A preliminary study on the effects of elevated CO_2 on aphid resistance of Tugela *Dn* and the population dynamics of the Russian wheat aphid (Homoptera: Aphididae), *Diuraphis noxia*

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DECLARATION

I, DAPHINE MUNDONDO, declare that this dissertation titled "A preliminary study on the effects of elevated CO_2 on aphid resistance of Tugela Dn and the population dynamics of the Russian Wheat Aphid (Homoptera: Aphididae), *Diuraphis noxia*" submitted for the award of the Master of Science degree in Biochemistry at the University of Fort Hare, is my own work that has never been submitted for any other degree at this university or any other university.

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Abstract

Food security is of major importance due to the increasing world population with 8.9 billion people expected by 2050 (Cohen, 2003). *Diuraphis noxia* (RWA), have caused aggravating, massive losses to wheat farmers in many areas of the world. If unchecked, RWA are able to destroy plants resulting in major economic impacts (Botha, 2013). Due to ineffective use of other control methods, the Small Grains Institute in Bethlehem, South Africa, have therefore developed resistant cultivars to the known RWA subtypes over the past decades through intensive breeding programmes (Tolmay *et al*., 2006). Climate change has however become a major factor threatening food security especially with the observed increase in CO_2 from less than 300 ppm in pre-industrial period to the current 385 ppm and is predicted to reach 550 ppm by 2050 (IPCC, 2007; Meehl *et al*., 2007). Elevated CO_2 concentration may affect individual species of a community hence the need to understand the wheat-aphid interactions.

In this study, population growth rates and virulence of RWA SA1 at ambient (385 ppm) and elevated (450 ppm) CO_2 concentration were evaluated on two wheat cultivars: Tugela Dn (resistant) and Scheepers (susceptible). Fluorescence microscopy techniques using aniline blue were used to investigate feeding related damage caused by RWA SA1 through an examination of callose deposition at the two CO_2 concentration. A two-dimensional gel electrophoresis method was developed in order to determine the effect of RWA SA1 on the wheat cultivars proteome at the two CO_2 concentration. Differentially expressed proteins that were up or down regulated more than two fold were identified using PDQuest™ Basic 2D Gel analysis software.

Populations of RWA SA1 increased significantly on the two wheat cultivars at both CO_2 concentration. Although the population growth rate for RWA SA1 on both cultivars was generally exponential at all treatments, growth at elevated CO_2 concentration was noticeably faster with populations increasing 3 fold in 14 days as compared to the 2 times at ambient CO_2 concentration. Hence, both cultivars provided a better quality host for RWA SA1 at 450 ppm than 385 ppm. There was no significant difference between RWA SA1 population on Tugela Dn and on Scheepers at elevated CO_2 concentration on day 14 after infestation which means there was a change in the resistance mechanism in Tugela Dn at this condition. Approximately 70% of the total leaf showed chlorosis by 21 days of aphid infestation for both cultivars although the susceptible cultivar was more vulnerable. There was low callose

deposition in the controls (uninfested plants) but heavy callose in infested plants due to aphid feeding.

A proteomics approach was used as a pilot study to investigate whether it would be possible to identify the changes in the resistance mechanism during aphid infestation under elevated CO_2 levels. The major changes in the proteome of the control group (uninfested Tugela Dn at ambient versus elevated CO_2 concentration) occurred in the early events (day 1-7) in the molecular weight range of approximately 25 kDa to 55 kDa are mainly within the basic to neutral pH range. This was suggested to be a result of mechanisms to adjust to the CO_2 concentration. Elevated CO_2 results in instant higher photosynthetic rates and C:N ratios as well as changes in expression levels of SA-dependant defense genes (Lindroth 1995; Hughes and Bazzaz, 2001; Sun *et al.*, 2013). Because most of these changes are directly regulated by proteins, it is expected that the most differential protein expression will occur immediately after the atmospheric changes (early events) as was shown in the study.

Infested plants under elevated and ambient conditions showed that the stress conditions gave rise to differentially regulated proteins within the wheat proteome. Most changes occurred elevated CO_2 levels. It can be suggested that the changes were a result of differentially regulated plant defence proteins which fall in this range (25 kDa - 80 kDa) such as peroxidases, chitinases and β-1.3-glucanases as well as protein kinases, heat-shock proteins and photosynthetic proteins. These results indicate that there has been changes in the resistance due to elevated CO_2 because of the evident changes in the proteome.

If so, then the results will be similar to those documented by Louw (2007) where up-regulation was due to putative storage proteins, proteins involved in photosynthesis, heat shock proteins and defense proteins. Of course, the pI value and molecular mass of the proteins and the identification of the proteins in these spots, must be determined in future work to specifically identify whether these suggestions are authentic. However, Louw (2007) also reports that the susceptible Betta wheat cultivar, displayed a defence response similar to the HR although it was unable to up-regulate specific defensive proteins against RWA infestation but proteins for broad resistance. Although the changes in the proteins in infested Tugela Dn under elevated CO_2 concentration were not accurately identified, the defense mechanism is similar to that portrayed by the susceptible Betta wheat cultivar which shows that the resistance mechanism had been overcome. Because this was a pilot study and

preliminary results were obtained due to limited funding and time constraints, suggestions were made on how to further develop the method to obtain statistically significant results.

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List of Abbreviations

^{o}C	Degrees Celsius
2-DE	Two Dimensional Gel Electrophoresis
ADP	Adenosine diphosphate
Amb	Ambient
ATP	Adenosine triphosphates
Avr	Avirulence factor
C:N	Carbon to Nitrogen ratio
$[CO_2]$	Carbon Dioxide Concentration
DAI	Days After Infestation
DAMP	Damage-Associated Molecular Patterns
Dn	*Diuraphis noxia*
DNA	Deoxyribonucleic Acid
Ele	Elevated
ET	Ethylene
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'triphosphate
Ha	Hectares
HR	Hypersensitive Response
HSP	Heat-Shock Protein
IEF	Iso-electric focussing
JA	Jasmonic Acid
LRR	Leucine-Rich Repeat
MES	2-[morpholino] ethanesulfonic acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction

qPCR	Real-Time Polymerase Chain Reaction
ROS	Reactive oxygen species
RWA	Russian Wheat Aphid
R-gene	Resistant gene
SA	Salycylic Acid
SAR	Systemic acquired resistance
Sch	Scheepers
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
Ser	Serine
Thr	Threonine
Tug Dn	Tugela Dn
UV	Ultraviolet

1 CHAPTER ONE

1.1 Literature Review

1.1.1 General Introduction

Plants are naturally sessile, and subject to diverse stresses. Plant stress is very complex and highly dynamic. It tends to inhibit normal systems from functioning optimally thus affecting growth, development and productivity (Atkinson and Urwin, 2012). Biotic and abiotic stress is the negative impact of living and non-living organisms respectively in a specific environment. Abiotic plant stress includes the climatic or environmental, physical or chemical insults such as light, temperature, salt and pollution. Whereas, biotic stress includes pests and pathogens such as insects, nematodes, bacteria and fungi. Major losses in crop productivity worldwide occur as a result of abiotic and biotic stresses. The frequent exposure of plants to these disturbing external pressures usually leads to the activation of complex response mechanisms within the plants.

The study of plant-insect interactions has been extensively studied by scientists for a while now with the integration of many fields which include: biochemistry, botany, physiology, ecology and evolution (Moran *et al*., 2002). Insects are a very diverse group and normally exist in large populations. This is due to their small size, high rates of reproduction, abundance of food supplies and adaptation to every land and freshwater habitat (Insect, 2014). Insect plant feeders are of a major threat to commercial agriculture. They can be classified depending on the way in which they cause harm to the plant e.g. sap-sucking insects such as aphids, gnawing insects such as locusts, tunnelling insects such as ants and pathogenic vector agents such as cockroaches. They are responsible for direct injury through their feeding as well as indirectly by transmittance of bacteria, viruses or fungi. For example the viral diseases of potatoes carried from plant to plant by aphids (Metcalf and Metcalf, 1993).

It has been discovered that plants experience changes in their chemical composition and morphological set-up in response to attack by pathogens and herbivores (Karban and Baldwin, 1997). Plants can perceive insect attacks either through metabolic changes in

injured tissues or binding of molecular compounds to certain receptors in the plant to initiate an opposing response (Saheed *et al*., 2008). To overcome this, insects have in turn evolved specialised mechanisms to evade sensitivity, down-regulate plant defence responses and gain maximum nutrition from its host. Hence, the interaction between plants and insects such as aphids are complex and dynamic.

1.1.2 Wheat

Wheat is of the genus *Triticum L* and belongs to the grass family Graminea. *T. aestivum* (also known as common or bread wheat) is a very important crop with abundant uses. For that reason, it is among the "big three" cereal crops alongside rice (*Oryza sativa*) and corn (*Zea mays*) (Shrewrey, 2009).

1.1.2.1 Origin and Evolution of Wheat

According to Shewrey (2009), wheat was first cultivated about 10 000 years ago. Its origin is debatable although most authors suggest that its cultivation originated in the middle east citing as evidence that the earliest remains of the crop were found in Turkey, Jordan and Syria (Gibson and Benson, 2002). Archaeological proof has drawn the conclusion that the domestication of wheat originated from Southern Levant spreading quickly into the rest of the Fertile Crescent dating back to 9, 600BC (Badr *et al*., 2000). The Fertile crescent was an area that ran through Mesopotamia (now Iraq) and Syria. The domestication of wheat brought enormous changes where people began growing their own food transitioning from hunting and gathering referred to as the Neolithic Revolution (Shewrey, 2009). Soon as production increased, trade between various cultures developed.

Modern day cultivated wheat evolved from the earliest wild forms. The genomic affinities of a large number of members of the *Tritecia* family where studied to try and determine the evolutionary steps of wheat. The earliest cultivated forms were diploid (genome AA i.e. 2n=2x=14 e.g. einkom) and tetraploid (AABB: 2n=4x=28 e.g. emmer) (Dubcovsky and Dvorak, 2007). There were a number of crossings with these wild species to the hexaploid (2n= 6x=42) bread wheat. *T. aestivum* (AABBDD) appeared when *T. turgidum* (AABB) crossed with *T. tauschii* (DD), a weed within the crops or around the margins of cultivation (Feldman, 2001).

The final major step in the evolution of bread wheat was the selection from the hulled forms where the enveloping glumes adhere tightly to the grain seed, to the free-threshing naked mutants (Dubkovsky and Dvorak, 2007). The early domesticated emmer, einkom and spelt forms were all hulled compared to the recent bread wheat which is now free-threshing. In these later plants, the grain can easily be separated from the glumes thus improving the texture of the flour. As early farmers probably set aside the best wheat plants to use as seeds for the coming years, the best wheat qualities were passed to new generations.

1.1.2.2 Overview of wheat production

Wheat is an annual grass which grows at areas from sea levels to altitudes over 300m. It prefers a habitat with well-drained, clay-loam soils of little rainfall and extreme temperatures (Gibson and Benson, 2002). It can be grown throughout temperate, mediterranean-type and subtropical regions of the world. It is well adapted to harsh environments and mostly grown on wind-swept, dry and cold areas as compared to rice and corn which are grown in warm regions with high rainfall as they require ample water.

More than 600 million tonnes of wheat are harvested annually worldwide (FAOSTAT, 2012). For example, approximately 671 million tonnes of wheat where produced worldwide in 2012 compared to 720 million tonnes of rice and 872 million tonnes of maize (FAOSTAT, 2012). The world leader of wheat production is China followed by the United States of America. In South Africa, there has been a general decrease in wheat production and area it is harvested during the period 2008 to 2012 (Table 1.1). There is a need therefore, to find ways in which wheat production can be enhanced.

1.1.2.3 Uses of Wheat

Wheat is mainly used as a human food source as well as a livestock feed. It is nutritious and can easily be stored, transported and processed into various foods. It is the major ingredient in most foods such as bread, biscuits, cakes, baby foods, and pasta. Wheat has become a major staple food and provides about 20% of the calories for the world's population (Gibson and Benson, 2002). Wheat is a high carbohydrate food with additional valuable minerals, vitamins and proteins.

Wheat is the only plant derived food that contains gluten protein which forms minute gas cells that hold carbon dioxide during fermentation, enabling dough to rise. The wheat grain is also used for fermentation to make alcoholic beverages such as beer. Wheat is therefore of a major economic importance. In 2012, the total world wheat production was worth over $79 Billion (FAOSTAT, 2012).

Table 1.1 - Overview of Wheat Production and the Area Harvested in South Africa from 2008 – 2012 (Adapted from FAOSTAT, 2012).

Year	Area Harvested (Ha)	Production (Tonnes)
2008	748 000,00	2 130 000,00
2009	642 500,00	1 958 000,00
2010	558 100,00	1 430 000,00
2011	604 700,00	2 005 000,00
2012	511 000,00	1 915 000,00

1.1.2.4 General Anatomy Of Wheat

The plant is generally made up of a root system, stem, leaves and a flower spike or head (Fig 1.1). Most grow to approximately 1 metre in height. The wheat plant has two types of roots, the seminal/seedling roots and the clonal/adventitious roots (Maiti *et al*., 2012). The seminal roots are the primary roots belonging to the embryo at germination. The clonal roots form the permanent root system replacing the seminal, arising from the basal nodes forming the vegetative crown. Roots function in gathering nutrients for sustenance from the soil and providing anchorage for the plant.

The stem is erect, cylindrical, hollow and jointed from which the leaves emerge at opposite sides. Bold joints called nodes separate plant into sections of repeating units called internodes. Each unit potentially comprises of a node, an elongated internode and a leaf with a bud on its axial (Kirby, 2002). The stem supports the head of the plant. The head of a wheat plant consists of kernels covered with bristle-like spikes called beards. The kernel is the seed/grain from which a wheat plant grows (Maiti *et al*., 2012).

The leaves of a wheat plant are long, thin, simple and alternate. The stem contains parallel venation arranged into rows. The leaf consists of two parts which form from different meristems: the leaf sheath which encircles the stem and the leaf blade (lamina) which bends away from the stem. The lamina has a visible midrib along which the major vascular bundles (primary site of aphid feeding) run.

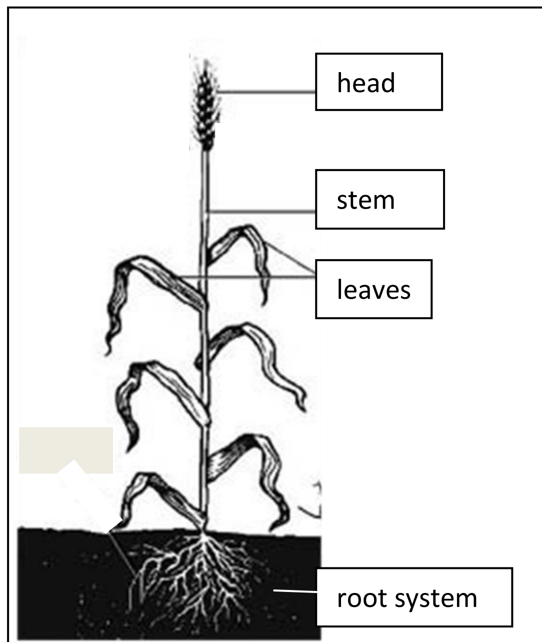


Fig.1.1: The General Structure of a Wheat Plant. (Taken from: http://www.bakeinfo.co.nz/Facts/Wheat-Milling/Wheat)

Further, comprehensive analysis of the anatomy of wheat leaves is crucial to understand the mechanisms of aphid feeding on the vascular bundles. Wheat plants grow several side shoots or lateral branches called tillers which have a basic structure similar to the main shoot (Kirby, 2002). These arise from the axils of the basal leaves and may grow a long stem which bears a flowering head at the top.

1.1.3 The Russian Wheat Aphid (Diuraphis noxia, Mordvilko)

The family Aphididae consists of aphid colonies specialized to feed on phloem sap. According to Blackman and Eastop (1994), this family comprises of more than 4300 species. The Russian Wheat aphid (RWA), *Diuraphis noxia* (Mordvilko) is a phloem-feeding pest of

wheat and barley causing large crop losses that lead to major economic losses (Vandenberg *et al*., 2001).

1.1.3.1 Morphology

It has a small lime-green spindle-shaped body, less than 2mm in size with very short cornicles, antennae and legs. Viewed from the side, its appendage above the cauda appears as a second tail (Michaud and Sloderbeck, 2005).

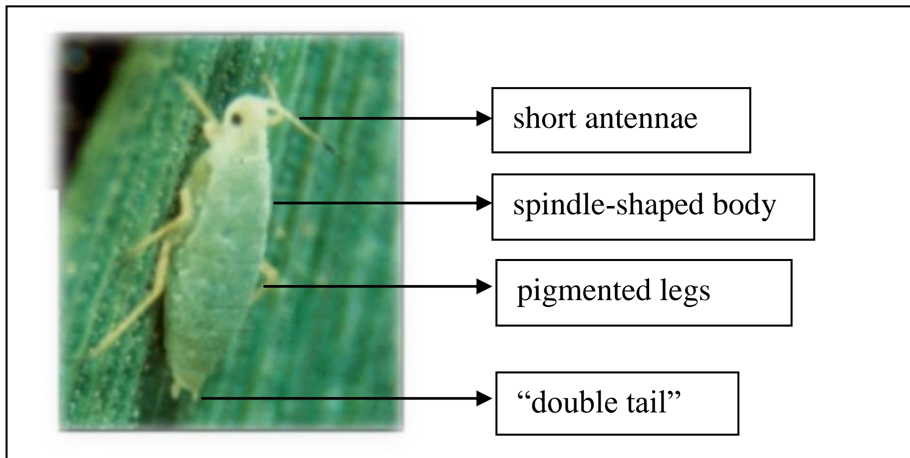


Fig. 1.2: The Russian Wheat Aphid: Wingless Female. (Taken from: http://iwheat.org/book/russian-wheat-aphid)

1.1.3.2 The Life Cycle of RWA

RWA reproduces asexually with females giving birth to daughters that are living at the same time carrying embryonic granddaughters (Michaud and Slonderbeck, 2005). Although there are male forms, they have not been reported locally because they reproduce parthenogenetically without fertilisation (Schotzko and Smith, 1991). Hence, there are two forms of RWA: a winged or wingless female (alate and apterous respectively) (Saheed, 2007). The winged form is responsible for relocating in search of a suitable host and initiating a new colony. Thus it usually develops when the conditions of the hosts become an unfavourable habitat likely due to stress. After settling in more favourable conditions, they start to reproduce new nymphs within a period of 14 days (Walters *et al*., 1980). These are relatively sedentary maturing from 9 days under favourable conditions and in turn produce up to 4 nymphs each day and live for a mean period of up to 70 days. Dense colonies form as

offspring infest new leaves of the host (inside the rolled up leaves). This mechanism to develop and reproduce fast, asexually gives rise to the bursting population growths achieved by this aphid, reducing the risk of individual attacks by natural enemies.

1.1.3.3 History and Distribution

RWA is native to Russia, from where its name is derived, and countries bordering the Mediterranean Sea such as Iran and Afghanistan (Hewitt *et al*., 1984) where it has been regarded as a cereal pest from 1912. It was first reported as a pest in the USA in 1986 (Burd and Burton, 1992) and in all wheat-producing countries by 1990, with Australia being the exception (Basky, 2003). The first sightings of RWA in South Africa were in 1978 in the Orange Free State (Walters *et al*., 1980). RWA caused up to 90% crop loss in field experiments especially in the summer rainfall production region of South Africa (Du Toit and Walters, 1984). Increasing economic losses of approximately $1 billion due to RWA were estimated in the US from the period 1987-2000 (Webster *et al*., 2000). It continues to be a persistent serious pest on wheat production thus has been depicted as a major pest in many countries of the world.

1.1.3.4 Host choice

Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are the most preferred hosts. There are a number of different host plants that play a major role of providing a food source between summer grain harvest and emergence of fall (the wheat producing seasons) (Karen, 1989). These include wild oats, rye, and volunteer wheat in road reserves, false barley, wild grasses around field margins, maize and at times sorghum. This study focuses on wheat as the host for RWA.

1.1.3.5 Feeding Habits

RWA has a narrow mouthpart called stylet, that enables it to pierce through leaves and suck phloem sap from its hosts. During feeding, it probes its stylet between cells (intercellular) until it reaches the phloem (Fouché *et al*, 1984) from which it gets its nutrient supply. RWA colonies aggregate on plant parts where food is of high quality and quantity specifically from

sieve tubes of the vascular bundles and preferentially from the thin-walled sieve tubes (Matsiliza and Botha, 2002).

1.1.3.6 Symptoms of infestation

RWA visible feeding damage includes distinct white or yellowish longitudinal streaks and severe leaf rolling. RWA induces two types of leaf rolling in cereals by inhibiting proper unfolding in newly developed immature leaves and inducing leaf folding in fully expanded mature leaves (Walters *et al*., 1980). This encloses the developing aphid colony in a tubular protective shelter making it difficult to use insecticides or parasitic insects against the aphids.

During probing and feeding activities, the aphid injects its saliva containing a toxin (Fouché *et al*, 1984) that causes the longitudinal white or yellowish streaks on the leaf. After several days of feeding, the chloroplast and cell membranes become disrupted leading to extensive chlorosis and necrosis (Marasas, 1999). During feeding, aphids can acquire viruses from infected plants and in turn, release and inoculate these into healthy plants. Damsteegt and colleagues (1992) reported the transmit ion of plant pathogenic viruses, such as the barley yellow dwarf virus, by RWA which results in stunting and loss of the green colour in leaves to usually bright yellow.

RWA feeding and associated symptoms therefore affects yield and quality of the wheat as reported by Girma *et al*. (1993), where they showed that plant height, shoot weight and number of spikes were significantly reduced as a result of the feeding of *D. noxia*. Botha and Matsiliza (2004), summarised that:

> "feeding by *D. Noxia* results in redirection of the assimilate flow through the formation of local sinks and also causes massive, possibly long-term, damage to cells and tissues, due to feeding-related pressure loss, through enhanced callose deposition in the damaged functional phloem in non-resistant wheat."

Hence, this reduction in carbohydrate translocation capacity of the phloem brings stress on plant development.

1.1.4 Aphid Primary Feeding Sites: The Vascular Bundles

Grasses of the Gramineae family have a common anatomy which can be extended amongst the family members (Saheed, 2007). The leaf blade of wheat – like barley, has parallel

longitudinal vascular bundles which are located in ridges and separated by the mesophyll. Abundant transverse veins interconnect the vascular strands (Blackman, 1971).

1.1.4.1 Longitudinal Veins Of Wheat Vascular Bundles

Three orders of the vascular bundles can be distinguished in the longitudinal veins of the wheat leaf blade. They are classified according to bundle size, presence or absence of large metaxylem vessels as well as protoxylem elements (Martre and Durand, 2001; Botha, 2013). The first order, known as the large bundles, are characterised by the presence of both protoxylem and protophloem (first formed primary xylem and phloem respectively). They have a distinct metaphloem with two large metaxylem vessels which are distributed on either sides of the protoxylem lacuna (air space) (Cutler *et al.*, 2008). The second order - intermediate bundles- lack a protoxylem and clear metaxylem (Matsiliza, 2003). Large and intermediate bundles are associated with strands of hypodermal sclerenchyma (supporting or protective tissue) (Cutler *et al.*, 2008). They always have metaphloem and often protophloem (Evert *et al.*, 1996). The third order -the small bundles, typically consists of only of metaxylem and metaphloem which are present in all the other orders. As noted by Cutler, Botha and Stevenson (2008), these bundles lack a protophloem and are not normally associated with either hypodermal strands or girders. They make up the marginal strands for most of the length of the leaf blade (Dannenhoffer *et al.*, 1990).

Vascular bundles are usually surrounded by two sheaths, an outer parenchymatous bundle sheath and an inner mestome sheath (between the metaxylem vessel and bundle of sheath) (Matsiliza, 2003). In C_3 and C_4 grasses, these orders also have been found to have different roles (Fritz et al., 1989). The small as well as intermediate bundles are mainly the loading bundles (Evert *et al.*, 1996) whilst large bundles are involved in longitudinal transport. These orders have been found to have different roles in water conductances (Altus *et al.,* 1985). According to Martre and Durand (2001), the small and intermediate bundles distribute water across the leaf by forming a distributing network connected by the transverse bundles. Large bundles dominate in the longitudinal direction of water conductance from the base to the tip of the leaf, distributing also to the mesophyll in their vicinity.

Phloem is the living tissue that is concerned mainly with the transport of soluble organic products of photosynthesis from the source to all parts of the plant where needed (sinks) (Cutler *et al*., 2008). Protophloem is momentary and is replaced by metaphloem (Botha, 2013). The longitudinal veins contains two types of metaphloem sieve tubes namely thin-walled (early) and thick-walled (late) sieve tubes basing on the times of differentiation and position within the phloem. According to Dannenhofer and colleagues (1990), thin-walled sieve tubes have a thin cell wall and are associated with companion cells and vascular parenchyma cells whilst thick-walled sieve tubes have a thicker cell wall and lack companion cell associations. They are in close proximity to metaxylem and connected to vascular parenchyma cells adjacent to metaxylem by means of pore-plasmodesma units (Botha, 2013). There is no proof of any plasmodesmal interconnections of the two metaphloem but are isolated from each other symplasmically.

1.1.4.2 The Function of the thick- and thin-walled sieve tubes

The functions of both sieve tube elements in the Poaceae family members have also been critically reviewed. There is evidence that ^{14}C-assimilates are almost exclusively translocated through the thin-walled not thick-walled sieve tubes. Frits *et al*., (1983) reported collective results from experiments with Z. *mays* fed on 14C-sucrose which supported that thin-walled sieve tubes where more functional in translocation and phloem loading than their correspondence. In addition, Matsiliza and Botha (2002) reported that the aphid *S. Yakini* feeds preferentially on the thin-walled sieve tubes of the small longitudinal vascular bundles as 96% stylets and stylet tracks terminated in those particular sieves tubes. Under normal and stress conditions, the data suggested that this aphid is more attracted to the thin-walled sieve tubes and are most likely more functional in terms of phloem loading and transport. Botha (2013) suggests that though it’s difficult to explain, the inclination towards the thin-walled sieve tubes by aphids could probably be due carbohydrate levels. These sieve tubes have a higher sucrose content which attracts the aphids. According him, the fact that aphids can infiltrate the lignified xylem vessels often and feed on watery solutes out-rules the rational that thick-walled sieve tubes have a tough wall structure for aphid stylets to penetrate.

1.1.5 Emergence of wheat resistance and RWA biotypes

Due to the destructive nature of the aphid's feeding behaviour on crops, methods where devised to try and control the damages. The primary means of controlling crop pests included using chemical controls e.g. pesticides, biological controls i.e. introducing natural enemies and cultural controls which involve the use of agronomic practices such as early maturing cultivars and crop rotation. Chemical controls are extremely expensive, damage the ecosystem and do not differentiate non-targeted beneficial insects. The secure shelter of rolled leaves also make biological controls less effective. Plant resistance is a defensive strategy e.g. against herbivores, which results in reduction of the amount of herbivore damage (Leimu and Korichera, 2006; Dogimont *et al*., 2010). There are resistant and non-resistant (susceptible) wheat varieties. The development of wheat resistant cultivars is the most sustainable, commercial and environmentally safe method generally acceptable to control RWA damage (Liu *et al*., 2001; Tolmay, 2008; Dogimont *et al*., 2010). Furthermore, natural enemies can also be utilised in support of host resistance as the cultivars have leaves that defy rolling taking away the ability of the aphids to nest inside them (Tolmay *et al*., 2007).

1.1.5.1 The Development of Resistant Wheat

The spread of RWA to the USA and Mexico during the 1980's intensified the need for the search for resistance genes in wheat. Resistant cultivars dramatically reduce aphid population in a field as it inhibits their growth and reproduction (Tolmay and Maré, 2000). Resistance to RWA in *T. aestivum* was first identified by Du Toit (1987). The first resistant wheat cultivar, Tugela DN which contains the Dn1 resistance gene, was released in 1992 (Van Niekerk, 2001). The resistance conferred by *Dn1* and *Dn2* resistance genes decrease the amount of tillers infested with aphids and minimizes the populations of RWA per infested tiller. Since then, several resistant cultivars, up to 27 (Jimor, 2011), have been released into the market which include Betta DN, Gariep, Limpopo and SST 333. A quick acceptance of the RWA resistant cultivars in South Africa occurred as their release brought a yield advantage over the non-resistant varieties grown by the farmers (Tolmay, 2008).

1.1.5.1.1 Backcross Breeding:

Different sources of plant resistance against RWA have been reported in bread wheat. In studies of resistance, Du Toit (1989) reported the identification the single dominant resistance genes *Dn1* and *Dn2* in wheat germplasms accessions PI 137739 and PI 262660 respectively. Examples of cultivars containing the *Dn1* gene are Tugela Dn, Betta Dn and Malopo Dn. Another, *Dn4* was discovered in PI 372129 (Nkongolo *et al*., 1991). *Dn5* resistance gene was found in PI 224994 (Zhang *et al*., 1998). Three types of inheritance were discovered in this accession: a single dominant independent gene, two dominant independent genes or one dominant and one recessive gene carrying resistance to RWA. Eleven resistant genes have been found in wheat and its relatives denoted: *Dn1* to *Dn9, Dnx* and *Dny* (Botha *et al*., 2006). Table 1.2 shows their source and mode of inheritance.

Table 1.2 Summary of the genes associated with RWA resistance, their resistance source and mode of inheritance (Adapted from Andrews Van Zyl, 2007).

Gene	Resistance Source	Mode of Inheritance	Reference
Dn1	PI127739	SD	Du Toit, 1989
Dn2	PI262660	SD	Du Toit, 1989
Dn3	*T. tauschii* line SQ24	R	Nkongolo, 1991
Dn4	PI372129	SD	Nkongolo, 1991
Dn5	PI294994	SD	Marais and Du Toit, 1993
Dn6	PI243781	SD	Dong and Quick, 1995
Dn7	94M370	SD	Marais *et al*., 1994
Dn8	PI294994	SD	Liu *et al*., 2001
Dn9	PI294994	SD	Liu *et al*., 2001
Dnx	PI220127	SD	Liu *et al*., 2001
Dny	PI220350	Unknown	Tolmay *et al*., 2012

SD: Single dominant; CD: Co-dominant; DI: dominant independent: R: Recessive

These genes where introduced into lines with more acceptable agronomic characteristics through backcross breeding. The donor parent is crossed with the recurrent parent and the progeny is further crossed with recurrent parent to create a line identical to the recurrent

parent with the addition of the gene of interest (Dogimont *et al*., 2010). At the Small Grain Institute (Bethlehem, South Africa), backcross breeding was used to transfer this resistance into 8 well adapted South African cultivars namely: Tugela, Betta, Malopo, Karee, Kariega, Letaba, Molen and Palmiet (Tolmay *et al*., 2006). During the process, plants were screened for resistance to RWA in a greenhouse bioassay. Live aphids were used in a tedious method to identify resistant plants until a reliable genetic marker became available to assist selection of plants containing the resistance genes.

1.1.5.1.2 Marker-assisted Breeding:

A genetic marker is a DNA fragment associated with a certain location within the genome and hence identifies that particular sequence in a pool of unknown DNA. Microsatellites are loci where short sequences are repeated in tandem arrays e.g. -CACACA-. Newly developed microsatellite markers are becoming widely used as a rapid and accurate DNA marker system (Plaschke *et al*., 1995). The development of such markers in wheat is however extremely expensive and time-consuming because of its large genome size (Roder *et al*., 1998). Several microsatellite maps of wheat have been constructed regardless, with microsatellite maps loci evenly distributed along the chromosome lengths to provide excellent coverage of the wheat genome (Liu *et al*., 2001).

Liu and colleagues (2001) reported an experiment where they used microsatellite markers to link 6 RWA resistance genes. DNA of wheat from near-isogenic lines and segregating F_2 populations were amplified with microsatellite primers of known chromosome location via PCR. The results showed that the locus for wheat microsatellite *GWM111* (located on wheat chromosome *7DS*- short arm) was tightly linked to *Dn1, Dn2* and *Dn5* as well as *Dnx* in PI 220127. Genetic linkage maps and markers of these genes have now been constructed for wheat chromosomes *1D* and *7D* and will prove to be useful in marker-assisted breeding for RWA-resistant wheat (Liu *et al*., 2001).

1.1.5.2 RWA Biotypes

A biotype is a population within an insect species that has been able to overcome plant resistance thereby causing damage (Smith *et al*, 1992). The emergence of new RWA biotypes

forced by selective pressures, poses an increased threat to the wheat industry as RWA resistant cultivars which offered a long-term solution to RWA control may no longer be effective. Furthermore, crops that are resistant to one biotype might be susceptible to another and vice versa. There are currently four RWA biotypes in South Africa: RWA SA1 identified in 1978, RWA SA2 identified in 2005 and RWA SA3 identified in 2009 (Jankielson, 2011) and RWA SA4 identified in 2011 (Jankielson, 2014). These biotypes are very similar in morphology but differ on the basis of which resistance gene of the wheat cultivar the biotype can overcome (see Table 1.3) and the severity of their attacks.

Table 1.3 Summary of the South African RWA biotypes, when they were first identified and the genes they are virulent against.

Biotype	**Year Identified**	**Virulent Against**	**Reference**
RWA SA1	1978	*Dn3*	Jankielson, 2011
RWA SA2	2005	*Dn1, Dn2, Dn3 and Dn9*	Jankielson, 2011
RWA SA3	2009	*Dn1, Dn2, Dn3, Dn4 and Dn9*	Jankielson, 2011
RWA SA4	2011	*Dn1, Dn2, Dn3, Dn4,Dn5 and Dn9*	Jankielson, 2014

A practical method to determine the difference between biotypes is to collect samples of RWA in the field and grow them in a greenhouse. When colonies are mature, they are then screened against a differential set of wheat cultivars containing the resistance genes from *Dn1-Dn9* (Tolmay *et al*., 2012). Jankielson (2011) screened 96 RWA clones and reported that infestations of RWA SA1 caused susceptible damage symptoms only in wheat cultivars containing the *Dn3* gene as compared to RWA SA2 which damaged wheat entries containing *Dn1*, *Dn2*, *Dn3* and *Dn9* resistant genes but not those containing *Dn4*. RWA SA3 was found to be virulent to the same resistance sources as RWA SA2 in addition to *Dn4*. In a study comparing the feeding related damages caused by RWA SA1 and RWA SA2 (Jimor, 2011), the results revealed that RWA SA2 was a more virulent and fast breeder than RWA SA1. Further work must be done therefore to successfully set up RWA resistance in wheat against these four biotypes and any that emerge in future.

1.1.6 Mechanisms Of Plant Resistance:

As mentioned earlier, plants are constantly being threatened by abiotic and biotic stress. Plants are not able to move away from any alerted danger and are without a circulatory system as in animals for defence, and have therefore evolved varied physical barriers and sophisticated chemical mechanisms to which their success depends upon. They are able to perceive an insect attack either through metabolic changes in injured tissues or binding of molecular compounds of attack to certain receptors in the plant to initiate an opposing response (Saheed *et al*., 2008).

Plants can employ two general strategies to defend themselves against herbivory. They can either reduce the amount of damage they experience known as resistance or tolerate the damage. Resistance is a heritable trait contained by a plant species and reduces insect populations in the field. To overcome this, insects have in turn evolved specialised mechanisms to evade sensitivity, down-regulate plant defence responses and gain maximum nutrition from its host. Hence, the interaction between plants and insects such as aphids are complex and dynamic. Studies to understand these interactions have gained major interest among scientists for decades and its research integrated into many fields of study such as biochemistry, ecology and physiology (Moran *et al*., 2002).

1.1.6.1 Tolerance, Antibiosis and Antixenosis Resistance

Painter (1951) categorised three host responses that occur after RWA infestation namely tolerance, antibiosis and antixenosis. Tolerance occurs when the plant survives under levels of infestation (nutrient drain) that will kill susceptible plants. Tolerance mechanisms for example; increased photosynthetic activity, utilization of stored resources and compensatory re-growth, leave the plant healthy with good yield despite the damage by herbivores. Antibiosis occurs when the resistant plant is able to affect the biology of the aphid. It causes injury, death, reduced longevity or reproduction of the aphids. Antixenosis occurs when the resistant plant affects the way an aphid perceives the host thus prevents it from settling on or colonizing it. The plant either provides stimuli that are unattractive to the aphid e.g. odour or texture or simply fail to provide stimuli that attract it thus affects the behaviour of the pest.

1.1.6.2 Plant Responses to Feeding Insects

Plant responses to aphid infestation are caused by agents known as elicitors defined as molecules able to induce a physiological and/or biochemical response associated with the expression of resistance. The signals which expose these plant responses are suggested to be derived from aphid saliva secreted during stylet penetration, probing and feeding activities (Gill and Metcalfe, 1997). During probing, aphid stylets puncture epidermal mesophyll and parenchyma cells causing a mechanical damage that influence plant responses (Tjallingii and Esch**,** 1993). Aphids secrete a proteinaceous salivary sheath that lines the stylet path as well as watery saliva that contains enzymes such as oxidases, pectinases and cellulases which have been suggested as eliciting agents (Miles, 1999). Elicitors from the wound and herbivore combined with abiotic stresses differentially activate various signalling pathways. These cascades intermingle to either directly produce volatile signals which serve as indirect defences or effect the expression of defence-related genes. This gives rise to either re-growth in tolerance or production of compounds that directly affect the herbivores or attracts natural enemies.

It is hypothesised that compounds from the deposited salivary sheath in the apoplastic fluid bind to receptor proteins on the cell membrane in resistant plants leading to an activation of the signalling cascade and onset of defence responses (Botha *et al*., 2005) which occurs within 1-2 hours after infestation. Plants have evolved mechanisms to detect this attack which includes the deposition of coagulating proteins onto and callose collars around the pores connecting sieve elements to plug and stop nutrient flow. In turn, aphids have also evolved mechanisms to suppress this plugging response. Its saliva contains calcium-binding proteins that reverses phloem occlusion triggered by calcium flux in response to wounding (Will *et al*., 2007) thus is able to prevent sieve tube plugging and aphid can remain at a single feeding site for hours.

1.1.6.3 Activation of resistant genes

Resistant genes (*R*-genes) allow certain plant genotypes to recognise specific pathogen effectors and prevent pests that can overcome non-host resistance by producing *R*-proteins. The physiological changes as a result of aphid feeding can activate or suppress defence-response genes (Thompson and Goggin, 2006). Most *R*-genes consist of a nucleotide-binding

domain (NB) and leucine-rich repeat (LRR) domains which provide resistance by direct interaction with specific Avr factors (Chisholm *et al*., 2006). NB binds either ATP/ADP or GTP/GDP whereas the LRR domain usually is involved in protein-protein interactions and ligand binding. An example is the RPS2 gene from Arabidopsis which confers resistance to Pseudomonas syringae protein 2 (Bent *et al.,* 1994). Other classes of R-genes are the Ser/Thr protein kinases for example Pto shown to exhibit protein kinase catalytic activity in vitro (Loh and Martin, 1995) and the exoplasmic LLRs (eLLR) .

R-gene mediated resistance occurs through different methods. The *R*-proteins can directly interact with the pathogen's avirulence (Avr) factor or it can guard another protein which detects degradation by an Avr gene or it may detect a Pathogen-Associated Molecular Pattern (PAMP) otherwise the *R*-protein encodes an enzyme that degrades toxins from the pathogen. The identification of Avr factors sets off the signalling cascades that rapidly activate a strong plant defence. In a previous study, the *R*-gene *Vat* was activated in Melon by the cotton-melon aphid, *A. Gossypii* (Dogimont *et al*., 2003). It confers resistance by reducing the aphids feeding and virus transmission. Because *R*-genes confer resistance against specific pathogens, they can be isolated and transferred from one plant to another making it resistant to a particular pathogen. The discovery of the structure of R-genes as well as their loci ought to give rise to new strategies for disease control (Hammond-Kosack and Jones, 1997) as well as insect control.

1.1.6.4 The Hypersensitive Response

The hypersensitive response (HR) which is seen as necrotic lesions on leaves of resistant plants (Fouche´ *et al*., 1984), is activated in response to, amongst others, pathogen infestation, whilst susceptible plants lack recognition and the response onset. The HR results in rapid, localised cell death at the site of infection with a release of anti-microbial compounds and induction of intense metabolic alterations in the cells surrounding the necrotic lesions. It mainly occurs in pathogen-induced defence responses while herbivores normally induce wounding responses. Although the HR is an effective defence, the suicide of cells has large disadvantages and requires complicated recognition systems to avoid un-programmed apoptosis (Kessler and Baldwin, 2002).

Most gene-for-gene interactions are characterised by the activation of a hypersensitive response (HR) (Jones and Dangl, 1996) as part of the overall defense response. The HR is further characterised by cell wall modifications through callose deposition, cellular ion influxes leading to invasions of calcium into the cytosol, oxidative burst of reactive oxygen species (ROS), generation of salycylic acid (SA), jasmonic acid (JA), ethylene (ET), nitric oxide, oxylipins, benzoic acids and induction of enzymes that participate in the overlapping signalling pathways and synthesis of signal molecules (Harris *et al*., 2003). It must be noted that in the absence of R-gene mediated resistance, many of these biochemical responses can also be induced during interactions where disease develops (Bent, 1996).

1.1.6.5 Callose Deposition

Callose is a linear β-1, 3-glucan plant polysaccharide which functions in dividing cell walls and is also found in pollen mother cell wall and pollen tubes. Deposition occurs between the plasma membrane and cell wall mostly within minutes of wounding (Nakashima *et al*., 2003) as a response to pathogen attack, aphid infestation, abiotic stress and metal toxicity. It serves as a matrix in which antimicrobial compounds can be deposited thus providing focused delivery of chemical defences at cellular sites of attacks (Luna *et al*., 2010). It has been shown that the formation of callose leads to a reduced rate of transport in phloem tissues of wheat leaves (Botha and Matsiliza, 2004). Studies have shown that some aphid species, including RWA can cause callose formation (De Wet and Botha, 2007). However, it has been suggested that aphids can prevent wounding reactions such as callose formation and protein plugging due to their salivary sheath which seals the stylet puncture site and its watery saliva which is believed to interact with sap constituents (Will *et al*., 2007). Callose is easily detected under UV light after staining with aniline blue fluorochrome forming an intense yellow fluorescence (Stone and Clarke, 1992).

Callose deposition is usually activated by conserved pathogen-associated molecular patterns (PAMPs) such as the potent callose-inducing PAMPs from fungal cell walls: chitin and chitosan (Gomez-Gomez and Boller, 2000). Endogenous elicitors from pathogen- or herbivore- damaged plant tissue also trigger callose deposition. Examples of damage-associated patterns (DAMPs) are oligogalacturonides (Luna *et al*., 2010). Callose is synthesised by complexes of β-1, 3-glucan (callose) synthase (Flors *et al*., 2005) which are

activated when there is plasma membrane disruption. Each complex contains one of the twelve β-1, 3-glucan synthase (*GSL*) - related genes from *Arabidopsis* whose sequences are similar to that of *FKSI*, a *GSL* (genes involved in callose formation) identified in yeast (Richmond and Somerville, 2000; Verma and Hong, 2001). The role for the *GSL* gene products have been confirmed by transformation with double-stranded RNA interference (dsRNAi) constructs which silence *GLS5* which inhibited formation of wound callose and papillary callose (Jacobs *et al*., 2003). Aphid species cause callose formation in different places as was supported by Saheed *et al.* (2007), using RWA and BCA aphid species. RWA caused formation of callose in sieve pores, plasmodesmata and plasmodesmata pores between companion cells and sieve tubes, whereas only sieve pores appeared to contain callose in the case of BCA.

These callose plugs obstruct mass flow and prevents nutrition flow to the aphids hence they must in-turn suppress the mechanisms triggering the plugging. The functions of sieve plate plugging are therefore to maintain the pressure conditions in the plant or a defence against phytopathogens. Callose is digested by β-1, 3-glucanases which has been shown to accumulate in higher levels in resistant cultivars when compared with susceptible cultivars during aphid infestation (Van der Westhuizen *et al*., 2002). It has been reported that wound callose disappears over the course of days (Saheed *et al*., 2008) due to degradation by β-1, 3-glucanases (Saheed *et al*., 2008). Not much work has been done on these degrading enzymes. Considering all the importance of callose, it can easily be concluded that knowledge of its functioning is crucial for understanding of varied processes that occur in plants especially resistance mechanisms.

1.1.6.6 Reactive Oxygen Species (ROS)

ROS are chemically reactive molecules that contain oxygen. They are formed as a natural by-product of normal metabolism of oxygen functioning as signalling molecules (Apel and Hurt, 2004) and involved in homeostasis (Sen, 2003). However under abiotic and biotic stress, ROS levels can increase dramatically, known as oxidative stress, resulting in significant cell structural damages (Sen, 2003). The major forms of ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) and they are highly reactive and toxic at high levels leading to the oxidative destruction of cells. The oxidative burst is when there is a

rapid accumulation of ROS following pathogen recognition (Mehdy, 1994). It is directly toxic to pathogens (Lamb and Dixon, 1997) and can trigger a HR resulting in host cell death preventing the spreading of pathogens and additionally involved in the activation of distinct signalling pathways and other defense mechanisms.

ROS production has been attributed to several mechanisms in plants. However, much attention has been given to the NADPH-dependant oxidase system in which the plasma membrane localised NADPH oxidases (NOX) catalyses the production of O_2^- by reduction of oxygen with NADPH as the electron donor (Maffei *et al.*, 2007). Because of this role, the level of NADPH-dependant oxidase is usually used as an indicator of oxidative defense. In a study, the concentration of NADPH-dependant oxidases and H_2O_2 increased after infestation with RWA (*D. Noxia*) suggesting that these oxidades are involved in ROS generation as a plant response to insect infestation (Moloi and Van der Westhuizen, 2006). A study where two Arabidopsis NADPH-dependant oxidase subunit genes, *AtrbohD* and *AtrbohF,* were mutated, resulted in largely reduced ROS production during disease resistance reactions to avirulent pathogens (Torres *et al.*, 2002). Other groups of oxidases which may be involved in ROS production such as germin-like oxalate oxidases (Hu *et al.*, 2003) and amine oxidases (Walters, 2003).

Elevated levels of ROS induce the biosynthesis of antioxidant molecules involved in ROS scavenging increase to limit its damage to host cells. They are thought to detoxify the cytotoxic effects of ROS. These include ascorbates, catalase, superoxide dismutase, polyamines and glutathione reductase (Liu *et al.*, 2010) many of which have been used to engineer plants that are tolerant of abiotic stresses (Umezawa *et al.*, 2006). The biological roles of ROS in plant defense against insects are yet to be understood.

1.1.6.7 System Acquired Resistance (SAR)

Apart from gene-to-gene resistance, plants have also evolved mechanisms of systemic immunity where local defences confer resistance against subsequent pathogen attacks for an extended period of time. This is known as systemic acquired resistance (SAR). Phloem feeding insects such RWA have been shown to modulate three signalling compounds that have major roles in plant defense regulation: jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Thompson and Goggin, 2006). Many studies using *Arabidopsis* have been crucial in trying to understand the pathways and how they interplay. SAR offers resistance

against a broad spectrum of pathogens and requires the phenolic signalling molecule SA (Durrant and Dong, 2004).

1.1.6.8 Defense Signalling Pathways

It has been revealed that SA is a signalling molecule that plays a central role in both local defense reactions and in SAR (Durner, 1997; Durrant and Dong, 2004). In higher plants, this molecule is produced from the shikimate-phenylpropanoid pathway (Métraux, 2002). SA levels increase dramatically after a pathogen infection (Malamy *et al*., 1990; Ryals *et al*., 1996). Studies on the genetic level have showed that SA regulates many pathogenesis-related (PR) genes (Glazebrook, 2001) including those encoding *PR1, PR2* and *PR5* and is required for the rapid activation of defense responses and the establishment of SAR. In wheat, SA was produced in incompatible in contrast to compatible interactions with RWA (Mohase and van der Westhuizen, 2002).

JA is a 12-carbon fatty acid-derivative synthesised from linoleic acid (18-carbon substrate) (Phillipe and Edwards, 1998). Synthesis of JA elicits the expression of wound inducible genes such as the protease inhibitor (PI-II) gene (Mùller and Chua, 1999). JA is involved in many aspects of plant biology including defense, seed and pollen development. It was shown to be important for defence of tomato against tobacco hornworm larvae as well as that for *Arabidopsis* against the fungal pathogen *A. brassicicola* (Howe *et al*., 1996; Thomma *et al*., 1998). The SA and JA signalling pathways are mutually antagonistic.

ET has different roles in regulation of plant processes. Its role in plant defense has been evaluated with mutants in the ethylene signal transduction pathway. It has been shown to contribute to resistance but in some cases (Thomma *et al*., 1999), promote disease production (Lund *et al*., 1998). JA and ET work together to regulate expression of many genes. Some JA-inducible genes are not inducible in plants that cannot produce or sense ET. For instance, expression of the JA-dependent defense genes *PDF1.2* and *THI2.1* require *EIN2* (Norman-Setterblad *et al*., 2000).

1.1.7 Climate Change

According to Intergovernmental Panel on Climate Change (IPCC, 2001), climate change is defined as “change in climate over time, either due to natural variability or as a human

activity". It brings about higher frequencies of abiotic disturbances. Evidence to this is given by the increase in global average temperature, changes in rainfall patterns, increase in atmospheric CO_2 concentration and extreme climatic events. These changes affect all trophic levels and potentially alter the interactions in food webs (Stenseth *et al*., 2002). They can result in reduced plant growth, survival or productiveness, being the major threats in agriculture that lead to poor yield. Environmental stresses such as drought, extreme temperatures and high salt concentrations in plants trigger molecular and biochemical responses. Signalling pathways are expressed after the detection of an external change to convert a physical stress into a biochemical response.

Change in the environment severely affects the pest population dynamics either directly or indirectly by altering the host physiology. Population dynamics is the part of population ecology that deals with factors affecting changes in population densities (Karuppaiah and Sujayanad, 2012). Because of their short generation time and low developmental threshold temperatures, aphids have been shown to be sensitive to climatic conditions (Harrington *et al*., 1995). The effects of climate change on communities can be short-term or long-term (van Baaren *et al*., 2010). The short-term effects include the direct effects of temperature on life history traits such as development time, metabolic rate and sex allocation while long-term effects involve genetic changes in populations associated with climatic adaptations.

1.1.7.1 Temperature

Climatic models predict an increase in the global mean temperature from 1.4-5.8^{o}C in the next century (Houghton *et al*., 2001). This change in the temperature will affect the biology of each of the component species of a system in various ways and specifically alters the relationship between plants and insects. Positive direct responses of insects to increasing temperature conditions such as enhanced reproductive potential may occur as long as the optima for development of the species is not exceeded. Temperatures above the specific optimum range lead to negative direct responses. It directly affects developmental rates, survival range, voltinism, population genetic composition, size and abundance of insects and indirectly via the effects on plant physiology and chemistry. The development time of the aphid *Aphis gossypii* ranged from 4.8 days at 20°C to 3.2 days at 30°C (Vansteenis and Elkhawass, 1995). High temperature is reported to play a detrimental role in the population

development of the rose grain aphid *Metopolophium dirhodum* for example constant temperature of 30°C reduced the survival of aphids to zero (Zhou and Carter, 1992).
Heat stress is associated with a greater risk of incorrect protein folding and denaturation of several intracellular proteins and membrane complexes. Several proteins are expressed in response to heat stress. Heat leads to enhanced accumulation of several proteins with chaperone functions, chiefly the heat-shock proteins (*HSPs*) e.g. *HSP100*s, *HSP90*s, *HSP70*s, and *HSPS* (*HSPs* <40kDa) (Trend, 1996). Up-regulation of *HSP*s plays a central role in abiotic stress responses in plants. Transgenic plants over expressing *HSP* genes exhibited improved tolerance to high temperature (Sanmiya *et al*., 2004).

1.1.7.2 Drought

Drought is a serious problem for agriculture worldwide. The need to improve drought tolerance in plant cultivars has become an important aim in scientific research. Plants may avoid drought stress by trying to maintain tissue water potentials, or alternatively, tolerate the water stress by decreasing their vulnerability. The mechanism of tolerance adapted by a plant can have effects to the herbivores that feed on it for example, changes in cell or phloem composition to compensate against water stress could affect phloem feeding insects like aphids. Under water stress, turgor pressure and water content of plant tissue decreases leading to tougher foliage. Many studies demonstrate that severe or prolonged drought not only has direct adverse effects on tree growth and survival, but on the other hand may also trigger more frequent or severe outbreaks of forest insects (Jactel *et al*,. 2012).

The response to drought of phloem feeders such as aphids remains vague. According to Vickers (2011), drought stress in plants can likely have a good or bad consequence on aphid feeding. "Under drought conditions, host plant sieve elements become more concentrated; hence increases in available amino acid concentrations and proportion of small nitrogenous molecules will potentially benefit aphids since they are frequently limited by available nitrogen in their diet. However, the increased need for aphid osmoregulation under drought, to deal with the osmotically challenging diet, may be detrimental to aphid performance" (Vickers, 2011).

Favourable changes have not always been found in experimental studies comparing aphid numbers and plant growth characteristics under drought stress and well watered conditions. Aslam et al., (2012) investigated how simulated summer drought affected the bird cherry–oat aphid, *Rhopalosiphum padi L.*, which feeds on barley, and its natural enemy the parasitoid wasp *Aphidius ervi*. Both drought and aphids reduced barley (*Hordeum vulgare*) dry mass by 33% and 39%, respectively. Drought reduced leaf and root nitrogen concentrations by 13% and 28%, respectively. Aphid numbers were unaffected by drought although population demography changed significantly.

1.1.7.3 Increased atmospheric CO_2 Concentration

Over the past 200 years, atmospheric CO_2 concentration has increased. IPCC (2007) reported that increases in CO_2, methane and nitrous oxides are a consequence of fossil fuel combustion and are responsible for most of the increase in global temperature levels that were observed since the middle of 20th century. It is predicted that the atmospheric CO_2 will most likely rise from the current 385 ppm to 550 ppm by 2050 (IPCC, 2007; Meehl *et al*., 2007) and is anticipated to have doubled over the next century (Watson *et al*., 1996). CO_2 is a substrate and a fundamental C-source for photosynthesis in plants, where other biomolecules originate (Drake *et al*., 1997). Due to CO_2 enrichment, many plants will increase in dry weight/ biomass (Idso *et al*., 2014). It has a direct "fertilizing" effect on important C_3 crops, such as wheat and rice, by stimulating photosynthesis and inhibiting photorespiration (Ainsworth and Rogers, 2007).

Many C_3 plants grown under CO_2–enriched atmospheres have instant higher photosynthetic rates, grow faster, increased yield and C:N ratios thus increasing the biomass of plant material available to herbivorous insects (Kimball *et al*., 2002; Idso and Idso, 1994; Drake *et al*., 1996). Increased CO_2 levels can also alter the water content of a plant resulting in tough leaves and concentrated defensive chemicals (Lindroth *et al.,* 1995). Rising CO_2 levels may affect herbivorous insects as they generally grow more slowly, take longer to mature, suffer great mortality and have higher consumption rates due to a decline in the nutritional quality of the host plant.

Phloem feeders may be less responsive to changes in the quality of the plant as they can avoid most of the plant-derived secondary metabolites that cause a reduction in the performance of herbivores. In two different studies, the populations of the aphids *Myzus persicae* and *Aphis rumicis* increased due to elevated CO_2 concentration (Bezemerb *et al*., 1999 and Whittaker 1999 respectively). Writing on the findings of their work, Awmack and colleagues (1996) say that "*Sitobion avenae* fed on winter wheat grown under elevated CO_2 concentration showed a small but significant increase in their productive rate". Some authors have not found any effect of CO_2 on the performance of phloem feeding insects (Butler, 1985).

In another study, Pleijel *et al*., (2000) report that they grew spring wheat in open-top chambers maintained at atmospheric CO_2 concentration of 340 ppm and 680 ppm for three consecutive years. In addition, they exposed some plants in each CO_2 treatment to ambient, 1.5 x ambient and 2 x ambient O_3 concentration. According to their findings, "the elevated O_3 concentration negatively influenced wheat yield at both atmospheric CO2 concentration. However, grain yield was always higher for the plants grown in the CO_2-enriched air, averaging 13% greater over the period of study". Thus, they concluded that "the positive effect of elevated CO_2 concentration could compensate for the yield losses due to O_3".

In another experiment, the effect of changing CO_2 concentration on resistant and susceptible barley cultivars to feeding by the two RWA biotypes (SA1 and SA2) was investigated (Jimor *et al*., 2013). Uninfested plants grew vigorously under elevated CO_2 concentration whilst infested plants significantly experienced biomass loss. The elevated CO_2 enrichment was of benefit to the aphids resulting in faster breeding rates with feeding depleting leaf nitrogen content. Sun and colleagues (Sun *et al*., 2013) determined using qPCR that elevated CO_2 up-regulated the expression of SA-dependant defense genes but down-regulated the expression of JA/ET- dependant defense genes in wild-type *Arabidopsis* infested by the green peach aphid *Myzus persicae*. Elevated CO_2 enhanced the ineffective defense-SA signalling pathway and reduced the effective defense-JA signalling pathway against the aphids resulting in their numbers increasing.

1.2 Problem Statement

Aphid feeding on cereal plants leads to extensive damage and serious loss of yield. South African wheat farmers spend millions on pesticides and herbicides to produce two million tons of cereals per year. The discovery of new RWA biotypes poses an increased threat to the *Triticum aestivum* (wheat) industry as RWA resistance which offered a long-term solution to RWA control may no longer be effective. The new and fast-evolving biotypes largely shorten effective periods of the existing resistance of cultivars in the agro-economic system. Climate change affects all trophic levels and potentially alters the interactions in food webs for example affect the resistance of wheat to aphid infestation. This situation further emphasises the importance of understanding the RWA biology and wheat defence responses leading to resistance. There is a need to extensively understand aphid and host plant adaptations during predicted climate changes. Poor understanding of this interaction hampers progress in the development of solutions and management strategies that will lessen the problem.

1.3 Hypothesis

Climate change consequences, specifically elevated CO_2 concentrations, will affect the resistance mechanism of the wheat cultivar Tugela Dn during RWA SA1 infestation

1.4 Aim

To identify the effects of elevated CO_2 concentrations on the resistance of Tugela Dn to RWA SA1 infestation.

1.5 Objectives

- To evaluate population growth rates of RWA SA1 under ambient and elevated CO_2 concentrations.

- To assess the relative virulence of RWA SA1 on resistant and susceptible wheat under ambient and elevated CO_2 concentrations.

- To examine callose deposition as a response to RWA SA1 feeding on resistant and susceptible wheat under ambient and elevated CO_2 concentrations.

- To identify any possible changes in the resistance of Tugela Dn to RWA SA1 infestation under elevated CO_2 concentrations.

- To identify any differentially regulated (up- or down-regulated) proteins in Tugela Dn as a result of RWA SA1 infestation under elevated CO_2 concentrations.

2 CHAPTER TWO

THE EFFECT OF ELEVATED CO2 concentration LEVELS ON POPULATION GROWTH RATE AND VIRULENCE OF RWASA1 ON RESISTANT AND NON-RESISTANT WHEAT CULTIVARS

2.1 Introduction

The Russian Wheat Aphid (RWA) *Diuraphis noxia* (Mordvilko) has been an insect pest to small grain farmers in South Africa since 1978, when it was first detected in the eastern Orange Free State (Du Toit, 1987). It has since spread and resulted in major yield losses especially in wheat production. RWA is a phloem-feeding insect that draws plant sap through its stylets and causes major plant damage during feeding. The aphids disrupt the phloem tissue, triggering the deposition of a β-1, 3-glucan carbohydrate compound known as callose (Saheed, 2009; Botha and Matsiliza, 2004). In response to wounding, callose is deposited in sieve pores and plasmodesmata, sealing the pores to reduce assimilate loss from the phloem.

The symptoms of RWA feeding include longitudinal whitish, yellowish or purplish streaking known as chlorosis, leaf rolling and stunted growth. It injects a phytotoxin, whilst feeding that result in the breakdown of chloroplast and cellular membrane in susceptible plants, which results in chlorophyll deficiency that can be fatal to the plants (Botha *et al*., 2006). RWA also causes leaf-rolling, which reduces the surface area where photosynthesis takes place. Feeding on the flag leaf results in entrapment of the developing head thus interferes with self-pollination and grain-filling (Louw, 2007). RWA has also been reported to transmit viruses, such as the Barley yellow Dwarf Virus (Damsteegt *et al*., 1992).

RWA feeds in dense colonies. The increase in population size and growth rate of these insects is attributable to its biology. Females give birth to live young ones which develop inside their bodies and are thus viviparous. These nymphs are able to produce embryos. Each female can produce up to 4 nymphs per day that mature approximately after 2 weeks (Dixon, 1998). This asexual reproduction allows for the explosive population growth achieved by the aphid. The development of wheat cultivars that are resistant to RWA feeding has been the

most practical, cost effective and sustainable method of reducing yield loss due to RWA (Budak, 1999). Resistant wheat cultivars are obtained by means of selective breeding of plants with the desired resistance and with plants which have the desired commercial traits such as high grain yield. Aphids can develop counter-resistance to resistant crops, thereby giving rise to various biotypes such as RWA SA1, SA2, SA3 and SA4, which are currently found in South Africa. The emergence of new RWA biotypes further magnifies the threat to wheat production. Poor understanding of the wheat resistance mechanism responses to RWA infestation hampers the development of effective resistant cultivars.

Adaptability of aphids to stress is species and ecotype specific (Vickers, 2011). Many studies have been made to try and understand the effects of environmental stresses such as drought, heat and cold on aphids (Kuo, 2006; Vickers, 2011). Global atmospheric CO_2 level has increased since the pre-industrial times from 280 ppm to 370 ppm today (Rosenzweig and Hillel, 1998) and is predicted to double by the end of the 21st century (IPCC, 2007). In future, elevated CO_2 environments are likely to have an effect on chewing insects. Many experimental studies have been published on the effects of elevated CO_2 concentration on plant-insect interaction (Coviella and Trumble, 1998; Hughes and Fakhri, 2001; Newman, 2003; Zhang *et al*., 2003, Ryan, 2012; Sun *et al.*, 2013).

Evidence regarding the responses of cereal aphids to elevated level of CO_2 has been contradictory, with some suggesting increased populations, while others suggest decreased levels. Although many do suggest an alteration of the nutritional quality of plants for insect herbivores, little is known about the responses of the aphids to the altered plants. While there have been a number of studies to measure the impact of elevated CO_2 on aphid performance, there is no published work to assess its effects on the feeding of RWA SA1 on resistant or susceptible wheat cultivars, such as Tugela Dn and Scheepers.

The following questions are addressed in this chapter:

(1) What are the effects of elevated CO_2 on population growth rates of RWA SA1 feeding on the wheat cultivars Tugela Dn and Scheepers?

(2) What are the effects of elevated CO_2 on the relative virulence of RWA SA1 on these cultivars?

(3) How will callose deposition in the plant change, as a response to RWA SA1 feeding under elevated CO_2 concentration.

2.2 Materials and Methods

2.2.1 Host Plant Material and Aphid Colony Maintenance

Susceptible (Scheepers) and resistant (Tugela Dn) wheat seeds (*Triticum aestivum*) to the RWA [*Diuraphis noxia* (Mordvilko)] cv. SA1, were sterilized in 1% bleach for 30mins then washed in distilled water. They were then placed on moist sterile filter paper in a Petri dish, covered by a lid and allowed to germinate in the conviron at 25ºC day/23ºC night and 14hr/10hr day/night cycle for one week with watering. Seedlings were then sown in plastic pots containing sterile soil mixture (river soil:vermiculite:compost in the ratio 2:1:1). Plants were watered twice a week with Long-Ashton nutrient solution (Hewitt, 1996) and every other day with tap water. They were grown to 3 leaf stage under a controlled environment in growth cabinets (Conviron S10H, Controlled Environments Limited, Winnipeg, Manitoba, Canada) set at a 14hr/10hr day/night cycle, 25ºC day/23ºC night temperature and 60% relative humidity (RH).

Colonies of RWA SA1 (*Diuraphis noxia* Mordvilko), obtained from the ARC (Small Grain Institute, Bethlehem, South Africa), were maintained separately on young feeder susceptible wheat plants (Scheepers) and kept in cylindrical insect cages in a separate growth cabinet under the same condition. New feeder plants were infested at 2 weekly intervals to maintain the colony, by removing heavily infested leaves and placing them on the plant.

2.2.2 Aphid Infestation and Experimental Set-up

Susceptible and resistant wheat plants were infested with adult apterae RWA SA1 at the 3-leaf stage above coleoptiles. Ten aphids were introduced to each experimental plant using a fine paint brush to carefully pick up each aphid individually from the growing colonies and placing them onto the new host's leaves. A cylindrical cage was used to cover each plant to prevent aphid escape as shown in Figure 2.1. Both experimental and control plants (aphid infested and uninfested respectively) were covered with insect cages and allowed to grow in the conviron under controlled environments, as described above, and various CO_2 levels, ambient CO_2 concentration (385 ppm) and elevated CO_2 concentration (450 ppm).

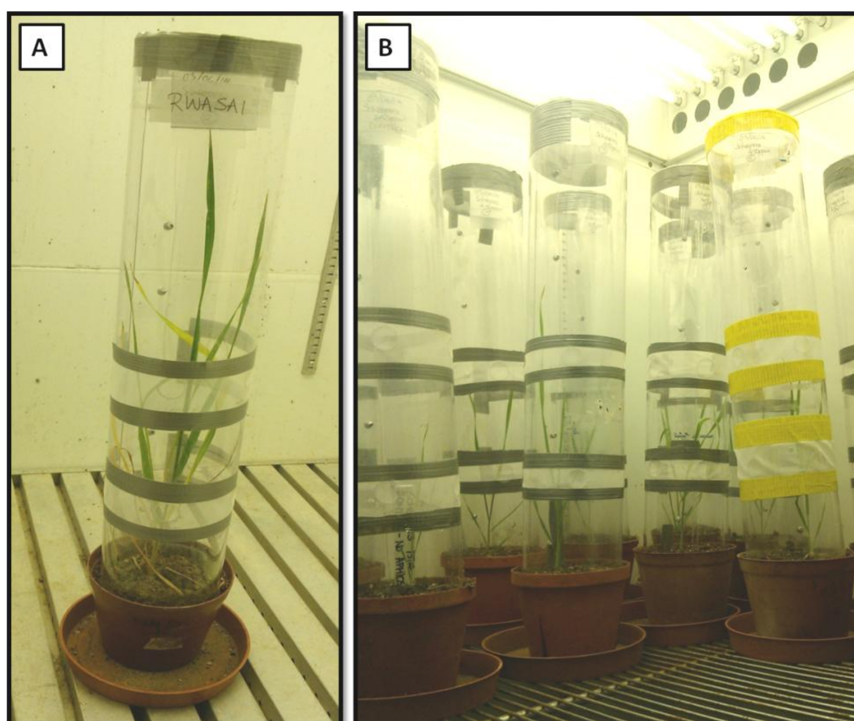


Fig. 2.1 Depiction of plants in the conviron covered with insect cages. A: - a single plant covered with a cylindrical cage and B: - both control and experimental plants covered with insect cages.

2.2.3 Population Growth and Virulence Studies

Ten adult RWA SA1, from the feeder plant, were transferred onto each uninfested experimental plant as explained in Section 2.2.2. In this experiment, 10 replicates with an additional two plants as controls (to ensure that there was no transfer of aphids between plants through the cages) were set-up per treatment. Two wheat cultivars were used, Tugela DN (resistant) and Scheepers (susceptible), under two CO_2 concentration, ambient (385ppm) and elevated (450ppm). Aphids were allowed to settle on the plant for 24hrs. They were allowed to feed and reproduce on the leaves of the experimental plants for 21 days. During this time, population increases from the original (10 aphids) were determined per plant. This was done by physically counting the total number of aphids on the abaxial and adaxial surfaces of each leaf (per plant), carefully with the aid of a hand lens on days 2, 4, 6, 8, 10, 12 and 14 days after infestation (DAI).

Visible manifestation of aphid feeding related damage was simultaneously assessed by examining chlorosis and leaf roll on each experimental plant under each treatment. The scoring system in Table 2.1 was used to give a rating for chlorosis and leaf roll per plant at 2,

7, 14 and 21 DAI. Population data obtained were statistically analysed using STATISTICA and ANOVA (Analysis of Variance) was used to determine significant differences between the means of each group (Jimor *et al*., 2011). Tukey's Post-Hoc test was performed on groups where the means were significantly different, to confirm significant difference between the groups.

Table 2.1 Virulence rating (chlorosis and leaf rolling) scales for assessing the effect of aphids on test plant.

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

NB- Chlorosis[1] scale adapted from Webster *et al*., (1987). Leaf roll[2] scale adapted from Burd *et al*., (1993).

2.2.4 Fluorescence Microscopy

Tugela Dn and Scheepers plants, at 3-leaf stage, where infested with 10 RWA SA1 as described in Section 2.2.2. and together with the uninfested controls, allowed to grow under two different levels of CO_2 , ambient (385 ppm) and elevated (450 ppm).. Leaves from infested plants and their respective controls were harvested at 7, 14 and 21 DAI and

examined for callose distribution using the method described by Jimor *et al*., (2011), as described in section 2.2.4.1. All experiments were performed in triplicate.

2.2.4.1 Preparation of Aniline Blue Fluorochrome

Fresh aniline blue fluorochrome (ABF, 4’4-[carbonyl bis(benzene,1-diyl) bis (imino)] bis benzensulphonic acid (Biosupplies Australia Pty Ltd) stock solutions were made up in 2ml microcentrifuge tubes by diluting 0.1mg aniline blue in 1.0ml distilled water. The vials were wrapped in tin foil. The stock dilution was immediately diluted (1:3 v/v) using distilled water to produce the working strength. The stock solution was kept at 4ºC until required.

2.2.4.2 Preparation of Leaf Material for Wound Callose Distribution Study

An infested leaf from each treatment was severed at the base and immediately placed in Ca^{+}- free MES buffer (2-[morpholino] ethanesulfonic acid, pH 7.2) at each time frame. The abaxial surface of the leaf was gently scraped on a glass plate under Ca^{+}- free MES buffer using a sharp single edged titanium razor blade. Caution was taken to remove the cuticle and epidermis to expose the mesophyll and shorten the pathway to the phloem. Immediately after scraping, the leaf was mounted on a glass slide, stained with drops of diluted aniline blue and covered with a cover slip. The slide was incubated in the dark for 30 minutes, after which the tissue was washed in fresh Ca^{+}- free MES buffer.

Under white light, aniline blue stained callose appears blue but fluoresces blue-green under UV light. The leaf specimens were viewed under UV light, using an Olympus BX61 wide-field fluorescence microscope (Olympus Tokyo Japan, Wirsam Scientific, Johannesburg, South Africa), fitted with an aniline blue specific filter cube (excitation range: 424-444nm, emission: 475nm). Images were saved in a database using the program analySIS (Soft Imaging System GmHb, Germany). Images representing the feeding damage under each treatment were selected for presentation.

2.3 Results

2.3.1 Aphid population growth rates on host plants:

RWA SA1 population growth increased on Scheepers and Tugela Dn from days 0 – 14 days after infestation (Fig 2.2 A and B). The population growth rate for RWA SA1 on all cultivars was generally exponential under ambient and elevated CO_2 concentration. Under ambient CO_2 concentration conditions, RWA SA1 population growth on both cultivars lagged initially from DAI=0 to DAI=2. This was probably due to the aphids trying to adapt and settle on the new host.

The greatest population numbers, during the 14 days of infestation, were obtained under 450ppm CO_2 for both cultivars as compared to 385 ppm. A rapid and sharp increase was observed in aphid numbers from 6 to 14 days after infestation, with RWA SA1 reproducing faster at elevated (450ppm) as compared to the ambient (385 ppm) CO_2 concentration for both wheat cultivars. More than double the number of RWA SA1 were recorded on Tugela Dn at 450 ppm as compared to Tugela Dn-385 ppm, 168 vs. 70 aphids, respectively. Aphid population also doubled 3 times within the 14 day period at 450 ppm as compared to 2 times at 385ppm for both Tugela Dn and Scheepers cultivars. However, the susceptible cultivar Scheepers was more heavily infested by RWA SA1 during the 14 day period as compared to the resistant cultivar, Tugela Dn, with almost double the aphid population on Scheepers as compared to Tugela Dn (218 vs. 168 respectively).

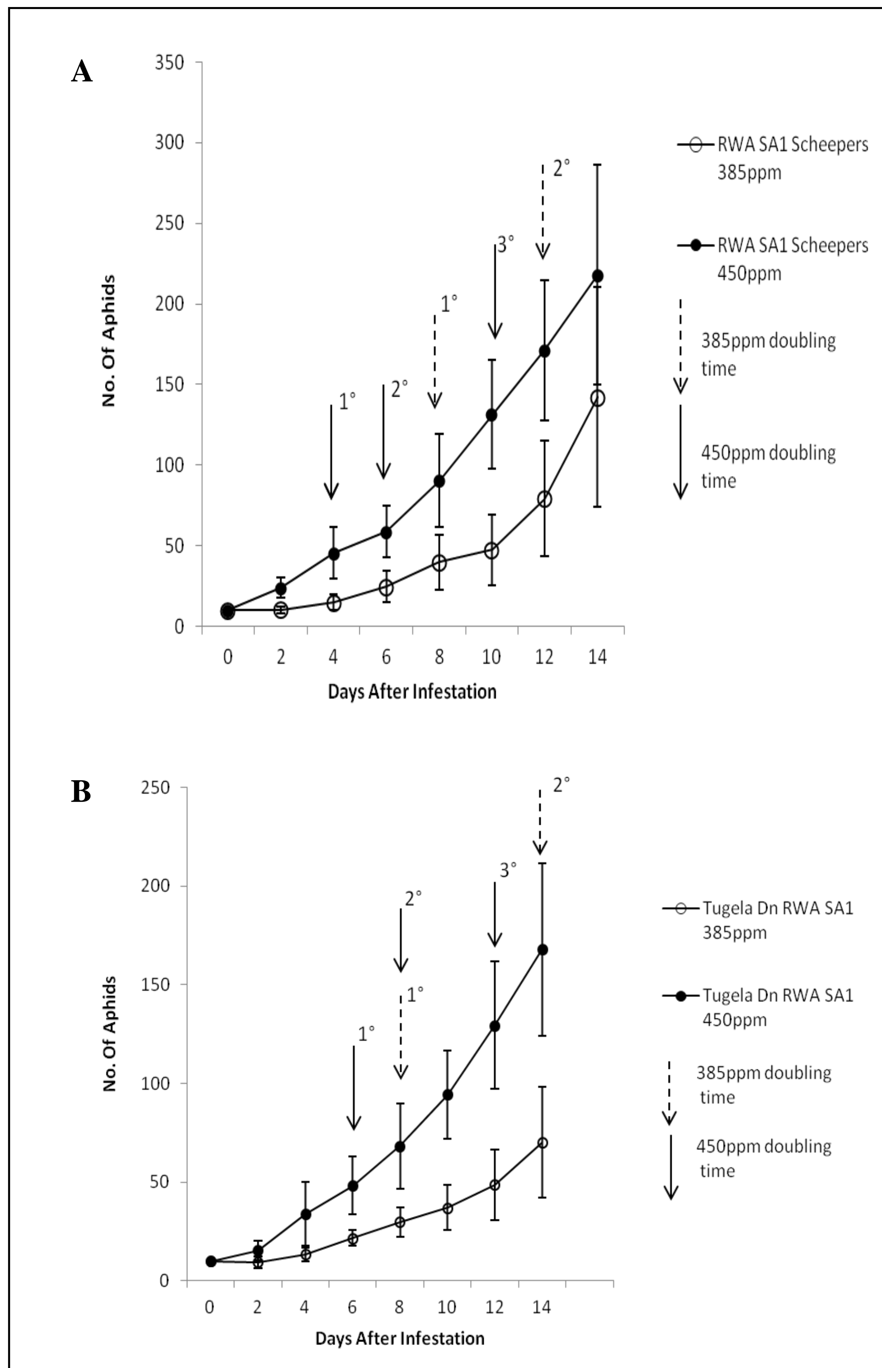


Fig.2.2 Population growth of RWA SA1 under ambient (385 ppm) and elevated (450 ppm) CO_2 concentrations feeding on A- Scheepers and B- Tugela Dn over 14 days. Arrows on graphs show the average time (days) it took for the aphid population to fold (denoted 1^0- primary doubling time; 2^0- secondary doubling time and 3^0- triple doubling time)**.** The error bars represent the variability of the 10 records of population made per day. Number of aphids were the averages of the 10 recordings per day.

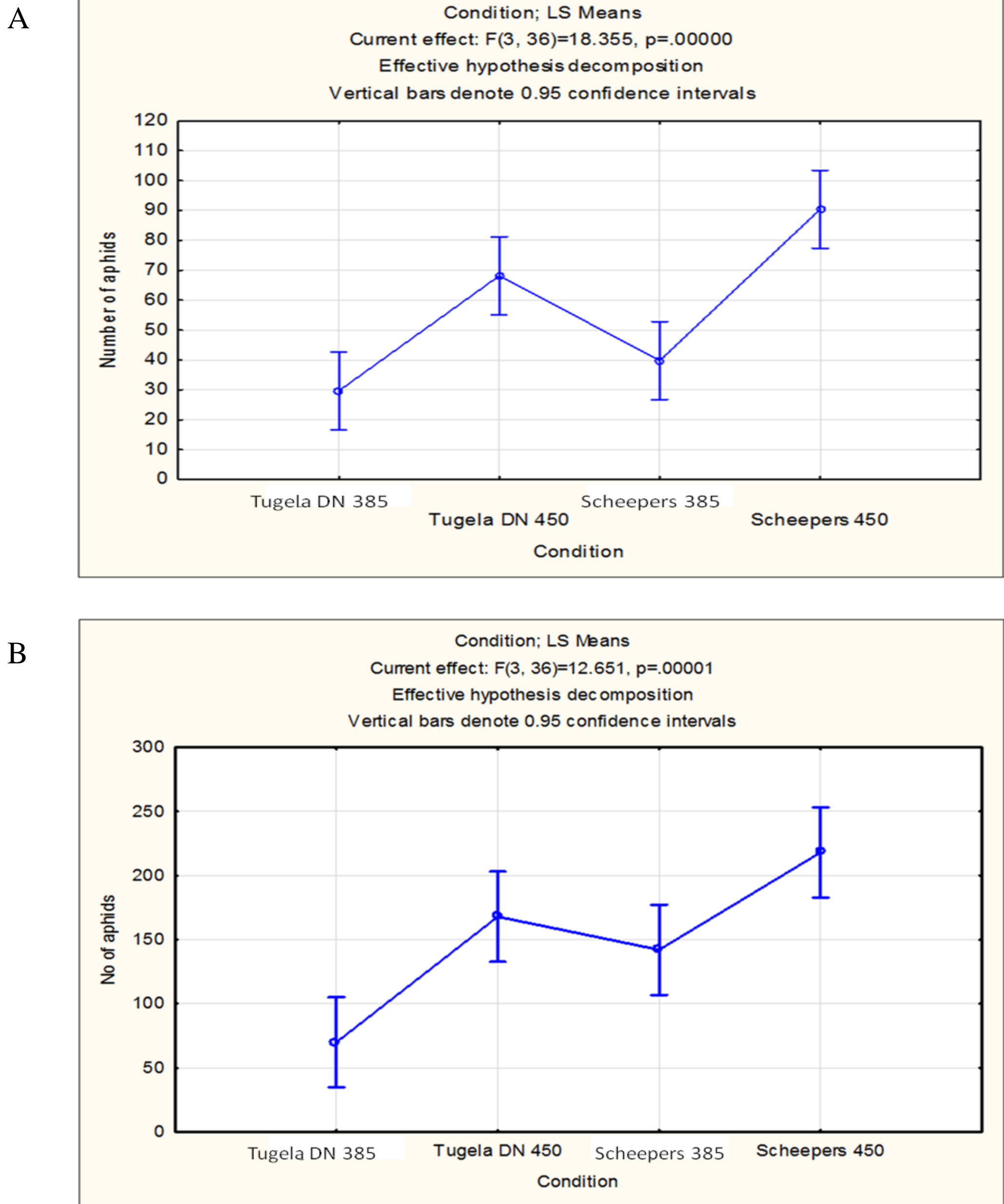


Fig. 2.3 Graphical results of ANOVA analysis for populations of RWA SA1 recorded under different conditions A: DAI=8 and B: DAI=14

The results of the ANOVA for populations recorded on DAI=8 and DAI=14 showed that the population of RWA SA1 after 8 days of infestation at elevated CO_2 concentration were significantly ($P<0.05$) higher than the numbers at ambient CO_2 concentration for both the susceptible and resistant wheat cultivars. However, the population numbers of the aphids on the resistant (Tugela Dn) were not significantly ($P>0.05$) different from those on the

susceptible (Scheepers) cultivars at both CO_2 concentration (Figure 2.3 A and B respectively).

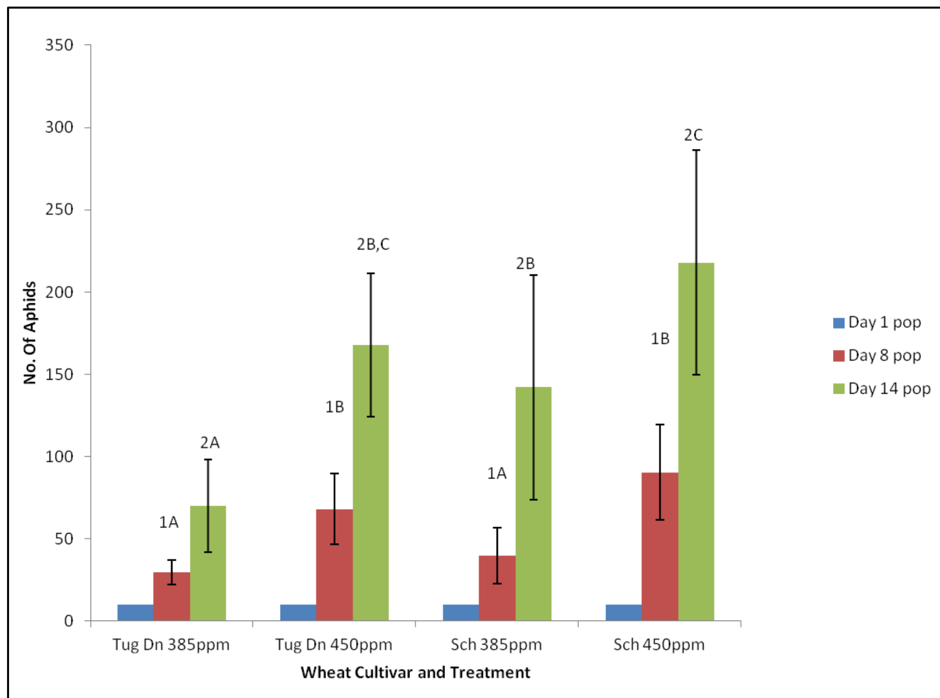


Fig. 2.4 Relative growth rate of the aphid populations over time (DAI=1, 8 and 14) on -Tugela Dn at ambient and elevated CO_2 concentration (Tug Dn 385 ppm and Tug Dn 450 ppm respectively) and Scheepers at ambient and elevated CO_2 concentration (Sch 385 ppm and Sch 450 ppm). Bars with different letters and numbers indicate significantly different homogenous groups using Tukey's *post hoc* test ($p<0.05$), same letters indicate no significant difference.

At 14 days after infestation, a significantly ($P<0.05$) lower number of aphids were recorded on Tugela Dn-385 ppm compared to the Tugela Dn-450 ppm and the susceptible cultivar at both CO_2 concentration. RWA SA1 also showed significantly ($P<0.05$) higher population numbers at elevated than ambient CO_2 concentration for both wheat cultivars. On the other hand, the populations of the aphids on the resistant (Tugela Dn) and susceptible (Scheepers) cultivars were significantly ($P<0.05$) different at ambient CO_2 concentration but not significantly ($P>0.05$) different at elevated CO_2 concentration. Although populations on Scheepers-385 ppm and Scheepers-450 ppm were significantly ($P<0.05$) different from each other, neither were significantly ($P>0.05$) different from Tugela Dn-450 ppm (Figure 2.3 and 2.4).

2.3.2 Visible symptoms of aphid virulence on host plants

Chlorosis, leaf rolling and necrosis associated with RWA SA1 feeding on the resistant and susceptible wheat cultivars are shown in Table 2.3 below. It can be seen that the treatments i.e. wheat cultivar and the CO_2 concentration had an effect on the aphids resulting on evident differences in leaf rolling and necrosis during infestation. The table shows the average damages caused by the aphid at each treatment during 21days of feeding.

Table 2.2 Chlorosis and leaf roll average ratings of Tugela Dn (Tug Dn) and Scheepers (Sch) infested with RWA SA1 under ambient and elevated CO2 concentration at 2, 7, 14 and 21 days after infestation.

Treatment (Cultivar + CO_2 concentration)	2 Days	7 Days	14 Days	21 Days
Tug Dn 385 ppm	A0B1	A1B1	A3B1	A5B2
Tug Dn 450 ppm	A0B1	A1B1	A4B2	A6B2
Sch 385 ppm	A0B1	A1B1	A4B2	A7B3
Sch 450 ppm	A0B1	A2B1	A4B2	A8B3

A= Chlorosis B= Leaf Roll.

After 2 days of infestation, RWA SA1 had the same effects on all treatments. No chlorotic spots were visible and leaves appeared healthy and flat. By 7 days of infestation, all treatments showed some level of symptoms of chlorosis but no leaf rolling. The susceptible cultivar (Sch) at 450 ppm had more noticeable spots as compared to the other 3 treatments. After 14 days of aphid infestation, all treatments were more adversely affected at both ambient and elevated CO_2 concentration. Streaking became more apparent with larger and more numerous chlorotic spots as well as loosely folded leaves. Tugela Dn at 385 ppm had fewer chlorotic spots and flatter leaves as compared to the other treatments.

After long-term infestation, 21 days, by RWA SA1, extensive chlorosis and leaf rolling was visible for all treatments. Damage was more developed in the susceptible Scheepers cultivar as compared to the resistant Tugela cultivar, at both CO_2 concentration. For most, chlorosis covered up to 70% of the total leaf area and leaves were dying back from the tips. Thirty percent of the susceptible plants at elevated CO_2 concentration were dead with no possible recovery as compared to 10% of the same cultivar at ambient CO_2 concentration (data not

shown in the table). Whereas, at elevated CO_2 concentration only 10% of the resistant plants died. It is clear from the results that leaf rolling, chlorosis symptoms and necrosis were more noticeable and extensive in the susceptible cultivar as compared to the resistant cultivar, which seems to be able to tolerate aphid infestation much better at both ambient and elevated CO_2 concentration.

2.3.3 Aphid feeding damage to host

The fluorescence images for callose deposition indicate that there was a progressive increase in wound callose synthesis from DAI=0 to DAI=21 i.e. from the short-term to long-term infestation by RWA SA1, for both the resistant and susceptible wheat cultivars. Callose deposition in the control leaves (uninfested) of both cultivars (Tugela Dn and Scheepers) at each treatment was very low as can be seen in Fig 2.5. Minimal wound callose deposition appeared within the 7 days of feeding by RWA SA1 for both the resistant and the susceptible wheat varieties (Fig 2.6). There is a visible difference in callose deposition for the controls and the plants infested for 7 days. The susceptible (Scheepers) cultivar had moderately higher callose deposition as compared to the resistant cultivar (Tugela Dn). In this case, Tugela Dn at ambient conditions had a greater amount of callose as compared to that of Tugela Dn at elevated conditions. This was the reverse for the Scheepers cultivar where the plants grown at elevated CO_2 treatment had more callose deposition as compared to the ambient CO_2.

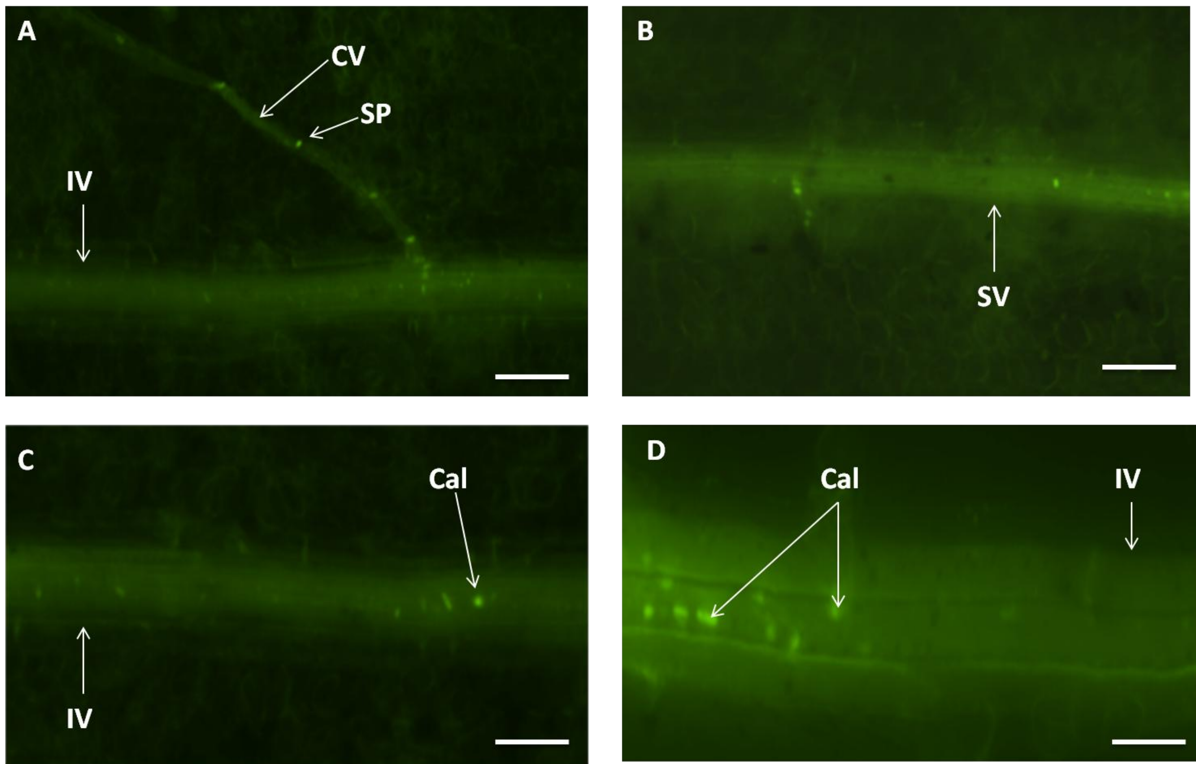


Fig. 2.5 Fluorescence images to show the level of callose in longitudinal sections of control leaves (uninfested plants) stained with aniline blue. A- an intermediate vein (IV) with a cross vein (CV) from Tugela Dn under elevated CO_2 concentration. Callose is present mostly in sieve plates (SP) only. B- a small vein (SV) from Tugela Dn under ambient CO_2 concentration. C- an intermediate vein (IV) from Scheepers under elevated CO_2 concentration with little callose (Cal) deposition. D- A large vein (IV) from Scheepers under ambient CO_2 concentration. (Bars: A-C = 200 µm and D=100 µm).

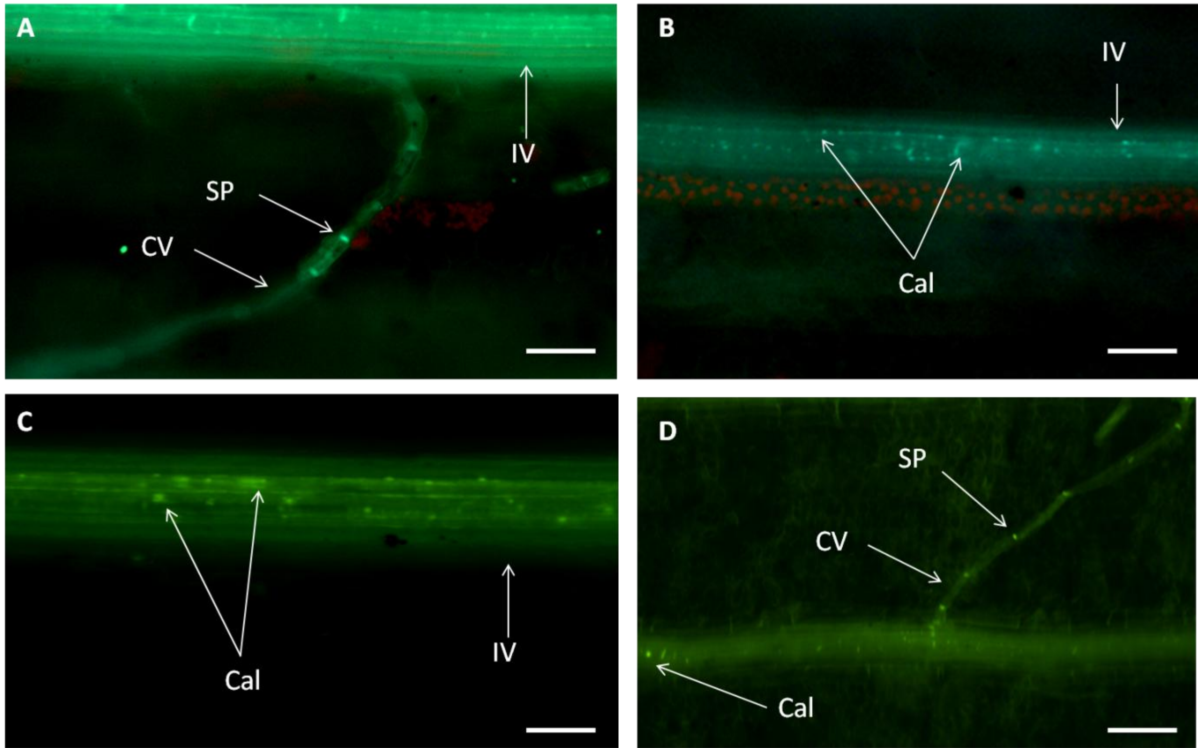


Fig. 2.6 Sections of wheat leaves after feeding by RWA SA1 for 7 days at ambient and elevated CO_2 concentration. Sections of scrapped leaves stained with aniline blue for wound callose distribution. A= Tug Dn at 450 ppm showing part of an intermediate vein (IV). B= Tug Dn at 385 ppm also part of an intermediate vein with callose (Cal) associated with RWA SA1 feeding. C= Sch at 450 ppm and D= Sch at 385 ppm showing a cross vein. All the images show moderate callose distribution observed after the 7 days of feeding. (Bars: A and C= 100 µm, B and D= 200 µm).

Wound callose becomes more evidently visible by day 14 and abundant by day 21 (Fig 2.7 and Fig 2.8 respectively) of infestation by RWA SA1. At day 14, RWA SA1 induced greater wound callose in Tugela Dn at elevated CO_2 concentration as compared to the ambient treatment (Fig 2.7 A and B respectively). There seemed to be not much of a difference in callose deposition in the Sch cultivar at both CO_2 concentration levels during this period (Fig 2.7- C and D).

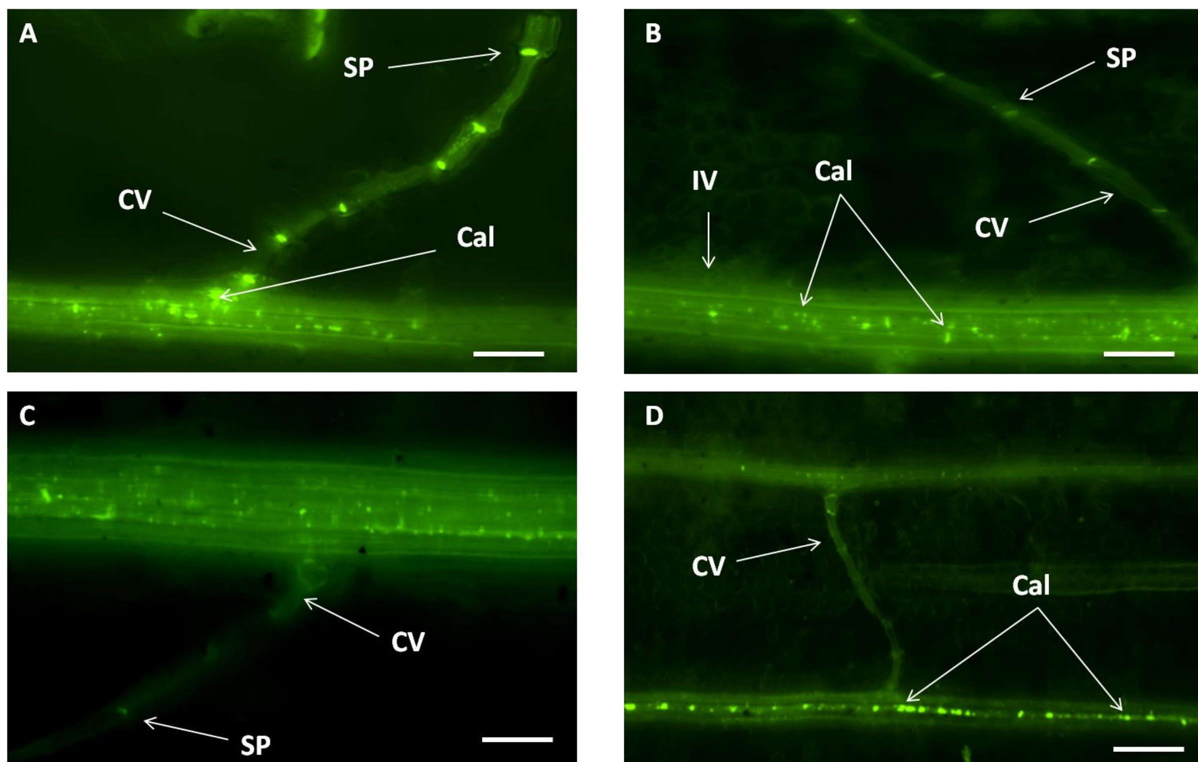


Fig. 2.7 Sections of wheat leaves after feeding with RWA SA1 for 14 days at elevated and ambient CO_2 concentration, stained with aniline blue for callose deposition. A= Tug *Dn* at 450ppm showing intense callose in the vein. B= Tug *Dn* at 385 ppm showing moderately lower callose (Cal) as compared to A. C= Sch at 450 ppm with numerous clusters of callose in the vein. D= Sch at 385 ppm showing callose deposition in the vein which obviously interferes with transportation. Extensive regions of wound callose can be seen as the bright fluorescence in the veins. (Bars: A-C= 100 µm and D= 200 µm).

By day 21, the damage to the vascular tissues of the host plants was more extensive due to long-term feeding by the aphid and may have lost their functionality. Where aphids probed and fed on the leaf, higher callose formation occurred. As expected (Tolmay, 2008), wound callose was more evident in the veins of the susceptible as compared to the resistant cultivar. There is massive callose deposition in the phloem of longitudinal intermediate veins resulting in blockage of transport in the susceptible cultivar (Sch) (Fig 2.8- C and D). Transport in the cross vein in Fig 2.8- D is blocked. Tugela Dn at elevated conditions sustained much more probing due to the many spots of callose deposition as compared to the ambient treatment (Fig 2.8- A and B respectively). Overall, there was greater damage due to RWA SA1 in the susceptible as compared to the resistant wheat cultivar.

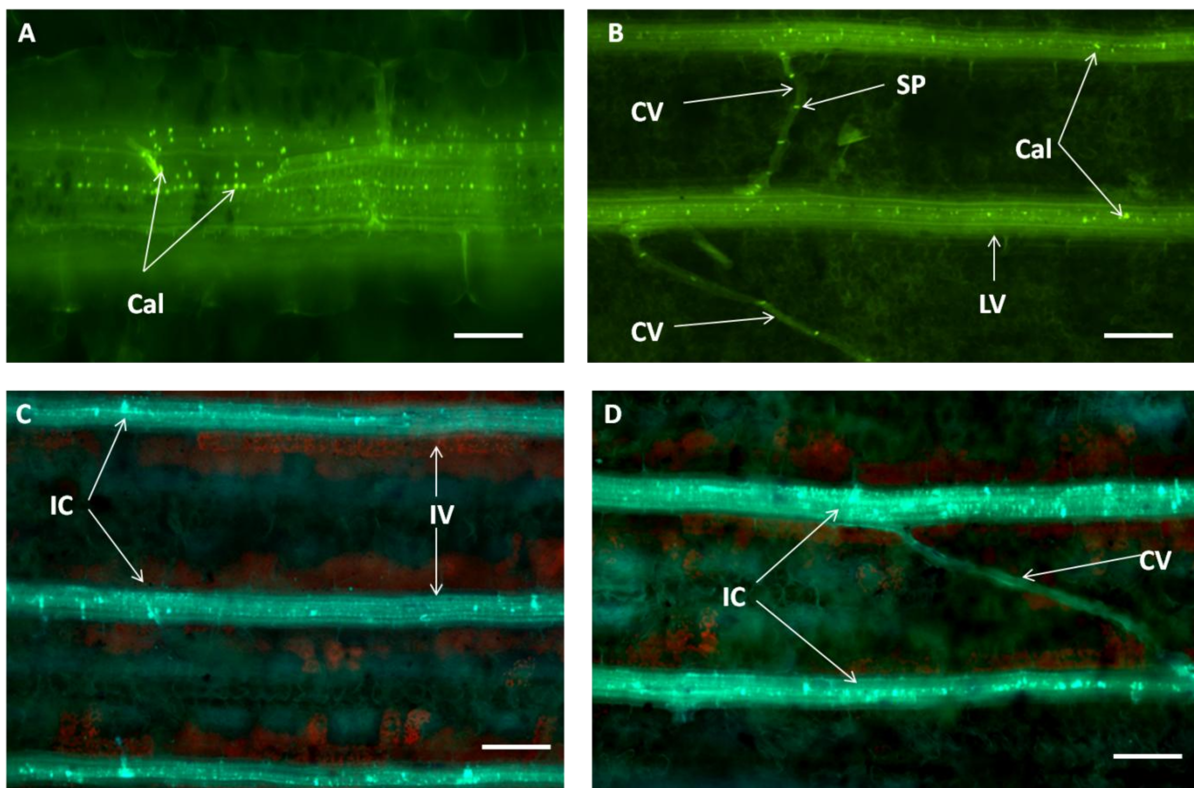


Fig. 2.8 Sections of wheat leaves after infestation by RWA SA1 for 21 days at elevated and ambient CO_2 concentration then stained with aniline blue for callose deposition. A= Tug *Dn* at 450 ppm. B= Tug *Dn* at 385 ppm showing two veins next to each other, connected by cross veins. C= Sch with three adjacent intermediate veins at 450 ppm. D= Sch at 385 ppm showing two adjacent intermediate veins connected by a cross vein. The images show extensive, intense callose distribution (IC) in the veins. The high amounts of callose present indicate a high disruption of transport and damage as a result of long-term aphid feeding.

2.4 Discussion and Conclusion

Diuraphis noxia (RWA) has been considered a major agricultural threat to the South African wheat industry. With food security being under pressure due to the increasing growth in population in South Africa, understanding the effect an insect pest on grain production and yields under changing climate condition is vitally important. Small grain farmers incur significant losses annually as a result of insect pests. Control by conventional methods, such as insecticides, has proved to be ineffective and damaging to the environment. Since 1992 researchers and plant breeders have been developing new aphid resistant cultivars of wheat in a quest to improve the yield of wheat farmers especially in the Free State. Although the resistant cultivars managed to keep RWA SA1 infestation levels on tillers to a minimum, new aphid biotypes were detected that had overcame the resistance of these cultivars.

A new biotype (RWA SA2) was reported in 2007 from wheat farms in the Free State (Tolmay *et al*., 2007). Four biotypes (RWA-SA1, SA2, SA3 and SA4) have emerged to date in South Africa (reference of Vicki and others at Bethlehem). The new biotypes are more aggressive feeders and reproduce faster than the RWA SA1 biotype. This poses a serious threat to the wheat industry and therefore it is necessary to fully understand the insect-host interactions, mechanisms of plant defence responses and try to identify potential sources of resistance against the new RWA biotypes. For this reason many researchers have been working in this field for several years and published results which when brought together can greatly affect the success of the new RWA biotypes.

Food production is also being affected by the changing climatic conditions. An increase in global average temperatures, changes in precipitation patterns and extreme climatic events. These climatic changes will affect the biodiversity on the earth, causing changes in plant and animal stress response and defence mechanisms and the ecosystems. The evidence of the rising atmospheric levels of CO_2 we are experiencing leads to issues that need to be dealt with by researchers and conservationists to maintain life on any area that might be affected (Bradley et al., 2012).

2.4.1 Population growth rate and development of aphid on host

In this study, the effect of RWA SA1 on a resistant and susceptible cultivar (Tugela Dn and Scheepers respectively) at elevated and ambient CO_2 concentration (450 ppm and 385 ppm)

was studied. Data from this study showed positive growth for RWA SA1 at ambient and elevated CO_2 concentration on both wheat cultivars (Fig 2.2). As can be elucidated from the results, elevated CO_2 showed some impact on the aphid population dynamics. RWA SA1 excelled under elevated CO_2 concentration as compared to the ambient CO_2 concentration for both the resistant and susceptible cultivars. This is deduced from the populations recorded at 450 ppm which were approximately double that at 385ppm after the 14 day period of infestation. This could be attributable to the fact that RWA SA1 was reproducing faster at 450 ppm with populations doubling 3 times in the period but was much slower at 385ppm, thereby appearing to have overcome some of the resistance of the plant resistance of Tugela Dn under growth at elevated CO_2 levels.

Although the population growth rate for RWA SA1 on all cultivars was generally exponential at all treatments, growth at elevated CO_2 concentration was noticeably faster as can be seen with the sharp rising curves (Fig 2.2 A and B). The aphids adapted to the environment much faster and easier at the elevated CO_2 concentration for both cultivars whereas a lag phase of growth was seen for the aphid populations under ambient CO_2 concentration. This makes it much easier and faster for aphids to infest, spread and surmount on new hosts in the predicted CO_2 concentration increases in the future.

It is widely accepted and well documented that plants grown under elevated CO_2 concentration have enhanced productivity due to the direct fertilizing effect on photosynthesis (Lindroth *et al*., 1993), increased total biomass, carbohydrates and C:N ratio (Pritchard et al., 2007). The effect of elevated CO_2 on growth and yield depends on the photosynthetic pathway. C_3 plants have increased photosynthesis and growth under elevated CO_2 concentration as compared to C_4 plants that respond modestly due to a mechanism that increases leaf CO_2 concentration resulting in CO_2 saturation of photosynthesis at ambient concentrations (Sage, 2002). Common C_3 plants include small grain cereals (wheat, rice, barley, oats and rye), various beans, peanuts, potato and yams. In contrast, common C_4 plants include maize, sugarcane, sorghum and millet.

The success in insect herbivore development also indirectly depends on climate as environmental changes have an impact on the plant host. Because elevated atmospheric CO_2 concentration is unlikely to alter all plant tissues in the same manner, the indirect effects on herbivores will vary among insects. Therefore, this would result in altered insect population dynamics. As a result of elevated CO_2 concentration, chewing insects develop slower, suffer

increased death rates and increased consumption. On the other hand, phloem-feeding insects have a more complex response to the changes in quantity and quality of the plant (Sun *et al.*, 2013). Their responses are species-specific and thus can be a positive or negative or neutral effect. These where the findings by Hughes and Bazzaz (2001), where elevated CO_2 concentration resulted in species-specific reactions when the abundances of five aphid types were tested on their hosts. One species (*M. persicae*) increased in population, one decreased (*A. pisum*) and the last three were unaffected (*A. nerii* , *A. oestlund* and *A. solani*).

This can explain the increase in performance and abundance of RWA SA1 at 450ppm observed in these experiments. This clearly shows that Tugela Dn and Scheepers are a better quality host for RWA SA1 at 450 ppm than 385 ppm. The fact that in this study the resistant cultivar Tugela Dn showed much resistance to RWA SA1 can be comforting to the small grain industry although it must be noted that a weaker, less aggressive biotype was studied. Although not beneficial for all insects, many researches with aphids at elevated CO_2 concentration have shown similar results. In one such experiment, the populations of RWA SA1 and RWA SA2 increased significantly on the four barley lines (STARS- 9301B, STARS -9577B, STARS -0502B and PUMA) at 450 ppm as compared to 380 ppm (Jimor, 2011). In another, the aphid *A. Solani* increased its daily rate of production of nymphs by 16% at elevated CO_2 concentration (700 ppm) as compared to the ambient (350 ppm) (Awmack *et al.*, 1997). In another recently published work, elevated CO_2 concentration (750 ppm) increased the abundance of the peach aphid *M. persicae* on four isogenic *A. thaliana* genotypes (a wild type and three SA-deficient mutants) (Sun *et al.*, 2013).

According to Hughes and Bazzaz (2001), a possible reason why most aphid species are not negatively affected at high CO_2 concentration is that they may be able to compensate sufficiently for changes in nutritional quality induced by changing the feeding positions, rate of uptake or post-ingestive metabolism. Thus they can easily adapt to the changes predicted for the future. In summary, this study showed that there is a difference in performance of RWA SA1 on both resistant and susceptible wheat cultivars when grown at elevated CO_2 concentration. The reproductive rate and virulence of the aphid was greater at elevated CO_2 concentration as compared to ambient CO_2 concentration on both cultivars. This may indicate a change in behavioural response of RWA SA1 to host plant interactions under elevated CO_2 concentration, thereby partly overcoming the resistance of Tugela Dn conferred by the *Dn1* gene.

2.4.2 Wound callose deposition

Callose is a β-1,3-glucan polysaccharide existing in cell walls of plants. It has roles in plant development and in response to stress (biotic and abiotic). In the later, it is deposited in sieve pores, plasmodesmata and between the plasma membrane and the cell wall as a response to wounding or infection. This occurs within minutes of wound initiation (Nakashima *et al.*, 2003). Wounding during aphid feeding results in a decreased rate of assimilate movement in the phloem and loss of turgor due to penetrated parenchymatic cells and elements (Botha and Matsiliza, 2004). The fast response is hence useful as a response against these effects by sealing damaged pores in the phloem. On the other hand, callose deposition slows down transport of solutes in the phloem thereby preventing leakage of nutritious solutes from the symplast into the apoplast.

This study demonstrates that RWA SA1 feeding resulted in the development of wound callose seen as the intense yellow deposits under UV light. Seeing as wound callose formation is associated with damage to the phloem cells, it is expected that greater amounts of wound callose would be deposited when the plant has been more actively probed and fed upon. This explains the build up of callose in the vascular tissue of the leaf in the long-term with very extensive callose deposition at day 21 after infestation as compared to day 7 and day 14. As can be seen, blockage of sieve plates, plasmodesmata pore units and cross veins by callose in infested plants from day 14 to 21 post infestation, can result in a huge decrease in transport of assimilates. This can be effective to discourage aphids from feeding from that site and forces them to move off and seek a new favourable succulent host. On the contrary, the continuous reduction of solutes vital for survival can have serious implications on the plant. This can explain plant deaths that occurred during the long-term infestation by RWA SA1. The results obtained in this study are supported by previous studies.

RWA has been found to rapidly induce the synthesis and hence deposition of wound callose by plants in response to infestation in many experiments (Matsiliza, 2003; Saheed, 2007 and Jimor *et al.,* 2013). For example, in a study, Jimor (2011) demonstrated that RWA SA1 and

RWA SA2 feeding induced wound callose formation within 24hrs of infestation and progressed through the 14 days on both resistant and susceptible barley. It was also revealed that the level and distribution of callose induced was dependant on whether the wheat cultivar tested was susceptible or resistant. Assimilates in plants are driven through a classical source-to-sink pathway. Data from a study demonstrated that aphids re-routed assimilate to themselves resulting in the formation of “strong local secondary sinks” as a continuous nutrient supply (Botha and Matsiliza, 2004). The large areas of wound callose examined were an indication of this.

The results of the study reported in this section shows that callose formation and distribution varied with feeding duration, wheat cultivar (resistant or susceptible) and as well as CO_2 concentration, a similar finding to that by De wet and Botha (2007). Although there seems to be a bit of variations in the amount and intensity of wound callose formation among the different days and treatments, it must be noted that different resolutions and colours were used to try and access the best image at each treatment. Generally wound callose became more evident and extensive the longer the plant was infested by the aphid. It is clear that there was more callose deposition at day 21 after infestation as compared to day 7 for all treatments (Fig 2.6 and 2.8).

As expected, continuous RWA SA1 feeding showed evidence of more callose deposition in the susceptible cultivar especially under elevated CO_2 concentration than at ambient levels. This could be because the *Dn1* resistance gene in Tug Dn reduced callose deposition due to RWA SA1 feeding. It cannot be clearly supported that callose production and distribution was proportional to the number of aphids feeding from the test plants. For example at day 14 (Fig 2.7), Tug Dn at ambient CO_2 concentration had more extensive, intense callose deposition as compared to Scheepers at the same conditions although the later was heavily infested by almost a two times- larger colony of RWA SA1 than the resistant cultivar (Fig 2.2). At elevated CO_2 concentration, callose deposition in the two cultivars is almost similar despite the major difference in total populations at day 14. It can be concluded that once a plant is damaged, wound callose is rapidly produced and deposited mainly in sieve elements irrespective of the aphid population or duration of infestation.

3 CHAPTER THREE

IDENTIFICATION OF DIFFERENTIALLY REGULATED PROTEINS IN RWA SA1 INFESTED WHEAT UNDER AMBIENT AND ELEVATED CO2 concentration

3.1 Introduction

Cereal grains are of major importance in the world as a source of food both for people and their livestock. Wheat is a source of many nutrients such as minerals, vitamins, carbohydrates, fats and of major interest: proteins. These proteins have both functional and nutritional roles. Gluten in wheat forms a continuous network of protein matrices which link cells together, giving bread dough their unique characteristic and strength (Shewry, 2009). Cereal proteins also play a major role in cereal stress response have therefore been studied for a long time (Shewry and Halford, 2001).

Proteomics is the comprehensive study of quantitative changes of the protein complement of a given cell or organism under well-defined conditions (Chen and Kim, 2006). According to Wilkins *et al*. (1996), it determines the relative abundance and posttranslational modification state of proteins in a sample. The results can be applied in fields such as drug discovery, diagnostics, therapy and genetic engineering. Structural proteomics determines the protein constitution of a cell. Proteomics therefore provides a more accurate picture of the state of a living cell under specific conditions, than genomics or transcriptomics do (Lubec *et al,* 1999). It is difficult to reproducibly separate complex protein mixtures and many combinations of techniques have been used in proteomics (Cutler *et al.,* 1999).

Proteome analysis consists of two steps: the separation of protein mixtures by 2-D electrophoresis followed by the identification of these separated proteins by analytical techniques. One of the most reproducible, accurate and proficient methods to separate complex proteins is Two-Dimensional Electrophoresis (2-DE). It is a powerful tool employed in proteomics for purification and characterization of proteins (Chinnasamy and Rampitsch, 2006). 2-DE can be used to map the proteome of a given sample, as it effectively resolves complex mixtures. This allows for the identification of differential protein regulation in a given tissue or organ under different conditions (Finnie *et al*., 2002).

The efficacy of 2-DE as a separating method, lies in its ability to do so in two dimensions. The first step in 2-DE is Isoelectric Focusing (IEF) whereby proteins are separated on the basis of differences in net charge. A current is applied across an immobilized pH gradient strip (IPG) containing a mixture of proteins, thereby forcing them to migrate down the strip until they reach their individual isoelectric points (when it has a zero charge) (Görg *et al.*, 2000). In the second step also known as the second dimension, these proteins are further separated on the basis of differences in molecular masses. The addition of the anionic detergent SDS gives all proteins a negative charge with a constant charge to mass ratio, causing them to migrate through the porous polyacrylamide gel under an electric field according to size.

The identification of the separated proteins resolved as spots on the second dimension can be identified by analytical techniques such as mass spectrometry (MS) and amino acid composition analysis as well as software such as PDQuest™ (Skylas *et al.*, 2005) for advanced image analysis that yields high reproducibility. The complexity of protein sample preparation is a major challenge prior to 2-DE. This is due to differences among tissues (both plants and animals). For more accurate resolution of the proteins during 2-DE, an efficient protein extraction method has to be employed and usually, requires extensive optimization prior to 2-DE (Louw, 2007).

Non-protein contaminants such as organic acids, polyphenols and pigments in plant extracts make it generally difficult to resolve proteins. Extraction processes usually begin with the precipitation of the protein which is then re-suspended in an appropriate buffer. Protein loss occurs when there is incomplete precipitation or resolubilization. Mainly two extraction methods have been employed in plants protein extraction: phenol-based and acetone- based (TCA) methods (Chen and Harmon, 2006). TCA method minimises degradation of proteins by instantly eliminating enzyme-activity. Both methods recover proteins that are difficult to resolubilize (Chen and Harmon, 2006). The high resolution of 2-DE makes it an efficient technique to study protein expression in response to various stress factors (Görg, 2000). This study reports the use of 2-DE with PDQuest™ analysis software to identify differentially expressed proteins in Tugela Dn infested by RWA SA1 under elevated and ambient CO_2 concentration as a result possibly of changes in the plants' resistance mechanism.

3.2 Experimental Overview

3.2.1 Materials

Host plant material and aphid colonies were grown and maintained as explained in Section 2.2.1. A total of 48 plant (24 Tugela Dn and 24 Scheepers) were grown half of each as tests (infested with RWA SA1) and the other half as controls (uninfested). Tugela Dn and Scheepers tests were then infested with RWA SA1 and allowed to grow in the conviron as outlined in Section 2.2.2.

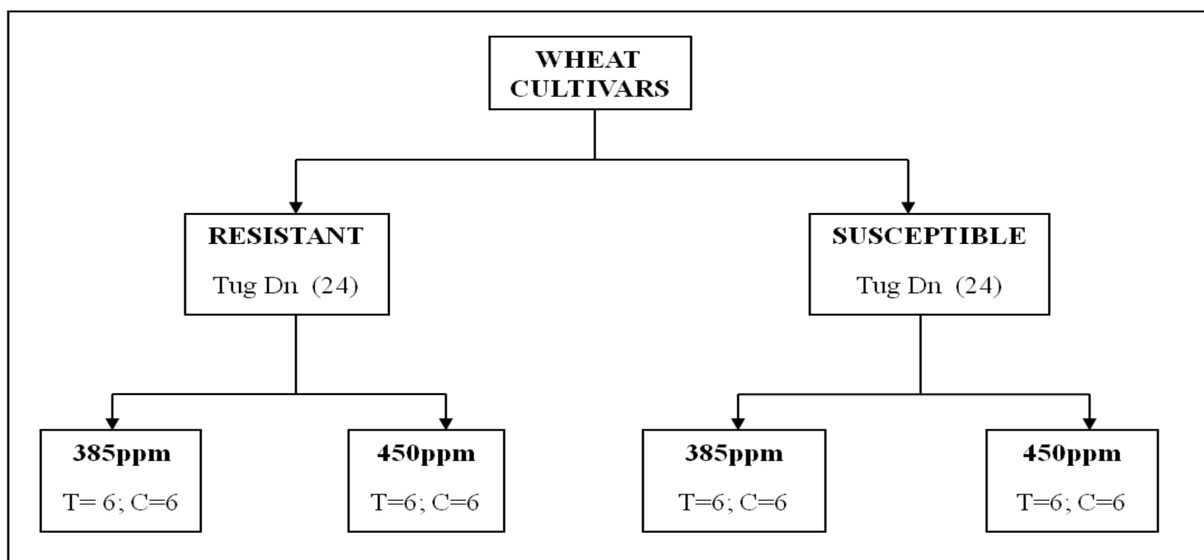


Fig. 33.1 Overview of wheat cultivars used (total number in brackets) as well as the number of plants used for tests (T) and control (C) at elevated (450 ppm) and ambient (385 ppm) CO_2 concentrations. A total of 48 plants were used.

Aphids were allowed to feed on leaves for 14 days with leaves harvested at different intervals: Day 0 (before infestation) then 1, 5, 7, 9 and 14 days after infestation. At each interval, 3 leaves were collected from each test plant and its corresponding control. Aphids on test plants were brushed off prior to harvesting. Leaves were immediately frozen in liquid nitrogen (Department of Chemistry, Rhodes University, Grahamstown) in 15ml Eppendorf tubes and stored in a -80°C freezer until further processed.

3.2.2 Total Protein Extraction

All equipment used for this extraction was autoclaved to ensure sterility before the process. Total protein was extracted using the ReadyPrep™ Protein Extraction Kit (Total Protein) from Bio-Rad (US). The kit was chosen as the protocol is simple, rapid and reproducible method for preparing total protein extracts for 2-D gel analysis. Harvested leaf samples were homogenized in liquid nitrogen using a mortar and pestle into a fine powder, quickly transferred into a 2ml microcentrifuge tubes and weighed. The homogenized leaf tissue was immediately suspended in 2-D Rehydration/Sample Buffer 1 (contains 7 M urea, 2 M thiourea, 1% (w/v) ASB-14 detergent, 40mM Tris base and 0.001% Bromophenol Blue) for cell lysis and complete solubilization proteins in a volume of 3ml buffer per 1 g plant tissue. Samples were sonicated (4 busts-30sec each) to disrupt cells prior to centrifugation (16, 000 x g, 30mins, room temperature) to pellet debris. The supernatant was stored at -80 ^{o}C while an aliquot was retained for protein quantification.

3.2.3 Determination of Protein Concentration of Samples

The *RC DC* Protein Assay kit from Bio-Rad (US) was used. It is a colorimetric assay for protein quantification based on the Lowry assay (Lowry *et al.*, 1951) but modified to be reducing agent compatible (*RC)* as well as detergent compatible (*DC*). The Microcentrifuge Tube Assay Protocol (1.5ml) was followed as per the manufacturer's instructions in the manual. Bovine serum albumen (BSA) protein standards were prepared (from 0.2mg/ml to 1.5mg/ml). After precipitating the proteins from samples following the protocol, absorbencies were read at 750nm. BSA standard curves were created using Microsoft Excel® and sample protein concentrations determined by interpolation from the curve.

3.2.4 2-D Cleanup of Sample Proteins

Protein extracts (from section 3.2.2) were cleaned up using the ReadyPrep™ 2-D Cleanup Kit from Bio-Rad. This kit serves to prepare low conductivity samples suitable for Isoelectric focusing (IEF) and 2-D gel electrophoresis as well as concentrate proteins from sample to improve spot detection (Bio-Rad, 2014c). Precipitation of proteins leaves behind substances that can interfere with IEF such as ionic detergents, salts and nucleic acids. Following the manufacturer's instructions throughout, the pellet was re-suspended in 100 µl of the 2-D Rehydration/ Sample buffer (containing 8M urea, 2% CHAPS, 50mM dithiothreitol (DTT),

0.2 (w/v) Bio-Lyte® 3/10 ampholytes and a trace of Bromophenol Blue). Any unused protein sample was stored in clean microcentrifuge tubes at -80°C.

3.2.5 Determination of Protein Concentration of Samples After 2-D Cleanup

The *RC DC* Protein Assay kit from Bio-Rad (US) was used again to quantify the protein after 2-D Cleanup. This was done to determine the actual protein concentrations in the samples so that the correct amount of protein could be loaded onto IPG strip for IEF assuming that protein concentrations might change during the cleaning process.

3.2.6 Isoelectric Focusing

A ReadyPrep™ 2-D Starter Kit from Bio-Rad (US) was used and it enables the protocol and reagents to successfully perform 2-D polyacrylamide gel electrophoresis (2-D PAGE). Broad-range pH 3-10 NL (11cm) ReadyStrip™ IPG strips (Bio-Rad, U.S.A) were rehydrated overnight in 185µl re-suspended protein sample (containing 250µg wheat leaf protein) sufficient to easily visualize protein spots when 2-D SDS-PAGE gel is stained. After rehydration, the IPG strips were then transferred into a clean, dry PROTEAN IEF focusing tray (11cm) and IEF ran at default PROTEAN IEF cell temperature following the program in Table 3.1.

Table 3.1 PROTEAN IEF cell program for IEF run

STEP	VOLTAGE	TIME (min)	Volt-Hours	RAMP
1	250	20	----	Rapid
2	8000	60	----	Linear
3	8000	----	26 000	Rapid
4	1500	∞	----	Hold

3.2.7 SDS- PAGE

Criterion™ TGX™ precast polyacrylamide gels (4-20%) where used to run SDS PAGE. IPG strips from IEF were equilibrated using buffers from the kit following the manufacturer's

instructions. After laying the strip on a SDS-PAGE gel and overlaying with molten agarose, they were vertically placed in a gel box (Criterion system) connected to a Power Station 200 (Labnet International, Inc., Edison, NJ, USA). Reservoirs were filled with 1X Tris/glycine/SDS running buffer and electrophoresis began under the set program in Table 3.2.

Table 3.2 Mini-PROTEAN program for SDS-PAGE run

Strip length	11cm
Electrophoresis Cell	Power Station 200
Conditions	200V (constant)
Approximate Run Time	35min

3.2.8 Staining

SYPRO® Ruby protein gel stain was used for the rapid detection of proteins on the 2-D PAGE gels. The gels were initially fixed for 30mins in a 50% methanol and7% acetic acid solution then washed in dH_2O. This was followed by staining with SYPRO® Ruby overnight on a gentle shaker, gels covered with aluminium foil. Gels were then de-stained in a 10% methanol and 7% acetic acid solution for 30mins then viewed and photographed using an Alliance 4.7 Transilluminator (UVITEC Ltd, Cambridge, UK).

3.2.9 Determination of differential protein expression in wheat in response to aphid herbivory under different CO2 concentration

Analysis of protein spots across different gels (infested and control groups) was performed using PDQuest™ Basic 2D Gel Analysis software (Bio-Rad Labs, USA) Version 8.0. Gels were analysed using the "Spot Detection Wizard" to setup the matching parameter as follows, Manual identification of faint, small and large clustered spots was done allowing for some user input in terms of parameters. Spots were normalized using the "Local regression model" parameter.

The difference in each spot between the gels was resolved from the "Quantity Graph Report" which is a collection of histogram graphs which can be used to quickly compare and identify

any absence of spot or changes in spot quantity (protein quantity) from gel to gel. Changes in the proteome, due to CO_2 elevation, were determined in both the uninfested and infested resistant cultivars. Finally, the effect of CO_2 on the resistance mechanism was evaluated by comparing the difference in the proteome of the infested and uninfested resistant cultivar, under elevated CO_2. It must be noted that although 2-DE was done from both Tugela Dn and Scheepers samples, protein spot analysis was done only on gels of the resistant cultivar because at this point, the proteins of interest were only those reflecting a change in the resistance mechanism and not those reflecting the occurrence of the resistance mechanism. Therefore, the resistance mechanism only takes place in the resistant cultivar and not the susceptible.

3.3 Results

3.3.1 Determination of Protein Concentration of Samples

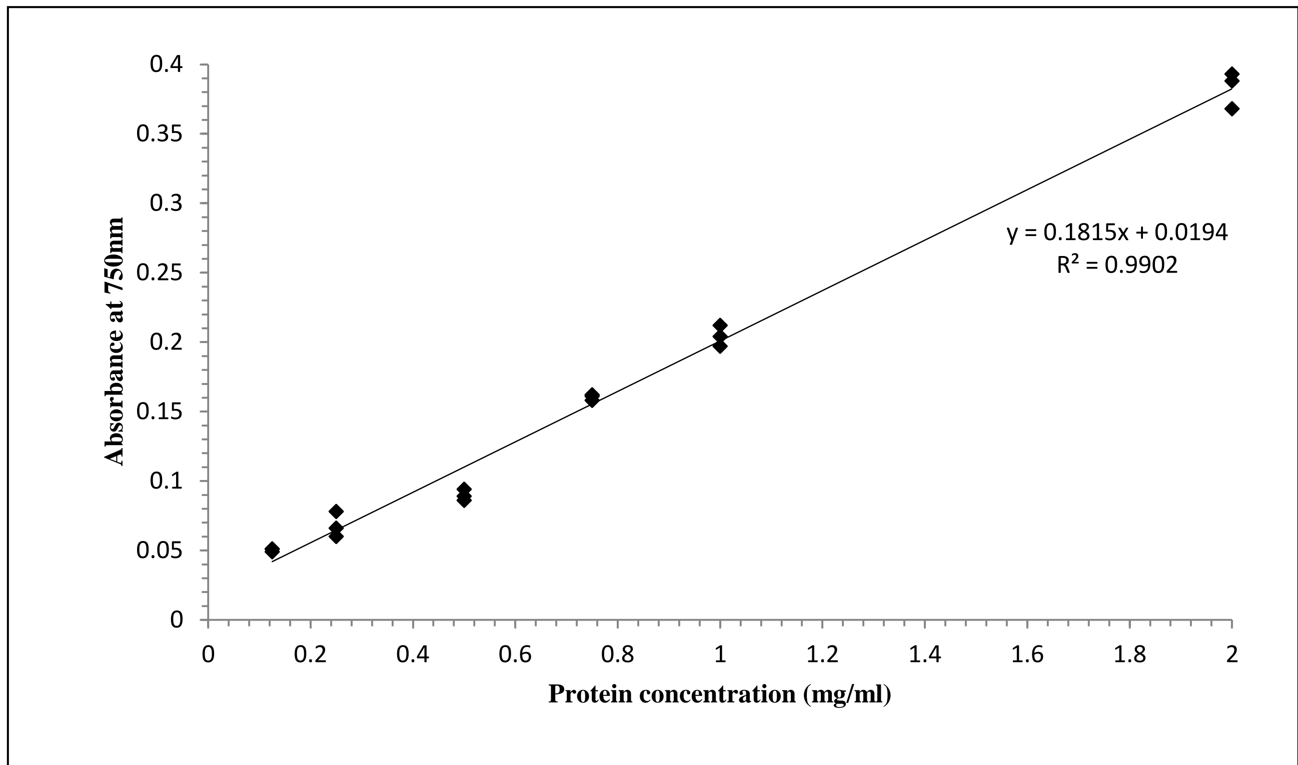


Fig. 3.2 Protein standard curve generated using the RC / DC protein assay (Bio-Rad) with bovine serum albumen protein standards from 0.125mg/ml to 2mg/ml. All readings were taken in triplicate.

3.3.2 Tugela Dn wheat total protein samples on 2DE gels stained with Sypro Ruby™

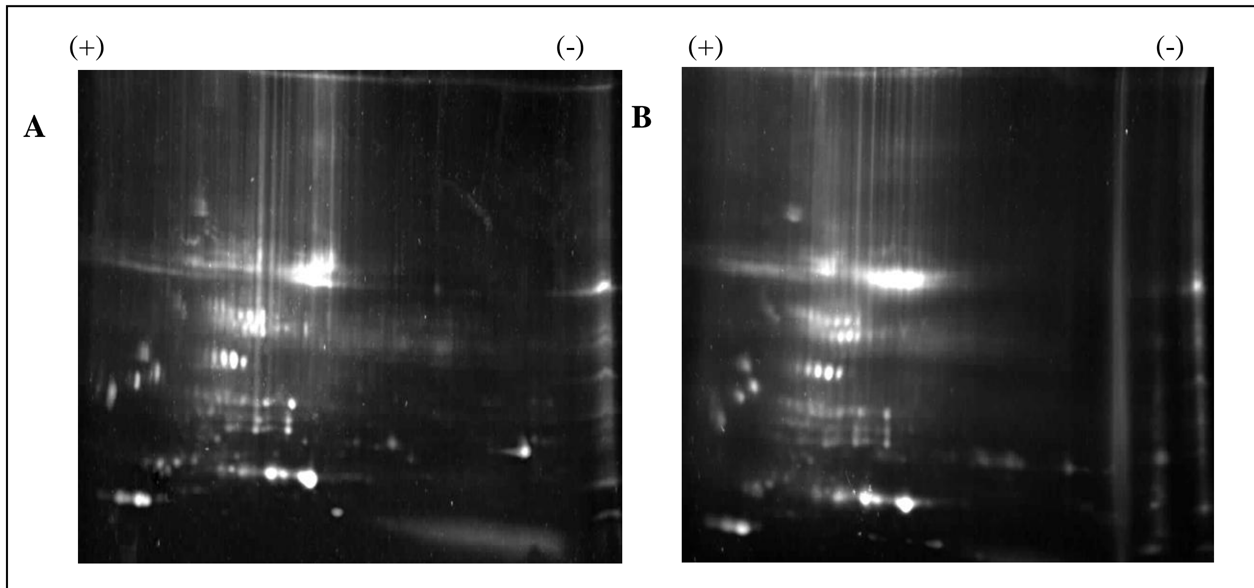


Fig. 3.3 2-DE gel samples showing differential protein expression in Tugela Dn wheat in response to different CO_2 concentration only. A: Tugela Dn grown under 450 ppm at day 14. B: Tugela Dn grown under 385 ppm at day 14.

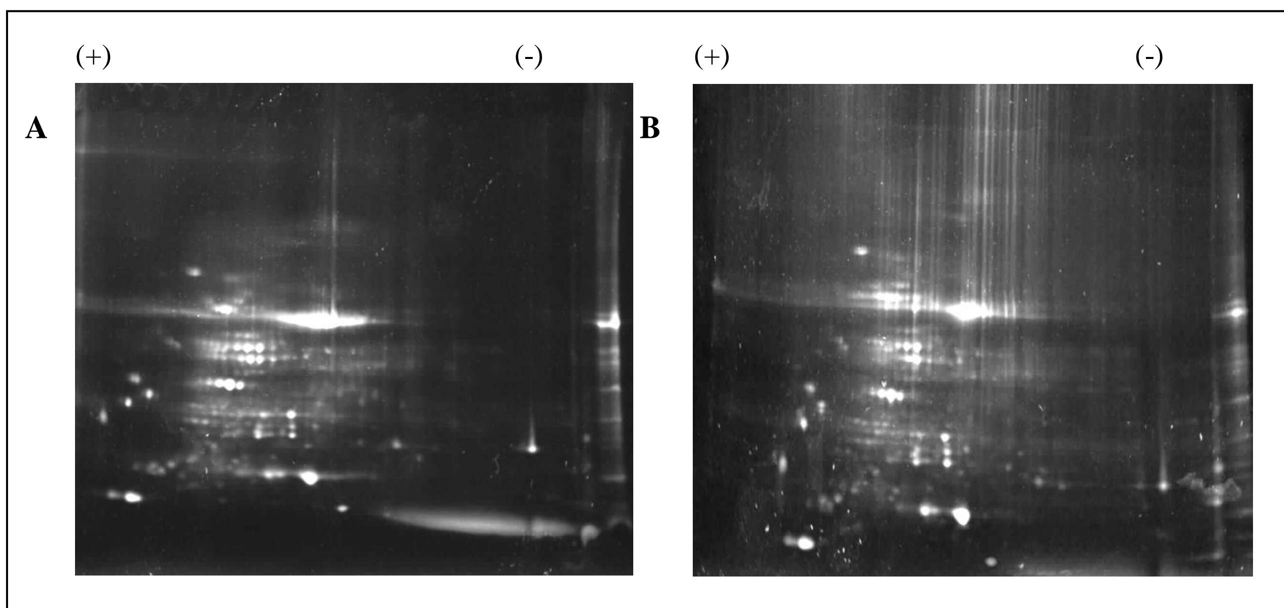
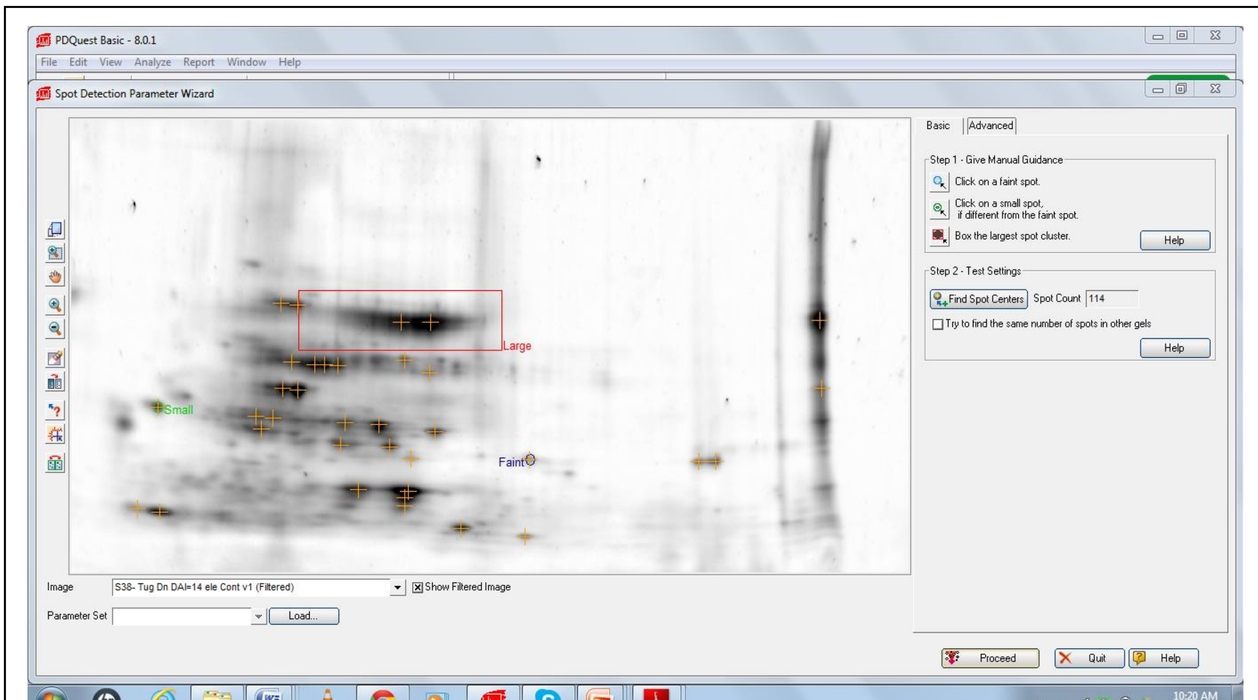


Fig. 3.4 DE gel samples showing differential protein expression in Tugela Dn wheat in response to RWA SA1 infestation under elevated and ambient CO_2 concentration. A: Tugela Dn infested with RWA SA1 at CO_2 concentration= 450 ppm, DAI= 14. B: Tugela Dn infested with RWA SA1 at CO_2 concentration= 385 ppm, DAI=14.

3.3.3 Analysis of 2-DE gels using PDQuest™ Basic Software

A



B

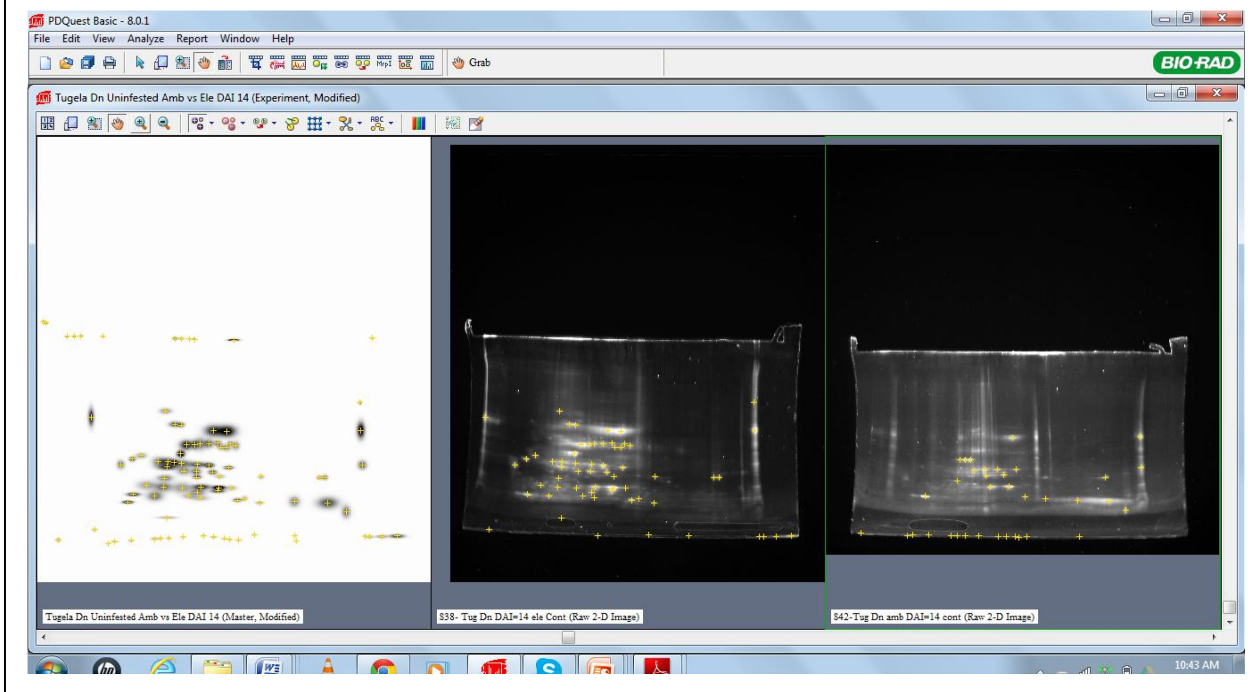


Fig. 3.5 PDQuest™ Basic Software (Bio-Rad) showing two steps taken during the analysis of the 2-DE gel samples showing differential protein expression in Tugela Dn wheat in response to RWA SA1 infestation and different CO2 concentration.

A- Screen print of inputted parameters for small, faint and large clustered spots. **B**- Screen print of the results of the image analysis obtained when comparing two gels (Tugela Dn DAI= 14 Uninfested: Ambient vs. Elevated CO2 concentration) with the master gel at the far left and the yellow crosses representing the matched spots.

3.3.4 PDQuest™ Basic Software gel matching results

3.3.4.1 Experiment Summary

After running the software, the Experiment Summary window is displayed (Fig 3.6). This is an interactive chart that shows the number of matched protein spots on the gels within the entire experiment based on the user settings. It also allows for changes to be made on some experimental settings such as matching and normalization. The spots column gives the number of spots detected per gel whilst the matched is the total of those matching. Match Rate 1 is the percentage of matched spots relative to total number on gel whilst Match Rate 2 is relative to those on the master gel. The summary in Fig 3.6 shows that 114 spots were detected in the gel for the uninfested Tugela Dn plant under elevated CO_2 levels at day 14 (DAI=14) with 78% of the matching spots relative to those on the master gel. 84 spots were detected on the gel containing the uninfested plants under ambient CO_2 levels with 57% matching spots relative to the master gel. Overall, 52 spots were matched to every member.

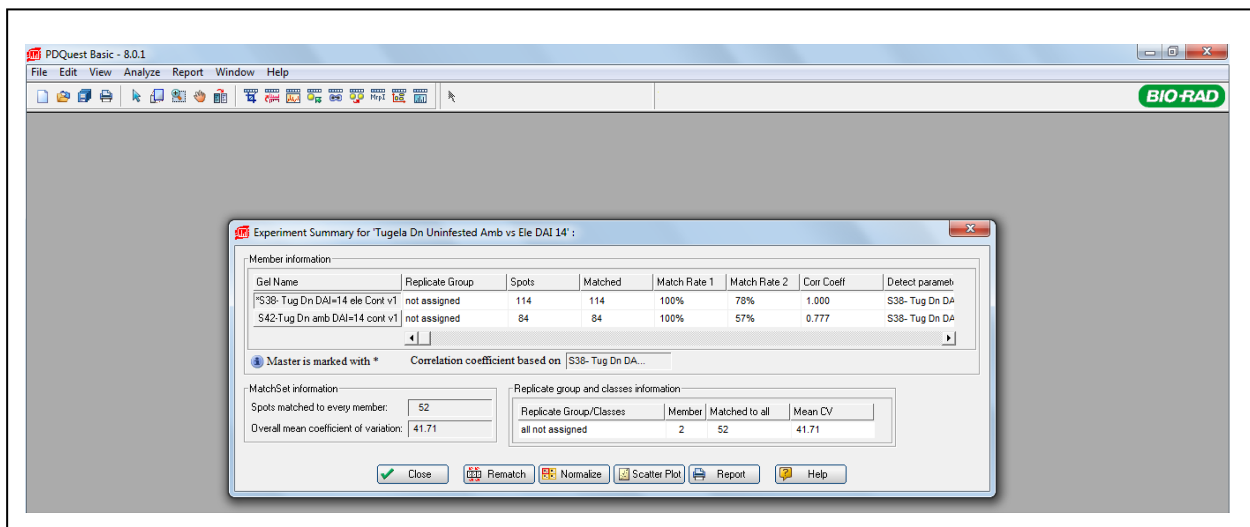


Fig. 3.6 Screen print of the "Experiment Summary" window for Tug Dn Uninfested: Amb vs. Ele DAI=14 showing 52 protein spots matched to every member.

3.3.4.2 Quantity Graph Report

Histogram graphs give a sense of the general trends in spot quantity. They are a quick and useful tools in detecting unmatched spots or differentially expressed spots. Each bar in the histogram represents the spot's quantity in a member of the match set drawn in proportion to

the highest bar. The quantitation of the maximum bar in the graph is shown on the upper right of the histogram. Each spot is automatically given a number (Standard Spot number: SSP) which is shown below each graph. Fig 3.7 is an example of a quantity graph report. Left bars represent the protein quantities for the uninfested Tugela Dn under elevated CO_2 whist right bars are for ambient CO_2. A single bar on the left side represents a protein that is expressed only in plants grown at elevated CO_2 and a single bar on the right side represents a protein expressed only under ambient CO_2. Up- regulation at elevated CO_2 level is detected when the left bar is longer than the right bar and down- regulation when the left bar is shorter than the one on the right. When the bars had approximately or exactly the same height, the quantity of the protein across the gels would be the same hence no change occurred.

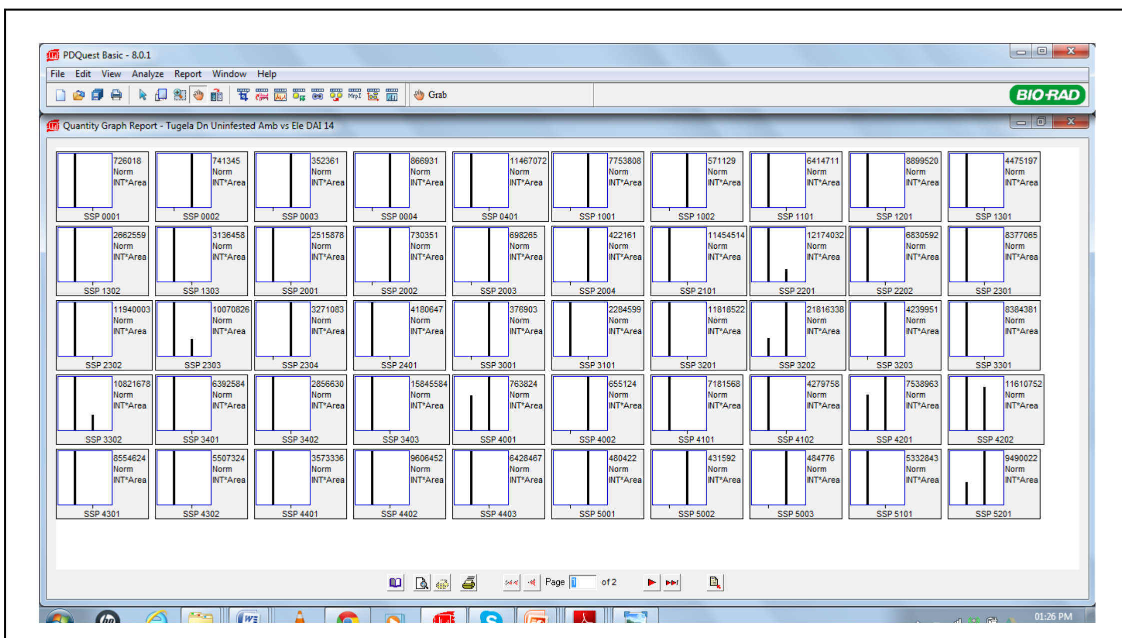


Fig. 3.7 Screen print of the Quantity Graph Report obtained after analysing the match-set: Tug Dn Uninfested - Ambient vs. Elevated CO_2 concentration - DAI=14 (only page 1 of 2 is shown).

3.3.5 Effect of CO2 concentration on the Proteome

The numbers of differentially regulated proteins (spots) were calculated as fractions of the total number of spots obtained for each match-set in order to construct pie charts. Changes in spot quantity were identified as either up- or down-regulation. Furthermore the presence or absence of spots in either gel of a match-set was also noted as well as spots that showed no changes between the two match-set gels.

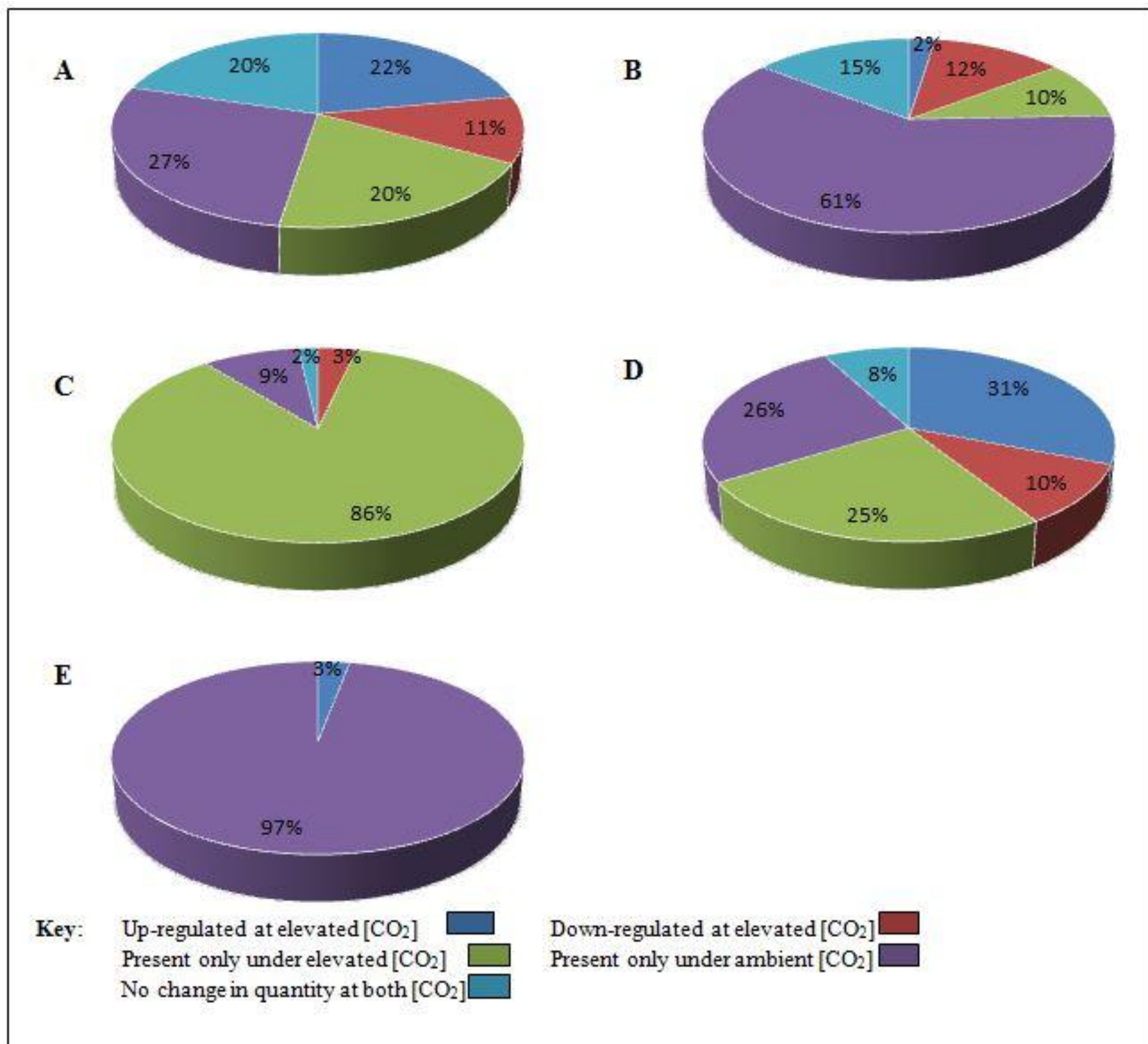


Fig. 3.8 Summary of results obtained from 2-DE analysis of "Tug Dn-Ambient CO_2 concentration-Uninfested" vs. "Tug Dn-Elevated CO_2 concentration-Uninfested". Where: [A] - DAI=1; [B] - DAI=5; [C] - DAI=7; [D] - DAI=9; [E] - DAI=14.

3.3.6 Effect of CO_2 on the Resistance Mechanism

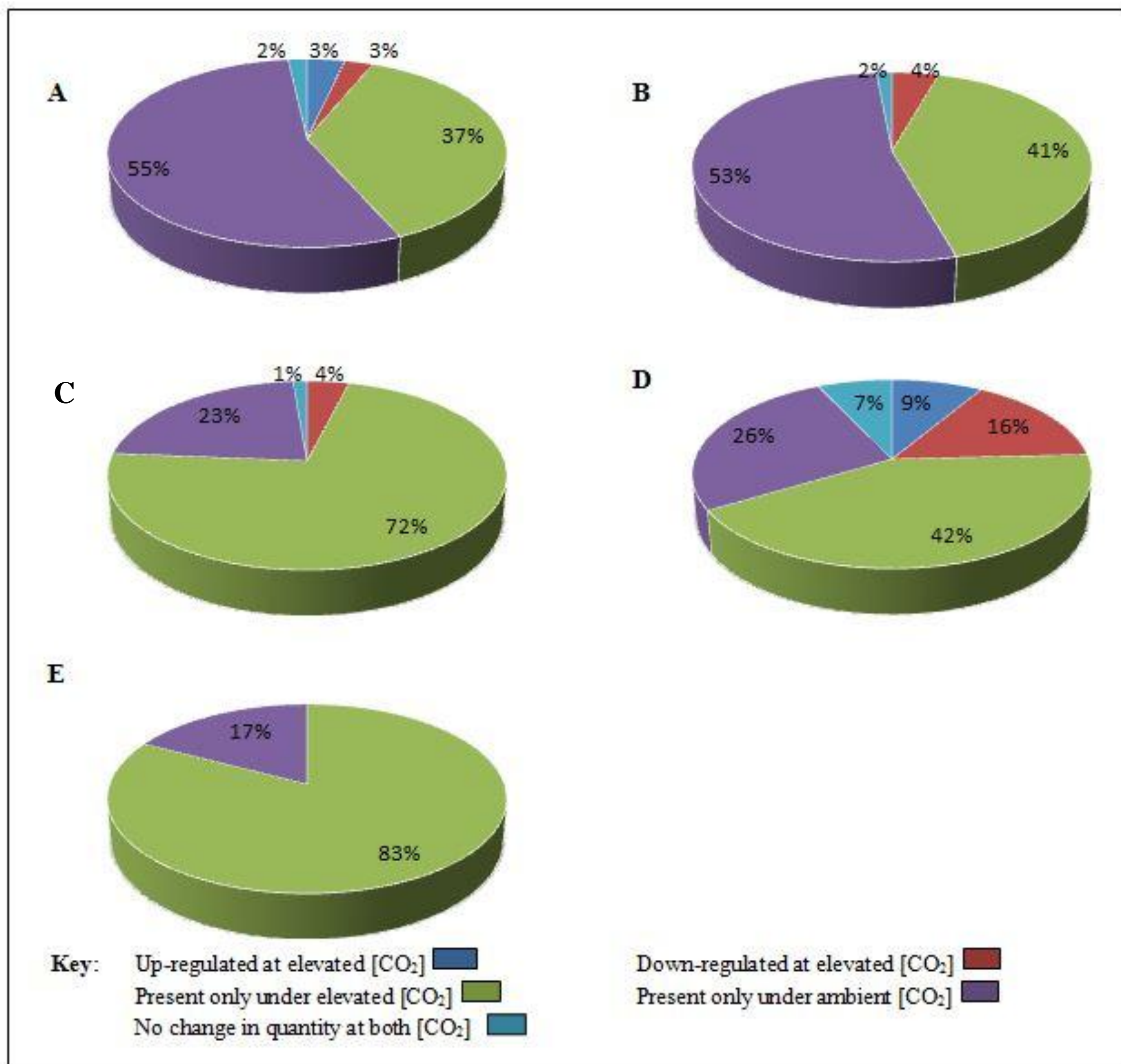


Fig. 3.9 Summary of results obtained from 2-DE analysis of "Tug Dn-Ambient CO_2 concentration-Infested" vs. "Tug Dn-Elevated CO_2 concentration - Infested". Where: [A] - DAI=1; [B] - DAI=5; [C] - DAI=7; [D] - DAI=9; [E] - DAI=14.

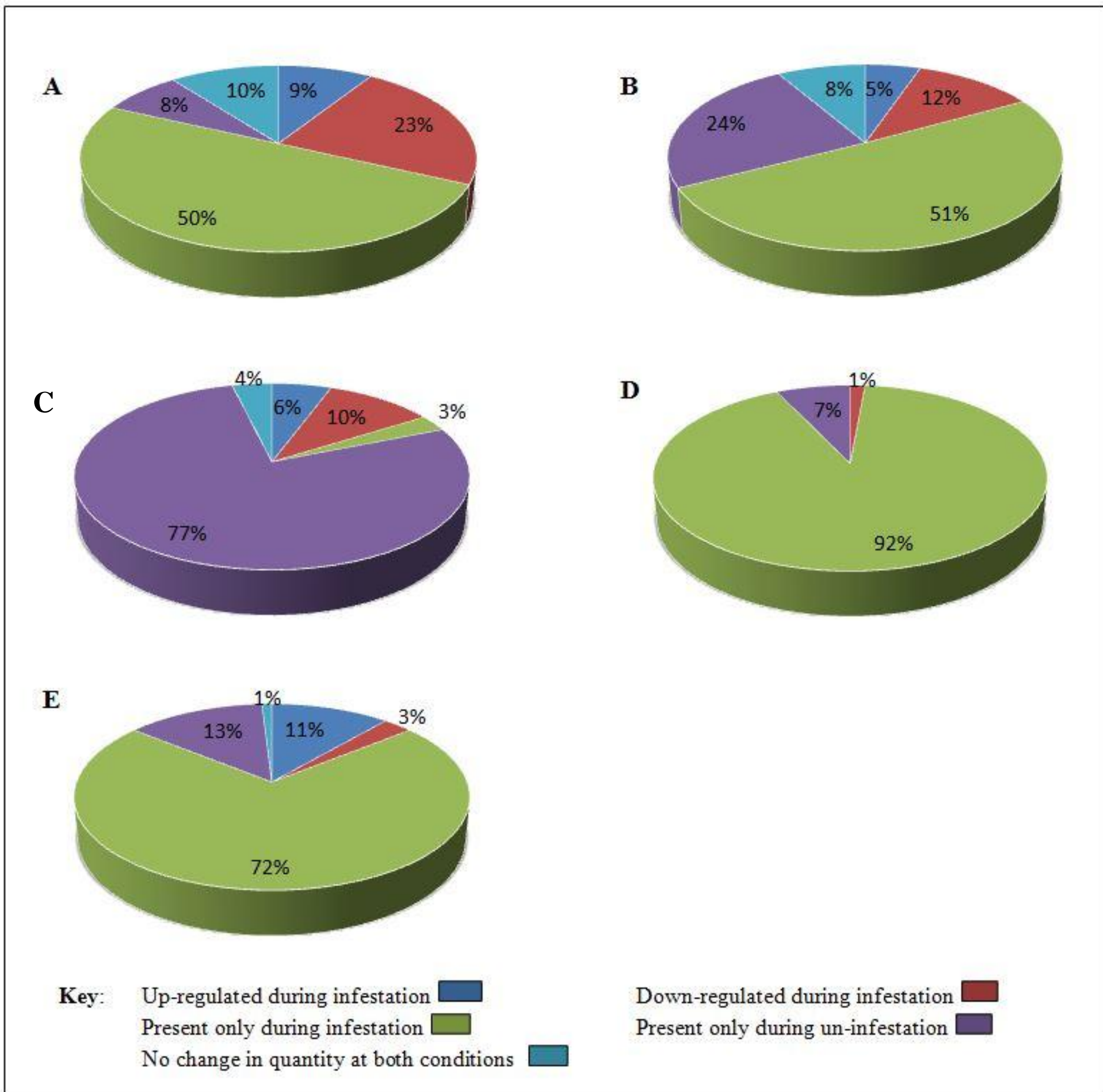


Fig. 3.10 Summary of results obtained from 2-DE analysis of "Tug Dn-Elevated CO_2 concentration-Uninfested" vs. "Tug Dn-Elevated CO_2 concentration - Infested". Where: [A] - DAI=1; [B] - DAI=5; [C] - DAI=7; [D] - DAI=9; [E] - DAI=14.

3.4 Discussion

3.4.1 Sample Protein Concentrations

The ReadyPrep^{TM} Protein Extraction Kit (Bio-Rad, U.S.A) contains agents that allow for extraction of maximum total cellular protein extracts from a given sample making it useful for 2-DE applications. The manufacturer instructs that 2-3 ml of buffer must be added to every 1 g of plant tissue for maximum protein extraction. Due to plant biomass constraints as young wheat plants do not yield large leaf masses, smaller leaf masses were used although the ratio of 3 ml of buffer for every 1g of leaf material was maintained throughout. Protease inhibitors were not required with the extraction kit used.

The protein concentrations ranged from 5.58 mg/ml to 10.83 mg/ml after protein extraction. 2-DE clean-up resulted in "protein loss" with concentrations ranging between 1.0 mg/ml to 3.3 mg/ml. The perceived loss of protein can however be accounted for as a result of the removal of substances that can interfere with reagents of the protein quantitation assays, thereby giving false high readings before the clean-up stage. This was further confirmed during the sample preparation optimization study, where the perceived protein loss was consistent, indicating that the clean-up stage consistently removed the interfering substances.

3.4.2 2-DE Gels for the Tugela Dn Wheat Proteome

Wheat has a large genome size and is about 35 times larger than that of rice. It is difficult to analyse the wheat proteome as the wheat genome project has not been finished (Shan-Shan *et al*., 2012). Sample preparation is very crucial for the success of 2-DE. Sample preparation and IEF were optimised to obtain gels with maximum, highly resolved protein spots with minimum streaking. Each IPG strip for IEF was loaded with 185µl of 250 µg protein. The choice of stain is also very crucial to visualize a maximum number of spots on 2-DE gels. SYPRO® Ruby Protein Gel Stain was desirable as it is easier to use and highly sensitive fluorescent stain and rapidly detects proteins on gels. Although cheaper, also easier to use and commonly used, Coomassie® Brilliant Blue Stain is not comparably sensitive as Sypro Ruby. There are also possibilities of over-staining as well as de-staining protein spots when using Coomassie.

The gel pictures indicate that the leaf proteins were successfully isolated as can be seen by the presence of protein spots. It can be generalised that negatively charged proteins (at the positive end) were mostly resolved on the gels. Although a protein molecular weight marker was not used, it can however be generalized that most of the proteins resolved in the relatively low molecular weight region from approximately 25kDa to 55kDa. This is in line with the reviews of 2-DE analysis by Mak *et al.* (2006) that stated that relatively few high molecular proteins (greater than 100 kDa) are present in wheat. The SwissProt database also shows the majority of wheat proteins to be smaller than 50kDa (Louw, 2007). In all gels, there was an outstanding band that was large in size and highly fluoresced. It occupies the same position in the gels and is thought to be RuBisCo (ribulose bisphosphate carboxylase oxygenase), the most prominent protein in plants. This is also in line with the findings of Louw (2007) in whose gels were a very prominent band occurring at approximately 55 kDa and was thought to be one highly abundant protein.

The distribution of most gel spots were similar in most 2-DE images indicating that the protein content in wheat leaves are similar as was suggested by Zhenhu and Jiangyan (2013). As can be observed from the gel pictures (Fig 3.3; 3.4 and appendix), there was a high similarity of protein profiles between the gels. This is consistent with the observations made by Shan-Shan and colleagues (2012), during the proteomic analysis of drought-responsive proteins in the grain development stage of two wheat varieties. This does not necessarily mean that the expression patterns were the same as shall be seen later. Also in line with observations by Zhenhu and Jiangyan (2013), only a few proteins existed at the extreme pH regions of 3-4 or 9-10, the majority of the spots were located within the narrow pH range of 5-7.

3.4.3 2-DE gels analysis using PDQuest^{TM} Basic Software (Version 8.0)

The selection of the faint, small and largest spots on the gel during the setting up phase of the analysis (Fig 3.5- A), established the input parameters to be used by the PDQuest^{TM} software. These were used to remove background particles that resemble spots from the gel and thereby enhance the detection of the actual protein spots. A master gel image was created by the PDQuest^{TM} software which is a virtual copy of all of the gels in the experiment and contains all the spots detected from all the gels in the specific experiment (see Fig 3.5-B). Therefore,

all the identified spots on all gels are included in the master gel. Each gel in the experiment was then compared to the relevant master gel.

Analysis on how well the spots on each individual gel match the total number of identified spots in the entire experiment was determined (i.e. Match Rate 2) and results given as an Experiment Summary (Fig 3.6) and a Quantity Graph Report (Fig 3.7). A spot that occurred in every gel in the analysis set can also be quantified (through the density of the spots) and then classified as either up- or down- regulated. The parameters for this study were set to identify only those spots that had a two-fold difference in the protein quantity. The software also identified spots that were present on one gel but missing on the other. A spot that occurred in every gel in the analysis set at approximately the same intensity was identified as being neither up- nor down- regulated (Figures 3.8, 3.9 and 3.10).

3.4.4 The Effect of CO2 concentration on the Proteome

It was necessary to determine the profile of the differentially expressed proteins during growth under elevated CO_2 concentration and to establish the changes due to CO_2 changes only and not aphid infestation. Fig 3.8 is a summary of results obtained from 2-DE analysis of the samples: "Tugela Dn-Ambient CO_2 concentration-Uninfested" vs. "Tugela Dn-Elevated CO_2 concentration-Uninfested" at 1, 5, 7, 9 and 14 days, which provided the baseline proteome information, for the effect of elevated CO_2 for uninfested plants.

The results of the analysis revealed that when Tugela Dn was grown under different CO_2 concentration, the expression levels of proteins were differentially regulated on each sampling day. The greatest differential protein expression was observed on days 5 and 7 (DAI=5 and 7) where 61% of the proteins were expressed only in the plants grown at ambient CO_2 levels (DAI=5) and 86% of the proteins expressed were found only in the plants exposed to elevated CO_2 levels (DAI=7). This could be a result of the plant trying to adjust to the new conditions in the presence of elevated CO_2 concentration. At day 1 (DAI=1), 22% of the proteins were up-regulated under elevated CO_2 conditions, 20% found only in plants grown at elevated CO_2, 20% found only in plants grown at ambient CO_2 and 20% undergoing no changes. This is very similar to the results obtained on day 9, indicating that at this stage the plants had adapted to the elevated CO_2 levels. The results obtained at day 14 cannot be

explained and appear to be more of an anomaly than a true reflection of the state of the proteome after 14 days hence must be investigated further. The proteins that are up-regulated only in the plants at ambient CO_2 levels were observed in the molecular weight range of approximately 25kDa to 55kDa are mainly within the basic to neutral pH range.

3.4.5 The Effect of CO_2 concentration on the Resistance Mechanisms

Aphids are seriously plant-damaging pests and the methods in which it can be controlled has been of major interest (Tolmay and Maŕe, 2000; Jankielsohn, 2014). In addition, the changes in climate also have an effect on the plant-aphid interaction. Not much is known about plant responses induced by aphid attacks especially under elevated CO_2 concentration making it crucial for further studies to be done. Previous studies have shown that aphid infestation may induce metabolic changes of the same nature as defense reactions (Karin *et al.,* 2000), for example the production of PR-proteins and proteinase inhibitors (Casaretto and Corcuera, 1998).

According to Van der Westhuizen and Pretorius (1996), RWA feeding induces the build up of specific proteins such as chitinase and some related to PR proteins in the intercellular fluids of resistant wheat cultivars. Van der Westhuizen *et al.* (1998b) report that a resistance response was induced by RWA infestation on susceptible and resistant wheat. It rapidly induced the activities of the enzymes peroxidase and chitinase. Immunologic studies confirmed that the stimulation of peroxidase was a result of up- regulated proteins.

This analysis was done to determine the nature of the differentially expressed proteins due to the change in the resistance mechanism under elevated CO_2 concentration. Fig 3.9 is a summary of the results obtained from 2-DE analysis of the samples: "Tugela Dn-Ambient CO_2 concentration-Infested" vs. "Tugela Dn-Elevated CO_2 concentration-Infested" at 1, 5, 7, 9 and 14 days, which provided the baseline proteome information, for the effect of elevated CO_2 for infested plants. The infested plants under ambient CO_2 gives the normal resistance response of the plant to infestation. The infested plants under elevated CO_2 gives the changes in the normal resistance response as a result of the elevated CO_2. This then identifies the aphid resistance proteins that are changed.

Like the summary on Fig 3.8, the results on Fig 3.9 (infested plants under elevated and ambient conditions) also show that the stress conditions gave rise to differentially regulated proteins within the wheat proteome. The greatest differential protein expression was observed on days 7 and 14 (DAI=7 and 14) where 72% (DAI=7) and 83% (DAI=14) of the proteins expressed were found only in the plants exposed to elevated CO_2 levels. At day 1 (DAI=1), 55% of the proteins were found only in the plants exposed to ambient CO_2 conditions. At day 2 (DAI=2), 41% of the proteins were found only in plants grown at elevated CO_2 and 53% found only in plants grown at ambient CO_2. Because most of the proteins are seen only under elevated CO_2 levels, analysis of these proteins will elucidate on the changes that are occurring in the resistance mechanism. These results indicate that there has been changes in the resistance due to elevated CO_2 because of the evident changes in the proteome.

As was established from the earlier results (Section 3.4.4), aphid infestation and altered CO_2 concentration induces changes in the proteome. Hence, comparing plants grown under identical conditions with the only difference in infestation gives results attributable to the changes in the proteome induced by the resistance mechanism. The summary on Fig 3.10 the analysis results when infested plants grown under elevated CO_2 are compared to uninfested plants under the same conditions. The uninfested plants under elevated CO_2 levels give the baseline proteome information of the plants normal state under high CO_2 levels. The infested plants under the same CO_2 levels give the plant response to infestation under elevated CO_2. This identifies aphid stress response proteins.

From the summary, the plant is adapting to the elevated CO_2 and infestation during day 1 (DAI=1) and 5 (DAI=5). Most proteins were found only in the infested plants at elevated CO_2 i.e. 50% at day 1 and 51% at day 5. After day 5, there seems to be a massive response where you see proteins only appearing under the infested plants this show a change in the resistance mechanism. At day 9 (DAI=9), 92% of the proteins were found only in the infested plants. The changes in the protein expressions after aphid-infestation and altered CO_2 concentration could be due these defence-related events as documented by Louw (2007) where up-regulation was due to putative storage proteins, proteins involved in photosynthesis, heat shock proteins and defense proteins. Of course, the pI value and molecular mass of the proteins and the identification of the proteins in these spots, must be determined in future work to specifically identify whether these results are valid for these conditions.

4 CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

Food security is of major importance due to the increasing world population with 8.9 billion people expected by 2050 (Cohen, 2050). Cereal proteins are the basis of human diet in the world. The three major cereals that serve as a staple food for humans are wheat, rice and maize (Botha, 2013). For this reason, there has been an increased interest by researchers to study their proteomic composition to enhance growth, development and yield capacity. Plants cannot escape from abiotic and biotic stresses but have to rely on proteomic modifications and sophisticated mechanisms in response to stress conditions for endurance and adaptation (Abu *et al*., 2010, Ahuja *et al*., 2010). Plant stress responses have led to changes in the structure at different levels namely the transcriptome, proteome and metabolome levels (Klára *et al.*, 2013) which can be qualitatively and quantitatively scrutinized. Proteins have important roles in plant stress response as they are directly involved in both structural and metabolic changes (Klára *et al.*, 2013). Because the proteome is not a static entity, comparative proteomic studies of plants before and after specific stresses have become a potent tool for understanding biochemical pathways and intricate responses (Abu *et al*., 2010).

Aphids and in particular the *Diuraphis noxia* (RWA), have caused aggravating, massive losses to farmers in many areas of the world. If unchecked, RWA are able to destroy 60-80% (reference) of a crop. Its economic impact from 1987-1993 was estimated to be approximately $800 million in the United States alone (Botha, 2013). The insect feeds from the phloem sieve elements of wheat plants damaging cell membranes resulting in impaired functioning of cells (Botha *et al*., 2005). Many methods of control have been tried including the use of pesticides which have had limitations due to the fact that RWA causes leaf rolling which protects the aphids. The most competent and efficient method of control is the use of resistant cultivars. The Small Grains institute in Bethlehem, South Africa, have therefore developed resistant cultivars to the known RWA subtypes over the past decades through intensive breeding programmes (Tolmay *et al*., 2006).

Climate change has however become a major factor threatening food security especially with the observed increase from less than 300 ppm in pre-industrial period to the current 385 ppm and is predicted to reach 550 ppm by 2050 (IPCC, 2007; Meehl *et al.*, 2007). Despite the great emphasis on stimulated photosynthesis that enhances growth and yield in C_3 plants which is an advantage of elevated CO_2, it is therefore essential to understand how this increase will affect wheat growth and resistance to pests such as the RWA (Van Zyl, 2007).

Elevated CO_2 concentration may affect individual species of a community. In the current investigation, the population growth studies showed that populations of RWA SA1 increased substantially on the two wheat cultivars at both CO_2 concentration. Although the population growth rate for RWA SA1 on all cultivars was generally exponential at all treatments, growth at elevated CO_2 concentration was noticeably faster with populations doubling 3 times in 14 days as compared to the 2 times at ambient CO_2 concentration. This suggests that both cultivars provided a better quality host for RWA SA1 at 450 ppm than 385 ppm. As expected, the susceptible cultivar (Sch) was more vulnerable to infestation at both CO_2 concentration due to the higher populations as a result of heavy infestation throughout the experiment period (14 days).

The results of this study are in line with Jimor's (2011) work where the populations of RWA SA1and RWA SA2 increased significantly on four barley lines (STARS- 9301B, STARS - 9577B, STARS -0502B and PUMA) at elevated CO_2 concentration (450 ppm) compared to ambient CO_2 concentration (380 ppm). In another study by Chen *et al*. (2004), aphid (*S. avenae*) population also increased with elevated atmospheric CO_2 concentration on spring wheat. They suggested that the increased plant productivity at elevated CO_2 concentration could compensate for the aphid damage acting as a natural control mechanism. In another recently published work, elevated CO_2 concentration (750 ppm) increased the abundance of the peach aphid *M. persicae* on four isogenic *A. thaliana* genotypes (a wild type and three SA-deficient mutants) (Sun *et al*., 2013).

The results of the virulence studies show that RWA SA1 inflicted severe and extensive chlorosis and leaf-roll on the susceptible cultivar at elevated CO_2 concentration in contrast to the resistant cultivar which seemed to tolerate aphid infestation much better than the susceptible cultivar. RWA SA1 also became more virulent under elevated CO_2 concentration

on both wheat cultivars as evidenced by larger chlorotic spots as compared to those on plants under ambient CO_2 concentration. It can be concluded that there is a more rapid damage to wheat under elevated CO_2 concentration.

Although the aphid population on both cultivars generally increased at elevated CO_2 concentration, the statistical analysis shows that on day 14, there was no significant difference between RWA SA1 population on Tugela Dn and Scheepers at elevated CO_2 concentration. It is evident that RWA SA1 was able to overcome the resistance mechanism in Tugela Dn and reproduced just as it did on the susceptible cultivar. Because there was a significant difference in populations for Tugela Dn and Scheepers under ambient CO_2 concentration at day 14, it means elevated CO_2 concentration resulted in a change in the resistance mechanism to RWA SA1. This contrasts the earlier findings by Jimor (2011) where the populations of the two aphids RWA SA1 and RWA SA2 on resistant barley lines where significantly different to those on susceptible barley lines at both CO_2 concentration. This shows that elevated CO_2 concentration did not change the resistance of the barley lines which is why he concluded that the use of these lines had a potential to decrease the expected negative effects of aphid infestation under this CO_2 concentration.

The physical damage to wheat plants caused by RWA feeding was confirmed in this study. RWA SA1 feeding on susceptible wheat plants under elevated CO_2 concentration resulted in more noticeable signs of chlorosis and leaf roll within seven days of phloem-feeding and maintained the highest level of aphid-virulence susceptibility in the 21 days. All plants exhibited extensive signs of chlorosis and leaf-roll after 21 days of aphid feeding. To sustain growth under aphid infestation, the plant has to recognize the attack and initiate a defensive response (Botha *et al*., 2005). Failure to do so under harsh infestations results in increased stress which triggers premature plant aging (senescence) that eventually leads to death (Botha *et al.,* 2014). This may be the reason why the susceptible cultivar deteriorated quicker resulting in plant deaths during the 21 days of infestation. It must be noted that at elevated CO2 concentration, the resistant cultivar however showed similar vulnerability and most plants were at risk of not recovering after infestation showing some level of change to the resistance of Tugela Dn at these conditions.

The resistance mechanism is a complex response and still needs to be resolved. According to Botha *et al.* (2014), it is proposed that the resistance against *D. noxia* conferred by the Dn

genes (currently eleven) works in a gene-for-gene manner similar to pathogen resistance genes. A defence response occurs when encoded proteins recognize aphid-specific effectors that activate signalling cascades as the onset of defence responses (Botha *et al*., 2005). Most gene-for-gene interactions are characterised by the hypersensitive response (HR) as part of the overall defense response (Jones and Dangl, 1996; Moloi and van der Westhuizen, 2006) which is localised, programmed cell and tissue death that occurs as a response to RWA infestation.

An effective defence response is mediated by an array of events (Moloi and van der Westhuizen, 2006). These include the oxidative burst of reactive oxygen species (ROS) such as HO^-, O_2^- and H_2O_2 (Moloi and van der Westhuizen, 2006); cell modifications due to callose deposition (Matsiliza, 2003; Saheed *et al*., 2007) and synthesis of signal molecules SA, JA and ET (Kunkel and Brookes, 2002; Mohase and van der Westhuizen, 2002; Thompson and Goggin, 2006). Much research interest has been on the downstream defence responses in aphid-wheat interactions to try and understand the resistance mechanism. Van der Westhuizen *et al*. (1998a), reports of an apoplastic rapid increase in activity of the oxidative enzymes; peroxidase and chitinase, due to aphid infestation. Infestation induced more activity in resistant as compared to susceptible wheat indicating that these two enzymes are part of the resistance response. In similar studies of the protein composition of wheat apoplastic fluid, RWA infestation also induced increase of pathogenesis-related (PR) proteins in resistant wheat cultivars such as chitinases and intercellular β-1,3- glucanases (Van der Westhuizen and Pretorius, 1996; Van der Westhuizen *et al.,* 1998b). β-1,3- glucanases is associated with the systemic acquired resistance hence ensures sustained long-term resistance (Botha *et al*., 2014).

Deposition of callose and sealing off of sieve elements interferes with aphid feeding. The induction of callose deposition is usually activated by conserved PAMPs (Gomez-Gomez and Boller, 2002). Some aphid species such as RWA have been shown to induce rapid callose formation (De Wet and Botha, 2007). It has been suggested that callose deposition differs according to aphid species (Saheed, 2007). The increase in callose deposition blocks sieve plates and plasmodesmata-pore units which results in a marked decrease and eventually termination of assimilate transport via the phloem and this is fatal to the plant. RWA infestation induced callose deposition which led to a reduced rate of transport in the phloem (Botha and Matsiliza, 2004). Callose is degraded and/or regulated by the β-1,3-glucanase

which have been reported by Van der Westhuizen *et al.* (2002) to have an increased accumulation in susceptible wheat cultivars than resistant cultivars.

The results of the current investigation show low levels callose in controls (uninfested plants) but heavy callose in infested plants. Hence, it can be elucidated that RWA SA1 infestation induced callose formation. This is in line with earlier findings by Matsiliza (2003) whereby *D. noxia* infestation caused heavy callose deposition not only in the phloem but also in neighbouring vascular parenchyma cell. Aphid feeding also induced more callose formation in susceptible wheat as compared to the resistant cultivar (De Wet and Botha, 2007). From the results of the structural studies of this research, it can be concluded that once a plant is damaged, wound callose is rapidly produced and deposited mainly in sieve elements irrespective of the aphid population or duration of infestation.

Although there was not a clear trend in the amounts of callose deposited at the different conditions, it has been shown that there was more callose deposition in the susceptible cultivar as compared to its resistant counterpart. Because higher levels of β-1,3-glucanase have been found to accumulate in resistant cultivars (Van der Westhuizen *et al.*, 2002; De wet and Botha, 2007), it would be expected that wound callose disappears over the course of time in these plants. The results show that in contrast, wound callose actually increased with time in the resistant cultivar under elevated CO_2 concentration which supports the results of the populations studies at day 14 i.e. the aphids overcame the resistance conferred by the *Dn*1 gene in Tugela Dn. Because it has been concluded that differences in callose deposition is probably caused by differences in the induction of β-1,3-glucanase between plants (Saheed *et al.,* 2008), further studies will be needed to confirm this suggestion.

Despite the increased interest in the field, the molecular basis of plant-aphid interactions remains poorly understood (Botha *et al*., 2006). Genes and proteins involved in the defense mechanism have been reported to be expressed as a result of aphid feeding (Moran *et al*., 2002; Van der Westhuizen and Pretorius, 1996). Therefore, a proteomics approach was used as a pilot study to investigate whether it would be possible to identify the changes to these various resistance mechanisms during aphid infestation and elevated CO_2 levels. The analysis of the control group i.e. uninfested Tugela Dn at ambient versus elevated CO_2 concentration gave the baseline of the effect of CO_2 concentration on the proteome of the resistant cultivar. The major changes occurred in the early events (day 1-7). This may have been a result of

mechanisms to try and adjust to the new conditions in the presence of elevated CO_2 concentration.

It has been reported that elevated CO_2 results in instant higher photosynthetic rates and C:N ratios as well as changes in expression levels of SA-dependant defense genes (Lindroth 1995; Hughes and Bazzaz, 2001; Sun *et al.*, 2013). Because most of these changes are directly regulated by proteins, it is expected that the most differential protein expression will occur immediately after the atmospheric changes (early events) as was shown in the study. Most changes were observed in the molecular weight range of approximately 25 kDa to 55 kDa are mainly within the basic to neutral pH range. This is a good range considering that most wheat proteins have been reported to be of lower molecular weights (less 100kDa) (Mak *et al.*, 2006; Louw, 2007).

Infested plants under elevated and ambient conditions showed that the stress conditions gave rise to differentially regulated proteins within the wheat proteome. Most changes occurred elevated CO_2 levels. These results indicate that there were changes in the resistance due to elevated CO_2 because of the evident changes in the proteome. It has been reported that resistant wheat cultivars undergo an initial HR-type response and a systemic acquired resistance type response (SAR) over time in response to RWA phloem feeding but that susceptible cultivars do not (Botha *et al.*, 2006). It can be suggested that the changes occurring are a result of differential regulation of plant defence proteins which fall in this range (25kDa-80kDa) such as peroxidases, chitinases and β-1.3-glucanases as well as protein kinases, heat-shock proteins and photosynthetic proteins.

If so, then the results will be similar to those documented by Louw (2007) where up-regulation was due to putative storage proteins, proteins involved in photosynthesis, heat shock proteins and defense proteins. Of course, the pI value and molecular mass of the proteins hence the name of each protein, must be determined in future work to specifically identify whether these results are valid for these conditions. However, Louw (2007) also reports that the susceptible Betta wheat cultivar, displayed a defence response similar to the HR but was unable to up-regulate specific defensive proteins against RWA infestation but proteins for broad resistance. Although the changes in the proteins in infested Tugela Dn under elevated CO_2 concentration were not accurately identified, the defense mechanism is

similar to that portrayed by the susceptible Betta wheat cultivar which shows that the resistance mechanism had been overcome.

Because the present study was a pilot study to develop, tryout and establish an experimental procedure that can be used for further analysis in the future to minimize costs, errors and time; many things need to be considered. Due to limited funding available, it was not possible to do multiple gels hence no statistics to evaluate whether the differences in protein quantities were significant. Also, protein spots on 2D gels were not identified hence could not make clear visualisations of the resistance mechanism but mere assumptions that are not backed by factual results. In further studies, it is suggested to repeat the population and virulence studies at least twice to obtain detailed, statistically significant results.

Examination of the formation and distribution of callose in response to aphid feeding can be made more detailed by also incorporating Transmission Electron Microscopy (TEM). Transmission electron micrographs give a detailed and clear image with regards to the extent of the actual damage on functional vascular tissue as a result of aphid feeding (Matsiliza and Botha, 2003; Matsiliza, 2003; Saheed, 2007; Jimor, 2011). Wound callose can also be quantitatively analysed to automatically measure the area it covers in relation to aphid feeding using phase analysis as described by Jimor (2011). Depending on time and resources, the experiment can be enhanced further by assessing the effects on transport in the phloem following the movement of the phloem mobile fluorophore, 5,6-carboxyfluoresein (5,6-CFDA) in a method described by Matsiliza (2003).

The outlined 2-DE method explained in this investigation must be improved in future studies to obtain improved results and to confirm the current ones. To begin with, the 2-DE method for the determination of differentially expressed proteins in wheat leaf tissue induced by aphid feeding must be optimised. Total protein extraction must be optimised using different masses of leaf tissue to determine the optimal amount for the maximum protein to be obtained. The most accurate method of protein quantity must be identified by comparing the accurateness of a test using standards of known concentration. To improve the resolution of spots, different IPG strips can be employed. One can start with a broad range IPG strip to get an outline of the distribution of proteins and makes it easier to make out the specific narrow range strip to use for an in-depth overview (Görg *et al.*, 2000). This then leads to the optimisation of the IEF conditions and the suitable stain to use.

Once the 2-DE method has been optimised to obtain improved spot resolutions, the next essential step is the identification and mapping of proteins separated by 2-DE. There is need for a protein molecular weight marker to identify the actual sizes of the resolved proteins. A mass spectrometer can be employed to analyse and identify proteins using suitable proteases in-gel digestion. Protein analysis software such as PDQuest^{TM} can also be used to analyse proteins that are differentially expressed. Protein databases such as SwissProt database can be used to identify the proteins after analysis. Optimization of the 2-DE method in conjunction with a perfect method of identifying the differentially regulated proteins will provide more reliable explanations concerning the resistance mechanism. Methods such as real time PCR, DNA microarray analysis and Northern and Western blots can be employed to confirm the connection of the identified proteins in the resistance mechanism.

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6 APPENDIX 1: RESULTS AND REAGANTS FOR CHAPTER TWO

Table 5.1 Population growth of RWA SA1 on Tugela Dn at elevated CO2 concentration (450 ppm)

Days After Infesting	Replication Number										Mean	Standard Deviation
	1	2	3	4	5	6	7	8	9	10		
2	23	10	11	20	12	13	9	19	19	18	**15.4**	**4.9**
4	43	19	17	47	28	18	16	55	56	39	**33.8**	**16.1**
6	65	34	36	58	38	35	32	68	63	54	**48.3**	**14.6**
8	93	48	43	87	55	45	50	82	87	92	**68.2**	**21.5**
10	131	79	62	108	73	77	86	98	106	123	**94.3**	**22.6**
12	189	93	87	152	122	105	120	118	146	162	**129.4**	**32.3**
14	249	152	114	204	148	127	142	140	188	215	**167.9**	**43.7**

Table 5.2 Population Growth of RWA SA1 on Scheepers under elevated CO2 concentration (450 ppm)

Days After Infesting	Replication Number										Mean	Standard Deviation
	1	2	3	4	5	6	7	8	9	10		
2	21	19	19	21	33	19	29	35	23	18	**23.7**	**6.3**
4	48	30	25	49	65	32	62	68	46	28	**45.3**	**16.1**
6	51	38	36	65	71	50	72	74	81	49	**58.7**	**15.9**
8	80	52	58	98	132	76	110	137	91	70	**90.4**	**29.4**
10	123	79	96	154	145	156	148	188	132	93	**131.4**	**33.9**
12	147	118	122	208	187	232	192	226	156	124	**171.2**	**43.5**
14	180	135	156	269	214	304	254	332	182	154	**218**	**68.2**

Table 5.3 Population growth of RWA SA1 on Tugela Dn at ambient CO2 concentration (385 ppm)

Days After Infesting	Replication Number										Mean	Standard Deviation
	1	2	3	4	5	6	7	8	9	10		
2	15	8	11	9	7	7	6	7	14	9	**9.3**	**3.1**
4	20	11	15	14	12	9	10	13	17	12	**13.3**	**3.3**
6	20	20	24	15	17	28	23	26	22	21	**21.6**	**3.9**
8	33	25	27	20	21	45	30	38	28	30	**29.7**	**7.6**
10	35	27	55	18	24	47	42	46	36	40	**37**	**11.4**
12	38	32	83	26	30	60	53	65	46	52	**48.5**	**17.8**
14	49	46	115	38	42	81	71	116	66	76	**70**	**28.1**

Table 5.4 Population Growth of RWA SA1 on Scheepers under ambient CO2 concentration (385 ppm)

Days After Infesting	Replication Number										Mean	Standard Deviation
	1	2	3	4	5	6	7	8	9	10		
2	8	10	13	7	10	12	8	9	13	11	10.1	2.1
4	28	14	13	12	15	16	10	13	14	13	14.8	4.9
6	32	43	15	20	19	20	18	38	24	16	24.5	9.8
8	42	84	25	28	30	34	38	50	35	32	39.8	17.1
10	57	93	26	32	35	36	50	72	49	25	47.5	21.8
12	116	122	48	54	52	50	77	142	85	46	79.2	35.7
14	204	208	80	105	89	94	136	281	146	78	142.1	68.3

Table 5.5 Virulence Ratings of RWA SA1 on Tugela Dn under ambient and elevated CO_2 concentration

Replication No.	CO2 concentration ppm	Day 2	Day 7	Day 14	Day 21
1	385	A0;B1	A1;B1	A3;B1	A4;B1
2	385	A0;B1	A1;B1	A1;B1	A5;B1
3	385	A0;B1	A1;B1	A2;B1	A7;B2
4	385	A0;B1	A1;B1	A1;B1	A4;B1
5	385	A0;B1	A1;B1	A3;B1	A4;B2
6	385	A0;B1	A1;B1	A3;B1	A5;B2
7	385	A0;B1	A2;B1	A4;B2	A7;B3
8	385	A0;B1	A1;B1	A4;B2	A5;B2
9	385	A0;B1	A1;B1	A4;B2	A6;B1
10	385	A0;B1	A1;B1	A2;B1	A6;B2
1	450	A0;B1	A2;B2	A4;B2	A8;B2
2	450	A0;B1	A3;B2	A4;B2	A6;B2
3	450	A0;B1	A2;B1	A3;B2	A6;B3
4	450	A0;B1	A1;B2	A3;B2	A5;B2
5	450	A0;B1	A1;B1	A4;B1	A6;B2
6	450	A0;B1	A1;B1	A2;B1	A5;B2
7	450	A0;B1	A1;B1	A2;B1	A4;B1
8	450	A0;B1	A2;B1	A5;B1	A7;B2
9	450	A0;B1	A1;B1	A3;B2	A6;B3
10	450	A0;B1	A4;B2	A6;B2	A8;B3

Table 5.6 Virulence Ratings of RWA SA1 on Scheepers under ambient and elevated CO_2 concentration

Replication No.	CO2 concentration ppm	Day 2	Day 7	Day 14	Day 21
1	385	A0;B1	A1;B1	A4;B2	A9;B3
2	385	A0;B1	A2;B1	A4;B2	A7;B2
3	385	A0;B1	A1;B1	A2;B2	A7;B3
4	385	A0;B1	A1;B1	A3;B2	A7;B3
5	385	A0;B1	A1;B1	A2;B1	A5;B1
6	385	A0;B1	A1;B1	A2;B1	A5;B2
7	385	A0;B1	A1;B1	A3;B2	A6;B2
8	385	A0;B1	A2;B1	A5;B2	A8;B3
9	385	A0;B1	A2;B1	A3;B1	A7;B2

10	385	A0;B1	A1;B1	A2;B1	A5;B1
1	450	A0;B1	A1;B1	A4;B2	A6;B2
2	450	A0;B1	A1;B1	A3;B1	A5;B2
3	450	A0;B1	A1;B1	A3;B2	A5;B2
4	450	A0;B1	A2;B1	A5;B2	A7;B3
5	450	A0;B1	A1;B1	A4;B2	A6;B2
6	450	A0;B1	A1;B1	A5;B2	A7;B2
7	450	A0;B1	A2;B2	A6;B2	A9;B3
8	450	A0;B1	A3;B1	A5;B2	A9;B3
9	450	A0;B1	A2;B2	A4;B2	A8;B3
10	450	A0;B1	A1;B1	A3;B1	A6;B2

Long-Ashton nutrient solution

Table 5.7 The complete Long-Ashton nutrient solution composition as adapted from Hewitt (1966)

SALT	WEIGHT USED (g)	VOLUME OF STOCK SOLUTION (ml)	VOLUME OF STOCK SOLUTION DILUTED IN 25L (ml)	CONCENTRATION IN FINAL VOLUME OF 25L (ml)
Macronutrients				
KNO_3	101	500	25	2
K_2SO_4	43	500	25	1
$Ca(NO_3)_2$	164	500	25	4
$CaCl_2$	111	500	25	4
$MgSO_47H_2O$	92	500	25	1.5
$NaH_2PO_4.2H_2O$	104	500	25	4
Micronutrients				
$MnSO_4.4H_2O$	11.20	500	2.5	0.02
$CuSO_4.5H_20$	1.25	500	2.5	0.002
$ZnSO_4.7H_20$	1.45	500	2.5	0.002
H_3BO_3	15.50	500	2.5	0.05
$NaMO_4.2H_2O$	0.605	500	2.5	0.0005
NaCl	29.30	500	2.5	0.1
Fe-Citrate ($3H_2O$)	29.30	500	2.5	0.6

Ca^{2+} - Free MES buffer solution (per Litre)

MES: 1.952 g/L

Mannitol: 22.775 g/L

KCl: 10 ml stock solution (Stock Solution: 0.373 g/100ml)

$MgCl_2$: 10ml stock solution (Stock Solution: 1.017 g/100ml)

$CaCl_2$: 10ml stock solution (Stock Solution: 0.735 g/100ml)

Li Cl: 12.717 g/100ml (3 Molar).

7 APPENDIX 2: RESULTS AND REAGANTS FOR CHAPTER THREE

Preparation of 1X SDS-PAGE gel running buffer

3.03 g Tris Base

14.40 g Glycine

1.00 g SDS

Dissolve and bring to total volume up to 1 L with deionised water.

Differential protein expression profiles in Tugela Dn under different conditions over a 14-day period

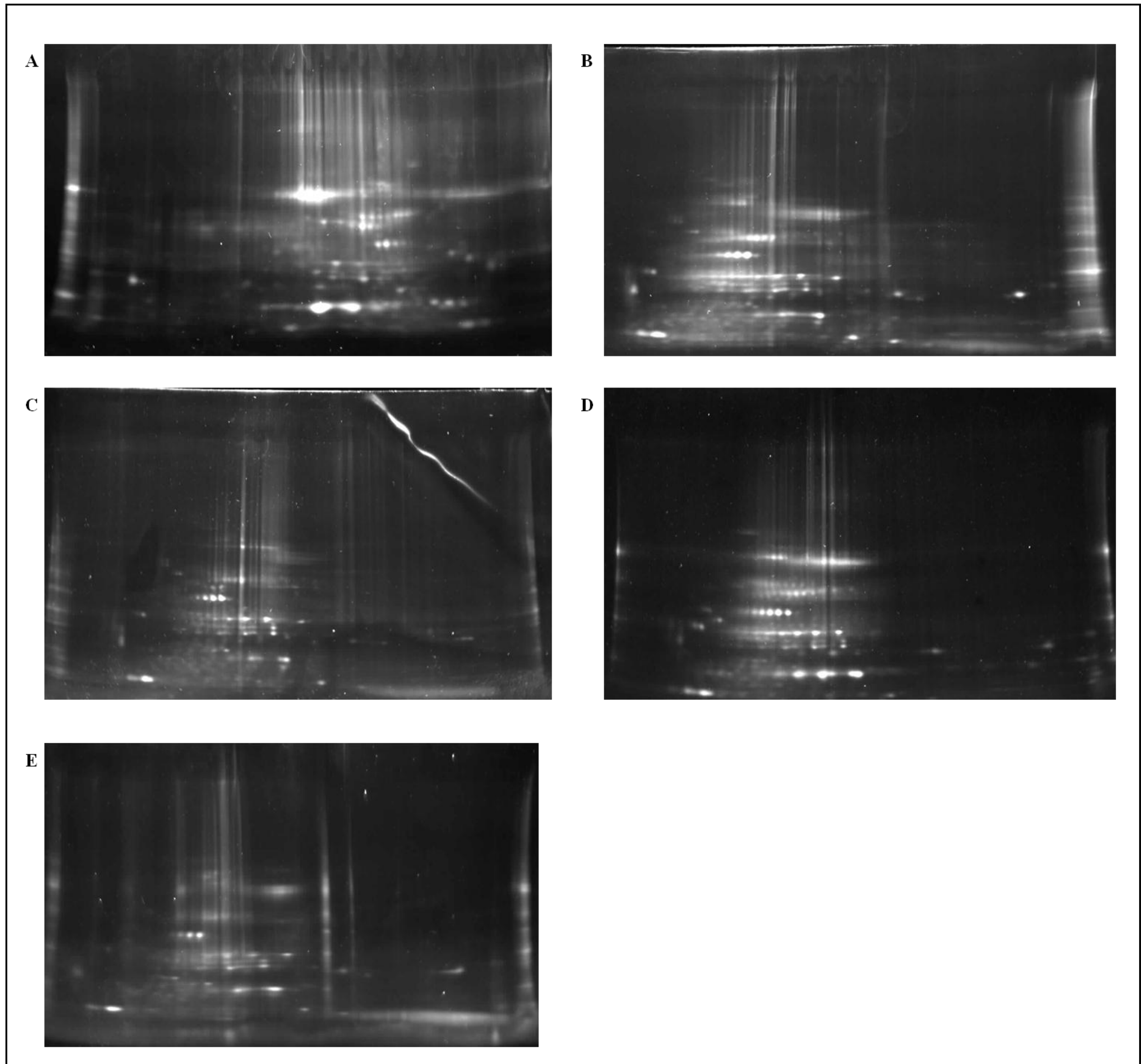

Fig 5.1 Differential protein expression profiles of uninfested Tugela Dn wheat under ambient CO2 concentration (385 ppm). Samples were obtained at the following periods: A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

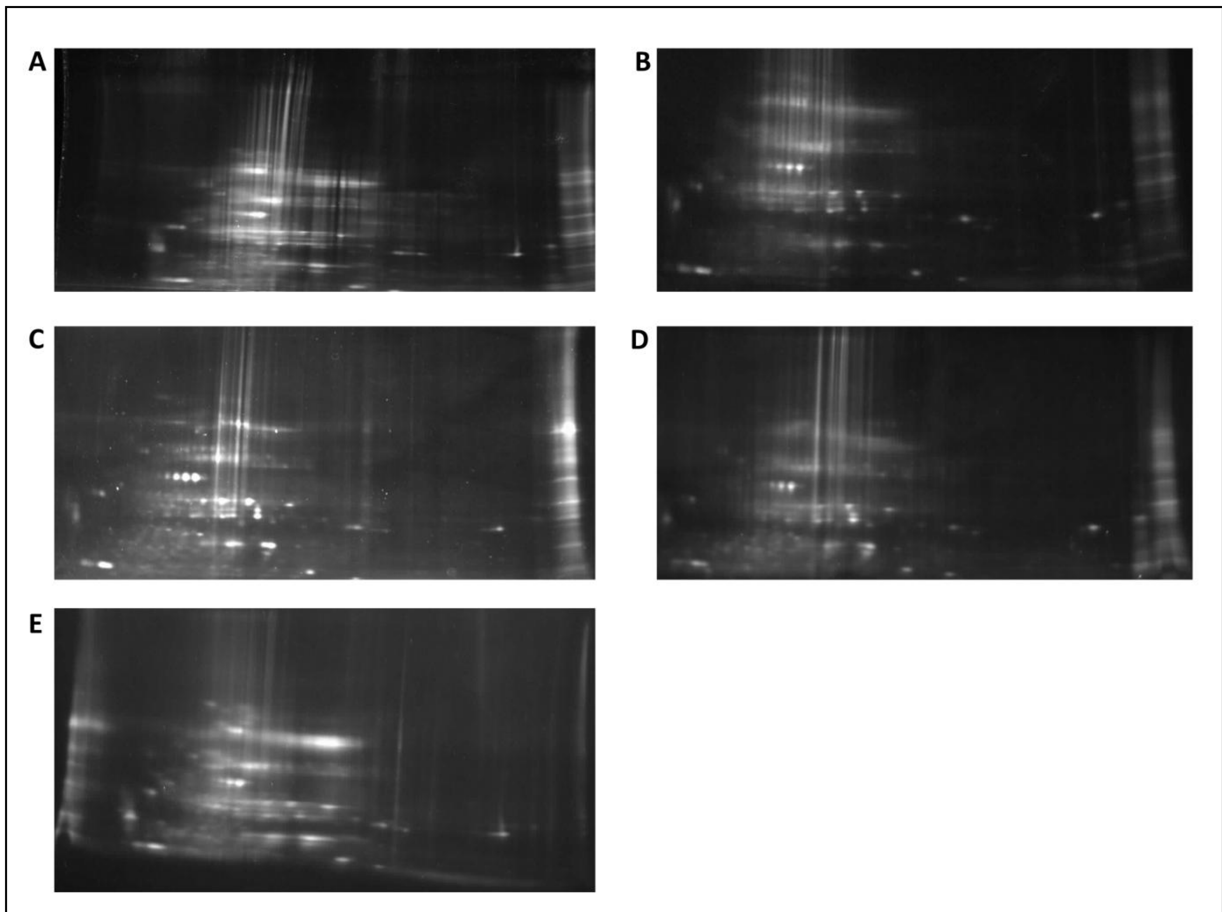

Fig 5.2 Differential protein expression profiles of Tugela Dn wheat in response to RWA SA1 infestation under ambient CO2 concentration (385 ppm). Samples were taken at the following days after infestation A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

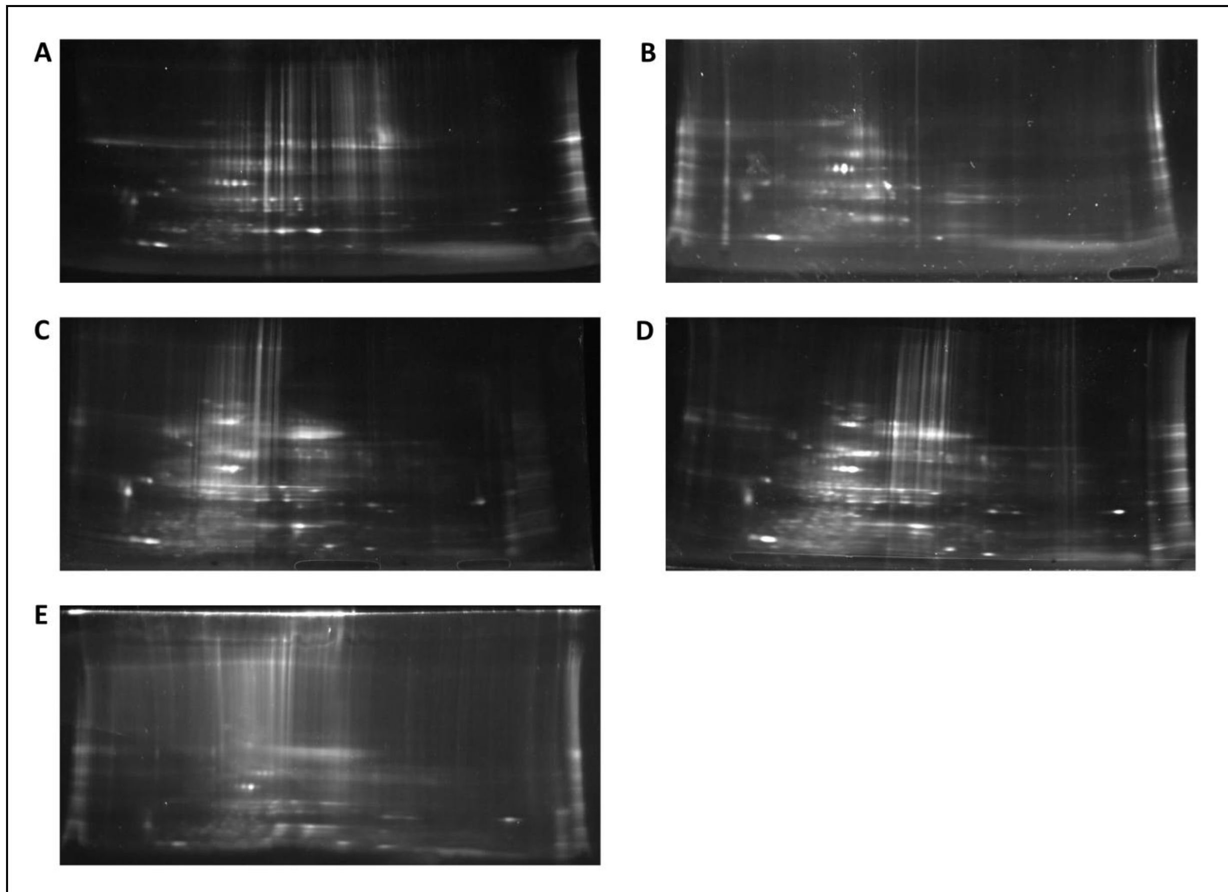

Fig 5.3 Differential protein expression profiles of uninfested Tugela Dn wheat at elevated CO2 concentration (450 ppm). Where: A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

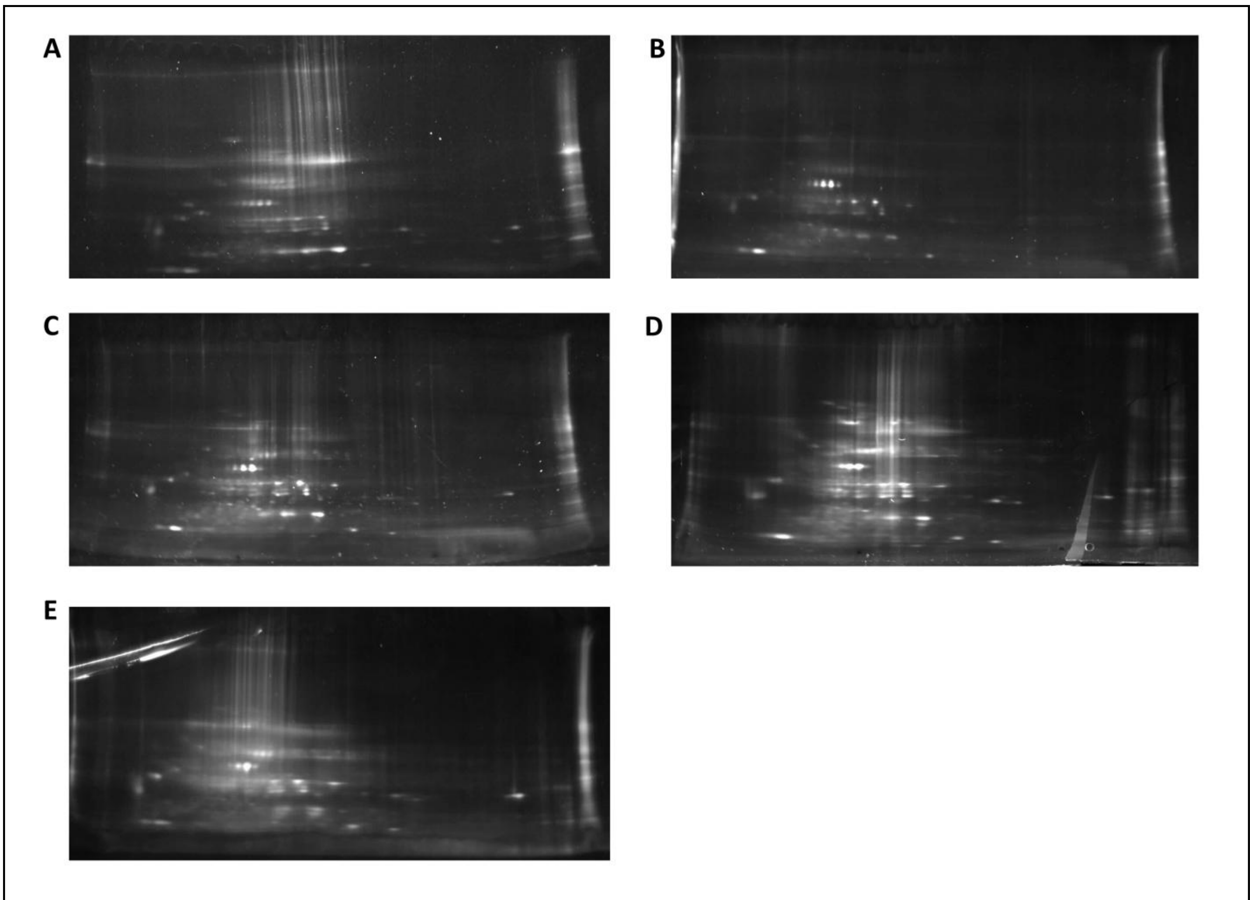

Fig 5.4 Differential protein expression profiles of Tugela Dn in response to RWA SA1 infestation under elevated CO_2 concentration (450 ppm). Samples taken at the following days after infestation: A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

Differential protein expression profiles in Scheepers under different conditions over a 14-day period

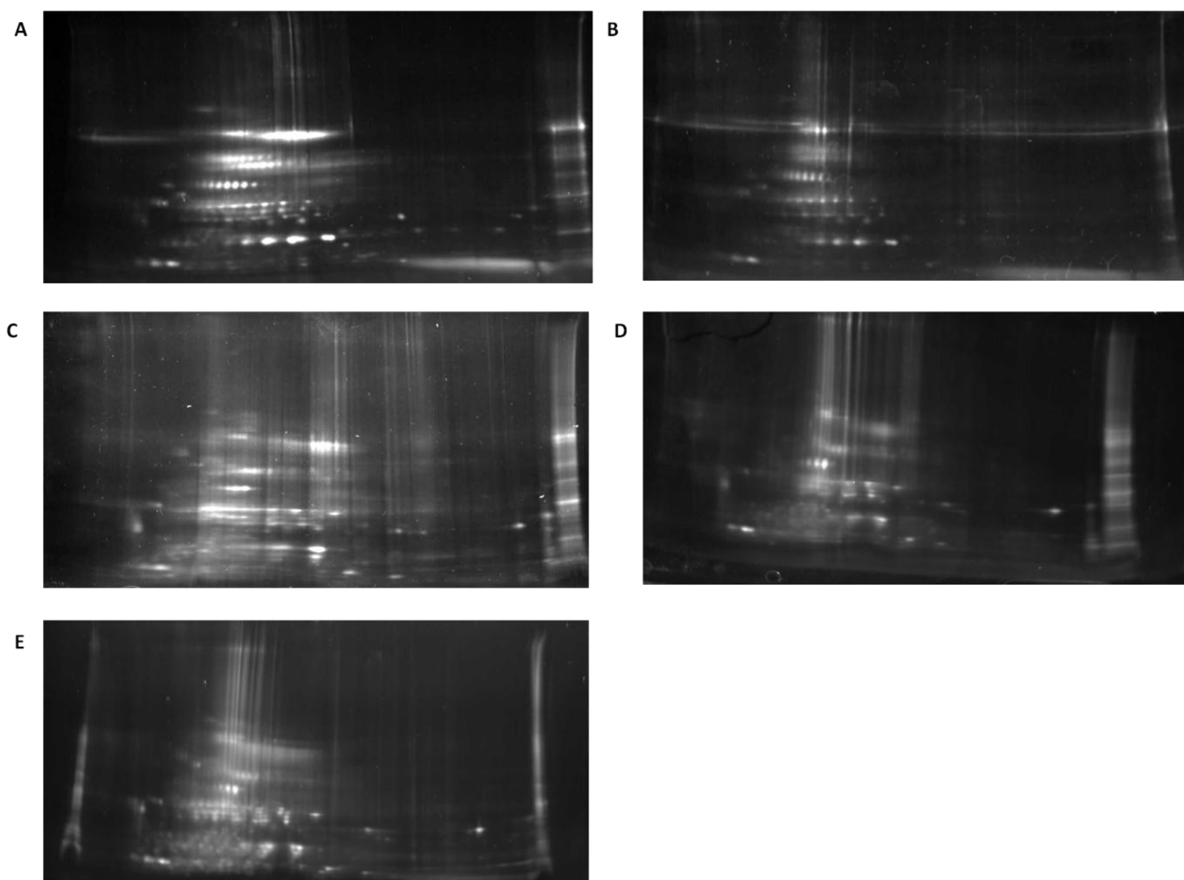

Fig 5.5 Differential protein expression profiles of uninfested Scheepers wheat at ambient CO_2 concentration (385 ppm). Samples were collected on: A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

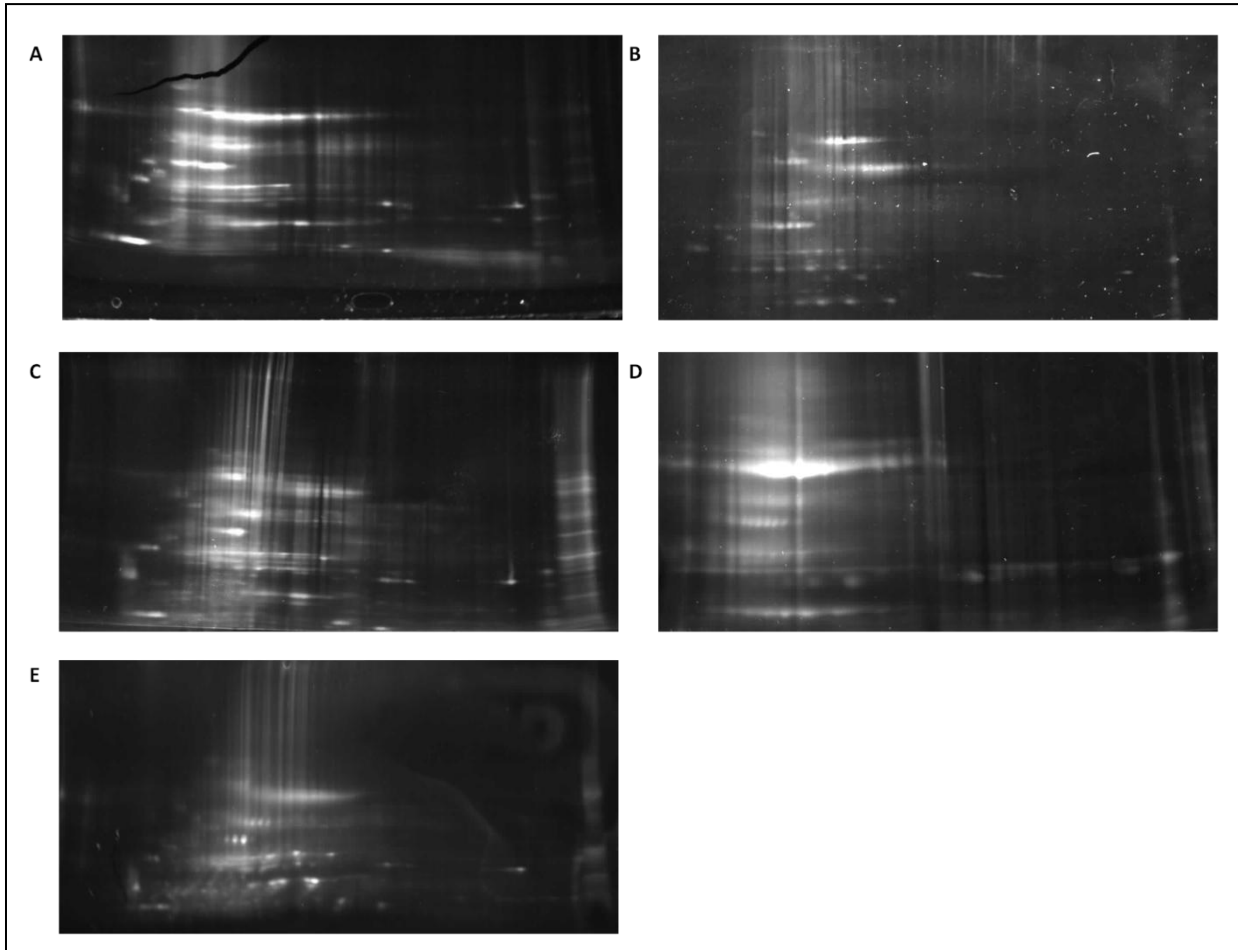

Fig 5.6 Differential protein expression profiles of Scheepers wheat in response to RWA SA1 infestation under ambient CO2 concentration (385 ppm). Samples were taken at the following days after infestation A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

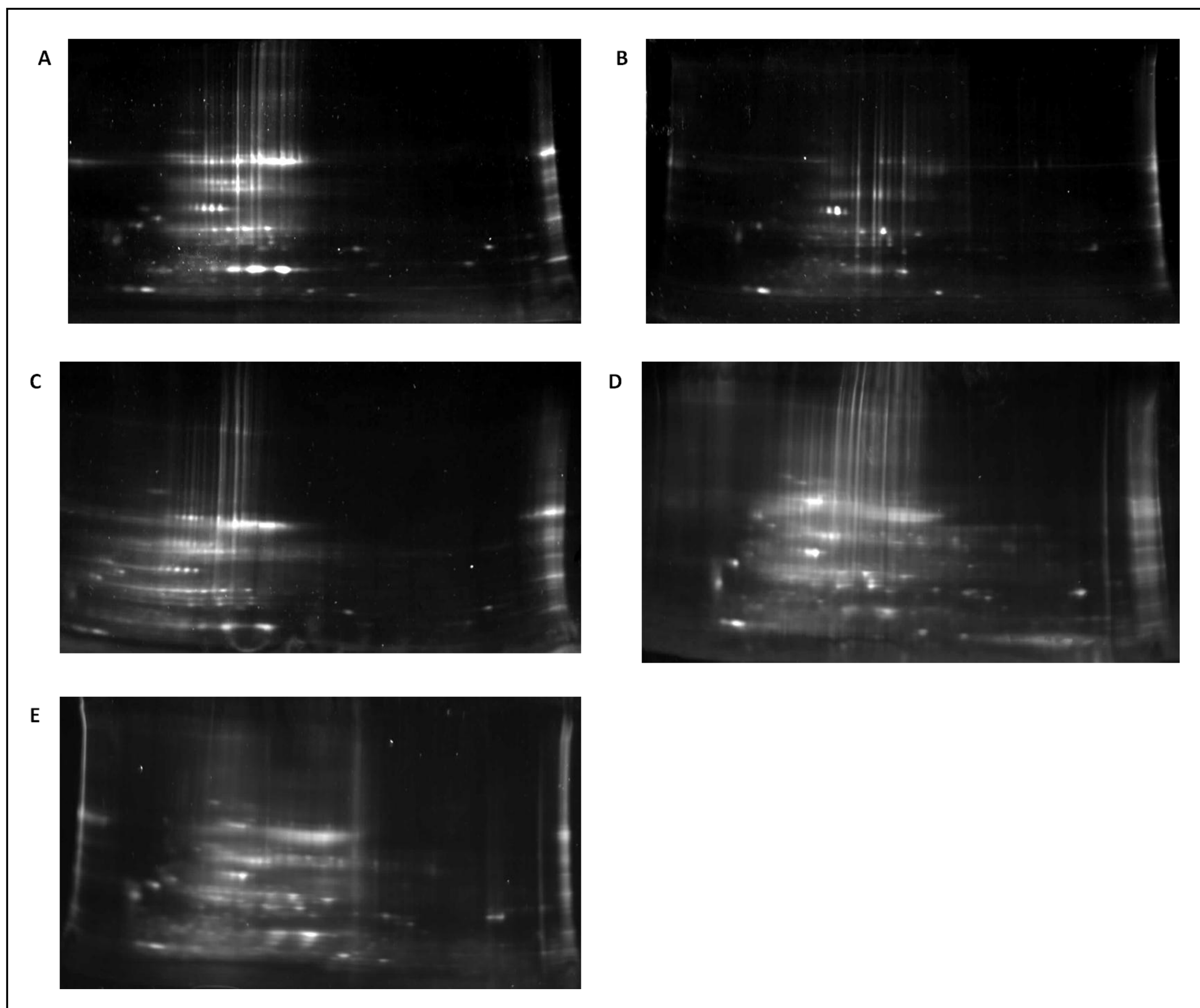

Fig 5.7 Differential protein expression profiles of uninfested Scheepers wheat at elevated CO_2 concentration (450 ppm). Where: A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

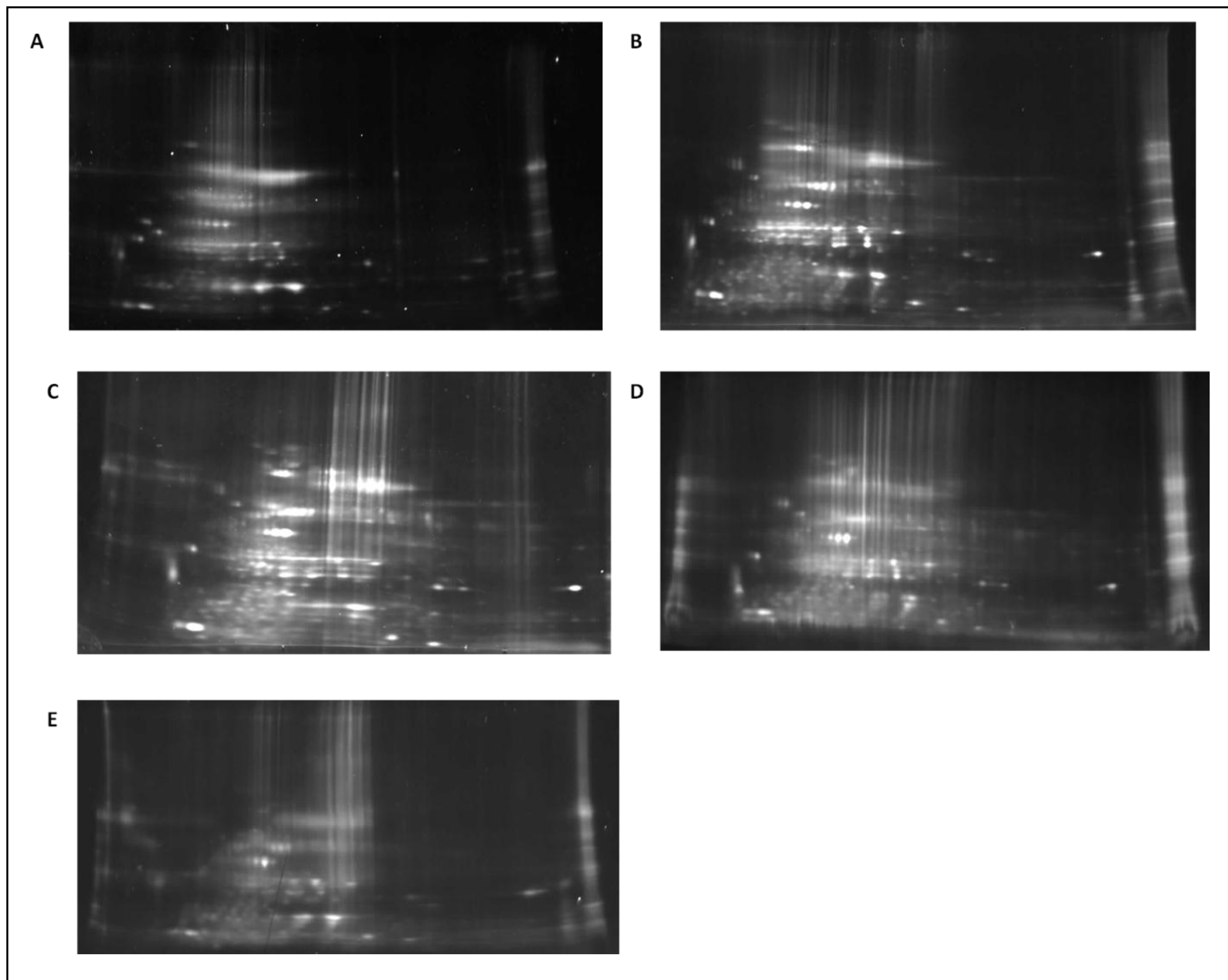

Fig 5.8 Differential protein expression profiles of Scheepers in response to RWA SA1 infestation under elevated CO_2 concentration (450 ppm). Where : A- Day 1 B- Day 5 C- Day 7 D- Day 9 E- Day 14

PDQuest™ Basic Software Version 8.01 analysis results of 2-DE gels

Uninfested Tugela Dn – at Ambient vs. Elevated CO2 concentration:

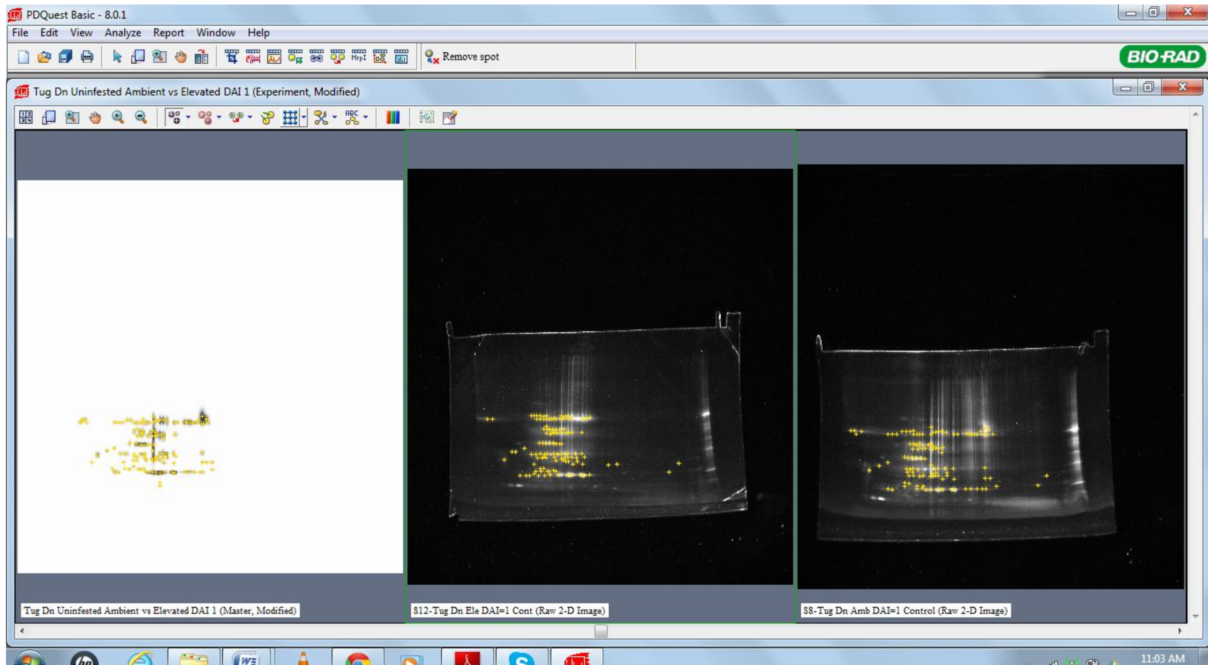

Fig 6.1 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in uninfested Tugela Dn at ambient CO2 concentration vs. uninfested Tugela Dn at elevated CO2 concentration. Day 1 (DAI=1)

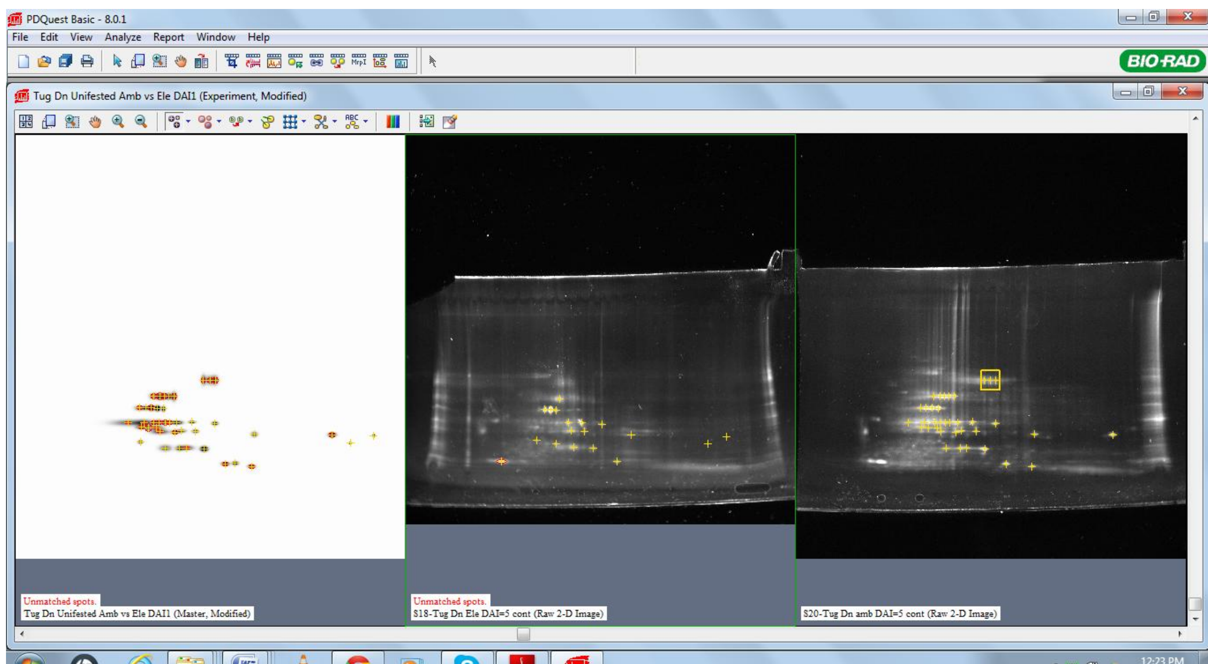

Fig 6.2 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in uninfested Tugela Dn at ambient CO2 concentration vs. uninfested Tugela Dn at elevated CO2 concentration. Day 5 (DAI=5)

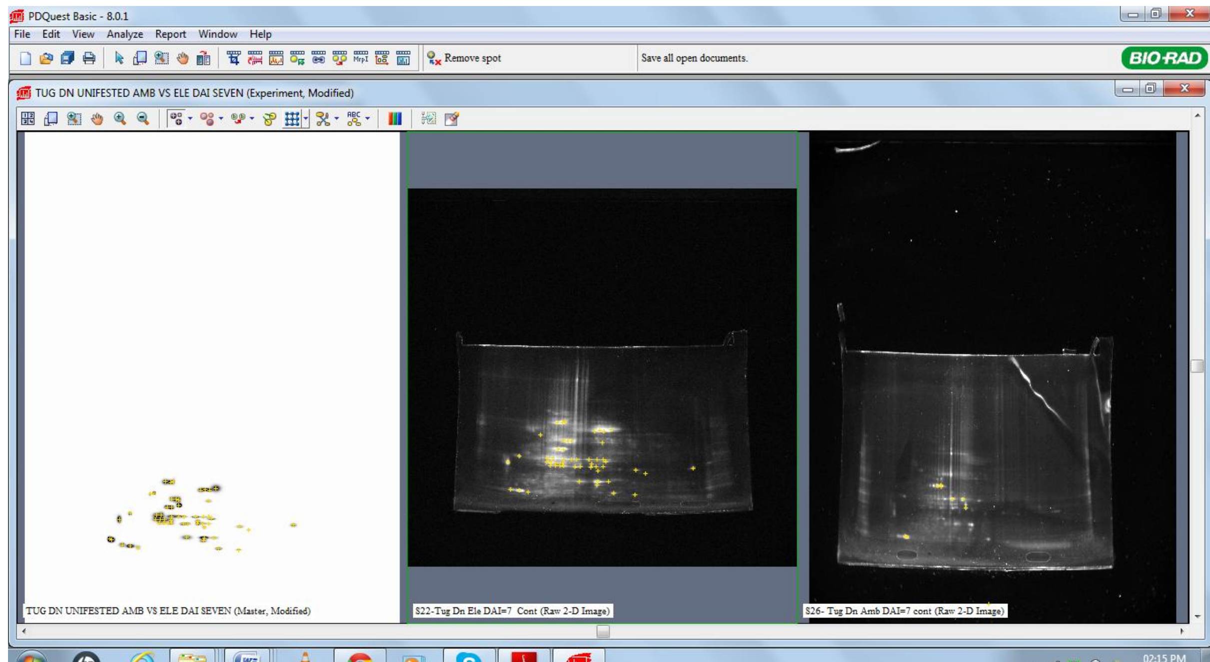

Fig 6.3 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in uninfested Tugela Dn at ambient CO2 concentration vs. uninfested Tugela Dn at elevated CO2 concentration. Day 7 (DAI=7)

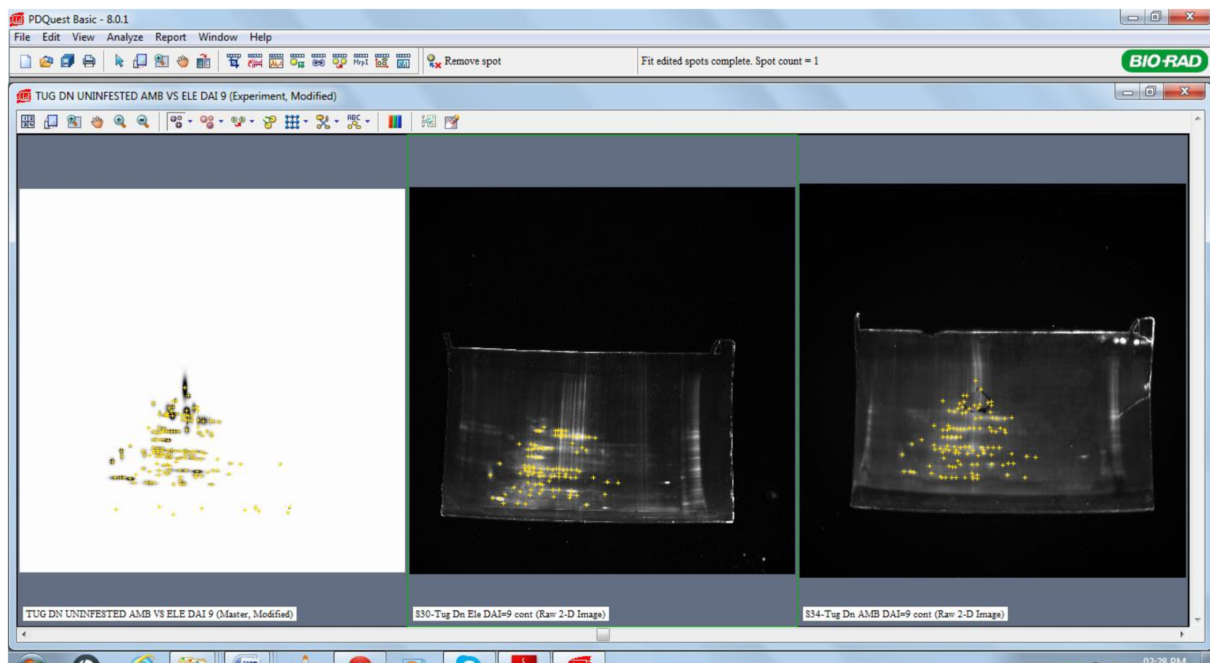

Fig 6.4 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in uninfested Tugela Dn at ambient CO2 concentration vs. uninfested Tugela Dn elevated CO2 concentration. Day 9 (DAI=9)

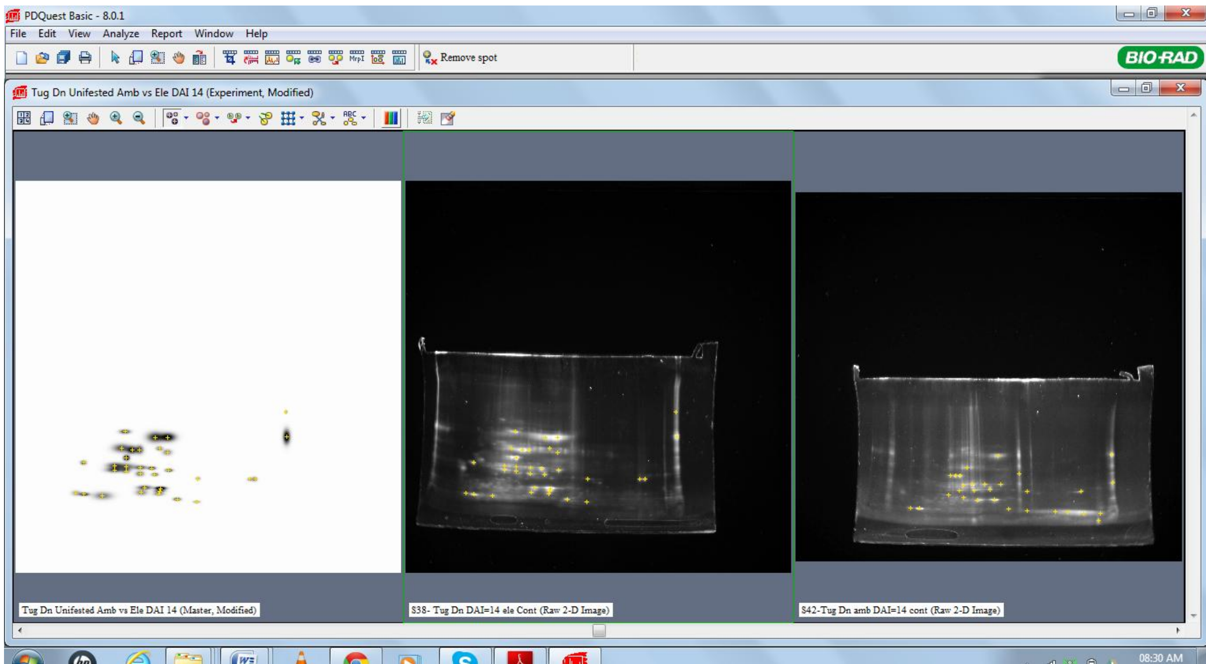

Fig 6.5 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in uninfested Tugela Dn at ambient CO2 concentration vs. uninfested Tugela Dn at elevated CO2 concentration. Day 14 (DAI=14)

RWA SA1 – Infested Tugela Dn: Ambient vs. Elevated CO2 concentration:

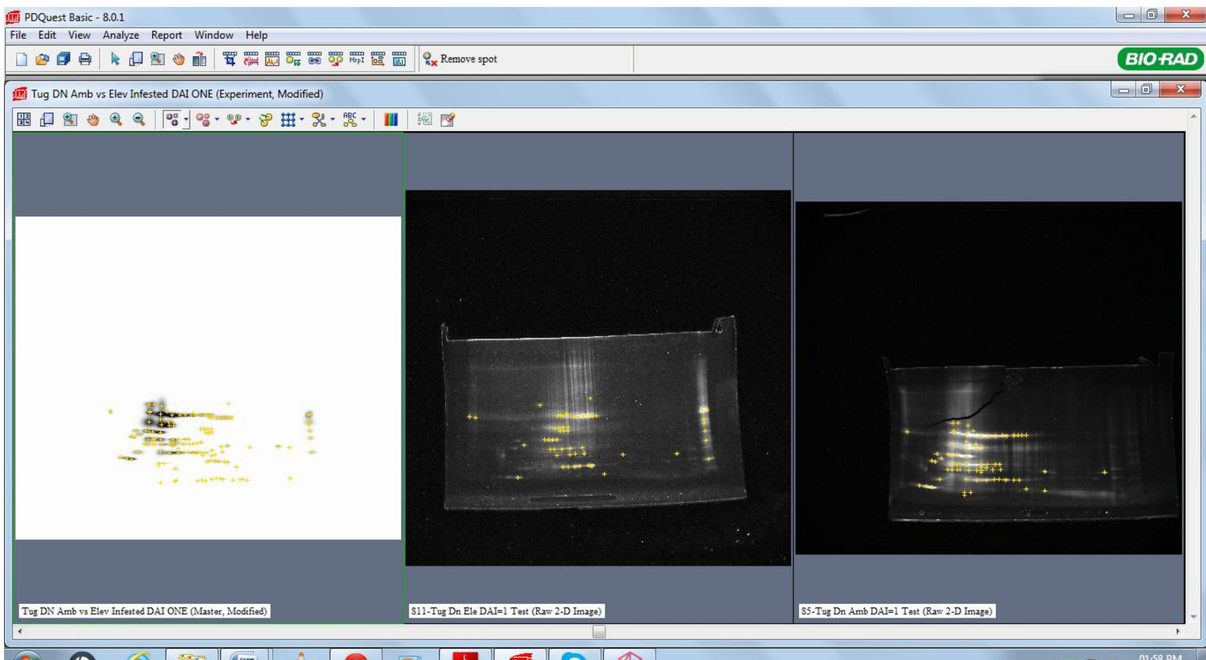

Fig 6.6 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in infested Tugela Dn at ambient CO2 concentration vs. elevated CO2 concentration. Day 1 (DAI=1)

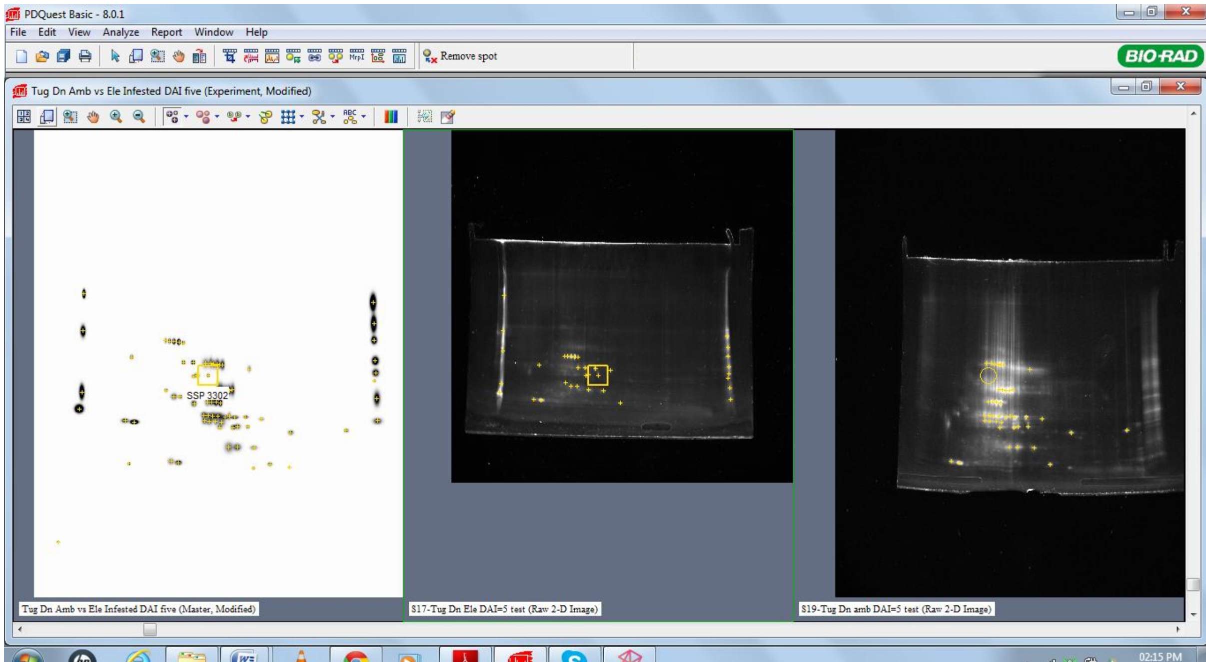

Fig 6.7 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in infested Tugela Dn at ambient CO2 concentration vs. elevated CO2 concentration. Day 5 (DAI=5)

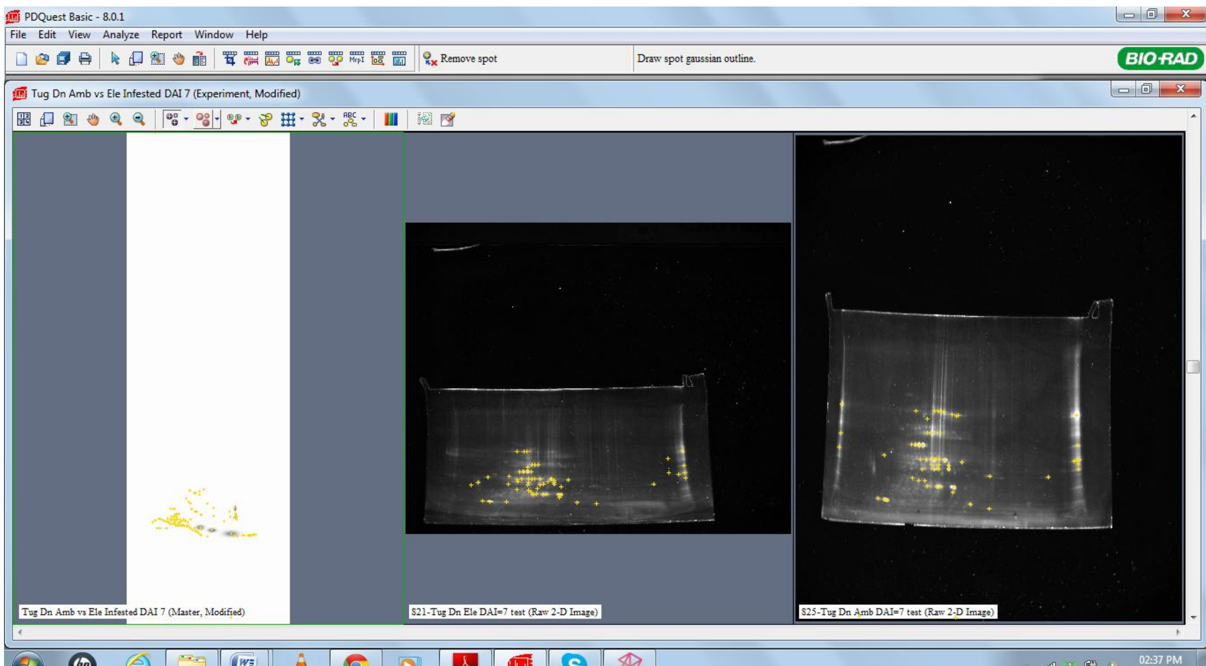

Fig 6.8 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in infested Tugela Dn at ambient CO2 concentration vs. elevated CO2 concentration. Day7 (DAI=7)

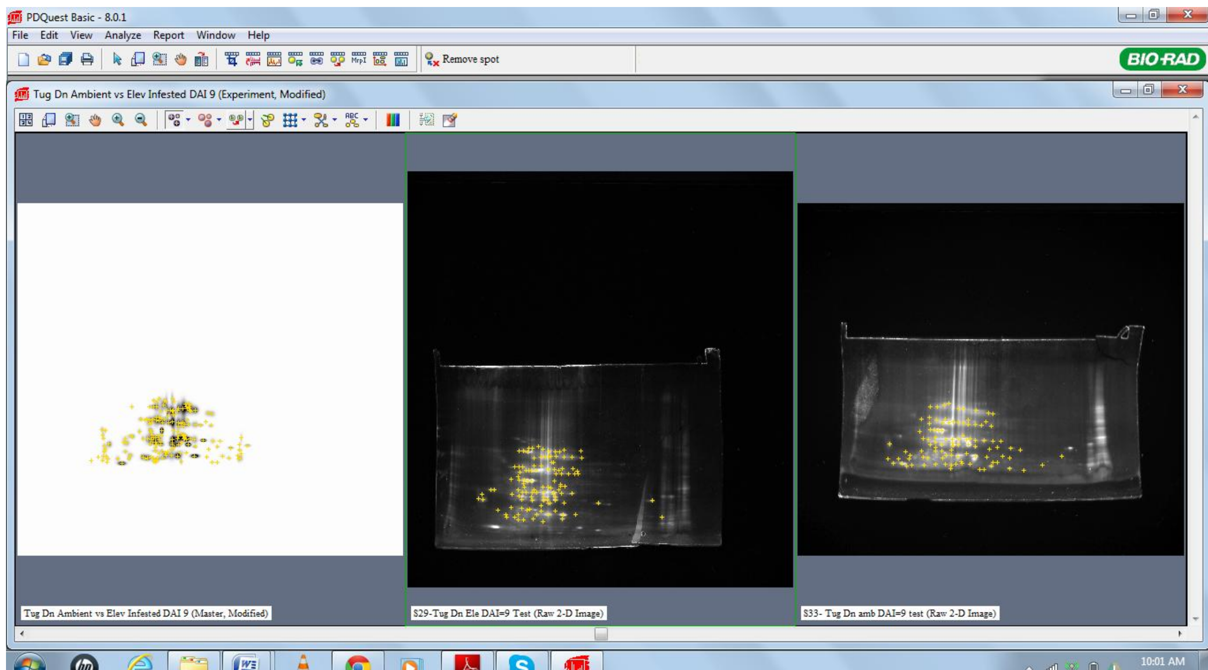

Fig 6.9 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in infested Tugela Dn at ambient CO2 concentration vs. elevated CO2 concentration. Day 9 (DAI=9)

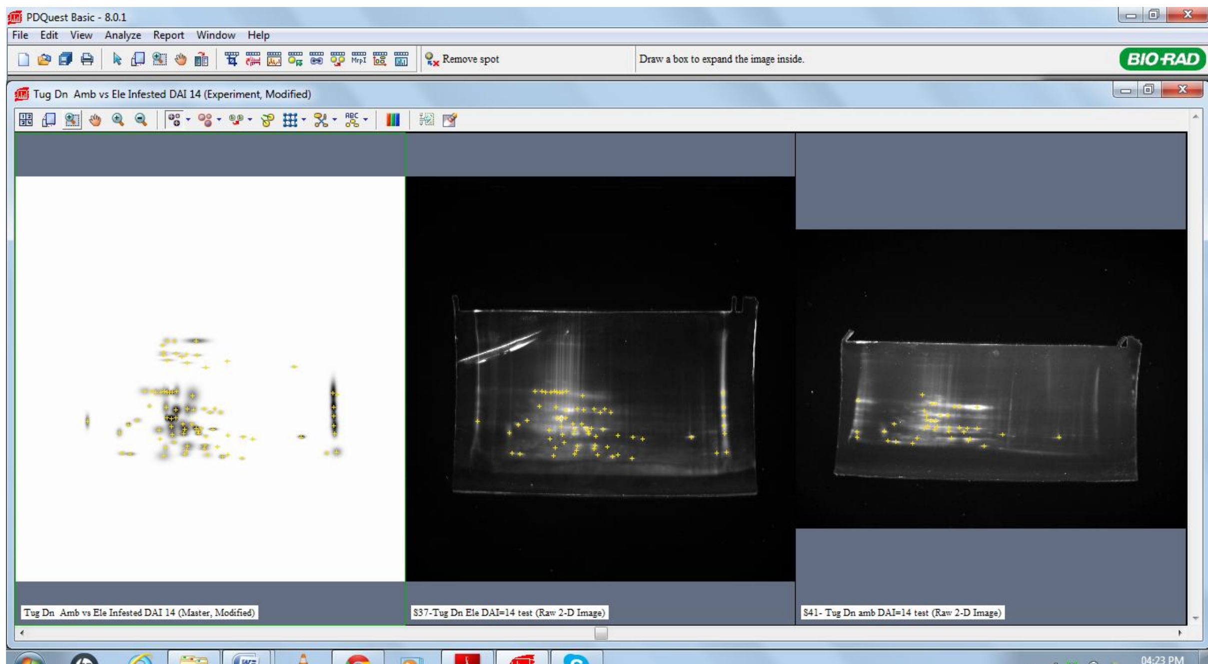

Fig 6.10 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in infested Tugela Dn at ambient CO2 concentration vs. elevated CO2 concentration. Day 14 (DAI=14)

RWA SA1-Infested Tugela Dn vs. Uninfested Tugela Dn at Elevated CO2 concentration:

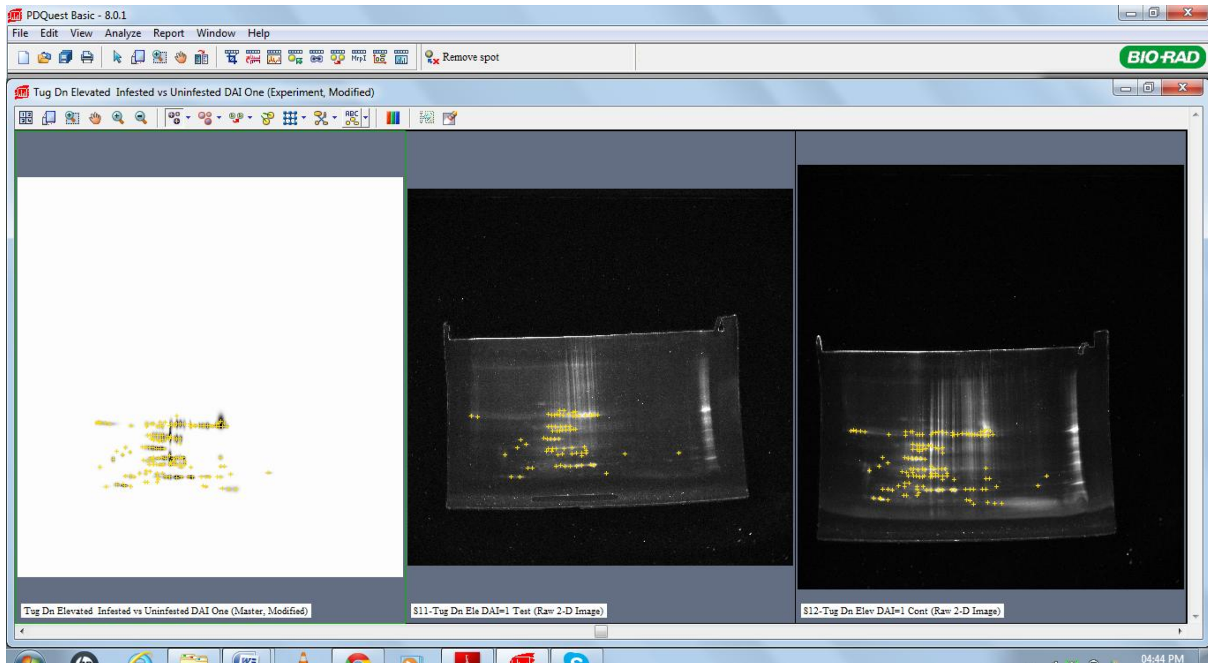

Fig 6.11 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in elevated CO2 concentration Tugela Dn CO2 concentration Infested vs. Uninfested. Day 1 (DAI=1)

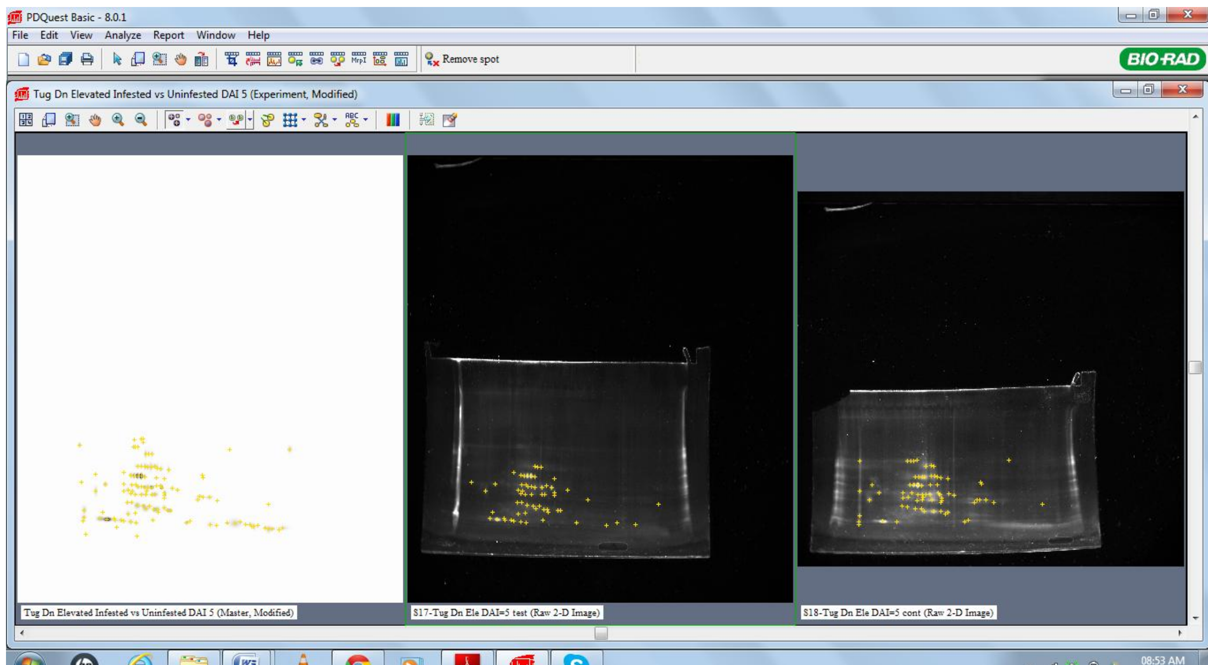

Fig 6.12 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in elevated CO2 concentration Tugela Dn CO2 concentration Infested vs. Uninfested. Day 5 (DAI=5)

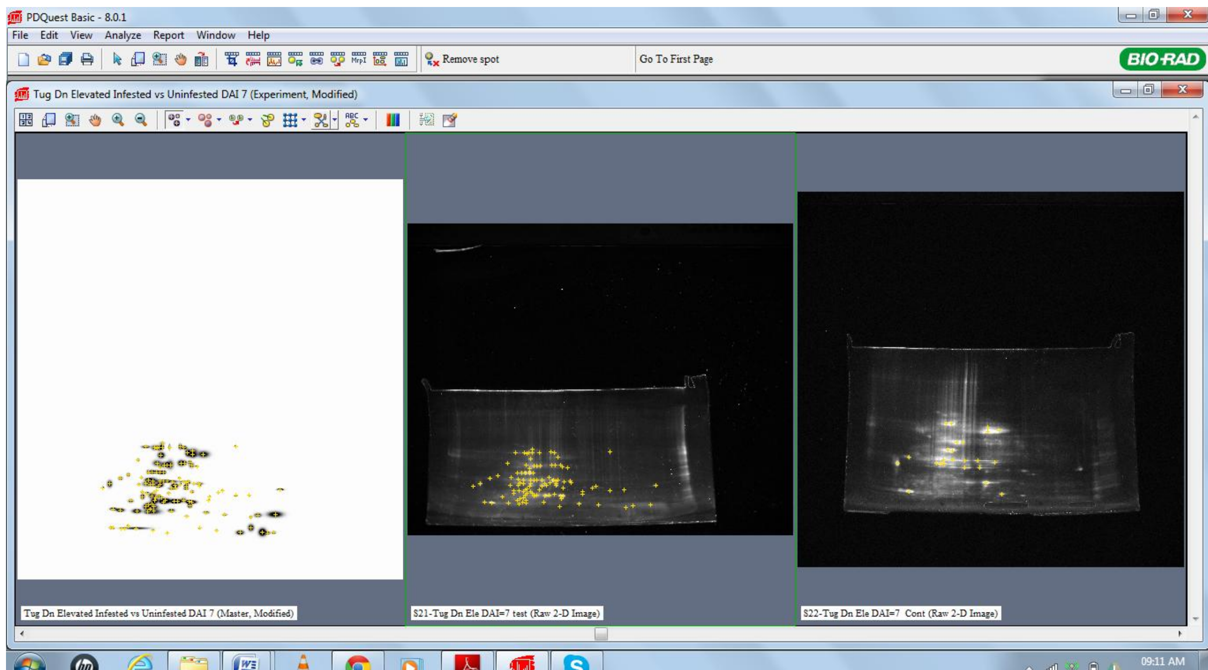

Fig 6.13 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in elevated CO2 concentration Tugela Dn CO2 concentration Infested vs. Uninfested. Day 7 (DAI=7)

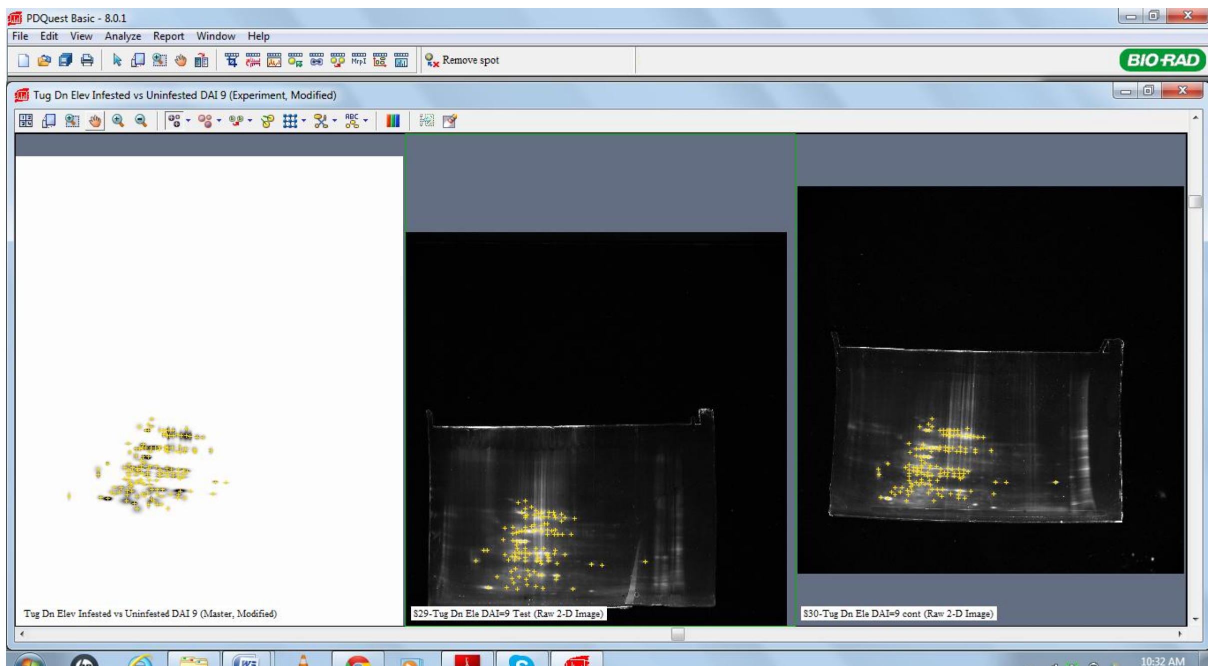

Fig 6.14 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in elevated CO2 concentration Tugela Dn CO2 concentration Infested vs. Uninfested. Day 9 (DAI=9)

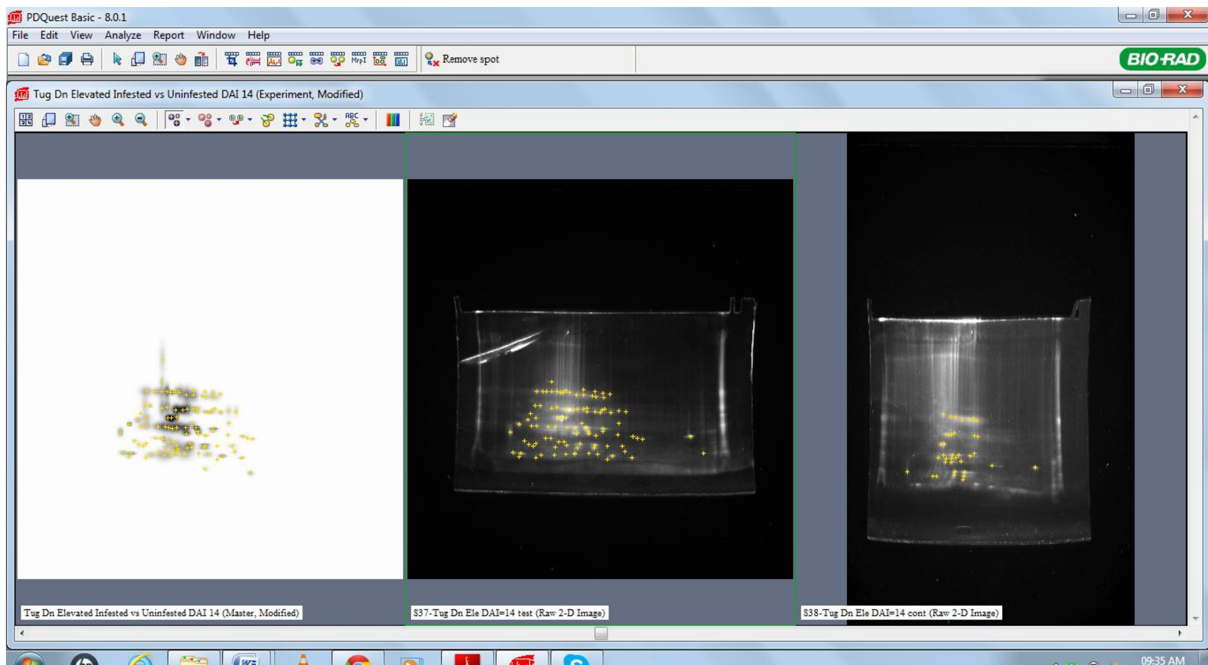

Fig 6.15 PDQuest™ Basic Software analysis of the 2-DE gel samples showing differential protein expression in elevated CO2 concentration Tugela Dn CO2 concentration Infested vs. Uninfested. Day 14 (DAI=14)

Results of analysis of differentially expressed proteins with PDQuest™ Basic Software Version 8.01: Quantity Graph Reports

Uninfested Tugela Dn at – Ambient vs. Elevated CO2 concentration:

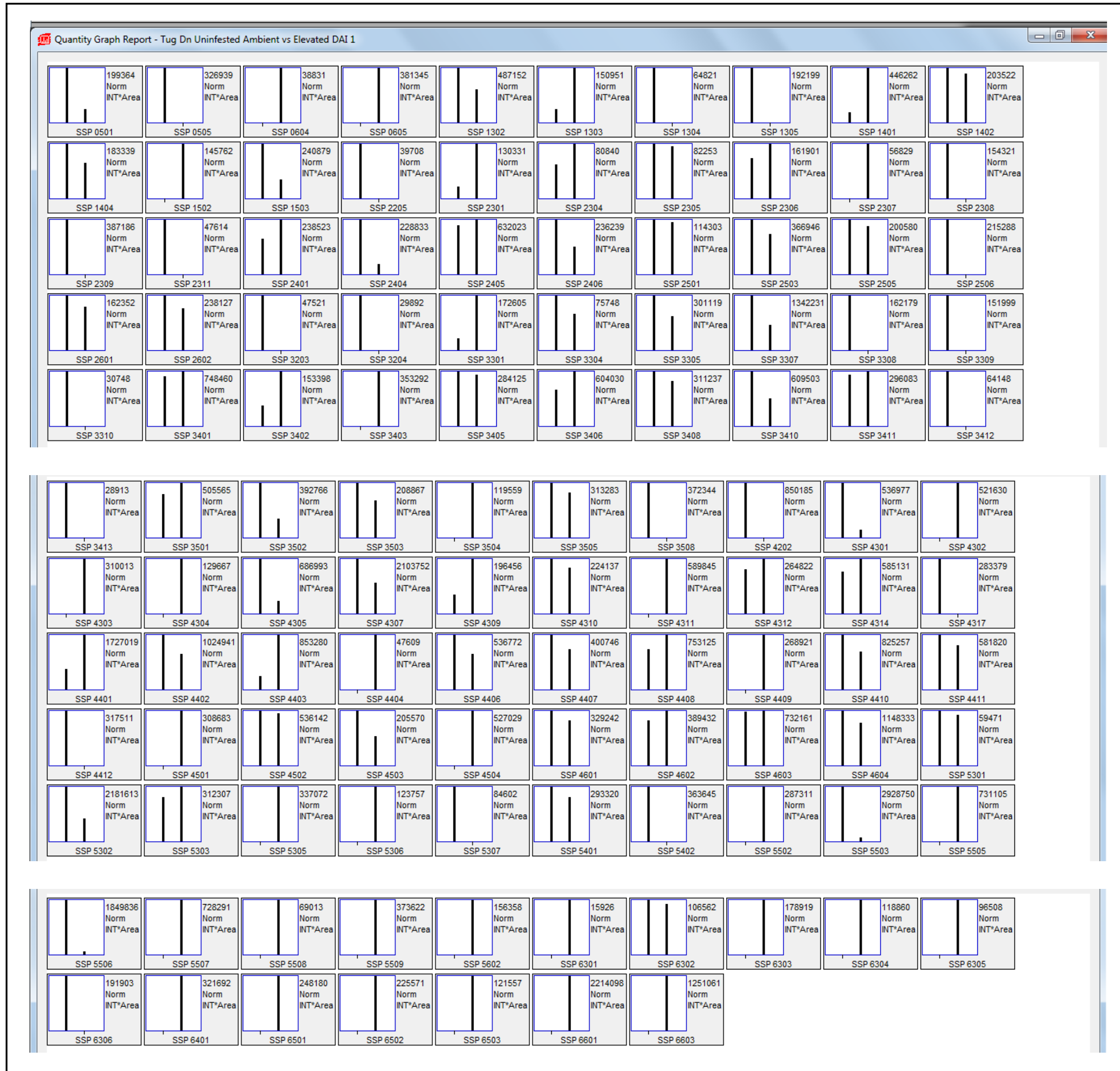

Fig 7.1 Quantity graph report from analysis of the gels: Uninfested Tugela Dn at ambient CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day=1

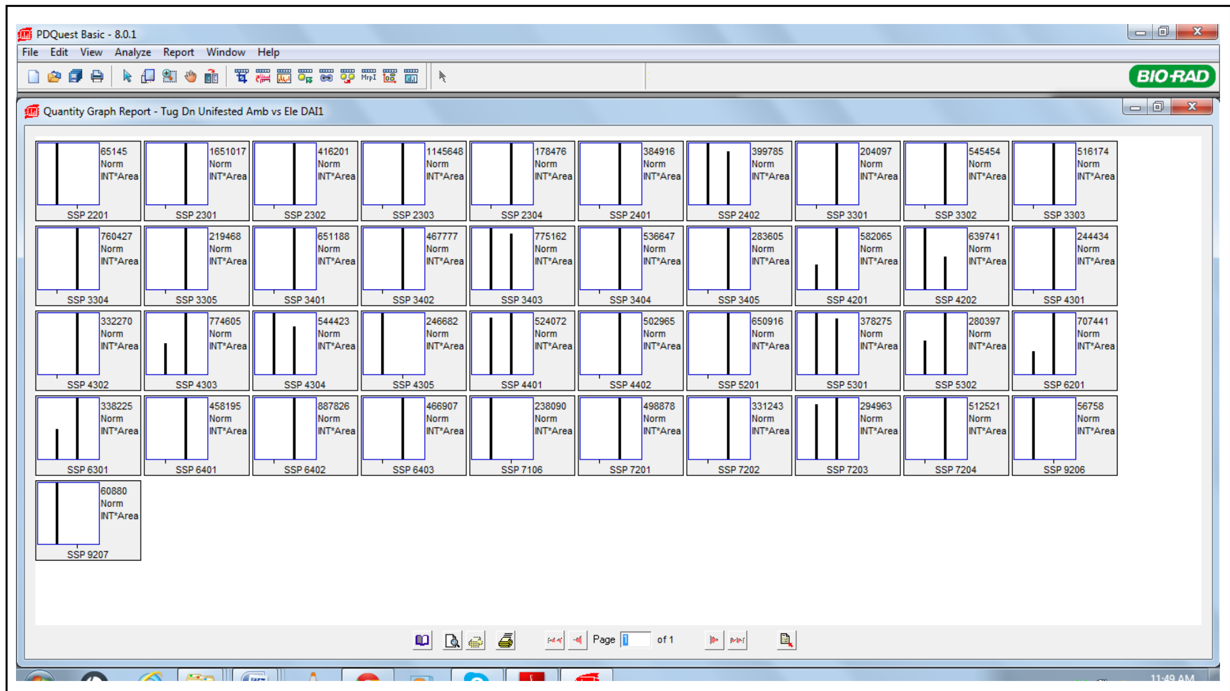

Fig 7.2 Quantity graph report from analysis of the gels: Uninfested Tugela Dn at ambient CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day=5

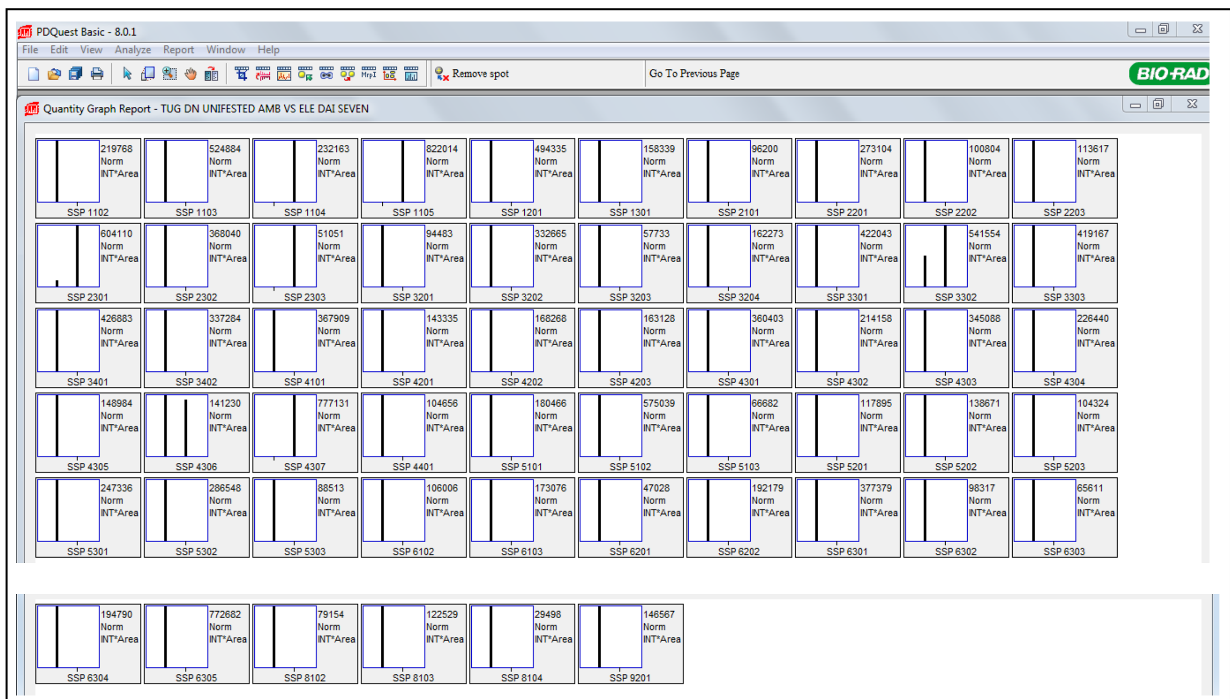

Fig 7.3 Quantity graph report from analysis of the gels: Uninfested Tugela Dn at ambient CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day=7

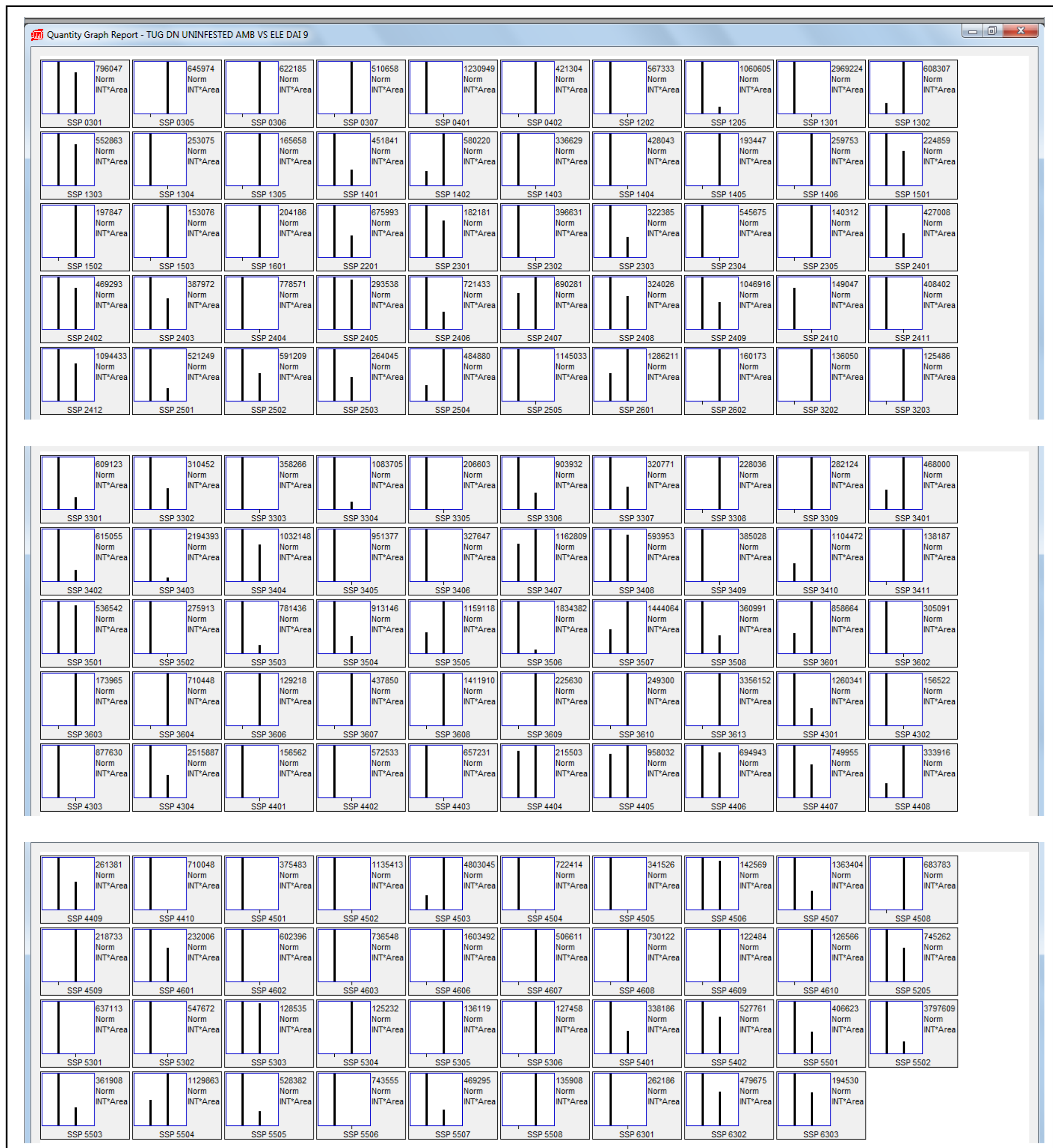

Fig 7.4 Quantity graph report from analysis of the gels: Uninfested Tugela Dn at ambient CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day=9

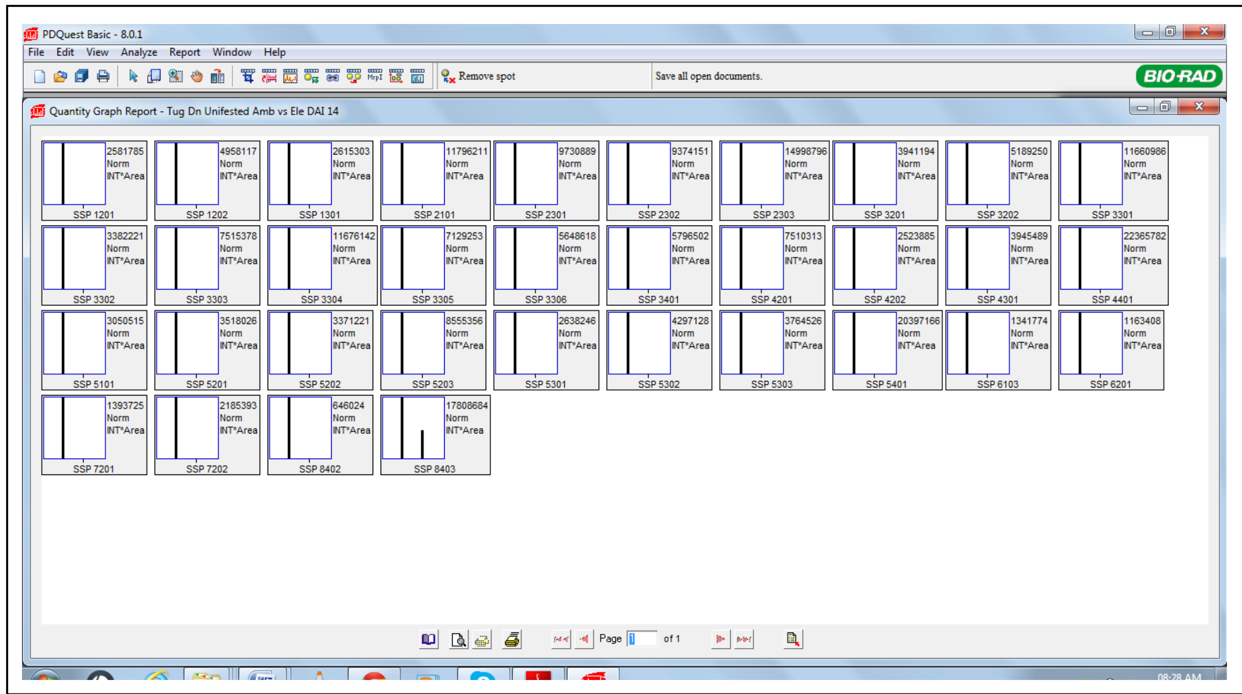

Fig 7.5 Quantity graph report from analysis of the gels: Uninfested Tugela Dn at ambient CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day=14

RWA SA1 – Infested Tugela Dn: Ambient vs. Elevated CO2 concentration:

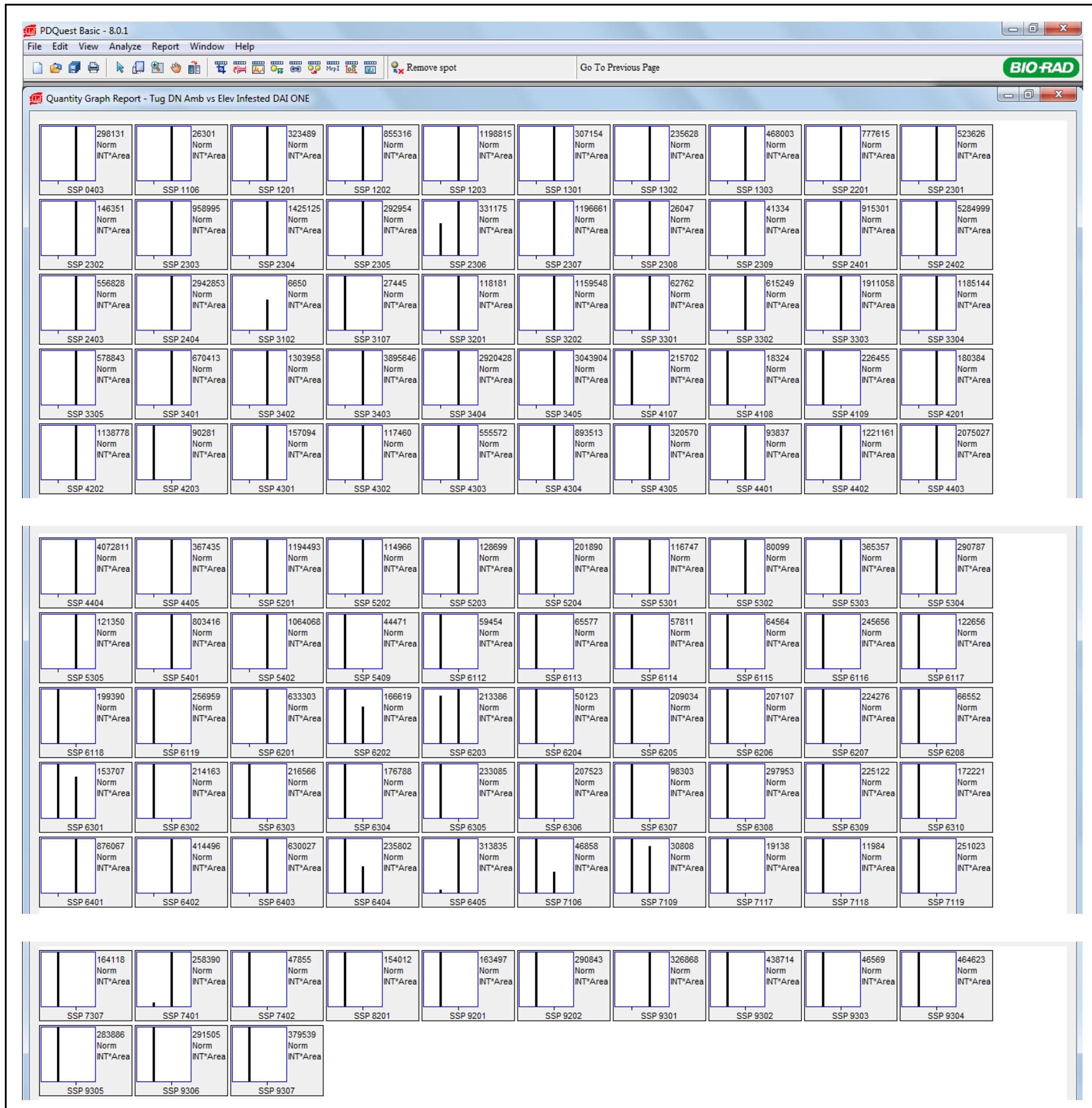

Fig 7.6 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at ambient CO2 concentration vs. RWA- SA1 - infested Tugela Dn at elevated CO2 concentration at Day 1 (DAI=1)

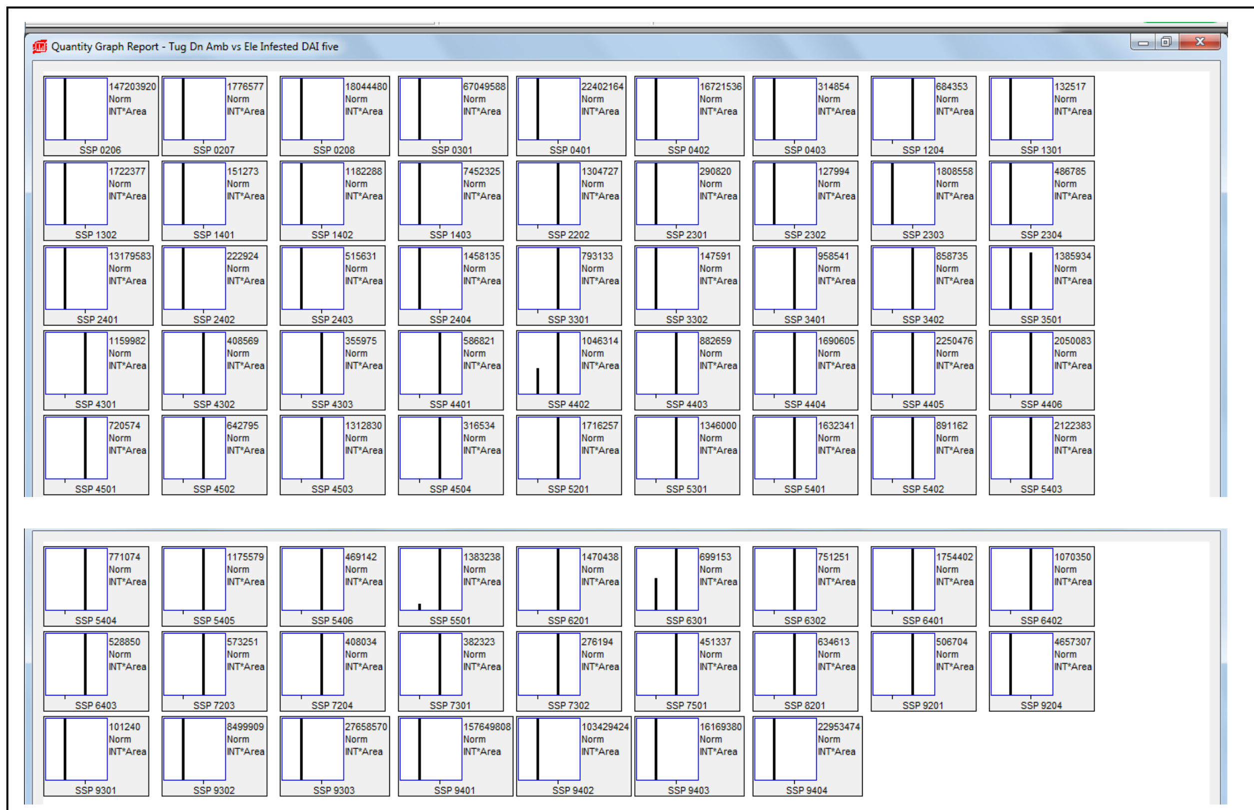

Fig 7.7 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at ambient CO2 concentration vs. RWA- SA1 - infested Tugela Dn at elevated CO2 concentration at Day 5 (DAI=5)

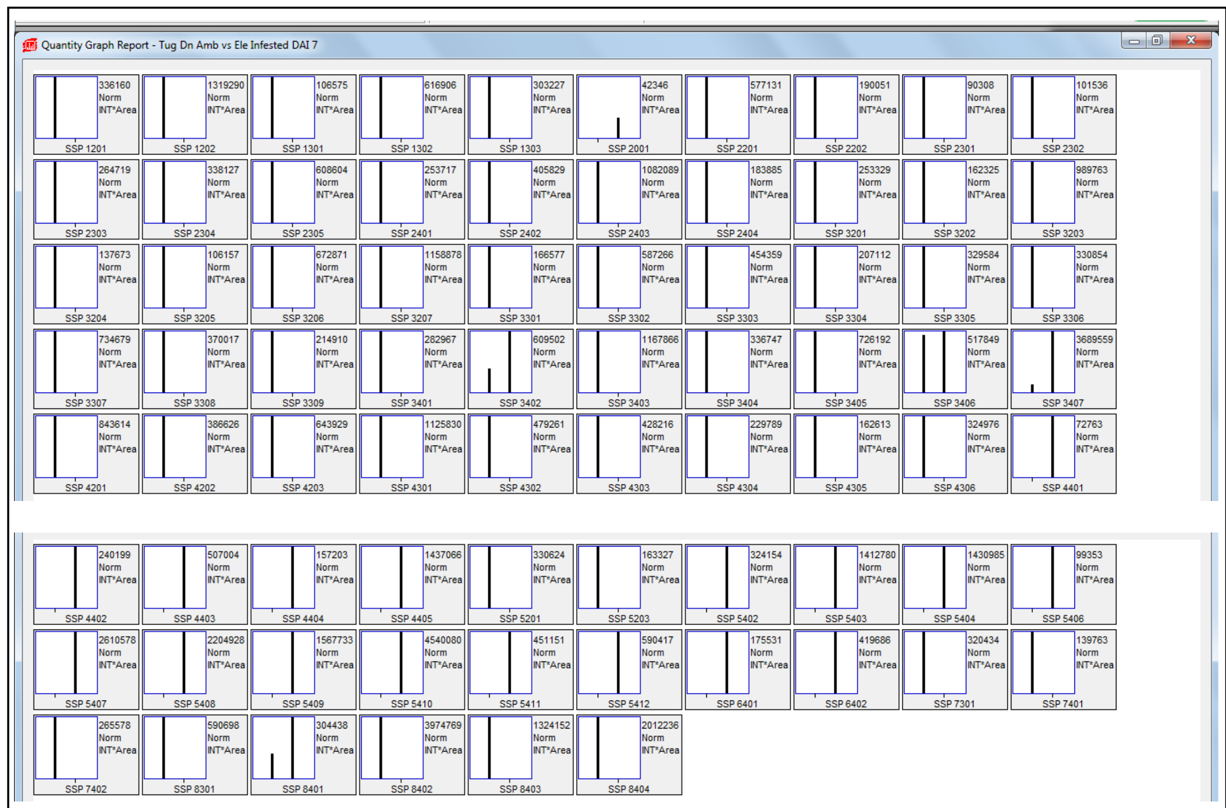

Fig 7.8 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at ambient CO2 concentration vs. RWA- SA1 - infested Tugela Dn at elevated CO2 concentration at Day 7 (DAI=7)

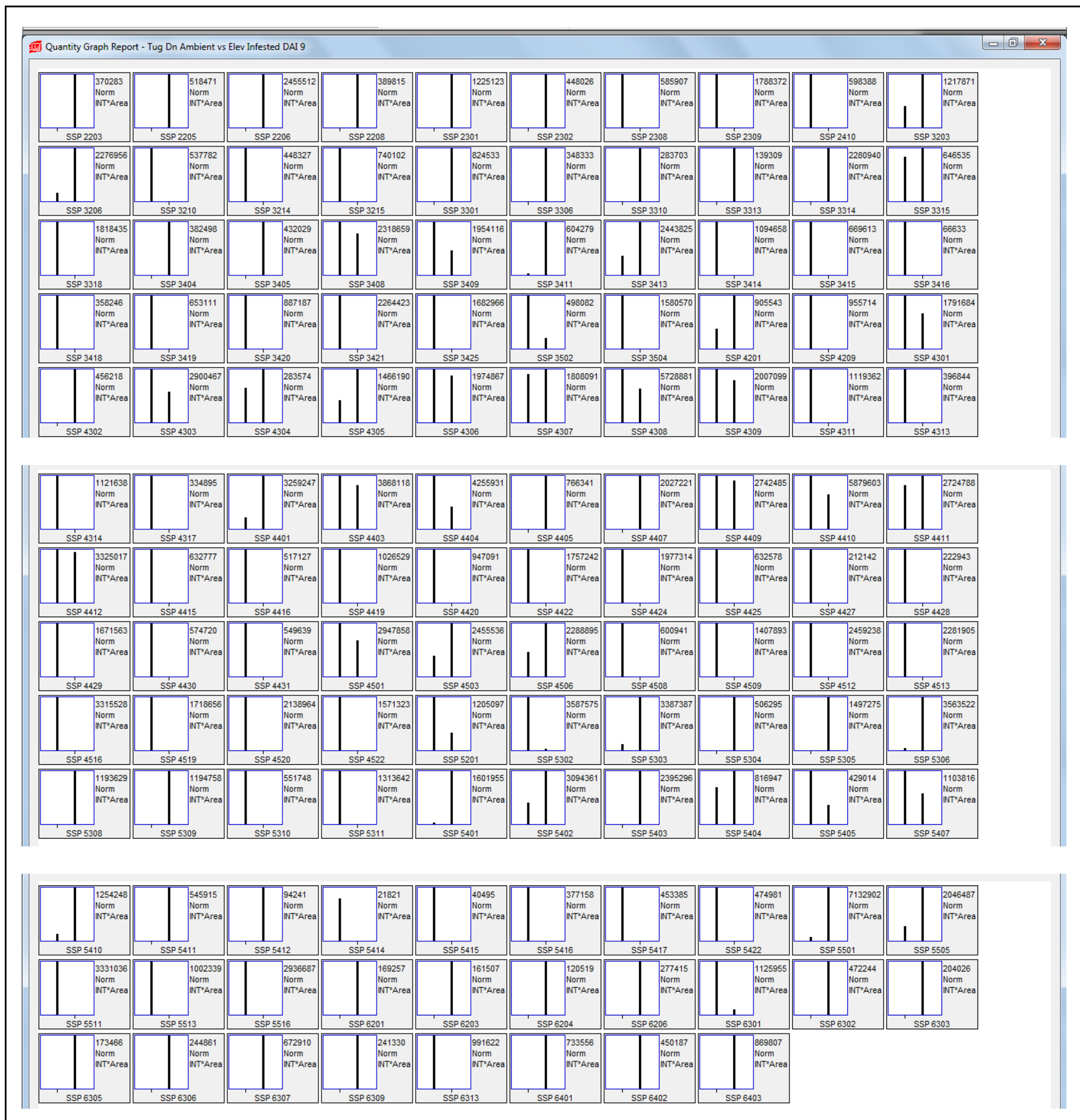

Fig 7.9 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at ambient CO2 concentration vs. RWA- SA1 - infested Tugela Dn at elevated CO2 concentration at Day 9 (DAI=9)

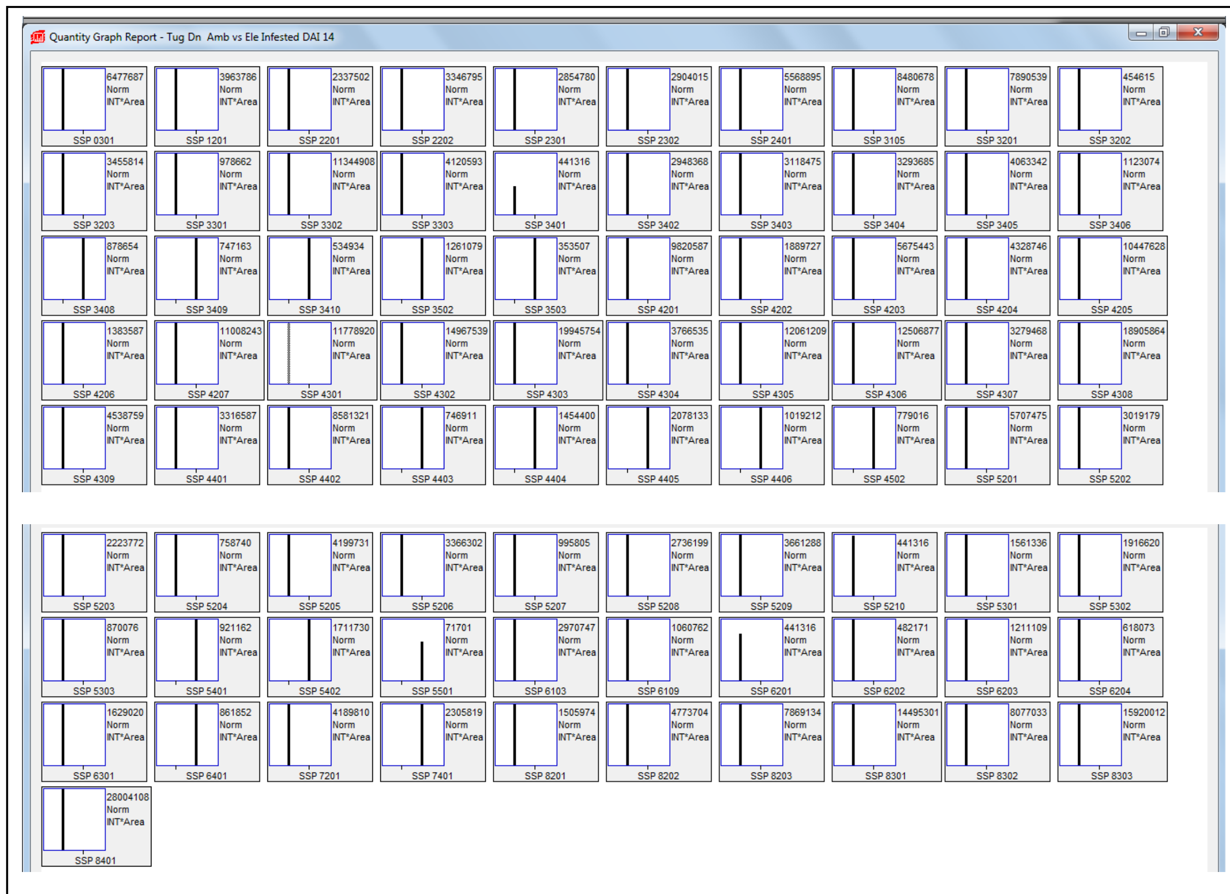

Fig 7.10 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at ambient CO2 concentration vs. RWA- SA1 - infested Tugela Dn at elevated CO2 concentration at Day14 (DAI=14)

RWA SA1 – Infested Tugela Dn at Elevated CO2 concentration vs. Uninfested Tugela Dn at Elevated CO2 concentration:

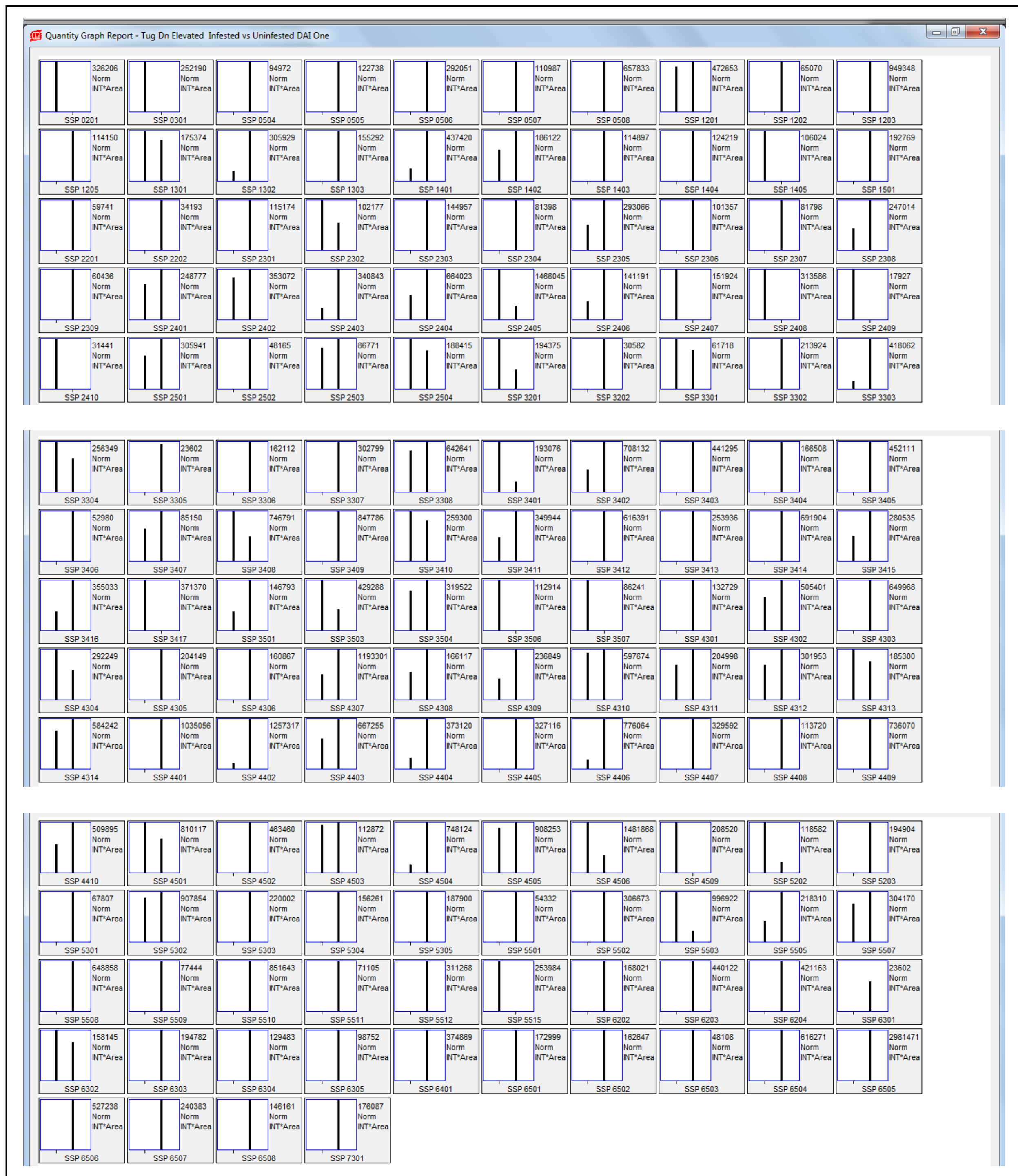

Fig 7.11 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at elevated CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day 1

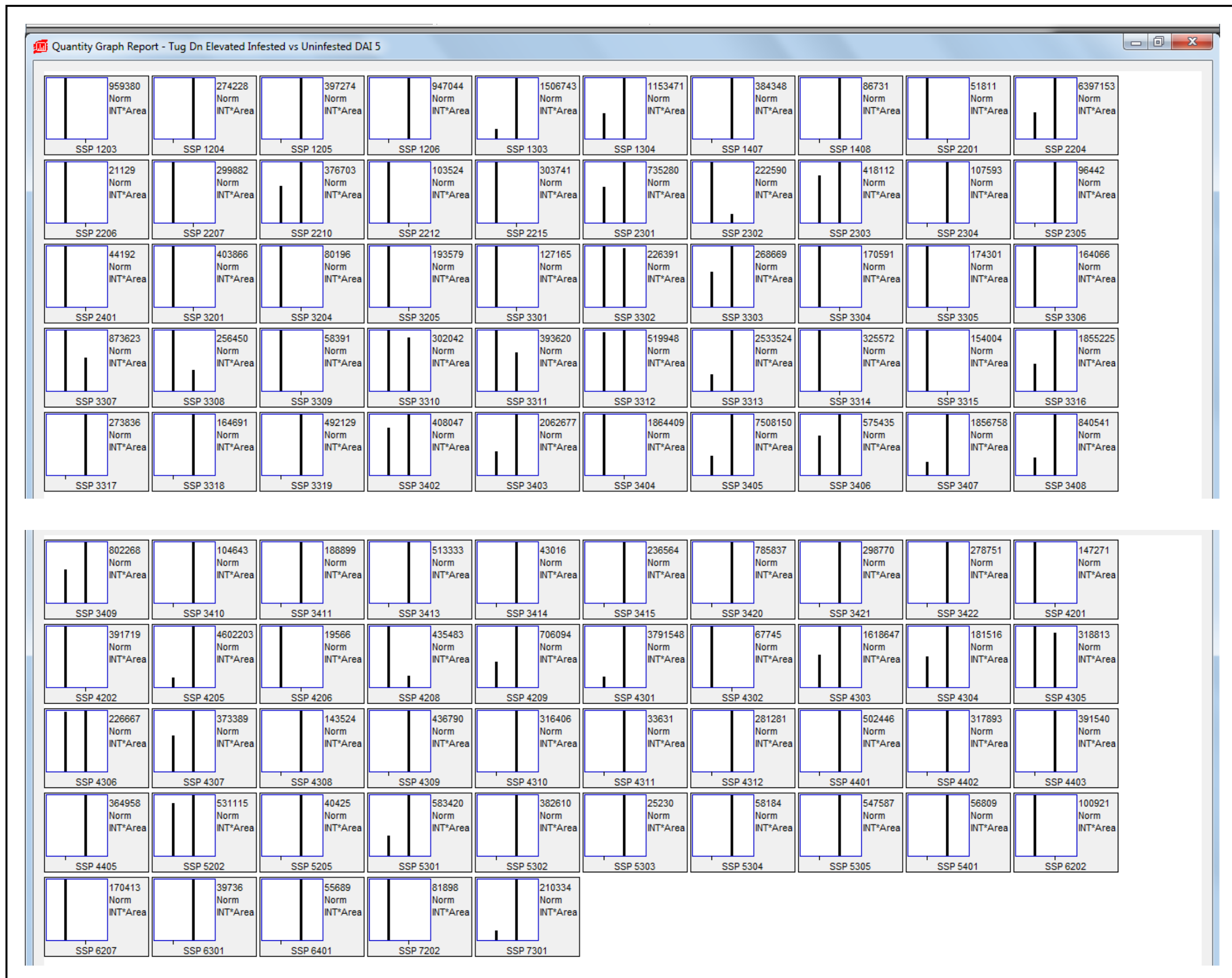

Fig 7.12 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at elevated CO_2 concentration vs. Uninfested Tugela Dn at elevated CO_2 concentration at Day 5

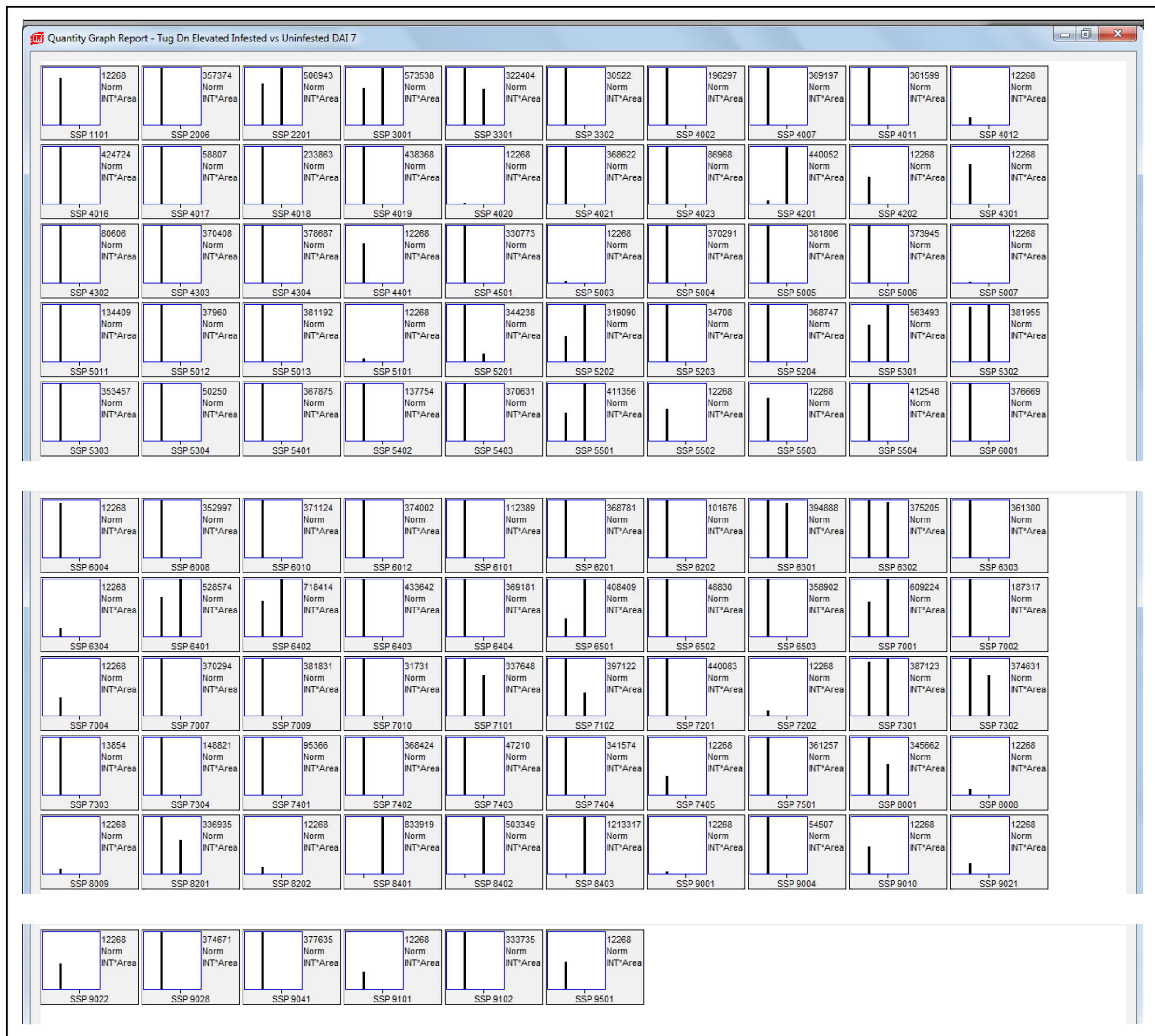

Fig 7.13 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at elevated CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day 7

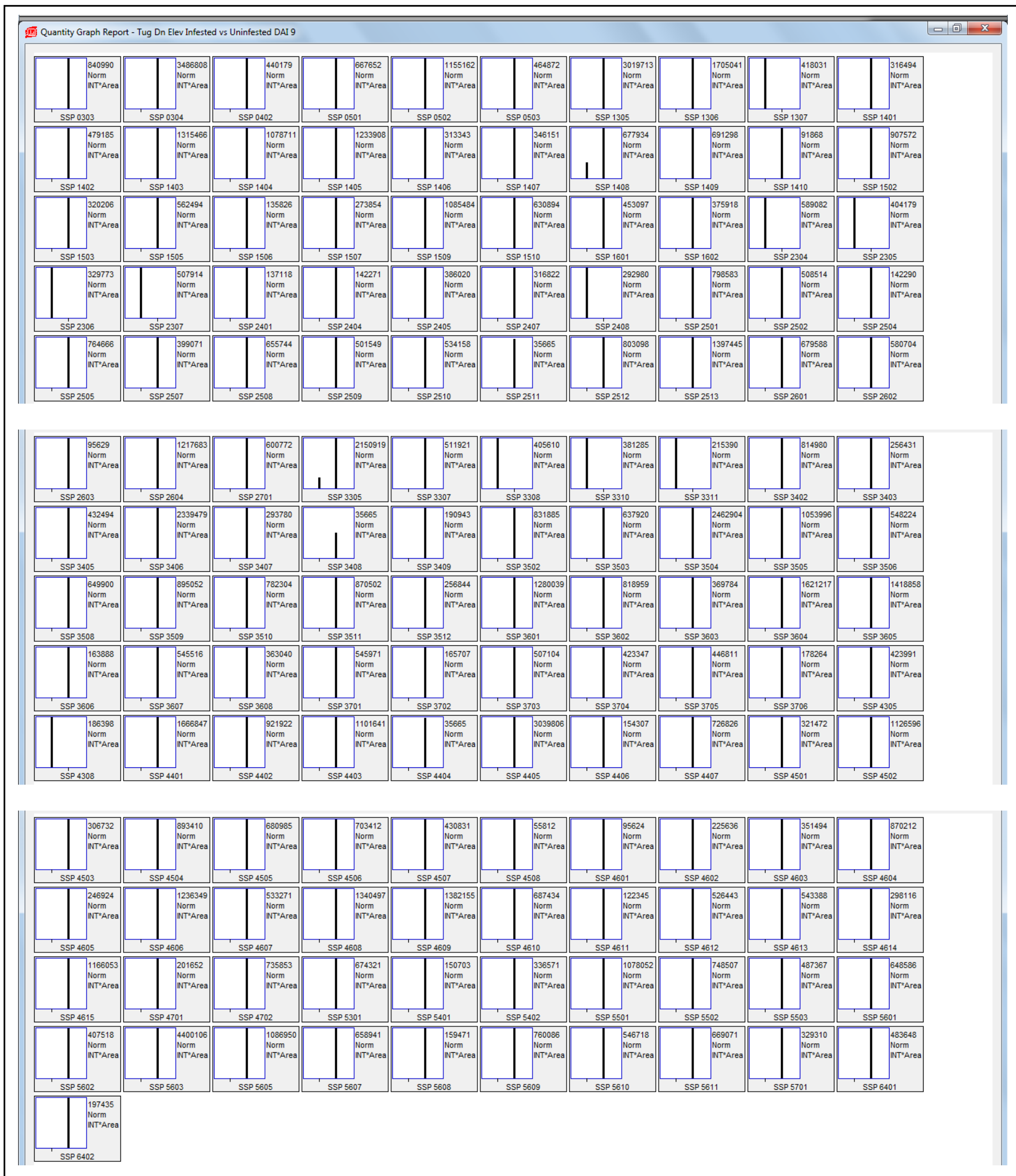

Fig 7.14 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at elevated CO2 concentration vs. Uninfested Tugela Dn at elevated CO2 concentration at Day 9

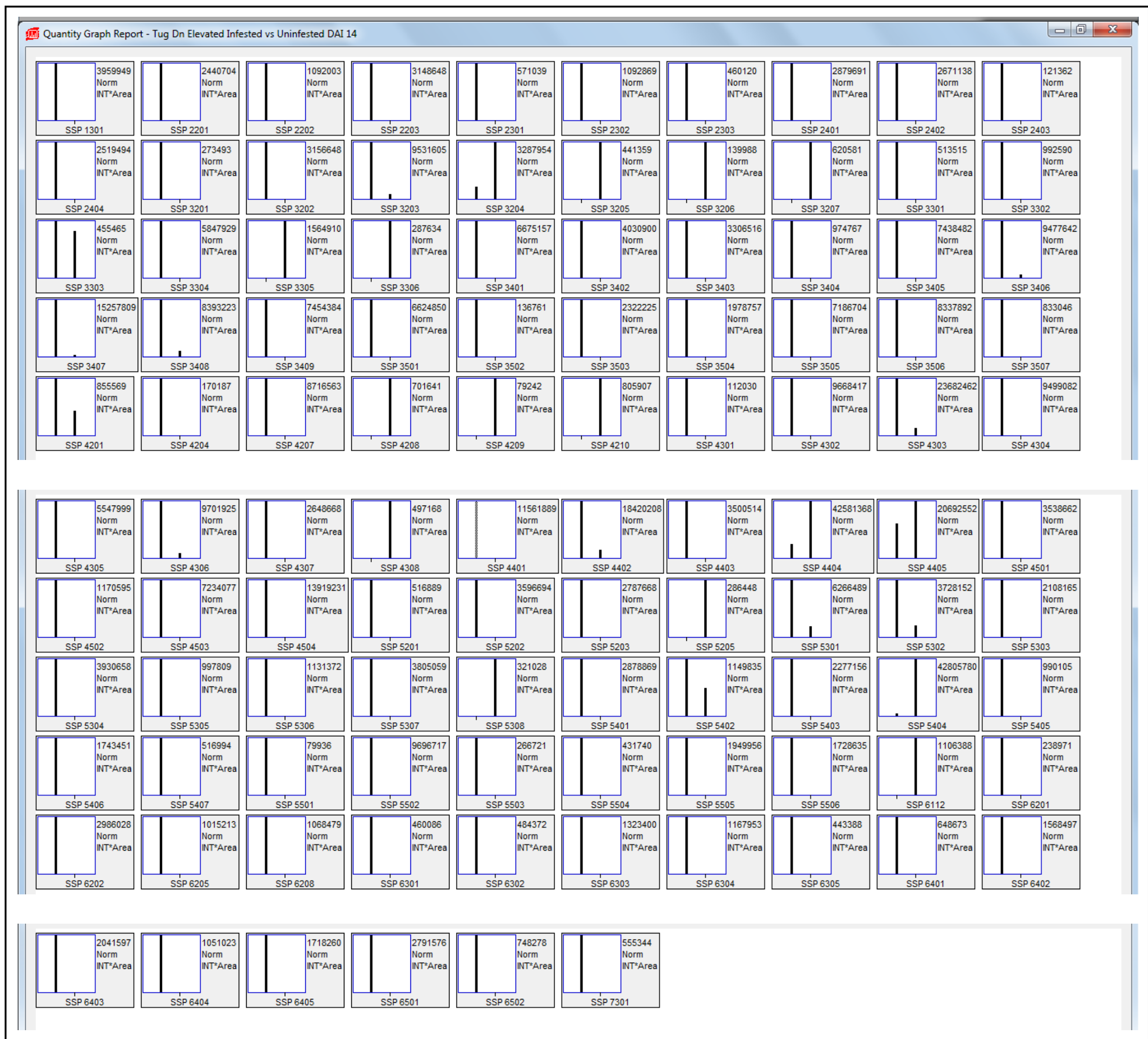

Fig 7.15 Quantity graph report from analysis of the RWA SA1- infested Tugela Dn at elevated CO_2 concentration vs. Uninfested Tugela Dn at elevated CO_2 concentration at Day 14