COMPARATIVE STUDY OF THE FEEDING DAMAGE CAUSED BY THE SOUTH AFRICAN BIOTYPES OF THE RUSSIAN WHEAT APHID (*Diuraphis noxia* Kurdjumov) ON RESISTANT AND NON-RESISTANT LINES OF BARLEY (*Hordeum vulgare* L.)

THESIS

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MAHBOOB ADEKILEKUN JIMOH

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Abstract

Cereal crop productivity is hampered when these plants are infested by phloem feeding aphids. A great deal of research has been carried out with the direct aim of a clearer understanding of the mechanism involved in the interaction between aphids and their host plants. Research has directly or indirectly been geared towards enhanced plant productivity and achieving sustainable agriculture. Barley (*Hordeum vulgare* L.) is an important small grain crop in South Africa, whose crop performance is negatively affected by fluctuations in weather patterns as well as by agricultural pests. One of the insect pests infesting barley is the Russian wheat aphid, *Diuraphis noxia* Kurdjumov (RWA), of which the two South African biotypes, code-named RWASA1 and RWASA2, were studied in this thesis. During dry spells, RWA infestation becomes a more serious threat to barley productivity. Resistant plants have been used to combat RWA infestation of small grains. In South Africa, 27 RWA-resistant wheat cultivars are currently used in commercial cultivation, but no resistant barley lines have, unfortunately, been developed, in spite of this grain's significant economic importance. This informed the study in this thesis, and this interest particularly focussed on three RWA-resistant lines developed by the USDA, testing their performance against South African RWA biotypes, for possible adaptation to South Africa. The aim was thus to examine the plant-aphid interactions, aphid breeding rates, plant damage

and sustainability, evidence of resistance or tolerance and finally potential performance under elevated CO_2 (a very real climate change threat).

Two major avenues of research were undertaken. The first aspect involved examination of structural and functional damage caused by RWASA1 and RWASA2 on the three resistant and a non-resistant line. Aphid population growth and damage symptoms (chlorosis and leaf roll) of infestation by these aphid biotypes were evaluated. This was followed by a structural and functional approach in which the effects of feeding on the transport systems (phloem and xylem) of barley were investigated. Fluorescence microscopy techniques (using aniline blue fluorochrome, a specific stain for callose and 5,6-CFDA, a phloem-mobile probe) were applied to investigate the feeding-related damage caused by the aphids, through an examination of wound callose formation and related to this, the resultant reduction in phloem transport capacity. Transmission electron microscopy (TEM) techniques provided evidence of the extent of the feeding-related cell damage. The second aspect involved a study of the effect of changing CO_2 concentrations ($[CO_2]$) on the resistant and susceptible barley cultivars to feeding by the two RWA biotypes. Leaves of plants grown at ambient and two elevated levels of $[CO_2]$ were analysed to investigate the effect of changing $[CO_2]$ on biomass, leaf nitrogen content and C:N ratio of control (uninfested) and infested plants.

The population growth studies showed that the populations of the two RWA biotypes as well as bird cherry-oat aphid (BCA, *Rhopalosiphum*

padi L.) increased substantially on the four barley lines. BCA was included here, as it had been the subject of several previous studies. RWASA2 bred faster than RWASA1 on all lines. The breeding rates of the two RWA biotypes were both suppressed and at near-equivalent levels on the three resistant lines, compared to the non-resistant PUMA. This suggests that the resistant lines possessed an antibiosis resistance mechanism against the feeding aphids. Feeding by the aphids manifested in morphological damage symptoms of chlorosis and leaf roll. The two biotypes inflicted severe chlorosis and leaf roll on the non-resistant PUMA. In the resistant plants, leaf rolling was more severe because of RWASA2 feeding compared to RWASA1 feeding. In contrast, chlorosis symptoms were more severe during RWASA1 feeding than was the case with RWASA2 feeding.

Investigation of the effect of aphid feeding on the plants showed that callose was deposited within 24h and that this increased with longer feeding exposure. Wound callose distribution is more extensive in the non-resistant PUMA than in the resistant plants. RWASA2 feeding on the resistant lines caused deposition of more callose than was evident with RWASA1 feeding. During long-term feeding, it was evident that variation in the intensity and amount of wound callose was visible in the longitudinal and transverse veins of the resistant plants. Of the three STARS plants, STARS-9301B had the least callose. Interestingly, wound callose occurred in both resistant and non-resistant plants, in sharp

contrast to what has been reported on resistant wheat cultivars that were developed in South Africa. The relative reduction in the wound callose deposited in the resistant line, when compared to the non-resistant lines, suggests the presence of a mechanism in the resistant lines, which may prevent excessive callose formation. Alternatively, the mechanism may stimulate callose breakdown. RWASA2 feeding on the resistant lines deposited more wound callose than RWASA1 feeding. This evidence supports the hypothesis that RWASA2 is a resistance breaking and more aggressive feeder than RWASA1 is; and further underscores the urgent need for development of RWA-resistant barley cultivars.

The ultrastructural investigation of the feeding damage showed that the two biotypes caused extensive vascular damage in non-resistant plants. There was extensive and severe cell disruption and often obliteration of cell structure of the vascular parenchyma, xylem and phloem elements. In sharp contrast, among the resistant plants, feeding-related cell damage appeared to be substantially reduced compared to the non-resistant PUMA. Low frequency of damaged cells indicated that majority of the cell components of the vascular tissues were intact and presumed functional. There was evidence of salivary material lining the secondary walls of the vascular tissue, which resulted in severe damage. Within xylem vessels, saliva material impregnated half-bordered pit pairs between the vessels and adjacent xylem parenchyma. This is believed to prevent solute exchange through this interface, thereby inducing leaf stress and

leaf roll. A notable finding is that RWASA2 effectively induced more cell damage to vascular tissues in the resistant lines than did RWASA1. In general the experimental evidence (see Chapter 5) suggests that the resistant lines are possibly more tolerant (or able to cope with) to RWA feeding. Evidence for this is the reduction of wound callose and at the TEM level, a comparatively less obvious cell damage in the resistant lines, which suggests that they possess antibiosis and tolerance capacity. The apparent reduction of feeding-related cell damage from the TEM study confirmed the disruptive action of the feeding aphids in experiments using the phloem-mobile probe, 5,6-CF. Results showed that feeding by RWASA1 and RWASA2 reduced the transport functionality of the phloem in all cases, but that RWASA2 feeding caused a more obvious reduction in the rate and distance that 5,6-carboxyfluorescein was transported, than did RWASA1.

Investigation of the effect of changing [CO_2] on the barley cultivars showed that in the absence of aphids and under elevated CO_2 conditions, the plants grew more vigorously. In this series of experiments, the infested plants suffered significant reduction in biomass under ambient (as was expected) and under the two elevated CO_2 regimes. Biomass loss was greater at elevated CO_2 than under ambient [CO_2]. The infested non-resistant PUMA plants showed a more significant biomass loss than did the resistant cultivars. Clearly, the benefits derived from elevated CO_2 enrichment was thus redirected to the now-advantaged aphids. Uninfested

plants showed an increase in leaf nitrogen under the experimental conditions. However, feeding aphids depleted leaf nitrogen content and this was more apparent on plants exposed to RWASA2 than was the case with RWASA1. The end result of this was that C:N ratio of infested plants were higher than uninfested plants. Clearly, the faster breeding rates of the aphids at elevated CO_2 caused depletion of N and a resultant deficiency exacerbated chlorosis as well as leaf rolling due to the higher aphid population density under elevated CO_2 than at ambient. By 28 days after infestation (DAI), majority of the plants exposed to enriched CO_2 treatments had died. A major finding here was thus that although this study demonstrated that elevated CO_2 resulted in an increase in biomass, this was detrimentally offset in plants infested by the aphids, with a decline in biomass and loss of functionality leading to plant death at 28DAI. The overriding conclusion from this study is a clear signal that the twin effects of CO_2 enrichment (a feature of current climate change) and aphid infestations may precipitate potential grain shortages. A disastrous food security threat looms.

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.....’O my Lord! So order me that I may be grateful for Thy favours which Thou hast bestowed on me and on my parents and that I may work the righteousness that will please thee and admit me by Thy Grace to the ranks of Thy righteous servants’ (HQ 27: 19).

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1 Chapter 1: General introduction

1.1 An overview of aphids as pests of cereals

Humankind depends on a wide variety of cereals such as wheat, barley, oat, rye and triticale, either as foodstuff, feeds for livestock, or raw materials for industries. The availability of cereals to meet these human needs has, for some time now, been under severe threat, because the productivity of cereal crops is hampered due to infestation by phloem feeding insects including aphids, in various parts of the world. As a result of serious damage these insects cause to crops and subsequent yield losses encountered, infestation of cereal crops by aphids has generated a great deal of research interest over the years.

The occurrence of aphids in farms where cereal crops are cultivated has been recorded as early as the 18th century (Vickerman and Wratten, 1979). They were then regarded as vectors of barley yellow dwarf virus disease (BYDV), a common viral disease of the grass family for example, but not as pests of economic importance. Aphids are however, an important group of insects, with many of their species occurring as serious pests of cultivated plants and have great impact on agriculture, horticulture and forestry, particularly in the temperate regions of the world (Minks and Harrewijn, 1987; Millar, 1990). As aphids lead a fully mobile life (Miles, 1999), they can spread over extensive areas, migrating between and within winter and summer crops, as well as on grasses, which serve as alternative host plants (Taylor, 1977). Owing to their

high reproductive and mobility rates, aphids have increased the incidences of their pestilence on cereal and fodder crops worldwide. Reports have shown that aphids can, and do cause yield losses in excess of 30% in crop fields, if left unattended (Walters et al., 1980). In fields where crop yield potential was estimated at 5 tons ha^{-1}, for example, losses of 400 – 1800 kg ha^{-1} have been recorded because of infestation by aphids (Anonymous, 2010).

The term “cereal aphids” was originally associated with a few aphids that affect small grain fields in Europe, Canada and Australia (Vickerman and Wratten, 1979). Nowadays, it has assumed a wider, more generic status, incorporating all aphids commonly found on cereal crops, among which are *Sitobion avenae, S. fragarie, S. yakini*, *Metopolophium dirhodum*, *M. festucae, Rhopalosiphum padi*, *R. Maidis, R. rufiabdominalis*, *Schizaphis graminum*, and *Diuraphis noxia*, the Russian wheat aphid (RWA), of which its two South African biotypes are used in studies reported in this thesis.

1.2 General features of aphids

In appearance, aphids are plump, ovoid, soft-bodied insects, measuring between 0.5-7.0mm in length (Dixon, 1978; Millar, 1990). As a single organism, aphids are small and innocuous, but as a colony of them develops (resulting from their pathenogenetic reproduction), they often become very numerous on infested plants. In the crop field, the number on leaves, shoots and roots on an acre of ground is estimated to be over two billion (Dixon, 1978).

Aphids are classified as a single superfamily, the Aphidoidea (Millar, 1990). Although there is a great deal of controversy with respect to their classification (Ilharco and van Harten, 1987), they are generally categorised as belonging to the order Homoptera (and some, Hemiptera) in the family Aphididae, based upon their feeding strategy as plant-sucking bugs (Dixon, 1978; Miles, 1999).

Aphids are most widely distributed in the temperate regions of the world. There is usually a prevalence of aphids in the northern temperate regions compared to their occurrence in the southern parts (Millar, 1990). Out of about 4000 species worldwide (Nault, 1997), only 600 species occur in the southern continents. About 220 of these occur in Africa south of the Sahara, out of which about 136 – the largest number of species – occur in South Africa alone (Millar, 1990). One of these is RWA, which is a destructive pest of small grains (Hein et al., 1990).

1.3 The Russian wheat aphid (RWA – Diuraphis noxia Kurdjumov)

1.3.1 Occurrence and distribution

RWA is indigenous to southern Russia, countries bordering the Mediterranean Sea, Iran and Afghanistan (Walters, 1984). It is widespread throughout Europe, Central Asia, the Middle East and North Africa from where it was introduced to Argentina, North America and South Africa (Stoetzel, 1987; Millar, 1990). Over the past 30 years, RWA has spread to all major cereal-

growing regions of the world (CSIRO, 2007). It was first reported as a pest of wheat in the Orange Free State Region of South Africa in 1978, from where it spread to all other wheat-producing areas (Walters et al., 1980). Several countries yet free of RWA, such as Australia, Argentina and New Zealand, are at serious risk to its invasion (Walters, 1984). RWA are rapidly distributed in the field. Reports have shown that farms of unattended susceptible crops recorded between 20% and 80% of infestation within two weeks, when feeding conditions are favourable (Walters et al., 1980).

1.3.2 Morphology and life cycle

RWA is a relatively small, lime-green to grey aphid (Walters et al., 1980). It has an elongated and spindle-shaped body, which is between 1.4 and 2.3mm in length (Millar, 1990). It has very short antennae, reduced cornicles or tailpipes (a characteristic projection above the tail, giving the appearance of a forked tail, the so-called “double tail”) and lacks siphunculi (a feature typical of other aphids), making it different from other Southern African wheat aphids (Walters et al., 1980). It exists in two forms, depending on the availability of resources (Kieckhefer and Elliot, 1989). These are the winged alate and the wingless apterous females. The latter is the most common morph, which spends most of its time feeding and reproducing. The winged alate female form emerges under specific conditions, such as when the apterous females reach maturity (Peairs, 2010); the host plant is under either biotic or abiotic stress or situation of diminishing food quality, due to growth stage (Baugh and Phillips, 1991). Then, the alate forms relocate to a more

favourable habitat nearby. Since they can only fly short distances on their own, they make use of prevailing convectional current to migrate over long distances (Walters et al., 1980; Kriel et al., 1984).

Upon alighting on suitable hosts, the alate females immediately begin to feed, producing nymphs, which grow into the apterous forms. Under favourable conditions, especially one of ample food quality, the apterae starts the characteristic pathenogenetic reproduction. Each newly emerged female can produce up to four nymphs per day and can live for 60 to 80 days, during which each adult can also produce approximately 80 offspring in their lifetime, maintaining a rate of about four nymphs per day (Walters et al., 1980; Peairs, 1998). The apterae continually infest young leaves as they emerge, forming dense colonies within rolled-up leaves, which, it is believed, are prevented from unrolling to keep their predators and parasitoids away from reaching the aphids, and coincidentally this helps the aphids to avoid being attacked by insecticidal spray (Webster et al., 1987). Once ears form on host plants and developing grains now become strong sinks, RWA population will tend to decline, become winged alates and migrate in search of more favourable host plants (Walters et al., 1980). The ability of RWA to reproduce quickly and spread rapidly within a short period gives it an advantage to re-infest areas where it may have been eliminated by lack of oversummering and alternative grass hosts, severe winter or any other unfavourable feeding and environmental factors (Aalbersberg et al., 1988a; Peairs, 1998).

1.3.3 Biotypes of RWA

Auclair (1987) defined a biotype as an individual or population that can be distinguished from the rest of its species by criteria other than morphology. Aphid biotypes evolve when an individual or population, which arises from an existing population, becomes virulent and successfully infests resistant plants developed against their earlier form (Formusoh et al., 1992; CSIRO, 2007). Such new aphid biotypes may differ from their older ones in a number of characteristics, such as parasite ability, feeding behaviour, digestive system activity, growth, reproduction, survival, nutritional requirements, polymorphism, virus transmission, insecticide resistance, components of saliva, among others (Auclair, 1987; CSIRO, 2007).

Studies have indicated that RWA biotypes are morphologically similar. They only differ in their feeding behaviour, reproductive capacity and virulence (Smith et al., 1992; Puterka et al., 1992, 2006; Basky, 2003; Haley et al., 2004; Walton and Botha, 2008). More recent reports now based RWA biotypic description on the phenotypic response of the host plant to the parasite ability of the aphid (Randolph et al., 2009) and the aphid's salivary component (CSIRO, 2007). This is because RWA has demonstrated the ability to overcome resistant crop varieties developed against them from time to time (Randolph et al., 2010; CSIRO, 2007). This has raised serious concern on the use of resistant cultivars. For instance, in the United States, wheat cultivars bearing *Dn*4 resistance gene are resistant to RWA1 and do not show

symptoms (Randolph et al., 2009), whereas these cultivars are susceptible to RWA2 in wheat farms (Haley et al., 2004).

Since the early 1990s, biotypic variation in RWA has been reported from many parts of the world (Puterka et al., 1992; Basky, 2003). In 2003, a second biotype of RWA, designated as RWA2, was reported from Colorado wheat farms, which was virulent to all existing RWA-resistant wheat varieties, except for those containing *Dn7* resistance gene (Haley et al., 2004). In subsequent years, six additional biotypes were further identified, and a standard nomenclature RWA1 to RWA8 was established (Burd et al., 2006; Weiland et al., 2008). Reports show that new RWA biotypes that are more virulent than older strains have also been identified in countries such as Mexico, Russia, Chile, Argentina and Ethiopia (CSIRO, 2007).

The mechanism behind the evolution of aphid biotypes is not yet fully understood. It has, however, been suggested that biotypic evolution is related to multiple mechanisms governing genetic recombination during the parthenogenetic reproduction form (Puterka et al., 1992). This reproduction mechanism may be responsible for an increase in the subsequent genetic diversity. Another theory postulated that aphid biotypes develop because of the deployment of resistant cultivars, which in turn exert pressure on the existing biotype(s), thereby causing the evolution of new, more virulent biotype(s) of the erstwhile form(s) (see Weiland et al., 2008). This could be true as evolution of several RWA biotypes are majorly restricted to the United States of America and South Africa, where many resistant cultivars of barley

and wheat have been developed and released for field use. However, this theory was debunked by the fact that biotypic diversity had occurred long before resistant cultivars were deployed (Basky, 2003). Moreover, host-adapted races of aphids exist, and have diverged on several noncultivated alternate hosts. As such, alternate hosts of aphids between harvest and planting of new crops seem to be an important factor in the generation of variant biotype(s). This was demonstrated by Weiland et al. (2008) when they collected RWA7 and RWA8 US biotypes from noncultivated grass hosts, both of which exhibited a unique virulence profile among known biotypes of RWA in Colorado. Viewed from another perspective, CSIRO (2007) suggested that the mechanisms controlling virulence in RWA is saliva-based, as it is the aphid's saliva that interacts with the host plant, during feeding. Several genes have been identified (CSIRO, 2007) that significantly differentiate various RWA biotypes and which, in the short-term, provide a molecular diagnostic tool that can be used for identifying various biotypes.

Tolmay et al. (2007) reported the existence of a second RWA biotype in South Africa. It was field-selected and virulent on wheat bearing resistance genes against the old RWA strain. The two biotypes are designated as RWASA1 and RWASA2, and are considered different from the US strains of RWA (Prinsloo, pers. comm.). This position is informed by previous laboratory studies, which demonstrated a high degree of biotypic variation in worldwide collection of RWA (Puterka et al., 1992) and specifically

illustrated the variation of pest status between Hungarian and South African RWA populations (Basky, 2003).

1.3.4 Host range and habitat of RWA

The preferred host plants for RWA are barley, wheat and triticale on which it breeds rapidly (Walters et al., 1980; Webster et al., 1987). Although *D. noxia* is commonly referred to as the Russian wheat aphid, it has been shown that it is more destructive on barley than wheat (see Webster et al., 1993). This view was predicated on the claims by Butts and Pakendorf (1984) that RWA prefers barley over wheat as host plant. Being polyphagous, RWA can feed on many species in the Graminae. It occurs as pest of 43 genera and more than 140 species of grasses found in and around crop fields, which can serve as alternate hosts on which they remain until preferred host plants are available (Walters et al., 1980; Clement et al., 1990; Millar, 1990). RWA tolerates low temperatures, which merely restrict the rate of reproduction, whereas high temperatures do cause high mortality and a drastic reduction in population. Barley, a secondary host to RWA, is the experimental plant selected for the studies reported in this thesis.

1.4 The host plant – barley

1.4.1 Origin, distribution and habitat of barley

Barley (*Hordeum vulgare* L.) is one of the most ancient cultivated grain crops known to man (Harlan and Zohary, 1966; Magness et al., 1971). This cereal belongs to the Graminae family- Poaceae. The origin of barley as a cultivated

plant is debatable. Some authors suggest that its cultivation originated in Egypt, citing as evidence the fact that barley grains were found in pits and pyramids that were built more than 5000 years ago (Young, 2001). Others believe that it was discovered as a wild grass in Asia, where it was cultivated around 1500 to 2000 BC (Young, 2001). It was also reported to have been cultivated much later around 3000 BC in northwestern Europe, and used as a trade item around 3500 BC in Mesopotamia (Young, 2001). Among cereal crops, barley is presently ranked as one of the world's most cultivated small grain, grown in approximately 100 countries worldwide, with annual world production of about 138 million tonnes in 2005 (Young, 2001).

Barley is an important crop of the temperate areas, where it is grown as a summer crop and of the tropical regions, where it is sown as a winter crop. It thrives well in areas that are particularly dry, wet, or cold and in areas in which soils are affected by salinity (Nesbitt, 2005). It is an annual grass that has two growing seasons – winter and spring. It performs best in the spring in temperate regions, with a 90-day growing season. It has a very good resistance to dry heat, compared to other small grains – a feature suitable for its cultivation in near-desert areas, such as North Africa (Young, 2001).

1.4.2 Economic importance of barley

Barley is rich in carbohydrates and moderate amounts of proteins, mineral salts such as calcium and phosphorus and the B vitamins. Barley is mainly grown for livestock feed and for brewing beer. It is used as feed for animals

such as cattle, swine and a wide variety of poultry birds. Its kernels may be rolled, ground or flaked before being rationed to these animals (Nesbitt, 2005). Livestock fed with barley feed generally yield good portions of firm fat and lean meat. Second, barley is processed into malting barley, an important raw material for breweries and food industries for the production of beer, alcoholic drinks, malted syrup, milk drinks and flavouring agents (Young, 2001). Its least common use is as food for humans, when it is processed into pearl barley after milling, after the outer hull and part of the bran layer have been removed. It may then be used to make soup, food dressing, flour, flakes and grits. Its flour is popular in making baby and breakfast cereal foods, bread, cookies and snack bars, either alone or in combination with wheat flour (Young, 2001).

1.4.3 Overview of barley production and its problems in South Africa

Barley is mainly grown in two regions in South Africa, namely the South Western Cape (SWC), where it is under dry land cultivation and the Northern Cape (NC), where it is grown under irrigation (Kotze, SAB Malting, pers. comm.). Available 2009 data on barley production in South Africa shows that area under barley cultivation covers an estimated 73,250 ha (Fig. 1.1), with annual production of 209,313 tons (Fig. 1.2), about 70% of which is produced in the SWC alone (Kotze, SAB Malting, pers. comm.). The major use of barley in South Africa is for the production of pearl barley and malt used for brewing beer. Local industrial consumption of barley is estimated at 270,000

tons per annum. Part of the locally produced barley crop is rated generally less suitable in quality for malting and are then used as livestock feed. The low quality of South African malting barley, shortfall in local production and industrial need to cater for specific brands of products make the Southern Association Maltsters (SAM), the major buyer of barley in South Africa, to rely on importation of higher quality malting barley from the European Union markets.

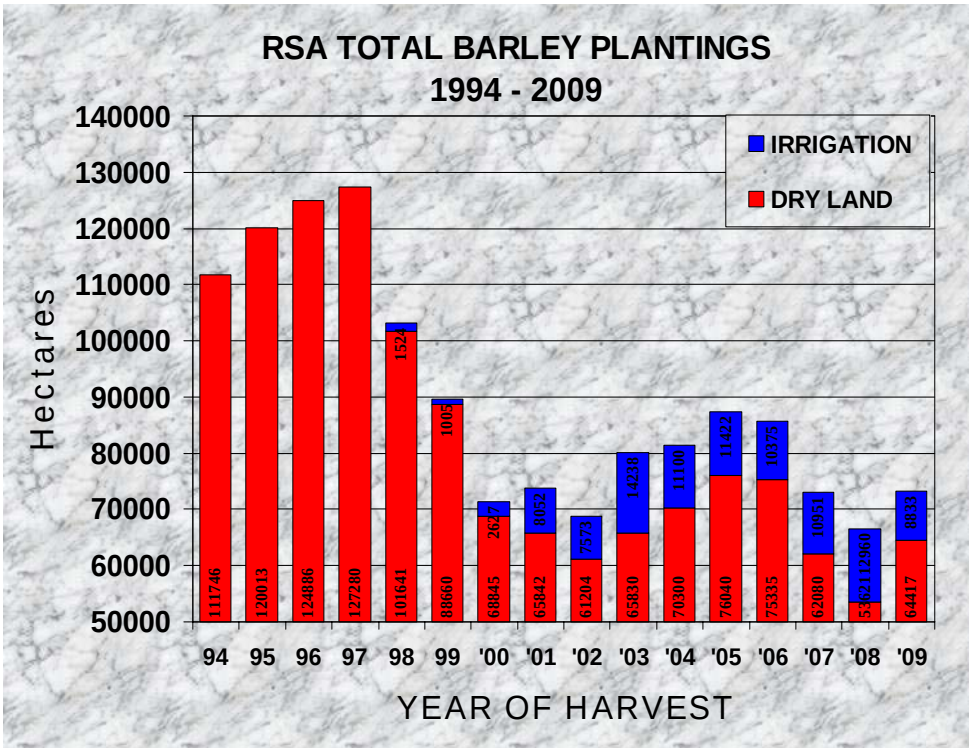

Fig. 1.1 Area of land under barley cultivation in South Africa

Source: SAB Malting, Caledon, South Africa

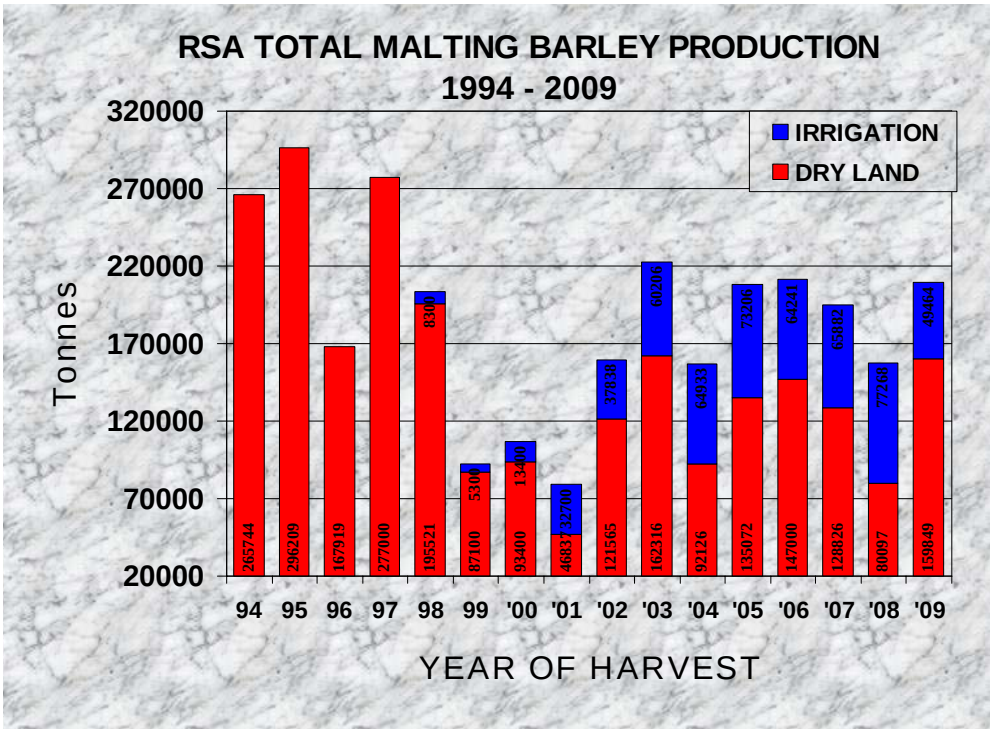

Fig. 1.2 Barley production in South Africa

Source: SAB Malting, Caledon, South Africa

The biggest problem of barley cultivation in the two areas is climatic conditions (Kotze, SAB Miller, pers. comm.). Barley is very sensitive to fluctuations in weather patterns, and this easily affects crop productivity. There are also variations in the two areas in terms of insect infestation and diseases. Available information shows that if the SWC [where majority of the barley is produced (Fig. 1.2)] get dryer due to climate, RWA can become an important pest (VL Tolmay, pers. comm.). Barley productivity is therefore potentially threatened by RWA infestation, particularly in the SWC where it occurs in the dry season (Kotze, pers. comm.). It can be a pest of focus if the region becomes dryer due to vagaries of climate, which is undergoing changes due to emission of greenhouse gases. Outbreak of RWA infestation may further aggravate the currently experienced deficit in production in relation to consumption. Presently, control of sporadic incidences of RWA in barley farms is done by application of insecticides, with the attendant problem of environmental pollution and undesirable barley product contamination. This necessitated studies on evaluation of available RWA-resistant lines, though from another geographical location, which can make adoption of appropriate control measures possible, in line with acceptable universal standards. In contrast to wheat, in which 27 RWA-resistant cultivars have been released for incorporation into integrated pest management schemes (Tolmay et al., 2007), no resistant barley lines have been developed against RWA in South Africa.

1.4.4 Anatomy of the barley leaf

The primary site of RWA feeding is the vascular bundles, which form the transport system of the host plant. A broad understanding of the anatomical features of the leaf is necessary, before discussing the finer details of feeding and feeding effects. An overview of the leaf anatomy provides a good grasp of the structural features of the plant, pre- and post- feeding damage, as well as the mechanisms involved in aphid feeding activities on the host plant.

A mature barley leaf, as typical of the Graminae, is an elongated structure that is morphologically divided into leaf blade and sheath. The anatomical features of mature barley leaves have been fully described by Dannenhoffer et al. (1990). The barley leaf has typical Poaceae anatomy (Blackman, 1971; Dannenhoffer et al., 1990; Dannenhoffer and Evert, 1994; Evert et al., 1996; Cutler et al., 2008). The leaf blade contains a system of longitudinal vascular strands that are net-linked by numerous transverse veins, which are separated by a loosely arranged mesophyll (Dannenhoffer et al., 1990; Dannenhoffer and Evert, 1994). The longitudinal strands are surrounded, entirely or partly, by two bundle sheaths - an outer parenchymatous and an inner mestome sheath (Evert et al., 1996).

Three orders of vein are visible in cross section. These occur in the longitudinal vascular strands (Botha et al., 1982; Dannenhoffer and Evert, 1994). These veins are classified as large (first order), intermediate (second order) and small (third order) veins (Dannenhoffer et al., 1990; Dannenhoffer

and Evert, 1994; Evert et al., 1996; Botha, 2005; Cutler et al., 2008). This categorization is based on the sizes, composition of the xylem and phloem tissues, as well as the nature of other contiguous tissues (Dannenhoffer et al., 1990; Evert et al., 1996). Most of the longitudinal strands individually intergrade structurally from one bundle size (or order) into another, as they descend the leaf, along their length, where the smaller bundles intergrade into intermediate and large bundles. The large and intermediate bundles are associated with girders or strands of hypodermal sclerenchyma that are often reduced and are not in contact with the vascular bundles (Botha et al., 1982; Dannenhoffer et al., 1990; Evert et al., 1996). Most of the longitudinal strands descend both blade and sheath, and enter the stem as large bundles (Dannenhoffer and Evert, 1994). The three different orders of vascular bundles carry out different functions. The small and intermediate bundles serve primarily as loading bundles while the large or first order bundles are mainly involved in longitudinal long-distance transport (Fritz et al., 1989).

1.4.5 Structure of the vascular bundles of barley leaves

In cross section, the vascular bundles of barley leaves show that large bundles are characterised by the presence of two large metaxylem vessels, one each on either side of the protoxylem, which is often represented by a protoxylem lacuna and abaxial to this is a distinct metaphloem region. The protophloem is obliterated in the lower abaxial side of the metaphloem (Cutler et al., 2008). Intermediate bundles lack protoxylem and do not have conspicuous metaxylem vessels. Though protophloem and metaphloem sieve tubes are

present, the protophloem is usually obliterated in mature bundles. The vascular bundles are surrounded by complete mestome sheath. The small bundles lack large metaxylem and protoxylem (Cutler et al., 2008), with the largest of the small bundle containing protoxylem (Evert et al., 1996). A mestome sheath that may be disrupted on the xylem side can surround bundles (Botha and Cross, 1997). They are not associated with either hypodermal strands or girders and they are embedded within the mesophyll.

The functional metaphloem typically consists of two distinct sieve tubes. These are the early-formed thin-walled sieve tubes, with associated companion cells, and the late-formed, thick-walled sieve tubes, which lack companion cells (Botha and Cross, 1997; Cutler et al., 2008). The thick-walled sieve tubes possess cellulosic walls, which may, in rare instances, undergo lignification. Many views have been expressed on the functions of the two types of sieve tubes. While Fritz et al. (1983) reported that the thick-walled sieve tubes are involved in the retrieval of photosynthates from the transpirational stream and/or from the apoplast, Matsiliza and Botha (2002) demonstrated that the thin-walled sieve tubes are more functional in transport and phloem loading than the thick-walled sieve. Evert et al. (1996) and Botha and Cross (1997) have earlier shown that the common wall between the thick-walled sieve tubes and other cell types contains very few plasmodesmata. They concluded that both sieve tube-companion cell complexes and thick-walled sieves might be symplastically isolated from the rest of the leaf. From another dimension, Haupt et al. (2001) reported that both the intermediate and

large bundles of sink leaves in barley contain large numbers of plasmodesmata between cells in the vascular bundles, which facilitate cytoplasmic connections between thick-walled sieve tubes and adjoining cells, situations that are not obtainable in source leaves. This implies that solute loading in source leaves is mostly apoplastic while that of sink leaves is symplastic.

1.5 Implications of aphid feeding on plant growth and productivity

Aphids mainly affect their host plants in three ways. First, aphids can damage plants when they feed directly on phloem sap, thereby denying the plants essential food materials they need for growth (Dixon, 1978; Miles, 1999). Second, as remarked earlier, they may act as vectors of viruses, spreading associated diseases in crop fields through their mode of feeding (Vickerman and Wratten, 1979; Miller, 1990). Lastly, after sustained feeding, aphids excrete honeydews on parts of the plant where they feed which, being sugary, promote growth of saprophytic and pathogenic fungi and bacteria, and indirectly predispose the affected part to infection (Manitoba Agriculture, Food and Rural Initiatives, 2010). Damage caused by direct feeding occurs when colonies of 10 or more aphids invade the plant, from the seedling stage through to the head filling stage (Hewitt et al., 1984). Later, the population of the aphids gradually builds, and this causes damage to the crop field. Evaluation of yearly costs of the feeding damage by RWA infestation on

small grains in the US alone, covering yield loss and procurement of insecticidal input, run into billions of US dollars (Webster et al., 2000).

1.5.1 Symptoms of RWA infestation

Symptoms of RWA infestation have been well documented (see Matsiliza, 2003; Saheed, 2007). As an aphid that predominantly feeds on younger leaves of host plants, it prevents normal unrolling of younger leaves, while the aphid colony densely builds up within the rolled, tube-like and tightly curled leaves (Hewitt et al., 1984). Heavy infestation by aphids causes wheat, barley, oat and triticale plants to turn yellow, become stunted and eventually unproductive (GRDC, 2010). Leaves of RWA-infested susceptible plants show extensive chlorosis and necrosis, which may include white, yellow, purple, or at times reddish-purple longitudinal streaks (Walters et al., 1980; Hewitt et al., 1984; Riedell, 1989; Saheed et al., 2007a). Heavily infested plants often become stunted, with the young tillers flattened, lying almost parallel to the ground (Walters et al., 1980). The ears of infested plants may become bent and turn white, which is indicative of poor yield. Infested resistant plants on the other hand, may develop chlorotic spots. They are able to maintain the chlorophyll content of their leaves longer than susceptible plants do (Van der Westhuizen and Pretorius, 1995). Their growth may, however, be slightly slowed down, as they manage to survive the continuous presence of the aphids. These symptoms result from aphid feeding activities, which impair normal functioning of the chloroplast and associated carbon flux (Botha et al., 2005, 2006; Gutsche et al., 2009; Saheed et al., 2007 a, 2007b).

Prolonged aphid feeding may cause destruction of cell and chloroplast membranes, degeneration of the chlorophyll content and disruption of vascular tissues of host plants (Fouche et al., 1984; Heng-Moss et al., 2003; Wang et al., 2004; Botha et al., 2005; Saheed et al., 2007a, 2007b).

1.5.2 Feeding-related damage caused by aphids

1.5.2.1 Physiological damage

Aphids are innocuous insects, which may individually cause little injury or damage to their host plants (Miles, 1999). When a single aphid feed from the phloem, it causes little damage to the host plant, compared to their chewing insect counterparts (Pollard, 1973; Miles, 1999). A major impact of their feeding damage can therefore be expected with increase in population. The general rule is that larger populations will cause a great deal more damage. Dixon (1978) states that feeding damage caused by aphids on host plants become substantial when aphids occur, as they do, in large numbers. Then, the drain on the plant sap becomes so enormous that it portends insidious damage, which becomes evident in the effects of overall reduction of assimilates available to other parts of infested plants (Dixon, 1971a, 1971b; Pollard, 1973).

During their feeding, aphids redirect assimilates into their gut instead of the more normal continuous flow within the vascular tissues of host plants. This redirection of assimilates turn aphids into secondary sinks (Botha and Matsiliza, 2004). This is detrimental to host plants, as the aphids then compete

with growing parts of the plants for essential nutrients normally transported via the phloem. These plants parts are therefore denied adequate quality and quantity of nutrients, which result in observable deficiency symptoms on infested plants. Girma et al. (1993) reported that plant height, shoot weight and number of spikes are significantly reduced as a result of RWA infestation on wheat.

Reports have shown that while feeding from the phloem, RWA periodically 'drink' water from the xylem (Saheed et al., 2007b). Earlier studies showed that RWA and the bird cherry-oat aphid (*Rhopalosiphum padi*, BCA) infestation decreased the water potentials in leaves of host plants (Riedell, 1989), which caused an increase in proline and glycine-betaine accumulation in leaves. Cabrera et al. (1994) reported that aphid infestation caused drought stress symptoms in leaves of barley, in spite of sufficient root moisture, which resulted in metabolic changes that affected the growth of barley cv Aramir. In addition, Gerloff and Ortmann (1971) observed that *Schizaphis graminum* infestation decreased rate of photosynthesis in barley. Effects of this physiological damage as enumerated above must have a negative impact on the productivity and yield of infested host plants.

1.5.2.2 Ultrastructural damage

Apart from the physiological damage, aphid feeding also causes structural and functional damage to cells and tissues of host plants. Feeding damage

commences after penetration of aphid's stylets into tissues of host plants. In the process of penetration, the stylets disrupt the structure and organisation of plant tissues. These become evident, as mechanical disturbance of tissue structure occurs along with cellular disorganisation, due to discharge of substances contained in the injected saliva (Miles, 1987). The stylets also cause laceration of plant tissues and cell walls, accompanied by occasional cellular penetration and irregular movement of the stylets to both sides, which rupture plasmodesmata (Pollard, 1973; Spiller et al., 1985). As the stylets penetrate inwards, two types of saliva – gelling and watery – are secreted and ejected into the host plant (Miles, 1999). These secretions contain enzymes, which break down the pectin content of the middle lamellae of the cellular organisation (Pollard, 1973; Miles, 1987, 1999; Cherqui and Tjallingii, 2000; Saheed et al., 2007a, 2007b). Continued 'wandering' of the stylets in the inter- and intracellular matrix of parenchyma tissues further disintegrate cellular arrangements in the epidermal and mesophyll tissues *en route* the actual feeding site, the vascular bundle (Spiller et al., 1985; Tjallingii and Hogen-Esch, 1993). It has been shown that damage to mesophyll and vascular tissues of wheat and barley by feeding RWASA1 and BCA caused cell wall destruction, disruption of cellular contents, splitting apart of mesophyll cells and cleavage and occlusion of plasmodesmatal fields (Saheed et al., 2007a, 2007b). Report by Miles (1989) showed that cells close to where there is tissue disturbance during stylets' penetration experience plasmolysis and increased cytoplasmic streaming. This was further corroborated by Saheed et

al. (2007a, 2007b) that plasmolysis of bundle and mestome sheaths occurred during RWASA1 and BCA infestation of barley and wheat.

1.5.3 Control measures against RWA in South Africa

Great efforts and resources have been committed globally to curb losses incurred due to RWA infestation. In South Africa, there was little information available regarding control of RWA at its introduction in 1978 (Smit et al., 2010). Thus, large-scale chemical control was adopted. By 1980, RWA became the target of an integrated control strategy (Tolmay et al., 2000). This strategy combines application of insecticides with the use of a variety of natural enemies, such as parasitic wasps (Prinsloo, 1988, 2000; Prinsloo and Du Plessis, 2000), predators (Aalbersberg et al., 1988b), entomopathogenic fungi (Hatting et al., 1999, 2000, 2004). In addition, some cultural practices, such as delayed planting, cultivation of non-host crops and eradication of oversummering or overwintering alternative and primary hosts (Walters, 1984; Du Toit, 1989a) were adopted. Pest control in modern agriculture is increasingly shifting away from reliance on exogenously applied pesticides, which are both costly and toxic, towards the use of a more environmentally-friendly resistant cultivars (Gatehouse, 2002) or at best, its incorporation into an integrated pest management scheme, using a workable combination (Tolmay et al., 2007). This brought about a drastic reduction in the frequency and quantity of pesticides.

1.6 An assessment of breeding programmes for RWA-resistant plants

Development of resistant variety of plants involves breeding a plant, using naturally occurring genes in its gene pool that would make it endogenously resistant to insects, pathogens or any other predator, after series of selection procedures, using phenotypically expressed indicators. In 1987, Du Toit conducted pioneering research works on development of wheat cultivars with genetic resistance to RWA in South Africa (see review by Smit et al., 2010). This was after a thorough selection process by which endogenous resistance genes were discovered in the common bread wheat accession PI 137739, which is native to Iran. Initial thought on this breakthrough in RWA control measure was based on the belief that genetic resistance should be present in the primitive wheat species and varieties from Asia, the original distribution area of both wheat and RWA (Du Toit, 1987, 1988). As a result, *Dn1* resistance gene was identified and incorporated into Tugela cultivar to develop the near-isogenic Tugela-*Dn* engineered wheat cultivar (Du Toit, 1989a). This RWA-resistant cultivar was released in South Africa in 1992 (Van Niekerk, 2001).

In addition to the above initial effort, Liu et al. (2002, 2005) identified 10 RWA resistance genes among small grains, excluding barley. Reports show that many RWA-resistant wheat cultivars have been developed, integrated into RWA pest management scheme and released to wheat growers in South Africa (see reviews by Tolmay and van Deventer, 2005; Tolmay et al., 2007;

Smit et al., 2010). Situation reports show that these cultivars are effective in controlling RWA infestations in wheat fields (Smit et al., 2010). The resistant wheat cultivars inhibited aphid growth and reproduction, leading to a reduction in both the percentage of infested tillers and number of RWA per tiller. Furthermore, their leaves do not roll closed, leaving the aphids exposed, thereby enhancing the success of the complimentary control strategies, such as modestly reduced application of pesticides and introduction of natural enemies (i.e. biological control), in an integrated pest management scheme (Smit et al., 2010).

Several resistance genes have also been identified and used in breeding RWA-resistant barley lines in other parts of the world. Robinson et al. (1991) and Webster et al., 1991 reported the availability of resistant barley lines in Mexico and the United States respectively. In 1992, CI 1412, the parent plant used to develop STARS-0502B (Mornhinweg et al., 2006b), and one of the resistant lines evaluated against RWA in this thesis, was identified along with three other lines (Webster et al., 1993). Two RWA resistance genes were also identified from two resistant barley lines (PI 366444 and PI 366453), which are native to Afghanistan (Nieto-Lopez and Blake, 1994). Several other breeding programmes have led to the development of many RWA-resistant barley lines, which have proved to be effective in the field (see Mornhinweg et al., 1995a, 1999, 2002, 2006a; Mornhinweg and Porter, 2006; Bregitzer et al., 2003, 2005, 2008; Mittal et al., 2008, 2009). However, these lines have been developed mainly against the US RWA biotypes. It is feared that direct

use of any of these US-developed resistant lines may not be effective against the South African strains of RWA (Tolmay, pers. comm.). This is due to identified worldwide biotypic variation in RWA, which is making resistant plant germplasm to be geographically limited, and the reported instances that RWA-populations in various parts of the world interact differently with resistant cultivars (Puterka et al., 1992). Up to this time, no RWA-resistant barley cultivar has been developed in South Africa, hence, the relevance of this work.

In this thesis, I have selected three US-developed RWA-resistant barley lines, using a commercial barley cultivar as control, to study their respective responses to the two South African biotypes of RWA under controlled environment.

1.7 Plant-aphid interaction in a changing environment

The enrichment of atmospheric carbon dioxide CO_2 due to emission from industries and automobiles, along with its attendant global warming effects is of great global concern. In recent decades, scientists have investigated divergent effects of this global phenomenon on different aspects of human and plants lives. The consequences of changes which global warming cause to natural ecosystems present a major challenge on the diversity of the interactions between plants and animals, particularly insect herbivores such as aphids.

Many studies on the effects of climate change and specifically elevated CO_2 on plant-aphid interactions were carried out with emphasis on the response from the aphids (Holopainen and Kössi, 1998; Holopainen, 2002; Peltonen et al. 2006). Few studies, such as those of Lindroth et al. (1995), Curtis and Wang, (1998) and Hughes and Bazzaz, (2001), placed emphasis on the effects on plants. These studies collectively show that plants raised under elevated CO_2 conditions have higher photosynthetic rates, increase in C:N ratios and that the water content in the foliage is considerably altered leading to tough-textured leaves and concentrations of defensive chemicals. There is a gap in the literature on how aphids' performances at elevated CO_2 will influence the widely reported increase in plant biomass of a given plant-aphid interaction. Furthermore, the mechanisms of these important physiological changes in plants were poorly understood.

1.8 Rationale for studying the effects of the two South African RWA biotypes' feeding on barley

Efforts have been committed towards combating the threats, discussed previously, which RWA poses to small grain production. Modern integrated pest management schemes has been employed, the arrowhead of which is the use of resistant plants, considered more efficient, safe and environmentally friendly, compared to the use of insecticides alone. Several RWA-resistant wheat and barley cultivars have been developed all over the world. In South Africa, 27 RWA-resistant wheat cultivars have been released (Smit et al., 2010). As stated earlier, no RWA-resistant barley cultivar has been developed

in South Africa despite its importance as industrial raw material for breweries, confectionary industries as well as livestock farmers. Yet, RWA is a potential threat to its production, which underscores the importance of this study.

As effective, environmentally safe and friendly as the use of resistant plants is, its limitation is that it is only effective against a specific biotype(s) of a given pest. The resistance held by a resistant variety of plant can be broken by evolution of a different pest biotype from those carrying the resistant gene, in much the same way that pests are known to evolve strains that cannot be affected by pesticides formulated to eradicate them (Gatehouse, 2002; Porter et al., 1997; Quick et al., 2001). Evidently, since 2003, virulent and resistance-breaking biotypes of RWA have evolved in the United States of America (Haley et al., 2004; Burd et al., 2006; Jyoti et al., 2006; Michaud et al., 2006; Puterka et al., 2006; Voothuluru et al., 2006; Puterka et al., 2007; Shufran et al., 2007; Merrill et al., 2008; Weiland et al., 2008), and in South Africa (Tolmay et al., 2007). This development has negative effect on the durability of plant resistance to RWA among small grains, raising deep concerns for future deployment in control measures (Randolph et al., 2010). The new South African RWA biotype is code-named RWASA2 (Tolmay et al., 2007). A preliminary comparative study on this biotype by Walton and Botha (2008) revealed that it breeds faster, causes more damage than its preceding form and virulent on a wheat line resistant to RWASA1.

Previous structural and ultrastructural studies have examined the pathway of aphids' stylets in the plant tissue as they feed (Evert et al., 1973; Matsiliza and

Botha, 2002; Botha, 2005; Saheed et al., 2007a, 2007b). Such studies have contributed to the present knowledge of the effects of aphid feeding on structure and function of the transport system of plants. It has, however, been felt that what is seen as morphological and physiological damage exhibited by plants in the various symptoms are products of damage occurring basically at the cellular level, as a result of feeding by aphids. Studies by Botha and Matsiliza (2004) and Saheed et al. (2007a, 2007b) gave insight into the damage done to the transport tissues of plants due to RWA infestation. Continued evolution of resistance-breaking biotypes of the aphid makes it necessary to update what is currently known about the long-term effect of the emerging biotypes on the transport system of their host plants.

The emergence of RWASA2 into the pest stage in South Africa calls for studies on the nature of its damage to host plant cells and tissues that makes it more virulent on host plants than RWASA1. This will improve our current level of knowledge on the mechanism and effects of RWA feeding on small grains. It would invigorate the cause-effect focus of breeders for plant resistance as soon as there is evolution of a new biotype; and from morphological considerations to the more desirable structure-to-function one.

1.9 Research objectives

The use of resistant cultivars have been developed in an attempt to control RWA, as this approach is a viable and environmentally-friendly alternative to the application of insecticides (Butts and Pakendorf, 1984; Webster et al., 1991). One of the current problems with controlling RWA is that the aphid is capable of adapting to, and subduing resistance genes incorporated into resistant varieties that are developed to control it (CSIRO, 2007). A virulent aphid biotype emerges when new strains of its existing form can successfully feed on resistant plants that have been developed against the previous strain. This biotypic variation had been identified worldwide, in cultures of RWA (Puterka et al., 1992). Many of these cultures are endemic, such that they are resistance breaking and virulent on resistant plants, developed outside their locality. The emergence of RWA biotypes poses a challenge to the use of resistant varieties, reducing the value of the efficacy of these resistant cultivars and raising new concerns about their future deployment in control measures (Randolph et al., 2010). Clearly, the use of resistant cultivars will have geographical limits because of biotypic variation in RWA (Puterka et al., 1992). RWASA2, the second RWA biotype in South Africa, is classified as resistance breaking and virulent to most of the available resistant wheat lines developed against the earlier biotype, RWASA1. Given its resistance breaking, potential, it was thought necessary to undertake baseline comparative studies of the effects of the feeding damage caused by these two

RWA biotypes on resistant and non-resistant barley lines. This forms the basis of this thesis, which focussed on the following two main areas:

1. Structural and functional studies of the feeding damage caused by RWASA1 and RWASA2:-

Previous studies have shown that feeding by RWASA1 primarily causes severe damage to the transport system of wheat and barley (Botha and Matsiliza, 2004; Saheed et al., 2007a, 2007b, 2009, 2010). Several reports have described the pathway of RWA stylets when it is feeding on its hosts (Evert et al., 1973; Matsiliza and Botha, 2002; Botha, 2005; Saheed et al., 2007a, 2007b). It has also been shown that RWA preferentially feeds in thin-walled sieve tubes (Matsiliza and Botha, 2002). In two separate studies, Saheed et al. (2007a, 2007b) demonstrated the structural damage caused by RWASA1 to the vascular tissues in leaves of a non-resistant barley cv Clipper. They also compared the damage this same aphid caused in non-resistant Betta and resistant Betta-*Dn* wheat cultivars. Other studies have shown that damage to vascular tissues resulted in the reduction of the capacity of the phloem to transport assimilates in barley and wheat hosts infested with RWASA1 (see Botha and Matsiliza, 2004; Saheed et al., 2009, 2010). However, it is important to stress that these studies only involve RWASA1 as they predate the appearance of the new, more virulent and more aggressive feeder, RWASA2.

There has, to date, been only one study (fluorescence microscopy-based, Walton and Botha, 2008), which involved RWASA1 and RWASA2 feeding on non-resistant Tugela and resistant Tugela-*Dn* wheat cultivars. There has been no comprehensive study, which details the structural and functional relationships of the damage caused by more than one biotype, using non-resistant and resistant barley lines. An in depth comparative study was thus necessary, to provide further more detailed evidence of relative damage study

2. Effects of CO_2 concentration on the barley lines infested by RWASA1 and RWASA2:-

The current interest in global climate change and its potential impacts on ecosystems in the broad sense, as well as its potential impacts triggered by even small changes in rainfall or, the effects of changing [CO_2] may well impact adversely on crop productivity and food security. Monitoring aphid effects occurring concurrently with [CO_2] change, is limited. It was thought that a second focus – that of plant-aphid interactions under changing CO_2 concentration – was important and paucity of available information was the driver for this part of the thesis. The Intergovernmental Panel on Climate Change (IPCC) declared that the biosphere is currently experiencing consistent increases in atmospheric carbon dioxide, reactive nitrogen, temperature and high variance in precipitation and disturbance pattern (IPCC, 2007). Among insects, aphids rank the most sensitive to changes in environmental quality, and these changes presage into their host plant quality (Docherty et al., 1997).

This thesis therefore involves a comprehensive comparison of the structural and functional relationships of the damage caused by the two biotypes, as well as the responses of the non-resistant and resistant barley hosts to the aphids, grown in an elevated CO_2 environment run at two higher levels of carbon dioxide concentrations aside the ambient level. These two major areas (structure-function relationships and [CO_2] change), developed into a thesis with five interest areas:-

1) An evaluation of the performances of RWASA1, RWASA2 and BCA on barley. Here, comparative population growth rates of the three aphids were assessed, which together with appraisal of relative virulence (measured as leaf roll and chlorosis), established the resistance-breaking, aggressive feeding and faster breeding characteristics of RWASA2, compared to RWASA1 and another known fast breeding aphid (BCA) (Chapter 3).
2) An investigation of the differences in the functional responses of the barley lines to feeding RWASA1 and RWASA2, by detailed examination of the formation and distribution of aphid-induced wound callose (Chapter 4).
3) An investigation of the structural damage caused by the two biotypes to their barley hosts. Here, a combination of wide-field fluorescence and transmission electron microscopy were used to compare damage to the vascular tissues of the plants due to probing and feeding by the two biotypes (Chapter 5).

4) A comparative study of the effects of the feeding aphids on the transport functionality of the barley lines was carried out. Here, I report on the results of experiments carried out to show the movement of the phloem mobile fluorophore, 5,6-carboxyfluoresein diacetate (5,6-CFDA) to assess the damage to the phloem in control and aphid-probed leaf material. (Chapter 6).
5) The effects of varying [CO_2] was evaluated, in relation to the breeding performance across various aphid-host plant combinations, under ambient (380 µmol mol^{-1}) and two elevated levels (450 and 550 µmol mol^{-1}) conditions. The aim of this section was to gain insight into the present and potential future impact that changing [CO_2] would have on RWA infestation of small grain crops (Chapter 7).

The entire study reported in this thesis combines a structural and functional approach and provides new knowledge to the field of plant-aphid interactions. This, together with experiments which illustrate the impacts of changing [CO_2] on plant growth, carbon/nitrogen balance, aphid population density changes and virulence provides baseline data on the complex interactions that exist between aphid biotype breeding and plant resistance, and in addition, information on the impact of changing [CO_2] on plant survival and food security is highlighted.

1.10 Hypotheses

The hypotheses upon which this study was based were:

1) That virulent and resistance-breaking RWASA2 would cause more severe structural and functional feeding-related damage on barley than the older RWASA1 biotype;
2) That the non-resistant barley line would show more extensive structural and functional damage to the vascular bundles than the experimental resistant STARS lines;
3) That the US-developed RWA-resistant STARS lines would retain (at least some of) their resistance against the two South African RWA biotypes;
4) That the population growth rates and virulence of the two RWA biotypes on the barley lines would be higher at elevated [CO_2] than at ambient level;
5) That the potential effects of aphid infestation on the four barley lines would be higher at elevated [CO_2] than at ambient level.

2 Chapter 2: Materials and methods

2.1 Host plant materials

Experiments reported in this thesis involved the use of five barley (*Hordeum vulgare* L.) lines. The first of these is cv Clipper, reported to be susceptible to the older South African biotype of the Russian wheat aphid (RWA, *Diuraphis noxia* Kurdjumov) i.e. RWASA1 (Saheed et al., 2007a). cv Clipper was used to generate feeder plants for maintaining cultures of the two RWA biotypes (i.e. RWASA1 and RWASA2). The remaining four were used as experimental plants for various studies. These include three resistant lines, STARS-0502B (PI 47541), STARS-9301B (PI 573080) and STARS-9577B (PI 591617), developed at the United States Department of Agriculture, Agricultural Research Station (USDA-ARS, Stillwater, Oklahoma, USA). They were demonstrated to be variously resistant to eight identified biotypes of RWA (i.e. RWA1 to RWA8) in the USA (Mornhinweg et al. 1995a, 1999, 2006b; Mornhinweg and Porter, 2006; Puterka et al. 2006). The fourth, PUMA, one of the most important barley cultivars grown commercially in South Africa and known to be susceptible to RWASA1 (VL Tolmay, pers. comm.), was used as a non-resistant line. All the seeds were obtained from the Agricultural Research Council (ARC), Small Grain Institute, Bethlehem, South Africa.

Seeds were pre-germinated on filter papers soaked with 0.75% hydrogen peroxide in Petri dishes. The dishes were placed in a refrigerator maintained at 8^{o}C for 2 days and then transferred to room temperature to germinate for another two days (Åhman et al., 2000). Seedlings were planted one per pot, in

17cm diameter plastic pots filled with potting soil (2:1:1; garden soil: compost: vermiculite mixture) in a greenhouse maintained at 20-30^{o}C for one week. The seedlings were sprayed with aerosol pyrethroid insecticide (SC Johnson and Sons (Pty) Ltd., South Africa) to kill any insects that may have colonised them while in the greenhouse. They were further exposed to fresh air for another 24h (Jyoti et al. 2006), then moved to the growth cabinets (Conviron) where they were grown for two weeks to adjust to preset growth conditions (see details in section 2.3). The young plants were allowed to reach 2 – 3 leaf stage before they were manually infested with the aphids. Positions of the experimental plants in the growth cabinets were always changed every day in a definite pattern in order to prevent any accumulative chamber effect (Ade-Ademilua and Botha, 2005). Half strength Long Ashton nutrient solution (Hewitt, 1966) was applied 3 times per week.

2.2 Aphid colonies and maintenance

The two South African biotypes of the Russian wheat aphid (RWASA1 and RWASA2) and Bird cherry-oat aphids (BCA, *Rhopalosiphum padi* L.) were also obtained from ARC, Small Grain Institute, Bethlehem, South Africa. Colonies of the two biotypes were maintained on young barley cv. Clipper plants and kept in insect cages in separate controlled environment cabinets, under growth conditions described in section 2.3 below. Fresh pots of two-week old feeder plants were introduced into the breeding cages of each aphid at intervals of two weeks to ensure that healthy feeder plants were always provided for the aphid colonies to feed from as well as sustain continuous

availability of aphids for various experiments. Each pot was usually infested with 30 apterous aphids on older leaf segments, which were placed at the axils of young feeder plants, thereby allowing aphids' free movement and settlement. In order to prevent release of the aphids into the environment, discarded treatments were placed in black polythene bags and sprayed with aerosol pyrethroid insecticide (SC Johnson and Sons (Pty) Ltd., South Africa).

2.3 Growth conditions

All experiments reported in this thesis were carried out under a controlled environment in growth cabinets (Conviron S10H, Controlled Environment Ltd., Winnipeg, Manitoba, Canada) maintained at a day time maximum of 24^{o}C and 66% relative humidity (RH) and at 22^{o}C, 60% RH (night), with a 14-h photoperiod. Plants were illuminated using a combination of fluorescent tubes (F48T12.CW/VHO 1500, Sylvania, Danvers, MA) and frosted incandescent 60W bulbs (Phillips, Eindhoven, The Netherlands), with a photosynthetic active radiation (PAR) level of 250 µmol^{-2} s^{-1} set at 30 cm below the light source. Experiments reported in Chapter 7 involved the investigation of the effects of elevated CO_2 levels on plant–aphid interactions. The experiments were carried out at three levels of [CO_2] – 380, 450 and 550 µmol mol^{-1} – in separate growth cabinets. Fluctuations in the two elevated [CO_2] as well as ambient levels were kept within ±15 µmol mol^{-1} of the [CO_2] set point. [CO_2] was regularly monitored using integrated computer-controlled Horiba APBA-250 indoor CO_2 monitor (Horiba Ltd., Japan).

2.4 Population growth studies

Population growth rates of the aphids were measured in Chapters 3, 4, 5 and 7 in order to:

(1) examine differences in the breeding rates of the two RWA biotypes on resistant and non-resistant barley lines and

(2) relate their breeding rates to other qualitative and quantitative parameters assessed under various studies.

Aphid population count was carried out in clip cages (Chapters 3, 4 and 5) as well as on whole plants (Chapters 3 and 7). The clipcage (Saheed et al., 2009) was used to enclose a 3-cm long leaf segment. The whole plant cage was a ventilated cylindrical plastic cage isolation, which can contain a whole plant. Assessments of aphid population growth in the clip cages as well as on whole plants in Chapter Three were carried out at 3, 5, 7, 9, 11, 13, and 15 days after infestation (DAI). The adaxial and abaxial surfaces of the leaf segment (clip cage) and on each leaf of every plant (whole plant) were carefully examined and the numbers of live aphids were non-destructively counted with the aid of a hand lens. Aphid population count on whole plant in Chapter 7 was carried out in a similar way except that assessments were carried out at 1, 7 and 14 DAI.

Clipcage population count was carried out at the expiration of various feeding exposure periods, which in Chapter 4 consisted of 1, 3, 7 and 14 DAI, and in Chapter 5 at 10 DAI. In each case, the portion of the leaf enclosed by the clipcage was gently marked with a soft tip marker. After this, clipcages were

carefully dismantled; all aphids feeding in the confinement were gently brushed onto a white paper using a camel hairbrush and counted with the aid of a hand lens. Population of the aphids were entered into spreadsheets and data analysed on Statistica version 9 using factorial ANOVA design. Prior to analysis, homogeneity of variances and normality of the data were examined using Levene's and Shapiro-Wilk's tests, respectively (Johnson and Wichern, 2002). Heterogeneity was eliminated after a $\sqrt{}$ transformation of the data.

2.5 Virulence studies

Effects of the feeding aphids on the experimental plants were assessed using a virulence scoring system (Table 2.1). Leaves of infested whole plants (Chapters 3 and 7) were carefully and continuously scrutinised for various levels of chlorosis and leaf roll at 1, 7, 14, 21 and 28 DAI. Each plant score was summarised and entered into a spreadsheet. Data were analysed using repeated measures ANOVA design.

Table 2.1 Virulence rating (chlorosis and leaf rolling) scales used for assessing effects of aphids on test plants

Scale	Description
Chlorosis[1]	
0	Plant appears healthy, no chlorotic or necrotic spot(s) on any leaf
1	Plant appears healthy, may have few isolated chlorotic or necrotic spots
2	Chlorotic spots become more noticeable, up to 5% of total leaf area
3	Chlorotic spots are larger and more numerous, up to 15% of total leaf area
4	Chlorosis covers up to 25% of the total leaf area;, some streaking may become apparent, especially along the midrib
5	Chlorotic spots may begin to coalesce or definite streaking may occur; chlorosis covers up to 40% of the total leaf area
6	Larger chlorotic areas form coalesced spots, leaves start to die back from tips; chlorosis covers up to 55% of the total leaf area
7	Further symptom development; chlorosis covers up to 70% of the total leaf area
8	Extensive chlorosis and necrosis; up to 85% of the total leaf area affected
9	Plant death or no recovery possible
Leaf rolling[2]	
1	Leaves are flat, no apparent rolling
2	Leaves are folded and/or loosely rolled at the margins
3	Tightly or completely rolled leaves

[1]Chlorosis scale adapted from Webster et al. (1987).
[2]Leaf rolling scale adapted from Burd et al. (1993).

2.6 Fluorescence microscopy – Aniline blue treatments

2.6.1 Experimental design and aphid infestation

In this thesis, two experiments were conducted which involved use of aniline blue fluorochrome to investigate the effects of feeding by RWASA1 and RWASA2 on leaves of the four barley lines. The first experiment is reported in Chapter 4, which involved comparative study of the feeding effect of the two RWA biotypes through examination of the distribution of aphid-induced wound callose in the phloem of non-resistant and resistant barley host plants, after short-term and sustained long-term feeding periods. The second experiment reported in Chapter 5, which also investigated comparative effects of feeding damage, served as a supplementary first step study before a more detailed ultrastructural investigation of differences in the feeding damage was caused by the two biotypes when they feed on host plants for 10 days.

These two experiments have similar experimental set-ups. Clipcages were used to enclose a 3cm-long segment in the mid-region of either the second or the third leaf above the coleoptile of each experimental plant. Control plants were also fitted with clip cages but were not infested with aphids. A leaf segment from the feeder plant, containing 10 apterous aphids, was carefully introduced into the clipcage for each biotype. The aphids were allowed 24h to transfer from the feeder leaf segment onto the 3cm-long confined leaf portion of the experimental plant before commencing counting the hours/days of feeding exposures. Ten replicates of each aphid treatment comprising 2 aphid

biotypes × 4 barley lines × 4 feeding exposures and 10 control (uninfested) plants were set up, making a total of 330 plants.

After the various feeding periods, the clipcages were carefully removed, aphid population count was carried out and the confined portion was gently marked with a soft tip marker. After this, the portions where the aphids had been feeding were processed for fluorescence microscopy investigation of feeding-related wound callose deposition (section 2.4 above). In the experiments to investigate transport of wound callose signals beyond (above or below) the aphids' feeding sites in both source and sink leaves, feeding exposure was limited to 7 days. After this, the leaves were harvested for the study of feeding-related wound callose formation using aniline blue treatments and fluorescence microscopy to visualize the wound callose.

2.6.2 Preparation of aniline blue fluorochrome

Stock solution of 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: 0.1mg of the compound was dissolved in 1ml of distilled water, foil-wrapped and kept in a refrigerator maintained at 4°C until required for use. The stock solution was diluted (1:3 v/v) using distilled water to make the working solution (427 µM aniline blue fluorochrome), kept wrapped in foil and stored at 4°C until needed.

2.6.3 Treatment of leaf material for study of wound callose distribution

The leaf segment covered by the clipcage where the aphids were confined for feeding (for infested plants) or without aphid (control) was excised and then transferred immediately to Ca^{2+}-free buffer (10 mM 2- [morpholino] ethanesulfonic acid (MES), 0.5mM $MgCl_2$, 0.5 mM KCl and 125 mM mannitol, adjusted to pH 7.2). The abaxial leaf surface was gently scraped under the MES buffer on a glass plate using a sharp single-edge carbon steel razor blade (Agar Scientific, USA) to remove the cuticle and the underlying epidermal tissue in order to expose "windows" into the mesophyll and underlying vascular tissues. The scraped leaves were mounted on glass slides and cover slips in Ca^{2+}-free MES buffer after staining with a few drops of the working strength of aniline blue fluorochrome, incubated in the dark for 30 min at 20^{o}C, and washed in fresh Ca^{2+}-free MES buffer. The tissues were thereafter examined for callose fluorescence using an Olympus BX61 wide-field fluorescence Digital Imaging Microscope (Olympus, Tokyo, Japan supplied by Wirsam Scientific, Johannesburg, South Africa), fitted with aniline blue specific filter cube (excitation of 425-444nm; emission of 475nm). Aniline blue fluorochrome dye specifically stains callose, which appears blue under white light but fluoresces blue-green under UV light. High-resolution images were collected and saved in a database using the programme analySIS (Soft Imaging System GmbH, Germany). Images which are representative of the feeding damage under each treatment were randomly

selected and were imported as bitmaps to CorelDraw version 12 for presentation.

2.6.4 Quantification of wound callose distribution

Damage caused by the feeding aphids was quantitatively assessed by randomly selecting ten high resolution images of the same magnification as samples for each of the feeding exposure treatments (24h, 72h, 7d and 14d) including the uninfested control. Quantitative analysis of the wound callose was carried out using phase analysis (analySIS program, Soft Imaging System GmbH). The programme automatically measures the area covered by the feeding–related callose, which is the magenta-coloured area (Fig. 2.1). Data for the area of callose in each selected image for each feeding exposure experiment and its respective uninfested control were entered into a spreadsheet and subjected to further statistical analysis using Statistica version 9. Three-way ANOVA, as described under 2.4 above, was used to examine the differences in the area of wound callose per image which now forms the dependent variable.

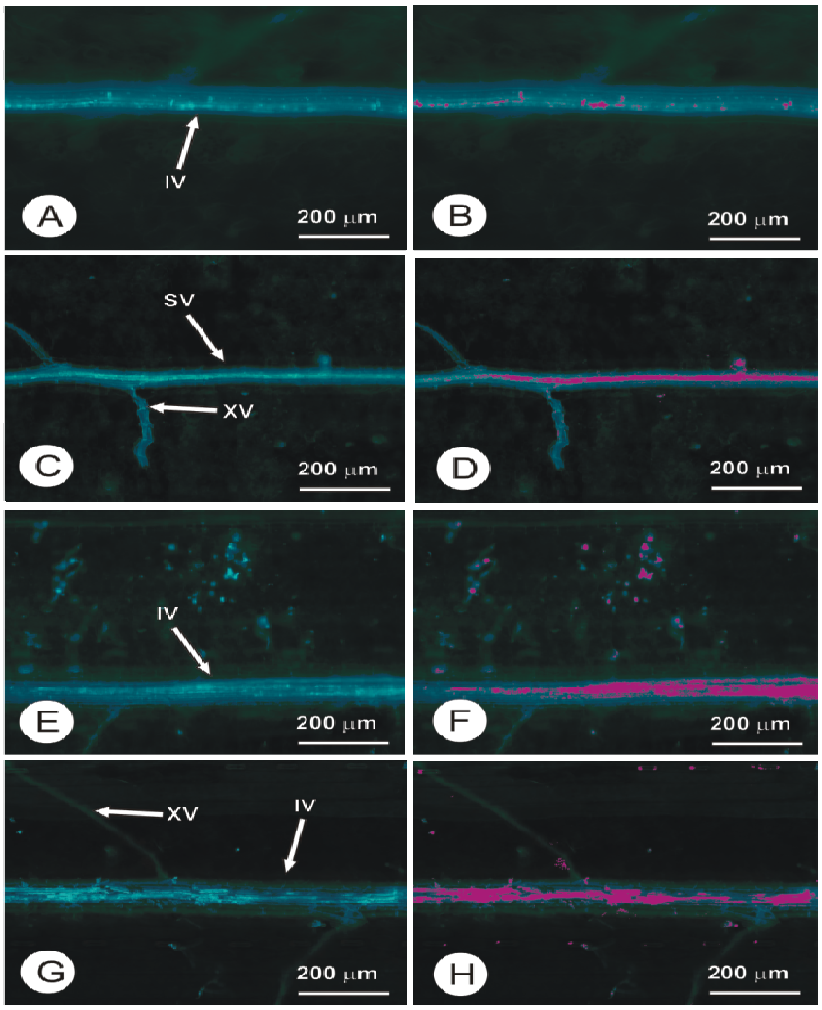

Fig. 2.1 Images showing application of phase analysis to measure area of wound callose formed in leaves

Fig. 2.1 A-H show samples of images to illustrate application of the phase analysis menu of analySIS programme to measure the area of the image of scraped leaf covered by wound callose formed in control (uninfested) as well as plants exposed to feeding aphids. **A**, **C**, **E** and **G** show feeding-related staining reaction under aniline blue fluorochrome treatment while **B**, **D**, **F** and **H** illustrate quantitative measurement of wound callose on the images after assigning the "false" colour magenta using the phase analysis menu.

2.7 Fluorescence microscopy – 5,6-CFDA treatments

2.7.1 Experimental set-up

Effects of aphids' feeding on phloem transport were investigated in Chapter Six, using the xenobiotic phloem-mobile fluorophore, 5,6-carboxyfluorescein diacetate (5,6-CFDA). Leaf segments of experimental plants enclosed in clipcages were infested with 10 apterous aphids. The aphids were allowed an initial 24h to transfer and settle on the confined experimental leaf area before commencing the counting of the hours/day for the four treatment regimes which consisted of 24h, 72h (short-term), 7d and 14d (long-term) feeding periods. Ten replicates of each treatment (2 aphid types, each infesting 4 different barley lines) and control (uninfested) plants were set up. These consisted of 90 plants per treatment with 360 plants in all, and these experiments were repeated twice.

2.7.2 Preparation of 5,6-CFDA

The stock solution of 5,6-CFDA (C-195, Molecular Probes, Eugen, Oregon, USA) was made by adding 1ml of 0.2% dimethylsulphoxide (DMSO) to 100mg of 5,6-CFDA. This was foil-wrapped and stored at -5°C until needed. A working solution, 217µM in distilled water, was prepared by adding 1µL aliquots of the stock solution to 1ml distilled water in propylene centrifuge tubes, foil-wrapped to prevent cleavage of the 5,6-CFDA by light, frozen at -5°C, defrosted when needed and used immediately.

2.7.3 Treatment of leaf material

Intact control (uninfested) and aphid-infested plants were used for all the treatments, using the flap feeding method (Saheed et al., 2010). After each feeding exposure period, the area to be abraded was rinsed with Ca^{2+}-free MES buffer (see section 2.6.3 above), before been gently abraded on the abaxial surface with a sterilized needle. To keep in line with the classical pattern of assimilates' acropetal and basipetal movements (Turgeon, 1989), source leaves were abraded on the part above the clipcage while sink leaves were abraded below the clipcage. Thereafter, 100µL working solution of 5,6-CFDA was applied to the abraded portion and covered with transparent polythene film (Housebrand, Brackenfell, South Africa) to prevent evaporation of the solution. The fluorophore was allowed 3h to be taken up and transported through the intact leaf. In the process, the non-polar 5,6-CFDA gets introduced into the damaged cells and moves across membranes (Botha, 2005). In the diacetate form, it does not fluoresce. Once the dye reaches living cells (by moving across membranes), the diacetate is cleaved from the molecule and it becomes 5,6-carboxyfluorescein (5,6-CF), a polar molecule. The 5,6-CF cannot move across membranes and is thus retained within the symplasmic transport system where it is transported largely within the phloem (Botha, 2005). After the expiration of the 3h allowed for the loading and transportation of the dye, the caged region of the leaf where the aphids were confined for feeding was marked with a soft tip marker. The clipcage was gently dismantled, experimental leaf excised at the base and

placed on a glass slide. The fluorescent front and the distance transported from the point of application of the dye in control (uninfested) as well as aphid-infested leaves was observed under UV light using Olympus BX61 wide-field fluorescence microscope fitted with U-YFP filter set (10C/Topaz 41028, Chroma Technologies, Battlebro, USA) with excitation of 513nm and an emission of 527nm. Immediately after recording the distance 5,6-CF trafficked in the phloem, the portion of the leaf within the clipcage was cut and its abaxial surface was gently scraped under silicone oil on a glass plate using a sharp single-edge carbon steel razor blade (Agar Scientific, USA), in order to remove the cuticle and expose the underlying vascular tissues. The scraped leaf tissue was promptly mounted on a glass slide in silicone oil, observed under the microscope and images taken were saved in a database using the programme analySIS (Soft Imaging System GmHb, Germany) and imported as bitmaps to Corel Draw 12 for presentation.

2.8 Transmission electron microscopy

2.8.1 Experimental set-up and aphid infestation

A leaf segment containing 10 apterous aphids was carefully introduced into the clipcage already fitted on the experimental plants for each RWA biotype treatment. Control (uninfested) plants were also fitted with clipcage but with no aphids. Ten replicates of each treatment combination (2 aphid types × 4 plant types) and 10 control plants were set up, making a total of 90 plants. Experimental procedures were repeated twice. The aphids were allowed 24h to transfer and settle on the confined experimental leaf. At 10 DAI, the

clipcages were carefully removed and aphid population was counted using a hand lens. Leaf segments from regions where the aphids had been feeding were processed for transmission electron microscopy (TEM) studies.

2.8.2 Treatment of leaf materials for TEM

Leaf segments from the control and infested plants were cut into strips in cold fixative made up of 6% paraformaldehyde-glutaraldehyde (v/v) in 0.05M sodium caccodylate buffer using a sharp, clean and single-edge razor blade. The strips were trimmed, diced into smaller pieces (approximately 2×3 mm in size), placed in small vials and subjected to a very slight vacuum (17000kg/m sec^2) for 1h after which the fixative was changed and the vials transferred to a refrigerator maintained at 4^oC and left overnight. The leaf tissues were washed in three changes of cold 0.05M sodium caccodylate buffer and transferred to cold 2% osmium tetroxide in 0.05 sodium caccodylate buffer in the refrigerator overnight, washed in cold buffer and dehydrated in a cold graded ethanol series, followed by two changes in 100% propylene oxide. Spurr's (1969) epoxy resin was used in embedding the leaf tissues. Ultrathin sections (silver to gold) were cut using a diamond knife (Drukker, The Netherlands). The sections were collected on 300 mesh copper grids (SPI Suppliers, Philadelphia, USA) and stained with 2% uranyl acetate in distilled water followed by Reynolds's lead citrate. They were viewed and imaged at 80kV using a JEOL JEM 1210 transmission electron microscope (JEOL, Tokyo, Japan). The images were imported into CorelDraw version 12 for presentation.

2.9 Plant biomass partitioning

The determination of total, above ground and below ground plant biomass of control (uninfested) and infested plants under the three levels of [CO2] reported in Chapter 7 were carried out at 28 DAI. The whole plant material in each pot was carefully removed. The root system was washed of soil by soaking the root mass in large volume of water and using screen mesh to recover loose roots (Reid and Fiscus, 2008). The entire vegetative material was enclosed in a medium-sized paper envelope and oven-dried to constant weight at 60◦C. Dried plant material was weighed to obtain total biomass. The material was then separated into the above ground (shoot) and below ground (root) components by removing the roots at the point of attachment to the base of the rhizome (Ripley et al., 2008) and each component separately weighed.

2.10 Determination of nitrogen concentration and C:N ratio of leave

In the experiments reported in Chapter Seven, five out of ten replicates of the treatments were randomly selected for determination of nitrogen concentrations and C:N ratios of leaves. Three centimetres long leaf segments were cut from the mid-leaf region of every leaf from each of the three treatments (i.e. uninfested, RWASA1, RWASA2). In the case of infested plants, leaf surfaces were carefully brushed using a fine paintbrush to remove remnants of dried aphids and guard against contamination from animal (aphid) matter. Leaf segments from each sample were ground and homogenised to a fine powder in a mortar and pestle that was cleaned

between samples. Ground sample was packed and stored in 1.5ml polyvinyl Eppendorf tube and further desiccated for 48h. An average of 1.45 – 1.65mg of the powder was weighed out into clean 9×5mm OEA tin capsule using an analytical semi-micro weighing balance (OHaus Discovery DV 215CD, Ohaus Corporation, Switzerland). Tin capsules were crimped and gently folded repeatedly into a compact ball and then stored in 96-cell well culture plates before analysis. The samples were analysed for % nitrogen concentration and C:N ratio of leaf tissues using a Europa Scientific Elemental Analyser (Model ANCA-SL, Europa Scientific, United Kingdom). Data for each measurement were separately analysed for each [CO_2] level on Statistica version 9 using factorial ANOVA design at 5% level of significance. Infestations, 3 {i.e. control (uninfested), RWASA1 and RWASA2} and barley lines (4) were the independent variables while % nitrogen or C:N ratio constituted the dependent variable.

3 Chapter 3: Evaluation of population growth rate and relative virulence of RWASA1, RWASA2 and BCA on resistant and non-resistant barley lines

Preamble

The data presented in this chapter are in part, as published in Jimoh et al., 2011 (Appendix A).

3.1 Introduction

Russian wheat aphid (RWA) and the bird cherry-oat aphid (BCA) are severe pests of wheat and barley in many parts of the world, causing substantial yield loss to small grain farmers (Du Toit and Walters, 1984; Riedell et al., 1999). The two aphid species cause quite different symptoms in cereals during feeding. RWA feeds on host plants in dense colonies within tightly curled leaves, causing rolling up of fully expanded leaves and prevent the normal unrolling of newly emerging leaves (Hewitt et al., 1984; Riedell, 1989). Its feeding often result in loss of effective leaf area, substantial reduction in chlorophyll content and a reduced photosynthetic ability of leaves of host plants (Walters et al., 1980; Fouchè et al., 1984; Kruger and Hewitt, 1984). Symptoms of RWA infestation are well documented (Saheed et al., 2007a; Tolmay et al., 2007). Local symptoms at the RWA feeding site include chlorosis and necrosis (Burd et al., 1993), as well as extensive damage to phloem sieve element, xylem, parenchyma, and mesophyll cells (Saheed et al., 2007a, 2007b) while systemic leaf symptoms are purple streaking and leaf

rolling (Burd et al., 1993). In contrast, BCA produces no visible symptoms on leaves of host plants (Reidell et al., 1999; Saheed et al., 2007a), except under heavy aphid loads when golden yellow streaking may occur on leaves (UCIPM, 2007).

Both aphid species have been the target of international breeding programs with the aim of identifying effective host plant resistance, with contrasting results. Several single, major dominant resistance genes have been identified against RWA in wheat (Botha et al., 2005). Several RWA resistance genes of major effect have also been identified in barley (Nieto-Lopez & Blake, 1994; Mornhinweg et al., 1995b, 2002; Mittal et al., 2008, 2009). BCA resistance in both wheat and barley is often not under simple genetic control (Weibull, 1994; Moharramipour et al., 1997; Åhman et al., 2000; Delp et al., 2009). As a consequence, RWA resistance but not BCA resistance has been deployed successfully as part of international breeding programs. A review of available literature has shown that up to date, no resistance has yet been identified in either wheat or barley that provides effective resistance against both aphid species. Cereal aphid resistance is usually species-specific (Migui and Lamb, 2003), and the RWA resistance genes deployed in wheat have been consistently shown to have no effect on BCA performance in the laboratory or field (Schotzko and Bosque-Perez, 2000; Messina and Bloxham, 2004).

Monogenic resistance genes are popular among breeders because they can be easily introgressed into commercial cultivars without affecting agronomic traits. Monogenic resistance to RWA in wheat shows all the characteristics of

classic *R*-gene resistance (Botha et al., 2005), and in recent years resistance-breaking 'virulent' RWA have appeared in the USA after the widespread deployment of resistant cultivars containing the *Dn4* gene (Haley et al., 2004; Puterka et al., 2007). As expected under the 'gene for gene' model of *R*-gene resistance, the virulence phenotype of these virulent RWA biotypes on resistant wheat in the USA was not observed on resistant barley (Puterka et al., 2006). The *Dn4* resistance gene in wheat does not affect the performance of other aphid species (Ni & Quisenberry, 2006). RWA resistance (*Dn1*) has also been deployed widely in South Africa. By 2006, 27 RWA-resistant wheat cultivars had been released for commercial cultivation in South Africa (Tolmay et al., 2007), none yet for barley (VL Tolmay, pers. comm.). As in the USA, a resistance-breaking biotype of RWA, RWASA2, has appeared in South Africa (Tolmay et al., 2007), which breeds faster and causes more damage to wheat than the original biotype RWASA1 (Walton and Botha, 2008).

The differential effects of the South African biotypes (RWASA1 and RWASA2) on barley have not yet been studied. As on wheat, RWA causes more damage to barley than does BCA, even when BCA exhibits a higher population growth rate (Saheed et al., 2007a). In this chapter, I compared the performance of the RWASA2 biotype to the less virulent RWASA1 and BCA on one susceptible and three selected RWA-resistant barley accessions. The use of BCA in this study was informed by the study remarked above that, it reproduces faster than RWASA1 (Saheed et al., 2007a). I measured the

population growth rates of the aphids and their relative damage to the four barley lines, in forms of chlorosis and leaf rolling. The objective of the study was to determine whether successful aphid reproduction is a good indicator of the level of damage. The aim was to develop a baseline for further studies of structural and functional damage by RWASA1 and RWASA2 in chapters that follow.

3.2 Experimental overview

Each aphid type (RWASA1, RWASA2, and BCA) was tested against the four barley accessions using two assays, a clip cage method and a whole-plant method. Ten replicates of each treatment combination (3 aphid types × 4 plant types × 2 assays) were set up, for a total of 240 plants. Experimental procedures were repeated twice. Each set up was infested by placing leaf segments containing 10 adult apterous aphids either in the clipcage or in the axils of leaves of the whole plant. Aphid population levels on each plant were assessed at 3, 5, 7, 9, 11, 13, and 15 DAI. Chlorosis and leaf roll ratings were also assessed for each of the test plants at 7, 14, 21, and 28 DAI, using the scoring system provided in Table 2.1 (Chapter 2).

Data were analysed using Statistica version 9 with barley lines, aphid types, and days after infestation included as independent variables, and the number of aphids per plant, the leaf rolling, and chlorosis ratings recorded as dependent variables. Statistical significance was determined using a repeated measures ANOVA design and homogenous means were grouped using Tukey's *posthoc* tests at 5% level of significance. Prior to analyses,

homogeneity of variances and normality of the data were examined using Levene's and Shapiro-Wilk's tests, respectively (Johnson and Wichern, 2002). Heterogeneity was eliminated after a $\sqrt{}$ transformation of the data.

3.3 Results

3.3.1 Aphid damage to host plants

Table 3.1 shows interactions among the aphids and the test plants for feeding damage, measured as chlorosis and leaf rolling, inflicted on the test plants at 7, 14, 21, and 28 DAI. The interaction between aphid type and plant cultivar is highly significant for both chlorosis rating and leaf roll rating ($P<0.01$).

Table 3.1 Repeated measures ANOVA of virulence[1] of the Russian wheat aphid biotypes RWASA1 and RWASA2, and the bird cherry-oat aphid on whole plant of four barley lines (n = 10 for each line) at 7, 14, 21, and 28 days after infestation (DAI)

		Chlorosis rating		Leaf roll rating	
Source of variation	d.f.[2]	F[3]	P[4]	F	P
Aphid	2	185.64	<0.001	276.40	<0.001
Barley line	3	11.40	<0.001	35.87	<0.001
Aphid×barley line	6	1.89	0.089	14.03	<0.001
Aphid×barley line error	108				
DAI	3	419.09	<0.001	63.76	<0.001
DAI×aphid	6	45.88	<0.001	16.27	<0.001
DAI×barley line	9	1.57	0.123	1.51	0.141
DAI×aphid×barley line	18	1.55	0.072	0.87	0.610
DAI×aphid×barley line error	324				

[1]Virulence assessed as chlorosis and leaf rolling.

[2]d.f. – degree of freedom; [3]F – F-value; [4]P – P-value.

Damage symptoms on test plants became noticeable in RWASA2-infested plants as early as 7 DAI, and continued to worsen until 28 DAI (Table 3.2). RWASA2 was most damaging on PUMA, on which complete leaf roll set in by 14 DAI and leaf death was apparent at 28 DAI. For RWASA1, feeding damage was higher on PUMA than on the resistant lines. BCA feeding recorded few chlorotic spots on PUMA, covering up to about 15% of total leaf area (see Table 2.1 in Chapter 2) and even less on the RWA-resistant lines. Leaves of all BCA-infested plants remained flat until 28 DAI. Trend of aphids' feeding damage is evidently RWASA2 > RWASA1 > BCA.

Table 3.2 Population growth rate and mean barley line ratings (n = 10 for each line) for chlorosis and leaf rolling at 7, 14, 21, and 28 days after infestation (DAI) with the Russian wheat aphid biotypes RWASA1 and RWASA2, and the bird cherry-oat aphid (BCA)

		No. aphids			Chlorosis rating			Leaf roll rating		
DAI	Barley Line	RWASA1	RWASA2	BCA	RWASA1	RWASA2	BCA	RWASA1	RWASA2	BCA
7	PUMA	39.10aA	82.30bA	56.40cA	0.60aA	1.80bA	0.00cA	1.70aA	2.30bA	1.00cA
	STARS-0502B	35.90aA	57.20bB	33.80aB	0.00a2	1.00b2	0.00aA	1.60aA	1.70aB	1.00 bA
	STARS-9301B	41.90aA	46.90aB	38.30aB	0.00a2	1.00b2	0.00aA	1.00aB	1.70bB	1.00aA
	STARS-9577B	35.20aA	39.30aC	38.40aB	0.00a2	1.00b2	0.00aA	1.00aB	1.70bB	1.00aA
14	PUMA	130.90aA	310.70bA	176.20cA	6.70aA	7.20aA	0.50bA	2.30aA	3.00bA	1.00cA
	STARS-0502B	102.90aB	174.40bB	91.40aB	4.20a2	3.20b2	0.00cA	2.00aB	2.00aB	1.00bA
	STARS-9301B	101.50aB	141.40bC	87.10aB	3.90a2	2.20b3	0.00cA	1.20aC	1.90bB	1.00aA
	STARS-9577B	105.30aB	155.00bC	138.70bC	3.10a3	3.10a2	0.50bA	1.30aC	2.00bB	1.00cA
21	PUMA				7.70aA	8.70aA	1.50bA	2.60aA	3.00bA	1.00cA
	STARS-0502B				6.80aA	4.70b2	1.80cA	2.30aB	2.40aB	1.00bA
	STARS-9301B				7.20aA	3.00b3	1.60cA	1.40aC	2.00bC	1.00cA
	STARS-9577B				6.90aA	4.30b2	2.00cA	1.50aC	2.20bBC	1.00cA
28	PUMA				8.70aA	9.00aA	3.30bA	2.70aA	3.00bA	1.00cA
	STARS-0502B				7.30aA	5.40b2	2.60cA	2.70aA	2.50aBC	1.00bA
	STARS-9301B				7.50aA	4.70b2	2.60cA	1.40aB	2.30bC	1.00cA
	STARS-9577B				7.30aA	5.40b2	2.60cA	1.70aB	2.60bB	1.00cA

Different lower case letters in rows and capital letters in columns indicate that means are significantly different following Tukey's *post hoc* test ($P<0.05$). Note: population growth data were not accumulated beyond 14 DAI.

3.3.2 Population growth rates of aphids on host plants

In both the clip cage and the whole plant experiments, RWASA2 formed the largest aphid population on all four barley lines, with the highest number on non-resistant PUMA: an average of 250 aphids per clipcage (Fig. 3.1) and about 320 aphids per whole plant at 15 DAI (Fig. 2). RWASA2 population size was lower on the three resistant lines in clip cages as well as on whole plants. RWASA1 colony size was also largest on PUMA, with an average of 185 aphids per clip cage and about 130 aphids per plant at 15 DAI. However, when exposed to the RWA-resistant lines, its colony sizes reduced to about 98 aphids in the two experiments. BCA population growth rates were similarly higher on PUMA and STARS-9577B than on STARS-0502B and STARS-9301B in the two set-ups. At 15 DAI, PUMA and STARS-9577B recorded an average of 160 aphids, whereas STARS-0502B and STARS-9301B produced approximately 80 aphids.

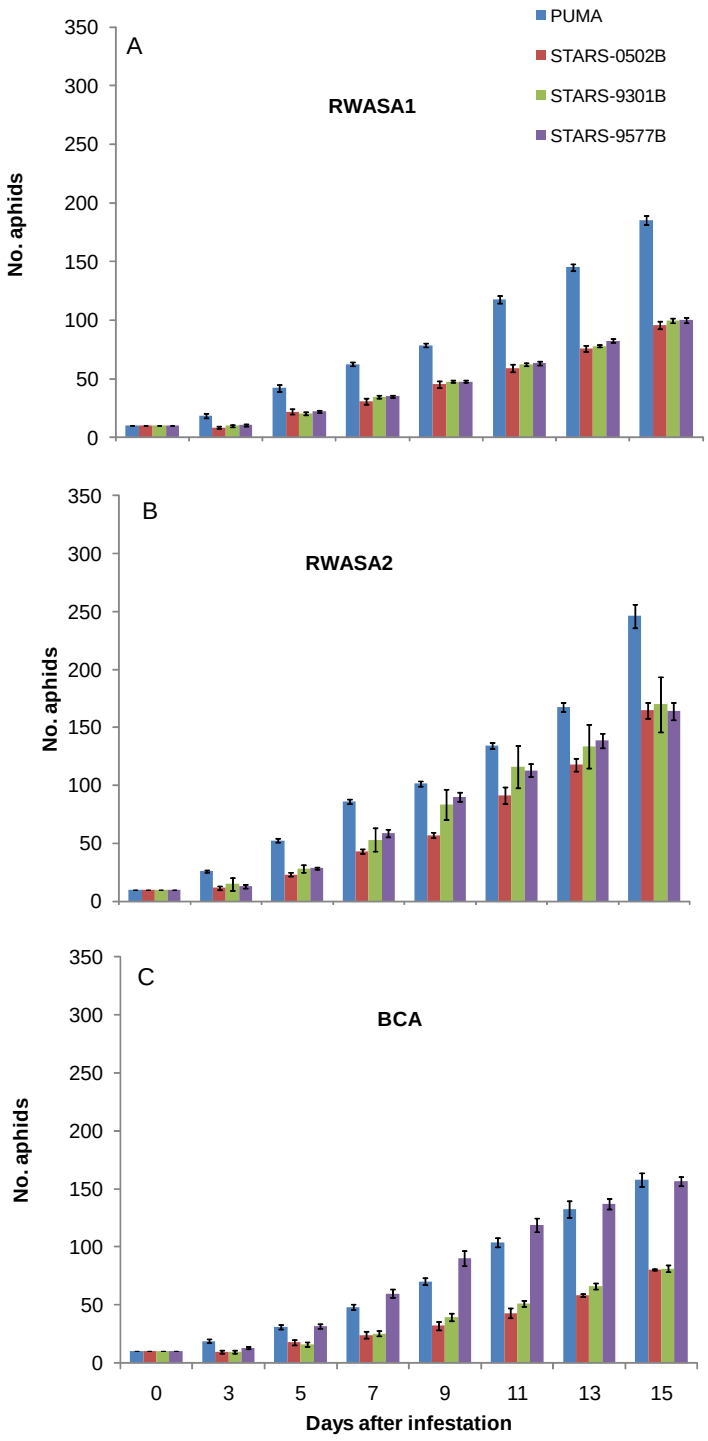

Fig. 3.1 Population growth rates of RWASA1 (A), RWASA2 (B) and BCA (C) on PUMA, STARS-0502B, STARS-9301B, and STARS-9577B, in the clipcage experiments n = 10 for each barley line.

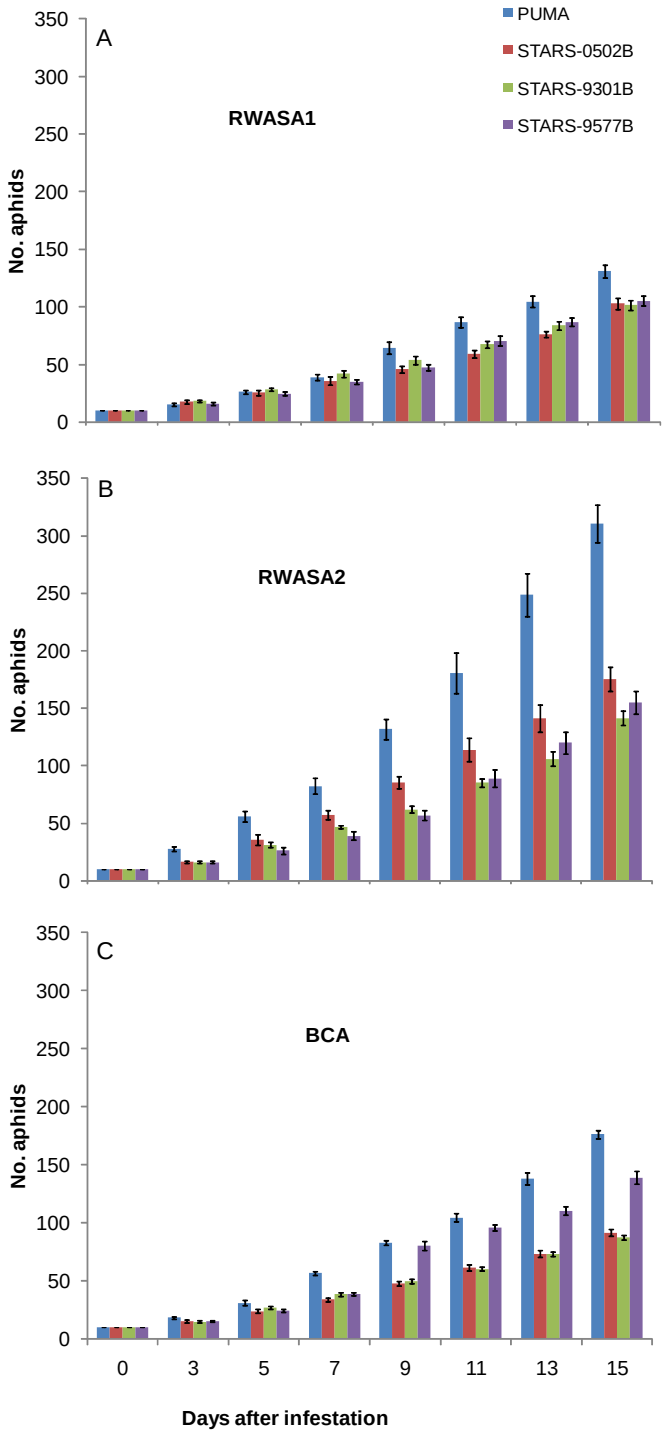

Fig. 3.2 Population growth rates of RWASA1 (A), RWASA2 (B) and BCA (C) on PUMA, STARS-0502B, STARS-9301B, and STARS-9577B, in the whole plant experiment

n = 10 for each barley line.

Table 3.3 shows the population growth rates of the aphids in the two experiments subjected to repeated measures of ANOVA. Interactions among aphids and barley lines on all days of observation were highly significant ($P<0.01$).

Table 3.3 Repeated measures ANOVA of population growth of the Russian wheat aphid biotypes RWASA1 and RWASA2, and the bird cherry-oat aphid in the clip cage and whole plant experiments of the four barley lines (n = 10 for each line)

		Clip cage		Whole plant	
Source of variation	d.f.	F	p	F	P
Aphid	2	157.74	<0.0001	100.78	<0.0001
Barley line	3	187.22	<0.0001	68.45	<0.0001
Aphid×barley line	6	30.81	<0.0001	17.19	<0.0001
Aphid×barley line error	108				
Days	6	4356.69	<0.0001	3112.43	<0.0001
Days×aphid	12	41.39	<0.0001	45.52	<0.0001
Days×barley line	18	19.41	<0.0001	32.27	<0.0001
Days×aphid×barley line	36	8.09	<0.0001		<0.0001
Days×aphid×barley line error	648				

3.4 Discussion

Results of the study reported in this chapter showed that a virulent aphid biotype, RWASA2, which was discovered in the field because of its ability to feed and reproduce on wheat containing the *Dn1* gene resistance developed against RWASA1, also exhibited an altered phenotype on RWA-resistant and susceptible barley lines. This result was surprising because initial studies of the RWASA2-*Dn1* relationship in wheat (Tolmay et al., 2007) suggested that it was a 'gene for gene' interaction characteristic of the NBS-LRR family of plant resistance (*R*) proteins. This family of resistance proteins mediates resistance by detecting the presence or function of a factor derived from the pest or pathogen, then triggering an effective defence response (Dangl and Jones, 2001). The only two cloned aphid resistance genes, the *Mi* gene in tomato and the *Vat* gene in melon, are both members of this family (Rossi et al., 1998; Dogimont et al., 2008), and many other aphid resistance genes have been mapped to clusters of *R*-genes in the plant genome (Klingler et al., 2005, 2007, 2009). One characteristic of *R*-gene resistance is that it is usually highly specific (Edwards and Singh, 2006), and can often select for the appearance of virulent pathotypes or biotypes that are able to successfully colonise resistant plants (Porter et al., 1997; Quick et al., 2001; Gatehouse, 2002). Because of this reason, it is expected that the performances of RWASA2 and RWASA1 would not differ on barley.

On the susceptible control PUMA, both RWA clones caused severe chlorosis and leaf roll symptoms, though they appeared earlier in response to RWASA2

feeding. In general, RWASA2 also caused earlier and/or more severe leaf roll symptoms on the resistant lines. Thus, earlier onset of chlorosis and an increase in leaf roll was a general pattern of response to RWASA2 compared to RWASA1, irrespective of the presence of resistance. I therefore believe that this can be attributed to the much higher reproductive rate that was observed for the RWASA2 clone compared to the RWASA1. A similar pattern is observed when RWASA2 and RWASA1 are confined to resistant (*Dn1*) and susceptible wheat plants (Walton and Botha, 2008), but this difference is not observed when aphids are allowed to move freely between resistant and susceptible plants (Tolmay et al., 2007). It is likely that the movement of aphids from resistant to susceptible plants masks the difference in reproductive rate between clones in choice experiments. Randolph et al. (2008) observed a difference in fecundity between USA biotypes of RWA on resistant and susceptible wheat, but only at lower temperatures (13-18 °C). Jyoti et al. (2006) found that the population growth rate of USA biotype 2 was consistently higher than that of USA biotype 1. Interestingly, RWASA2 had a higher rate of reproduction than BCA, as previous studies have shown that BCA usually reproduces faster than RWA (Messina et al., 2002; Saheed et al., 2007a).

It is equally interesting that though chlorosis symptoms appeared earlier on RWA-resistant plants after RWASA2 feeding, these symptoms developed much faster during RWASA1 feeding on all three RWA-resistant varieties. Hence, unlike the leaf roll symptoms, chlorosis was not well correlated with

aphid population numbers. Previous studies on RWA have also demonstrated that chlorosis-based damage scores are often not associated with aphid population performance (Puterka et al., 2006). The difference in chlorosis caused by RWASA2 and RWASA1 in this study may indicate a difference in behavioural response between the two clones. Increased salivation is a typical aphid response to an occluded phloem stream (Will et al., 2007), and the response is quite variable among aphid species and individuals (Will et al., 2009). As chlorosis is thought to be a direct effect of RWA saliva (Miles, 1990; Ni and Quisenberry, 1997; Saheed et al., 2007b), the increased chlorosis caused by RWASA1 may indicate an increased salivation response by this clone in response to sieve element occlusion resulting from the action of the resistance gene. It is also possible that there are differences between the clones in the components of the saliva that are responsible for the chlorosis response.

Throughout this study, all the barley lines were far less affected by BCA feeding than by the feeding of either RWA biotype. Leaves of BCA-infested plants did not show leaf roll, which is consistent with previous observations that suggest that BCA does not cause visible damage to host plants, except under heavy infestation (Leather et al., 1989; Riedell et al., 1999; Saheed et al., 2007a; UCIPM 2007). Interestingly, at least two of the three RWA-resistant barley lines tested showed an effect on BCA population growth equivalent to that observed on the RWA clones in both the clipcage and whole plant tests. For the third RWA-resistant line (STARS-9577B), a significantly

smaller population reduction effect on BCA population growth was observed on whole plants, but there was no effect observed in the clip cage experiments. Additional experiments are needed to determine whether the same resistance genes mediate the effects on BCA and RWA. If this is so, this particular resistance would be very attractive to breeders in countries, where these species are important pests. It is rare though, to find resistance that has a simple underlying genetics and is effective against multiple aphid species.

Data from this study showed positive population growth for all three aphids on all the lines over the 15-day period (Figures 1 and 2). Puterka et al. (2006) also found that five USA biotypes of RWA continued to develop successfully on STARS-9301B and STARS-9577B. In contrast to results of this study, Puterka and colleagues found no evidence of antibiosis effects of these resistant lines on any of the five USA biotypes. Earlier, Webster & Starks (1987) found only a modest antibiosis effect of STARS-9301B on the original USA RWA biotype 1. These and other authors have concluded that resistance to RWA in barley is due primarily to tolerance of aphid feeding (Puterka et al., 1992, 2006; Webster et al., 1996). The antibiosis effects, which were observed in these lines against both South African biotypes, suggest that there may be fundamental differences in the genetics and physiology of Russian wheat aphid feeding biology between these two locations.

Although it has been observed that aphid performance can be negatively affected by the use of clipcages (Kift et al., 1996), I could not detect any consistent differences between the clipcage and whole plant experiments in

this study. Differences in RWA performance between the two assays were only observed on the susceptible line PUMA. Strangely, RWASA1 population growth seemed higher in clip cages, whereas RWASA2 population growth was higher on whole plants. In contrast to RWA, BCA performance on PUMA was not affected by the assay but, as mentioned above, resistance to BCA in STARS-9577B was not detected using clipcages. There are several reasons why the use of clipcages may affect the results of aphid performance assays. Aphid population growth could be adversely affected if clipcages caused diminished nutrient supply or local sink strength (Davis and Radcliffe, 2008). Clipcages can cause a reaction in the leaf resembling senescence (Crafts-Brandner and Chu, 1999), which could result in increased assimilate levels in the phloem and improved aphid performance. Clipcages could also induce wound-associated defences that could negatively affect aphid performance. It is not clear what caused the variable clipcage effects in this experiment, but induction of the hormone ethylene by the clip cages could cause both senescence and defence responses (Quirino et al., 2000).

In summary, this study showed that there is a difference in performance of the two RWA biotypes on both susceptible and resistance barley that respond differently to a resistance gene (*Dn1*) in wheat. These results demonstrated that the two biotypes differ not only in their interaction with *Dn1*, but also with respect to some important aspects of their biology. In this study, it was clearly shown that RWASA2 had a higher reproductive rate on all barley lines which were tested, supporting previous results on wheat (Tolmay et al., 2007;

Walton and Botha, 2008), and that this difference accounted for much of the increase in damage symptoms it caused. The results also showed that RWASA2 not only breed faster than RWASA1, but it also reproduces more quickly than BCA contrary to earlier reports on BCA and RWASA1 (Saheed et al., 2007a). RWASA1 caused greater levels of chlorosis on RWA-resistant plants, which could be due to a difference in feeding behaviour or salivary biochemistry in this biotype. The reproductive rate of both biotypes was reduced to an almost equivalent degree on all three resistant lines and, unlike the case in the USA biotypes; this antibiosis appears to be an important mode of action against South African biotypes of RWA. Interestingly, the antibiosis effects of at least two of these lines had a similar effect on the population growth of a second aphid species, BCA. Hence, these lines should also be evaluated for breeding BCA-resistant barley lines particularly, in countries where both aphid species are pests.

4 Chapter 4: Distribution of wound callose in response to feeding damage by RWASA1 and RWASA2

4.1 Introduction

Aphids feed in the phloem of their host plants, removing essential food materials and growth substances (Dixon, 1978; Miles, 1999; Saheed et al., 2007a, 2007b). This action has been shown to result in stunted growth and low crop yield (Blackman and Eastop, 2006). While feeding, aphids damage the phloem tissue, a development which initiates cascades of wound responses from the host plants, which includes formation and deposition of wound callose (Botha and Matsiliza, 2004; de Wet and Botha, 2007; Saheed et al., 2009). Callose is a β-1, 3-glucan carbohydrate compound that is reported to be rapidly deposited in sieve pores and plasmodesmata between the sieve tube-companion cell complex in response to wounding (Nakashima et al., 2003; Saheed et al., 2009). Deposition of callose is a defence response by host plants, which effectively seal sieve plates, pore plasmodesmal areas and sieve area pores all in the phloem tissue to the feeding aphids, in order to reduce assimilate loss from the phloem (Sjölund, 1997).

Recent studies have shown that aphid-induced wound callose is deposited in phloem tissues of susceptible wheat and barley cultivars infested with the Russian wheat aphid (Botha and Matsiliza, 2004; Saheed et al., 2007a, 2009; Walton and Botha, 2008). Formation of this wound response is reduced in resistant wheat carrying *Dn1* gene (De Wet and Botha, 2007; Walton and

Botha, 2008). These engineered wheat cultivars, carrying *Dn1* gene, produce an antibiotic effect against RWA feeding, suppressing its population growth, fecundity and biomass (Du Toit, 1987, 1989b; Budak et al., 1999; Heng-Moss et al, 2003).

A more virulent biotype of RWA, RWASA2, recently reported from South Africa, appears not to be affected by *Dn1* gene in wheat (Tolmay et al., 2007; Walton and Botha, 2008). Studies by Botha and Matsiliza (2004), De Wet and Botha (2007) and Saheed et al. (2009) provided visual presentations of damage inflicted by feeding aphids in vascular tissues of host plants. Furthermore, Walton and Botha (2008) quantified wound callose deposited by the less virulent RWASA1 biotype and the more virulent RWASA2, which demonstrated that RWASA2 not only bred faster but also caused greater wound callose deposition in the leaf veins of both non-resistant and resistant wheat cultivars than RWASA1.

In this chapter, I gave report of experiments conducted to further comparatively evaluate the levels of structural damage caused by RWASA1 and RWASA2 in the vascular tissues of host plants. The study focused on the expected differences between the feeding effects of the two biotypes, through examination of the deposition and distribution of wound callose they formed in the phloem tissues while feeding on the leaves of non-resistant and resistant barley host plants. In addition, morphometric analysis data were presented, which I used to quantify the level of damage caused by RWASA1 and RWASA2 feeding within the sieve tube lumina of the test plants.

The objectives of the study were to determine whether callose production and deposition is proportional to number of aphids feeding on the test plants. In Chapter 3, I showed that there is a difference in the performances of the two RWA biotypes on both non-resistant and resistant barley lines. It was demonstrated that RWASA2 not only bred faster than RWASA1 but this faster breeding capacity translated into more serious leaf rolling damage symptoms recorded on RWASA2-infested plants. The hypothesis behind this investigation is two-fold – first that RWASA2 will inflict more severe structural damage by causing greater wound callose deposition in phloem tissues of test plants than RWASA1 and second that in response to each of the two biotypes, the non-resistant line will show more extensive structural and functional damage than the experimental resistant lines.

4.2 Experimental overview

RWASA1 and RWASA2 were allowed to feed for short- (24h and 72h) and long- (7d and 14d) term on test plants of the four barley lines. After each feeding treatment, the experimental plants were studied as follows:

(a) Population growth (from the initial infested 10 aphids) of the aphids was counted on each plant;

(b) Formation and deposition of wound callose during in infested leaves of test plants using the callose-specific aniline blue fluorochrome. Details on use of this fluorochrome are given in Chapter 2 sections 2.6.2 and 2.6.3;

(c) Morphometric analysis of the deposited wound callose in the leaves of test plants. Measurement of area covered by wound callose in randomly selected images was carried out as described in Chapter 2 section 2.6.4 using phase analysis (analySIS program, Soft Imaging System);

(d) Determination of movement of wound callose signals beyond the aphids' feeding sites in source as well as in sink leaves.

4.3 Results

4.3.1 Visible symptoms of damage

Characteristic symptoms of RWA infestation were associated with the feeding of the two biotypes in the non-resistant PUMA as well as in the three resistant lines (Fig. 4.1). These included chlorosis, necrosis, leaf rolling and longitudinal leaf streaking. Differences in the physical evidence of leaf damage due to feeding by RWASA1 and RWASA2 on resistant and non-resistant strains were evident.

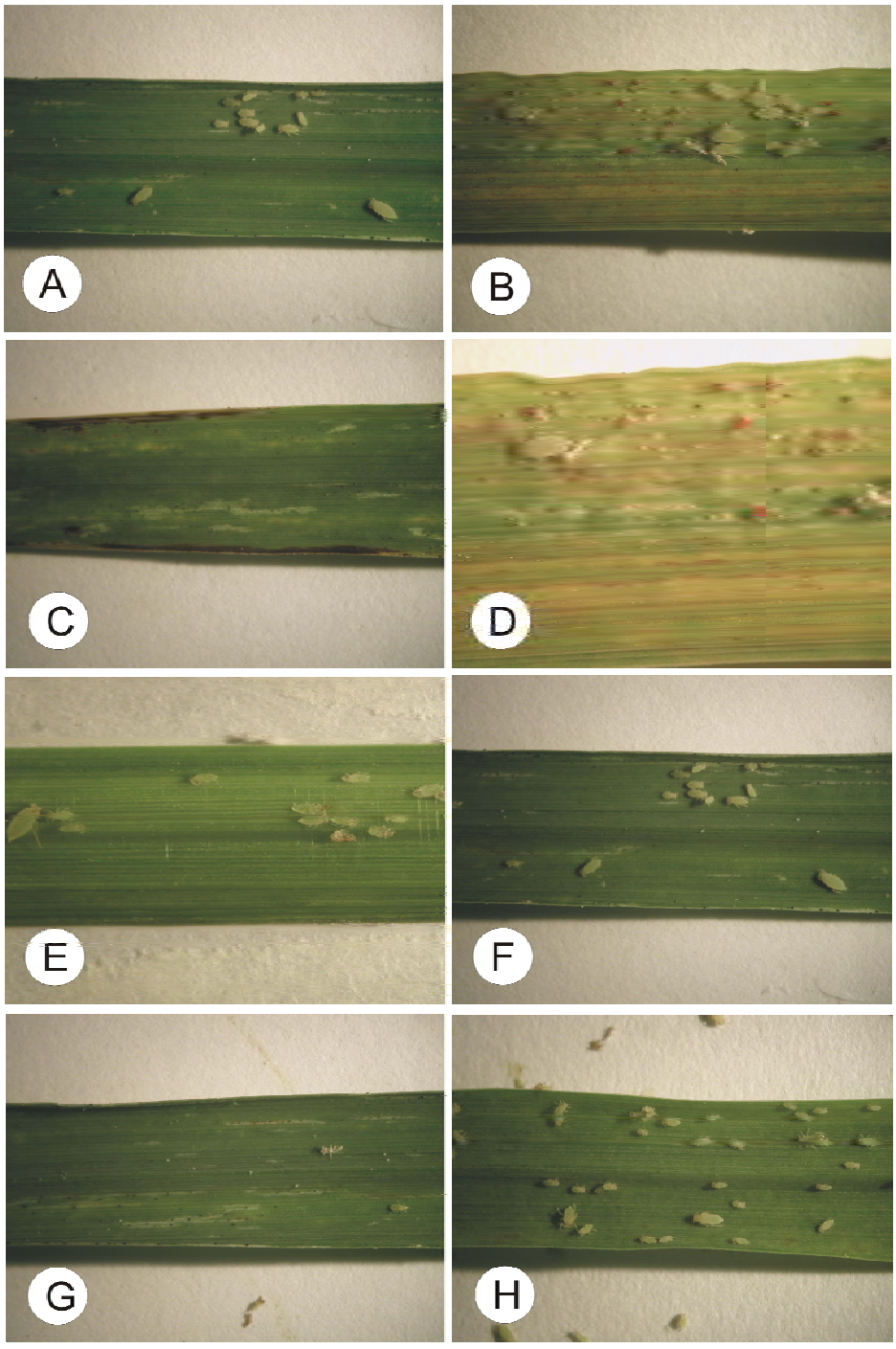

Fig. 4.1 Symptoms of RWASA1 and RWASA2 infestation on resistant and non-resistant barley lines

Table 4.2 Mean† ± SE population growth‡ of RWASA1 and RWASA2 on barley lines at 1, 3, 7 and 14DAI[*]

	RWASA1	RWASA2
Barley line	1 DAI	
PUMA	12.60 ± 0.79 ab	17.10 ± 0.96 abc
STARS-0502B	10.40 ± 0.50 ab	10.80 ± 0.76 ab
STARS-9301B	10.60 ± 0.54 ab	12.60 ± 0.62 ab
STARS-9577B	10.40 ± 0.72 ab	12.20 ± 0.98 ab
	3 DAI	
PUMA	18.70 ± 1.93 abcd	25.80 ± 0.81 bcd
STARS-0502B	8.50 ± 1.08 a	14.90 ± 1.74 abc
STARS-9301B	9.90 ± 0.96 ab	11.50 ± 1.85 ab
STARS-9577B	10.70 ± 0.96 ab	12.90 ± 1.87 ab
	7 DAI	
PUMA	62.50 ± 1.42 g	86.10 ± 2.13 h
STARS-0502B	35.00 ± 1.10 de	58.40 ± 3.99 fg
STARS-9301B	30.90 ± 2.50 cde	43.20 ± 1.89 ef
STARS-9577B	34.40 ± 1.32 de	53.00 ± 3.21 fg
	14 DAI	
PUMA	185.30 ± 4.09 j	246.00 ± 9.85 k
STARS-0502B	100.10 ± 2.27 h	169.80 ± 7.49 ij
STARS-9301B	95.80 ± 3.47 h	164.10 ± 4.22 i
STARS-9577B	99.60 ± 1.88 h	164.80 ± 6.86 i

† Values are means of 10 replicates. ‡ Mean values followed by same letters are not significantly different at the 0.05 level using Tukey *posthoc* test after a 3-way ANOVA.

[*]DAI - Days after infestation.

Fig. 4.1A-H show typical symptoms associated with feeding of RWASA1 (LHS) and RWASA2 (RHS) on non-resistant PUMA and resistant STARS-9301B lines. **A.** Leaf of PUMA on which RWASA1 fed for 72h. Few longitudinal chlorotic lines and leaf rolling from the margins are visible. **B.** PUMA leaf fed on by RWASA2 for 72h. Note extensive longitudinal yellow streaks, necrosis and leaf rolling due to RWASA2 feeding. **C** shows PUMA leaf on which RWASA1 fed for 14d. In contrast to Fig. 4.1A, more extensive chlorosis, necrosis and leaf rolling developed during the long-term feeding. **D.** 14d feeding by RWASA2 on PUMA shows a severely damaged leaf. Note extensive chlorosis and reddish-yellow longitudinal necrotic streaks leaf death. **E.** Leaf of resistant STARS-9301B under RWASA1 72h feeding exposure. The leaf appears more or less normal, fully expanded, though with some chlorotic spots. **F** shows leaf of STARS-9301B fed on by RWASA2 after 72h. There are chlorotic streaks and noticeable leaf rolling at the margins when compared to same leaf under RWASA1 feeding. **G** illustrates leaf of STARS-9301B exposed to RWASA1 feeding for 14d. **H.** In contrast, leaf of STARS-9301B exposed to RWASA2 feeding for 14d sustained more severe damage compared to those under RWASA1 feeding. Note more extensive and spread of chlorotic patches and leaf rolling at the margins. Generally, more visible signs of damage are associated with the non-resistant PUMA line than the resistant STARS lines. Also, plants exposed to RWASA2 feeding developed more severe damage symptoms than those fed on by RWASA1.

Figure 4.1 (A-D) show relative physical damage visible on the non-resistant PUMA line infested with RWASA1 (Figs. 4.1 A and C) and RWASA2 (Figs. 4.1 B and D). During short-term (72h, Figs. 4.1 A and B) and long-term (14d, Figs. 4.1 C and D) feeding exposures. Figures 4.1 A and B show physical damage to PUMA leaves fed upon by RWASA1 and RWASA2 for 72h respectively. On both treatments, the leaves have commenced to roll. Few chlorotic spots are visible in Fig.4.1A while in Fig. 4.1B for RWASA2 feeding damage, extensive chlorosis, necrosis and yellow streaking are visible. After 14d of infestation, damage to the leaves is greater. Figure 4.1C shows more chlorotic spots and some necrosis towards the leaf margins when compared to Fig.4.1A. Greater damage is shown in the PUMA leaf infested with RWASA2 for 14d (Fig. 4.1D). This is characterised by extensive chlorosis and reddish yellow longitudinal necrotic streaks indicative of a dying leaf. Plants exposed to RWASA2 showed more visible signs of physical damage than plants fed upon by RWASA1.

Figures 4.1 E-H show typical feeding-related damage visible on one of the resistant barley lines (STARS-9301B). Similar results were observed on the other two resistant lines for the same duration of infestation (data not shown). Figure 4.1E shows a leaf infested with RWASA1 for 72h. Here, the leaf appears normal but several chlorotic spots are evident. Figure 4.1F is the leaf of the same line when exposed to RWASA2 for 72h. Here, margins of the leaf roll and chlorotic spots can be seen as signs of aphid feeding damage. In Fig. 4.1G when feeding exposure was 14d with RWASA1, extensive chlorosis and

leaf rolling at the margin are visible, which became more extensive due to RWASA2 feeding (Fig. 4.1H). As expected, damage was more developed in the non-resistant PUMA line compared to the three resistant lines.

4.3.2 Aphid population growth

Results of the three-way factorial analysis of variance (ANOVA) of the differences in the means of the population growth of the two aphid biotypes on the four barley lines at 24h, 72h, 7d and 14d feeding exposures, prior to excising the leaves for fluorescence microscopy is shown in Table 4.1. The two aphid biotypes, the four barley lines and the four days of infestation are all significantly different ($p<0.01$).

Table 4.1 General linear model (GLM) results of comparison of levels of interactions on aphid population growths and areas of wound callose recorded at 1, 3, 7 and 14 days of feeding

Interactions	Aphid population growth	Area of wound callose
Aphids	$F_{1,288} = 419.08$ *	$F_{1,288} = 16.46$ *
Barley lines	$F_{3,288} = 210.08$ *	$F_{3,288} = 34.30$ *
Days of infestation	$F_{3,288} = 3476.52$ *	$F_{3,288} = 77.03$*
Aphids × Barley lines	$F_{3,288} = 0.65$ n.s.	$F_{3,288} = 1.63$ n.s
Aphids × Days of infestation	$F_{3,288} = 174.59$ *	$F_{3,288} = 0.27$ n.s.
Barley lines × Days of infestation	$F_{9,288} = 64.85$ *	$F_{3,288} = 8.61$ *
Aphids × Barley lines × Days of infestation	$F_{9,288} = 0.69$ n.s.	$F_{3,288} = 0.35$ n.s.

Levels of significance are indicated as: n.s. (not significant) as $p> 0.01$ and * (significant) at $p< 0.01$.

It is also clear that interactions between the aphids and days of infestation on one hand and the four barley lines and days of infestation on the other hand, are significantly different ($p<0.01$). However, interactions between the aphids and the barley lines as well as the three-way interaction of the aphids, barley lines and days of infestation are not significantly different at $p<0.01$ (Table 4.1). A Tukey *posthoc* test subsequently carried out to identify homogenous groups in the three-way interaction effect shows that RWASA2 population increased more rapidly on all barley lines than was the case with RWASA1 (Table 4.2). It also shows that populations of the two RWA biotypes were higher on the non-resistant PUMA line than on any of the three resistant lines. During sustained long-term 7 and 14d feeding periods, least number of RWASA1 and RWASA2 were recorded on STARS-9301B.

4.3.3 Feeding damage

4.3.3.1 Formation of wound callose in control leaf tissues

As expected, very little callose was found in the control (uninfested) leave tissues of all lines. Development of wound callose due to scraping the leaves was minimized utilizing Ca^{2+}-free MES buffer at pH 7.2. Figure 4.2 (A-F) shows a segment of leaf from control (uninfested) non-resistant PUMA and resistant STARS-9301B lines at 72h. Results for controls taken throughout the study showed little or no callose-associated fluorescence, except that associated with sieve plates (SP) and lateral sieve area pores in longitudinal and cross (XV) veins.

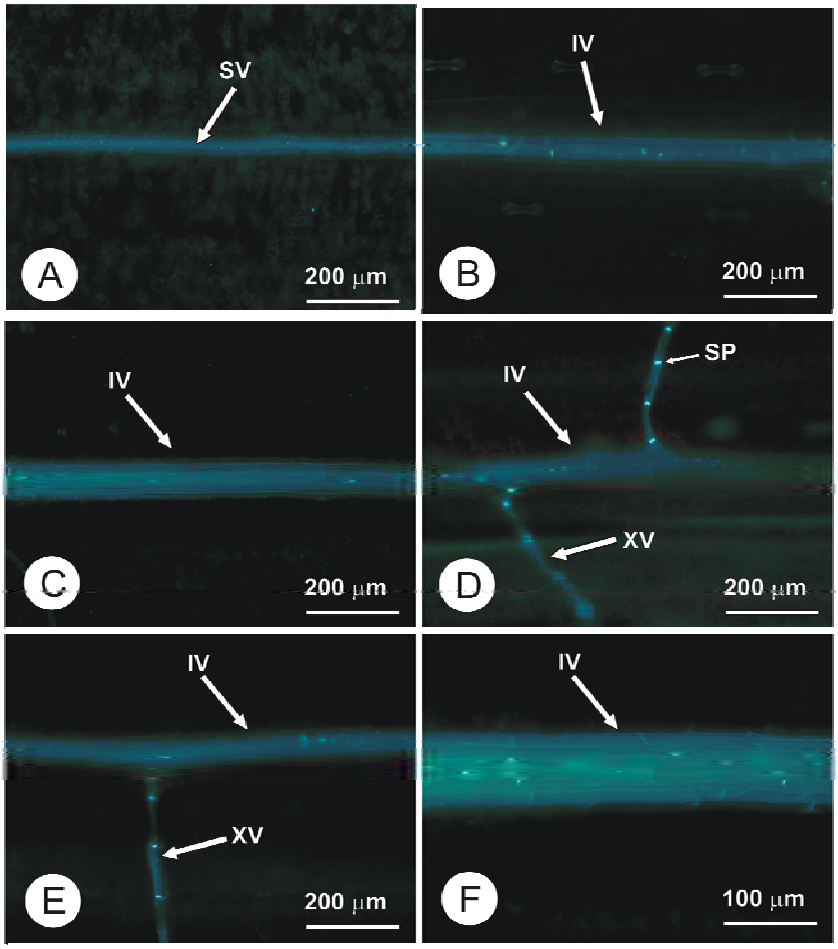

Fig. 4.2 Callose formation in control (uninfested) barley leaves

Fig. 4.2 A-F illustrate longitudinal sections of scraped leaves of control (uninfested) leaves from non-resistant and resistant barley lines. **A and B** repectively show small (SV) and intermediate (IV) veins from the non-resistant PUMA line. **C-F** show intermediate and associated cross (XV) veins obtained after scraping leaves of uninfested resistant STARS lines. Note that development of callose is minimal, restricted to sieve plates (SP) and lateral sieve area pores along the various veins.

4.3.3.2 Formation of wound callose after 24h feeding exposure

Formation of wound callose appeared within 24h of feeding by RWASA1 and RWASA2, in both resistant and non-resistant barley lines. Figures 4.3 A-H illustrates the 24h (short-term) pattern of RWASA1- (Fig. 4.3 left hand side) and RWASA2- (right hand side) induced wound callose distribution in the veins of resistant and non-resistant lines. Aphid stylets (ST) were inserted into the veins (Figs. 4.3A, B and H) and wound callose associated with stylets or stylet tracks was visible. Majority of the wound callose was however associated with the sieve plates (SP) and pore-plasmodesmata units in the common walls between the sieve tube members and their associated parenchyma elements. Visible development at 24h feeding exposure relates to commencement of feeding by the aphids. There was no evidence of difference in wound callose formation either due to the two aphid biotypes or to the barley lines (non-resistant vs. resistant).

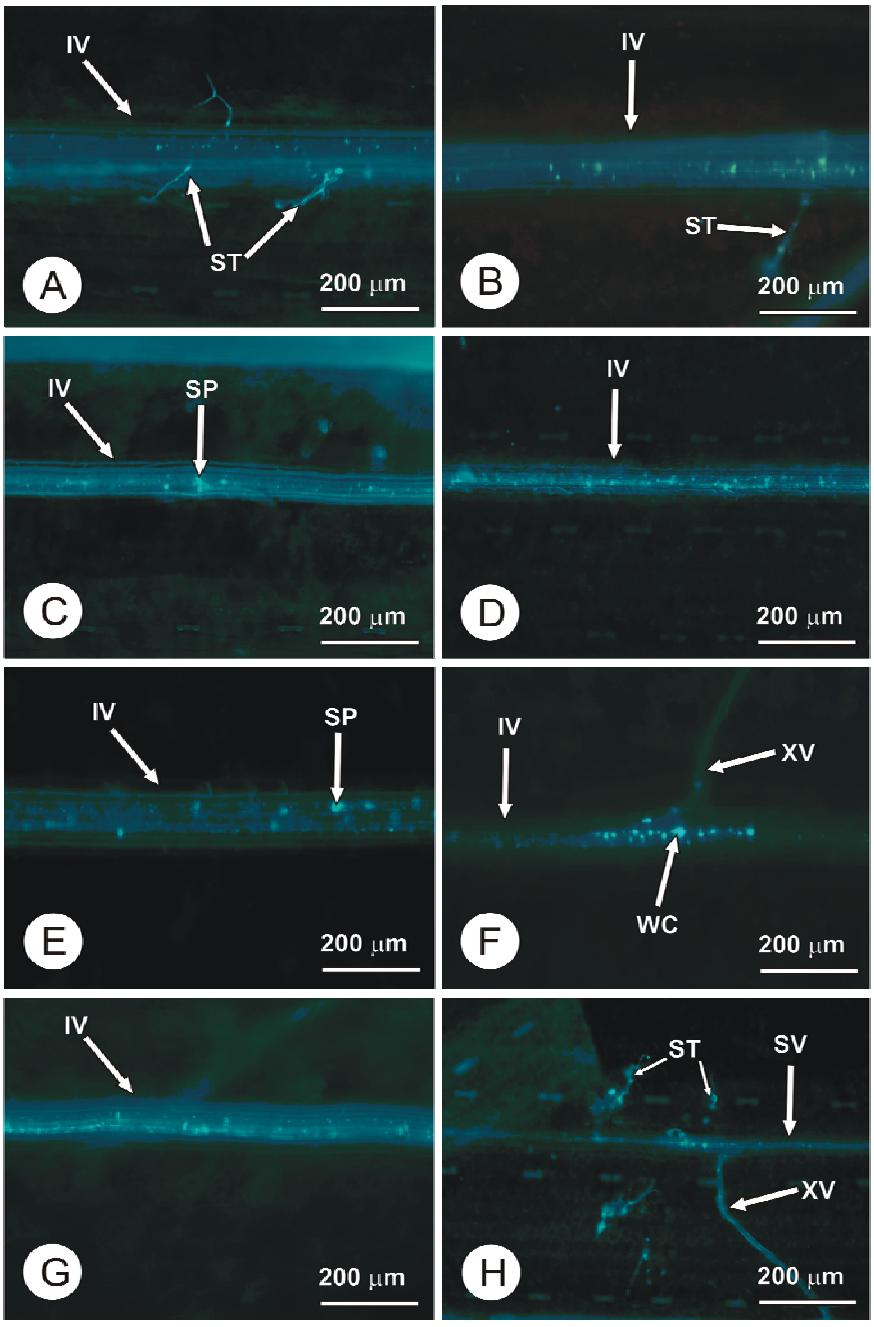

Fig. 4.3 Wound callose formations by RWASA1 and RWASA2 after 24h feeding on barley leaves

Fig. 4.3A-H show aspects of wound callose formation by RWASA1 (LHS) and RWASA2 (RHS) after a short-term 24h feeding on barley leaves. **A**. Feeding by RWASA1 colony show aphid stylets (ST) inserted in the intermediate vein (IV) of non-resistant PUMA leaves. **B**. Part of an intermediate vein of PUMA showing the stylet of feeding RWASA2. In both A and B, wound callose formation is minimal and appears to be confined to sieve plates within the veins. **C**. Small intermediate vein from resistant STARS-0502B fed on by RWASA1 for 24h. Intense fluorescence was seen only on sieve plates (SP) and pore-plasmodesmata units within the veins. **D.** In contrast, an intermediate vein of the same barley line exposed to RWASA2 feeding shows more intense fluorescence of wound callose along its length. **E.** Part of an intermediate vein from leaf of resistant STARS-9301B fed on by RWASA1. Sieve plates contain limited fluorescence. **F.** Corresponding intermediate vein from a leaf of STARS-9301B exposed to RWASA2 feeding showing greater level of wound callose (WC) formation within the vein. **G.** Small intermediate vein of a STARS-9577B leaf on which RWASA1 fed. There is sparse distribution of wound callose. **H.** In contrast, the image of a STARS-9577B leaf exposed to RWASA2 shows stylets inserted in the mesophyl area, the action of which resulted in formation of wound callose that can be seen in the small vein (SV).

4.3.3.3 Formation of wound callose after 72h

Wound callose becomes more evident by 72h of feeding by the two aphid biotypes (Figs. 4.4 A-H). In the non-resistant PUMA, RWASA2 induced greater wound callose than RWASA1 (see Fig. 4.4B and Fig. 4.4A respectively). As expected, wound callose was more evident in the veins of the non-resistant line (Figs. 4.4 A and B) compared to those of the resistant lines (Figs. 4.4 C-H). In the resistant lines, RWASA2 induced greater wound callose (Figs. 4.4 D, F and H) than RWASA1 (Figs. 4.4 C, E and G). No difference among the three resistant lines due to RWASA1 and RWASA2 feeding was evident.

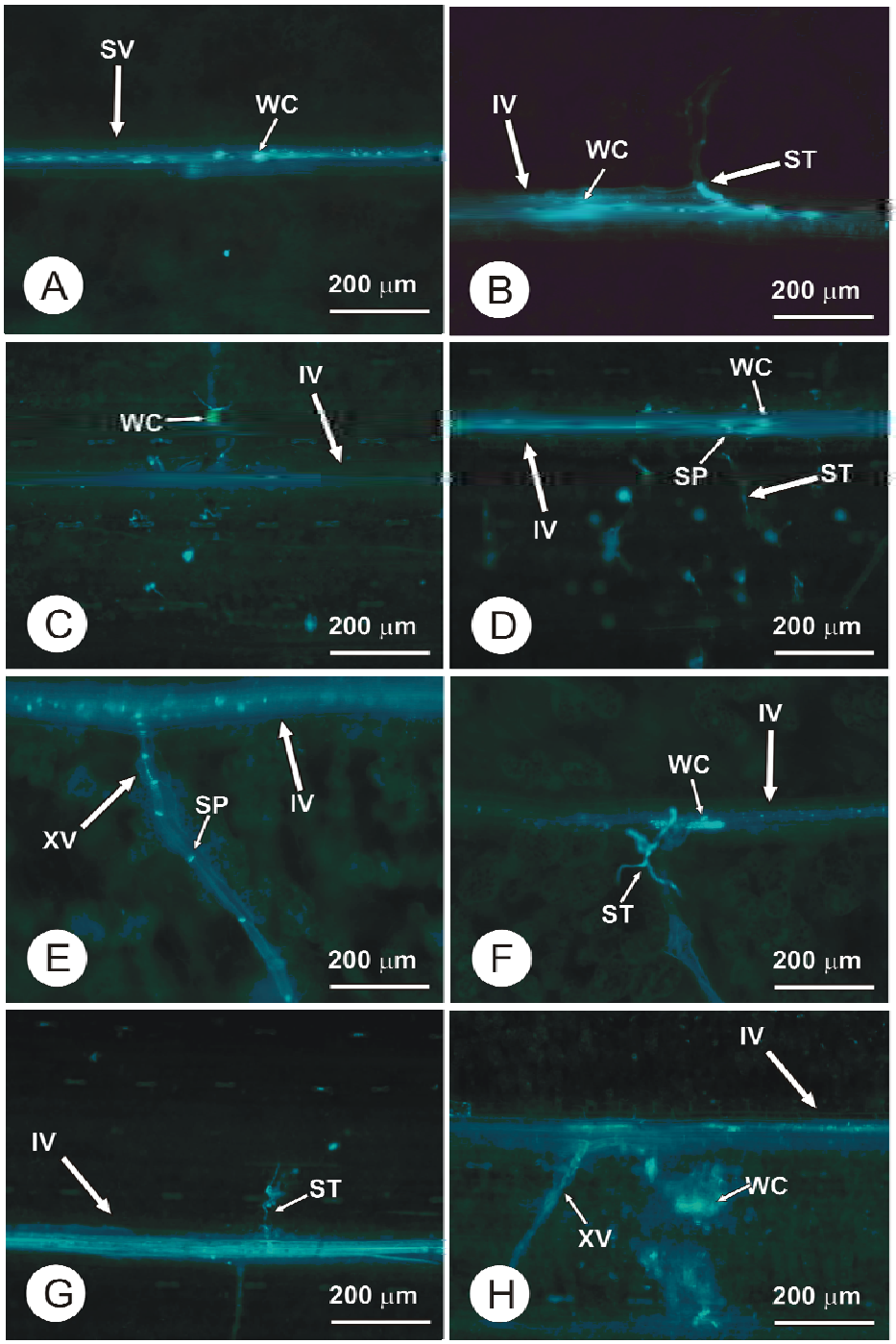

Fig. 4.4 Wound callose formation by RWASA1 and RWASA2 after 72h feeding on barley leaves

Fig. 4.4 A-H illustrate patterns of wound callose formation by RWASA1 (LHS) and RWASA2 (RHS) after 72h feeding on barley leaves. **A.** After 72h exposure to RWASA1, development of wound callose within the small vein (SV) of the non-resistant PUMA line has intensified. More regions of wound callose (WC) are visible within the vein. **B.** An intermediate vein (IV) from a PUMA leaf exposed to RWASA2. Part of a stylet (ST) lodged within the vein shows intense fluorescence. Wound callose (WC) is spread extensively along the vein. **C.** A small intermediate vein from a resistant STARS-0502B leaf fed on by RWASA1. Little wound callose could be seen in the vein when compared to the situation in the PUMA line. **D.** In contrast, an intermediate vein of STARS-0502B leaf under RWASA2 feeding showed more intense wound callose formation within the vein. **E.** An intermediate vein and associated cross veins(XV) from a leaf of STARS-9301B fed on by RWASA1. Wound callose formation is restricted to sieve plates and pore plasmodesmata units and is not as intensive as that observed in the non-resistant line. **F** shows part of an intermediate vein from resistant STARS-9301B leaf in which the stylet lodged within the phloem caused intense wound callose formation. **G.** An intermediate vein from a leaf of STARS-9577B line exposed to RWASA1 feeding. Feeding stylet inserted in the sieve tube showed intense fluorescence on the sieve plate. **H.** Intermediate vein of a STARS-9577B leaf fed on by RWASA2 showed extensive wound callose formation within the vein and associated part of the vascular tissue probed by the aphid.

4.3.3.4 Formation of wound callose at 7d

By 7d, damage to the vascular tissue was more extensive (Figs. 4.5 A-H). In the non-resistant line, RWASA2 induced a greater area of wound callose in the mesophyll tissue (Fig. 4.5B) than RWASA1 feeding (Fig. 4.5A). Resistant lines exposed to feeding by RWASA2 (Figs. 4.5D, F and H) show more damage compared to feeding by RWASA1 (Figs.4.5C, E and G). A more intense wound callose fluorescence is visible in the leaves of STARS-0502B and STARS-9577B infested with RWASA2 (Figs. 4.5D and H) than in STARS-9301B (Fig. 4.5F). Overall, the non-resistant PUMA line was more severely affected by both aphid biotypes (Figs. 4.5A and B) than any of the resistant lines (Figs. 4.5C – H).

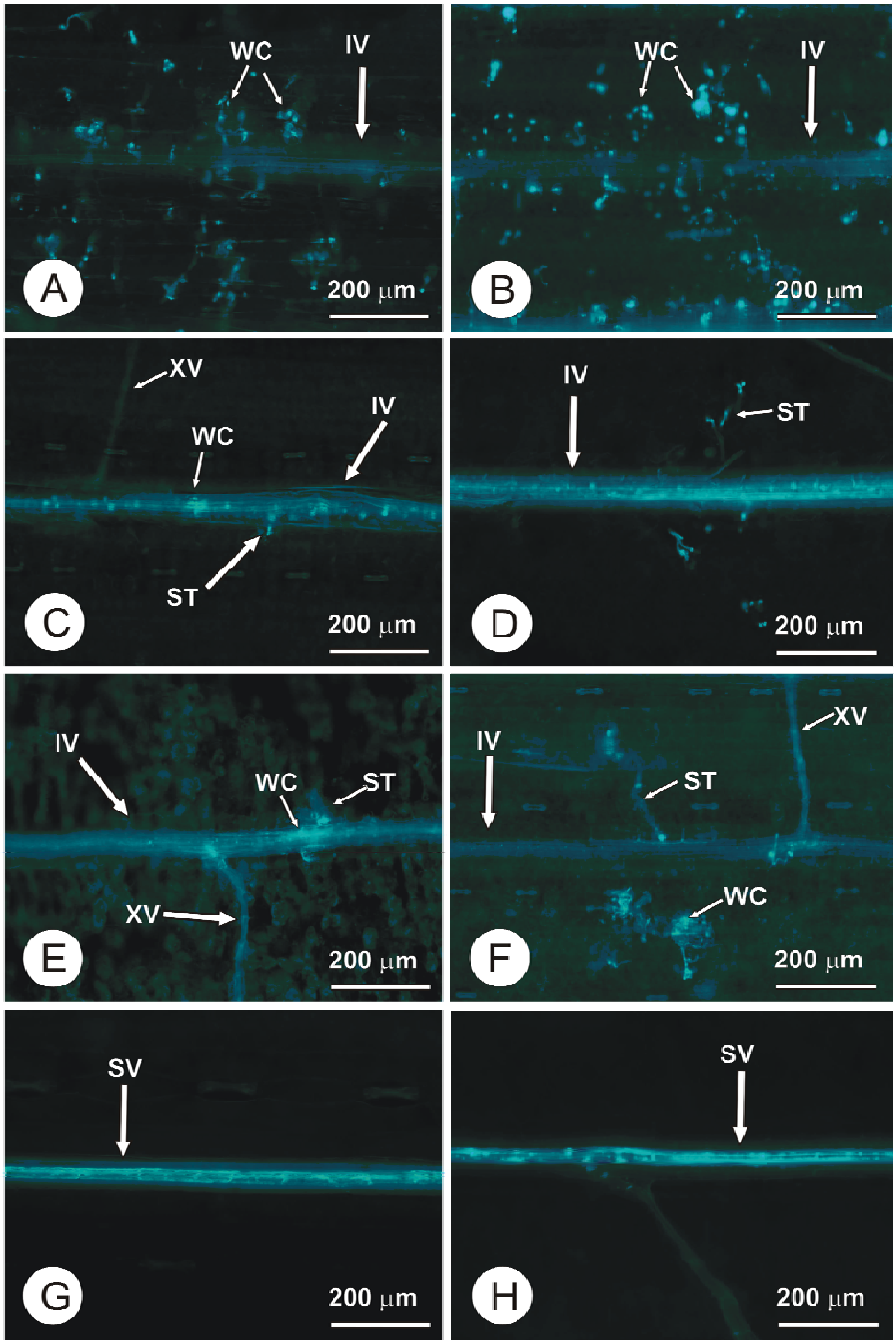

Fig. 4.5 Wound callose formation by RWASA1 and RWASA2 after 7d feeding on barley leaves

Figs. 4.5 A-H. Aspects of the effect of feeding by RWASA1 (LHS) and RWASA2 (RHS) on barley during long-term feeding of 7d. **A.** Shows widespread wounding on a PUMA leaf by feeding of RWASA1. Note numerous wound callose sites (WC) throughout the mesophyll, possibly showing a blocked intermediate vein (IV). **B.** More intense fluorescence of wound callose developed on a PUMA leaf exposed to RWASA2. **C.** An intermediate vein of resistant STARS-0502B showing part of a stylet (ST) lodged in the sieve tube. Deveolpment of wound callose has apparently intensified compared with the 72h exposure but not as greatly as in the corresponding non-resistant PUMA line above. **D.** Intermediate vein from a STARS-0502B leaf exposed to RWASA2 feeding for 7d. The vein appears blocked by extensive wound callose. **E.** Part of an intermediate vein of a STARS-9301B leaf on which RWASA1 fed. Although wound callose is visibly formed in the vein, it is not as widespread as in those of other lines (see D and H). **F** shows a largely blocked intermediate vein of STARS-9301B leaf resulting from RWASA2 feeding. Note wound callose formed by probing stylets in the mesophyll. **G.** Here, a small vein (SV) from a leaf of STARS-9577B fed on by RWASA1 is shown. **H** shows a corresponding small vein of the same line fed on by RWASA2, indicating more intense and widespread wound callose than that formed by RWASA1, shown in G.

4.3.3.5 Formation of wound callose at 14d

Long-term feeding-associated wound callose formation in the leaves of test plants after a sustained 14d feeding exposure to the two aphid biotypes are illustrated in Fig. 4.6 (A-H). In the non-resistant line, there is extensive wound callose formation in the phloem of longitudinal intermediate veins fed on by RWASA1 (Fig. 4.6A). Sustained feeding in the leaves of the barley lines by RWASA2 results in the deposition of wound callose, which completely blocked the small veins (SV) (Fig. 4.6B). Extensive wound callose distribution is evident where aphids have probed and fed on the leaf. Interestingly, feeding by each of RWASA1 and RWASA2 on the resistant lines does not show much intense reaction as was observed in the corresponding non-resistant line (compare Figs. 4.6A and B with Figs. 4.6 C-H). However, probed and punctured leaves of the resistant lines also show more extensive wound callose formation at 14 DAI (Figs. 43.6C-H) compared to feeding for seven days (see Figs. 4.5C-H). Feeding by RWASA2 (Figs. 4.6D, F and H) resulted in higher callose formation and deposition in the phloem of the intermediate veins (IV) compared to corresponding feeding by RWASA1 shown in Figs. 4.6C, E and G. It is evidently shown that STARS-0502B and STARS-9577B (see Figs. 4.6C, D, G and H) are more affected than STARS-9301B (Figs. 4.6E and F). The sieve tubes are progressively callosed such that the veins appear to be blocked (Fig. 4.6B) and may have consequently lost their functionality. These data indicate that there is progressive increase in wound callose formation from the 24h treatments up

to the 14-day feeding exposure due to feeding by the two aphid clones in both the non-resistant and resistant barley lines.

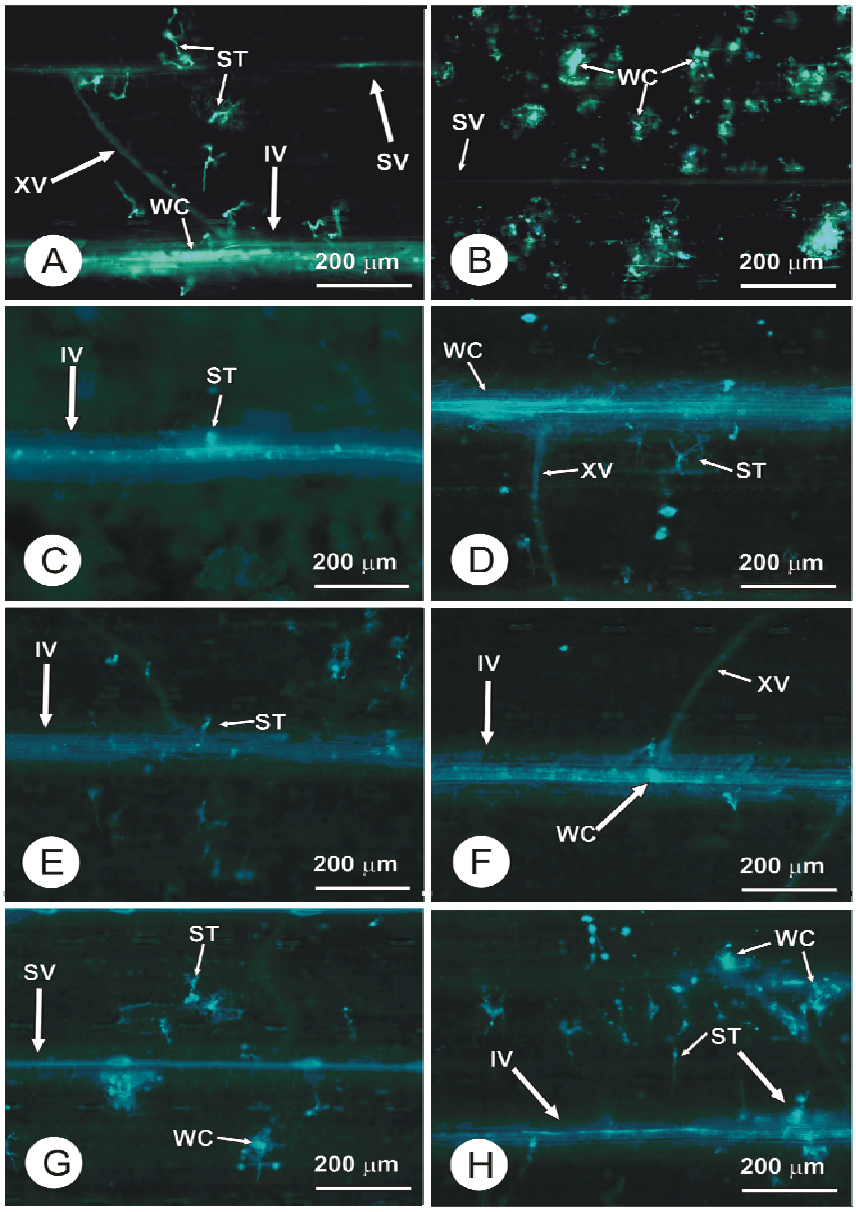

Fig. 4.6 Wound callose formation by RWASA1 and RWASA2 after 14d feeding on barley leaves

Fig. 4.6 A-H show patterns of wound callose formation in barley leaves on which RWASA1 (LHS) and RWASA2 (RHS) have fed after sustained long-term 14d exposure. **A.** Part of a small vein (SV), an intermediate vein (IV) and a connecting cross vein (XV) from a PUMA leaf fed on by RWASA1. Feeding sites of the aphids show intense callose-associated fluorescence. The intermediate vein contains extensive spread of wound callose within it. **B.** A sustained long-term feeding by a large colony of RWASA2 on PUMA formed widespread wound callose. **C.** A small intermediate vein from a leaf of STARS-0502B fed on by RWASA1. Part of a stylet (ST) is embedded in a sieve tube where it caused severe wounding and callose deposition. **D.** An intermediate vein of STARS-0502B leaf exposed to RWASA2 feeding. Wound callose is extensively formed and deposited in the sieve tubes. **E.** An intermediate vein from a leaf of STARS-9301B fed on by RWASA1. Image shows widespread probed area and the vein possibly blocked by wound callose. **F.** Intermediate vein from a leaf of STARS-9301B exposed to RWASA2 feeding. Although the vein contains wound callose, the reaction in this case is not as widespread as can be seen on other images. **G** shows a small vein from STARS-9577B leaf on which RWASA1 fed. Aphid probes caused wound callose which may have blocked the vein. **H.** Here, copious wound callose associated with probing by a large colony of RWASA2 is shown. On a general note, sustained long-term feeding led to formation of large colonies of the two aphid clones on all the lines. This is accompanied by deposition of extensive wound callose in vascular parenchyma and phloem elements in the longitudinal sections of the various veins. There were extensive damage to all barley lines irrespective of whether resistant or not.

4.3.3.6 Movement of the wound callose signal beyond the aphids' feeding sites

Of interest was the determination of callose signal transmission in damaged leaves. Typical results obtained during investigation of transport of the wound callose signals beyond part of the leaves where the aphids were confined for feeding for 7d in both source and sink leaves are illustrated in Figs. 4.7 and 4.8. Little or no wound callose was formed in longitudinal veins of the source leaves of non-resistant and resistant lines above the feeding sites of the two aphid biotypes (Figs. 4.7A-D). However, wound callose was formed in leaf portions below the clipcages (Figs. 4.7E-H).

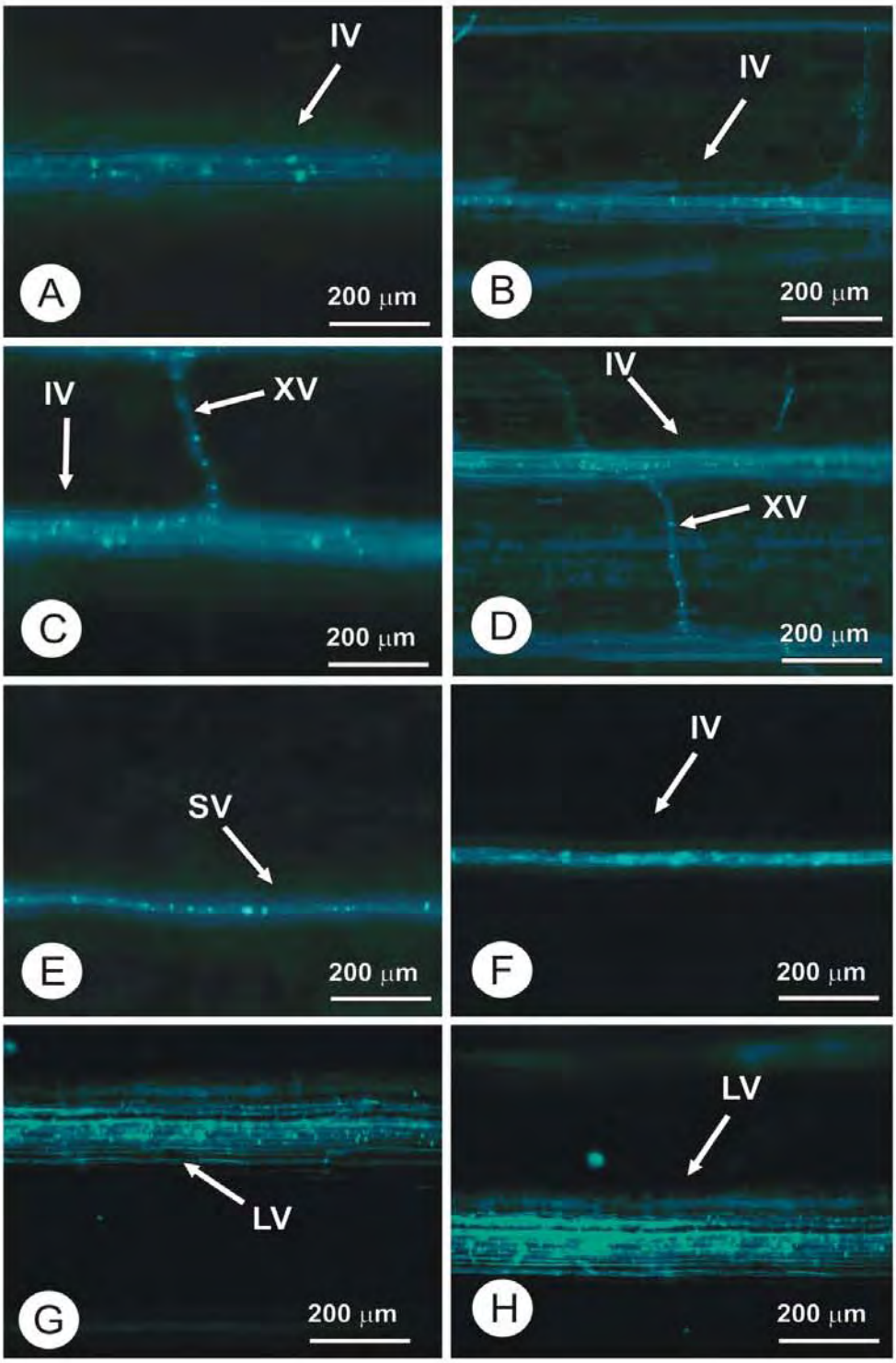

Fig. 4.7 Transport of wound callose signal beyond feeding sites of aphids in source leaves

Fig. 4.7 A-H illustrate patterns of movement of wound callose signal above and below the feeding sites of aphids in source leaves of non-resistant PUMA and resistant STARS-9301B after 7d feeding exposure. **A** and **B** show parts of the intermediate vein above the clipcage in the leaf blade of non-resistant PUMA line infested with RWASA1 and RWASA2 respectively. **C** and **D** denote corresponding areas in the resistant STARS-9301B for RWASA1 and RWASA2 respectively. Note that little or no wound callose was formed within the intermediate (IV) and associated cross (XV) veins except fluorescence of the sieve plates. **E** and **F** respectively are images of the scraped source leaf blade of PUMA below the sites where RWASA1 and RWASA2 were confined for feeding. **G** and **H** are the corresponding sites below the clipcages for STARS-9301B. In contrast to portions above the clipcages, it is interesting to note here that wound callose were formed in the small intermediate and large veins.

The reverse was obtained in sink leaves where wound callose was formed above the clipcages (Figs. 4.8A-D), which was not formed below the clipcages (Figs. 4.8E-H). This pattern was observed in all the treatments of both short- and long-term feeding among the test plants infested with the two aphid biotypes.

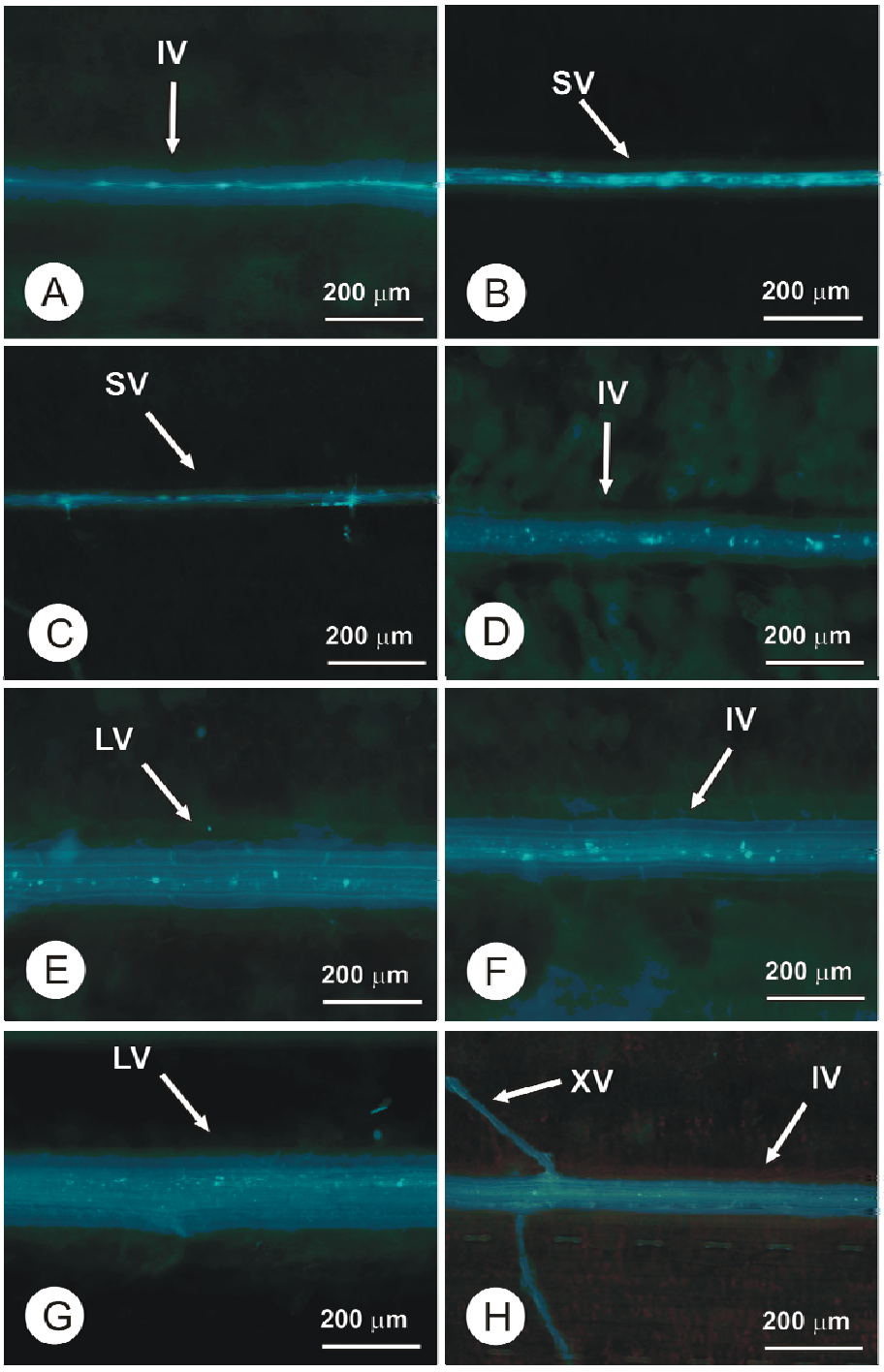

Fig. 4.8 Transport of wound callose signal beyond feeding sites of aphids in sink leaves

Fig. 4.8 A-H show the pattern of transport of wound callose signal above and below the clipcages in sink leaves of PUMA and STARS-9301B fed on by RWASA1 and RWASA2 for 7d. **A** and **B** show portions of the leaf above the sites of feeding RWASA1 and RWASA2 respectively. **C** and **D** are the respective corresponding images for the two aphid clones in STARS-9301B. Wound callose are formed in the phloem tissues. As expected, more wound callose are formed in veins of non-resistant PUMA than in those of resistant STARS-9301B. **E** and **F** illustrate parts of large and intermediate veins of the leaf blade of PUMA below the feeding sites of RWASA1 and RWASA2 while **G** and **H** are the corresponding images in STARS-9301B line. Little or no wound callose are formed in these veins.

4.3.3.7 Distribution of wound callose in leaf tissues

Results of the three-way ANOVA at various levels of interactions for the area of callose, measured morphometrically in randomly selected images for each feeding treatment is shown in Table 4.1. It is indicated that the effects of the two aphid biotypes, the four barley lines and days of feeding exposure are all significantly different ($p<0.01$) with respect to area of wound callose measured on images. It also shows that the effect of the interaction between the barley lines and days of infestation is significantly different ($p<0.01$). However, interactions between the aphids and the barley lines, aphids and days of infestation as well as the three-way interaction effects of the aphids, barley lines and days of infestation are all not significantly different at $p<0.01$ (Table 4.1).

Figures 4.9A and B show the trend of wound callose distribution in the leaves of the four barley lines fed upon by RWASA1 and RWASA2 during short- (1 and 3 DAI; Fig. 4.9A) and long-term (7 and 14 DAI; Fig. 4.9B) feeding treatments. The figures also contain Tukey's *posthoc* tests conducted to identify homogeneous groups in the three-way interaction effects (shown as letters in Figs. 4.9A and B) post ANOVA. Each aphid infestation caused a progressive increase in the area of wound callose with increasing days of infestation. RWASA2 had higher values on each barley line than RWASA1. At 24h, there was no significant difference (at the 5% level) in wound callose formed in leaf tissues of the four barley lines due to RWASA1 feeding. Interestingly, after 24h of RWASA2 feeding, the pairs of PUMA: STARS-

0502B and STARS-9301B: STARS-9577B behaved similarly (Fig. 4.9A). At 3DAI, there was no significant difference in the area of wound callose of PUMA and STARS-0502B infested with RWASA1. Similarly, there was no significant difference in the area of wound callose in STARS-9301B and STARS-9577B. However, on this same day under RWASA2 infestation (Fig. 4.9A), area of wound callose formed in the non-resistant PUMA was higher and significantly different from those formed in the three resistant lines, which all behaved similarly. At 7DAI, there was no significant difference in the area of wound callose recorded for the three resistant lines infested with RWASA1 and as well for those of STARS-9301B and STARS-9577B infested with RWASA2 (Fig. 4.9B). At 14DAI, it was observed that there is no significant difference in the area of wound callose observed in STARS-9301B and STARS-9577B infested with RWASA1. Interestingly, areas of wound callose formed in leaves of the four barley lines were all significantly different from one another at 5% confidence limit using Tukey *posthoc* test.

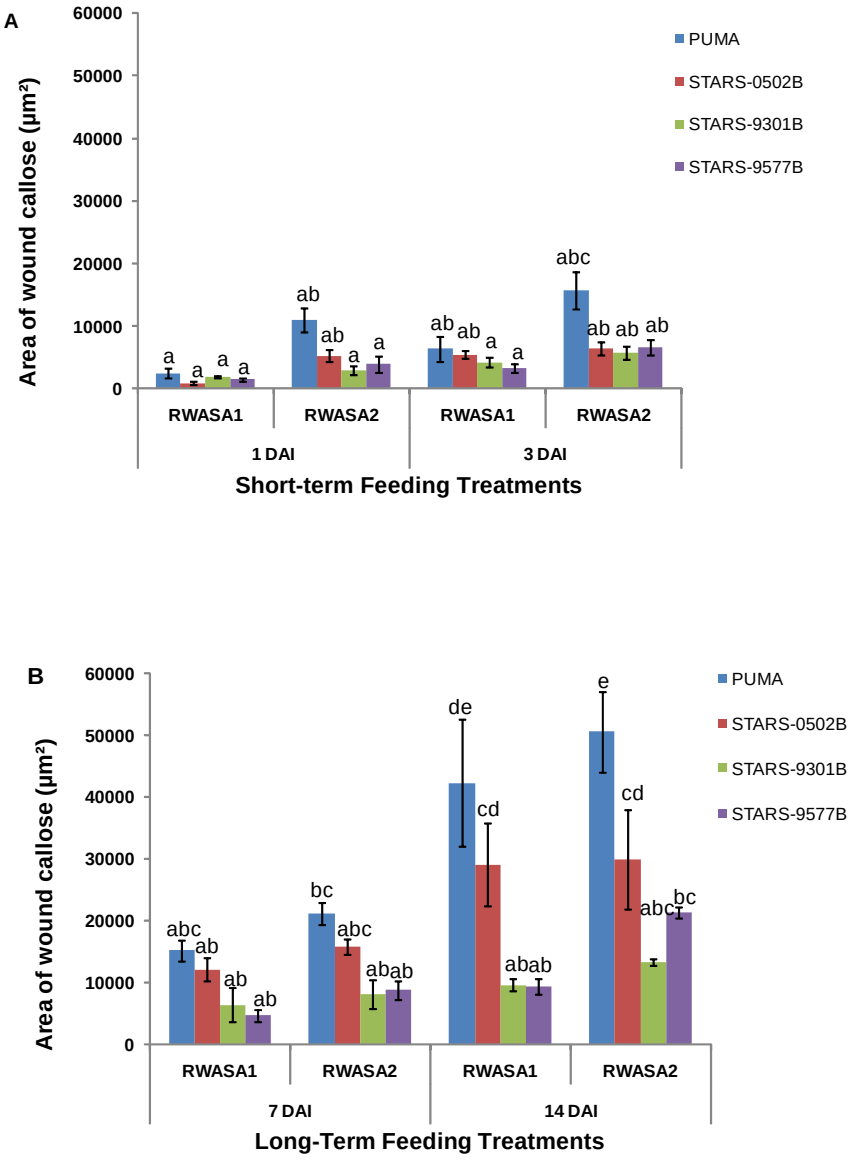

Fig. 4.9 Distribution of wound callose in barley leaves during short-(A) and long-term (B) feeding by RWASA1 and RWASA2

Fig. 4.9 Area of wound callose (μm^2) due to infestation by RWASA1 and RWASA2 on the four barley lines. Letters above each bar indicate results of Turkey *posthoc* test at 5% level of significance to identify homogeneous groups. n=10. **A.** Shows trend of wound callose distribution after the aphids have fed for 24h and 72h (short-term feeding exposure). Feeding by RWASA1 on the four barley lines give similar area of wound callose whereas RWASA2 feeding produced more wound callose particularly on the non-resistant PUMA and STARS-0502B. At 3DAI, greater wound callose were formed compared to 1DAI. Feeding by RWASA2 at 3DAI shows that wound callose response in PUMA is significantly higher than in the three STARS lines. **B.** In comparison, trend of wound callose distribution after sustained long-term feeding (7 and 14 DAI) revealed variability in reactions to feeding by the two biotypes among the four lines. At 14DAI it was obviously shown that RWASA2 is more virulent than RWASA1.

4.3.3.8 Relationship between aphid population and total area of wound callose

Table 4.3 shows the results of the Pearson's Product Moment correlation between the population of each aphid biotype and the corresponding area of wound callose measured on each cultivar for each of 1, 3, 7 and 14 DAI. Overall, the results suggest that the number of aphids on a leaf does not necessarily determine the area of wound callose developed. All the correlation coefficient values show weak correlation between the two variables, half of which are weakly positively correlated and half weakly negatively correlated except in STARS-9577B fed upon by RWASA2 at 7DAI, which showed a stronger positive correlation between aphid population and area of wound callose. There is no significant difference in any of the correlation coefficient values ($p < 0.05$).

Table 4.3 Correlation between aphid population and area of wound callose formed in barley leaves at 1, 3, 7 and 14 days of feeding exposure to RWASA1 and RWASA2

	RWASA1	RWASA2
Barley line	1 DAI	
PUMA	-0.148	-0.107
STARS-0502B	-0.299	0.199
STARS-9301B	0.263	0.097
STARS-9577B	0.342	-0.216
	3 DAI	
PUMA	0.054	0.018
STARS-0502B	0.266	0.255
STARS-9301B	-0.541	-0.238
STARS-9577B	-0.214	0.130
	7 DAI	
PUMA	0.273	0.035
STARS-0502B	-0.098	-0.109
STARS-9301B	0.018	0.298
STARS-9577B	-0.134	0.612
	14 DAI	
PUMA	-0.095	0.289
STARS-0502B	0.376	0.256
STARS-9301B	-0.255	-0.436
STARS-9577B	-0.449	-0.024

4.4 Discussion

Formation of callose (β-1, 3-glucan) is a recognised plant response to wounding and other physiological stress (Donofrio and Delaney, 2001). Wounding, particularly during aphid feeding not only decreases the rate of assimilate transport in the vascular tissue of plants (Botha and Matsiliza, 2004), but also causes turgor loss due to puncturing of parenchymatic cells and elements of the phloem (Evert et al., 1968). Callose is formed rapidly after wounding and is deposited between the plasma membrane and the cell wall (Radford et al., 1998; Nakashima et al., 2003). Its rapid formation and deposition in phloem sieve pores is therefore an efficient wound response to prevent assimilate and turgor loss (Sjölund, 1997). It is a safety mechanism, elicited as a plant response to wounding as a result of aphid feeding. It serves to mitigate the detrimental effects of wounding created on the punctured phloem by stylets of the feeding aphids. It thus serves to quickly and effectively seal off the puncture and prevent leakage of assimilates from the symplast. Radford et al. (1998) surmised that callose might block plasmodesmatal pores completely, thereby altering the size exclusion limit of the plasmodesmata primarily to reduce sap loss from punctured phloem. Furthermore, Botha and Cross (2001) reported that callose formation has an integral effect on the regulation of plasmodesmatal pore size when it is deposited in the neck region of the plasmodesmata. However, recent reports show that even though aphid stylet penetration damages sieve elements which leads to formation of wound callose, aphids still manage to feed from the

phloem (Botha and Matsiliza, 2004; Saheed et al., 2009). This was attributed to a special feeding mechanism which prevents wounding reactions such as protein plugging of their feeding site (Will and van Bel, 2006). In addition, aphids inject watery saliva into the sieve element, which enables them to continue sucking assimilates from host plants.

4.4.1 Formation of wound callose in sieve elements

Studies have shown that the RWA causes callose deposition in cereals (Botha and Matsiliza, 2004; Saheed et al., 2009). It has also been reported that aphid-induced wound callose formation and distribution vary with feeding duration as well as susceptibility or resistance of the wheat cultivars studied (De Wet and Botha, 2007; Walton and Botha, 2008). Due to aphid feeding, wound callose deposition is reduced in resistant *Dn1* wheat cultivars compared to susceptible cultivars. However, little is known about the feeding damage and plant response to different RWA biotypes in barley. This study demonstrates that RWASA1 and RWASA2 feeding caused formation of wound callose within 24h of infestation in resistant and non-resistant barley lines (Fig. 4.3). Though restricted to sieve plates and pore-plasmodesmal units, wound callose is visible within the sieve elements (Fig. 4.3) when compared to control aphid-free plants (Fig. 4.2). This is in agreement with an earlier study by De Wet and Botha (2007), using the aphid *Sitobion yakini* on non-resistant Betta and resistant Betta-*Dn* wheat cultivars.

Data from this study suggest that wounding progressed from 1d through 14d. Wound callose formation became extensive after 3d of infestation (Fig. 4.4).

This result confirms an earlier report by Saheed et al. (2009). This was more evident in the non-resistant PUMA (Figs. 4.4 A and B) than in the resistant lines (Figs. 4.4C-H). RWASA2 feeding caused greater callose deposition among the resistant lines (Figs. 4.4D, F and H) than RWASA1 feeding (Figs. 4.4C, E and G). Statistically, no variation in wound callose formed was apparent among the three resistant lines due to either RWASA1 or RWASA2 feeding ($p<0.05$) (Fig. 4.9A). During long-term feeding treatments of 7d (Fig. 4.5) and 14d (Fig. 4.6), callose was more extensively distributed in probed areas in longitudinal and transverse veins. As expected, long-term feeding showed evidence of more callose deposition in non-resistant PUMA from RWASA2 feeding (Figs. 4.5B and 4.6B) than was the case under RWASA1 infestation (Figs.4.5A and 4.6A). However, there are variations in amount and intensity of wound callose formation among the three resistant lines due to both RWASA1 and RWASA2 long-term feeding. STARS-9301B generally had less callose (Figs. 4.5E, F; Figs. 4.6E, F) than did either STARS-0502B (Figs. 4.5C, D; Figs. 4.6C, D) or STARS-9577B (Figs. 4.5G, H; Figs. 4.6G, H). Longer feeding exposure thus induced far greater callose deposits when RWASA2 probed the leaves (Figs. 4.5D, F and H; Figs. 4.6D, F and H) than what was observed during RWASA1 feeding (Figs. 4.5C, E and G; Figs. 4.6C, E and G).

Previous studies by De Wet and Botha (2007) and Walton and Botha (2008) showed that wound callose, as a result of aphid feeding, may be absent or greatly reduced in the resistant wheat cultivars bearing *Dn* resistance genes.

Interestingly, this is in stark contrast to the results reported here where wound callose is present in both resistant and non-resistant barley lines. This supports the idea that plant response to feeding is aphid species-specific (see Gill and Metcalf, 1977; Saheed et al., 2009). While De Wet and Botha (2007) used *S. yakini* on wheat, Walton and Botha (2008), though used RWASA1 and RWASA2 (same as in this study) but worked with wheat cultivars carrying *Dn* gene resistance that are developed against RWASA1 in South Africa as well. There is currently no resistant barley cultivar, developed in South Africa that is resistant to RWA (Tolmay, pers. comm.). It is possible that the two aphid biotypes produced signals that elicited diverse responses in the barley lines when compared with the wheat hosts. In addition, there is the possibility that some salivary components in the South African RWA biotypes are resistance-breaking on the USDA developed barley lines which are resistant to the US RWA biotypes. It has also been expressed that the South African strains of RWA are different from those in the US (Prinsloo, pers. comm.) against which the three resistant barley lines used in this study have been developed.

4.4.2 Transmission of callose signals beyond aphid feeding sites

Transport of assimilates in plants is driven by a classical source-to-sink pathway (Turgeon, 1989). As such, assimilates move from the leaf lamina tip basipetally in source leaves and acropetally in sink leaves. Results of the investigation on possible transport of wound callose signals showed that

where aphids fed on source leaves, the sieve elements below the clipcages contained wound callose (see Figs. 4.7E-H) while those above appeared less damaged (Figs. 4.7A-D), suggesting that part of the 'wound signal' travels along with the assimilates in the phloem. The same trend was established for sink leaves in which more wound callose occurred above the aphids' feeding sites (Figs. 4.8A-D), whereas portions below the feeding sites contain little or no callose (Figs. 4.8E-H).

4.4.3 Distribution of wound callose

The data on distribution of wound callose in leaf tissues expressed as area covered by the wound callose on the leaves investigated (see Fig. 2.1 in Chapter 2), show variation in the formation of wound callose observed in resistant and non-resistant lines fed upon by either RWASA1 or RWASA2. With respect to callose distribution, the RWA biotypes reacted differently to different barley lines ($p = 0.184$, > 0.001). The analysis also confirmed that the number of days of exposure of the four barley lines to the two biotypes (1, 3, 7 and 14d) affected distribution of wound callose ($p = 0.958$, > 0.001) as wound callose deposition on an area basis increases in each of the four barley lines (Figs. 4.9A and B). Irrespective of aphid biotype, area of wound callose is higher in PUMA than in any of the three resistant lines. This supports Walton and Botha (2008) data that callose deposition is higher in the non-resistant Tugela than in its near-isogenic resistant Tugela-*Dn* counterpart. However, PUMA fed upon by RWASA2 showed more evidence of callose than those fed upon by RWASA1.

The trend of callose distribution among resistant lines was STARS-0502B > STARS-9577B > STARS-9301B. The relative reduction in the levels of wound callose in the resistant lines recorded in this study (Figs. 4.5C-H; Figs. 4.6C-H) when compared to the non-resistant PUMA (Figs. 4.5A, B; Figs. 4.6A, B) suggests that the mechanism of resistance may reside in the relative potency of the resistant lines to cause breakdown of callose as soon as it is formed. Clearly, RWASA2 feeding on resistant lines produced a larger area of wound callose than RWASA1 (Figs. 4.9A and B). This supports the report by Walton and Botha (2008) that RWASA2 deposited more callose in resistant Tugela-*Dn* wheat than RWASA1. This indicates that RWASA2 is both a resistance-breaking biotype as well as a more aggressive feeder than RWASA1.

Earlier reports have shown that distribution of wound callose vary between different aphid species (Saheed et al., 2009) and between biotypes of the same aphid (Walton and Botha, 2008). This may imply that aphid virulence can be attributed to their respective ability to affect callose formation or its breakdown in host plant tissues regardless of whether susceptible or resistant. Though not much is known about plant responses induced during aphid attack, few available reports have shown that plants react to aphid infestation through production of PR-proteins, proteinase inhibitors (Casaretto and Corcuera, 1998) and/or some secondary compounds (Niemeyer et al., 1989; Gianoli and Niemeyer, 1998). This was later identified to be β-1, 3-glucanase (Callase, EC 3.2.1.6; Will and van Bel, 2006) which is reported to degrade

callose (Van der Westhuizen et al., 1998; Moran and Thompson, 2001; Will and van Bel, 2006). Elevated levels of β-1, 3-glucanase were reported in resistant wheat cultivars (Forslund et al., 2000; Van der Westhuizen et al., 2002). It can therefore be hypothesized that with respect to the resistant lines, these RWA biotypes may vary in their ability to cause up-regulation in the synthesis of β-1, 3-glucanase, an enzyme capable of removing aphid-induced wound callose as it is progressively formed during sustained feeding. As the aphids induce callose formation, it is possible that RWASA2 feeding does not have the capacity to up-regulate β-1, 3-glucanase formation as much as RWASA1 feeding does. Alternatively, synthesis of β-1, 3-glucanase may be down-regulated in the plant during RWASA2 feeding, in which case callose formation becomes up-regulated, thus confirming that this new biotype is simply a more aggressive feeder than RWASA1. These results show that there is an obvious difference in the wounding responses elicited by RWASA1 and RWASA2 on susceptible and resistant barley lines. This outcome will enable a further examination of the difference in the β-1, 3-glucanase activities of the two aphid biotypes.

4.4.4 Aphid population growth

Both aphid biotypes bred faster on the non-resistant PUMA compared to any of the three resistant barley lines (Table 4.2). This is in agreement with results of similar studies on aphid population growing on non-resistant and resistant cultivars of barley (Gutsche et al., 2009; Jimoh et al., 2011). It also demonstrates that RWASA2 breeds faster than RWASA1 on both resistant

and non-resistant lines. This is in support of the report by Jimoh et al. (2011) and Walton and Botha (2008) on these biotypes growing on susceptible and resistant barley and wheat cultivars respectively. The trend of population growth of the two biotypes on the resistant lines, particularly during longer feeding exposures (3d, 7d and 14d), demonstrates that the resistance gene they contained may have affected the fecundity of the aphids. This supports the idea that the resistant lines may be antibiotic in their reactions to the infesting aphids (Leszcynski et al., 1989; Nkongolo et al., 1990).

4.4.5 Correlation between aphid population and development of wound callose

This study shows that there is no clear correlation between aphid population and deposition of wound callose irrespective of aphid biotype, plant cultivar, or duration of feeding exposure (Table 4.3). Deposition of wound callose is a rapid response to wounding, which impacts on phloem transport as soon as it occurs (Radford et al., 1998; Saheed et al., 2009, 2010). It is formed within minutes of wound initiation by stylets of the feeding aphids and is largely produced in sieve elements in response to elevated calcium levels (Botha and Cross, 2001; Will and van Bel, 2006). Once damaged, even by a small aphid population, vascular tissues are blocked, which must affect phloem functionality and the transport of photoassimilates. As such, population of an infesting aphid may not necessarily determine the level of damage sustained, contrary to expectations based on trends of the results on aphid population growth and distribution of wound callose. A small aphid population may be

capable of initiating signals, which can translate into wound callose deposition in an attempt by the plant to minimize damage to sieve elements. The signals may be amplified with time as the aphids continue to feed and reproduce. For instance, whilst I used 10 aphids per leaf for the initial infestations and recorded callose deposition in veins within 24h (Fig. 4.3), a previous study by Walton and Botha (2008) used only five aphids, which equally induced wound callose deposition within 24h and the callose deposition increases with increase in time and aphid population.

In conclusion, results from this study showed that wound callose formation occurred in both resistant and non-resistant barley lines and that its intensity increased with infestation time. Extensive callose was formed in non-resistant compared to the resistant lines. The study further showed that RWASA2 feeding induced greater wound callose than RWASA1. This further lends support to the findings in Chapter 3 that RWASA2, which bred faster, caused more leaf roll as well as earlier onset of chlorosis damage symptoms than RWASA1. This might be due to variations in the salivary components of each of the two biotypes either by up-regulating the activity of callose synthase in the plant or by suppressing the β-1, 3-glucanase activities when they respectively feed on both resistant and non-resistant barley lines. This is further in agreement with the earlier suggestion that there are biological differences in the feeding behaviours of RWASA1 and RWASA2. Interestingly though, in spite of the findings above, statistical evidences show that the number of infesting aphids does not correlate well with the amount of

wound callose deposited by each aphid biotype in the phloem of either resistant or non-resistant plants. This may be due to instantaneous nature of wound callose deposition irrespective of aphid biotype, number of infesting aphids and duration of feeding exposure. This is the first known report, which both qualitatively and quantitatively details and relates wound callose deposition and distribution to structural damage caused by two biotypes of an aphid species to the vascular tissues of resistant and non-resistant of a plant.

5 Chapter 5: Is feeding-related cell damage proportional to reproduction rates of RWASA1 and RWASA2 on resistant barley lines?

5.1 Introduction

The Russian wheat aphid (RWA) is a very destructive pest of small grains causing major economic losses to their producers (Walters et al., 1980; Kovalev et al., 1991). Infestation results in loss of effective leaf area, substantial reduction in chlorophyll content and reduced photosynthetic capacity of leaves of host plants, all of which culminate in yield loss (Walters et al., 1980; Fouché et al., 1984; Kruger and Hewitt, 1984). Symptoms of RWA infestation are well documented (see Saheed et al., 2007a; Tolmay et al., 2007).

Previous studies have copiously shown that the effects of feeding by RWASA1 primarily causes severe damage to the phloem as well as to the xylem transport systems in leaves of host plants (Botha and Matsiliza, 2004; De Wet and Botha, 2007; Saheed et al., 2007a, 2007b; 2009; 2010). Several reports have described the pathway of its stylets (see Botha, 2005 and literature cited) to their preferred feeding site, the thin-walled sieve tubes, inflicting severe damage to phloem as well as xylem tissues (Botha and Matsiliza, 2004). Studies by Saheed et al. (2007a, 2007b) in particular, highlighted the structural damage caused by RWASA1 to the vascular tissues of its wheat and barley hosts. These authors showed that RWA, in addition to feeding preferentially on thin-walled sieve tubes, also probes the xylem for

water in non-resistant plants and symptomatic leaf streaking, leaf rolling and chlorosis result. Feeding damage by RWASA1 was reduced in resistant Betta-*Dn* wheat cultivar compared to its susceptible Betta counterpart (Saheed et al., 2007b).

Tolmay et al. (2007) reported the appearance of a second RWA biotype in South Africa. The new biotype is now understood to be resistance-breaking and virulent on existing RWA-resistant wheat lines. A preliminary population study (Walton and Botha, 2008) indicated that RWASA2 not only bred faster but also caused more damage to wheat than did RWASA1. RWASA2 is apparently unaffected by the *Dn1* resistance gene (Walton and Botha, 2008) and may therefore pose a serious threat to small grain production in South Africa.

As previously shown in Chapter 3 (Jimoh et al., 2011), RWASA2 breeds faster than RWASA1 on resistant and non-resistant barley lines. It was also reported that the severe damage caused by RWASA2 than RWASA1 on the non-resistant PUMA might be associated with differences in their respective reproductive rates on this line. The study further demonstrated that the development of leaf rolling, an important symptom of RWA feeding, correlated well with the relative population levels of RWASA2 on the resistant lines. Leaf roll symptom and its development have been suggested to be as a result of RWA probing the xylem tissue for water (Saheed et al., 2007a, 2007b). Leaf chlorosis, a second symptom of RWA feeding, did not correlate with aphid population levels. Chlorosis symptoms appeared earlier,

but were more severe on plants fed upon by RWASA1, which is the more slowly reproducing of the two biotypes (Jimoh et al., 2011). It was inferred that the increased level of chlorosis caused by RWASA1 might be due to differences existing either in the behavioural responses or in components of the saliva of each of the two RWA biotypes when feeding on the resistant lines.

The study reported in this chapter focuses attention on the marked differences in the breeding rates of RWASA1 and RWASA2 noted in Chapter 3. Experiments were carried out to examine the relationship between the reproductive rates of the two biotypes and the feeding-related cell damage, which they caused on infested barley lines after a 10d feeding exposure. Comparative wide-field fluorescence microscopy, coupled with in-depth transmission electron microscopy (TEM) investigations was employed to provide ample evidences of the damage sustained by plants infested by the two RWA biotypes. The aim of this study was to evaluate the reproductive rates of RWASA1 and RWASA2 on three resistant lines, known to confer resistance to several RWA biotypes in the US, in relation to the cell damage caused by these aphids. From the study, it would be possible to appraise the differences in the structural damage caused by the two RWA biotypes on the three selected resistant lines. This was to evaluate the potential resistance in each of these three lines to RWASA1 as well as the more virulent RWASA2 biotype. This is with the belief that results obtained from this study will be a good working template for plant breeders in their efforts to develop RWA-

resistant barley cultivars that are presently non-existing in South Africa, to the two RWA biotypes.

5.2 Experimental overview

In the experiments reported in this chapter, the second or third leaves above coleoptiles were fitted with clipcages at the mid–length of each leaf blade, to enclose a 3cm-long leaf segment. Ten adult apterae of either RWASA1 or RWASA2, contained on a leaf segment from the feeder plant, were introduced into each clipcage. Control (uninfested) plants were also fitted with clipcages. Ten replicates of each treatment were set up and experimental procedures were repeated twice. The aphids were allowed to feed and reproduce on the leaf segments for 10 days after which they were counted. Population density per cm^2 of leaf area was calculated by dividing the number of aphids recorded on each leaf segment by the area of the leaf segment enclosed within the clip cage (i.e. 2.1 cm^2). The confined leaf segments, in both infested and control plants, were thereafter processed for either fluorescence or transmission electron microscopy investigations of feeding-related damage.

5.3 Results

5.3.1 Infestation symptoms

The feeding activity of RWASA1 and RWASA2 resulted in visible damage to the leaves of non-resistant PUMA, but was less evident in the three resistant lines. Symptoms such as chlorosis, necrosis, longitudinal yellow streak and leaf rolling were observed on PUMA at 5 DAI, while the resistant lines only

showed few chlorotic and necrotic spots within the 10-day experimental period (data not included). These results were consistent with symptoms observed at this stage of infestation in the experiments reported in chapters three and four.

5.3.2 Aphid population growth

Population growth (Fig. 5.1) and aphid population density per cm^2 of leaf area (Table 5.1) for RWASA1 and RWASA2 on the non-resistant and resistant lines show the means of 10 replicates recorded at 10 DAI, when experiments were terminated for collection of leaf material for the fluorescence microscopy and TEM studies of vascular damage. Two-way ANOVA showed that there were significant differences in the mean number of aphids between the two aphid biotypes ($F_{1,72}$ = 343.4, p= 0.0001) and among the four barley lines ($F_{3,72}$ = 159.2, p= 0.0001). However, there was no significant difference in the interaction between the aphids and cultivars ($F_{3,72}$ = 2.3, p= 0.082). The Tukey *posthoc* test showed that each of the four barley lines responded differently to infestation by the two aphids (Fig. 5.1). Populations of the two biotypes on the test plants increased substantially from the initial 10 apterous aphids at the beginning of the experiments. Of interest was that RWASA2 reproduced much faster and had higher aphid density than RWASA1 on all barley lines (Table 5.1). Among the three resistant lines, STARS-9301B had the lowest aphid density for the two biotypes. As expected, the non-resistant PUMA line supported the largest population for both biotypes after the 10-day experimental period. In contrast, RWA feeding on STARS-9301B resulted in

the lowest population (Fig. 5.1) for both RWASA1 and RWASA2 (about 50 and 85 aphids respectively). PUMA (infested with RWASA1), STARS-0502B (infested with RWASA2) and STARS-9577B (infested with RWASA2) were not significantly different at 5% confidence level. Similarly, there was a lack of significance for both STARS-9301B and STARS-9577B infested with RWASA1. The trend of the population growth of the two biotypes among the resistant lines was thus STARS-0502B>STARS-9577B>STARS-9301B.

Table 5.1 RWASA1 and RWASA2 population density (cm^{-2} of leaf area) on barley leaves at 10 DAI ± standard error of mean

	Aphid Biotype	
Barley line	RWASA1	RWASA2
PUMA	48.67±0.90	65.33±1.12
STARS-0502B	34.05±1.15	47.14±1.12
STARS-9301B	24.95±0.74	39.81±1.91
STARS-9577B	26.38±1.37	45.62±1.09

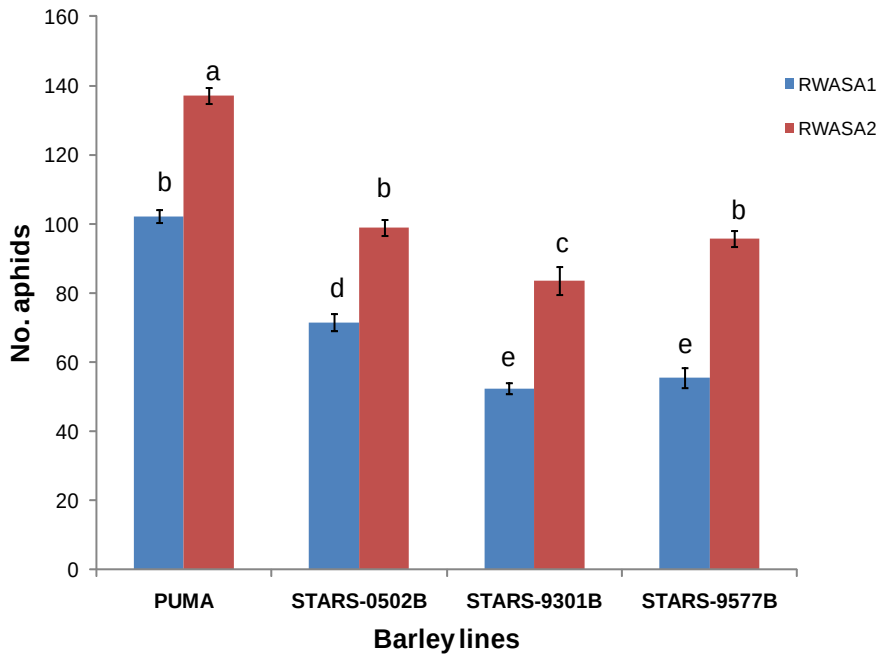

Fig. 5.1 Population growth data for RWASA1 and RWASA2 on the four barley lines after a 10-d infestation period

Fig. 5.1 shows the population growth data for RWASA1 and RWASA2 on four barley lines over a 10-d infestation period, all treatments starting with 10 aphids. As expected, RWASA2 reproduced faster than RWASA1 on non-resistant as well as on the resistant lines. Highest aphid numbers were recorded on non-resistant PUMA for both RWASA1 and RWASA2, but were reduced on STARS-9301B. Bars with different letters indicate significantly different homologous groups at the 0.05 level using Tukey *posthoc* test of a 2-way ANOVA (n=10).

5.3.3 Callose distribution

Figures 5.2 – 5.3 provide a broad view of the damage sustained due to aphid infestation as shown by the distribution of callose within control (uninfested) and infested leaves when viewed with a wide-field fluorescence microscope. In longitudinal veins of uninfested non-resistant PUMA plants (Fig. 2A), what was observed was the normal (developmental) callose deposition which are usually associated with sieve plates, lateral sieve areas, pore plasmodesmal units between sieve elements as well as their associated parenchymatous elements (including companion cells). A similar observation like in uninfested PUMA was found in uninfested resistant STARS-9301B (Fig. 2B). In contrast, during exposure of non-resistant PUMA to RWASA1 (Fig. 2C) or RWASA2 (Fig. 2D), callose deposition and distribution was generally similar and extensive in sieve tubes as well as the sieve tube lumina.

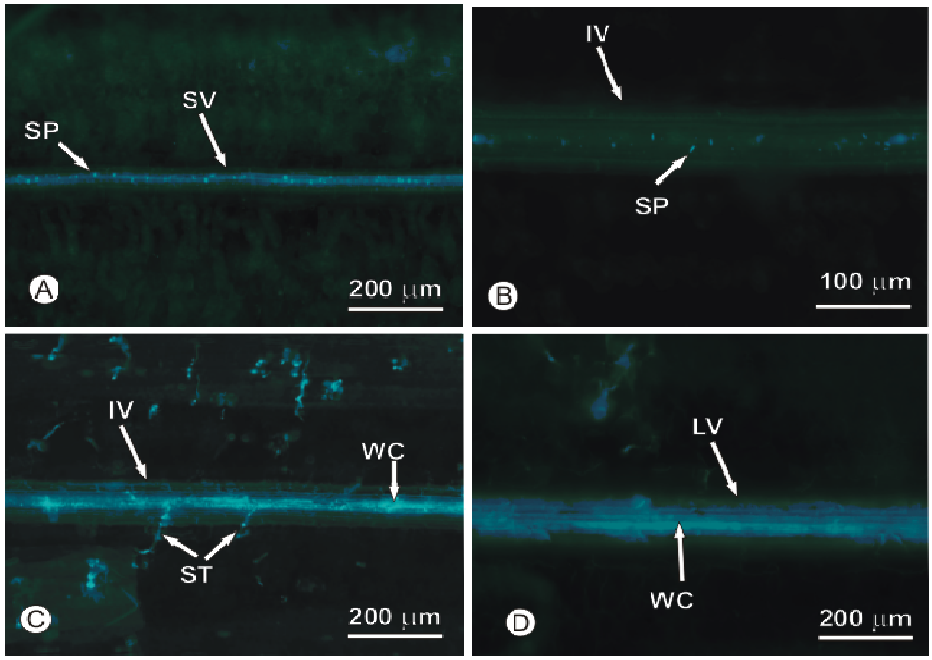

Fig. 5.2 Callose formation in control (uninfested) leaves of a non-resistant and a resistant line (A and B) and infested leaves of the non-resistant PUMA line (C and D)

Fig. 5.2: A and **B** show longitudinal sections of scraped control (uninfested) leaves, stained in aniline blue for callose. **A** shows a small vein (SV) from the leaf of the non-resistant PUMA line. **B.** Part of an intermediate vein (IV) from STARS-9301B leaf. Callose formation in the two images is minimal and restricted to the sieve plate regions along the lengths of the veins. **C - D.** Wound callose distribution after 10d feeding exposure on the non-resistant PUMA leaves, showing that extensive damage was inflicted by RWASA1 (**C**) and RWASA2 (**D**), due to sustained feeding.

The distribution of callose in small and intermediate veins of the resistant STARS lines is illustrated in Figs. 5.3 A-F. Feeding on the resistant lines by RWASA1 (Figs. 5.3A, C, & E) and RWASA2 (Figs. 5.3B, D & F) showed substantial aphid-induced wound callose deposition within the veins; positive callose reactions were associated with the stylet tracks (see ST, Figs. 5.3A – F) in all sections examined. The similarity of salivary deposition reactions caused by RWASA1 and RWASA2 infestations is intriguing. Neither leaves with high aphid population (typified by STARS 0502B, Fig. 5.3A and 5.3B respectively) nor those leaves on which smaller aphid populations existed (typified by STARS 9301B, Figs. 5.3C and 5.3D respectively) showed contrasting callose distribution at the end of the 10-day experimental period. It was however evident that the sieve tubes in small, intermediate and large veins contained massive callose deposits, which is an indication of severe damage to the vascular tissues and that the probed sieve tubes may well have lost functionality. When aphids probed near to, or in cross veins, all sieve tubes, sieve plates and pore plasmodesmal units contained callose (see Figs. 5.3A – F).

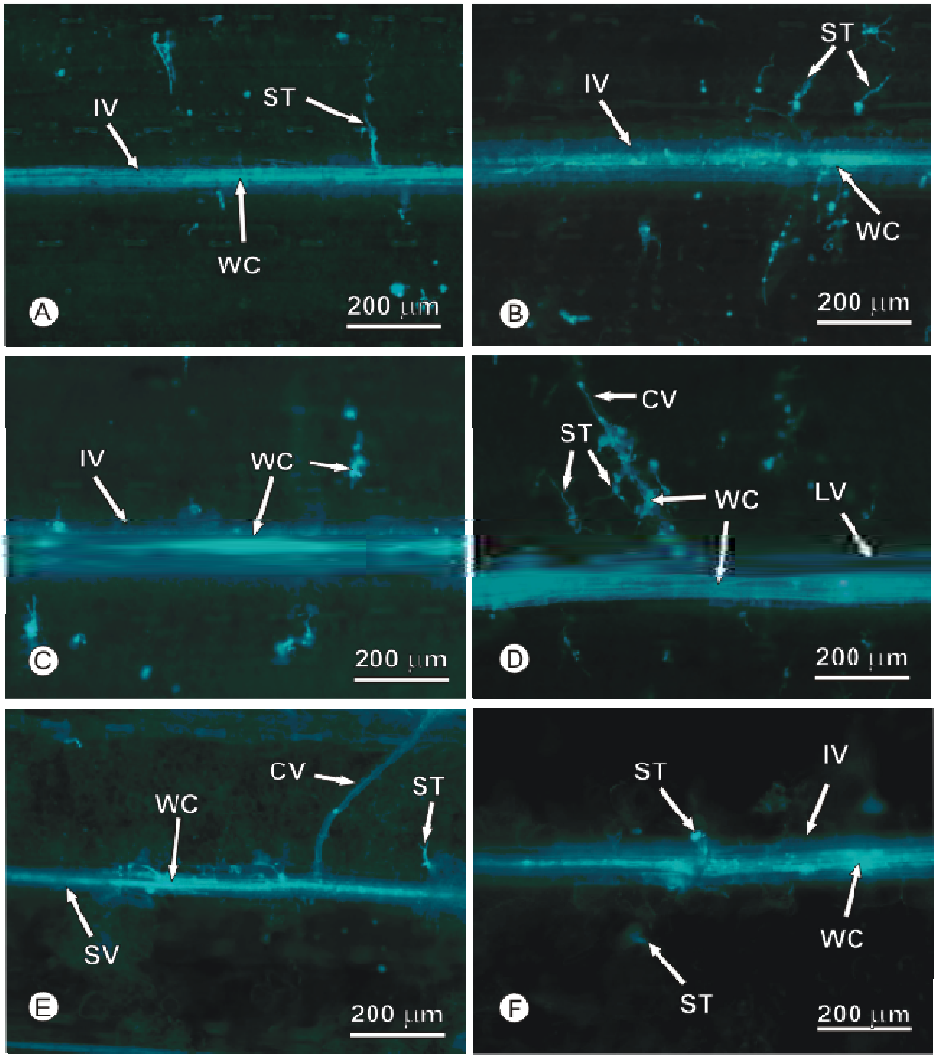

Fig. 5.3 Wound callose formation in leaves of resistant lines after 10 days of feeding by RWASA1 (LHS of plate) and RWASA2 (RHS of plate)

Fig. 5.3 (**A - F**). Aspects of the distribution of wound callose in the leaves of the resistant STARS lines, on which RWASA1 (LHS of plate) and RWASA2 (RHS of plate) fed for 10d. **A.** Part of an intermediate vein (IV) from STARS-0502B leaf fed on by RWASA1. Stylet track (ST) of a feeding stylet inserted into the vein, resulted in an extensive deposit of wound callose in the vein. **B.** Intermediate vein from a STARS-0502B leaf, exposed to RWASA2 feeding. The large aphid colony probed extensively, through the mesophyll, as evidenced by several callose-positive stylet tracks. These probes induced the formation of more extensive and widespread wound callose within this vein (compare callose distribution in Figs. 5.3A & B). **C.** Intermediate vein, from a STARS-9301B leaf; fed on by RWASA1. **D.** Part of a large vein (LV) and a connecting cross vein (CV) from STARS-9301B fed on by RWASA2. Again, more extensive and intense staining reveals aggressive feeding by RWASA2 on this cultivar than was the case with RWASA1. **E.** A small vein from the leaf of STARS-9577B fed on by RWASA1. Whilst intense callose fluorescence (WC) is evident in this vein, RWASA2 feeding on this cultivar (Fig. 5.3F) shows more widespread callose formed within the intermediate vein in STARS-9577B, where all visible sieve tubes and associated vascular parenchyma cells have been obliterated.

5.3.4 Ultrastructural damage

5.3.4.1 Control tissue

Figures 5.4 A-C show details of the vascular tissue in a large intermediate vascular bundle from control (uninfested) leaves. Two thick-walled (solid black dots) and several thin-walled sieve tubes (S) are visible (Fig. 5.4A). Details of the ultrastructure reveal that the cell structures are intact, normal and presumably functional (Fig. 5.4B-C). Characteristic thin-walled sieve tube-companion cell complex (S and CC respectively) form the bulk of the phloem tissues in the vascular bundles (Figs. 5.4A-C). These images are typical of those obtained from the control tissues in non-resistant as well as resistant lines.

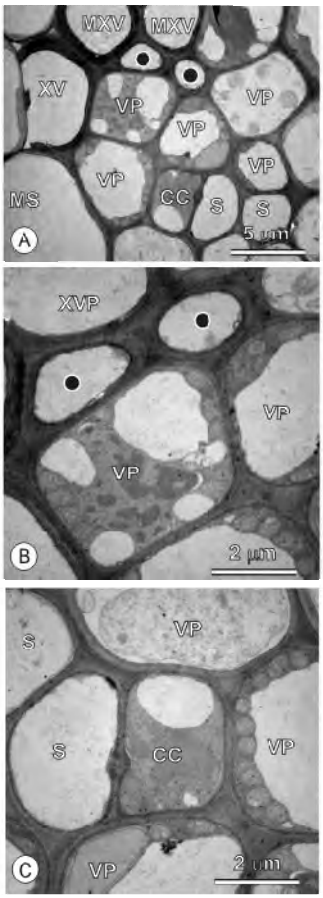

Fig. 5.4 Transmission electron micrographs illustrating ultrastructural details in an intermediate vascular bundle in uninfested control leaves

Fig. 5.4 Transmission electron micrographs illustrating ultrastructural details in an intermediate vascular bundle in uninfested control leaves. **A.** Part of the phloem, showing two thick-walled sieve tubes (solid dots) which abut the metaxylem vessels (MXV). Several thin-walled sieve tubes (S), an associated companion cell (CC) and vascular parenchyma (VP) are also visible**. B.** Detail showing two thick-walled sieve tubes and associated vascular parenchyma. **C.** Thin-walled sieve tubes to the left and a companion cell with lateral sieve area interconnecting to a sieve tube. Note that there is no visible evidence of plasmolysis or cell disruption in these control images.

5.3.4.2 Feeding damage by RWASA1 and RWASA2 on non-resistant PUMA

Feeding-related damage attributable to RWASA1 feeding and probing of vascular tissues of leaves of the non-resistant PUMA are shown in Fig. 5.5 (A-D). The aphids primarily probed and fed in the sieve tubes of the phloem. As in earlier studies, I observed that RWA feeds more extensively from the thin-walled than from the thick-walled sieve tubes. RWASA1 feeding resulted in severe damage to the phloem and copious evidence of salivary material (SM) deposition in thin-walled sieve tubes (Fig. 5.5A) occurs which in most cases, resulted in disruption of the sieve tube cytoplasmic matrix. Stylet sheath material (SS) completely obliterated a vascular parenchyma (VP) cell and saliva was present in a thin-walled sieve tube as well (Fig. 5.5B). Probing of the xylem, irrespective of the aphid and barley cultivar, always had similar effects – watery ejecta preceded presumed water ingestion and the resultant watery saliva rapidly coated the inner face of vessels as well as half-bordered pits between xylem and associated parenchyma (Figs. 5.5C and E; 5.6A, B, D and E). The electron-dense material can be easily identified in Fig. 5.5C due to RWASA1 feeding and in Fig. 5.5E due to RWASA2. Little, if any, discernible difference exists in salivary material deposition unlike the reported difference between BCA and RWASA1 (Saheed et al. 2007a). Thick-walled sieve tube lumen (Fig. 5.5C) contains saliva, suggesting that the aphid in question also probed the cell as well. Thin-walled sieve tubes are often plasmolysed (Fig. 5.5D) and contain granular material. Companion cells (CC)

and phloem parenchyma (VP) contain saliva (dark matrix) and are plasmolysed.

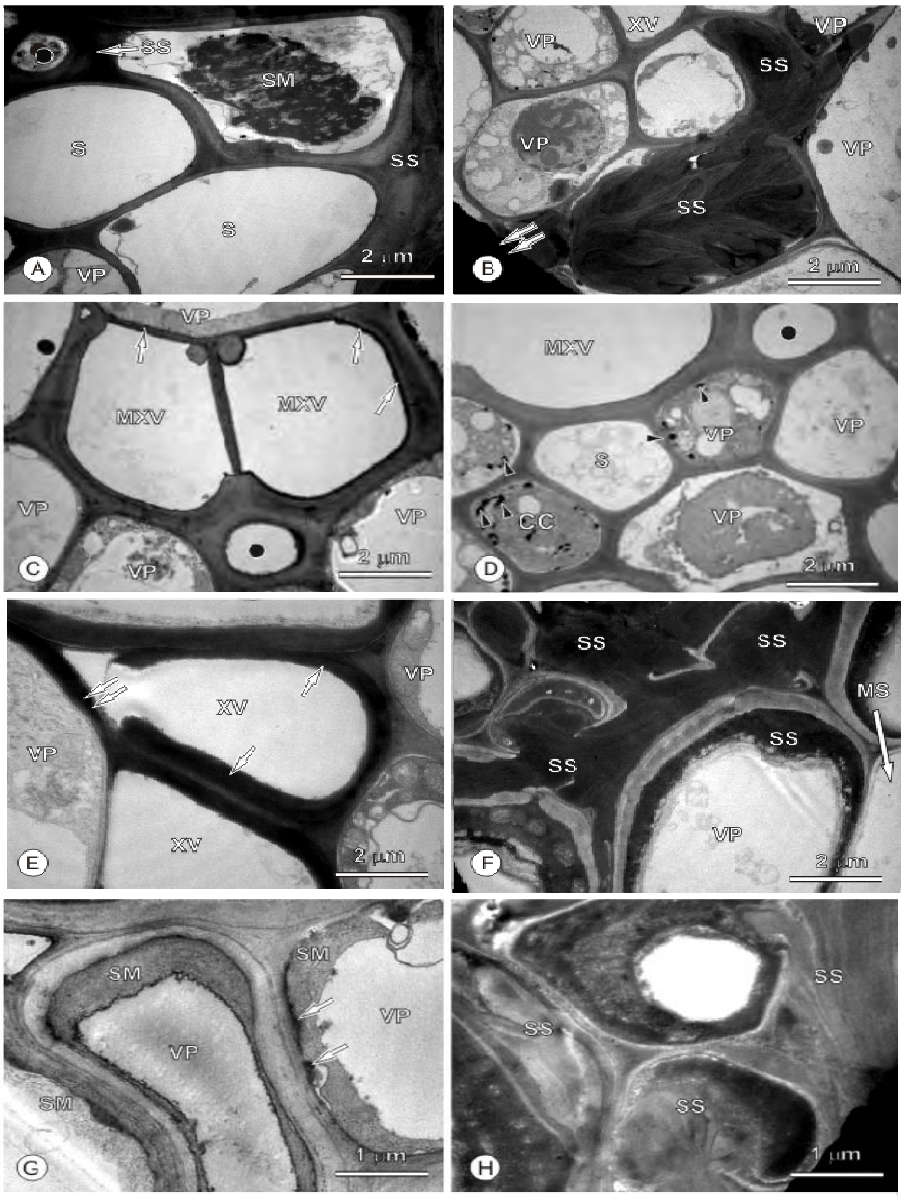

Fig. 5.5 Transmission electron micrographs illustrating aspects of the typical feeding-related damage caused by RWASA1 and RWASA2 in non-resistant line

Fig. 5.5 TEM images illustrating aspects of the typical damage caused by RWASA1 (Figs. 5.5A - D) and RWASA2 (Figs. 5.5 E - H) feeding on non-resistant PUMA leaves. **A.** A salivary sheath (SS, left and right) shows the intercellular passage of stylets next to a punctured saliva-containing thick-walled sieve tube (left, solid dot) and thin-walled sieve tube (right). Note granular salivary deposit (SM) in the adjacent sieve tube. The two thin-walled sieve tubes below are undamaged. **B. S**hows massive saliva deposits (SS) resulting from an extensive inter- and intracellular probe of the cells within the vascular tissues in the bundle. The probe continues (double arrow at lower left) under the TEM grid. Note the disruption in the vascular parenchyma cell and an adjacent thin-walled sieve tube due to the aphid's probing. **C.** Part of a small intermediate vascular bundle, in which the metaxylem is lined by electron-dense saliva (arrows), deposited as a result of saliva ejection prior to the aphid drinking xylem sap. Note the pit membrane between the metaxylem vessels is electron-dense, as it has been impregnated by the watery saliva ejected during the probe of these xylem vessels. **D.** Thin-walled sieve tubes and associated parenchyma cells, including companion cells show evidence of damage and disruption of their cytoplasm (electron-dense regions in cytoplasm (arrowheads). **E.** This xylem vessel in an intermediate vein contains salivary ejecta, again deposited prior to uptake of xylem sap and water by the aphid. Watery saliva has impregnated the cell walls. Note that the pit membrane between the vessels is also occluded (double arrow). **F.** Shows extensive and massive damage to the vascular tissue in the large vein. All the cells in this view are obliterated and contain copious salivary deposits. Based upon this and other images the vascular tissue was classified as non-functional. **G.** Granular, part electron-lucent salivary deposits (SM) line the wall of the parenchyma cell. The saliva was deposited on and in the wall and has stained the plasma membrane within this vascular parenchyma cell. **H.** Copious salivary ejection has resulted in cell obliteration.

Aspects of feeding and resultant cell damage in vascular tissues probed by RWASA2 are illustrated in Figs. 5.5E - H. Cells are generally more extensively probed by RWASA2 than under RWASA1 feeding. Figures 5.5E and F show salivary sheaths that mark the path of an extensive aphid probe. Xylem cell walls (arrows, Fig. 5.5E) and the half-bordered pit pairs (double arrows) contain electron-dense saliva. In Fig. 5.5F, salivary sheath material (SS) obliterates all cells in view. Phloem parenchyma (VP) which were probed during penetration by the stylet sheath of RWASA2 are obliterated and ensheathed with salivary material (SM and arrows, Fig. 5.5G). Inter- and intracellular probes are evident (Fig. 5.5H).

5.3.4.3 Feeding damage by RWASA1 and RWASA2 on resistant lines

Figure 5.6 shows examples of feeding-related cell damage caused by RWASA1 in the resistant STARS lines. Here, damage to xylem (Figs. 5.6A, B, D and E) and phloem elements (Figs. 5.6C, E and F) as well as deposition of salivary sheaths (SS, Figs. 5.6E and F) are evident, but not as extensive, as observed for both biotypes in the non-resistant PUMA (see Fig. 5.5). Cells in the probe pathway though contain saliva as well as granular material; many of the observed cell plasmolysis and disruption are less severe (Figs. 5.6A and C), compared to RWASA2 feeding damage (Figs. 5.7A-F). Xylem probes show amorphous electron-dense salivary deposits lining the lumen of vessels, which again effectively seal these vessels from surrounding vascular parenchyma (Figs. 5.6B and E) and phloem tissues (see Figs. 5.6E and F).

However, xylem vessels of STARS-9301B appear unaffected (Fig .5.6D), the half-bordered pit pairs and their pit membranes are not occluded when compared to those of STARS-0502B (Fig. 5.6B) and STARS-9577B (Fig. 5.6E).

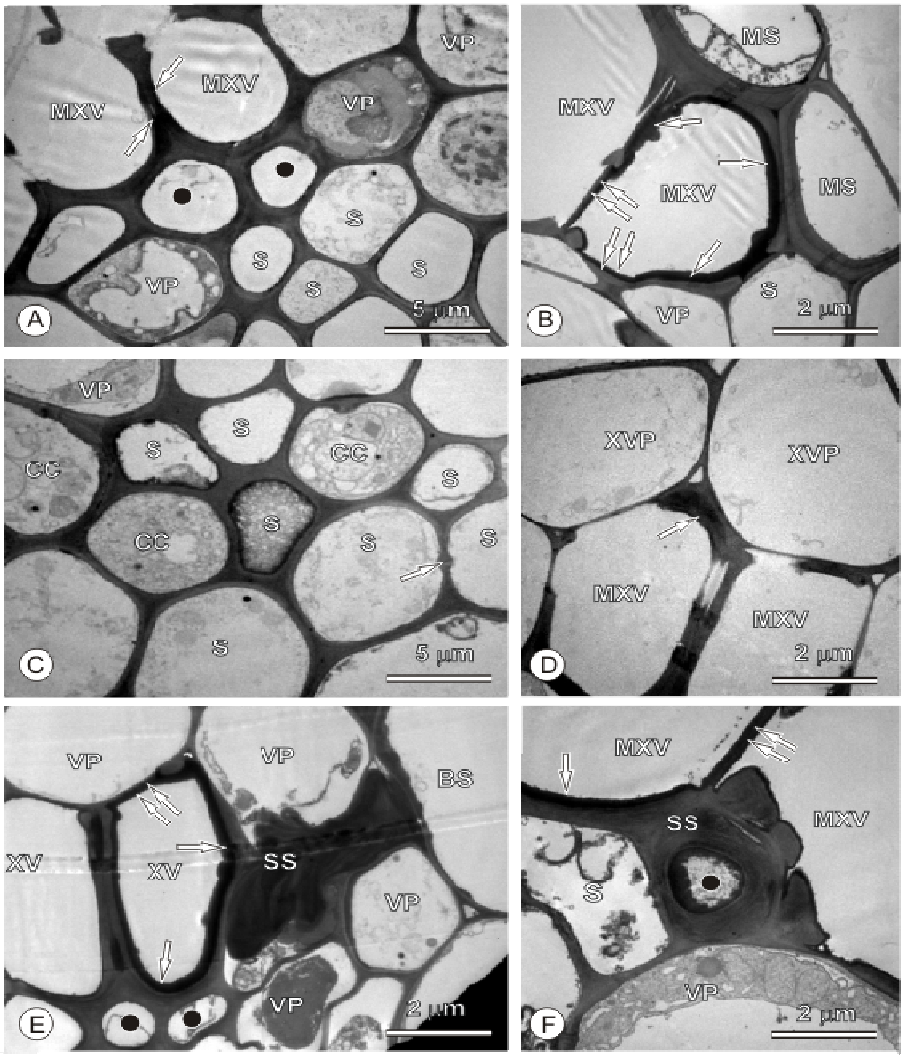

Fig. 5.6 Transmission electron micrographs from RWASA1-infested resistant STARS lines

Fig. 5.6 illustrates the damage caused by RWASA1 feeding on the resistant STARS lines. **A.** STARS-0502B. Detail of part of a large vein. Note several thick-walled sieve tubes (solid dots) appear undamaged while a vascular parenchyma (VP, lower left) cell is plasmolysed, presumably as a result of stylet puncture. The metaxylem shows evidence of probing as electron-dense saliva lines the walls (arrows), but pit membranes between the pair of metaxylem vessels (top centre) are unoccluded. **B.** STARS-0502B. Electron-dense saliva lines the walls of these metaxylem vessels (arrows). Pit membranes (double arrow) are occluded by saliva. Plasmolysed mestome sheath cell (upper right) was possibly punctured during this xylem probe. Surrounding xylem parenchyma cells appear unaffected. **C.** STARS-9301B. Detail of phloem of a large or intermediate bundle with evidence of RWASA1 feeding. The two central thin-walled sieve tubes contain electron dense saliva and the surrounding vascular parenchyma cells exhibit varying degrees of plasmolysis. **D.** STARS-9301B. Detail of metaxylem vessels in an intermediate vascular bundle, with salivary deposits along the inner face of the cell walls. Pit membranes are occluded with electron-dense saliva. **E.** STARS-9577B. Detail of the salivary sheath (SS) associated with inter- and intracellular probe, which terminated at the xylem. Several cells were obliterated during this probe and thick wall sieve tubes as well as associated vascular parenchyma cells are plasmolysed. Xylem vessels are lined and pit membranes are occluded with electron dense smooth saliva (arrows); pit membranes are occluded (double arrow). **F.** STARS-9577B. Detail showing an obliterated thick-walled sieve tube, lined with saliva, adjacent to a pair if metaxylem vessels (above). Saliva is located in walls and pit membranes (double arrow) of these vessels.

RWASA2 feeding-related damage on STARS lines was more extensive than under RWASA1 feeding (Figs. 5.7A-F). Here, xylem damage (Figs. 5.7A, B) and obliteration of a thick-walled sieve tube (SS, Figs. 5.7B, and D) are visibly more extensive compared to the situation under RWASA1 feeding (see Figs.5.6B, E and F). Both thin-walled sieve tubes and parenchyma are obliterated or are ensheathed by saliva (see sieve tubes, S in Figs. 5.7C and E) - often resulting in dense saliva-related aggregates in sieve tubes and companion cells (CC, Fig. 5.7E). Cytoplasm shows evidence of extensive plasmolysis (Figs. 5.7B, C, and D) than under RWASA1 infestation (Figs. 5.6A and C). Probed thin-walled sieve tubes of STARS-9577B show severe plasmolysis (see Fig. 5.7F) and occlusion of pore- plasmodesmatal units (arrows, Fig. 5.7E) and sieve area pores (arrows, Fig. 5.7F).

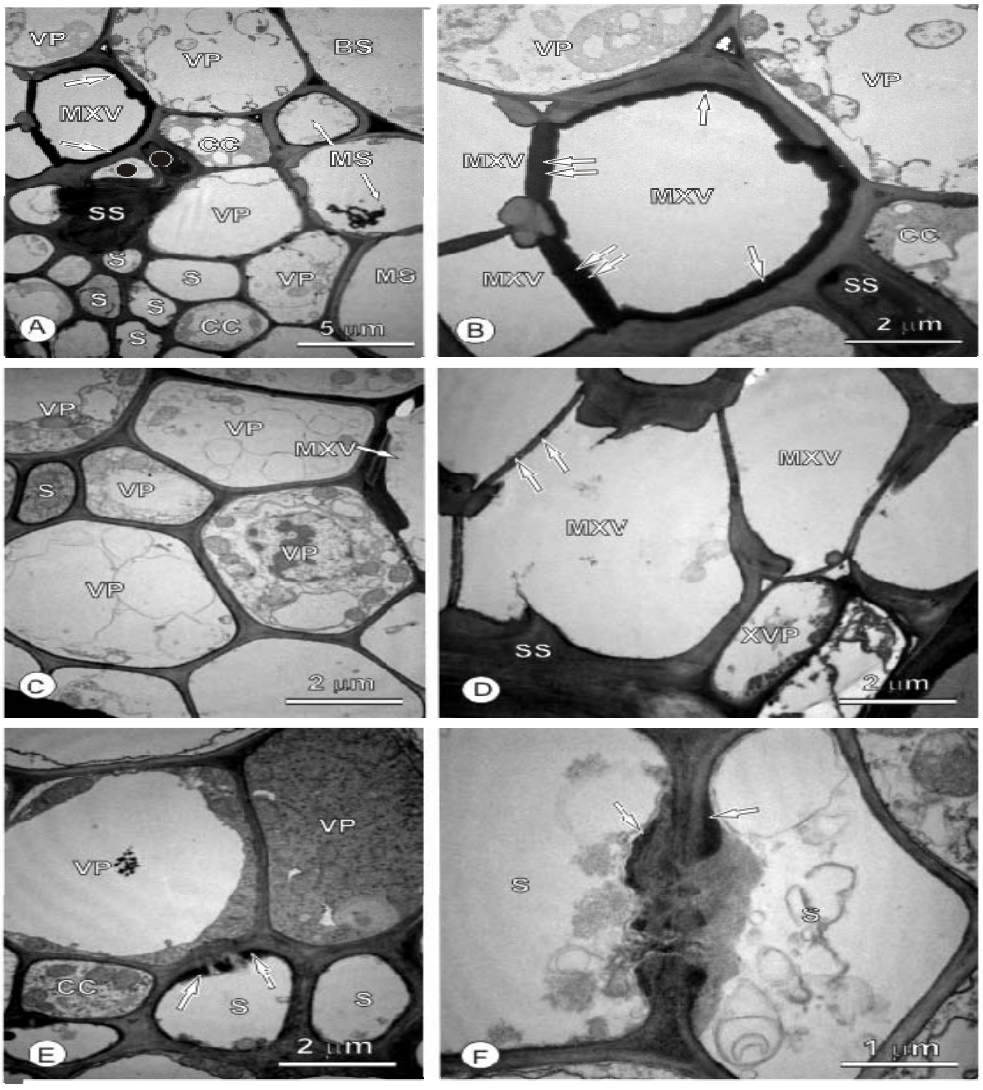

Fig. 5.7 Transmission electron micrographs showing effects of RWASA2 feeding on resistant STARS lines

Fig. 5.7 Effects of RWASA2 feeding on resistant STARS lines. **A.** STARS-0502B. Thick-walled sieve tubes (solid dots) and adjacent cells – presumably vascular parenchyma – were obliterated by saliva (SS) during this probe of an intermediate vascular bundle. The surrounding phloem tissue including sieve tubes show evidence of partial plasmolysis associated with this probe. Xylem vessels as well as pit membranes are occluded by saliva. **B.** STARS-0502B. Detail of probed xylem vessel from Fig. 5.7A. The thick, electron-dense saliva has encrusted walls (arrows) and the pit membranes plugged completely (double arrow). The thick-walled sieve tube (centre left) was punctured and is filled with saliva (SS). Surrounding vascular parenchyma cells are severely plasmolysed. **C.** STARS-9301B. Detail of part of a vascular bundle showing extensive, general plasmolysis of parenchyma and thin-walled sieve tubes (S). Metaxylem (MXV, right) cell walls and pit membranes are encrusted with electron dense saliva. **D.** STARS 9301B. Detail, showing metaxylem in a large vascular bundle, with saliva lining the cell walls (SS), which contain occluded pit membranes (double arrow). **E.** STARS-9577B. The large vascular parenchyma cells (above) as well as thin-walled sieve tubes and associated companion and vascular parenchyma cells are extensively damaged which led to complete disruption of the cytoplasm in the companion cell (CC). The sieve tube cell walls are lined with saliva and callose is associated with the lateral sieve pores (arrows). **F.** Detail from Fig. 5.7E. Shows disrupted appearance of a pair of sieve tubes, resultant from RWASA2 feeding. Material associated with the lateral sieve pores is presumably a mixture of callose and saliva.

5.4 Discussion

5.4.1 Population growth of the aphids on host plants

The growth of the colony size for the two biotypes increased substantially over the 10-day experimental period (Fig. 5.1), which agrees with the results obtained by Jimoh et al. (2011) on the same barley lines. RWASA2 reproduced faster than RWASA1 on both resistant and non-resistant lines in a manner similar to their relative performance on susceptible and resistant wheat (Walton and Botha, 2008). None of the virulent RWA biotypes studied in the US show a difference in performance on barley and wheat (Puterka et al., 2007). This suggests that the virulence mechanism exhibited by RWASA2 over the existing RWASA1 is unique to South Africa. Aphid populations increased from the initial 10 aphids, with a range of about five-fold in a resistant STARS-9301B infested with RWASA1 to about 14-fold in the non-resistant PUMA infested with RWASA2. These results are consistent with other studies for RWASA1 (Saheed et al., 2007a) and the 15-day time-course study presented in Chapter Three, which showed positive population growth of the two biotypes and the bird cherry-oat aphid (*Rhopalosiphum padi*, BCA) on the four barley lines. In this study, the non-resistant line sustained faster population growth and therefore, higher density per cm^2 of leaf area (Table 5.1) of both RWA biotypes than was the case with the resistant lines. These results are in conformity with earlier reports by Walton and Botha (2008) for the non-resistant Tugela and resistant Tugela-*Dn* wheat infested with RWASA1 and RWASA2 and for the US RWA Biotype 1 on a susceptible and

resistant barley cultivars (Gutsche et al., 2009). The performance of both biotypes was reduced on all three resistant lines, but the population levels of RWASA2 on the resistant lines were similar to that of RWASA1 on the non-resistant PUMA (Fig. 5.1).

5.4.2 Cellular damage on the non-resistant line

Vascular damage caused by RWASA1 and RWASA2 on the non-resistant PUMA after 10d feeding exposure was so extensive that it was impossible to determine whether there were any differential effects by the two biotypes. Previous studies have shown that aphids feed on susceptible hosts, using their slender stylets to tap the vascular tissues (Evert et al., 1973; Matsiliza and Botha, 2002). Stylets usually penetrate leaves of host plants sequentially beginning with the bundle sheath, the vascular parenchyma, xylem elements and phloem tissues (Evert et al., 1973; Matsiliza and Botha, 2002; Botha and Matsiliza, 2004; Botha, 2005; Saheed et al., 2007a, 2007b). RWA saliva has been observed in both xylem and phloem elements of wheat and barley hosts (Botha and Matsiliza, 2004; Saheed et al., 2007a, 2007b). In this study, aggressive inter- and intracellular probing and penetration of vascular tissues by the stylets of the two biotypes in the non-resistant line resulted in severe cell disruption and frequent cell obliteration of the vascular parenchyma, xylem and phloem tissues (Fig. 5.5). This is similar in magnitude to what was reported caused by RWASA1 in a susceptible barley cv Clipper (Saheed et al., 2007a) and a susceptible wheat, Betta (Saheed et al., 2007b).

Previous studies have shown that aphids probe the xylem for water (Tjalingii, 1994; Saheed et al., 2007a, 2007b). RWA is reported to always eject watery and sometimes smooth saliva prior to drinking water from the xylem (Saheed et al., 2007b). The ejecta usually encrust the inner cell wall face of xylem vessels, which results often in blockage of the half-bordered pit pairs (Figs. 5.5C and E). This is thought to impair water and solute exchange between the xylem vessels and associated parenchyma tissues (Saheed et al., 2007b), which may contribute to the leaf rolling symptoms caused by the feeding biotypes.

Similar to the situation in xylem probes, salivary material is ejected into sieve tubes, when the aphids sample the cell contents. This ejected material gels and forms a stylet sheath that often completely obliterates cells in the probe pathway (see Figs. 5.5B, F, H). The evidence presented here is indicative of extensive cell damage, which apparently is more severe under RWASA2 feeding (Figs. 5.5F and H) than with RWASA1 infestation (Figs. 5.5A and B). The salivary ejecta may also cover lateral sieve area pores through which solute transport is carried out. This is with the attendant proportional impact on sieve tube functionality and consequently, a reduction in the efficacy of the transport function of phloem tissues. The resultant reduction in phloem transport capacity would exacerbate leaf chlorosis, local necrosis and longitudinal streaks commonly found on leaves and associated with prolonged RWA feeding as suggested by Saheed et al. (2007b).

5.4.3 Reduced cellular damage on the resistant lines

The vascular cell damage caused by each biotype was substantially reduced on all three resistant lines compared to what was observed on PUMA described above. In these lines, some cell disruption were evident (see Figs. 5.6A, C and E; Figs. 5.7A and B) but majority of the vascular parenchyma cells appeared relatively unaffected. Though cell plasmolysis was often evident (Figs. 5.6A, C and E; Figs. 5.7A, C-F), suggesting that functional disruption had taken place, this is not as severe as obtained in non-resistant PUMA (see Figs. 5.5A, B, D, F and H).

With overall damage levels reduced as described above, it was possible to compare the damage caused by the two biotypes as well as contrast among the three resistant lines. Probe-related xylem damage, shown by saliva encrusting xylem vessel walls is evidently more severe with RWASA2 Figs. 5.7A, B and D) than under RWASA1 feeding (Figs. 5.6A, B, D and E). Among the three resistant lines, blockage of the half-bordered pit pairs and their pit membranes is more severe in STARS-0502B (Fig. 5.6B for RWASA1 and Fig. 5.7B for RWASA2) and STARS-9577B (Fig. 5.6E for RWASA1) than in STARS-9301B (Fig. 5.6D for RWASA1 and Fig. 5.7D for RWASA2). The same goes for phloem cells. Damage to sieve tubes by RWASA2 (Figs.5.7A, C and E) is more severe than during RWASA1 feeding (Figs. 5.6A, C and E). Apparently, there is similarity of cell damage in STARS-9577B infested by RWASA1 (Fig. 5.6E) and STARS-0502B infested by RWASA2 (Fig. 5.7A). Salivary

ejecta of RWASA2 visibly occluded lateral sieve area pores between adjacent sieve tube members (Figs. 5.7E and F). These scenarios would cause a relative reduction in rates of water and solute transport, first due to each RWA biotype and second, among all three resistant lines, which is particularly more reduced in STARS-9301B than in the other two lines. Relative reduction in cell damage on these resistant lines compared to the non-resistant PUMA is similar to the report by Saheed et al. (2007b) in which the isogenic resistant Betta-*Dn* wheat reduced damage to xylem and phloem tissues compared to the susceptible Betta cultivar.

5.4.4 Similarity in callose deposition in resistant and non-resistant lines

When the vascular damage was assessed using aniline blue, results obtained varied from those of previous studies. It was evident that large swaths of vascular tissue - irrespective of aphid population and barley line - contained aphid-induced callose within the sieve plates, lateral sieve areas and pore plasmodesmal units (Figs. 5.2 and 5.3). RWASA1 and RWASA2 appear to induce callose formation during probing and feeding on both non-resistant and resistant barley lines. This is contrary to reports of previous studies on aphids feeding on resistant wheat cultivars containing the *Dn1* resistance gene, where callose deposition as well as feeding damage was lessened in the resistant lines, when compared to the non-resistant lines (see De Wet and Botha 2007; Saheed et al. 2007b; Walton and Botha 2008). The development in the current study is not entirely unexpected – wheat lines bearing *Dn*1 genes are specifically developed against RWASA1. Reduced callose deposition can be

(as demonstrated in Chapter 4), but is not by necessity an indicator of resistance to RWA feeding. In the instance reported in this Chapter, the pervasive callose was obtained after a sustained 10d feeding exposure and the bottom-line is that these resistant barley lines are not specifically developed against these South African as is done for wheat.

The positive callose reaction obtained in this study on the resistant lines (Fig. 5.3) may be due (in part) to an increase in β-1, 3-glucan synthase activity. Studies have shown that this is associated with increased salivation, particularly when aphids find it difficult to feed on their hosts (Will et al., 2007, 2009), when they probe more frequently. This could cause more wounding by the aphids, which up-regulates β-1, 3-glucan synthase activity; hence more callose deposition in sieve tubes. I suggest that RWASA1 and RWASA2 may have precipitated unequal but high levels of salivation while feeding on the resistant plants. This probably accounts for wound callose formation within their sieve tubes (Fig. 5.3), of which the resultant knock-on effect being a reduction in phloem transport capacity, which culminates in a decline in aphid feeding and reproductive capacity and consequently, a small colony size.

Increased levels of salivation by the two biotypes while feeding on these resistant lines suggests that *Dn* resistant gene plays no role in the lines. This is in stark contrast to the differential levels of salivation by these biotypes when they fed on Tugela-*Dn* wheat cultivar (Walton and Botha, 2008). The inherent *Dn* resistance gene in this wheat may have suppressed RWASA1 salivation

and thus caused smaller callose deposition as against higher salivation by RWASA2, which resulted in higher callose deposition. This made Tugela-*Dn* behave as if it were a susceptible cultivar when infested with RWASA2. Further studies are necessary on the feeding behaviour and salivary components of these aphids.

5.4.5 Relationship between population growth and feeding damage

Damage resulting from aphid feeding occurs when they puncture cells with their stylets and subsequently deposit salivary materials (Miles, 1999). It may then be assumed that as the aphid population increases, so is the attendant increase in the number of the stylets penetrating the vascular system will lead to a larger volume of deposited saliva. This results into more severe damage to leaves and eventually, the entire plant. In this study, the trend of population growth of the aphids shows that RWASA1 and RWASA2 reproduced faster (Fig. 5.1) and had higher aphid density per cm^2 of leaf (Table 5.1) on the non-resistant PUMA than on any of the three resistant lines. A previous report obtained a positive population growth of these biotypes on these barley lines over a 15-day time course (Jimoh et al., 2011). As a result, damage on the non-resistant PUMA is more severe (Fig. 5.5) than those observed on the resistant lines (Figs. 5.6 and 5.7).

Of interest is the fact that STARS-0502B and STARS-9577B appeared ‘less resistant’ than STARS-9301B. The former two resistant lines supported

higher populations and hence higher density of the two biotypes than STARS-9301B (Fig. 5.1). Larger feeding population density of RWASA2 than did RWASA1 on the resistant lines reflected in its (RWASA2) greater feeding-related cell damage during ultrastructural assessments (Figs. 5.6 and 5.7). Cross correlation of results of this study with those in Chapter 3, where leaf roll damage correlated with aphid population number corroborates the findings of this study. It is possible that the occlusion of xylem vessels by aphid saliva, which is more pronounced in both STARS-0502B and STARS-9577B infested by RWASA2 (Fig. 5.7), may have exacerbated cell damage in these two resistant lines than in STARS-9301B.

From the presentations above, it can be deduced that aphid population growth was significantly influenced by two factors tested in this study to wit: resistance and aphid biotype. Cellular damage resulting from aphid feeding was also affected by both factors, but to a different degree. The presence of resistance in the resistant lines suppressed RWASA2 population to a level resembling those of RWASA1 on the resistant PUMA. In contrast, cell damage by RWASA2 on the resistant lines was lower than that caused by RWASA1 on PUMA, when considered on the same yardstick. Thus, the results of this study support the conclusion in Chapter 3 that resistance in these STARS lines has an antibiosis effect on the two RWA biotypes. However, the findings in this study seemingly suggest that the resistant lines are more tolerant of the RWA biotypes feeding on them. This is because the level of suppression of cell damage on the resistant lines exceeds what one

would expect from the antibiotic effects arising from suppression of aphid population growth. In Chapter 3, I showed that the amount of leaf roll, a visible symptom of RWA feeding, could be explained simply by aphid population levels, irrespective of biotype. However, the amount of leaf roll caused by RWASA2 on the resistant lines in that study approached the maximum leaf roll score of '3', such that higher measurements for RWASA1 on the non-resistant PUMA was not possible. Therefore, the current conclusion that there are antibiosis and tolerance effects of resistance in the STARS lines is supported by results obtained in Chapter 3 and that of the present study. Tolerance was suggested as the major component of resistance in these lines when they were tested against the US RWA biotypes (Mornhinweg et al., 2006a; Puterka et al., 2006).

This study demonstrates that RWASA1 and RWASA2 induced callose formation when feeding on the susceptible cultivar (PUMA) as well as on the resistant STARS lines developed against US strains of RWA (see Figs. 5.2 and 5.3). This is contrary to results obtained in other studies using wheat cultivars containing the *Dn* resistance genes against RWASA1 in South Africa (see Saheed et al., 2007b; Walton and Botha, 2008). Ultrastructural studies show that cell damage was extensive on the non-resistant PUMA but was suppressed on the resistant lines. This study also shows that feeding-related damage caused by RWASA2 to the vascular systems of the four barley lines, was more noticeable and severe compared to RWASA1 feeding-related damage.

The RWASA2 biotype was discovered in the field because of its ability to feed and reproduce effectively on RWSA1-resistant wheat containing the *Dn1* gene (Tolmay et al., 2007). These authors suggested that the RWASA2 virulence on *Dn1* wheat plants arose from a "gene for gene" interaction between a characteristic R-gene and a modified avirulence protein in the aphid's saliva (see Edwards and Singh, 2006). Widespread deployment of R-genes in crops can often select for virulent biotypes that are capable of successfully colonising resistant plants (Porter et al., 1997; Quick et al., 2001; Gatehouse, 2002). R-gene resistance implies that virulence is dependent on the individual aphid's specific ability to colonize host plants, regardless of whether the plants are susceptible or resistant, given that these plants do not carry resistant gene(s) specific to them. If this was the case, RWASA2 should not outperform RWASA1 on plants that do not contain the *Dn1* gene. RWASA2 grows faster than RWASA1 on susceptible Tugela wheat, whereas RWASA1 population, but not RWASA2 population, was suppressed on the resistant Tugela-*Dn* counterpart (Walton and Botha, 2008). The two biotypes grow faster, though unequally, on resistant and non-resistant barley lines (Jimoh et al., 2011). It is possible that the only biological difference between RWASA2 and RWASA1 is a higher reproductive rate.

The critical outcomes of this study are that RWASA2, the recently evolved South African RWA biotype, is more virulent than the older RWASA1 biotype on the US-developed resistant barley lines. Secondly, RWASA2 reproduces more rapidly and has higher aphid density per cm^2 of leaf area

compared with RWASA1. This is with the knock-on effect that the damage sustained by the vascular systems in barley leaves infested by RWASA2 is more severe when compared to RWASA1 feeding. However, suppression of feeding-related vascular damage on these STARS lines shows that there is a tolerance component to the resistance in them, in addition to the antibiosis effect previously suggested in Chapter 3. Based on this, I suggest that these lines should be studied further at the molecular and breeding levels to unravel the resistance factor as they represent an excellent potential source of durable resistance to RWA for the South African barley industry.

6 Chapter 6: Disruption of phloem transport

6.1 Introduction

Transport of photoassimilates in plants from regions of synthesis to regions of utilization and storage is an essential process without which growth and development would be impossible (Yeo, 2007; Pritchard, 2007). Effective translocation of the assimilates from the photosynthesising tissues, which are mostly in the leaves (i.e. source), to growing tissues in leaves, stem, roots and storage organs such as seeds and fruits (i.e. sinks) is the basis of plant performance and agricultural yield (Komor, 2000). Translocation is carried out in the vascular tissue system comprising of xylem and phloem, both of which occur in the veins of leaves. The process has been extensively studied in the vascular bundles of several monocots (see Evert et al., 1978; Altus and Canny, 1982; Fritz et al., 1989; Botha and van Bell, 1992).

The major constituent of the solutes translocated in the phloem of most plants is sucrose, and this is transported essentially by the phloem (as phloem sap) from source sites to sink tissues (Turgeon and Wolf, 2009). This sucrose-rich phloem sap is tapped by aphids in their quest for other nutrients moving passively in the phloem sap. Aphids' stylets inflict damage on the pressurized sieve tubes of the phloem, breaching it in order to take up and process the sap (Saheed et al., 2010). Aphids usually aggregate on the young parts of the plant, such as newly unrolling leaves, where the sap is abundant. Because this sap is low in protein contents, aphids have to ingest large quantities of sap

(Kennedy and Forsbrook, 1971) so as to acquire sufficient amino acids needed for their survival, the excess of the sugars which they excrete in form of 'honeydew' (Douglas, 1993). Aphids therefore become strong secondary sinks, by diverting energy- and nutrient- rich assimilates primarily meant for distribution to growing plant tissues and sink organs (Girousse et al., 2003). The damage to sieve tubes must affect the transport capacity of the phloem adversely in its primary function of distributing assimilates throughout the plant body (Nielsen et al., 1990; Botha and Matsiliza, 2004; de Wet and Botha, 2007; Saheed et al., 2010).

Dannenhoffer and Evert (1994) have described the phloem within the leaves of barley. As in all angiosperms, the sieve elements making up the sieve tubes and their associated companion cells form a functional complex with the surrounding phloem parenchyma and other parenchyma within the tissue matrix connected by plasmodesmata (Turgeon and Wolf, 2009). Anatomy of sieve tubes is structured to facilitate bulk flow of the photoassimilates (Pritchard, 2007). Sieve tubes of the Poaceae are of two types – thin-walled and thick-walled. Thin-walled sieve tubes are associated with and closely connected to companion cells by pore-plasmodesmata (Evert et al., 1978; Botha and Evert, 1988; Pritchard, 2007). The second type, thick-walled sieve tubes, due to its thickened walls, occurs singly or sometimes in pairs in close proximity to the xylem, and they lack companion cells (Botha and Evert, 1988). Reports have shown that aphids feed specifically from sieve tubes and preferentially from the thin-walled sieve tubes, as greater quantities of

assimilates are transported in them (Matsiliza and Botha, 2002; Saheed et al., 2007a, 2007b). This suggests that thin-walled sieve tubes are more functional in phloem loading and transport than thick-walled sieve tubes (Matsiliza and Botha, 2002; Saheed et al., 2007b).

Aphids' feeding activities affect phloem transport in two ways. First, aphids become a strong secondary sink, resulting in pressure loss in the phloem transport pathway below the points of aphid stylet insertions. Evert et al., (1968) clearly showed that aphids' stylet puncturing parenchyma cells and elements of the phloem during feeding caused turgor loss and reduced phloem transport capacity. Botha and Matsiliza (2004) suggested that the RWA feeding on a susceptible wheat cultivar might have resulted in feeding-related pressure loss, when they observed reduced phloem capacity to transport assimilates. Secondly, aphids probing and feeding on plants cause the formation and deposition of wound callose in sieve tube elements of the phloem. These effects contribute to substantial decrease in the rate of transport across the vascular parenchyma to the companion cell-sieve tube complexes. De Wet and Botha (2007) reported that the grass aphid (*Sitobion yakini*) feeding on susceptible Betta wheat cultivar, caused extensive callose deposition in phloem tissues, which was manifested in a reduction of phloem transport capacity. Furthermore, Saheed et al. (2009) demonstrated the blockage of sieve plate pores and pore plasmodesma units by aphid-induced callose, which caused marked decrease and possible cessation in transport of

assimilates by phloem (Saheed et al., 2010) in a RWA-susceptible barley cultivar.

Response of plants to aphid feeding has been described to be largely species-specific which could be either aphid or host plant dependent (Gill and Metcalfe, 1977). For instance, Saheed et al. (2007a, 2009, 2010) demonstrated that two aphid species, RWA and *Rhopalosiphum padi* (BCA) evoked different feeding effects on a susceptible barley cultivar. A study by De Wet and Botha (2007) indicated that *S. Yakini* elicited different feeding effects on susceptible and resistant wheat cultivars. These studies present significant findings that have explained the mechanism of interaction between plants and aphids.

Considerable progress has been recorded in understanding the physiological responses of both resistant and non-resistant wheat cultivars to RWA (Burd and Elliot, 1996; MacEdo et al., 2009). Recently, Walton and Botha (2008) reported that RWASA1 and RWASA2 exhibited markedly different feeding-related damage on susceptible (Tugela) and resistant (Tugela-*Dn*) wheat cultivars. However, limited progress has been made in understanding the physiological responses of barley hosts to aphid feeding except those of Miller et al. (1994) and Gutsche et al. (2009), particularly when it involves two biotypes of the same aphid species. Report of Walton and Botha (2008) that RWASA2 breeds faster and is more virulent on susceptible and resistant wheat cultivars than RWASA1 informed the present cross-relational study of

the feeding effects of the two biotypes on phloem transport capacity of resistant and non-resistant barley lines.

I have established that feeding by RWASA1 and RWASA2 evoked diverse levels of damage to resistant and non-resistant barley lines (see Chapter 3; Jimoh et al., 2011) and that RWASA2 caused more severe damage symptoms than RWASA1. I have also demonstrated that RWASA2 feeding formed higher amounts of wound callose than RWASA1 in the sieve elements of these barley lines during short- and long-term feeding exposure (Chapter 4). Furthermore, I have reported that RWASA2 caused more extensive feeding-related cell damage than RWASA1 in the vascular tissues of the three resistant barley lines (Chapter 5).

The focus of the experiments reported in this chapter is the effects, which the feeding damage caused by RWASA1 and RWASA2 have on phloem transport functionality of these barley lines. The aim of these experiments was to explore the connectivity of various reports on effects of feeding damage caused by the two biotypes on the barley lines, contained in Chapters 3, 4 and 5 (as highlighted above), to their separate and collective effects on the phloem transport functionality in the experimental resistant lines as well as the non-resistant control. I have used the phloem-mobile fluorophore, 5,6-CFDA, which has proven reliable in studying phloem transport capacity of both wheat and barley cultivars (see Botha and Matsiliza, 2004; DeWet and Botha, 2007; Saheed et al., 2010), to examine the effects RWASA1 and RWASA2 feeding have on the capacity of the phloem to transport photoassimilates. I

hypothesized that RWASA2, which is the more aggressive feeder (Walton and Botha, 2008), would cause a greater reduction in phloem transport of the four barley lines than RWASA1 and that the non-resistant line would exhibit an increased reduction in rate of phloem transport than any of the three resistant lines. It is expected that results from this study would offer explanations to possible differences in trend of phloem transport capacities existing among the three resistant lines infested with the two aphid biotypes, with the view of identifying the most 'vigorous' in relation to RWA infestation.

6.2 Experimental overview

Clipcages were used to confine 10 adult apterae of either RWASA1 or RWASA2 on 5cm long leaf segments at the mid-region of fully expanded source or sink leaves of each barley experimental plant. The aphids were allowed 24h, 72h (short-term), 7d and 14d (long-term) feeding periods. Following the classical acropetal and basipetal patterns of assimilates movement in plants, flap feeding method was used to apply the phloem-mobile fluorophore, 5,6-CFDA on the experimental plants after the expiration of each feeding treatment. Details of the experimental procedure were given in Chapter 2. The dye was allowed 3h to be taken up and the cleaved product, 5,6-CF transported within the phloem of aphid-infested and control (uninfested) leaves. The leaves were removed to investigate the rate (measured as the distance moved by 5,6-CF from the point of initial application of the fluorophore) and the amount (in terms of fluorescence

distribution and intensity) of phloem transport in longitudinal veins of the experimental plants.

6.3 Results

6.3.1 Transport of 5,6-CF in control (uninfested) leaves

Three hours after the application of 5,6-CFDA, the cleaved product, 5,6-CF, moved into the leaf mesophyll, got loaded into the bundle sheath cells and thereafter into the vascular bundles (Fig. 6.1). The dye front was observed in unscraped leaves at approximately 5cm from the point of application of 5,6-CFDA. Similar results were obtained in experiments involving all control (uninfested) leaves of the four barley lines carried out after 24h, 72h, 7d and 14d treatment periods. Movement of the phloem-mobile fluorophore (5,6-CF) in uninfested control as well as in infested leaves always took place from the site of application of the fluorochrome towards the leaf base (basipetal) in source leaves, and towards the lamina tip (acropetal) in sink leaves (data not shown). Figures 6.1A-D illustrate movement of 5,6-CF in longitudinal and cross veins of leaf blade material of uninfested PUMA. Continuous undisrupted band of bright fluorescence 5,6-CF was observed in longitudinal as well as cross veins.

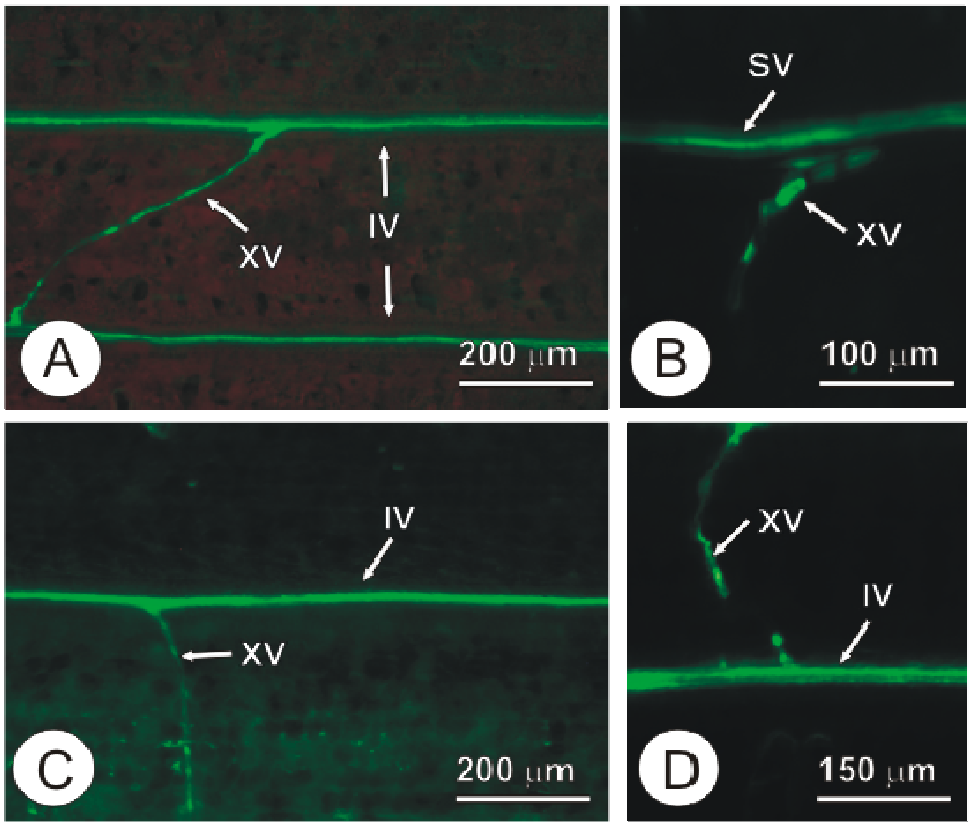

Fig. 6.1 Transport of 5,6-CFDA in control barley leaves

Fig. 6.1 A-D Transport of the phloem-mobile fluorophore, 5,6-carboxyfluorescein (5,6-CF), in control (uninfested) leaves of barley. **A** and **C** show continuous, uninterrupted flow of the cleaved 5,6-CF along two parallel intermediate veins (IV) joined by a cross vein (XV). **B** and **D.** Details of uninterrupted trafficking of the fluorophore. Bright fluorescence in the cross veins indicate movement of the dye in files of sieve tubes.

6.3.2 Transport of 5,6-CF in infested leaves

Figures 6.2-6.3 illustrate typical wide-field fluorescence images which show movement of 5,6-CF in vascular bundles of infested leaves of non-resistant and resistant barley lines after short-term (24h and 72h) and long-term (7d and 14d) feeding exposures.

6.3.2.1 Infested non-resistant leaves

After 24h of feeding by both RWASA1 and RWASA2, the distance moved by 5,6-CF as shown in Figs. 6.2A and B was less than that in corresponding control leaves (Figs. 6.1A and C). During longer feeding periods of 72h, 7d and 14d, there is progressive reduction in the intensity and distance moved by 5,6-CF front (Figs. 6.2 C-H). Discontinuous bands of 5,6-CF fluorescence were observed after 72h of RWASA2 feeding (Fig. 6.2D). Prolonged feeding for 7 and 14 days by both biotypes resulted in a patchy and uneven fluorophore distribution pattern in longitudinal veins (Figs. 6.2E and G). These illustrate a reduction in the intensity of fluorescence of transported assimilates in sieve tubes (Fig. 6.2H) which becomes more obvious when compared to what was obtained in control leaves (Fig. 6.1B).

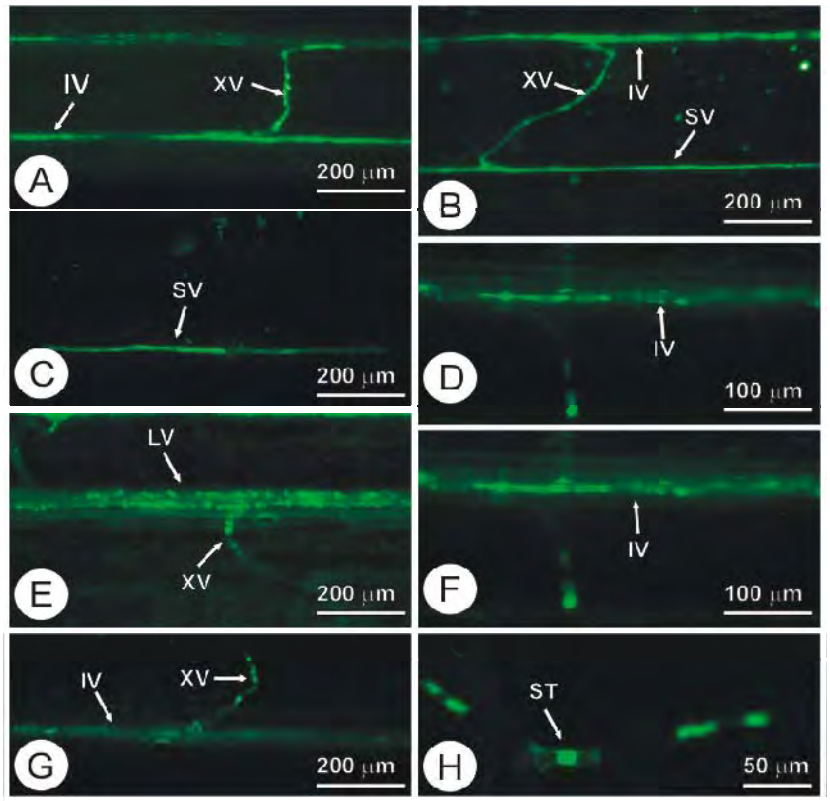

Fig. 6.2 Transport of 5,6-CFDA in infested leaves of non-resistant barley line

Fig. 6.2 A-H Transport of cleaved 5,6-CF in veins of non-resistant PUMA line infested with RWASA1 (left hand side) and RWASA2 (right hand side) after short- and long-term feeding exposures. **A** and **B** illustrate movement of 5,6-CF in intermediate and small veins after 24h of RWASA1 and RWASA2 feeding respectively. Note that flow of the fluorochrome is not as continuous as observed in the control leaf (Fig. 6.1 A and C). Some part of the tissue (B) show leakage of the fluorophore into the mesophyll tissue where the aphids probed. **C** shows mobility of cleaved 5,6-CF in a small vein (SV) after 72h feeding by RWASA1. **D** shows detail of 5,6-CF transport in an intermediate vein from which RWASA2 fed. Disruption to flow became more noticeable at this point. **E** illustrates patchy distribution of the dye in the large vein (LV) after 7d of RWASA1 sustained feeding. There is greater reduction in dye transport compared to patterns observed during short – term feeding periods. **F** shows detail from **D.** When compared to RWASA1, feeding by RWASA2 caused more reduced rate of fluorochrome movement. **G.** An intermediate vein which appears to have been blocked after a long-term 14d continuous feeding by RWASA1. **H.** Detail of a cross vein showing reduction in fluorescence brightness of sieve tubes from a leaf blade material fed upon by RWASA2.

6.3.2.2 Infested resistant leaves

Figures 6.3A-H show typical images of movement and distribution of 5,6-CF in longitudinal veins of infested resistant lines. The three resistant lines infested with either RWASA1 or RWASA2 generally showed reduction in the transport of the fluorophore in phloem tissues in a manner similar to results obtained in the non-resistant PUMA. Distribution of 5,6-CF is progressively reduced as days of aphid feeding increases. The patchy appearance and uneven distribution of cleaved 5,6-CF in the phloem tissues of these resistant lines suggest that both biotypes caused structural damage to the vascular tissues which adversely affected phloem functional capacity to transport assimilates.

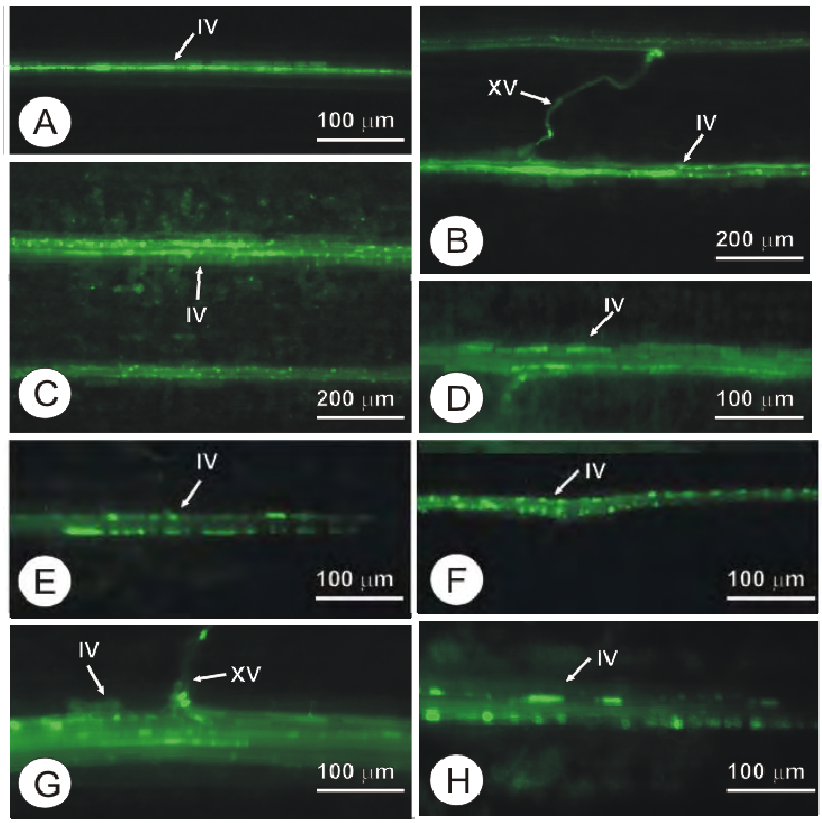

Fig. 6.3 Transport of 5,6-CFDA in infested leaves of resistant barley lines

Fig. 6.3 A-H Generalised view of the pattern of distribution and movement of 5,6-CF among the three resistant lines infested with RWASA1 (Left Hand Side) and RWASA2 (Right Hand Side) after short – and sustained long – term feeding periods. **A.** Short – term (24h) feeding by RWASA1 shows marginal disruption to flow of the cleaved 5,6-CF in an intermediate vein. **B** shows a more discontinuous flow of the fluorochrome due to RWASA2. **C.** More patchy distribution of 5,6-CF in intermediate vein upon RWASA1 feeding for 3d. **D.** shows detail of RWASA2 3d feeding indicating discontinuous flow of the fluorophore in the intermediate vein. **E** shows aspects of reduced transport of 5,6-CF during long – term 7d feeding by RWASA1. **F** Detail, illustrating RWASA2 feeding for 7d, which caused uneven distribution of the dye in the intermediate vein arising from partial blockade to the phloem sieve elements. **G** and **H.** Details of large intermediate veins with greatly reduced transport of 5,6-CF in the phloem after sustained long-term 14d feeding by RWASA1 and RWASA2 respectively. Note that disruption to flow of the cleaved product of 5,6-CF continued to exacerbate as from 3DAI and became worse after 14d of RWASA1 and RWASA2 feeding.

6.3.3 Comparison of the distance moved by 5,6-CF in infested leaves of non-resistant and resistant barley lines

Figures 6.4 and 6.5 show the effects of feeding by RWASA1 and RWASA2 on phloem transport capacity of the four barley lines, during short- and long-term feeding treatments respectively. A three-way factorial analysis of variance (ANOVA) of the differences in the means of the transformed data on movement of 5,6-CF, measured as a percentage of the uninfested control treatments, was carried out and subsequently confirmed with Tukey *posthoc* tests at 95% level of confidence.

Table 6.1 shows results of ANOVA at various levels of interactions. In the two treatments, it was established that the two aphids, the four barley lines and days of feeding exposures were significantly different ($p<0.01$). It was also established that interactions between the aphids and the barley lines on one hand and days of feeding exposures and barley lines were significantly different ($p<0.01$). However, interactions between the aphids and their respective days of feeding as well as the three-way interaction of the aphids, barley lines and days of feeding were not significantly different ($p<0.01$) (see Table 6.1).

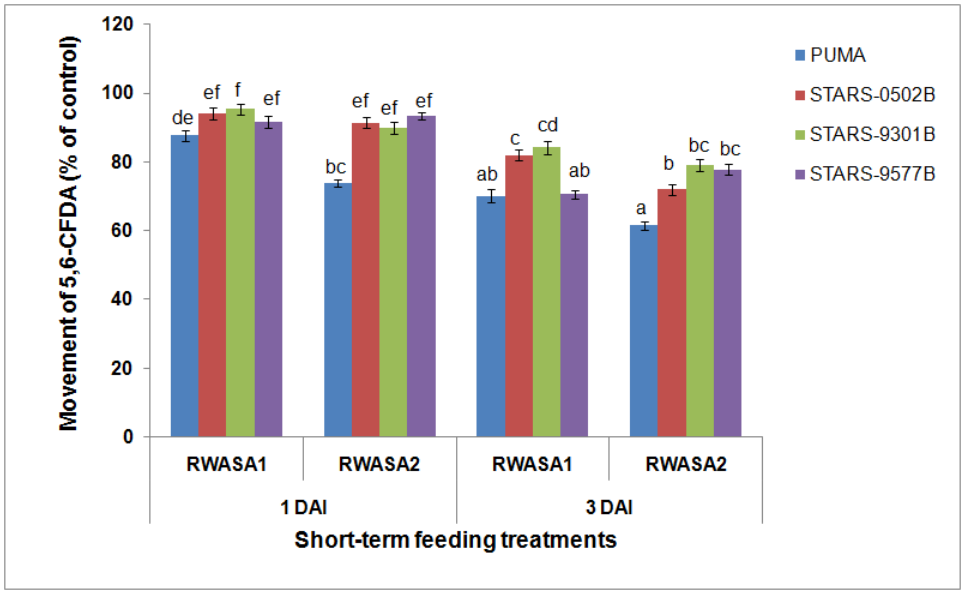

Fig. 6.4 Comparison of the distance moved by 5,6-CF from point of application measured as percentage of control in leaves of the four barley lines infested with RWASA1 and RWASA2 during short-term (24h and 72h) feeding treatments
Letters above each bar indicate results of Tukey *posthoc* test at 5% level of significance to identify homogenous groups. n=10.

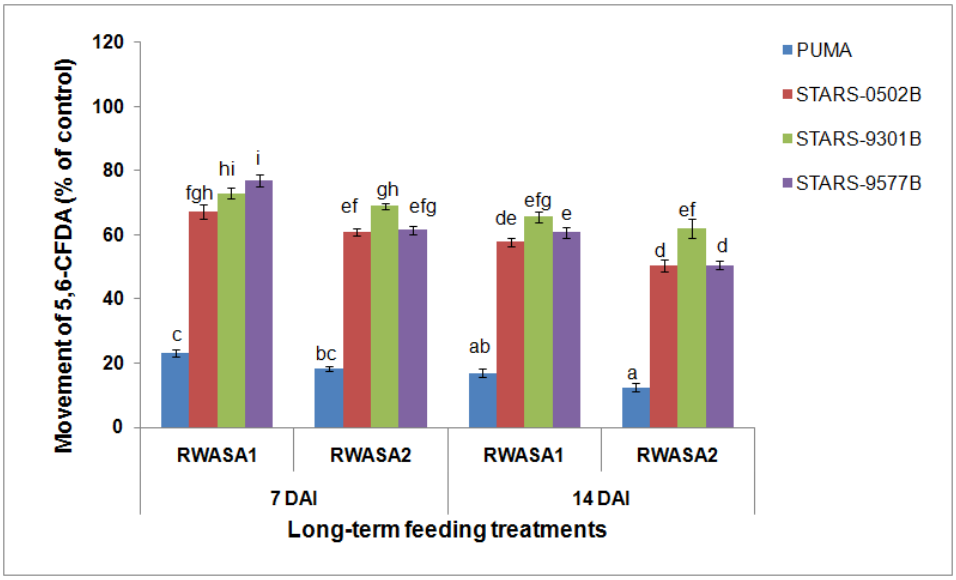

Fig. 6.5 Comparison of the distance moved by 5,6-CF from point of application measured as percentage of control in leaves of the four barley lines infested with RWASA1 and RWASA2 during long-term (7d and 14d) feeding treatments
Letters above each bar indicate results of Tukey *posthoc* test at 5% level of significance to identify homogenous groups (n=10).

Table 6.1 General Linear Model (GLM) results of comparison of various levels of interactions on movement of 5,6-CF measured as percentage of control during short- and long-term treatments†

Interaction	Short – term	Long – term
Aphid	$F_{1, 144}= 29.53^{*}$	$F_{1, 144}= 78.59^{*}$
Line	$F_{3, 144}= 48.41^{*}$	$F_{3, 144}= 841.97^{*}$
Day	$F_{1, 144}= 442.64^{*}$	$F_{1, 144}= 185.05^{*}$
Aphid × Line	$F_{3, 144}= 12.83^{*}$	$F_{3, 144}= 5.47^{*}$
Aphid × Day	$F_{1, 144}= 1.51$ n.s.	$F_{1, 144}= 0.80$ n.s.
Line × Day	$F_{3, 144}= 3.04^{*}$	$F_{3, 144}= 3.37^{*}$
Aphid × Line × Day	$F_{3, 144}= 2.32$ n.s.	$F_{3, 144}= 0.99$ n.s.

† Separate analyses were conducted for short– and long–term feeding treatments. Levels of significance are indicated as: n. s. (not significant) when $p>0.01$ and * (significant) when $p<0.01$.

Feeding by RWASA1 and RWASA2 for 24h significantly reduced the distance moved by the fluorochrome in leaves of the non-resistant PUMA compared to the three resistant lines (Fig. 6.4). However, RWASA2 feeding for 24h significantly reduced transport of 5,6-CF, when compared to RWASA1 feeding for the same period on PUMA. Largely however, there was no significant difference ($p<0.01$) in the distance moved by the fluorophore due to feeding by the two biotypes for 24h in the three resistant lines. During longer 72h feeding period, further reduction in distance moved by the fluorochrome was recorded when compared to 24h feeding period (Fig. 6.4). There was no significant difference in the distance the 5,6-CF moved in RWASA1-infested PUMA and STARS-9577B. Movement of the fluorophore was significantly greater in STARS-0502B and STARS-9301B than in both PUMA and STARS-9577B under RWASA1 infestation for 72h. With the exception of STARS-9577B, feeding-related phloem damage inflicted by RWASA2 was greater in all barley lines, when compared to RWASA1 feeding. It was clearly shown that transport of 5,6-CF during short-term treatments of 24h and 72h by the two RWA biotypes is above 60%. It was evident that among the three resistant lines, STARS-9301B appeared, at this stage, to be least affected by feeding by the two RWA biotypes, with an approximate 20% reduction in distance moved by 5,6-CF.

Infestation by the two RWA biotypes for 7 and 14 days greatly reduced transport of 5, 6-CF in non-resistant PUMA compared to the three resistant plants (Fig. 6.5). On either the non-resistant line or the resistant lines,

RWASA2 feeding reduced movement of the fluorochrome more than RWASA1 feeding. Generally, similar to the situation during short-term feeding (Fig. 6.4), STARS-9301B remained least affected by both aphids with respect to phloem transport capacity.

6.4 Discussion

It is well known that phloem loading follows a well-defined pathway through bundle sheath-vascular parenchyma as well as companion cell-sieve tube complexes as described in previous reports (see Turgeon and Beebe, 1991; Evert et al., 1996; Botha and Cross, 2001; Botha, 2005). Visualization of this process for example, by application of phloem mobile 5, 6-CFDA and subsequent observation of the movement of its cleaved product, 5, 6-CF, in and through the phloem, allows for comparisons, and gives a clear visual indication of the effects of feeding by the two RWA biotypes on the phloem transport capacity of the phloem. It also allows comparison of the relative damage inflicted by the two biotypes (see Botha and Matsiliza, 2004; Walton and Botha 2008; Saheed et al., 2010). It is important to note that though visual interpretation may appear subjective, nonetheless, it gives a strong overview of the effects of aphid infestation on the transport of assimilates in the phloem. Measurement of the distance moved by the fluorophore would further strengthen the results obtained.

Results of this study showed that the rate of phloem transport in uninfested control barley tissues is about 2cm per hour, similar to that measured by Botha and Matsiliza (2004) and Saheed et al. (2010). When the four barley

lines were infested with the aphids, the rate of phloem transport was reduced relative to the uninfested control, depending, however, on the aphid biotype, plant genotype (whether resistant or non-resistant) and duration of infestation (Figs. 6.4 and 6.5). For instance, RWASA2 feeding reduced fluorochrome movement more than RWASA1 feeding did. This indicates that RWASA2 feeding must have slowed down assimilate flow more than RWASA1 did. This is statistically confirmed by the ANOVA summary (Table 6.1), which showed that the two aphid biotypes are significantly different from one another. The results also showed that the rate of assimilate flow, as indicated by the movement of the fluorochrome, reduces as the number of days of infestation by the aphids increases (Figs. 6.4 and 6.5), this is in agreement with the report of the work by Saheed et al. (2010). The study further showed that phloem transport is greatly reduced, after aphid infestation, in non-resistant PUMA compared to any of the three resistant lines, particularly after sustained feeding. This is statistically consistent with the ANOVA results shown in Table 6.1 which indicated that the four barley lines are significantly different ($p<0.01$). I need to state here that this is the first study that compares and quantifies the disruption of phloem transport in resistant and non-resistant host plants exposed to feeding by two RWA biotypes.

Results presented in this chapter indicate that when the transport of assimilates is not affected by biotic factors such as aphid feeding, its flow is not disrupted, but confined within the vascular system as shown in the longitudinal transport of 5,6-CF in the veins of control (uninfested) plant

tissues (Figs. 6.1A-D). Feeding by either RWASA1 or RWASA2 on resistant and non-resistant barley lines affected phloem capacity to transport assimilates as indicated by reduction in the intensity and distribution of 5,6-CF in the longitudinal veins of infested plants (Figs. 6.2 and 6.3). During short-term feeding (24h or 72h), the two biotypes caused reduction in the intensity of the fluorochrome contained in the veins of non-resistant (Figs. 6.2A-D) and resistant (Figs. 6.3A-D) barley lines. The assumed disruption of phloem transport, as indicated by 5,6-CF movement, was noticeable after 24h of aphid feeding (see Figs. 6.2A and B, non-resistant; and resistant, Figs. 6.3A and B). Disruption to phloem transport became worse with longer aphid feeding for 72h (non-resistant, Figs. 6.2C and D; resistant, Figs. 6.3C and D). Track of the fluorochrome showed a patchier pattern after 72h than after 24h of feeding which connotes severer disturbance within the flow pathway. This may be due to deposition of wound callose, a wounding response attributable to feeding by aphids (Botha and Matsiliza, 2004; de Wet and Botha, 2007; Saheed et al, 2009). A clear description of this development is better understood in Figs.6.4-6.5, where the movement of 5,6-CF in non-resistant PUMA infested with RWASA2 for 24h was about 70% of observations in control, compared to about 90% with RWASA1. Whereas the corresponding values in the resistant lines are approximately 95% of uninfested control (Fig. 6.4), these values were further reduced during longer feeding for 72h.

Sustained long-term feeding (7d and 14d) resulted in greater reduction in phloem transport capacity, the trend of which were RWASA2 > RWASA1

and non-resistant line > resistant lines (Fig. 6.5). A more patchy distribution pattern as well as reduced intensity of the fluorochrome were visible in non-resistant (Figs. 6.2E and G) and resistant (Figs. 6.3E-H) lines. Severe feeding by RWASA2 on non-resistant PUMA for 14 days resulted in complete cessation of transport (Fig. 6.2H). These results lend support to findings by Botha and Matsiliza (2004) that infested leaves of a susceptible wheat cultivar showed little longitudinal or transverse trafficking of 5,6-CF. The feeding aphids in this case have massively damaged vascular tissues and rerouted assimilates containing the fluorochrome to themselves.

This study demonstrates that feeding by the two RWA biotypes alter the phloem transport functionality of resistant and non-resistant barley lines. Once their stylets penetrate functional phloem, aphids become local sinks, redirecting and tapping products of photosynthesis into their guts. Hill (1962) showed that if draining of photoassimilates by feeding aphids is sufficiently strong and localised, host plant reacts to it in such a way as if the feeding aphid were its bud. In this manner, aphids compete directly with the primary sink organs of the plant, denying them of essential nutrients normally supplied during assimilates transport through the phloem. This position is again illustrated by Saheed et al. (2010) in which 5,6-CF ingested by RWA and BCA were visibly present in honeydew excreted after feeding. Similar phloem feeding insects have been shown to disrupt and redirect assimilates in their respective host plants (Nielsen et al., 1990; Watanabe and Kitagawa, 2000). The alteration to the transport system leads to a decline in the flow rate, the

quality and the availability of assimilates loaded and transported in source leaves, and eventually unloaded in sink leaves or storage organs.

The experiments reported in this chapter shows that RWASA2 feeding caused greater disruption to phloem transport functionality than RWASA1. This suggests that RWASA2 inflicts more damage than RWASA1 feeding. This observation confirms the position of earlier studies that showed that RWASA2 is not only a resistance-breaking biotype but also a more aggressive feeder than RWASA1 (Tolmay et al., 2007; Walton and Botha, 2008). As callose deposition is promoted by aphid infestation (see Saheed et al., 2009 and literature cited), the two biotypes elicit differential callose deposition (see Walton and Botha, 2008), with different effects on movement of 5,6-CF illustrated in Figs. 6.4 - 6.5). Possibly, components of the saliva of the two aphids differ, which may result in differences in the damage to the phloem and to phloem transport capacity of the infested plants. Clearly, further investigation into the inherent qualities of the saliva of the two RWA biotypes is warranted, as it is hereby suggested that differences in salivary components may make RWASA2 more devastating to crops than the RWASA1 biotype. Furthermore, this study showed that the levels of damage suffered by non-resistant is greater compared to resistant lines. With the resistance factor in them, the resistant lines might be able to cope with aphid infestation, and suffer less damage than non-resistant cultivars (Nkongolo et al., 1990; De Wet and Botha, 2007).

In this chapter, I have demonstrated that long distance transport of assimilates in barley hosts becomes disrupted by the two currently known RWA biotypes in South Africa. The disruption is quicker and more devastating as a result of RWASA2 feeding than RWASA1. Disruption to phloem transport functionality by the feeding aphids could result into reduction in photoassimilate translocation, which can adversely affect barley productivity. Reduction in the damage to phloem tissues recorded on the resistant lines (STARS-0502B, STARS-9301B and STARS-9577B) shows they are promising and may be utilised in the development of barley lines that can be used to control RWA infestation in South Africa.

7 Chapter 7: Effect of changing CO_2 concentrations on plant and aphid interactions

7.1 Introduction

Anthropogenic activities such as increased fossil fuel consumption, increased industrial activities, increased atmospheric pollution through the release of greenhouse gasses, coupled to deforestation, has resulted in an alarming increase in the rate at which concentration of carbon dioxide ([CO_2]) in the atmosphere is increasing. It has risen from about 285ppm since the Industrial Revolution era of 1750 to about 385ppm in 2005 (Forster et al., 2007; Stiling and Cornelissen, 2007; Ryan et al., 2010). The [CO_2] is expected to continue to rise well into the next century, to above 500ppm depending on the magnitude of the world economic growth and energy use (IPCC, 2010). Concerns raised over this development have led to investigations into the primary responses of plant communities to rising [CO_2]. Available evidence suggests that plants have responded to the 25% increase in atmospheric [CO_2] that has occurred since the onset of the Industrial Revolution (Woodward, 1987; Overdieck et al., 1988; Dippery et al., 1995; Duquesnay et al., 1998).

There are many studies which show that [CO_2] influences both photosynthetic and developmental processes in plants (Bassi et al., 1976; Hicklenton and Jolliffe, 1980). For example, many C_3 plants grown at elevated [CO_2] attain higher photosynthetic rates and thus grow faster (Hughes and Bazzaz, 1997; Owensby et al., 1999). This results in a large increase in plant biomass, due to

the accumulation of both structural and non-structural carbohydrates, the imbalance in the C:N ratio, and the resulting reduction in the nitrogen content of foliage, all affect plant quality (Lindroth et al., 1995; Poorter et al., 1997; Bezemer and Jones, 1998). Changes in plant quality must affect the feeding patterns of herbivores, which has the potential to induce far-reaching effects on the patterns of plant biodiversity and resultant agricultural productivity (Theurillat and Guisan, 2001; Fuhrer, 2003). In general, most organisms do not rapidly adapt to sudden increase in [CO_2] from the ambient levels (Holopainen, 2002). What is of interest in this chapter is the effect that elevated CO_2 has on the aphid-plant interaction.

Aphids are one of the most important groups of insect pests in temperate regions, where small grains, such as wheat and barley are cultivated on a large scale (Vickerman and Wratten, 1979). Aphids inflict direct damage on their host plants by removing large quantities of sap and indirectly by serving as virus vectors (Risebrow and Dixon, 1987). Their parthenogenetic reproduction often results in an elevated regeneration rate (Dixon, 1998), which has been demonstrated using the South African RWA biotypes (see Chapter 3 of this thesis; Walton and Botha, 2008; Jimoh et al., 2011). If their breeding rate is either sustained or increases with climate change, then they may heighten their pest status (Harrington et al., 1995). It is likely that increased herbivory would effect and modify plant responses to climate change. For instance, aphids form strong secondary sinks when feeding (Nielson, et al., 1990; Botha and Matsiliza, 2004; Saheed et al., 2010), which

may be an important factor when considering plant acclimation responses under elevated CO_2 (Rogers et al., 1995).

Aphids are regarded as one of the most sensitive insect groups that respond quickly to changes in plant quality, due to altered plant environment such as climate change (Docherty et al., 1997). Studies on the effects of changing [CO_2] on aphids have provided variable results (see review by Holopainen, 2002). Of the 28 available aphid-host plant data in the literature, 6 support increased aphid performance, 6 had reduced performance at elevated [CO_2] and in the remaining 16 cases, the population at elevated [CO_2] did not differ from control experiments carried out at ambient [CO_2] (Holopainen, 2002; Awmack et al., 2004; Sudderth et al., 2005, Flynn et al., 2006). There are many reasons for the variability. It could be because of marked change(s) in the environmental conditions of host plants (such as nutrient availability and light conditions) and/or differential feeding behaviour of various aphids; some are generalists, while others are species-specific (Dixon, 1998; Bezemer et al., 1999). In addition, fecundity and nymph size are not always reflected in final population sizes of aphids, primarily due to changes in host plant quality, which alter feeding behaviour of apterae and alates in later phases of aphids' population development (Docherty et al. 1997).

Hughes and Bazzaz (2001) commented that many studies on plant-aphid interaction at elevated [CO_2] have focused only on aphid performance, without considering the corresponding host plant responses. This has created a gap in our understanding and raises questions about aphids' potential pest

performance under elevated [CO_2] with respect to the reported changes in plant quality of a given herbivory relationship (see Ehleringer et al., 2002). Of interest is that few reports have shown that aphid infestation caused reduction in host plant productivity at elevated [CO_2] than at ambient level (Awmack and Harrington, 2000; Hughes and Bazzaz, 2001; Flynn et al., 2006; Himanen et al., 2008). These data suggest that aphid infestation may negate the (expected) beneficial effects of plant growth and productivity at elevated [CO_2].

Surprisingly, RWA has not been used in experiments to determine the effect of elevated [CO_2] on small grains until now. In this chapter, I describe experiments, which are the first attempt, baseline study on the effects of RWA infestation on barley lines at elevated [CO_2] levels. I examined the effects which the two South African RWA biotypes would have on plant biomass, C:N ratio, leaf nitrogen concentration, plant damage symptoms (chlorosis and leaf roll) and aphid population growth rates under three [CO_2] levels [the ambient (380 µmol mol^{-1}) and two elevated levels (450 and 550 µmol mol^{-1}], using four barley host combinations.

7.2 Experimental overview

All extraneous climatic variables were eliminated as experiments were conducted a under controlled environment. Within the growth cabinets, [CO_2] was maintained at 380 (ambient) and elevated (450 or 550 µmol mol^{-1} [CO_2] levels). Details of the growth conditions are provided in section 2.3 of Chapter 2. Each [CO_2] level required 120 plants, with 10 plants for each of control

(uninfested) and for each RWASA1 and RWASA2 treatment. Whole plant of each barley line was enclosed under a ventilated plastic cylindrical isolation cage. Experimental plants (with the exception of the controls) were infested with 10 adult apterae of either RWASA1 or RWASA2. Responses of the experimental plants to each [CO_2] treatment were investigated as follows:

(a) Population growth of the aphids on infested plants at 1, 7, 14 DAI (see Chapter 2, section 2.4 for details).

(b) Virulence of the two RWA biotypes on the infested plants, measured as chlorosis and leaf rolling, at 1, 7, 14, 21 and 28 DAI, using the scoring system described in section 2.5 of Chapter 2.

(c) Determination of the total biomass as well as its above and below the ground components, of the control (uninfested) and infested plants at 28 DAI (see Chapter 2, section 2.9 for details).

(d) Determination of foliar nitrogen concentration and C:N ratio of control and infested plants, details of which are given in Chapter 2, section 2.10.

7.3 Results

7.3.1 Effect of [CO_2] on biomass components

Elevated [CO_2] had significant effect on the growth of the four barley lines that were not infested by the aphids. Total (above-ground and below-ground) biomass of each of the four barley lines increased significantly under the two

elevated [CO_2] levels when compared to their respective values at ambient [CO_2] level (Fig.7.1). The increase in biomass is proportional to [CO_2] increase, as the greatest increase was recorded in plants grown at 550 µmol mol^{-1}.

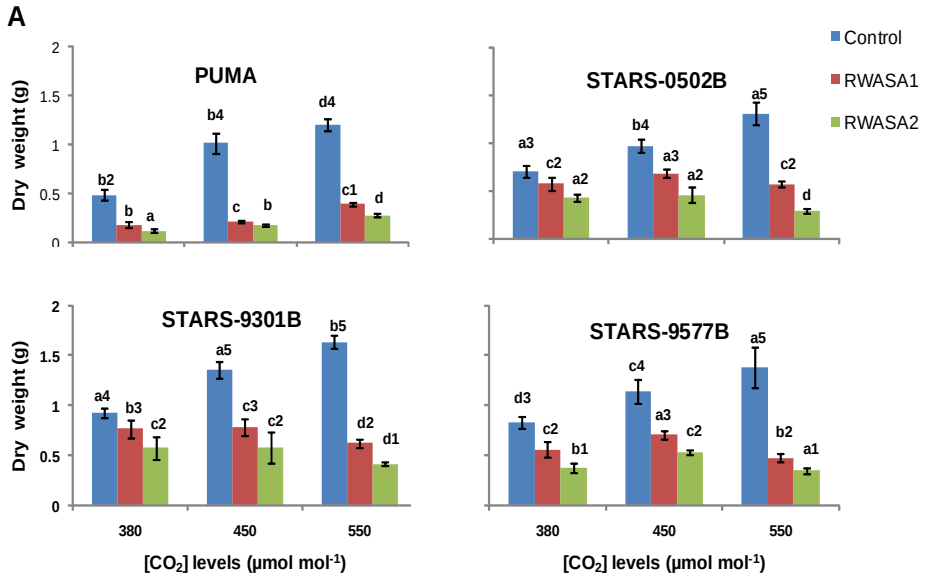

Fig. 7.1 Relative changes in total biomass [dry weight (g)] of control and infested plants grown at ambient and elevated [CO2]
Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey *posthoc* test (n=10)

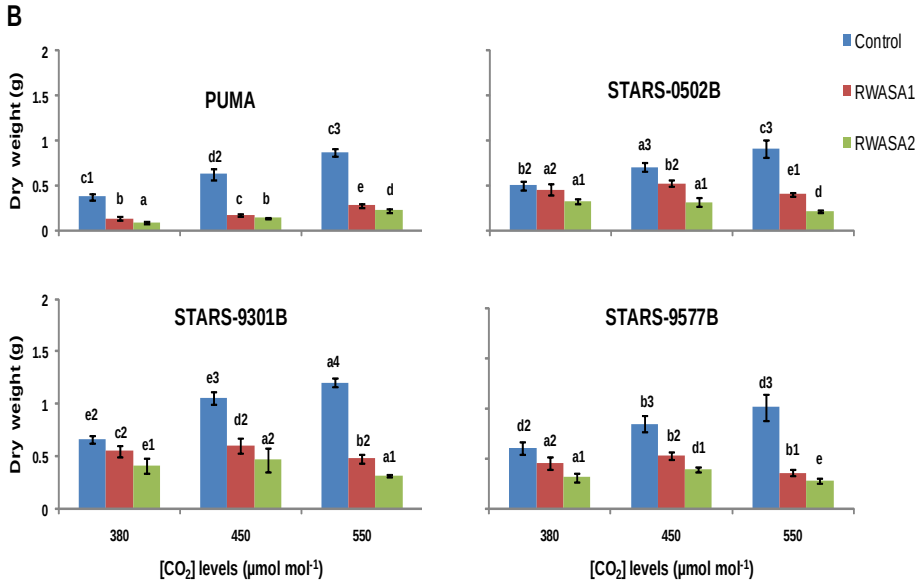

Fig. 7.2 Relative changes in above-ground biomass [dry weight (g)] of control and infested plants grown at ambient and elevated [CO2]
Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey posthoc test (n=10)

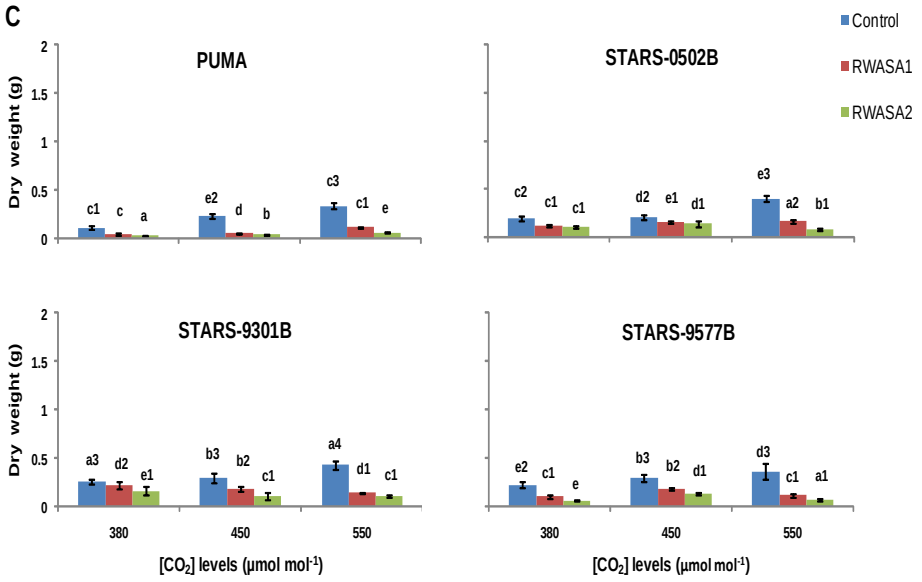

Fig. 7.3 Relative changes in below-ground biomass [dry weight (g)] of control and infested plants grown at ambient and elevated [CO2]
Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey posthoc test (n=10)

Elevated CO_2 also resulted in increase in above- (Fig. 7.2) and below-ground (Fig 7.3) biomass of uninfested plants above the ambient values for all four barley lines.

Table 7.1 shows that the total biomass of each of the four lines recorded percentage increases ranging from 38% to 108% between ambient [CO_2] and 450 µmol mol^{-1}; and from 66% to 145% between ambient [CO_2] and 550 µmol mol^{-1} [CO_2] level. However, percentage increase recorded for the total biomass between the two elevated CO_2 levels (450 and 550 µmol mol^{-1}) is smaller than those observed between the ambient level and each of the two elevated [CO_2] levels, ranging from 18% to 35%.

Table 7.1 Relative percentage increase in total biomass of control (uninfested) barley lines for the three [CO2] levels

Barley line	Ambient – 450 (%)	Ambient – 550 (%)	450 – 550 (%)
PUMA	108	145	18
STARS-0502B	38	86	35
STARS-9301B	47	78	22
STARS-9577B	39	66	20

ANOVA data (Table 7.2) showed clearly that interactions between barley lines and [CO_2] were not significantly different for each of the biomass components ($p<0.001$), which indicates that each line reacted independently to [CO_2]. ANOVA results also showed that the four barley lines are different from one another ($p<0.001$).

Table 7.2 Results of Multiple-way ANOVA of biomass (g dry weight) of infested and uninfested barley lines exposed to the three levels of [CO2] at 28 DAI

		Total biomass		Above-ground biomass		Below-ground biomass	
Source of variation	d.f	F	p-value	F	p-value	F	p-value
Aphid types	2	325.074	<0.001	292.863	<0.001	179.922	<0.001
Barley lines	3	59.670	<0.001	65.675	<0.001	23.896	<0.001
[CO2] levels	2	24.945	<0.001	22.760	<0.001	16.009	<0.001
Aphid types * Barley lines	6	3.038	0.007	2.940	0.008	0.690	0.658
Aphid types * [CO2] levels	4	20.879	<0.001	22.115	<0.001	16.223	<0.001
Barley lines * [CO2] levels	6	4.314	<0.001	4.126	<0.001	3.852	<0.001
Aphid types * Barley lines * [CO2]	12	1.149	0.320	0.566	0.869	1.222	0.267

In contrast to the data for control plants, as a result of feeding by the two RWA biotypes on all four barley lines, significant loss in biomass was recorded under ambient as well as the two elevated CO_2 levels (Figs. 7.1-7.3). The two biotypes caused greater reduction in the biomass of the non-resistant PUMA line than on any of the three resistant lines. When the reduction in the total biomass of infested plants was expressed as a percentage of the control (uninfested) biomass of each barley line at each [CO_2] level, the reduction in biomass was massive in ambient as well as in elevated CO_2 (Table 7.3). Loss in biomass in the non-resistant line was 63% at ambient CO_2 when exposed to feeding RWASA1 and rose to as high as 82% at 450 µmol mol^{-1} when these plants were exposed to feeding RWASA2 aphids. Interestingly, in plants grown at a CO_2 concentration of 550 µmol mol^{-1}, biomass loss dropped to 68 and 77% respectively on plants being fed on by RWASA1 and RWASA2. Although at each [CO_2] level, RWASA2 caused greater reduction in total biomass than RWASA1, the trend in the percentage reduction of total biomass suffered by PUMA infested by either RWASA1 or RWASA2 among the three [CO_2] levels is 450>550>ambient (Table 7.3). However, among the resistant lines, loss in biomass due to feeding by the two biotypes is generally lower than in non-resistant PUMA. Reduction in biomass increases with increase in [CO_2]. RWASA2 feeding caused greater loss in biomass than RWASA1 feeding in each of the three lines.

Table 7.3 Reduction in the total biomass of barley lines infested with RWASA1 and RWASA2 at the three levels of [CO2] expressed as a percentage of the values for uninfested plants

	Ambient (%)		450 µmol mol^{-1} (%)		550 µmol mol^{-1} (%)	
Barley Lines	RWASA1	RWASA2	RWASA1	RWASA2	RWASA1	RWASA2
PUMA	63	76	79	82	68	77
STARS-0502B	18	38	29	53	57	77
STARS-9301B	16	36	42	57	62	74
STARS-9577B	32	54	38	54	65	75

7.3.2 Effect of [CO_2] on total leaf nitrogen

The results of the analyses of the leaves of control (uninfested) and infested plants after 28d of RWASA1 and RWASA2 feeding to determine the leaf nitrogen concentration per gramme of leaf tissue at the three levels of [CO_2] are shown in Fig. 7.4. In uninfested plants of the four lines, leaf nitrogen concentration increases as [CO_2] increases. Based on this trend, percentage increases in leaf nitrogen concentration among the three levels of CO_2 were calculated for the four barley lines as shown in Table 7.4.

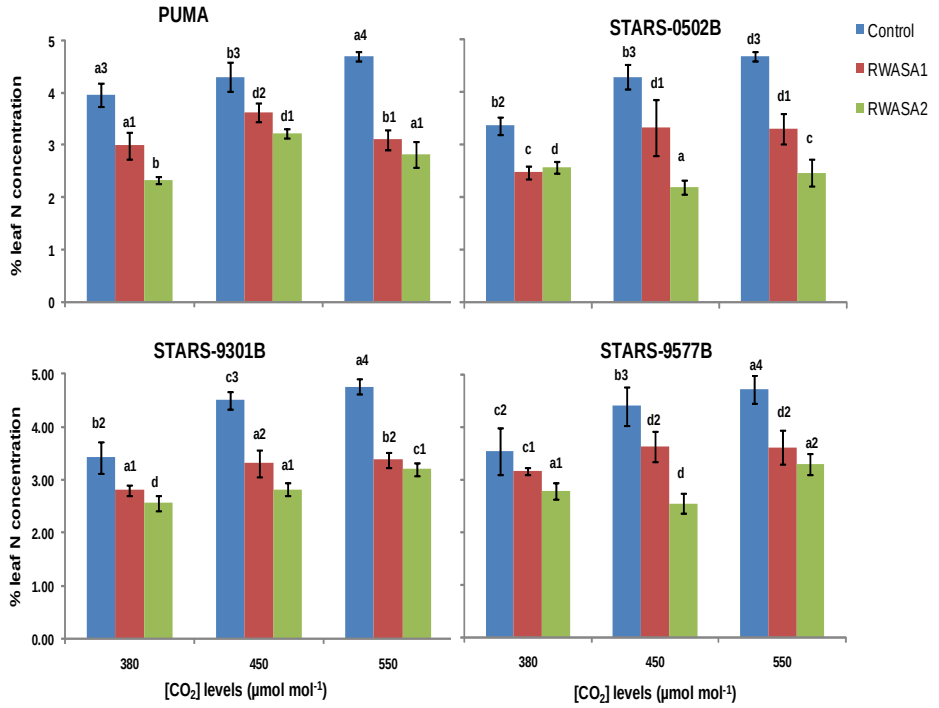

Fig. 7.4 Percentage foliar nitrogen concentrations ($g^{-1}$of leaf) of control and infested barley leaves grown under the three levels of [CO2] at 28 DAI
Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey *posthoc* test (n=5).

Table 7.4 Percentage increase in leaf nitrogen concentration of control (uninfested) barley lines at ambient and the two levels of [CO2]

Barley line	Ambient - 450 (%)	Ambient - 550 (%)	450 - 550 (%)
PUMA	9	19	9
STARS-0502B	28	39	9
STARS-9301B	32	39	6
STARS-9577B	24	33	7

Among the four barley lines, percentage increases in leaf nitrogen concentrations of these uninfested plants range from 9% to 32% between ambient [CO_2] and 450 µmol mol^{-1}, 19% to 39% between ambient and 550 µmol mol^{-1} and 6% to 9% between the two elevated CO_2 levels (Table 7.4). The increase in foliar nitrogen concentrations between the two elevated [CO_2] levels is smaller than those obtained between the ambient level and each of the two elevated [CO_2] levels.

Results of ANOVA on foliar nitrogen concentrations showed that the interaction between barley lines and [CO_2] were significantly different (p=0.33) (Table 7.5). This indicates that increase in [CO_2] from ambient level significantly affected leaf nitrogen concentrations of these experimental plants.

Table 7.5 ANOVA of % leaf nitrogen concentrations (g-1 of leaf) and C:N ratio of infested and uninfested barley leaf tissues exposed to the three levels of [CO2] at 28 DAI

		%N		C:N	
Source of variation	df	F	p-value	F	p-value
Aphid types	2	126.30	<0.001	87.5	<0.001
Barley lines	3	5.21	0.002	15.6	<0.001
[CO2] levels	2	26.55	<0.001	4.3	0.016
Aphid types * Barley lines	6	1.13	0.349	3.3	0.004
Aphid types * [CO2] levels	4	3.19	0.015	3.8	0.005
Barley lines * [CO2] levels	6	1.16	0.330	4.7	<0.001
Aphid types * Barley lines * [CO2] levels	12	1.54	0.115	4.0	<0.001

Feeding by the two RWA biotypes on the four barley lines resulted in significant depletion of nitrogen concentration of leaves, under each of the three CO_2 levels (Fig.7.4). Based on this result, percentage reduction in leaf nitrogen concentration as a result of aphid infestation was calculated relative to the values for uninfested plants as shown in Table 7.6. Reduction in leaf nitrogen concentration due to aphid feeding gave variable results among the four barley lines at the three levels of CO_2. It is evidently shown that the two biotypes depleted leaf nitrogen content of both resistant and non-resistant plants. With the exception of STARS 0502B at ambient CO_2 level, RWASA2 caused greater depletion of leaf nitrogen than RWASA1 under the three CO_2 levels in all barley lines (Table 7.6).

Table 7.6 Reduction in the leaf nitrogen concentration of barley lines infested with RWASA1 and RWASA2 at the three levels of [CO2] expressed as a percentage of the values for uninfested plants

	Ambient (%)		450 µmol mol^{-1} (%)		550 µmol mol^{-1} (%)	
Barley Lines	RWASA1	RWASA2	RWASA1	RWASA2	RWASA1	RWASA2
PUMA	25	41	16	25	34	40
STARS-0502B	26	24	22	49	30	47
STARS-9301B	18	25	26	37	29	33
STARS-9577B	11	22	18	42	23	30

7.3.3 Effect of elevated CO2 on leaf C:N ratio

The C:N ratios among uninfested plants of the four lines were variable (Fig. 7.5). Of the four lines, only STARS-9301B showed a consistent (but insignificant) increase in C:N ratios. The percentage increase in C:N ratio between ambient and 450 µmol mol^{-1} was 6%, between ambient and 550 µmol mol^{-1} was 10% while the increase between the two elevated [CO_2] levels was 6% (Table 7.7; Fig. 7.5). In PUMA and STARS-9577B the C:N ratio increased between ambient and each of the two elevated CO_2 levels. However, in these two lines, C:N ratio decreased between the two elevated CO_2 levels (Fig. 7.5; Table 7.7). In STARS-0502B line, C:N ratios generally decreased among the three CO_2 levels (Fig. 7.5; Table 7.7). There was no significant barley line and [CO_2] interactions on C:N ratios ($p<0.001$) (Table 7.5).

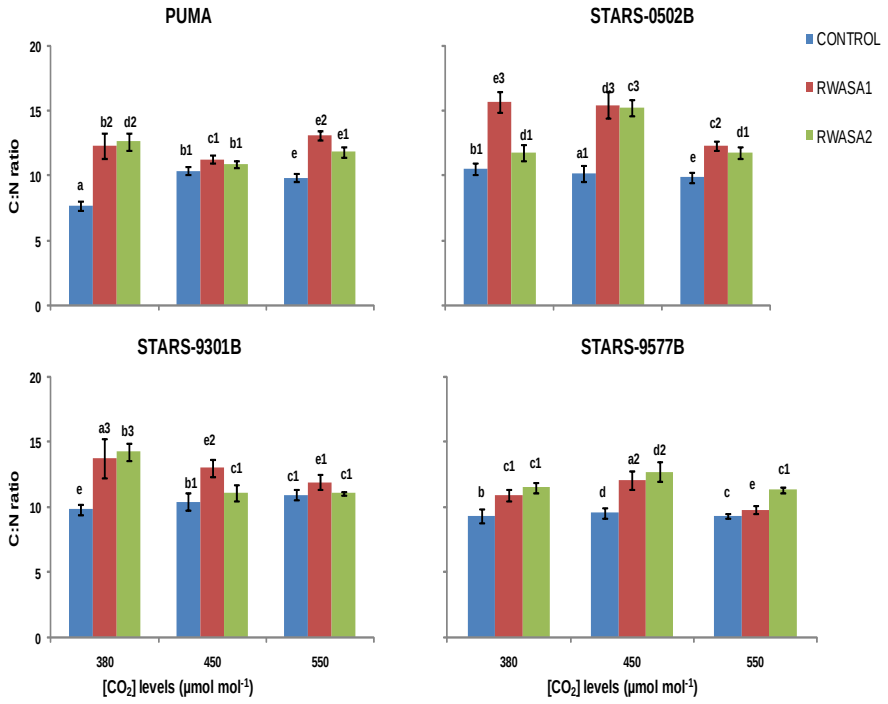

Fig. 7.5 C:N ratios of control (uninfested) and infested barley leaf tissues exposed to the three [CO2] levels at 28 DAI

Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey posthoc test (n=5).

Table 7.7 Percentage change in leaf C:N ratios of control (uninfested) barley lines at ambient and elevated CO_2 levels

Barley line	Ambient - 450 (%)	Ambient - 550 (%)	450 - 550 (%)
PUMA	36 ↑	29 ↑	5 ↓
STARS-0502B	4 ↓	6 ↓	3 ↓
STARS-9301B	6 ↑	11 ↑	6 ↑
STARS-9577B	3 ↑	0.4 ↑	2 ↓

Where: [↑] represents increase and [↓] represents decrease.

There was also no significant difference in the 3-way interactions of barley line, [CO_2] and aphids ($p<0.001$) (Table 7.5). Feeding by the two RWA biotypes caused considerable increases the leaf C:N ratios in the four barley lines, when compared to levels recorded on uninfested plants, under each of the three [CO_2] levels (Fig.7.5). The trend of the increase in C:N ratio, as a result of feeding by the two biotypes, gave variable results on the four barley lines, under each of the three CO_2 levels (Table 7.8).

Table 7.8 Increase in the leaf C:N ratios of barley lines infested with RWASA1 and RWASA2 at the three levels of [CO2] expressed as a percentage of the values for uninfested plants

	Ambient (%)		450 µmol mol^{-1} (%)		550 µmol mol^{-1} (%)	
Barley Lines	RWASA1	RWASA2	RWASA1	RWASA2	RWASA1	RWASA2
PUMA	60	65	9	5	33	20
STARS-0502B	49	12	52	50	25	19
STARS-9301B	40	45	25	7	9	1
STARS-9577B	17	24	26	33	5	21

7.3.4 Aphid damage to host plants at varying levels of [CO2]

The experiments to assess damage symptoms on the four barley lines under the three CO_2 levels showed that feeding by RWASA1 and RWASA2 resulted in visible symptoms associated with RWA infestation on host plants. These include chlorosis, necrosis, longitudinal streak and leaf rolling. These observations are consistent with those reported in Chapters 3 and 5 of this thesis. In the current study, two among the observed damage symptoms, chlorosis and leaf rolling, reported to be important visible criteria for evaluating extent of damage on host plants during RWA infestation were assessed in a similar approach reported in Chapter 3 (Jimoh et al., 2011).

7.3.4.1 Chlorosis

Results of the repeated measures of ANOVA for chlorosis rating on the test plants infested with the two RWA biotypes and grown under the effects of the three [CO_2] at 1, 7, 14, 21 and 28 DAI are shown in Table 7.9. There was no significant difference in the interactions among the barley lines infested by the two aphids at the three [CO_2] levels at various days of infestation ($p<0.001$). The results show that chlorosis damage symptom aggravates as days of infestation advances, getting worse on all barley lines under elevated CO_2 treatments than ambient level as from seven DAI (Table 7.10). Non-resistant plants displayed extensive chlorosis symptom under the three CO_2 levels at 14 DAI. By 21 DAI, some of these non-resistant plants under the elevated CO_2 treatments infested by RWASA2 have died. This development became

extensive, covering plants infested by the two biotypes at the three CO_2 levels at 28 DAI. The resistant plants displayed less chlorotic symptoms when compared to the non-resistant plants. Chlorosis coverage on the resistant plants range from 55 to 85% at 28 DAI, when experiments were terminated. It is evident that RWASA2 was more destructive than RWASA1 at elevated [CO_2] levels as days of infestation increases (Table 7.10).

Table 7.9 Repeated measures ANOVA of virulence* of RWASA1 and RWASA2 feeding at 1, 7, 14, 21 and 28 DAI on the barley lines under the three levels of [CO2]

		Chlorosis rating		Leaf roll rating	
Source of variation	d.f	F	p-value	F	p-value
Aphid types	1	19.77	<0.001	132.43	<0.001
Barley lines	3	385.11	<0.001	160.84	<0.001
[CO2] levels	2	218.58	<0.001	21.72	<0.001
DAI	4	6996.65	<0.001	527.30	<0.001
Aphid types * Barley lines	3	18.00	<0.001	2.67	0.046
Aphid types * [CO2] levels	2	87.97	<0.001	9.33	<0.001
Barley lines * [CO2] levels	6	30.53	<0.001	0.88	0.505
Aphid types * DAI	4	7.18	<0.001	8.63	<0.001
Barley lines * DAI	12	36.54	<0.001	13.40	<0.001
[CO2] levels * DAI	8	41.96	<0.001	8.23	<0.001
Aphid types * Barley lines * [CO2] levels	6	6.01	<0.001	5.68	<0.001
Aphid types * Barley lines * DAI	12	3.76	<0.001	0.97	0.473
Aphid types * [CO2] levels * DAI	8	12.16	<0.001	1.49	0.155
Barley lines * [CO2] levels * DAI	24	7.27	<0.001	1.86	0.008
Aphid types * Barley lines * [CO2] levels * DAI	24	2.48	<0.001	0.62	0.923

*Virulence assessed as chlorosis and leaf rolling

Table 7.10 Mean ratings for chlorosis of barley lines infested with RWASA1 and RWASA2 (n=10)

		380 µmol mol^{-1}		450 µmol mol^{-1}		550 µmol mol^{-1}	
DAI	Barley line	RWASA1	RWASA2	RWASA1	RWASA2	RWASA1	RWASA2
1	PUMA	0.10a	0.10a	0.00a	0.00a	0.00a	0.00a
	STARS-0502B	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	STARS-9301B	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	STARS-9577B	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
7	PUMA	1.30c	1.80f	3.00e1	4.70c3	1.30c	2.10a1
	STARS-0502B	1.00b	1.00b	3.90e2	5.00e3	2.50d1	2.90e1
	STARS-9301B	1.00b	1.00b	1.40d	1.70e	1.00b	1.20c
	STARS-9577B	1.00b	1.00b	1.70e	1.90f	1.00b	1.00b
14	PUMA	6.70e4	7.20c5	5.50a4	7.10b5	4.60b3	6.70
	STARS-0502B	4.20e2	3.20a2	6.10b4	6.70e4	3.70d2	4.70b3
	STARS-9301B	3.90c2	2.20b1	3.00e1	4.00e2	2.40c1	1.90f
	STARS-9577B	3.10f1	3.10f1	3.10f1	4.40a3	1.90f	2.50d1
21	PUMA	7.70a6	8.70e6	7.80b6	8.70e6	6.40c4	8.60e6
	STARS-0502B	6.80f4	4.70c3	7.60f5	8.50e6	5.40f3	7.50e5
	STARS-9301B	7.20c5	3.00f1	5.40f3	6.40c4	3.50b2	3.80d2
	STARS-9577B	6.90a5	4.30e2	4.80d3	6.40c4	3.40b2	4.60f2
28	PUMA	8.70e6	9.00f6	9.00f6	9.00f6	8.00c6	9.00f6
	STARS-0502B	7.30d5	5.40f3	8.60e6	9.00f6	8.10c6	8.80e6
	STARS-9301B	7.50e5	4.70c3	7.50e5	8.20d6	5.00e3	6.70e4
	STARS-9577B	7.30d5	5.40f3	6.50d4	8.20d6	5.30f3	6.80f4

Values are means of 10 replicates. Values followed by different notations are significantly different following Tukey's *posthoc* test ($p<0.05$).

7.3.4.2 Leaf roll

Results of repeated measures ANOVA for leaf roll ratings are shown on Table 7.9. There was no interaction effect among the barley lines, [CO_2] levels and aphid biotype at various days of infestation. Leaves of the non-resistant PUMA and STARS-0502B (one of the resistant lines) infested by the two aphids were loosely folded at seven DAI (Table 7.11). Leaf folding continued and exacerbated until 28 DAI on these two lines. Leaves of STARS-9301B and STARS-9577B showed no apparent rolling symptoms at seven DAI (Table 7.11). These two resistant lines displayed a simple infolding of leaves only at 14 DAI and apparently remained so, until 28 DAI when experiments were terminated. It is evident that in all cases, RWASA2 caused more leaf roll symptom damage than RWASA1 at all [CO_2] levels.

Table 7.11 Mean ratings for leaf roll damage symptoms of barley lines infested with RWASA1 and RWASA2 (n=10)

		380 µmol mol^{-1}		450 µmol mol^{-1}		550 µmol mol^{-1}	
DAI	Barley line	RWASA1	RWASA2	RWASA1	RWASA2	RWASA1	RWASA2
1	PUMA	1.00a	1.10a	1.00a	1.00a	1.00a	1.00a
	STARS-0502B	1.00a	1.00a	1.00a	1.00a	1.00a	1.00a
	STARS-9301B	1.00a	1.00a	1.00a	1.00a	1.00a	1.00a
	STARS-9577B	1.00a	1.00a	1.00a	1.00a	1.00a	1.00a
7	PUMA	1.70a1	2.30f1	1.60f	1.70a1	1.00a	1.50e
	STARS-0502B	1.60f	1.70a1	1.50e	1.80b1	1.30c	1.30c
	STARS-9301B	1.00a	1.70a1	1.00a	1.00a	1.00a	1.00a
	STARS-9577B	1.00a	1.70a1	1.00a	1.00a	1.00a	1.00a
14	PUMA	2.30f1	3.00a3	2.30f1	2.60c2	2.20e1	2.70d2
	STARS-0502B	2.00d1	2.00d1	1.70a1	2.00d1	1.70a1	1.90c1
	STARS-9301B	1.20b	1.90c1	1.30c	1.60f	1.30c	1.30c
	STARS-9577B	1.30c	2.00d1	1.60f	1.70a1	1.20b	1.30c
21	PUMA	2.60c2	3.00a3	2.80e2	3.00a3	2.40a2	3.00a3
	STARS-0502B	2.30f1	2.402a	2.20e1	2.50b2	2.10e1	2.60c2
	STARS-9301B	1.40d	2.00d1	1.90c1	2.00d1	1.40d	1.80b1
	STARS-9577B	1.50e	2.20e1	1.90c1	2.20e1	1.50e	1.80b1
28	PUMA	2.70d2	3.00a3	2.90f2	3.00a3	2.60c2	3.00a3
	STARS-0502B	2.70d2	2.50b2	2.50b2	2.80e2	2.40a2	2.90f2
	STARS-9301B	1.40d	2.30f1	2.50b2	2.70d2	1.70a1	2.30f1
	STARS-9577B	1.70a1	2.60c2	2.20e1	2.70d2	1.90c1	2.40a2

Values are means of 10 replicates. Values followed by different notations are significantly different following Tukey's *posthoc* test ($p<0.05$).

7.3.5 Effects of [CO_2] on the population growth of RWASA1 and RWASA2

The general observation is that as the number of days of infestation increases, populations of RWASA1 and RWASA2 on each of the four barley lines increase progressively. This seems to be irrespective of the level of [CO_2] (Figs. 7.6 A-D). Populations of the two RWA biotypes on the non-resistant PUMA were significantly larger than the average of their respective populations on the three resistant lines ($p<0.01$) (Table 7.12). Irrespective of the barley line, [CO_2] level and days of infestation, RWASA2 bred faster than RWASA1 (Fig. 7.6).

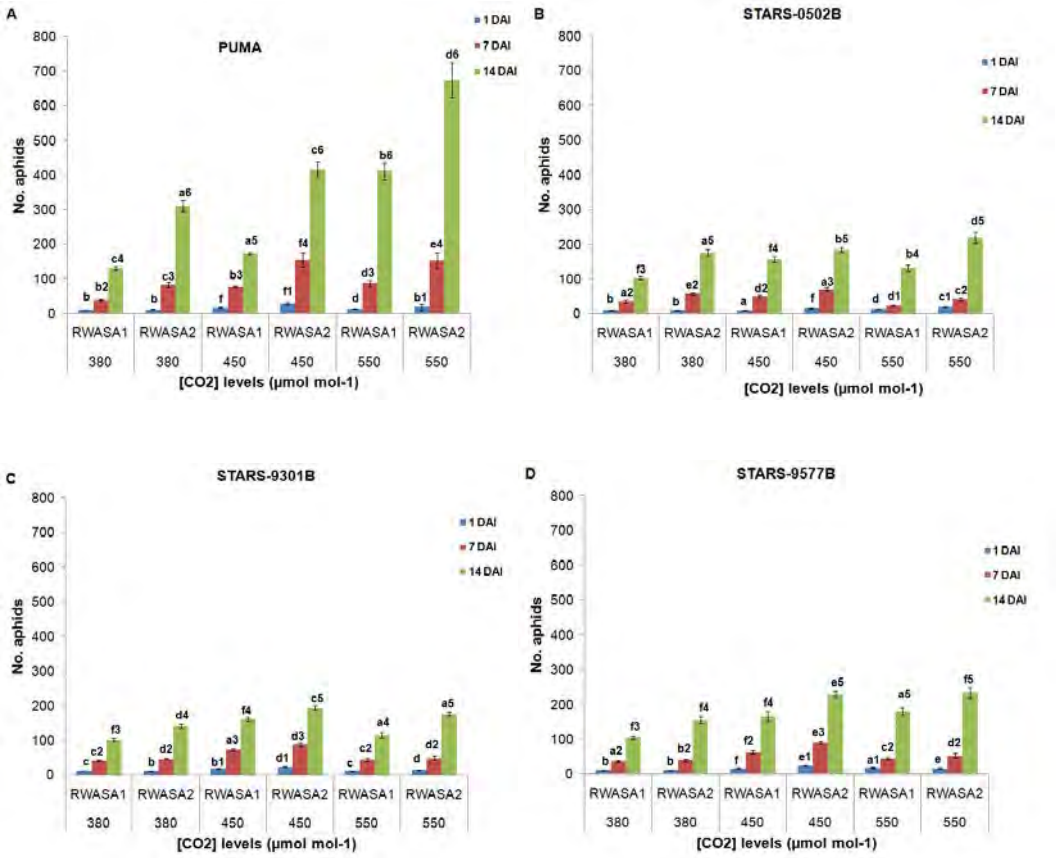

Fig. 7.6 Population growth of RWASA1 and RWASA2 on the four barley lines at 1, 7 and 14 DAI grown under the three levels of [CO2]

Bars with different letters and numbers indicate significantly different homologous groups at 0.05 level using Tukey *posthoc* test (n=10).

Table 7.12 Repeated measures of ANOVA of population growths* of RWASA1 and RWASA2 on the four barley lines exposed to the three levels of [CO2] after 1, 7 and 14d of feeding

Sources of variation	d.f.	F value	p- value
Aphid types	1	287	<0.001
Barley lines	3	137	<0.001
[CO_2] levels	2	188	<0.001
Days after infestation (DAI)	2	5801	<0.001
Aphid types * Barley lines	3	19	<0.001
Aphid types * [CO_2] levels	2	3	0.074
Barley lines * [CO_2] levels	6	24	<0.001
Aphid types * DAI	2	17	<0.001
Barley lines * DAI	6	26	<0.001
[CO_2] levels * DAI	4	23	<0.001
Aphid types * Barley lines * [CO_2] levels	6	3	0.003
Aphid types * Barley lines * DAI	6	2	0.098
Aphid types * [CO_2] levels * DAI	4	6	<0.001
Barley lines * [CO_2] levels * DAI	12	7	<0.001
Aphid types * Barley lines * [CO_2] levels * DAI	12	1	0.454

*Population growth data were not accumulated beyond 14 DAI

Notwithstanding the aphid biotype, barley line or days of infestation, aphid populations at the two elevated [CO_2] levels were significantly larger than their populations at ambient [CO_2] level ($p<0.01$). At ambient [CO_2], populations of the two biotypes were significantly larger on the non-resistant line than the average for the three resistant lines for each day of data collection and clearly, RWASA2 bred faster than RWASA1 in each case. However, a comparison of aphid population at each of the two elevated [CO_2] levels gave variable results between the two biotypes. The trend of their population growth on each of the four barley lines is 14d > 7d > 1d. Among

the resistant lines, STARS-0502B bred less aphids than both STARS-9301B and STARS-9577B across the three [CO_2] levels after 1 and 7 DAI.

7.4 Discussion

7.4.1 Biomass changes under changing [CO_2]

Not surprisingly, all uninfested plants grown at elevated [CO_2] achieved higher biomass (total, above- and below-ground) compared with those grown at ambient [CO_2] (see Figs. 7.1-7.3 and Table 7.1). The data presented here are thus in agreement with previous studies. Newman et al. (1999) noted that plant dry matter of *Festuca arundinacea* was 37% greater under elevated [CO_2] than under ambient level. Awmack and Harrington (2000) showed that shoot and root weight of uninfested bean (*Vicia faba*) plants were greater at elevated [CO_2] than at ambient. Also, Hughes and Bazzaz (2001) reported that elevated [CO_2] increased the biomass of *V. Faba*, *Asclepias syriaca*, *Oenothera biennis*, *Nicotiana sylvestris* and *Solanum dulcamara* when compared with their biomass at ambient [CO_2]. These results conform to expected increase in plant productivity at CO_2 atmospheres higher than ambient level. It is clear from several sources that plant growth is accelerated and improved under elevated CO_2 (Barbehenn et al., 2004; Flynn et al., 2006; Reich et al., 2006). As atmospheric [CO_2] increases, so gaseous CO_2 that is available in the immediate plant environment also increases and that it becomes uniformly distributed, unlike water vapour (Schlesinger, 1997). It follows that the higher concentration of atmospheric CO_2 creates a steeper air-leaf mesophyll gradient, which will facilitate entry of a higher [CO_2] into the

leaf (Rowland-Bamford et al., 1991). This is in favour of photosynthetic carbon reduction over oxygenation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Stitt, 1991; Drake et al., 1997). Thus, the increased net rate in photosynthetic capacity leads to increased carbohydrate synthesis (Rogers and Dahlman, 1993; Conroy et al., 1994; Woodrow, 1994).

7.4.2 Effect of [CO_2] and aphid feeding on plant biomass

The data presented here show that the reduction in biomass of aphid-infested plants was greater on non-resistant PUMA than on the three resistant lines (Table 7.3). It is clear that RWASA2 caused more damage (inferred here as a reduction in biomass) than did RWASA1 (Table 7.3), supporting the study by Awmack and Harrington (2000). These authors reported that *V. faba* infested with the pea aphid *Acyrthosiphon pisum* induced a reduction in shoot fresh weight at both ambient and elevated [CO_2] levels. The five plant-aphid interaction study (Hughes and Bazzaz 2001) also showed that infested plants suffered substantial reduction in total biomass at ambient and elevated [CO_2]. What is clear from this study is that under elevated [CO_2], infestation by the two RWA biotypes had a major effect on the growth of the four barley lines. A significant reduction in total, above-ground and below-ground biomass occurred under elevated [CO_2] (see Figs. 7.1-7.3). These data are consistent with that for chewing herbivorous insects where consumption rates by the insects often increase at elevated [CO_2] (Lincoln et al., 1986; Johnson and Lincoln, 1990; Docherty et al., 1996). However, the effects of aphids are generally more complex and variable (Bezemar and Jones, 1998). The

negative effects of aphid infestation seen in the present study on plant growth were greater than the positive effects induced through elevated photosynthetic rate under elevated [CO_2] (see Table 7.3). This view is supported by the Flynn et al. (2006) who demonstrated that aphid infestation reduced plant biomass under ambient and elevated [CO_2] environments. According to Sun and Ge (2010), aphids spend more time on a leaf, probing and ingesting under elevated CO_2 than they do under normal CO_2 conditions. Among the resistant lines, it was observed that the higher the [CO_2], the greater the reduction in biomass components irrespective of RWA biotype.

It is understood from this study that there is an increase in biomass in all controls where elevated CO_2 is supplied (Table 7.1). When aphids feed on these plants, there is a very significant loss in biomass (Figs. 7.1-7.3, Table 7.3). This points to a positive flux of assimilates to aphids, as they become a large and significant diversionary sink at the expense of the plant. This is because assimilates are diverted to the aphids' guts instead of the needy parts of the plants. The results presented in this chapter are, I believe, one of the first to highlight effects of elevated [CO_2] on plant-aphid interaction, comparing resistant and non-resistant lines of a plant species that were infested by distinct biotypes of the same aphid.

7.4.3 Effect of changing [CO_2] on leaf nitrogen

Results of this study however shows an increase in percentage foliar nitrogen concentrations in uninfested plants at elevated [CO_2] levels, compared to ambient level (Fig. 7.4; Table 7.1). Many studies have reported that carbon

dioxide enrichment induce a decline in leaf nitrogen (Dixon et al., 1993; Cotrufo et al., 1998; Hughes and Bazzaz, 2001; Stiling and Cornelissen, 2007). Most of these studies however, were conducted using either open-topped chambers (Newman et al., 1999; Morgan et al., 2001), or techniques of Free-Air Carbon dioxide Enrichment (FACE) (see Awmack et al., 2004; Taub, 2010). The decrease in nitrogen level is as a result of increased photosynthetic rates, which tend to increase the C:N ratio of leaves due to accumulation of non-structural carbohydrates (Lindroth et al., 1995). However, in the views expressed by Stitt and Krapp (1999), responses of photosynthesis and growth to elevated [CO_2] will depend on availability of other inorganic nutrients and the way in which they are utilised by the plant. Riviere-Rolland et al. (1996) showed that decrease in leaf nitrogen may be expected in plants grown under conditions of limited nitrogen supply, but not when the plants were supplied abundant nitrogen in form of nitrates. When nitrogen supply is adequate (as is the case in this study), there may be no major decrease in the internal concentration of nitrogen or the levels of nitrogen metabolites expected under enriched [CO_2]. After all, increased rates of growth, as a result of increased synthesis of carbohydrates under elevated [CO_2] as recorded in this study, will require higher rates of inorganic nitrogen uptake and its subsequent assimilation into plant tissues (Stitt and Krapp, 1999), which may have contributed to the high leaf nitrogen concentration measured in the uninfested plants. N fertilization may have caused the increase in leaf N concentration obtained in this study. Taub (2010) states that

while elevated [CO_2] makes carbon more available than under ambient, the benefitting plants would also require other resources including minerals obtained from the soil. While it is certain that CO_2 enrichment does not in any way make mineral elements such as N available, it however affects their utilization during plant metabolic processes, depending of course on the conditions to which the plants are subjected. For instance, plants grown with low amounts of N fertilization or when N is entirely limited in supply (such as in field situations) would show a decrease in tissue N concentrations. Whereas, when plants are raised with application of N or higher N fertilization, plant tissue nitrogen may differ (Taub et al., 2008). In the experiments reported in this thesis, Long Ashton nutrient solution (Hewitt, 1966) is applied to experimental plants at 3d intervals (see Chapter 2, section 2.1), which may have increased soil N available to the plants, hence, increased the foliar N concentrations obtained in this study.

7.4.4 The effect of changing [CO_2] and aphid on leaf nitrogen

Leaf nitrogen concentrations of infested plants were lower than those of uninfested plants (Fig. 7.4). Aphids have specific requirements for certain amino acids which may be present in phloem sap (Risebrow and Dixon, 1987). As nitrogen content of the host plant is a crucial factor in insect herbivore diet (Mattson, 1980), CO_2-induced changes in levels of nitrogen content of host plants would have a major impact on herbivore (which in this study are the aphids) feeding (Bezemer and Jones, 1998). The effect of

elevated [CO_2] on phloem amino acid composition may have influenced the performances of these aphids, since the nutritional quality of phloem sap is an important limiting resource for the growth, development and performance of feeding aphid populations (Bezemer and Jones, 1998). However, the phloem sap is reported to be low in protein (Douglas, 1993). This situation may have prompted the aphids to ingest large quantities of the sap, in order to gain enough amino acids which are essential for their survival and excrete the excess water and sugars as 'honeydews' as demonstrated by Saheed et al. (2010). No wonder then, the massive reduction in plant biomass as a result of feeding by the two biotypes noted above. RWASA2 caused greater reduction in leaf nitrogen concentration than RWASA1 at all [CO_2] levels across the four barley lines (Table 7.6). This might be due to its higher breeding capacity than RWASA1 on all the lines than RWASA1 which I established in Chapter 3 (Jimoh et al., 2011). However, this species-specific difference in the response of aphids to foliar nitrogen should be investigated further.

7.4.5 Effect of changing [CO_2] on C:N ratio

C:N ratios recorded in this study were variable. It is only in STARS-9301B that C:N ratio of uninfested plants increased at elevated [CO_2] levels (Fig. 7.5; Table 7.7). Uninfested PUMA and STARS-9577B recorded increases in C:N ratios from ambient to both 450 and 550, but not between the two elevated CO_2 levels (Table 7.7). Studies have shown that increase in the rates of photosynthesis at elevated [CO_2] leads to increase in C:N ratio of plants (Lindroth et al, 1995; Hughes and Bazzaz, 2001; Stiling and Cornelissen,

2007). Though increases in C:N ratio were obtained due to feeding by the two RWA biotypes, it is only in two instances that the percentage increase was striking (Table 7.8). All the same, this depicts that the feeding aphids depend on the barley hosts for some of their essential amino acid nutrition as enunciated in section 7.3.1.3 above. Wilkinson and Douglas (2003) reported that aphid dependence on host plant for amino acid is species-specific. They transform the sourced amino acids into proteins for their nutrition (Sun and Ge, 2010). This would deplete the nitrogen content of the host, which must increase the C:N ratio. High amounts of amino acids were found in cotton aphids which fed on cotton grown under elevated CO_2 conditions while in cotton plants, a higher C:N ratio is recorded (Sun et al., 2009), similar to what is observed in the current study.

7.4.6 Virulence of the aphids on plants under changing [CO2]

Chlorosis and leaf roll are two damage symptoms that have been identified as important visible criteria, which are useful both for evaluating damage caused by RWA (Burd et al., 1993) and for establishing biotypic variation among RWA biotypes in different geographical locations on resistant and non-resistant hosts (Puterka et al., 1992). I employed these two damage criteria in the current study, by adapting the rating scales developed for chlorosis by Webster et al. (1987) and for leaf roll by Burd et al., (1993), as previously used in Chapter 3 (see Table 2.1 in Chapter 2).

This study shows that development of chlorosis is gradual and it worsens with time (Table 7.8). Chlorosis became noticeable on leaves of all barley lines irrespective of aphid biotype or levels of [CO_2] at 7 DAI. This lends support to Deol et al., (2001) who reported that RWA feeding on host plants required 7d of infestation before chlorosis became noticeable, spreading and worsening thereafter. RWASA2 caused more severe chlorosis on PUMA than RWASA1 at all levels of [CO_2] at 21 DAI. By 28 DAI, death of many PUMA plants were recorded (Table 7.10). However, the three resistant lines developed reduced chlorotic symptoms compared with PUMA and RWASA2 was visibly more destructive than RWASA1 at both elevated [CO_2] at 28 DAI (Table 7.10). However, elevated [CO_2] levels alone did not affect development of leaf roll symptoms in any of the four barley lines, whereas it did with interaction with aphid biotype and days of infestation (Table 7.9). Leaf rolling generally commenced on PUMA and STARS-0502B by 7 DAI under all three [CO_2] levels, worsening as days of aphid infestation increased. The faster rate of reproduction of RWASA2 resulted in a higher leaf roll damage rating than RWASA1, in all cases and under all three [CO_2] levels. The results on these damage symptoms are consistent with the report in Chapter 3 (Jimoh et al., 2011).

7.4.7 Effects of changing [CO_2] on population growth of the aphids

Elevated [CO_2] levels affected the population growth of the two biotypes on the four barley lines. Populations of the two biotypes on the non-resistant line

at 1, 7 and 14 DAI under the three [CO_2] (Fig. 7.6A) were larger than their respective populations on each of the three resistant lines (Figs. 7.6C-D). Mondor et al. (2005) reported that elevated [CO_2] affects the population abundance of aphids. Increase in the net photosynthetic capacities of plants, resulted in an increase in biomass under enriched CO_2 conditions and more assimilates were available to the feeding aphids This impacted positively on their breeding capacities, which is higher at elevated CO_2 levels than under ambient CO_2, with the resultant effect of plant death. Results demonstrated here for the two South African biotypes of RWA agree with previous others such as on *Macrosiphum euphorbiae* (Flynn et al., 2006) on *Myzus persicae* (Hughes and Bazzaz, 2001; Bezemer et al., 1998, 1999) as well as on *Aphis rumicis* reported by Whittaker (1999) which showed that aphid populations grew faster at elevated [CO_2] than at ambient level.

Previous studies on effects of elevated [CO_2] on aphid performance have given varying and inconsistent results. In a five aphid-plant interaction study, Hughes and Bazzaz (2001) reported that elevation of [CO_2] negatively affected population growth of *Acyrthosiphum pisum*, positively affected that of *Myzus persicae* and had no significant effect on those of *Aphis nerii*, *A. oenotherae* and *Aulacorthum solani*. Docherty et al. (1997) also reported inconsistencies in the response of aphid performance at elevated [CO_2] levels. However, results of this study showed that CO_2 enrichment substantially increased the populations of RWASA1 and RWASA2 on the four barley lines. It also showed that aphid populations were higher on non-resistant PUMA

than the three resistant lines and that RWASA2 out-performed RWASA1 in each case.

7.5 Effects of elevated [CO_2] and aphid infestation on plant survival: the potential disaster

Climate change has the potential to alter the face of agriculture, which will possibly, may have a yet unknown but potentially colossal global dimension, that could negatively impact on, and seriously affect global food economy and food security. Many view this as the "albatross" of the 21st century. Climate change will affect natural and agricultural ecosystems – causing and or effecting perturbations of yet unknown proportions. Pritchard et al. (2007) identified the three dominant variables of climate change as elevated CO_2 concentration, altered rainfall patterns and potential changes in predicted global mean temperature from 1.4^{o}C to an upper projection of about 5.8^{o}C over current mean temperatures (global warming). Global warming is a reality, but it is yet unclear, as to its impact or the magnitude of its effect on agriculture.

Strategies related to the predicted increase in photosynthesis, resulting directly from increased atmospheric CO_2 content must be balanced by what will happen to the insect pest populations, which feed on crop plants. Given that the main focus of this thesis was the effect of the aphid populations (RWASA1 and RWASA2) on selected, supposedly resistant barley lines, it seemed important to be able to make a first attempt at examining the effect of

relatively small [CO_2] changes, in order to examine the potential impact of climate change on the interaction between crops and their pests.

Several questions drove this aspect of the investigation:-

1. Would relatively small changes in [CO_2] (450 and 500 µmol mol^{-1}) have any influence on the plant-insect relationships that I reported under the ambient as well as the changing CO_2 concentrations (see Chapters 3 and 7)?

2. How much crop enhancement would changing CO_2 concentration elicit?

3. Would changes in CO_2 concentration impact on aphid (RWA in this study) population growth rate (down-regulation or up-regulation), colony size and aphid mortality rate?

4. Would the plants (barley in this study) survive the onslaught effect of a combination of elevated CO_2 and aphid infestation?

Previous studies indicate that an elevated [CO_2] has the capacity to enhance photosynthesis and a *de facto* increase in crop yield should therefore result (Hughes and Bazzaz, 1997; Owensby et al. 1999; Awmack and Harrington, 2000; Donnelly et al., 2001; Ehleringer et al., 2002; Flynn et al., 2006; Reid and Fiscus, 2008). Elevated [CO_2] leads to an increase in carbon available in plant tissues, which results in an increased net rate in photosynthetic capacity of the plant. This is with the consequential effect of alteration to, and increase in the C:N ratio of the plant. This should impact on, and cause a decline in the nitrogen concentration of plant tissues, as a result of the resultant phenomenon

of ‘nitrogen dilution effect’ (Cotrufo et al., 1998). Nitrogen dilution implies a lowered concentration of leaf protein and an eventual reduction in plant quality. This in turn would affect not only on food quality but also on livestock feed and industrial raw materials. Even though elevated [CO_2] is linked to increased plant productivity, Goudriaan and Zadoks (1995) cautioned that this potential yield is a mirage, as it is almost never achieved, in the face of crucial yield limiting factors such as low soil moisture and nutrient availability and on top of this, other yield reducing factors such as pests, pathogens and weeds. Among the itemised yield reducing factors, pests, particularly insect pests, are the focus of this thesis. The insect pest considered here is RWA.

Experiments were set up in which I investigated the effects of changing [CO_2] on the growth characteristics of four barley lines (resistant and non-resistant) infested with the two South African biotypes of RWA. Barley is cultivated as a monoculture in cereal fields, where it has evolved worldwide, and has become an important habitat to insect pests and in this case, the RWA. One of the areas of interest in this thesis was focussed on obtaining baseline data on rates of population growth, virulence and feeding-related damage of the two South African RWA biotypes – RWASA1 and the recently emerged RWASA2, which is more damaging to plants as a whole (see chapters 3, 4, 5 and 6 for details) – on non-resistant and resistant barley lines. In controlled environment experiments under ambient CO_2 conditions, I demonstrated that RWA negatively affects the plants, and is thus a serious threat to small grain

crops productivity. Also under controlled environment experiments (although limited in scope), under changing CO_2 concentrations, I demonstrated that plant productivity decreased over and above the effect of aphid feeding, even under a realistic 70 µmol mol^{-1} change. The aphids bred faster (as there was an increase in N- based nutrients in the phloem system) at elevated [CO_2]. The increasing population of the aphids had a very serious, major knock on effects: the damage to leaf vasculature becomes severe; much of the xylem/phloem transport system becomes dysfunctional; the increased aphid population size increases the local (plant to aphid) sink pathway and negates the normal leaf to plant sink pathway; all to the detriment of the plant. RWASA2 is even more threatening, if RWASA1 infestations do result in yield losses of between 30 to 60% (Du Toit and Walters, 1984).

I have established that infestation by the two RWA biotypes have detrimental effects on some growth parameters of the barley lines examined under changing [CO_2]. In particular, feeding by these biotypes caused a more significant reduction in biomass at elevated CO_2 levels than under ambient level. Under these elevated CO_2 conditions, I believe that RWA might have ingested more phloem sap than at ambient. The increased in photosynthesis thus turns into a huge new bonus for the aphids: higher photosynthetic rate means a larger volume of assimilate passing by and into their stylets, to the advantage of the feeding aphids to the detriment of the plants.

RWA infestation depletes the nitrogen content of the plants. Where nitrogen supply is a limiting factor, such as in the field, CO_2 enrichment causes

reduction in leaf nitrogen concentration (Cotrufo et al., 1998) and would have a major impact on herbivore feeding (Bezemer and Jones, 1998). Under ambient CO_2 levels, phloem sap is low in protein content (Douglas, 1993). Further reduction in nitrogen content due to CO_2 enrichment would induce intensified feeding by the aphids, to take up larger quantities of the sap than would be the case under ambient CO_2 (Sun and Ge, 2010). Therefore, a CO_2-enriched atmosphere, coupled to aggressive aphid infestation will impact negatively on bioproductivity, grain quality and yield; and the net result would be compromised grain food supply as well as compromised food security. Perhaps the single most disturbing factor, is that some cultivars will not survive the twin impact of elevated [CO_2] and increased pest populations (Jimoh et al., 2011; Sacranie and Botha, unpublished), and inability to survive long enough for grain set and maturation to occur, will create an even more serious threat to agricultural productivity.

Is there a solution?

It should be noted that the biotic or abiotic factors which currently limit plant productivity and yield have the potential to become more significant and more important in agroecosystems in a future climate driven by increased temperature and elevated [CO_2]. By then, the positive effects of increased [CO_2] may be of lesser consideration, compared to the potential negative effects of these yield-limiting factors. The plant's capacity to cope with the increased aphid feeding pressure (higher assimilation rate → more phloem sap → more nutrient for the aphid population → higher breeding rates →

increased plant damage) may have a major impact. What is needed is a combination of approaches, which will limit the envisaged loss in plant productivity. Several options exist (see Fuhrer, 2003). The problem is, we do not know what management practice, or a combination thereof that would lessen the impact of the combined effects of the pest (RWA) and the increased CO_2, together with those of other yield limiting factors, such as soil moisture and available nutrients.

Some options are:

1. Irrigation management, in order to adapt to changes in precipitation patterns (Fuhrer, 2003);

2. Land management, in order to preserve soil quality (Williams et al., 2001);

3. Selection of crop varieties for cultivation, in order to shift crops to shorter growing seasons, early-maturing and earlier planting dates (Dale, 1997);

4. Nutrient management such as timing, amount, type and appropriate fertilizer application to cope with the demands of CO_2 enrichment (Fuhrer, 2003);

5. Use of plant cultivars with improved resistance and tolerance to aphid feeding (here, resistance genes against RWA), as well as enhance specific agronomical, morphological and physiological traits, which may mitigate problems of crop survival in a stressful environment (Kobiljski and Dencic, 2001).

6. Some research currently being undertaken shows that there may be merit in using barley and wheat cultivars whose middle eastern origins mean that they

are exposed to a similar RWA genome, like RWASA2 in South Africa (Sacranie and Botha, unpublished);

Whatever strategy is adopted, there is clearly, an urgent need for assessment of known resistant lines (such as those developed by the USDA) against RWA under elevated CO_2 environments. It is my contention that this is a way forward, which should be a core function in a continuous breeding programme for genetic improvement of available and performing crops, to keep in tune with changing CO_2 environments and biotypes of pests such as RWA.

7.6 Conclusions

The results reported here serve to support arguments presented elsewhere that plants grown at elevated [CO_2] levels will attain greater biomass than those grown under ambient [CO_2] level. In the absence of aphids, an enriched CO_2 atmosphere will have beneficial effects on crops (see Newman et al., 1999; Awmack and Harrington, 2000; Hughes and Bazzaz, 2001). In this study, infestation by the two RWA biotypes has been conclusively shown to have a detrimental effect on the growth characteristics of the four barley lines. In the presence of aphids, a significant biomass reduction was recorded at elevated [CO_2] levels than was the case under ambient [CO_2]. Awmack and Harrington (2000), corroborated by Sun and Ge (2010) hypothesized that aphids ingested more phloem sap at elevated [CO_2] than at ambient [CO_2]. Biotype notwithstanding, what the plant benefitted in growth under enriched [CO_2] clearly advantaged the aphids to the detriment of the host plants.

The study showed that the detrimental effects of the two RWA biotypes on plants' growth characteristics measured were greater on the non-resistant PUMA than on any of the three resistant lines, under the experimental conditions. This is a positive outcome for the possibility of using these resistant lines as promising sources of resistance to RWA now and in the future. Though it is evident that RWASA2 caused greater biomass reduction than RWASA1, the fact that the populations of the two biotypes were suppressed on the resistant lines at elevated [CO_2] levels shows that the these lines have the potential for abating the anticipated negative effect of aphid infestation, which this study as well as some previous ones have shown. The current study has provided additional evidence in support of the need for simultaneous measurements of both aphid and plant responses to elevated [CO_2] initiated by Hughes and Bazzaz (2001). Further studies are necessary to provide ultrastructural and physiological explanations on the effects of elevated [CO_2] on infestation of biotypes of aphids on susceptible and resistant plants to compliment what is known at ambient [CO_2] level.

8 Chapter 8: General Discussion and Conclusion

8.1 Preamble

The experiments reported in this thesis were carried out largely to evaluate the responses of three selected resistant barley lines – STARS-0502B, STARS-9301B and STARS-9577B – to feeding by the two currently identified South African biotypes of RWA, RWASA1 and RWASA2. PUMA, which is one of the most important barley cultivars grown commercially in South Africa, served as a non-resistant control. The three resistant plants were developed at USDA-ARS, Stillwater, Oklahoma, USA, and were shown to be resistant to all currently known biotypes of RWA in the USA. There are no commercial barley cultivars that are resistant to the two South African biotypes (RWASA1 and RWASA2). The approach used, was to investigate the structural and functional damage caused by the two RWA biotypes while feeding on the four barley lines as well as the responses of the plant-aphid interaction so set up, in a pilot baseline CO_2 enrichment studies.

Barley is an important small grain crop, second only to wheat as livestock feed and industrial raw material in South Africa. An estimated 270,000 tons is consumed annually out of which about 209,313 tons are locally produced (Kotze, SAB Malting, pers. comm.). RWA is more destructive on barley than wheat (see Webster et al., 1993). Barley is very sensitive to fluctuations in weather patterns, which do affect its performance and productivity. Majority

of South African barley cultivation is in the South Western Cape (SWC), which is presently affected by vagaries of weather. Dryer climate than what obtains presently can predispose barley cultivation in the SWC to RWA infestation (Tolmay, pers. comm.). Thus, RWA is a potential threat to the production of this important industrial mainstay.

At its introduction to South Africa in 1978 (Walters, 1984), little information was available, regarding the control of RWA. Wheat farmers suffered extensive losses. Insecticides registered for other prevalent cereal aphids such as *Schizaphis graminum* were tested but found to be largely ineffective. When the economic loss threshold as a result of RWA damage was established by Du Toit in 1986, large-scale use of systemic insecticides such as dimethoate and demeton-s-methyl as well as vapour action insecticides such as chlorpyriphos and parathion were used (with potential knock on environmental cost), but these still had little success (Smit et al., 2010). A strategy, which incorporated several integrated control measures was adopted and remains in use to this day (Tolmay et al., 2000). It involves the use of resistant cultivars. By 2006, 27 RWA-resistant wheat cultivars have been developed and released to farmers (Tolmay et al., 2007), but none to date for barley, despite its economic importance (highlighted in Chapter 1).

Development of resistant plant cultivars basically involves crosses between identified resistance donors or germplasms and locally adapted and widely grown cultivars (Tolmay and van Deventer, 2005). The use of RWA-resistant wheat cultivars in South Africa was largely successful that in 2001, 70-85%

of area planted to wheat in the Free State as well as in other wheat producing areas was covered by resistant cultivars (Smit et al., 2010). However, this feat was short-lived. In 2005, high population threshold of RWA was discovered in majority of the above-mentioned wheat farms. Trials conducted at ARC-Small Grain Institute in Bethlehem confirmed the emergence of a second, more damaging and faster-breeding RWA biotype designated as RWASA2 (Tolmay et al., 2007). This is the primary reason that informed the use of the three selected resistant lines in this study. One of the underlying aims of this research programme is to identify one or more sources of resistance, against the South African RWA biotypes as must have been done against the US RWA strains, which led to the development of many resistant barley lines yonder. Such may then be incorporated into an anticipated RWA-resistant barley breeding programme.

This study had two main directions. The first was an examination of the structural and functional components relating to aphid feeding. Here, I evaluated the population growths of these biotypes and related these to the damage they caused on the four experimental barley plants. These were further related to structural and functional damage the aphids caused to the transport system of the resistant and non-resistant barley plants. The second research focus was informed by the contemporary issue of climate change that is making the biosphere to experience continuous increases in greenhouse gases, altered rainfall patterns and global warming. All these are making the climate warmer and dryer than normal, which have far-reaching future

implications on plants and their herbivores, with the attendant effect on world food supplies. This second focus area attempts to address the question – how will changing [CO_2] affect aphid population and virulence, and plant survival? To achieve this, experiments were conducted at ambient (380 µmol mol^{-1}) and two higher (450 and 550 µmol mol^{-1}) levels of [CO_2], to investigate the responses of the two RWA biotypes and the four barley lines to these changing [CO_2].

8.2 Population growth rates of the RWA biotypes

Investigations were carried out to determine the relative breeding capacities of RWASA1 and RWASA2 on resistant and non-resistant barley lines, under controlled environment. In Chapter 3, the rates of population growth of these RWA biotypes as well as an additional aphid species, the bird cherry-oat aphid (BCA), which is commonly found on small grains in South Africa, were studied over a 15-day time-course, using two assays. The first involved aphids confined in clip cages, and the second involved a free ranging whole-plant method. BCA was used, as there is evidence that it reproduces faster than RWASA1 (Saheed et al., 2007a). I therefore felt it necessary to compare the new biotype, RWASA2, with RWASA1 and BCA, as data concerning their breeding rates existed from a previous study by Saheed et al. (2007a).

The population study shows that RWASA1, RWASA2 and BCA have different reproductive rates on non-resistant and resistant barley lines. In both clip cage and whole-plant assays, the three aphids showed strong population growth rates on all barley plants (see Figs.3.1 and 3.2). This data agrees with

an earlier report that five US RWA biotypes continued to develop successfully on resistant and non-resistant barley (Puterka et al., 2006). In the present study, RWASA2 outperformed RWASA1, as had been reported by Walton and Botha (2008) for resistant and non-resistant wheat cultivars, but contrasts with findings that emerging virulent US RWA biotypes did not show a difference in their performances on barley as they did on wheat (Puterka et al., 2007). This suggests that the faster breeding capacity of RWASA2 than RWASA1 is unique to South Africa, supporting the notion that RWA biotypes are geographically limited (Puterka et al., 1992; Basky, 2003). This study demonstrated that the breeding rates of the two RWA biotypes were both suppressed and at near-equivalent levels on the three resistant lines, when compared to the non-resistant PUMA population data (see Figs.3.1 and 3.2). This suggests that the resistant plants possessed an antibiosis resistance mechanism against these aphids. This is in agreement with an earlier position by Webster et al. (1987), who reported a modest antibiosis effect of STARS-9301B on the US RWA1. However, the finding is contrary to the suggestion by Puterka et al. (2006) that there was no evidence of an antibiosis effect using STARS-9301B and STARS-9577B to any of the five US RWA biotypes. Several other authors (Puterka et al., 1992, 2006; Webster et al., 1996) also reported that ‘resistance’ to US RWA biotypes by resistant barley lines is due primarily to tolerance of aphid feeding. The antibiosis effects observed on the three resistant lines reported in this study (see also Jimoh et

al., 2011) suggest that there may be fundamental differences in the genetics and physiology of RWA feeding biology between the US and RSA biotypes.

In summary, findings of this study presented evidence that showed the differential effects which each of the four barley lines had on the breeding capacities of the three aphids. The study showed that RWASA2 population growth rate exceeded that of RWASA1 and BCA on both resistant and non-resistant lines. However, the reproductive rates of the two RWA biotypes were significantly reduced on the resistant lines when compared to the non-resistant PUMA.

8.3 Comparative effects of feeding damage caused by RWASA1 and RWASA2 on non-resistant and resistant barley lines

Aphids are known to induce extensive but variable damage, dependent on the exposure to resistant or non-resistant plants (see Saheed et al., 2007a, 2007b; de Wet and Botha, 2008; Walton and Botha, 2008; Jimoh et al., 2011). Visible morphological damage symptoms occur usually on the leaves, whereas structural damage takes place in the underlying tissues and cells of the vascular system, while functional damage is evidenced through reduction in the transport capacity of the phloem. Experiments were conducted to investigate the extent of damage inflicted by RWASA1 and RWASA2 in non-resistant and resistant barley lines to cover these three broad areas.

8.3.1 Virulence of RWASA1 and RWASA2 on barley

The virulence indicators measured in Chapter 3 were leaf rolling and chlorosis, both of which are characteristic feeding damage symptoms of RWA (see discussion in Walters et al., 1980; Hewitt et al., 1984; Webster et al., 1987; Burd et al., 1993). Feeding damage symptoms, determined using the virulence indices, were correlated to the breeding rates of the aphids. The two biotypes inflicted severe leaf roll and chlorosis symptoms on the non-resistant PUMA. These symptoms appeared earlier in response to RWASA2 feeding than RWASA1 feeding (Table 3.2). RWASA2 caused earlier and more severe leaf rolling on the resistant plants than RWASA1. The faster breeding attribute of RWASA2 than RWASA1 might be responsible. Development of leaf roll symptom correlated well with population growth of the aphids. The trend obtained here is similar to the one obtained when the same biotypes were raised separately on resistant and non-resistant wheat cultivars (Walton and Botha, 2008). However, these differential rates of development of leaf roll symptom between the two biotypes were not observed, when they were allowed to move freely on either resistant or non-resistant wheat cultivars (Tolmay et al., 2007). Based on this, the consequences of the co-occurrence of free-ranging RWASA1 and RWASA2 in wheat and barley fields would be serious.

In contrast to the leaf-roll damage symptoms, the development of chlorosis in resistant lines showed poor correlation to aphid breeding rate. Chlorosis symptoms only appeared earlier on the resistant plants after RWASA2

feeding, but more severe during RWASA1 feeding (Table 3.2). A previous study on RWA also demonstrated that chlorosis-based scores do not often associate with aphid population performance (Puterka et al., 2006). The difference in the development of chlorosis by the two aphids indicates a difference in the behavioural responses between the two biotypes, when they feed on the resistant plants. Will et al. (2006) reported that increased salivation is a typical response to an occluded phloem stream. Also, development of chlorosis symptom is reported to be a direct effect of aphid saliva activities in plant tissues (Miles, 1990; Ni and Quisenberry, 1997; Saheed et al., 2007a, 2007b). RWASA1, which caused more severe chlorosis than RWASA2, may have salivated more, in response to sieve element occlusion resulting from the action of the resistance genes in these plants.

In summary, the study demonstrated that the two biotypes caused severe chlorosis and leaf roll symptoms on non-resistant PUMA. It is evidently shown that there is a difference in the development of these damage symptoms when the two biotypes fed on resistant plants. The faster reproductive rate of RWASA2 than RWASA1 might account for the increase in the leaf roll damage it caused compared to RWASA1 feeding damage. The study also showed that RWASA1 caused greater levels of chlorosis on the resistant plants than RWASA2. This may be because of the difference in the feeding responses of the two biotypes to the presence of the resistance gene in the resistant lines, which might have made RWASA1 to salivate more than RWASA2.

8.3.2 Evaluation of structural damage: Examination of wound callose formation and distribution in the phloem

When aphids feed on leaves of host plants, they not only tap essential assimilates needed for plant growth (Miles, 1999), they also damage the vascular tissues, particularly the phloem (Botha and Matsiliza, 2004; Saheed et al., 2007a, 2007b). While feeding from the phloem, a cascade of wound responses are induced by the host plant primarily as a defence mechanism to forestall aphid feeding, so as to prevent or at best reduce assimilate loss from the plant (Sjölund, 1997). This was demonstrated more recently to be by formation and deposition of aphid-induced wound callose in sieve tube lumina and its associated areas (Botha and Matsiliza, 2004; de Wet and Botha, 2007; Saheed et al., 2009). As a recap, in Chapter 3, I reported the different effects and responses of the two RWA biotypes on the four barley lines. Those experiments revealed clearly that the two biotypes performed differently with respect to population changes as well as feeding-related damage symptoms on the test plants. The import of these is that these plants must have responded differently to the aphids' diverse feeding mechanisms alluded to earlier. It therefore becomes necessary to investigate the responses of the plants to the feeding aphids.

A principal focus in this thesis was the structural and functional study of feeding damage caused by RWASA1 and RWASA2. In Chapter 3, I established the baseline for further examination of the reactions of the experimental plants to infestation by the two RWA biotypes. These were in

the forms of their breeding rates as well as consequences of this rapid reproductive rate on the well-being of the plant. Related to the consequences of rapid breeding rate, I investigated wound callose formation, and quantified the distribution of callose in sieve tubes of non-resistant and resistant barley plants caused by probing by RWASA1 and RWASA2 (see Chapter 2 section 2.6.4 for details). My focus here was two-fold. First, I explored the differences between RWASA1 and RWASA2 feeding effect through the formation, deposition and distribution of wound callose. Second, I made an appraisal of the variation in wound callose distribution among the barley lines, especially the STARS lines. To my knowledge, there is no known report on the feeding damage and plant's response via callose formation, deposition and distribution as a result of feeding by different RWA biotypes in barley lines.

I have shown that less than 24h of feeding by either RWASA1 or RWASA2 induced formation of wound callose in all four barley lines (Fig.4.3). This is similar to the observation by Saheed et al. (2009), though in a susceptible barley cultivar (Clipper) infested by RWASA1. The result obtained, however, conforms to earlier position that wound callose formation is a rapid response in wounded cells (Radford et al., 1998) as well as in aphid-infested plants (Nakashima et al., 2003). Rapid response to injury must serve to quickly and effectively seal punctured sieve elements of the phloem (Sjölund, 1997). In this study, wounding became more obvious from 1d through 14d. Botha and Matsiliza, (2004) showed that wound callose persisted even after removal of aphid. When aphid infestation was sustained through to 14d after initial

infestation, the wound callose formed over time accumulated. Aphid-induced callose, unlike mechanically induced wound callose (Currier and Webster, 1964), does not disappear (Saheed et al., 2009). The response became more extensive after 3d of infestation and more evident in the non-resistant PUMA than in the resistant lines. Among the resistant lines, the response to RWASA2 feeding, viewed through wound callose formed, was slightly greater than RWASA1 feeding. Largely, there was no variation in wound callose formed among the three resistant lines due to either RWASA1 or RWASA2 during short-term feeding exposures ($p<0.05$) (Fig.3.9A).

During long-term feeding exposures however, the response of the barley lines, expressed as callose formation, became more extensive than observed during short-term feeding. In non-resistant PUMA, RWASA2 feeding caused more damage, thus more extensive callose deposition than RWASA1 (see Figs.3.5A, B; 3.6A, B). There was variation in the amount and intensity of wound callose formed among the three resistant plants, when subjected to sustained feeding by the two RWA biotypes ($p = 0.184, > 0.001$) (see Fig.3.9B). Of note was the fact that STARS-9301B generally contained less callose than the other two. Number of days of exposure of the four lines to feeding by the two biotypes also affected distribution of wound callose ($p = 0.958, > 0.001$). Wound callose deposition increases as days of aphid infestation advances in each of the four barley plants (Fig.3.9B). This study showed that aphid-induced wound callose occurred in both resistant and non-resistant lines. This is in sharp contrast to resistant wheat cultivars – Betta-*Dn*

and Tugela-*Dn* – both bearing resistance genes against RWA, in which suppression of wound callose formation by *Sitobion yakini* (De Wet and Botha, 2007) and RWASA1 and RWASA2 (Walton and Botha, 2008) occurred.

The present study supports the suggestion that the plant response to feeding is aphid species-specific (Gill and Metcalfe, 1977; Saheed et al., 2009). Equally possible is that the two aphid biotypes (RWASA1 & RWASA2) produced different signals that induced diverse responses in the resistant barley lines when compared with the resistant wheat hosts. Another tempting suggestion could be that the South African biotypes of RWA are resistance breaking on the USDA barley lines (which are resistant to the US RWA biotypes). It is also possible that the two aphids possess different salivary components (O. Edwards, pers comm.).

The relative reduction in wound callose deposited in the resistant plants when compared to the non-resistant PUMA, suggests that the operative mechanism of resistance may involve the ability of the resistant lines to inhibit or prevent an excessive formation of callose, or that they upregulate β-1, 3 glucanases, which may be involved in callose degradation. The response to RWASA2 is the formation of more callose, which suggests that it is a resistance breaking biotype, as well as being a more aggressive feeder than RWASA1. Thus, aphid virulence can as well be linked to the ability of the aphid to effect callose formation or its breakdown in host plant tissues, regardless of whether the plants are susceptible or resistant. Synthesis of callose (a β-1, 3-glucan), is

carried out by callose synthase complexes (Verma and Hong 2001). It has also been reported that elevated β-1, 3-glucan synthase activity can result in increased callose levels (Ostergaard et al., 2002). As mentioned, callose degradation is controlled by β-1, 3-glucanases (Van der Westhuizen et al., 1998; Moran and Thompson, 2001; Will and van Bell, 2006). Interestingly, elevated β-1, 3-glucanase levels have been reported in resistant wheat cultivars (Forslund et al., 2000; Van der Westhuizen et al., 2002).

I would argue that as the RWA biotypes and the barley lines interact during infestation, the aphids have variable capacity to up-regulate the synthesis of β-1, 3-glucanase. I would thus speculate that RWASA2 feeding does not support up-regulation of β-1, 3-glucanase formation, as may be the case with RWASA1 feeding. Alternatively, the synthesis of β-1, 3-glucanase may be down-regulated during RWASA2 feeding, resulting in an increase in callose formation, suggesting that RWASA2 is simply a more virulent and an aggressive feeder than RWASA1. The study also showed that deposition of wound callose did not correlate well with population of the infesting aphid, be it RWASA1 or RWASA2, in either non-resistant or resistant lines. Given that wound callose is formed in sieve elements within minutes of wounding by aphids' stylets, and that elevated Ca^{2+} stimulates wound callose formation (Botha and Cross, 2001; Will and Van Bell, 2006), feeding damage by even a small aphid population and resultant Ca^{2+} leakage from punctured vascular parenchyma could trigger callose synthesis and blockage of the phloem.

Thus, the experimental evidence in Chapter 4, shows that

1. The response of the plant, through wound callose formation, occurred in both resistant and non-resistant barley plants; and that the amount and intensity of wound callose increased with infestation time.

2. RWASA2 feeding induced greater wound callose than was the case with RWASA1.

3. The difference in the wound callose formed by the two biotypes could be due to yet ill-defined and poorly understood variation in the interactions between RWASA1 and RWASA2 salivary components in the control of the up-regulation of callose synthase in the plant, or by suppression of β-1, 3-glucanase activity.

4. There is no clear relationship between the number of aphids in a population (aphid density) and deposition of wound callose.

I have therefore demonstrated through the experiments reported in this section, that the four barley lines responded to infestation by the two RWA biotypes by forming wound callose in sieve elements as a reaction to wounding of vascular tissues. In addition, I have shown that STARS-9301B, which produced less callose, could be an important link in the development of a local RWA-resistant barley line.

8.3.3 Ultrastructural damage to vascular tissues

The results discussed to this point, clearly demonstrate that the relationship between population growth rates of the two biotypes and their respective virulence (Chapter 3) are reflected in the results observed in the deposition and distribution of wound callose (Chapter 4). As a follow up to the callose

study reported in Chapter 4, I examined the relationship between the breeding rates of the two biotypes and the feeding-related cell damage they caused to resistant and non-resistant barley lines after a sustained 10d feeding exposure. Why 10d? Results of the feeding response via wound callose deposition showed that during sustained feeding, high amounts of wound callose was deposited in both resistant and non-resistant lines (Chapter 4). This indicates that formation of wound callose may no longer be a good diagnostic criterion to distinguish between resistant and non-resistant lines after bouts of continuous RWA feeding on them during sustained feeding exposure. I therefore resorted to the novel tool of leaf ultrastructure to evaluate presumed diversity in feeding-related cell damage among the four barley lines.

The two aphid biotypes probed and fed in sieve tubes of the phloem in non-resistant PUMA. As expected, the aphids fed extensively from thin-walled sieve tubes (Fig.7.5), which has been reported elsewhere (Matsiliza and Botha, 2002; Saheed et al., 2007a, 2007b). Ultimately, both RWASA1 and RWASA2 caused such extensive cell damage in this susceptible plant that it was not possible to differentiate between the feeding effects due to each biotype at the light or fluorescence microscope level.

Among the STARS plants, the vascular cell damage caused by the two biotypes appeared to be substantially reduced (Figs.7.6 and 7.7), when compared to what was observed on the susceptible PUMA (Fig.7.5). Some vascular cell disruption was evident but majority of the cells appeared relatively unaffected. Plasmolysis of cells was evident, which suggested that

functional disruption occurred. It appeared that xylem damage was not as extensive among the resistant lines compared to non-resistant PUMA. The results suggested that xylem damage was particularly more reduced in STARS-9301B (Figs. 7.6C and 7.7D) than in the other two resistant lines. RWASA2 caused more feeding-related cell damage than RWASA1 feeding. RWASA2 probing, which resulted in the xylem damage (Figs. 7.7A and B) and obliteration of sieve tubes and associated parenchyma (Figs. 7.7B and D) suggested a more severe damage than under RWASA1 feeding (Figs. 7.6B, D and E). Interestingly, this relatively reduced level of cellular damage on the resistant plants when compared to that of non-resistant PUMA was not reflected in the extent of wound callose deposition experiment simultaneously conducted at 10 DAI. There were no obvious differences in callose distribution in sieve elements between the PUMA (Fig. 7.2 C and D) and the resistant plants (Fig. 7.3), or as regards RWASA1 and RWASA2 feeding. The result here is similar to my report on callose distribution during sustained feeding in Chapter 4. In that experiment, wound callose distribution was recorded on both infested resistant and non-resistant lines and as a consequence of feeding by the two biotypes.

Results of the ultrastructural studies reported in this thesis generally support previous reports of severe damage and salivary materials lining the secondary walls of the vascular tissue (see Saheed et al., 2007b). What decided the level of damage, especially to the xylem vessels, is the presence of saliva material, impregnating half-bordered pit pairs between the vessels and adjacent xylem

parenchyma (Figs.5.5C, E; 5.6B, E), which has been reported to interfere with or preclude solute exchange at this interface, which may, as a result, be rendered non-functional. Leaf stress and leaf roll would follow (Saheed et al., 2007a, 2007b).

Based on the relationship between population growth of the infesting aphids and the corresponding feeding-related cell damage suffered by the plants, findings from this study suggest that the resistant lines exhibited both antibiosis and tolerance resistance to RWA feeding. I reported in Chapter 3 (Jimoh et al., 2011) that aphid population growth was significantly influenced by two factors: plant resistance and aphid biotype. Results of the ultrastructural study presented here showed that feeding-related cell damage was also affected by both factors: vascular damage was more extensive in non-resistant PUMA than in the resistant lines; and RWASA2 caused more extensive cell damage among the resistant lines than RWASA1. I have demonstrated that RWASA2 population levels were higher than those of RWASA1 on all barley lines (Table 5.1) and that the presence of resistance in the three resistant lines suppressed RWASA2 populations to a level similar to those of RWASA1 on the resistant lines (Fig.5.1; Jimoh et al., 2011). Here in this study, cell damage caused by RWASA2 on the resistant lines was far lower than that caused by RWASA1 on the non-resistant PUMA. Viewed side by side, outcome of Chapter 5 seems to support conclusion from Chapter 3 that these resistant lines have antibiotic effect on the RWA biotypes. Alternatively, it can be argued that the resistant plants appeared to be tolerant

to RWA feeding. This is based on experimental evidence that the level of suppression of feeding-related cell damage on resistant plants exceeds what one would expect based on the high breeding rates of the two biotypes on them. I therefore suggest that there are both antibiosis and tolerance effects of resistance in the STARS lines. Previous evaluation of the resistance in these lines against US RWA biotypes had suggested tolerance effect (Mornhinweg et al., 2006a; Puterka et al., 2006).

In conclusion, the results of the experiments reported in Chapter 5 provide evidence which showed that the breeding rates of each biotype is related to the feeding-related damage it caused in the vascular tissues of non-resistant and resistant lines. There was no effect of aphid biotype in the vascular damage of the non-resistant line. In the resistant plants, the two factors of aphid biotype and plant resistance significantly influenced the reduction in cell damage observed in them compared to the non-resistant PUMA. Also, I reported that RWASA2 caused more extensive cell damage in the resistant lines than RWASA1. These results suggest the presence of both antibiosis and tolerance effects in the three resistant lines.

8.3.4 Overall effects of RWA biotypes' feeding damage on phloem transport functionality

The phloem is the primary feeding site of aphids when they infest their host plants and the assimilate stream becomes diverted to the aphids themselves. In Chapter 6, I reported experiments in which I investigated the effects of the feeding activities of RWASA1 and RWASA2 on the transport capacity of the

phloem in the four barley lines. The disruptive action of the feeding aphids was confirmed by exposure to the phloem-mobile dye, 5,6-CF (applied experimentally as 5,6-CFDA). The distance travelled by this fluorophore per unit time in the phloem was used as a visual indicator of rates at which assimilates move in control as well as infested attached leaves.

The results demonstrated that when the four barley lines were infested with the aphids, the capacity for transport (of the 5,6-CF) as well as the distance transported was reduced, when compared to the rate recorded in uninfested control plants (see Fig.7.1A-D). This confirms earlier studies on wheat and barley by Botha and Matsiliza (2004) and Saheed et al. (2010) respectively. Feeding by either RWASA1 or RWASA2 had an adverse effect on phloem translocation (see Figs.7.2 and Figs.7.3). However, RWASA2 feeding had a greater impact than did RWASA1. Here, the feeding and probing activities adversely affected phloem transport, redirecting assimilates away from the plant into the gut of the feeding aphids (Nielson et al., 1990; Watanabe and Kitagawa, 2000; Saheed et al., 2010). The results also demonstrated that phloem transport is more reduced in infested non-resistant than in infested resistant plants, especially as from longer feeding exposures of 3d through 14d. Thus, as the number of days of exposure to the RWA biotypes increases, so a progressive degeneration of the vascular tissue takes place. For example, by 14 DAI, there was complete cessation of phloem transport (Fig.7.2H) in non-resistant PUMA.

When the results in the transport experiments are considered together with those of wound callose deposition (Chapter 4) and ultrastructural damage (Chapter 5), it becomes clear that the structural damage correlated well with aphid feeding and together, they provide good evidence of the negative impact that feeding has on phloem transport capacity.

Several points arising from all the results and the short discussion above are:

1. That aphid feeding for as little as 24h resulted in differences in the feeding-related damage caused to the transport through the phloem in the vascular systems of leaves of resistant and non-resistant lines, which was confirmed using 5,6-CF.

2. Among the resistant plants, STARS-9301B is the least affected and was observed to be more vigorous than the remaining two lines against the aphids under the experimental conditions.

3. The faster breeding RWASA2, being a more aggressive feeder, caused greater reduction in phloem transport than RWASA1.

4. That by 14 DAI, phloem transport in probed vascular bundles ceases.

8.4 Changing CO_2 concentrations and the effect on plant-aphid interactions

The continuing increase in the level of atmospheric CO_2 concentration has generated a lot of research as well as concern about the potential responses of the plant communities particularly, plant-herbivore interactions. Carbon dioxide is the primary determinant of the photosynthetic rate in plants.

Elevation in its concentration is usually accompanied by an increase in the relative carbon composition of plants (see Farrar and Williams, 1991). This may cause an imbalance in the plant's C:N ratio because as the C component becomes higher, a reduced N components results. The decrease in the nitrogen content of plants has implications for plant quality (Cotrufo et al., 1998). The change in plant quality will and must have considerable effect on the feeding patterns of herbivores such as RWA.

It is well known that aphids depend on phloem sap for their supply of amino acids essential for their survival (Douglas, 1993). As phloem sap is low in protein, aphids have to ingest large amount of phloem sap, and thus, have to excrete excess sugar and water as 'honeydew'. The hypothesis is that CO_2 enrichment would further reduce the protein content of the plant sap and aphids would have to ingest even a larger amount when feeding under elevated [CO_2] than they would under ambient [CO_2]. It is logical that this CO_2-induced feeding behaviour by the aphids, would have serious effects and that it will affect the plant's growth capacity adversely. In addition, increased food supply must have a positive effect on aphid population growth, which will, in turn, impact negatively on yield. Their pest status will then become even more serious.

In Chapter 7, the effects that changing CO_2 concentration had on the barley lines infested with the two RWA biotypes, was investigated. The results clearly demonstrated that all uninfested barley lines grown under elevated CO_2 achieved higher biomass than those grown at ambient level (Figs.7.1-

7.3). This is in agreement with several previous reports (see Newman et al., 1999; Awmack and Harrington, 2000; Hughes and Bazzaz, 2001; Flynn et al, 2006). In sharp contrast to the control plants, infestation resulted in a significant reduction in biomass, under ambient as well as the two elevated CO_2 levels, though reduction was greater at the two elevated CO_2 levels. As expected, the reduction in biomass of aphid-infested non-resistant PUMA was greater than was recorded for the three resistant lines. This is consistent with the results in Chapters 3, 4 and 5, where I showed that these aphids inflicted more significant damage on PUMA, than on the resistant cultivars. The results also indicated that the aphids caused greater feeding damage as a result of lengthier feeding under elevated CO_2 than under ambient level (Sun and Ge, 2010).

In contrast to the report by Cotrufo et al. (1998) that elevated CO_2 causes reduction in leaf nitrogen concentration, my experiments showed an increase in percentage foliar nitrogen concentrations in uninfested plants grown under elevated [CO_2] (compare data in Fig.7.4; Table 7.1). Possibly, more nitrogen was taken up under high CO_2 conditions, to accommodate the faster growth rates within control plants. Plants were well-supplied with Long Ashton nutrient solution (Hewitt, 1966), which contains about 6% N in solution, at intervals of three days. Most of the studies that show a decline in foliar N, were experiments conducted in N-limited environments (Riviere-Rolland et al., 1996; Newman et al., 1999; Morgan et al., 2001 Awmack et al., 2004; Taub, 2010). When aphids were feeding, the leaf nitrogen concentrations were

significantly reduced (Fig.7.4, Table 7.6). Aphids obtain their amino acids requirements from the phloem sap of their hosts (Risebrow and Dixon, 1987), thus N content of the host plant must be a crucial factor in insect herbivore diet (Mattson, 1980). CO_2-induced changes in levels of nitrogen content of host plants will have a major impact on the herbivore feeding (Bezemer and Jones, 1998). Feeding by RWASA2 caused greater N reduction than did RWASA1 at all [CO_2] levels and across the four barley lines (see Table 7.6). Faster breeding rates of the aphids (Deol et al., 2001) would expose the plants to a new stress – N deficiency – therefore raising the C:N ratios of infested plants, compared to the uninfested plants, which supports Sun et al.'s (2009) findings.

An imbalance in C:N exacerbated chlorosis, and leaf roll followed, due to the higher aphid population density under elevated [CO_2] (see Tables 7.10 and 7.11 of this thesis and Deol et al., 2001, respectively). Results of this study on population growth rates of the aphids showed that their breeding rates were faster at the elevated CO_2 levels than at ambient. This lends support to the report by Mondor et al. (2005) that CO_2 enrichment enhances abundant growth of aphids. Increase in photosynthetic capacities of the plants at elevated CO_2, which increased their carbohydrate content, has been shown to have a positive impact on the breeding capacities of the aphids (see Bezemer et al., 1998, 1999; Whittaker, 1999; Hughes and Bazzaz, 2001; Flynn et al., 2006).

In conclusion, changing [CO_2] resulted in

1. All uninfested plants had higher leaf nitrogen concentration at elevated CO_2 than at ambient level.

2. Acclimation of the four barley lines to [CO_2] change led to their gain in biomass above those recorded on them at ambient CO_2 level.

3. Infestation by the two aphids resulted in a significant reduction in the biomass of all lines at the three CO_2 levels.

4. Reduction in biomass of infested plants was greater at elevated CO_2 levels than at ambient [CO_2] (this shows that the aphids must have fed more aggressively under elevated CO_2 levels).

5. The reduction in biomass of infested plants was significantly greater in the non-resistant plants than in the resistant plants.

6. RWASA2 caused greater biomass reduction than RWASA1 as a result of feeding.

Elevated [CO_2] was shown to result in increased biomass. Under the relatively high N supply made available to the plants, made up of about 6% in the Long Ashton nutrient solution (Hewitt, 1966), the amount of N (free and bound) within the plant material actually increased. This suggests that N might have been oversupplied to the plants. However, the significant biomass increases point to favourable plant growth conditions. No doubt, increased

photosynthetic rate resulted in more carbon partitioning to rapid growth. However, introduction of either RWASA1 or RWASA2, results in a loss of the ameliorative effect of CO_2 enrichment. High population numbers and rapid breeding led to increased damage, early appearance of chlorosis and leaf roll, and earlier plant death recorded at 28 DAI. Indeed this situation alerts us to a potential 'double tragedy' with a potential unsustainable crop losses and declining food security, going into the future.

As a general observation, the resistant lines showed some indication of the startling results, compared with what was obtained with non-resistant PUMA. Here, I point to the urgent necessity to fast track a breeding programme, perhaps testing new lines, known to be resistant to the US RWA biotypes, here in South Africa. Controlled environment studies – although limited and often criticised – present perhaps the only chance to fast track a RWA resistance breeding programme while there is still time.

8.5 Reflections on the experimental conditions

All experiments reported in this thesis were conducted under a controlled environment, using plant growth cabinets, which were maintained at 380 (ambient), 450 and 550 µmol mol^{-1} CO_2 levels as may be appropriate for each experiment. Details of the growth conditions in the cabinets are provided in section 2.3 of Chapter 2. The use of the growth cabinets made it possible for me to eliminate all extraneous variables that would have been difficult to control either in the greenhouse or in the field. There is the obvious sentiments expressed by some researchers on studies carried out under

controlled environment conditions, that the studies do not represent the 'real' or field environment of the experimental insects (aphids in this instance) and plants. While I agree that this is true, there can be little argument with the premise that being able to model parameters such as temperature, humidity, daylight (length and intensity) and CO_2 levels in growth cabinets provide all necessary wherewithal for obtaining reliable baseline results, on which we can proceed with the more traditional (and indeed essential) field trials. Furthermore, there are some peculiar limitations to carrying out these studies in open field or even in the greenhouse. I' am under a licence agreement to guard against release of the aphids to the larger environment; and the release of seeds of the barley lines for commercial use without prior approval of the ARC-Small Grain Institute, Bethlehem, South Africa and the USDA-ARS, Stillwater, Oklahoma, USA. Limitation of space in greenhouses may lead to transfer of these objectionable aphids onto other plants, which might disperse them into the outer environment unnoticed. However, I wish to add here that a shortcoming of growth cabinets though is the chamber effect. Growth chambers affect the metabolism of plants (Flynn et al., 2006). The environment of growing conditions in growth chambers only mimic, but do not perfectly match natural conditions (Morgan et al., 2001; Flynn et al., 2006). Flynn et al. (2006) objected to light levels in growth chambers that more often are relatively lower than what obtains under natural conditions. Morgan et al. (2001) contested that chambers, however manipulated, do

provide warmer temperatures other than what they were set, to simulate that of natural conditions.

8.6 Overall conclusion

The results presented in this thesis reflect the two areas of focus I addressed in my study. First, it establishes the threat, which the feeding damage, caused by RWASA1 and RWASA2 poses to the barley industry in South Africa. Second, it catalogues the twin detrimental effects of aphid infestation and changing CO_2 concentrations on the barley test plants that is feasible under climate change. The results of various investigations evidently showed that the two biotypes displayed distinctive features in their reproductive rates, feeding behaviours and feeding damage they caused on the non-resistant as well as resistant barley lines. The responses of the three selected USDA resistant lines to infestation by the two biotypes indicate that there is disparity in these biotypes and their US counterparts against which these lines were developed. Furthermore, the study on changing [CO_2] indicates the gloomy prospects of the quality and quantity of barley, when there is the interactive effect of aphid infestation and climate change in the future.

The results of the population study show that the two RWA biotypes increased with time on all barley lines. However, RWASA2 bred faster than RWASA1 on resistant and non-resistant barley lines. It is evidently shown that the reproductive rates of the two biotypes were significantly reduced on the resistant lines when compared to the non-resistant lines. I surmised that the resistant lines possess an antibiosis resistance mechanism. The two RWA

biotypes exhibited different performances concerning chlorosis and leaf roll damage symptoms on both non-resistant and resistant barley lines. It is evidently shown however, that RWASA2, which recorded both earlier and faster development of leaf rolling as well as earlier onset of chlorosis, is more virulent because of its higher reproductive rates on all barley lines than RWASA1. The fact that RWASA1 caused greater levels of chlorosis on the resistant lines than RWASA2 indicates differences in their respective feeding behaviour and/or biochemistry of the saliva of the two biotypes. These developments may have caused the variations in the structural and functional damage to vascular tissues, which are the main feeding sites of the aphids.

I have shown that both resistant and non-resistant barley lines respond to feeding by the two RWA biotypes through formation of wound callose in sieve elements. This suggests that the two biotypes are resistance-breaking on the USDA-developed resistant lines, which were expected to prevent feeding-related damage to the vascular tissues. During short-term feeding of 24 to 72h, the amount of wound callose is higher in non-resistant PUMA than on any of the three resistant lines. However, during sustained feeding exposure of 7d through to 14d, high amounts of wound callose was deposited in the plants, though higher in non-resistant than resistant lines, and in RWASA2-infested plants than in RWASA1 infested plants. This shows that amount and intensity of wound callose increased with duration of feeding exposure. It also demonstrates that aphid-induced wound callose is not degraded or removed with time, but get accumulated to exacerbate reduction of phloem capacity to

transport assimilates. I also reported that RWASA2 feeding induced greater wound callose than RWASA1. This evidently shows that RWASA2 is a more aggressive feeder than RWASA1. Lesser amounts of callose in the resistant lines (least with STARS-9301B) than non-resistant control shows that the resistant lines may have used a resistance mechanism to prevent excessive formation of wound callose, which the non-resistant plant is not capable of doing. This sustained my earlier suspicion of the presence of antibiosis resistance in the three resistant lines. I also reported that the breeding rate of each biotype is related to the feeding-related damage it caused in the vascular tissues of non-resistant and resistant lines. I observed that there was no effect of aphid biotype in the vascular damage of the non-resistant line. In the resistant lines, the two factors of aphid biotype and plant resistance significantly influenced the reduction in cell damage observed in them compared to the non-resistant line. Because the evidence obtained from the feeding damage is not commensurate with the population growth rates of the two biotypes, I suggest that the three resistant lines are also tolerant to the feeding aphids. This led to the conclusion that both antibiosis and tolerance effects are present in the three resistant lines. I demonstrated that aphid infestation causes damage to vascular tissues of host plants and therefore reduces the capacity of the phloem to transport assimilates. Results indicate that non-resistant plants suffered greater reduction in phloem transport capacity than the resistant lines. I concluded that the faster rates of reproduction of RWASA2, which was responsible for its more extensive

feeding-related cell damage, must have caused the greater reduction in phloem transport than RWASA1 in the barley lines.

When subjected to changing [CO_2], the four barley lines acclimated to this change – substantial increases in biomass and leaf nitrogen concentrations above those recorded at ambient CO_2 level were achieved at the two elevated CO_2 levels. However, when aphids fed on the plants, there was a very significant loss of both biomass and leaf nitrogen concentration at the three CO_2 levels. I showed that reduction in biomass was greater at the elevated CO_2 levels than at ambient level. This indicates that the aphids fed better under elevated CO_2. This is reflected in the increase in the population of the aphids at elevated CO_2, when compared to ambient level. Aggressive feeding RWASA2 recorded the highest population growth, caused greater biomass and leaf nitrogen loss, impacted negatively on plant well-being and resulted in more severe damage symptoms than RWASA1.

Results presented in this thesis show that in the absence of aphids, an enriched CO_2 atmosphere will have beneficial effects on crops. However, infestation by the two RWA biotypes has a detrimental effect on the growth characteristics of the four barley lines. Since aphids depend on their host plants for their nutrition, the aphids, which are more populous at elevated [CO_2], must have ingested more phloem sap than at ambient [CO_2]. This shows that whatever the plant may have benefitted in these growth parameters under enriched [CO_2] clearly advantaged the infesting aphids to the detriment of the host plants.

The outcome of various studies reported in this thesis must have provided useful insight into the effects of the two RWA biotypes and their interaction under changing CO_2 concentrations on barley. It is my fervent belief that the suggestions and conclusions raised in each chapter would be useful to researchers in the field of plant-aphid interactions, breeders and the South African barley industry on what to expect due to raising of the biotypes to a destructive status of pests of barley and under human-created problem of climate change. I' am of the hope that more research efforts would be directed towards addressing the issues raised in this thesis to abate the threats that both the aphids and climate change factors pose to world food security.

8.7 Proposals for future research

Two contemporary issues led to conceptualisation of the studies reported in this thesis. These are the emergence of the second RWA biotype, RWASA2, in South Africa (Tolmay et al., 2007) and the global phenomenon of climate change. A combination of well-entrenched techniques, involving a strong structural and functional approach was used to gain a clearer understanding of the dimensions of the damage caused by the two RWA biotypes on a crop, barley, to which no RWA-resistant cultivars have been developed in South Africa. In the present study, using the structural and functional techniques, I established clear-cut baseline information on the three selected US-developed RWA-resistant lines. Results therefrom demonstrated their relative potentials as sources of resistance to the RWA biotypes. These should be explored further at the breeding levels towards developing resistant barley lines against these South African strains of RWA. Further studies should be carried out to investigate the effect of the damage caused by the two RWA biotypes on the photosynthetic capacity of barley or wheat. Recently, a wheat cultivar, PAN 3144, was developed and reported to be resistant to RWASA2 (Smit et al., 2010). It may be necessary to extend the structural and functional studies to this cultivar, to fill the existing gap on the feeding damage caused by RWASA1 and RWASA2, as had been done on other resistant wheat such as Betta-*Dn* (De Wet and Botha, 2007; Saheed et al., 2007b) and Tugela-*Dn* (Walton and Botha, 2008).

Based upon the data presented here, urgent attention should be focussed on elevated CO_2; and elevated temperature studies are critical, if we are going to get a better understanding of the potential severity of the effects, which relatively mild atmospheric [CO_2] increases will have on food security. In addition, it is essential that further studies be carried out to assess the structural and functional damage caused by these biotypes on host plants under elevated CO_2 levels, to compliment what is already known at ambient level. This thesis has presented a comparative study of the breeding capacity, feeding activity and extent of feeding-related damage caused by RWASA1 and RWASA2 on barley under changing [CO_2]. Further detailed studies are needed to investigate the biological differences between the two biotypes, such as the salivary biochemistry of the aphids under the various environments. The feeding behaviour of RWASA2 should be studied using the electrical penetration graph (EPG) technique, to add to that for RWASA1 carried out by Tolmay et al. (2008). Another essential aspect that must be investigated is the effect of the co-occurrence of the two biotypes on growth and yield parameters of small grain crops, as in reality, the two biotypes co-exist in the field of small grain crops.

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