Interactions between heavy metals and glucosinolates as defense mechanisms in *Thlaspi caerulescens*

By

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Declaration

I hereby declare that the work presented in this thesis is my own work and any part of this work has not been submitted for any other degree. Relevant sources of information have been acknowledged by reference to the authors.

Saeed Ahmad Asad

Dedication

This work is dedicated to my parents and family.

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ABSTRACT

Hyperaccumulator plant species grow in metalliferous soils and accumulate exceedingly high concentrations of metals. They are increasingly studied because of their potential for cleaning up land contaminated with heavy metals, but another aspect of study relates to the reason for hyperaccumulation. The most accepted hypothesis over the last few decades is the 'elemental defence' hypothesis, which states that high levels of metals defend the plant against herbivores. Whilst some of the literature is contradictory, some is supportive. An added complication is that many hyperaccumulators belong to the Brassicaceae and produce glucosinolates as organic defences against herbivory. The question to be answered is whether metals or glucosinolates act as the primary defence in these plants and the most recent suggestion is the 'joint effects' hypothesis, which states that both classes of chemical work together to benefit the plant and protect it from herbivores.

This study investigates these hypotheses and utilized three experimental systems. The hyperaccumulator studied was *Thlaspi caerulescens* (Gange ecotype) which hyperaccumulates zinc. Plants were grown in a series of glasshouse experiments at a range of soil zinc amendments. There was a positive relationship between soil and foliar zinc; optimum growth occurred at 2000 mg Zn kg^{-1} soil and this equated to approximately 8000 mg Zn kg^{-1} shoot, although plants took up as much as 14000 mg Zn kg^{-1} shoot tissue at higher levels of soil amendment.

The herbivore systems studied were generalist thrips (*Franklinella occidentalis*) and the specialist cabbage whitefly (*Aleyrodes proletella*). In addition, artificial damage caused by clipping served as a positive control.

Four aromatic glucosinolates were extracted from *T. caerulescens* and two were identified as benzyl and p-OH-benzyl. Glucosinolates were synthesized 32 hours after damage occurred and reached a maximum concentration after 48 hours.

Generally, lower concentrations of glucosinolates were observed in plants with higher foliar Zn concentrations and *vice versa*. However, when plants were subjected to a sustained and heavy herbivore attack, as was the case when thrips infested the plants, glucosinolate production occurred irrespective of foliar Zn concentration. This observation supports the 'joint effects' hypothesis, which states that both defences work in tandem and enhance overall defence.

Nitrogen was an important component that directed herbivore response. Thrip feeding damage was negatively correlated with foliar nitrogen whilst cabbage whitefly (CWF) benefitted from higher N. Nitrogen was positively correlated with glucosinolate concentrations and glucosinolate content negatively affected the generalist thrips but not the specialist CWF. Data were analysed by accumulated general linear regression and the explanatory model for thrip feeding was C/N ratio + GS + Zn whilst the explanatory model for CWFs was C/N ratio + Zn.

Use of the specialist feeder (CWF) allowed for study of the effects of zinc without glucosinolates confounding the results since the CWF was unaffected by foliar glucosinolates. Zinc acted as a defence against CWF but only at high concentrations.

The data taken together show that zinc acts as a defence against herbivores that are unaffected by glucosinolates, but only at high concentrations. Zinc also defends the plant against generalist thrips, but glucosinolates are more influential in this case. This might be because of the severe and sustained damage that these plants suffered and systemic effects (i.e. higher concentrations of glucosinolates in undamaged leaves relative to attacked leaves) suggests flexibility in the Zn-glucosinolate relationship.

The overall conclusion is in support of the joint effects hypothesis.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Heavy metals in soil

Transition elements with a specific gravity of >5 and an atomic mass of over 20 are toxic to both plants and animals even at very low concentrations and are often termed “heavy metals” (Rascio and Navari, 2011). Heavy metals form only 1% of the earth’s crust, the other 99% is constituted by major elements, Mg, Na, Ti, P, K, Fe, Al, Ca, O and Si (Alloway, 1995). Individual concentrations of these trace elements are always <0.1%. Heavy metals that are ecotoxic and of great concern to agriculture and human health include As, Pb, U, Hg, Se and Cd. These are also termed non-essential, because none of the known physiological functions requires their presence. Not all heavy metals are ecotoxic at all concentrations; some trace elements are essential for normal growth of fauna and flora and without the presence of these elements it is impossible for plants and animals to complete their life cycle. For example crops need Mn, Zn and Cu and livestock need Mn, Co, Cu and Zn for normal growth and productivity (Alloway, 1995). However, their supra-optimal concentrations may lead to the poisoning in both plants and animals (Rascio and Navari, 2011).

Heavy metal pollution and contamination are terms most often used interchangeably to discuss the heavy metal concentration in the environment. However, Holdgate (1979) defined pollution as “ the introduction by man into the environment of substances or energy, liable to cause hazards to human health, harm to living

resources and ecological systems, damage to structures or amenity, or interference with legitimate uses of the environment".

Studies of ecosystems have revealed that areas adjacent to mining activities, highways, urban and peri-urban sites have noticeably higher concentrations of non-essential heavy metals, especially Cd, Pb, Hg and As. Despite this, manufacturing of industrially important metals, mining and metal disposal remains a major cause of soil and ecosystem pollution. Soil pollution by heavy metals on a global scale is summarized in Table 1.1 which indicates the production of industrially important heavy metals and concentration reaching the soil.

Table 1.1: The worldwide changes in the production of heavy metals and concentrations reaching soil (10^3 tonnes yr^{-1}).

Metal	Year				Global emissions to soil
	1975	1980	1985	1990	1980s
Cd	15.2	18.2	19.1	20.2	22.0
Cu	6739.0	7204.0	7870.0	8814.0	954.0
Hg	8.7	6.8	6.1	5.8	8.3
Ni	723.8	658.2	687.3	836.9	325.0
Pb	3432.2	3448.2	3431.2	3367.2	796.0
Sn	232.2	247.3	180.7	219.3	----
Zn	3975.4	4030.3	4723.1	5570.9	1372.0

Source: World resources institute (world resources, 1992/93) Nriagu and Pacyna, (1988).

As is shown in Table 1.1, Cd production in the last 50 years has almost tripled from 6000 tonnes in 1950. This is because of its presence as a contaminant in many ores and dispersion from metalliferous industries and phosphatic fertilizers. Along with Cd other heavy metals like Cu and Zn have increased, but Hg and Pb production is gradually declining due to less mining activity resulting from an awareness of their hazardous effects in ecosystems. Huge amounts of all these metals are extracted

from ores each year and >50% of this is either recycled or dispersed in the environment causing toxicity in plants and animals (Alloway, 1995).

Although some trace elements in the ecosystem may result in pollution and toxicity, a few are essential for normal growth of plants and/or animals and reduced agricultural productivity in some parts of the world has been attributed to trace element deficiency. These elements/metalloids include Se, Cr and Co for animals and Mn, Zn and Cu for both plants and animals. For example, Alloway (1995) observed that deficiency of of Se and Zn has seriously affected human health in China and the USA respectively. Zn deficiency is a serious problem in rice crops, especially in south Asia.

1.1.1 Sources of metal contamination

The primary sources of heavy metal inputs to the soil body are the mineral rocks. Geochemical origin of heavy metals results from weathering of different rock minerals and total content of soil heavy metal depends on the composition of the parent material. For example, wurtzite and ZnS are the major sources of Zn release in soil (Lindsay, 1972). The lithosphere has been reported to contain ~80 mg kg^{-1} of Zn (Lindsay, 1972). Magmatic rocks have a uniform distribution of Zn but concentrations vary from 40-100 mg kg^{-1} in acidic and basaltic rocks respectively (Lindsay, 1991). Clayey sediments and shales have the highest Zn contents (80-120 mg kg^{-1}), while dolomites, sandstones and limestones have lower Zn contents (10-30 mg kg^{-1}) among the sedimentary rocks (Kebata-Pendias and Pendias, 1992). Cadmium is mostly extracted as a by-product of Zn smelting. Due to non-selective extraction and release of Cd, its geochemistry resembles Zn so major sources of Cd

in the soil are the same as those of Zn i.e. ZnS, secondary minerals and wurtzite. Secondary minerals like $ZnCO_3$ contain Cd concentrations of 0.2-0.4% (Alloway, 1995) while Rose *et al*. (1979) reported as high as 50% Cd in secondary minerals. Ni being the 24th most abundant element constitutes concentrations of approximately 75 mg Ni kg^{-1} rock in the earth's crust (McGrath, 1995). Important sources of Ni are Ni-silicates, especially garnierite $(Ni, Mg)_6 Si_4O_{10}(OH)_8$. Other minerals contributing Ni to the soil body are laterites resulting after prolonged weathering of ultrabasic rocks.

Although heavy metals are naturally present in the rock minerals (parent material), human activities including smelting and mining of ore bodies, agricultural inputs, use of fossil fuels and sewage sludge, metallurgical and electronics industries, waste disposal and warfare and military operations have also resulted in significant additions of trace elements to soil particularly in regions where intensive farming occurs. According to the MAFF (1993) soil contamination with Zn, Cd, and Pb results from impurities in fertilizers and by using waste-derived compost. The use of phosphatic fertilizers contributes 50-1450, 0.1- 170 and 7-38 mg kg^{-1} of Zn, Cd and Ni respectively to the soil. Similarly Cu, Zn and Pb contamination of soil is caused by the use of sewage sludge and pesticides. Heavy metals in soil may ultimately pass into the food chain in addition to causing yield losses, reduced microbial activity and decreased fertility (McGrath *et al*. 1995). According to Murtaza *et al*. (2008), these heavy metals may result in either direct hazardous effects to the human health or indirectly by leaching which contaminates both surface and underground water. Inputs of heavy metals from different sources contribute varying amounts of these toxic substances into the soil-plant systems and a huge range of concentrations of

these elements can cause toxicity in soils and to plants. Table 1.2 summarizes different concentration ranges for the most studied heavy metals in the ecosystem.

Table 1.2 Concentrations of heavy metals in soils and plants

	Soils		**Plants**		
Element	Normal range (total) in soil (µg g^{-1} d. wt) [1]	Conc. (toxic) (total) in soil (µg g^{-1} d. wt.) [2]	Normal range in plants (µg g^{-1} f. wt.) [3]	Conc. in contaminated plants (µg g^{-1}) [2]	Plant uptake (kg ha^{-1} yr^{-1}) [4]
Zn	10-300	70-400	8-400	100-400	0.01
Ni	10-1000	100	0.02-5	10-100	nd
Cd	0.01-7	3-8	0.2-0.8	5-30	nd
Cr	5-1000	75-1000	0.03-15	5-30	nd
Cu	2-100	60-125	4-15	20-100	0.006
Co	1-70	25-50	0.05-0.5	15-50	0.0006
Mn	200-2000	1500-3000	15-1000	300-500	1.0
Hg	0.02-0.2	0.3-5	0.005-0.5	1-3	nd
Pb	2-200	100-400	0.1-10	30-300	nd
Sn	<5	50	0.2-6.8	60	0.001

Source: [1]Swaine (1955); [2] Kabata-Pendias and Pendias (1984); [3]Allaway (1968); [4]Bohn *et al*. (1985)

As is evident from Table 1.2, Ni, Cr and Mn are present in highest concentrations in the soil while Hg and Cd are present in smallest concentrations. Weathering of parent material determines the amount of these elements released in soil in the form of complex inorganic ligands depending on the solubility of minerals and pH conditions.

Although parent material is the major source of heavy metals, certain trace elements are also derived from other sources (Ross, 1994); for example, Zn and Pb from atmospheric deposition and Cd from agricultural inputs. Two main anthropogenic sources of heavy metal inputs into soil are: 1) Burning of coal releasing 62.86%, 34.2%, 51.3% and 33.3% of annual inputs of Mn, Cd, Ni and Hg respectively; 2) corrosion of commercial waste which contributes 36.17%, 55.8%, 47% and 42% of

Zn, Cu, Cr and Pb respectively apart from 22.9% of Zn released from agricultural inputs and 20.5% of Cd coming from urban waste.

In addition to causing toxicity in soils and plants, heavy metals leach down into the soil and inhibit the microbial activity (McGrath *et al*. 1995). For example, Tyler (1989) reported that Cu and Zn aerosol accumulations in the vicinity of a brass foundry in Sweden inhibited plant nutrient recycling and conifer litter decomposition. This reduced microbial activity resulted in retarded tree growth due to a deficiency of macronutrients. Similar observations were made by Doelman and Haanstra (1979) who observed inhibitory effects of Pb on dehydrogenase activity and microbial respiration in contaminated soil. Despite the presence of tolerant populations of microbes in contaminated soil, the balance of soil biota is changed and might have an impact on soil fertility. About 50% less microbial biomass was observed by Brooks and McGrath (1984), in soils with a higher content of heavy metals than adjacent low metal concentration soils; both soils had been treated with sewage sludge.

Obbard and Jones, (1993) while working on nitrogen fixing Rhizobia bacteria observed reduced bacterial populations in Cd contaminated soils in the UK, but not in an area of the USA with the same heavy metal concentrations (Angle and Chaney, 1991). According to Chaudri *et al*. (1993), elevated concentrations of Zn in sludge and sludge-treated soil are more toxic to nitrogen fixing bacteria especially Rhizobia than any other heavy metal, although it is not very toxic to crops grown on sludge-treated soils. *Rhizobium leguminosum* is significantly affected at Zn concentrations even within the permissible Zn limits in the EC and UK (300 mg Kg^{-1}).

1.1.1.1 Scale of metal contamination in plants

Plants need a number of elements to complete their life cycle. These include heavy metals, which are also micronutrients (required in trace amounts), such as Zn, Ni, Fe and Cu (Broadley *et al*. 2001). Plants also accumulate non-essential heavy metals, such as Pb and Cd that are not needed for plant growth (Broadley *et al*. 2001; Thomine *et al*. 2002). However the occurrence of heavy metals in soil can be both beneficial and harmful to the plant. For example, Zn acts as a cofactor for several enzymes like oxidases, peroxidases, anhydrases and dehydrogenases (Hewitt, 1983) and regulates nitrogen metabolism, cell multiplication, photosynthesis and hormone (auxin) synthesis in plants (Shier, 1994). Zn also has several important key roles in protein and nucleic acid synthesis, and helps in the utilization of nitrogen and phosphorus during seed formation. On the other hand Zn may produce toxicity symptoms when present in high concentrations. When plants assimilate zinc at early developmental stages, it may be highly phytotoxic and inhibit growth (Collins, 1981). Specifically, Barcelo and Poschenrieder (1990) reported zinc-related root thickening, reduced cell division and cell elongation. Sresty and Madhava (1999) concluded that radicle elongation is more adversely affected than plumule extension with enhanced Zn concentrations. Stunting of shoots, curling and rolling of young leaves, leaf tip chlorosis and death are also common symptoms of Zn toxicity.

Metal toxicity in plants is mainly a function of metal movement from soil to root and subsequent absorption and translocation to the shoot. The metal toxicity resulting from increased Zn and Ni supply to the root of pigeon pea resulted in the disintegration of cell organelles, disruption of membranes, condensation of chromatin material and an increase in the number of nucleoli (Sresty and Madhava,

1999). Godbold and Hutterrmann (1985) observed that increasing zinc concentrations in culture solution decreased shoot to root ratios. As a result of this imbalanced equilibrium, translocation of P, Mg, Ca, Fe, Zn, and K is disturbed and results in the accumulation in the roots of *Picea abies* (Karst). According to Pearson and Rengel (1995), root and leaf morphology of wheat plants (*Triticum aestivum*) is adversely affected at higher concentrations of zinc. Malea *et al*. (1995) tested the lethal effect of increased Zn concentration on the leaf cells of *Halophila stipulacea* and observed that increased zinc concentration (10^{-7} – 10^{-4} M), lengthened the incubation time from 2 to 12 days and increased cell mortality.

Zinc also plays an important role in nuclear activity. Ernst (1998) compared cell division and nuclear activity in Zn resistant and Zn sensitive ecotypes of the perennial grass *Festuca rubra* at different Zn levels. He observed that on the 4^{th} day after exposure to 3 µM Zn, the length of the cell cycle was doubled and nuclear volume decreased by 30% in Zn sensitive ecotypes. However Powell *et al*. (1986) observed a 50% increase in nuclear volume at the same Zn level. Davis *et al*. (1995) reported disturbed mitochondrial structure and reduced energy production in *Festuca rubra* exposed to high concentrations of zinc. Ochi *et al*. (1983) and Michaelis *et al*. (1986) demonstrated that heavy metals like nickel, cadmium and zinc had genotoxic activity through oxidative pathways involving free radicals.

Cd is not involved in any known physiological function within the plant body, so unlike Zn it is regarded as a non-essential element and has been reported to cause deleterious effects on plants. For example, Cd resulted in stunted growth and chlorosis in plants, symptoms that resemble those induced by iron (Fe) deficiency.

Chlorosis caused by the presence of excess cadmium appears to result from direct or indirect interactions with iron as reported by Haghiri (1974), where excessive Cd content in the substrate suppressed iron uptake by plants. In some other studies, cadmium toxicity appeared to induce phosphorus deficiency or reduce manganese transport and interfere with uptake and transport of several essential nutrients (Ca, Mg, P and K) and water by plants (Godbold and Hutterrmann, 1985).

Although Ni is required by some plant species it is not really essential, unlike Zn, Cu, and Mg and its elevated concentrations have been reported to be toxic for plants (Seregin *et al*. 2006). For example, excess Ni in the environment lowers the uptake of Mg and Fe, because of the chlorosis caused by it (Piccini and Malavolta, 1992). Seregin *et al*. (2006) reported that excess Ni concentrations specifically affect the ionic balance in different plant organs. Barsukova and Gamzikova (1999) observed decreased contents of Zn, Mn and Fe in the leaves of *Triticum aestivum* and reduced Mn content in the roots of same plant due to excessive Ni concentrations.

At high Ni concentrations (0.1 to 1 mM), the concentration of macro- and micro-nutrients in plant tissues is usually lowered because of disordered absorption and transport (Pandolfini *et al*. 1992; Rubio *et al*. 1994). At the same time, at low environmental Ni concentrations (1 to 10 μM), Ca and Mg contents did not change and in some cases even increased (Piccini and Malavolta, 1992; Barsukova and Gamzikova, 1999). This phenomenon was described as the concentrating effect, which is the result of growth inhibition in plants grown in nutrient solutions with low Ni, while the rate of metal absorption stays the same as in the control plants; consequently, the contents of heavy metals increase per unit of dry matter.

Ni^{2+} decreases leaf area by ~40% in pigeon pea plants exposed to 1mM nutrient solution of $NiCl_2$ (Sheoran *et al*. 1990). Similarly, Molas, (1997) observed the diminishing effect of Ni on leaf area of *Brassica oleraceae* plants grown in agar at concentration of 5–20 g m^{-3} $NiSO_4$. Ni induced decreases in moisture content and stomatal conductance is one reason for reduced photosynthetic capacity (Molas, 1997). According to Sresty and Madhava, (1999); Seregin and Ivanov, (2001); and Seregin *et al*. (2006) inhibition of plant growth is the obvious result of heavy metal toxicity.

1.1.1.2 Scale of metal contamination in insects

Human activities including industrialization, mining and advancements in technology are slowly reducing the number of animal and plant species in nature (Ives and Cardinale, 2004; Florea and Busselberg, 2006). The most efficient route for metal entrance into the food chain is through plants and insect herbivores. Insects from polluted environments accumulate heavy metals (Zvereva *et al*. 2003; Boyd, 2007, 2009) and in some cases these heavy metals are present in higher concentrations in the insect body than in the host plant. For example, Crawford *et al*. (1996) and Merrington *et al*. (2001) observed that the phytophagous aphid (*Rhopalosiphium padi*) accumulated more heavy metals (Zn and Cd) than the host plants, wheat and beans.

Accumulated metals in insects may defend them against potential predation (Boyd, 1998; Boyd and Wall, 2001; Vickerman and Trumble, 2003), but in most cases negative impacts have been observed on the herbivore throughout their life cycle. For example, Culliney and Pimentel (1986) noted significantly reduced fecundity

and survival of aphids (*Myzus persicae*) when reared on *Brassica oleracea* plants growing on contaminated sewage sludge. Similarly, Gorur (2007) studied effects of Cu and Pb on life history traits of cabbage aphids by feeding them on metal-treated brassica plants (cabbage and radish) and noticed the high death rate and low fecundity compared to aphids on untreated controls. Heavy metals and metalloids, not only affect the rasping and phloem feeding invertebrates, but leaf chewer performance is equally affected by heavy metal toxicity, either by direct exposure or by feeding on metal accumulating plants (Heliovaara *et al*. 1989). According to Koricheva *et al*. (1998) decreased performance of leaf chewers is more likely to be a result of direct exposure to pollutants rather plant mediated effects.

In laboratory studies Boyd and Martens (1994) observed 75% larval mortality and no pupation in cabbage white butterfly caterpillars (*Pieris rapae*) when reared on an artificial diet containing 1000 µg g^{-1} Ni. When high-Ni leaves of the Ni hyperaccumulator *Thlaspi montanum* were presented to *Pieris rapae* caterpillars in no-choice experiments, 100% mortality was observed after 12 days. In contrast, 21% mortality occurred in caterpillars fed on low-Ni leaves of the same plant species. When *P. rapae* larvae were fed with leaves of the Ni hyperaccumulator *Streptanthus polygaloides* grown on Ni rich soil, they failed to pupate and died.

Apart from inducing mortality in invertebrates (Mitterbock and Fuhrer, 1988) sub-lethal effects include reduced growth (Warrington, 1987) and altered immunity (Galloway and Depledge, 2001; Rickwood and Galloway, 2004; Sorvari *et al*. 2007). Van Ooik *et al*. (2007) observed that increased Cu levels decreased the encapsulation rate in the moth *Epirrita autumnata* and when fed on Ni, the immune response of the

moth was reduced compared with moths reared in Ni-free conditions. Larval mortality was also significantly greater when Ni was included in an artificial diet. Sorvari *et al*. (2007) observed altered immune response in wild ant populations living in a contaminated area and similarly in aquatic invertebrates the immune system is temporarily up-regulated when exposed to low levels of metals, but higher concentrations inhibit the immune response (Van Ooik *et al*. 2007). Maintaining an up-regulated immune response in insects is metabolically expensive and is associated with reduced fitness.

1.2 *Thlaspi caerulescens*

Thlaspi caeruescens is commonly called Pennycress and is a member of the Cruciferae (Brassicaceae) family. The name Pennycress is derived from the two common characters of the plant i.e. 'Penny' because of the flat round fruit pods and 'cress' for the cross shaped flowers observed in all the crucifers. *T. caerulescens* is a biannual diploid with chromosome number 2n=14. *Thlaspi* species consist of a raceme character, with purple and white flowers. The fruit valves are usually white with two locules, each containing 1-8 seeds. The genome size of *Thlaspi* is almost double (0.7 pg) that of model plant *Arabidopsis thaliana* (0.34 pg) and ~88% of sequenced nucleotide is similar to that of *Arabidopsis* (Peer *et al*. 2003, 2006; Krämer, 2010). Divergence from the *Arabidopsis* lineage occurred ~20 million years ago (Clauss and Koch, 2006).

Al-Shehbaz (1986) described the genus *Thlaspi* as containing more than 80 species but Meyer (1973, 1979) split the genus into 12 segregated genera consisting of 104

species. These genera (clades) including *Raparia, Noccaea, Thlaspiceras, Microthlaspi, Neurotropsis, Vania* and *Thlaspi* and were classified based on seed coat morphology and seed characteristics. *Thlaspi caerulescens* J. and C. Presl. belongs to the *Noccaea* clade and is distributed in the temperate and subarctic regions of North and South America (Koch *et al*. 1998) and Europe, stretching from the Czech Republic to the Rhine river and southern France (Ganges ecotype) and Belgium (Prayon ecotype) (Meyer, 1979). *Thlaspi* is assumed to be distributed by anthropogenic activities in Scandinavian countries (Meyer, 1979; Elven and Fremstad, 1996). In Britain it appears in patches and is classified as an absolute metallophyte (Baker and Proctor, 1990). It grows on metalliferous soils across the country and several species, i.e. *occitanicum* Jordan, *virens* Jordan, and *sylvestre* Jordan have been recognised from Yorkshire, Derbyshire and Teesdale respectively (Jordan, 1846).

Reeves and Brooks (1983) and Reeves (1988) described all species belonging to the *Noccaea* clade as accumulating high concentrations of heavy metals in their tissue but especially Zn, Ni and Cd. *T. caerulescens* is of great interest and is recognised as a model hyperaccumulator (Baker *et al*. 1994; Brown *et al*. 1994, 1995; Vasquez *et al*. 1994; Huang and Cunningham, 1996; Lasat *et al*. 1996).

Brassicales (*Alyssum, Arabidopsis* and *Thlaspi* etc.) are well known for their ability to hyperaccumulate and detoxify heavy metals (Baker and Brooks, 1989; Salt *et al*. 1995; Sanità di Toppi *et al*. 2001; Bert *et al*. 2003). Storing heavy metals in the plant body without showing any toxicity symptoms is an extraordinary phenomenon, where per cent quantities of metals are stored in the above ground parts rather than

roots (Brooks, 1987; Rascio and Navari, 2011). Hyperaccumulators usually contain two to three orders of magnitude (of dry mass) of inorganic element more than "normal" plants (Boyd, 2010). For example Robinson *et al*. (1998) observed >0.01% Cd and >3% Zn in the shoots of *T. caerulescens* on a dry weight basis and according to Baker *et al*. (1994) wild populations of *T. caerulescens* in Britain have been reported to accumulate 164 µg g^{-1} and 21000 µg g^{-1} of Cd and Zn respectively on a dry weight basis. Generally, the threshold value that differentiates hyperaccumulators is approximately ten fold higher than the level found in non-accumulating (normal plants) plants growing in the same habitat (Bert *et al*. 2002).

Plants that acquire concentrations of 10,000 µg g^{-1} for Mn and Zn, 100 µg g^{-1} for Cd and 1000 µg g^{-1} for As, Cu, Co and Ni are considered to be hyperaccumulators (Reeves and Baker, 2000; Boyd, 2004). Hyperaccumulation potential of the plant also depends on soil metal concentrations, i.e. if soil is low in metal concentrations then it is very unlikely for hyperaccumulators to reach these accumulation thresholds. Out of ~500 known species of hyperaccumulators, 25% belong to the Brassicaceae family (Krämer, 2010), the majority (75%) of which are nickel hyperaccumulators (Brooks, 1987; Reeves & Baker 2000). Zn hyperaccumulators are far less common than nickel hyperaccumulators (Brooks, 1998) and according to Macnair (2003) only 11 species from the Brassicaceae are documented as Zn accumulators, while 5 species has been recognised as Cd hyperaccumulators (Sun and Zhou, 2006; Rascio and Navari, 2011).

Hyperaccumulation of heavy metals by *Thlaspi* species has attracted much attention during the last decades (Freeman *et al*. 2004; Papoyan and Kochian, 2004;

Hammond *et al*. 2006), due to the potential of this plant for remediating heavy metal contaminated soils (Chaney *et al*. 2005; McGrath *et al*. 2006). The majority of hyperaccumulators grow in serpentine soils (Proctor, 1999) and have been reported across continents except Antarctica (Boyd, 2010).

1.3 Plant herbivory and defence mechanisms

Plants and insect herbivores in an ecosystem have very complex interactions which affect the life cycle of each. Survival depends on their ability to circumvent or counter the adaptations of the other. Plants are always exposed to a variety of attackers including pathogens, invertebrate and vertebrate herbivores that threaten their survival and fitness. To overcome these attacks and to safeguarding themselves, plants have developed a plethora of defence mechanisms ranging from mechanical (e.g. spines and armour coating), visual (crypsis), and chemical defences (Mello and Silva-Fillho, 2002; Boyd, 2010). Chemical defences are the most studied and range from constitutive to induced defences in response to attack (Takabayashi and Dicke, 1996; Karban and Baldwin, 1997; Pare and Tumlinson, 1999; Dicke and Hilker, 2003; Boyd 2010). Other defence tactics include direct and indirect defences such as induction of secondary metabolites, production of volatiles, synthesis of defence proteins and trichomes to reduce the insect attack as observed by Ahuja *et al*. (2010). These defence compounds defend the plants either through direct toxicity or by hindering digestion of the tissue after being ingested.

Functioning of these defences can be individual, additive or synergistic and antagonistic; however, synergistic effects are more effective than individual defence

(Boyd, 2010). These defence compounds (organic) are naturally present in the plant but are also induced when plants are attacked. Inducible defences can formulate the defensive strategy of the plant (Agrawal *et al*. 1999), i.e. the nature of attack by herbivores, parasites and predators can give an indication of future attack. Once the plant tissue is damaged by herbivores, defences are induced and reach the climax within a certain time period and according to Haukioja (1999) induced defences are crucial when the attack is unpredictable.

Plant responses that affect herbivores by inducing toxic metabolites are categorized as direct defences while others such as secretion of volatiles (Hilker and Meiners, 2002; Kessler and Baldwin, 2002; Dicke *et al*. 2003; Rohlof and Bones, 2005) that attract herbivore predators are called indirect defences (Mattiacci *et al*. 2001). Release of volatiles is considered as a "call for help" from predators to reduce the pressure of herbivores. Volatiles released from the damaged plant may be synthesized by any of three biosynthetic pathways (Hilker and Meiners, 2002), namely, the fatty acid, the shikimic acid and isoprenoid pathways that produce volatiles from (Z)- jasmine, indole and methyl salicylate and terpenes respectively. According to Heil, (2008) further information is needed to establish the ecological and evolutionary relationships related to indirect plant defences.

Brassica plants naturally contain a group of non-volatile secondary metabolites called glucosinolates (Fahey *et al*. 2001). They occur in almost all brassica plants of economic value (Tripathi and Mishra, 2007). The amount of glucosinolate varies with species, depending on the cultivar, environmental factors and agronomic practices (Clossais- Bernard and Larher, 1991; Rangkadilok *et al*. 2002; Font *et al*.

2005; Tripathi and Mishra, 2007). These non-volatile defence compounds mediate relationships between plant and herbivore and according to Zukalova and Vasak, (2002) these compounds have been recognised as natural pesticides due to their toxicity and repellent effects towards pests and diseases.

Although intact glucosinolates in the plant body deter insect attack (Kim and Jander, 2007), their defence capabilities are enhanced after the plant tissue is damaged. Once the tissue is damaged, thioglucosidase (myrosinase) enzyme comes in contact with the glucosinolates to form hydrolysis products. As a result of glucosinolate hydrolysis, apart from the release of sulphate and glucose, toxic products like thiocyanates, isothiocyanates, oxazolidinethiones and nitriles are produced (Wittstock and Halkier, 2002; Bones and Rossiter, 2006). Among these hydrolysis products isothiocyanate is known to be the most toxic compound whilst the others are relatively less toxic. According to Burow *et al*. (2006) the hydrolysis products formed depend on the amino acid side chain apart from pH and ferrous ion concentrations.

Apart from the plant species the concentration of induced glucosinolates depends on the type of damage and insect species and herbivore feeding mode (Koristas *et al*. 1991; Bodnaryk, 1992; Hopkins *et al*. 1998). The induction of glucosinolates as a result of herbivore feeding can be detrimental to both generalist herbivores and specialists and according to several authors glucosinolates can be effective both as stimulants (specialists) and deterrents (generalists) (Li *et al*. 2000; Agrawal and Kurashige, 2003; Kusnierczyk *et al*. 2007, 2008; Gols *et al*. 2008).

1.3.1 Importance of plant herbivory for driving plant communities

Evolution of defence by individual plants affects plant community structure; both result from herbivore pressure (Martin and Baltzinger, 2002; Trembley *et al*. 2007). A huge number of plant species invest resources in producing defence metabolites and these resources are diverted away from growth. According to Herms and Mattson (1992) and Strauss *et al*. (2002), if herbivores are absent the ability of a plant to compete with neighbours is reduced. This is because the allelopathic effect of induced chemicals is not present. In the absence of insect herbivores not only growth, but also development is negatively affected. For example, Vourch *et al*. (2002) observed a slower growth rate due to higher concentrations of secondary metabolites and monoterpenes in the leaves of *Thuja plicata*. Similar conclusions were made by Baldwin (1998) for *Nicotiana attenuata* where reduced seed production was attributed to increased nicotine. Like folivores, rhizovores also play a key role in driving the plant communities and are important in the food web (Williams *et al*. 1993; Blossey and Hunt, 2003). Their role varies from potentially positive to negative as observed by Muller and Steinger (1990) in *Centaurea maculosa* plants attacked by *Agapeta zoegana*. Attacked plants produced more flowers than the control plants. Kentucky blue grass produced more biomass after grubs attack (Crutchfield and Potter, 1995). The only drawback of herbivory is that a critical threshold for the positive influence on plants communities has not been determined yet (Hunt *et al*.1992; Crutchfield and Potter, 1995; Cormier and Martel, 1997). Although low levels of herbivory have little effect on plant performance, higher levels can result in collapse and failure of the entire plant population (Strong *et al*. 1995).

Herbivores can affect the plant community in many ways like hormone synthesis, carbohydrate storage and production of plant secondary metabolites (Harris *et al*. 1994; Larcher, 1995). For example, herbivores with a chewing mode of feeding can damage the plant organs involved in nutrient adsorption, water or carbohydrate storage belowground and affect the vascular connections between roots and shoots causing disruption in nutrient flow to aerial parts. Murray and Clements (1998) noticed that *Medicago sativa* plants became dormant and water stressed after attack by the root feeder *Sitona discoideus* and *Capsella bursa-pastoris* showed water deficiency symptoms as a result of feeding by scarab beetles, even though the plants were well watered (Gange and Brown, 1989). Herbivores with a mining habit of feeding have different effects on plant populations. For example larvae of *Hylobius transversovittatus* mining in the rootstock of *Lythrum salicaria* did not result in any symptoms of drought in the attacked plants, but led to enhanced carbon assimilation in the leaves (Hunt, 2002), perhaps because of improved changes in source-sink ratio in the plant body. Tissue damaging herbivores can enhance the source-sink ratio and may increase translocation of carbohydrates to the sink and in return slow down photosynthesis. However, Neales and Incoll (1968) reported enhanced photosynthesis due to increased translocation of carbohydrates from leaves.

Brassica plants naturally produce secondary metabolites (glucosinolates), and other defence compounds. Belowground parts often contain higher concentrations than shoots in order to deter root herbivore attack (Hara *et al*. 2000), but these defence compounds are also induced as a result of herbivory (Karban and Baldwin, 1997). For example corn root-worm damage resulted in increased concentration of hydroxamic acid, which resulted in the plant producing more biomass than control

plants (Assabgui *et al*. 1995). Schmelz *et al*. (1998) observed 30% increase of 20-hydroxyecdysone in spinach plants after root damage by vine weevils. Hydroxyecdysone restricts herbivore feeding and also hinders insect development (Champs, 1991; Tanaka *et al*. 1994).

Research so far has focussed only on interactions between host and herbivore, ignoring other biological interactions in the community (Law and Dieckmann, 1998; Dillon and Dillon, 2004). Symbionts affect the phenotype of their host and ultimately affect the interactions with other species. For example according to Moore (2002) some symbiotic species enhance their host susceptibility to predators while some protect the host; e.g. locust gut bacteria defend the host from fungal infection by producing phenolic compounds (Dillon and Dillon, 2004). Similar observations were recorded by Currie *et al*. (2003); Actomycetes protect leaf-cutting ants from pathogen attack. Mycorrhizal and endophytic fungi provide a defence to their hosts from pathogens and herbivorous insects (Gange and West, 1994; Gehring and Whitham, 2002; Arnold *et al*. 2003). Herbivores have also been used as biological agents in weed control programmes to decrease competition between host plant population and weeds and enhance the development of plant community (Blossey and Hunt-Joshi, 2003).

1.3.2 Carbon:Nutrient Balance (CNB) Hypothesis

Carbon, nitrogen, trace elements and defensive metabolites constitute the food quality of the host plant. This idea led to the formation of **Carbon-Nutrient Balance (CNB)** hypothesis by Bryant *et al*. (1983) stating that "the relative availability of C and N in the environment influences production of C-based plant defence

compounds (e.g. iridoid glycosides)". The CNB hypothesis is actually a new version of the "overflow metabolism" hypothesis that postulates that secondary metabolites are produced to reduce abnormal concentrations of cellular constituents (Haslam, 1986; Berenbaum, 1995). For example when plant growth is limited due to reduced nitrogen, the CNB hypothesis explains that carbohydrates will accumulate in plant tissues. As a result of this increased accumulation of carbohydrates in the plant tissue, carbon-based secondary metabolites (terpenes and phenolics) are synthesized to the maximum concentration.

The logical extension of the CNB hypothesis is that if C-based defence compounds are dependent on C/N availability in the medium, then N-based defence compounds (e.g. glucosinolates) will be affected by foliar C/N availability as well. For example when light is limited, all available carbohydrates will be diverted to growth resulting in reduced concentrations of carbohydrates and ultimately lower the C/N ratio in the plant tissue. This unbalanced C/N ratio will result in decreased synthesis of carbon-based secondary metabolites. Because of increased availability of nitrogen, synthesis of nitrogen-containing secondary compounds such as alkaloids, cyanogenic glycosides and glucosinolates will be enhanced. Production of C-based or N-based defence compounds depends on the plant species involved.

Host plant quality influences the plant-insect relationship during their entire life cycle. According to Awmack and Leather (2002) selection of oviposition sites and quality and size of the egg are influenced by host plant quality. They also postulated that interactions between higher-trophic levels (predators and parasitoids) might also depend on the host plant quality. Nitrogen is a vital component in determining food

quality and has a direct influence on the fecundity of herbivorous insects. The phloem feeding sycamore aphid (*Drepanosiphum platanoidis*) only feeds on sycamore plants (*Acer pseudoplatanus*). Dixon (1970) observed that sycamore aphids are highly reproductive and larger in size at the start of growing season because the amino acid content is high in the phloem sap. With the maturity of the plant, aphid reproduction ceases due to a reduction in the proteinacious content of the sap. Different herbivores respond differently to changes in the availability of nitrogen in the diet. Strauss (1987) observed an increase in phloem-feeding and seed herbivores, but no effect was observed on leaf chewing insect herbivores as a result of nitrogen fertilization to *Artemisia ludoyiciana*. Similarly the increased supply of nitrogen to barley and wheat plants resulted in the increased performance of *Metopolophium dirhidum* (leaf feeding aphid) but did not affect the ear-feeding aphid species (Honek, 1991).

Initially the CNB hypothesis provided the basis to understand the variations in the production of phenolic compounds (Bryant, 1983; Koricheva *et al*. 1998) depending on the availability of carbon and nitrogen. Later on, the extension of this hypothesis resulted in the formation of the 'Protein Competition Model' (PCM), which predicts that under reduced concentrations of nutrients, growth protein demand will be decreased. As a result phenolic synthesis will increase the phenylpropenoids concentration (Jones and Hartley, 1999).

Production of secondary metabolites is also affected by environmental factors (Herms and Mattson, 1992; Koricheva *et al*. 1998) linked with the genetic makeup of the plant, but the CNB hypothesis nullifies such interactions. Apart from

environment-genotype interactions, environment might have direct effects on gene expression for suppression or induction of defence compounds. Combined study of the environment and genetics (Karban, 1992) can resolve the criticisms of the CNB hypothesis. Hamilton *et al*. (2001) observed that even if the production of defence compounds is not heritable, these compounds still have implications on plant fitness and herbivores. Impacts of environmental factors on defence compound production do not affect the behavior of insect herbivores in a predictable manner (McDonald *et al*. 1999; Hemming and Lindroth, 1999; Hunter and Forkner, 1999; Mutikainen et al. 2000).

1.3.3 Plant chemical defences

Plants possess a wide array of organic chemical defences such as terpenes, tannins, alkaloids and glucosinolates (Fraenkel, 1959; Whittaker and Feeny, 1971; Kessler and Baldwin, 2002). The contents of many organic plant defences fluctuate in response to plant age (Coley and Aide, 1991), resource availability (Coley *et al*. 1985; Bryant *et al*. 1987) and herbivore damage (Karban and Baldwin, 1997).

Herbivores can greatly influence the amount of specific organic defences within plants (Karban and Baldwin, 1997). Variation in the degree to which plant defences respond to herbivory has led to the classification of plant defences as either constitutive or inducible (Howe and Westley, 1988). Large compounds (e.g. tannins), which are metabolically expensive to synthesize are considered to be constitutive defences. Relatively small compounds that are synthesized or activated in direct response to herbivore damage (e.g. glucosinolates and cyanogenic glycosides) are categorized as inducible defences. With the exception of the work by

McNaughton and Tarrants (1983), no studies have attempted to place elemental plant defences into either category. These researchers showed that silica content of several African savanna grass species increased after exposure to simulated herbivory treatments; thus classifying silicification as an inducible defence.

Brassica plants naturally possess a wide range of chemical defences which defend the plants in one way or the other. The Glucosinolates myrosinase defence system has been studied most extensively in the last two decades. Apart from the glucosinolate-myrosinase system, plants also secrete volatiles and according to Baldwin *et al*. (2002) and Kishimoto *et al*. (2005) each volatile released has specific biological functions relevant to plant-pathogen, plant-insect, plant-stress or plant-to-plant interactions. Phytoanticipins and phytoalexins are another group of chemicals defending brassica plants from herbivores and diseases, particularly phytoanticipins act as infection inhibitors (Rouxel *et al*. 1991; Dixon, 2001). Dixon (2001) also reported that phytoalexins and phytoanticipins behave in different ways in different species, i.e. phytoalexins in one species might be phytoanticipins in other species. Like glucosinolates phytoalexins and phytoanticipins are also considered as natural pesticides with different modes of action (Zukalova and Vasak, 2002).

Sulphur is an integral part of nearly all the brassica defence compounds (Williams and Cooper, 2004) and all sulphur containing chemicals like glucosinolates, phytoalexins and phytoanticipins, antifungal proteins protect the plants against pathogens and insect herbivores (Dubuis, 2004). Antimicrobial activity of elemental sulphur has been reported by Cooper *et al*. (1996) in *Theobroma cacao* and

inorganic sulphur compounds like cyclooctasulphur have been reported in brassicas by Rohloff and Bones (2005).

1.3.4 Glucosinolates

Glucosinolates (also called mustard oils) are nitrogen and sulphur containing natural plant secondary metabolites found mainly in the order Brassicales, which includes agriculturally important crop plants of the Brassicaceae family such as oilseed rape (*Brassica napus*), fodder and vegetable crops, the model hyperaccumulator *Thlaspi caerulescens* and the model plant *Arabidopsis thaliana* (Halkier, 1999).

The first observation on the properties of glucosinolates or mustard oils, dates back to the17th century while investigating the sharp taste of mustard seed oil. Challenger (1959) reviewed the discovery and early history of glucosinolates including the role of the enzyme myrosinase in the hydrolysis of glucosinolates to isothiocyanates and other hydrolysis products such as epithionitriles and nitriles. According to Fahey *et al*. (2001) the glucosinolates such as sinalbin (4-hydroxybenzyl glucosinolate) and sinigrin (2-propenyl or allyl glucosinolate) were perhaps the first isolated compounds as early as 1830 from white (*Sinapis alba*) and black (*Brassica nigra*) mustard seeds, respectively (Fig. 1.2, Marsh and Waser, 1970). The parent structure of glucosinolates consists of a ß-D glucose residue which is linked to N-hydroximinosulfate ester *via* a sulphur atom and a variable side chain of one of the 8 amino acids (Halkier and Gershenzon 2006).

Gadamer (1897) proposed the first general structure for glucosinolates in which the side chain was linked to the nitrogen rather than carbon (Fig.1.1).

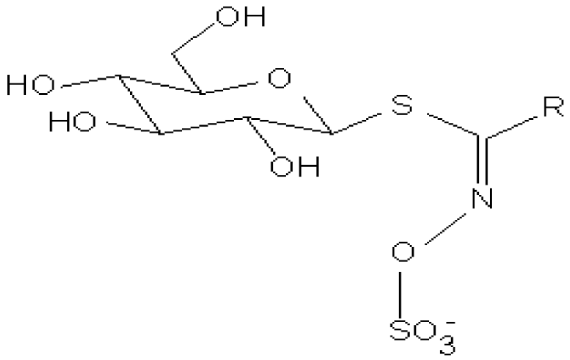
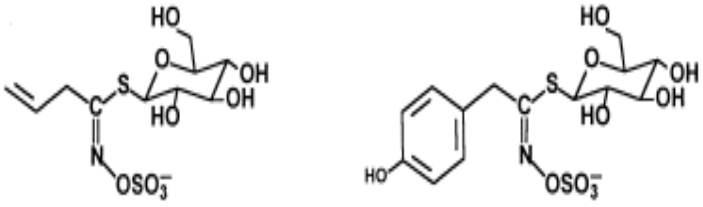


Fig. 1.1: The initial structure of glucosinolates proposed in the 19th century, in which the side chain was linked to nitrogen rather than carbon. Source: (http://boneslab.chembio.ntnu.no/Paal/glucosin.htm)

This structure was assumed to be correct until 1956, when Ettlinger and Lundeen (1956, 1957) while working on the chemical properties and origin of glucosinolates pointed out and suggested the "now correct structure" in which the side chain was linked to carbon rather than nitrogen. The first chemical synthesis of glucosinolates was also described by Ettlinger and Lundeen (1957).



(a) **(b)**

Fig. 1.2: The structure of (**a**) Sinigrin (2- propenyl glucosinolate) and (**b**) Sinalbin (4-hydroxybenzyl glucosinolate), originally isolated in the 1830s from *Brasssica nigra* and *Sinapis alba* respectively (Marsh and Waser, 1970).

On the basis of structural similarities, Fahey *et al*. (2001) grouped glucosinolates into three classes based on side chain amino acids (Table 1.2). According to these

researchers aliphatic glucosinolates are most obvious in all brassicas constituting about 50% of the known structures and are derived from methionine. Indolic and aromatic glucosinolates are derived from tryptophan and phenylalanine precursors of amino acids respectively and each group formulates 10% of the known structures (Fahey *et al*. 2001; Mithen, 2001). The biosynthetic origin of the other 30% of known structures of glucosinolates is not yet known. The most extensively studied glucosinolates in brassicas are listed in Table 1.3.

Glucosinolate content is approximately 1-10 % (of dry weight) in some tissues of the Brassicaceae (Kushad *et al*. 1984; Rosa *et al*. 1997; Farnham *et al*. 2000). Most species contain a limited number of glucosinolates (generally fewer than 12) although as many as 23 different glucosinolates have been identified in *Arabidopsis thaliana* (Hogge *et al*. 1988; Haughn *et al*. 1991). Distribution of the glucosinolates varies between plant organs, with both quantitative and qualitative differences between roots, leaves, stems and seeds. For example, Fahey *et al*. (1997) observed 70-100 mmol total glucosinolates g^{-1} f. wt. in the young sprouts of broccoli (*Brassica oleraceae* var. *italica*) out of which >99% were aliphatic (glucoraphanin, glucoerucin and glucoiberin) and the remaining ~1% were indole glucosinolates. In contrast, aliphatic and indole glucosinolates were present in a similar ratio in the late vegetative to reproductive stage plants of the same cultivar (Fahey *et al*. 1997; Fahey and Stephenson, 1999) and overall concentrations were much lower. Plant age is therefore a major determinant of the quantitative or qualitative glucosinolate composition of plants. Environmental factors such as soil fertility (Booth and Walker, 1992; Fahey and Stephenson, 1999), pathogen challenge (Butcher *et al*. 1974), wounding (Bodnaryk, 1992) or plant growth regulators (Bodnaryk, 1994;

Bodnaryk and Yoshihara, 1995) also have significant effects on level and distribution of glucosinolates between plant organs.

Glucosinolate hydrolysis is catalysed by the enzyme thioglucosidase (myrosinase EC 3.2.1.147) to release sulphate and glucose, resulting in the formation of an isothiocyanate, thiocyanate, amine, epithionitriles, nitriles (Chew, 1988; McGregor, 1988). Bussy (1840) first reported the enzyme myrosinase while studying *Brassica nigra* glucosinolates. It is most likely that plants containing glucosinolates also have the glucosinolate-degrading enzyme myrosinase. Myrosinase has also been reported in some bacteria, fungi and insects (Rask *et al*. 2000).

Table 1.3 Chemical and common names of glucosinolates identified in higher plants.

Chemical names	Common names
3-Butenyl	Gluconapin
4-Hydroxybenzyl∗	[Gluco] sinalbin
2(R)-2-Hydroxy-3-butenyl	Progoitrin
2(S)-2-Hydroxy-3-butenyl	Epiprogoitrin
2-Hydroxy-4-pentenyl	[Gluco] napoleiferin
Indol-3-ylmethyl	Glucobrassicin
1-Methoxyindol-3-ylmethyl	Neoglucobrassicin
1-Methylethyl	Isopropyl
1-Methylpropyl	Glucococchlearin
4-Methylsulfinyl-3-butenyl	Glucoraphenin
4-(Methylsulfinyl) butyl	Glucoraphanin
5-(Methylsulfinyl) pentyl	Glucoalyssin
3-(Methylsulfinyl) propyl	Glucoiberin
4-(Methylthio) butyl	Glucoerucin
5-(Methylthio) pentyl	Glucoberteroin
3-(Methylthio) propyl	Glucoiberverin
1-Pentenyl	Glucobrassicanapin
4-Pentenyl	Glucobrassicanapin
2-Phenylethyl	Phenethyl
2-Propenyl∗	Allyl, Sinigrin

∗: The first identified glucosinolates in *Sinapis alba* and *Brasssica nigra* (Marsh and Waser, 1970). Source; Fahey *et al*. (2001).

1.3.4.1 Biosynthesis

From the plant defence perspective against herbivores, glucosinolates have been most repeatedly discussed due to several reasons. First, they are derived from low molecular weight amino acids and have a high sulphur content. The basic structure of these metabolites consists of a glucose molecule, a sulphur atom and variable side chain of amino acids (Halkier and Gershenzon, 2006). Second, all 120 glucosinolates discovered so far (Fahey *et al*. 2001) are almost completely confined to the Brassicaceae, Caparaceae and 14 other families of the order Brassicales. Third, intact glucosinolates have a very limited biological role, but upon tissue damage, they are acted upon by the enzyme myrosinase to hydrolyse them into isothiocyanates, nitriles, thiocyanates and epithionitriles (Bones and Rossiter 1996). These products are responsible for the deterrence of herbivores, the taste of cruciferous vegetables and anti-cancer activities (Halkier and Gershenzon, 2006) associated with brassicas.

During the past two decades, extensive research has been carried out on the model plant *Arabidopsis,* to understand the mechanism of glucosinolate biosynthesis. According to Wittstock and Halkier, (2002) use of techniques like reverse genetics, functional genomics, mapping of genetic loci to determine glucosinolate profiles, and mutant screens have not only complemented biochemical investigations but also have been the driving force for the rapid progress in the field.

The biosynthesis of glucosinolates comprises three steps. First, some amino acids (e.g. tryptophan for indolic; alanine, methionine, valine, leucine or isoleucine for aliphatic; phenyl alanine or tyrosine for aromatic glucosinolates) are elongated by one or multiple methylene groups. In the second step, the precursor amino acids are

converted into the parent glucosinolates and finally, the parent glucosinolates undergo secondary modifications. This observation is based on *in vivo* studies (Graser *et al*. 2001*)*. The identification of *Arabidopsis* genes involved in biosynthetic steps of all three stages and the characterization of the relevant enzymes confirmed this concept (Wittstock and Halkier, 2002). Subsequent studies with *Arabidopsis thaliana* and *Brassica napus* have examined genetic variants in side chain length. This has led to the mapping of a number of 'Gsl-elong' loci, allelic variation responsible for determining the length of the glucosinolate side chain.

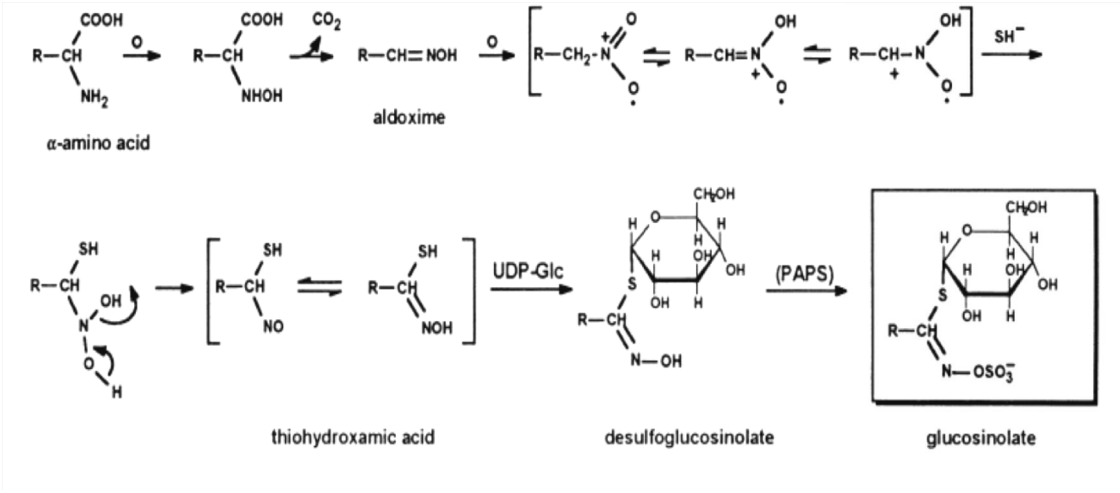
According to Halkier and Du, (1997), Halkier, (1999) and Mithen *et al*. (2000) glycone biosynthesis is initiated by the conversion of protein amino acids or chain elongated amino acids (e.g. homomethionine) to aldoximes. Involvement of cytochrome P450 in the conversion of amino acids to aldoximes has emerged recently (Fahey *et al*. 2001). The process is also performed by cyanogenic glycoside-producing plants (Hull *et al*. 2000; Wittstock and Halkier, 2000). After aldoxime formation biosynthetic steps are the conversion to a thiohydroximic acid and addition of cystein derived thioglucoside sulphur.

No biochemical evidence for the proposed intermediates between aldoxime formation and thiohydroximic acid involved in these steps has been attained (Fahey *et al*. 2001). Thiohydroximic acid is S-glycosylated by the soluble enzyme uridine diphosphate glucose (UDPG): thiohydroximate glucosyltransferase resulting in the formation of desulfoglucosinolate. This enzyme has been purified from *Brassica juncea, Brassica oleracea, Brassica napus* and *Arabidopsis thaliana* (Jain *et al*. 1990; Reed *et al*. 1993; GrootWassink *et al*. 1994; Guo and Poulton, 1994). Among

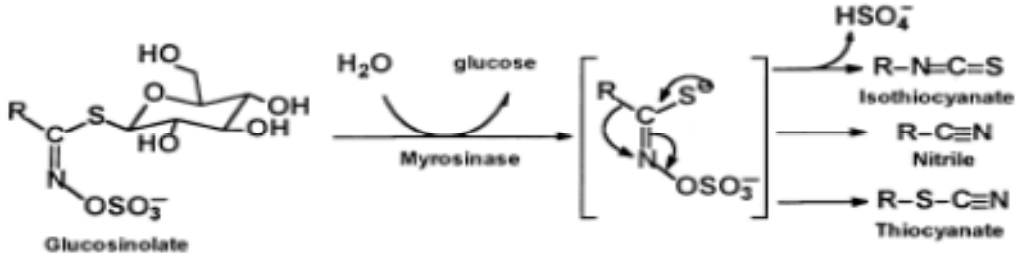
these, *B. oleraceae* and the *B. napus* enzyme had very high substrate-specificity for thiohydroximate, but had very low specificity for the side chain structure. Desulfoglucosinolates are sulphated to form glycine, catalysed by the soluble enzyme 30-phosphoadenosine 50-phosphfghhosulfate (PAPS); desulfoglucosinolate sulfotransferase. This enzyme has also been purified and characterized but it is extremely unstable with highly variable and substrate specific (Glendening and Poulton, 1988; Jain *et al*. 1990).

Very little research work has been carried out to explore the mechanism of side chain modifications of glucosinolates. Initial oxidation of the side chain sulphur of methionine and its chain-elongated homologues is expected to give rise to the large family of methylsulfinyl- and methylsulfonyl-glucosinolates. Mithen *et al*. (1995) and Mithen and Campos (1996) proposed models for side chain modification of aliphatic and alkylthioalkyl glucosinolates based upon allelic variation at three loci: Gsl-oxid, Gsl-alk and Gsl-oh, resulting in oxidation of the methylthio group, desaturation of alkyl to alkenyl side chains and hydroxylation of alkenyl groups respectively (Parkin *et al*. 1994; Giamoustaris and Mithen, 1996).

Since indolic and aromatic glucosinolates exhibit the same structural diversity as the aliphatic glucosinolates then presumably similar oxidations, hydroxylations and desaturation occur on their branched chains. It has been suggested that benzyloxyalkyl glucosinolates arise from the conjugation of a hydroxylalkyl glucosinolate with benzoic acid (Mithen *et al*. 2000).



(a)



(b)

Fig. 1.3: Biosynthesis of glucosinolates. **(a)** Glucosinolate production from an α-amino acid. **(b)** Hydrolysis of glucosinolate by myrosinase. **Source**: Fahey *et al*. (2001).

1.3.4.2 Herbivore- induced production

Glucosinolates are present constitutively in members of the Brassicaceae (Wittstock and Halkier, 2002) but are also induced as a result of herbivore feeding. Herbivores from different feeding guilds and feeding modes alter the level of induced glucosinolates. The same glucosinolates which act as deterrents for generalist herbivores behave as attractants for specialist feeders. For example Traw and Dawson (2002) observed no change in foliar glucosinolates in black mustard 12 hours after feeding by the specialist *Pieris rapae* with a chewing habit, but Travers and Muller (2007) recorded enhanced indole and aromatic glucosinolates 24 hours after feeding by the generalist leaf chewer *Spodoptera exigua.* Glucosinolate

induction triggered by herbivores can be site specific, systemic, or it can occur throughout the plant body. According to Chew (1988) as a result of herbivore attack, the quantity of at least one class of glucosinolates increases which reduces the further attack by herbivore insect and formulates the defence strategy of the plant.

Herbivore induced production of glucosinolates can be spontaneous occurring within days, or long lasting (Hopkins *et al*. 2009), and significant differences between herbivore damaged and undamaged plants can be observed from the start of damage to eight weeks later. As a result of this long lasting induction of glucosinolates Poelman *et al*. (2008) and Bartlet *et al*. (1999) postulated that early season insect herbivores may affect late season herbivores by altering food quality. Moreover, due to the systemic induction of defence metabolites, root herbivores may alter the quantity of glucosinolates in the shoot. For example, Bezemer and van Dam (2005) observed reduced performance of shoot feeding specialist *P*. *rapae*, due to enhanced glucosinolate level as a result of root herbivory by the endoparasitic nematode (*Pratylenchus penetrans* Cobb).

1.3.4.3 Glucosinolates in Thlaspi caerulescens

In *Thlaspi caerulescens* 5 glucosinolates have been discovered, of which one is aliphatic, two are aromatic and two are indolic glucosinolates (Tolra *et al*. 2001) (Table 1.4). Different glucosinolates respond differently to pathogen or herbivore attack and defence against specialist insects depends on the concentration of individual glucosinolates (Wallsgrove *et al*. 1999). So far there is no evidence about the fate of glucosinolates in hyperaccumulator plants, but according to Tolrà *et al*. (2001) changes in glucosinolates in the Zn hyperaccumulator *Thlaspi caerulescens*

can have detrimental effects on defence. Doughty *et al*. (1991) observed enhanced concentrations of indolic and aromatic glucosinolates and decreased levels of aliphatic glucosinolates in hyperaccumulating *Brassica napus*. Similar findings were recorded by Tolra *et al*. (2001) in Zn treated *Thlaspi caerulescens* with decreased concentrations of indolyl and benzyl glucosinolate in the shoots.

Table 1.4 Glucosinolates discovered in *Thlaspi caerulescens*

Trivial name	Enzyme class	Systematic name	Plant organ
Sinalbin	Aromatic	p-hydroxybenzyl	Whole plant
Gluconasturtin	Aromatic	2-phenylethyl	Shoot
4-MeO-Glucobrassicin	Indolic	4-methoxy-3-indolylmethyl	Shoot
1-MeO-Glucobrassicin	Indolic	1 -methoxy-3-indolylmethyl	Root
Gluconapin	Aliphatic	3-butenyl	Whole plant

Source: Tolra *et al*. (2001).

According to Tolra *et al*. (2001) only the aromatic glucosinolate sinalbin was found in the whole plant (roots and shoots) while gluconasturtin was found in the shoots of certain plants. Similarly, indolic glucosinolates 4-meo-glucobrassicin 1-meo-glucobrassicin was observed in shoot and root respectively and gluconapin was detected in roots in all plants grown with different Zn treatments. Ernst (1990) reported high concentrations of total glucosinolates in metal hyperaccumulating Brassicales and Mathys (1977) observed elevated concentrations of total glucosinolates in Zn tolerant populations of *Thlaspi alpestre*, compared to Zn-sensitive populations.

Glucosinolates are synthesized and maintained at a metabolic cost (Mauricio and Rausher, 1997). Therefore, metal hyperaccumulating species (e.g. *Thlaspi caerulescens* and *Arabidopsis thaliana*) are thought to be better defended from

herbivores as a result of the high metal concentrations in tissues (Boyd and Martens, 1992), meaning that glucosinolates may not be required in these plants. For example in choice experiments by Pollard and Baker, (1997) slugs preferred to eat *T. caerulescens* plants grown on low-Zn rather than high-Zn plants due to deterrence imposed by the metal concentration in the plant. The literature indicates that this observation has also been reported in other heavy metal (e.g. Ni and Cd) hyperaccumulators as well. Despite the work carried out to date to verify the idea of 'elemental plant defence', extensive research is needed to further understand metals as a means of defence and also the interactions between metals and glucosinolates in these hyperaccumulating brassicas.

1.3.4.4 Metal hyperaccumulation as a defence mechanism

Plants with exceptionally high concentrations of heavy metals have attracted the attention of researchers to investigate the reasons behind this phenomenon and address three basic questions: (1) Why some plants species hyperaccumulate heavy metals, (2) what physiological functions are attributed to these accumulated heavy metals and (3) what benefits do these hyperaccumulators reap from the metals or metalloids (Rascio and Navari, 2011)? Boyd and Martens (1992) hypothesised different possible answers to these questions. For example, the 'metal disposal' hypothesis postulates that metal hyperaccumulation allows plants to translocate these toxic heavy metals and metalloids from roots to aerial biomass and eliminating them by shedding the leaves. The 'drought resistance' hypothesis correlates hyperaccumulation with a water conserving role in cell walls and may benefit the plant by increasing drought resistance. Allelopathy or the 'interference' hypothesis suggests that hyperaccumulating plants may enrich the surface soils by shedding off

their metal-containing leaves and hinder the growth of less tolerant plant species under their canopies. However, none of these hypotheses have any acceptable experimental verification yet (Boyd and Jaffré, 2001; Morris *et al*. 2009) and much debate about the reason for hyperaccumulation is still going on. For example, Zhang *et al*. (2007) observed no 'toxic zone' under the canopy of the Ni hyperaccumulator *Alyssum murale* to hamper the germination of less tolerant plant species. By contrast, Boyd and Jaffré (2001) noticed that another Ni hyperaccumulator *Sebertia acuminata* increased the Ni level in the surface soil which ultimately interfered with the neighbouring plant species.

Another hypothesis called the 'defence' hypothesis (Boyd and Martens, 1992) describes the role of hyperaccumulated elements as defence compounds against a wide range of insect herbivores and pathogens and so far, this hypothesis has received the most supporting evidence despite some contradictory results (Boyd, 2007). Almost all the hyperaccumulated heavy metals have proved to be effective against herbivores in different hyperaccumulator species. For example, Zn (Behmer *et al*. 2005), Se (Galeas *et al*. 2008), Cd (Jiang *et al*. 2005), As (Rathinasabapathi *et al*. 2007), and Ni (Jhee *et al*. 2006) all have been reported to be involved in plant defence. Assunçao *et al*. (2003) described the elemental defence hypothesis to be the most plausible of the hypotheses put forward to explain hyperaccumulation.

Inorganic defences are thought to be a more effective defence tactic than organic defences due to three reasons: 1) Heavy metals are acquired from soil and not synthesized by the plant and concentrations can be modified, 2) metals cannot be degraded and thus prevent the evolution of a herbivore defence strategy and 3)

elemental defences are not metabolically expensive unlike organic defences (Boyd, 2010). Although most of the elemental defences are bound to organic complexes, usually small organic acids (Kramer *et al*. 1996; Sagner *et al*. 1998; Verbruggen *et al*, 2009) it is their inorganic components i.e. flourine, nickel, cadmium or zinc that deter herbivores.

Hyperaccumulated elements can provide defence in two ways, either through avoidance (deterrence) of feeding on the hyperaccumulated plant or through toxicity of the hyperaccumulated metal once ingested (Boyd and Jhee, 2005). A number of experiments have shown that toxicity only occurs when insects are given high concentrations of elements in hyperaccumulator plants or artificial medium under no choice conditions (Boyd and Martens, 1999; Boyd *et al*. 2002; Vickerman *et al*. 2002; Hanson *et al*. 2003, 2004; Zidar *et al*. 2004; Behmer *et al*. 2005; Boyd and Jhee, 2005; Coleman *et al*. 2005; Jhee *et al*. 2005; Scheirs *et al*. 2006; Jhee *et al*. 2006 a,b; Notten *et al*. 2006 a,b; Gonçalves *et al*. 2007; Boyd, 2007). For example high Ni leaves of *S. polygaloides* are acutely toxic to polyphagous herbivore *Spodoptera exigua* larvae resulting in 96% mortality (Boyd and Moar, 1999).

In order to observe if elevated metal concentrations have a direct effect on herbivores, experiments using artificial foods run as separate tests or to complement hyperaccumulation tests in plants should be conducted. However according to Macnair (2003), while showing the toxic effects of the elements in insects is an important step to prove the elemental defence hypothesis, in practice the plant is damaged by the herbivore until it has ingested a lethal dose which limits the effectiveness of the defence. While plants are young, herbivores can do extensive

damage before toxicity has a benefit to the plant, so plant defence from an early stage is of immense value. Huitson and Macnair, (2003) studied the ability of young *Arabidopsis halleri* seedlings to accumulate zinc, and observed that even when the seedlings were grown at 100 μM the foliar Zn concentration was well below levels shown to protect against herbivores.

Therefore another way that metal hyperaccumulation can give protection is through avoidance of the hyperaccumulator, with herbivores preferring plants with lower concentrations of metals. Complete deterrence is rare and has been shown only in two experiments, one by Pollard and Baker (1997) and the other by Jhee *et al*. (2005). These researchers reported complete rejection of high Zn and Ni leaves respectively by caterpillars, cabbage-maggot and cross-striped cabbage-worm. According to Boyd (1998), such deterrence is very important and is the most effective defence strategy preventing initial herbivory damage. Numerous experiments have also shown aversion responses in a number of different invertebrates in choice experiments (Jhee *et al*. 1999; Hanson *et al*. 2003, 2004; Boyd and Jhee, 2005; Behmer *et al*. 2005; Jhee *et al*. 2005, 2006b; Gonçalves *et al*. 2007).

Overall it appears that hyperaccumulation of elements plays a significant role in plant defence from herbivores and pathogens. The broad effectiveness of the elements within a plant species will heighten the evolutionary pressure on increasing the elemental concentrations within the plant (Boyd *et al*. 1994). Co-evolution of insects and pathogens that can tolerate increased elemental concentration also gives much weight towards the fact that elements do have a protective role; otherwise they

would not have to evolve tolerance mechanisms. However more detailed research in this area, with respect to the direct effects of the metals is required.

The hypothesis that metal hyperaccumulation is an evolutionary adaptation for metal tolerance has been proposed in several studies (Boyd and Martens, 1994; Kramer *et al*. 1997). The possibility that hyperaccumulation is an adaptation for tolerance assumes that hyperaccumulation is not an independent character and if so, then tolerance and hyperaccumulation would be pleiotropic (Macnair *et al*. 1999). To address this aspect Macnair *et al*. (1999) showed that genes segregated independently from F2 generations between the hyperaccumulator, *Arabidopsis halleri*, and the related non-accumulator, *Arabidopsis petraeae* indicated that that zinc tolerance and hyperaccumulation were conferred by separate genes, which argues against the proposal that metal hyperaccumulation is a method of tolerance. It is therefore possible that multiple selection pressures acting concurrently or sequentially might have driven the evolution of the metal hyperaccumulation trait (Whiting *et al*. 2003). It must also be remembered that metal hyperaccumulation has evolved independently in a number of different species and widely spaced geographic locations, therefore it can be assumed that different selection pressures have affected the evolution of this trait (Boyd, 1998). Huitson and Macnair (2003) reported that even if it is proved that certain selective forces are currently acting on the ability to hyperaccumulate (e.g. pathogens and herbivores) other factors might have been involved in its initial evolution.

Previous research has concentrated on either organic defence or inorganic defence of plants. A few preliminary reports have also been cited on the possible trade-off

between organic and inorganic defences, but one aspect of plant defence, the 'synergistic effect' of both defences has been missing in those studies. More recently Boyd (2007) and Rascio and Navari, (2011) postulated that 'joint effects' of both organic and elemental compounds may actually work in tandem for effective overall plant defence. According to this joint effects hypothesis more than one hyperaccumulated element in the same plant as well as the organic compounds and heavy metals might also be involved in the effective deterrence of herbivore and pathogens. For example Jhee *et al*. (2006) noticed that heavy metal and multiple organic compounds work together against the specialist Lepidoptera herbivore *Plutella xylostella* in *Streptanthus polygaloides*. However, experimental evidence is needed to broaden the knowledge about element-element and element-organic compound joint effects.

Further research is needed to determine the heritability of the hyperaccumulation trait and prove or disprove the hypothesis. Also research on interactions between different types of defences (organic and inorganic) would be beneficial, as it would be interesting to observe if it is hyperaccumulation that provides better defence or if secondary metabolites play a role. It would be worth investigating the effects of natural and simulated herbivory on the production of defence metabolites and levels of defence. Answers to these questions will not only determine the evolution of the trait but also help reach a conclusion regarding why hyperaccumulation is been maintained in a wide range of different species (Bert *et al*. 2000) and the possible role of organic metabolites in plant defence in these plants.

1.4 Aims of the project

The main aim of this project was to determine if there was a link between metal uptake in the hyperaccumulator *Thlaspi caerulescens* and production of glucosinolates. The main **hypothesis** tested was that a Zn hyperaccumulating plant would contain lower levels of glucosinolates when tissue Zn concentrations were high, than when they are low. Therefore, Zn uptake would result in a metabolic saving to the plant. A corollary of this is a test of the relative effectiveness of the 'elemental' and 'joint effects' hypotheses.

Since *T. caerulescens* naturally hyeraccumulates zinc, it was chosen as the study plant. It is an interesting species from an evolutionary standpoint, but also from a practical aspect since interest in its potential to remediate contaminated land is gaining momentum, although its small biomass is restricting.

After a basic 'range-finding' set of experiments (Chapter 3), the main experiments conducted to test the hypothesis consisted of:

(1) An experiment to test the effects of artificial damage (to simulate a chewing insect) on glucosinolate production by *T. caerulescens* growing over a range of soil Zn concentrations (Chapter 4).

(2) An investigation of the effects of foliar Zn concentrations on natural infestation by thrips, which are generalists and have a rasping/sucking mode of feeding (thus causing foliar damage). Infestation levels and glucosinolate production were measured (Chapter 5).

3) An investigation of the effects of foliar Zn concentrations on natural infestation by cabbage whitefly, which are specialist phloem feeders. Infestation levels and glucosinolate production were measured (Chapter 6).

Three questions were posed and the aim was for answers to be generated:

(1) Does Zn hyperaccumulation protect the plant from herbivores?

(2) Are glucosinolates produced in plants containing high levels of Zn?

(3) If there are interactions between GS and Zinc, are they responsive?

These questions will be addressed at the end of the thesis.

CHAPTER 2: GENERAL METHODS

2.1 Plant Growth and Maintenance

2.1.1 Germination and growth conditions of plants

Thlaspi caerulescens J&C Presl Ganges ecotype seeds were purchased from Guy Delmont, a commercial grower in southern France. Prior to sowing, seeds were soaked in deionised water for 48 hours for stratification (this softens the seed coat to enhance germination). Pre-soaked seeds were then sown in plug trays filled with a mixture of potting compost (John Innes No. 2, Norwich, UK), sand and grit in the ratio of 3:1:1 respectively.

Seed trays were placed in a glasshouse for germination and subsequent growth under supplementary lights to support the natural radiation at 16h/8h (day/night) photoperiod. The temperature in the glasshouse was 20/16ºC (day/night) although this varied somewhat depending on time of year. Maximum summer temperatures sometimes reached 28^{o}C on occasion. Irrigation was applied from tap water when required. Germination was generally completed 10 days after sowing.

All experimental plants were grown in the glasshouse as described, but the germination conditions varied as experiments progressed and experience was gained. Deviations from the germination procedure are explained in the relevant Chapters if they occurred. Experimental plants were always grown in a randomised block design in the glasshouse.

2.1.2 Pest control

Sciarid fly was controlled by drenching the potting medium with the insecticide, Intercept 70 WG (Scotts, Ipswich, UK) at a rate of 0.02 g L^{-1} deionized water. Adult sciarid fly and other flying insects from the neighboring plants in the glasshouse were controlled with yellow sticky traps, deployed throughout the experiment.

2.1.3 Harvest technique and storage

On completion of experiments, shoots of experimental plants were destructively harvested and washed in tap water to remove any dust particles from the surface and dried with paper towels. After gently patting dry, the shoots were dipped in liquid nitrogen for 10 - 15 seconds and stored frozen at -80ºC. Prior to analysis, these shoots were then lyophilized for 17 hours at -40ºC using a Hem Lab SB4 freeze drier. After lyophilisation, shoot dry weights were recorded after which they were stored in desiccators containing silica gel. Prior to analyses, dried leaves were ground manually with a pestle and mortal and passed through sieve of 2 mm mesh size.

2.2 Experimental Procedures

2.2.1 Zinc application to soil

All experimental plants were grown in zinc-amended soil (apart from controls which did not receive any supplementary zinc). Two weeks from germination, seedlings were transplanted into pots filled with potting compost amended with the Zn treatments. Zinc was applied as $ZnSO_4.7H_2O$, dissolved in deionised water and

mixed with compost before filling the pots. Zinc concentrations varied depending on the experiment and these are specified in each experimental Chapter.

2.3 Soil and Plant Chemistry

2.3.1 pH and soil moisture

To determine pH, 10 g soil was weighed into a beaker and Milli-Q water was added to make slurry. The ratio of soil: water was 1:3. The slurry was shaken overnight at 100 rpm to mix the soil and then centrifuged at 2000 rpm. The supernatant was collected and pH determined using a digital pH meter calibrated before use.

Soil moisture content was determined gravimetrically (Jury *et al*. 1991; Maier *et al*. 2000). Approximately 30g soil was oven dried at 105ºC for 24 hours. Moisture content was calculated by the loss of weight after oven drying according to the equation 2.1.

$$\theta g = \frac{m-d}{d}$$

(Eqn. 2.1)

Where m denotes the weight (g) of moist soil prior to drying and d is the dry weight (g) of same soil after drying. θ_g is the mass of water per unit mass of oven-dried soil.

2.3.2 Plant metal analysis

Approximately 50mg of ground plant material was weighed (exact weights recorded) and digested in concentrated (70% v/v) HNO_3, H_2O_2 and H_2O in the ratio of 3:2:3

respectively using a microwave digestion system (Model Multiwave 3000, Anton Paar Ltd., Hertford, UK).

After digestion, the volume of digested plant material was made up to 20ml by adding Milli- Q H_2O. For analysis, digestates were further diluted to 1:10 with Milli-Q water and analysed using Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific- x series II ICP-MS) against appropriate standards.

2.3.3 Carbon, nitrogen and sulphur (CNS) determination

Approximately 15-20mg of finely ground plant material was carefully weighed into a tin capsule. Five mg of vanadium pentoxide was added to each sample. Capsules were folded over using forceps and crimped tightly to avoid spillage of the sample. Reference materials (tomato leaves, cystein) with known CNS concentrations were treated similarly. One of the reference materials was chosen for calibration of the instrument while the remaining ones were run as samples. A capsule containing only vanadium pentoxide was used as a blank. CNS analysis was undertaken using a CE instruments Flash EA1112 Elemental Analyser. Samples were dropped into the combustion tube packed with approximately 25g copper oxide and 70g electrolytic copper and heated to 900^{o}C. The resulting gas was then passed through an absorption filter packed with magnesium perchlorate to remove any water before passing through a PTFE separation column and onto a thermal conductivity detector.

2.3.4 Total glucosinolate analysis

2.3.4.1 Solution preparation

Pyridine acetate buffers (0.5 and 0.02 M)

To make a 0.5 M pyridine acetate buffer, 40 ml pyridine and 30 ml glacial acetic acid were measured into a one litre volumetric flask. Milli-Q water was added to make up to volume and pH adjusted to pH 5 with NaOH. The 0.02 M solution was made from dilution by taking 40ml of the 0.5 M and making to 1 L with Milli-Q water; pH was adjusted as before.

DEAE-Sephadex A-25 suspension

DEAE-Sephadex A-25 suspension was prepared by mixing and stirring the dry gel in an excess of 0.5 M pyridine acetate buffer. This suspension was filtered through No. 40 Whatman filter paper and washed with 0.02 M pyridine acetate buffer. The residue was then suspended in 0.02 M pyridine acetate buffer and the final volume was made up to twice that of the settled gel. The solution was stored at 4ºC.

Myrosinase (thioglucosidase, EC 3.2.3.1. Sigma St. Louis, MO, USA)

Myrosinase was prepared using the method of Appelqvist and Josefsson (1967). The enzyme was dissolved @ 2.5 mg per 1ml of 0.02 M pyridine acetate buffer and 250 µL of this solution was used for each determination. The active part of the final prepared enzyme solution was not less than 0.1 units regardless of the formulation of the enzyme, as suggested by Wilkinson *et al*. (1984).

2.3.4.2 Glucosinolate extraction

For the extraction of total glucosinolates, 200 mg of freeze-dried ground plant material (section 2.1.3) was weighed in a disposable centrifuge tube and 3ml of aqueous methanol (70% v/v) heated to boiling temperature was added to it. The test tube was put in a boiling water bath for 5 minutes and then stirred continuously for 4-5 minutes using a whirl mixer. Following mixing, samples were then centrifuged at 3500 rpm for 15 minutes using a bench top centrifuge (MSE, Centaur 2, Sanyo, UK). After centrifugation the supernatant was collected and the pellet was re-extracted using the same method as before. The extraction procedure was done three times in total for each sample. The three liquid phases were amalgamated and concentrated using a rotary evaporator (Rotvapor-R, Buchi, Switzerland) at 45ºC to evaporate the methanol. After evaporation, the remaining sample (2.5-3.0 ml) was collected and the total volume made up to 7.5 ml by adding deionised water. This solution was marked as “Solution A”.

2.3.4.3 Myrosinase treatment

Four ml of “Solution A” were loaded onto a DEAE-Sephadex A-25 column and allowed to drain slowly so that glucosinolates were absorbed onto the Sephadex ion exchange filter. After draining, the column was washed through with deionised water and subsequently with 0.02M sodium acetate buffer. These ‘drainings’ were discarded. Myrosinase (250µl, prepared as described above) was added to the column and left at room temperature for 15 hours. After 15 hours, liberated glucose was eluted by adding two aliquots of 0.5 ml deionised water to the column; this solution was collected for analysis. Final volume of 1.25 ml (0.25 + 0.5 + 0.5 ml)

was collected in screw capped plastic vials and stored at -20ºC until analysed. This solution was marked as "Solution B".

2.3.4.4 Quantification of total glucosinolates

A measured volume of "Solution B" was incubated with the assay reagents provided in the GAGO20 kit obtained from Sigma, St. Louis, MO, USA. Released glucose was determined according to the manufacturer's instructions. Solutions were mixed thoroughly and absorbance was recorded against the reagent blank at 540 nm using a spectrophotometer (CECIL instruments, CE 1011, 1000 series). Total glucosinolates in the plant and reference materials (2.3.4.5) were quantified by using equation 2.2.

$$\textbf{Glucosinolates} = \frac{\textbf{Glucose in solution B} \times 1.25 \times 7.5 \times 1000}{\textbf{M} \times \textbf{V} \times 180}$$

(Eqn. 2.2)

where M is the weight (mg) of plant sample, V is the volume of "solution A" applied to the Sephadex column and 7.5 is the total volume (ml) of solution after methanol evaporation, 1.25 is the volume (ml) collected from the Sephadex column after incubation with myrosinase enzyme and 180 denotes the relative molecular mass of glucose.

***Glucose standard curve*:** Glucose concentration was quantified from a standard curve. Aliquots of 0.02, 0.04, 0.06, and 0.08 ml of glucose standard (Sigma, St. Louis, MO, USA) were pipette out into appropriately marked test tubes and 0.98, 0.96, 0.94 and 0.92 ml of deionised water added to the respective tubes to make a final volume of 1ml in each tube. Two ml of assay reagent (provided in the glucose

assay kit) was added to the first tube at time zero and thereafter at intervals of 30-60 seconds. The reaction was started at time zero by putting the tubes into a water bath at 37±1ºC, where they remained for 30 minutes. After 30 minutes, the reaction was stopped at 30-60 seconds intervals by adding 2 ml of 12N H_2SO_4 to each tube. The solution was mixed thoroughly and absorbance measured against the reagent blank at 540 nm with spectrophotometer (CECIL instruments, CE 1011, 1000 series). A typical standard curve is shown in Fig. 2.1. The procedure was repeated for each set of new glucose assays.

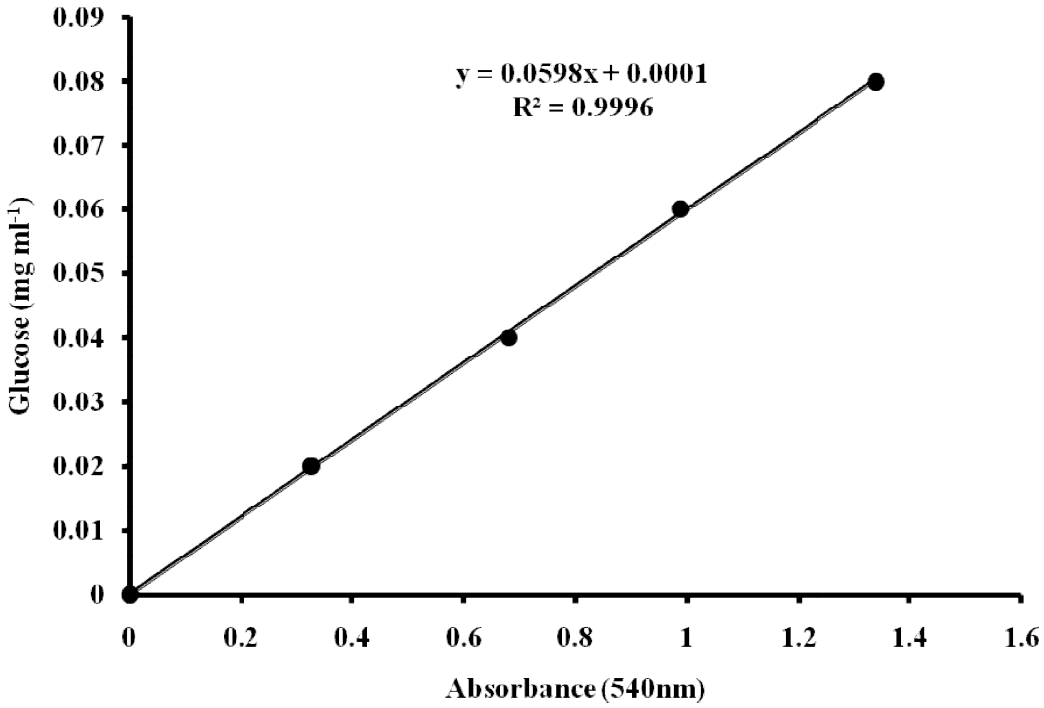


Fig. 2.1: Example of a glucose standard curve used to calculate the liberated glucose in leaves of *Thlaspi caerulescens*.

2.3.4.5 Validation of total glucosinolate method

Reference material (rape seed) for total glucosinolates was purchased from the Institute for Reference Materials and Measurements (IRMM), European Reference

Material (ERM) Belgium. For checking the performance of the method for extracting and quantifying total glucosinolates from *T. caerulescens*, the unopened sample was allowed to warm at room temperature to avoid moisture condensation on cold seed. The contents of the sachet (20 g of whole seed) was ground at once and stored at 4ºC.

Ground reference material was weighed as 100, 250 and 500 mg and glucosinolates were extracted as outlined in section 2.3.4.2 in order to optimise the amount of material to run alongside the experimental plant material. Total glucosinolates were calculated by recording the absorbance of sample against the glucose standard curve.

The certificate of analysis provided with the reference material, indicated a glucosinolate content of 99 mmol kg^{-1}. In the analysis performed, the closest value obtained was 90.76 mmol kg^{-1} with 0.03 ml of extract and a seed weight of 100 mg. This amount of reference material was run along with all the subsequent analyses carried out on *T. caerulescens*.

2.3.5 Individual glucosinolate analysis of Thlaspi caerulescens

2.3.5.1 Preparation of solutions

Sodium acetate

Acetic acid (1.2ml) was pipette out in 990 ml of deionised water in a one litre volumetric flask and pH was adjusted to 5 with sodium hydroxide. Volume was made to 1L by adding deionised water to obtain a final concentration of 0.02 M. The solution was stored at 4ºC.

DEAE-Sephadex (A-25, C-25) columns

Sephadex A-25 and C-25 were prepared as before; 13 g were weighed out and added to 100 ml of 0.02 M sodium acetate and left to expand. Suspended gel was filtered and fresh buffer was added to the residue. The suspension was filtered again followed by washing with water. The residue was then resuspended in excess sodium acetate and stored at 4ºC.

Sinigrin (2 propenyl glucosinolate)

To prepare 16 mM sinigrin (*Sinapis alba*, VWR, Leicestershire, UK) solution, 0.0664 g of sinigrin was dissolved in 10 ml of deionised water and stored at -20ºC.

Sulphatase purification

Approximately 300 mg sulphatase from *Helix pomatia* (Sigma-Aldrich, St. Louis, USA) was weighed into a flask and 12 ml cold water added. This was stirred continuously whilst 12 ml cold ethanol was also added in order to assist in dissolving the sulphatase. The solution was centrifuged at 3000 rpm for 6 minutes in a temperature controlled centrifuge to keep the temperature at 4ºC. The supernatant was collected and 1.5 ml of cold ethanol added to the supernatant, stirred and centrifuged as before. This time the supernatant was discarded and the residue dissolved in 8 ml of deionised water. This suspension was passed, in 2 ml batches, first through an A-25 and then through a C-25 Sephadex column and the filtrate stored at 4ºC.

2.3.5.2 Glucosinolate extraction

Approximately 150 mg of freeze-dried ground plant material was weighed (exact mass was recorded). Methanol (70%) was heated to boiling and 5 ml of methanol followed by 50 µl of sinigrin as an internal standard was added to the sample. The sample was incubated at 70ºC for 30 minutes and vortexed twice during incubation. After incubation, the sample was allowed to cool to room temperature and centrifuged at 3500 rpm for 6 minutes and the supernatant was collected.

2.3.5.3 Production of desulphoglucosinolates

Three ml of supernatant (section 2.3.5.2) was added to the prepared A-25 exchange column and allowed to drain slowly so that glucosinolates were absorbed to the ion exchange column. (The C-25 column was used to purify the sulphatase only.) The A-25 column was washed with two aliquots (0.5 ml each) of deionised water followed by washing with the same volume of 0.02M sodium acetate. After draining the column, collecting vials was placed under the columns and 75 µl of prepared sulphatase was layered on the surface of the sephadex without disturbing the gel bed and left overnight at room temperature. The next day glucosinolates were eluted from the column by adding three aliquots (0.5, 0.5 and 0.25 ml) of water and a final volume of 1.25 ml collected and stored at -20ºC until analysis.

2.3.5.4 Mass spectrometric analysis

A liquid chromatograph equipped with a C18 reversed phase column and a diode array detector (Agilent 1100, Hewlett Packard, CA, USA) was used to analyse the samples. The equipment was fitted with an auto sampler and injection volume was adjusted to 20 µl. The mobile phase to elute the sample was 100% water in “bottle

A" and 100% acetonitrile in "bottle B". Gradient time profile was made up from 100% water and 100% HPLC grade acetonitrile to 0 min 0% B, 2 min 0% B, 25 min 50% B, 27min 50% B, 29 min 0% B and 39 min 0% B at a flow rate of 1ml m^{-1} for a total run of 39 minutes. Glucosinolates were quantified by using the following equation (Eqn.2.3).

$$\textbf{Glucosinolates} = \frac{\textbf{(Peak area of glucosinolate/peak area of sinigrin)}\times\textbf{0.8}\times\textbf{0.95}}{\textbf{Mass of tissue used(mg)/1000}}$$

(Eqn. 2.3)

Where 0.8 is the concentration of sinigrin (µmol) used as internal standard and 0.95 is the correction factor.

CHAPTER 3: EFFECT OF GROWTH STAGE, TRANSIENT DAMAGE AND CONTINUAL COMPETITIVE STRESS ON GLUCOSINOLATES IN *THLASPI CAERULESCENS*

3.1 Introduction

Due to the sessile nature of plants, they are particularly vulnerable to the actions of pathogens and predators. Since plants are constantly exposed to external dangers posed by animals (grazers, insects) or by pathogenic organisms (viruses, bacteria and fungi), they have developed a series of mechanisms to combat the attackers. These mechanisms include physical exclusion, containment of the attacking organism or induction of toxic compounds (Mayer, 2004). Plants contain and synthesise a huge variety of toxic compounds called "secondary metabolites". This arsenal of secondary metabolites formed through different metabolic pathways includes glucosinolates, terpenoids, phenolics, carbohydrates (oxalic acid), quinones and alkaloids (Harborne, 1988) and their defensive role against herbivores and pathogens is well established (Rosenthal and Berenbaum, 1991). In addition to the organic chemical defences, some plants have developed the ability to take up toxic substances, such as cations of Zn, Cd, Ni and anions of Se and Cl from their habitat to make them less prone to enemies (Terry *et al*. 2000; Kupper and Kroneck, 2005).

Glucosinolates are nitrogen and sulphur containing organic defence compounds naturally present in the majority of brassica plants. Apart from being constitutively present in the plant, glucosinolates are also induced as a result of insect, pathogen or

mechanical damage to the plant. The degree of glucosinolate induction depends on the plant species being attacked, plant organ, type of damage, time of the day, age of the plant and ecological niche (Bellostas *et al*. 2007; Falk *et al*. 2007). Considerable variation in glucosinolate profiles is observed within plant species (Hopkins *et al*. 2009). For example Kliebenstein *et al*. (2001) observed variations in the quantity and quality of glucosinolates analysed from seeds and leaves of different ecotypes of *Arabidopsis thaliana* and Poelman *et al*. (2008) described overlapping glucosinolate profiles in cabbage (*Brassica oleracea*). Thus diversity in glucosinolates occurs under both natural and artificial selection.

Bidart-Bouzat *et al*. (2005) reported that ecotypes and cultivars (Birch *et al*. 1992, 1996) within the same species induce different concentrations of glucosinolates in response to herbivory. According to Gols *et al*. (2008), herbivory induced higher glucosinolate concentrations in wild populations of *Brassica oleracea* than in a *Brassica oleracea* cultivar.

Although concentrations vary within plant species, the situation is complicated because 120 glucosinolates have been identified and the profile of each plant species (Brassicaceae) may differ (Fahey *et al*. 2001). Further, this diversity of profiles may increase with the discovery of new species able to produce glucosinolates (Mithen, 2001).

Concentrations of induced glucosinolates depend on the type of damage and species involved. For example chewing insects trigger higher induction of glucosinolate profiles than sucking insects (e.g. aphids) and specialist herbivores induce lower

concentrations than generalists. Birch *et al*. (1992), Hopkins *et al*. (1998), van Dam and Raaijmakers (2006) observed 60% reductions in aromatic or aliphatic glucosinolates and increases in indole glucosinolates in *Arabidopsis* plants when populated with *Delia floralis* (Turnip Root Fly) and *D. radicum* (Cabbage Root Fly). Soler *et al*. (2005) noticed a 50-70% reduction in leaf glucosinolates in *Brassica nigra* plants when infested with two specialist herbivores (*D. radicum* and *Pieris brassicae*).

Glucosinolate content is also triggered by mechanical damage or other stressors to the plant tissue (Koritsas *et al*. 1989; Griffiths *et al*. 1994), but the level of increase in glucosinolates is less than that induced by herbivory. This might be due to damage frequency as herbivory involves repeated wounding and hence more tissues are damaged, whilst mechanical wounding is usually administered once (Mithofer *et al*. 2005). Bodnaryk, (1992) observed more glucosinolate induction in the cotyledons of *Brassica napus* after repeated mechanical damage on two consecutive days rather than after damaged on a single day.

3.2 Aims and Objectives

The aims of the experiments described here were to identify the glucosinolate (GS) profile and quantify GS concentrations in *Thlaspi caerulescens* growing under experimental glasshouse conditions. Speed of induction after artificial damage (clipping) and effects of long-term stress (intraspecific competition) were also measured. A broad understanding of the responsiveness of the plant and effects of Zn on GS production was essential for the successful implementation of the

remainder of the PhD research. Three small 'range-finding' experiments are described in this Chapter.

3.3 Materials and Methods

3.3.1 Experiments undertaken

Three experiments were carried out as follows:

1) Effect of time on glucosinolate concentrations following artificial damage

This experiment was designed to investigate the effect of clipping damage on glucosinolate concentrations over a range of time spans, i.e. from 2 hours to seven days after damage. Seedlings were germinated in potting compost (John Innes, Norwich, UK) amended with 2000 mg Zn kg^{-1} of soil. Seedling trays were placed in a constant temperature room at 5ºC until germination was completed. Two weeks after germination, plants were transferred to the experimental pots containing potting compost amended with 2000 mg kg^{-1} Zn and placed in the glasshouse. Fourteen days after transplanting, plants were artificially damaged by clipping. Plants were harvested at the time intervals of 2, 4, 8, 24, 32, 48, 56, 72, 80, 96, 104, 168, hours after clipping. Harvested plant material was dipped in liquid nitrogen and stored at -80ºC until analysis.

2) Effect of growth stage and soil Zn amendments on glucosinolate profiles

This experiment was conducted to observe the effect of simulated herbivory on GS (total as well as individual) in 17 day old *Thlaspi* (cotyledons, 1-2 true leaves) and at maturity (35 day old plants). *Thlaspi caerulescens* seeds were soaked overnight in water, drained and sown into plug trays (47.0×27.5×2.0cm) filled with Zn treated

compost (John Innes, Norwich, UK). Zn was applied in the form of $ZnSO_4.7H_2O$ to give the concentrations of 1500 and 3000 mg Zn kg^{-1} of soil. Plug trays were placed in a cold room at 5ºC until germination was completed. After germination, trays were moved to the glasshouse (as outlined in Chapter 2, section 2.1.2). After one week in the glasshouse, half of the seedlings were transplanted into pots (8.0×7.5cm) filled with potting compost (John Innes, Norwich, UK), sand and grit in a ratio of 3:1:1 respectively and treated with the same concentrations of Zn as used for germination. The remaining un-transplanted seedlings were harvested and processed as detailed above. The potted plants were harvested after a further 28 days.

3) Effect of continual stress (planting density) on glucosinolate concentrations

The aim of this experiment was to determine the effect of planting density (intraspecific competition) on glucosinolate content of *T. caerulescens*. Seedlings were obtained by sowing seeds in unamended seedling compost (John Innes, Norwich, UK) and maintaining them at 5ºC. Sixteen days after germination, seedlings were transplanted into compost filled pots treated with $ZnSO_4.7H_2O$ solution to obtain a single treatment of 2000 mg Zn Kg^{-1} of soil. Seedlings were transplanted to obtain a population density of 1, 2, 4, 8 or 16 plants pot^{-1}. There were 3 replicates for each population density. Irrigation was applied with tap water as required. Six weeks after transplanting (5 true leaves) all the plants were harvested, washed with tap water and immediately dipped in liquid nitrogen for 12-15 seconds and stored at -80ºC until analysis.

3.3.2 Extraction and purification of glucosinolates

Total GS in the plant material (all 3 experiments) was extracted and quantified by enzymatic hydrolysis of glucosinolates to glucose using the methods of Heaney *et al*. (1988) and Makkar and Becker, (1997) (Chapter 2, Section 2.3.4). Individual GS (experiment 2) were quantified by High Performance Liquid Chromatography (HPLC) as described in Chapter 2 (Section 2.3.5).

3.3.3 Metal analysis

Zn in the plant tissue was quantified by ICPMS as described in Chapter 2 (Section, 2.3.2) for plants in experiment 3.

3.3.4 Statistical analyses

Data were analysed using GenStat release 13.1 as follows: Experiment one, one-way ANOVA using time as the factor; Experiment 2, two-way ANOVA using soil Zn amendment and plant age as factors; Experiment 3, one-way ANOVA using planting density as the factor. Normality was checked by plotting the residuals against the expected normal quantiles.

3.4 Results and Discussion

3.4.1 Effect of time on glucosinolate concentrations following artificial damage

Glucosinolates (GS) are metabolically expensive to synthesise. It has been estimated that 90 ATP and 12 $NADH_2$ are required in addition to 9 enzymatic steps, but the real costs involve myrosinase production, compartmentalisation, transport and

maintaining production (e.g. Wallace and Eigenbrode, 2002). More to the point, synthesis is complex and the speed of production is likely to be essential to successful defence. The data below (Fig. 3.1) show that when *T. caerulescens* was artificially damaged it took 32 hours before GS synthesis increased in response and 48 hours for maximum concentrations to be detected. Thereafter GS concentrations returned (almost) to the 'starting' values. GS production here was in response to one damage event. In reality, herbivores are likely to attack the plant multiple times and/or over a period of time. Nevertheless, an increase was observed. In contrast to these data, Martin and Muller (2007) reported a 3-fold increase in GS after just 24 hours from wounding *Sinapsis alba*.

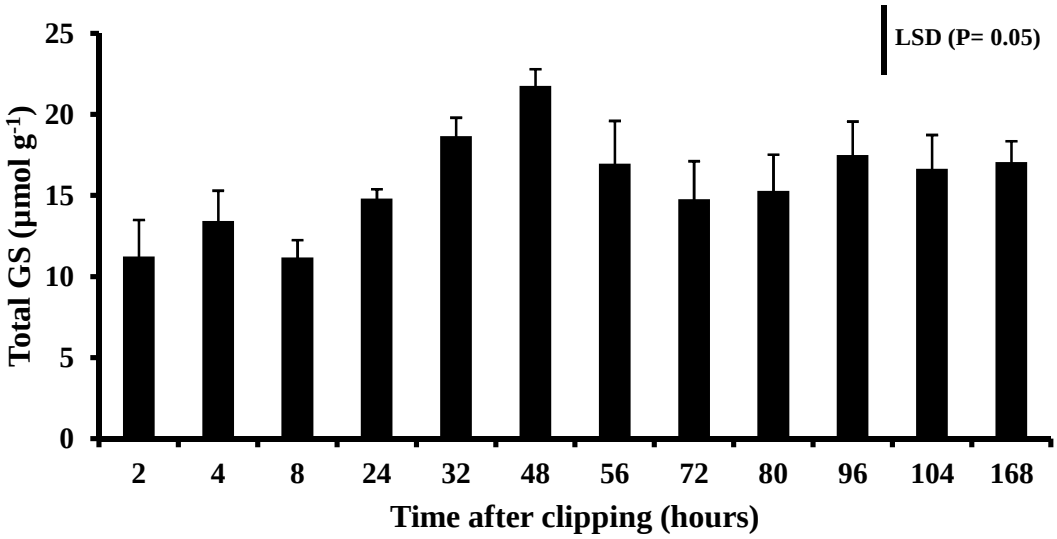


Fig. 3.1: Total glucosinolate concentrations in *Thlaspi caerulescens* shoots. Leaves were clipped 14 days after transplanting and plants harvested at regular time intervals thereafter. Soil was amended with 2000 mg kg^{-1} Zn. ANOVA for time as a single factor; P=0.003; LSD= 4.9 (P=0.05).

The plants here were damaged at a relatively young stage (14 days from transplanting). In a subsequent experiment, plants were allowed to grow for 6 weeks following transplanting before being clipped. The GS concentrations in these older plants were low with an overall average of 3.4 µmol g^{-1} rather than the average of

15.8 µmol g^{-1} observed for the younger plants. It is known that GS concentrations may fall with developmental stage (Pongrac *et al.* 2008) and this would explain the differences. However, the two experiments were temporally separated and different environmental conditions may have affected the GS levels. The response profile in the older plants in the current work was faster than that in the younger plants. GS concentrations were lower 72 hours from clipping than those after 24 hours. This contrasts with the younger plants above which did not return to pre-24 hour GS levels even after 168 hours. One explanation might be that the older plants had higher foliar Zn concentrations and therefore when no further damage was experienced organic defences did not need to be maintained. A further alternative explanation might be related to the N content of the soil; since glucosinolates are N-based defences a shift away from defence production may have occurred if N was limiting.

3.4.2 Effect of growth stage and soil zinc amendment on glucosinolate profiles

Seedlings (with cotyledons and 1-2 true leaves) and plants that had formed rosettes (both grown on soil amended with two Zn concentrations) were compared for their glucosinolate content. Four different aromatic glucosinolates were extracted, two were identified as benzyl (glucotropaeolin) and p-OH-benzyl (sinalbin) whilst 2 remained unidentified (other than they were aromatic glucosinolates) and hereafter will be referred to as glucosinolate 'A' (GS 'A') and glucosinolate B (GS 'B'); examples of chromatograms are available at the end of the Chapter (Figs 3.4 to 3.7). No indole or aliphatic glucosinolates were observed which is contrary to the findings of Tolra *et al.* (2001) who found two indolic GS (although in very low concentrations) along with sinalbin in *T. caerulescens*. GS 'A' and GS 'B' were

present in much smaller concentrations than the benzyls present and GS ‘B’ was not affected by either age of plant or zinc amendment. In contrast, the total glucosinolates, benzyl and p-OH-benzyl were present in higher concentrations in the older plants than in the seedlings and specifically in older plants grown at the lower soil Zn concentration. However, GS ‘A’ is more prevalent in the younger plants (seedlings) than in the older ones, at both soil Zn concentrations. Therefore taken together, the seedlings have a different GS profile than the older plants. Different profiles have been shown in *Arabidopsis* seedlings and Wentzell and Kliebenstein (2008) suggested that these seedlings produce simpler nitrile structures compared to more mature rosettes as these are easily metabolised.

The different concentrations and profiles might reflect relative palatability at different growth stages. The seedlings may require more protection – GS ‘A’ might be biologically more active as a defence than the other glucosinolates, but there is no evidence for this and herbivory tests would need to be carried out. If Zn has a protective role then GS ‘A’ might be redundant in larger plants which will likely have higher foliar Zn concentrations than the seedlings. Plants under natural conditions will be attacked by a range of different herbivores and pathogens over their lifetime and the optimum defence theory (ODT) hypothesises that glucosinolates will be synthesised and allocated in proportion to the relative importance of the plant tissue (Wallace and Eigenbrode, 2002). Wallace and Eigenbrode (2002) suggest that the cotyledon stage is important and that allocation of glucosinolates to the cotyledons should decline as the true leaves develop. It is possible that the decline in GS ‘A’ in this experiment was due to allometric dilution as the plant grew, although if this were the only reason for its decline, less would be

expected in the 35 day old plants than was actually observed. The fact that benzyl, p-OH-benzyl and total GS were present in greater concentrations in older plants grown with less Zn is interesting (and also contrasts with the previous experiment above).

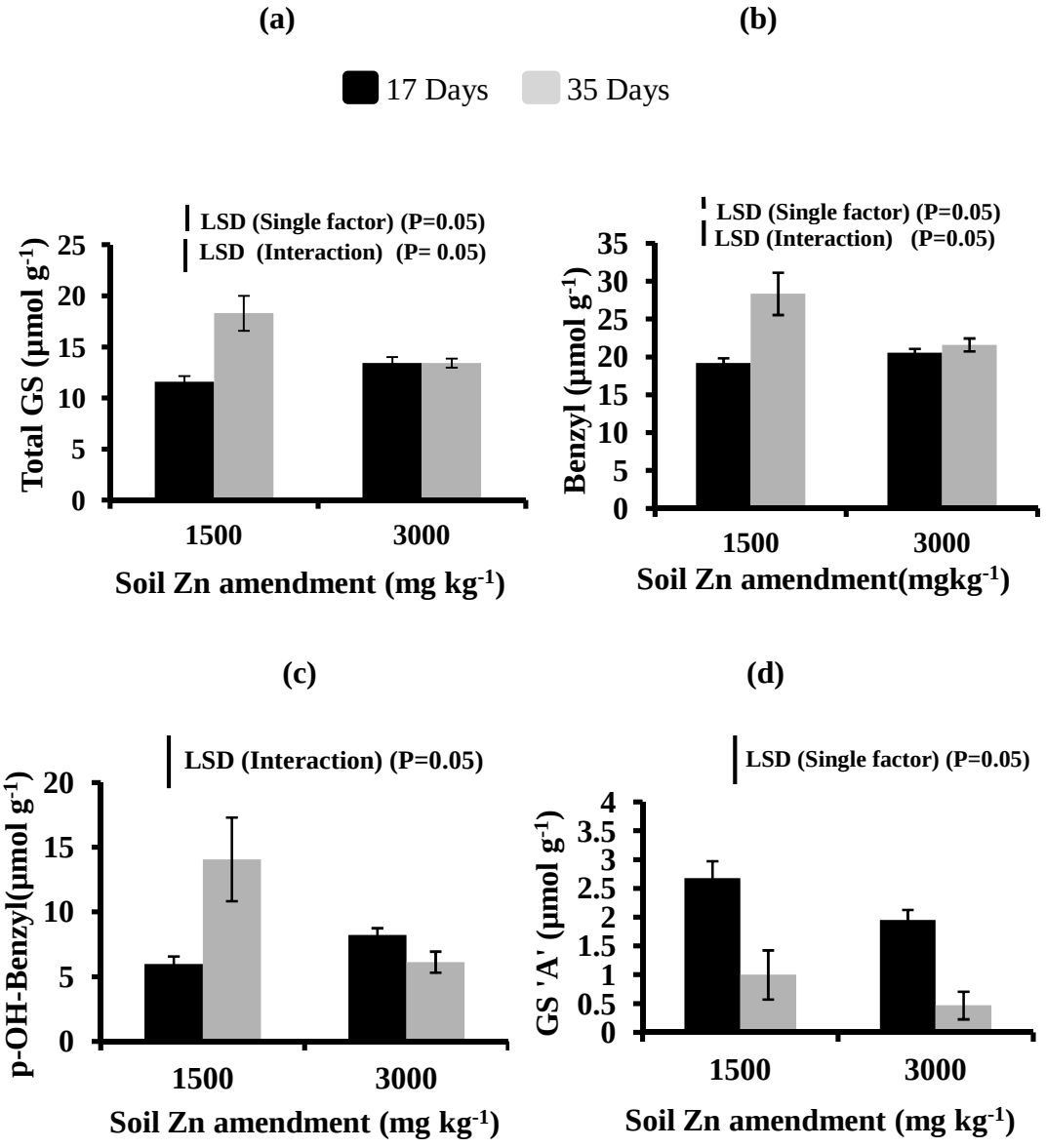


Fig. 3.2: Effects of growth stage on shoot glucosinolate concentration and profiles in *T. caerulescens*. Plants were grown in soil amended with either 1500 or 3000 mg kg^{-1} Zn and harvested either 17 days or 35 days from germination. ANOVA: **(a)** Total GS; Growth stage, P=0.004; LSD= 2.152; Zn amendment, NS; Growth stage x Zn interaction, P=0.005; LSD= 3.044. **(b)** Benzyl; Growth stage, P<0.001; LSD= 0.243; Zn amendment, P=0.032; LSD= 0.243; Growth stage x Zn interaction, P=0.003; LSD=3.448. **(c)** p-OH-Benzyl; Growth stage, NS; Zn amendment, NS; Growth stage x Zn interaction, P=0.007; LSD= 4.978. **(d)** GS 'A'; Growth stage, P<0.001; LSD= 0.648, Zn amendment, P=0.055; LSD= 0.648; Growth stage x Zn interaction, NS. LSDs (P=0.05) are shown when significant treatment effects or interactions are present.

These plants would have had lower foliar Zn concentrations than those grown with the higher Zn amendment. This suggests that Zn and GS complement each other in the defence of these plants. A further interesting observation is that of the relatively few glucosinolates present in the shoots of *T. caerulescens*. Most brassicas contain a variety of glucosinolates designed to respond to the wide range of herbivores that comprise a range of feeding guilds (Fahey *et al*. 2001; Hopkins *et al*. 2009). *T. caerulescens* shoots only contain 4 GS and all are aromatics. This lack of GS diversity is possibly linked to the Zn hyperaccumulation strategy adopted by this species. This lack of GS diversity supports the 'trade-off' hypothesis to some extent if the metabolic 'saving' resulting from hyperaccumulation is in terms of limited structural variation rather than reduced GS concentrations *per se*. However, Boyd (2007) proposed the 'joint effects' hypothesis and the data here support the idea of both defences working together, whether this is in terms of 'trade-off' or 'joint effects'.

3.4.3 Effect of continual stress (planting density) on glucosinolate concentrations

Experiment 1 above focussed on one damage (clipping) event. Therefore, to determine the effects of continual stress, *T. caerulescens* was subjected to intraspecific competition for 6 weeks. Theoretically stress should be induced because of competition for limiting resources (soil nutrients and/or light) with a concomitant increase in GS production. Roots were not considered so it is not known if there was a change in biomass partitioning, but shoot dry weight generally decreased with increasing competition. The difference in dry weight between each treatment was significant (LSD = 0.05). The plants were grown in soil amended with 2000 mg kg^{-1} Zn; plants grown singly illustrate the accumulative capacity of the species (Fig. 3.3

a). The plants in competition all had lower foliar Zn concentrations than those in monoculture (LSD = 1190). Relative to the density-related decrease in shoot dry weight, the Zn concentrations remained remarkably consistent (Fig. 3.3 b). The observation is clearly not a concentration effect and suggests that plants increased allocation of Zn to shoots with increasing intensity of competition. The GS concentrations appear to increase with plant density but only GS in the monoculture are significantly different from all other treatments. Plants grown in densities of 2, 4 and 8 plants per pot contain similar GS concentrations and those grown in pots with 4, 8 and 16 plants were also statistically the same (LSD = 1.72). The average individual plant grown at a density of 16 per pot was very small compared to plants in the other treatments, yet the GS concentrations were similar to those of plants grown at other lower densities. Nevertheless, the plants in competition contained higher concentrations of GS than those in monoculture. The increase in GS with competition is probably a stress effect, but subsequent proportional effects with increasing density were not seen, either in terms of increased production or dilution effects. Nitrogen is a key soil nutrient and under this level of competition it will have been limiting.

Glucosinolates are N-based compounds and it is likely that N was allocated to growth and maintenance rather than to defence (van Dam and Baldwin, 2001) in plants growing under extreme N limitation (i.e. 16 plants per pot). Such phenotypic plasticity is widely reported and enables plants to maximise their growth/reproduction under harsh conditions (Fenner, 1986).

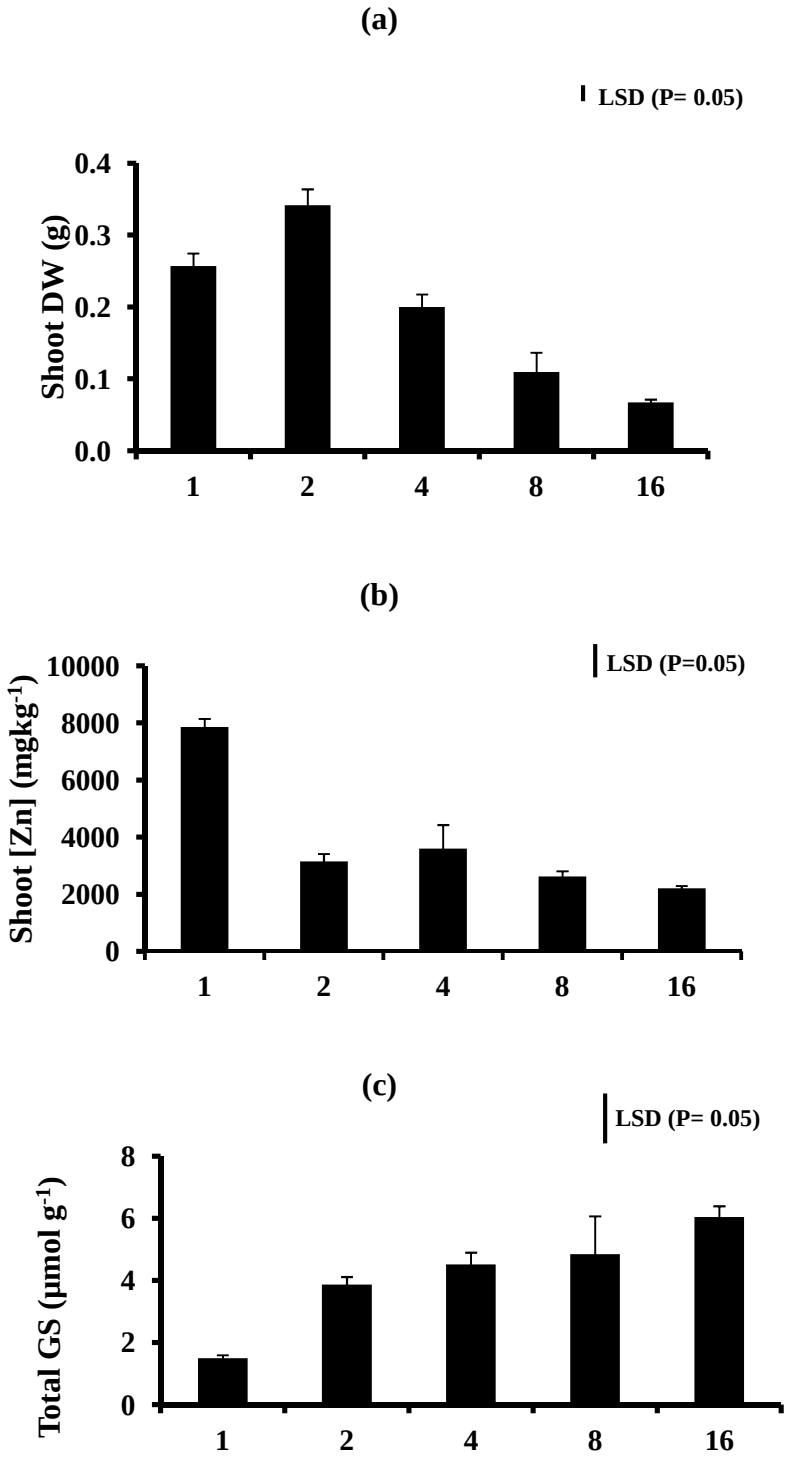


Fig. 3.3: Effects of long-term stress on **(a)** shoot biomass of *T. caerulescens*, P<0.001; LSD= 0.054 (P=0.05). **(b)** Shoot Zn concentration, P<0.001; LSD= 1193 (P=0.05) **(c)** Total glucosinolate concentration in shoots (P=0.004); LSD= 1.722 (P=0.05). Plants were grown in soil amended with 2000 mg kg^{-1} Zn and at different intraspecific densities for 6 weeks before harvest. Data are average values per plant and not 'pot biomass'. Data were analysed by ANOVA.

3.5 Key Points

- Total glucosinolates were induced 24 - 48 hours after clipping in *Thlaspi caerulescens* plants. Damage occurred only once. Maximum concentrations were present 48 hours from initial damage after which they decreased to a constant level slightly higher than the 'starting' concentrations. Measurements stopped 168 hours after clipping.

- Increasing plant intraspecific competition created a continual stress particularly at high planting densities. Shoot weight was severely reduced but total GS did not show the same proportional change. This suggests a degree of phenotypic plasticity aimed at conserving nutrients.

- Four aromatic glucosinolates were extracted from *T. caerulescens* shoots and two were identified as benzyl (glucotropaeolin) and p-OH-benzyl (sinalbin).

- GS profiles were different in seedlings (cotyledons and 1-2 true leaves) than in 35 day old plants (rosettes). GS concentrations were higher in the older plants grown in low-Zn soil but not in those grown in the high-Zn soil (1500 and 3000 mg kg^{-1} respectively).

3.6 Appendix – Figs 3.4 to 3.7

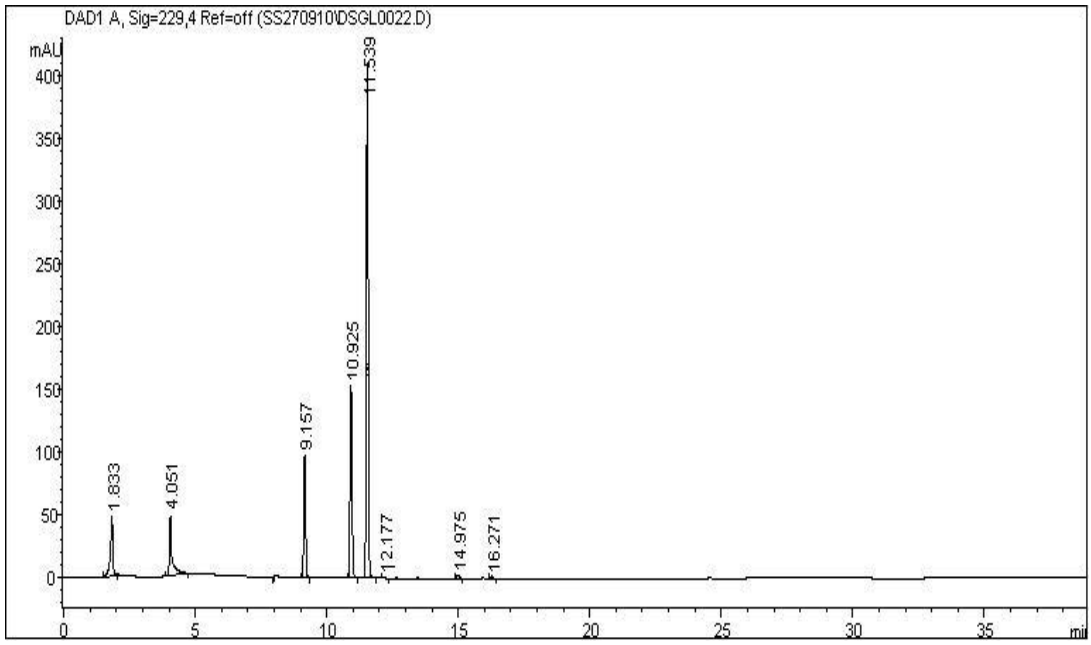


Fig. 3.4: Chromatogram of GS 'A', GS 'B', sinalbin and glucotropaeolin (retention time 1.83, 4.05, 10.92 and 11.539 min. corresponding to peak areas of 305.1, 214.6, 790.4 and 2038.7 respectively) in **17 day old** *Thlaspi caerulescens* grown in soil amended with **1500 mg kg^{-1} Zn**. Chromatogram derived from the chromatographic analysis (HPLC) of individual glucosinolates.

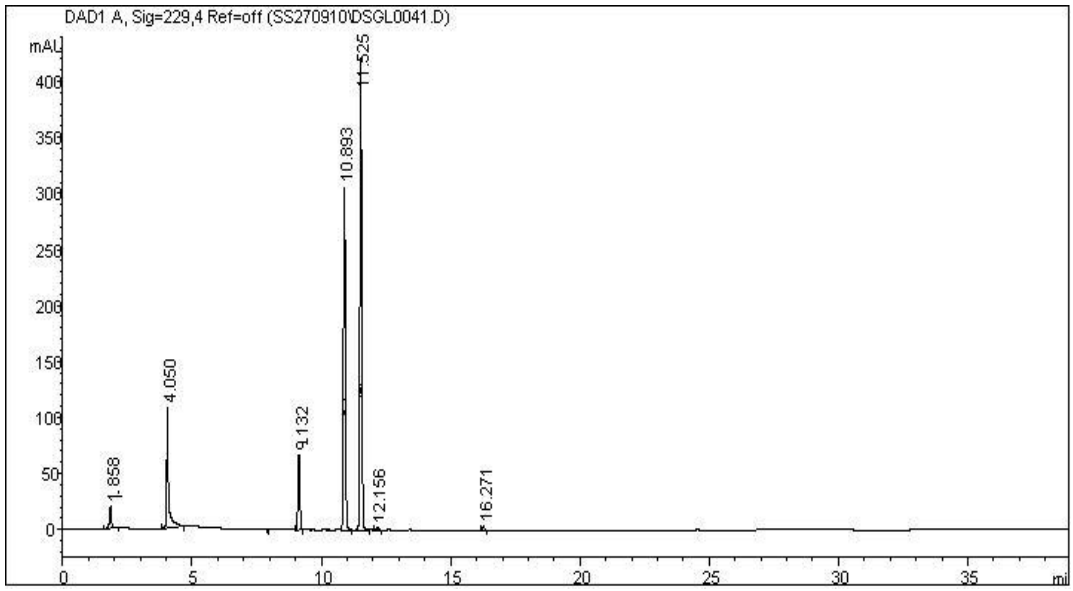


Fig. 3.5: Chromatogram of GS 'A', GS 'B', sinalbin and glucotropaeolin (retention time 1.856, 4.050, 10.893 and 11.525 min. representing peak areas of 110.2, 553.9, 1570, and 2081 respectively) in **35 day old** *Thlaspi caerulescens* plants grown in soil amended with **1500 mg kg^{-1} Zn**.

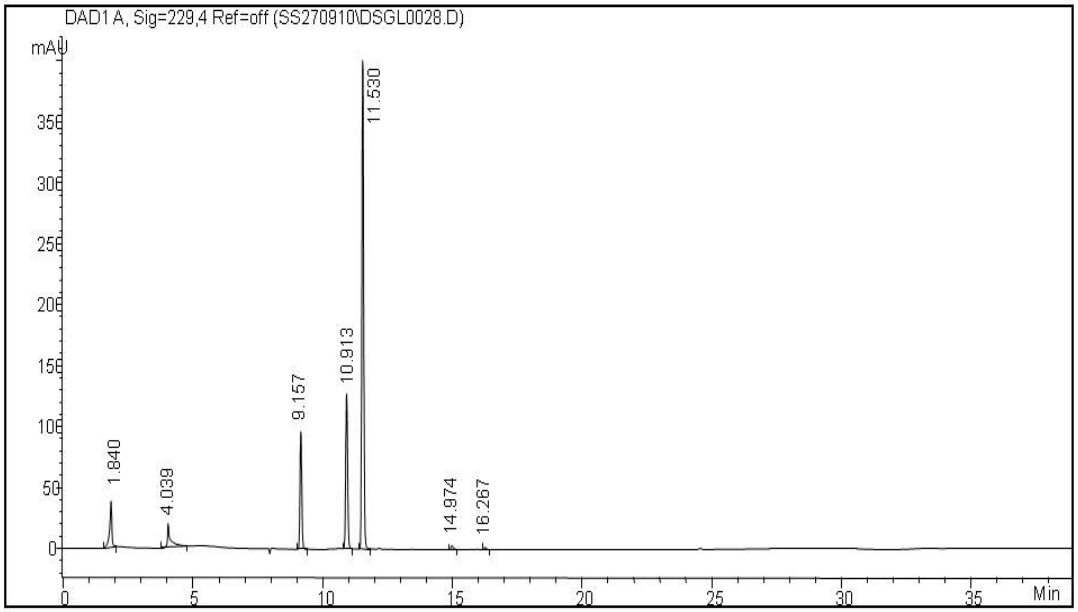


Fig. 3.6: Chromatogram of GS 'A', GS 'B', sinalbin and glucotropaeolin (retention time 1.840, 4.039, 10.913 and 11.530 min. representing peak areas of 224.7, 172.7, 659 and 1994.5 respectively) in **17 day old** *Thlaspi caerulescens* grown in soil amended with **3000 mg kg^{-1} of Zn**.

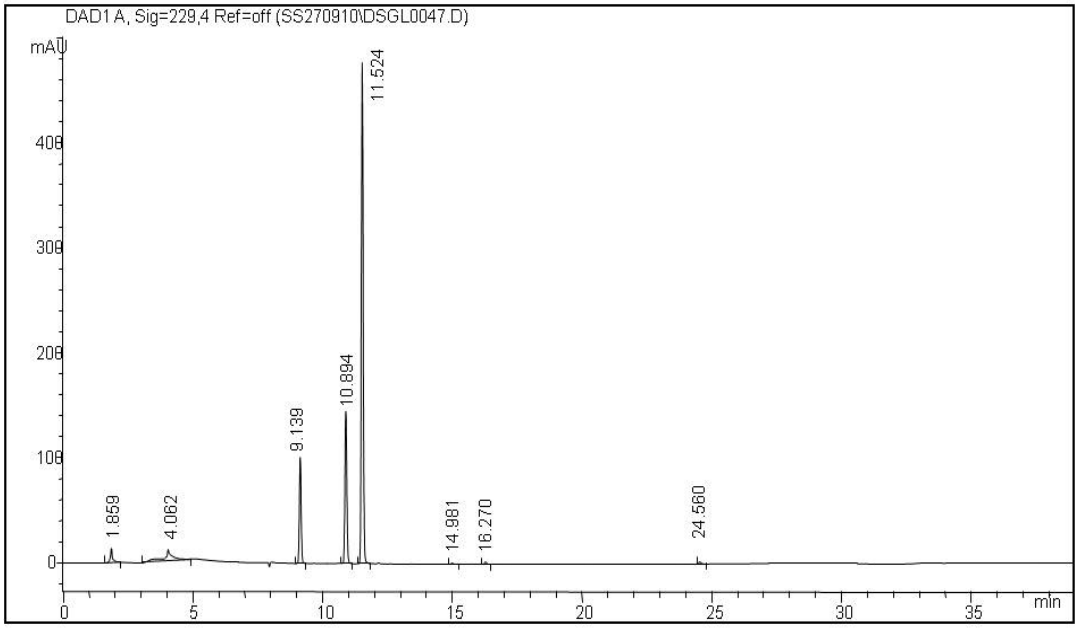


Fig. 3.7: Chromatogram of GS 'A', GS 'B', sinalbin and glucotropaeolin (retention time 1.859, 4.062, 10.893 and 11.525 min correspond to peak areas of 82.9, 231.5, 746.8 and 2357.9 respectively) in **35 day old** *Thlaspi caerulescens* plants grown in soil amended with **3000 mg kg^{-1} of Zn**.

CHAPTER 4: EFFECT OF ARTIFICIAL HERBIVORY AND ZINC AMENDMENT ON GLUCOSINOLATE INDUCTION

4.1 Introduction

Abiotic and biotic stress factors in the environment include herbivory, pathogen attack, high metal concentrations and light amongst others, all of which may activate a plant's defence mechanisms. These defence mechanisms ultimately result in quantitative and/or qualitative changes in metabolite production. Growth and post-harvest conditions have also been known to affect organic defence production (Jahangir *et al*. 2009), as the signalling molecules (jasmonic acid and salicylates) are triggered, thus resulting in activation of metabolic pathways. This activation of metabolic pathways affects the induction of phytochemicals including amino acids, phenols, carbohydrates and glucosinolates.

As plants exhibit a combination of constitutive and inducible metabolites (Chinnusamy *et al*. 2004; Bruce and Pickett, 2007; Pedras *et al*. 2008), the responses are specific depending on the type of stress involved. For example, total glucosinolates in susceptible and tolerant varieties of brassicas differed significantly after infestation with the pathogen *Plasmodiophora brassicae* (cause of club root disease in Brassicaceae); aliphatic glucosinolates were induced in susceptible varieties while aromatic glucosinolates were induced in tolerant varieties (Ludwig-Muller *et al*. 1997). The amount and composition of secondary metabolites also fluctuates depending on the type of stress-inflicting herbivore or pathogen as observed by Mewis *et al*. (2005) in *Arabidopsis thaliana*, where methylsulfinyl (an

aliphatic glucosinolate) increased when attacked by a generalist herbivore (*Myzus persicae*) or by a specialist, *Brevicoryne brassicae*. Stress responses are coordinated by signalling hormones including salicylic acid, ethylene and jasmonic acid, as observed by Mewis *et al*. (2005) in *A. thaliana*. Similarly, *Brassica napus* plants accumulated indole glucosinolates when treated with methyl jasmonate (Doughty *et al*. 1995) and the concentration of induced glucosinolates was dependent on the concentration of hormone.

Apart from the biotic stress factors and signalling hormones physical damage to the plant also influences the concentrations of plant metabolites. Bodnaryk (1992) observed that mechanical wounding with scissors caused a threefold increase in indole glucosinolates in the cotyledons of three brassica species (*B. rapa, B. napus* and *B. juncea*). Similarly, Bartlet *et al*. (1999) noted induced systemic changes in the concentration of indole glucosinolates as a result of damage to *B. napus*. Furthermore, brassica cultivars have higher amino acid contents than wild species due to exposure to different stress conditions (Cole, 1997).

An imbalance of macronutrients in soil (e.g. nitrogen and phosphorus) forms another important array of abiotic stresses affecting the concentration of total glucosinolates as well as glucosinolate profiles in Brassicaceae. For example, Kaur *et al*. (1990) observed an increase in the individual glucosinolates, sinigrin, gluconapin and progoitrin in the seeds of *B. juncea* with the application of phosphatic fertilizers. Similar observations were made by Kim *et al*. (2002) in *B. rapa* with enhanced use of phosphorus. Elevated levels of nitrogen in the soil resulted in enhanced concentrations of aliphatic glucosinolates in oilseed rape, whereas sulphur deficiency

in the soil decreased the level of alkenyl glucosinolate in the same plant (Zhao *et al*. 1994). Likewise, the presence of low nitrogen in the soil resulted in an increase in total as well as individual glucosinolates in *Brassica juncea* (Kaur *et al*. 1990).

Heavy metal content in brassica plants also influences the level of glucosinolates. For example, application of selenium in broccoli cultivation enhanced the level of glucosinolates along with phenolic compounds (Robbins *et al*. 2005), but stress caused by Cd application did not alter the level of glucosinolates in *B. rapa* (Siemens *et al*. 2002). Other abiotic factors involved in the induction of glucosinolates include light and temperature (Schonhof *et al*. 2007; Volden *et al*. 2008). Diverse levels of indolic, aliphatic and aromatic glucosinolates were observed in *B. oleracea* grown in different seasons due to different radiation and temperature regimes (Cartea *et al*. 2008). Canola shoots accumulated glucosinolates when growth temperature was increased to 40ºC for two weeks (Aksouh *et al*. 2001). Glucosinolate profiles (aliphatic glucosinolates) were increased in broccoli shoots when subjected to a temperature range of 7-13ºC and radiation of 10-13 mol m^{-2} day^{-1} (Schonhof *et al*. 2007). According to Reifenrath and Müller (2007), UV light effectively induced glucosinolates in different brassica species.

Other plant secondary products, flavonoids (anthocyanins) are induced as a result of stress (Winkel-Shirley, 2002). Their appearance in plants may be spontaneous or permanent depending on the developmental stage of the plant. Like glucosinolates, anthocyanins are naturally present in the plants, but also induced as a result of environmental stresses like chilling, UV radiation and water stress (Chalker-Scott, 1999); they are metabolically ‘expensive’ to synthesise. Kaliamoorthy and Rao

(1994) observed that *Zea mays* roots accumulate anthocyanins when exposed to salt (NaCl) stress. Similar observations were made by Ramanjulu *et al*. (1993) and Dutt *et al*. (1991) in white mulberry (*Morus alba*) and *Casuarina equisetifolia* respectively. *A. thaliana*, when submitted to osmotic stress accumulated anthocyanins relative to control plants (Mita *et al*. 1997).

The aim of the current experiment was to establish whether artificial damage induced glucosinolate production in *Thlaspi caerulescens* and whether there was any interaction between zinc amendment (and therefore foliar Zn concentrations) and GS induction.

4.2 Aims and Objectives

The prime aim of this study was to observe the effects of simulated herbivory (clipping) at a range of soil Zn concentrations. The reason for this was to determine if there was any 'trade-off' between glucosinolate induction and concentration of Zn in the soil (and therefore the plant). The 'trade-off' hypothesis states that glucosinolates (GS) should not need to be synthesised if high Zn concentrations prevent herbivory, thus reducing the metabolic costs of defence. The Zn concentrations chosen were within the range where active uptake was observed and biomass of the plant increased accordingly. In this Chapter, anthocyanin production as an indicator of stress (clipping) was measured in addition to glucosinolate profiles. Clipping was selected as a mimic of natural herbivory so that it could be applied to the plants uniformly.

4.3 Materials and Methods

4.3.1 Rationale and experimental setup

Two experiments were conducted with different aims and objectives. The first short trial aimed to determine the optimum level of soil Zn amendment that was required to generate maximum plant growth and highlight limiting Zn conditions likely to stress the plant, either by inhibiting growth or by inducing Zn toxicity. This was the starting point for the second experiment within the Chapter. The aim of the second experiment was to determine whether soil Zn amendment influenced the glucosinolate response in clipped and unclipped plants. Clipping was used here to simulate herbivory.

The methodology was broadly the same for both experiments. Seedlings were obtained by sowing the seeds in plug trays (47.0×27.5×2.0cm) filled with seedling compost (John Innes, Norwich, UK), amended with Zn. To amend the potting compost with zinc, $ZnSO_4.7H_2O$ was dissolved in deionised water and mixed with compost to obtain concentrations of 0, 100, 1000 and 2000 mg Zn kg^{-1} on a dry weight equivalent basis for the second experiment; seedling compost was unamended for the first exploratory trial. Control plants (experiment 2) received no metal applications, but did receive an equivalent volume of deionised water. Plug trays were placed in a dark cold room at 5ºC until germination was completed. After germination (10 days), the plug trays (with seedlings) were placed in a glasshouse (see Chapter 2, section 2.1.2). After one week, seedlings were transplanted into the experimental pots (8.0×7.5cm) filled with Zn-treated soil-based potting compost. Zinc concentrations ranged from 0-4000 mg Zn kg^{-1} for experiment 1 and from 0-2000 mg kg^{-1} for experiment 2. There were 6 replicates for each Zn treatment. In

experiment 1, plants were allowed to grow for 7 weeks and were then harvested. In experiment two, 7 weeks from germination (6 true leaves), three replicates from each Zn treatment were damaged by clipping with scissors and three were left unclipped. Clipping was synchronized by making an equal number and size of cuts on both sides of the mid vein depending on the leaf size. Extra care was taken not to remove any leaf material during clipping. After 24 hours of clipping, both damaged and undamaged plants were harvested, washed in tap water and immediately dipped in liquid nitrogen to stop the metabolic processes. Plant material was stored at -80ºC until further analysis.

4.3.2 Anthocyanin determination

Anthocyanins were determined in experiment 2 plants by using the method of Drumm- Herrel and Mohr (1982). Five ml of HCl: methanol (1:99 v/v) were added to 100 mg of ground, freeze-dried plant sample and kept at 5ºC for 24 hours in the dark. After 24 hours, the samples were centrifuged at 3000 rpm for 30 minutes using a bench top centrifuge (MSE, Centaur 2, Sanyo, UK). After centrifugation, the supernatant was decanted and absorbance of the extract determined at 525 nm using a spectrophotometer (CECIL instruments, CE 1011, 1000 series).

4.3.3 Zinc, glucosinolate and biomass determinations

Zinc, total glucosinolates and individual glucosinolates were determined in experiment 2 plants as described in Chapter 2, Sections 2.3.2, 2.3.4 and 2.3.5 respectively. Plant biomass was determined after freeze drying at -50^{o}C for both sets of experimental plants.

4.3.4 Statistical analyses

Analyses of variance were performed on data from experiment 2 using soil Zn (4 levels) and damage (+ or -) as factors. Since leaves were uniformly damaged and there was a relationship between foliar Zn and level of soil amendment, use of ANOVAs and in particular of soil Zn as a factor rather than foliar Zn is justified. Linear and polynomial regressions were carried out on Zn uptake data as appropriate. Data were analysed using GenStat release 13.1 (Lawes Agricultural Trust).

4.4 Results and Discussion

4.4.1 Effects of zinc application on plant growth and Zn uptake

In the initial trial *Thlaspi caerulescens* responded positively to soil Zn amendment and biomass increased along with foliar Zn (Fig. 4.1). The soil Zn amendments ranged from 0-4000 mg kg^{-1} soil; the highest level resulted in foliar uptake of ~14000 mg kg^{-1} Zn. However, the linear phase of biomass accumulation occurred at much lower foliar Zn concentrations. Based on these data, soil Zn amendments of 0-2000 mg kg^{-1} were used in the follow-up experiment in which simulated herbivory was the main focus. However, plants grown for this trial did not perform as well as the plants in the previous test. A soil Zn amendment of 2000 mg kg^{-1} resulted in plants with a foliar Zn concentration of ~3400 mg kg^{-1}, considerably less than previously observed (Fig. 4.2 a and b). Although Zn uptake did not reach its maximum potential in this trial, foliar accumulation still occurred. Enhanced Zn uptake relative to application is because *Thlaspi caerulescens* is a Zn hyperaccumulator. According to Brooks (1987), plants accumulating 10,000 mg kg^{-1}

of Zn, 100 mg kg^{-1} of Cd and 1000 mg kg^{-1} of Ni are considered hyperaccumulators. More recently, Papoyan and Kochian (2004) observed concentrations of 30000 mg kg^{-1} Zn and 1000 mg kg^{-1} Cd in shoots of *Thlaspi* without any toxicity symptoms.

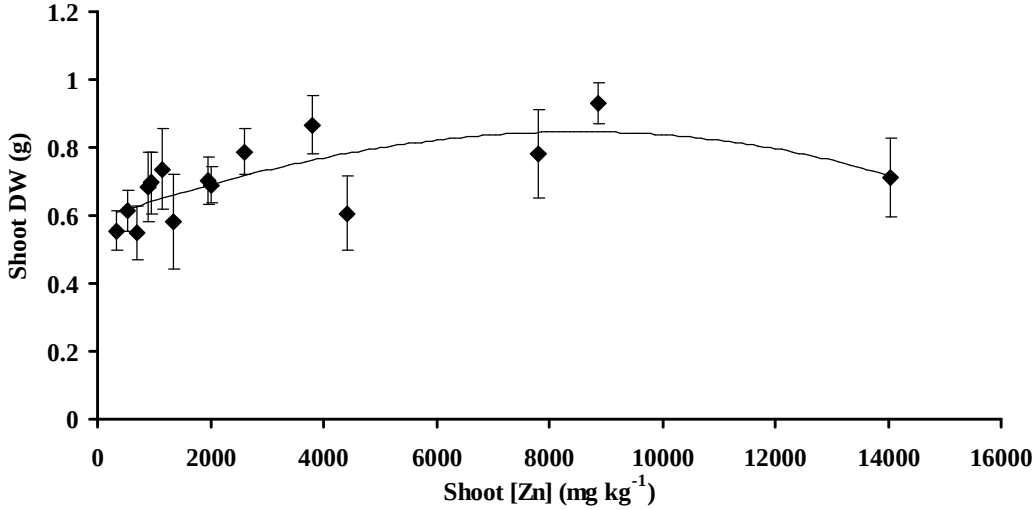


Fig. 4.1: Relationship between shoot Zn concentration and shoot dry weight. Data are for plants grown in a range-finding trial (experiment 1). Regression (2^{nd} order polynomial), P=0.016.

Brown *et al*. (1994) recorded concentrations of up to 51000 mg kg^{-1} for Zn and 1740 mg kg^{-1} for Cd in plants grown on Zn and Cd amended soils. Due to this hyperaccumulation characteristic, *Thlaspi* has been highly studied to determine its phytoremediation potential. Nevertheless, relatively little work has been done on the physiology of metal hyperaccumulation in *Thlaspi,* but its homolog *Arabidopsis thaliana*, has been extensively studied and genes involved in metal hyperaccumulation identified (Papoyan and Kochian, 2004). These authors also observed that a metal ATPase in *Thlaspi* called TcHMA4 mediated metal tolerance in yeast *via* efflux of different heavy metals, but not in *Arabidopsis*. Based on these observations, Papoyan and Kochian (2004) postulated that this gene may be involved in xylem loading of metals and thus an important factor in hyperaccumulation in *T.*

caerulescens. Ingrouille and Smirnoff (1986) concluded that Zn accumulation and tolerance by *T. caerulescens* may be inherited characteristics.

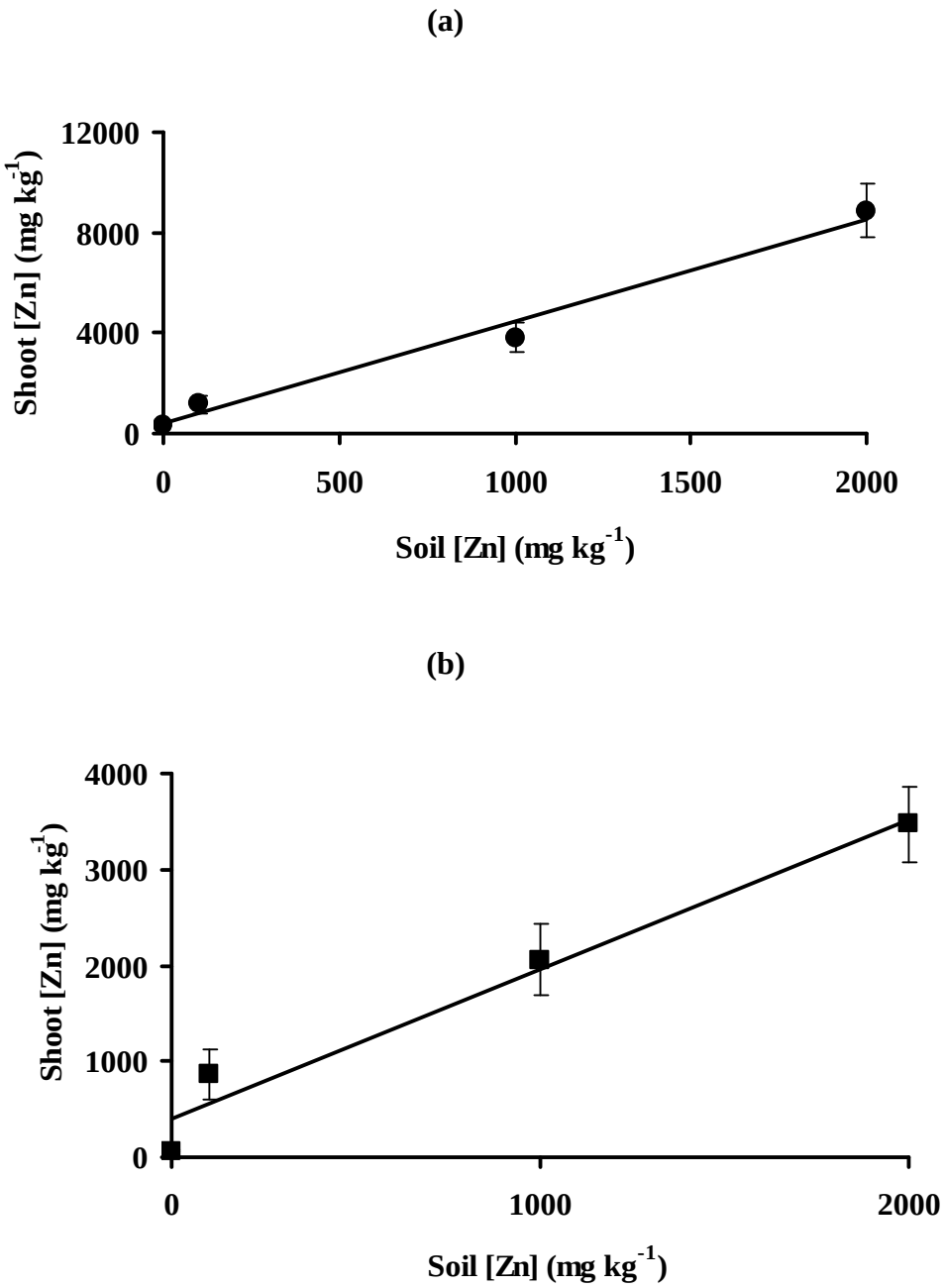


Fig. 4.2: (a) Foliar Zn concentration as a function of soil Zn amendment; experiment 1. Only data up to 2000 mg kg^{-1} soil Zn are shown to aid comparison with **(b)** foliar Zn concentration as a function of soil Zn amendment in experiment 2.

The reason for the relatively ineffective Zn accumulation in the second experiment is unknown, but is likely to be related to subtle temperature differences in the

glasshouse or changes in day length and/or natural light intensity between the two experiments since there was at least a two month gap between both trials. It is even possible that some form of undisclosed glasshouse maintenance (e.g. insecticide treatment) could be responsible. This illustrates the importance of considering foliar Zn concentration when performing studies such as this rather than relying on soil Zn data only, particularly if comparative studies are done. It is possible that the magnitude of Zn uptake will affect GS production but clipping did not affect uptake.

4.4.2 Glucosinolate profiles

Clipping treatment did not affect foliar Zn concentrations so if the limited uptake observed in experiment 2 was due to a physiological problem it is not related to the clipping treatment. In experiment 2, neither simulated herbivory (clipping) nor Zn application had any significant effect on total glucosinolate concentration. However, the data show (Fig. 4.3) an apparent reduction in GS concentration in clipped plants grown at 2000 mg kg^{-1} soil Zn application relative to unclipped plants grown at the same Zn concentration.

Mechanical damage is known to induce production of secondary metabolites in members of the Brassicaceae (Koritsas *et al*. 1991), but the induction level might not be the same as that for natural herbivores (Textor and Gershenzon, 2009). Mithöfer *et al*. (2005) speculated that less GS induction as a result of artificial damage might be due to a lack of repeated wounding as it is generally administered only once, whereas natural herbivory occurs repeatedly and often by a range of herbivores, each subjecting the plant to a different form of damage. Furthermore, whilst artificial clipping is an efficient means of regulating experimental damage, it does not

adequately reflect damage incurred by herbivores; e.g. damage patterns, timing of damage, inclusion of pathogens, inclusion of waste products such as honeydew and faeces and effects of saliva (Walling, 2000).

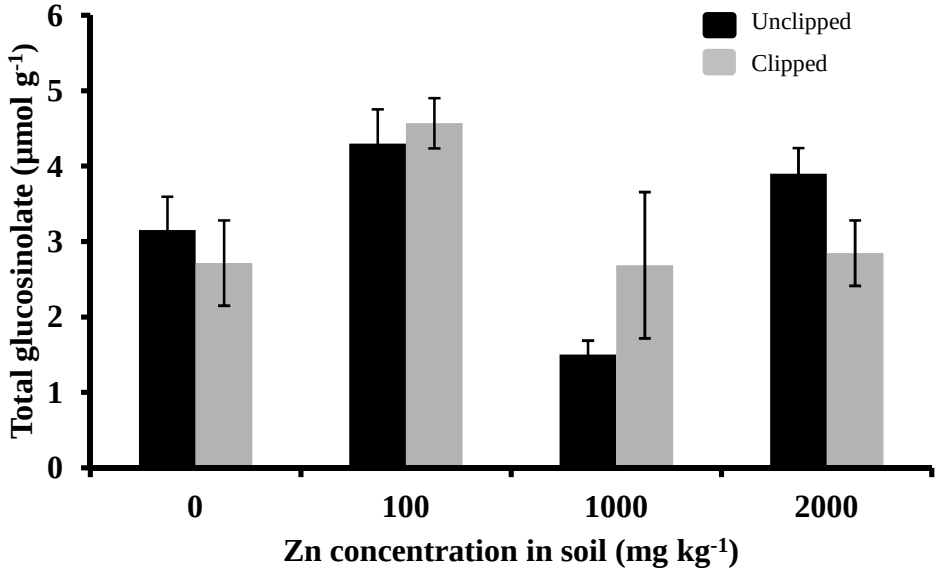


Fig. 4.3: Foliar glucosinolate concentrations in shoots of *T. caerulescens* grown over a range of soil Zn concentrations and either left unclipped or clipped. Data are means ± SE. No significant treatment effects were observed.

Indeed Agrawal (1998) found that clipping with scissors did not induce production of indole glucosinolates but natural herbivory did. This author suggested that clipping resulted in a reduced area of actual damage compared to natural herbivory (he removed one leaf per plant at the petiole), but also the absence of herbivore saliva was a key factor. In this current experiment, *T. caerulescens* was subjected to a greater level of more uniform damage than were Agrawal's plants.

In this study (Fig. 4.3) the concentration of GS was apparently less in artificially damaged leaves compared to the undamaged foliage but only at the highest concentration of applied Zn. This is in line with the results of Griffiths *et al*. (1994),

who noticed significant reductions in GS in artificially damaged roots of oilseed rape compared with the effects of natural herbivory by *Delia floralis* (Turnip root fly). An alternative view is that the results obtained in general may support the defence hypothesis since no additional glucosinolates were induced as a result of clipping, or it may be indicative of some unmeasured factor such as limiting soil sulphur. The possibility of reduced GS due to limiting sulphur is in line with the findings of Zhao *et al*. (1994), who found lowered concentrations of alkenyl and indolic glucosinolates in oilseed rape (*Brassica napus*) when grown on sulphur deficient soils as compared with plants grown on sulphur rich soil. GS levels are known to be positively correlated with soil sulphur concentrations (Falk *et al*. 2007) and Heiss *et al*. (1999) commented that glucosinolates may provide an additional S-source in plants suffering from metal stress. There is no reason to suspect S as a limiting factor here, although it is accepted that brassicas require higher concentrations than non-brassica plants (Ernst, 2000).

It is most likely that the reduction in GS in clipped plants grown at the highest rate of soil Zn is a Type 1 error, particularly in light of the level of variability in the data, but it is probably erroneous to conclude that or to focus on one data point since the effect of clipping overall was not significant (P=0.510). The overall result with regard to total GS concentrations is therefore in agreement with Agrawal (1998), clipping did not induce (or otherwise) foliar GS. Similarly, soil and foliar Zn concentrations did not affect total GS concentrations.

Two major glucosinolates identified in *T. caerulescens* were benzyl and p-OH-benzyl. Both glucosinolates belong to the aromatic class which constitute about 10% of known glucosinolates. A large degree of variability in the concentration of these

two glucosinolates over a range of Zn and clipping treatments was observed between replicates (Fig. 4.4 a and b). However, Zn amendment had a significant effect (P=0.024) on benzyl; lower concentrations were observed in plants growing at higher Zn concentrations (1000 and 2000 mg kg^{-1}) compared to plants growing at 100 mg kg^{-1} Zn amendment irrespective of clipping treatment. The benzyl present may actually be constitutive and intact within the plant rather than induced since there is no evidence of clipping-related induction. It is possible that the higher level of benzyl observed in shoots of plants grown in the low-Zn soil was because of limiting Zn causing nutrient stress and inducing benzyl as a result. However, production of GS is metabolically costly and an alternative explanation might be related to the 'trade-off' hypothesis. This states that plants containing high concentrations of metals do not need to produce secondary metabolites as defences.

The effectiveness of metal defences has been shown by Mathew *et al*. (2009) who observed significantly reduced herbivory in arsenic-accumulating *Pteris vittata*. Similarly Tolrà *et al*. (2001), observed less GS in a metallicolous population of *Thlaspi caerulescens* compared to a non-metallicolous one. Contrary to these observations, Noret *et al*. (2005) found no heavy metal related defence in *T. caerulescens*; rather the GS was a more effective defence against snails than was hyperaccumulated Zn, Cd and Ni.

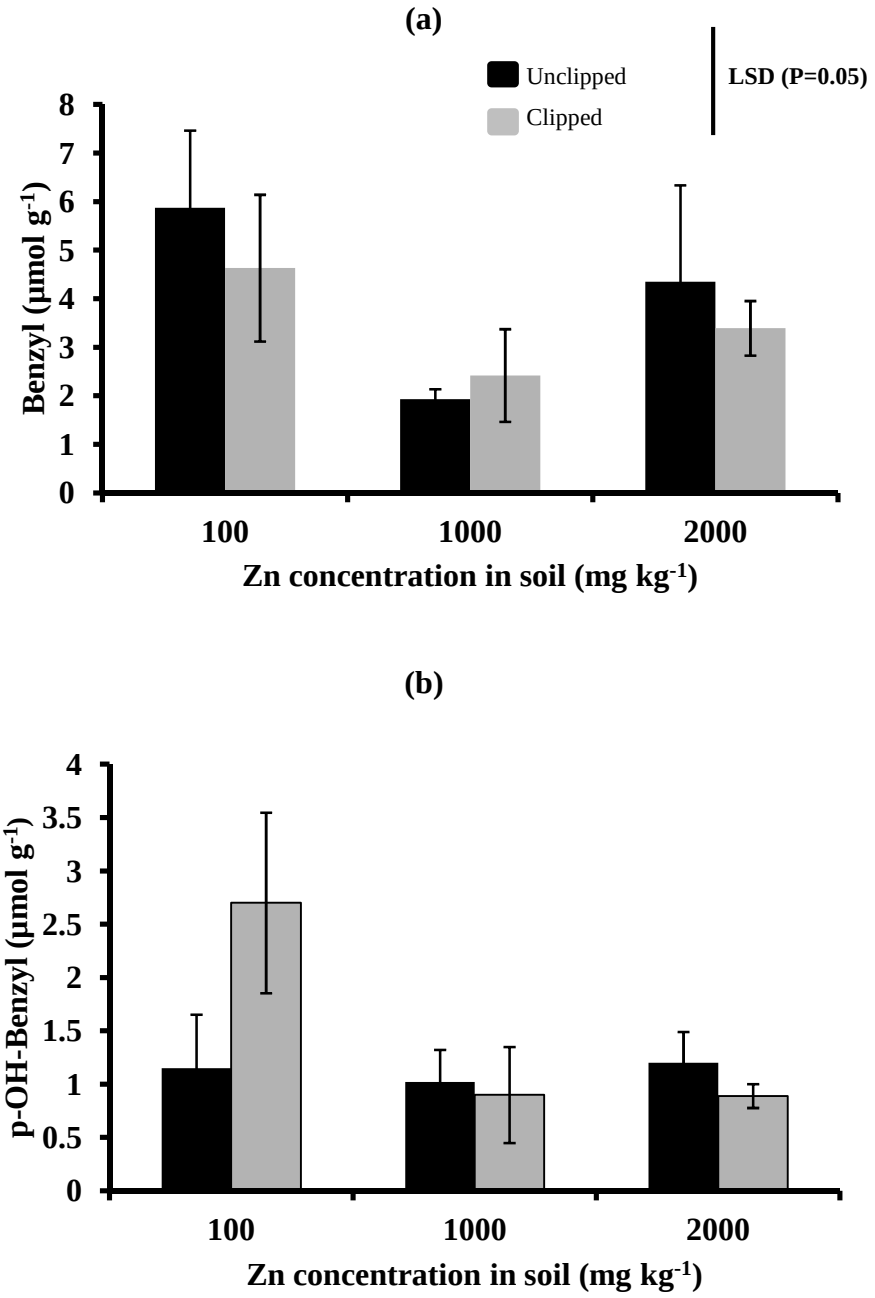


Fig. 4.4: (a) Effect of soil Zn application on foliar benzyl glucosinolate. ANOVA: Zn, P=0.024; LSD=2.2 (P=0.05), Damage, NS; Zn x Damage interaction, NS. **(b)** Effect of soil Zn application on foliar p-OH-benzyl glucosinolate. Key as before. ANOVA: Zn, NS; Damage, NS; Zn x Damage interaction, NS. LSDs (P=0.05) shown when significant effects were present.

Although no significant treatment effects were observed on p-OH-benzyl glucosinolate concentration, wounding induced foliar p-OH-benzyl at soil Zn

concentration of 100 mg kg^{-1} (Fig. 4.4 b). No induction of this GS was observed in plants growing at higher soil Zn concentrations (1000 and 2000 mg kg^{-1}). Tolrà *et al*. (2001) quantified less aromatic glucosinolate (p-OH-benzyl) in the shoots of *T. caerulescens* when exposed to higher levels of Zn in the substrate which is broadly in line with the findings here if clipped and unclipped data are amalgamated. Tolra *et al*. (2001) also observed a significant increase in aromatic GS in *Thlaspi* roots when exposed to high Zn concentrations. It can be hypothesized that aromatic glucosinolates are effective against soil borne pathogens or root herbivore insects because they are reduced in aerial biomass and are predominantly present and induced in roots. Schreiner and Koide (1993) noticed p-OH-benzyl hydrolysis products as the major antifungal compound in *Brassica kaber*. However, there are many contradictory findings in the literature particularly with regard to hyperaccumulator plants. For example, Pollard and Baker (1997) observed preferential feeding on low Zn treated *Thlaspi caerulescens* and similarly Jhee *et al*. (2005) observed that Ni hyperaccumulation in *Streptanthus polygaloides* defended the plants and not the glucosinolates. This Ni dependent defence might be due to disruption of cell vacuoles and release of Ni by tissue feeders, where Ni is present in complex ligands with organic acids like malate (Kramer *et al*. 2000; Salt and Kramer, 2000). Making a straightforward distinction between the effectiveness of metals versus glucosinolates is not easy and likely not appropriate.

Surprisingly, the accumulative amount of both individual GS was almost double that of the value for total GS in the respective tissue of both damaged and undamaged plants although variability existed. No reason was found for this variation in glucosinolate concentration, apart from the different methodologies applied for

extraction and quantifying both categories of glucosinolate. This is in contrast to the observations of Verkerk *et al*. (2001) who reported that increases in individual glucosinolates resulted in elevated levels of total GS in white cabbage (*Brassicaceae*). They also reported that not all the individual glucosinolates increased as a result of chopping the leaves of cabbage, some declined.

In the current experiment the concentration of benzyl glucosinolate was 2-9 folds higher than the p-OH-benzyl. This may be the 'natural' ratio of the two GS in this plant species or it may be the result of the growing conditions. Different GS are induced at different temperatures and other environmental factors like pH and humidity also affect GS ratios (Verkerk *et al*. 2001). Kushad *et al*. (1999) reported huge variations in individual GS in brassicas growing under different environmental conditions. Significant variations in indolic glucosinolates were observed by Verkerk *et al*. (2001) in cabbage, where significant induced increases were noted in the concentration of 3 indolyl glucosinolate, while 4-OH 3- indolyl methyl and other indolic glucosinolates remain unchanged in the same tissue of cabbage.

The use of clipping here may be the cause of a lack of response to damage as discussed above. It is possible that plants can discriminate between artificial damage and damage caused by herbivores. In a comparison of data in the literature relating to artificial and natural plant damage, Lehtilä and Boalt (2004) concluded that physiological responses were more likely to be similar between the two forms of damage than biochemical responses. However, use of artificial damage in this experiment was beneficial because it allowed for a balanced design and consistent

measurable damage. Furthermore, these data serve as a 'positive control' for the natural herbivory work that follows.

4.4.3 Response of anthocyanins to Zn concentration and clipping

Anthocyanins were strongly influenced by Zn application and clipping damage to the plants (Fig. 4.5). Soil Zn application significantly decreased (P=0.020) the level of pigment in shoots, while clipping damage increased the concentration compared to the undamaged plants (P=0.040). The maximum concentration of anthocyanins (2.44 abs. at 525 nm) in tissue was observed in the clipped control plants (with no Zn amendment) which was about 1 fold higher than the concentration in clipped plants (1.33) grown at the highest Zn concentration (2000 mg kg^{-1}). In undamaged plants, the concentration of anthocyanins responded in a similar way as for damaged plants, where maximum pigments were reported in control plants (0 mg kg^{-1} Zn) with a gradual decrease to the lowest concentrations in the plants grown with 2000 mg Zn kg^{-1} of soil. These observations are in agreement with Pongrac *et al*. (2009), who showed that anthocyanins were lowered with increasing Cd concentration in the substrate in the Cd/Zn hyperaccumulator *Thlaspi praecox*. This is an interesting observation since it could be hypothesised in the current experiment that increasing Zn availability to *T. caerulescens* alleviates stress, since this species has a requirement for Zn. However, *Thlaspi* spp. do not have the same requirement for Cd, so in the experiment conducted by Pongrac *et al*. (2009), differentiating between increased Cd and Zn uptake is likely to be difficult although these authors also observed significant reductions in leaf chlorophyll content of *T. praecox* at high doses of Cd. As a result, they speculated that anthocyanin production was a result of Zn toxicity. Anthocyanin production is often associated with nutritive stress and

Hoch *et al.* (2003) suggested that these compounds facilitate nutrient recovery in senescing tissue. Bao and Cormier (1991) and Rajendran *et al*. (1992) observed that nitrogen deficiency caused anthocyanin accumulation in grapes and later, Bongue-Bartelsman and Philips (1995) reported the same effect in tomato plants. Phosphorus deficiency has also been associated with increased anthocyanin production in grapes (Dedaldechamp *et al*. 1995; Trull *et al*. 1997).

It has also been suggested that anthocyanins have a defensive role and Karageorgou *et al*. (2008) found a relationship between anthocyanins and production of phenolics (defence compounds). It is possible in the current experiment that anthocyanin production at low soil Zn levels was either associated with defence because Zn levels were low (this would make sense if Zn is indeed accumulated as a constitutive defence), or as a result of limited nutrients resulting from relative Zn-deficiency.

These flavonoid-derived anthocyanins primarily have been reported to be induced by photo-induction in the ultraviolet, far red and visible light regions (Chalker-Scott, 1999). This is further supported by the reduced synthesis of pigments in the dark (Camm *et al*.1993; Dong *et al*. 1998), but other environmental factors have also been linked to the induction or inhibition of anthocyanins (Moll *et al*. 1996; Graham, 1998). For example, Suzuki (1995) observed enhanced levels of anthocyanins accumulation in grape cells (*Vitis hybrid*) when exposed to low pH. Similarly fungal pathogens (Dixon *et al*. 1994), signalling hormones like methyl jasmonate (Franceschi and Grimes, 1991), drought stress (Balakumar *et al*. 1993) and flooding (Anderson *et al*. 1984) are all associated with increased anthocyanins. Nevertheless, the literature is contradictory since fungal elicitors can suppress anthocyanin

production (Glabgen *et al*. 1998; Lo and Nicholson, 1998), or induce it (Dixon *et al*., 1994).

The significant increase in anthocyanin production as a result of clipping the plants with scissors agrees with the findings of Ferreres *et al*. (1997) who observed increased anthocyanin accumulation in *Lactuca sativa* (red pigmented lettuce), when shredded with a knife for storage.

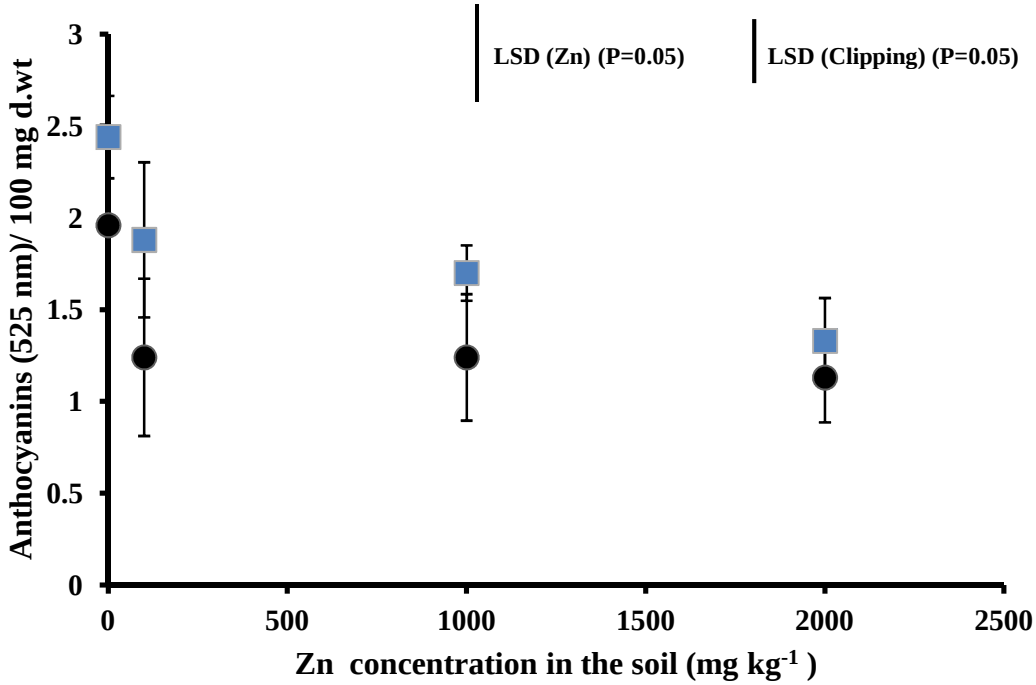


Fig. 4.5: Effect of soil Zn application and clipping on anthocyanins in *T. caerulescens*. Squares represent the clipped plants and circles represent the unclipped plants. Data are means of three replicates ± SE. ANOVA: Zn treatment, P=0.024; LSD=0.612 (P= 0.05) Clipping, P= 0.045; LSD= 0.433 (P=0.05); Zn x Clipping interaction, NS.

4.5 Key Points

- Foliar Zn uptake was lower in the second of the two experiments undertaken. No explanation is offered apart from changes in light intensity/day length or

temperature that affected the second set of plants. However it is likely that differences related to Zn addition at the seedling stage may have been a contributing factor.

- There was a significant trend towards lower concentrations of benzyl in plants grown with higher Zn amendments. This might be because plants growing in conditions of low Zn are nutrient stressed, or it might be because plants with high foliar concentrations of Zn do not need to synthesise glucosinolates. If this was the case, it would give credence to the trade-off hypothesis.

- Anthocyanin production was higher in plants growing in soil with low levels of Zn amendment. This suggests these plants were indeed stressed.

- Clipping (artificial damage) did not result in glucosinolate induction. This corroborates the findings of others (see Lehtilä and Boalt, 2004) and highlights the negative aspects of simulating herbivory. However, it also contrasts with the data presented in Chapter 3 of this thesis.

CHAPTER 5: EFFECT OF THRIPS ON INDUCTION OF GLUCOSINOLATE

5.1 Introduction

Due to their sessile nature plants are vulnerable to attack by herbivores and pathogens and have evolved many defence mechanisms as a result. Physical defences against herbivores include spines, cuticular deposits, stinging hairs and non-glandular trichomes amongst others (e.g. Harborne, 1988; Traw and Feeny, 2008). Nevertheless, chemical defences are far more important for plant survival than physical defences and these include a wide range of secondary metabolites.

Chemical defences can be classified as either inorganic or organic, depending on the chemical involved for insect deterrence. Among the inorganic defence chemicals, heavy metals are known to defend the plant against herbivores and pathogens as summarized by Boyd and Martens (1992). Another category of plant chemical defences include secondary metabolites such as glucosinolates and alkaloids. Glucosinolates as such do not hinder attack, but hydrolysis products such as nitriles and isothiocyanates have been reported to defend the plant (Halkier and Gershenzon, 2006).

Glucosinolate hydrolysis products may result in lower food use efficiency, decreased growth and reduced palatability of food (Anilakumar *et al*. 2006). Despite being present constitutively in plant organs of nearly all members of the Brassicaceae, glucosinolates are also induced by damage inflicted on the plant. However, the concentration of induced secondary metabolites depends on the type of damage

involved and the type of herbivore involved (Hopkins *et al*. 2009). Similarly the role of glucosinolates as defence compounds is very complicated and their presence in the plant challenges insect herbivores in different ways. For examples, Li *et al*. (2000) observed that the generalist herbivore *Spodoptera eridania* spent less time on, and damaged a smaller area of cotyledons of *Brassica juncea* compared to the specialist herbivore *Plutella xylostella* which spent more time on cotyledons. Therefore, metabolites that attract specialist insects for breeding or food, may also act as repellents and poisons for generalist herbivores (Rojas 1999; Miles *et al*. 2005).

Among the three major classes of glucosinolates i.e. indolic, aliphatic and aromatic glucosinolates, the indolic glucosinolates are associated with the majority of herbivore attacks irrespective of herbivore involved, while the others either decrease to undetectable levels or increase as a result of herbivory (Hopkins *et al*. 1998; Muller and Sieling 2006; van Dam and Raaijmakers 2006; Gols *et al*. 2008). The main indolic glucosinolate induced in response to herbivore attack is indol-3-ylmethyl glucosinolate. Other indolic glucosinolates such as 1-methoxyindol-3-ylmethyl increase in very small amounts after herbivory or after treatment with salicylic or jasmonic acid (Kliebenstein *et al*. 2002; Mikkelsen *et al*. 2003; Liang *et al*. 2006; Kim and Jander 2007) and according to Kim and Jander (2007), these induced levels of indolic glucosinolates have different roles in plant defence.

Herbivores from different guilds have different induction patterns of glucosinolates. For example, aphids are sucking insects and inflict less damage on plants than chewing insects (e.g. caterpillars). Phloem feeders such as aphids have been reported

to reduce the concentration of total glucosinolates in their hosts. According to Barth and Jander (2006), since sucking insects do relatively little damage to the plant they may not trigger myrosinase to come in contact with glucosinolate because both the substrate and enzyme are compartmentalized in separate cells; therefore hydrolysis to poisonous products (isothiocyanates and nitriles) may not occur. Pontoppidan *et al*. (2003) and Kusnierczyk *et al*. (2007) reported a decline in myrosinase induction after sucking based aphid feeding, so clearly myrosinase is induced to varying concentrations by different herbivores. For example, in contrast to the observations above, chewing herbivores increased the myrosinase many fold as reported by Siemens and Mitchell-Olds (1998), Pontoppidan *et al*. (2005), Martin and Müller (2007), and Travers-Martin and Müller (2007). Despite differences between sucking and chewing insects, myrosinase induction is not affected by specialist or generalist herbivores (Textor and Gershenzon, 2009). Nevertheless, it has been argued that generalists and specialists induce similar patterns of gene expression as observed by Moran *et al*. (2002) and Reymond *et al*. (2004) in the model plant species *Arabidopsis thaliana*; this relates to glucosinolates rather than to myrosinase. In some plant species aliphatic and aromatic glucosinolates are induced less readily than indolic glucosinolates by specialist herbivores relative to generalists. For example, feeding of *Brassica nigra*, *B. oleracea* and *B. napus* by two specialist herbivores *Delia radicum* and *D. floralis* increased the concentration of indolic glucosinolates (Hopkins *et al*. 1998; van Dam and Raaijmakers 2006), but caused 60% reductions in aromatic and aliphatic glucosinolates. Similarly Soler *et al*. (2005) observed 50-70% reductions in glucosinolates in *B. nigra* after feeding by two specialist herbivores, one root feeder (*D. radicum*) and one leaf feeder (*Pieris brassicae*).

Little work has been carried out on the effects of glucosinolates on the generalist herbivore, the Western flower thrip (*Frankliniella occidentalis*) and none to date on hyperaccumulators such as *Thlaspi caerulescens*. Thrips are ubiquitous generalists and therefore ideal organisms to test the elemental and joint defence hypotheses. *Frankliniella occidentalis* belongs to the insect order Thysanoptera and family Thripidae. Adults are about 1mm long and thin, red or yellow in colour, while nymphs are yellowish with red eyes. Male adults are very rare so females reproduce by parthenogenesis. Typical thrip damage includes silver discolouration of leaves (Fig.5.1).

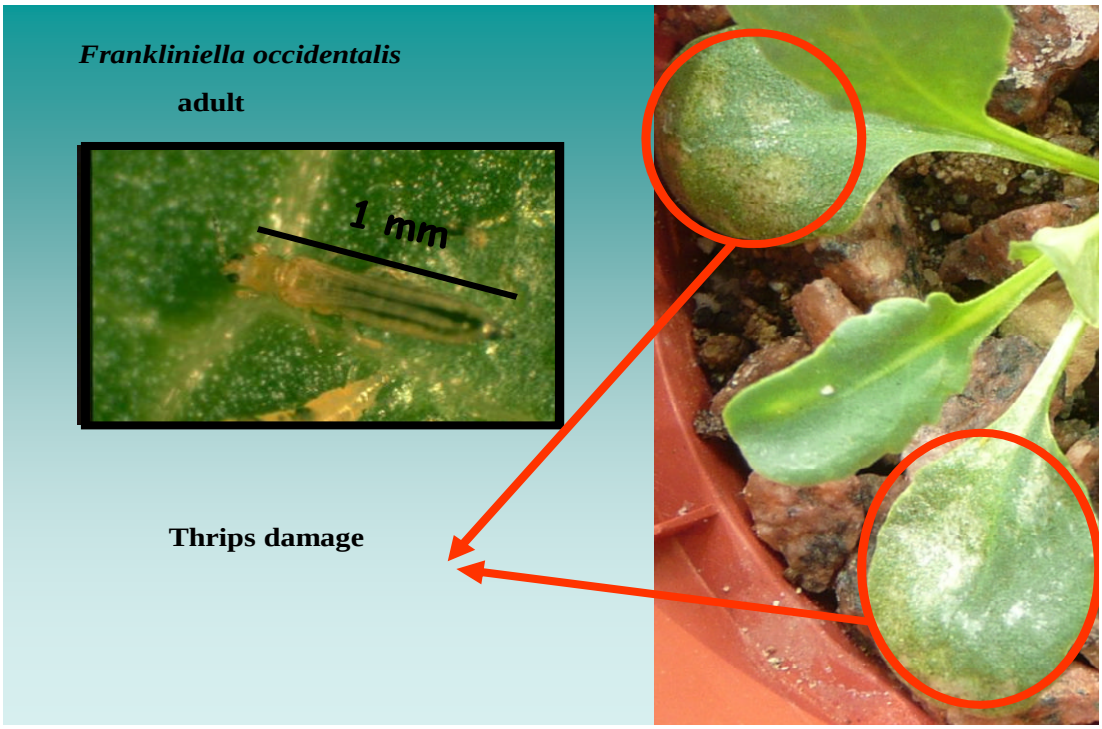


Fig. 5.1: Thrips (*Frankliniella occidentalis*) damage on *Thlaspi caerulescens* growing in the glasshouse.

The life cycle is 2-4 weeks long depending on the environmental conditions. *F. occidentalis* is a generalist folivore with worldwide distribution, including southern France, where the Ganges ecotype of *Thlaspi caerulescens* originated; so both plant

and insect naturally occur in the same ecological niche. It is a general pest for most of the agricultural crops ranging from fruits and vegetables to ornamental and cash crops.

5.2 Aims and Objectives

The main aims were to:

1) Determine whether hyperaccumulation of zinc deterred *Frankliniella occidentalis* from feeding on *T. caerulescens*.

2) Establish whether thrip damage induced glucosinolate production in *T. caerulescens.*

3) Determine if there was a relationship (trade-off) between foliar Zn and glucosinolate concentrations and if one existed, determine the effect on herbivory.

These aims were addressed by growing *T. caerulescens* at a range of Zn concentrations (0-10,000 mg Zn kg^{-1} soil) under glasshouse conditions in a thrip-infested glasshouse and observing thrip feeding preference which was quantified by degree of leaf damage. Total glucosinolates were measured in addition to individual glucosinolates in shoots of *T. caerulescens.* Measurements of foliar N, C, S, Zn and anthocyanins were made and leaf feeding damage index (LFDI) estimated.

5.3 Materials and Methods

5.3.1 Experimental setup

Thlaspi caerulescens seeds were soaked in water overnight for stratification (this softens the seed coat and enhances germination). Pre-soaked and drained seeds were germinated in a dark constant temperature room (5ºC) in seedling compost-filled (John Innes Norwich, UK) plug trays (47.0×27.5×2.0cm) without any Zn treatment. After germination was completed (10 days), the plug trays were placed on benches in a thrip-free glasshouse (as described in Chapter 2, section 2.1.1). After 17 days from germination, seedlings of uniform vigour were transplanted at one seedling pot^{-1} in experimental pots (8.0×7.5 cm), filled with potting compost (John Innes No. 2, Norwich, UK) and maintained in the same glasshouse as used for the seedlings. After transplanting, Zn was applied as $ZnSO_4.7H_2O$ to each pot to give soil concentrations of 0, 10, 16, 25, 40, 63, 100, 158, 251, 398, 631, 1000, 1585, 2512, 3981, 6310, 10,000 mg Zn kg^{-1} of compost (on a dry weight equivalent basis). Zn was surface-applied in liquid form (rather than mixing with the compost before potting) so that each plant received an individually measured amount of metal. There were 20 replicates for each treatment. Pots were laid out in a randomized block design.

Thrips were introduced to the glasshouse on some contaminated ornamental plants 10 days after transplanting. Two weeks after infestation (5 weeks from germination), the first observation of leaf feeding damage index (%LFDI) was carried out with a further measurement two weeks later (just prior to harvest). After calculating the damage index (section 5.3.2) plants were harvested and washed with tap water. Plants were gently dried on paper towels and damaged and undamaged leaves on the

same plant were separated. Harvesting was staggered to ensure that leaves were dipped into liquid nitrogen and stored at -80°C as quickly as possible after harvest. Following storage at -80^{o}C, leaves were freeze dried and stored frozen (-80^{o}C) prior to chemical analysis.

5.3.2 Leaf feeding damage index (LFDI)

Leaf feeding damage index (%LFDI) was determined by the method of Jiang *et al*. (2005). All leaves per plant were counted and observed for thrip damage. Leaves were grouped into six classes of 0, 1, 2, 3, 4 and 5, each class representing the percentage damage by thrips. Thus class 0 included leaves with no visible damage, class 1 with 0-1% damage, class 2 with 1-5%, class 3 with 5-25%, class 4 with 25-50% and class 5 with >50% damage. LFDI was calculated using equation 5.1 below.

$$\textbf{LFDI\%} = \frac{\textbf{100} \times (\sum \textbf{class value} \times \textbf{Corresponding leaf numbers})}{\textbf{(Total number of leaves} \times \textbf{6)}}$$

(Eqn. 5.1)

5.3.3 Measurement of foliar chemistry

Extraction and measurement of shoot glucosinolates, C, N, S, and Zn was carried out as described in Chapter 2 and anthocyanins were quantified using the method of Drumm-Herrel and Mohr (1982), as described in Chapter 4, section 4.2.2.

5.3.4 Statistical analyses

Leaf feeding index and foliar chemistry were analysed by accumulated Generalized Linear Models; benzyl glucosinolate concentration versus zinc application was analysed by quadratic polynomial regression and comparisons of within-plant foliar chemistry (in damaged and undamaged leaves) were compared by two-sample paired t-tests. All analyses were performed using GenStat version 13.1 (Lawes Agricultural Trust).

5.4 Results and Discussion

5.4.1 Zn application vs. uptake by plant

Thlaspi caerulescens was grown in soil amended with different Zn concentrations ranging from 0-10,000 mg kg^{-1}. The majority of the seedlings grown at concentrations greater than 3981 mg kg^{-1} died, but those in soils containing 0-3981 mg kg^{-1} Zn grew well. There was a positive relationship between foliar and soil Zn concentrations ($F_{1, 88}$ = 352.06; P<0.001) which is in line with results from previous Chapters. In this experiment, shoot concentrations were 2-4 folds higher than the corresponding soil concentrations. Interestingly at the 0 mg Zn kg^{-1} soil treatment, shoot concentration was ~2 mg Zn kg^{-1} dry weight, reflecting the background soil Zn concentration in addition to illustrating the plant's efficiency in extracting Zn. Root Zn concentrations were not determined here because roots were too fine to extract from the soil, but McGrath *et al*. (1997) observed significantly higher concentrations of Zn in *T. caerulescens* shoots than in roots, highlighting the translocation efficiency of Zn from root to shoot.

Maximum Zn uptake (14036 mg kg^{-1}) was observed in plants growing in soil amended with the highest Zn concentration (3981mg kg^{-1}); in this treatment, shoot Zn concentration was ~4 times higher than that of the soil. It would seem that *Thlaspi* could potentially accumulate concentrations of Zn beyond the maximum observed here (Fig. 5.2) but seedling death at higher soil Zn concentrations was the limiting factor in this experiment. Papoyan and Kochian (2004) recorded Zn concentrations of 30,000 mg kg^{-1} in *T. caerulescens* shoots. Similarly, Brown *et al*. (1995) and Shen *et al*. (1997) observed far higher Zn concentrations in *T. caerulescens* shoots growing in solution culture compared to those in the current investigation. *T. caerulescens* therefore has greater hyperaccumulation potential than that observed here.

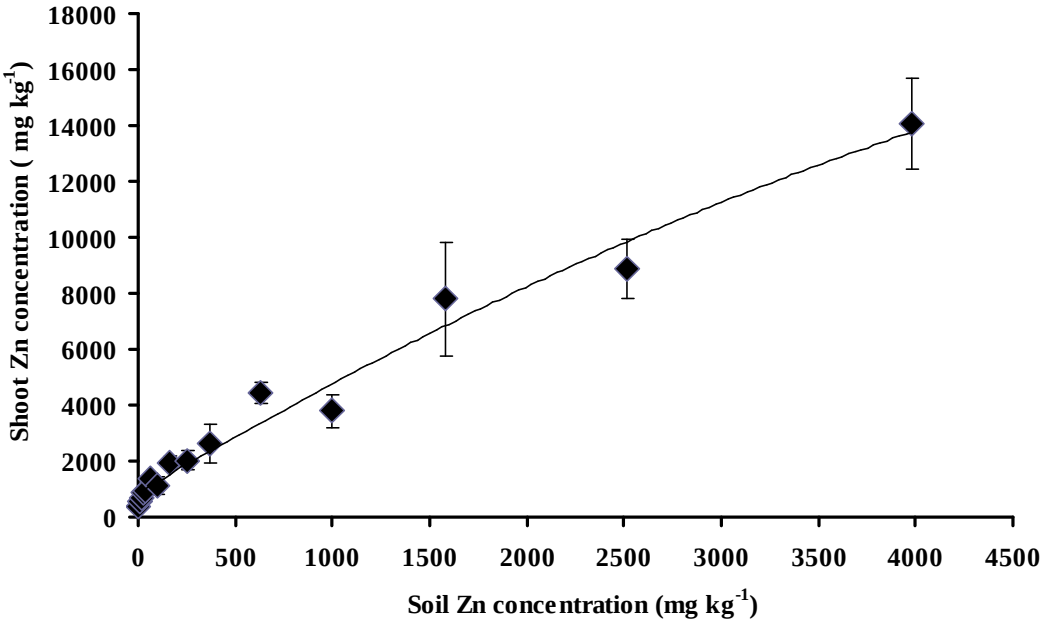


Fig. 5.2: Relationship between soil Zn application and uptake by *Thlaspi caerulescens*. Data are means of six replicates ±SE. Regression: P<0.001.

5.4.2 Thrip damage: Leaf feeding damage index

The *Thlaspi*-thrips system is complex and it is not possible to draw simple conclusions from the data because feeding activity is related to several variables including foliar Zn, glucosinolates and plant nutrition (e.g. N) and possibly *vice versa*. Therefore no one factor can unequivocally explain the feeding patterns. Furthermore, *Thlaspi* is a very variable species and this adds a further challenge. Final harvest data were therefore analysed by accumulative generalised linear models (GLM) that contrasted the effects of several variables on feeding damage. Correlations were first carried out to identify those parameters which were correlated variables. Variables from damaged and undamaged leaves of the same plant fell into this category so whole shoot data are used here. Per cent shoot N was negatively correlated with the C/N ratio; models were therefore run with either C/N or %N and %C. Initially all parameters measured were included in the model: foliar Zn concentration, foliar GS concentration, shoot dry weight, %S, C/N ratio or %N and %C, replicate block. Similarly, shoot dry weight was positively correlated with %N and C/N ratio so was not included in the analyses with these variables. When plant dry weight was included it explained a significant amount of the variation, but no more than %N and %C combined (data not shown). Replicate block was included in the model because it was significant for LFDI (although not for other variables measured). This was likely to have occurred because of directionality of movement across the blocks as thrips relocated from other plants in the glasshouse. The model was repeatedly run until the best fit was obtained; the aim was to obtain the minimum explanatory model (Table 5.1).

Table 5.1: Accumulated GLM output showing explanatory variables for LFDI (feeding damage caused by thrips). (a) The most parsimonious model, parameters were dropped following Wald F Tests to leave foliar Zn, total GS and block (position in glasshouse) as explanatory variables. (b) The model showing explanatory variables based on probability generated by regression (accumulated GLM) and not on the basis of Wald Tests.

(a)

	d.f.	s.s.	m.s.	F	P
+ Shoot Zn (mg kg^{-1})	1	1579.99	1579.99	39.81	<0.001
+ Total GS (µmol g^{-1})	1	967.70	967.70	24.38	<0.001
+ Replicate Block	5	34747.15	6949.43	175.10	<0.001
Residual	68	1944.77	39.69		
Total	75	39239.61	700.71		

(b)

	d.f.	s.s.	m.s.	F	P
+ C/N ratio	1	3550.46	3550.46	87.95	<0.001
+ Total GS (µmol g^{-1})	1	2649.02	2649.02	65.62	<0.001
+ Shoot Zn (mg kg^{-1})	1	820.48	820.48	20.32	<0.001
+ Replicate Block	5	30281.83	6056.37	150.02	<0.001
Residual	48	1937.82	40.37		
Total	56	39239.61	700.71		

The most parsimonious model (Table 5.1a) illustrates that shoot Zn and total GS both influenced thrip feeding in the current trial; total GS accounted for a significant degree of variation once that accounted for by Zn had been considered. However, Table 5.1 (b) shows that Zn accounts for less of the variation in the data if the C/N ratio is included in the model but its position in the model nevertheless indicates it is a significant factor. High foliar Zn concentrations reduced LFDI but a relationship also existed between feeding and increasing concentrations of total glucosinolates (Fig. 5.3). The efficacy of glucosinolates as a defence mechanism may be affected by foliar nutritional value and according to Hopkins *et al*. (2009) GS levels should be considered in that context. Indeed, Table 5.1(b) shows that most of the variation in LFDI is accounted for by foliar C/N ratio, with GS accounting for a lesser (but nevertheless significant) proportion of the variation in the model. This may explain

the apparently unconvincing trend when values for total GS are plotted against LFDI (Fig. 5.3 b). Foliar zinc accounts for less of the variation in LFDI than C/N ratio or total GS, yet in the first model its effect was greater than that of the total GS. This illustrates the importance of accounting for nutritional aspects when considering insect feeding trends.

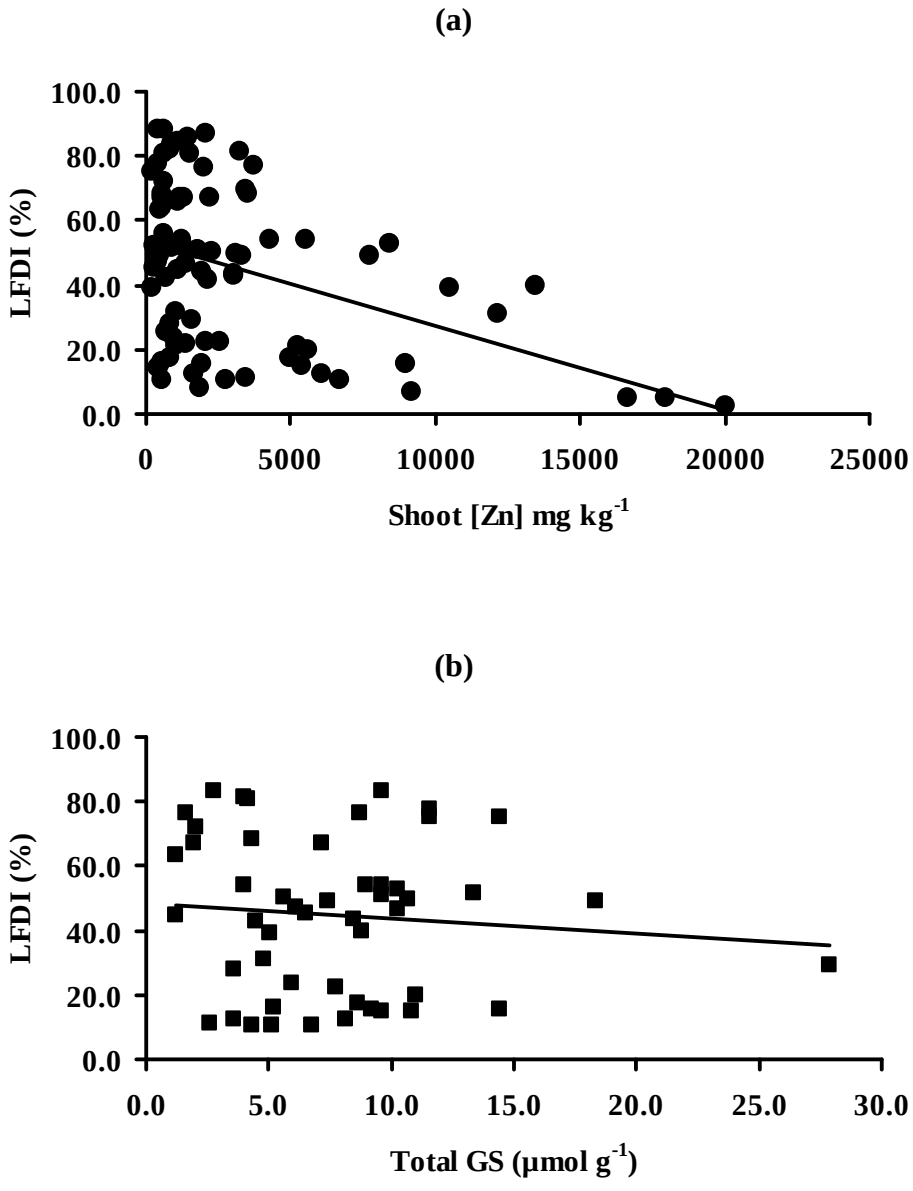


Fig. 5.3: (a) Effect of shoot Zn concentration on leaf feeding damage index (%LFDI) by thrips (*F. occidentalis*) in *T. caerulescens*. **(b)** Relationship between %LFDI and concentration of total GS in plant shoots.

Whilst foliar nitrogen was influential, it was not included in the final model because C/N ratio accounted for more variation in thrip damage than either %N or %C individually and since these correlated with C/N ratio they were omitted. Nevertheless, it should be noted that there was a significantly negative relationship (P<0.001) between %N and LFDI whilst that between C/N ratio and LFDI was positive (Fig. 5.4).

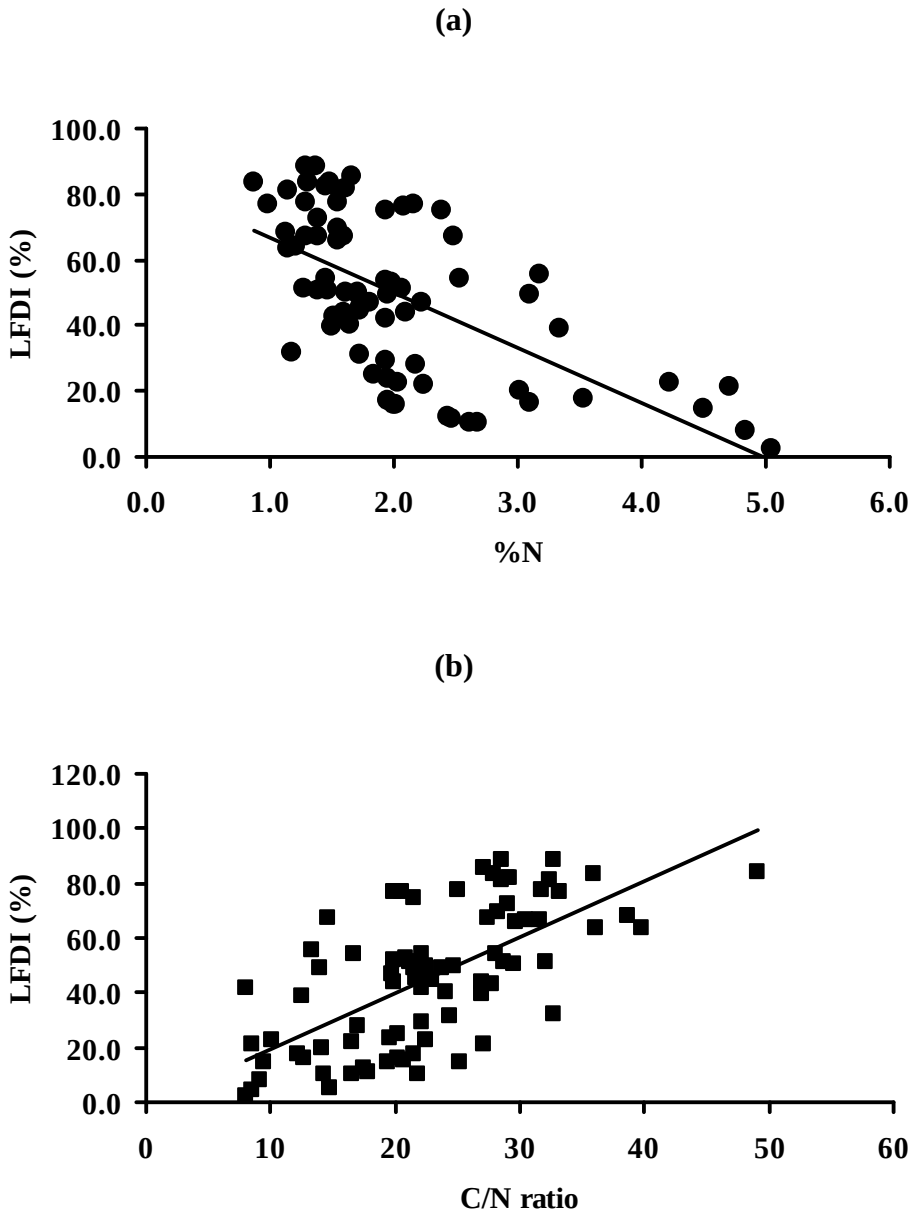


Fig. 5.4: (a) Relationship between LFDI and shoot %N (Regression: P<0.001). **(b)** Relationship between LFDI and foliar C/N ratio (Regression: P<0.001).

All plants within the experiment suffered from thrip damage, but not all leaves on each plant were attacked. By week 7, an average of 62% of leaves exhibited some degree of scarring. Undamaged leaves contained substantially higher concentrations of total GS than damaged leaves (Table 5.2). This may reflect a systemic effect arising from the damaged leaves; systemic effects are well documented (e.g. van Dam and Raaijmakers, 2006).

Table 5.2: Different foliar chemistry of thrip damaged and undamaged shoots of *T. caerulescens*. Data are means ± SE with corresponding t-test output (two-sample paired t-test). Total GS = glucosinolates; C/N ratio = carbon/nitrogen ratio.

Variable	Portion of shoot		t- value	d.f.	P
	Damaged	**Undamaged**			
Total GS (µmol g^{-1})	4.58 ± 0.49	7.81 ± 0.61	5.07	56	<0.001
Nitrogen (%)	1.84 ± 0.09	2.43 ± 0.11	-10.61	73	<0.001
Carbon (%)	40.01 ± 0.28	41.33 ± 0.28	-12.69	73	<0.001
C/N ratio	25.29 ± 1.00	19.21 ± 0.68	9.03	73	<0.001
Sulphur (%)	0.83 ± 0.05	1.11 ± 0.04	10.75	72	<0.001
Anthocyanins (Abs. 525 nm)	1.30 ± 0.07	0.74 ± 0.02	8.45	41	<0.001

However, it has recently been shown that systemic increases in GS levels are due to enhanced gene transcription (Mewis *et al*., 2006), therefore a possible explanation is that natural variability within the plant system here resulted in selection and avoidance of feeding sites by the thrips. If so, this represents considerable within-plant variability. To date, most recorded variability in GS concentrations or profiles has been between individual plants (Bidart-Bouzat and Kliebenstein, 2008) or within-leaf variations (Shroff *et al*. 2008). Generally between-leaf variability is associated with leaf age, with younger leaves containing higher concentrations of GS (Lambdon and Hassall, 2005). However, no obvious preference by the thrips for older leaves was observed, nor was there evidence of thrips preferentially eating already damaged leaves rather than moving onto undamaged leaves i.e. over a two

week period, thrip damage worsened on already damaged leaves and thrips moved onto previously undamaged leaves. Kim and Jander (2007) observed local reductions in overall GS concentrations in *Arabidopsis* leaves on which aphids were feeding, although they also reported increased production of 4-methoxyindol-3-ylmethylglucosinolate induced by aphids. Since the concentration of total GS in unscarred *Thlaspi* leaves in the current experiment was 1.7 times that of damaged leaves, it is possible that thrips caused localised alterations in foliar GS. Since glucosinolates are mobile and can be redistributed within the plant (Chen *et al*., 2001) it is feasible that *Thlaspi* either relocated GS to undamaged portions or induction in these portions occurred. It is therefore impossible to say whether GS concentrations in undamaged leaves increased due to herbivory of other portions of the shoot (i.e. a systemic effect) or instead, whether concentrations in damaged leaves fell as a result of feeding activity. Due to the small amount of plant material it was not possible to compare individual glucosinolates in scarred and unscarred tissues, but the individual glucosinolates identified in *Thlaspi* in *whole shoots* did not alter proportionally with level of feeding damage. This is to be expected since all plants had some degree of damage. It is worth noting that indole glucosinolates were not identified in *Thlaspi* and it is these glucosinolates that were altered in Kim and Jander's (2007) study; however, this does not mean they were not present, although the values for total glucosinolates (8.33 μmol g^{-1}) and the sum of the individual glucosinolates (8.53 μmol g^{-1}) were similar, which suggests if others were present they would be there in trivial concentrations. Since total glucosinolates and individual GS were extracted and quantified using different techniques, the consistency in the overall mean values is pleasing. In the current experiment, there is a weak (but significant) trend on a whole plant basis for increased thrip damage with

decreasing GS concentration. When LFDI and GS concentrations in only the damaged leaves are considered, the positive trend is lost and there is no relationship between levels of herbivory and GS concentration within the damaged components of the shoots.

Table 5.3: Models (accumulated GLM) showing explanatory variables for LFDI (feeding damage caused by thrips). Details are described in Table 5.1 above. The key difference between these data and those in Table 5.1 is the output here is generated from data relating to thrip-damaged leaves only.

(a)

	d.f.	s.s.	m.s.	F	P
+ Shoot Zn (mg kg^{-1})	1	2884.13	2884.13	64.57	<0.001
+ Total GS (µmol g^{-1})	1	24.94	24.94	0.56	<0.001
+ Replicate Block	5	43504.72	8700.94	194.79	<0.001
Residual	63	2814.16	44.67		
Total	70	49227.95	703.2		

(b)

	d.f.	s.s.	m.s.	F	P
+ C/N ratio	1	5280.77	5280.77	115.04	<0.001
+ Shoot Zn (mg kg^{-1})	1	1376.53	1376.53	29.99	<0.001
+ Total GS (µmol g^{-1})	1	682.69	682.69	14.87	<0.001
+ Replicate Block	5	35336.15	7067.23	153.96	<0.001
Residual	60	2754.16	45.90		
Total	68	45430.29	668.09		

If regressions are repeated using data from the scarred (eaten) leaves only, then total glucosinolates account for less of the variation than zinc (Table 5.3b) so in this case the explanatory model is C/N ratio + shoot Zn + GS. This difference between damaged-only and whole shoot data is not surprising since GS concentrations were greater in the unscarred leaves and there was no herbivory. It could be argued that intuitively GS levels explain the pattern of thrip damage since leaves with maximum GS concentrations were thrip-free (Table 5.2). Clearly GS concentrations were markedly higher in undamaged leaves. However, in simple linear regressions of whole shoot data, foliar Zn was a significant single explanatory variable (P=0.011)

for LFDI whilst GS was not significant, further exemplifying the complexity of the GS system, but also illustrating that zinc plays a role in reducing thrip damage.

The only work to date to consider tolerance of *F. occidentalis* to glucosinolates was carried out by Abe *et al*. (2008) but these authors actually focused on jasmonic acid in *Arabidopsis* rather than on GS *per se*. The authors found that jasmonic acid enhanced resistance against thrips and concluded this was *via* a jasmonic acid-regulated defence response. Since glucosinolate production is regulated by jasmonic acid (Sasaki-Sekimoto *et al*. 2005), it is likely that glucosinolates were at least in part responsible for reducing thrip damage in their study. In the current investigation, foliar sulphur concentrations explained a small amount of the variation after that accounted for by GS (in whole shoot data), but since glucosinolates contain sulphur, it was not included along with glucosinolates in the model.

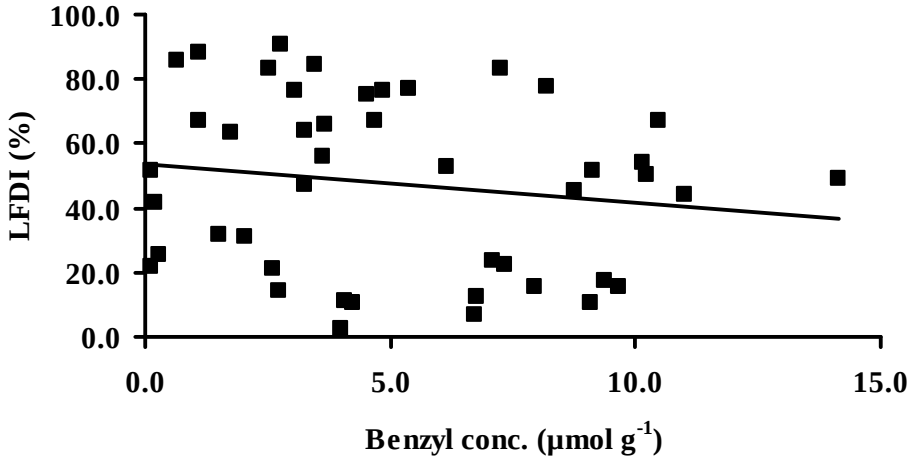


Fig. 5.5: Reduction in thrip damage associated with increasing benzyl concentration. (P=0.056).

There was a weakly significant relationship (P=0.056) between foliar benzyl concentration (one of the individual glucosinolates quantified) and LFDI, whereby

reduced damage was observed in plants with higher concentrations of benzyl. The effect was only observed after the variation caused by blocking was accounted for in the accumulated GLM analysis (block + benzyl).

Hopkins *et al*. (2009) concluded that different glucosinolates have variable activity depending on insect species. None of the other individual glucosinolates (p-OH-benzyl, 'GS-A' or 'GS-B') showed any significant relationship to LFDI although there was a trend for herbivory to decrease with increasing p-OH-benzyl concentrations. Benzyl was present in the highest concentration in the plants (overall average of 5.14 µmols g^{-1}) so it is perhaps not surprising that it should have an effect on LFDI. An overall mean of 3.06 µmols g^{-1} of p-OH-benzyl was observed, with 'GS-A' and 'GS-B' present in relatively low concentrations (0.13 and 0.41 µmols g^{-1} respectively). Benzyl and p-OH-benzyl are both aromatic glucosinolates and there was a significant positive (P<0.001) relationship between the two (Fig. 5.6).

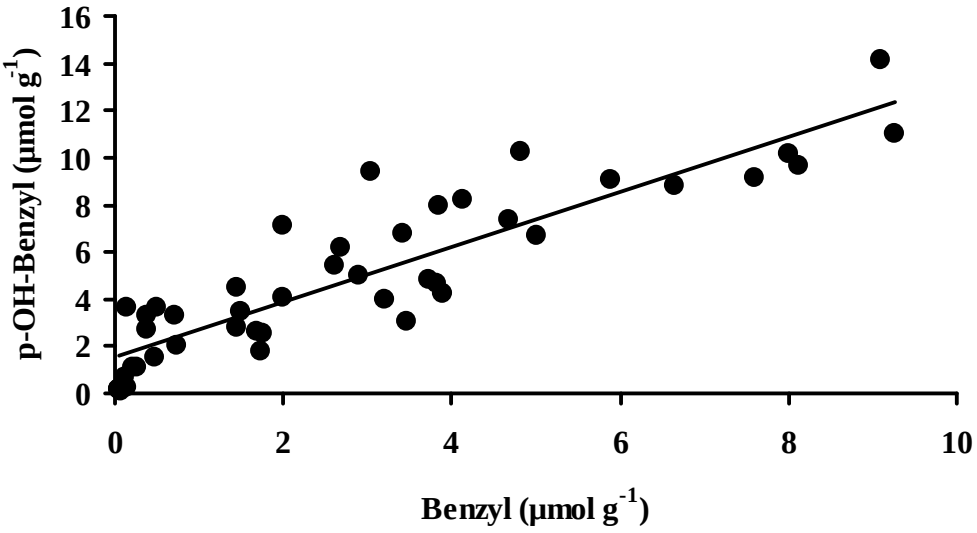


Fig. 5.6: The positive relationship between two aromatic glucosinolates identified in *T. caerulescens* shoots, benzyl and p-OH-benzyl (Regression: P<0.001).

Similarly, there was a positive relationship between unidentified 'GS-B' and p-OH-benzyl ($P=0.007$) and 'GS-B' and benzyl ($P<0.001$). Whilst only benzyl was 'statistically responsible' for reducing LFDI, it would therefore be incorrect to separate the biological effects of the individual glucosinolates.

Whilst higher concentrations of glucosinolates reduced thrip damage, the ability of the thrips to apparently thrive on the plants is interesting. It is possible that damage caused by the thrips was insufficient to activate myrosinase; however this seems unlikely since there was a significant relationship between GS production and LFDI and total glucosinolates accounted for a large proportion of the variation in the feeding data (Table 5.1). Furthermore, the higher concentration of total GS present in undamaged leaves is indicative of GS mobilisation in response to feeding (Chen *et al*. 2001), although there could be other explanations as outlined above. Tolrà *et al*. (2006) demonstrated that cadmium increased the level of total glucosinolates in *T. praecox* and attributed this to enhanced benzyl production. In the current investigation, increased total GS concentrations were associated with reductions in herbivory but this relationship was only significant when other parameters were included in the analysis (e.g. foliar Zn and/or C/N ratio), whereas the effect of benzyl on herbivory was only dependent on replicate block (position in glasshouse). This suggests that GS-related reductions in LFDI were primarily a consequence of benzyl synthesis.

No work to date has been carried out to determine how thrips detoxify glucosinolates, although the generalist aphid *Myzus persicae* excretes GS and favours older leaves, i.e. those with lower GS concentrations (Merritt, 1996). Thrips

suck the contents of epidermal cells and their liquid excrement is visible on leaves; it is therefore possible that *F. occidentalis* is also able to excrete glucosinolates, although further work would need to be conducted to verify this hypothesis. The glucosinolate concentrations in the *Thlaspi* are in line with those measured in several accessions of *T. caerulescens* by Noret *et al*. (2005); these authors demonstrated that GS concentrations affected snail herbivory in choice tests to a greater extent than Zn concentrations. The individual glucosinolates identified (benzyl and p-OH-benzyl) were also extracted from *T. caerulescens* by Tolrà *et al*. (2001, 2006) together with alkenyl and indole, although only aromatic glucosinolates were identified in the current study. In many plant species aromatic glucosinolates are usually present in lower concentrations than the indolic glucosinolates; the indolic GS is the group most often recorded as increasing due to herbivory although responses by the aromatic class are also known (see Textor and Gershenzon, 2009 and references therein). It is clear from Tolrà *et al*. (2006) that different types of GS are observed in different species of *Thlaspi*.

T. caerulescens hyperaccumulates Zn and approximately 80% of foliar Zn is in water-soluble form (Zhao *et al*. 1998). Mechanisms for detoxification of Zn by plants revolve around vacuolar storage and in particular the mesophyll is the main storage site because of its mass relative to the upper and lower epidermis (Ma *et al*. 2005). Despite most of the Zn being present in the mesophyll, the upper epidermis actually contains a higher concentration of Zn than other portions of the leaf (Küpper *et al*. 1999; Ma *et al*. 2005). It would be expected therefore, that thrips would be subjected to high concentrations of Zn when feeding on *Thlaspi* due to their scraping and sucking means of feeding but despite this; they were only deterred at the highest

Zn concentrations. Since *Thlaspi* requires Zn for healthy growth, it is likely that Zn uptake influenced foliar N and other nutrients which in turn would have affected resource quality and subsequently herbivory. Accumulated GLM showed that increased shoot dry weight was related to foliar Zn only after %N had been accounted for. Simple linear regression showed a positive relationship between foliar %N and foliar Zn ($P=0.043$) inferring healthier plants with increasing Zn concentration. Nevertheless, this apparent improvement in foliar nutrition did not benefit the thrip populations since increasing N actually reduced damage (Fig. 5.4 above). This contrasts to the findings of Brodbeck *et al*. (2001) and Atakan (2006) who stated that *F. occidentalis* populations respond positively to nitrogen fertilisation of crops. The observation that Zn is detrimental at very high shoot concentrations corroborates the findings of Noret *et al*. (2007) who suggested a threshold of between 2700 and 5400 mg kg^{-1} Zn below which herbivory was unaffected. Various studies have demonstrated antifeedant properties of high concentrations of Zn in *Thlaspi* (Pollard and Baker, 1997; Jhee *et al*, 1999). It should be noted that these studies considered generalist chewing and rasping invertebrates (e.g. locusts and slugs) and apart from the paper by Jiang *et al*. (2005), no other work has been conducted into the effects of foliar Zn on thrip feeding activity. Interestingly Jiang *et al*. (2005) found no correlation between shoot Zn and leaf damage by thrips in *T. caerulescens* although they did show that foliar Cd reduced feeding. The highest Zn concentration in their study was <10,000 mg kg^{-1} DW compared to a maximum of >14,000 mg kg^{-1} DW in the current investigation (Fig. 5.3). These authors did not consider other aspects of plant nutrition (e.g. %N) and it is clear from the current investigation that both glucosinolates and C/N ratio should be considered too. Jiang *et al*. (2005) did not relate feeding to glucosinolate levels in

their plants, but instead measured sulphur as a proxy to GS. They found no relationship between feeding and foliar S concentrations. Wild populations of *T. caerulescens* (Ganges) accumulate between 1,000-53,000 mg Zn kg^{-1}, depending on the availability of metal in the soil medium (Reeves *et al*. 2001); at these levels it is likely that Zn acts as a deterrent and the uptake values observed in the current study are realistic.

Leaf feeding damage index (LFDI) was determined on two occasions; the first was 5 weeks after germination and was compared to *soil* Zn amendment (rather than *foliar* Zn concentration) because it was a non-destructive measure which could be carried out part-way through the experiment. The damage assessment (LFDI) seven weeks after germination coincided with the final destructive harvest so these data were included in the model above (i.e. LFDI and *foliar* Zn). Initial determination showed that increasing Zn application to soil led to a reduction in LFDI (Fig. 5.7). The average LFDI in week 5 was 7.8% whilst in week 7 it was 47.6%. *F. occidentalis* is a highly invasive species and it is not surprising that high levels of leaf damage occurred relatively quickly. What is interesting is the level of feeding activity by a generalist species on plants containing both high concentrations of Zn and also glucosinolates. Neither thrip numbers nor reproduction on the *Thlaspi* plants was measured and it is possible that thrips were breeding elsewhere in the glasshouse. However, the explosion in feeding damage over a two week period (5 to 7 weeks) suggests that thrips were certainly thriving (and likely reproducing) on the plants. Perhaps intraspecific competition was such that less favourable leaves were utilised, thus masking observations of any localised feeding preferences; nevertheless, the

species of thrip here is a generalist and should have been deterred by the glucosinolates.

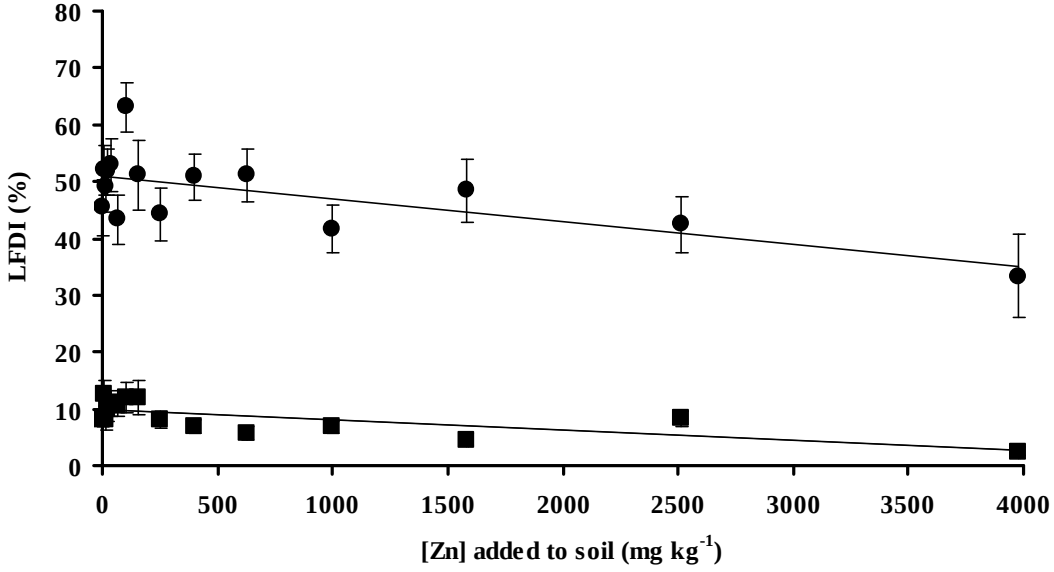


Fig. 5.7: Effect of Zn concentration on Leaf Feeding Damage Index (%LFDI) by thrips (*Frankliniella occidentalis*). Squares represent %LFDI, 5 weeks after germination and circles represent %LFDI, 7 weeks after germination. Values are means of 20 replicates ±SE. Regression: P< 0.001 for both 5th and 7th week after germination.

5.4.3 Effects of zinc application and foliar N, C and S on glucosinolates

The main aim of this investigation was to determine whether glucosinolate induction would be modified by increasing foliar zinc concentration and what impact changes in foliar chemistry would have on thrip feeding activity. However, it is clear that other aspects of foliar chemistry are linked to GS content. The selected model for leaf herbivory which explained the highest proportion of variance was C/N ratio + GS + Zn (Table 5.1a). These parameters are inter-related and this is illustrated (Fig. 5.8b) by the relationship between total GS and shoot C/N ratio (Regression: P=0.042). There was no effect of leaf C on GS concentration, but %N and GS were

positively correlated (Fig. 5.8a). When LFDI was included in an accumulated regression with C/N ratio (C/N + LFDI) the level of significance of the C/N ratio (P=0.037) was increased and C/N ratio and LFDI explained a similar degree of the variation in GS concentrations. However, including LFDI in the regression with %N was only marginally better than the simple regression with %N and total GS. Both %N and the C/N ratio influenced total GS concentration and both are good indicators of foliar value in this system. Glucosinolates are considered to be nitrogen-based secondary defence compounds and it is therefore not surprising that %N should affect concentrations. The carbon/nutrient hypothesis (Bryant *et al*. 1993) states that enhanced C/N ratio will lead to increased production of C-based defence compounds, but the corollary of this is that in plants (such as *Thlaspi* and other crucifers) where N-based defences are important, a decrease in C/N ratio will result in greater production of N-based compounds. This is borne out by the data in this experiment and furthermore it may explain why LFDI was higher in plants with lower leaf N concentrations (Fig. 5.4).

Although glucosinolates are N-based defences, only 0.60% of the foliar N was incorporated into GS in the experimental *Thlaspi* (and 0.25% of C). This is in line with the findings of Traw and Feeny (2008) who estimated the percentage of N and C incorporated into GS to be 0.45% and 0.38% respectively in *Brassica nigra*.

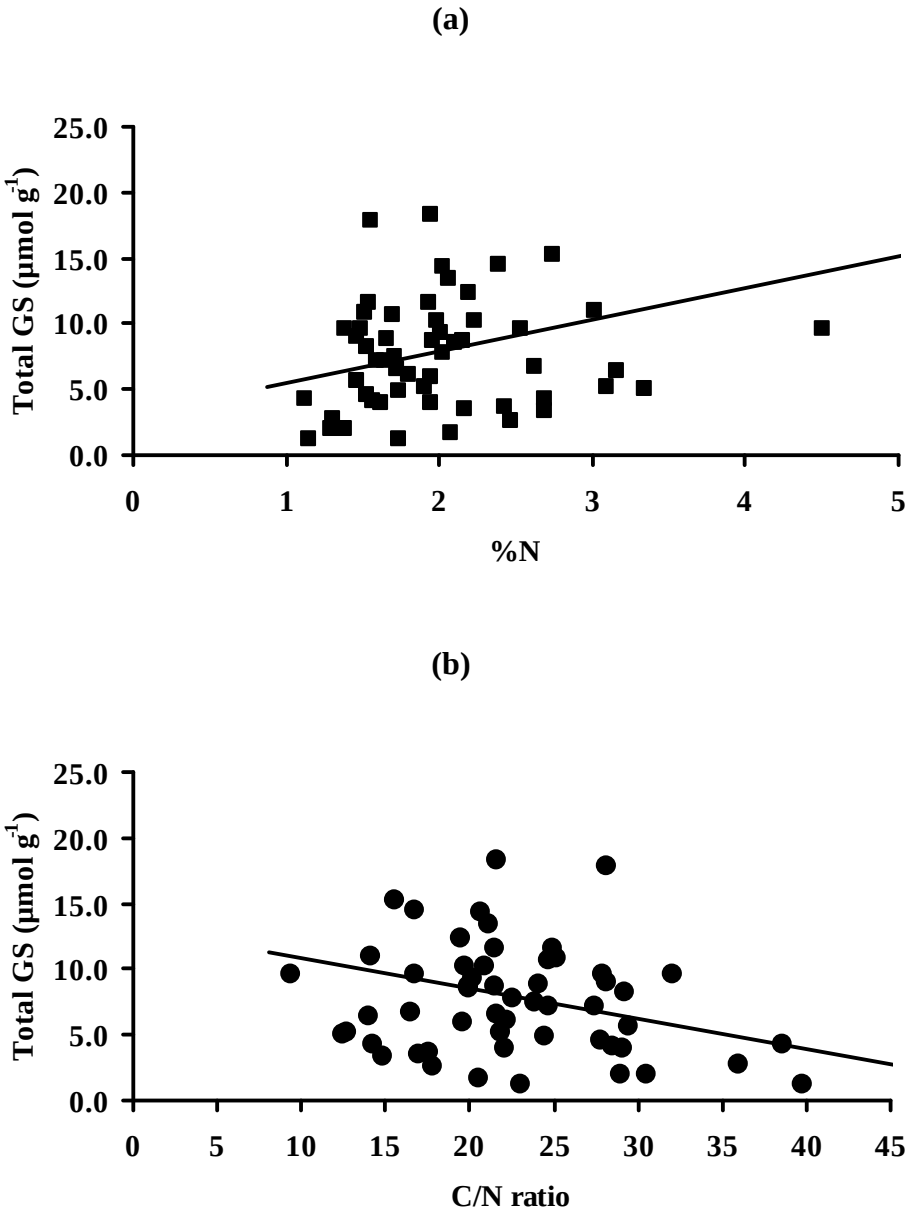
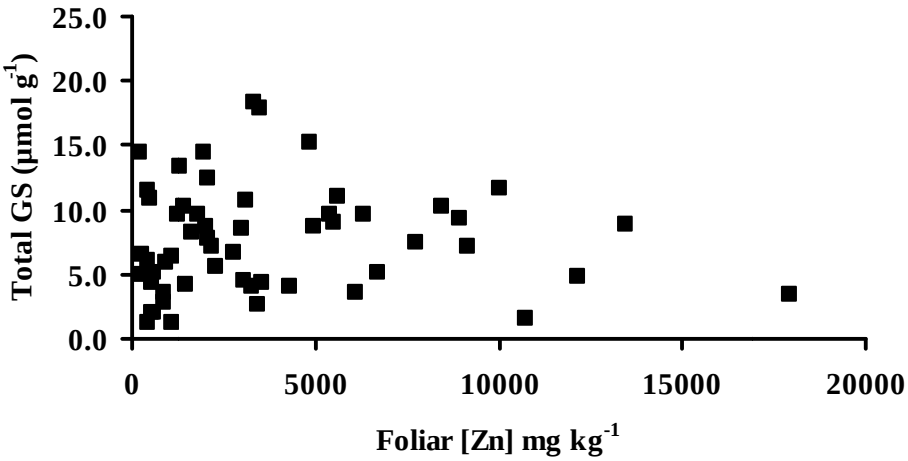


Fig. 5.8: Relationship between **(a)** total GS concentrations and %N in *T. caerulescens* shoots and **(b)** total GS and C/N ratio in *T. caerulescens* shoots (Regression: P=0.015 and P= 0.042 respectively).

Estimations of the N and C fractions allocated to GS in the current study were based on the method described by Traw and Feeny (2008). One modification to the method was made and that was the inclusion of S; each GS molecule was presumed to contain 2 atoms of S and the per cent total S partitioned into glucosinolates was calculated as for N and C. It was estimated that 6.0% of the sulphur in *T.*

caerulescens in this investigation was incorporated into total GS. Despite glucosinolates containing a substantial amount of S (some estimates state as much as 55% sulphur, e.g. Jiang *et al*. 2005) there was no relationship here between %S and foliar GS concentration. Foliar zinc did not appear to affect total GS concentrations (Fig. 5.9a) but if foliar Zn is categorised into broad concentration ranges, a trend towards decreasing GS with increasing Zn is observed (Fig. 5.9b).

(a)



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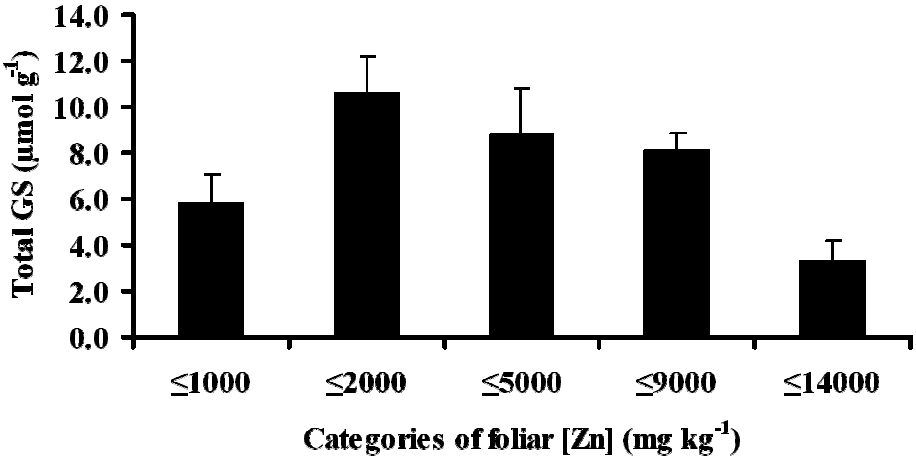


Fig. 5.9: (a) Total foliar glucosinolates versus foliar Zn. No significant relationship was observed. **(b)** Total foliar GS present within categories of foliar Zn ± SE.

The lower levels of GS at foliar concentrations of <1000 mg kg^{-1} likely reflect the nature of the plant species. *T. caerulescens* has a requirement for Zn and shoot growth is stunted at soil concentrations of less than 500 mg kg^{-1} (~2000 mg kg^{-1} foliar Zn) (Chapter 3). Since glucosinolates are metabolically expensive to produce because biosynthesis involves a series of complex pathways (Mithen, 2001), it follows that GS production will be reduced in these plants. Further examination indicates that foliar benzyl concentrations increase with increasing soil Zn application and decrease with higher concentrations of applied Zn (second order polynomial regression, P<0.001; Fig. 5.10).

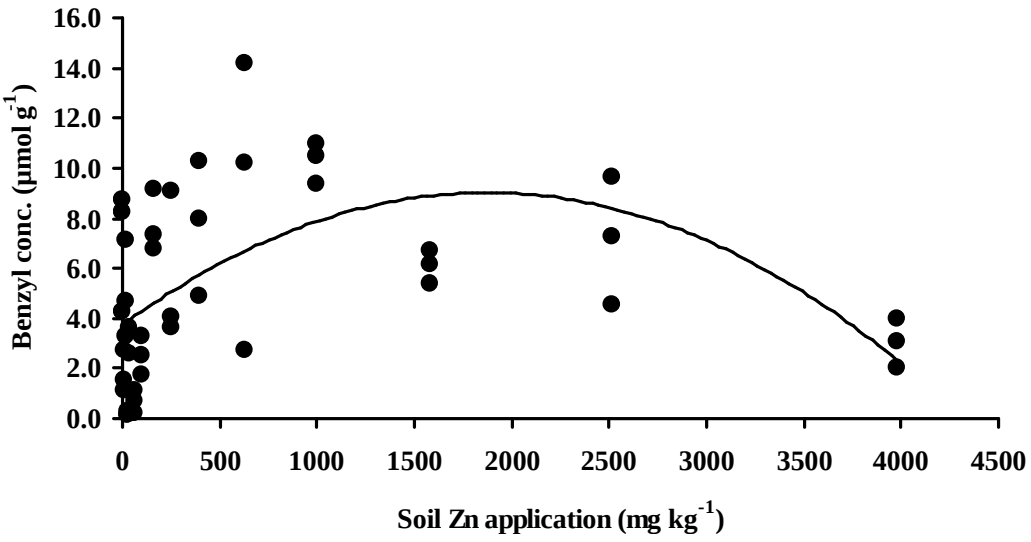


Fig. 5.10: Relationship between foliar benzyl glucosinolate in *T. caerulescens* and soil Zn application. (Second order polynomial regressions: P<0.001).

Foliar Zn was not measured in these plants because of insufficient material, but the correlation between soil Zn application and foliar Zn (Fig. 5.2) content lends weight to the argument that at very high foliar Zn concentrations GS production is less and also supports the earlier suggestion that an optimum foliar Zn concentration is

required for glucosinolate synthesis to occur. p-OH-benzyl followed a similar trend (second order polynomial regression, P=0.015, data not shown).

5.4.4 Anthocyanin production and effects of zinc treatment and thrip damage

Determination of anthocyanin production showed that *T. caerulescens* leaves subjected to thrip feeding produced higher concentrations than leaves that had not been damaged by thrips (Table 5.2 above); therefore there were significant within-plant differences related to the level of herbivory. This is interesting because other foliar parameters measured, e.g. total GS, %N and C/N ratio were all higher in the undamaged leaves.

Table 5.4: Model (accumulated GLM) showing explanatory variables for LFDI (feeding damage caused by thrips). Anthocyanins were included in the analysis. These data were analysed separately from those in Tables 5.3above, because anthocyanin data were not available on a whole plant basis. This analysis is for the damaged portions of the leaves only.

	d.f.	s.s.	m.s.	F	P
+ C/N ratio	1	1742.16	1742.16	66.07	<0.001
+ Anthocyanin (525 nm)	1	806.92	806.92	30.60	<0.001
+ Shoot Zn (mg kg^{-1})	1	270.43	270.43	10.26	<0.004
+ Total GS (µmol g^{-1})	1	214.33	214.33	8.13	<0.009
+ Replicate Block	2	5151.19	2575.59	97.67	<0.001
Residual	24	632.87	26.36		
Total	30	8817.90	293.93		

Anthocyanin data are not available for the whole plant, but they are available for damaged and undamaged leaves of the same plant. Analysis of the damaged leaves by accumulated GLM regression indicated that anthocyanins explained more of the variation in the feeding data than did glucosinolates. Despite this, only foliar zinc and C/N ratio were significant as single factors in simple linear regressions. Three replicate blocks were analysed rather than 6 for logistical reasons.

Such a finding is perhaps not surprising since it has already been established above that GS in the damaged leaves had little effect on herbivory relative to Zn. What is interesting is the effect of anthocyanins on LFDI. Previous work by Irwin *et al.* (2003) demonstrated that *F. occidentalis* did not differentiate between anthocyanin-recessive or -dominant morphs of wild radish; therefore the anthocyanin levels did not influence the thrips. They observed a significant interaction between damage and anthocyanin-producing morphs in which plants partially eaten by *Pieris rapae* (cabbage-white butterfly) larvae induced higher concentrations of indole glucosinolates than damaged recessive morphs. This is interesting since it illustrates some relationship/interaction between GS and anthocyanins. It cannot be implied from their results that *F. occidentalis* was unaffected by induced indole glucosinolates because the caterpillars were fed on leaf material whilst the thrips were flower feeders. Therefore, whilst conclusions can be drawn with regard to anthocyanins and thrip preferences, similar conclusions cannot be made for GS content and thrip feeding.

The reason for the increase in anthocyanins in the damaged leaves in this current experiment is possibly related to nitrogen mobilisation into undamaged leaves, presumably for GS production. Certainly, both N and GS concentrations were higher in undamaged leaves so this lends some credence to the hypothesis. Hoch *et al.* (2003) suggested that in senescing tissue, anthocyanins reduce photo oxidative stress on the chloroplasts and facilitate nutrient recovery. There is much debate in the literature about the role of anthocyanins and it has been suggested that they act as a signal to warn insects that nutrient levels are low; this benefits the plant by deterring herbivores. Karageorgou *et al.* (2008) demonstrated (providing different

developmental stages were accounted for) a relationship between anthocyanins and production of phenolics (defence compounds). Brassicas are known contain phenolics although they were not quantified in this investigation. It is interesting to speculate about the importance of these compounds. In most studies of *Thlaspi*, phenolics and/or anthocyanins have generally been ignored, although Pongrac *et al*. (2009) measured anthocyanins produced by *T. caerulescens* in response to Zn and Cd. They demonstrated that anthocyanins increased with increasing soil Zn amendment (50, 250 and 500 mg kg^{-1}, which equated to around 2500 – 3000 mg kg^{-1} foliar Zn). They speculated that anthocyanin production was a result of Zn toxicity. However, in the current experiment, anthocyanin levels in damaged leaves were primarily explained by LFDI (P=0.009) but only after the variation resulting from foliar Zn and total GS was accounted for (although these were not significant factors; GLM). If the data are analysed by ANOVA with herbivory reduced to two levels (damage or no damage) and soil Zn application used as factors instead, the trends are clearer (Fig. 5.4). Zn treatment had little effect on anthocyanin production which is in contrast to the findings of Pongrac *et al*. (2009), but this is not surprising since the plant is a hyperaccumulator and Zn should not act as an abiotic stressor at these experimental levels. Nevertheless, there is a trend towards an increase in anthocyanins at lower concentrations of Zn although ANOVA did not show an herbivory x Zn interaction. This is likely to be the cause of the foliar Zn effect when data were analysed by GLM.

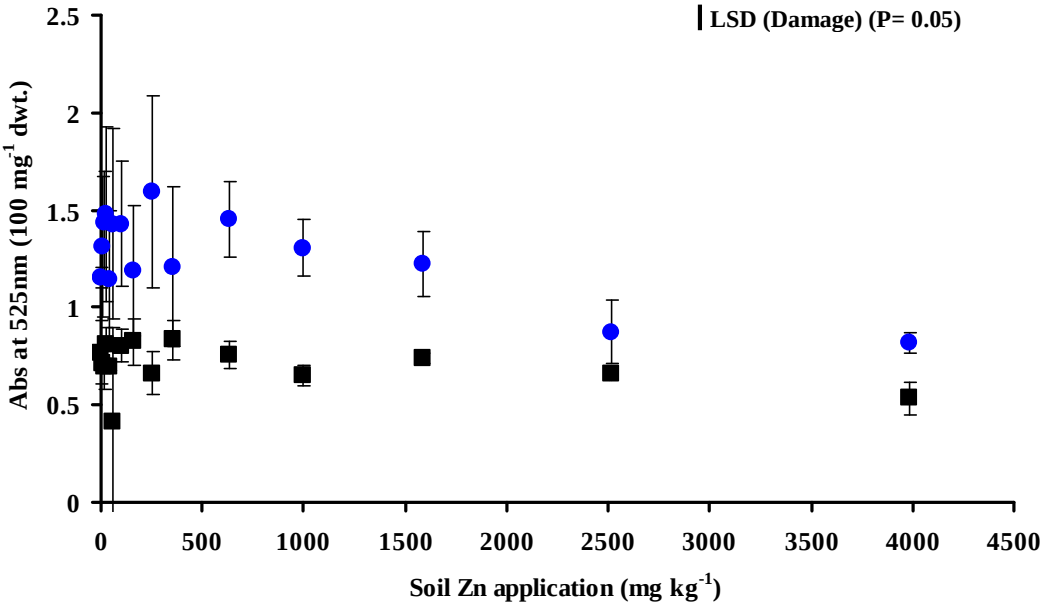


Fig. 5.11: Effect of natural herbivory by thrips (*Frankliniella occidentalis*) on *Thlaspi caerulescens* growing in soils amended with a range of Zn concentrations. Blue circles represent damaged leaves and black squares represent undamaged leaves on the same plant. ANOVA: Leaf damage was a significant single factor, $F_{1, 58}$ = 46.28, P<0.001; LSD= 0.164 (P= 0.05).

In contrast, many heavy metals have been reported to inhibit anthocyanin biosynthesis by inactivating PAL (L-phenylalanine ammonia-lyase) (Holton and Cornish, 1992), which is the main enzyme involved in flavonoid biosynthesis (Dube *et al*. 1992; Krupa *et al*. 1996). According to Sakihama *et al*. (2002), flavonoids are involved in plant responses when hyperaccumulating heavy metals because of their antioxidant properties (Gould *et al*. 2002). Holton and Cornish (1992) explained observed decreases in anthocyanin production (particularly under Ni stress) by a redirection of synthesis away from anthocyanins to phenolics.

It is clear from the data that the simple 'elemental defence hypothesis' or indeed the 'joint defence hypothesis' will not fully explain the mechanisms that determine feeding preferences and outcomes by thrips on *Thlaspi caerulescens*. Multiple

factors influence feeding and also foliar chemistry and these factors are interwoven and likely to vary with season and environmental conditions. To complicate the situation, anthocyanins may play a defensive role and these are not produced without a metabolic cost. It would seem prudent in further studies to consider other likely defences in addition to Zn hyperaccumulation and GS production.

5.5 Key Points

- Shoot Zn concentration was positively correlated with soil Zn concentration. More than 14000 mg Zn kg^{-1} was observed in the dry mass of *T. caerulescens*, while soil Zn concentration was 3981 mg kg^{-1}.

- Soil and plant Zn concentrations both negatively influenced leaf feeding damage by thrips (*Frankliniella occidentalis*). Feeding damage was >40 higher in older plants (7 weeks from germination) as compared to the younger ones (5 weeks from germination).

- Analysis of feeding data (LFDI) by accumulated GLM showed the model that explained most of the variation in the data to be: C/N ratio + total GS + shoot Zn.

- Efficiency of glucosinolates as a defence is affected by C/N ratio.

- Undamaged leaves had higher glucosinolate concentrations than damaged leaves on the same plant. This may be because of remobilisation or due to selective feeding.

- Data show an increase in glucosinolates (especially benzyl) with increasing soil Zn and a decrease at the highest Zn concentrations.

- Four individual GS (benzyl, p-OH-benzyl, ‘GS-A’ and ‘GS-B’) were quantified and benzyl was by far the most abundant GS.

- Anthocyanins were present in damaged leaves at higher concentrations than in undamaged leaves on the same plant, possibly playing a role in N mobilisation to undamaged leaves. Zn application did not affect anthocyanin production.

- The data show that foliar nutrition, Zn and GS all play a role in responding to herbivory. It is possible that anthocyanins also play a defensive role in this system but further experiments need to be carried out to determine this. Whilst Zn deterred thrips at high concentrations, the involvement of the other factors suggest that the simple ‘elemental defence hypothesis’ is unlikely to be upheld with this system.

CHAPTER 6: EFFECTS OF ZINC HYPERACCUMULATION ON *ALEYRODES PROLETELLA* INFESTATION AND ON FOLIAR GLUCOSINOLATES

6.1 Introduction

Thlaspi caerulescens is a zinc hyperaccumulator and as such can take up concentrations of Zn that would be toxic to non-hyperaccumulator species. For instance Zn concentrations may be 1000-fold higher in hyperaccumulators than in non-hyperaccumulatng species. One hypothesis to explain hyperaccumulation is that of 'plant defence' (Boyd and Martens 1992; Boyd 1998). Hyperaccumulated metals may defend plants against attack by a plethora of herbivores including both generalists (Pollard and Baker 1997; Jhee *et al*. 1999) and specialists (Jhee *et al*. 2006). For example, Pollard and Baker (1997) observed preferentially less feeding by generalist locusts (*Schistocerca gregaria*) and by the specialist caterpillar (*Pieris brassicae*) on *T. caerulescens* plants containing high Zn concentrations, compared with plants containing lower Zn concentrations. Similarly Boyd and Moar, (1999) observed slow development of *Spodoptera exigua* larvae when fed with leaves of the Ni hyperaccumulator *Streptanthus polygaloides*, as compared to those animals fed on leaves with lower Ni levels. According to Ghaderian *et al*. (2000), hyperaccumulation of heavy metals (especially Zn, Ni and Cd) by plants has proved to be an effective deterrent against herbivores and pathogens.

Organic metabolites (e.g. glucosinolates) are another effective defence against herbivores, but due to high biosynthetic costs (Ernst, 1990), substitutive metal based inorganic defences may be beneficial for the host plant (Boyd, 1998). According to

the ‘trade-off’ hypothesis, hyperaccumulator plants induce less glucosinolate (GS) because the excess heavy metal accumulation acts as a constitutive defence. Sasse (1976) and Mathys (1977) observed a substantial decrease in GS in Zn and Ni hyperaccumulating metallophytes.

Despite the high biosynthetic cost of GS, they are an effective deterrent against herbivores. As already discussed in Chapter 1, glucosinolates are naturally present in plant species, especially members of the Brassicaceae but are also induced as a result of damage to the host plant. Textor and Gershenzon (2009) observed that GS inducibility varies depending on the type of damage, time and herbivore inflicting the damage. According to Bidart-Bouzat *et al*. (2005) even the ecotypes within species differ in GS induction in response to herbivory and Gols *et al*. (2008) found more induced GS in wild populations of *Brassica oleracea* after herbivory than in cultivated *B. oleracea*.

Glucosinolates, like other anti-herbivore defences, do not provide a defence against all herbivores and specialists are generally able to avoid or detoxify them (Textor and Gershenzon, 2009). Rojas (1999) and Miles *et al*. (2005) observed that defence compounds against generalists function as oviposition attractants for specialist feeders and glucosinolate hydrolysis products may attract and provide feeding cues for specialist herbivores (Renwick, 2002). Specialist herbivores have developed mechanisms to detoxify the glucosinolates by blocking their conversion to noxious products or by converting them to less toxic compounds. For example, the specialist herbivore *Plutella xylostella* cleaves the sulphate part from the glucosinolate by sulphatase in its gut (Ratzka *et al*. 2002) and blocks the hydrolysis of glucosinolate

by myrosinase. Specialist herbivores have also been reported to absorb glucosinolates very rapidly from digestive tracts before they are hydrolysed. This sequestration of glucosinolates may be favourable for the self defence of herbivores from predators (Muller *et al*. 2002; Vlieger *et al*. 2004; Winde and Wittstock, 2011).

On the other hand, plant exposure to heavy metals, particularly Zn can result in the synthesis of diverse primary metabolites (e.g. amino acids) (Ghanaya *et al*. 2010) and these amino acids indirectly affect the primary metabolism of various compounds like carbohydrates (Rai, 2002). For example, Ghanaya *et al*. (2010) observed decreased levels of soluble sugars (glucose, fructose and sucrose) in Zn treated rape seed (*Brassica napus*) plants as compared to untreated controls. Similarly, Jahangir *et al*. (2008), observed a decreasing tendency of primary (carbohydrates and amino acids) and secondary metabolites (glucosinolates) at elevated concentrations of Cu and Fe in *Brassica rapa*.

As a result of herbivore attack plants face a dilemma in resource allocation, either towards growth or towards the formation of defence metabolites. Plant defences are closely linked with primary metabolites (e.g. amino acids, carbohydrates) and depend on the herbivore feeding mode as well as on the host plant. Chewing herbivores, severely damage the photosynthesizing organ of the plant resulting in the induction of jasmonic acid (Ozawa *et al*. 2000; Leitner *et al*. 2005) which may close the stomata and alter photosynthesis (Beltrano *et al*. 1998). This altered photosynthesis will ultimately hinder the primary metabolism and plant growth. Herbivore development is also dependent on the availability of primary metabolites, but unlike plants herbivores development and growth is most affected by the amino

acids rather than carbohydrates. For example, Morehouse and Rutowski, (2010) observed normal growth of *Pieris rapae* larvae feeding on carbohydrate deficient plants of *Brassica napus* and postulated N as a key factor for the development of this animal rather than carbohydrates.

In contrast to the chewing insects, relatively little work has been conducted on sucking insects, yet phloem feeders create a unique stress on the host plant because of their piercing mode of feeding; they cause relatively little damage to the plant compared to chewing or rasping/sucking insects. For example, Gomez *et al*. (2006) showed that cotton plants recover from inflicted damage if whitefly attack is not repeated. Glucosinolates measured in plants attacked by whitefly are likely to be constitutive GS rather than induced GS because cellular damage will be limited. Therefore myrosinases are less likely to be released from myrosin cells and the glucosinolate moiety less likely to be hydrolysed to nitriles, isothiocyanates and other toxic metabolites. Silver leaf whitefly (*Bemisia argentifolii*) rarely damage epidermal or mesophyll cells prior to puncturing phloem cells (Freeman *et al*. 2001). Most of the focus to date has been on the generalist Silver leaf whitefly and there is very little information on the Cabbage whitefly (*Aleyrodes proletella*) which is a brassica specialist. Cabbage whiteflies (CWF) have a continuous interaction with the host plant and cause relatively little damage relative to a chewer. This makes them an ideal model species to study and to compare with the effects of the rasping/sucking thrips (Chapter 5).

Description of insect species used

Brassica whitefly (*Aleyrodes proletella*) was used in the current experiment. Both adults and nymphs are phloem feeders with sucking mouth parts and belong to the family *Aleyrodidae* of the sub-order Homoptera. Adults are 1.5 mm long having yellow to white colouring with dark flecks on the wings (Fig. 6.1). Male whiteflies are slightly smaller than females. They live in colonies on the underside of host plant leaves. Females lay eggs on the underside of leaves, usually in circles around feeding sites. Severe attack of whitefly may cause defoliation, deformation of cells and ultimately the plant death while moderate attack results in leaf wilting. They are native to Brazil and Europe, but now have a worldwide distribution and are recognised as serious pests for most of the economically important brassica crops (Byrne and Bellows, 1991). Whitefly damages the plant in four ways: Direct feeding damage, honeydew secretion which stimulates fungal growth, transmitting plant pathogens including viruses and by causing physiological disorders in the host plant.

Fig. 6.1: *Aleyrodes proletella* adult and nymphs.

6.2 Aims and Objectives

In the current experiment, the effects of Zn hyperaccumulation on infestation of *Thlaspi caerulescens* by the specialist Cabbage whitefly (*Aleyrodes proletella*) were studied. It was hypothesised that because this species is a brassica specialist, any effects of induced glucosinolates should be negligible and therefore infestation levels could be directly attributed to foliar Zn concentrations.

The main aims were to:

1) Determine whether hyperaccumulation of zinc deterred *Aleyrodes proletella* from infesting *T. caerulescens*.

2) Establish whether CWF induced glucosinolate production in *T. caerulescens*; it was hypothesised that it should not.

3) Determine if there was a relationship (trade-off) between foliar Zn and glucosinolate concentrations and establish if plant nutrition is more or less important than Zn concentrations in directing infestation levels.

These aims were addressed by growing *T. caerulescens* at a range of Zn concentrations (0-1000 mg Zn kg^{-1} soil) under glasshouse conditions. The Zn concentrations spanned a lower range than those in Chapter 5. This was because originally the experiment was set up for a feeding trial using *Pieris rapae* and an earlier pilot study indicated that the caterpillars would not survive high Zn concentrations. Unfortunately the stock caterpillars died and CWF were utilised instead. Measurements of foliar N, C, S, Zn, soluble carbohydrates, starch and total GS were made and numbers of eggs and nymphs counted.

6.3 Materials and Methods

6.3.1 Insect collection and maintenance

Whitefly adults (*Aleyrodes proletella*) were collected from *Brassica juncea* leaves growing in field margins near the Sutton Bonington campus of the University of Nottingham. CWF were collected by aspiration and maintained in Universal tubes with leaf material for 2 days prior to use.

6.3.2 Experimental setup

This glasshouse based investigation was carried out to observe the effects of hyperaccumulated Zn and glucosinolates on *A. proletella*, while feeding on *Thlaspi caerulescens* grown at a range of Zn concentrations. *T. caerulescens* seeds were prepared as described in Chapter 5 except the seedling compost was amended with Zn in the form of $ZnSO_4.7H_2O$ to obtain concentrations of 0, 1, 2, 4, 6, 8, 10, 16, 25, 40, 63, 100, 158, 251, 398, 631 and 1000 mg kg^{-1} of soil. After germination (10 days), the seedling trays were transferred to the glasshouse (see Chapter 2, section 2.1.2) where one week old seedlings of uniform vigour and size were transplanted at one seedling pot^{-1} into experimental pots (8.0×7.5cm) filled with soil-based potting compost (John Innes, Norwich, UK) amended with Zn ($ZnSO_4.7H_2O$) as above. There were 10 replicates for each treatment. Glasshouse conditions were similar to those described in Chapter 2 (section 2.1.2). Ten days after transplanting, plants were infested with adult whiteflies by releasing them from vials on top of the growing plants. Two weeks after infestation, egg and nymph numbers were counted on each leaf of each plant using a stereo microscope (Wild Heerbrugg, M3 Switzerland). After data collection, plants were harvested and stored at -80ºC. Plant material was lyophilized before analyses were carried out.

6.3.3 Zn and total glucosinolate concentrations in the shoot

Zn and total glucosinolate concentrations in the shoot tissue were determined as detailed in Chapter 2, in Sections 2.3.2 and 2.3.4.

6.3.4 Carbon, nitrogen and sulphur concentrations in plant tissue

Carbon, nitrogen and sulphur concentrations were determined in the plant shoots by the method described in Chapter 2, section 2.3.3.

6.3.5 Non-structural carbohydrate determination

Soluble neutral sugars (i.e. glucose, fructose and sucrose) and starch were quantified in experimental plants using the methods of Farrar (1980) and Dubois *et al*. (1956).

6.3.5.1 Soluble sugars

Plant material was oven dried at 40ºC until a constant mass was obtained. Dried plant material was ground with a pestle and mortar and approximately 25-30 mg weighed into a test tube and 6ml of 95% ethanol added. A marble was placed on top of the test tube and the sugars extracted at 80ºC in a water bath for 1 hour. The liquid was allowed to boil until 3 ml had evaporated, the remaining 3 ml was then collected and dispensed into a separate test tube and 6 ml of 95% ethanol was added again to the original sample. The process was repeated until the colour disappeared from the plant material. The collected liquid was made to 10 ml with 95% ethanol.

6.3.5.2 Starch

After the soluble sugars had been extracted, the remaining pellet of plant material was further extracted in 10 ml of 0.2 M sodium acetate buffer (pH 4.5) containing amyloglucosidase (Sigma) for 24 hours. Enzyme was mixed with deionised water to give 12.8 units per 6 ml of buffer. After 24 hours, extracts were collected with Pasteur pipettes and final volumes made to 10 ml with sodium acetate/acetic acid buffer.

6.3.5.3 Performing the assays

Sucrose standard solutions (containing 10, 20, 30, 50, 70, 100 and 120 µg sugar) and plant sample extracts (0.5 ml for soluble sugars and 1.0 ml for starch) were pipetted into separate clean dry test tubes. The volume in the test tubes was made up to 2 ml with deionised water. Phenol solution (5% w/v) was added to each test tube, whirl-mixed and then 5 ml of concentrated sulphuric acid was added rapidly to each tube and whirl-mixed immediately. Tubes were left at room temperature for 20 minutes. Absorbance was recorded at 483 nm using a spectrophotometer (CECIL instruments, CE 1011, 1000 series). Results were expressed as sucrose equivalents (mg g^{-1} dry weight of plant material).

6.3.6 Statistical analyses

All data were analysed using GenStat release 13.1. Egg and nymph counts were $\log_{10}$ (n+1) transformed to normalise the data. Other parameters were normally distributed and did not require transforming. Regressions (linear and polynomial) and generalised accumulated linear regressions were performed where appropriate.

6.4 Results and Discussion

6.4.1 Metal concentrations in soil versus uptake by Thlaspi caerulescens

Zinc concentration in the plant tissue was positively correlated with the amount applied in the soil ($P<0.001$) in line with the trends observed in previous Chapters. The difference here was the lower soil application rates and these (0-1000 mg kg^{-1}) resulted in shoot Zn concentrations ranging from 132 to 7226 mg kg^{-1}.

6.4.2 Effects of nitrogen, carbon, sulphur and glucosinolates on Aleyrodes proletella

Plant nitrogen is considered a host quality indicator and affects survival and development of homopterans (van Emden, 1966; Jauset *et al*., 2000). Specifically, nitrogen has been reported to increase female weight and fecundity in *A. proletella* (Iheagwam 1981) although nitrogen may not have the same effect on other species within the Aleyrodidae family (Jauset *et al*., 2000).

Jauset *et al*. (2000) studied the effect of nitrogen fertilisation on the glasshouse whitefly (*Trialeurodes vaporariorum*) and concluded there was no effect of nitrogen on development time of this species. The observations in the current study agree with this since no treatment-related difference in proportion of 4^{th} instar nymphs relative to the total number was observed (data not shown). However, differentiation between different instars is not easy, and plants were not kept free of adults after the original introduction, so there would be overlap of different age structures. Plants were not maintained for long enough for the overlap to be generational, although the original adult whiteflies would have matured and/or began laying eggs at different times following initial release. Therefore, data presented in this Chapter are for total number of eggs and for nymphs at the time of harvest, irrespective of stage of

development. Adults were counted but no treatment-related differences in numbers were observed, but there would have been some movement of adults away from the plants during the counting process.

Jauset *et al*. (2000) reported increased egg density on plants fertilised with their highest level of N (308 mg kg^{-1}); in the current study there was a trend towards a greater number of eggs laid on plants containing higher concentrations of foliar N than on those with less N (linear regression, P=0.014; Fig. 6.2 a) and also between foliar N and the number of nymphs (linear regression, P=0.012; Fig. 6.2 b). This infers that adults chose to lay eggs on plants with higher N contents and this is possibly reflected in nymph numbers although it should be noted that eggs counted were those present at the time of harvest and will have been laid after initial infestation, whilst nymphs present will have resulted from earlier egg batches. The slope of the relationship between nymphs and N concentration is double that of the slope of the relationship between egg numbers and N concentration (0.6 and 0.3 respectively). This suggests that nitrogen has a more profound effect on nymph survival than on choice of adult oviposition site, although since the eggs counted at harvest will have been laid after and during CWF nymph feeding, it is possible that some other factor such as feeding-related changes in glucosinolates influenced egg deposition.

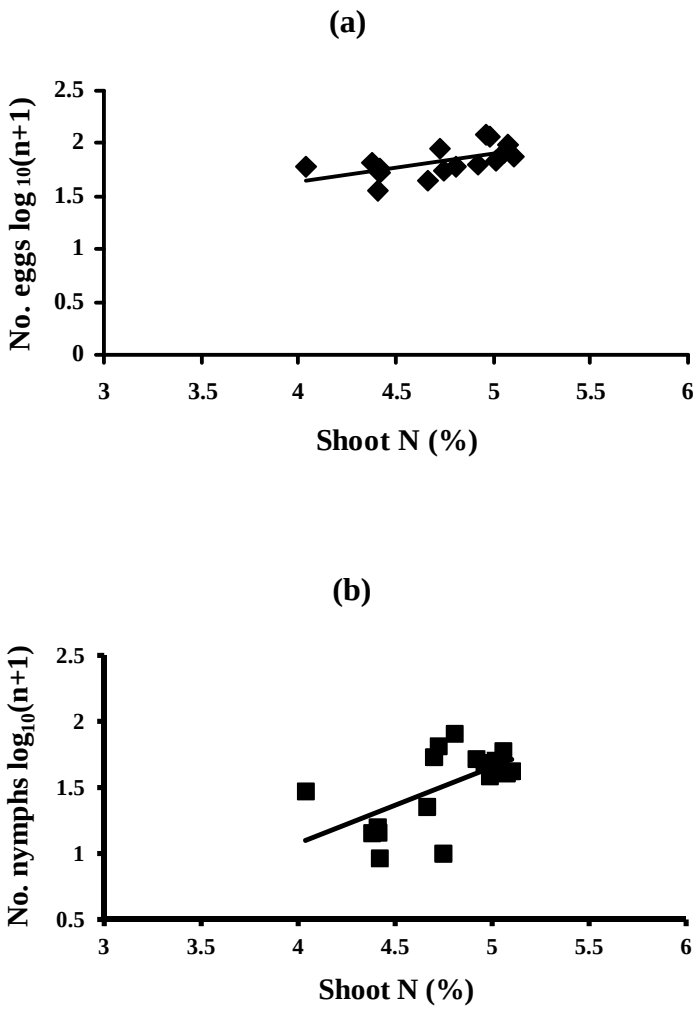


Fig. 6.2 The relationship between per cent total shoot N and (a) *Aleyrodes proletella* egg numbers and (b) *A. proletella* nymph numbers on *Thlaspi caerulescens* plants. Data are $\log_{10}$ (n+1) values; linear regression P=0.014 and P=0.012 for eggs and nymphs respectively.

Viability of the eggs was not determined here. However, it is possible that reduced nymph numbers at lower N concentrations (Fig. 6.2 b) resulted from increased egg mortality on plants with lower N contents in addition to fewer eggs being laid on lower-N plants. This can only be speculation, but Jauset *et al*. (2000) observed higher egg mortality on low-N plants and greater numbers of crawler nymphs (early stage) on high-N plants.

The number of CWF nymphs was not affected by soluble carbohydrate content of the leaves. It should be emphasised that the carbohydrate content of the phloem sap was not measured; only that of the whole leaves. Since CWF feed on phloem sap some caution should be taken when interpreting the data. Nevertheless, it is estimated that soluble carbohydrates made up approximately 55% of the total leaf C content in the experimental *T. caerulescens*. If the carbohydrate content of the phloem was equally high, then no Zn-related increase in carbohydrates would affect nymph numbers because sucrose content would be more than adequate even at the lowest value in the carbohydrate range (45-65%). According to Salvucci and Crafts (2000), 50% of the sucrose ingested is excreted when dietary concentrations exceed 10% sucrose. These authors worked on the Silver leaf whitefly (*Bemisia argentifolii*) but it is accepted that phloem feeders tolerate molar concentrations of sucrose in their diets (Fisher *et al*. 1984). In the current experiment there was no relationship between foliar carbohydrates and total N. However, the relationship between N and carbohydrate content can be complex as demonstrated by Bi *et al*. (2005). These authors showed in field grown cotton that Silver leaf whitefly (SLWF) numbers in late season were positively correlated with glucose and fructose in late-planted cotton but not in early-planted cotton. Since whitefly mainly utilise sucrose, the authors suggested that the correlation between SLWF numbers and the sugars may not be diet-related and referred to other work by Lin *et al*. (2000) who showed that SLWF limits export of carbohydrates from source leaves, thereby increasing their carbohydrate content. In the current experiment the method used to determine soluble carbohydrates did not allow for differentiation between sucrose, fructose and glucose. However, since there was no relationship between nymph numbers and soluble carbohydrates, the most parsimonious explanation is that there was sufficient

sucrose in the phloem sap not to make a difference. Even if sucrose constituted only a third of the total soluble carbohydrates, the 10% value mentioned by Salvucci and Crafts, (2000) would be exceeded. Neither starch nor total foliar carbon had an effect on nymph numbers, but there was a negative relationship between C/N ratio and nymph numbers (linear regression, P=0.013, Fig.6.3). This emphasises the importance of shoot nitrogen as discussed above.

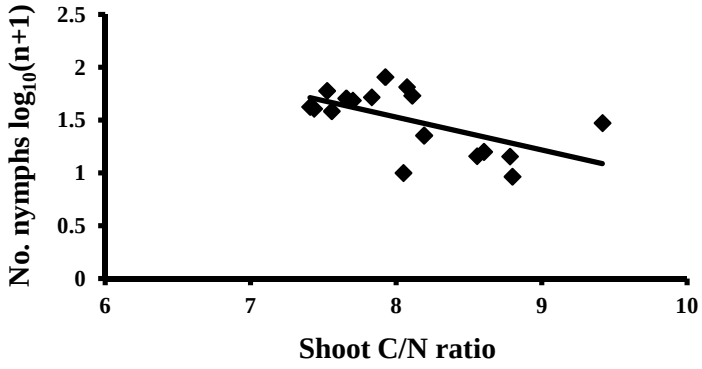


Fig. 6.3 Relationship between the number of *A. proletella* nymphs and shoot C/N ratio of *T. caerulescens* plants. Linear regression, P=0.013.

Since phloem feeders cause relatively little damage to the plant the glucosinolates (GS) measured in this current experiment are likely to be intact and therefore limited effects of GS on nymph numbers would be expected. According to van de Ven *et al*. (2000) plants can differentiate between SLWF nymphs and adults and between closely related biotypes. The key difference between SLWF and CWF is the former is a generalist whilst CWF is a specialist. Differences between generalist and specialist chewers are well documented (e.g. Hopkins *et al*. 2009) but phloem-feeding whitefly have largely been ignored in comparison. However Kempema *et al*. (2007) demonstrated that in Arabidopsis (*Arabidopsis thaliana*), SLWF influenced RNA levels of 4 genes related to sulphur- and glucosinolate-metabolism (two were

up-regulated and two down-regulated); in contrast the chewer *Pieris rapae*, increased 14 GS biosynthesis/catabolism gene RNAs. Interestingly these authors showed the plant response to the biotrophic fungus, *Erysiphe cichoracearum* was similar to that for the SLWF. In contrast to the minimal changes in glucosinolate-metabolism gene RNAs induced by SLWF nymphs, the phloem-feeding aphid *Myzus persicae* actively repressed many of the genes. The authors concluded that the plant perceives the two insects differently, or the insects have different deterrence mechanisms.

Aphid attack of brassicas has been studied and in particular the specialist *Brevicoryne brassicae* was shown to target inflorescence stems of white mustard (*Sinapsis alba*) rather than leaves, and this was linked to higher concentrations of sinalbin in epidermal cells than in inflorescence stems (Gabrys *et al*. 1997). The generalist *M. persicae* excretes glucosinolates in its honeydew (Merritt, 1996). Behaviourally, aphids are more mobile than CWF; the latter remain on the same plant for up to 28 days once the second instar is reached after which the insects are no longer mobile until they reach adulthood.

The specialist chewer (*P. rapae*) is able to protect itself from GS and can therefore feed on brassicas without any apparent negative consequences. According to Wittstock *et al*. (2004) the caterpillars do this by producing a nitrile-specifier protein which directs production of less toxic nitriles rather than more toxic isothiocyanates when plant material is ingested.

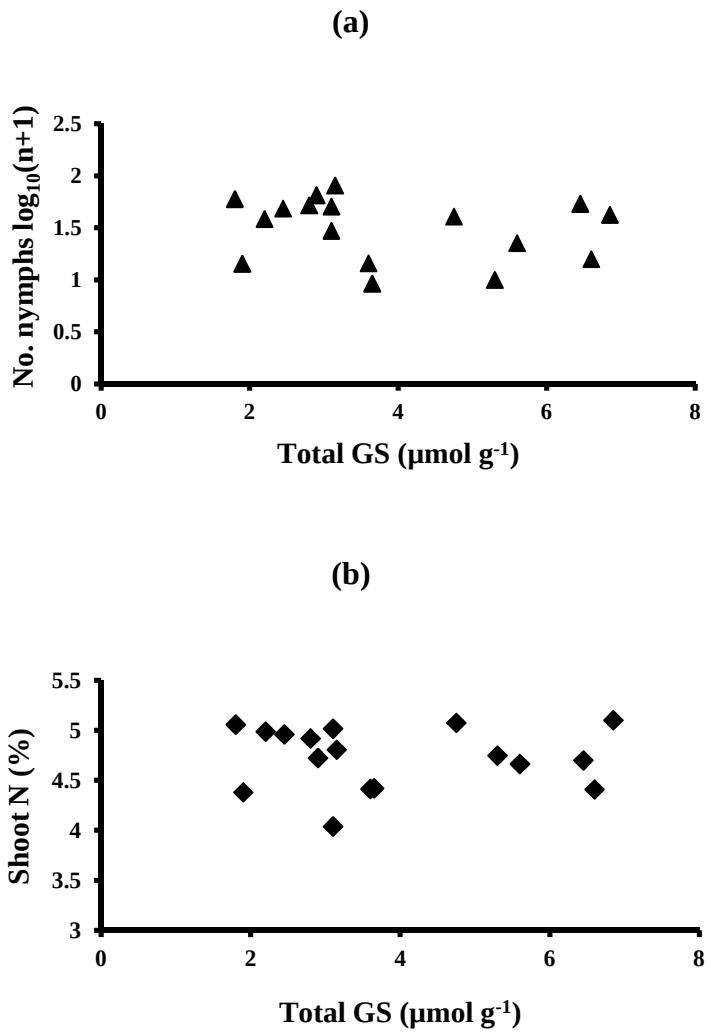


Fig. 6.4. No relationship was observed between (a) total glucosinolates and the number of *A. proletella* nymphs on *T. caerulescens* or (b) between total glucosinolates and total shoot nitrogen.

Whether specialist CWFs have any similar detoxification strategies is unknown. Whilst it is likely, the CWF may nevertheless simply evade the GS-defences by avoiding or limiting tissue damage. The glucosinolates measured in this current experiment are likely to be intact GS (because of the piercing mouthparts of CWF) rather than induced GS and/or breakdown products, as would be the case for thrips (scraping and sucking mouthparts). The data obtained for CWF are in contrast to those observed for thrips (Chapter 5) where feeding damage was negatively correlated with glucosinolates and foliar nitrogen and positively correlated with C/N

ratio. The contrast here is that there was no relationship between total GS and nymph numbers, or between foliar nitrogen (%) and total GS (Fig. 6.4 a, b).

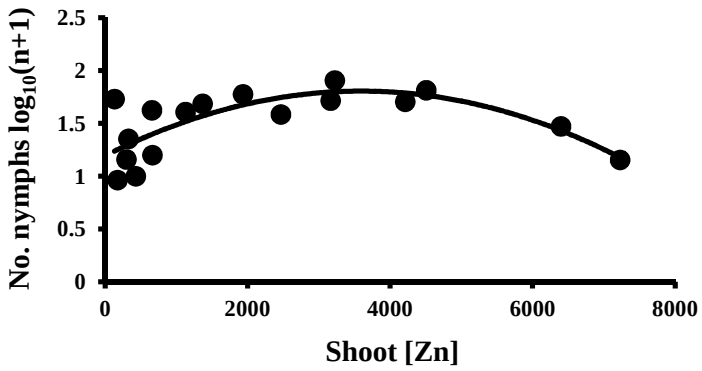
Interestingly, *A. proletella* was unaffected by progoitrin in wild cabbage populations (*Brassica oleracea*) but infestation was consistently negatively correlated with the proportion of plants containing sinigrin (Newton *et al*. 2010). This was a between-population effect and did not scale down to within-population trends. Therefore the lack of GS effects observed in this current experiment may not be translated to between-population effects under field conditions.

Foliar sulphur did not influence nymphs or glucosinolate profiles. Sulphur concentrations remained constant with a mean total shoot concentration of 0.9%.

6.4.2 Relationship between Zn, glucosinolates and nymph numbers

Since CWF is unaffected by glucosinolate content, it is an ideal organism with which to test whether Zn hyperaccumulation by *T. caerulescens* inhibits infestation. One of the problems with thrips and other herbivores is differentiating between the effects of GS and Zn. One way around this problem is to use a plant like Arabidopsis and modify the genetic pathways or alternatively, to use a natural system such as CWF and *Thlaspi*. This system should allow testing of the 'elemental defence' hypothesis without GS induction confounding the results; however, nitrogen was an important influence in the system.

(a)



(b)

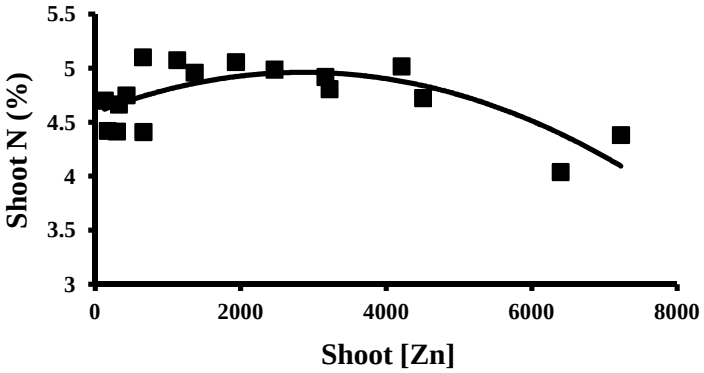


Fig. 6.5 Relationship between (a) numbers of *A. proletella* nymphs and shoot zinc concentration (Regression, 2nd order polynomial, P=0.003) and (b) shoot N and shoot Zn concentrations (Regression, 2nd order polynomial, P=0.005).

The number of nymphs was related to foliar Zn (2nd order polynomial regression, P=0.003, Fig. 6.5 a) and the N content was also related to foliar Zn (2nd order polynomial regression, P=0.005, Fig. 6.5 b). Since the number of nymphs was highly influenced by foliar N (Fig. 6.2 b) a generalised accumulated linear regression was carried out which produced the explanatory model: **Foliar C/N + foliar Zn** (P=0.006 and P=0.036 respectively). C/N ratio explained more of the variation in the data than %N in GLM. Therefore, foliar Zn affected nymph numbers only after the variation

due to foliar C/N had been accounted for. This corroborates the findings in Chapter 5 for the thrips experiment, i.e. the effects of metal hyperaccumulation on insect herbivory should be considered in context of plant nutrition.

Interestingly, foliar Zn significantly affected total GS concentration in the shoots. No other parameter influenced this relationship (Fig. 6.6; P=0.008) so the data were not confounded by %N, %S or the C/N ratio.

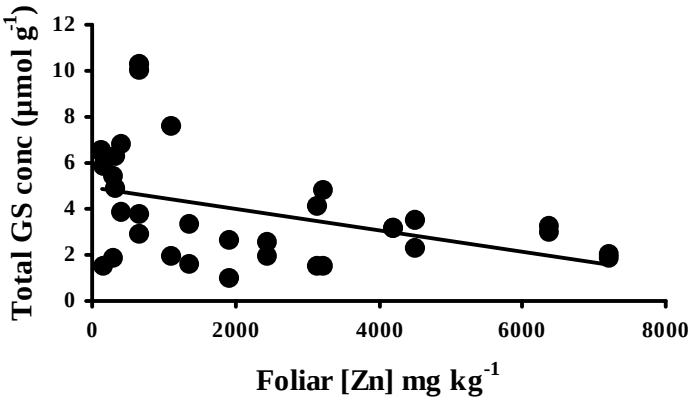


Fig. 6.6 Relationship between total glucosinolates and foliar Zn concentration. Linear regression, P=0.008.

What is particularly interesting here is that these data show a relationship between foliar glucosinolates and Zn in which there were higher concentrations of GS in plants containing lower concentrations of Zn and the GS concentrations fell as foliar Zn increased. This trend may support the 'trade-off' hypothesis which states that hyperaccumulated metals negate the requirement for GS production, thereby reducing the metabolic costs of defence (Martens and Boyd, 1994). However, the observation that nitrogen concentration increased with increasing foliar Zn may indicate that at low levels of foliar Zn the plants were suffering stress as a result of insufficient available Zn; such nutritional stress may induce GS production.

However, the GS concentrations fell at around 1000 mg kg^{-1} foliar Zn which is before maximum Zn accumulation and before maximum N concentrations were reached. Therefore, the changes in GS concentrations are unlikely to be entirely due to stress due to limited Zn or N. Furthermore, there was no relationship between GS and N (Fig. 6.4 b) which supports the view that nutrient stress did not induce GS. These data contrast those obtained in Chapter 5 where there was no (or very little) support for the 'trade-off' hypothesis when GS concentrations only fell at exceedingly high foliar Zn concentrations (>14000 mg kg^{-1}). Foliar Zn concentrations were considerably less in the current experiment. Apart from that, the key differences between the two experiments (thrips, Chapter 5 and CWF, this experiment) are that in the previous thrips experiment, plants were harvested 7 weeks from potting whilst plants in this experiment were harvested 3 weeks from potting. In addition, in the thrips experiment, GS were induced in all plants whilst it is unlikely that GS were induced in the current experiment because of the feeding method of the CWF. Indeed average GS concentrations were lower here than in the thrips experiment. These data support the findings in Chapter 3 (e.g. Fig. 3.7) which show that GS concentrations were higher in 28 day old undamaged plants when soil had been amended with 1500 mg kg^{-1} Zn compared to plants growing in soil amended with 3000 mg kg^{-1} Zn. The age of the plant is likely to be influential; young plants are often cited as containing higher concentrations of GS than older plants (Lambdon and Hassall, 2005), but it is possible that metabolic costs of maintaining constitutive GS are unnecessary and better directed towards growth. If this apparent 'trade-off' is lost after induction of GS (e.g. after thrip attack) then it would appear that in *Thlaspi* a defensive enhancement of glucosinolates occurs which tentatively

supports the 'joint effects' hypothesis of Boyd (2007), although further work needs to be carried out to fully test this theory.

In the current experiment, increasing foliar Zn was initially associated with enhanced nymph numbers followed by a decrease at higher Zn concentrations. Zn concentration in the phloem was not measured and it is this that the CWF would have been subjected to. Zn is relatively mobile in the phloem of young wheat plants (Riesen and Feller, 2005). These authors found that the mobility of ^{65}Zn and ^{109}Cd was different, with Zn being exported in higher concentrations than Cd and further, Zn was transferred from the xylem to phloem more efficiently than Cd. This is important in this context because by its nature the Gange ecotype of *T. caerulescens* hyperaccumulates Cd. Although no Cd was applied to the potting soil, the plants nevertheless contained an average concentration of 7 mg kg^{-1}. Cadmium is considerably more toxic to herbivores than Zn, but since it is relatively less mobile in the phloem than Zn, it is unlikely to have affected the current results. This is supported by (a) that it was not a significant variable in the GLM conducted here and (b) the concentrations of <10 mg kg^{-1} cited by Jiang *et al*. (2005) had little effect on thrip feeding, so even lower foliar concentrations experienced here would have negligible effect on phloem feeding CWF.

6.5 Key points

- A linear relationship was observed between Zn application and foliar concentrations. This corroborates data in earlier Chapters.

- More CWF nymphs were observed on plants as the foliar Zn concentration increased, up to a maximum of approximately 4000 mg kg^{-1} after which numbers declined. Zn was a significant variable in GLM after the variation due to the C/N ratio had been accounted for. Therefore both variables affect nymph numbers.

- Nymph numbers declined with increasing C/N ratio and increased with increasing N concentration. This is the opposite of what was observed in Chapter 5 and can be explained by the lack of influence that either nymph feeding or C/N ratio or %N had on GS concentrations.

- Highest concentrations of foliar GS were observed at the lowest shoot Zn concentrations and GS levels were lower in plants with increased Zn contents. This infers a 'trade-off' between Zn hyperaccumulation and GS production. This was not observed in the thrips experiment (Chapter 5) and may be because the plants in the current experiment were younger or because GS were not induced by herbivory.

CHAPTER 7: GENERAL DISCUSSION

7.1 General Findings of the Experimental Work

Three experimental systems were used within this project to attempt to determine (a) whether zinc hyperaccumulation by the brassica *Thlaspi caerulescens* protects the plant from foliar herbivores, (b) whether glucosinolates are induced in plants containing high concentrations of Zn and (c) whether there are interactions between Zn and glucosinolate concentrations that enhance plant protection. The experimental systems employed included (a) artificial damage (clipping), (b) natural attack by a generalist herbivore (thrips) and (c) natural attack by a specialist herbivore (cabbage whitefly). All experiments were carried out under glasshouse conditions. *T. caerulescens* is a difficult plant to work with because it is relatively slow growing and germination can be erratic, although methods were developed to maximise germination. One of the problems with a study such as this is actually differentiating between zinc and glucosinolate toxicity. Use of the specialist cabbage whitefly (CWF) was an attempt to in effect 'remove' the glucosinolates from the system so the effects of Zn on the herbivore could be established. Although there is evidence in the literature for Zn hyperaccumulation being an effecting herbivore deterrent (Pollard and Baker, 1997; Behmer *et al*. 2005), many studies have not accounted for the actions of glucosinolates and in those that have, the evidence in support of Zn is often lacking (Huitson and Macnair, 2003; Noret *et al*. 2005;Noret *et al*. 2007). There appears to be more evidence in favour of cadmium and nickel having a defensive function than Zn (Boyd and Martens, 1994; Jhee *et al*. 2005). This is perhaps not surprising since Zn is an essential nutrient, but nevertheless if the

'elemental defence' hypothesis of Boyd and Martens (1992) has any credence, it should be validated against Zn hyperaccumulation in *T. caerulescens* because of the exceedingly high concentrations found in this species. Despite the difficulty involved with growing *T. caerulescens*, the vagaries associated with working with insect infestations and the fact that experiments were conducted over a three-year period, certain findings were consistent across all experiments. In particular, there was a significant trend towards lower concentrations of glucosinolates in plants with higher foliar Zn concentrations than in those with lower amounts of Zn (Chapters 4, 5 and 6). This supports the idea of a 'trade-off' between glucosinolates (GS) and Zn uptake, but the data do not necessarily **prove** this hypothesis. For instance, it is possible that the trend is actually related to plant stress resulting from Zn toxicity since in Chapter 5 (thrip experiment) the phenomenon was only detected at very high levels of foliar Zn. Nevertheless, data from Chapter 6 (CWF experiment) show that glucosinolates were reduced at much lower Zn concentrations than in Chapter 5 and at that point plants had not reached their maximum size so it is unlikely to be due to a 'dilution' effect.

The findings in Chapter 3 (artificial damage) suggest the differences are related to plant age with plants of around 35 days demonstrating this GS reduction at high Zn concentrations for total glucosinolates, benzyl and p-OH-benzyl. Plant age is likely to be an influential factor here since the plants in the CWF investigation were 28 days old and those in the thrip experiment were 49 days old. It would therefore appear that GS production is less in plants with high foliar Zn during the active growing (vegetative) phase, but this is not the case in seedlings and older plants. The GS profiles of seedlings were different from those of older plants so the fairest

comparison is between the vegetative age groups (28 – 49 days). Whilst it is known that GS profiles alter with plant developmental stage, plants within this age range are all vegetative, although growth will have slowed by day 49. However on balance, the most likely explanation for the differences in GS/Zn interaction between the plants attacked by thrips and those subjected to CWF and artificial clipping is the level of damage and length of time over which the damaged was sustained. Plants subjected to thrip attack tolerated a sustained and increasing level of infestation over a 7-week period, whilst the artificially damaged plants were only clipped once and the plants subjected to CWF suffered less apparent damage than those eaten by thrips; furthermore the CWF experiment was terminated sooner. During the thrips experiment it seems logical to presume that the plants needed to initiate the organic defences irrespective of foliar Zn and the systemic effects observed in undamaged leaves are supportive of this. Therefore in conclusion, there was a relationship between GS and foliar Zn in which at high levels of Zn, GS production was less than at low levels of Zn when damage was at a tolerable level. However, when plants were subjected to a sustained and heavy herbivore attack, GS production occurred irrespective of foliar Zn concentration. This observation and conclusion supports the 'joint effects' hypothesis (Boyd, 2007), which states that both defences work in tandem and enhance overall defence. Glucosinolates were produced within 32 hours of attack and it took 48 hours for maximum concentrations to be reached (Chapter 3). In this experiment, attack was transient and production fell again, but after 168 hours concentrations were still slightly higher than pre-clipping values. Travers and Muller (2007) reported enhanced levels of aromatic and indolic glucosinolates in *Sinapis alba* within 24 hours of damage, followed by significant decreases. Hopkins

et al. (2009) speculated that GS induction is spontaneous and long lasting depending on the type of damage and inducing agent.

Textor and Gershenzon (2009) observed that in 90% of cases after biotic or abiotic stress, the most induced glucosinolates were indolic. This is in contrast to the findings here because only aromatic GS were extracted from *T. caerulescens* and this was consistent over a range of experiments that were separated temporally (Chapters 3 and 5). It is interesting to speculate that the reason this species contains relatively few GS compared to other brassicas is because of the hyperaccumulating activity and the use of Zn as a defence.

In both the natural herbivory experiments (Chapters 5 and 6), plant nitrogen was an important determinant of level of damage. It was suggested by Loader and Damman (2001) and Schoonhoven *et al*. (2005) that the length of time an herbivore spends feeding depends on food quality. The quality of the foliage is defined by the nitrogen content, usually high N reflects palatable food. However, in this investigation high foliar N concentration had different consequences for the generalist thrips than for the specialist CWF. Thrip feeding damage was negatively correlated with foliar nitrogen whilst CWF benefitted from higher N. The reason for this is that nitrogen content was positively correlated with GS production and GS content affected the generalist thrips but not the specialist CWF. Therefore, the CWF responded positively to leaf N-content. The general linear models run on the data for both experiments reflect this. The explanatory model for thrip feeding was C/N ratio + GS + Zn whilst the explanatory model for CWFs was C/N ratio + Zn. The data also suggest that the outcomes of herbivore attack are influenced by the plant nutrients

and this should be taken into account when studies of GS are undertaken. The use of a generalist and a specialist herbivore was valuable in this study. Ideally a chewing insect would have been included but for logistical reasons (i.e. the caterpillars died) clipping was used to simulate chewing. Whilst clipping is commonly used and is excellent for producing uniformly damaged leaves, it can be criticised because the process does not include chemicals from insect saliva. Nevertheless, the method provided useful data and acted as a positive control for the natural herbivory experiments. Also, trends observed in the artificial trial were also observed in the natural feeding experiments. The lack of effect of glucosinolates on the CWF was pleasing because it allowed for an evaluation of Zn as a defence mechanism without the confounding input from GS production. It seems that Zn had a detrimental effect at the higher concentrations used. CWF is a phloem feeder and if the chewer *Pieris rapae* had survived, more extreme results might have been obtained.

7.2 Conclusions

Three questions were posed at the start of the work: (1) Does Zn hyperaccumulation protect the plant from herbivores? (2) Are glucosinolates produced in plants containing high levels of Zn? (3) If there are interactions between GS and Zinc, are they responsive?

The data obtained go some way to answering them:

1) In both natural herbivore experiments Zn played a defensive role but only at high concentrations. Glucosinolates were more important defences against the generalist thrips than Zn, but the latter did reduce feeding at high concentrations. The specialist CWF was reduced by high Zn concentrations and unaffected by GS.
2) The overall trend across all experiments was that glucosinolate concentrations were lower in plants with high Zn concentrations and *vice versa*.
3) In the thrips study, GS were produced irrespective of Zn content except at the very highest concentration. Severe and sustained attack by thrips induced GS production. This suggests that GS production is responsive to damage and will be maintained even at high Zn levels if necessary, but not if unnecessary.

The data obtained favours the 'joint effects' hypothesis of Boyd (2007) rather than the original 'elemental defence' hypothesis.

Table: 7.1 Key points from the experimental work

Chapter	Key points
Chapter 3	• Total glucosinolates were induced 24 - 48 hours after clipping in *Thlaspi caerulescens* plants. • Four aromatic glucosinolates were extracted; two were identified as benzyl (glucotropaeolin) and p-OH-benzyl (sinalbin). • GS profiles were different in seedlings (cotyledons and 1-2 true leaves) than in 35-day-old plants (rosettes). • GS concentrations were higher in older plants grown in low-Zn soil but not in those grown in the high-Zn soil (1500 and 3000 mg kg^{-1} respectively).
Chapter 4	• *Thlaspi caerulescens* accumulated >14,000 mg Zn kg^{-1}, but the threshold value for normal growth was observed as 2000 mg Zn kg^{-1} of soil. • There was a significant trend towards lower concentrations of benzyl in plants grown with higher Zn amendments. • Anthocyanin production was higher in plants growing in soil with low levels of Zn amendment. This suggests these plants were stressed or that anthocyanins were produced as defence compounds. • Clipping (artificial damage) did not result in glucosinolate induction. This corroborates the findings of others (see Lehtilä and Boalt, 2004).

Chapter 5	• High foliar Zn concentrations negatively affected leaf-feeding damage by thrips (*Frankliniella occidentalis*). • The GLM model that explained most of the variation in the feeding data was C/N ratio + total GS + shoot Zn. • Efficiency of glucosinolates as a defence is affected by C/N ratio. • Undamaged leaves had higher glucosinolate concentrations than damaged leaves on the same plant. This may be because of remobilisation (systemic effect) or due to selective feeding. • Benzyl concentration decreased at the highest Zn concentrations. • Four individual GS (benzyl, p-OH-benzyl, 'GS-A' and 'GS-B') were quantified and benzyl was the most abundant. • Anthocyanins were present in damaged leaves at higher concentrations than in undamaged leaves on the same plant. • The data show that foliar nutrition, Zn and GS all play a role in responding to herbivory. • Zn deterred thrips at high concentrations, but the involvement of the other factors suggest that the simple 'elemental defence hypothesis' is unlikely to be upheld with this system.
Chapter 6	• Zn was a significant variable in GLM after the variation due to the C/N ratio had been accounted for. Therefore both variables affect nymph numbers. • Nymph numbers declined with increasing C/N ratio and increased with increasing N concentration. This is the opposite of what was observed in Chapter 5 and can be explained by the lack of influence that either nymph feeding or C/N ratio or %N had on GS concentrations. • Highest concentrations of foliar GS were observed at the lowest shoot Zn concentrations and GS levels were lower in plants with increased Zn contents.

7.3 Future work

Whilst conditions in the glasshouse were changeable during extreme weather events, in general, heating and lighting maintained a reasonably constant environment and plants were watered as required. Whilst this is good for temporal comparisons, the plants were not subjected to the same environmental variables that would be experienced in the field. Apart from variations in temperature, light intensity and water availability, in the field, plants would experience intra- and inter-specific competition and a myriad of herbivores encapsulating different feeding guilds. Constant stress may be more likely to induce GS and 'cloud' any Zn/GS interactions. Future work must therefore include a field element.

An interesting question that arose from this work is that of the role of anthocyanins. It is unclear both from the data presented here and from the literature whether they were produced as a stress-response or as an alternative defence mechanism. Detailed studies of the biosynthetic pathways of these pigments may provide evidence for their involvement in plant defence. Given the complexity of the system, teasing apart the effects of GS, Zn and anthocyanins in this system will be difficult and initially *Arabidopsis* might be the species to use in the first instance.

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