APHID RESPONSES TO DROUGHT: A COMBINED PHYSIOLOGICAL AND TRANSCRIPTOMIC APPROACH

By

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Abstract

Drought is arguably one the greatest future challenges for agriculture. The response of phloem feeders, such as aphids, to increased drought expected under climate change is still relatively undefined. The effect on aphid feeding of drought stress in plants can be viewed as potentially positive and negative. It is currently accepted that under drought conditions, host plant sieve elements will become more concentrated; hence increases in available amino acid concentrations will potentially benefit aphids. However, the increased need for aphid osmoregulation under drought, to deal with the osmotically challenging diet, may be detrimental to aphid performance.

Using the electrical penetration graph technique (EPG), the probing behaviour of two clones of *Myzus persicae (*O Clone and B Clone*), Brevicoryne brassicae, Rhopalosiphum padi* and *Sitobion avenae* on the host plants *Brassica nigra* and *Lolium perenne* (respectively), were analysed under a defined drought regime.

Drought treatment had a significant effect on the behaviours of all aphid species and clones, except *S. avenae*. In *B. brassicae* and B clones of *M. persicae*, xylem feeding was significantly increased on droughted host plants. Furthermore, both clones of *M. persicae* and *R. padi* spent significantly less time ingesting sieve element sap, and more time not probing or in plant pathway activities whilst on droughted host plants. These results suggest that drought stress may cause a reduced palatability of host plants.

In addition, fecundity measurements showed that drought resulted in a reduction in aphid reproductive performance in *M. persciae* (O and B clone)*, B. brassicae, R. padi*

and *S. avenae*. However, fecundity was only significantly reduced in the *M. persicae* (O clone only), *B. brassicae* and *R. padi*.

To understand the aphid response to droughted *B. nigra* at the genetic level, the gene expression of *M. persicae* exposed to different drought regimes was analysed using microarrays. Gene expression analysis showed up regulation of the osmoregulation associated enzyme, sucrase, as well as the up regulation of other enzymes such as amylases, cytochrome P450s, Heat Shock Proteins, and an aquaporin when exposed to droughted hosts. Furthermore, it was found that the level of drought had a noticeable effect on gene expression in *M. persicae*, showing aphids have a very adaptable response to drought stress.

The combined physiological and transcriptomic approach of this study gives a complementary whole organism assessment of aphid responses to drought. This study has helped to confirm xylem feeding and sugar polymerisation as important mechanisms of aphid osmoregulation, as well as providing support for the hypothesis of water cycling within the body of the aphid. This study has highlighted that aphids respond heterogeneously to water stress, and although it has been possible to identify some general trends, this study has emphasised that the adaptability of aphids to stress is species and even ecotype specific.

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Glossary of terms

Abaxial: Facing away from the stem of the plant, i.e. lower surface of leaves

Alate: A winged aphid

Apterous: An aphid without wings

Buccal Cavity: the cavity between the jaws and the cheeks

Cauda: a small projection at the tip of the abdomen

Cibral Pump: Food can be sucked into the buccal cavity and into the pharynx by contractile action of cibarial muscles (located between the head capsule and the anterior wall of the pharynx) which create suction by enlarging the volume of the pharynx, this "suction pump" mechanism is called the cibarial pump.

Epipharynx: A structure that overlaps the mouth of certain insects

EST: Expressed Sequence Tag

Fundatrix: (aka stem-mother) the aphid which emerges from the winter egg and gives rise to the first generation in spring

Gametogenesis: the production of cells specialised in sexual reproduction

Gynoparae: Parthenogenetic females which produce sexual females

Heteroecious: Requiring more than one host species to complete a life cycle

Hypopharynx: The bottom part of the pharynx

Labium: (aka rostrum) Lower lips that surround the probiscus containing the stylet. During piercing, the labium encasing the stylet remains outside the plant tissue

Labrum: Upper lips that surround the probiscus containing the stylet.

Monoecious: Completes whole life cycle on one host

Oviposition: The process of laying eggs by oviparous animals

Parthenogenetic: A form of asexual reproduction found in females, where growth and development of embryos occurs without fertilisation by a male

Proboscis: In aphids the mandibles and maxillae are modified into a proboscis, which in turn is sheathed within a modified labium

Siphunculi: (aka cornicles) Paired protruding organs near the end of the abdomen of aphids, through which a sugary secretion is extruded

Stylectomy: A technique to obtain sieve tube sap by severing the stylets of feeding aphids

Stylet: Paired mandibles and maxillae together form the aphid stylet, which is used to pierce the plant tissue

Type I error: false positive, when the statistical test rejects a true null hypothesis

Type II error: false negative, when the test fails to reject a false null hypothesis

Virginoparae: female aphids giving birth to young by parthenogenesis

Viviparous: method of reproduction in which the embryo develops inside the body of the female from which it gains nourishment (opposite of oviposition)

List of Abbreviations

RIN – RNA Integrity Number
EST – Expressed Sequence Tag

cDNA – Complementary DNA

mRNA – Messenger RNA

aRNA – amplified RNA

UTP - uridine 5'-triphosphate.

DMSO - Dimethyl sulfoxide

NCBI – National Center for Biotechnology Information

GO Analysis – Gene Ontology Analysis

Nt – Single stranded unit of DNA/RNA

Chapter 1: Literature Review

1.0 Climate Change and drought stress

The “warming of the climate system is unequivocal, as is now evident from observations of increases in global average air and ocean temperatures, widespread melting of snow and ice and rising global average sea level” IPCC (2007). Anthropogenic increases in greenhouse gases are predicted to cause changes in global precipitation profiles. In the UK a warming in atmospheric temperature is expected of ≈2-3.5^{o}C, in addition to a predicted fall in summer precipitation levels by up to 50% by 2080 (Hulme *et al*, 2002). Extremely warm seasons and years are predicted to increase in frequency by 30% by 2050, with a 90% chance of two warm dry years occurring consecutively (CCIRG 1996). In the southeast of the UK, prolonged periods of drought are likely, even in years where average rainfall has occurred (CCIRG 1996).

Under the current climate change scenario, it is anticipated that the UK agricultural industry could suffer from increased insect outbreaks related to the global rising temperatures, and crop losses from increased incidences of drought and flooding events. The effect of frequent droughts on one of agriculture’s most significant insect pests, the aphid, is not well understood. It can be certain that the interaction between aphids and their host plants will alter under drought conditions, as drought affects the host-plant and consequently phloem composition. However, whether we can predict these changes for the whole feeding guild is questionable. In a recent review on this subject, it was suggested that the species specific adaptability among the *Aphididae,* might make any predictions only valid if made on an individual species level (Pritchard *et al*, 2007).

This study aims to examine the effect of drought on the physiology of two common British plant species, the herbaceous *Brassica nigra* and the grass species *Lolium perenne*, with the aim of creating a repeatable drought regime. This thesis will then explore the effect of this drought regime on the behaviour and fecundity of the aphid species, *Brevicoryne brassicae*, *Rhopalosiphum padi*, *Sitobion avenae* and two clones of *Myzus persicae*. In chapter five this thesis will also investigate whether the aphid species *M. persicae* alters gene expression in response to varying levels of drought stress.

It is hoped that this work will determine whether the response of aphids to drought stress is predictable across the feeding guild, as well as highlight aphid mechanisms of osmoregulation. Therefore, the following sections in this chapter will introduce a background to aphid evolution, biology, diet, osmoregulation and their relationships with plants and conspecifics in attempt to understand the complexity of the aphid-plant relationship, as well as providing an introduction on the current work on aphid responses to drought.

1.1 The Aphid

Over 400 million years ago the three phyla of vascular plants diverged from early land plants (Campbell and Reece, 2002), these early vascular plants revolutionised the colonisation of plants to terrestrial habitats. Their vascular systems enabled the transport of solutes as well as control of cell water content vital for the turgor pressure that drives cell expansion and maintains structure. The two types of vascular tissue in vascular plants are xylem and phloem tissue. The xylem carries minerals and water from the roots to the shoots in a unidirectional manner, whilst phloem tissue transports nutrients such as sugars and amino acids bi-directionally. The emergence of this vascular tissue opened up a niche for specialist vascular feeding insects, and 220 million years ago in the Carboniferous period the first aphids, phloem feeders, are thought to have appeared (Evans, 1956). Currently, the oldest known aphid fossil is from the Triassic period, is a species called *Triassoaphis cubitus* (Evans, 1956). These early ancestors of aphids were relatively unspecialised lacking a proboscis and wing venation structures until the appearance of angiosperms 160 million years ago, which provided the evolutionary drive for aphid speciation.

Parthenogenesis, the ability to reproduce asexually without fertilisation of eggs has been estimated to have evolved in aphids around 200 million years ago (Heie, 1967). Other special adaptations such as viviparae, the reproduction of live offspring, as well as wing venation and proboscis structures are believed to have appeared around 146 million years ago. Structures such as the cauda and siphunculi appeared later in the Cretaceous around 65 million years ago (Shaposhnikov, 1977).

Today there are 10 different families of aphid; *Adelgidae, Anoeciidae, Aphididae, Drepanosiphidae, Greenideidae, Hormaphididae, Mindaridae, Pemphigidae, Phloeomyzidae, Phylloxeridae, Thelaxidae* and *Lachnidae* consisting of around 4,000 described species (Dixon, 1998), of which approximately 250 species are classified as pests (Blackman and Eastop, 2000). In the UK there are more than 500 species and although the distribution of aphids is global, the majority of species are found in temperate environments.

1.1.1 Importance of Aphids as Agricultural Pests

Aphids are among the most important agricultural pests in temperate agriculture systems, attacking all types of plants, woody trees, shrubs, herbs and grasses (Hill, 1997). The damage they cause can be direct via extraction of phloem sap which can cause a reduction in photoassimilates and crop yield, or indirect in the transmission of plant viruses (Dedryver *et al*, 2010). In a study by Nault *et al*, (1997), it was found that a staggering 50% of insect borne plant viruses were transmitted by aphids. Additionally, aphids can cause damage to crops via their sucrose rich excreta, termed honeydew, which can attract other pests species (Gratwick, 1992) as well as being ideal for the growth of sooty moulds that cover host-plant foliage and hinder the photosynthetic capacity of the plant (Jones and Jones, 1974).

The success of the aphid can be attributed to its lifecycle. The aphid's lifecycle can vary between species and they have adapted unique ways to coping with seasonal and climatic variation. Some species are monophagous, and feed only on one species of plant, whereas others are polyphagous. Aphids are also capable of two different types of reproduction: oviposition (eggs) or viviparae (live young). Depending on the cues the aphid receives the young can be born gamogenetically (sexual reproduction) or agamogenetically (asexual reproduction), and become apterous (wingless) or alate (winged) aphids as adults. Aphids also display the phenomena known as the 'telescoping of generations', whereby the female aphid will have a daughter within her body that despite not being born yet, is already parthenogenetically producing her own daughter (fig 1.1). This 'telescoping of generations' not only allows aphids to pass on important environmental information through generations, but also increases their intrinsic rate of population increase (*rm*). For example, in the summer a single viviparous reproducing adult female of the pea aphid species, *Acyrthosiphon pisum*, can reproduce 50+ progeny in less than a month, which in turn can complete maturation and commence reproducing in as little as ten days (Baumann *et al*, 1997).

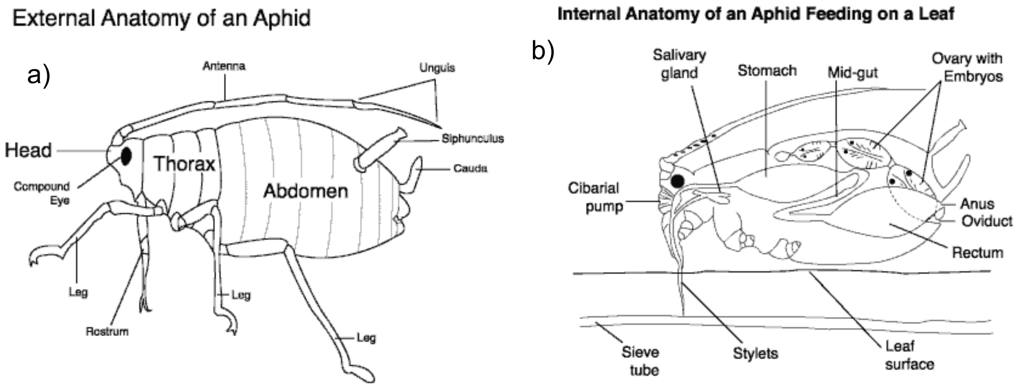


Figure 1.1 Image taken from The University of Arizona, Centre for Insect Science Education Outreach (http://insected.arizona.edu/gg/resource/internal.html). The image shows the basic external a) and internal b) anatomy of an aphid. Diagram b) shows the ovaries with developing embryos, demonstrating viviparity and the adaptive physiology that allow aphids to increase their *rm*.

Aphids are largely phloem feeders and the nature of the diet of aphids requires specialist equipment to feed. The stylet bundle (fig 1.2) consists of a pair of outer mandibular stylets and inner maxillary stylets. The maxillary stylets meet at the hypopharynx and form two canals, an anterior food canal and posterior salivary canal. The stylets are typically driven into the plant and moved around the mesophyll layer in the plant in an attempt to locate the phloem sieve element.

The size of the stylet bundle can vary in width and length in different species. In *Myzus persciae* the average diameter of the stylet bundle is 3µm, the food canal being a diameter of 0.5µm near the head and 0.35µm near the stylet tips, whereas in *Tuberolachus saligans* the diameter of the food canal is 3.6µm near the aphids head, and 1.2µm near the tips.

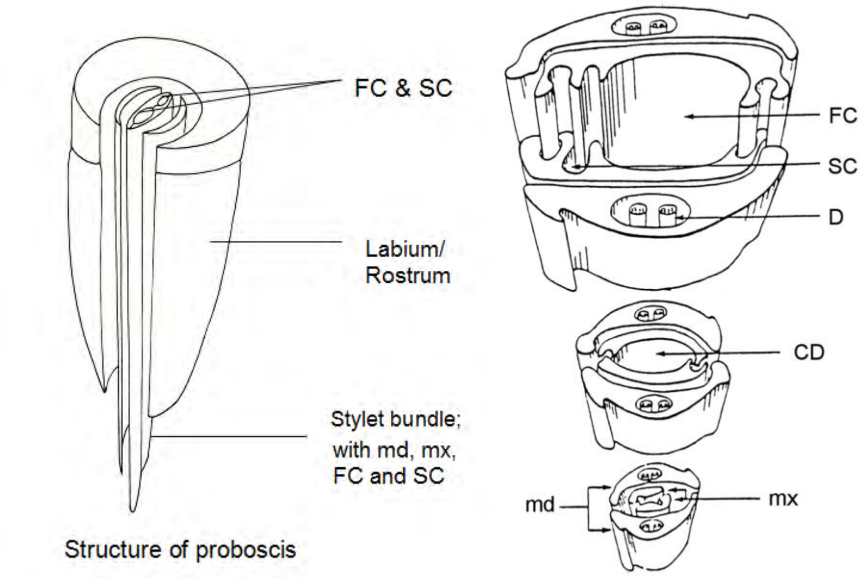


Figure 1.2 Schematic representation of the stylets bundle anatomy of **M. persicae** a) image taken from Biology Teaching Resources, Insect Life-Cycles Educational Material by © D.G. Mackean (http://www.biology-resources.com/insects-01.html) b) Image reproduced from Taylor and Robertson (1974). Image a) and b) show the mandibular stylets (md) located on the outside of the bundle, protecting the maxillary stylets (mx). The inner architecture of maxillary stylets is a large food canal (FC) and a narrow salivary canal (SC). Image b) also shows along several hundred micrometers of the stylet's length the narrowing of the inner architecture to form a fusion between the FC and SC into single common duct (CD)

The overall length of the stylet in *M. persicae* is 300µm, whereas in *Adelges piceae* the length can be up to 1.5 mm (Auclair, 1963). The variation in stylet width and length between aphid species is one of the features that make aphids specially adapted to host plants. Small stylets would restrict penetration depth within plant tissue, whilst long, thick stylets would require a large labium (lower 'lips' that surround the probiscus) to hold them during periods when the aphid is not feeding. In addition, the size of the stylet bundle can vary between instars and in some cases morphs (Balch, 1952; Amman, 1970). Young nymphs of *Aphis gossypii* do not have stylet bundles long enough to reach the phloem from the upper surface of cotton leafs (Pollard, 1958), and so are found in larger concentrations in the abaxial regions of leaves. These compromises demonstrate how aphid size and physiologically are evolutionarily intertwined with the physiology of their host plants.

In selecting plant hosts aphids are thought to have an array of different mechanisms that are initiated on plant contact. Due to their specificity of feeding it is vital for survival that aphids are able to recognise suitable host plants, to ensure successful reproduction and colonisation.

1.1.3 Host Detection

Alate (winged) aphids (during flight) use visual cues to locate plants, with most species displaying a preference to land on yellow surfaces (Prokopy and Owens, 1983; Roberts, 1987), whilst the polyphagous species *R. padi* and *A. fabae*, have been found to be more responsive to green wavelengths (Hardies, 1989; Nottingham *et al*, 1991). In addition to visual cues, aphids can also use chemical cues in the form of olfaction detection. Aphids can detect plant volatiles such as nitriles, green leaf volatiles, henzaldehydes, isothiocyanates and monoterpenes (Visser and Piron, 1997). The exact role of plant volatiles in host selection has been subject to debate in the literature and although it is not well understood in alate morphs, walking apterae have been shown to respond to host-plant odours and even avoid non-host odours when in olfactometers (Pettersson, 1970; 1973; Petterson *et al*, 1994;

Nottingham *et al*, 1991b; Hori, 1999). Thus aphids can utilise both visual and chemical cues when locating a suitable host plant.

It has been suggested that aphids also use mechanical information about a plant, such as leaf waxiness, hairiness, glandular trichrome absence or presence, and epidermal thickness before deciding whether to attempt probing. At the distal end of an aphid’s probiscus there is a ring of hairs, which could be a sensory mechanism to determine plant texture and to find a suitable place for stylet insertion. In addition, an organ located over the epipharynx has been suggested to play a role in host acceptance. The organ consists of specialised hypodermal cells with epipharynx nerve endings, and this is thought to be a ‘tasting organ’ that enables aphids to taste plant sap as it enters the pharynx (Auclair, 1963). The ability to taste plant sap can provide vital information to the aphid such as host palatability and host condition.

1.1.4 Sieve Element Location and Feeding

Once aphids insert their stylets into plant tissue they perform a series of cell punctures. It is thought that during these cell punctures aphids sample cell sap to acquire information about their host plant (Martin *et al*, 1997). Although the exact mechanisms in sieve element location are not well understood, it has been suggested that gustatory monitoring of ingested sap samples allows aphids to follow potential apoplastic gradients in pH and sugar leading to the phloem.

Upon sieve element location aphids always start with a brief cell puncture; it is thought that this event is crucial in the aphid deciding whether or not to commit to phloem feeding. Phloem sap is typically made up of water, carbohydrates often as sucrose, amino acids, inorganic ions, proteins, hormones, mRNA, lipids and secondary compounds (Van Bel, 2003). Once aphids decide to commit to phloem feeding they commence salivating into the sieve element for a short duration (known as E1 waveforms) and then move onto the second phase of phloem activity, sap ingestion (known as E2 waveforms, these waveforms and the feeding behaviours of aphids will be discussed in more detail in chapter three).

During ingestion the hydrostatic pressure of the phloem forces sap up into maxillary food canal of the aphid. In addition to the hydrostatic driving force of sap, aphids also have the cibarial pump which is located between the head capsule and anterior wall of the pharynx. The cibarial pump is a mass of muscles, which act on the pharynx enlarging the volume of the pharynx. This action causes a pump action drawing sap from the buccal cavity into the pharynx. It is believed that this 'sucking action' is how aphids assimilate additional sap, than what is driven hydrostatically.

Phloem feeding aphids have also been shown to pierce the xylem of the plant and acquire water and ions. Xylem feeding is rare and the majority of studies have shown xylem feeding displayed in aphids experiencing dehydration (Spiller *et al*, 1990; Ramirez *et al*, 2000; Daniels *et al*, 2009), under these conditions to acquire xylem sap in watered stressed plants aphids would require the cibarial pumping action.

The discovery of the importance of plant turgor pressure in the feeding of aphids was enabled by the development of the technique called stylectomy (Kennedy and Mittler, 1953). This technique involved the cutting of feeding aphid stylets *in situ*, and measuring the rate of sap exudation from the remaining severed stylet. Sap exudation from severed stylets can continue sometimes for several days in the absence of the aphid. The importance of hydrostatic pressure in the feeding of aphids is undisputed. However, techniques developed to measure sap ingestion have shown that aphids can assimilate more sap than the amount exuded out of the plant. By measuring the frequency and volume of honeydew droplets, calculations of honeydew excretion can be obtained. It has been found in some studies that the amount of honeydew excreted by aphids has exceeded the amount exuded from severed stylets (Mittler, 1957). When we consider the loss of water and nutrients from ingested sap for processes involving growth, development, respiration and reproduction, it can be concluded that sap exudation rates are lower than the actual rate of intake of sap by the aphid, especially on plants with lower hydrostatic pressures such as herbaceous species. In support of this, work involving the feeding of aphids on artificial diets where there is very little hydrostatic pressure has shown that aphids have a physiological adaptation to ingest sap in the absence of these forces. This adaptation takes the form of the cibarial pump. Interestingly a study into the plant resistance in two lucerne (*Medicago sativa*) genotypes suggested that resistance was linked to sap viscosity. Genotypes where aphid performance was reduced did not vary in sugars, amino acids or GABA concentrations but in exudation rates when compared to susceptible genotypes. Resistant genotypes had exudation rates that were 2.8 and 2.3 times lower than susceptible genotypes (Girousse and Bournoville, 1994). This suggests that there is a cost in aphids overcoming the difficulty in phloem acquisition. This may be a result of resource allocation to mechanisms such as a cibarial pump.

1.2 Aphid Interactions: Plant defence and disarmament

To understand the complexity of the aphid-plant relationship, this section will explore the ways in which plants have adapted defensive strategies against herbivory and then will specifically consider aphid examples.

1.2.1 Plant Strategies

Natural selection by herbivores on plants has resulted in a broad array of plant defences, ranging from constitutively expressed to induced defences. Plant defences can be structural, such as cell wall modifications, or chemical. The induction of plant defences can be a universal response to herbivory damage via the initiation of a signalling pathway, or species (even ecotype specific) with R gene induction.

Plants have also adapted different defensive strategies, with some species adapting to escape herbivores in space and time by growing in an inaccessible location, or adapting growing seasons to avoid herbivores. For plant hosts unable to avoid their herbivore in space and time, tolerance is another defensive strategy that can be adopted; this sometimes involves diverting resources to damaged areas to assist in recovery after herbivory. Strategies of avoidance and tolerance are strategies that act on the plant as opposed to the herbivore; however, antibiosis and antixenosis are defence strategies that affect herbivores in the interaction.

If a plant has antibiosis resistance it means that it affects the biology of the herbivore, and therefore herbivore abundance by reducing longevity, fecundity or increasing mortality. Antixenosis resistance is where the plant affects the behaviour of the herbivore, usually this manifests as a deterrent towards colonisation on the host plant, and thus the herbivore typically demonstrates a preference for non-resistant plants. Antixenosis or antibiosis strategies may take the form of chemical defence molecules like secondary metabolites, which are produced by plants to influence the behaviour, growth, or survival of a herbivore. These defences can be repellents or toxins and can affect the herbivore directly or reduce the palatability of the plant.

1.2.2 Defence against aphids: Antibiosis and antixenosis

Antibiosis and antixenosis are widespread defence mechanisms utilised against aphid herbivores. Some of these are constitutively expressed whereas others may be induced. Inducible antibiosis defence was demonstrated in *Arabidopsis thaliana* in response to feeding by the aphid *M. persicae*. Plants converted one indole glucosinolate, a secondary metabolite (indol-3-ylmethylglucosinolate), into another (4-methoxyindol-3-ylmethylglucosinolate) to provide a more effective toxic defence. This induced defence was localised and not systemic (Kim and Jander, 2006). In soybean resistant genotypes, soybean aphids, *Aphis glycines matsumur,* showed a reduction in feeding and longer maturation periods. The resistance mechanism is thought to be due to the production of potentially toxic compounds yet to be fully investigated in the resistant genotypes (Li *et al*, 2004). Other plant secondary metabolites involved in defence include saponins, which act as feeding deterrants to pea aphid, *Acyrthosiphon pisum*, reducing the aphid’s ability to ingest phloem or xylem sap (Golawska, 2007). Saponins in *A. pisum* have also been found to reduce growth and reproductive rates (Sylwia *et al,* 2006). In the plant alfalfa, saponin defence is constitutive, with some varieties containing higher concentrations than their more susceptible counterparts.

Plants can adopt a constitutive defence by simply absorbing chemicals from the environment. Selenium (Se) is an essential trace element; however, in high concentrations the element is toxic to biological functions. The aphid *M. persicae* showed no preference in choice tests towards the Indian mustard plant, *Brassica juncea*, when grown in a medium containing selenium. Aphid population growth was also inversely correlated with Se leaf concentration. This study is intriguing as it opens up the possible function of plant defence in hyperaccumulation varieties.

Although studies so far show how the accumulation of toxic chemicals within leaves can affect aphids, other studies into aphid-plant systems have shown resistance can also be localised to the phloem. In lupin, resistance against the cowpea aphid, *Aphis craccivora*, was in the form of a feeding deterrence, with aphids exhibiting more time in pathway activities (i.e. in mesophyll tissue layers) on narrow leafed lupin (*Lupinus angustifolius*). This deterrence mechanism was thought to be from fluctuating phloem alkaloid concentrations (Zehnder *et al*, 2001) however, further investigation is required to prove this.

Phloem based resistance is not limited to Lupins, but is demonstrated to be a strategy employed across the plant kingdom. As aphids are phloem feeders, they cause little mechanical tissue damage when inserting their stylets, and pose detection problems for the host plants. Detection of aphid presence by the host plant therefore, may only be once the aphid has reached the phloem. Phloem based defence would mean that the defence could be specialised to the phloem feeding guild and induced only on attack by the aphid. This strategy could save resource allocation costs as the plant is only initiating a defence upon aphid infestation, but it would not be effective against potential virus transmission by the aphid.

A study on the resistant breeding line of melon, *Cucumis melo*, AR5, concluded resistance against the cotton-melon aphid, *Aphis gossypii*, was located within the phloem sieve elements. Resistance resulted in a slower population growth and acted throughout aphid development. The behaviour of aphids on the resistant line showed longer salivation periods, reduced phloem ingestion and consequently secretion (Klinger *et al*, 1998). Increased salivation by aphids is usually associated with reduced palatability of the phloem sap, or changes in its composition. It is possible that these results are the consequence of an induced phloem defence response on resistant lines of melon.

1.2.3 Defence against aphids: Recruitment

The last type of defence plants have in their chemical warfare is recruitment. Plants can recruit the colonisation of herbivore natural enemies by releasing chemical attractant signals. Plants from the *Brassicaceae* family are renowned for their accumulations of secondary metatoblites, especially glucosinolates, following aphid herbivory. Glucosinolates are toxic to aphid herbivores and can be hydrolysed into isothiocyanates compounds, which are involved in indirect defence through recruitment of parasitoids of herbivores. The braconid wasp, *Diaeretiella rapae*, is an endoparasitoid that attacks aphids on *Brassica* hosts including species of aphids that are *Brassica* specialists. Infested turnip plants have been shown to release higher levels of isothiocyanates than uninfested plants, promoting recruitment of *D. rapae*

(Blande, 2004). Furthermore, *D. rapae* individuals appear to have an innate evolved recognition of certain plant volatiles, and additionally *D. rapae* can also be conditioned by (plant emitted) chemical cues whilst developing and emerging out of the aphid mummy. It is using combinations of these chemical signals that plants are able to recruit aphid natural enemies (Pope *et al*, 2008).

1.2.4 Plant Signalling Pathways and Aphids

An adaptation of plants to deal with the multitudes of abiotic and biotic stresses is to utilise similar messaging pathways to activate responses to stimuli. Hormones such as jasmonic acid (JA), salicylic acid (SA), ethylene, absisic acid (ABA), gibberellic acid (GA), nitric oxide (NO) and auxin (IAA), can activate and induce responses to generalist/specialist herbivory, or even to drought and temperature stress. This universal response in plants provides a mechanism whereby an attack below ground would influence responses aboveground, and *vice versa*. Using these systemic signalling pathways plants can mount a multiple layered defence throughout the plant, involving production of secondary metabolites, modification of leaf or phloem composition, cell wall modification, metabolism changes, recruitment of natural enemies and signalling to neighbouring plants. Systemic defence also enables defence enhancement, resulting from prior attack by other organisms against future herbivores (Mauch-Mani and Métraux, 1998).

Microarrays of *A. thaliana* have shown how systemic defence can enhance defence over a period of time. After a 2h infestation period by *M. persciae*, it was shown two genes had altered expression. After an infestation period of 36h, *A. thaliana* had significantly altered expression of twenty two genes. Twenty of the genes were up regulated whilst two were down regulated. These genes were involved in cell wall modification, carbon metabolism, signalling, oxidative stress and defence (Couldridge *et al*, 2007). In species of *Triticum, Sorghum* and *Nicotiana*, genes associated with the disruption of cell wall tissues are shown to be expressed during aphid feeding (Smith & Boyko, 2007). Whilst in celery, *Apium graveolens*, feeding by *M. persicae* resulted in an increase in RNA transcripts for 126 genes in the phloem. These genes belonged to cell wall modification, vitamin biosynthesis, carbon assimilation, carbon mobilisation, nitrogen modification, water transport and photosynthesis. The response was found to vary from that driven by a pathogen infection, suggesting a degree of specificity and a role for the phloem in signalling and recruiting metabolic pathways at the site of aphid feeding (Divol *et al*, 2005).

In some plant species the release of signalling molecules involved in signal pathways has been favoured for defence against aphids. The lettuce aphid, *Nasonovia ribisnigri*, when sensing JA will avoid the host, this is in part due to the attractive nature JA has on aphid antagonists such as parasitoids. The plant stress signal, methyl salicylate (MeSA), has also been suggested as a deterrent in aphid host selection (Chamberlain *et al*, 2001). Interestingly, neighbouring plants took up JA released from infested plants. These non-infested plants then became more attractive to parasitoids. The release of plant volatiles as a defence against aphids has been researched in other species and seems to be another defence mechanism evolved against these specialist feeders (Verheggen *et al*, 2008).

Systemic signalling of herbivorous attack in plants is largely mediated by the JA pathway and its derivatives. JA signalling is highly conserved and shown to be involved in not only defence against herbivores but also against microbial pathogens and stress responses to abiotic stressors such as UV radiation and ozone (Howe and Jander, 2008). JA defences are widespread throughout the plant kingdom, which may reflect that induced defences have lower costs associated with them than constitutive defence mechanisms. Although mechanical damage is often viewed as the trigger for JA-herbivore related defences, mechanical damage is not always sufficient to trigger JA production and induced responses. As aphids cause little mechanical tissue damage when feeding, detection and activation of JA related defences are therefore limited.

Some studies have shown that aphids are able to avoid activation of the JA pathways. In a study on the greenbug aphid, *Schizaphis graminum*, it was found that feeding caused exclusive activation of gene transcipts on the plant host sorghum, *Sorghum bicolor*, that were independent of SA or JA pathways. Aphid feeding was found to activate SA signalling and a weak induction of methyl jasmonic acid (MeJA) regulated defences. It was hypothesised that aphids avoid activation of the effective plant machinery characteristic of a JA activation defence, by inducement of SA and MeJA pathways that act antagonistically with JA-mediated responses (Zhu-Salzman *et al*, 2004). This antagonistic approach is also shown in other phloem insects such as the silver whitefly, which adopts repression of JA defences associated with basal defence (Zarate *et al*, 2007). It is also suggested that the absence of JA mediated defences is due to the way in which plant hosts perceive aphids, i.e. comparable to that of fungal hyphae growth or viruses, causing elicitation of different defence responses than chewing herbivores or mechanical damage (Fidantsef *et al*, 1999).

1.2.5 Gene-to-gene recognition systems: R gene induced aphid resistance

Increasing evidence is gathering over aphid resistance in host plants being attributable to gene-to-gene recognition systems, like those in pathogen defence. In these systems plants acquire resistance by specific resistance genes (R genes), that recognise elicitors from aphid-related compounds and initiate defence responses. Plant identification of aphid salivary components is thought to be the main mechanism of recognition to identify attack (Howe and Jander, 2008). Studies into this area have shown that after exposure to aphid feeding plants alter gene expression levels, in particular in aphid species specific genes. In some plant species aphid R genes have been identified.

In the aphid-plant system, *A. thaliana* and *M. persicae*, genetic profiling showed after infestation for 72 and 96h that plant responses to aphid feeding included genes involved in oxidative stress, calcium dependent signalling, pathogenesis related responses (PR), and signalling (Moran *et al*, 2002). Using *Arabidopsis* mutants, defence against aphid feeding induced premature chlorosis and cell death, caused by an increased expression of *SENESCENCE ASSOCIATED GENES* (*SAG*s). Hypersenescence in *A. thaliana*, was also correlated with enhanced resistance against *M. persicae* feeding. Further investigation revealed that the gene *PAD4* modulates activation of senescence in aphid infested leaves, and it is this R gene mechanism that contributes to basal resistance not its association with SA signalling (Venkatramana *et al*, 2005).

Identification of other R genes in other host- aphid systems include the tomato gene *Mi-1.2* against the potato aphid *Macrosiphum euphorbiae* (Rossi *et al*, 1998), the *Sd1* dominant gene conferring resistance to biotypes of the rosy leaf curling aphid *Dysaphis devecta* (Roche *et al*, 1997), and wheat *Pto* and *Pti1*-like kinase genes against the Russian wheat aphid, *Diuraphis noxia* (Boyko *et al*, 2006). Finally in melon the *Vat* gene not only confers resistance against the cotton aphid, *A. gossypii*, but also against plant viruses transmitted by the species (Garzo *et al*, 2001). Hopefully further work in these systems will soon elucidate aphid specific elicitors, and potential aphid avirulence genes mimicking gene-to-gene systems in plant-pathogen interactions.

1.2.6 Plant Defence: multi-strategies, pathway crosstalk and timing

In certain circumstances conflicting elicitors from different species may reduce defence. In *Brassica nigra*, the glucosinolate sinigrin is positively correlated to damage caused by specialist aphids, whilst being negatively correlated to generalist aphid damage. This suggests that specialist and generalist herbivores exert opposing selection pressures on chemical defences (Lankau, 2007). It was also found in *B. nigra* that selection for chemical defence was favoured when under the influence of herbivores experiencing interspecific competition and not intraspecific competition (Lankau & Strauss, 2008). Selection for chemical defences it seems is dependent upon the prevalence or rarity of certain species.

In some plants resistance involves both an antixenosis and antibiosis response, with parts being constitutively expressed and induced. In the resistant variety kondoi of *Medicago truncatula*, migratory aphids showed a significant deterrence in their settling behaviour, showing a preference to settle on susceptible plants. When aphids were given no choice of host, antibiosis resistance in *M. truncatula* was found to be enhanced by prior infestation, located in the sieve element and in need of an intact plant. Resistance was also shown to be inherited by a single dominant gene, *AKR* (*Acrythosiphon kondoi* resistance). This work highlights a combined defence system, with initial defence being antixenosis with aphid deterrence, followed upon colonisation to antibiosis if the host has the appropriate R gene *AKR* (Klinger *at al*, 2005). Defences multifaceted like these are most likely the standard across the plant kingdom.

Although multifaceted defence strategies seem an effective but costly defence strategy by plants, effective timing and organisation of these defences is paramount. Natural selection has resulted in a step wise defence initiation. Studies into the timing of defence initiation in *A. thaliana* infested with *Brevicoryne brassicae*, showed early defence mechanisms involved reactive oxygen species (chemically reactive molecules containing oxygen involved in signalling) and calcium signalling. Regulation of initial defences was controlled by the SA and JA pathways. Later defence included callose deposits at feeding sites and the production of secondary metabolites, which accumulated after 48h infestation (Kus'Nierczyk *et al*, 2008).

Similarities in plant defence mechanisms against aphids can be found across different major plant taxa; however, due to the specificity and diversity of aphids there are also examples of unique aphid-plant interactions. When three ecotypes of *A. thaliana* are exposed to specialist aphid feeding by *B. brassicae*, and generalist feeding by *M. persicae*, each ecotype showed different defence glucosinolate profiles. Furthermore, infestation by each aphid species resulted in differential regulation of 60 genes in some of the ecotypes; these genes encoded proteins involved in JA and tryptophan synthesis pathways as well as PR proteins. Aphid fitness of the specialist *B. brassicae*, also varied on each ecotype, but not for the generalist feeder *M. persicae*. This research reveals insect specific induction of the indole glucosinolate synthesis pathway in different ecotypes of *Arabidopsis* (Kus'nierczyk *et al*, 2007).

Confirmation of aphid-plant defences being interaction specific was also obtained in studies using the bird-cherry oat aphid, *Rhopalosiphum padi*. Microarray analysis of resistant and susceptible lines of barley, *Hordeum vulgare*, gave raise to potential resistance gene candidates that reduce aphid growth. Gene induction between the susceptible and resistance lines had substantial variation in induced responses even in closely related genotypes. These variations were related to defence, primary metabolism and signalling. Two of the plant lines tested also revealed differences in constitutive defence gene expression (Delp *et al*, 2008). Constitutive levels of peroxidase and polyphenol oxidase activity, proteinase inhibitors and soluble phenolics also differed among five varieties of Lupin (*Lupinus*) when exposed to feeding by *M. persicae* (Cardoza *et al*, 2005).

1.2.7 Aphids Overcoming Plant Defences

Aphids have a complex array of mechanisms to overcome plant defences, some co-evolved against a specialist host defence. The relationship between host and herbivore is an evolutionary arms race. Aphids have detection methods to identify potential hosts, which they are adapted to feeding on as choosing a different host for most aphid species could risk survival.

Detection of a host involves a wide array of host features. Host surface texture and 'taste' may be external mechanisms of identification, whilst internal cell elicitors and secondary metabolites contribute to internal recognition. Once the host is recognised the aphid begins to assimilate the plant resources by tapping into the sieve element of the plant with stylets. Once in the phloem the aphids must overcome the phloem defences of the plant. The phloem wound response involves the release of coagulating proteins in the phloem sieve element of the plant. Coagulating proteins may also pose a problem directly to aphids by blocking the aphid's food canal (Tjallingii, 2006). To address this aphids have two main forms of saliva, gelling saliva to produce the salivary sheaths that protect the stylets and keep sieve elements open following puncture by stylets (Walling, 2008), and watery saliva which is injected into sieve elements prior to sap ingestion (Prado and Tjallingii, 1994). To overcome coagulating proteins blocking the aphid's food canal the ejection of watery saliva plays a pivotal role. Behavioural analysis using the electrical penetration graph has shown aphids perform E2 salivation, which is a combination of the behaviours E1 salivation (involving watery saliva) and E2 sap ingestion. This behaviour of continuous ingestion and salivation is thought to be a mechanism to prevent blockage of the aphid's food canal (Tjallingii, 2006).

In other studies into aphid feeding it has been noticed that before sap ingestion aphids always salivate into the phloem sap and that this salivation rate increases when aphids experience changes in phloem sap composition. In response to water stressed barley seedlings the aphid *R. padi* was shown to increase sieve element salivation (Ponder *et al*, 2000), and this behaviour has also been exhibited where aphids are exposed to reduced amino acid concentrations (Hunt *et al*, 2009). This behaviour is believed to be the aphid using salivary compounds to condition phloem sap and the sieve element so it is more favourable.

In response to injury the phloem has a natural occlusion mechanism, which is calcium triggered. This defence mechanism involves forisomes that undergo calcium regulated conformational changes, thus blocking the phloem. It has been shown that aphid saliva *in vitro* reverts forisomes in the phloem into a non-plugging contracted state. Initiation of sieve tube occlusion causes aphid behaviour to change from ingestion to watery salivation. It has further been shown that *M. persicae* aphid saliva has calcium binding proteins that competitively bind calcium in the sieve element, preventing plugging by calcium bound forisomes (Will *et al*, 2007). This is considered a crucial adaptation in providing aphids with continuous phloem sap. Evolutionary success of the aphid thus depends on the calcium binding abilities of aphid saliva proteins, when compared with plant forisomes.

Aphid saliva is made up of many proteins. A proteomic study into the salivary proteins of *M. persicae* showed the presence of glucose oxidase, glucose dehydrogenase, NADH dehydrogenase, α-glucosidase, α–amylase and many unknown proteins (Harmel *et al*, 2008). Further studies into the functions of salivary proteins are uncovering the extent of importance salivary proteins bear on aphid growth and survival. Using novel RNA interference (RNAi) methods, knockouts of the abundant saliva encoded transcript C002, function unknown, caused premature mortality and a change in feeding behaviour in the pea aphid, *A pisum* (Mutti *et al*

2006; Mutti *et al*, 2008). These studies may lead to the uncovering of aphid saliva proteins that are important for overcoming plant defences.

Earlier it was mentioned that plants have an indirect defence in the form of recruitment of natural enemies of aphids, using signals in the form of plant volatiles. In response to attack by recruited natural enemies, aphids can release an alarm pheromone that causes conspecifics in the population to cease feeding and disperse; this is also supplemented by avoidance and aggressive behaviour (Verheggen *et al*, 2008).

Finally, the release of secondary compounds such as glucosinolates by hosts has been cleverly utilised by aphids to compliment their own defence against predators. The aphid *B. brassicae* has been shown to accumulate the secondary metabolite glucosinolate, sinigrin as a defense against *Coccinellid* species. For example, *Adalia bipunctata*, is unable to survive first instar stages when fed on aphids that have been reared on *B. nigra*, a sinigrin producer (Pratt *et al*, 2008 & Kanzana *et al*, 2007).

1.2.8 Summary

In summary, although plant hosts elicit defences specific to aphid species, and even aphid ecotypes, activation of general stress related defences still has an important role. Hosts have evolved to display a wide range of defence strategies; tolerance, avoidance, antixenosis and antibiosis. In many systems defence is multifaceted encompassing a multilayer of defences that are timed, systematic and enhanced during periods of continued attack.

Despite these defences aphids are still major agricultural pests, causing devastating economic losses from virus transmission and plant damage. Observed aphid population growth year on year can only lead to the conclusion that aphids are highly adaptable insects with their own developed defence system. The adaptability of aphids to overcome plant defences may be of benefit in overcoming environmental stresses such as drought. However, the aphid-plant interaction is very complex and any predictions with regards to performance to drought would be extremely difficult.

1.3 Aphid Interactions: Aphid responses to conspecifics

Certain herbivores also have the ability to overcome defences of plants, making hosts more susceptible to attack from other organisms. Some aphids will colonise a plant more easily if it has already been colonised by conspecifics. The aphid *Sipha flava* induces red spots on infested leaves of the host plant *Sorghum halepense*. This attack was shown to induce plant susceptibility that benefited additional *S. flava* colonisers with enhanced feeding and growth (Gonz'ales *et al*, 2002). The aphid *M. euphorbiae* also appears to benefit pre-infestation as it demonstrated to prefer preinfested potato plants, *Solanum tuberosum* (Ameline *et al*, 2007). This suggests that the preinfestation by conspecifics may aid the aphids in overcoming the plant's natural defences, hence the preference of aphids for pre-infested plants. The discovery of differentiation between aphid probing behaviour on plants previously damaged abiotically or by heterospecifics, than preinfested plants by conspecifics, has lead to the increasing support for the idea that in some aphid species saliva has a pivotal role in combating host defences, and even altering phloem composition. This hypothesis would underpin the selective advantage for the majority of aphid species to fulfil a monophagous lifestyle.

The presence of other herbivores can also have the opposite effect, resulting in a decrease in population fitness. Variation was observed in the ability of two clones of pea aphid, *Acyrthosiphon pisum*, to outcompete the vetch aphid, *Megoura viciae*. One clone was able to outcompete *M. viciae* whilst the other was overcome. The differences were contributed to the differential population growth rates between the clones, and potential intra and inter-specific behavioural responses to predators (Hazel *et al*, 2006). In a comparative study it was found that specificity occurs in plant response to preinfestation by two aphid species. The cereal aphid *Rhopalosiphum padi*, which usually shows no behavioural response to prior colonisation (Prado and Tjallingii, 1997; Messina *et al*, 2002), resulted in reduced plant quality making it less nutritious to future conspecific colonisation. Whereas *Diuraphis noxia* enhanced plant quality for conspecifics, and *Aphis fabae* resulted in conspecifics displaying reduced salivation and continuous sap ingestion.

The change in aphid feeding behaviour suggests that prior colonisation by some species of aphid, can result in saliva induced changes in sieve element composition. The ability of aphids to induce changes in sieve element composition to benefit conspecifics suggests the possibility that aphids could also manipulative sieve elements in water stressed plants to negate any detrimental drought induced effects. The effect of drought, therefore, may not be detrimental to aphid performance. However, these studies into the effects of prior colonisation have also yielded the observation that not all aphid species respond homogenously. This diversity of responses may also be observed in the aphid's response to drought.

1.4 Aphid Osmoregulation: Diet? A struggle even for aphids

Aphids are highly specialised insects adapted to their nutritionally poor and osmotically challenging diet of phloem sap. Phloem sap has a high C: N ratio with a low concentration of essential amino acids, vital for aphid growth, reproduction and development. Aphids are thought to combat this by exploiting a highly evolved symbiotic relationship with bacteria (Sasaki & Ishikawa, 1995).

1.4.1 Phloem sap assimilation

The bacteria *Buchnera* ia a vertically transmitted obligate symbiotes that lives within aphids in specialised cells called bacteriocytes. It has been found that they provide aphids with essential amino acids, which are unavailable to aphids from their natural diet. *Buchnera* are therefore responsible for the capacity of aphids to utilise phloem sap. In *A. pisum*, *Buchnera* are responsible for producing 90% of the essential amino acids required for aphid nutrition and physiology (Douglas, 2006). The effect of amino acids on aphid performance is therefore, largely determined by the relationship between the aphid and its bacterial symbiont, *Buchnera* (Douglas, 2003). Due to this being a highly specialised relationship, the *Buchnera* are ineffective on non-host plants adding complexity to the plant-aphid interaction (Wilkinson *et al*, 2001; Douglas, 2003), and provides a possible insight into why some species of aphid remain monophagous.

Phloem sap contains large quantities of sugar in the form of sucrose. Although sucrose concentration in sap changes with seasons, time of day, plant tissue and species, sucrose is still dominant in sap composition. Its large quantities make it an ideal candidate for respiration and aphids have been shown to preferentially oxidise sucrose than amino acids for respiration (Rhodes *et al*, 1996).

Phloem sap also has a sucrose attributed high osmotic potential (-0.6 to -3.0MPa) which can vary between and within plants (Downing, 1978; Wilkinson *et al*, 1997; Fisher, 2000). The osmotic potential of phloem sap from a host plant can be three fold that of the aphid haemolymph, moreover, the aphid haemolymph and excreted honeydew are iso-osmotic (Wilkinson *et al*, 1997). The aphid's ability to maintain water potentials less than the food they ingest can be attributed to various osmoregulation mechanisms.

1.4.2 Osmoregulation Mechanisms

To deal with the osmotic challenge of ingesting phloem sap aphids have demonstrated an array of potentially important osmoregulation mechanisms. Increasing respiration rates of sucrose was suggested as a possible mechanism, but shown not to be, as altering sucrose concentrations in artificial diets did not result in altered respiration rates (Rhodes *et al*, 1996). However, acquisition of water from the xylem of host plants and producing metabolic water from processes such as flight has been suggested as other potential mechanisms of osmoregulation. The production of metabolic water was first suggested after aphids showed that post a six hour flight the body water content in the aphid had increased by 7%, whilst the dry weight had decreased by 15%, indicating the elevated respiration rates had resulted in a release of metabolic water (Cobain, 1961). However, a proteomics study of *M. euphorbiae* fed on droughted plants showed an overall decrease in the abundance of energy metabolism enzymes, and this was suggested as a function to conserve energy and prioritise survival (Nguyen *et al*, 2007).

The mechanism of metabolic water as an osmoregulatory mechanism is not likely when we consider apterous parthenogenetic morphs. More likely is that apterous aphids may instead place a dependence on other osmoregulatory mechanisms, such as sugar polymerisation or xylem water acquisition. Studies have shown that aphids starved for 24h spent 26% of their time xylem feeding when placed back onto a host plant, whereas those starved for only 1h displayed no xylem feeding (Spiller *et al*, 1990). Another study showed that starved *Sitobion fragariae* increased xylem feeding on wheat and oats (Ramirez *et al*, 2000). These observations are consistent with the process of removing aphids from plants resulting in water loss, these studies show that dehydrated aphids increase xylem feeding, and thus the act of water acquisition from the xylem is the aphid's means of rehydration.

Starvation is not the only scenario in which an aphid may find itself dehydrated, alate black bean aphids, *A. fabae*, were shown to frequently ingest from the xylem of broad bean plants, *Vicia faba*. The study showed that aphids are predisposed to xylem feeding as a result of fasting during the teneral period (Powell and Hardie, 2002). It was also suggested that alate aphids may reduce their weight before take off, giving aerodynamic benefits, but resulting in dehydration which makes xylem feeding a priority following plant contact. These results may also explain the observed increase in body water content of aphids following flights, as they break down fat stores to release energy and produce metabolic water to cope with physiological challenges of flight (Cobain, 1961).

If xylem feeding is important for osmoregulation, then if feeding becames unfavourable aphids will be denied an essential rehydration source and perform worse. TMX, a xylem borne insecticide, was used at sub lethal doses to make the xylem unpalatable to aphids starved for 5h. The results showed that there was a reduction in the proportion of time spent xylem feeding, from 25% in control plants to 0.6% in TMX treated plants. The aphids on TMX plants also showed a reduced growth, performance and water content (Daniels *et al*, 2009), indicating that xylem feeding is an important part of an aphids diet.

Alternatively, work into the importance of sugar metabolism has provided a strong case for sugar polymerisation being one of the major methods of aphid osmoregulation. Modification of phloem sap to reduce the osmotic pressure is postulated via sucrase mediated hydrolysis of phloem sucrose into glucose and fructose; the glucose can then be polymerised by transglucosidase into oligosaccharides of low osmotic pressure per hexose unit. In support of this studies into honeydew analysis showed that under high dietary sucrose concentrations, aphids excreted higher concentrations of oligosaccharides (Wilkinson *et al*, 1997), whilst *A. pisum* fed on artificial diets with 14-C labelled sucrose showed showed that glucose polymerisation resulted in a 34% reduction in sugar molarity of the honeydew (Rhodes *et al*, 1997).

Research into the area of aphid osmoregulation is unearthing enzymes involved in this sugar hydrolysis and transglucosidase activity. In *A.pisum,* sucrase activity has been localised to the gut distal to the stomach (Ashford *et al*, 2000; Cristofoletti *et al*, 2003). One identified sucrase APS1 was found in aphid guts and shown to have α-glucosidase activity to form the hexose units for oligosaccharide synthesis (Price, et al. 2007). Application of α-glucosidase inhibitor acarbose to inhibit sucrase activity in aphids gut resulted in aphids being unable to osmotically control haemolymph water potential when exposed to a hyperosmotic diet. It was concluded that the aphid gut sucrase activity is essential for osmoregulation of aphids when ingesting diet hyperosmotic to their haemolymph (Karley *et al,* 2005).

Other suggested mechanisms of osmoregulation is water cycling from the distal to proximal regions of the gut. This water flux would require membrane associated aquaporins to mediate water movement. A putative aquaporin, ApAQP1, was identified in *A. pisum;* when expressed in *Xenopus oocytes* ApAQP1 increased osmotic water permeability was demonstrated. ApAQP1 was also localised in aphids to the stomach and distal intestine, and an RNAi-mediated knockout for AqAQP1 expression resulted in an increase in haemolymph osmotic pressure (Shakesby *et al*, 2009). These advances in the literature aid to uncover aphid osmoregulation mechanisms, and provide evidence supporting the important role for aquaporins, sucrases and polymerising enzymes in osmoregulation.

Despite phloem sap being costly to aphids, with resource allocation to the array of osmoregulation mechanisms, the presence of sucrose gradients from sieve elements may play a role in host acceptance in some aphid species. Aphid species *M. persicae* and *Aulacorthum solani* performed equally or superior when reared on potato plants with a two fold reduction in sucrose, due to treatment with antisense for a sucrose-

H+ transporter (*StSUT1*), than when fed on wild types. This indicates the osmoregulation cost to aphid performance from high sucrose concentrations. Interestingly, the opposite was observed in the aphid *M. euphorbiae,* which struggled to locate sieve elements and withdrew stylets prematurely from sieve elements reduced in sucrose concentration. These results demonstrate the importance of sucrose concentration gradients, and sieve element composition, in sieve element location and acceptance in some species of aphids (Pescod *et al*, 2007).

1.5 Aphid Interactions: Response to Drought Stress

Studies into the effects of watering regimes on the cowpea aphid, *Aphis craccivora*, showed significant negative linear correlations between water stress on host plant and aphid population, shoot biomass and seed yield (Agele *et al*, 2006). We can conclude from this study that drought has a detrimental effect upon both plant and herbivore. Although other studies have indicated that we can expect this not to be true of the whole feeding guild. The performance of *R. padi* and *S. avenae* aphid clones sourced from England and Spain on drought stressed wheat, demonstrated no initial difference between clone performance on drought stressed plants. No effect of water stress was observed in aphid development time, nymphal mortality and embryo number in first generation teneral adults at the onset of reproduction. However, in subsequent reproduction, there was a significant reduction, indicating sustained water stress was needed to cause detrimental affects on aphid performance. There was also an observed clonal difference in *R. padi* clones, with clones from Spain performing better than English clones. This was not observed in *S.*

avenae clones, although English *S. avenae* clones did produce significantly more alates on water stressed plants (Pons and Tatchell, 1995).

Ultimately under drought stress, hosts plant water potentials become more negative and aphids would place an increased dependence on osmoregulation mechanisms to cope with the extreme osmotic challenge posed by concentrated phloem sap. Water stress has been associated with net increases in sieve element solutes (Smith and Milburn, 1980) to maintain positive phloem turgor pressure. Although solutes other than sucrose, i.e. ions, can play important roles in phloem osmotic pressure, such as potassium ions found to have important role in transport of photo-assimilates (Gould *et al*, 2004), it is thought that phloem sap carbohydrate concentrations are mainly responsible for adjustments in phloem osmotic pressure. Phloem carbohydrates in water stressed Eucalyptus trees, *Eucalyptus globulus*, can increase in concentration by over 50% from 550mM to 850mM (Cernusak *et al*, 1980). Similarly, in more recent literature using tomato plants, *Solanum lycopersicum* cv. Marmara, carbohydrate concentrations were shown to increase by 20-40% (Najla *et al*, 2010). Although these values are dependant on the level of water stress, location in the plant, plant developmental stage and growth conditions, it is indicative that carbohydrates play a dominant role in the adjustment of phloem osmotic pressure under water stress.

Alterations in organic ions composition, such as amino acids, in sieve element sap of water stressed plants have been have been suggested as potentially beneficial to aphids. Although the symbiont *Buchnera* provide aphids with a mechanism to overcome low essential amino acids content, increasing available amino acids in drought stressed plants may increase aphid performance and may even promote colonisation of non-hosts. In droughted *B. napus* there is an observed increase in essential amino acids (Good and Zaplachinski, 1994). Drought was also shown to increase the quality of phloem sap in four grass species with an observed increase in osmotic pressure and essential amino acids (Hale *et al*, 2003). The aphid *R. padi* on drought stressed hosts reduced sap ingestion rates and where amino acid concentrations were higher, the performance of the aphid correlated with the accessibility of essential amino acids, although this correlation seems species dependant.

In the *Arabidopsis* mutant AAP6 where amino acid levels were reduced in leaves, aphids hardly showed any differences in feeding. These results suggest that amino acid levels may not have been reduced sufficiently below threshold limits to have an effect. This also highlights the importance of endosymbionts in altering the quality of phloem sap to benefit the aphid (Hunt *et al*, 2009).

A lot of current research has shown aphid responses to droughted hosts under laboratory conditions. Aphid population performance in fields may then vary considerably, with aphids simply moving to more drought tolerant or physiologically favourable hosts to avoid water stress. A field study into the effects of drought on populations of pea aphids, *A. pisum*, on pea pants, reported that after three weeks of moderate drought, significantly fewer aphids were found on droughted plants to controls. There were also an observed higher proportion of alate aphids on the droughted plants, than controls, by week four. Post week four, the study did not observe any proportional difference in the number of alatoid nymphs, suggesting that the process of plant senescence rather than drought stress was important in determining nymph physiology (McVean and Dixon, 2001). The primary observation that populations were significantly smaller on drought stressed to well watered hosts illustrates the sensitivity of aphids to water stress in the natural environment.

However, the differences observed in all these discussed studies may reflect more the level and duration of water stress imposed. Alternatively they may demonstrate how drought performance in aphids can vary between species and genotypes, with some species more equipped to adjust to the heterogeneity of plant quality.

1.5.1 Current Debate & Summary

There are currently two main hypothesises that attempt to explain how aphids will respond to drought stress. The first hypothesis is that the osmotic stress from feeding on severely droughted hosts would be detrimental to aphid performance, as aphids have to reallocate their energy resources from growth and reproduction into osmoregulation mechanisms. Alternatively, the plant stress hypothesis (White, 1984) proposes that under water stress host plants may become more susceptible to herbivores, with aphids benefiting from the change in plant physiology, such as increases in amino acid concentrations in host plant species (Good and Zaplachinski, 1994; Hale *et al*, 2003; also see Chapter two results). Plant defences may also become reduced in water stressed plants (Rhoades, 1985) making plants more susceptible to insect attack. So far there have been a number of studies (discussed in more depth in chapter four) that support the plant-stress hypothesis (Wearing, 1967; Wearing and van Emden, 1967; Wearing, 1972; Miles *et al*, 1982; Oswald and Brewer, 1997), and conversely, a number of studies that support the notion that resource allocation to osmoregulation mechanisms, as a result of feeding on drought stressed hosts, would be detrimental to aphid performance (Larsson, 1989; Pons and Tatchell, 1995; Bethke *et al*, 1998; Honěk *et al*, 1998; Kennedy *et al*, 1958; Kennedy and Booth, 1959).

In conclusion, this chapter has highlighted that the aphid-plant interaction is very complex and multifaceted. Differences observed in the discussed studies may reflect the differences in the level and duration of water stress imposed, or they may represent the variation between species and genotypes. Alternatively, these studies indicate that the response of aphids to water stress may be too complex to summarise within one simple hypothesis. It is possible that the variation in results in the literature may be due to the differential plant responses to water-stress, and subsequent differential insect responses to plant stress.

Thus, this study will set out to establish a repeatable drought regime using water withdrawal in two plant species *Brassica nigra* and *Lolium perenne*. Once a drought regime has been defined this study will try to find out if there is variation in aphid behavioural and reproductive responses to drought, and by imposing a similar level of drought for all the species, attempt to establish if the response is predictable across the feeding guild. To establish the latter four aphid species and two clones will be used. Furthermore this study will later go on to try and elucidate whether aphids respond to drought stress by altering their gene expression, particularly in genes related to osmoregulation functions like sucrase and aquaporins. It is hypothesised that aphid responses may vary between species and even clones. It is also hypothesised that aphids under drought stress may place an increased dependence on osmoregulation functions such as xylem feeding and sugar polymerisation.

Chapter 2: Development of a reproducible drought regime and its effects on plant performance

2.0 Abstract

Plants may avoid drought stress by trying to maintain tissue water potentials, or alternatively, plants may simply tolerate the water stress by decreasing their vulnerability to it. In this chapter the physiological changes in *Brassica nigra* and *Lolium perenne* were quantified under a progressive drought regime.

Results showed that *B. nigra* was able to maintain water potential for the first 9 days of water withdrawal, whereas, in *L. perenne* it progressively dropped throughout the drought regime. Interestingly, both species displayed similar water potential and water content levels at the beginning and end of the drought treatment. Measurements of water content demonstrated that water content fell faster in *L. perenne* than *B. nigra*. Tissue sap from both *L. perenne* and *B. nigra* had increased levels of reducing sugars and amino acids over the course of the drought treatment, except by day seven in *B. nigra*, where amino acids concentrations started to fall. Furthermore, cations K^+, Mg^+ and Ca^{2+} were significantly altered in B. nigra, and NH_4^+, Na^+, K^+, Mg^+ and Ca^{2+}, significantly altered in *L. perenne* over the drought treatment.

This chapter suggests that the water stress strategy of both plants is slightly different. *B. nigra* is predicted to use osmotic adjustment and leaf shedding to minimise water loss, whilst, osmotic adjustment is also thought to play a role in *L. perenne*. Furthermore, controlled water withdrawal is concluded as suitable for a repeatable drought regime in both of these plant species.

2.1 Introduction

Drought is a serious problem for agriculture worldwide. Improving drought tolerance in plant cultivars has become an important aim of scientific research. Fortunately, many drought resistance traits are heritable and work has been carried out on breeding drought tolerant varieties. Ultimately how tolerant a plant is to drought could have knock-on effects to the herbivores that feed on it. Changes in cell or phloem composition to compensate against water stress could affect phloem feeding insects like aphids.

This chapter will seek to briefly review plant adaptations to drought stress and their potential effect on phloem feeding insects. The work in this chapter will then develop a repeatable drought regime in *Brassica nigra* and *Lolium perenne*, that can then be used in subsequent chapters to determine the effect of drought on aphids. During the establishment of a repeatable drought regime, physiological data will be collected in an attempt to assess the plants' physiological status during water stress. This physiological data is important in gauging the type of diet the aphids are ingesting, which can later inform chapters involving aphid responses to water stressed host-plants (Chapter three-five). In quantifying the drought regime and its effect on *B. nigra* and *L. perenne*, this chapter is not only just setting up a drought experimental system, but also defining the level of drought and subsequent changes experienced by plant hosts.

2.1.1 Early Adaptations – from the seas to the land

Early ancestors of plants made a transition from aquatic to terrestrial environments over 500 million years ago. In the absence of an aquatic environment, plants were exposed to the problem of transporting and controlling water loss. Adaptations such as a cuticle coated epidermal layers helped prevent excessive water loss from above ground plant tissue (Campbell and Reece, 2002). Special organs called stomata provided a mechanism for gas exchange in and out of the plant tissue to supply photosynthesis, with the flexibility to close and prevent water loss in arid conditions.

Over 400 million years ago the three phyla of vascular plants diverged from early land plants. These were: pteridophytes, gymnosperms and angiosperms (Campbell and Reece, 2002). The vascular systems in these phyla revolutionised the colonisation of plants to terrestrial habitats. The vascular system enabled the transport and division of solutes as well as control of cell water content vital for turgor pressure, which drives cell expansion and maintains structure. The two types of vascular tissue in vascular plants are xylem and phloem tissue. The xylem consists of dead conducting cells that carry minerals and water from the roots to the shoots. The xylem transport system operates in a unidirectional manner unlike the living phloem tissue that can transport nutrients such as sugars and amino acids bi-directionally. The emergence of this vascular tissue also opened up a niche for specialist vascular feeding insects, and 220 million years ago in the Carboniferous period the first aphids are thought to have appeared (Evans, 1956). Aphids feed directly from the phloem tissue with their specialist stylet mouthparts and utilise cues, such as the sugar species and concentration, to locate the phloem and commence feeding (Hewer *et al*, 2010). Changes in phloem composition or gradients within the tissue of the plant could affect the ability of the aphid to locate the phloem and feed successfully from the plant.

2.1.2 Plant strategies to deal with drought stress

Under drought stress land plants demonstrate a variety of physiological changes ranging from the physical, metabolomic and chemical. Although some mechanisms appear conserved throughout the plant kingdom, certain combinations of mechanisms involved in drought avoidance and tolerance are species specific, depending upon the strategy of the plant.

Drought resistance strategies are classically divided into three categories: escape, avoidance and tolerance (Levitt, 1972). These strategies are not mutually exclusive and can occur in a range of combinations (Chaves *et al*, 2003). An example of a drought escape strategy would be desert ephemerals (short lived plants), which are able to escape drought conditions by germinating and completing their life cycle whilst water is available. Drought avoidance is where plants avoid tissue dehydration by maintaining tissue water potential, maximising water uptake and minimising water loss. Stomatal closure, lowering cuticular conductance and leaf shedding are all mechanisms that plants can use to minimise water loss, whilst utilising water storage and investment in roots (Jackson *et al*, 2000) are ways to maximise water uptake. Drought tolerance is defined as decreasing vulnerability to water stress by continuing water transport by osmotic adjustment, increasing the ability of meristem cells to survive at low water potentials and attempting to reduce occurrence of xylem cavitation (Tyree *et al*, 2002). It is the strategy of drought tolerance and drought avoidance that could potentially affect aphids the most. Leaf shedding could prove beneficial to some species of aphids as they preferentially feed on dying plant tissue. However, continuing water transport by lowering water potentials could have a detrimental effect on aphids, which would be ingesting a more osmotically challenging diet of altered phloem sap. Additionally, synthesis of osmoprotectants could prove beneficial to phloem feeders, if those osmoprotectants were essential amino acids required by the aphids for growth.

2.1.3 Physiological Adaptations to Drought stress

Under progressive drought plants can demonstrate a variety of physiological alternations. For example, in response to drought, leaf physiology can change with younger developing leaves maintaining water uptake remaining turgid whilst older leaves wilt (Bray *et al*, 2000). Water stress can also result in a number of physiological phenomena in different plant species. Root alterations have been observed in members of the *Cruciferae* family in response to progressive drought. Under drought conditions short, hairless, tuberized roots emerge, which survive under extreme soil desiccation. Upon rehydration these tuberized roots rapidly develop hairs and elongate (Vartanian *et al*, 1987).

However, the main and arguably most important physiological response to drought stress is osmotic adjustment. The ability of plants to increase or maintain water uptake from the soil, by lowering their internal root water potential, is fundamental and a process that is likely to have the greatest impact on aphids. Osmotic adjustment is usually achieved by the translocation and alteration of internal solute concentrations.

Water stress has been associated with net increases in sieve element solutes (Smith and Milburn, 1980), to maintain positive phloem turgor pressure. Although there are other solutes, i.e. ions, which can play important roles in phloem osmotic pressure, it is thought that phloem sap carbohydrate concentrations are mainly responsible for adjustments in phloem osmotic pressure.

Phloem carbohydrates in water stressed Eucalyptus trees, *Eucalyptus globulus*, can increase by over 50% from 550mM to 850mM (Cernusak *et al*, 2003). Similarly in tomato plants, *Solanum lycopersicum*, carbohydrate concentrations were shown to increase by 20-40% (Najla *et al*, 2010). Although these values are dependant on the level of water stress, location in the plant, plant developmental stage and growth conditions, it is indicative that carbohydrates play a dominant role in the adjustment of phloem osmotic pressure under water stress. Aphids already feed on a diet with a high in C:N ratio and an increase in carbohydrates via osmotic adjustment may prove to be detrimental to growth of the aphid, which will subsequently have to allocate more resources to osmoregulation.

Water stress in plants is also shown to have an effect on altering levels of other organic ions; in droughted *Brassica napus* there has been observed increases in essential amino acids (Good and Zaplachinski, 1994). Drought was also shown to change the quality of phloem sap in four grass species with an observed increase in osmotic pressure and essential amino acids (Hale *et al*, 2003). Meanwhile, potassium ions have been shown to play an important role in phloem osmotic pressure adjustment and transport of photo-assimilates (Gould *et al,* 2004). It is not just amino acids, carbohydrates and ions that are altered in response to water stress, in oilseed rape leaves, *Brassica rapa*, drought induced decreases in galactolipid and phospholipid concentration and increased neutral lipids (Dakhma *et al*, 1995). Unlike increases in carbohydrates, increases in amino acids may be beneficial to aphids, which are limited by the available essential amino acids in phloem sap. The bird cherry-oat aphid, *Rhopalosiphum padi,* on drought stressed hosts with higher amino acid concentrations demonstrated greater performance, showing that aphid performance is correlated with the accessibility of essential amino acids (Hale *et al*, 2003).

Additionally, alterations in certain solutes used in osmotic adjustment can influence the susceptibility of plants to aphid infestation. Some osmotic adjustment solutes are referred to as ‘compatible solutes’ and have a dual role as osmoprotectants, protecting cell membranes from dehydration. Usually these osmoprotection mechanisms are only functional when severe dehydration occurs, indicating that they are critical to survival (Chaves *et al,* 2003). Proline is one such compatible solute, with high proline concentration in cells being associated with prevention of protein denaturation, preservation of enzyme structure and activity, and protection from drought induced damage by reactive oxygen species (Rajendrakumar *et al*, 1994; Samuel *et al*, 2000; Saradhi *et al*, 1995).

Proline is synthesised by two successive reduction reactions catalysed by the enzyme P5C synthetase (P5CS) and P5C reductase (PC5R). In plants, genes encoding P5CS and P5CR have been found to be up-regulated by water stress, whilst the catabolism of proline by the enzyme P5C dehydrogenase (P5CDH) is repressed under water stress (Deuschle *et al*, 2001). Up regulation of genes encoding P5CS and P5CR have been found to be initiated by both the ABA-independent (abscisic acid) and ABA-dependant pathways (Hare *et al*, 1999). Water deficit exposure generally results in the initiation of one or both of these signalling pathways in plants (Kizis *et al* 2001).

Alterations in proline content have been shown to influence the performance of aphids beyond altering the quality of diet. Susceptible cultivars of pea have show increased amounts of all free amino acids except proline, which was reduced, suggesting that aphid infestation is related with the proline content of plant hosts (Auclair *et al*, 1957). CCC-induced resistance to aphids has also been shown to be accompanied by increased proline concentrations (Van Emden, 1964), whilst reduced transmission of cabbage black ringspot virus was associated with free proline concentration alterations (Selman and Kandiah, 1971).

The degree at which osmotic adjustment can allow a plant species to tolerate drought is variable between species and genotypes. Interestingly, a study of the desiccation-tolerant *Licania platypus* showed individuals did not die until leaf water potentials fell to ⁻7.5 MPa and relative water content fell to 0.14 under progressive drought. In addition it was also found that whilst whole stem hydraulic conductance declined by 85% in dead *L. platypus*, whole-root hydraulic conductance declined by only 50% in plants with dead shoots (Tyree et al, 2002), displaying an active preference to maintaining hydraulic conductance in root tissue than shoot tissue. This drought-induced prioritisation in some plants to maintain hydraulic conductance in roots could be hypothesised to be more beneficial to root feeding aphid species than shoot feeding species.

Drought studies in plants have so far used leaf water potential as a reliable parameter for quantifying the plant water stress response. However, in separate observations water potential can differ among genotypes under drought stress (Singh *et al,* 1990) and so it is suggested that leaf relative water content (RWC) would be a better indicator of water status (Sinclair and Ludlow, 1985). To reliably determine the drought response of *B. nigra* and *L. perenne* in this chapter, both leaf water potential and water content assessments in the form of fresh weight to dry ratios will be used.

It is important to quantify the extent at which each plant species adapts to drought, as there is inter- and intra-species plasticity for each drought response. Interestingly, studies into the genotypic plasticity of water stressed silver birch, *Betula pendula,* showed that although there was no clonal differences in leaf water potential, there were marked differences in other physiological parameters such as stomatal conductance, net photosynthesis and leaf carbon isotope composition ($\delta^{13}C$). Reduction of stomatal conductance was found to be the first and most plastic response to drought in all genotypes before osmotic adjustment (Aspelmeier and Leuschner, 2003). In addition, work on three tropical plant species *Eperua falcate*, *Diplotropis purpurea* and *Virola michelii*, found species had a differential stomatal sensitivity to atmospheric drought (Bonal and Guehl, 2001).

What these studies demonstrate is that plant species and genotypes can differ in their plasticity of response and value in certain drought adaptations and responses. Other physiological responses to drought besides osmotic adjustment such as altering stomatal conductance, hydraulic conductance and photosynthesis rates may be more heavily weighted upon in some species, suggesting that in fact any mechanism that influences the process of water cycling in the plant may be enrolled in some form as part of the plant's physiological response to water stress.

This chapter will attempt to quantify the variation of individual responses to drought stress, and the extent to which each drought tolerance mechanism is weighted in *L. perenne* and *B. nigra*. Through biological replication the aim of this chapter is to have a defined repeatable drought regime for use in later chapters. Although it is beyond the scope of this study to examine the detailed molecular responses of these two plants to water stress, by utilising basic physiological protocols to examine total amino acid, cation and reducing sugar concentrations, it is hoped that an insight will be gained into the types of solutes in which these two plants species use in osmotic adjustment. In comparing water potentials and fresh weight : dry weight ratios we hope to be able to monitor the level of water stress both species experience, and gain an understanding of the level of drought resistance each species has. In addition, this information will enable us to gain an insight into the changes of diet that aphids will experience when exposed to drought stressed hosts in later chapters.

2.2 Materials and Methods

2.2.1 Plant Species

The two plant species that were chosen for this investigation were *B. nigra* and *L. perenne*. *B. nigra* is a weedy annual plant cultivated for its seeds commonly referred to as black mustard. The plant is a dicot and can be found growing naturally throughout England. *B. nigra* belongs to the *Brassica* genus, in the family *Cruciferae* (also known as the *Brassicaceae*). One characteristic of the *Cruciferae* family is their ability to produce glucosinolates, these are sulphur containing compounds that play an important role in plant defence. Although toxic to non-brassica feeding insects, the presence of the glucosinolates appears to be no problem for the aphid species *Brevicoryne brassicae* and *Myzus persicae*, which can be found naturally feeding on B. nigra from March or April respectively, until November (Kirk, 1992). It is thought that the specialist feeding mechanism of aphids allows them to avoid the toxic effect of glucosinolates.

B. nigra was chosen as the dicotyledon host plant for the following studies due to its membership in a commercially important crop family, its palatability to both generalist and specialist aphids *M. persicae* and *B. brassicae*, the recent expansion in available species specific genomic resources, and the ease and quantity of which UK wild native seeds could be sourced. Seeds used in experiments were obtained from John Chambers Wildflower Seeds Co. and were from a wild seed stock in Northamptonshire, UK.

L. perenne, is from the family *Poaceae* and is widely cultivated as a pasture and forage plant. It is commonly referred to as perennial ryegrass and has been cultivated in the UK for over 300 years. A monocot plant species, *L. perenne* can be found in old pastures, meadows, road sides and waste land throughout the UK, and typically flowers from May to August. *L. perenne* easily hybridises with other species of *Lolium*, as well as *Festuca*, and can be very variable in spike structure (Hubbard, 1984). Although *L. perenne* can serve as a grass host for the aphid species *Sitobion* *avenae* and *Rhopalosiphum* padi, it is a relatively unsuitable winter host in comparison to other grass species (Leather and Dixon, 1982) or indeed to wheat species. However, *L. perenne* was chosen as the monocotyledon host plant for the following studies as it is often the commonest grass species on farmland and around the UK (Hubbard, 1984). In addition, due to its commonness it is very easy to get a supply of wild UK native seeds. Thus, seeds used in experiments were also obtained from John Chambers Wildflower Seeds Co. and were from a wild seed stock in Northamptonshire, UK.

2.2.2 Plant culturing

Plants were grown in the insect free plant growth rooms with a 16:8 light cycle at a temperature of 22^{o}C. Growth room PAR was between 80-120mol m^{-2} s^{-1} with a relative air humidity 70-80%. Plants were grown in John Innes peat based compost with mixed in Silvaperl in 100-150ml plastic pots (for *L. perenne* and *B. nigra* respectively).

2.2.3 Drought regime

Plants pots were stood in a tray and drought stress was produced by withholding water from the tray for a prolonged period of between 1 to 13 days. Subsequent experiments described below determined plant age and pot size used in the drought regime for each species. Plants were randomised within the growth room. To gather data on the plants physiological status during water withdrawal, a total of two large experiments were conducted involving 100 plants. At each time point individual plants randomly selected were destructively sampled to assess solute concentrations and water status.

2.2.4 Plant growth scoring & determining age of plants for experiments

To decide the age at which plants should experience water withdrawal so that they would survive long enough under water stress in subsequent aphid fecundity experiments (Chapter 4), plant growth data was scored. Plants were measured using a 15cm ruler at regular intervals. Data was recorded on leaf width, leaf length and number of leaves (fig 2.1 & 2.2).

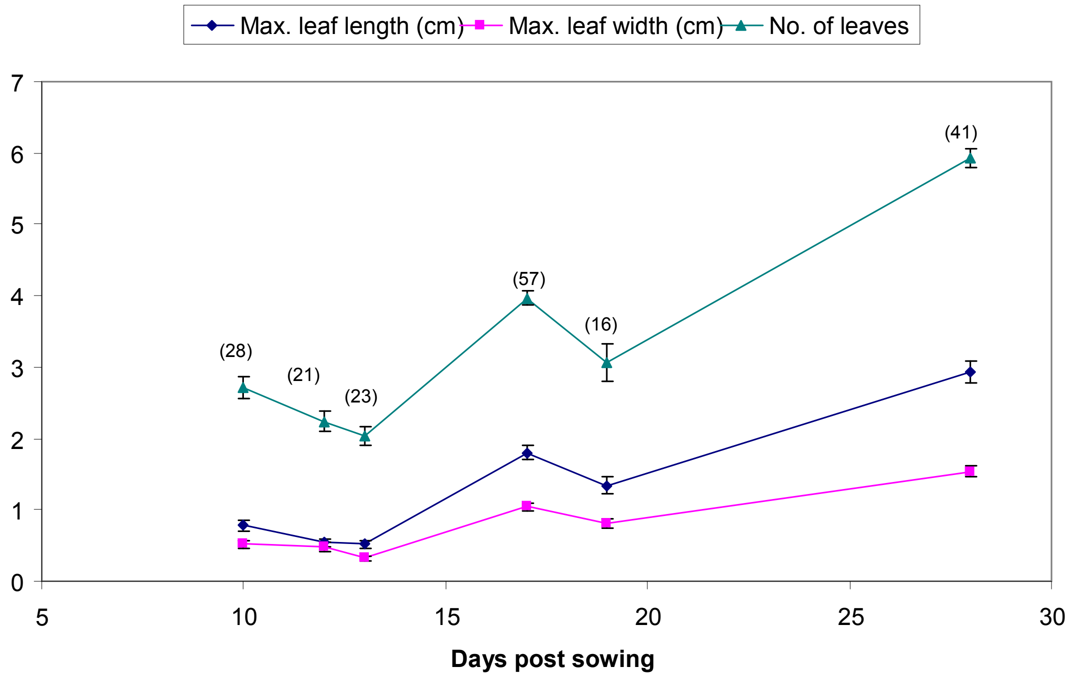


Fig 2.1 - The mean (±SE) growth *B. nigra* over 30 days in growth room conditions (22^{o}C 16:8 LD). Growth was assessed using maximum leaf length and width and total number of leaves, all measurements increased significantly over time (one way ANOVA P<0.001 $_{F69, F46.92, F116.20}$ respectively), N is shown in brackets on graph.

Growth of *B. nigra* quantified as maximum leaf length, maximum leaf width and number of leaves increased significantly with time post sowing (one-way ANOVA P<0.001 $_{F69, F46.92, F116.20}$ respectively) under growth room conditions (22^{o}C 16:8 LD). Growth of *L. perenne*, quantified as maximum leaf length and number of leaves also increased significantly with time post sowing (one-way ANOVA P<0.001 $_{F57.40, F81.82}$

respectively). For the drought regime to have a visible effect on both species the optimum time for drought treatment to commence should be whilst the plant is still growing. If drought treatment was imposed just before or during flowering then observed alterations could be related to the developmental stage of the plant and not the drought treatment. Using simple visual observations it was found that both species did not reach flowering within the first 28-30 days of growth under growth room conditions. The growth data (fig 2.1 & 2.2) shows a continued steady growth during the first 28-30 days post sowing. Therefore it was decided that plants would be grown in well watered growth room conditions for 20 days post sowing, before imposing a drought treatment of water withdrawal. This length of time is sufficient for plants to reach a reasonable size for experimental handling, whilst ensuring plants are still in the growth stage of development.

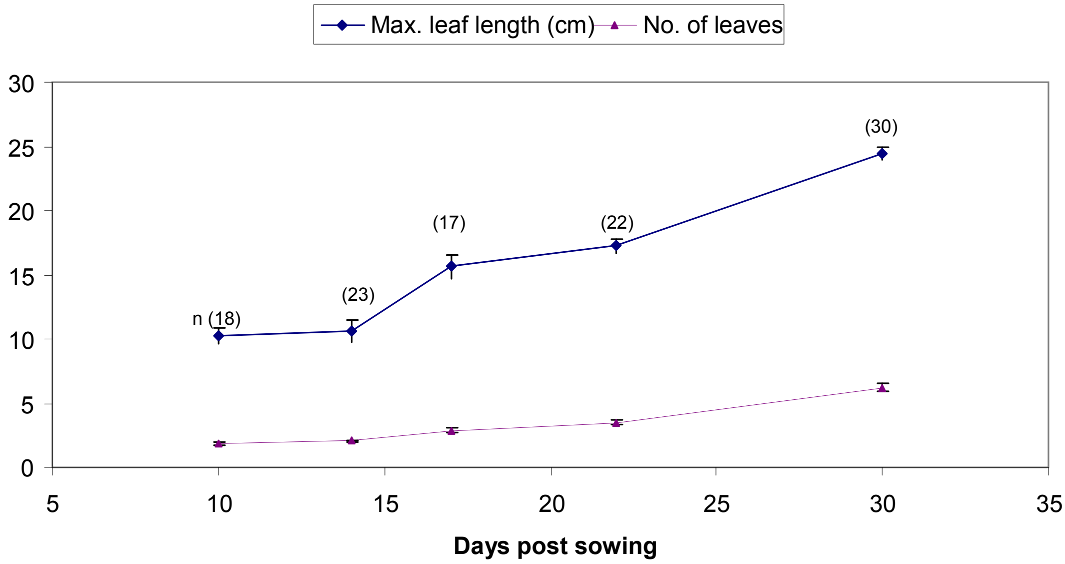


Fig 2.2 – The mean (±SE) growth *L. lolium* over a period of a month in growth room conditions (22^{o}C 16:8 LD). Growth was assessed using maximum leaf length and total number of leaves, both measurements increased significantly over time (one way ANOVA P<0.001 $_{F57.40\ ,\ F81.82}$ respectively), N is shown in brackets on graph.

2.2.5 Chardakov technique and calculating leaf water potential (MPa)

The Chardakov technique was used to verify drought stress via leaf water potential measurements. Test tube stock solutions of sucrose were made up with distilled water to 0, 0.5, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 M concentrations. 0.5ml of each solution was placed in 1.5ml microfuge tubes with added methylene blue dye (approximately 0.001g) using a small paintbrush. Leaf tissue from the mid-top leaves of the plant were cut using a scalpel into 10 small pieces (ea piece ~ $2mm^2$) and placed into microfuge tubes with methylene dyed solutions. Tissue was incubated in solution for approximately 10 minutes. Using a glass pipette a drop of the solution bathing the leaf tissue was withdrawn and released in test tubes. Records were made of whether the droplets sank, rose or remained suspended in the solution. Suspension means the tissue sample is isosmotic with the test tube solutions. If the droplet sank, the tissue had a lower water potential than the solution, and if it rose it had a higher water potential. Water potential was calculated using the standard that 500M sucrose is the equivalent to –1.25MPa and therefore a 250M sucrose solution is the equivalent to –0.625MPa water potential.

2.2.6 Pot Size

Plants were grown in plastic pots, although pot size has previously been found to have no significant effect on the transpiration response to drought (Ray and Sinclair, 1998), *B. nigra* was potted in 4 different pot sizes (‘pot 1’= 100ml, ‘pot 2’= 150ml, ‘pot 3’= 250ml, and ‘pot 4’= 400ml) at 20 days old and exposed to progressive drought until desiccated. At intervals of 1-2 days plants were assessed to determine tissue water potential (fig 2.3) using the Chardakov method described above.

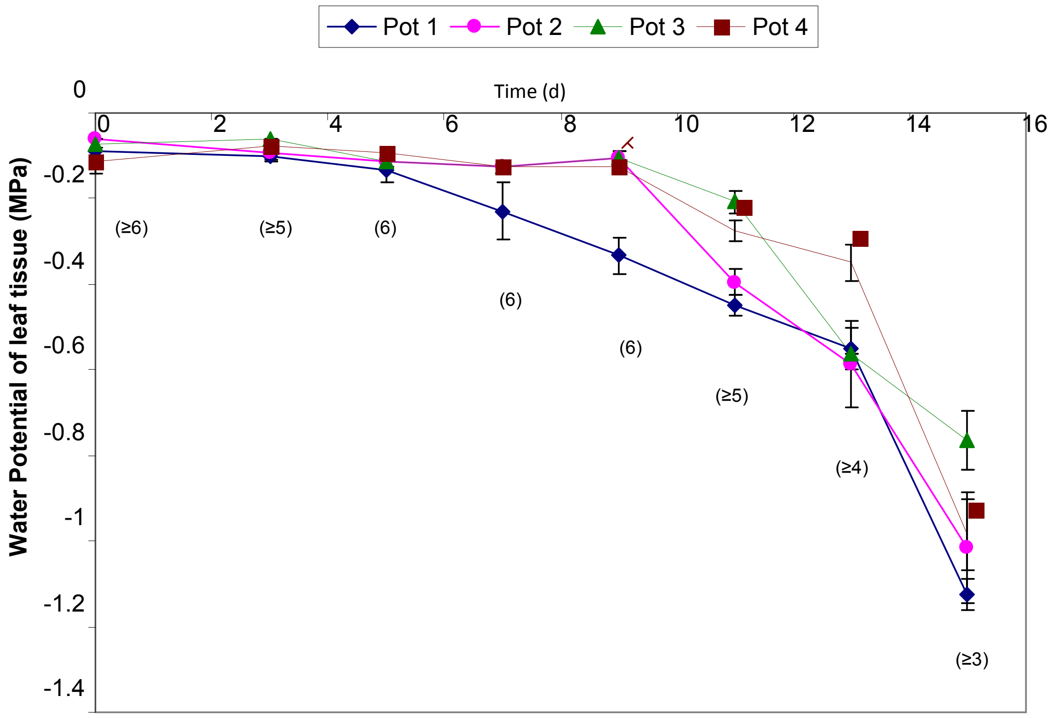


Fig 2.3 – The mean (±SE) leaf water potential (MPa) of 20 day old *B. nigra* potted in four different pot sizes (‘pot 1’= 100ml, ‘pot 2’= 150ml, ‘pot 3’= 250ml, and ‘pot 4’= 400ml) during a progressive drought regime of water withdrawal in growth room conditions (22^{o}C 16:8LD). MPa did not vary significantly between pot sizes (Kruskal Wallis P>0.0.5), N is shown in brackets on graph.

There was a significant effect of time on the water potential. However, pot size was shown to have had no significant effect on the rate of water loss on the plants (Kruskall Wallis P value 0.215). Thus pot size chosen for consecutive experiments with *B. nigra* was ‘pot 2’ (150ml), with ‘pot 1’ (100ml) used for *L. perenne*, these pots were chosen because they were the smallest pot sizes that provided adequate room for plant growth, without limiting space in the growth room for other experiments.

2.2.7 Calculating Fresh weight: Dry weight Ratios

To estimate water loss in plants over the progressive drought regime fresh: dry weight ratios were calculated. Empty 1.5ml microfuge tubes were weighed and then weighed again once plant tissue (a mixture of stem and leaves from all over the plant) was inserted to calculate fresh weight. The microfuge tubes were then placed for 24h in an oven set at approximately 50°C with their lids open. The microfuge tubes were weighed again and dry weight was calculated.

2.2.8 Collection of sap from whole tissue

Sugar, cation and amino acid analysis was analysed from sap extracted from whole tissue. To extract sap, leaves from the top and middle of *B. nigra* and *L. perenne* were placed in a 0.5ml microfuge tubes. Tubes were sealed and placed in a freezer; once tissue was frozen it was macerated with a small plunger. A small hole was placed in the bottom of the microfuge tube, and then it was placed in a 1.5ml microfuge tube. The tissue was then centrifuged at 13,000rpm for 10 minutes. The leaf sap collected in the lower tube and was frozen for later analysis.

2.2.9 Sugar Quantification and the Somogyi-Nelson Method

Reducing sugars (glucose, fructose, glyceraldehyde and galactose) were quantified using the Somogyi-Nelson method (Wager, 1954). Sugar standards were made up using 1mg/ml glucose in distilled water. Glucose standards were transferred in aliquots of 5 to 75 µl into test tubes whereas aliquots of 20 µl of samples were used. To each test tube 1ml of distilled water and 1ml of low alkalinity copper reagent (made up according to the protocol in Wager, 1954) was added to the test tubes. Samples were placed into a boiling water bath for 10 minutes and then transferred to a fume cupboard where 1ml of arsenomolybdate reagent (Wager, 1954) was added. Samples and standards were then transferred to curvettes and read on spectrophotometer set at absorbance A500. Samples were diluted when absorbance was over 1.0 OD. Standards were plotted in Excel and the linear equation (y=mx+b) was used to calculate the concentrations of reducing sugar in each of the samples.

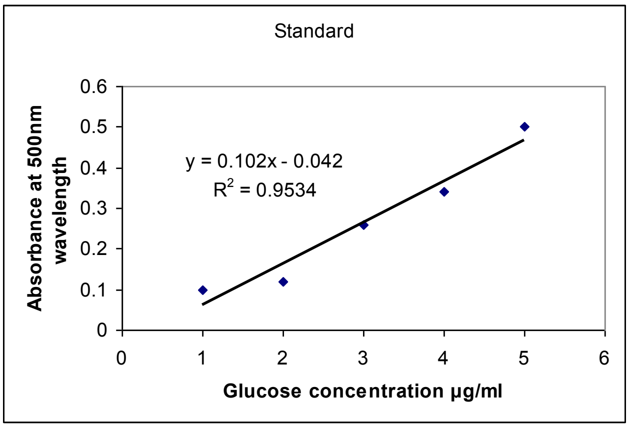


Fig 2.4 – A typical standard curve for sugar analysis. Glucose standards were plotted against absorbance. MS Excel generated linear equations was used to work out sugar concentrations of unknown total sap samples. N=1 per standard, five standard concentrations were used to create curve. Standard curves were repeated each time analysis took place

2.2.10 Using the HPLC for analysing cation concentrations

Total cell sap was collected, via the bulk sap collection method described earlier, from both *L. perenne* and *B. nigra* plants. Extracted total cell sap was then diluted in de-ionised water 1:200. This was usually done by adding 497.5µl of eluent to 2.5µl of sample (eluent was made by adding 0.7ml of 1M H_2SO_4 to 1l of de-ionised water). Standards were diluted 1:40 by adding 12.5µl standard solution to 487.5µl eluent. The standard solution was a 1M solution of each cation in salt form (i.e. 1mM of $MgCl_2$, NaCl, KCl, $CaCl_2$, NH_4Cl). Once diluted 500µl of standards and samples was pipetted into the Dionex polyvial tube (1.5ml) and covered with a Dionex polyvial filtercap (0.5ml). These samples were placed into the autosampling cartridges and placed in the autosampler (Dionex AS40 Automated Sampler). The HPLC (Dionex DX-120 Ion Chromatograph) was switched on and primed to ensure the column had not dried up. The HPLC was set at flow rate 1.0, pressure <2000MPa but >1500MPa and total conductivity <5. The programming schedule detailing the order of samples was programmed into the computer using PeakNet software. The program was run using the schedule, which had every six samples followed by a sample of solely eluent, to ensure no cations got stuck to the column during the sampling process. In addition, after every six samples a standard was re-analysed to cater for any detection changes in the process. The area under each cation peak was interpreted within the context of the standard results.

2.2.11 Amino Acid Analysis

Sample sap was diluted 1:20 with distilled water in a microfuge tube. In a fluorimeter glass curvette (with light path 10mm, 12.5 mm width and length, 45mm height and four polished sides), 100µl of sample was added to 1.5ml of 0.5M Sodium borate buffer (boric acid and sodium tetra-borate, pH 8.5), 1ml distilled water and 0.5ml Fluorescamine solution (25mg Fluram dissolved in 100ml acetone). Fluorescence was read on the fluorimeter (Perkin Elmer LS-5 Luminescence Spectrometer) at 390 excitation and 490 emission. Leucine at 0, 0.2, 0.4, 0.6, 0.8 and 1.0mM concentrations was used to create a standard curve calibration. In the assay 100µl of standard was used instead of sample sap to create the standard calibration. Standards were plotted in Excel (fig 2.5) and a linear equation (y=mx+b) used to calculate the concentrations of amino acids in the samples.

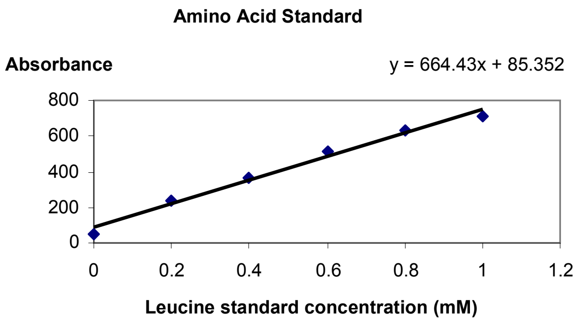


Fig 2.5 – A typical standard curve for amino acid analysis. Leucine standards (0, 0.2, 0.4, 0.6, 0.8 and 1.0) were plotted against absorbance. N=1 per standard, five standard concentrations were used to create curve. Standard curves were repeated each time analysis took place

2.2.12 Statistical Analysis

Data was entered into MINITAB and analysed using the Anderson-Darling Normality Test. If the P value was ≤0.05 then the data was not normally distributed. In cases where data is normally distributed parametric tests utilising the means, such as the t-test (to compare two datasets with one variable) and ANOVA (to compare multiple datasets with one or more variable) were used. Where data was not normally distributed, non-parametric statistical tests that use the median like the Mann-Whitney and Kruskal Wallis test were used respectively.

2.3 Results

2.3.1 Drought induced water potential (MPa) changes

Leaf water potential decreased significantly over the drought period (fig 2.6) in both *B. nigra* and *L. perenne* (Kruskal Wallis P<0.001, P<0.001 respectively). Interestingly, in *B. nigra* the leaf water potential was maintained during the first nine days of water withdrawal, not dropping significantly until after day 9 of the drought treatment, in contrast to *L. perenne*. After 9 days from withholding water, leaf water potential (MPa) had dropped by only 66% (-0.062 ± 0.0 SE n=7 to -0.104 ± 0.01 SE n=4 MPa) in *B. nigra*, but by approximately 740% in *L. perenne* (-0.062 ± 0.0 SE n=8 to -0.527 ± 0.01 SE n=4 MPa). The maintenance of water potential in *B. nigra* suggests a well established osmotic adjustment mechanism or control of water loss. However, by end of the drought treatment both species displayed similar leaf water potentials, with -0.588 (± 0.1 SE, n = 4) MPa in *B. nigra* and -0.625 (± 0.0 SE n= 4) MPa in *L. perenne*, by day 13 and day 12 of drought treatment respectively. Visual observations of both species at ten days water withdrawal confirmed both species were severely wilted, as they both had a substantial amount of 'crispy' dead older leaf tissue with a similar surface area.

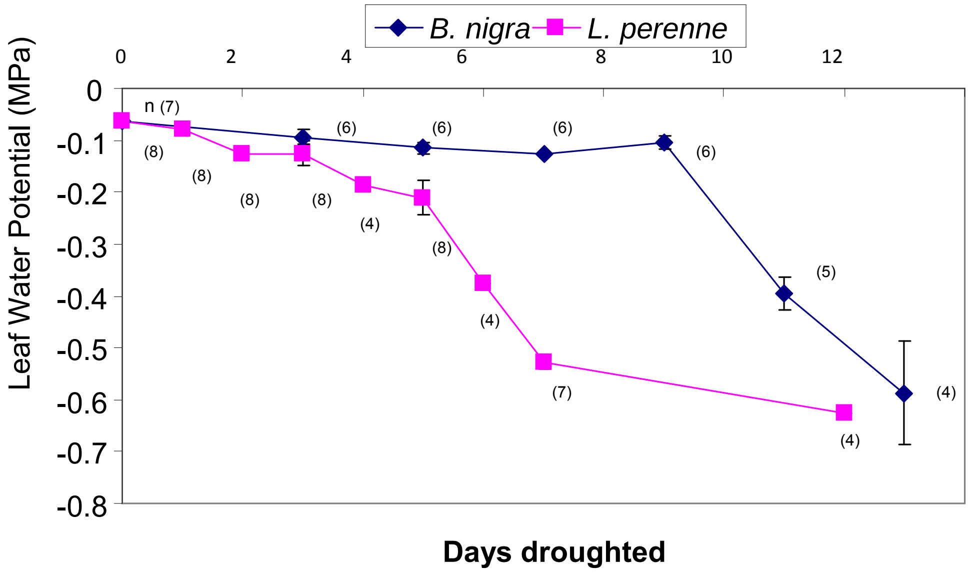


Fig 2.6 – The mean (±SE) leaf water potential (MPa) of 20 day old *B. nigra* and *L. perenne* during a progressive drought regime of water withdrawal in growth room conditions (22°C 16:8LD). MPa dropped significantly (Kruskal Wallis $P<0.001$ and $P<0.001$) in both *B. nigra* and *L. perenne* (respectively), N is shown in brackets on graph.

2.3.2 Fresh: Dry weight ratio (fw:dw) changes under drought

The fresh weight to dry weight ratio (fw/dw) decreased significantly over the course of the drought treatment (fig 2.7) in both plant species (Kruskal Wallis P<0.001, P<0.001 respectively). Despite both plants having a similar fw/dw ratio when under well watered conditions (*B. nigra* 10.1 ± 0.19 SE n=4 and *L. perenne* 10.8 ± 4.16 SE n=8), *L. perenne* fw/dw ratios dropped within the first day of the drought treatment (fw/dw 9.6 ± 0.18 SE n=8), whereas in *B. nigra* it initially rose (fw/dw 12.4± 1.37 SE n=8) and did not drop until day 6 of the drought treatment (fw/dw 9.5 ± 1.44 SE n=9).

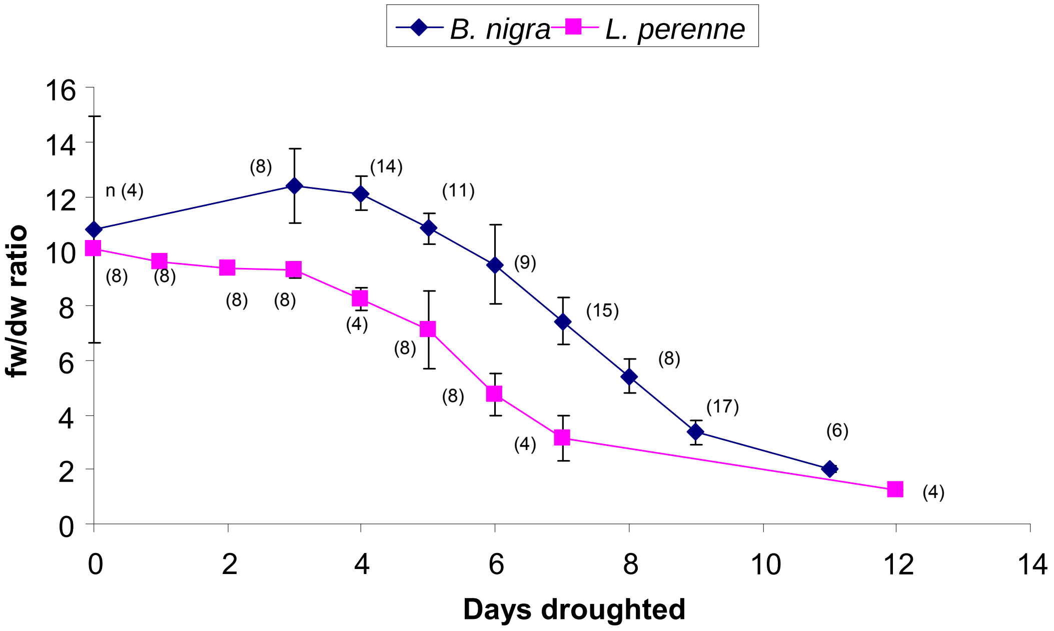


Fig 2.7 – The mean (±SE) fresh: dry weight (fw:dw*)* ratio of 20 day old *B. nigra* and *L. perenne* during a progressive drought regime of water withdrawal in growth room conditions (22°C 16:8LD), fw:dw ratio dropped significantly (Kruskal Wallis P<0.001 and P<0.001) for *B. nigra* and *L. perenne* (respectively), N is shown in brackets on graph

Interestingly, *B. nigra* displays a slightly higher fw:dw ratios than *L. lolium*; with a ratio of 1.23 (± 0.02 SE n=6) in *L. lolium,* and 2.03 (± 0.12 SE n=4) in *B. nigra* by day 12 and 11 of drought treatment respectively. However, these results demonstrate that overall the drought regime has a detrimental effect on the relative water content of both plant species.

2.3.3 Amino acids

B. nigra and *L. perenne* total average leaf sap amino acid concentrations significantly (one way ANOVA P<0.005 $_{F3.65}$, Kruskal Wallis P<0.001 respectively) altered during the droughting regime. *B. nigra* individuals showed average higher concentrations (fig 2.8) when subjected to between 3 and 8 days water withdrawal. The maximum increase was at 5 days water withdrawal ($^{+}$27%) and the minimum increase was at 7 days water withdrawal ($^{+}$7.7%). At nine days water withdrawal *B. nigra* had reduced concentrations ($^{-}$29%) compared to well watered controls. In contrast amino acid concentrations showed an increase throughout the drought regime in *L. perenne* (fig 2.9), with the max increase on day 9 of drought treatment ($^{+}$233%). Overall total cell sap amino acid concentrations were found to be higher in *L. perenne*, with well watered individuals containing 58.95 mM (± 7.7 SE n=12) in comparison to well watered *B. nigra* individuals which had average amino acid concentrations of much lower at 17.6mM (± 0.84 SE n=15). Leaf sap was only analysed post nine days after the commencement of the drought treatment as after this leaves were to dry too extract cell sap adequately.

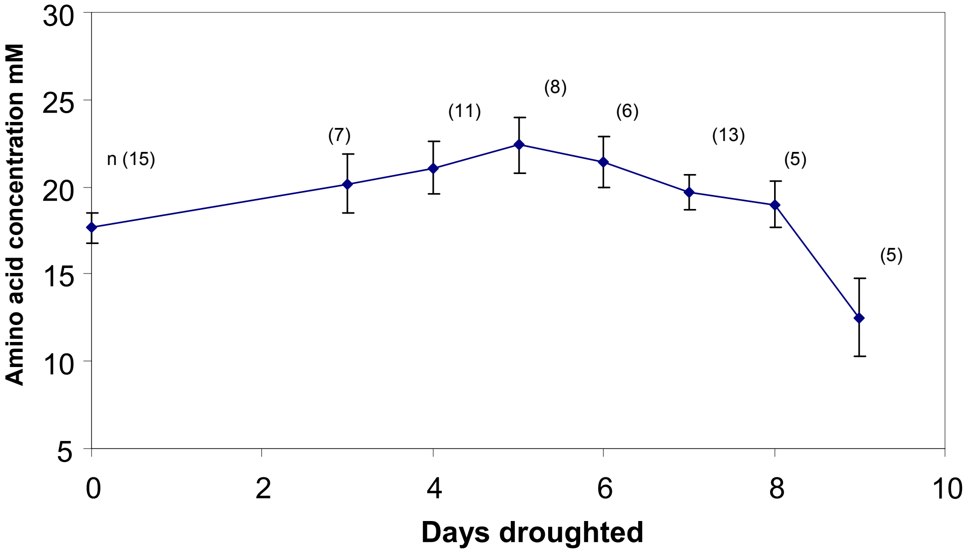


Fig 2.8 – The effect of drought on the mean amino acid concentration (mM) of *B. nigra* cell sap (±SE); drought treatment consisted of water withdrawal at age 20d from plants for up to 9 days in growth room conditions (22^{o}C 16:8LD). Amino acid concentrations increased significantly (one way ANOVA $P< 0.005_{F3.65}$) with drought until day 9 when amino acids dropped below levels in well watered controls, N is shown in brackets on graph.

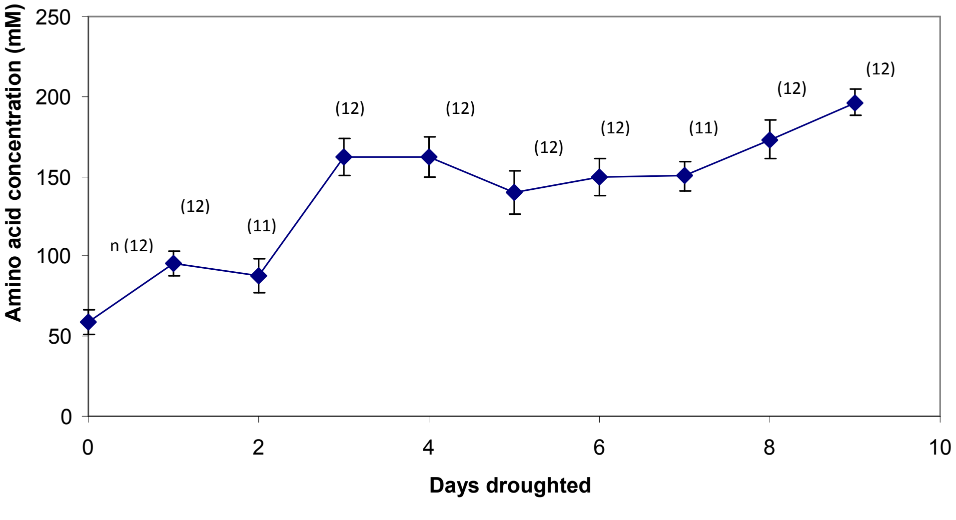


Fig 2.9 – The effect of drought on the mean amino acid concentration (mM) of *L. perenne* cell sap (±SE); drought treatment consisted of water withdrawal at age 20d from plants for up to 9 days in growth room conditions (22^{o}C 16:8LD). Amino acid concentrations increased significantly (Kruskal Wallis P< 0.001), N is shown in brackets on graph.

2.3.4 Sugars

Cell sap absorbance measurements showed that *B. nigra* and *L. perenne* average reducing sugar concentrations (glucose, fructose, glyceraldehyde and galactose etc) significantly (Kruskal Wallis P<0.001, P<0.001 respectively) increased during the droughting regime. *L. perenne* individuals showed average higher concentrations (fig 2.10) of reducing sugars throughout the drought regime particularly when subjected to between 7 and 9 days water withdrawal. The max increase was at 9 days water withdrawal ($^{+}$783%) and the min increase was at 4 days water withdrawal ($^{+}$90%). *B. nigra* (fig 2.10), also showed a max increase on day 9 of drought treatment when compared to well watered ($^{+}$409%). Despite *L. perenne* over the course of the drought regime displaying higher increases in concentrations of reducing sugars in leaf tissue, both well watered *L. perenne* and *B. nigra* leaves display similar concentrations of reducing sugars (1.54µg/ml ± 0.08 SE n=5 and 1.53 µg/ml ± 0.08 SE n=5 respectively).

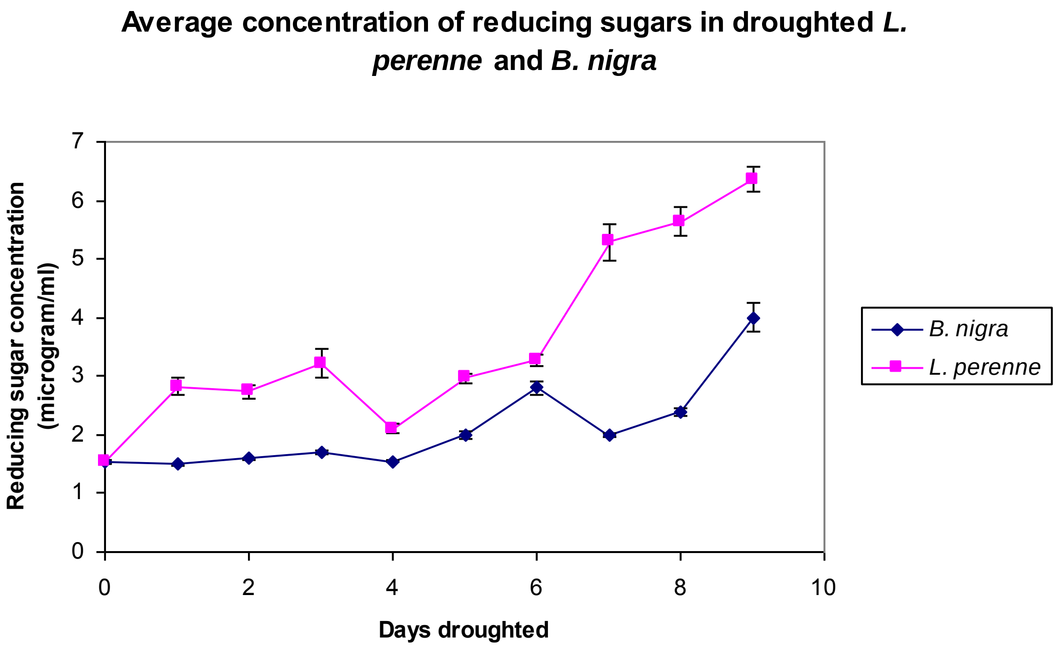


Fig 2.10 – The mean (±SE) absorbance of reducing sugars in leaves of 20 day old *B. nigra* and *L. perenne* during a progressive drought regime of water withdrawal in growth room conditions (22°C 16:8LD), sugar concentrations rose significantly (Kruskal Wallis $P<0.001$ and $P<0.001$) for *B. nigra* and *L. perenne* (respectively), N is 5 for each point on graph.

2.3.5 Cations

The concentration of the cations magnesium (Mg^+), potassium (K^+), calcium (Ca^{2+}), ammonium (NH_4^+) and sodium (Na^+) were quantified in total cell sap of droughted *B. nigra* plants. Only K^+, Mg^+ and Ca^{2+} were significantly increased (fig 2.11 & 2.12) over the droughting period (one way ANOVA $P<0.001$ $_{F6.73}$, $P<0.05$ $_{F2.26}$ and $P<0.05$ $_{F2.60}$ respectively). NH_4^+ and Na^+ levels (fig 2.13) did not vary significantly in *B. nigra* leaves (one way ANOVA $P>0.05$ $_{F1.84}$ and $P>0.05$ $_{F1.30}$ respectively) over the droughting period indicating that only K^+, Mg^+ and Ca^{2+} ions accumulated in droughted leaves. However, the accumulation of K^+, Mg^+ and Ca^{2+} ions did not start until around day 9 of the drought regime.

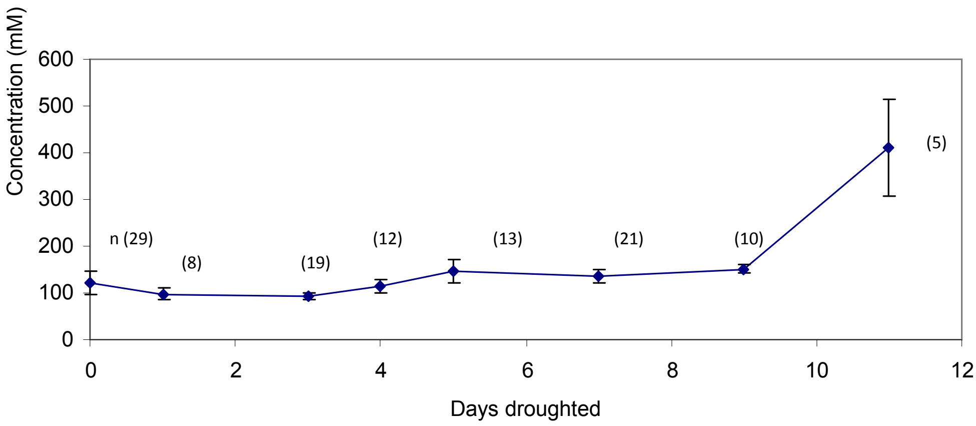


Figure 2.11: Changes in mean K^+ cation concentrations (mM) in cell sap of *B. nigra* subjected to a progressive drought (±SE) under growth room conditions (22°C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 11 days. Ion concentrations increased significantly over the drought regime (one way ANOVA $P<0.001$ $_{F6.73}$), N is shown in brackets on graph.

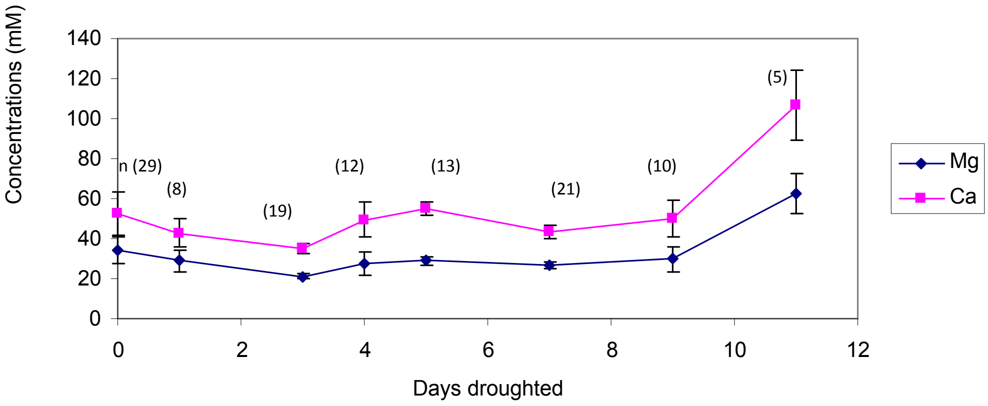


Figure 2.12: Changes in mean Mg^+ and Ca^{2+} cation concentrations (mM) in cell sap of *B. nigra* subjected to a progressive drought (±SE) under growth room conditions (22°C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 11 days. Ion concentrations increased significantly over the drought regime (one way ANOVA $P<0.05$ $_{F2.26}$ and $P<0.05$ $_{F2.60}$ respectively), N is shown in brackets on graph.

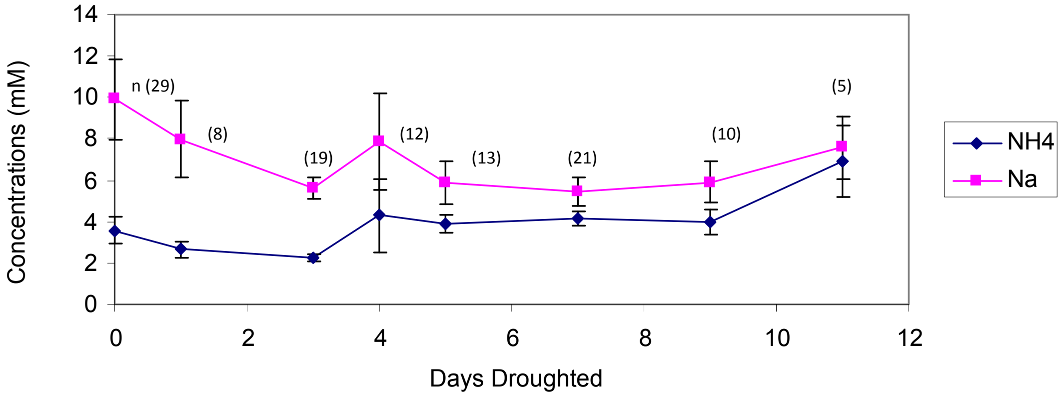


Figure 2.13: Changes in mean NH$_4^+$ and Na$^+$ cation concentrations (mM) in cell sap of *B. nigra* subjected to a progressive drought (±SE) under growth room conditions (22^{o}C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 11 days. Ion concentrations did not change significantly over the drought regime (one way ANOVA P>0.05 $_{F1.84}$ and P>0.05 $_{F1.30}$ respectively), N is shown in brackets on graph.

Whilst in the grass species *L. perenne,* all the cation concentrations (Mg$^+$, K$^+$, Ca^{2+}, NH$_4^+$ and Na$^+$) were significantly altered (one way ANOVA P<0.001, $_{F12.77}$, $_{F11.42}$, $_{F10.76}$, $_{F5.79}$ and $_{F15.86}$ respectively). Levels of K$^+$ cations (fig 2.14) dropped at day four of the drought regime (¯5%) and droughted leaves maintained a lower average K$^+$ concentration then well watered *L. perenne* for the rest of the drought regime.

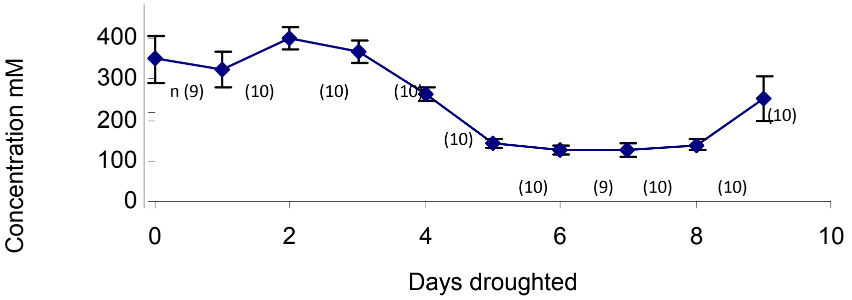


Figure 2.14: Changes in mean K$^+$ cation concentrations (mM) in cell sap of *L. perenne* subjected to a progressive drought (±SE) under growth room conditions (22^{o}C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 9 days. Ion concentrations altered significantly over the drought regime (one way ANOVA P<0.001 $_{F11.42}$), N is shown in brackets on graph.

A similar pattern was seen in levels of NH_4^+ cations (fig 2.15) which maintained a drop in droughted leaves at day six ($^{-}$38%) until day eight ($^{-}$30%) of the drought regime, however by day nine droughted leaves had a higher average NH_4^+ concentration than well watered leaves ($^{+}$30%, 3.8mM ± 0.6 SE n=10). In addition unlike K^+, the NH_4^+ cations initially rose in droughted leaves at the start of the drought regime with a peak average of 5.5mM (± 0.59 SE n=10), an 86% higher concentration than in well watered *L. perenne* leaves (2.9mM ± 0.34 SE n=9).

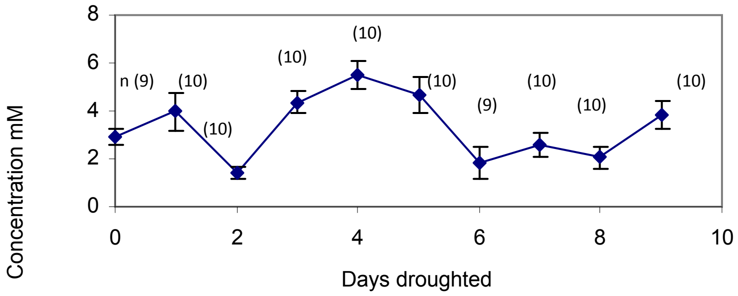


Figure 2.15: Changes in mean NH_4^+ cation concentrations (mM) in cell sap of *L. perenne* subjected to a progressive drought (±SE) under growth room conditions (22°C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 9 days. Ion concentrations altered significantly over the drought regime (one way ANOVA P<0.001 $_{F5.79}$), N is shown in brackets on graph.

In contrast, the average concentrations of cations Mg^+, Ca^{2+} and Na^+ initially stayed the same until day seven of the drought regime when they significantly rose by 111%, 74% and 55%, respectively (fig 2.16). The highest average concentration of each cation in *L. perenne* was in leaves subjected to nine days drought; whereby concentrations of Mg^+, Ca^{2+} and Na^+ increased by 294%, 222% and 258%, respectively, when compared to well watered leaves.

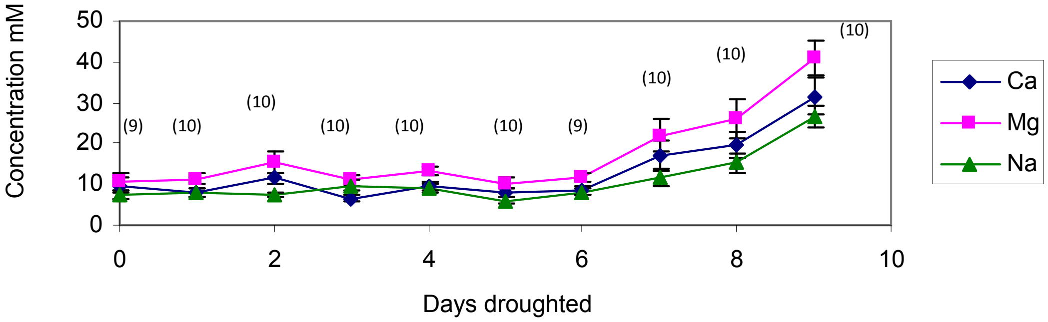


Figure 2.16: Mean Mg^+, Ca^{2+} and Na^+ concentrations (±SE) significantly increased in cell sap of *L. perenne* (one way ANOVA $P<0.001$, $F_{12.77}$, $F_{10.76}$ and $F_{15.86}$ respectively) subjected to a progressive drought under growth room conditions (22°C 16:8LD). Drought treatment consisted of water withdrawal at age 20d for up to 9 days. Ion concentrations increased after day 6 of the drought regime, N is shown in brackets on graph.

These results demonstrate that drought has a significant effect on cation concentrations in cell sap of droughted leaves of *L. perenne* and *B. nigra*. In particular extreme drought stress results in an increase of Mg^+ and Ca^{2+} cations, which increased significantly in both *B. nigra* and *L. perenne*, with K^+ cations also being increased in *B. nigra* and Na^+ only being increased in *L. perenne*.

2.4 Discussion

Simply withholding water from plants as a drought methodology has the issue that the level of drought stress is hard to quantify. Although pot size seemed to have no effect on water loss through transpiration in some studies (Ray and Sinclair, 1998), keeping pots at constant weights for the treatment is required to keep drought stress treatment reproducible (Socias *et al*, 1997; Sanguineti *et al*, 1999). The results of this chapter have demonstrated that in utilising an optimum pot size and plant growth rates, a reproducible drought regime can be established. The drought regime showed significant differences between treatments via a variety of physiological parameters.

Measurements of leaf water potentials show that both plant species *B. nigra* and *L. perenne* display significantly lower water potentials as the drought treatment progressed. However, *B. nigra* appears not to undergo significant osmotic adjustment for the first nine days of the drought regime as leaf water potential values are maintained. This is in contrast to *L. perenne*, which lowers its leaf water potential within a day of water withdrawal. The maintenance of leaf water potential in *B. nigra* may be due to the plant maintaining water loss through stomatal closure, leaf shedding or other mechanisms that increase water acquisition, such as root investment. Although not recorded, leaf shedding of older leaves was observed during drought regimes in *B. nigra*. It could be hypothesised that maintaining water potential during drought could benefit aphids which would otherwise be osmotically challenged if leaf water potential dropped. Despite these differences both plant species show a similar water potential level in leaf tissue around 12-13 days of water withdrawal, indicating that at this point of the drought regime neither plant species can remedy the effect of the water stress on leaf water potential.

Drought stress has typically been associated with a reduction in plant growth (Ashenden *et al*, 1974; Thomas, 1986; Thomas, 1991; Khalil and Grace, 1992; Volaire *et al*, 1998; Clarke *et al*, 1999), as reductions in plant turgor detrimentally affect plant cell growth (Wu and Cosgrove, 1999). Measurements of percentage water content proceeding drought in a variety of grass species (Hale *et al,* 2003) showed that drought stress also induces lower fresh weight to dry weight ratios (fw:dw) than in well watered plants. The results in our study confirm this, with the fw:dw ratio lowering in both the grass species *L. perenne*, as well as in the dicotyledon, *B. nigra*.

Interestingly, despite both plant species having similar fw:dw ratios at the beginning and end of the drought regime, *B. nigra* displayed slightly higher fw:dw ratios than *L. perenne* during the drought treatment*,* with *L. perenne* fw:dw ratios dropping more rapidly over the course of the regime. Unlike the measurements in leaf water potential, fw: dw ratio calculations show a dynamic change in the water status of both plant species over the drought time course. The drop in fw:dw ratio in *B. nigra* individuals is interesting as it occurs before the drop in leaf water potential. From this information it could be hypothesised that leaf water potential is maintained in the younger leaves via leaf shedding, as measurements of the fw:dw ratio involved a mixture of plant tissue from all over the plant, whereas leaf water potential measurements were taken from the younger mid to top leaves on the plants. With regards to aphid performance a leaf shedding drought strategy from the host plant *B. nigra,* could be predicted to be less detrimental than the strategy of osmotic adjustment that *L. perenne* appears to favour, that is unless the changes as a result of osmotic adjustment were advantageous to aphids.

Leaf sap had a significant increase in amino acid concentration compared with well watered controls in *L. perenne* and *B. nigra*. In *L. perenne* amino acid concentrations increased throughout the drought regime confirming other studies which have observed increases in the osmotic pressure and concentration of essential and total amino acids in four grass species (Hale *et al*, 2003). This increase in amino acids in *L. perenne* may be part of the plants osmotic adjustment mechanism, or a by product of water loss from transpiration, given that it occurs at the same time as the leaf water potential becomes more negative it could also be a result of loss of membrane integrity.

Interestingly, despite other studies showing observed increases in essential amino acids in droughted *B. napus* (Good and Zaplachinski, 1994), in *B. nigra* our results showed the initial increase in amino acids was relatively small compared to *L. perenne.* Furthermore, at nine days water withdrawal there was a reduction in amino acid concentrations, which was followed closely by lowering of leaf water potential. Observations of *B. nigra* during the drought regime suggest that at this stage individuals are fatally affected by the drought stress. Therefore, it can be hypothesised that as this initial increase in amino acid concentrations is small it may not be due to osmotic adjustment in *B. nigra*, but part of a drought resistance mechanism that protects cell membranes, or a result of leaf shedding as part of the strategy to reduce water loss. The delayed reduction in MPa and slower decline in fw:dw in *B. nigra* compared to *L. perenne* suggest that *B. nigra* individuals are able to reduce water loss. Whereas, in *L. perenne* the increase of amino acids in cell sap at day four of the drought regime also coincides with the reduction in leaf water potential, leading to the hypothesis that amino acids play a role osmotic adjustment in *L. perenne*.

However, usually it is phloem sap carbohydrate concentrations that are mainly responsible for adjustments in phloem osmotic pressure, with increases in sieve element solutes such as sugars (Smith and Milburn, 1980) used to maintain positive phloem turgor pressure. The amount at which carbohydrate concentrations increase is dependant on the level of water stress, location in the plant, plant developmental stage, growth conditions and how dominant the role of carbohydrate is in adjustment of phloem osmotic pressure. In water stressed *Eucalyptus globulus*, carbohydrates increased by over 50% (Cernusak *et al*, 2003), whilst in *Solanum lycopersicum*, carbohydrate concentrations increased by 20-40% (Najla *et al*, 2010). Our results have shown that *B. nigra* and *L. perenne* are able to greatly exceed this value, with maximum increases in reducing sugar concentrations in cell sap of around 410% and 780%, respectively. However, these increases were observed by day nine of the drought regime, which due to the wilting and frequently fatal level of water stress to the plants will be classified as severe drought. Although it could be argued that these changes could be an artefact of simple water loss the data has shown changes in solute concentrations that would make this unlikely unless there has been active transport of solutes out of the shoots.

Overall, analysis of the reducing sugar concentrations (glucose, fructose, glyceraldehyde and galactose etc) in the cell sap of *L. perenne* and *B. nigra* shows us that both species increase the concentration of reducing sugars throughout the drought regime. Interestingly, *L. perenne* undergoes a greater increase in reducing sugar concentrations during water stress than in *B. nigra*, despite well watered individuals of both species displaying similar concentrations of reducing sugars. These results further suggest that the strategy between the two species is different with regards to water stress.

Aside from amino acids and carbohydrates there are many other solutes that can play an important role in osmotic adjustment and drought tolerance. Ions can play roles in phloem osmotic pressure, with potassium ions found to have an important role in transport of photo-assimilates in *B. napus* (Gould *et al* 2004). Interestingly, studies into water stress perception have also shown that signal transduction cascades involve changes in cytoplasmic Ca^{2+} (Luan 1998; Lee *et al*, 1999), whilst in *Eucalyptus camaldulensis* a high affinity K^+ transporter was identified that shows the properties of being an osmosensor (Liu *et al*, 2001), suggesting that ion levels within/outside cells is important in sensing and communicating water stress.

Our results have confirmed that ion levels alter during water stress, suggesting they may have an important role. HPLC analysis showed significant increases in the ions magnesium, potassium and calcium in *B. nigra,* and significant changes in the levels of potassium, ammonium, magnesium, calcium and sodium in *L. perenne.* These results demonstrate that water stress significantly alters the levels of cations in *B. nigra* and *L. perenne,* and that magnesium and unsurprisingly calcium accumulation is an effect of drought on both species.

2.4.1 Conclusion & The Experimental System

It is clear from the data that both *L. perenne* and *B. nigra* undergo a series of physiological changes in response to water stress. So far the data has enabled many hypotheses to be made about the type of mechanisms and strategies each species uses to address water stress, but it is not in depth enough to make any clear statement about how each species divides resources between drought mechanisms. For example both species show an increase in amino acids, however, the exact role of this increase is debatable with only small increases observed in *B. nigra* compared with *L. perenne.* Detailed analysis via HPLC would enable us to pinpoint which amino acids are increased in concentration preferentially during water stress. Previous studies have already shown that certain amino acids such as proline are associated with prevention and protection of damage to protein structure and activity, and protection from drought induced damage by reactive oxygen species (Rajendrakumar *et al*, 1994; Samuel *et al*, 2000; Saradhi *et al*, 1995). Interestingly, increased levels of proline have also been associated with resistance to aphid infestation (Auclair *et al*, 1957; Van Emden, 1964; Selman and Kandiah, 1971). HPLC analysis of the amino acid composition changes in *B. nigra* and *L. perenne* would enable an assessment to ascertain which amino acids increase. This additional work would provide information to make more informed hypotheses as to the effect of drought on aphids, as increases in plant sourced essential amino acids could be beneficial to aphids, whereas increases in amino acids associated with resistance would be detrimental.

Overall, *B. nigra* and *L. perenne* have demonstrated that water withdrawal at 20 days of age results in a decrease of fresh mass relative to dry mass and a drop in leaf water potential. Both species undergo an increase in sap sugar concentrations, altering the sugar: amino acid ratios, as well as alterations in cation concentrations. It can be hypothesised that this alteration in sap composition could be unpalatable to specialist aphid species that rely on specific plant cues to establish phloem feeding (Wensler and Filshie, 1969, Martin *et al,* 1997), as well as resulting in aphids displaying poor reproductive performance as energy is reallocated to osmoregulation mechanisms, such as sugar polymerisation to deal with the increased sugar concentrations ingested.

To conclude the data from this chapter has shown that simple water withdrawal can be a repeatable drought treatment. Using a progressive drought regime also gives access to different levels of drought stress. For the rest of the thesis two levels of drought will be defined, mild drought and extreme drought, where plants are droughted for 3-6 and 6-9 days at 20 days post sowing respectively. Mild drought is defined by both species showing alterations in levels of amino acids and sugars without the plants showing substantial water loss or osmotic adjustment. Extreme drought is defined as both plant species showing increases in sugar levels, alterations in amino acid and cation levels with substantial water loss quantified in the form of fw: dw ratios. The extreme drought treatment also results in a substantial drop in the leaf water potential of *L. perenne* and is just before a substantial drop in leaf water potential in *B. nigra*, indicating that it is just before the water potential maintenance mechanisms no longer function in *B. nigra*.

Chapter 3: The feeding and settling behaviours of *Myzus persicae, Brevicoryne brassicae, Rhopalosiphum padi* and *Sitobion avenae*, on water stressed hosts *Brassica nigra* and *Lolium perenne*

3.0 Abstract

Aphid behaviour can be affected intrinsically by genes and biochemical processes and externally by environmental and social cues. Many variables can affect the quality of host plants and in turn affect the feeding behaviour of aphids. Using the electrical penetration graph technique the feeding behaviours of four aphid species and two clones was quantified in response to water stress.

Results showed that there was no significant effect of water stress on the aphid *Sitobion avenae* when fed on *Lolium perenne*. However, *Brevicoryne brassicae* and B clones of *Myzus persicae*, did show a significant increase in xylem feeding when fed on droughted *Brassica nigra* plants. Furthermore, both O and B clones of *M. persicae* and *Rhopalosiphum padi* (fed on *B. nigra* and *L. perenne*, respectively), showed significantly less time ingesting sieve element sap on droughted host plants.

The results are consistent with the view that xylem drinking is an important osmoregulation mechanism in *B. brassicae* and B clones of *M. persicae*. This chapter has hypothesised that the reduction in sieve element sap ingestion by *M. persicae* and *R. padi* may be an artefact of the drought induced increases in amino acids in plant sap, or conversely a reduced palatability of water stressed hosts.

3.1 Introduction

Behaviour is a very complex aspect of an organism's response, being influenced intrinsically via genes and biochemical processes and externally via environmental and social driving forces. Behaviour has arguably had a large influence on evolution, especially in sexual selection. It is therefore vital to study the behavioural responses of organisms in addition to their molecular and physiological responses. This chapter will set out to examine how aphids locate, and then subsequently accept suitable hosts. It will then set out to address the behavioural responses of aphids to water stress, using two techniques. Firstly, observational scoring and secondly, the electrical penetration graph (EPG) technique. By understanding the behavioural adaptations of aphids to water stress, it is hoped that the function of genetic responses examined later will be better understood.

3.1.1 Aphid Behaviour – Locating and Accepting suitable Plant-Hosts

Aphids live in colonies; colonial existence may benefit aphids by resulting in a less viable plant stress response, or a change in phloem composition that improves host quality (Telang *et al*, 1999). Colonial existence can however cause other issues such as attracting natural enemies, increasing the risk of infection from contagious pathogens, and in some cases eventual exhaustion of plant host. Therefore, in such instances aphid survival depends on the dispersal of colony members to infest alternative suitable hosts.

The aphid colonial dispersal mechanism is the result of host and social cues. In *R. padi* increased mobility of aphids has been attributed to an increase, over threshold levels, of aphid originated volatile semiochemicals (Quiroz *et al,* 1997). Conversely *Drepanosiphum platanoidis* increased mobility after an increase in tactile disturbance from other members of the colony (Dixon, 1998). This type of social cue can also promote production of winged individuals, called alata(e). Additionally feeding interruption and decreased food quality are all stimuli associated with alate production (Harrewijn, 1972; Dixon, 1998; Muller *et al*, 2001). Development of alate individuals is a crucial mechanism in the aphid dispersal strategy to find suitable hosts.

Alate aphids are able to cover considerable distances as well as remain airborne for many hours being transported by air currents (Wiktelius, 1984). Despite some flight weaknesses, alatae aphids are able to control to some degree where they land. Most species of aphids show a preference to land on yellow surfaces (Prokopy and Owens, 1983; Roberts, 1987) although the more polyphagous species such as *A. fabae* and *R. padi* have been found to be more responsive to wavelengths in the green region of the spectrum (Hardie, 1989; Nottingham *et al*, 1991). With 95% of aphid species being monophagous (Blackman and Estop, 1984), aphids must have effective mechanisms that enable them to detect host suitability

3.1.2 Olfactory influence on plant-host selection

The development of the electroantennogram (EAG) technique has resulted in a range of studies that show aphids can detect plant volatiles, indicating that olfactory cues are important in host plant selection. Aphid's olfactory organs are mainly situated on the antennae; EAG has shown that main olfactory organ present in all morphs and life stages is the primary rhinaria, which is located on the last antennal segment (Bromley *et al*, 1980). Electrophysiological recordings have shown that the rhinaria in adult aphids contain receptors for common leaf volatiles (van Giessen *et al,* 1994; Park and Hardie *et al,* 2002; 2004). Although aphids can detect plant volatiles such as nitriles, green leaf volatiles, henzaldehydes, isothiocyanates and monoterpenes (Visser and Piron, 1997), the exact role of plant volatiles as a cue for host selection in alate morphs is not well understood. Previous studies have shown that alate aphids from the species *Myzus persicae*, *Brevicoryne brassicae* and *Aphis fabae* showed no distinction between host and non host plants when landing. These findings are unsurprising considering the polyphagous nature of *M. persicae,* but are when considering the specialist species *B. brassicae*. This indicates that at a distance these aphids do not show discrimination between host plants using olfactory cues (Kennedy *et al,* 1963 a & b). However, several studies in walking apterae have shown aphids respond to host plant odours when in olfactometers (Pettersson, 1970; 1973; Petterson *et al*, 1994) with some experiments showing that apterous aphids even avoid non host odours (Nottingham *et al*, 1991b; Hori 1999). Olfactory sensory cells in aphids may be more involved in host plant selection once grounded and the initiation of probing behaviours may be dependant on host recognition by these sensory cells on the antennae.

If olfactory cues play a role in the location of host plants, then responses to certain plant volatiles may differ between species with different host plants. It can be hypothesised that different morphs of the same species may alter in their sensitivity to certain plant odours as the season dictates they switch plant host.

Comparisons between the specialist aphid *B. brassciae* and the generalist aphid *M. persicae*, showed differences in response to 35 out of 80 (58%) plant volatiles when reared on the same host plant (Visser and Piron, 1997). In *M. persicae*, summer morphs of apterous and alate aphids differed by 16% in their responses to tested plant volatiles. When compared to *M. persicae* reared on artificial diet, alate morphs reared on host plants differed in response to 20% of the tested plant volatiles, whilst apterae differed by 12% suggesting that sensitivity to plant volatiles varies with species, morph and previous experience. In the aphid *R. padi*, significant differences were observed in EAG responses between winged virginoparae and gynoparae (Park *et al,* 2000), with gynoparae being sensitive to benzaldehyde, a prominent volatile from the aphids winter host, bird cherry (*Prunus padus*). Aphid morphal differences in plant volatile sensitivity may contribute to successful host plant location for aphid species that seasonally alternate host plants. However, morphal differences are not universal across species, with *A. fabae* showing no difference between apterae and alatae in response to 35 plant volatiles (Hardie *et al,* 1994). Moreover *R. padi* host acceptance and growth, was affected by plant volatiles in resistant varieties of *Hordeum vulgare* (Ninkovic and Åhman, 2009), suggesting that certain plant volatiles which are also connected with aphid resistance add to the need for early detection when aphids are selecting suitable hosts.

It can be concluded that plant volatiles play a role in host plant location, providing a rapid host plant screening mechanism in the field (Pettersson *et al*, 2007). The importance of plant volatile cues may vary between species with some aphids relying on volatiles as a mechanism to locate winter hosts.

3.1.3 Influence of Initial Plant Contact in Host Acceptance

When attempting to locate host plants aphids can utilise visual cues in the form of light wavelengths, and olfactory cues in form of detection of plant volatiles. These stimuli although useful in locating host plants, may not suffice in giving the aphid sufficient information as to the feeding quality of the host plant. The gathering of other information is therefore required before complete host acceptance.

Aphids can utilise both chemical and mechanical information about the plant before deciding whether to attempt probing. Host plant waxiness, hairiness, glandular trichrome absence or presence, and epidermal thickness are all important morphological features that could affect aphid colonisation. Hairiness has the potential to limit aphid movement, whilst glandular trichomes have the potential to physically entrap aphids. Host plants with a genotype for waxy cuticles can pose grip problems for aphid's tarsal claws and result in aphids falling off plants more frequently, whilst genotypes with thicker epidermal cell walls could pose additional probing resistance. In a study the aphid *Lipaphis pseudobrassicae* (mustard aphid) was placed on genotypes of *Brassica* with different waxy cuticles, it was found that on waxy genotypes the tarsal claws of the aphid *L. pseudobrassicae* were too short to sufficiently grip the leaf cuticle wax and so the aphids fell off the leaves (Åhman, 1990). It has also been found that when cultivars that produce glandular trichromes were washed, aphids were able to probe them as easily as cultivars that lacked them altogether (Alvarez *et al,* 2006). This demonstrates that glandular trichrome presence has a repellent effect on aphid feeding, which can be attributed to the trichrome production of volatile repellents and sticky membranes.

3.1.4 Chemoreceptors or Mechanoreceptors or both?

When arriving on a potential host plant, chemoreception is thought to be important in determining host suitability. However, aphids have a reduced number of external chemoreceptors when compared to other phytophagous insects. The aphid labial tip and other labia sensilla were found to contain only mechanoreceptors (Wensler, 1977; Tjallingii, 1978), suggesting that mechanoreceptors play the dominant role in host acceptance upon initial plant contact. However, additional sensory organs located on the antennae are thought to be involved in olfaction detection of plant volatiles. Ultra-structural imaging evidence has been used to suggest that it is chemoreceptors present on the apical antennal hairs that play a role in surface chemoreception (Bromley *et al,* 1980; Powell *et al,* 1995). This coincides with observations that when walking on a host plant aphids wave their antennae, and the tips of the antennae touch the plant surface (Powell *et al,* 1995). This behaviour could be to maximise the detection on non-volatile cuticle chemical cues. Aphids can also detect non-volatile epicuticular lipids (Powell *et al,* 1999), but what chemoreceptors are attributable for lipid detection is unknown. What is clear is that aphids have mechanisms for external chemoreception.

Thus, although we know that mechanoreceptors and chemoreceptors play a role in external assessment of host suitability, the exact relationship needs further investigation. Ultimately, the less time aphids use to decide if a host is suitable, the more time an aphid can spend feeding and reproducing on suitable hosts.

3.1.5 Probing Host-Plants and Feeding

Upon landing and satisfaction with the exterior qualities of a plant host an aphid will start penetrating behaviour. This begins with a series of ‘test probes’, these are short stylet penetrations into the epidermal and mesophyll cells of the host. It is believed that during these initial probes the aphid ‘samples’ the internal plant chemical composition allowing for assessment of the internal quality of a host. This mechanism of gustatory monitoring may involve the pharyngeal gustatory organ, which contains 60 taste cells (Wensler and Filshie, 1969). After satisfactory ‘test probes' aphids begin a sustained period of penetration in an attempt to locate the phloem.

In addition, during probing aphids may produce a gelling saliva to produce the salivary sheaths that protect the stylets in the plant tissue and keep sieve elements open following puncture by stylets (Walling, 2008). This gelling saliva is excreted by the accessory glands and forms a protective sheath that assists in stabilising the stylet within plant tissue *(*Pollard, 1973; Douglas, 2003). Aphids also produce watery saliva, which is injected into sieve elements prior to sap ingestion (Prado and Tjallingii, 1994) and will be discussed in more depth later on.

3.1.6 Phloem Location

Although aphid stylets penetrate exclusively intercellularly (Tjallingi and Hogen Esch, 1993) they are also found to puncture nearly every cell intracellularly through plant cell walls on route to the phloem sieve elements, although this will depend upon the host plant and its physiological state. There can typically be 50-100 cell punctures before reaching the phloem, each penetration lasting 5-10s (Tjallingii, 1985). Aphids are thought to sample small amounts of sap during puncture events (Martin *et al*, 1997). These small samples are thought to provide the aphid with information about their host.

The mechanism(s) of sieve element location are not well understood but it has been suggested that location is a result of the gustatory monitoring of ingested sap samples (Wensler and Filshie, 1969). The intracellular puncturing of plant cells along the stylet pathway provides aphids with the ability to follow potential apoplastic gradients, in pH and sugar leading to the phloem, these gradients are a result of the combined effects of symplastic loading and leakage into the apoplast. It is also possible that tasting the allelochemicals contained in vacuoles aids plant host selection

3.1.7 Phloem Acceptance

Once reaching the sieve element, initial acceptance is determined by brief cell punctures of the sieve tubes and companion cells. Typically phloem activities always start with a brief cell puncture; it is thought that this event is crucial in the aphid deciding whether or not to commit to phloem feeding. In support of this hypothesis electron microscopy has shown punctures into sieve elements where no subsequent phloem phase activities where detected using the Electrical Penetration Graph Technique (EPG) (Tjallingii and Hogen Esch, 1993). Upon initial phloem acceptance aphids without exception commence salivating into the sieve element; this behaviour is defined as E1 in EPG. However, it is not the only saliva produced by aphids during plant penetration, the first saliva is a lipoproteinous gelling saliva mentioned earlier, which is secreted during the phloem locating phase of plant penetration (aka pathway phase). The saliva secreted during E1 behaviour into the sieve element is known as watery saliva (Miles, 1999; Tjallingii, 2006) and is secreted by the principle gland of the aphid.

On host plants the secretion of watery saliva lasts for roughly one minute before the aphids move onto the second phase of phloem activity, termed E2, which is phloem sap ingestion coupled with watery salivation. Unsuitable hosts may result in aphids not proceeding onto E2 behaviour and withdrawing their stylets from the sieve element entirely. The exact stimuli that results in successive E2 after E1 behaviour is currently unknown. E1 salivation is thought to be associated with suppressing potential phloem wound responses, and phloem resistance mechanisms that can hinder feeding. *Aphis gossypii* showed extended periods of E1 when fed on resistant melon cultivars (Garzo *et al*, 2002), whilst aphids involved in phloem ingestion (E2) resorted back to phloem salivation (E1) when forisomes (proteins involved in the phloem wound response) were activated (Will and van Bel, 2006). Thus, continued salivation during the sap ingestion (E2) is thought to be associated with preventing stylet canal blockage (Tjallingii and Hogen Esh, 1993).

Complete phloem acceptance can then be defined as when aphids display ‘committed phloem ingestion’, instead of merely displaying an absence or presence of each behaviour. Committed phloem ingestion is generally accepted as when E2 behaviour occurs continuously for longer than fifteen minutes (Montllor *et al*, 1983; Tjallingii, 1990), and if the host plant is suitable aphids can then sustain E2 for many hours.

To summarise aphids have an array of mechanisms that assist them in finding suitable hosts, from visual cues when in flight, to chemical cues detected within the tissues of plants. The dependence on each of these host selection mechanisms may alter depending on the aphid-plant species combination. Central in the elucidation of these mechanisms has been the study of aphid behaviour, and one technique which changed the way aphids were studied was the electrical penetration graph technique.

3.1.8 Assessing aphid probing behaviour using the Electrical Penetration Graph Technique

EPG is a method used to qualitatively and quantitatively assess aphid's feeding behaviour through the visualisation and recording of waveforms that correlate with specific feeding behaviour. The EPG technique makes an aphid and plant part of an electrical circuit; this is done by inserting a wire into the soil of the plant and attaching a thin wire to the dorsum of the aphid. As the aphid pierces the plant tissue with its stylet mouthparts the circuit is completed, and a fluctuating voltage sent through the system, called the 'EPG signal', is amplified and recorded to form distinct patterns known as 'waveforms'. Analysis of these waveforms allows the differentiation between different feeding behaviours.

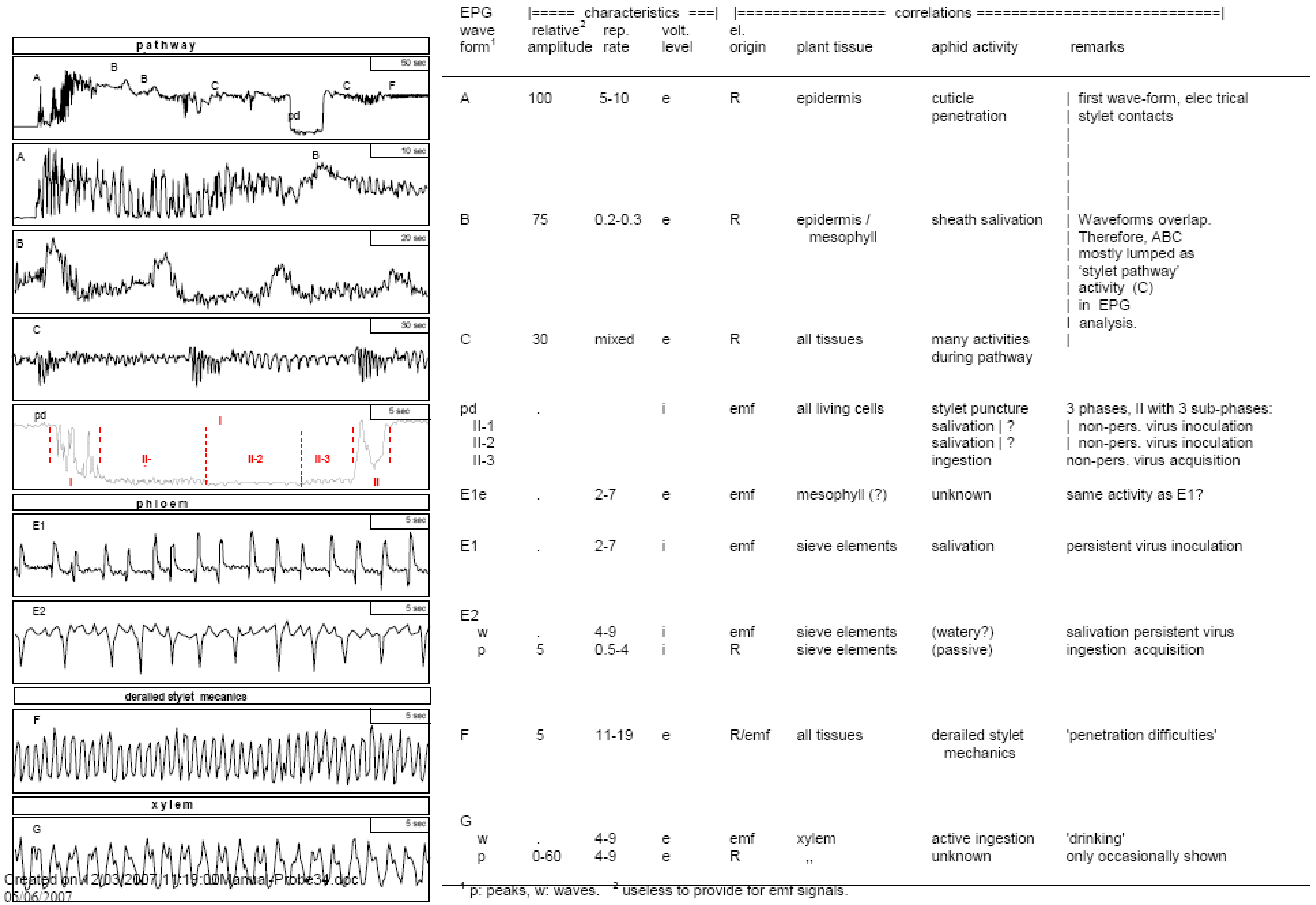
Waveform patterns are produced by a variety of different components. The fluctuating voltage is affected by the changes in electrical resistance in the stylet of the aphid; this is known as the resistance component (R). In addition, the fluctuating voltage is also affected by voltages generated in the aphid-plant interaction called *emf* components. These are a consequence of plant cell punctures by the aphid's stylet resulting in changes in membrane potentials, causing a drop in measured voltage as well as streaming potentials in the stylet canal of the aphid.

The EPG system was originally designed by McLean and Kinsey (1964), but the early AC system lost *emf* components in the signal generation. As a result, the DC system was developed by Freddy Tjallingii from Wageningen University (Tjallingii, 1988), which could record both R and *emf* components. It is this system that is used in this study.

EPG has provided a lot of information on aphid feeding behaviour especially in regards to plant resistance (van Helden and Tjallingii, 1993; Klinger et al, 1998; Kaloshian et al, 2000). For example, EPG allows the experimenter to assess whether plant resistance is phloem based, with the ability to assess the amount of time aphids spend feeding from the phloem, xylem or in pathway (defined in this thesis as probing in non-vascular plant tissues). Short durations of phloem feeding detected by EPG followed by rejection of plant host (often defined as non-penetration) may suggest a plant has unsuitable phloem quality and or phloem based resistance. Phloem resistance may make the phloem more difficult to locate or result in phloem feeding being unsustained (Garzo *et al*, 2002; Marchetti *et al*, 2009).

Criticism of the EPG technique is that the process of wiring may have adverse affects on aphid behaviour (Annan *et al*, 1997). However, as it is mainly used in comparative studies it has been counter argued that the effects of EPG tethering are constant, and therefore, do not negate the reliability of the results (Tjallingii, 1978). In a study to test the effects of EPG tethering (Annan *et al*, 1997), it was found that tethered and un-tethered aphids displayed no significant differences in survivorship, reproductive period, r_m (intrinsic rate of increase) and fecundity. Therefore, it is maintained that EPG is an effective method for study, as any detrimental impacts of EPG are the same for both experimental and control trials.

In order to disseminate the information produced by the EPG system it is important that feeding behaviours are clearly correlated with the waveforms produced by EPG. Over the last few decades there have been many studies using a variety of techniques to correlate waveforms to behaviours. Video observations coupled to EPG systems have been used to observe when food uptake and salivatory release takes place (Hardie *et al*, 1992; Hardie and Powell, 2000; Lösel *et al*, 1992), whilst stylectomy and honeydew production have been used to correlate EPG patterns to feeding. However, histological sectioning has probably been the most important tool in assigning waveforms to behaviours. Sections taken during particular waveforms have allowed experimenters to view where aphids were feeding at that point in time (Tjallingii, 1978; Tjallingii and Hogen Esh, 1993; Tjallingii and Gabrys, 1999). As a result at least six feeding waveforms have now been distinguished using the EPG system (Tjallingii, 1978; 1985; 1990) (fig 3.0). In this thesis EPG data acquired shall be defined in less subcategories as the software allows. For simplicity data will be categorised as non-penetration, pathway defined as aphid feeding/probing in non-vascular tissue layers, cell penetrations, xylem feeding and phloem feeding defined in two catagories, salivation (defined as E1) and ingestion (defined as E2).



EPG	|===== characteristics		===|	|================= correlations			===========================|
wave form[1]	relative[2] amplitude	rep. rate	volt. level	el. origin	plant tissue	aphid activity	remarks
A	100	5-10	e	R	epidermis	cuticle penetration	| first wave-form, elec trical | stylet contacts | | | | |
B	75	0.2-0.3	e	R	epidermis / mesophyll	sheath salivation	| Waveforms overlap. | Therefore, ABC | mostly lumped as | 'stylet pathway' | activity (C) | in EPG I analysis.
C	30	mixed	e	R	all tissues	many activities during pathway	|
pd	.		i	emf	all living cells	stylet puncture	3 phases, II with 3 sub-phases:
II-1						salivation | ?	| non-pers. virus inoculation
II-2						salivation | ?	| non-pers. virus inoculation
II-3						ingestion	non-pers. virus acquisition
E1e	.	2-7	e	emf	mesophyll (?)	unknown	same activity as E1?
E1	.	2-7	i	emf	sieve elements	salivation	persistent virus inoculation
E2							
w	.	4-9	i	emf	sieve elements	(watery?)	salivation persistent virus
p	5	0.5-4	i	R	sieve elements	(passive)	ingestion acquisition
F	5	11-19	e	R/emf	all tissues	derailed stylet mechanics	'penetration difficulties'
G							
w	.	4-9	e	emf	xylem	active ingestion	'drinking'
p	0-60	4-9	e	R	,,	unknown	only occasionally shown

[1] p: peaks, w: waves. [2] useless to provide for emf signals.

Figure 3.0: Diagram showing the EPG waveforms and correlations to aphid feeding behaviours, as defined in the 'Probe 3.4 Manual: Software manual for EPG acquisition and analysis in Windows', by Tjallingii, W. F. (2007), distributed by EPG systems, Wageningen the Netherlands. Definitions of waveforms in this guide were used to interpret waveforms obtained in EPG traces for this chapter.

3.1.9 Influences of drought and environmental stressors on aphid behaviour

Aphids when on non hosts usually display reduced phloem feeding (Gabrys and Pawluk, 1999; McLean and Kinsey, 1968) and more non-penetration waveforms. Furthermore, they may tend to probe more frequently and for shorter amounts of time (McLean and Kinsey, 1968). On resistant plant varieties aphids have been found to alter their feeding displaying longer salivation (E1) periods (Ramirez and Niemeyer, 1999; Caillaud et al, 1995), lower total plant penetration times (van Helden and Tjallingii, 1993; Paul et al, 1996) reduced phloem ingestion and consequently secretion (Klinger et al, 1998). Increased salivation by aphids is usually associated with reduced palatability of the phloem sap or changes in its composition.

Additionally, studies have shown aphids alter their feeding behaviour in response environmental stressors. In response to water stressed barley seedlings, the aphid R. padi, was shown to increase sieve element salivation (Ponder et al, 2000), this behaviour has also been exhibited where aphids are exposed to reduced amino acid concentrations (Hunt et al, 2009). Dehyrated and water stressed aphids have also been known to exhibit more xylem feeding. Studies have shown that aphids starved for 24h spent 26% of their time xylem feeding when placed back onto a host plant, whereas those starved for only 1h displayed no xylem feeding (Spiller et al, 1990). In another study starved Sitobion fragariae increased xylem feeding on wheat and oats (Ramirez et al, 2000). Recently TMX, a xylem borne insecticide, was used to determine the importance of xylem feeding in aphids. TMX was used at sub lethal doses to make the xylem unpalatable to aphids starved for 5h. The study showed that there was a reduction in xylem feeding from 25% in control plants to 0.6% in

TMX treated plants. However, aphids on TMX plants showed a reduced growth, performance and water content (Daniels et al, 2009), indicating that xylem feeding is an important aspect of an aphids diet, in particular in response to dehydration and water stress.

3.1.10 Chapter Aims

It can be concluded that, many variables that affect the quality of host plants, can also affect the feeding behaviour of aphids. This study aims to quantify aphid feeding behaviour in response to drought stress. This chapter will analyse the settling and probing behaviour of Myzus persicae, Brevicoryne brassicae, Rhopalosphium padi and Sitobion avenae on droughted and well watered plant hosts Brassica nigra and Lolium perenne, to observe if drought stress is detected upon initial plant contact or upon aphid probing.

The two techniques that will be used to meet this aim will also provide two levels of behavioural analysis in response to droughted and well watered host plants. The first approach is observational scoring (OS), which provides an overview of aphid behaviour in the initial ten minutes of host plant contact, for example, whether the aphid is walking or not. In contrast the EPG technique will provide more specific data on aphid feeding at the level of the stylet and cell for the first six hours of plant contact.

EPG is able to record in detail what the aphid is doing with its stylet mouthparts once it is in the plant tissue, but EPG does not give an account of behaviour prior to penetration. Behavioural scoring is an effective tool to study the initial behaviour of aphids before host plant probing. Using behavioural observations data can be obtained on aphid behaviour during periods where no penetration is taking place.

3.2 Methods and Materials

3.2.1 Plant Species

B. nigra and L. perenne were obtained from John Chambers Wildflower Seeds Co. and from wild seed stocks in Northamptonshire, UK.

3.2.2 Aphid Species

Myzus persicae clones used in the experiment were the red type B clones obtained from Brian Fenton's cultures in SCRI and a green clone obtained from the University of Birmingham, School of Biosciences. Brevicoryne brassicae, Rhopalosiphum padi and Sitobion avenae were obtained from Phil Northing culture stocks at FERA.

3.2.3 Plant Culturing

Plants were grown in the insect free plant growth rooms with a 16:8 light cycle at a temperature of 22oC. Growth room PAR was between 80-120mol m-2 s-1 with a relative air humidity 70-80%. Plants were grown in John Innes peat based compost with mixed in Silvaperl in 100-150ml plastic pots (for B. nigra and L. perenne respectively).

3.2.4 Aphid Culturing

'Tents' (Megaview Bugdorm 3120) were used to isolate the cultures from other insects and to prevent the escape of insects from the culture. Plants were grown in the insect free plant growth rooms before being placed into tents to be fed on by the insects. The constant removal of old dying plants and replacement with young new plants into the cultures ensured that the culture was healthy with an abundant supply of insects. The insect rooms was held at a constant temperature of 18oC under a 16:8 light: dark cycle.

3.2.5 Electrical Pentration Graph (EPG) Technique

A gold wire (0.25mm diameter, 2-3cm long) was attached to the dorsum of an adult apterous aphid using silver conductive paint (RS Components). Aphids were previously cultured on either B. nigra or L. perenne, depending on species. This wire was then connected via a pin to the EPG probe. An output probe was inserted into the soil of the potted plant. When the aphid’s stylets penetrated the plant, the circuit was completed, and a fluctuating voltage was amplified using the Giga-8 DC amplifier and recorded by Probe on a Windows platform (Wageningen Agricultural University, The Netherlands). The voltage fluctuations appear in a number of distinct patters, referred to as 'waveforms'. These waveforms correlate with the feeding activity of the aphid, i.e. whether it is xylem or phloem feeding etc. All plants and insects were contained in a Faraday cage surrounded by reflective foil to minimise electronic noise disturbance. All experiments were conducted at ambient room temperature of 18oC.

EPG traces were acquired and analysed using Windows XP OS system and Probe software developed by Freddy Tjallingii, (Wageningen Agricultural University, The Netherlands). The acquisition of traces was using software ACQ-8, and analysis was conducted on ANA-34. Data was imported from ANA-34 to MS Excel, where cumulative time for each behaviour per hour was calculated.

3.2.6 Electrical Penetration Graph Statistical Analysis

Care must be taken when analysing this type of data, as not all the categories are independent of each other. For example, one exhibited behaviour could have a negative correlation with the occurrence of another behaviour, not through association but simply because there is less time for the other behaviour to occur (Cane, 1961; Sackett et al, 1978). Thus, data was analysed with MINITAB using the Anderson-Darling Normality Test. In cases where data was normally distributed parametric tests utilising the means, such as the t-test (to compare two datasets with one variable) and ANOVA (to compare multiple datasets with more then one variable) were used. However, data was usually not normally distributed and could not be normalised, therefore non-parametric statistical tests using the median such as the Mann-Whitney (compare two datasets), Kruskall Wallis (two compare datasets with one variable) and Scheirer-Ray-Hare test (multiple datasets with two variable) were used. Where results were deemed not significant by the Schierer-Ray-Hare test but figures plotting averages suggested otherwise, datasets was re-analysed using the non-parametric Mann-Whitney test to simply compare drought vs well watered.

3.2.7 Behavioural Scoring

To study the behaviour of aphids upon intial plant contact and prior to plant penetration with stylets, a simple experimental system using behavioural observations was set up. Only the first ten mintues upon first plant contact and before initial plant penetration was chosen for observational analysis, this is because during preliminary experiments using Myzus persicae, it usually took ten minutes for an aphid on a suitable host to commence probing when wired up to the EPG system. To avoid effects of starvation, aphids were transferred directly from the culture plant to the host plant in the experiment.

Each individual aphid was observed for a standard duration, and the frequency of categories of behaviour recorded (focal sampling). The sampling duration for this study was ten minutes. Behaviours were categorised and the frequency of occurrence measured at set ‘instances’ of time (instantaneous sampling). Adult aphids were removed from the culture and immediately transferred onto experimental plants in the laboratory. Aphid behaviour was scored at the point of contact with the host plant and successively every 30 seconds from then on, for a total of ten minutes. Behaviours scored were; aphid movement, defined as walking or still, and the position of the antennae, back, straight up or forward (see table 3.1). In total behavioural data was collected for 21 time points over the ten minute period. Each experimental plant had a maximum of three aphids present to avoid aphid crowding and displays of conspecific induced behaviour. Plant host species were L. perenne and B. nigra, that had been subjected to one of two different types of pre-treatments, namely control or extremely drought stressed (as defined in chapter two). All plants were approximately 29 days old, with drought stressed plants experiencing total water withdrawal for 9 days prior to the experiment.

With regards to the above experimental design, the categories of antennae orientation and locomotion can occur simultaneously, thus both categories are truly independent of each other. However, within each category the observed behaviours are not independent, as the aphids can either do one or the other and so they must be treated with caution during analysis using appropriate statistics.

	Behaviour
Category 1: Walking	**1**
Still	**2**
Category 2: Antennae facing forwards	**1**
Antennae facing upright	**3**
Antennae facing backwards	**2**

Table 3.1: List of the numerical values assigned to behaviours recorded in Observational Scoring experiments, involving the aphid species *M. persicae* (red type B clone and green clone), *B. brassicae*, *S. avenae* and *R. padi*. Aphids were scored for every 0.5 minutes during the first ten minutes contact with droughted (extreme drought defined in Chapter 2) and well watered host plants *B. nigra* and *L. perenne* respectively.

3.2.8 Observational Scoring Statistical Analysis

Data was analysed with MINITAB using the Anderson-Darling Normality Test. In cases where data is normally distributed parametric tests utilising the means, such as the t-test (to compare two datasets with one variable) and ANOVA (to compare multiple datasets with more than one variable) were used. However, where data was not normally distributed and could not be normalised, non-parametric statistical tests that use the median such as the Mann-Whitney (compare two datasets), Kruskall Wallis (two compare datasets with one variable).

3.3 Results

3.3.1 Effect of time on feeding behaviour

Analysis of the EPG results showed that time had a major effect on most feeding behaviours in the four aphid species and two clones studied. As a general rule the longer the aphid was on a host plant the more time it would spend per hour ingesting sieve element sap (E2) (fig 3.4). Conversely the longer the aphids were exposed to plant hosts the less time they would spend per hour not probing at all and probing non vascular tissue (referred to as pathway from now on) (fig 3.1 and 3.2).

In *R. padi*, *S. avenae*, *B. brassicae* and *M. persicae* (red type B and green clone) the amount of time spent per hour not probing and ingesting sieve element sap was significantly affected by the length of time the aphid had spent on the plant (see table 3.2 for significance values). Aphids spent less time not probing as time went on (fig 3.1), then as they settled, they displayed more sieve element ingestion (fig 3.4). The length of time aphids were on host plants also significantly affected the amount of time spent per hour in pathway (fig 3.2) in *S. avenae*, *B. brassicae, R. padi* and one clone of *M. persicae* (red type B clone only). Overall there was a decrease in none probing behaviour over time (fig 3.1), as aphids became more settled on host plants and increased the time spent ingesting sap from the sieve element (E2). In red (type B) clones of *M. persicae* the length of time spent on host plants also had a significant effect on the duration spent each hour salivating into the sieve element, with a decrease over time in red clones of *M. persicae* (fig 3.3). Interestingly, in green clones of *M. persicae*, time had neither an effect on the duration each aphid spent per hour in E1 or pathway. These results indicate that there are significant clonal differences in the feeding behaviour of aphids.

3.3.2 Effect of drought on feeding behaviour

EPG analysis showed there was no significant effect of drought treatment on feeding behavious of *S. avenae* individuals over the six hour period (fig 3.1-3.5). However, *S. avenae* proved a difficult species to obtain traces from, and consequently the replication for this species was only eight and four traces for well watered and droughted treatments respectively. However, drought did have a significant effect on behaviours in all the other species of aphids tested. Red clones of *M. persicae* and *B. brassicae* fed significantly longer from the xylem on droughted hosts than on well watered host plants (fig 3.5). Green *M. persicae* showed no significant effect of treatment of time spent xylem feeding. Nevertheless, both red and green clones of *M. persicae* and *R. padi* showed significant differences in the amount of time spent not-probing (fig 3.1), in pathway (fig 3.2) and ingesting from the sieve element, when on droughted than well watered hosts (fig 3.4). Overall, aphids on droughted hosts spent less time ingesting sieve element sap, and more time over the six hours not probing or in pathway (fig 3.1 & fig 3.2). Interestingly, both *M. persicae* clones spent a greater time per hour salivating (E1) into the sieve element of droughted hosts (fig 3.3), however these findings were not significant when tested using an non parametric version of ANOVA the Schierer-Ray-Hare test (red clone P>0.05, green clone P>0.05). However, when the data for both *M. persicae* clones was analysed using (a non parametric t-test equivalent) the Mann-Whitney test, there was a significant effect of drought (Mann-Whitney P<0.05) on time spent salivating in only red clone of *M. persicae.* Furthermore, there was a significant effect of drought ($P<0.05$) on the time spent ingesting sieve element sap in *B. brassicae* when using the Mann-Whitney non-parametric test directly to compare data from aphids feeding on well watered and droughted hosts, but not with the Schierer-Ray-Hare test ($P>0.05$).

3.3.3 Proportional changes in EPG behaviour over the 6 hours

Data collected over the six hour period was collated to examine the average change in the proportion of time aphids spent engaged in each of the feeding behaviours on droughted hosts (fig 3.6 & 3.7). Drought increased the time aphids spent not probing in all species and clones (*M. persicae* red clone $^{+}$76%, green clone $^{+}$50%, *B. brassicae* $^{+}$82% and *R. padi* $^{+}$162%) except in *S. avenae*, which showed hardly any difference ($^{-}$3%). This was partially confirmed by statistical analyses (table 3.2), which found significant increases in the time not probing in both *M. persicae* clones and *R. padi*. Time spent probing in pathway, was increased in both M*. persicae* clones ($^{+}$42% in the red clone, $^{+}$32% in the green clone) and *R. padi* ($^{+}$33%) when on feeding on droughted host plants, whereas no substantial difference was observed for *B. brassicae* ($^{-}$4%) and *S. avenae* ($^{+}$5%).

Sieve element salivation (E1) was increased in both *M. persicae* clones (+147% in red type B clone, +78% in green clone), but reduced in *R. padi* and *B. brassicae* (-50% and -27% respectively) feeding on droughted plants. *S. avenae* showed little difference between treatments ($^{-}$6%). Interestingly, previous statistical analysis indicated the changes in E1 under drought in the green *M. persicae* were not significant whereas, it was significant for *R. padi*.

Overall average E2 ingestion was reduced in all species and clones over the six hour period when feeding on droughted host plants. The largest reduction was observed in the green *M. persicae* clone ($^{-}$65%, $P<0.001$), followed by *R. padi* ($^{-}$58% $P<0.001$), *M. persicae* red clone ($^{-}$34% $P<0.005$), *B. brassicae* ($^{-}$29% $P>0.05$) and *S. avenae* ($^{-}$8% $P>0.05$) respectively.

The proportion of time spent xylem feeding was increased in most aphid species on droughted hosts. The largest increases in xylem feeding behaviours were in *B. brassicae* ($^{+}$208904%, $P<0.05$) and the red clone of *M. persicae* ($^{+}$1203%, $P<0.05$) where the behaviour of xylem feeding was rare when fed on well watered hosts. Increases in xylem feeding were also displayed by the green clone of *M. persicae* ($^{+}$123%, $P>0.05$) and *S. avenae* ($^{+}$25%, $P>0.05$). Surprisingly, xylem feeding over the whole period was reduced in *R. padi* ($^{-}$43%, $P>0.05$). Collectively this analysis suggests that xylem feeding is significantly increased in *B. brassicae* and red clones of *M. persicae* in response to host plant water stress.

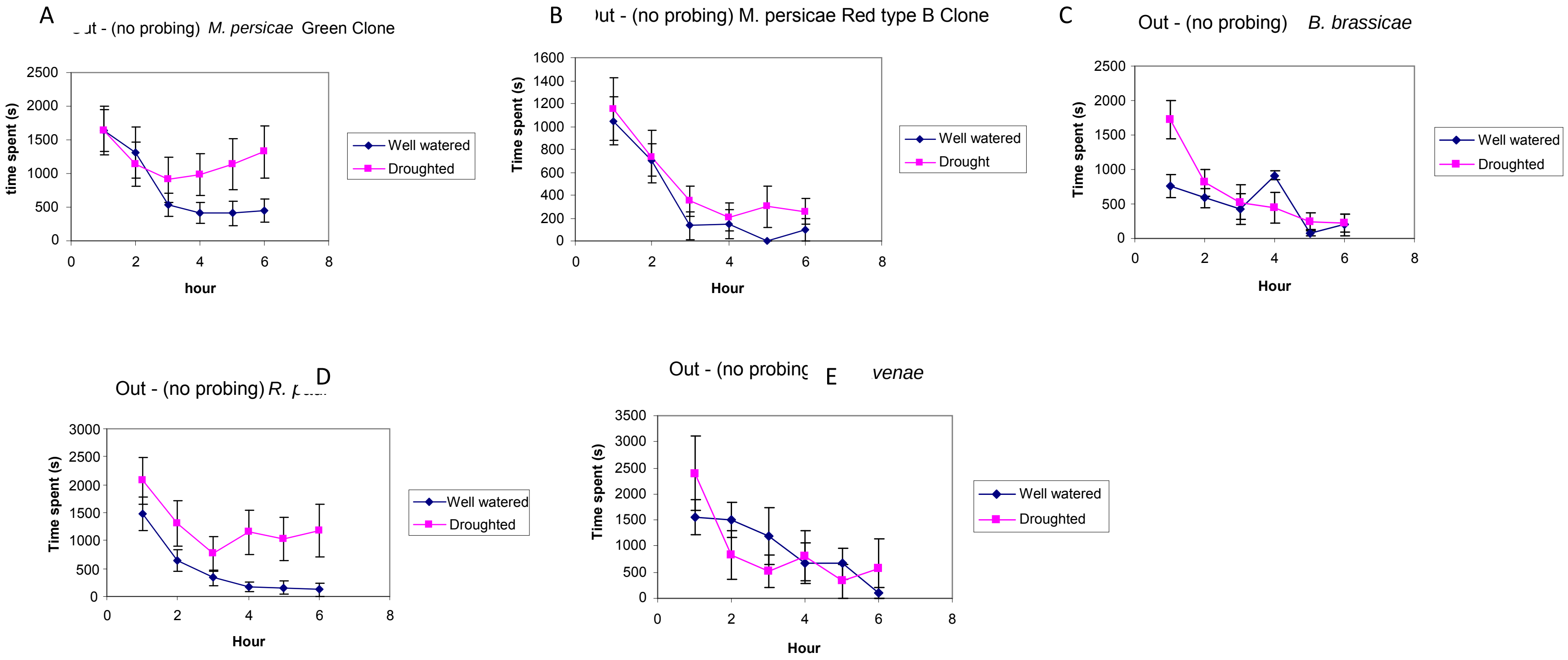


Figure 3.1: Average amount of time (s) recorded via the Electrical Penetration Graph (EPG) technique that aphids spent not probing the plant-host (±SE) on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (green O clone (A) and red type B clone (B)), *Brevicoryne brassicae* (C), *Rhopalosphium padi* (D) and *Sitobion avenae* (E) over a six hour period. Pink lines denote aphids fed on droughted treatments whilst blue lines denote well watered treatments. Plants were between 27-29 days old, with droughted plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. Statistical analysis of this data can be found in table 3.2. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

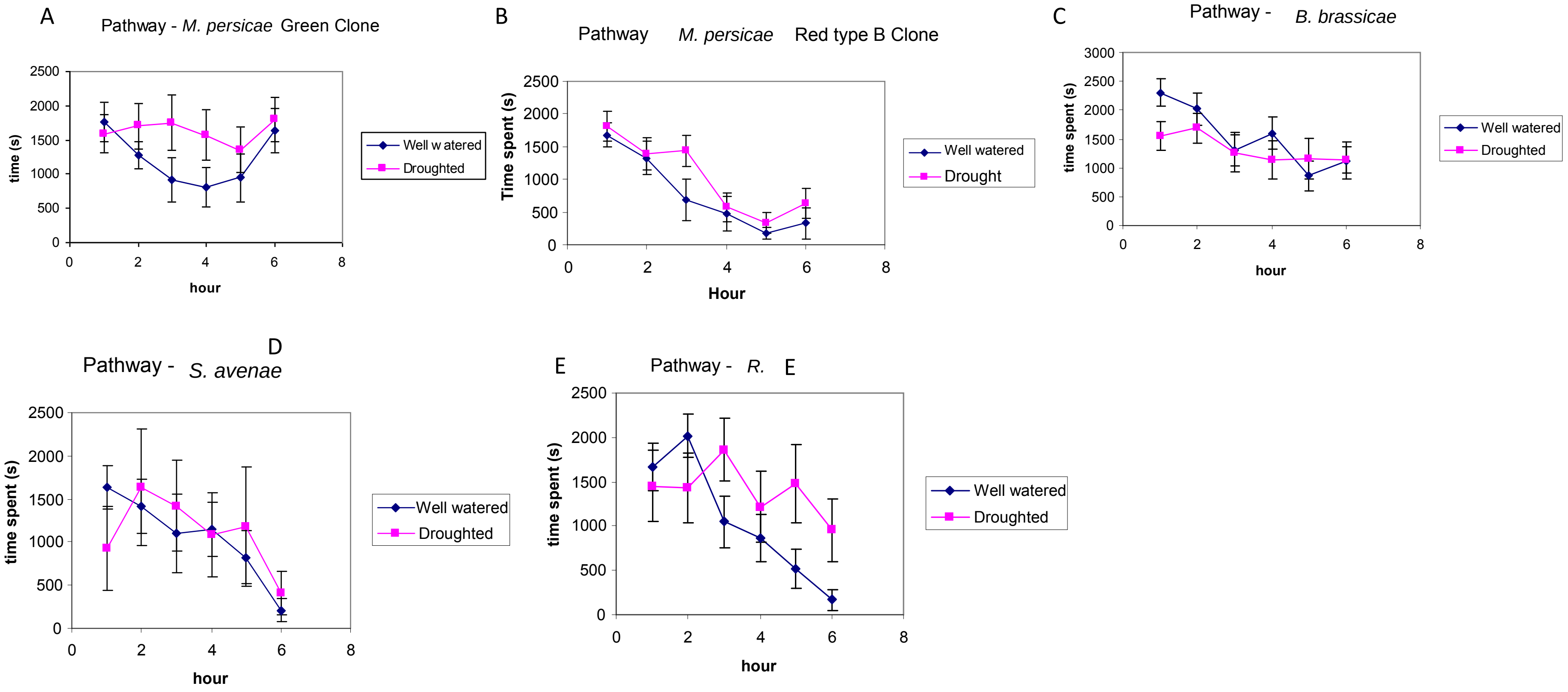


Figure 3.2: Average amount of time (s) recorded via the Electrical Penetration Graph (EPG) technique that aphids spent probing the plant-host but not in xylem or phloem tissue (±SE) on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (green O clone (A) and red type B clone (B)), *Brevicoryne brassicae* (C), *Rhopalosphium padi* (D) and *Sitobion avenae* (E) over a six hour period. Pink lines denote aphids fed on droughted treatments whilst blue lines denote well watered treatments. Plants were between 27-29 days old, with droughted plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. Statistical analysis of this data can be found in table 3.2. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

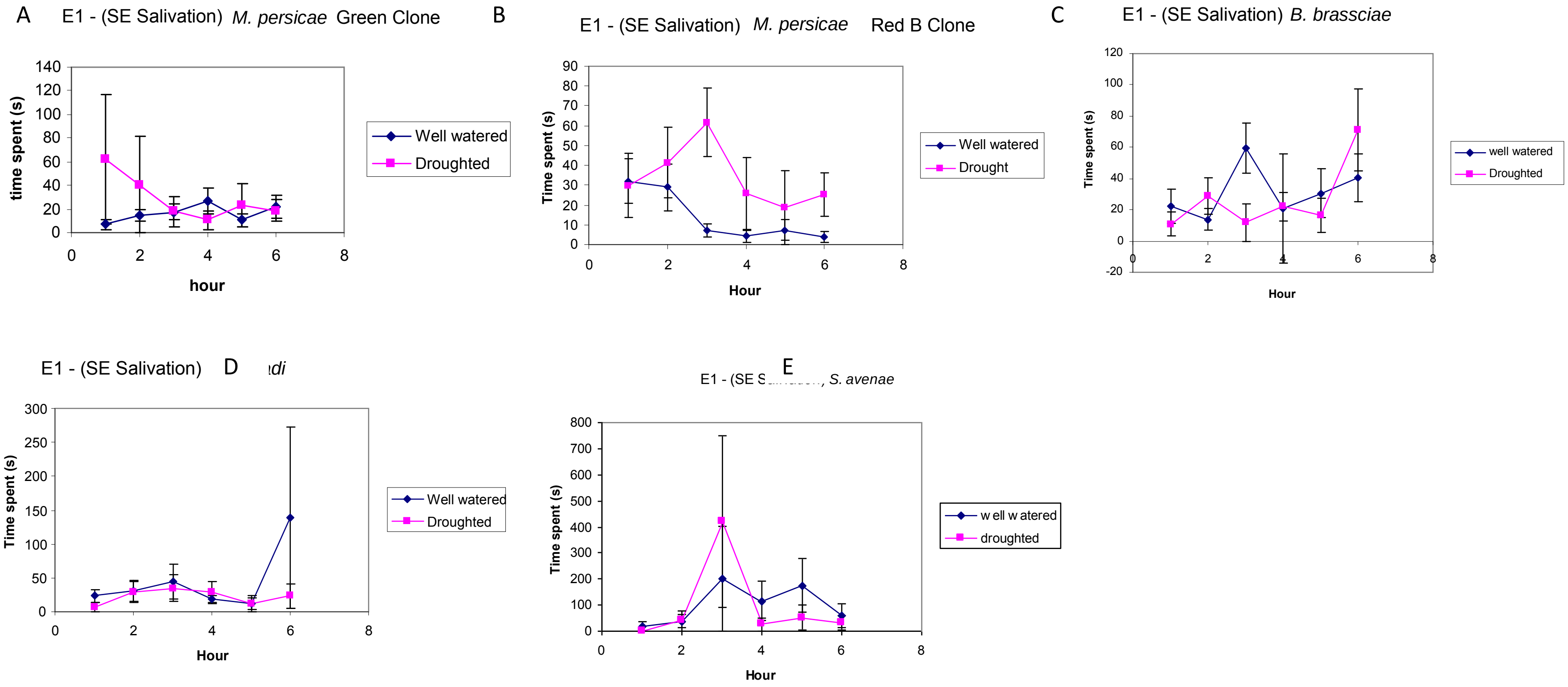


Figure 3.3: Average amount of time (s) recorded via the Electrical Penetration Graph (EPG) technique that aphids spent salivating in the sieve element (±SE) on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (green O clone (A) and red type B clone (B)), *Brevicoryne brassicae* (C), *Rhopalosphium padi* (D) and *Sitobion avenae* (E) over a six hour period. Pink lines denote aphids fed on droughted treatments whilst blue lines denote well watered treatments. Plants were between 27-29 days old, with droughted plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. Statistical analysis of this data can be found in table 3.2. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

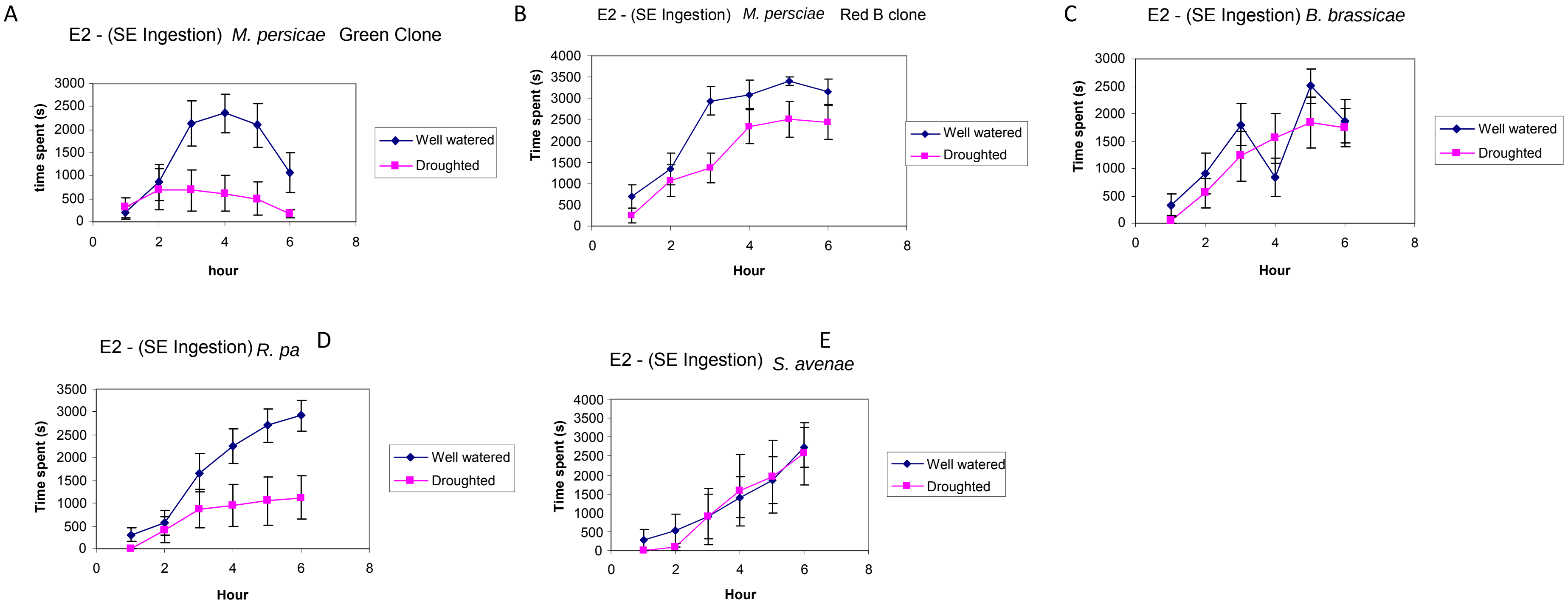


Figure 3.4: Average amount of time (s) recorded via the Electrical Penetration Graph (EPG) technique that aphids spent ingesting sieve element sap (±SE) on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (green O clone (A) and red type B clone (B)), *Brevicoryne brassicae* (C), *Rhopalosphium padi* (D) and *Sitobion avenae* (E) over a six hour period. Pink lines denote aphids fed on droughted treatments whilst blue lines denote well watered treatments. Plants were between 27-29 days old, with droughted plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22°C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. Statistical analysis of this data can be found in table 3.2. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

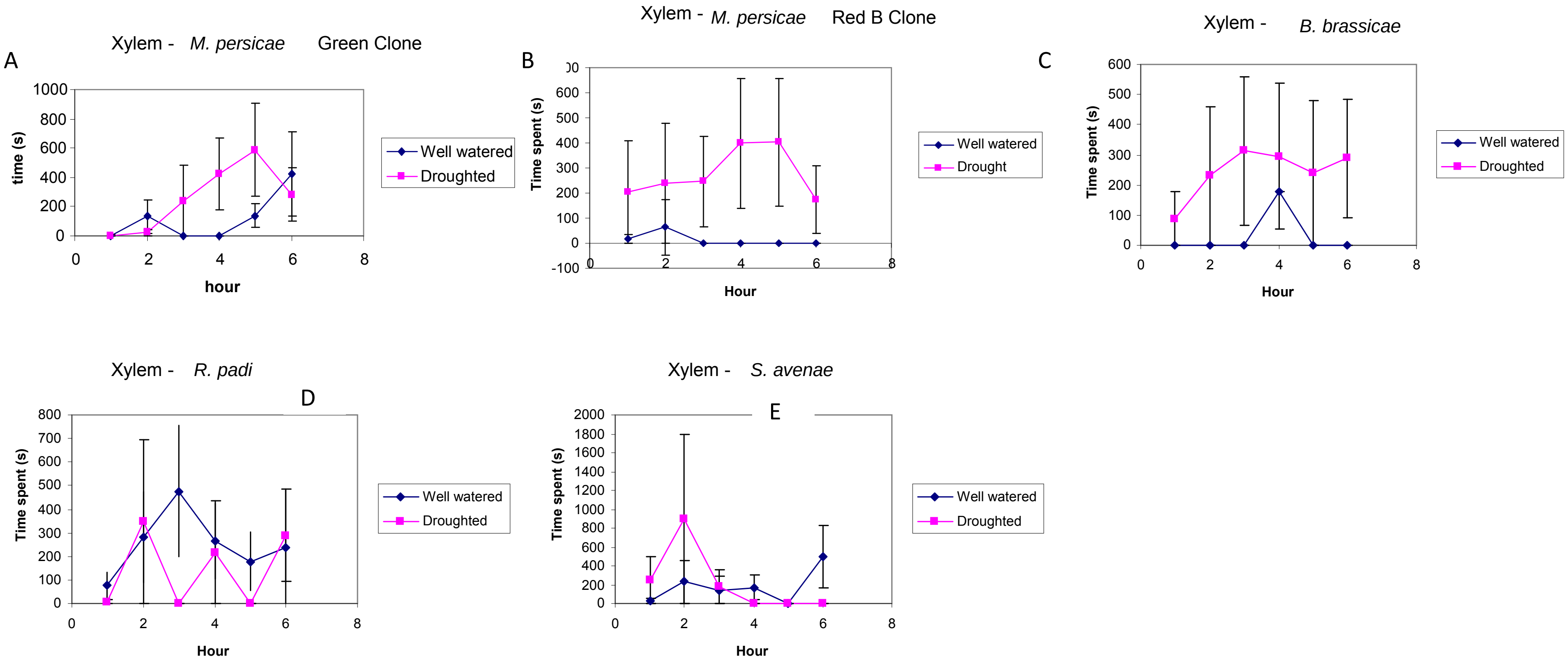


Figure 3.5: Average amount of time (s) recorded via the Electrical Penetration Graph (EPG) technique that aphids spent xylem feeding (±SE) on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (red type B clone and green O clone), *Brevicoryne brassicae*, *Rhopalosphium padi* and *Sitobion avenae* over a six hour period. Pink lines denote aphids fed on droughted treatments whilst blue lines denote well watered treatments. Plants were between 27-29 days old, with droughted plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. Statistical analysis of this data can be found in table 3.2. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

Table 3.2

Statistical test: Scheirer-Ray-Hare Test (balanced design)

Behaviour	**Species**	**Total SS**	**Total DF**	**Total MS**	**Factor**	**SS**	**SS/Total MS**	**Chi**	**P**
Non-probing time	*B. brassicae*	444900	179	2485.47	Treatment	3809	1.5325	0.784	0.216
					Hour	100171	40.3026	1	0.000
					interaction	9786	3.9373	0.441	0.558
Non-probing time	*M. persicae* red type B	362531	179	2025.31	Treatment	12087	5.968	0.985	0.015
					Hour	85580	42.2552	1	0.000
					interaction	4978	2.4579	0.217	0.783
Non-probing time	*M. persicae* green	140995	119	1184.83	treatment	9883	8.3413	0.996	0.004
					hour	18306	15.4503	0.991	0.009
					interaction	4561	3.8495	0.428	0.571
Non-probing time	*R. padi*	General Linear Model			treatment	21281583	21281583	23.37	0.000
					hour	26443659	5288732	5.81	0.000
					interaction	1738149	347630	0.38	0.861
Non-probing time	*S.avenae*	General Linear Model			treatment	26062	26062	0.03	0.873
					hour	18515527	3703105	3.65	0.006
					interaction	5137643	1027529	1.01	0.419
Probing time	*B. brassciae*	476179	179	2660.22	Treatment	1176	0.4421	0.493	0.506
					Hour	45735	17.1922	0.995	0.004
					interaction	8596	3.2313	0.335	0.664
Probing time	*M. persicae* red type B	436599	179	2439.1	Treatment	12650	5.1863	0.977	0.023
					Hour	128677	52.7559	1	0.000

					interaction	8297	3.4017	0.361	0.631
Probing time	*M. persicae* green	142838	119	1200.32	treatment	8927	7.43719	0.993	0.006
					hour	1820	1.51626	0.088	0.911
					interaction	1275	1.06222	0.042	0.957
Probing time	*R. padi*	General Linear Model			treatment	4447617	4447617	3.95	0.049
					hour	22571887	4514377	4.01	0.002
					interaction	11722438	2344488	2.08	0.071
Probing time	*S. avenae*	General Linear Model			treatment	41436	41436	0.05	0.83
					hour	931885	1863771	2.09	0.079
					interaction	2154463	430893	0.48	0.788
E1 - Salivation in sieve element	*B. brassciae*	327107	179	1827.41	Treatment	6055	3.31343	0.931	0.069
					Hour	7935	4.3422	0.498	0.501
					interaction	14699	8.04361	0.846	0.154
E1 - Salivation in sieve element	*M. persicae* red type B clone	315295	179	1761.42	Treatment	2509	1.4244	0.767	0.233
					Hour	22747	12.914	0.975	0.024
					interaction	17281	9.8108	0.919	0.081
E1 - Salivation in sieve element	*M. persicae* green	98048	119	823.933	treatment	935	1.1348	0.713	0.287
					hour	1056	1.28166	0.063	0.937
					interaction	4325	5.24921	0.613	0.386
E1 - Salivation in sieve element	*R. padi*	General Linear Model			treatment	17628	17628	0.59	0.443
					hour	76192	15238	0.51	0.767
					interaction	64674	12935	0.43	0.824

E1 - Salivation in sieve element	*S. avenae*	General Linear Model			treatment	637	637	0.01	0.929
					hour	646920	129384	1.64	0.163
					interaction	191977	38395	0.49	0.785
E2 - Ingestion of sieve element sap	*B. brassciae*	446048	179	2491.89	Treatment	8708	3.4945	0.938	0.062
					Hour	98242	39.4247	1	0.000
					interaction	4239	1.7011	0.111	0.889
E2 - Ingestion of sieve element sap	*M. persicae* red type B clone	433596	179	2422.32	Treatment	24036	9.9227	0.998	0.002
					Hour	136127	56.1969	1	0.000
					interaction	2452	1.0123	0.038	0.962
E2 - Ingestion of sieve element sap	*M. persicae* green	127792	119	1073.88	treatment	25056	23.3322	1	0.000
					hour	14678	13.6682	0.982	0.018
					interaction	8306	7.7346	0.828	0.171
E2 - Ingestion of sieve element sap	*R. padi*	General Linear Model			treatment	36211237	36211237	21.97	0.000
					hour	69228224	13845645	8.4	0.000
					interaction	14359295	2871859	1.74	0.129
E2 - Ingestion of sieve element sap	*S. avenae*	General Linear Model			treatment	162976	162976	0.08	0.78
					hour	49855436	9971087	4.81	0.001
					interaction	734033	146807	0.07	0.996
Xylem Feeding	*B. brassciae*	76581	179	427.827	Treatment	2952	6.89999	0.991	0.009
					Hour	349	0.81575	0.023	0.976
					interaction	853	1.9938	0.15	0.850

Xylem Feeding	*M. persicae* red type B	131703	179	735.771	Treatment	3320	4.51227	0.966	0.034
					Hour	494	0.6714	0.015	0.985
					interaction	4389	5.96517	0.690	0.310
Xylem Feeding	*M. persicae* green	52938	119	444.857	treatment	875	1.96692	0.839	0.161
					hour	3153	7.08767	0.785	0.214
					interaction	1611	3.62139	0.394	0.605
Xylem Feeding	*R. padi*	General Linear Model			treatment	425702	425702	0.91	0.342
					hour	1369300	273860	0.58	0.712
					interaction	1194252	238850	0.51	0.769
Xylem Feeding	*S. avenae*	General Linear Model			treatment	30574	30574	0.08	0.774
					hour	2070984	414197	1.13	0.357
					interaction	2035799	407160	1.11	0.367

Table 3.2: Statistical analysis of the amount of time (recorded via the Electrical Penetration Graph (EPG) technique), that aphids spent not-probing, probing non-vascular tissue, xylem feeding, ingesting and salivating into plant sieve elements on well watered and drought host-plants. Aphid species tested were *Myzus persicae* (red type B clone and green O clone), *Brevicoryne brassicae*, *Rhopalosphium padi* and *Sitobion avenae* over a six hour period. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone; 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively. The Scheirer Ray Hare non-parametric test was used to compare the effects of time and water treatment on each of the behaviours for both *M. persicae* clones and *B. brassicae*. However, due to the unbalanced number of replicates per treatment, a General Linear Model was used to analysis of the behaviour of *R. padi* and *S. avenae*.

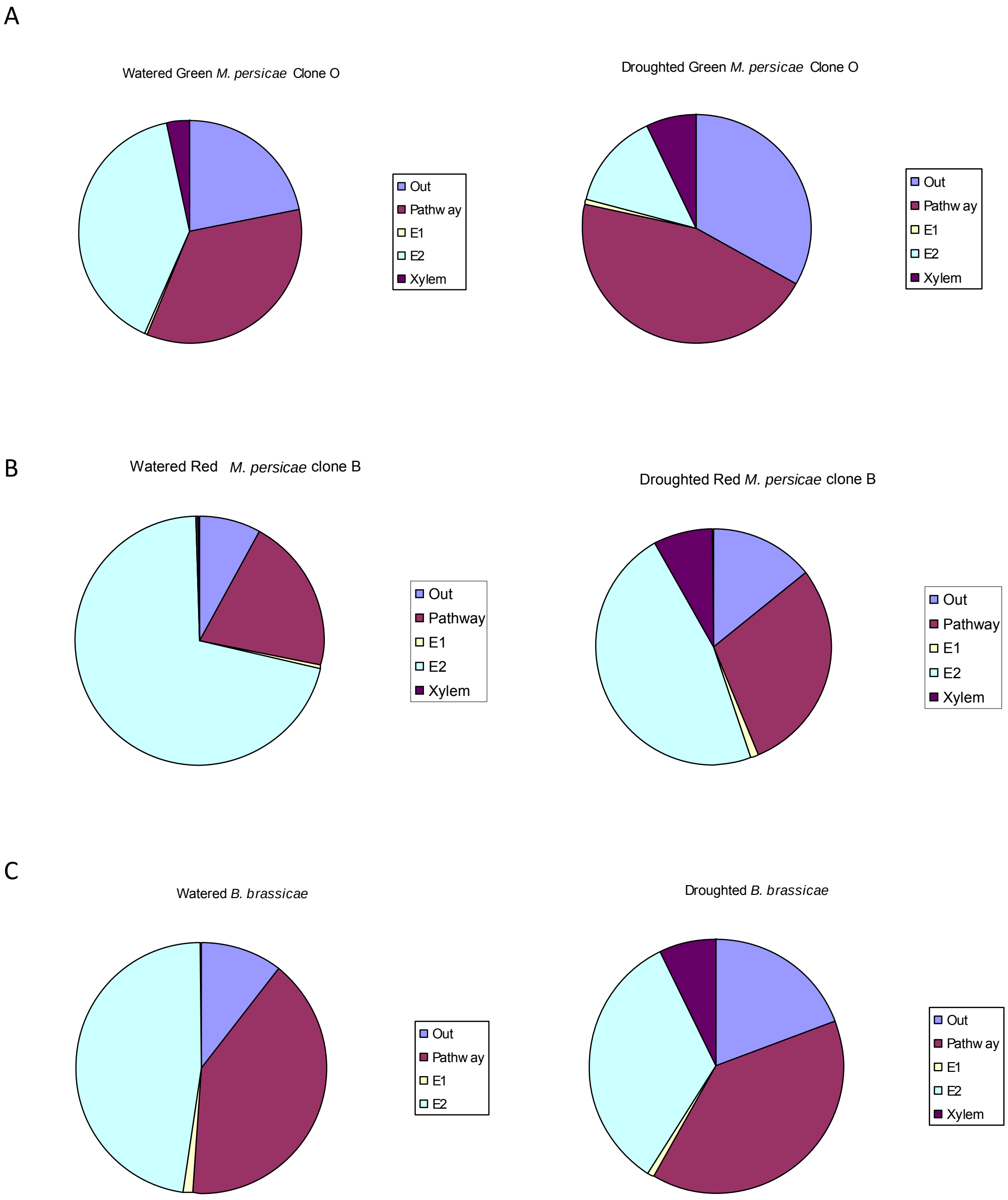


Figure 3.6: Average proportion of time (s) each hour (recorded via the Electrical Penetration Graph (EPG) technique) that aphids spent not-probing (defined as 'out'), probing non host vascular tissue (defined as 'pathway') and in vascular tissue (defined as 'xylem' feeding, salivating into the sieve element 'E1' and ingesting sieve element sap 'E2') when on well watered and drought host-plants. Aphids species tested were *Myzus persicae* (red type B clone and green O clone) and *Brevicoryne brassicae*, over a six hour period. Plants were between 27-29 days old, with droughted *Brassica nigra* plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22°C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. N = 15 per treatment for *M. persicae* red type B clone and *B. brassicae*; 10 per treatment for *M. persicae* green clone.

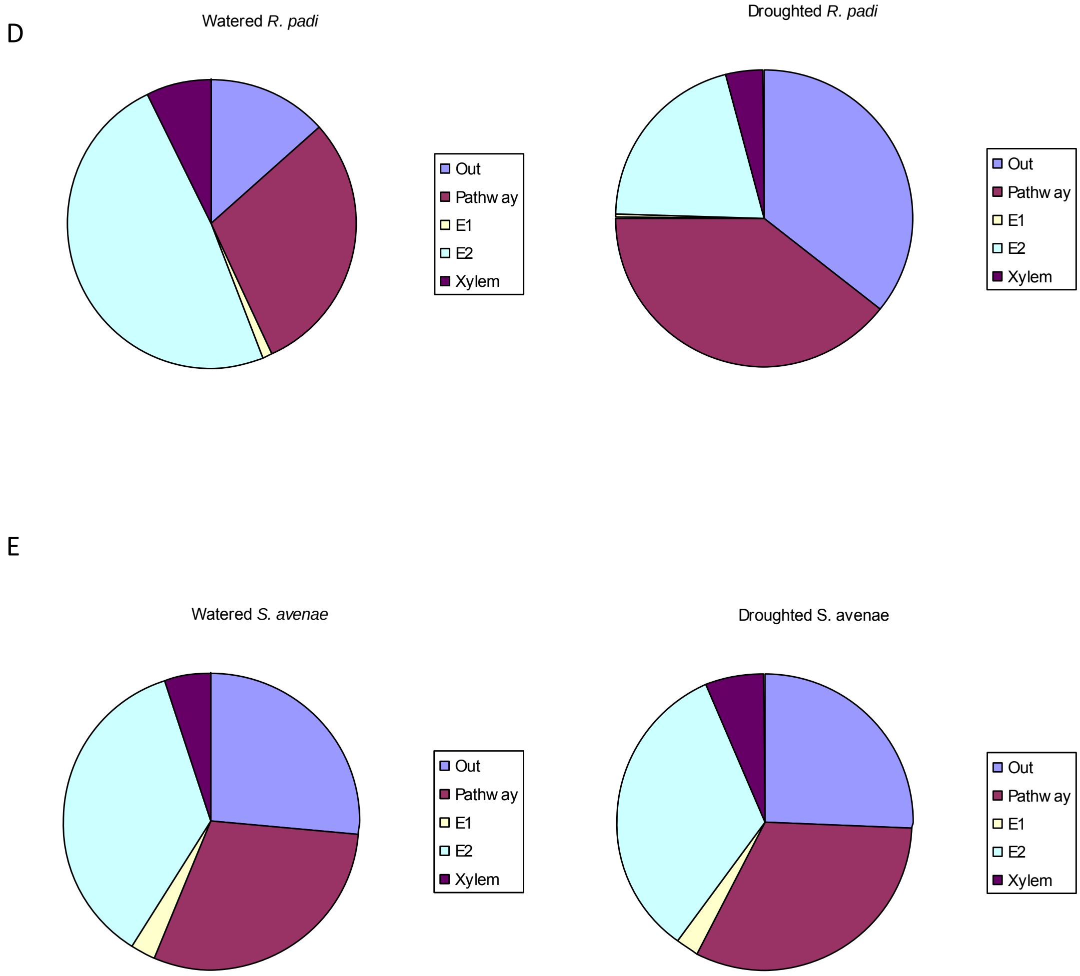


Figure 3.7: Average proportion of time (s) each hour (recorded via the Electrical Penetration Graph (EPG) technique) that aphids spent not-probing (defined as ‘out’), probing non host vascular tissue (defined as ‘pathway’) and in vascular tissue (defined as ‘xylem’ feeding, salivating into the sieve element ‘E1’ and ingesting sieve element sap ‘E2’) when on well watered and drought host-plants. Aphids species tested were *Rhopalosiphum padi* and *Sitobion avenae*, over a six hour period. Plants were between 27-29 days old, with droughted *L. perenne* plants having experienced prior to experimentation 7-9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). Aphids were removed from culture and wired straight up to the EPG system. N = 15 and 10 for well watered and drought treatment of *R. padi*; 8 and 4 for well watered and drought treatment of *S. avenae* respectively.

3.3.4 Analysis of aphid locomotion and antennae movements upon initial host plant contact

Aphid locomotion was defined as ‘walking’ or ‘still’. Analysis showed that there was no significant effect of treatments on aphid locomotion and no significant difference in locomotion activity between aphid species for the first ten minutes of contact with host plants. Similarly there was no significant difference in antennae movements between treatments. Antennae movements were categorised as forward facing (in front of aphid body), upright (vertical to aphid body) and backward facing (over aphid body). There was a significant difference in antennae movements between species. Frequencies of a forward facing antenna ($P<0.05$) as well as a backward facing antenna ($P<0.05$) differed between aphid species. *R. padi* and *M. persicae* (green and red clones) tended to have backward facing antennae, whereas *S. avenae*, showed no distinction (fig 3.8). *B. brassicae* showed exlusively backward facing antennae on droughted hosts; however effect of treatment on antennae positions was found not to be significant ($P>0.05$).

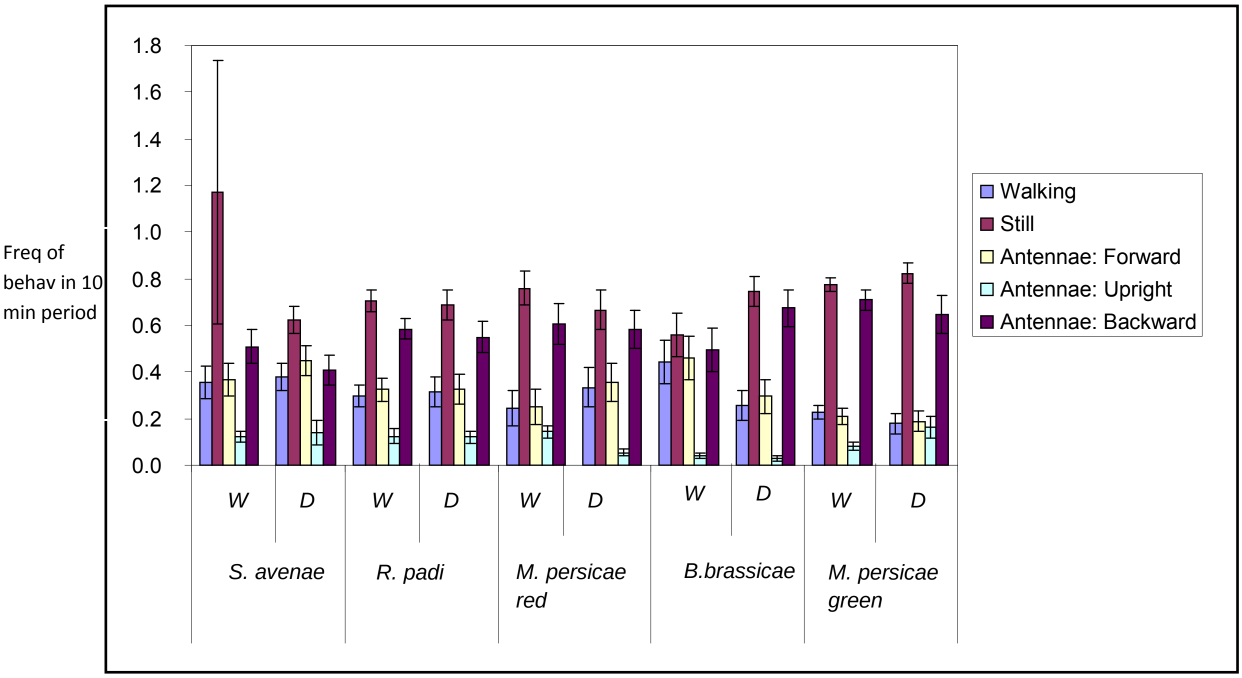


Figure 3.8: Observational behaviour scoring of aphid species *Myzus persicae* (red type B clone and green O clone), *Brevicoryne brassicae*, *Rhopalosphium padi* and *Sitobion avenae.* Behaviour responses were recorded every 0.5 minutes for a 10 minute duration on initial contact with host plant. Recorded behaviours were locomotion defined as walking and still, and antennae movements, defined as forward, upright or backward facing. Adult aphids were removed from the culture and placed onto the host plants. Plants were 29 days old, with droughted plants having experienced prior to experimentation 9 days water withdrawal in growth room conditions (22^{o}C 16:8LD). N = 15 per treatment. Statistical analysis of this data can be found in table 3.3. X axis denotes species, clone and treatment; W=well watered and D=droughted host plants

Behaviour	**Total SS**	**Total DF**	**Total MS**	**Factor**	**SS**	**SS/ TotalMS**	**Chi**	**P**
Walking	279857	149	1878.23	treatment	600	0.31945	0.428061	0.571939
				species	15168	8.07567	0.911155	0.088845
				interaction	8891	4.7337	0.684271	0.315729
Still	279804	149	1877.88	treatment	812	0.4324	0.489188	0.510812
				species	16442	8.75562	0.932493	0.067507
				interaction	8635	4.59827	0.668947	0.331053
Antennae: Forward	279695	149	1877.15	treatment	0	0	0	1
				species	21679	11.5489	0.978959	0.02104
				interaction	8548	4.5537	0.663775	0.33623
Antennae: Upward	271914	149	1824.93	treatment	5210	2.85491	0.908905	0.091095
				species	9659	5.29282	0.74145	0.25855
				interaction	3072	1.68336	0.206261	0.793739
Antennae: Backward	280172	149	1880.35	treatment	11	0.0058	0.060967	0.939033
				species	22489	11.96	0.982349	0.01765
				interaction	6945	3.6935	0.550923	0.44908

Table 3.3: Statistical analysis of locomotive and antennae behaviour frequencies recorded in the aphid species *Myzus persicae* (red type B clone and green O clone), *Brevicoryne brassicae*, *Rhopalosphium padi* and *Sitobion avenae* when placed on well watered and droughted host plants for 10 minutes. N = 15 per treatment. The Scheirer Ray Hare non-parametric test was used to assess if behaviour frequency was affected by species or water treatment.

3.4 Discussion

The EPG results in this chapter have shown that over the six hours all of aphid species altered the proportion of time spent exhibiting certain behaviours. The longer aphids were exposed to plant hosts the less time they spent not probing and probing non vascular tissue. This is consistent with the view that aphids begin feeding on a new host plant with a series of ‘test probes’, which penetrate into the epidermal and mesophyll cells of the host. During these initial probes the aphid can sample the plant chemical composition and assess the internal quality of a host. After satisfactory test probes aphids begin a sustained period of penetration in an attempt to locate the phloem (Tjallingii, 1985). In this study *S. avenae*, *B. brassicae* and one clone of *M. persicae* (red type B clone only), aphids showed a significant decrease in time spent per hour probing non-vascular tissue (aka pathway) as they became more settled on host plants. Additionally in *R. padi*, *S. avenae*, *B. brassicae* and *M. persicae* (red type B and green clone), the longer aphids were on host plants then more time was invested in E2 activities. This confirms findings in the literature that upon initial phloem acceptance aphids usually commence salivation into the sieve element (E1) before the aphids move onto the second phase of phloem activity; phloem sap ingestion (E2) coupled with watery salivation. This second phase of E2 behaviour on suitable hosts can be sustained for many hours (Montllor *et al*, 1983; Tjallingii, 1990).

Interestingly red (type B) clones of *M. persicae*, showed a significant effect of time on the duration spent each hour salivating into the sieve element (E1). Overall as time progressed, decreases in E1 activities were observed in red clones of *M.*

persicae. Typically E1 waveforms represent watery saliva which is injected into sieve elements prior to sap ingestion (Prado and Tjallingii, 1994). These observed changes in E1 salivation may be associated with the phloem's natural occlusion mechanism found in legumes, which is a calcium triggered response to injury. This occlusion mechanism involves proteins called forisomes that undergo calcium regulated conformational changes blocking the sieve tube upon injury. It has been shown that aphid saliva *in vitro* can revert forisomes into a non-plugging contracted state. Initiation of sieve tube occlusion also causes aphid behaviour to change from ingestion (E2) to watery salivation (E1) (Will *et al*, 2007). Furthermore, studies into the effects of conspecifics and heterospecifcs on feeding behaviour have given support for the idea that aphid saliva may have a pivotal role in combating host defences and altering phloem composition to be more palatable (Ameline *et al*, 2007). For example *M. persicae* host acceptance, was enhanced on leaves pre-infested by heterospecifics *Macrosiphum euphorbiae* (Dugravot *et al*, 2007), whilst *Diuraphis noxia* enhanced plant quality for conspecifics (Messina *et al*, 2002), and *Aphis fabae* resulted in conspecifics displaying reduced salivation and continuous sap ingestion (Prado and Tjallingii, 1997).

Thus, the alterations in E1 behaviour seen in *M. persicae* red clones in this study may be a product of aphid phloem conditioning. If E1 salivation is the aphid's mechanism to condition the phloem then these results suggest that the *M. persicae* red clones are more efficient at phloem conditioning, reducing their salivation rates with time. Furthermore this trend was not observed for *M. persicae* green clones, indicating that there are clonal differences in behaviour within this species. Interestingly, the red type B clone was first detected in 1996 and associated with insecticide control failure reported by Rothamsted. It was consequently one of the first MACE clones discovered in the UK (unpublished correspondence Fenton, 2010). The differential feeding behaviour of B clones to green clones may indicate that there could be indirect trade-offs between MACE insecticide resistance and other aphid fitness parameters (like feeding).

3.4.1 Effect of Drought on Feeding Behaviour

Drought treatment had a significant effect on behaviours in all aphid species and clones except *S. avenae*. In *B. brassicae* and red clones of *M. persicae*, xylem feeding was significantly increased on droughted host plants. These results confirm other studies, which have shown xylem feeding to be an important behavioural response to dehydration (Spiller *et al*, 1990; Ramirez *et al*, 2000). Studies involving TMX, a xylem borne insecticide, have highlighted that xylem feeding is an important part of an aphid's diet especially when dehydrated (Daniels *et al*, 2009), with aphids deprived of xylem feeding experiencing higher mortality rates. Additionally, in contrast to phloem feeding there is little nutrition value to be gained from xylem feeding for an aphid (Pettersson *et al*, 2007). The xylem elements, unlike sieve elements, are under negative hydrostatic pressures during stomatal opening in the day, this means that to aquire xylem sap aphids have to actively suck. This technicality has resulted in the literature referring to 'xylem drinking' as opposed to 'xylem feeding' (Pettersson *et al*, 2007).

The results in this chapter are consistent with the view that xylem drinking is an important osmoregulation mechanism in *B. brassicae,* and red clones of *M. persicae*.

However, xylem drinking was not found to be significant in all the species tested in this chapter, for example, it was not found in *R. padi*, *S. avenae* or green clones of *M. persicae*. The dependence on xylem drinking as an osmoregulation mechanism appears to vary depending on the level of water stress experienced, aphid species, clone and even morph. For example on host plants *Aphis fabae* winged morphs displayed a high percentage of xylem drinking, whilst apterous *A. fabae* displayed no xylem drinking behaviour (Powell and Hardie, 2002). In contrast, xylem drinking in *R. padi* apterous morphs was found to be common (Prado and Tjallingii, 1997), as well as in apterous morphs of *B. brassicae* and *A. pisum* (Gabrys *et al*, 1997; Tjallingii, 1986). As this study in this chapter has used only apterous morphs and similar levels of drought stress across species (defined in chapter two as higher reducing sugar levels, altered ionic and amino acid levels, and severe water loss defined as a fw: dw ratio <6), differential preference for xylem drinking behaviour can be attributed to aphid species and clonal variation, which affects the dependence aphid may place on xylem drinking as an osmoregulation mechanism. However, as stylectomy was not used to directly assess the phloem osmotic pressure, we cannot rule out the possibility of variation in plant phloem osmotic pressures also attributing to this observation.

Other significant effects of drought in this study are changes in the amount of time spent not probing, in pathway and ingesting from the sieve element. Overall, both (red and green) clones of *M. persicae* and *R. padi* spent less time ingesting sieve element sap, and more time over the six hours not probing or in pathway on droughted host plants. Drought has previously been shown to reduce sap ingestion rates in *R. padi*, when feeding on drought stressed grasses (Hale et al, 2003). This reduction in sap ingestion may have been due to the observed increase in phloem osmotic pressure and other phloem factors. However, the strength of this association seems species dependent, for example, this effect was not observed in all the species in this study. It has been hypothesised that drought induced increases in available amino acids in the sieve element could result in a reduction of sap ingestion in aphids as aphids would require less sap to obtain the required amount of amino acids for growth and development. However, in the *Arabidopsis thaliana* loss of function mutant AAP6 (amino acid permease) lowered sieve element amino acid levels but had no effect on the feeding behaviour of *M. persicae* (Hunt *et al*, 2009).

Typically aphids display reduced phloem feeding (Gabrys and Pawluk, 1999; McLean and Kinsey, 1968) and more non-penetration waveforms when fed on non host plants. Additionally, they tend to probe more frequently and for shorter amounts of time (McLean and Kinsey, 1968). The increase in non-penetration and pathway activities observed in both (red and green) clones of *M. persicae* and *R. padi* may be a result of the reduced palatability of droughted host plants. Alternatively, changes within the plant such as, the reduction in water content coupled with increases in solutes (such as sugar species, amino acids and certain cations concentrations) may have altered the gradients and chemical cues relied upon by aphids to locate the phloem. However, a reduction in the time spent in sieve element ingestion may not result in less sap uptake by the aphid.

Although this study has demonstrated an effect of time on E1 salivation in red clones of *M. persicae*, there was no significant effect of drought treatment on E1 behaviour in any of the aphid species tested. Increased salivation is usually associated with instances where sieve element sap is unpalatable to the aphid such as on resistant plant varieties (Ramirez and Niemeyer, 1999; Caillaud *et al*, 1995), in response to water stress (Ponder *et al,* 2000) or reduced amino acids concentrations (Hunt *et al*, 2009). However, the absence of significant alterations in E1 behaviours in any of the species in this study, indicate that perhaps the complexity of the plant-aphid interaction defies attempts for behavioural generalisation and that a species by species approach is required (Pritchard *et al*, 2007).

Finally, the observation scoring experiments in this study revealed that quantitative behavioural responses to drought stress occur once aphids have commenced plant penetration. Within the first ten minutes of contact with the plant host none of the aphid species tested had displayed any responses specific to drought stressed hosts. Locomotion and antennae movements were unchanged in response to host condition; however, there were species dependant behavioural preferences with regards to antennae positions with *R. padi* and both *M. persicae* clones showing a preference for facing their antennae backwards over the body. Interestingly, settled aphids typically face their antennae backwards, and whilst moving aphids face their antennae forwards and vertically. Furthermore, aphids have been observed to ‘wave’ their antennae in front of them allowing them to touch the leaf surface (Powell *et al,* 1995). This behaviour has been suggested to maximise detection of non-volatile chemical cuticle cues of the plant. Aphid antennae contain sensory organs, which are thought to be involved in olfaction detection of plant volatiles. The lack of any observed significant changes in behaviour during initial plant contact, and the preferred backwards facing antennae positions recorded in *R. padi* and *M. persicae* in this study, suggests that these aphid species did not detect any surface differences between well watered and droughted host plants to warrant further investigation.

3.4.2 Conclusion

The data in this chapter demonstrate that drought stress is detectable by aphids following host plant penetration. Alterations in the chemical composition of plants as a result of drought can result in behavioural changes in aphid feeding. Aphids feeding on droughted plant hosts typically ingest less sieve element sap and spend more time not penetrating at all or in pathway activities. Xylem drinking appears to be an established osmoregulation mechanism in some species of aphids. However, drought stress does not affect aphid species or clones homogeneously. The complex relationship between plants and aphids make general predictions above the behavioural responses to drought even at a clonal level.

Chapter 4: Reproductive performance of *Myzus persicae*, *Brevicoryne brassicae, Rhopalosiphum padi* and *Sitobian avenae* on water stressed hosts *Brassica nigra* and *Lolium perenne*

4.0 Abstract

Water stress in plants is associated with net increases in sieve element solutes, as plants try to maintain positive turgor pressure. The subsequent affect on aphid performance may be negative, as aphids have to reallocate resources from growth and reproduction to osmoregulation mechanisms. Alternatively it may be positive, as water stress makes plants more susceptible to herbivores and changes phloem composition to the benefit of phloem feeders.

The results in this chapter show that *Brevicoryne brassicae* and O clones of *Myzus persicae* have significantly reduced fecundity when fed on droughted *Brassica nigra*. In addition, *Rhopalosiphum padi* fed on droughted *Lolium perenne* also had significantly lowered fecundity. Interestingly, although B clones of *M. persicae* and *Sitobion avenae* showed reduced fecundity when fed on droughted *B. nigra* and *L. perenne* (respectively), it was found to be not significant. Furthermore, honeydew analysis of both *M. persicae* clones found aphids significantly reduced their rate of honeydew production when on droughted host plants.

The conclusion of this chapter is that the level of drought used in this study is detrimental to aphid reproduction. It is also suggested that the level of drought influences aphid performance and that there is heterogeneity in the adaptability of aphids to water stress.

4.1 Introduction

The ultimate goal of all organisms is reproduction, which accounts for a large proportion of resource allocation. An organism's reproductive fitness is determined by the amount of progeny that the organism produces and so the number of genes reaching the next generation.

Reproductive success depends on many factors such as an organism's body size, its' physiological state as well as the environmental conditions. Stress, whether abiotic and biotic can be detrimental to an organism's reproductive success and strategies used to cope with a stressor can use up resources and energy that would ordinarily be utilised in maximising reproductive output. Generally energy investment in reproduction follows two different patterns; organisms may be r-selected or K-selected. In K-selected organisms offspring are relatively large and require high resource input by the parents, resulting overall in fewer offspring.

Invertebrates such as aphids, often have an r-selected reproductive strategy. Smaller offspring require a smaller amount of energy from parents, which can then produce large numbers of progeny. In the aphid species considered in this thesis, each individual is capable of producing hundreds of progeny in a lifetime. This reproductive strategy does come with the trade-off that there is less energy in parental care. In the case of aphids there is yet no record of any type of parental care, with progeny able to commence feeding from the plant host immediately.

4.1.1 Aphid life cycles

Aphids have evolved a highly adaptable lifecycle that consists of both parthenogenetic and sexual reproduction. Embryogenesis can be initiated in the grandparent; this is known as the telescoping of generations. Some species of aphids can have a development time from first instar nymph to adulthood that lasts as little as nine days (OzgÖkqe and Athhan 2005). Once into adulthood within a week of the onset of reproduction an aphid can produce 50 progeny. This reproductive capacity is a classic example of an r-selected organism, as well as being a major reason why aphids have become such prominent agricultural pests globally.

The evolutionary advantage of a rapid generation time is that the organism can rapidly increase population numbers under favourable environmental conditions. In the case of aphids, the annual sexual phase is usually initiated in response to a number of environmental and genetic factors. Photoperiod was the first environmental cue to be discovered that acted as a stimulus in initiating sexual morphs, and it was later found that specifically the length of night as opposed to the length of day was the stimulus for sexual morph production (Lees, 1961; 1963; 1964). Temperature was also later identified as being an important factor in sexual morph production (Dixon and Glen, 1971). Changes in host plant growth, such as a halt in plant growth have also been found to be important cues in root feeding aphids, as they cannot be influenced directly by night or day length (Hille Ris Lambers, 1960; Forrest, 1970).

In addition to environmental factors it was also found that aphids had an intrinsic clock that could delay the production of sexual morphs. Offspring of *Megoura viciae* fundatrices (stem mothers that hatch from the egg after winter), do not produce sexual morphs before 80-90 days has elapsed, despite being reared in conditions optimal for sexual morph production (Lee, 1960). All of these mechanisms are important in ensuring that the holocyclic oviparae (sexual female) is produced in time to mature to adulthood with egg laying capabilities, just before the autumn induces leaf senescence in temperate environments. However, not all aphids have sexual morphs which are produced under the correct environmental cues (holocycly), some aphids genotypes are purely asexual (anholocycly). Furthermore, aphid species can differ in host specificity with some species alternating hosts throughout the year, whereas others are monophagous, feeding on one host all year round. In order to understand the adaptability and plasticity of the aphid species studied in this chapter, it is important to have an understanding of each aphid species lifecycle.

4.1.2 Myzus persicae

Myzus persicae (the peach-potato aphid) is a cosmopolitan, holocyclic, polyphagous aphid, which has gained pest status due to its ability to spread plant viruses and its rapid development time. The aphid overwinters as an egg stage on *Prunus* spp. In the spring the eggs hatch and the emerging nymphs feed on the young plant foliage, after several generations (winged) alates are produced (late April-early June), which disperse from *Prunus* to summer hosts. *M. persicae* has many summer hosts spread over 40 dicotyledon plant families and include many economically important plants (Blackman, and Eastop, 1984). In temperate climates *M. persicae* returns to *Prunus* in the autumn (late September-early October), where mating occurs and eggs are deposited. All generations except the egg producing autumn generation are parthenogenetic (asexual). Although *M. persicae* overwinters as eggs on peach, overwintering in mild winter climes can also occur as parthenogenetic clones on herbaceous plants, weeds and brassicas. *M. persicae* unlike other species do not form dense colonies, instead when aphid density increases *M. persicae* disperses to other host plants and therefore spreads disease making it an effective virus vector. Generally in the UK *M. persicae* numbers peak around July, its dispersal mechanisms contribute to it being an efficient virus transmitter (van Emden *et al*. 1969).

4.1.3 Brevicoryne brassicae

The mealy cabbage aphid, *Brevicoryne brassicae*, is a specialist aphid species that does not reproduce as rapidly as *M. persicae. B. brassicae* also requires the cue of sinigrin, a glucosinolate in *Brassica* plants, to initiate a feeding response, so as a result its entire life cycle is restricted to the *Brassicaceae* (*Cruciferae)* family (Gabryś *et al*, 1997). *B. brassicae* is holocyclic, and can overwinter on cruciferous crops as eggs which hatch between February to April. Summer migration occurs in May-July as (winged) alate forms are produced, populations usually peak around July to August and autumn migrations follow (September-October) which result in egg laying. If mild winters are experienced, like *M. persicae*, *B. brassicae* can also spend the winter as parthenogenetic mobile forms resulting in earlier colonisation of cruciferous crops the following spring.

4.1.4 Sitobion avenae

The majority of *Sitobion avenae* (grain aphid) species no longer have host alternation. It has evolved to spend its entire life cycle on cereals and grasses. *S. avenae* can also produce sexual morphs, and a small proportion of the population will lay overwintering eggs, which will hatch in March on species of Poaceae in Europe. However, where winters are mild, the majority of the population will continue through the winter parthenogenetically, similarly to *B. brassicae* and *M. persicae* (Blackman and Eastop, 2007).

4.1.5 Rhopalosiphum padi

A major pest of cereal crops, *Rholpalosiphum padi,* the bird cherry-oat aphid feeds on all grasses and cereals, as well as some *Poaceae* species. It can colonise many monocotyledonous plant species and even some dicotyledons. *R. padi* also has a broader range than *S. avenae*, and lays eggs overwinter on **Prunus padus**, which hatch from April onwards. Alate forms are produced in May-June and migrate to species of *Gramineae*. Upon infestation *R. padi* settle on lower leaves and stems, moving higher up the host as the population size increases. Summer migrations of the parthenogenetic clones usually result in the movement away from cereals to wild grasses, until the autumn migration of *R. padi* to the winter host **P. padus.** Like the other aphid species discussed, mild winters mean *R. padi* can overwinter as eggs on *P. padus* and parthenogenetic forms on grasses (Blackman and Eastop, 2007).

4.1.6 Measuring Insect Performance

There are a variety of methods to measure insect performance, most of which will depend upon the experimental system being used. Fecundity is probably one of the most frequently used parameters to assess insect performance (Wearing, 1972; Li *et al*, 2004), whilst counting the number of unborn aphid nymphs can even give a measure of potential fecundity (Pons and Tatchell, 1995). Relative growth rates (RGR) and developmental weight increases are also parameters used to assess aphid performance (Castle and Berger, 1993; Mesfin *et al*, 1995). Low RGRs can be correlated with unsuitable hosts (Van Emden and Bashford, 1971), whilst measuring insect size, weight, longevity, survival and developmental rates can also be useful in assessing insect performance (Llewellyn and Brown, 1985; Kennedy and Kishaba, 1977; Miles *et al*, 1982; Grüber and Dixon, 1988). The duration of the pre-reproductive, reproductive and post-reproductive lives can also be measured (Williams, 1995; Kift *et al*, 1996; Bethke *et al*, 1998).

The intrinsic rate of natural increase (r_m) is another method to measure performance but unlike the others it has the advantage of factoring the survival and fecundity of an insect during each day of its reproductive life (Birch, 1948; Wyatt and White, 1977). The r_m provides a single statistical value that summarises fecundity, survival and development; this provides a useful performance index to compare the performance of insects exposed to different treatments. The disadvantage with this method is that it can be time consuming.

Special care must be taken to avoid any sources of variability when measuring insect performance. For example, variation in the host plants such as leaf age or exposure to prior aphid feeding can influence future aphid performance as well as the type of aphid clone, morph, birth weight or age (Dixon, 1987; Sandström, 1994a). Interestingly, marked differences in performance have been found between and within aphid clones (Sandström, 1994a; Sandström 1994b; Sandström and Pettersson, 1994). However, whether the *M. pesicae* clones used in this chapter will vary in performance to drought stress is yet to be established.

4.1.7 Measuring Honeydew

Although EPG used in chapter three can define if an aphid is spending on average more time in phloem ingestion feeding activities, it cannot be used to determine if aphid ingestion rates are greater. However, by studying the frequency and quantity of honeydew production (honeydew being the liquid excretion of the remaining phloem sap ingested by aphids), we can determine the rate of secretion. Honeydew rate of production is defined as the volume of honeydew produced over a given period of time, and the frequency of production is the number of droplets produced in any unit of time (Mittler, 1958).

The volume, dry mass and composition of honeydew is likely to be affected by the solute potential and composition of ingested phloem sap, as well as the gut metabolic activity within the aphid. This makes honeydew analysis an important aspect of understanding how the quality of assimilated nutrients can affect aphid performance.

There are a variety of techniques that can be used to determine volume and frequency of honeydew droplets. Honeydew frequency can be recorded using collection in oil (Mittler and Meikle, 1991), circles or paper (Auclair, 1958), or honeydew clocks (Smith, 1937; Broadbent, 1951; Ajayi and Dewer, 1982). Honeydew clocks consist of a motor that rotates at a constant velocity to which a disc is placed on top to collect honeydew droplets. The advantage of honeydew clocks is that excreted droplets land on clean sections over the duration of the experiment so each droplet is discrete from other droplets. To determine volume, collection in oil is the easiest method, as both frequency and volume can be calculated by measuring droplet diameter and occurrence.

4.1.8 Effect of drought on plants

Water stress in plants is associated with net increases in sieve element solutes (Smith and Milburn, 1980), to maintain positive phloem turgor pressure. Carbohydrate concentrations can increase by 20-40% (Najla *et al*, 2010), and cause increases in amino acids and osmotic pressure (Good and Zaplachinski, 1994; Thomas, 1991; Hale *et al*, 2003; Volaire *et al*, 1998). Specifically water deficit can cause increase in levels of serine and GABA, valine, isoleucine, leucine, threonine, glutamate, aspartate and proline (Tully and Hanson, 1979; Girousse *et al*, 1996). However, it is not just amino acids and carbohydrates that can alter in response to water stress, ions can also increase as they play a role in phloem osmotic pressure adjustment and the transport of photo-assimilates (Gould *et al*, 2004).

In chapter two, it was shown that water withdrawal in *B. nigra* and *L. perenne* at 20 days of age resulted in a drop in fresh weight. Furthermore, total water withdrawal for 6-9 days resulted in increases in reducing sugar levels, alterations in amino acid and cation levels, and a substantial water loss quantified in the form of fw: dw ratios in both species.

4.1.9 Effect of diet on aphids

As the composition of sieve element sap varies in host plants in response to drought, so may the performance of aphids feeding on the hosts. Studies have shown that the concentrations of essential amino acids, arginine, threonine, aspartate, glutamate, glycine, tyrosine, proline and valine are positively correlated with aphid performance (Sandström and Pettersson, 1994; van Emden and Bashfod, 1971; Holopainen *et al*, 1997; Srivastava *et al*, 1983), whilst methionine, histidine and phenylalanine are essential for normal aphid growth (Leckstein and Llewellyn, 1973). Interestingly, enhanced levels of glutamate can also be correlated with inhibition of aphid feeding (Sandström and Pettersson, 1994). This is also the case with proline, which has been shown to be in higher concentrations in plant resistant varieties (van Emden, 1964).

Aphids also respond to the levels of carbohydrates in their diet. Low concentrations of sucrose have been shown to cause a reduction in feeding rates (Srivastava and Auclair, 1971); this is thought to be due to a lack of phagostimulation. Additionally, if the level of sucrose is too high, then this can also lead to a reduction in aphid feeding rate (Mittler and Meikle, 1991). Aphids ordinarily reduce the osmotic pressure of phloem sap by assimilating and transforming sugars (Mittler and Meikle, 1991; Wilkinson *et al*, 1997). Hsowever where levels of sucrose are higher than normal, the increased osmotic stress may reduce aphid performance, as body water is lost to the gut (Hendrix *et al*, 1992).

4.1.10 Plant Stress Hypothesis

One hypothesis is that aphids feeding on droughted *B. nigra* and *L. perenne*, would have to reallocate their energy resources from growth and reproduction into osmoregulation mechanisms to cope with the osmotic stress from feeding on droughted host plants. This resource allocation is predicted to be observed via an overall reduction in fecundity in aphids fed on droughted host plants, when compared with aphids on well watered host plants. In addition the reduction in performance between species may vary, with species that are more specialised with slower reproductive rates like *B. brassicae*, displaying a greater reduction in performance than species that are cosmopolitan, highly polyphagous, with higher reproductive rates, like *M. persicae*.

Alternatively, the plant stress hypothesis (White 1984) proposes that under water stress host plants may become more susceptible to herbivores, with aphids benefiting from the change in plant physiology, such as the increase in amino acid concentrations in both host plant species (see Chapter 2 results). Amino acids are essential to insect development and growth (Brodbeck and Strong, 1987), and an increase in free amino acids, sugars and minerals may result in host plants being more attractive to insect herbivores. Additionally plant defences may be reduced in water stressed plants (Rhoades, 1985), making plants more susceptible to insect attack. So far a number of studies have supported this idea, with higher fecundity, faster development, and longer adult reproductive lives being observed under drought stress for the aphid species; *Myzus persciae* (Wearing, 1967; Wearing and van Emden, 1967; Wearing, 1972), *Brevicoryne brassicae* (Wearing, 1972; Miles *et al*,

1982) and *Diuraphis noxia* (Oswald and Brewer, 1997). Additionally the grasshopper, *Melanoplus differentialis*, was shown to prefer wilted to turgid plants (Lewis, 1982); further studies found that grasshoppers fed more on plants enriched with proline and valine (Haglund, 1980). Thus, increases in free amino acids, especially essential amino acids in drought stressed plants could lead to proliferation of herbivores on water stressed plants, as herbivores benefit from the increased availability of amino acids.

In contrast there have been many studies that contradict the plant-stress hypothesis, and instead support the notion described earlier, that resource allocation to osmoregulation mechanisms as a result of feeding on drought stressed host, should lead to a detrimental effect on aphid performance. More recently the plant-stress hypothesis has come under direct attack (Larsson, 1989), with many studies showing evidence that there is no effect of drought stress on development time, adult weight, nymphal mortality or number of embryos in the aphid species *S. avenae* and *R. padi* when fed on drought stressed wheat, *Triticum aestivum* (Pons and Tatchell, 1995). No effect on fecundity, longevity and r_m was also observed in *Aphis gossypii* (Bethke *et al*, 1998) when fed on drought stressed chrysanthemums, *Dendranthema grandiflora*. In addition, residence time of *R. padi*, *S. avenae* and *Macrosiphum euphorbiae* (Honěk *et al*, 1998) and feeding, survival and oviposition of *Aphis fabae* (Kennedy *et al*, 1958; Kennedy and Booth 1959) were all negatively affected by drought stress.

These studies suggest that the response of aphids to water stress may be too complex to summarise within one simple hypothesis. It is possible that the variation in results in the literature may be due to the differential plant responses to water stress, and subsequent differential insect responses to plant stress. Additionally, in previous studies the level of drought has not always been defined in a way that can be compared to other studies. It may be that aphid species benefit from low level water stress situations and consequential rises in certain amino acid concentrations, but under extreme water stress scenarios, the cost of aphid osmoregulation and overcoming drought induced responses such as, leaf waxiness, prove detrimental to aphid performance.

In this chapter the aim is to assess aphid performance in response to a defined extreme level of water stress (defined in chapter 2) in the form of fecundity measurements. In addition, measurements of honeydew frequency and volume will allow an assessment on the rate of phloem sap ingestion in aphids subjected to a drought regime. Honeydew analysis will be interpreted within the context of the earlier EPG analysis (chapter three) and fecundity results.

To ascertain an overview of the fecundity response of aphids to drought four aphid species (*Myzus persicae, Brevicoryne brassicae, Sitobian avenae* and *Rhopalosiphum padi)* and two genotypes (*M. persicae* green ‘O’ clone and red ‘B’ clone) were analysed. Aphid species range from strictly monocotyledonous non-host alternating species like *S. avenae* to dicotyledonous host-alternating species like *M. persicae*. For honeydew measurements in response to drought only the two *M. persicae* clones (O and B) shall be measured.

4.2 Materials and Methods

4.2.1 Plant Species

B. nigra and *L. perenne* used in experiments were seeds obtained from John Chambers Wildflower Seeds Co. from wild seed stocks in Northamptonshire, UK

4.2.2 Aphids

Myzus persicae clones used in the experiment were the red type B clone and the green O clone, which were obtained from Brian Fenton's cultures in SCRI. *Brevicoryne brassicae*, *Rhopalosiphum padi* and *Sitobion avenae* were obtained from Phil Northing culture stocks at FERA.

4.2.3 Plant Culturing

Plants were grown in the insect free plant growth rooms with a 16:8 light cycle at a temperature of 22^{o}C. Growth room PAR was between 80-120mol m^{-2} s^{-1} with a relative air humidity 70-80%. Plants were grown in John Innes peat based compost with mixed in Silvaperl in 100-150ml plastic pots (*L. perenne* and *B. nigra* respectively).

4.2.4 Aphid Culturing

Special tents are used to isolate the cultures from any infestation from other insects as well as to prevent the escape of insects from the culture. Plants are reared up in the insect free plant growth rooms before being placed into tents (Megaview Bugdorm 3120) to be fed on by the insects. The constant removal of old dying plants and replacement with young new plants into the cultures ensures that the culture is kept healthy with an abundant supply of insects. Growth rooms are run at a constant temperature of 18^{o}C under a 16:8 light cycle.

4.2.5 Fecundity

Adult aphids were removed from the culture and immediately transferred onto experimental plants in the insect room with a constant temperature of around 18^{o}C and 16:8 light cycle. Plant hosts species were *B. nigra* and *L. perenne* for *M. persicae* (green O clone and red type B clone) / *Brevicoryne Brassicae* and *Rholashipon padi* / *Sitobion avenae* respectively. The host plants had been subjected to one of two different types of treatments, control or extremely drought stressed (as defined in the discussion of chapter two). All plant hosts were 26 days old, with drought stressed plants experiencing total water withdrawal for 6 days prior to the experiment. Aphids were left on the plants for 72 hours, thus droughted plants experienced an additional 72 hours of water withdrawal (totalling 9 days of water withdrawal at the end of the experiment), whilst well watered plants had access to water. Droughted plants of both species had increased levels of sugars, calcium and magnesium, reduced water content and changes in amino acid concentrations to well watered controls (see results in chapter 2). Aphids during this period were contained on the plant host using an inverted clear plastic cup with mesh for ventilation; this prevented aphid dispersal to other plants. After 72 hours the aphids were removed and the total number of progeny produced for each aphid was recorded.

4.2.6 Honeydew

The frequency and volume of honeydew drops excreted by adults feeding on well watered and droughted plants was determined using a honeydew clock (Wilkinson and Douglas, 1995). Adult reproducing aphids were taken from culture and immediately placed (using a small paint brush) onto plant leaves, for drop frequency measurements one aphid per plant was used, whereas for drop volume measurements aphids were placed onto the plants in groups of 5. Aphids were left for an hour to settle before the plant was positioned over the honeydew clock to collect droplets. Only aphids that had fully settled were used for honeydew collection, this reduced the risk of using aphids that subsequently dispersed half way through the experiment. To measure honeydew rate, each plant leaf was placed horizontally above a plastic petri dish (14 cm diameter) and connected to the hour-hand spigot of a kitchen clock. This ensured a rotation of 360^{o} in a 12 h period. In the petri dish a circle of filter paper was placed (125 mm diameter; Fischer Scientific, UK) pre-treated with 0.1% bromophenol blue (Sigma-Aldrich Company Ltd., UK) and 0.01 M HCl (Sigma-Aldrich Company Ltd.). As the aphids excreted the honeydew it was visualised by a colour change on the dyed filter paper from yellow to blue, after 12 hours the droplets could be counted for each hour from the filter paper to work out honeydew production rate. Only results where aphids were still in the same position post and prior to the experiment were used. In addition a silanized dish (which prevents the adsorption of the honeydew droplets to the glass surface) is filled with oil and placed under the aphids. The honeydew drops collected in the dish can be later quantified for volume by measuring the diameter of the sphere which would not stick to the glass and alter shape.

4.2.7 Statistical Analysis

Data was entered into MINITAB and analysed using the Anderson-Darling Normality Test. If the P value was ≤0.05 then the data was not normally distributed. In cases where data is normally distributed parametric tests utilising the means, such as the t-test were used. In examples where data was not normally distributed, non-parametric statistical tests that use the median like the Mann-Whitney test were used.

4.3 Results

Adult green (O) clones of *M. persicae* showed a significant reduction (t-test P< 0.01) of approximately 19% in fecundity over the 72 hour period, when fed on droughted *B. nigra* plants as opposed to well watered controls (fig 4.1). The average nymph production fell from 14.9 per adult (± 0.64 SE N=10) in green *M. persicae* clones on well watered *B. nigra* to 12.0 (± 1.77 SE N=11) when fed on droughted *B. nigra* in the 72 hour period.

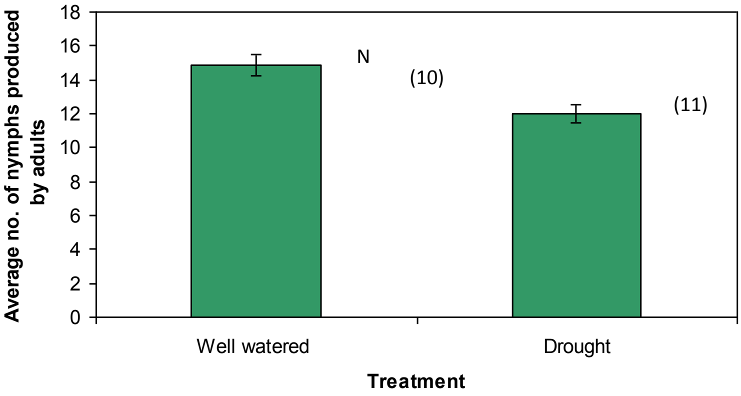


Figure 4.1: Average fecundity (±SE) over a 72 hour period in adult green O clones of *M. persicae* fed on well watered and droughted *B. nigra.* Plants were grown in growth room conditions (22^{o}C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 6 days before experimentation, and 3 days during the experiment. Fecundity experiments were conducted at 16^{o}C and under a 16:8 LD cycle. Fecundity significantly fell in aphids fed on droughted *B. nigra* (t-test value 0.003), N is shown in brackets on chart.

A drop in fecundity was also observed in the adult red (B) clones of *M. persicae.* However, the fall in fecundity was not as large with an average drop of approximately 9% in fecundity (fig 4.2) over the 72 hour period*,* from 4.6 (± 0.36 SE N=11) to 4.2 (± 0.42 SE N=13), on well watered to droughted *B. nigra* respectively. This reduction was not significant.

These results demonstrate that an extreme level of drought has a negative effect on the fecundity of *M. persicae*. It also demonstrates clonal differences within the species, *M. persicae*, with the green O clone and red B clone displaying different fecundity rates on *B. nigra* and a differential degree of response to the water-stressed hosts.

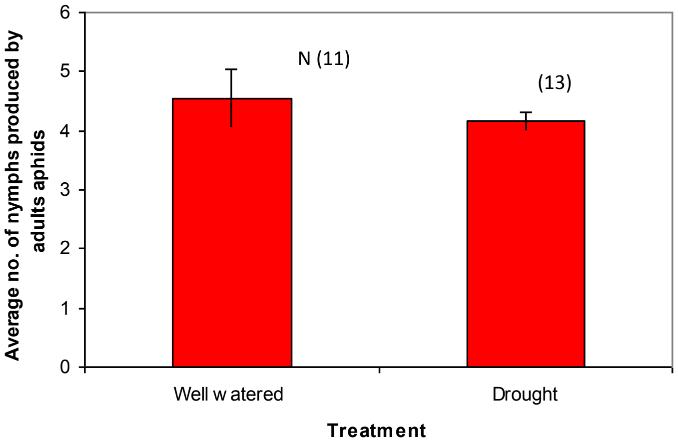


Figure 4.2: Average fecundity (±SE) over a 72 hour period in adult red B clones of *M. persicae* fed on well watered and droughted *B. nigra.* Plants were grown in growth room conditions (22^{o}C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 6 days before experimentation, and 3 days during the experiment. Fecundity experiments were conducted at 18^{o}C and under a 16:8 LD cycle. Fecundity did not significantly alter in aphids fed on droughted *B. nigra* when compared to well watered *B. nigra* (t-test value 0.49), N is shown in brackets on chart.

In *Brevicoryne brassicae* there was a significant (t-test P<0.001) marked decrease in fecundity of 37% over the 72 hours (fig 4.3). Adult apterous *B. brassicae* fed on well watered *B. nigra* produced an average of 9.3 (±0.56 SE N=15) nymphs over the 72 hour period, whilst only 5.9 (±0.42 SE N=19) nymphs were produced on droughted *B. nigra* hosts. This data also suggests that water stress affects the reproductive performance of the specialist *B. brassicae* greater than the polyphagous aphid *M.*

persicae, as reductions of 37% were observed in the fecundity of *B. brassicae*, compared to only 19% in (green O clone) *M. persicae*.

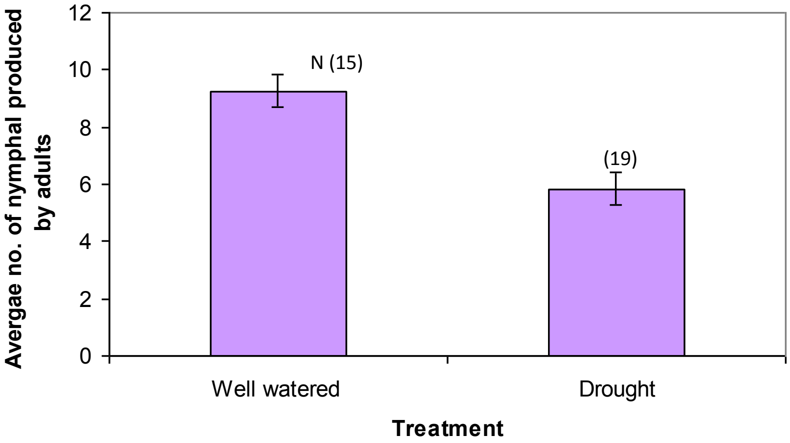


Figure 4.3: Average fecundity (±SE) over a 72 hour period in *B. brassicae* fed on well watered and droughted *B. nigra.* Plants were grown in growth room conditions (22°C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 6 days before experimentation, and 3 days during the experiment. Fecundity experiments were conducted at 18°C and under a 16:8 LD cycle. Fecundity significantly decreased in aphids fed on droughted *B. nigra* when compared to well watered *B. nigra* (t-test value $P<0.001$), N is shown in brackets on chart.

The trend of reduced fecundity in aphids feeding on water stressed plants was also seen in *Rhopalosiphum padi* and *Sitobion avenae. R. padi* adult fecundity was reduced by an average of 35% in aphids fed on drought stressed *Lolium perenne.* Average fecundity over the 72 hour period in adult *R. padi* aphids was 4.0 (±0.49 SE N=10) nymphs when fed on well watered *L. perenne* plants. However, on drought stressed *L. perenne,* nymphal production dropped to an average of 2.6 (±0.16 SE N=10) nymphs over the 72 hour period. This observed reduction in reproductive performance in *R. padi* was also statistically significant (t-test $P<0.05$). In contrast, *S. avenae* showed no statistically significant differences in reproductive output, despite a visible overall reduction of 48% in nymphal production on drought stressed *L. perenne*. This could be attributed to the low number of biological replicates for this species, as only 5 individuals per treatment were successfully recorded on *L. perenne*. The low biological replication was due to difficulties encountered in culturing *S. avenae* on *L. perenne* prior to experimentation. Despite this average nymphal production of *S. avenae* on well watered *L. perenne* was 5 (±0.89 SE N=5) nymphs over the 72 hour period, whereas on droughted *L. perenne* it was 2.6 (±0.81 SE N=5).

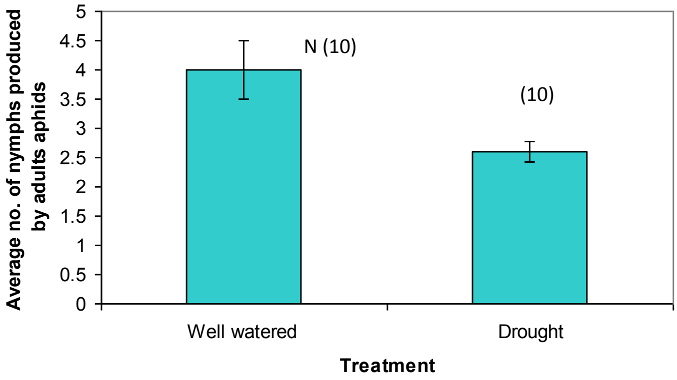


Figure 4.4: Average fecundity (±SE) over a 72 hour period in *R. padi* fed on well watered and droughted *L. perenne.* Plants were grown in growth room conditions (22°C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 6 days before experimentation, and 3 days during the experiment. Fecundity experiments were conducted at 18°C and under a 16:8 LD cycle. Fecundity significantly decreased in aphids fed on droughted *L. perenne* when compared to well watered controls (t-test value 0.015), N is shown in brackets on chart.

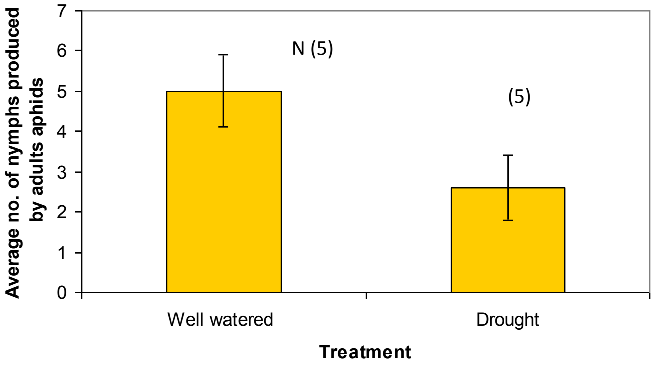


Figure 4.5: Average fecundity (±SE) over a 72 hour period in *S. avenae* fed on well watered and droughted *L. perenne.* Plants were grown in growth room conditions (22°C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 6 days before experimentation, and 3 days during the experiment. Fecundity experiments were conducted at 18°C and under a 16:8 LD cycle. Fecundity did not significantly alter in aphids fed on droughted *L. perenne* when compared to well watered controls (t-test value 0.08), N is shown in brackets on chart.

Honeydew frequency was significantly affected by drought treatment in both *M. persicae* clones (O and B types). Honeydew frequency (Mann-Whitney $P<0.05$) and droplet volume ($P<0.001$) was reduced in green *M. persicae* (O) clones (fig 4.6), when subjected to extreme drought, by 54% and 17% (respectively). However, whilst red *M. persicae* (B) clones (fig 4.7) displayed a reduction in honeydew frequency by 140% in response to drought ($P<0.001$), honeydew droplet volume was significantly increased ($P<0.001$) by 4%.

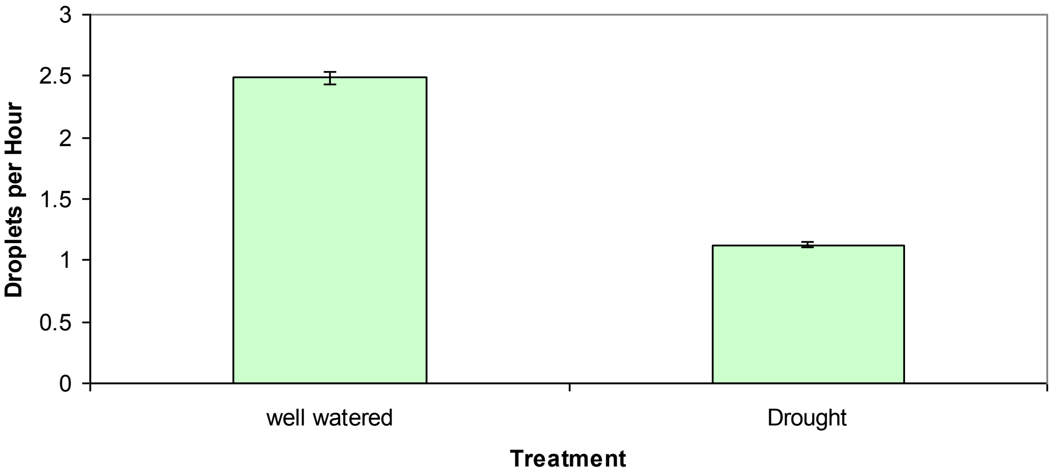


Figure 4.6: Average honeydew frequency (±SE) over a 12 hour period in green O clones of *M. persicae* fed on well watered and droughted B*. nigra.* Plants were grown in growth room conditions (22^{o}C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 8 days prior to experimentation. Honeydew experiments were conducted at approximately 18^{o}C. Honeydew frequency significantly reduced in aphids fed on droughted hosts (Mann-Whitney value 0.032, N 15 per treatment). Additionally droplet volume also significantly reduced in response to drought ($P<0.001$, $N=64$ and 53 for well watered and drought treatment, respectively).

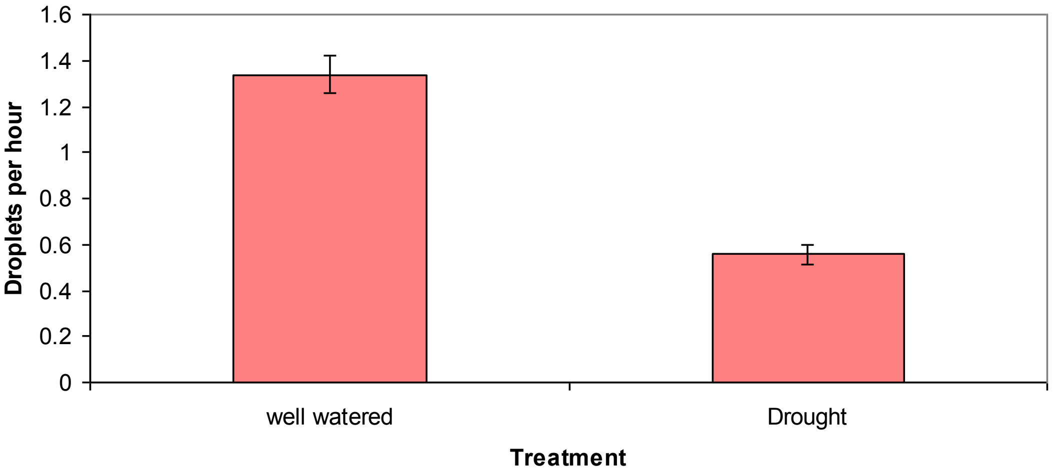


Figure 4.7: Average honeydew frequency (±SE) over a 24 hour period in red B clones of *M. persicae* fed on well watered and droughted B. *nigra.* Plants were grown in growth room conditions (22°C 16:8LD) until 20 days old prior to experiment, drought treatment consisted of water withdrawal for 8 days prior to experimentation. Honeydew experiments were conducted at approximately 18°C. Honeydew frequency was significantly reduced in aphids fed on droughted hosts (Mann-Whitney value 0.000, N is 10 for drought treatment and 15 for well watered treatment), whilst droplet volume significantly increased ($P<0.001$, N 100 per treatment) in response to drought, however the increase in droplet size was marginal at 4%

	Control *M. persicae* green O clone	Drought stressed *M. persicae* green O clone	Control *M. persicae* Red B clone	Drought stressed *M. persicae* Red B clone
Honeydew Droplet Average Volume mm	0.93	0.64	0.73	0.79
N	64	53	100	94

Table 4.1: Average honeydew droplet volume of *M. persicae* green O and red B clones when fed on well-watered and drought stressed *B. nigra* over a 12 hour period at 16°C and under a 16:8 LD cycle. Plants were grown in growth room conditions (22°C 16:8LD) until 20 days old prior to experiment; drought treatment consisted of water withdrawal for 6-8 days before experimentation.

4.4 Discussion

Fecundity was reduced in all species of aphid; however it was only significantly reduced in the green O clone of *M. persicae*, *B. brassicae* and *R. padi*. In *S. avenae* although fecundity was reduced by the drought treatment, it was not a statistically significant reduction. Interestingly, the two clones of *M. persicae* responded differently to the drought stressed host, with no significant effect of drought treatment on red *Myzus* clone ‘B’, but a significant effect of drought on the green *Myzus* clone ‘O’. As many laboratory lineages are derived from a single parthenogenetic female aphid, the observed differences between *M. persicae* clones could be due to clonal variation. Additionally studies into *M. persicae* and aphid species *Aphis craccivora*, *Sitobion miscanthis* and *Acyrthosiphon pisum*, have shown genetic attributed variation in growth and development rates even when reared on host plants of similar quality (Edwards, 2001; Sandström, 1994; Sunnucks *et al*, 1998). Field studies into clonal composition using molecular markers have also revealed that aphid clones proliferate differentially during the summertime (Tomiuk and Woehrmann, 1981; Haack *et al*. 2000). Individual reproductive rates may also differ over the lifetime of the aphid, with aphids not producing nymphs at the same constant rate throughout adulthood. Studies into the dynamics of nymphal production showed that *A. pisum* produced more nymphs during the daytime (Zeng *et al*, 1993) than during the night. The observed difference between fecundity of both *M. persicae* clones in this study, is further evidence that genetic variation between clones can affect clonal performance responses sto certain host plants and environmental conditions. This makes it difficult to predict precisely the response of other clones of aphids. However, the data does show that under extreme drought none of the clones or species showed an increase in fecundity, thus demonstrating that although precise responses cannot be predicted it may be possible to infer an overall expected response based on this information.

Aphids can alter their reproductive rate due to changes in plant quality (Leather, 1987) or environmental conditions (Leather *et al*, 1983; Grüber and Dixon, 1988). Additionally, environmental changes may affect reproductive rates but not growth rates, and vica versa. In *S. avenae*, doubling atmospheric CO_2 affected fecundity but not development rates (Awmack *et al*, 1996). Whilst in the greenhouse potato aphid, *Aulacorthum solani*, aphids demonstrated decreased development time and increased r_m values when reared on *Tanacetum vulgare* under elevated CO_2, but not on *Vicia faba,* instead increased fecundity was the only effect of increased CO_2 on aphids fed on *V. faba* (Awmack *et al*, 1997). These studies highlight the importance of not inferring other aspects of aphid performance based on fecundity or growth results, as the relationship between aphid size, fecundity and r_m can become uncoupled. If aphids are exposed to starvation, they may resorb embryos to release energy and essential nutrients (Ward and Dixon, 1982), altering their future reproductive capacity.

The results from this chapter do not support the plant stress hypothesis (White, 1984). The plant stress hypothesis proposes that water stressed host plants may benefit aphids in their stress induced change in physiology. The most obvious change in the host plants in this chapter would be the increase in amino acid concentrations described in chapter 2. Amino acids are essential to insect development and growth (Brodbeck and Strong, 1987), and increases in concentrations of free amino acids could promote accelerated growth in aphids. However, the results in this chapter suggest that an extreme level of water stress is detrimental to the aphids species almost universally, with no aphids displaying a benefit in reproductive performance.

Whilst we can conclude aphid reproduction is affected by this level of drought, it must be noted that this chapter has not explored aphid growth. There is the possibility that the effect of drought stress on reproduction may not be concurrent with any affects this level of stress would have on growth. Studies have shown that MRGR is more correlated with r_m than fecundity in *R. padi*, despite MRGR increases usually correlating with fecundity increases (Leather and Dixon, 1984). Conversely, more recently another study has shown correlations between MRGR, r_m and fecundity as not significant in *Aphis fabae* on different cultivars of *Vicia faba* (Wojciechowicz-Zytko and Emden, 2009). Ultimately, it is more likely that growth would be detrimentally affected by an extreme level of drought, as nutrient resources in the host plant become scarce due to leaf shedding and tissue dehydration. In a study into the effects of drought in four grass species on the aphid *R. padi,* it was found that drought detrimentally affected the r_m of *R. padi* for all but one species of grass. On the species of grass where *R. padi* did not display a reduced r_m value, there was also no record of any increase in r_m either (Hale *et al*, 2003).

The results in this chapter so far support many other studies showing evidence that drought has either no effect or a negative effect on aphid performance (Agele *et al*, 2006; Bethke *et al*, 1998; Hale *et al,* 2003; Honěk *et al*, 1998; Kennedy *et al*, 1958; Kennedy and Booth, 1959). In contrast other studies indicate that this may not be true of the whole feeding guild (Wearing, 1967; Wearing and van Emden, 1967; Wearing, 1972; Miles *et al*, 1982; Oswald and Brewer, 1997). It is possible that the variation in the literature may be due to the level of drought used in the other studies, and are not representative of intra- and interspecies plasticity and adaptability. It can therefore be concluded from this study that an extreme level of drought has a detrimental effect upon aphid herbivores.

Interestingly, the fecundity reduction when exposed to droughted host was greater in *B. brassicae* than in both green and red *M. persicae* clones. Overall, fecundity on well watered *B. nigra* was also higher in the green clone of *M. persicae* than in *B. brassicae*. These results suggest that the polyphagous life cycle of *M. persicae* may influence the aphids' ability to tolerate plant hosts of differential quality, whereas the specialist life cycle of *B. brassicae* restricts plasticity in environmental adaptation. If this hypothesis is correct then it would be expected that *R. padi* would also out perform *S. avenae* on drought stressed *L. perenne*. However, despite *S. avenae* displaying a greater reduction in fecundity than *R. padi* on *L. perenne*, this reduction was not significant.

A consideration to be taken into account, when interpreting this data, is that any conclusion drawn from laboratory based studies into aphid performance may not reflect the performance of a wild population of aphids. Wild aphid populations will interact with many other abiotic and biotic factors in addition to drought, natural populations are rarely uniform possessing a greater genetic variability than a laboratory culture derived from a few parthenogenetic females (Edwards, 2001; Loxdale, 1998; Sandström, 1994, Wilson *et al*, 2003). Although aphid growth and development has been successfully recorded in field conditions (Cannon, 1984), it was beyond the scope of this study. Another consideration is that with the exception of *M. persicae* O clones and *B. brassicae*, aphid fecundity was found to be quite low in both treatments, however this is consistent with observations of the aphid culturess. Furthermore, the results of *R. padi* and *S. avenae* in this study suggest that *L. perenne* was not a preferred host of these aphid species.

Finally, honeydew results showed that droplet frequency was significantly reduced in response to drought in both *M. persicae* clones. This reduction in honeydew frequency was also accompanied by a reduction in droplet volume in the green (O) clones of *M. persicae*. In contrast, droplet volume in red (B) clones of *M. persicae* increased under drought stress, however, the observed increase was only marginal (+4%) when compared to the reduction in droplet frequency (-140%). These findings support behavioural analysis in chapter three, which showed both clones reduce sieve element ingestion times whilst on droughted host plants. We can therefore conclude that *M. persicae* reduces both the time spent in the sieve element and the volume of sap ingested from sieve elements when on fed droughted host plants.

4.4.1 Conclusion

Extreme drought (defined in chapter two as higher reducing sugar levels, altered amino acid profiles, altered cation profiles and severely decreased water content; fw: dw ratio <6), has a detrimental effect on the fecundity in most of the aphid species and clones in this study. Where a significant reduction in fecundity was not observed, aphid species/clones did not display any evidence to contradict these findings. Based on this data, we can reject the idea that aphids will reproductively perform better under drought due to increases in free amino acids in the host plant. However, we can only reject this hypothesis when aphids are subjected to extreme levels of drought. Furthermore as aspects of aphid performance such as growth, developmental rates, r_m and fecundity can be uncoupled in certain circumstances it is not possible to infer that all aspects of aphid performance would be detrimentally affected by extreme drought in the same way as fecundity. To conclude although it is easier to assess aphid performance by a simple fecundity count over 72 hours, perhaps the most reliable way to investigate drought effects on aphids would be to use populations in field conditions. This way the effects of genetic variation, crowding, competition, predation, disease and sink induction would also be incorporated into any results.

Chapter 5: Analysis of gene expression by microarray of *Myzus persicae feeding* on *Brassica nigra* subjected to differing water regimes

5.0 Abstract

The osmotic potential of phloem sap is thought to be three fold that of the aphid haemolymph. The ability of aphids to maintain water potential hypo-osmotic to their diet, is thought to be due to an array of osmoregulation mechanisms. Xylem drinking, water cycling, metabolic water production and sugar polymerisation are all suggested mechanisms of aphid osmoregulation. It is thought that under drought stress aphids will have to place an increased dependence on these mechanisms.

This chapter has investigated the gene expression, using microarray technology, of *Myzus persicae* under two different drought levels on *Brassica nigra*. Results revealed that under extreme drought, aphids increased their regulation of a putative aquaporin AqAQP1 and sucrases, supporting the hypothesis that sugar polymerisation and water cycling play an important role in osmoregulation. It was also found that the salivatory enzyme alpha-amylase was increased in response to extreme drought. Aphids also heterogeneously expressed stress-related genes depending on the level of drought experienced, with cytochrome P450s being expressed preferentially when exposed to a mild drought treatment, and heat shock proteins when exposed to an extreme drought treatment.

To summarise this chapter has helped to confirm and elucidate genes induced under drought stress, enabling us to start to understand a complex response with many functions still unknown.

5.1 Introduction

The results of chapter 3 (in this thesis) have showed that extreme drought in the host plant (as defined in chapter 2), has a significant effect on the feeding behaviours of *Rhopalosiphum padi*, *Brevicoryne brassciae* and two clones of *M. persicae*. In *B. brassicae* and red clones of *M. persicae*, xylem feeding was significantly increased on droughted host plants, confirming other studies which have shown xylem feeding to be an important behavioural response to dehydration and water stress (Spiller *et al*, 1990; Ramirez *et al*, 2000). Additionally on droughted hosts, both clones of *M. persicae* and *R. padi* demonstrated reduced sieve element ingestion time, and an increase in time spent not probing or probing non-vascular tissue. Reductions in sap ingestion and increases in non-penetration waveforms can be associated with non-host plants (Gabrys and Pawluk, 1999; McLean and Kinsey, 1968), increases in sap osmotic pressure, increases in sap essential amino acids concentrations (Hale *et al*, 2003), reduced phloem palatability or difficulty in phloem location.

Increased salivation is a behaviour associated with instances where sieve element sap is unpalatable, such as on resistant plant varieties (Ramirez and Niemeyer, 1999; Caillaud *et al*, 1995), in response to water stress (Ponder *et al*, 2000) or in response to reduced amino acids concentrations in sieve elements (Hunt *et al*, 2009).

Aphid saliva is comprised of amino acids, haemolymph and salivary gland secreted proteins (Miles, 1999). Proteins found in aphid saliva are amylases, cellulases, glucosidases, oxidases, dehydrogenases and pectinesterase (Cherqui and Tjallingii, 2000; Miles, 1999; Urbanska and Leszczynski, 1997; Hamel *et al*, 2008). Cellulases, pectinesterases and glucosidases are all enzymes that can be involved with breaking down plant cell wall components such as cellulose, pectin and glycoproteins, and this may be their function in aphid saliva. Oxidases and dehydrogenases are common catalytic enzymes associated with many functions including respiration; interestingly amylases are only involved in the breakdown of starch, which in plants is only found in the plastids not in the cell wall or cytoplasm.

In the vetch aphid, *Megoura viciae*, watery aphid saliva was also found to contain calcium binding proteins that caused contraction of the injury induced forisomes, found in sieve elements (Will *et al*, 2007). This suggests that aphid saliva plays a role in altering the phloem composition to prevent the plant's injury induced natural occlusion mechanism blocking the sieve element. Despite these recent advances there are still many proteins in aphid saliva with unknown functions. In some recent work, RNAi was used to knock out the expression of an unknown aphid saliva protein, which was found in large abundance in aphid saliva, called C002. The result was increased aphid mortality (Mutti *et al*, 2006). Further experimentation showed that loss of function in C002 resulted in a change in foraging and feeding behaviour in aphids associated with increased mortality (Mutti *et al*, 2008). This uncovering of a molecular relationship between saliva and feeding behaviour is particularly interesting, when considering that increased salivation is a behaviour that has previously been characterised as a response to unsuitable hosts. These studies suggest that the process of salivation and its composition is important for successful feeding.

Studies so far have elucidated the importance of saliva and found that aphids may increase salivation on unsuitable hosts; however, none of these studies have investigated if aphids change the composition of saliva in response to drought. The behavioural work in chapter 3 and other studies (Hale *et al*, 2003; Ponder *et al*, 2000) have shown that *M. persicae*, *B. brassicae* and *R. padi* all alter their feeding behaviours on water stressed host plants. Although none of these species in this study have been found to significantly alter the amount of time they spend salivating, it could be hypothesised that aphids may instead alter the composition of saliva to drought stress. In the literature aphid saliva has already been found to be important in phloem conditioning for conspecifics (Prado and Tjallingii, 1997), and as a defence against the phloem's natural injury occlusion mechanism (Will *et al*, 2007). More recently, it has been found that salivatory proteins are important for survivorship (Mutti *et al*, 2006; 2008) and that salivation is a behaviour associated with instances where sieve element sap is unpalatable (Ramirez and Niemeyer, 1999; Caillaud *et al*, 1995; Ponder *et al*, 2000; Hunt *et al*, 2009). It can therefore, be hypothesised that saliva may play a role against drought stress as observed in Ponder *et al* (2000), and that under droughted conditions aphids may either increase the time spent salivating or potentially alter the composition of saliva.

5.1.1 Aphid Osmoregulation

Aphids are highly specialised insects adapted to their osmotically challenging diet of phloem sap. Phloem sap has a high osmotic potential (-0.6 to -3.0MPa) which can vary between and within plants (Downing, 1978; Wilkinson *et al*, 1997; Fisher, 2000). The osmotic potential of phloem sap from a host plant can be three fold that of the aphid haemolymph, moreover, the aphid haemolymph and excreted honeydew are iso-osmotic. The ability of aphids to maintain a water potential hypo-osmotic to their diet can be attributed to various osmoregulation mechanisms.

Phloem sap modification to reduce osmotic pressure is thought to occur in aphids through sucrase mediated hydrolysis of phloem sucrose into glucose and fructose. This glucose can then be polymerised into oligosaccharides of low osmotic pressure per hexose unit (Rhodes *et al*, 1997). Honeydew analysis has also shown that under high dietary sucrose concentrations the excreted oligosaccharide levels significantly increased (Wilkinson *et al*, 1997).

In the pea aphid, *Acyrthosiphon pisum,* sucrase activity has been localised to the gut, distal to the stomach (Ashford *et al*, 2000; Cristofoletti *et al*, 2003). More recently, an identified sucrase APS1 was found in aphids gut and shown to have an α-glucosidase activity to form the hexose units for oligosaccharide synthesis (Price *et al*, 2007). Furthermore, the application of α-glucosidase inhibitor acarbose to inhibit sucrase activity in aphids gut resulted in aphids being unable to osmotically control haemolymph water potential when exposed to a hyperosmotic diet. Aphid gut sucrase activity is therefore essential for osmoregulation of aphids when ingesting diet hyperosmotic to their haemolymph (Karley *et al*, 2005).

Other mechanisms of osmoregulation are water cycling from the distal to proximal regions of the gut. This water flux would require membrane aquaporins to mediate water movement. A putative aquaporin, ApAQP1, was identified in *A. pisum,* which when expressed in *Xenopus oocytes* increased osmotic water permeability. ApAQP1 has been localised in aphids to the stomach and distal intestine, and using an RNAi-mediated knockout for AqAQP1, demonstrated that it is important in maintaining the osmotic pressure of the haemolymph (Shakesby *et al*, 2009).

Currently, advances in the literature are helping to elucidate aphid osmoregulation mechanisms and provide evidence to support the important role for aquaporins, sucrases and polymerising enzymes in osmoregulation. Under drought stress, host plant water potentials would increase and aphids would place an increased dependence on these various mechanisms to cope with the extreme osmotic challenge posed by concentrated phloem sap. It can be hypothesised, that under these conditions resultant increases in the expression of sucrases, aquaporins and other sugar glucosidases would occur.

Furthermore previous studies have suggested that aphid saliva is important in phloem conditioning (Ramirez and Niemeyer, 1999; Caillaud *et al*, 1995; Ponder *et al*, 2000; Hunt *et al*, 2009; Prado and Tjallingii, 1997; Will *et al*, 2007) and survivorship (Mutti *et al*, 2006; 2008). It could therefore be hypothesised that saliva may play a role in drought stress, and that under droughted conditions aphids may alter the composition of saliva resulting in altered expression of salivary enzymes, such as amylases, cellulases, pectinesterases, oxidases and dehyrogenases.

5.1.2 Microarray Technology

Microarrays consist of thousands of microscopic spots containing short olignucleotides called 'features' that represent sections of genes, these are used as probes to hybridise to target cDNA or cRNA samples under controlled conditions. The samples are usually pre-labeled with fluorescence to allow for detection of relative abundance of target within a sample. Microarray technology evolved from Southern Blotting, and is used commonly today to detect and measure changes in gene expression level or to detect single nucleotide polymorphisms (SNPs) in genotyping. As microarrays contain thousands of probes, it means one single experiment can achieve many genetic tests in parallel (Voelckel *et al*, 2004; Voelckel and Baldwin, 2004). This has meant that special software and statistics have had to be developed to deal with the sheer amount of data generated by microarrays.

Microarray technology allows thousands of probes to be tested simultaneously, these probes could originate from an EST or cDNA library. Arrays can be designed so that each feature represents a single gene. The technology means that the experimenter can test for changes in thousands of genes between treatments. This chapter will attempt to elucidate whether (and which) gene expression changes underlie aphid responses to osmotic stress. This information will help to inform determine whether aphids place an increased dependence on genetically controlled osmoregulation mechanisms when fed on extremely droughted host plants. To achieve this would require the screening of many genes, thus microarray technology shall be used to analyse whole aphid body tissue, with 'features' representing an EST library developed for *M. persicae* by Ramsey *et al* (2007).

Microarrays can be fabricated using a variety of technologies; the main two different technologies are spotted arrays and oligonucleotide arrays. In spotted arrays the probes can be oligonucleotides, cDNA or small PCR fragments, and can be synthesised prior to being spotted onto the glass array surface. The advantage of this technology is that it can be done 'in house' in a lot of labs, and is a relatively low-cost way of studying gene expression without having other genes on the microarray of no-interest to the researcher. The disadvantage of spotted arrays is that they may experience printing inefficiencies and misalignment that can result in a lack of specificity.

In oligonucleotide arrays, the oligonucleotides are synthesised directly onto the array surface, instead of being deposited intact as in spotted arrays. With this technology the sequences can be longer up to 60 mer with companies such as Agilent. In this chapter Agilent oligonucleotide arrays were used, with the probes synthesised from an EST library (Ramsey *et al*, 2007) as the genome of the aphid *M. persicae* is yet to be sequenced.

Another variation in the technology is whether to use a one-dye/colour (one-channel) or two dye/colour (two channel) experimental design. With both these designs the sample cDNA is typically hybridised to a fluorescent label. The most common fluorescent dyes are Cy 3 and Cy 5, containing cyanine. Cy 3 has an emission of 570nm (corresponding to the green part of the spectrum) whereas Cy 5 has an emission wavelength of 670nm (corresponding to the red part of the spectrum). In this project the dyes 555 and 647 were used, as the name suggests dye 555 had an emission wavelength of 555nm and corresponded to the green part of the spectrum whereas, 647 had an emission of 647nm and corresponded the red part of the spectrum. In two colour arrays the two fluorescently labeled cDNA samples (control and treatment) are mixed together and hybridised to one single microarray, which is then scanned in a microarray scanner to visualise the intensity of fluorescence. Each sample in a two colour array will be labelled with a different fluorescent dye. Therefore using Cy3 (green) and Cy5 (red) dyes as an example if equal amounts of gene expression were found in the control and treatment samples, then there would be equal amounts of hybridisation of fluorescently labelled cDNA to the array, this would generate a yellow colour. If the spot on the array appeared more red in colour then more cDNA labelled with Cy5 had bound to the spot, showing a higher expression of gene product in that sample. Therefore the relative intensities of each different fluorophore are used to determine ratios, which then inform whether each gene was up-regulated or down-regulated from a comparison between the intensities of control and treatment fluorescence. In one colour arrays the design is more focused on giving an absolute level of gene expression, as opposed to a measure of difference as in the two colour arrays. This is due to a single dye being used per sample per array. The strength in one-colour arrays is that one aberrant sample cannot affect the raw data from another sample. Another benefit is that the data is generally more comparable to other experiments, even those conducted years apart. A drawback is that twice as many arrays are needed for each experiment.

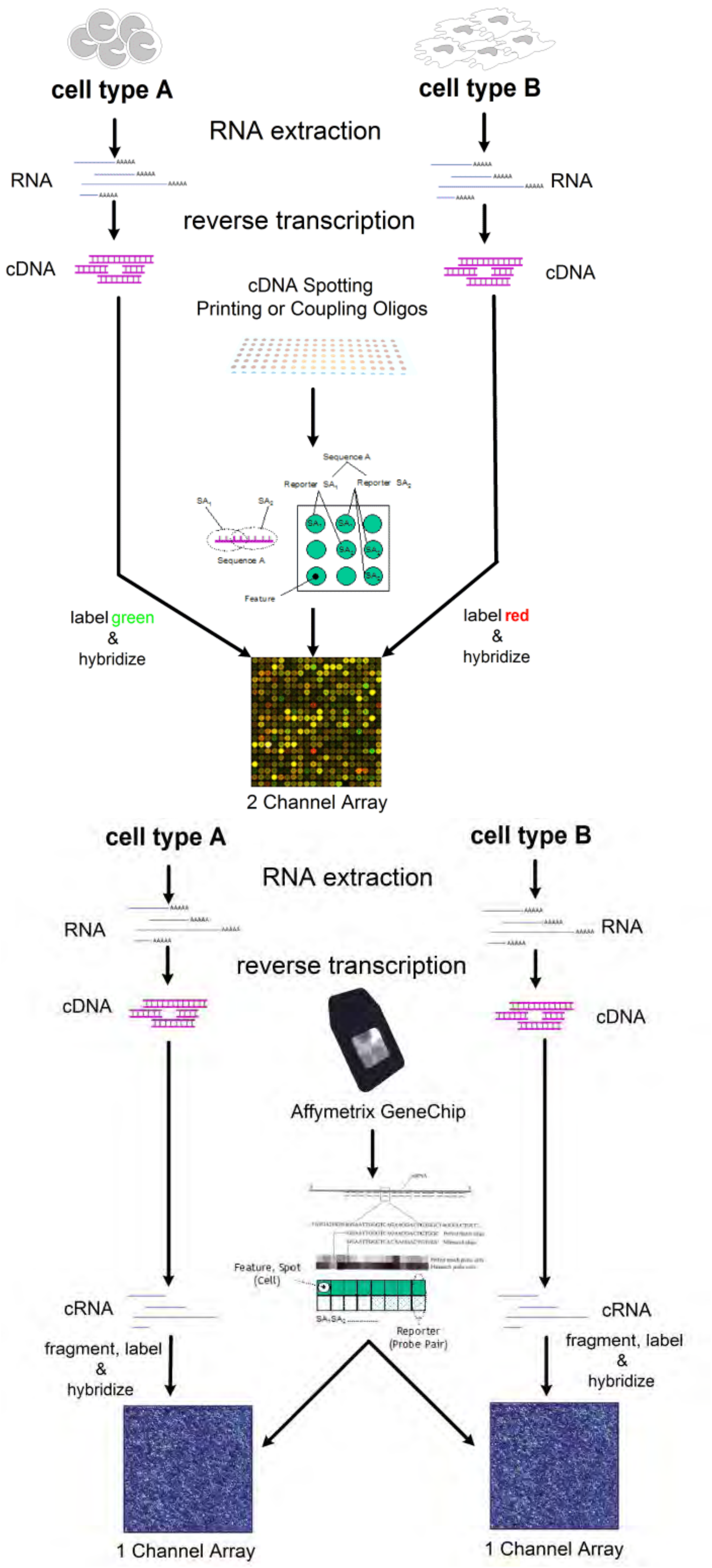


Figure 5.1: Example of a two-colour/channel microarray experimental workflow taken from the NCBI website and an Affymetrix (single-channel) microarray experimental workflow also taken from the NCBI website

5.1.3 Quality Control in Microarray Design

The three most important factors to consider in array experiments are biological replication, technical replication and spot replication. The custom designed arrays that are being used in this experiment, contain duplicate probes of certain EST sequences addressing spot replication, whilst cost limitations mean it will not be possible to conduct technical replication in this chapter.

In two-colour experiments there are a number of different experimental designs to consider (Stekel, 2003). These are:

1. Direct comparison: the results of two samples being directly compared via hybridization to the same array
2. Reference Design: Each sample is compared to a standard reference that is common to all arrays
3. Loop Design: comparisons which link together to form a loop allowing indirect comparison between at least two other conditions i.e. A-B, B-C, C-A

Reference design is the most cost effective whilst loop designs and direct comparisons are the most effective (Stekel, 2003). As this project involves two different drought treatments and their relative controls the most effective design for this experiment would be direct comparison.

Whilst there are some problems, which are unsolvable in the design process such as the reliance on a genomic EST library to fully represent and be associated with every gene there are other problems that can be avoided or catered for in the experimental design. By adding in control probes on the array design it is possible to check the degree of hybridization to assist in normalization of the other target probes. This is achieved by mixing in RNA ‘Spike-ins’ with the samples to hybridise with the control probes on the Agilent oligonucleotide arrays used in the project. Another possible area of error is the binding affinity and resulting intensity of each of the dyes. By alternating which dye hybridises with the control and treatment samples this can be overcome, this is called dye swapping and a regular practice in microarray design.

Dye incorporation rates, even with dye swapping techniques, can be an issue in microarray design. A technique to overcome this is the conversion of complementary DNA (cDNA) into amplified RNA (aRNA). Fluorescent dyes are easily incorporated into aRNA with greater efficiency than into cDNA and mRNA, overcoming this problem. Therefore in this study aRNA was used at the stage of dye incorporation.

Other control tools are the implementation of special statistical tests during microarray analysis. When a significant gene list has been populated, the sheer amount of genes being analysed can sometimes result in the probability of genes being generated out of chance increasing. This is called the ‘false discovery rate’. Statistical methods have been tailored to adjust for this such as the Bonferonni correction, first used in a paper by Paulson (1952). The Bonferroni adjustment works by lowering the alpha value (experiment error rate), as the number of comparisons made increases. For an observation to be significant, the P value would be corrected to be lower than 0.05 in respect to the increasing number of comparisons. This makes it harder to claim a significant result and in so doing decreases the chance of making a Type I error (false positive) to acceptable levels. However in doing this it can make it too hard to claim a significant result, and the strictness of the test can have the possibility of identifying genes as false positive, when in fact they are false negatives (Type II error).

5.1.4 Chapter aims

This chapter aims to test if under drought stress aphids place an increased dependence the sucrases, aquaporins and other sugar glucosidases as part of their osmoregulation mechanisms, and if aphids alter saliva composition by changing the abundance in expression of known salivatory associated proteins such as amylases, pectinases etc.

At the end of this study we will have:

1. Examined gene expression of whole body tissue of *Myzus persicae* when fed on well watered, mild and extremely droughted plant host *Brassica nigra.*
2. Studied changes in expression of predicted aquaporin, sucrase, amylase, cellulase, glucosidase, oxidase, dehydrogenases and pectinesterase sequences.
3. Used two different levels of drought stress to ascertain the threshold of water stress needed to induce expression changes.

5.2 Methods and Materials

5.2.1 Aphids

M. persicae was from the Lab culture at The University of Birmingham. Culture is kept at a constant temperature of 18^{o}C under a 16:8h light cycle.

5.2.2 Plants

Brassica nigra seeds used in the experiments were seeds obtained from John Chambers Wildflower Seeds Co. from wild seed stocks in Northamptonshire, UK. *B. nigra* plants were potted up and grown in a plant quarantine room at the University of Birmingham with a 16:8h light cycle at a temperature of 22°C. Growth room PAR was between 80-120mol m^{-2} s^{-1} with a relative air humidity of 70-80%. Plants were grown in John Innes peat based compost with mixed in Silvaperl in 150ml plastic pots.

5.2.3 Aphid Treatments

Ten individual reproducing adult *M. persicae* were placed on *B. nigra* individuals subjected to one of four treatment types (see below). Aphids had previously been cultured on *B. nigra* for at least three generations in growth rooms. Aphids prior to experimentation had been kept in relatively un-crowded colonies in bug dorms under a 16:8h light cycles at 16-18°C. Aphids were left for 72 hours and then removed and immediately frozen in microfuge tubes in liquid nitrogen for later total RNA extraction.

The treatments were as follows:

- Well watered (1) – Individual well watered *B. nigra* plants aged 23 days old were exposed to approximately 5-10 adult *M. persicae* individuals each. After 72 hours these aphid individuals were removed for genetic analysis. *B. nigra* plants had water available to them throughout the 72 hours of aphid exposure.

- Mild Drought – Individual *B. nigra* plants were grown for 20 days with unlimited water supply. At 20 days plants experienced total water withdrawal for 6 days. After the first initial 72 hours of drought plants were also exposed to approximately 5-10 adult *M. persicae* individuals each that remained on the plant for the final 72 hours of drought, after which the aphids were removed for genetic analysis.
- Well watered (2) – Individual well watered *B. nigra* plants aged 26 days old were exposed to approximately 5-10 adult *M. persicae* individuals each. After 3 days these individuals were removed. *B. nigra* plants had water available to them throughout the 3 days of aphid exposure.
- Extreme Drought – Individual *B. nigra* plants were grown for 20 days with unlimited water supply. At 20 days plants experienced total water withdrawal for 9 days. After the first 6 days of drought plants were also exposed to approximately 5-10 adult *M. persicae* individuals to each plant that remained on the plant for the 3 days of drought, after which the aphids were removed for genetic analysis.

During each treatment aphids did reproduce, however, only the original adult aphids were removed from plants for extraction. For each treatment there were four biological replicates used for array analysis, each replicate had the surviving aphids grouped together from 2-3 *B. nigra* plants, totalling twelve plants used for each treatment. In each replicate there were approximately 10-20 aggregated frozen aphids.

5.2.4 RNA extraction

The frozen aphids were processed to remove the RNA using a TRIzol based total RNA extraction (Ambion RiboPure™ Kit). The quality of the total RNA was evaluated using A_{260}/A_{280} ratios determined via a Nanodrop. Only RNA with a ratio value of 1.9 was used. The RNA was also quantified by electrophoresis on the Agilent Bioanalyser. RNA extractions used had to have 11 µl of RNA at a concentration ranging from 100-1000ng and a RIN (RNA Integrity Number) number no lower than 5 to proceed, this was to ensure the quality of the RNA was suitable to use for subsequent amplification/conversion without sequence degradation.

5.2.5 cDNA conversion and aRNA synthesis

The isolated RNA was then converted into cDNA and then transcribed into aRNA using the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit. The kit reverse transcribes the total poly(A) RNA into cDNA using a oligo(dT) primer containing a T7 RNA polymerase promoter sequence. During second strand cDNA synthesis the enzyme RNase H is added to cleave mRNA template into small fragments which act as primers to produce the double-stranded cDNA. This cDNA is purified to remove rRNA, mRNA and primers. The purified cDNA is then transcribes into aRNA in vitro by Ambions MEGAscript® technology, with the additional incorporation of amino allyl UTP's. The above was all conducted following the protocols set out in each of the kits.

5.2.6 Quantification check and Labelling

The resulting aRNA is quantified again using the NanoDrop to ensure aRNA presence. The aRNA is then coupled to the dye using the protocol set out in Alexafuor 555 and

647 Reactive Dye Decapack. 5 µg of aRNA is then coupled to the NHS ester label dyes 555 (cy) and 647 (cy). This is done via aliquoting 5 µg into a new tube and placing in a speed vac on low heat until dry. The chosen fluorescent dyes (555 and 647) were re-suspended in 11µ l of DMSO, vortexed and kept in the dark at room temperature for up to 1 hour. 9µl coupling buffer was used to re-suspend the dry RNA samples and then the 11µl of re-suspended dye was added and left for 30 minutes in the dark at room temperature. The coupling reaction is quenched with 4.5µl of 4M hydroxylamine and incubated for 15 minutes in the dark at room temperature. To this 5.5µl of pre-heated nuclease free water is added and the labelled aRNA is purified, using the method and columns in the aRNA Amplification Kit. This labelled aRNA once purified by the columns is then quantified again along with determining A_{260}/A_{280} ratios via a NanoDrop technology to ensure quality control. The absorbance values read off the Nanodrop at 260nm and either 555nm or 647nm was used to work out the dye incorporation rate for each sample. The dye incorporation rate was calculated using the equation and information below. The dye incorporation rate should be around 30-60 dye molecules/1000nt. If the samples did not equate to the range of these values then the samples were not used in the next step.

#dye molecules/1000nt = [(555nm OR 647nm absorbance value depending on dye used)/Absorbance at 260nm] x [9010/dye extinction coefficient] x 1000

Dye 555 extinction co-efficient: 150000

Dye 647 extinction co-efficient: 239000

5.2.7 Fragmentation and Hybridisation to Array

The dye labelled aRNA is re-quantified to ensure there is at least 300ng in 9 µ l of dye labelled aRNA for use in hybridisation, again using A_{260}/A_{280} ratios quality control was also enforced at this point using the NanoDrop.

The aRNA is ready for fragmentation and hybridisation to the Agilent custom chips. The fragmentation involved 9.5µl of 300ng of 555 labelled control sample and 9.5µl of 300ng 647 labelled treatment sample being mixed together along with 5µl 10X blocking agent, 1µl 25x fragmentation buffer. This was incubated in a pre-heated water bath at 60^{o}C for 30 minutes. To stop the fragmentation reaction 25µl 2xGEx Hybridisation Buffer HI-PRM was added. The samples were then placed on ice and transferred for hybridisation to the Agilent service at Cornell University (NY, USA), and this was followed by the proceeding scanning of the arrays. The chips used were eight arrays chips, each array containing approximately 15,000 oligonucleotides formed from a *M. persicae* EST library (Ramsey *et al*, 2007), and both treatment and corresponding control were hybridised together in the two-dye experiment.

5.2.8 Data Analysis and Quality Control

As part of quality control the microarray chip used had 15744 features, of which 14555 features relate to *M. persicae* EST sequences from an EST database created by Ramsey *et al*, 2007. There was a total of 1189 control features on each chip, randomly distributed around the array that correspond to sequences in other organisms. In addition, there were approximately 4000 repeated *M. persicae* sequences on each array. Thus out of the 14555 EST features for *M. persicae*, there were only 4000 features that were duplicated on the array and only 6555 features that were not, totalling 10555 *M. persicae* EST sequences represented on each array.

The results from the microarray were imported into GeneSpring software and background corrections were performed. All samples were normalised using the method 'loess' before further analysis and subjected to basic quality control parameters. To test if any samples were outside the mean of the other replicates of the same treatment box plots were formed; it was found that one sample meet this criteria and so one replicate for extreme drought was disregarded. Quality control parameters on the remaining samples were; intensity values must be no lower than 100 and probes outside the range of 20-100% intensity, meaning that probes had to give between 20-100% intensity to be used. In addition unless probes in 3/7 samples were consistent then they were disregarded. This means that probes had to be consistent in three out of three of my biological replicates for the extreme drought treatment and at least three out of the four replicates for the mild drought treatment. In addition a stdev< 1 filter was also applied. Statistical analysis was conducted using the unequal t-test and probe lists were blasted on the NCBI database for information on function. Where function was assigned, sequence homology had to be 90% or more identical to the homologous sequence from the NCBI database.

Due to the repetition of *M. persicae* EST sequences on each array, the Bonferroni statistical correction was not used in the analysis. Although computing multiple t-tests has risk of type I errors, the Bonferroni for this array comes with a high risk of type II errors as it will be unable to take into account the duplicity of some of the EST sequences on the array.

Phylogenetic analysis of significant genes was done using PhylomeDB (Huerta-Cepas *et al*, 2008; http://phylomedb.org), a database that contains pregenerated phylogenetic trees for each *A. pisum* aphid gene. The phylogenetic trees contain paralog gene information obtained by sequencing data (International Aphid Genomics Consortium, 2010) and information on homologous genes in other arthropods with sequenced genomes (Huerta-Cepas et al, 2010; 2011). Investigation into similarity and relatedness between EST sequences was done using ClustalW2 online software (http://www.ebi.ac.uk/Tools/clustalw2/) by EMBL-EBI, Wellcome Trust Genome Campus, Cambridgeshire.

5.3 Results

5.3.1 Mild drought

5.3.1.1 Quality Control

Arrays for mild drought treatment were filtered using GeneSpring software selecting only features that had intensities of 20-100% and a minimum fluorescence of 100. Filtering removed 3862 features, which did not meet this criterion leaving 11882 for further statistical analysis (fig 5.2). Box plots of means were also used to check that none of the arrays for mild drought treatment were outside the means for the group of samples. As a result all four arrays were used giving a biological replication of four for the mild drought treatment.

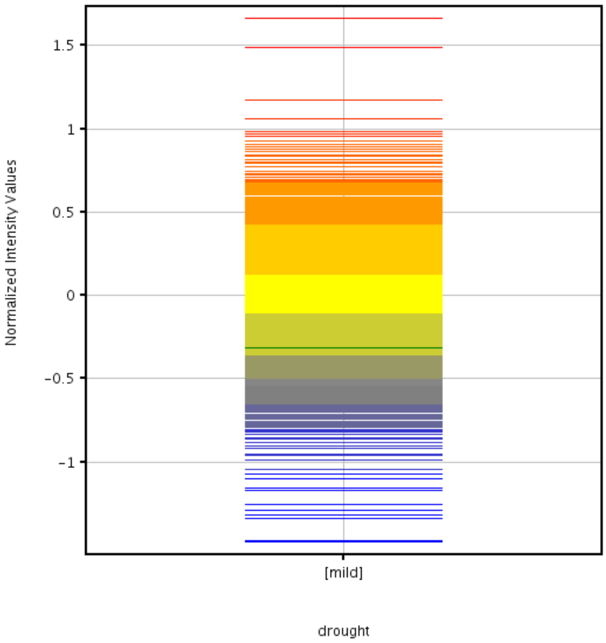


Figure 5.2 Profile plot using GeneSpring software of adult *M. persicae* fed on *B. nigra* individuals subjected to the mild drought treatment defined in Chapter 2, data has been filtered to contain intensities 20-100% and a minimum fluorescence of 100. Each line represents a difference feature (EST probe) in a total of 11882, the colour scale indicates the degree of up regulation (red) or down regulation (blue)

5.3.1.2 Significant Genes

When the Bonferroni adjustment was applied in GeneSpring to array data, none of the EST sequences were found to be significantly up or down regulated in *M. persicae* in response to a mild drought treatment. The Bonferroni correction can also be calculated manually online via the SISA Bonferroni correction tool (www.quantitativeskills.com/sisa/calculations/bonfer.htm). An alpha value of 0.05 was used with N being the filtered number of probes (11882). The manual calculation indicated that the Bonferroni correction would require the alpha value for any one feature on the array to be lowered to 4.2E-6 in order to rule out a type I error (aka false positive, when the statistical test rejects a true null hypothesis). Consequently by using the Bonferroni correction in GeneSpring, only features that have a significance value of 4.2E-6 would be considered as significantly altered genes, of which none fitted this criterion.

However, if we were to calculate instead the potential number of false positive genes that could occur by chance from a 0.05 alpha level in our 11882 filtered probes (by simple multiplication), then we can estimate that there would be 594 features that would appear significant but were actually the consequence of a type I error.

Statistical analysis without a Bonferroni correction in GeneSpring highlighted that there were 736 feature spots (fig 5.3) that had significantly different intensities between treatments according to an alpha level of 0.05. This indicates that in applying the Bonferroni correction a type II error would occur (aka false negative, when the test fails to reject a false null hypothesis), with potentially 141 genes being classified as false positives by the correction when they are actually biologically significant.

Furthermore, the 736 probe features highlighted as significant to an alpha level of 0.05 by GeneSpring (fig 5.3), contained 94 EST sequences that had duplicated spots whereby both spots were found significant in the treatment. These duplicated spots are therefore accountable for a total of 188 features out of the 736 highlighted by GeneSpring.

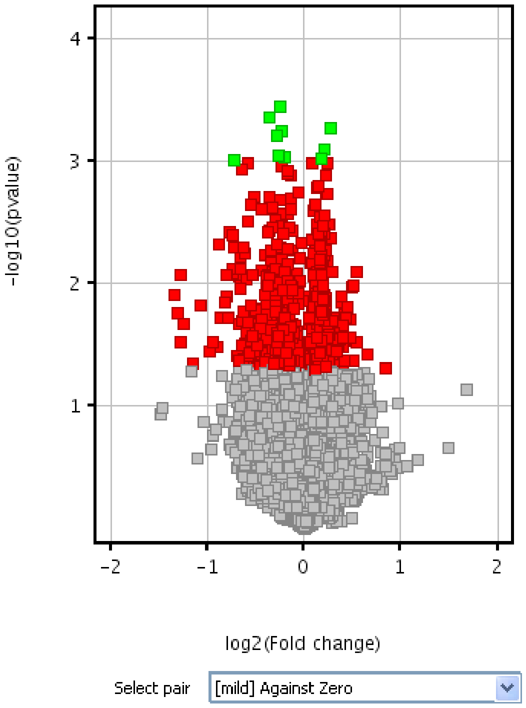


Figure 5.3 Volcano plot using GeneSpring software of the expression of adult *M. persicae* fed on *B. nigra* individuals subjected to the mild drought treatment defined in Chapter 2, data has been filtered to contain intensities 20-100% and a minimum fluorescence of 100. Each square represents a different feature (EST probe), the red squares (736) represent each EST which was significantly (P=0.05) differentially expressed in individuals fed on droughted plant hosts than the controls whilst the green squares (11) are those significant to a P value of <0.001

As the Bonferroni cannot take into account the duplicity within the array, the data has been analysed without the application of a Bonferroni correction, therefore, where any probes are presented as being significant without their duplicate probes, they are considered as false positives. Therefore any significant probes found to also have high fold changes and be highly significant (<0.005) will be considered as biologically significant regardless of the Bonferroni correction.

The 736 EST sequences highlighted as significant to an alpha level of 0.05 by GeneSpring were blasted in NCBI and categorised according to predicted gene functions. Genes were manually classified into the following categories (as the genome of *M. persicae* is still unsequenced, and no GO Annotation has been made for the EST sequences used in this microarray):

- unknown function
- aquaporins
- amino acid processing/transport
- sugar hydrolysis/transport
- metabolism
- sensory, nervous and hormonal messaging/control, oxygen transport, haemostasis and immunity
- solute carriers/exchangers/transport, transmembrane proteins/receptors
- DNA replication/transcription/translation and chromosome (dis)assembly, DNA-RNA interactions, chromosome interaction and regulation
- Development and Growth
- Aphid communication, defence and behaviour
- Cell-cell communication, membrane formation and membrane trafficking

Analysis found (fig 5.4) that most of the EST sequences identified as significant were of an unknown function (391 EST sequences). Furthermore, 94 EST sequences were present with their duplicated features accounting for 188 out of the 736 EST sequences found significantly altered in the mild drought treatment.

Conversely 135 EST sequences out of the 736 were found without their duplicate probes, indicating type I errors within the analysis. Interestingly, no EST sequences relating to aquaporins were found significantly altered in regulation between control and mild drought treated aphids. Unsurprisingly, the category of DNA/RNA/chromosome regulation and control represented the second largest group of EST sequences found altered in response to mild drought stress, with 110 EST sequences out of the 736 EST sequences found significantly altered. Amino acid and sugar related EST sequences accounted for 46 significantly altered features, whilst metabolism (including amino acid and sugar metabolism) accounted for 115 sequences out of the 736 found significantly altered.

Furthermore, only 11 EST sequences were directly related to stress, and these were mostly cytochrome proteins (8 ESTs). Sensory, nervous and hormonal messaging accounted for 20 EST sequences, whilst there were only 3 EST sequences directly related to communication, behaviour and defence. However, it is worth being cautious when interpreting these groups as some EST sequences may be related to these categories but due to being dehyrogenases or DNA regulation proteins they have been categorised into other categories above. This highlights one of the issues with interpreting data without an automated GO analysis that can cross reference every sequence.

Sequences related to solute movement, membrane proteins/receptors, cell communication and membrane trafficking/formation accounted for 52 EST sequences whilst EST sequences related to development, growth and movement accounted for 34 out of the 736 significantly altered ESTs.

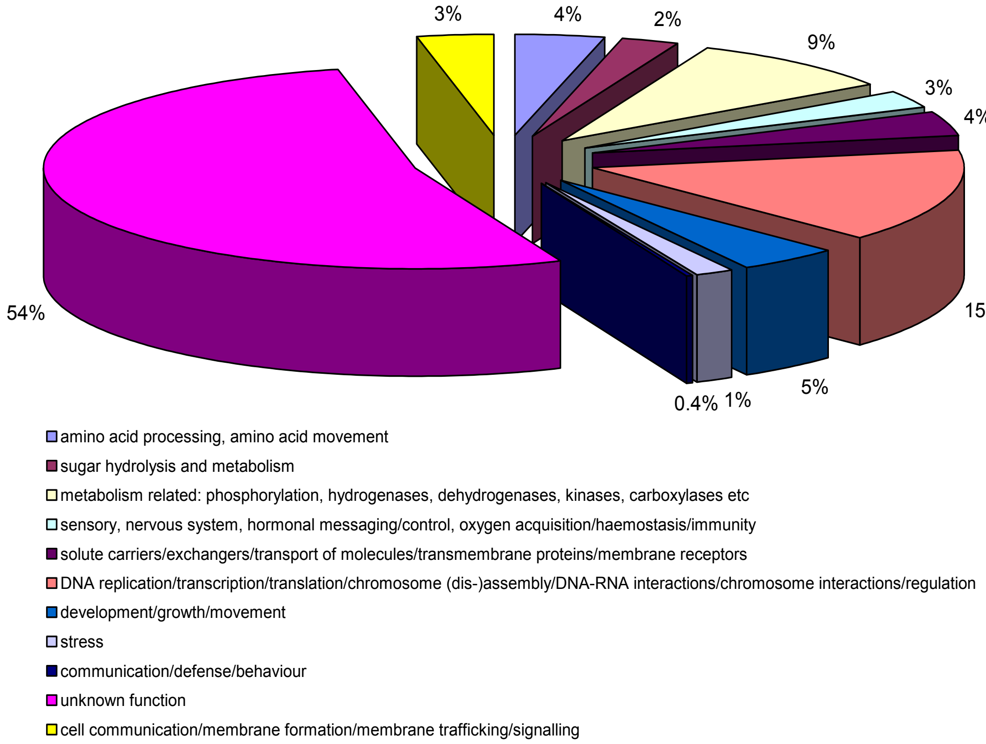


Figure 5.4 Piechart showing portions of EST sequences found significantly altered in an expression microarray of adult *M. persicae* fed on *B. nigra* individuals subjected to the mild drought treatment defined in Chapter 2. The EST sequences found significantly (t-test, alpha value=0.05) up/down regulated between aphids on control and treatment host plants were blasted in NCBI and manually categorised according to function. The piechart shows the percentage each group occupied out of the 736 EST sequences.

5.3.2 Extreme drought

5.3.2.1 Quality Control

Arrays for extreme drought treatment were filtered using GeneSpring software in the same way as the mild drought treatment. Filtering was set so that only features which had intensities of 20-100% and a minimum fluorescence of 100 were left. Filtering removed 3778 features that did not meet this criterion, leaving 11966 features for statistical analysis (fig 5.5). Box plots of means were also used to check that none of the arrays for extreme drought treatment were outside the means for the group of samples. As a result to prevent anomalous results later in analysis, one array was removed from the group, as it was not within the group's means, this gave three biological replicates for the extreme drought treatment.

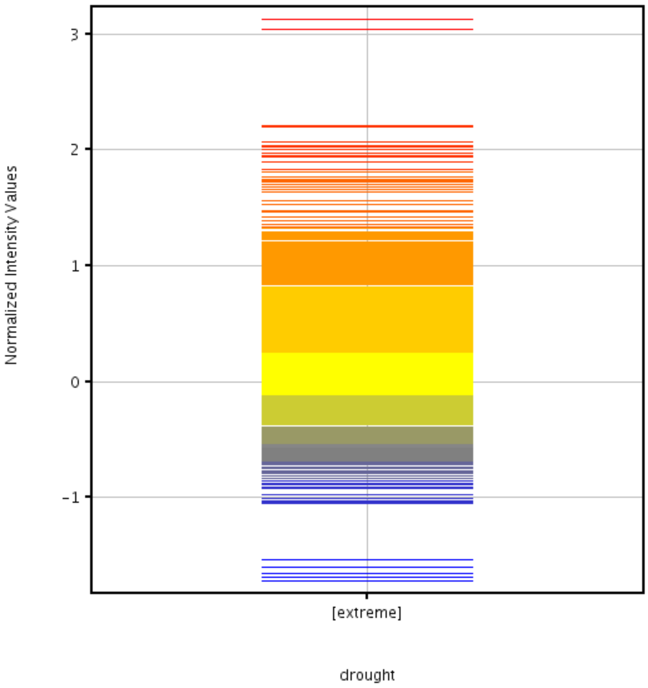


Figure 5.5 Profile plot using GeneSpring software of adult *M. persicae* fed on *B. nigra* individuals subjected to the extreme drought treatment defined in Chapter 2, data has been filtered to contain intensities 20-100% and a minimum fluorescence of 100. Each line represents a difference feature (EST probe) total of 11966, indicates the degree of up regulation (red) or down regulation (blue)

5.3.2.2 Significant Genes

When the Bonferroni adjustment was applied in GeneSpring to the extreme drought array data, like the mild treatment, none of the EST sequences were found to be significantly up or down regulated in *M. persicae* in response to the drought stress. Using the online SISA Bonferroni correction tool (www.quantitativeskills.com/sisa/calculations/bonfer.htm), the alpha value for any one feature on the array would have to be lowered to 4.2E-6 in order to rule out a type I error, this is based on N being the number of filtered probes (11966) after quality control (fig 5.5). In addition, it was also estimated that in the 11966 filtered array features there would be 598 that would appear significant but would be down to chance and a consequence of a type I error.

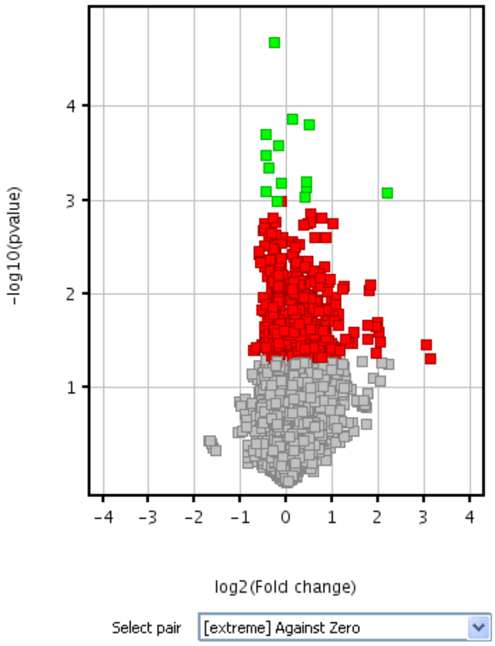


Fig 5.6 Volcano plot using GeneSpring software of the expression of adult *M. persicae* fed on *B. nigra* individuals subjected to the extreme drought treatment defined in Chapter 2, data has been filtered to contain intensities 20-100% and a minimum fluorescence of 100. Each square represents a different feature (EST probe), the red squares (690) represent each EST which were significantly (P=0.05) differentially expressed in individuals fed on droughted plant hosts than the controls, whilst the green squares (15) are those significant to a P value of <0.001

However, statistical analysis in GeneSpring highlighted that there were 690 feature spots (fig 5.6) that had significantly different intensities between treatments according to an alpha level of 0.05. This indicates that in applying the Bonferroni correction a type II error would occur, with potentially 91 genes being classified as false positives by the correction when they are biologically significant.

Furthermore, out of the 690 probe features highlighted as significant to an alpha level of 0.05 by GeneSpring, 52 EST sequences were present with their duplicate spots. These duplicated spots are therefore accountable for a total of 104 features out of the 690 highlighted by GeneSpring. As the Bonferroni correction cannot take into account the duplicity within the array, as in the mild drought data, this data has been analysed without a Bonferroni correction. In addition (as in mild drought data) where any probes are presented as being significant without their duplicate probes, they are considered as false positives. Additionally where significant probes have high fold changes and are highly significant (<0.005)s then they will be considered as biologically significant regardless of the Bonferroni correction.

The 690 EST sequences highlighted as significant to an alpha level of 0.05 by Genespring were blasted in NCBI and categorised according to predicted gene functions. EST sequences were manually classified into the same categories used in analysis of the mild drought microarray data (see section 5.3.1.2).

Compared to the mild drought treatment (736) there were less EST sequences significantly altered in expression level in the extreme drought treatment (690). However, analysis found (fig 5.7) that like in the mild drought treatment most of the EST sequences identified as significant were of an unknown function (341 EST sequences). Additionally, 52 EST sequences were present with their duplicated features accounting for 104 out of the 690 EST sequences found significantly altered in the mild drought treatment.

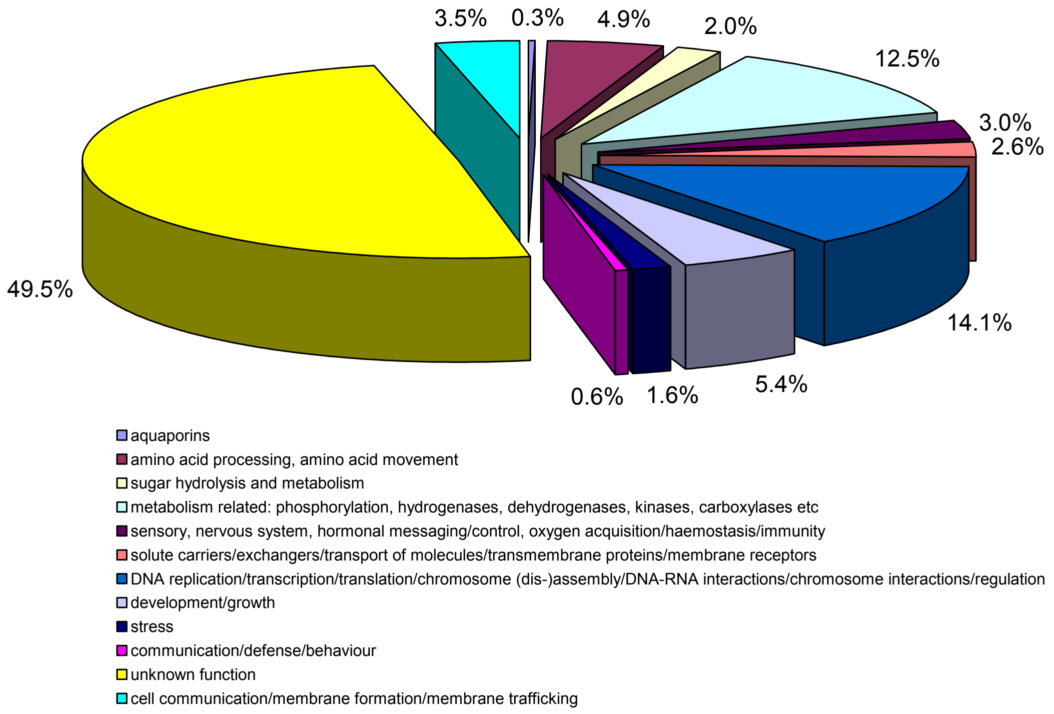


Figure 5.7 Piechart showing portions of EST sequences found significantly altered in an expression microarray of adult *M. persicae* fed on *B. nigra* individuals subjected to the extreme drought treatment defined in Chapter 2. The EST sequences found significantly (t-test, alpha value=0.05) up/down regulated between aphids on control and treatment host plants were blasted in NCBI and manually categorised according to function. The piechart shows the percentage each group occupied out of the 690 EST sequences.

Conversely, 96 EST sequences out of the 690 were found without their duplicate probes, indicating like in analysis of the mild data that type I errors had occurred within the analysis. Interestingly fewer ESTs without their duplicate probes were found in the extreme drought treatment (96) than the mild drought treatment (135), however, this may correlate with there being overall less EST sequences significantly altered in expression levels in the extreme (690) than mild drought treatment (736).

Again the category of DNA/RNA/chromosome regulation and control represented the second largest group of EST sequences found altered in response to mild drought stress, with 97 EST sequences out of the 690 EST sequences found significantly altered. Amino acid and sugar related EST sequences accounted for 48 significantly altered features, whilst metabolism (including amino acid and sugar metabolism) accounted for 134 sequences out of the 690 found significantly altered. Extreme drought treatment therefore has a higher proportion of metabolism ESTs (19.4%) significantly altered, than the mild drought treatment (15.6%).

Interestingly, exactly like the mild drought treatment only 11 EST sequences were directly related to stress, although unlike the mild drought treatment only 2 were cytochrome proteins, whilst 6 were heat shock proteins associated proteins. Sensory, nervous and hormonal messaging accounted for 21 EST sequences, whilst there were 4 EST sequence directly related to communication, behaviour and defence. Sequences related to solute movement, membrane proteins/receptors, cell communication and membrane trafficking/formation accounted for 42 EST sequences, and EST sequences related to development, growth and movement accounted for 37 out of the 690 significantly altered ESTs.

Interestingly, only in the extreme drought treatment were aquaporin related ESTs significantly altered. Two features (duplicated probes) representing an EST sequence for AQP1 Aquaporin were found significantly up regulated (probe: M_persicae2387a P=0.045 and M_persicae2387b P=0.033, t-test) in the extreme treatment.

5.3.3 Comparison of treatments

Overall, the amount of significant ESTs in the extreme drought treatment was lower then the mild treatment. However, the extreme drought treatment displayed more (134) EST sequences involved in metabolism, and specifically in amino acid processing (28 ESTs in mild treatment and 34 ESTs in extreme treatment), than the mild drought treatment (115), although ESTs specifically involved in sugar metabolism were reduced (18 ESTs in mild treatment and 14 ESTs in extreme treatment) in the extreme drought treatment.

Significant EST sequences involved in DNA/RNA/chromosome regulation and control were also more abundant in the mild treatment (110) than extreme drought treatment (97). This may be related to the increase in metabolism related ESTs in the drought treatment, indicating that a period under mild drought conditions may trigger gene regulation pathways. These pathways may be involved in increasing expression of metabolic related proteins, explaining the increase in abundance of metabolism ESTs in the extreme drought treatment.

The difference in expression between treatments indicates that there is an effect of the level of drought on gene expression in *M. persicae*. This is highlighted by absence of any significantly altered aquaporin ESTs in the mild drought treatment, and presence of an AQP1 EST aquaporin sequence in the extreme drought treatment.

Finally despite both mild and drought treated aphids expressing 11 stress associated ESTs, the composition of ESTs in these categories varies between treatments, with mild droughted aphids significantly expressing more cytochrome related ESTs and drought stressed aphid expressing heat shock related ESTs (not found in significantly altered in the mild drought treatment).

5.3.4 Candidate Genes

5.3.4.1 Identification

So far the analysis has indicated that aphids change gene expression in response to the level of drought. The purpose of this study was to also evaluate if aphids change the expression levels of genes potentially involved in osmoregulation, specifically genes such as glucosidases, sucrases and aquaporins (Shakeby, et al 2009; Ashford et al, 2000; Cristofoletti et al, 2003; Price, et al. 2007; Karley, et al 2005). In addition, this study was to investigate if expression levels altered in salivatory related genes, such as cellulases, glucosidases, oxidases, dehydrogenases and pectinesterase (Cherqui and Tjallingii, 2000; Miles, 1999; Urbanska and Leszczynski, 1997; Hamel *et al*, 2008).

To achieve this ESTs relating to these functions were searched for in the lists of significant ESTs for both array datasets (mild and extreme drought treatment). Interestingly, ESTs (M_persicae3682a/b, M_persicae3987; M_persicae8280; M_persicae3812, M_persicae2868a/b) relating to three putative amylase genes *ACYPI007122*, *ACYPI007753*, *ACYPI009042* (respectively) were identified as significantly increased (*ACYPI007122* $P<0.05_{FC:3.363/3.856}$ $0.03_{FC:3.415}$; *ACYPI007753* $P=0.034_{FC:1.165}$; *ACYPI009042* $P<0.05_{FC:4.022}$ $0.035_{FC:\ 8.215}$, t-test) in the extreme drought treatment, but did not show changes in expression in the mild drought treatment. Furthermore, a duplicated EST sequence (M_persicae2387a/b) relating to the aquaporin AQP1 (*ACYPI006387*) was significantly up regulated only in the extreme drought treatment ($P<0.05_{FC:1.8\text{-}1.95}$, t-test).

Further analysis of the mild drought treatment found significant up regulation of aN ortholog to the *A. pisum* gene *ACYPI001296*, encoding for a peroxisomal n1-acetyl-spermine/spermidine oxidase ($P<0.05_{FC:1.132}$, t-test), whilst a significant down regulation ($P<0.05_{\sim FC:1.25}$, t-test) was observed in ten EST probes (M_persicae15aP1-10) for a NAD-dependent methanol dehydrogenase (similar to *ACYPI009841*).

These observations were not consistent in aphids subjected to an extreme drought regime. Aphids under an extreme drought regime displayed no changes in expression of oxidases, however, they did display a significant up regulation ($P<0.05_{FC:1.317}$, t-test) in an EST encoding an alcohol dehyrogenase similar to the *A. pisum* gene *ACYPI003387*.

Whilst no alterations in expression levels of glucosidases were detected in either treatment, one EST sequence related to the *A. pisum* sucrase gene *ACYPI000002* was significantly up regulated in the extreme drought treatment ($P<0.05_{FC:1.5}$, t-test) and down regulated in the mild drought treatment ($P<0.05_{FC:1.6}$, t-test). However, in each array there were a total of four EST sequence probes that showed orthology to the *A. pisum* sucrase gene *ACYPI000002* (M_persicae4022a/b, M_persicae7717, M_persicae7867, M_persicae8379). Only one of these EST sequences wass significantly (M_persicae8379) altered in this analysis may signify that *M. persicae* has a family of paralogous sucrases which share homology to *ACYPI000002*, conversely it could also indicate that this EST sequence is an artefact of a type I error in the analysis.

Additionally, an EST sequence relating to the sucrase gene *ACYPI001436* was also found up regulated in aphids from the extreme drought treatment ($P<0.05_{FC:1.785}$, t-

a/b: denoted duplicated probe

FC: Fold change on array

test) and down regulated in aphids from mild drought treatment ($P<0.05_{FC:1.241}$, t-test). As with *ACYPI000002*, *ACYPI001436* was represented by two EST sequences M_persicae10020 and M_persicae933, however only the EST M_persicae10020 was found significantly altered in expression in the extreme drought analysis, whilst EST M_persicae933 was found significantly altered in the mild drought analysis. Although, these results support the data for *ACYPI000002*, that sucrase activity is down regulated in aphids during the mild drought treatment and up regulated during extreme drought treatment, these results also further raise the question of whether these EST sequences are representative of one gene or multiple gene paralogs.

To investigate the overlap between sucrase EST sequences, ClustalW2 (multiple sequence alignment) online software was used; this software enables analysis between EST sequences to determine the extent of EST sequence homology. If the sucrase EST sequences used in this array overlap or share similarities with each other then this will show up in the ClustalW2 analysis and EST sequences will score a high alignment scoring. However, where alignment scores are low between the ESTs (eg M_persicae10020 and M_persicae933, predicted to encode the sucrase gene *ACYPI001436* in *A. pisum*), then this would suggest that in *M. persicae* there is gene redundancy and multiple paralogs of that gene. Alternatively, it may suggest that the ESTs for *ACYPI001436* encode different regions of the gene.

A phylogram tree created through ClustalW2 analysis showed that EST M_persicae10020 predicted to encode *ACYPI001436* and M_persicae7717 encoding *ACYPI000002* were closely related, unsurprisingly EST sequences M_persicae8279

a/b: denoted duplicated probe

FC: Fold change on array

and M_persicae7867 both predicted to encode *ACYPI000002* were also closely related (fig 5.8). In contrast the other EST predicted to encode *ACYPI001436*, M_persicae933, was not closely related to M_persicae10020. Therefore, it can be concluded that these EST sequences may encode different areas of a homologous gene to *ACYPI001436*, or alternatively, may indicate that these ESTs represent two sucrase gene orthologs of *ACYPI001436*. The latter is supported by observed differential expression of each ESTs in the array, with M_persicae10020 was only found significantly up regulated in the extreme drought analysis, whilst EST M_persicae933 was only found significantly down regulated in the mild drought analysis.

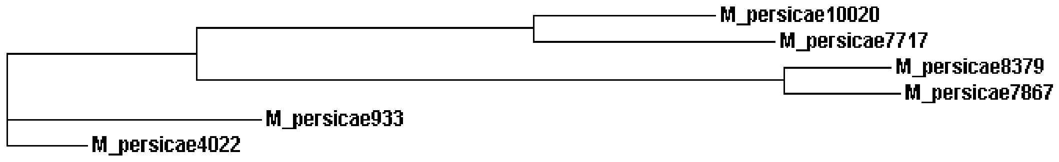


Figure 5.8 Phylogram tree of *M. persicae* ESTs created using online multiple sequence alignment software, ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). Tree shows the sequence homology between ESTs M_persicae10020 (sequence 1), M_persicae7717 (sequence 2), M_persicae8379 (sequence 3), M_persicae7867 (sequence 4), M_persicae933 (sequence 5) and M_persicae4022 (sequence 6). All EST sequences have predicted sucrase functions. M_persicae10020 and M_persicae933 share homology with the *A. pisum* gene *ACYPI001436*, whilst the other ESTs share sequence homology with *A. pisum* gene *ACYPI000002*. Alignment scores from ClustalW2 are as follows: Sequence 1:2=65, 1:3=56, 1:4=61, 1:5=69,1:6=45, 2:3=58, 2:4=56, 2:5=57, 2:6=69, 3:4=74, 3:5=74, 3:6=54, 4:5=58, 4:6=61, 5:6=64.

Finally, in the array there were differences in expression of amino acid metabolism related genes between treatments. ESTs predicted to encode for *ACYPI009247* a homocysteine S-methyltransferase (M_persicae562a/b $P<0.05_{FC:1.345}$, t-test), *ACYPI006227* an alanine-glyoxylate aminotransferase (M_persicae2724a/b

a/b: denoted duplicated probe

FC: Fold change on array

$P<0.05_{FC:1.453}$, t-test), *ACYPI004184* an amino acid transporter (M_persicae3389a/b $P<0.05_{FC:1.553}$, t-test), *ACYPI000561* an alpha-aminoadipate aminotransferase (M_persicae3127a/b $P<0.05_{FC:2.676}$, t-test) and ACYPI007549 an trypsin-like serine protease (M_persicae4618a $P<0.05_{FC:1.33}$, t-test) were significantly up regulated in the extreme drought treatment but absent from the mild drought treatment. Most of these EST sequences featured duplicated spots on the array and were observed to have both spots significantly up regulated.

5.3.4.2 Candidate Genes relationships (model *A. pisum* genome)

So far blasting the EST sequences from *M. persicae* in NCBI has enabled predicted functions for around half the EST sequences contained on the array. However, using PhylomeDB tree explorer means predicted *M. persicae* sucrase and amylase sequences can be compared against other gene orthologs within the *A. pisum* genome and in other arthropods species (Huerta-Cepas *et al*, 2008; 2010; 2011; International Aphid Genomics Consortium, 2010).

Phylogenetic analysis using PhylomeDB can provide an insight into the size and diversity of the amylase and sucrase family in *A. pisum*. This information will be important for predicting the diversity and similarity of these genes within *M. persicae*.

Analysis of a pre-generated phylogenetic tree containing the *A. pisum* alpha amylase gene *ACRYPI007122* (homologous to EST sequences significantly up regulated in extreme drought treated *M. persicae*), showed that the amylase gene family (in *A. pisum*) was very closely related to the sucrase gene family (fig 5.9). Analysis showed

a/b: denoted duplicated probe

FC: Fold change on array

A. pisum has nine paralogous alpha amylase genes (*ACYPI005645, ACYPI006558, ACYPI003132, ACYPI007122, ACYPI002724, ACYPI009754, ACYPI001772, ACYPI009042* and *ACYPI007753*). Four of these genes were detected with sequence homology in the *M. persicae* EST database, *ACYPI007122, ACYPI002724, ACYPI009042* and *ACYPI007753*. Additonally, three of these genes (*ACRYPI007122, ACYPI009042* and *ACYPI007753*) were found to alter in response to extreme drought stress in *M. persicae*.

Furthermore, phylogenetic analysis revealed *A. pisum* has seven sucrase paralogs (*ACYPI001436, ACYPI007467, ACYPI000002, ACYPI009924, ACYPI002020, ACYPI003310* and *ACYPI002659*), of which two of these were detected with sequence homology to sequences in the *M. persicae* EST database (*ACYPI001436*, and *ACYPI000002*), both of which were found to significantly alter in both mild and extreme drought treatment.

The presence of multiple paralogous amylase and sucrase genes within the *A. pisum* genome leads to the suggestion that *M. persicae* may also have further paralogs that are yet to be uncovered. Alternatively, *A. pisum* may be unique in the *Aphididae* family with the extent of gene redundancy in amylases. Interestingly, this analysis has shown that alpha amylase and sucrase genes in *A. pisum* are closely related phylogenetically, and this information may help to explain the unexpected increase in alpha amylase expression in drought stressed in *M. persicae*.

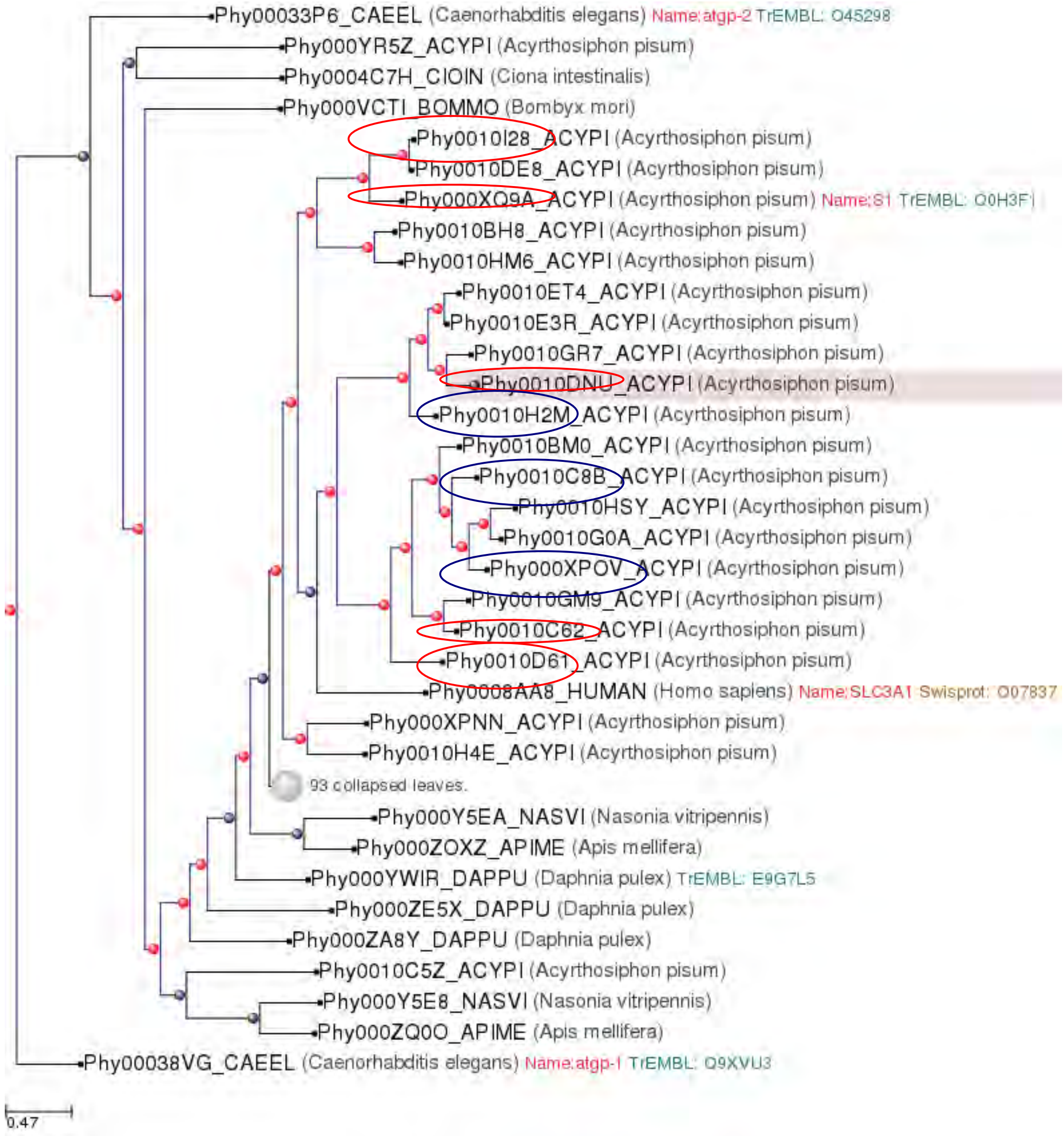


Figure 5.9 Phylogenetic tree generated using PhylomeDB (http://phylomedb.org) showing amylase and sucrase genes in *A. pisum* and orthologs in other organisms. Genes with sequence homology to *M. persicae* ESTs are circled blue; genes with sequence homology to *M. persicae* ESTs and found with altered expression in response to drought stress in *M. persicae* are circled red. Names for genes were pre-generated by PhylomeDB, thus a translation to gene names in NCBI with a summary of gene function is given for the following: Phy000YR5Z=*ACYPI054809* Glean peptide; Phy0010I28=*ACYPI001436* sucrase; Phy0010de8=*ACYPI007467* sucrase; Phy000XQ9A=*ACYPI000002* sucrase; Phy0010BH8= *ACYPI009924* sucrase; Phy0010HM6=*ACYPI002020* sucrase; Phy0010ET4=*ACYPI005645* alpha-amylase; Phy0010E3R=*ACYPI006558* alpha-amylase; Phy0010GR7=*ACYPI003132* alpha-amylase; Phy0010DNU=*ACYPI007122* alpha-amylase; Phy0010H2M=*ACYPI002724* alpha-amylase; Phy0010BM0=*ACYPI009754* alpha-amylase; Phy0010C8B=*ACYPI008964* CG14935; Phy0010HSY=*ACYPI001772* alpha amylase; Phy0010G0A= *ACYPI004099* alpha-glucosidase isozyme; Phy000XPOV=*ACYPI000732* CG14935; Phy0010GM9=*ACYPI003310* sucrase; Phy0010C62=*ACYPI009042* alpha-amylase; Phy0010D61=*ACYPI007753* alpha-amylase; Phy0008AA8=Human basic amino acid transport protein; Phy000XPNN= *ACYPI000768* GA13362 and Phy0010H4E=*ACYPI002659* sucrase

5.4 Discussion

Unfortunately, the genome for *M. persicae* is not yet fully sequenced and the use of an EST library (Ramsey *et al*, 2007) in this study means that there is the possibility that not all the genes in *M. persicae* genome have been represented in the array. It is also a possibility that there may be redundancy in the EST sequences on the array, with multiple EST sequences representing one gene.

Despite this, analysis of array data has shown that *M. persicae* alters gene expression in response to the level of water stress. For example, an *M. persicae* EST encoding the putative aquaporin, ApAQP1, was significantly up regulated in aphids undergoing the extreme drought treatment, but absent from those undergoing a mild drought treatment. Interestingly, ApAQP1 was originally identified in *A. pisum,* and was localised to the stomach and distal intestine area, where it was suggested to function in maintaining haemolymph osmotic pressure (Shakesby *et al*, 2009). The data in this chapter has indicated that ApAQP1 plays an important role in the aphid response to drought stress. In this study ApAQP1 expression was increased in response to extreme drought stress in *M. persicae*. Therefore, this study supports the hypothesis that water cycling may be an important osmoregulation mechanism in aphids. An RNAi knockout of ApAQP1 in drought stressed *M. persicae* followed by a measurement of aphid performance would enable an assessment of the importance of water cycling, and ApAQP1 in osmoregulation, which currently the data in this chapter can only speculate on.

Additionally, aphids also heterogeneously expressed stress related genes depending on the level of drought experienced. Heat shock transcripts were only up regulated in *M. persicae* experiencing extreme drought through their plant host. Heat shock proteins are a class of proteins that are involved in the unfolding and folding of proteins, their expression is usually increased in the heat shock response or in response to other stresses. In two resistant cotton varieties, heat shock protein (HSPs) homologs were increased in expression in response to water deficit. One of the presumed functions of HSPs is that they prevent protein denaturation during cellular dehydration (Nepomuceno *et al*, 2002). Furthermore, HSPs have been found to be increased in expression in other organisms, with water stress inducing the production of heat-shock genes in parasitic protozoa and arthropods (Petersen and Mitchell, 1985; Van der Ploeg *et al,* 1985). The increased expression of HSPs in extremely droughted *M. persicae* suggests that under severe drought aphids may utilise HSPs to prevent protein denaturation. However, this would have to be further investigated.

Interestingly, heterogeneous expression was also observed between treatments for cytochrome proteins. Eight cytochrome proteins were up regulated in aphids on mildly droughted hosts, whilst only two were up regulated in aphids on extremely droughted hosts. This emphasises that aphids are able to distinguish levels of drought and adjust expression accordingly. This may partly explain the adaptability of aphids to drought stress, and why aphid fecundity was only reduced by a maximum of 37% (in *B. brassicae*) on extremely droughted hosts (in chapter four).

Cytochromes are membrane-bound hemoproteins that carry out electron transport and contain heme groups. The cytochromes that were up regulated in *M. persicae* during this study were mainly cytochrome P450 oxidase proteins, which function as oxidising catalysts (monooxygenases) and are the largest and most functionally diverse class of insect detoxification enzymes (Scott, 1999; Li *et al*, 2007). Studies have shown that cytochrome P450 (P450), in *M. persicae* resistant clones, mediate detoxification of neonicotinoid insecticides (Puinean *et al*, 2010). Moreover, it has been estimated that the P450 gene family in *M. persicae* is around 40% larger than that of the sequenced *A. pisum* (Ramsey *et al*, 2010). This expansion of P450s is believed to reflect the host range of *M. persicae*, which feeds on 40 different plant families, and requires a greater variety of detoxification enzymes compared to *A. pisum*, which only feeds on the plant family *Fabaceae*. Thus, as P450s are typically associated with toxicity stress, it is interesting that P450s were found increased more under mild drought stress than under extreme drought. It may be that under mild drought the host plant *B. nigra* responds to drought stress by activating stress pathways that influence leaf toxicity, resulting in an increase in P450s in *M. persicae*. Under extreme drought *B. nigra* may divert more resource allocation specifically to osmoregulation mechanisms resulting in less P450s being up regulated in aphids feeding on extremely droughted *B. nigra* plants. However, to confirm any of this speculation further investigation into *B. nigra* leaf toxicity under water stress would be required.

Increased salivation in the literature is associated with overcoming plant defences and reduced palatability in host plants (Ramirez and Niemeyer, 1999; Caillaud *et al*, 1995; Ponder *et al*, 2000; Hunt *et al*, 2009; Hale *et al*, 2003; Prado and Tjallingii, 1997; Will *et al*, 2007). Recently there have been many studies attempting to elucidate the composition of aphid saliva. In *Sitobion avenae*, oxidoreductases (polyphenol oxidase and peroxidase) have been found in the gelling saliva forming the aphid’s stylet sheath, whilst polyphenol oxidase has been located in watery saliva (Urbanska *et al*, 1998). Oxidoreductases have been suggested to function in mediating oxidative detoxification of plant allelochemicals or promoting the gelling of the sheath protein (Carolan *et al,* 2009). Additionally, phosphatases have been putatively identified in the saliva of the Russian wheat aphid, *Diuraphis noxia,* using gel electrophoresis and mass spectrophotometry. In *D. noxia*, other peptides were also identified as putative dehydrogenases and RNA helicases from their similarities to ESTs identified from other aphids (Cooper *et al*, 2010). In *A. pisum*, a homolog of an angiotensin-converting enzyme (an M2 metalloprotease), an M1 zinc-dependant metalloprotease, a glucose-methanol-choline-oxidoreductase and a homolog to regucalcin (a putative calcium-binding protein) have been identified in saliva. Furthermore, metalloproteases and regucalcin have been predicted as functioning by inactivating plant protein defences and inhibiting the calcium-mediated occlusion mechanism in phloem sieve elements (respectively) (Carolan *et al*, 2009).

Earlier in this chapter it was hypothesised that aphids may potentially alter saliva composition in response to drought. If aphid salivation composition alters as a response to drought then it could be expected that differential expression levels in *M. persciae* salivatory associated genes such as amylases, cellulases, glucosidases, oxidases, dehydrogenases and pectinesterase (Cherqui and Tjallingii, 2000; Miles, 1999; Urbanska and Leszczynski, 1997; Hamel *et al*, 2008) would be observed. However, the microarray data gathered in this chapter showed no significant changes in the expression of pectinesterases, cellulases or glucosidases.

In contrast, there were some oxidases and dehydrogenases that were up regulated heterogeneously between the two drought treatments. A peroxisomal n1-acetyl-spermine/spermidine oxidase was significantly up regulated in the mild drought treatment whilst ten EST probes (M_persicae15aP1-10) for a NAD-dependent methanol dehydrogenase (similar to ACYPI009841) were significantly down regulated. Interestingly, none of these genes were altered in expression levels in the extreme drought treatment, furthermore, aphids under an extreme drought regime displayed no changes in expression of any oxidases. However, an EST encoding an alcohol dehyrogenase similar to the *A. pisum* gene, ACYPI003387, was up regulated in aphids under the extreme drought treatment.

Peroxisomal n1-acetyl-spermine/spermidine oxidase is a polyamine oxidase, which oxidises spermine involved in cellular metabolism, whilst methanol dehydrogenase catalyses methanol into formaldehyde and participates in methane metabolism. Interestingly, in plant cells of sycamore, **Acer pseudoplatanus*,*** methanol has been shown to be metabolised to serine, methionine, and phosphatidylcholine (Gout *et al*, 2000). Methanol dehydrogenase may play a similar role in aphids.

Although the differential expression of these genes highlights that aphids must perceive the level of drought, and adapt expression levels accordingly, the protein products from these genes may not be exclusively expressed in saliva. Without tissue localisation experiments, it is impossible to conclude if the saliva composition has altered under drought. It is possible that the alterations in expression levels of these dehydrogenases and oxidases, are in fact an artefact of other changes in metabolism relating to osmoregulation.

Interestingly, analysis of amylase expression provides the most support for the hypothesis of aphids having an alternative salivation composition in response to drought stress. The enzyme α-amylase catalyses the hydrolysis of 1, 4-alpha-D-glucosidic linkages in oligosaccharides and polysaccharides (Hamel *et al*, 2009). In honeybees, amylase is thought to be needed to convert plant starch (found in nectar) into glucose, which can then be converted in gluconic acid (Ohashi *et al*, 1999). Alpha-amylase has also been found with glucosidase in mosquito salivary glands (Effio *et al*, 2003) and in the saliva of *M. persicae* (Harmel *et al*, 2008).

Unlike dehydrogenases and oxidases, there are fewer putative amylases found in the EST library of *M. persicae*, this may be because amylase is involved in fewer pathways. This study has shown that *M. persicae* under an extreme drought treatment significantly increased the expression of three putative amylase genes. Furthermore, phylogenetic analysis shows that amylase genes in *A. pisum* are closely related to sucrase genes.

In the pea aphid, *Acyrthosiphon pisum,* sucrase activity has been localised to the gut, distal to the stomach (Ashford *et al*, 2000; Cristofoletti *et al*, 2003). Gut sucrase APS1 in *A. pisum* was shown to have an α-glucosidase activity to form the hexose units for oligosaccharide synthesis (Price, et al. 2007). Phylogenetic analysis has shown *A. pisum* has seven sucrase genes, of which, two orthologs were detected up regulated in *M. persicae* fed on extremely droughted plants. This indicates that sucrase and amylases play important roles in *M. persicae* osmoregulation, in their role in breaking down sugars into hexose units for polymerisation into polysaccharides.

Finally, around half of the genes found in both treatments to be significantly up/down regulated were of an unknown function. Although it is beyond the scope of this study to elucidate the function of these genes, they should not be disregarded. Recently, C002 a salivatory protein of unknown function, has been identified as having a significant effect on aphid survivorship (Mutti *et al*, 2006; 2008). This demonstrates that there is still a lack in the body of knowledge surrounding gene functions in aphids. However, despite this it is hoped the data in this study will aid in any further investigation into the function of some of these genes. Additionally as the focus of this chapter has been to study the changes in gene expression in relation to osmoregulation and salivary processes, the results found here would require further validation by RT-PCR, particularly for the sequences relating to predicted sucrases and aquaporins.

In summary, this chapter has helped us to identify and confirm genes that are induced under drought stress, enabling us to start to understand a response that is very complex, with many functions still unknown. The increased expression of sucrases and the ApAQP1 aquaporin, under drought stress, supports the current hypothesis of sugar polymerisation and water cycling as important aphid osmoregulation mechanisms. In addition, this chapter has highlighted that the level of drought plays an important role in observed expression changes. Therefore, it is suggested that for future comparative studies it is important for the same level of drought to be adopted.

Chapter 6: General Discussion

6.0 Discussion

The complexity of the plant-aphid interaction was highlighted at the beginning of this thesis. Ultimately, how it responds to stressors will depend on the species in the interaction and the influences of past experience, environmental and social cues. This study set out to establish if the response of the plant-aphid interaction was predictable, or whether there was variation in aphid behavioural, reproductive and transcriptomic responses to drought.

Currently, there are conflicting hypothesises on how aphids might respond to drought and the predictability of this relationship. The plant-stress hypothesis states that plant defences may become reduced in water stressed plants and that drought induced composition changes may make them more palatable or susceptible to herbivory. In support of this hypothesis are a number of studies (Wearing, 1967; Wearing and van Emden, 1967; Miles *et al*, 1982; Oswald and Brewer, 1997) recorded in the literature, however, there are a number of studies that support an alternative notion.

This notion is that the osmotic stress resulting from drought will result in resource re-allocation from growth and reproduction into osmoregulation mechanisms. Therefore, aphid performance would be reduced. In addition, drought induced changes in the plant host may make the host less palatable to aphid herbivores, for instance changes in sugar/pH gradients may result in aphids facing difficulty in locating the phloem. In support of this hypothesis there has also been a number of studies (Larsson, 1989; Pons and Tatchell, 1995; Bethke *et al*, 1998; Honěk *et al*, 1998; Kennedy *et al*, 1958; Kennedy and Booth, 1959) recorded in the literature.

So far the differences observed in these studies may be attributable to the differences in the level and duration of water stress imposed, or to the variation between species and genotypes. In order to establish if these differences are attributed to the level and duration of water stress, this thesis set out to establish a droughting regime that was repeatable in two plant species, *Brassica nigra* and *Lolium perenne* and to test the responses of four aphids species. Chapter two defined a mild and extreme drought regime, in extreme drought both *B. nigra* and *L. perenne* showed altered levels of ions and amino acids, increased reducing sugar concentrations and fresh weight: dry weight ratios of <6. Interestingly, transcriptomic analysis showed that the level of drought affected gene expression of *Myzus persicae* feeding on drought treated *Brassica nigra*. Under mild drought treatment, cytochromes associated with detoxification (Scott, 1999; Puinean *et al*, 2010) were abundantly upregulated, and this observation was absent in extreme drought. However, under extreme drought Heat Shock Proteins (HSPs) were abundantly upregulated, and again this observation was not observed in the mild drought treatment. HSPs have the predicted function of preventing protein denaturation during cellular dehydration, and so this seems to be a response to osmotic stress, whilst the upregulation of cytochrome may be a response to drought triggered plant defences.

It was predicted that to cope with the extreme level of drought established in chapter two, aphid species would have to utilise their well evolved array of osmoregulation mechanisms. These mechanisms under normal conditions allow aphids to cope with diets that have three-fold greater osmotic pressures than their haemolymph. These mechanisms have been identified as sugar polymerisation, xylem feeding, water cycling and production of metabolic water.

Subsequent analysis of these mechanisms in this thesis showed they were not utilised homogenously under water stressed conditions. Upon plant contact and initial probing, *Brevicoryne brassicae* and *Myzus persicae* (B clone) placed a greater bearing upon behavioural osmoregulatory mechanisms such as water acquisition from the xylem.

In the field aphids may behaviourally attempt to avoid water stressed hosts spatially, by migration. Whilst it was beyond the scope of this thesis to assess the extent aphids simply avoid drought stress spatially. If it was shown that in *Rhopalosiphum padi*, *Brevicoryne brassicae* and two clones of *Myzus persicae* (O and B clone), drought resulted in reduced sieve element sap ingestion and significantly more time spent not-probing plant tissue. These results suggest that aphids are unsettled on droughted hosts, and if subsequent field studies were conducted, we may find these species behaviourally avoided droughted hosts by migration.

When aphids are faced with no host alternative, adaption to water stress may be an upregulation of enzymes associated with sugar polymerisation, arguably the most important osmoregulation mechanism in aphids. If aphids have the ability to upregulate sugar polymerisation enzymes then, despite aphids feeding on an osmotically extreme diet, they may still have an adaptive capability to withstand further osmotic stress.

It is thought that sugar polymerisation of sieve element sap in aphids is mediated by sucrase enzymes that hydrolyse sucrose into glucose and fructose units (Wilkinson *et al*, 1997; Ashford *et al*, 2000; Cristofoletti *et al*, 2003). These units can then be polymerised into oligosaccharides of low osmotic pressure per hexose unit (Rhodes *et al*, 1997), inhibition of sucrase activity has been shown to result in aphids being unable to control haemolymph water potential (Karley *et al*, 2005). The results of this thesis showed that sucrases in *M. persicae* are upregulated in response to extreme drought stress, indicating that aphids have the potential physiological plasticity to cope with drought stress.

Furthermore, the upregulation of a putative aquaporin ApAQP1 in extreme drought treated *M. persicae* highlighted that water cycling is also important in the aphid's response to drought stress. ApAQP1 has been demonstrated to increased osmotic water permeability and play an important role in maintaining the osmotic pressure of the haemolymph (Shakesby *et al*, 2009). The upregulation of ApAQP1 in *M. persicae* indicates that like with sucrases, there is regulation of these mechanisms and that the genes controlling them are not expressed at stress threshold levels under normal conditions.

However, aphid adaptability appears to vary between species, with some species exhibiting more plasticity then others. Aphid performance in response to water stress has also been shown to vary depending on the clone of aphid. The results of this thesis showed that fecundity was reduced in both *M. persicae* clones but only significantly in green (O) clones, which displayed a reduction of 19% whilst red (B) clones of *M. persicae* displayed a reduction of 9%. Studies into development rates of aphid species have shown that variation in growth and development rate can occur within the species *M. persicae, Sitobion avenae, Aphis craccivora* and *Acyrthosiphon pisum* (Edwards, 2001; Sandström, 1994; Sunnucks *et al*, 1998). Additionally field studies have also shown that aphid clones can proliferate differentially during the summertime (Tomiuk and Woehrman, 1981; Haack *et al*, 2000). Whilst other studies regarding clonal temperature tolerance have shown clonal variation in response to stress is not unusual (Alford *et al*, 2011; Powell and Bale, 2005).

In conclusion aphids are highly adaptable to their mode of feeding. Water stress is without a doubt generally detrimental to aphid hosts, but its' affect on aphids depends heavily on the level of water stress experienced as much as the aphid species and clone. In fecundity experiments in this chapter, fecundity was reduced in all species; *M. persicae, S. avenae, R. padi* and *B. brassicae*. However, fecundity was only significantly reduced *in R. padi, B. brassicae* and the *M. persicae* green (O) clone. Furthermore, the percentage reduction in fecundity varied between species, with the specialist *B. brassicae* showing a reduction of 37% compared to 19% and 9% in the two *M. persicae* clones (O and B clone respectively), this is despite both species being exposed to a similar drought level on the same host plant.

So far our study has shown that the plasticity in adaption to water stress in different aphid species, is dependant on the ability to upregulate sugar polymerisation, increase aquaporin expression to increase water cycling, or even the ability of an aphid to locate the xylem. Furthermore, this study has shown that the response of the plant-aphid interaction is predictable only in the sense that an extreme level of drought is detrimental to aphid performance. However, under varying levels of drought, and with different aphid-plant combinations, this thesis suggests that the response of the aphid-plant interaction is not predictable. This is due to the different responses of plant hosts to drought stress, and the differential adaptability and preference for certain osmoregulation mechanisms in aphid clones and species.

Finally, although this study has found some convincing behavioural and genetic evidence in support of some of the aphid osmoregulation mechanisms, there were still many genes in *M. persicae* that were found significantly upregulated in response to drought stress, but of an unknown function. These genes demonstrate that there is a lack in the body of knowledge, with an unknown gene recently being identified as vital for survivorship (Mutti *et al*, 2006; 2008). If the work in this thesis was continued then an obvious route would be gene cloning and use of RNAi to knockout function, in the hope to reveal the importance and function of some of these unknown genes.

Chapter 7: References

7.0 References

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