Wheat stress responses during Russian Wheat Aphid and Bird Cherry Oat Aphid infestation: An analysis of differential protein regulation during plant biotic stress responses

A thesis submitted in fulfilment of the requirements for the degree of

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Abstract

Plants possess a complex and poorly understood network of defence mechanisms that enable them to counteract the effects of abiotic and biotic stress. Aphid phloem feeding is source of biotic stress in plants. Russian wheat aphid and Bird Cherry-Oat aphid feeding cause significant losses in the annual wheat crop, and control by conventional methods such as pesticide application, has proved to be ineffective. Infestation by the Russian wheat aphid has a particularly devastating effect in South Africa. Aphid-resistant wheat cultivars have been identified but an incomplete understanding of the mechanism of the plant's resistance thwarts the development of improved cultivars.

A two-dimensional gel electrophoresis method was developed, partially optimised and validated in order to determine the effect of Russian wheat aphid and Bird Cherry-Oat aphid phloem feeding on the Betta and Betta DN wheat proteome. Differentially expressed proteins that were up or down regulated more than two fold were identified using PDQuest™ Basic software and matched to known wheat proteins stored in the SwissProt protein database on the basis of their molecular mass and isolectric point. Initial analysis of the differential protein expression of Betta and Betta DN wheat in response to Russian wheat aphid and Bird Cherry-Oat aphid phloem feeding at different growth stages revealed that younger plants display higher levels of resistance than older plants. Feeding by the Bird-Cherry Oat aphid does not result in the upregulation of proteins implicated in a defence response, which indicates that the damage incurred by the plant due to feeding by this aphid is not enough to trigger a classic defence response. Feeding by the more

damaging Russian wheat aphid resulted in a stress response in susceptible wheat cultivar Betta, and a defence response in resistant wheat cultivar Betta DN. The infestation of Betta DN resulted in the upregulation of putative thaumatins and amylase trypsin inhibitors, indicating that the Betta DN resistance response could be due to the combined effect of protease inhibitors that discourage aphid phloem feeding and the activation of the salicylic acid and jasmonic acid plant defence pathways.

Keywords: plant defence responses, Russian wheat aphid, Bird Cherry-Oat aphid, jasmonic acid pathway, salicylic acid pathway

Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science in Rhodes University. It has not been submitted before for any degree or examination in any other university.

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Cassandra Alexandrovna Louw
January 2007
Grahamstown

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List of Abbreviations

ABA	Abscisic acid
Agt	*Agrobacterium tumefaciens*
APS	Ammonium persulphate
ARC	South African Agricultural Research council
ASB-14	Amidosulfobetaine-14
Avr gene	Avirulence gene
BCA	Bird Cherry-Oat aphid
BSA	Bovine Serum Albumin
Bt	*Bacillus thuringiensis*
CC	Coiled-coil
CDPK	Calcium-dependent protein kinase
Cry proteins	Crystal proteins
cv.	Cultivar
°C	Degrees Celsius
2DE	Two-dimensional gel electrophoresis
DNA	Deoxyribonucleic acid
ESTs	Expressed sequence tags
ET	Ethylene
g	Gram
GM	Genetically modified
HR	Hypersensitive response
IEF	Isoelectric focussing
IF4E1	Eukaryotic translation initiation factor 4E-1
IPG	Immobilised pH gradient
JA	Jasmonic acid
JIP	Jasmonate-induced protein
kDa	kilodalton
mA	milliampére
MAPK	Mitogen-activated protein kinase

mg	milligram
mm	millimetre
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBS-LRR	Nucleotide-binding site and leucine-rich repeat domains
NL	Non-linear
NO	Nitric oxide
PEG	Polyethylene glycol
pI	Isoelectric point
PR	Pathogenesis related proteins
R gene	Resistance gene
RDR	Required for disease resistance
ROS	Reactive oxygen species
RWA	Russian wheat aphid
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TBP	Tributylphosphine
TEMED	N,N,N’N;-tetramethylethylenediamine
TIR	Toll / interleukin – 1 – receptor
Tris	Trishydroxymethylaminomethane
V	Volts
V-hr	Volt-hours
µg	microgram
µl	microlitre

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Chapter 1

Literature Review

Plant signalling mechanisms during responses to biotic stress

1.1. The nature of biotic stress

Plants possess a complex and poorly understood network of defence mechanisms that enable them to counteract the effects of abiotic and biotic stress. These defence strategies are essential for survival as the sedentary nature of plants precludes movement away from sources of stress, and can comprise physical or interactive molecular and cellular changes within affected plants (Hammond-Kosack and Jones, 2000; Knight and Knight, 2001). The stress response can take either an active or passive form. An active defence response involves the early detection of an invading pathogen followed by a suitable and rapid response, while a passive defence response takes more time and is usually a result of downstream activation from the active processes (Hammond-Kosack and Jones, 2000). When plants perceive stress, a response is generated that relays information along a signal transduction pathway that facilitates the initiation of appropriate physical and physiological responses. It appears that the various signalling pathways, that are implicated in different types of stress responses, all link up to form a highly complex network that allows for a significant amount of cross-talk in terms of stress responses between the different pathways, particularly in the case of the salicylic acid pathway (associated with pathogen infection) and the jasmonic acid pathway (associated with insect herbivory) (Maleck and Dietrich, 1999). This cross-talk benefits the plant as it reduces the energy cost required for developing multiple defence mechanisms (Knight and Knight, 2001).

Plant stress can be derived from both abiotic and biotic sources. Both types of stress cause major crop damage and result in significant annual losses of valuable crops worldwide (Rushton and Somssich, 1998). Abiotic stress includes drought, heat, cold, salinity, extreme light intensity and mechanical damage. Biotic stress results from pathogen attack or insect herbivory. While plant responses to these stress factors often overlap, it is important to note that different stress factors may occur simultaneously in the field and that the molecular response of affected plants takes place in a unique rather than cumulative fashion (Mittler, 2006).

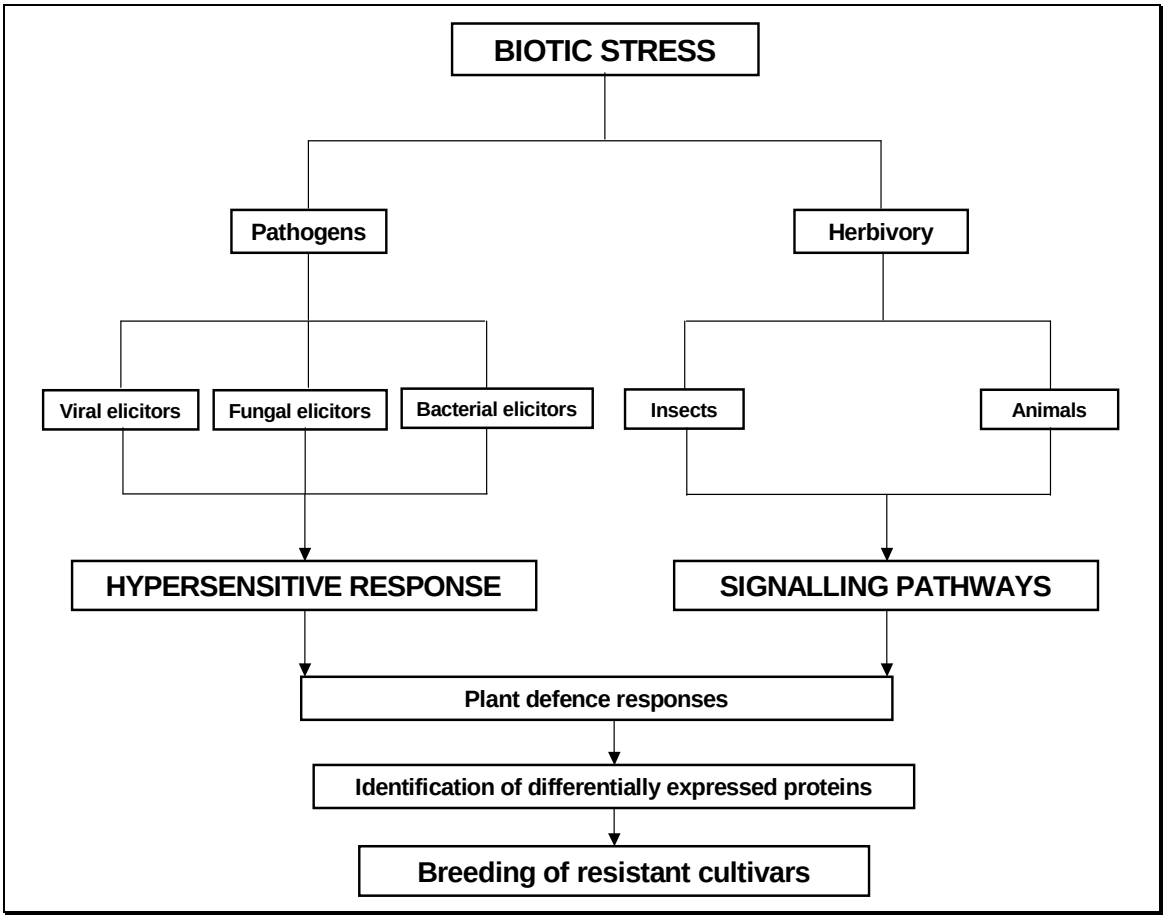


Figure 1.1. Analysis of plant responses to biotic stress factors leading to the development of novel resistance strategies in transgenic plants (Rushton and Somssich, 1998; Hammond-Kosack and Jones, 2000).

Plant disease susceptibility or resistance can be described in terms of compatible or incompatible relationships between the plant *R gene* and the pathogen *Avr* gene. Pathogen *Avr* genes are also known as avirulence genes while virulence genes are known as *avr* genes. In the same way, *R* genes encode resistance to various pathogens in plants while *r* genes occur in plants that do not contain resistance factors (Hammond-Kosack and Jones, 2000). An incompatible reaction between a plant and a pathogen due to the plant possessing a *R* gene that corresponds to a complementary *Avr* gene in the pathogen leads to an unsuccessful infection and subsequent resistance to disease symptoms, while a compatible reaction leads to a successful infection that results in the onset of disease, as outlined in Figure 1.2. Plant resistance to a given pathogenic factor is often inherited as a single dominant or semi-

dominant trait from the parental plant. This hypothesis of plant resistance to stress factors is known as the gene-for-gene model that was initially proposed by Harold Flor (Flor, 1955). This theory states that a plant will have resistance to the effect of a given pathogen if it possesses a single dominant resistance gene (R) corresponding to a complementary *Avr* gene in the pathogen leading to an incompatible relationship (Hammond-Kosack and Jones, 2000).

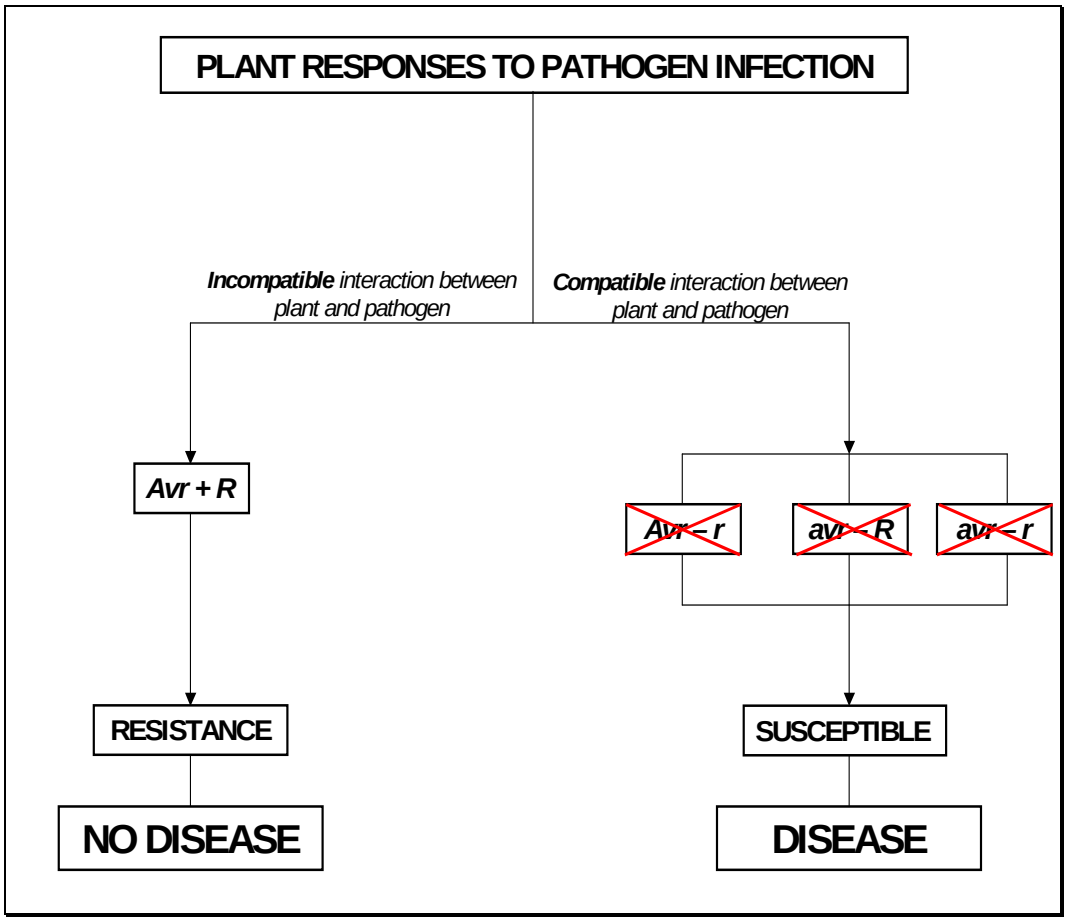


Figure 1.2. Illustration of the route of plant disease responses in terms of compatible and incompatible relationships between plant *R* genes and pathogen *Avr* genes (Heath, 2000).

Constitutive stress responses consist of a number of preformed peptides, proteins and non-proteinaceous secondary metabolites that act as deterrents against stress caused by insect herbivory and certain pathogens. In general, these responses tend to be non-specific (Heath, 2000). The regulation of plant stress response proteins occurs mainly at the transcriptional

level (Rushton and Somssich, 1998). Three families of transcription factors that have been implicated in plant stress responses include ethylene-responsive-element-binding-factors (ERF), basic-domain leucine-zipper (bZIP) and WRKY proteins (Rushton and Somssich, 1998). Protein kinase cascades are involved in plant stress responses as well as the induction of plant immunity (Romeis, 2001; Peck, 2003). Two classes of stress-activated protein kinases have been implicated in the integration of several environmental stresses by undergoing rapid activation subsequent to exposure of the host plant to a number of biotic and abiotic stresses. These two classes of kinases include mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) (Jonak *et al*., 2002).

The evolution of plant resistance responses facilitated by R proteins is proposed to have come about by means of natural selection (Meyers *et al*., 2005). Plants possess genetically defined *R* gene clusters on their genomes. These clusters normally result from tandem duplications of paralogous sequences (Meyers *et al*., 2003). It is proposed that new *R* genes evolve when crossing over occurs between such genes that are situated on the same locus. In support of this, it has been found that genes that are closely related or co-localized exchange sequences on a frequent basis. However, no evidence exists to indicate that such sequence exchange occurs between genes that are related but situated on different loci (Baumgarten *et al*., 2003). When studying the mechanisms of plant resistance and defence to stress responses, it is important to take into account the effect of resistance costs that are accrued by such responses. Resistant plants often have lowered fitness as compared to susceptible cultivars, which is displayed by diminished size and poor seed production. It is often assumed that fitness costs in resistant plants are due to the channelling of energy resources away from reproduction and seed production to the synthesis and maintenance of chemical and structural defence mechanisms. This is largely an inaccurate assumption as fitness costs due to resistance are caused by a combination of factors (Purrington, 2000). It is also important to appreciate that the interaction between different plant defence pathways can be either synergistic or antagonistic depending on the type of stress experienced by the plant and the particular pathogen that is involved. Many of these interactions are also known to be concentration-dependent (Feys and Parker, 2000). The extent of fitness costs incurred by the production of a given R protein plays a significant role in the evolution of *R genes* due to the fact that the plant has to maintain a suitable balance between the benefit of enhanced resistance and the fitness cost due to R protein production (Bergelson, 2001).

Plant resistance can lead to various ecological impacts, most notably that plants, which possess extensive chemical defences, are visited less frequently by pollinators. This leads to reduced pollen and / or seed production (Agrawal *et al*., 1999). The type of fitness cost incurred largely depends on the type of defence employed by the plant. Chemical defence mechanisms, for instance, can result in allocation and opportunity costs in addition to storage costs and self-toxicity to the plant if the defence chemicals are produced in excess quantity. Resistance to one pest type can also often result in increased susceptibility to another, more specialised type of pest (Purrington, 2000). Resistance costs also vary depending on whether the resistance type is constitutive or inducible; the latter tends to be less costly as it only comes into play during pathogen attack and not throughout the plant's life cycle (DeWitt *et al*., 1999; Cipollini, 1998).

1.2. Plant responses to pathogen attack

During, or shortly after, pathogen attack, plant resistance proteins (R) recognise pathogen avirulence (*Avr*) determinants, which results in the triggering of signal transduction cascades that cause rapid mobilization of defence factors (Dangl and Jones, 2001). It has been proposed that this reaction is of a receptor-ligand type in which the Avr protein binds to a suitable R protein to initiate defence responses. Current research, however, indicates that the co-localization of Avr and R proteins is essential for their function, in addition to a growing awareness that R proteins are not the exclusive primary receptors for Avr proteins (Bonas and Lahaye, 2002). The precise function of *avr* genes in pathogens remains unknown, although it has been observed that the presence of these genes confer a selective advantage to any pathogens in which they occur. This advantage, however, is only apparent when the pathogens containing *avr* genes attack plants without corresponding *R* genes. With these observations in mind, it has been suggested that *avr* genes are virulence factors and that plants have evolved to counteract this virulence by producing *R* genes that produce resistance (Kjemtrup et al., 2000; Laugé and De Wit, 1998). *R* genes, for their part, have been defined as: "the polymorphic component between susceptible and resistant plant genotypes without sequence knowledge of the loci involved." In other words, if a given species is found to be inactive in a susceptible cultivar, its active counterpart in a resistant species is defined as an *R* gene (Flor, 1971).

The tertiary structures of R proteins are well conserved across both monocotyledonous and dicotyledonous species (Moffet *et al*., 2002). Each plant genome possesses a high number of *R* genes that encode different classes of R proteins. These different R proteins are able to recognise various Avr determinants in a range of pathogens / pests and, in so doing, confer resistance to the plant containing them (Tang *et al*., 1996; Nimchuck *et al*., 2003). In terms of their structure, the most abundant R proteins contain a nucleotide-binding site and leucine-rich repeat domains (NBS-LRR). The NBS-LRR proteins contain three distinct domains, namely a variable N-terminus, a nucleotide-binding site and leucine rich repeats (Pan *et al*., 2000). LRRs are known to mediate protein-protein interaction, which is consistent with the proposed role of R proteins in plant defence (Kajava, 1998; Jones and Jones, 1997). R proteins that contain an intracellular LRR are also known to contain a putative nucleotide binding domain that occurs in conjunction with either an amino-terminal Toll / interleukin-1-receptor (TIR) homologous region or a coiled-coil (CC) domain (Dangl and Jones, 2001). LRR classes of R proteins are largely responsible for the recognition of specific residues (Wang *et al*., 1998). When taking their cellular localizations into account, it is readily apparent that R proteins and their matching Avr proteins are spatially interdependent, i.e. they are found to occur within the same organelles (Staskawicz *et al*., 2001). In addition to this, mutational studies have revealed that pathways mediated by different R proteins converge in common downstream pathways (Feys and Parker, 2000).

It is important to note that most plants are resistant to most pathogens. However, economically valuable crop plants are more vulnerable to pathogens than plants grown in the wild due to the fact that they are generally grown as uniform monocultures (Jones, 1994). For a pathogen to successfully infect a given plant, the plant and pathogen must interact in a specific manner. When a pathogen with a given *Avr* gene acts upon a plant that has a corresponding *R* gene locus, the affected plant is resistant. However, in cases where either the *R* gene on the plant or the matching *Avr* gene on the pathogen genome is absent or inactive, pathogen infection results in disease (Flor, 1971). This model is based on the fact that the production of plant R proteins is dependent on recognition of the appropriate Avr from the pathogen. It is increasingly being suggested, however, that a significant number of Avr proteins do not interact directly with R proteins but that an R protein functions as a type of guarding system that identifies and responds to an attack on a specific host protein by the

pathogenic Avr protein and subsequently triggers the plant's defence response (Dangl and Jones, 2001).

The rapid mobilization of plant defence responses following pathogen attack is known as the hypersensitive response (HR) and occurs at the site of invasion, effectively leading to necrosis of the tissue surrounding the pathogen and, in so doing, isolating it from the rest of the plant (De Wit, 1997). The HR is a form of programmed cell death that is accompanied by a burst of superoxide production and the expression of defence genes (Richberg, *et al*., 1998; Rushton and Somssich, 1998). It occurs at and around the area that is directly affected by a given pathogen and is accompanied by the induction of plant defence response pathways that facilitate the confinement of the pathogen to the infection site (Heath, 2000). The HR, despite being a highly effective and rapid form of defence, results in high fitness costs to the plant and sophisticated response networks are required to ensure that it is only deployed when necessary (Lam *et al*., 2001). Defence gene activation, however, plays an even more important role than the HR, in the response of plants to pathogen attack (Jakobek and Lindgren, 1993).

Plant protective strategies against pathogen attack generally consist of biochemical defence mechanisms such as enzymes or secondary metabolites (phytoalexins), as opposed to physical defence mechanisms such as thorns (Mithöfer *et al*., 2004). While the presence of waxy, cuticular layers protects plants from pathogens in a non-specific manner, it does not suffice to defend affected plants in the long term and only serves as a temporary defence unlike the more specialized biochemical defence systems (Dangl and Jones, 2001). The production of *de novo* synthesised phytoalexins is specific to pathogen attack that takes place during plant-microbial interactions and does not occur as a result of physical wounding or mechanical damage. Phytoalexins can, however, be produced in response to insect herbivore feeding, indicating a potential overlap between defence systems induced during insect and pathogen attack (Mithöfer *et al*., 2004). In addition to phytoalexins that are synthesised in response to pathogen attack, other defensive compounds such as phytoanticipins are pre-formed infectional inhibitors (VanEtten *et al*., 1994). The Shikimic acid pathway is central to the production of defensive secondary metabolites in plants (Dixon, 2001).

1.3. The Hypersensitive Response (HR)

Plant pathogens are divided into two categories, depending on the strategy of infection that they employ. Pathogens that kill the host and feed on it are known as necrotrophs, while pathogens that utilize a living host to complete their life cycle are known as biotrophs. Necrotroph infestation is often accompanied by the production of toxins in the affected plant (Dangl and Jones, 2001).

The HR is a rapid response by plants that utilizes programmed cell death to restrict the mobility of a pathogen in plant tissue. It has been defined as "the rapid death of plant cells in association with the restriction of pathogen growth" (Goodman and Novacky, 1994). The progression of HR in infected plants is usually indicated by the presence of dead cells at the site of infection. If a large number of cells are involved in the programmed cell death, the formation of a visible brown lesion may result (Heath, 2000). The HR can be induced by non-biotrophic pathogens, indicating that it also plays a role in defence gene expression although it is important to note that inducible defence responses and disease resistance do occur in plants in the absence of programmed cell death. The study of HR responses remains elusive because several natural processes within the plant life cycle can mimic the symptoms of the HR. These processes include events such as successful pathogen infection or genetic defects that resemble natural cell death in the HR (Molina *et al*., 1999). There are currently no known specific marker genes for the HR (Heath, 2000).

1.3.1. Role of the HR in plant defence responses

Despite being present in many reactions involving plant resistance to various stress factors, the HR is by no means involved in every reaction (Lam *et al*., 2001). It has been proposed that because some pathogens can be effectively resisted without recourse to the HR, it may represent a terminal stage in the resistance response that is only activated once a certain threshold level of defence stress signal is produced (Morel and Dangl, 1997). One of the most important questions currently facing researchers investigating HR responses is whether it is really significant in certain plant responses to attacks by specific fungal or pathogen enemies or whether it forms part of a more generalized response in plant defence systems, regardless of a specific attack (Lam *et al*., 2001).

1.3.2. The role of *avr* genes in the HR

The basis of resistance via the HR response is dependent on an *avr* gene in the pathogen that matches a suitable *R* gene in the host plant. While it has been proposed that there is a unique *R* gene for every *avr* gene in a pathogen that the plant is resistant to (the gene-for-gene hypthesis), recent research indicates that a more complex relationship exists with single *R* genes having the ability to match to several different *avr* genes (Hammond-Kosack and Jones, 1997). In terms of their structure, many R proteins contain leucine-rich repeats (LRR) or a serine-threonine kinase-domain. It is proposed that these proteins form part of signalling systems in the plant as the predominant class of R proteins contain a nucleotide-binding site (Hammond-Kosack and Jones, 1997). While at least one R gene is induced during the HR, it is generally assumed that others are constitutively expressed (Seehaus and Tenhaken, 1998).

1.3.3. Specific and non-specific elicitors in the HR

Because *R* genes are so closely associated with the HR, it is possible to forget the other lesser-known genes that are involved in this highly complex network. *RDR* (required for disease resistance) genes operate closely with *R* genes and may be different for each different *R* gene irrespective of the type of pathogen against which they confer resistance (Morel and Dangl, 1999). It is presumed that these genes enable both susceptible and resistant plants to undergo an HR (Falk *et al*., 1999). The nature of potential elicitors remains elusive, however, with only a few specific elicitors positively identified to date. Viral elicitors can include the viral helicase domain of the replicase gene, coat proteins or movement proteins, while fungal elicitors are largely peptides with no known function (Dawson, 1999; Ebel and Scheel, 1997). Bacterial elicitors of the HR have been cloned but the protein products formed are difficult to identify due to the fact that they appear to be secreted directly into the plant via a type III secretion system. It is important to note that *avr* gene products are sufficient on their own to cause cell death (He, 1998). While *avr* products produce specific elicitors to induce the HR, several other non-specific elicitors are known that can elicit plant defence responses (Ebel and Scheel, 1997). These include compounds such as arachidonic acid, cell wall carbohydrates, glycoproteins and proteins (Chen and Heath, 1994).

1.3.4. Biochemistry of the HR

Plant responses to stress are characterised by an influx of cytosolic calcium. This appears to be vital in facilitating the HR although additional signals are thought to be necessary in order to mediate successful defence (Ebel and Scheel, 1997; Malhó *et al*., 1998). It is proposed that calcium signals are translated via protein phosphorylation events (Pike *et al*., 1998). Membrane dysfunction is often the first symptom of a bacterial pathogen attack. Incompatible bacteria have been shown to cause membrane depolarization in which a K^+ / H^+ exchange appears depend on H^+ - ATPase activity (Blumwald *et al*., 1998). The HR is further characterized by the generation of reactive oxygen species (ROS), which have been implicated in programmed cell death in plants and apoptosis in animals (Green and Reed, 1998). It is widely accepted that ROS trigger the HR and allow it to proceed in stressed cells (Baker and Orlandi, 1995). Most ROS in plants are generated by means of wall-bound peroxidase or plasma membrane-bound NADPH oxidase and the role of ROS in HR has been found to vary across different plant-pathogen combinations (Higgins *et al*., 1998; Heath, 2000). ROS primarily consist of the superoxide (0_2^-) ion and hydrogen peroxide (H_20_2). These are toxic intermediates that are formed during successive steps in the reduction of molecular 0_2. Several plant species display what is known as biphasic ROS generation; initially a burst of ROS is observed 10-30 minutes after pathogen attack, while a second phase follows after approximately 1-3 hours (Bolwell and Wojtaszek, 1997). ROS perform various functions in plant stress responses, most notably in strengthening of cell walls by means of cross-linking of structural protein and lignin polymers, hypersensitive cell death and induction of defensive compounds within the plant (Levine *et al*., 1994).

1.4. Plant responses to fungal infection

Fungi are potentially the most damaging of all pathogens affecting plants. It is estimated that there is more than 13 000 plant pathogens in the United States while 12 of the 19 most dangerous pathogens listed in that country are fungal pathogens (Madden and Wheelis, 2003). The first defence that plants possess against fungal pathogens is the plant cell wall. In order to prevent penetration by fungi, plants are known to physically reinforce their cell walls; this is thought to be accomplished by means of the deposition of a sugar polymer (callose) containing (1,3)-β-D-glucan subunits that occurs between the plasma membrane and the cell

wall. Callose is deposited in the area surrounding the site of pathogen attack (Kudlicka and Brown, 1997). Certain fungi have managed to override this defence mechanism by possessing highly developed haustoria that avoid activation of plant responses. This is accomplished by the encapsulation of haustorial complexes by callose, and is an example of a pathogen utilizing a plant defence mechanism against the plant that is attempting to defend itself from attack. It has been proposed that the tendency of callose to encapsulate haustoria was initially used by plants to accommodate certain beneficial types of fungi such as mycorrhiza (Maor and Shirazu, 2005).

Syntaxins comprise another form of defence against fungal pathogens that is situated at the cell wall. These receptors are involved in fusion events and are members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) super family. It is proposed that papillae formation by a SNARE complex is a vital mechanism in the prevention of fungal penetration although the components of this system have yet to be identified and characterized (Collins *et al*., 2003; Assaad *et al*., 2004).

If the fungal pathogen manages to circumvent the first layer of defence and enter the plant cell, a response involving phytoalexins is triggered. Phytoalexins are produced in response to signals indicating the presence of a pathogen infection (Thomma *et al*., 1999; Yang, *et al*., 2004). For example, Camalexin is a major phytoalexin in *Arabidopsis thaliana*, which is important for resistance against several necrotrophic fungi such as *Alternaria brassicicola* and *Botrytis cinerea* (Thomma *et al*., 1999). Saponins are another means of defence against fungal pathogens. These preformed inhibitors are glycosylated compounds that have a potent antifungal activity due to their role in defence against pathogenic microbes (Hammond-Kosack and Jones, 2000; Osbourn, 2003).

When considering the biochemistry of plant stress responses, it is important to bear in mind that hormones regulate plant responses to stress. Certain pathogens have adapted to take advantage of this by producing plant hormones that alter the way the host plant responds to them (Yurekli *et al*., 2003; Lahey *et al*., 2004; Tudzynski, 2005). Some bacterial pathogens are known to overproduce plant growth hormones such as auxin and cytokinin, which can subsequently lead to the formation of tumour or gall formation in plants (Manulis *et al*., 1998; Escobar *et al*., 2001 and Vandeputte *et al*., 2005). Certain fungal pathogens are also known

to produce ethylene that can be distinguished from that produced by plants by using an inhibitor specific to the ethylene pathway in plants (Chague *et al*., 2002). The reason for the production of the fungal ethylene is not entirely clear at present; it is thought that it could either act as an agent required for the growth of the fungus or as virulence factor by interfering with plant ethylene-dependent resistance responses (Kim *et al*., 2000). In addition to ethylene, fungal pathogens are capable of producing auxins by means of fungal-specific biosynthetic pathways (Maor *et al*., 2004).

1.5. Plant responses to insect herbivory

Insect herbivory can result in both mechanical damage and pathogen transfer to affected plants. This form of biotic stress is responsible for losses of up to 20 % of valuable food crops per annum. It is a significant factor limiting food production at present (Ferry *et al*., 2004). Unlike pathogen-induced responses, plant responses to insect herbivory are often quite dynamic due to the rapid evolution of insect defences against plant protective systems. This interaction between plants and insects often results in a type of competition in terms of defence capability with insects rapidly adjusting to plant defences that limit the efficacy of their feeding and plants in their turn being forced to develop new strategies to limit damage caused by insect herbivory (Stotz *et al*., 1999). Defence responses to insect feeding are often the result of cross-talk between various defence pathways in which the affected plant adapts its response to a specific insect pest by eliciting various signalling molecules from the different pathways to differing degrees (Walling, 2000). Plant defence against insect herbivory can comprise either physical or biochemical means. Insects induce a wound response as a result of damage to the plant caused by chewing. This response is characterized by the production of anti-feedants such as protease inhibitors and alkaloids. Plant volatiles that attract insects that are antagonistic to the plant's current insect pest are also secreted at this stage of attack (Dangl and Jones, 2001). Plant volatiles can act either directly or indirectly to guard plants against mechanical damage inflicted by various insect species. Direct action by volatiles repels certain insect species and in so doing protects the emitting plant while indirect volatile action promotes indirect defences, often by attracting enemies of the feeding insect (Berkov *et al*., 2000; Brouat *et al*., 2000). While wounding that is not caused by herbivory also results in the production of volatiles, a significant fact is that these volatiles are different to those produced in response to insect herbivory (Baldwin *et al*., 2002).

The wound response pathway is mediated by Jasmonic acid (JA) and ethylene which act in concert, stimulating their own respective biosyntheses in addition to facilitating the induction of wound response genes (Xu *et al*., 1994; O'Donell *et al*., 1996). In addition to this, JA and ethylene are reported to aid in pathogen resistance and reduced JA synthesis in certain plant species results in greater susceptibility to certain fungi and bacteria (Rance *et al*., 1998). The role of ethylene in plant defence may be more significant in terms of controlling disease symptom expression than in determining absolute plant resistance or susceptibility phenotypes (Hoffman *et al*., 1999). This lipid-based cascade pathway is elicited by the action of a chemical messenger called systemin (Bergey *et al*., 1996). Subsequent to insect herbivory, the 200 amino acid systemically induced protein, prosystemin, is processed to form systemin, which is translocated via the phloem tissue. Once systemin binds to a receptor complex, an increase in the concentration of the precursor to JA, linolenic acid, is detectable. JA continues the defence response by forming amino acid conjugates that induce wound-response genes, as illustrated in Figure 1.3. (O'Donnell *et al*., 1996; Ryan, 1992).

One set of genes induced by the defence responses in solanaceous species, are those encoding proteinase inhibitors. Proteinase inhibitors are thought to occupy various roles in plants, including acting as storage proteins and as regulators of endogenous proteolytic activity (Ryan, 1990). They are also implicated in various developmental processes, including programmed cell death and the resistance of plants against a variety of insects and pathogens (Solomon *et al*., 1999; Lu *et al*., 1998; Pernas *et al*., 1998).

Plant wounding due to insect herbivory results in the systemic induction of proteinase inhibitors that protect the affected plant against the digestive proteases of herbivorous insects (Ryan, 1992). Proteinase inhibitors are small proteins that inhibit the digestive proteinases of feeding insects. This is highly detrimental to the insect's digestive system and reduces protein uptake to the point that affected insects grow much more slowly and can even die as a result of compromised nutrition. Certain insects, however, circumvent this defence mechanism by increasing their own proteolytic activity or inducing a different set of

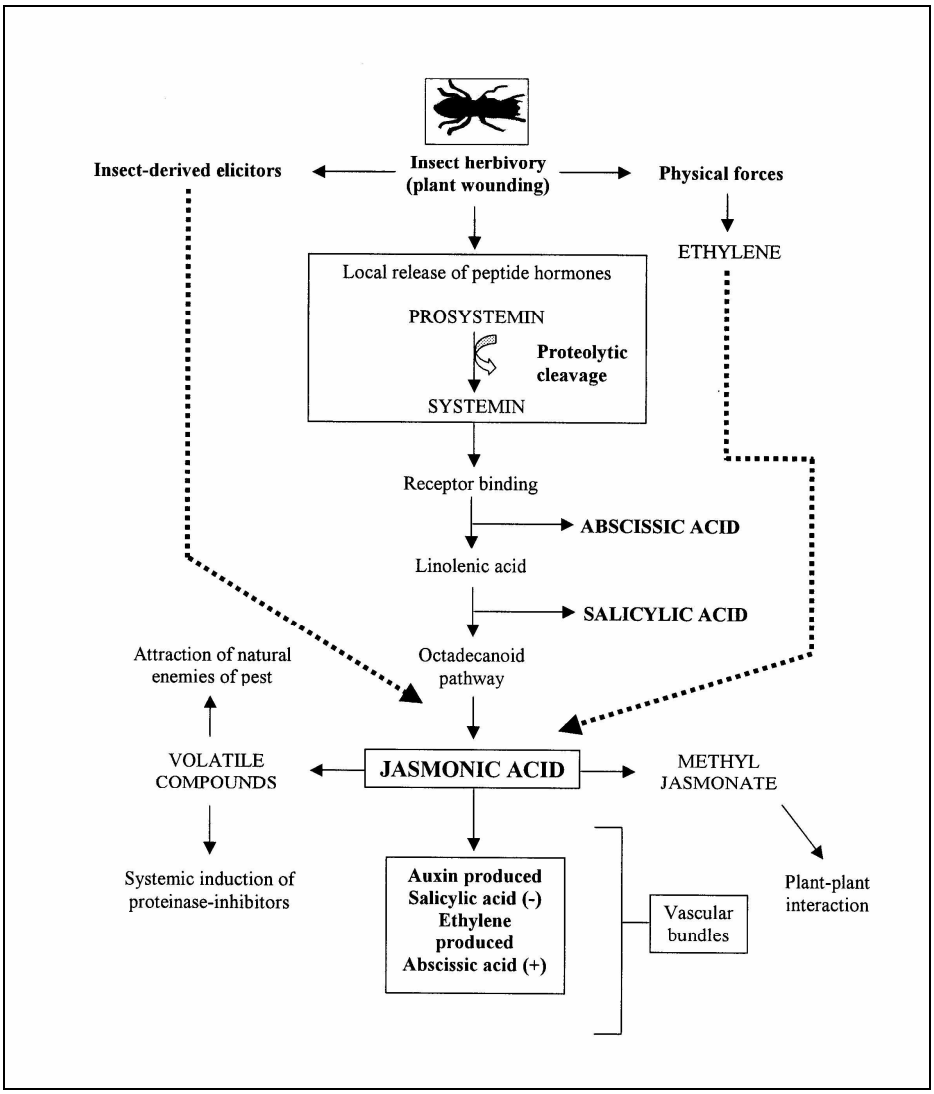


Figure 1.3. Wound response pathway initiated upon insect herbivory. Figure adapted from Ferry *et al.* (2004).

proteolytic enzymes that are insensitive to the action of the proteinase inhibitors produced by the plant that they are currently feeding on (Girard *et al.*, 1998; Gruden *et al.*, 1998). Other investigations indicate that this pathway can also be triggered by fungal elicitors (Boller, 1991).

1.6. Plant responses to stress from phloem feeding insects

Phloem feeding insects are a highly diverse group of insects that feed on plants. At present, the amount of information on the molecular and physiological responses by plants to phloem feeding insect herbivory is very limited (Moran and Thompson, 2001). Current literature indicates that plant responses to aphid feeding are more similar to that induced by bacterial infection as opposed to regular responses to insect herbivory (Botha *et al*., 2006).

1.6.1. Aphids

Aphids (Hemiptera, Aphidoidea) comprise the largest group of phloem feeding insects. There are approximately 4000 aphid species worldwide. These species are characterized by significantly different types of feeding behaviour (Blackman and Eastop, 1994). Aphids utilize specialized stylet-like mouthparts to probe intercellularly through both epidermal and mesophyll cell layers in order to feed on photoassimilates contained in the plant's phloem sieve elements (Pollard, 1972). They cause significant damage to valuable crop plants for two reasons (Dixon, 1998). Firstly, due to their role as vectors of various pathogens that enter the host plant's tissues while various affected aphid species feed on it, and secondly because of the secretion of watery saliva that contains several potential signal-generating enzymes. Some of these include peroxidases, β-glucosidases and a number of as yet uncharacterized enzymes (Matthews, 1991; Miles, 1999). Plants that aphids feed on are known to have higher mRNA levels of *PDF1.2* (the gene encoding defensin) and *LOX2* (the gene encoding lipoxygenase). Both of these genes are induced upon wounding and are involved in the JA signalling pathway (Walling, 2000). An increase in aphid population density has been linked to a resultant decrease in water potential (Zuñiga and Corcuera, 1987). This is significant due to the fact that the lipid composition of membranes may change under the influence of altered water potential caused by aphid feeding (Lynch and Steponkus, 1987; Todd *et al*., 1971). Aphid feeding has been reported to cause a decrease in the efficiency of photosynthesis, an inhibition of plant growth and the decreased synthesis of many metabolites (Todd *et al*., 1971).

While the probing action of phloem feeders such as aphids does not directly constitute herbivory, studies have proposed that phloem feeding insects trigger similar responses in

plants to that of feeding insects in general. This is indicated by the fact that plants that have been exposed to phloem feeders also experience an activation of JA and SA-mediated pathways. However, more current results indicate that plants such as Arabidopsis may be able to distinguish between phloem feeding and insect herbivory and that the responses generated from phloem feeding are specific. It has been suggested that the response of plants to insect herbivory is similar to that occurring during plant-pathogen interactions. Plant responses to aphid feeding include the production of reactive oxygen species (ROS), the cellular accumulation of SA and the subsequent expression of PR proteins (Moran *et al*., 2002). In addition to this, phloem feeding by aphids is characterized by the introduction of foreign substances such as oligosaccharides and glycoproteins occurring in aphid saliva (Moran and Thompson, 2001). Aphids also possess the ability to divert the plant enzyme system in order to produce tryptophan and related metabolites such as hydroxamic acids and indoleacetic acid (Venis, 1979). Hydroxamic acids are constitutive secondary metabolites that are associated with plant resistance to pathogenic fungi and bacteria in addition to certain insects such as the European corn borer (*Ostrinia nubilalis*). The abnormal growth of plants in response to aphid feeding has been linked to levels of the hormone indoleacetic acid (Forrest, 1987).

1.6.2. The Russian Wheat Aphid (*Diuraphis noxia*)

The Russian Wheat Aphid (RWA), *Diuraphis noxia*, is a serious pest of important agricultural crops such as barley and wheat (Du Toit, 1990; Moloi and van der Westhuizen, 2005). In South Africa, it is currently regarded as the most destructive insect pest in wheat (van der Westhuizen *et al*., 1998a). If an RWA infestation is left untreated, crop yield losses between 60 % and 90 % can occur (Du Toit and Walters, 1984).

RWA (Figure 1.4) infestation in *Triticum aestivum L.* ('bread wheat') results in a number of physically apparent symptoms, most notably a prostrate growth habit caused by a rolled flag leaf and the inability of leaves to unfurl normally (Cabrera *et al*., 1995; Messina and Sorenson, 2000). Aphid feeding on the flag leaf causes the developing grain head to become trapped, which interferes with self-pollination and grain-filling (van der Westhuizen *et al*., 1998a). Further symptoms include longitudinal streaking and stunted growth, which leads to a drastic reduction in effective leaf area that subsequently interferes with photosynthesis,

while the injection of a phytotoxin through aphid stylets causes the breakdown of the chloroplast and cellular membrane in susceptible plants. RWA feeding also results in a chlorophyll deficiency, which is often fatal in affected plants (Botha *et al*., 2005; Burd and Elliot, 1996).

Figure 1.4. The Russian wheat aphid (*Diuraphis noxia*) on wheat. Images taken from Internet 1 and Internet 2. See Appendix Five for full webpages.

Structurally, the main area that is affected by RWA feeding is the stacked region in the thylakoid membranes. Although the precise location of damage within these membranes is not known, it is thought to be in the light harvesting complex II (Heng-Moss *et al*., 2003). Physiologically, one of the most readily apparent symptoms of RWA infestation is a marked increase in levels of β-1,3-glucanase and its substrate (Benhamou, 1992). PR proteins are

also found to be upregulated in resistant cultivars in quantities exceeding that found in susceptible varieties. Osmotic shock triggered by injury sustained from aphid probing causes callose deposition at the site of injury. It has been proposed that the differences in metabolites produced in resistant and susceptible wheat cultivars in response to aphid feeding results from the fact that aphids tend to probe more, but feed less, when placed on resistant cultivars (Botha and Matsiliza, 2004). This results in the formation of a greater number of lesions on resistant plants as opposed to susceptible ones (Smith *et al*., 1992). Studies performed by van der Westhuizen and Pretorius have confirmed that RWA infestation on wheat results in differential intercellular protein expression. This was particularly the case in resistant cultivars, where the difference in protein expression was mainly quantitative rather than qualitative (van der Westhuizen and Pretorius, 1996).

Initially, farmers attempted to control RWA infestations by spraying vulnerable crops such as wheat and barley with insecticides (Du Toit, 1989a). This approach was met with limited success due to increased aphid resistance to insecticides as well as the fact that aphid feeding results in curled leaves which shelter the aphids from applied insecticides (van der Westhuizen *et al*., 1998a). The practical challenges in aphid control outlined above have indicated that the development of wheat and barely cultivars which are resistant to RWA feeding may be the most feasible approach in crop protection in terms of efficacy, cost effectiveness and environmental sustainability (Budak, 1999). Previous research on wheat resistant to RWA infestation has focussed mainly on the identification of resistance sources in plants that display tolerance to aphid infestation and observing and documenting the inheritance of this resistance in future generations of wheat (Quick *et al*., 1996). After this fashion, the United States released the first wheat cultivar that is resistant to RWA in 1994 (Quick *et al*., 1996). It has been established that wheat resistance to RWA feeding employs a combination of antibiosis and antixenosis (Du Toit, 1989b). Despite relatively extensive studies, however, the precise defence mechanism of resistant plants remains unknown at present. This greatly hampers progress in the development of new and highly efficient resistant cultivars (Budak *et al*., 1999; van der Westhuizen *et al*., 1998a). The fact that wheat constitutes one of South Africa's major staple crops and, at present, accounts for 21 % of crops on national arable land increases the need for the development of crops displaying effective resistance to RWA infestation (Marasas, 1999).

The development of resistant wheat cultivars is proposed to be the most effective form of crop protection due to the failure of conventional pest control methods, such as insecticides, in the control of RWA infestation on valuable crops (Budak *et al.*, 1999). Resistant wheat cultivars are obtained by means of selective breeding of plants with the desired traits (RWA resistance in the present case). The different types of DN genes have been found to confer resistance to RWA infestation in wheat in a variety of ways. The DN4 gene is known to confer tolerance to RWA feeding in plants that contain it (Quick *et al*., 1991). In contrast, the DN1 gene is known to confer antibiosis resistance towards RWA infestations in plants that contain it (Ni and Quisenberry, 2000). At present, a poor understanding of the mechanism of wheat resistance responses to RWA infestation is hampering the development of effective resistant cultivars, as aphids are able to develop resistance to resistant crops containing only one type of DN gene (Quick *et al.*, 1996).

More recent studies by Botha and colleagues (2006) have offered a more comprehensive outlook on the effect of RWA infestation on resistant and susceptible wheat cultivars. Expressed sequence tags (ESTs) and microarray technology were used to determine differential gene regulation in Tugela (susceptible) and Tugela DN (resistant) wheat stressed by RWA feeding at the 4-leaf stage and their findings are summarised in Figure 1.5. Genes involved in cell maintenance, growth and regulation, plant defence and signalling, photosynthesis, energy production as well as those of unknown function, were found to be differentially regulated in response to RWA feeding on Tugela and Tugela DN wheat (Botha *et al*., 2006). Further studies involving Northern Blot analysis revealed that 29 wheat transcripts were directly involved with the aphid feeding response. These included transcripts for genes encoding proteins involved in direct defence and signalling, oxidative burst (ROS), cell wall degradation, cell maintenance, photosynthesis as well as energy production (Botha *et al*., 2006). A study to determine the nature of these proteins would be useful in elucidating the mechanism of wheat resistance responses to aphid infestation. One of the biggest challenges involved in combating the effect of aphid feeding in wheat and barley plants is the fact that aphids shelter in the abnormally folded leaves and often manage to escape the effects of any pesticides that may be applied to affected plants. Chemical control is thus largely ineffective against aphid predation and the development of resistant plants is viewed as an attractive alternative, both in terms of cost and environmental impact. Due to the complex and often poorly understood nature of plant stress responses, it has been found that an

individual *R gene* occurring in a crop monoculture has little effect on long term resistance in such crops due to the corresponding mutations adopted by plant pathogens.

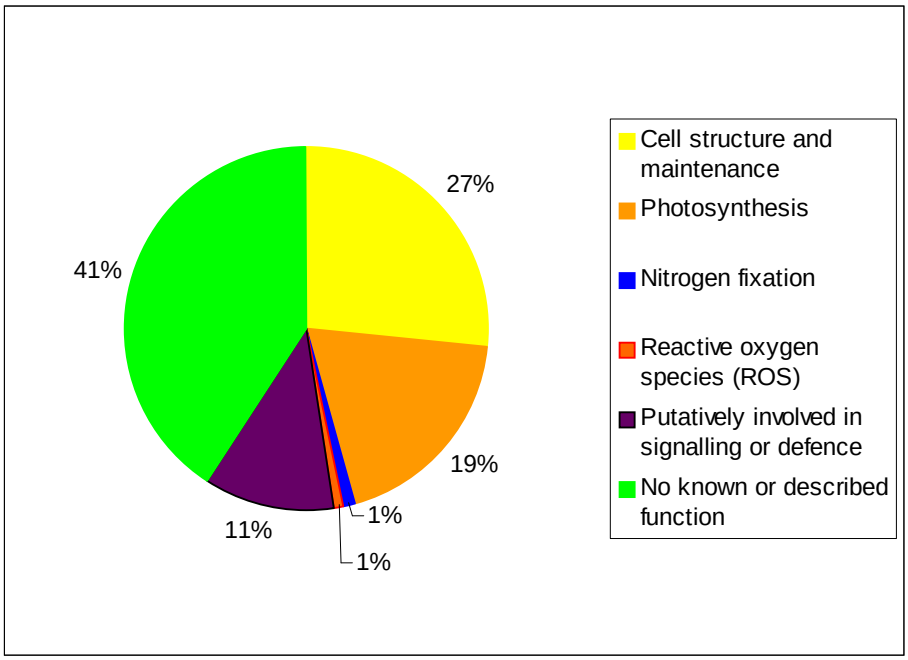


Figure 1.5. Profile of differential gene regulation in Tugela and Tugela DN wheat subjected to RWA feeding. Differential regulation was determined using expressed sequence tags (ESTs) and a combination of suppression subtractive and microarray technologies. ESTs were classified into groups based on their sequence homologies with known sequences from GenBank. Information taken from Botha *et al*. (2006).

It is thus essential to develop resistant crops containing a number of different *R* genes that will allow for a more general resistance to a wider range of pests and facilitating a more enduring remedy for crop losses (Tai *et al*., 1999). Despite contributing only a small percentage of wheat production in South Africa, the first wheat cultivars that have been bred to have resistance to RWA feeding display a significant lowering in economic losses (Du Toit, 1989b). While certain wheat cultivars such as Tugela DN and Betta DN are known to be resistant to the effects of RWA feeding, the development of new improved wheat cultivars is hampered by a poor understanding of the biochemistry of plant resistance to aphid feeding. A detailed investigation of this plant defence system could lead to an enhanced understanding of plant stress responses in general while enabling the development of improved cultivars possessing resistance to the effects of RWA infestation (Moloi and van der Westhuizen, 2005).

1.6.3. The Bird Cherry – Oat aphid (*Rhopalosiphum padi*)

The Bird Cherry-Oat aphid (BCA) occurs in nearly all parts of the world and is a pest of small grains such as wheat and barley (Blackman and Eastop, 2000). BCAs often occur as the dominant species on plants when they infest alongside other species such as the RWA (Elliott *et al*., 1994). Wheat, barley and other small grains infested with BCA incur a yield loss due to the reduction of yield components such as the number of spikelets and seeds (Kieckhefer *et al*., 1995). In addition to this, the BCA is a vector for barley yellow dwarf virus, thus causing further damage to the plants it feeds on (Chapin *et al*., 2001).

Figure 1.6. The Bird Cherry-Oat aphid (*Rhopalosiphum padi L.*) while feeding on the phloem sap of small grain leaves. Images taken from Internet 3 and Internet 4. See Appendix Five for full webpages.

BCA phloem feeding is not as damaging to crops as is RWA feeding. One of the most significant differences between the damage caused by RWA feeding and BCA feeding is that RWA feeding causes a chlorotic reaction in affected plants, which results in compromised photosynthesis, while BCA feeding does not (Ni *et al*., 2001).

1.6.4. Resistance responses to aphid feeding

The resistance response of wheat to aphid feeding is thought to be elicited mainly in the apoplast where several defence-related products accumulate (Bowles, 1990). At present, the plant response to RWA feeding has been found to be a typical HR response that includes the induction of intercellular β-1,3-glucanase (a PR protein), peroxidases and chitinases. This closely resembles the typical plant response to pathogen attack (van der Westhuizen *et al*., 1998b). It has been proposed that the apoplastic accumulation of β-1,3-glucanases is part of the wheat defence response against RWA infestation. RWA feeding on resistant wheat cultivars results in a marked increase in the levels of β-1,3-glucanase which appears to form part of a general HR-type response conferring resistance to affected plants. This could serve as a useful marker to determine whether or not a new cultivar is resistant to the effects of RWA infestation prior to large-scale agricultural use (van der Westhuizen *et al*., 1998a). In addition to β-1-3-glucanases, chitinases are also thought to be involved in the wheat defence response against RWA feeding, as substantially higher amounts of the enzyme's isoforms are known to be present in resistant Tugela DN wheat as opposed to the susceptible Tugela cultivar (Botha *et al*., 1995; van der Westhuizen and Pretorius, 1996). Wheat resistance to RWA infestation is constitutively expressed, and the extent of resistance varies between different cultivars (van der Westhuizen *et al*., 1998b). Resistance proteins are known to be induced within 6 days of the initial aphid infestation, while alterations in the affected plant's ethylene concentrations were observed within 24 hours. Changes in the transcript expression (corresponding to the HR) in wheat stressed with RWA determined by means of microarray technology, have been found to occur within 24 hours after the aphids have commenced feeding (Botha *et al*., 1998). A study on induced protein alterations in wheat (Tugela and Tugela DN) by Bahlmann in 2002 revealed that a 20 kDa band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of wheat stressed by RWA feeding separated into three protein spots during two dimensional gel electrophoresis (2DE). These

spots corresponded to as yet unidentified proteins with isoelectric points (pI) of 5.0, 5.2 and 5.8 (Bahlmann, 2002).

Despite developments in this area of research, the signalling events that lead to the induction of secondary defence reactions during a RWA infestation in wheat are poorly understood at present (Moloi and van der Westhuizen, 2005). A significant point in the study of wheat responses to aphid feeding is that plant responses to a given stress condition differ based on their age and developmental stage of the affected plant (Acreman and Dixon, 1985). It is therefore important to do an analysis of the effect of aphid feeding at different stages of wheat growth in order to gain a more complete understanding of the biochemistry of RWA resistance in wheat. Due to the lack of effective control of aphids on valuable crops by means of conventional methods, it has been proposed that the development of improved resistant cultivars could prove a more effective and economically sound approach. However, the development of such cultivars cannot be achieved as long as the mechanism of aphid resistance in plants is not fully elucidated and understood (Panda and Kush, 1995).

1.7. Plant stress response pathways

Plant stress response pathways are multifunctional and highly complex systems that are either basally transcribed or activated in response to stress conditions. The following section aims to give a brief overview of the current literature on the most significant plant stress response pathways known. Figure 1.7. illustrates the main pathways involved in plant stress responses to biotic stress (induced by either pathogens or herbivores) as well as the cross talk involved between the different pathways.

1.7.1 The Salicylic acid pathway

Salicylic acid (SA) is a key-signalling component in systemic acquired resistance (SAR), the plant defence that confers broad-spectrum resistance to various pathogens (viruses, bacteria and fungi) throughout the whole plant (Durner *et al*., 1997). SA is derived from the shikimate-phenylpropanoid pathway in higher plants (Métraux, 2002). SAR is proposed to confer resistance to plants in which it is activated by either interfering with virus replication

and / or inhibiting the systemic movement of viruses throughout the plant (Naylor *et al.*, 1998).

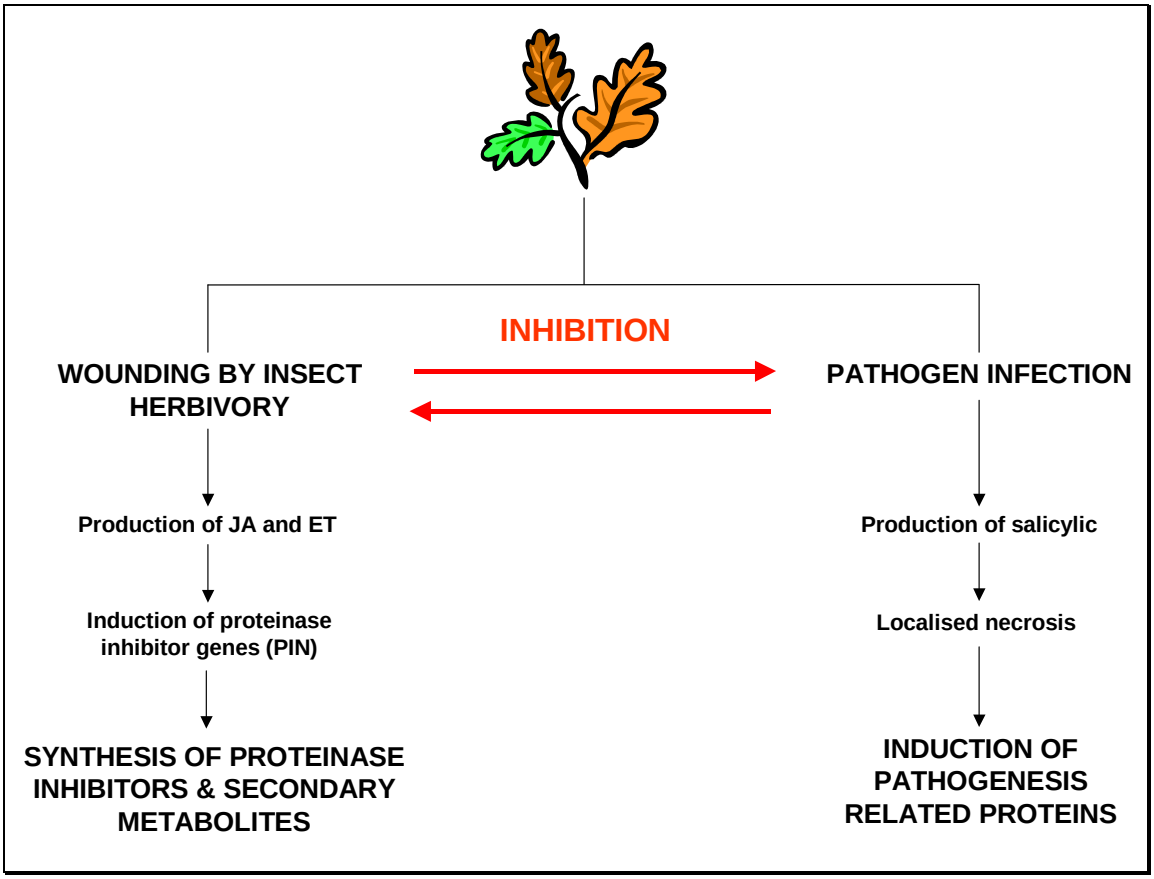


Figure 1.7. Plant stress response pathways in response to pathogen attack or insect herbivory. Note the antagonistic interaction between the salicylic acid and jasmonic acid pathways. JA = jasmonic acid and ET = ethylene. Adapted from Felton and Korth (2000) and Maleck and Dietrich (1999).

SAR usually follows after the initial and rapid HR response that is characterised by responses such as the formation of necrotic lesions around the pathogen and sustained bursts of ROS (Lamb and Dixon, 1997). SAR results in "broad-spectrum, long-lasting immunity in non-infected tissues" in certain plants that have suffered necrogenic pathogen infections (Hunt *et al.*, 1996).

The HR response follows immediately after pathogen attack when elicitors recognise the pathogen at the site of infection, resulting in differential ion fluxes and production of reactive oxygen species (ROS) prior to the induction of a signalling cascade that causes transcription factors for defence genes to be transcribed and activated (Métraux, 2002). SAR can also occur subsequent to necrosis resulting from a compatible interaction between the pathogen and the host plant (van Loon, 1985). The onset of SAR is characterised by an early increase in the levels of salicylic acid throughout the affected plant, resulting in the subsequent expression of pathogenesis related (PR) genes both locally (at the site of infection) and throughout the rest of the plant (Malamy *et al*., 1990; van Loon, 1985; van Loon and Antoniw, 1982). This facilitates the establishment of SAR, which aids in triggering a response on repeated encounters between the infected plant and further pathogens of the same kind, conferring broad-spectrum and enduring immunity in uninfected tissues (Mur *et al*., 1997; Hunt *et al*., 1996). This immunity extends to the pathogen that caused the original infection in addition to a range of pathogens that include viruses, bacteria and fungi (Ryals *et al*., 1995). Exogenous SA is able to induce a resistance that is characterized by reduced virus yields and delays in the onset of symptoms in plants lacking the required genes for resistance to a given pathogen (White *et al*., 1983).

1.7.1.1. Systemic acquired resistance (SAR) responses

Like many other aspects of plant defence, the SAR response is not very well understood. It has long been observed that plants that survive pathogen infections often manage to acquire an increased resistance to any subsequent type of infection (Ryals *et al*., 1995). SAR is a common plant response to necrogenic pathogen infection and results in a more long-term immune response than that provided by the HR, which has a broad-spectrum mode of action. This mode of action enables the host plant to defend uninfected tissue against a range of pathogens including viruses and bacteria (Ryals *et al*., 1995). Salicylic acid and hydrogen peroxide have been reported to play an essential role in this signalling response pathway, although their precise roles remain unknown. It has been proposed that salicylic acid accumulation is crucial to SAR due to the fact that it acts as a catalase inhibitor. The inhibition of catalase is implicated in elevated levels of hydrogen peroxide, which is proposed to act as a secondary messenger of salicylic acid in SAR signal transduction (Chen *et al*., 1993a,b). This was questioned by Hunt and colleagues who proposed that hydrogen peroxide

does not play such a role (Hunt *et al*., 1996). According to Ryals and coworkers, SAR can be conceptually divided into two phases, namely the initiation and the maintenance phase (Figure 1.8.). The initiation phase is transient and allows for all the effectors required for SAR to be activated, while the maintenance phase is more enduring and is involved in maintaining the acquired resistance (Ryals *et al*., 1996).

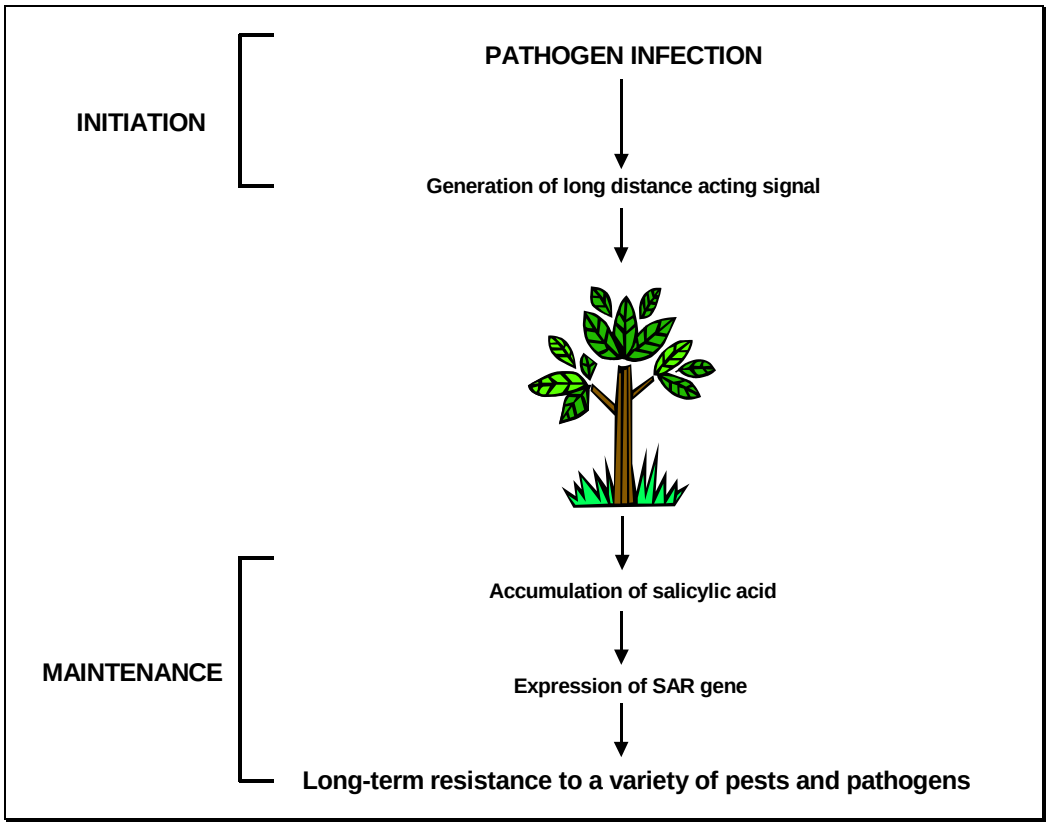


Figure 1.8. Model of the current understanding of the SAR response in plants exposed to pathogen attack. Adapted from Ryals *et al*. (1996).

In order for SAR to be initiated, a plant must first recognise that pathogen infection has taken place. The reaction triggered by pathogens leads to the production of signals in tissues both adjacent to, and distant from, the site of pathogen attack resulting in some form of resistance. Compatible interactions between plants and pathogens also lead to the induction of the SAR response. It is important to note that not all interactions between plants and pathogens lead to the induction of SAR (Kuć, 1982).

Cell death that is associated with pathogen-attack is proposed to be a requirement for the activation of the SAR response as it is often observed in plants after the initial HR response ends with cell death (Hecht and Bateman, 1964). It is therefore proposed that salicylic acid stimulates the SAR pathway downstream from cell death (Hunt *et al*., 1996).

1.7.2. The Jasmonic acid pathway

Jasmonic acid (JA) is involved in plant signal transduction and is synthesized in a seven step enzymatic pathway originating from linolenic acid via an inducible octadecanoid pathway (Schaller and Ryan, 1995). The principle of octadecanoid signalling using JA is conserved in plant stress responses although there are certain exceptions notably those of 12-oxo-phytodienoic acids and 15,16-dihydro-12-oxo-phytodienoic acid (Koch, *et al*., 1999). While occurring ubiquitously in plant tissue, the highest concentration of jasmonates are found in growing tissues such as the shoot apex, root tips, immature fruits and young leaves. They are responsible for a variety of pleitropic effects and cause leaf senescence. The responses attributed to the JA pathway are purported to be caused by alterations in gene expression (Sembdner and Parthier, 1993).

Studies on the differential protein expression of plants displaying JA-induced gene products revealed several significant differences in terms of soluble proteins. Levels of important pre-existing proteins such as Rubisco decreased while several unique polypeptides were synthesized *de novo*, including vegetative storage proteins, cell wall proteins, proteinase inhibitors and enzymes involved in phytoalexin synthesis (Weidhase, *et al*., 1987; Staswick, 1990; Creelman *et al*., 1992; Schaller and Ryan, 1995; Blechert *et al*., 1995). The JA pathway is implicated in a variety of stress conditions including wounding, pathogen and fungal attack, desiccation, osmotic stress, salt stress and nitrogen deficiency, resulting in an appropriate response to the type of stress experienced by the plant. For instance wounding by insect herbivory results in the expression of proteinase inhibitors which interfere with insect digestion and discourage further feeding Pathogen attack and fungal elicitation result in the production of pathogenesis-related proteins (PR) and phyoalexin-synthesizing enzymes, respectively (Wasternack and Parthier, 1997). It is important to note that many jasmonate-induced proteins are highly species specific (Hause *et al*., 1996).

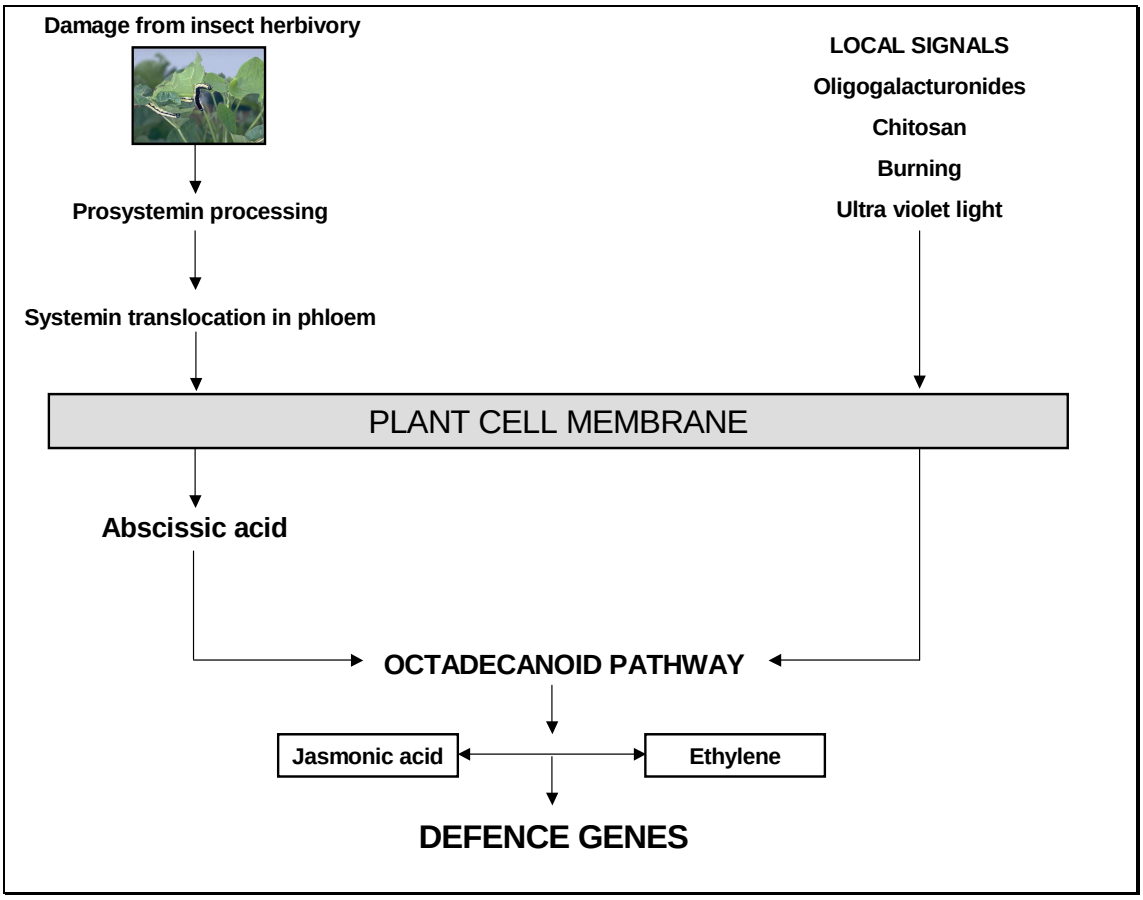


Figure 1.9. The octadecanoid signalling pathway for defence gene expression showing the formation of Jasmonic acid that was proposed after studies of wound responses in Tomato plants. Adapted from Wasternack and Parthier (1997).

JA acts as a mediator between stress perception and stress response by facilitating Jasmonate-induced protein (JIP) synthesis. Experiments using JA inhibitors have shown that the expression of stress proteins is dependent on the *de novo* formation of endogenous jasmonates in sufficient quantity to facilitate the induction of specific polypeptides that are important in various developmentally and environmentally regulated processes (Lehmann *et al*., 1995; Xu *et al*., 1994). Despite the fact that JA plays a central role in plant defense responses against diverse sources of stress that result in membrane damage, such as wounding by insect herbivory, ultraviolet damage and osmotic stress, it plays no significant role in damage incurred by means of light, heavy metals or temperature stress (Blechert *et al*., 1995). The regulators of both salicylic acid and jasmonic acid are present at very low concentrations

under physiological conditions. These regulators are often masked by a wide range of primary and secondary metabolites that occur in far higher quantities (Chiwocha *et al*., 2003).

1.7.3. Ethylene and other volatiles

Ethylene is a phytohormone that acts in concert with JA as a systemic signal of wound-induced gene activation and is found to accumulate upon plant wounding (O'Donnell *et al*., 1996; Chung *et al*., 2001). It is known to induce the accumulation of pathogenesis-related proteins and induces the synthesis of PR-1, β-1,3-glucanase, chitinase, Phe ammonia-lyase, Hyp-rich glycoproteins, osmotin and other defence related proteins (Boller, 1991; Deikman, 1997). Interestingly, ethylene has also been implicated as a causative agent in increasing the severity of disease symptoms in plants. Correlations have been detected in levels of ethylene production and the development of chlorotic and necrotic symptoms (Ben-David *et al*., 1986). In addition to its role in plant defence, ethylene regulates a variety of plant processes including growth and development. These include ripening and senescence (Matoo and Suttle, 1991). Despite the fact that elicitor-induced production of some secondary metabolites requires ethylene as an integral signal, it is not a common signal for the induction of plant secondary metabolites in general (Chung *et al*., 2001; Pan *et al*., 2000).

1.7.4. The role of abscisic acid in plant protection

In contrast to JA, SA and ethylene, which play a significant role in plant defence systems, the role of abscisic acid (ABA) in plant defence is more obscure. Unlike the other plant defence hormones, ABA occupies an important role in several aspects of plant development (Thomma *et al*., 2001; Turner *et al*., 2002). These developmental aspects include: regulation of stomatal opening and closing and the initiation of processes that allow the plant to adapt to various changes in its environment (Shinozaki *et al*., 2003). In spite of this, ABA-controlled signalling appears to have several features in common with various biotic-stress signalling elements that are regulated by JA, SA and ethylene, as summarised in Figure 1.10 (Thomma *et al*., 2001; Turner *et al*., 2002).

Various studies that investigated the effect of exogenously applied ABA, inhibition of ABA biosynthesis and the use of ABA-deficient mutants revealed that decreased levels of ABA

below wild type levels resulted in increased resistance to several pathogens. Increased levels of ABA in plants have been linked to a heightened level of susceptibility to pathogen attack (Henfling *et al*., 1980; Li and Heath, 1990). For example, ABA accumulation increases the susceptibility of rice plants to *Magnaporthe grisea* after a period of cold stress (Koga *et al*., 2004). Increased levels of ABA have, however, also been linked to disease resistance in plants such as tobacco (Whenham *et al*., 1986).

Current research suggests that ABA acts as an indirect interfering agent in disease resistance. Several reactions have been proposed as a result of investigation into ABA levels in stressed plants, although the mechanism whereby ABA levels influence disease progression in plants is obscure. Reduced synthesis of phytoalexins that are implicated in plant disease resistance is one of the factors that could result in decreased resistance on the part of plants that contain high levels of ABA (Henfling *et al*., 1980). It has been proposed that elevated concentrations of ABA exert an antagonistic effect on signalling pathways that are mediated by means of SA action and therefore high ABA concentrations inhibit resistance against pathogens that are controlled by SA signalling pathways (Leon and Sheen, 2003). ABA has also been shown to interact with the ethylene-signalling pathway by inhibiting the production of ethylene (LeNoble *et al*., 2004).

In relation to the JA signalling pathway, ABA displays both synergistic and antagonistic effects as elevated ABA concentrations have been shown to lower transcript levels of JA (and ethylene) responsive defence genes, whereas ABA deficient mutants showed an increase in these genes (Anderson *et al*., 2004). Despite the fact that ABA largely appears to act in an antagonistic fashion to plant stress response pathways, several factors are held in common by plant stress response pathways and ABA signalling. For instance, both the ABA pathway and the HR pathways generate ROS by means of the same NADPH-dependent respiratory burst oxidase homologs (Torres *et al*., 2002; Kwak *et al*., 2003). The highly reactive nitric oxide (NO) molecule has also been found to play a role in both signalling pathways (Wedehenne *et al*., 2004). At present, the ABA pathway is understood to mainly interfere with common plant defence systems against biotic stress. However, in some cases, ABA can also appear to play a defensive role in plant stress responses, as can be seen in the formation of a protective callose during certain stress conditions (Mauch-Mani and Mauch, 2005).

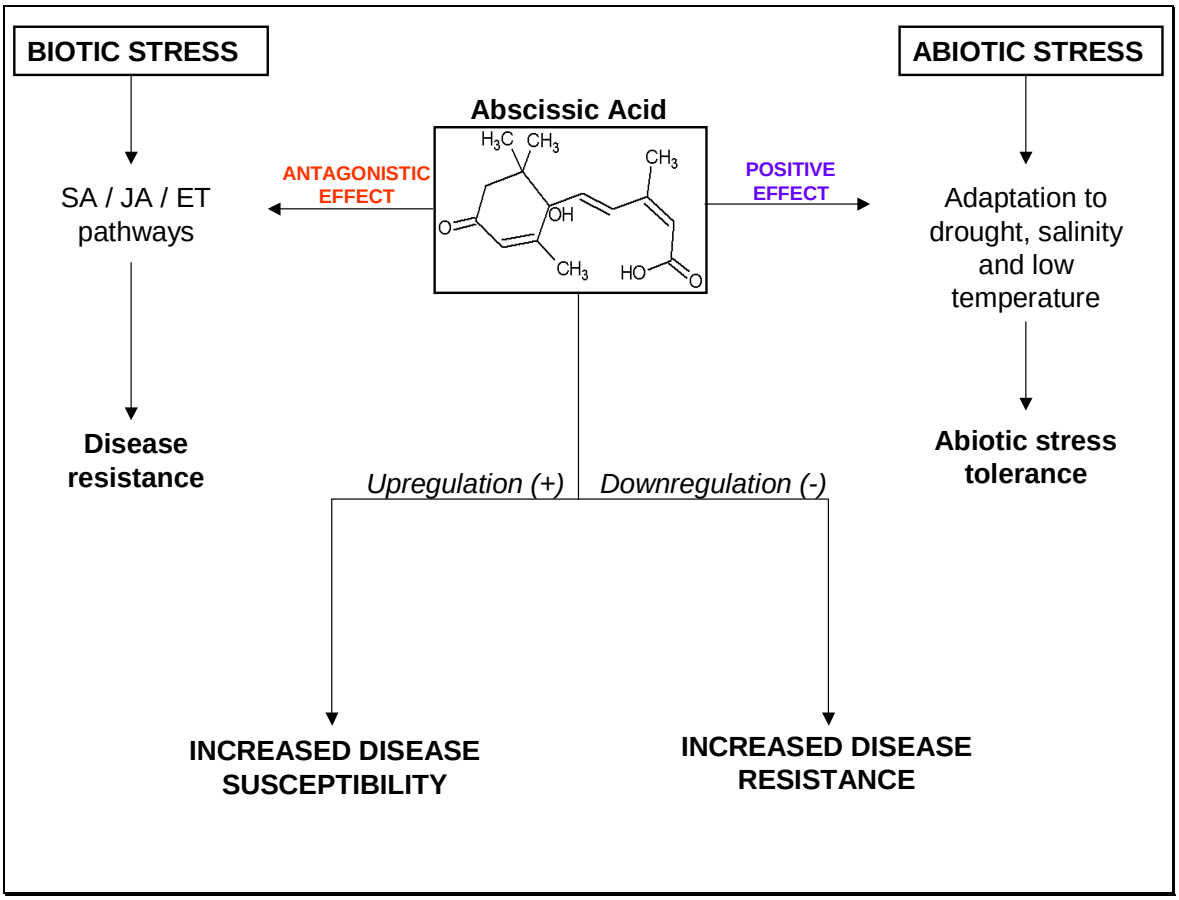


Figure 1.10. The role of abscisic acid in plant defence responses. Adapted from Mauch-Mani and Mauch (2005).

1.8. Advances in the cultivation of genetically modified wheat

1.8.1. Current strategies employed in crop protection

In the year 2000, the global population was estimated to have exceeded 6 billion. If the population growth increases at the same rate, the world's population is estimated to reach approximately 8.5 billion by 2025. This rapid population growth is placing an increased burden on commercial farmers to supply adequate amounts of food for consumers. At present, there is a high demand for crop varieties that possess an increased yield while being resistant to various stress factors in a stable and consistent manner (Khush, 2001). Pests and pathogens cause significant losses in valuable agricultural crops. At present, chemical pesticides comprise the most commonly used method of crop protection used by commercial

farmers. Chemical methods of pest management are often costly and ineffective. An estimated $ 10 billion was spent on chemical insect control methods in 1994. Despite this global crop losses amounted to between 20 – 30 % of the amount produced (Estruch *et al.*, 1996). Increasing pressure from concerned groups worldwide is encouraging researchers to look at developing novel methods of crop protection due to the long-term damage that chemical pesticides exert on both the environment and sometimes, on the wellbeing of humans and animals. In order for a plant defence system to be compatible with modern requirements and standards, it must be beneficial in terms of sustained agriculture in addition to being environmentally friendly (Boulter, 1993).

In modern agriculture, pest control in plants is achieved by means of chemical pesticides, natural biocontrol factors, preventative pest control strategies or transgenic insect-resistant crops, or a combination of these factors (Haq *et al*., 2004).

1.8.2. Alternative methods of crop protection: the role of transgenic plants in modern agriculture

Traditional farming practices have relied on selective breeding to develop improved crops by means of a crossing program between different cultivars which subsequently allows for the systematic selection of plants bearing new and useful traits (Sahrawat, 2003). While being moderately effective, this method does not supply plants with sufficient immunity to fully withstand a variety of pests and pathogens. At present, selective breeding of crops is no longer considered useful due to the inability of this technique to produce plants with effective resistance to various pests whilst maintaining a suitably high yield (Huang *et al*., 2004). The recent advances in molecular biology have opened new avenues to scientists attempting to produce improved crops. These new techniques have enabled the cultivation of transgenic crops containing foreign resistance genes. Such crops are known as genetically modified (GM) crops and are thought to be a promising solution to global food shortages due to insect and pathogen stress (Babu *et al*., 2003). At present, several methods of gene transfer into agriculturally valuable crops are being explored. These include the use of a modified Ti plasmid system in *Agrobacterium tumefaciens* (*Agt*), direct gene transfer including PEG-induced DNA uptake, microinjection of DNA into cells in culture or electroporation and microprojectile bombardment (De Leo and Gallerani, 2002). These techniques have been utilized to produce and cultivate transgenic crops from a number of different species,

including maize, wheat, tomatoes and potatoes, among others (Khush and Brar, 1998). Because insects are able to develop resistance to plant defence mechanisms, the development of resistant crops that contain a multiplicity of defence genes has been emphasised (Datta *et al*., 2002).

1.8.2.1. *Bt* crops and plant resistance

In the development of alternative methods of crop protection, the use of transgenic crops that express foreign insecticidal genes is gaining popularity. Current methods of transgenic crop production rely heavily on the use of transgenic *Bacillus thuringiensis* (*Bt*) crops that contain a *Bt* gene for resistance against several insects. In fact, roughly 98 % of all biopesticides have a *Bt* basis (Benedict and Ring, 2004). The first *Bt* expressing plant was developed approximately 20 years ago. It was developed by introducing an entomotoxic protein from the bacterium *Bacillus thuringiensis* into tobacco plants (Andrews *et al*., 1987). The *Bt* crops that are available at present express Cry protein genes (crystal proteins) and are able to target certain important pests including those that are resistant to conventional pesticides. The advantages of Cry proteins include their high specificity, rapid toxic activity and a short life in the environment. In general, the advantages of such resistant crops are quite numerous, especially in terms of environmental impact. These include the absence of pesticide drift and / or pesticide remnants in the soil in addition to lack of effect observed in non-target species. Possibly the main advantage of transgenic *Bt* crops is the fact that the *Bt* toxin is expressed throughout the plant, and subsequently provides protection to roots, shoots and stems (Benedict and Ring, 2004).

Despite a growing number of *Bt*-containing products on the market, both in the United States and several other countries, some concerns have been raised over the safety of *Bt* proteins for mammals in addition to a number of unanswered questions about the environmental impact of this type of bioinsecticide. Many consumers, scientists and interest groups have expressed concerns about the safety of introducing foreign genes into plants that are meant for human consumption (Vazquez-Padron *et al*., 1999). In addition to this, the use of *Bt* toxin to develop resistant plants is not without its demerits. One of these is the fact that the toxin remains in the plant throughout the growing season, thus selecting for the development of insect resistance (Moar *et al*., 1995). A further limitation of *Bt* crops is the fact that they are

known to effectively control relatively few insect species. For instance, no *Bt* toxin has yet been discovered with aphid toxicity, leaving much to be desired in areas of agriculture focussing on crops that regularly experience aphid infestations (Oppert *et al*., 1997).

An alternative strategy to the use of foreign genes in the development of resistant plants is to utilize the plants endogenous defence systems and manipulating these systems by upregulating the production of defence proteins. The introduction of insect control genes from other plants into a commercial crop of interest in order to facilitate the development of resistance to a given pest is an alternative technique to the use of *Bt* toxins (Peferoen, 1997).

1.8.2.2. Advances in the development of resistant wheat (*Triticum aestivum L.*)

Wheat is a member of the Triticeae cereal family and is one of the most valuable food crops in the world. It is arguably the most significant source of plant protein in the modern diet (Sahrawat, 2003). Biotic pests, such as aphids, are responsible for significant losses in wheat production every year. This loss, both in terms of economic and social impact, increases the urgency to develop suitable cultivars that are resistant to biotic pests such as RWA and which are able to maintain this resistance in subsequent generations. Traditional methods of crop improvement and engineering for resistance rely heavily on an extensive crossing programme, which requires a lengthy screening process to identify cultivars with the desired traits (Bedó *et al*., 1998; McIntosh, 1998). While some success has been achieved with this method in wheat, the efforts of plant breeders are currently reaching their limit as some aspects of the plants, especially yield are not improved even with extensive breeding (Huang *et al*., 2002). It is in light of this that new methods are being employed in the development of resistant wheat crops.

Weeks and colleagues reported the introduction of genes into immature bread wheat (*Triticum aestivum L*.) embryos (Weeks *et al*., 1993). This was accomplished by means of particle bombardment. While this process was effective, it was by no means highly efficient, and current research focuses on optimizing this transformation process to increase its efficiency (Becker *et al*., 1994; Alpeter *et al*., 1996). Transferring foreign DNA into wheat by means of *Agrobacterium tumefaciens* (Agt) is an attractive alternative to particle bombardment. This technique has several advantages, the most important being that it allows for the stable

integration of long DNA molecules with defined ends. Another attractive feature of transformation with Agt is that its use allows for the generation of lines that are free from selectable marker genes, thus minimizing concern of any possible harmful effects if such crops are used for human consumption (Sandström *et al*., 1989; Jones *et al*., 2005).

Resistance genes against RWA infestation are located mainly on the D-genome of wheat and are introduced into various cultivars in order to develop resistance (Liu *et al*., 2001). Wheat resistance or tolerance to RWA infestation comprises antibiosis and / or antixenosis although the mechanism whereby this is achieved is unknown at present (Du Toit, 1989b). Betta DN is an isogenic cultivar of Betta wheat that is resistant to RWA phloem feeding due to the presence of a DN gene. It has a moderate yield potential (10 % higher than the RWA susceptible cultivar Betta) and a very good milling and baking quality. The Wheat Technical Committee in South Africa uses Betta DN as a quality standard when new cultivars are released and is marketed in South Africa by Sensako (Internet 5). An understanding of the mechanism of resistance in this cultivar and the difference in response to RWA feeding between Betta and Betta DN at the proteomic level could play an important role in facilitating the development of new improved resistant cultivars.

The design of resistant cultivars of valuable and economically significant crops should ideally be supported by a sound knowledge and understanding of plant stress responses and resistance mechanisms. This is largely due to the fact that insects rapidly develop resistance to the foreign genes that have been inserted in a given plant and the insertion of multiple resistance genes can be used to overcome this problem (Sharma and Ortiz, 2000).

1.9. Conclusions

Plant stress responses are highly complex and poorly understood systems that enable plants to overcome stresses from biotic and abiotic sources. The great annual losses of valuable crops to insect pests and abiotic stresses, and the inefficacy of current methods of insect pest control, necessitate the development of plants possessing resistance to a variety of stress factors. Wheat is one of the most important and economically valuable crops produced globally. RWA and BCA infestation results in huge crop losses every year and it has been proposed that the cultivation of wheat cultivars that are resistant to the effects of aphid feeding may be a viable alternative to the use of ineffective, expensive and damaging

pesticides. At present, the mechanism of wheat defence responses against aphid infestation have not been well characterised. A study such as the one outlined in this thesis will serve as a foundation to enhance the present understanding of such defence responses, and subsequent development of aphid resistant wheat cultivars.

RESEARCH MOTIVATION AND HYPOTHESIS

Problem statement

Plants possess physical and biochemical defence mechanisms to prevent damage induced by abiotic and biotic stress. Insect pests such as the Russian Wheat Aphid cause significant damage to commercially valuable crops such as barley and wheat. Despite the identification of ten different genes as agents of resistance to RWA infestation and damage, the mechanism of the defence reaction as well as the identification of proteins involved is unknown. In addition, the molecular basis of plant resistance mechanisms to phloem-feeding insects has not been well characterized.

Hypothesis

Russian wheat aphid infestation on *Triticum aestivum L.* plants results in a stress response that causes the differential regulation of various proteins within the plant. Bird Cherry-Oat aphid infestation results in less significant damage to wheat structure than Russian wheat infestation, but also results in the differential expression of proteins. The identification and determination of the function of differentially expressed proteins in response to stress imposed by phloem-feeding insects would enhance our understanding of plant stress response pathways and thereby facilitate the development of new crop species possessing resistance genes.

Aim

The identification of differentially expressed proteins in susceptible and resistant wheat (*Triticum aestivum L.* cv Betta and Betta-Dn) plants in response to stress induced by Russian wheat aphid (*Diuraphis noxia, Mordvilko*) and Bird Cherry-Oat aphid (*Rhopalosiphum padi*) feeding.

Objectives

1. Cultivation of *Triticum aestivum L.* cultivar that is susceptible to feeding damage by the Russian wheat aphid in addition to a resistant cultivar that can withstand the damage caused by aphid feeding
2. Establishment of a homogenous and viable Russian wheat aphid colony
3. Optimization of protein extraction from wheat plants
4. Optimization of 2DE gel electrophoresis on proteins obtained from stressed and unstressed susceptible and resistant wheat cultivars (Betta and Betta DN)
5. Comparative 2DE gel electrophoresis on total protein samples from stressed and unstressed resistant and susceptible wheat cultivars (Betta and Betta DN)
6. Hypothetical identification of differentially expressed proteins in stressed and unstressed wheat cultivars (both susceptible and resistant) in response to stress induced by Russian wheat aphid and Bird Cherry-Oat aphid feeding

Chapter 2

Optimization of a two-dimensional gel electrophoresis (2 DE) method to determine the differential protein expression in wheat

2.1. Introduction

Two-dimensional gel electrophoresis (2DE) is a powerful tool employed in proteomics for the purification and characterization of proteins (Chinnasamy and Rampitsch, 2006). 2DE can be used to visualise and map the proteome of a given tissue or organ sample. A maximum number of protein spots and a high level of resolution characterize optimal gels. The use of 2DE facilitates the resolution of complex mixtures of protein which allows for the identification of differential protein regulation during various conditions within the life of a given tissue, organ or plant (Finnie *et al.*, 2002). The power of this technique lies in its ability to separate protein mixtures on the basis of both their net charge and their molecular mass. Separation in the first dimension occurs on the basis of net charge; the purified protein mixture of interest is rehydrated onto an immobilized pH gradient strip (IPG strip) containing an immobilised pH gradient in which proteins migrate to their isoelectric points under an applied electrical potential difference. A high voltage ramp is applied and proteins migrate down the strip and resolve at their individual isoelectric points (Görg *et al.*, 2000). The isoelectric point of a protein is a point at which the protein in question possesses an overall zero net charge. This value is unique for each protein, and thus forms a useful basis for separation of proteins in a complex mixture. Separation in the second dimension relies on a difference in mass between the different proteins in a complex mixture by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Görg *et al.*, 2000; Herbert *et al.*, 1997). The different proteins resolve as spots on the second dimension gel and can subsequently be identified and analysed by using either mass spectrometry (MS) or N-terminal sequencing coupled with bioinformatic tools and software such as PDQuest™ or Progenesis PG 200 v2006 (Skylas *et al.*, 2005).

2DE has previously been employed for various analyses of the wheat proteome. This technique has been employed to study wheat endosperm proteins (Hurkman and Tanaka, 2004; Mak *et al.*, 2006), the differential expression of wheat root proteins in response to different levels of nitrogen in feeding solutions (Bahrman *et al.*, 2005), mitochondrial proteins from different wheat tissues (Rios *et al*, 1991) as well as aspects of wheat genetics (Thiellement *et al.*, 1987). A qualitative and quantitative analysis of differential protein expression in wheat in response to heat shock as well as cold and drought has also been

undertaken (Bahrman *et al*., 2004). Most of the studies above have been carried out on milled wheat grain (flour) and few, if any, have made use of wheat leaf tissue. This is due to the fact that protein content is closely related to the quality of flour for bread making, which is of considerable interest to commercial farmers (Dougherty *et al*., 1989).

Certain factors such as the method employed to extract total proteins from the sample of interest and solubilization buffers used to resuspend these proteins, are crucial to the success of any 2DE analysis (Chinnasamy and Rampitsch, 2006). Sample preparation is often the most important step and must be optimized in order to obtain efficient extraction of the maximum number of proteins in a given sample, while retaining the fewest number of contaminating compounds possible. Traditional extraction methods rely on acetone and trichloroacetic acid precipitation (Skylas *et al*., 2000). Successful 2DE analysis is highly dependent on reproducible methods leading to low variability between gels in the same experiment. Minimisation of variability in sample preparation can be facilitated by using protein extraction and 2DE clean-up kits from suppliers dealing in products specifically designed for proteomics research applications. 2DE is a useful technique to study the differential protein expression in response to various stress factors because of its high resolution, (Thiellement *et al*., 1987). For the purposes of the present study, we used 2DE to identify the differential protein expression that occurs when susceptible and resistant wheat plants (*Triticum aestivum* L.) cv. Betta and Betta DN are exposed to phloem feeding by the Russian wheat aphid (RWA; *Diuraphis noxia*, Mordvilko) and the Bird Cherry-Oat aphid (BCA; *Rhopalosiphum padi*). This chapter outlines the development and optimization of a 2DE method used to determine the differential protein expression obtained in response to aphid feeding on resistant and susceptible wheat.

2.2. Materials and Methods

2.2.1. Materials

Wheat seeds (*Tritcum aestivum L*.) cv. Betta and Betta DN were obtained from the Agricultural Research Council of South Africa (Bethlehem, South Africa). One of several *DN* genes (*DN1-DN6* and a number of uncharacterised *DN* genes) confer resistance to the

Russian wheat aphid (*Diuraphis noxia;* Mordvilko) and wheat cultivars bred to contain any one of these *DN* genes or a number of them are said to be RWA-resistant wheat cultivars (Heyns *et al*., 2006; Liu *et al*., 2005). Betta DN is an RWA- resistant wheat cultivar because it has been bred to contain a *DN* gene, while Betta is an RWA-susceptible cultivar due to the absence of a *DN* gene. Potting soil was purchased from Green Fingers Nursery in Port Elizabeth. The total protein extraction, reduction alkylation, 2D clean up and protein assay kits were purchased from Bio-Rad (U.S.A.). Liquid nitrogen was obtained from the Rhodes University Department of Chemistry (Grahamstown, South Africa). Bovine Serum Albumen Fraction V was purchased from Roche (Switzerland). PageBlue™ protein staining solution was a free sample from Fermentas (Canada) while the SilverSnap® Stain kit II silver staining kit was purchased from Pierce (U.S.A.). Isolectric focussing equipment was from Invitrogen (France) while immobilized pH gradient strips were from Bio-Rad (U.S.A.). Protein molecular mass markers were purchased from PEQLab (Germany). All other reagents for buffers and gel reactions were purchased from Sigma and Bio-Rad (U.S.A.).

2.2.2. Overview of the procedure employed to optimise a 2DE protocol for the determination of differential protein expression in wheat leaf tissue

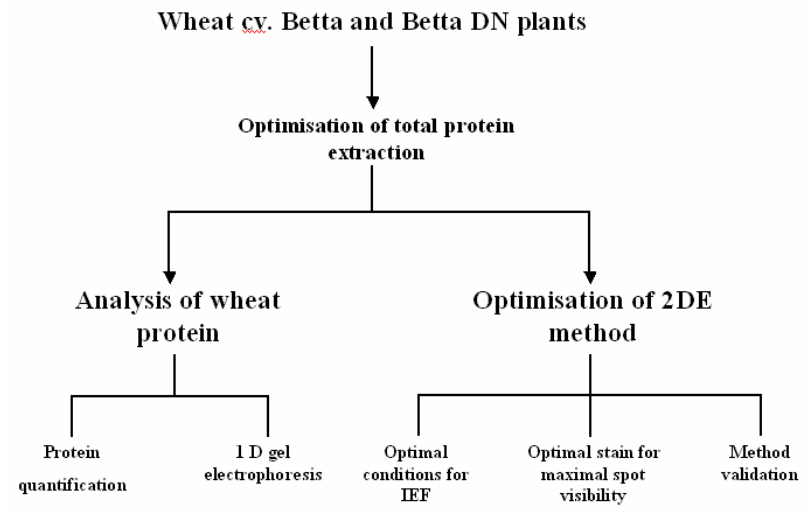


Figure 2.1. Overview of factors investigated during the optimisation of a 2DE method for the determination of differentially expressed proteins in wheat leaf tissue in response to aphid feeding.

2.2.3. Cultivation of wheat cv. Betta and Betta DN plants

Wheat seeds were germinated overnight in a petri dish on filter paper prior to planting in a 70:30 mix of potting soil (Green Fingers Nursery, Port Elizabeth) and vermiculite. Each pot contained roughly 20 wheat seeds and plants were grown under set conditions in a controlled environment chamber (Conviron S10H; Controlled Environments Limited, Winnipeg, Manitoba Canada; Analytical Scientific Instruments CC South Africa) at the Rhodes University Botany Department. The Conviron was maintained at a constant temperature of 24 ± 1°C at a relative humidity of 70 % with a 16 hour photoperiod. Plant nutrient status was maintained with a 75 % solution of Long Ashton nutrient mix (Appendix 3, Table 8.1.), which was administered every two days.

2.2.4. Optimization of total protein extraction from wheat

Two-leaf stage wheat (14 day old wheat plants) samples were homogenized in liquid nitrogen prior to extraction of total protein from different masses of leaf tissue (0.1g, 0.25g, 0.5 g and 0.75 g) using the ReadyPrep™ Protein Extraction Kit (Total Protein) (Bio-Rad, U.S.A.) as per kit manufacturer's instructions. Briefly, homogenized leaf tissue was suspended in 1 ml ReadyPrep 2-D Rehydration / Sample buffer 1 (1 ml; 7 M urea, 2 M thiourea, 1 % (w / v) amidosulfobetaine-14 (ASB-14) detergent, 40 mM Tris base and 0.001 % Bromophenol blue) to which 10 µl TBP reducing agent (200 mM tributylphosphine in 1-methyl-2-pyrrolidinone) was added. Samples were sonicated in four bursts of 30 seconds each prior to centrifugation at 14 000 g for 30 minutes. After centrifugation, the supernatant was retained and reduced and alkylated using the ReadyPrep™ Reduction-Alkylation Kit (Bio-Rad, U.S.A.), while the pellet was discarded. Proteins were reduced and alkylated to disrupt protein disulphide bonds and in order to prevent different oxidation states in sample proteins (Herbert *et al*., 2001; Taylor *et al*., 2000). Protease inhibitors were not added as they are not required with the protein extraction kit used in this experiment. The optimal amount of leaf tissue for the purpose of protein extraction was determined by assaying the protein obtained from each leaf mass and determining which was optimal in terms of the maximum amount of protein extracted in terms of sample manageability, as illustrated in Figure 2.2. In terms of sample

size, manageability and the highest amount of protein extracted, 0.1g leaf sample yielded the best results and was used in all subsequent experiments. Higher amounts of wheat leaf tissue did yield higher amounts of protein but were difficult to manage and obtain in terms of the plant size used during the course of the present investigation.

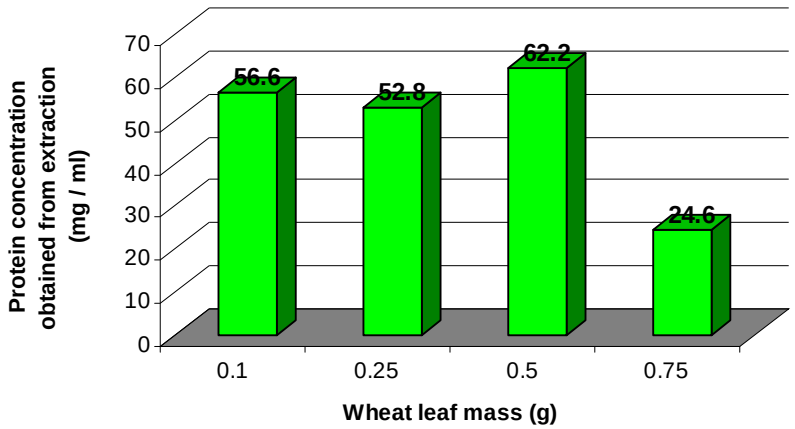


Figure 2.2. Protein yield from variable wheat leaf mass using the ReadyPrep™ Protein Extraction Kit (Total Protein) from Bio-Rad (U.S.A.)

2.2.5. Determination of protein content of wheat samples

The protein content of all wheat samples, both before and after the reduction-alkylation step was determined by means of the RC / DC protein assay (Bio-Rad, U.S.A.), which is a modification of the Folin-Lowry assay (Lowry *et al.*, 1951). Sample protein content was determined by interpolation from a standard curve using bovine serum albumen Fraction V as a standard.

2.2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total protein from wheat leaves

Wheat protein samples were resolved using SDS-PAGE according to the method outlined in the Mini-Protean® 3 Cell Instruction Manual (Bio-Rad, U.S.A.) which is a modification of the method outlined by Laemmli (Laemmli, 1970). SDS-PAGE and second dimension 2DE

gels were resolved using the Bio-Rad Mini-Protean® 3 gel electrophoresis system. Total protein from wheat leaf extract was added in a ratio of 1:2.5 to SDS-PAGE sample buffer (0.0625 M Tris, pH 6.8, 10 % (v/v) glycerol, 2 % SDS, 5 % β-mercaptoethanol, 0.05 % bromophenol blue) prior to resolving on 12 % acrylamide separating gels (0.375 M Tris, pH 8.8, 0.1 % SDS, 12 % acrylamide) prepared as outlined in Appendix 2 (Chapter 7). Gel polymerisation was induced upon addition of 0.05 % ammonium persulphate (APS) and 0.005 % N,N,N'N'-tetramethylethylenediamine (TEMED). Gels were resolved for 45 minutes at 200 V using a Bio-Rad PowerPac™ HV power supply in running buffer (25 mM Tris, 192 mM glycine and 1 % SDS) prior to staining with Coomassie (0.1 % Coomassie Brilliant Blue G 250 in 45 % methanol and 10 % acetic acid) and destaining (25 % methanol and 10 % acetic acid). Duplicate gels were resolved and stained with the SilverSNAP® Stain Kit II (Pierce, U.S.A.). Gels were scanned on an HP Scanjet 3800 and dried prior to analysis.

2.2.7. Two dimensional gel electrophoresis optimization

2.2.7.1. Sample preparation for 2DE

Wheat samples were prepared as described in 2.2.4. and cleaned up using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, U.S.A.). Subsequently, the total protein pellet obtained from the 2DE clean up reaction, containing 4.416 mg of total wheat protein, was resuspended in 2-D rehydration buffer (250 µl; 9 M Urea, 2 % Triton-X 100, 2 % Bio-Lyte 3-10 buffer, 0.1% Bromophenol Blue and 0.02 g dithiothreitol) and applied to the chambers of a Zoom® IPGRunner™ Cassette prior to careful insertion of an immobilised pH gradient (IPG) strip, with the gel side up, into the protein and buffer solution in the chamber. The broad-range (7 cm; pH 3-10 NL) ReadyStrip™ IPG strips were rehydrated overnight in 160 µl of 2-D rehydration buffer containing the wheat total protein sample after clean-up (containing between 124 – 150 µg protein), as per manufacturer's instructions. Once adequately rehydrated, the IPG strips were focussed by performing isoelectric focussing (IEF) in distilled water under variable electrical potential difference conditions as outlined in 2.2.7.2. The amount of protein loaded onto the IPG strips was optimised in order to avoid overloading of gels and poor spot resolution. All optimised 2DE gels were resolved containing 124 – 150 µg

protein (0.124 – 0.150 mg) of protein, which was found to be an optimal protein load for the silver stain used during the course of the 2DE experiments. The addition of the Bio-Lyte 3-10 (ampholyte) buffer to the 2DE sample rehydration buffer facilitated to compensate for a lack of salt in the sample which might cause certain proteins to precipitate at their given pI value. Ampholytes added to the first dimension sample buffer greatly improve the resolution of the second dimension gel by allowing for the formation of reproducible, linear pH gradients. The wide-range Bio-Lyte 3-10 employed during the course of this work has a working pH range of 3.5-9.5 and is useful for determining the over all distribution of proteins in terms of their pI values between these pH values although optimisation would require the use of a narrow-range buffer for improved resolution and spot identification.

2.2.7.2. Determination of optimal conditions and power source for isoelectric focussing

Rehydrated broad-range ReadyStrip™ 7 cm, pH 3-10NL (Bio-Rad, U.S.A.) IPG strips were subjected to IEF under different conditions to determine the optimal focussing parameters for maximal protein spot resolution. Initially, samples from 21-day old wheat (4-leaf stage) were prepared as outlined above in section 2.2.7.1 and protein content was determined as outlined in section 2.2.5. IEF was performed using a Consort E815 power supply with a step voltage. The applied electric potential (V) was increased in a step-wise fashion as follows: 200 V (10 minutes), 450 V (10 minutes), 750 V (10 minutes) and 1000 V (480 minutes). A second IEF method utilized a Bio-Rad PowerPac™ HV power supply, and featured a reduced focussing time. The applied electric potential (V) was increased in a continuous ramp fashion as follows: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The current was maintained at 5mA throughout the procedure. The detailed conditions for the above procedures are listed in Appendix 6.

2.2.7.3. Determination of optimal staining method to maximise protein spot visibility

The choice of a suitable stain is crucial for the success of 2DE experiments. Ideally, a 2DE stain must allow for qualitative analysis while simultaneously being sensitive enough to allow for the visualisation of low abundance proteins. In addition to this, a successful stain should possess a wide linearity and dynamic range while being affordable to the researcher

(Westermeier and Naven, 2002). A number of stains were used to visualise the 2DE gels in order to determine which stain would allow for the detection of a maximum of spots under the given running conditions. The following stains were investigated: Coomassie Brilliant Blue (40 % methanol, 0.7 % glacial acetic acid, 0.075 % Coomassie brilliant blue R250), PageBlue™ Protein Staining solution (Fermentas, Canada) and SilverSnap® Stain Kit II (Pierce, U.S.A.). The detailed method for these staining procedures is outlined in Appendix two in Chapter 8. Coomassie is an inexpensive staining method but lacks the sensitivity of silver stain and is considered an unreliable staining method for 2DE applications due to the fact that some protein spots destain faster than the gel background during the destaining step, which adds to a loss of protein spots prior to analysis (Neuhoff *et al*., 1988). PageBlue™ Protein Staining solution (Fermentas, Canada) contains Coomassie Brilliant Blue but does not contain hazardous substances such as methanol and is rapid as gels do not require destaining. It is also more sensitive than Coomassie stain. Silver staining is much more sensitive than Coomassie Brilliant Blue, but does present some difficulties due to its limited linear quantification range in addition to the formation of artefacts on certain gels (Rabilloud, 1992). The stain thus yielded the highest spot visibility at the lowest cost was used for all subsequent analyses.

2.2.7.4. Determination of the reproducibility of the 2DE protocol used in this study

Unstressed two-week-old Betta wheat was treated as outlined in 2.2.7.1. The total protein extracts were reduced and alkylated and cleaned up as described prior to performing IEF in triplicate on a Bio-Rad PowerPac™ HV power supply. The protein load on each strip was between 1.38-1.5 mg protein. The applied electric potential (V) was increased in a continuous ramp fashion as follows: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The second dimension was resolved on 12 % SDS-PAGE gels at 200 V for 45 minutes as outlined in 2.2.6. Gels were stained using the SilverSnap® Stain Kit II (Pierce, U.S.A.) prior to imaging on a ChemiDoc ™ EQ gel documentation system (Bio-Rad, U.S.A.). The reproducibility samples were performed in triplicate to ensure that the 2DE protocol utilized yielded stable and reproducible results. The images obtained were analysed using PDQuest™ Basic Software Version 8.0 (Bio-Rad, U.S.A.).

2.2.7.5. 2DE gels analysis using PDQuest™ Basic Software Version 8.0 (Bio-Rad)

Triplicate gels were studied in order to determine the number of visible spots as well as the spot variance between gels. Gels were grouped and analysed using the Spot Detection Parameter Wizard with a sensitivity of 0.49 while allowance was made for horizontal and vertical streak removal. The gel software analysis package relied on manual identification of faint spots, large spots and the largest spot cluster, allowing for some user input in terms of parameters. A speckle filter set at 50 was applied in order to remove speckles from the gel. This was considered necessary despite the fact that some small and faint spots may have been lost in the process. A Gaussian statistical model was used to detect and fit spots while a local regression model was selected for data normalisation. A spot that was not up or down regulated in either the control or stress gels at any point during the procedure was selected as a loading control in order to verify the validity of the assignation of up or down regulation by the PDQuest™ software.

2.3. Results

2.3.1. Wheat plants

2.3.1.1. Plant growth

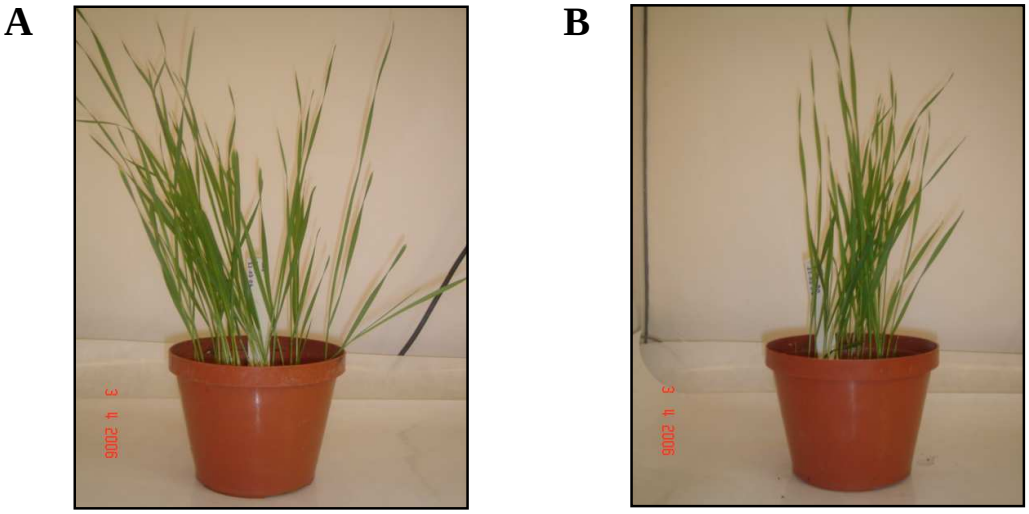


Figure 2.3. Wheat (*Triticum aestivum L.)* cv. Betta (A) and Betta DN (B) plants at the two-leaf stage (14 days after planting).

Plants were maintained at 24 ± 1 °C in a Conviron S10H growth cabinet with a light / dark cycle of 16 / 8 hours and a relative humidity of 70 %. Plant nutrient status was maintained with 75 % Long Ashton nutrient mix on a 48-hr basis. Betta plants (A) had an average length of 34 cm, while their resistant counterparts, Betta DN (B) had an average length of 26.3 cm from root to tip.

According to the South African Agricultural Research Council (ARC), Betta DN is a cultivar that is suited to low to medium potential conditions. It is resistant to RWA infestation and has an average grain yield that is 10 % higher than that of its susceptible counterpart under regular field conditions (Internet 1). The wheat plants exhibited stable growth, although the resistant plants were slightly smaller in size than the susceptible ones, as shown in Figure 2.3. above. Since Betta and Betta DN are isogenic cultivars, this size difference could be attributed to

resistance costs due to the incorporation of the *DN* resistance gene in Betta DN as discussed in the materials section (section 2.2.1).

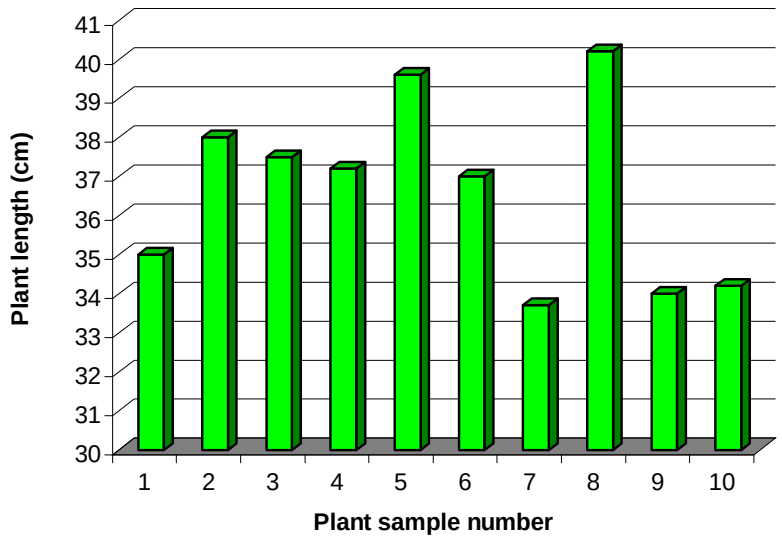


Figure 2.4. Length variability in susceptible Betta wheat plants taken from the same pot and grown under identical conditions at the 4-leaf (30 day) growth stage. Resistant Betta DN plants showed similar variance in height but were shorter on average (data not shown.

In order to study the variability of the wheat plants investigated, the length of all plants occurring in the same pot after a 30-day period were determined by measuring the plants from root to tip. Due to time constraints, and the secondary nature of this investigation in terms of the project, this was only performed once and hence no statistical data is available. A large variation in height was found (Figure 2.4.) indicating that not all plants grown under the same conditions would reach the same size. It was, however, observed that all wheat plants at the 4-leaf growth stage would have a height of no less than 33.5 cm and of no more than 40 cm indicating that a maximum discrepancy of around 10 cm can be anticipated under optimized growing conditions. It is important to note that each wheat seed planted was unique and that the difference in height with time could be the result of various genetic factors. These differences result during breeding of the wheat cultivars (Sharma *et al*., 2005). A high density of plants in each pot was maintained in order to maximise wheat production for the

optimization of protein extraction that was performed prior to the commencement of 2DE analysis. Short leaves were observed to contain roughly the same amount of total protein as long leaves did when equivalent leaf masses were analysed for their total protein content. The protein content of the wheat leaf tissue did, however, vary with time. Both Betta and Betta DN cultivars had higher protein content after 4 weeks growth than they did at 2 weeks, as shown in Table 2.1. below, although it was noted that Betta DN had a higher extractible protein content throughout the duration of the current procedure.

Table 2.1. The relationship between wheat age and wheat leaf protein content for Betta and Betta DN wheat at two different growth stages

Wheat cultivar	**Days after planting**	**Length (root to tip [cm])**	**Mass used for total protein extraction (g)[a]**	**Protein concentration (mg / ml)**
Betta	15	31	0.104	30.77
Betta	29	47.5	0.122	45.16
Betta DN	15	29.5	0.104	39.19
Betta DN	29	35	0.121	49.87

a- Refers to wet leaf weight

2.3.2. Optimization of total protein extraction from wheat

The ReadyPrep™ Protein Extraction kit (Bio-Rad, U.S.A.) is useful for the reproducible extraction of total cellular protein extracts from a given sample. This is due to the presence of the zwitterionic detergent, ASB-14, which acts as a powerful solubilising agent that allows for the extraction of membrane proteins in addition to cellular proteins, making it particularly useful for 2DE applications. The manufacturer recommends a ratio of 2-3 ml of sample extraction buffer per gram of plant leaf tissue. However, due to financial and plant biomass constraints, a smaller leaf mass was desirable for protein extraction purposes. The amount of wheat leaf sample required in order to extract a maximum amount of protein was optimized. The results are summarised in Figure 2.2. The optimisation procedure attempted to obtain a maximum protein content per 1 ml ReadyPrep™ sample buffer with the lowest wheat leaf mass possible due to the fact that young wheat plants do not yield a high leaf mass and individual plants were being sampled.

The highest protein content was obtained with 0.5 g (protein yield of 62.2 mg / ml) of wheat leaf tissue, but this mass was not adopted for the purposes of this study due to fact that it resulted in a very large and unstable pellet during the latter parts of the protein extraction procedure which complicated the subsequent reduction-alkylation and 2DE clean up steps. Leaf masses higher than 0.5 g (e.g. 0.75 g) appear to render the protein extraction solution incapable of performing its function and were subsequently not investigated any further. These findings were confirmed by means of analysis performed using denaturing SDS- PAGE (results not shown). It is clear that the efficiency of protein extraction and the manageability of the sample itself for further treatment depended on a smaller amount of leaf mass in the initial sample. A leaf mass of 0.1 g was subsequently used for the duration of the 2DE analysis outlined in this work.

2.3.3. Optimization of 2DE method for analysis of differential protein expression in Betta and Betta DN wheat

2.3.3.1. Determination of optimal conditions for isoelectric focussing

Wheat protein samples obtained after extraction of total proteins from leaf tissue were analysed using SDS-PAGE in order to determine whether the wheat leaf protein had been successfully isolated in addition to the relative abundance of various major proteins as well as their relative molecular masses. The gel analysis was performed in duplicate and stained with Coomassie or the SilverSnap® Stain Kit II (Pierce, U.S.A.) in order to visualise proteins after 2DE clean up which resulted in a significant, yet apparently consistent, protein loss. The results are shown in Figure 2.5 on page 54.

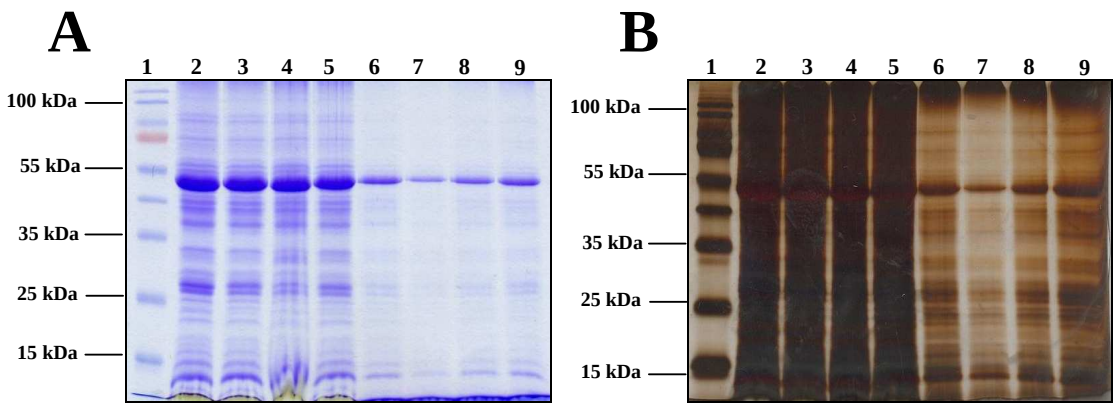


Figure 2.5. Protein profile of wheat leaf total protein obtained for Betta and Betta DN wheat at the two-leaf growth stage

Wheat proteins were resolved using SDS-PAGE gels in duplicate and stained with Coomassie [A] and the SilverSnap® Stain Kit II (Pierce, U.S.A.) [B] to visualise faint bands. Lane 1 – peqGold Protein-Marker IV (PEQLab, Germany); Lane 2 – Betta total protein extract; Lane 3 – Betta DN total protein extract; Lane 4 – Betta after reduction alkylation; Lane 5 – Betta DN after reduction alkylation; Lanes 6 – 9 – Betta after reduction alkylation and 2DE clean up with ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, U.S.A.).

The results indicate that wheat leaf protein was successfully isolated and that a relatively even distribution of proteins in terms of their molecular masses occur in the wheat leaf proteome, although the very prominent band occurring at approximately 55 kDa was of special interest as it was thought to be either one highly abundant protein or several proteins of the same molecular mass but different pI values that would resolve into discrete spots on a 2DE gel. Further analysis with 2DE confirmed the latter hypothesis to be correct. The amount of protein that was lost during 2DE clean-up (lanes 6-9 in Figure 2.5. A) was a matter of concern but numerous attempts to prevent this protein loss were unsuccessful (data not shown). This was overcome for the purposes of 2DE analysis by optimising the concentration of protein loaded onto the IPG strip during rehydration until a suitable amount of protein for maximum spot visibility was obtained. As the amount of protein in the pellet after 2DE clean-up could not be quantified accurately due to experimental constraints in terms of sample size and potential interference of the 2DE rehydration buffer with the protein assay, the total protein remaining in the sample after reduction alkylation was quantified and the loss due to 2DE clean up was considered to be consistent enough for the purposes of this study. The amount of protein was thus optimised in terms of loss after the reduction alkylation step.

As mentioned previously, sample preparation is crucial to the success of 2DE applications. To this end, all samples were reduced and alkylated using the ReadyPrep™ Reduction-Alkylation Kit (Bio-Rad, U.S.A.) in order to disrupt protein disulphide bonds so that proteins could be analysed as single subunits (Bio-Rad manual). Sample preparation and IEF were optimised in order to obtain gels that displayed a maximum of spots with a minimum of streaking. IPG strips were rehydrated overnight in 160 µl of rehydration buffer containing the protein sample of interest. This was to facilitate the absorption of high molecular weight proteins onto the IPG strip as this is known to require more time than the absorption of low molecular weight and high abundance proteins (Westermeier and Naven, 2002).

Because the present study was a pilot study in terms of the aphid-stressed wheat leaf proteome, broad-range (pH 3-10 NL) IPG strips were used in order to display most proteins in a single gel. Broad-range 2DE gives an indication of the overall proteome of a given tissue, which allows for further analysis of specific proteins of interest using narrow-range IPG strips once the method is established and optimised. Non-linear strips were used which condense protein spots on the outer pH extremes (pH 3 and 10 in this instance) and extends the spots in the median pH range (pH 5-8 in this instance). These were selected as most proteins are found to occur in the non-extreme pH range and their visualisation was considered significant to this study (Bio-Rad Manual).

Attempts at IEF optimisation relied on optimisation of applied current and potential difference, as the Zoom® IPGRunner™ system used in this study does not feature a device to control temperature. Initial IEF was performed using a Consort E815 power supply with a total accumulated V-hr of 8 233 V-hr over an 8 hour period. This did not yield satisfactory focussing, as indicated in Figure 2.6. (A) by excessive amounts of streaking and poor spot resolution. A number of different power supplies, focussing times and focussing voltage gradients were attempted (data not shown) but the most successful IEF obtained was when the Zoom® IPGRunner™ system was connected to a Bio-Rad PowerPac™ power supply with a total accumulated V-hr of 2 366.6 V-hr over a 90-minute period as shown in Figure 2.6. (B). This was achieved by means of a continuous ramp in the applied potential difference (V) as follows: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). Although the V-hr mentioned here are very low for IEF purposes (average

focussing occurs between 10 000 and 50 000 V-hr) it was found to be sufficient for the available system, as focussing times exceeding this increased temperatures beyond acceptable levels and resulted in damage to the IPG strip leading to very poor focussing. The second dimension gel pore size selected for optimal spot visualisation was 12 %, as both higher and lower percentage gels had reduced spot visibility (data not shown).

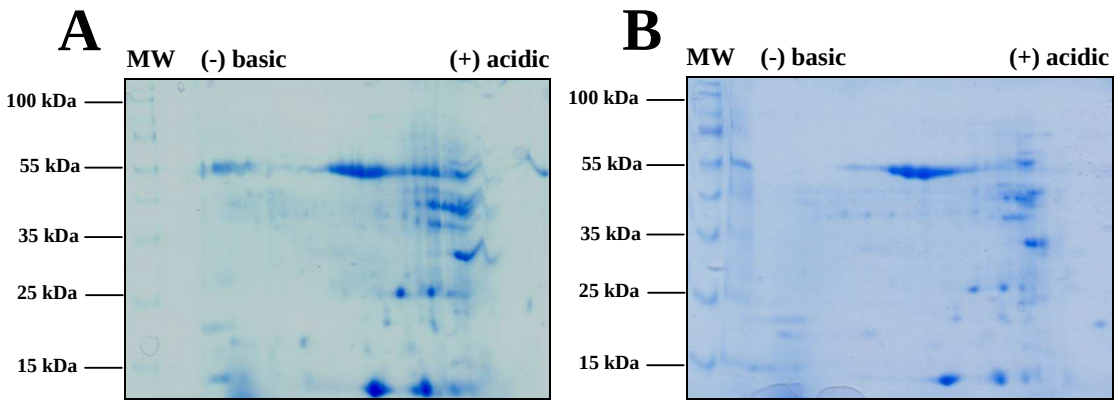


Figure 2.6. Optimization of isoelectric focussing of 2DE gels under different running conditions using alternate power supplies.

[A]: Isoelectric focussing and second dimension of a two-week old Betta total protein extraction was run on a Consort E815 power supply with a total accumulated V-hr of 8 233 V-hr. [B]: Isoelectric focussing and second dimension of a two-week old Betta total protein extraction was run on a Bio-Rad PowerPac™ power supply with a total accumulated V-hr of 2 366.6 V-hr. Both gels were stained with PageBlue™ Protein Staining Solution (Fermentas, Canada).

2.3.3.2. Optimisation of conditions to visualise wheat leaf protein spots

The choice of stain is crucial to the success of any 2DE application. Several different stains were used to visualise a maximum number of spots on the second dimension 2DE gels, as shown in Figure 2.7. on page 57.

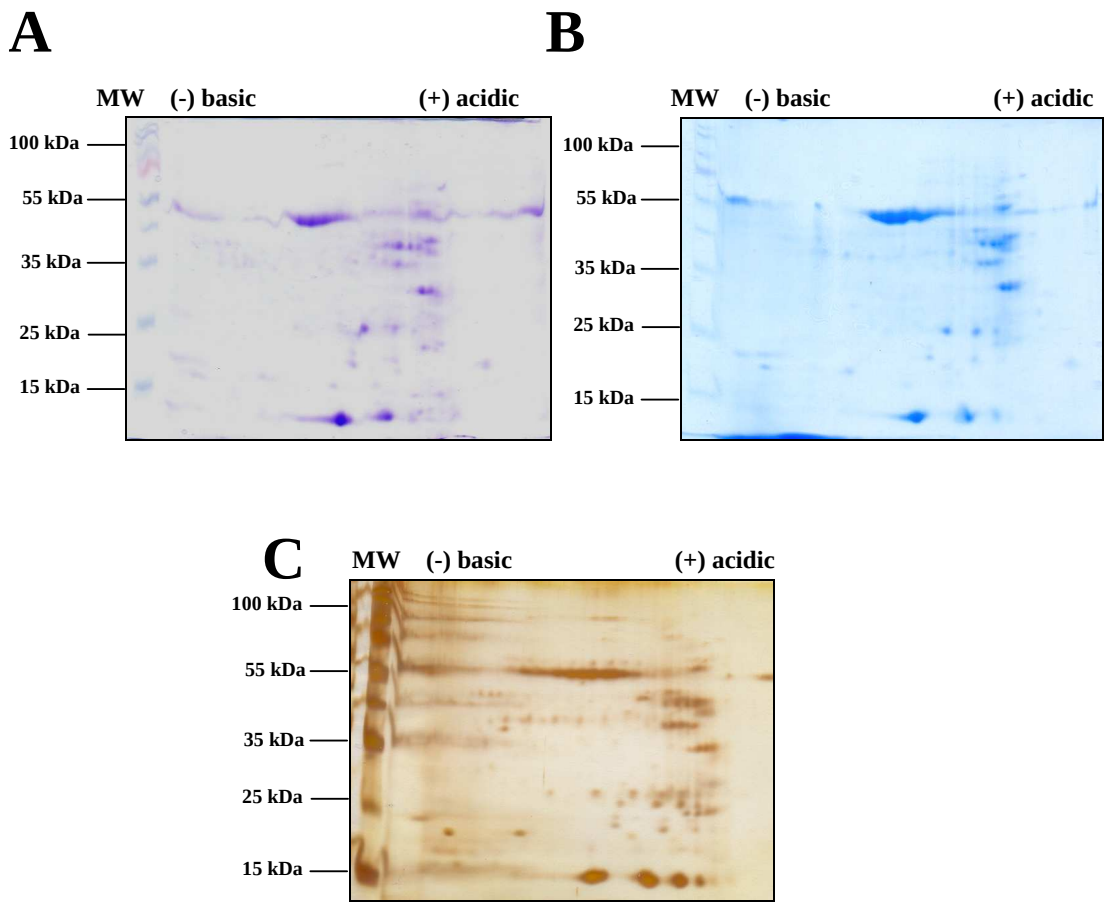


Figure 2.7. Determination of a suitable staining method to allow for the visualisation of a maximum number of spots on 2DE gels

Betta wheat samples at the two-week growth stage were resolved under optimized conditions. The wheat leaf protein concentration was maintained between 1.38-1.5 mg / ml prior to 2DE clean up and a final amount of 124 – 150 µg protein was loaded on each gel. The IEF was performed using a Bio-Rad PowerPac™ HV power supply with a continuous ramp: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The second dimension was resolved on 12 % gels at 200 V for 45 minutes. Gels were stained with Coomassie brilliant blue [A], PageBlue™ Protein Staining Solution (Fermentas, Canada) [B] and SilverSnap® Stain Kit II (Pierce, U.S.A.) [C] respectively.

Because Coomassie stain is cheap, easy to use and widely available, its usefulness as a protein stain for 2DE applications using wheat leaf protein was investigated. It proved to be reasonably ineffective, as shown in Figure 2.7. (A) as only the most abundant proteins were visible on the second dimension gel stained with Coomassie, and great care had to be taken in

order to avoid destaining protein spots corresponding to abundant proteins when destaining the gel background. The PageBlue™ Protein Staining Solution (Fermentas, Canada) was not much more effective than Coomassie in aiding the visualisation of low abundance proteins, as indicated in Figure 2.7. (B) and a more sensitive stain was subsequently sought for analytical purposes. PageBlue™ Protein Staining Solution was, however, deemed to give sufficient levels of staining for the purposes of method optimisation and validation and was used extensively during the 2DE method optimisation stage due to its rapid staining ability and cost effectiveness. The SilverSnap® Stain Kit II (Pierce, Canada) yielded good spot visualisation and was found to be both cost effective and highly reproducible thus limiting variability between gels and lowering the requirement for replicates during the course of the analysis. Figure 2.7. (C) shows a 2DE gel stained with the SilverSnap® Stain Kit II. The improvement in spot visualisation as compared to Coomassie Blue and PageBlue™ is readily apparent.

2.3.3.3. Optimization of protein loading on 2DE gels

IPG strips can tolerate a protein load ranging from a few micrograms to more than a milligram. The optimal protein load for each system needs to be optimised according to the protein sample in question and the type of stain to be used (Bjellqvist *et al.*, 1993). Protein overloading results in streaking and poor spot visualisation as illustrated in Figure 2.8. (A) in which a protein concentration of approximately 4 mg / ml was employed prior to 2DE clean-up. As mentioned previously, 2DE clean-up resulted in a significant protein loss which was difficult to quantify due to the interference of the resuspension buffer with the protein assay in addition to the difficulty of assaying the pellet obtained after clean-up. Thus all protein loads mentioned refer to the amounts that were in solution prior to 2DE clean-up, after reduction-alkylation. The best gels in terms of maximal spot visibility and minimal streaking were obtained when the protein concentration was maintained around 1.38-1.5 mg / ml prior to 2DE clean-up as indicated in Figure 2.8. (B) which resulted in a final protein load of 124-150 µg in each 2DE gel. It was proposed that the amount of protein lost during clean-up would remain constant as the method remained identical throughout the course of the experimental procedure due to the use of a clean-up kit as opposed to precipitation with trichloracetic acid (TCA) which could lead to variable results. The protein concentration of all samples was

subsequently maintained at around 124-150 µg for all 2DE gels that were resolved during the course of this study.

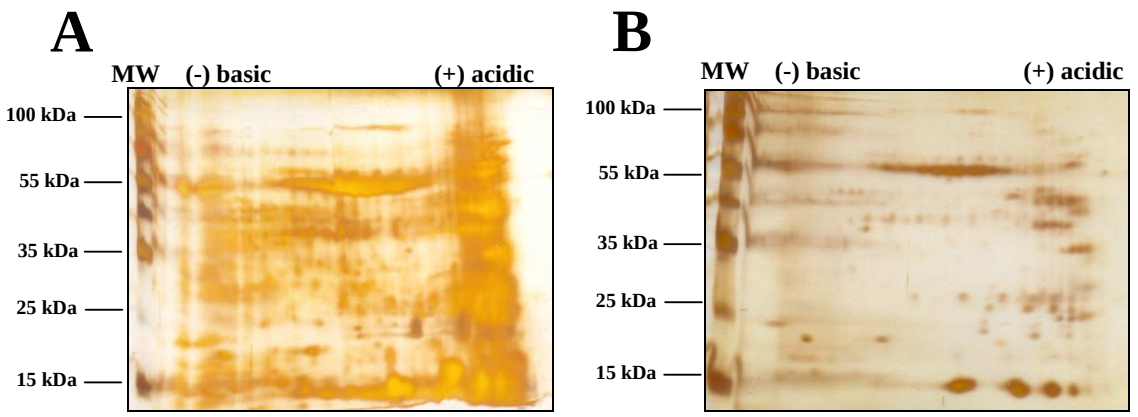


Figure 2.8. Determination of optimal protein concentration for 2DE gels using total protein from Betta wheat at the two-week stage of growth.

Betta wheat samples at the two-week growth stage were resolved under optimized conditions. The IEF was performed using a Bio-Rad PowerPac™ HV power supply with a continuous ramp: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The second dimension was resolved on 12 % gels at 200 V for 45 minutes. The initial protein load applied to the IPG strip after 2DE clean up was 4.416 mg [A] while improved protein loads of 124-150 µg [B] were used for all subsequent experiments. Gels were stained with the SilverSnap® Stain Kit II (Pierce, U.S.A.)

2.3.3.4. Determination of reproducibility of 2DE gel electrophoresis protocol

In spite of the wealth of data that can be obtained using 2DE, the technique is prone to high levels of variability, which must be determined and accounted for in order to obtain meaningful results from any given experiment. The variability can result from differences in technique, spot detection capability and spot volume measurements (Choe and Lee, 2003).

The reproducibility of the 2DE method outlined in the current chapter was determined by resolving wheat total protein samples on 2DE gels and staining with SilverSnap® Stain Kit II (Pierce, U.S.A.). After the second dimension gels were stained and imaged, they were analysed using PDQuest™ Basic Software Version 8.0 (Bio-Rad, U.S.A.).

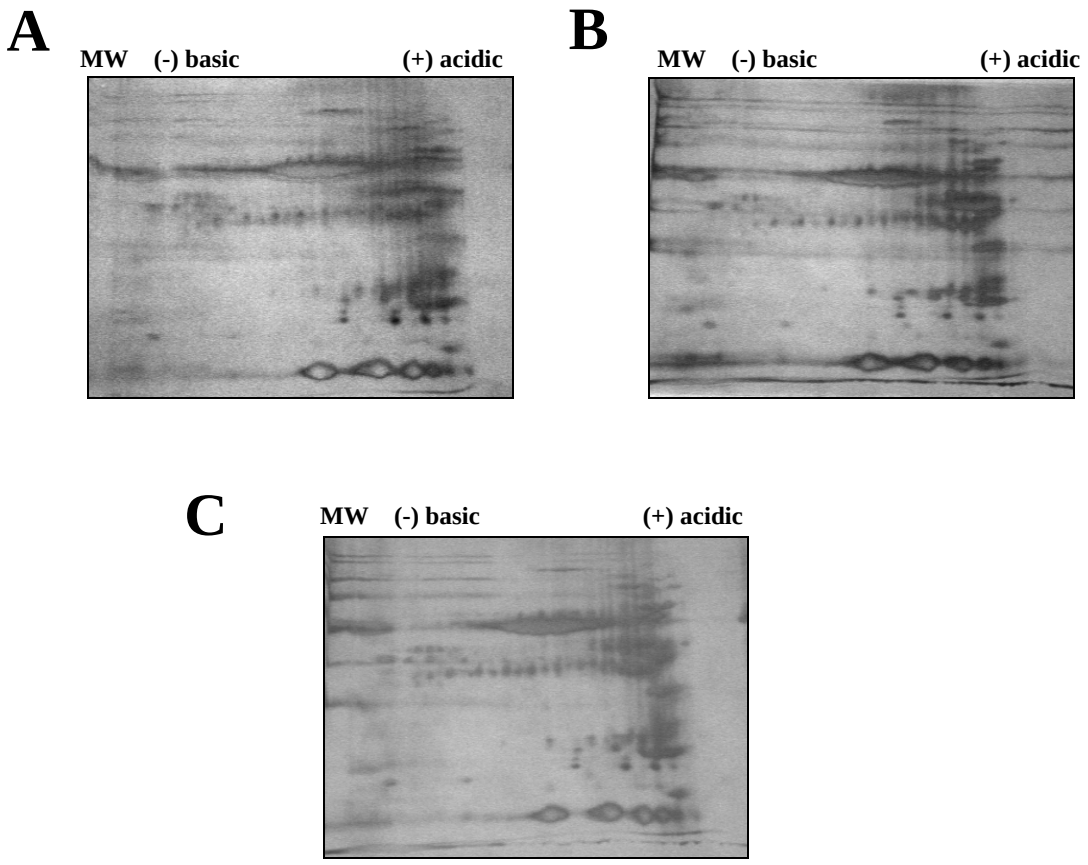
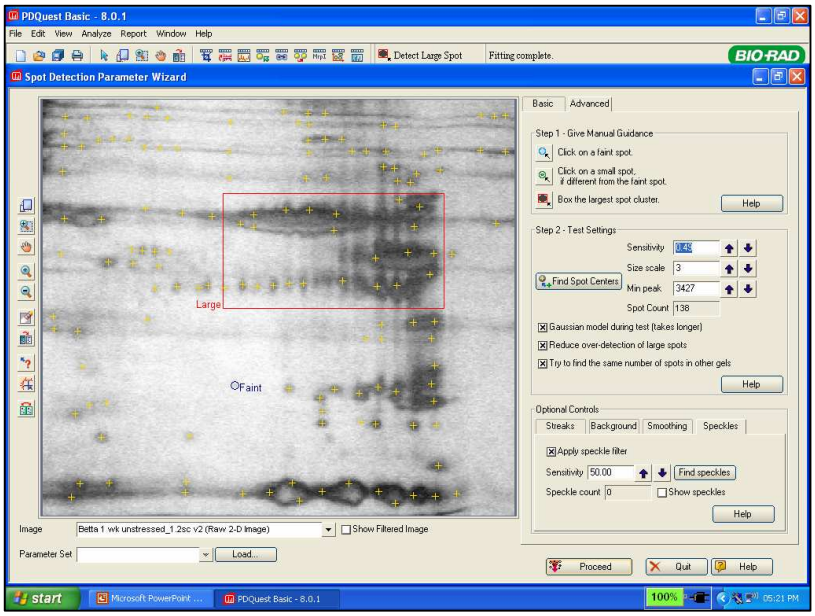


Figure 2.9. Determination of reproducibility of 2DE method used in the determination of differential protein expression in wheat.

Unstressed Betta wheat (2 week old) samples after total protein extraction and reduction alkylation were run under identical first and second dimension conditions and visualised using the SilverSnap® Stain Kit II (Pierce, U.S.A.). Sample protein amounts were maintained between 124-150 µg per gel. The IEF was performed using a Bio-Rad PowerPac™ HV power supply with a continuous ramp: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The second dimension was resolved on 12 % gels at 200 V for 45 minutes. Gels were cropped prior to analysis in order to minimise errors due to molecular weight markers. Figures A, B and C represent wheat protein extracts that were produced in triplicate and that were focussed and resolved on 12 % SDS-PAGE gels prior to staining and imaging with a ChemiDoc™ EQ gel documentation system. The reproducibility analysis was performed using PDQuest™ Basic Software Version 8.0 (Bio-Rad, U.S.A.).

A



B

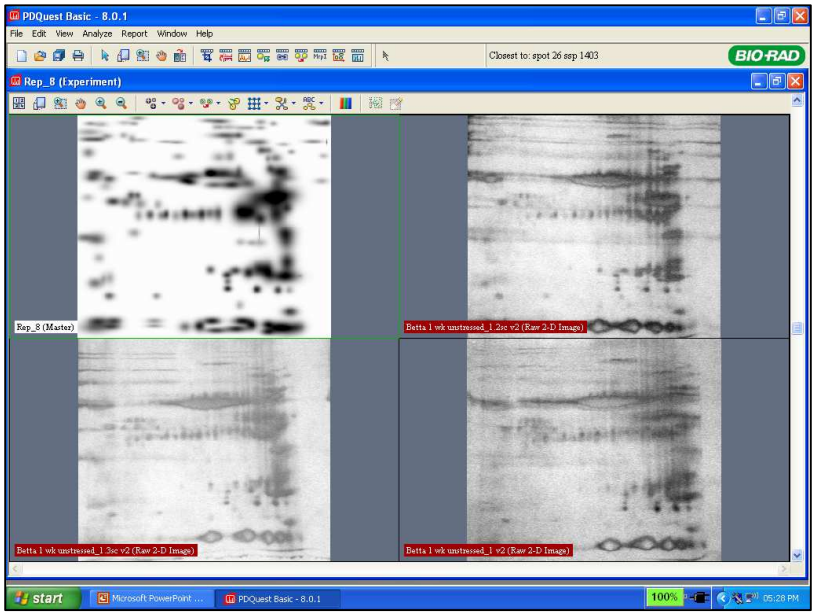


Figure 2.10. PDQuest™ Basic Software Version 8.0 (Bio-Rad) showing two of the steps taken during the determination of the reproducibility of the 2DE method developed to determine the nature of differential protein expression in wheat in response to aphid herbivory.

[A] – Screen-print of parameters entered for PDQuest™ analysis, including the settings used for sensitivity, minimum peak size and the spot count; [B] – Screen-print of the results obtained for the reproducibility study including the master gel in the top left hand corner.

2DE can be used for both quantitative and qualitative applications (Choe and Lee, 2003). In order to develop a method for the determination of differential protein expression in susceptible and resistant wheat in response to aphid feeding, only qualitative changes were taken into account. This is due to the fact the present study is a pilot study in order to develop a method that can be used for further analysis in future and it was aimed to minimise time and cost during the development of the method itself. The screen-print results obtained from triplicate gel analysis on independent protein samples using PDQuest™ Basic Software Version 8.0 (Bio-Rad) are shown in Figure 2.10. (B). The results obtained indicate that the 2DE system used in our laboratory is prone to high but acceptable levels of variability, as shown by the correlation coefficient of 0.83 in Figure 2.11. and the spot matching results in Table 2.2.

Table 2.2. Results obtained from triplicate gel matching performed using PDQuest™ Basic Software Version 8.0 (Bio-Rad)

Gel replicate number	**Number of spots detected**	**Number of spots matched to master gel**	**Match rate 1[a]**	**Match rate 2[b]**	**Correlation coefficient**
1.1	141	141	100	100	1.000
1.2	135	60	44	42	0.836
1.3	90	57	63	40	0.764

a- Match rate 1 = total number of spots matched to master gel / total number of spots on actual gel x 100
b- Match rate 2 = total number of spots matched to master gel / total number of spots on master gel x 100

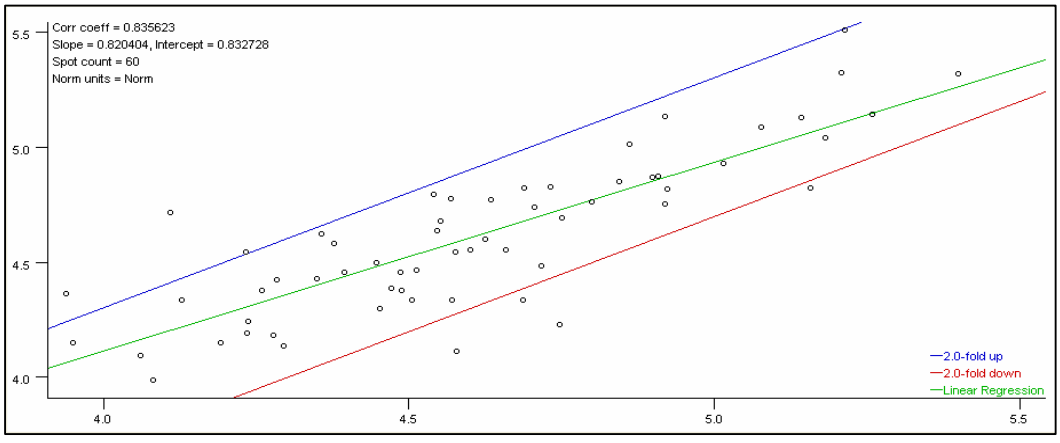


Figure 2.11. Linear regression of reproducibility of 2DE gels in triplicate after analysis with PDQuest™ Basic Software Version 8.0 (Bio-Rad).

2.4. Discussion and Conclusions

A 2DE method for the determination of the differential protein expression in Betta and Betta DN wheat cultivars was developed. This method does require further optimization to improve the reproducibility of the gels in addition to maximising spot visibility and minimise streaking and variability, but as a pilot study the results obtained for this section were considered sufficient in order to continue with the study on the differential expression of proteins during Russian wheat aphid and Bird Cherry-Oat aphid feeding on wheat. Commercially available kits have been utilised in order to remove some of the variability that is a problem in many 2DE applications. Sample preparation for the optimised 2DE method made use of 0.1 g homogenised wheat leaf tissue from which proteins are extracted using the ReadyPrep™ Protein Extraction Kit (Total Protein) (Bio-Rad, U.S.A.) prior to reduction and alkylation with the ReadyPrep™ Reduction-Alkylation Kit (Bio-Rad, U.S.A.). Contaminating compounds were removed from samples using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, U.S.A.) prior to performing isoelectric focussing on a Zoom® IPGRunner system that was powered by a Bio-Rad PowerPac™ HV power supply with an applied electric potential (V) which was increased in a continuous ramp fashion as follows: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). A protein load of 124-150 μg per IPG strip was used. The current was maintained at 5mA throughout the procedure. After focussing, the IPG strips were applied to 12 % second dimension gels prior to staining with the SilverSNAP® Stain Kit II (Pierce, U.S.A.). Gels were analysed using PDQuest™ Basic Version 8.0 software from Bio-Rad. While the procedure outlined in this chapter was sufficient in terms of forming part of a pilot study of differences in the wheat proteome in response to biotic stress, it must be stressed that this method still requires further optimisation in terms of future work.

The one-dimensional gel analysis (SDS-PAGE) indicated that total wheat leaf proteins had been successfully isolated as the one dimensional protein profile obtained for wheat revealed that a range of proteins from all molecular masses was obtained. The most abundant protein in the unstressed wheat protein profile appeared at approximately 53 kDa and was also observed in stressed wheat, although the intensity of the band on SDS-PAGE gels was

considerably diminished in stressed wheat samples (results not shown). Further prominent bands were observed in the molecular weight range of 35-55 kDa, which is where many proteins implicated in plant stress responses are anticipated to be found. This was an encouraging finding as an analysis of all the wheat proteins characterised to date on the SwissProt database revealed that an overwhelming majority of characterised wheat proteins deposited in the SwissProt database were smaller than 50 kDa. Relatively few high molecular weight proteins, i.e. those greater than 100 kDa were observed, indicating that there are not many such proteins present in unstressed Betta and Betta DN wheat or that the protein extraction method of choice did not successfully isolate them from the plant leaf matrix. Once again, however, this corresponds to what is expected for wheat proteins based on the SwissProt database. Literature indicates that there are relatively few high molecular proteins (molecular mass greater than 100 kDa) present in wheat. Reviews of 2DE analysis published by Dougherty *et al*. (1989) and Mak *et al*. (2006) indicate that very few protein spots occur in regions of the gel corresponding to high molecular masses. Thus it appears that the one dimensional protein profile obtained corresponds to that expected from literature.

The optimization of IEF conditions presented several challenges. Optimal IEF conditions rely on very high voltages for a relatively short period of time, which necessitates temperature regulation in order to prevent the gel on the IPG strip from melting (Westermeier and Naven, 2002). The Zoom® IPGRunner™ system (Invitrogen, France) powered by a Bio-Rad PowerPac™ HV power supply used in this study did not allow for either high voltages (above 5000 V) or controlled temperature therefore conditions had to be optimised in order to obtain the best possible gels on this system. The The Zoom® IPGRunner™ system was used due to budget constraints. However, in future, the use of a more costly temperature-controlled system must be investigated in order to improve the quality of gels in terms of the efficiency and reproducibility of the IEF procedure, as well as adequate spot resolution. The second dimension 2DE gels displayed high levels of streaking which could be eliminated by using increased focussing times and a higher potential difference during focussing.

Literature pertaining to 2DE analysis recommends that analytical gels be stained with a sensitive stain such as silver or fluorescent stains (Corhals *et al*., 2000). It is significant to note that all available stains interact differently with different proteins, which necessitates the

optimisation of staining for proteins from a given tissue like wheat leaf tissue in the present case (Carrol *et al*., 2000). Coomassie Blue appears to stain the broadest spectrum of proteins according to the available literature and was initially employed to visualise protein spots in the second dimension gels in our present (Carrol *et al*., 2000). Despite its ease of use and cost effectiveness, one of the major drawbacks in using Coomassie Blue is its lack of sensitivity relative to other stains such as SYPRO Ruby (commonly used in 2DE applications) and Silver Stain. Coomassie Blue R-250 typically has a detection limit of approximately 40 ng (100 ng for BSA) while stains regularly used in 2DE applications studying low abundance proteins require a detection limit around 1 ng (Westermeier and Naven, 2002). PageBlue™ Protein Staining Solution (Fermentas, Canada) is a relatively inexpensive and rapid staining solution that is reported to have a detection limit of 5ng, which is higher than that of Coomassie Blue. However, for the purposes of this study, it was not significantly more effective than Coomassie. The use of a fluorescent stain for future work would result in the visualisation of a greater number of spots, as would the use of IPG strips with different pH ranges which would facilitate the enhanced visualisation of more obscure spots, particularly for acidic proteins which occur as very dark and poorly defined spots under the present conditions. Silver staining with the SilverSnap® Stain kit II silver staining kit from Pierce (U.S.A.) was found to be the most sensitive, reliable and cost-effective method of staining for the purposes of this experiment, despite the fact that silver staining is known to have certain drawbacks such as a limited dynamic range despite its relatively high sensitivity (Lopez *et al*., 2000).

In terms of the method validation and reproducibility, literature pertaining to the variation in 2DE analyses reveals that errors as high as 32 % are quite common and standard deviations of 28 % have been reported (Choe and Lee, 2003). The method outlined here is prone to variation but this has been found to be within the acceptable limits that are anticipated for a technique such as 2DE. The match rate between the gels and the master gel used for the reproducibility study was not very high (44 and 63 % respectively) but this was found to be acceptable in terms of the fact that the current study was a pilot study and that 2DE is a technique that is prone to high levels of variation. The correlation coefficient of 0.83 obtained from the scatter plot for the triplicate gel analysis was encouraging in terms of method reproducibility as the coefficient value is a measure of the quantitative similarity between gels. A value of 1.00 indicates a perfect match while a value of 0.40 or less suggests that the

replicate gels are not very similar. As the value obtained was closer to 1.00 than to 0.40, it can be indicated that the method outlined is adequately reproducible (PDQuest Instruction manual).

In order to further optimise the method outlined throughout this chapter, the effect of several interfering factors in 2DE analysis will have to be minimised or eliminated. One of the most important factors to be considered would be to dramatically increase the V-hr used during the IEF stage. This could be accomplished by purchasing a system that has a built-in temperature control feature, which will allow for increased focussing with a constant temperature, which eliminates overheating and subsequent melting of the gel on the IPG strip. This will increase the efficiency with which IEF is performed and, apart from improved spot resolution will greatly diminish the appearance of streaking on the second dimension gels. Other factors such as choice of stain would also have to be investigated in order to develop a better method. Several fluorescent stains of high sensitivity (such as Flamingo fluorescent gel stain from Bio-Rad) are available commercially but a method using these would have to be carefully investigated in terms of feasibility due to their high cost and the fact that they require excitation at specific wavelengths that would require the purchasing of additional imaging equipment.

The 2DE method outlined in this Chapter is adequate for the determination of the type and distribution of differential protein expression in wheat plants subjected to aphid phloem feeding. It shows great promise in future proteomic studies of wheat provided that it is developed and extended to include supplementary techniques such as tandem mass spectroscopy (MS / MS) and N-terminal sequencing for the positive identification of protein spots of interest.

Chapter 3

The effect of Russian wheat aphid and Bird Cherry-Oat aphid feeding on the wheat proteome at different stages of wheat development

3.1. Introduction

Aphids are phloem-feeding insects that cause significant damage to valuable crops. They feed from phloem tissue at a rapid rate by probing the thin-walled sieve tubes of the plant with specialised mouthparts (Matsiliza and Botha, 2002; Evert *et al*., 1973). Prior to the commencement of feeding, aphids inject watery saliva into the plant, which is believed to be a medium for the transmission of viruses in addition to being the eliciting agent for plant defence responses (Moran and Thompson, 2001; Kimmins and Tjallingii, 1985). Plants respond to aphid feeding by producing pathogen-resistance proteins such as glucanases, which are linked to the deposition of callose. This phenomenon is particularly noted in wheat cultivars that are resistant to aphid feeding; susceptible cultivars do not produce glucanases in response to aphid feeding (Moran and Thompson, 2001).

The Russian wheat aphid (RWA; *Diuraphis noxia*) and the Bird Cherry-Oat aphid (BCA; *Rhopalosiphum padi*) are pests of small grains, most notably wheat. The RWA originated in Southern Russia and was initially identified as a threat to wheat and barley in South Africa in 1978 in the northern Free State, where significant crop losses occurred due to subsequent infestation during 1979 and 1980 (Du Toit and Walters, 1984; Kovalev *et al*., 1991). This aphid is small (less than 2 mm in length) and light green in colour and is distinguishable by its short antennae and 'forked-tail' appearance due to a projection above the caudal segment (Walters et al., 1980). The RWA is considered to be a serious pest of wheat causing devastating crop losses every year (Lukaszewski *et al*., 2001; Botha *et al*., 2006). RWA feeding causes extensive structural damage to affected plants, including chlorosis, longitudinal streaking in new leaves as well as a general failure of new leaves to unroll (Miller *et al*., 1994). Wheat seedlings infested with RWA cease growth and affected plants eventually die as a result of infestation unless it is checked in time (Burd and Burton, 1992). The stunting in plant growth in response to RWA infestation is largely due to the stunting of leaves / leaves not unfolding which causes a significant reduction in the photosynthetic leaf area (Burd and Burton, 1992). The double membrane of the affected plant's chloroplast is typically degraded within 10 days of the commencement of aphid feeding, which causes the characteristic streaking on the leaves of RWA-infested plants (Fouché *et al*., 1984).

Despite being a pest of great economic importance in Europe, the BCA is considered a much lesser threat to wheat than the RWA (Nielsen and Steenberg, 2004). It does not cause significant structural damage and its effects are not seen on affected plants (Internet 7). Recent research, however, indicates that the BCA may be a more serious pest than had been previously suggested and ongoing research aims to determine the effects of BCA feeding on winter wheat. BCAs are larger than RWAs and are olive-green in colour, with more mature aphids appearing almost black (Internet 7). BCA feeding on host plants does not result in any visible macroscopic changes, which is unlike the physical response observed during RWA feeding. At the physiological and molecular level, the host plant's response to BCA feeding also differs from that experienced during RWA feeding. The phloem sap of plants subjected to RWA infestation displays an increased concentration of amino acids while that of plants subjected to BCA infestation displays no marked difference (Sandström *et al*., 1999). It is important to note that plants infested with BCA do exhibit a stress response, in spite of the differences between the aphid species above. This occurs by means of the induction of defensive chemicals against aphid feeding (Gianoli and Niemeyer, 1998).

It is known that plants are not equally susceptible to the effect of insect pests at every stage in their development. Younger wheat plants are more resistant to the effects of certain aphid types than are the older plants (Acreman and Dixon, 1985). In light of this, a study was performed to determine the differential protein expression in Betta and Betta DN wheat at two different growth stages in response to infestation by *Diuraphis noxia* (Mordvilko) and *Rhopalosiphum padi* (L.).

3.2. Materials and Methods

3.2.1. Materials

Wheat seeds (*Triticum aestivum L*. cv. Betta and Betta DN) and Russian wheat aphids (*Diuraphis noxia*) were obtained from the Agricultural Research Council of South Africa (Bethlehem, South Africa) while potting soil was purchased from Green Fingers Nursery in Port Elizabeth. The Bird Cherry-Oat aphids (*Rhopalosiphum padi*) were obtained from an existing colony at the Rhodes University Department of Botany (Grahamstown, South Africa). The total protein extraction, reduction alkylation, 2D clean up and protein assay kits

were purchased from Bio-Rad (U.S.A.). Liquid nitrogen was obtained from the Rhodes University Department of Chemistry (Grahamstown, South Africa). Bovine Serum Albumen Fraction V was purchased from Roche (Switzerland). PageBlue™ protein staining solution was a free sample from Fermentas (Canada) while the SilverSnap® Stain kit II silver staining kit was purchased from Pierce (U.S.A.). Isolectric focussing equipment was from Invitrogen (France) while immobilized pH gradient strips were from Bio-Rad (U.S.A.). Protein molecular mass markers were purchased from PEQLab (Germany). All other reagents for buffers and gel reactions were purchased from Sigma and Bio-Rad (U.S.A.).

3.2.2. Wheat plants

Wheat seeds (*Triticum aestivum L.*) cv. Betta and Betta DN were germinated overnight in a petri dish on filter paper prior to planting in a 70:30 mix of potting soil (Green Fingers Nursery, Port Elizabeth) and vermiculite. Each pot contained roughly 20 wheat seeds and plants were grown under controlled conditions in a Conviron S10H growth cabinet at the Rhodes University Department of Botany. The Conviron was maintained at a constant temperature of 26 ± 1°C at a relative humidity of approximately 70 % while plant nutrient status was maintained with a 75 % solution of Long Ashton nutrient mix which was prepared as outlined in Appendix 1 (Chapter 8) and was administered every two days. All plants were subjected to a 16-hour photoperiod. Stressed plants and their unstressed controls were kept separate in order to eliminate the possibility of aphid contamination of control plants. Furthermore, the RWA and the BCA colonies were maintained in separate growth cabinets in order to prevent cross-contamination of plants involved in the study.

3.2.3. Development of *Diuraphis noxia* (Mordvilko; Russian wheat aphid) and *Rhopalosiphum padi* (Bird Cherry-Oat aphid) colonies

Russian wheat aphids were obtained from the Agricultural Research Council's Small Grain Institute in Bethlehem, South Africa where the parental aphid colony had been allowed to develop while feeding on susceptible wheat cultivars. Aphids were received on wheat leaves in glass containers and were immediately transferred to two-week-old susceptible wheat (*Triticum aestivum* cv. Betta) plants in a Conviron S10H at the Rhodes University Department of Botany. The plants and aphids were maintained under controlled conditions that included a temperature of 26 ± 1°C, 70 % relative humidity and a 16-hour light: dark cycle. The wheat

nutrient status was maintained by application of 75 % Long-Ashton solution every two days. The Bird Cherry-Oat aphid colony was cultivated from an existing colony in the Department of Botany, Rhodes University (South Africa). The same procedure as for the cultivation of the Russian wheat aphid colony was followed. Care was taken not to damage aphids when transferring them between plants by moving one leaf containing a sufficient amount of either type of aphid from each wheat plant and placing it in a pot containing between 10 – 20 previously uninfested Betta wheat plants at the 2-leaf stage (two weeks old). This process was repeated until the study commenced, with aphids being placed on fresh plants every two weeks.

Figure 3.1. Cages used to restrict aphid movement between different experimental plants.

Experimental plants subjected to BCA feeding were kept in a separate controlled-temperature growth chamber to those subjected to RWA feeding. Aphid cages (seen in the left hand figure) consisting of plastic and gauze restricted aphid movement between different experimental plants. Pots containing aphids restricted by aphid cages were kept in wooden boxes as seen in the figure on the right in order to further restrict movement.

Plants infested with Russian wheat aphids and Bird Cherry-Oat aphids were placed inside aphid 'cages' as shown in Figure 3.1. on the left which were then placed inside wooden

containers (Figure 3.1. right) in separate convirons in order to prevent the co-existence of different aphids on any of the experimental or feeder systems.

3.2.4. Total protein extraction from stressed and unstressed wheat leaves (Betta and Betta DN)

Betta and Betta DN wheat plants were maintained in the absence of aphids (controls) or subjected to aphid feeding (experiment). Plants were kept in a Conviron S10H growth cabinet at 26 ± 1°C and 70 % relative humidity. Watering plants with 75 % solution of Long Ashton solution every two days maintained adequate nutrient status. In order to develop a profile of the wheat proteome response to aphid feeding at different stages of development, RWA or BCA (10-12 aphids per plant) were placed on wheat plants at 7, 14 and 21 days after planting. The RWA and BCA were then allowed to feed on plants at the given growth stage for 1 week prior to extraction of total protein from stressed wheat leaves which took place after all aphids were removed from the leaves with a paintbrush. The control samples were maintained under identical conditions in the absence of RWA and BCA.

Total protein extraction proceeded as outlined in the method development section in Chapter 2. Briefly, plant samples were collected after all aphids were carefully removed with a paintbrush and immediately frozen in liquid nitrogen prior to homogenizing them in the same. The extraction was performed using the ReadyPrep™ Protein Extraction Kit (Total Protein) from Bio-Rad (U.S.A.) as per manufacturer's instructions. Homogenized leaf tissue was suspended in 1 ml ReadyPrep 2-D Rehydration / Sample buffer 1 (7 M urea, 2 M thiourea, 1 % (w/v) ASB-14 detergent, 40 mM Tris base and 0.001 % bromophenol blue) containing TBP reducing agent (200 mM tributylphosphine in 1-methyl-2-pyrrolidinone). Samples were sonicated in four bursts of 30 seconds each prior to centrifugation at 14 000 g for 30 minutes at room temperature. After centrifugation, the supernatant was retained and reduced and alkylated using the ReadyPrep™ Reduction-Alkylation Kit from Bio-Rad (U.S.A.), while the pellet was discarded. The protein content of all samples was determined using the RC / DC assay (Bio-Rad, U.S.A.) which is a modification of the method of Lowry *et al.* (1951). The experimental layout is shown in Figure 3.1.

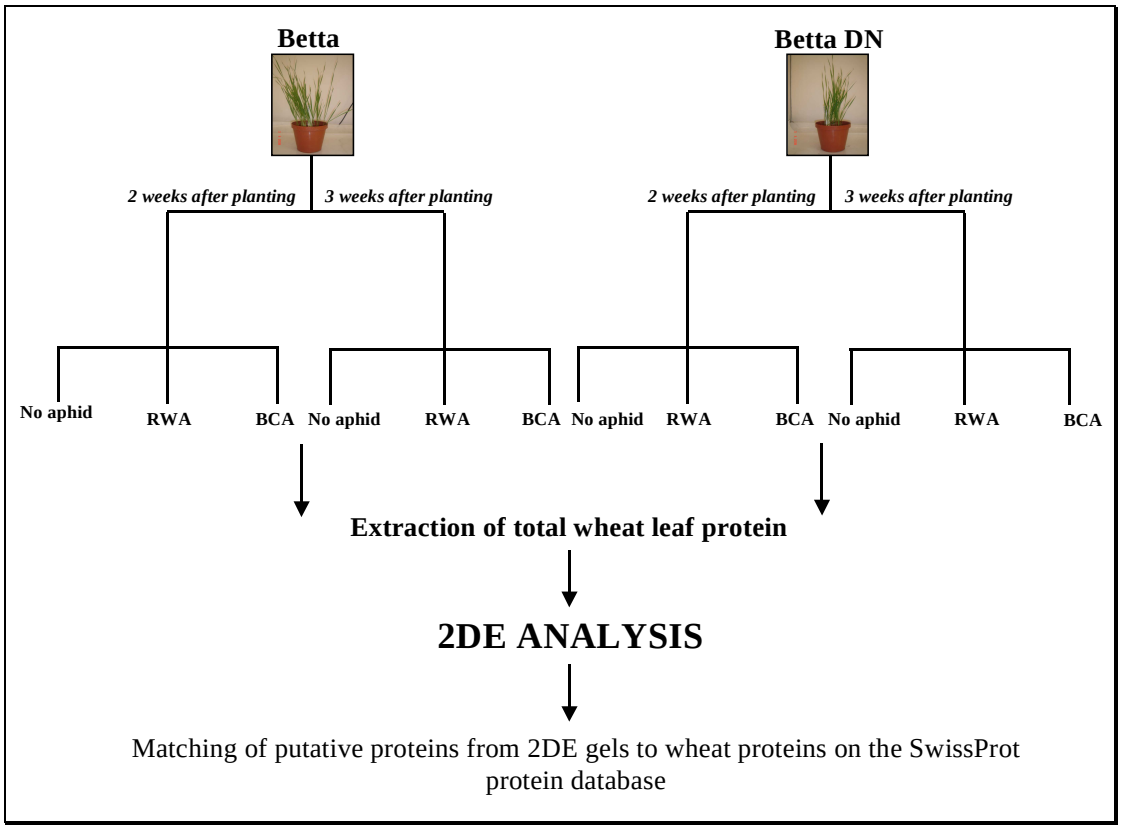


Figure 3.2. Schematic representation of experimental approach for the determination of the differential protein expression in Betta and Betta DN wheat in response to RWA and BCA phloem-feeding.

3.2.5. Two dimensional gel electrophoresis of stressed and unstressed wheat (Betta and Betta DN) at different growth stages

Wheat leaf samples were prepared as described in 3.2.3. and cleaned up using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, U.S.A.). Subsequently, the total protein pellet obtained was resuspended in 2-D rehydration buffer (250 µl; 9 M Urea, 2 % Triton-X 100, 2 % Bio-Lyte 3-10 buffer, 0.1% Bromophenol Blue and 0.02 g dithiothreitol). Protein pellets that dissolved with difficulty were sonicated for 15 seconds. Broad-range pH 3-10 NL (7 cm) ReadyStrip™ IPG strips (Bio-Rad, U.S.A.) were rehydrated overnight in 160 µl resuspended protein sample (containing 124-150 µg wheat leaf total protein) in a Zoom® IPGRunner™

Cassette (Invitrogen, France) prior to performing isoelectric focussing (IEF) using a Zoom® IPGRunner™ system (Invitrogen, France) powered by a Bio-Rad PowerPac™ HV power supply (Bio-Rad, U.S.A.) in water. The applied electric potential (V) was increased in a continuous ramp as follows: 200 V (20 minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The current was maintained at 5 mA throughout the duration of the IEF procedure. The second dimension was resolved on 12 % SDS-PAGE gels at 200 V for 45 minutes, using the same power supply as was used for the IEF step.

3.2.6. Staining, visualisation and analysis of 2DE gels

Gels were stained with the SilverSNAP® Stain Kit II (Pierce, U.S.A.) as per manufacturer's instructions prior to imaging with a ChemiDoc ™ EQ gel documentation system (Bio-Rad, U.S.A.). Stained gels were analysed using PDQuest™ Basic Software Version 8.0 (Bio-Rad) as described in Chapter 2. Gels were grouped and analysed using the Spot Detection Parameter Wizard with a sensitivity of 0.49 while allowance was made for horizontal and vertical streak removal. In each case, the gel corresponding to the control reaction for the given experimental group served as the master gel for the purposes of the software analysis. PDQuest™ Basic Software Version 8.0 (Bio-Rad) relies on manual identification of faint spots, large spots and the largest spot cluster, allowing for some user input in terms of parameters. A speckle filter set at 50 was applied in order to remove speckles from the gel. This was considered necessary despite the fact that some small and faint spots may have been lost in the process. A Gaussian statistical model was used to detect and fit spots while a local regression model used by the software was selected for data normalisation. A spot that occurred in every gel in the analysis set, both stressed and unstressed, at roughly the same intensity and was identified as being neither two fold up or down regulated by the software - was selected and putatively identified to comprise a validation for the software's analysis of up or down regulated proteins.

Once the 2DE gels were analysed using the gel analysis software, the relative molecular mass and pI of spots identified as being potentially up or down regulated, was determined. The molecular mass of proteins corresponding to spots of interest was assigned based on the molecular weight markers that were resolved alongside the sample in each gel. The pI of proteins corresponding to spots of interest was determined by using a standard graph of pI

values for a pH 3-10NL IPG strip as given in Westermeier and Naven (2002) and reading off the appropriate pI value corresponding to the distance migrated along the IPG strip. The putative molecular mass and pI value obtained for each protein spot of interest was subsequently compared to a list of the known and characterised wheat proteins on the SwissProt database (http://expasy.org/sprot/) and an attempt to match the unique pI value and molecular mass of the protein spots of interest (putatively up or down regulated proteins) to known wheat proteins was made in order to determine the nature of wheat stress responses to aphid infestation at the protein level.

3.3. Results

3.3.1 Physical responses of Betta and Betta DN wheat to stress caused by RWA and BCA phloem feeding

Although both the RWA and the BCA are classified under the division of Homoptera: Aphididae, their effect on the plants that they feed on is markedly different. The RWA is considered one of the most damaging cereal pests in the world (Labuschagne and Maartens, 1999). Despite being a pest of great economic importance in Europe, the BCA is not considered as serious a pest as the RWA (Nielsen and Steenberg, 2004). This is largely due to the effect RWA feeding has on it's host plant. The physical damage to wheat plants caused by RWA feeding was confirmed in the current study. Susceptible wheat plants subjected to RWA phloem-feeding exhibited signs of chlorosis within five days after feeding commenced, and plants displayed advanced signs of chlorosis and leaf-curl after two weeks of aphid feeding, as shown in Figure 3.3. (A), relative to control plants of the same age, as shown in Figure 3.3.(C). After four weeks of continuous feeding by the RWA, the susceptible wheat plants were yellowed and desiccated, as shown in Figure 3.3.(B). Resistant plants sustained fewer aphids but did exhibit some chlorosis after a four-week period (results not shown). It appears that the effect of RWA feeding is somewhat ameliorated in younger plants (1 week old) but that plants past the two-week growth stage are not as well equipped to counter the damage incurred from RWA herbivory.

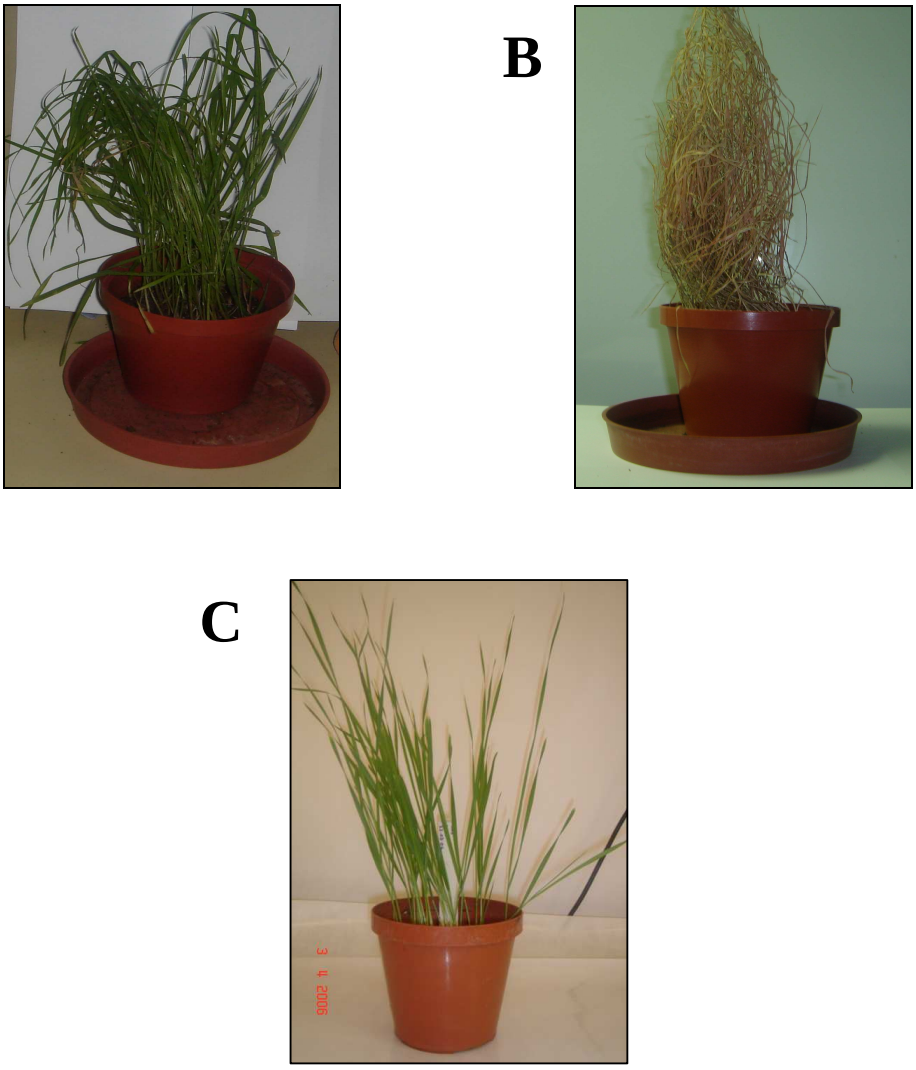


Figure 3.3. Damage to Betta wheat infested with RWA at the 2-leaf stage of development.

[A] – Betta wheat (susceptible) after a 2-week infestation with RWA. Note the appearance of leaf curl; [B] - Betta wheat (susceptible) four weeks after infestation with RWA; [C] – Betta wheat (susceptible) plant from the control group at two weeks in the absence of any RWA infestation.

In contrast to plants subjected to RWA feeding, plants exposed to BCA phloem-feeding did not display any symptoms of chlorosis, even after four weeks of constant feeding and despite the fact that BCA density on the plants was roughly three times that of RWA on the

susceptible wheat plants, as they appear to multiply much faster than the RWA (Figure 3.4.A and B). An interesting observation made during the course of this study was that BCA appear to prefer feeding on Betta plants, which are susceptible to RWA feeding and did not feed as well on Betta DN plants which are resistant to the RWA. However, after a period of approximately two weeks, the BCA appeared to settle on the resistant plants and feed in the same manner as they did on the susceptible plants.

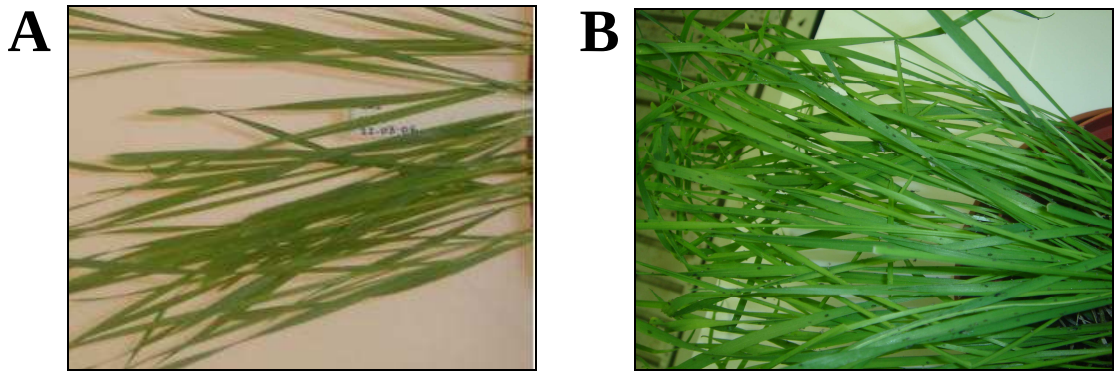


Figure 3.4. Damage to Betta wheat infested with BCA at the 2-leaf stage of development

Betta (susceptible wheat) shows no physical symptoms such as chlorosis upon BCA infestation. [A] – Betta wheat (susceptible) after a 2-week infestation with BCA. Note the lack of leaf curl and chlorosis; [B] - Betta wheat (susceptible) four weeks after infestation with RWA.

A protein spot occurring on all gels irrespective of age or stress condition was sought in order to normalise gels and serve as a basis of comparison for proteins that were thought to be up or down regulated in further analysis of the data. A spot corresponding to a molecular mass of 20 kDa and a pI of approximately 5.4 was selected (as shown by the small black arrow in Figures 3.5 and 3.6) as it appeared in all gels, irrespective of stress or non-stress conditions and appeared to be regulated at the same level in each case, according to the PDQuest™ analysis (results shown in the Appendix 1). A search of the protein databases for a wheat protein matching the criteria outlined above in addition to being necessary to the plant during all stages of growth and not only under stress conditions revealed the eukaryotic translation initiation factor 4E-1 (IF4E1) as a possible match. The protein spot of interest had a

molecular mass of 20-25 kDa and a pI of 5.0-5.5 while IF4E1 has a molecular mass of 23.9 kDa and a pI of 5.39.

3.3.2. Two-week old Betta wheat responses to RWA and BCA phloem feeding

The 2DE analysis performed revealed that unstressed Betta wheat controls yielded a higher number of spots than the RWA stressed experimental samples. The controls for the current experiment were unstressed Betta and Betta DN wheat at two and three week growth stage. It was necessary to determine the nature of proteins up or down regulated during normal wheat growth from two weeks to three weeks in order to determine whether or not the upregulated proteins in stressed plants could be attributed the stress condition or proteins upregulated during normal growth. Detailed results and analysis of 2DE gels are recorded in Appendix 1.

Figure 3.5.(A). (1 and 2) shows the 2DE gel analysis of Betta wheat at the two-week growth stage. Figure 3.5.(A). shows the control gel for experiment, which is unstressed Betta wheat at the two-week growth stage. The magnified gel inserts on the right (Figure 3.5.A.1 and 3.5.A.2.) are sections of interest taken from 2DE gels of two-week old Betta wheat exposed to RWA phloem feeding and BCA phloem feeding, respectively. The gel sections in this case show areas of the highest differential protein expression that may be valuable in future studies. The complete gel images that these sections were taken from are shown in Figures 6.1.(C) and 6.3.(C). in Appendix 1. Two-week old Betta wheat that is stressed with the RWA exhibits the expression of 16 proteins in the section of interest circled in black (shown by 16 spots on the 2DE gel) as opposed to only 8 spots in the control gel. The greatest differential protein expression from control conditions was observed in areas of the gel corresponding to a neutral to basic (pI of 7.0-9.0) in spots corresponding to proteins in the 38-55 kDa range. Several putative matches for these proteins, as well as others on the gel that were identified as being upregulated proteins by the PDQuest™ software, were obtained on the SwissProt protein database. These putative matches are detailed in Table 6.1. in Appendix 1. These proteins include putative storage proteins, proteins involved in photosynthesis, heat shock proteins and defence proteins.

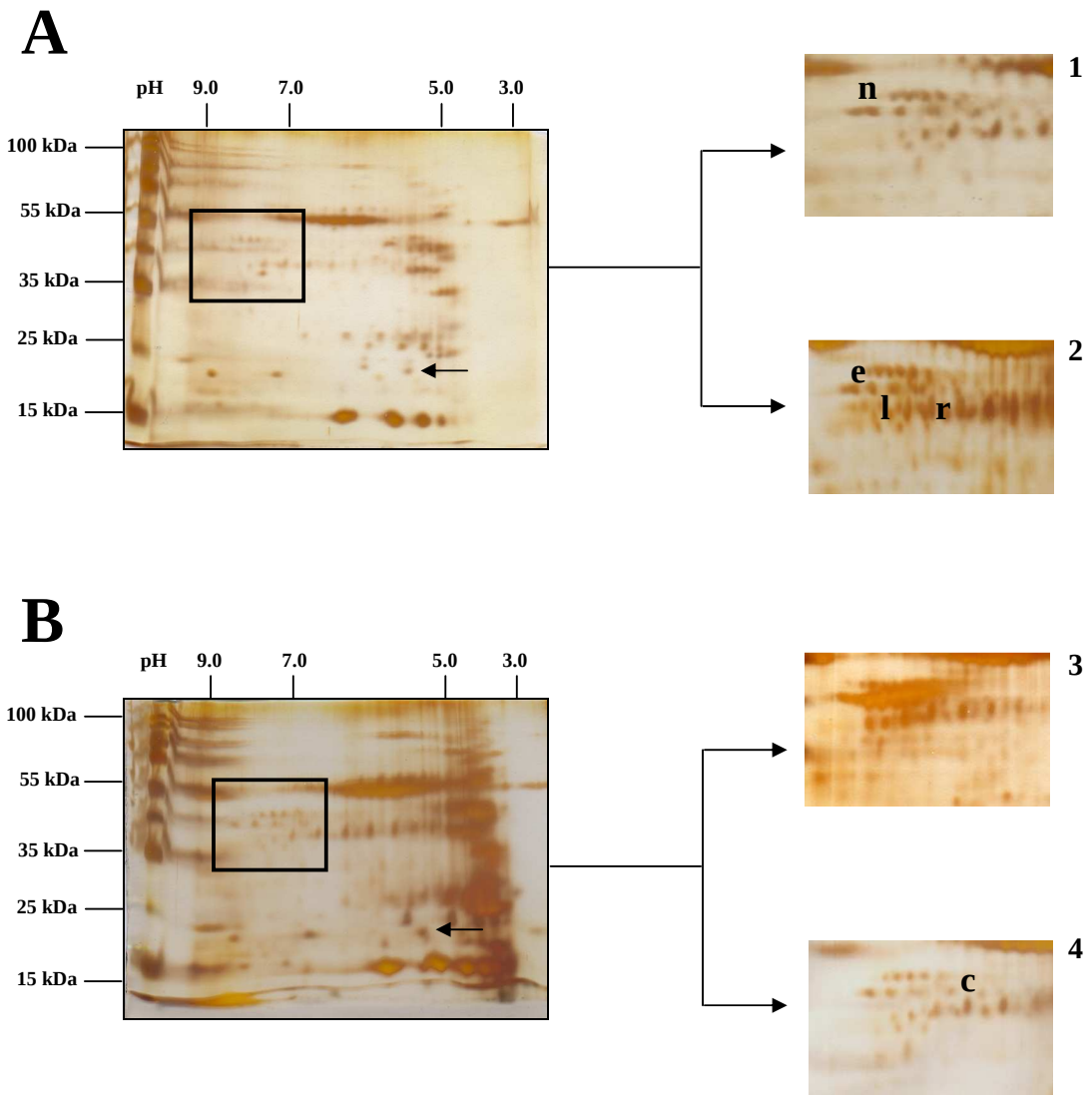


Figure 3.5. Summary of differential protein expression in Betta wheat in response to RWA and BCA phloem feeding

Areas of most pronounced differential protein expression are circled in black in the figures on the left. Magnification of corresponding areas of RWA and BCA stressed gels are inserted on the right. Detailed gel images and analysis are recorded in Appendix 1. Black arrows indicate the spot used for gel normalisation. [A] – Betta wheat at the 2 week (3-leaf) growth stage in the absence of aphid phloem-feeding (control); [B] - Betta wheat at the 3 week (4-leaf) growth stage in the absence of a phloem-feeding (control); [1] – Two-week old-Betta wheat stressed with RWA; [2] – Two-week old Betta wheat stressed with BCA; [3] – Three-week-old Betta wheat stressed with RWA; [4] – Three-week-old Betta wheat stressed with BCA. Upregulated proteins of interest are marked with letters.

The protein corresponding to spot n in Figure 3.5.(A).1. is proposed to be a low molecular weight glutenin storage protein with a molecular mass of 41.307 kDa and a pI of 8.69. When two-week old Betta wheat is stressed with the BCA (Figure 3.5.A.2), the number of protein spots observed in the area of interest increases to 18-22 spots and the increased levels of streaking and smearing on the gel could allude to higher protein concentrations being found in this region of the gel. Once again, the most proteins of interest were found in the neutral to basic (pI of 7.0-9.0) range, in spots corresponding to proteins in the 38-55 kDa molecular weight region. The putative matches of protein spots to proteins in the SwissProt protein database include proteins that are involved in growth and development. The spots e, l and r in Figure 3.5.(A).2. are proposed to be NADH dehydrogenase subunit 2 (MW: 53 kDa; pI: 8.98), a leucine zipper protein (MW: 47.913, pI: 8.88) and a cysteine proteinase (MW: 40.807; pI: 6.8). It is important to note that no proteins that are specifically involved in plant stress or defence responses were matched to any of the upregulated protein spots in this case.

3.3.3. Three-week old Betta wheat responses to RWA and BCA phloem feeding

When three-week old Betta wheat is exposed to RWA phloem feeding (Figure 3.5.B.3.), a greater number of proteins are differentially expressed than was done in the two-week old cultivar. The greatest level of differential protein expression is observed in the same region of the gel as was observed in two-week old Betta wheat and the putative upregulated proteins from the SwissProt database include kinases and proteins involved in normal growth and development. The effect of BCA phloem feeding on three-week old Betta wheat showed a lesser extent of differential protein expression (Figure 3.5.B.2.) than did BCA phloem feeding on two-week old wheat. The putative protein matches from SwissProt include proteins involved in growth and development, proteins involved in photosynthesis as well a few proteins of unknown function as shown in Table 6.2. in Appendix 1.

3.3.4. Two-week old Betta DN wheat responses to RWA and BCA phloem feeding

In Betta DN wheat exposed to the RWA and the BCA, a different region of the proteome experiences marked differential regulation in response to stress, as shown in Figure 3.6.A and 3.6.B. In this case, the low molecular weight proteins appear to be more active in response to

both RWA and BCA phloem-feeding at both growth stages, although three-week old Betta DN wheat appears to express a greater number of proteins in response to feeding from both aphid types than two-week old Betta DN does, as shown in Figure 3.6.3 and 3.6.4.

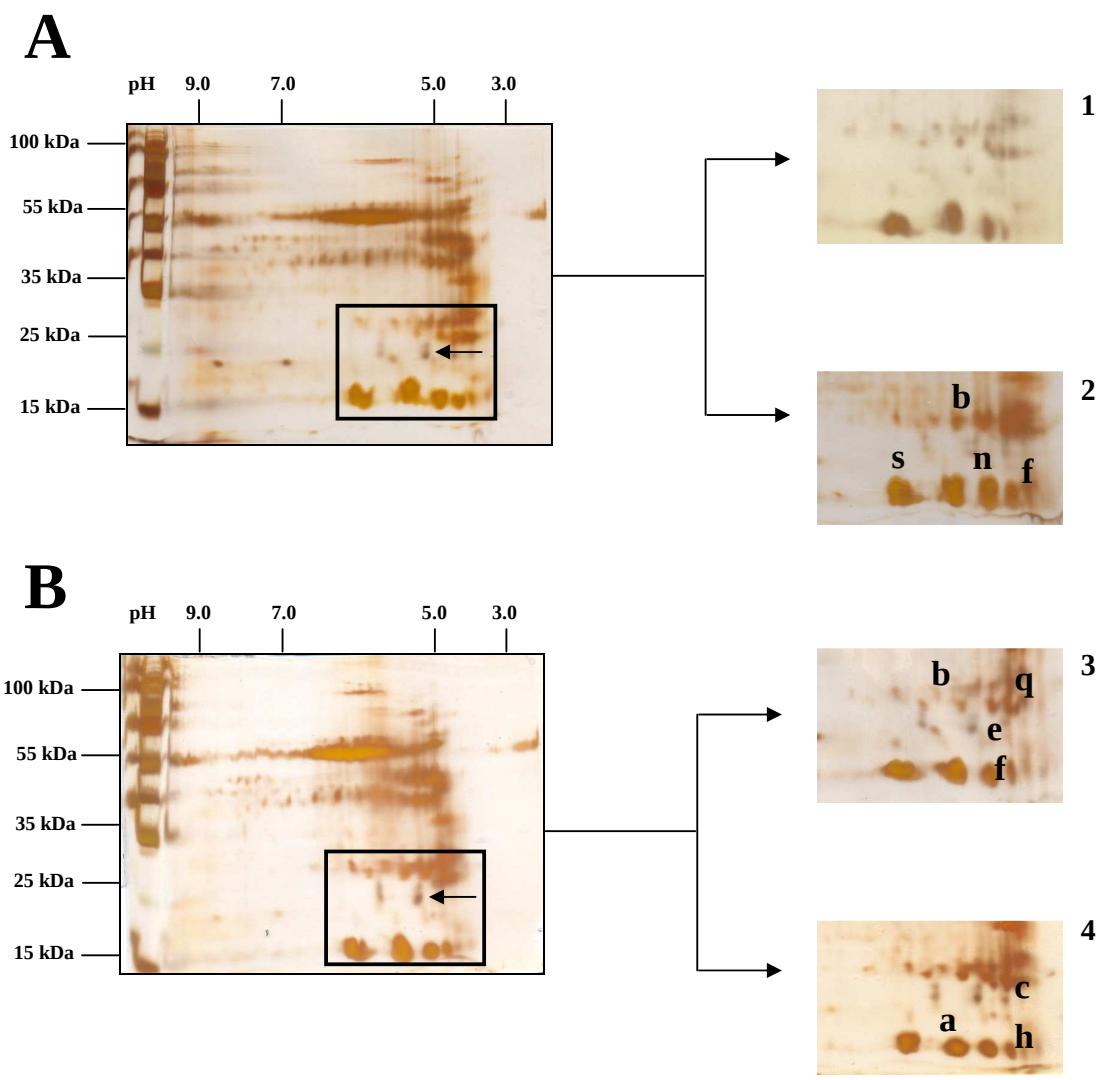


Figure 3.6. Differential protein expression in Betta DN wheat in response to RWA and BCA phloem feeding

Areas of most pronounced differential protein expression are circled in black in the figures on the left. Magnification of corresponding areas of RWA and BCA stressed gels are inserted on the right. Detailed gel images and analysis are recorded in Appendix 1. Black arrows indicate the spot used for gel normalisation. [A] – Betta DN wheat at the 2 week (3-leaf) growth stage in the absence of RWA phloem-feeding (control); [B] – Betta DN wheat at the 3 week (4-leaf) growth stage in the absence of RWA phloem-feeding (control); [1] – Two-week old-Betta DN wheat stressed with RWA; [2] – Two-week old Betta DN wheat stressed with BCA; [3] – Three-week-old Betta DN wheat stressed with RWA; [4] – Three-week-old Betta DN wheat stressed with BCA. Upregulated proteins of interest are marked with letters.

The Betta DN response to RWA and BCA phloem feeding appears to involve low molecular weight (below 20 kDa) and acidic (pI values lower than 6) proteins. Putative proteins upregulated in two-week old Betta DN wheat in response to RWA feeding included heat shock proteins and some defence related proteins as shown in more detail in Table 6.1. BCA phloem feeding results in the upregulation of proteins putatively involved in heat shock responses, defence, kinases and storage as shown in Table 6.2. in Appendix 1. None of the upregulated proteins occur in the highlighted area of interest in Figure 3.6.A.1. These proteins (indicated by spots n, f and s) are proposed to include a putative ubiquitin-conjugating enzyme (MW: 17.301; pI: 5.67), a 16.9 kDa class I heat shock protein (MW: 16.878 kDa; pI: 5.83) and an alpha-2-purothionin precursor fragment (MW: 14.558; pI: 5.13).

3.3.5. Three-week old Betta DN wheat responses to RWA and BCA phloem feeding

At the three-week growth stage, Betta DN wheat shows a higher level of differential protein expression characterised by a greater number of spots on the 2DE gels (Figures 3.6.B.3 and 3.6.B.4). Despite this, a similar amount of proteins are upregulated in three-week old Betta DN as was found in two-week old Betta DN in response to RWA feeding (Table 6.1 in Appendix 1). The upregulated proteins in response to RWA phloem feeding putatively include proteins involved in heat shock responses, photosynthesis and growth and development. These proteins, identified by spots b, e, f, and q are thought to include a putative chlorophyll a-b binding protein (MW: 28.264; pI: 5.67), ubiquitin conjugating enzyme (MW: 21.126; pI: 4.40), heat shock protein 20 (MW: 16.882; pI: 4.67) and a putative translationally-controlled tumour protein homolog (MW: 18.806; pI: 4.55). All the proteins that were upregulated in response to BCA phloem feeding at the three-week growth stage in Betta DN wheat were not putatively identified, as they did not have any close matches in terms of molecular mass and pI on the SwissProt protein database.

3.3.6. Comparison of Betta and Betta DN responses to RWA and BCA phloem feeding

Spots which corresponded to proteins that were found to be up or downregulated by a value of at least twice the spot intensity obtained from the control sample (master gel) were matched to

the specifications (pI value and molecular mass) of known wheat proteins from the SwissProt protein database (http://expasy.org/sprot/) where possible, in order to determine putative protein matches to spots from the 2DE gel analysis. This was only performed for proteins of interest that were found to be up or downregulated as opposed to for all differentially expressed proteins in the 2DE analysis. Figure 3.7. portrays a summary of the findings obtained from this study (detailed results are given in Table 6.1. and 6.2. in Appendix 1.

When two-week old Betta wheat was exposed to RWA phloem feeding, 5 proteins were putatively upregulated, as represented in Figure 3.7.1 and detailed in Figures 6.5. and 6.7. in Appendix 1. These were putatively identified as proteins involved in photosynthesis, storage, signalling and defence / stress in addition to one unidentified protein (Figure 3.7.1). At the three-week growth stage, the response of Betta wheat to RWA feeding changes (Figure 3.7.2.), with only 2 proteins being upregulated. These were putatively identified as a stress protein and a defence protein (Figure 3.7.2.). In terms of the response of Betta wheat to BCA phloem feeding (Figures 3.7.3 and 3.7.4 as well as Figures 6.5. and 6.7. in Appendix 1), the response between two and three week old Betta wheat is similar to that found for RWA feeding, although the types of proteins upregulated are different. When the BCA feeds on two-week-old Betta wheat, 3 proteins were upregulated. These were putatively identified to be a photosynthesis-related protein, a signalling protein and an unidentified protein (Figure 3.7.3.). Similar results were found for the three-week-old Betta wheat exposed to the BCA (Figure 3.7.4.).

The response of the resistant Betta DN wheat cultivar to RWA and BCA phloem feeding appears to rely on a greater diversity of proteins than does that of the susceptible Betta cultivar. Two proteins were upregulated in response to RWA feeding on two-week-old Betta DN wheat. These were putatively identified as a signalling protein and a protein involved in plant growth and development (Figure 3.7.5 and Figure 6.6. in Appendix 1). When three-week-old Betta DN wheat is subjected to RWA feeding for one week (Figure 3.7.6 and Figure 6.6. in Appendix 1), three proteins appear to be upregulated. These have been putatively identified as proteins involved in heat shock response, photosynthesis and one protein that plays a role in normal plant growth and development. In contrast to this, three-week-old Betta DN wheat exposed to the BCA putatively displayed upregulation of only one type of type protein, which remained provisionally unidentified.

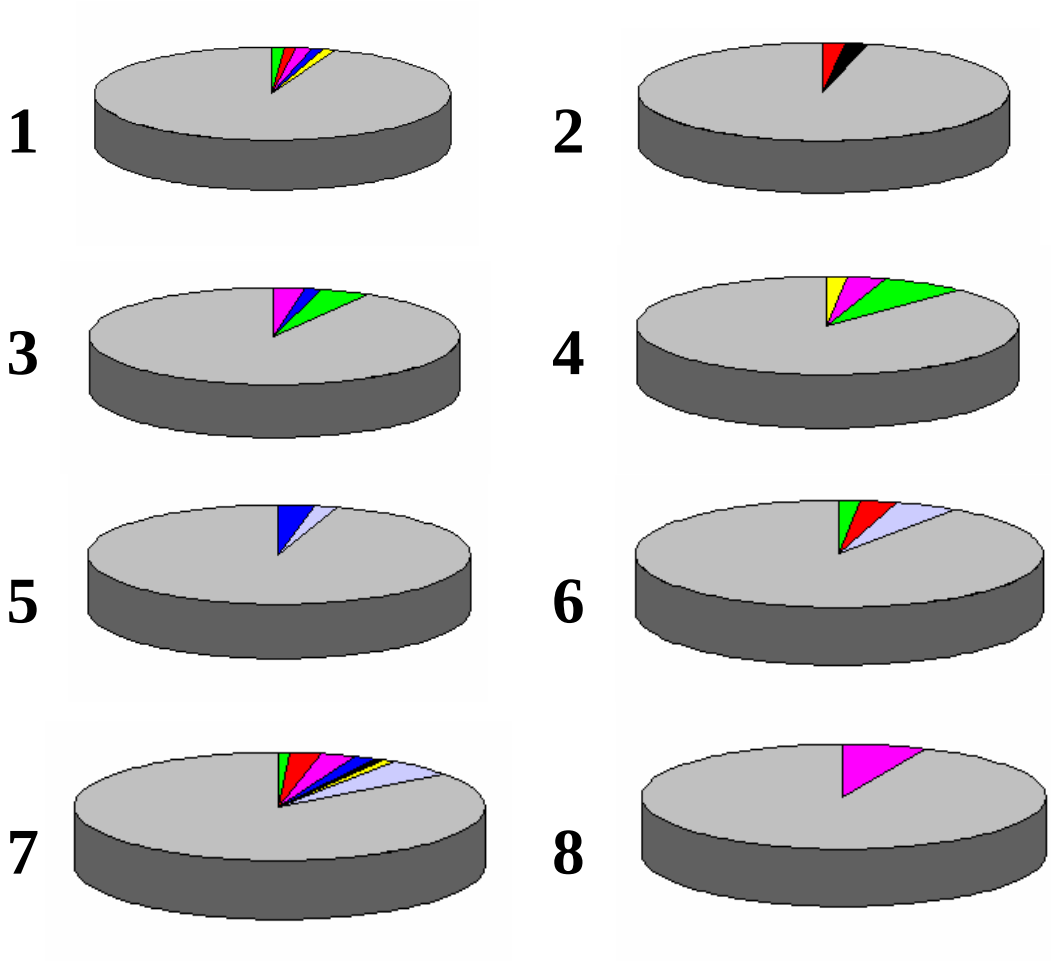


Figure 3.7. Summary of results obtained from 2DE analysis of Betta and Betta DN wheat subjected to RWA and BCA phloem feeding

Putatively upregulated spots were taken as fraction of the total number of spots obtained for each stress condition in order to construct pie charts. Different classes of protein, grouped according to interest are represented by the following colours: Photosynthesis-related protein - ; Heat shock protein / Stress protein - ; Unidentified protein (no match exists in SwissProt database) - ; Kinase - ; Protease Inhibitor - ; Defence protein - ; Storage protein - ; Other (proteins not related to stress / plant defence responses) - ; Proteins not found to be upregulated by PDQuest™ software (remainder of protein spots on gel) -

[1] – Two-week-old Betta wheat stressed with RWA; [2] - Three-week-old Betta wheat stressed with RWA; [3] - Two-week-old Betta wheat stressed with BCA; [4] - Three-week-old Betta wheat stressed with BCA; [5] - Two-week-old Betta DN wheat stressed with RWA; [6] - Three-week-old Betta DN wheat stressed with RWA; [7] - Two-week-old Betta DN wheat stressed with BCA; [8] - Three-week-old Betta DN wheat stressed with BCA

The largest number of upregulated proteins were observed when two-week old Betta DN was exposed to BCA phloem feeding for one week (Figure 6.8. in Appendix 1). These were putatively identified as proteins involved in stress, plant defence responses, storage, signalling, plant growth and development in addition to some unidentified proteins.

3.4. Discussion and Conclusions

The 2DE analysis of Betta and Betta DN wheat stressed with RWA and BCA yielded interesting and somewhat unexpected results. Betta wheat, like all known cultivars lacking the DN gene, is susceptible to RWA infestation. It has been proposed that resistance against RWA phloem feeding is obtained when plants upregulate the synthesis of proteins that are involved in defence responses to bacterial and fungal pathogens (Botha *et al*., 2006; Reymond *et al*., 2000). DNA microarray studies performed on the effects of RWA feeding on wheat indicate that several genes are up and down regulated in response to aphid feeding. These include genes encoding proteins that are involved in defence and signalling, oxidative burst (relating to the hypersensitive response), cell wall degradation, cell maintenance, photosynthesis and energy production (Botha *et al*., 2006). Although DNA microarray studies are useful in identifying genes that are upregulated during stress responses, they do not identify actual changes occurring in the proteome. A proteomic approach was therefore followed in this study to investigate whether specific wheat proteins could be identified that are differentially regulated during aphid stress responses and to potentially validate the results observed in the published DNA microarray studies. It is important to note that this is only a pilot study to investigate the efficacy of a proteomic approach in identifying these differentially expressed wheat proteins during aphid infestation and therefore only putative identification of differentially expressed proteins was attempted.

The present study investigated potential differences in proteins that were upregulated in the susceptible and resistant wheat cultivars Betta and Betta DN during aphid infestation in addition to determining what effect, if any, plant age and aphid type may have on the observed stress response. Unstressed Betta wheat did not upregulate any stress proteins during its normal growth. Physical observations of plants subjected to aphid herbivory in our laboratory indicated that the plants withstood the negative effects of aphid feeding for a

longer period if the plants were infested when they were younger. The plants used to maintain the developing RWA colony were grown to the three weeks stage prior to transferral of the RWA and showed the damaging effects of aphid feeding much sooner than the experimental two-week old plants.

The control protein IF4E1, is crucial during the early stages of protein synthesis and serves to facilitate ribosome binding to mRNA sequences by assisting in the unwinding of mRNA secondary structures (Metz *et al*., 1992). As this protein putatively occurs in every gel in the current experiment and despite the fact that slight differences in the intensity of the spot thought to correspond to it do not indicate up or down regulation according to PDQuest™ analysis, it can be proposed to serve as a form of internal control to verify that differences in spot intensity detected by the software and identified as up or down regulated proteins was indeed such.

The response at the two growth stages appears to be quite different. Two-week old Betta wheat stressed with RWA for a week-long period displays upregulation of proteins involved in photosynthesis, storage, signalling and stress responses. These proteins are proposed to play a role in countering the initial symptoms of aphid herbivory. For instance, aphid feeding is known to disrupt normal photosynthesis that would necessitate the upregulation of proteins involved in photosynthesis such as the Photosystem II P680 chlorophyll A apoprotein, which would attempt to compensate for a shortage of nutrients in the plant (Botha *et al*., 2006). This would also explain the upregulation of the putative glutenin storage protein and Hsp90; the latter is thought to play a role in plant stress conditions by regulating the activities of various proteins with which it comes into contact and could potentially be involved in as yet unknown pathways of plant defence (Krishna *et al*., 1997). Three-week old Betta wheat displays the upregulation of only two stress proteins, viz., a small heat shock protein and a thaumatin-like protein as shown Table 6.1 in Appendix 1. Thaumatin-like proteins play an important role in plant defences, most notably in plant antifungal activity. They are pathogenesis proteins (PR-5) that occur in monocotyledonous and dicotyledonous species and have been shown to act as protease and reverse-transcriptase inhibitors (Vitali *et al*., 2006). The upregulation of thaumatins in response to increased concentrations of salicylic acid (SA) within the plant, indicate the possibility that some form of systemic acquired resistance (SAR) has been acquired by means of the SA-mediated pathway. SAR is common in plant-pathogen

interactions and confers broad-spectrum disease resistance to the affected plant, which protects it from future pathogen encounters (Jayaraj *et al*., 2004). The upregulation of thaumatin and possible development of SAR has not been previously reported during RWA feeding on susceptible wheat cultivars and therefore will require further investigation in future studies in order to elucidate the possible role and function of thaumatin in wheat stress responses during plant and aphid interactions. The presence of SAR in plants in response to RWA infestation has been confirmed from the literature but this is thought to largely belong to resistant plant cultivars, so further work would have to be performed in order to confirm the possibility of such a response in susceptible Betta wheat (Botha *et al*., 2005).

During normal growth and development from the two to the three-week growth stage, Betta wheat displays the upregulation of a storage protein (putatively identified as a low molecular mass glutenin of 41 kDa with a pI of 8.69) and several signalling proteins as shown in Table 6.1. in Appendix 1. The absence of any upregulated stress / defence proteins in the unstressed Betta wheat indicates that it was not experiencing any form of stress during its normal growth process and could also indicate that proteins putatively involved in plant defence responses are not constitutively expressed in the susceptible Betta wheat. The expression of genes that are involved in plant defence responses against fungal and bacterial pathogens has been reported in wheat that has been exposed to RWA phloem-feeding (Botha *et al*., 2006). This type of response is not associated with plant responses to chewing insects and is considered more similar to the type of response obtained in plant-pathogen interactions, which may be due to an elicitor that behaves like a pathogen that is contained in the watery saliva that aphids inject into plants prior to feeding (van der Westhuizen *et al*., 1998 a,b). The literature appears to indicate that defence responses such as the production of glucanases and PR proteins only occurs in resistant wheat cultivars such as Betta DN (van der Westhuizen *et al*., a).

When the resistant cultivar, Betta DN was subjected to RWA feeding, a larger number of plant defence proteins were upregulated. As with the Betta cultivar two-week old Betta DN displayed a higher number (7 proteins) of upregulated proteins than three-week old Betta DN. In the case of Betta DN, the thaumatin protein precursor was upregulated at the two-week growth stage but not at three weeks. This is in contrast to what would be expected if the Betta DN plants had developed SAR. When the BCA is allowed to feed on Betta wheat various

proteins that are implicated in plant growth and development are thought to be upregulated. It would appear that the observation that BCA is not as harmful to Betta wheat is confirmed by the nature of the proteins that are upregulated as no putative stress proteins were found to have been upregulated. This could indicate that the damage caused by BCA phloem feeding is not sufficient to trigger a defence response in affected plants. When the BCA feeds on the RWA resistant Betta DN cultivar, a great diversity of other proteins are putatively upregulated, including a number of heat shock proteins and one resistance protein of uncertain function.

It appears that differences do exist in plant responses to aphid phloem feeding at different growth stages. Both resistant and susceptible wheat cultivars show greater diversity of protein upregulation when stressed at the two-week as opposed to the three-week growth stage. A clear mechanism of RWA or BCA resistance in wheat plants could not be determined from the present findings. However, several possible candidate proteins have been identified and their possible function in RWA and BCA resistance will need to be investigated in future studies. A general observation is that infestation with the BCA results in the upregulation of a greater number of proteins than does RWA infestation, in both Betta and Betta DN wheat. However, these proteins appear not to be involved in known stress responses. This could serve to explain why the effect of BCA feeding is not as detrimental to plants as RWA feeding. It also serves to confirm the findings in literature which state that BCA phloem-feeding is asymptomatic and nonchlorosis-eliciting and that the main damage resulting from BCA phloem-feeding is due to it acting as a vector of barley yellow dwarf virus as opposed to causing significant structural damage of itself (Hestler and Tharp, 2005; Xinzhi and Quisenberry, 2006). In closing, it can be stated that these preliminary results suggest that RWA feeding on susceptible and resistant wheat results in a possible common upregulation of thaumatin protein which is indicative of the development of a salicylic acid-dependent SAR type response. However, the resistant wheat cultivars showed an upregulation of several other proteins involved in plant stress / defence responses, including heat shock proteins and defence proteins. This indicates that the development of resistance to RWA involves several other pathways in addition to the SAR response for the BCA. The role of plant defence proteins in Betta and Betta wheat in response to RWA phloem feeding will be discussed in greater depth in Chapter 4.

Chapter 4

Differential protein expression in Betta and Betta DN wheat cultivars in response to feeding by the Russian wheat aphid

4.1. Introduction

Diuraphis noxia (Russia wheat aphid, RWA) is a very serious pest of both wheat and barley, which causes significant losses to farmers, most notably in the United States, Canada and South Africa, each year (Budak *et al*., 1999). It is currently regarded as the most destructive pest of wheat in South Africa (van der Westhuizen *et al*., 1998a). Symptoms of RWA feeding on susceptible wheat cultivars include longitudinal leaf chlorosis, leaf curl / rolling, prostrate growth habit caused by a rolled flag leaf (Cabrera *et al*., 1995; Messina and Sorenson, 2000). In addition, RWA feeding on the flag leaf of wheat causes the developing grain head to become trapped, which interferes with self-pollination and grain-filling, while the reduction in effective leaf area that subsequently interferes with photosynthesis (van der Westhuizen *et al*., 1998b). RWA are also known to inject a phytotoxin through their stylets, which causes the breakdown of the chloroplast and cellular membrane in susceptible plants. RWA feeding also results in a chlorophyll deficiency, which can often be fatal in affected plants (Botha *et al*., 2006; Burd and Elliot, 1996).

Studies have revealed that the wheat apoplast plays a significant role in defence against both pathogens and aphids (van der Westhuizen *et al*., 1998a). In fact, the plant response to aphid infestation is proposed to be more similar to that exhibited in response to pathogen infection than to that displayed upon insect herbivory (Bowles, 1990; van der Westhuizen *et al*., 1998b). Resistant wheat cultivars appear to compensate for RWA infestation and feeding by means of the accumulation of certain proteins in their intercellular fluid. Defence proteins are also found to accumulated in the apoplast, among which are the pathogenesis-related (PR) proteins and peroxidases (Bowles, 1990). Studies have suggested that the defence response of resistant wheat cultivars is induced upon RWA feeding as opposed to being constitutively expressed (van der Westhuizen *et al*., 1998a). Analyses using one dimensional protein electrophoresis (SDS-PAGE) have indicated that RWA infestation of wheat results in the differential expression of proteins in resistant cultivars only. These proteins were found to be of predominantly low molecular weight, ranging from 17 – 28 kDa, and at least some are proposed to be serologically related to PR proteins (van der Westhuizen, 1996). Due to the limitations of SDS-PAGE, it was not possible to develop a comprehensive understanding of the wheat proteome upon RWA infestation in the study mentioned above as some proteins of

similar molecular mass may have migrated as a single band, thus effectively obscuring one another.

No detailed proteomic analysis of wheat subjected to aphid stress has been performed to date. Bahlmann conducted a study on Russian wheat aphid (*Diuraphis noxia*) induced protein alterations in Tugela and Tugela DN wheat. Some differential protein expression was identified, but no detailed analyses of these were conducted (Bahlmann, 2002). Two dimensional gel electrophoresis studies (2DE) of wheat have focussed largely, but by no means exclusively, on the quality of grain to be used in bread making as well as aiding in the identification of potential allergens prior to human consumption of grain (Kitta, 2005; Skylas *et al*., 2000). Other factors such as the characterization of protein expression during development in mature grains and the effect of heat stress in tolerant and susceptible wheat have been investigated using a proteomics approach (Clarke et al., 2000; Majoul et al., 2004). Most proteomic studies of wheat have focussed on the differential analysis of proteins within wheat grain. Little is known about the wheat leaf proteome and wheat developmental studies. The present work aimed to determine the nature of the RWA-induced stress response in both resistant and susceptible wheat cultivars over time, by sampling the wheat leaf proteome at set periods of time during an RWA infestation. It was hoped that this would give a clearer indication of the nature of wheat defence responses against RWA phloem feeding.

4.2. Materials and Methods

4.2.1. Materials

Wheat seeds and aphids were obtained from the Agricultural Research Council of South Africa (Bethlehem, South Africa) while potting soil was purchased from Green Fingers Nursery in Port Elizabeth. The total protein extraction, reduction alkylation, 2D clean up and protein assay kits were purchased from Bio-Rad (U.S.A.). Liquid nitrogen was obtained from the Rhodes University Department of Chemistry (Grahamstown, South Africa). Bovine Serum Albumen Fraction V was purchased from Roche (Switzerland). PageBlue™ protein staining solution was a free sample from Fermentas (Canada) while the SilverSnap® Stain kit II silver staining kit was purchased from Pierce (U.S.A.). Isolectric focussing equipment was

from Invitrogen (France) while immobilized pH gradient strips were from Bio-Rad (U.S.A.). Protein molecular mass markers were purchased from PEQLab (Germany). All other reagents for buffers and gel reactions were purchased from Sigma and Bio-Rad (U.S.A).

4.2.2. Cultivation of wheat plants

Wheat seeds (*Triticum aestivum L.*) cv. Betta and Betta DN were germinated overnight in a petri dish on filter paper prior to planting in a 70:30 mix of potting soil (Green Fingers Nursery, Port Elizabeth) and vermiculite as described in Chapters 2 and 3. Each pot contained roughly 20 wheat seeds and plants were grown under controlled conditions in a Conviron S10H growth cabinet at the Rhodes University Department of Botany. The Conviron was maintained at a constant temperature of 26 ± 1°C at a relative humidity of approximately 70 % while plant nutrient status was maintained with a 75 % solution of Long Ashton nutrient mix which was prepared as outlined in Appendix 1 and was administered every two days. All plants were subjected to a 16-hour photoperiod.

4.2.3. Subjection of Triticum aestivum L. plants at the 3-leaf growth stage to stress by *Diuraphis noxia (Mordvilko)* feeding for predetermined time periods

Aphids were obtained from the Agricultural Research Council's Small Grain Institute in Bethlehem, South Africa. The parental colony was allowed to develop on susceptible wheat (*Triticum aestivum* cv. Betta) plants and plants were stressed with aphids after they had adjusted sufficiently to the growth conditions in the controlled environment. Wheat plants (Betta and Betta DN) were grown in a Conviron S10 H at the Rhodes University Department of Botany at a temperature of 26±1°C and a relative humidity of 70 % in the absence of aphids. Adequate nutrient status was maintained by watering plants with 75 % Long Ashton nutrient solution every two days. Plants that had reached the 3-leaf stage of development (two weeks old) were stressed with RWA over a set time period of 9 days. Ten aphids were placed on each plant in the study for the duration of the study and were carefully removed with a paintbrush prior to harvesting. Susceptible and resistant wheat plants were stressed with RWA for periods of 2, 3, 5, 7 and 9 days in order to determine the differential protein expression over the first week in response to stress incurred due to aphid feeding as outlined

in Figure 4.1. Control plants were grown under the same conditions as the test plants, but were kept isolated from any contact with aphids. The control plants were sampled at the same time intervals as the test plants in order to develop a protein profile of wheat plants at the 3-leaf growth stage. Stressed plants and their unstressed controls were kept separate in order to eliminate the possibility of aphid contamination of control plants. The possibility of RWA contamination of control plants was eliminated by keeping aphid-infested plants in aphid cages (as shown in Chapter 3) and keeping control and experimental plants in separate convirons. The aphid cages allowed for sufficient ventilation of aphids and plants but restricted aphid movement. Plants for each time study were harvested and placed directly into liquid nitrogen prior to the extraction of total proteins from the frozen leaf tissue.

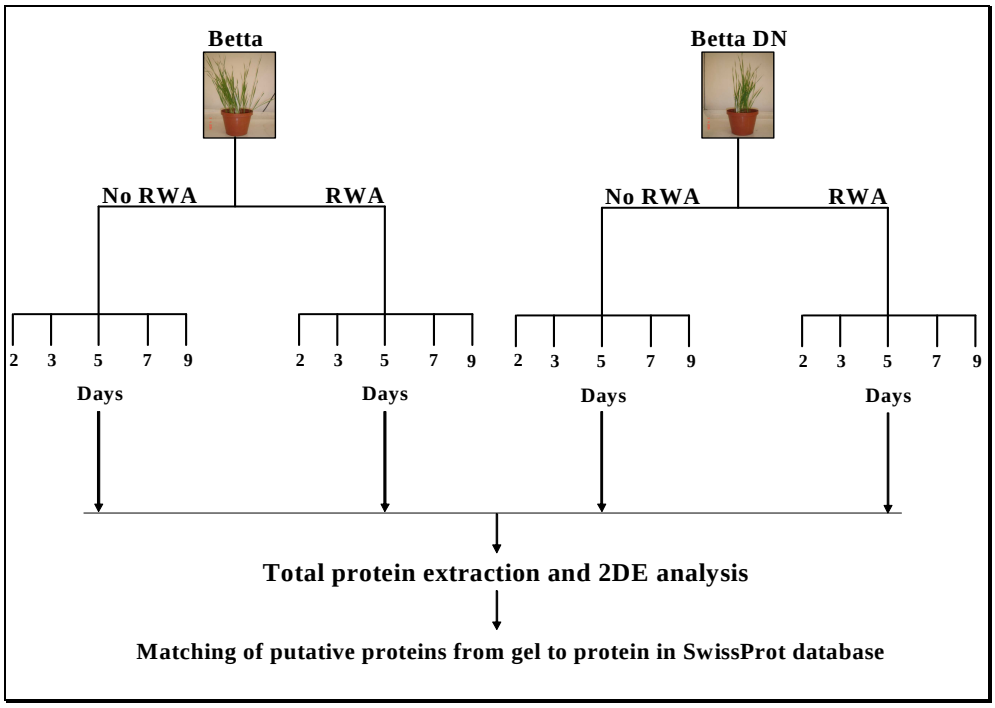


Figure 4.1. Schematic representation of the experimental approach undertaken for the determination of the differential protein expression in Betta and Betta DN wheat in response to RWA phloem feeding over various time periods (in days after feeding commenced)

4.2.4. Total protein extraction from stressed and unstressed wheat leaves (Betta and Betta DN)

Control and experimental plants were harvested simultaneously, with care being taken not to bring any uninfested wheat samples into contact with the RWA. The controls were harvested and immediately placed into sterile falcon tubes that were snap-frozen in liquid nitrogen. In the case of infested plants, aphids were gently removed with the aid of a paintbrush prior to total protein extraction as outlined in Chapter 2. The extraction of total protein was performed using the ReadyPrep™ Protein Extraction Kit (Total Protein) from BioRad (Catalogue, U.S.A.) as per kit manufacturer's instructions. Total protein extraction proceeded as outlined in Chapter 2. The extraction was performed using the ReadyPrep™ Protein Extraction Kit (Total Protein) from Bio-Rad (U.S.A.) as per manufacturer's instructions. Homogenized leaf tissue was suspended in 1 ml ReadyPrep 2-D Rehydration / Sample buffer 1 (7 M urea, 2 M thiourea, 1 % (w/v) ASB-14 detergent, 40 mM Tris base and 0.001 % bromophenol blue) containing TBP reducing agent (200 mM tributylphosphine in 1-methyl-2-pyrrolidinone). Samples were sonicated in four bursts of 30 seconds each prior to centrifugation at 14 000 g for 30 minutes at room temperature. After centrifugation, the supernatant was retained and reduced and alkylated using the ReadyPrep™ Reduction-Alkylation Kit from Bio-Rad (U.S.A.), while the pellet was discarded. The protein content of all samples was determined using the RC / DC assay (Bio-Rad, U.S.A.).

4.2.4. Two dimensional gel electrophoresis of stressed Betta and Betta DN wheat at different stages after aphid infestation as well as of unstressed controls (Betta and Betta DN)

Wheat samples were prepared as described in 4.2.3. and cleaned up using the ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, U.S.A.). Subsequently, the total protein pellet obtained was resuspended in 2-D rehydration buffer (250 µl; 9 M Urea, 2 % Triton-X 100, 2 % Bio-Lyte 3-10 buffer, 0.1% Bromophenol Blue and 0.02 g dithiothreitol). Broad-range pH 3-10 NL (7 cm) ReadyStrip™ IPG strips were rehydrated overnight in 160 µl of 2-D rehydration buffer. The IPG strips were rehydrated in Zoom® IPGRunner™ Cassettes prior to performing isoelectric focussing (IEF) using a Bio-Rad PowerPac™ HV power supply. The applied electric potential (V) was increased in a continuous ramp fashion as follows: 200 V (20

minutes), 450 V (15 minutes), 750 V (15 minutes) and 2000 V (60 minutes). The current was maintained at 5 mA throughout the duration of the IEF procedure and the IEF was resolved in water.

4.2.5. Staining, visualisation and analysis of 2DE gels

Gels were stained with the SilverSNAP® Stain Kit II (Pierce, U.S.A.) as per manufacturer's instructions prior to imaging with a ChemiDoc ™ EQ gel documentation system (Bio-Rad, U.S.A.). Stained gels were analysed using PDQuest™ Basic Software Version 8.0 (Bio-Rad) as described in Chapter 2. Gels were grouped and analysed using the Spot Detection Parameter Wizard with a sensitivity of 0.49 while allowance was made for horizontal and vertical streak removal. In each case, the gel corresponding to the control reaction for the given experimental group served as the master gel for the purposes of the software analysis. PDQuest™ Basic Software Version 8.0 (Bio-Rad) relies on manual identification of faint spots, large spots and the largest spot cluster, allowing for some user input in terms of parameters. A speckle filter set at 50 was applied in order to remove speckles from the gel. This was considered necessary despite the fact that some small and faint spots may have been lost in the process. A Gaussian statistical model was used to detect and fit spots while a local regression model was selected for data normalisation.

Once the 2DE gels were analysed using the gel analysis software, the relative molecular mass and pI of spots identified as being potentially up or down regulated, was determined. The molecular mass of proteins corresponding to spots of interest was assigned based on the molecular weight markers that were resolved alongside the sample in each gel. The pI of proteins corresponding to spots of interest was determined by using a graph of pI values from a pH 3-10NL IPG strip as given in Westermeier and Naven (2002) and plotting the appropriate distances corresponding to a given pI value along the gel. The putative molecular mass and pI value obtained for each protein spot of interest was subsequently compared to a list of the known and characterised wheat proteins on the SwissProt database (http://expasy.org/sprot/) and an attempt to match the unique pI value and molecular mass of the protein spots of interest (putatively up or down regulated proteins) to known wheat proteins was made in order to determine the nature of wheat stress responses to aphid infestation at the protein level.

4.3. Results

4.3.1 Differential protein expression in Betta wheat (RWA-susceptible) in response to RWA infestation over set time periods

Select 2DE gels of Betta and Betta DN wheat exposed to RWA phloem feeding over different time periods are shown in Figures 4.2 and 4.3. Detailed results and analysis of all 2DE gels are shown in Appendix 2. An average of 70 spots was observed on each gel in the analysis, although some stress conditions (such as Betta DN exposed to RWA feeding) appeared to result in the production of more proteins than others. The spot corresponding to IEF4 was again selected as an internal control and served as a basis of relative comparison for proteins that were thought to be up or down regulated in further analysis of each gel. Once again, a spot proposed to correspond to the eukaryotic translation initiation factor 4E-1 (IF4E1) as a possible match (shown by the white arrows in Figures 4.2. and 4.3.). IF4E1 has a molecular mass of 23.9 kDa and a pI of 5.39. This protein is crucial during the early stages of protein synthesis and serves to facilitate ribosome binding to mRNA sequences by assisting in the unwinding of mRNA secondary structures (Metz *et al*., 1992). As this protein putatively occurs in every gel in the current experiment and despite the fact that slight differences in the intensity of the spot thought to correspond to it do not indicate up or down regulation according to PDQuest™ analysis, it can be proposed to serve as a form of internal control to verify that differences in spot intensity detected by the software and identified as up or down regulated proteins was indeed such.

As outlined in Chapter 3, spots corresponding to up or down regulated proteins were putatively identified by matching their molecular mass and pI to known wheat proteins found in the SwissProt protein database in an attempt to gain a knowledge of the possible changes in the wheat proteome in response to RWA feeding as well as an understanding of the mechanism of plant stress responses to aphid phloem-feeding. Only proteins that were up or down regulated with a two-fold or higher value were investigated, as a manual investigation of all differentially expressed protein spots was not feasible in terms of the time available to complete this work. These detailed results are given in Figures 7.1-7.4 and Tables 7.1-7.4 in Appendix 2 and summarised in Figure 4.2. and 4.3. RWA-stressed Betta wheat displayed the

highest level of differentially expressed proteins in low molecular weight and acidic regions of the 2DE gels.

A marked difference in the Betta wheat proteome was observed after only 2 days of RWA phloem feeding (Figures 7.1.E and 4.2.A). Five proteins were detected by the PDQuest software to have been up or down regulated at this stage as shown in Table 7.1 and by the yellow triangles in Figure 7.3.B.1. The upregulated proteins putatively include proteins involved in photosynthesis and an unidentified protein. A downregulated stress protein found at this stage is proposed to be a β-1,3-endoglucanase. After 3 days of RWA phloem-feeding (Figure 4.2.B), the putative β-1,3-endoglucanase was found to be upregulated while two unidentified proteins (proteins with no matches in terms of molecular mass and pI to proteins deposited in the SwissProt database) were found to be downregulated. The upregulation of a putative β-1,3-endoglucanase is also seen after 5 days of RWA phloem feeding on Betta wheat (Figures 7.3.A.3. and 4.2.C). Two putative heat shock proteins were also found to be upregulated at this point. An interesting finding at this point, is that there is a marked upregulation of higher molecular mass (greater than 40 kDa) and basic proteins (indicated by the dotted box in Figures 4.2.C – 4.2.E). It appears that the Betta wheat leaf proteome undergoes greater changes after 5 days of RWA herbivory than during the initial stages of phloem feeding (2 days – 3 days). A further two days of RWA phloem feeding on Betta wheat (Figure 4.2.D) resulted in the proposed upregulation of a number of unidentified proteins and a number of proteins that were putatively assigned as heat shock proteins, storage proteins and proteins involved in photosynthesis. After 9 days of continuous RWA phloem feeding, the Betta wheat was already showing some of the damaging physical symptoms of RWA phloem feeding, including chlorosis and leaf curl as outlined in Chapter 3. At this stage, 13 proteins were found to be up or down regulated, and include putative heat shock proteins, defence proteins (β-1,3-endoglucanase and a type-5 thionin precursor), storage proteins and several proteins that could not be provisionally identified.

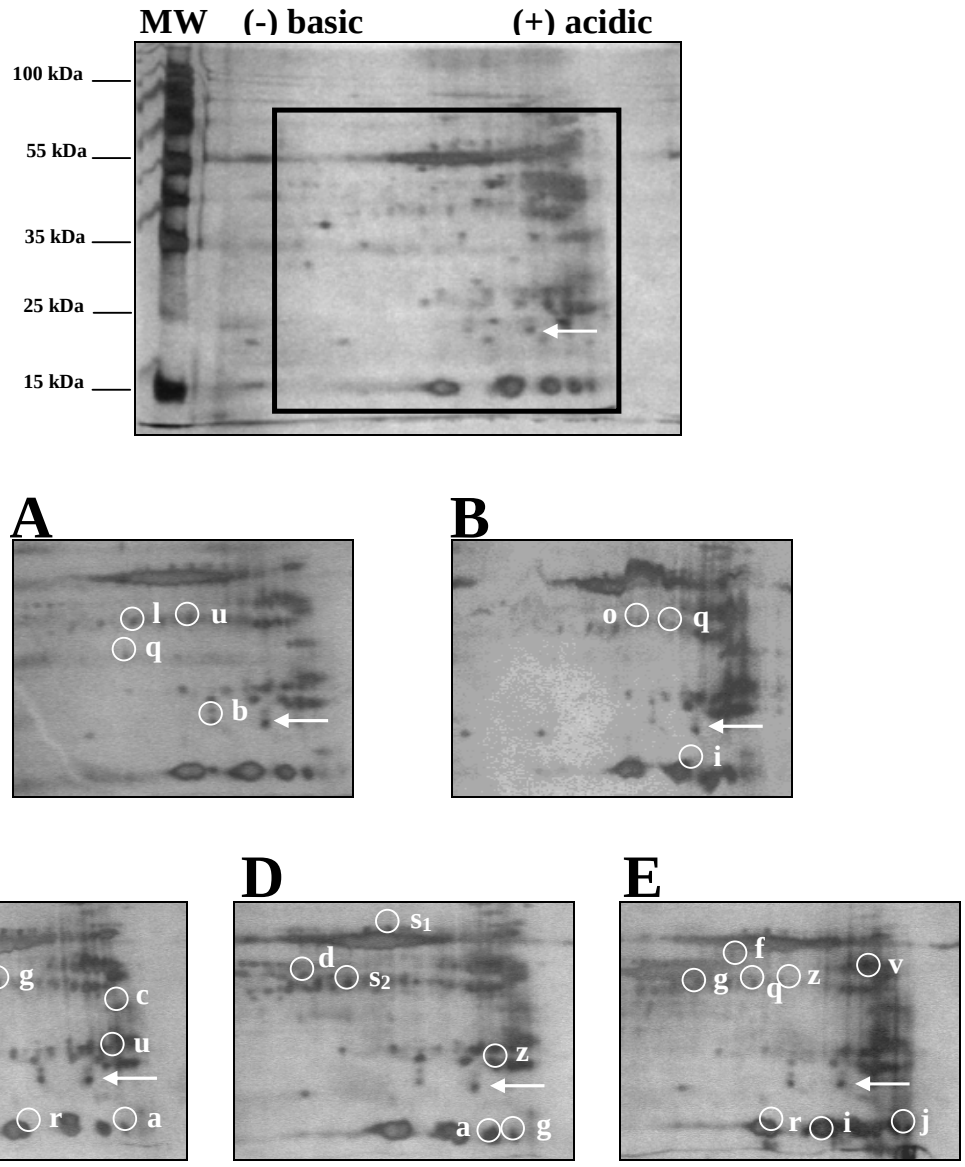


Figure 4.2. Summary of 2DE gel electrophoresis results representing differential protein expression in Betta wheat in response to RWA phloem feeding observed at fixed intervals over a 9 day period

The experimental control (master gel) of unstressed two-week-old Betta wheat is the annotated gel at the top of the figure. Figure A – Betta wheat after 2 days of RWA feeding; Figure B – Betta wheat after 3 days of RWA feeding; Figure C – Betta wheat after 5 days of RWA feeding; Figure D – Betta wheat after 7 days of RWA feeding; Figure E – Betta wheat after 9 days of RWA feeding. Gels were cropped to include the area of interest highlighted on the master gel. The internal control protein, IF4E1 is indicated by the white arrows and key up or downregulated proteins are circled in white and identified by their lettering as given in Figure 7.3. in Appendix 1

4.3.2 Differential protein expression in Betta DN (RWA-resistant) wheat in response to RWA infestation over set time periods

Betta DN is the isogenic RWA-resistant cultivar of Betta. Due to its resistance to RWA phloem feeding, it was anticipated that the response of Betta DN to RWA phloem feeding would differ from that of Betta at the proteomic level. After 2 days of RWA phloem feeding (Figures 7.4.E and 4.3.A) the Betta DN wheat leaf proteome already displayed the upregulation of putative defence proteins, including a putative endo-beta-1,3-glucanase and a thaumatin-like protein. The other upregulated proteins at this stage of infestation are suspected to be kinases, proteins involved in photosynthesis and a number of unidentified proteins. After exposure to the RWA for 3 days (Figures 7.4.F and 4.3.B), it would appear that the defence response undergoes a change (Figure 7.6.B.3). Fewer proteins are upregulated than at 2 days after RWA feeding commenced, including fewer putative defence proteins (Table 7.4. in Appendix 2). However, one defence protein, the putative endo-beta-1,3-glucanase is still found to be upregulated at this point.

At 5 days of RWA feeding, the number of upregulated proteins is even lower than in the previous two stages of feeding (2 days and 3 days as shown in Table 3.4.), but it is interesting to note that of the five proteins that were observed to be upregulated, three are defence proteins, including the putative endo-beta-1,3-glucanase seen in the previous two sampling stages. The additional two defence proteins were putatively identified as an alpha amylase trypsin inhibitor. The putative alpha amylase trypsin inhibitor is even more markedly upregulated after 7 days of RWA phloem feeding as shown in Figures 7.6.5. and 4.3.D.

It is interesting to note that the same marked change that took place in the Betta wheat leaf proteome after 7 days of RWA feeding also occurs in the resistant Betta DN cultivar (Table 7.2. and 7.4. At this stage, like in Betta wheat, a marked upregulation of proteins with molecular weights above 40 kDa and with basic pI's is found (Figure 4.3.C).

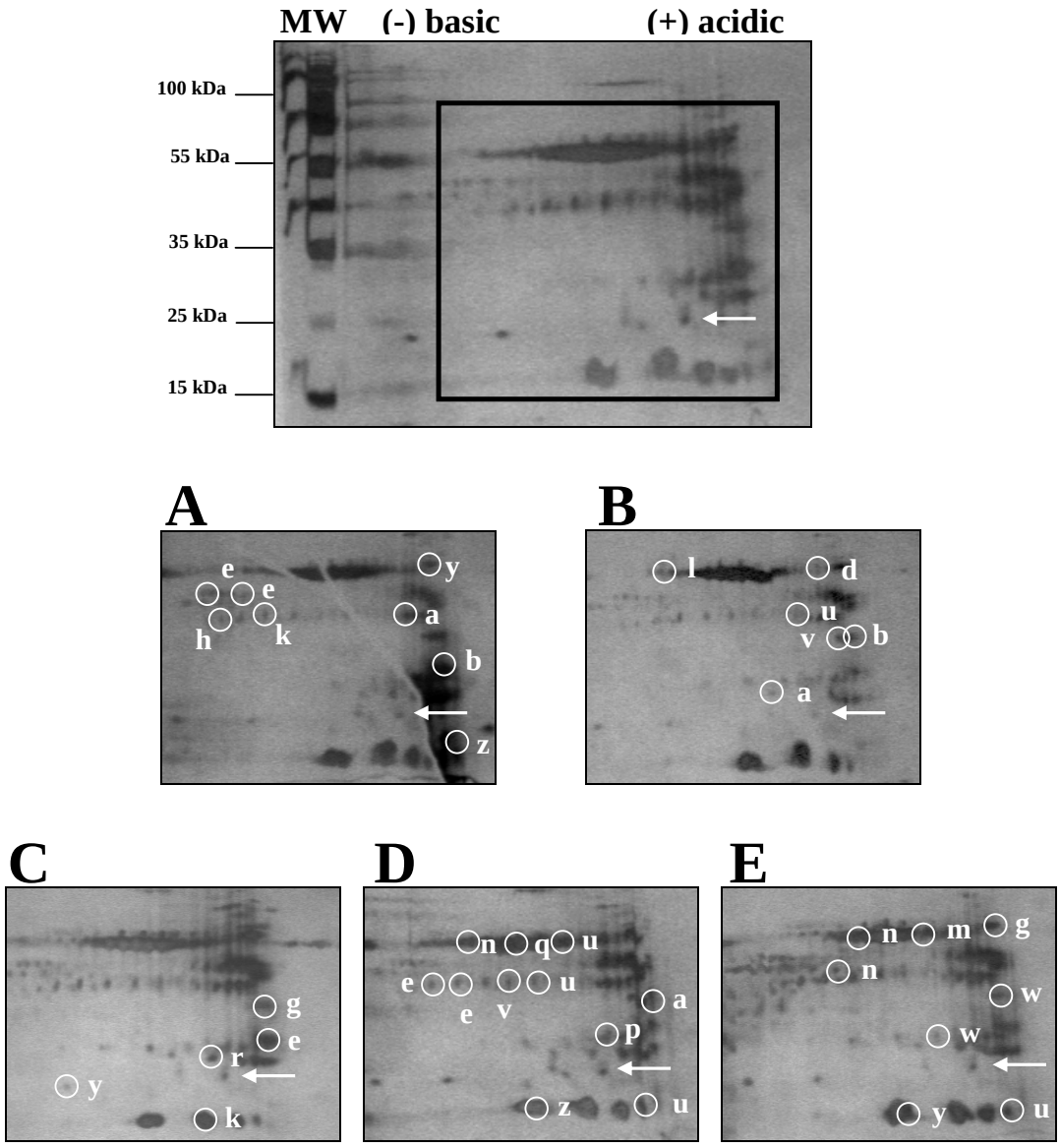


Figure 4.3. Summary of 2DE gel electrophoresis results representing differential protein expression in Betta DN wheat in response to RWA phloem feeding observed at fixed intervals over a 9 day period

The experimental control (master gel) of unstressed two-week-old Betta DN wheat is the annotated gel at the top of the figure. Figure A – Betta DN wheat after 2 days of RWA feeding; Figure B – Betta DN wheat after 3 days of RWA feeding; Figure C – Betta DN wheat after 5 days of RWA feeding; Figure D – Betta DN wheat after 7 days of RWA feeding; Figure E – Betta DN wheat after 9 days of RWA feeding. Gels were cropped to include the area of interest highlighted on the master gel. The internal control protein, IF4E1 is indicated by the white arrows and key up or downregulated proteins are circled in white and identified by their lettering as given in Figure 7.3. in Appendix 1

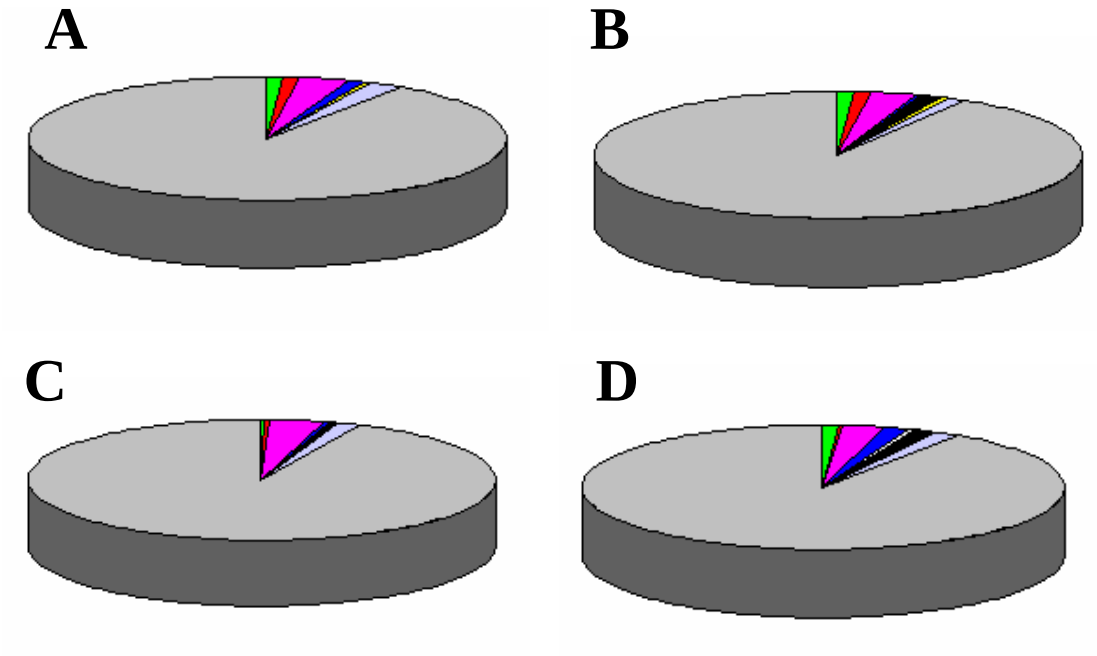


Figure 4.4. Summary of results obtained from 2DE analysis of Betta and Betta DN wheat subjected to RWA phloem feeding

Putatively upregulated spots were taken as fraction of the total number of spots obtained for each stress condition in order to construct pie charts. Different classes of protein, grouped according to interest are represented by the following colours: Photosynthesis-related protein - ; Heat shock protein / Stress protein - ; Unidentified protein (no match exists in SwissProt database) - ; Kinase - ; Protease Inhibitor - ; Defence protein - ; Storage protein - ; Other (proteins not related to stress / plant defence responses) - ; Proteins not found to be upregulated by PDQuest™ software (remainder of protein spots on gel) -

Putatively upregulated spots were taken as fraction of the total number of spots obtained for each stress condition in order to construct pie charts. In order to simplify the analysis, the results over the entire stress period were taken into account, to give an overview of protein upregulation in response to RWA feeding. Figure A – Unstressed Betta wheat; Figure B – Betta wheat stressed with RWA; Figure C – Unstressed Betta DN wheat; Figure D – Betta DN wheat stressed with RWA

A large number of proteins are up or downregulated after 7 days of RWA feeding on Betta DN wheat, making it appear that the plant has entered a second phase of its defence response, potentially catalysed by the upregulation of the putative alpha amylase trypsin inhibitor after 5 days of RWA feeding. Many of the proteins at this stage were found to be unidentified proteins, however the two putative defence proteins (β-1,3-endoglucanase and the alpha amylase trypsin inhibitor) were found to still be upregulated in addition to several signalling

intermediates (e.g. protein kinases). After 9 days of RWA feeding (Figure 4.3.D), the Betta DN wheat leaf proteome appears to reach a plateau in terms of its defence responses. At this stage, a putative β-1,3-endoglucanase and ethylene responsive element binding protein are proposed to be upregulated, but no upregulation of putative protease inhibitors were found. Several putative stress proteins, including two heat shock proteins were upregulated as well as a number of proteins that appear to be involved in growth and photosynthesis (Table 7.4. and Figure 7.6.6.). The results obtained are summarised in Figure 4.4.

4.4. Discussion and Conclusions

The results obtained indicate differences between the Betta and Betta DN wheat cultivars in response to RWA phloem feeding at different time intervals. The first week of a plant's exposure to a given pest or pathogen is the most crucial, as this is time during which plant defence responses such as the hypersensitive response (HR) and systemic acquired resistance (SAR) come into play (Botha *et al*., 2006; Moran *et al*., 2002). Previous studies have indicated 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). From the results obtained however, it would appear that the susceptible Betta cultivar also undergoes an HR type response to RWA phloem feeding as suggested by the putative upregulation of a calcium-dependent protein kinase and β-1,3-endoglucanase after 2 days of plant exposure to aphids. This is confirmed by the literature which indicates that barley undergoes an HR response upon RWA phloem feeding (Mohase and van der Westhuizen, 2002). The putative β-1,3-endoglucanase remains upregulated after 3 and 5 days of aphid feeding and is thought to form part of the plant's general disease resistance mechanism (Mauch *et al*., 1988). β-1,3-endoglucanases are anti-microbial compounds that function to degrade pathogen cell walls and are PR proteins that have no clear role in insect attack but are induced in response to pathogen infection (Slusarenko, 1996; Collinge et al., 1993 and Van der Westhuizen, 1998).

The proteins that are upregulated after 3 days of RWA feeding on Betta wheat do not appear to indicate the presence of systemic acquired resistance (SAR), which is a form of induced resistance that has a broad mode of action, is induced by increased levels of salicylic and

jasmonic acids and follows the HR response in most plants (Jayaraj *et al*., 2004; Ryals *et al*., 1994). Most of the proteins putatively upregulated after this point are proteins involved in photosynthesis, heat shock proteins and proteins that were not provisionally identified. The Betta leaf proteome changes markedly after 5 days of RWA infestation, but due to factors of time and cost, tandem mass spectroscopy (MS / MS) was not performed to identify these proteins, which may well be the key to the Betta response to RWA stress responses. After 9 days of RWA feeding an ethylene-responsive element is upregulated, indicating that the ethylene defence pathway may come into play at some point. In general, however, it would appear that the defence responses displayed by Betta wheat after RWA phloem feeding are relatively few and non-specific. This finding was anticipated based on the current literature on the subject. The finding that Betta wheat may, however, undergo an HR-type response upon RWA herbivory is in contrast with current literature and warrants a further investigation in terms of future work on this project as this could serve to enhance the current understanding of plant-aphid interactions as well as of the HR mechanism itself.

The resistant Betta DN cultivar, on the other hand, responds to RWA herbivory by upregulating putative glucanases, thaumatin-like proteins and plant proteinase inhibitors, which are proposed to discourage aphid feeding and possibly restrict aphid movement to resistant plants. This finding supports the observation that aphids probe more but feed less on resistant plants, opting to spending more time on nonfeeding activities instead (Kindler *et al*., 1992). This could be the key to understanding plant defence responses to RWA feeding as well as serving as a basis for the design of resistant cultivars in the future. Unstressed Betta DN wheat displays upregulation of a number of proteins involved in normal plant growth and regulation, a great number of which remained unidentified. After 2 days of RWA phloem-feeding, the RWA-resistant Betta DN cultivar displayed the upregulation of proteins putatively involved in photosynthesis, a putative endo-β-1,3-glucanase and a thaumatin-like protein precursor, indicating that it underwent an HR response like Betta. The putative thaumatin-like protein's upregulation indicates increased levels of salicylic and / or jasmonic acid within the affected plant, as these compounds are known to upregulate thaumatins that are also proposed to be PR proteins (Gaudet *et al*., 2003; Jayaraj *et al*., 2004). This indicates the possibility of an SAR response in Betta DN plants upon exposure to RWA phloem feeding and could also suggest the involvement of the SA and JA pathways in Betta DN defence responses against RWA feeding.

A great diversity of proteins is upregulated in response to RWA feeding in Betta DN wheat, which was not the case in Betta wheat. Once again, proteins such as a putative β-1,3-glucanase that are associated with plant responses to pathogen infection, were found to have been upregulated in addition to various putative kinases and unidentified proteins. The most interesting result obtained from this study was that after 5 and 7 days of exposure to RWA phloem-feeding, Betta DN wheat displays upregulation of a putative alpha amylase trypsin inhibitor, which is directly involved in plant responses to chewing insects. It has previously been suggested by Tran and colleagues (Tran *et al.*, 1997) that plant proteinase inhibitors could serve as useful compounds in deterring aphid feeding on valuable crops, but the upregulation of proteinase inhibitors in response to RWA phloem feeding in Betta wheat has not yet been shown. This finding has exciting applications in the development of improved resistant cultivars for commercial use.

Figure 4.4. portrays a summary of the results obtained for this study. From the pie charts, it is apparent that unstressed Betta wheat (Figure 4.4.A) does not display the upregulation of any defence proteins, but in contrast displays the upregulation of proteins that are putatively involved in plant growth and development such as proteins involved in photosynthesis and storage, as well as putative kinases and proteins involved in normal cellular signalling pathways and functions. Once Betta wheat is exposed to RWA phloem feeding (Figure 4.4.B) the upregulation of putative defence proteins is observed while the upregulation of proteins thought to be involved in photosynthesis and heat shock responses appears to remain constant. The number of putative kinases and proteins involved in other functions is somewhat lowered, indicating that the plant may allocate more resources to the production of defence proteins in response to the aphid infestation and that regular cellular functions may take second place in light of the perceived emergency afflicting the plant while the RWA feed on it.

Unstressed Betta DN wheat appears to upregulate fewer proteins putatively involved in heat shock and photosynthesis. It is interesting to note that some putative defence proteins were found in unstressed Betta DN wheat, indicating that part of the defence response to aphids and other pests / pathogens might be constitutively expressed as opposed to being inducible. When the RWA commences phloem-feeding on Betta DN plants, the number of putative

defence proteins upregulated increases markedly in addition to the putative upregulation of protease inhibitors known to be involved in the defence against herbivorous insects and pests. The number of putatively unidentified proteins in all cases is high. It is interesting to note that Betta DN wheat did not appear to upregulate storage proteins (Figure 4.4.C. and 4.4.D.) as was observed for Betta wheat (Figure 4.4.A. and 4.4.B.) and that the putative upregulation of heat shock proteins in Betta DN wheat was only observed after 9 days, indicating that the RWA feeding on Betta DN wheat does not elicit a stress response as observed for the susceptible Betta wheat, but rather a defence response that allows it to counteract the effects of RWA feeding. The Betta stress response to RWA feeding also appears to favour the upregulation of proteins involved in plant defence against fungal infestation, such as glucanases rather than protease inhibitors (Moran *et al*., 2002). The resistance of Betta DN cultivars would therefore appear to be dependent on the constitutive expression of β-1,3-glucanases and the inducible expression of specific plant protease inhibitors.

This preliminary investigation appears to indicate that Betta wheat does display an HR-type response to RWA phloem feeding but is rendered susceptible to the effects of aphid feeding due to its inability to upregulate suitable defensive proteins in response to RWA herbivory. As outlined in literature, the proteins upregulated are largely proteins that are involved in plant stress responses to pathogen infection. Betta DN undergoes upregulation of proteins that are involved in pathogen resistance in addition to proteins (proteinase inhibitors) that are involved in plant defence against insect herbivory. This double-barrelled approach appears to be necessary for plant defence against aphids. This appears to form the basis of its resistance to RWA phloem feeding and paves the way for future studies in this area, as well as plant stress responses in general. The results obtained correspond to what was anticipated from the literature, namely that RWA feeding causes the upregulation of proteins involved in cell maintenance, growth and regulation, defence and signalling as well as photosynthesis and energy production (Botha *et al*., 2006). These findings thus confirm what is known from the literature and serve as a basis for future studies in this field.

Chapter 5

General discussion of results and future work

General discussion

The Russian wheat aphid (RWA), Diuraphis noxia, is a serious pest of important agricultural crops such as barley and wheat (Du Toit, 1990; Moloi and van der Westhuizen, 2005). In South Africa it is currently regarded as the most destructive insect pest in wheat (van der Westhuizen *et al*., 1998a). While certain wheat cultivars such as Tugela DN and Betta DN are known to be resistant to the effects of RWA feeding, the development of new improved wheat cultivars is hampered by a poor understanding of the biochemistry of plant resistance to aphid feeding (Budak *et al*., 1999; van der Westhuizen *et al*., 1998a). A detailed investigation of these resistance mechanisms could therefore lead to an enhanced understanding of wheat stress responses to aphids in general, thereby enabling the development of improved cultivars possessing resistance to the effects of RWA infestation (Moloi and van der Westhuizen, 2005).

This study was therefore undertaken to develop and evaluate a proteomic method to identify differentially expressed proteins in susceptible and resistant wheat (*Triticum aestivum L*. cv Betta and Betta DN) plants in response to stress induced by RWA and Bird Cherry-Oat aphid (BCA), *Rhopalosiphum padi* feeding, and thereby attempt to identify possible resistance mechanisms.

Initial studies on the effect of plant growth stage and subsequent response to aphid feeding revealed that a higher number of proteins were upregulated in the two week old wheat plants as compared to three week old plants when subjected to aphid feeding. However, the two week old plants displayed a typical stress response while the three week old plants displayed a resistance response. Two week old plants however, appeared to withstand the aphid infestation for a longer period before showing signs of chlorosis and physical damage, which is why two week old plants were used in all subsequent studies. RWA phloem feeding on Betta wheat resulted in a systemic acquired resistance (SAR) type of response that is typically seen in plant responses to pathogen attack. When the RWA fed on Betta DN wheat it resulted in the upregulation of a number of defence proteins. In the case of BCA phloem feeding, the proteins that were found to be upregulated were involved in normal plant growth responses and this could indicate that the damage plants sustain due to BCA feeding is not enough to trigger a defence response. In addition to this, it could also indicate that BCA phloem feeding

differs from that of RWA phloem feeding in that no watery saliva containing defence elicitors is present. An alternative hypothesis to this could be that the BCA has developed certain mechanisms to prevent triggering of plant defence responses. The RWA was therefore used in all subsequent studies on the effect of aphid phloem feeding on the composition of the proteome of Betta and Betta DN wheat cultivars. The RWA was also of greater interest due to the extensive damage it causes to important agricultural crops such as barley and wheat, which is much greater than the effect of the BCA.

Unstressed Betta wheat displayed upregulation of a number of proteins putatively involved in normal plant growth and development. No putative stress proteins were found in the absence of RWA feeding on Betta plants. A few putative stress proteins in Betta DN wheat in the absence of stress responses indicate that the Betta DN resistance response could be due to constitutively expressed proteins. The detection of differences in the putative identity of proteins expressed in unstressed and stressed wheat samples together with the putative identification of several stress or defence-related proteins in the proteome of stressed wheat indicated that the proteomic method described here could be used to identify possible wheat resistance mechanisms. However, it is recognised that the results are very preliminary and that further studies are required to validate these results.

RWA feeding on the susceptible Betta wheat cultivar resulted in the upregulation of proteins involved in plant stress responses as well as plant responses to fungal pathogens, including putative heat shock proteins and β-1,3-glucanases. RWA feeding on the resistant Betta DN wheat cultivar resulted in the upregulation of putative defence proteins, including putative thaumatins, alpha amylase trypsin inhibitors and glucanases. It appears that RWA phloem feeding elicits a defence response in both the susceptible and resistant wheat cultivars. A typical pathogen defence response was observed for the Betta wheat (Mohase and van der Westhuizen, 2002). However, the resistant Betta DN cultivar displays both insect herbivory and pathogen like defence responses incorporating putative protease inhibitors and defence proteins which helps it to prevent damage from RWA phloem feeding and, possibly also, to discourage aphid feeding on the resistant plants. This finding supported the observation that aphids probe more but feed less on resistant plants, opting to spending more time on nonfeeding activities instead (Kindler *et al*., 1992). This could be the key to understanding

plant defence responses to RWA feeding as well as serving as a basis for the design of resistant cultivars in the future.

Preliminary results obtained from this pilot study suggest that Betta wheat is susceptible to RWA feeding because it does not upregulate sufficient types and quantities of defence proteins, except for glucanases, which on their own appear to be an inadequate defence against the effects of RWA phloem-feeding. The upregulation of a number of proteins relating to the plant's photosynthetic processes indicate that Betta wheat tries to respond to RWA-incurred damage by repairing rather than preventing it; a strategy that proves largely ineffective as evidenced by the devastating damage that the plants incur in response to RWA feeding. It has been suggested that the wheat response to RWA herbivory is one that is more characteristic to that found in cases of pathogen infection rather than the feeding of chewing insects. This is based on the observation that plants subjected to RWA feeding display the expression of genes that are known to be involved in plant defence responses against bacterial and fungal pathogens (Moran *et al*., 2002). The first stage in the initiation of plant defence responses against pathogen infection is the hypersensitive response (HR) that is thought to be initiated by the influx of cytosolic calcium and is characterised by a rapid response that includes the generation of reactive oxygen species (ROS) (Ebel and Scheel, 1997). It is proposed that the calcium signals are translated by means of protein phosphorylation events and that the HR comprises a vital front line defence against fungal and bacterial pathogens (Pike *et al*., 1998; Moran *et al*., 2002). The ROS produced in the HR cause cellular salicylic acid (SA) to accumulate, giving rise to the expression of a number of pathogenesis related (PR) proteins such as β-1,3-glucanases, chitinases and peroxidases (Van der Westhuizen *et al*., 1998). Previous studies have indicated 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). From the results obtained however, it would appear that Betta wheat does undergo an HR type response to RWA phloem-feeding as suggested by the upregulation of a putative calcium-dependent protein kinase and β-1,3-endoglucanase after 2 days of plant exposure to aphids.

The putative thaumatin-like protein's upregulation in Betta DN wheat is indicative of increased levels of salicylic (SA) and / or jasmonic acid (JA) within the affected plant, as these compounds are known to upregulate thaumatins that are also proposed to be PR proteins

(Gaudet *et al*., 2003; Jayaraj *et al*., 2004). It is uncertain at this stage, however, whether or not Betta DN wheat undergoes a SAR response after RWA phloem-feeding. Further studies would have to be performed in order to determine this. It can be proposed, however, from the putative protein identification performed in the present study that Betta DN wheat's defence response relies on the combined action of protease inhibitors and defence pathways. Due to the upregulation of SA and JA indicated by the putative presence of upregulated thaumatins, it can be proposed that the plant defence pathways that are involved in wheat defence responses to RWA phloem feeding include the JA and SA pathways as the plant response to RWA feeding is indicative of plant responses to both pathogens and insect herbivory. This hypothesis is confirmed by literature (Botha *et al*., 2005). It has previously been suggested by Tran and colleagues (Tran *et al*., 1997) that plant protease inhibitors could serve as useful compounds in deterring aphid feeding on valuable crops, but the upregulation of protease inhibitors in response to RWA phloem feeding in Betta wheat has not yet been shown. This finding has exciting applications in the development of improved resistant cultivars for commercial use.

It appears that Betta wheat does display an HR-type response to RWA phloem feeding but is rendered susceptible to the effects of aphid feeding due to its inability to upregulate suitable defensive proteins in response to RWA herbivory. As outlined in literature, the proteins upregulated are largely proteins that are involved in plant stress responses to pathogen infection. Betta DN undergoes upregulation of proteins that are involved in pathogen resistance in addition to proteins (protease inhibitors and thaumatins) that are involved in plant defence against insect herbivory. This double-barrelled approach appears to be the basis of the aphid resistance mechanisms of the Betta DN wheat cultivar. This appears to form the basis of its resistance to RWA phloem feeding and paves the way for future studies in this area, as well as plant stress responses in general.

Future work

The 2DE method outlined in this work was adequate in order to form part of a pilot study. However, in terms of future studies, improved reproducibility and gel resolution must be

achieved in order to confirm the current findings as well as to serve as a platform for future studies in wheat proteomics.

A continued optimisation of the method developed in this work would focus on the use of IPG strips with different pH ranges (3-6; 5-8 and 7-10) in order to improve the resolution of spots in the second dimension gel, particularly in the acidic region. Broad range IPG strips give a good overview of the overall distribution of proteins in terms of their unique pI values but more detailed studies require the use of narrow range strips that allow for the study of proteins in a certain range and improved spot resolution (Görg, 1991). Another method that could improve spot resolution is to increase the length of the IPG strip and the size of the second dimension gel from the regular 7 cm gel to a 16 cm gel (Langen *et al*., 1997).

A further aspect of the work that must be addressed in order to improve the 2DE method and protein spot resolution is the length of focussing times of the IPG strips. The length of the IEF stage and the extent of the potential difference experienced by the IPG strip are crucial to the success of the 2DE experiment. In order to accomplish this, a focussing system that utilises a temperature control function would have to be utilised in order to eliminate damage to IPG strips from increased temperatures when a higher potential difference is applied (Westermeier and Naven, 2002). In order to improve the visualisation of protein spots in the second dimension gels, a number of different stains, particularly the more sensitive fluorescent stains, should be investigated. This would also overcome the problems of saturation observed from silver staining in this study. Once the 2DE method is sufficiently optimised to give high quality, reproducible gels, a method to confirm protein spot identity should be employed. The identity of protein spots of interest that are provisionally identified by matching their molecular weight and pI from the SwissProt database could be confirmed by excising spots of interest and performing tandem mass spectroscopy (MS/MS) and / or N-terminal sequencing on them. This will allow for positive identification of protein spots of interest that are up or down regulated. The involvement of specific proteins in the resistance mechanism will need to be further confirmed by using complementary techniques such as the real time polymerase chain reaction (real time PCR), DNA microarray analysis and Northern and Western blots.

Appendix 1:

Results for Chapter 3

6.1. Images of original 2DE gels analysed in Chapter 3

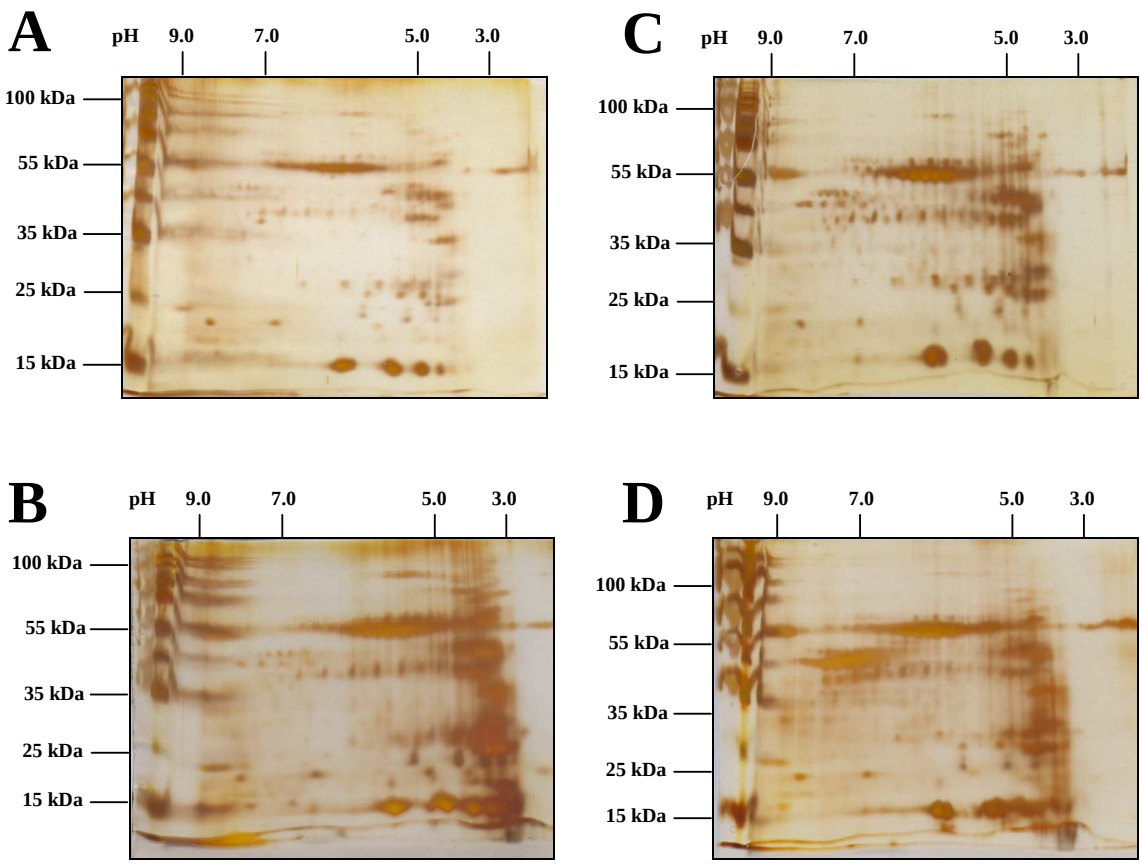


Figure 6.1. Differential protein expression profiles in Betta wheat (susceptible) in response to stress resulting from RWA phloem-feeding over a seven-day period.

[A] – Betta wheat at 2 weeks (3-leaf) growth stage in the absence of RWA phloem-feeding; [B] – Betta wheat at 3 weeks (4-leaf) growth stage in the absence of RWA phloem-feeding; [C] - Betta wheat at 2 weeks (3-leaf) growth stage that was subjected to RWA phloem-feeding for one week; [D] - Betta wheat at 3 weeks (4-leaf) growth stage that was subjected to RWA phloem-feeding for one week.

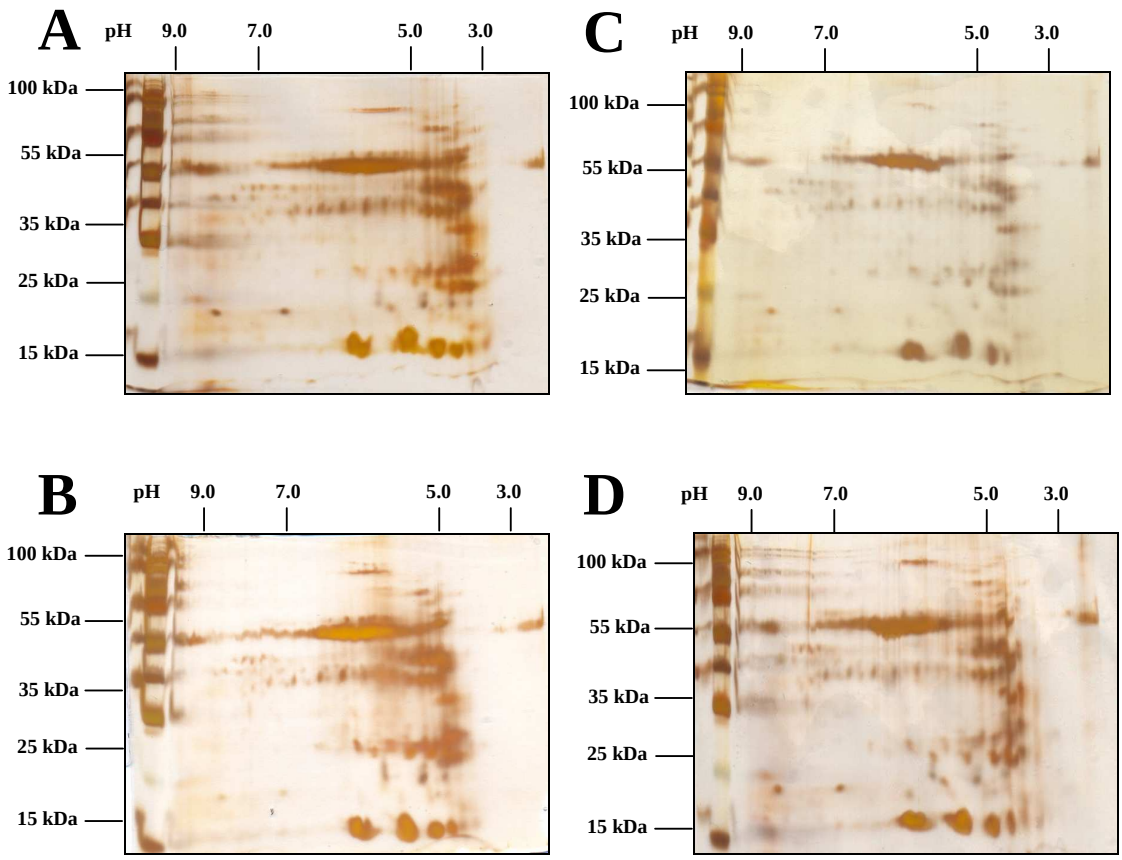


Figure 6.2. Differential protein expression profiles in Betta DN (resistant) wheat in response to stress resulting from RWA phloem-feeding over a seven-day period.

[A] – Betta DN wheat at 2 weeks (3-leaf) growth stage in the absence of RWA phloem-feeding; [B] – Betta DN wheat at 3 weeks (4-leaf) growth stage in the absence of RWA phloem-feeding; [C] - Betta DN wheat at 2 weeks (3-leaf) growth stage that was subjected to RWA phloem-feeding for one week; [D] – Betta DN wheat at 3 weeks (4-leaf) growth stage that was subjected to RWA phloem-feeding for one week.

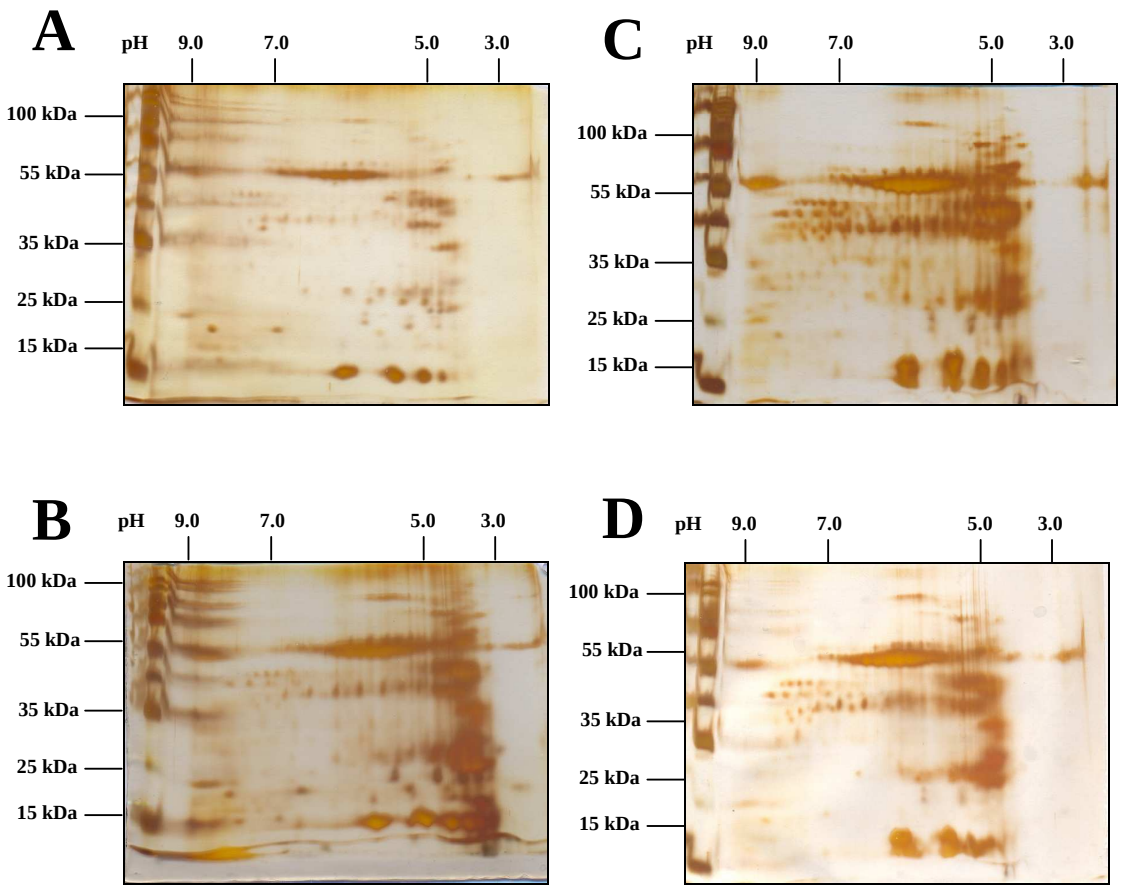


Figure 6.3. Differential protein expression profiles in Betta wheat in response to stress resulting from BCA phloem-feeding over a seven-day period.

[A] – Betta wheat at 2 weeks (3-leaf) growth stage in the absence of BCA phloem-feeding; [B] – Betta wheat at 3 weeks (4-leaf) growth stage in the absence of BCA phloem-feeding; [C] - Betta wheat at 2 weeks (3-leaf) growth stage that was subjected to BCA phloem-feeding for one week; [D] - Betta wheat at 3 weeks (4-leaf) growth stage that was subjected to BCA phloem-feeding for one week.

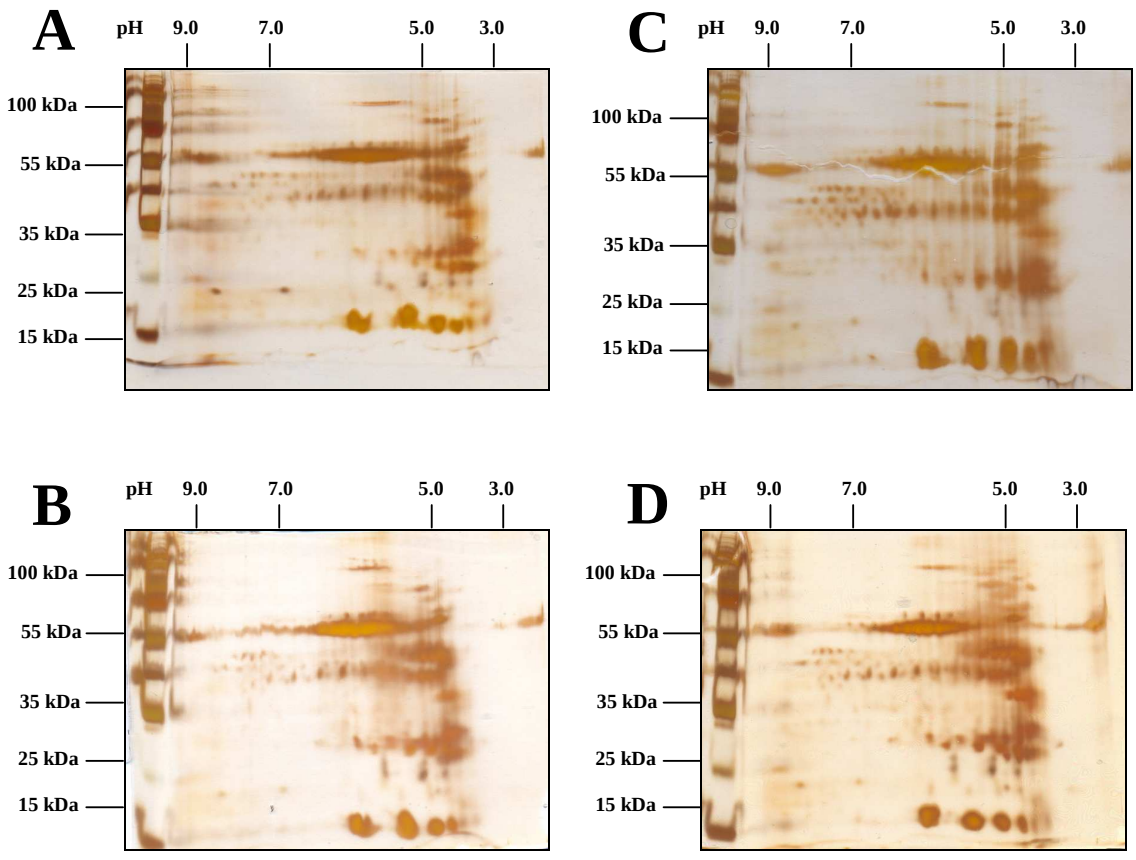


Figure 6.4. Differential protein expression profiles in Betta DN wheat in response to stress resulting from BCA phloem-feeding over a seven-day period.

[A] – Betta DN wheat at 2 weeks (3-leaf) growth stage in the absence of BCA phloem-feeding; [B] – Betta DN wheat at 3 weeks (4-leaf) growth stage in the absence of BCA phloem-feeding; [C] - Betta DN wheat at 2 weeks (3-leaf) growth stage that was subjected to BCA phloem-feeding for one week; [D] – Betta DN wheat at 3 weeks (4-leaf) growth stage that was subjected to BCA phloem-feeding for one week.

6.2. PDQuest™ Basic Software Version 8.0 analysis of 2DE gels

Upregulated proteins are indicated by yellow circles, mismatched / differentially expressed proteins by red circles and matched proteins by green symbols. Each set of gels shows the control (left) alongside the stress condition (right).

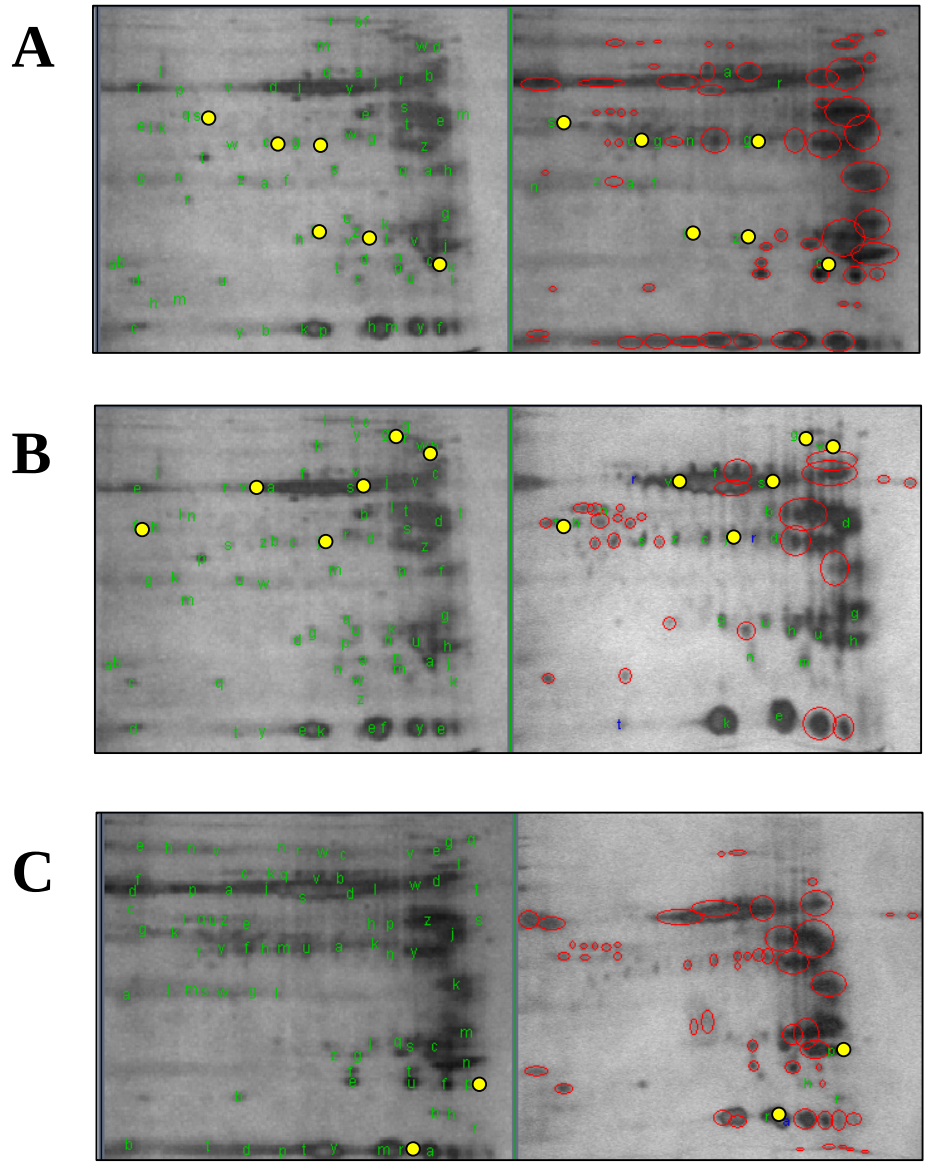


Figure 6.5. Analysis of differential protein expression profiles in Betta wheat subjected to RWA phloem feeding with PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Each set of gels represents a control condition (left) and a test condition (right). The control gels were used as master gels for the purposes of software analysis. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow circles indicate proteins that are 2-fold up or down regulated under the test conditions. [A] (left) – Betta wheat at the two-week growth stage (unstressed); [A] (right) – Betta wheat at the three-week growth stage (unstressed); [B] (left) – Betta wheat at the two-week growth stage (unstressed); [B] (right) – Betta wheat at the two-week growth stage stressed with RWA; [C] (left) - Betta wheat at the three-week growth stage (unstressed); [C] (right) - Betta wheat at the three-week growth stage stressed with RWA.

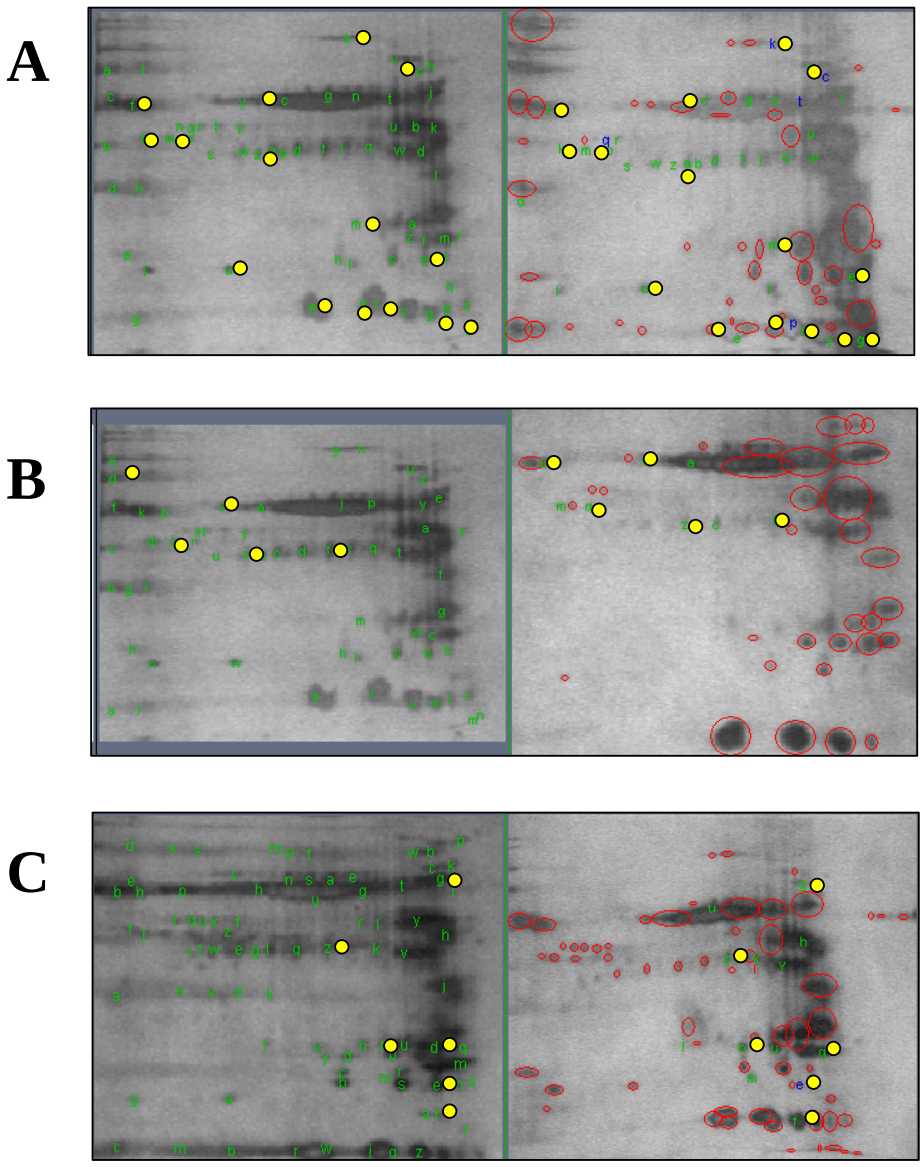


Figure 6.6. Analysis of differential protein expression profiles in Betta DN wheat subjected to RWA phloem feeding with PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Each set of gels represents a control condition (left) and a test condition (right). The control gels were used as master gels for the purposes of software analysis. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow circles indicate proteins that are 2-fold up or down regulated under the test conditions. [A] (left) – Betta DN wheat at the two-week growth stage (unstressed); [A] (right) – Betta DN wheat at the three-week growth stage (unstressed); [B] (left) – Betta DN wheat at the two-week growth stage (unstressed); [B] (right) – Betta DN wheat at the two-week growth stage stressed with RWA; [C] (left) – Betta DN wheat at the three-week growth stage (unstressed); [C] (right) – Betta DN wheat at the three-week growth stage stressed with RWA.

Table 6.1. Proteins putatively upregulated in response to RWA feeding in Betta and Betta DN wheat

Wheat cultivar[1]	Aphid type	Figure number	Spot symbol	Putative protein match from SwissProt database[2]	MW (kDa)	pI
Betta (2)	None[3]	6.1.A	c_1	Low molecular weight glutenin storage protein	41.307	8.69
Betta (2)	None	6.1.A	g	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta (2)	None	6.1.A	s	Cyclin dependent protein kinase	48.174	9.10
Betta (2)	None	6.1.A	l	Cyclin dependent protein kinase	25.934	6.5
Betta (2)	None	6.1.A	z	Chlorophyll a-b binding protein	28.267	5.67
Betta (2)	None	6.1.A	c_2	Unidentified	-	-
Betta (2)	RWA	6.1.C	t	Low molecular weight glutenin storage protein	41.307	8.69
Betta (2)	RWA	6.1.C	v	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta (2)	RWA	6.1.C	g	Heat shock protein 90	80.460	4.96
Betta (2)	RWA	6.1.C	w	Unidentified		
Betta (2)	RWA	6.1.C	s	Calcium-dependent protein kinase	56.788	5.34
Betta (2)	RWA	6.1.C	j	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta (3)	RWA	6.1.D	r	16.9 kDa class I heat shock protein	16.878	5.83
Betta (3)	RWA	6.1.D	p	Thaumatin-like protein PWIR2 (precursor)	17.605	4.64
Betta DN (2)	None	6.2.A	k_1	Unidentified		
Betta DN (2)	None	6.2.A	v	Heat shock protein 90 (fragment)	75.674	5.09
Betta DN (2)	None	6.2.A	f	NADH dehydrogenase subunit 2	53.704	8.98
Betta DN (2)	None	6.2.A	k_2	Proline-rich protein	42.119	9.01
Betta DN (2)	None	6.2.A	g	Low molecular weight glutenin storage protein	41.307	8.69
Betta DN (2)	None	6.2.A	a	Unidentified	-	-
Betta DN (2)	None	6.2.A	u	Unidentified	-	-
Betta DN (2)	None	6.2.A	m	Abscicic acid inducible protein kinase (fragment)	37.516	5.68
Betta DN (2)	None	6.2.A	e_1	Unidentified	-	-
Betta DN (2)	None	6.2.A	e_2	Alpha-amylase trypsin inhibitor	15.460	6.86
Betta DN (2)	None	6.2.A	p	16.9 kDa class I heat shock protein	16.878	5.83
Betta DN (2)	None	6.2.A	r	Thaumatin-like protein PWIR2 precursor	17.605	4.64
Betta DN (2)	None	6.2.A	y	Alpha-1-purothionin precursor (fragment)	13.526	4.76
Betta DN (2)	None	6.2.A	g	Unidentified	-	-
Betta DN (2)	None	6.2.A	x	Unidentified	-	-
Betta DN (2)	RWA	6.2.C	k	Protein kinase	60.974	9.14
Betta DN (2)	RWA	6.2.C	v	Mitogen-activated protein kinase	65.214	6.87
Betta DN (2)	RWA	6.2.C	z	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN (2)	RWA	6.2.C	f	Serine-threonine protein kinase	44.191	5.97
Betta DN (2)	RWA	6.2.C	q	Leucine zipper protein zip 1	47.193	8.88
Betta DN (3)	RWA	6.2.D	g	Heat shock protein 90	80.460	4.96
Betta DN (3)	RWA	6.2.D	z	Cysteine proteinase	40.807	6.80
Betta DN (3)	RWA	6.2.D	h	Chlorophyll a-b binding protein	28.264	5.67
Betta DN (3)	RWA	6.2.D	q	Ubiquitin conjugating enzyme	21.126	4.40
Betta DN (3)	RWA	6.2.D	f	Heat shock protein 20	16.882	4.67
Betta DN (3)	RWA	6.2.D	e	Translationally-controlled tumor protein homolog	18.806	4.55

1- The number in brackets refers to the age of the wheat in weeks after planting
2- The protein from the SwissProt database that matched a given spot of interest most closely in terms of its molecular mass and pI
3- The control reaction in which no aphids were present

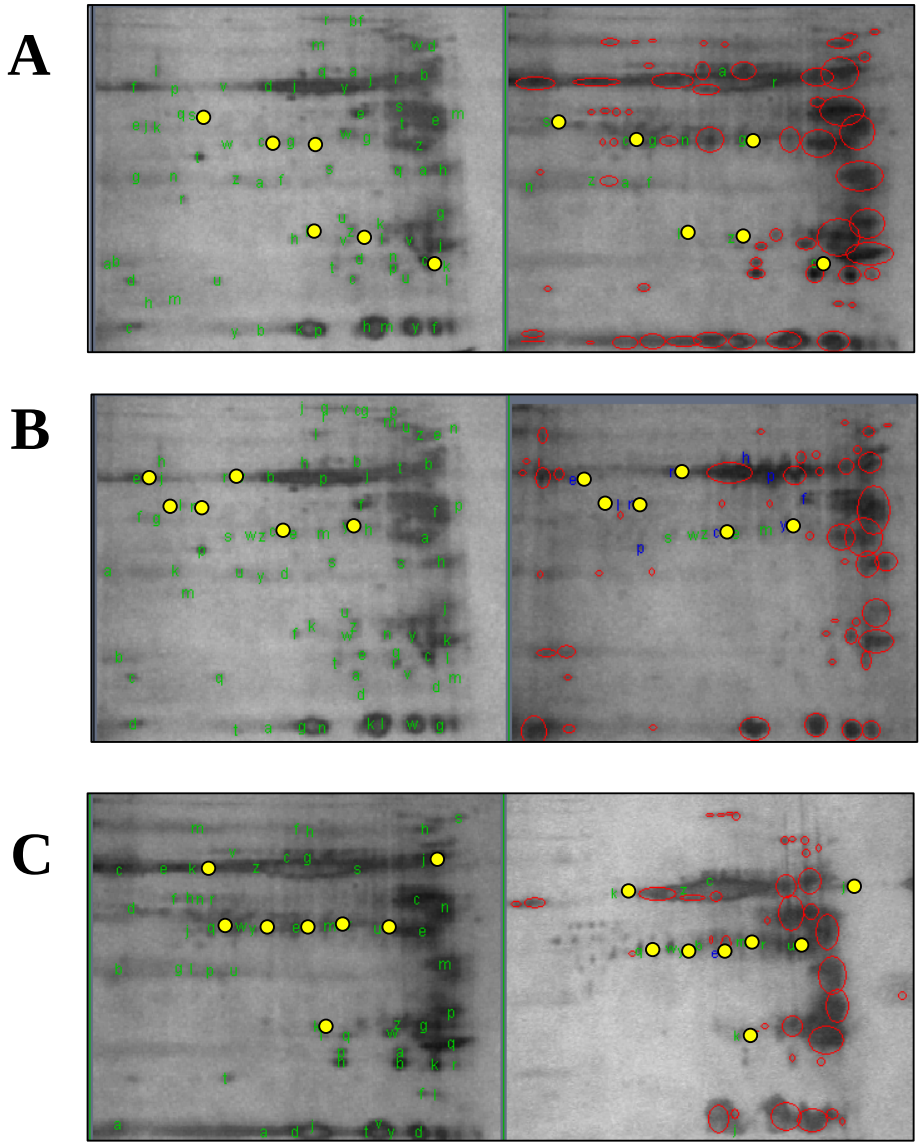


Figure 6.7. Analysis of differential protein expression profiles in Betta wheat subjected to BCA phloem feeding with PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Each set of gels represents a control condition (left) and a test condition (right). The control gels were used as master gels for the purposes of software analysis. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow circles indicate proteins that are 2-fold up or down regulated under the test conditions. [A] (left) – Betta wheat at the two-week growth stage (unstressed); [A] (right) – Betta wheat at the three-week growth stage (unstressed); [B] (left) – Betta wheat at the two-week growth stage (unstressed); [B] (right) – Betta wheat at the two-week growth stage stressed with BCA; [C] (left) - Betta wheat at the three-week growth stage (unstressed); [C] (right) - Betta wheat at the three-week growth stage stressed with BCA.

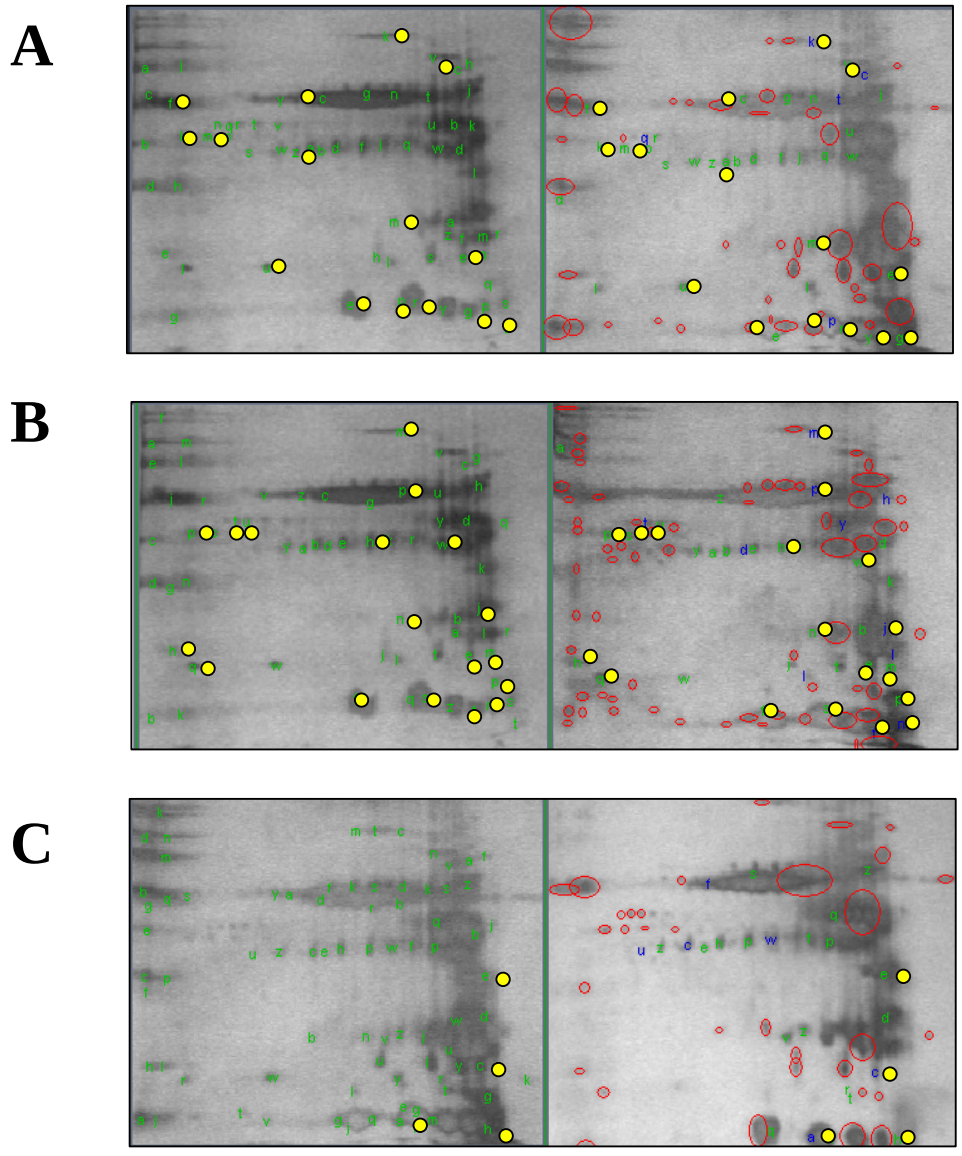


Figure 6.8. Analysis of differential protein expression profiles in Betta DN wheat subjected to BCA phloem feeding with PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Each set of gels represents a control condition (left) and a test condition (right). The control gels were used as master gels for the purposes of software analysis. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow circles indicate proteins that are 2-fold up or down regulated under the test conditions. [A] (left) – Betta DN wheat at the two-week growth stage (unstressed); [A] (right) – Betta DN wheat at the three-week growth stage (unstressed); [B] (left) – Betta DN wheat at the two-week growth stage (unstressed); [B] (right) – Betta DN wheat at the two-week growth stage stressed with BCA; [C] (left) – Betta DN wheat at the three-week growth stage (unstressed); [C] (right) – Betta DN wheat at the three-week growth stage stressed with BCA.

Table 6.2. Protein putatively upregulated in response to BCA feeding in Betta and Betta DN wheat

Wheat cultivar[1]	Aphid type	Figure number	Spot symbol	Putative protein match from SwissProt database[2]	MW (kDa)	pI
Betta (2)	BCA	6.3.C	r	Unidentified	-	-
Betta (2)	BCA	6.3.C	e	NADH dehydrogenase subunit 2	53.704	8.98
Betta (2)	BCA	6.3.C	l	Leucine zipper protein	47.913	8.88
Betta (2)	BCA	6.3.C	n	Unidentified	-	-
Betta (2)	BCA	6.3.C	c	Cysteine proteinase	40.807	6.8
Betta (2)	BCA	6.3.C	y	Serine-threonine protein kinase	44.191	5.97
Betta (3)	BCA	6.3.D	k	NADH dehydrogenase subunit 2	53.704	8.98
Betta (3)	BCA	6.3.D	y	Cysteine proteinase	40.807	6.8
Betta (3)	BCA	6.3.D	j	Unidentified	-	-
Betta (3)	BCA	6.3.D	k_2	Chlorophyll a-b binding protein	28.264	5.67
Betta (3)	BCA	6.3.D	u	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta (3)	BCA	6.3.D	m	Vesicle associated membrane protein	49.282	6.41
Betta (3)	BCA	6.3.D	q	Unidentified	-	-
Betta DN (2)	BCA	6.4.C	m	Heat shock protein 90	80.680	4.96
Betta DN (2)	BCA	6.4.C	p_1	Calcium-dependent protein kinase	56.788	5.34
Betta DN (2)	BCA	6.4.C	u	Low-molecular weight glutenin storage protein	41.307	8.69
Betta DN (2)	BCA	6.4.C	p_2	Cyclin-dependent protein kinase	48.174	9.10
Betta DN (2)	BCA	6.4.C	t	Leucine zipper protein	47.913	8.88
Betta DN (2)	BCA	6.4.C	h	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN (2)	BCA	6.4.C	w	Ethylene-responsive element binding protein	39.157	4.65
Betta DN (2)	BCA	6.4.C	n_1	Chlorophyll a-b binding protein	28.224	5.30
Betta DN (2)	BCA	6.4.C	j	Unidentified	-	-
Betta DN (2)	BCA	6.4.C	e	Protein-L-isoaspartate O-methyltransferase	24.708	4.90
Betta DN (2)	BCA	6.4.C	m	Ubiquitin-conjugating enzyme E2	17.301	5.67
Betta DN (2)	BCA	6.4.C	h_2	Small heat shock protein (chloroplast)	26.596	9.64
Betta DN (2)	BCA	6.4.C	q	Resistance protein	20.518	8.98
Betta DN (2)	BCA	6.4.C	f	16.9 kDa class I heat shock protein	16.878	5.83
Betta DN (2)	BCA	6.4.C	s	Alpha-2-purothionin precursor (fragment)	14.558	5.13
Betta DN (2)	BCA	6.4.C	p	Unidentified	-	-
Betta DN (2)	BCA	6.4.C	n_2	Unidentified	-	-
Betta DN (2)	BCA	6.4.C	f_2	Unidentified, potentially a kinase	-	-
Betta DN (3)	BCA	6.4.D	e	Unidentified	-	-
Betta DN (3)	BCA	6.4.D	c	Unidentified	-	-
Betta DN (3)	BCA	6.4.D	a	Unidentified	-	-
Betta DN (3)	BCA	6.4.D	h	Unidentified	-	-

1 - The number in brackets refers to the age of the wheat in weeks after planting

2- The protein from the SwissProt database that matched a given spot of interest most closely in terms of its molecular mass and pI

Appendix 2:

Results for Chapter 4

7.1. Differential protein expression in Betta wheat in response to RWA infestation over set time periods

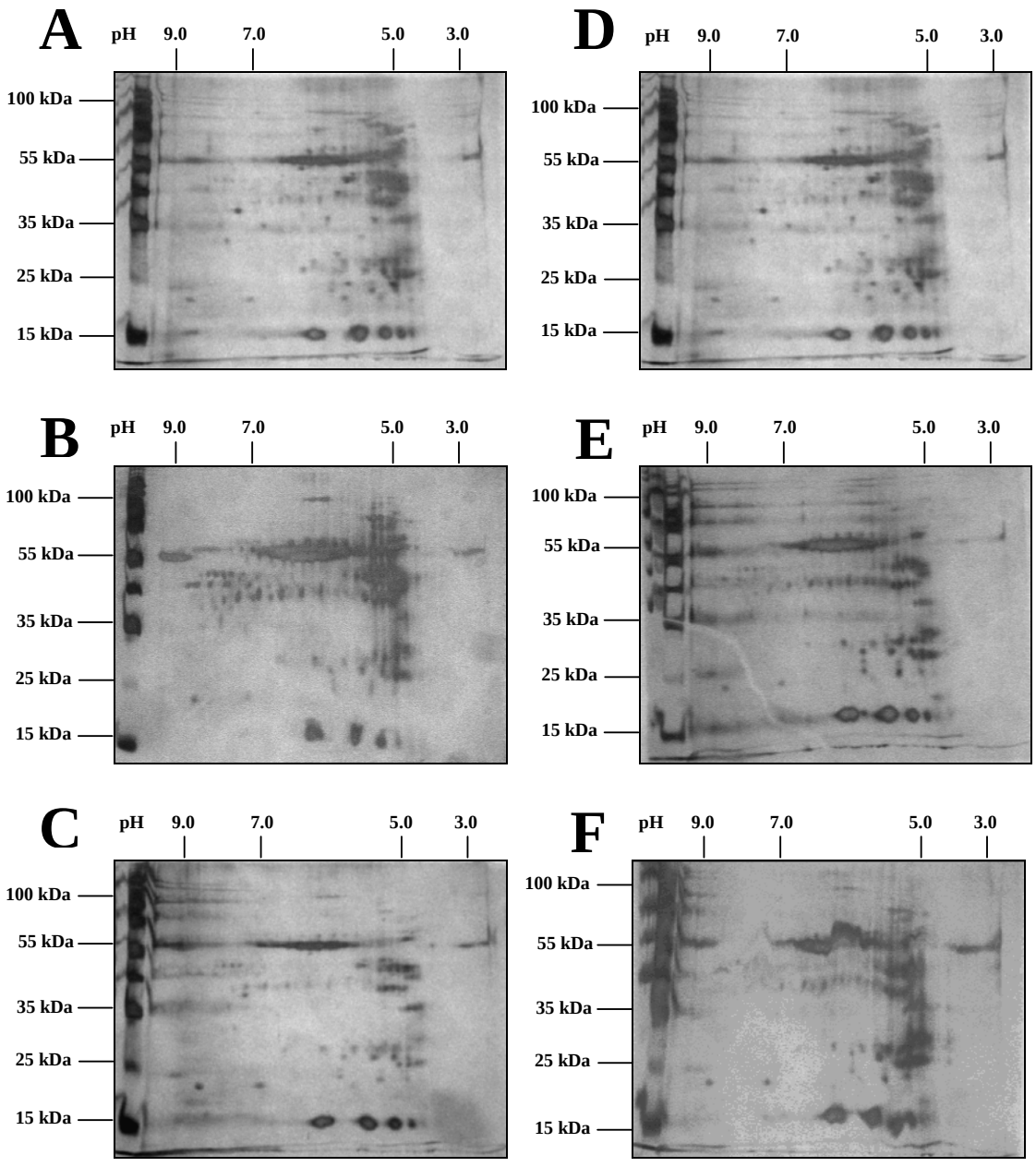


Figure 7.1. Differential expression of proteins in Betta wheat after 2 or 3 days of RWA infestation

All wheat samples were two weeks old at the start of the study. Time periods given refer to the duration of the experimental period. [A] – Unstressed Betta wheat; [B] – Unstressed Betta wheat at 2 days; [C] – Unstressed Betta wheat at 3 days; [D] – Betta wheat subjected to aphid feeding at two-weeks; [E] – Betta wheat subjected to aphid feeding for 2 days; [F] – Betta wheat subjected to aphid feeding for 3 days.

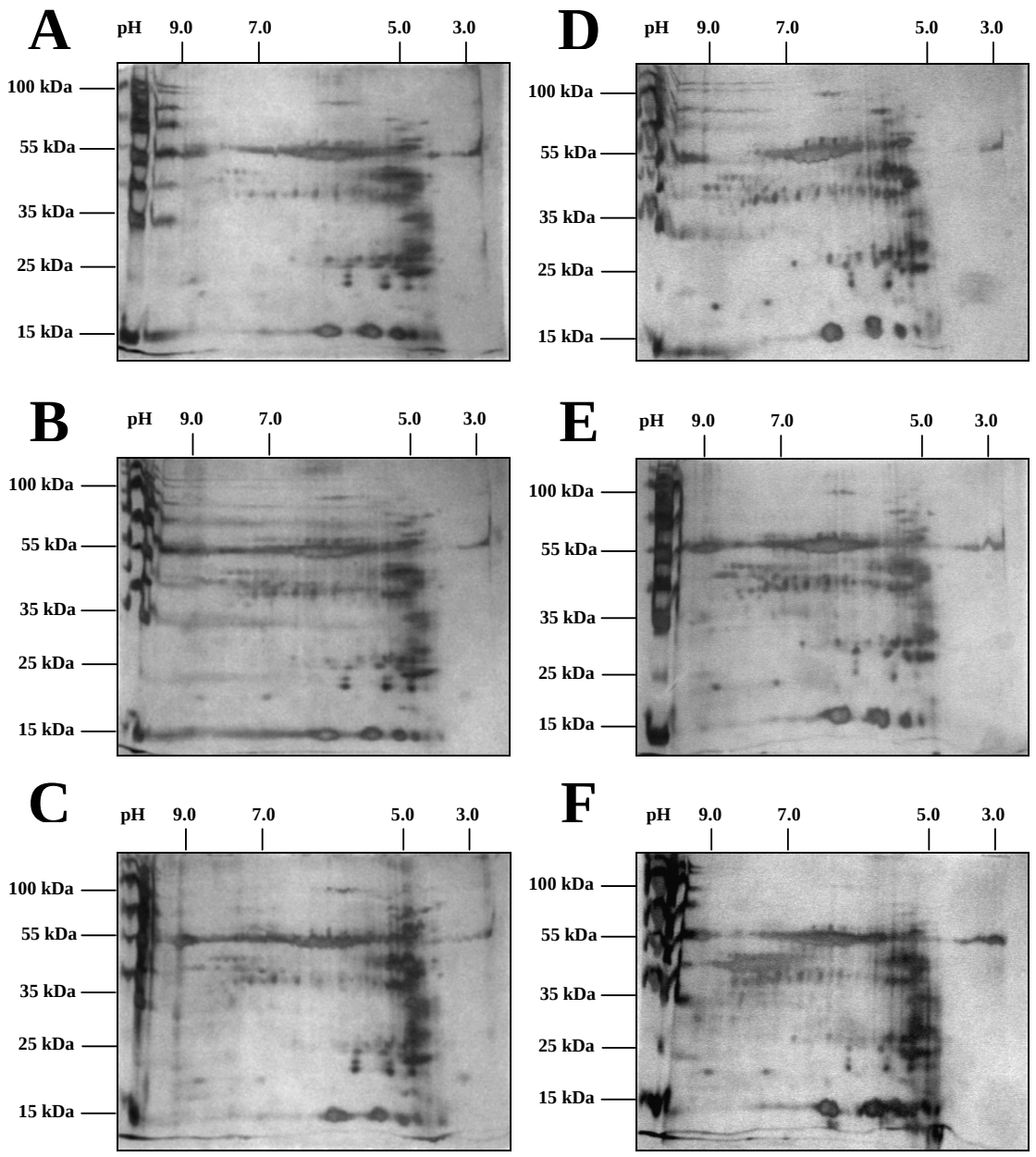


Figure 7.2. Differential expression of protein in Betta wheat after RWA infestation over different time periods.

All wheat samples were two weeks old at the start of the study. Time periods given refer to the duration of the experimental period. [A] – Unstressed Betta wheat at 5 days; [B] – Unstressed Betta wheat at 7 days; [C] – Unstressed Betta wheat at 9 days; [D] – Betta wheat subjected to aphid feeding for 5 days; [E] – Betta wheat subjected to aphid feeding for 7 days; [F] – Betta wheat subjected to aphid feeding for 9 days.

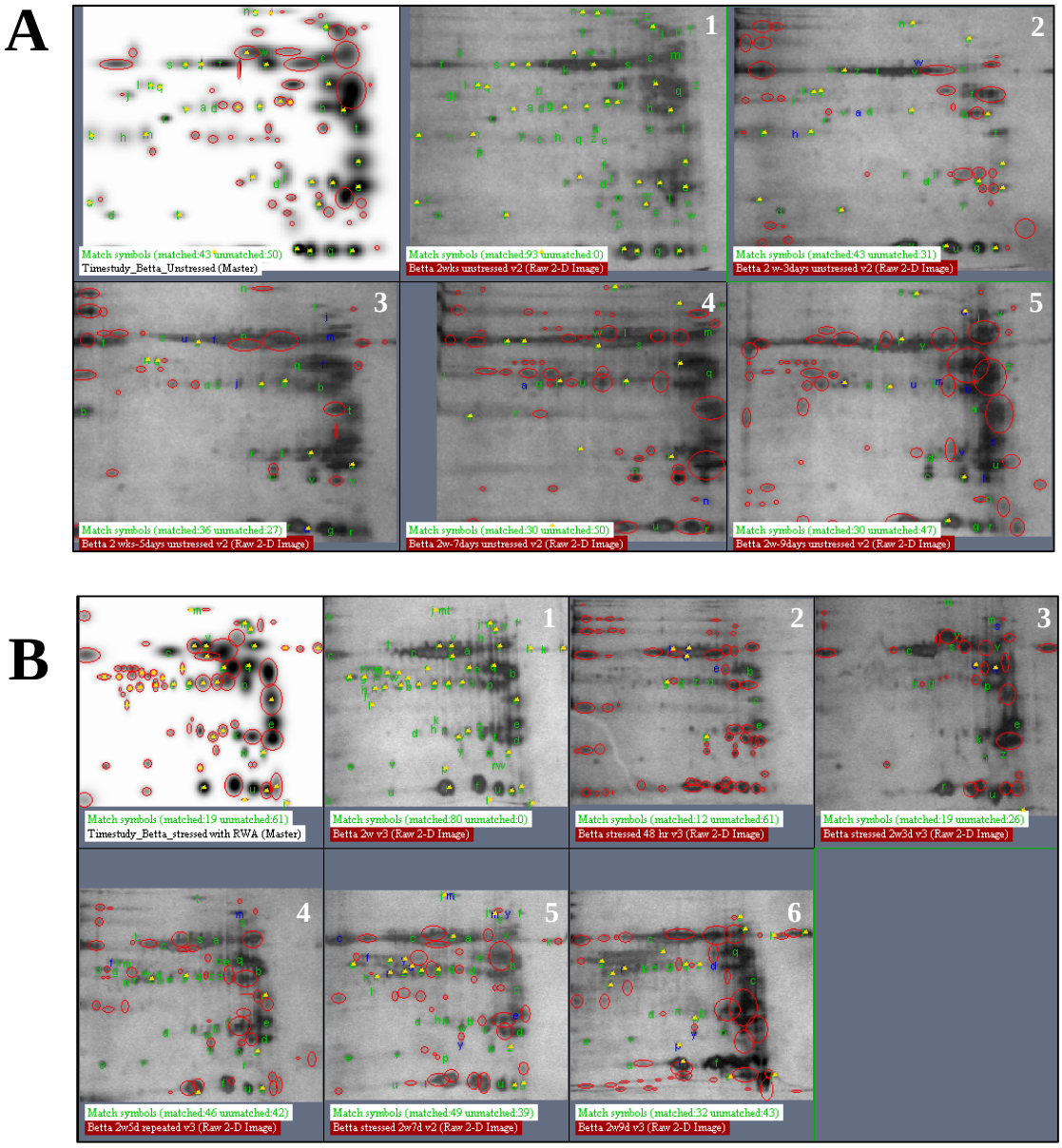


Figure 7.3. Analysis 2DE gels of Betta wheat (susceptible) exposed to RWA phloem-feeding over different time periods using PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Figure A shows unstressed controls and Figure B shows the experimental 2DE gels. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow triangles indicate up and down regulated proteins. [A] – (1) - Two-week old Betta wheat (control and master gel); (2) – Betta at three days; (3) – Betta at five days; (4)- Betta and seven days; (5) – Betta at nine days. [B] – (1) - Two-week old Betta wheat (control and master gel); (2) – Betta wheat 2 days after RWA infestation; (3) - Betta three days after infestation; (4) - Betta at five days after infestation; (5) - Betta seven days after RWA infestation; (6) - Betta at nine days after RWA infestation.

Table 7.1. Putative identification of proteins up or downregulated in unstressed two-week-old Betta wheat (RWA-susceptible) over a 9 day growth period

Wheat cultivar	D.A.I. [1]	Figure number	Spot symbol	Putative protein	MW (kDa)	pI
Betta	2w3d u	7.1.C	s_1	Unidentified	-	-
Betta	2w3d u	7.1.C	d	Della protein RHT-1	65.337	4.99
Betta	2w3d u	7.1.C	u_1	Unidentified	-	-
Betta	2w3d u	7.1.C	n	Cyclin dependent protein kinase	48.174	9.10
Betta	2w3d u	7.1.C	q	Leucine zipper protein zip 1	47.193	8.88
Betta	2w3d u	7.1.C	b	Ribosomal protein S2	40.892	9.43
Betta	2w3d u	7.1.C	m	Low molecular weight glutenin storage protein	41.307	8.69
Betta	2w3d u	7.1.C	u_2	Serine-threonine protein kinase	44.191	5.97
Betta	2w3d u	7.1.C	k	Unidentified	-	-
Betta	2w3d u	7.1.C	s_2	Unidentified	-	-
Betta	2w3d u	7.1.C	h	Ethylene-responsive element binding protein	31.791	4.53
Betta	2w3d u	7.1.C	u_3	Unidentified	-	-
Betta	2w3d u	7.1.C	a	Small heat shock protein chloroplast (precursor)	26.596	9.64
Betta	2w3d u	7.1.C	t	Cyclin dependent protein kinase	25.934	6.5
Betta	2w3d u	7.1.C	i	Translationally-controlled tumour protein homolog	18.806	4.55
Betta	2w3d u	7.1.C	r	Unidentified	-	-
Betta	2w5d u	7.2.A	z	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta	2w5d u	7.2.A	n	NADH dehydrogenase subunit 2	53.704	8.98
Betta	2w5d u	7.2.A	q	Unidentified	-	-
Betta	2w5d u	7.2.A	u_1	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta	2w5d u	7.2.A	g	Serine-threonine protein kinase	44.191	5.97
Betta	2w5d u	7.2.A	v	Endo-beta-1,3-glucanase	35.005	4.86
Betta	2w5d u	7.2.A	u_2	Unidentified	-	-
Betta	2w5d u	7.2.A	u_3	Heat shock protein 20	16.882	4.67
Betta	2w7d u	7.2.B	b	Unidentified	-	-
Betta	2w7d u	7.2.B	d	Unidentified	-	-
Betta	2w7d u	7.2.B	u	Unidentified	-	-
Betta	2w7d u	7.2.B	z	Photosystem II 44 kDa reaction centre protein	52.001	6.93
Betta	2w7d u	7.2.B	y	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta	2w7d u	7.2.B	f	Unidentified	-	-
Betta	2w7d u	7.2.B	j	ATPsynthase a chain	42.992	6.19
Betta	2w7d u	7.2.B	m_1	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta	2w7d u	7.2.B	m_2	DNA binding protein EMBP-1	36.193	9.08
Betta	2w7d u	7.2.B	r	Chlorophyll a-b binding protein	28.264	5.67
Betta	2w7d u	7.2.B	h	Protein-L-isoaspartate O-methyltransferase	24.708	4.90
Betta	2w9d u	7.2.C	b	Unidentified	-	-
Betta	2w9d u	7.2.C	d	Heat shock protein 90	80.460	4.96
Betta	2w9d u	7.2.C	p	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta	2w9d u	7.2.C	v	Unidentified	-	-
Betta	2w9d u	7.2.C	j	Unidentified	-	-
Betta	2w9d u	7.2.C	y	Heat shock protein 20	16.882	4.67

1- Age of the wheat used in the control study, for instance 2w3d is two weeks and three days. This was done in order to have controls of the same age as the experimental plants.

Table 7.2. Putative identification of proteins up or downregulated in two-week-old Betta wheat (RWA-susceptible) over a 9 day growth period

Wheat cultivar	D.A.I. [1]	Figure number	Spot symbol[2]	Putative protein	MW (kDa)	pI
Betta	2w2dRWA	7.1.E	l	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta	2w2dRWA	7.1.E	s	Calcium-dependent protein kinase	56.788	5.34
Betta	2w2dRWA	7.1.E	u	Unidentified	-	-
Betta	2w2dRWA	7.1.E	b	β-1,3-endoglucanase	48.873	5.88
Betta	2w2dRWA	7.1.E	g	Chlorophyll a-b binding protein	28.267	5.67
Betta	2w3d RWA	7.1.F	o	β-1,3-endoglucanase	48.873	5.88
Betta	2w3d RWA	7.1.F	q	Unidentified	-	-
Betta	2w3d RWA	7.1.F	i	Unidentified	-	-
Betta	2w5d RWA	7.2.D	z_1	Low molecular weight glutenin storage protein	41.307	8.69
Betta	2w5d RWA	7.2.D	g	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta	2w5d RWA	7.2.D	c	Endo-beta-1,3-glucanase	36.117	4.54
Betta	2w5d RWA	7.2.D	z_2	Unidentified	-	-
Betta	2w5d RWA	7.2.D	r	16.9 kDa class I heat shock protein	16.878	5.83
Betta	2w5d RWA	7.2.D	a	Heat shock protein 20	16.882	4.67
Betta	2w7d RWA	7.2.E	m_1	Unidentified	-	-
Betta	2w7d RWA	7.2.E	m_2	Heat shock protein 90	80.460	4.96
Betta	2w7d RWA	7.2.E	s_1	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta	2w7d RWA	7.2.E	u	Proline-rich protein	42.119	9.01
Betta	2w7d RWA	7.2.E	s_2	Leucine zipper protein	47.913	8.88
Betta	2w7d RWA	7.2.E	y	Unidentified	-	-
Betta	2w7d RWA	7.2.E	a_1	Unidentified	-	-
Betta	2w7d RWA	7.2.E	n	Low molecular weight glutenin storage protein	41.307	8.69
Betta	2w7d RWA	7.2.E	z_1	Unidentified	-	-
Betta	2w7d RWA	7.2.E	e	Unidentified	-	-
Betta	2w7d RWA	7.2.E	g_1	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta	2w7d RWA	7.2.E	z_2	Unidentified	-	-
Betta	2w7d RWA	7.2.E	a_2	Heat shock protein 20	16.882	4.67
Betta	2w7d RWA	7.2.E	g_2	Unidentified	-	-
Betta	2w9d RWA	7.2.F	s	Heat shock protein 90	80.460	4.96
Betta	2w9d RWA	7.2.F	h	Unidentified	-	-
Betta	2w9d RWA	7.2.F	l_1	Unidentified	-	-
Betta	2w9d RWA	7.2.F	f	Vesicle associate membrane protein	49.282	6.41
Betta	2w9d RWA	7.2.F	g	Cyclin dependent protein kinase	48.174	9.10
Betta	2w9d RWA	7.2.F	r	Low molecular weight glutenin storage protein	41.307	8.69
Betta	2w9d RWA	7.2.F	n	Unidentified	-	-
Betta	2w9d RWA	7.2.F	l_2	β-1,3-endoglucanase	35.356	8.50
Betta	2w9d RWA	7.2.F	q	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta	2w9d RWA	7.2.F	z	Ethylene-responsive element binding protein	39.157	4.65
Betta	2w9d RWA	7.2.F	w	Chlorophyll a-b binding protein	28.267	5.67
Betta	2w9d RWA	7.2.F	p	2-cys Periredoxin Bas1 chloroplast precursor	23.327	5.71
Betta	2w9d RWA	7.2.F	r_2	16.9 kDa class I heat shock protein	16.878	5.83
Betta	2w9d RWA	7.2.F	l_3	Type –5 thionin precursor	13.748	4.41
Betta	2w9d RWA	7.2.F	j	Unidentified	-	-

1- D.A.I. refers to the days after RWA feeding commenced in addition to the age of the wheat plants. For example, 2w3d refers to RWA feeding for 3 days on two-week-old wheat and 2w5d refers to RWA feeding for 5 days on two-week-old wheat

2- Spot symbol refers to the reference symbol assigned to each spot by the PDQuest™ 2DE analysis software in Figure 7.3.

7.2. Differential protein expression in Betta DN wheat in response to RWA infestation over set time periods

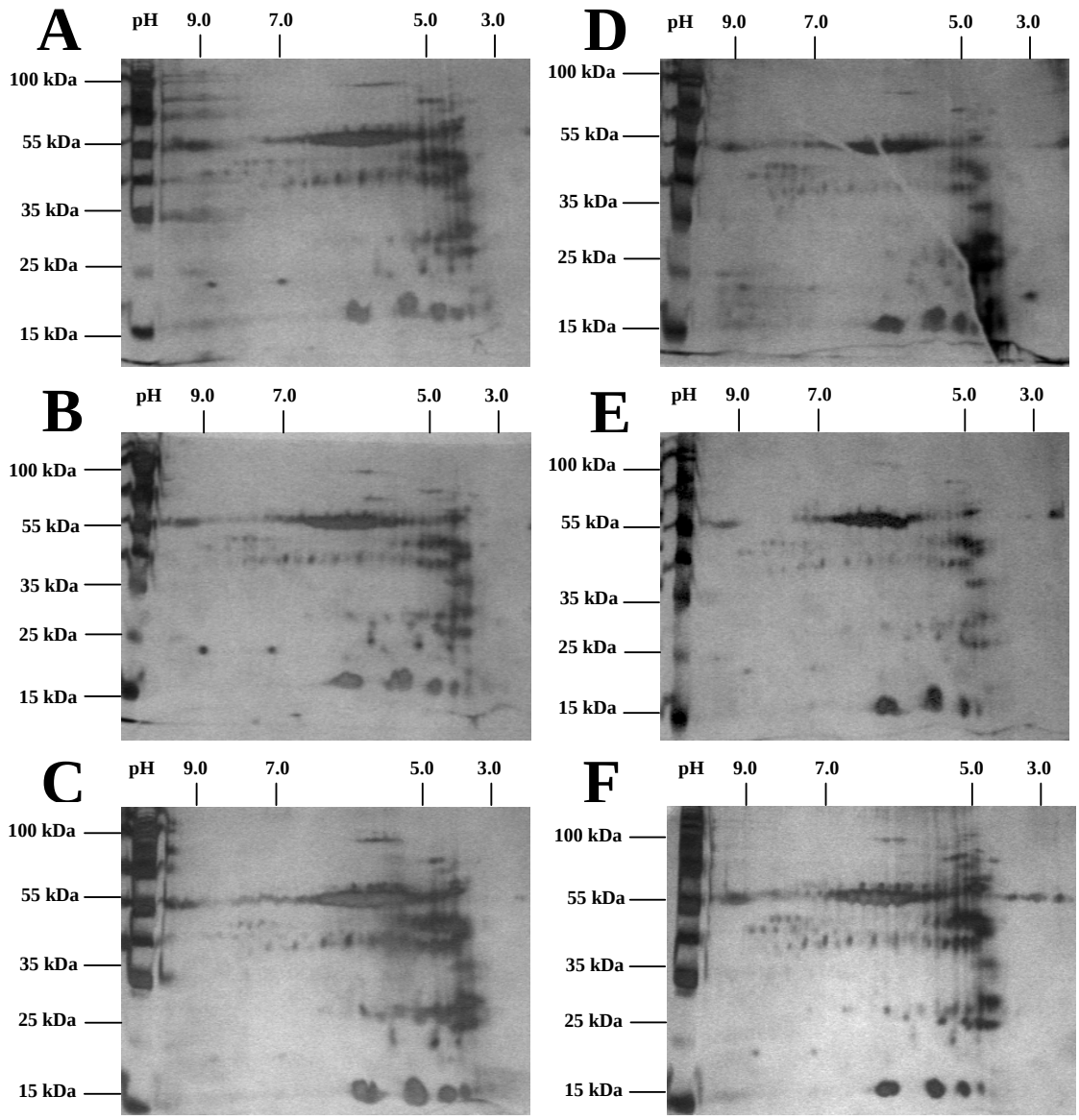


Figure 7.4. Differential expression of proteins in Betta DN wheat after RWA infestation over 9 days.

All wheat samples were two weeks old at the start of the study. Time periods given refer to the duration of the experimental period. [A] – Unstressed Betta DN wheat; [B] – Unstressed Betta DN wheat at 2 days; [C] – Unstressed Betta DN wheat at 3 days; [D] – Betta DN wheat subjected to aphid feeding at two-weeks; [E] – Betta DN wheat subjected to aphid feeding for 2 days; [F] – Betta DN wheat subjected to aphid feeding for 3 days.

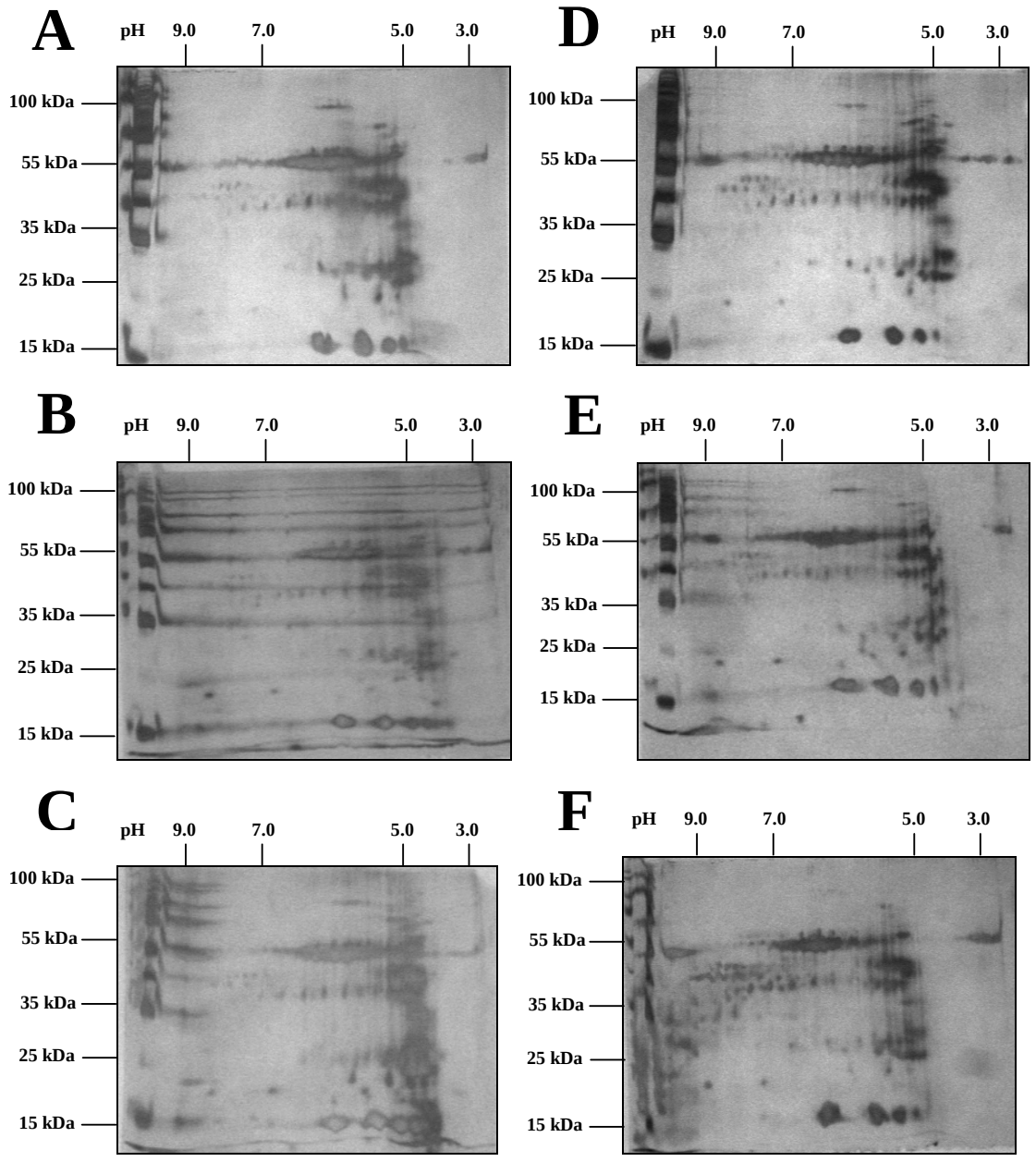


Figure 7.5. Differential expression of proteins in Betta DN wheat after RWA infestation over 9 days.

All wheat samples were two weeks old at the start of the study. Time periods given refer to the duration of the experimental period. [A] – Unstressed Betta DN wheat at 5 days; [B] – Unstressed Betta DN wheat at 7 days; [C] – Unstressed Betta wheat DN at 9 days; [D] – Betta DN wheat subjected to aphid feeding for 5 days; [E] – Betta DN wheat subjected to aphid feeding for 7 days; [F] – Betta DN wheat subjected to aphid feeding for 9 days.

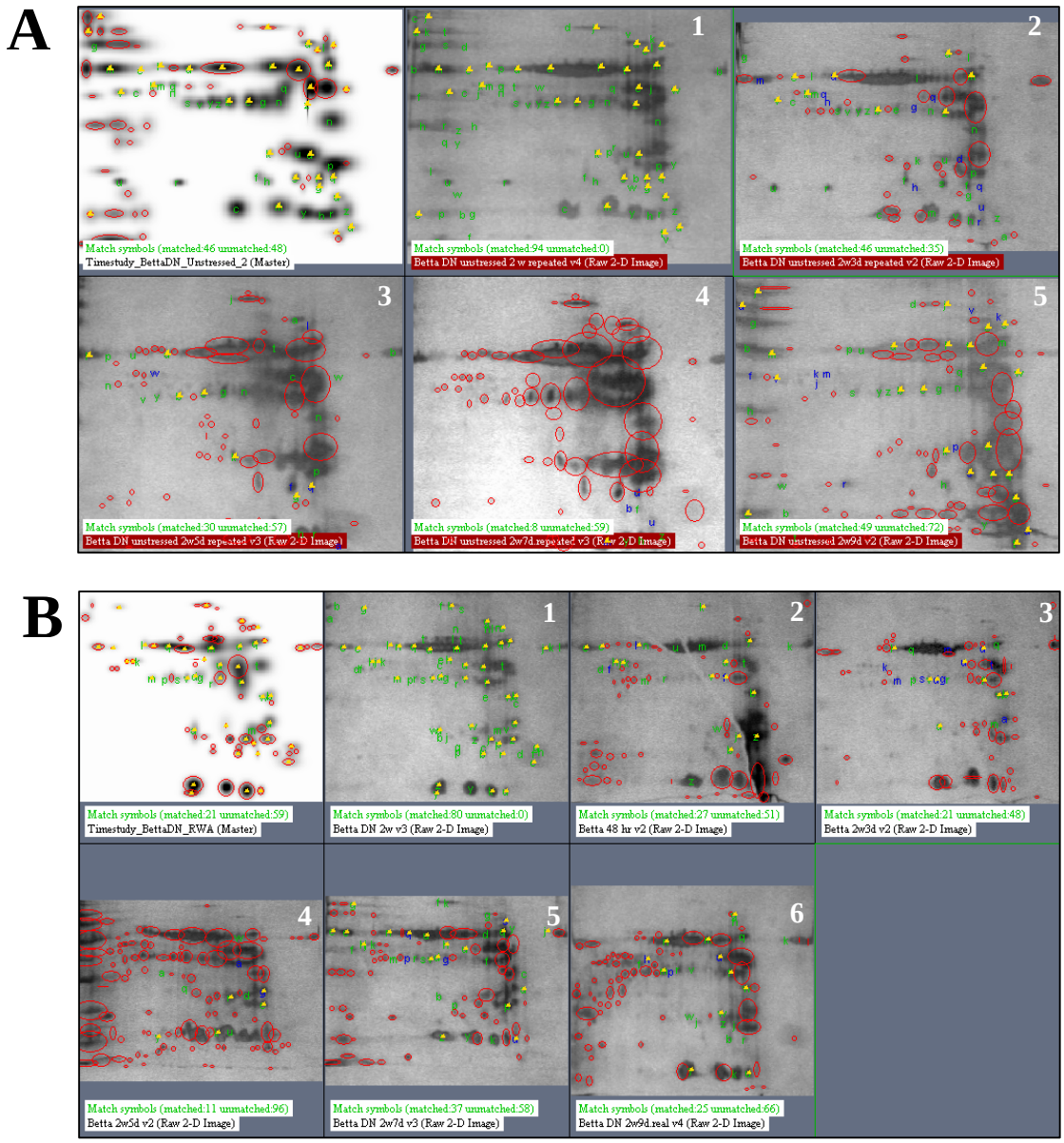


Figure 7.6. Analysis 2DE gels of Betta DN wheat (resistant) exposed to RWA phloem-feeding over different time periods using PDQuest™ Basic Software Version 8.0 (Bio-Rad).

Figure A shows unstressed controls and Figure B shows the experimental 2DE gels. [A] – (1) - Two-week old Betta DN wheat (control and master gel); (2) – Betta DN at two weeks and three days; (3) – Betta DN at two weeks and five days; (4)- Betta DN wheat at two weeks and seven days; (5) – Betta DN at two weeks and nine days. [B] – (1) - Two-week old Betta DN wheat (control and master gel); (2) – Betta DN wheat 2 days after RWA infestation; (3) – Betta DN at two weeks and three days after infestation; (4) – Betta DN at two weeks and five days; (5) – Betta DN wheat at two weeks and seven days after RWA infestation; (6) – Betta DN at two weeks and nine days after RWA infestation. Green letters indicate matched proteins, red circles indicate differentially expressed proteins and yellow triangles indicate up and down regulated proteins.

Table 7.3. Putative identification of proteins up or downregulated in unstressed two-week-old Betta DN wheat (RWA-resistant) over a 9 days

Wheat cultivar	D.A.I. [1]	Figure number	Spot symbol[2]	Putative protein	MW (kDa)	pI
Betta DN	2w3d u	7.4.C	a_1	Heat shock protein 90	80.460	4.96
Betta DN	2w3d u	7.4.C	a_2	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta DN	2w3d u	7.4.C	e	Unidentified	-	-
Betta DN	2w3d u	7.4.C	v	Cyclin-dependent protein kinase	48.174	9.10
Betta DN	2w3d u	7.4.C	b	Gibberellin oxidase 20 1-D	40.293	6.07
Betta DN	2w3d u	7.4.C	z	Ethylene-responsive element binding protein	39.157	4.65
Betta DN	2w3d u	7.4.C	m	Unidentified	-	-
Betta DN	2w3d u	7.4.C	k	Leucine zipper protein	47.913	8.88
Betta DN	2w5d u	7.5.A	l	NADH dehydrogenase subunit 2	53.704	8.98
Betta DN	2w5d u	7.5.A	a	Photosystem II 44kDa reaction centre protein	52.001	6.93
Betta DN	2w5d u	7.5.A	b	ATP synthase a chain	42.992	6.19
Betta DN	2w5d u	7.5.A	c	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN	2w5d u	7.5.A	k	β-1,3-glucanase	34.882	5.70
Betta DN	2w5d u	7.5.A	g	Unidentified	-	-
Betta DN	2w5d u	7.5.A	q	Unidentified	-	-
Betta DN	2w7d u	7.5.B	-	None	-	-
Betta DN	2w9d u	7.5.C	j_1	Unidentified	-	-
Betta DN	2w9d u	7.5.C	d	Unidentified	-	-
Betta DN	2w9d u	7.5.C	m	NADH dehydrogenase subunit 2	53.704	8.98
Betta DN	2w9d u	7.5.C	v	Cyclin dependent protein kinase	48.174	9.10
Betta DN	2w9d u	7.5.C	e	DRP6 protein (fragment)	16.604	8.54
Betta DN	2w9d u	7.5.C	a	Unidentified	-	-
Betta DN	2w9d u	7.5.C	b	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN	2w9d u	7.5.C	c_1	Ethylene-responsive element binding protein	39.157	4.65
Betta DN	2w9d u	7.5.C	j_2	Unidentified	-	-
Betta DN	2w9d u	7.5.C	l	Unidentified	-	-
Betta DN	2w9d u	7.5.C	t	Protein disulphide isomerase	56.533	4.99
Betta DN	2w9d u	7.5.C	c_2	Serine threonine protein kinase	44.191	5.97
Betta DN	2w9d u	7.5.C	w	Unidentified	-	-
Betta DN	2w9d u	7.5.C	e	Unidentified	-	-
Betta DN	2w9d u	7.5.C	s	Unidentified	-	-
Betta DN	2w9d u	7.5.C	k	Chlorophyll a-b binding protein	28.264	5.67
Betta DN	2w9d u	7.5.C	d	Unidentified	-	-
Betta DN	2w9d u	7.5.C	f	Unidentified	-	-
Betta DN	2w9d u	7.5.C	q	Unidentified	-	-
Betta DN	2w9d u	7.5.C	a	Unidentified	-	-
Betta DN	2w9d u	7.5.C	t	Unidentified	-	-
Betta DN	2w9d u	7.5.C	e	Unidentified	-	-

1- Age of the wheat used in the control study, for instance 2w3d is two weeks and three days. This was done in order to have wheat of the same age as the experimental plants
2- Spot symbol refers to the symbol assigned to each spot by the 2DE analysis software

Table 7.4. Putative identification of proteins up or downregulated in two-week-old RWA stressed Betta DN wheat (RWA-resistant) over a two-week growth period

Wheat cultivar	D.A.I. [1]	Figure number	Spot symbol	Putative protein	MW (kDa)	pI
Betta DN	2w2dRWA	7.4.E	k	Unidentified	-	-
Betta DN	2w2dRWA	7.4.E	e_1	Protein kinase	61.700	9.12
Betta DN	2w2dRWA	7.4.E	e_2	Protein kinase	60.974	9.14
Betta DN	2w2dRWA	7.4.E	l_1	NADH dehydrogenase subunit 2	53.704	8.98
Betta DN	2w2dRWA	7.4.E	l_2	Unidentified	-	-
Betta DN	2w2dRWA	7.4.E	j_1	Photosystem II 44 kDa reaction centre protein	52.001	6.93
Betta DN	2w2dRWA	7.4.E	a	Putative vesicle associated membrane protein	49.282	6.41
Betta DN	2w2dRWA	7.4.E	v	Serine-threonine protein kinase	44.191	5.97
Betta DN	2w2dRWA	7.4.E	f	Unidentified	-	-
Betta DN	2w2dRWA	7.4.E	b_1	Endo-beta-1,3-glucanase	36.117	4.54
Betta DN	2w2dRWA	7.4.E	j_2	Chlorophyll a-b binding protein	28.264	5.67
Betta DN	2w2dRWA	7.4.E	z	Unidentified	-	-
Betta DN	2w2dRWA	7.4.E	b_2	Thaumatin-like protein PWIR2 (precursor)	17.605	4.64
Betta DN	2w3dRWA	7.4.F	l	Photosystem II 44 kDa reaction centre protein	52.001	6.93
Betta DN	2w3dRWA	7.4.F	d_1	Unidentified	-	-
Betta DN	2w3dRWA	7.4.F	d_2	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN	2w3dRWA	7.4.F	q	ATP synthase a chain	42.992	6.19
Betta DN	2w3dRWA	7.4.F	v	Endo-beta-1,3-glucanase	36.117	4.54
Betta DN	2w3dRWA	7.4.F	s	Unidentified	-	-
Betta DN	2w3dRWA	7.4.F	a	Cyclin dependent protein kinase	25.934	6.50
Betta DN	2w3dRWA	7.4.F	u	Serine threonine protein kinase	44.191	5.97
Betta DN	2w5dRWA	7.5.D	r	Abscisic acid inducible kinase (fragment)	37.516	5.68
Betta DN	2w5dRWA	7.5.D	e	Unidentified	-	-
Betta DN	2w5dRWA	7.5.D	g	Endo-beta-1,3-glucanase	35.005	4.86
Betta DN	2w5dRWA	7.5.D	k	Unidentified	-	-
Betta DN	2w5dRWA	7.5.D	y	Alpha amylase trypsin inhibitor	15.460	6.86
Betta DN	2w7dRWA	7.5.E	g	Unidentified	-	-
Betta DN	2w7dRWA	7.5.E	s	Unidentified	-	-
Betta DN	2w7dRWA	7.5.E	e_1	Protein kinase	61.700	9.12
Betta DN	2w7dRWA	7.5.E	e_2	Protein kinase	60.974	9.14
Betta DN	2w7dRWA	7.5.E	n	NADH dehydrogenase subunit 2	53.704	8.98
Betta DN	2w7dRWA	7.5.E	q_1	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta DN	2w7dRWA	7.5.E	u_1	Photosystem II 44 kDa reaction centre protein	52.001	6.93
Betta DN	2w7dRWA	7.5.E	q_2	RuBisCo large subunit	57.521	4.83
Betta DN	2w7dRWA	7.5.E	j_1	Unidentified	-	-
Betta DN	2w7dRWA	7.5.E	j_2	β-1,3-endoglucanase	48.873	6.32
Betta DN	2w7dRWA	7.5.E	h	Putative vesicle associate membrane protein	49.282	6.32
Betta DN	2w7dRWA	7.5.E	u_2	β-1,3-endoglucanase	48.873	6.32
Betta DN	2w7dRWA	7.5.E	v	Serine threonine protein kinase	44.191	5.97
Betta DN	2w7dRWA	7.5.E	d	Gibberellin 20 oxidase 1-D	40.293	6.07
Betta DN	2w7dRWA	7.5.E	a	Unidentified	-	-
Betta DN	2w7dRWA	7.5.E	p	Unidentified	-	-
Betta DN	2w7dRWA	7.5.E	z	Alpha amylase trypsin inhibitor CM2 precursor	15.460	6.86
Betta DN	2w7dRWA	7.5.E	u_3	Unidentified	-	-
Betta DN	2w9dRWA	7.5.F	g	Heat shock protein 90	80.460	4.96
Betta DN	2w9dRWA	7.5.F	n	Photosystem II P680 chlorophyll A apoprotein	56.092	6.06
Betta DN	2w9dRWA	7.5.F	m_1	Calcium-dependent protein kinase	56.788	5.34
Betta DN	2w9dRWA	7.5.F	h	Unidentified	-	-
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	Beta-1,3-endoglucanase	48.873	5.66
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	Gibberellin 20 oxidase 1-D	40.293	6.07

Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	Ethylene responsive element binding protein	39.157	4.65
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	Beta-1,3-glucanase	34.660	4.352
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	2-cys periredoxin BAS 1 chloroplast precursor	23.327	5.71
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	16.9 kDa class I heat shock protein	16.878	5.83
Betta DN	2w9dRWA	4.3.3.B 6	7.5.F	Unidentified	-	-

1- D.A.I. refers to the days after RWA feeding commenced in addition to the age of the wheat plants. For instance, 2w3d refers to RWA feeding for 3 days on two-week-old wheat and 2w5d refers to RWA feeding for 5 days on two-week-old wheat
2- Spot symbol refers to the symbol assigned to each spot by the 2DE analysis software

Appendix 3: Long Ashton nutrient solution

Table 8.1. The full Long-Ashton nutrient solution as taken from Hewitt (1966).

Type of Salt	Weight used (g)	Volume of stock solution (ml)	Volume of stock solution Dilute in 25 L (ml)	Concentration in final volume of 25 L (ml)
Macronutrients				
KNO_3	101	500	25	2
K_2SO4	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
$NaH_2PO4.2H_2O$	104	500	25	4
Micronutrients				
$MnSO_4.4H_2O$	11.20	500	2.5	0.02
$CuSO4.5H_2O$	1.25	500	2.5	0.002
$ZnSO_4.7H_2O$	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

Appendix 4: Reagents and protocols used

8.2.1. Determination of protein concentration

Sample protein standard curve generated with the RC / DC protein assay (Bio-Rad; Catalogue # 500-0120) using bovine serum albumin fraction V (Roche; Catalogue # 735 078) as a standard. All samples were diluted 100 x prior to assaying and dilutions were taken into account in all subsequent calculations of protein content.

The scaled-down assay for microtitre plates was used. A standard curve was generated using a bovine serum albumin fraction V (Roche; Catalogue # 735 078) stock solution with a concentration of 1 mg / ml that was diluted to give concentrations ranging from 0.2 – 1.0 mg / ml. The assay was performed according to the manufacturer's instructions and absorbencies were read on a Power Wave x microplate reader from Bio-Tek Instruments on Kcjunior software. Standard curves were created using Microsoft Excel ®.

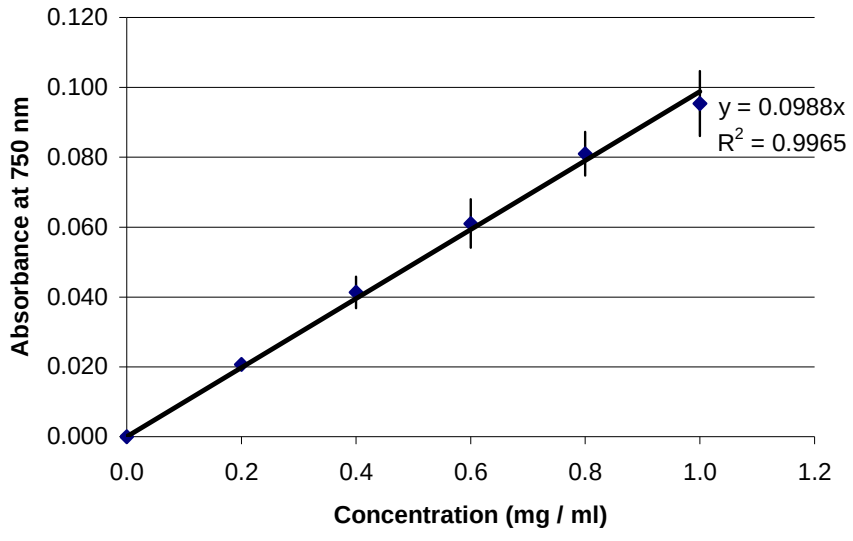


Figure 8.1. Sample standard curve generated using the RC / DC protein assay (Bio-Rad) with a bovine serum albumen (Fraction V) stock solution of 1 mg / ml. All readings were taken in triplicate and standard deviations are shown by the vertical black lines on the figure.

8.2.2. Sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method according to the Mini-Protean® 3 Cell Instruction manual (modified protocol of the method according to Laemmli, 1970).

10 x Running buffer (pH 8.3)

30.3 g Tris Base
144.0 g Glycine
10.0 g SDS

Make up to 1 L with distilled water and do not adjust pH. Store at 4°C, if precipitation occurs warm up to room temperature prior to use. Dilute 50 ml stock in 450 ml distilled water prior to use.

Sample buffer (SDS reducing buffer)

7.10 ml deionized water
2.50 ml Tris-HCl buffer (0.5 M, pH 6.8)
5.0 ml glycerol
4.0 ml SDS solution (10 % w / v)
0.4 ml bromophenol blue (0.5 % w / v)

Store at room temperature. Add 50 µl β-mercaptoethanol to 950 µl sample buffer prior to use. Dilute protein samples at least 1:2 with sample buffer and boil for 4 minutes prior to loading onto SDS-PAGE gel.

10 % Ammonium persulphate (APS)

Add 100 mg (0.1 g) APS to 1 ml deionised water. Prepare fresh daily.

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Dissolve 6 g Tris(Hydroxymethyl)Aminomethane (Merck) in 100 ml distilled water. Adjust the pH to 6.8 with Hydrochloric acid (HCl).

Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

Dissolve 18.15 g Tris(Hydroxymethyl)Aminomethane in 100 ml distilled water. Adjust the pH to 8.8 with Hydrochloric acid (HCl).

Gel formulations

SDS-PAGE stacking gel (4 %)

Reagent	**Volume (ml)**
Distilled water	6.1
30 % Degassed Acrylamide / Bis	1.3
0.5 M Tris-HCl, pH 6.8	2.5
10 % w / v SDS	0.1
10 % APS	0.05
TEMED	0.01

SDS-PAGE resolving gel (12 %)

Reagent	**Volume (ml)**
Distilled water	3.4
30 % Degassed Acrylamide / Bis	4.0
0.5 M Tris-HCl, pH 6.8	2.5
10 % w / v SDS	0.1
10 % APS	0.05
TEMED	0.005

Gel stains

Coomassie Stain

Add 0.075 % Coomassie brilliant blue R250 to 40 % methanol and 0.7 % glacial acetic acid and store in a dark container. Stain gels for one hour or overnight while shaking.

Coomassie Destain

Add 40 % methanol and 0.7 % glacial acetic acid. Destain gels stained with Coomassie stain until destaining solution is no longer dark purple and a clear background is observable on the gel.

PageBlue™ Protein staining solution (Fermentas)

Add approximately 25 ml of solution to each gel, shake for one hour or overnight and destain with water.

Appendix 5: Two-dimensional gel electrophoresis reagents

2 D sample rehydration buffer

Add 13.5 g Urea (9 M) (Merck; Catalogue # 1.08487.0500), 2 % PlusOne Triton-X 100 (0.5 ml) (Amersham; Catalogue # 17-1315-01), 2 % Ampholytes in the form of Bio-Lyte 3-10 buffer (0.5 ml) (Bio-Rad; Catalogue # 163-2094) and 0.5 ml of a 1 % solution of Bromphenol Blue before making up to 25 ml. Aliquot 1 ml volumes and store at – 20 °C. Add 0.02 g DTT (Roche; Catalogue # 708 984) to 1 ml buffer prior to use.

SDS equilibration buffer

Add 6 M Urea (90.09 g), 0.375 M Tris-HCl (62.5 ml; pH 8.8), 2 % SDS (50 ml of a 10 % stock solution) and 20 % glycerol (50 ml) in 250 ml distilled water. Add a pinch of bromophenol blue and store at 4°C. Add 2 % w / v DTT prior to use. Use approximately 5 ml per IPG strip and incubate for 30 minutes before running second dimension on 12 % SDS-PAGE gels.

Second dimension gels (12 % SDS-PAGE)

Makes 2 x mini gels

Reagent	**Volume (ml)**
Distilled water	5.1
30 % Degassed Acrylamide / Bis Acrylamide	6.0
1.5 M Tris-HCl (pH 8.8)	3.75
10 % w / v SDS solution	0.15
10 % APS	0.210
TEMED	0.03

Appendix 6: Isoelectric focussing protocols for two-dimensional gel electrophoresis

Protocol A

Protocol A utilized a Consort E815 power supply. The IPG strips were subject to a total of 8 233 V-hr. The number of V-hr were calculated using the formula given below:

V-hr on IPG strips = Applied electric potential (V) x [time (mins)] / 60

Table 8.2. Isoelectric focussing conditions for broad-range IPG strips rehydrated with total protein from 14-day old wheat leaves on a Consort E815 power supply

Program	Duration (mins)	Current (mA)	Voltage	V-hr
P1	10	0.5	200	33
P2	10	0.9	450	75
P3	10	0.4	750	125
P4	480	0.4	1000	8 000
Total duration	510 (8.5 hours)	--	--	8 233

Protocol B

Protocol A utilized a Bio-Rad PowerPac™ HV power supply. The IPG strips were subject to a total of 2 366.6 V-hr. The current was maintained at 0.5 mA throughout the focussing period.

Table 8.3. Isoelectric focussing conditions for broad-range IPG strips rehydrated with total protein from 14-day old wheat leaves on a Bio-Rad PowerPac™ power supply

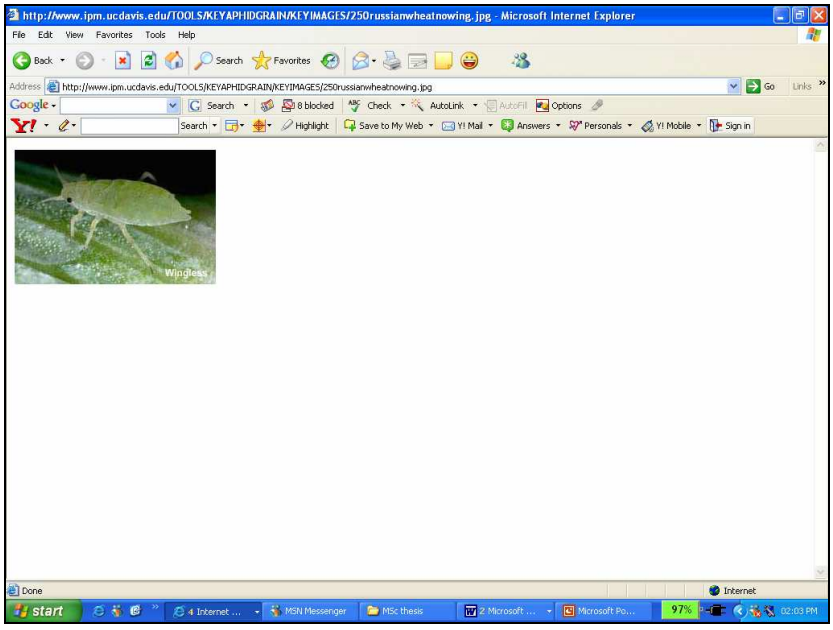
Voltage (V)	Duration (mins)	V-hr
200	20	66.6
450	15	112.5
750	15	187.5
2000	60	2 000
Total V-hr	2 366.6	

Appendix 7: Copies of Internet references used in Chapter 1

Internet 1: http://www.invasive.org/images/3072x2048/1357013.jpg

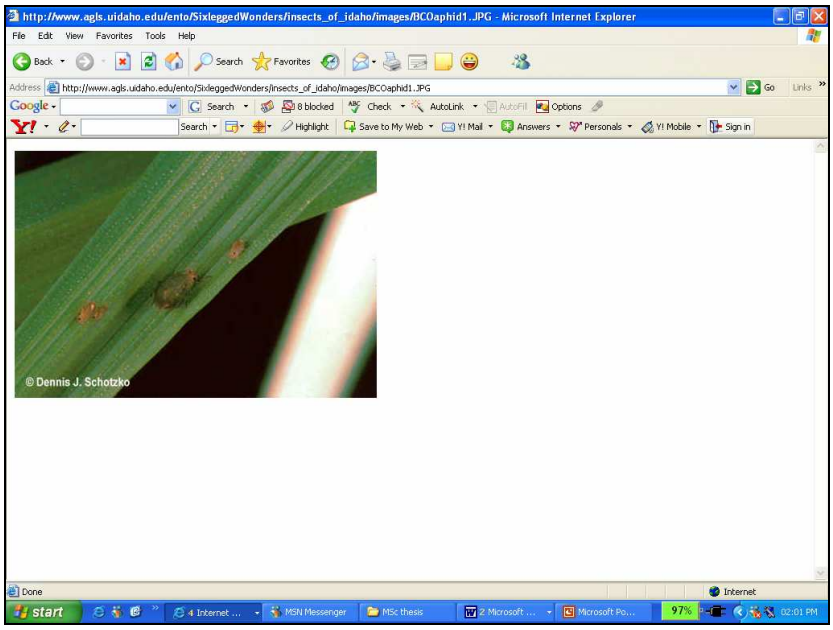
Internet 2:

http://www.ipm.ucdavis.edu/TOOLS/KEYAPHIDGRAIN/KEYIMAGES/250russianwheatnowing.jpg

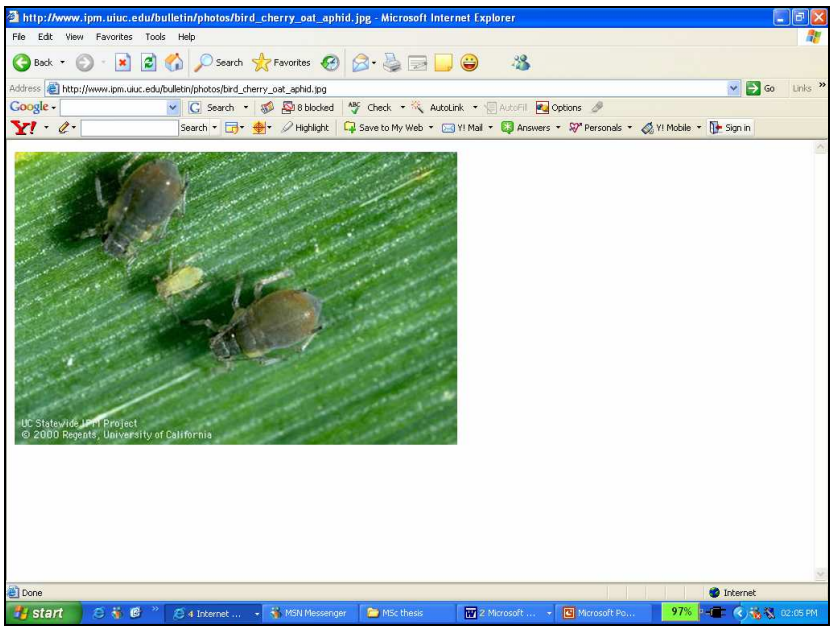


Internet 3:

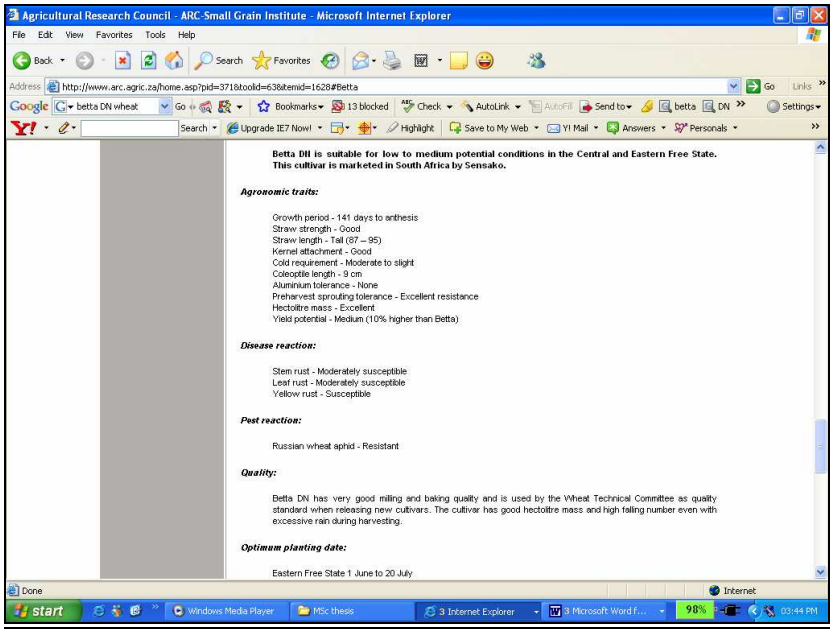
http://www.agls.uidaho.edu/ento/SixleggedWonders/insects_of_idaho/images/BCOaphid1.JPG



Internet 4: http://www.ipm.uiuc.edu/bulletin/photos/bird_cherry_oat_aphid.jpg



Internet 5: http://www.arc.agric.za/home.asp?pid=371&toolid=63&itemid=1628#Betta



Appendix 8: Copies of Internet references used in Chapter 2

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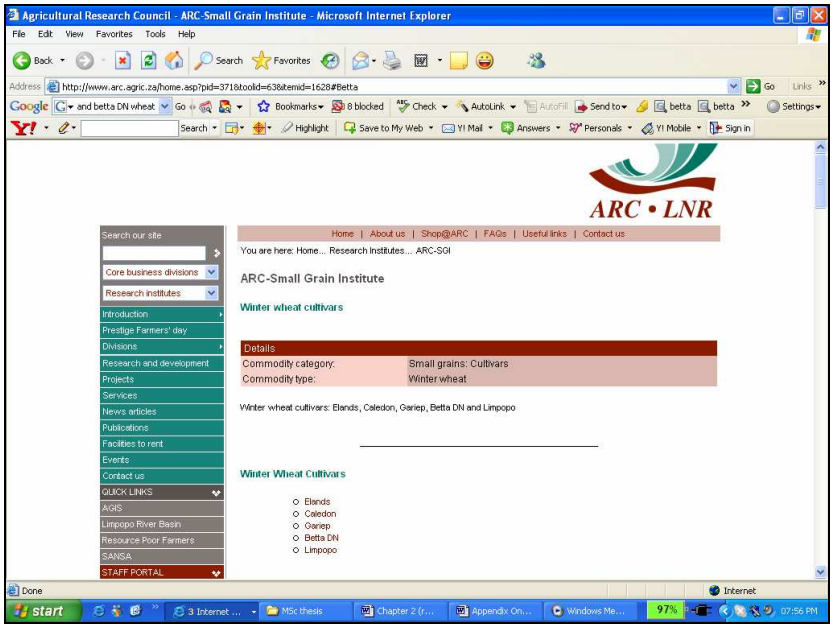
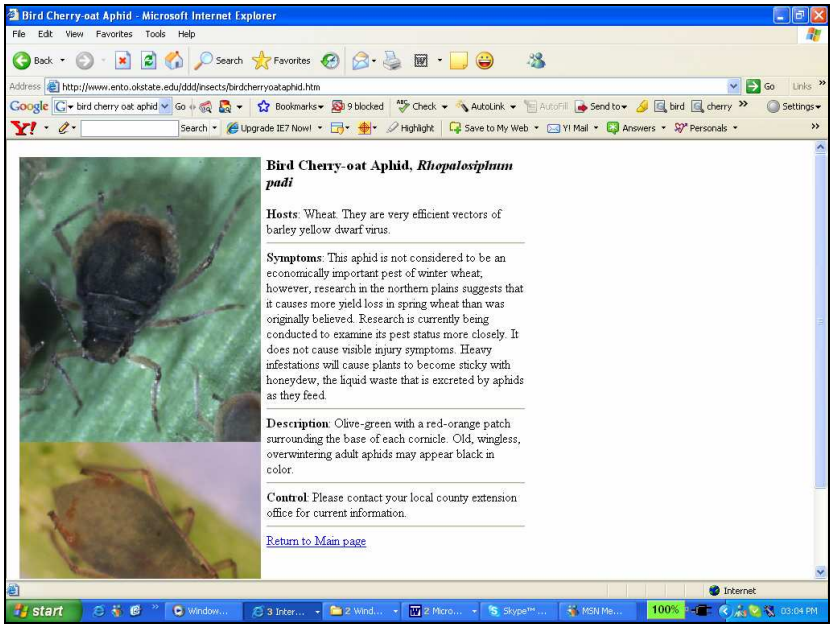
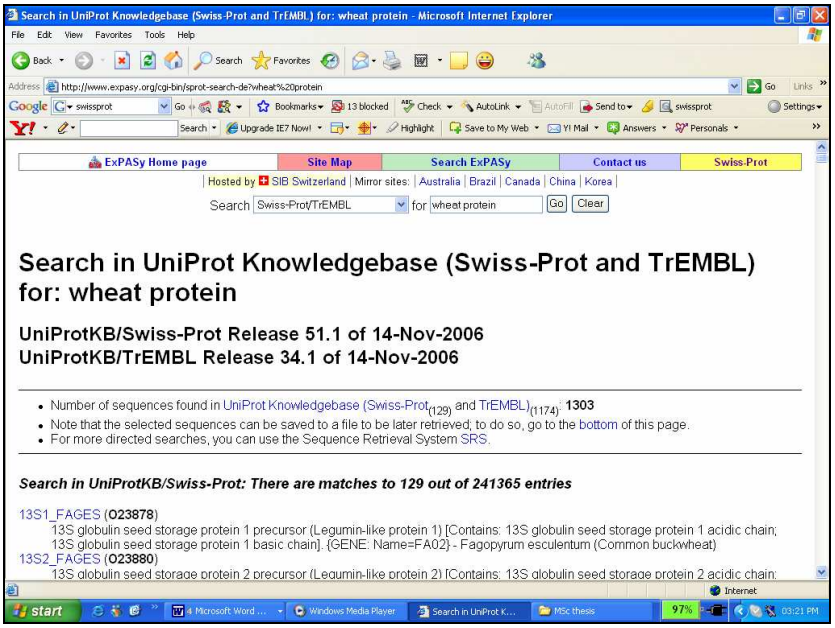


Appendix 9: Copies of Internet references used in Chapter 3

Internet 1: http://www.ento.okstate.edu/ddd/insects/birdcherryoataphid.htm



Internet 2: http://www.expasy.org/cgi-bin/sprot-search-de?wheat%20protein



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Internet References

Internet References for Chapter 1

(Full copies of webpages used attached in Appendix 7)

Internet 1:
http://www.invasive.org/images/3072x2048/1357013.jpg

Internet 2:
http://www.ipm.ucdavis.edu/TOOLS/KEYAPHIDGRAIN/KEYIMAGES/250russianwheatnowing.jpg

Internet 3:
http://www.agls.uidaho.edu/ento/SixleggedWonders/insects_of_idaho/images/BCOaphid1.JPG

Internet 4:
http://www.ipm.uiuc.edu/bulletin/photos/bird_cherry_oat_aphid.jpg

Internet 5:
http://www.arc.agric.za/home.asp?pid=371&toolid=63&itemid=1628#Betta

Internet References for Chapter 2

(full copies of web pages used attached in Appendix 8)

Internet 1:
http://www.arc.agric.za/home.asp?pid=371&toolid=63&itemid=1628#Betta

Internet References for Chapter 3

(Full copy of this reference in Appendix 9)

Internet 1:

http://www.ento.okstate.edu/ddd/insects/birdcherryoataphid.htm

Internet 2:
http://www.expasy.org/cgi-bin/sprot-search-de?wheat%20protein