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PLANT MEDIATED EFFECTS OF EARTHWORMS ON APHID DYNAMICS

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PLANT MEDIATED EFFECTS OF EARTHWORMS ON APHID DYNAMICS

BY

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Plant mediated effects of earthworms on aphid dynamics

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The individual and combined effects of the endogeic and epigeic groups of earthworms on the growth of Chinese cabbage (*Brassica rapa*), and on the subsequent growth and development of the generalist sap-sucking herbivore *Myzus persicae* were determined in separate pot experiments.

Many previous studies have investigated the relationship between soil biodiversity and aboveground plants, but few researches have considered the indirect interaction between soil biota and above-ground aphids. In this study the individual effects of *Aporrectodea rosea* and *Allolobophora chlorotica* and the combined effects of *A. rosea* and *A. chlorotica*, *Aporrectodea caliginosa* and *Satchellius mammalis*, *A. chlorotica* and *S. mammalis*, and *A. rosea*, *A. caliginosa*, *Lumbricus rubellus* and *S. mammalis* on plant morphology and physiology and aphid development (nymphs day⁻¹, fecundity and number of adults) were investigated.

Plant growth was affected by the presence of *A. rosea* which caused increases in plant biomass, height of plant, leaf surface area and specific leaf area (SLA). Mean number of leaves per plant was unaffected by worm density. In contrast, increasing density of *A. chlorotica* had no effect on any aspect of plant performance. The combined effect of *A. rosea* and *A. chlorotica* resulted in a similar increase in plant biomass to *A. rosea* alone. While the combined effects of *A. caliginosa* and *Satchellius mammalis*, *A. chlorotica* and *S. mammalis* and *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* caused increases in all plant parameters except leaf number. Additionally, the individual effect of *A. rosea* and the combined effects of *A. caliginosa* and *S. mammalis*, *A. chlorotica* & *S. mammalis* and *A. rosea* and *A. caliginosa*, *L. rubellus* and *S. mammalis* resulted in increased leaf nitrogen concentration.

Aphid development was also affected by the presence of earthworms. The nymphs day⁻¹, fecundity and numbers of adults were significantly increased with increases in earthworm densities. The interaction between all groups of earthworms and their influence on aphid growth showed that the combined effect of two different groups was greater than the individual groups.

Proteomic techniques were used to compare protein patterns in the plants. The combined effects of *A. caliginosa* and *S. mammalis*, *A. chlorotica* and *S. mammalis*, *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* earthworms on plant resulted in differences in number and kind of protein between plant treated with earthworms and the control, but no significant difference in proteins volume.

Effects of earthworms on plant growth and aphid development are shown to be modified by increasing density and interactions between different species and functional groups of earthworms.

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List of abbreviation

Abbreviations used	Glossary of terms used
SLA	Specific leaf area
G	Gram
cm	Centimeter
Cm ²	Square centimeter
DIASTM	Delta-T image analysis system-type
2-DE	Two dimensional gel electrophorsis
TCA	Trichloroacetic acid
DTT	Dithiothreitol
IEF	Isoelectric focusing
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
IPG	Immobilized pH gradient
μL	Microliter
GE	General electric
ANOVA	Analysis of variance
CHCA	α-Cyano-4-hydroxycinnamic acid
TFA	Trifluoroacetic acid
MS	Mass spectrometry
MALDI TOF/TOF	Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight
GPS	Global Proteome Server
PMF	Probability mass function

LC-MC	Liquid chromatography mass spectrometry
MW	Molecular weight
PI	Isoelectric point
KDa	Kilo Dalton
SE	Standard error
LSD	Least significant difference
GLM	General linear model
Mn	Manganese
JA	Jasmonic acid
SA	Salicylic acid
N	Nitrogen
C	Carbon
ml	Millilitre
D.W	Distilled water
V	Volt
W	Watt
µg	Microgram
DGGE	Denaturing gradient gel electrophoresis

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Finally, I also extend my thanks to all my friends for their ongoing support.

Dedication

This thesis is dedicated in memory of my father.

My beloved mother and to my beloved family:

My Wife Aras

My lovely daughter, Pana

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of The Ministry of Higher Education and Scientific Research (MOHESR), Iraq.

During the course of the study, relevant postgraduate courses were attended to gain transferable and research skills. Relevant scientific seminars and conferences were regularly attended at which work was often presented.

Word account of main body of thesis: 30.043 words (42.577 including references and appendix).

Signed.....

Date.....

CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Soils have been known to support a great number of organisms for many decades (Darwin, 1859). More recently however, the interactions between soil-dwelling organisms and functionally important aspects of above-ground ecology have become increasingly apparent (Bardgett & Wardle, 2003; Fragoso *et al.*, 1997; Wardle *et al.*, 2004b). The presence of soil organisms, for example via their ability to mineralize organic matter, can increase crop production (Bardgett & Wardle, 2003; Fragoso *et al.*, 1997; Giller *et al.*, 1997; Scheu, Theenhaus & Jones, 1999).

The role of soil organisms as decomposers of organic materials is well established (Brussaard, de Ruiter & Brown, 2007; Fitter *et al.*, 2005; Reichle, 1977), but it has also been proven that they have a key role in interactions between below-ground and above-ground ecosystem processes (Bardgett, Hopkins & Usher, 2005; Werner, 1990), such as herbivory (Ke & Scheu, 2008; Letters, 2005; Masters, Jones & Rogers, 2001; Wurst *et al.*, 2003). By increasing organismal biomass in poor soils, plant production is increased by more than one-third, consequently below-ground processes play a significant role in the regulation of productivity (Sackett, Classen & Sanders, 2010).

The economic importance of studying the interplay between these different environments comes by linking producers and decomposers with above-ground herbivores that feed on crop plants (Bezemer *et al.*, 2005; Poveda *et al.*, 2005; Wardle, 2002). This is particularly relevant to soil biota whose activities provide essential elements for plant growth such as

carbon and nitrogen (Alban & Berry, 1994; Callaham & Hendrix, 1998; Fitter *et al.*, 2005), and those whose activity may affect plant chemistry and impact on the activity of above-ground insect herbivores (Bezemer & van Dam, 2005; Bezemer *et al.*, 2003).

Studies on the interaction between soil biodiversity, plant growth and above-ground ecosystem processes have varied according to the taxonomy and relationship between different soil organisms (Bardgett, Hopkins & Usher, 2005). These studies have demonstrated that soil biodiversity is very important to ecosystem stability and function (Andr n & Balandreau, 1999; Hunt & Wall, 2002). However, there remains some ambiguity regarding the role of soil organisms in above-ground processes (Bowker, Maestre & Escolar, 2009), in particular the impact of soil fauna like earthworms on above-ground insects, such as aphids which have a wide range of commercially important plant hosts, whether crops or fruits (Dixon, 1977; Guerrieri & Digilio, 2008). In addition, to their direct impact on plant cropping, aphids also transfer viruses to the plant (Sylvester, 1989; Will *et al.*, 2007), and increase plant exposure to infection by fungi (Fokkema *et al.*, 1983; Rabbinge *et al.*, 1981).

1.2 Functional biodiversity

1.2.1 Biodiversity and ecosystems

Given the prominent role of soil biota in ecosystem function, a large number of studies have addressed this interaction (Brussaard, 1997; Fitter *et al.*, 2005; Hunt & Wall, 2002; Wolters, 2001). Soil biota have a great role in sustaining ecosystems through their impact on the

distribution of nutrients and water sources (Eriksen-Hamel & Whalen, 2008; Whitford, 1996). In addition, the role of soil biodiversity extends beyond decomposition, as it provides support and protection to the plant via increasing nutrients in the soil (Altieri, 1999). Furthermore, in agroecosystem soils biota perform ecosystem services in pest management via plant stimulation to attract natural enemies of above-ground insects (Arimura, Kost & Boland, 2005; Bezemer *et al.*, 2005; Masters, Jones & Rogers, 2001; Soler *et al.*, 2007).

Hunt & Wall, (2002), observed that the loss of some soil-dwelling functional groups greatly influenced ecosystem functioning. The loss of the microflora group such as bacteria and fungi for example reduced available nitrogen for the plant. However, this decline could be compensated by changes to the abundance of other functional groups. This result is important as it forms the basis of any investigation of the role of macrofauna such as earthworms in influencing plant growth and above-ground interactions with herbivores.

Also, Scheu, (2001), stressed that soil organisms are the foundation for the provision of food for the plant, but compared with root -feeding herbivores such as nematodes and insects, which have a direct effect on plants, the role of earthworms, Collembola and protozoans are more important for plant development.

Earthworms, Collembola and mites contribute to the improvement of the quality and sustainability of the soil through plant litter decomposition and

nutrient recycling; hence they are responsible for providing nutrients for plant growth via the facilitation of the microbial breakdown of organic materials (Eaton *et al.*, 2004; Smetak, Johnson-Maynard & Lloyd, 2007).

Therefore, organisms such as earthworms act as ecosystem engineers, as they directly or indirectly provide the resources and habitats for other species, particularly through physical changes to the soil (Jones, Lawton & Shachak, 1994; Thompson *et al.*, 1993). Microbial diversity also provides a healthy soil; mycorrhiza associations are a commonly understood way in which plant access to soil nutrients is increased (Brussaard, de Ruiter & Brown, 2007). Kardol *et al* (2009), highlighted the role of soil biodiversity in restoring ecosystem function and stability, though with some ambiguity about their ability to restore after human intervention.

Diversity and function, especially in arable ecosystems, is greatly influenced by practices such as tillage and the use of pesticides, which through their negative impacts upon soil biota directly or indirectly affect plant growth (Ammer *et al.*, 2006; Bongers, van der Meulen & Korthals, 1997; Dmowska & Kozłowska, 1988; Lal, 1988). In forests, removal of trees also has great negative effects on soil organisms, which cause soil disturbance, including; imbalance in the ratio of available nutrients such as nitrogen and carbon (Mariani, Chang & Kabzems, 2006).

1.2.2 The ecology of soil organisms

Wardle, (2002) proposed a soil food web structure that shows the major groups of soil fauna; (microfood-web, litter transformers and ecosystem engineers), and the complex interactions between different soil organisms, and how each group provides the energy to other groups (Figure 1.1). Soil biodiversity describes both the range of taxa involved in these food webs and their functionality.

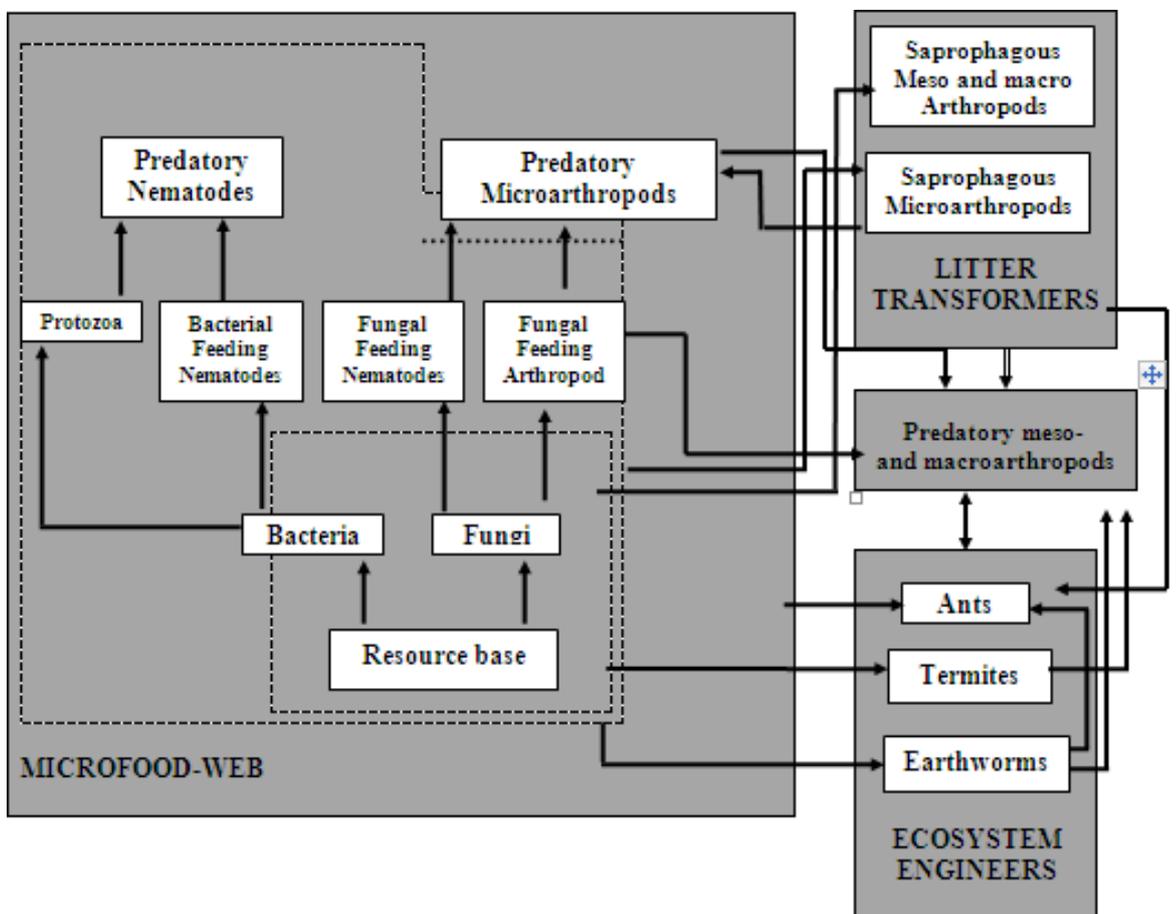


Figure 1.1 Structure of the soil food web, and the interactions between microfood-web and ecosystem engineers (after Wardle, 2002).

The physical and chemical characteristics of the environment and the kind of microbes available play an important role in the abundance of soil organisms in the soil (Beare *et al.*, 1992).

However, in soil the numbers of bacteria found is higher than other groups even at small scales (Grundmann & Gourbiere, 1999). This diverse microbial community including bacteria and fungi, fulfils various roles in the ecosystem, including nitrogen fixation, and are a major nutrient route to the plant (Beare *et al.*, 1991; Flanagan & Van Cleve, 1983).

As one of the principal decomposers, earthworms exert a tremendous influence on ecosystem functions in the soil (Eriksen-Hamel & Whalen, 2007; Paoletti, 1999; Smetak, Johnson-Maynard & Lloyd, 2007; Uvarov, 2009). Blackshaw (1983), for example highlighted the major role of earthworms play in regulating plant growth and development through their ability to mix soil between the surface and deeper layers, in addition to breaking up the soil and increasing ventilation.

Beside their role in plant growth, however, earthworms also provide habitats for other smaller soil biota, via burrowing (Lavelle, 1997). They are also beneficial to agriculture, as they offer an alternative to mechanical soil tillage (Metzke *et al.*, 2007; Römbke, Jänsch & Didden, 2005). Therefore they affect soil properties and composition, through increasing ventilation and the redistribution of soil nutrients (Christensen, 1988; Eisenhauer & Scheu, 2008).

Earthworms are associated indirectly with other organisms that live in the soil such as mycorrhiza, and thus indirectly stimulate plant growth by increasing nitrogen uptake (Eisenhauer & Scheu, 2008; Wurst *et al.*, 2004). Wurst, (2010), found that earthworms reduced the negative effects of nematodes on plant growth by changing soil properties (e.g. enhancing soil microorganisms that are beneficial for plant growth and defense). In a laboratory experiment, the negative effect of the aphid *Myzus persicae* Sulzer on two plant species (*Poa annua* Linnaeus and *Trifolium repens* Linnaeus) were reduced when two different earthworms *Aporrectodea caliginosa* (Savigny 1826) and *Octolasion tyrtaeum* (Savigny 1826) were added to the soil (Scheu, Theenhaus & Jones, 1999).

Earthworms are affected by environmental factors such as temperature and soil moisture (Berry & Jordan, 2001; Wever, Lysyk & Clapperton, 2001), in addition to various agricultural activities such as tillage (Lee, 1985). In soil rich in organisms, especially earthworms, it is not necessary to use chemical material or activities which affect the biotic and abiotic soil characteristics.

In the mesofauna group, Collembola are globally widespread across many different habitats (Hopkin, 1997), and as decomposers, have a pivotal role in ecosystem function (Rusek, 1998). Moreover, it is also known that they can affect plant growth via reducing nitrogen uptake by plants, due to changes in soil nutrients (Scheu, Theenhaus & Jones, 1999; Schütz, Bonkowski & Scheu, 2008).

Nematodes are classified in the group of soil microfauna. They are widespread and abundant in soils, and show varying food preferences, ranging from plant roots to soil microbes (Bongers & Bongers, 1998; Ingham, Moldenke & Edwards, 2000). Nematodes have an important role in soil food webs as they increase soil nitrogen concentrations by feeding on bacteria, nematodes release nutrients into the soil (Anderson *et al.*, 1981; Ingham, Moldenke & Edwards, 2000).

1.2.3 The complex interactions between soil organisms

The effects of soil organisms on plant and above-ground herbivores differ in terms of their individual or combined presence in the soil. The role of earthworms goes beyond that of a decomposer of organic materials, since they directly and indirectly affect soil organisms via their ability to change physical and chemical soil properties (Blanchart *et al.*, 1999; Brown, 1995; Reich *et al.*, 2005). Elmer (2009), highlighted the effects that earthworms have on soil microbial communities, and in reducing plant disease. Daniel & Anderson (1992), showed that earthworms support some bacteria in their gut; which act as decomposers of organic matter and provide nutrients to the earthworm.

Earthworms are known to play an important role in regulating the number and activity of soil arthropods, via their activities and decomposition processes. The epigeic earthworms *Eisenia fetida* (Savigny, 1826) caused increases in the numbers of different soil arthropods; springtails, spiders and astigmatid, prostigmatid, mesostigmatid and oribatid mites,

while psocids were negatively influenced by the presence of *E. fetida* worms (Monroy, Aira & Domínguez, 2011).

Many previous studies have investigated the interactions between earthworms and nematodes (Boyer, Michellon & Reversat, 1998; Senapati, 1992; Tao *et al.*, 2009; Yeates, 1981).

Lohmann, Scheu & Muller (2009) showed how nematodes reduced plant growth, but that the presence of earthworms reduced the negative effects of nematodes on the plant by increasing plant defences (plant biomass and glucosinolates concentration). In the presence of nematodes, the earthworm biomass also increased because of increasing nutrient availability for the earthworms.

It is known that the effects of earthworms on nematodes depend on nematode identity and feeding behaviour. For example while the impact on root-feeding nematodes is negative, earthworms have a positive impact on microorganism-feeding nematodes (Senapati, 1992). Furthermore earthworms suppress the negative effects of root-feeding nematodes, in addition to reducing above-ground insect herbivory (Wurst, 2010). Plant-feeding nematodes were also negatively influenced by the presence of the epigeic *Lumbricus rubellus* (Hoffmeister, 1843) earthworm (Ilieva-Makulec & Makulec, 2002). This results in improved plant performance via improved soil conditions and changes in plant chemistry (nitrogen, phosphorus and carbon).

There are interactive relationships between Collembola and other soil biota (Gange, 2000; Ke & Scheu, 2008; Larsen & Jakobsen, 1996; Scheu, Theenhaus & Jones, 1999). The interaction between Collembola, earthworms and litter distribution has been investigated, and it has been shown that plant growth increased as a result of activities, such as mineralization and the distribution of soil litter so that nitrogen concentration positively increased in the soil (Ke & Scheu, 2008). As decomposers, Collembola have different effects on the number and activity of bacteria and fungi on the soil surface because removal of surface litter by Collembola decreased bacterial and fungal standing crops (Hanlon & Anderson, 1979).

1.3 Relationships between below-ground biodiversity and above-ground insect herbivores

1.3.1 Interactions between soil biota and plants

Soil fauna have a great role in the maintenance of plant productivity (Hunt & Wall, 2002). The interaction between soil organisms and plants are at least two-fold; first there is a direct effect via plant roots, and second an indirect effect via decomposition of soil matter. For example, Van Der Heijden *et al.*, (2008) highlighted that bacteria and fungi have an effect on plant production, which directly impacts on plants and free-living microbes as decomposers, indirectly providing nutrients to the plant. Williams, Birkhofer & Hedlund (2014), confirmed this by demonstrating a significant effect of the microbial community on crop production.

Most below-ground insects have a direct interaction with plants (Brown & Gange, 1989; Gavloski, Whitfield & Ellis, 1992; Riedell, 1990). Larvae of western corn rootworm *Diabrotica virgifera virgifera* (LeConte, 1868) cause reductions in root biomass of Maize *Zea mays* (Linnaeus). (Dunn & Frommelt, 1998). Also, Roubíčková, Mudrák & Frouz (2012) found a negative effect of the wireworm *Agriotes lineatus* (Linnaeus) on *Calamagrostis epigejos* (L.) (Roth, 1788) root and shoot biomass.

Earthworms as a main decomposer in the soil also have indirect influences on plant growth. Scheu (2003), mentioned different mechanisms of the effect of earthworms on plants such as; organic matter mineralization, physical changes of soil, and nutrient availability by interaction with microorganisms. Also earthworms stimulated soybean and maize growth, via mineral matter nitrogen concentration increases in the soil (Eriksen-Hamel & Whalen, 2007).

The effect of earthworms on plant performance is different depending on their species and functional groups. Edwards & Bate (1992), compared two different earthworm groups, and found that the endogeic group *A. caliginosa* and *Allolobophora chlorotica* (Savigny 1826) increased the cereal seedling biomass but much less than the anecic group (*L. terrestris* and *A. longa*).

Plant species also differ in their responses to the presence of earthworms. Scheu & Jones (1999), found that in the presence of *A. caliginosa* and *O. tyrtaeum* the shoot and root biomass of annual meadow grass (*Poa*

annua) increased more than that in white clover (*Trifolium repens* L.). Similarly, the endogeic *A. jassyensis* earthworm positively influenced foliage biomass and nitrogen content of a grass (*Lolium perenne* L.) and a legume (*T. repens*), while a forb (*Plantago lanceolata* L.) showed no response to the presence of earthworm (Wurst, Langel & Scheu, 2005).

Generally, most below-ground herbivores have direct negative effects on the plant, via removal of root biomass and changes to plant physiology (Bezemer *et al.*, 2003; Blossey & Hunt-Joshi, 2003). These studies show that root-feeding herbivores cause reduced plant growth and delayed development via reducing plant viability to absorb nutrients and water, and thereby reducing plant defences against above-ground insect herbivory. Nonetheless, their effects are often species-specific; for example, root-feeding nematodes have direct impacts on plants, via feeding on roots and changing plant biomass (Verschoor, 2002), while bacterial and fungal feeding nematodes have indirect effects on plants via the manipulation of microflora communities (Griffiths, 1994).

However, further research is needed to investigate the interaction between soil fauna and their effects on plant growth, plant defences and insect herbivores.

1.3.2 Plant defences against above-ground insects

Plants are regarded as hosts for different insect herbivores from different orders. In order to overcome the enemy attacks they have developed three different defence mechanisms against these herbivores (Jongsma &

Bolter, 1997) and these include structural (Eigenbrode & Espelie, 1995; Hanley et al., 2007; Price et al., 1980), chemical (Arimura, Kost & Boland, 2005; Broadway *et al.*, 1986; Duffey & Stout, 1996; Ryan, 1990), and biotic (Heil *et al.*, 2000; Wink, 1988) defences.

Plant chemical defences are known to regulate interactions between below and above-ground herbivores, (Bezemer & van Dam, 2005; Howe & Jander, 2008). These chemical responses can occur in any part of the plant (roots and foliage) (Bezemer & van Dam, 2005) either directly by releasing toxic compounds (Agrawal, Tuzun & Bent, 1999; Karban & Baldwin, 2007) or indirectly by releasing chemical compounds to attract natural enemies of herbivores (Arimura, Kost & Boland, 2005; Poecke & Dicke, 2004; Price *et al.*, 1980; Soler *et al.*, 2007; Van Tol *et al.*, 2001), and these defences increase following attacks by herbivores. Foliage defence levels can be changed as a response to attack by root herbivores, and vice versa (Bezemer & van Dam, 2005).

The organisms directly associated with plant roots such as insects, nematodes and pathogens have an influence on above-ground plant defence compounds (e.g. terpenoids or glucosinolates), which frequently have a negative impact on above-ground herbivores (Bezemer *et al.*, 2004; Manninen, Holopainen & Holopainen, 1998). On the other hand, as a response to insect attacks, a plant releases specific compounds to attract natural enemies. For example, cabbage root fly (*Delia radicum* L.) causes chemical compound release from *Brassica nigra*, stimulating natural enemies to attack insects feeding on above-ground tissues (Soler

et al., 2007). Also, the roots of a coniferous plant (*Thuja occidentalis* L.) release a chemical compound, as an SOS signal to attract the entompathogenic nematode *Heterorhabditis megidis* (Van Tol et al., 2001). Similarly, above-ground cotton and corn plants emit chemical signals to attract herbivores natural enemies, e.g. parasitic wasps against caterpillar (Turlings et al., 1995).

Decomposers such as earthworms which are indirectly associated with root plants, also have an effect on above-ground plant defences, either negatively or positively depending on the species. For example, the endogeic *A. caliginosa* earthworm causes an increase in aromatic glucosinolate concentrations in the leaves of white mustard (*Sinapis alba* L.) (Lohmann, Scheu & Müller, 2009). In contrast, the glucoiberin concentrations decrease in the shoot of *B. oleracea* in the presence of the endogeic *O. tyrtaeum* earthworm (Wurst et al., 2006).

On the other hand, the enzyme lipoxygenase (Lox) increases when young rice seedlings are exposed to the endogeic *Millsonia anomala* (Omodeo and Vaillaud, 1967) earthworm (Blouin et al., 2005), it is known that lipoxygenase causes the release of jasmonic acid which is responsible for plant defence by producing pathogenesis-related (PR) proteins as a defensive mechanism (Farmer, Alméras & Krishnamurthy, 2003; Stratmann, 2003; Zhao, Davis & Verpoorte, 2005).

Different physical changes occur in plants in order to defend themselves against herbivores. For instance, the yellow star thistle (*Centaurea*

solstitialis L.) develops spines to avoid the attacks by legitimate and illegitimate flower visitors (Agrawal *et al.*, 2000). While, blackberry (*Rubus bogota* L.) plants defend themselves against butterfly larvae by developing scleromorphic structures on toughened leaves (Björkman & Anderson, 1990).

Other plants deter insect attacks by developing a layer of hairs (trichomes) to prevent or interfere with herbivore feeding. For instance, Woodman & Fernandes (1991) investigated the role of leaf hairs as a structural defence against grasshopper on the common mullein (*Verbascum Thapsus* L.) plant. Also, the trichomes of (*Arabidopsis thaliana* L.) contribute to resistance against the diamondback moth (*Plutella xylostella* L.) (Handley, Ekbom & Ågren, 2005).

Many previous studies have investigated the role of plant structural traits (Agrawal & Fishbein, 2006; Hanley *et al.*, 2007; Herms & Mattson, 1992) and chemical compounds changes (Arimura, Kost & Boland, 2005; Kessler & Baldwin, 2002; Poecke & Dicke, 2004; Stout, Thaler & Thomma, 2006; Zhang & Turner, 2008) as plant defences against herbivores.

However, few studies to date have investigated the role of protein in plant defences against herbivores, where they found that pathogenesis-related (PR) proteins produced as a defensive protein in response to herbivory attacks (Farmer & Krishnamurthy, 2003; Zhao & Verpoorte, 2005).

There is a dynamic biochemical interaction between aphids and plants, since they develop a strategy to counter each other (Miles, 1999; Will & van Bel, 2006). For example, plants defend themselves by producing phenolic compounds which combine with proteins in insect guts that can be very toxic to insects (Miles, 1999). However, the grain aphid (*Sitobion avenae*) has the ability to overcome these toxic compounds in cereals by secreting saliva which contains polyphenol oxidases to detoxify phenolic compounds (Urbanska *et al.*, 1998).

1.3.3 The effect of soil fauna on above-ground insects

There are strong relationships between above and below-ground communities mediated by how the composition and activity of soil decomposers affects above-ground herbivory (Gange, Bower & Brown, 1999; Poveda *et al.*, 2005; Scheu, 2001; Wardle *et al.*, 2004a; Wurst *et al.*, 2003). Plants perform a great role in this relationship by linking between two different habitats (Bardgett, Wardle & Yeates, 1998; Bezemer *et al.*, 2003; Van der Putten *et al.*, 2001). Although most previous studies have focused on above-ground interactions, below-ground herbivores could be an important driver for above-ground processes (Van der Putten *et al.*, 2001), particularly via changing plant chemistry (Brown & Gange, 1990; van Dam, Raaijmakers & van der Putten, 2005; Wurst *et al.*, 2003).

The relationship between above and below-ground herbivores via the mediation of plant defence was demonstrated by Kaplan *et al.*, (2008). Root-feeding nematodes increased the larval weight of two insects

feeding on tobacco, the cabbage looper worm *Trichoplusia ni* (Hubner, 1800) and tobacco hornworm (*Manduca sexta* L.), because the nematodes interfered with foliar nicotine dynamics. Kaplan, Sardanelli & Denno, (2009), also, found that root-feeding nematodes decreased the effect of aphids *M. persicae* on *N. tabacum*. These results suggest that root-feeding nematodes have positive effects on insects with chewing mouth parts and negative effects on insects with sucking mouth parts. It seems that these effects are connected with leaf thickness; it is difficult for insects with sucking mouth parts to penetrate a thicker leaf surface.

Also, the effect of belowground herbivores on aboveground insects depended on their species, since, in the presence of cabbage root fly (*D. radicum*), the number of the cabbage aphid (*Brevicoryne brassicae* L.) was significantly increased in comparison to the cabbage butterfly (*Pieris brassica* L.) and the cabbage moth (*Plutella xylostella* L.) (Pierre *et al.*, 2013).

A further direct effect of soil herbivory on plant performance/herbivory was reported by Bezemer *et al.*, (2003), who showed that in the presence of click beetles (*Agriotes lineatus* L.) which feed on the roots of cotton (*Gossypium herbaceum* L.), the larval performance of beet armyworm *Spodoptera exigua* (Hubner, 1808) was reduced by more than half in comparison with control plants, while plant root biomass also decreased.

The development of the small white butterfly *P. rapae* is slower in the presence of nematodes *Pratylenchus penetrans* (Stekhoven, 1941), and

cabbage root fly (*D. radicum*), but the effect of nematodes in reducing the food quality for the insect on black mustard (*B. nigra*) was higher than that from the root fly (van Dam, Raaijmakers & van der Putten, 2005). However, Kaplan, Sardanelli & Denno (2009), found different interactions between nematodes and above-ground insects depending on herbivore feeding behaviour. Nematodes caused a reduction in numbers of the aphid *M. persica*, but increased growth and abundance of insects with chewing mouth parts such as tobacco hornworm (*M. sexta*). Furthermore, the diversity of the soil biota influences insect herbivores. Wurst & van der Putten (2007), found that nematodes reduced (*M. persicae*) fecundity, while aphids were unaffected by the presence of *A. lineatus*.

Scheu, Theenhaus & Jones (1999), examined interactions between different species of earthworms and Collembola. Aphid development was negatively influenced by the presence of earthworms and Collembola, while their effect on plant biomass depended on species and in combination earthworms and Collembola affected numbers and biomass of each other. Also collembolan-mediated activity caused a reduction of above-ground herbivory by their effect on plant growth, Collembola reducing plant biomass via delaying ear production (Schütz, Bonkowski & Scheu, 2008).

Wurst (2010), pointed to the existence of two mechanisms for how earthworms affect above-ground herbivores: indirectly through chemical changes of plant and directly through the modification of the herbivores habitat via mixing the plant litter in the surface with the soil (Figure 1.2).

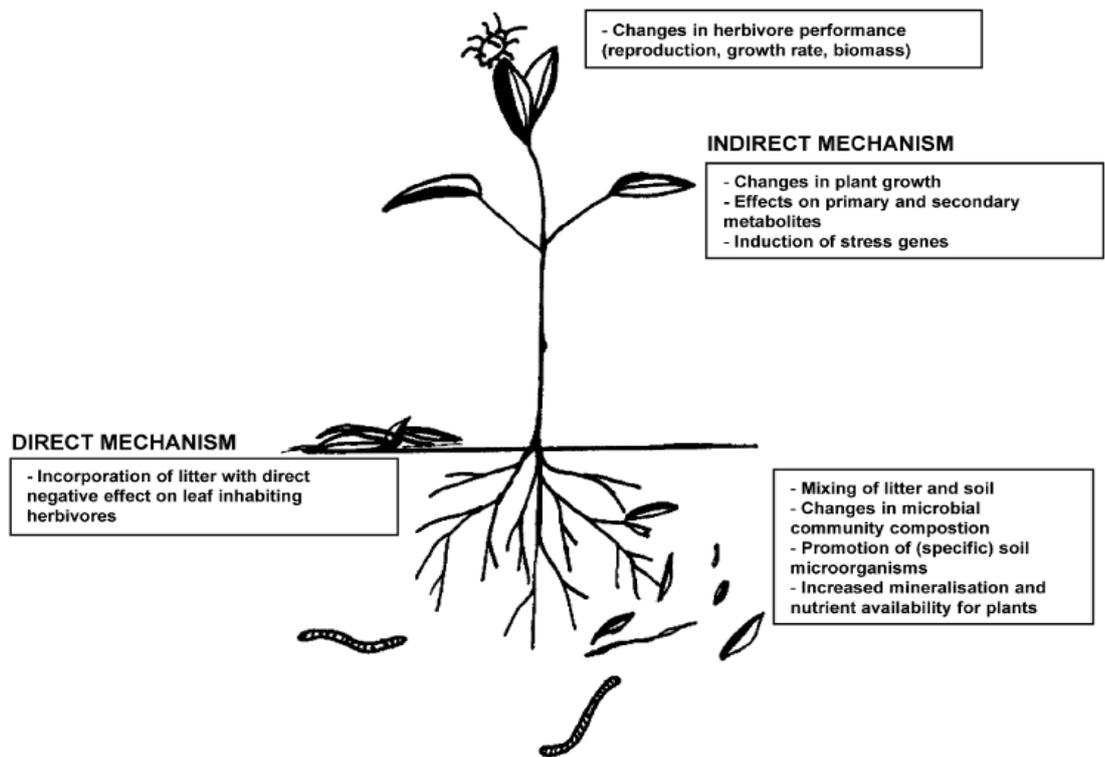


Figure1.2 The indirect effect of earthworms on above-ground communities via interactions with soil microorganisms and litter and resulting changes in plant chemistry, adapted from Wurst, 2010.

By contrast, in the relationships between above and below-ground herbivores, Sinka, Jones & Hartley (2009), found that grain aphids (*S. avenae*) affected the spatial distribution of the soil surface-dwelling Collembolan *Folsomia candida* due to honeydew deposition. The number of Collembola also increased in the presence of the aphid *S. avenae*, which caused a decrease in *P. annua* biomass.

These previous results showed that the presence of different functional groups (e.g. earthworms with nematodes or root-feeding insects) may adversely affect each other and above-ground communities. However, studies need to investigate the effect of soil biota within the same group on the plant and above-ground herbivory.

1.4 Soil biodiversity

Soil is an important habitat for large numbers of organisms, which have been associated with each other and with above-ground herbivores in interactions that are functionally important (Giller *et al.*, 1997; Scheu *et al.*, 1999; Bardgett *et al.*, 2005). Soil biodiversity refers to the variety of living organisms that are located in soil (Table 1.1), and they are divided into size groups; micro, meso and macro organisms. New techniques have helped in the identification of several organisms that were unknown in the past, and DNA technologies have a role in obtaining much information on soil biota, such as bacteria (Tiedje *et al.*, 1999).

The size distribution of soil biota varies from < 200 μm to > 2mm (Swift, Heal & Anderson, 1979; Wallwork, 1970), and their distribution depends on soil pore size (Whitford, 1996), and the presence and activity of macrofauna which provide microhabitats for microfauna (Bursaad,1997).

However, in the soil the numbers of bacteria found is higher than other groups even at small scales (Grundmann and Gourbiere 1999), followed by fungi, protozoa and invertebrate animals respectively (Giller *et al.*, 1997). This diverse soil microbial community differs in its impact on other

organisms and on the ecosystem, but are known to have an effect on soil heterogeneity (Marilly and Aragno 1999). Generally, among the metazoa, nematodes are the most abundant (Bongers and Marina 1998). Soil nematode biodiversity is widespread in the soil, especially in the tropical temperate forests, but it is lower near the poles (Boag and Gregor 1998).

Table 1.1 Global abundance of selected soil organisms and their species number in the world that have significant roles in the soil and maintain agroecosystems.

Taxa	Number of species	References
Earthworms	1200	(Edwards & Bohlen, 1996)
Nematodes	100000	(Bongers, 1990)
Collembola	6500	(Rusek, 1998)
Bacteria	60000	(Gordon, 1967)
Fungi	74000	(Hawksworth, 2001)
Enchytraeids	30-50	(Didden, 1993)
Oribatid Mites	7000	(Balogh & Balogh, 1992)

The functional importance of earthworms has received considerable attention (Brown, 1995; Decaëns & Jiménez, 2002; Lee, 1985). There are three ecological groups of earthworms (Figure 1.3), classified according to their life style; epigeic, endogeic and anecic (Bouché, 1977; Fragoso *et al.*, 1997). Epigeic earthworms live on the soil surface, feed on the leaf litter and tend not to make burrows, Endogeic earthworms are soil feeders, making horizontal burrows through the soil, and anecic

earthworms live in soil, feed on surface leaves that they drag into soil, and make vertical burrows (Bouché, 1977).

The great interactions between soil organisms have been investigated by many researchers (Brown, 1995; Fitter & Garbaye, 1994; Groffman *et al.*, 2004; Gryndler, 2000; McLean & Parkinson, 2000). Earthworms as a macrofauna have either a direct (Monroy, Aira & Domínguez, 2008) or indirect (Edwards & Bohlen, 1996) influence on other micro and macro organisms in the soil, and via these activities soil organisms regulate the number and activity of microorganisms (Lavelle & Spain, 2001). Soil microorganisms also play a great role in nutrient cycling, are involved in the decomposition process and release nutrients which are beneficial to plants (Altieri & Nicholls, 2004; Groffman & Bohlen, 1999; Groffman *et al.*, 2004; Torsvik & Øvreås, 2002).

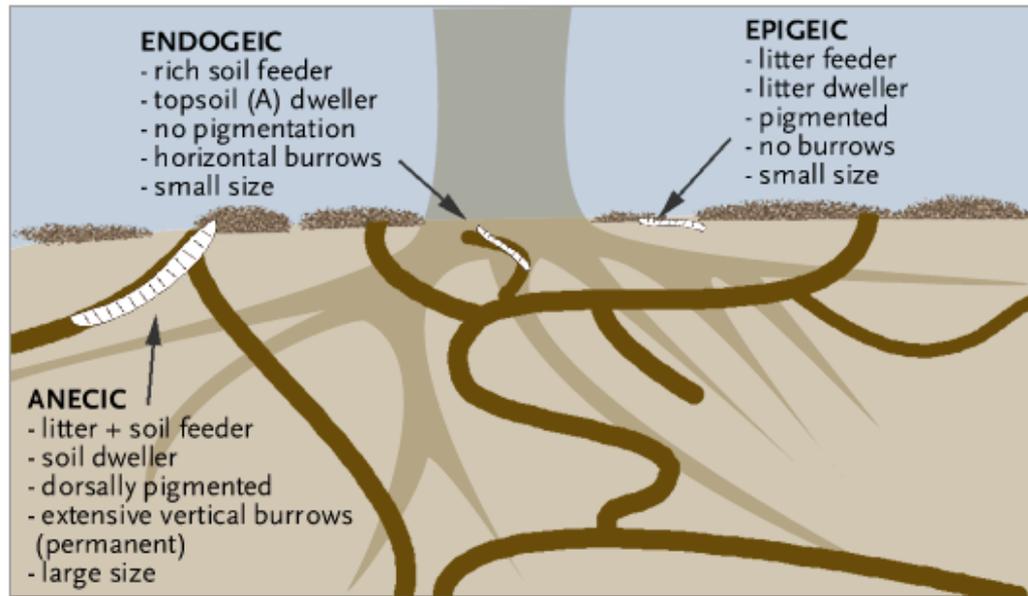


Figure 1.3 Three functional groups of earthworms (epigeic, endogeic and anecic) based on their life behavior (Bouche 1977).

1.5 Plant physiology

Plants are the main source of food for many organisms, because they are rich in different minerals which are essential nutrients for humans, animals and insect herbivores. Plant proteins are one of the main nutrients and are of great value, being directly involved in the chemical processes essential for plant growth.

The changes in plant chemistry by soil organisms have been investigated by much research. For example, Ingham *et al.*, (1985), found that the nitrogen uptake and growth of Blue grama grass (*Bouteloua gracilis*) increases through the interactions between bacteria, fungi and nematodes in the soil. Also, soil microbes have a positive effect on birch seedling (*Betula pendula*) growth by increasing the amount of nitrogen and

phosphorus in the plant (Setälä & Huhta, 1991). Similarly, Sheehan (2006) and Subler, Baranski & Edwards (1997), mentioned the role of the earthworm community in increasing nitrogen concentration in the soil.

While a number of previous studies have investigated the impact of below-ground organisms on plant elements, none have yet examined the role of plant proteins in the indirect interactions between soil organisms and aboveground insects, since the function of most of the phloem sap proteins are still poorly understood (Kehr, 2006).

These changes are important in terms of plant defences against insects, such as aphids, which feed on sap in new growth buds and other delicate tissues, and these insects have a wide range of host plants. Beside their direct damage, aphids also have the ability to transmit virus diseases to the plant (Edwards & Bohlen, 1996; Govier & Kassanis, 1974; Ng & Perry, 2004). Therefore they are regarded as being economically very important (Guerrieri & Digilio, 2008; Halbert, Irwin & Goodman, 1981).

Leaves are the main part of plants for accumulating proteins (Pirie, 1986) each protein consists of different amino acids, and their functions differ according to the amino acid composition and sequence. There is a link between nitrogen and protein content in the plant, which tends to be plant specific (Fujihara, Kasuga & Aoyagi, 2001; Yeoh & Wee, 1994).

Many previous studies have investigated the role of plant proteins in insect development (Bernays & Woodhead, 1984; Horie & Watanabe, 1983; Wicker & Nardon, 1982). Also, Broadway & Duffey (1988), found

that the larva of beet armyworm (*S. exigua*) was positively influenced by adding an artificial protein (casein) to its diet.

The relationship between plant proteins and herbivores has been previously investigated. Broadway & Duffey (1988) and Horie & Watanabe (1983) tested the effect of artificial plant proteins on insect herbivores. Also, Pierre *et al.* (2013), found that the response of insects to jasmonic acid (JA) differed according to their species, since JA increases the numbers of cabbage aphids and cabbage butterfly larvae, while the numbers of cabbage moth (*Mamestra brassicae* L.) was lower. On the other hand Glazebrook (2005) and Walling (2000), suggested that the role of JA in plant defense was against leaf chewing herbivores, while salicylic acid (SA) is responsible for defense against phloem sap herbivores.

Earthworms as mineral decomposers have an influence on plant chemistry, *M. anomala* earthworms positively influenced on young rice seedling growth (increasing total biomass) by chemical changes (increasing in lipoxygenase and decreasing in cysteine protease) in the plant (Blouin *et al.*, 2005).

While a number of previous studies have investigated the impact of below-ground organisms on plant physiology and insect development, none have yet examined the interaction between earthworms (from different functional groups), plant protein changes and aphid development. Plants contain different proteins, some of them repeated in different plant species. Table 1.2 shows the different identified phloem sap proteins from different plant species and their functions.

Table 1.2 Compilation of phloem sap proteins that are potentially involved in plant wound and defence reactions A.th., *Arabidopsis thaliana*; B.n., *Brassica napus*; C.m., *Cucurbita maxima*; C. melo, *Cucumis melo*; C.s., *Cucumis sativus*; L.e., *Lycopersicon esculentum*; O.s., *Oryza sativa*; P.v., *Phaseolus vulgaris*; R.c., *Ricinus communis*. Adapted from Kehr (2006).

Function	Protein	Species	Reference(s)
Reactive oxygen/redox related	Ascorbate peroxidase	B.n.	(Giavalisco et al., 2006)
	Copper homeostasis factor	A.th., B.n.	(Giavalisco et al., 2006; Mira H, 2001)
	Dehydroascorbate reductase	B.n., C.m., C.s.	(Walz et al., 2004; Walz et al., 2002)
	Ferredoxin	B.n.	(Giavalisco et al., 2006)
	Glutaredoxin	B.n., R.c.	(Szederkényi, Komor & Schobert, 1997)
	Glutathione reductase	C.m.	(Alosi, Melroy & Park, 1988)
	Glutathione-S-transferase	B.n., O.s.	(Fukuda et al., 2004)
	Iron transport protein	R.c.	(Krüger et al., 2002)
	Metallothionein	R.c.	(Barnes et al., 2004)
	Monodehydroascorbate reductase	B.n.	(Giavalisco et al., 2006)
	Peroxidase	B.n., C.m., C.s.	(Walz et al., 2002)
	Peroxiredoxin	B.n.	(Giavalisco et al., 2006)
	Superoxide dismutase, Cu/Zn	C.m., C.s.	(Walz et al., 2004; Walz et al., 2002)
	Thioredoxin h	B.n., C.m., O.s.	(Ishiwatari et al., 1995)
	Annexin	B.n., R.c.	(Barnes et al., 2004)
	Calcium-related	C2 domain-containing protein	B.n.
Calmodulin		B.n., R.c.	(Barnes et al., 2004; Yoo, Lee & Lucas, 2002)
Kinases		C.m., O.s.	(Avdiushko et al., 1997; Nakamura et al., 1993; Yoo et al., 2002)
Phytohormone-related	ACC oxidase	C.m., C.s.	(Walz et al., 2004)
	ACC synthase	C.m., C.s.	(Walz et al., 2004)
	Allene oxide cyclase ^a	L.e.	(Hause et al., 2003)
	Allene oxide synthase ^a	L.e.	(Hause et al., 2003)
	Lipoxygenase	C.m., C.s.	(Avdiushko et al., 1994; Hause et al., 2003; Walz et al., 2004)
SE plugging	SAM synthetase	B.n.	(Giavalisco et al., 2006)
	Systemin	L.e.	(Narváez-Vásquez et al., 1995)
	Forisome	P.v.	(Knoblauch et al., 2001)
	PP1	C.m.	(Clark et al., 1997)
	PP2	B.n., C.m., C.melo, Cs	(Bostwick et al., 1992; Gomez, Torres & Pallas, 2005; Walz et al., 2004)
Protase inhibitors	Aspartic protease inhibitor	C.m.	(Christeller et al., 1998; Walz et al., 2004)
	Chymotrypsin inhibitor	C.m.	(Murray & Christeller, 1995; Walz et al., 2004)
	Cystatin	B.n., C.m., C.s., R.c.	(Barnes et al., 2004; Haebel & Kehr, 2001; Walz et al., 2004)
	Serpin-1	C.m.	(Walz et al., 2004; Yoo et al., 2000)
Lectins	Trypsin inhibitor	C.m., C.s.	(Murray and Christeller, 1995; Walz et al., 2004)
	Cm lectin 17	C.melo, C.s.	(Dinant et al., 2003; Gomez, Torres & Pallas, 2005; Walz et al., 2004)
	Cm lectin 26	C.s.	(Dinant et al., 2003; Walz et al., 2004)
Others	Lectin, mannose-binding	C.m.	(Walz et al., 2004)
	CSF-2	C.m., C.s.	(Haebel & Kehr, 2001; Walz et al., 2004)
	Epithiospecifier protein	B.n.	(Giavalisco et al., 2006)
	Myrosinase	B.n.	(Giavalisco et al., 2006)
	Myrosinase-binding protein	B.n.	(Giavalisco et al., 2006)
	SN-1	C.s.	(Walz et al., 2004)
	SWF-1 (peptidase)	C.m.	(Walz et al., 2004)
SWF-3 (b-glucosidase)	C.m.	(Walz et al., 2004)	

^a Immunological detection

1.6 Implications for pest management

Above-ground insects, mostly have a negative impact on plant via feeding on or transferring microbes to the plant which causes economic losses. Different control methods; chemical, physical, biological and natural have been applied in order to suppress pest populations below the economic injury.

Insects have the ability to overcome most of the traditional control methods through hiding from enemies or resistance to pesticides.

Recently, efforts have been focused on finding alternative methods which benefit of ecosystems by maintaining a balance between soil animals.

Soil organisms, individually or combined interact with above-ground communities, and these interactions - either positive or negative - depend on the kind of soil biota.

Most researchers have investigated individual taxa or combined effects of different soil biota (e.g. Alpei, Bonkowski & Scheu, 1996; Araujo, Luizão & Barros, 2004; Ke & Scheu, 2008; Lohmann, Scheu & Müller, 2009; Noguera *et al.*, 2010), leaving the interactions between earthworms within or between different functional groups on above-ground herbivores unstudied. Studies on the influence of earthworms on aphids are needed to understand the physical and chemical changes in the plant as defence methods against above-ground insects. This will provide the insight about the role of soil organisms and how to maintain the soil environment with an appropriate balance between soil fauna, plants and above-ground insects.

Despite the many studies which have been conducted in this area, some questions remain unanswered related to the relationships between soil biota and above-ground insects. Several hypotheses include:

- 1- We know that soil fauna can affect above-ground herbivores but we do not know whether this is either consistent or there is a set of rules that will allow us to predict what the effect will be.
- 2- We also know that soil biota has important effects on plant growth through direct and indirect effects. What we do not know is whether this is linked to ecological function or is taxa specific.
- 3- It is known that changes in plants can affect above-ground herbivores, but we do not know whether they are affected by the chemical or physical changes in the plants.

1.7 Aims and objectives

- 1- Investigate the relationships between earthworms and above-ground insects, especially the influences of earthworms on aphids.
- 2- To assess the response of plants to these relationships, via known chemical and morphological changes in the plant, by investigating the effect of different species and density of earthworms on the morphology and nitrogen changes in the plant (as described in Chapter three).
- 3- To study the interactions between different functional groups of earthworms and their influences on above-ground aphid development, by investigating the differences between species (individually & combination) and functional groups of earthworms and their influence on aphid (Chapter five is described this achievement).
- 4- To investigate the role of nitrogen and protein changes in the plant and aphid development. Using 2-DG technique in order to determine the proteins in the plants under the influence of different earthworms and understanding the role of earthworms in protein changes and aphid development (as described in Chapter six).

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 Introduction

The work described in this thesis attempts to elucidate the influence of earthworms on aphid development, and to investigate the role of the plant in this interaction via their chemical and morphological changes. To do this a suite of methods were utilised, subsequent chapters. The general methods are described below, including earthworm sampling locations, aphid culturing, experimental design and the laboratory work.

2.2 Earthworm collection

Individuals of the endogeic earthworm *A. rosea* were collected from a grassland site near Tavistock, Devon (50° 32' 44" N 4° 8' 40" W). The endogeic earthworm *A. chlorotica* was collected from rough ground in central Plymouth, Devon (50° 22' 17.03" N 4° 8' 23" W).

The endogeic earthworm *Aporrectodea caliginosa*, the epigeic earthworms *Satchellius mammalis* (Savigny 1826), and *L. rubellus* were collected from Schumacher College in Dartington, South West, Devon (50° 45' 11" N 3.7099° W).

Earthworms were retained in species groups in four-litre containers containing John Innes No. 2 potting compost in a glasshouse at 20°C ± 2°C pending further use.

2.3 Aphid culture

The aphid *Myzus persicae* Sulzer was maintained at a maximum of 25-30 individuals in Blackman boxes at 21°C ± 2°C and L/D 16:8 (Foster *et al.*, 1997) using excised leaves from Chinese cabbage (*Brassica rapa* subspecies *pekinensis*) grown under glasshouse conditions. The Blackman boxes were stood upright in trays of water (to prevent escape from the incubator if aphids escaped from the boxes).

Plants were kept in a Light/Dark 16hr L: 8hr D incubator at ~ 21°C.

2.4 Experimental treatments

This study focused more on the endogeic species to investigate the individual effect of earthworms on above-ground plants and aphids because the species of this group tend to live in the same level of *B. rapa* roots.

The experimental design consisted of the following treatments (Table 2.1):

Table 2.1 The experimental design, earthworm species from different functional groups and densities.

Earthworm species	Treatments
<i>A. rosea</i> (endogeic)	0, 2, 4 and 8 worms
<i>A. chlorotica</i> (endogeic)	0, 2, 4 and 8 worms
<i>A. rosea</i> (endo.) + <i>A. chlorotica</i> (endo.)	0, 2, 4 and 8 worms
<i>A. caliginosa</i> (end.) + <i>S. mammalis</i> (epigeic)	0, 2, 4 and 8 worms
<i>A. chlorotica</i> (endo.) + <i>S. mammalis</i> (epi.)	0, 2, 4 and 8 worms
<i>A. rosea</i> (endo.) + <i>A. caliginosa</i> (endo.) + <i>L. rubellus</i> (epi.) + <i>S. mammalis</i> (epi.)	0, 4, 8, 12 (multiples of 4)

Five replicates were used for each treatment.

All experiments were conducted in a glasshouse in ambient day- light at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Each experimental unit consisted of a two-litre plastic plant pot with 1 mm plastic mesh placed over the drainage holes to prevent earthworm escape. Each pot was filled with John Innes No 2 potting compost. Chinese cabbage (*Brassica rapa* L.) seeds (obtained from Rothamsted Research Centre) were sown and germinated approximately one week later. Earthworms were introduced to the pots following germination to establish the treatments set out above. Four weeks later individual aphid nymphs (third instar) were transferred from cultures to leaves of the growing plants and enclosed in clip-cages (20 mm diameter and 10 mm height) (Scheu, Theenhaus & Jones, 1999). A plastic sleeve (placed over the plant/clip cages) was used for extra protection against escape, though this made it difficult to monitor the aphids.

Plants were grown for a further four weeks before destructive sampling (Figure 2.1).



Figure 2.1 Photograph to illustrate the design of experiment in greenhouse at Plymouth University

2.5 Plant harvest and analysis

At harvest the following parameters were quantified:

- 1- Leaf number per plant.
- 2- Plant height.
- 3- Leaf surface area (all leaves on each plant), using a leaf area meter [Delta-T Image Analysis System-Type (DIASTM)].
- 4- Specific leaf area (SLA), calculated as the projected leaf area per unit dry mass. (Poorter & Remkes, 1990).
- 5- Total shoots biomass, (above-ground biomass dried at 80°C for 48 hours prior to weighing).

2.6 Nitrogen analysis

Kjeldahl analysis (Bremner *et al.*, 1996) was used to determine total nitrogen in the dried plant material. This procedure is based on the digestion of the sample with concentrated sulphuric acid in the presence of sodium sulphate and a copper catalyst which converts nitrogen compounds to ammonium sulphate (Anon, 1988). In order to determine the ammonium content of the digested solution a distillation process (Vapodest 50s, Gerhardt UK Ltd.) was used.

This procedure was completed according to the following steps:

The dried shoot was ground to a powder and 0.01-0.015gm was weighed (three replicate readings per sample). 3.5gm potassium sulphate (K_2SO_4), 0.105gm copper(II) sulphate ($CuSO_4 \cdot 5H_2O$) and 0.105gm titanium dioxide (TiO_2) were added to each sample in the digest tube, then 10 ml of sulphuric acid was added in a fume cupboard. Samples were gradually heated to 370 °C for 3-4 hours in the digestion block and then left at this temperature for 30 minutes.

2.7 Statistical analysis

Conventional statistical methods were used to calculate the means, coefficient of variance (CV), standard deviation (SD) and standard error (SE). A one-way ANOVA multiple comparisons were applied to test for any significant differences. Differences among treatments were compared by Tukey's mean and least significant difference (L.S.D) tests at the 0.05

probability level. The statistical analysis was carried out using MiniTab statistical software v.16.

For the protein analysis, the raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific). MASCOT Database search engine v2.1 (Matrix Science Ltd, London, UK) was used for Combined PMF and MS/MS queries. Progenesis SameSpots software was used to analysis 2-DE gel images.

**CHAPTER 3
THE INFLUENCE OF EARTHWORM ON PLANT
PERFORMANCE**

3.1 Abstract

In this study the individual effect of the endogeic *Aporrectodea rosea* and *Allolobophora chlorotica* and the combined effects of *A. rosea* & *A. chlorotica*, *Aporrectodea caliginosa* & *Satchellius mammalis*, *A. chlorotica* & *S. mammalis* and *A. rosea*, *A. caliginosa*, *Lumbricus rubellus* and *S. mammalis* earthworms on the growth of Chinese cabbage (*B. rapa*) was determined in separate pot experiments.

Plant growth was affected by the presence of *A. rosea* which caused increases in plant biomass, height of plant, leaf surface area and specific leaf area (SLA). Additionally, leaf nitrogen concentration significantly increased in the highest density of *A. rosea* individuals. The mean number of leaves per plant was unaffected by worm density. In contrast, increasing the density of *A. chlorotica* had no effect on any aspect of plant performance. The combined effect of *A. rosea* and *A. chlorotica* resulted in a similar increase in plant biomass to *A. rosea* alone. The combined effect of *A. caliginosa* and *S. mammalis*, *A. chlorotica* and *S. mammalis* and *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* caused increases in all plant parameters except leaf number.

Additionally, the individual effect of *A. rosea* and the combined effect of *A. caliginosa* and *S. mammalis*, *A. chlorotica* and *S. mammalis* and *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* resulted in increases in leaf nitrogen concentration. In contrast, *A. chlorotica* alone had no significant effect on nitrogen concentration.

3.2 Introduction

By virtue of their influence on the distribution of soil organic matter, and rates of decomposition and nutrient recycling (Blackshaw, 1983; Ke & Scheu, 2008), plant morphology (Haimi, Huhta & Boucelham, 1992; Scheu, 2001), and plant anti-herbivore defence (Little *et al.*, 2011; Lohmann, Scheu & Müller, 2009), earthworms have attracted the attention of researchers to investigate their role in chemical changes in the plant and the interaction between plant and above-ground herbivores.

Earthworms cause different changes in the soil (e.g. biological, physical and chemical) (Edwards & Bohlen, 1996), via burrowing and other activities, and so provide services to other soil organisms and plants. Brown (1995) pointed out the role of earthworms in creating voids in the soil, thus providing dwellings for other organisms such as Collembola and mites. Moreover these pores and spaces facilitate gas exchange which is necessary to promote microbial activities (Kretzschmar & Monestiez, 1992). Also, feeding activities result in chemical changes to the food in the earthworm gut, hence increasing nitrogen levels in the soil through the casts (Araujo, Luizão & Barros, 2004; Lee, 1985).

There are a multitude of interactions between earthworms and other soil biota (Baogui, 1997; Monroy, Aira & Domínguez, 2011; Scheu, Theenhaus & Jones, 1999), either positive or negative (Senapati, 1992). These interactions lead to different physical and chemical changes in the soil, and thus affect plant growth (Doube *et al.*, 1994). The interaction between different species of earthworms needs more study in order to

investigate whether they positively or negatively influence above-ground communities.

Chinese cabbage was chosen as the experimental plant because it has no associated nitrogen fixing bacteria and is easily grown from seed. Any change in plant growth could therefore be attributed to the earthworm treatments. Thus the hypothesis for this study is that earthworms will promote plant growth by making more nitrogen available.

Several previous studies pointed out that the plant responds to the presence of earthworms. Ke & Scheu (2008) and Haimi, Huhta & Boucelham (1992), found that *A. caliginosa* caused increases in nitrogen uptake by plants, hence increasing plant biomass. Also, Eriksen-Hamel & Whalen (2007), mentioned the positive role of the endogeic *A. caliginosa* and anecic *L. terrestris* earthworms in plant growth via increasing mineral matter nitrogen concentration in the soil. On the other hand, the combined effects of earthworms and other organisms that live in the soil has been investigated by Wurst *et al.*, (2004), where they found plant growth was positively affected by the presence of earthworms and mycorrhiza together in the soil. Ke & Scheu (2008), mentioned the interactions between earthworms, Collembola and litter distribution and their role in increasing plant growth. The influence of earthworms on plants depends on plant species. For example, in the presence of earthworms, the biomass of *P. annua* increased more than twofold in comparison to *T. repens* (Scheu, Theenhaus & Jones, 1999). Also, the foliage biomass and nitrogen content of *L. perenne* and *T. repens* were positively affected by

the presence of the endogeic *A. jassyensis*. In contrast, *P. lanceolata* showed no response to the presence of *A. jassyensis* (Wurst, Langel & Scheu, 2005).

3.3 Aims

This experiment aims to investigate:

- 1- Plant responses to the presence of earthworms in the soil via morphological changes.
- 2- The influence of earthworms on nitrogen content in the plant.

3.4 Materials & methods

This experiment was carried out in a glasshouse. The individual and combined influences of five different species of earthworms from two different functional groups were assessed to investigate the morphological and chemical changes in the Chinese cabbage plant. Kjeldahl analysis was used to determine total nitrogen in the dried plant materials (see chapter 2, section 2.6).

The experimental design of all the six experiments is described in chapter 2 (section 2.4).

3.5 Results

3.5.1 The influence of earthworms on *B. rapa* performance

One-Way ANOVA (Table 3.1) revealed that two aspects of *B. rapa* morphology were affected by increasing *A. rosea* density. These included; plant height and leaf surface area. Post-hoc Tukey tests showed a substantial increase in all three traits when plants were grown in the presence of eight *A. rosea*, compared to the other density treatments. Leaf number and specific leaf area were unaffected by worm density.

In addition to morphological changes, there was a substantial increase in above-ground *B. rapa* plant biomass in the presence of eight *A. rosea* individuals. Similarly, the amount of nitrogen within plant leaves was also substantially greater in plants grown in the eight worm treatment than for all other treatment groups (Table 3.1).

In the presence of *A. chlorotica* the results revealed that all aspects of *B.rapa* morphology, plant biomass and nitrogen concentration were unaffected by increasing *A. chlorotica* density (Table 3.2). Post-hoc Tukey tests showed no significant differences ($P>0.05$) between all treatments.

One-Way ANOVA (Table 3.3) revealed that all aspects of *B.rapa* morphology were unaffected by increasing *A. rosea* and *A. chlorotica* density. Post-hoc Tukey tests showed no significant differences ($P>0.05$) between all treatments in each morphological aspect.

However, above-ground *B. rapa* plant biomass did increase with increasing earthworm density. Post-hoc Tukey tests showed a substantial increase in above-ground biomass in the presence of eight individuals compared to two worm density treatments (two and control), while there was no significant difference ($P>0.05$) between the eight and four earthworm treatments. The amount of nitrogen was unaffected by increasing earthworm density (Table 3.3).

In the presence of *A. caliginosa* and *S. mammalis* the results revealed that several aspects of *B. rapa* morphology were affected by increased *A. caliginosa* and *S. mammalis* density (Table 3.4). These included; plant height, leaf surface area and specific leaf area. Post-hoc Tukey tests showed a substantial increase in plant height and leaf surface area when plants were grown in the presence of eight *A. caliginosa* and *S. mammalis*, compared to the other density treatments, while the specific leaf area was increased in plant growth in the control treatment compared to the other density treatments with no significant differences ($P>0.05$) between the control and two earthworm treatments. Leaf number was unaffected by worm density (Table 3.4).

In addition to morphological changes, there was a substantial increase in above-ground *B. rapa* plant biomass in the presence of eight *A. caliginosa* and *S. mammalis*, with no significant differences ($P>0.05$) between the eight and four earthworm treatments. Similarly, the amount of nitrogen within plant leaves was also substantially greater in plants grown in the eight worm treatment than for all other treatment groups (Table 3.4).

One-Way ANOVA (Table 3.5) also revealed that two aspects of *B. rapa* morphology were affected by increasing *A. chlorotica* and *S. mammalis* densities (Table 3.5). These included; plant height and leaf surface area. Post-hoc Tukey tests showed a substantial increase in plant height and leaf surface area when plants were grown in the presence of eight *A. chlorotica* and *S. mammalis*, compared to the other density treatments. Leaf number and specific leaf area were unaffected by worm density.

In addition to morphological changes, there was a substantial increase in above-ground *B. rapa* plant biomass in the presence of eight *A. chlorotica* and *S. mammalis*, with no significant differences ($P > 0.05$) between the four and two earthworm treatments. Similarly, the amount of nitrogen within plant leaves was also substantially greater in plants grown in the eight worm treatment than for all other treatment groups (Table 3.5).

In the presence of four different species from two different functional groups, the results revealed that several aspects of *B. rapa* morphology were affected by increasing *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* density (Table 3.6). These included; plant height, leaf surface area and specific leaf area. Post-hoc Tukey tests showed a substantial increase in plant height and leaf surface area when plants were grown in the presence of 12 *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*, compared to the other density treatments, while the specific leaf area was increased in the presence of 4 *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*, compared to other density treatments with no

significant differences between eight, four and control treatments. Leaf number was unaffected by worm density.

In addition to morphological changes, there was a substantial increase in above-ground *B. rapa* plant biomass in the presence of 12 *A. rosea*, *A. caliginosa*, *L. rubellus* and *S.mammalis*, compared to the other density treatments. Similarly, the amount of nitrogen within plant leaves was also substantially greater in plants grown in the 12 worm treatment than for all other treatment groups (Table 3.6).

The relationship between plant morphology, plant biomass and nitrogen amount shows that several aspects of plant morphology (leaf number, plant height and leaf surface area), plant biomass and nitrogen amount were increased with increasing earthworms worm density except SLA where decreased in the presence of *A. rosea* and *A. chlorotica*, *A. caliginosa* and *S. mammalis*, *A. chlorotica* and *S. mammalis* and in the presence of *A. rosea*, *A. chlorotica*, *L. rubellus* and *S. mammalis* (Appendix 1).

Table 3.1 Mean (\pm SE) variation in *B. rapa* traits in response to increasing *A. rosea* earthworm densities. The results of a one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
8 worms	14.0 \pm 0.1	22 \pm 1.2 ^a	114 \pm 6.8 ^a	37.0 \pm 2.8	3.11 \pm 0.2 ^a	7.66 \pm 0.1 ^a
4 Worms	11.2 \pm 0.5	18.6 \pm 1.5 ^b	96.6 \pm 16.9 ^b	53.9 \pm 7.37	1.88 \pm 0.4 ^b	3.83 \pm 0.5 ^b
2 Worms	11.4 \pm 1.3	16 \pm 1.1 ^b	73.7 \pm 10.5 ^b	90.4 \pm 37.7	1.29 \pm 0.4 ^b	3.21 \pm 0.04 ^b
Control	9.60 \pm 1.3	15.8 \pm 0.8 ^b	68 \pm 3.7 ^b	61.3 \pm 11.8	1.32 \pm 0.3 ^b	3.69 \pm 0.5 ^b
One-Way ANOVA	F=2.85 (19)	F=6.10 (19)	F=3.95 (19)	F=1.23 (19)	F=7.09 (19)	F=29.2 (11)
F= (df)	P=0.070	P=0.006	P=0.028	P=0.333	P=0.003	P=0.001
P=						

Table 3.2 Mean (\pm SE) variation in *B. rapa* traits in response to increasing *A. chlorotica* earthworm densities. The results of a one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
8 worms	11.2 \pm 0.4	21.2 \pm 2.3	122 \pm 4.0	49.6 \pm 2.26	2.49 \pm 0.2	4.34 \pm 0.1
4 Worms	11.2 \pm 0.7	21.4 \pm 1.3	124.7 \pm 3.6	50.2 \pm 4.44	2.54 \pm 0.2	4.59 \pm 0.1
2 Worms	10.8 \pm 0.4	21.2 \pm 0.9	124.1 \pm 4.1	45.7 \pm 3.70	2.76 \pm 0.1	4.18 \pm 0.1
Control	10.6 \pm 0.6	18.4 \pm 1.03	121.6 \pm 1.7	43.6 \pm 2.23	2.81 \pm 0.1	3.87 \pm 0.4
One-Way ANOVA	F=0.31 (19)	F=0.19 (19)	F=0.19 (19)	F=0.90 (19)	F=0.99 (19)	F=2.16 (11)
F= (df)	P=0.82	P=0.46	P=0.90	P=0.46	P=0.42	P=0.17
P=						

Table 3.3 Mean (\pm SE) variation in *B. rapa* traits in response to increasing *A. rosea* and *A. chlorotica* earthworm densities. The results of one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc. Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
8 worms	10.8 \pm 0.9	19.2 \pm 1.85	129.3 \pm 0.98	48.5 \pm 4.85	2.75 \pm 0.22 ^a	3.37 \pm 0.01
4 Worms	10.0 \pm 0.0	21.2 \pm 0.58	132.1 \pm 6.66	57.1 \pm 3.19	2.32 \pm 0.08 ^{ab}	3.48 \pm 0.33
2 Worms	10.0 \pm 0.8	19.6 \pm 1.17	112.4 \pm 8.10	58.7 \pm 3.8	1.97 \pm 0.26 ^b	3.84 \pm 0.58
Control	11.0 \pm 0.7	20.0 \pm 0.32	126.6 \pm 6.76	63.3 \pm 2.8	2.1 \pm 0.11 ^b	3.15 \pm 0.44
One-Way ANOVA	F=0.57 (19)	F=0.57 (19)	F=1.95 (19)	F=2.76 (19)	F=3.95 (19)	F=0.52 (11)
F= (df)	P=0.64	P=0.64	P=0.61	P=0.08	P=0.03	P=0.68
P=						

Table 3.4 Mean (\pm SE) variations in *B. rapa* traits in response to increasing *A. caliginosa* and *S. mammalis* earthworm densities. The results of one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc. Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
8 worms	20.2 \pm 0.4	21.0 \pm 0.78 ^a	137.4 \pm 1.52 ^a	18.7 \pm 0.39 ^a	7.37 \pm 0.44 ^a	2.19 \pm 0.006 ^a
4 Worms	20.0 \pm 0.3	19.6 \pm 0.51 ^{ab}	131.1 \pm 1.06 ^b	19.7 \pm 0.8 ^{ab}	6.71 \pm 0.25 ^a	1.82 \pm 0.013 ^b
2 Worms	20.0 \pm 0.3	19.8 \pm 0.37 ^{ab}	128.8 \pm 1.68 ^b	21.8 \pm 0.5 ^{bc}	5.94 \pm 0.51 ^b	1.72 \pm 0.008 ^c
Control	19.9 \pm 0.3	18.0 \pm 0.71 ^c	120.6 \pm 0.77 ^c	22.7 \pm 0.78 ^c	5.32 \pm 0.15 ^b	1.72 \pm 0.003 ^c
One-Way ANOVA	F=1.49 (19)	F=4.05 (19)	F=28.38 (19)	F=8.64 (19)	F=27.22 (19)	F=562.5 (11)
F= (df)	P=0.26	P=0.02	P< 0.001	P=0.001	P< 0.001	P< 0.001
P=						

Table 3.5 Mean (\pm SE) variations in *B. rapa* traits in response to increasing *A. chlorotica* and *S. mammalis* earthworm densities. The results of one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
8 worms	19.8 \pm 0.4	20.2 \pm 0.37 ^a	129.2 \pm 3.5 ^a	18.3 \pm 0.93	7.10 \pm 0.26 ^a	2.49 \pm 0.003 ^a
4 Worms	19.8 \pm 0.2	19.0 \pm 0.45 ^b	119.3 \pm 2.98 ^b	18.3 \pm 1.26	6.59 \pm 0.27 ^{ab}	2.39 \pm 0.006 ^b
2 Worms	19.6 \pm 0.3	18.8 \pm 0.37 ^b	105.4 \pm 2.45 ^c	18.1 \pm 1.18	5.90 \pm 0.29 ^b	2.23 \pm 0.010 ^c
Control	19.4 \pm 0.3	17.0 \pm 0.32 ^c	102.5 \pm 2.95 ^c	21.6 \pm 0.99	4.77 \pm 0.13 ^c	1.92 \pm 0.003 ^d
One-Way ANOVA	F=0.73 (19)	F=12.02 (19)	F=17.35 (19)	F=2.34 (19)	F=18.30 (19)	F=629.5 (11)
F= (df)	P=0.55	P=0.25	P< 0.001	P=0.112	P<0.001	P=0.02
P=						

Table 3.6 Mean (\pm SE) variations in *B. rapa* traits in response to increasing *A. rosea*, *A. caliginosa*, *L. rubellus* & *S. mammalis* earthworm densities. The results of one-way ANOVA are given and differences ($P \leq 0.05$) between treatments means determined using post-hoc Tukey tests are denoted by different letters.

Treatment	Leaf No.	Plant height (cm)	Leaf surface area (cm ²)	S L A	Plant biomass (gm)	Nitrogen concentration (%)
12 worms	20.8 \pm 0.4	22.0 \pm 0.37 ^a	177.5 \pm 3.73 ^a	22.4 \pm 0.53 ^b	7.93 \pm 0.14 ^a	2.15 \pm 0.02 ^a
8 Worms	20.6 \pm 0.4	20.4 \pm 0.51 ^b	163.3 \pm 2.41 ^b	24.9 \pm 0.9 ^a	6.59 \pm 0.27 ^b	1.81 \pm 0.02 ^b
4 Worms	20.4 \pm 0.5	19.8 \pm 0.37 ^b	153.5 \pm 2.39 ^c	25.6 \pm 0.58 ^a	6.01 \pm 0.07 ^{bc}	1.70 \pm 0.00 ^c
Control	20.4 \pm 0.3	18.4 \pm 0.25 ^c	134.1 \pm 2.06 ^d	24.1 \pm 0.6 ^a	5.60 \pm 0.17 ^c	1.71 \pm 0.01 ^c
One-Way ANOVA	F=0.24 (19)	F=15.88 (19)	F=44.64 (19)	F=4.21 (19)	F=33.64 (19)	F=208.01 (11)
F= (df)	P=0.87	P<0.001	P<0.001	P=0.023	P<0.001	P<0.001
P=						

3.6 Discussion

This investigation confirmed that *B. rapa* responds positively to the presence of earthworms in the soil. These results were consistent with the general view that soil modification by meso fauna changes plants growth (Haimi & Einbork, 1992; Lohmann, Scheu & Müller, 2009; Scheu, Theenhaus & Jones, 1999; Wurst *et al.*, 2006).

Increasing plant growth in the presence of earthworms (although varying according to earthworm species), concurs with the results of Schmidt & Curry (1999), where they found that earthworms from two different species; *A. rosea* and *A. trapezoides* enhanced wheat growth through increasing shoot dry weight. Also, Doube & Willmott (1997), indicated that earthworms from the two species *A. rosea* and *A. trapezoides* had a positive influence on wheat, barley and faba bean growth.

In the current study, increasing earthworm density significantly increased plant growth. Several morphological plant aspects and plant biomass were influenced by the epigeic and endogeic earthworms which were consistent with the results of others (Scheu, 2003; Schmidt & Curry, 1999; Zhang *et al.*, 2010).

Haimi & Boucelham (1992) used a laboratory microcosm experiment to show that the epigeic *L. rubellus* increased leaf and stem biomass of birch seedlings by 33 and 24% respectively.

Also, Blouin & Laffray (2007), highlighted the role of the endogeic earthworm *M. anomala* in increasing shoot biomass of rice (*Oryza sativa*

L.) plants by 40 %. Similarly, Scheu & Jones (1999), found that endogeic (*A. caliginosa* and *O. tyrtaeum*) earthworms increased shoot and root biomass of *P. annua* by more than 100%, while in white clover (*T. repens*) the shoot and root biomass increased by 18% and 6% respectively compared to a control. On other hand, Lohmann & Müller (2009), found that the endogeic *A. caliginosa* earthworm had no effect on plant morphology, but he attributed that to the short duration of the experiment. Nitrogen concentration was increased according to earthworm densities. This result indicated that earthworm activities make nitrogen more available for uptake by the plant, consistent with the results of many studies (Subler, Baranski & Edwards, 1997; Tomati *et al.*, 1990; Wurst & Jones, 2003; Zhang *et al.*, 2010).

Tomati & Galli (1995), stated that the epigeic *Eisenia fetida* (Savigny, 1826) and the endogeic *A. caliginosa* earthworm casts increased the nitrogen content of lettuce (*Lactuca sativa* L.). A similar result was also reported by Wurst *et al.*, (2006), who found that the endogeic (*O. tyrtaeum*) earthworm activity caused increases of nitrogen uptake by *B. oleracea*.

In contrast, Hale *et al.*, (2005), reported that there is a negative relationship between earthworms and nitrogen, with increasing earthworm biomass causing decreases in the amount of nitrogen in plant tissue. These results were also not consistent with the results of Scheu & Jones (1999), who reported that *A. caliginosa* and *O. tyrtaeum* earthworm activities have no effect on nitrogen concentration in *P. annua* and *T.*

repens plant tissues. Similarly, the endogeic *A. jassyensis* has no effect on the shoot biomass and nitrogen content in a ribwort plantain (*P. lanceolata*) (Wurst, Langel & Scheu, 2005). On the other hand, Blouin, Barot & Lavelle (2006), reported that the positive effect of the endogeic *Millsonia anomala*, (Omodeo and Vaillaud, 1967) on rice seedlings *O. sativa* growth is constant despite the nitrogen amount available in the soil. This contradiction in the results indicates that the difference in plant species and the functional groups of earthworms plays an important role in the changes that occur in plant growth.

This was mentioned by Edwards & Lofty (1980) when they added two different functional groups (endogeic and anecic) to the soil to investigate their effect on cereal crops and they found the deep-burrowing worms (anecic) were more effective in supplying nutrients to the cereal plant compared with the endogeic worms. This means that the nature of the interaction between earthworm and plant depends on the depth of their penetration into the soil with the same level of root growth, where the burrows provide nutrients around the plant roots.

Finally, the results from this study showed that increasing earthworm density caused significant decreasing in the specific leaf area (SLA) in the presence of *A. caliginosa* and *S. mammalis* also in the *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* treatment. It seems to be that there is a relationship between, SLA, plant biomass and plant nutrient. Poorter and de Jong, (1999), indicated that low SLA with high biomass means the efficient of nutrients conservation in the plant. Also, plants with

low specific leaf area have the ability to overinvest in photosynthetic nitrogen content (Poorter & Evan 1998).

CHAPTER 4
THE INFLUENCE OF EARTHWORMS ON ABOVE-GROUND
APHID DEVELOPMENT

4.1 Abstract

In this study the individual effect of *Aporrectodea rosea* and *Allolobophora chlorotica* and the combined effects of *A. rosea* and *A. chlorotica*, *Aporrectodea caliginosa* and *Satchellius mammalis*, *A. chlorotica* and *S. mammalis* and *A. rosea*, *A. caliginosa*, *Lumbricus rubellus* and *S. mammalis* earthworms on aphid (*M. persicae*) development was determined in separate greenhouse pot experiments (using pair-wise combinations between members of different functional groups) to investigate the differences in their influences between species from the same and different functional groups. It seems that the combined impact is greater due to some kinds of synergy between worms from different species.

Aphid development was affected by the presence of earthworms. The numbers of aphids were increased by increasing earthworm density. The combined influences of *A. rosea* and *A. chlorotica*, *A. caliginosa* and *S. mammalis*, *A. chlorotica* and *S. mammalis*, and *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* on nymphs day⁻¹, fecundity and number of adults resulted in significant increases in aphids over two generations. In contrast, the individual effects of *A. chlorotica* and *A. rosea* showed no significant increase in the numbers of adults in the 1st and 2nd generation respectively.

Additionally, the increase in aphid fecundity was significantly correlated with increases in the leaf nitrogen concentration in all experiments except the individual influence of *A. chlorotica*.

4.2 Introduction

Soil decomposers, either individually or combined are associated with many activities in the soil and by their activity they increase soil nitrogen, which is important for nutrient cycling (Aina, 1984; Parmelee & Crossley Jr, 1988; Smetak, Johnson-Maynard & Lloyd, 2007), and thus indirectly for above-ground insect development via plant chemical changes (Newington *et al.*, 2004; Poveda *et al.*, 2005; Wurst *et al.*, 2004). The manipulation of the soil animal community is a major experimental approach to determining possible links between decomposers and above-ground communities. In this study, changes in aphid development are investigated. In this investigation, earthworms are included as representative of the major decomposers in the soil (Knight, 1989; Römbke, Jänsch & Didden, 2005).

Many previous studies have investigated the individual effects of earthworms on above-ground herbivores, with many contrasting results. For instance, Eisenhauer & Scheu, (2008); Poveda *et al.*, (2005) and Wurst & Jones, (2003), found the positive effects of earthworms on aphids. In contrast, Ke & Scheu (2008), found that the endogeic *A. caliginosa* earthworm reduces the numbers of aphid (*Rhopalosiphum padi*) on wheat (*Triticum aestivum*), also Wurst & Forstreuter (2010), have described the negative influence of the endogeic *Aporrectodea spp* earthworms on aphid (*M. persicae*) development. Wurst *et al.*, (2004), found that *A. caliginosa* had no effect on *M. persicae* development.

In the current study both endogeic and epigeic earthworms were used to increase functional variability. The endogeic group feeds on subsurface material and makes horizontal burrows, while the epigeic group feeds on surface material.

Aphids (*M. persicae*) were chosen as a model system to study the effect of earthworms on them, because they have a wide range of host plants and a short generation interval, with more than 20 annual generations, (Van Emden *et al.*, 1969), and reproduce pathenogenetically *in vitro*. Also, aphids have a strong response to soil nutrients in comparison to chewing insects (Butler, Garratt & Leather, 2012). However recent studies have confirmed that the soil biota has an indirect impact on above-ground aphids. Poveda *et al.*, (2005), mentioned the role of soil fauna individually in increasing aphid abundance. Also, *M. persicae* reproduction was positively affected in the presence of two endogeic earthworms *A. caliginosa* and *O. tyrtaeum* (Scheu, Theenhaus & Jones, 1999). In contrast, *A. caliginosa* caused a decrease in *M. persicae* on ribwort plantain *P. lanceolata* (Wurst *et al.*, 2004; Wurst & Jones, 2003), and *Rhopaslosiphum padi* reproduction was negatively affected in the presence of *A. caliginosa* on wheat *Triticum aestivum* (Ke & Scheu, 2008).

4.3 Aims

1- To investigate the influence of earthworms from two different functional groups on above-ground aphids.

2- To determine if nitrogen concentration in the plant affects aphid fecundity.

4.4 Materials & Methods

This experiment was conducted in a greenhouse (see Chapter 2, section 2.4), the individual and combined influences of five different species of earthworms from two different functional groups were introduced to investigate their influence on aphid development. Earthworm collection and aphid culture are described in Chapter 2 (sections 2.2 and 2.3).

Aphid development was evaluated using the following parameters:

1- Nymphs day⁻¹; the numbers of nymphs produced each day.

2- Fecundity; the total numbers of nymphs produced by adults.

3- No. adult; the total numbers of adults developed from nymphs.

4.4.1 Statistical analysis

Data from (nymph daily production, fecundity and numbers of aphids) were analysed by one-way ANOVA. Significant differences between treatments were determined using Tukey's mean and least significant difference (L.S.D) tests at the 0.05 level. The statistical analysis of data was performed using MiniTab statistical software v.16.

The relationship between increasing numbers of aphids and plant nitrogen concentration was analysed using curvilinear regression. Based on the Shapiro-Wilk test the data was normally ($P>0.05$) distributed. SigmaPlot graphing software version 12.5 was used.

4.5 Results

4.5.1 One species of endogeic earthworm

4.5.1.1 The influence of *A. rosea* on *M. persicae* development

The development of aphid populations in the different treatments of *A. rosea* in the first generation is shown in (Figure 4.1). Post-hoc Tukey tests ($P<0.05$) revealed a substantial increase in the numbers of nymphs produced in the presence of eight worms, compared to worm density treatments four, two and zero, with significant ($P<0.05$) differences between all treatments (Figure 4.1 A). In addition to increasing daily production of nymphs, the fecundity of aphids was also affected by increasing the density of *A. rosea*. Post- hoc Tukey tests showed a substantial increase in the fecundity of aphids in the eight worm density compared to the four two and zero worm treatments, with no significant differences ($P>0.05$) between all treatments (Figure 4.1 B). The numbers of adults developed from the nymphs was substantially higher in the presence of eight worms compared to the four, two and control treatments, with no significant differences ($P>0.05$) between the four, two and control treatments (Figure 4.1 C).

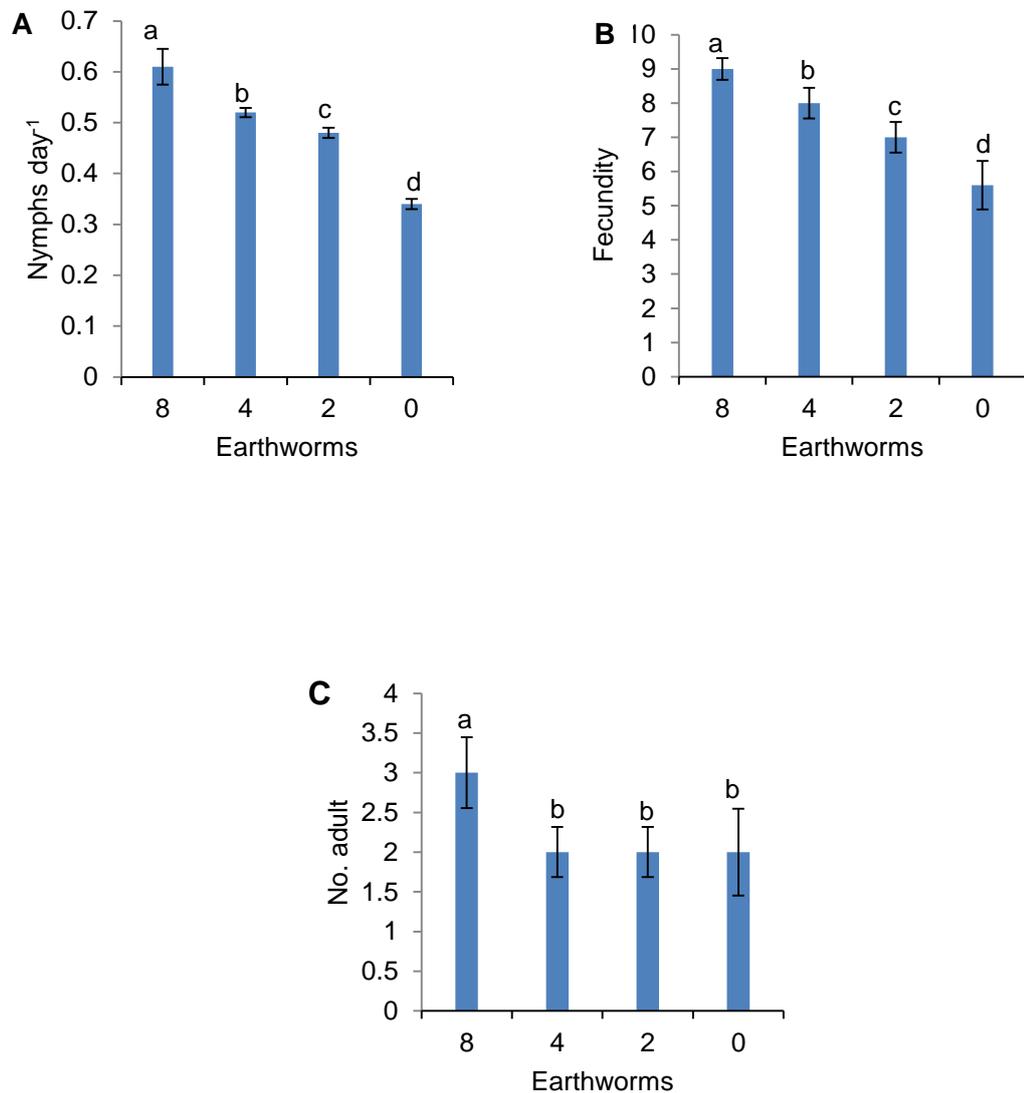


Figure 4.1 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

In addition to the first generation, aphid development was also affected by increasing *A. rosea* density in the second generation (Figure 4.2). Post-hoc Tukey tests showed higher daily production of nymphs in the presence of eight *A. rosea* individuals compared to the four, two and zero worm treatments, with significant differences ($P < 0.05$) between the four two and zero treatments (Figure 4.2 A). Similarly, the fecundity of nymphs was also substantially higher in plants grown in the eight and four worm treatments than for the two and control treatments with significant differences ($P < 0.05$) between two and control treatments (Figure 4.2 B). The numbers of adults developed from nymphs in the second generation were not affected by increasing worm densities (Figure 4.2 C).

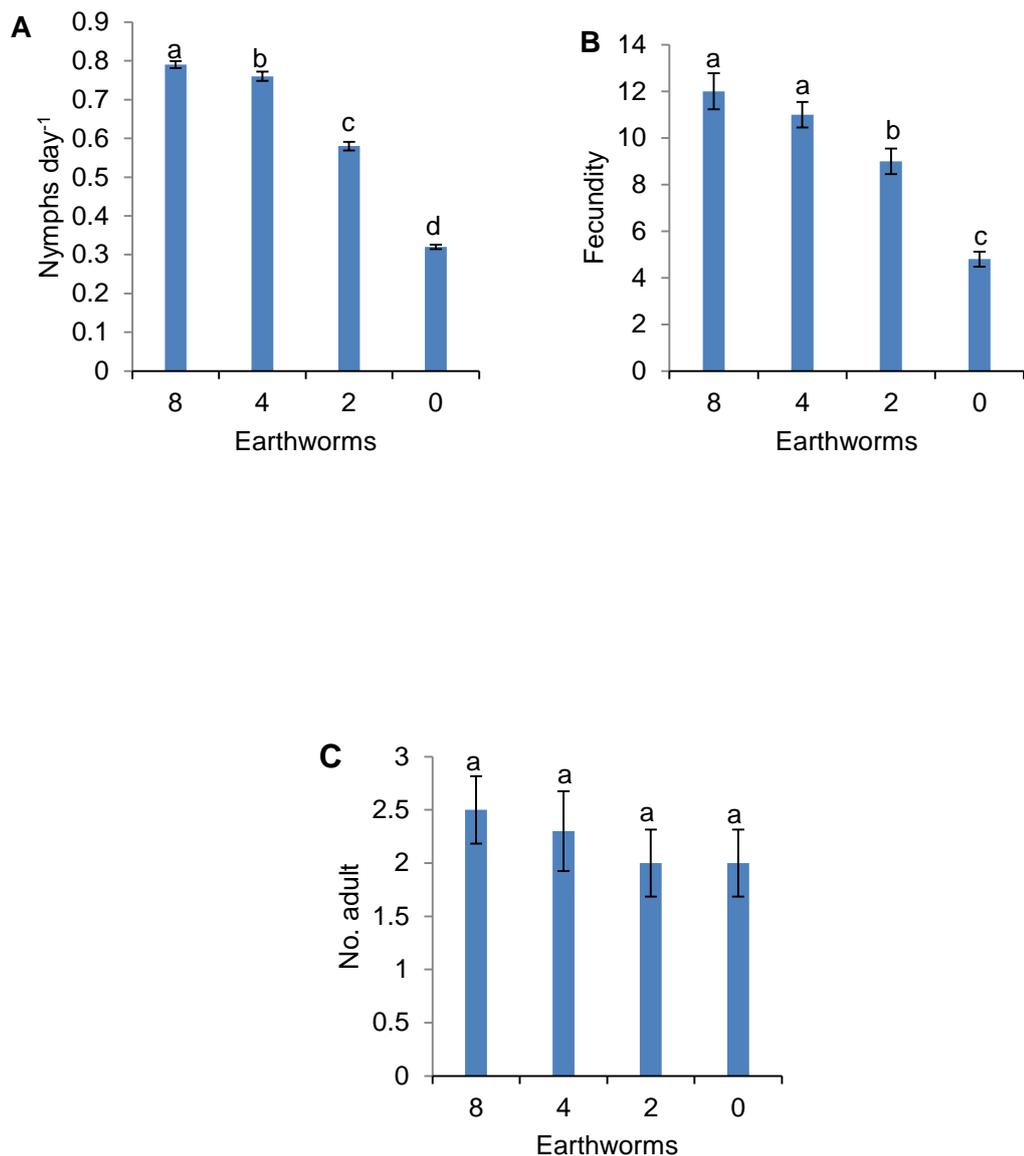


Figure 4.2 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea*) earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

The increases in the numbers of aphid offspring with time as a result of adding (8, 4, 2 and 0) of *A. rosea* in the both generations is shown in Figure (4.3). There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope of the regression

line for 8 worms was steeper than for the other treatments in both generations (Figure 4.3).

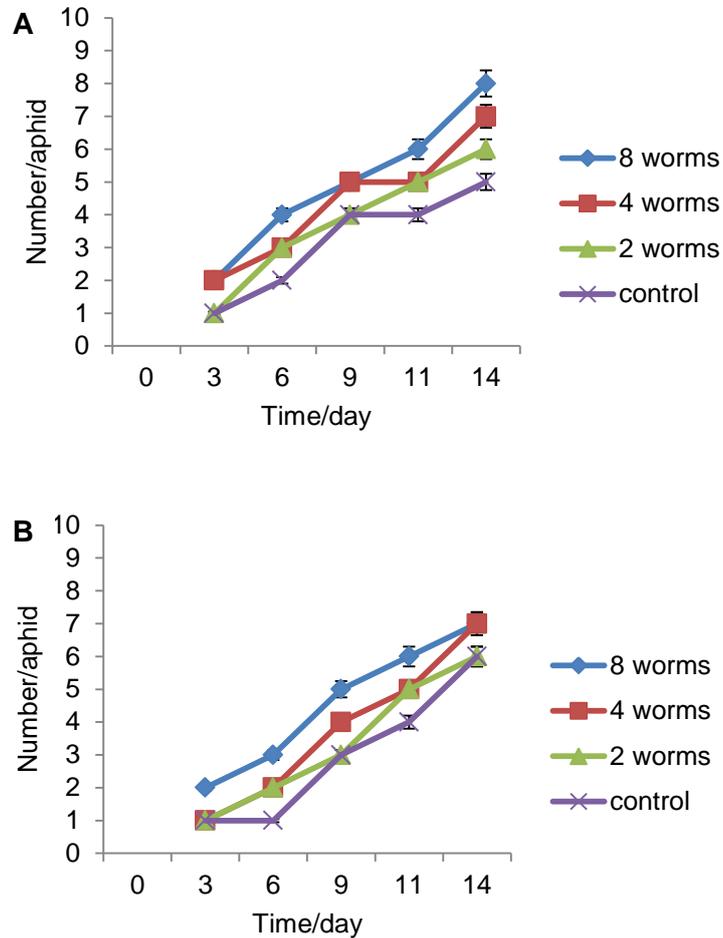


Figure 4.3 The increase of aphid population over a two week period on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea*) earthworm densities. (A) 1st generation and (B) 2nd generation. Vertical error bars ± 1 SE.

Adult increase with the time as a result of adding (8, 4, 2 and 0) of *A. rosea* in the both generations is shown in Figure 4.4. The results revealed that the numbers of adult recruited each day increased regularly in the first generation.

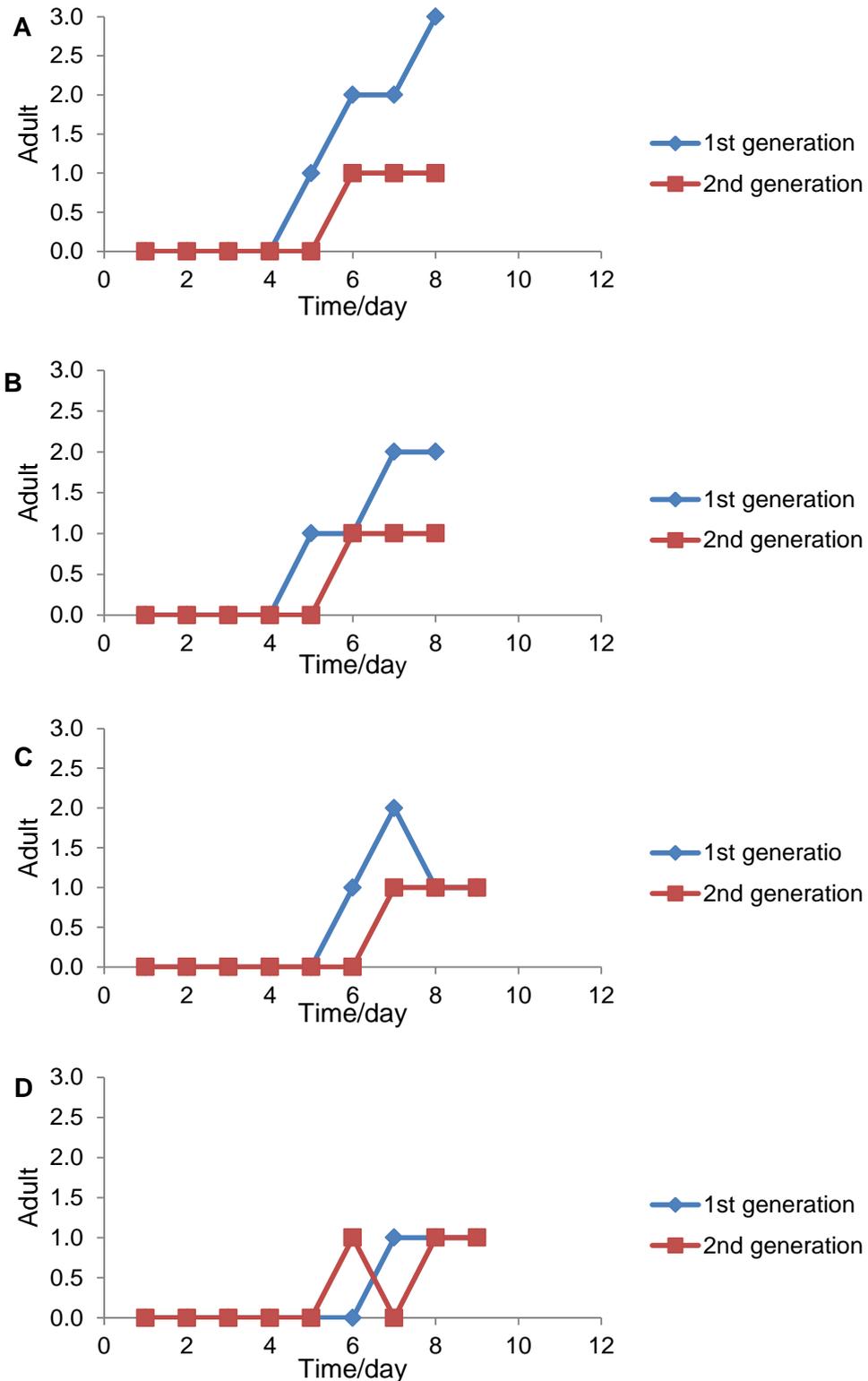


Figure 4.4 Daily adult increases in two generations on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea*) earthworm densities. (A) 8 worms, (B) 4 worms, (C) 2 worms and (D) control (no worms).

4.5.1.2 The influence of *A. chlorotica* on *M. persicae* development

The results of aphid development in the first generation are shown in Figure 4.5. Post-hoc tests showed that the numbers of nymphs produced each day were higher in the presence of eight *A. chlorotica* compared to worm density treatments of four, two and zero which had no significant differences ($P>0.05$) between them (Figure 4.5 A). The highest fecundity of aphids was in the presence of eight worms compared to all other treatments (Figure 4.5 B). While the numbers of adults developed from nymphs were not affected with increasing the worm densities with no significant differences between them (Figure 4.5 C).

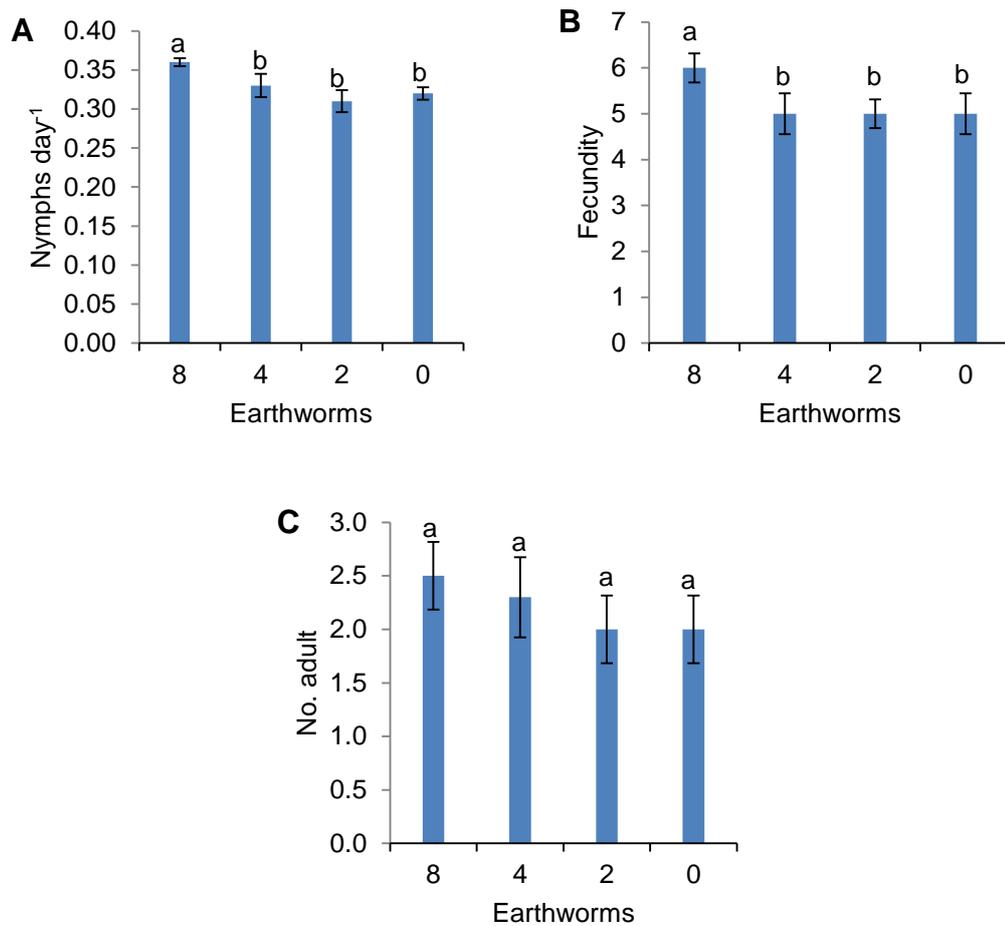


Figure 4.5 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

The results of aphid development in the second generation (Figure 4.6) revealed that daily nymph production was significantly increased in the presence of eight *A. chlorotica* individuals compared to the four, two and zero worm treatments, with significant differences ($P < 0.05$) between all treatments (Figure 4.6 A). Similarly, the fecundity of nymphs was also substantially higher in plants grown in the eight and four worm treatments than for the two and control treatments with significant differences

($P < 0.05$) between the two and control treatments (Figure 4.6 B). The numbers of adults developed from nymphs were also substantially higher in plants grown in the eight and four worm densities than for the two and zero worm treatments, with no significant differences ($P > 0.05$) between the latter treatments (Figure 4.6 C).

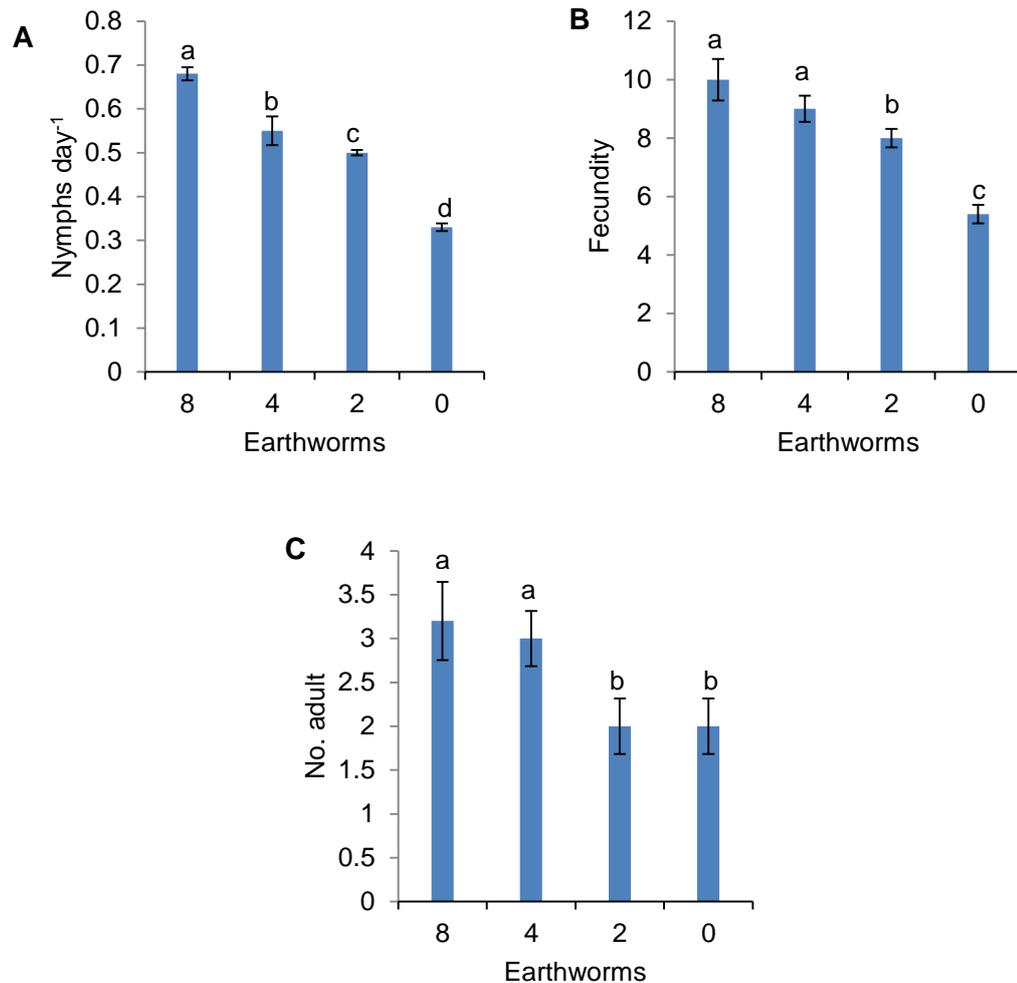


Figure 4.6 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

The increases in the numbers of aphid offspring with time as a result of adding (8, 4, 2 and 0) of *A. chlorotica* in both generations is shown in (Figure 4.7). There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope of the regression line for 8 worms was steeper than for the 4, 2 and control treatments in both generations (Figure 4.7).

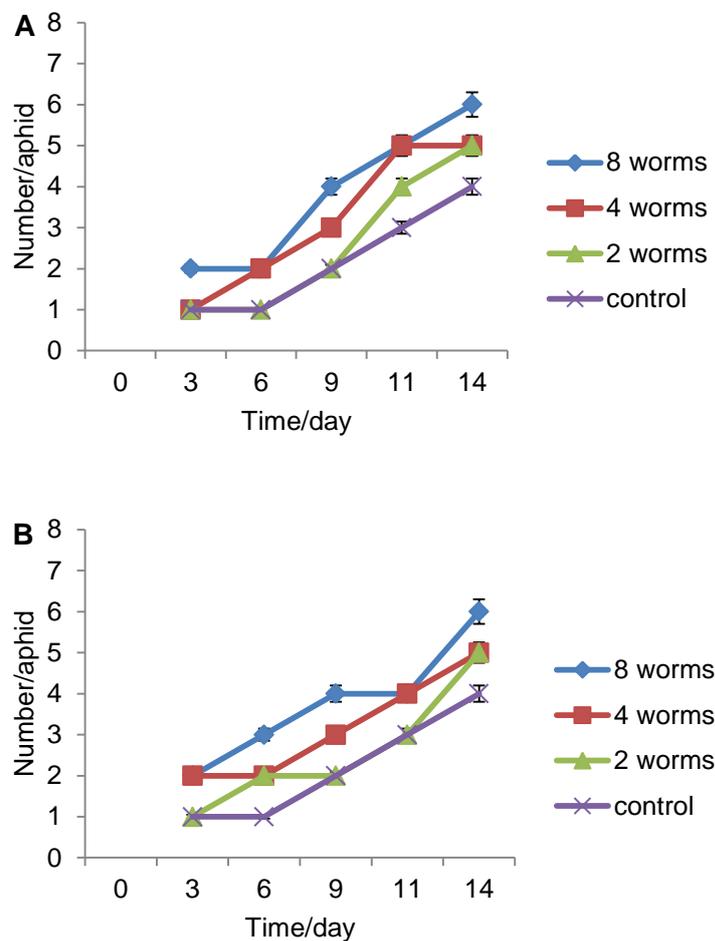


Figure 4.7 The increase of aphid population over a two week period on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) earthworm densities. (A) 1st generation and (B) 2nd generation. Vertical error bars $\pm 1SE$.

Adult increase with the time as a result of adding (8, 4, 2 and 0) of *A. chlorotica* in the both generations is shown in Figure 4.8. The results revealed that the numbers of adults recruited each day increased regularly in the first generation.

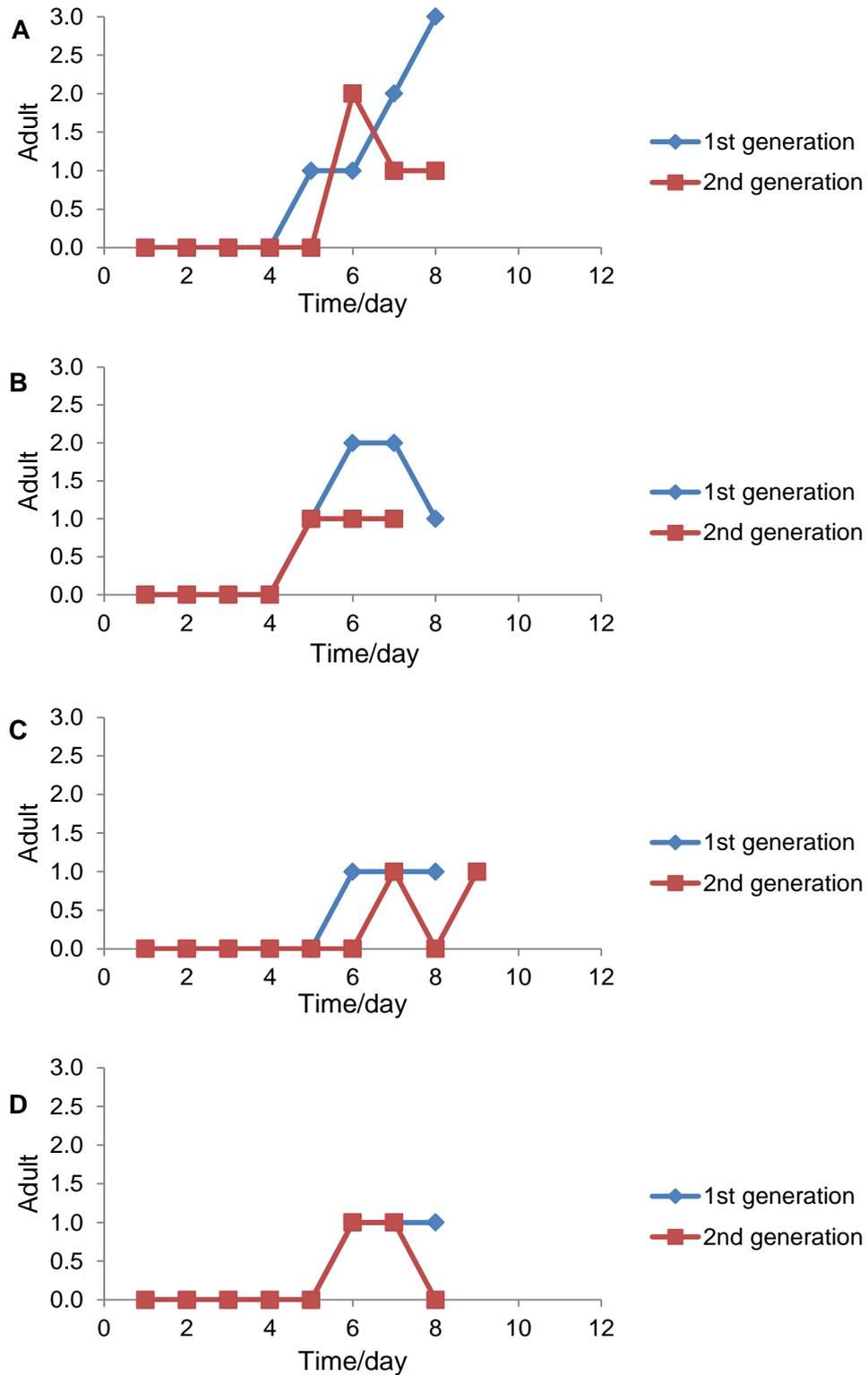


Figure 4.8 Daily adult increases in two generations on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) earthworm densities. (A) 8 worms, (B) 4 worms, (C) 2 worms and (D) control (no worms).

4.5.2 Two species of endogeic earthworms

4.5.2.1 The combined influence of *A. rosea* and *A. chlorotica* on *M. persicae* development

The development of aphid populations in the different treatments of *A. rosea* and *A. chlorotica* in the first generation is shown in (Figure 4.9). Post-hoc Tukey tests showed a substantial increase in the number of nymphs produced in the presence of eight worms, compared to worm density treatments four, two and zero, with significant differences ($P < 0.05$) between them (Figure 4.9 A). In addition to increasing daily production of nymphs, the fecundity of aphids was also affected by increasing the density of *A. rosea* and *A. chlorotica*. Post- hoc Tukey tests showed a substantial increase in the fecundity of aphids in the eight worm density compared to the four two and zero worm treatments, which had no significant differences ($P > 0.05$) between four and two treatments (Figure 4.9 B). Similarly, the numbers of adults developed from the nymphs was substantially higher in the presence of eight worms compared to the four, two and control treatments, with no significant differences between the four and two treatments (Figure 4.9 C).

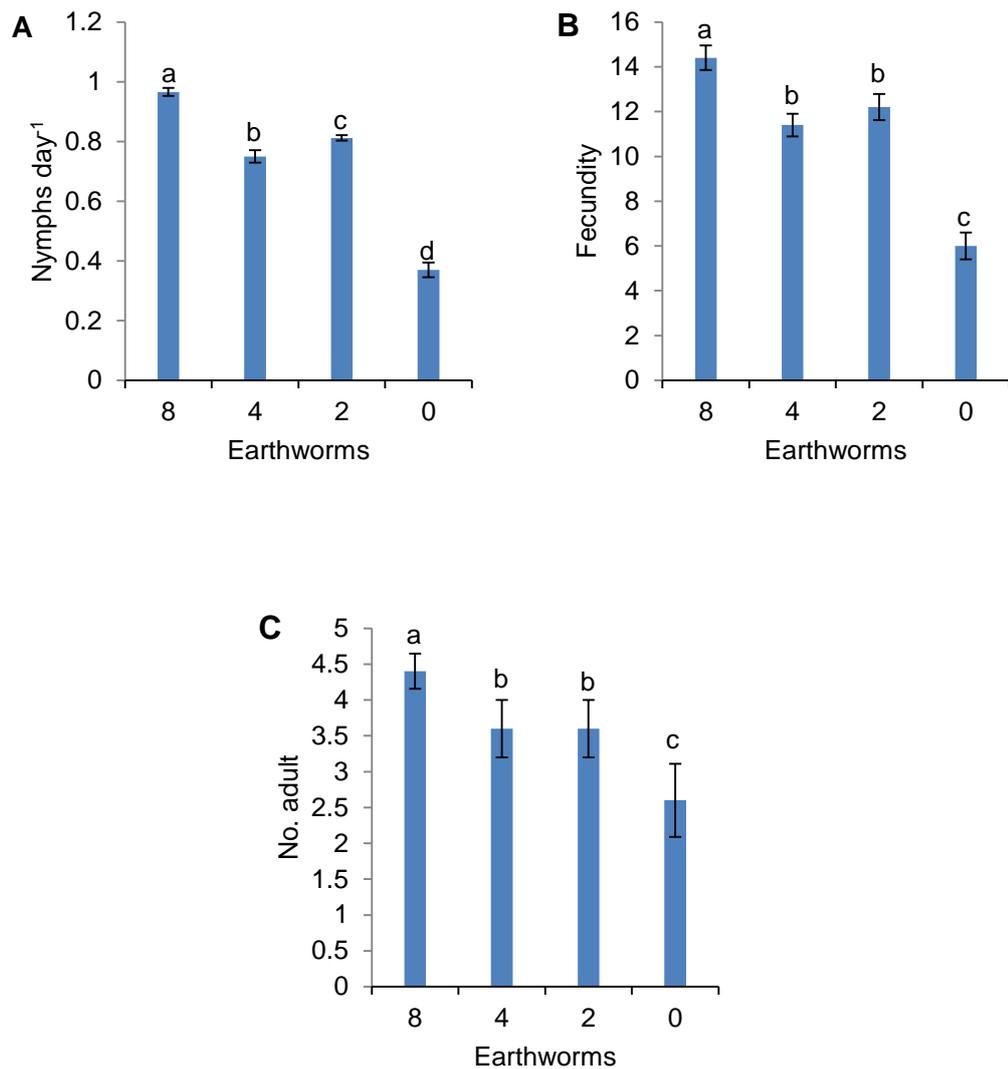


Figure 4.9 Mean (\pm SE) values for aphids development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* and *A. chlorotica*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

In addition to the first generation, aphid development was also affected by increasing *A. rosea* and *A. chlorotica* density in the second generation (Figure 4.10). Post-hoc Tukey tests showed higher daily production of nymphs in the presence of eight *A. rosea* and *A. chlorotica* individuals

compared to the four, two and zero worm treatments, with no significant differences ($P>0.05$) between the four and two treatments (Figure 4.10 A). The fecundity of nymphs was higher in plants grown in the eight, four and two worm treatments than for the control, with significant differences ($P<0.05$) between the control and all other treatments (Figure 4.10 B). The numbers of adults developed from nymphs were higher in plants grown in the eight and four worm densities with no significant differences ($P>0.05$) between them, while there were significant differences ($P<0.05$) between eight and the two and zero worm treatments (Figure 4.10 C).

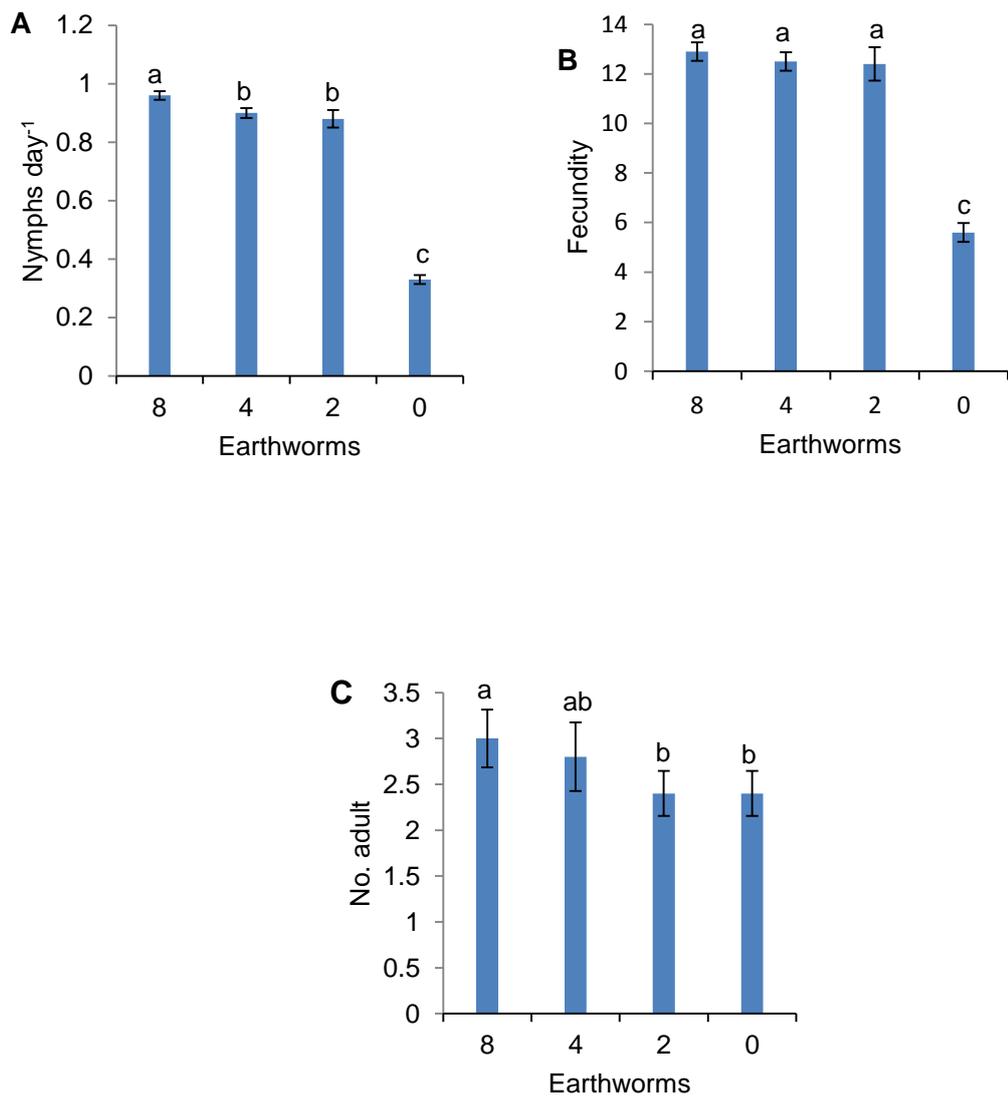


Figure 4.10 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* and *A. chlorotica*) earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

The increases in the numbers of aphid offspring with time as a result of adding (8, 4, 2 and 0) of *A. rosea* and *A. chlorotica* in both generations is shown in Figure 4.11. There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope

of the regression line for 8 worms was steeper than for the other treatments in both generations (Figure 4.11).

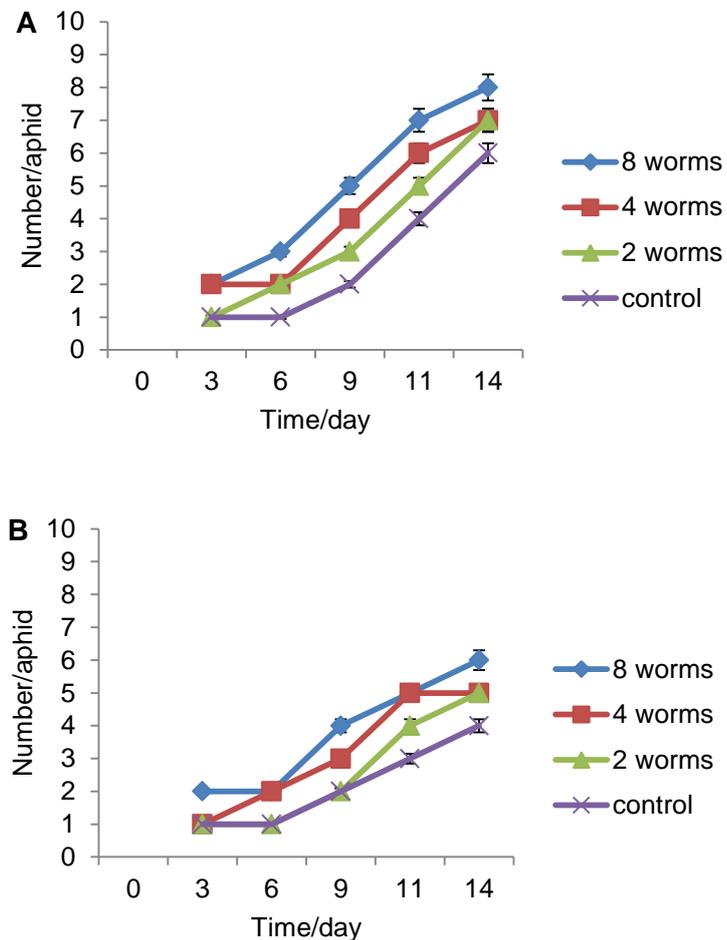


Figure 4.11 The increase of aphid population over a two week period on Chinese cabbage *B. rapa* under four different endogeic *A. rosea* and *A. chlorotica* earthworm densities. (A) 1st generation) and (B) 2nd generation. Vertical error bars ± 1 SE.

Adult increase with the time as a result of adding (8, 4, 2 and 0) of *A. rosea* and *A. chlorotica* in both generations is shown in Figure 4.12. The results revealed that the numbers of adult recruited each day increased regularly in the first generation.

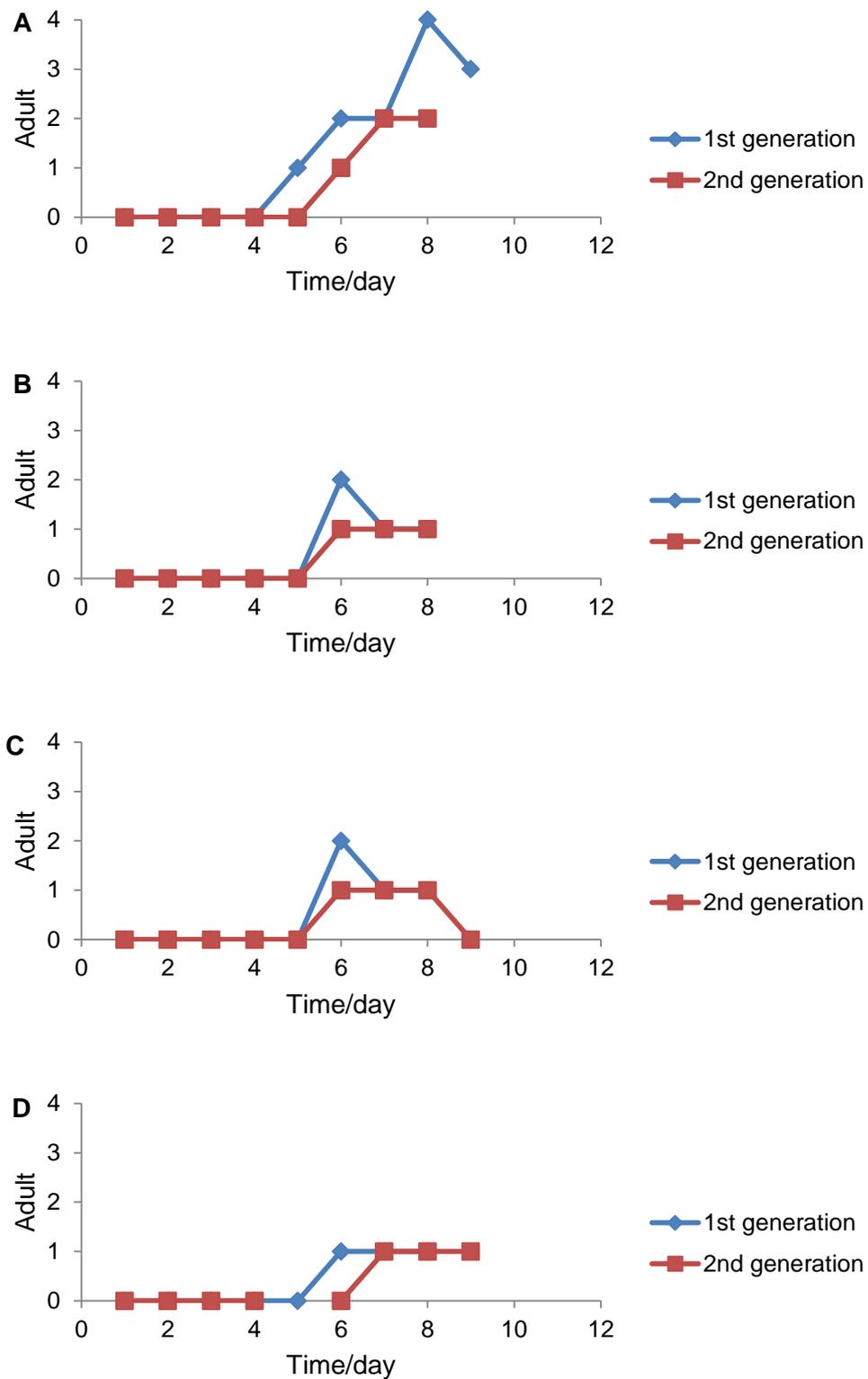


Figure 4.12 Daily adult increases in two generations on Chinese cabbage (*B. rapa*) under four different endogeic *A. rosea* & *A. chlorotica* earthworm densities. (A) 8 worms, (B) 4 worms, (C) 2 worms and (D) control (no worms).

4.5.3 Two species of endogeic and epigeic earthworms

4.5.3.1 The combined influence of the endogeic *A. caliginosa* and epigeic *S. mammalis* earthworms on *M. persicae* development.

The development of aphid populations in the different treatments of *A. caliginosa* and *S. mammalis* in the first generation is shown in Figure 4.13. Post-hoc Tukey tests showed a substantial increase in the number of nymphs produced in the presence of eight worms, compared to worm density treatments four, two and zero, with significant differences ($P < 0.05$) between all treatments (Figure 4.13 A). In addition to increasing daily production of nymphs, the fecundity of aphids was also affected by increasing the density of *A. caliginosa* and *S. mammalis*. Post- hoc Tukey tests showed a substantial increase in the fecundity of aphids in the eight and four worm densities compared to the two and zero worm treatments, which had significant differences ($P < 0.05$) between them (Figure 4.13 B). The numbers of adults developed from the nymphs was substantially higher in the presence of eight worms compared to the four, two and control treatments, with significant differences ($P < 0.05$) between them (Figure 4.13 C).

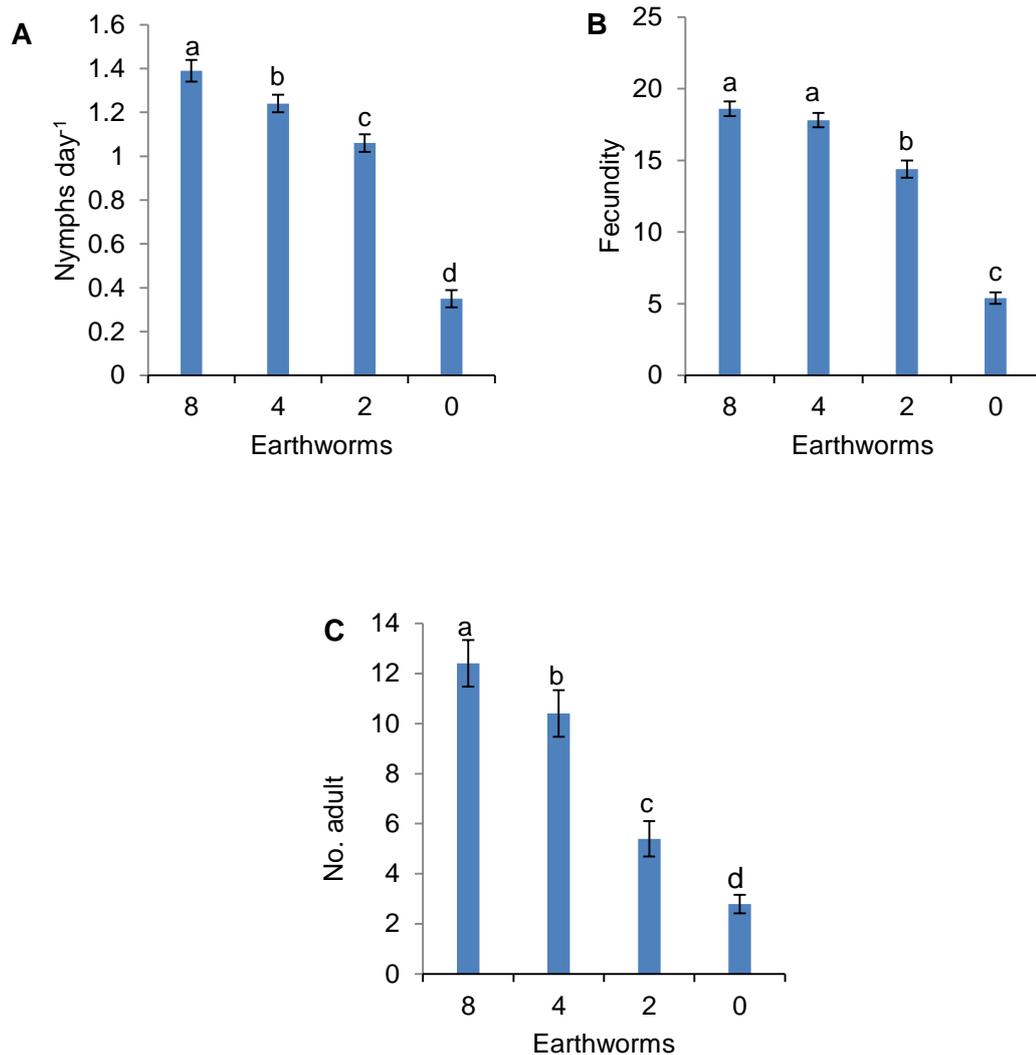


Figure 4.13 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*Brassica rapa*) under four different endogeic (*A. caliginosa*) and epigeic (*S. mammalis*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

In addition to the first generation, aphid development was also affected by increasing *A. caliginosa* and *S. mammalis* density in the second generation (Figure 4.14). Post-hoc Tukey tests showed higher daily production of nymphs in the presence of eight *A. caliginosa* and *S.*

mammalis individuals compared to the four, two and zero worm treatments, with significant differences ($P < 0.05$) between all treatments (Figure 4.14 A). Similarly, the fecundity of nymphs was also substantially higher in plants grown in the eight worm treatment than for all other treatment groups, with significant differences ($P < 0.05$) between all treatments (Figure 4.14 B). The numbers of adults developed from nymphs were higher in plants grown in the eight and four worm densities than for the two and zero treatments, with significant differences ($P < 0.05$) between them (Figure 4.14 C).

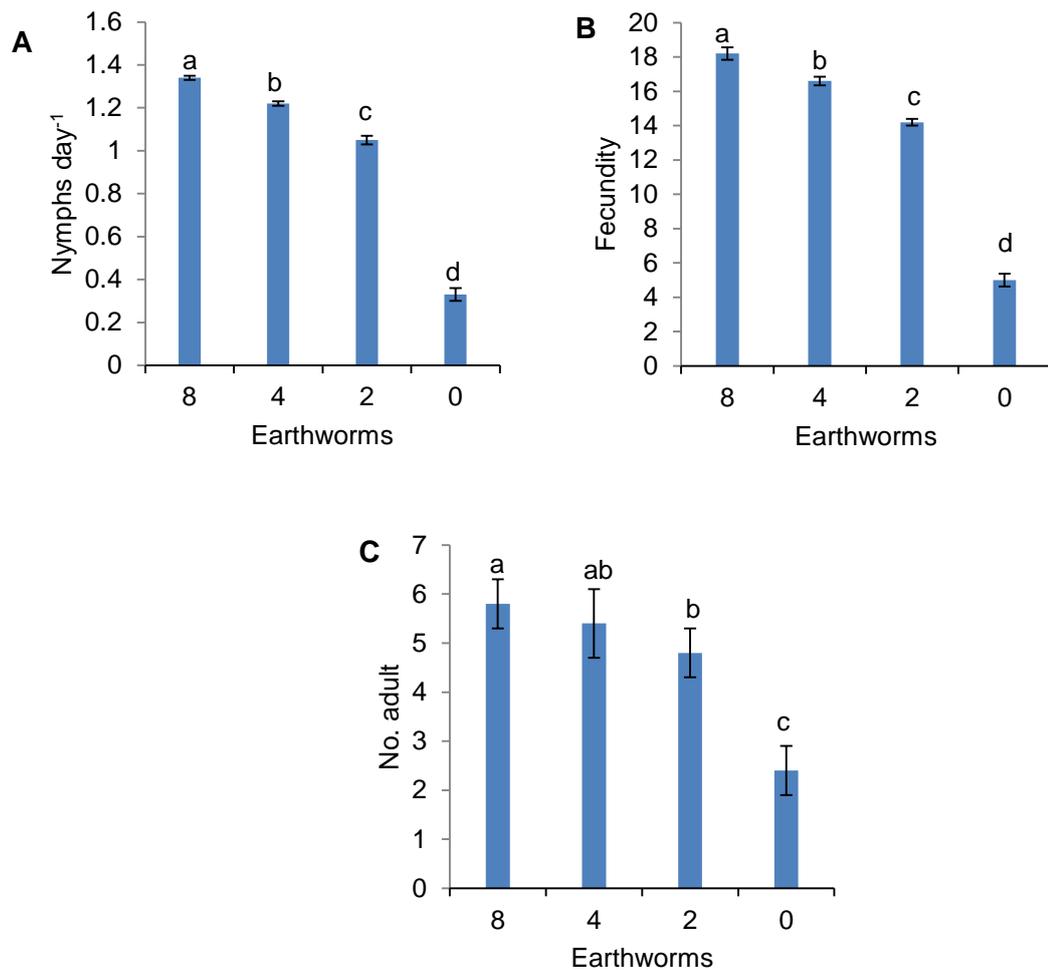


Figure 4.14 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. caliginosa*) and epigeic (*S. mammalis*) earthworm densities, (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

The increases in the numbers of aphid offspring with time as a result of adding (8, 4, 2 and 0) of *A. caliginosa* and *S. mammalis* in both generations is shown in Figure 4.15. There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope of the regression line for the control was less steep than for the other treatments in both generations (Figure 4.15).

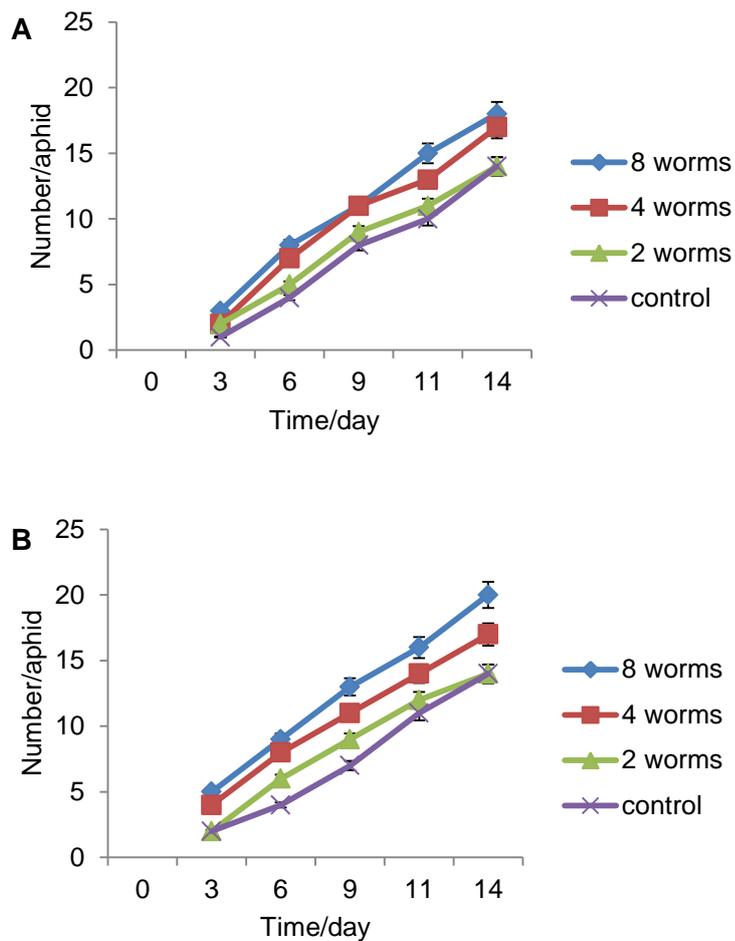


Figure 4.15 The increase of aphid population over a two week period on Chinese cabbage (*B. rapa*) under four different endogeic (*A. caliginosa*) and epigeic (*S. mammalis*) earthworm densities. (A) 1st generation) and (B) 2nd generation. Vertical error bars ± 1 SE.

Adult increase with time as a result of adding (8, 4, 2 and 0) of *A. caliginosa* and *S. mammalis* in the two generations is shown in Figure 4.16. The results revealed that the numbers of adults increased regularly in the first generation.

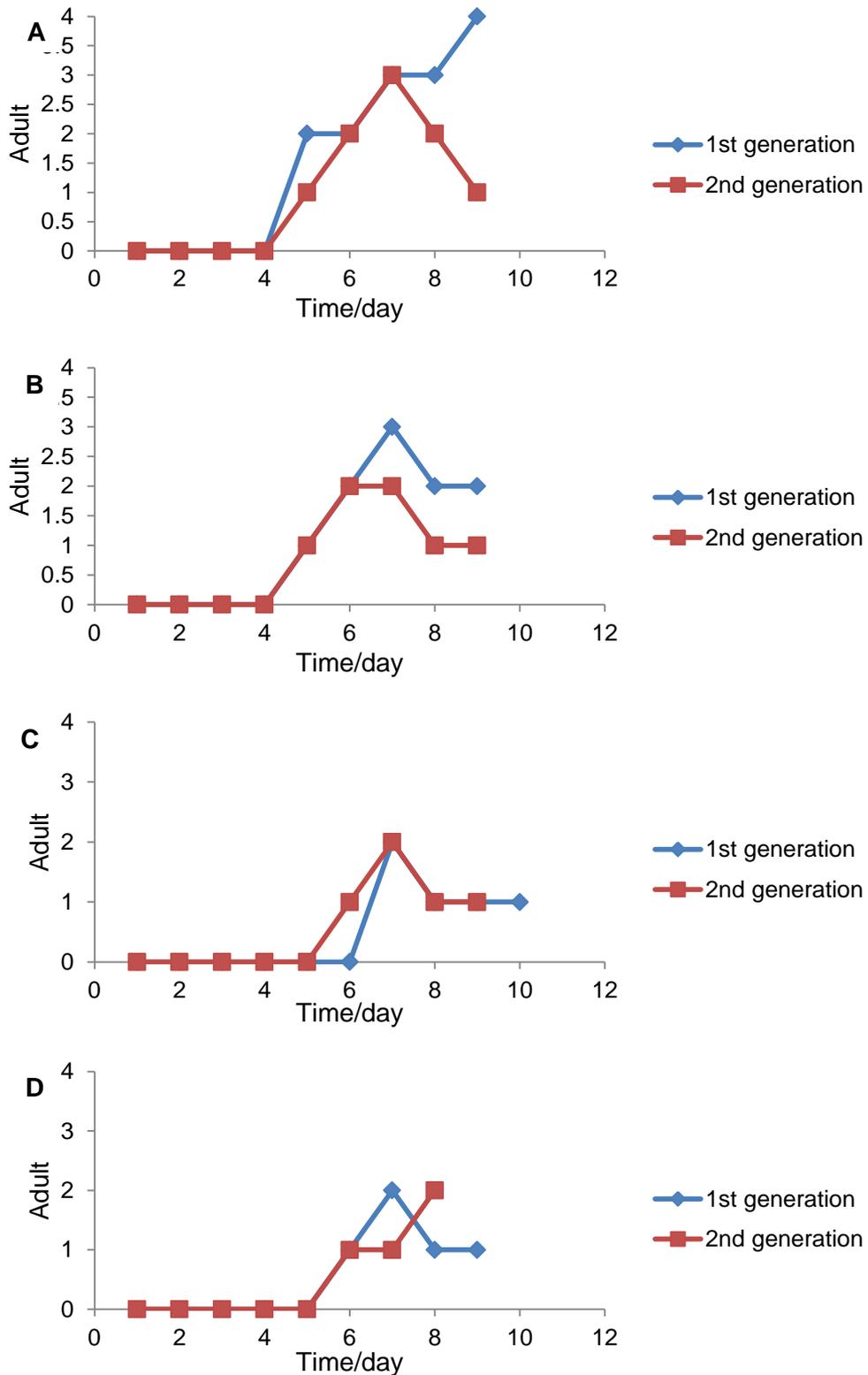


Figure 4.16 Daily adult increase in two generations on Chinese cabbage (*B. rapa*) under four different endogeic (*A. caliginosa*) and epigeic (*S. mammalis*) earthworm densities. (A) 8 worms, (B) 4 worms, (C) 2 worms and (D) control (no worms).

4.5.3.2 The combined influence of the endogeic *A. chlorotica* and epigeic *S. mammalis* earthworms on *M. persicae* development

The results of aphid development in the first generation are shown in Figure 4.17. Post-hoc tests showed that the number of nymphs produced each day were higher in the presence of eight *A. chlorotica* and *S. mammalis* compared to worm density treatments of four, two and zero which also had significant differences ($P < 0.05$) between them (Figure 4.17 A). The highest fecundity of aphids was in the presence of eight worms compared to all other treatments, with significant differences ($P < 0.05$) between all treatments (Figure 4.17 B). Similarly, the numbers of adults developed from nymphs were also substantially higher in plants grown in the eight worm treatment than for other treatment groups, with significant differences ($P < 0.05$) between all treatments (Figure 4.17 C).

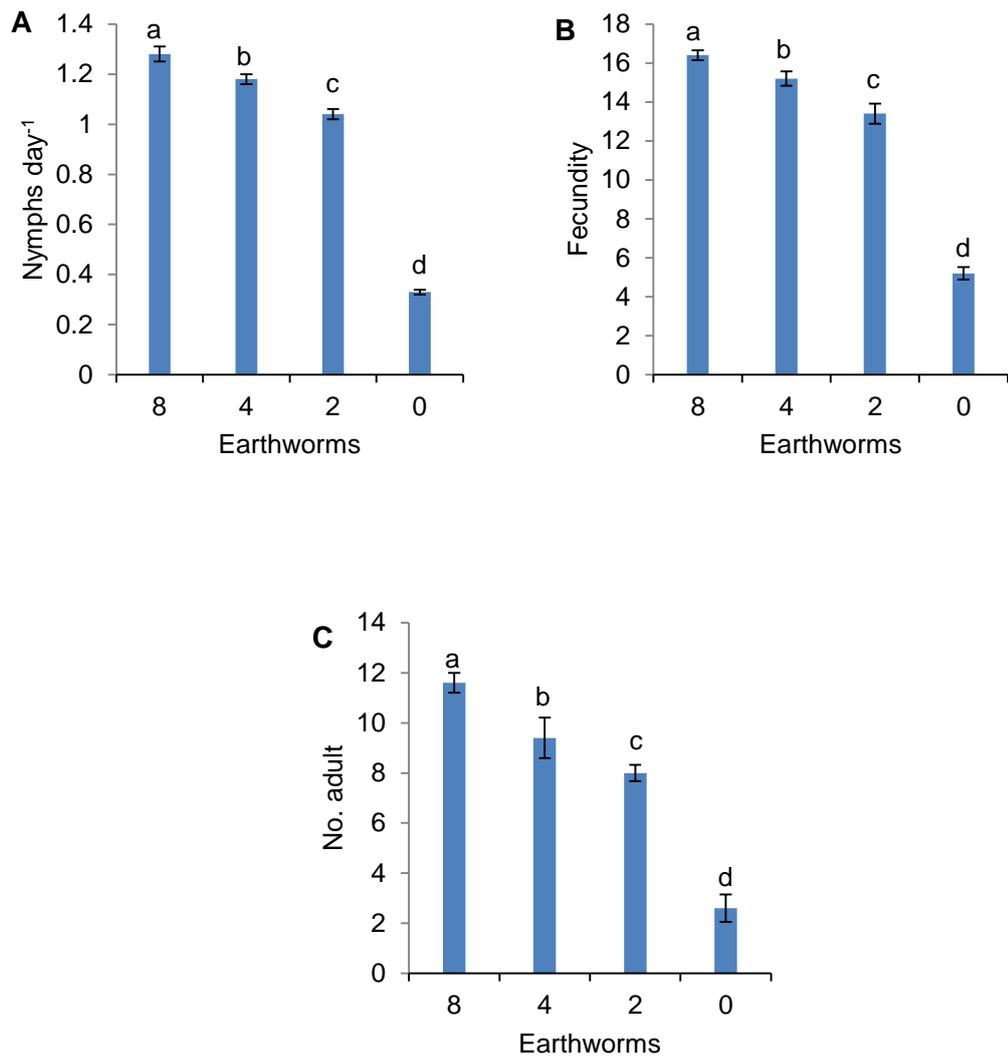


Figure 4.17 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) and epigeic (*S. mammalis*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

The results of aphid development in the second generation (Figure 4.18) revealed that daily nymph production and nymph fecundity were significantly increased in the presence of eight *A. chlorotica* and *S. mammalis* individuals compared to the four, two and zero worm treatments, with significant differences ($P < 0.05$) between all treatments

(Figure 4.18 A, B). The numbers of adults developed from nymphs were higher in the presence of eight, four and two treatments compared to the control treatment (Figure 4.18 C).

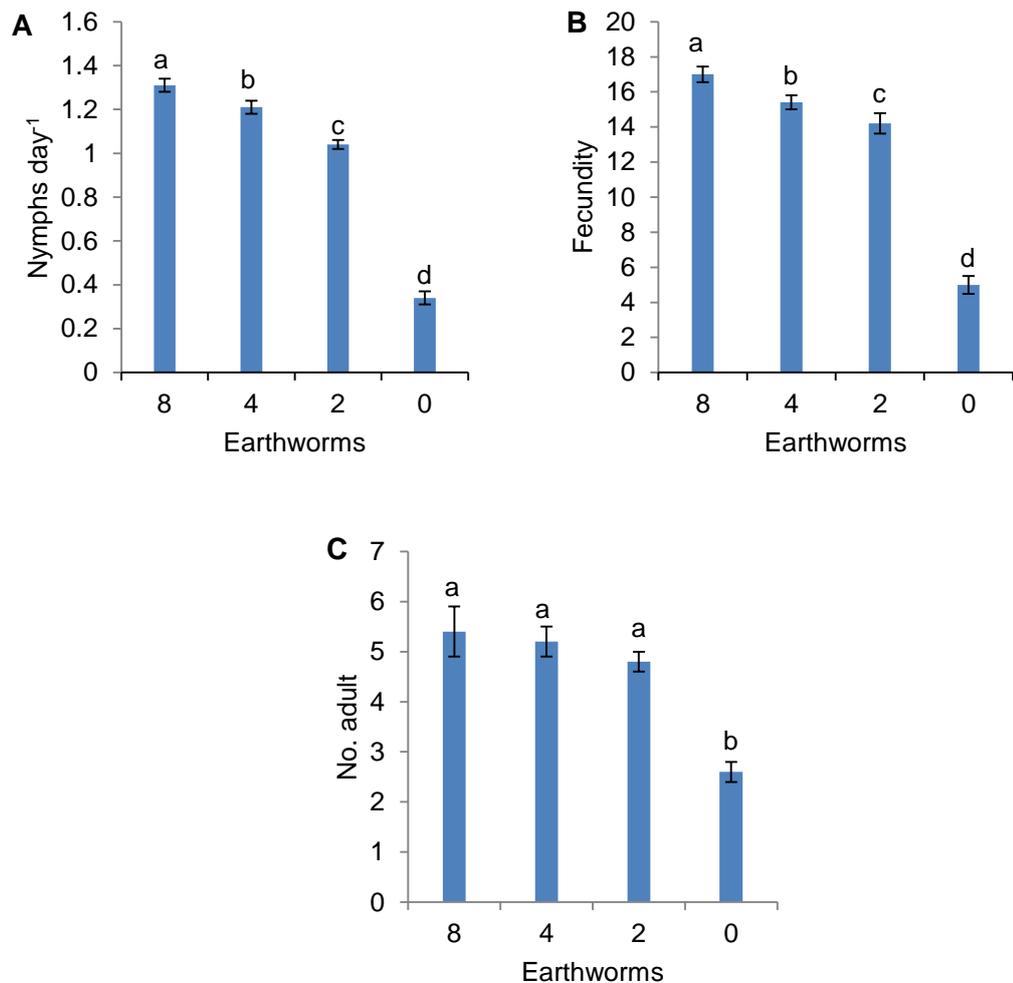


Figure 4.18 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) and epigeic (*S. mammalis*) earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

Increases in the numbers of aphid offspring with time as a result of adding eight, four, two and zero of *A. caliginosa* and *S. mammalis* in both

generations is shown in (Figure 4.19). There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope of the regression line for 8 worms was steeper than for the other treatments in both generations (Figure 4.19).

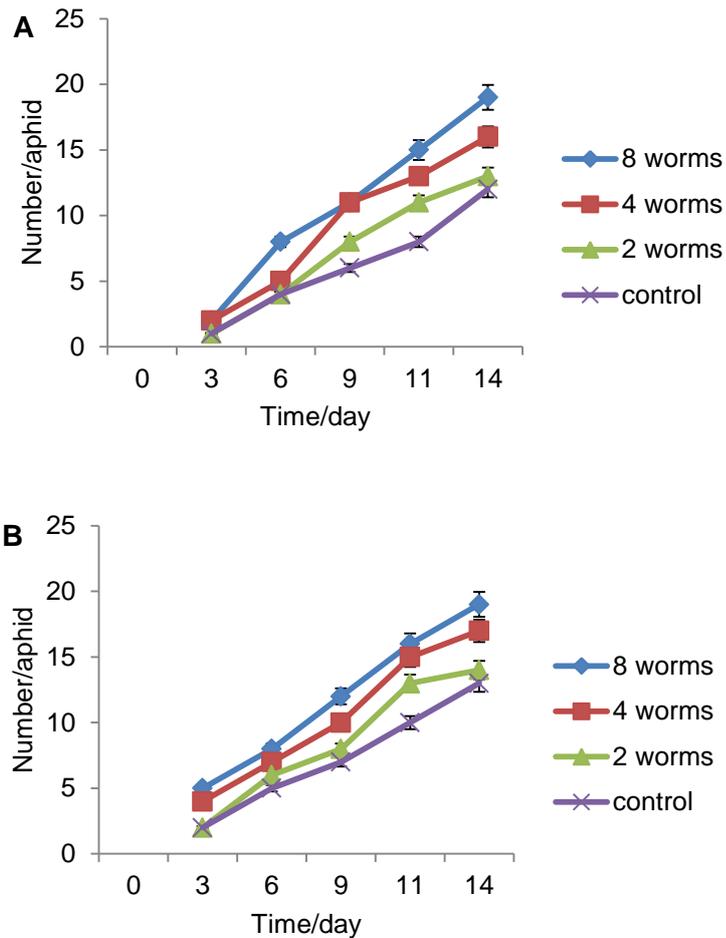


Figure 4.19 The increase of aphid population over a two week period on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) and epigeic (*S. mammalis*) earthworm densities. (A) 1st generation and (B) 2nd generation. Vertical error bars $\pm 1SE$.

Adult increase with time as a result of adding (8, 4, 2 and 0) of *A. chlorotica* and *S. mammalis* in the two generations is shown in Figure 4.20. The results revealed that the numbers of adults increased regularly in the first generation.

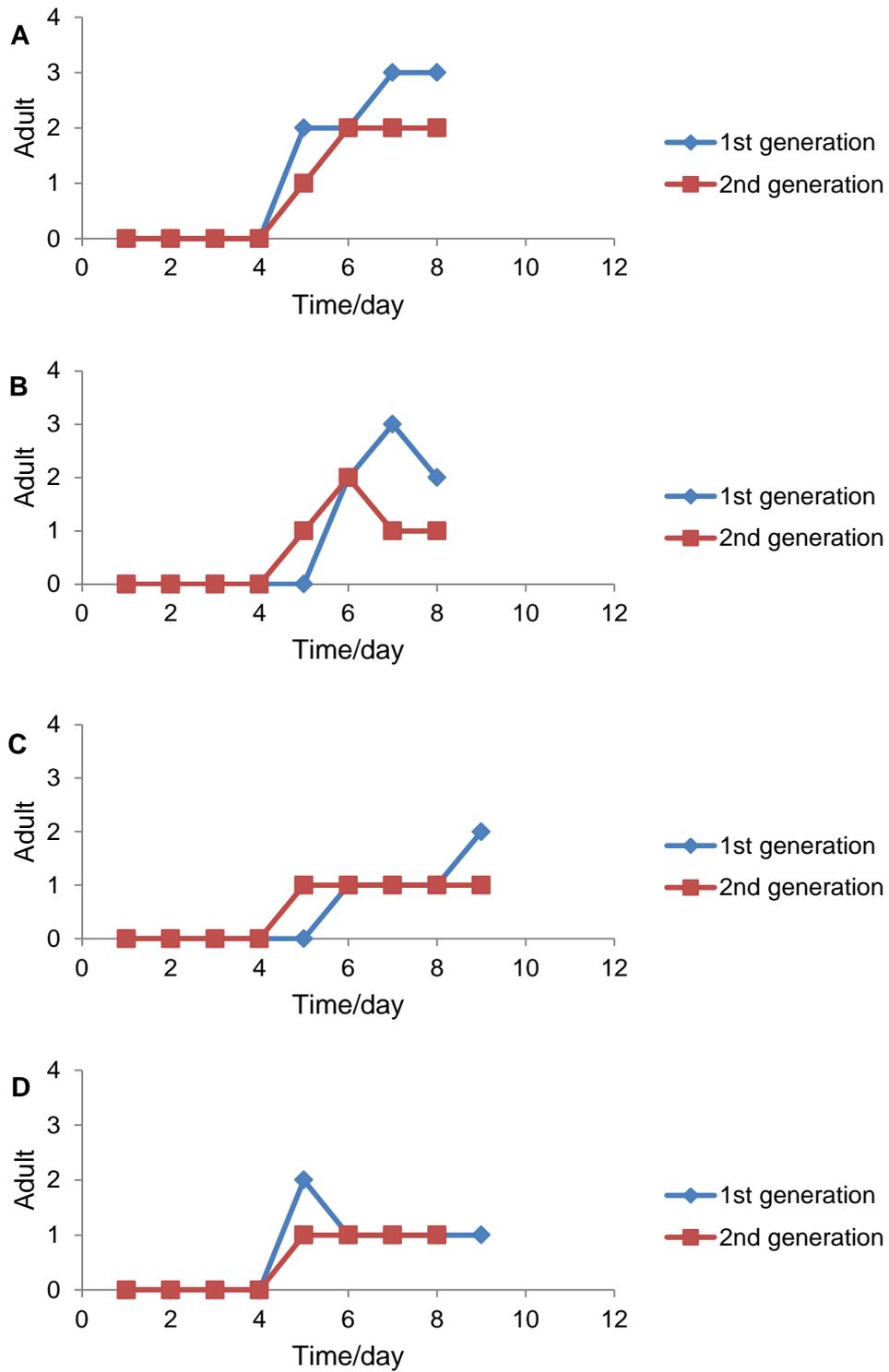


Figure 4.20 Daily adult increase in two generations on Chinese cabbage (*B. rapa*) under four different endogeic (*A. chlorotica*) and epigeic (*S. mammalis*) earthworm densities. (A) 8 worms, (B) 4 worms, (C) 2 worms and (D) control (no worms).

4.5.4 Four species of endogeic and epigeic earthworms

4.5.4.1 The combined influence of the endogeic *A. rosea* & *A. caliginosa* and the epigeic *L. rubellus* & *S. mammalis* earthworms on *M. persicae* development

The results (Figure 4.21) revealed that aphid development was affected by increasing *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* density in the first generation. These included; nymphs day⁻¹, fecundity and numbers of adults. There was a substantial increase in the daily production of nymphs in the presence of the 12 earthworm density compared to the eight, four and zero worm treatments, with significant difference ($P < 0.05$) between all treatments (Figure 4.21 A). Additionally fecundity was higher in the presence of 12 individuals compared with the eight, four and zero treatments, with significant differences ($P < 0.05$) between all treatment groups (Figure 4.21 B). Similarly, the numbers of adults developed from nymphs were also substantially higher in plants grown in the 12 worm density than for the eight, four and zero treatments, with significant differences ($P < 0.05$) between all treatments (Figure 4.21 C).

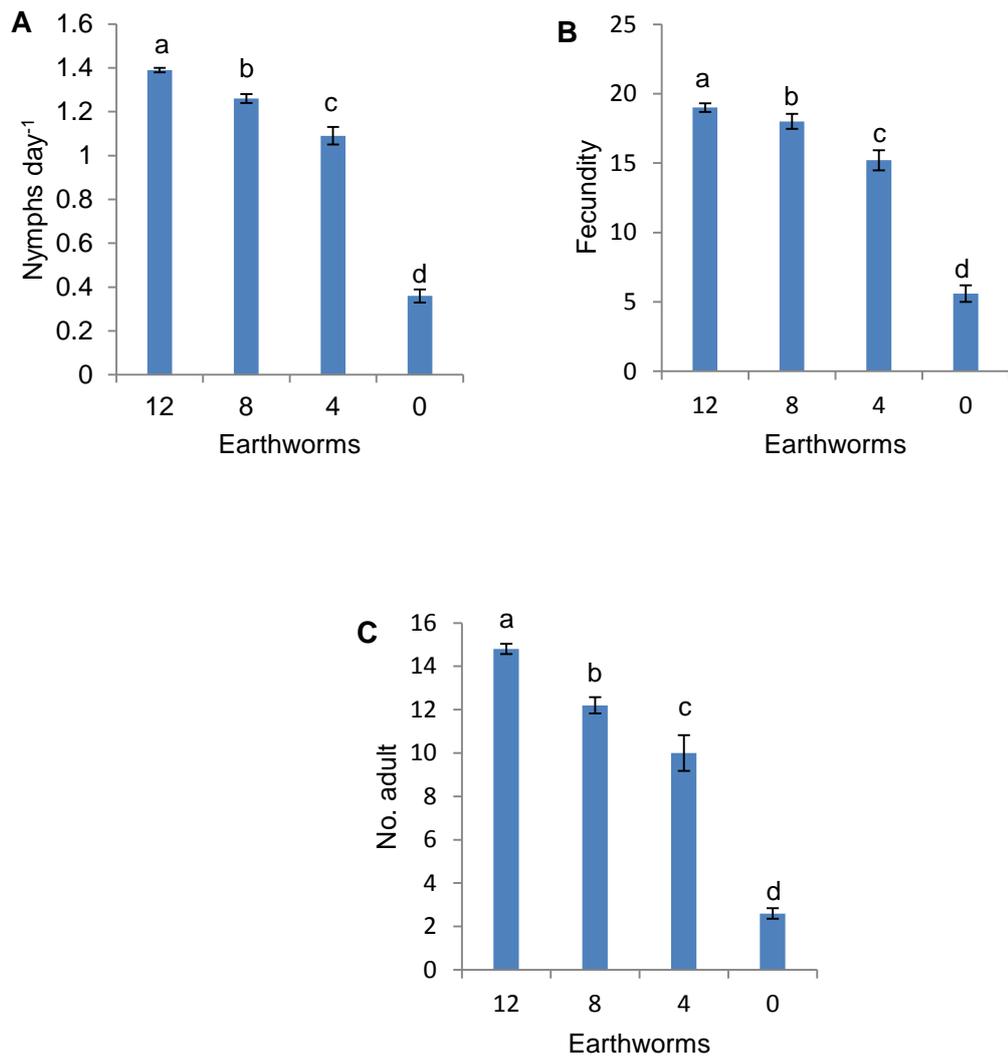


Figure 4.21 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) number of adults produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* and *A. caliginosa*) with epigeic (*L. rubellus* and *S. mammalis*) earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each parameter.

In addition to the first generation, in the second generation (Figure 4.22), the numbers of *M. persicae* nymphs day⁻¹ were clearly higher in the 12 *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* earthworm treatment, while there was no significant difference ($P > 0.05$) between the eight and four worm treatments (Figure 4.22 A). Fecundity was also affected by

increasing earthworm density. Post-hoc Tukey tests showed a substantial increase in numbers of nymphs in the presence of 12 worms compared to the eight, four and zero treatments, with significant differences ($P < 0.05$) between all treatments (Figure 4.22 B). Similarly, the numbers of adults developed from nymphs were also substantially higher in plants grown in the 12 worm density than for the eight, four and zero treatments, with no significant differences ($P > 0.05$) between eight, four worm treatments (Figure 4.22 C).

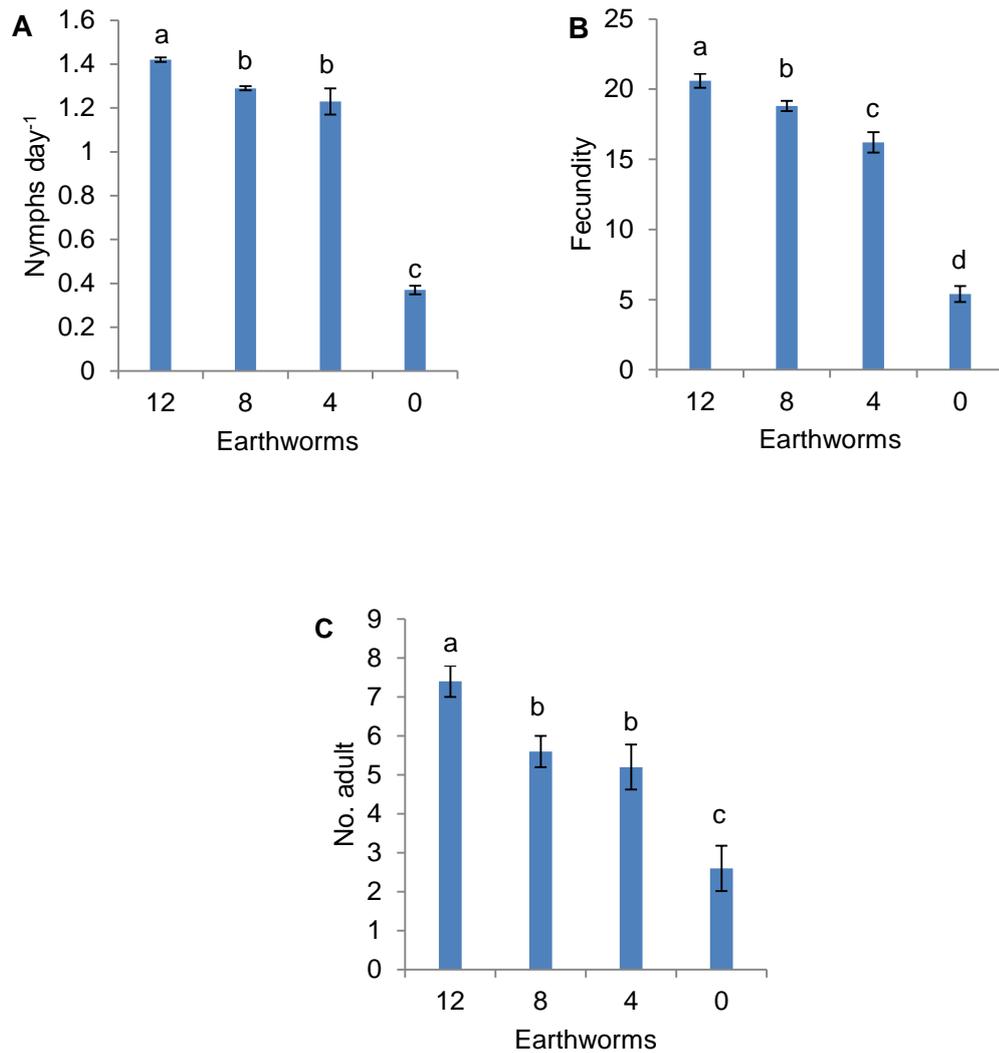


Figure 4.22 Mean (\pm SE) values for aphid development (A) nymphs day⁻¹, (B) fecundity and (C) numbers of adult produced on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* and *A. caliginosa*) with epigeic (*L. rubellus* and *S. mammalis*) earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each parameter.

Figure 4.23, shows increasing numbers of *M. persicae* nymphs with time as a result of adding twelve, eight, four and zero *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* earthworms in both generations. There are differences in the slopes between treatments. The results from the scatter diagrams showed that the slope of the regression line for 12 worms was

steeper than for the 8, 4 and control treatments in both generations (Figure 4.23).

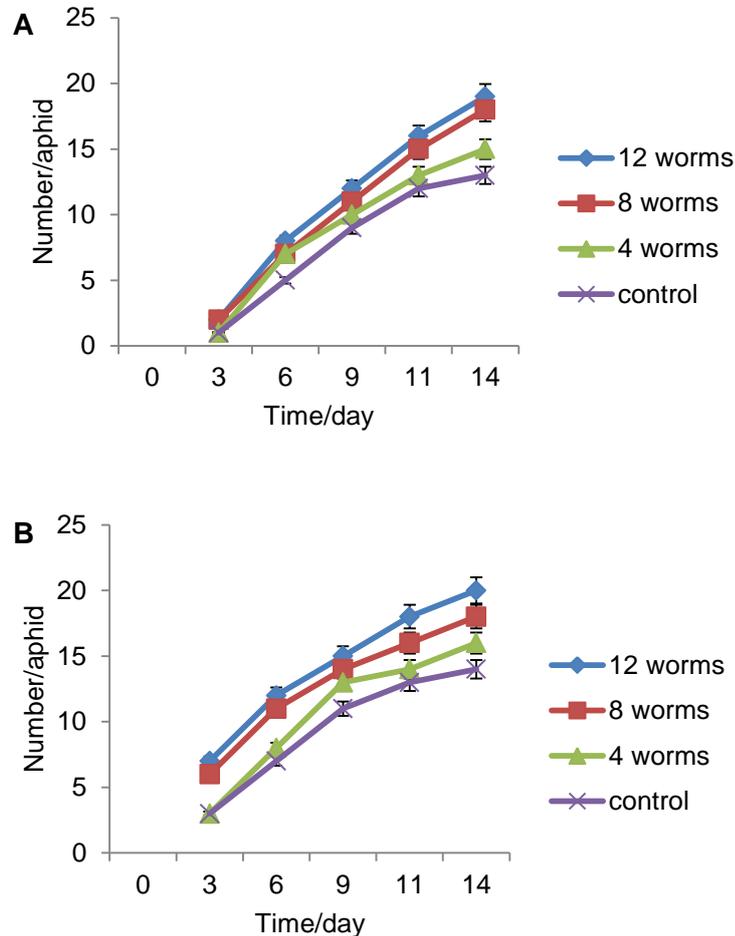


Figure 4.23 The increase of aphid population over a two week period in Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* and *A. caliginosa*) with epigeic (*L. rubellus* and *S. mammalis*) earthworm densities. (A) 1st generation) and (B) 2nd generation. Vertical bars \pm 1SE.

Adult increase with time as a result of adding (12, 8, 4 and 0) of *A. rosea*, *A. chlorotica*, *L. rubellus* and *S. mammalis* in the two generations is shown in Figure 4.24. The results revealed that the numbers of adults increased regularly in the first generation.

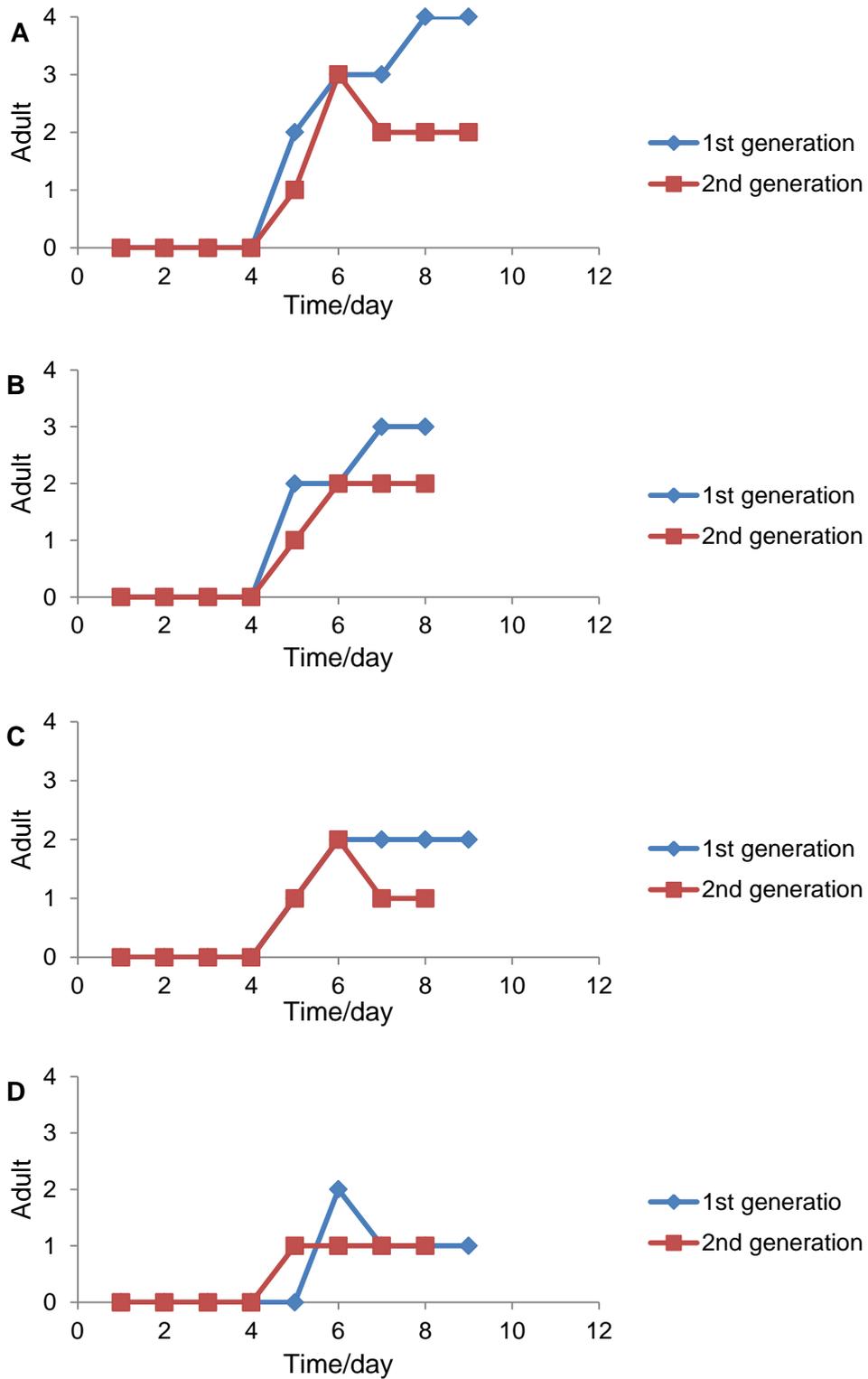


Figure 4.24 Daily adult increase in two generations on Chinese cabbage (*B. rapa*) under four different endogeic (*A. rosea* & *A. chlorotica*) with epigeic (*L. rubellus* & *S. mammalis*) earthworm densities. (A) 12 worms, (B) 8 worms, (C) 4 worms and (D) control (no worm).

4.5.5 The relationship between aphid performance and leaf nitrogen content

The relationship between nitrogen concentration and aphid development in Chinese cabbage shows a correlation between increasing the amount of nitrogen and numbers of aphids. Increased daily production of nymphs, fecundity and numbers of adults were generally correlated with increasing nitrogen concentration ($R^2 = 0.72$, $P=0.0031$; $R^2 = 0.79$, $P=0.0008$; $R^2 = 0.71$, $P=0.0032$) respectively (Figure 4.25).

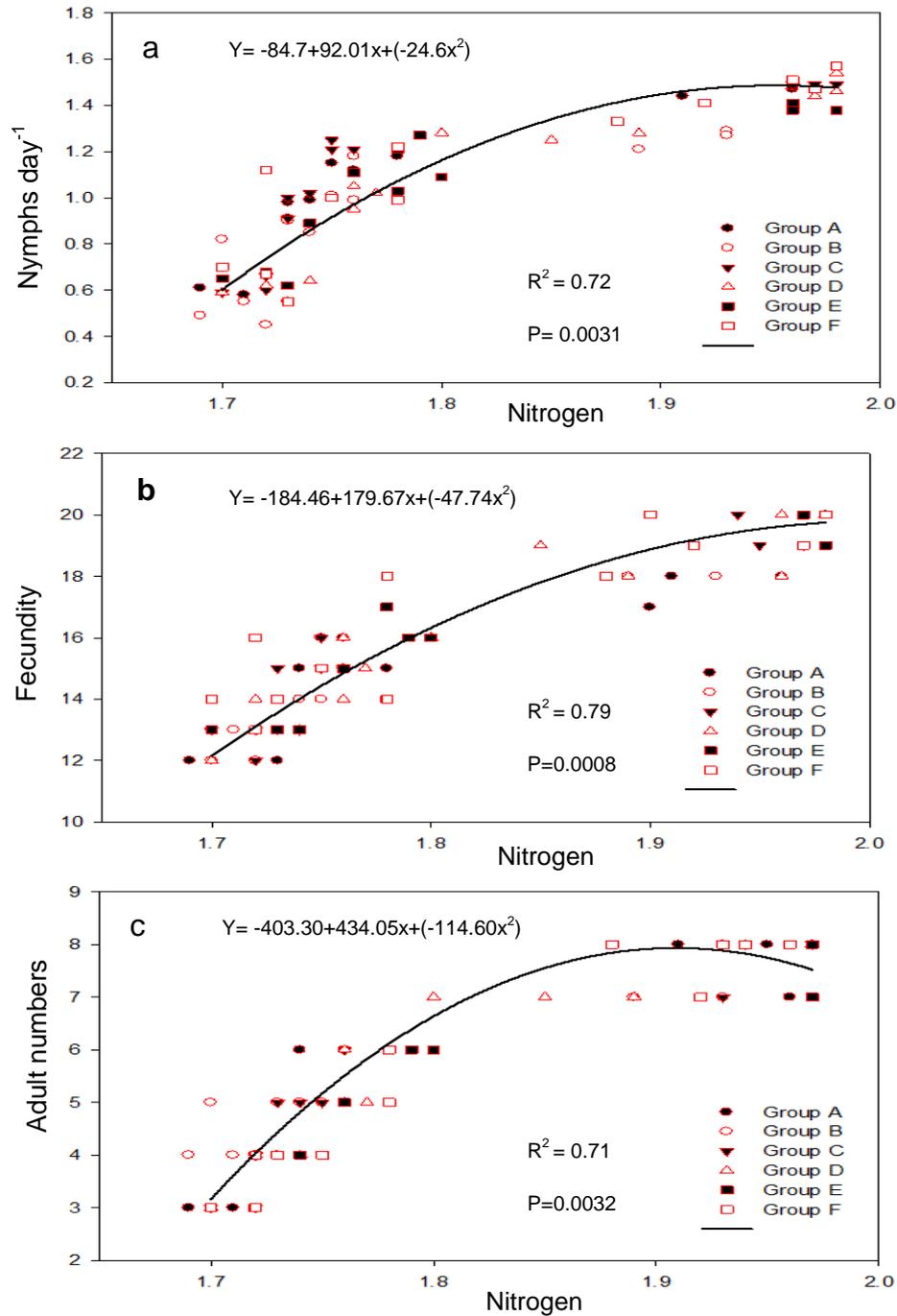


Figure 4.25 Relationship between increasing nitrogen concentration in *B. rapa* and increasing (a) nymphs day⁻¹ (b) fecundity (c) adult numbers under different (A): *A. rosea*, (B): *A. chlorotica*, (C): the combined effect of *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis*, (E): *A. chlorotica* & *S. mammalis* and (F): *A. rosea*, *A. caliginosa*, *S. mammalis* and *L. rubellus* earthworm groups.

4.6 Discussion

The results achieved in this investigation have shown the important role of earthworms in influencing aphid population dynamics and the interaction between aphids and the host plant.

In this study there are three possible effects of earthworms on aphid development; daily production of offspring, fecundity - measured by the number of nymphs produced- and growth of adults developed from nymphs in two generations.

The results here suggest that the highest number of daily offspring recorded were consistently in the presence of the highest worm density, and the number of new born nymphs increased daily with increasing the earthworm densities.

This study also indicated that aphid fecundity and the numbers of adults developed from nymphs positively increased in the highest (eight and 12) earthworm densities.

The results obtained in this study confirmed these findings; *M. persicae* was positively influenced by the presence of the endogeic *A. caliginosa* earthworms, as reported in a similar study by Wurst & Jones (2003). Furthermore, by week 16 of their study *M. persicae* reproduction had been significantly increased on *P. annua* plants by the presence of endogeic *A. caliginosa* and *O. tyrtaeum* individuals.

In contrast, Wurst *et al.*, (2003) suggested that earthworms decreased the number of aphids. However, there were differences in aphid reproduction depending on the plant functional groups; the number of nymphs was

lower in *L. perenne* (grass) compared to *P. lanceolata* (weedy forb) and *T. repens* (legume).

As further evidence of differences in plant-aphid systems, Wurst & Forstreuter (2010) found that in the presence of an endogeic *Aporrectodea* sp. earthworm the number of aphids were decreased on Tansy (*Tanacetum vulgare* L.), which could be attributed to differences in plant species, since this plant is known as an aromatic plant (Hussey, 1974), and is repellent against insects (Nottingham *et al.*, 1991).

Furthermore Razmjou *et al.*, (2012), reported that adding vermicompost to the soil caused decreases in aphid numbers on cucumber cultures. The reason behind that may be due to the source of vermicompost which produce from different species of earthworms, possibly this species has a negative effect on aphid.

On the other hand, results in the current study indicated that aphid development was positively correlated with nitrogen concentration (Figure 4.25). There are contrasting results about the role of nitrogen on insects; Dixon, (1998) found that in the presence of earthworms, nitrogen concentration increased in the plant, thereby positively influencing aphid reproduction. A similar result was reported by Wurst *et al.*, (2004). Under conditions where nitrogen concentration increased the numbers of aphids also increased on grass. Simply by increasing soil nitrogen levels Aqueel & Leather (2011), were able to increase the numbers of adults and their fecundity for *S. avenae* and *Rhopalosiphum padi* L.

In contrast Scheu & Jones (1999), found a weak relationship between increased nitrogen concentration and *M. persicae* development. However this seems to be due to the differences in plant species because there was a positive correlation in week 16 on *P. annua*, but it was absent on *T. repens*.

Generally, in all treatments, increased rates of aphid development were found in both generations. However the same level of development was not achieved, where the numbers of nymphs in the second generation were higher than the first generation. The explanation for this may be due to differences in nitrogen concentrations over the period of plant growth, since the earthworm activities increased with time via increasing their sizes.

In agreement with this study, Wurst & Forstreuter (2010) found that the number of aphids changed with time (increased gradually from week 9 to week 11).

Finally, this experiment indicated that earthworms individually or in combination positively influenced above-ground aphid development, and these influences increased with increasing earthworm densities, as the results from chapter five showed that the effect of earthworms on aphid depend on their species and functional group and the interaction between them.

CHAPTER 5
THE INTERACTIONS BETWEEN DIFFERENT EARTHWORM
SPECIES AND FUNCTIONAL GROUPS AND THEIR
INFLUENCES ON APHID DEVELOPMENT

5.1 Abstract

The interaction between different earthworms; *Aporrectodea rosea*, *Allolobophora chlorotica*, *A. rosea* & *A. chlorotica*, *Aporrectodea caliginosa* & *Satchellius mammalis* and *A. chlorotica* & *S. mammalis* from two different functional groups and their influence on aphid growth (nymphs day⁻¹, fecundity and number of adults) showed that the combined effect of two different functional groups (epigeic & endogeic) of earthworms was more effective in increasing aphids numbers than individual groups.

1-The interaction between individual earthworms and their influences on daily nymph production, fecundity and numbers of adults:

The difference in the effect of eight worms between all treatment groups showed that the daily nymph production in both generations was significantly higher in the presence of the endogeic and epigeic *A. caliginosa* & *S. mammalis* earthworms compared to other earthworm treatments. In the four worms' density, in the first generation the daily nymph production was significantly higher in the presence of *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis*, while in the second generation it was higher in the presence of *A. chlorotica* with *S. mammalis*. In the two worm density in both generations the daily nymph production was significantly higher in the presence of *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis*. While in the control treatment there were no significant differences between all groups in both generations.

In the effect of eight worms in both generations, the fecundity was significantly higher in the presence of *A. caliginosa* with *S. mammalis*. Similar results were shown in the effect of four worms on the fecundity in both generations. In the effect of two worms, the fecundity was significantly higher in the presence of *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* in both generations. In the controls, there were no significant differences between all groups in both generations.

The differences in the influence of eight worms on the numbers of adults in both generations showed that the numbers of adults were significantly higher in the presence of *A. caliginosa* with *S. mammalis*. A similar result was shown in the presence of four worms in both generations. Also, in the two worm density, the numbers of adults in both generations were significantly higher in the presence of *A. caliginosa* with *S. mammalis* earthworms. In the control treatment there were no significant differences between all groups.

2-The interaction between two aphid generations (nymphs/day, fecundity and numbers of adults) in response to five different groups of earthworms: The total daily nymph production, fecundity and numbers of adults were significantly higher in the presence of *A. caliginosa* with *S. mammalis* group compared to other groups in both generations.

3-The interaction between five treatment groups of earthworms and their influences on (nymphs/day, fecundity and numbers of adults):

The combined influences of endogeic *A. rosea* with *A. chlorotica*, endogeic *A. caliginosa* with epigeic *S. mammalis* and endogeic *A.*

chlorotica with epigeic *S. mammalis* earthworms on daily nymph production and fecundity showed no significant differences between the first and second generations, while they were significantly higher in the second generation when *A. rosea* and *A. chlorotica* were individually present. In contrast, the numbers of adults were significantly higher in the first generation in the *A. rosea* with *A. chlorotica*, *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* earthworm treatments, while they were higher in the second generation in the presence of *A. chlorotica* alone, and there were no significant differences between both generations in the *A. rosea* treatment.

5.2 Introduction

Earthworms are well-known to be associated with many chemical and physical activities in the soil (Brown, 1995; Edwards & Bohlen, 1996), and thus, promote plant growth (Lee, 1985). The role of earthworms in the decomposition of organic matter depends on their species and functional group, and the interactions between species within the same group or different groups are important to understand their functions in the soil (Uvarov, 2009).

Earthworms have been divided into three functional groups including; endogeic, epigeic and anecic groups, and each group has a different life style (Bouché, 1977). The endogeic species are most commonly found (Werner, 1990), and they are more important for soil function than other groups especially in intensified agroecosystems (Fragoso *et al.*, 1997). However, all groups are involved in decomposition in the soil (Werner, 1990).

Many previous studies have focused on the effect of individual earthworms on above-ground insects (Eisenhauer & Scheu, 2008; Haimi, Huhta & Boucelham, 1992; Ke & Scheu, 2008; Poveda *et al.*, 2005; Wurst & Jones, 2003), and others have studied the interaction between earthworms and other soil organisms, e.g. earthworms and protozoa (Bonkowski *et al.*, 2001; Tao *et al.*, 2009), earthworms and Collembola (Salmon, Geoffroy & Ponge, 2005; Scheu, Theenhaus & Jones, 1999; Wickenbrock & Heisler, 1997), earthworms and nematodes (Senapati, 1992; Tao *et al.*, 2009), and earthworms with microorganisms (Doube *et*

al., 1994; Lachnicht & Hendrix, 2001; Sheehan *et al.*, 2008; Wurst *et al.*, 2008; Wurst *et al.*, 2004). A few studies have investigated the interaction between different earthworm species (Doube, Williams & Willmott, 1997). Sheehan *et al.* (2006), examined the interaction between individuals from functional groups and their influences on soil nitrogen, also, Capowiez (2000), studied the spatial interaction between the endogeic *A. chlorotica* and the anecic *A. nocturna* earthworms, however no research has yet examined the individual and combined influences of earthworms within the same functional group or different groups on above-ground aphid performance and the interactions between all of them.

The study of such interactions is important in terms of understanding whether or not there is a relationship between root growth and earthworms biodiversity.

5.3 Aims

This study aimed to investigate the interactions between different species and densities from two different functional groups of earthworms and their influence on aphid growth.

5.4 Materials & Methods

5.4.1 Experimental treatments

In this experiment individuals and combined earthworm treatments from endogeic and epigeic functional groups were used to investigate the interaction between them and their influences on aphid's growth.

The experimental design consisted of the following treatments (groups):

- 1- Group (A): the individual influence of *A. rosea* on aphid growth
- 2- Group (B): the individual influence of *A. chlorotica* on aphid growth
- 3- Group (C): the combined influence of *A. rosea* and *A. chlorotica* on aphid growth.
- 4- Group (D): the combined influence of *A. caliginosa* and *S. mammalis* on aphid growth.
- 5- Group (E): the combined influence of *A. chlorotica* and *S. mammalis* on aphid growth.

In all groups treatments consisted of two, four and eight individuals of earthworm added to a pot plus a control with no earthworms. For combination groups, an equal number of each species was used. There were five replicates per treatment.

For details regarding earthworm collection and aphid culture, see Chapter 2 (sections 2.2 and 2.3).

5.4.2 Statistical analysis

The data from five different experiments were pooled. Generalized linear models (GLM) from SPSS statistical software version 21 were used to

analyse the data and test the differences between individuals and groups of earthworms. For count data which is the response variable (aphids), a Poisson distribution of errors and log link function model were assumed. The full model was assumed with two main effects; continuous variable coefficients (density of earthworms) and the earthworm groups as a factor. To test the normality distribution of residuals, a linear regression was assumed based on the Studentized and Standardized residuals applying Kolmogorov-Smirnov and Shapiro-Wilk tests. This showed that the residuals were normally ($P > 0.05$) distributed (Appendix 2-A).

For the non-integer variable data (nymph day^{-1}), the results are expressed as mean values \pm SE. Data were analysed using GLM to compare differences between treatments. Probability values ($P < 0.05$) were considered statistically significant.

To examine the differences between two generations of aphid, data were analysed using Minitab version 16 (Minitab Ltd, Coventry, UK). A GLM was used to compare different treatment groups followed by an appropriate multiple test (Tukey). Factors were Individuals of earthworms and aphid generations were the response variables ($y = a + bx + \epsilon$). Data are shown as mean \pm SE and $P < 0.05$ is considered significant.

5.5 Results

5.5.1 The interaction between individual earthworms from five groups

5.5.1.1 The interactions between different numbers and species of earthworms and their influences on daily nymph production

The results of daily nymph production in the first generation (Figure 5.1) revealed that in the control treatments there was no significant ($P>0.05$) difference between controls among all groups. The overall mean value for all the controls was (0.32 ± 0.006). In the comparison between eight individual worms, daily nymph production was significantly ($P<0.05$) higher by 128, 286, 43, 8 and 334% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica*, *A. chlorotica* with *S. mammalis* and the control respectively. In the presence of four individual worms, the daily nymph production was significantly ($P<0.05$) higher by 138, 276, 63 and 288% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica* and the combination of *A. rosea* with *A. chlorotica* and control respectively with no significant differences ($P>0.05$) between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* treatments. Similarly, in the presence of two individual worms, daily nymph production was significantly ($P<0.05$) higher by 121, 242, 31 and 239% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica* and the combination of *A. rosea* with *A. chlorotica* and the control respectively with no significant ($P>0.05$) differences between *A.*

caliginosa with *S. mammalis* and *A. chlorotica* with *S. mammalis* treatments.

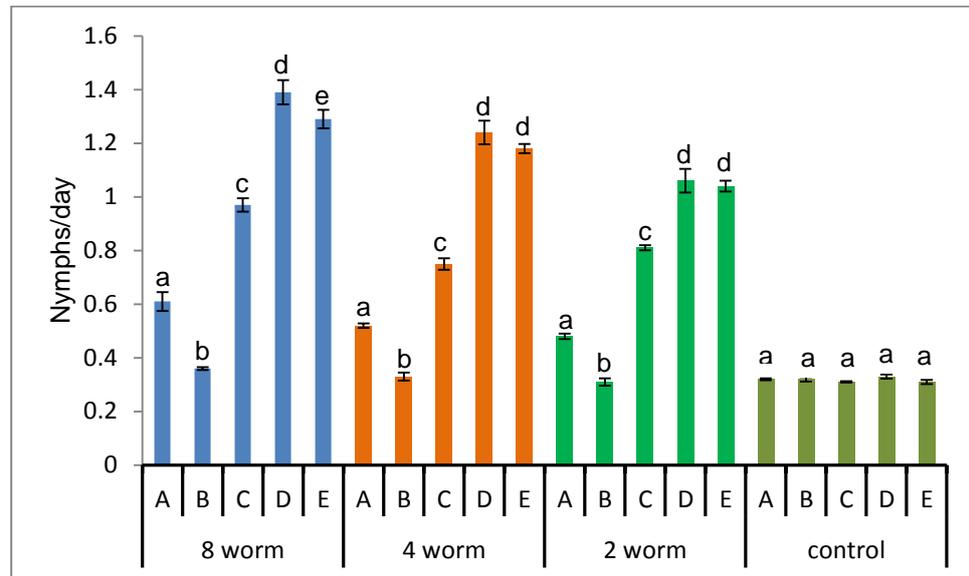


Figure 5.1 The interactions between individual worms regarding their influence on the daily nymph reproduction under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each earthworm density.

As with the first generation, there was no significant ($P > 0.05$) differences between controls among all groups in the second generation. The overall mean value for all the controls was (0.33 ± 0.006). In the presence of eight individual worms, daily nymph production in the second generation was also significantly ($P < 0.05$) higher by 69, 97, 40 and 306% in the *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively

with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* treatments. In the presence of four individual worms, the daily nymph production was significantly ($P<0.05$) higher by 59, 120, 34, 9 and 268% in the *A. chlorotica* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica*, *A. caliginosa* with *S. mammalis* and the control respectively. In the presence of two worms the daily nymph production was significantly ($P<0.05$) higher by 86, 112, 20.5 and 221% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* treatments (Figure 5.2).

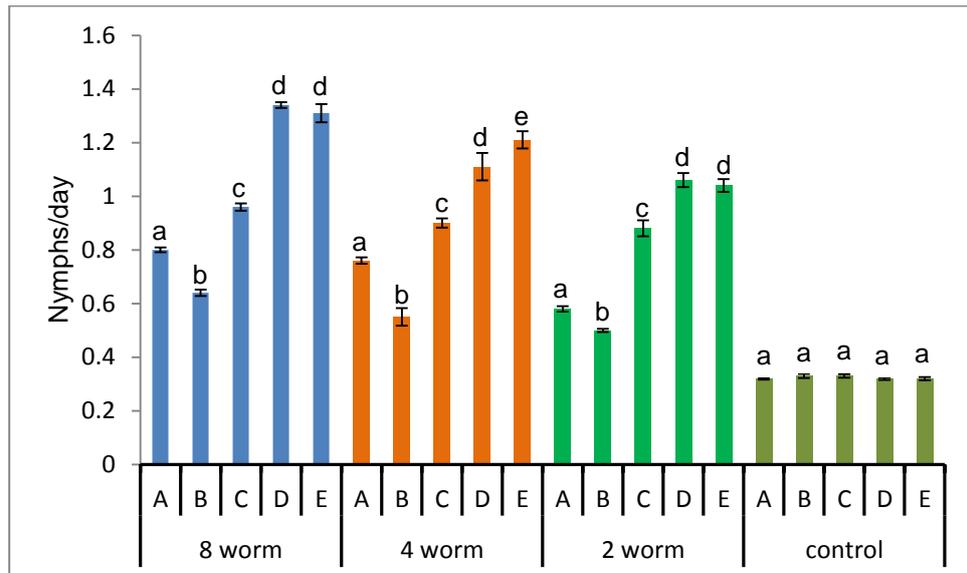


Figure 5.2 The interactions between individual worms regarding their influence on the daily nymph reproduction under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each earthworm density.

5.5.1.2 The interactions between different individuals and species of earthworms and their influences on aphid fecundity.

The interaction between different individual earthworms and their influence on the fecundity in the first generation is shown in Figure 5.3. The results revealed that in the control treatment, there were no significant differences ($P > 0.05$) between all groups. The overall mean value for all the controls was (5.19 ± 0.31) . In the presence of eight individual worms, fecundity was significantly ($P < 0.05$) higher by 107, 210, 29, 13 and 258% in the *A. caliginosa* with *S. mammalis* treatment compared to *A. rosea*, *A. chlorotica*, combined *A. rosea* / *A. chlorotica*, *A.*

chlorotica / *S. mammalis* and the control respectively. In the presence of four individual worms, fecundity was significantly ($P < 0.05$) higher by 123, 256, 56, 17 and 243% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combinations of *A. rosea* with *A. chlorotica*, *A. chlorotica* with *S. mammalis* and the control respectively. In the two worm density, fecundity was significantly ($P < 0.05$) higher by 14, 20, 8 and 177% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica* the combined *A. rosea* / *A. chlorotica* and the control respectively (Figure 5.3).

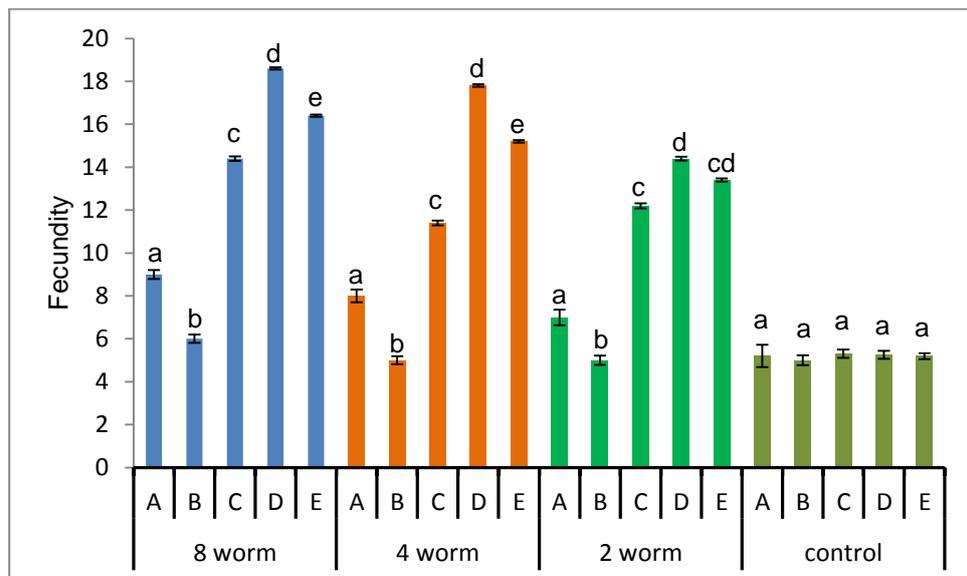


Figure 5.3 The interactions between individual worms regarding their influence on the aphid fecundity under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each earthworm density.

In the second generation, the results revealed that in the control treatment, there was no significant ($P>0.05$) differences between all control treatments. The overall mean value for all the controls was (4.96 ± 0.09). In the presence of eight worms the fecundity was significantly ($P<0.05$) higher by 52, 82, 40 and 267% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control treatments respectively with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. Similarly, in the presence of four individual worms, the fecundity was significantly ($P<0.05$) higher by 51, 84, 26 and 235% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* & *A. chlorotica* and the control respectively with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. In the two individual worms, the fecundity was significantly ($P<0.05$) higher by 58, 78, 15 and 186% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively with no significant ($P>0.05$) differences between the combined *A. caliginosa* / *S. mammalis*, *A. rosea* / *A. chlorotica* and *A. chlorotica* / *S. mammalis* worm treatments (Figure 5.4).

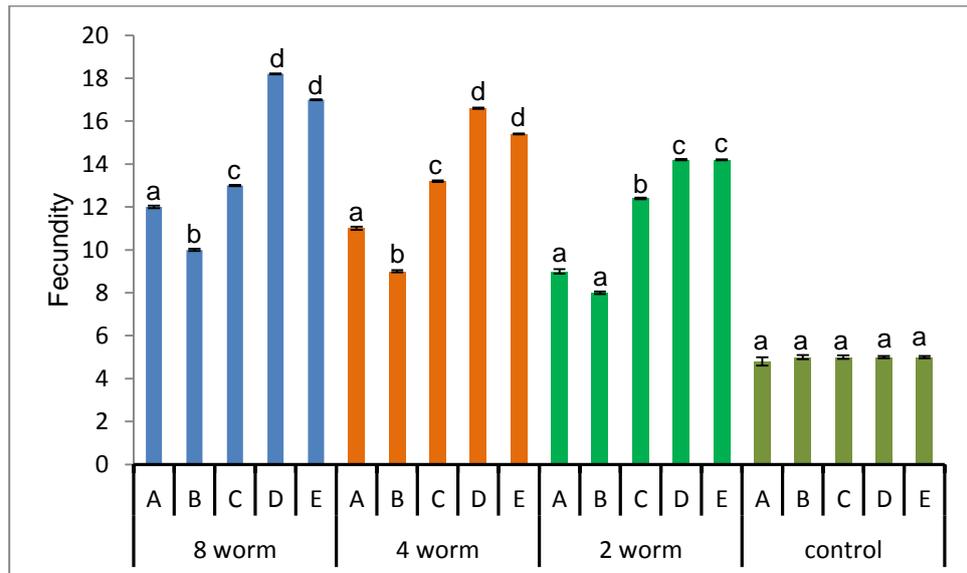


Figure 5.4 The interactions between individual worms regarding their influence on the aphid fecundity under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each earthworm density.

5.5.1.3 The interactions between different individuals and species of earthworms and their influences on adult numbers.

The interaction between different individual worms from five groups and their influence on the numbers of adults in the first generation is shown in (Figure 5.5). The results revealed that in the control treatment, there were no significant differences ($P > 0.05$) between all groups. The overall mean value for all the controls was (2.00 ± 0.31). While in the presence of eight worms, the numbers of adults developed from nymphs were significantly ($P < 0.05$) higher by 313, 451, 23 and 520% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combinations of *A. rosea* with *A. chlorotica* and the control respectively,

with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. There were also no significant ($P>0.05$) differences between *A. rosea* and *A. chlorotica*. Similarly, in the presence of four worms, the numbers of aphids developed from nymphs were also substantially ($P<0.05$) higher by 240, 373, 189 and 240% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and control treatments respectively, with no significant differences ($P>0.05$) between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. Again there was no significant ($P>0.05$) difference between the *A. rosea* and *A. chlorotica* treatments. Additionally, in the presence of two worms the numbers of adults were significantly ($P<0.05$) higher by 170, 170 and 50, 170% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combined *A. rosea* with *A. chlorotica* and the control treatments, with no significant ($P>0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. There was no significant ($P>0.05$) differences between the *A. rosea* and *A. chlorotica* treatments (Figure 5.5).

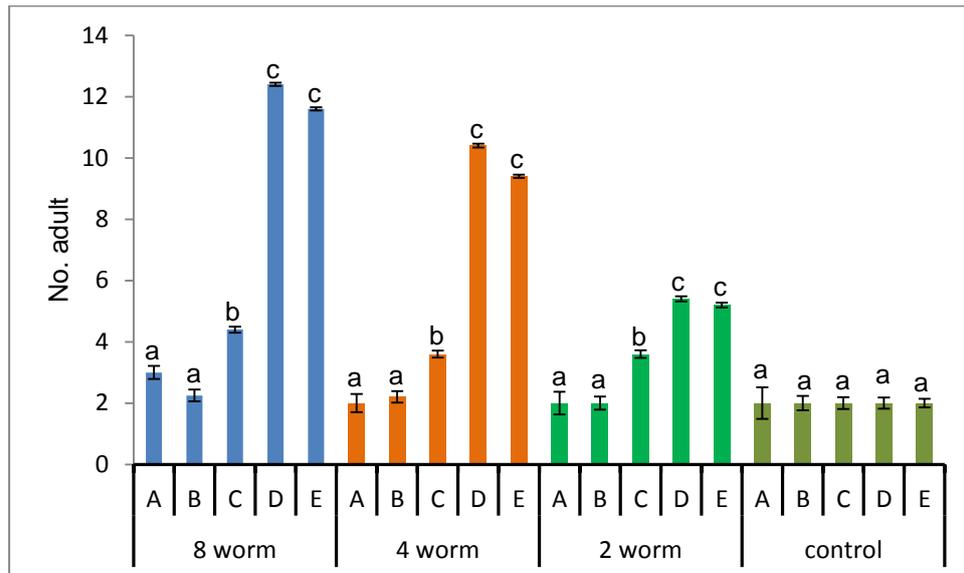


Figure 5.5 The interactions between individual worms regarding their influence on the numbers of adults under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (1st generation). Columns annotated with the same letter are not significantly different within each earthworm density.

In addition to the first generation, the results revealed that in the control treatment in the second generation there were no significant ($P > 0.05$) differences between all groups. The overall mean value for all the controls was (1.9 ± 0.22) . In the presence of eight individual worms the numbers of adults were also significantly ($P < 0.05$) higher by 93, 81, 93 and 205% in the *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively, with no significant differences ($P > 0.05$) between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments. There was no significant ($P > 0.05$) difference between *A. rosea*, *A. chlorotica* and the combined *A. rosea* / *A. chlorotica*. Similarly, the numbers of adults in

the four worm treatments were also significantly ($P < 0.05$) higher by 80, 80, 93 and 184% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively with no significant ($P > 0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments, and between *A. rosea*, *A. chlorotica* and the combined *A. rosea* / *A. chlorotica* treatments. In the presence of two worm densities the numbers of adults were significantly ($P < 0.05$) higher by 130, 130, 42 and 142% in the presence of *A. chlorotica* with *S. mammalis* worms compared to *A. rosea*, *A. chlorotica*, the combination of *A. rosea* with *A. chlorotica* and the control respectively with no significant differences ($P > 0.05$) between *A. rosea*, *A. chlorotica* and combined *A. rosea* / *A. chlorotica* treatments. There were also no significant ($P > 0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* worm treatments (Figure 5.6).

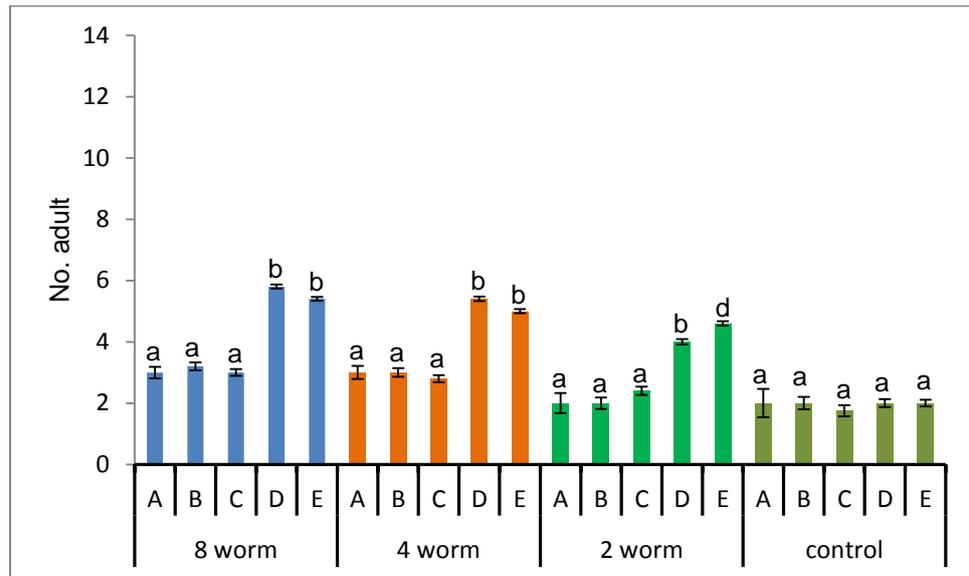


Figure 5.6 The interactions between individual worms regarding their influence on the numbers of adults under five different groups of (A): *A. rosea*, (B): *A. chlorotica*, and the combinations of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (2nd generation). Columns annotated with the same letter are not significantly different within each earthworm density.

5.5.2 The interactions between five epigeic and endogeic groups of earthworms and their influences on aphid development across two generations.

The total daily nymph production and the interaction between two generations in five groups of earthworms are shown in Figure 5.7. The results revealed that the number of nymphs produced per day in the first generation was significantly ($P < 0.05$) higher by 110, 205 and 41.9% in the *A. caliginosa* with *S. mammalis* group compared to the *A. rosea*, *A. chlorotica* and combined *A. rosea* with *A. chlorotica* groups respectively with no significant ($P > 0.05$) differences between *A. caliginosa* with *S.*

mammalis and *A. chlorotica* with *S. mammalis* groups, and between the two endogeic *A. rosea* and *A. chlorotica* groups (Figure 5.7.a). In the second generation the total daily nymph production was significantly ($P < 0.05$) higher by 58, 87.7 and 26.6% in the *A. chlorotica* with *S. mammalis* group compared to the *A. rosea*, *A. chlorotica* and combined *A. rosea* with *A. chlorotica* groups respectively, with no significant ($P > 0.05$) differences between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* groups, while there were significant ($P < 0.05$) differences between *A. chlorotica* and the combination of *A. rosea* with *A. chlorotica* groups (Figure 5.7.b).

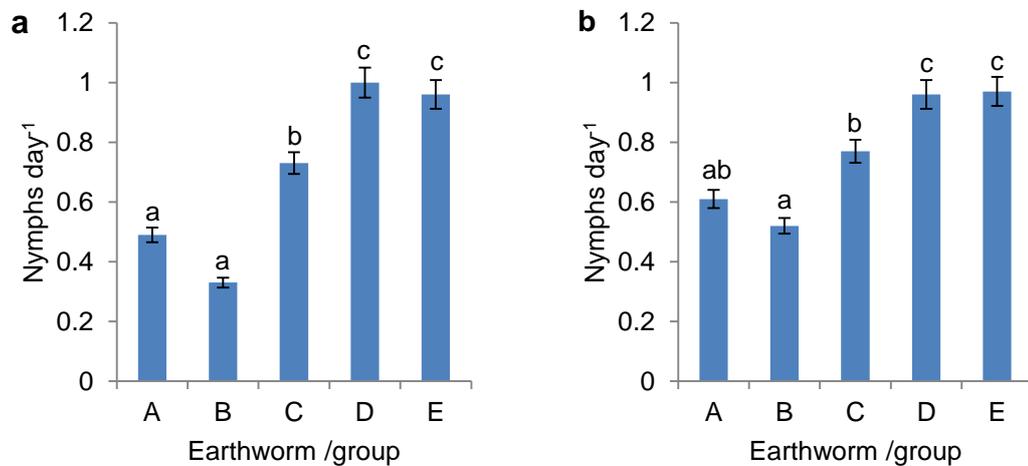


Figure 5.7 Mean (\pm SE) values of daily nymph production, the interaction between five groups of (A): *A. rosea*, (B): *A. chlorotica* and the combined effects of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (a) 1st generation and (b) 2nd generation. Columns annotated with the same letter are not significantly different within each generation.

The results of total fecundity (Figure 5.8) revealed that the fecundity in the first generation was substantially ($P < 0.05$) higher by 103, 179 and 26% in the *A. caliginosa* with *S. mammalis* group compared to *A. rosea*, *A. chlorotica* and the combined *A. rosea* / *A. chlorotica* groups respectively with no significant ($P > 0.05$) differences between the combination of *A. rosea* with *A. chlorotica*, and *A. chlorotica* with *S. mammalis*, and between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* (Figure 5.8 a). In the second generation the total fecundity was significantly ($P < 0.05$) higher by 40, 65 and 18% in the *A. caliginosa* with *S. mammalis* group compared to the *A. rosea*, *A. chlorotica* and combined *A. rosea* / *A. chlorotica* groups, with no significant differences ($P > 0.05$) between *A. chlorotica* and the *A. rosea* / *A. chlorotica* groups, and between the combination of *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* (Figure 5.8 b).

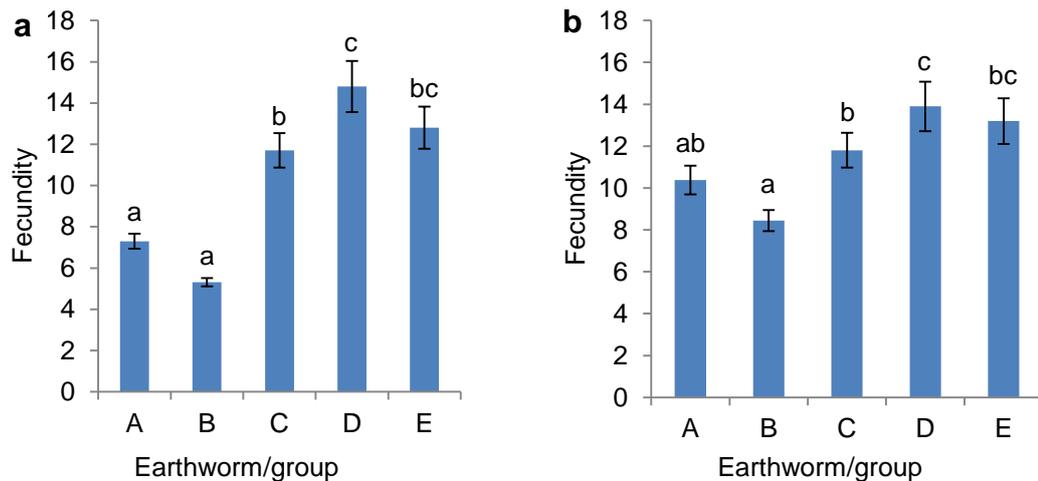


Figure 5.8 Mean (\pm SE) values of nymph fecundity, the interaction between five groups of (A): *A. rosea*, (B): *A. chlorotica* and the combined effects of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (a) 1st generation and (b) 2nd generation. Columns annotated with the same letter are not significantly different within each generation.

The total numbers of adults and the interaction between five groups of earthworms is shown in (Figure 5.9). In the first generation the numbers of adults that developed from nymphs were significantly ($P < 0.05$) higher by 228, 260 and 122% in the *A. caliginosa* with *S. mammalis* group compared to the *A. rosea*, *A. chlorotica* and combined *A. rosea* with *A. chlorotica* groups respectively, with no significant differences ($P > 0.05$) between *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis*, and between the *A. rosea* and *A. chlorotica* groups (Figure 5.9 a). In the second generation the numbers of adults were substantially ($P < 0.05$) higher by 72, 69 and 72% in the presence of *A. caliginosa* with *S. mammalis* compared to *A. rosea*, *A. chlorotica* and the combined *A. rosea* / *A. chlorotica* groups respectively, with no significant ($P > 0.05$)

differences between *A. rosea*, *A. chlorotica* and the combined *A. rosea* with *A. chlorotica* groups, nor between *A. chlorotica* with *S. mammalis* and *A. caliginosa* with *S. mammalis* (Figure 5.9 b).

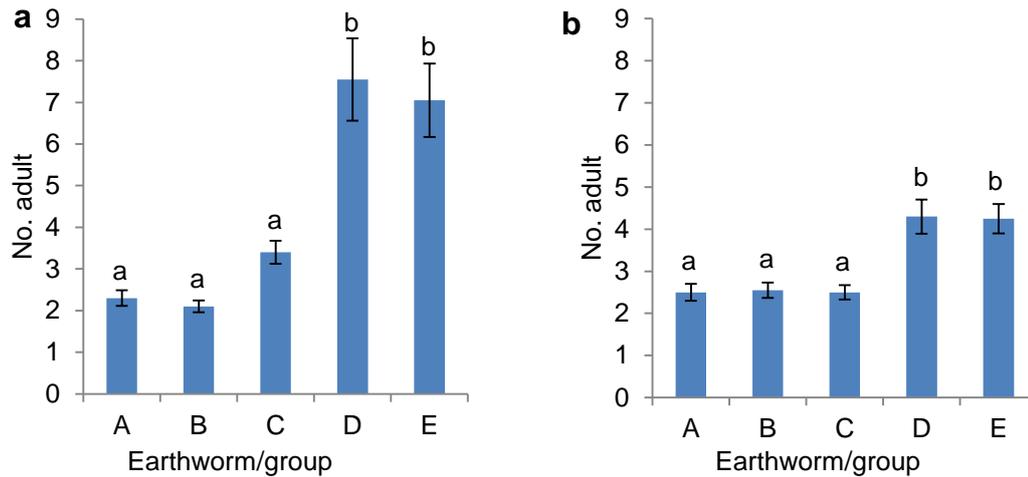


Figure 5.9 Mean (\pm SE) values of adults, the interaction between five groups of (A): *A. rosea*, (B): *A. chlorotica* and the combined effects of (C): *A. rosea* & *A. chlorotica*, (D): *A. caliginosa* & *S. mammalis* and (E): *A. chlorotica* & *S. mammalis* earthworm densities (a) 1st generation and (b) 2nd generation. Columns annotated with the same letter are not significantly different within each generation.

5.5.3 The interaction between two aphid generations (nymphs day⁻¹, fecundity and numbers of adults) in response to five different groups of earthworms.

The daily nymph production and the interaction between the two generations under the effect of different earthworm treatments showed that increasing daily nymph production was significantly correlated with increasing earthworm densities in all groups (Figure 5.10). The results revealed that daily nymph production in the presence of *A. rosea* (Figure 5.10 a) and *A. chlorotica* (Figure 5.10 b) were significantly higher ($P < 0.05$) in the second generation compared to the first generation, while, there were no significant differences ($P > 0.05$) between the two generations in the presence of *A. rosea* with *A. chlorotica*, *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* (Figure 5.10 c, d and e).

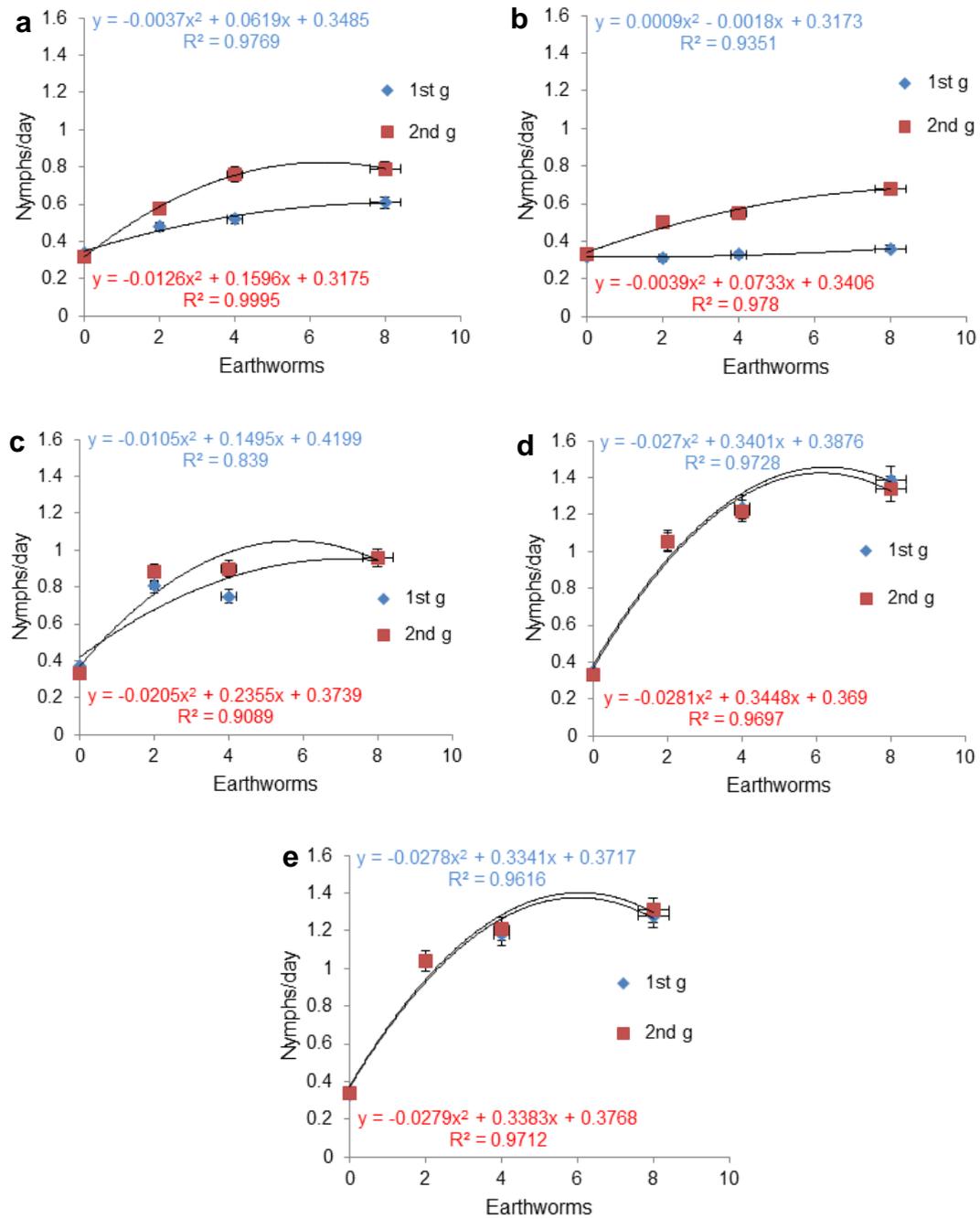


Figure 5.10 Mean (\pm SE) values of daily nymph production, the interaction between two aphid generations under the influences of different (a): *A. rosea*, (b): *A. chlorotica*, and the combined effects of (c): *A. rosea* & *A. chlorotica*, (d): *A. caliginosa* & *S. mammalis* and (e): *A. chlorotica* & *S. mammalis* earthworms.

In addition to the daily nymph production, increasing fecundity was also significantly correlated with increasing earthworm densities in all groups (Figure 5.11). The results from this experiment revealed that fecundity in the presence of *A. rosea* (Figure 5.11 a) and *A. chlorotica* (Figure 5.11 b) was significantly higher ($P < 0.05$) in the second generation compared to the first generation, while there were no significant differences ($P > 0.05$) between the two generations in the presence of *A. rosea* with *A. chlorotica*, *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* (Figure 5.11 c, d and e).

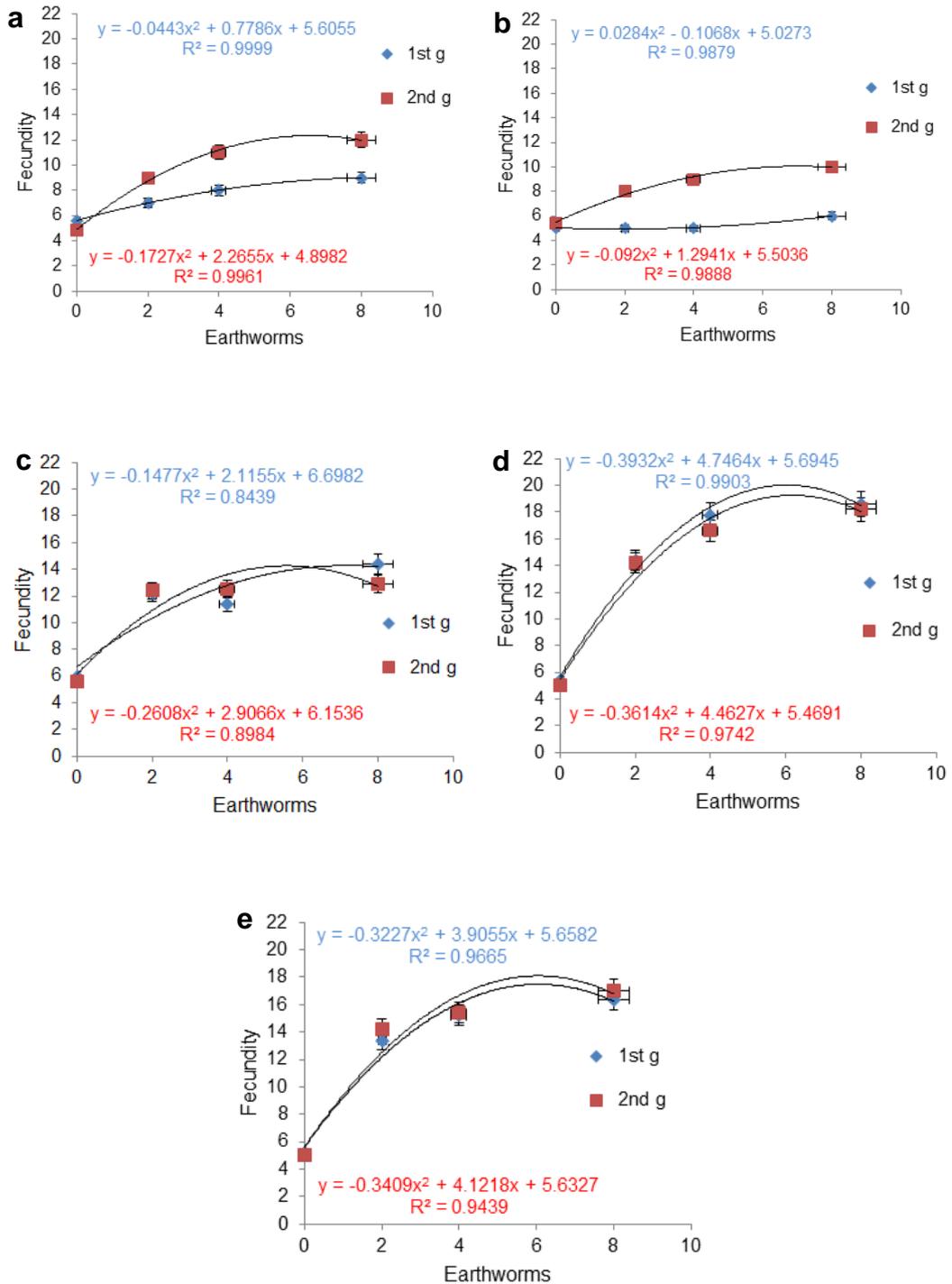


Figure 5.11 Mean (\pm SE) values of fecundity, the interaction between two aphid generations under the influence of different (a): *A. rosea*, (b): *A. chlorotica* and the combined effects of (c): *A. rosea* & *A. chlorotica*, (d): *A. caliginosa* & *S. mammalis* and (e): *A. chlorotica* & *S. mammalis* earthworms.

In contrast to the daily nymph production and nymph fecundity, the numbers of adults were significantly higher ($P < 0.05$) in the first generation in the presence of *A. rosea* with *A. chlorotica*, *A. caliginosa* with *S. mammalis* and *A. chlorotica* with *S. mammalis* compared to the second generation (Figure 5.12 c, d and e), while in the presence of *A. chlorotica* and *A. rosea* there were no significant differences ($P > 0.05$) between the two generations (Figure 5.12 a).

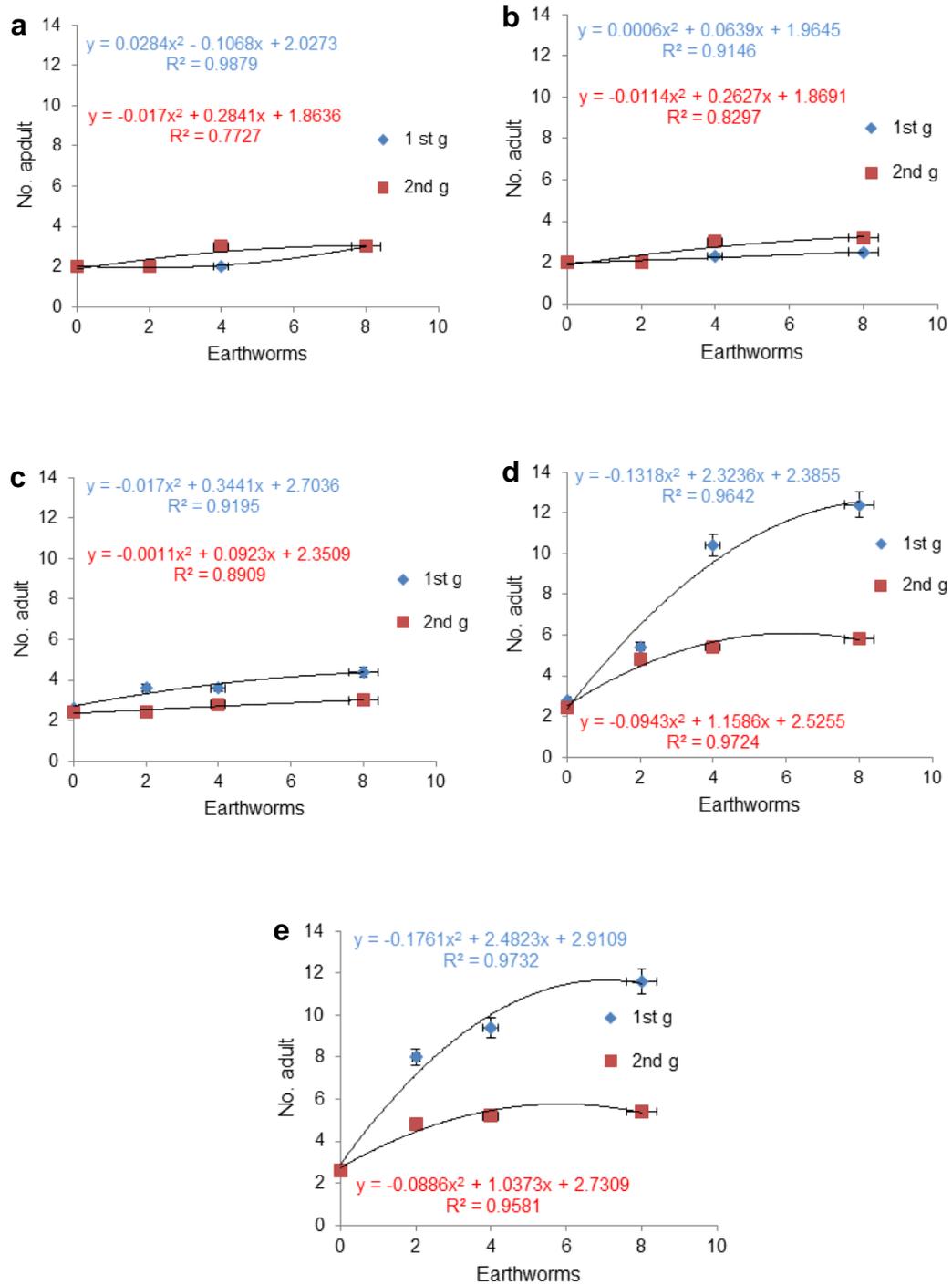


Figure 5.12 Mean (\pm SE) adult production arising from two aphid generations under the influence of different (a): *A. rosea*, (b): *A. chlorotica*, (c): the combined effect of *A. rosea* & *A. chlorotica*, (d): *A. caliginosa* & *S. mammalis* and (e): *A. chlorotica* & *S. mammalis* earthworms.

5.6 Discussion

This study investigated the individual and combined effects of earthworms from five groups on aphid development namely nymphs day⁻¹, fecundity and numbers of adults in two generations and the interaction between them.

The results here suggested differential effects of earthworms on aphid growth depending on their species and the functional group to which they belong. It seems that the lifestyle of earthworms in the soil have a critical role on aphid development, through their activities around plant roots, they could affect the nutrients uptake by plants. Uvarov (2009) found that earthworm species differentially affect organic matter.

Similar results have been reported for the effect of different earthworm species on the population growth of *M. persicae*, it being enhanced by the presence of *A. caliginosa*, while *O. tyrtaeum* had no such effect (Scheu, Theenhaus & Jones, 1999).

In contrast, Wurst *et al.*, (2003), found that the number of *M. persicae* reduced in the presence of *A. caliginosa* and *O. tyrtaeum* earthworms. Also, *Aporrectodea spp* negatively affected *M. persicae* development (Wurst & Forstreuter, 2010).

This experiment also showed that daily nymph production and fecundity increased with time, while the number of adults in the second generation was lower than in the first generation (Figure 5.12), possibly indicating higher fecundity but slowed development of aphids in the presence of

earthworms. This could be due to decreasing the nutrients available in the soil for plant growth and aphid development.

The results from this chapter suggested that there is relationship between increasing the number of offspring and the time under different earthworm density, as the results showed the differences in the slopes of increasing aphid offspring between all treatments. This regular increasing in aphid offspring could be related to increasing the activity of earthworms with the time, thus increasing the nutrients available for plants and aphid growth. Also the results obtained in this investigation demonstrated that the combined effect of two species had greater impact on aphid development than the individual worm species alone. Hale *et al.*, (2005), in their investigation on the effect of earthworm species on plants, reported that earthworms have either negative or positive effects on nitrogen availability in plants depending on species which they attributed to diversity in burrowing habits, or to differences in earthworm biomass.

In addition, the results in the present study showed that the combined influence of two species from different functional groups had greater impacts on aphid growth than two species from the same functional group.

Finally, in the present study, there was a significant interaction effect of the epigeic and endogeic groups on aphid performances. The current results show that adding earthworms from different functional groups seems to have a greater effect on the above ground community.

In conclusion, earthworms either individually or combined have indirect effect on aphid development through their activities in the soil they act to change the plant physiology, thus aphid development.

CHAPTER 6
FOLIAR PROTEIN ANALYSIS

6.1 Abstract

Identification of proteins and determination of their functions in plant tissues is an essential step to elucidate aphid-Chinese cabbage interactions. In this study, proteomic techniques (2D-GE patterns) were used to compare protein patterns in the different (*B. rapa*) plants treated with different *Aporrectodea caliginosa* and *Satchellius mammalis*, *Allolobophora chlorotica* and *S. mammalis*, *Aporrectodea rosea*, *A caliginosa*, *S. mammalis* and *Lumbricus rubellus* earthworm densities and a control (no worms added). Among all the detected spots, 40 spots were selected for protein identification. 22 proteins were identified, 11 proteins were differentially expressed between the plant treated with 12 worms and a control. The probable fructose-Bisphosphate aldolase 2, ATP synthase subunit beta, oxygen-evolving enhancer protein 1-1, albumin, ribulose bisphosphate carboxylase large chain, ribulose bisphosphate carboxylase large chain OS, ATP synthase subunit alpha, ATP synthase subunit beta, ribulose bisphosphate carboxylase large chain, uncharacterized protein and annexin proteins were found in the plants treated with different earthworm densities. Albumin was found only in the plant treated with twelve *A. rosea*, *A. caliginosa*, *S. mammalis* and *L. rubellus* earthworms. The remaining eleven proteins were found in all treatments including the control.

6.2 Introduction

The genus *Brassica* is an economically important crop, used as a food source for animals and humans (Bennett & Wallsgrove, 1994; Mun *et al.*, 2010), because their leaves are rich in proteins (Font *et al.*, 2005; Pirie, 1986). Different kinds of protein have been discovered in the plants depending on the species. Protein composition differs according to the kind, number and sequence of amino acids that make up the polypeptide backbone (Fujihara, Kasuga & Aoyagi, 2001; Kehr, 2006; Yeoh & Wee, 1994).

The role of proteins in growth and driving the interaction between plants and insects has been reviewed by (Kehr, 2006), although, the mechanism(s) of their effect on insects has not yet been investigated. The composition, but not the amount, of amino acid has an effect on aphid performance (Karley, Douglas & Parker, 2002). Previous studies (Chiozza, O'Neal & MacIntosh, 2010; Karley, Douglas & Parker, 2002; Kehr, 2006) in this area focused on the role of proteins in the interaction between plants and insects. However the role of earthworms in manipulating the proteins in plants and their interaction with above-ground herbivores need more study, since earthworms exert a tremendous impact on plant physiology (Haimi & Einbork, 1992; Scheu & Parkinson, 1994; Wurst *et al.*, 2003).

The use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been the main method used in determination of proteins in biological tissues. The first use of this technique was by O'Farrell (1975). Recently,

separating proteins by the 2-DE method has increased for plant tissues (Carpentier *et al.*, 2005; Wang *et al.*, 2003), and also in insects (Bezdi *et al.*, 2012; Harmel *et al.*, 2008). In this technique proteins are separated in two ways, firstly, according to their isoelectric points (IEF), and then according to the molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The use of the Kjeldahl method for nitrogen analysis can give an estimate of the amount of proteins depending on nitrogen-to-protein conversion factors (Yeoh & Wee, 1994), but 2-DE remains the best way to identify and characterise proteins.

Plant phloem sap is the main source of proteins for piercing and sucking insects (Kehr, 2006). Since the plants vary in the quality and quantity of accumulated proteins (Giavalisco *et al.*, 2006), the composition of proteins is one of the main factors affecting the preference of insect feeding on the plant, and so it can be concluded that proteins are driving the interaction between plant and insect (Karley, Douglas & Parker, 2002; Kehr, 2006).

In the present study the initial hypothesis was that earthworms cause a significant change in nitrogen concentration in the plant. What is not known is whether earthworms have an effect on plant proteins, and if there is any relationship between protein changes and aphid development.

6.3 Aims

This study aimed to investigate the chemical changes in the Chinese cabbage (*B. rapa*) via protein identification. In order to understand the role of earthworm in protein changes in the plant 2D-GE technique was applied to determine the differences in plant proteins.

6.4 Materials and Methods

6.4.1 Experimental treatments

The details of earthworm collection and aphid culture are fully described elsewhere; see Chapter 2 (section 2.2 and 2.3). In summary, in this experiment foliar tissues were taken from Chinese cabbage under the following treatments (Table 6.1):

Table 6.1 The experimental design, earthworm species from different functional groups and densities.

	Earthworm species	Treatments
A	<i>A. caliginosa</i> (end.) + <i>S. mammalis</i> (epigeic)	0, 2, 4 and 8 worms
B	<i>A. rosea</i> (endo.) + <i>A. caliginosa</i> (endo.) + <i>L. rubellus</i> (epi.) + <i>S. mammalis</i> (epi.)	0, 4, 8, 12 (multiples of 4)
C	<i>A. chlorotica</i> (endo.) + <i>S. mammalis</i> (epi.)	0, 2, 4 and 8 worms

6.4.2 Plant sample preparation and protein extraction

Fresh harvested leaves were collected from plants. The leaves were ground with a pestle and mortar (pre-cooled to -20°) under liquid nitrogen. The TCA precipitation method was used to extract the proteins according to Damerval *et al.*, (1986) with some modification given by Carpentier *et al.*, (2005).

0.15 g of homogenate plant sample was precipitated with 20% TCA/0.2% DTT in pre-chilled acetone (-20°) overnight (at -20°), followed by centrifugation at $16000 \times g$ for 30 minutes at 4°C . After removing the supernatant the pellet was washed twice in ice-cold acetone/0.2% DTT. Between the two washing steps, the sample was incubated for one hour at -20° . After air-drying, the pellet was re-suspended in 100 μL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% IPG-buffer, 1% DTT), and vortexed for one hour at room temperature.

6.4.3 Protein clean-up

A 2-D clean up kit was used to prepare plant samples with pure proteins, otherwise poor results would be produced because plant tissues contain high levels of interfering compounds. This procedure was:

50 μL of protein sample transferred into 1.5 ml micro-centrifuge tube, 300 μL of precipitant (this solution renders proteins insoluble) added to the protein samples and mixed well by vortex and incubated on ice ($4-5^{\circ}\text{C}$) for 15 minutes. Then 300 μL of co-precipitant (this solution contains reagents that co-precipitate with proteins and enhances their removal

from solution) added to the mixture of protein and precipitant and mixed briefly by vortex. After centrifugation at 12000g for 5 min, the supernatant was removed as soon as the centrifugation was completed. Without disturbing the pellet, 40 μ L of co-precipitant was added to the tube which was then placed on ice for 5 min. The tube was then centrifuged as before for 5 min and the supernatant removed. 25 μ L of de-ionized water was added with 5-10 s vortex till the small pellet dispersed. After that, 1 ml of wash buffer (pre-chilled for at least 1 h at -20 °C. This is used to remove non-protein contaminants from the protein precipitate) and 5 μ L of wash additive (this solution contains a reagent that promotes rapid and complete resuspension of the sample proteins) were added and vortex used to make sure that the pellet was fully dispersed but not dissolved. The tube was incubated at -20 °C for 30 min and vortexed for 30 s once every 10 min, then located in the micro centrifuge at 12000g for 5 min. The supernatant was then discarded and the pellet was allowed to air dry for 5 min.

Rehydration solution [200 μ L (7 M urea, 2 M thiourea, 4% CHAPS)], 1.0 μ L IPG buffer (Bio-Rad, pH3 - 10), 0.00123g plus One DTT and 0.4 μ L (1% Bromophenol Blue) was used to re-suspend the pellet with 30 s vortex. Finally, the micro-centrifuge was used at speed (12000 \times g) for 5 min to remove any insoluble material.

6.4.4 Rehydration

The individual IEF strip (11 cm, pH 3-10) was rehydrated in 200 μ L of rehydration solution by mixing the following reagents:

- 200 μ L (7 M urea, 2 M thiourea, 4% CHAPS)
- 1.0 μ L IPG buffer (Bio-Rad pH3-10)
- 0.00123g plus One DTT
- 0.4 μ L (1% Bromophenol Blue)

The strip was covered with GE mineral oil and left overnight with the lid on.

6.4.5 First dimension IEF (Isoelectric focusing)

A Protean IEF cell (Bio-Rad) device was used to separate the proteins according to their electrical charges. The strip was placed gel-side down in a single well of the tray (manifold), then the sample was loaded. After an hour each well was covered by 1 ml mineral oil to avoid drying the strip and left with lid on at room temperature with a current limit of 50 μ A/strip: 3 h at 300 V (sample enters strip), 6 h at 1000 v (desalting), 3 h at 8000 v (gradient), and final focussing with 20000 Vh at 8000 V. Each single focused strip was equilibrated for 15 min in equilibration buffer (1.8 g urea, 0.5 ml tris, 1.5 ml glycerol, 1 ml D.W, 0.5 g SDS, and 0.05 g DTT, followed by a further 15 min in a second equilibration buffer which was the same as the previous buffer but with DTT substituted by 0.0625 g iodoacetamide and 12.5 μ L of 1% Bromophenol Blue.

6.4.6 SDS-PAGE (second dimension).

SDS- Page was performed on a 11cm wide Criterion platform (Bio-Rad). The IPG strip was subjected to second dimension electrophoresis (separating proteins according to their molecular weight) using a 1.4 - 12% SDS-polyacrylamide gel: (60 V, 1 W for 1 h) and followed by (120 V, 4 W for 2 h).

Beforehand the gel was covered with 5% XT MOPS running buffer 20X (dilution factor) (Bio-Rad).

6.4.7 Staining

Coomassie brilliant blue (CBB) R-250 (Fisher Scientific, UK Ltd.) was used for protein visualization. The gels were stained overnight in a staining solution (0.2 g Coomassie, 10% acetic acid and 30% ethanol). Before scanning, the gels were de-stained overnight in (10% acetic acid, 40% methanol).

For the full 2-DE protocol, see Appendix 3.

6.4.8 Image analysis

Gels from the ten treatments were scanned with ultraviolet (UV) light (Universal Hood11, BIO-RAD Laboratories, Italy). Images were captured by Grayscale Digital Camera (CFU-1312M, Japan). The Progenesis SameSpots software was used to analysis the 2D gel images. Image analysis included the following stages of quality control of images, reference image selection (most appropriate image), applying a mask of disinterest to exclude no spot detection area, alignment of all images with

the reference image, and image filtration by the deletion of non-matching spots. Significantly changed spots between all gels were determined according to the fold differences between treatments.

6.4.9 Protein identification (protein analysis)

Gel plugs (1.5 mm diameter) containing protein bands were manually excised and placed in a 96-well plate and peptides recovered following trypsin digestion using a slightly modified version of the Shevchenko *et al.*, (1996) method. Sequencing grade modified trypsin (Promega UK Ltd) was used at 6.25 ng/ μ l in 25 mM NH_4HCO_3 and incubated at 37°C for 3 hours. Finally the dried peptides were re-suspended in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA; 5 μ l) for mass spectrometry (MS) analysis and an aliquot corresponding to 10% of the material (0.5 μ l) was spotted onto a 384 well MS plate. The samples were allowed to dry and then overlaid with α -cyano-4-hydroxycinnamic acid (CHCA, Sigma, Dorset, UK; 0.5 μ l prepared by mixing 5mg matrix with 1 ml of 50% (v/v) acetonitrile in 0.1% (v/v) TFA).

Mass spectrometry was performed using a MALDI TOF/TOF mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyser; Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355nm (Bienvenut, 2002; Brennan *et al.*, 2009; Glückmann *et al.*, 2007; Medzihradszky *et al.*, 2000) MALDI mass spectra and subsequent MS/MS spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other

were excluded from the selection and the peaks were analyzed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6×10^{-6} Torr) and default calibration.

Combined PMF and MS/MS queries were performed using the MASCOT Database search engine v2.1 (Matrix Science Ltd, London, UK) Perkins (1999) embedded into Global Proteome Server (GPS) Explorer software v3.6 (Applied Biosystems) on the Swiss-Prot database.

6.4.10 Statistical analysis

Two dimensional gels were analysed using Progenesis SameSpots software (v 4.5.4325.32621, Nonlinear Dynamics Ltd, Newcastle Upon Tyne, UK). For the quantitative analyses, spots were considered significantly altered if fold changes was ≥ 2.5 .

6.5 Results

Proteins from Chinese cabbage (*B. rapa*) foliage were extracted and separated by 2-D and visualized with CBB. Protein expression patterns were analysed using image analysis and the protein spots were detected. MALDI mass spectrometry and LC-MS were performed in order to identify the Chinese cabbage proteome. A total of 427 protein spots were detected, forty spots from 10 different gels were selected and picked for their identification (Figure 6.1). Twenty two different proteins were

identified (Table 6.2). From the total proteins identified, all twenty two proteins (100%) were found in the in the presence of 12 endogeic (*A. rosea* & *A. caliginosa*) and epigeic (*L. rubellus* & *S. mammalis*) earthworms (B12), whereas only eleven proteins (50%) were found in the control treatment (Table 6.2).

Eleven (50%) of the identified proteins were up-regulated in the presence of 12 endogeic (*A. rosea* & *A. caliginosa*) and epigeic (*L. rubellus* & *S. mammalis*) earthworms (Table 6.2). Albumin was found only in the plant growth in the presence of 12 endogeic (*A. rosea* & *A. caliginosa*) and epigeic (*L. rubellus* & *S. mammalis*) earthworms. For the remaining 10 differentially expressed spots; 1, 2, 3, 4, 6, 11, 12, 13, 14 and 18 were identified as a probable Fructose- Bisphosphate aldolase 2, ATP synthase subunit beta, Ribulose bisphosphate carboxylase small chain, Oxygen-evolving enhancer protein1-1, Ribulose bisphosphate carboxylase large chain, Ribulose bisphosphate carboxylase large chain (Fragment), ATP synthase subunit alpha, ATP synthase subunit beta, Ribulose bisphosphate carboxylase large chain, and Annexin, respectively. Spots 15, 16, 17, 19 and 21 were identified as uncharacterized proteins.

The isoelectric point (pI) of the majority of discovered proteins ranged between five and seven (Figure 6.2 A), while the molecular weights ranged between 50 and 60 KDa (Figure 6.2 B).

PI 3 → 10

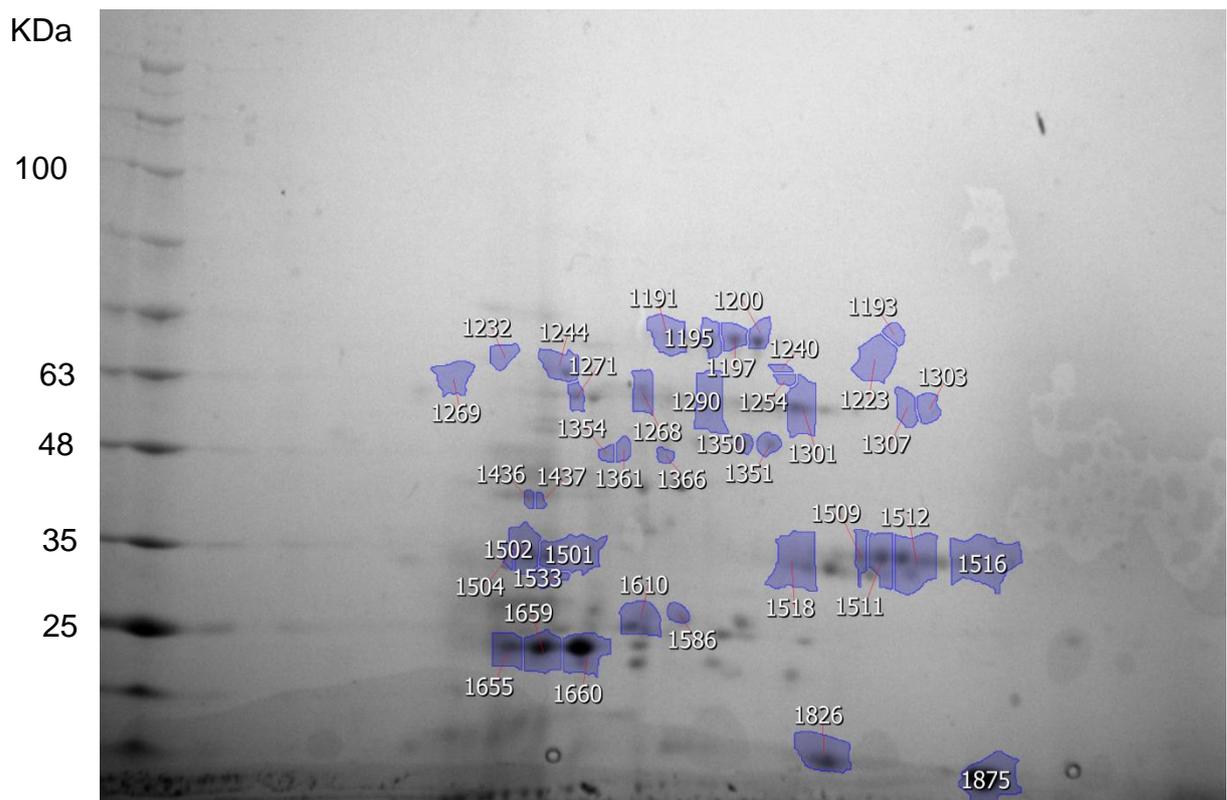


Figure 6.1 Composite gels from the image analysis software showing all picked protein spots in all treatments.

Table 6.2 Proteins identified from Chinese cabbage (*B. rapa*) with their isoelectric points (PI) and molecular weights (MW) under different earthworm treatments A: (*A. caliginosa* & *S. mammalis*), B: (*A. rosea* & *A. caliginosa*, *L. rubellus* and *S. mammalis*) and C: (*A. chlorotica* & *S. mammalis*) earthworm densities. The numbers refer to the total number of earthworms.

Spot	Protein	pI	MW	Treatment
1	Probable Fructose- Bisphosphate aldolase 2, chloroplastic	7.34	43	B12, B8, A8, C8, and B4
2	ATP synthase subunit beta, chloroplastic OS	5.24	52	B12 and B8
3	Ribulose bisphosphate carboxylase small chain F1, chloroplastic	8.19	20	All
4	Oxygen-evolving enhancer protein1-1, chloroplastic OS	5.29	35	B12, B8, A8, C8, B4, A4, C4, A2 and C2
5	Albumin OS	6.00	68	B12
6	Ribulose bisphosphate carboxylase large chain OS	6.24	53	B12, B8, A8, C8, B4, A4 and C4
7	Ribulose bisphosphate carboxylase large chain OS / <i>B. oleracea</i>	6.24	53	All
8	Ribulose bisphosphate carboxylase large chain (Fragment) OS	7.01	52	All
9	Ribulose bisphosphate carboxylase large chain (Fragment) OS	6.15	52	All
10	Ribulose bisphosphate carboxylase large chain OS	6.68	53	All
11	Ribulose bisphosphate carboxylase large chain OS	6.58	53	B12, B8, A8, C8, B4, A4, C4, A2 and C2
12	ATP synthase subunit alpha, chloroplastic OS	5.00	52	B12, B8, A8, C8, and B4
13	ATP synthase subunit beta, chloroplastic OS	5.13	52	B12, B8, A8, C8, B4 and A4
14	Ribulose bisphosphate carboxylase large chain OS	6.70	53	B12, B8, A8, C8, B4, A4 and C4
15	Uncharacterized protein OS= <i>Brassica rapa</i>	5.92	41	B12, B8, A8, C8, B4, A4, C4 and A2
16	Uncharacterized protein OS= <i>Brassica rapa</i>	6.29	53	All
17	Uncharacterized protein OS= <i>Brassica rapa</i>	5.43	24	All
18	Annexin OS= <i>Brassica rapa</i>	5.31	37	B12, B8, A8 and C8
19	Uncharacterized protein OS= <i>Brassica rapa</i>	5.43	22	All
20	Ribulose bisphosphate carboxylase small chain OS/ <i>B. rapa</i>	8.05	18	All
21	Uncharacterized protein OS= <i>Brassica rapa</i>	6.01	52	All
22	Adenosylhomocysteinase OS= <i>Brassica rapa</i>	5.97	53	All

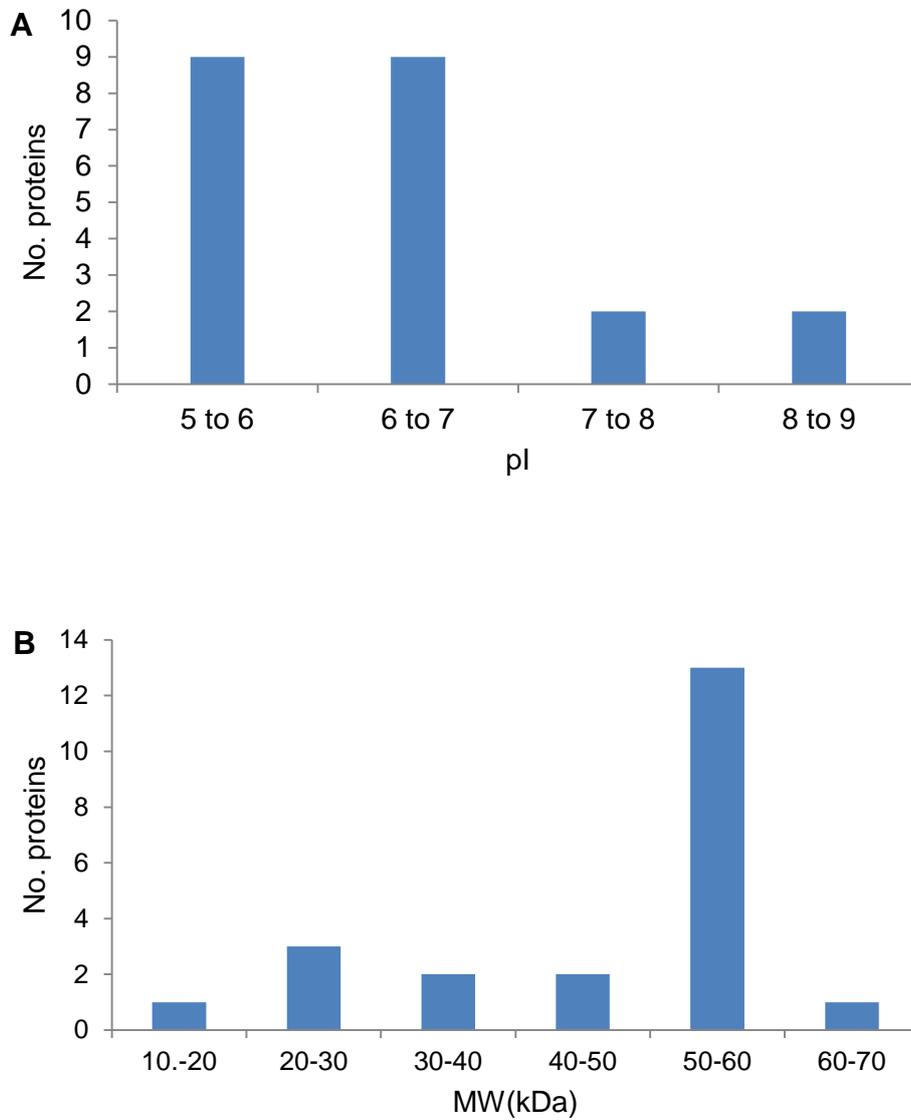


Figure 6.2 The molecular weights and isoelectric point of identified proteins in *B. rapa*. (A) the PI and (B) the MW of proteins.

The quantitative analysis of some selected spots (Table 6.3) showed differences in spots volumes between treatments. The results revealed that only in spot 1655 were there significant (Fold changes ≥ 2.5) differences between the control and 12 & 8 *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis* earthworm treatments (Table 6.3) (Appendix 2-B).

Table 6.3 Differentially expressed proteins in the *B. rapa* treated with different species and densities of (A): *A. caliginosa* & *S. mammalis*, (B): *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*) and (C): *A. chlorotica* & *S. mammalis* earthworms. Significance is based on the fold change ≥ 2.5 . Spot numbers correspond with the numbers in Figure 6.1.

Spot id	Fold change relative to control							
	B12	B8	B4	A8	A4	A2	C8	C4
1437	+2.3	+2.4	+2.3	+1.2	+1.7	+1.8	+0.8	+1.6
1660	-0.3	-0.6	-0.2	-0.3	-0.2	-0.4	-0.3	-0.5
1655	+2.5	+3.1	+1.7	+1.3	+1.1	+1.7	+2.2	+2.4
1826	+1.3	+1.2	-0.4	-0.14	+1.03	-0.3	-0.14	-0.11
1659	+1.4	+2.2	+1.3	-0.01	-0.05	-0.3	-0.04	+1.5
1351	+1.8	+1.3	-0.02	-0.16	-0.19	-0.3	+1.2	+1.1
1504	+1.2	-0.10	-0.06	-0.12	-0.02	-0.08	-0.12	+2.4
1191	+2.1	+1.4	+1.2	+1.7	+1.1	-0.05	+2.3	+1.1

6.6 Discussion

Earthworms have a critical role in protein changes in the plant. The results showed that changing worm density and species has an effect on plant proteins. Chinese cabbage *B. rapa* growth and aphid development were affected by the protein quality and quantity. Albumin was only expressed in the plant treated with twelve endogeic *A. rosea* and *A. caliginosa* and epigeic *L. rubellus* & *S. mammalis* earthworms (B12 treatment), while ATP synthase subunit beta chloroplastic was expressed in the presence of twelve (B12) and eight (B8) endogeic (*A. rosea* and *A. caliginosa*) and epigeic (*L. rubellus* & *S. mammalis*) earthworms.

In addition, Annexin was found in four treatments; (B12, B8, A8 and C8).

Albumin is compounds of C, H, O and (usually) sulphur elements, it is the main protein in the blood serum of humans and mammals, also it is found in some plant chloroplasts such as tobacco (Millán *et al.*, 2003), and also, in the seeds of some plants; beans, peas and peanuts (website: <http://science.howstuffworks.com>).

The function of this protein in the human is to help maintain the osmotic pressure in the blood, but the role in the plants is not clearly understood. This is what was mentioned by (Kehr, 2006) about the poor understanding of the functions of most phloem sap proteins in the plants.

In the current study, there were differences in aphid development between B12 and the control treatment on the *B. rapa* plant, with the daily nymph production, fecundity and number of adults were significantly higher. Concurrently, the albumin content was also higher in the B12 than

the control treatment. However, a direct causal link cannot be made from this study (Table 6.2).

Annexin has a role in the plant regulation growth via regulation H₂O₂ accumulation, which has a role in C₄ fixing during photosynthesis process. The current results showed that annexin content was higher in the B12, B8, A8 and C8 than in the control treatment. Also the results from Chapter four showed that the daily nymph production, fecundity and number of adults were significantly higher in the (B12, B8, A8 and C8) treatments than the control. These results provide an area for future research to investigate whether there is any relationship between increasing specific proteins in the plant and aphid development or not.

ATP synthase subunit beta, chloroplastic, known as a membrane protein that plays a key role in the energy metabolism, it is an essential component of the photosynthetic process in the plant chloroplasts (Howe *et al.*, 1985).

It is well known that increasing the energy flow leads to an increase of new vegetative growth and phloem sap (Hopkins & Hüner, 1995), which is the main food source for aphids. It is also a site of protein accumulation that can potentially influence insect - plant interactions (Kehr, 2006). However, in the present study the results showed that ATP synthase subunit beta, chloroplastic was higher in the plant treated with earthworms. Also the results from Chapter four showed that the numbers of daily nymph production, fecundity and the numbers of adult were

significantly higher in the plant treated with earthworms in comparison to the control treatment.

There is a little information known about Probable Fructose- Bisphosphate aldolase 2 (Cooper *et al.*, 1996), however aldolase enhances the formation of sugars (^{13}C labelled, N or F containing and high carbon sugars) (Wong & Whitesides, 1994).

Oxygen-evolving enhancer protein 1-1, chloroplastic was found in all treatments except the control, it is regarded as a manganese stabilizing protein which is required for photosystem II, and water splitting in the chloroplast thylakoid membrane of plants (Yi *et al.*, 2005). Results from the literature show that body fluids and casts of earthworms contain different mineral elements such as manganese (Beyer, Hensler & Moore, 1987; Zhenjun *et al.*, 1997; Zou, 1993), and many researchers have investigated the effect of Mn in plant defences against diseases. This effect depends on the host plant and pathogen species; e.g. Chhillar & Verma (1985), found that Mn has a negative influence on the aphid *Rhopalosiphum maidis* on Barley. While, Edwards (1983), investigated the effect of Mn on the same plant with a different disease (Mildew) and he found a positive effect. Also, Falcon, Fox & Trujillo (1984), reported an increase in root rot (*Phytophthora cinnamomi*) on Avocado.

The results showed that earthworms have a positive effect on protein content in the plant, and nitrogen amount was increased with increasing earthworm densities (Chapter one), it seems to be there is a positive relationship between the protein and nitrogen content in the plant. On the

other hand this positive relationship may have positively affected aphid development, since, daily nymph production, fecundity and numbers of adults increased with increasing earthworm densities, as well as nitrogen and protein content in the plants.

These results were consistent with the general view that phloem sap proteins influence the plant insect interactions (Bernays & Woodhead, 1984; Hogenhout & Bos, 2011; Horie & Watanabe, 1983; Kehr, 2006; Wicker & Nardon, 1982). Also, Miles (1999) mentioned the role of most phloem sap proteins identified in plant defences against insects. When, Broadway & Duffey (1988) tested the effect of different artificial plant proteins on the growth of beet armyworm larvae (*Spodoptera exigua*), they found that the effect of proteins depended on the kind of protein where casein shown more significant influences on the larval development compared to other proteins (soybean, tomato foliar protein, gluten and zein). On the other hand, Pierre *et al.*, (2013), found that the response of broccoli (*Brassica oleracea*) plants treated by Phytohormone to aboveground herbivores depended on the insect species, specialization degree of herbivore and the root treatment, since, aphid cabbage (*B. brassicae*) and butterfly cabbage (*P. brassicae*) were significantly influenced by roots treated with jasmonic acid (JA), while the numbers of the moth (*P. xylostella*) found was low.

The results showed that differences in protein volumes generally did not vary with treatment (Table 6.3). Only spot 1655 showing a significant difference between increasing earthworm density and protein volume. In

this case, there are two possible explanations; the first one that plants are already produce the proteins and earthworms have an effect on the kind of amino acid via increasing nitrogen concentration. In agreement with this achievement, Lam *et al.*, (1996) mentioned the role of nitrogen in plant growth, since, nitrogen assimilated into the amino acids. Also, Karley, Douglas & Parker (2002) found that the aphid responds to the composition but not the amount of amino acids. The second explanation, is that aphid feeding may provide the plant with essential amino acids, and the aphids obtain amino acids from symbiotic bacteria called *Buchnera* (Douglas, 1998), which are secreted into the plant through saliva during their feeding on sap (Cherqui & Tjallingii, 2000; Harmel *et al.*, 2008; Hogenhout & Bos, 2011; Miles & Harrewijn, 1991).

Galli *et al.*, (1990), investigated the role of earthworm casts in increasing protein in the white mushroom *Agaricus bisporus*. They found that the proteins L-¹⁴C- leucine incorporation and carpophores were increased about 34% and 25% respectively.

Generally, the plant sap contents very few essential amino acids because unlike nitrogen, plants cannot uptake protein from the soil. However, the plants are able to synthesise the amino acids via metabolism processes, Access to these proteins is very important for aphid development because aphids are unable to synthesise amino acids, therefore, it obtains the required amino acid from the plant sap (Douglas, 2006).

Furthermore, Douglas (2003) has pointed out the significant role of phloem sugar as a source of energy, because it is the basis for respiration in aphids (Febvay *et al.*, 1999; Rhodes, Croghan & Dixon, 1996), therefore, aphids prefer phloem containing sugar (Pescod, Quick & Douglas, 2007), and they can overcome the osmotic pressure problem by transforming disaccharides into long chain oligosaccharides (Douglas, 2006).

Finally, the results showed that the molecular weights of the majority of proteins ranged between 50-60 KDa, and the isoelectric point ranged between 5 -7 (Figure 6.2). Fisher *et al.*, (1992), found that the majority of wheat sap proteins collected from aphid stylets range between 60-70 KDa.

**CHAPTER 7
GENERAL DISCUSSION**

7.1 Introduction

Extensive studies of earthworms and their physical and chemical influences on the soil and above-ground community has led to much knowledge about their role in agro-ecosystems (Amador & Görres, 2005; Clapperton *et al.*, 2001; Kennel, 1989; Logsdon & Linden, 1992; Pashanasi *et al.*, 1992; Stephens & Davoren, 1997; Topoliantz *et al.*, 2002). The interaction between earthworms, plants and above-ground aphids is complex, since many contrasting results have been achieved by several researchers who investigated the morphological changes in the plant and their influence on aphid development (Wurst *et al.*, 2003), and some have focused on the relationship between earthworms and aphids through chemical changes in the plant (Bonkowski *et al.*, 2001; Scheu, Theenhaus & Jones, 1999; Wurst *et al.*, 2003). However, there has been limited study on the effect of earthworms on protein changes in the plant (Tomati *et al.*, 1990; Tomati, Grappelli & Galli, 1988), and only a few have investigated the role of plant proteins on aphid growth [e.g. (Sandström & Moran, 1999)], and non-have investigated the influence of earthworm on aphids through protein changes in the plant. A specific gap in the research, addressed in this study, is the interaction between individuals and combinations of earthworms from different functional groups and their influence on above-ground aphids.

The relatively recent introduction of new tools, such as two dimensional electrophoreses (2-DE) for protein analysis, has allowed greater

understanding of the role of earthworms in protein changes in the plants and aphid development.

Furthermore studies are also required to investigate the protein changes in aphid as well by using the same technique (2-DE) to understand the relationship between protein changes in the plant and aphids.

7.2 Plant growth

Earthworms markedly modified plant growth, although their effects differed between earthworm species. There was, however, evidence that some earthworm species in this case *A. rosea* can have a stimulating effect on plant morphology (plant biomass, height of plant, leaf surface area and SLA). While, in the presence of *A. chlorotica* there were no such changes in plant growth even though they belong to the same functional group.

The results presented in Chapter 3 provided evidence about differences between earthworm species and their influences on plant growth, these differences may be due to the difference in earthworm size providing a small amount of nitrogen for plants and proof of this is that their impacts on plants increased with increasing their densities, which may also be linked to increased casting.

On the other hand, these differences in the individual effects of *A. rosea* and *A. chlorotica* from the same functional group on plants raises doubts about the validity of their belonging to the same functional group.

Also, the results from this study showed that earthworms have no effect on the numbers of plant leaves, it seems that earthworms act by

increasing nitrogen, possibly also stimulating the photosynthesis process in the plant, thus increasing phloem sap which leads to increased leaf surface area and plant biomass. In addition, by their activities in the soil, earthworms may be having an effect on microbial community, thus it is very important to consider the soil analysis in the future work.

A similar result was reported for the effect of earthworms on plant performance by Scheu, Theenhaus & Jones (1999) when plant biomass significantly increased with the presence of the endogeic *A. caliginosa* & *O. tyrtaeum* earthworms. Also, the leaf and stem biomass of *Birch* seedlings increased with the presence of the epigeic *L. rubellus*. In contrast, Lohmann, Scheu & Müller (2009), found that the endogeic earthworm *A. caliginosa* had no effect on plant morphology.

The differential modification in plant growth is also consistent with our expectations, since we assumed that the earthworm influences were related to their densities; increasing worm density significantly increases plants growth.

Several morphological plant aspects and plant biomass were influenced by increasing earthworm densities which was consistent with the results of Callaham and Hendrix (1998), who reported that the biomass of *Pinus palustris* seedlings increased with increasing *Diplocardia mississippiensis* earthworm density. Also, increasing the endogeic *Pontoscolex corethrurus* earthworms biomass caused increases in *Achiote Bixa orellana* biomass (Pashanasi *et al.*, 1992). In contrast, Doube *et al.* (1997), found that the responses of plants to earthworms varied with plant

species and soil types. Also, in the presence of the endogeic *A. caliginosa* & *O. tyrtaeum* earthworms the shoot biomass of *L. perenne* increased by 49% while in *P. lanceolata* increased by 20% (Wurst *et al.*, 2003).

Also, the interactions between earthworms from two different groups were more effective on plants performance due to their activities in different soil layers and supplying the food around the roots, according to their functional groups, the epigeic worms tend to live and feeding in the topsoil layer, while the endogeic worms, they tend to make horizontal burrows in 10 cm depth, and through their activities in different layers they mix the nutrients around the plant roots (Bouche 1977). Sheehan *et al.*, (2008), mentioned the important role of the interaction between earthworm species from different functional groups in increasing the microbial community and their distribution in the soil. Since, increasing the nutrient in any layer of soil depends on the earthworm community composition (Sheehan *et al.*, 2006).

Edwards and Lofty (1980) pointed out the importance of the presence of earthworms at the same level as the roots in order to be more influential on plant growth, because the nutrients accumulate in the channels provided by worms more than the surrounding soil. Also, Edwards and Bater (1992), found that the effect of earthworms on plants depends on the depth of root growth and the activity zones of earthworms; e.g. the endogeic *A. caliginosa* and *A. chlorotica* earthworms affected cereal seedlings roots in the upper 15 cm of the soil profiles, while the anecic *L. terrestris* and *A. longa* promoted root growth in the deeper soil layers.

This study indicated that plant nitrogen content increased in the presence of earthworms, possibly through their activities enhancing mineralization, or due to organic nitrogen coming from their mucus or casts.

The role of nitrogen in accelerating plant growth might be through increasing photosynthesis process and thus increasing phloem sap. In agreement, Schütz, Bonkowski & Scheu, (2008), found that soil nitrogen has a positive effect on plant growth (leaf length and area) of maize.

The results achieved from this study showed the differences in the effect of worm species on plant physiology may be also due to the differences in their size, since increasing earthworm density leads to increases in their casts, nutrients and nitrogen uptake by plant. In agreement with this study, Sheehan *et al.*, (2006), found that the soil nitrate increases with increasing earthworm densities. Also, increasing the endogeic *Pontoscolex corethrutus* earthworm biomass positively increased nitrogen mineralization through their impact on microbial biomass in the soil (Pashanasi *et al.*, 1992). The specific lifestyle of worm species may be having a critical effect on the availability of plant nutrients. Stephens *et al.*, (1994), found that earthworm species differ in supplying elements to the plant, since the endogeic *A. rosea* worm causes increase in Ca, Cu, K, N, Na and P concentration in the leaves of wheat *T. aestivum* crops, while the anecic *A. trapezoides* earthworm caused increase in Al, Ca, Fe, Mn, N and Na.

Double *et al.*, (1994), reasoned that the positive influence of earthworms on nitrogen content may be correlated to their interactions with soil

microorganisms (nitrogen-fixing bacteria) at a small spatial scale (earthworm casts or channel walls). Also, through their activities and interaction with microbial community, the endogeic *D. mississippiensis* earthworm increased the nutrient availability in the soil (Lachnicht & Hendrix, 2001).

7.3 Aphid development

The relationship between plants and aphids is complex, since many factors affect this relationship, either positively or negatively. The morphological and chemical changes in the plants are the more important factors, and these changes in the plants occur as a result of changing soil nutrients through organisms' activities.

Despite many previous studies about the effect of earthworms on above-ground aphids (Bonkowski *et al.*, 2001; Scheu, Theenhaus & Jones, 1999; Wurst, 2010), there are still some ambiguities about the effect of earthworms on above-ground community because of the discrepancy in the results, in particular the chemical changes in plants and their influence on aphids (Eisenhauer & Scheu, 2008; Ke & Scheu, 2008; Wurst *et al.*, 2003).

Additionally, the morphological changes in the plants such as increasing the leaf surface area may considerably influence the growth of aphids through providing a wider area of leaf surface for aphid feeding, also increasing the plant biomass led to increases in phloem sap which has a critical role in the relationship between plants and aphids. Increasing

phloem sap might help aphids' growth and development for successive generations.

The interaction between different species from different functional groups of earthworms could influence aphid development.

The results from this study showed that there is distinct variation among the functional groups of earthworms in their effects on aphid development.

The results here suggested that the effects of earthworms on aphid fecundity might be more important than those on development (slow growth from birth to adult). What are noticeable though are the differences in the individual effect of the *A. rosea* and *A. chlorotica* on the numbers of adults between first and second generations. Since both are members of the Bouché (1977) endogeic group. The intra-group difference revealed in this study might explain why there are contrasting results in the literature. Scheu & Jones (1999), found that aphids developed significantly in the presence of the *A. caliginosa* and *O. tyrtaeum* but, Wurst *et al.*, (2003) and Little *et al.*, (2011), found that using soil treated with earthworms reduced numbers of nymphs and adults of aphids. The evidence from this study is that observed effects may be linked to quantitative changes in plant growth but this does not rule out qualitative changes such as the inducement of defence chemicals.

The results showed that the combined influence of two different functional groups of earthworms had a greater effect on aphid development than those from a single group. By their activities in different zones in the soil, earthworms could provide more nutrients around the plant roots through

their casts. There is also the possibility that these activities may positively affect the photosynthesis process, thus making phloem sap more available for aphid. In agreement, Edwards & Bate (1992), found that the effect of earthworm on plant growth depends on the activity zones of earthworms and the level of root growth, where the anecic *L. terrestris* & *A. longa* worms caused greater increase in cereal seedling growth than endogeic *A. caliginosa* and *A. chlorotica* worms.

The results from this study suggested that there are differences in the influences of earthworms on aphid growth between the two generations.

The combined effect of earthworms on daily nymph production and fecundity were not different between the two generations while in their influence on adults the numbers of adults in the first generation were higher than in the second generation. The potential explanation for these differentials in their effect may be due to decreasing in the phloem sap. Chinese cabbage features a very short growing season thus the failure of nymphs to develop into adults in the second generation.

Furthermore, the results showed that there are differences in the effects of earthworms on aphid development between two generations, where the results from chapter 5 showed that in the individual effect of *A. rosea* and *A. chlorotica* the daily nymph production and fecundity in the first generation were higher than in the second generation. It may be that the amount of nitrogen provided by earthworms is not commensurate with the size and needs of plant and aphids in the second generation.

This may be because the competition between them may increase with time because they belong to the same species and functional group, affecting their efficiency in providing nitrogen. But here a question arises as to why this does not happen when *A. rosea* and *A. chlorotica* are present together despite their belonging to the same group. These differences raise doubts about the validity of their belonging to the same functional group.

The increases in the numbers of adults developed from nymphs with time (Chapter 4) showed that the numbers of adults recruited each day increased regularly in the first generation compared to the second generation. It suggests that the nutrient availability from plants decreased with time as plant leaves became thicker and harder for nymphs sucking phloem sap.

The results from this study also showed positive relationship between increasing earthworm density, plant nitrogen and aphid development. Increasing nitrogen content in the plant maybe affects phloem sap composition through changes in protein content and amino acids.

Annan *et al.*,(1997), Butler *et al.*, (2012), Megahed (2005) and Schütz *et al.*, (2008), found that sucking insects (e.g. aphids) have a strong response to the nitrogen content in the soil compared to chewing insects. Furthermore, Mattson (1980), explained that the high responsiveness of sucking insects to nitrogen is due to the low-levels of nitrogen in the phloem sap.

Ponder *et al.*, (2000), reported that aphids prefer to feed on plant growth with nitrogen, and there is a significant correlation between nitrogen amount and amino acid concentration. The total amino acid concentration increases in the phloem sap of barley seedlings grown with nitrogen.

In contrast Scheu, Theenhaus & Jones (1999), found a weak relationship between nitrogen concentration and *M. persicae* development in the annual meadow grass (*P. annua*).

Finally, this study showed that increasing biodiversity (single species < members of the same functional group < members of different functional groups) enhanced their influences on plant growth and aphid development. These results provide the evidence about the importance of the interactions between soil organisms in terms of their influences on above-ground community and ecosystems in general.

7.4 Protein changes in the plants and aphid development

Knowledge about the proteins changes in the plants is an important tool to understand their role in the interaction between earthworms, plants and aphids. Chapter six investigated the protein changes in the plants. In this study 2-DE technique was applied for protein determination, and gave evidence about the role of earthworms in protein changes in plants despite the few numbers of spots which were detected in each gel due to difficulty in solubilisation and high levels of interfering substances and salts in the plant foliage. Similarly, Wang *et al.*, (2003), in their study on the protein extraction mentioned the high levels of interfering compounds in olive leaf.

The results from this study showed that aphid development was positively associated with protein changes in the Chinese cabbage. Aphid growth depends on the kind of protein. Also the current study indicated that there is no evidence about the relationship between increasing the protein volume in the plant and aphid growth. It seems that increasing nitrogen in the presence of earthworm may affect the metabolism of protein through changes in amino acids composition, and these changes might be in the benefit of aphid development. Karley, Douglas & Parker (2002), found that only the composition of amino acids has an effect on aphid growth. Also Kawashima & Tamaki (1967), found small but remarkable changes in proteins composition (amino acids quality) of tobacco leaves as a result of adding fertilizer.

Stratmann (2003), pointed out the role of Lipoxygenase as a defence compound in plants, and how it is concentrated in the leaves as a reaction to the presence of earthworms (Blouin *et al.*, 2005).

High fecundity and slow development may result from decreasing soil nutrients with time. In agreement, Dixon and Watson (1970), investigated the sycamore aphid *Drepanosiphum platanoides* development on sycamore tree and found high aphid fecundity at the beginning of growth, but as the leaves matured, aphids produced fewer numbers of nymphs due to decreasing the amount of phloem amino acids. In addition, William (1980) found that young plant tissues are rich in all amino acids and soluble proteins.

Finally, the results showed that the isoelectric point of the majority of identified proteins ranged between 5-7, and the molecular weight between 50-60 KDa, it seems that Destree (2008), found that the highest numbers of aphids on bean were when the pH of sap ranged between 5.3-5.7. It is possible that these ranges make the plant healthier and aphids have preferentially adapted to them.

7.5 Conclusions

The use of a novel technique for studying the protein changes in plants in the presence of earthworms has led to the acquisition of new information on the role of proteins in the interaction between plant and aphid, opening new avenues of investigation in this area for future research. The identification of proteins in the plants and the differences between them in the presence of different earthworm species, led to a number of important outcomes: that the protein changes in the plant depended on the earthworm species and densities, and aphid growth was associated with these protein changes, as well as with increased foliar nitrogen, while earthworms had little or no effect on protein quantity in the plant.

Several morphological aspects of *B. rapa* were affected by the presence of earthworms. However, their influences were differing according to their species and density. More study required in this area in particular the differences in the leaves thickness between treatments.

The results from this study showed differences in aphid development between treatments. These differences have occurred due to the changes

in earthworm density and species. In particular it has shown enhanced effects with increased diversity of both taxonomic and functional groups.

For future work, my suggestion is extracting proteins from aphids to investigate the differences in the protein between aphids and plants, also using denaturing gradient gel electrophoresis (DGGE) technique to investigate the differences in the soil microbial diversity in the presence and absent of earthworms.

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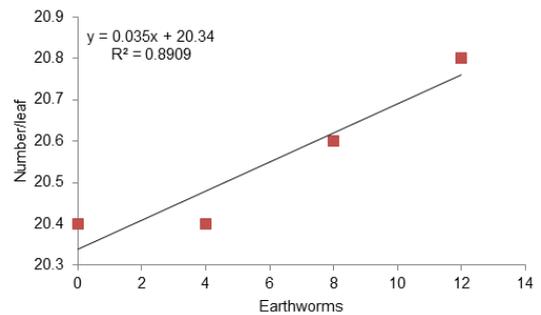
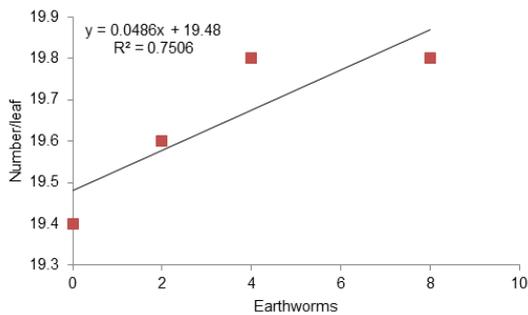
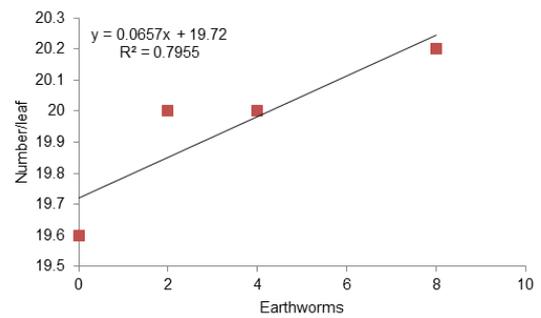
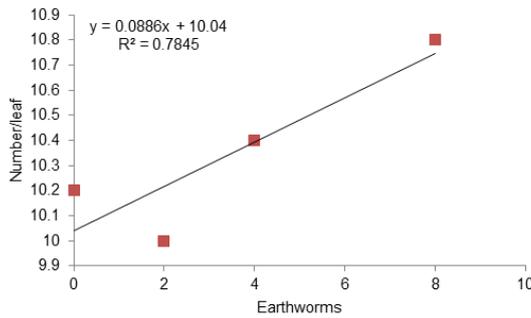
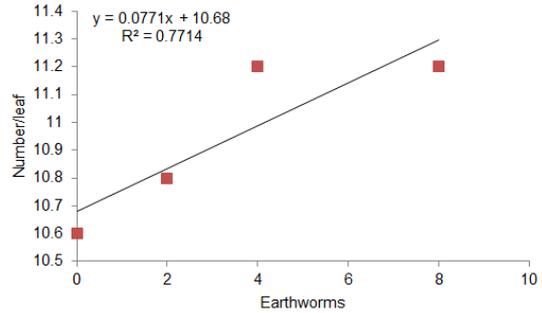
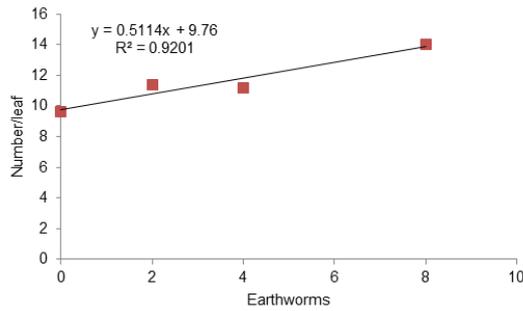
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Zou, W. C. (1993) 'Nutritive composition of earthworm's body fluids and its exploitation and utilization'. *Chinese Journal of Zoology*, 28 (3). pp 7-11.

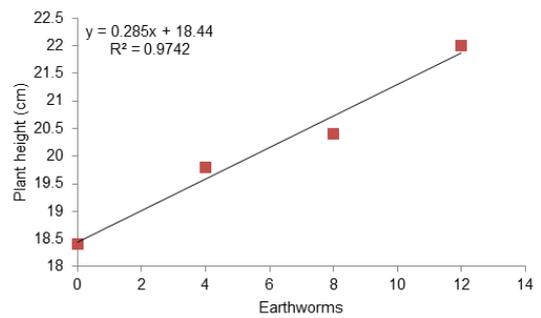
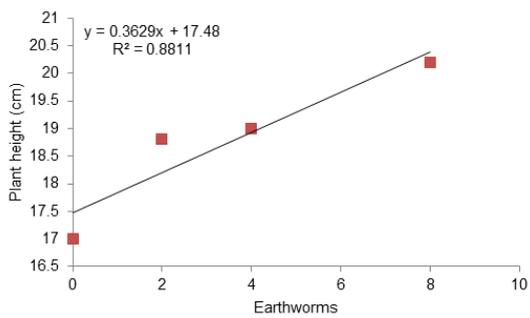
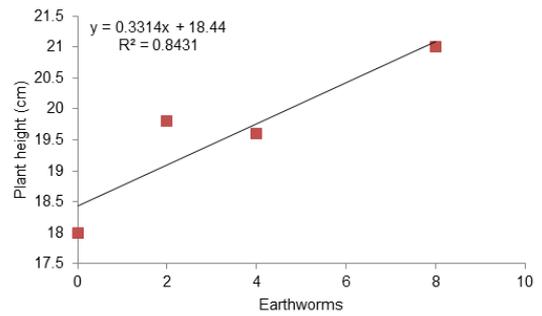
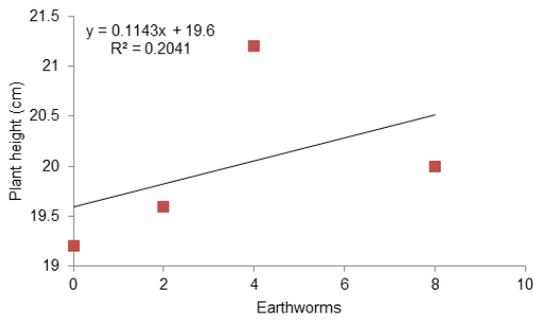
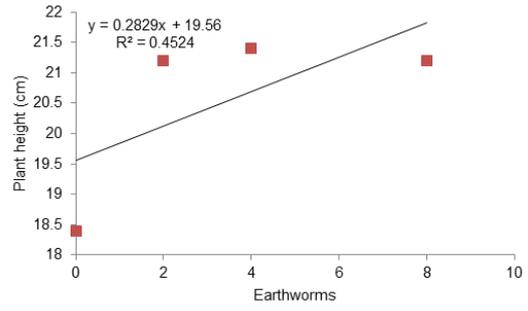
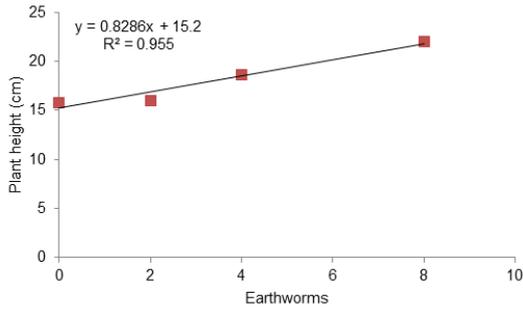
APPENDICES

Appendix 1

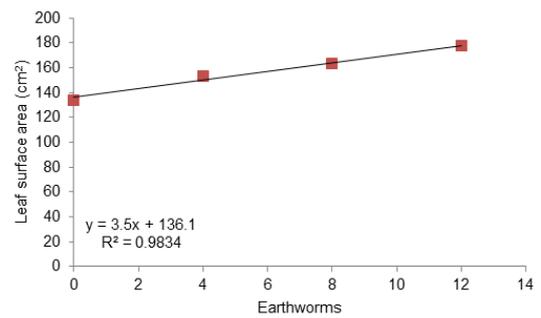
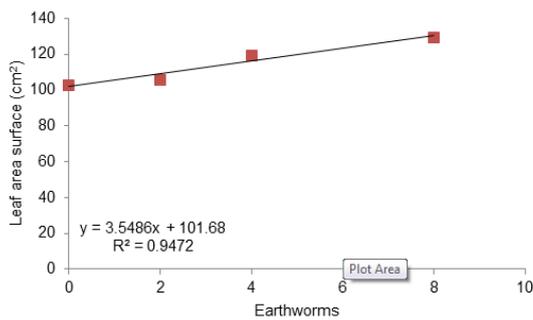
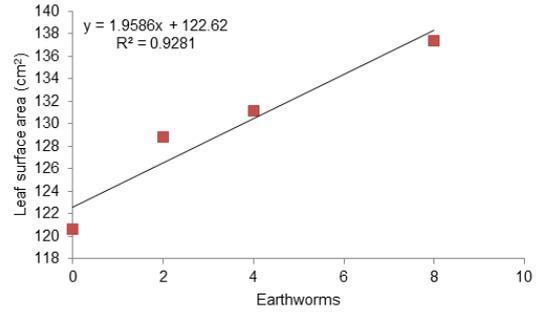
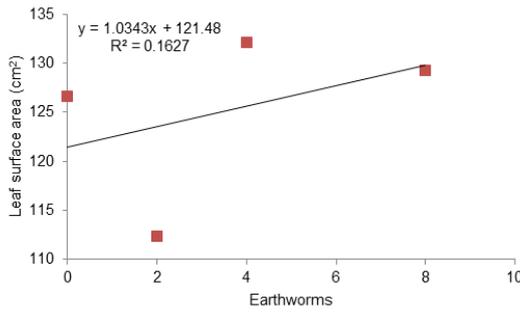
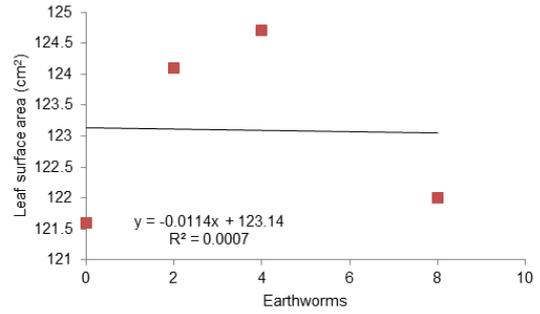
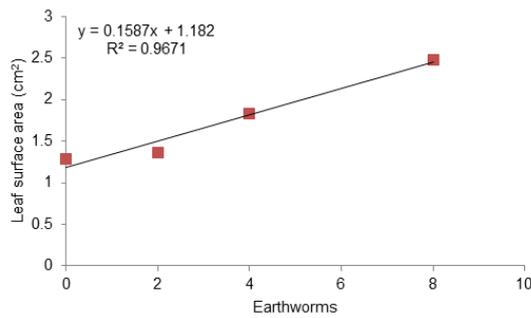
Appendix (1-A) Relationship between increasing earthworms density and leaf number in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.



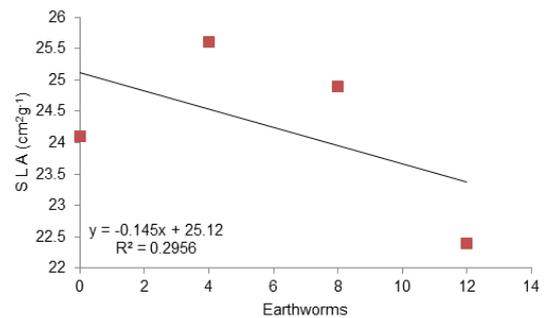
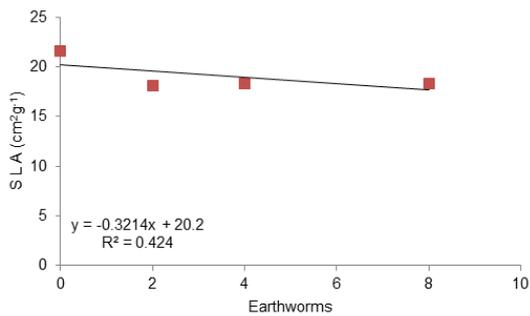
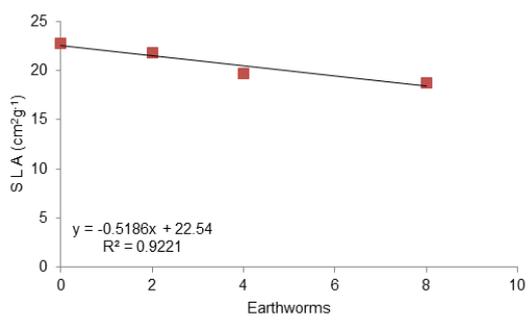
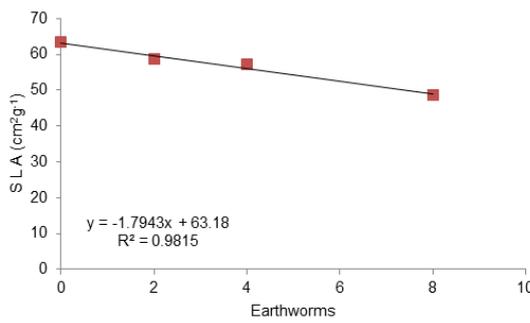
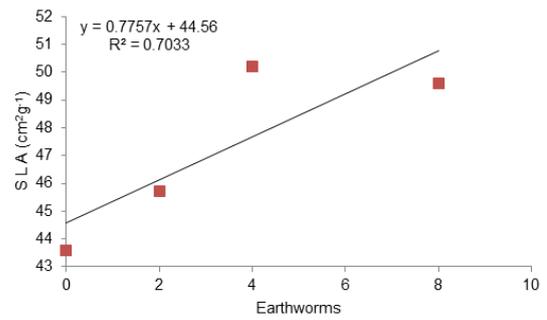
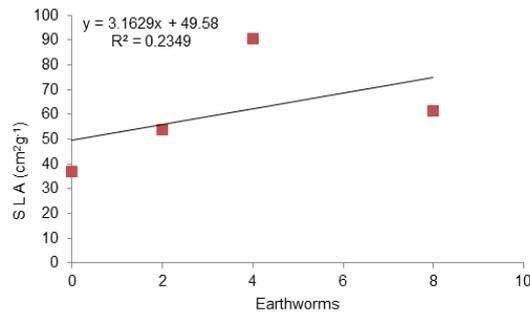
Appendix (1-B) Relationship between increasing earthworms density and plant height in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.



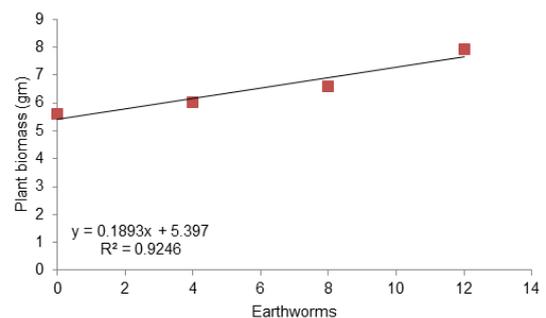
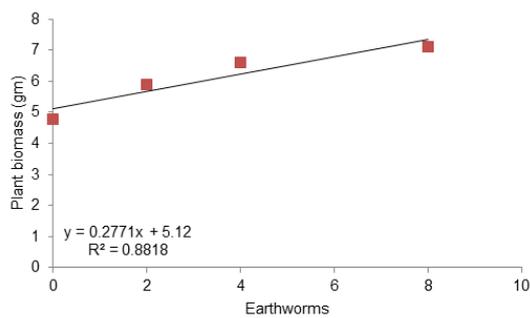
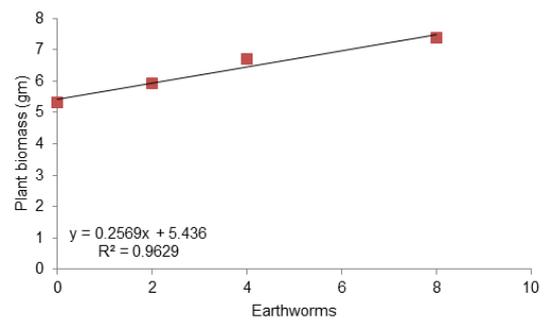
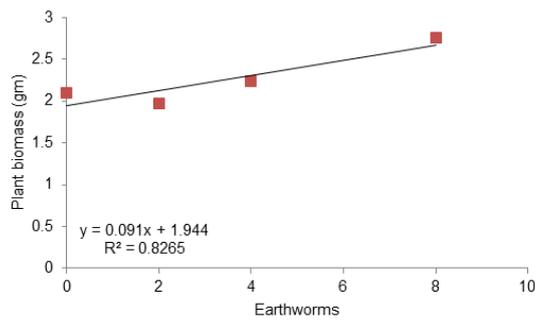
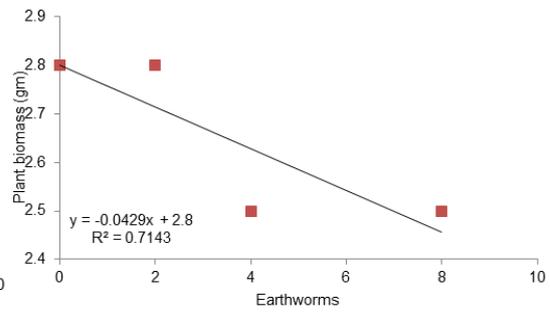
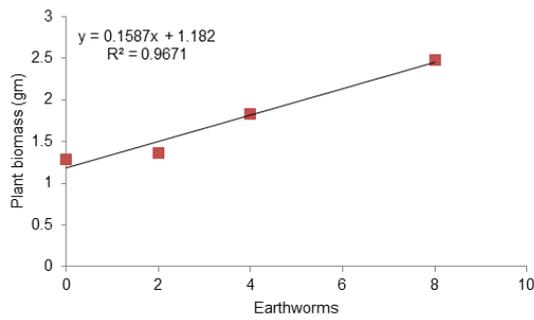
Appendix (1-C) Relationship between increasing earthworms density and leaf surface area in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.



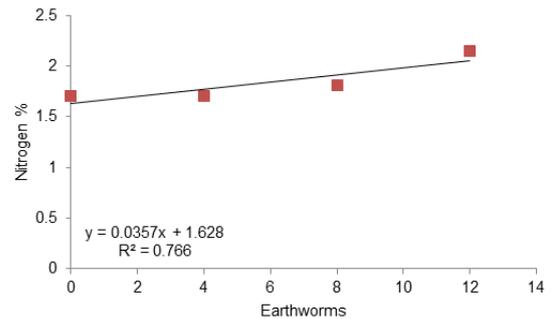
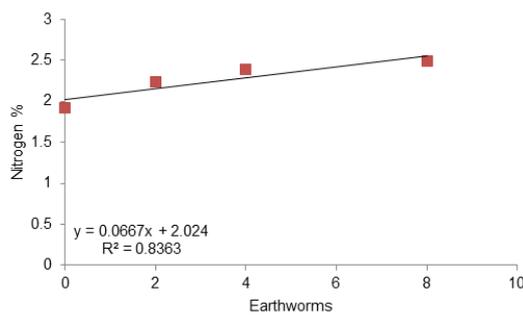
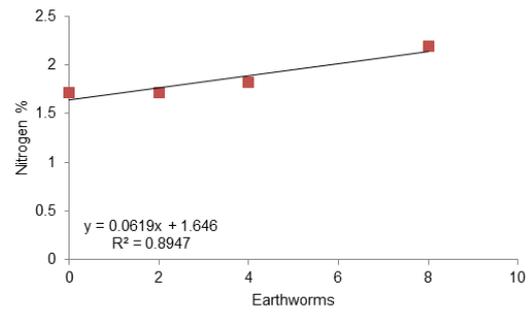
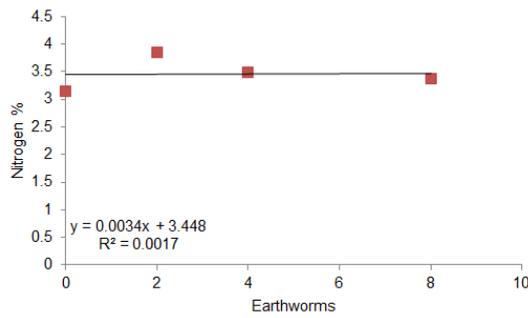
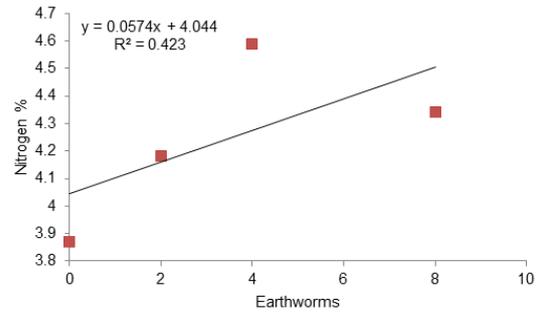
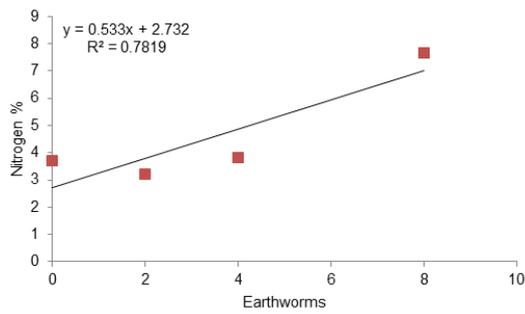
Appendix (1-D) Relationship between increasing earthworms density and SLA in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.



Appendix (1-E) Relationship between increasing earthworms density and plant biomass in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.

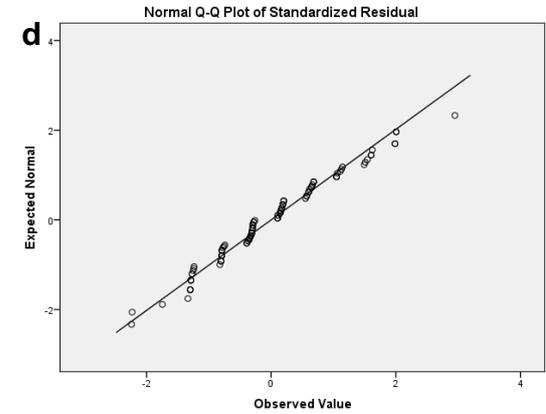
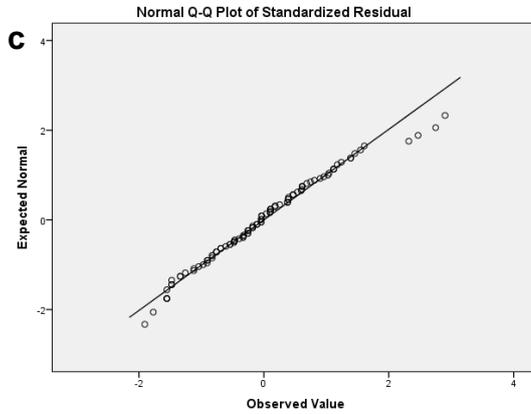
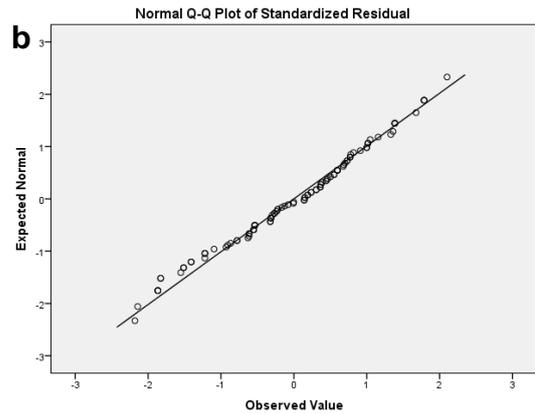
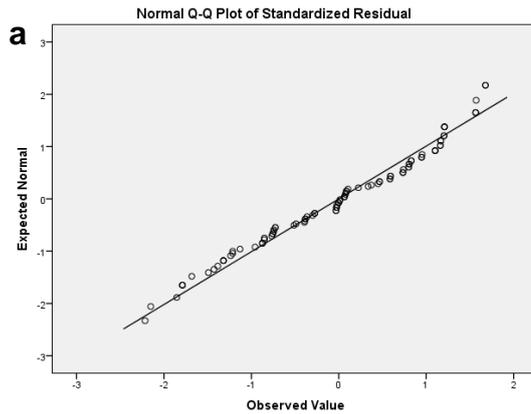


Appendix (1-F) Relationship between increasing earthworms density and nitrogen concentration in *B. rapa* (a) *A. rosea*, (b) *A. chlorotica*, (c) *A. rosea* and *A. chlorotica*, (d) *A. caliginosa* and *S. mammalis*, (e) *A. chlorotica* and *S. mammalis* (f) *A. rosea*, *A. caliginosa*, *L. rubellus* and *S. mammalis*.

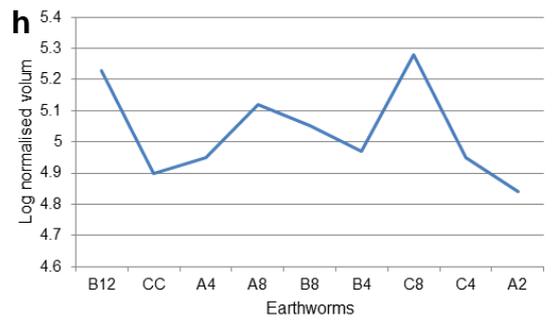
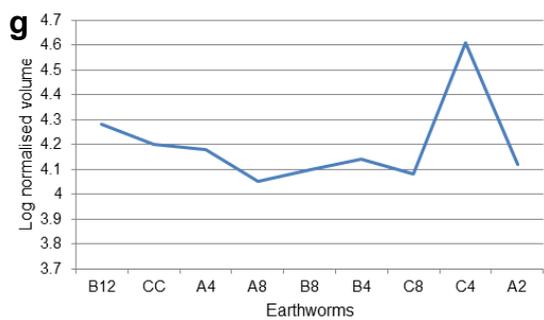
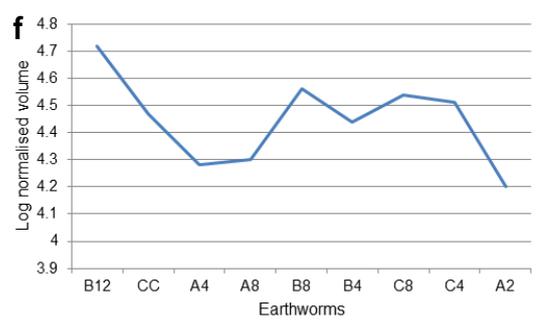
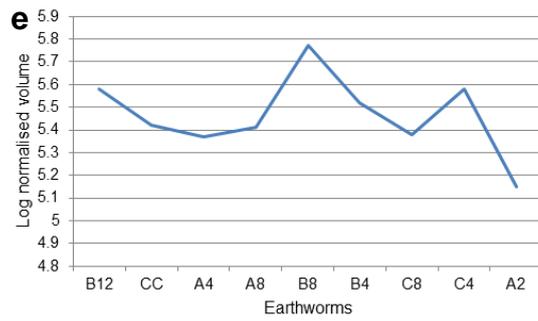
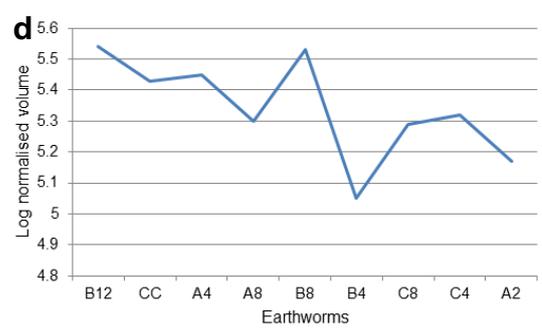
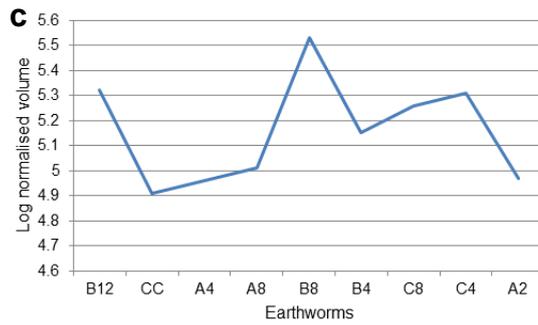
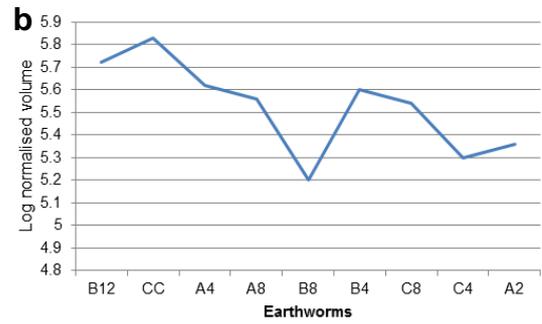
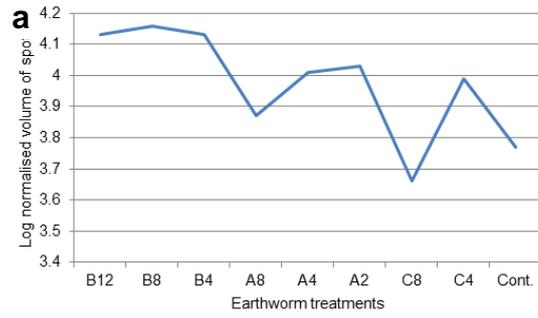


Appendix 2

Appendix (2-A) Q-Q plot of the residual distribution, fecundity (a) 1st generation, (b) fecundity 2nd generation, (c) adult 1st generation and (d) adult 2nd generation.



Appendix (2-B) The quantitative analysis of some selected spots, (a) 1437, (b) 1660, (c) 1655, (d) 1826, (e) 1659, (f) 1351 (g) 1504 and (h) 1191.



2-DE Protocol

For one strip

1) Put 200 μ l rehydration solution strip holder

How to prepare rehydration solution (Prepare fresh rehydration solution):

200 μ l (7 M urea +2M Thiourea+4% CHAS (Plus from GE) prepare in 100 ml
(42.04 g urea, 15.2 g thiourea, 20% of CHAPS 2g in 10

1.0 μ l Ipg buffer Bio-Rad PH 3-10.

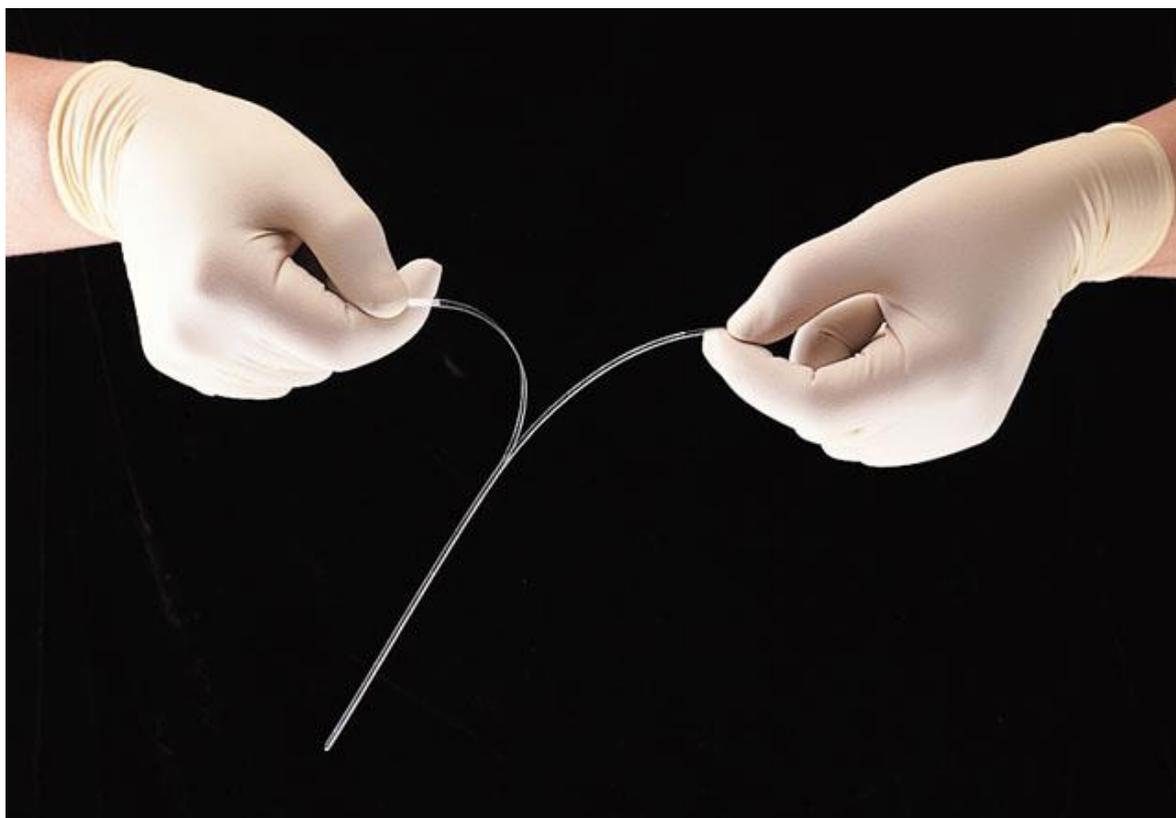
0.00123 g plus one DTT

0.4 μ l 1% bromophenol blue

Mix well

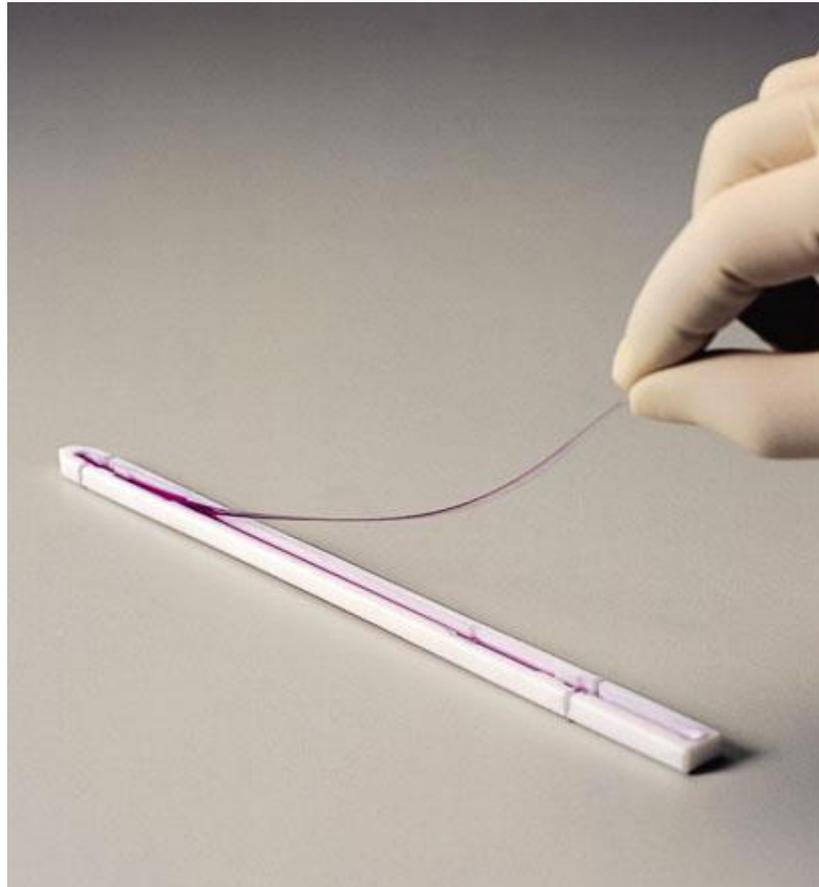
2) Prepare your IPG strip

(Remove the protective cover from immobilized pH gradients (IPG) strip.



Put the prepared rehydration solution in strip holder

Wet entire length of IPG strip in rehydration solution by placing IPG strip in strip holder (gel facing down).



- 3) Overlay each IPG strip with mineral oil fluid to minimize evaporation and Urea crystallization (1 ml).
- 4) Leave the strip holder overnight
- 5) Removal the strip from the strip holder and but the same rehydration solution in the strip holder of protein isoelectric focusing cell after this step put wet wicks in the end of the holder (wicks is small pieces like filter paper) . add 10 μ l of 2D water to each wick to make them wet.
- 6) Put each wick in the end of the strip holder then put 200 μ l of rehydration solution put your IPG strip and put the holder in isoelectric focusing machine

Put IPG strip positive in positive

- 7) Prepare your sample (30 μ l of rehydration solution +2.5 μ l sample)
- 8) Put 30 μ l of prepared sample under the IPG strip

- 9) Put 1.4 IPG strip with mineral oil Fluid
- 10) Put 1ml mineral oil Fluid to other in the holder close the lid. Turn on the machine for overnight.
- 11) Once the program is complete, turn off the power and remove your samples
- 12) IPG strips can either be stored in a plastic Petri dish at -80°C (gel side facing up) or directly following two equilibration steps for the further SDS-PAGE analysis.
- 13) If your strips are stored at -80°C, remove the IPG strips from the freezer and allow them to thaw for 10 to 20 minutes prior to equilibration of strips.
- 14) Equilibration Buffer:

10 ml	5 ml
1) 1.0 ml of 0.5 M Tris, pH 6.8	0.5 ml
3.6 g of urea	1.8 g
3 ml of glycerol	1.5
2 ml 2D water	1 ml
1 g of SDS	0.5

Put the tube in gycr rocker to mix for 20 min

Put all above in tube if the chemical prepare in 5 ml complete the volume to 5ml by 2D water divided into two part add 0.05 g DTT to the and to second part 0.0625 g of iodoacetmide + 12.5 µl Bromophenol Blue.

14) transfer the strip the holder and clean the strip whit d water than add the equilibration Buffer + DDT (2.5 ml) incubation for 15 mint.

15) Clean the strip with d water and put second part of Equilibration solution For 15 mint.

16) Than rinse the holder with ruining buffer MOP 20 x stocks (950 ml +50 ml)

17) Prepare your gel but the put the strip in the gel the + end in the – of gel.

- 18) Put running buffer in the top of the gel and then full the tank with running buffer and connected the tank and the run 1 W for 1.5 h then 4 W for 2 h then turn off the machine
- 19) Pour off the running buffer broke the plat of the gel by the top of end of the lid.
- 20) Place gel in a plastic container. Cover with fixing solution and shake at room temperature 15 mint.
- 21) Pour off fixing solution. Cover with Coomassie blue staining solution and shake at RT for 1 hr.
- 22) Pour off staining solution. Wash gel with 10% acetic acid to destain, shaking at RT ON.

1 Courses and workshops

- English language summer school (intensive course), academic writing, 27th September 2009 to 30th March 2010. Plymouth University, UK.
- Postgraduate research skills and methods in biology (BIO 5124), October 2010 to January 2011. Plymouth University, UK.
- Laboratory based teaching methods and practices (ENV 5101), October 2010 to January 2011. Plymouth University, UK.
- General Teaching Associates Course (GTAC), 5th September to 9th September 2010. Plymouth University, UK.
- Getting the most from conference workshop, Plymouth University, UK 30th March 2012.
- GLIM, GLAM & GLUM Statistics course, Plymouth University, UK. 1 - 2nd May 2013.
- GLIM, GLAM & GLUM Statistics course, Plymouth University, UK. 4- 5th June 2013.
- Hands on Molecular Biology Techniques, Plymouth University, UK. 26- 29th June 2013.
- Hands on Genomics and Proteomics, Plymouth University, UK. 1-3rd July 2013.

2 Taught sessions

- Endnote session. 22nd April 2010.
- Overview of the intranet for PGRs. 23rd April 2010
- Induction day for development of academic research. 27th April 2010.
- Getting started with qualitative research. 19th May 2010.
- Introduction to my sites. 1st June 2010.
- Preparing for the Viva. 7th June 2010.
- Overview of the intranet for PGRs. 15th June 2010.
- Introduction to applying for research. 18th June 2010.
- Transfer process. 25th June 2010.
- Avoiding Plagiarisms referencing. 25th October 2010.
- Presentation skills (Structure & Signposting). 9th November 2010.
- Microsoft PowerPoint 2007. 15th November 2010.
- Developing professional writing skills for the PhD. 17th November 2010.
- Preparing to Transfer. 22nd November 2010.
- Creating graphics using paint shop pro photo x2. 3rd December 2010.
- Writing up for PhD. 19th January 2010.
- Introduction to Qualitative research methods. 7th February 2011.
- Creative and reflexive reading. 8th February 2011.
- Introduction to Qualitative research methods. 10th February 2011.
- Introduction to endnote. 14th February 2011
- Introduction to interviews and Assessment centre. 22nd February 2011.

- La Tex. 1st March 2011.
- GIS-Geographical Information Systems-Introduction. 14th March 2011.
- Overview to researching and accessing information. 15th March 2012.
- La Tex. 16th March 2011.
- Careers: The UK Labour Market. 21st March 2011.
- Introduction to apply for research funding. 27th March 2011.
- Getting to most conference. 30th March 2011.

3 Conference registrations

- The Postgraduate Society Conference. Plymouth University, UK. 26th May 2010.
- The Postgraduate Society Conference. Plymouth University, UK. 28th May 2010.
- The Postgraduate Society Conference Series. Plymouth University, UK. 20th December 2010.
- The Postgraduate Society Conference Series. Plymouth University, UK. 17th March 2011.
- 1st Annual Conference, Plymouth University, UK. 4th April 2011.
- Annual Research Day, Plymouth University, UK. 5th April 2011.
- The Royal Entomology Society and Soil Ecology Society Conference. National Marine Aquarium. Plymouth. UK. 14-16th September 2011.
- The Postgraduate Society Conference, Plymouth University. UK. 14th March 2012.
- The Postgraduate Society Annual Conference, Plymouth University. UK. 26th June 2012.

- The Royal Entomology Society and Soil Ecology Society, Cambridge.
UK. 18-20th July 2012.
- The Postgraduate Society Conference, Plymouth University. UK. 27th
November 2012.
- The Postgraduate Society Annual Conference, Plymouth University.
UK. 27th November 2012.
- CARS Postgraduate Symposium, New Continental Hotel, Plymouth.
UK. 10th December 2012.
- The Post graduate Society Conference Series, Plymouth University.
UK. 19th March 2014.