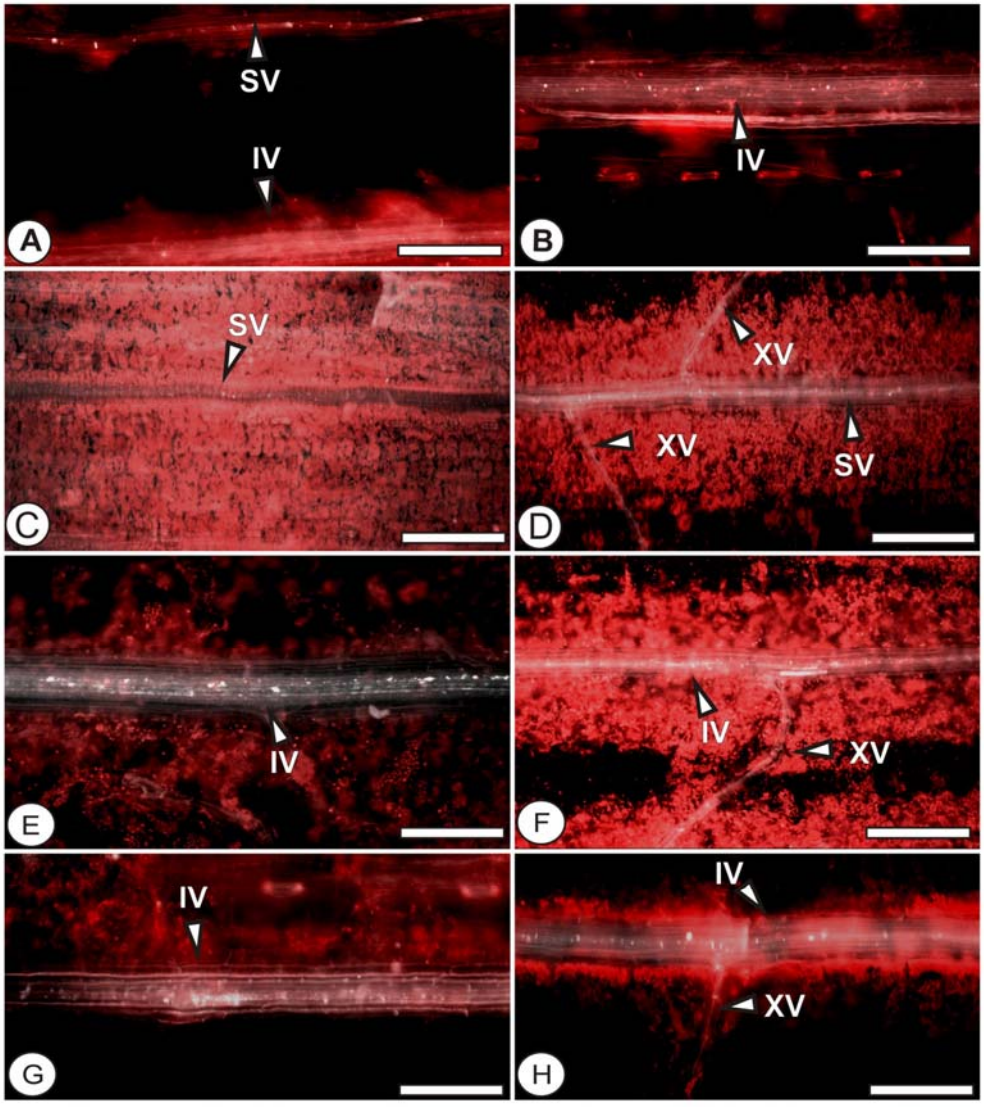

Fig. 5.2 Wound callose formation during short- and long-term feeding by BCA in barley leaves

Figure 5.2 (A-H) illustrates aspects of wound callose formation by BCA, after 72h (short-term) as well as after 14d (long-term) of feeding on barley leaves.
Figs. A and C show the portion of the leaves where the aphids were feeding after 72h in the source leaves, while similar portion in sink leaves are shown in **Figs. B and D.** There is no wound callose formation in the tissues of these leaves, except those associated with sieve plate pores and PPU. This is similar to the formation found in the control, uninfested leaves (see Fig. 5.1).
Figs. E-H show aspects of wound callose formation after 14d of feeding (long-term feeding). The tissues of these leaves show limited wound callose in longitudinal intermediate veins (IV), as well as cross vein in the area of aphid feeding, and within sieve plates and PPUs.
Scale bars: A = 200 μm; B = 100 μm; C = 200 μm; D = 200 μm; E = 200 μm; F = 200 μm; G = 100 μm; H = 100 μm.

5.3.3 Formation of wound callose in RWA-infested leaves

Formation of wound callose starts appearing within 24h of infestation by RWA, and these formations become more evident by 72h of feeding. Figure 5.3 (A-E) illustrates the 72h (short-term) pattern of wound callose distribution due to RWA feeding. Wound callose formation occurs in longitudinal intermediate veins (IV) as well as cross vein (XV) in the portion of the leaves where the aphids fed from after 72h of continuous feeding in source (Figs. 5.3 A and C) and sink (Figs. 5.3 B and E) leaves. The aphid stylet track (ST) was strongly reactive and showed evidence of callose (Figs. 5.3 C and D). These results show that RWA induces severe wound callose formation within 72h of feeding, whereas BCA does not induce wound callose formation within the same period (see Figs. 5.2 A-D for comparison). The experiments indicate that there is a progressive increase in wound callose formation, from the start of RWA feeding through the 7-14d period. Long-term (14d) feeding-associated wound callose in RWA-infested leaves was illustrated in Figs. 5.3 (F-L). There is extensive wound callose formation in the phloem of longitudinal intermediate veins (Figs. 5.3 F, G and J), as well as in other classes of vein, including cross veins (Figs. 5.3 K-L) in the infested leaves. The micrographs clearly demonstrate severe damage to the phloem system as a result of long-term RWA feeding. As mentioned, stylet tracks (ST) or stylet track terminations were also heavily callosed (Figs. 5.3 H-I), as were sieve plates and pore plasmodesmal units (Figs. 5.3 K-L).

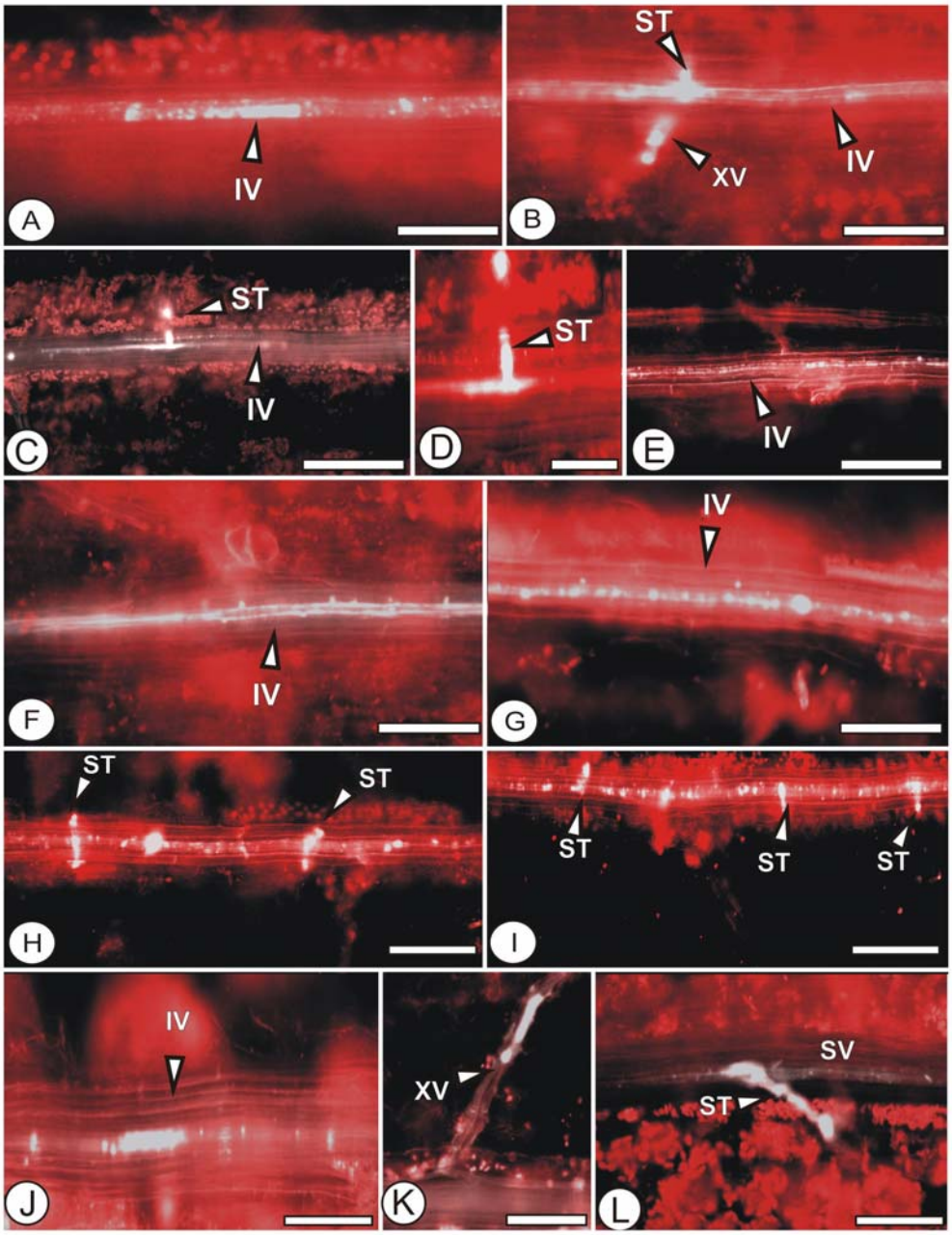

Fig. 5.3 Wound callose formation during short- and long-term feeding by RWA in barley leaves

Figure 5.3 (A-L) illustrate patterns of callose formation in RWA infested leaves after 72h and 14d of intensive feeding. The portions of leaves where the aphids were feeding after 72h (short-term) are illustrated in **Figs. A-E.** Note the extensive formation of wound callose in the longitudinal, intermediate veins of both source (Figs. A and C) and sink leaves (Figs. B and E), as wound callose completely blocked the aphid stylet track (ST). Fig. D shows the high magnification of the blocked stylet track from Fig. C.
Figs. F-L illustrate wound callose formation after 14d (long-term) in RWA-infested leaves. Sites where RWA was feeding can be seen in these images where stylet tracks (ST) show strong callose-associated fluorescence. Note the severe formation of wound callose in vascular parenchyma and phloem elements along longitudinal intermediate veins (Figs. F-J) as well as cross veins (Figs. K-L). This is a sign of extensive damage to the plant. Many stylet tracks (ST) in these cases are completely occluded with callose (Figs. H-I) heavily deposited in these veins.
Scale bars: A = 100 μm; B = 100 μm; C = 100 μm; D = 50 μm; E = 100 μm; F = 100 μm; G = 100 μm; H = 100 μm; I = 100 μm; J = 50 μm; K = 50 μm; L = 50 μm.

5.3.4 Wound callose formation resulting from high BCA populations

In contrast to smaller population feeding in barley leaves, feeding by higher populations of BCA resulted in formation of wound callose along longitudinal as well as cross veins. The two different experimental procedures (see Chapter 2) show that wound callose was deposited by the feeding aphids and stylet tracks were callosed. In the first procedure, wound callose is deposited and located mainly in sieve tubes after prolonged feeding over a 21d period (Figs. 5.4 A-B). The formation of wound callose along longitudinal veins increases between 14d and 21d and several stylet tracks were callosed. The second procedure, involving feeding by 50 adult aphids over 72h, shows formation of wound callose. This is illustrated in Figs. 5.4 C-D, where significant formation along longitudinal intermediate veins is evident. Stylet tracks were also callosed here.

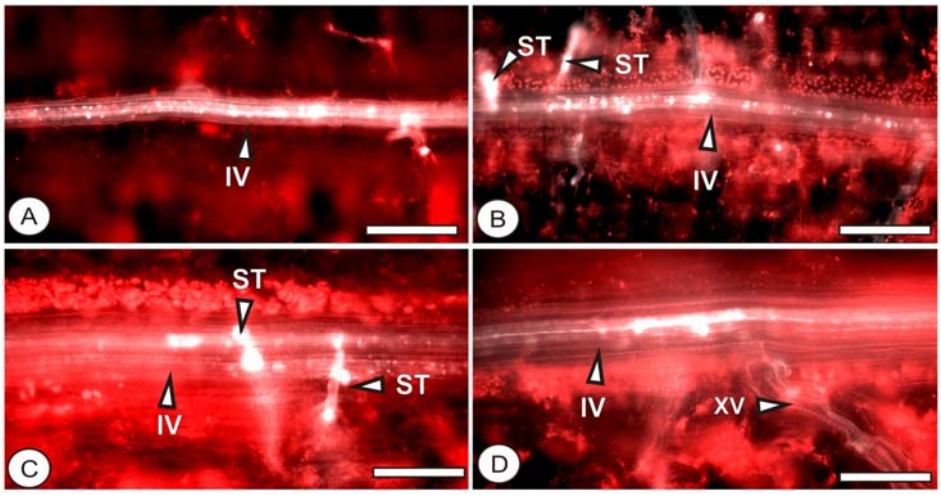

Fig. 5.4 Wound callose formation by higher BCA populations feeding on barley leaves

Figure 5.4 (A-D) illustrates patterns of wound callose formation after prolonged feeding by BCA (21d) and after 72h of higher aphids population feeding.
Figs. A-B shows extensive formation of wound-induced callose in longitudinal intermediate veins (IV) after 21d of feeding.
Figs. C-D shows wound callose deposited in longitudinal intermediate veins and stylet tracks after 72h of feeding by higher aphid populations. Note that the sieve plates and pore-plasmodesmal units are associated with wound callose; stylet tracks (ST) are extensively callosed.
Scale bars: A = 200 µm; B = 200 µm; C = 100 µm; D = 100 µm.

5.3.5 Wound callose persistence after aphid feeding

The response of the fed leaves and formation of wound-induced callose during the aphids' feeding appears to be a long-term effect. It is important to mention here that what is interpreted as feeding-related symptoms continue to develop after the removal of the aphids. Observations of wound callose deposited up to 120h after the aphids have been removed (at 24h intervals) revealed that wound callose formations persisted in all classes of longitudinal vein orders, as well as in cross veins in the portion of leaves where the aphids fed from for up to 96h, after the removal of the aphids. There was no obvious reduction in the intensity of the fluorescence associated with the callose deposited over the time interval investigated. Figure 5.5 illustrates the formation of wound callose in intermediate veins (Figs. 5.5 A and C) and cross vein (Fig. 5.5 B) in source leaves 0h after the removal of the aphids. Callose distribution 120h after aphid removal is shown in longitudinal veins (Figs. 5.5 D and F), as well as cross veins (Fig. 5.5 E) of source leaves. Both source and sink leaves appear to exhibit similar callose formations at 24, 48, 72 and 96h intervals (data not shown). No difference was observed in the intensity or distribution of callose in phloem tissue in either source or sink leaves.

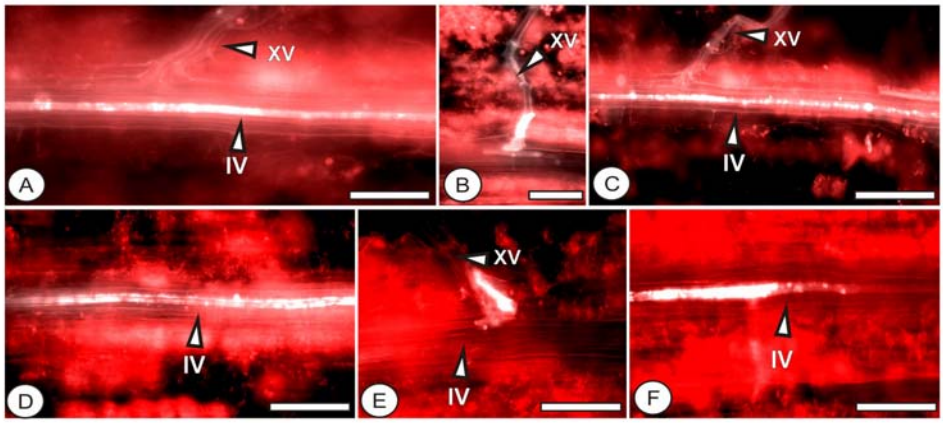

Fig. 5.5 Wound callose persistence after feeding aphids' removal in barley leaves

Figure 5.5 (A-F) shows formations of wound callose 0h and 120h after aphid removal.
Figs. A-C show formation of wound callose in intermediate longitudinal veins (A and C) and cross vein (B) 0h after the aphids were removed from the leaves.
Figs. D-E show details of the wound callose formation in intermediate veins (D and F) as well as cross vein (E) 120h after the feeding aphids were removed. Note that wound callose was deposited in sieve plates and pore plasmodesmal units, and that there seems to be no observable reduction in the amount of callose deposited.
Scale bars: A = 100 µm; B = 50 µm; C = 100 µm; D = 100 µm; E = 50 µm; F = 100 µm.

5.3.6 Transport of the wound callose signal

The movement of wound callose-related formation, and presumably the signal from part of the leaves where the aphids were feeding, to the portions of the leaves either below or above the feeding site in source as well as sink leaves, showed an interesting pattern. Figure 5.6 illustrates part of the leaf below and above the point of aphid feeding in source and sink leaves of the control and infested plants. It is clear that more wound callose occurs along longitudinal intermediate veins as well as cross vein in the portion of the leaves above the feeding site (Fig. 5.6 F) in sink leaves than in the portion below the feeding site (Fig. 5.6 H). Interestingly, the reverse was true in source leaves, where more wound callose appeared below the feeding site (Fig. 5.6 G) than above the site of aphid feeding (Fig. 5.6 E). This pattern occurred in all treatments, in both short- and long-term feeding experiments. However, no such formation was observed in similar leaf regions of the control experiments (Figs. 5.6 A-D), nor in short- or long-term feeding treatments involving low-level population of BCA infestations.

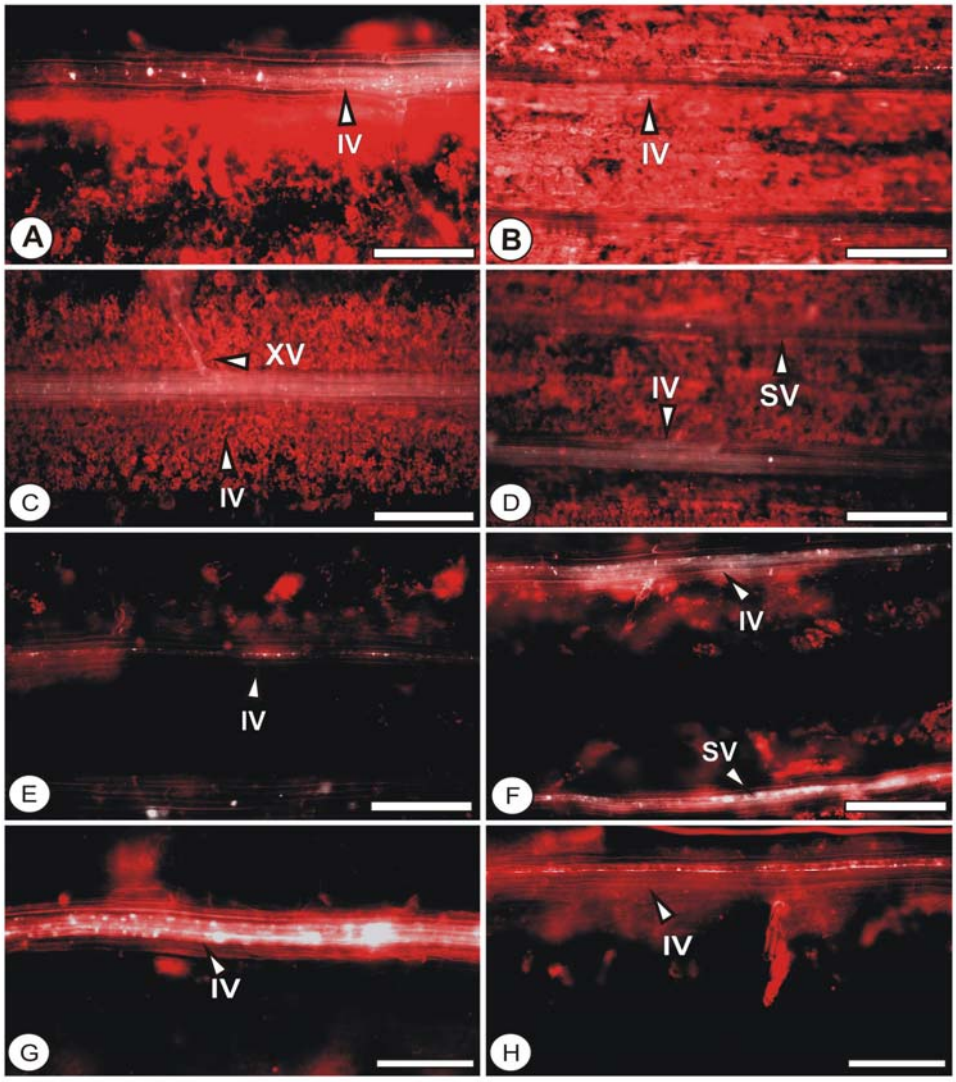

Fig. 5.6 Transport of wound callose signal in source and sink leaves of barley

Figure 5.6 shows the pattern of wound callose formation in leaf tissue above and below the area of aphids feeding in both source and sink leaves of control, as well as aphid infested leaves.

Fig. A shows part of the leaf blade above the aphid cage in a source leaf of the control plant, while a similar area in a sink leaf is shown in **Fig. B.** The areas below it are as shown in **Figs. C and D** for source and sink leaves respectively. Note that there is little or no evidence of wound callose formation in intermediate vein (IV) and cross vein (XV) in these non-infested leaves except callose associated with sieve plates and PPUs.

Fig. E shows the portion of the leaf above the site of aphid feeding in a source leaf. Note that there is evidence of less wound callose formation in the intermediate vein (IV) shown compared to similar portion of the sink leaf (**Fig. F**) where severe formation of callose occurs in the small vein below. There is more wound callose formation below the site of aphid feeding in source leaves as evident in **Fig. G,** compared to the portion above it (Fig. E). The reverse is the case in sink leaves where the portion below the feeding site **(Fig. H**) shows less callose formation when compared to the formation found in portion above the site of feeding (Fig. F).

Scale bars: A = 100 μm; B = 200 μm; C = 100 μm; D = 200 μm; E = 200 μm; F = 200 μm; G = 100 μm; H = 100 μm.

5.4 Discussion

The responses of plants to aphid infestation have been suggested to be species-specific, which is the case for most of the aphid species (Gill and Metcalf, 1977). This is because the signals that initiate these responses are aphid-derived, especially from the saliva ejected during probing and feeding activities. The response of the barley cultivar to feeding by RWA and BCA through wound callose formation varies, and this response may be induced by the saliva-associated signals from these aphids. In RWA-infested source and sink leaves, wound callose occurred along longitudinal veins, cross veins and stylet tracks, within 24h of infestation, during short-term feeding experiments. Callose formation became extensive after 72h (Figs. 5.3 A-E) of infestation. In contrast, feeding by BCA in similar leaves for up to 72h did not lead to any visible wound callose formation (Figs. 5.2 A-D), despite the fact that wound callose deposition seems to be a rapid response (Radford et al., 1998; Nakashima et al., 2003). Interestingly, the formation found in BCA-infested leaves was similar to that observed in control tissue (Figs. 5.1 A-D). The results presented in this chapter confirm earlier reports of wound callose being induced by RWA in wheat (Botha and Matsiliza, 2004) and lend support to the different deposition pattern of callose observed in various cells at the ultrastructural level, caused by the two aphid species in the same plant (Chapter 3; Saheed et al., 2007a).

Long-term feeding for 14d resulted in extensive wound callose formation in longitudinal and cross veins in leaves infested with RWA. Damage to sieve elements commenced as early as 24h after infestation, with the intensity of the callose formation increasing through to 14d (Figs. 5.3 F-L). In contrast, little wound callose formation was observed after 14d of extensive feeding by BCA (Figs. 5.2 E-H). Stylet tracks were always associated with callose, suggesting that the saliva itself must triggers callose formation. Formation of wound callose in all vein classes,as well as stylet tracks, is evident only when leaves are exposed to a higher BCA population, which was confirmed in extended long-term feeding (21d experiments; Figs. 5.4 A-B) and short-term feeding (feeding for 96h; Figs. 5.4 C-D) experiments.

The extensive formation of wound callose by both aphid species in the susceptible barley cultivar reported in this study confirms the results of Forslund et al. (2000) and Van der Westhuizen et al. (2002). The two studies have reported that β-1, 3 glucanase (an enzyme that seems to digest and/or regulate callose formation) selectively accumulates in higher quantities in the resistant barley and wheat cultivars than in their susceptible counterparts. It was therefore entirely expected that wound callose would develop in the susceptible barley cv Clipper used in this study, either earlier and by smaller feeding populations as it occurs in RWA-infested leaves, or only with higher feeding population, as reported for BCA-infested plants. Of importance was the observation that wounding – as evidenced by callose formation – appears

to be a sustained or possibly permanent effect. No evidence of rapid recovery (removal of callose) was seen, as wound callose formation persisted for up to 120h after the removal of the feeding aphids (see Figs. 5.5 A-F). This further justifies the continuous development of the feeding symptoms observed after the aphids were removed. Progressive spread of the wound callose suggests that once triggered, the wounding response to feeding continues for an indeterminate period and that wound callose, once deposited as a result of aphid feeding, could be an almost permanent process.

Blockage of sieve plate pores, pore plasmodesmata and plasmodesmata themselves must result in a marked decrease and possibly cessation of transport of assimilates via the phloem. This will lead to other symptomatic expressions and eventual death of the plant. In Chapter 3 and in Saheed et al. (2007a), it was reported that RWA-infested barley expressed symptoms of chlorosis and necrosis, while BCA-infested leaves looked healthy within the same period. It was suggested that the differences in the damage to ultrastructures might be responsible for the differences in the observed symptoms. The results presented in this chapter lend strong support to the ultrastructural investigation, where TEM evidence of severely damaged phloem and parenchyma cells must result in the reduction in transport of assimilates, which commence with wound callose formation. However, the observed wound callose formation when higher number of BCA feed on the plant (Fig. 5.4) could be partly responsible for the reported golden yellow

streaking symptoms that occur during heavy infestation of crops by BCA (Agronomy Guide, 2002; UCIPM, 2002).

Transport of assimilates in plants are known to follow classical source to sink pattern; basipetal (lamina tip to base) movement occurs in source leaves, while acropetal (lamina base to tip) movement occurs in sink leaves, and leaves of intermediate age are capable of bidirectional transport in some certain instances (see review by Turgeon, 1989). Based on the results of this investigation, it is suggested that the wound callose signals both defence and anti-defence, are transported in the phloem tissue, and are dependent on the direction of assimilate flow. They will move basipetally from source leaves and acropetally in sink leaves. For example, in source leaves, extensive callose formation was observed below the area of feeding (Fig. 5.6 G), compared to the area above it (Fig. 5.6 E). The reverse is true for sink leaves, where more callose formation occurs above feeding sites (Fig. 5.6 F) than in the area below (Fig. 5.6 H). Bidirectional transport, however, could account for some levels of wound callose formation found in areas above the feeding site in source leaves and the area below it in sink leaves.

In conclusion, it is possible that wound callose formation is aphid species-specific in the barley cultivar studied. The RWA is the more aggressive feeder with the formation starting within 24h of infestation by small RWA population. Equivalent levels of callose are only attained with BCA when

significantly larger populations (10 RWA vs 50 BCA aphids) feed from the leaves. This is the first known report of wound callose formation by BCA. This suggests that notwithstanding any "wounding" or "response" signals generated in response to BCA feeding, the callose manifestation could well be a pressure-related response (i.e. a drastic drop in phloem), rather than a signal related response. It is therefore suggested that wound callose development might be partly responsible for the symptomatic expressions observed during RWA-infestation, as well as the reported symptoms when a higher population of BCA infest a cereal field, and the absence of such symptoms during low-level infestation of BCA in plants.

6 Chapter 6: Callose genes regulation in response to RWA and BCA feeding in Barley leaves

Preamble

The data presented in this chapter are published as part of data presented in Saheed et al., 2007c; Physiologia Plantarum.

6.1 Introduction

The carbohydrate callose (β-1, 3-glucan) occurs in intercellular connections (Currier, 1957) in plants. Callose is known to be synthesised in response to both biotic and abiotic stress, as well as during plant tissue development (Verma and Hong, 2001). The synthesis is initiated when β-1, 3-glucan synthase is activated subsequent to the disruption of plasma membrane integrity (Kauss, 1985), much as when aphids feed on plant tissues, resulting in the formation of wound-induced callose. However, I have reported that there are differences in the wound callose formation as a result of RWA or BCA feeding on a susceptible barley cultivar (Chapter 5; Saheed et al., 2007c). The observed differences in formation of callose could be as a result of differences either in the biosynthesis or in degradation of the callose.

Callose synthesis has been reported to be carried out by callose synthase complexes (Verma and Hong, 2001). These complexes contain twelve β-1, 3-glucan synthase (*GSL*)-related genes from *Arabidopsis* and these sequences

are similar to that of *FKS1*, a *GSL* which has been identified in yeast (Richmond and Somerville, 2000; Verma and Hong, 2001). It is proposed that the different callose synthases might be active in different tissues during the developmental stages in plants, or could be activated in response to a variety of stresses, such as those induced by feeding aphids. For example, in *Arabidopsis*, one *GSL* gene, *AtGsl5 (GSL5*) is expressed at highest levels in flowers, indicating a role for callose deposition in pollen, but the expression of *AtGsl5* was also strongly elevated in a *mpk4* mutant, which exhibited systemic acquired resistance, elevated β-1,3-glucan synthase activity and increased callose levels (Ostergaard et al., 2002). A role for this gene in wound callose formation and the role for the *GSL* gene products in callose formation in general has been confirmed by transformation with double-stranded RNA interference (dsRNAi) constructs silencing *GSL5*, which inhibited the formation of wound callose and papillary callose (Jacobs et al., 2003). However, in barley, a gene homologous to the yeast *FKS* gene, designated as *HvGSL1*, has been identified. It is member of a family of at least six genes and was linked by biochemical evidence to callose synthesis activity (Li et al., 2003).

The degradation of callose has, in contrast, been poorly investigated. Earlier studies reported that wound callose disappears over the course of days (Currier and Webster, 1964). The enzymes involved in this degradation would be β-1, 3-glucanases. A tobacco mutant deficient in a Class I β-1, 3-glucanase

showed a reduced plasmodesmatal size exclusion limit and enhanced callose deposition (Iglesias and Meins Jr, 2000). In addition, it was shown that the expression of a fibre-specific β-1, 3-glucanase gene, *GhGluc1*, became evident at the time of callose degradation in cotton and this coincides with plasmodesmata reopening during cotton fibre elongation (Ruan et al., 2004). The β-1, 3-glucanases belong to the group of pathogenesis-related (PR)-proteins that have been widely studied in connection with stress-related conditions (Van Loon and Van Strien, 1999; Muthukrishnan et al., 2001). Both RWA (Van der Westhuizen et al., 1998, 2002; Botha et al., 2006) and BCA (Forslund et al., 2000) have been found to induce β-1, 3-glucanases, in wheat and barley respectively. Interestingly, both cases shows that the levels of enzyme activity or β-1, 3-glucanase protein were higher in resistant plant cultivars than the susceptible counterparts (Van der Westhuizen et al., 1998b, 2002; Forslund et al., 2000). This chapter further examines the earlier reported differences in wound callose formation between RWA and BCA at the fluorescence microscopic level. The principal aim is to see whether the observed callose synthesis and deposition is regulated at the gene (transcript) level. Therefore, the expression of genes for callose synthase (enzymes responsible for callose synthesis) as well as β-1, 3-glucanase (the enzyme responsible for callose degradation) was investigated. The results obtained are reported in this chapter.

6.2 Experimental overview

The experiments reported in this chapter involved infesting ten-day-old plants with 20 aphids each of RWA and BCA and covering them with clipcages around the mid-region of expanded second leaves. 24 replicate plants were set up for each treatment; the control plant, as usual, carried an empty cage. The infested leaves and control were harvested at different time intervals (0, 24, 48 and 72h). Total RNA was extracted from the plant materials thus harvested and subjected to RT-PCR as well as quantitative real-time RT-PCR. Details of the procedures are described in Chapter 2.

6.3 Results

6.3.1 Regulation of callose synthase genes at the RNA level

The expression of callose synthase genes by RT-PCR on 1.2% agarose gel are illustrated in Figure 6.1, and subsequent confirmation of the observed products using real-time RT-PCR procedure are illustrated in Figure 6.2. The three Primers that were designed and used in these experiments are callose synthase sequences that were present on the Barley1 GeneChip (Affymetrix; see Table 2.1). The first primer, Contig8428_at is the callose synthase *HvGSL1* identified by Li et al. (2003), it is most similar to callose synthase *TaGSL8* from wheat (Voigt et al., 2006). The identity is 95% at nucleic acid level over the 580 bp partial cDNA representing *TaGSL8* (see Table 6.1). The second primer contig13152_at is again most similar to *TaGSL12* (91% identity over the 527 bp partial cDNA sequence of *TaGSL12*). The third, which is Contig4949_at, is most similar to *TaGSL3* (75% over the 481 bp partial cDNA of *TaGSL3)*. Analysis of similarities at amino acid level between the callose synthase sequences annotated in the rice genome (Yamaguchi et al., 2006) and the barley sequences used in this study revealed that *HvGSL1* is 88% identical to *OsGSL8*, contig4949 is 88% identical to *OsGLS7* and contig13152 is 85% identical to *OsGSL1*. However, among the three barley sequences investigated, only two sequences (*HvGSL1* with similarity to *TaGSL8* and *OsGSL8* and contig13152 with similarity to

TaGSL12 and *OsGSL1*) were expressed in the tissue (Fig. 6.1), and contig4949 was not expressed in the leaf tissue.

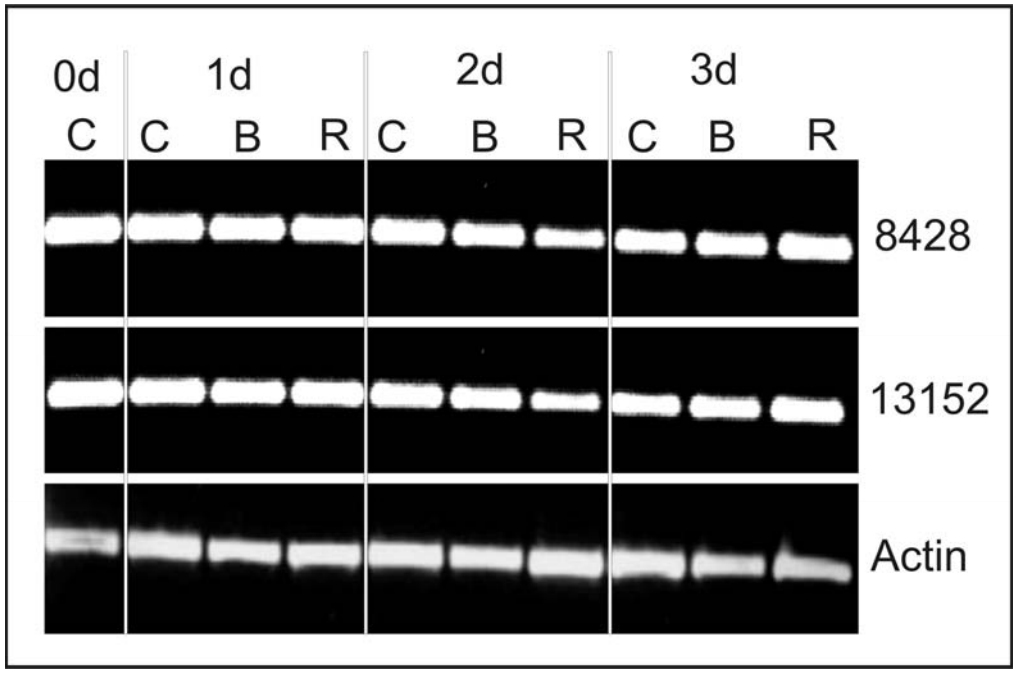

Fig. 6.1 RT-PCR expression of callose synthase genes after aphid infestation of barley leaves

Figure 6.1 illustrates callose synthase genes expression after 0, 1, 2 and 3 days of aphid infestation. Control plants were marked with (C), while BCA-infested samples were marked with (B) and RWA-infested with (R). One-step RT-PCR was performed on 30 ng RNA extracted from barley leaves and primers specific for callose synthases contig8428 and contig13152 were used. Actin serves as loading control.

None of the two expressed sequences showed any change in their expression upon aphid infestation (Fig. 6.1). The real-time RT-PCR equally shows no change in expression between the two primers upon aphid infestation. Only contig13152 was presented (Fig. 6.2), since contig8428 shows similar expression levels. The differences in the relative transcript levels in the control and aphid infested tissues over the three-day period is not even up to 1x fold, indicating that there is no difference in the expression levels of all the leaf tissues examined.

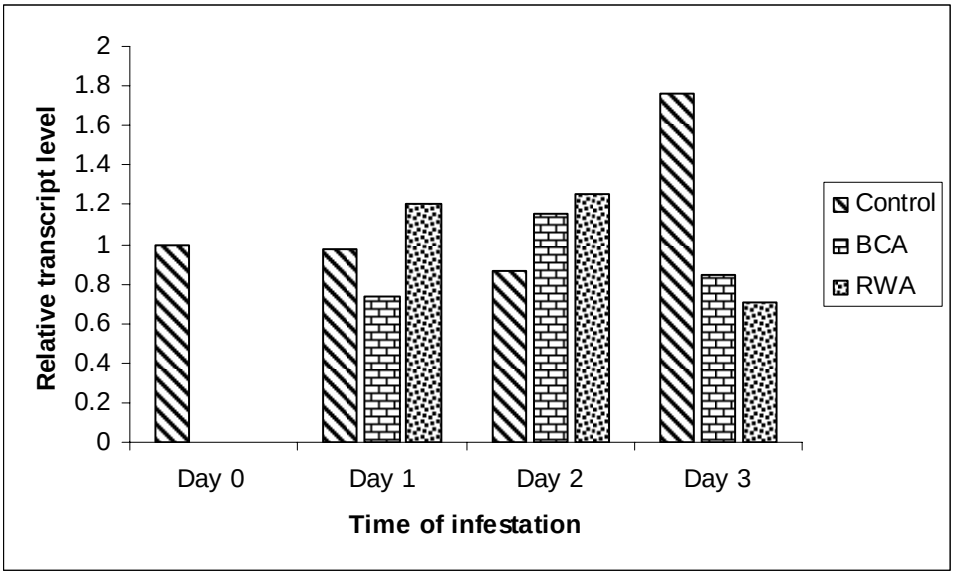

Fig. 6.2 Real time RT-PCR expression of callose synthase genes contig13152_at

Figure 6.2 shows callose synthase genes expression in control barley leaves as well as those infested with BCA and RWA over a period of 0-3 days. Real-time RT-PCR using primer contig13152_at and 30 ng RNA was extracted from barley leaf tissue. The relative transcript levels of contig13152_at were calculated by using actin as the reference gene.

Table 6.1 Similarities of *β*-1, 3-glucanases (at nucleotide level) and callose synthase-like sequences (at amino acid level) found on the Barley1 GeneChip and used in this study with sequences in nucleic acid databases

	Contig no	Most similar to Acc no	% identity	Expressed in leaves	Induced by aphids
Callose synthases	4949	NM001055136.1	86%	yes	no
	8428	AY177665.1	99%	yes	no
	13152	NM01063131.1	93%	no	no
β-1,3-glucanases	1636	X67099	99%	yes	yes
	1637_s	AJ271367	99%	yes	yes
	1639	AK248899.1	97%	yes	yes
	10477	U96096	99%	yes	no

NB: AY177665.1 is coding for *HvGSL1* (Li et al., 2003).

6.3.2 Regulation of β-1, 3-glucanase genes at RNA level

The expression of β-1, 3-glucanases by RT-PCR, in control plants as well as upon infestation by BCA and RWA is illustrated in Fig. 6.3. Real-time RT-PCR confirmation of this expression is shown in Fig 6.4. The four primer pairs that were designed and used in these experiments (Table 2.1) are based on those sequences from the Barley1 GeneChip (Affymetrix) which putatively code for β-1, 3-glucanases. These four were found to be expressed in barley leaves with or without aphids in microarray experiments (Delp G, Gradin T, Åhman I, Jonsson LMV, unpublished); the others were classified as absent. The four sequences were contig1636_at (99% identical to isoenzyme GIII Accession No. X67099, Wang et al., 1992), contig1637_at (99% identical to isoenzyme GII, Accession No. AJ271367, Hoj et al., 1989), contig1639_at (97% similarity to Accession No. AK248899.1) and contig10477_at (Accession No. U96096, Litts et al., 1990). The results with RT-PCR show that out of all the four primers used, contig10477_at was expressed at equal levels during aphid infestation (Fig. 6.3). However, contig1636_at, contig1637_at and contig 1639_at showed induced expression upon infestation by RWA and BCA (Fig. 6.3).

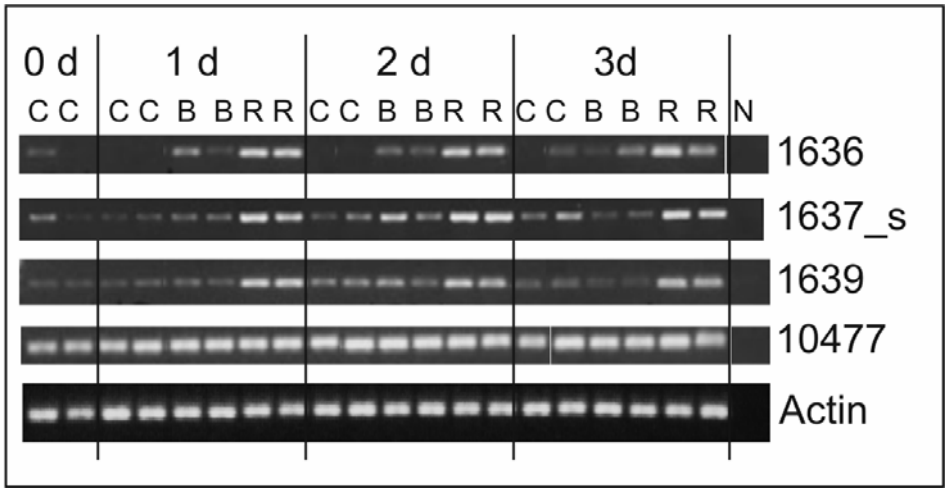

Fig. 6.3 RT-PCR expression of β-1, 3-glucanases in control, BCA and RWA-infested barley leaves

Figure 6.3 shows the expression of *β*-1, 3-glucanase after 0, 1, 2 and 3 days of infestation with BCA and RWA. Control plants are marked with (C), leaves infested with BCA marked with (B), those infested with RWA marked with (R) and negative control without template marked (N). One-step RT-PCR was performed on 30 ng RNA extracted from the leave tissues of barley cv. Clipper. Primers specific for glucanases 1636, 1637_s, 1639, 10477 and actin (as loading control) were used.

The three genes were induced after one day of infestation, but to different degrees, depending on the aphid species. All three genes were more strongly induced by RWA (Fig. 6.3). This expression, however, was further confirmed with real-time RT-PCR, where similar observations occur with the expressed primers, only data for contig1637_at is presented (Fig. 6.4). In these experiments, both genes were induced after one day of infestation; the expression levels increased after two and three days; while the most strongly induced β-1, 3-glucanase, contig1637_at, was 50 times higher (strongly induced) in RWA-infested tissue (Fig. 6.4), compared to BCA-infested tissue.

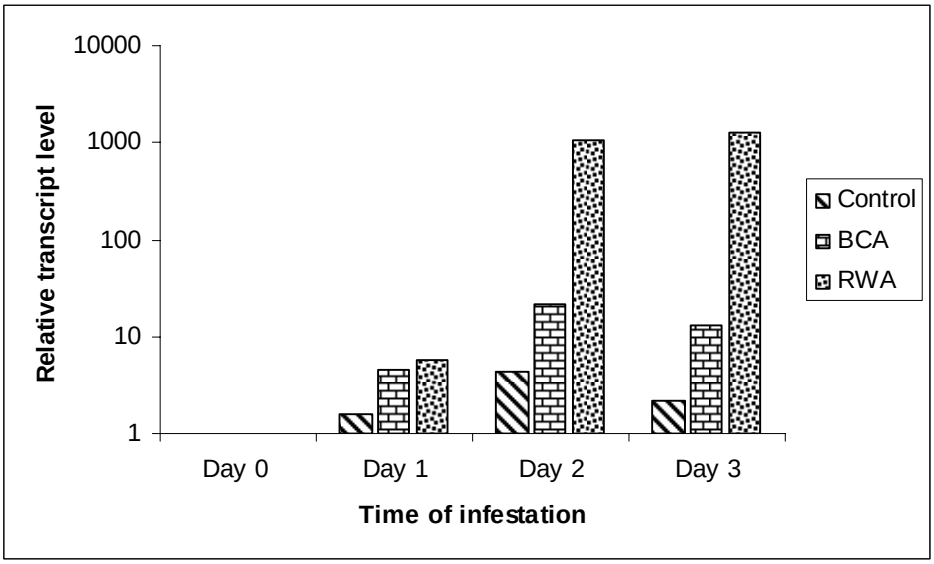

Fig. 6.4 Real time RT-PCR expression of β-1, 3-glucanases contig1637_at

Figure 6.4 shows expression of β-1, 3-glucanases in control, BCA- and well as RWA-infested barley leaves. The real-time RT-PCR was performed using glucanase primer contig1637_at and 30 ng RNA extracted from barley leaves. The relative transcript levels were calculated using actin as the reference gene. The scale is logarithmic.

6.4 Discussion

6.4.1 Regulation of callose synthase genes

The induction, formation and deposition of wound callose were expected to be as a result of increased expression of *GSL* genes in barley leaf blades. Out of the three analysed gene sequences, only two were expressed in leaf blades (contig8428 and contig13152), while contig4949 was not expressed at all. The two sequences expressed did not show any increase or clear pattern of regulation between control and aphid-infested tissues (Figs. 6.1 and 6.2). However, the possibility that the biosynthesis of callose in barley leaf blades upon aphid infestation might be caused by another *GSL* gene other than the three genes examined in this study cannot be excluded. Li et al. (2003) have suggested that at least six *GSL* genes can be expected to be present in barley, based on barley EST libraries. A candidate might be a gene with sequence similarity to *GSL6,* the only Arabidopsis *GSL* gene that was induced by phloem-feeding silverleaf whitefly nymphs, in tissue where callose deposition was found at feeding sites and in vascular tissue (Kempema et al., 2007). As mentioned earlier, in *Arabidopsis* twelve genes for GSL have been identified (Richmond and Somerville, 2000; Verma and Hong, 2001), of which *GSL5* has been functionally characterized and shown to be specifically involved in wound and papillary callose formation (Jacobs et al., 2003). The Arabidopsis *GSL* genes have been positioned in a phylogenetic tree with callose synthase genes in rice, where 10 *OsGSL*s were identified (Yamaguchi et al., 2006).

None of the three barley sequences studied here, nor their rice orthologues are close to *GSL5* or *GSL6* from Arabidopsis. This indicates that there might be more callose synthase genes expressed in barley leaves, which could be induced by the aphids.

In any case, the results presented in this chapter rather support the idea that wound callose synthesis is not regulated at the RNA-level. An additional strong argument for this process being regulated at the protein level is that it is quite rapid (within 5 minutes) (Nakashima et al., 2003) and that callose synthesis can be activated by changes in the intracellular distribution of a glucoside activator (Ohana et al., 1993). Sound evidence exists that callose synthase is located in membrane-associated complexes with several other proteins and it has been reported that release, or binding of separate units from this complex, could rapidly switch on and off callose synthesis, with no need for *de novo* synthesis of glucan- β-1, 3-synthase (Verma and Hong, 2001). *In vitro* studies have demonstrated that callose synthesis is activated by calcium and by proteases (Kauss, 1985; Botha and Cross, 2001; Nakashima et al., 2003). It is possible that penetration of aphid stylets causes leakage of extracellular calcium into sieve elements, thereby causing an increase of internal calcium levels (Will and Van Bel, 2006). However, the synthesis of callose occurs on the outside of the plasma membrane where calcium levels are high. An interesting suggestion put forward by Nakashima et al. (2003) is that β-1,3-glucan (callose) synthesis and β-1,4-glucan (cellulose) synthesis is

linked, such that during wounding, cellulose synthesis at the plasma membrane is terminated (within minutes) and callose synthesis initiated (within 30 sec). Nakashima et al. (2003) have used antibodies and shown that β-1, 4-glucan synthase ceased completely in mung bean plasma membranes within 5 min of wounding, whereas β-1, 3-glucan synthase appeared. Nakashima et al., (2003) proposed that an endogenous protease immediately regulate the inactivation or attenuation of β-1, 4-glucan synthase activity, and that a protease directly or indirectly activates callose synthase. Using this model, the difference between RWA and BCA in callose induction could either be due to exudation of proteases from RWA (which would initiate the degradation of cellulose synthase) and/or exudation of protease inhibitors from BCA (which would prevent this from happening). Whatever the case may be, the aphid effect involves components that are transported, probably via phloem; since callose induction was seen further away from aphid infestation sites in both source and sink leave tissues (see Chapter 5).

6.4.2 Regulation of β-1, 3-glucanases

It was hypothesised that the difference in callose induction between RWA and BCA could be due to a stronger induction of the callose-degrading enzyme, β-1, 3-glucanase, which could lead to a more efficient breakdown of callose in BCA-infested tissue. In contrast to this expectation, there was an increase in the expression of all the three aphid-induced β-1, 3-glucanases and this expression was much higher in RWA-infested tissue than in BCA-infested

tissue (see Fig. 6.3). Two of the induced β-1, 3-glucanases are almost identical to earlier studied barley β-1, 3-glucanases: contigs1636_at (X67099) and 1637_s_at (AJ271367), which both have a potential N-terminal signal peptide and basic pI; 9.8 for AJ271367 (isoenzyme GII) and 10.3 for X67099 (isoenzyme GIII) (Hrmova and Fincher, 1993). The AJ271367 sequence (isoenzyme GII) is expressed in the aleurone (Wang et al., 1992), but was induced in leaf tissue by barley powdery mildew (Xu et al., 1992) and the pathogen *Bipolaris sorokiniana* (Jutidamrongphan et al., 1991). The X67099 sequence (isoenzyme GIII) is expressed in young developing leaves (Wang et al., 1992). It was not induced by *Blumeria graminis* (Xu et al., 1992), but more recently it has been shown that activation of the promoter is salicylic acid-dependent and that the gene is induced by salicylic acid (Li et al., 2005). Both isoenzymes GII and GIII were also induced in barley leaves infected with the leaf scald fungus (*Rhynchosporium secalis*) (Roulin et al., 1997). There are three different β-1, 3-glucanases (two basic and one acidic), which have been shown before to be induced by BCA in barley at protein level (Forslund et al., 2000). These proteins probably correspond to the gene sequences and proteins now identified.

Thus, there are no strong indications as to the function of these β-1, 3-glucanases in the degradation of aphid-induced callose in leaf tissue, since two of them are induced by various pathogens and there is no indication that any of them is involved in the degradation of callose caused by aphids. The

possibility that other β-1, 3-glucanases are involved in this process, which may show stronger induction in BCA-infested leaves than in RWA-infested leaves, cannot be excluded. Nevertheless, based on the present data, it is suggested that the differences between these two aphids are caused by a stronger synthesis of callose in RWA-infested leaves. It is possible that the very strong expression of the three induced β-1, 3-glucanases in RWA-infested tissue manifest at 48h is linked to the callose induction seen already at 24h via a feedback mechanism. The fact that callose persisted throughout the length of the experiments in spite of increased levels of β-1, 3-glucanase may be due to the heavy infestation, with an increasing number of aphids within a limited space. It has been reported before that callose induced by RWA feeding in wheat persisted for up to 48h (Botha and Matsiliza, 2004) and for up to 120h in barley (see Chapter 5) after removal of feeding aphids. This is contrary to earlier reports that wound callose disappears over the course of days (Currier and Webster, 1964).

7 Chapter 7: Differences in the rate of phloem transport caused by RWA and BCA infestation in barley leaves

7.1 Introduction

Transport of assimilates in the phloem of different orders of vascular bundles in monocots to other sink regions of plants has been extensively studied previously (see Evert et al., 1977, 1978, 1988; Fritz et al., 1989; Botha and Van Bel, 1992 among others). Barley leaves, as in other typical monocots, contain three vein orders of vascular bundles, which are interconnected by cross veins (Evert et al., 1996; Botha and Cross, 1997). These vascular bundles in the leaf blades are known to serve either as loading bundles (in the cross, small and intermediate bundles) or as longitudinal transport of assimilates (in the large bundle) across different plant tissues (Lush, 1976; Altus and Canny, 1982; Fritz, et al., 1989; Botha and Cross, 1997). The major constituents of the transported assimilate is sucrose, which is known to be compartmentalised into transport (mesophyll and vascular tissues) and vacuolar pools in barley leaves (Farrar and Farrar, 1986).

It was surmised that phloem-feeding aphids derive their nutrient supply from these pools, as aphids mostly aggregate on the parts of the plant where food is of high quality (Kennedy and Booth, 1951). Since phloem sap is low in protein, aphids need to ingest large quantities of the phloem sap in order to gain enough amino acids, which are essential to their survival, while excess

water and sugars are excreted as 'honeydew' (Douglas, 1993). Aphids feed specifically from the sieve tubes of the vascular bundles and preferentially from the thin-walled sieve tubes (Matsiliza and Botha, 2002). Seemingly, they select their feeding sites according to the quality and quantity of the food that the feeding site yields. Obviously, feeding activities adversely affect the transport capacity of the infested plants. A number of authors have reported that aphids become strong secondary sinks due to the diversion of assimilates meant for other growing plant tissues to the aphid's guts. Diversion results in several symptoms in infested plants (see Cagampang et al., 1974; Hicks et al., 1984; Nielsen, et al., 1990 and Botha and Matsiliza, 2004).

The study of the feeding mechanisms of aphids during infestation of plants as it relates to the observed symptoms is the focus of this thesis. This was further investigated with respect to the effects that RWA and BCA feeding have on the transport capacity of the phloem in barley leaves. I have used the phloem mobile fluorophore, 5, 6 carboxyfluorescein (5, 6-CF), to investigate the potential differences in damage to the phloem caused by BCA and RWA. 5, 6-CF and some other fluorescein compounds have been used *in situ* on several occasions to study phloem transport in plants and they were reported to provide reliable information (Turgeon and Beebee, 1991; Farrar et al., 1992; Botha et al., 2000 among others). 5, 6-CFDA is non-polar. When applied to damaged cells, it is taken up by plant cells and then moved across cell walls and membranes. Once in physiologically intact tissues, the diacetate is

cleaved, resulting in polar free 5, 6-CF, which is non-permeable to cell membranes. It fluoresces while moving symplasmically within contiguous cell of the phloem. Therefore, 5, 6-CFDA is very useful in studying phloem transport, a position that was supported by Grignon et al., (1989). 5, 6-CF derived from 5, 6-CFDA have been used recently to study phloem transport during RWA infestation in wheat (Botha and Matsiliza, 2004), and during infestation of wheat cultivars by *Sitobion yakini* (de Wet and Botha, 2007). These two studies provide background information concerning the reduction of phloem transport capacity during RWA feeding. However, literature on the effect of feeding by BCA on the transport capacity of the phloem seems to be non-existent.

I have established at the TEM level (see Chapters 3 and 4; Saheed et al., 2007a and b), that RWA inflicts more severe damage than does the BCA, especially to the thin-wall sieve tubes of the phloem. RWA probing leads to the formation of wound callose in the phloem tissue of the infested leaves under short- and long-term infestation (Chapter 5; Saheed et al., 2007c), even with small (5-10 aphids) feeding populations. In contrast, BCA feeding does not lead to extensive formation of wound callose in phloem tissues during the same period and with similar-sized feeding populations. Formation of wound callose only occurs when a larger population (50 aphids or more) of BCA feeds on barley leaves (see Chapter 5).

The experiments reported in this chapter were conducted to examine the effects the feeding aphids had on the transport capacity of the phloem and to relate it to the visible symptoms expressed by the infested plants. The aim of these experiments was to compare the differences in the damage caused by RWA and BCA to conducting sieve tubes (at TEM level), as well as the observed differences in the pattern of wound callose formation to their possible effects on the phloem transport. These results will hopefully further elucidate the effects of aphid feeding on the transport capacity of the phloem and relate it to visible symptoms shown by infested plants.

7.2 Experimental overview

In all cases, five adult aphids were confined using clipcages, to feed on either sink or source leaves of barley for 72 h (short-term) and 14d (long-term). The leaves used in these experiments were about 12 cm long, while the clipcages, placed at the mid-region of the fully expanded leaves, were about 5 cm in length. The xenobiotic 5, 6-CFDA was applied to the attached leaves using the flap feeding method (see Chapter 2 for details of the procedure), was allowed to be taken up and the cleaved product, 5, 6-CF transported for three hours. The leaves were removed to investigate the amount (fluorescence intensity) and the rate (measured by the distance moved by the cleaved 5, 6-CF from the point of application over a three-hour period) of phloem transport in aphid-infested leaves as well as in control leaves. The results presented are based on acropetal and basipetal movements of the 5, 6-CF in the longitudinal bundles only.

7.3 Results

7.3.1 Transport of 5, 6-CF in control barley leaves

The movement of the dissociation product of 5,6-CFDA (5, 6-CF) occurs from the site of application in control, uninfested source as well as sink leaves, and thus shows that the fluorochrome moved acropetally in sink leaves and basipetally in source leaves. Three hours after the application of the 5, 6-CFDA, the fluorochrome front had usually moved approximately 5cm from the point of application. Through repeated experiments, 5, 6-CF uptake starts with the mesophyll, then moves through the bundle sheath and then loads into the vascular bundles (Fig. 7.1A). Loading was seen to start with small and intermediate bundles, after which the fluorochromes move towards the large exporting bundles. In control leaves, the transport of 5, 6-CF appears continuous and undisturbed (Figs. 7.1 B-C) up to the fluorochrome front (Fig. 7.1D). The unloading sequence of the fluorochrome, as expected, shows a movement from the sieve tubes into the mesophyll tissue via the vascular parenchyma and bundle sheath. All these movements are purely symplasmic.

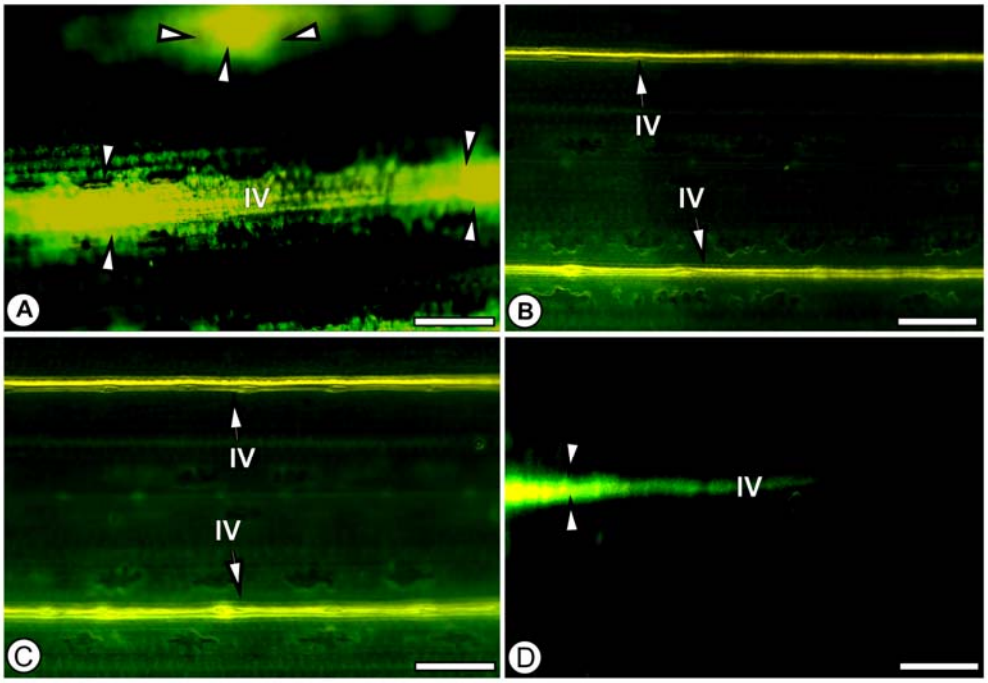

Fig. 7.1 Transport of 5, 6-CFDA in control barley leaves

Fig. 7.1 illustrates transport of cleaved 5, 6-CF in control leaves of barley. **Fig. A** shows the point of application of the fluorochrome (arrowheads). Note that the 5, 6-CF is being taken up and transported in the intermediate vein. **Figs. B and C** show undisrupted transport of the cleaved 5, 6-CF along intermediate veins. Note that the transport is smooth, even and continuous. **Fig. D** shows the fluorochrome front (arrowheads) in an intermediate vein. Scale bars: A = 100 µm; B = 200 µm; C = 200 µm; D = 150 µm.

7.3.2 Transport of 5, 6-CF in BCA-infested leaves

After 72h (short-term) of feeding by BCA, distribution of the 5, 6-CF was patchy. Figure 7.2A illustrates this distribution pattern after short-term feeding. Despite this patchiness in the distribution, there is no apparent reduction in the intensity or the distance moved by 5, 6-CF from the point of application when compared to the control tissue.

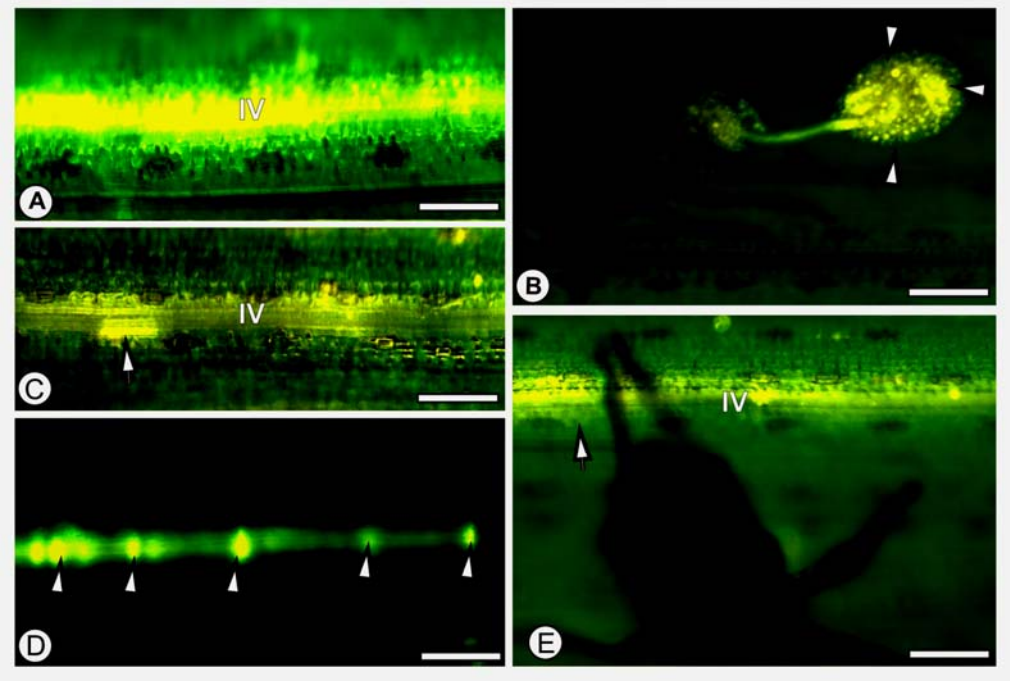

Fig. 7.2 Transport of 5, 6-CFDA in BCA infested barley leaves

Figure 7.2 illustrates transport of cleaved 5, 6-CF in BCA-infested barley leaves.
Fig. A shows patchy transport pattern of the fluorochrome during short-term feeding by the aphids in intermediate vein (IV).
Fig. B shows the fluorochrome in the honeydew (arrowheads) ejected by the aphid.
Figs. C-E illustrates a gradual reduction in the transported 5, 6-CF during long-term feeding by BCA. Note: stylet points (arrowheads) show a higher concentration of the fluorochrome.
Scale bars: A = 100 µm; B = 150 µm; C = 100 µm; D = 150 µm; E = 200 µm.

However, after 14d (long-term) of continuous feeding, there is an apparent reduction in the intensity and distance moved by the fluorochrome from the point of application in the infested leaves when compared to the control leaves. Figures 7.2 C-E illustrates the observed gradual reduction in the intensity of the transported 5, 6-CF, and points of stylet penetrations show an increased fluorochrome concentration. Interestingly, traces of ingested 5, 6-CF can be seen in the honeydew excreted by the aphids (Fig. 7.2B).

7.3.3 Transport of 5, 6-CF in RWA-infested leaves

In contrast to the results obtained in BCA experiments, transport of 5, 6-CF in RWA-infested barley leaves even after short-term feeding (72h), shows a dramatic reduction in the distance moved as well as in the intensity of the fluorochrome. Figure 7.3 A-B illustrate transport of the 5, 6-CF after 72h of feeding. The images show that the intensity of the fluorochrome before the point of aphid feeding (arrows) is much higher than after the aphid feeding point (arrowheads). Prolonged feeding (long-term) by RWA results in a greater reduction in the intensity and well as the distance moved by 5, 6-CF and in most cases, cessation of the transport ensues. Figures 7.3 C-E illustrate transport of 5, 6-CF after 14d (long-term) feeding by RWA, while Fig. 7.3C shows apparent leakages of the cleaved 5, 6-CF through many points of stylet penetrations (arrowheads). Fig. 7.3D shows the presence of the fluorochrome in the abdomen of an aphid, while Fig. 7.3E illustrates the total cessation in the movement of 5, 6-CF which occurs on many occasions.

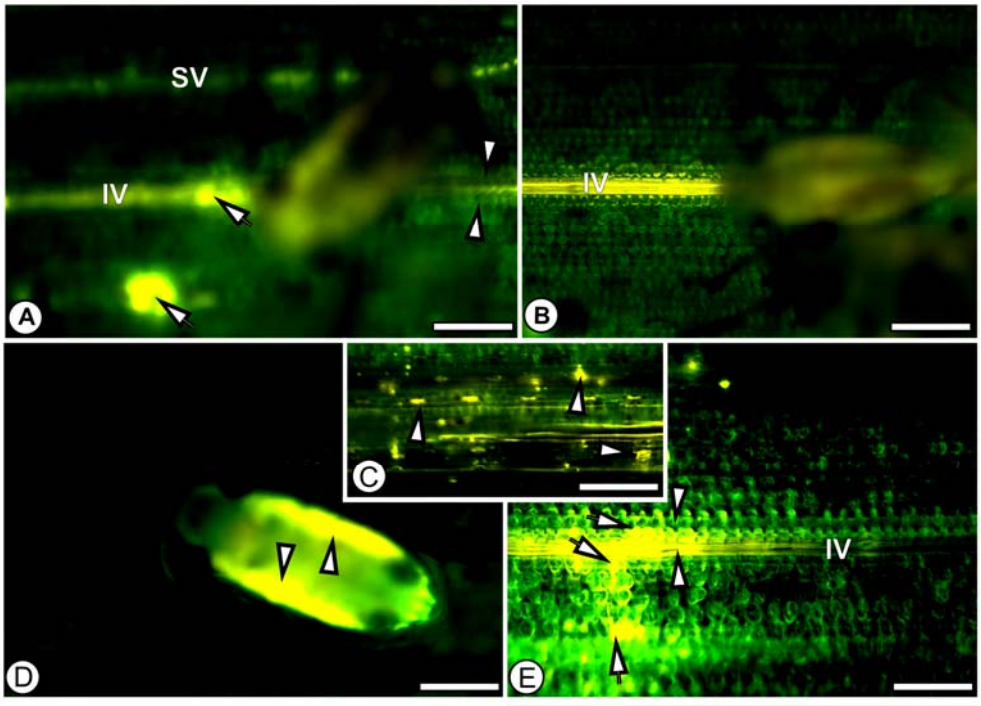

Fig. 7.3 Transport of 5, 6-CFDA in RWA infested leaves

Figure 7.3 shows the pattern of transport and distribution of 5, 6-CF in RWA-infested barley leaves.
Fig. A shows reduction in the transported 5, 6-CF after short-term feeding (72h) by RWA. Note the feeding points (arrows) and reduced transport after the points (arrowheads).
Fig. B shows feeding in an intermediate vein, reduced transport of 5, 6-CF was observed after the point of aphid feeding.
Fig. C illustrates several stylets' points (arrowheads) after long-term feeding.
Fig. D shows cleaved 5, 6- CF in the abdomen of an aphid.
Fig. E illustrates complete cessation of further transport of the fluorochrome after severe, long-term feeding by the aphids. Note the leakages of the 5, 6-CF through many stylet tracks (arrows) out of the intermediate vein and stoppage of further movement (arrowheads) of the 5, 6-CF from the feeding points.
Scale bars: A = 150 µm; B = 200 µm; C = 50 µm; D = 150 µm; E = 150 µm.

7.3.4 Comparison of the distance moved by 5, 6-CF in control and infested leaves

Analysis of variance (ANOVA) was used to show differences in the distance moved by 5, 6-CF in control, BCA- and RWA-infested leaves, and homogenous groups were identified using Tukey *post hoc* at a 95% level of confidence. From 20 replicates taken per treatment, there was no significant difference between the control and BCA-infested leaves (Fig. 7.4) during the short-term feeding experiments. However, control and BCA-infested leaves were significantly different ($F_{2, 57}$ = 300.98, $p < 0.0001$) from the RWA-infested leaves (Fig 7.4). RWA infestation lead to a significant reduction in the distance moved by the fluorochrome after 72h of feeding when compared to the distance moved in both control and BCA-infested tissue.

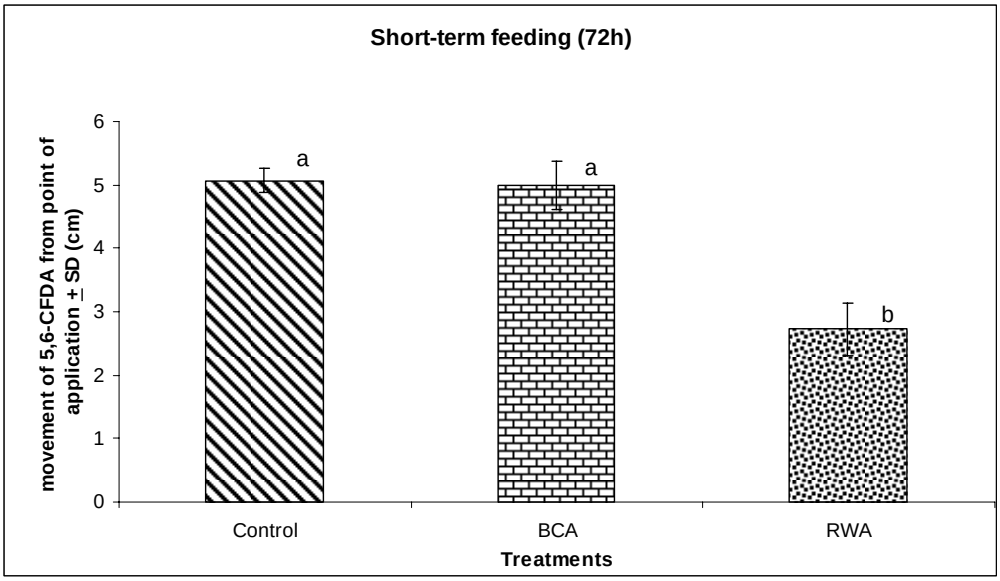

Fig. 7.4 Comparison of the means of distance moved by 5, 6-CF from point of application in control, BCA- and RWA-infested leaves after short-term (72h) feeding by the aphids(± Standard deviation); ANOVA: $F_{2, 57}$ = 300.98; $p < 0.0001$

After 14d of feeding by the aphids (long term), significant difference ($F_{2, 57}$ = 621.94, $p < 0.0001$) was found in the distance moved by 5, 6-CF between the control, BCA and RWA-infested leaves. The movement of 5, 6-CF in the leaves infested with BCA was reduced when compared to what was observed in the control leaves (Fig. 7.5). Whereas transport of 5, 6-CF in RWA-infested leaves is further reduced (Fig. 7.5) when compared to the movement in control and BCA-infested leaves, and on many occasions no transport or total cessation of transport occurs.

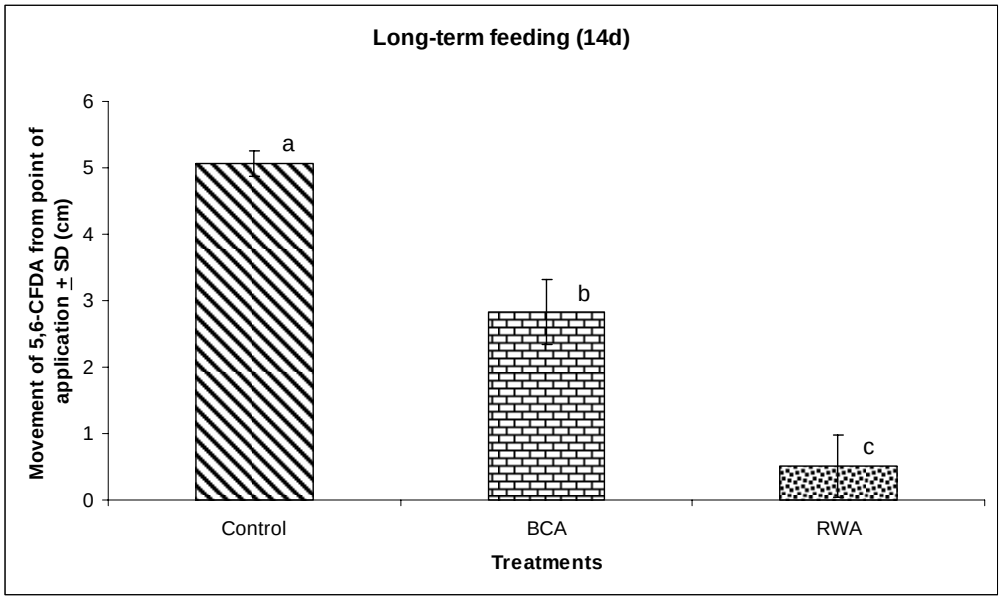

Fig. 7.5 Comparison of the means of distance moved by 5, 6-CF from point of application in control, BCA- and RWA-infested leaves after long-term (14d) feeding by the aphids (± standard deviation); ANOVA: $F_{2, 57}$ = 621.94; $p < 0.0001$

7.4 Discussion

I have demonstrated again that the phloem transport can still be visualised by the transport of cleaved product of 5, 6-CFDA (5, 6-CF). The results presented in this chapter deal only with longitudinal phloem transport as visualised by 5, 6-CF transport and its fluorescence. Transport is generally acropetal in sink leaves and basipetal in source leaves. Longitudinal transport of assimilates (visualised by transport of 5, 6-CF) in control barley leaves shows that the rate (the distance moved from the point of application of 5, 6-CFDA to the 5, 6-CF front in 3h) of phloem transport is at an average of 5cm over 3h period in all classes of veins. This observation is true in both source and sink leaves. The movement of the phloem-mobile 5, 6-CF follows classical source-sink transport patterns. The data presented here thus lend strong support to an earlier report that demonstrated similar movement of the fluorochrome in wheat leaves (Botha and Matsiliza, 2004). Once applied, the symplasm of the mesophyll tissues takes up the fluorochrome (Fig 7.1A), and subsequently loads it into the phloem through the many symplasmic connections between bundle sheath-vascular parenchyma and companion cells-sieve tube complexes (Evert et al., 1996; Botha and Cross, 2001; Botha, 2005) and then moved longitudinally. This transport is unrestricted and confined within the transporting veins of the control tissues (Figs. 7.1 B-C).

However, this movement pattern is severely disrupted upon feeding by the aphids. The focus of this chapter is thus on the impact on the longitudinal

transport of assimilates when RWA and BCA feed on barley leaves. The observed differences in the damage caused by the two aphid species during TEM (see Chapter 3; Saheed et al., 2007a) and wound callose formation (see Chapter 5; Saheed et al., 2007c) experiments are further confirmed with the present phloem transport results. In short-term (72h) feeding experiments, infestation by BCA does not result in obvious reduction in the capacity of the phloem to transport assimilate (Fig. 7.2A), as there was no significant difference in the transport of 5, 6-CF in the assimilate stream of control and BCA-infested leaves (Fig. 7.4). On the other hand, RWA infestation led to a significant reduction in the 5, 6-CF transported in the assimilate stream (Fig. 7.3A-B) and phloem transport capacity (Fig. 7.4) within 72h of feeding. However, some reduction in the 5, 6-CF transported (Figs. 7.2C-E) and a significant reduction in the phloem transport capacity (Fig. 7.5) occurs when BCA fed for 14d (long-term) as compared to the control plant. Obviously, RWA infestation resulted in a pronounced reduction in the 5, 6-CF transported: in most cases, complete cessation of transport ensued (Fig. 7.3E). This infestation caused a greater reduction in phloem transport capacity (Fig. 7.5) when compared to observations in both control and BCA-infested leaves. Interestingly, the ingested 5, 6-CF in the assimilates were visible in the egested honeydew of BCA (Fig. 7.2B) and also in the abdomen of RWA (Fig. 7.3D). Importantly, the data presented here show that feeding by RWA resulted in patchiness in 5, 6-CF distribution (Figs. 7.3C and E), much more than in observations in BCA infested leaves (Figs. 7.2C-E).

The results presented in this chapter clearly show that the aphids redirected assimilates that would normally be transported in the veins of the leaves, thereby depressing the sink strength in growing regions of the plant. Diversion and disruption of transported assimilates have been reported in different species of phloem feeding insects. Nielsen et al., (1990) have reported the disruption of assimilates' transport when potato leafhopper (*Empoasca fabae*) feeds on *Medicago sativa* (alfalfa). Feeding of planthopper (*Nilaparvata lugens*) on leaf blades of rice have also been shown to results in removal of assimilates and reduction in photosynthesis (Watanabe and Kitagawa, 2000), thereby causing a reduction in rice growth and yield. Hill (1962) had earlier shown that if the redirection of assimilates by the phloem feeding insects is strong and localised, the plant will react in some respects as if the aphids were a bud.

The present study revealed that diversion and disruption of the assimilate transport pathway in barley leaves is more apparent due to RWA feeding than BCA feeding. This suggests that the combined effects of aggressive feeding and damage sustained by leaves that were inflicted by RWA are more severe than that caused by BCA. Therefore, the results reported in this chapter support data in previous chapters that:

1. RWA is a more aggressive feeder than is BCA.

2. RWA disrupts the phloem system thereby reducing the transport capacity of the phloem, as evidenced by reduced transport of the phloem-mobile xenophore, 5, 6-CF.
3. RWA may redirect more assimilate to itself than does BCA, which supports 1-2 above, and finds support in earlier chapters in which ultrastructural damage was more severe and callose formation (wounding effects) was more intense in RWA than BCA infestations.

The results reported in this chapter suggest that the greater disruption and diversion of assimilates in RWA-infested barley leaves appears to be responsible for higher yield losses (30% to 60%) encountered during RWA-infestation (Du Toit and Walters, 1984), while a reduced disruption and diversion of assimilates results in reduced (21%) yield loss that occurs during BCA-infestation (Riedell et al., 1999). The suggestion finds support in earlier observation of rice, where disruption and diversion of assimilates caused yield losses in infested plants (Watanabe and Kitagawa, 2000). The data presented in this chapter further elucidates the mechanism behind aphid feeding and responses of the infested plants by revealing more reasons why RWA-infested leaves show symptoms of chlorosis, necrosis and leaf roll while leaves infested by BCA do not.

In conclusion, the symptoms shown by RWA-infested plants that eventually lead to the subsequent greater losses in yield when compared to observations of BCA-infested plants, are suggested to be partly due to ability of RWA to inflict severe damage to phloem transport of the infested plant. This damage leads to noticeable reduction in the transport of assimilates within 72h of RWA infestation and significant reduction during prolonged feeding, which, in most cases, results in total cessation of phloem transport. Whereas BCA feeding does not appear to cause as much serious damage to the transport capacities of the phloem, only a reduced assimilate transport occurs during its prolonged infestations.

8 Chapter 8: General Discussion and Conclusion

8.1 Preamble

Global efforts to achieve sustainable agriculture require research aimed at addressing severe problems such as aphid infestation of crop plants. Of particular importance is a clearer understanding of the mechanisms of plant-aphid interaction; otherwise, efforts aimed at sustainable agriculture will be very difficult to achieve. Many researchers have used different approaches, such as ecological, behavioural, physiological, structural, aphid evolution, biochemical and molecular studies (see Krischik, 1991; Karban and Baldwin, 1997; Van der Westhuizen et al., 1998a, b; Miles, 1999; Moran and Thompson, 2001; Messina et al., 2002; Moran et al., 2002; Botha and Matsiliza, 2004 among others for details). The underlining mechanism of the interaction that occurs during aphid infestation of crop plants is not yet fully understood. Detailed knowledge of these interactions will assist plant breeders, as breeding resistant crop cultivars is the most economically and environmentally safe and effective option for controlling aphid pests (Zhang et al., 1998; Tolmay and Mar'e, 2000).

The potential for resistance to RWA first identified in ancestral diploid wheat species (Butts and Pakendorf, 1984; Du Toit and Van Niekerk, 1985; Du Toit, 1987), before it was later found in rye and some triticale lines (Nkongolo et al., 1989; Webster, 1990). To date, breeding efforts have led to identification

of 10 different genes that shows resistance against RWA in wheat and other cereals (Liu et al., 2002, 2005). The success of these breeding approaches may eventually result in a greater reduction in the heavy cost that result from yield losses and chemicals used in spraying these pests, which was estimated to be more than 1 billion US dollars in United States alone (Morrison and Peairs, 1998). Breeding resistant plant cultivars will also reduce or eliminate the hazardous effects the sprayed chemicals potentially have on human, plants, livestock, and the whole environment.

The experiments reported in this thesis will hopefully contribute to a better understanding of the aphid effects on crops, and in general, towards a greater insight into the interaction between aphids and cereal crops, thereby contributing to our present knowledge of this interaction. The approach used was to investigate the structural and functional aspects of feeding by BCA and RWA, as the two aphid species are serious pests of cereal crops but with diverse effects on their host plants. This diversity forms the basis of this thesis, in an effort to provide answers to their feeding habit on barley and wheat cultivars.

8.2 Morphological symptoms of infestation

Infestation by the aphids investigated in this thesis have revealed that there are divergent visible responses to aphid feeding by the host plants. In Chapter 3, I reported that the infestation of barley cv Clipper populated by the same number of BCA and RWA will, over the same time period, result in different

observed symptoms. RWA infestation caused extensive chlorosis, necrosis and rolling of leaves of infested plants, while BCA infestation does not lead to any observable symptoms (reported in Saheed et al., 2007a, Fig. 3.1). This confirms earlier reports that whilst RWA induced severe symptoms on host plants, BCA did not, especially when the level of BCA infestation was low (Walters et al., 1980; Riedell, 1989; Wiktelius et al., 1990). This result was even more interesting given the higher breeding rate of BCA, and given the same population at the start of the experiments where BCA reproduced faster (Table 3.1), resulting in larger feeding population in comparison to the number of RWA produced at the end of the two-week experimental period. This observation supports that of Messina et al., (2002). However, the observation is contrary to the logical assumption that damage would be caused by the aphid stylet puncturing cells during probing and feeding, as well as by the destructive effects of the salivary material constituents deposited; this was supported by Miles, (1999). Based on this assumption, it was expected that BCA, with its higher feeding population sizes, would cause more cell-related damage than RWA. However, leaves fed on by higher BCA populations were visibly healthier, which was in sharp contrast to RWA-infested leaves, where symptoms such as chlorosis and necrosis occurred despite their smaller feeding populations (Saheed et al., 2007a).

The experiments involving the use of susceptible and resistant wheat cultivars reported in Chapter 4 conform to the expected results. Damage symptoms

were more evident in the susceptible Betta cultivar than in the resistant Betta-*Dn1* cultivar. In these experiments, the susceptible cultivar shows symptoms of chlorosis, necrosis as well as leaf rolls, while the resistant cultivar exhibited only a few chlorotic patches and necrotic spots (see Saheed et al., 2007b). This is in line with the known effects of the *Dn1* gene contained in the resistant cultivar, which is mostly antibiotic. Antibiosis results in reduction of growth rate, reduced fecundity, and reduced aphid biomass (Du Toit, 1987; 1989b; Heng-Moss et al., 2003). Clearly, in these experiments, it shows that more feeding aphids caused more severe observable symptoms than where the feeding aphids were less.

8.3 Ultrastructural damage during aphid feeding

Experiments were set up to investigate damage at the cellular level during aphid feeding. The first series of experiments explore the differences in the damage caused by BCA and RWA in a susceptible barley cv Clipper; most importantly, the damage caused to the vascular bundles – the primary site of aphid feeding and drinking. I investigated the structural damage caused by these aphids in the vascular bundles and then related the data to the observable effects on host plants. The results of this investigation provided a convincing insight into the feeding patterns of these two aphid species. In the second series of experiments, I investigated the feeding damage caused by RWA in susceptible and resistant wheat cultivars. This was to highlight the feeding mechanism with respect to the effects of resistant genes, in an attempt to

improve our understanding of the aphid feeding mechanism with respect to the response(s) of genetically modified crop plants.

8.3.1 Damage to vascular tissue in susceptible barley leaves

Because aphids feed from the phloem and drink from the xylem tissues of the vascular bundles, they have to probe through some other tissues before they reach their primary target – the vascular bundles. However, the results obtained from the investigation into the patterns of damage caused by BCA and RWA in the vascular bundles leave no doubt that RWA induces more severe damage to conducting tissues than did the BCA. In Chapter 3, I showed that both RWA and BCA puncture vascular parenchyma cells during their search for functional sieve tubes to feed from, but sustained feeding by RWA appears to result in more damage to sieve tubes-companion cell complex (Figs. 3.3 D, M and N) and these cells may be rendered non-functional. This is in contrast to similar phloem probes by BCA (Figs. 3.4 F and H) which do not seem to lead to such severe damage (see Saheed et al., 2007a). In addition, during RWA-drinking processes, the aphids ejects a large quantity of electron-dense but watery saliva that appears to completely seal the xylem vessels by blocking all membrane pit fields between xylem vessels and their associated parenchyma cells (see Fig. 3.3A). This blocking of membrane pit fields appears to isolate the xylem elements from surrounding xylem parenchyma. This action precludes the exchange of vital water, mineral nutrients and / or

solutes with adjacent cells in RWA-infested leaves. In contrast, in leaves infested with BCA, the aphids only deposit what can be described as a dense, granular saliva into the xylem vessels (see Figs. 3.4 A-C), which does not appear to occlude the membrane pit fields, and thus the exchange process of vital nutrients and solutes might not be hampered. In general, damage inflicted by RWA mostly involved complete destruction of the cells, and which could lead to rapid pressure loss in those cells. This might precede irreversible physiological damage that may induce degradation of the apoplasmic and symplasmic transport systems, apart from the reported effects of saliva deposition. This is in contrast to a lesser physiological damage observed in BCA infested leaf tissues.

The result of the ultrastructural damage study revealed that damage to the vascular tissues presented in this thesis clearly provide benchmark information on aphid feeding in vascular bundles of barley as well as in wheat leaves, and the data can be extended to similar grain crops. Previous ultrastructural studies involving RWA (Fouché, 1983 and Fouché et al., 1984), address only the changes that occurred to mesophyll cell organization and specifically to chloroplast contents, after they treat wheat leaves with whole body RWA extract. Apart from these publications and this thesis, literature that addresses feeding and drinking of RWA and BCA with ultrastructural studies seem to be non-existent. Tjallingii (1994) used electrical puncturing graphs (EPG) and reported that aphids also drink from xylem, but the results

presented in this thesis, I believe, might be the first ultrastructural evidence and confirmation of such feeding and drinking in xylem elements by the two aphid species reported here.

The primary aim of this thesis was to provide more insight into the mechanism behind aphid feeding, and these experiments were set up to investigate the damage to vascular bundles in order to relate it to the observed symptoms inflicted by these aphid species on their host plant. I therefore conclude here that leaf rolling and streaking symptoms shown by plants infested with RWA might, in part, be due to the damage caused to vascular system which lead to the apoplasmic and symplasmic isolation of both xylem and phloem. This is because of the limitation in vital nutrient exchange from these tissues to their adjacent cells. Specifically, chlorosis and necrosis may appear during probing, where puncturing of cells may induce oxidative stress, while the sealing of xylem and phloem might generate leaf rolling and streaking symptoms.

8.3.2 Damage to leaf tissues in susceptible and resistant wheat cultivars

The effects of resistance genes incorporated into cultivated crops to enhance their resistance to insect pests have been under investigation for years, yet the mechanism of these resistance genes is poorly understood. Resistant cultivars – containing resistant genes – have been shown to cope better and exhibit less physical damage when compared to susceptible counterparts during aphid

infestation (Nkongolo et al., 1990; Saheed et al., 2007b). Many resistance genes have been identified and inserted into crop genomes: 10 of such genes that are resistant to RWA have been identified in wheat and related cereals (Liu et al., 2002; 2005). Resistance genes are sustainable and retained over several generations within the incorporated crop plants (Mohan Babu et al., 2003). In addition, these genes exhibit different levels of resistance and show diverse resistance effects during aphid infestations (Du Toit 1989a, b; Budak et al., 1999).

In Chapter 4, I reported on the outcome of the experiments conducted using two near-isogenic wheat cultivars – the susceptible Betta and the resistant Betta *Dn1* – during RWA infestation. The *Dn1* genes incorporated into the resistant cultivar show antibiosis effects, which result in reduced aphid growth, fecundity and biomass (Du Toit, 1987; 1989b; Budak et al., 1999; Heng-Moss et al., 2003). Investigation at the ultrastructural level provided more insights into the action of these resistance genes and the mechanism of feeding by RWA in these cultivars. With the results presented in this thesis, new evidence now exists, which shows that RWA is capable of both intra- as well as inter-cellular probing within mesophyll cells (Figs. 4.2 A-E). This is contrary to over 23 years of belief that probing by RWA in the mesophyll cell is entirely intercellular (Fouché, 1983; Fouché et al., 1984). In susceptible Betta cultivar, inter- as well as intra-cellular probing in mesophyll cells by RWA results in severe cellular damage (Figs. 4.2 B-D). The punctured cells,

with evidence of salivary material deposits, exhibit severe damage to the cell cytoplasm and its organelles (Fig. 4.2 A). However, the case is different with the resistant Betta-*Dn1* cultivar, where intracellularly probed mesophyll cells do not show any signs of damage either to the cytoplasm or constituent organelles. Lowe (1967) had earlier reported inter- as well as intra-cellular penetration of mesophyll cells during *Myzus ornatus* infestation of *Vicia faba*. Reports of similar intra- and inter-cellular penetrations exist for some other aphid-plant interactions (see Pollard, 1973; Tjallingii and Hogen Esch, 1993). Prado and Tjallingii (1994) and Martin et al. (1997) have shown that the puncturing of cells like parenchyma and others during aphid probing activities involves discharge of watery saliva, ingestion of small amounts of sap from these cells, and formation of stylet sheath materials. Intracellular puncturing of mesophyll cells suggest that aphids, and in this case RWA, are capable of penetrating other cells on its way to the vascular bundle. This puncturing of the mesophyll cells appears to provide the aphids with clues regarding the substrate contained in the cells being penetrated (see also Klingauf, 1987; Tjallingii, 1995; Miles, 1999). The differences in the cellular damage that occurs between resistance and susceptible wheat cultivars upon infestation with RWA find support in an earlier work with greenbug aphids by Al-Mousawi et al. (1983). In this study, feeding by greenbug aphids results in extensive damage to mesophyll cells, vascular bundles and cellular organelles in susceptible cultivars after 10 days of infestation. Yet most cell organelles in

the mesophyll of the resistant cultivars, as well as vascular tissues, appears normal within the same time frame of infestation.

Feeding from the phloem of the two cultivars by RWA showed a similar pattern of damage that occurred during probing in mesophyll cells. The preferred site of feeding in the phloem is the thin-walled sieve tubes (Botha and Matsiliza, 2004). The thin-walled sieve tubes showed more severe damage in the susceptible Betta cultivar (Figs. 4.2 H-I) in comparison to the resistant Betta-*Dn1* cultivar, where this damage appears less severe (Figs. 4.2 J-L). It should be emphasised here that damage to thick-walled sieve tubes appears to be minimal in both susceptible and resistant cultivars. This supports earlier evidence that thick-walled sieve tubes are symplasmically isolated from the thin-walled sieve tube-companion cell complexes (Botha, 2005) and they are not a preferred site of feeding for the aphids (Matsiliza and Botha, 2002). Feeding also results in occlusion of plasmodesmata and deposition of callose within sieve tubes. This is a common occurrence in susceptible Betta cultivars and it affects the symplasmic transport of assimilates across adjacent cells. However, limited plasmodesmatal disruptions were commonly observed in resistant cultivar. This result suggests that symplasmic transport could still take place in the resistant cultivar, though it could be reduced due to redirection during aphid feeding. But reduction of phloem-related transport might be greater in the non-resistant Betta cultivar and, in most cases, total cessation could occur. The apparent lack of occlusion of plasmodesmata in the

Betta-*Dn1* cultivar could be attributed to β-1, 3-glucanase (an enzyme suspected to degrade callose), which has been shown to accumulate to a greater extent in the resistant cultivars (Van der Westhuizen et al., 1998a). Ectodesmata formation (generally taken as an indicator of severe damage to plasmodesmata) is usually associated with plasmolysis and it is often observed in Betta cultivars, and not in Betta-*Dn1*.

However, drinking from xylem resulted in characteristic occlusion of metaxylem vessels by copious deposition of saliva by RWA. This was the case with all the classes of veins investigated in Betta and Betta-*Dn1* cultivars. I have earlier discussed the implications of metaxylem vessels' occlusion and resultant, extensive blocking of pit membranes (see section 8.2.1 above and Saheed et al., 2007a, b). This complete sealing of the xylem vessels lends strong support to the suggestion that streaking and wilting in Betta and Betta-*Dn1* appears to result from the prevention of water and nutrient flow to parenchymatous elements from the xylem. It is important to mention here that during probing, feeding and drinking activities of RWA in both cultivars, all probed cells with evidence of salivary material deposit, or those cells that were adjacent to salivary material deposit, exhibit significant damage in susceptible Betta cultivars, whereas similar cells in Betta-*Dn1* cultivars do not show as damage as severe. It appeared as if cell disruption in resistant cultivars was an isolated event in all these instances, with individual cells destroyed, rather than whole groups of cells, during probing, feeding and

drinking. A similar phenomenon occurs during greenbug infestation of susceptible and resistant wheat cultivars (Al-Mousawi et al., 1983). These results suggest that the resistant genes' *Dn1* function by enhancing reduced cell and organelle damage (resistance to cell damage) in the resistant cultivar. I therefore proposed that this action might be due to higher levels of accumulation of PR-proteins, peroxidase and some other defence-related products reported to occur in the cell apoplast of some resistant wheat cultivars, including Betta-*Dn1* (Van der Westhuizen and Pretorius, 1996; Van der Westhuizen et al., 1998a). Al-Mousawi et al. (1983) similarly suggest that resistance to greenbug infestation of wheat results from physiological and biochemical factors, and that those changes in the cellular structures reflect only the changes in both physiological and chemical states. It follows that PR-proteins, peroxidase and other defence-related products may act by limiting penetration of the aphid's salivary components, as part of hypersensitive response (Moloi and Van der Westhuizen, 2006), and eventually conferred some protection to probed cells in the resistant cultivar, thereby preventing subsequent damage to the cells and their organelles.

The primary aim of these experiments was to elucidate the effects of aphid feeding and the mechanism of the action of the resistant genes selected against aphid infestation. I am of the opinion that the results presented in Chapter 4 have achieved this by providing strong evidence for this mechanism. I therefore conclude that RWA inflicts severe cellular and subcellular damage

to mesophyll and vascular leaf tissues in susceptible Betta cultivars, while significantly reduced damage occurs (due to the influence of the resistant genes *Dn1*) in the leaves of resistant Betta-*Dn1*. The reduced damage observed in the resistant Betta-*Dn1* seems to be a combination of a reduced number of feeding aphids (antibiosis), an established effect of *Dn1* genes, as well as enhanced cell wall strengthening effects that could be associated with the activity of some PR-proteins such as peroxidase.

8.4 Functional damage during aphid feeding

The second focus in this thesis is the functional effects of feeding by the selected aphid species on barley leaves. There are two principal approaches employed to study this. In the first approach, I examined the response of the plant to aphid feeding by formation of aphid-induced wound callose, after which I examined the regulation of callose genes (callose synthase and β–1, 3 glucanase) at the transcript level. Callose formation during infestation of plants by aphids is an effective plant response to the wounding of its cells. Formation and deposition of callose, reported to be a rapid plant response, could occur within a minute of cell wounding (Radford et al., 1998; Nakashima et al., 2003). The deposition of callose helps seal the pores of damaged phloem tissue, thereby preventing the loss of assimilates (Sjölund, 1997). The second approach was to investigate the effects of aphid feeding on the transport capacity of the phloem in barley leaves. Since aphids are known

phloem feeders, they feed on the transported assimilates in the phloem tissue; specifically, the sap transported within the sieve tubes. This feeding activity means the aphids becoming strong secondary sinks as a result of assimilate diversion during feeding (Cagampang, 1974; Hicks et al., 1984; Nielsen, et al., 1990).

8.4.1 Wound callose formation during RWA and BCA feeding

The barley cv Clipper investigated in this study again shows a diverse response to feeding by RWA and BCA with respect to formation of aphid-induced wound callose. In Chapter 5, I reported on the experiments conducted to investigate the wounding response of the barley cultivar to feeding aphids by examination of wound callose formation. The first experiments involved investigation of both short- and long-term effects of feeding. Aphid-induced wound callose in longitudinal and cross veins occurs within 24h of feeding by RWA and formation of callose become extensive after 72h (Figs. 5.3 A-E). This is in sharp contrast to BCA feeding, where no callose formation was observed within the same period. The aphid-induced wound callose in RWA-infested leaves increases, forming extensive deposits in longitudinal as well as cross veins (Figs. 5.3 F-L) during the long-term (14d) feeding experiments. Interestingly, little wound callose appeared even after 14d of extensive feeding by BCA (Figs. 5.2 E-H). However, wound callose formation increased with larger feeding populations of BCA on barley leaves (Figs. 5.4 A-D). This is the first known report of wound callose formation and distribution for this

aphid species. In all cases, I observed that stylet tracks were callosed, suggesting that the saliva triggers callose formation. Aphid saliva seems to play a crucial role in eliciting critical changes in plant's morphological, physiological and genetic changes (see reviews by Tjallingii, 1995; Miles, 1999).

The known effect of callose deposition is a decrease in the volume of transported assimilates, but if deposition is extensive, total cessation in the transportation of synthesised assimilates through the phloem tissues could occur, achieved by the blocking of sieve plate pores and plasmodesmata. Reduction or cessation in phloem transport must lead to series of symptomatic expressions, but if severe callose formation occurs, cessation of transport and eventual death of the plant could ensue. The results of the experiments conducted in Chapter 5, therefore, further illustrate the mechanism of aphid feeding and the response of the plant to such aphid feeding. The results suggest that the formation of wound callose in RWA-infested tissue appears to be partly responsible for the development of chlorosis and necrosis symptoms observed during RWA-infestation and lack of such formation in BCA-infested tissue results in lack of such symptoms (see Chapter 3; Saheed et al., 2007a). It is therefore logical to suggest that the formation and deposition of wound callose that occurs during infestation by higher BCA populations are in part responsible for the reported golden yellow streaking that occur during heavy infestation of crops by BCA (Agronomy Guide, 2002; UCIPM, 2002). In

addition, aphids drinking from the xylem induce severe damage and the apoplasmic distribution of water becomes impaired (Saheed et al., 2007a, b).

Wounding of leaf tissues during aphid infestation as evidenced by wound callose formation appears to be permanent. The experiments conducted to investigate the possible removal of callose after formation demonstrated that once deposited, callose remained in the leaf tissues 120h after the aphids were removed (Figs. 5.5 A-F). This lends support to the observed development of feeding symptoms even after the removal of the aphids. Results of the experiments provide evidence that wound callose signals, both defence and anti-defence, are transported in the phloem tissue (Figs. 5.6 E-H) and are dependent on the direction of assimilate flow. I further suggest that wound callose formation and manifestation are pressure-related and that a drop in phloem pressure during aphid puncturing of the functional (pressurised) sieve tubes could trigger an effect rather than a signal-related response. Whatever the case may be, I have been able to demonstrate that aphid-induced wound callose development is aphid species-specific in the barley cultivar studied. Deposition appears to be partly responsible for the symptomatic expressions observed during RWA-infestation, as well as the symptoms reported when higher populations of BCA infest the plants. Consequently, the absence of any observable symptoms during low-level infestation of BCA appears to be due to the lack of observable formation and deposition of wound callose during

such infestation. These observations and suggestions might well hold true for similar infestations in other cereal crops and possibly other aphid species.

8.4.2 Regulation of callose genes during aphid infestation

The differences in the formation and eventual deposition of wound callose during RWA and BCA infestation reported in Chapter 5 (see further discussion in section 8.4.1 above), lead me to speculate that the differences in the formation could be as a result of variation in the induction and regulation of callose genes. The two genes considered in this thesis are callose-synthase gene complexes that are involved in the synthesis of callose (Verma and Hong, 2001) and β-1, 3-glucanases, suspected to be involved in callose degradation. The questions raised were: does the formation of wound callose during RWA-infestation mean that callose synthase is up-regulated and / or that β-1, 3-glucanases are down-regulated in such tissues? Alternatively, does it mean that the reverse is the case in BCA-infested tissues, i.e. that β-1, 3-glucanases are up-regulated by either the plant or the aphid while callose synthase is down-regulated?

In Chapter 6, I showed that three glucan synthase genes (*GSL*) sequences were analysed (see Table 2.1 for sequence details), and that only two sequences were expressed in the barley leaf blades (contig8428 and contig13152), and contig4949 was not expressed at all. However, the two expressed sequences did not show any significant increase or regulation upon aphid infestation

(Figs. 6.1 and 6.2). Based on the investigated sequences, this suggests that wound callose synthesis is not regulated at the RNA-level. There is a possibility that apart from the three genes investigated in this study, other *GSL* genes could be responsible for the biosynthesis of callose. Li et al. (2003) reported that at least six *GSL* genes were expected to be present in barley, based on barley EST libraries, and that there might even be more that are not yet identified. So, it is possible that the genes responsible for callose induction are among those not yet investigated. Another possibility is that the regulation of the observed callose formation is not at the transcriptional level, but instead might occur at the protein level. The reason for this suggestion is that callose formation is quite a rapid process that occurs within minutes of wound initiation (Radford et al., 1998, Nakashima et al., 2003). There is also other evidence suggesting that callose synthase occurs in co-association with several other membrane-associated protein complexes (Verma and Hong, 2001) and that any release or binding of any separate units from such complexes could lead to a rapid switching on or off of callose synthesis without *de novo* synthesis of β-1, 3-glucan synthase. Irrespective of the pathway of callose induction, it is clear that callose synthesis occurs upon activation of calcium and proteases (Kauss, 1985; Botha and Cross, 2001; Nakashima et al., 2003). It has been suggested that the aphids' probing causes extracellular calcium leakage into sieve elements that results in increased cellular calcium levels (Will and van Bel, 2006; Will et al., 2007). Since endogenous protease has been shown to directly or indirectly activate callose

synthase (Nakashima et al., 2003). The differences now observed between RWA and BCA infestation could be due to endogenous proteases' activity. Therefore, the differences could either be due to exudation of proteases from RWA that perhaps initiate the degradation of cellulose synthase and increases callose synthase activity and / or exudation of protease inhibitors from BCA that prevent this synthesis from taking place.

The second gene group investigated were the β-1, 3-glucanases, which were suspected to cause degradation of synthesised callose. The hypothesis was that perhaps a stronger induction of β-1, 3-glucanase occurs in BCA-infested tissue, which could be responsible for the breakdown of callose, and so would explain why little or no wound callose occurs in such tissues. Interestingly and in contrast, all the three aphid-induced β-1, 3-glucanases showed a higher expression in RWA-infested tissue than in BCA-infested tissue (see Fig. 6.3). The function of the β-1, 3-glucanases investigated are not yet clear, but the results presented in Chapter 6 clearly show that they are not involved in callose degradation as was speculated. As suggested for callose synthase genes above, I cannot exclude the possibility that some other β-1, 3-glucanases might be involved in this process and they could be those expressed at higher levels in BCA-infested leaves than in RWA-infested leaves. In Chapter 6, I suggest that the stronger expression of the three induced β-1, 3-glucanases in RWA-infested tissue, as compared to the BCA-infested tissues, might have manifested at 48h and could be linked to the

callose induction seen already at 24h via a feedback mechanism. It is equally a possibility that the callose that persisted throughout the length of the experiments in spite of increased levels of β-1, 3-glucanase could be due to the heavy infestation, as a result of an increasing number of aphids within a limited space. It is important to state here that at the time of writing, further work continues in our research groups (CEJB and LJO) to unravel the involvement of the callose synthase and β-1, 3-glucanase genes. This investigation is, however, beyond the scope of this thesis.

In conclusion, the results presented in Chapter 6 do not yet explain the involvement of callose synthase genes (β-1, 3-glucan) and β-1, 3-glucanase genes in the formation of aphid-induced wound callose. However, what is clear from the experiments conducted is that the differences in the wound callose formation observed is due to a stronger synthesis of callose as a result of RWA infestation in leaf tissues than is the case in BCA-infested tissue at the same level of infestation and within the same time frame. Further studies could focus on the involvement of these genes, following leads from suggestions made in this thesis. However, I would like to state here that there seems to be a great difference between the actual mechanisms involving plant-aphid interactions and the molecular background presently known of such interactions, despite the global interests in molecular understanding of both induced as well as constitutive aspects of plant-aphid interaction. Tjallingii (2006) also championed this position. It appears that the salivary composition,

the relative population density of the feeding aphids, as well as length or time of infestation, are crucial in this interaction, which might account for the differences observed in the results reported in my thesis.

8.4.3 Effects of aphid feeding on transport capacity of the phloem

Aphids ingest large quantities of phloem sap to gain enough amino acids that are essential to their survival, because the sap is low in protein content (Douglas, 1993). This action transforms the aphids into strong secondary sinks (Hicks et al., 1984; Nielsen et al., 1990) through diversion of assimilates meant for other plant tissues into their gut (Botha and Matsiliza, 2004). This results in the disturbance of the assimilates' transport and by extension, the transport efficiency of the phloem of infested plants. The investigation of the effects of feeding aphids on the transport capacity of the phloem, especially on the longitudinal transport of assimilates in barley leaves as visualised by the transport of 5, 6-CF, was carried out in Chapter 7. The results show that during short-term feeding experiments (72h), BCA-infestation does not lead to a significant reduction in the intensity (amount) and distance travelled in the phloem transport (rate) of 5, 6-CF. In contrast, RWA-infested leaves showed considerable reduction in the intensity and transport of 5, 6-CF (Fig. 7.4). However, after long-term feeding (14d) by BCA, a considerable reduction in the intensity and transport of 5, 6-CF was observed in infested tissues. In contrast, a marked reduction in the intensity and transport of 5, 6-CF was

observed in RWA-infested leaves (Fig. 7.5), and in most cases, complete cessation of transport was recorded (Fig. 7.3).

These transport experiments results confirms the ultrastructural and wound callose formation investigations (see Chapters 3, 5 and Saheed et al., 2007a, c). In the case of RWA infestation, severe damage to vascular bundles and other tissues, along with extensive wound callose formation, occurs when compared to the reduced damage effects observed with BCA infestation. It is logical to state that the damage to vascular tissues and subsequent deposition of wound callose in phloem tissues would lead to the disruption and reduction in the transport capacity of the phloem in the infested plant, with RWA being the most damaging. The situation is quite different when BCA feed on barley leaves, where limited damage to the vasculature and little or no formation of wound callose occurs during prolonged feeding and low levels of infestation. Reduction in phloem transport capacity occurs only during long-term feeding by BCA. Watanabe and Kitagawa (2000) have reported that infestation of rice by the planthopper (*Nilaparvata lugens*) results in reduction in photosynthetic capacity, removal of assimilates and yield loss. I therefore suggest that the disruption of the vascular system and the diversion of assimilate which occurs during infestation by RWA and BCA is, in part, responsible for the yield losses reported for these aphids. The degree of these diversions might explain the higher yield losses (30 – 60%) reported during RWA infestation (Du Toit

and Walters, 1984) compared to the reduced yield loss (21%) that occurs during BCA infestation (Riedell et al., 1999).

In conclusion, the differences observed in the damage to the transport systems appears to be responsible in part for the chlorosis, necrosis and leaf roll which occurs in RWA-infested plants, these damage effects eventually leading to higher yield losses encountered when this aphid infest crops. The reduction in the damage to transport system is less severe in BCA infestation – the plants, as a result of lesser damage, might be able to tolerate low infestations better than they would under RWA infestation. This reduced damage to phloem transport appears to be partly responsible for the absence of visible damage symptoms and reduced yield losses recorded during BCA infestation of crop plants.

8.5 Overall conclusion

The results presented in this thesis show that RWA is a serious and most destructive phloem feeder, when compared to the BCA. Their mechanism of feeding involved damage to probed tissues such as mesophyll cells and eventually to the vascular tissues from where they feed and drink. I have shown that severe damage to all cellular tissues occurs when RWA feeds on host plants, which result in apoplasmic and symplasmic isolation of xylem and phloem tissues. Reduced damage results when BCA feeds on the same host for the same time and same population levels. Resistant genes, in this case *Dn1* gene, appear to function by conferring resistant to cell damage on the cultivar containing such genes during aphid feeding. I also report that host plants respond to aphid feeding effectively by reducing the amount of assimilate available to the aphids through formation of wound callose. This response has been demonstrated to be species-specific, a quick response results when RWA feeds even at a very low population level, whilst a response occurs only at a higher infestation level by BCA. Probing and feeding by RWA and BCA resulted in differences in damage to the cellular tissues. A difference in damage, among other things, comes with a diverse pattern of wound callose formation and varying degrees of damage to the transport capacity of the phloem. These differences in the damage signatures, in turn, caused the differences in the observed symptoms and resulting yield losses upon infestation by these aphids. Plant breeders can work on constructing cultivars with increased cellular resistant genes, especially those

that can confer resistance to salivary damage in vascular bundles' tissue; most importantly, xylem vessels and phloem sieve tubes. This will prevent the characteristic apoplasmic and symplasmic isolation of these tissues shown in this thesis as the significant mechanism of aphid-induced damage in infested plants. The results presented in this thesis have provided useful insight into the feeding by the two aphid species on barley and wheat cultivars investigated here. The suggestions and conclusions raised in each chapter can thus be useful in predicting how other, similar aphid species infest and feed on other cereal crops. I also strongly believe that these results will have spin-off and that more effort should be directed towards elucidating the interaction between the two aphid species and the cereal crops investigated in this thesis at the molecular level. Nevertheless, this work will hopefully serve as template for future research insight into aphid feeding, given that the focus in this thesis was on a structural and functional assessment of aphid feeding, and the plants' responses to that feeding.

8.6 Future research

The usefulness of combined structural and functional studies such as that reported in this thesis is that they promote better understanding of plant-aphid interactions as related to cell damage. I believe that this approach could be used to investigate the effects of aphid feeding and the resultant damage on the metabolic activities of the plant, such as down- or up-regulation of photosynthetic capacity. Given the current high level of awareness of climate change, studies could be extended to conduct comparative studies under ambient and elevated CO_2. Questions that might arise include: will aphids reproduce faster or slower under elevated CO_2? Will plants cope better with aphid infestation during such conditions? Will resistant cultivars remain resistant under elevated CO_2? These questions are of fundamental importance and their answers will give rise to modelling and prediction of future effects of aphid feeding on crop plants.

This study has provided some insight about the regulation of callose synthase and β-1, 3-glucanase during infestation of the two aphid species with respect to callose formation. Further studies could examine the role of the already identified gene sequences on the callose formation during aphid feeding.

The emergence of new aphid biotypes with resistance-breaking capacity clearly calls for research into their interaction between aphids and cereal crops. A new biotype of RWA, RWA SA2 (Tolmay et al., 2007) has emerged in South Africa, which has resistance-breaking capacity to present resistant

wheat cultivars. Haley et al. (2004) had earlier reported a similar resistant-breaking biotype in Colorado, USA. The results of the experiments presented in this thesis reflect the effects of the original South African biotype, now known as RWA-SA1. The appearance of the new biotype of the Russian wheat aphid (RWA SA2) presents a completely new opportunity and new scope for studies of the effects that the biotype will have on small grain industry. A preliminary study (Walton, 2007) shows that RWA-SA2 breeds faster and as a result it has the ability to become an even greater pest than RWA SA1 already is. The results presented in this thesis could be used as a template for further study of the mechanism of feeding of this new biotype and can be extended to other aphid biotypes and cultivars in the future.

9 REFERENCES

Aalbersberg KY, van der Westhuizen MC, Hewitt PH (1988) Occurrence of the Russian wheat aphid *Diuraphis noxia* between wheat seasons in the Orange Free State. Phytophylactica **20**: 87-88

Agrawal AA, Sheriffs MF (2001) Induced plant resistance and susceptibility to late-season herbivores of wild radish. Annals of the Entomological Society of America **94**: 71-75

Agronomy Guide (2002) Cereals: cereal aphid. Ministry of Agriculture, Food and Rural Affairs Ontario. Publication 811.
http://www.omafra.gov.on.ca/english/crops/pub811/6aphid.htm#history
(Accessed February 9 2007)

Al-Mousawi AH, Richardson PE, Burton RL (1983) Ultrastructural studies of greenbug (Homoptera: Aphididae) feeding damage to susceptible and resistant wheat cultivars. Annals of the Entomological Society of America **76**: 964-971

Altus DP, Canny MJ (1982) Loading of assimilates in wheat leaves. The specialization of vein types for separate activities. Australian Journal of Plant Physiology **9**: 571-581

Badr A, Müller K, Schäfer-Pregl R, El Rabey H, Effgen S, Ibrahim HH, Pozzi C, Rohde W and Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). Molecular Biology and Evolution **17**: 499-510

Blackman E (1971) The morphology and development of cross veins in the leaves of bread wheat (*Triticum aestivum*, L.). Annals of Botany **35**: 653-665

Blanco RL, Adamson HY, Hales DF (1992) *Proceedings of the XIX International Congress of Entomology*. Beijing, China; p. 167

Botha A-M, Lacock L, van Niekerk C, Matsioloko MT, du Preez FB, Loots S, Venter E, Kunert KJ, Cullius CA (2006) Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'TugelaDN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? Plant Cell Reports **25**: 41-54

Botha CEJ, Mabindisa SEW (1977) Observations on the penetration of the phloem in young stems of *Nerium oleander* (Linn) by stylets of the aphid, *Aphis nerii* (B. de F.). South African Journal of Science **73**: 276-277

Botha CEJ, Malcom SB, Evert RF (1977) An investigation of preferential feeding habit of four Asclepiadaceae by the aphid, *Aphis nerii* (B. de F.) Protoplasma **92**: 1-19

Botha CEJ, Evert RF (1978) Observations of preferential feeding by the aphid, *Rhopalosiphum maidis* on abaxial phloem of *Cucurbita maxima*. Protoplasma **96**: 75-80

Botha CEJ, Evert RF, Cross RHM, Marshall DM (1982) Comparative anatomy of *Themeda triandra* Forsk. Leaf blades. A correlated light and electron microscopy study. Journal of South African Botany **48**: 311-328

Botha CEJ, Evert RF (1988) Plasmodesmatal distribution and frequency in vascular bundles and contiguous tissues of the leaf of *Themeda triandra*. Planta **173**: 433-441

Botha CEJ, Cross RHM (1997) Plasmodesmatal frequency in relation to short-distance transport and phloem loading in leaves of barley (*Hordeum vulgare*). Phloem is not loaded directly from the symplast. Physiologia Plantarum **99**: 355-362

Botha CEJ, van Bel AJE (1992) Quantification of symplastic continuity as visualized by plasmodesmograms: diagnostic value for phloem-loading pathways. Planta **187**: 359-366

Botha CEJ, Cross RHM, Van Bel AJE, Peter CI (2000) Phloem loading in the sucrose-export-defective (SXD-1) mutant maize is limited by callose deposition at plasmodesmata in bundle sheath-vascular parenchyma interface. Protoplasma **214**: 65-72

Botha CEJ, Cross RHM (2001) Regulation within the supracellular highway-palsmodesmata are the key. South African Journal of Botany **67**: 1-9

Botha CEJ, Matsiliza B (2004) Reduction in transport in wheat (*Triticum aestivum*) is caused by sustained phloem feeding by Russian wheat aphid (*Diuraphis noxia*). South African Journal of Botany **70**: 249-254

Botha CEJ (2005) Interaction of phloem and xylem during phloem loading – Functional symplasmic roles for thin- and thick-walled sieve tubes in monocotyledons? In: Holbrook NM, Zweiniecki MA (Eds.), Vascular Transport in Plants. Elsevier, London, pp 115-130

Bowles DJ (1990) Defense-related proteins in higher plants. Annual Review of Biochemistry **59**: 873-907

Budak S, Quisenberry SS, Ni X (1999) Comparison of *Diuraphis noxia* resistance in wheat isolines and plant introduction lines. Entomologia Experimentalis et Applicata **92**: 157-164

Burd JD, Burton RL (1992) Characterization of plant damage caused by Russian wheat aphid (Homoptera: Aphidae). Journal of Economic Entomology **85**: 2017-2022

Butts PA, Pakendorf KW (1984) The utility of the embryo count method in characterizing cereal crops for resistance to *Diuraphis noxia*. In: Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* (Mordvilko) research in the Republic of South Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, pp 53-57

Cagampang GB, Pathak MD, Juliano OB (1974) Metabolic changes in the rice plant during infestation by brown planthopper, *Nilaparvata lugens* Stål. (Hemiptera: Delphacidae). Applied Entomology and Zoology **9**: 174-184

Canning ESG, Penrose MJ, Barker I, Coates D (1995) Improved detection of barley yellow dwarf virus in single aphids using RT-PCR. Journal of Virological Methods **56**: 191-197

CAPS Program (1994) Fact sheet: The Russian when aphid, *Diuraphis noxia* (Mordvilko). http://ceris.purdue.edu/napis/pests/rwa/facts.txt (accessed February 4, 2007)

Cabrera HM, Argandoña VH, Corcuera LJ (1994) Metabolic changes in barley seedlings at different aphid infestation levels. Phytochemistry **35:** 317–319

Castro AM, Ramos S, Clua AA, Vasicek A, Gimenez DO (1996) Genetic variability in greenbug and RWA populations collected throughout Argentina. In Proceedings of the IV Chilean Congress of Entomologist, Temuco, Chile, p 118

Chamberlain K, Guerrieri E, Penacchio F, Pettersson J, Picket JA, Poppy GM, Powell W, Wadhams LJ, Woodcock CM (2001) Can aphid-induced plant signals be transmitted aerially and through the rhizosphere? Biochemical Systematics and Ecology **29**: 1063-1074

Chan CK, Forbes AR, Raworth DA (1991) Aphid-transmitted viruses and their vectors of the world. Agriculture Canada Research Branch Technical Bulletin. 1991-3E, pp 216

Cherney JH, Marten GC (1982) Small grain crop forage potential. II. Interrelationships among biological, chemical, morphological and anatomical determinants of quality. Crop Science **22**: 240-245

Colbert JT, Evert RF (1982) Leaf vasulature in sugarcane (*Saccharum officinarum* L.) Planta **156**: 136-151

Currier HB (1957) Callose substance in plant cells. American Journal of Botany **44**: 488-496

Currier HB, Webster DH (1964) Callose formation and subsequent disappearance: studies in ultrasound stimulation. Plant Physiology **39**: 843-847

Damsteegt VD, Gildow FE, Hewings AD, Carroll TW (1992) A clone of the Russian wheat aphid (*Diuraphis noxia*) as a vector of the Barley Yellow Dwarf, Barley Stripe Mosaic and Brome Mosaic Viruses. Plant Disease **76**: 1155-1160

Dannenhoffer JM, Ebert Jr. W, Evert RF (1990) Leaf vasculature in barley, *Hordeum vulgare* (Poaceae). American Journal of Botany **77**: 24-34

Dannenhoffer JM, Evert RF (1994) Development of the vascular system in the leaf of barley(Hordeum vulgare L.). International Journal of Plant Sciences **155**: 143-157

De Wet LR, Botha CEJ (2007) Resistance or tolerance: An examination of aphid (*Sitobion yakini*) Phloem feeding on Betta and Betta-Dn wheat (*Triticum aestivum* L.). South African Journal of Botany **71**: 35-39

Dixon AFG (1971) The life cycle and host preferences of the bird cherry-oat aphid *Rhopalosiphum padi* L., and their bearing on the theories of host alternation in aphids. Annals of Applied Biology **68**:135-147

Donofrio NM, Delaney TP (2001) Abnormal callose response phenotype and hypersusceptibility to *Peronospora parasitica* in defense-compromised Arabidopsis nim1-1 and salicylate hydroxylase-expressing plants. Molecular Plant-Microbe Interaction **14**: 439-450

Douglas AE (1993) The nutritional quality of phloem sap utilized by natural aphid populations. Ecological Entomology **18**: 31-38

Du Toit F, Walters MC (1984) Damage assessment and economic threshold values for chemical control of the Russian wheat aphid *Diuraphis noxia* (Mordvilko) on winter wheat. In: Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* (Mordvilko) research in the Republic of South

Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, pp 58-62

Du Toit F, Van Niekerk HA (1985) Resistance in Triticum species to the Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Hemiptera: Aphididae). Cereal Research Communications **13**: 371-378

Du Toit F (1987) Resistance in wheat (*Triticum aestivum*) to *Diuraphis noxia* (Homoptera: Aphididae). Cereal Research Communications **15**: 175-179

Du Toit F (1988) A greenhouse test for screening wheat seedlings for resistance to the Russian wheat aphid, *Diuraphis noxia* (Homoptera: Aphididae). Phytophylactica **20**: 321–322

Du Toit F (1989a) Inheritance of resistance in two *Triticum aestivum* lines to Russian wheat aphid (Homoptera: Aphididae). Journal of Economic Entomology **82**: 1251-1253

Du Toit F (1989b) Components of resistance in three bread wheat lines to Russian wheat aphid (Homoptera: Aphidiae) in South Africa. Journal of Economic Entomology **82**: 1779–1781

Ellis RP (1987) A review of comparative leaf blade anatomy in the systematics of the Poaceae: the past twenty-five years. In Soderstrom TR, Hilu KW, Campbell CS, Barkworth ME (Eds.), Grass systematics and Evolution. Smithsonian Institution Press, Washington, DC, pp 3-10

Evert RF, Derr WF (1964) Callose substance in sieve elements. American Journal of Botany **51**: 552-559

Evert RF, Eschrich W, Medler JT, Alfeiri FJ (1968) Observation of the penetration of linden branches by the stylets of the aphid *Longistigma caryae* (Ham.) American Journal of Botany **55**: 860-874

Evert RF, Eschrich W, Eichhorn SE, Limbach ST (1973) Observation on penetration of barley leaves by the aphid *Rhopalosiphum maidis* (Fitch). Protoplasma **77**: 95-110

Evert RF, Eschrich W, Heyser W (1977) Distribution and structure of the plasmodesmata in mesophyll and bundle-sheath cells of *Zea mays* L. Planta **136**: 77-89

Evert RF, Eschrich W, Heyser W (1978) Leaf structure in relation to solute transport and phloem loading in *Zea Mays* L. Planta **138**: 279-294

Evert RF, Mierzwa RJ, Eschrich W (1988) Cytochemical localization of phosphatase activity in vascular bundles and contiguous tissues of the leaf of Zea *mays* L. Planta **159**: 193-206

Evert RF, Russin WA, Botha CEJ (1996) Distribution and frequency of plasmodesmata in relation to photoassimilate pathways and phloem loading in the barley leaf. Planta **198**: 572-579

FAO (1998) World cereal production – Challenges and opportunities. FAO, 1998

Farrar SC, Farrar JF (1986) Compartmentation and fluxes of sucrose in intact leaf blades of barley. New Phytologist **103**: 645-657

Farrar J, van der Schoot C, Drent P, van Bel AJE (1992) Symplastic transport of Lucifer yellow in mature leaf blades of barley: potential mesophyll-to-sieve-tube transfer. New Phytologist **120**: 191-196

Feng MG, Johnson JB, Nowierski RM, Halbert SE (1992) Population trends and biological aspects of cereal aphids (Homoptera: Aphididae), and their natural mortality factors on winter wheat in southwestern Idaho. Pan-Pacific Entomologist **68**: 248-260

Forslund K, Pettersson J, Bryngelsson T, Jonsson L (2000) Aphid infestation induces PR-proteins differently in barley susceptible or resistant to the bird cherry-oat aphid (*Rhopalosiphum padi*) Physiologia Plantarum **110**: 496-502

Fouché A (1983) Voedingskade veroorsaak deur die Russiese koringluis, *Diuraphis noxia*, op korin en verwante gasheerplante. MSc. Thesis, University of Free State, South Africa

Fouché A, Verhoeven RL, Hewitt PH, Walters MC, Kriel CF, De Jager J (1984) Russian aphid (*Diuraphis noxia*) feeding damage on wheat related cereals and a Bromus grass species. In: Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* (Mordvilko) research in the Republic of South Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, pp 22-33

Fritz E, Evert RF, Heyser W (1983) Microautoradiographic studies of phloem loading and transportation in the leaf of *Zea mays* L. Planta **159**: 193-206

Fritz E, Evert RF, Nasse H (1989) Loading and transport of assimilates in different maize leaf bundles. Digital image analysis of ^{14}C-microautoradiographs. Planta **178**: 1-9

Gange AC, Brown VK (1989) Effects of root herbivory by an insect on a foliar-feeding species, mediated through changes in host plant. Oecologia **81**: 38-42

Gerloff ED, Ortmann EE (1971) Physiological changes in barley induced by greenbug feeding stress. Crop Science **11**: 174-176

Gibson L, Benson G (2002) Origin, History, and Uses of Oat (*Avena sativa*) and Wheat (*Triticum aestivum*). Iowa State University, Department of Agronomy. http://www.agron.iastate.edu/courses/agron212/Readings/Oat_wheat_history.htm (accessed February 17, 2007)

Gill CC, Metcalfe DR (1977) Resistance in barley to corn leaf aphid *Rhopalosiphum maids*. Canadian Journal of Plant Science **57**: 1063-1070

Girma M, Wilde GE, Harvey TL (1993) Russian wheat aphid (Homoptera: Aphididae) affects yield and quality of wheat. Journal of Economic Entomology **86**: 594-601

Grignon N, Touraine B, Durand M (1989) 6(5) Carboxyfluorescein as a tracer of phloem sap translocation. American Journal of Botany **79**: 265-274

Gunning BES, Steer MW (1996) Plant Cell Biology – Structure and Function. Jones and Barlett Publishers

Haley SD, Peairs FB, Walker CB, Rudolph JB, Randolph TL (2004) Occurrence of a new Russian wheat aphid biotype in Colorado. Crop Science **44**: 1589-1592

Haupt S, Duncan GH, Holzerberg S, Oparka KJ (2001) Evidence for symplastic phloem unloading in sink leaves of barley. Plant Physiology **125**: 209-218

Hein G, Brooks L, Johnson G, Massey B, McBride D, Morrison WW, Schultz JT, Spackman E, Peairs F (1990) Economic impact of the Russian wheat aphid in the western United States, 1988-1989. Great Plains Agricultural Council Publication, No. 134

Heng-Moss TM, Ni X, Macedo T, Markwell JP, Baxendale FP, Quisenberry SS, Tolmay V (2003) Comparison of chlorophyll and carotenoid concentrations among Russian wheat aphid (Homoptera: Aphididae)-infested wheat isolines. Journal of Economic Entomology **96**: 475-481

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Technical Communications No.: 22, 2nd Edn. Commonwealth Agricultural Bureau, Farnham, England

Hewitt PH, van Niekerk GJJ, Walters MC, Kriel CF, Fouché A (1984) Aspects of the ecology of the Russian wheat aphid, *Diuraphis noxia*, in the Bloemfontein district. I. The colonization and infestation of sown wheat, identification of summer hosts and cause of infestation symptoms. In: Walters MC, ed. Progress in Russian wheat aphid *(Diuraphis noxia* (Mordw) research in the Republic of South Africa. Technical Communication No. 191, Department of Agriculture, Republic of South Africa, 3-13

Iglesias VA, Meins F Jr (2000) Movement of plant viruses is delayed in a β-1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition. The Plant Journal **21**: 157-166

Hicks PM, Mitchell PL, Dunigan EP, Newsom LD, Bollich D (1984) Effect of three-cornered alfalfa hopper (Homoptera: Membracidae) feeding on translocation and nitrogen fixation in soybeans. Journal of Economic Entomology **77**: 1275-1277

Hill GP (1962) Exudation from aphid stylets during the period of dormancy to bud break in Tili americana (l). Journal of Experimental Botany **13**: 144-151

Ilharco FA, van Harten A (1987) Systematics. In Aphids: their biology, natural enemies and control, vol 2A, Minks AK, Harrewijn P (ed) Elsevier, Amsterdam, pp 51-77

Hoj PB, Hartman DJ, Morrice NA, Doan DNP, Fincher GB (1989) Purification of (1$\rightarrow$3)-β-glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone. Plant Molecular Biology **13**: 31-42

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. The Plant Cell **15**: 2503-2513

Jutidamrongphan W, Andersen JB, Mackinnon G, Manners JM, Simpson RS, Scott KJ (1991) Induction of β-1,3-glucanase in barley in response to infection by fungal pathogens. Molecular Plant-Microbe Interaction **4**: 234-238

Karban R, Baldwin IT (1997) Induced Responses to Herbivory. University of Chicago press, Chicago

Kauss H (1985) Callose biosynthesis as a Ca2+- regulated process and possible relation to the induction of other metabolic changes. Journal of cell science (Supplement) **2**: 89-103

Kempema LA, Cui X, Holzer FM, Walling LL (2007) Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. Plant Physiology **143**: 849-865

Kennedy JS, Booth CO (1951) Host alternation in *Aphis fabae* Scoop. I. Feeding preferences and fecundity in relation to the age and kind of leaves. Annals of Applied Biology **38**: 25-64

Klingauf FA (1987) Feeding, adaptation and excretion. In Aphids: their Biology, Natural enemies and Control, vol. 2A, Minks AK, Harrewijn P (ed) Elsevier, Amsterdam, pp 225-253

Kovalev OV, Poprawski TJ, Stekolshchikov AV, Vereshchagina AB, Grandabur SA (1991) *Diuraphis aizenberg* (Homoptera: Aphididae): key to apterous viviparous females, and a review of Russian language literature on the natural history of *Diuraphis noxia* (Kurdjumov 1913). Journal of Applied Entomology **112**: 425-436

Kriel CF, Hewitt PH, De Jager J, Walters MC, Fouché A, van de Westhuizen MC (1984) Aspects of ecology of the Russian wheat aphid, D. noxia in the Bloemfontein district II. Population dynamics. In: Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* Mordw.) research in the Republic of South Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, 14-21.

Krischik VA (1991) Specific or generalized plant defense: reciprocal interactions between herbivores and pathogens. In Barbosa P, Krischik VA, Jones CG (Eds). Microbial mediation of plant-herbivore interactions. John Wiley and Sons, New York, p 309-340

Leather SR, Walters KFA, Dixon AFG (1989) Factors determining the pest status of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae) in Europe: a study and review. Bulletin of Entomological Research **79**: 345-360

Leszczynski B, Wright LC, Bakowski T (1989) Effects of secondary plant substances on winter wheat resistance to grain aphid. Entomologia Experimentalis et Applicata **52**: 135-139

Li J, Burton RA, Harvey AJ, Hrmova M, Wardak AZ, Stone BA, Fincher GB (2003) Biochemical evidence linking a putative callose synthase gene with (1→3)-*β*-D-glucan biosynthesis in barley. Plant Molecular Biology **53**: 213-225

Li Y-F, Zhu R, Xu P (2005) Activation of the gene promoter of barley β-1,3-glucanase isoenzyme GIII is salicylic acid (SA)-dependent in transgenic rice plants. Journal of Plant Research **118**: 215-221

Litts JC, Simmons CR, Karrer EE, Huang N, Rodriguez RL (1990) The isolation and characterization of a barley 1,3-1,4-*β*-glucanase gene. European Journal of Biochemistry **194**: 831-838

Liu XM, Smith CM, Gill BS, Tolmay V (2001) Microsatellite markers linked to six Russian wheat aphid resistance genes in wheat. Theoretical and Applied Genetics **102**: 504-510

Liu XM, Smith CM, Gill BS (2002) Identification of microsatellite markers linked to Russian wheat aphid resistance genes *Dn4* and *Dn6*. Theoretical and Applied Genetics **104**: 1042-1048

Liu XM, Smith CM, Friebe BR, Gill BS (2005) Molecular mapping and allelic relationships of Russian wheat aphid -resistance genes. Crop Science **45**: 2273-2280

Lowe HJB (1967) Interspecific differences in the biology of aphids on leaves of *Vicia faba* I. Feeding behaviour. Entomologia Experimentalis et Applicata **10**: 347-357

Lush WM (1976) Leaf structure and translocation of dry matter in a C_3 and C_4 grass. Planta **130**: 234-244

Matsiliza B, Botha CEJ (2002) Aphid (*Sitobion yakini*, Eastop) investigation shows thin-walled sieve tubes to be more functional than thick-walled sieve tubes. Physiologia Plantarum **115**: 137-143

Martin B, Collar JL, Tjallingii WF, Fereres A (1997) Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. Journal General Virology **78**: 2701-2705

Mehdy MC (1994) Active oxygen species in plant defense against pathogens. Plant Physiology **105**: 467-472

Messina FJ, Taylor R, Karren ME (2002) Divergent responses of two cereal aphids to previous infestation of their host plant. Entomologia Experimentalis et Applicata **16**: 43-50

Messina FJ, Bloxham AJ (2004) Plant resistance to the Russian wheat aphid: effects on a nontarget aphid and the role of induction. The Canadian Entomologist **136**: 129-137

Miles PW (1999) Aphid saliva. Biological Reviews **74**: 41-85

Mohan Babu R, Sajeena A, Seetharaman K, Reddy MS (2003) Advances in genetically engineered (transgenic) plants in pest management – an overview. Crop Protection **22**: 1071-1086

Moloi MJ, Van der Westhuizen AJ (2006) The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. Journal of Plant Physiology **163**: 1118-1125

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiology **125**: 1074-1085

Moran PJ, Youfa C, Jeffery LC, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Archives of Insect Biochemistry and Physiology **51**: 182-203

Morrison WP, Peairs FB (1998) Response model concept and economic impact. In SS Quisenberry and FB Peairs (Eds) A response model for an introduced pest – The Russian wheat aphid, p1-11

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2001) Pathogenesis-related proteins and their genes in cereals. Plant Cell, Tissue and Organ Culture **64**: 93-114

Nakashima J, Laosinchai W, Cui X, Brown Jr RM (2003) New insight into the mechanism of cellulose and callose biosynthesis: proteases may regulate callose biosynthesis upon wounding. Cellulose **10**: 369-389

Ni X, Quisenberry SS, Heng-Moss T, Markwell J, Sarath G, Klucas R, Baxendale F (2001) Oxidative responses of resistant and susceptible cereal leaves to symptomatic and non-symptomatic cereal aphid (Hemiptera: Aphididae) feeding. Journal of Economic Entomologist **94**: 743-751

Nielsen GR, Lamp WO, Stutte GW (1990) Potato leafhopper (Homoptera: Cicadellidae) feeding disruption of phloem translocation in alfalfa. Journal of Economic Entomology **83**: 807-813

Nkongolo KK, Quick JS, Meyer WL, Peairs FB (1989) Russian wheat aphid resistance of wheat, rye and triticale in greenhouse tests. Cereal Research Communications **17**: 227-232

Noble MD (1958) A simplified clip cage for aphid investigations. Canadian Entomologist **90**: 760

Ohana P, Benziman M, Delmer DP (1993) Stimulation of callose synthesis in vivo correlates with changes in intracellular distribution of the callose synthase activator β-furfuryl-β-glucoside. Plant Physiology **101**: 187-191

Ortego J, Delfino MA (1994) *Diuraphis noxia* (Mordvilko) (Homoptera: Aphididae) in Argentina. Revista de la Facultad de Agronomía **70**: 51-55

Ostergaard L, Petersen M, Mattsson O, Mundy J (2002) An *Arabidopsis* callose synthase. Plant Molecular Biology **49**: 559-566

Peairs FV, Brooks L, Hein G, Johnson G, Massey B, McBride D, Morrison WP, Schultz JT, Spackman E (1989) Economic impact of the Russian wheat aphid in the western United States: 1987-88. Great Plain Agricultural Council Publication, No. 129

Peairs FB (1998) Russian Wheat Aphid Management. Proceedings of The Inaugural National Wheat Industry Forum, San Diego, California

Pettersson J (1994) The bird cherry-oat aphid, *Rhopalosiphum padi* (L.) (Homoptera: Aphididae) and Odours. In: SR Leather, A Wyatt, NAC Kidd and KFA Walter (Eds.), Individuals, populations and patterns in ecology, Intercept Ltd. Andover, pp 3-12

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research **29**: 2002-2007

Pike KS (1988) Russian wheat aphid (*Diuraphis noxia*). Insect answers. Washington State University Extension Services Publication EB 1468. Pullman

Pike K, Allison D (1991) Russian wheat aphid biology, damage and management. Pacific Northwest Research Bulletin 371. Washington State University, Pullman

Pollard DG (1973) Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review. Bulletin Entomol Res 62: 631-714.

Prado E, Tjallingii WF (1994) Aphid activities during sieve element punctures. Entomologia Experimentalis et Applicata **72**: 157-165

Prado E, Tjallingii WF (1997) Effects of previous plant infestation on sieve elements acceptance by two aphids. Entomologia Experimentalis et Applicata **82**: 189-200

Quick JS, Stromberger JA, Clayshulte S, Clifford B, Johnson JJ, Peairs FB, Rudolph JB, Lorenz K (2001) Registration of 'Prairie Red' wheat. Crop Science **41**: 1362-1363

Radford JE, Vesk M, Overall RL (1998) Callose deposition at plasmodesmata. Protoplasma **201**: 30-37.

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters **339**: 62-66

Richmond TA, Somerville CR (2000) The cellulose synthase superfamily. Plant Physiology **124**: 495-498

Riedell WE (1989) Effects of Russian wheat aphid infestation on barley plant response to drought stress. Physiologia Plantarum **77**: 587-592.

Riedell WE, Kieckhefer RW, Haley SD, Langham MAC, Evenson PD (1999) Winter wheat responses to bird cherry-oat aphids and barley yellow dwarf. Crop Science **39**: 158-163

Roulin S, Xu P, Brown AHD, Fincher GB (1997) Expression of specific (1→3)-β-glucanase genes in leaves of near-isogenic resistant and susceptible barley lines infected with the leaf scald fungus (*Rhynchosporium secalis*). Physiological and Molecular Plant Pathology **50**: 245-261

Rovenska B, Natr L (1981) The effect of nitrogen deficiency on leaf anatomy of young spring barley plants. Biologia Plantarum **23**: 291-295

Ruan Y-L, Xu S-M, White R, Furbank RT (2004) Genotypic and developmental evidence for the role of plasmodesmatal regulation in cotton fiber elongation mediated by callose turnover. Plant Physiology **136**: 4104-4113

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell **8**: 1809-1819

Rybicki EP, von Wechmar MB (1984) Serological, Biophysical and Biochemical Investigations of Aphid-Transmitted Viruses of Small Grains. In:

Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* (Mordw.) research in the Republic of South Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, pp 42-46

Saheed SA, Botha CEJ, Liu L, Jonsson L (2007a) Comparison of structural damage caused by Russian wheat aphid (*Diuraphis noxia*) and Bird cherry-oat aphid (*Rhopalosiphum padi*) in a susceptible barley cultivar, *Hordeum vulgare* cv. Clipper. Physiologia Plantarum **129**: 429-435

Saheed SA, Liu L, Jonsson L, Botha CEJ (2007b) Xylem – as well as phloem – sustains severe damage due to feeding by Russian wheat aphid. South African Journal of Botany **73**: 593-599

Saheed SA, Larsson KAE, Delp G, Bradley G, Jonsson LMV, Botha CEJ, (2007c) Russian wheat aphid induces stronger callose deposition than bird cherry-oat aphid when feeding on barley. Physiologia Plantarum (in press)

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA

Sandstrom J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids – a comparison of three aphid species on grasses. Journal of Insect Physiology **46**: 33-40

Schotzko DJ, Smith CM (1991) Effects of preconditioning host plants on population development of Russian wheat aphid (Homoptera: Aphididae). Journal of Economic Entomology **84**: 1083-1087

Shufran KA, Margolies DC, Black WC IV (1992) Variations between biotype E clones of *Schizaphis graminum* (Homoptera: Aphididae). Bulletin of Entomological Research **92**: 407-416

Sjölund RD (1997) The phloem sieve element: a river runs through it. The Plant Cell **9**: 1137-1146

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research **26**: 31-43

Stoetzel MB, Miller GL (2001) Aerial feeding aphids of corn in the United States with reference to the root-feeding *Aphis maidiradicus* (Homoptera: Aphididae). Florida Entomologist **84**: 83-98

Stone BA, Evans NA, Bonig I, Clarke AE (1985) The application of Sirofluor, a chemically defined fluorochrome from aniline blue for histochemical detection of callose. Protoplasma **122**: 191-195

Stone BA, Clarke AE (1992) Chemistry and Biology of (1→3)-β-D-Glucans. La Trobe University Press, Victoria, Australia

Tjallingii WF, Hogen Esch T (1993) Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. Physiological Entomology **18**: 317-328

Tjallingii WF (1994) Sieve element acceptance by aphids. European Journal of Entomology **91**: 47-52

Tjallingii WF (1995) Regulation of phloem sap feeding by aphids. In Regulatory Mechanisms in Insect Feeding, Chapman RF, ge Beor G (Ed) Chapman and Hall, New York. pp 190-209

Tjallingii WF (2006) Salivary secretions by aphids interacting with proteins of the phloem wound responses. Journal of Experimental Botany **57**: 739-745

Tolmay V, Mar'e R (2000) Is it necessary to apply insecticides to Russian wheat aphid resistant cultivars? In CIMMYT. 2000. The eleventh regional wheat workshop for Eastern, Central and Southern Africa. Addis Ababa, Ethiopia. CIMMYT. 183-190

Tolmay V, Lindeque RC, Prinsloo GJ (2007) Preliminary evidence of a resistance-breaking biotype of the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae), in South Africa. African Entomology **15**: 228-230

Turgeon R (1989) The sink-source transition in leaves. Annual Review of Plant Physiology **40**: 119-138

Turgeon R, Beebe DU (1991) The evidence for symplastic phloem loading. Plant Physiology **96**: 349-345

UCIPM (2002) University of California Agriculture and Natural resources, Statewide Integrated Pest Management Programme, Small Grain, Bird cherry-oat aphid. <http://www.ipm.ucdavis.edu/PMG/r730300311.html (accessed on February 13, 2007)

Underwood NC (1998) The timing of induced resistance and induced susceptibility in the soybean-Mexican bean beetle system. Oecologia **114**: 376-381

Van der Westhuizen AJ, Pretorius Z (1996) Protein Composition of Wheat Apoplastic Fluid and Resistance to the Russian Wheat Aphid. Australian Journal of Plant Physiology **23**: 645-648

Van der Westhuizen AJ, Qian X-M, Botha A-M (1998a) Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Reports **18**: 132-137

Van der Westhuizen AJ, Qian X-M, Botha A-M (1998b) β-1,3-glucanases in wheat and resistance to the Russian wheat aphid. Physiologia Plantarum **103**: 125-131

Van der Westhuizen AJ, Qian X-M, Wilding M, Botha A-M (2002) Purification and immuno-cytochemical localization of a wheat *β*-1,3 -glucanase induced by Russian wheat aphid infestation. South African Journal of Science **98**: 197-202

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology **55**: 85-97

Verma DPS, Hong Z (2001) Plant callose synthase complexes. Plant Molecular Biology **47**: 693-701

Voigt CA, Schäfer W, Salomon S (2006) A comprehensive view on organ-specific callose synthesis in wheat (*Triticum aestivum* L.): glucan synthase-like gene expression, callose synthase activity, callose quantification and deposition. Plant Physiology and Biochemistry **44**: 242-247

Walling LL (2000) The myriad plant responses to herbivores. Journal of plant Growth Regulation **19**: 195-216

Walters MC, Penn F, Du Toit F, Botha TC, Aalbersberg K, Hewitt PH, Broodryk SW (1980) The Russian wheat aphid. Farming in South Africa, Leaflet Series, Wheat **G3**, 1-6

Walters MC (1984) Introduction. In: Walters MC, ed. Progress in the Russian wheat aphid (*Diuraphis noxia* (Mordw.) research in the Republic of South Africa. Technical Communication, No. 191, Department of Agriculture, Republic of South Africa, p 2

Walton SM (2007) The effects of two biotypes (SA-1 and SA-2) of Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae) on non-resistant and resistant wheat cultivars Tugela and Tugela-Dn (*Triticum aestivum*). B.Sc. thesis, Rhodes University, Grahamstown, South Africa

Wang J, Xu P, Fincher GB (1992) Purification, characterization and gene structure of (1→3)-*β*-glucanase isoenzyme GIII from barley (*Hordeum vulgare*). European Journal of Biochemistry **209**: 103-109

Wang T, Quisenberry SS, Ni X, Tolmay V (2004) Aphid (Hemiptera: Aphididae) resistance in wheat near-isogenic lines. Journal of Economic Entomology **97**: 646-653

Washington Barley Commission (2007) Washington Barley Facts. http://www.washingtonbarley.org/washingtonbarley/index.html (accessed February 17, 2007)

Watanabe T, Kitagawa H (2000) Photosynthesis and translocation of assimilates in rice plants following phloem feeding by planthopper *Nilaparvata lugens* (Homoptera: Delphacidae). Journal of Economic Entomology **93**: 1192-1198

Webster JA, Starks KJ, Burton RL (1987) Plant resistance studies with *Diuraphis noxia* (Homoptera: Aphididae), a new United States wheat pest. Journal of Economic Entomology **80**: 944-949

Webster JA (1990) Resistance in triticale to Russian wheat aphid (Homoptera: Aphididae). Journal of Economic Entomology **83**: 1091-1095

Webster J, Treat R, Morgan L, Elliot N (2000) Economic impact of the Russian wheat aphid and greenbug in the western United State 1993-1994, 1994-1995, and 1997-1998. USDA-ARS service report PSWCRL Rep. 00-001

Wiktelius S, Weibull J, Pettersson J (1990) Aphid host plant ecology: The bird cherry-oat aphid as a model. In: Campbell RK, Eikenbary RD (Eds.), Aphids-plant genotype interactions, Elsevier Science Publishers, BV, Amsterdam, pp 21-35

Will T, Van Bel AJE (2006) Physical and chemical interactions between aphids and plants. Journal of Experimental Botany **57**: 729-737

Will T, Tjallingii WF, Thönnsessen A, van Bel AJE (2007) Molecular sabotage of plant defense by aphid saliva. Proceeding of The National Academy of Science **104**: 10536-10541

Wraight SP, Poprawski TJ, Meyer WL, Peairs FB (1993) Natural enemies of Russian wheat aphid (Homoptera: Aphididae) and associated cereal aphid species in spring-planted wheat and barley in Colorado. Environmental Entomology **22**: 1383-1391

Xu P, Wang J, Fincher GB (1992) Evolution and differential expression of the (1→3)-β-glucan endohydrolase-encoding gene family in barley, *Hordeum vulgare*. Gene **120**: 157-165

Yamaguchi T, Hayashi T, Nakayama K, Koike S (2006) Expression analysis of genes for callose synthases and Rho-type small GTP-binding proteins that are related to callose synthesis in rice anther. Bioscience, Biotechnology, and Biochemistry **70**: 639-645

Zhang Y, Quick JS, Liu S (1998) Genetic variation in PI 294994 wheat for resistance to Russian wheat aphid. Crop Science **38**: 527-530