An investigation on the effect of Russian wheat aphid (*Diuraphis noxia* Kurdjumov) population growth and feeding damage on selected barley (*Hordeum vulgare* L.) cultivars under ambient and elevated CO_2

Thesis submitted in fulfilment of the requirements for the degree of
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By

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Abstract

The Russian wheat aphid (RWA) (*Diuraphis noxia* Kurdjumov) is a major pest of cultivated small grains. It is particularly devastating because of is high reproductive rate which results in the growth of large populations which become damaging to its host plants. Development of resistant barley (*Hordeum vulgare* L.) cultivars is complicated as resistance is polygenic. As a result, the industry remains at risk now that the RWA has spread throughout South Africa. It has, as recently as, 2013, been identified in the SW Cape, which was previously geographically isolated. This is South Africa's principle barley growing region. Now a potentially huge problem exists. Therefore, it is imperative that an alternative to pesticide use is found. Testing potential innate resistance in barley cultivars is thus, critical.

In this thesis, I present data on four barely cultivars where I have examined their resistance/ lack of resistance to three known RWA biotypes, RWASA1, RWASA2 and RWASA3. The barley varieties used were two economically important South African malt barley cultivars (S5 and SSG 564) along with two potentially RWA resistant Afghan accessions (CIho 4125 and CIho 4159). The RWA biotype population growth rates on each of the plants were determined over a 14 day period. The aim was to establish baseline data of the effects of RWA population growth on the host plants under ambient CO_2 (380 – 400 ppm) conditions. The extent of RWA feeding damage was investigated at the cell level by examining saliva deposition and cell disruption using Transmission Electron Microscopy; at the tissue/vascular level using fluorescence microscopy, to determine the extent of callose formation; at a whole leaf level by recording percent chlorosis and leaf roll; and finally, at a whole plant level by measuring biomass loss.

The experiments were repeated under elevated CO_2 (450 ppm) to model any changes in RWA/plant interaction with respect to future climate change. The effects of an elevated CO_2 environment and RWA feeding on host plant foliar N and C:N ratio were compared to ambient CO_2 conditions, to provide a clearer picture of the potential nutrient drain that a feeding RWA colony exacts on its host.

Of the varieties tested, the CIho accessions performed better than the two SA barley cultivars as the CIho accessions appeared to express a mild antibiosis resistance response as RWA populations, particularly those of RWASA1, were smaller than those observed on either S5 or SSG 564. In addition, less damage was evident in the two CIho accessions due to RWA feeding.

RWASA2 was the most virulent of the three RWA biotypes tested, followed by RWASA3 while RWASA1 was the least virulent.

Under elevated CO_2 conditions, RWA feeding damage was exacerbated but the trend of biotype virulence remained the same. Higher aphid population sizes were recorded under elevated CO_2, meant that even the more resistant CIho accessions were overcome by the increased demand made by the larger aphid colonies on the host plants.

The % foliar N data showed that under elevated CO_2 aphid-free control plants had increased N levels in their leaves. Increased "food" supply (as shown by the increased N levels) therefore allowed significantly larger aphid populations to develop on the plants exposed to elevated CO_2, due to improved nutrient status of the phloem sap taken up by RWA. The knock-on effect of a higher aphid population was increased cell disruption as a result of extensive probing, extensive formations of wound callose, with the result that phloem damage impeded nutrient flow through the vascular tissues which contributed to chlorosis and (eventually plant) death.

The major conclusion from this study is that even a mild CO_2 elevation resulted in an increase aphid population which may pose a severe and very real threat to a barley crop. Therefore, without effort to identify and deploy resistant barley cultivars, it could well be possible that future barley cultivation in South Africa may no longer be viable.

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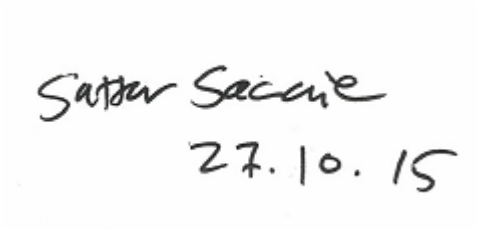
Declaration

This dissertation, submitted for the degree of Master of Science in the Department of Botany, Rhodes University, represents original work by the author and has not been submitted in any form to any other institution. Where mention has been made of the work of others, it has been duly acknowledged in the text.



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I certify that the above statement is correct.

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Prof. C.E.J. Botha

Supervisor

Chapter 1: Introduction

1.1 The Russian wheat aphid (Family Aphididae)

The Russian wheat aphid (*Diuraphis noxia)* is a member of the family Aphididea of which comprises over 4300 species. Like all members of the family it is specialized to feed on phloem sap (Goggin, 2007; Giordanengo et al., 2010). Many aphid species have become specifically adapted to their host plants. This results in specialization by most host plants, which become adapted to aphid feeding and are thus able to effectively combat and reduce the impact of the parasites. For example, *Macrosiphum euphorbiae* (Thomas) can infest tomato plants (*Solanum lycopersicum* L.) in large numbers, without causing any severe damage to the plant (Guerrieri and Digilo, 2008). However, some generalist feeders such as *Aphis fabae* (Scopoli) may have devastating effects on the un-adapted plants that they infest (Guerrieri and Digilo, 2008). A consequence of their rapid reproductive capacity means that aphid infestation impact negatively on host plants. This translates to exacerbated damage as a result of probing for phloem sap (Goggin, 2007). Aphids reproduce via viviparous parthenogenesis, resulting in live birth of genetic parental clones. Embryonic development of new clones begins inside foetal clones yet to be born which results in rapid turnover of generations. Depending on the availability of resources, either highly fecund wingless nymphs or alternatively winged aphids will morph, which then disperse great distances to locate and infest any nearby or remote viable food sources (Zhang et al., 2001; Goggin, 2007). Aphid dimorphism promotes alternation of host plants which coincides with winter and summer seasons thereby providing an excellent survival and colonisation strategy. Should an aphid colony experience high levels of disturbance they may release alarm pheromones that promote the production of winged nymphs, so that the colony can effectively escape to safety and begin rebuilding (Kunert et al., 2005). Aphid adaptive strategies are augmented further by monoculture, with the result that almost every major crop is host to one or other aphid species (Goggin, 2007).

The RWA's small body size and soft cuticle means that they are at constant risk of dehydration. To survive, they must have prolonged interactions with their host plants as they probe the xylem and phloem to ingest their dietary requirement (Saheed et al., 2007b; Giodanengo et al., 2010). Continuous infestation (10-20 days) usually induces non-reversible chlorois and necrosis, as well as the formation of longitudinal streaks along the veins of leaves, which vary from white, yellow to purple in colour. Leaf-roll is known to retard leaf

development reduce photosynthetic leaf area and much damage is done to the veins as well (Riedell, 1989; Tolmay et al., 2007; Saheed et al., 2007b: Belay and Stauffer, 2007). Heavy infestation will interfere with seed production and may lead to deformation of kernels (Tjallingii, 2006). RWA, like other suctorial insects probes the vascular tissues for nutrients and the primary response by the host plants is to initiate wound callose deposition (Tjallingii, 2006; Saheed et al., 2007a; de Wet and Botha, 2007). Tapping the xylem can result in a non-reversible reduction of nutrient transport capacity, which is followed by substantial decreases in leaf chlorophyll. Lower photosynthetic output and decreasing plant growth follows (Tjallingii, 2006; Belay and Stauffer, 2007; Saheed et al., 2007b; Tolmay et al., 2007; Smith et al., 2010; Jimoh et al., 2011b).

It has been suggested that much of the damage that leaves sustain is as a direct result of saliva injected into the xylem. This saliva lines cell walls and blocks half-bordered pit-pairs, thus limiting or partially curtailing transfer of water and nutrients from the xylem to the xylem parenchyma. This interference compromises the surrounding cells as they are starved of resources (Saheed et al., 2007b; Botha, 2009; Jimoh et al., 2011a; Jimoh et al., 2011b). During probing RWA continuously secretes a gelling saliva that serves as a lubricant and which subsequently hardens to form a sheath around the stylets. This sheath remains in the host plant tissues even after the aphids have withdrawn their stylets. TEM of infested plant tissue clearly shows the saliva sheaths and stylet tracks that have breached and plasmolysed the plants cells which may cause complete loss of functionality (Will et al., 2007; Saheed et al., 2007b; Jimoh et al., 2011a; Jimoh et al., 2011b). Gelling saliva is known to contain a cocktail of proteins and enzymes, which effectively degrade and damage the plant's cells and are also believed to activate any defensive responses from host plants (Will et al., 2007; Giordanengo et al., 2010).

In summary, whilst many of the aphid species have adapted to their hosts, RWA has not. This translates to severe damage to growing plants especially so, where the winters are not severe enough to force migration and dormancy, as in the case of Africa, South American and parts of the USA, where RWA is classified as a severe agricultural pest. Add to this the effects of climate change and the situation of severe crop loss is intensified.

1.2 RWA origin, distribution and spread

The Russian wheat aphid is indigenous to central Asia, the Caucasus and Northwest China. It has spread over time to other grain producing areas. The advent of large scale farming and the

availability of alternative host plants has contributed to RWA's invasion of other regions (Jankielsohn, 2013), with the result that it has become a serious and widespread pest over the past 40 years, it subsequently spread to, and invaded Central America, USA, Canada, Eastern Europe, Northern Africa and South Africa (SA) (Turanli et al., 2012; Zhang et al., 2001). The aphid follows a holocyclic lifecycle in the regions where it occurs naturally, and thus not is considered to be a serious pest. However, in regions where RWA have achieved pest status winters are much milder in comparison to that in their native territories and RWA reverts to an anholocyclic life cycle resulting in massive infestations of small grains as the aphids feed all season (Zhang et al., 2001; Zhang et al., 2014). The holo/anholocyclic life cycles are discussed in greater detail in section 1.4.

Analysis of microsatellite markers, mitochondrial DNA and endosymbiont genes between populations indicates that the RWA populations in SA and North America share a common ancestry with the populations from Turkey and Syria (Turanli et al., 2012). It is thought that the initial invasions radiated outwards from Turkey to SA. The similarity between the North American and SA RWA biotypes suggests that both these invasions were initially caused by the same genetic clone (Turanli et al., 2012; Zhang et al., 2014). Furthermore, soon after it reached SA it successfully invaded North America. RWA was most likely transported these distances on contaminated plant material more likely linked to human commerce than natural migration (Turanli et al., 2012).

RWA was first detected in the Free State in 1978 and has now reached severe pest status (Du Toit, 1990; Legg and Brewer, 1995; Tolmay et al., 2007). RWA has proved to be so detrimental to crops because of its high reproductive potential which results in swift and unmanageable spread. An initial low frequency infestation may escalate rapidly and it may result in crop production decline and economic loss (Chander et al., 2006; Saheed et al., 2007b). Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) producers have no alternative but to implement insecticide spraying routines or to face crop losses as high as 60% (Du Toit, 1990).

1.3 Evolution of biotypes

Many aphid species together with other pest insects have developed resistance to insecticides. In addition to pesticide resistance RWA feeding causes leaf roll, and these rolled leaves shelter RWA colonies from the elements, and also provide a protective barrier against contact-based pesticides. Therefore, effective control of heavy RWA infestation can only be

achieved through multiple applications of expensive and environmentally unfriendly systemic pesticides (Smith and Boyko, 2007, Dahleen et al., 2012). Genetic variability in RWA populations occurs through mutations in genetic sequence in the process of parthenogenesis. The rapid turnover of RWA generations means that even with a very low percentage of genetic mutation there is still sufficiently high genetic diversity in the overall population. High genetic diversity increases the fitness and adaptive potential of RWA populations. With selection pressures such as pesticides and host plant quality, the evolution of new RWA biotypes has been forced in a "survival of the fittest" scenario (Puterka et al., 2007; Weiland et al., 2008). Consequently there are multiple biotypes of RWA in each of the regions it currently inhabits, which are a direct consequence of anthropogenic selection pressures.

1.4 Breeding RWA resistant plants

It is widely accepted that host plant resistance is the best means to combat RWA infestation and is a viable alternative to the use of pesticides. However, it has also been argued that by imposing different selection pressures this can contribute to biotype evolution (Smith and Boyko, 2007; Weiland et al., 2008). Plant resistance comes in two defined forms, namely antixenosis and antibiosis. Antixenosis is defined as low host acceptability to the parasite, the host plant is able to manipulate the aphids behaviour and thus reduce infestations. This is caused by altering chemical products so that the plant no longer meets the aphids' dietary requirements. It is theorised that wounding causes a redistribution of plant enzyme elicitors and secondary metabolites in an attempt to repel feeders. The host plant is then rejected by the aphids and the potential for colonization is reduced (Hesler and Dashiell, 2011)

Antibiosis involves the production of signals/chemicals that directly affects the predators. In most cases the antibiosis response by the plant is to promote production of secondary products which could be toxic, which would be unsuitable nutrient components which would result in reduced fecundity and higher aphid mortality (Pointeau et al., 2013). A resistant host plant will exhibit both antibiotic and antixenotic responses in varying proportions, depending on the makeup of resistance genes. Observations have shown that a combined antibiosis/xenosis response negatively affect nymph settlement and survival along with development and reproductive rate (Pointeau et al., 2013; Webster et al., 1996).

However a major concern regarding the deployment of highly resistant cultivars has been the fear of forcing the development of more virulent biotypes. In any biological 'warfare' scenario the insects will, with time, develop virulence and overcome resistant plants. RWA

biotypes have evolved in SA, and the new virulent biotypes are highly detrimental to susceptible crops. Virulent biotypes successfully infest previously resistant cultivars. It is well-known that susceptible plants sustain greater cell damage and may have a high mortality when exposed to the newly evolved biotypes (Haile et al., 1999; Tolmay et al., 2007; Jimoh et al., 2011a; Jimoh et al., 2011b).

Plant tolerance could be a valuable trait in breeding programs. It is believed that tolerant plants do not impose selection pressures on RWA colonies. These plants have more efficient metabolic systems that allow for drain of aphid feeding while limiting reductions to fitness. The concept of plant tolerance is closely related to aphid/plant interactions where both parties have had ample time to adapt to one another, thus the host plants are able to sustain themselves even as they harbour large aphid infestations (Guerrieri and Digilo, 2008). Through the use of resistance and tolerance genes with integrated pesticide management it could be possible to deploy crops with increased fitness that could potentially reduce the threat of RWA (Haile et al., 1999).

1.5 The implications of holocyclic and anolocyclic life cycles

The Russian wheat aphid can successfully survive by making use of one of two of its life cycles variants; holocycly or anholocycly (See Figure 1.1). The holocyclic pattern involves parthenogenic reproduction during the warmer summer months, which is normally followed by a sexual phase, producing males and oviparous females. After mating, the females lay eggs that that remain dormant during winter periods. RWA populations should thus; become dormant over winter, and thus the initial populations are small when the viviparous females emerge in spring to recommence the life cycle over (Zhang et al., 2001). In stark contrast, when RWA follow an anholocyclic pattern they produce no males or oviparous females, only viviparous females of both winged and wingless forms are produced, which result in massive populations as individuals are simply cloned via parthenogenesis (Zhang et al. 2001). The key to whether a RWA population follows a holocyclic or anholocyclic lifecycle is largely determined by environmental factors such as minimum temperature during winter. Harsher, colder winters favour holocycly while more temperate winter conditions favour an anholocyclic lifecycle (Zhang et al., 2001; Zhang et al., 2014). Under laboratory conditions RWA colonies have been shown to be able to switch between lifecycles. Through the use of controlled environments and by raising minimum temperatures to over 15°C it is possible to induce a lifecycle change from holocycly to anholocycly, Zhang et al., 2001 found that after

80 generations eggs were no longer produced and males and oviparae had been replaced by viviparous females and nymphs.

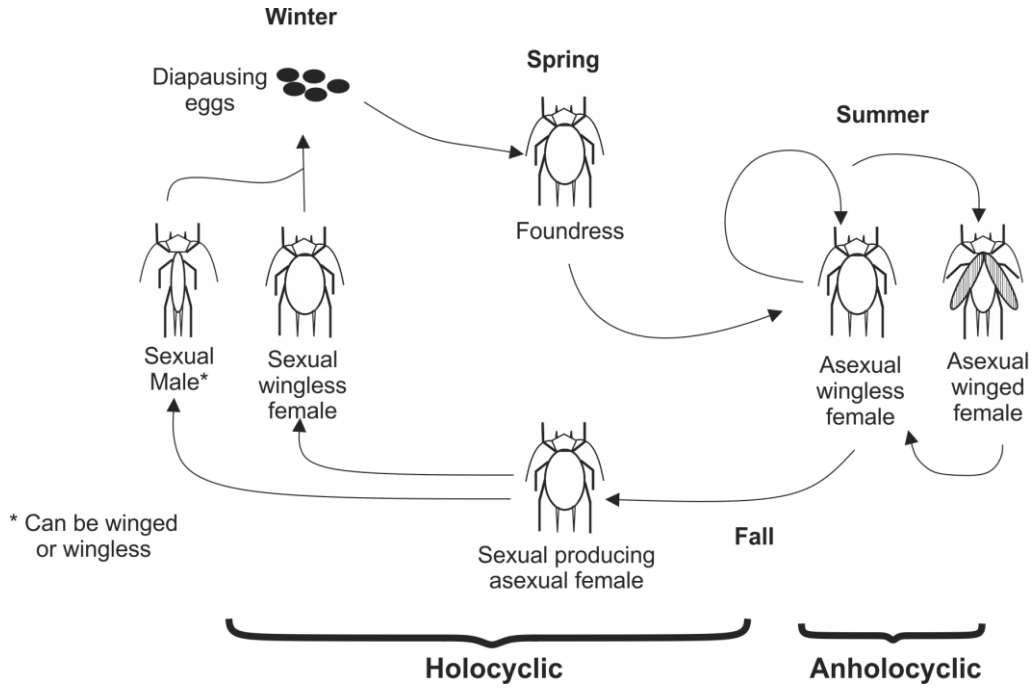

Figure 1.1 RWA holocyclic and anholocyclic life cycles over seasons (Adapted from Zhang et al., 2001).

1.6 The implications of barley domestication on RWA

1.6.1 The domestication of barley and its early interactions with RWA

Barley was domesticated at approximately the same time as wheat. Early humans lifestyles changed from nomadic hunter gatherers and began settled agriculture around 8000-1000 B.C. (Badr et al., 2000; Tanno and Wilcox, 2006; Saisho and Purugganan, 2007; Shewry, 2009). Archaeological evidence indicates that early agriculture started in the Fertile Crescent (Israel, Jordan, Southern Turkey and Iraqi Kurdistan) and wild landraces of both wheat and barley occurred naturally in this region. The Fertile Crescent is where the first forms of early civilization took root. Domestication no doubt began when early famers selected wheat and barley mutant strains with higher yields and indehiscent ears which did not shatter to form dispersal spikelets. By inadvertent selection of mutant strains, early farmers applied different selection pressures, thereby and unknowingly began an early form of selective breeding in wild varieties. It is an evolutionary advantage for ears to shatter at maturity in wild varieties as this improves the chances of effective seed dispersal, the evolution/selection of non-brittle ears meant that fewer seeds were lost during harvesting (Badr et al., 2000; Tanno and Wilcox,

2006; Shewry, 2009). Domestic barley differs from its wild ancestors and has different taxonomic nomenclature with wild barley being classified as (*Hordeum spontaneum* L.*)*. Once domesticated, wheat and barley cultivars had to then be threshed for their seed, subsequent crops would have to be manually planted as they lost the capability to self establish without human intervention. The original distribution of *Hordeum spontaneum* stretches from the west of the Fertile Crescent in Egypt across Asia to the Himalayas (Badr et al., 2000). Examination of the genetic loci of non-brittle barley ears has indicated that there may have been a second event of domestication of barley outside of the Fertile Crescent in the regions of the Himalayas. Modern American and European cultivars originate from the Fertile Crescent while Asian cultivars are descended from Asian landraces from the second event of barley domestication. Regardless of where the strains and wild landraces were first domesticated, they were selected for the common traits primarily being high grain yield and brittle ears (Badr et al., 2000: Morrel and Clegg, 2007). Thus over the course of its domestication, barley plants and humans developed a mutually benefiting relationship where the barley plants would produce high grain yields in return for effective human-induced seed dispersal and successful planting. All modern accessions of cultivated barley are descended from early domesticated cultivars from approximately 8000 BC (Morrel and Clegg, 2007).

Considering the original distributions of *Hordeum spontaneum* and Russian wheat aphid, it is likely that RWA fed on early wild barley as one of many host species. It is however paradoxical that RWA is so detrimental to crops that it should have had a long and well established host-parasite relationship with. It is counter-adaptive to the parasite if it rapidly kills off its host and then leaves itself in a situation where it must locate a new food source or perish (Jankeilsohn pers comm.). Among the USA RWA biotypes it has been shown that crested wheatgrass (*Agropyron cristatum* L.) and Canada wild rye (*Elymus canadensis* L.) are dominant hosts between winter wheat seasons for RWA. Minor host species include downy brome (*Bromus tectorum* L.) and the intermediate wheat-grass (*Thinopyrum intermedium* (Host) Barw. and D.W.); interestingly most of the alternative hosts screened have been found to be completely susceptible to RWA feeding (Armstrong et al., 1991; Weiland et al., 2009).

1.6.2 The implications of monoculture

Small grains monoculture provides such an abundant food source for RWA and thus, all other potential alternative hosts are ignored, in favour cultivated wheat and barley fields (Goggin, 2007). Jankielsohn, 2013 sampled RWA colonies throughout the major wheat growing regions of SA (See Table 1.1) and screened for potential alternative hosts.

Though RWA was predominantly found on dryland wheat it was also found infesting irrigation wheat, volunteer wheat, barley, rescue grass (*Bromus catharticus* Vahl), false barley (*Hordeum murinum* L.*)*, oats (*Avena sativa* L.), wild oats (*Avena fatua* L.) and rye (*Secale cereal* L.). Though cultivated wheat and barley can be considered to be the primary hosts in SA, the alternative hosts play an important role in maintaining RWA numbers, during intervals between harvest, planting and emergence of new crops. Hosts such as *B. catharticus* are found bordering fields and in road reserves and these may assist RWA populations to overwinter. As temperatures rise in summer, RWA will spread to seasonally cultivated small grains and thus become serious pests (Jankeilsohn, 2014).

The feeding and life pattern observed RWA can be compared to those of the greenbug (*Schizaphis graminum* Rondani), which is in itself a serious pest of cultivated grasses such as wheat, barley and sorghum. *S. graminum* has been found to have approximately 70 host species of wild and cultivated varieties across 29 genera. It is believed that host alternation allows the greenbugs to maintain successful temporal and spatial population reservoirs. Generalised feeding may result in increased genetic diversity which in turn, could harbour virulence genes and lead to emergence of new biotypes (Anstead et al., 2003).

Table 1.1 Alternative RWA hosts in South Africa (adapted from Jankielsohn, 2014)

Host Plant		Biotype					
		RWASA1		RWASA2		RWASA3	
Common Name	Scientific name	No.	%	No.	%	No.	%
Drylan wheat	Triticum aestivum	56	76.71	59	55.66	42	58.33
Irrigation wheat	T. aestivum	3	4.11	6	5.66	5	6.95
Volunteer wheat	T. aestivum	4	5.48	17	16.04	10	13.89
Rescue grass	Bromus Catharticus	4	5.48	5	4.72	11	15.28
Barley	Hordeum vulgare	1	1.37	2	1.89	1	1.39
False Barley	Hordeum murinum	3	4.11	0	0	1	1.39
Oats	Avena sativa	0	0	13	12.26	1	1.39
Wild Oats	fatua	1	1.37	3	2.83	0	0
Rye	Secale cereale	1	1.37	1	0.94	1	1.39
Total		73		106		72	

1.6.3 The resistance conundrum

There are no known resistant barley cultivars available in SA at this time, and the identification and deployment of resistance in barley is not as simple as it is with wheat. The resistance responses in barley are polygenic and it is thus not governed by single *Dn* genes as in the case in wheat relatives. As barley resistance comes from quantitative trait loci, breeding resistant barley cultivars becomes a very complex and difficult process and the result is that determination of resistant traits is extremely challenging (Dahleen et al., 2012). Currently the most effective means of determining resistant qualities is to test the host plant survivability against the different RWA biotypes (Tolmay et al., 1999; Dogimont et al., 2010).

1.7 The barley plant – agricultural importance

Barley is ranked fifth-most produced of all crops across the world, after maize, wheat, rice and soybean. Historically barley was cultivated predominantly as a human food source, though its use has changed over time (Baik and Ullrich, 2008: Newman and Newman, 2006). It is no longer a staple crop/food like wheat or maize, but is rather principally used in the production of livestock feeds and as a malt for the beer brewing industry. Barley is the most adaptable of the cereal grain species and can be cultivated at higher latitudes and altitudes,

and further into deserts than other small grain crops. Due to its hardiness it still remains the principle food source in more extreme environments such as the Himalayan nations, Morocco and Ethiopia (Newman and Newman, 2006; Baik and Ullrich, 2008). Barley produces kernels that are rich in carbohydrates; it also contains some proteins, minerals and vitamins. Currently 65% of the global barley crop is used to make feed, 33% for beer malting and approximately two percent is used directly in food (Baik and Ullrich, 2008). Such is the versatility of the barley kernels that they can be rolled, ground or flaked to be suitable for cattle, pigs or poultry (Newman and Newman, 2006)

1.8 Barley cultivation in South Africa:

Barley is cultivated in two regions of SA, the South Western and Northern Cape regions (See Fig 1.2). The South Western Cape (SWC) is a region of fairly high rainfall and therefore, the barley farmed there does not require irrigation. The Northern Cape (NC) is more a arid environment, meaning that, irrigation is necessary to successfully farm barley there (Potgieter, SABBI, pers. comm. 2014).

Most of the barley produced in SA is used in malting by SA Breweries (SABM). SABM uses approximately 270,000 tonnes of barley per annum. Due to strict quality control requirements, the 391 farmers in the SWC and the 104 emergent and 193 commercial farmers from the NC are only able to produce 222,000 tonnes of malting barley per annum (See Figure 1.1). This amounts to 82% of SABM yearly usage the remaining 18% is imported from Canada, Central Europe, Argentina and Australia (Macfarlan, 2011, SABM). The SWC is the primary barley growing region and RWA infestations are controlled through the use of expensive pesticide spray regimes. Currently it is believed that the wet conditions of the SWC may provide a buffer against RWA infestation (Potgieter, SABBI, pers. comm. 2014). Heavy torrential rain hinders the spread of winged RWA nymphs. Of concern, is that as future climate change models predict the SWC to become a drier environment, which would be much more conducive to (massive) RWA invasions (Potgieter, SABBI, pers. comm. 2014).

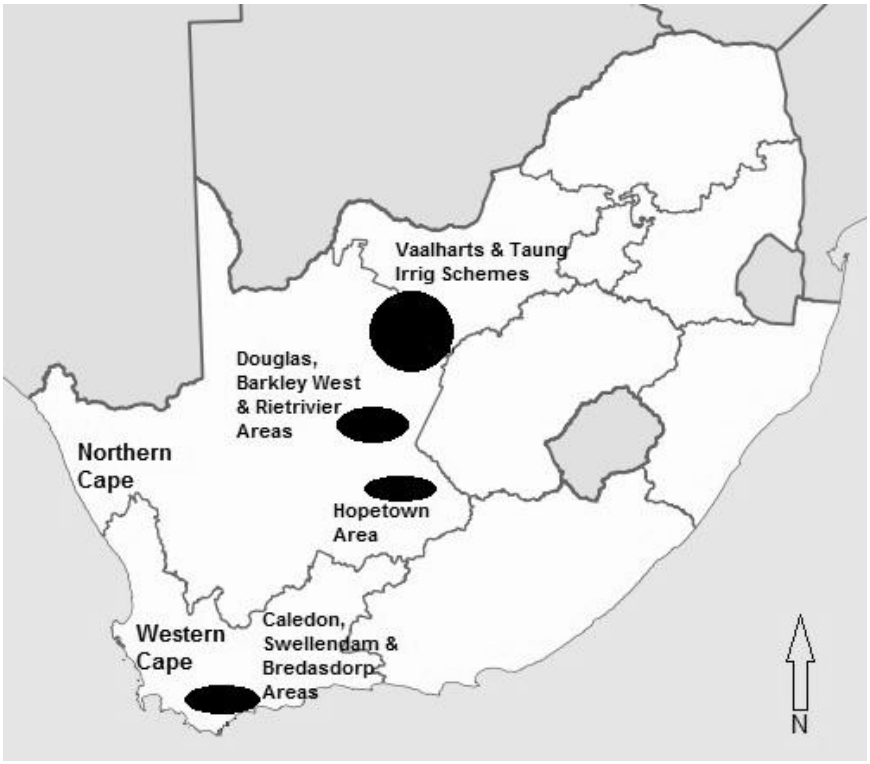

Figure 1.2 Map of South Africa showing main barley cultivation areas in Northern and Western Cape provinces (adapted from Angus McFarlan SABM 2011).

1.9 Global climate change and rising CO_2: The future scenario

1.9.1: The implications of rising CO_2

It is important to note that the nature and potential viability of barley farming in SA will be directly influenced by rising CO_2 levels. In 2014 the average global CO_2 concentration reached 400 ppm, when the Rhodes University Botany Dept. began experiments with elevated CO_2 the ambient concentration was between 370-375 ppm, thus a 25 ppm rise in ambient CO_2 over the last 25 years has been experienced. It is projected that atmospheric CO_2 concentrations will rise past 500 ppm by 2050 and reach 700 ppm by 2100 (Leakey et al., 2009). Ever increasing coal-fired electric generation in developing nations such as India and China and limited action taken to reduce emissions in developed countries mean that current CO_2 levels are the highest they have been since the early Miocene (Leakey et al., 2009). Initially it was thought that elevated CO_2 would not have a significant effect on the plant/aphid interactions. However subsequent research carried out at Rhodes University has shown the RWA will become a serious threat to SA barley cultivars (See Jimoh et al., 2013). It is of great importance to understand how plants will respond to the rapid changes in the

CO_2 environment, and therefore I will expand more on the effects of elevated CO_2 on plants and RWA in the following sections.

1.9.2 Plant response to elevated CO_2

The majority of small grain crops utilize the C_3 carbon fixation pathway. In the C_3 cycle, CO_2 and O_2 are in a competitive equilibrium with each other, competition for the active sites of Rubisco results in lower photosynthetic efficiency (PE) than is the case in C_4 plants. A rise in ambient CO_2 will initially improve C_3 plant PE. Viewed in broader terms CO_2 is but one of the many inorganic substrates required by plants. Many researchers have found that under prolonged exposure to elevated CO_2 C_3 plants will undergo acclimation. The resultant photosynthetic down-regulation is characteristic of plants that reduce Rubisco activity and lower their carboxylation capacity (Stitt and Krapp, 1999; Sage, 2004). It is important to note though that the boost of photosynthetic activity caused initially by the elevated CO_2 causes a net increase in growth but then long-term down regulation will result (Leakey et al., 2009). One should ask the question why this is so? The primary reason for down-regulation is believed to be due to nutrient and mineral limitation. Increased metabolic demand resulting from increased photosynthesis rapidly exhausts the plants supplies of other metabolically important inorganic substrates (see Stitt and Krapp, 1999). In contrast optimally fertilized plants do not suffer down-regulation, but decreased carboxylation and reduction in Rubisco is more marked, specifically in nitrogen-limited plants. Plants with sufficient N to meet increased plant requirements under elevated CO_2, have an improved N-use efficiency and there is thus a limited change in the C:N ratio (Stitt and Krapp, 1999; Arp, 1991). In contrast, low N in barley plants at elevated CO_2, causes severe nutrient shortfalls which result in chlorois of leaf tissues. Barley plants with optimal N show increased photosynthetic efficiency and limited modification to metabolic function (Sicher, 2001). So, the precondition is that fertilizer must be in ample supply, then increased CO_2 equates to increased vegetative growth and grain yield, by as much as 10-16% in wheat and barley (Hughes and Bazzaz, 2001; Högy et al., 2010; Manderschied et al., 1995; Sæbø and Mortensen, 1996). However this scenario results in a significant increase in the cost of agricultural fertilizer.

1.9 RWA and Rising CO_2 Concentrations: who benefits?

As mentioned, anthropogenic activities in play means the atmospheric CO_2 may well rise to 450 ppm within the next 10-20 years. Whilst this could have a positive impact in the absence of aphids, modelling of elevated CO_2 on plants which are RWA infested under controlled

environment has demonstrated that increased CO_2 will negatively impact host plants. Previous research by Jimoh et al., 2013 revealed a scenario which pointed to the fact that elevated CO_2, while increasing host plant photosynthetic rates would also promote aphid population growth rates. PUMA a barley cultivar commonly deployed in SA and used in malting by SABM showed increased damage (chlorosis and leaf roll) and high mortality at higher CO_2 levels as a result of increased RWA population. American cultivars STARS-0502B, STARS-9301B, and STARS-9577B also showed similar results, biotypes RWASA1 and RWASA2 caused reductions in plant biomass and increase damage at higher CO_2 (Jimoh et al., 2013). There is good evidence to suggest that as CO_2 concentrations increase and climate patterns change that there may well be extensions to crop growing seasons. This in turn could potentially affect the geographic distribution, over-wintering patterns, population growth rates, the number of generations produced per season and most importantly, crop-pest synchrony of RWA (Porter et al., 1991; Bezember et al., 1998; Rötter et al., 2011; Jimoh et al., 2013). Over the last 30 years climate change has favoured aphids, Europe has experienced a 20% increase in the number of aphid species, due to increased average seasonal temperatures, higher CO_2 and increases in global plant transport that facilitated new invasions by aphids. The take home message is that climate is the most important factor in determining the success and viability of invading colonies (Hulle et al., 2010).

Climate change will undoubtedly expose agriculture to new and as yet relatively unknown threats with respect to aphid invasions. What is certain is that knowledge and understanding of resistant cultivars will be needed. Resistant genes are commonly deployed in SA wheat cultivars to combat and manage RWA infestations. It is clear that this practise has contributed to the emergence of new RWA biotypes. As of 2014 three new biotypes of RWA had been indentified, RWASA2, RWASA3 and RWASA4, each with different virulence ratings to the *Dn* genes 1, 2, 3, 4, 5, 7, 9 and, *x* and *y* (Jankielsohn, 2014). RWASA1 was first discovered in SA in 1978, RWASA2 in 2005 and RWASA3 in 2009 and RWASA4 was identified in 2014 (Jankielsohn, 2014). The interval between the evolution of new biotypes has accelerated faster than the development and deployment of resistant cultivars. It is important to note that it takes approximately 5 years to develop, trial, and finally deploy a cultivar which generally stays in circulation for approximately 8 years. With the current rate of biotype evolution by the time a resistant cultivar is deployed there will already be a new RWA biotype in the field which could potentially harbour virulence (Jankielsohn, 2011; Potgieter, SABBI, pers. comm. 2014).

A possible explanation for this is the large degree of diversity in the RWA populations which facilitates increased number of virulent biotypes.

SA barley breeders are yet to deploy resistant barley cultivars. Resistant cultivars have been deployed in other regions with mixed results (Dahleen et al., 2012). Instead farmers must rely on expensive pesticides to eliminate or control RWA infestation. However, there are significant drawbacks to pesticides 1) the cost of spraying; 2) timing constraints as to when a crop can be prayed prior to harvesting in order for the grain to be fit for human consumption. At this time RWA populations and outbreaks are low in the main barley growing areas in the SWC. Of concern is that SA does not produce sufficient harvests of barley to meet the total demands of the breweries, which increase on a yearly basis (Potgieter, SABBI, pers. comm. 2014). SABM is reliant on imports to cover the annual short-fall. Considering the climate and environmental changes that are taking place, a scenario where RWA becomes a major pest to SA barley is possible. Should the barley industry be negatively impacted through RWA infestation, many of the people whose livelihoods depend on this crop through agriculture and malting would be at risk. Modelling the effects of RWA on current cultivars is thus essential.

1.10 Controversy over the use of FACE, open-topped chamber or controlled environments

There is a great deal of research effort directed to a clearer understanding of the effects elevated CO_2 concentrations will have on plants in natural and managed ecosystems. Much of the foundation knowledge was obtained by conducting experiments in controlled environments (CE) or through the use of open-topped chambers. It has more recently been argued that these techniques are limited, and have been replaced by using free-air CO_2 enrichment (FACE) rings. Whilst it has been argued that controlled environments provide results that cannot be easily extrapolated into the real world as they do not mimic environmental variability, the absolute strength of controlled environment experiments is that single variables can be manipulated to provide a clear indication of the effects of that variable (Ainsworth and Long, 2005). CE systems allow models to be constructed that possible provide clearer insight into otherwise highly complex biological interactions. Open topped chambers introduce more variables in that they are open to outside weather conditions. Many argue that the FACE ring is the best way of implementing enriched CO_2 experimentation. Plants, mature trees or potentially even whole fields can be studied. The FACE rings surround the test plants and emit jets of CO_2 in order to create a high CO_2 atmospheric environment in

a natural setting (Ainsworth and Long, 2005; Leakey et al., 2009). FACE rings might be ideal for eco-physiology studies, however they fall short a basic level interactions and modelling. Without doubt FACE rings have a highly variable CO_2, and being exposed to the elements other abiotic factors are also variable; as a result it is difficult to clearly interpret findings in FACE conditions. However all three methods (CE, open topped chambers and FACE) have shown the same basic conclusions. The take home message is that elevated CO_2 causes increased uptake of carbon and improved carboxylation, N use efficiency increases and finally in conditions where N is limited then plants will acclimate to cover the nutrient short fall (Ainsworth and Long, 2005; Leakey et al., 2009). Mjwara et al., 1996 used CE to show that enriched CO_2 induces significant changes in N availability in *Phaseolus vulgaris* L., in this cases the extent and deviation from the control groups could be easily quantified and analysed because the CE allowed meticulous control of experimental design.

The experiments conducted in this thesis are also carried out using. The predominant reason for using CE is the ability to isolate one particular environmental variable, in this case the effects elevated CO_2 could effectively be modelled. CE also provides secure containment for RWA while experiments are running, thus reducing the risk of nymphs escaping.

1.11 Research objectives:

Barley like wheat is one of the prime feeding target plants of the RWA. Given the lack of resistance it does not take much imagination to realize that the potential threat presented by RWA is huge. Continued mutation of the original RWASA1 biotype brings with it the potential threat of crop devastation. Monoculture provides an immense feeding resource for these insects. RWASA2, SA3 and SA4 have been shown to be increasingly virulent on wheat and barley yet no work has been done to determine if these biotypes will become more detrimental to resistant cultivars as environmental conditions change.

It is imperative to model the interactions between the biotypes and the main commercial barley cultivars of SA. This would allow “worst case” scenarios to be acknowledged and also would test the integrity and inherent resistance of current barley cultivars should they become a target of RWA. Additionally through the manipulation of carbon dioxide concentrations it becomes possible to model future interactions between insect and host. Long-term viability of current cultivars can be trialled to ascertain if they will meet productivity requirements in an uncertain future.

This thesis examines two SA cultivars (S5 and SSG 564) and comparison is made between them and two Afghan accessions (CIho 4125 and CIho 4159) that are assumed to be drought tolerant/resistant and could potentially be resistant to RWA. S5 and SSG 564 are two modern cultivars developed by SABBI. They are both deployed in the SWC dry land conditions (non-irrigated land) and have been specifically produced for high quality malt at high yield. Examining the original RWA distributions and its routes of invasion led us to select barley accessions that were produced and deployed in regions where the RWA naturally occurs. CIho 4125 and CIho 4159 are drought resistant accessions from Afghanistan provinces of Wardak and Herat and have been used as partial source accessions in some STARS cultivars. According to the USDA these accessions have been classified as highly tolerant to RWA infestionInherent tolerance or resistance to RWA was expected as these accessions were developed within the RWA home range (Mornhinweg et al., 2007). The main thrust of this thesis is to trial the resistance (or tolerance) potential of these accessions against the South African RWA biotypes; RWASA1, SA2 and SA3.

As ambient global CO_2 levels rise, the interactions between RWA and its host plants will change. After having tested the resistance qualities of these four varieties at ambient CO_2 we wished to test them at 450 ppm CO_2 to ascertain if there would be any resistance breaking effect. This would provide potential insights into future viability of these cultivars. Should the CIho accessions show that they have an inherent resistance to RWA they could then potentially be used in breeding programs to try and confer some of their traits to SA barley cultivars. These research questions will be explored using controlled environments which can precisely manipulate one independent variable which in this thesis is CO_2 concentration.

This research is separated into the following chapters:

Materials and methods (Chapter 2)

Aphid population dynamics at ambient and elevated CO_2 (Chapter 3)

The effects of aphid infestation on the host plants biomass, chlorosis and leaf roll under ambient and elevated CO_2 (Chapter 4)

The effects of aphid infestation on host plants % nitrogen and C:N ratios at ambient and elevated CO_2 (Chapter 5)

Florescence microscopy and transmission electron microscopy examining physical damage caused by aphid feeding (Chapter 6)

General discussion and conclusion (Chapter 7)

The use of controlled environments does not necessarily recreate a “true world” scenario but it does provide a baseline of plant/aphid interactions without the added complexity of confounding variables.

1.12 Outline of hypotheses

In this thesis it is hypothesized that:

1: The population growth rate of all RWA biotypes (RWASA1, RWASA2, and RWASA3) would be higher at elevated CO_2 than under ambient CO_2.

2: The three RWA biotypes tested would have different population dynamics on the different host plants, under elevated CO_2 compared to ambient CO_2.

3: Host plants would sustain more feeding damage and have lower biomasses when exposed to aphids at elevated CO_2 than at ambient.

4: Given that they are grown in regions of low rainfall, suggests a potential drought resistance together with the known tolerance to the USA RWA biotypes, the Afghan accessions (CIho 4125 and CIho 4159) will show greater resistance to RWA feeding than either of the SA cultivars (S5 and SSG 564).

Chapter 2: Methods and materials

2.1 Host plant propagation:

In this investigation the effect of feeding (28 days) by the Russian wheat aphid was examined. Two of the four barley cultivars (CIho 4125 and CIho 4159) tested, are of Afghanistan origin. CIho 4125 originates from Herat province while CIho 4159 is from Wardak province. Both accessions were obtained from United States Department of Agriculture, Agricultural Research Station (USDA-ARS, Stillwater, Oklahoma, USA). CIho 4125 was used as a source accession by the USDA to produce the barley line STARS 0626B, of which seedlings are rated as resistant to the USA RWA biotypes (Ullrich, 2010). Prior to this both CIho accessions were classified as tolerant to the USA RWA biotypes (USDA).

Two cultivars (S5 and SSG 564) used were of South African (SA) origin and, were supplied by SA Barley Breeders Institute (SABBI). Both S5 and SSG 564 were developed by SABBI; they are commercially used in the non-irrigated lands of South Western Cape (SWC) for the production of beer malt. The cultivar S5 is a high yielding cultivar that is recommended for the high production areas of the SWC. S5 is susceptible to leaf blotch, net-form net blotch and spot-form net blotch, and is resistant to leaf rust and immune to physiological leaf spot (SABBI, 2012, S5 cultivar guide). SSG 564 is considered a more agriculturally versatile cultivar with average yield that is suitable for all production areas of SWC. According to SABBI, SSG 564 is moderately resistant to leaf blotch, moderately susceptible to net-form net blotch and spot-form net blotch, susceptible to leaf rust and immune to physiological leaf spot (SABBI, 2012, SSG 564 cultivar guide). Neither of these cultivars were bred specifically to be resistant to RWA, and thus farmers who deploy them are reliant on pesticides to control aphid infestation.

Seeds of all four cultivars were washed in a 0.05% commercial bleach solution as sterilization against fungus before germination. The seeds were then transferred to Petri dishes with water soaked filter paper and germinated at room temperature. Once the seeds had germinated they were planted in individual pots which contained a 2:1:1 potting soil, compost and vermiculite mixture. Potted seedlings were maintained in a greenhouse at 25-30°C for one week then moved into a controlled environment (Conviron S10H, Controlled Environment Ltd., Winnipeg, Manitoba Canada) to grow to the three leaf stage. At the three leaf stage the plants were ready for experimentation and were manually infested with the RWA. The plants were

watered with 50% Long Ashton nutrient solution every two days to limit the development of nutrient deficiencies in the plants.

2.2 RWA colonies:

The three RWA biotypes (RWASA1, RWASA2 and RWASA3) were obtained from the Agricultural Research Council, Small Grain Institute, Bethlehem, Free State, SA (ARC). The aphid colonies were maintained on potted plants within Perspex insect cages air flow holes sealed with fine gauze. Colonies were maintained on young wheat (Scheepers cultivar) and barley (Clipper and Puma cultivar) plants. Feeder plants were alternated so that no particular selection pressures could be applied or would develop in the RWA colonies, continuous exposure to resistant cultivars could potentially increase virulence and vice versa with susceptible feeders. The aim, therefore, was to maintain unaffected colonies. Feeder plants were regularly changed to maintain large and healthy aphid colonies for experimental use. Aphid infested material that was no longer of use was disposed of in black plastic polythene bags and sprayed with insecticide to ensure that no aphids could escape into the environment.

2.3 Controlled environmental conditions:

All experiments reported in this thesis, were carried out within S10H Convirons. The Convirons were set to a maximum mid-day temperature of 25°C and 66% relative humidity (RH) with a night time temperature and RH of 22°C and 60%. A combination of fluorescent and incandescent lighting provided a 14 hour photoperiod with an average photosynthetic active radiation level (PAR) of 250 μmol^{-2} s^{-1} at 30cm below the lights. The Convirons were calibrated with 0% span gas (Nitrogen) and 1000 ppm [CO_2] before experiments were commenced. The experiments were carried out at ambient (380 ppm) and elevated (450 ppm) [CO_2] conditions.

2.4 RWA population studies:

The population growth rate of the aphids was determined to examine differences between biotypes and to monitor any resistance qualities of the cultivars. Once the experimental plants had reached the 3 leaf stage, ten apterous aphids were gently removed from the feeder hosts plants with a fine horse hair brush and placed on the 2^{nd} and 3^{rd} leaves of each of the experimental plants which was then fully enclosed in a ventilated Perspex plastic aphid cage. The cages allowed aphids free roam over the experimental plants. A ten replicate plant treatment was established for each experimental condition in which infested experimental

plants were placed in either a 380 ppm or a 450 ppm [CO_2] environment for the duration of the experiment.

Over the course of 28 days two studies were conducted on the plants, one to measure aphid population growth and the other to determine the damage sustained by the plants as a direct result of aphid feeding. Population growth data was collected for 14 days, the aphid populations grew at such a rate that counting accuracy could not be assured beyond 14 days. However, the assessment of plant damage continued to 28 days to better assess host plant survivability.

Total aphid population size was determined by counting living aphids on each of the infested plants under each of the experimental conditions. Counts were made on days 1, 2, 4, 6, 8, 10, 12, and 14 post infestation. A magnifying glass was used to aid in the identification of living aphids on the plants. The data was imported into Microsoft Excel 2007 and used to derive the aphid population doubling times and relative growth rate (RGR). Though winged morphs did develop as the experiments progressed, their small size and high mobility made it virtually impossible to accurately count their numbers. Thus only wingless morphs feeding on the plants were counted.

Previous population studies of the RWA make use of the intrinsic rate of growth (see Jankielsohn, 2013). The intrinsic rate of reproduction is derived by counting the reproductive capacity of a single apterous aphid. This method does not give indication regarding the population growth rates of a whole RWA colony which, in terms, of the research being carried out in this thesis was of greater interest.

Doubling times were calculated by: Average population for Day x – standard deviation Day x = Dpop.

Dpop was cross checked with 1°- 4° population's sizes when Dpop was higher than a value of a population size class, it was regarded that the population would beyond doubt have doubled by that specific time. For example on Day 6 Dpop = 24 then the 1° doubling time has been reached (1° time = 2 x 10 (initial starting population).

Table 2.1 Aphid population Dpop values required to meet doubling time tiers

Doubling time	Minimum Dpop value required to reach doubling time
1°	≤20
2°	≤40
3°	≤80
4°	≤160

Relative growth rate formula used: RGR = (ln P2 – ln P1)/(t2 –t1)

P = population, t = time.

RWA data was further analysed in Statistica 10 (Stat Soft Inc. Tulsa, USA, 2010) using an ANOVA to test for significance between the control group and the elevated [CO_2] group – the ANOVA results where then used to perform a post hoc Tukey-test to further investigate any potential significance in the data. Sigma Plot 11.0 (Systat Software Inc. San Jose, CA, USA, 2008) was used to graph the findings. From 14d onwards only the damage assessment of the host plants continued until 28d.

2.5 Host plant damage:

On days 7, 14, 21, and 28 post infestation the plant leaves were closely examined and rated on a scale of chlorosis and leaf roll (See Table 2.2). RWA feeding causes progressive chlorosis, yellow spots and streaks along the leaves which are indicative of functionality and health of the host plant. The chlorosis scale (0-9) is rated according to the estimated percentage cover of chlorotic tissues.

The second symptom of RWA feeding stress is that the host plant leaves begin to roll, a leaf that is continually wounded begins to fold and loses photosynthetic area, making a microhabitat for the aphids to hide in. Thus, the leaf roll rating (0-3) provides insight into the relative functionality of the plant leaves. These ratings are the same as used by Jimoh et al., 2011 and the determined virulence data of the RWA could be cross compared directly to the ARC biotype differential data.

Table 2.2 Chlorosis and leaf roll rating used to assess the levels of damage RWA cause to experimental plants. Damage rating from Jimoh et al., (2011).

Scale:	Description:
Chlorosis	
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 apparent, especially along the midrib.
5	Chlorotic spots may begin to coalesce or definite streaking may occur. Chlorosis covers 40% of leaf area.
6	Larger chlorotic areas form coalesced spots, leaves die back from tips. Chlorosis covers 55% of the 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 Roll	
1	Leaves are flat, no apparent rolling.
2	Leaves are folded and/or loosely rolled at the margins
3	Tightly or completely rolled leaves.

2.6 Host plant dry biomass:

Following aphid population study the whole plants, including the entire root mass, were removed from their pots and cleaned of soil and debris. Any aphids that were on the leaves were removed using a fine horse hair paint brush. Plants were then placed in a 60°C drying oven for 48h, to remove all moisture. A control group which had not been exposed to any aphid feeding were grown in conditions identical to the aphid infested plant and subjected to the same cleaning and drying treatments. The control plants provided baseline biomass data for comparative purposes, and also provided data showing the effects of [CO_2] on total biomass. As dry biomass relates to, and is indicative of, plant productivity, it follows that if the feeding aphids are virulent, biomass would be reduced as a result of the excessive draw-down by the feeding aphids (which form local nutrient sinks) and the resulting diversion of nutrient and assimilate giving rise to a heavy cost on the plants' resources.

2.7 C:N ratio and percentage N of leaves:

These methods follow those used in Jimoh et al., 2013. After weighing the dry plants (after 28DAI), five replicates from each experimental condition (control plants, and plant infested by RWASA1, SA2 and SA3 for both ambient and elevated [CO_2]) were randomly chosen for

C:N and percentage nitrogen analysis. Three cm long sections were cut from the mid-leaf sections of each of the selected plants. The sections were thoroughly cleaned to remove any potential aphid contamination. The leaf sections were placed in eppendorf tubes and homogenised into a fine powder using a TissueLyser II bead-mill, (QIAGEN, Valenica, California, USA) then placed in a drying oven for 48 hours to fully desiccate. 1.45-1.65 mg of homogenised leaf powder were measured out using an analytical semi-micro weighing balance (OHaus Discovery DV 215CD, Ohaus Corporation, Switzerland) and placed in sterile 9x5 mm organic elemental analysis tin capsules. Loaded tin capsules were carefully crimped using forceps and tweezers and folded into small balls then analysed using a Europa Scientific Elemental Analyser (Model ANCA-SL, Europa Scientific, United Kingdom) to provide % N g^{-1} and C:N ratio of leaf tissues. Data was further analysed using ANOVA at 5% level of significance in Statistica 10 and results were graphed using Sigma Plot 11.0.

2.8 Aniline blue (fluorescence microscopy)

Concurrently as the populations studies were running additional plants (5 replicates) for each condition were set up together along with 5 control plants. These plants were used for microscopic examination (light microscopy, fluorescence and TEM). At 14 days post infestations, leaves where the aphids were feeding were removed with a razor blade and prepared for fluorescence microscopy where callose depositions caused by RWA feeding were investigated. Excised leaf segments were placed in a Ca^{2+} free MES buffer (10 mM $^{2-}$ [morpholino] ethanesulfonic acid (MES), 0.5 mM $MgCl_2$, 0.5 mM KCl and 125 mM mannitol, adjusted to pH 7.2). Leaf abaxial surfaces were scraped using carbon steel razor blades (Aga Scientific, USA) to remove the dermal tissues and present the vascular tissues and mesophyll of the inner leaf. Scraped sections were mounted in calcium free MES buffer on glass microscope slides and stained with a solution of Gurr's aniline blue, (4'4-[carbonyl bis (benzene 4, 1-diyl) bis (imino)] bis benzensulphonic acid) diluted to 427 μM with distilled water, then covered with cover slips. Callose deposition was then examined using Olympus BX61 wide-field fluorescence Digital Imagine Microscope (Olympus, Tokyo, Japan supplied by Wirsam Scientific, Johannesburg, SA). The microscope was fitted with an aniline blue filter cube (excitation of 425 - 444 nm; emission of 475 nm). High resolution photos were taken using analySIS (Soft Imaging System GmbH, Germany) and then the later edition CellSens (Soft Imaging System GmbH, Germany). Pictures were imported into CorelDraw for presentation. Both programs were used for phase analysis to provide quantitative measures of feeding related damage. Phase analysis allows measurement of the percentage of a colour in

an image – in this the case the green blue of wound callose was measured. Data was collected and analysed with ANOVA in Statistica 10 and results was presented in Sigma Plot 11.

2.9 TEM (Transmission electron microscopy):

Following the fluoresce microscopy studies the original experimental design was repeated using the same cultivars (S5, SSG 564, CIho 4125 and CIho 4159) with control plant, RWASA1, SA2 and SA3 groups at elevated and ambient [CO_2]. 10 replicates for each condition were used, and after 14 days plants would be sacrificed and their leaves would be removed and prepared for TEM. The resin embedding protocol used was adapted from the Rhodes University Electron Microscopy Unit handbook on the preparation of biological material for electron microscopy (Cross, Botha and Pinchuck, 2001).

Sections of leaf were cleaned of aphids and cut into small 1 mm x 1 mm squares with a razor blade then placed in a pre-fix solution of 2.5 M glutaraldehyde and 0.1 M sodium phosphate buffer, with two drops of tween to break surface tension. The leaf squares were then placed in a vacuum oven (Model 19, Precision Scientific, Chennai, India) at 17000 kg/m sec^2 for one night to allow the pre-fix to impregnate the tissues. The leaf squares were then washed in 0.1 M sodium phosphate buffer solution and then placed into a 4% osmium tetroxide solution for secondary fixing then washed again in 0.1 M sodium phosphate buffer solution. The squares were then sequentially dehydrated in 30%, 50%, 70%, 80%, 90% and absolute ethanol solutions and then washed in propylene oxide. For the following four days the leaf squares were then embedded overnight in propylene oxide/ araldite resin mixtures, the first night being a 75:25 propylene oxide/resin mixture, the second a 50:50 mix, the third a 25:75 propylene oxide / resin mixture and finally the forth night pure resin.

Once in pure resin the leaf squares were placed in resin block moulds and baked in an oven at 65°C for 36 hours to cure. Hardened blocks were trimmed with glass knives made in a knife maker (LKB Type 7801B, Laboratoire Kastler Brossel, Paris, France) and sections of leaf tissue were cut using an Ultra Diatome diamond knife (45°, MF 646, Diatome, Hatfield, Pennsylvania, USA) on a ultra-microtome (RMC MT7, Tucson, Arizona, USA). Leaf sections were mounted on 200 mesh grids (SPI Suppliers, Philadelphia, USA). The sections on the grids were stained with uranyl acetate then washed then stained a second time with lead citrate. Stained grids were then viewed with Zeiss Libra (Libra, TEM, Zeiss, Oberkochen, Germany) at 120 kv. Digital images were imported into Corel Draw version 12 (Corel Corporation Ottawa Ontario, Canada) which was used to arrange and label images for

presentation. Images were exported as high-resolution images for incorporation into the thesis.

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Chapter 3: RWA population dynamics at ambient and elevated CO_2

3.1 Introduction:

Wheat plants containing the *Dn* genes which provide resistance to RWASA1 are susceptible to RWASA2. Since its discovery in 2007 RWASA2 was an established virulent biotype, which caused more damage to host plants compared to RWASA1. A major contributing factor to RWASA2 virulence is its much higher reproductive capacity, which leads to larger populations which may overwhelm plant defences (Tolmay et al., 2007; Jankielsohn, 2011; Jimoh et al., 2013). Subsequently two more biotypes RWASA3, discovered in 2011, and RWASA4, discovered in 2014, can be found in South Africa (SA) (Jankielsohn, 2014). However, until this thesis, the population dynamics of RWASA3 have not been determined or reported. Based on the wheat *Dn* resistance and susceptibility traits examined by the ARC, I hypothesized that RWASA3 could well be more destructive and that it could well have higher population growth rates than RWASA1. RWASA3's higher virulence ratings mean that it could possibly produce a level of damage similar to RWASA2 (Jankielsohn, 2011).

RWASA2 has been shown to have a greater population growth rate on certain resistant and susceptible barley cultivars than RWASA1 (Jimoh et al., 2013). RWASA2 is also more virulent than RWASA1 according to the ARC differential. RWASA3 has a slightly different virulence profile than RWASA2 and is not necessarily more virulent (Jankielsohn, 2014). The ARC differential if formed from data of RWA and wheat with the *Dn* genotypes, it does not include barley cultivars. It is possible that the virulence differential is inaccurate when examining the differences of RWA biotypes when they infest barley plants. RWA population dynamic experiments provide a simple yet effective way to determine RWA biotype performance and host plant resistance. On previously untested barley cultivars RWA population dynamics provide baseline data about the host/aphid interaction and highlight if the barley cultivars have any innate antibiosis traits.

Jimoh et al., (2013) showed that RWASA1 and SA2 population growth on barley is influenced by changing the CO_2 environment. Both biotypes were found to have increased population growth rates under elevated CO_2, while the effects of CO_2 enrichment on RWASA3 were unknown. Considering that little is known about barley resistance to RWA feeding it is imperative to trial untested barley cultivars and against RWA biotypes under ambient and elevated CO_2 conditions. This would provide preliminary population data of

RWA biotypes under current and future CO_2 conditions so that accurate projections can be made with respect to the RWA biotype virulence and barley resistance and viability.

3.2 Aims and objectives:

The aim of these experiments was to establish baseline population data for each of the three biotypes chosen (RWASA1, SA2 and SA3) on four previously untested barley cultivars. Two barley cultivars from SA (S5 and SSG 564) were trailed, both cultivars are economically important in the malt production industry in SA (SABBI). One of the major problems facing SA breeders is the fact that RWA resistant barley cultivars are rare and difficult to produce because of complexities with the resistance genes (Dogimont et al., 2010) and some resistant cultivars are still susceptible to more virulent RWA biotypes. S5 and SSG 564 are believed to be susceptible to RWA feeding in that they have no known inherent resistance to RWA because they were developed purely for their growth and malt production capabilities (SABBI). Two other barley accessions from Afghanistan (CIho 4125 and CIho 4159) were chosen for trials in the hope of finding evidence of potential innate resistance to RWA feeding as they are both known to possess drought resistance genes. The CIho accessions are also much older and would have possibly had long standing interactions with natural RWA biotypes, and could have potentially adapted some innate resistance to RWA feeding.

Having established the RWA population growth rate performances of the three biotypes on each of the cultivars under ambient CO_2 (380 ppm) the experiments were repeated under elevated CO_2 (450 ppm) conditions. Changes in population dynamics would infer that the aphid/host plant interactions could be directly influenced with changes in CO_2.

The populations studies reported in this thesis directly examine the performance of the aphids on the test cultivars, this allowed preliminary virulence to be studied from a different perceptive. Evidence of any innate resistance, primarily in the form of antibiosis, in the experimental cultivars was also obtained.

The results of the experiments reported in this chapter provide a comparison of aphid biotype population dynamics under the two CO_2 regimes. The RWA population data was used to calculate the rate at which population doubling time occurred, which reflects the time taken for the colony (of living aphids) to double in size. Sequential doubling times provided an indication of the relative virulence of each of the RWA biotypes on the cultivars tested. Calculated relative growth rate (RGR) enabled the cultivars to be ranked according to their

resistance (antibiosis and/or antixenosis) qualities. The Reader is referred to the experimental methodology chapter where details of the procedures are reported in section 2.4.

It was hypothesised that RWASA2 and RWASA3 would have the higher population growth rates than RWASA1, and the growth rates of all three biotypes would be further increased under elevated CO_2 conations. Furthermore, I hypothesised that the SA barley cultivars S5 and SSG 564 would support higher RWA populations than either of the Afghan CIho accessions due to the potential that they may harbour some innate RWA feeding resistance

3.3 Results:

3.2.1 Biotype population growth at ambient CO_2:

Of the three aphid biotypes tested at ambient CO_2, RWASA2 produced largest populations on all test cultivars. This was followed by RWASA3 then RWASA1 (see Fig 3.1 and Fig 3.2). The general trend amongst the biotype population growth (RWASA2>RWASA3>RWASA1), was significant ($p = <0.001$) after ANOVA analysis (see Table 3.1) at both 8 and 14 days after infestation (DAI).

Little difference was noted between CIho 4125 and CIho 4159, with respect to aphid biotype population sizes at ambient CO_2 (see Table 3.1). Similar results were recorded for RWA populations on S5 and SSG 564. The performance of three RWA biotypes appeared to be constant. It is however clear that cultivars S5 and SSG 564 appear to be less resistant to all three RWA biotypes than the CIho accessions.

Figs 3.1 and 3.2 A, C, E and G reveal that under ambient CO_2 S5 and SSG 564 sustained significantly higher aphid populations than did either CIho 4125 or CIho 4159 after 14 DAI. Over the course of the experiments when RWASA1 colonies were established on either/or CIho 4125 and CIho 4159 they only reached their 1° doubling time while colonies on S5 and SSG 564 reached their 2° doubling. RWASA3 on the CIho accessions reached 2° doubling while on S5 and SSG 564 the populations got as far as 3° doubling. RWASA2 even got as far as 4° doubling on S5 and SSG 564, but in contrast only reached 3° on the CIho accessions.

Clearly RWA biotypes performance on CIho accessions is poor compared to the data from either S5 or SSG 564. Thus it would appear that the CIho accessions may well exhibit some form of antibiosis.

Table 3.1 Summary of aphid populations after 14 DAI infestation of accessions CIho 4125 and CIho 4159 and cultivars S5 and SSG 564. Table shows average aphid population and standard deviation. The rightmost column shows the percentage difference between RWA populations under elevated and ambient CO_2.

Treatment	No. aphids at Ambient CO_2 (380ppm)	No. aphids at Elevated CO_2 (450ppm)	Percentage increase between elevated and ambient aphid populations
CIho 4125			
RWASA1	40.8 ± 12.9	167.2 ± 26.50	309.80%
RWASA2	94.8 ± 14.77	161.3 ± 13.08	70.14%
RWASA3	85.5 ± 17.1	129.2 ± 19.60	51.11%
CIho 4159			
RWASA1	41.6 ± 13.66	55.5 ± 15.13	33.41%
RWASA2	96.1 ± 9.96	187.7 ± 16.97	95.59%
RWASA3	77.1 ± 9.57	146.2 ± 16.88	89.62%
S5			
RWASA1	74.75 ± 15.02	166 ± 18.564	122.07%
RWASA2	215.75 ± 10.22	296 ± 16.943	37.2%
RWASA3	163.21 ± 21.23	208 ± 14.543	27.60%
SSG 564			
RWASA1	82.75 ± 14.34	185 ± 17.323	123.56%
RWASA2	202.00 ± 26.43	265 ± 21.223	31.19%
RWASA3	142.5 ± 25. 05	217 ± 17.096	52.28%

Table 3.2 Results of ANOVA tests for day 8 and day 14 RWA populations and results of ANOVA of relative growth rate experiments.

Univariate tests of significance for Day 8 Pop	Degr. of freedom	F value	p value
Intercept	1	2145.19	<0.001
Aphid Condition	27	60.54	<0.001
Error	223		
Univariate tests of significance for Day 14 Pop	Degr. of freedom	F value	p value
Intercept	1	4787.21	<0.001
Aphid Condition	23	190.628	<0.001
Error	216		

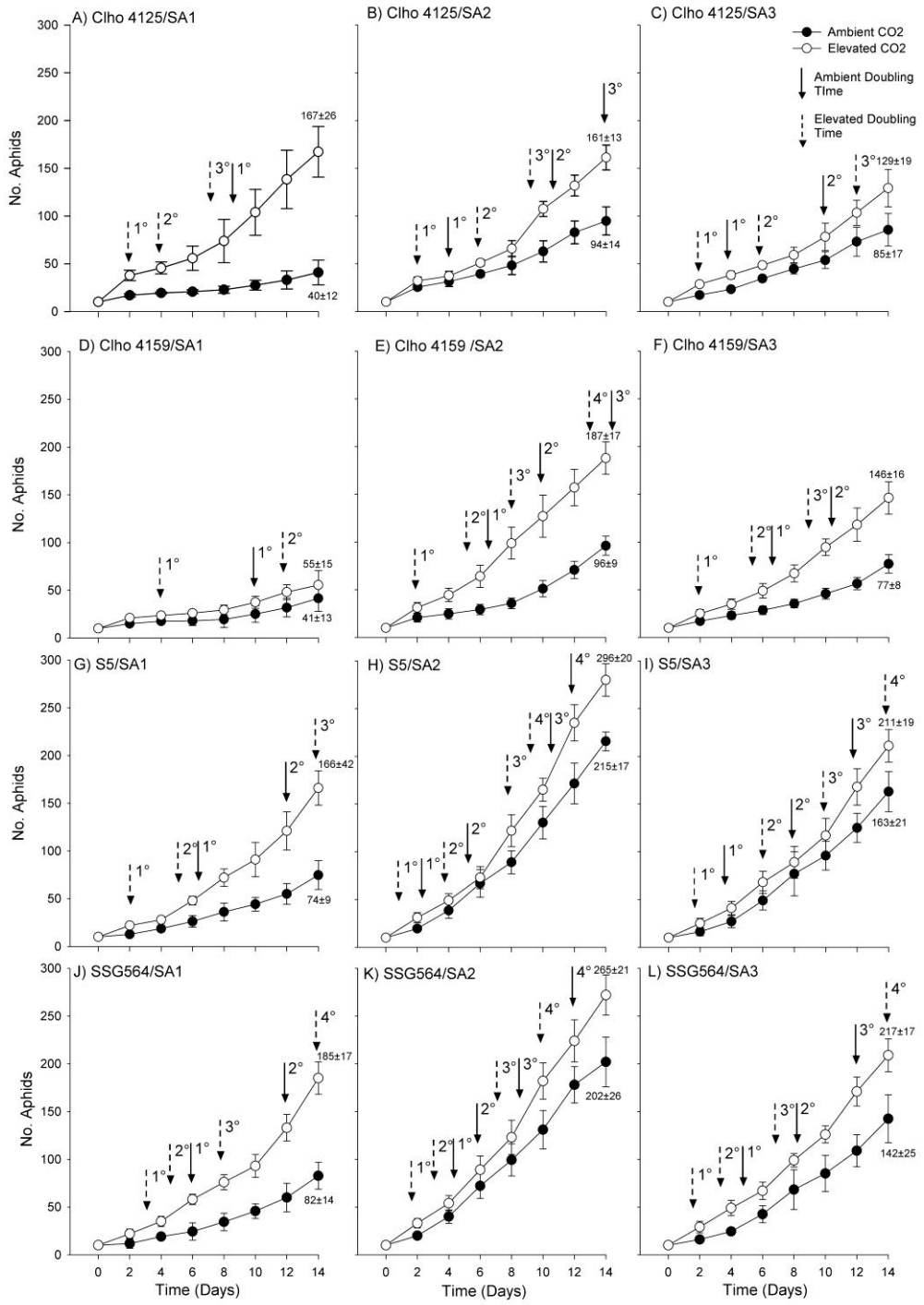

Figure 3.1 RWA population growth showing average doubling times and total number of aphids at 14 DAI (based on counts of living aphids) of RWASA1, SA2 and SA3 over 14 days on accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 at ambient and elevated CO_2 (n = 10). Solid down arrow = doubling time at ambient CO_2; dotted down arrow = doubling time at elevated CO_2, the arrows are labelled 1°- 4° depending which doubling has been reached. Final populations of the aphids are shown in small print on the last plots. Graphs A-L show the effect of CO_2 on aphid population growth: RWASA1, RWASA2 and RWASA3 infesting A-C) CIho 4125; D-F) CIho 4159; G-I) S5; J-L) SSG 564, under ambient and elevated CO_2.

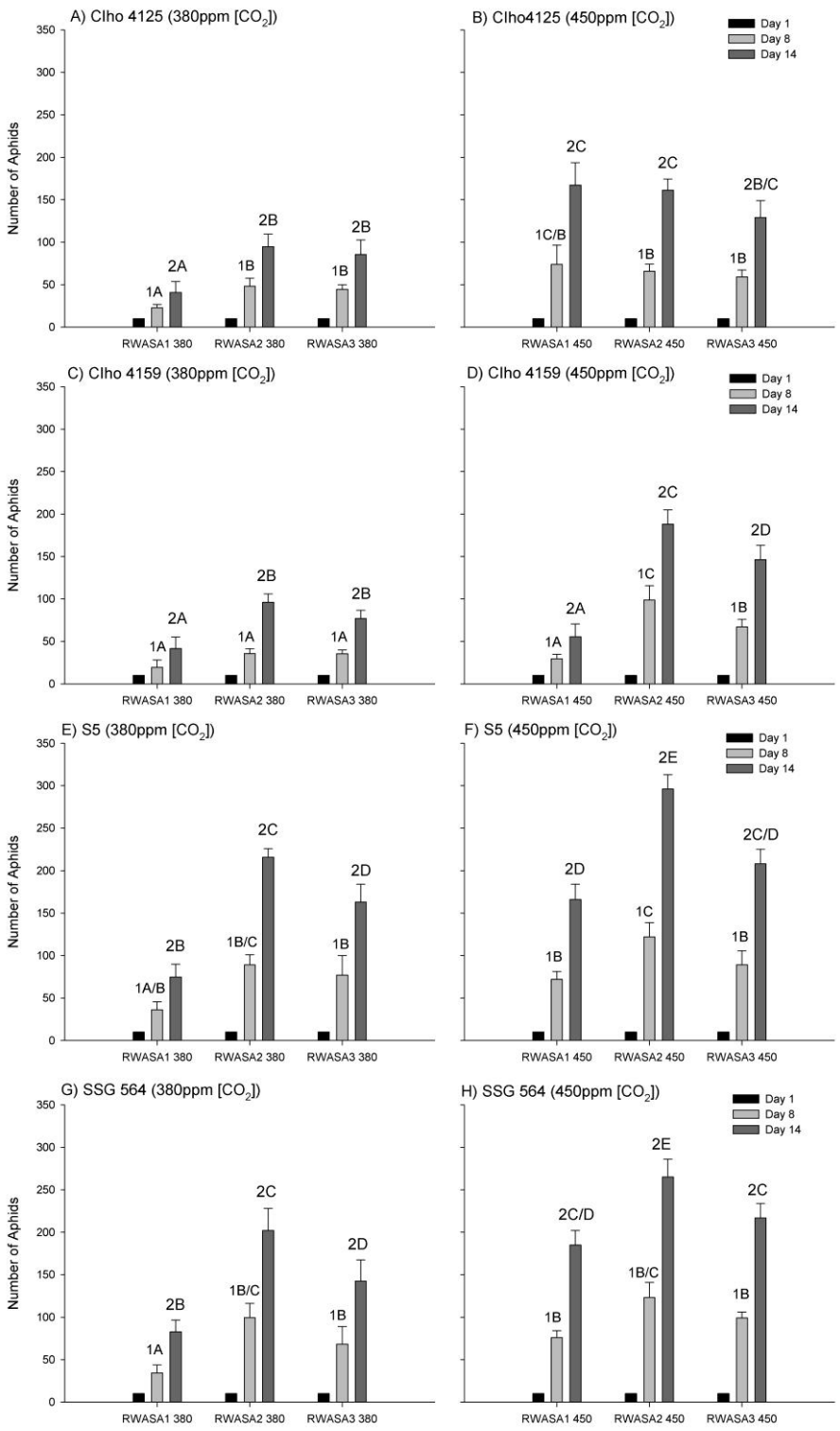

Figure 3.2 RWA average population at Day 8 and Day 14 on accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 at elevated and ambient CO2. The results of post-hoc Tukey analysis show levels of significant difference between populations. Significance levels labelled "1" refer to 8 day ANOVA results and those labelled "2" signify 14 day ANOVA results. Graphs A-H show the effect of CO_2 on RWA population growth with 8d and 14d levels of significance: RWASA1, RWASA2 and RWASA3 infesting: A-B) CIho 4125; C-D) CIho 4159; E-F) S5; G-H) SSG 564, under ambient and elevated CO_2.

3.2.2 The effects of elevated CO_2 on RWA population growth:

Figs 3.1 and 3.2 show the aphid population growth under both CO_2 regimes. The data indicates that elevated CO_2 caused an increase in aphid population in all treatments which was obvious from the first day of counting (day 2) and fast reproduction was maintained throughout the duration of the experiments. Comparison of the growth data under ambient and elevated CO_2 showed that RWASA1 was, under elevated CO_2, potentially breaking the resistance of CIho 4125. At 14 DAI plants grown under ambient conditions had an average population of 40 ± 12 aphids, whereas 167 ± 26 occurred on plants grown under elevated CO_2. This translates to an increase in population by over 300% (see Table 3.1).

Under ambient CO_2 aphid population growth rates were slower. In contrast, elevated CO_2 resulted in significant increases in population growth (p value = <0.001 see Table 3.2). For example RWASA1 saw an increase of 122-123% on S5 and SSG 564 (Table 3.1, Fig 3.1 G, J and Fig 3.2 E-H). The only exception was CIho 4159 which seemed to maintain resistance to RWASA1.

RWASA2 on S5 and SSG 564 at ambient CO_2 (see Fig 3.1 H and K) achieved its 4° doubling time by 12 DAI, but under elevated CO_2 the 4° was reached by 10 DAI. RWASA2 14 DAI populations recorded under elevated CO_2 were significantly higher than those recorded under ambient across all cultivars. The CIho accessions saw increases of 70-90%, while the SA barley cultivars saw population growth increases 31-33% (See Table 3.1).

RWASA3 infesting S5 and SSG 564 reached 4° population doubling time by 14 DAI under elevated CO_2 while under ambient CO_2 the aphid population only got a far as the 3° doubling point (see Fig 3.1 H and K). The CIho accessions infested with RWASA3 reached 3° under elevated CO_2, while only achieving 2° under ambient CO_2. There were also significantly higher RWASA3 populations under elevated than at ambient CO_2 across all cultivars, with population increases of 51-89% for the CHLO cultivars and 27-52% for S5 and SSG 564 respectively (See Table 3.1)

Of the cultivars, the CIho pair show a level of resistance, and clearly reduced aphid performance when compared to S5 or SSG 564. However, elevated CO_2 clearly contributes to resistance breaking as aphids seem to overcome the CIho plants under elevated conditions.

3.2.3 The effects of elevated CO_2 on relative growth rate (RGR):

The RGR allows standardisation of raw population data and meaningful comparisons to be made between the reproductive performances of the RWA biotypes and experimental cultivars. The RGR values are arbitrary and thus have a limited frame of reference; I therefore calculated the median of the RGR values of RWA under ambient CO_2 which rounded down to "30". Fig 3.3 A-C shows a line indicating a RGR value of 30 this represents a boundary line and should the RGR of any of the test RWA biotypes exceed this line then they could be considered virulent to the cultivar in which they were infesting. The RGR 30 line serves to provide an extra frame of reference and aid in comparing the RGR of the biotypes. The interactions between biotype and cultivar were significant, p value = <0.001 (see Table 3.3 for ANOVA results).

Under ambient CO_2 conditions all four cultivars appear to somewhat contain RWASA1 RGR below RGR 30 (Fig 3.3 A). However all varieties with exception to CIho 4159 experienced increases in RWASA1 population growth under elevated CO_2, with a doubling of populations on CIho 4125. The RGR boost conferred by elevated CO_2 seems to make RWASA1 hyper virulent to three of the four test cultivars.

On CIho 4125 and CIho 4159, under ambient CO_2, both RWASA2 and RWASA3 saw RGR values close to the RGR 30 line (see Fig 3.3 B and C), elevated CO_2 caused the RGR for both biotypes to outstrip the RGR 30 marking these biotypes as virulent, based on population performance. What little resistance CIho 4125 and CIho 4259 might have had to RWASA2 and RWASA3 was completely lost under elevated CO_2 conditions. In the remaining cases, under ambient CO_2, RWASA2 and RWASA3 mostly had RGR values averaging above the RGR 30 line, therefore they were considered virulent to the test cultivars. However, elevated CO_2 caused a further increase in RGR and thus potentially made an already virulent biotype potentially even more damaging to host plants.

In summary elevated CO_2 elicited significant changes to the reproductive performance of these aphids. RWASA1 became virulent to cultivars which showed some resistance quality, while both RWASA2 and RWASA3 became potentially more virulent under elevated CO_2.

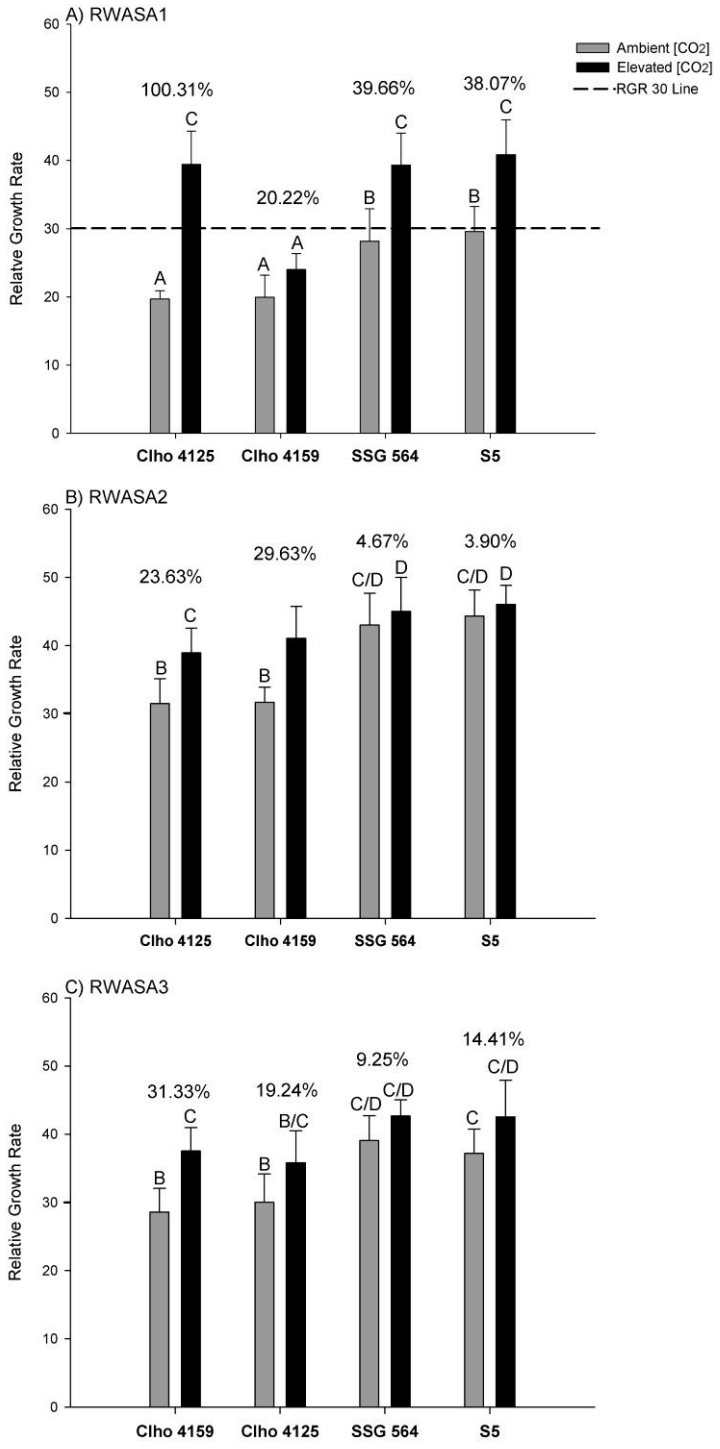

Figure 3.3 Average relative growth rate (RGR) of RWASA1, SA2 and SA3 on accessions CIho 4125, CIho 4159, and cultivars, S5 and SSG 564, at ambient and elevated CO_2.

$RGR = (\ln P_2 - \ln P_1)/(t_2 - t_1)$ P = population, t = DAI.

Percentage difference between ambient and elevated RGR is shown above the plots. Dotted line indicates RGR value of 30 (median of RWA RGR values under ambient CO_2), should RWA growth rate exceed this line then there evidence of plant susceptibility. Accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested by; A) RWASA1; B) RWASA2; C) RWASA3, under ambient and elevated CO_2.

Table 3.3 Results of ANOVA tests for relative growth rate.

Univariate Tests of significance for RGR	Degr. of freedom	F value	p value
Intercept	1	2254.335	<0.001
Aphid Condition	24	89.91	<0.001
Error	216		

3.2.4 RWA RGR as a means of ranking test cultivars:

Comparisons of RGR of each biotype allow the host plants to be ranked according the reproductive rate of the aphids infesting them. In this sense RGR gives an indication of the plants resistance, primarily through antibiosis (though antixenosis may also have a small influence), these plants would be able to withstand RWA infestations or not. Fig 3.4 shows RWA RGR for the cultivars, these are ranked from lowest to highest. These RGR values are the same as those shown in Fig 3.3 however they have been re-ordered to make presentation of cultivar ranking clearer and easier to explain. The RGR 30 line is also shown in Fig 3.4 and is derived in the same manner as in Fig 3.3.

Under ambient CO_2 the CIho accessions show significantly lower RGR across all RWA biotype treatments, with CIho 4125 performing best when infested by RWASA1 and SA2 and coming second in the ranking to CIho 4159 with RWASA3. SSG 564 and S5 were found to be insignificantly different from each other across the experimental conditions but alarmingly, neither performed well, always supporting the highest RWA RGR. This trend is carried across under elevated CO_2. When CIho 4125 was infested with RWASA1, it showed signs of total collapse of any resistance and thus is ranked 3rd of the four cultivars tested (see Fig 3.4 Graph B).

The RGR 30 lines on Fig 3.4 clearly illustrated the failure of resistance under elevated CO_2. Most alarming is that S5 and SSG 564 show virtually no resistance to RWASA2 and RWASA3 under ambient CO_2 while showing only very mild resistance to RWASA1. Under elevated CO_2 all three biotypes can be considered highly virulent to both S5 and SSG 564.

Though the CIho accessions perform better than the SA barley cultivars they too are at severe risk under elevated CO_2. With RWASA2 and RWASA3 having increased virulence to both Afghan cultivars and CIho 4125 resistance to RWASA1 completely collapsing, CIho 4159 is

the only cultivar to show any resistance quality under elevated CO_2 and that is to only one biotype; RWASA1.

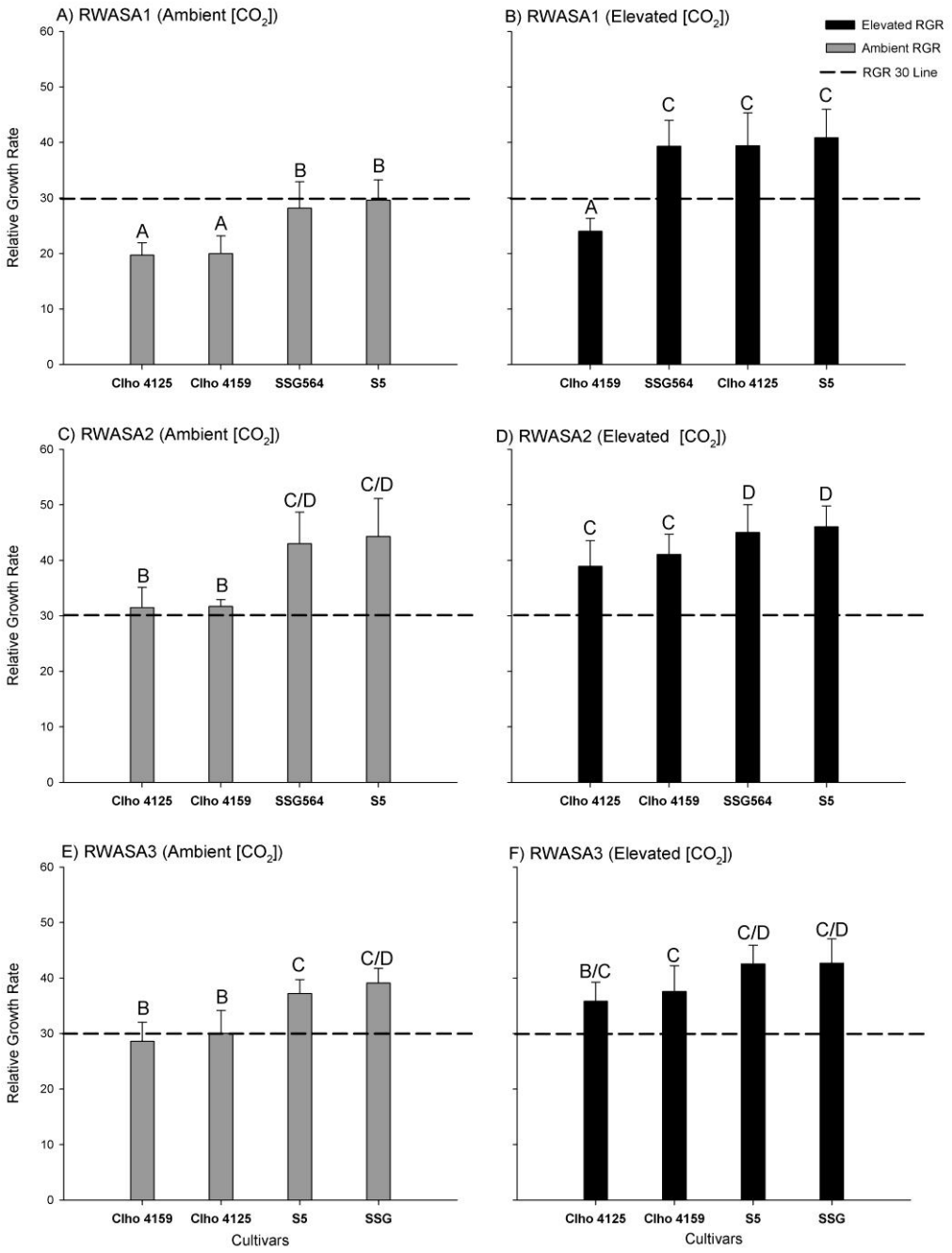

Figure 3.4 Ranking of CIho 4125, CIho 4159, S5 and SSG 564 according to RGR of RWA biotypes RWASA1, SA2 and SA3 at ambient and elevated CO_2. The graphs shows the results of Post-hoc Tukey results to show levels of significance between plots, n=10. Dotted line indicated RGR value of 30 (median of RGR values under ambient CO_2), should RWA growth rate exceed this line cultivar susceptibility is suggested. Graphs A-H showing ranking of test plants according to RWASA1, SA2 and SA3 RGR. Accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested by; A-B) RWASA1; C-D) RWASA2; E-F) RWASA3, under ambient and elevated CO_2.

3.4 Discussion

3.4.1 RWA biotypic differences

The data presented in this chapter show that biotype RWASA2, presents by far the greatest threat to the SA barley industry as it generally appears to be a more aggressive feeder and the host plants sustain larger colonies compared with those of RWASA1 or RWASA3. This conclusion supports the results of Jimoh et al., (2013) where RWASA2 was shown to have a higher reproductive rate than RWASA1 and that it could successfully infest potentially resistant barley cultivars. With respect to the data presented in this chapter the concept of host plant resistance is explored in terms of aphid population sizes, thus an accession or cultivar that supports a larger RWA colony is considered less resistant than one that is infested with fewer RWA. RWASA2 has also been reported to successfully colonise many of the resistant wheat cultivars that have been deployed including those bred with *Dn* 1-3, 8, 9 genes (Tolmay et al., 2007; Jankielsohn, 2014).

The differential utilised by the ARC to determine RWA biotypes suggests that RWASA3 is not as virulent as RWASA2 but that it is more virulent than RWASA1 (Jankielsohn, 2011), the findings presented in this thesis support this, as RWASA3 population growth rate fell between RWASA2 and SA1. Differences in fecundity between the biotypes have been previously documented in the USA with biotype RWAUSA2 having a higher population growth rate than does biotype RWAUSA1 (see Qureshi and Michaud, 2005; Qureshi et al., 2005; Jyoti and Michaud, 2005).

3.4.2 The effects of host cultivar of RWA populations under ambient CO_2

Whilst the general biotypic trend differs little across cultivars, the overall population size and RGR of the RWA biotypes were typically higher on S5 and SSG 564. It is of concern that there have been no attempts made yet, to breed resistance into any of the currently grown SA barley cultivars. As S5 and SSG 564 are susceptible to RWA feeding, it is expected that they would carry higher aphid populations and that the RWA will inflict heavy crop losses. The RGR data shows some support for the USDA resistance classification of the CIho accessions. The Afghan accessions may well harbour antibiosis quality as they consistently outperformed the SA barley in all cases.

3.4.3 The effect of elevated CO_2 on RWA population dynamics

The data presented in this thesis reveal that elevated CO_2 is responsible for an increase in RWA population growth. This is most likely due to enhanced host plant photosynthetic rate which would improve the food supply available to probing RWA. Under elevated CO_2 the host plants will undergo numerous biochemical changes attributable to and as a direct result of increased photosynthesis. Changes such as increases in photo-assimilate stream capacity, the reallocation of plant resources and shifts in C:N ratio, will all contribute to change the nutrient status of the host plant from the RWA perspective (Smith and Boyko, 2007; Sun and Ge, 2011).

In general the trends observed between the aphids and cultivars at ambient remained much the same at elevated CO_2, though at a significantly higher scale. The only exception was CIho 4125 infested with RWASA1 at elevated CO_2 where the RGR of the aphid more than doubled. Though I have argued that CIho 4125 may have some resistance qualities the elevated CO_2 data suggests a collapse of resistance and thus CIho 4125 performs poorly under 450ppm CO_2. Michaud et al., (2006) have shown that the larger an aphid colony the higher the fitness of the individual aphids. This could be the reason elevated CO_2 facilitates huge colonies of RWA, particularly on CIho 4125, as the host plants provide a more suitable dietary solution at the start of the infestation, therefore the fitness of the pioneer colony is high which creates a positive feedback loop. The aphid population then grows, with a colony comprised of high fitness nymphs which eventually overcome any host plant resistance.

3.4.5 Conclusion

The data presented in this chapter is thus, RWASA2 has the greatest reproductive capacity, in line with the ARC differential and can be considered the most virulent biotype, followed by RWASA3 then RWASA1. The SA cultivars S5 and SSG 564 perform poorly showing little resistance if any at ambient CO_2 and this is then exacerbated at elevated CO_2 as the cultivars become hosts to huge RWA populations. Whilst the CIho accessions certainly show a measure of resistance to RWASA1 feeding, they are susceptible to RWASA2 and SA3. Elevated CO_2 leads to a breaking of resistance of RWASA1 on CIho 4125. Further examination into the plant response and the effects of aphid feeding is required to fully confirm this, it is covered in greater detail in the following chapters. Findings of plant damage and leaf roll caused by RWA feeding are presented in Chapter 4, and the effects of RWA feeding on host plant N metabolism are presented in Chapter 5. Chapter 6 presents

microscopy findings detailing the effects of RWA feeding on wound callose deposition as well as the effects of RWA saliva on cell ultra-structure.

In conclusion and irrespective of data analysis the evidence is clear: elevated CO_2 will undoubtedly have the potential to cause rapid spread of large colonies of RWASA1-SA$^{\chi}$ with potentially devastating results and impacts on an already threatened industry.

Chapter 4: RWA feeding related damage and plant biomass loss

4.1 Introduction

RWA biotypes are initially identified by assessing the effect they have on selected wheat cultivars, i.e. if one RWA population is able to effectively feed and cause damage to a known resistant cultivar while another population cannot then they are defined as separate biotypes.

In effect, little attention is paid to the performance (population growth rates) of the aphids themselves but rather the focus is on the damage they inflict on the host plants (Jyoti and Michaud, 2005; Tolmay et al., 2007; Jankeilsohn, 2011). The ARC virulence differential was created by screening the resistance or susceptibility of series wheat cultivars containing of *Dn* genotypes ranging from *Dn*1-9, *Dnx* and *Dny* to indentify the separate RWA biotypes via their *Dn* virulence profile (Jankielsohn, 2014). The four RWA biotypes currently found in South Africa (SA) have unique virulence profiles for instance, *Dn* 1, 4-9, x and y genotypes are resistant to RWASA1, while *Dn* 4-7, *x* and *y* are resistant to RWASA2 (Jankielsohn, 2014). Thus it is through wheat resistance and susceptibility that the different RWASA biotypes have been indentified and studied.

Irrespective of the fact that current biotypes are identified based on their interactions with wheat, there is very little known about the virulence of these RWA biotypes on barley. For example, RWASA2 has been shown to have a higher population growth rate, as well as the potential to inflict more damage on barley cultivars than RWASA1 (Jimoh et al., 2011a; Jimoh et al., 2013). As a result, RWASA2 was considered more virulent than RWASA1 on barley. However, nothing is known about the virulence of RWASA3 on barley. Unlike wheat genetic resistance to RWA, in barley cultivars this is not governed by a single dominant gene, in fact resistance is polygenic with quantitative trait loci (Dogimont et al., 2010). Resistant barley cultivars are therefore rare and not actively engineered as are wheat strains (Dogimot et al., 2010). Thus it is imperative to establish a baseline of which barley cultivars have any innate resistance to RWA feeding so that breeding programs could be used to potentially create RWA resistant barley cultivars.

RWA feeding causes progressive chlorosis and leaf roll, and it is these symptoms that are used to screen cultivars for resistance of susceptibility (Hewitt et al, 1984; Burd et al., 1993; Saheed et al., 2007a, 2007b; Tolmay et al., 2007; Jimoh et al 2011a; Jimoh et al 2013). Chlorosis is believed to be caused by increased Mg-dechelatase activity (Jyoti and Michaud,

2005) which causes accelerated chlorophyll catabolism resulting in bleached streaks appearing on leaves. It is also just as likely that chlorosis and leaf roll can be induced as a result of vascular damage (Saheed et al., 2007a) Leaf roll is another consequence of RWA feeding which results from prolonged probing and, the leaf then enfolds the RWA colony in a protected microhabitat (Jyoti and Michaud, 2005; Qureshi and Michaud, 2005).

Screening chlorosis and leaf roll provides insight into the virulence of the RWA biotypes. However when this data together with host plant biomass is examined a more accurate assessment of the drain that the RWA feeding effects on the host plants is obtained. Plant biomass also provides useful information about the potential innate resistance that the cultivars may have, as was shown in Jimoh et al., (2013) where the different RWA biotypes were shown to cause different biomass losses in barley cultivars.

In the previous chapter I hypothesized that, based on the virulence differentials utilised by the ARC, that RWASA3 would have higher populations than did RWASA1 on the same cultivar. The results of that study confirmed this hypothesis; the logical step therefore was to test the damage rating scales of chlorosis and leaf roll (Jimoh et al., 2011b) to see if it could be cross-compared with ARC virulence differential. This would reveal if there are any similarities in the wheat based differential profiles to those which appeared in barley cultivars (see Tolmay et al., 2007; Jankiesohn, 2011 for method). In order to obtain a clearer understanding of the barley/RWA interaction without preconceptions and definitions based on wheat/RWA interaction it was imperative to cross compare the RWA population findings with barley damage ratings and determine if the currently accepted virulence profiles would be useful on barley.

The biotype classification screenings were carried out under ambient CO_2 conditions, Jimoh et al., (2013) showed that both RWASA1 and RWASA2 had increased population growth rates under elevated CO_2. Both biotypes were also shown to inflict more damage to their host plants under elevated CO_2. Given that biotype virulence is based on the damage that the aphids inflict on their hosts it followed that changes in CO_2 environment it would affect RWA biotype virulence/ and or the resistance qualities of the host plants. Given these findings it became clear that baseline data on the current and potential future virulence of the RWA biotypes with respect to barley cultivars was necessary.

4.2 Aims and objectives

The aim of these experiments was to determine if the current virulence profiles of the RWA biotypes used for wheat could be applied to the four test barley cultivars. Given that RWASA2 is known to be more virulent than RWASA1 on barley (Jimoh et al., 2013) and that the virulence of RWASA3 on barley cultivars was unknown, experiments to test this were set up (see Chapter 2 for methods). Chlorosis and leaf roll data were collated with plant biomass data to determine the virulence of the RWA biotypes and the resistance/susceptibility of two commonly deployed SA malting barley cultivars and two drought resistant Afghan cultivars.

Once the virulence of the biotypes and the resistance qualities of the experimental cultivars under ambient CO_2 the experiments had been established, the experiments were then repeated under elevated CO_2 to investigate potential changes to the virulence/resistance profiles of either the aphids or the barley cultivars.

The hypothesis driving this particular investigation was that there would be differences in the damage that the three RWA biotypes inflict on the experimental cultivars. Furthermore, the biotypes that inflict the greatest damage would also cause the greatest biomass loss in the barley cultivars. Given their drought resistance the Afghan barley were investigated to test if they held some innate RWA resistance and thus, if they had it would result in decreased damage and biomass loss compared to either of the SA cultivars. Finally, I hypothesize that baseline damage profiles obtained under ambient CO_2 would not be reflected under elevated CO_2, as the interactions between RWA and the barley plants would differ with increased CO_2 and thus biotype virulence and host plant resistance would change.

4.3 Results

4.3.1 Host plant damage- chlorosis and leaf roll ratings under ambient CO_2

Table 4.1 shows the results of the chlorosis and leaf roll studies. From the data it is clear is that RWASA2 induced more damage than RWASA1 and RWASA3 across all varieties tested. This is borne out by the chlorosis and leaf roll ratings of RWASA2 infested plants show that it was the most damaging biotype, followed by RWASA3 then RWASA1. The symptoms of chlorosis only became evident on the test plants from 14 days after infestation (DAI) and that by day 21 the damage related differences between the biotypes became more pronounced and that they followed the trend of RWASA2>RWASA3>RWASA1 (see Table 4.1).

The experimental data clearly showed that across all the cultivars, the overall degrees of damage differed between the cultivars as well. The SA cultivars S5 and SSG 564 sustained the most damage followed by CIho 4125 and CIho 4159. At 28 DAI both S5 and SSG 564 plants infested with RWASA2 had reached critical damage levels and were rated A9B3, here the host plants were almost totally chlorotic, and that photosynthetic area was severely limited. In addition leaves were also tightly rolled; the plants were either dead or in state of no possible recovery. CIho plants under the same conditions were rated A7B2 after 28 DAI, indicating 70% chlorosis and loosely folded leaves. Though these plants can scarcely be considered healthy, they did last longer than their SA counter-parts. RWASA1 infestation resulted in scores of A7B2 and A6B2 for SSG 564 and S5 respectively while CIho 4125 and CIho 4159 were both scored at A5B1. The original hypothesis that the Afghan cultivars had innate RWA resistance was correct as damage was limited to chlorosis cover of 40% and no apparent leaf roll.

RWASA3 did cause more damage than RWASA1. RWASA3 infested SSG 564 and S5 plants were severely damaged with ratings of A8B3 and A8B2 respectively, this signified 85% chlorosis and advanced leaf roll. Again the CIho accessions fared slightly better but still showed the symptoms of RWA feeding with ratings of A6B2 (55% chlorotic cover) for both cultivars (see Table 4.1)

From the above it is clear that that S5 and SSG 564 appear to be susceptible cultivars hence they also sustained the most damage. The better performance of the CIho accessions certainly suggests that they have some inherent resistance qualities especially to RWASA1, however, only under ambient CO_2.

4.3.2 The effect of elevated CO_2 on chlorosis and leaf roll

At ambient CO_2 the biotype damage trend was RWASA2<RWASA3<RWASA1 it is clear that the CIho accessions survive better than the SA barley cultivars. Table 4.1 shows that under elevated CO_2 the damage which the host plants sustained was higher than at ambient CO_2. Additionally varieties which showed poor resistance performance at ambient CO_2 showed signs of significant damage from 14 days of infestation under elevated CO_2 conditions. Alarmingly S5 and SSG 564 infested with RWASA2, showed signs of dying (ratings of A9B2 and A8B2) at 21 DAI under elevated CO_2, and by 28 DAI the plants had long since succumbed to the RWA feeding. RWASA3 and RWASA1 were also highly

damaging to S5 and SSG 564 under elevated CO_2. By 28 DAI S5 and SSG 564 reached damage ratings of A8B3 and A9B3 from RWASA3 and A7B2 and A8B2 from RWASA1.

The CIho accessions also suffered under elevated CO_2 as both RWASA2 and RWASA3 effectively killed their host plants. However, CIho 4159 did maintain a semblance of resistance against RWASA1 under elevated CO_2 with a damage score of A6B1. CIho 4125 on the other hand showed a complete breakdown of resistance to RWASA1. Under ambient CO_2 CIho 4125 scored A5B1 after 28 days however under elevated CO_2 RWASA1 caused increased damage resulting in a score of A8B2 signifying that the plant was in a state close to death. The data and trends represented here mirror the biotypic trends observed from the previous chapter. Additionally the increased damage that the host plants sustain under elevated CO_2 mirrors the data reporting the effects of elevated CO_2 on increased aphid population growth.

Table 4.1 Average chlorosis and leaf roll ratings of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564, infested by RWASA1, SA2 and SA3 at ambient and elevated CO_2, taken at seven day intervals, n = 10. The letter "A" is representative of chlorosis and the letter "B" represents leaf roll.

Treatment	2 days	7 days	14 days	21 days	28 days
CIho 4125 at 380ppm					
RWASA1	A0B1	A0B1	A1B1	A3B1	A5B1
RWASA2	A0B1	A1B1	A2B1	A5B1	A7B2
RWASA3	A0B1	A1B1	A2B1	A4B1	A6B2
CIho 4125 at 450ppm					
RWASA1	A0B1	A2B1	A3B1	A6B1	A8B2
RWASA2	A0B1	A2B1	A3B1	A7B2	A9B3
RWASA3	A0B1	A2B1	A3B1	A6B2	A8B2
CIho 4159 at 380ppm					
RWASA1	A0B1	A0B1	A2B1	A3B1	A5B1
RWASA2	A0B1	A1B1	A2B1	A4B1	A7B2
RWASA3	A0B1	A1B1	A2B1	A3B1	A6B2
CIho 4159 at 450ppm					
RWASA1	A0B1	A1B1	A2B1	A3B1	A6B2
RWASA2	A0B1	A3B1	A4B1	A6B2	A9B3
RWASA3	A0B1	A2B1	A3B1	A6B1	A8B2
S5 at 380ppm					
RWASA1	A0B1	A1B1	A2B1	A4B1	A6B2
RWASA2	A0B1	A4B1	A6B2	A7B2	A9B3
RWASA3	A0B1	A3B1	A4B2	A6B2	A8B2
S5 at 450ppm					
RWASA1	A0B1	A2B1	A4B2	A5B2	A7B2
RWASA2	A0B1	A5B1	A7B2	A9B2	A9B3
RWASA3	A0B1	A4B1	A5B2	A7B2	A8B3
SSG 564 at 380ppm					
RWASA1	A0B1	A2B1	A3B1	A5B2	A7B2
RWASA2	A0B1	A3B1	A6B2	A8B2	A9B3
RWASA3	A0B1	A3B1	A5B2	A7B2	A8B3
SSG 564 at 450ppm					
RWASA1	A0B1	A3B1	A5B2	A6B2	A8B2
RWASA2	A0B1	A5B2	A8B2	A9B3	A9B3
RWASA3	A0B1	A4B2	A6B2	A8B3	A9B3

4.2.3 Cross correlation of chlorosis/leaf roll damage rating to ARC grading system

As mentioned previously, the resistance screening system utilised by the ARC measures the performance of RWA infested plants and then categorises them into resistant, moderately resistant or susceptible classes (Tolmay et al., 2007). The chlorois and leaf roll data functions in the same way and thus allows direct comparisons to be made and facilitates better descriptions of the plant resistance quality. The rating system used in this thesis is essentially the same as that used by the ARC and thus it is possible to use our rating system with the

ARC definitions of host plant resistance (Tolmay et al., 2007). A merger of the ARC definitions and the resistance qualities of the experimental barley varieties used here can be equated at least to the ambient CO_2 data (see Figure 4.2). It is important to note that in terms of these experiments resistance simply means that the plants will survive longer. One must take into account that the aphids are confined to one plant. Given this limitation as the aphids are infesting singular plants the resistance quality of these host plants is limited to 28 DAI.

After determining the resistance qualities of these plants using the ARC ratings, the biotype trends described by the population and chlorosis/leaf roll data remained robust when comparing biotype virulence and plant resistance qualities (see Table 4.2). Based on this analysis, both the CIho accessions were classified as moderately resistant to RWASA1; however both were susceptible to RWASA2 and SA3 under ambient CO_2. S5 and SSG 564 were susceptible to all three biotypes and 28DAI RWASA2 infestation killed SSG 564.

That elevated CO_2 conditions lead to resistance breaking is very clear. For example, RWASA1 infesting CIho 4125 under ambient conditions was classified as moderately resistant however under elevated CO_2 its classification changed to susceptible, these classifications certainly mirror the RWASA1 population data findings (Tables 4.1 and 4.2). The effect that elevated CO_2 had on plant damage was striking as RWASA2 damage was intensified to killing the host plant, often regardless of variety, usually outright by 28 DAI (Tables 4.1 and 4.2). RWASA3 also experienced increased virulence and SSG 564 plants suffered a similar fate by 28 DAI.

Table 4.2 Inherent resistance of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested by RWASA1, SA2 and SA3 at ambient and elevated CO_2. Resistance terms are based on damage rating from day 28 post infestation cross compared with damage rating system utilised by ARC for determination of RWA biotype profiles. Should the plants sustain little to no damage after 28 DAI then they would be classified as "resistant", and a damage rating from A4B1 to A6B1 was designated as moderately resistant, any damage ratings higher were classified as susceptible. Aphid virulence is shown is opposition to plant resistance (denoted by italics). The aphid population at 14 DAI is shown in brackets.

Treatment	Host plant performance/ aphid virulence based on ARC damage conversion	
	Ambient CO_2	Elevated CO_2
CIho 4125 RWASA1	Moderately Resistant/ *Non-virulent* (40 ± 12)	Susceptible/ *Virulent* (167 ± 26)
RWASA2	Susceptible/ *Virulent* (94 ± 14)	Susceptible (plant death)/ *Virulent* (161 ± 13)
RWASA3	Susceptible/ *Virulent* (85 ± 17)	Susceptible/ *Virulent* (129 ± 19)
CIho 4159 RWASA1	Moderately Resistant/ *Non-virulent* (41 ± 13)	Susceptible/ *Virulent* (55 ± 15)
RWASA2	Susceptible/ *Virulent* (96 ± 9)	Susceptible (plant death)/ *Virulent* (187 ± 17)
RWASA3	Susceptible/ *Virulent* (77 ± 8)	Susceptible/ V*irulent* (146 ± 16)
S5 RWASA1	Susceptible/ *Virulent* (74 ± 9)	Susceptible/ *Virulent* (166 ± 42)
RWASA2	Susceptible (plant death)/ *Virulent* (215 ± 17)	Susceptible (plant death)/ *Virulent* (296 ± 20)
RWASA3	Susceptible/ *Virulent* (163 ± 21)	Susceptible/ *Virulent* (211 ± 19)
SSG 564 RWASA1	Susceptible/ *Virulent* (82 ± 14)	Susceptible/ *Virulent* (185 ± 17)
RWASA2	Susceptible/ *Virulent* (202 ± 26)	Susceptible (plant death)/ *Virulent* (265 ± 21)
RWASA3	Susceptible/ *Virulent* (142 ± 25)	Susceptible (plant death)/ *Virulent* (217 ± 17)

4.2.4 The effect of elevated CO_2 on control plant biomass

Having clearly established that RWA feeding did more damage to host plants under elevated CO_2, further investigation into the complex interaction between aphid and host were required. The current definitions of resistance and susceptibility are subject to change depending on CO_2 environment, the effects of aphid feeding on plant biomass and nitrogen metabolism would perhaps shed more light on complex barley/RWA interactions. Below are the findings of the biomass study and the following chapter refers the reader to experimental plant C:N and %N results.

Figure 4.1 shows the dry biomass of control plants exposed to ambient and elevated CO_2. Results of ANOVA (p value = <0.001) show that [CO_2] had a significant influence on plant dry biomass. Across the four varieties, the elevated CO_2 groups had significantly higher dry biomasses than the plants at ambient CO_2. It was possibly a result of selective breeding that the SA cultivars produced almost twice as much biomass as either of the Afghan accessions under ambient CO_2. In fact the biomass accumulated by the CIho accessions under elevated CO_2 was approximately the same as the biomass of S5 and SSG 564 under ambient conditions. Under elevated CO_2 the biomass increase of the SA cultivars was not boosted to the same extent as the Afghan accessions, however they still accumulated significantly more biomass (see Table 4.3)

Table 4.3 shows that the enriched CO_2 environment caused the CIho 4125 and CIho 4159 biomass to increase by 75% and 114 % respectively. Additionally elevated CO_2 caused further accumulation of biomass by S5 and SSG 564 cultivars resulting in increases of 45% and 30% respectively.

Table 4.3 Average dry biomass and percentage biomass difference between control (uninfested) accessions CIho 4125, CIho 4159, S5 and cultivars SSG 564 at ambient and elevated CO_2.

Cultivar	Dry biomass at Ambient CO_2 (grams)	Dry biomass at elevated CO_2 (grams)	Percentage increase from ambient to elevated CO_2
CIho 4125	0.784±0.104	1.378±0.124	75.77%
CIho 4159	0.542±0.140	1.164±0.218	114.76%
S5	1.2±0.177	1.75±0.125	45.83%
SSG 564	1.4±0.139	1.83±0.175	30.71%

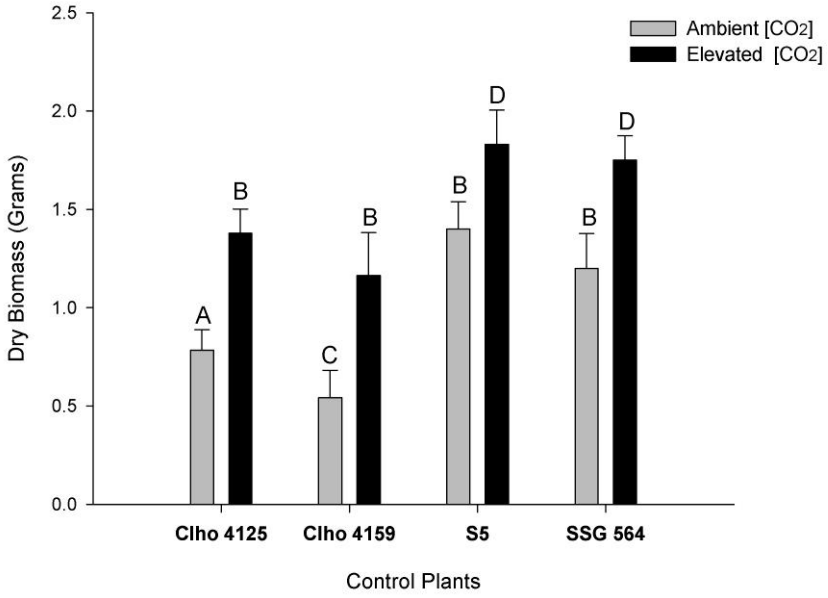

Figure 4.1 Average dry biomass of control plants CIho 4125, CIho 4159, S5 and SSG 564 at ambient and elevated CO2. Letters above the plots denote significant differences reported by post-hoc Tukey results. CIho accessions produced the lowest biomass, while the SA barley's had significantly higher biomasses under ambient CO_2. Elevated CO_2 caused significant increase in biomass for all accessions. The biomasses produced by the CIho accessions were still significantly smaller than those of S5 and SSG 564 under elevated CO_2.

4.2.4 The effect of RWA infestation on plant dry biomass:

As much as elevated CO_2 boosted the control plants, RWA infestation was found to cause significant reductions in plant biomass (p value = <0.001, see Table 4.5) under elevated CO_2. Figure 4.2 clearly demonstrates the extent in which RWA infestation inhibits biomass accumulation. Recorded biomass values show limited differences in terms of significance between the accessions however when this is coupled with the percentage decreases caused by RWA feeding it becomes alarming (see Figure 4.2 and Table 4.4)

RWA feeding at elevated CO_2 resulted in all varieties having lower biomasses compared with the biomass values recorded under ambient CO_2. Table 4.4 shows that S5 and SSG 564 suffered the greatest loss of biomass ranging from 73-85% depending on biotype infestation at ambient CO_2. The trends observed in the population and damage studies are once again confirmed here with RWASA2 causing the greatest biomass decline followed by RWASA3 then RWASA1. Elevated CO_2 exacerbated the trends observed at ambient, and the biomass decline for S5 and SSG 564 was increased, ranging from 84-91%.

Interestingly, CIho 4159 had the smallest biomass loss. RWA infestation under ambient CO_2 caused CIho 4159 a biomass loss of 22-56%, which then increased to 69-85% under elevated CO_2. CIho 4125 performed better than either S5 or SSG 564 under ambient CO_2, where RWA feeding caused losses ranging from 60-87%. However in under elevated CO_2 CIho 4125 had massive losses in accumulated biomass by as much as 92%. These results showed that though CIho accessions were better suited to withstand RWA feeding under ambient CO_2 than either of the SA cultivars they were affected. Elevated CO_2 seemed to amplify the effects of RWA feeding but at the same time it maintained the general trends observed by the population and damage studies.

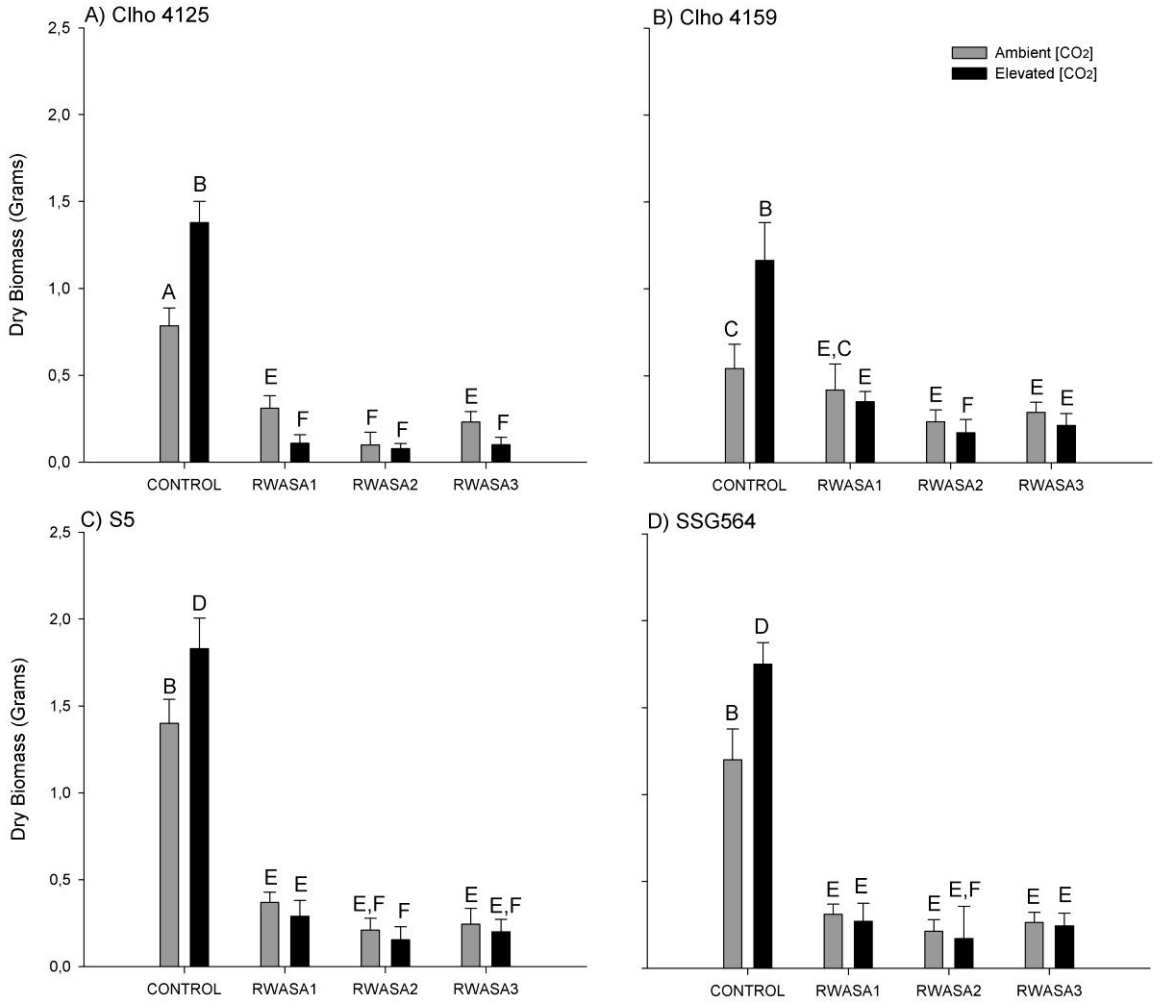

Figure 4.2 Average dry biomass of accessions CIho 4125, CIho 4159, S5 and SSG 564 infested by RWASA1, SA2 and SA3 at ambient and elevated CO_2. Post-hoc Tukey results depicted as letter above the plots show levels of significant difference.

Figure 4.2 Legend continued:

Graphs A-D show the effect of elevated CO_2 and RWA feeding on host plant biomass: RWASA1, RWASA2 and RWASA3 infesting: A) CIho 4125; B) CIho 4159; C) S5; D) SSG 564, under ambient and

elevated CO_2.

Table 4.4 Percentage decrease in plant dry biomass of RWA infested plants against uninfested control plants for accessions CIho 4125, CIho 4159, S5 and SSG 564.

Treatment	Percentage decrease in infested plant biomass from control plant biomass	
	Ambient CO_2	Elevated CO_2
CIho 4125		
RWASA1	↓60.46%	↓92.16%
RWASA2	↓87.37%	↓94.34%
RWASA3	↓70.66%	↓92.74%
CIho 4159		
RWASA1	↓22.88%	↓69.93%
RWASA2	↓56.46%	↓85.22%
RWASA3	↓46.68%	↓81.70%
S5		
RWASA1	↓73.57%	↓84.15%
RWASA2	↓85.00%	↓89.34%
RWASA3	↓82.50%	↓89.01%
SSG 564		
RWASA1	↓74.17%	↓84.57%
RWASA2	↓82.33%	↓91.18%
RWASA3	↓78.00%	↓86.00%

Table 4.5 Results of ANOVA test of dry biomasses of control and experimental plants.

Univariate tests of significance for Biomass	Degr. of freedom	F value	p value
Intercept	1	331.774	<0.001
Exp Condition	22	154.547	<0.001
Error	243		

4.3 Discussion

4.3.1 Resistance qualities of the experimental accessions under ambient CO_2

The trends reported in Chapter 3 between the RWA biotype populations are supported by the plant damage and biomass results. Population data (Chapter 3) suggested that S5 and SSG 564 are very likely to be susceptible cultivars. Under ambient CO_2 the CIho accessions appear to have some resistance to RWASA1, and CIho 4159 sustained less damage and also lost the least biomass with RWASA1 infestation under ambient CO_2. It can be concluded that

RWASA1 was not virulent to either of the CIho accessions under ambient CO_2. However both CIho accessions were susceptible to RWASA2 and RWASA3.

The RWA that initially invaded SA and became designated RWASA1 originated in Asia, it is highly likely that the landraces in which the CIho accessions evolved had prolonged interactions with RWA (El Bouhssini et al 2011; Turanli et al. 2012; Zhang et al., 2014). It is therefore, likely that the current CIho accessions have inherited some resistance to RWA, which would account for RWASA1 not being virulent on these two accessions. Both wheat and barley have wild ancestors in modern day Afghanistan (Liu et al., 2001; El Boussini et al., 2011). The USDA has found that there are some Afghan landraces which contain the RWA resistance gene *Dnx* (Smith et al., 2010). Zhang et al., (2012) argue that host plant adaptation is common in regions where there is interaction between the native populations of aphid and host plants; hence it is probable that the CIho accessions are better adapted to RWA.

That the SA barley cultivars S5 and SSG 564 performed poorly, regardless of which biotype infests them is of great concern, as both damage and the biomass data indicate that these cultivars possessed limited inherent resistance qualities under ambient CO_2. For example S5 is completely overcome by RWASA2 under ambient CO_2 and by 28 DAI the plants had died. The S5 and SSG 564 cultivars were created specifically for the beer brewing qualities of their grain and their yield, hence the greater biomass accumulation of the control plants when compared to the CHLO controls. It can be concluded that S5 and SSG 564 are not adapted to RWA feeding and are fully susceptible and at severe risk to all three of the SA RWA biotypes tested.

4.3.2 The effect of elevated CO_2 on host plant resistance

The significant effect that elevated CO_2 had on aphid population mirrors the damage and biomass results. Clearly elevated CO_2 will exacerbate the effect that RWA feeding on host plants. Increased damage will result along with and lower biomasses accumulation, with a similar biotypic trend to ambient CO_2, but enriched CO_2 conditions increase the damage effect related to RWA feeding. All three RWA biotypes become more virulent on all of the host plants.

This is most likely as a result of increased photosynthetic rates (Balaguer et al., 1995; Bezemer et al., 1998; Amthor, 2001; Högy et al., 2010). Furthermore, evidence exists to show that under normal fertilization regimes, elevated CO_2 causes an increase in vegetative growth

and grain yield by as much as 10-16% (Manderscheid et al., 1995; Sæbø and Mortensen, 1996; Högy and Fangmeier, 2008; Högy et al., 2010). However, without N supplementation the standard fertilizer regime would (eventually) result in photosynthetic down-regulation under elevated CO_2 as plants will become N limited (Stitt and Krapp, 1999). The plants in these experiments were supplied with nutrient solution every two days to avoid N and other nutrient stress. Clearly the elevated CO_2 environment changes the dynamics of the RWA/barley interaction. Porter et al., (1991) Hunter, (2001), and Coviella and Trumble, (2000) suggest that increased biomass loss is a direct result of changes in that favour aphid fecundity and propagation and thus more insects exact a greater toll on the host plant resources.

Jimoh et al., (2013) found that aphid feeding caused massive losses of biomass under elevated CO_2 which mirrors the findings of our experiments. Elevated CO_2 conditions made a bad scenario under ambient CO_2 worse. Under ambient conditions, without the use of pesticides, the threat that RWA poses under elevated CO_2 is significantly increased. More biomass was lost and damage was sustained by the CIho accessions under elevated CO_2 than under ambient CO_2. Elevated CO_2 caused a change of resistance rating as both CIho accessions changed from "moderately resistant' to "susceptible" to RWASA1 with increased [CO_2].

4.3.3 Conclusion

The close correlations of the aphid population growth (Chapter 3), plant biomass loss and plant damage show that elevated CO_2 has a significant effect on the complexity of this insect/plant interaction. In conclusion it is possible to predict a future scenario where the SA barley cultivars become redundant with climate changes as it is evident that they currently cannot survive without the use of pesticides. Thus, it is critical that barley cultivars, especially those that may hold innate resistance, be trailed based on current and future CO_2 regimes so that a breeding stock of effective RWA resistant cultivars can be produced. The CIho accessions were resistant to RWASA1 under ambient CO_2 but under elevated CO_2 RWASA1 was virulent, thus these accessions would be ineffective in future CO_2 environments. The SA barley cultivars are already in peril and it seems that this will only get worse as CO_2 enrichment favours RWA virulence.

Chapter 5: The effect of elevated CO_2 and RWA feeding on host plant nitrogen accumulation and C:N ratio

5.1 Introduction

Chapter 4 of this thesis demonstrated the effects that elevated CO_2 had on control plants in terms of increased biomass accumulation, due to an increased photosynthetic rate. Under the elevated CO_2 environment the experimental plants would have had an increased accumulation of structural and non structural carbohydrates (Stitt and Krapp, 1999; Sicher, 2001; Leakey et al., 2009).

Elevated [CO_2] results in increased Pn which in turn, puts pressure on available N. Therefore additional nutrient supplementation is necessary to ensure optimal growth and maintenance of C:N balance. N depletion down regulation would result in reduced photosynthetic capacity (Stitt and Krapp, 1999; Leakey et al., 2009). Previous studies have shown that elevated CO_2 caused a decrease in % N in plant leaves (Hughes and Bazzaz, 2001; Awmack et al., 2004; Stiling and Cornelissen, 2007; Taub, 2010). However, Long Ashton nutrient solution (Hewitt, 1966) was applied to the plants every two days to remove any potential nutrient stress. The direct effects of prolonged elevated CO_2 on plant C:N ratio and % N could therefore be examined without the risk of additional variables such as C:N imbalance.

The previous chapters have shown that RWA feeding becomes more detrimental under elevated CO_2 as aphid population growth rates increases and plant biomass decreases. Nitrogen in the host plant is essential for the production of amino acids, and thus a higher % N would directly influence the dietary solution that the aphids probe for (Bezemer et al., 1998). Since nutrients were regularly applied to the experimental plants, I was confident that any changes in C:N ratio of % N (over that of control plants) would be a direct result of RWA feeding.

5.2 Aims and objectives:

The aim of these experiments was to determine the effects of elevated CO_2 on % N (percentage of N per mg of dry leaf tissue) and C:N ratio (ratio of C to N per mg of dry leaf tissue) of four barley varieties (CIho 4125, CIho 4159, S5 and SSG 564). Furthermore, the effects of RWA feeding on host plant % N and C:N ratio were investigated under ambient and elevated CO_2.

The hypothesis tested in this chapter was that RWA feeding would lower % N as the aphid populations become a plant nutrient sink which would have a negative effect on the C:N ratio. Given that in Chapters 3 and 4 the South African (SA) barley cultivars S5 and SSG 564 supported larger aphid colonies and suffered more damage than either of the Afghan barley accessions (CIho 4125 and CIho 4159), it is hypothesised that S5 and SSG 564 would show a greater N loss than either of the CIho accessions .

5.3 Results

5.3.1 The effects of elevated CO_2 on host plant C:N ratio and % N

Fig 5.1 A shows the effects of elevated CO_2 on leaf C:N ratio of control plants. Both cultivars and accessions showed a decrease in C:N ratio, however, the decrease (range from 3-11%) was not significant with the control plants under ambient and elevated CO_2 (see Table 5.1). The limited effect of elevated CO_2 on C:N ratio of the experimental plants is most likely due to the fertilizer regime.

However, there was a significant interaction with respect to plant variety and C:N ratio, with the SA cultivars having significantly higher C:N ratios than either of the CIho accessions under both CO_2 regimes (Table 5.1). C:N ratios shown in Fig 5.1 A, show that carbon accumulation in S5 and SSG 564 outperformed the CIho accessions; both of the SA barley cultivars have been bred to be fast growing with high productivity and thus would have higher C accumulation capacity.

Figure 5.1 B shows the effects of elevated CO_2 on % N of control plant leaves. There were significant interactions between the plant varieties with CIho 4159, S5 and SSG 564 all having significantly higher % N accumulation than CIho 4125 under ambient CO_2. CIho 4125 had the lowest % N under ambient CO_2. Elevated CO_2 caused a significant increase in % N across all varieties. CIho 4125 increased nitrogen accumulation by 37% while CIho 4159, S5 and SSG 564 increased by 15-20% (see Table 5.1). Under elevated CO_2 the % N values for all for experimental plants were insignificantly different from one another as all had ± 5% foliar N. Elevated CO_2 caused increases in % N as the plants demand for N increased with higher photosynthetic rates. The % N data shows that the decrease in C:N ratio is a result of higher N accumulation rates.

Table 5.1 Percentage changes in C:N ratio (ratio C to N /mg leaf tissue) and % N (% N/mg leaf tissue between ambient and elevated $CO_2$for control (aphid free) accessions CIho 4125, CIho 4159 and cultivars, S5 and SSG 564. Downwards arrows representing decrease and upwards arrows representing increase in percentage

Accessions and Cultivars	C:N ratio of control plants under ambient CO_2	C:N ratio of control plants under elevated CO_2	Percentage decrease in average C:N ratio between ambient and elevated CO_2
CIho 4125	6.10 ± 0.97	5.89 ± 0.51	↓3.34%
CIho 4159	5.77 ± 1.09	5.51 ± 0.92	↓4.67%
S5	7.66 ± 1.14	6.81 ± 0.93	↓3.78%
SSG 564	7.22 ± 0.81	6.95 ± 0.47	↓11.03%
	% N ratio of control plants under ambient CO_2	% N ratio of control plants under elevated CO_2	Percentage increase in average % N between ambient and elevated CO_2
CIho 4125	3.60 ± 0.22	4.95 ± 0.18	↑37.5%
CIho 4159	4.22 ± 0.17	4.88 ± 0.17	↑15.64%
S5	4.22 ± 0.22	5.05 ± 0.21	↑19.55%
SSG 564	3.98 ± 0.31	4.81 ± 0.23	↑20.73%

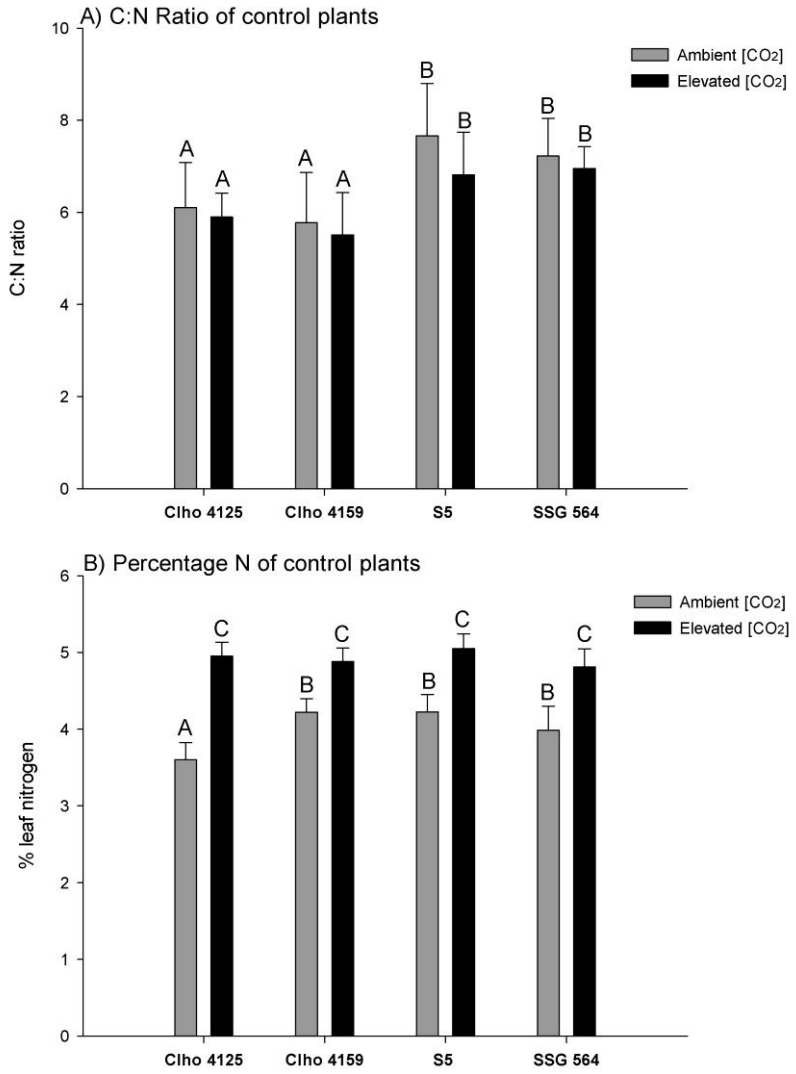

Figure 5.1 C:N ratio (ratio C to N /mg leaf tissue) and % N (% N/mg leaf tissue) of control plants under ambient and elevated CO_2. Letter above plots show levels of significant from post-hoc Tukey results (n = 5).

A) C:N ratio of control plants under ambient and elevated CO_2; **B)** % N of control plants under ambient and elevated CO_2.

5.3.2 The effect of RWA feeding on % leaf N of host plants under ambient and elevated CO_2

Examination of the data presented in Fig 5.2 shows that all plant varieties had a loss in % N when infested with any of the RWA biotypes; however the SA barley cultivars consistently had the greatest losses of N. Overall the interaction was significant ($p = <0.001$, see Table 5.3). RWASA2 caused the greatest decreases in % N in the host plants though in some cases (Figure 5.2 C) the difference between RWASA2 and the other biotypes was not significant. Under ambient CO_2 RWASA2 caused reductions of 43%, 40% and 45% on CIho 4159, S5 and SSG 564 respectively. In exception CIho 4125 suffered a reduction of 24% to RWASA2 feeding (see Figure 5.2 A). RWASA3 feeding caused greater losses of N in the host plants than RWASA1. Furthermore, in some cases the differences between the RWASA2 and SA3 were insignificant (see Figure 5.2 B, C, D).

RWA feeding under elevated CO_2 caused significant decreases in foliar N of the host plants. RWASA2 overall caused the greatest N reductions under elevated CO_2 and both the SA barley cultivars suffered the highest % N losses. SSG 564 had a N reduction of 65% and S5 was severely drained of N with a 77% loss (see Figure 5.2 C and D and Table 5.2).

Overall the main trends of % N loss caused by RWA feeding observed at ambient CO_2 were still evident under elevated CO_2. However, in all cases under elevated CO_2, RWA feeding translated to increased loss of N in plant leaves. RWASA2 caused the greatest N loss followed by RWASA3 then RWASA1. The only exception was RWASA1 on CIho 4125 under elevated CO_2 where N losses were similar to those caused by RWASA2.

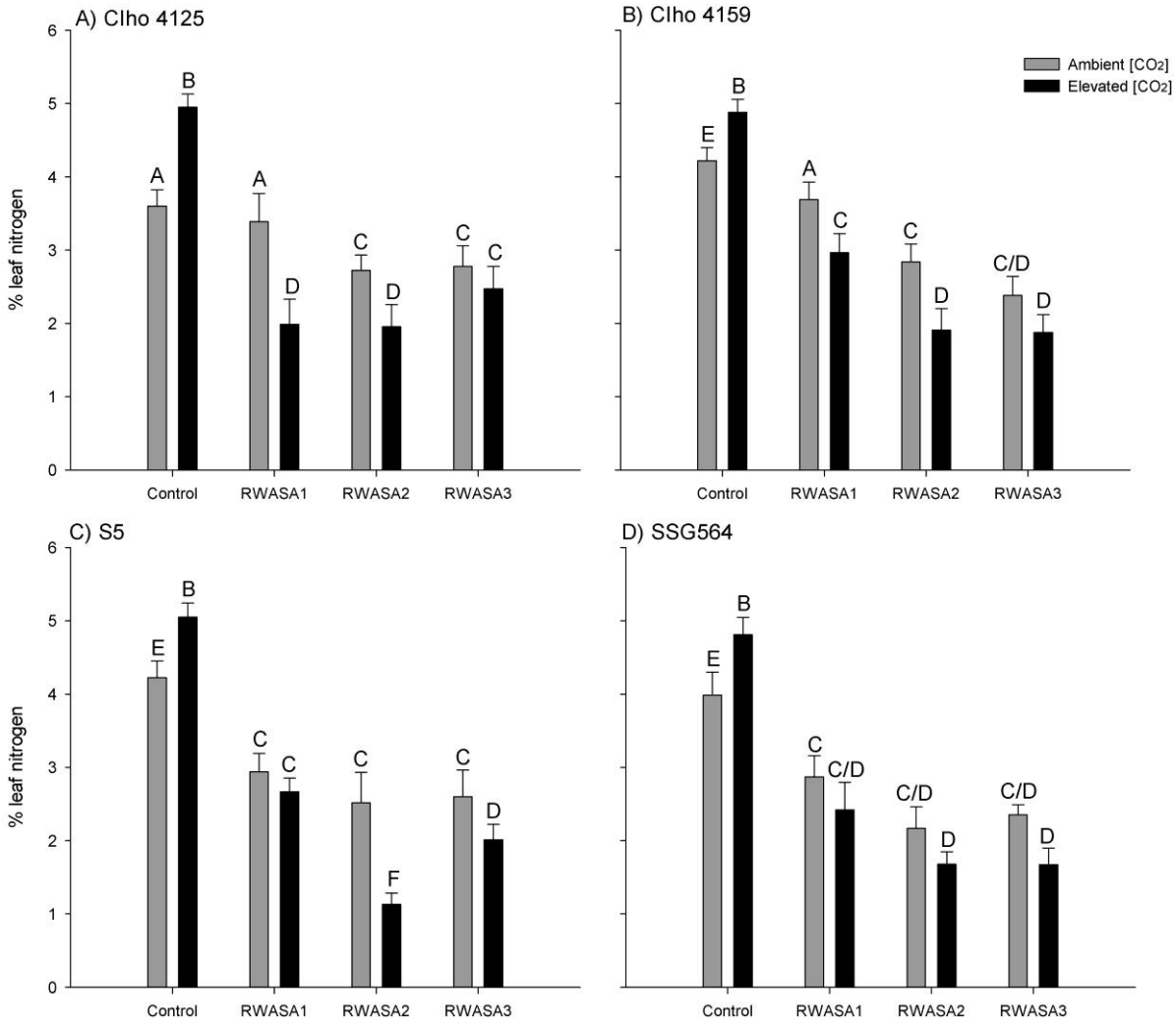

Figure 5.2 % N of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested with RWA biotypes RWASA1, SA2 and SA3 under ambient and elevated CO_2. Letters above plots indicated levels of significance from post-hoc Tukey (n = 5)

A-D shows the effects of elevated CO_2 and RWA infestation on host plant % N. RWASA1, RWASA2, RWASA3 infesting: **A**) CIho 4125; **B**) CIho 4159; **C**) S5; **D**) SSG 564, under ambient and elevated CO_2.

Table 5.2 Percentage difference (decrease) in % leaf N of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested with RWASA1, SA2 and SA3 from control plant % N

Treatment	Ambient [CO_2]		Elevated [CO_2]	
	%N per mg of leaf tissue	Percentage difference between % N of RWA infested from %N of control plant leaves	%N per mg of leaf tissue	Percentage difference between % N of RWA infested from %N of control plant leaves
CIho 4125				
Control	3.60 ± 0.22		4.95 ± 0.18	
RWASA1	3.38 ± 0.38	↓5.94%	1.98 ± 0.34	↓59.85%
RWASA2	2.72 ± 0.20	↓24.33%	1.95 ± 0.30	↓60.53%
RWASA3	2.77 ± 0.28	↓23.83%	2.47 ± 0.31	↓50.10%
CIho 4159				
Control	4.22 ± 0.17		4.88 ± 0.17	
RWASA1	3.69 ± 0.23	↓12.56%	2.96 ± 0.26	↓39.22%
RWASA2	2.83 ± 0.24	↓43.55%	1.87 ± 0.24	↓60.90%
RWASA3	2.38 ± 0.26	↓32.75%	1.90 ± 0.29	↓61.55%
S5				
Control	4.22 ± 0.22		5.05 ± 0.21	
RWASA1	2.93 ± 0.25	↓30.45%	2.66 ± 0.18	↓47.21%
RWASA2	2.51 ± 0.41	↓40.44%	1.13 ± 0.15	↓77.58%
RWASA3	2.60 ± 0.36	↓38.45%	2.01 ± 0.21	↓60.14%
SSG 564				
Control	3.98 ± 0.31		4.81 ± 0.23	
RWASA1	2.87 ± 0.28	↓27.91%	2.41 ± 0.38	↓49.73%
RWASA2	2.16 ± 0.29	↓45.58%	1.67 ± 0.17	↓65.11%
RWASA3	2.35 ± 0.13	↓40.93%	1.74 ± 0.23	↓65.20%

Table 5.3 Results of ANOVA test for % N of control and experimental plants.

Univariate tests of significance for C:N ratio	Degr. of freedom	F value	p value
Intercept	1	4697.31	<0.001
Aphid Condition	28	186.528	<0.001
Error	268		

5.3.3 The effect of RWA feeding on C:N ratio of host plants at ambient and elevated CO_2

RWA feeding caused significant increases in C:N ratio of experimental plant leaves under both ambient and elevated CO_2 in comparison to control plants ($p = <0.001$ see Table 5.5). Increased C:N ratios infer a decrease in N and thus it was expected that the C:N ratio data would be, to some extent, inversely proportional to the % N data.

Under ambient CO_2 significant differences between the RWA biotypes were relatively few. Under elevated CO_2, RWASA2 caused the greatest imbalance to host plant C:N ratios followed by RWASA3 then RWASA1. RWASA3 and RWASA1 did not imbalance the C:N ratios of the host plants to the same extents as did RWASA2. RWASA1 consistently had the least impact on the C:N ratio the plants it was infesting.

RWASA2 feeding under ambient CO_2 caused the highest C:N ratios in the plants. CIho 4125 and CIho 4159 had significantly higher C:N ratios with percentage increases of 84% for both accessions (See Figure 5.3 A and B and Table 5.4). S5 and SSG 564 had lower % increased in C:N ratio of 35% and 53% respectively (See Figure 5.3 C and D and Table 5.4). Under elevated CO_2, RWASA2 became even more detrimental, and caused further C:N ratio imbalances. All plants infested by RWASA2 showed significant increases in C:N ratio resulting in percentage increases of 136% and 122% for CIho 4125 and CIho 4159 and 117% and 106% for S5 and SSG 564 respectively (See Figure 5.3 A-D and Table 5.4).

What is most striking is that there were not many differences between the plants under ambient CO_2, when the differences between aphid populations are not as large (as shown in Chapter 3), however under elevated CO_2 the RWA populations were much larger on the SA barley cultivars than the Afghan accessions, and this is reflected in both the C:N ratios and the % N data. Elevated CO_2 and RWA infestation resulted in the C:N ratios of the host plants to almost double in most cases, across all plant varieties. It is clear that under elevated CO_2 conditions RWA feeding severely imbalances the host plant C:N ratios. These results compliment those of % N as foliar N drops with aphid feeding then the ratio of C to N should increase, this is demonstrated in Tables 5.3 and 5.4.

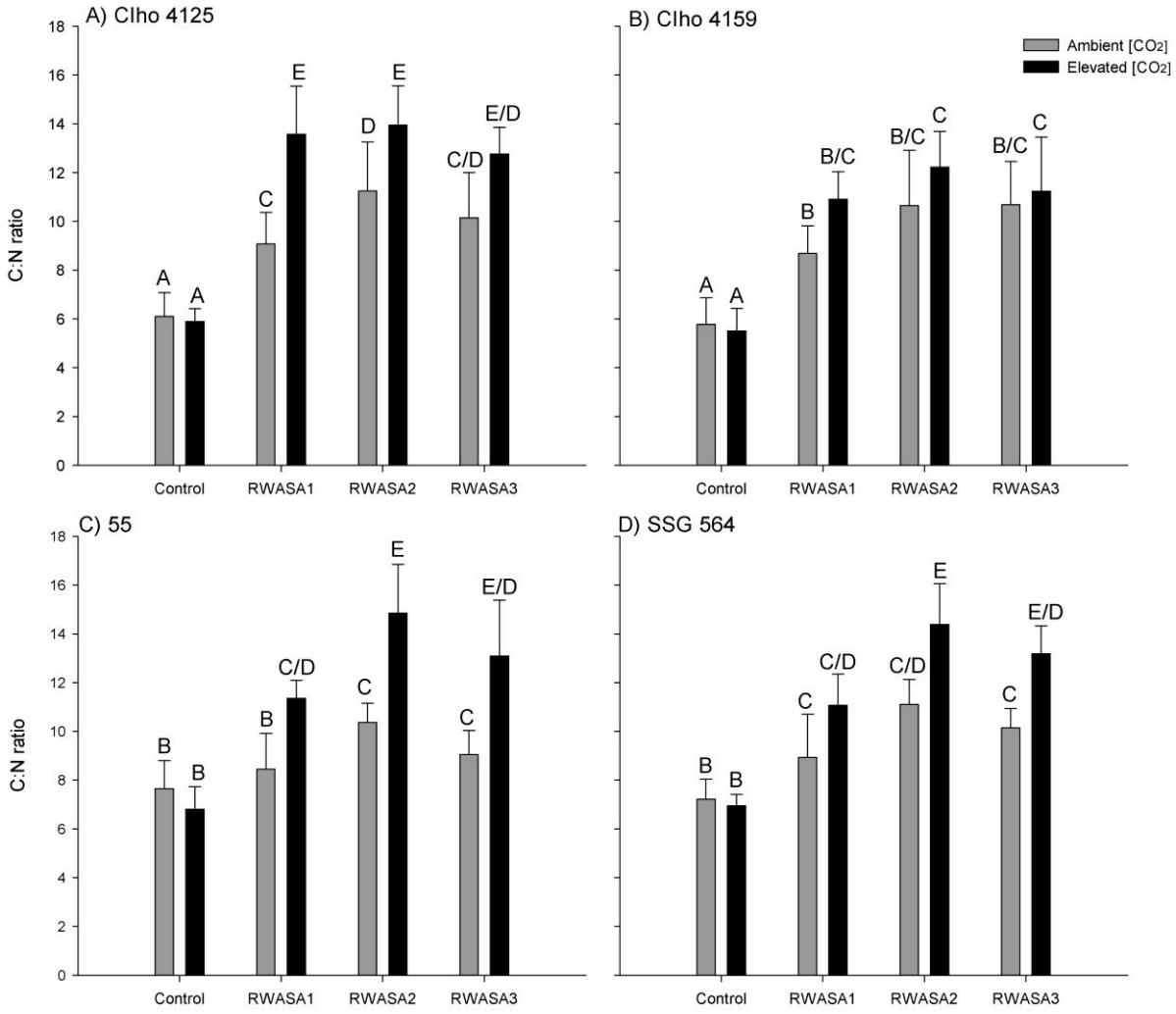

Figure 5.3 C:N ratio of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested with RWA biotypes RWASA1, SA2 and SA3 under ambient and elevated CO_2. Letter above plots indicated levels of significance from post-hoc Tukey (n = 5)

A-D shows the effects of elevated CO_2 and RWA infestation on host plant C:N ratio. RWASA1, RWASA2 and RWASA3 infesting: **A**) CIho 4125; **B**) CIho 4159; **C**) S5; **D**) SSG 564, under ambient and elevated CO_2.

Table 5.4 Percentage increase in C:N ratio of accessions CIho 4125, CIho 4159, and cultivars S5 and SSG 564 infested with RWASA1, SA2 and SA3 from control plant C:N ratio.

Treatment	Ambient [CO_2]		Elevated [CO_2]	
	C:N ratio per mg of leaf tissue	Percentage difference between C:N ratio of RWA infested from C:N ratio of control plant leaves	C:N ratio per mg of leaf tissue	Percentage difference between C:N ratio of RWA infested from C:N ratio of control plant leaves
CIho 4125				
Control	6.10 ± 0.97		5.89 ± 0.51	
RWASA1	9.08 ± 1.29	↑48.74%	13.58 ± 1.96	↑130.21%
RWASA2	11.25 ± 2.00	↑84.43%	13.95 ± 1.61	↑136.47%
RWASA3	10.15 ± 1.84	↑66.34%	12.76 ± 1.09	↑116.36%
CIho 4159				
Control	5.77 ± 1.09		5.51 ± 0.92	
RWASA1	8.68 ± 1.12	↑50.33%	10.91 ± 1.11	↑98.08%
RWASA2	10.64 ± 2.26	↑84.25%	12.23 ± 1.46	↑122.04%
RWASA3	10.68 ± 1.77	↑84.91%	11.28 ± 2.22	↑104.03%
S5				
Control	7.66 ± 1.14		6.81 ± 0.93	
RWASA1	8.54 ± 1.46	↑10.42%	11.35 ± 0.73	↑66.59%
RWASA2	10.37 ± 0.78	↑35.35%	14.85 ± 1.20	↑117.94%
RWASA3	9.05 ± 0.98	↑18.19%	13.08 ± 2.29	↑92.02%
SSG 564				
Control	7.22 ± 0.81		6.95 ± 0.47	
RWASA1	8.93 ± 1.77	↑23.66%	11.06 ± 2.28	↑59.02%
RWASA2	11.10 ± 1.02	↑53.72%	14.38 ± 1.68	↑106.93%
RWASA3	10.14 ± 0.81	↑40.40%	13.18 ± 1.14	↑89.72%

Table 5.5 Results of ANOVA test C:N ratios for control and experimental plants.

Univariate tests of significance for % N	Degr. of freedom	F value	p value
Intercept	1	2215.19	<0.001
Aphid Condition	28	56.57	<0.001
Error	225		

5.4 Discussion

5.4.1 The effect of elevated CO_2 on host plant N metabolism

As expected under elevated CO_2, control plants showed increases in % N, this supports the results of Jimoh et al., 2013. Under elevated CO_2 the C:N ratio of the host plants decreased, previous studies have suggested that elevated CO_2 leads to an increase in C:N ratio (Hughes and Bazzaz, 2001), however, these studies were conducted under circumstances where additional nutrients were not added to compensate for increased plant metabolism under elevated CO_2. Thus it follows that as the control plants had increased % N under elevated CO_2 translating to a decrease in C:N ratio. That the differences between the recorded values under ambient and elevated CO_2 were insignificantly different was expected. It is possible that as the photosynthetic rate increased under elevated CO_2 the well fertilized plants would have increased their N uptake from the soil to maintain a balance between the C:N ratios.

5.4.2 The effect of RWA feeding and elevated CO_2 on host plant N metabolism

RWA feeding caused significant decreases in % N and this is reflected in the changes in the C:N ratio of the host plants under ambient CO_2. Under elevated CO_2 this was further exacerbated, most likely as a direct result of increased aphid numbers creating a larger nutrient sink. It is evident from the control plants that the nutrient status of the host plants was altered drastically with elevated CO_2. It has been suggested that the amino acid composition of the host plant phloem sap is a limiting resource that influences RWA colony growth and development (see Porter et al., 1991; Bezemer et al., 1998; Bezemer and Jones 1998). An elevated CO_2 environment changes the nutrient quality of the host plants and thus a larger aphid population may result (see Chapter 3 for RWA population growth) this drains the nutrient supply of the plant even more. Jimoh et al., (2013) showed similar results by demonstrating that RWA feeding under elevated CO_2 caused a significant decrease in foliar % N. Thus, % N results reported here complement the C:N data for RWA infested plants, as the larger aphid colonies drained the host plants of their functional N compounds more rapidly, causing changes to the C:N ratio of the host plants. Previous research using the pea aphid (*Acyrthosiphon pisum* Harris) has shown that feeding on Alfafa (*Medicago sativa*) causes an imbalance and increase in plant C:N ratio (Girousse et al., 2005). The results presented in this chapter are further supported by those of Lindroth et al., (1995) and Hughes and Bazzaz (2001).

5.4.3 Virulence, biotype and N

In the previous chapters it has been shown that RWASA2 is the most virulent of the three biotypes tested followed by RWASA3 and RWASA1 this was further confirmed when analysing the % N and C:N data of the host plants. RWASA2 feeding consistently caused the greatest reduction in foliar N. This translated to a greater increase in plant C:N ratio. The ARC classifies RWASA2 as the "most virulent" followed by RWASA3 and then RWASA1 (Tolmay et al., 2007; Jankielsohn, 2011). This is supported in the current results.

5.4.4 Conclusion

The effects of aphid feeding on the % N and C:N ratio of the experimental plants provides insight into the resistance qualities of these barley plants. The SA barley cultivars fared poorly with S5 and SSG 564 losing more N to RWA compared to the Afghan accessions under both CO_2 regimes. Having already established that the SA barley cultivars are susceptible, it is not surprising that they suffered the greatest N-source drain and thus have the greatest increases in C:N ratio. There is also evidence of the resistance breaking with elevated CO_2 in which the CIho accessions under go as they tended to lose significantly more N to RWA feeding under elevated CO_2 while under ambient CO_2 they lost comparatively less.

Based on the results I infer that resistance breaking will occur as the aphid populations increase under elevated CO_2. This leads to heavy feeding and therefore, a higher N draw-down from the host plants. The aphids potentially become more damaging to the host plants and this would potentially increase their virulence classification.

Chapter 6: Wound callose accumulation and damage to cell ultra-structure as a result of RWA feeding under ambient and elevated CO_2

6.1 Introduction:

The Russian wheat aphid probes the phloem (and in so doing, other vascular tissues, as well) to obtain their dietary requirements during which they remove nutrients that are essential to the host plants (Saheed et al., 2007a, 2007b). It is well documented that feeding causes damage to the vascular tissues and that these elicit a series of wound responses, which include the formation of callose (Botha and Matsiliza, 2004; de Wet and Botha, 2007). Callose is a β-1,3 glucan carbohydrate compound produced to seal damaged regions of the phloem including sieve plates and sieve plate pores so that assimilate leakage and loss is reduced. There is evidence that resistant wheat cultivars produce less callose than susceptible cultivars. This is due to resistant cultivars inducing antibiosis and antixenosis responses. This lowers RWA populations and thus decreases the collective physical damage which the aphids inflict on their host plants (de Wet and Botha, 2007; Saheed et al., 2007a; Saheed et al., 2007b; Tolmay et al., 2007). Walton and Botha, (2008) quantified wound callose accumulation as result of feeding by biotypes RWASA1 and RWASA2 and found that as RWASA2 had higher populations and it also induced the formation of more callose in both resistant and non-resistant wheat cultivars than did RWASA1.

At the cellular scale RWA feeding has been shown to cause plasmolysis to cells. In addition, saliva and salivary sheath deposits compromise and disrupt tissue function as the aphids probe inter and intra-cellularly for nutrients (Saheed et al., 2007a). As mentioned, RWASA2 feeding elicits greater callose deposition and causes more vascular disruption than does RWASA1. This is most likely due to the high reproductive rate of RWASA2 (Jimoh et al., 2011).

In the preceding chapters, I demonstrate that RWASA2 is the most virulent of the three biotypes tested and that RWASA1 is the least damaging. Therefore it comes as no surprise that RWASA3 caused extensive callose damage as shown in the micrographs that follow, the fact that damage was exacerbated by elevated CO_2 is also born out in this chapter.

6.2 Aims and objects

The aim of the experiments presented in this chapter is to present the results of an examination of the effects of feeding related damage caused by RWA biotypes RWASA1, RWASA2 and RWASA3 feeding on the four barley varieties (CIho 4125, CIho 4159, S5 and SSG 564).

The simple hypothesis was that RWASA3 would cause less vascular disruption than RWASA2 and that RWASA1 would inflict the least amount of cell damage given the relative virulence.

Given their innate resistance to RWA the CIho 4125 and CIho 4159 accessions would sustain less damage than the (non-resistant) SA cultivars S5 and SSG 564. In addition, I hypothesized that the plants would develop more callose as a result of RWA feeding under elevated than under ambient CO_2 growth conditions.

6.2 Results

6.3.1 RWA feeding related callose deposition under ambient CO_2

Fig 6.1 shows the distribution of wound callose in control plants. In control sections, longitudinal intermediate veins (IV) and the branching cross veins (CV) containing the sieve plates (SP) are likely sites in which RWA would probe which would cause tissue damage which results in callose formation. Some callose was evident (Fig 6.1 C-D) as local wound callose formed even though the sections were scraped in a solution of MES buffer (Ca^{2+} free) at pH 7.2 to minimise damage.

The effect of aphid feeding under ambient CO_2 on the four barley varieties is illustrated in Figs 6.2 and 6.3. A high proportion of callose deposition is evident in the intermediate and small veins with little callose visible in the cross veins which was limited due to limited stylet penetration.

RWASA2 resulted in the largest formations of wound callose (Fig 6.2 D). RWASA1 and RWASA3 did not seem to cause as much callose deposition on either of the Afghan accessions (Fig 6.2 A, B and E, F) and thus the veins do not seem to be completely disrupted. In contrast, the leaf sections from the SA cultivars showed a higher density of wound callose formation than either of the Afghan barley accessions (Fig 6.2 A-F). Callose was clearly visible in the cross veins and mesophyll parenchyma (Fig 6.2 A-F). SSG 564 in particular had

large formations of wound callose (Fig 6.2 B, D, and F) in the veins which would have translated to highly impaired functionality.

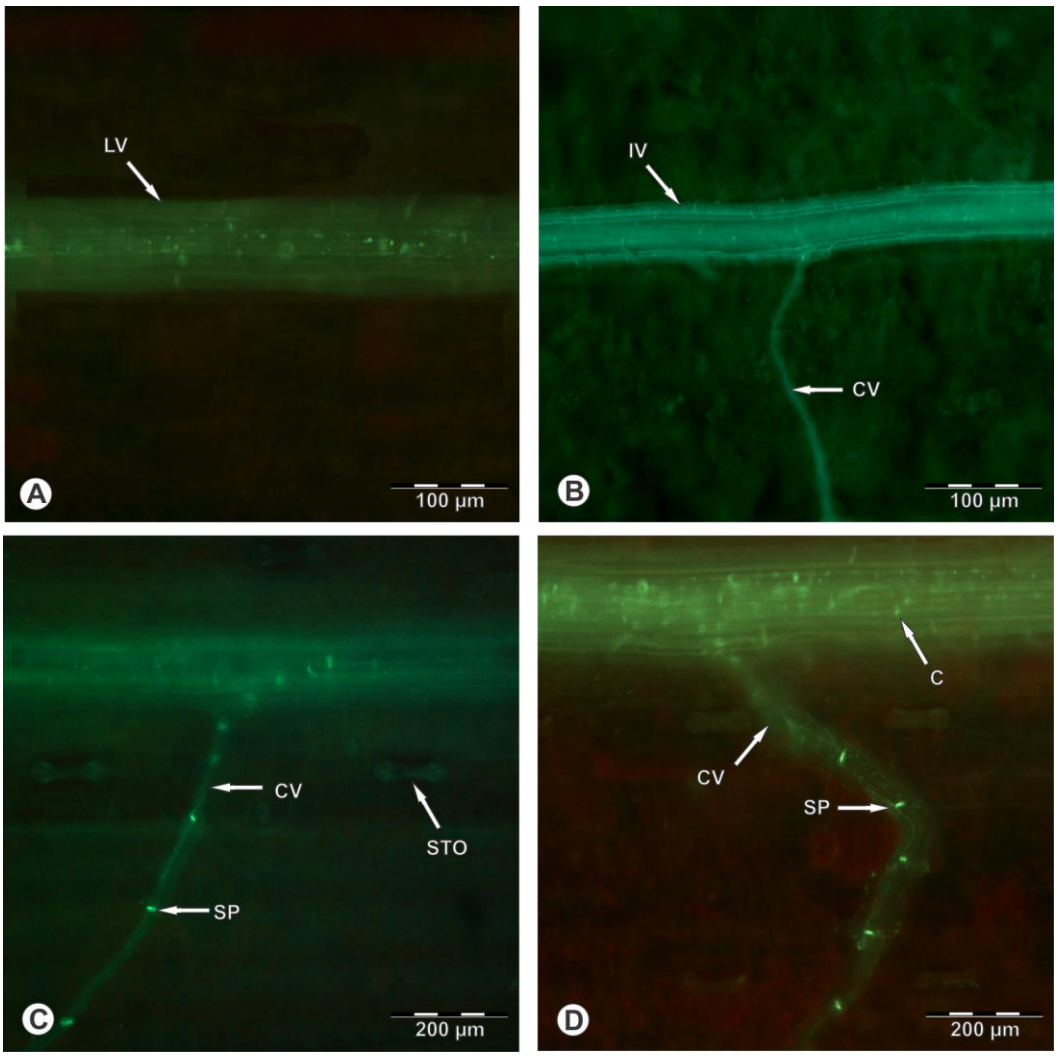

Figure 6.1 Fluorescence photomicrographs shows little effect of scraping related damage and thus limited wound callose formations in leaf tissue of control plants.

Florescence photomicrographs **A-D** show longitudinal sections of aniline blue stained sections of control (RWA free) plants leaves. The photos show large vein (LV) of CIho 4125 (**A**), and intermediate vein (IV) of CIho 4159 (**B**), S5 (**C**) and SSG 564 (**D**) fluorescence associated with cross veins (CV) and sieve plates (SP) and stomata (STO). **D)** Here, some callose (C) has formed this is likely to be associated with the scraping away of dermal tissues to expose "windows" in the mesophyll.

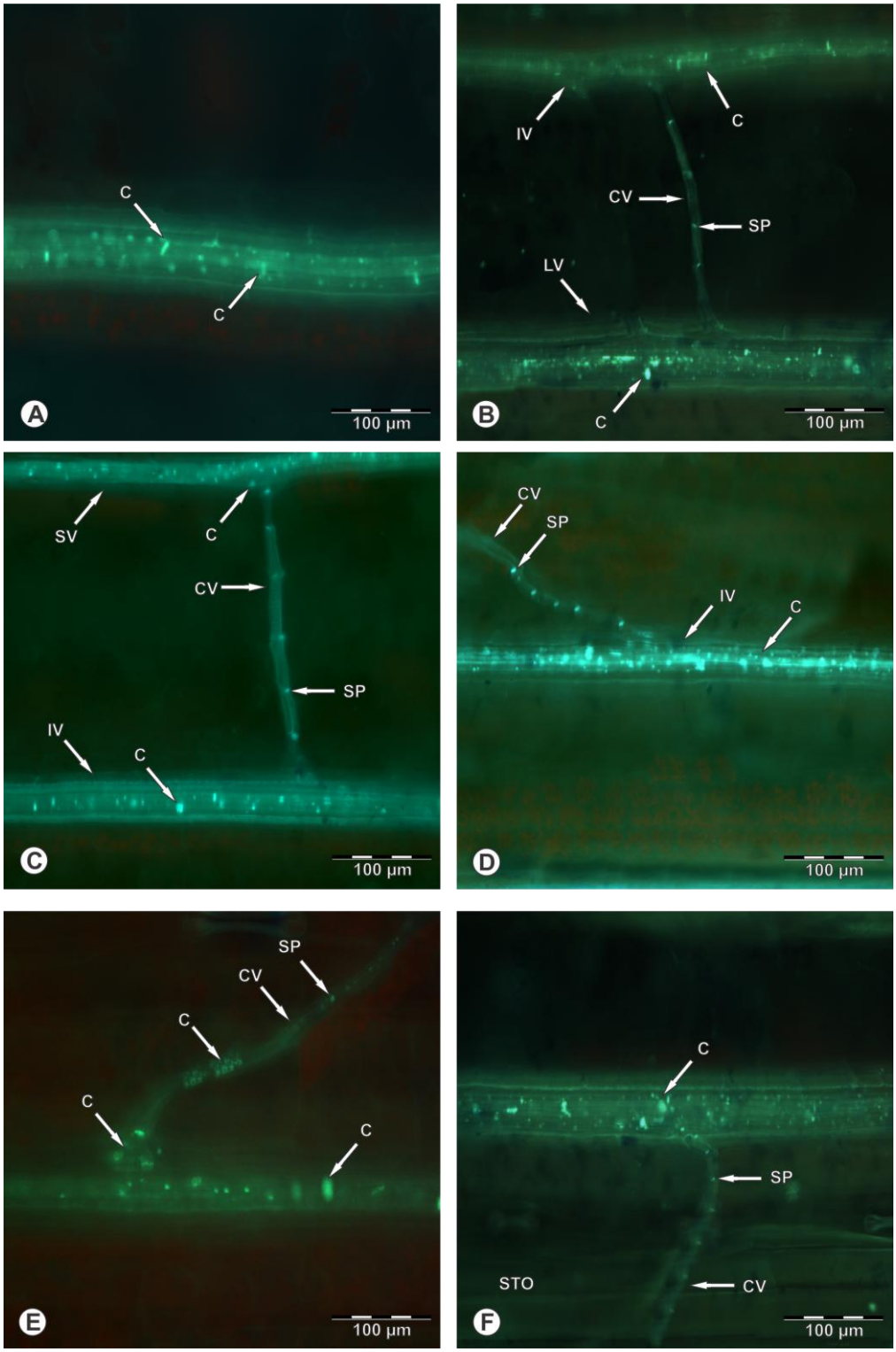

Figure 6.2 Fluorescence photomicrographs showing wound callose formations in sections of leaf tissue from CIho 4125 and CIho 4159 formed as a direct result of probing and feeding by RWASA1, SA2 and SA3 for 14 days under ambient CO_2.

Figure 6.2 Legend continued:

Representative fluorescence photomicrographs which show feeding related damage sustained by probing of the leaf and vascular tissue by RWASA1 on CIho 4125 (A) and CIho 4159 (B); RWASA2 on CIho 4125 (C) and CIho 4159 (D); RWASA3 on CIho 4125 (E) and CIho 4159 (F). Average RWA population was 73 ± 14 on CIho 4125 and 71 ± 11 on CIho 4159 at 14 DAI. Note that in all cases the longitudinal veins contain large proportions of callose. All three longitudinal vein classes were probed and fed on. Sieve tubes in the longitudinal veins (C, D) appear to have suffered a high level of damage judging by the propensity of callose present.

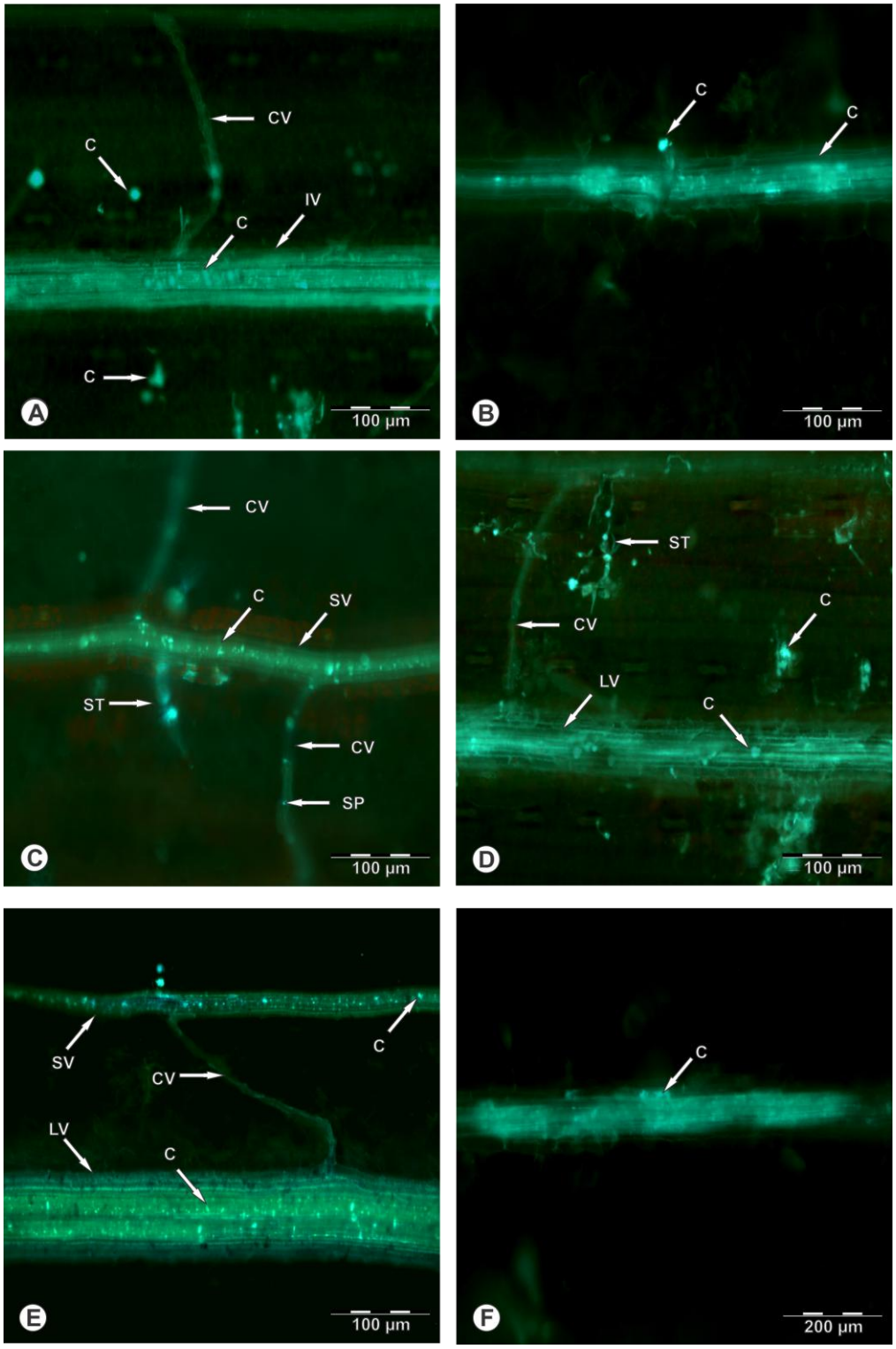

Figure 6.3 Fluorescence photomicrographs of wound callose formations in sections of leaf tissue from S5 and SSG 564 formed as a direct result of probing and feeding by RWASA1, SA2 and SA3 for 14 days under ambient CO_2.

Figure 6.3 Legend continued:

Representative fluorescence photomicrographs which show feeding related damage sustained by probing of the leaf and vascular tissue by RWASA1 on S5 (**A**) and SSG 564 (**B**); RWASA2 on S5 (**C**) and SSG 564 (**D**); RWASA3 on S5 (**E**) and SSG 564 (**F**). Average RWA population was 151 ± 15 on S5 and 141 ± 21 on SSG 564 at 14 DAI. Note there were extensive callose formations on all longitudinal veins, additionally there were callose formations in the cross veins. Stylet tracks (ST) (C and D), where common, through the leaf mesophyll leading to where aphids have probed the sieve tubes. Both of these cultivars show signs of having suffered severe damage from large numbers of RWA probes.

6.3.2 RWA feeding related callose deposition under elevated CO_2

The micrographs presented in Figs 6.1-6.5 clearly demonstrate that little wound related callose appears in control (uninfested) leaf tissue, yet, after prolonged feeding (14 days) there is evidence that RWA inflicts a great deal of visible (physical) damage to leaf tissue as the level of wound callose is high. It is, as expected, greatest with RWASA2 on both Afghan accessions and SA cultivars.

In general the CIho accessions showed an increase in callose formation under elevated CO_2 compared to ambient. Callose formations in the intermediate, small veins and cross veins were more widespread (Fig 6.4 A, C and D). Callose was also evident in the mesophyll parenchyma (Fig 6.4 A and F) and stylet tracks were more commonly evident under elevated CO_2 (Fig 6.4 E). It is also important to note that increased aphid populations had a marked effect on the veins of the leaves which veins had greater accumulations of callose which reduced the veins capacity to carry photo assimilates and water from source to sink.

Fig 6.5 A-F show veins of SA cultivars that are seriously disrupted by callose formation and supporting parenchyma was obliterated by stylet tracks (Fig 6.5 B, C and F). All three RWA biotypes caused large callose formations.

Overall all four strains fared poorly under elevated CO_2 compared to ambient. Further evidence for the susceptibility of the S5 and SSG 564 cultivars to RWA feeding is shown by the large callose formations in all test scenarios. The increasingly poor performance of CIho 4125 and CIho 4159 accessions under elevated CO_2 is further evidence for the concept of resistance breaking under elevated CO_2.

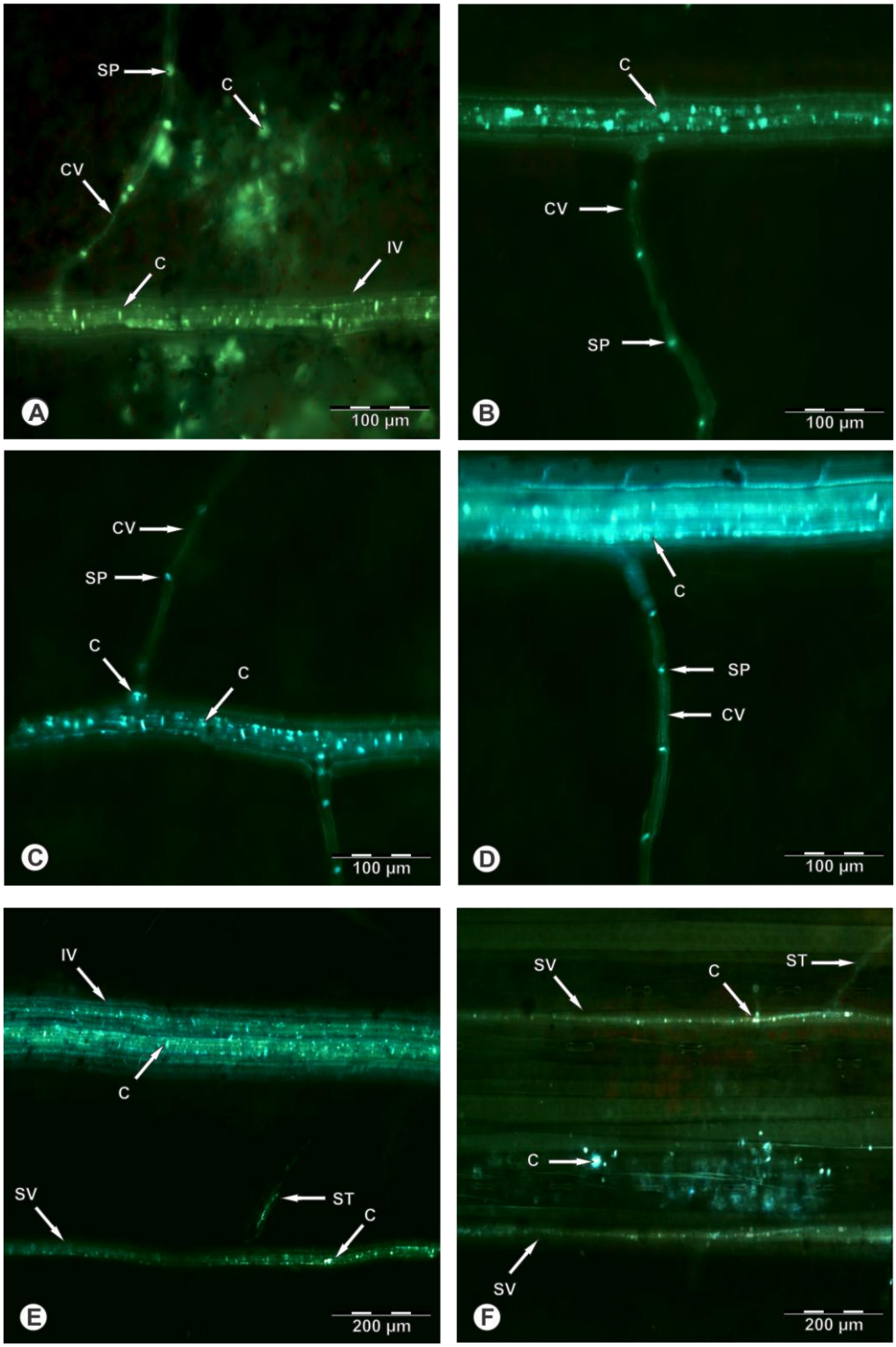

Figure 6.4 Fluorescence photomicrographs of wound callose formations in sections of leaf tissue from CIho 4125 and CIho 4159 formed as a direct result of probing and feeding by RWASA1, SA2 and SA3 for 14 days under elevated CO_2.

Figure 6.4 Legend continued:

Representative fluorescence photomicrographs which show feeding related damage sustained by probing of the leaf and vascular tissue by RWASA1 on CIho 4125 (**A**) and CIho 4159 (**B**); RWASA2 on CIho 4125 (**C**) and CIho 4159 (**D**); RWASA3 on CIho 4125 (**E**) and CIho 4159 (**F**). Average RWA populations were 152 ± 19 on CIho 4125 and 129 ± 16 on CIho 4159 at 14 DAI. Note that 14 days of RWA feeding under elevated CO_2 there were massive callose deposits that indicated extensive, possibly multiple probes of the same vein. Callose formations in the leaf mesophyll and around the longitudinal vein indicating multiple aphid probing events, additionally note that cross veins and all three vein classes were heavily probed indiscriminately. Stylet tracks (E and F) indicated probing of sieve tubes that caused damage in to leaf tissues.

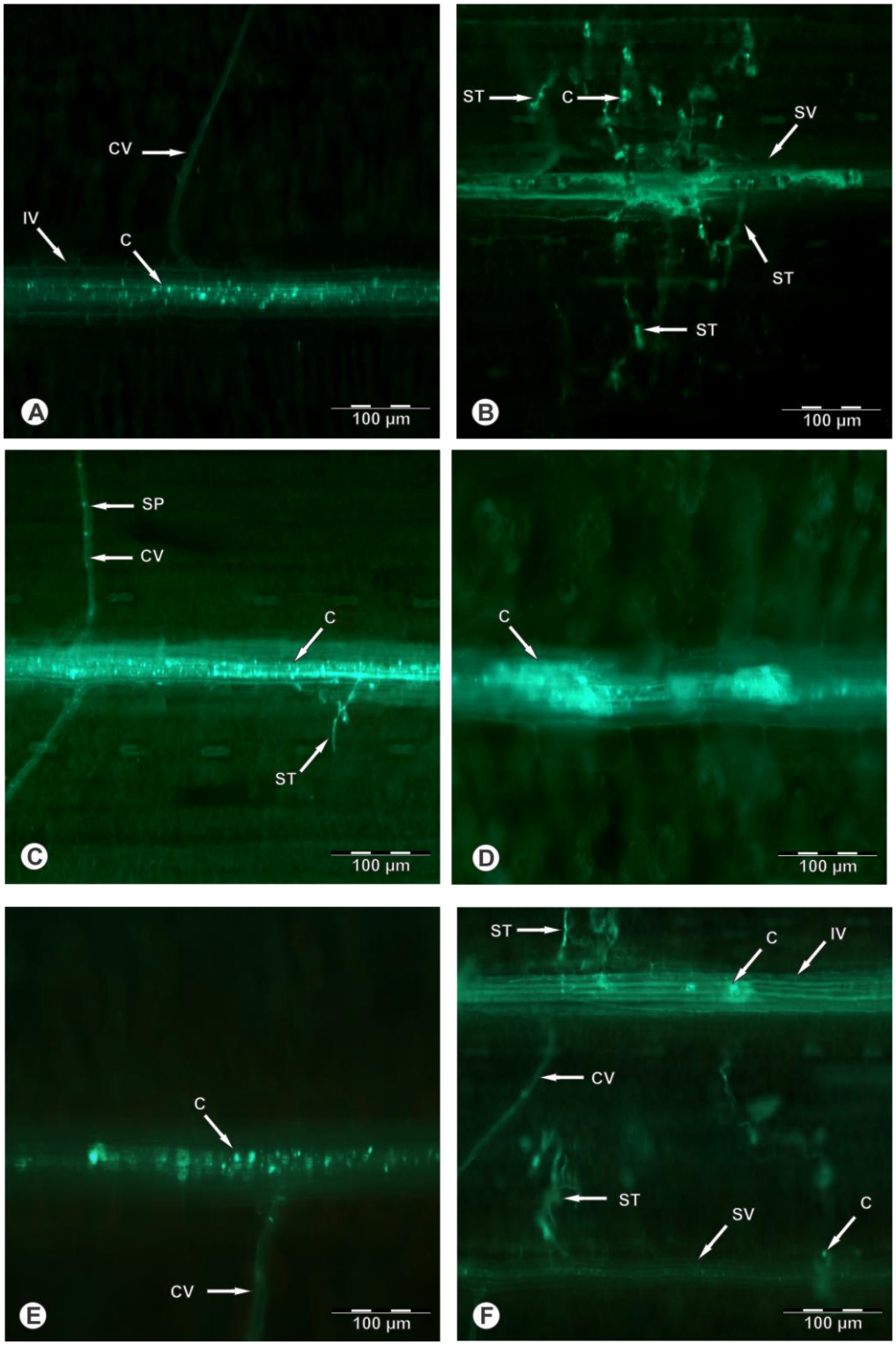

Figure 6.5 Fluorescence photomicrographs of wound callose formations in sections of leaf tissue from S5 and SSG 564 formed as a direct result of probing and feeding by RWASA1, SA2 and SA3 for 14 days under elevated CO_2.

Figure 6.5 Legend continued:

Representative fluorescence photomicrographs which show feeding related damage sustained by probing of the leaf and vascular tissue by RWASA1 on S5 (**A**) and SSG 564 (**B**); RWASA2 on S5 (**C**) and SSG 564 (**D**); RWASA3 on S5 (**E**) and SSG 564 (**F**). Average RWA populations were 223 ± 17 on S5 and 222 ± 18 on SSG 564 at 14 DAI. Note that callose formations on these (SA) cultivars under elevated CO_2 were the largest of those observed under all experimental conditions. Stylet tracks were evident breaching the sieve tubes and adjacent leaf mesophyll tissues (B, C, F). The extensive callose formations are indicative of large numbers of aphids probing the leaf. It is important to note that the average RWA populations were highest under these particular experimental conditions.

6.3.3 Phase analysis of wound callose photomicrographs

Phase analysis is the percentage of area that reacted to the aniline blue stain vs. the area which showed no fluorescence. It is therefore a powerful technique that can help in the assessment of the level of damage sustained. It provides a convincing argument when relating RWA populations to callose formations. Figure 6.6 shows an example of the application of the phase analysis software used to measure wound callose formation in the host plant leaves.

Figure 6.7 shows the results of the phase analyses of callose-related damage vs. undamaged tissues in the representative sections of the barley cultivars. Note in all cases that there was a significant interaction ($p = <0.001$) with respect to percentage area of leaf tissue that contains callose when a) aphids are present and b) when [CO_2] is elevated. On CIho 4125 and CIho 4159 under ambient CO_2 RWASA2 caused significantly larger areas of callose deposition than either RWASA3 or RWASA1 (Fig 6.7 A and B). RWASA1 caused the least callose formation.

The SA barley cultivars under ambient CO_2 with RWA infestation produced significantly more callose than either of the CIho accessions (Fig 6.7 C and D). Elevated CO_2 caused significant increases in callose formation for CIho 4125 and CIho 4159 across all three RWA biotypes (Fig 6.7 A and B). These findings suggest that the CIho accessions experience resistance breaking particularly with RWASA1 as the callose formation dramatically increases as a result of increased probing under elevated CO_2. The elevated CO_2 environment seemed to induce the RWA colonies to cause large callose formations on S5 and SSG 564 (Fig 6.7 C and D). These findings suggest that S5 and SSG 564 were already susceptible to RWA feeding under ambient CO_2. It is important to note that all increases in callose formations occurred concurrently with increases in RWA population.

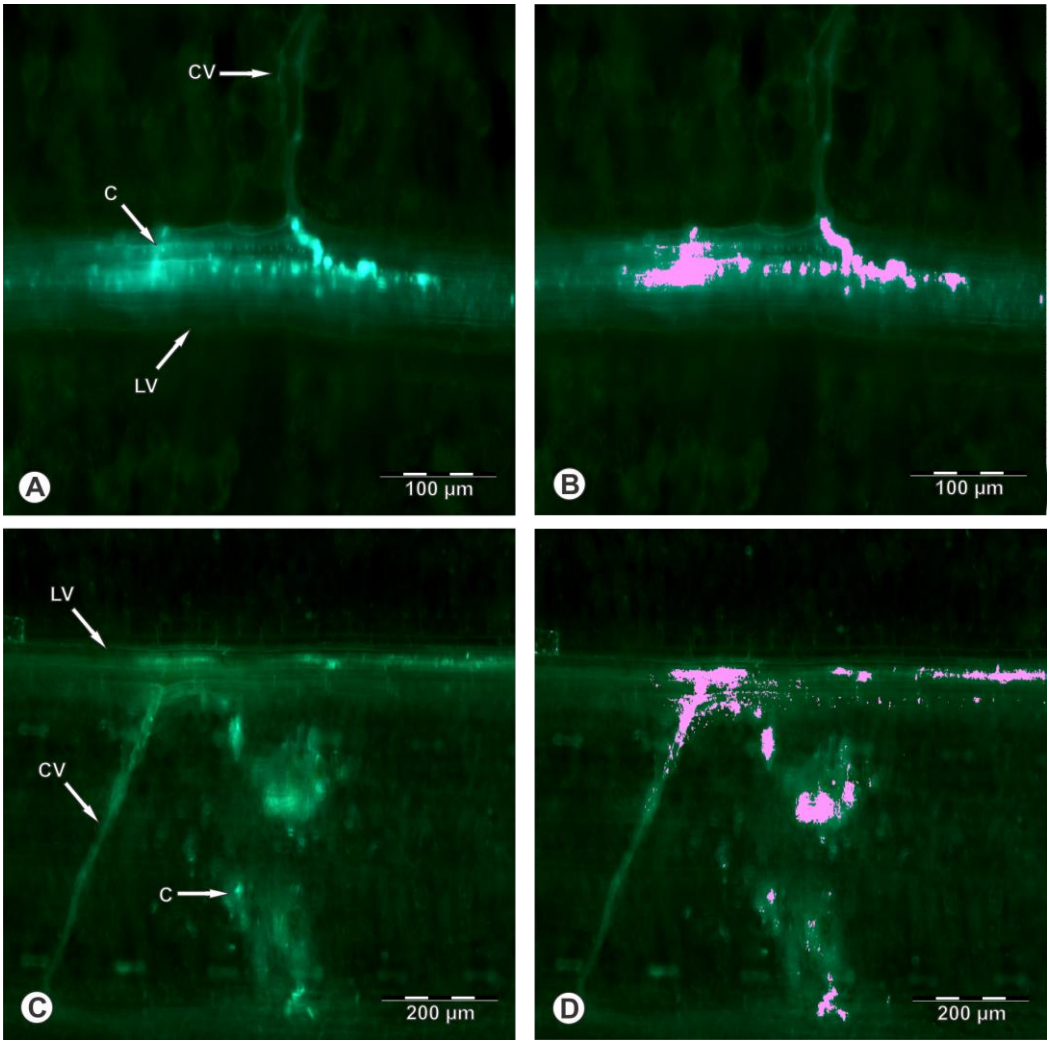

Figure 6.6 Example of fluorescence photomicrographs used for phase analysis of wound callose formation in host plant leaves.

A-D show examples of fluorescence photomicrographs that have been subject to phase analysis. A and C show longitudinal sections of leaf tissue before phase analysis, while B and D show the same sections after phase analysis where callose has been measure and assigned a false colour (light green).

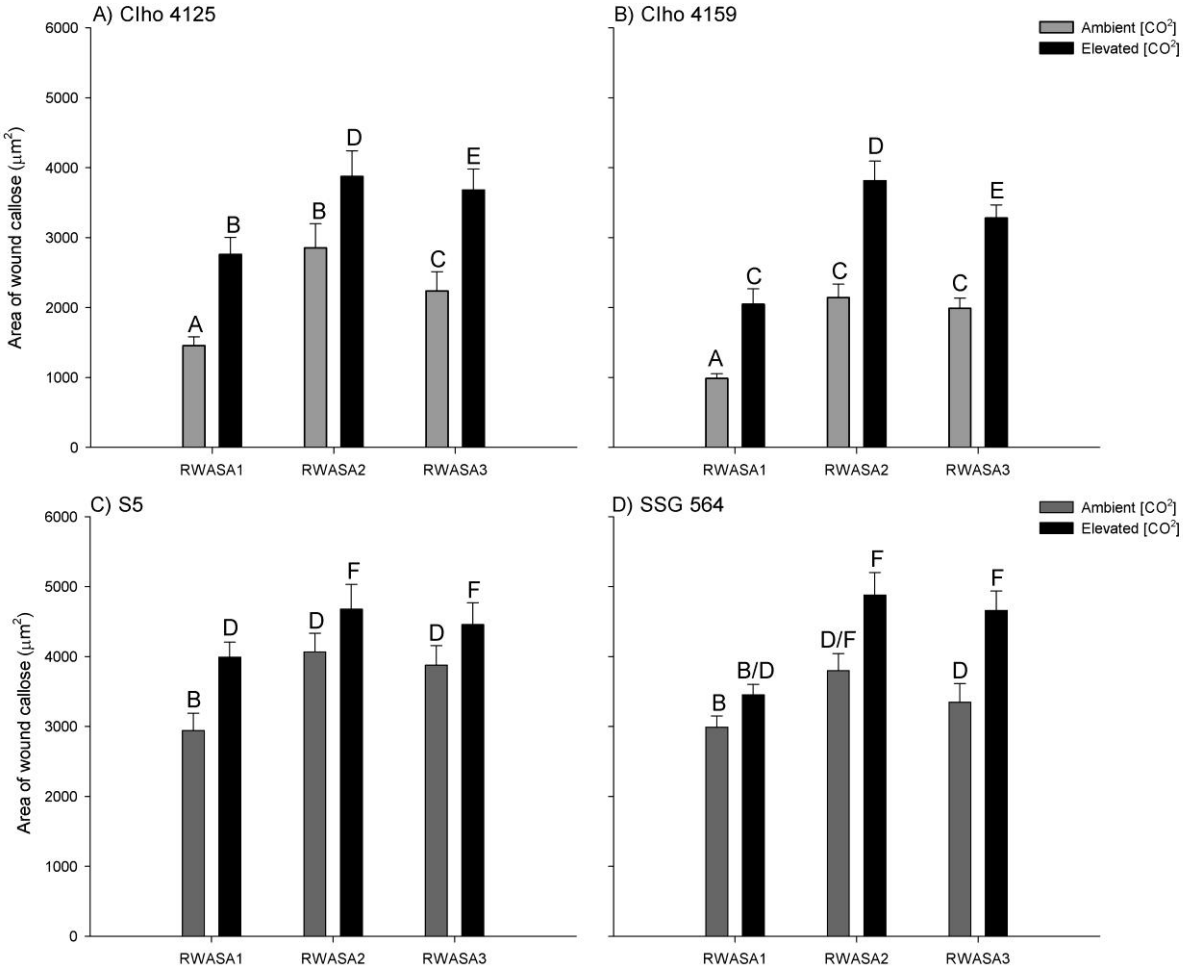

Figure 6.7 Phase analysis of wound callose on CIho 4125, CIho 4159, S5 and SSG 564 after 14 days of infestation by RWASA1, SA2 and SA3 under ambient and elevated CO_2.

Letters above plots denote levels of significance from post-hoc Tukey n = 10. Wound callose area of caused by RWASA1, RWASA2 and RWASA3 feeding on **A**) CIho 4125; **B**) CIho 4159; **C**) S5; **D**) SSG 564 under ambient and elevated CO_2.

Table 6.1 Results of ANOVA tests for significance in area of wound callose in experimental plant leaf sections

Univariate tests of significance for area of wound callose	Degr. of freedom	F value	p value
Intercept	1	429711	<0.001
Aphid Condition	24	136.573	<0.001
Error	269		

6.3.4 Evidence of RWA feeding damage under ambient CO_2

Fig 6.8 shows the vascular tissues of control plants denoting vascular parenchyma (VP), sieve tubes (ST), companion cells (CC), metaxylem vessels (MXV), thick-walled sieve tubes (TST) and xylem vessels (XV). Note there was no disruption causes by aphid feeding, and thus there were no signs of saliva deposits nor have any of the cells become compromised or plasmolysed.

Fig 6.9 shows the effects of RWA feeding on CIho 4125 and CIho 4159 under ambient CO_2. Saliva sheaths (SS) show where regions of the vascular bundles were penetrated (Fig 6.9 B, D and F) Many cells appear to be occupied by extensive deposits of electron dense granular saliva (SG) (Fig 6.9 C and E) the saliva lines the lumen of the cells and effectively seals them from surrounding cells. The bottommost arrow in Fig 6.9 F indicates some half-bordered pits which have become blocked with aphid saliva. Certain cells also became plasmolysed and occluded (Fig 6.9 A and F) and lost functionality due to the disruption. Xylem vessels, vascular parenchyma and sieve tube have been probed for RWA dietary solution and water.

Fig 6.10 shows the effects of RWA feeding on the S5 and SSG 564 damage was more evident in these cultivars than in the CIho accessions. The large black saliva sheaths and saliva deposits were evident spreading through all tissues types including the mesophyll parenchyma (Mes) and bundle sheaths (BS) adjacent to the vascular tissues, and subsequently compromising the functionality of the cells that were breached (Fig 6.10 C, D, E and F). The granular saliva lining and damaging the cells is evident (Fig 6.10 A and B) where cells become isolated from their neighbours and lose functionality.

Differences between the ultra structural damage in the plant tissues caused by the different biotypes is not obvious, however it does seem that the SA barley cultivars sustained more extensive damage than the CIho accessions under ambient CO_2. S5 and SSG 564 had larger deposits of saliva and salivary sheaths were more common throughout the tissues, the areas that had been heavily probed were more extensively damaged than those of either CIho accession and there were more S5 and SSG 564 cells that had been plasmolysed and compromised.

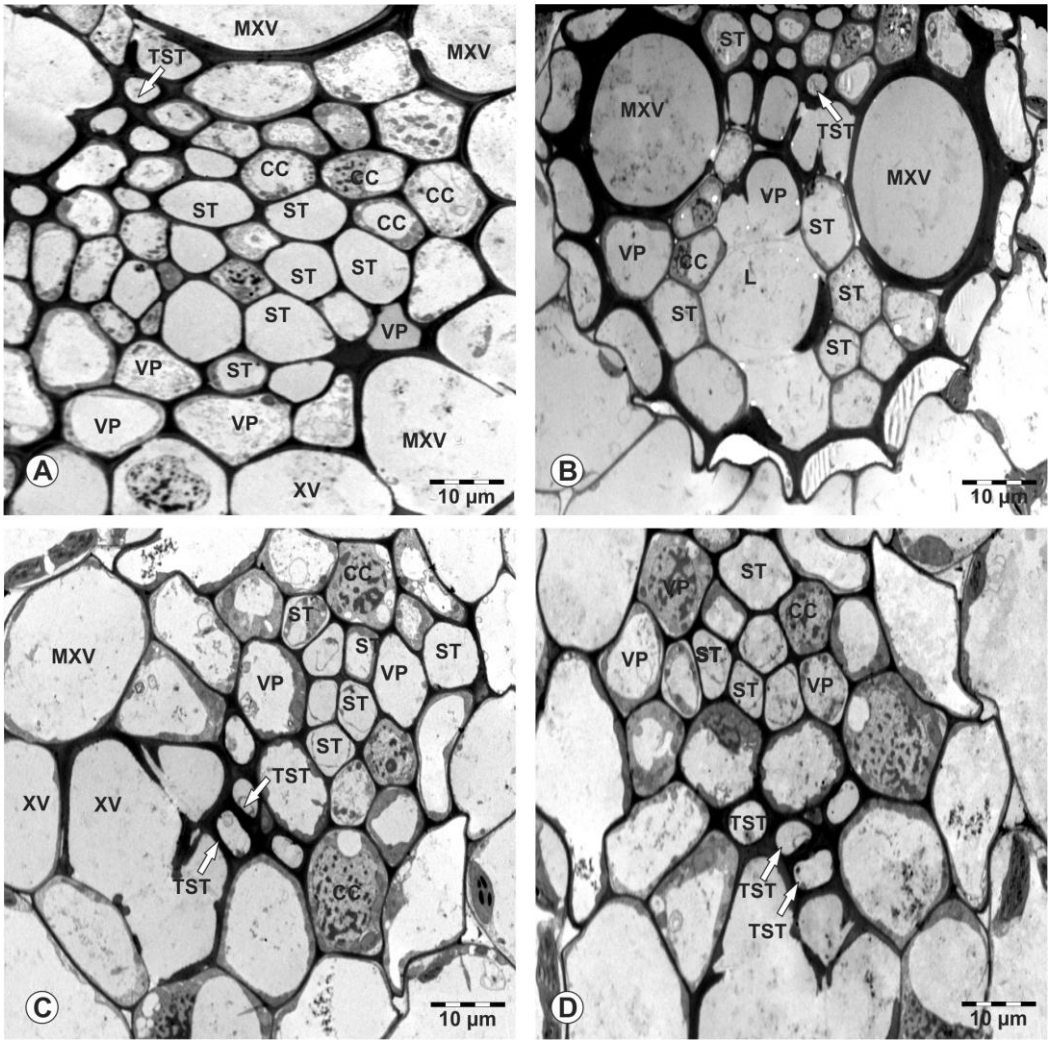

Figure 6.8 TEM images illustrating representative vascular tissue and cell structure in uninfested control plant leaves, in transaction.

A-D) Longitudinal veins showing the metaxylem vessels (MXV) and xylem vessels (XV) bordering thick-walled sieve tubes (TST), vascular parenchyma (VP) companion cells (CC) and sieve tubes (ST) and lacuna (L). Note there is no evidence of cell damage or plasmolysis nor is there any evidence of disruption to the vascular tissues.

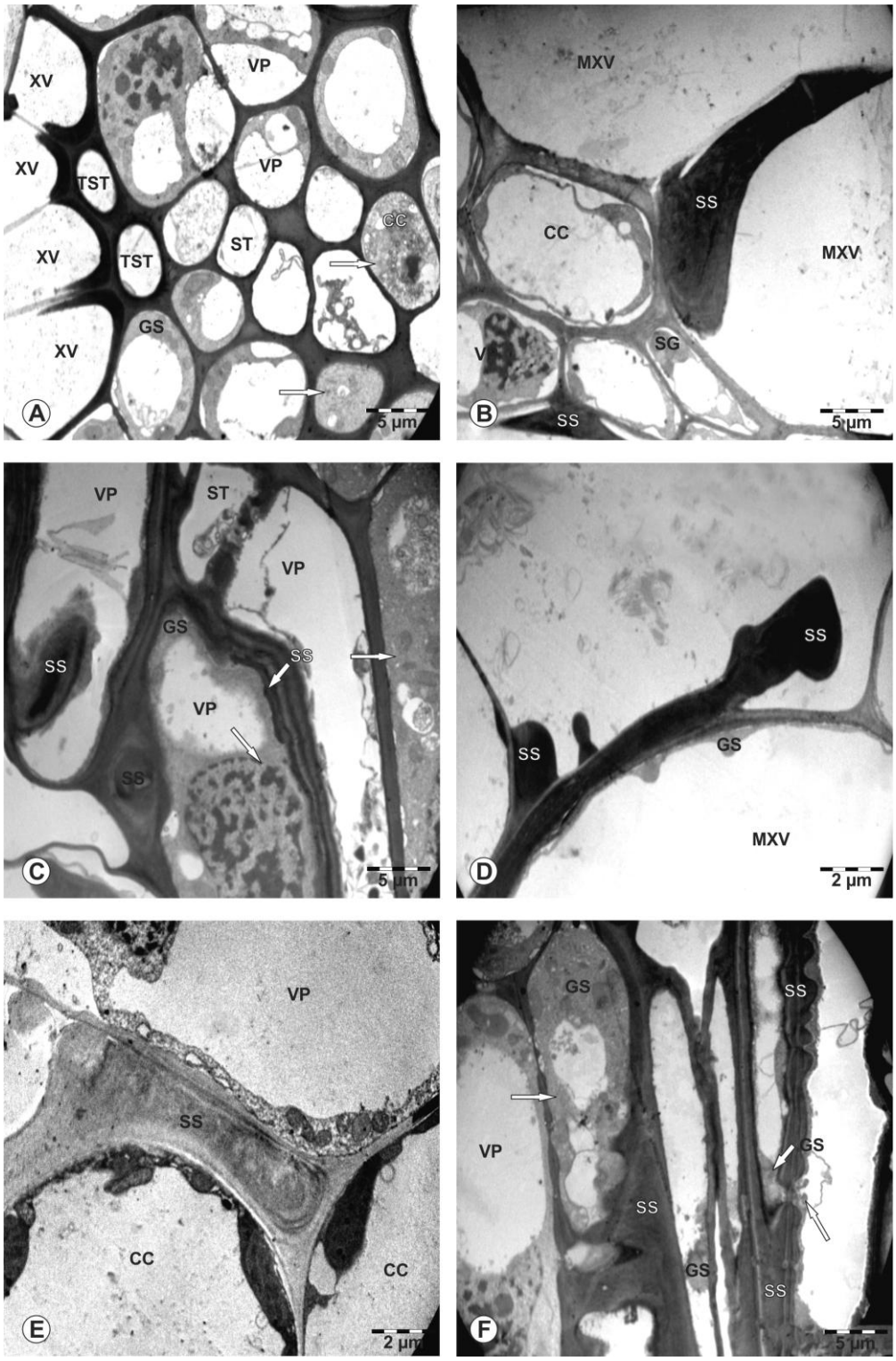

Figure 6.9 Cellular damage caused by feeding of RWASA1, SA2 and SA3 on accessions CIho 4125 and CIho 4159 after 14 days under ambient CO_2

Figure 6.9 Legend continued:

Representative TEM images which show feeding related damage sustained by probing by RWASA1 on CIho 4125 (**A**) and CIho 4159 (**B**); RWASA2 on CIho 4125 (**C**) and CIho 4159 (**D**); RWASA3 on CIho 4125 (**E**) and CIho 4159 (**F**). Low power TEM shows that the majority of the vascular tissue has been disrupted and damage by stylet penetration. Nearly all cells show plasmolysis, restriction and disruption of plasma membranes. A) Cells show accumulations of granular saliva (GS) from aphid feeding have become plasmolysed (indicated with arrows. B) Showing a metaxylem vessel (MXV) with a salivary sheath (SS) penetrating it. C) Showing disruption of vascular parenchyma with arrows indicating plasmolysed cells as salivary sheaths permeated the tissues and sieve tubes. D-E) Shows large saliva sheaths disrupting xylem tissue and vascular tissue. F) Details large accumulations of granular saliva are visible in vascular parenchyma and cells are also plasmolysed. Bottom arrow indicates granular saliva (GS) blocking half bordered pits.

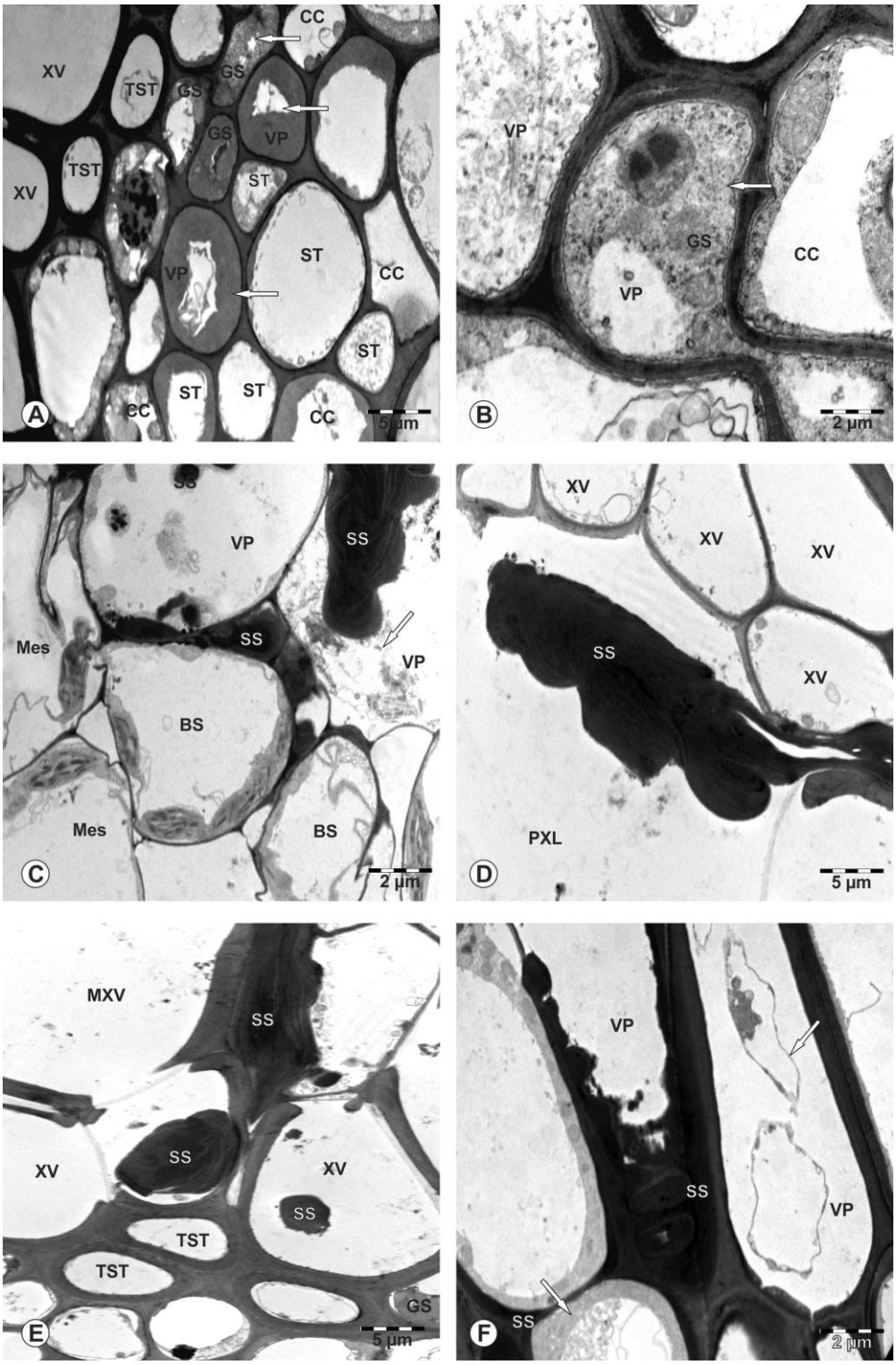

Figure 6.10 Cellular damage caused by feeding of RWASA1, SA2 and SA3 on cultivars S5 and SSG 564 after 14 days under ambient CO_2

Figure 6.10 Legend continued:

Representative TEM images which show feeding related damage sustained by probing by RWASA1 on S5 (**A**) and SSG 564 (**B**); RWASA2 on S5 (**C**) and SSG 564 (**D**); RWASA3 on S5 (**E**) and SSG 564 (**F**). 14 days exposure to RWA biotypes results in extensive damage to the vascular tissue. Electron dense granular salivary (GS) deposition was evident (A and B) and evidence of extensive probing for functional phloem is evidenced by saliva sheaths (SS). The aphids probed both intra and intercellularly in the phloem and xylem tissues. A-B) Vascular parenchyma (VP) contains granular saliva deposits. C) Severe damage is evident from the penetration of mesophyll (M) and adjacent vascular bundle (VB). D) Stylet track in large metaxylem vessel penetrated from right hand side. E-F) Shows penetration of xylem and vascular tissue in S5 and SSG 564 respectively.

6.3.5 Evidence of feeding damage under elevated CO_2

Fig 6.11 shows the effects of RWA feeding on CIho 4125 and CIho 4159 grown under elevated CO_2. The differences between these micrographs and those of tissues trialled under ambient CO_2 are striking. Extensive damage was sustained to vascular parenchyma (VP), sieve tubes (ST), companion cells (CC) and xylem vessels (XV). Numerous saliva sheaths and massive saliva deposits (SS) were evident throughout the tissues (Fig 6.11 A, C, D and F). Vascular tissues were compromised with granular saliva (SG) and many sieve tubes had become occluded (Fig 6.11 B). Both xylem and phloem had been damaged through probing (Fig 6.11 B and C) and isolated cells had subsequently lost their ability to function.

Although differences between the biotypes are not obvious in the micrographs of all four barley strains the RWA appear to be more virulent most likely as a result of increased population density. RWASA2 and RWASA3 seem to cause the largest deposits of the dark electron dense saliva in the host plant tissues. What is certain though is that all three RWA biotypes have the capability to cause more obvious and extensive damage under elevated CO_2.

Fig 6.12 shows the effects of RWA feeding on S5 and SSG 564 under elevated CO_2. The SA barley cultivars sustained more damage under elevated CO_2 than under ambient. They also sustained more damage than either of the two Afghan accessions across all three biotypes. Whole vascular bundles were compromised and virtually bisected by saliva sheaths breaching and flowing through the mesophyll (Mes) and bundle sheaths (BS) to reach the vascular tissues (Figs 6. 12 A, C, and E). Along with the vascular tissues becoming damaged and plasmolysed the mesophyll parenchyma surrounding the vascular bundles were also obliterated (Fig 6.12 A and E). Whole xylem cells were filled with saliva deposits which would render then completely useless as transport conduits (Fig 6.12 B and F).

Overall under elevated CO_2 all four test plants sustained more damage as a result of increased probing and large deposits of saliva which disrupts cell functions. The concentration of probing by RWASA3 on S5 (Fig 6.12 E) exemplified how increased aphid populations would negatively impact the host plant tissues as it shows how one mesophyll parenchyma cell had been breached on three sides by three distinct salivary sheaths, these tissues were highly disrupted and heavily damaged by the sheer number RWA of probes.

However, the susceptible cultivars S5 and SSG 564 sustained more damage than either of the CIho accessions under both CO_2 conditions. This follows the trends observed in the previous chapters and provides further evidence that RWA population is a driving factor in aphid biotype virulence.

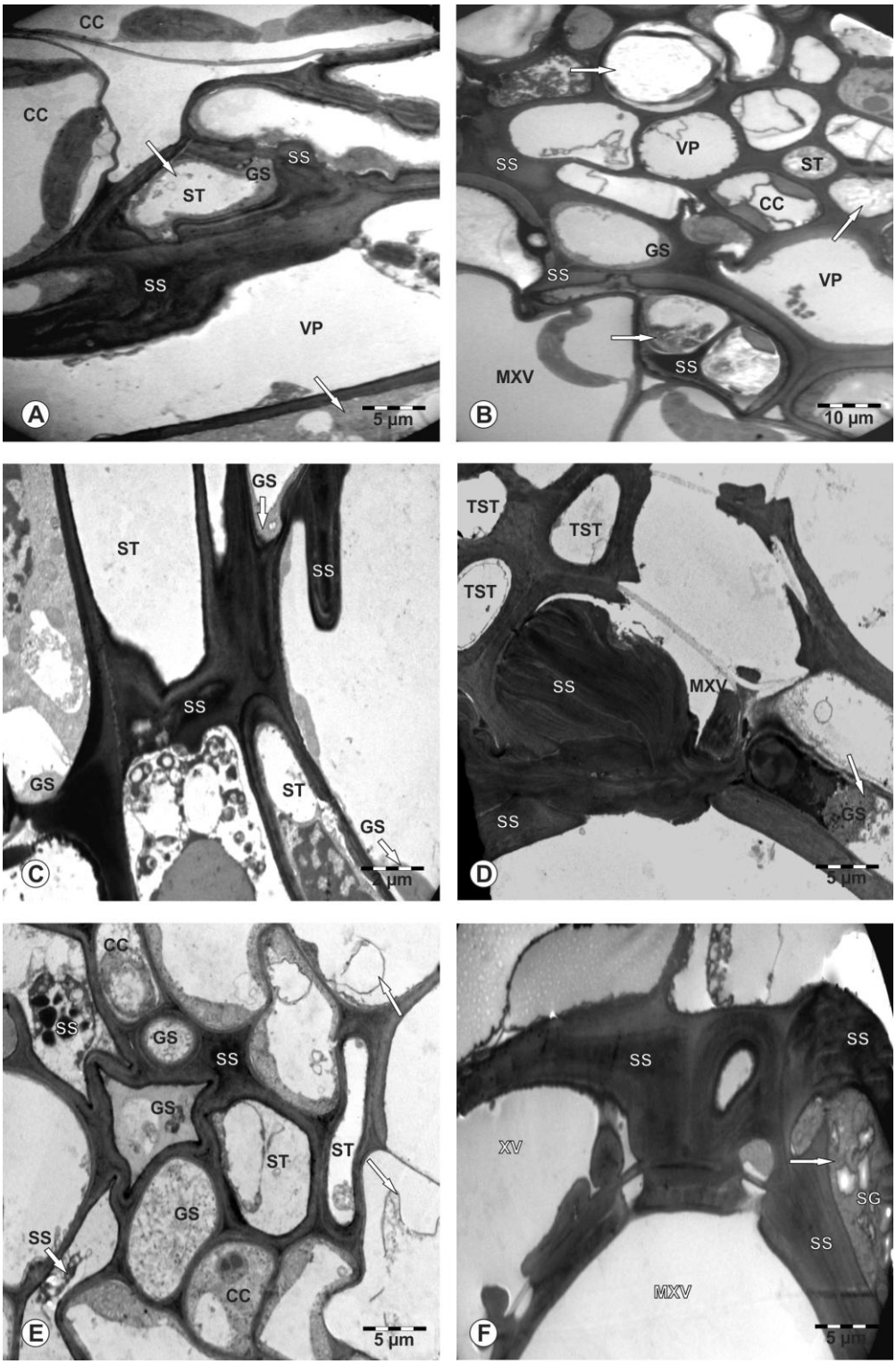

Figure 6.11 Cellular damage sustained by CIho 4125 and CIho 4159 by feeding of RWASA1, SA2 and SA3 after 14 days under elevated CO_2

Figure 6.11 Legend continued:

Representative TEM images which show feeding related damage sustained by probing by RWASA1 on CIho 4125 (**A**) and CIho 4159 (**B**); RWASA2 on CIho 4125 (**C**) and CIho 4159 (**D**); RWASA3 on CIho 4125 (**E**) and CIho 4159 (**F**) under elevated CO_2. Extensive damage was sustained by vascular tissues after 14 days of RWA feeding. Extensive probing was evident as were depositions of salivary sheath (SS) material and the resultant plasmolysis of parenchymatous elements including the sieve tubes. Nearly all living cells showed signs of plasmolysis. Probed xylem elements became compromised to the point complete lack of functionality. A-B) Stylet sheath deposition in vascular tissues and resultant plasmolysis of cells. C-F) Stylet tracks in xylem and phloem elements causing disruption, cellular plasmolysis and loss of functionality.

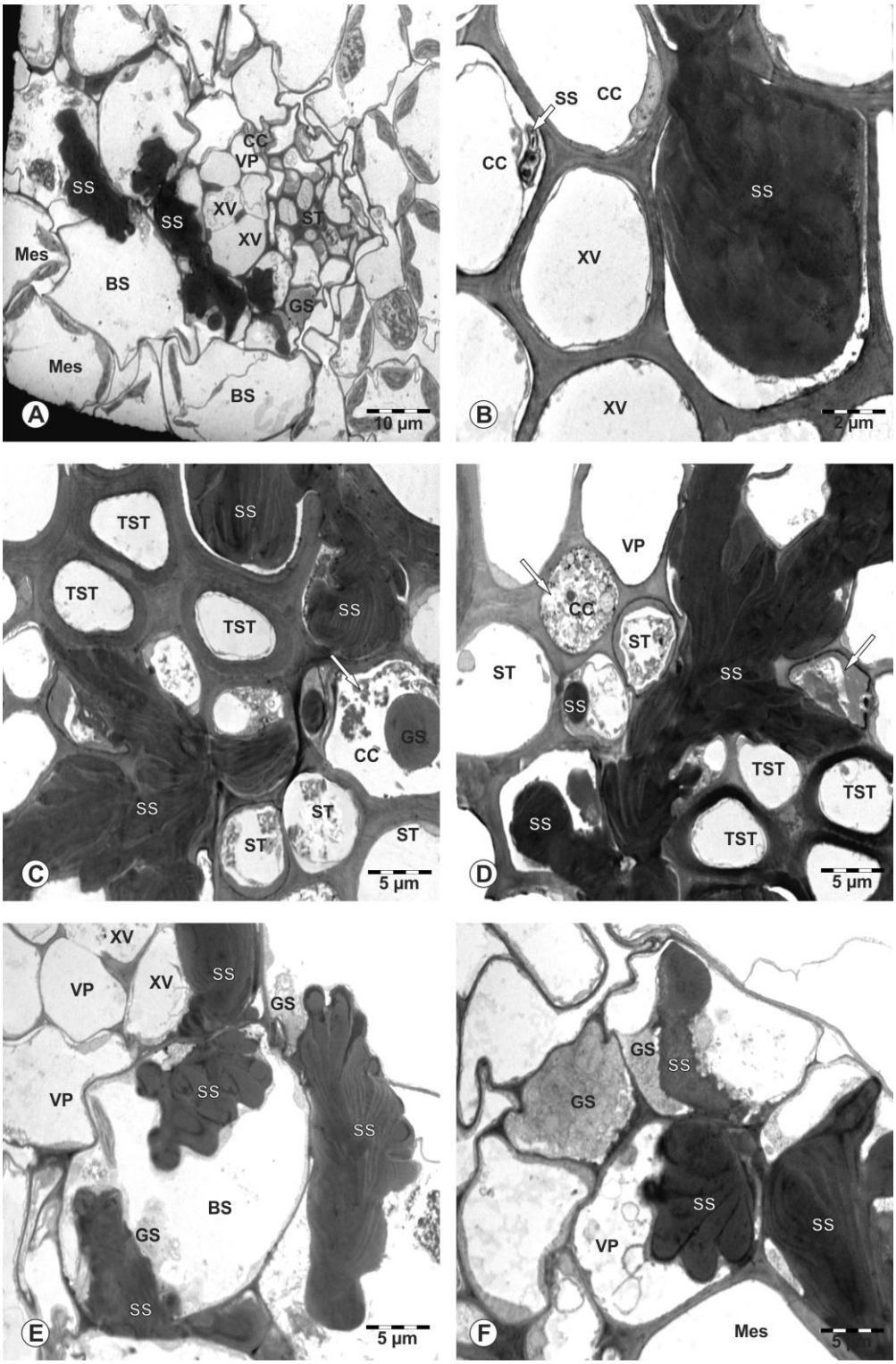

Figure 6.12 Cellular damage sustained by S5 and SSG 564 by feeding of RWASA1, SA2 and SA3 after 14 days under elevated CO_2

Figure 6.12 Legend continued:

Representative TEM images which show feeding related damage sustained by probing by RWASA1 on S5 (**A**) and SSG 564 (**B**); RWASA2 on S5 (**C**) and SSG 564 (**D**); RWASA3 on S5 (**E**) and SSG 564 (**F**) under elevated CO_2. It is evident from the TEM images here that the leaf tissues of S5 and SSG 564 have been totally compromised after 14 days of RWA feeding. The extensive destruction is a result of multiple inter- and intra-cellular probing events which leave large deposits of electron dense saliva (SS, GS) and cause plasmolysis of living cells. A) saliva deposition as a result of probing the vascular bundle through the mesophyll and the bundle sheath. B-D) Saliva deposition in vascular tissues, specifically xylem and phloem. E-F) Saliva deposition in mesophyll and bundle sheath adjacent to vascular bundles.

6.4 Discussion

6.4.1 The effects of RWA feeding on wound callose formation

RWA feeding causes severe wounding to vascular tissue of host plant leaves, which translates to physiological damage in terms of turgor loss and photosynthetic assimilate loss. Callose rapidly forms after wounding in an attempt to seal of the damage tissues and to limit assimilate loss and maintain turgor (Botha and Matsiliza, 2004; Saheed et al., 2009). The results presented here, confirm that the more virulent RWASA2 biotype causes significantly more callose formation than RWASA1 (supporting the findings of Jimoh et al., 2011). As expected RWASA3 feeding resulted in larger callose formations than RWASA1, but less than RWASA2.

All four plant varieties sustained callose damage at 14 DAI as a result of RWA feeing, which contrasts with the results presented by De Wet and Botha, (2007) and Walton and Botha (2008) who found that wheat cultivars containing the *Dn*1 resistance gene have greatly reduced or no visible callose as a result of aphid feeding. Even given that *Dn*1 is no longer classified as resistant in wheat due to its susceptibility to RWASA2 and RWASA3 (Jankielsohn, 2014). However, the observed interactions between the RWA and wheat do not necessarily apply to barley. (Saheed et al., 2009; Jimoh et al., 2011) The visible wound callose formations observed in the two Afghan accessions was similar as was the wounding effect in the two SA barley cultivars. The damage which S5 and SSG 564 sustained was similar to that inflicted by RWA on the susceptible barley cultivar Clipper (Saheed et al., 2007a). Based on these findings it would seem that CIho 4125 and CIho 4159 may be more resistant to RWA feeding than either S5 or SSG 564. However, given that all strains showed visible signs of wound callose under ambient CO_2, callose may not necessarily be the best indicator of innate plant resistance.

Elevated CO_2 caused a significant increase in callose formation in all experimental plants.. This was expected as elevated CO_2 conditions causes increased population growth which translated to increased feeding pressure from all three RWA biotopes, thus supporting the findings of Jimoh et al., (2013) who showed that there was a close correlation between wound callose and aphid population density. The results presented in chapters 3 and 4 demonstrate the close relationship between increased aphid populations under elevated CO_2, which together resulted in increased chlorosis and leaf roll. Thus it is not surprising to observe increased callose deposition in the host plant leaves following RWA infestation.

Under elevated CO_2 conditions all four barley varieties showed increases in callose deposition; however it could be argued that the SA cultivars were the most susceptible as they supported the largest callose depositions. Furthermore, it could be argued that the CIho accessions also became susceptible to larger RWA colonies feeding under elevated CO_2 because of the significant increases in callose depositions in comparison to ambient CO_2. These results conform to those of Chapter 4 where elevated CO_2 conditions caused and increase in aphid number which translates to a decrease in the observable resistance quality of the test barley plants.

6.4.2 The effects of RWA feeding on cell structure

Previous work has shown that even though callose density increases in sieve tubes, aphids are still able to feed from the phloem (Botha and Matsiliza, 2004; Saheed et al., 2009). All three biotypes are invasive when feeding, as inter- and intracellular probes of vascular tissues cause severe disruption, and frequently whole cells were completely obliterated. The saliva is known to block half-bordered pit pairs causing inhibition of water and solute exchange between xylem and vascular parenchyma transport interfaces (Saheed et al., 2007b). Saliva that remains in the cells after probing either form a stylet sheath that than compromises cell function, or remains permanently as ejecta in the cells, thereby reducing the efficacy of transport tissues. The punctured tissues then become isolated and localised necrosis and chlorosis will result, which supports what was shown in susceptible wheat and barley cultivars (Saheed et al., 2007a; Saheed et al., 2007b).

Damage levels were comparable between the host plants, the Afghan accessions seemed to sustain less damage than either S5 or SSG 564. The SA barley cultivars had more cellular disruption and plasmolysis; there was also more evidence of saliva which had been deposited than in the CIho accessions. Comparison between the three biotypes was difficult as they all showed the capability to cause terrible damage at the cellular level, suggesting that the major difference between the biotypes is simply reproductive rates.

The increase in aphid population under elevate CO_2 (as reported in Chapter 3) resulted in massive damage to the host plant cellular structures, with widespread increase in vascular disruption, cell isolation and plasmolysis. Based on the micrographs it could be argued that under elevated CO_2 as all test plants sustain such extensive damage that none of them showed any resistance qualities to RWA feeding. Furthermore, one could argue that the damage sustained is permanent.

6.4.3 Conclusion

The callose deposition results reported in this chapter support what has been shown in the previous chapters where RWASA2 is the most virulent of the three biotypes tested. RWASA3 is the next most virulent and RWASA1 is the least. In addition both the callose and cell TEM micrographs suggest that the CIho accessions have some resistance quality to RWA feeding under ambient CO_2, they sustain less damage than either S5 or SSG 564. However under elevated CO_2 all four varieties sustain severe cellular damage and support large formations of callose. This illustrates clearly the potential devastating effects that increased CO_2 environments would have on barley cultivation.

In conclusion and based on the fluorescence microscopy as well as TEM, it is clear that elevated CO_2 promotes and increase in aphid population, and that this results in an increase in probing-related damage as the ever increasing aphid population (possibly) struggles to find viable and functional sieve elements to feed on and functional xylem to sustain their needs for water.

Chapter 7: General discussion and conclusion

7.1 Preamble

The experiments reported in this thesis were carried out to identify and, if present, try to quantify any innate resistance qualities in two commercially important SA barley cultivars (S5 and SSG 564) to feeding by three South African (SA) Russian wheat aphid biotypes (RWASA1, RWASA2 and RWASA3). Jimoh et al., (2011a, 2013) reported that three American barley cultivars (STARS-0502B, STARS-9301B and STARS-9577B) had been identified as resistant to USA RWA biotypes, sustained heavy damage from RWASA1 and RWASA2. As a result it was decided that USA RWA resistant barley cultivars were not fully protected against the SA RWA. Given that the STARS cultivars were susceptible to RWASA1 and RWASA2, it was decided to examine other cultivars for potential resistance. Several Afghan accessions were noted by their nature and hardiness from their geographical origins. As a result CIho 4125 and CIho 4159 were selected together with S5 and SSG 564, two economically important SA barley cultivars, for an evaluation of resistance traits.

All current virulence data on the SA RWA biotypes, is based on RWA's interactions with the *Dn* resistance gene-containing wheat cultivars, but as of yet, no commercially-grown RWA resistant barley cultivars are available in SA. Since the RWA's discovery in SA many different management strategies have been attempted, including a control measure in which the application of systemic pesticides, such as dimethoate and demeton-s-methyl and vapour action pesticides, such as chlorpyriphos and parathion have been used (Du Toit, 1987; Smit et al., 2010). To limit the use of harmful pesticides, an integrated control measure was first implemented using resistant wheat cultivars, this system remains in force and the ARC constantly trials and deploys new resistant wheat cultivars (Tolmay et al., 1999; Jankielsohn, 2014). Initially this strategy worked very well as by 2001 70-85% of the cultivated wheat in the Free State province of SA was of a *Dn* carrying variety. The year 2005 saw the appearance of a second RWA biotype, which was noted to be virulent on certain of the resistant wheat cultivars (see Tolmay et al., 2007; Smit et al., 2010). In 2011 a third biotype designated RWASA3, and a fourth RWASA4, in 2014, were identified in SA, both with different virulence characteristics to RWASA2 (Jankielsohn, 2011; Jankielsohn, 2014).

Given that there are still no resistant barley cultivars in SA, and with RWA resistance known to be polygenic on quantitative trait loci, development of resistant barley is very difficult (see Dogimont et al., 2010; Dahleen et al., 2012). Therefore RWA control on barley cultivation

must still be carried out by a complete reliance on expensive and potentially harmful pesticides. The simplest way to develop RWA resistant barley cultivars would be to trial other currently utilised cultivars and to identify any innate resistance to RWA. Following this would be to to start breeding programs to carry these resistance genes into new RWA resistant commercially viable barley cultivars. Hence the inclusion of the two Afghan accessions in this study. It was thought, CIho 4125 and CIho 4159 were a good starting point.

In this thesis, the four barley varieties (CIho 4125, CIho 4159, S5 and SSG 564) were tested vigorously using a number of different experimental approaches to attempt to provide a clear picture of each barley plants potential resistance qualities to each of the three of the RWA biotypes (RWASA1, RWASA2 and RWASA3) tested. Aphid population growth rates were established, as was biomass loss due to RWA feeding along with chlorosis and leaf roll, callose formation and cell-ultra structure disruption. In addition the effects of RWA feeding on N content of the plants was investigated to provide a clear picture of each of the selected barley plants in terms of interactions with RWA.

Given that climate change is a reality, it was imperative to model the same barley/RWA interactions under a higher CO_2 concentration. This produced baseline data relating to elevated CO_2 conditions which might influence RWA or barley in any way that could lead to a change in aphid virulence or alternatively potential barley resistance.

7.2 RWA population growth rates on the experimental barley plants

Experiments were carried out to determine the absolute population growth and the relative growth rate (RGR) of RWASA1, RWASA2 and RWASA3 on the two Afghan barley accessions (CIho 4125 and CIho 4159) as well as the two SA cultivars (S5 and SSG 564) over 14 days under ambient and elevated CO_2 (see Chapter 3). Briefly, I found that:

A) Across both CO_2 regimes, the RWA biotypes had different population growth rates to each other; RWASA2 attained the highest population numbers and had the highest RGR, whereas RWASA1 had the lowest population growth rate. The "new" biotype, RWASA3 was confirmed to have a higher population growth rate and RGR than RWASA1, but was not as virulent as RWASA2.

B) The RWA populations differed depending on cultivar over the 14 days experimental period, with larger aphid population growth on the SA barley cultivars.

C) When the experiments were repeated at 450 ppm [CO_2], RWA populations of all three biotypes were significantly larger than those observed under ambient CO_2.

Differences in biotype reproductive rates have been found in other aphid species such as the greenbug (*Schizaphis graminum*) and among the USA biotypes of the RWA (Reese et al., 1994; Qureshi et al., 2005; Qureshi et al., 2006; Michaud et al., 2006; Smith et al., 2010). Amongst the SA RWA biotypes, RWASA2 has been conclusively established as virulent and outperforms RWASA1 in terms of reproductive capacity (Saheed et al., 2007; Walton and Botha, 2008; Jimoh et al., 2013). Given the prior research, it was not surprising when RWASA2 was shown to have higher reproductive rates that RWASA1. However, prior to the work I report here, there have been no attempts made to study the reproductive capacities of RWASA3, which as it turns out is surprisingly different from either RWASA1 or RWASA2.

7.2.1 CIho accession antibiosis resistance under ambient CO_2

Antibiosis is defined as a plants resistance response which occurs in reaction to RWA feeding, resulting in the production of secondary products, which may increase aphid mortality and hereby reduce aphid population growth (see Reese et al., 1994; Smith et al., 2010). Wheat cultivars containing the *Dn* series of resistance genes, are known to up-regulate processes involved with reactive oxygen species (ROS), pathogen defence and arthropod allelochemical defence (Smith et al., 2010). The antibiosis resistance quality that affects different RWA biotypes are well documented in many different wheat cultivars (see Haile et al., 1999; Tolmay et al., 1999; Michaud et al., 2006; Smith et al., 2010). The United States Department of Agriculture (USDA) have released barley cultivars with combinations of resistance genes, sourced mainly from the STARS series of germplasms. These cultivars have been shown to elicit an antibiosis effect on RWAUSA1 though this is limited on RWAUSA2 (Marimuthu and Smith, 2012). Jimoh et al., (2013) also showed that the STARS barley tested had some antibiosis effect on RWASA1, while RWASA2 was not influenced. Results reported in this thesis, thus strongly support previous work which demonstrates that magnitude and efficacy of host plant antibiosis differs between biotypes. Based on the above, it appears that there is some evidence of antibiosis by both CIho accessions, tested in this thesis, as they supported much smaller RWA populations than did the SA barley cultivars. Between the biotypes RWASA2 was least affected by antibiosis while RWASA1 was most affected.

The effect of antibiosis that was observed with RWA infestations on the CIho accessions under ambient CO_2 was non-existent under elevated CO_2 conditions. Other evidence of a

resistance breaking scenario was reported by Jimoh et al., (2013) where, USA RWA biotype resistant, STARS cultivars supported significantly larger RWASA1 and RWASA2 populations at 450 and 550 ppm [CO_2] than those populations observed at 380 ppm [CO_2].

The effects of elevated CO_2 on aphid fecundity have been subject to numerous investigations, and though the interactions are species specific, generally most aphid species have demonstrated increased fecundity under elevated CO_2 conditions (see Awmack et al., 1997; Hunter, 2001; Hullé et al., 2010; Yu et al., 2014). For example the peach potato aphid (*Myzuz persicae*), bird cherry-oat aphid (*Rhopalosiphum padi*), grass aphid (*Sitobion avenae*), glass-house potato aphid (*Aulacorthum solani*) and RWA have all been shown to exhibit higher reproductive rates under elevated CO_2 (Bezemer et al., 1998; Hunter, 2001; Xing et al., 2003; Sun and Ge, 2012)

7.3 Resistance qualities of the experimental plants

This thesis demonstrates that any potential inherent resistance quality in barley cultivars can be identified by examining the damage that the RWA biotypes inflict. Plant resistance was assessed according to three measures of damage. Damage visible to the naked eye in the forms of chlorosis and leaf roll ratings, was carried out over 28 days (see Chapter 4). Dry biomass of aphid infested plants was compared to that of aphid-free control plants (see Chapter 4). And finally, cell, tissue and morphological, foliar damage was assessed using fluorescence microscopy to examine callose formation and TEM was used to investigate the effects of aphid probing on plant visual bundles (See Chapter 6).

7.3.1 RWA feeding related chlorosis and leaf roll

Based on the population experiments it was expected that the CIho accessions would sustain less chlorosis and leaf roll than the SA cultivars, as it seemed that the Afghan accessions demonstrated some antibiosis resistance. The chlorosis and leaf roll ratings reported in Chapter 4 are summarised below:

A) As expected the visible damage recorded mirrored the population sizes and RWASA2 infested plants showed the most damage. RWASA2 infestations lead to the premature death of the host plant over the course of the experimental duration. RWASA1 feeding caused the lowest chlorosis and leaf roll ratings on the host plant leaves. RWASA3 feeding resulted in damage ratings similar to, but slightly lower than, those recorded for RWASA2.

B) The CIho accessions had lower chlorosis and leaf roll damage ratings than was determined for either of the SA barley cultivars. CIho 4125 and CIho 4159 appear to be moderately resistant to RWASA1, but they were susceptible to both RWASA2 and RWASA3. S5 and SSG 564 were susceptible to all three RWA biotypes.

The data presented in this thesis strongly supports the ARC differential and confirms that RWASA2 is the most virulent of the three RWA biotypes tested, followed by RWASA3 then RWASA1. Chlorosis and leaf roll are widely used to determine relative resistance in both wheat and barley, and to separate RWA biotypes. (see Reed et al., 1991; Burd et al., 2006; Gutsche et al., 2009; Jimoh et al., 2011a; Dahleen et al., 2012; Franzen et al., 2007). It has even been used to quantify resistance and virulence in other aphid species such as the greenbug (*S. graminum*) (Cabrera et al., 1995). The SA biotypes were identified and classified using similar methods (Tolmay et al., 2007; Jankielsohn, 2011; Jankielsohn, 2014).

Based on the experimental evidence presented in this thesis, the CIho accessions demonstrated some innate resistance to RWASA1. This was expected, considering the origin of the CIho accessions and that there are "natural" populations of RWA endemic to Central Asia. In the their natural ranges, the RWA is not considered to be a pest, because they undergo seasonal holocycly followed by anholocycly (Zhang et al., 2001; Zhang et al., 2012; Zhang et al., 2014). The Afghan accessions appear to have some effective antibiosis quality against RWASA1. It is most likely that RWASA1 is closely related to some of the wild RWA strains native to Afghanistan, and thus it is possible that the CIho accessions have become adapted to RWASA1 infestation. However, RWASA2 and RWASA3 evolved in an environment comprised of resistant wheat, and thus they are far more virulent than any biotypes that the CIho accessions would have encountered in their native ranges. Thus the CIho 4125 and CIho 4159 were rated as moderately resistant to RWASA1 and susceptible to RWASA2 and RWASA3.

Both S5 and SSG 564 were developed as high yielding cultivars, however they have been shown to be susceptible to all three RWA biotypes. Chlorosis and leaf roll damage is prevalent in these cultivars as they likely have none of the advantages of pre-adaptation. Thus pesticides are the only solution when using these cultivars. According to the US Environmental Protection Agency (EPA) whose pesticide tolerance guide cultivars are used by the USDA, the tolerance limits on barley are 0.2-1.7 ppm depending pesticide application. Any barley products where pesticide residue exceeded its tolerance values would be rendered

unfit for human consumption. This means that some pesticides must be applied at least 3 weeks before harvest; however, the data shows that under elevated CO_2 RWASA2 and RWASA3 cause severe damage after 21 days of infestation.

7.3.2 Decrease in plant biomass as a result of RWA infestation

To recap the plant biomass data reported in Chapter 4 showed that:

A) RWA feeding caused significant decreases to all plants biomass compared to the aphid free control plants. RWASA2 caused the greatest decreases in plant biomass followed by RWASA3 then RWASA1. Differences in biomass loss caused by the different biotypes was however insignificantly different.

B) Differences between the experimental plants were observed under control conditions with the SA barley accumulating more biomass than either of the Afghan accessions. This gain became a large loss when infested by RWA; there was little significant difference between the dry biomasses of all strains tested.

7.3.3 The effect of RWA feeding on plant tolerance

Reese et al. (1994) classified tolerance as the ability for plants to increase biomass despite insect attack. Based on this definition none of the plants used in this thesis, showed any tolerance to RWA feeding. The biomass loss was evident and the SA cultivars suffered the worst loss. The data in this thesis suggests that the CIho accessions antibiotic effect reduced RWA population growth rate and therefore comparatively less biomass was lost compared to the S5 and SSG 564, this is in agreement with Jimoh et al. (2013); Reed et al. (1991); Strauss and Agrawal, (1999); Girousse et al. (2005) where biomass decrease was noted to be more apparent more as a result of aphid feeding on susceptible cultivars than in resistant cultivars. One of the flaws of this approach is that biotypic differences were difficult to discern, thus I suggest that whole plant biomass is too broad as a measure of these interactions. However, it does provide insight of the plant functional burden that aphid feeding places on plant development resources.

7.3.4 RWA feeding related wound callose formation and cell disruption

The experimental evidence reported in Chapter 6 shows that:

A) It was difficult to separate and assess the level and extent of wound-related callose formations, without phase analysis (see Fig 6.6). RWASA2 caused the greatest wound callose

formations while RWASA1 feeding caused the least callose formation in the host plant leaves.

B) Fluorescence photomicrographs show that Afghan accessions had less extensive formations RWA related wound callose at 14 days of infestation (DAI) than did either S5 or SSG 564.

C) The TEM study showed that RWA feeding was highly invasive to host plant vascular tissues, however, any differences between RWA biotypes was difficult to establish, but the two CIho accessions suffered less extensive damage than S5 and SSG 564 at 14 DAI.

Given the population growth data shown in Chapter 3, it was expected that S5 and SSG 564 rather than CIho 4125 and CIho 4159 would have the more extensive callose formations. Susceptible wheat and barley cultivars have been shown to have larger wound callose formations than resistant cultivars (Belefant-Miller et al., 1994; de Wet and Botha, 2007; Will et al., 2007; Dinant et al., 2010; Bonnemain, 2010). Though RWASA2 caused more callose than the other biotypes tested, differences in the callose formations expressed in the leaf tissues infested by all three biotypes, this suggests that the callose responses were the same regardless of biotype infestation (Jimoh et al., 2011a) and that different population densities are responsible for the differences in callose formation across the RWA biotypes, this supports the phase analysis and TEM studies.

In general, the TEM micrographs showed that RWA feeding was highly invasive and that saliva sheaths and deposits caused disruption to phloem and xylem tissues alike, which lends support to the results of Saheed et al. (2007b) and Jimoh et al. (2011b). However, given the limited scope of the TEM study, the TEM analysis did not reveal any clear differences between the RWA biotypes tested.

As expected, chlorosis and leaf roll, TEM and fluorescence microscopy and biomass all demonstrated that RWA population size was the dominant factor determining the magnitude of the effect of RWA feeding. Chlorosis and leaf roll ratings, however, were the most insightful and provided better comparisons between the RWA biotypes but again it can be argued that damage is related directly to population size. An alternative would be to examine the host plants leaves at perhaps 7 DAI as RWA populations would be smaller and more scattered which could potentially provide a clearer picture of biotypic differences in RWA feeding.

7. 4 The effects of CO_2 environment on host plant N metabolism

The results reported in Chapter 5 demonstrated:

A) Control plants had increased % weight N under elevated CO_2 compared to ambient CO_2..

B) RWA feeding caused significant decreases to plant foliar N which resulted in increased C:N ratios.

7.4.1 Changing [CO_2] needs additional fertilizer

Much of the research conducted on plants under elevated CO_2 demonstrates that the increased carboxylation rates rapidly exhaust soil N supply. This results primarily in photosynthetic acclimation, which translates to changes in C:N ratio (Lindroth et al., 1995; Hughes and Bazzaz, 2001). The controlled (optimal nutrient) environments meant that N was not in short supply therefore, more N was available as part of the food source for the RWA. This would have direct effects on population growth rate as low N is believe to be a limiting factor to RWA fecundity (Porter et., 1991; Bezemer et al., 1998; Bezemer and Jones 1998). More food translates to higher aphid populations which translate to increased damage. A possible future experiment could be to examine the effect of N limitation on RWA populations.

7. 5 Changes in host plant symptoms of RWA infestation under elevated CO_2 conditions

To briefly recap, the findings of the elevated CO_2 experiments reported in Chapters 4, 5 and 6 showed that:

A) All experimental barley plants suffered more extensive chlorosis and leaf rolling under elevated CO_2 than at ambient.

B) Control plants biomasses were significantly higher under elevated than under ambient CO_2. However when infested with RWA under elevated CO_2 reductions in plant biomass were greater than those observed under ambient CO_2; though the differences were not statistically significant.

There was a correlation between the percentage increase of plant biomass (see Table 4.3) and aphid population growth under elevated CO_2 (see Table 3.1). For RWASA2 and RWASA3 the increase in population is similar to the percentage increase in biomass of all four barley varieties (see Table 7.1) However for RWASA1 its percentage population increase far

outstrips the plants' percentage biomass increase in three out of the four varieties tested, the exception being CIho 4159.

Table 7.1 Comparison between plant dry biomass data and increase in RWA population, both under elevated CO_2 conditions

Plant Varieties	% Dry Biomass Increase	% Increase in RWA population		
		RWASA1	RWASA2	RWASA3
CIho 4125	**76%**	310%	70%	51%
CIho 4159	**115%**	33%	96%	90%
S5	**46%**	122%	37%	28%
SSG 564	**31%**	124%	31%	52%

C) Control plants had higher foliar N under elevated CO_2 compared to ambient. However RWA feeding under elevated CO_2 caused massive decreases in plant N which translated to imbalances in C:N ratio.

D) Under elevated CO_2 the host plants callose formations were extensive and significantly larger than those observed under ambient CO_2. TEM evidence shows that the vascular tissues of all four barley strains become more disrupted and saliva tracks and deposits are larger and more common with RWA feeding under elevated CO_2.

7.5.1 Modelling the experimental results

Figure 7.1 shows a model that combines the principle findings of this thesis to provide a clear picture of the interactions between the RWA and the host barley plant under ambient and elevated CO_2. Under ambient CO_2, RWA probing is responsible for cell membrane plasmolysis and vascular disruption, which lead to localised chlorosis and leaf roll which decrease photosynthetic area and result in low biomass production. Furthermore, under elevated CO_2 the host plants have increased N metabolism and thus provide a more nutrient rich dietary solution which, when ingested by the RWA reduced limitations on their reproductive rates. Increased aphid numbers translates to increased probing events on the plants leaves which results in increased saliva deposition and vascular disruption. Longitudinal veins become blocked as a result of excess formations of wound callose, disrupting xylem and phloem transport (Botha and Matziliza, 2004). Following this areas of the leaves become isolated and die causing necrosis and chlorosis. The final result is reduced biomass accumulation and thus poor plant development and in the cases of the more virulent RWASA2 and RWASA3 biotypes the plants die within 28 days of infestation.

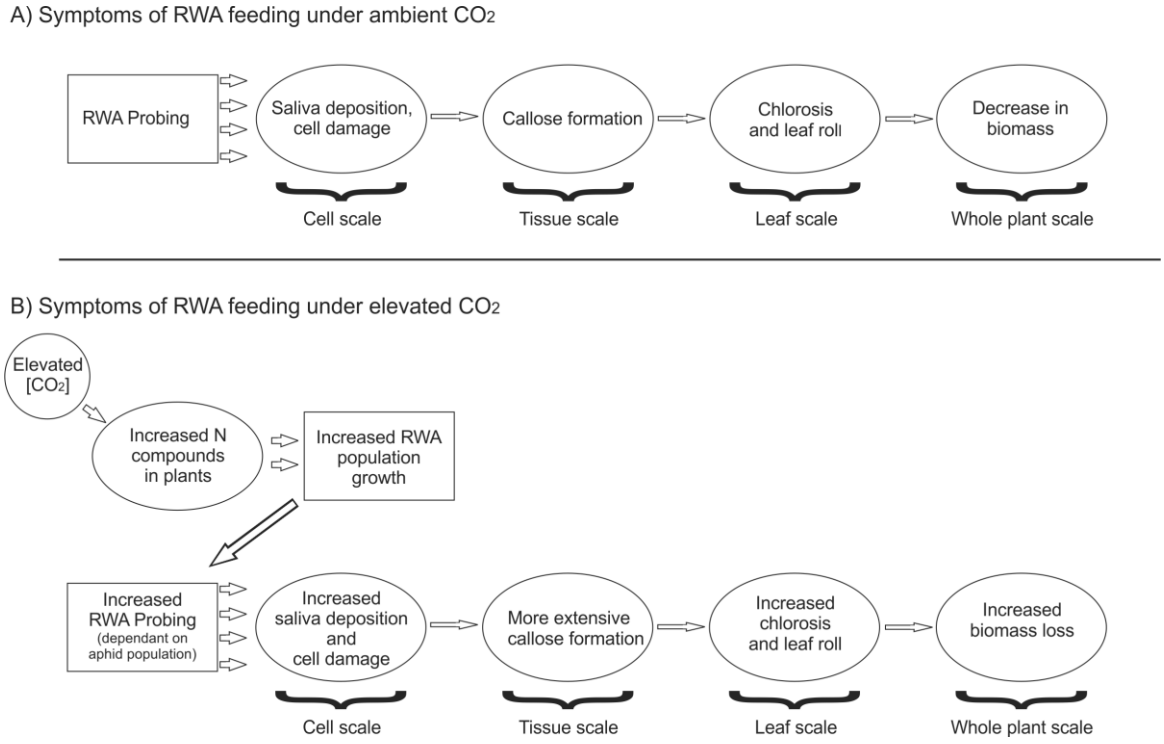

Figure 7.1 Model illustrating the knock-on effects of RWA feeding under ambient (A) and elevated (B) CO_2 conditions. As aphid population increases, so probing of xylem and phloem is intensified exacerbating the wound response (callose formation). Lack of supply of water and nutrients as a result of reduced phloem transport capacity (see Saheed et al., 2010). This form of inhibition of phloem transport leads to chlorosis and tissue death.

7.6 Concluding remarks

The research which I have presented in this thesis casts a somewhat gloomy picture for small grains including barley farming in SA. There are no RWA resistant cultivars and the projections based on the performance of S5 and SSG 564 suggests that RWA will increasingly threaten barley production, and thus food security as well. Finding resistant cultivars to the SA RWA biotypes is a difficult task and there is no evidence that barley producers are aware of the magnitude of the problem.

Elevated CO_2 conditions exacerbate the magnitude of the problem, so as CO_2 levels increase the aphids will potentially destroy/ reduce yields more. The costs will increase primarily, as the reliance on pesticides will become stronger, until the point it no longer becomes financially viable to farm barley. For example as of 2104 there were no more small scale wheat farmers in the North Eastern Free State, because the crop was no longer viable in that region.

A measure of resistance may be inherent in Afghan accessions, therefore these need to be explored and gene mapped to check for potential *R* genes.

7.7 Reflections on experimental conditions

The use of controlled environments (CE) have been criticised, and it can be argued that the scenarios presented in this thesis may not be a "true" reflection. Therefore, the aim and sole purpose of using CE was exactly that: to control the environment and then manipulate it to create a "worst case" scenario and to test, specifically, the effects of elevated CO_2 on the three RWA biotypes and the selected barley plants.

The cages that confined the aphids to the test plants could be considered to be a flawed approach as they remove (or mask) any potential antixenosis effects. Antixenosis is defined as a plant based defence response through which the host plant prevents colonization by aphids (Heslar, 2011). Using cages means that the aphids have no alternative host, or means of escape, leaving them but one option -to infest the given host plants regardless of any potential antixenosis effects. Even though the aphids successfully colonised the host plants it would be nonetheless interesting to provide the aphids with a choice of cultivars using CE. It follows therefore that the plant with the strongest antixenosis expression would (theoretically) have the lowest aphid population size. In addition, as host plants weaken the RWA colonies begin the production of winged nymphs, which would then search out new viable hosts (Goggin, 2007). It would therefore be an interesting study to include the number of winged morphs produced as the experiments progress. It is a possibility that the massive RWA populations observed under these experimental circumstances were a result of the RWA confinement, whereby they were unable to relocate to healthier hosts.

Furthermore it would be of interest to carry out the same series of experiments with plants under standard fertilization, and perhaps analyse photosynthetic assimilation rates to analyse the impact of RWA population size on photosynthesis.

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