INVESTIGATIONS INTO INSECT-INDUCED PLANT RESPONSES OF WATER HYACINTH (*EICHHORNIA CRASSIPES* (MART.) SOLMS-LAUB.) (PONTEDERIACEAE)

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ABSTRACT

The water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laub (Pontederiaceae)) biological control programme makes use of tight plant-insect interactions to control the weed, by re-establishing the interactions between the plant and its natural enemies. Since the beginning of the water hyacinth biological control initiative, the impact of biological control agent herbivory on water hyacinth's population growth and fitness have been well documented; however, very few investigations have been conducted to determine whether herbivory elicits insect-induced responses by water hyacinth.

Studies were conducted to determine the presence and function of water hyacinth insect-induced responses, using the plant activator, BION®, in attempt to determine the plant hormone-mediated pathways regulating the final expressions of insect-induced defences in response to herbivory by the phloem-feeder, *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae) and the leaf chewer, *Neochetina bruchi* Hustache (Coleoptera: Curculionidae). BION® (Syngenta, acibensolar-S-methyl (benzothiadiazole)) is a dissolvable, granular formulation that contains a chemical analogue of the plant hormone, salicylic acid (SA), which typically regulates defences against pathogens. The application of BION® results in the induction of the SA-mediated defence pathways in plants (activation of defences against pathogens), and consequently the inhibition of the jasmonic acid (JA)-mediated defence pathways (de-activation of defences against insect herbivores).

To test for induced defence responses in water hyacinth, plants treated with BION® and then subjected to herbivory, were compared to un-treated plants that were also subjected to herbivory, BION®-only treated plants and control plants. The application of BION® did not confer resistance against the two insect herbivores, as herbivory, reductions in chlorophyll content and plant growth (leaf production and second petiole lengths) significantly increased in comparison to non-BION® treated plants. Furthermore, palatability indices significantly increased (>1.00) in BION® treated plants, reflecting increased weevil preferences for SA-induced water hyacinth plants. This concluded that SA-mediated defences are not effective against insect herbivory in water hyacinth plants, but are in fact palatable to insect herbivores, which reflects ecological and physiological costs of SA-mediated defences (pathogen defences) in water hyacinth.

Biochemical analyses of leaves exhibited increases in nitrogen content in BION® treated plants. These elevated levels of nitrogenous compounds account for the increases in mirid and weevil preferences for BION® treated plants. The increases in nitrogenous compounds are probably structural proteins (e.g. peroxidises), because leaves treated with BION® increased in toughness, but only when exposed to herbivory. Regardless, insect herbivory was elevated on these leaves, probably because the nitrogenous compounds were nutritionally viable for the insects.

Non-BION® treated plants exposed to herbivory remained comparable to control plants, in terms of biomass accumulation and leaf production, indicating that water hyacinth employed some form of defence strategy to deter insect herbivory. Interestingly, water hyacinth did not toughen its leaves in response to herbivory by the mirid and weevil. In addition, water hyacinth exhibited different responses to the mirid and the weevil, indicating species-specific responses. JA-mediated defence pathways most likely contributed to *N. bruchi*-induced water hyacinth defences in younger leaves, because in the presence of JA-mediated defence pathways, younger water hyacinth leaves significantly increased in thickness and weevil herbivory declined significantly. On the other hand, plants exposed to *E. catarinensis* did not alter leaf structural traits. Thus, JA-mediated defence pathways are likely to play a minimal or no role in *E. catarinensis*-induced water hyacinth defences. Water hyacinth did, however, exhibit a compensatory growth response to mirid herbivory, whereby water hyacinth plant biomass accumulation, leaf production and chlorophyll content remained unchanged, and the lengths of the second petioles significantly increased.

Ultimately, SA plays no role in water hyacinth insect-induced responses, and is most likely to confer resistance against water hyacinth pathogens. In comparison, JA-induced responses play an important role in water hyacinth insect-induced responses, namely in response to *N. bruchi* feeding. The role that JA-mediated defence pathways play in response to feeding by *E. catarinensis* is unclear. However, this study identified a clear compensatory growth response when water hyacinth was fed on by the mirid, highlighting the species-specific responses water hyacinth employs against the biological control agents. The results obtained in the present study conforms to plant-hormone defence theory in the literature, where JA-mediated defence pathways generally confer resistance against insect herbivores. This seminal work on water hyacinth places the biological control of water hyacinth into a theoretical context of plant-insect interactions. Subsequently, we can apply the knowledge of

fundamental plant-insect interactions in the applied field of biological control to better understand the physiological basis for successful biological control.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INSECT-PLANT INTERACTIONS

Plants are the principal primary producers on earth, providing considerable resources to consumers (Bernays and Chapman, 1994), while insects are the most species rich group of organisms, contributing to a remarkably large proportion of all extant species (Price, 2002). For 350 million years, plants and insects have developed perpetual and highly evolving relationships, where each organism takes opportunity of the available resources and variety of ecological services that another organism has to offer (Price, 2002, Gatehouse, 2002, Mello and Silva-Filho, 2008, War *et al*., 2012). The interactions between plants and insects are highly dynamic and may be beneficial to both organisms, as is the case with pollination and seed dispersal (Mello and Silva-Filho, 2008), or beneficial only to one, as with herbivory. Insects are major herbivores, making up roughly half of all extant insect species (Wu and Baldwin, 2010), feeding on plants in various ways, according to different feeding guilds (Strauss and Zangerl, 2002). A variety of feeding guilds exists, such as chewers (which mainly includes the orders Lepidoptera, Orthoptera and Coleoptera), sap suckers (order Hemiptera), miners (orders Diptera, Lepidoptera and Coleoptera) and borers (orders Lepidoptera and Coleoptera), all of which injure plants in various ways. In order to endure injury from herbivory and ensure survival and production of progeny, plants have evolved various response mechanisms to deter herbivores; however, insect herbivores have subsequently evolved ways to overcome the plants' defence responses, which operate as a selective pressure on plants to improve defence responses (Stamp, 2003). Plants and insects

that compete in this manner subsequently co-evolve and maintain tight relationships, which have contributed to the evolution of complex plant chemistry (the theory of coevolutionary arms race) (Ehrlich and Raven, 1964, Bernays and Graham, 1988).

1.1.1 Defence theory: costs and benefits

Studies of plant-insect interactions and associated plant anti-herbivore defences have paved way for the formulation of plant defence theories, in order to explain why vast interspecific differences in plant defences occur (Bazzaz *et al*., 1987, Endara and Coley, 2011). To ensure survival and the production of progeny, plants allocate resources to growth, reproduction and defence (Bazzaz *et al*., 1987); however, investment of limited resources into one component of fitness (e.g. growth) typically precludes the allocation of resources to other components of fitness (e.g. reproduction and defences) (Rhoades and Cates, 1976, Herms and Mattson, 1992). Consequently, in the event that a plant is under attack, the resources that could otherwise be used to maintain growth (vegetative and reproductive) and physiological functioning are reallocated to the production of defences to minimise the deleterious impacts of herbivory (Mole, 1994). Numerous studies have identified these negative relationships between plant growth and plant defences (Smedegaard-Petersen and Stølen, 1981, Coley, 1986, Herms and Mattson, 1992, Sagers and Coley, 1995).

In effect, the production of defences is costly and because herbivory is random, allocation costs are invariably dependent on the timing of defence deployment (Wu and Baldwin, 2010). Defences produced regardless of the presence of herbivores or injury (Wu and Baldwin, 2010) are termed constitutive defences (e.g. tannins, Sagers and Coley, 1995). Conversely, induced defences are defences only initiated after attack by herbivores (Walling, 2000, Wu and Baldwin, 2010). The most costly defences are generally those that are

constitutive, as they are produced continuously; therefore it is hypothesised that inducible defences are an evolutionary result of plant resource optimization.

The mechanism behind which a plant allocates resources to defences is largely dependent upon resource availability in the environment (the Resource Availability Hypothesis) (Coley *et al*., 1985). In addition, a plant's investment into defences is also largely dependent on its inherent growth rate and whether the benefits outweigh the costs ("growth-rate theory") (Coley, 1988, Endara and Coley, 2011). In resource limited environments, plants with slow growth rates are better adapted to herbivory than plants with fast growth rates, as they invest heavily in constitutive defences (Coley *et al*., 1985). On the other hand, in resource rich environments, plants with fast growth rates are capable of outgrowing herbivore injury, and thus typically invest less in constitutive defences; however, plants with fast growth rates in resource rich environments may also invest in induced defences.

1.1.2 Insect-induced plant responses

Plants have evolved a wide array of defence mechanisms in response to attack, which help reduce the impact of herbivores. A plant trait that reduces an insect's performance and preference for the plant, and ultimately results in increased fitness to the plant, is characterised as a form of defence (Howe and Schaller, 2008, Karban and Myers, 1989). Biochemical processes within plants mediate plant defences and these defence mechanisms are typically chemical and/or physical in nature (Bernays and Chapman, 1994, Stamp, 2003), and are employed either directly and/or indirectly.

Chemical defences

Plant chemical defences generally entail the accumulation and/or release of biochemical compounds in response to specific herbivore attack, which may contribute to a nutritional and/or non-nutritional component of chemical defences (Reese, 1979). Primary metabolites are products of plant biochemical processes that contribute largely to plant growth and metabolism. However, primary metabolites are also used as a form of defence in plants (Steinbrenner *et al*., 2011); by altering the concentrations of primary metabolites, the plant quality changes. For example, low nutritive plant quality is a direct strategy employed by plants to deter herbivores (Rhoades and Cates, 1976, Moran and Hamilton, 1980). This is achieved by altering the concentrations of primary metabolites (e.g. nitrogen), which effectively reduce the nutritional value of the plant tissue, thereby interfering with the herbivores' dietary needs (Lundberg and Åström, 1990).

On the other hand, secondary metabolites contribute very little to plant growth and metabolism, but play large and essential roles in plant defence strategies. Typically, secondary metabolites are classified into various compounds with specific functions, such as cyanogenic glucosides, alkaloids, phenols, terpenes and sterols (Bennett and Wallsgrove, 1994). Many of these secondary metabolites are responsible for the specific odours, colours and tastes in a variety of plants (Bennett and Wallsgrove, 1994). In addition to the low nutritive quality defence strategy brought about by primary metabolites, secondary metabolites also contribute to reduced plant quality, by increasing the concentration of digestibility-reducing toxins or compounds within the plant tissue (Lundberg and Åström, 1990), effectively reducing the palatability of the plant. Leaf carbon and nitrogen content, and the ratio of carbon to nitrogen (C:N) in leaves, are regarded as good indicators of leaf quality and predictors of the amount and type of herbivore defences (secondary metabolites)

(Bryant and Klean, 1983,). Large leaf C:N ratios indicate reductions in leaf palatability, because the availability of nutritional protein (nitrogen) is surpassed by the availability of increased, indigestible, carbon-based structural compounds, thus deterring herbivores seeking more nutritious plants (carbon-based defences) (Herms and Mattson, 1992). On the other hand, smaller leaf C:N ratios indicate larger allocations of nitrogenous-based compounds in the leaf in relation to carbon-based compounds, and these increases in nitrogen may be of nutritional value to one type of herbivore, but detrimental to another, because the synthesis of nitrogenous compounds may have toxic properties (nitrogen-based defences, e.g. alkaloids, glucosinolates and amino acids) (Herms and Mattson, 1992).

Secondary metabolites also may be released as distinct volatile organic compounds in response to insect herbivory, which act as an indirect defence, attracting natural enemies of the herbivores, such as parasitic and predacious insects (De Moraes *et al*, 1998, Hare, 2011). By attracting parasitoids and predators, injury to the plant is reduced through the removal and/or eventual death of the insect herbivore. Depending on the species of herbivore, the plant species, growth conditions and the suite of herbivore species attacking the plant, the volume and the composition of the volatile compounds released may vary (Hare, 2011).

Physical defences

Physical defences are derived from the same biochemical processes that form chemical defences within plants, but are the final expressions of these processes (Schoonhoven *et al*., 2005). Plant physiological and morphological traits contribute to a direct form of defence by deterring insect herbivores. Spines, thorns and hairs (trichomes) act as physical barriers on the plant that influence a herbivore's ability to utilise the plant for nutrition and/or oviposition, and essentially reduce the extent of further herbivory (Karban and Myers, 1989, Howe and Schaller, 2008). Leaf toughness and waxy leaves are two other forms of physical

defence that plants employ, ultimately reducing the ability of insect herbivores to penetrate and chew the plant tissue (Howe and Schaller, 2008).

1.1.3 Induced plant defence pathways

As discussed above, plant defences are costly, as they incur allocation costs in terms of growth and reproduction. Taking into account the resource demands of plant defence production, plants have evolved highly sophisticated regulatory systems to balance growth against defence. Induced plant defences are flexible cost-effective defences that are mediated by a variety of plant hormones. Depending on the guild type of insect feeding on the plant (chewers, sap suckers, miners), the type of hormone defence pathway employed varies. Salicylic acid (salicylates, SA) and jasmonic acid (jasmonates. JA) are the most extensively studied defence-mediating plant hormones (Achuo *et al.*, 2004, Smith *et al.*, 2009). The salicylate defence pathway mediates the initiation of a hypersensitive response and the production of phytoalexins and pathogenesis-related proteins that confer resistance against biotrophic pathogens (pathogens which feed on living plant tissue) (Gaffney *et al.*, 1993, Farouk *et al.*, 2008). On the other hand, the jasmonate defence pathway mediates the production of secondary metabolites and induced volatiles that confers defence against herbivores and necrotrophic pathogens (pathogens which feed on decaying plant tissue) (Traw *et al.*, 2003, Smith *et al.*, 2009). However, it is not always clear-cut; some herbivores may induce SA-mediated defence pathways, and some pathogens may induce the JA-mediated defence pathway (Smith *et al.*, 2009). Generally, it is the level of damage caused by the herbivores that dictates the defence pathway employed; some sap suckers (phloem feeders) cause less tissue damage than chewing insects which is often comparable to the damage caused by pathogens, and consequently the resultant defence mechanisms are similar

to pathogen-induced defences (SA-mediated defences) (Moran and Thompson, 2001, Smith *et al*., 2009). There is substantial cross-talk between the SA pathway and JA pathway; the up-regulation of one pathway may inhibit the induction of the other pathway (Walling, 2000).

For example, the synthesis of JA in tomato (*Lycopersicon esculentum* Mill. (Solanaceae)) is inhibited with the external application of SA (Doares *et al*., 1995). In tobacco (*Nicotiana tabacum* L. (Solanaceae)), the same antagonistic relationships are observed with the external applications of SA and JA (Felton *et al*., 1999). This negative cross-talk between the defence pathways allows plants to suppress the up-regulation of unnecessary defence mechanisms in order to conserve energy costs for appropriate defence mechanisms elicited against specific herbivores (Cipollini *et al*., 2003, Smith *et al*., 2009).

Scientists have developed methods for inducing specific plant defence mechanisms in order to investigate insect-plant interactions. Simply, the saliva of insect herbivores may be applied to plants to mimic insect induced releases of volatiles (Baldwin and Preston, 1999). However, numerous studies have used chemical elicitors to induce insect defence responses in plants (Cipollini *et al*., 2003). Volicitin, a herbivore-specific elicitor isolated from the saliva of beet armyworm (*Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae)), has been used to induce the release of volatile compounds in *Zea mays* L. (Poaceae) seedlings (Alborn *et al*., 1997). A study by Brown (1988), investigating the costs of chemical defences through proteinase inhibitors in tomato, induced proteinase inhibitor production by injecting chitin (aqueous ethylene glycol chitin, 1mg/ml, Sigma) into the parenchymal tissue of the stems. More recently, the exogenous application of chemical analogues of SA and JA has been more frequently used.

Thaler *et al.* (1996) exogenously applied JA and its methyl ester to tomato (*L. esculentum*), which in effect resulted in reduced suitability of the foliage to the two insect herbivores, *S. exigua* and *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae). Furthermore, Qiu *et al.* (2012) used exogenous applications of SA to induce feral cabbage plant defences, and observed the foraging behaviour of *Cotesia glomerata* (Linnaeus, 1758) (Hymenoptera: Braconidae), a parasitoid of *Pieris brassicae* (Linnaeus, 1758) (Lepidoptera: Pieridae), the large cabbage white butterfly. Results of the study exhibited increased parasitism rates of *P. brassicae* on plants with SA applied to the shoots. Chemical analogues of SA, namely benzothiadiazole, have been used to mediate plant defences against pathogens in numerous agronomic studies (e.g. Dietrich *et al.*, 2004, Heil *et al*., 2000, Patricio *et al*., 2007, Sarwar, *et al.*, 2005). An exogenous application of SA enables the expression of pathogen resistance genes and subsequent systemic acquired resistance, which are effective against necrotrophic, and biotrophic pathogens and some insects (Smith *et al*., 2009). Additionally, the external application of SA to plants also inhibits the biosynthesis of ethylene and therefore ethylene-mediated defence pathways (Leslie and Romani, 1986).

Agronomists are increasingly investigating the roles and practical uses of chemical elicitors in pest resistance. One particular registered plant activator, BION® (Syngenta), has gained notable use within field and laboratory studies. BION® is a granular formulation that contains the active ingredient acibensolar-S-methyl (benzothiadiazole), a chemical analogue of SA. The application of BION® results in the induction of the SA-pathway (activation of defences against biotrophic pathogens and some insect herbivores), and the inhibition of the JA pathway (de-activation of defences against herbivores), whereby the application of SA ceases the production of allene oxide synthase, a essential enzyme to produce JA (Iverson *et al*., 2001). Achuo *et al*. (2004) investigated the role of SA in the defence and resistance against powdery mildew (*Oidium neolycopersici*) and grey mould (*Botrytis cinerrea*) in

tomato and tobacco. The plant activator, BION®, was used to induce the SA defence pathway and results of the study showed that SA played no role in the defence of tobacco against *B. cinerrea*, but played a role in tomato defence against the same pathogen. The opposite results were observed for the role of SA in the resistance against *O. neolycopersici*, where tomato exhibited increased resistance, but tobacco did not. Chemical elicitors, such as BION®, have expanded opportunities for investigations into insect-plant interactions, not only for agronomy and the subsequent biological control of pests, but possibly for other fields of study, namely the biological control of invasive weeds.

1.1.4 The role of insect-induced plant responses in weed biological control

The applied field of classical weed biological control makes use of the tight relationship between a plant species and its natural enemies to elicit control. The foundation of weed biological control essentially relies on the interactions between an invasive plant species and its introduced natural enemies, with the anticipation that the herbivores will feed without hindrance and consequently result in a reduction of a weed's population growth (e.g. Hoffman and Moran, 1998, Albright *et al.*, 2004). Decades of research have gone into developing weed biological control programmes worldwide (McFadyen, 2000, Olckers, 2004), and many programmes have been developed to target emerging weeds (e.g. in South Africa (Olckers, 2004)), but the mechanisms involved in these specific plant-insect relationships are often not understood. Very little has been done to investigate the mechanisms whereby invasive plants alleviate herbivory, despite the extensive documentation of insect-induced plant responses within the literature, and the fact that effective control depends on the optimal feeding by natural enemies. Effectively, weed biological control programmes provide suitable platforms for investigating insect-plant

interactions, particularly for freshwater macrophytes, as one of the most active weed biological control programmes in the world targets a highly noxious, invasive free-floating macrophyte species, water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laub (Pontederiaceae)).

1.2 FRESHWATER MACROPHYTE HERBIVORY

Freshwater macrophyte herbivory has rarely been investigated by freshwater ecologists (Lodge, 1991), despite numerous acknowledgments within the literature that macrophytes play important ecological roles in freshwater ecosystems (Cronin and Lodge, 2003). A study by Ratushnyak *et al.* (2010) investigated the role of SA in macrophyte (*Typha angustifolia* L.) adaptation to nitrate nitrogen, but did not investigate the role of SA on *T. angustifolia*-herbivore relationships.

The biological control of aquatic weeds is one field of applied research that investigates plant-herbivore interactions associated with freshwater plants. A variety of exotic aquatic plants have invaded freshwater systems worldwide by means of the unregulated aquarium and ornamental trade (Jacot Guillarmod, 1979, Sheppard *et al.*, 2006, Martin and Coetzee, 2011). Biological control, which is the use of natural arthropod enemies (particularly insect herbivores) to reduce the spread of invasive plant species, is one of the control methods employed to control these invasive weeds. *Myriophyllum aquaticum* (Vell.) Verdcourt (Haloragaceae), *Salvinia molesta* D. Mitch. (Salviniaceae), and *Azolla filiculoides* var. *rubra* (R.Br.) Strasb. (Azollaxeae) are a few of the most dominant invasive weed species in Africa (Jacot Guillarmod, 1979, Hill and Coetzee, 2008), all of which have been re-introduced to one or more of their natural enemies in their adventive range. Biological control programmes have increased the research scope for macrophyte herbivory, and provide suitable platforms

for investigating plant-insect interactions. One of the most noteworthy biological control programmes, aimed at controlling an aquatic macrophyte, is that against the notorious invasive weed, water hyacinth. Water hyacinth has been studied considerably since the 1960s, as it is a significant problem throughout the tropical and subtropical world; however, much of the water hyacinth research has focused specifically on its ecological and economic impacts and the dynamics of the relationships with its natural enemies at the macro-scale (e.g. Matthews, 1967, Center, 1981, Forno, 1981, Center and Durden, 1986, Xie *et al*., 2004, Coetzee *et al.*, 2005, Coetzee *et al.*, 2007b, Ajuonu *et al*., 2009). Despite the knowledge that insect-plant interactions at the cellular level are the crux of weed biological control programmes, little has been done to examine the insect-plant interactions at a micro-scale, namely the ability of water hyacinth to respond to the biological control agents through defence mechanisms.

1.3 WATER HYACINTH

1.3.1 Origin and spread

Originating from neotropical South and Central America (Penfound and Earle, 1984), water hyacinth is currently recognised as one of the most aggressive freshwater weeds worldwide (Center *et al.*, 2002). The first record of its deliberate transfer into areas outside its area of origin was in the early 1880s (Penfound and Earle, 1948). Since then, it has spread to various countries throughout the tropical and sub-tropical regions of the world, making its way as far as 40°C North and South of the equator (Holm *et al*., 1977, Gopal, 1987). Water hyacinth was first recorded in Africa in the 1870s along the Nile River (Williams *et al.*, 2005); however, many of the spreading infestations were only observed in the 1980s (Coetzee *et al.*, 2009). In several of the developing countries across Africa, the weed is still spreading

(Center *et al.*, 2002) and it is in such developing countries, which are resource limited, where the impacts of water hyacinth on aquatic ecosystems and the associated livelihoods are more notably severe (Holm, 1969).

1.3.2 Morphology and life history

Water hyacinth is an erect, free-floating macrophyte, with a lavender-purple flower, and aerial leaves arranged in a whorl (Center and Spencer, 1981). Each plant typically carries 6 – 10 leaves comprising a spongy, aerenchyma-filled petiole and a smooth, waxy lamina (Julien *et al.*, 2001, Coetzee *et al*., 2009). The life span of each leaf extends up to 6-8 weeks, before senescence occurs (Center *et al*., 2002). Water hyacinth exhibits considerable variation in morphology, ranging from a tall, slender form (grows up to 1m in height) to a short, bulbous form (no more than 30cm in height) (Coetzee *et al*., 2009). The morphological forms alter according to the conditions in which the plant grows. The bulbous form is characteristic of younger, colonising plants found in less dense mats in open water, or along the edges of dense infestations. The slender, elongated form is found in dense, crowded stands, where horizontal expansion is limited (Coetzee *et al*., 2009).

Water hyacinth is capable of reproducing at rates faster than it can be eliminated. Its main method of reproduction is asexual, through the production of ramets, alternately called daughter plants, allowing it to double in biomass every 11-18 days (Edwards and Musil, 1975). Water hyacinth also propagates through seed germination (Coetzee *et al*., 2009). Each flower is capable of producing numerous and long-lived seeds, which can remain dormant in bank sediments for up to 15-20 years (Matthews, 1967). Water hyacinth proliferates optimally in conditions characteristic of high light intensities, high nutrient levels, warm temperatures ranging from 28 to 30°C and a neutral pH level (Chadwick and Obeid, 1966,

Tipping *et al*., 2008). In extreme, low temperature conditions, the plant dies back, but is capable of regrowing if the submerged rhizome survives the frost or through seed germination along the river banks in the warmer months (Owens and Madsen, 1995, Tipping *et al*., 2008).

1.3.3 The biological control of water hyacinth

Various methods have been employed to control water hyacinth, but biological control is the most sustainable and effective programme (Julien, 2001). The first surveys for candidate biological control agents began in 1962 (Julien, 2001). During the 1970s, the first biological control agents were released into North America by the United States Department of Agriculture, which included the two weevils *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae), *N. bruchi* Hustache (Coleoptera: Curculionidae) and the moth *Niphograpta* (=*Sameodes*) *albigutallis* (Warren) (Lepidoptera: Pyralidae) (Cordo, 1999, Tipping *et al*., 2008). To date, a total of eight arthropod water hyacinth biological control agents have been released worldwide, with the latest introductions being *Cornops aquaticum* (Bruner) (Orthoptera: Acrididae) into South Africa in 2011, and *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) into the United States of America and South Africa in 2010 and 2013, respectively (Tipping *et al*., 2008, Coetzee *et al*., 2011, Coetzee JA, pers. comm) (Table 1.1). Fungal pathogens also have been shown to be highly effective agents, acting alone or in combination with the insect biological control agents (Charudattan, 1995). Numerous pathogens have been identified on water hyacinth around the globe, and various studies have focused on the potential development of bio-herbicides (Charudattan, 1995, Shabana *et al*., 1997, Shabana *et al*., 2001).

Table 1.1 A list of the 7 arthropod biological control agents used in South Africa to control water hyacinth, detailing the feeding modes they employ and the niches they occupy on the weed.

		Feeding mode		Niche occupied	
Biological control agent	**Year**	**Adults**	**Larvae/nymphs**	**Adults**	**Larvae/nymphs**
Neochetina eichhorniae	1974	leaf chewer[1]	petiole borers[1]	leaves[1]	petiole and crown[1]
Orthogalumna terebrantis	1989	sap sucker[4]	sap sucker[4]	leaves[4, 5]	leaves[4, 5]
Neochetina bruchi	1990	leaf chewer[1]	petiole borers[1]	leaves[1]	petiole and crown[1]
Niphograpta albiguttalis	1990	n/a[2]	petiole borers[2]	n/a[2]	petiole and crown[2]
Eccritotarsus catarinensis	1996	sap sucker[3]	sap sucker[3]	leaves[3]	leaves[3]
Cornops aquaticum	2011	leaf chewer[6]	leaf chewer[6]	leaves[7]	leaves[7]
Megamelus scutellaris	2013	sap sucker[8]	sap sucker[8]	leaves[8]	leaves[8]

[1]Deloach and Cordo, 1976, [2]Julien *et al*., 2001, [3]Hill *et al*., 1999, [4]Del Fosse *et al*., 1975, [5]Coetzee *et al*., 2009, [6]Coetzee *et al.*, 2011, [7]Silveira Guido and Perkins, 1975, [8]Tipping *et al*., 2008

The *Neochetina* spp. weevils, particularly *N. eichhorniae*, are considered the most effective agents, contributing solely to the complete control of water hyacinth in a number of regions around the world (Center *et al.*, 2002, Heard and Winterton, 2000), including Papua New Guinea (Julien and Orapa, 2001) and Lake Victoria and Kyroga River Basin, Uganda (Albright *et al.*, 2004). However, in some regions the successful control of water hyacinth has been variable, despite the use of a suite of biological control agents. In South Africa, a total of seven biological control agent species have been released, more than anywhere else in the world (Coetzee *et al*., 2011). Biological control agents have suppressed the spread of the weed in some regions in South Africa, for example New Years Dam (Eastern Cape Province) (Hill and Cilliers, 1999) and Clairewood quarry (Kwa-Zulu Natal) (Hill, 2003), but also have been unsuccessful in other regions, such as the Highveld, along the Vaal River (Hill, 2003, Coetzee *et al.*, 2007a). To increase the herbivore pressure on water hyacinth,

additional candidate species are being investigated for release into South Africa (Coetzee *et al*., 2011).

1.3.4 Water hyacinth insect-plant associations

1.3.4.1 Biological control agent host utilisation and damage

Although all the water hyacinth biological control agents, during some part of their lifecycle, exhibit considerable niche overlap either on the lamina, petiole or both, their feeding modes do differ. *Neochetina eichhorniae* and *N. bruchi* share the same feeding mechanism and inflict damage upon water hyacinth in a similar manner (Julien, 2001); however, because *N. bruchi* has faster development rates than *N. eichhorniae*, water hyacinth mortality is faster when *N. bruchi* feeds on the plant (Deloach and Cordo, 1976). The adult weevils are leaf chewers, removing the upper epidermal layer of the leaf, forming distinctive feeding scars (Deloach and Cordo, 1976). The exposure of the internal leaf tissue results in an increase in water loss and susceptibility to pathogen infection (Julien, 2001, Moran, 2005). A recent study showed that the water hyacinth weevils are vectors for fungi and bacteria and that these microbes greatly influence the impact of the weevils on water hyacinth photosynthesis (Venter *et al*., 2013). *Neochetina* spp. weevil larvae mine the petioles and the crown of the plant, causing internal tissue to rot, a reduction in growth and the prevention of flowering (Deloach and Cordo, 1976, Julien, 2001).

Niphograpta albiguttalis larvae are the only life stage of the moths which feed on water hyacinth; adult moths only use the plant for oviposition. Like the larvae of *Neochetina* spp., *N. albiguttalis* larvae tunnel and feed within the petiole (Julien *et al*., 2001). Damage inflicted results in waterlogging and tissue rot (Julien, 2001, Julien *et al*., 2001). In contrast,

Eccritotarsus catarinensis (Carvalho) (Hemiptera: Miridae) is a sap-sucking mirid. The adults and nymphs feed gregariously on the under surface of the lamina, drawing sap from the palisade parenchyma within the leaf (Hill *et al*., 1999). Herbivory by the mirid results in the removal of chlorophyll from the leaf, which in due course causes leaf chlorosis and possible leaf death (Hill *et al*., 1999b). Water hyacinth growth rate and biomass production is reduced under severe mirid herbivory, due to the decline in the plant's photosynthetic capabilities (Coetzee *et al*., 2007b). The weevils, mirid and moth only are discussed in this chapter as they have been investigated extensively in the literature and they have largely contributed to the control of water hyacinth in South Africa. The remaining biological control agents have contributed to negligible control of water hyacinth or have yet to make an impact because of their recent release (post release evaluations have yet to be conducted (e.g. *M. scutellaris*)).

1.3.4.2 Water hyacinth insect-induced responses

Biological control practitioners have been releasing arthropod biological control agents onto water hyacinth for 40 years and throughout this time, very little research has focused on water hyacinth's responses to the agents, despite knowing that the water hyacinth plant-insect interactions are the crux of the weed biological control programme. A few studies have paved the way to identifying various water hyacinth chemical and physical responses to insect herbivory.

Field explorations of water hyacinth herbivory would suggest that the weed is unattractive or unpalatable, as very few generalist herbivores, throughout the global distribution of water hyacinth, feed on the weed (Center and Wright, 1991). Center and Wright (1991) investigated the phytochemical composition of leaves after feeding by *N. eichhorniae* and the generalist herbivore, *Spilosoma virginica* (F.) (Lepidoptera: Arctiidae). Weevil feeding

was predominant on young leaves, and *S. virginica* feeding was limited to older, mature leaves. Center and Wright (1991) attributed these differences to possible defence mechanisms within the younger leaves, where younger leaf tissue was higher in nitrogen compounds; in effect, the generalist herbivore was incapable of overcoming the possible nitrogenous defences employed by water hyacinth in the younger leaves, while the specialist herbivore, *N. eichhorniae*, was most likely adapted to overcome the barriers. Furthermore, Center and Wright (1991) ruled out the possibility of phenolic compounds (carbon-based) playing a role in water hyacinth responses to herbivory in the younger leaves, but suggest the possibility of terpenes playing a role in defences. Investigations in the role of terpenes in water hyacinth have not been conducted to date. The presence and function of a kairomone produced by water hyacinth was investigated by Del Fosse and Perkins (1977). While the allelochemical is a phagostimulant and oviposition stimulant for the *Neochetina* spp., it may be a feeding deterrent (an allomone) for generalist species, or it may act as an attractant for parasitoids. The functions of allelochemicals are not mutually exclusive (Del Fosse and Perkins, 1977), and water hyacinth may release the chemical studied by Del Fosse and Perkins (1977) as an allomone in response to herbivory, possibly to attract natural enemies of the *Neochetina* spp. Further investigations into the role of this kairomone in water hyacinth indirect defences have not been conducted. Phytochemical analyses of three aquatic angiosperms were conducted by Vasu *et al.* (2009), one of which was water hyacinth. Although phytochemical compounds, such as alkaloids, phenols, tannins, saponins and steroids were present in water hyacinth, the study did not investigate the dynamic roles of these phyotbhemical compounds in response to herbivory. The presence of these phytochmeicals is likely to have roles in water hyacinth insect-induced chemical and physical responses.

An induced physical response to herbivory was observed in water hyacinth after feeding by *N. eichhorniae* in a study by Wright *et al.* (1989). Leaf hardness, a physical barrier to herbivory, significantly increased in the youngest leaf after weevil feeding. In addition, moisture content, a component of plant quality contributing to digestibility-reducing defences, was found to have significantly reduced after weevil feeding (Wright *et al.*, 1989). Similarly, a study by Wright and Bourne (1986) found that water hyacinth petiole hardness significantly influenced the entry of *N. albiguttalis* larvae, and concluded that petiole hardness may be a reflection of leaf age or induced plant defences, such as cuticle thickness and accumulation of phenolics. Furthermore, Wright and Bourne (1986) made mention of investigations into altering the epidermal hardness of water hyacinth leaves by the application of plant growth regulators to favour attack by *N. albiguttalis* as a concluding remark. Research into the use of plant hormone activators on water hyacinth plants has not been attempted and therefore substantiates the purpose of this thesis.

1.4 THESIS AIMS AND OBJECTIVES

Biological control practitioners have been releasing arthropod biological control agents onto water hyacinth for 40 years. Ultimately, the premise of the water hyacinth biological control programme relies on the re-establishment of the interactions between the biological control agents and the weed. Therefore, it is crucial to understand the dynamics of these relationships, particularly water hyacinth's ability to defend itself against the biological control agents. Ecological and physiological parameters, such as resource availability, habitat type and plant growth rates, are valuable predictors of the level of defences employed by plants (Rhoades and Cates, 1976, Coley *et al.*, 1985, Bazzaz *et al.*, 1987). In addition, the Resource Availability Hypothesis provides an applicable framework to investigate insect-

induced responses (Endara and Coley, 2011). Plants with rapid growth rates and colonizing traits, inhabiting resource-rich environments are typically associated with low levels of constitutive defensive compounds. Essentially, resource allocations to plant growth and/or inducible defences are favoured by colonising plants. As discussed in section *1.3.2 Morphology and life history*, water hyacinth fits this profile. While a plant's responses to a myriad of natural enemies are highly complex, it is hypothesised that water hyacinth may employ induced defences in response to some, but not all, biological control agents. With the availability of a suite of water hyacinth biological control agents, varying in feeding mode, and the availability of chemical elicitors to induce defence pathways, further investigations into insect-induced water hyacinth defences are plausible.

The present study aimed to elucidate the possible insect-induced defences employed by water hyacinth in response to herbivory, by inducing SA up-regulation through the use of the chemical plant elicitor, BION®. By inducing SA-mediated defence pathways in water hyacinth, JA-mediated defence pathways were invariably inhibited (Walling, 2000, Smith *et al*., 2009).

Before initiating experimental trials, the appropriate plant measures and insect densities to be used throughout the present study were investigated, taking into consideration the possible impact of water nutrient availability and exogenous applications of BION® (Chapter 2). The following objectives were the scope of this thesis:

Objective 1: Determine the effect of salicylic acid on water hyacinth growth, physiological and biochemical parameters

Herbivore-induced plant defences are costly and defence theory posits that investment of limited resources into one component of fitness precludes the allocation of resources to other

components of fitness (Rhoades and Cates, 1976). In this study, water hyacinth was manipulated to induce the SA-mediated defence pathway, via the exogenous application of BION®, presumably precluding resource allocation to plant growth and/or flower and/or ramet production. Therefore, the effects of SA induction in water hyacinth on plant growth (e.g. plant biomass, leaf production, and ramet production) and physiological and biochemical parameters (e.g. chlorophyll content, carbon and nitrogen content, and moisture content) were investigated in Chapter 3 of this thesis.

Objective 2: Evaluate the effect of salicylic acid on herbivory by insects with different feeding modes and the resultant impact on water hyacinth growth, physiological and biochemical parameters

Insect-induced plant responses are species- and feeding mode-specific (Gaffney *et al*., 1993, Moran and Thompson, 2001, Traw *et al*., 2003, Farouk *et al*., 2008, Smith *et al*., 2009). Therefore Chapter 3 also investigated the effect of SA-induced water hyacinth plants on herbivory by two biological control agents with varying feeding modes; the highly damaging leaf chewer and petiole borer, *N. bruchi,* and the sap sucker, *E. catarinensis*. The impact of these herbivores on SA-induced water hyacinth growth, physiological and biochemical parameters was also investigated in Chapter 3.

Objective 3: Assess the effect of salicylic acid on water hyacinth leaf quality traits

Plant defence pathways (SA- or JA-mediated pathways) regulate the expression of insect-induced plant defences, which can be expressed as chemical and/or physical plant traits. The effect of SA induction on water hyacinth leaf quality traits was investigated in Chapters 3 and 4. Chapter 3 dealt with the changes in carbon content, nitrogen content and C:N ratios as possible expressions of chemical components of leaf quality (i.e. leaf nutrition and

moisture), however, these biochemicals may contribute to structural components of leaf quality (i.e. physical defences). Therefore, Chapter 4 investigated the possible expression of leaf toughness, leaf thickness and epidermal cell layer thickness in SA-induced water hyacinth leaves.

Investigations into the expression of water hyacinth insect-induced defences in response to herbivory by the biological control agents are limited. Identifying how water hyacinth responds to herbivory using the application of BION® will contribute to the general field of insect-plant interactions in aquatic plants, in addition to encompassing applied implications for the biological control of water hyacinth in South Africa, and potentially other biological control programmes around the world.

CHAPTER 2

THE EFFECT OF HERBIVORY BY DIFFERENT POPULATION DENSITIES OF THE BIOLOGICAL CONTROL AGENTS ON WATER HYACINTH GROWTH

2.1 INTRODUCTION

The study of ecology fundamentally aims to understand the abundance, distribution, and the associated interactions of organisms in varying ecosystems. Subsequently, the knowledge gained from such ecological studies facilitates the management of artificial and natural ecosystems (Gutierrez *et al*., 1994). In most naturally occurring ecosystems, conditions promoting optimal growth are infrequent; conditions are generally limiting. Biological interactions, such as plant-insect relationships, are simultaneously regulated by such limiting factors, namely top-down and bottom-up control processes (Hairston *et al*., 1960, Polis and Strong, 1996). In a typical plant-insect relationship, the plant population is limited by abiotic factors such as light, nutrients, and water availability (bottom-up control) (Coley *et al*., 1985). The plant population is also constrained by biotic factors such as competition (intraspecific and/or interspecific) (Ungar, 1992, Bank, 2010) and by the population density and impact of insect herbivores (top-down control) (Louda, 1982, Hoffmann, 1990, Hoffmann and Moran, 1998, Carson and Root, 1999). In turn, the insect population is regulated by competition (intraspecific and/or interspecific), predation, and parasitism (top-down control) (Denno and Roderick, 1992, Kursar *et al*., 2006), and limited by host plant quality, quantity, and defence mechanisms (bottom-up control) (Center and Durden, 1986,

Bach, 1988, Crawley, 1989, Denno *et al.*, 2002, Rodriguez-Saona and Thaler, 2005, Stiling and Moon, 2005).

Numerous studies have attempted to investigate the role of insect herbivory as a top-down regulatory process on plant growth and population dynamics (Cantlon, 1969, Wilbur *et al.*, 2013). A study by Wilbur *et al*. (2013) investigated the effect of insect herbivory on the growth and fitness of common mullein, *Verbascum thapsus* L. (Scrophulariaceae), and found that reductions in insect herbivory increased *V. thapsus* survival, height and seed set. Seed set increased by 50% (~48, 000 to ~98, 000 seeds) under reduced herbivore pressure, notably increasing plant fitness. This experiment demonstrated the effective role of herbivores as a top-down control on a plant population outside the plant's area of origin. Moreover, classical weed biological control has represented many successful ecological studies on plant-insect interactions, particularly the impact of insect herbivory (top-down) on plant population growth (Crawley, 1989, Keane and Crawley, 2002). When exotic plants enter new environments, they are exposed to altered top-down and bottom-up controls and, in many cases, the plants are released from their natural enemies. Subsequently, the plants proliferate and become invasive due to the reduction in herbivore pressure. This process whereby the plant escapes its natural enemies in new environments is termed the enemy release hypothesis (ERH) (Keane and Crawley, 2002). Classical weed biological control programmes essentially aim to restore the top-down control of an invasive host plant by re-introducing the natural enemies responsible for the regulation of the plant species in its area of origin (Pearson and Callaway, 2005, Pratt *et al.*, 2005).

The impacts of insects at varying densities on their host plants have been frequently reported in the scientific literature, and it is generally concluded that higher insect densities result in greater reductions in plant growth and reproductive parameters and considerable increases in

injury and, in some cases, plant death (Ladd and Buriff, 1979, Center and Van, 1989, Briese, 2000, Briese *et al*., 2002, Rhainds and English-Loeb, 2003, Briese *et al*., 2004, Ding *et al*., 2006, Schooler and McEvoy, 2006). Briese *et al.* (2002) investigated the impact of the weevil *Trichosirocalus briesei* Alonso-Zarazaga and Sanchez-Ruiz (Coleoptera: Curculionidae) on *Onopordum* spp. thistles (Asteraceae: Cardueae) and found that high densities of the weevil increased plant mortality to 40%, compared to 10% mortality in the control treatment. Additionally, high densities of the root-feeding beetle larvae, *Popillia japonica* Newman (Coleoptera: Scarabaeidae) resulted in a reduction in the yield of Kentucky bluegrass, *Poa pratensis* L. (Poaceae), and eventual death (Ladd and Buriff, 1979).

The impact of different densities of water hyacinth biological control agents on water hyacinth have been investigated, namely for the mite, *Orthogalumna terebrantis* Wallwork (Acari: Galumnidae) (Marlin *et al.*, 2013) and the weevil, *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) (Center and Van, 1989). Center and Van (1989) investigated the impact of plant density and varying densities of the weevil *N. eichhorniae* on water hyacinth leaf dynamics. This study examined high and low water hyacinth densities exposed to three *N. eichhorniae* density treatments; zero weevils, 25 weevils and 250 weevils. Center and Van (1989) did not provide the insect:plant ratio used within the experiment, but it is concluded from their results that crowded water hyacinth plants exposed to high densities of *N. eichhorniae* resulted in faster water hyacinth mortality rates than the lower density treatment, as reductions in plant growth parameters exceeded leaf production. Therefore, lower densities of *N. eichhorniae* and less crowded experimental plants provide measurable, but non-lethal effects on water hyacinth growth. Studies such as these are lacking for the weevil *N. bruchi* Hustache and *Eccritotarsus catarinensis* (Carvalho) (Heteroptera: Miridae), the most effective water hyacinth biological control agents in South Africa (Hill pers. com.).

A study by Moran (2005) attempted to correlate adult *Neochetina* spp. leaf scarring with insect densities and dead plant biomass. While increased leaf scarring was correlated with high dead plant biomass, scarring was not associated with weevil densities. This was largely due to low adult weevil densities (0-0.2 adults per leaf). Although this study marks an attempt at determining the impact of weevil densities on water hyacinth plant biomass, the study did not examine species specific density impacts on water hyacinth, particularly by *N. bruchi*. A study by Heard and Winterton (2000), investigating the interaction between nutrient status and weevil herbivory in the biological control of water hyacinth, did not investigate the effects of varying densities of *N. eichhorniae* and *N. bruchi* on water hyacinth, but used a total of 10 *Neochetina* spp. per batch of 20 water hyacinth plants (0.5 insects per plant) for experimentation. Heard and Winterton (2000) used 0.5 weevils per plant, because during preliminary trials, stands of water hyacinth inoculated with one adult weevil per plant, *N. eichhorniae* or *N. bruchi*, resulted in near destruction of test plants. However, statistical results for the preliminary trials were not provided and experiments were not conducted on individual water hyacinth plants. Studies investigating the impact of the mirid, *E. catarinensis*, on water hyacinth commonly use 15 individuals per water hyacinth plant to provide significant herbivore pressure without causing premature plant death (Coetzee *et al*., 2005, Coetzee *et al*., 2007b, Ajuono *et al.*, 2009, Weyl and Hill, 2012).

2.1.1 Experimental aims and objectives

The purpose of this chapter was to formulate the methodology for the forthcoming experimental chapters (Chapters 3 and 4), by determining the damaging density of *N. bruchi* on water hyacinth. A damaging density represents a density of insects that will have a measurable impact on water hyacinth growth parameters, but will not result in premature

plant mortality. Additionally, because the application of BION® should ideally result in increased susceptibility of water hyacinth to injury by herbivores, a minimum number of *N. bruchi* must be used. The minimum density of *N. bruchi* weevils has yet to be statistically proven. Subsequently, this chapter investigated the impact of three different densities of *N. bruchi* per water hyacinth plant to identify the minimum number of weevils, to determine what density of weevils should be used to observe non-lethal changes in water hyacinth growth parameters. Ultimately, this should ensure that time and insect and plant resources are not wasted due to premature plant death and insufficient sampling time.

It was hypothesised that at higher *N. bruchi* densities, weevil feeding/damage would increase and plant growth should be increasingly adversely impacted. It was predicted that a low density of *N. bruchi* would be required to cause measurable damage to water hyacinth without resulting in premature death. The plant hormone activator, BION® (Syngenta) (see Chapter 1, *1.1.3 Induced plant defence pathways*) was used in subsequent trials to investigate the role of JA and SA in water hyacinth insect-induced defences against mirid and weevil herbivory. Therefore, possible increases in rates of herbivory and insect performance were both expected, and taken into account when selecting the appropriate *N. bruchi* density. Ultimately, the aim of this study was to determine the minimum number of pairs of *N. bruchi* required to observe non-lethal changes in water hyacinth growth and physiological parameters, grown in water nutrient conditions indicative of South African field conditions.

2.2 METHODS AND MATERIALS

2.2.1 Experimental insect and plant cultures

Water hyacinth plants used for this trial were grown in a plastic lined pool at the Department of Zoology and Entomology, Rhodes University, Grahamstown, South Africa (33°18'49"S 26°31'11"E). Plants were maintained under insect free conditions using insecticide (Mercaptothion 500 EC, Dow AgroSciences, used according to manufacturer's guidelines) and isolated in a polyethylene covered greenhouse. The plastic covering of the greenhouse allowed for ample light penetration and zero insect contamination. Experimental plants were used two weeks after an insecticidal application and after being thoroughly rinsed with water. Insecticide application had no adverse impacts on water hyacinth plants.

In total, 120 *N. bruchi* were collected from cultures at the mass rearing facility at the South African Sugar Cane Research Institute (SASRI), Durban, South Africa (29°42'28"S 31°02'52"E) and couriered to Rhodes University. The 120 *N. bruchi* were divided into three density treatments with a 1:1 sex ratio; High density (12 individuals per plant, ergo 6 mating pairs), Medium density (6 individuals per plant, ergo 3 mating pairs) and Low density (2 individuals per plant, ergo 1 mating pair). Mating weevil pairs were used in order to mimic the full spectrum of weevil damage (adult and larval damage).

The density trial took place in the Working for Water Biological Control polyethylene covered greenhouse at Rhodes University. In total, 24 healthy water hyacinth plants of a similar size with five to six healthy leaves were collected from the plant cultures and placed individually in 6*l* plastic square tubs filled with 5*l* of water. The tubs were covered and secured with a fine mesh sleeve to prevent herbivore contamination of control plants and the escape of experimental insects from the treated plants. Plants were left to acclimate for a

week before the initiation of the trial. The water in the tubs was changed every 2 weeks and maintained at a medium nutrient level throughout the trial, by adding 0.00252g of potassium dihydrogen orthophosphate (KH_2PO_4) and 0.416g of potassium nitrate (KNO_3) to obtain total phosphorous and nitrogen concentrations of 0.025mgP.l^{-1} and 2.5mgN.l^{-1}. The required nutrients were weighed out per plant using an Ohaus® Adventurer™ analytical balance. These nutrient levels simulate mesotrophic conditions in South African water bodies (Byrne *et al*., 2010). Each plant was tagged for identification and assigned to one of four treatments and the appropriate insect numbers were released (Table 2.1). Each treatment was replicated six times.

Table 2.1 The 7 experimental treatments formulated for the density trial and the corresponding number of individual insects inoculated in each treatment (LD = Low density, MD = Medium density, HD = High density).

Treatment		Denotation	Number of Individuals per plant
Control	-	Cont.	0
N. bruchi	**Low Density**	LD	2
	Medium Density	MD	6
	High Density	HD	12

2.2.2 Plant and insect parameters

After the week long acclimation period, plant parameters were measured prior to the release of the insects, and were denoted as data for Week 0. Thereafter, the relevant numbers of insects were released onto their respective treatment plant, and plant and insect parameters were measured every 7 days, for a total period of 6 weeks.

Plant parameters

The following plant parameters were measured and collated; total dry plant biomass (including the ramets (daughter plants)), number of leaves produced, leaf turnover, length of the second petiole, and number of new ramets produced.

Total dry plant biomass was measured at the end of the trial. Each plant was cut up and plant parts separated (roots, petioles, leaves and ramets), and dried in an oven at 60°C. All dried plant parts per plant were weighed using an Ohaus® Adventurer™ analytical balance and weights were added together to obtain a total dry weight per plant. Dry plant biomass was chosen as opposed to wet plant biomass, as it provides a more accurate estimate of the total reduction or increase in plant biomass in response to herbivory (Vandermeer and Rice, 1980). Furthermore, herbivory by the weevils causes water logging of the petioles (Julien, 2001), thus influencing the weight of the plant and creating a misleading result.

The number of leaves produced per plant was simply measured as the total number of leaves present on each plant, and the leaf turnover is the number of new leaves produced per week per water hyacinth plant. The leaf turnover was measured by labelling leaf 1 at Week 0 and noting its shift in position each week as the plant aged. Leaf 1 is the newly unfurled leaf in the centre of the plant, closest to the crown (Center, 1981). As the plant grows, new leaves are produced and subsequently take the place of the now second oldest leaf, moving down the crown of the water hyacinth plant. The quantification of leaf turnover and leaf production rates are regarded as the most informative and straightforward measure for the study of water hyacinth population biomass (Center, 1981, Center *et al.*, 1999).

Ramet production also is an indicator of plant growth and proliferation. The second petiole is young and still developing (Center, 1981) and are indications of enhanced water hyacinth

growth rates. These plant parameters were chosen as they have been used throughout water hyacinth studies (Coetzee *et al.*, 2007b, Bownes *et al.*, 2010, Marlin *et al.*, 2013) and reflect changes in water hyacinth growth and functioning.

Insect parameters

Adult weevil feeding was recorded by estimating the percentage surface area damaged on leaves 1, 2, 3 and 4. Leaves 1 to 4 on a typical water hyacinth plant are the first four leaves in the whorl. Leaf 2 is the second youngest leaf and is typically fed upon by the weevils; the *Neochetina* spp. weevils have a recorded preference for leaf 2 (Center and Wright, 1991). Leaf 3 is simply the third youngest leaf and leaf 4 is generally fed upon by the mirids (Hill *et al.*, 1999a). To minimise sampling and estimation differences caused by observer bias, the same observer conducted measurements at each sampling week. The numbers of adult weevil feeding scars were also counted on leaves 1 to 4.

2.2.3 Statistical analyses

Generalised linear/non-linear models (GLZ) in STATISTICA 10.0 (© StatSoft, Inc. 2011) analysed differences in the plant and insect parameter data. On examination of the residuals, all plant and insect parameter data exhibited a normal and homoscedastic distribution around the regression line.

2.3 RESULTS

2.3.1 Plant parameters

2.3.1.1 Total dry plant biomass

The low weevil density treatment exhibited greater total dry plant biomass, however no differences in dry plant biomass were observed between the four weevil density treatments at the end of the trial period (Wald's $X^2_{(3)} = 4.12$, $P = 0.248832$) (Figure 2.1).

2.3.1.2 Number of functional leaves and leaf turnover

At the beginning of the experiment, each plant had a mean number of six leaves. As the trial progressed, the number of functional leaves did not differ over time (Wald's $X^2_{(1)} = 2.43$, $P = 0.12$) (Figure 2.2). Additionally, the leaf turnover was not different between the four density treatments (Wald's $X^2_{(3)} = 4.07$, $P = 0.25$). Subsequently, at the end of the trial period (Week 6), no differences were observed in the number of functional leaves among the density treatments (Wald's $X^2_{(3)} = 4.45$, $P = 0.22$). Both the leaf turnover (Wald's $X^2_{(1)}=3.41$, $P = 0.06$) (Figure 2.3) and the rate of change (Wald's $X^2_{(3)} = 0.36$, $P = 0.95$) did not differ among the density treatments.

2.3.1.3 Length of petiole 2

The initial mean (+SE) length of the second petiole for all plants in all treatments WAS 7.65±0.29cm. As the trial progressed, the length of the second petiole decreased significantly between weevil densities over time (Wald's $X^2_{(1)}=133.46$, $P<0.05$), but no differences were observed among the treatments (Wald's $X^2_{(3)}=3.654$, $P = 0.30$) (Figure 2.4). However,

significant differences in the length of the second petiole existed between weevil densities at the end of the trial (Week 6) (Wald's $X^2_{(3)}$=9.57, P<0.05). Second petioles were significantly longer in the control treatment compared to the medium and high density treatments. The low density treatment was not significantly shorter than the control.

2.3.1.4 Number of ramets

The number of ramets per plant changed significantly over the sampling period (Wald's $X^2_{(1)}$=12.95, P<0.05) and differences were observed among the four density treatments at each sampling event (Wald's $X^2_{(3)}$=31.17, P<0.05) (Figure 2.5). The low weevil density and control treatments produced higher numbers of ramets per plant than the high and medium weevil density treatments. However, there were no differences in the number of ramets produced per plant at the end of the trial (Week 6) (Wald's $X^2_{(3)}$=4.30, P = 0.23).

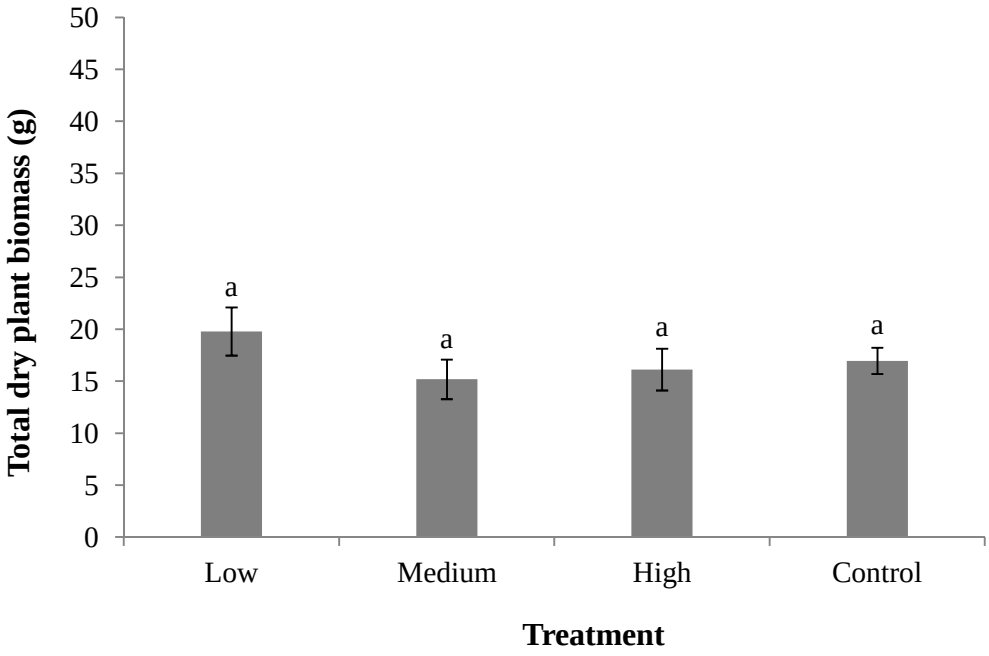

Figure 2.1 Mean dry plant biomass for plants in all four weevil density treatments (Treatments: Control = 0 *N. bruchi*, Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*), at the end of the sampling period. Error bars represent the standard error of the mean. Means followed by the same letter are not statistically different, $P > 0.05$ (n=6 plants/treatment).

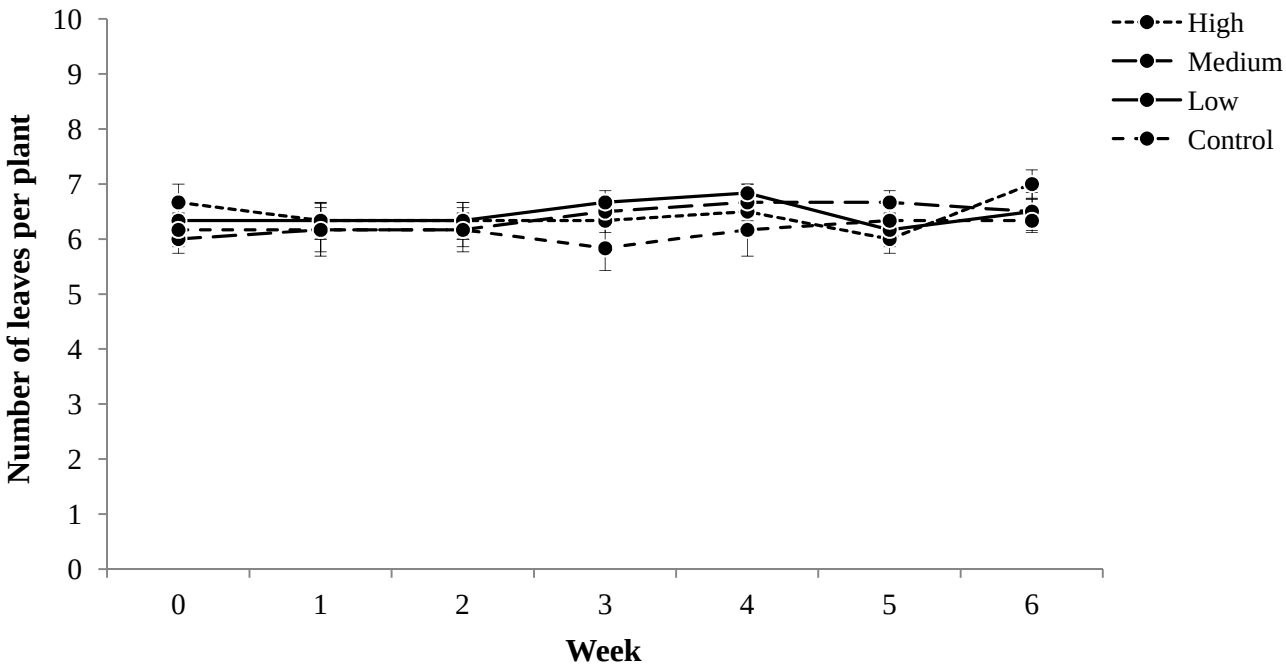

Figure 2.2 The effect of weevil density on the number of functional leaves per plant throughout the sampling period (Treatments: Control = 0 *N. bruchi*, Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*). Error bars represent the standard error of the mean (n=6 plants/treatment).

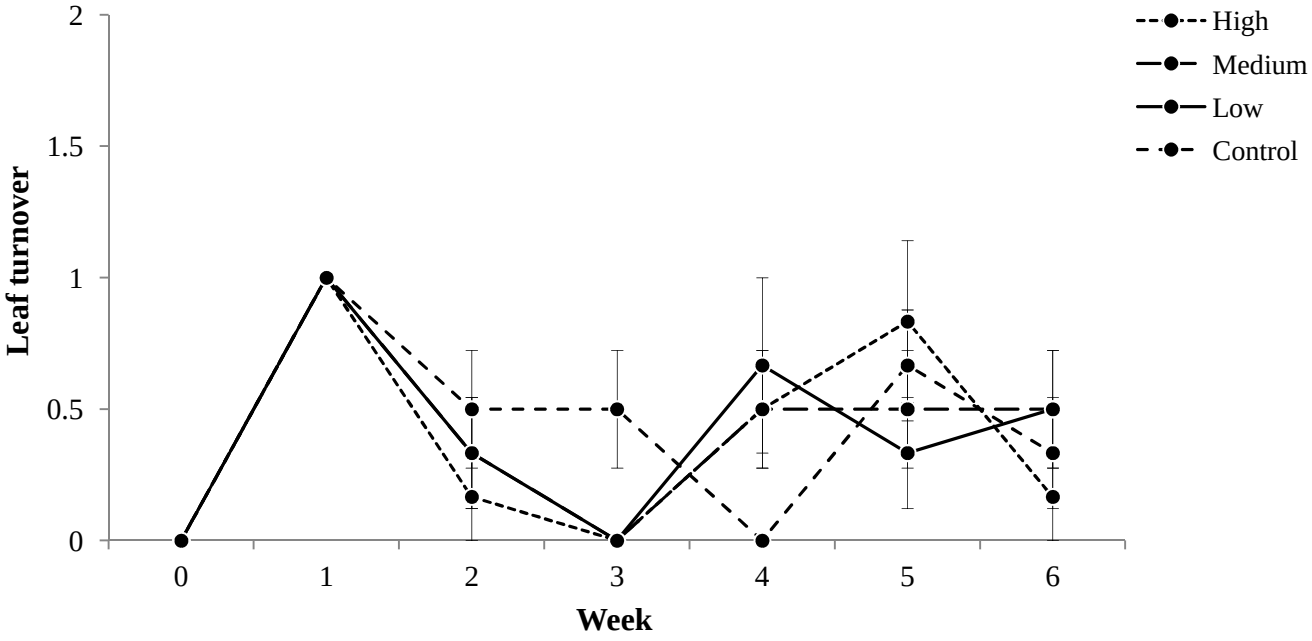

Figure 2.3 Number of water hyacinth leaves that turned over at each week, over the six week sampling period, for plants in all four density treatments. Error bars represent the standard error of the mean (n=6 plants/treatment).

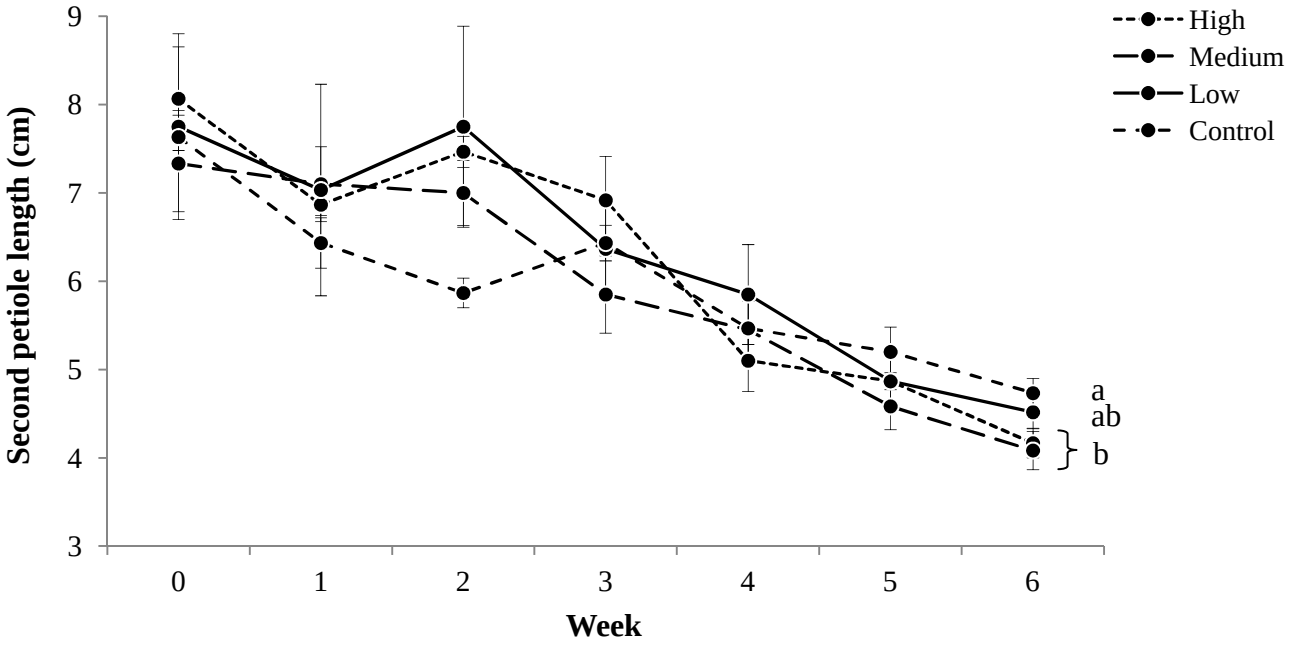

Figure 2.4 The effect of weevil density on the length of the leaf 2 petiole over the 6 week trial (Treatments: Control = 0 *N. bruchi*, Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*). Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

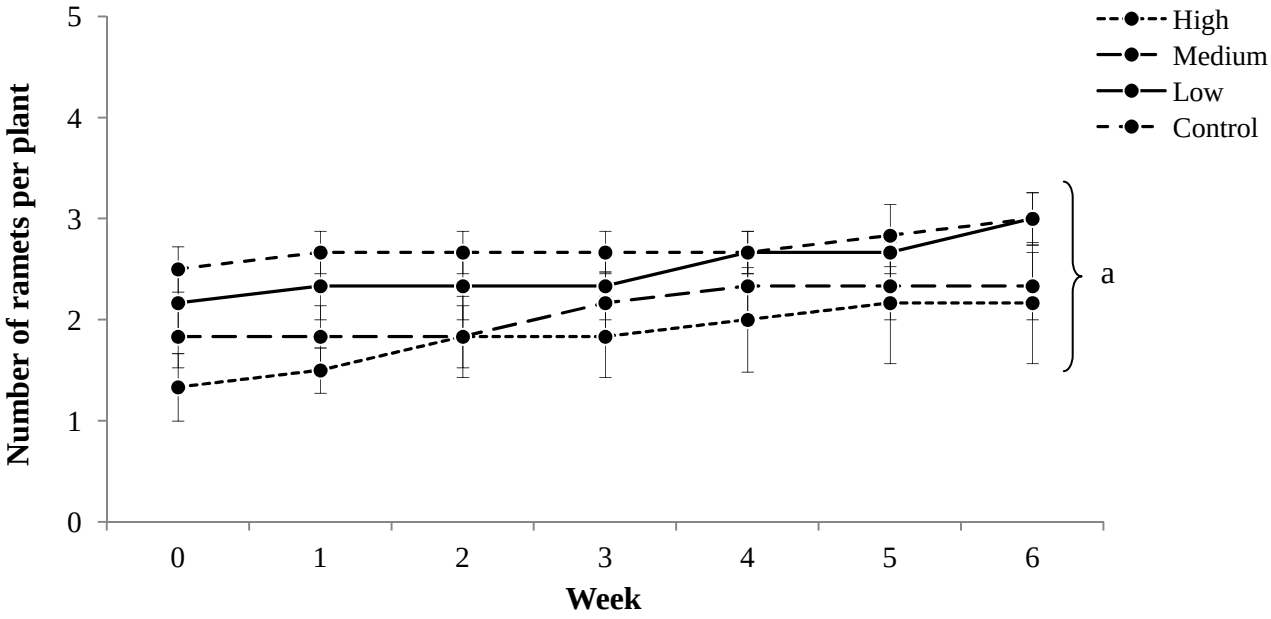

Figure 2.5 The effect of weevil density on ramet production throughout the sampling period (Treatments: Control = 0 *N. bruchi*, Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*). Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

2.3.2 Insect parameters

2.3.2.1 Percentage feeding area

Measurable differences in the percent area damaged by *N. bruchi* were observed between sampling weeks, treatments, and leaf position (Table 2.2). However, the relationship between leaf position and treatment was not significant.

There were differences in the percentage area damaged by *N. bruchi* weevils for each leaf position over the trial period (Figure 2.6 – 2.9) between sampling weeks and between the four density treatments (Table 2.3). A general increase in the percentage leaf area damaged by *N. bruchi* was observed for all leaf positions in all density treatments. As expected, the high density treatment presented leaves with significantly greater percentage leaf area

damage than the medium and low density treatments for all leaf positions. Clearly, a greater density of insects result in greater damage inflicted upon a host plant.

At the end of the trial (Week 6), differences were observed in percentage feeding area between the three density treatments for all leaf positions (Figure 2.10) (Table 2.4). Each leaf position exhibited significant increases in percentage feeding area as insect density increased, where the high density treatment typically resulted in more than double the amount of feeding than the low density treatment. Additionally, the younger leaves (Leaf 1 and 2) were the most damaged for the high and medium density treatments. Differences in the percentage feeding area were not significant between leaf positions for each of the density treatments at the end of the trial (Figure 2.10) (Table 2.5).

2.3.2.2 Number of adult weevil feeding scars

Significant changes in the number of adult weevil feeding scars were observed over the sampling period, and the rate of change was different between the four density treatments, but not between the four leaf positions (Table 2.2). Furthermore, the relationship between leaf position and treatment was not significant.

Visible differences in the number of adult weevil feeding scars for leaves 1 to 4 were found between the sampling weeks and between the four density treatments and leaf positions (Table 2.3). As with the percentage area damage, a general increase in the number of feeding scars was observed in all leaf positions for all density treatments (Figure 2.11 – 2.14), however, the differences observed between the treatments were more noticeable in the older leaves (Leaf 3 and 4). Additionally, the high density treatment produced a significantly greater number of feeding scars than the medium and low density treatments for all leaf

positions (Figure 2.15) (Table 2.4). The number of feeding scars did not differ between leaf positions for each of the density treatments at the end of the trial (Figure 2.15) (Table 2.5).

Table 2.2 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the number of *Neochetina bruchi* feeding scars and the percentage feeding area for all leaf positions and all three density treatments over the sampling period. *P*-values in bold indicate significant differences, $P < 0.05$ (n=6 plants/treatment).

	Sampling weeks		**Density Treatment**		**Leaf**		**Leaf*Treatment**	
	$\mathbf{X^2_{(1)}}$	***P-value***	$\mathbf{X^2_{(1)}}$	***P-value***	$\mathbf{X^2_{(3)}}$	***P-value***	$\mathbf{X^2_{(3)}}$	***P-value***
Nb % feeding area	114.66	**<0.001**	99.58	**<0.001**	54.27	**<0.001**	2.30	0.89
Nb feeding scars	271.70	**<0.001**	190.45	**<0.001**	4.09	0.25	10.66	0.10

Table 2.3 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage area damaged and number of adult weevil feeding scars, exhibiting significant differences between sampling weeks and treatments for leaves 1 to 4. *P*-values in bold indicate significant differences between means, $P < 0.05$ (n=6 plants/treatment).

		Sampling weeks		**Treatments**	
	Leaf	$\mathbf{X^2_{(1)}}$	***P*-values**	$\mathbf{X^2_{(2)}}$	***P*-values**
% Feeding area	**1**	21.56	**<0.001**	49.29	**<0.001**
	2	29.51	**<0.001**	51.38	**<0.001**
	3	39.11	**<0.001**	43.38	**<0.001**
	4	67.63	**<0.001**	42.85	**<0.001**
# Feeding scars	**1**	36.15	**<0.001**	53.20	**<0.001**
	2	77.42	**<0.001**	91.51	**<0.001**
	3	68.55	**<0.001**	63.22	**<0.001**
	4	123.93	**<0.001**	72.90	**<0.001**

Table 2.4 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage area damaged and number of adult weevil feeding scars, exhibiting significant differences between treatments for each leaf position. *P*-values in bold indicate significant differences, $P < 0.05$ (n=6 plants/treatment).

Parameter		Leaf 1	Leaf 2	Leaf 3	Leaf 4
% Feeding area	$\mathbf{X^2_{(2)}}$	8.63	12.32	17.26	7.66
	***P*-value**	**<0.001**	**<0.001**	**<0.001**	**0.02**
# Feeding scars	$\mathbf{X^2_{(2)}}$	4.99	22.29	17.93	9.74
	***P*-value**	0.08	**<0.001**	**<0.001**	**0.01**

Table 2.5 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage area damaged and number of weevil feeding scars, exhibiting significant differences between leaf position for each of the density treatments at the end of the trial. *P*-values in bold indicate significant differences between means, $P < 0.05$ (n=6 plants/treatment).

		Source of variation	
Parameter	**Density**	$\mathbf{X^2_{(3)}}$	***P*-values**
% Feeding area	**High**	1.22	0.77
	Medium	5.29	0.15
	Low	5.56	0.13
# Feeding scars	**High**	5.56	0.14
	Medium	1.44	0.70
	Low	4.47	0.21

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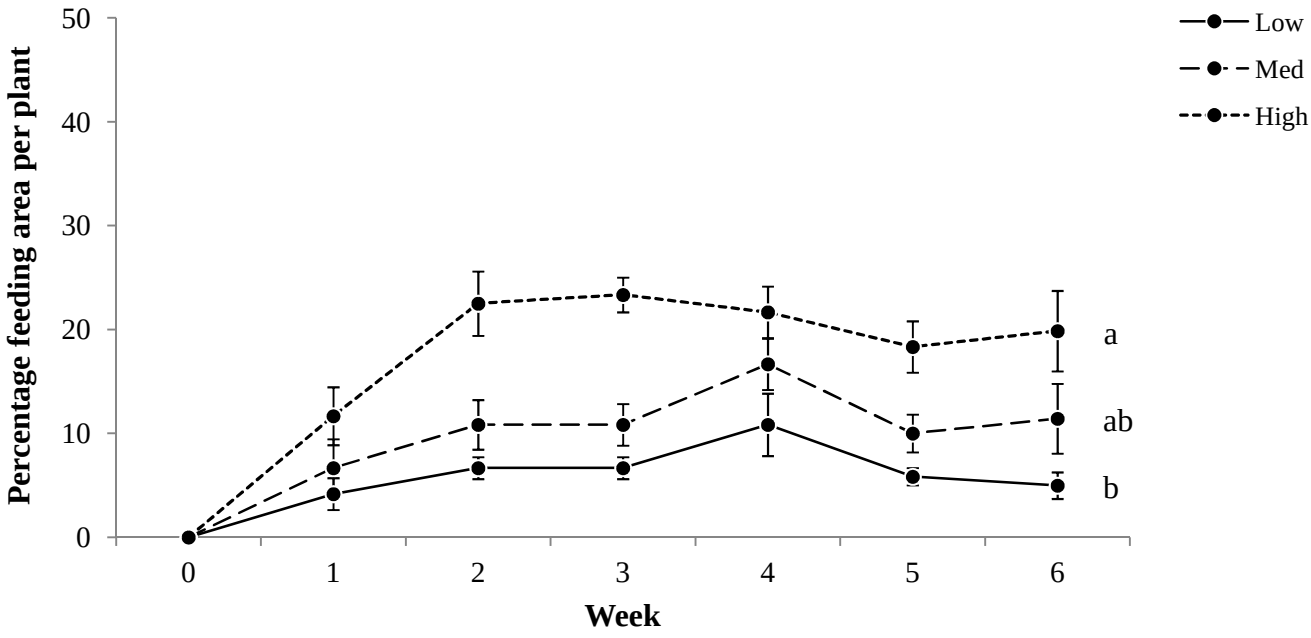

Figure 2.6 The effect of weevil density on the mean percentage feeding area of *Neochetina bruchi* on leaf 1. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

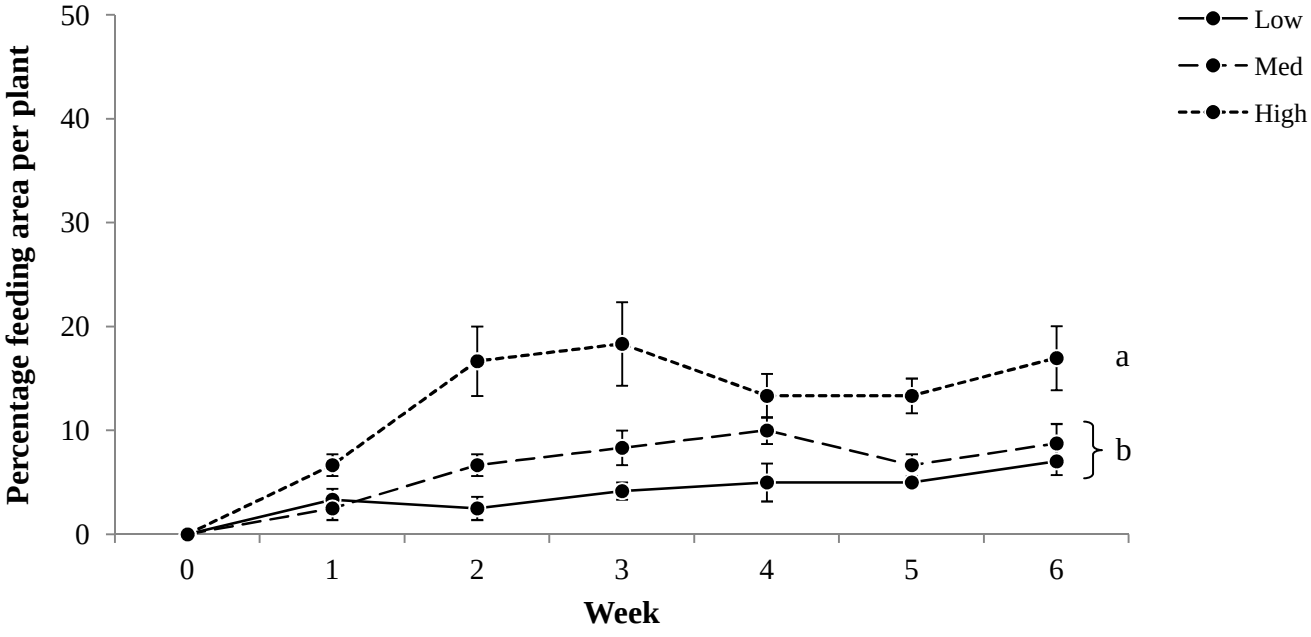

Figure 2.7 The effect of weevil density on the mean percentage feeding area of *Neochetina bruchi* on leaf 2. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

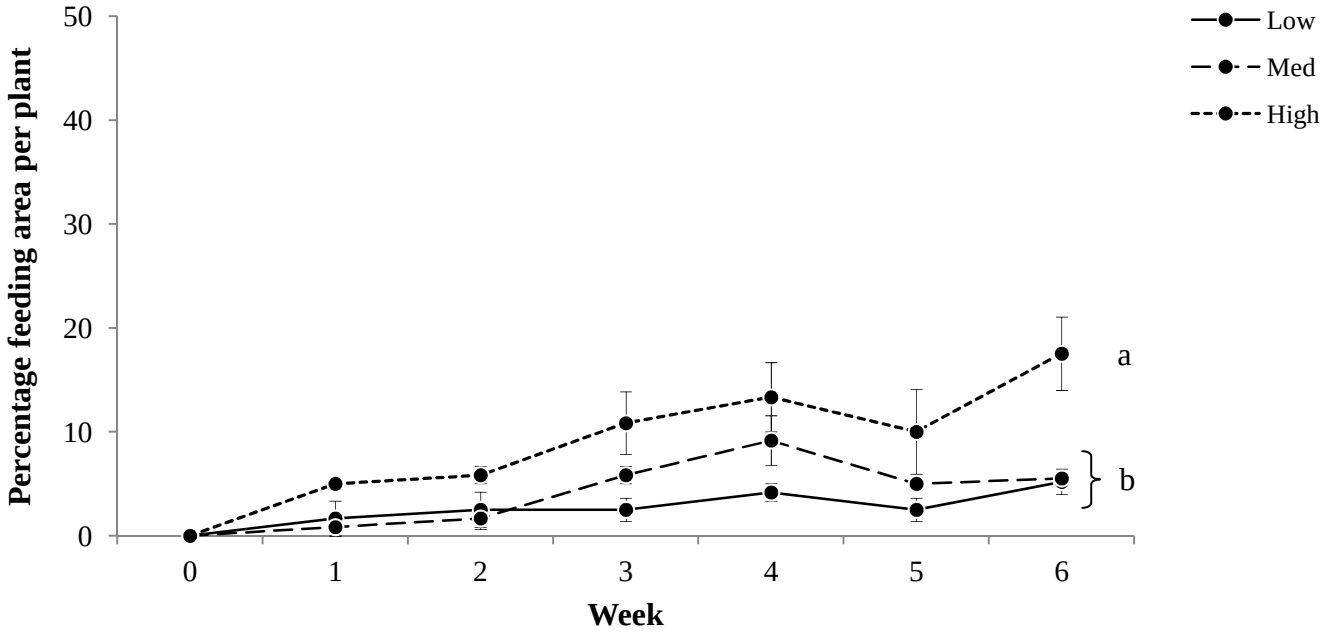

Figure 2.8 The effect of weevil density on the mean percentage feeding area of *Neochetina bruchi* on leaf 3. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

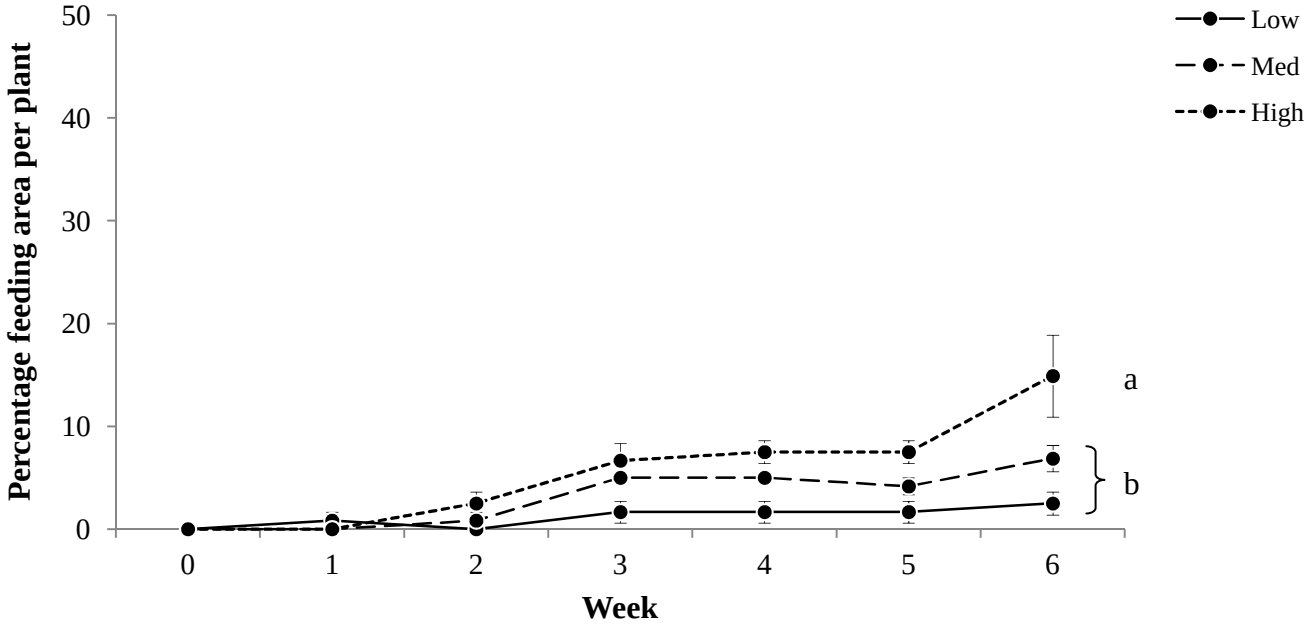

Figure 2.9 The effect of weevil density on the mean percentage feeding area of *Neochetina bruchi* on leaf 4. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

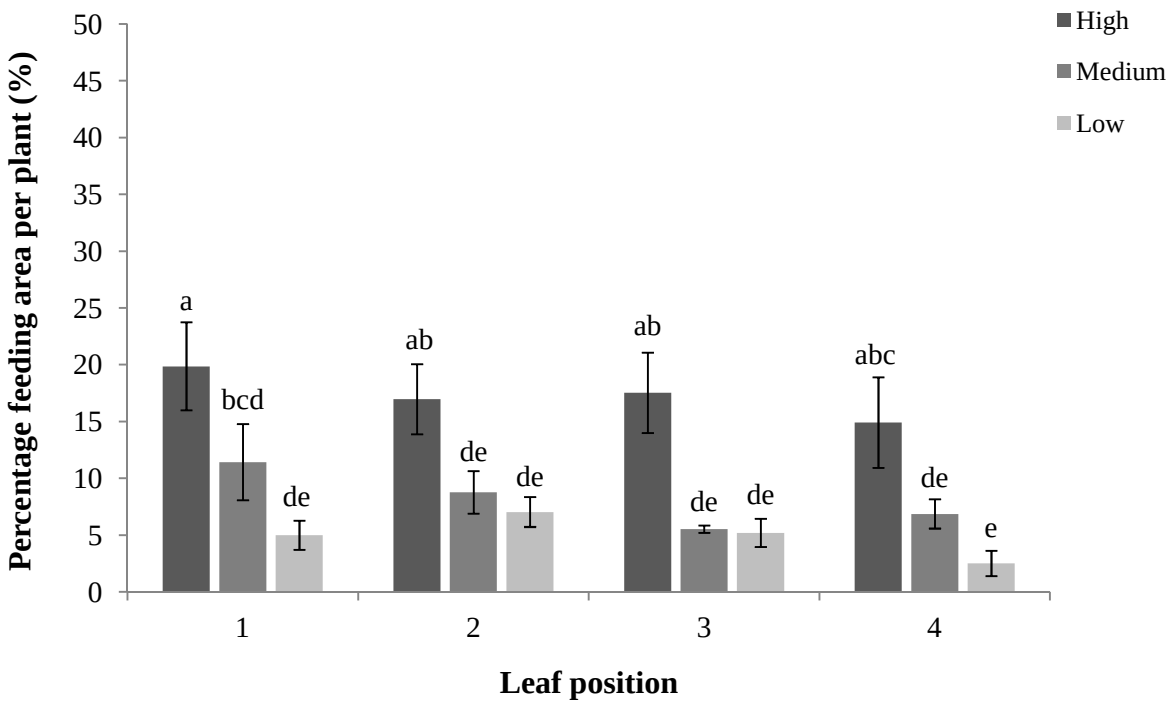

Figure 2.10 The effect of weevil density on the mean percentage feeding area of *Neochetina bruchi* on each leaf position at the end of the trial (Week 6). Treatments: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter are not statistically different, *P*>0.05 (n=6 plants/treatment).

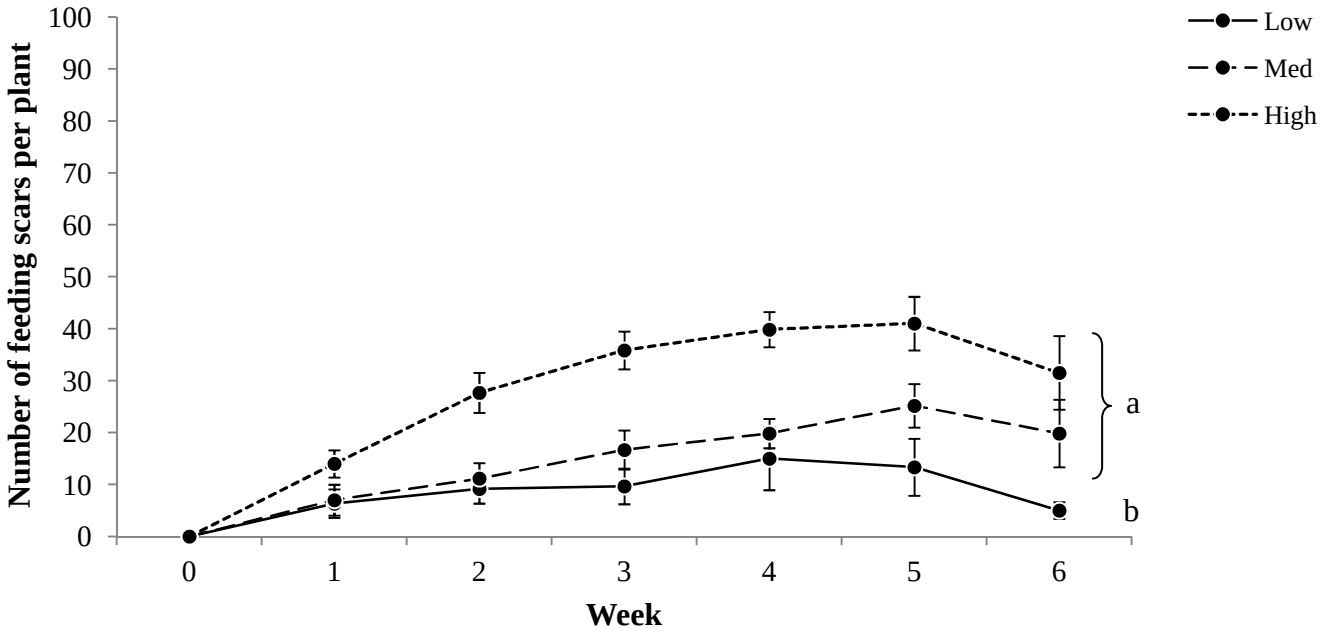

Figure 2.11 The effect of weevil density on the mean number of adult weevil feeding scars on leaf 1 over the sampling period. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, *P*>0.05 (n=6 plants/treatment).

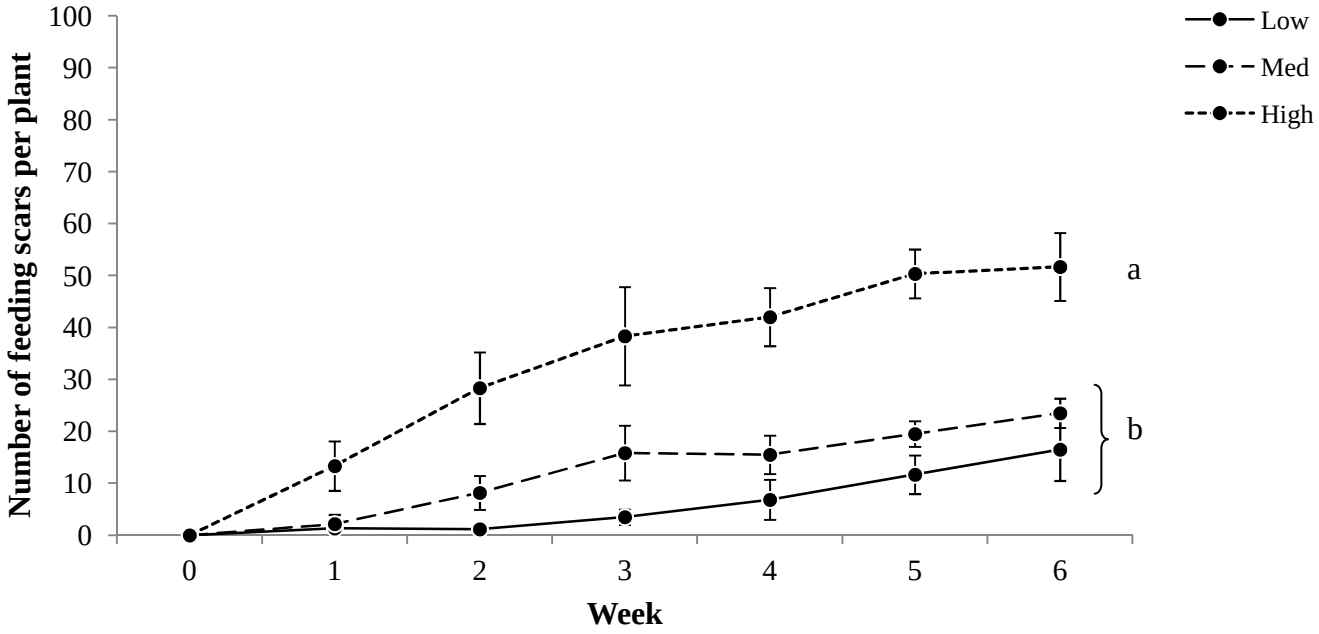

Figure 2.12 The effect of weevil density on the mean number of adult weevil feeding scars on leaf 2 over the sampling period. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

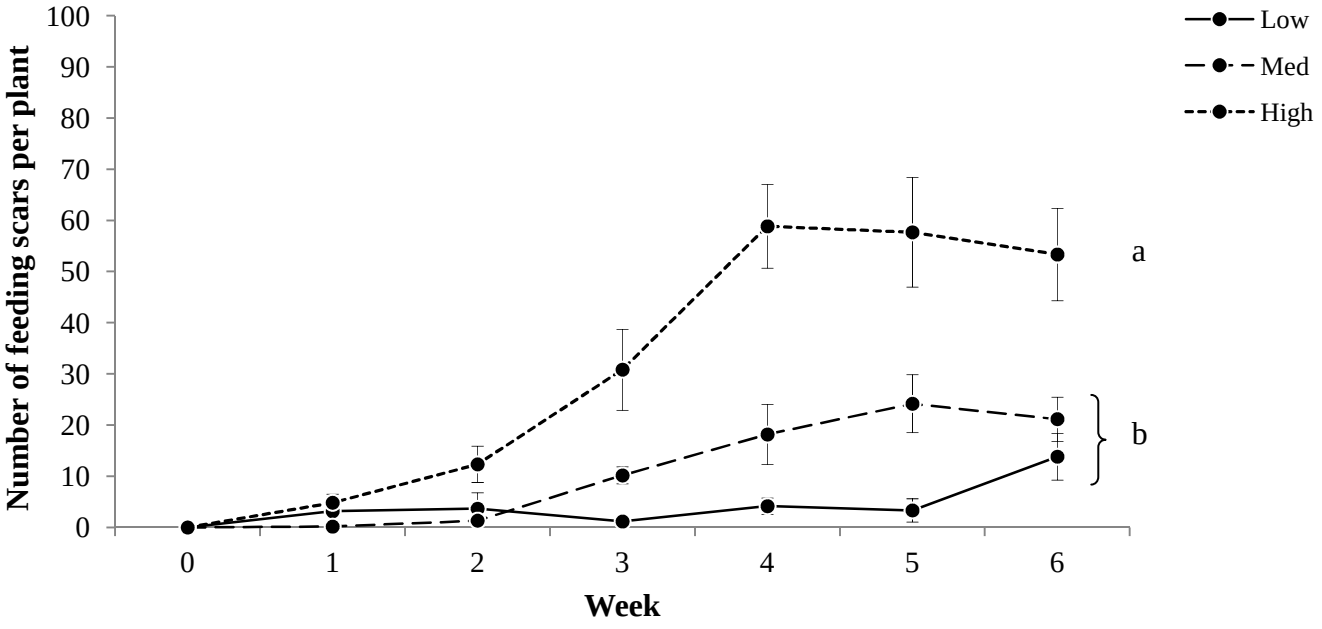

Figure 2.13 The effect of weevil density on the mean number of adult weevil feeding scars on leaf 3 over the sampling period. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

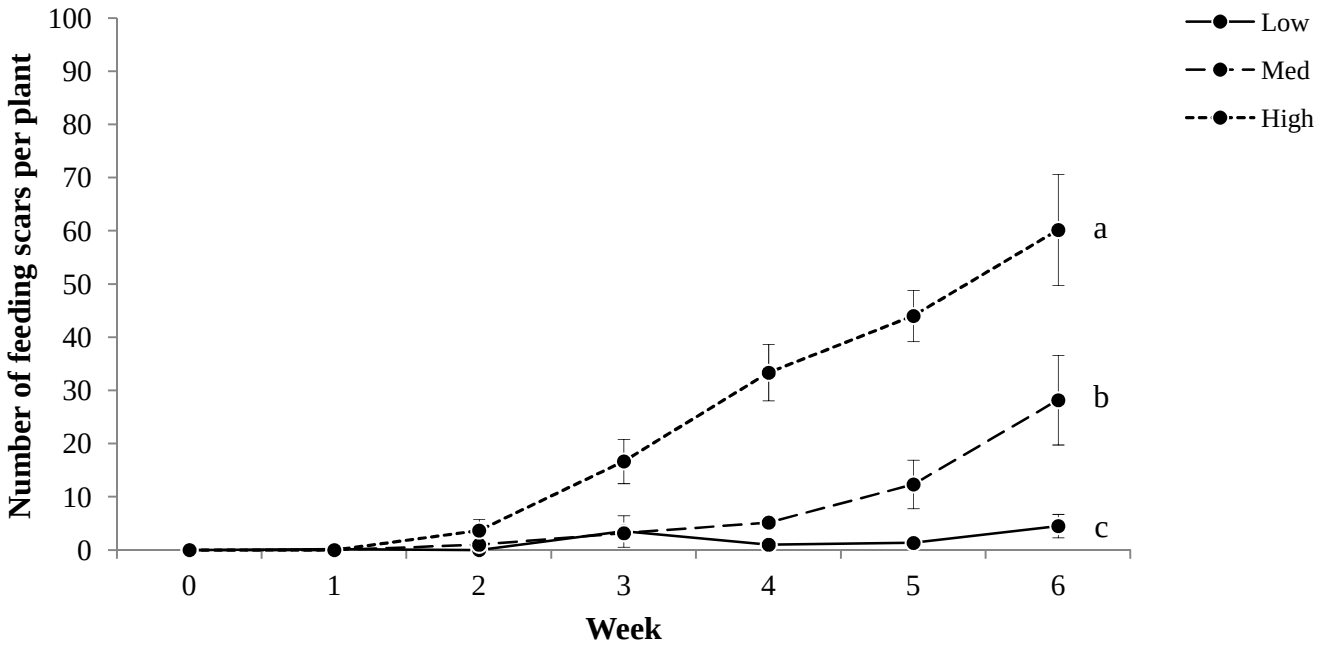

Figure 2.14 The effect of weevil density on the mean number of adult weevil feeding scars on leaf 4 over the sampling period. Treatment: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter at Week 6 are not statistically different, $P>0.05$ (n=6 plants/treatment).

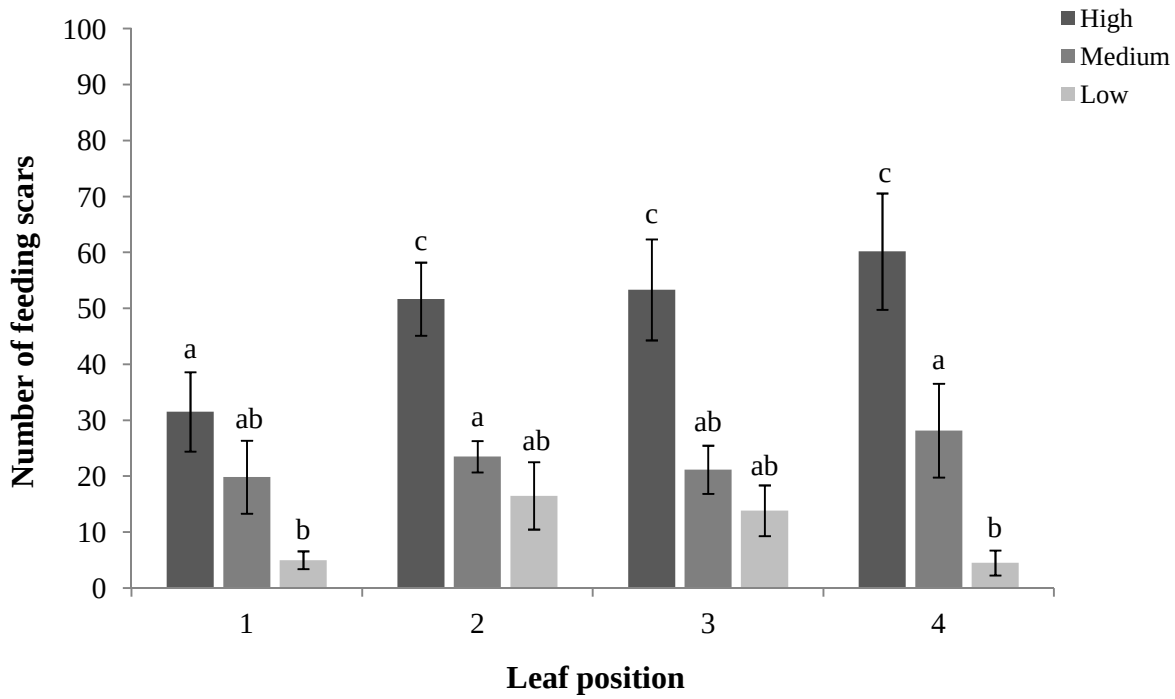

Figure 2.15 The effect of weevil density on the mean number of adult *Neochetina bruchi* feeding scars on each leaf position at the end of the trial (Week 6). Treatments: Low = 2 *N. bruchi*, Medium = 6 *N. bruchi*, High = 12 *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter are not significantly different, $P>0.05$ (n=6 plants/treatment).

2.3.3 Effect of *Neochetina bruchi* on water hyacinth plant growth

As dry plant biomass increased, percentage leaf surface area damage for leaves 2 and 3 decreased significantly in the high density treatment (Table 2.6). Conversely, no correlations were found between percentage leaf surface area damaged and dry plant biomass for the remaining density treatments for all leaf positions. There were no significant relationships between the number of adult weevil feeding scars and dry plant biomass for all leaf positions in all density treatments (Table 2.7). Strong negative correlations were found between leaf production and the number of adult feeding scars for leaves 1 and 2 when exposed to high densities of weevils, but none was found for the remainder of the leaf positions and density treatments (Table 2.8). As with the relationship between leaf production and weevil feeding scars, strong negative correlations were found between ramet production and weevil feeding scars for leaves 1 and 2 when exposed to high densities of weevils (Table 2.9); as adult weevil feeding scars increased in the high density treatment, decreases in leaf and ramet production occurred. Additionally, a strong correlation was observed between ramet production and the percentage feeding area for leaf 2 for the high density treatment ($y = 5.13 – 0.07*x$, $R = -0.90$, $P<0.05$, $R^2 = 0.80$). No correlations were found for the remainder of the leaf positions and low and medium density treatments. Furthermore, no significant relationships were observed between the length of the second petiole and both percentage feeding area and the number of weevil scars for all leaf positions in all density treatments.

Significant strong and positive relationships were found between adult weevil percentage feeding area and the number of scars for all leaf positions, but not for all treatments (Table 2.10).

Table 2.6 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between dry plant biomass and percentage feeding area by *N. bruchi* for leaves 1 to 4 for all weevil density treatments at the end of the trial. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 1	**High**	y = 225.34 + 1.64*x	0.19	0.73	0.03
	Medium	y = 305.58 – 6.44*x	-0.66	0.15	0.44
	Low	y = 292.82 – 1.90*x	-0.11	0.83	0.01
Leaf 2	**High**	y = 425.20 – 9.86*x	-0.88	**0.02**	0.78
	Medium	y = 301.93 – 7.99*x	-0.46	0.36	0.21
	Low	y = 281.61 + 0.25*x	0.02	0.98	0.0002
Leaf 3	**High**	y = 416.98 – 9.07*x	-0.93	**0.01**	0.87
	Medium	y = 442.46 – 38.13*x	-0.39	0.45	0.15
	Low	y = 292.57 – 1.78*x	-0.10	0.85	0.01
Leaf 4	**High**	y = 300.84 – 2.87*x	-0.33	0.52	0.11
	Medium	y = 107.16 + 18.19*x	0.72	0.10	0.52
	Low	y = 287.67 – 1.73*x	-0.09	0.87	0.01

Table 2.7 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between dry plant biomass and the number of adult *N. bruchi* feeding scars for leaves 1 to 4 for all weevil density treatments at the end of the trial. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 1	**High**	y = 332.79 – 2.37*x	-0.49	0.32	0.24
	Medium	y = 285.18 – 2.68*x	-0.54	0.27	0.29
	Low	y = 278.60 + 0.95*x	0.07	0.90	0.005
Leaf 2	**High**	y = 412.60 – 3.00*x	-0.57	0.24	0.32
	Medium	y = 419.31 – 7.97*x	-0.69	0.13	0.48
	Low	y = 279.35 + 0.24*x	0.07	0.90	0.005
Leaf 3	**High**	y = 284.87 – 0.50*x	-0.13	0.80	0.02
	Medium	y = 268.48 – 1.72*x	-0.23	0.66	0.05
	Low	y = 316.79 – 2.42*x	-0.51	0.30	0.26
Leaf 4	**High**	y = 235.10 + 0.38*x	0.12	0.83	0.01
	Medium	y = 148.45 + 2.97*x	0.77	0.08	0.59
	Low	y = 297.25 – 3.09*x	-0.32	0.54	0.10

Table 2.8 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf production and the number of adult *N. bruchi* feeding scars for leaves 1 to 4 for all weevil density treatments at the end of the trial. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 1	**High**	y = 8.00 – 0.03*x	-0.87	**0.02**	0.76
	Medium	y = 6.93 – 0.02*x	-0.4	0.42	0.17
	Low	y = 6.70 – 0.04*x	-0.28	0.59	0.08
Leaf 2	**High**	y = 8.65 – 0.03*x	-0.81	**0.05**	0.65
	Medium	y = 7.74 – 0.05*x	-0.43	0.39	0.19
	Low	y = 6.16 + 0.02*x	0.56	0.25	0.31
Leaf 3	**High**	y = 7.46 – 0.01*x	-0.30	0.56	0.09
	Medium	y = 7.28 – 0.04*x	-0.46	0.35	0.21
	Low	y = 6.65 – 0.01*x	-0.21	0.68	0.05
Leaf 4	**High**	y = 6.57 + 0.01*x	0.29	0.58	0.08
	Medium	y = 5.88 + 0.02*x	0.54	0.27	0.29
	Low	y = 6.39 + 0.02*x	0.24	0.65	0.06

Table 2.9 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between ramet production and the number of adult *N. bruchi* feeding scars for leaves 1 to 4 for all weevil density treatments at the end of the trial. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 1	**High**	y = 4.37 – 0.07*x	-0.83	**0.04**	0.68
	Medium	y = 2.47 – 0.01*x	-0.13	0.80	0.02
	Low	y = 2.80 + 0.04*x	0.24	0.64	0.06
Leaf 2	**High**	y = 6.13 – 0.08*x	-0.84	**0.04**	0.70
	Medium	y = 3.42 – 0.05*x	-0.39	0.44	0.15
	Low	y = 3.56 – 0.03*x	-0.79	0.06	0.63
Leaf 3	**High**	y = 4.05 – 0.04*x	-0.53	0.28	0.28
	Medium	y = 2.88 – 0.03*x	-0.33	0.52	0.11
	Low	y = 3.47 – 0.03*x	-0.60	0.21	0.36
Leaf 4	**High**	y = 2.00 + 0.003*x	0.05	0.93	0.002
	Medium	y = 1.51 + 0.03*x	0.73	0.10	0.54
	Low	y = 3.37 – 0.08*x	-0.70	0.12	0.49

Table 2.10 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between percentage leaf area damage and the number of adult *N. bruchi* feeding scars for leaves 1 to 4 for all weevil density treatments at the end of the trial. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 1	**High**	y = 9.50 + 1.11*x	0.60	0.20	0.37
	Medium	y = 2.79 + 1.49*x	0.77	0.07	0.59
	Low	y = -0.54 + 1.11*x	0.89	**0.02**	0.79
Leaf 2	**High**	y = 20.41 + 1.84*x	0.87	**0.03**	0.75
	Medium	y = 19.21 + 0.49*x	0.33	0.53	0.11
	Low	y = -14.58 + 4.42*x	0.97	**0.002**	0.93
Leaf 3	**High**	y = 40.46 + 0.73*x	0.29	0.58	0.08
	Medium	y = 4.79 + 2.97*x	0.23	0.67	0.05
	Low	y = -2.73 + 3.19*x	0.87	**0.03**	0.75
Leaf 4	**High**	y = 32.42 + 1.86*x	0.71	0.11	0.51
	Medium	y = -10.63 + 5.65*x	0.87	**0.02**	0.76
	Low	y = 0 + 1.8*x	0.91	**0.01**	0.82

2.3.4 Summary of results

At the end of the density trial, differences in plant growth parameters between the control and the three density treatments were negligible (Table 2.11). Although no statistical differences were observed in dry plant biomass, the number of functional leaves and ramet production between the density treatments, these plant parameters were strongly negatively related to weevil feeding in the high density treatment (specifically for leaves 1, 2 and 3), suggesting that water hyacinth growth was negatively affected by high weevil densities.

Decreases in the length of the second petiole were observed in all density treatments at the end of the trial, compared to the control treatment, but were only statistically different between for the medium and high weevil density treatments. Water hyacinth characteristically exhibits shortening of the second petiole when exposed to weevil herbivory (Forno, 1981). The lack of a statistical difference in the length of the second petioles between the control and low density treatment suggests a compensatory response to nominal herbivory.

Different weevil densities resulted in different levels of damage for all leaf positions. However, a general trend was evident; weevil feeding in the high density treatment resulted in significantly greater surface area damage than the low density treatment, with the medium density treatment as an intermediate of the high and low treatments for all leaf positions. Percentage feeding was typically greatest on the youngest leaves (1 and 2); however, differences in surface area feeding between leaf positions for each density treatment did not differ. Much like percentage feeding area, the number of weevil feeding scars increased with an increase in weevil density; the high density treatment resulted in a significantly greater number of feeding scars than the low density treatment, with the medium density treatment as an intermediate.

Table 2.11 A summary of all the differences observed in the four experimental treatments for all plant and weevil parameters measured, in relation to the control treatment. o = no significant change in comparison to the control treatment, + = increases observed, but not necessarily significant, - = decreases observed, but not necessarily significant. Symbols followed by * indicate significant increases or decreases observed. *Effect of weevil herbivory* displays significant relationships between plant and insect parameters, $P < 0.05$.

	Treatment			
	Low	**Med**	**High**	**Control**
Plant parameters				
Total dry plant biomass	+	-	o	o
Number of functional leaves	+	o	+	o
Leaf turnover	+	+	-	o
Length of petiole 2	-	-*	-*	o
Number of ramets	o	-	-	o
Insect parameters				
% Feeding area	+	+	+*	o
Weevil feeding scars	+	+	+*	o
Effect of weevil herbivory				
Dry plant biomass vs % feeding area	o	o	-*	o
Dry plant biomass vs # feeding scars	o	o	o	o
Leaf production vs # feeding scars	o	o	-*	o
Ramet production vs % feeding area	o	o	-*	o
Ramet production vs # feeding scars	o	o	-*	o
% feeding vs # feeding scars[i]	+*	o	+*	o

[i] Summarised for leaf 2

2.4. DISCUSSION

2.4.1 Effect of *Neochetina bruchi* herbivory on water hyacinth growth parameters

This study aimed to determine an ideal density of weevils for subsequent experiments investigating the role of water hyacinth insect-induced defence pathways in response to biological control agent herbivory. This study showed that different densities of weevils have varied impacts on water hyacinth growth parameters and insect feeding, where high densities of *N. bruchi* are generally more impacting than lower densities. A significant negative relationship between dry plant biomass and percentage weevil feeding area was observed for the high density treatment, suggesting that with increasing insect density and damage, water hyacinth biomass accumulation declines. This is further supported by the significant negative relationships seen for leaf and ramet production; increased weevil feeding through high weevil densities results in decreased leaf and ramet production. In addition, the lengths of the second petioles decreased over time in response to *N. bruchi* herbivory, with the high and medium density being significantly shorter. Herbivory by the *Neochetina* spp. weevils typically results in the shortening of water hyacinth petioles (Forno, 1981), particularly because the second leaf is the leaf of preference and petiole mining by the larvae remove vital leaf tissues for nutrient and water transport (Center and Van, 1989). However, no significant negative correlations were found between weevil herbivory and the shortening of the second petioles (see section 2.3.3). Similarly, the study by Briese *et al*. (2002) identified adverse impacts of increasing *T. briesei* densities on *O. acanthium* total plant biomass (dry weight (g)), and seed and flower production. *Onopordum acanthium* exposed to one pair of weevils resulted in reduced plant biomass accumulation by 59%, seed production by 58%, and as weevil densities increased to a maximum of 8 weevils per plant, the impact of herbivory became lethal, where no plant production occurred.

Although none of the densities of *N. bruchi* weevils used in the present study resulted in lethal impacts on water hyacinth, the results obtained in the present study corroborate with the trends identified by Briese *et al.* (2002) and numerous other studies (Ladd and Buriff, 1979, Center and Van, 1989, Briese, 2000, Rhainds and English-Loeb, 2003, Briese *et al.*, 2004, Ding *et al*., 2006, Schooler and McEvoy, 2006), where increasing herbivore densities invariably result in greater reductions in host-plant growth and physiological parameters. The consequential reductions in host-plant growth and physiological parameters in response to increasing herbivore densities and injury could possibly be a result of reallocation of resources to insect-induced defences as opposed to growth and reproduction. To ensure individual fitness, plants allocate limited resources to three crucial functions; growth, reproduction and/or defence. However, investment into one component of fitness incurs allocation costs to the other components (Bazzaz *et al*., 1987). In effect, water hyacinth most likely increased investment into insect-induced responses and incurred allocation costs in terms of growth and reproduction with increasing insect density. The observed negatively correlated relationships between weevil feeding and ramet production and dry plant biomass in the high density treatment is consistent with this notion, as well as examples in the literature. A study by Coley (1986) found that with increasing herbivory by the generalist army-worm, *Spodoptera latifascia* Walker, 1856 (Lepidoptera: Noctuidae), on the neotropical tree, *Cercropia peltata* L. (Moraceae), leaf tannin concentrations increased significantly. Subsequently, increases in tannin concentrations conferred resistance against the army-worm, as leaves with higher tannin concentrations had reduced leaf damage. However, the production of leaf tannins in *C. peltata* leaves incurred a significant cost in terms of leaf production. Ultimately, under medium water nutrient availability, herbivory by *N. bruchi* at increasing insect densities most likely induced a resource reallocation response to defences in water hyacinth.

If weevil densities were to increase higher than 12 *N. bruchi* per plant, it is predicted that undesirable reductions in plant growth, ineffective insect-induced responses and premature plant death would occur; thus it is possible that water hyacinth insect-induced responses will be negated against higher weevil densities. Water chestnut, *Trapa natans* L. (Trapaceae), host plant of the leaf beetle, *Galerucella birmanica* Jacoby (Coleoptera: Chrysomelidae), exhibited reduced or ceased biomass production when inoculated with high densities of *G. birmanica* (Ding *et al*., 2006). A study by Ladd and Buriff (1979), investigating the influence of larval feeding by the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), on Kentucky bluegrass, *P. pratensis*, yields, found similar results, where higher densities of the larvae resulted in reductions in bluegrass yields and eventual death of the plant.

Heard and Winterton (2002) observed almost total destruction of water hyacinth when plants in a stock stand (a number of plants grown in one container or pool) were exposed to one *N. bruchi* per plant during preliminary trials, and therefore used 0.5 weevils per plant. The low density treatment in the present study comprised one mating pair of *N. bruchi* and did not result in premature plant mortality. This was most likely due to differences in water nutrient concentrations and exposure time to herbivory. Nutrient availability (mainly nitrogen and phosphorous) is an important abiotic factor that influences plant communities (Koerselman and Meuleman, 1996). Reduced nitrogen concentrations can have negative impacts on plant growth and development, physiological functioning, and synthesis of secondary defence compounds (Bazzaz *et al.,* 1987, Koerselman and Meuleman, 1996, Winter and Rostás, 2010). This in turn may impact a plant's resistance to herbivores, and the amount of injury (associated with herbivore density) a plant can tolerate. Furthermore, under increasing nutrient conditions, water hyacinth has been observed to increase ramet production, biomass accumulation, and leaf nitrogen concentrations (Xie *et al*., 2004). Heard and Winterton

(2000) used high (1.0mgP.l^{-1} and 1.6mgN.l^{-}1) and medium (0.025mgP.l^{-1} and 0.4mgN.l^{-1}) water nutrient concentrations during their experiment. In comparison to the water nutrient concentrations used in the present study (0.025mgP.l^{-1} and 2.5mgN.l^{-1}), the water nutrient levels used by Heard and Winterton (2000) were nitrogen poor. Plants grown under high water nutrient concentrations in Heard and Winterton's (2000) study, individually weighed less (control treatment: ~9.5g dry weight) than the plants grown under medium water nutrient concentrations used in the present study (control treatment: ~16.96±1.27g dry weight). When plants grown under high nutrient concentrations were exposed to *N. bruchi* herbivory and subjected to 0.5 weevils per plant in Heard and Winterton's (2000) study, they weighed less (*N. bruchi* treatment: ~6g dry weight) than the plants grown under medium water nutrient concentrations and exposed to one pair of weevils in the present study (low density treatment: ~19.78±1.75g dry weight). Therefore, it is possible that the higher nitrogen concentration used in the present study resulted in greater water hyacinth growth and development than that observed by Heard and Winterton (2000), and consequently the ability for water hyacinth to resist weevil densities greater than 0.5 weevils per plant. Moreover, as discussed above, the low density treatment in the present study probably induced a compensatory response where water hyacinth reallocated resources to reproductive growth to negate nominal injury.

The present study also used a shorter exposure time to herbivory in comparison to the study by Heard and Winterton (2002), which was conducted over a period of eight weeks. It is possible that the six week exposure time employed in the present study did not provide comparable cumulative impacts of herbivory on water hyacinth, particularly by the larvae, in comparison to an eight week trial. Given more exposure time to herbivory in the present study, it is likely that notable differences between the low and medium density treatments would have arisen. Furthermore, the effect of time was significant for all plant growth

parameters, excluding the number of functional leaves and leaf turnover, so it's possible that with an extended sampling period, measurable differences in plant growth parameters between the lower density treatments would ultimately occur; especially as water hyacinth leaves have a life-span of approximately two months (Center, 1981, Coetzee *et al*., 2009). In addition to exposure time, temperature may have played an important role during the trial. *Neochetina bruchi* development occurs optimally at ambient temperatures of about 30°C and ceases below 15°C (Julien, 2001). Although, ambient temperatures were not recorded for this trial, the study took place in a polyethylene covered greenhouse, providing warmth. The study was conducted late summer and perhaps the temperatures at this time were too low for optimal *N. bruchi* development and feeding. Furthermore, it is likely that the fine mesh sleeves enclosing the plants and insects reduced the photosynthetically active radiation (PAR). Weyl and Hill (2012) documented a 36% reduction in PAR when using fine mesh sleeves. However, all of the treatments, including the controls, were exposed to these temperatures and light deficits and that the mesh sleeves were necessary to prevent weevil escape and contamination from greenhouse pests.

2.4.2 Effect of *Neochetina bruchi* herbivory on leaf surface area damage

The different weevil densities resulted in different levels of leaf surface area damage, but a general trend was identified; the high density treatment resulted in greater surface area damage, followed by the medium density and low density treatment. A similar result was found by Center and Van (1989), where the percentage leaf area eaten by high *N. eichhorniae* densities (250 weevils) was higher than the low weevil densities (25 weevils); however, this result was found for plants maintained in high densities (tank dimensions of 0.8m by 2.2m). Another study, by Schooler and McEvoy (2006), found that increasing adult

and larval densities of the golden loosestrife beetle, *Galerucella pusilla* Duftschmid (Coleoptera: Chrysomelidae), positively correlated with increasing purple loosestrife leaf damage (*Lythrum salicaria* L. (Lythraceae)).

Interestingly, leaf position had no effect on *N. bruchi* weevil leaf surface area damage in this study, which is inconsistent with results shown by Center and Wright (1991). Younger water hyacinth leaves were more attractive to adult *Neochetina eichhorniae* weevils than older water hyacinth leaves (Center and Wright, 1991). In the same study, Center and Wright (1991) found that the chemistry of water hyacinth leaves is age specific and suggest that leaf phytochemistry influences the feeding behaviour of *N. eichhorniae*, and the generalist herbivore, yellow woollybear (*Spilosoma virginica* (F.) (Lepidoptera: Arctiidae)). The specific leaf constituents affecting *N. eichhorniae*'s preference for younger water hyacinth leaves was not confirmed.

The number of weevil feeding scars increased with an increase in weevil density; the high density treatment resulted in a larger number of feeding scars and the low density treatment with the lowest number of feeding scars. Furthermore, the number of feeding scars increased with leaf position, but only from leaf 1 to leaf 2 for the high density treatment. This result does not follow the same trend as the percentage feeding area, most likely due to the cumulative damage on older leaves (3 and 4) having greater leaf surface areas (Center and Wright, 1991) and longer exposure times to herbivory. Conversely, Center and Van (1989) found that low densities of *N. eichhorniae* (25 weevils) resulted in a greater number of feeding scars per leaf over time than the higher weevil density (250 weevils) which may have been the result of decreased competition or increased defences.

2.4.3 Effect of *Eccritotarsus catarinensis* on water hyacinth growth

Numerous experiments investigating the impact of the mirid on water hyacinth have used a total of 15 mirids (Coetzee *et al*., 2005, Coetzee *et al*., 2007b, Ajuonu *et al*., 2009, Weyl and Hill, 2012), as a result a density trial was not conducted for *Eccritotarsus catarinensis* in the present study and 15 mirids per plant were used in the forthcoming trials. Subjecting water hyacinth to 15 mirids per plant has typically resulted in neutral and/or positive impacts on biomass accumulation and growth rates without premature plant death, therefore the impact of the mirid on BION®-treated plants was predicted to be non-lethal.

2.5 CONCLUSION

The low and medium weevil densities had negligible adverse impacts on water hyacinth growth parameters. In contrast, the high density treatment presented significant negative impacts, although only evident in dry plant biomass, the lengths of petiole two, ramet production, and leaf production. Therefore, the hypothesis that increasing *N. bruchi* densities would result in increasingly adversely impacted water hyacinth growth was accepted. In accordance, the hypothesis that weevil feeding and damage increases significantly with a higher density of weevils also was confirmed. The results of this study found that a higher density of weevils results in greater and more rapid impacts on water hyacinth plant growth parameters. Although the high density treatment exhibited the only significant relationships between weevil herbivory and water hyacinth growth parameters, high densities of *N. bruchi* were not used for the forthcoming trials, as the plant activator, BION®, was used to induce SA-mediated defences in water hyacinth. It was predicted that the application of BION® would increase the susceptibility of water hyacinth to herbivory by the mirid and weevil; therefore, densities higher than one pair of weevils and 15 mirids on BION®-treated plants

would have resulted in premature plant death. The following experimental set-up was therefore used in the forthcoming trials:

1) ***Exposure time***. The low and medium weevil density treatments had very little impact on water hyacinth growth parameters and this was largely attributed to inadequate exposure time. Given more time, a lower density of weevils was expected to provide measurable changes (Center and Van, 1989), especially taking into account the intended application of BION® and allowing the weevils to complete larval development. An extended exposure time was also predicted to allow for significant changes in leaf turnover and leaf production to be observed. Additionally, plants were acclimated prior to insect inoculation for an additional week under the exact nutrient concentration used in the present study.

2) ***Temperature***. The experimental set-up employed within the present study did not create unfavourable conditions; however, low temperatures may have influenced weevil development and feeding. Subsequent trials were started in late spring to early summer, and in the event of limited daylight, plant growth lights were used to supplement the lack of PAR.

3) ***Insect densities***. The range of weevil densities chosen for the present trial was estimated and provided sufficient insight into formulating the methodology for future trials. Wheras results showed that the high density treatment promoted measurable changes in plant growth and insect parameters, densities equal to or higher than 12 individuals would result in possibly plant death. This was predicted due to the intended application of BION®, the extended exposure times (8-9 weeks) and increased ambient temperatures and day lengths. It was predicted that a low density of *N. bruchi* would be required. Therefore, *N. bruchi* was

used in low densities of two individuals per plant. As mentioned in section 2.4.3, 15 mirids per plant, with a 7:8 sex ratio (male: female), were used in subsequent trials.

4) *Plant growth parameters*. The same methodology and experimental set-up employed for *N. bruchi* was conducted for the mirid, so as to minimise constraining variables and to allow for comparisons between feeding guilds. Although measures of the length of the second petiole, leaf turnover and leaf production did not provide insight in the current experiment, these parameters were included in the next experiment. Leaf quality traits and relative chlorophyll content also were measured in subsequent trials, so as to obtain a more detailed insight into the effect of herbivory on water hyacinth physiological parameters.

5) *Insect parameters*. The same method of determining percentage feeding area for *N. bruchi* was used for *E. catarinensis*, as it is a method commonly used for determining the extent of mirid feeding (Coetzee *et al*., 2005, Coetzee *et al*., 2007b) and allowed for comparisons with the surface area damage by the weevil.

CHAPTER 3

DOES SALICYLIC ACID PLAY A ROLE IN WATER HYACINTH INSECT-INDUCED RESPONSES?

3.1 INTRODUCTION

The most common insect-plant interaction involves insects feeding on plants, and plants employing defences against insect herbivores (Gatehouse, 2002). Because of the high diversity of plants and insects, and the long established relationships between these two groups, plants have developed an array of diverse defence and resistance mechanisms to alleviate and/or tolerate injury. Plants may elicit defensive strategies that are chemical (e.g. secondary metabolites functioning as toxins, deterrents and anti-digestives) and/or physical in nature (e.g. cuticlular thickening, trichomes and thorns) (Karban and Myers, 1989, Lundberg and Åström, 1990, Bernays and Chapman, 1994, Wu and Baldwin, 2010). These responses can be maintained with or without herbivore pressure (constitutive defences) and/or induced only after injury has occurred (Wu and Baldwin, 2010). Whereas, induced defences are disadvantageous with respect to the time-lag between injury and the final expression of the defence strategies, they may aid in conserving energy costs by limiting the production of defences to isolated events where herbivory is in progress, if the defences employed incur costs (Tollrian and Harvell, 1999). Cost-benefit analyses of induced plant responses, and whether defences incur any costs, are still under substantial experimentation and revision (Tollrian and Harvell, 1999, Baldwin, 2001, Heil and Baldwin, 2002, Accamando and Cronin, 2012).

Induced defences are described by Wu and Baldwin (2010) as a three component process: 1) At the plasma membrane, plants will identify the threats, either through wounding, movement on the plant and/or compounds within the insect's oral secretions, such as fatty acid-amino acid conjugates (Alborn *et al*., 1997, Halitschke *et al*., 2001, Yoshinga *et al*., 2007), and will initiate a series of signaling cascades; 2) once the threat has been identified and the signals have been activated, the signals will then activate the up-regulation of plant hormones, such jasmonic acid (JA) and salicylic acid (SA); 3) these plant hormones are involved in the complex regulatory networks, that induce and mediate gene expressions that are responsible for the biosynthesis of plant metabolites, which ultimately form part of a plant's defensive responses. For example, protease inhibitors, which contribute to reducing digestibility in herbivores, are generated after wounding, activating the JA signalling pathway, which results in the up-regulation of protease inhibitor gene expression (Koiwa *et* al., 1997). Insect herbivore-derived elicitors (namely molecules isolated from insect oral secretions, like volicitin (Alborn *et al*., 1997)) and chemical analogues of signalling hormones, such as JA and SA, have been used in studies aimed at investigating insect-plant interactions, in order to replicate the biochemical processes involved in inducing plant responses.

Although a variety of studies have identified numerous insect-induced plant responses, and the underlying signalling pathways and gene expressions that result in such defences, the biochemical pathways employed are not always the same for every insect-plant relationship and vary in complexity (Baldwin and Preston, 1999, Wu and Baldwin, 2010). Herbivory by the silver leaf whitefly (SLWF), (*Bemisia tabaci* type B; *Bemisia argentifolii*) (Homoptera: Aleyrodidae)), induces the SA signalling pathway in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) (although this pathway does not induce defence against SLWF) (Zarate *et al*., 2007), whereas rhizobacteria (Pieterse *et al*., 2002) and the generalist herbivore, beet

armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), induce JA signalling pathways in *Arabidopsis* (van Oosten *et* al., 2008). Essentially, the signalling pathways deployed may vary according to the host plant species, and the insect feeding mode (e.g. phloem-feeding versus leaf chewing), which impacts the extent of injury inflicted (Walling, 2000). Therefore, insect-induced plant responses within particular insect-plant relationships cannot simply be predicted, especially when the plant is host to a variety of insect herbivores. This may be particularly significant for the biological control of weeds where one or more host-specific, herbivorous insect species is used to control invasive plants (Julien and Griffiths, 1998, McFadyen 1998, Klein, 2011). The success of such control programmes relies on the re-establishment of the interactions between the insects and the exotic plant, with the insect exerting a significant top-down pressure on the weed. However, despite the knowledge that classical biological control employs time-honoured insect-plant relationships, which undoubtedly include plant responses, little has been done to examine the significance and the presence of possible insect-induced plant responses and the biochemical processes involved.

The free-floating aquatic macrophyte, water hyacinth (*Eichhornia crassipes* (Mart.) Solms-Laub (Pontederiaceae)), is invasive in several countries around the world (Gopal, 1987). Subsequently, biological control initiatives, using one or more of the eight released arthropod natural enemies, have been implemented worldwide (Tipping *et al.*, 2008, Coetzee *et al.*, 2011, Coetzee pers. com.). As outlined in Chapter 1, the water hyacinth biological control programme provides a suitable platform for investigating insect-induced aquatic plant responses and the pathways responsible for their expression. The reasons for this are largely due to the limited investigations into water hyacinth responses and the suite of insect herbivores released on water hyacinth comprise varying genera from various feeding guilds.

3.1.1 Experimental aims and objectives

The aim of this chapter was to investigate insect-induced responses of water hyacinth, and the roles that SA and JA play in contributing to the expressed defences, through the external application of BION®. BION® induces SA-mediated defence pathways, and subsequently inhibits JA-mediated defence pathways within a plant. The insect herbivores are hypothesised to feed optimally and unhindered on SA-induced water hyacinth plants, resulting in increased plant damage and reduced physiological performance, indicating the potential role of JA in water hyacinth insect induced responses.

3.2 METHODS AND MATERIALS

3.2.1 Experimental insect and plant cultures

Water hyacinth plants were collected from the uMgeni River (upper), KwaZulu-Natal, South Africa, and were maintained in fibreglass pools at the South African Sugar Cane Research Institute (SASRI), Durban, South Africa (29°42’28”S 31°02’52”E). Plants were maintained insect free by spraying with insecticide (Mercaptothion 500 EC, Dow AgroSciences) and covering the pools in mesh netting. The mesh netting allowed for ample light penetration, but zero insect contamination. Experimental plants were used two weeks after an insecticidal application and after being thoroughly rinsed with water. Two weeks provide sufficient time for insecticidal toxic residues to become ineffective (Hill pers. comm.). The present study was conducted at SASRI, because this is where recent research using BION® as an activator of pathogen resistance in sugarcane occurred. Furthermore, the Department of Water Affairs and Forestry’s Working for Water insect mass rearing facility is located on the SASRI

property, where the necessary insects for this trial, *N. bruchi* and *E. catarinensis*, are mass reared.

The weevil *Neochetina bruchi* and the mirid *Eccritotarsus catarinensis* were collected from cultures at the mass-rearing facility at SASRI. In total, 24 weevils and 180 mirids were collected and used for the BION® bioassay. A pair of weevils (1:1 sex ratio, female:male) was placed onto single plants for the *N. bruchi* treatments, whereas 15 mirids were placed onto single plants for the *E. catarinensis* treatments (8:7 sex ratio). The insect densities used in this trial are based on the preliminary density trial and experimental methods adapted from literature, as discussed in Chapter 2.

The trial took place in a glass greenhouse at SASRI. In total, 36 plants of a similar size were selected from the plant cultures and used for the BION® bioassay trial. Plants were placed individually in thirty-six 30*l* round tubs, filled with 23*l* of water and covered with a mesh sleeve. The mesh sleeve prevented contamination of control plants and the escape of experimental insects from the treatment plants. Plants were left to acclimate for two weeks before initiating the trial. The water was changed every two weeks and maintained at a medium nutrient level (0.025mgP.l^{-1} and 2.5mgN.l^{-1}) throughout the trial, as discussed in the experimental protocol for Chapter 2. Each plant was assigned to one of six treatments; *N. bruchi* only, *E. catarinensis* only, exogenous application of BION® only, *N. bruchi* with the exogenous application of BION®, *E. catarinensis* with the exogenous application of BION®, and an insect-free control treatment with the exogenous application of distilled water. Each treatment was replicated six times. The six treatments and the potential defence pathways available are shown in Table 3.1.

Table 3.1 The six experimental treatments and the corresponding number of insects inoculated on each plant in each treatment.

Treatment		Denotation	Resultant pathways
Control	**Distilled water**	dH_2O	Insect free, SA and JA not induced
	BION®	BION	Insect free, SA induced, JA not induced
N. bruchi	**Non- BION®**	Nb	2 weevils, SA and JA induced
	BION®	BNb	2 weevils, SA induced, JA not induced
E. catarinensis	**Non- BION®**	Ec	15 mirids, SA and JA induced
	BION®	BEc	15 mirids, SA induced, JA not induced

3.2.2 Exogenous application of BION® bioassay

A particular advantage of using plant activators, such as BION®, is that they induce the desired plant defences, regardless of the presence of herbivores, which may naturally induced other plant defences (Dietrich *et al*., 2004); therefore herbivory by the experimental insects in the present study did not induce other unknown plant defence pathways, subsequently eliminating confounding factors. Dosage was formulated according to manufacturer's recommendations as a low to medium cover spray (12.5g/100*l* water). The BION® solution used in this trial was prepared by adding 0.0625g of BION® granules to 500ml of distilled water in a spray bottle.

3.2.2.1 BION® toxicity trial

Toxicity trials with BION®, showed non-toxic effects to birds, bees, rats, earthworms and insect/mite predators and parasites, at least up to an external application rate of 30g a.i./ha (Australian Pesticides and Veterinary Medicines Authority (APVMA)). Initial ecological toxicity trials of standard acute exposure to Acibenzolar-S-methyl indicated toxicity to

aquatic organisms, such as fish, invertebrates and algae/aquatic plants, but the environmental risk assessment concluded that chronic toxicity of Acibenzolar-S-methyl to non-target animals is unlikely (United States Environmental Protection Agency (EPA), office of Prevention, Pesticides and Toxic Substance). Doses used within this trial were lower than the doses used within the BION® and Acibenzolar-S-methyl toxicity trials. However, preliminary trials were conducted to test for negative effects on the experimental insects, as some insect species may be more sensitive to certain compounds.

Before the initiation of the BION® bioassay, a preliminary toxicity trial was conducted on the biological control agents, *N. bruchi* and *E. catarinensis*, using the manufacturer's recommended dosage (12.5g/100*l* water). In total, 20 weevils and 23 mirids were individually placed in petri-dishes (9cm diameter) lined with filter paper and a section of water hyacinth lamina. Twelve of the replicates, six weevils and six mirids, were assigned to the control treatment, which were sprayed with distilled water instead of BION®. The remainder of the insects were sprayed exogenously with 1ml each of BION® solution (0.000125g a.i./ml) for every hour for four hours and once again the following day for two consecutive days. Insects were observed hourly for mortality on the first day of BION® application. Thereafter, four times a day for the remainder of the toxicity trial.

For the main BION® bioassay experiments on water hyacinth plants, BION® solution was applied one week before insect inoculation (at Week 0), and then once every week after inoculation, for a period of 8 weeks. In total, 10 millilitres of BION® solution (0.000125g a.i./ml) was exogenously sprayed using a hand held spray bottle (calibrated at 1 ml (0.000125g a.i./ml) per spray), from 1m above the plant, over the entire canopy of each plant in each of the BION® required treatments. The 10 millilitres of BION® was sufficient to cover the whole water hyacinth canopy.

3.2.3 Plant and insect parameters

After the acclimation period of seven days to BION®, plant parameters for all the plants used within the trial were measured prior to the release of the insects and further application of BION® and distilled water, and were denoted as data for Week 0. Thereafter, the insects were released onto the requisite plants, and the application of BION® and distilled water continued. Thereafter, plant and insect parameters were measured every seven days, for a total period of eight weeks.

Plant parameters

The following plant parameters were measured and collated; dry and dead plant biomass, the number of functional leaves produced, leaf turnover, the length of the second petiole, the number of flowers produced, relative chlorophyll content of leaves 1 to 4, percentage leaf moisture, and the percentage carbon and nitrogen content of leaves 1 to 4. Table 3.2 provides a summary of the plant and insect parameters measured during this study and the underlying principle behind their use. Additionally, Table 3.2 includes the timing of each parameter measurement, because certain plant parameters were measured weekly, and others measured at the end of the trial. *Eccritotarsus catarinensis* were temporarily removed from the relevant treatments using an aspirator, prior to data collection, in order to minimise insect losses, because the mirid is highly mobile.

Total dry plant biomass was measured at the end of the trial. Each plant was cut up and plant parts separated (roots, petioles, leaves and ramets), and dried in an oven at 60°C. The leaf turnover and the number of leaves produced for each plant was measured by labelling the youngest leaf at Week 0 and noting its shift in position each week as the plant aged. Relative leaf chlorophyll content was measured every week using a Minolta SPAD-502 chlorophyll

meter. Mean leaf chlorophyll content was obtained for each leaf, 1 to 4, by taking readings from six different positions on each leaf. A measure of chlorophyll content is an index for predicting changes in photosynthetic productivity (Vasu *et al.*, 2009). Leaf moisture was determined at the end of the trial by excising leaves 1, 2, 3 and 4 from each replicate in each treatment, and immediately weighing each leaf using an analytical balance to obtain the wet weight (Adventurer™, Ohaus Ltd.). Once the wet weights were obtained, leaves were individually placed in labelled brown paper bags and dried in an oven at 60°C for no less than 48 hours, and weighed again to obtain the dry weight. Using the same formula used in Chapter 2, the percentage leaf moisture was determined:

$$\% \text{ leaf moisture} = \frac{(\text{wet weight} - \text{dry weight}) \times 100}{\text{wet weight}}$$

Percentage carbon and nitrogen content was obtained by excising a leaf disc, 20mm in diameter, from the centre of each leaf. This procedure was done after obtaining the wet weights for the leaf moisture evaluation. Each leaf disc was individually placed in labelled Eppendorf vials and dried in an oven at 60°C, and then weighed using the analytical balance to obtain the dry weight (the dry weight of each leaf disc was added to the dry weight of the leaf from where it was excised). Samples were then ground using a pestle and mortar. Ground samples were analysed for percentage carbon and nitrogen using a C-N analyser (Europa Scientific, ANCA-SL) at IsoEnvironmental, Rhodes University, South Africa.

Insect parameters

Adult weevil feeding was recorded using the same method used and calibrated in Chapter 2; estimating the percentage leaf area damage. Additionally, the number of adult weevil feeding scars was counted on leaves 1 to 4. *Eccritotarsus catarinensis* percentage feeding damage

was recorded in a similar manner to that of the weevil. The number of mirids on each plant was counted each week, and the numbers of weevils per plant (adults, larvae, and pupae) were collected at the end of the trial. Measuring population sizes provides an indication of fecundity and resource acquisition; if insect numbers increased, it would suggest resources and nutrients are more readily available to the insects, possibly unhindered due to lowered defences. In addition to percentage feeding, a palatability test was conducted for *N. bruchi* on BION® and non-BION® treated plants using the following equation:

$$\textbf{P.I.} = \frac{\textbf{Log of total amount of treated leaves eaten}}{\textbf{Log of total amount of control leaves eaten}}$$

The estimations of percentage leaf area damage were log transformed and inserted into this formula, where a palatability index (P.I.) was produced. A P.I. less than 1.00 (<1.00) indicates an insect's preference for the control plant, >1.00 indicates a preference for the treated plants (in this case BION® treated plants), and a P.I. of 1.00 indicates no preference.

3.2.4 Statistical analyses

Generalised linear/non-linear models (GLZ) in STATISTICA 10.0 (© StatSoft, Inc. 2011) were used to analyse the plant and insect parameter data. On examination of the residuals, all plant and insect parameter data exhibited a normal and homoscedastic distribution around the regression line. To test for a correlation between relative chlorophyll content and percentage nitrogen content of water hyacinth leaves, a simple regression was performed.

Table 3.2 The plant and insect parameters measured during the present study, the timing of their measurements and the underlying rationale for their use.

Plant parameters	**Parameter measured**	**Rationale**
Weekly measurements	Number of functional leaves	Plant growth
	Leaf turnover	Plant growth rate
	Length of the second petiole	Plant growth
	Number of flowers	Plant reproduction
	Relative chlorophyll content	Photosynthetic productivity
End of trial measurements	Total dry plant biomass	Plant growth
	Total dead plant biomass	Plant longevity or damage
	% Leaf moisture	Leaf nutrition
	% Carbon content	Leaf nutrition
	% Nitrogen content	Leaf nutrition
Insect parameters		
Weekly measurements	Ec % feeding area	Leaf damage and insect performance
	Nb % feeding area	Leaf damage and insect performance
	Number of adult weevil feeding scars	Leaf damage and insect performance
	Number of mirids and nymphal stages	Insect performance
	Nb palatability test	Insect performance and plant quality
End of trial measurements	Number of weevil pupae and larvae	Insect performance
	Pupal weight	Insect performance

3.3 RESULTS

3.3.1 Toxicity trial

No mortality was recorded from the BION® toxicity trial for both the weevils and mirids, after the exogenous application of the BION® solution. This finding indicated that BION® was suitable for use, because it had no toxic effect on the biological control agents after 48 hours of periodic application.

3.3.2 Plant parameters

The induction of the SA-mediated defence pathway had clear effects; throughout the trial, plants exhibited varying responses to the different treatments, particularly by week 8, where the differences in plant appearance were visible (Figure 3.1).

3.3.2.1 Total dry plant biomass and dry dead biomass

A difference in total dry plant biomass was observed between the six treatments (Wald's $X^2_{(5)}=21.95$, $P<0.05$) (Figure 3.2), with dH_2O and Ec having significantly higher dry biomass than treatments BION and BNb at the end of the experiment. The difference observed between dH_2O and BION may be a result of the lowered JA levels in the BION treatment. BION and BNb treatments did not differ and had the lowest recorded dry biomass. Biomass of plants inoculated with insects and treated with BION® (BEc and BNb) were not different from their respective counter-plants treated with insects only (Ec and Nb). Significant differences were also observed in the total dead dry biomass of plants between treatments, which gives an indication of plant longevity, leaf turnover or the severity of herbivore damage (Wald's $X^2_{(5)}=48.54$, $P<0.05$) (Figure 3.3).

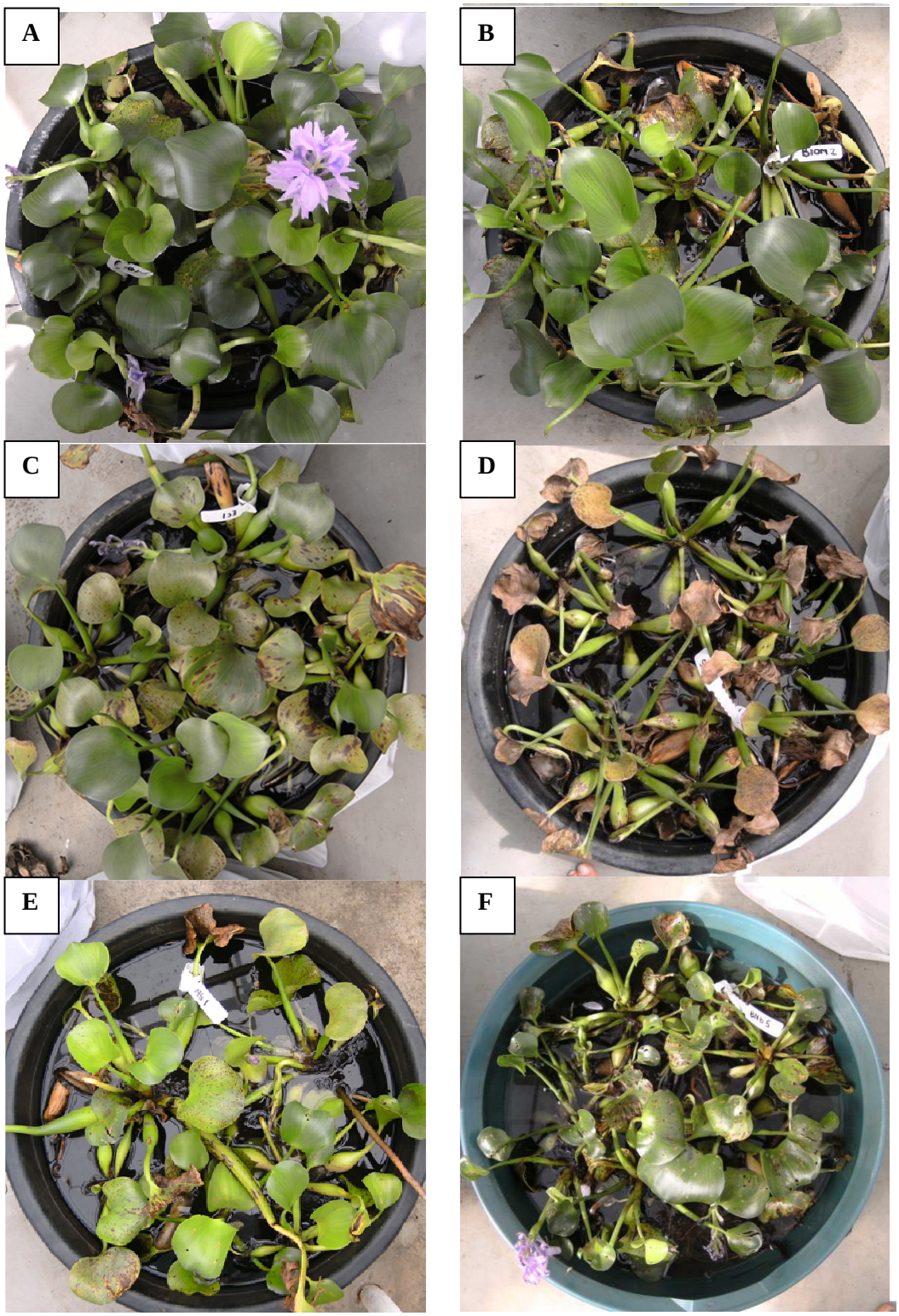

Figure 3.1 Water hyacinth at the end of the BION® bioassay, exhibiting the overall condition of the plants; A) Control plants (dH_2O), B) BION®-only treated plants (BION), C) *Eccritotarsus catarinensis*-only treated plants (Ec), D) BION® and *E. catarinensis* treated plants (BEc), E) *Neochetina bruchi*-only treated plants (Nb), F) and BION® and *Neochetina bruchi* treated plants (BNb).

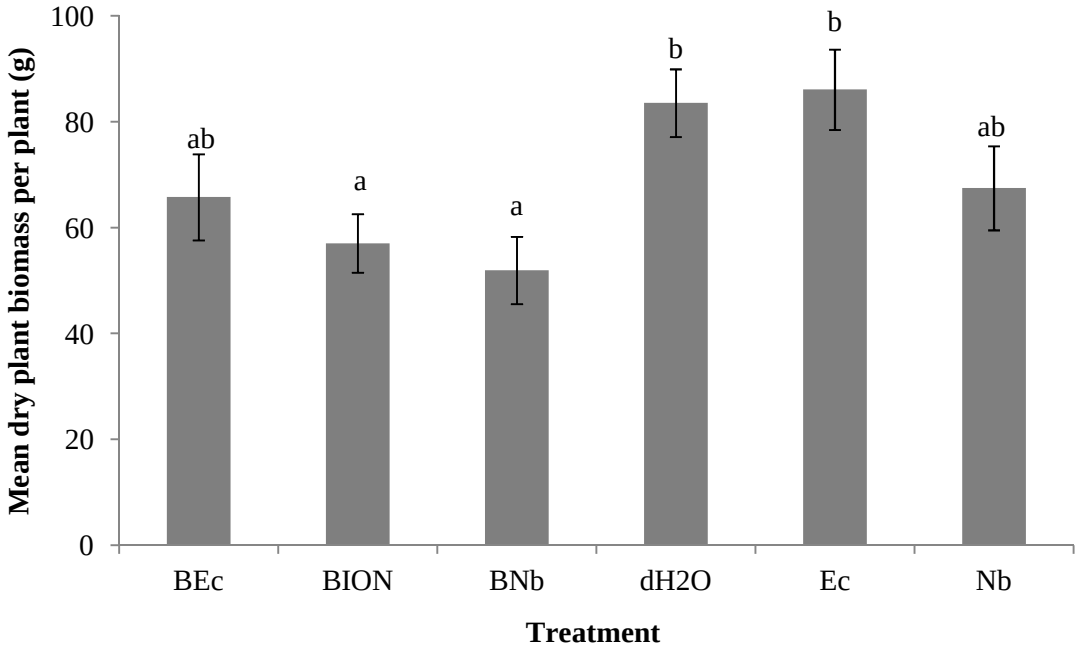

Figure 3.2 Dry biomass of water hyacinth plants in all six treatments (Treatments: BEc = BION® + *E. catarinensis*, BION = BION® only, BNb = BION® + *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only), at the end of the sampling period. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, $P < 0.05$ (n=6 plants/treatment).

BEc had the highest dead dry biomass compared to the other five treatments, and was significantly higher than Ec. This suggests that the lower levels of JA in BEc plants have compromised the ability for water hyacinth to defend itself against the mirid and maintain growth. On the other hand, BNb did not differ from Nb. Dead plant material produced of BION®-treated plants was significantly less than the dead plant material for BION®-treated plants exposed to the mirid, most likely due to weevil larvae removing large amounts of internal plant tissue in the petioles.

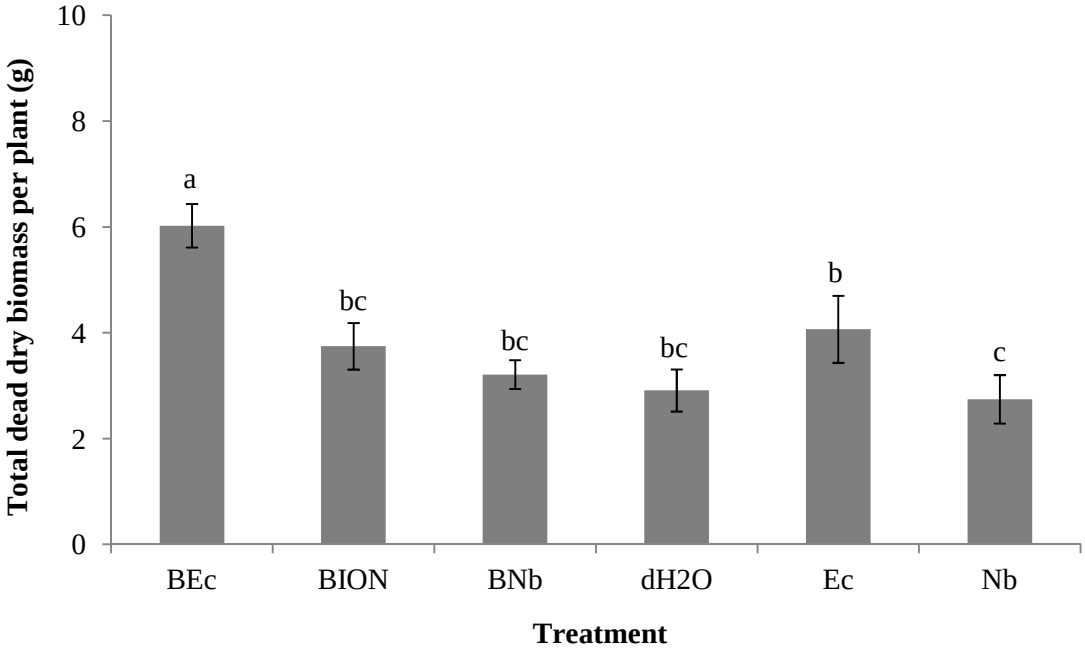

Figure 3.3 Dead dry biomass of water hyacinth plants in all six treatments (Treatments: BEc = BION® + *E. catarinensis*, BION = BION® only, BNb = BION® + *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only), at the end of the sampling period. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, P <0.05 (n=6 plants/treatment).

3.3.2.2 Number of functional leaves and leaf turnover

At the outset of the experiment, each plant had a mean number of six leaves, which changed significantly from their starting points by the end of the experiment (Wald's $X^2_{(1)}$=27.67, P <0.05) (Figure 3.4). Differences in the number of leaves were observed within treatments between sampling weeks (Wald's $X^2_{(1)}$=52.96, P <0.05) and between treatments within sampling weeks (Wald's $X^2_{(5)}$=85.89, P <0.05). Significant differences between the six treatments were observed at Week 4 (Wald's $X^2_{(1)}$=13.25, P <0.05). The largest differences in leaf number were observed between treatments at the end of the experiment (Wald's $X^2_{(5)}$=51.77, P <0.05). The number of leaves produced in treatments Ec, Nb, and dH_20 increased significantly from 6 to a mean of 9 leaves per plant at the end of the trial. BION® treated plants had significantly fewer functional leaves towards the end of the trial, in

comparison to dH_2O, Ec, and Nb. The number of leaves produced by treatments BNb and BION at the end of the trial did not differ from Week 0, indicating no increase in leaf production. Treatment BEc exhibited a decline in the number of leaves from Week 5, from a mean of 8 leaves to a mean of 4 leaves per plant by the end of the trial. BEc had the lowest number of leaves at Week 8.

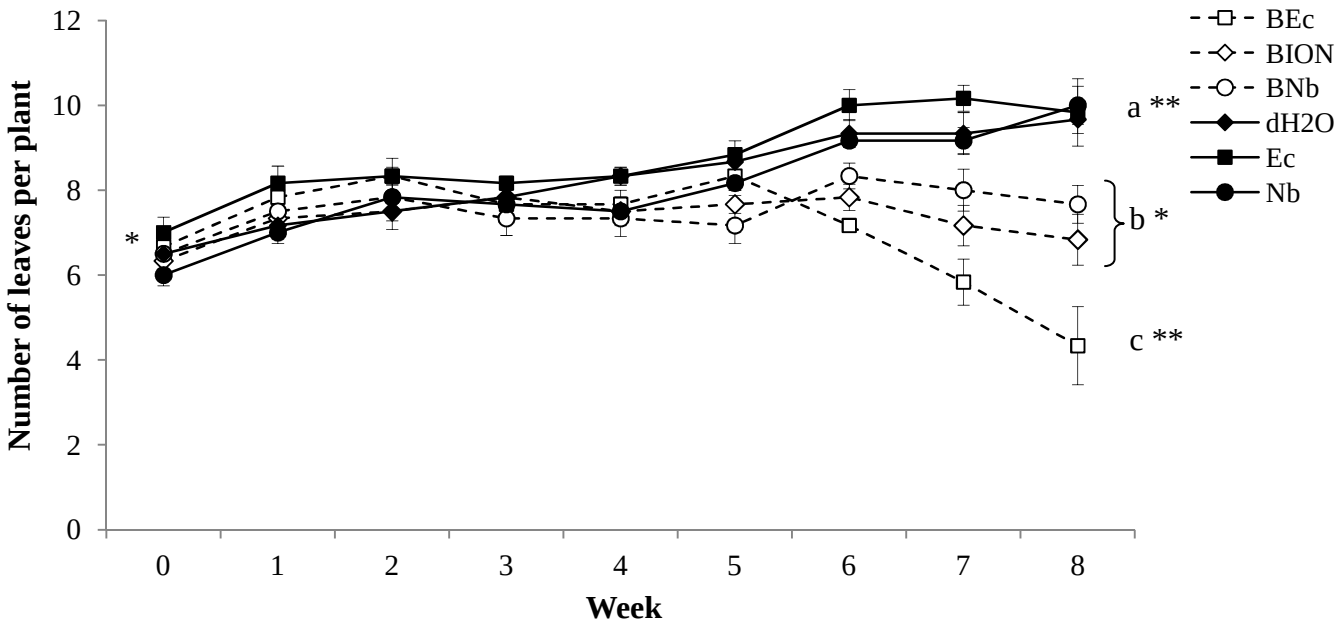

Figure 3.4 Number of functional leaves of each water hyacinth plant at each week throughout the sampling period for plants in all treatments (BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only). Means followed by the same letter at Week 8 are not significantly different, P <0.05. Means followed by the same number of asterisks at Week 0 and Week 8 are not significantly different (n=6 plants/treatment).

The leaf turnover for all treatments throughout the trial demonstrated similar trends (Figure 3.5). However at Week 8, there was a significant decline in turnover of leaves for BEc in comparison to the remaining treatments, which had general increases in leaf turnover (Wald's $X^2_{(5)}$=12.01, P <0.05). Differences between sampling weeks were observed (Wald's $X^2_{(1)}$=5.5120, P <0.05); however, there was no difference in the rate of change between treatments (Wald's $X^2_{(5)}$=4.39, P =0.51).

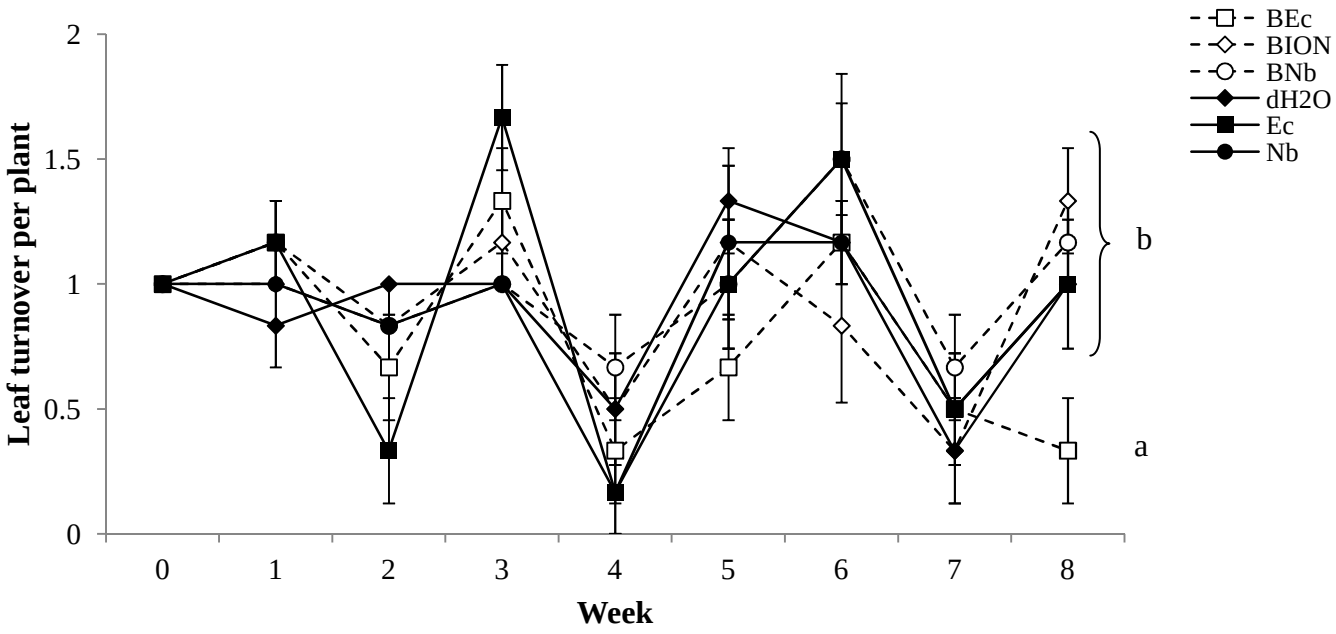

Figure 3.5 Number of water hyacinth leaves that turned over at each week, over the nine week sampling period, for plants in all treatments. Error bars represent the standard error of the mean. Means followed by the same letter at Week 8 are not significantly different, P <0.05 (n=6 plants/treatment).

3.3.2.3 Length of petiole 2

Over the trial period, differences in the length of petiole 2 were observed among treatments within sampling weeks (Wald's $X^2_{(5)}$=104.12, P <0.05), but no differences were observed within treatments between sampling weeks (Wald's $X^2_{(1)}$=1.62, P =0.20). The mean length of petiole 2 for all plants within all treatments initially measured 17.22±0.58cm (mean ± se) and a general decline in length for all treatments was observed up to Week 3 (Figure 3.6). Significant changes between the six treatments were observed from Week 3 (Wald's $X^2_{(5)}$=15.77, P <0.05). Week 8 showed the largest differences between treatments, where the lengths of petiole 2 in treatments BEc, BION, and Ec were significantly longer than Nb, BNb, and dH_20 (Wald's $X^2_{(5)}$=47.68, P <0.05).

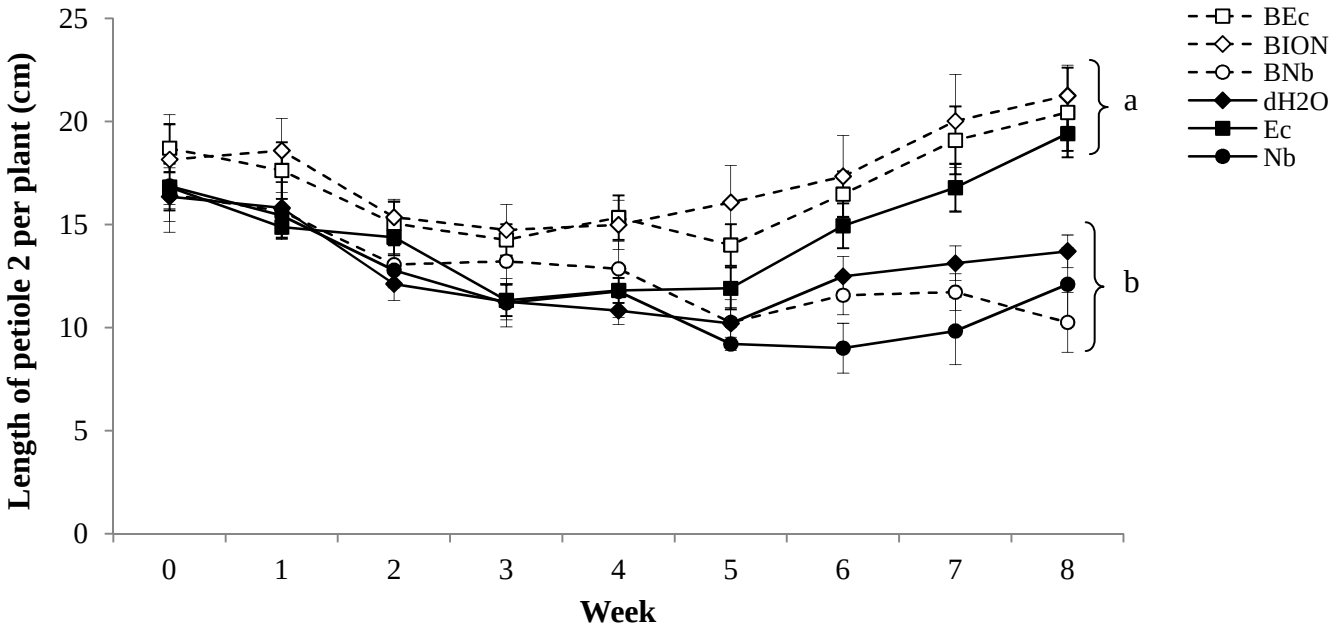

Figure 3.6 Changes in length of leaf 2 petiole over 9 weeks, when water hyacinth plants were exposed to the various treatments. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error of the mean. Means followed by the same letter at Week 8 are not significantly different, P <0.05 (n=6 plants/treatment).

3.3.2.4 Number of flowers

All plants within all treatments began to flower at Week 4 (Figure 3.7) and differences between sampling weeks (Wald's $X^2_{(1)}$=30.31, P <0.05) and treatments (Wald's $X^2_{(5)}$=18.71, P <0.05) were observed. There were no significant differences between treatments at Week 8 (Wald's $X^2_{(5)}$=10.76, P =0.06).

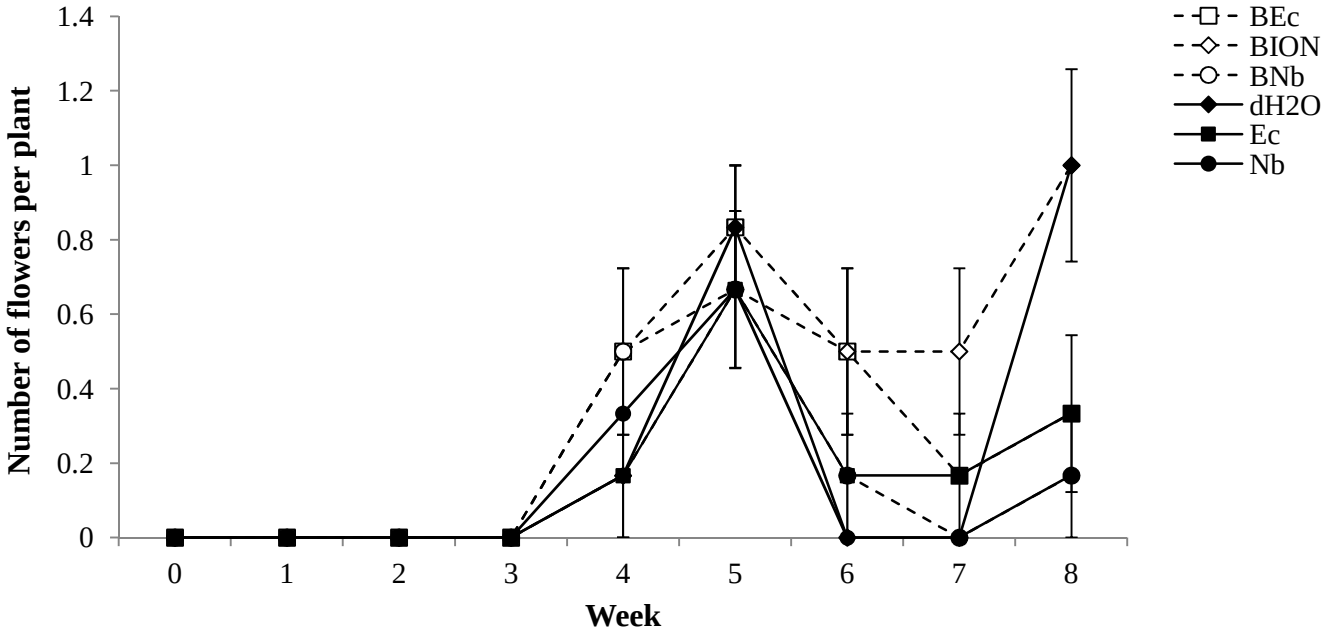

Figure 3.7 The number of flowers produced each week, over the nine week sampling period, for plants in all treatments. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean (n=6 plants/treatment).

3.3.2.5 Chlorophyll content

The initial relative chlorophyll content for all leaves within all treatments was high and declined as the experiment progressed (Figure 3.8). Differences were observed between sampling weeks (Wald's $X^2_{(1)}=5339$, P <0.05), between treatments (Wald's $X^2_{(5)}=32$, P <0.05) and between the four leaf positions (Wald's $X^2_{(3)}=1230$, P <0.05) over the trial period. The relationship between treatment and leaf position was significant (Wald's $X^2_{(15)}=84$, P <0.05).

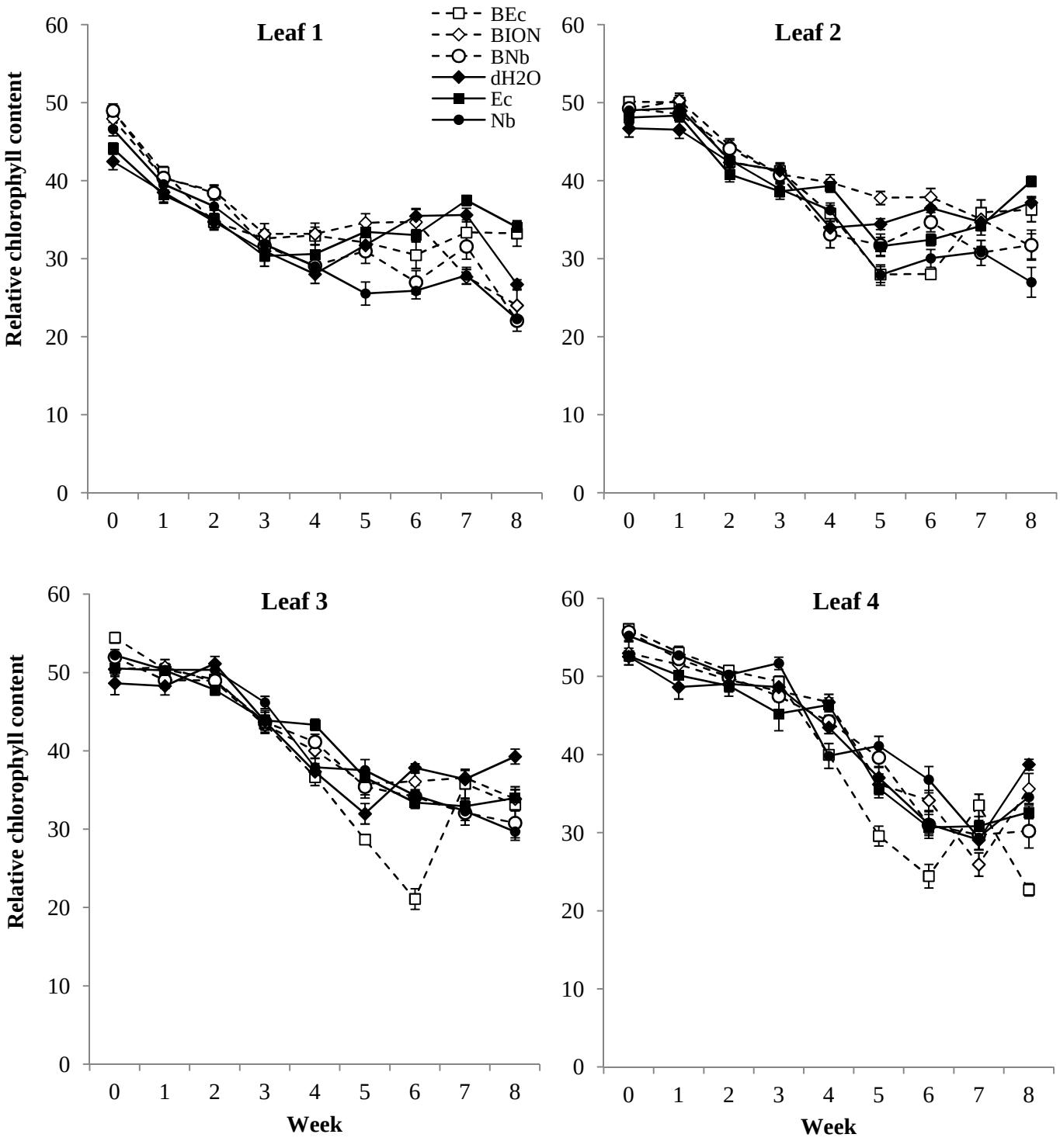

Figure 3.8 Changes in the relative chlorophyll content of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, over 9 weeks. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean (n=6 plants/treatment).

Leaves 1, 2, 3 and 4 exhibited differences between sampling weeks and treatments (Table 3.3). Significant changes in relative chlorophyll content between treatments for all leaves occurred from Week 4 and 5. In leaves 2, 3 and 4, treatment BEc exhibited significantly lower levels of chlorophyll at Week 6 in comparison to the remaining treatments, particularly in leaves 3 and 4. In these same leaves, an increase in chlorophyll content for BEc was observed a week later, followed by another decline, while the other treatments increased or levelled off in chlorophyll content.

Table 3.3 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the relative chlorophyll content data, exhibiting significant differences between sampling weeks and treatments for leaves 1 to 4. *P*-values in bold indicate significant differences between means.

	Sampling weeks		**Treatments**	
Leaf	$\mathbf{X^2_{(1)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**
1	647.6	**<0.001**	38.7	**<0.001**
2	1201.5	**<0.001**	37.6	**<0.001**
3	1677.6	**<0.001**	17.6	**<0.001**
4	1903.9	**<0.001**	20	**<0.001**

At the end of the trial (Week 8), the total chlorophyll content significantly differed between treatments for all leaf positions (Wald's $X^2_{(5)}$=66.7, P <0.05). Weevil treated plants (BNb and Nb) did not differ from each other, but were significantly lower in chlorophyll content than the other treatments. Treatments BEc and Ec differed from each other, with treatment Ec having higher chlorophyll content. Interestingly, treatments Ec and dH_2O were not statistically different, suggesting water hyacinth's photosynthetic productivity is

uncompromised when exposed to herbivory by *E. catarinensis.* Treatment BION was significantly lower in chlorophyll content than the control treatment.

Significant differences were observed between treatments for all leaves at the end of the trial (Table 3.4). In leaf 1, treatments BEc and Ec were not different from each other, but were significantly higher in chlorophyll content compared to the remaining treatments, and were significantly different from dH_2O. Treatments BNb and Nb were not different from each other, but were significantly different from dH_2O. No differences in chlorophyll content between treatments dH_2O and BION were observed in leaf 1. In leaf 2, treatment Nb had a significantly lower chlorophyll content compared to BNb and the remaining treatments, whereas Ec had a significantly higher content compared to BEc and the remaining treatments. Treatment dH_2O was significantly higher in leaf 2 chlorophyll content than treatment BION. In leaf 3, dH_2O was significantly higher in chlorophyll content compared to the other five treatments, which were all not different from each other. Treatments BNb and Nb were lower in chlorophyll content, although not significantly different. In leaf 4, BEc had significantly lower chlorophyll content compared to EC and the remaining treatments. This may indicate that with unimpeded feeding due to lowered JA levels and the mirid being a phloem feeder, the mirid apparently reduced the level of chlorophyll content in its leaf of preference, leaf 4. Treatments dH_2O and BION were not different in chlorophyll content from each other. Treatment BNb was significantly different from treatment Nb, with BNb being lower in chlorophyll content.

Table 3.4 Mean (± S.E.) relative chlorophyll content of leaves 1 to 4 from all treatments at the end of the trial period (week 8). Means followed by (^{o}) indicate no significant differences between the control treatment (dH_2O) and the remaining treatments (*P* >0.05), ($^{+}$) = significant increases in chlorophyll content, ($^{-}$) = significant decreases in chlorophyll content in relation to dH_2O (n=36). *P*-values in bold indicate significant differences between means.

Treatment	Leaf 1	Leaf 2	Leaf 3	Leaf 4
BEc	33.26 ± 1.63 $^{+}$	36.28 ± 1.52 ^{o}	33.14 ± 1.94 $^{-}$	22.7 ± 0.78 $^{-}$
BION	24.02 ± 2.00 ^{o}	31.60 ± 1.61 $^{-}$	33.90 ± 1.55 $^{-}$	35.61 ± 1.97 ^{o}
BNb	22.06 ± 1.63 ^{o}	31.77 ± 1.92 $^{-}$	30.8 ± 1.90 $^{-}$	30.22 ± 2.17 $^{-}$
dH_2O	26.72 ± 0.60	37.20 ± 0.78	39.28 ± 0.95	38.73 ± 0.68
Ec	34.03 ± 0.79 $^{+}$	39.93 ± 0.68 ^{o}	33.97 ± 1.06 $^{-}$	32.62 ± 0.80 $^{-}$
Nb	22.26 ± 1.54 ^{o}	27.01 ± 1.91 $^{-}$	29.70 ± 1.09 $^{-}$	34.55 ± 0.81 $^{-}$
Source of variation				
$X^2_{(5)}$	65.13	45.7	28.31	45.76
***P*-value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

3.3.2.6 Percentage leaf nitrogen and carbon content, and C:N ratio

Leaf nitrogen and carbon content varied between treatments and leaf positions (Table 3.5). Significant differences were observed in leaf nitrogen content between treatments and between the four leaf positions. Although leaf carbon content did not vary between leaf positions, significant differences were observed among treatments. The interaction between leaf position and treatment was significant for leaf nitrogen content, but was not significant for leaf carbon content; leaf nitrogen content significantly changed with leaf age (or position), but carbon content did not. The ratio of leaf carbon to nitrogen (C:N) was significantly different between leaf positions and between treatments. In addition, leaf C:N ratios changed significantly with leaf position.

Table 3.5 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage nitrogen and carbon content and C:N of water hyacinth leaves. *P*-values in bold indicate significant differences between means, $P < 0.05$.

	Leaf		Treatment		Leaf * Treatment	
Parameter	$X^2_{(3)}$	***P*-value**	$X^2_{(5)}$	***P*-value**	$X^2_{(15)}$	***P*-value**
% Nitrogen	45.62	**<0.001**	71.48	**<0.001**	45.82	**<0.001**
% Carbon	2	0.67	73	**<0.001**	17	0.31
C:N	52.33	**<0.001**	46.72	**<0.001**	44.33	**<0.001**

The application of BION® resulted generally in elevated levels of leaf nitrogen content in all BION® treated plants in comparison to treatment dH_2O (Figure 3.9a), although not all statistically significant. The BION treatment produced leaves with significantly high concentrations of nitrogen. Non-BION® treated plants exposed to herbivory (Ec and Nb) had lower levels of nitrogen in the leaves, but were not different from the control. Treatment BEc resulted in leaves with significantly higher nitrogen content than leaves in treatment Ec. All treatments resulted in significantly lower leaf carbon content than treatment dH_2O, except for treatment BEc, which exhibited a slight increase, although not statistically different (Figure 3.9b). Interestingly, BION® treated plants exposed to herbivory (BEc and BNb) resulted in significantly high leaf carbon content compared to the respective non-BION® treated plants exposed to herbivory (Ec and Nb); however, the BION treatment showed significantly low leaf carbon content compared to the control (dH_2O). Negligible differences were observed in the C:N ratios for all treatments, except for treatment BION, which exhibited a significant decline in C:N ratios (Figure 3.9c). These results suggest that the application of BION®, and the subsequent induction of salicylates, altered the accumulation of macronutrients in water hyacinth leaves, possibly relating to leaf quality plant defences.

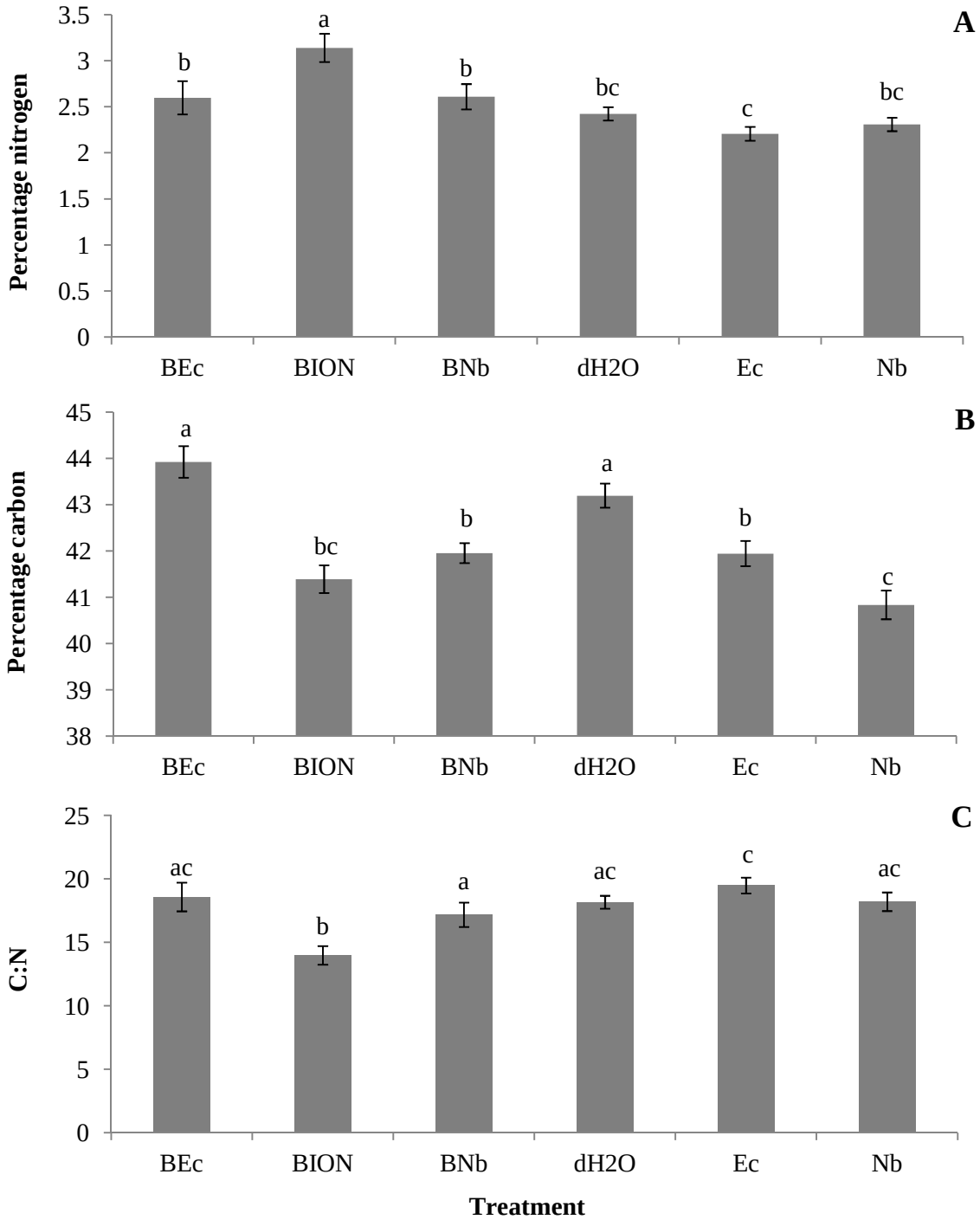

Figure 3.9 a) Mean percentage leaf nitrogen, b) mean percentage leaf carbon and c) mean carbon to nitrogen ratios for all treatments at the end of the experiment. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, P <0.05 (n=24).

Leaf nitrogen content differed significantly between treatments for each leaf position (Table 3.6), with leaf 1 displaying the greatest variation (Figure 3.10). Plants treated with BION® (BEc, BION and BNb) were not different from each other for leaf 1, but were significantly higher in nitrogen content compared to their respective non-BION® treated plants. Treatments dH_2O, Ec and Nb also were not different from each other. Very few differences were observed between treatments for leaf 2, however, the BION treatment was significantly higher in nitrogen content than treatments dH_2O, Ec and Nb. Interestingly, treatment BNb was significantly lower than treatment BION, suggesting the weevils removed the SA-induced nitrogenous compounds in leaf 2. Leaf 3 nitrogen content did not differ greatly between treatments, however, the BION treatment was significantly higher in nitrogen content compared to treatments BEc, BNb, Ec and Nb. Treatment dH_2O did not differ from all the other treatments. In leaf 4, treatments BION and dH_2O were not different from each other; however these treatments exhibited the highest levels of nitrogen content compared to the remaining treatments, although not all significant. No differences were observed between the insect only and insect in combination with BION® treatments on leaf 4. These results suggest that the BION treatment consistently had elevated levels of nitrogen within the leaves. Additionally, the youngest leaves (leaves 1 and 2) had higher allocations of nitrogen, suggesting that when the SA pathway is induced in water hyacinth, nitrogen is increasingly allocated to the younger leaves, perhaps as a form of defence.

In contrast to leaf nitrogen content, leaf carbon content exhibited greater variation between treatments for each leaf (Figure 3.11) and significant differences were observed between treatments for all leaves (Table 3.6). Leaf carbon content was consistently higher in treatment BEc than the other treatments for all leaf positions, except for leaf 4, where treatment dH_2O maintained the highest level of carbon content. The BEc Treatment was higher in carbon content than treatment Ec for all leaf positions, although not statistically

different for leaves 1 and 4. Conversely, treatments BNb and Nb did not vary in leaf carbon content, except for leaf 4, where treatment Nb was significantly lower in carbon content. Treatment BION was significantly lower in carbon content compared to treatment dH_2O for all leaf positions, excluding leaf 2. These results suggest that the application of BION® results in lower leaf carbon content, and when plants sprayed with BION® are exposed to herbivory, carbon content is elevated in the younger leaves (leaf 1 to 3). This was particularly evident for mirid treated plants.

Leaf C:N ratios differed significantly between treatments for all leaf positions, except for leaf 2 (Figure 3.12, Table 3.6). In leaf 1, leaf C:N was significantly lower in all BION® treated plants, suggesting the application of BION® induces increases in leaf nitrogen, which is supported by the percentage nitrogen content obtained for leaf 1. Significant differences between BION® treated plants and the respective non-BION® treated plants were only observed in leaf 1 and as leaves aged, differences between treatments became less consistent with the results obtained for leaf 1. Leaf C:N ratios increased in treatment BION with leaf age, which is supported by the increases in carbon content and decreases in nitrogen content. This suggests that the application of BION® induced carbon-based defences in older leaves, and nitrogen-based defences in younger leaves. Similar to treatment BION, treatments BEc and BNb followed a similar pattern; as leaves aged, the C:N ratios increased. In the control treatment, C:N ratios decreased with leaf age and the C:N ratios of plants solely exposed to herbivory (treatments Ec and Nb) increased with leaf age. The trends observed in the non-BION® treated plants indicate that water hyacinth maintains a relatively high C:N ratio which may be regulated by JA.

Table 3.6 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage nitrogen and carbon content and C:N for each leaf position. *P*-values in bold indicate significant differences between means, $P < 0.05$.

	% Nitrogen		% Carbon		C:N	
Leaf	$X^2_{(5)}$	***P*-value**	$X^2_{(5)}$	***P*-value**	$X^2_{(5)}$	***P*-value**
1	56.27	**<0.001**	17.0	**0.004**	67.19	**<0.001**
2	14.52	**0.01**	25.0	**0.0001**	10.82	0.06
3	19.89	**0.001**	23.7	**0.0002**	12.35	**0.03**
4	17.61	**0.003**	35.1	**<0.001**	19.11	**0.002**

3.3.2.7 Percentage leaf moisture

Significant differences were observed in the percentage leaf moisture between treatments (Wald's $X^2_{(5)}=78.4$, $P < 0.05$) (Figure 3.13), but no differences were observed between leaf positions (Wald's $X^2_{(3)}=6.4$, $P=0.095$). Furthermore, the relationship between leaf position and treatment was not significant (Wald's $X^2_{(15)}=16.4$, $P=0.36$). Treatment dH_2O yielded the greatest leaf moisture, which was significantly higher than treatments BEc, BNb and Ec. Percentage leaf moisture was lower in treatments BNb and BEc; however leaf moisture of treatment BEc was significantly lower than the other five treatments, and was significantly different from Ec. Leaf moisture of treatment BNb was significantly lower than treatment Nb. The mirid had the greatest impact on leaf moisture content in BION® treated plants.

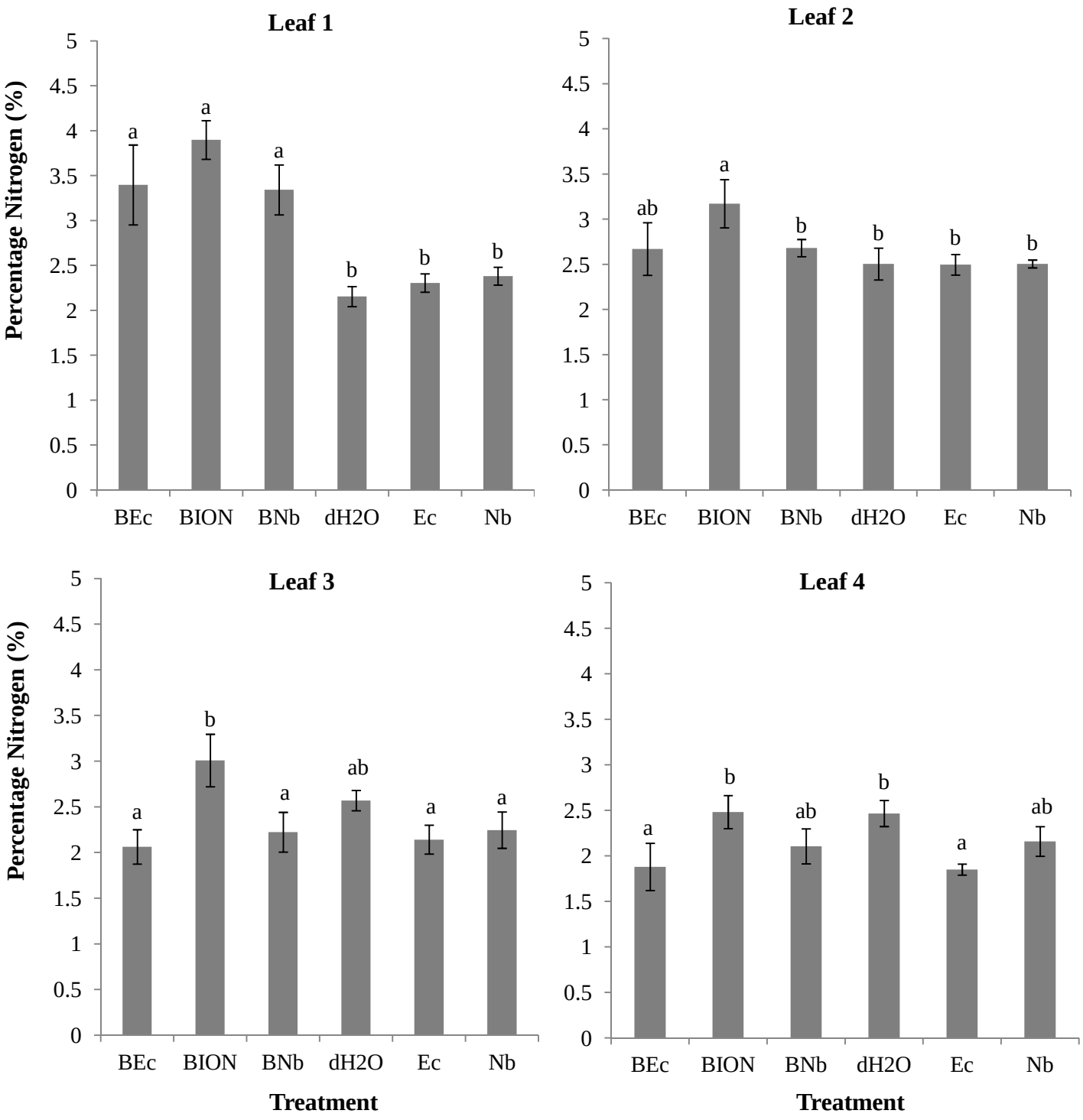

Figure 3.10 Mean percentage leaf nitrogen content for leaves 1 to 4, for all treatments at the end of the experiment. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, P <0.05 (n=6 plants/treatment).

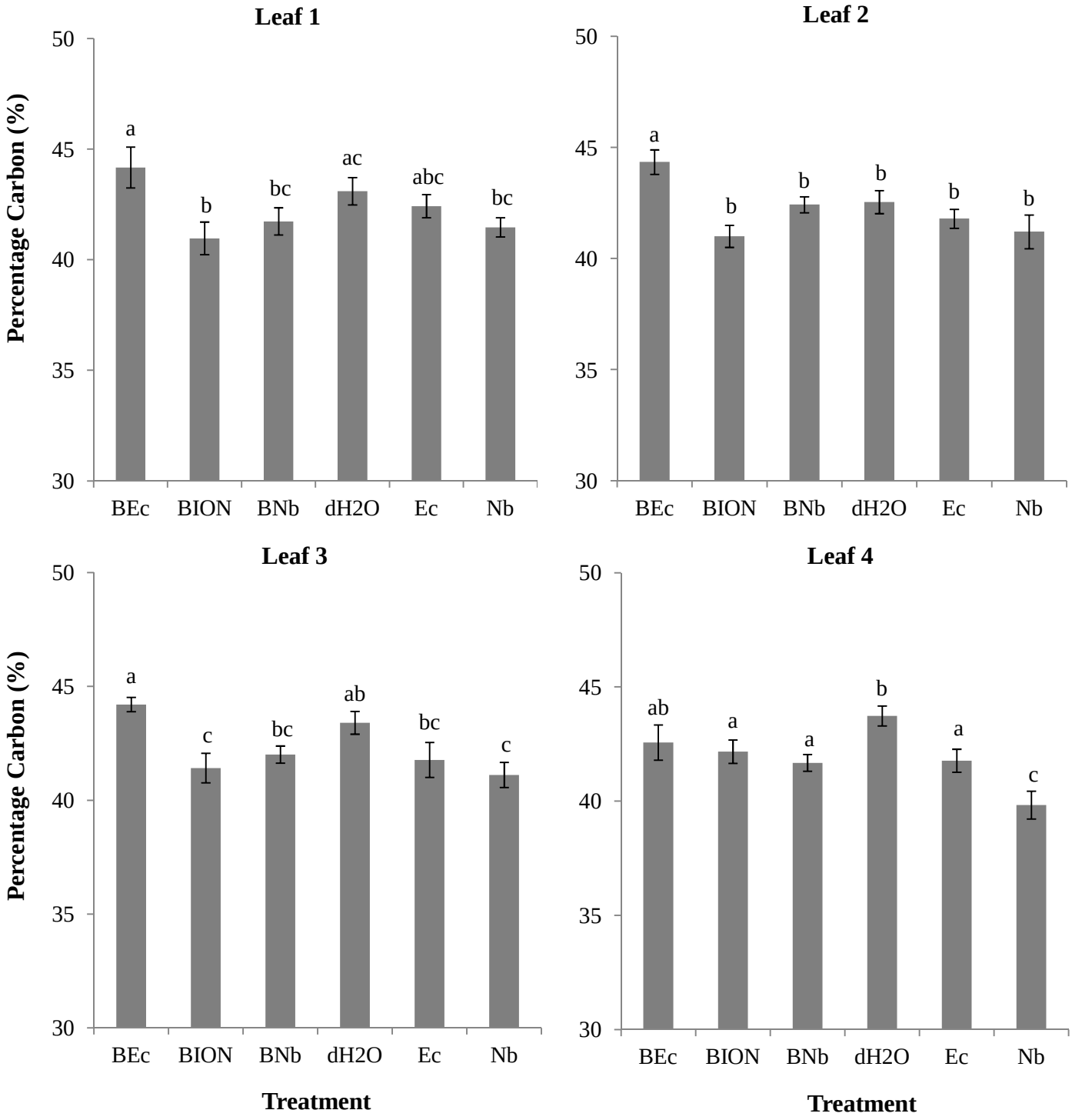

Figure 3.11 Mean percentage leaf carbon content for leaves 1 to 4, for all treatments at the end of the experiment. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, P <0.05 (n=6 plants/treatment).

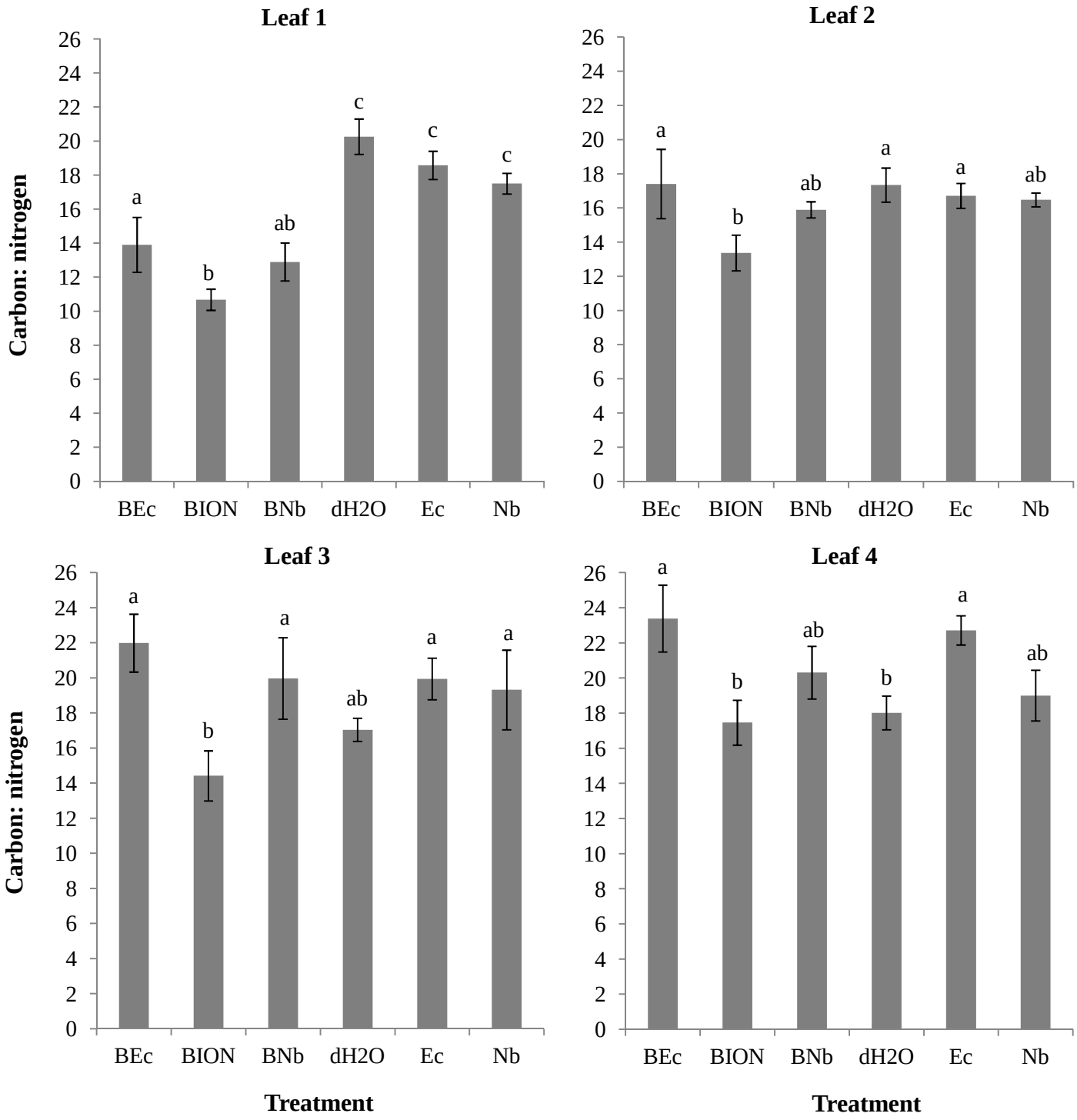

Figure 3.12 Mean carbon to nitrogen ratio for leaves 1 to 4, for all treatments at the end of the experiment. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, $P < 0.05$ (n=6 plants/treatment).

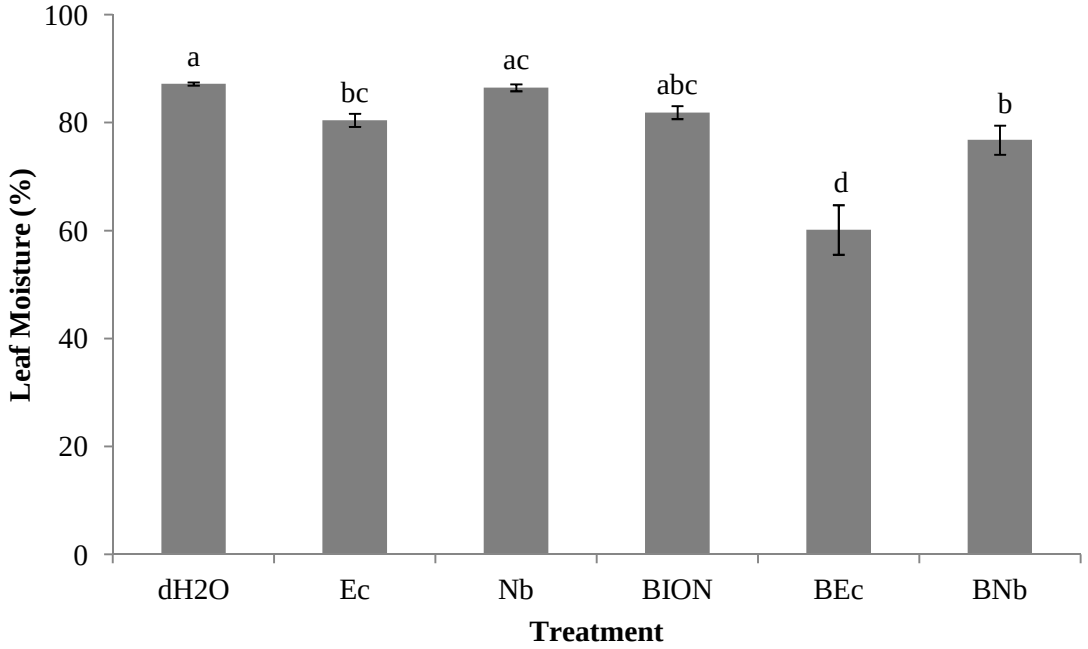

Figure 3.13 Mean percentage leaf moisture for all leaf positions within all treatments at the end of the experiment. Treatments: BEc = BION® and *E. catarinensis*, BION = BION® only, BNb = BION® and *N. bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not significantly different, $P < 0.05$ (n=6 plants/treatment).

3.3.2.8 Investigating relationships between insect and plant parameters

3.3.2.8.1 Relative chlorophyll content vs total dry plant biomass

Strong positive correlations between chlorophyll content and dry plant biomass were only observed for BION® treated plants exposed to *N. bruchi* herbivory (Table 3.7). This finding suggests that in the absence of JA up-regulation, photosynthetic productivity is related to water hyacinth plant growth when exposed to herbivory by the weevil. Reductions in chlorophyll content due to feeding by the weevil will result in reductions in biomass.

Table 3.7 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between the relative chlorophyll content and plant biomass. *P*-values in bold indicate significant positive relationships.

Treatment	Equation	*R*	*P*-value	R^2
BEc	y = 26.61 + 1.40*x	0.40	0.43	0.16
BION	y = 41.88 + 0.60*x	0.44	0.38	0.20
BNb	y = 0.66 + 1.96*x	0.91	**0.01**	0.82
dH_2O	y = 7.40 + 2.23*x	0.48	0.33	0.23
Ec	y = -36.58 + 3.61*x	0.68	0.14	0.47
Nb	y = 75.80 – 0.20*x	-0.03	0.95	0.001

3.3.2.8.2 Relative chlorophyll content vs length of petiole 2

Strong positive correlations between chlorophyll content and 2nd petiole lengths were only observed for non-BION® treated plants exposed to *E. catarinensis* herbivory (Table 3.8), indicating that elongation of the second leaf results in increased water hyacinth chlorophyll content.

Table 3.8 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between the relative chlorophyll content and the length of the second petiole. *P*-values in bold indicate significant positive relationships.

Treatment	Equation	*R*	*P*-value	R^2
BEc	y = -1.78 + 0.69*x	0.75	0.09	0.56
BION	y = 14.40 + 0.22*x	0.60	0.20	0.36
BNb	y = 7.18 + 0.11*x	0.23	0.66	0.05
dH_2O	y = 13.63 + 0.002*x	0.004	0.99	0.00
Ec	y = 2.14 + 0.49*x	0.85	**0.03**	0.72
Nb	y = -13.25 + 0.88*x	0.76	0.13	0.58

3.3.2.8.3 Relative chlorophyll content vs percentage nitrogen content

There was no correlation between the relative chlorophyll content and percentage leaf nitrogen for all treated plants (Table 3.9). However, in the absence of herbivory and BION® (treatment dH_2O), there was a significant positive relationship between chlorophyll and nitrogen content, suggesting that when water hyacinth is exposed to stress, the relationship between nitrogen and chlorophyll content is disturbed.

Table 3.9 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between the relative chlorophyll content and percentage leaf nitrogen content. *P*-values in bold indicate significant positive relationships.

Treatment	Equation	*R*	*P*-value	R^2
BEc	y = 18.36 + 5.64*x	0.50	0.05	0.25
BION	y = 37.37 – 1.94*x	-0.13	0.53	0.018
BNb	y = 37.35 – 3.35*x	-0.24	0.35	0.06
dH_2O	y = 15.27 + 8.34*x	0.45	**0.03**	0.21
Ec	y = 28.31 + 3.10*x	0.22	0.30	0.05
Nb	y = 21.31 + 3.19*x	0.15	0.54	0.022

3.3.3 Insect parameters

3.3.3.1 Percentage feeding area

Significant differences in percentage feeding area were evident mainly between the mirid treated plants, BEc and Ec, than the weevil treated plants, BNb and Nb (Table 3.10 and 3.11). Significant differences were observed between sampling weeks, treatments and leaf position for both the mirid and weevil treated plants (Table 3.10).

Percentage feeding area differed significantly between treatments BEc and Ec for all leaf positions (Table 3.11). Significant differences between the two treatments were largely in leaf 4 (Figure 3.15), where at the end of the trial, mean feeding area of treatment BEc was approximately 84% (± 8.00 (± S.E.)), whereas the feeding area of the Ec treatment was 39.2% (± 7.7). This is particularly interesting, as it suggests that with the application of BION®, water hyacinth's ability to defend against herbivory may have been compromised, resulting in increased feeding by the mirid on its leaf of preference. In leaf 2 (Figure 3.14), treatment BEc exhibited greater feeding compared to Ec. Furthermore, a gradual decline in percentage feeding area for leaf 2 for both mirid treatments was observed at week 7. This has been attributed to a decline in insect numbers, reported in section 3.3.3.3.

Table 3.10 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the number of *Neochetina bruchi* feeding scars and the percentage feeding area by the two experimental insects, *Eccritotarsus catarinensis* and *N. bruchi*, for both insect only and insect + BION treated plants. *P*-values in bold indicate significant differences, $P < 0.05$.

	Sampling weeks		**Treatment**		**Leaf**		**Leaf * Treatment**	
	$\mathbf{X^2_{(1)}}$	***P*-value**	$\mathbf{X^2_{(1)}}$	***P*-value**	$\mathbf{X^2_{(3)}}$	***P*-value**	$\mathbf{X^2_{(3)}}$	***P*-value**
Ec % feeding area	155.81	**<0.001**	45.09	**<0.001**	12.30	**<0.001**	5.36	0.15
Nb % feeding area	102.75	**<0.001**	6.19	**0.01**	12.22	**<0.01**	35.32	**<0.001**
Nb feeding scars	191.59	**<0.001**	11.28	**<0.001**	9.35	**0.03**	723.02	**<0.001**

Table 3.11 Means (± S.E.) of the percentage feeding area for leaves 1 to 4 for *Eccritotarsus catarinensis* and *Neochetina bruchi* treated plants and the number of feeding scars for weevil treated plants. Means for a particular leaf position followed by different letters are significantly different, $P < 0.05$.

Parameter		Leaf 1	Leaf 2	Leaf 3	Leaf 4
% Feeding area	**BEc**	19.63 ± 2.43 [a]	24.72 ± 3.34 [a]	25.83 ± 3.45 [a]	30.47 ± 4.04 [a]
	Ec	6.85 ± 0.53 [b]	14.07 ± 1.73 [b]	16.48 ± 1.76 [b]	14.81 ± 1.56 [b]
	$X^2_{(1)}$	13.01	11.19	8.42	36.70
	***P*-value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	BNb	8.98 ± 0.80 [a]	9.4 ± 1.77 [a]	7.22 ± 0.60 [a]	7.87 ± 0.77 [a]
	Nb	9.17 ± 0.91 [a]	7.11 ± 1.21 [a]	6.67 ± 0.55 [a]	5.37 ± 0.43 [b]
	$X^2_{(1)}$	0.08	0.04	0.61	6.07
	***P*-value**	0.77	0.84	0.44	**0.01**
Feeding scars	**BNb**	27.50 ± 2.37 [a]	25.00 ± 2.64 [a]	24.37 ± 2.72 [a]	18.09 ± 2.41[a]
	Nb	22.93 ± 2.41 [b]	23.06 ± 2.61 [a]	20.70 ± 2.40 [b]	16.85 ± 2.47 [a]
	$X^2_{(1)}$	3.87	2.09	6.23	0.52
	***P*-value**	**<0.05**	0.15	**0.01**	0.47

Differences in percentage weevil feeding area between treatments BNb and Nb for each leaf position were not significant, excluding the differences observed in leaf 4, which were significant (Figure 3.17, Table 3.11), namely at Weeks 6 and 7. At the end of the trial, there were no differences between treatments in leaf 2 (Figure 3.16), however the trend suggests that weevil feeding was greater and increasing on plants treated with BION®. Although no significant differences in percentage feeding area were observed between BNb and Nb in the younger leaves (leaf 1 and 2) (Table 3.11), the total area consumed by the weevils was greater in these leaves than the older leaves (Table 3.10 and 3.11). This finding suggests that the weevils showed a preference for the younger leaves.

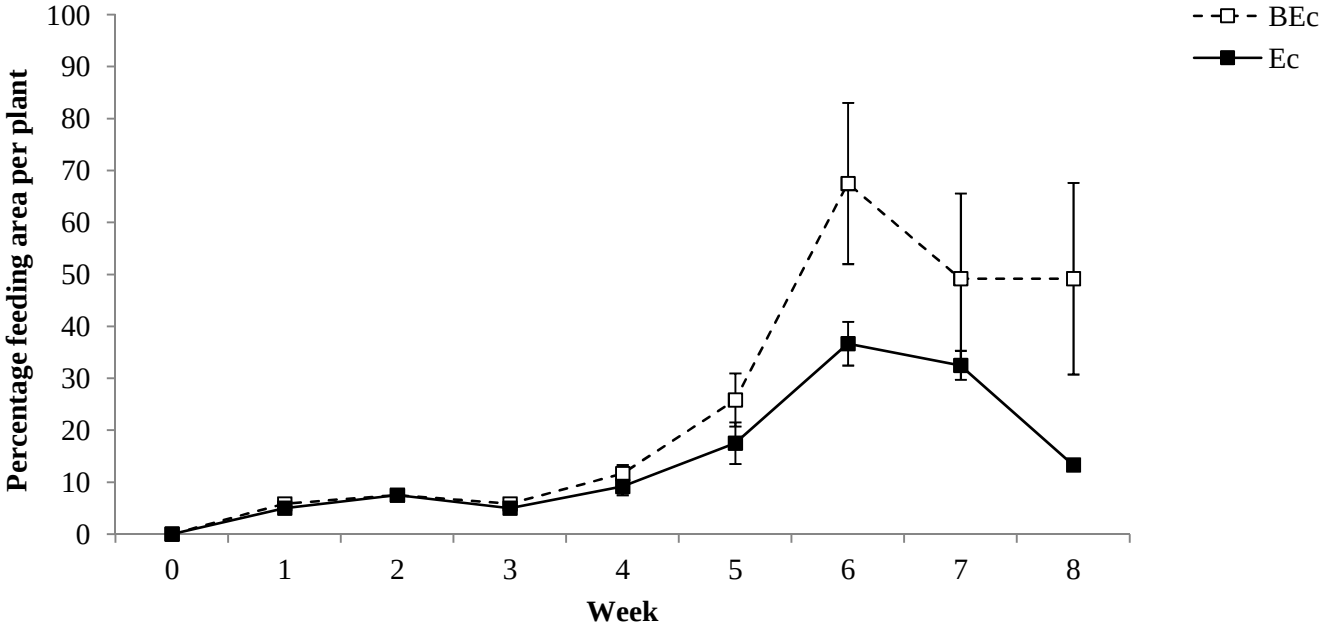

Figure 3.14 The percentage feeding are of *Eccritotarsus catarinensis* on leaf 2 at each week for plants treated with *E. catarinensis* (Ec) and *E. catarinensis* with BION® (BEc). Error bars represent the standard error of the mean (n=6 plants/treatment).

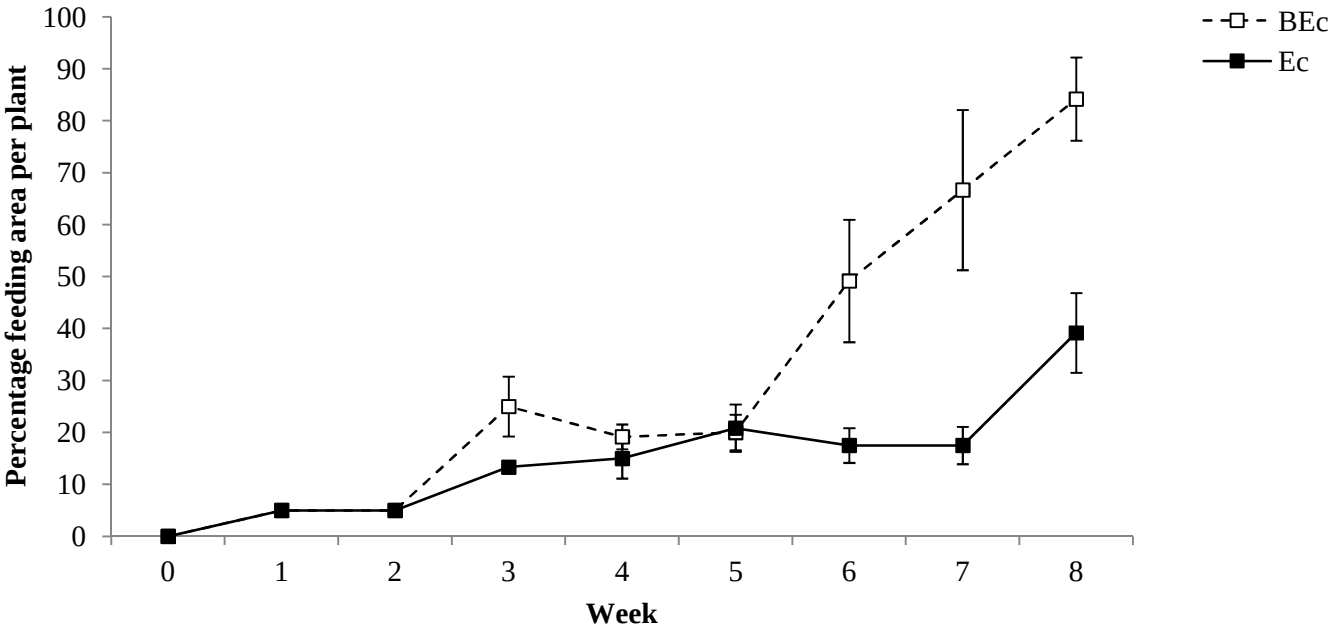

Figure 3.15 The percentage feeding are of *Eccritotarsus catarinensis* on leaf 4 at each week for plants treated with *E. catarinensis* (Ec) and *E. catarinensis* with BION® (BEc). Error bars represent the standard error of the mean (n=6 plants/treatment).

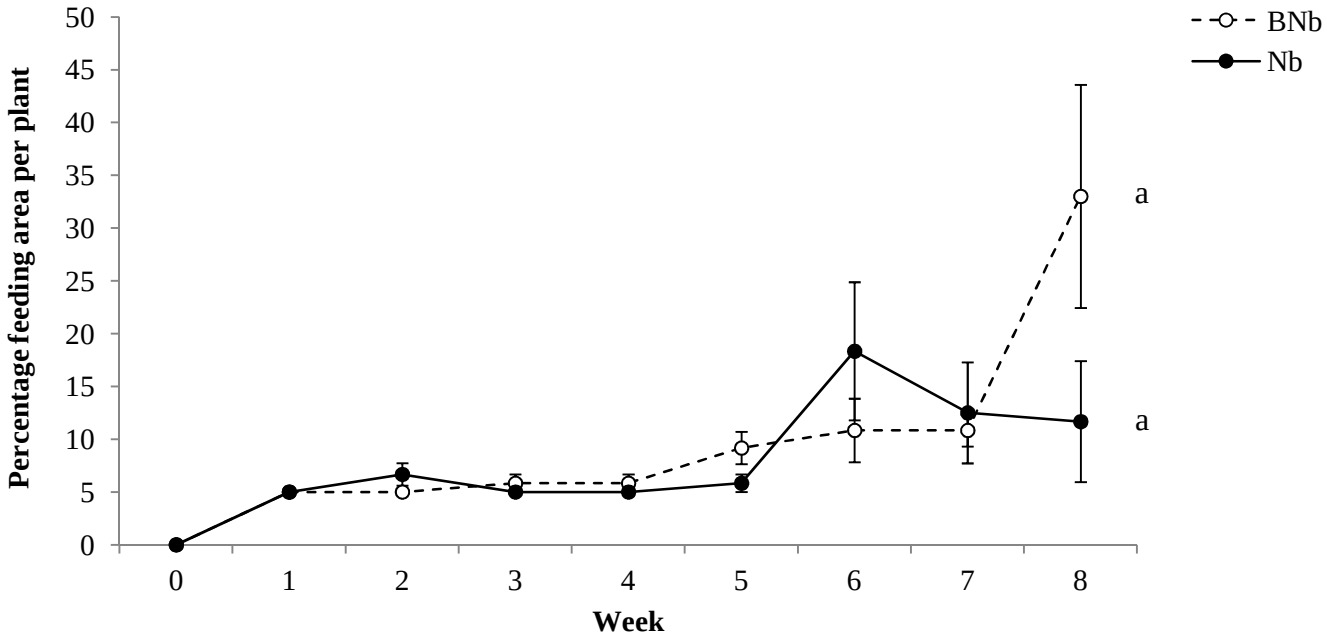

Figure 3.16 The percentage feeding are of *Neochetina bruchi* on leaf 2 at each week for plants treated with *N. bruchi* (Nb) and *N. bruchi* with BION® (BNb). Error bars represent the standard error of the mean (n=6 plants/treatment).

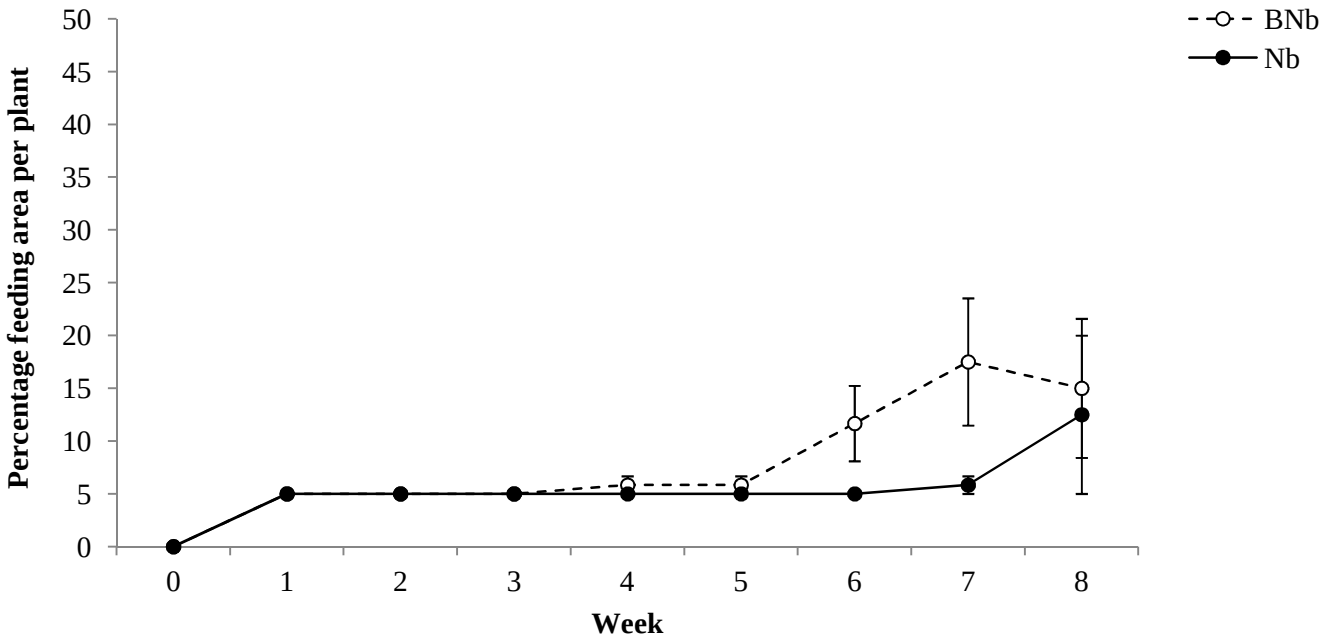

Figure 3.17 The percentage feeding are of *Neochetina bruchi* on leaf 4 at each week for plants treated with *N. bruchi* (Nb) and *N. bruchi* with BION® (BNb). Error bars represent the standard error of the mean (n=6 plants/treatment).

3.3.3.2 Number of adult weevil feeding scars

The number of adult weevil feeding scars was significantly different between sampling weeks, leaf positions, treatments and the interaction between treatment and leaf position (Table 3.10). Treatment BNb typically had a greater number of feeding scars than treatment Nb (Figure 3.18), and at the end of the experiment, the mean number of feeding scars was significantly lower in the Nb treatment compared to BNb (Wald's $X^2_{(1)}$=4.90, P<0.05). Differences in the number of scars between treatments BNb and Nb were only significant for leaves 1 and 3 (Table 3.11, pg 100). Similar to the results obtained for the percentage feeding by the weevils, the number of adult weevil feeding scars was significantly greater in the younger leaves than the old leaves (Table 3.10 and 3.11).

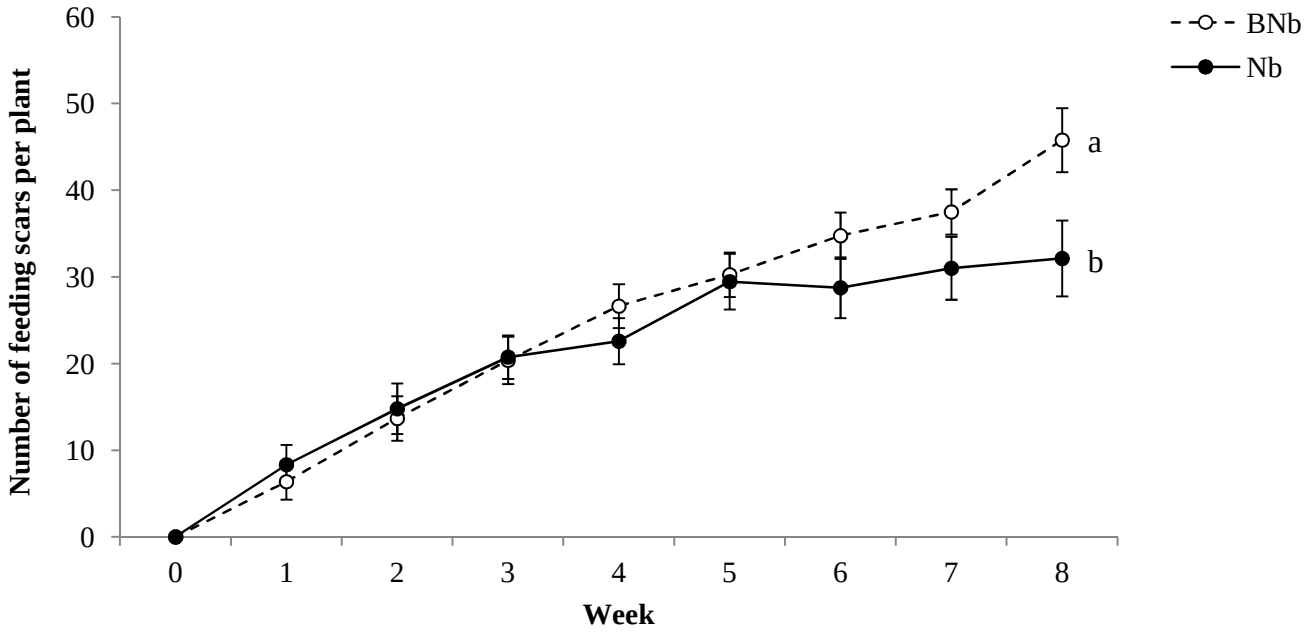

Figure 3.18 Mean number of adult *Neochetina bruchi* weevil feeding scars recorded each week on water hyacinth plants exposed to *N. bruchi* only (Nb) and *N. bruchi* with BION® (BNb). Error bars represent the standard error of the mean. Means at Week 8 followed by the same letter are not significantly different, P >0.05 (n=6 plants/treatment).

3.3.3.3 Palatability index

Mean palatability was significantly different between sampling weeks for *N. bruchi* (Wald's $X^2_{(1)}$=29.09, P<0.05), but did not differ between leaf position (Wald's $X^2_{(3)}$=1.25, P=0.74). At the onset of the trial, the palatability indices for *N. bruchi* were 1 (± 0), indicating very little or no preference between BION® treated plants and non-BION® treated plants, but as the trial progressed, the P.I. indices were greater than 1.00, indicating that a distinct preference for BION® treated plants occurred (Table 3.12). This suggests that salicylic acid-mediated water hyacinth responses produced leaf quality traits that were palatable to the weevil.

Table 3.12 Mean palatability of water hyacinth plants for *N. bruchi* and sampling week. Indices in bold indicate values greater than 1.00 and therefore a preference for BION® treated plants. Values less than 1.00 indicate a preference for non-BION® treated plants and a P.I. of 1.00 indicates no preference between control and treated plants.

Week	Mean P.I. ± se
1	1 ± 0
2	0.94 ± 0.04
3	**1.05 ± 0.03**
4	**1.10 ± 0.04**
5	**1.14 ± 0.08**
6	**1.14 ± 0.10**
7	**1.27 ± 0.11**
8	**1.45 ± 0.13**

3.3.3.4 Insect numbers

Number of Eccritotarsus catarinensis

Significant differences in the number of *E. catarinensis* individuals (adults and nymphs) were observed between sampling weeks (Wald's $X^2_{(1)}$=28.81, P<0.05) (Figure 3.19). The number of *E. catarinensis* increased from the beginning of the trial to about week 5, where a marked decline in individuals was observed towards the end of the trial. The decline in insect numbers from week 5 is likely due the population carrying capacity being met.

Interestingly, treatment BEc accumulated a greater number of *E. catarinensis* from week 3 to week 5 than treatment Ec, and at week 4 a significant difference was observed (Wald's $X^2_{(1)}$=5.408, P<0.05). This suggests that before the carrying capacity was met, *E. catarinensis* produced greater numbers of progeny in treatment BEc than Ec, most likely due to unhindered access to leaf nutrients resulted from the application of BION®. Towards the end of the trial, although insect numbers decreased in both treatments, treatment BEc maintained lower relative insect numbers, most likely due to depleted leaf nutrients from unhindered herbivory and/or limited viable oviposition sites (leaves 1 to 4 were severely damaged or dead). At week 8, treatment Ec accumulated a significantly greater number of *E. catarinensis* than treatment BEc (Wald's $X^2_{(1)}$=5.9.4, P<0.05).

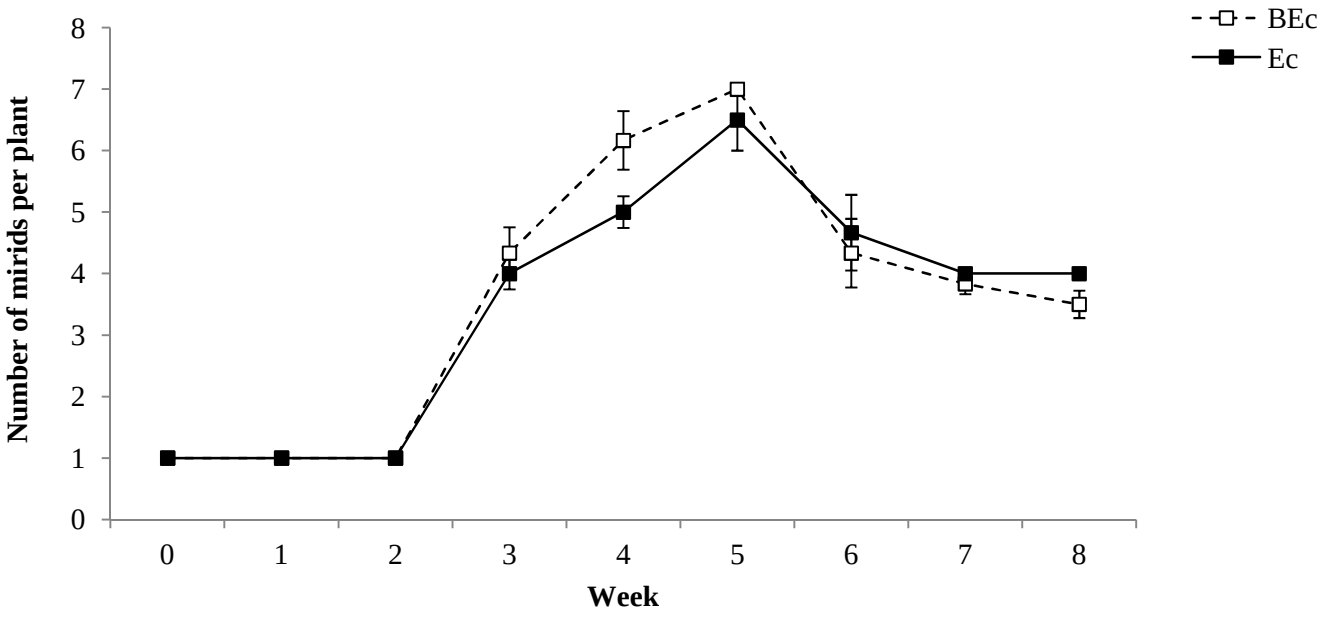

Figure 3.19 Mean number of *Eccritotarsus catarinensis* (adults and nymphs) collected from water hyacinth plants subjected with *E. catarinensis* only (Ec) and *E. catarinensis* sprayed with BION® (BEc) at the end of the trial. Error bars represent the standard error of the mean (n=6 plants/treatment).

Number of Neochetina bruchi

The number of *N. bruchi* individuals (larvae, pupae and adults) collected at the end of the trial did not differ between treatments BNb and Nb (Wald's $X^2_{(1)}$=2.12, *P*=0.15); however, treatment BNb yielded an average of eight more individuals per plant (Figure 3.20a). The mean dry weight of pupae collected at the end of the trial did not differ between treatments BNb and Nb (Wald's $X^2_{(1)}$=0.98, *P*=0.32) (Figure 3.20b).

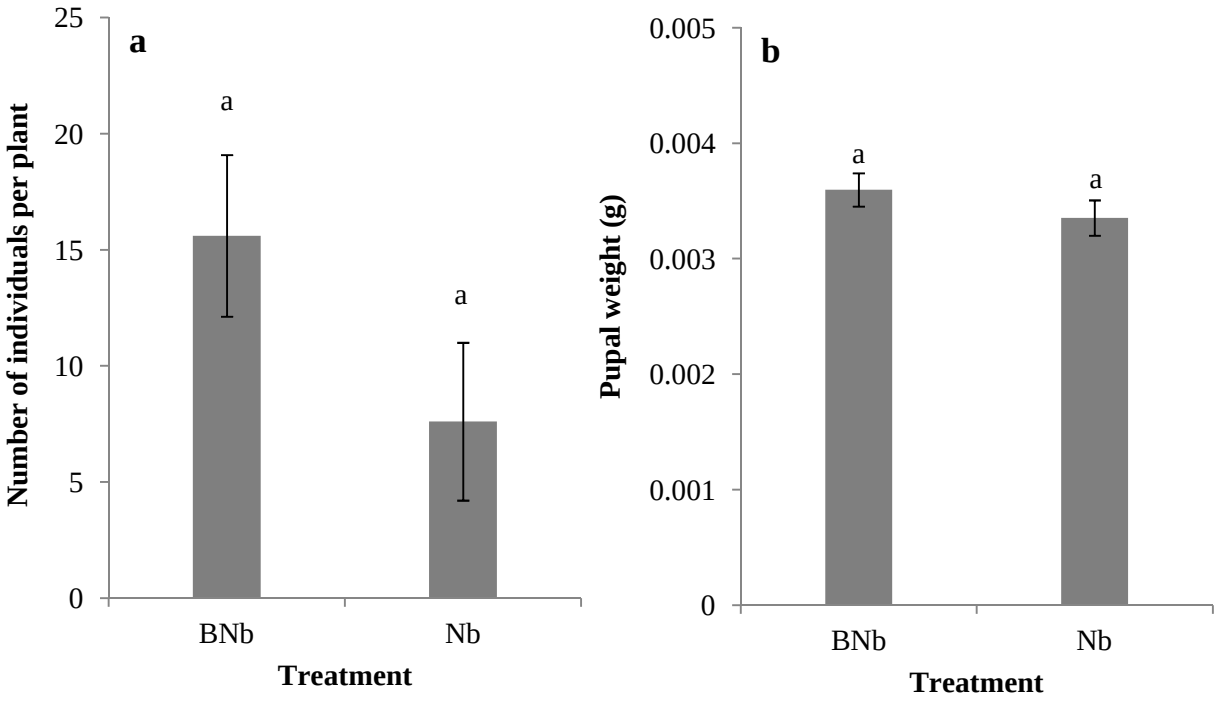

Figure 3.20 a) Mean number of *Neochetina bruchi* immatures (pupae and larvae) per plant and b) mean dry weight of *N. bruchi* pupae collected from water hyacinth plants subjected with *N. bruchi* only (Nb) and *N. bruchi* with BION® (BNb) at the end of the trial. Error bars represent the standard error of the mean. Means followed by the same letter are not significantly different, $P > 0.05$ (n=6 plants/treatment).

3.3.4 Effect of insect herbivory and BION® on water hyacinth plant parameters

Effect of Eccritotarsus catarinensis and BION®

Herbivory by *Eccritotarsus catarinensis* on leaf 2 was not significantly correlated to any of the water hyacinth plant growth parameters, even when plants were sprayed with BION® (Table 3.13). Mirid herbivory on leaf 4 had minimal effect on water hyacinth plant growth (Table 3.14); however, significant correlations between percentage feeding and dead biomass, and the number of leaves produced were found for non-BION® treated plants (Ec). Increases in mirid percentage feeding area correlated with increases in dead plant material for treatment Ec. Surprisingly, no significant relationship between herbivory and dead plant

biomass was observed for treatment BEc. A strong positive correlation was expected to have occurred, especially as treatment BEc accumulated significantly more dead plant biomass at the end of the trial (see 3.3.2.1). Conversely, the number of leaves produced by non-BION® treated plants was negatively correlated with mirid herbivory on leaf 4; as herbivory increased the number of leaves produced deceased. Similar to the results obtained for dead plant material, a strong negative correlation between mirid herbivory and leaf production was expected to have occurred in treatment BEc, as the number of leaves produced by plants in treatment BEc was significantly lower than treatment Ec (see 3.3.2.2). No significant relationships were observed between herbivory and total dry plant biomass, leaf turnover, the lengths of second petioles and flower production. In general, negative relationships were observed for all plant growth parameters, excluding total dead plant biomass.

The majority of the significant correlations observed between mirid herbivory and water hyacinth physiological parameters was found for plants treated with BION® (BEc) (Table 3.15); suggesting the application of BION® amplified the relationship between mirid feeding and water hyacinth physiological parameters. Strong correlations between mirid herbivory and leaf nitrogen content were observed in leaves 2 and 4 for treatment BEc. In leaf 2, decreases in leaf nitrogen content significantly correlated with increases in mirid percentage feeding area. This suggests that with lowered nitrogen content, *E. catarinensis* feeding increased on leaf 2, or increased feeding by the mirid resulted in a lowering of leaf nitrogen. Interestingly, a strong positive correlation was observed between mirid feeding and C:N ratios for leaf 2, possibly indicating that with increased leaf 2 C:N ratios, mirid herbivory increased. An increase in C:N ratios indicates a greater leaf carbon content to nitrogen content, which typically results in unpalatable leaves. Conversely, in leaf 4, increases in nitrogen content significantly correlated with increases in mirid herbivory. This suggests that with increased leaf nitrogen content, *E. catarinensis* feeding increased on leaf 4, suggesting

that the up-regulation of SA-induced nitrogenous based defence compounds did not impact mirid herbivory on leaf 4. Strong negative correlations were found between relative chlorophyll content and mirid herbivory for leaves 1 and 2 for treatment BEc; decreases in chlorophyll content were associated with increases in mirid percentage feeding area. A significant negative relationship also was observed between relative chlorophyll content and mirid herbivory for leaf 3 for treatment Ec; suggesting decreases in chlorophyll content were associated with increases in mirid percentage feeding area. Effectively, increased mirid feeding on leaf 3 resulted in decreased chlorophyll content, which is most likely due to acquisition of phloem from the leaf tissue by *E. catarinensis.* Leaf moisture content was strongly correlated with mirid herbivory for leaves at positions1 to 3in treatment BEc; leaf moisture decreased as percentage feeding area increased. This also is probably due to the increased acquisition of phloem from leaf tissue by the mirid. No significant relationships were observed for percentage leaf carbon content for both BION® and non-BION® treated plants.

Table 3.13 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between mirid herbivory and water hyacinth growth parameters for leaf 2. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Treatment	Equation	*R*	*P*-value	R^2
Total dry biomass	**BEc**	y = 180.80 – 1.83*x	-077	0.07	0.59
	Ec	y = 4.30 + 0.10*x	0.19	0.72	0.04
Total dead biomass	**BEc**	y = 140.43 – 15.15*x	-0.48	0.33	0.23
	Ec	y = 26.13 – 3.15*x	-0.35	0.50	0.12
Number of leaves	**BEc**	y = 111.32 – 14.34*x	-0.67	0.15	0.44
	Ec	y = 6.37 + 0.71*x	0.14	0.79	0.02
Leaf turnover	**BEc**	y = 71.25 – 66.25*x	-0.71	0.12	0.50
	Ec	-	-	-	-
2nd petiole length	**BEc**	y = 194.21 – 7.10*x	-0.78	0.07	0.61
	Ec	y = 27.79 – 0.75*x	-0.15	0.77	0.02
Number of flowers	**BEc**	y = 26.25 + 68.75*x	0.73	0.10	0.54
	Ec	y = 10 + 10*x	0.53	0.28	0.28

Table 3.14 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between mirid herbivory and water hyacinth growth parameters for leaf 4. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Treatment	Equation	*R*	*P*-value	R^2
Total dry biomass	**BEc**	y = 125.31 – 0.57*x	-0.60	0.21	0.35
	Ec	y = 81.42 – 0.47*x	-0.46	0.36	0.21
Total dead biomass	**BEc**	y = 74.09 + 1.67*x	0.13	0.80	0.02
	Ec	y = -19.61 + 14.46*x	0.83	**0.04**	0.69
Number of leaves	**BEc**	y = 111.25 – 6.25*x	-0.72	0.11	0.52
	Ec	y = 117.92 – 8.01*x	-0.83	**0.04**	0.68
Leaf turnover	**BEc**	y = 90 – 17.5*x	-0.46	0.36	0.21
	Ec[i]	-	-	-	-
2nd petiole length	**BEc**	y = 108.85 – 1.21*x	-0.33	0.53	0.11
	Ec	y = 166.95 – 6.59*x	-0.71	0.11	0.51
Number of flowers	**BEc**	y = 86.25-6.25*x	-0.16	0.76	0.03
	Ec	y = 40 – 2.5*x	-0.07	0.90	0.004

[i] Insufficient replicates to perform the simple regression method

Table 3.15 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between mirid herbivory and water hyacinth physiological parameters. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Leaf	Treatment	Equation	*R*	*P*-value	R^2
Nitrogen content	**1**	**BEc**	y = 108.87 – 23.80*x	-0.62	0.27	0.38
		Ec	y = -0.11 + 2.58*x	0.32	0.54	0.10
	2	**BEc**	y = 215.34 – 70.81*x	-0.97	**0.03**	0.94
		Ec	y = 32.30 7.50*x	-0.23	0.67	0.05
	3	**BEc**	y = 149.50 – 55.52*x	-0.56	0.44	0.331
		Ec	y = -29.27 + 29.24*x	0.45	0.37	0.21
	4	**BEc**	y = 7.99 + 32.13*x	1.00	**0.04**	0.99
		Ec	y = 40.67 – 0.81*x	-0.006	1.00	0.001
C:N ratio	**1**	**BEc**	y = -64.11 + 6.63*x	0.68	0.21	0.46
		Ec	y = 10.50 – 0.25*x	-0.25	0.63	0.06
	2	**BEc**	y = -122.03 + 8.52*x	1.00	**0.004**	0.99
		Ec	y = 2.40 + 0.65*x	0.12	0.82	0.01
	3	**BEc**	y = -76.02 + 5.05*x	0.55	0.45	0.30
		Ec	y = 122.57 – 4.47*x	-0.52	0.29	0.27
	4	**BEc**	y = 140.07 – 3.07*x	-0.99	0.09	0.98
		Ec	y = 83.93 – 1.97*x	-0.21	0.69	0.05
Chlorophyll content	**1**	**BEc**	y = 185.85 – 4.39*x	-0.90	**0.01**	0.82
		Ec	y = 3.85 + 0.06*x	0.11	0.83	0.01
	2	**BEc**	y = 223.06 – 4.80*x	-0.89	**0.02**	0.79
		Ec	y = 14.58 – 0.03*x	-0.01	1.00	0.0001
	3	**BEc**	y = 92.64 – 1.35*x	-0.33	0.59	0.11
		Ec	y = 173.59 – 4.13*x	-0.95	**0.004**	0.90
	4	**BEc**	y = 132.34 – 2.82*x	-0.08	0.95	0.006
		Ec	y = 132.15 – 2.85*x	-0.68	0.14	0.46
Leaf Moisture	**1**	**BEc**	y = 199.06 – 2.36*x	-0.90	**0.02**	0.80
		Ec	y = -0.70 + 0.08*x	0.17	0.74	0.03
	2	**BEc**	y = 165.84 – 2.08*x	-0.92	**0.009**	0.84
		Ec	y = 39.90 – 0.33*x	-0.14	0.79	0.02
	3	**BEc**	y = 118.15 – 1.22*x	-0.85	**0.03**	0.71
		Ec	y = 86.39 – 0.66*x	-0.11	0.83	0.01
	4	**BEc**	y = 115.71 – 0.28*x	-0.30	0.81	0.089
		Ec	y = 90.90 – 0.67*x	-0.32	0.53	0.11

Effect of Neochetina bruchi and BION®

Herbivory on leaf 2 by *N. bruchi* also was not significantly correlated with any of the water hyacinth plant growth parameters, even when plants were sprayed with BION®, except for leaf turnover, where a strong positive correlation was found between weevil herbivory on leaf 2 and leaf turnover for plants treated with BION® (BNb) (Table 3.16). This relationship was not anticipated as increases in herbivory were expected to decrease the rate of leaf production, even more so when insect defences were inhibited through the application of BION®. Increased weevil percentage feeding on leaf 4 was significantly correlated with increases in total dead plant biomass and the length of the second petiole for plants treated with BION® (BNb) (Table 3.17). The significant positive relationship observed between weevil herbivory and dead plant material was predicted for treatment BNb, however, the significant positive relationship between weevil herbivory and petiole 2 lengths was not expected; weevil herbivory typically stunts water hyacinth growth (Center *et al*., 1999, Heard and Winterton, 2000). The positive correlation between increased weevil herbivory and increased petiole lengths may be a response induced by the application of BION®.

Very few significant relationships were found between weevil percentage feeding and water hyacinth physiological parameters (Table 3.18). A strong correlation was observed between leaf 2 nitrogen content and weevil herbivory for non-BION® treated plants (Nb); increased weevil feeding correlated to increased levels of leaf 2 nitrogen. This suggests that water hyacinth may respond to weevil herbivory with elevated nitrogen levels, which are likely to be defence related. This relationship was predicted, particularly for leaf 2, as weevil herbivory is concentrated on younger leaves. In addition to nitrogen content, relative chlorophyll content for leaf 2 also was strongly correlated with weevil herbivory; however this relationship was observed only for plants treated with BION® (BNb). Presumably, this

observation is a result of water hyacinth's response to the application of BION® and not herbivory, as it is a positive response. Weevil herbivory on leaf 4 strongly correlated with leaf moisture for plants treated with BION®. This is most likely due to adult weevil feeding causing water hyacinth leaves to dry out (Oberholzer, 2001), however, this relationship was predicted to be more evident in the younger leaves, where weevil feeding is more predominant.

Table 3.16 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between weevil herbivory and water hyacinth growth parameters for leaf 2. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Treatment	Equation	*R*	*P*-value	R^2
Total dry biomass	**BNb**	y = -2.14 + 0.42*x	0.25	0.63	0.06
	Nb	y = 10.45 + 0.06*x	0.071	0.89	0.01
Total dead biomass	**BNb**	y = 46.72 – 8.06*x	-0.34	0.51	0.11
	Nb	y = 16.92 – 0.70*x	-0.03	0.96	0.001
Number of leaves	**BNb**	y = 50.45 – 3.86*x	-0.18	0.74	0.03
	Nb	y = -43.33 + 5.83*x	0.36	0.48	0.13
Leaf turnover	**BNb**	y = -101.12 + 369.13*x	0.99	**0.0001**	0.99
	Nb	y = 15.60 – 2.15*x	-0.02	0.97	0.0004
Leaf 2 petiole length	**BNb**	y = 53.11 – 1.96*x	-0.23	0.66	0.05
	Nb	y = -15.45 + 2.60*x	0.58	0.30	0.34
Number of flowers	**BNb**	y = 23 - 13*x	-0.20	0.71	0.04
	Nb	y = 17 - 12*x	-0.28	0.59	0.08

Table 3.17 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between weevil herbivory and water hyacinth growth parameters for leaf 4. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Treatment	Equation	*R*	*P*-value	R^2
Total dry biomass	**BNb**	y = -12.43 + 0.50*x	0.46	0.36	0.21
	Nb	y = 8.41 + 0.06*x	0.06	0.91	0.004
Total dead biomass	**BNb**	y = -34.06 + 15.28*x	0.97	**0.001**	0.95
	Nb	y = -0.23 + 4.64*x	0.17	0.75	0.03
Number of leaves	**BNb**	y = 41.14 – 3.41*x	-0.23	0.65	0.06
	Nb	y = -62.5 + 7.5*x	0.45	0.37	0.20
Leaf turnover	**BNb**	y = 37.51 – 68.15*x	-0.28	0.59	0.08
	Nb	y = 10.29 + 7.87*x	0.07	0.90	0.004
Leaf 2 petiole length	**BNb**	y = -60.98 + 4.62*x	0.83	**0.04**	0.69
	Nb	y = -15.38 + 2.43*x	0.53	0.36	0.28
Number of flowers	**BNb**	y = 17 - 12*x	-0.28	0.59	0.08
	Nb	y = 14 - 9*x	-0.20	0.70	0.04

Table 3.18 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationships between weevil herbivory and water hyacinth physiological parameters. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

Parameter	Leaf	Treatment	Equation	*R*	*P*-value	R^2
Nitrogen content	**1**	**BNb**	y = 24.34 – 3.09*x	-0.23	0.70	0.05
		Nb[i]	-	-	-	-
	2	**BNb**	y = -8.06 + 6.74*x	0.32	0.68	0.10
		Nb	y = -450.24 + 186.14*x	0.93	**0.02**	0.86
	3	**BNb**	y = 9.33 + 0.75*x	0.18	0.77	0.03
		Nb	y = -0.78 + 4.80*x	0.23	0.66	0.05
	4	**BNb**	y = 97.48 – 38.02*x	-0.79	0.21	0.62
		Nb	y = 42.18 – 13.76*x	-0.30	0.57	0.09
C:N ratio	**1**	**BNb**	y = 3.95 + 0.78*x	0.26	0.67	0.07
		Nb[i]	-	-	-	-
	2	**BNb**	y = 17.62 – 0.48*x	-0.14	0.86	0.02
		Nb	y = 252.82 – 14.38*x	-0.73	0.16	0.54
	3	**BNb**	y = 12.93 – 0.10*x	-0.25	0.69	0.06
		Nb	y = 18.49 – 0.44*x	-0.24	0.64	0.06
	4	**BNb**	y = -68.66 + 4.24*x	0.84	0.16	0.71
		Nb	y = -1.08 + 0.71*x	0.14	0.79	0.02
Chlorophyll content	**1**	**BNb**	y = 22.23 – 0.10*x	-0.04	0.94	0.001
		Nb[i]	-	-	-	-
	2	**BNb**	y = -5.46 + 0.49*x	0.99	**0.01**	0.98
		Nb	y = -15.73 + 1.17*x	0.63	0.26	0.39
	3	**BNb**	y = 5.11 + 0.19*x	0.74	0.15	0.55
		Nb	y = -21.20 + 1.05*x	0.52	0.30	0.27
	4	**BNb**	y = 38.91 – 0.87*x	-0.57	0.31	0.33
		Nb[i]	-	-	-	-
Leaf Moisture	**1**	**BNb**	y = -0.81 + 0.25*x	0.25	0.75	0.06
		Nb[i]	-	-	-	-
	2	**BNb**	y = 9.78 + 0.003*x	0.02	0.98	0.0003
		Nb	y = -440.62 + 5.32*x	0.61	0.27	0.37
	3	**BNb**	y = 10.42 + 0.01*x	0.05	0.94	0.002
		Nb	y = 37.64 – 0.32*x	-0.13	0.80	0.02
	4	**BNb**	y = 89.11 – 0.97*x	-0.96	**0.04**	0.92
		Nb[i]	-	-	-	-

[i] Insufficient replicates to perform the simple regression method

3.3.5 Summary of results

Results of all the experiments are summarised in Table 3.19. The exogenous application of BION® on water hyacinth plants resulted in various effects on water hyacinth plant growth and physiological parameters, and herbivory by the two biological control agents, *E. catarinensis* and *N. bruchi*.

Mean dry plant biomass was significantly lower in the BION treatment, compared to the control treatment (dH_2O), suggesting that the induction of SA-mediated defence pathways precluded resource allocation to water hyacinth plant growth. Reductions in the number of functional leaves and relative leaf chlorophyll content at the end of the trial in the BION treatment accounted for the reductions in total biomass loss. On the other hand, the lengths of the second petiole were significantly longer in the BION treatment, signifying the need for increased photosynthetic production. Percentage leaf nitrogen content was significantly higher in the BION treatment in comparison to the control treatment, but only for leaves 1 and 2. This result suggests that the induction of SA-mediated defence pathways resulted in increased production or reallocation of nitrogenous compounds, most likely defence related, to the younger water hyacinth leaves.

Weevil herbivory had no effect on dry plant biomass, dead dry plant biomass, the number of functional leaves and relative leaf chlorophyll content in BION® treated plants; however, weevil herbivory significantly reduced the lengths of the second petiole. Conversely, positive correlations between leaf chlorophyll content and dry plant biomass for treatment BNb indicate that weevil herbivory had an effect on the relationship between photosynthetic productivity and water hyacinth plant growth. The exogenous application of BION® had no effect on percentage weevil feeding area, but at the end of the trial, the number of adult

weevil feeding scars was significantly higher in the BNb treatment than the Nb treatment; while the area of damage did not increase significantly, the adult weevil feeding rate did increase. This most likely suggests that plants treated with BION® were palatable for the weevils and therefore the weevils increased their rate of feeding. This is supported by the significant decrease in leaf carbon content and increase in palatability indices.

Feeding by the mirid resulted in contrasting results. Plants treated with BION® were severely impacted when exposed to mirid feeding. Dry plant biomass did not differ between treatments BION and BEc, but dead dry plant biomass was significantly greater in treatment BEc, suggesting the mirid caused severe leaf death. The significant decline in number of functional leaves and leaf turnover rate at the end of the trial in treatment BEc accounted for this. Conversely, the lengths of the second petiole did not differ between treatments BION, BEc and Ec, and were the longest petioles in comparison to treatments BNb, dH_2O and Nb. Mirid percentage feeding area significantly increased on BION® treated plants, on all leaf positions, which accounts for the 20% reduction in leaf moisture content in BION® treated plants. *E. catarinensis* population growth was significantly greater on BION® treated plants.

The negative impact of mirid herbivory on BION® treated plants indicates that with the induction of SA-mediated pathways, and subsequent inhibition of JA-mediated pathways, SA does not contribute to water hyacinth mirid-induced responses. On the other hand, the nominal differences in weevil feeding, and the effect of weevil herbivory on plant growth and physiological parameters suggests that SA-mediated pathways may play a role in weevil-induced water hyacinth responses. The possible mechanisms behind the contrasting effects of *E. catarinensis* and *N. bruchi* herbivory, and the exogenous application of BION®, on water hyacinth growth and physiological parameters are discussed below.

Table 3.19 A summary of all the differences observed in the 6 experimental treatments for all plant and insect parameters measured, in relation to the control treatment (dH_2O). o = no significant change in comparison to the control treatment (dH_2O), + = increases observed, but not necessarily significant, - = decreases observed, but not necessarily significant. Symbols followed by * indicates significant increases or decreases observed.

	Treatment					
	BEc	**BION**	**BNb**	**dH_2O**	**Ec**	**Nb**
Plant parameters						
Total dry plant biomass	-	-*	-*	o	+	-
Total dry dead biomass	+*	+	o	o	+	o
Number of functional leaves	-*	-*	-*	o	o	o
Leaf turnover	-*	o	o	o	o	o
Length of petiole 2	+*	+*	-	o	+*	-
Number of flowers	-	o	-	o	-	-
Relative chlorophyll content	-*	-*	-*	o	o	-*
% Nitrogen content	+	+*	+	o	-	-
% Carbon content	+	-*	-*	o	-*	-*
C:N	o	-*	-	o	+	o
% Leaf moisture	-*	o	-*	o	o	o

	Treatment			
	BEc	**Ec**	**BNb**	**Nb**
Insect parameters				
% feeding	+*	o	+	o
Weevil feeding scars	n/a	n/a	+*	o
Nb palatability test	n/a	n/a	+*	o
Number of Ec	+	o	n/a	n/a
Weight of Nb pupae	n/a	n/a	+	o
Number of Nb	n/a	n/a	+	o

3.4 DISCUSSION

3.4.1 Post-experimental procedure evaluation

Abiotic stresses, such as nutrient limitation, drought or high soil/water salinity, contribute readily to reduced defences in plants (Bostock *et al*., 2001, Dietrich *et al*., 2004). Such abiotic stresses and the resultant impact on a plant's responses to herbivores were not relevant to the results obtained in this trial. Water hyacinth plants were not nutrient, water, salinity or light limited. Additionally, no plants died prematurely, therefore the exposure time and the insect densities chosen were tolerable.

Initial shortenings of the second petioles observed from Week 0 to Week 3 for all treatments are possibly due to water hyacinth responding to the new, experimental environment. The water hyacinth used in the present study originally came from densely populated and nutrient enriched river systems in the Durban area. Once the plants were exposed to a medium nutrient concentration and had more space to develop, the plants developed laterally. Over the experimental trial, the lengths of the second petiole differed between treatments, proving the length of the second petiole to be an excellent predictor of plant growth in the present study.

Much like the initial reductions observed in the lengths of the second petiole, reductions in chlorophyll content were observed for all treatments for the initial weeks of the experimental trial (Week 0 to Week 4). This also was most likely due to the change in water nutrient concentrations from the eutrophic uMgeni River (Walmsley, 2003) to the mesotrophic nutrient conditions during the experiment. That being said, plants acclimated to the nutrient differences, as evidenced by the equalised chlorophyll content observed from Week 4, and differences between treatments were distinguishable from then on. Water hyacinth adapted

to the altered nutrient regime and the changes in growth and physiological parameters were not influenced by the changes in nutrient status at the beginning of the trial.

Water hyacinth flowering is affected by water nutrient status (Coetzee *et al.,* 2007b). It is possible that flowering was simply a response to the lowered nutrient status of the water during the experiment, considering that the experimental water hyacinth originally came from a eutrophic river system in Kwa-Zulu Natal (Walmsley, 2003). The number of flowers did not differ between treatments, proving that this plant measure was not effective for evaluating the impact of herbivory and the application of BION® on water hyacinth in the present study.

3.4.2 Effect of BION® on water hyacinth growth, physiological and biochemical parameters

Plants treated with BION® and excluded from herbivory exhibited changes in plant growth (dry plant biomass, number of functional leaves, and length of the second petiole), physiological (leaf chlorophyll content) and biochemical parameters (percentage leaf nitrogen and carbon content) in comparison to the control treatment. BION® treated plants, in the absence of herbivory, appeared stressed.

The BION treatment exhibited significant reductions in leaf production and total dry plant biomass. In addition to reduced plant growth, relative chlorophyll content also declined. The reduced levels of chlorophyll content, which reflect reductions in photosynthetic activity (Cottrell *et al.,* 2009, Vasu *et al*., 2009), most likely translated into the observed reductions in plant biomass of plants in the BION treatment. The mechanism whereby this occurred is probably through the down-regulation of photosynthetic genes. For instance, in tobacco

plants (*Nicotiana attenuata* L. (Solanaceae)), photosynthetic genes unrelated to defence mechanisms are strongly down-regulated in response to herbivory by tobacco hornworm larvae (*Manduca sexta* L. (Lepidoptera: Sphingidae)) (Hermsmeier *et al*., 2001). In the present study, the induction of SA in water hyacinth plants (plants treated with BION®) most likely down-regulated photosynthetic activity. This strategy is speculated to allow plants to allocate resources otherwise used in photosynthetic productivity and growth to the synthesis of defence compounds (Gatehouse, 2002). Pancheva *et al.* (1996) found that SA-induced barley seedlings (*Hordeum vulgare* L. (Poaceae)) exhibited reduced plant growth and photosynthetic rates. These reductions were a result of altered chloroplast anatomy when barley seedlings were exogenously treated with SA (Uzunova and Popova, 2000). The changes in chloroplast anatomy were presumably due to the SA-induced down-regulation of photosynthetic genes, which are not used in defences (Uzunova and Popova, 2000). Additionally, increased investment into defences, namely nitrogen-based plant defences, is predicted to reduce photosynthetic capacity, because large proportions of nitrogen may be allocated to the synthesis of defence compounds (Gleadow *et al.*, 1998, Simon *et al*., 2010). Therefore, it is speculated that BION®-induced SA-mediated defences impeded photosynthesis activity by down-regulating photosynthetic genes unrelated to defence mechanisms, ultimately precluding a large proportion of resources being allocated to plant growth in the BION® treated water hyacinth plants.

Defence mechanisms are costly and there is typically a trade-off between the synthesis of defences and plant growth (Mithöfer and Boland, 2012). A study by Zangerl and Bazzaz (1992), investigating the allocation of resources to defences in the neotropical tree *Cecropia palata* L. (Moraceae), found that a 33% reduction in tree growth was likely due to the allocation of 6% of leaf biomass constituents to plant defences. It can be deduced that in the event of SA up-regulation in field conditions, water hyacinth is likely to experience reduced

resource allocation to plant growth. In contrast to total biomass loss, the second petioles significantly increased in length for plants in the BION treatment. Lengthened second petioles are a measure of actively growing water hyacinth plants (Center and Van, 1989) and the need to acquire more light for photosynthetic function (Center and Dray, 2010). Therefore, whereas resources are likely to be allocated to the up-regulation of SA-mediated defences, water hyacinth also is disproportionately allocating resources to petiole growth to maintain a measure of physiological function to fuel the production of SA-induced defences.

The notion that the application of BION® and the subsequent induction of SA mediated defences reprioritise resource use towards plant defences is further supported by the significant changes in water hyacinth leaf biochemical compositions; a significant increase in nitrogen content, a significant decrease in carbon content and therefore a significant decrease in C:N ratios were observed in the BION only treatment. Significant decreases in carbon and increases in nitrogen content in water hyacinth leaves, mediated by the induction of SA (BION®) are likely to be defence related (Dietrich *et al*., 2004). Decreases in C:N ratios within leaves of plants are associated with nitrogen-based herbivore defences (i.e. toxins), and increases in C:N ratios are associated with carbon-based defences (i.e. phenolics, which invariably contribute to reduced palatability of leaves) (Herms and Mattson, 1992). By these criteria, the biochemical results obtained for the BION treatment suggest that the elicitation of SA through the application of BION® increased the accumulation of nitrogen-based, defence related compounds in water hyacinth leaves. Although, the form in which some defence related nitrogenous compounds occur may be palatable to some insect herbivores. Center and Wright (1991) attributed observed high leaf nitrogen concentrations in younger water hyacinth leaves, possibly in the form of amino acids, as a favourable form of nitrogen for *Neochetina* spp. weevils, but also an unfavourable form of nitrogen for the generalist species, the yellow woolly bear (*Spilosoma virginica* (F.)

(Lepidoptera: Arctiidae)). This was supported by the preference for younger leaves by the weevil, and the preference of older leaves by the yellow woolly bear.

3.4.3 Effect of BION® on herbivory by insects with different feeding modes and the resultant impact on water hyacinth growth, physiological and biochemical parameters

Rates of herbivory and subsequent percentage leaf area damage increased only on BION® treated plants exposed to *E. catarinensis* herbivory (BEc > Ec). The difference between the two mirid treatments (BEc and Ec) is attributed to the elevated nitrogen content within BION® treated water hyacinth leaves (BION treatment), and supported by the resultant declines in nitrogen (particularly leaves 3 and 4) and moisture content in mirid treated plants (BEc), while the elevated levels of nitrogenous compounds in BION® treated water hyacinth leaves are most likely herbivore defence-related, as a result of SA-induction, the form in which these compounds occurred was favoured and removed by the mirid. Invariably, increases in leaf nitrogen content would be attractive and beneficial to insects (Athey and Connor, 1989, Casotti and Bradley, 1991, Huberty and Denno, 2006, Kraft and Denno, 1982), as nitrogen is a crucial plant nutrient for insect development and reproduction, and typically occurs in low concentrations (Slansky and Feeny, 1977, Mattson, 1980, Lodge, 1991). Furthermore, nitrogen and water are considered the most important diet components for insect herbivores (Coley *et al.*, 2006). Consequently, rates of herbivory have been found to increase with increases in leaf nitrogen and water content (Athery and Connor, 1989, Casotti and Bradley, 1991, Coley *et al*., 2006). Soo Hoo and Fraenkel (1966) propose that plants that maintain leaf nitrogen concentrations lower than 3% are substandard food quality for insect herbivores. In the present study, plants treated with BION® maintained approximately 3.14% leaf nitrogen, making the leaves more nutritionally attractive than

control plants (dH_2O). Furthermore, the mirid's positive response to nitrogen-rich BION® treated leaves can be attributed to a general trend observed in phloem-feeders, where they consistently respond to the addition of nitrogen to its host plant in a positive way (Speight *et al.*, 2008). *Eccritotarsus catarinensis* is a phloem feeder (phloem contains water, chloroplasts and nitrates crucial for plant productivity (Lalonde *et al.*, 2004)) and phloem feeders are regarded to have rapid consumption rates (Mattson, 1980).

Weevil herbivory also reduced leaf nitrogen content on BION® treated plants. While *N. bruchi* percentage feeding area damage did not differ between non-BION® and BION® treated plants (BNb = Nb), the weevil showed an initial feeding response to BION® treated plants, but over time weevil percentage feeding area on BION® treated plants equalled to non-BION® treated plants and is attributed to cumulative damage. Although trends suggest that percentage feeding area damage was greater on BION® treated plants at the end of the trial. Increases in adult weevil feeding scars on BION® treated plants (BNb > Nb) may account for the reductions in leaf nitrogen content of BION® treated plants, suggesting the weevil favoured and removed the SA-induced nitrogenous compounds in BION® treated water hyacinth leaves, similarly to BION® treated plants exposed to *E. catarinensis* herbivory. Significant increases in mirid individuals, and no difference in weevil individuals, between BION® and non-BION® treated plants, indicate that the insects were not nutritionally limited. Plants with lower nutritional quality leaves typically result in lower relative growth rates, biomass accumulation, fecundity and population growth rates of insect herbivores (Feeny, 1970, Casotti and Bradley, 1991, Hunter *et al.*, 1992). The observed increases in nitrogen content of BION® treated plants could have supplemented *N. bruchi* and *E. catarinensis* growth rates and fecundity.

When considering nitrogen content for each leaf position, nitrogen content was highest in leaf 1 for all plants treated with BION® in comparison to their respective control treatments (Ec, Nb and dH_2O); leaf 1 exhibited the only notable differences between all treatments, indicating a leaf age effect on biochemical composition (Center and Wright, 1991). The observed allocation of nitrogen to younger water hyacinth leaves is in agreement with many results found in the literature (Mattson, 1980, Kursar and Coley, 1991, Coley and Kursar, 1996). Typically, higher concentrations of nitrogen in younger leaves are defence related (Bazzaz *et al*., 1987). Therefore, the induction of SA mediates the reallocation of nitrogen to younger water hyacinth leaves as an anti-herbivory response. However, as discussed above, SA-induced nitrogenous-based compounds in water hyacinth leaves had a positive effect on *E. catarinensis* and *N. bruchi* herbivory

In contrast to the observed increases in nitrogen content in BION® treated plants, nitrogen content did not significantly differ in the non-BION® treated plants exposed to insect herbivory (treatments Ec and Nb) in comparison to the control treatment (dH_2O). The unchanged nitrogen content may suggest minimal acquisition of protein (nitrogen) from water hyacinth leaves by the mirid and weevil, possibly because the nitrogenous-compounds have no nutritional value (e.g. non-nutritive amino acids) (Rhoades and Cates, 1976). The inability to acquire larger amounts of nitrogen from water hyacinth plants is most likely due to water hyacinth's intact insect-induced response systems regulated through JA-mediated defence pathways. Interestingly, leaf carbon content declined significantly in treatments Ec and Nb in comparison to the control treatment. This result is in accordance with results recorded in the literature. Low concentrations of phenolic compounds (carbon-based) have been detected in younger water hyacinth leaves and have been attributed to *Neochetina* spp. weevil preference for leaf 2 (Center and Wright, 1991). It is likely water hyacinth was

investing resources into nitrogen-based compound synthesis, incurring a cost in terms of carbon-based defences.

The reallocation of nitrogen in water hyacinth leaves was influenced by leaf age (or leaf position), which corroborates with other studies (Center and Van, 1989, Center and Wright, 1991). It is argued that the allocation of nitrogenous compounds to younger leaves confers resistance against herbivores, namely generalist herbivores, but specialist herbivores such as *N. bruchi* and *N. eichhorniae* negate these defences (Center and Wright, 1991). While the trends in carbon allocation to each leaf position are unclear, leaf C:N ratios indicated a greater accumulation of carbon than nitrogen in the older leaves than the younger leaves, suggesting the concentrations of nitrogenous compounds in relation to carbon compounds increased in the younger leaves. These biochemical leaf allocations explain the preference for younger leaves by *N. bruchi.* On the other hand, the mirid has a preference for leaf 4 (Hill *et al.,* 1999a), which has been examined in the present study. This suggests that the mirid avoids younger leaves, most likely due to the greater allocation of nitrogenous-compounds. Furthermore, the mirid has the ability to negate the increases in carbon-based compounds, namely phenolics (Center and Wright, 1991), in older leaves.

However, the decreases in C:N ratios in water hyacinth leaves treated with BION® did not confer resistance to the mirid and the weevil, largely due to the function of SA-mediated pathways (pathogen resistance) and the observed increases in insect feeding and population growth on BION®-treated plants. Therefore, the observed increases in nitrogen and decreases in carbon content increased water hyacinth leaf palatability (Furlan *et al.,* 1999).

3.4.2.1 *Eccritotarsus catarinensis*-induced water hyacinth responses

While insect herbivores typically induce jasmonate-mediated defences (Traw *et al*., 2003), the phloem feeder, *E. catarinensis*, is predicted to induce either the JA- or the SA-mediated defence pathway, because some phloem feeders, such as aphids (Zhu-Salzman *et al*., 2004) and whiteflies (Zarate *et al*., 2007), may emulate pathogen damage and can induce salicylate-mediated defences (Smith *et al*., 2009). Interestingly, *E. catarinensis* had a greater negative impact on water hyacinth growth and physiological parameters when plants were treated with BION® than plants not treated with BION®. Notable differences were observed particularly for dry dead plant biomass, the number of leaves produced, leaf turnover, carbon content and leaf moisture. Furthermore, BION® treated plants exposed to mirid herbivory had the lowest leaf production in comparison to all treatments. This suggests that in the presence of SA-mediated defence pathways and the limited allocation of resources to growth, the mirid feeds unhindered due to water hyacinth's responses being severely compromised. This further suggests that the mirid is unlikely to induce the up-regulation of SA in water hyacinth in the field.

Therefore, by a process of elimination, jasmonate-mediated defences are likely to play a role in water hyacinth defences against *E. catarinensis*. Herbivory by *E. catarinensis* had a nominal impact on non-BION® treated dry plant biomass (Ec, ~90.15g) compared to the control treatments (dH_2O, ~86.46g). Additionally, the lengths of the second petioles did not differ to the control treatment. Typically, the impact of *E. catarinensis* on water hyacinth biomass accumulation is negligible, regardless of nutrient conditions (Ripley *et al*., 2006, Coetzee *et al*., 2007b). Observations of unchanged number of leaves and increases in the lengths of petiole 2 corroborate with the unchanged plant biomass in treatment Ec.

Furthermore, leaf moisture content did not decrease with or inversely relate to mirid feeding, suggesting that water hyacinth had maintained physiological function.

Essentially, two conclusions can be made; 1) the unhindered up-regulation of JA is regulating resistance against the mirid, or 2) water hyacinth is tolerating mirid herbivory by allocating resources to plant growth to compensate for damage to plant tissue. While the former is highly probable, the present study cannot confirm or deny the proposition; further investigations into gene-coding are required. However, according to Bazzaz *et al* (1987), ecological parameters, such as resource availability and habitat type, are valuable tools in predicting the level of defences employed by plants. For example, slow growing plants in nutrient limited environments allocate greater resources to insect-induced responses than plant growth (the Resource Availability Hypothesis) (Coley *et al*., 1985, Orians and Ward, 2010). Subsequently, in nutrient unlimited environments, fast growing colonial plant species inherently allocate resources to growth instead of insect-induced defences (Coley *et al.*, 1985). Water hyacinth characteristically exhibits rapid plant growth in nutrient enriched water (Heard and Winterton, 2000, Coetzee *et al*., 2007b). Therefore, the limited impact that *E. catarinensis* has on water hyacinth biomass accumulation can be attributed to water hyacinth's allocation of resources to growth instead of induced defences in response to herbivory by the mirid. This response is commonly termed compensatory growth, which is defined as a plant's positive response to injury (Belsky, 1986). More specifically, Belsky (1986) formulated and defined three sub-categories of compensatory growth; 1) overcompensation, 2) exact compensation, and 3) undercompensation. Exact compensation arises when the differences in total dry plant weight between the treated and control plants are equal (Belsky, 1986). In effect, water hyacinth exhibited exact compensation in response to herbivory by *E. catarinensis*, by allocating resources to growth in order to alleviate the detrimental effects of tissue damage (McNaughton, 1983).

As expected, leaves in the control treatment were high in chlorophyll content at the end of the trial in comparison to all treatments, but the chlorophyll content of plants solely exposed to *E. catarinensis* herbivory remained consistent with the control treatment. Furthermore, the observed increases in the lengths of the second petioles for non-BION® treated plants exposed to mirid herbivory (Ec) were significantly related to leaf chlorophyll content in the present study; increases in petiole lengths correlated with increases in chlorophyll content. This suggests that the lengthened second petioles in treatment Ec most likely fuel the compensatory growth response; as the second petiole lengthens, photosynthetic productivity enhances, and growth is subsequently maintained. The differences in Leaf 4 chlorophyll content between BION® and non-BION® treated plants exposed to *E. catarinensis* herbivory further supports the notion that water hyacinth is capable of tolerating mirid herbivory and maintaining growth and photosynthetic function under herbivore pressure. The chlorophyll content of Leaf 4 was significantly lower in the BION® treated plants at the end of the trial, presumably due to the mirid's preference for Leaf 4 (Hill *et al*., 1999a), as reflected in the percentage area damage on Leaf 4; *E. catarinensis* feeding was greatest on Leaf 4 in comparison to the younger leaves.

As mentioned above, *E. catarinensis* is a phloem-feeder, and phloem sap contains chloroplasts and photosynthates crucial for photosynthetic productivity (Lalonde *et al.*, 2004), and consequently the reduced chlorophyll content in Leaf 4 is most likely due to the mirid removing significant amounts of chlorophyll and nutrients (nitrogen) from the leaf tissue (Hill *et al.*, 1999a, 1999b). Additionally, leaf moisture content significantly declined in treatment BEc compared to treatments Ec and BION, and mirid percentage feeding was inversely related to decreases in moisture content. Photosynthetic responses to phloem-feeders have been largely characterised for aphids, and results have been variable; aphids have been found to have positive, negative or have no impact on photosynthetic output

(Peterson and Higley, 1993). Interestingly, Ripley *et al*. (2006) found that mirid herbivory significantly reduced leaf chlorophyll content when plants were exposed to a similar nutrient supply to the concentrations used in the present study (0.01 mgP.l^{-1} and 2 mgN.l^{-1} vs. 0.025 mgP.l^{-1} and 2.5mg N.l^{-1} respectively). The difference between the results obtained by Ripley *et al*. (2006) and the present study is most likely due to the slight differences in nutrient concentrations; the lower nitrogen and phosphorus concentrations used by Ripley *et al*. (2006) most probably impacted chlorophyll content. Nutrient status directly impacts photosynthetic productivity, particularly nitrogen supply, which is closely associated with leaf chlorophyll content and RuBisCO activity (Cao *et al*., 2007, Evans, 1983, Evans, 1989), and plant leaf quality (Estiarte *et al*., 1994).

Unexpectedly, BION® treated plants exposed to mirid herbivory (BEc) also exhibited significant increases in the lengths of the second petioles (BEc > dH_2O), and were statistically similar in lengths to treatment Ec (BEc = Ec). This may indicate a trend where water hyacinth is re-allocating resources to growth to overcome injury caused by the mirid. While resources are predominantly being allocated to salicylate-mediated defences induced by the application of BION®, resources are being allocated disproportionately to leaf growth for the replenishment of photosynthetic productivity. This notion is supported by the significant increase in Leaf 2 chlorophyll content for BION® treated plants exposed to mirid herbivory.

Leaf turnover was only significant for treatment BEc, where leaf turnover significantly declined, while the other treatments remained unchanged. The reduced turnover accounts for the low numbers of leaves and the significant amount of dead plant material for BEc at the end of the trial. Reductions in leaf production and increases in dead leaf material indicate shortening of water hyacinth leaf life-spans. Non-BION® treated plants had longer leaf life-

spans than plants treated with BION® and subsequently were fed on by the biological control agents; young leaves accounted for in the beginning of the trial moved down the crown and were still present and photosynthetically active at the end of the trial (leaves were green) (pers. obs.). Water hyacinth leaf life-span is typically 2 months (8 weeks) (Center, 1981), therefore resource allocation to growth and maintenance of leaves was restricted in BION® treated plants, further supporting the concept that water hyacinth responses to *E. catarinensis* feeding were compromised when resources were allocated to BION®-induced SA-mediated defences.

3.4.2.2 *Neochetina bruchi*-induced water hyacinth responses

Whereas *E. catarinensis* induced a compensatory response from water hyacinth, the weevils did not follow suit. Non-BION® treated plants exposed to *N. bruchi* did not differ in biomass, second petiole length and the number of leaves to the control treatment (Nb = dH_2O), although trends show that the presence of the weevil reduced water hyacinth dry plant biomass. When taking into account the significant reductions in photosynthetic productivity (relative chlorophyll content) in non-BION® treated plants exposed to *N. bruchi*, it is likely that water hyacinth biomass accumulation would significantly reduce. The reduced levels of chlorophyll content in treatment Nb, which reflect reductions in photosynthetic activity (Cottrell *et al.*, 2009, Vasu *et al*., 2009), are most likely a result of the removal of the epidermal layer and photosynthetic tissue by the leaf chewing weevils. However, it is also likely that these reductions are a result of photosynthetic gene down-regulation in the remaining undamaged leaf tissue. As described earlier, the induction of defence hormones typically result in the down-regulation of photosynthetic genes (Hermsmeier *et al*., 2001, Uzunova and Popova, 2000). This is seen in treatment BNb, where the removal of the epidermal layer by the weevil exacerbated reductions in relative leaf

chlorophyll content, which were already impacted by the down-regulation of photosynthetic genes due to SA-induction (BNb < BION).

Despite these reductions in photosynthetic productivity, reductions in the lengths of the second petioles (BNb < BION), and the adverse appearances of water hyacinth plants in treatment BNb in comparison to treatments BION and Nb, *N. bruchi* did not exacerbate the effect of BION® on water hyacinth biomass accumulation and leaf production (BION = BNb). Furthermore, the effect of *N. bruchi* on non-BION® treated plants is equal to the effect of *N. bruchi* on BION® treated plants. Therefore, it is possible that *N. bruchi*-induced defence hormone up-regulation in the field may similarly result in reductions in photosynthetic productivity and plant biomass, much like the effect of the application of BION® on water hyacinth, but water hyacinth was capable of maintaining vigour in treatment Nb, due to intact JA-mediated defence pathways.

While water hyacinth susceptibility to weevil herbivory was not exacerbated by the absence of JA up-regulation (BNb), *N. bruchi* herbivory and preference increased on BION® treated plants. Additionally, trends suggest weevil populations were increasing on BION® treated plants. Collectively, these results show that in the absence of JA up-regulation in water hyacinth plants, *N. bruchi* activity enhanced, suggesting that JA-mediated defence pathways may play a role in reducing *N. bruchi* performance on water hyacinth. This is in agreement with a few studies in the literature, where insect herbivore activity is reduced by the jasmonate defence pathway (Traw *et al*., 2003, Smith *et al*., 2009). Iverson *et al* (2001) investigated the effect of surface applied SA on tomato plants (*Lycopersicon esculentum* Mill. (Solanaceae)), exposed to herbivory by the tobacco hornworm (*M. sexta*)). Not only did the application of SA result in greater susceptibility to tobacco hornworm herbivory, tobacco hornworm larvae also showed greater preference for SA-treated tomato plants, and

consequently greater growth. Effectively, the inhibition of JA up-regulation in tomato plants promoted tobacco hornworm activity.

3.5 CONCLUSION

The present study revealed that water hyacinth mounts different insect-induced responses in response to the two biological control agents, *N. bruchi* and *E. catarinensis*. In response to the mirid, it was found that water hyacinth expresses a compensatory growth response instead of an inducible defence response via jasmonic-acid mediated defence pathways. By reallocating resources to vegetative growth the plant can outgrow the adverse impacts of *E. catarinensis*. Conversely, JA-mediated defence pathways were found to play a role in reducing *N. bruchi* performance, because in the absence of JA (BNb), *N. bruchi* performed better, although this did not significantly translate into adversely impacted water hyacinth biomass accumulation. To confirm these notions, further investigations into gene-coding are required.

Moreover, the present study exposed an ecological cost of the induction of SA-mediated defence pathways in water hyacinth, as it opened the plant up to mirid and weevil attack. In the event that other insect or pathogen attackers induce the up-regulation of SA in naturally occurring conditions, the mirid and the weevil are capable of negating the SA-mediated defences and in fact favour SA-induced leaves over non-SA-induced leaves.

The elevated levels of leaf nitrogen found in BION® treated water hyacinth plants, were readily available for weevil and mirid consumption and were effectively non-deterrent, particularly for the mirid. In comparison to BION® treated water hyacinth plants, non-BION® treated plants were nutritionally poor. This suggests that JA may regulate changes in

structural components of leaf quality (e.g. leaf toughness and leaf thickness), as poor nutritional values are associated with structural compounds (Mortensen, 2013). Consequently, Chapter 4 of this thesis investigates structural components of leaf quality, namely the toughness and thickness of water hyacinth leaves, in response to herbivory and BION® application.

CHAPTER 4

THE EFFECT OF BION® ON WATER HYACINTH STRUCTURAL LEAF QUALITY AND THE ASSOCIATED IMPACT ON BIOLOGICAL CONTROL AGENT FEEDING

4.1 INTRODUCTION

Salicylic acid-induced water hyacinth plants were shown to have reduced responses (plant growth and leaf production) to herbivory by the biological control agents *Neochetina bruchi* and *Eccritotarsus catarinensis*, and the biological control agents exhibited intensified feeding behaviour, suggesting that the up-regulation of SA-mediated defence pathways do not play a significant role in water hyacinth defences against these particular insects, especially for the mirid (Chapter 3). Through the process of elimination, salicylic acid (SA) is predicted to play some role in water hyacinth's defence against the weevil and the mirid. However, the specific defence mechanisms induced through the up-regulation of SA are unknown. Chapter 3 of this thesis investigated water hyacinth's growth and biochemical responses to agent herbivory. Subsequently, this chapter investigates water hyacinth leaf structural (or physical) responses as final expressions of SA- and SA-mediated defence pathways.

The root and aerial plant surface structures are the first regions of contact between a herbivorous insect and a plant. Therefore, it is important for a plant to maintain these surface structures as the foremost mechanisms of defence (Peter and Shanower, 2001, Müller, 2008). The biological control agents, *N. bruchi* and *E. catarinensis*, occupy and utilise the leaves of

water hyacinth, particularly *E. catarinensis*, as it oviposits and spends its entire life-cycle on the leaves (*N. bruchi* larvae do not use the leaf as they are petiole miners (Chapter 1)); for this reason, water hyacinth is speculated to express forms of defence mechanisms predominantly at the leaf surface, in response to herbivory by the biological control agents.

As outlined in Chapter 1, defence mechanisms at the leaf surface can be in the form of deterring or toxic chemical compounds, or in the form of physical barriers that reduce mobility, accessibility and attachment (Müller, 2008). All defence mechanisms, including components of plant quality, are the final expressions of genetically regulated biochemical processes within the plant (Bernays and Chapman, 1994, Schoonhoven *et al*., 2005). Plant or leaf quality traits can act as barriers or deterrents to herbivory (structural and/or chemical defences), ultimately altering the performance of herbivores (Kursar *et al*., 2006). Such traits include the presence of trichomes, nutritive quality, leaf thickness, epidermal cell wall thickness, cuticular thickness and leaf hardness and/or toughness (Wright *et al*., 1989, Lundberg and Åström, 1990, Wright and Cannon, 2001, Schoonhoven *et al*., 2005, Hanley *et al*., 2007, Williams *et al*., 2011, War *et al*., 2012).

Leaf nutritive quality is a chemical component of leaf quality. Water hyacinth's leaf nutritive quality and the influence it had on herbivory by the biological control agents was investigated in Chapter 3, by examining the nitrogen, carbon and moisture content of leaves. The nitrogen content and leaf moisture content (water) are two of the most important dietary requirements for insect herbivores (Mattson, 1980, Coley *et al*., 2006), therefore any reduction in these dietary components is thought to deter or reduce herbivory and insect performance. The induction of SA through the application of BION® significantly increased the nutritional value of water hyacinth leaves (increases in leaf nitrogen content were observed), and the mirid and the weevil expressed a greater preference for BION® treated

leaves over nutritionally poorer non-BION® treated leaves. Whereas the elevated nitrogen levels were nutritionally viable for the mirid and the weevil, in the line of pathogen defence, the SA-induced changes in the chemical composition of water hyacinth leaves may be toxic or contribute to structural properties of leaf quality.

Leaf toughness, on the other hand is a structural component of leaf quality and may deter and hinder the feeding, development and oviposition of a herbivorous insect, thereby reducing damage (Wheeler and Center, 1996, Graça and Zimmer, 2005, Hanley *et al.,* 2007). Changes in nutritional quality (e.g. increases in carbon content) are associated with structural compounds, such as lignin, fibres and structural carbohydrates which contribute to leaf toughness (Coley, 1983, Mortensen, 2013). For instance, larvae of the fly *Hydrellia pakistanae* Deonier (Diptera: Ephydridae) exhibited increased mortality, prolonged development time and decreased female biomass when reared on the submerged invasive weed *Hydrilla verticillata* (L.f.) Royale (Hydrocharitaceae), specifically on plants with higher leaf toughness and low nitrogen content (Wheeler and Center, 1996). Similarly, a reduced growth rate and prolonged development time were observed in the Australian plague locust, *Chortoicetes terminifera* (Walker, 1870) (Orthoptera: Acrididae), when provided with Mitchell grass (*Astrebla lappacea* (Poaceae)) with higher toughness (Clissold *et al*., 2009). Bjorkman and Anderson (1990) showed that the butterfly larva of a yet-to-be-identified Ithomiid (Lepidoptera) avoided feeding on tougher blackberry leaves (*Rubus bogotensis* H. B. K. (Rosaceae)). Of relevance to this study is that water hyacinth leaf toughness has been measured in response to feeding by the weevil *N. eichhorniae.* Wright *et al.* (1989) observed increases in leaf toughness (measured using a force-gauge penetrometer (grams)) of the youngest leaf and the petioles of leaves positioned 1 to 3 in response to feeding by the weevils. Leaf thickness also is a physical trait, which largely contributes to the toughness of a leaf and is often measured in conjunction with dry leaf density to obtain a measure of leaf

hardness (specifically referred to as leaf specific mass (LSM)) (Witkowski and Lamont, 1991, Hanley *et al*., 2007, Westbrook *et al*., 2011). However, a study by Witkowski and Lamont (1991) found that a measurement of leaf thickness alone provides an adequate measure of a plant's response to environmental stresses, such as insect herbivory and associated insect-induced changes in leaf quality.

Exogenous applications of chemical analogues of SA and JA have been used to observe changes in plant leaf anatomy and leaf quality in response to the induction of the hormonal pathways. A study solely examining the effect of SA on leaf anatomy and chloroplast ultrastructure on barley seedlings (*Hordeum vulgare* L. cv. *Alfa*) found reductions in barley seedling leaf thickness and the dimensions of the epidermal layers in response to the treatment of SA, with the most notable reduction in leaves treated with the highest concentration of SA (Uzunova and Popova, 2000). However, these higher concentrations may represent unnaturally occurring concentrations of SA in barley seedlings, and therefore an exaggerated plant response to the induction of SA mediated pathways; high concentrations of SA had severe impacts on barley seedling leaf quality and functioning. The manufacturer's recommended dosage of BION® was lower and used at a greater spatial scale on adult water hyacinth plants in this thesis; therefore the concentrations of SA had non-lethal impacts on water hyacinth leaf quality and functioning.

Prior to the investigations carried out in Chapter 3, aspects of leaf quality have rarely been investigated for water hyacinth leaves in response to herbivory, but have been investigated using two biological control agents, *N. eichhorniae* and *N. albiguttalis* (Wright and Bourne, 1986, Wright *et al*., 1989, Center and Wright, 1991). As discovered in Chapter 3, the most noteworthy changes in water hyacinth growth and physiological parameters in response to the exogenous application of BION® and exposure to herbivory by *E. catarinensis* and *N.*

bruchi were the significant changes in leaf biochemical compositions (percentage nitrogen and carbon, and C:N ratios). These changes in leaf quality have been discussed in the context of nutritive quality as a chemical component of leaf quality, but have not been investigated and discussed as structural components of leaf quality (e.g. leaf toughness, leaf and epidermal thickness). Poor nutritional values of plant leaves (e.g. large C:N ratios) are associated with structural components of leaf quality, and typically result in reduced palatability and access to leaf tissues (Mortensen, 2013). In addition to the lack of investigations into insect-induced water hyacinth leaf quality responses, attempts to identify the hormonal pathways responsible for the induction of these responses in water hyacinth have been lacking. The availability of chemical analogues of SA and JA for example, will allow for a greater insight into water hyacinth's structural responses to feeding by the biological control agents.

4.1.1 Experimental aims and objectives

Because water hyacinth does not have any leaf trichomes, leaf toughness and leaf thickness, and their relationships to nitrogen and moisture content were the only components of leaf quality addressed and measured in this chapter. Reflecting on the nitrogen content, carbon content and C:N ratio analyses conducted in Chapter 3, it is hypothesised that BION® treated water hyacinth plants would express limited structural changes in leaf quality, because the observed increases in nitrogen content and decreases in C:N ratios do not fit the criterion of structural defences, although it has already been established that these leaf quality changes had no effect on mirid and weevil herbivory. Non- BION® treated water hyacinth plants exposed to herbivory are hypothesised to respond to herbivory with increases in structural components of leaf quality, because SA-mediated pathways are uninhibited.

4.2 METHODS AND MATERIALS

4.2.1 Experimental insect and plant cultures

The water hyacinth plants used for this trial were grown in a plastic lined pool at the Department of Zoology and Entomology, Rhodes University, Grahamstown, South Africa. Plants were maintained under insect free conditions; by insecticide application and sheltering the pool in an isolated greenhouse. The plastic covering of the greenhouse allowed for ample light penetration and zero insect contamination. Experimental plants were used two weeks after an insecticidal application and after being thoroughly rinsed with water to ensure no toxic residues remained. Due to unfavourable day lengths, plant growth lights were set up to simulate optimal day lengths.

Eccritotarsus catarinensis were mass reared and collected from cultures at Rhodes University; *N. bruchi* were collected from cultures at the mass rearing facility at SASRI and couriered to Rhodes University. In total, 750 mirids and 100 weevils were collected and used for the leaf quality trial. Like the protocol followed in Chapter 3, 15 mirids were placed onto each plant for the *E. catarinensis* treatments (7:8 sex ratio, male:female), whereas a pair of weevils (1:1 sex ratio) was placed onto each plant for the *N. bruchi* treatments.

The leaf quality trial took place in the Working for Water Biological Control greenhouse at Rhodes University. In total, 150 similar sized plants were selected from the insect-free water hyacinth plant culture and placed singly into a 6*l* square tub. The tubs were filled with 5*l* of water, covered with a mesh sleeve and plants were allowed to acclimate for a week before starting the trial. The water in each tub was replaced every 14 days and maintained at a medium nutrient level (0.025mgP.l^{-1} and 2.5mgN.l^{-1}) throughout the trial period. Because the trial required plants to be destroyed on a periodical basis, 30 plants were assigned to one of

five destruction weeks (week 1, week 3, week 5, week 7 or week 9). All 150 plants were set up on the same date and as the trial progressed, every two weeks 30 plants were subsampled and destroyed to obtain leaf toughness and thickness measurements. Each of the 30 plants assigned to each destruction week were assigned to one of six treatments; *N. bruchi* only (Nb), *E. catarinensis* only (Ec), exogenous application of BION® only (BION), *N. bruchi* with the exogenous application of BION® (BNb), *E. catarinensis* with the exogenous application of BION® (BEc), and an insect-free control treatment with the exogenous application of distilled water (dH_2O). Each treatment was replicated five times.

4.2.2 Exogenous application of BION®

One week prior to insect inoculation, BION® solution was applied to all plants requiring BION® treatment (at Week 0), and then once every week after inoculation, for a period of 9 weeks. The same concentration used in Chapter 3 was used in this trial; ten millilitres of BION® solution was exogenously sprayed using a pump hand sprayer, from 1m above the plant, over the entire canopy of each plant in each of the BION® required treatments (at $0.0042ml/cm^2$).

4.2.3 Plant and insect parameters

Prior to the release of insects and further application of BION® and distilled water, all the plants used within the trial were weighed using a scale (Denver Instrument Company, XL-3100), and were denoted as data for Week 0. Thereafter, once the insects were released onto the requisite plants, the application of BION® and distilled water continued. Plant and insect parameters were measured weekly, for a total period of 9 weeks.

Insect parameters

Adult *N. bruchi* feeding was recorded using the same method used in chapter 2 and 3, where an estimate of the percentage surface area damaged on leaves 1, 2, 3 and 4 was recorded each week. Additionally, the number of adult weevil feeding scars was counted on the abaxial and adaxial surfaces of leaves 1 to 4. *Eccritotarsus catarinensis* feeding damage was recorded in a similar manner, by estimating the percentage surface area damage on leaves 1, 2, 3 and 4 (Coetzee *et al*., 2007b).

Plant parameters

At each of the assigned destruction weeks, leaves positioned 1 to 4 were sampled from each plant in each treatment and the leaf toughness measured. Leaf toughness was measured using an Imada® DPS-1 force gauge mounted on a stand. Leaves were placed in a custom-made stand to maintain consistency between measurements. The mean leaf toughness was subsequently measured from five points along the leaf tip-to-leaf base axis (Figure 4.1 a). The leaf toughness was measured in grams (g).

Leaf thickness and upper epidermal layer thickness also was measured, using the same leaves used for measuring the leaf toughness. Leaves 1 to 4 from each plant in each treatment, for each destruction week were prepared for stereo microscope examination. A section of leaf was excised from the middle of each leaf, with a 1x5cm area (Figure 4.1 b) and one 0.1µm thick leaf section was cut using a microtome. The leaf section was mounted onto a slide in a drop of silicone to avoid desiccation, and a cover slip placed over the slide sample. The slides were examined under a stereo microscope (Olympus SZX) and images, with the scale imprinted on them, were taken of the leaf sections using a digital camera (Olympus DP72). Using ImageJ 1.45s (National Institutes of Health, USA), three random

points across the leaf cross section were selected and measured to obtain the mean thickness of the leaves (from the upper epidermis to the lower epidermis) and thickness of the upper epidermal layer (Figure 4.1 c).

4.2.4 Statistical analyses

Generalised linear/non-linear models (GLZ) in STATISTICA 10.0 (© StatSoft, Inc. 2011) analysed the differences in leaf toughness and leaf and epidermal layer thickness of the different water hyacinth treatments. On examination of the residuals, all data exhibited a normal and homoscedastic distribution around the regression line. To test for correlations between leaf toughness and thickness and percentage carbon content, moisture content and insect feeding, simple regressions were performed. Data for water hyacinth leaf percentage nitrogen content, percentage carbon content, C:N ratios and moisture content were obtained from Chapter 3.

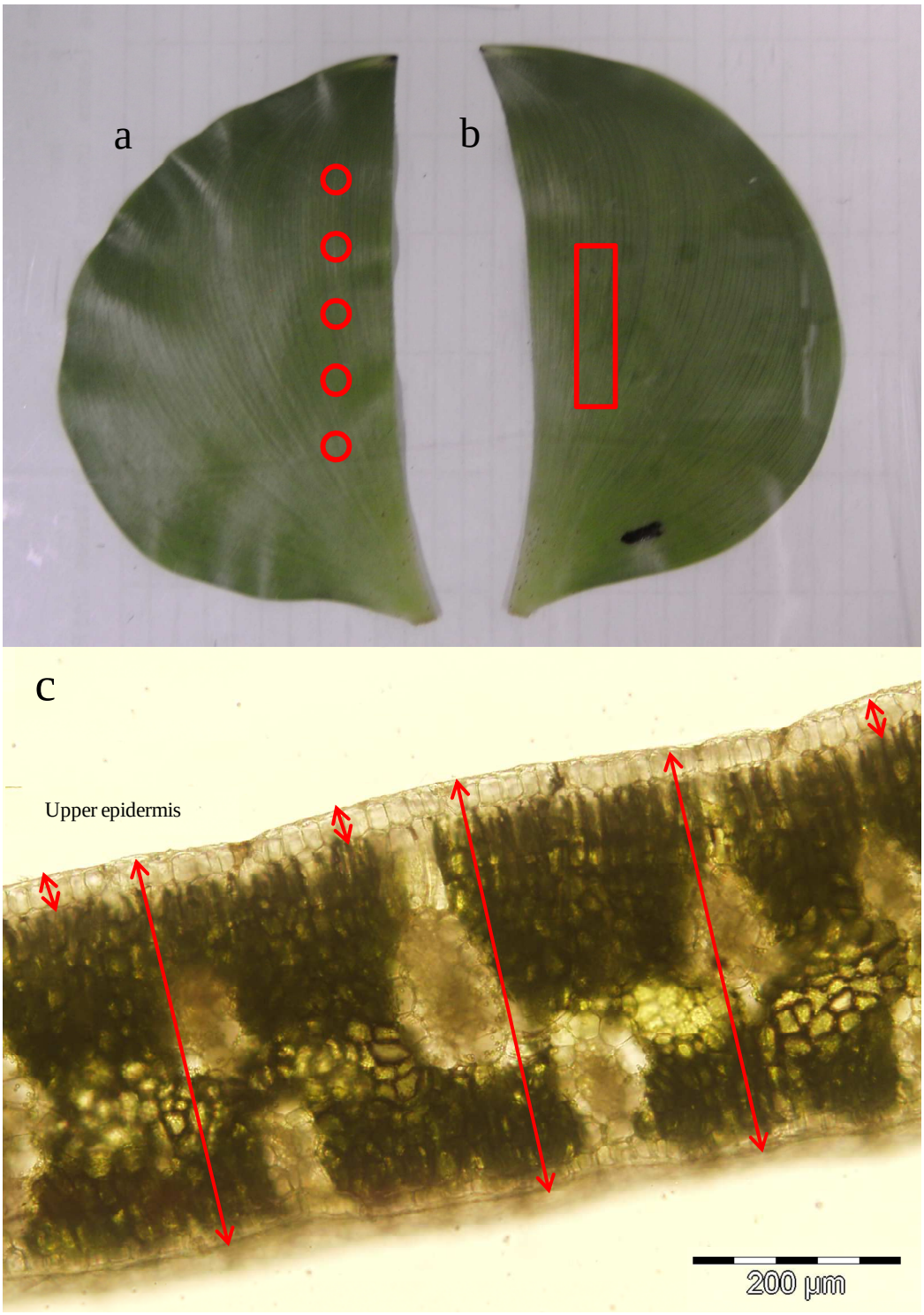

Figure 4.1 Image explaining the use of each leaf to obtain the leaf toughness, leaf thickness and upper epidermal layer thickness. a) One half of the water hyacinth leaf dedicated to obtaining an average measurement of leaf toughness from 5 points along the axis of the leaf, indicated by the red circles. b) A section of water hyacinth excised from the other half of the leaf, indicated by the red rectangle, to measure leaf and upper epidermal leaf thickness. c) A cross section of water hyacinth leaf obtained from b) with 3 random points of measurement, as indicated by the red arrows, to obtain the thickness of the leaf (long red arrow) and upper epidermal layer (short red arrow).

4.3 RESULTS

4.3.1 Insect parameters

4.3.1.1 Percentage feeding area

Throughout the trial, minimal differences in percentage feeding area were observed between the mirid treated plants, BEc and Ec (Figure 4.1), and the weevil treated plants, BNb and Nb (Figure 4.2). Although percentage feeding increased over time, there was no difference in mirid feeding over the sample period, except at Week 7, where feeding in the BEc treatment was significantly higher than treatment Ec (Table 4.1). In addition, there was no difference in mirid feeding between the different leaf positions. Feeding by *N. bruchi* did not increase over time and did not differ between treatments BNb and Nb, or between leaf positions (Table 4.1).

Table 4.1 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the percentage feeding area by the two experimental insects, *Eccritotarsus catarinensis* (Ec) and *Neochetina bruchi* (Nb), and the number of *N. bruchi* feeding scars for both insect only and insect + BION treated plants. *P*-values in bold indicate significant differences, $P < 0.05$.

	Sampling weeks		**Treatment**		**Leaf**		**Leaf*Treatment**	
	$\mathbf{X^2_{(1)}}$	***P*-value**	$\mathbf{X^2_{(1)}}$	***P*-value**	$\mathbf{X^2_{(3)}}$	***P*-value**	$\mathbf{X^2_{(3)}}$	***P*-value**
Ec % feeding area	55.21	**<0.001**	0.02	0.89	7.38	0.06	2.90	0.41
Nb % feeding area	1.98	0.16	1.17	0.28	2.84	0.42	3.10	0.38
Nb feeding scars	40.66	**<0.001**	0.038	0.85	27.72	**<0.001**	5.92	0.12

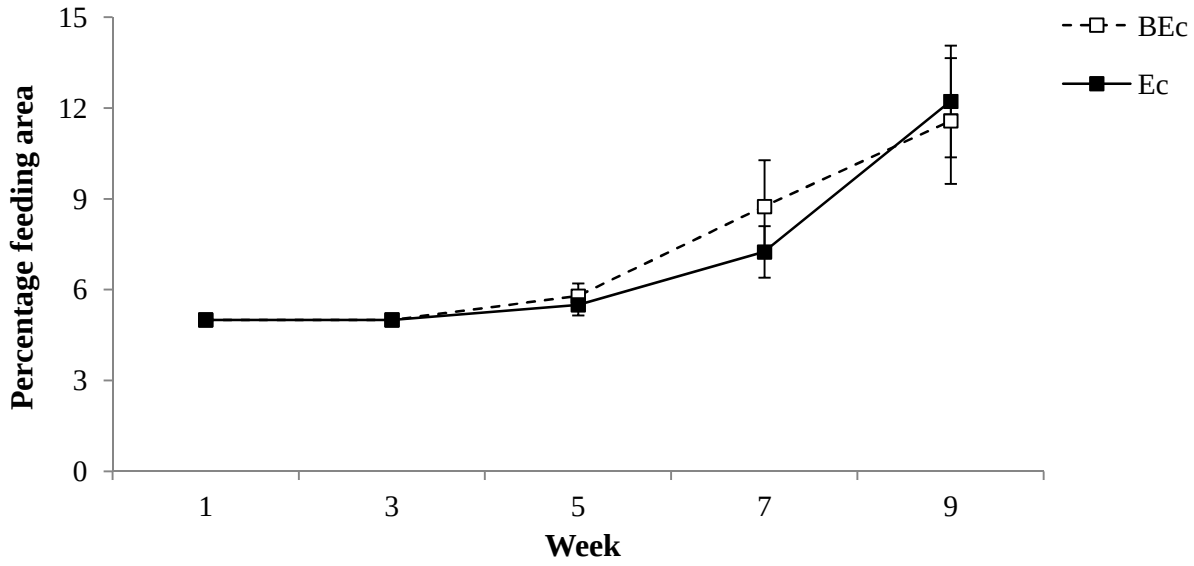

Figure 4.2 The percentage feeding area of *Eccritotarsus catarinensis* at each week for plants treated with *E. catarinensis* (Ec) and *E. catarinensis* with BION® (BEc). Error bars represent the standard error of the mean (n=5 plants/treatment).

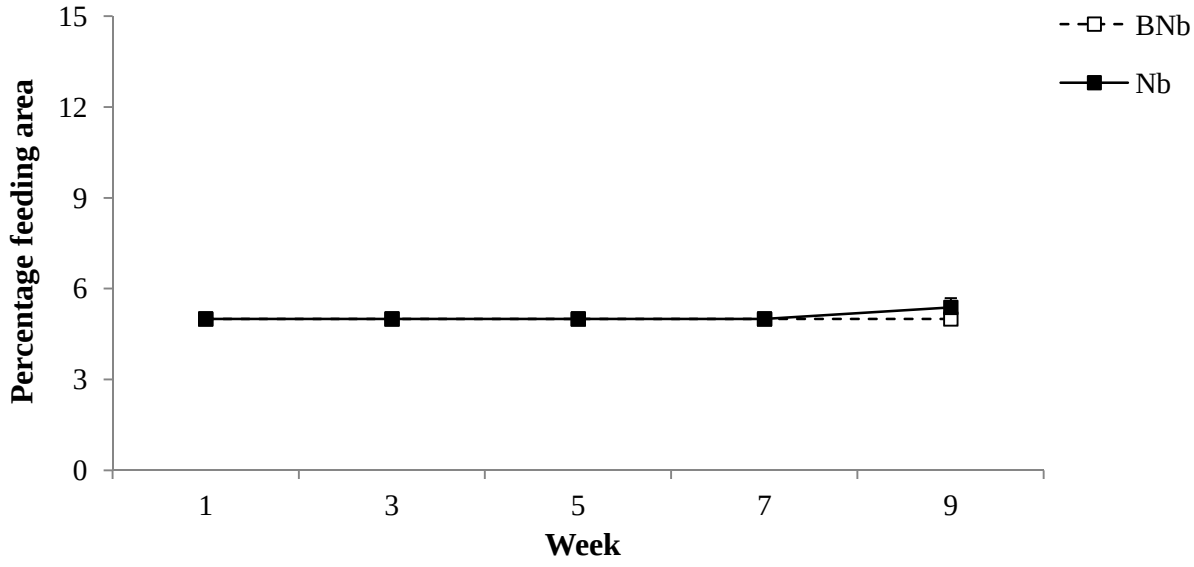

Figure 4.3 The percentage feeding area of *Neochetina bruchi* at each week for plants treated with *N. bruchi* (Nb) and *N. bruchi* with BION® (BNb). Error bars represent the standard error of the mean (n=5 plants/treatment).

4.3.1.2 Number of adult weevil feeding scars

Even though percentage feeding area did not increase, the number of adult weevil feeding scars increased significantly over time in both BNb and Nb treatments (Figure 4.4); however the number of feeding scars did not differ between treatments BNb and Nb (Table 4.1, pg 147). The younger leaves (leaf 1 and 2) had a significantly greater number of feeding scars than the older leaves (leaf 3 and 4) (Figure 4.5). This result corresponds with the weevils known preference for younger water hyacinth leaves.

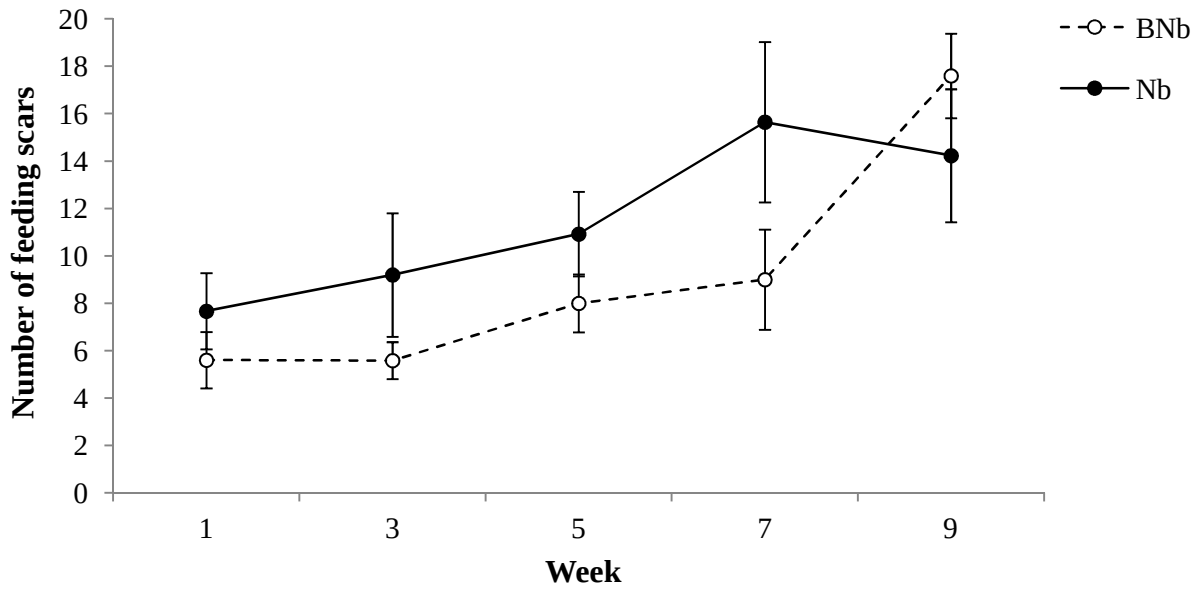

Figure 4.4 Mean number of adult weevil feeding scars produced by *Neochetina bruchi*, recorded each week on water hyacinth plants treated with *N. bruchi* only (Nb) and *N. bruchi* with BION® (BNb). Error bars represent the standard error of the mean (n=5 plats/treatment).

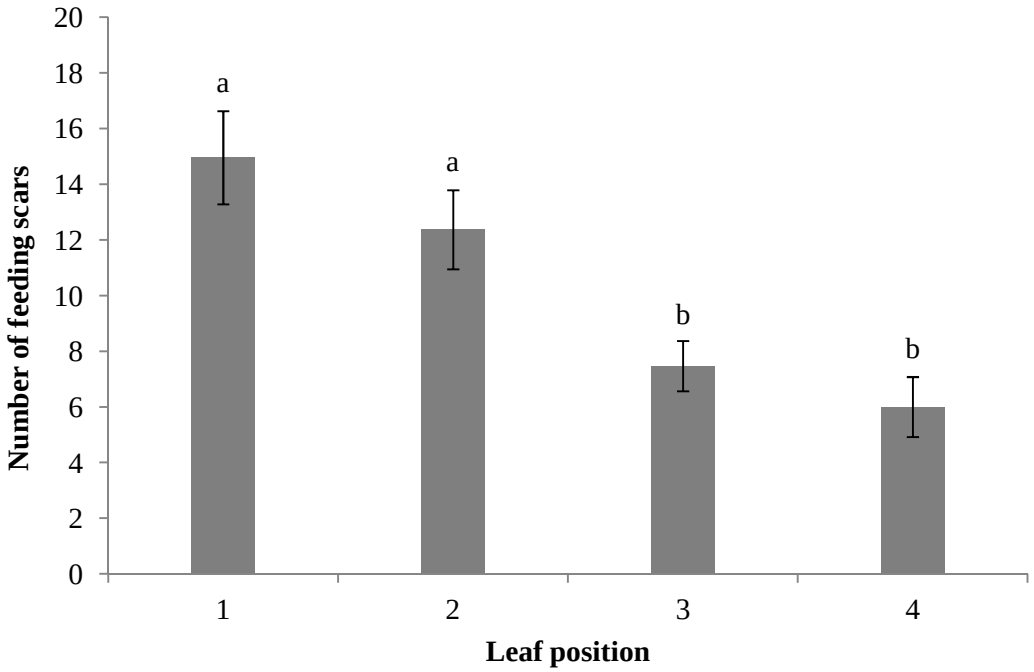

Figure 4.5 Mean number of adult weevil feeding scars by *Neochetina bruchi* recorded on each leaf position for all water hyacinth plants treated with *N. bruchi*. Error bars represent the standard error of the mean. Means followed by the same letter are not statistically different (n=5 plants/treatment).

4.3.2 Plant parameters

4.3.2.1 Leaf toughness

The toughness of water hyacinth leaves in all treatments remained relatively unchanged over the sample period, until week 7, where leaf toughness was significantly greater in the BION treatment (Figure 4.6 and 4.7), than the other treatments (Table 4.2). At the end of the trial (Week 9), significant differences in leaf toughness were observed between treatments (Wald's $X^2_{(5)}$=101.9, P <0.05) (Figure 4.6, 4.7 and 4.8). Treatments BION and dH_2O were not different from each other, and treatment dH_2O did not differ from non-BION® treated plants exposed to herbivory (Ec and Nb). Treatment BION was speculated to have softer leaves than the control treatment, as the C:N ratios for leaves in the BION treatment were significantly lower than the control treatment (Chapter 3). Additionally, treatments Ec and

Nb were hypothesised to be softer than treatment dH_2O, because the carbon contents for treatments Ec and Nb were significantly lower than the control treatment (Chapter 3). Although the induction of SA-mediated defence pathways in water hyacinth plants makes the leaves more palatable, water hyacinth is capable of maintaining leaf toughness comparable to the control treatment (BION vs dH_2O). The same result was observed in non-BION® treated plants exposed to herbivory (Ec and Nb), where the toughness of leaves remained comparable to the control treatment. This may suggest that leaf toughness is either a constitutive structural response mechanism employed by water hyacinth in naturally occurring conditions, or a structural response not employed by water hyacinth. However, when BION® treated plants were exposed to mirid and weevil herbivory (BEc and BNb), leaf toughness significantly increased in comparison to the BION treatment, and their respective non-BION® treated counterparts (Ec and Nb) (Figure 4.8). It is evident that the external application of BION® resulted in tougher leaves only when water hyacinth was exposed to herbivory, and this may be an induced response after continuous exposure to herbivory. Essentially, in the absence of herbivory, SA did not constitutively result in tougher leaves; leaf toughness was induced by herbivory. The results obtained for tougher leaves in treatments BEc and BNb are supported by the significant decreases in leaf moisture (Chapter 3), but no other biochemical analyses (C, N and C:N).

Table 4.2 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the leaf toughness data. *P*-values in bold indicate significant differences between means.

	Sampling weeks		**Treatment**		**Leaf**		**Leaf*Treatment**	
	$\mathbf{X^2_{(1)}}$	***P-value***	$\mathbf{X^2_{(5)}}$	***P-value***	$\mathbf{X^2_{(3)}}$	***P-value***	$\mathbf{X^2_{(15)}}$	***P-value***
Leaf toughness	69	**<0.001**	76.1	**<0.001**	98.2	**<0.001**	25.7	**<0.001**

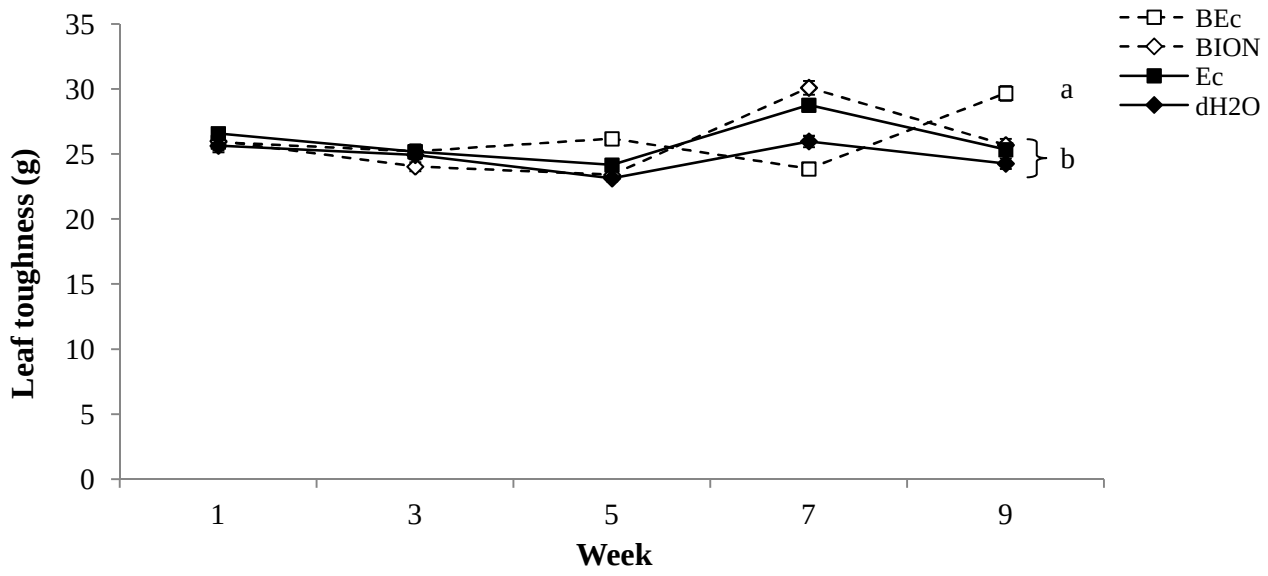

Figure 4.6 Changes in the leaf toughness, for all leaves, in water hyacinth plants in all treatments, at each of the destruction weeks. BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, dH^2O = distilled water, and Ec = *E. catarinensis* only. Error bars represent the standard error around the mean. Means at Week 9 followed by the same letter are not statistically different, *P* >0.05 (n=5 plants/treatment).

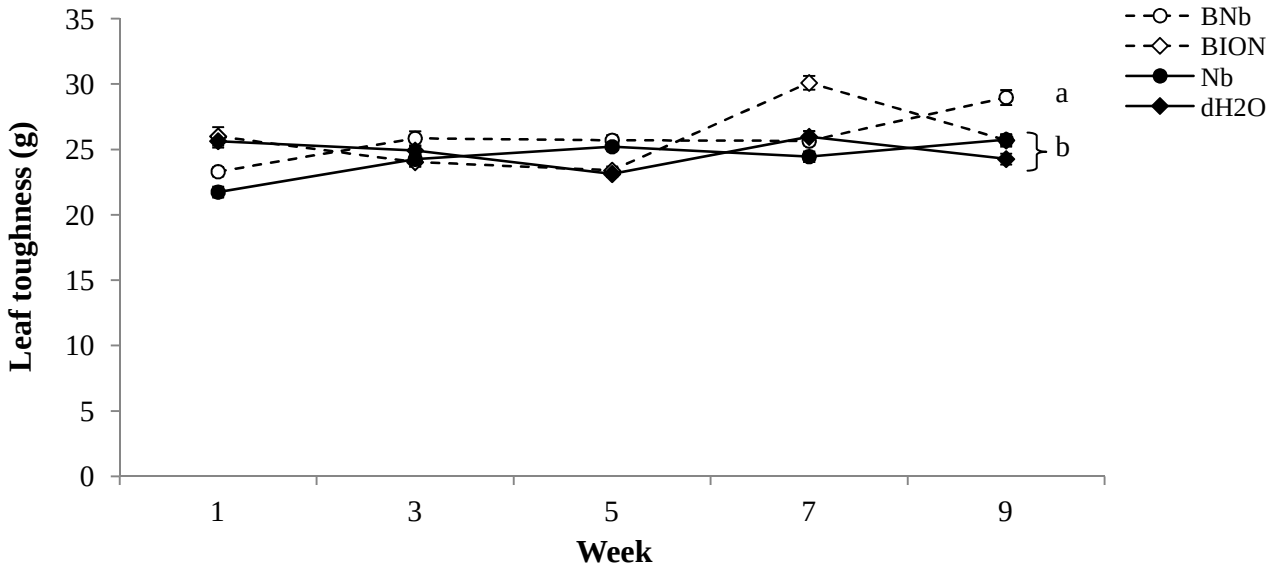

Figure 4.7 Changes in the leaf toughness, for all leaves, in water hyacinth plants in all treatments, at each of the destruction weeks. BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH^2O = distilled water, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means at Week 9 followed by the same letter are not statistically different, *P* >0.05 (n=5 plants/treatment).

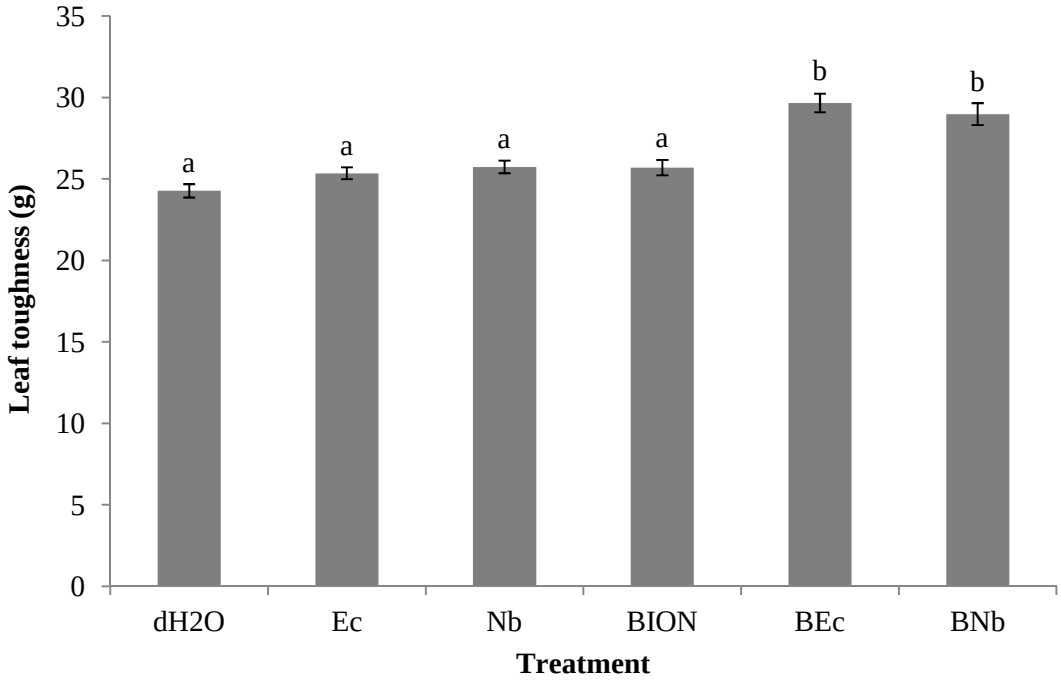

Figure 4.8 Mean leaf toughness (g) for all leaves in all six treatments (Treatments: BEc = BION® + *E. catarinensis*, BION = BION® only, BNb = BION® + *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only), at the end of the sampling period (Week 9). Means followed by the same letter are not statistically different, *P*>0.05 (n=5 plants/treatment).

When examining the differences in leaf toughness for each leaf position over the trial period, significant differences were observed between sampling weeks for all leaf positions, excluding leaf 3, and between treatments for all leaf positions (Figure 4.9, Table 4.3). Generally, over time the toughness of the leaves in all treatments remained unchanged or gradually increased. Interestingly, at Week 7, treatments BION and Ec exhibited significant spikes in leaf toughness for most of the leaf positions (BION: leaf 1, 2 and 3, Ec: leaf 2, 3 and 4). At the end of the trial, significant differences in leaf toughness between treatments for all leaf positions were observed (Figure 4.10, Table 4.3). Leaves positioned 1 to 4 for treatment BEc were significantly tougher than the leaves in treatments Ec and dH_2O.The same is seen for treatment BNb, which had significantly tougher leaves than treatments Nb and dH_2O, but only for leaves positioned 1, 2 and 4. Treatment BION was not different from

treatment dH_2O for all leaf positions. These results parallel the above results, suggesting that in the absence of herbivory, SA did not constitutively result in tougher leaves; leaf toughness was induced by herbivory. In addition, the younger leaves (leaf 1 and 2) were generally tougher than the older leaves (leaf 3 and 4), which was not expected (Table 4.4).

Table 4.3 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the leaf toughness data, exhibiting significant differences between sampling weeks and treatments for leaves 1 to 4, and differences between treatments for leaves 1 to 4 at the end of the trial (Week 9). *P*-values in bold indicate statistical differences between means, $P < 0.05$ (n=5).

	Sampling weeks		Treatments		Week 9	
Leaf	$\mathbf{X^2_{(1)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**
1	43.39	**<0.001**	31.37	**<0.001**	34.78	**<0.001**
2	12.32	**<0.001**	22.39	**<0.001**	58.66	**<0.001**
3	0.01	0.94	19.11	**<0.001**	25.68	**<0.001**
4	44.65	**<0.001**	31.97	**<0.001**	32.79	**<0.001**

Table 4.4 The Wald X^2-statistic and *P*-values for the generalized linear/non-linear model analysing the toughness of leaves, comparing the toughness of all leaves positioned 1 to 4 for treatments BEc and Ec, BNb and Nb, and BION and dH_2O. *P*-values in bold indicate significant differences ($P < 0.05$), and means followed by the same letter are not statistically different ($P > 0.05$) (n=5).

Treatment	Leaf 1	Leaf 2	Leaf 3	Leaf 4	$\mathbf{X^2_{(1)}}$	***P*-value**
BEc	31.73 ± 1.03 ^{a}	31.86 ± 1.01 ^{a}	28.77 ± 1.21 ^{ab}	26.37 ± 0.93 ^{b}	14.57	**<0.001**
Ec	25.48 ± 0.86 ^{a}	26.20 ± 0.68 ^{a}	25.75 ± 0.68 ^{a}	23.06 ± 0.58 ^{b}	7.75	**0.05**
BNb	33.41 ± 1.67 ^{a}	29.75 ± 0.66 ^{b}	24.48 ± 1.37 ^{c}	28.69 ± 0.88 ^{b}	25.96	**<0.001**
Nb	28.09 ± 0.51 ^{a}	25.77 ± 0.52 ^{ab}	24.73 ± 0.60 ^{b}	24.45 ± 1.18 ^{b}	9.20	**0.03**
BION	28.86 ± 1.00 ^{a}	26.70 ± 0.54 ^{a}	23.92 ± 0.78 ^{b}	23.56 ± 1.06 ^{b}	22.34	**<0.001**
dH_2O	26.11 ± 1.14 ^{a}	25.36 ± 0.84 ^{a}	22.78 ± 0.60 ^{b}	23.13 ± 0.47 ^{b}	12.88	**<0.001**

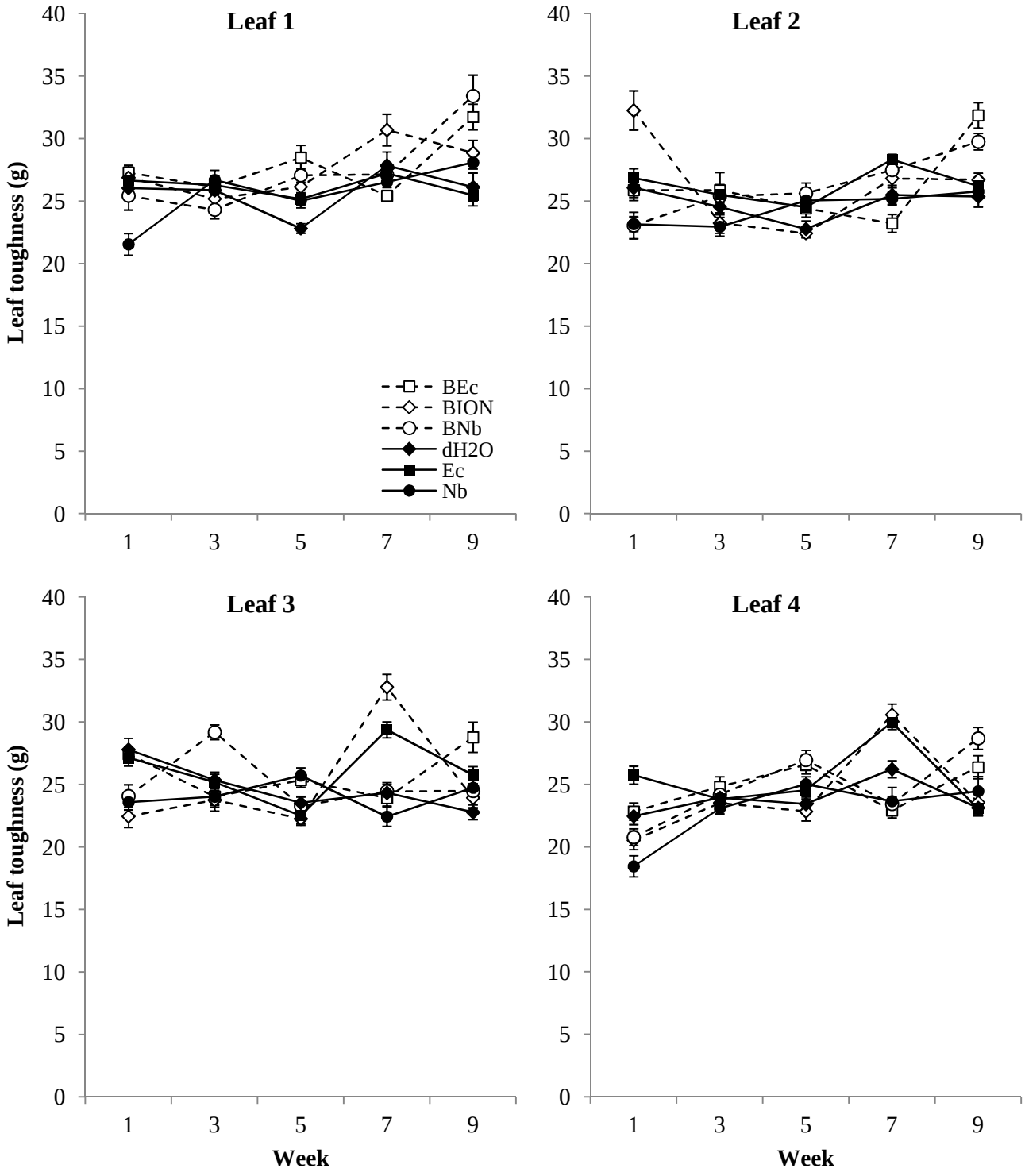

Figure 4.9 Changes in the leaf toughness (g) of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, at each of the destruction weeks. Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean, $P > 0.05$ (n=5 plants/treatment).

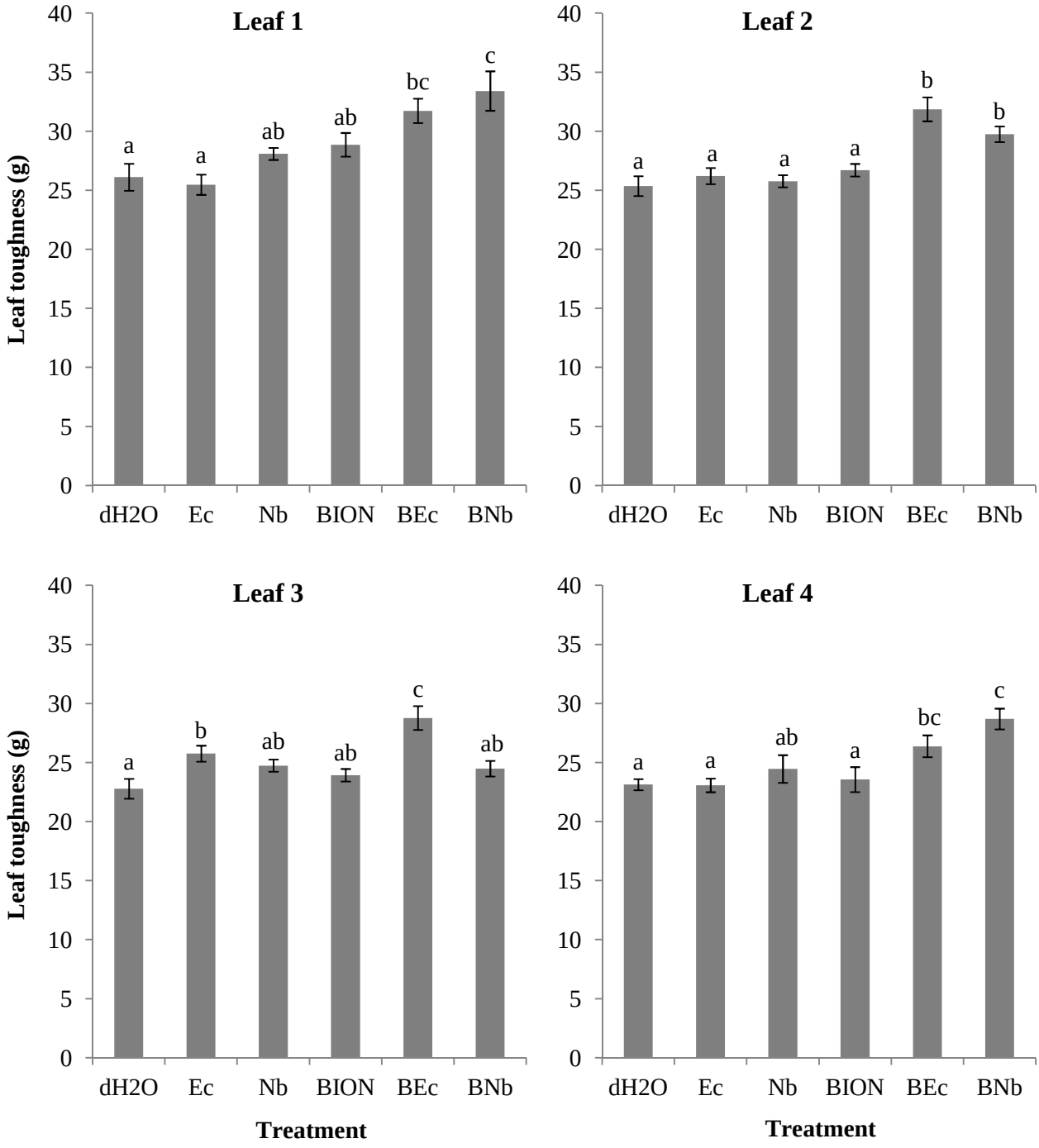

Figure 4.10 Mean leaf toughness (g) for leaves 1 to 4, for all treatments at the end of the experiment (Week 9). Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not statistically different, $P>0.05$ (n=5 plants/treatment).

4.3.2.2 Leaf thickness and upper epidermal layer thickness

Notable changes in water hyacinth leaf thickness (Figure 4.11 and 4.12) and upper epidermal cell layer thickness (Figure 4.13 and 4.14) were observed during the trial period. Significant differences in leaf thickness were observed between sampling weeks, between treatments and between leaf positions (Table 4.5). Similar results were observed for the thickness of the upper epidermal layer (Table 4.5). The interaction between leaf position and treatment was significant for both leaf thickness and epidermal layer thickness. At the end of the trial (Week 9), significant differences in leaf thickness were observed between leaf position (Wald's $X^2_{(3)}$=363.8, P<0.05) and between treatments (Wald's $X^2_{(5)}$=23.7, P<0.05) (Figure 4.15). The interaction between leaf position and treatment was significant (Wald's $X^2_{(15)}$=51.3, P<0.05). The exogenous application of BION® did not result in significantly thicker water hyacinth leaves (BION = dH2O), although trends suggest that leaves in treatment BION were thickening. Despite this, BION® treated plants significantly reduced in leaf thickness when exposed to herbivory by both biological control agents. *N. bruchi* herbivory resulted in significantly thicker leaves in comparison to the control treatment, whereas *E. catarinensis* did not, suggesting varying insect-induced water hyacinth responses to different feeding modes. Leaves in treatment Ec were not thicker than the leaves in treatment BEc. In contrast, leaves in treatment Nb were significantly thicker than leaves in treatment BNb. This may suggest that in the absence of the JA-mediated pathway (BNb), water hyacinth plants were incapable of thickening leaves as a form of defence against mechanical injury by the weevil.

Significant differences in the thickness of the upper epidermal cell layer also were observed between leaf position (Wald's $X^2_{(3)}$=83, P<0.05) and between treatments at the end of the trial (Wald's $X^2_{(5)}$=23, P<0.05) (Figure 4.16). The interaction between leaf position and

treatment also was significant (Wald's $X^2_{(15)}$=33, P<0.05). The application of BION® did not result in significantly thicker water hyacinth upper epidermal cell layers (BION = dH2O), and unlike leaf thickness, exposure to insect herbivory did not result in thinner upper epidermal cell layers. No differences in epidermal thickness were observed between treatments Ec and BEc; however, significant differences were observed between treatments Nb and BNb, with non-BION® treated plants having thicker upper epidermal cells than BION® treated plants exposed to weevil herbivory. The thicker upper epidermal cells in treatment Nb corresponds with the observed increases in leaf thickness.

Table 4.5 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the leaf thickness and epidermal cell layer thickness data. *P*-values in bold indicate statistical differences between means, P <0.05.

	Sampling weeks		**Treatment**		**Leaf**		**Leaf*Treatment**	
	$\mathbf{X^2_{(1)}}$	***P-value***	$\mathbf{X^2_{(5)}}$	***P-value***	$\mathbf{X^2_{(3)}}$	***P-value***	$\mathbf{X^2_{(15)}}$	***P-value***
Leaf thickness	38.4	**<0.001**	33.7	**<0.001**	602.8	**<0.001**	28.3	**0.02**
Epidermal thickness	22	**<0.001**	21	**<0.001**	330	**<0.001**	28	**0.02**

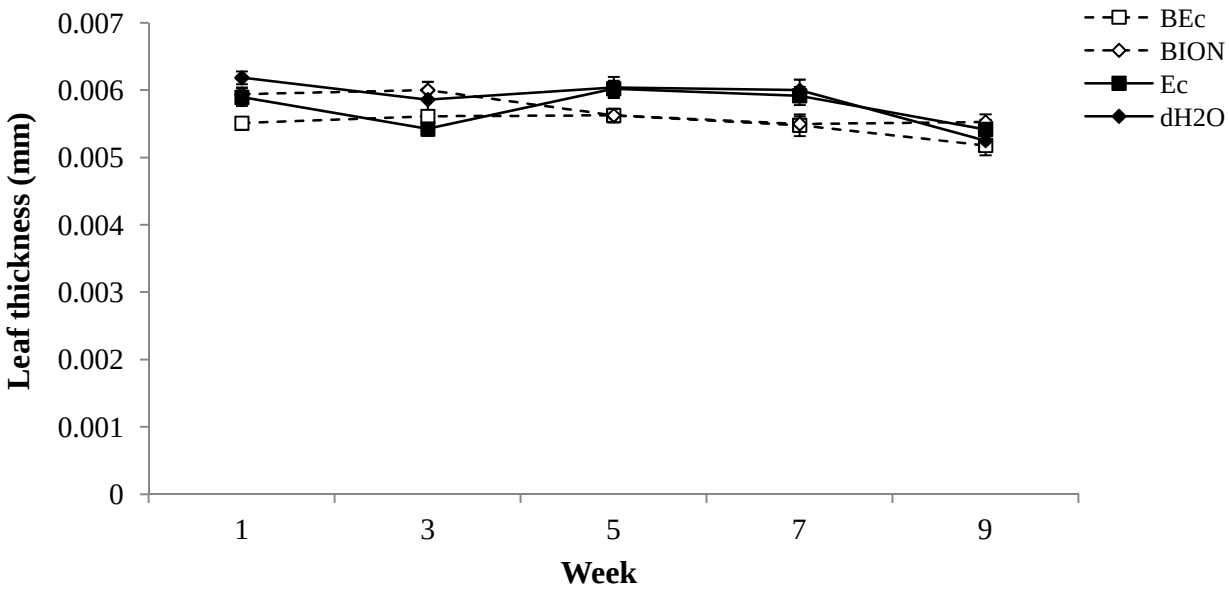

Figure 4.11 Changes in the leaf thickness, for all leaves, from water hyacinth plants in all treatments, at each of the destruction weeks. BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, dH_2O = distilled water, and Ec = *E. catarinensis* only. Error bars represent the standard error around the mean (n=5 plants/treatment).

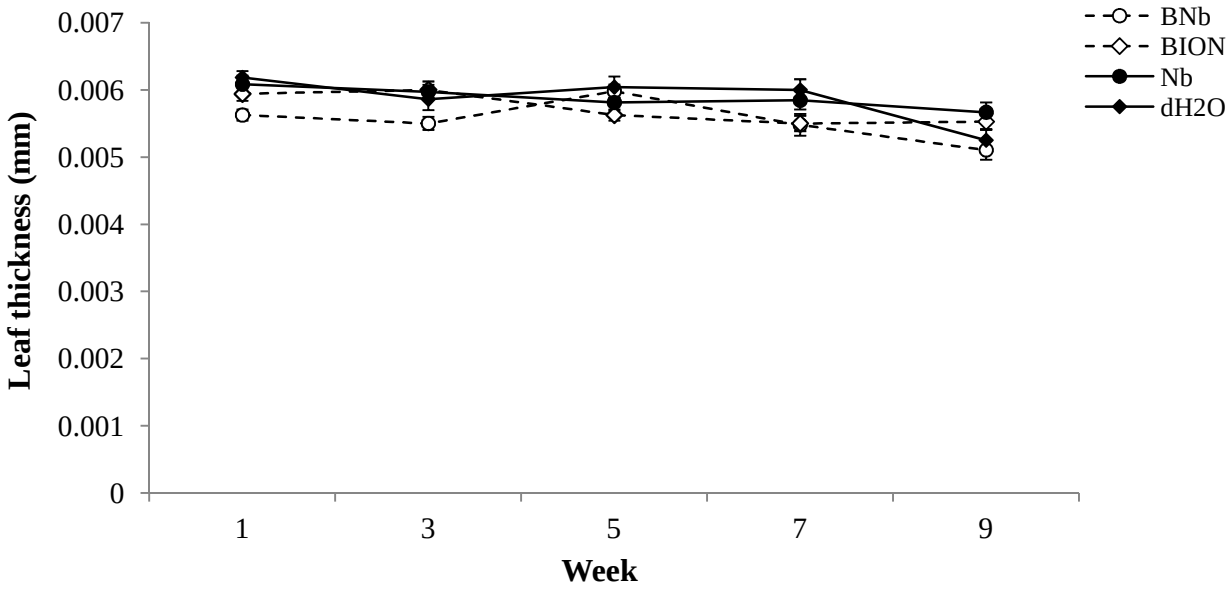

Figure 4.12 Changes in the leaf thickness, for all leaves, from water hyacinth plants in all treatments, at each of the destruction weeks. BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean (n=5 plants/treatment).

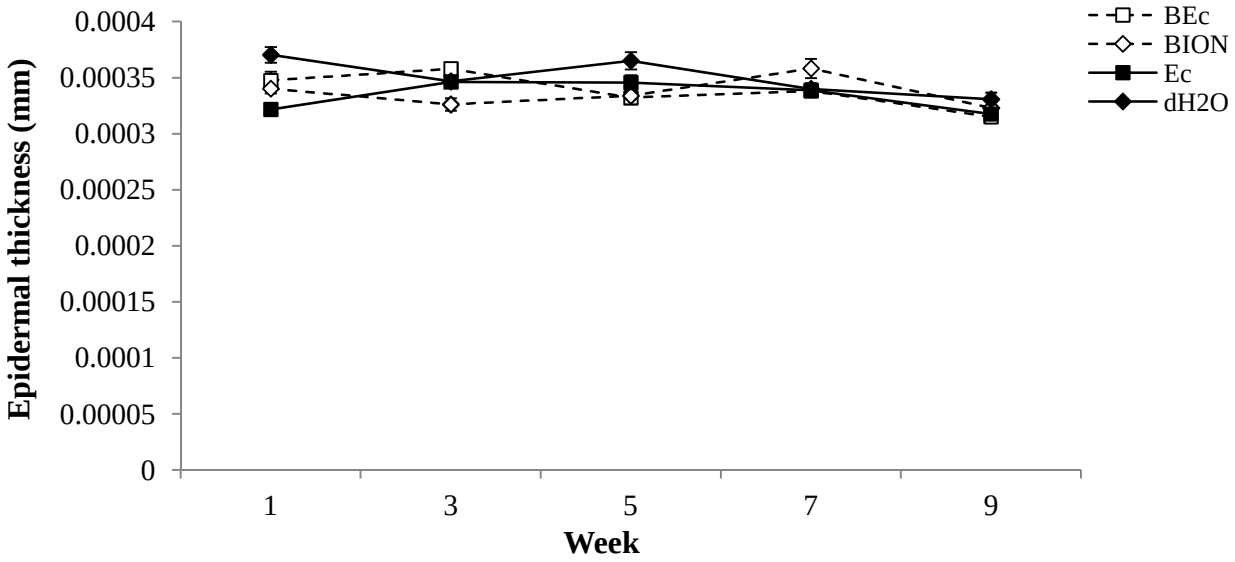

Figure 4.13 Changes in the upper epidermal layer thickness, for all leaves, from water hyacinth plants in all treatments, at each of the destruction weeks. BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, dH_2O = distilled water, and Ec = *E. catarinensis* only. Error bars represent the standard error around the mean (n=5 plants/treatment).

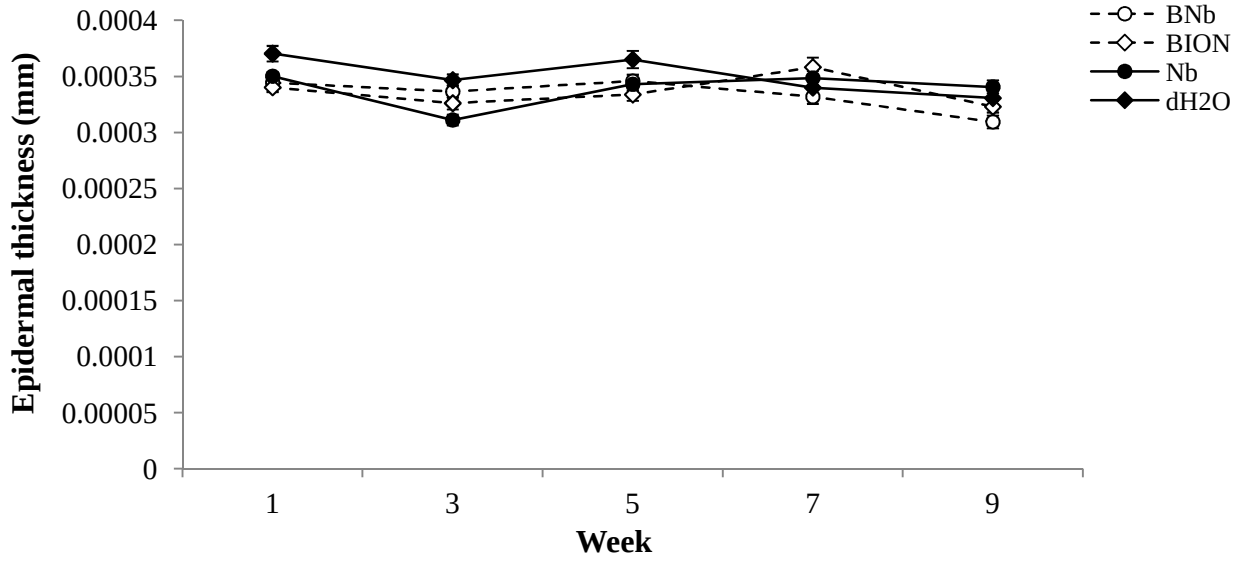

Figure 4.14 Changes in the upper epidermal layer thickness, for all leaves, from water hyacinth plants in all treatments, at each of the destruction weeks. BNb = BION® and *Neochetina bruchi*, BION = BION® only, dH_2O = distilled water, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean (n=5 plants/treatment).

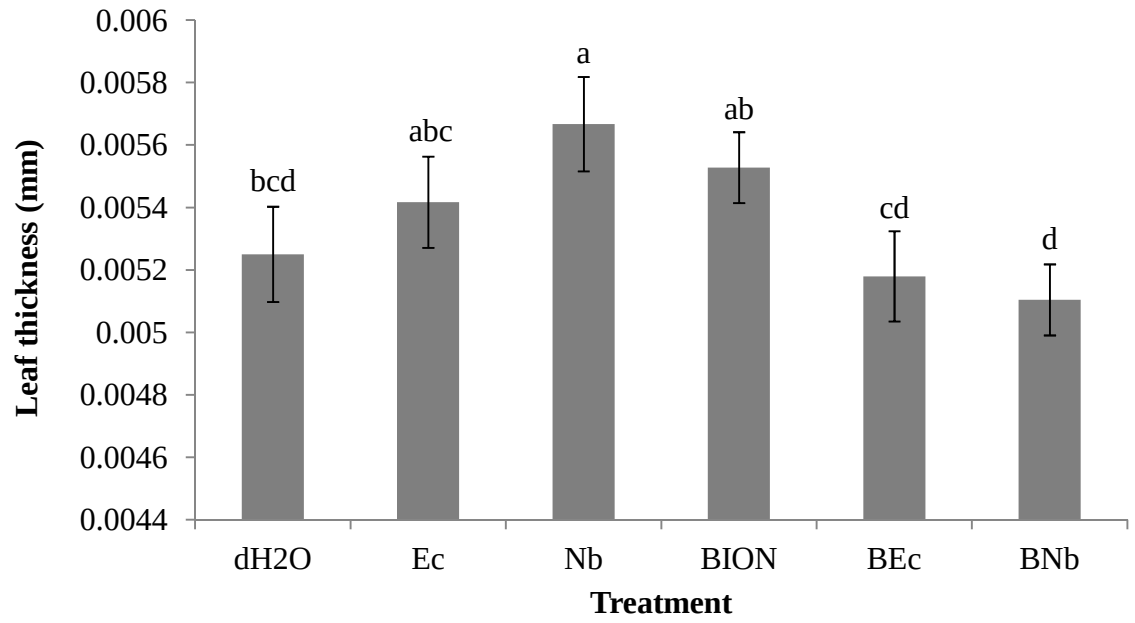

Figure 4.15 Mean leaf thickness (mm) for all leaves in all six treatments (Treatments: BEc = BION® + *E. catarinensis*, BION = BION® only, BNb = BION® + *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only), at the end of the sampling period (Week 9). Means followed by the same letter are not statistically different, *P*>0.05 (n=5 plants/treatment).

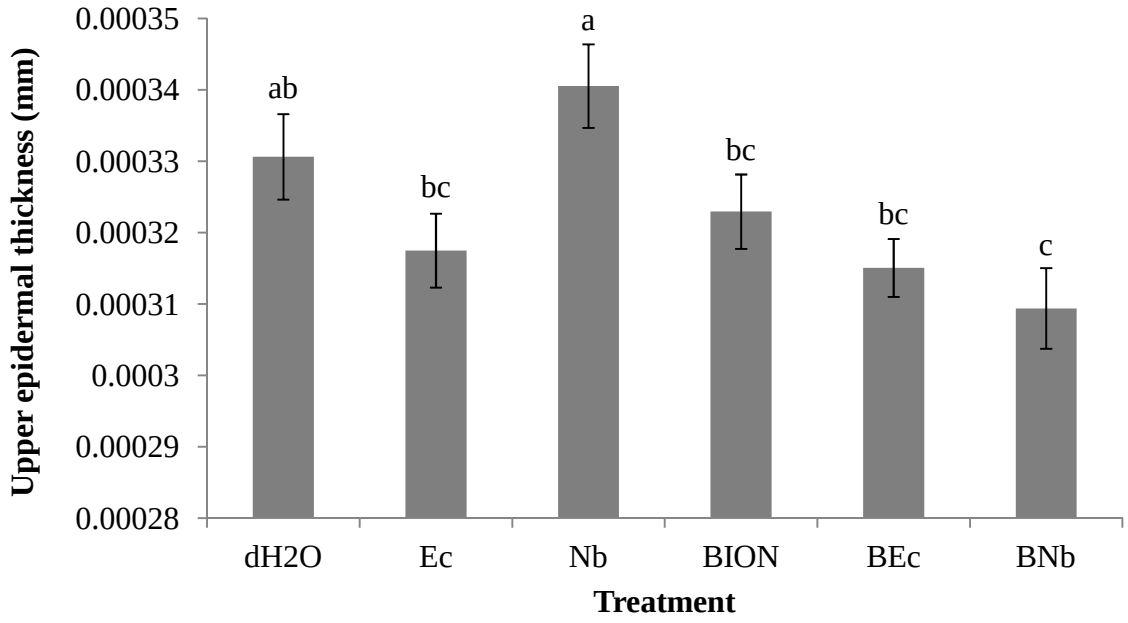

Figure 4.16 Mean upper epidermal layer thickness (mm) for all leaves in all six treatments (Treatments: BEc = BION® + *E. catarinensis*, BION = BION® only, BNb = BION® + *Neochetina bruchi*, dH_2O = distilled water, Ec = *Eccritotarsus catarinensis* only, and Nb = *N. bruchi* only), at the end of the sampling period (Week 9). Means followed by the same letter are not statistically different, *P*>0.05 (n=5 plants/treatment).

Over the course of the trial, the leaf thickness and the thickness of the upper epidermal cell layer for all treatments for each leaf position differed. Leaves 1 to 4 exhibited significant differences in leaf thickness (Figure 4.17) and upper epidermal layer thickness (Figure 4.18) between sampling weeks and between treatments (Table 4.6); however, no differences were observed between treatments for the thickness of the upper epidermal cell layer for leaf 2.

Table 4.6 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the leaf thickness and upper epidermal layer thickness data, exhibiting significant differences between sampling weeks and treatments for leaves 1 to 4. *P*-values in bold indicate significant differences between means, $P < 0.05$.

	Leaf thickness				**Upper epidermis thickness**			
	Sampling weeks		**Treatments**		**Sampling weeks**		**Treatments**	
Leaf	$\mathbf{X^2_{(1)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**	$\mathbf{X^2_{(1)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**
1	57.2	**<0.001**	17.6	**<0.001**	14.7	**<0.001**	11.8	**0.04**
2	92.6	**<0.001**	20.6	**<0.001**	25.9	**<0.001**	6.4	0.27
3	6.23	**0.01**	15.35	**0.01**	13.8	**<0.001**	16.8	**<0.001**
4	8.34	**<0.001**	18.36	**<0.001**	6.5	**0.01**	12.8	**0.02**

At the end of the trial (Week 9), significant differences in leaf thickness were observed between treatments for leaves 1 and 3 (Figure 4.19, Table 4.7), but leaves 2 and 4 did not differ. However, results for leaf 2 were reported, as the *P*-value was close to 0.05. All BION® treated plants exhibited thicker leaves at position 1 in comparison to the control treatment (dH_2O), indicating that the induction of SA results in thicker young leaves. However, treatments BEc and BNb were not significantly thicker than their non-BION® treated counterparts (Ec and Nb). Interestingly, in leaf 2, treatments BEc and BNb were thinner than their respective non-BION® counterparts, Ec and Nb; however, treatment dH_2O

was significantly thinner than treatment BION. In leaf 3, treatments BEc and BNb were significantly thinner than treatments Ec and Nb, respectively. This further suggests that in the absence of the SA-mediated pathway (BEc and BNb), water hyacinth does not thicken its lamina in response to herbivory by the mirid and weevil. This is particularly evident when observing the weevil treated plants; treatment Nb was significantly thicker than all the other treatments, including treatments BNb and dH_2O. In leaf 4, no differences were observed between treatments, although a trend was observed, where BION® treated plants were thinner than the non-BION® treated plants. Perhaps, given more time or a greater sample size, these differences would have been significant.

Even though there were differences in leaf thickness, very few differences were observed between treatments for the thickness of the upper epidermal layer for each leaf position (Figure 4.20), particularly in leaf 1 and 4. However, in leaf 2 and 3, treatment BNb had significantly thinner upper epidermal layers than treatment Nb. Treatment BION was not significantly thinner than treatment dH_2O for all leaf positions, excluding leaf 3.

Table 4.7 The Wald's X^2-statistic and *P*-values for the generalized linear/non-linear model performed on the leaf thickness and upper epidermal layer thickness data, exhibiting significant differences between treatments for leaves 1 to 4 at the end of the trial (Week 9). *P*-values in bold indicate statistical differences between means, $P < 0.05$.

	Leaf thickness		**Upper epidermis thickness**	
Leaf	$\mathbf{X^2_{(5)}}$	***P*-values**	$\mathbf{X^2_{(5)}}$	***P*-values**
1	29.6	**<0.001**	7.8	0.17
2	10.6	0.06	8.1	0.15
3	43	**<0.001**	30.2	**<0.001**
4	6.6	0.25	11.1	0.05

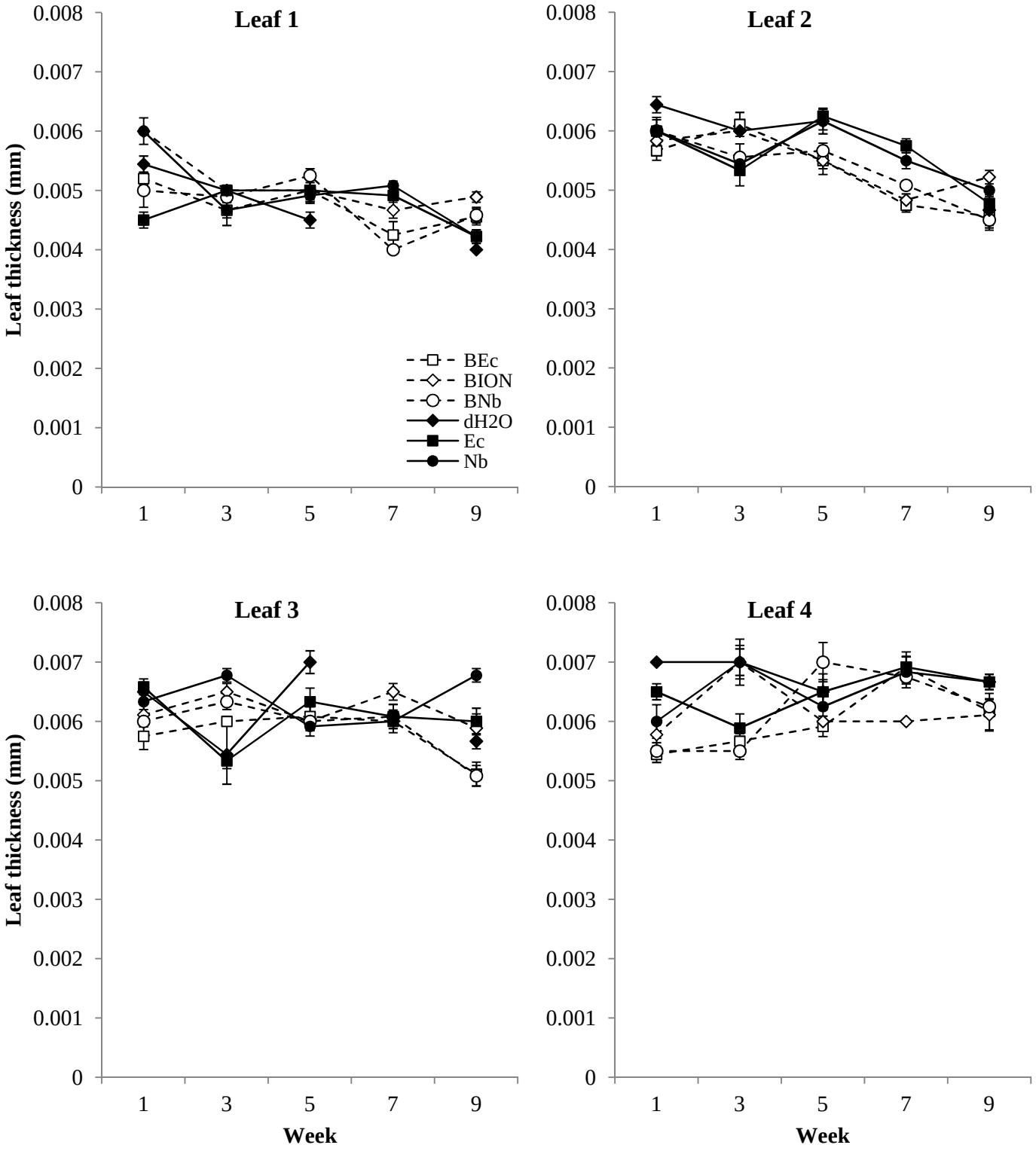

Figure 4.17 Changes in the leaf thickness of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, at each of the destruction weeks. Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Data for Week 7, treatment dH^2O is not present. Error bars represent the standard error around the mean, $P > 0.05$ (n=5 plants/treatment).

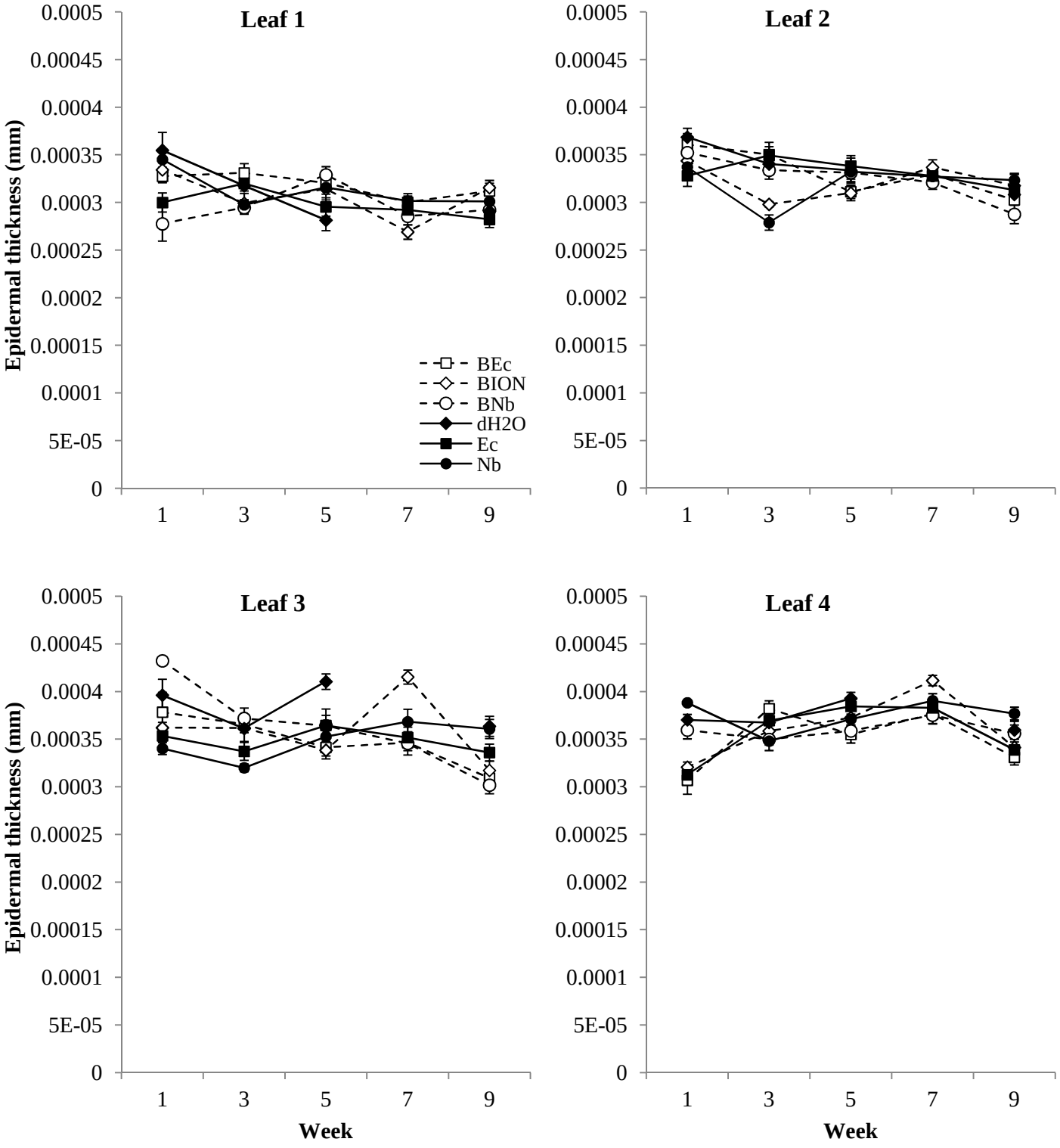

Figure 4.18 Changes in the thickness of the upper epidermal cell layer of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, at each of the destruction weeks. Treatments: Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean, $P > 0.05$ (n=5 plants/treatment).

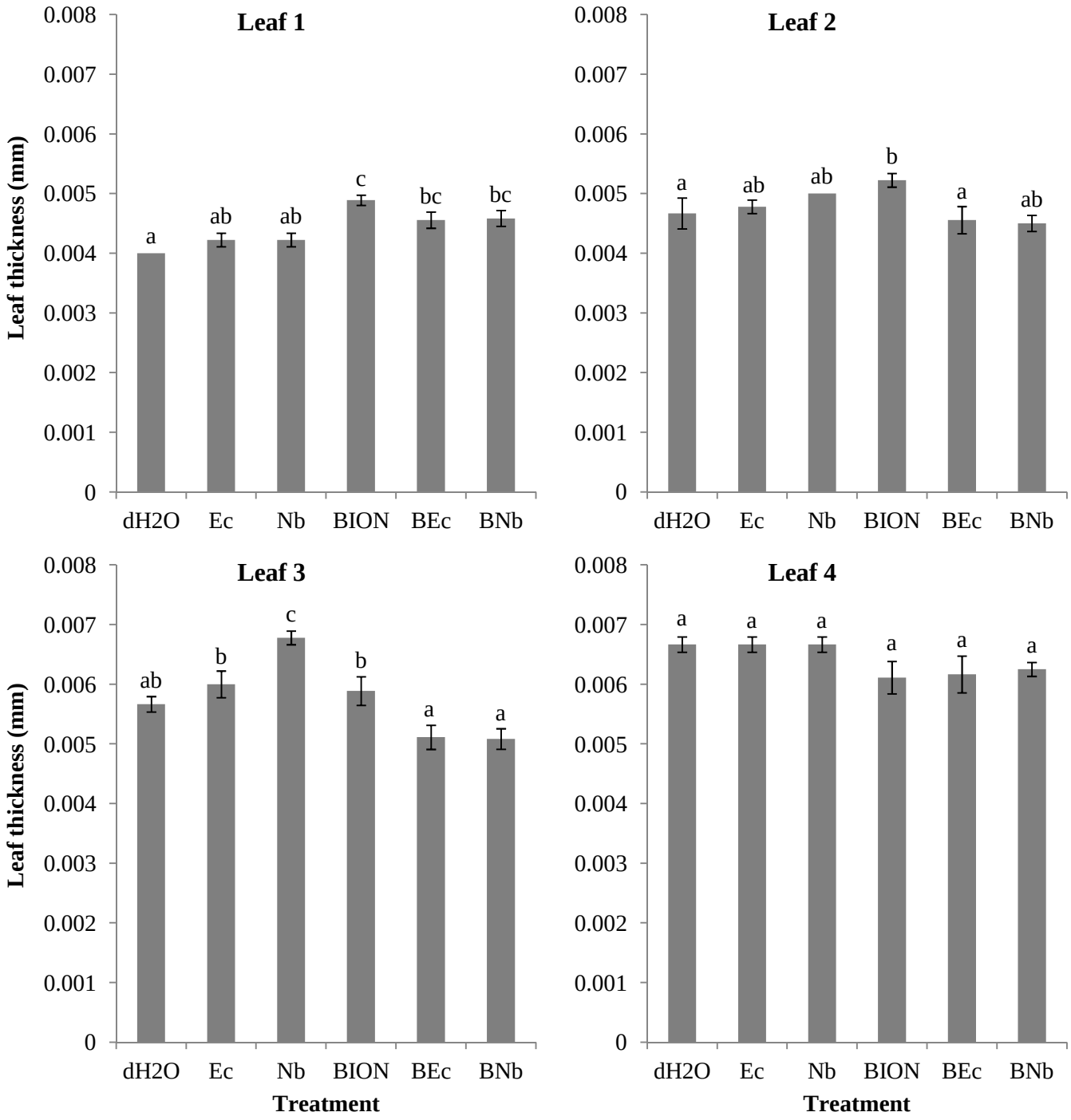

Figure 4.19 The leaf thickness of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, at the end of the experimental period (Week 9). Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not statistically different, $P>0.05$ (n=5 plants/treatment).

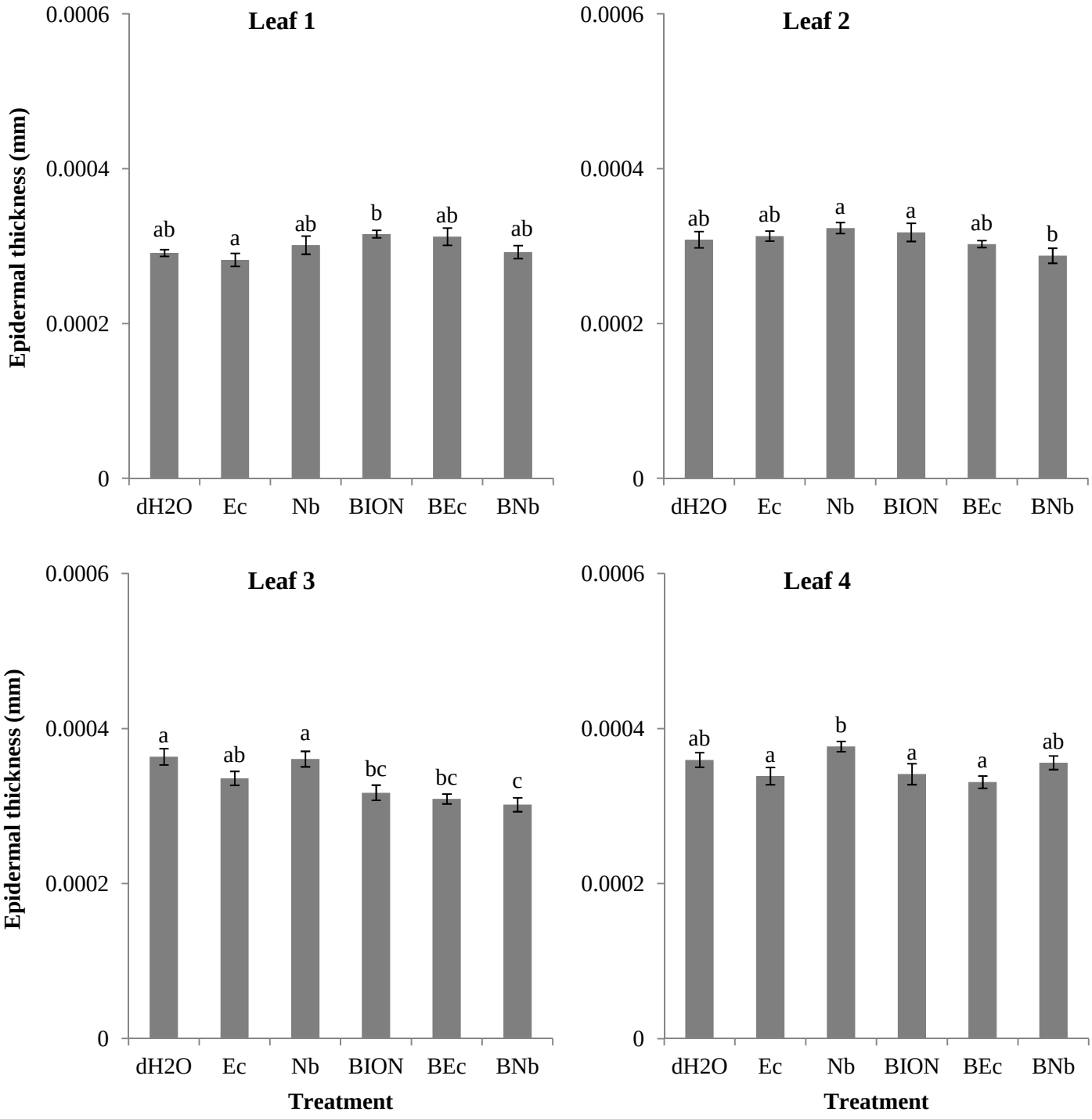

Figure 4.20 The thickness of the upper epidermal cell layer of leaves 1, 2, 3 and 4 of water hyacinth plants in all treatments, at each of the destruction weeks (Week 9). Treatments: BEc = BION® and *Eccritotarsus catarinensis*, BION = BION® only, BNb = BION® and *Neochetina bruchi*, dH_2O = distilled water, Ec = *E. catarinensis* only, and Nb = *N. bruchi* only. Error bars represent the standard error around the mean. Means followed by the same letter are not statistically different, $P>0.05$ (n=5 plants/treatment).

4.3.3 Investigating the relationships

4.3.3.1 Leaf toughness and thickness vs biochemical compositions

No correlations were observed between the leaf toughness and the thickness of the leaves and leaf toughness and the thickness of the epidermal cell layer for leaves positioned 2 and 4 (Table 4.8). However, a strong, significant negative relationship was observed between leaf toughness and leaf thickness for leaf 3 in treatment BNb; an increase in leaf thickness resulted in a decrease in leaf toughness, suggesting a SA-induced resource allocation cost in terms of leaf toughness when investing in leaf thickness in response to weevil feeding ($y = 72.76 - 92.71*x$, R=-0.98, P<0.05, R^2=0.95). Significant relationships between leaf toughness and the percentage leaf carbon content for leaves positioned 2 and 4 for all treatments were not observed, except for leaf 4 in treatment Ec, where a strong positive correlation was found (Table 4.9); as leaf 4 carbon content increased, the toughness of leaf 4 increased. This suggests that herbivory by the mirid on leaf 4 induced increases in leaf 4 carbon content, resulting in increased leaf toughness. Interestingly, whole leaf thickness was strongly and positively related to percentage leaf carbon content in treatment Nb for leaf 4 (Table 4.10), suggesting that as leaf 4 carbon content increased, leaf 4 thickness increased when exposed to *N. bruchi* herbivory on non-BION® treated plants. This result may be leaf age related, as *N. bruchi* adults infrequently feed on older leaves. In addition, the upper epidermal layer thickness was strongly and positively correlated with leaf carbon content in treatment BEc for leaf 4 (Table 4.10); as leaf carbon content increased, leaf 4 epidermal layer thickness increased in the presence of *E. catarinensis* herbivory. Relationships between leaf carbon content and both leaf thickness and upper epidermal thickness in the remaining treatments were not significant. No correlations were observed between the leaf toughness and percentage moisture content for leaves positioned 2 and 4 for all treatments (Table 4.11).

Table 4.8 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf toughness and both leaf thickness and upper epidermal layer thickness for leaf 2 and 4. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf thickness					
Leaf 2	**BEc**	y = 0.0041 + 2.83E-5*x	0.99	0.07	0.99
	BION	y = 0.0016 + 0.0002*x	0.98	0.13	0.96
	BNb	y = -0.0038 + 0.0003*x	0.94	0.21	0.90
	dH_2O	y = -0.0031 + 0.0003*x	0.93	0.24	0.86
	Ec	y = 0.0078 – 4.32E-5*x	-0.29	0.82	0.08
	Nb	y = 0.0002 + 0.0003*x	0.89	0.30	080
Leaf 4	**BEc**	y = 0.004 – 7.82E-7*x	-0.70	0.51	0.50
	BION	y = 0.0052 + 2.16E-6*x	0.01	1.00	0.0001
	BNb	y = 0.005 – 1.57E-6*x	-0.37	0.76	0.13
	dH_2O	y = 0.0058 – 3.67E-6*x	-0.02	0.99	0.0003
	Ec	y = 0.0028 + 0.0001*x	0.85	0.35	0.72
	Nb	y = 0.0076 – 3.72E-5*x	-0.34	0.78	0.11
Upper epidermal layer thickness					
Leaf 2	**BEc**	y = 0.0003 – 8.41E-7*x	-1.00	0.07	0.99
	BION	y = -0.0002 + 1.83E-5*x	0.78	0.43	0.61
	BNb	y = -1.58E-5 + 1.31E-5*x	0.98	0.14	0.95
	dH_2O	y = 8.08E-5 + 8.90E-6*x	0.65	0.55	0.42
	Ec	y = 4.10E-5 + 1.30E-5*x	0.86	0.34	0.74
	Nb	y = -5.48E-5 + 1.82E-5*x	0.90	0.29	081
Leaf 4	**BEc**	y = 0.0003 – 1.50E-6*x	-0.54	0.64	0.29
	BION	y = 0.0006 – 8.76E-6*x	-0.38	0.75	0.15
	BNb	y = 0.0006 – 1.17E-5*x	-0.74	0.47	0.55
	dH_2O	y = 0.0007 – 1.33E-5*x	-0.85	0.36	0.72
	Ec	y = 0.0002 + 6.54E-6*x	0.81	0.40	0.66
	Nb	y = 0.0003 + 1.47E-6*x	0.80	0.41	0.64

Table 4.9 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between percentage leaf carbon content and leaf toughness for leaves 2 and 4. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 2	**BEc**	y = 154.89 – 2.76*x	-0.72	0.28	0.52
	BION	y = 52.82 – 0.63*x	-0.41	0.50	0.17
	BNb	y = 94.97 – 1.53*x	-0.93	0.06	0.87
	dH_2O	y = 2.00 + 0.55*x	0.13	0.84	0.02
	Ec	y = 50.94 – 0.60*x	-0.29	0.64	0.08
	Nb	y = -9.25 + 0.87*x	0.90	0.29	0.80
Leaf 4	**BEc**	y = 30.54 – 0.06*x	-0.06	0.96	0.003
	BION	y = 1.99 + 0.52*x	0.13	0.84	0.02
	BNb	y = 526.02 – 12.02*x	-0.82	0.39	0.68
	dH_2O	y = 33.94 – 0.25*x	-0.19	0.76	0.04
	Ec	y = -47.39 + 1.69*x	1.00	**0.05**	0.99
	Nb	y = 65.82 – 1.03*x	-0.34	0.78	0.12

Table 4.10 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf carbon content and both leaf thickness and upper epidermal layer thickness for leaf 2 and 4. *P*-values in bold indicate significant positive relationships.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf thickness					
Leaf 2	**BEc**	y = 39.18 + 1090.71*x	0.69	0.51	0.48
	BION	y = 45.83 – 852.5*x	-0.31	0.62	0.09
	BNb	y = 44.04 - 325*x	-0.31	0.80	0.09
	dH_2O	y = 39.8 + 455*x	0.52	0.37	0.27
	Ec	y = 42.36 – 149.23*x	-0.18	0.78	0.03
	Nb	y = 36.79 + 813.21*x	0.42	0.73	0.17
Leaf 4	**BEc**	y = 37.70 + 767.86*x	0.62	0.57	0.39
	BION[i]	-	-	-	-
	BNb	y = 42.75 - 245*x	-0.38	0.75	0.14
	dH_2O	y = 46.02 - 465*x	-0.52	0.48	0.27
	Ec	y = 47.26 – 1099.29*x	-0.50	0.50	0.25
	Nb	y = 35.54 + 810*x	0.86	0.06	0.73
Upper epidermal layer thickness					
Leaf 2	**BEc**	y = 40.55 + 12520.15*x	0.37	0.76	0.14
	BION	y = 41.92 – 1905.88*x	-0.07	0.92	0.004
	BNb	y = 49.42 – 21648.95*x	-0.82	0.38	0.68
	dH_2O	y = 39.64 + 7990.03*x	0.31	0.61	0.10
	Ec	y = 44.61 – 9570.60*x	-0.33	0.58	0.11
	Nb	y = 42.14 – 1017.52*x	-0.01	0.99	0.0002
Leaf 4	**BEc**	y = 13.95 + 89725.43*x	1.00	**0.003**	1.00
	BION[i]	-	-	-	-
	BNb	y = 43.23 – 5877.24*x	-0.77	0.44	0.60
	dH_2O	y = 45.19 – 4610.40*x	-0.07	0.93	0.004
	Ec	y = 81.86 – 1.30E-5*x	-0.79	0.21	0.63
	Nb	y = 35.47 + 14648.20*x	0.59	0.29	0.35

[i] Insufficient replicates to perform the simple regression method

Table 4.11 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf moisture content and leaf toughness for leaves 2 and 4. *P*-values in bold indicate significant positive relationships, $P < 0.05$.

	Treatment	Equation	*R*	*P*-value	R^2
Leaf 2	**BEc**	y = 28.06 + 0.08*x	0.46	0.43	0.22
	BION	y = 5.33 + 0.26*x	0.79	0.11	0.62
	BNb	y = 54.00 – 0.30*x	-[i]	-	-
	dH_2O	y = -24.22 + 0.56*x	0.30	0.63	0.09
	Ec	y = 12.30 + 0.17*x	0.26	0.67	0.07
	Nb	y = -2.53 + 0.32*x	0.99	0.09	0.98
Leaf 4	**BEc**	y = -39.11 + 0.82*x	0.82	0.39	0.67
	BION	y = -42.95 + 0.82*x	0.53	0.35	0.29
	BNb	y = -23.68 + 0.63*x	0.47	0.53	0.22
	dH_2O	y = -14.05 + 0.43*x	0.58	0.31	0.33
	Ec	y = 63.29 -0.51*x	-0.97	0.17	0.93
	Nb	y = 85.33 – 0.69*x	-0.76	0.45	0.57

[i] Insufficient replicates to perform the simple regression method

4.3.3.2 Leaf toughness versus agent feeding

No correlations were observed between the leaf toughness and the percentage feeding area by *E. catarinensis* for leaves positioned 2 and 4 for treatments BEc and Ec (Table 4.12). The percentage feeding area by *N. bruchi* for leaves 2 and 4 for treatments BNb and Nb was very little with minimal variances between data; therefore, correlations between leaf toughness and percentage feeding area by *N. bruchi* could not be computed. The number of *N. bruchi* feeding scars was then used to test for correlations between leaf toughness and *N. bruchi* feeding. No correlations were observed between the leaf toughness and the number of *N. bruchi* feeding scars for leaves positioned 2 and 4 for treatments BNb and Nb (Table 4.12).

Table 4.12 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf toughness and insect herbivory for leaves 2 and 4. *P*-values in bold indicate significant relationships. The correlation between leaf toughness and *Neochetina bruchi* feeding scars for leaf 2 was omitted as the result obtained was negligible.

		Treatment	Equation	*R*	*P*-value	R^2
Leaf 2	**% Feeding**	**BEc**	y = 28.65 + 0.30*x	0.83	0.08	0.70
		Ec	y = 28.40 – 0.16*x	-0.80	0.10	0.64
	# Scars	**BNb**	y = 34.16 - 0.20*x	-0.55	0.33	0.31
		Nb[i]	-	-	-	-
Leaf 4	**% Feeding**	**BEc**	y = 26.37 + 0.0003*x	0.0002	0.10	0.00
		Ec	y = 27.74 – 0.35*x	-0.94	0.23	0.88
	# Scars	**BNb**	y = 32.71 – 0.28*x	-0.59	0.30	0.01
		Nb	y = 31.61 – 1.24*x	-0.36	0.76	0.13

[i] Insufficient replicates to perform the simple regression method

4.3.3.3 Leaf thickness versus agent feeding

No correlations were observed between the leaf thickness and the percentage feeding area by *E. catarinensis* for leaves positioned 2 and 4 for treatment BEc (Table 4.13). A significant negative correlation was observed between the leaf thickness and the percentage feeding area by *E. catarinensis* for Leaf 4 for treatment Ec (Table 4.13), suggesting that with increased feeding by the mirid, Leaf 4 thickness decreased. This result was not surprising, as water hyacinth mounted compensatory growth in response to *E. catarinensis* (Chapter 3), suggesting that water hyacinth is reducing leaf structural defences and reallocating resources to another function, possibly growth, in response to the mirid (compensatory growth vs. defence).

No correlation was observed between the leaf thickness and the percentage feeding area by *E. catarinensis* for Leaf 2 for treatment Ec. As explained in the previous section, the number of *N. bruchi* feeding scars also was used to test for correlations between leaf thickness and *N. bruchi* feeding. No correlation was observed between the leaf thickness and the number of feeding scars by *N. bruchi* for Leaf 2 for treatment BNb. However, a significant positive correlation was observed between the leaf thickness and the number of feeding scars by *N. bruchi* for Leaf 4 for treatment BNb (Table 4.13). An increase in feeding scars correlated with an increase in leaf thickness. This result was not expected; treatment BNb was hypothesised to exhibit a negative relationship between leaf thickness and the number of *N. bruchi* feeding scars, where an increase in feeding scars correlates with a decrease in leaf thickness. However, the positive correlation observed between the leaf thickness and the number of feeding scars by *N. bruchi* for Leaf 4 for treatment BNb (where an increase in feeding scars correlated with an increase in leaf thickness), may suggest a possible response to infection by pathogenic organisms; adult weevil feeding scars expose the internal leaf tissue, which has been shown to facilitate pathogenic infection (Moran, 2005). Through the induction of the SA pathway using BION®, the pathway typically up-regulated in response to pathogen attack, it is speculated that with the allowance of pathogenic related defences, water hyacinth leaf laminae thickened in response to increased pathogen infection. No correlation was observed between the leaf thickness and the number of feeding scars by *N. bruchi* for Leaf 4 for treatment Nb (Table 4.13).

Table 4.13 The straight line equations, *R*, *P*-values and R^2 obtained from the simple regression method for analysing the relationship between leaf thickness and insect herbivory for leaves 2 and 4. *P*-values in bold indicate significant relationships. The correlation between leaf thickness and *Neochetina bruchi* feeding scars for leaf 2 was omitted as the result obtained was negligible.

		Treatment	Equation	*R*	*P*-value	R^2
Leaf 2	**% Feeding**	**BEc**	y = 0.005 – 2.78E-5*x	-0.50	0.67	0.25
		Ec	y = 0.005 – 2.22E-5*x	-0.50	0.67	0.25
	# Scars	**BNb**	y = 0.0021 + 0.0001*x	0.94	0.06	0.89
		Nb[i]	-	-	-	-
Leaf 4	**% Feeding**	**BEc**	y = 0.01 – 0.0003*x	-0.60	0.40	0.36
		Ec	y = 0.008 – 0.0001*x	-1.00	**0.00**	1.00
	# Scars	**BNb**	y = 0.005 + 7.90E-5*x	0.97	**0.03**	0.95
		Nb	y = 0.0083 – 0.0003*x	-0.76	0.45	0.57

[i] Insufficient replicates to perform the simple regression method

4.3.4 Summary of results

A summary of results is presented in Table 4.14. In contrast to the results obtained in Chapter 3, herbivory by the mirid and weevil did not intensify on BION® treated plants. Adult weevil feeding scars exhibited negligible differences between BION® and non-BION® treated plants exposed to weevil herbivory. The similarities in insect feeding between BION® and non-BION® treated plants may be a result of inadequate environmental conditions, namely temperature.

Regardless of the negligible differences in weevil and mirid feeding, herbivory and the exogenous application of BION® had notable impacts on water hyacinth structural leaf quality (i.e. leaf toughness, leaf thickness and epidermal layer thickness). The independent

applications of BION® (BION treatment) and inoculations of insects (Ec and Nb treatments) had no impact on water hyacinth leaf toughness; leaf toughness remained comparable to the control treatment. However, when BION® treated plants were exposed to herbivory, water hyacinth leaves toughened significantly, suggesting that the elicitation of SA did not constitutively result in tougher leaves; toughness was induced after continuous exposure to herbivory. The same result was observed in all leaf positions; treatments BEc and BNb produced tougher leaves, 1 to 4, than the other 4 treatments. Unexpectedly, the younger leaves were tougher than older leaves in all treatments. However, the difference in leaf toughness between young and old leaves sprayed with BION® was intensified by the exposure to herbivory, further suggesting an induced structural response regulated through SA-mediated defence pathways. The comparable leaf toughness of non-BION® treated plants exposed to *E. catarinensis* and *N. bruchi* herbivory (Ec and Nb) to the leaves in the control treatment (dH_2O) was expected, due to the significant reductions in leaf percentage carbon content recorded in Chapter 3.

On the other hand, leaves on plants treated with BION® and exposed to weevil herbivory were significantly thinner than the non-BION® treated leaves. Additionally, non-BION® treated plants exposed to *N. bruchi* herbivory were thicker than the control treatment (dH2O). This suggests that JA-mediated defence pathways play a role in leaf thickening in response to weevil herbivory. While, non-BION® treated plants exposed to mirid herbivory (Ec) did not significantly differ in leaf thickness in comparison to the control treatment, a significant negative relationship between leaf 4 thickness and percentage mirid feeding was observed; as *E. catarinensis* feeding increased, leaf thickness decreased.

The same trends and observations were observed in upper epidermal cell layer thickness, where non-BION® treated plants exposed to weevil herbivory (Nb) produced thicker upper

epidermal layers than plants treated with BION® (BNb), further suggesting an induced structural response by way of SA-mediated pathways in response to feeding by the weevil. This was particularly evident in leaves 2 and 3, where leaf upper epidermal layers in treatment Nb were significantly thicker than leaves in treatment BNb. Non-BION® treated plants exposed to weevil herbivory had significantly thicker epidermal layers than plants exposed to mirid herbivory (Ec). This result was expected and suggests that water hyacinth mounts different structural responses when attacked by different insect feeding guilds; *N. bruchi* herbivory induced a structural response, while *E. catarinensis* did not.

In comparison to the leaf age effects exhibited in leaf toughness (younger leaves were tougher than older leaves, especially when treated with BION®), leaf thickness and epidermal layer thickness increased with age in all treatments. In the youngest leaf (leaf 1), BION® treated plants were thicker than non-BION® treated plants, specifically in comparison to the control treatment (dH_2O).

The possible mechanisms behind the contrasting effects of *E. catarinensis* and *N. bruchi* herbivory, and the exogenous application of BION®, on water hyacinth structural leaf quality traits are discussed below.

Table 4.14 A summary of all the differences observed in the 6 experimental treatments for all plant and insect parameters measured, in relation to the control treatment. o = no significant change in or no significant relationship, + = increases observed, but not necessarily significant, - = decreases observed, but not necessarily significant. Symbols followed by * indicates significant increases or decreases observed.

	Treatment					
	BEc	**BION**	**BNb**	**dH_2O**	**Ec**	**Nb**
Nutritive quality[i]						
% Nitrogen content	+	+*	+	o	-	-
% Carbon content	+	-*	-*	o	-*	-*
C:N	o	-*	-	o	+	o
% Leaf moisture	-*	o	-*	o	o	o
Plant parameters						
Leaf toughness	+*	o	+*	o	o	+
Leaf thickness	o	o	o	o	o	+*
Upper epidermal thickness	-	-	-*	o	-	+
Leaf toughness vs thickness	o	o	o	o	o	o
Leaf toughness vs epidermal thickness	o	o	o	o	o	o
Leaf toughness vs % carbon	o	o	o	o	+*	o
Leaf thickness vs % carbon	o	o	o	o	o	+(4)*
Epidermal thickness vs % carbon	+(4)*	o	o	o	o	o
Leaf toughness vs % moisture	o	o	o	o	o	o

	Treatment			
	BEc	**Ec**	**BNb**	**Nb**
Insect parameters				
% Ec feeding	o	o	n/a	n/a
% Nb feeding	n/a	n/a	o	o
Weevil feeding scars	n/a	n/a	o	o
Leaf toughness vs % Ec feeding	o	o	n/a	n/a
Leaf toughness vs Nb scars	n/a	n/a	o	o
Leaf thickness vs % Ec feeding	o	+(4)*	n/a	n/a
Leaf thickness vs Nb scars	n/a	n/a	+(4)*	o

[i] Summarised data taken from Chapter 3. Numbers in parentheses indicate leaf position.

4.4 DISCUSSION

In the previous chapter (Chapter 3), the biological control agents, *E. catarinensis* and *N. bruchi* exhibited differing preferences and performances on BION® and non-BION® treated water hyacinth plants. Both insects showed a significant preference for, and better performance, on BION® treated plants, which resulted in adversely impacted plants in comparison to non-BION® treated plants, particularly for mirid treated plants. The discernible increases in mirid and weevil preference and performance were attributed to the increased availability of nitrogen in the leaves. The increases in leaf nitrogen content were a result of the application of BION®, eliciting SA up-regulation. Non-BION® treated water hyacinth plants were not as adversely impacted, likely due to insect-induced resistance mechanisms. The plant's response to the biological control agents was species-specific; water hyacinth mounted a compensatory response to outgrow *E. catarinensis* herbivory, and mounted an induced defence response to reduce the impact of *N. bruchi* herbivory.

The aim of this chapter was to investigate these responses further by examining the final expressions of the induced defence pathways. Due to the improved nutritive quality of water hyacinth leaves (increases in nitrogen and decreases in carbon indicates good plant quality (Center and Dray, 2010) (Chapter 3)) by means of the elicitation of SA up-regulation, it was hypothesised that SA mediates nitrogen-based defences, incurring a cost in terms of carbon-based defences (Herms and Mattson, 1992) and water hyacinth growth and reproduction (Mithöfer and Boland, 2012). In effect, it was speculated that BION® treated water hyacinth plants would exhibit decreases in structural components of leaf quality, such as leaf toughness, leaf thickness and upper epidermal cell layer thickness. On the other hand, non-BION® treated water hyacinth plants were expected to exhibit increases in structural defences in response to the weevil, as the SA-mediated defence pathways were intact. In

response to mirid herbivory, non-BION® treated plants were predicted to show reductions in structural components of leaf quality, as resources would have been reallocated to plant growth (compensatory response).

Unexpectedly, feeding by *E. catarinensis* and *N. bruchi* did not demonstrate increases in leaf percentage area damage on BION® treated plants in the present study. In addition, no differences in feeding between leaf positions were observed. However, plants exposed to herbivory by *E. catarinensis* appeared more stressed than plants exposed to *N. bruchi* herbivory. In addition to percentage feeding area, the number of adult weevil feeding scars did not differ significantly between BION® and non-BION® treated plants, but the preference for younger leaves (leaf 1 and 2) was still evident, which corroborates the results obtained in the previous chapter and in previous studies (Wright *et al.*, 1989, Center and Wright, 1991). The non-significant results obtained for insect feeding is attributed to temperature effects; the present study took place in late summer-early autumn, when temperatures may have been below optimal for insect performance. Declines in temperatures have been shown to reduce biological control agent performance (Del Fosse, 1977, Julien *et al.*, 1999, Coetzee *et al*., 2007a). Regardless of the negligible differences observed between agent feeding on BION® and non-BION® treated plants, changes in structural components of leaf quality were obvious, yet variable, when plants were treated with BION® and exposed to herbivory, in combination and independently. For the purpose of this chapter, references will be made to the observed changes in insect performance and preferences recorded in Chapter 3.

Leaf toughness

Leaf toughness contributes to structural properties of leaf quality and may mechanically hinder an insects feeding and entry (chewing or piercing), and reproductive performance

(limited oviposition) (e.g. Tanton, 1962, Wright and Bourne, 1986, Wheeler and Center, 1996); therefore, insects exhibit a preference for softer leaves. Carbon-containing compounds, such as lignin, hemicellulose and cellulose (expressed as cell wall fibre) and phenolic compounds, largely contribute to leaf toughness and cell wall strengthening, and are generally costly for the plant to produce (Coley, 1988, Eraso and Hartley, 1990, Graça and Zimmer, 2005, Westbrook *et al.*, 2011, Santiago *et al.*, 2013). Furthermore, these compounds cannot be reclaimed before leaf senescence; therefore they are often produced in plants with longer leaf life-spans (Coley, 1988). Silicon deposits in plant leaves in response to herbivory also have been recorded to contribute to leaf toughness, through cell wall strengthening (Keeping and Meyer, 2002, Yamaji *et al.*, 2008, Ranger *et al.*, 2009, Moles *et al.*, 2013).

Non-BION® treated water hyacinth plants did not increase the toughness of their leaves significantly in response to herbivory by the mirid, when compared to the control treatment. This result was expected, because water hyacinth exhibited a compensatory growth response in response to herbivory by *E. catarinensis* (Chapter 3); therefore, resources that were reallocated to growth to effectively outgrow the impact of mirid herbivory most likely incurred a cost in terms of inducible structural defences (Rhodes and Cates, 1976, McKey, 1979, Fincher *et al.*, 2008). This was supported by the decreases in carbon content of non-BION® treated leaves exposed to mirid herbivory in Chapter 3. Trade-offs between plant growth and leaf toughness have been reported in the literature. A study by Coley (1988) investigated the growth and defence properties of 41 neotropical tree species and found that investment into leaf toughness was negatively correlated with growth rate. In addition, carbon compound synthesis requires large amounts of energy, and leaves with high carbon concentrations consequently incur high resource costs (Unwin and Kriedemann, 2000); therefore, the reductions in leaf carbon content observed in non-BION® treated plants

exposed to mirid herbivory are attributed to water hyacinth reallocating resources to plant growth instead of leaf structural traits, as observed by the increases in water hyacinth plant growth observed in Chapter 3. This notion also is supported by the "growth-rate theory" as discussed in Chapter 1. Plants with rapid growth rates and subsequent short leaf life-spans do not invest in defences that are costly and permanent (irretrievable), such as lignins and tannins (Coley, 1988); water hyacinth follows suit in response to the mirid.

A significant positive relationship was observed between carbon content and leaf toughness of leaf 4 in non-BION® treated plants exposed to *E. catarinensis* herbivory, suggesting that increased carbon accumulation in leaf 4 subsequently resulted in increased leaf toughness in response to mirid feeding. This result was considered to be the result of leaf age; as water hyacinth leaves age, they typically get tougher and phenolic concentrations increase (Wright and Bourne, 1986, Center and Wright, 1991). It is evident that the increase in phenolic compounds did not deter *E. catarinensis*, as the mirid exhibited a preference for older leaves, particularly leaf 4 (Hill *et al*., 1999a).

In contrast, BION® treated plants exposed to *E. catarinensis* herbivory exhibited significant increases in leaf toughness compared to treatments Ec and dH_2O, for all leaf positions. A similar result was observed for BION® treated plants exposed to *N. bruchi* herbivory; leaves in treatment BNb were significantly tougher than leaves in treatments Nb and dH_2O. This suggests that the elicitation of SA-mediated defence pathways induced a leaf toughness response in water hyacinth; however, plants independently treated with BION® had leaf toughness comparable to the control treatment. Therefore, despite the induction of SA, water hyacinth was capable of reserving resources spent on unnecessary defences and only mounted increased leaf toughness in response to herbivory. The mechanisms behind this are unclear. Leaf toughness as a defence strategy is commonly associated with pathogen

defences regulated through SA mediated defence pathways (e.g. lignification, Freeman and Beattie, 2008). Moreover, according to the percentage carbon content and C:N ratio results obtained in Chapter 3, it appears that the increases in leaf toughness for plants treated with BION® and exposed to herbivory were not a result of carbon-based structural changes. Although not investigated here, silicon deposits may explain the increased toughness in BION® treated plants exposed to herbivory (Keeping and Meyer, 2002, Ranger *et al.,* 2009), particularly as water hyacinth constitutively comprises 50% silicon (Makhanu, 1997). Therefore, it is possible that the plant concentrated these silicon deposits in response to herbivory when only SA-mediated plant defences were activated. Silicon depositions in water hyacinth in response to herbivory and BION® application thus merits further investigation.

Interestingly, Takashima *et al*. (2004) recorded an increase in nitrogen investment into leaf cell walls of evergreen tree species, consequently reducing nitrogen availability for photosynthesis. It was postulated that the investment of greater amounts of nitrogen in cell walls increase leaf toughness and subsequent leaf life-span (Takashima *et al*., 2004). The significant increases in leaf toughness in water hyacinth leaves treated with BION® may be a result of increased allocations of nitrogenous compounds into cell walls (e.g. peroxidases (Passardi *et al*., 2004)). This notion is supported by the significant increases in leaf nitrogen content of BION® treated plants, reported in Chapter 3. Ultimately, SA-mediated defence pathways regulated the increases in nitrogenous compounds in water hyacinth leaves, which are most likely pathogen defence related; although, whether these nitrogenous-based compounds are structural or toxic to herbivores is unclear. However, these nitrogenous-based compounds clearly have nutritive value with regards to insect herbivory, as evidenced by increased mirid and weevil preference for BION® treated plants (Chapter 3). Once again, further investigation into biochemical analyses, such as protein activity, is required.

In comparison to herbivory by the mirid, herbivory by *N. bruchi* did not induce increases in leaf toughness in non-BION® treated plants. This result was un-expected, as it was hypothesised that weevil feeding induced changes in leaf structural traits by means of SA-mediated defence pathways; however, leaf carbon content significantly declined in non-BION® treated plants exposed to *N. bruchi*. Regardless, as was found in a study on *Zinnia elegans* (Asterales: Asteraceae) resistance to aphids, silicon deposition could be responsible for the leaves toughening in response to weevil herbivory (Ranger *et al*., 2009) and could explain the differences in weevil feeding on BION® and non-BION® treated plants observed in Chapter 3, although potential silicon deposition was ineffective against weevil herbivory.

In effect, water hyacinth did not toughen its leaves in response to *N. bruchi* herbivory in the present study. This was particularly interesting as studies investigating water hyacinth leaf hardness in response to herbivory by *N. eichhorniae* exhibited otherwise. For example, the study by Wright *et al*. (1989) found that water hyacinth mounted an induced physical response in terms of leaf hardness in response to herbivory by *N. eichhorniae*, in addition to reductions in moisture content, which also were not observed in BION® treated plants exposed to *N. bruchi* in Chapter 3. The conflicting results obtained in the present study for *N. bruchi* and the results obtained by Wright *et al*. (1989) for *N. eichhorniae* may suggest a species-specific plant response by water hyacinth. While numerous plants exhibit species-specific insect-induced responses (Karban and Myers, 1989), the varying water hyacinth responses to the two *Neochetina* spp. weevils deserve further investigation.

Interestingly, in the present study, the younger leaves in all treatments were nominally tougher than the older leaves, which also conflicts with other results in the literature (Bazzaz *et al*., 1987, Pinto *et al*., 2009, Magalhães *et al*., 2012) and observed increases in C:N ratios with leaf age in Chapter 3. As discussed above, silicon deposits may explain the increased

toughness in younger leaves (Keeping and Meyer, 2002, Ranger *et al.*, 2009). Due to water hyacinth comprising 50% silicon (Makhanu, 1997), it is possible that water hyacinth concentrates these silicon deposits in younger leaves. This has been shown in young leaf blades of *Pleioblastus chino* Makino (Poaceae, Bambusoideae) which exhibited significant accumulations of silicon before leaf opening (Motomura *et al*., 2006). It is speculated that the increased toughness of young water hyacinth leaves in all non-BION® treated plants may be a result of leaf silicon deposition, which may constitutively provide structural support and in turn confer resistance against potential herbivores; however further investigations into changes in leaf silicon content are needed to elucidate this. Another hypothesis is that of structural nitrogen compounds, as nitrogen content was significantly higher in younger leaves

Leaf thickness and upper epidermal layer thickness

Leaf thickness and upper epidermal layer thickness are structural properties of leaf quality that play vital roles in plant-insect interactions, namely defence and physiological functioning (Glover, 2000). Leaf thickness contributes to the overall toughness of a leaf (Kitajima and Poorter, 2010); by increasing the thickness of the photosynthetic mesophyll in leaves, plants enable thickening and strengthening of leaves (Read *et al*., 2009). Plant species adapted to open sunny environments (e.g. open fields or bodies of water) incur very few costs in thickening leaves (Roderick *et al*., 1999, Read *et al*., 2009). By deduction, plants in open, well-lit environments should, on average, have leaves with greater structural leaf thickness and thus leaf toughness. However, the contribution of leaf thickness to leaf toughness (i.e. the relationship), is variable, as recorded in numerous studies (e.g. Kitajima and Poorter, 2010, Westbrook *et al*., 2011). Furthermore, leaf thickness is related to leaf carbon content and carbon isotope composition (Teeri *et al.*, 1981).

Whole water hyacinth leaf thickness results varied across treatments. At the end of the trial, BION® treated plants did not exhibit reductions in leaf thickness in comparison to the control treatment. Interestingly, a study by Uzunova and Popova (2000) investigating the effect of exogenous applications of SA on barley seedling leaf anatomy (*H. vulgare*), found that seedlings treated with SA exhibited reductions in laminae thickness. Uzunova and Popova (2000) attributed these changes in leaf anatomy to SA induced leaf growth and photosynthetic inhibition properties. Although, similar to leaf toughness, the reductions in leaf thickness of BION®-treated water hyacinth were induced by herbivory and not by the application of BION®, further suggesting an insect-induced structural response by means of SA-mediated defence pathways. The mechanisms behind this response are unclear. While leaf thickness in BION®-treated plants exposed to herbivory decreased at the end of the trial, there were clear leaf age effects. In the youngest leaf (leaf 1), treatments in BION®-treated plants were significantly thicker than the control treatment.

The reductions in leaf thickness negate the notion that the thickening of leaves contributes to the over-all toughness of leaves, because significant increases in leaf toughness were observed in treatments BEc and BNb. Furthermore, the relationships between leaf toughness and leaf thickness were negligible in the present study. Therefore, the toughness of leaves in BION® treated plants exposed to insect herbivory was most likely not a result of leaf thickening and carbon-based defences (no significant relationships were observed between leaf carbon content and leaf toughness, except for treatment Ec), but possibly a result of other structural components (Westbrook *et al.*, 2011). As proposed above, silicon and/ or nitrogen deposits in leaf cell walls may have contributed to the increases in leaf toughness of treatments BEc and BNb. Therefore, the role of SA-mediated silicon and/or nitrogenous-based compounds in water hyacinth leaf toughness deserves attention.

Leaf thickness increased in non-BION® treated plants exposed to *N. bruchi* herbivory (Nb) in comparison to BION® treated plants exposed to weevils (significant increase). Upper epidermal layer thickness also increased in comparison to BION® treated plants exposed to weevils. However, these results were largely an effect of increases in leaf 3 thickness, further indicating a leaf age effect. Whereas weevil herbivory was negligible in the present study, notable differences in weevil herbivory were observed between BION® and non-BION® treated plants in Chapter 3, particularly for adult weevil feeding scars on leaf 3, where weevils produced significantly fewer feeding scars on non-BION® treated plants (Nb). These reductions in comparison to treatment BNb are attributed to the significant increases in leaf 3 thickness, possibly reducing leaf tissue accessibility. The increases in leaf 3 thickness may be attributed to adult weevil feeding preferences on leaf 2 and when sampling occurred the leaf had moved to position 3 (Center, 1985, Center and Wright, 1991). According to Center (1985), *Neochetina* spp. weevils oviposit their eggs in mature leaves, namely leaf 3 and 4. As a result, the increased thickness in leaf 3 indicates a structural response by water hyacinth to minimise weevil feeding and oviposition. Oviposition-induced plant responses have frequently been recorded in the literature (Seino *et al.*, 1996, Colazza *et al.*, 2004, Little *et al*., 2007). These results indicate an induced structural response in water hyacinth leaves in response to weevil feeding and reproductive behaviour through the SA-mediated defence pathway. Water hyacinth leaves increased in whole leaf thickness and epidermal cell layer thickness, most likely to make leaf tissue less accessible to the weevil. Numerous studies have measured leaf thickness and the role it plays in insect-induced responses, and have subsequently found that leaf thickness contributes to the deterrence of herbivory (Gianoli and Hannunen, 2000, Krings *et al*., 2007).

On the other hand, no discernible trends were observed for water hyacinth leaf thickness and upper epidermal cell layer thickness when plants were exposed to *E. catarinensis* herbivory,

which would support the notion that water hyacinth responds to mirid herbivory through compensatory growth (e.g. biomass accumulation and chlorophyll content comparable to the control treatment, Chapter 3) and that there is most likely a trade-off between enhanced structural defences and compensatory plant growth (Mithöfer and Boland, 2012).

4.5 CONCLUSION

Structural components of leaf quality varied across treatments, exhibited various trends and supported numerous defence strategies; however clear-cut strategies cannot be specified from the results obtained in this chapter. As concluded in Chapter 3, the results observed in the present study substantiate the species-specific insect-induced responses mounted by water hyacinth, the ineffectiveness of SA on anti-herbivore defences in terms of *E. catarinensis* and *N. bruchi* resistance in water hyacinth, and the effect of leaf age on biochemical and structural components of leaf quality. SA, in response to insect herbivory, induced the toughening of water hyacinth leaves. Interestingly, leaf toughness was not correlated with leaf carbon content and leaf thickness (properties contributing to over-all leaf toughness). Therefore the observed increases in leaf toughness were probably a result of increased concentrations of nitrogenous compounds in the cell walls, as supported by the significant increases in leaf nitrogen in BION® treated plants observed in Chapter 3. Whether these compounds are toxic, structural, or both, in function is unclear and requires further investigation. Regardless of their purpose, their role in water hyacinth plant defences was not applicable to mirid and weevil herbivory in the present study.

The nominal changes (i.e. leaf thickness and upper epidermal thickness), or the lack thereof (leaf toughness), of structural components of leaves exposed independently to *E. catarinensis* herbivory corroborates the notion that water hyacinth mounts a compensatory

growth response in response to mirid herbivory. Ultimately, resources were reserved for plant growth instead of structural defences. Conversely, herbivory by *N. bruchi* induced a structural response in water hyacinth in terms of leaf and upper epidermal layer thickness, but not in leaf toughness. Although the thickening of the leaves did not contribute to leaf toughness, it is postulated that the thickening of the upper epidermal cell layer contributed significantly to over-all thickness of the leaves and subsequent reductions in weevil feeding. Epidermal cell layers contribute significantly to plant fortification and protection from biotic and abiotic stress (Hardham *et al.*, 2007). Therefore, the thickening of the upper epidermal cell layer, namely in leaf 3, in response to *N. bruchi* activity is a structural response mounted by water hyacinth.

Although further analyses into the biochemical compositions of water hyacinth leaves in response to herbivory and the application of BION® are required to make definitive statements about the roles silicon and nitrogenous-based compounds play, the present study exhibits the impact of insect herbivory on water hyacinth leaf quality traits, the different responses water hyacinth mounts in response to the mirid and the weevil, and the first attempt at identifying the roles that SA and JA play in water hyacinth defences.

CHAPTER 5

GENERAL DISCUSSION

5.1 INTRODUCTION

The expression of chemical and physical insect-induced plant responses and the subsequent insect-plant interactions, are mediated by plant hormones. In particular, salicylic acid (salicylates) (SA) and jasmonic acid (jasmonates) (JA), which typically regulate defences against pathogens and insect herbivores, respectively (Traw *et al*., 2003, Reymond *et al.*, 2004, Smith *et al*., 2009). Of relevance to the present study, the use of chemical analogues of SA and JA (plant activators or chemical elicitors, e.g. BION®) have been frequently studied in the field of agronomy, where the exogenous applications of these plant activators elicit appropriate plant resistance against certain pests of food crops (e.g. Dietrich *et al.*, 2004, Heil *et al*., 2000). The synthesis and production of chemical elicitors, such as BION®, have provided researchers with a novel tool to investigate insect-plant interactions, not only in agricultural systems, but in natural or invaded systems.

The applied field of weed biological control makes use of tight plant-insect interations to control invasive weeds, by re-establishing the interactions between an exotic plant and its natural enemies (McFadyen, 1998). Regardless of this crucial criterion, insect-induced plant responses and the ability of invasive weeds to alleviate injury are rarely investigated in weed biological control programmes. Subsequently, this study investigated the insect-induced responses of the world's most invasive aquatic plant, water hyacinth (*E. crassipes*) in

response to herbivory by the mirid *E. catarinensis*, and the weevil *N. bruchi*. Biological control practitioners have been releasing arthropod natural enemies onto water hyacinth for over 40 years yet during this time very little research has focused on water hyacinth's induced responses to the agents, particularly at the cellular level.

The motivation behind this thesis was to further investigate water hyacinth-insect interactions, using the plant activator, BION®, in an attempt to determine the role of plant hormones in regulating the insect-induced defences in response to herbivory by *E. catarinensis* and *N. bruchi* through elicitation of the production of salicylic acid. In addition, the final expressions, biochemical and/or structural, of these regulatory processes were determined. Various conclusions have been drawn and are categorised as follows:

1. *The role of salicylic acid in water hyacinth insect-induced responses*. Salicylic acid does not play a role in mirid- and weevil-induced water hyacinth defences.
2. *Water hyacinth defence allocation costs.* Certain insect-induced responses incur allocation costs to water hyacinth growth and other insect-plant-related responses.
3. *Species-specific insect-induced water hyacinth responses.* Water hyacinth responds differently to each of the biological control agents tested in this thesis -compensatory growth versus jasmonic acid-mediated defences.
4. *The effect of leaf age on water hyacinth insect-induced responses.* Leaf age had clear differentiation effects on defence allocation within water hyacinth plants.

The above results are discussed and placed in the context of insect-induced plant responses, and the ensuing impact these responses have on the plant-insect interactions between water hyacinth and the two specialist herbivores, *E. catarinensis* and *N. bruchi*, within the water hyacinth biological control programme.

5.2 THE ROLE OF SALICYLIC ACID IN WATER HYACINTH INSECT-INDUCED RESPONSES

The results obtained in this study confirmed that the induction of SA-mediated pathways in water hyacinth plants, through the exogenous application of BION®, did not confer resistance against *E. catarinensis* and *N. bruchi* herbivory (Chapter 3). *Eccritotarsus catarinensis* and *N. bruchi* expressed an increased preference for SA-induced plants, and enhanced performance (e.g. increased palatability indices (*N. bruchi* only), feeding and damage, and increases in population growth (*E. catarinensis* only)), consequently resulting in adversely impacted water hyacinth growth and physiological function (e.g. reduced leaf production, leaf turnover (*E. catarinensis* only), second petiole lengths (*N. bruchi* only) and reduced photosynthetic productivity (i.e. chlorophyll content)). These results suggest that SA-mediated defences in water hyacinth do not confer resistance against insect herbivores, particularly phloem feeders and leaf chewers, which is in accordance with SA-dependent defence theory and related studies in the literature (e.g. Gaffney *et al.*, 1993, Ruuhola *et al.*, 2001, Traw *et al.*, 2003, Achuo *et al.*, 2004, Sarwar *et al.*, 2005, Liu *et al.*, 2006, Berger *et al.*, 2007, Farouk *et al.*, 2008, Röder *et al.*, 2011). SA-mediated defence pathways generally confer resistance against pathogens, whereas JA-mediated defence pathways confer resistance against insect herbivores (Glazebrook *et al.*, 2005). In support of these results, a study by Röder *et al.* (2011) found that the alpine plant, *Adenostyles alliariae* (Gouan) A. Kern. (Asterales: Asteraceae), suffered increased attack rates by two species of leaf beetle, *Oreina elongata* (Suffrian, 1851) and *O. cacaliae* (Schrank, 1785) (Coleoptera: Crysomelidae), when plants were treated with BTH (acibenzolar-S-methyl, SA) in comparison to the control treatments. On the other hand, pathogenic infection rates by the rust fungus, *Uromyces cacaliae* (Uredinales: Pucciniaceae), were significantly lower in BTH treated *A. alliariae* plants.

Although the present study confirmed that SA does not play a role in mirid and weevil-induced defences, the biochemical processes involved in the SA induction of water hyacinth plants shed light on the potential defences employed by water hyacinth in response to pathogen infection, by means of SA-mediated defence pathways. SA-induced water hyacinth plants elicited increases in total nitrogen content in all leaves, particularly in the youngest leaves. These increases in nitrogen were nutritionally beneficial to the mirid and the weevil; BION® treated plants, having higher leaf nitrogen levels, were favoured by the biological control agents (as seen in the increased palatability indices, Chapter 3). Whereas these increases in nitrogen were favoured by the insects, it is most likely that these increases play a role in deterring pathogen infection (e.g. protein based defences (Passardi *et al*., 2004)). The nature of these nitrogenous compounds is unclear and requires further biochemical analyses.

Numerous plant proteins contributing to plant pathogen defence have been recognised and classified (van Loon *et al*., 2006). It is likely that the elevated levels of total leaf nitrogen in water hyacinth plants is a result of defence related protein synthesis and accumulation. One particular plant protein, PR-9, which is a specific type of peroxidise, may play a role in SA-induced pathogen defence in water hyacinth, particularly as it acts in plant cell wall reinforcement. The significant increases in leaf toughness in BION® treated plants, in response to mirid and weevil herbivory (Chapter 4), may be a result of PR-9 activity, promoting the deposition of structural compounds, such as lignin and suberin (Hiraga *et al*., 2001). As discussed in Chapter 4, the increases in leaf toughness content in BION® treated plants exposed to insect herbivory were attributed to structure-enhancing nitrogenous compounds (Takashima *et al*., 2004). Peroxidases are a common form of pathogen resistance recorded in the literature, which provides minimal to no resistance against insect herbivores (e.g. Baker and Orlandi, 1995, Young *et al*., 1995, Traw *et al*., 2003, Asthir *et al*., 2009). The only result confounding this scenario is the comparable toughness of water hyacinth

leaves independently treated with BION® (treatment BION) to the control treatment (dH_2O); however this was attributed to the inducible nature of plant protein activity. Peroxidases occur constitutively, but increases in activity occur when stimulated by herbivory or any other damage (Dietrich *et al.*, 2004). Naturally, PR-9 is not the only plant protein associated with pathogen resistance (Passardi *et al*., 2004). However, PR-9 explains the elevated levels of nitrogen and increases in leaf toughness of SA-induced water hyacinth plants. Analyses of protein activity could determine the possible proteins associated with water hyacinth pathogen defence (Fernandes *et al*., 2006), particularly in response to the prevalent pathogens, *Cercospora piaropi* Tharp and *Acremonium zonatum* (Sawada) Gams (Harely, 1990).

As discussed in Chapter 4, possible silicon deposits in water hyacinth leaves in response to herbivory also may have resulted in the increases in leaf toughness, through cell wall strengthening (Keeping and Meyer, 2002, Yamaji *et al*., 2008, Ranger *et al.*, 2009, Moles *et al*., 2013), particularly as water hyacinth constitutively comprises 50% silicon (Makhanu, 1997). Cell wall silicon deposition and subsequent increases in leaf toughness have been postulated as a defence mechanism employed by plants to increase pathogen resistance (Jones and Handreck, 1967). Interestingly, a study by Massey *et al*. (2006) found that increases to leaf abrasiveness in four perennial grass species, *Agrostis capillaries* L., *Brachypodium pinnatum* L., *Festuca ovina* L. and *Lolium perenne* L. (Poaceae), as a result of silicon deposition, deterred herbivory by two chewing insects, the African army worm, *Spodoptera exempta* Walker (Lepidoptera: Noctuidae), and the desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae), but was consequently ineffective against a phloem feeder, the grain aphid, *Sitobion avenae* Fabricius (Hemiptera: Aphididae). In the present study, potential SA-induced silicon deposits did not confer resistance against both chewing

(*N. bruchi*) and phloem-feeding (*E. catarinensis*) insects, but may play a role against particular pathogens.

5.3 WATER HYACINTH SA-INDUCED DEFENCE ALLOCATION COSTS

The expression of insect-induced plant responses requires resources which are typically limited in numerous environments (Agrawal, 2005). Consequently, induced defences are costly, because their production generally results in the reallocation of limited resources away from plant growth and reproduction (Hoffland *et al.*, 1996, Baldwin and Preston, 1999, Cipollini *et al*., 2003, Mithöfer and Boland, 2012). Ultimately, there is a trade-off between induced defences and plant growth. The cost of an induced response is measured by observing reductions in plant survivorship, vegetative growth and seed production and survival in the absence of herbivores (Cipollini *et al*., 2003).

The present study revealed a physiological cost of water hyacinth SA induction, where plant biomass accumulation, leaf production and leaf chlorophyll content significantly declined in plants treated with BION® only in comparison to the control treatment. A reduction in water hyacinth leaf chlorophyll content is an indication of reduced photosynthetic productivity (Vasu *et al*., 2009), which could account for the reductions in water hyacinth growth. A few studies have demonstrated the down-regulation of photosynthetic genes, unrelated to defence production, with the treatment and induction of SA (Pancheva *et al*., 1996, Uzunova and Popova, 2000). Although it is likely that the induction of SA resulted in reduced photosynthetic productivity in water hyacinth leaves, the present study did not investigate photosynthetic activity at the gene level; therefore, further investigations are required to confirm the down-regulation of photosynthetic genes in SA-induced water hyacinth plants.

In addition to a physiological cost of water hyacinth SA induction, an ecological cost of SA induction occurred. The induction of SA and the subsequent increases in leaf nitrogen content made water hyacinth plants more palatable and attractive to the insect herbivores (Chapter 3), which resulted in increased plant damage. This enhanced insect activity incurred a cost to water hyacinth growth and physiological function, as significant reductions in plant biomass, leaf production and leaf chlorophyll content were observed in plants treated with BION® and exposed to herbivory in comparison to the non-BION® treated plants exposed to herbivory (Chapter 3). In addition, the significant increases in SA-induced toughness of leaves did not confer resistance against insect herbivory (Chapter 4). Therefore, resources that were reallocated to the up-regulation of SA-mediated pathogen defences, namely the elicitation of nitrogenous compounds (thought to be structural proteins) and leaf toughness (induced by herbivory), exposed water hyacinth to greater insect attack. In a similar study, Heil *et al*. (2000) observed slower growth rates and lower biomass accumulation in BION® treated wheat plants (*Triticum aestivum, cv,* 'Hanno' (Poaceae)) in comparison to untreated control plants, which grew faster and accumulated greater biomass. In addition, wheat seed production was negatively impacted when plants were treated with BION®, illustrating the trade-off in resource allocation. In another study investigating the costs associated with pathogen resistance, radish cultivars (*Raphanus sativus* L.) were exposed to fungal wilt disease, *Fusarium oxysporum* Schlecht. Emend. Snyder & Hansen (Nectriaceae), and consequently resulted in negative relationships between radish growth rate and resistance, concluding that high levels of *F. oxysporum* infection are associated with resource costs.

Whereas trade-offs between plant growth and insect-induced defences are common, trade-offs also are observed between particular defence mechanisms, e.g. trade-offs between leaf trichome production and leaf toughening (Bjorkman and Anderson, 1990). In the present study, a possible trade-off between leaf thickening and an unidentified mechanism for leaf

toughening was identified in SA-induced water hyacinth plants. An increase in induced leaf toughness, with decreases in leaf thickness, was observed in BION® treated plants exposed to insect herbivory, although not significantly correlated (Chapter 4). Results in Chapter 4 confirmed that leaf thickening, which contributes to the over-all toughness of a leaf, did not play a role in the toughening of SA-induced water hyacinth leaf thickness. This suggests that resources were allocated to another form of water hyacinth leaf toughening. As discussed above in section *5.1.1 The role of salicylic acid in water hyacinth insect-induced response*, the increases in SA-induced water hyacinth leaf toughness may be a result of protein activity or silicon deposition.

5.4 SPECIES-SPECIFIC INSECT-INDUCED WATER HYACINTH RESPONSES

Insect-induced plant responses are species- and feeding mode-specific (Achuo *et al.*, 2004, Speight *et al.*, 2008), because plant defences exhibit differential efficacy against certain herbivores (Traw *et al*., 2003). Water hyacinth mounted species-specific responses to each of the biological control agents tested in this thesis.

Jasmonic-acid mediated defences against N. bruchi

JA-mediated defence pathways most likely contributed to *N. bruchi*-induced water hyacinth defences, because in the absence of JA-mediated defence pathways (BION® treated plants), water hyacinth plants were severely compromised (i.e. reduced second petiole lengths, leaf production and chlorophyll content, Chapter 3), and in the presence of JA-mediated defence pathways, water hyacinth plants maintained comparable growth to control treatments. In addition, leaf structural responses were initiated, namely leaf thickness, when JA-mediated defence pathways were not inhibited (Chapter 4). Plants thicken leaves, which contribute to

the over-all toughness of leaves, in response to herbivory to prevent further damage (Potter and Kimmerer, 1988). Significant reductions in plant palatability indices, and adult weevil feeding scars on water hyacinth plants (Chapter 3), are attributed to the significant increases in water hyacinth leaf thicknesses (Chapter 4). The role that JA played in *N. bruchi*-induced water hyacinth defences follows JA-dependent defence theory (Smith *et al*., 2009) and related studies in the literature (e.g. Iverson *et al*., 2001).

Even when a plant is attacked by insect species within a single feeding guild, plant responses may vary (Speight *et al.*, 2008). Water hyacinth did not initiate leaf toughening in response to *N. bruchi*, despite leaf thickening (Chapter 4). Conversely, water hyacinth leaves hardened in response to herbivory by *N. eichhorniae* (Wright *et al*., 1989), which highlights the species-specific properties of water hyacinth insect-induced defences. Moreover, the differences in the weevil-induced responses exhibited by water hyacinth may be due to the level of damage inflicted by each weevil at particular water nutrient levels. *Neochetina bruchi* performs better on plants grown in high water nutrient levels compared to *N. eichhorniae* (Heard and Winterton, 2000). In effect, the type and intensity of damage an insect inflicts on a plant determines the plant's response.

Compensatory growth in response to E. catarinensis

Numerous plants have evolved the ability to tolerate insect herbivory through compensatory mechanisms, by adjusting various physiological parameters of growth (Baldwin and Preston, 1999). JA-mediated defence pathways are likely to play a minimal to no role in *E. catarinensis*-induced water hyacinth defences, because water hyacinth displayed a compensatory growth response when attacked by the mirid; biomass accumulation, leaf production and chlorophyll content remained comparable to the control treatment. According to the theory of allocation costs, the reallocation of resources to growth most likely

precluded resources for jasmonate activity (Rhodes and Cates, 1976, Herms and Mattson, 1992). This was supported by the lack of leaf structural changes, regulated by JA (Chapter 4), observed in plants exposed to mirid herbivory only (Chapter 4). Moreover, JA is typically unimportant for vegetative growth (Wu and Baldwin, 2010).

The relative chlorophyll content in non-BION® treated plants exposed to mirid herbivory, which was somparable to the control treatment signifies the maintenance of the integrity of the photosynthetic system (Netto *et al.*, 2005, Vasu *et al.*, 2009, Biradar *et al.*, 2010). Increases or maintenance in photosynthetic activity after herbivore attack may occur, because many plants function below their maximum capacity (Doust, 1989). Therefore water hyacinth allocated resources to photosynthetic function to achieve maximum leaf functioning capacity in order to outgrow the negative impacts of *E. catarinensis* herbivory.

5.5 THE EFFECT OF LEAF AGE ON WATER HYACINTH INSECT-INDUCED RESPONSES

Leaves differ in life-span and function, and will exhibit different insect-induced responses, accordingly (Feeny, 1970, Rhoades and Cates, 1976). Generally, young leaves are more highly defended than old leaves, because they are of higher quality and are therefore preferred by herbivores (Coley, 1983, 1986). Leaf age had clear effects on water hyacinth insect-induced responses and insect preferences investigated in the present study. In most plant species, younger leaves are higher in nitrogen (i.e. amino acids/protein) and lower in structural reinforcements, such as lignin, in comparison to older leaves (Rhoades and Cates, 1976, Bazzaz *et al.*, 1987). Water hyacinth follows suit, with young leaves characteristic of higher nitrogen content, lower carbon content and softer tissues (Center and Wright, 1991). Interestingly, although water hyacinth leaves in the present study exhibited increased

nitrogen levels in the younger leaves, the younger leaves were nominally tougher than the older leaves. This was attributed to one of two chemical expressions; 1) the increases in nitrogen content reflected increases in structural forming proteins, such as PR-9, or 2) increases in silicon deposition. Either option is highly likely and merits further investigation. Ultimately, water hyacinth actively defends its younger leaves against biotic and most likely abiotic stresses.

The differences in biochemical compositions of leaves and the associated expressions of plant responses resulted in species-specific preferences for particular aged leaves. *Eccritotarsus catarinensis* generally feeds on older leaves, namely leaf 4 (Hill *et al*., 1999a), which has been attributed to the low concentrations of nitrogen in older leaves; therefore, the mirid preferentially feeds on older leaves to avoid nitrogenous toxins. This may be a mirid-induced water hyacinth response to deter the mirid from feeding on younger, photosynthetic leaves. *Eccritotarsus catarinensis* preferences remained similar in the present study, where feeding was greatest on leaf 4; particularly on BION® treated plants, because leaf 4 was softer and lower in nitrogen content.

On the other hand, *N. bruchi* expressed a preference for younger leaves, most likely because of their nutritional value (higher allocations of nitrogenous compounds, which are ineffective against the weevil), especially on BION® treated plants. Furthermore, regardless of the toughened younger leaves, weevil herbivory still did not decline. This was particularly interesting as toughened leaves can have negative impacts on insect mandibular structure (e.g. Raupp, 1985). However, the percentage feeding results obtained for the weevil did not significantly differ between the BION® and non-BION® treated plants, but the number of adult feeding scars increased in the BION® treated plants; feeding scars were smaller, but greater in number in the BION® treated plants. A few studies have shown that some insect

feeding patterns are driven by nutritional benefits rather than structural insect-induced defences (e.g. Kouki and Manetas, 2002, Magalhaes *et al.*, 2012). Ultimately, *N. bruchi* may have sacrificed mandibular wear for access to nutritional plant tissues, and that the access to nutrition outweighed the cost of mandibular wear; however further investigations into *N. bruchi* feeding behaviour is required.

5.6 CONCLUSION

Collectively, the results of this study clearly demonstrate that water hyacinth insect-induced responses are dynamic and complex, which is not unlike many of the plants in the plant kingdom. Water hyacinth protects itself from attack by *N. bruchi* by responses activated by the JA defence cascade, probably inducing leaf structural alterations (e.g. leaf thickness), which subsequently reduced weevil herbivory. Conversely, water hyacinth outgrows the impact of *E. catarinensis* herbivory through compensatory growth; however, it is not definitively clear that the JA defence cascade did not play a role in mirid-induced water hyacinth responses. Lastly, although SA-induced defences are likely to be aimed at pathogen infection, it was clear that induction of SA-mediated defence pathways incurs ecological and physiological costs to water hyacinth, which may be beneficial to the biological control of water hyacinth programme.

The negative effect of the plant activator, BION®, on water hyacinth plant growth and the associated insect-induced defences clearly had an impact on water hyacinth integrity, and for this reason the applied use of BION® in the water hyacinth biological control programme would be highly effective for obtaining greater control of the weed. In addition, numerous SA-induced plants exhibit reduced seed set and viability (e.g. Heil *et al.*, 2000), and water hyacinth may very well exhibit a similar response. Investigations into the impact of SA-

induction of water hyacinth seed set and lifetime viability requires investigation, and may have implications on the accumulation and longevity of seeds in bank sediments.

Clearly, no plant defence strategy provides perfect defence, as various factors influence the mechanism by which resistance traits are regulated and expressed. Nutrient availability, environmental conditions, insect herbivore densities, and plant densities are a few factors impacting insect-induced plant responses (Mattson and Addy, 1975, Karban and Myers, 1989, Schoonhoven *et al*., 2005). The insect densities investigated in Chapter 2 and used in this thesis (Chapter 3 to 4), exhibited non-lethal changes in water hyacinth growth and physiological function when plants were not treated with BION®, and grown in mesotrophic water nutrient conditions. However, when plants were treated with BION®, the lowest mating pair caused significant amounts of damage. It is likely that these low densities would provide effective water hyacinth control in combination with BION® application, which would reduce the number of weevils required during mass rearing and releasing strategies. However, water nutrient levels of aquatic systems vary from system to system, and these varying conditions undoubtedly impact water hyacinth insect-induced responses differently in the field, as they have been shown to impact water hyacinth growth and reproduction (Center and Spencer, 1981, Xie *et al*., 2004). Plant responses different from the ones observed in this thesis may occur, and some may have a negative effect on insect herbivory (e.g. reduces herbivory). Therefore, the use of BION® under field conditions to aid the biological control of water hyacinth requires further investigation into the impacts of different nutrient loads on SA-induced water hyacinth responses when exposed to insect herbivory, in addition to non-target effects and cost-benefit analyses. Furthermore, this study did not determine the specific nitrogenous compounds present within BION® and non-BION® treated plants, and determining whether the elevation of leaf nitrogenous compounds

were nutritive or defensive (e.g. amino acids/proteins versus various toxic compounds), could elucidate the results obtained in the present study.

In conclusion, water hyacinth is likely to induce species-specific defence responses in response to the biological control agents released for the control of the weed. Not only does this study contribute to the limited knowledge of aquatic macrophyte insect-induced defences, but it reveals for the first time the mechanisms driving water hyacinth plant-insect interactions. Furthermore, it underscores the importance of understanding the dynamic relationships between an invasive weed and its natural enemies in order to achieve effective control of the target weed.

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