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The use of Brassica Species for the Management of Potato Cyst Nematode Infestations of Potatoes

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The use of Brassica Species for the Management of Potato Cyst Nematode Infestations of Potatoes

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BSc. (Hons), MSc.

Thesis submitted in partial fulfilment of the requirements for the award of the degree of:

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Declaration

The work presented in this thesis is an original compilation of the author and is in line with the registered title of the research project. All the relevant sources of information referred to in this thesis are cited within the text and details presented in the references section. None of the findings herein have been previously presented elsewhere for application or award of a degree or other qualification in another institution.

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Abstract

The potato cyst nematodes (PCN), *Globodera pallida* and *G. rostochiensis* are economically the most important nematode problems of potatoes within the EU, costing the UK potato industry over £36 million based on yield loss and nematicides application. Historically, farmers within the UK have relied heavily on soil fumigant and granular nematicides for PCN control. However, increasing political pressure on pesticide usage means that alternative and sustainable strategies are required. Biofumigation, the suppression of soil borne pests and pathogens by biocidal compounds released when brassicaceous tissues are hydrolysed, offers a potential sustainable alternative in managing PCN.

In this study, summer cultivated *B. juncea* and *R. sativus* reduced PCN population post-potato harvest. *Eruca sativa* proved to be cold tolerant following overwinter cultivation, but did not reduce PCN multiplication. Glucosinolate concentrations in the brassicas varied significantly between different plant regions and cultivation seasons. *B. juncea* leaves produced high 2-propanyl levels while *R. sativus* produced predominantly 4-methylsulfinylbutyl and 2-phenylethyl GSL in foliage and root respectively. *Eruca sativa* produced a blend of GSL in both above and below grown tissues, most of which were either low or non-ITC producing GSL. Metconazole application enhanced 2-propanyl GSL production in *B. juncea* tissues. Glucosinolate concentrations correlated positively with PCN mortality in the summer-cultivated experiments.

In glasshouse experiments, sinigrin was significantly degraded in brassica cultivated soil pre- and post-incorporation of the brassicas. Positive relationships were observed between PCN mortality and microbial activity, while GSL concentration was found to be inversely related to microbial activity. Finally, the LD_{50} for *B. juncea* and

R. sativus against PCN were determined *in-vitro* as 0.027/0.032 and 0.546/0.035 mg ml^{-1} for leaf/root extracts respectively.

This study has demonstrated that using *B. juncea* and *R. sativus* can play an important role in PCN management, particularly if included in an integrated pest management scheme in ware potato production.

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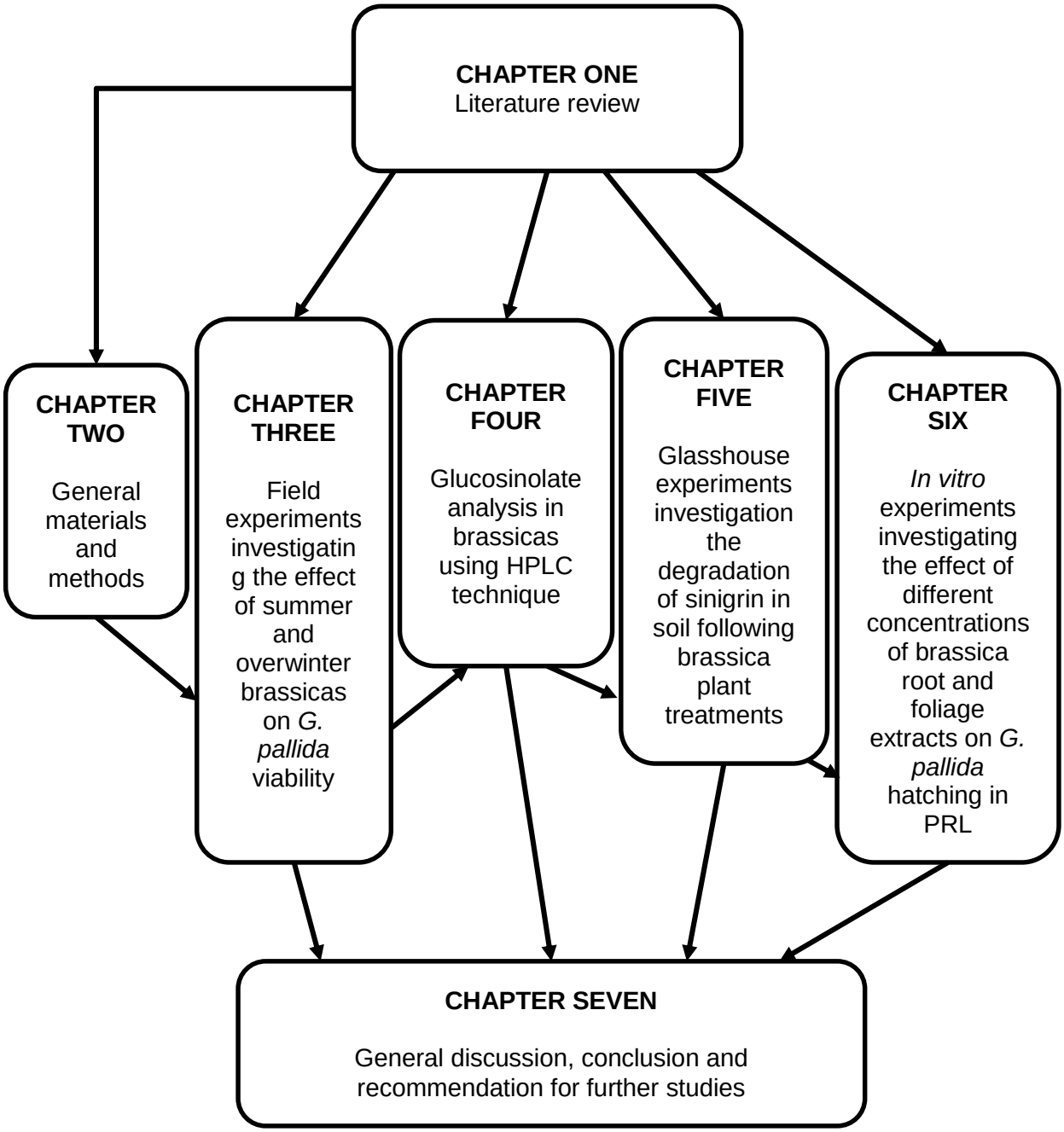
Outline of thesis chapters



Statement of advanced studies

During the period of this project, the author has published in a refereed journal and presented experimental results at scientific meetings as detailed below.

Research articles:

Published:

Ngala BM, Woods S, Back MA. (2015b). Sinigrin degradation and *G. pallida* suppression in soil cultivated with brassicas under controlled environmental conditions. *Applied Soil Ecology* **95:** 9-14. DOI 10.1016/j.apsoil.2015.05.009.

Ngala BM, Woods S, Back MA. (2015a). *In vitro* assessment of *Brassica juncea* and *Raphanus sativus* leaf and root extracts on the viability of *Globodera pallida* encysted eggs. *Nematology* **17**: 543-556. DOI 10.1163/15685411-00002888.

Ngala BM, Haydock PPJ, Woods S, Back MA. (2014). Biofumigation with *Brassica juncea*, *Raphanus sativus* and *Eruca sativa* for the Management of Field Populations of the Potato Cyst Nematode *Globodera pallida*. *Pest Management Science* **71**: 759–769 - DOI: 10.1002/ps.3849.

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Scholarly awards:

- Scientific poster prize of the 64th International Symposium of Crop Protection (ISCP) in 2012, Ghent University, Ghent, Belgium.
- Scientific poster prize for the SCI BioResources Young Researchers 2012: Crop Productivity, Sustainability and Utility.
- Student presentation prize for the Advances in Nematology Meeting organised by the Association of Applied Biologist, Linnaean Society of London 11th December 2012.

Conference presentations

Oral presentation at:

- The 31th International Symposium of the European Society of Nematologists (ESN) 23rd – 27th September, 2012. Adana, Turkey.
- Annual Advances in Nematology Meeting organised by the Association of Applied Biologist, Linnaean Society of London, 11th December 2012.
- Annual postgraduate colloquium held at Harper Adams University, 28 November 2013.
- Lunch time Research seminar held at Harper Adams University, 31st May 2013.
- The 6th International Congress of Nematology, 5 - 9 May 2014, Cape Town South Africa.
- The 5th International Symposium of Biofumigation, 9 – 12 September 2014, Harper Adams University, Newport, Shropshire

Poster:

- The 64th International Symposium of Crop Protection (ISCP) in 2012, Ghent University, Ghent, Belgium.
- The SCI BioResources Young Researchers 2012: Crop Productivity, Sustainability and Utility, University of Reading, 2 June 2012
- CropWorld Global 2012, Queen Elizabeth II Conference centre, London, 6 – 7 November 2012.
- The 5th International Symposium of Biofumigation, 9 – 12 September 2014, Harper Adams University, Newport, Shropshire.
- Annual Advances in Nematology Meeting, Association of Applied Biologist, Linnaean Society of London, 16th December 2014.

List of abbreviations

µ	micro
ANOVA	Analysis of Variance
C.I.H	Commonwealth Institute of Helminthology
CV	Coefficient of Variation
cv.	Cultivar
d.f.	degrees of freedom
DEFRA	Department for Environment Food and Rural Affairs
DW	Distilled Water
dw	dry weight
EC	European Council
ELISA	Enzyme-Linked Immunosorbent Assay
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
FAA	Formalin Acetic Acid
FAOSTAT	Food and Agriculture Organisation, Statistics
FDA	Fluorescein Di-acetate
Fera	Food and Environment Research Agency
g	grams
GPS	Global Positioning System
GSL	Glucosinolate
IPM	Integrated Pest Management
ITC	Isothiocyanate
ITS	Internally Transcribed Spacer
J2	Second stage juvenile
J2s	Second stage juveniles
J3	Third stage Juvenile

1	J4	Fourth stage Juvenile
2	ml	millilitres
3	mol	moles
4 5	OEPP	Organisation Européenne et Méditerranéenne pour la Protection des Plantes
6	OMC	Organic Matter Content
7	*P*	probability
8	PC	Potato Council
9	PCN	Potato Cyst Nematode
10	PCR	Polymerase Chain Reaction
11	Pf	Final Population Density
12	Pi	Initial Population Density
13	PLRV	Potato Leaf Roll Virus
14	PPN	Plant Parasitic Nematodes
15	PRL	Potato Root Leachate
16	RCBD	Randomised Complete Block Design
17	rDNA	Ribosomal Deoxyribonucleic Acid
18	RFLP	Restriction Fragment Length Polymorphism
19	SEM	Standard Error of Mean
20	TGG	Thioglucoside Glucohydrolase
21	UNFAO	United Nation Food and Agriculture Organisation
22	w/v	weight by volume
23	wt	weight

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CHAPTER ONE

1. Chapter 1: Literature review

1.1 Introduction

The potato cyst nematodes (PCN), *Globodera pallida* (Stone) and *Globodera rostochiensis* (Wollenweber) are the most economically important nematode problems to the UK potato industry. They occur in 64% of UK potato land, with *G. pallida* alone present at 92% of these sites (Minnis *et al*., 2002). Potato cyst nematodes inflict an annual cost of approximately £36 million on UK potato farmers in terms of yield lost and nematicides application (Twining, 2009) and have become a threat to the future of the potato crop for many famers. Breeding for PCN resistance since the 1950's has produced only a few acceptable varieties with both commercial value and partial resistance to *G. pallida*, although recently, there have been a greater number available to the processing industries (e.g. cvs. Ambassador, Accord, Vales Everest) (PCL 2013, Variety database). Effective management of *G. pallida* is a vital requirement to uphold the effectiveness of the Grate British potato production.

The management and control of this group of soil borne organisms has always been a challenge because of their cryptic environment. During the past three decades, farmers in the UK have relied heavily on granular nematicides and soil fumigants to control PCN. These fumigants, although effective, pose a variety of negative environmental effects, which has led to certain products being de-registered (E.g. 1,3-dichloropropene) or having restricted use. The broad spectrum and commonly used methyl bromide has been recognized as a contributor to the depletion of the stratospheric ozone layer. As a consequence it was listed for worldwide phase-out in 2010 (Noling, 2002; Schneider *et al*., 2003). Two former nematicides; aldicarb and 1, 3-dichloropropene, have now been withdrawn from use within the European Union. Recent EU legislation (EC 1107/2009) could result in the withdrawal of lone approved soil fumigant, metam sodium or the reduction in the rate of application by end of 2014. Therefore, researchers are constantly searching for phytochemical-based

sustainable approaches for nematode management. Biofumigation is a potential alternative to synthetic fumigants for efficiency against soil-borne pest and disease (Matthiessen & Kirkegaard, 2006).

A number of researchers have investigated biofumigation for PCN control. Buskov *et al*. (2002) found that when myrosinase was mixed with 1 mg l^{-1} of phenethylglucosinolate at pH 6.5, it could cause 100% mortality of *G. rostochensis* juveniles (J2) within just 16 h *in vitro*. Aires *et al*. (2009) conducted a glasshouse study that demonstrated that, PCN suppression in soil was dependent on total glucosinolate concentration and the type of *Brassica* species extract used. They found that a total glucosinolate concentration of just 0.2 µmoles 100 g^{-1} of dry weight was sufficient to cause a significant reduction in the number of new *G. rostochiensis* cysts forming on potatoes, 29 days post incorporation of the extracts. Recently Lord *et al*. (2011) reported that three *Brassica juncea* lines (Nemfix, Fumus, and ISCI99) containing high concentrations of 2-propenylglucosinolate were responsible for over 95% mortality of encysted eggs of *G. pallida* in polyethylene-covered soil after incorporation. However, these findings have been based on glasshouse and laboratory experiments. To date there is no published data to testify the effects of incorporated *Brassica* residues on PCN populations under field conditions.

The review reported herein therefore begins with a brief examination of the host crop *Solanum tuberosum*, its economic importance and major pests and diseases. The biology of PCN is reviewed in relationship with the host crop and biofumigation system to understand the life cycle stage of PCN at which biofumigation with Brassicaceae to control this nematode pest would fit from a field perspective. Previous research using biofumigation for nematodes and other soil borne pest and

disease management are discussed and 'grey' areas are identified while recommendations to effectively utilise the biofumigation system are suggested.

1.1.1 The potato plant: origin and importance

The potato plant is a perennial species belonging to the genus *Solanum* and is a member of the Solanaceae family. Potatoes originated in a southern Peruvian region (Spooner *et al*., 2005) where they were first domesticated between 8000 BC and 5000 BC (Office of International Affairs, 1989). The first introduction of potatoes outside its originated Andes region was four centuries ago, and today it has become an important part of the world's principal food. It is the world's fourth most consumed food crop after rice, wheat, and maize in that order (FAOSTAT, 2013). The introduction of the plant to Europe dates back to the second half of the 16th century by the Spanish following their conquest of the Inca Empire. The adoption of the potato crop by European famers was slow. However, this crop later became an important staple food crop playing a major role in the population boom for the 19th century Europe (John, 2005). As a result of the limited number of varieties during the initial introduction of this crop, there was the absence of genetic varieties, thus an increased vulnerability of the crop to disease. The year 1845 was marked by the Great Irish Potato Famine as a result of the late blight disease, caused by the oomycete *Phytophthora infestans* which spread rapidly through the poorer Irish communities, leading to the crop failure.

The United Nations Food and Agricultural Organization (2009) estimated that the annual diet of an average individual in the early 21st century includes approximately 33 kg of potato. Potatoes provide an important source of vitamin B6, potassium, copper and vitamin C. The crop is also known to be rich in a variety of nutrients with antioxidant activity such as flavonoids and carotenoids.

In the United Kingdom (UK), a potato census conducted by the Potato Council Ltd (PCL) on 97% of registered potato growers in the country revealed that as of January 2013, there were over 122,000 ha of potato grown in the UK (PCL, 2013). This figure shows a 5% increase from the 2012 data of 121,800 ha. Since 1960, there has been a 53% drop in the planted area and the number of growers have dropped by 97% over this period and now presently stands at 2,575 from the 1995 value of 13,395 (PCL, 2013).

Maris Piper commonly referred to as the multipurpose variety, remains the dominant variety in Great Britain, accounting for 16% (18,643 ha) of the total planted area, alongside Markies, Maris Peer, Lady Rosita and Estima making the top 5 varieties in that order. Maris Piper is generally used for pre-packing as well as in the fresh chipping and processing. Since 2008, Maris Piper and Estima have witness a 26 and 66% fall in planted area respectively (PCL, 2013).

In Great Britain, the potato crop is mostly planted from March to May, with a bulk of the crop being planted in April depending on the weather conditions. Optimum yields are obtained under mean daily temperature of 18 to 20°C, and the plants usually take up to 150 days from establishment till harvest. Harvesting in the UK typically begins in June in some areas and continues throughout the country until late October.

1.1.2 Major pests and diseases of potato

Phytophthora infestans (Plate 1.1B) remains an on-going problem in Europe and the US, accounting for and estimated £55 million on an annual basis in the UK alone (Twining *et al*., 2009). In untreated situations, the losses are estimated at a value of ca. £363 million per annum, which is approximately half the cost of potato production in the UK (Twining *et al*., 2009). Other diseases of potato include *Helminthosporium*

solani (silver scurf) (Plate 1.1A), *Rhizoctonia solani* (black scurf) (Plate 1.1E), *Sclerotinia* sp, *Pectobacterium atrosepticum* (black leg) (Plate 1.1F), *Streptomyces scabiei* (common scab), *Spongospora subterranea* (powdery scab) (Plate 1.1D), potato leaf roll virus (PLRV) and *Verticillium dahliae* (verticillium wilt) (Plate 1.1C). Some insects are known to disseminate potato diseases and/or cause damage to the plants. Those of economic importance to the UK potato industry include *Myzus persicae* (the peach potato aphid) and *Macrosiphum euphorbiae* (potato aphid). Nematode problems of potatoes cause great reduction in both crop yield and quality and the potato cyst nematode is the most important pest of potatoes within the UK and worldwide. Other nematode pests of potatoes in the UK include *Ditylenchus destructor* (potato tuber nematode), *Pratylenchus* spp. (root lesion nematodes) as well as the *Longidorus* and *Trichodorus* spp. that transmit potato viruses.

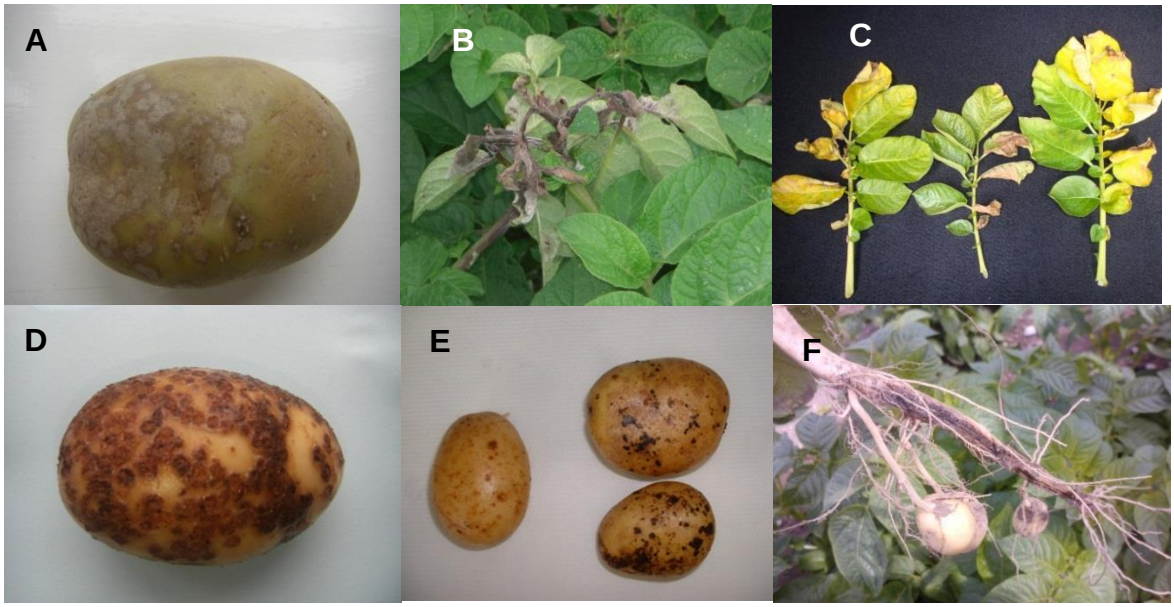


Plate 1.1: Major potato diseases; (A) *Helminthosporium solani* (silver scurf), (B) Phytophthora *infestans* (late blight), (C) *Verticillium dahliae* (verticillium wilt), (D) *Spongospora subterranean* (powdery scab) (E) *Rhizoctonia solani* (Black scurf) and (F) *Pectobacterium atrosepticum* (Black leg). (Photos kindly supplied by Dr. M. Back)

1.1.3 The potato cyst nematode (PCN)

A systematic classification of PCN (Table 1.1), places them under the order Tylenchida which encompasses most of the plant parasitic nematodes of agricultural

importance. They are classified under the generic name *Globodera.* Hitherto, PCN were classified under the genus *Heterodera,* until 1975 when Behrens (1975) proposed that they should be assigned to the genus *Globodera* to differentiate them from *Heterodera* species. However, Skarbilovich (1959) had previously designated the genus *Globodera* to describe these spherical cyst nematodes. They are known to parasitize members of the Solanaceae (Hesling, 1978) and thus have a narrow host range.

Potato cyst nematodes exist as two different species; *Globodera pallida* (Stone) (pale potato cyst nematode) and *G. rostochiensis* (Wollenweber) (yellow or golden potato cyst nematode). Before 1972, PCN was considered as a single species (*Heterodera rostochiensis*) until 1970 when Guile, (1970) observed differences between the species in terms of biology and morphology, thus raising the British pathotype B and E to *G. pallida*. The term “pathotype” is generally used as the International PCN Pathotype Scheme (Kort *et al*., 1977). The two PCN species have a number of different pathotypes (Kort, 1974). The characterization of these pathotypes is based on the nematode’s ability to reproduce on particular clones and crossbreeds of tuberous *Solanum* spp. used in breeding. For *G. rostochiensis*, five pathotypes are recognized as Ro1-Ro5 (international notation) whereas for *G. pallida* three pathotypes exist which include Pa1-Pa3 (Kort *et al*., 1977). All these pathotypes of PCN have been identified in Europe (Turner, 1985). Stone *et al*. (1986) suggested the use of the designation Pa2/3 to refer to Pa2 and Pa3 due to the difficulties in distinguishing between these two pathotypes.

Globodera pallida has been suggested to represent a species complex following phylogenetic analysis of the ITS-rRNA gene of the cyst forming nematode species parasitizing members of Solanaceae (Subbotin *et al*., 2000). In the UK, three PCN

pathotypes have been identified and these include Ro1, Pa1 and Pa2/3 (Turner, 1985). The system of pathotypes classification that is being recognized internationally applies principally to those found within Europe and this may possibly not be applicable to the South American populations (Kort *et al*., 1977). There is a likelihood of existence of some pathotypes in South America which were never transferred from the Andean region (Canto & Mayer, 1978).

Table 1.1: Taxonomy of the potato cyst nematode

Class:	Nematodea
Subclass:	Secenentia
Order:	Tylenchida
Suborder:	Tylenchina
Superfamily:	Heteroderidae
Family:	Heteroderinae
Subfamily:	Heteroderidae
Genus:	*Heterodera* (up to 1975)
	Globodera (Behrens, 1975**)**
Species:	*pallida* (Stone, 1972)
	rostochiensis (Wollenweber)
Common names:	*G. rostochiensis* **-** Yellow/golden potato cyst nematode *G. pallida* **-** White/pale potato cyst nematode Nématode doré de la pomme de terre (French)
Pathotypes:	Ro1, Ro2, Ro3, Ro4, Ro5
	Pa1, Pa2/3

1.1.4 Origin, distribution and economic importance of PCN

Potato cyst nematodes are considered among the most economically important nematode pests of potatoes (*Solanum tuberosum* L.) and are the subject of strict quarantine regulations in many countries. The origin of PCN can be traced to the origin of the host plant from the Andean region of South America, where these nematodes were isolated from wild potatoes and other species of *Solanum* (Evans & Stone, 1977). As a result of human activities, PCN have spread from South America into many regions of the world. Although potatoes were introduced into Europe in the

second half of the 16th century, it is assumed that the introduction of PCN was around 1850, possibly via tubers brought from South America (Evans *et al*., 1975). Potato cyst nematodes are responsible for yield losses of approximately 12% or more worldwide (Bates *et al.*, 2002). Damage caused by PCN in the EU is estimated at approximately €440 million (Ryan *et al.*, 2000a) and to the UK potato industry alone, is estimated to be in excess of £26 million on an annual basis (Twining *et al*., 2009).

An integrated approach to PCN management is usually based mainly on chemical control, crop rotation and resistant cultivars (Trudgill *et al.*, 1987). However, the two PCN species can react differently when subjected to the same control measures. For instance, Whitehead *et al.* (1984) reported that on field plots, the nematicide oxamyl controlled *G. pallida* less effectively than *G. rostochiensis.* The absence of cultivars with complete resistance to *G. pallida* on the UK National Variety List (Turner *et al*., 2006) makes the control of this species more difficult, unlike *G. rostochiensis* where various resistant varieties exist. Therefore, the continuous cultivation of potato cultivars that confer resistance to *G. rostochiensis* have led to a reduction in populations of *G. rostochiensis* and an increase in *G. pallida* in mixed populations. As a result, *G. pallida* now predominates in most of the potato-growing regions in the UK accounting for approximately 90% of infested fields (Minnis *et al*., 2002). In the UK and Ireland researcher are attempting to develop potato cultivars with improved and long-lasting resistance to *G. pallida* using germplasm derived from *S. tuberosum* spp. *andigena* CPC accession 2802 (Moloney *et al*., 2010).

In addition to the absence of *G. pallida* resistant potato cultivars in the UK, this species unlike *G. rostochiensis* is known to persist in the soil for a longer period in the absence of the host crop during crop rotation due to their low rate of spontaneous

hatch (Whitehead, 1992). Moreover, hatching is normally delayed in *G. pallida* (Deliopoulos *et al*., 2007) and the hatching period is extended (Haydock & Evans, 1998), making their control with nematicides such as oxime carbamates which decays faster in the field (Evans, 1993) difficult. Therefore, an ideal control strategy for *G. pallida* will be that which will target encysted eggs (Lord *et al.,* 2011) since PCN invade potato roots for several weeks after potatoes start growing (Whitehead, 1992).

Understanding the basic biology, physiology and biochemistry of phytoparasitic nematodes can form the basis for the determination of appropriate control strategies (Perry, 1994). For instance, many phases of the nematode lifecycle such as hatching, host location, movement to the feeding site and mate finding behaviour can be disrupted. Nematologists have used chemotaxis and hatching assays in a bid to evaluate the properties of phytochemicals to inhibit the hatching and/or affect the sensory response of nematodes. Hewlett *et al*. (1997) demonstrated that the inclusion of tannic acid on water agar could alter the behaviour of *Meloidogyne* spp. Wuyts *et al*. (2006) reported similar effects when the plant-parasitic root knot nematode (*M. incognita*) was exposed to phenylpropanoid products using chemotaxis and hatch inhibition assays.

1.1.5 Biology of PCN

Morphology

Females of PCN are sedentary, globose and have a small projecting neck. The smoothly rounded cysts have a diameter of approximately 450 µm and range in colour from white to yellow after emergence from the roots. Cysts of PCN are similar in shape with a tanned brown skin and the cuticle surface is in a zig-zag pattern of ridges. The perineal area is characterized by a single circumfenetration around the

vulva slit. The anus is sub-terminal without fenestra while the vulva is in a vulval basin. Eggs of PCN are retained within cyst and there is no formation of egg-mass. The non-sedentary second-stage juveniles (J2) are vermiform in shape, and tapered at both ends. The J2 body length ranges from approximately 445 - 510 µm while the stylet length is between 19 - 25 µm. The tail length measures between 37 - 55 µm and the hyaline tail part is between 21 - 31 µm.

The use of cyst and J2 characteristics (as demonstrated in Figure 1.3 below) is recommended for a reliable identification. These two stages are usually present in most soils infested with PCN. The two PCN species, *G. rostochiensis* and *G. pallida* are morphologically closely related. Species differentiation is mostly based on morphological characters. For instance, the perineal area can provide the most important cyst differentiation between the two species (Turner & Rowe, 2006). Diagnostic features include the Granek's ratio (that is, the distance between the anus to the edge of the vulval basin divided by the diameter of the vulval basin) as well as the number of cuticular ridges in between vulva and anus. Granek´s ratio is considered to be the most informative nematode morphometric measurement currently available, principally for differentiating *G*. *pallida* and *G*. *rostochiensis* (Fleming & Powers, 1998). Mean Granek´s ratio values below 3 are usually associated to *G*. *pallida*, whereas values above 3 are associated to *G*. *rostochiensis* (García *et al*., 2009). The most reliable J2 diagnostic features include the stylet length and stylet knob shape. Figure 1.1 shows some illustrations of the different stages of the two PCN species.

Advances in diagnostics have allowed for species identification by the use of more sophisticated methods such as isoelectric focussing (Fleming & Mark, 1983), ELISA (Robinson *et al*., 1993) and PCR (Mulholland *et al*., 1996). PCR-ITS-RFLP and PCR

with specific primer techniques are currently applied in many laboratories for the identification of cyst nematodes, including *Globodera*, and these techniques were recently reviewed by Subbotin *et al.* (2010).

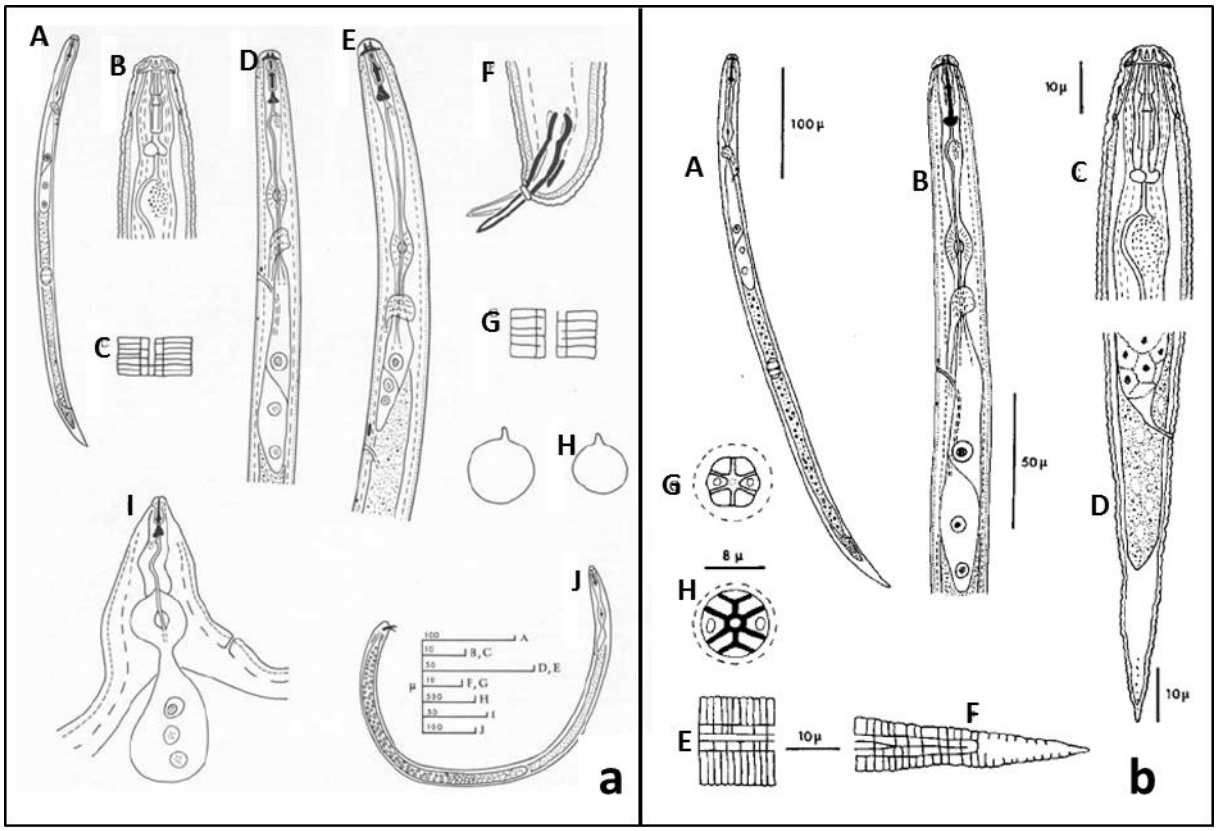


Figure 1.1: (a) *Globodera rostochiensis* (A= second stage juvenile (J2), B=head region of J2, C=J2 lateral field of mid-body region, D=pharyngeal region of J2, E= male pharyngeal region, F=male tail region, G=male lateral field of mid body region, H=cysts, I=female head and neck region, J=entire male). Figure adapted from the Commonwealth Institute of Helminthology's (C.I.H.) Descriptions of Plant-Parasitic Nematodes, (b) *G. pallida* (A=J2, B= anterior region, C=head region, D=tail, E= lateral field of mid-body region, F=lateral field of tail region, G=head and face at lips level, H=head and face at base level). Figure adapted from Stone, (1973a) (OEPP/EPPO, 2009)

1.1.5.1 Lifecycle

The life cycle of PCN (as illustrated on Figure 1.2) is characterized by two phases which are categorized as pre-parasitic and parasitic. The pre-parasitic phase, also referred to as the infective stage, occurs mostly as a free-living stage in the soil. The beginning of the parasitic phase is marked by the location and invasion of the definitive host. Cysts are tanned bodies of dead female nematodes protecting the eggs and are the most visible stage of PCN life cycle. For the completion of the

lifecycle, PCN requires between 38 - 48 days depending on soil temperature (Chitwood & Buhrer, 1945).

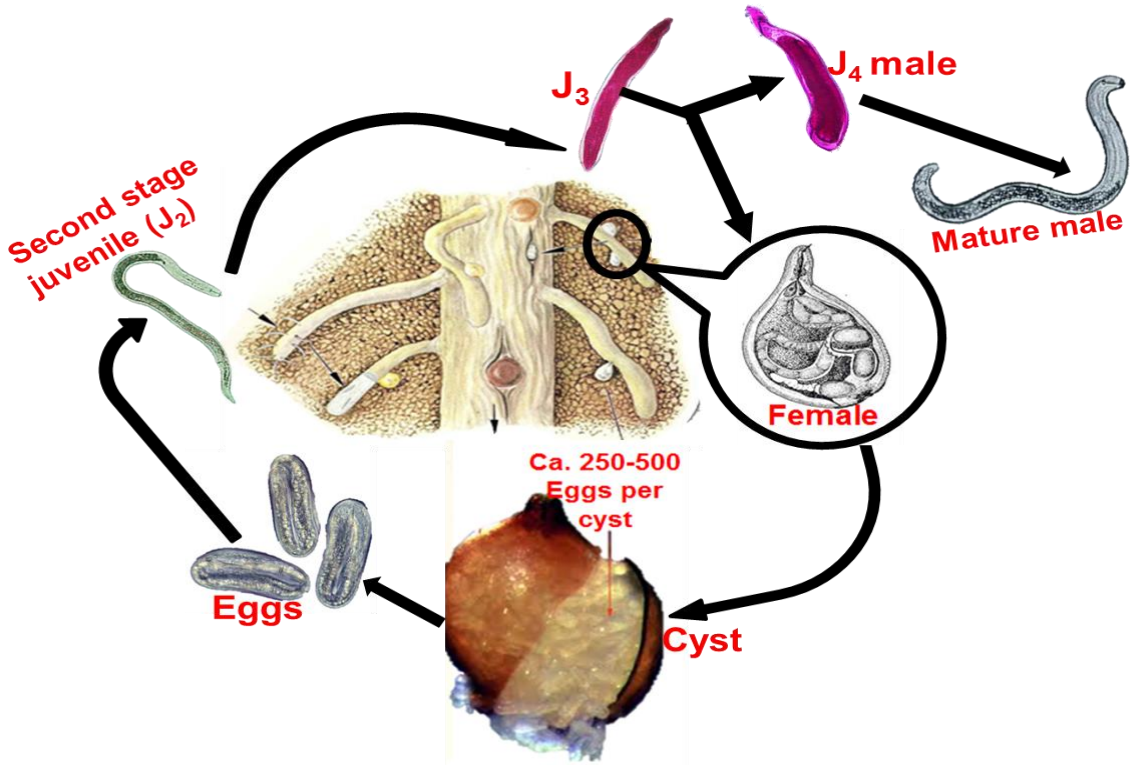


Figure 1.2: Lifecycle of potato cyst nematode (adapted from Evans & Stone, 1977)

Free-living stage (hatching and emergence)

The stimulus for hatching in *Globodera pallida* and *G. rostochiensis* emanates from host roots, as hatching factors in root diffusates (Perry, 2002; Wright & Perry, 2006) and this marks the beginning of the active phase of the life cycle. Hatch is stimulated in the majority (approximately 75%) of eggs by potato root leachates (Rawsthorne & Brodie, 1986), while the rest of the proportion that fail to hatch (Trudgill *et al*., 1996) remain in diapause (see later) and are carried over to the next host cropping season. The diapaused eggs constitute an important part of the PCN survival mechanism and ensure persistency in the soil over time.

The availability of adequate moisture and the presence of potato roots leachates play an important role in the initiation of PCN juvenile hatching from encysted eggs.

Distilled water can also stimulate hatch, but the proportion of hatch in this case is very low (< 30%) for both PCN species (Evans, 1983). The potato plant roots secrete a moderately strong organic acid in their root leachates (Perry, 1989) which is required in relatively small quantities to stimulate hatching of the J2 from the eggs. This is achieved through the alteration of the permeability of the cyst thus reducing the osmotic pressure on the un-hatched J2 within the egg as well as increasing the metabolic activity of the hatching J2.

Temperature and soil type plays an important role in the hatching of PCN and each species responds differently to the range of temperatures under which they hatch. In general, *G. pallida* shows a better adaptation to lower temperatures than *G. rostochiensis* with optimum thermal conditions for hatching of 16°C and 20°C respectively (Robinson *et al.*, 1987b). However, under field conditions *G. rostochiensis* can adapt to hatch at low temperatures (Ellenby & Smith, 1975; Hominick, 1979). Crops grown in sandy soils tend to suffer more damage due to the ease of movement of J2 in these soils (Trudgill *et al.,* 1998). *Globodera pallida* is also known for its slower utilisation of its lipid reserves (Storey, 1984; Robinson *et al.,* 1987a) which contributes to a longer persistence in the soil in the absence of the host plant.

Parasitic stage (root invasion)

Upon successful hatching, J2's need to locate their host as soon as possible because they are highly vulnerable to extreme environmental conditions (Perry, 1998) such as lack of moisture and extreme soil temperatures. Attractants such as potato root leachates play an important role in attracting and directing the nematode toward the root tips which is the most metabolically active region of the root. Robinson *et al.* (1987a) estimated the infective life for both *G. rostochiensis* and *G*

pallida to be within six to eleven days. After host location, invasion of the roots takes between 50 to 60 minutes followed by intercellular migration. With the use of a robust stylet, J2's cut their way through the cells to reach the vascular cylinder where they remain sedentary, forming a feeding site referred to as a syncytium (Von Mende, 1997).

The J2 feeds and moults into the J3 stage where sexual differentiation begins. The ratio of males to females is determined by environmental conditions as well as food availability. In conditions with adequate food supply, there is a high population of females and vice versa (Trudgill, 1967), ensuring the maximum reproduction rate for the food available. After sexual differentiation, males develop into vermiform nematodes and migrate out of the roots, while the females will continue feeding and thus increase in size. Eventually, the posterior region of the female enlarges and erupts to the surface of the root while the head remains embedded in the root. Eggs are then produced and can be fertilized by the free-living male within 40 days. The male has a life span of up to 20 days and once they emerge from the roots, they remain fertile for up to 10 days. Males are attracted to females by sex pheromones produced by the female (Evans, 1970) and a single male can fertilize several females.

After fertilization, the embryos develop into eggs inside the females. At this stage, the female dies and its cuticle tans to form a protective cyst around the eggs. A single cyst can contain 200 to 500 eggs (Brodie *et al*., 1993). The encysted eggs undergoes a period of dormancy (Hominick, 1986; Muhammad, 1994) in which their development is suspended allowing them to survive unfavourable conditions (Wright & Perry, 2006).

Dormancy in PCN can be differentiated as quiescence and diapause (Evans & Perry, 1976). Quiescence refers to a reversible arrest in development induced in response to unfavourable conditions, whilst diapause is a state of arrested development which cannot be resumed unless specific requirements have been satisfied, even when favourable conditions return (Perry, 1997; Wright & Perry, 2006). In PCN, diapause is initiated by signals passed from the host plant to the nematode during the growing season (Hominick *et al.*, 1985; Hominick, 1986). In this case, the photoperiod acting on the host plant affects the females in development which influences subsequent hatch of J2 (Hominick, 1986). This is considered as obligate diapause which can be terminated by a fixed period of cold temperature (Wright & Perry, 2006). Perry (1989) referred to this diapause of un-hatched J2 as a sophisticated way of ensuring survival in the absence of host plant and synchronizing parasite emergence with host availability. Facultative diapause in PCN can also be associated with conditions such as day length, light intensity (Hominick, 1986) and low temperatures (Hominick, 1979; Wright & Perry, 2006).

Quiescence in PCN may occur in the absence of the host and thus can be broken by the host root diffusate. This type of dormancy allows PCN populations to persist in the soil for about 20 years in the absence of their host and to hatch in few days once the host is present in the field (Jones *et al.*, 1998).

1.1.6 Symptoms

Potato cyst nematode infestations are visible on the plant aerial parts and may be associated with patches of poor growth across the field (Figure 1.3). Affected plants appear stunted as a result of root infection which induces water stress and mineral deficiency. Plants may also exhibit yellowing, wilting or death of foliage (MAFF, 1989). In addition, the root system is reduced and shows abnormal branching. At

flowering, tiny white, brown or yellow cysts can be seen on the root epidermis (Brodie *et al*., 1993). Potential yeild is reduced by PCN yields even in small infestations, with lateral roots being the most vulnerable to nematode attack (Trudgill *et al*., 1975). Heavy PCN infestations are charecterised by significantly reduction in the tuber size and number in potatoes. Disease complexes involving PCN and soil-borne pathogens have been recorded with *Rhizoctonia solani* (Back *et al*., 2006; Bhattarai *et al*., 2010).

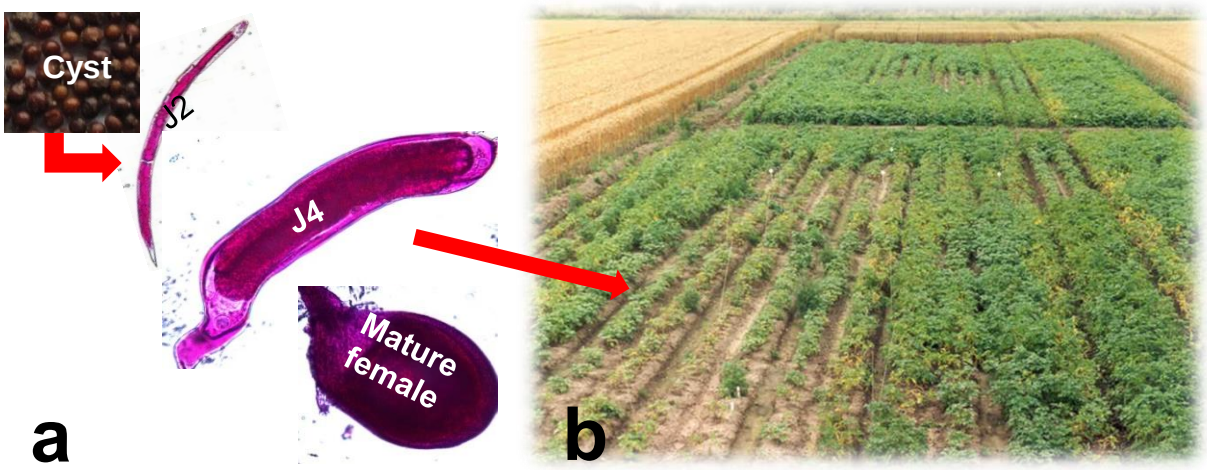


Figure 1.3: (a) Different stages of PCN (magnification= x60), (b) extensive root damage caused by PCN resulting in stunting and premature senescence of potatoes (source: M. Back).

1.2 Current management and control of PCN in the UK

The aim of any control method employed for PCN is to prevent significant yield losses in vulnerable crops and in the long term to keep populations below threshold levels (Whitehead & Turner, 1998). As of July 2010, the control of PCN became a legal requirement under the EU directive 9365/07. The control of this pest remains a challenge to the potato farmers considering the increased incidence of these nematode pests. Farmers have relied on a number of control options, some of which are discussed below.

Cultural practices.

Cultural control can be categorised into the prevention of spread, selection of nematode-free propagating materials and crop rotation (Brown, 1978). The prevention of nematode dissemination can be achieved both though quarantine regulations (at international level) and utilization of clean materials on uninfected lands (at farm level). Once established in the field, PCN population multiplies rapidly and can be spread through several means such as transferring infested plant material from one part of the field to another and movement of machinery and farm tools across the field.

Crop rotation is a very important means of control since PCN are known to have a narrow host range (Haydock & Evans, 1998). This method allows a sufficient cropping interval between successive potato cropping therefore allowing for natural decline in PCN populations (Brown, 1978). This decline results from a combination of factors such as spontaneous hatching, predation and reduction in food reserve within J2, thus preventing hatching (Turner, 1996; Perry, 1998).

Whitehead (1995) estimated a 20 - 40% and 10 - 30% decline for *G. rostochiensis* and *G. pallida* respectively on an annual basis as a result of the absence of the host and this is said to be most evident during the first year of rotation as the cysts becomes more sensitive to hatching factors (Devine & Jones, 2000). It has been suggested that, in fields with high populations of *G. pallida* (more than 60 eggs g^{-1} soil) a rotation of one potato crop in 10 to 13 years is required to reduce infestation levels to 5 eggs g^{-1} soil (Whitehead, 1998; Lane & Trudgill, 1999). However, such long rotations are commercially unviable due to the specialised nature of potato growing coupled with the high cost of machinery (Whitehead, 1998). A one in four

rotation is commone (depending on species) with the integration of other control strategies to reduce PCN populations.

Use of cultivar tolerance against PCN

Trudgill (1991) referred to tolerance as the capacity of plants to yield well regardless of the damage inflicted by PCN. This can result from attributes such as prolific haulm and root growth, a reduction in the production of hatching agent, localized root reaction in response to PCN invasion and their interaction with other antagonists. Some PCN tolerant potato varieties include Maris Piper and Cara (Trudgill & Cotes, 1983). Tolerant varieties can only be effective if used in combination with other methods as they allow for multiplication and population increase of PCN without any reduction in the long term management of the nematodes (Brodie *et al*., 1993)

Use of cultivar resistance against PCN

Resistance of a potato cultivar to PCN is measured by the ability of the nematodes to feed, develop, and reproduce on the host potato cultivar. A cultivar is termed susceptible if it allows free multiplication of nematodes; a partially resistant cultivar will allow some multiplication, whereas a resistant cultivar will not allow for any multiplication (Minnis *et al*., 2002). The wild diploid potato, *Solanum vernei* Bitt. *et* Wittm was the first to be reported as being resistant to PCN (Ellenby, 1954). Maris Piper was the first *G. rostochiensis* resistant cultivar to be commercialized in 1966 and has since then been widely cultivated in the UK. The mechanism for resistance is conferred by a single dominant gene (H_1) from *Solanum tuberosum* ssp. *andigena*. The underlying mechanism for resistance to PCN functions by preventing the establishment of feeding sites through walling off necrosis or by vascularization of the syncytium (Cook & Evans, 1987). The H_1 gene is, however, ineffective against *G. pallida* as this species of PCN is capable of selecting for virulent strains to overcome

resistance by the H_1 gene (Lane & Trudgill, 1999). Consequently, the wide spread cultivation of varieties with the H_1 gene (such as Cara and Maris Piper) by most of the UK potato growers has led to a shift in PCN population to *G. pallida* which now dominates ware production areas.

In the UK, the search for resistance to *G. pallida* among the widely grown potato cultivars with high market value has been less successful. Despite all the effort that has been put in place, no cultivar is found to confer more than about 90% resistance against *G. pallida* relative to non-resistant control cultivars. Also, the degree of resistance is said to vary with nematode population. So far, partially resistant cultivars to *G. pallida* Pa 2 and Pa 3 such as Aveka, Aviala, Darwina, Kantara, Nomade, Producent, Seresta, Agria, Hommage, Innovator, Elles, Maritiema, Santé, Vechtster (Schomaker & Been, 2005) do not have wide market demand. These cultivars have proved successful in limiting the multiplication of field populations of *G. pallida,* and in some cases, reducing the populations when granular nematicides were used at just half the recommended rate (Alphey *et al*., 1988). In order to avoid the selection for virulence by *G. pallida*, it is important that different partially resistant cultivars are alternated. However, due to the poor market demand, few growers use even one of such cultivars, thus the chances of two or more cultivars being considered suitable is unlikely.

Most recently, Arsenal, a crisping and processing variety with resistance to *G. pallida*, Ro1 and Ro4 has been developed by breeder and seed supplier Agrico UK Ltd. This virety is reportedly in its fouth year of field trials and may be available in the neares future for crisping and processing company as per the director of Agrico UK, Archie Gibson (Agrico UK Newsetter, Spring 2014).

The use of trap cropping against PCN

The use of non-host or poor host crops referred to as trap crops has generated substantial interest from researchers and growers since the recognition of PCN as being economically important in the production of potatoes. This area has been extensively researched since the 1930s (Carroll & McMahon, 1937; 1939) and has been used successfully to achieve PCN population reductions of up to 87% (Lamondia & Brodie, 1986) or more (Halford *et al*., 1999). This control strategy has the advantages of being relatively short in duration (between five and six weeks, depending on soil temperature) and flexible in terms of use within the growing season. The efficacy of a trap crop is dependent on the length of time required for the crop to be left in the ground in order to trigger hatching in as many cysts as possible, without the completion of the life cycle.

Scholte (2000a) reported that the potato itself can be used as a trap crop. However, potatoes are not an ideal trap crop for PCN due to drawbacks such as the stimulation of other potato diseases, the exact timing of crop destruction and the possibility of volunteers in the next cropping season. Volunteer potato plants, if not effectively controlled, will become point sources of PCN multiplication.

Solanum sisymbriifolium (Sticky Nightshade) has been selected as a promising candidate after extensive screening of non-tuber bearing *Solanaceae* for their potential as trap crops for PCN (Scholte, 2000b). This *Solanum* species has been reported earlier by Roberts and Stone (1981) to attain a level of hatch stimulation slightly less than that of *Solanum tuberosum* cv Bintje.

Solanum sisymbriifolium triggers hatch of PCN J2's and root invasion but does not allow multiplication of the nematodes. The advantages of using *S. sisymbriifolium* in trap cropping for PCN are twofold amongst which includes its capacity to trigger

hatch coupled with its complete resistance to PCN. *Solanum sisymbriifolium* is tolerant to frost and resistance to blight and has been demonstrated to reduce PCN population densities by more than 75% (Scholte & Vos, 2000; Scholte, 2000c). However, it also has some drawbacks such as its slow establishment and requires low pH for effective growth. The crop does not grow well in sandy loam which predominate the UK potato cultivated areas. *Solanum sisymbriifolium* may sometimes require irrigation to establish and generally requires fertilizer application as well as weed control.

The use of chemical nematicides against PCN

Nematicides by definition refer to chemicals which kill nematodes. Two broad categories of nematicides are recognized and are classified on the basis of their mobility in the soil (Table 1.2). These include soil fumigants formulated as liquids which rapidly volatilise and move through open air spaces in soil as a gas, and non-fumigant nematicides generally formulated as either micro-granules or liquids and move by percolation in soil water (Noling, 2002). Non-fumigant nematicides are further grouped as contact or systemic nematicides on the basis of whether they kill nematodes in soil by contact or affect the nematodes within the plant during feeding.

The mode by which nematicides acts on specific and vital life processes within tissues of the nematodes varies depending on the type of formulation. For instance, broad spectrum soil fumigant nematicides directly penetrate the body wall of the nematode without necessarily being consumed during nematode parasitism of plants. After diffusion into the body cavity of the nematode, different internal organs are affected. Upon hydrolysis, metam sodium decomposes rapidly, releasing methyl-isothiocyanate (MITC) as by-products which directly penetrates the nematode body wall and simultaneously interferes with many different vital processes, including

enzymatic, nervous and respiratory systems (Noling, 2002). In this case the death of the nematode occurs rapidly. Generally, fumigant concentrations within the soil frequently attend equilibrium with the concentrations in the nematode body within approximately 30 min to 4 h of exposure.

Non-fumigant nematicides also penetrate the body wall of nematodes. However, unlike the broad spectrum soil fumigants, these chemicals have little or no effects on fungal or bacterial pathogens, but may be insecticidal. Examples include carbamates (carbofuran, Oxamyl) and the organophosphates (Ethoprophos, Fenamiphos, Fosthiazate) which act as acetyl cholinesterase inhibitors, interfering with normal nerve impulse transmission within the central nervous system of insects. This leads to abnormal behaviour, paralysis and death. The primitive nature of the nematode's nervouse system means these compounds are not as toxic to the nematodes as they do on insects pests, thus, are not generally considered to be true nematicides. Therefore, the mortality of nematodes is due rather to a 'narcotic' effect and behavioural modification rather than direct killing. At high concentrations and over extended time period of exposure, disruption of nerve impulses, which ultimately may be lethal, affect primarily the nematode behaviour and their development in soil. This activity may sometimes reduce body movement, mobility in soil and mating behaviour as well as root penetration and feeding. As a result, there is often delayed egg hatch, moulting and subsequently reduced development within plant tissues. The observed reductions in nematode population densities following non-fumigant nematicide treatment is therefore as a result of the reduced nematode infection, development and reproduction in the plant.

Systemic nematicides such as Aldicab, Oxamyl and Fenamiphos are absorbed and translocated into roots and they are known to impede feeding, cause temporal

inactivation or repel the nematodes from the roots and its surrounding areas (Noling, 2002). Hence, death in this situation occurs as a result of disorientation and starvation of the nematodes.

Two former commercially available nematicides (aldicarb and 1,3-dichloropropene) which were widely used for the control of PCN in the UK and other EU countries, are no longer applicable within the European Union (EU) (European Council Directive 91/414/EEC). Further EU legislations (EC 1107/2009) could possibly result in the phaseout or limitation in application of the remaining approved granular nematicides (fosthiazate, ethoprophos, and oxamyl). The application of granular nematicides takes place prior to planting followed by soil incorporation through cultivation (Haydock & Evans, 1998). Granular nematicides have preference over fumigants due to their level of specificity, lower rates and ease of application as well as the absence of phytotoxic effects.

The biological nematicide DiTera® (Copping, 2004) which is a by-product from the fermentation of *Myrothecium verrucaria* has been used in PCN management. This nematicide affects nematodes by reducing their movement and by inhibiting the sensory perception of potato root diffusates (Twomey *et al.*, 2000). Presently, the application of this biocidal product is limited to the USA (Anon, 2009) and its full mode of action is unknown for commercial reasons.

Table 1.2: Important nematicides used for the management of potato cyst nematodes (adapted from Haydock *et al*., 2006)

Active substance	Chemical group	Year of discovery	Example trade name	State of formulation	Manufacturer
Aldicarb	Oxime carbamate	1965	Temik 10G & 15G	Microgranule	Bayer CropScience
Carbofuran	Carbamate	1965	Furadan 15G Furadan 4F	Microgranule Liquid	FMC Corporation
Cadusafos	Organophosphorus	1982	Rugby 200 CS Rugby 10G	Liquid Microgranule	FMC Corporation
Dazomet**	MITC liberator	1897	Basamid	Microgranule	BASF Corporation
1,3-Dichloropropene	Halogenated hydrocarbon	1956	Telone II Telone EC	Liquid Liquid	Dow AgroSciences
Ethoprophos	Organophosphorus	1966	Mocap 10G Mocap EC	Microgranule Liquid	Bayer CropScience
Fenamiphos	Organophosphorus	1967	Nemacur 15G Nemacur 3	Microgranule Liquid	Bayer CropScience
Fosthiazate	Organophosphorus	1992	Nemathorin 10G	Microgranule	Syngenta
Metam sodium**	MITC-liberator	1951	Vapam Vapam HL	Liquid Liquid	Amvac Chemical Corporation
Oxamyl	Oxime carbamate	1974	Vidate 10G Vydate L	Microgranule Liquid	Du Pont

**fumigant nematicide

Biological control of PCN

Biological control is an environmentally healthy and effective way of mitigating pests and their effects by using natural enemies. A lot of proposals have been suggested to define biological control. However, the most widely used definition is that of Eilenberg *et al*. (2001) which refers to biological control as the use of living organisms to suppress the population density or effect of a specific pest organism, reducing it ability to damage than it would otherwise be. With respect to cyst nematodes, Crump and Kerry (1987) came to a conclusion that, the biological control agent that can be most effective against this group of nematodes would be those that parasitize young female nematodes. Most often, the development of biological control agents has relied on empirical experiments (Stirling, 1991), but the development of successful strategies utilising biological agents requires a careful selection of active isolates and an understanding of the factors affecting the epidemiology of the agent and pest.

Although biologicals are considered environmentally safe, they are often slow in action, inconsistent and less effective when compared with conventional control methods. These limitations seem apparent for most biological control agents and thus, integration with other control strategies would be more promising for their success in managing plant pests. The integration of biologicals with organic amendments has been demonstrated to be a promising approach (Lang *et al*., 2012). For instance, the combination of neem cake with *Pseudomonas fluorescens* has been suggested to enhance the control of *Fusarium* wilt on banana (Zhang *et al*., 2011). Also, *Bacillus* spp. has been used synergistically with agricultural compost to suppress disease in soils (Zhang *et al*., 2011; Wei *et al*., 2011; Qiu *et al*., 2012). Therefore, a careful consideration of the mode of actions and favourable conditions for the biological control agents that have been identified for their activities against

plant-parasitic nematodes would be a promising approach to the success of these agents. Some examples of potential biological control agents and their respective mode of actions on plant-parasitic nematodes are shown on Table 1.3.

Table 1.3: Examples of potential biological control agents and the modes of action by which they affect plant-parasitic nematodes (PPN)

Type of biological control agent	Mode of action on PPN
Facultative parasites Trapping fungi	Traps produced on modified mycelium give rise to infective trophic hyphae
Paecilomyces lilacinus	Hyphal penetration
Verticitlium chlamydosporium	Hyphal penetration
Obligate parasites *Pasteuria* spp.	Adhesive spores
Hirsutella spp.	Adhesive spores
Rhizosphere bacteria	Toxins or modification of root exudates
Endophytic fungi (non-pathogenic root-infecting fungi and mycorrhizae)	Competition in roots and modification of root exudates

Sources: Kerry, (1987); Stirling, (1991); Sikora, (1988)

Integrated control strategies for PCN

The integration of different control methods is widely accepted by researchers as being the most effective for the control of PCN (Haydock & Evans, 1998). Strategies such as those that increase the activity of the indigenous flora and fauna has been used for the exploitation of nematodes natural enemies. The manipulation of the indigenous antagonists practically has been largely restricted to the use of soil amendments and crop rotation. Some examples of integrated control strategies for PCN include exclusion (quarantine), reduction of initial inoculum density, suppression of nematode reproduction, and restriction of damage to the current crop.

A combination of different methods such as the use of resistant cultivars, trap cropping, rotation with non-host crops, biological control and reduced rates of nematicides are recommended for a successful PCN control. The simultaneous

application of two or as many of these options as would fit the situation would compensate for the limitations of individual management options for PCN which ultimately will improve yield and reduce the initial population densities of the nematode.

1.2.1 The use of brassicaceous crops for the control of PCN

Considering the limitations of other available control methods coupled with the costs of using products such as soil fumigants against PCN, potato farmers in the UK and around the EU are developing interest in growing brassica cover crops as green manures that could serve as biofumigants. Such biofumigants, if effectively managed, are not only capable of reducing the soil nematode populations (Aires *et al*., 2009; Lord *et al*., 2011) but also ensuring an optimal environment for beneficial soil microorganisms (Wang *et al*., 2014). For instance, metham sodium is detrimental to these soil microbial populations and is thus capable of reducing soil processes such as nitrogen and carbon mineralization (Toyota *et al*. 1999; Ibekwe *et al*., 2001).

Studies on the biocidal effect of brassicaceous crops on nematodes date back to 1925 when Morgan (1925) noticed a reduction in the populations of the eelworm (cysts nematodes) on roots of potato plants grown in closed proximity to mustard plants. This effect was later confirmed by Triffitt (1929, 1930) who observed reduced hatching of PCN J2's in leachates from mustard plant roots. However, these authors failed to note the species of mustard used in these experiments.

Ellenby (1945) was motivated by the findings of Triffitt (1929; 1930) to conduct small scale field experiments with black and white mustard, turnip, rape, watercress, Brussels sprouts and lettuce (an additional non-brassicaceous plant). Ellenby (1945) found that when the root leachates of potato plants were mixed with selected

mustard plant leachates, there were significant effects in the emergence of second stage juveniles (J2s) of the potato eelworm. This phenomenon has been observed for decades with little interest, perhaps due to the availability of more effective chemical control measures. However, it has recently gained renewed interest due to the need to seek alternatives to soil fumigation as well as reducing the dependency on other synthetic pesticide due to the cost of their applications and their negative environmental effects.

Plants belonging to the Brassicaceae family have received special focus based on their human health benefits (Traka & Mithen, 2009; Verkerk *et al*., 2009) as well as their ability to control nematodes and other soil-borne pests (Mojtahedi *et al*., 1993; Potter *et al*., 1998; Chitwood, 2002; Zasada & Ferris, 2004; Aries *et al*., 2009; Lord *et al*., 2011). In agricultural practice, brassica crops are used as green manures. Their effectiveness in the control of nematodes and other soil borne pests and pathogens has been attributed to their ability to produce a range of volatile biocidal compounds when tissues are macerated and incorporated (Bones & Rossiter 1996; Fahey *et al*., 2001). These volatile compounds are referred to as biofumigants and the phenomenon is termed biofumigation (Kirkegaard *et al*., 1993).

1.2.2 Biofumigation process: definition and mechanism

The term biofumigation was originally coined as the suppression of soil-borne plant pests, weeds and pathogens by biocidal compounds, primarily isothiocyanates, released when plant residues are hydrolysed and incorporated into soils (Kirkegaard *et al*., 1993). In the UK and other parts of Europe, the use of brassicaceous plants as green manures is becoming a common practice. Elsewhere, the potential of the biofumigation process has been demonstrated under field experiments to control phytoparasitic nematodes such as *Meloidogyne incognita* in zucchini crop production

(Lazzeri *et al*., 2009) and *M. javanica* in vineyards (Rahman & Somers, 2005). Nevertheless, there have been inconsistencies in the level of pest or pathogen suppression achieved with the brassica green manures. Some researchers have reported moderate to high levels of control following soil incorporation of brassicaceous residues (Rahman & Somers, 2005; Motisi *et al.*, 2009; Lord *et al*., 2011), whereas others have shown little or no effects on target organisms (Johnson *et al*., 1992; Friberg *et al*., 2009, Valdes *et al.,* 2011; Vervoort *et al*., 2013) and in some cases stimulatory effects have been shown (Stephens *et al*., 1999). These inconsistences now need to be addressed and improved through a comprehensive understanding of the mechanism of action underlying the biofumigation process.

1.2.3 Glucosinolates and their occurrence:

The unique properties of glucosinolates (GSL) and their hydrolysis products, was first observed and recorded in the early 17th century due to research examining chemical origin behind the sharp taste of mustard seeds (Fahey *et al*., 2001). Sinigrin (2-propenyl or allyl glucosinolate) and sinalbin (4-hydroxybenzyl glucosinolate) were the first glucosinolates to be isolated from *Brassica nigra* (black mustard) and *Sinapis alba* (white mustard) respectively. Gadamer (1897) proposed the first general structure for GSL which was later quashed and revised by Ettlinger and Lundeen (1956a) who identified the limitations of the structure proposed by Gadamer to explain some properties of these compounds. They proposed the structure that is presently in used, and followed by describing the first chemical synthesis of a gGSL (Ettlinger & Lundeen, 1957) (Figure 1.4)

During the last 5 decades, GSL have been a subject of intense focus, and a succession of reviews have addressed the chemistry and biology of these secondary plant metabolites (e.g. Kjaer, 1961, 1974; Ettlinger & Kjaer, 1968; Kjaer & Larsen,

1973, 1976; Underhill *et al*., 1973; Underhill, 1980; Fenwick *et al*., 1983; Chew, 1988; Duncan & Milne, 1989; Brown & Morra, 1997; Halkier, 1999; Mithen *et al*., 2000; Fahey *et al.*, 2001; Chitwood, 2002; Bjorkman *et al*., 2011). Chemically, they exist as ß-thioglucosides, derived from amino acids and are grouped according to the structure of their side-chain (R). They are limited to the order Capparales, which includes the agriculturally important Brassicaceae (Crucifereae), Capparaceae, Resedaceae and Moringaceae (Rodman, 1981; Brown *et al*., 2003). The limitation of glucosinolates to this single order is believed to be associated with a comparatively new biosynthetic pathway in the plant kingdom. They all share the same basic structure with variation in the R-group which represents the precursor amino acids involved in their biosynthesis (Schonhof *et al*., 2004). To date approximately 132 glucosinolates have been identified (Agerbirk & Olsen, 2012) and their additional diversity is as a result of the secondary modifications of the side-chains by hydroxylation, glycosylation and desaturation after synthesis of the parent glucosinolate (Rask *et al*., 2000).

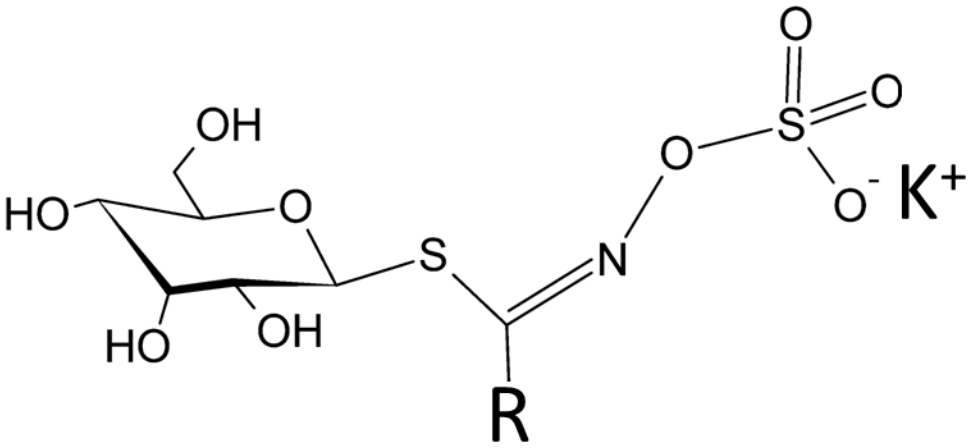


Figure 1.4: The general structure of glucosinolates (Fenwick *et al*., 1983). R=side chain

The first two stages of glucosinolate biosynthesis (side chain elongation and formation of the core structure) appear to have arisen principally by enzyme activity

(Graser *et al.,* 2000). On the other hand, side chain modification has involved the evolution of new enzyme activities after gene duplication (Kliebenstein *et al*., 2001a). Kliebenstein (2009) noted that the plant glucosinolate content is a quantitative trait under polygenetic control and the influence of environmental factors. The ability of plants to biosynthesize glucosinolates has been used by taxonomists to support the classification schemes based on evolution (Rodman, 1981, 1991a, b; Mithen *et al*., 1987a; Rodman *et al*., 1993). Some examples include the absence of methyl glucosinolate in the Brassicaceae, which is a distinctive factor of the closely related Capparaceae. The appearance of glucosinolates with glycosylated R-groups is limited to the Moringaceae and Resedaceae.

Attention has been given to glucosinolates belonging to the genus *Brassica* (Kjaer, 1974, 1976; Fenwick *et al*., 1983; Chew, 1988; McDanell *et al*., 1988; Duncan & Milne, 1989; Stoewsand, 1995; Rosa *et al*., 1997) probably due to the effectiveness of their hydrolytic products against soil-borne pests (Lord *et al*., 2011) as well as their nutritional and health benefits to humans and animals (Cartea & Velasco, 2008).

1.2.4 Origin and diversity of the genus Brassica

The family, Brassicaceae, alone contains more than 350 genera and 3000 species. The genus *Brassica* encompasses numerous species of agronomic importance, such as *B. napus* (rape seed or swede rape), *B. oleracea* (cabbage), *B. rapa* (turnip rape), *B. nigra* (black mustard), *B. juncea* (brown, yellow or Indian mustard) and *B. carinata* (Ethiopian or Abyssinian mustard). The genomic relationship between these species is referred to as the triangle of U (Nagaharu, 1935) (Fig 1.5), where the basic diploid species have been classified cytogenetically as *B. rapa* (AA; 2n=20), *B. nigra* (BB; 2n=16) and *B. oleracea* (CC; 2n=18). These species interbreed interspecifically with

one another to form three new allotetraploid species; *B. juncea* (AABB; 2n=36), *B. carinata* (BBCC; 2n=34) and *B. napus* (AACC; 2n= 38).

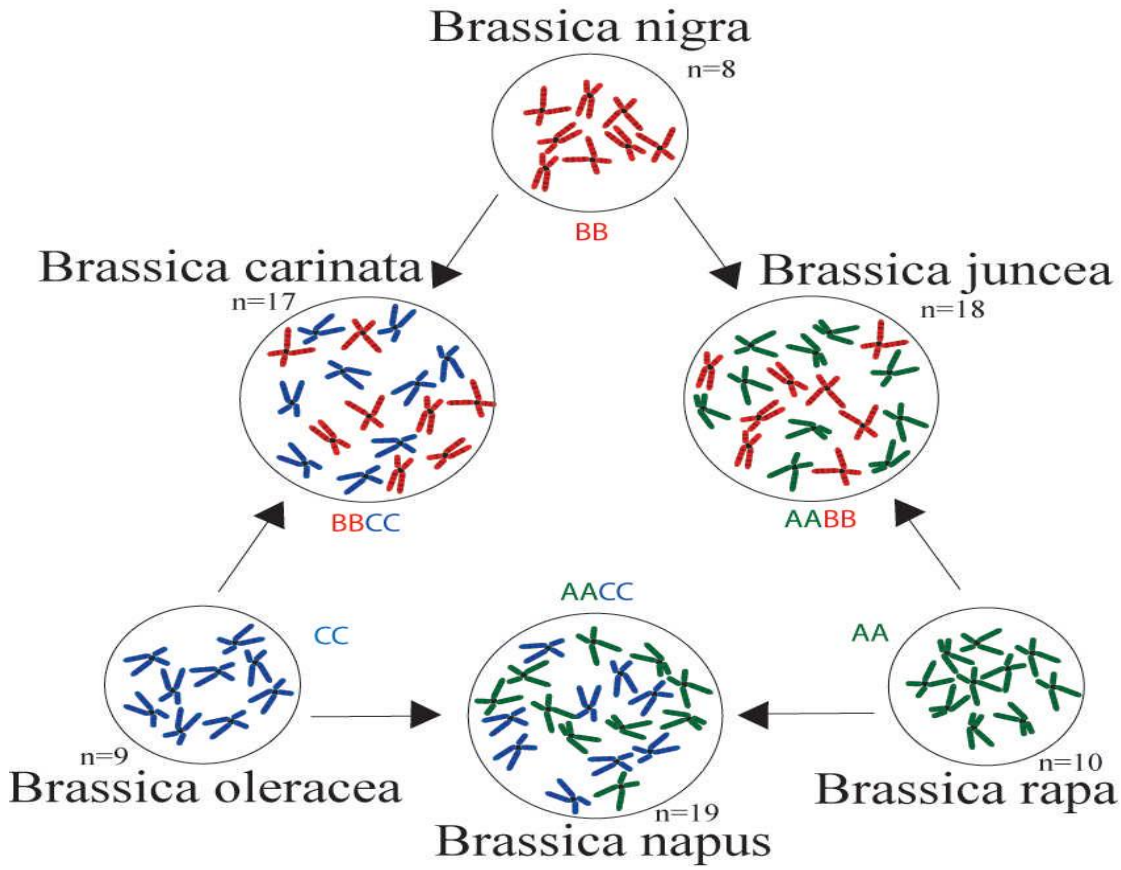


Figure 1.5: The "Triangle of U", an illustration of the genetic relationships between the six species of the genus *Brassica*. Chromosomes from each of the genomes A, B and C are represented by different colours (Nagaharu, 1935, cited in Downey & Rakow, 1987).

Out of the hundreds of *Brassica* species that have been investigated, all are endowed with the capacity to synthesize glucosinolates (Kjaer, 1976). The biosynthesis of glucosinolates among the Brassicacaea is suggested to have been as a result of an evolutionary detoxification mechanism in plants expressing cyanogenic glucosides (Wittstock & Halkier, 2002). The types of glucosolinates (Table 1.4) can vary greatly between species but are consistent within species.

Mixtures are common within the same species, although aliphatic glucosinolates predominantly appear in shoots whereas aromatic glucosinolates are more common

in roots (Kirkegaard & Sarwer, 1998). Quantitatively, individual glucosinolates vary greatly between and within the same *Brassica* species and this can be accounted for partly by the different biosynthetic pathways leading to a hypothesis that this content is subject to both genetic and environmental control (Mithen, 2001; Li & Quiros, 2003; Windsor *et al*., 2005). Aliphatic glucosinolates are said to be derivatives of methionine, aromatic glucosinolates from tyrosine or phenylalanine, while indole glucosinolates are derivatives from tryptophan (Schonhof *et al*., 2004).

1.2.5 Glucosinolates and their breakdown products

The hydrolysis of glucosinolates is catalyzed by myrosinase (thioglucoside glucohydrolase enzyme; EC 3.2.1.147) (Bor *et al*., 2009). In intact plant tissues, the protein myrosinase is contained in myrosin organelles which are confined to parenchymatous tissue of the green parts of different plants of Brassicaceae, especially in the epidermal cells of leaves. Myrosinase is separated from glucosinolates, which is localized in the vacuoles, by cell organelles (Bennett *et al*., 2006). The loss of this compartmentalization as a result of mechanical or physical damage to plant tissue during slow freezing, thawing, chopping or chewing, results in the myrosinase-catalyzed hydrolysis of glucosinolates (Song *et al*., 2005). The catalytic hydrolysis enhances the conversion of these compounds to the corresponding aglycone which then decomposes into isothiocyanates, oxozolidine-2-thiones, nitriles, epithionitriles, and thiocyanates depending on the R-group and the chemical conditions such as availability of ferrous ions and most importantly pH (Figure 1.6) (Bennett *et al.,* 2004; Grubb & Abel, 2006). Methyl isothiocyanate is the simplest form of these numerous isothiocyanates that are being produced when *Brassica* plant tissue is disrupted. Although the mechanism of degradation for

thiocyanate still requires elucidation (Hasapis & MacLeod 1982) those for isothiocyanate and nitrile formation have been studied in some detail (Benn, 1977).

Table 1.4: Glucosinolates nomenclature, molecular structure, acronyms and vegetable sources (Wathelet *et al*., 2004)

Glucosinolates	Vegetable source	Side chain	Acronym
Category 1: aliphatic & arylaliphatic			
Glucocapparin	*Capparis spinosa*	methyl	GCA
Sinigrin	*Brassica juncea*	2-propenyl or allyl	SIN
Gluconapin	*Brassica rapa*	3-butenyl	GNA
Glucobrassicanapin	*Brassica rapa*	4-pentenyl	GBN
Glucotropaeolin	*Lepidium sativum*	benzyl	GTL
Gluconasturtiin	*Barbarea verna*	2-phenylethyl	GST
Glucolimnanthin	*Limnanthes alba*	3-methoxybenzyl GLI	
Sinalbin	*Sinapis alba*	4-hydroxybenzyl	SNB
Glucobarbarin	*Barbarea vulgaris*	(R)-2-hydroxy-2-phenylethyl	GBB
Category 2: hydroxylated aliphatic			
Glucosisymbrin	*Sisymbrium loesilii*	2-hydroxy-1-methylethyl	GSY
Glucoconringiin	*Conringia orientalis*	2-hydroxy-2-methylpropyl	GCN
Glucocleomin	*Conringia orientalis*	2-hydroxy-2-methylbutyl	GCL
Progoitrin	*Brassica napus*	(R)-2-hydroxy-3-butenyl	PRO
epi-Progoitrin	*Crambe abyssinica*	(S)-2-hydroxy-3-butenyl	ePRO
Gluconapoleiferin	-	(R)-2-hydroxy-3-pentenyl	GNL
Category 3: thiofunctionalized			
Glucoibervirin	*Thlaspi sempervirens*	3- methylthiopropyl	GIV
Glucoiberin	*Iberis amara*	3-methylsulfinylpropyl	GIB
Glucocheirolin	*Cheirantus annuus*	3-methylsulfonylpropyl	GCH
Glucoerucin	*Eruca sativa*	4-methylthiobutyl	GER
Glucoraphanin	Broccoli	4-methylsulfinylbutyl	GRA
Glucoraphasatin	*Raphanus sativus*	4-methylthio-3-butenyl	GRH
Glucoraphenin	*Raphanus sativus*	4-methylsulfinyl-3-butenyl	GRE
Glucoalyssin	-	5-methylsulfinylpentyl	GAL
Category 4: indole-type			
Glucobrassicin	*Isatis tinctoria*	3-indolylmethyl	GBS
4-OH Glucobrassicin	-	4-hydroxy-3-indolylmethyl	4-OHGBS
4-OMe Glucobrassicin	-	4-methoxy-3-indolylmethyl	4-OMeGBS
Neo-glucobrassicin	-	1-methoxy-3-indolylmethyl	neo-GBS

It has been demonstrated that in the absence of active myrosinase proteins, intact glucosinolates have no biocidal activity against nematodes (Buskov *et al.*, 2002). Therefore, the biocidal activity is as a result of the myrosinase-generated hydrolysis

(Lazerri *et al*., 1993, 2004; Kirkegaard & Sarwar 1998; Serra *et al*. 2002; Buskov *et al*., 2002). Although ITC's have been shown to have biocidal activity against plant parasitic nematodes, their effectiveness varies depending on the type and/or structure (Serra *et al*., 2002) with the most effective being 2-Phenethyl-isothiocyanate (PEITC) (Rosa *et al*., 1997; Kirkegaard & Matthiessen, 1997; Pinto *et al*., 1998; Buskov *et al*., 2002; Serra *et al*., 2002; Lazerri *et al*., 2004; Aires & Rosa, 2009).

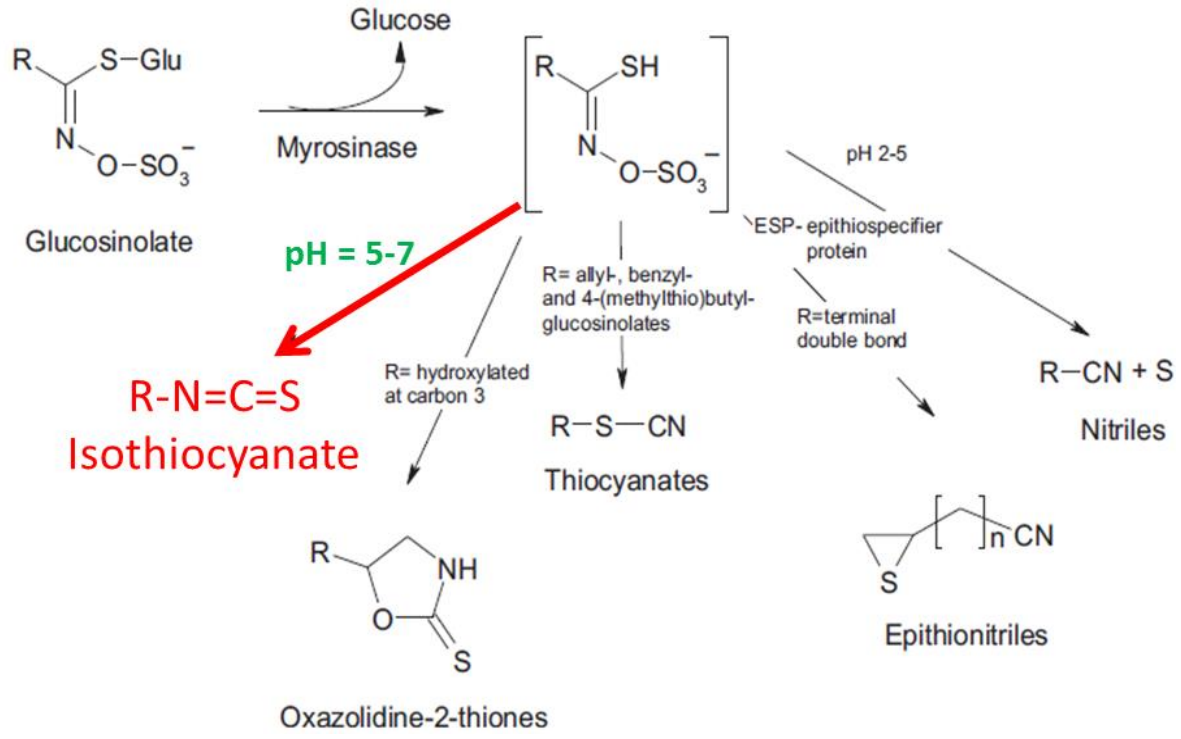


Figure 1.6: An illustration of the hydrolysis of glucosinolates upon plant tissue damage, shown at different pH values with the structures of possible glucosinolate breakdown products (Wittstock & Halkier, 2002)

1.3 The myrosinase enzyme

Myrosinase and glucosinolates are thought to have always co-existed in plant tissues and they were first discovered in mustard seeds by Bussy (1840). The myrosinase enzyme has been noted for its presence in all Brassicaceae species that have been examined (Rodman, 1991). Some *Brassica* species are said to produce higher quantities of myrosinase compared to others. For instance, Bones (1990) found that myrosinase activity was about ten times higher in *Sinapis alba* (Figure 1.7) than in

Brassica campestris. The occurrence and distribution of myrosin cells have been used as one of several criteria for the classification of the order Capparales (Jørgensen, 1981). The fungi *Aspergillus niger* (Ohtsuru *et al.,* 1973) and *Aspergillus sydowi* (Reese *et al.,* 1958) have been reported to contain enzymes with myrosinase activity. Myrosinase have also been identified in the aphids *Lipaphis erisimi* and *Brevicoryne brassicae* (MacGibbon & Beuzenberg, 1978).

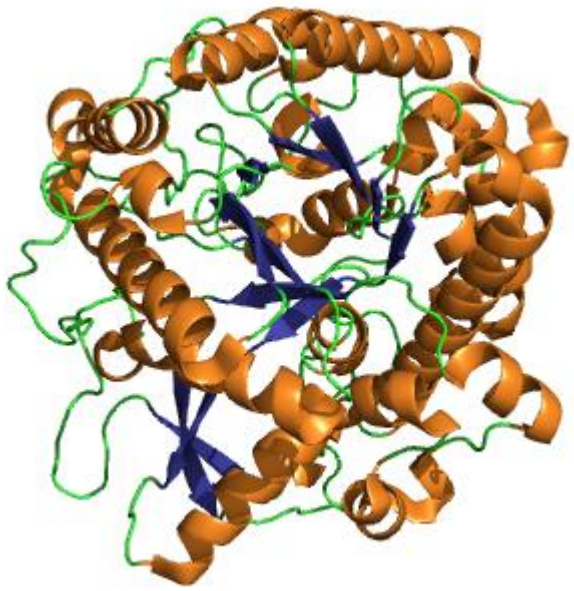


Figure 1.7: The structure of Myrosinase (thioglucosidase) from *Sinapsis alba* (Burmeister *et al*., 2000)

1.3.1 Isothiocyanates

Natural isothiocyanates (ITCs) (R-CH_2-N=C=S) are the products from the enzymatic hydrolysis of glucosinolate that are characterised by volatile, pungent and aromatic odours (Higdon *et al.,* 2007). Generally, they are biocides with activity resulting from irreversible interactions with proteins (Brown & Morra, 1997). Isothiocyanates are known to be the most toxic glucosinolate catabolites (Angus *et a*l., 1994; Lazzeri, 2004) and the biocidal activity of brassica green manures is generally attributed to the production of these volatile toxins (Gamliel & Stapleton, 1993; Mayton *et al*., 1996; Matthiessen & Kirkegaard, 2006; Taylor *et al*., 2014; Woods *et al*., 2014). They

have a broad spectrum of toxicity, ranging from mammals through birds, insects (Borek *et al*., 1995b), molluscs, aquatic invertebrates, nematodes (Buskov *et al.,* 2002; Lazzeri *et al*., 2004 and Lord *et al*., 2011; Woods *et al*., 2014), fungi (Motisi *et al*., 2009; Taylor *et al*., 2014) and bacteria (Brown & Morra, 1997; Ulmer *et al*., 2001; Noret *et al*., 2005,).

A typical example is the presence of allyl-isothiocyanate in mustards and horseradish (*Armoracia rusticana*) which is known to be responsible for much of the odours, thus they are sometimes referred to as mustard oils. The production of ITCs is predetermined by the type and concentration of glucosinolates and this is vital for the biofumigation process.

Buskov *et al*. (2002) postulated that the high mortality rate in PCN J2s observed in their laboratory bioassay with brassicaceous extracts depended on the type of compound released during the myrosinase-catalyzed hydrolysis of the glucosinolates. They obtained the strongest effects with phenethyl- and benzylglucosinolates which are both capable of producing isothiocyanates upon hydrolysis. Lord *et al*. (2011) observed a close correlation between the toxicity of brassicaceous green manures to *G. pallida* and their isothiocyanate producing glucosinolate content, and cited isothiocyanates as the main cause of toxicity. They also noticed that, *B. vulgaris* and *Moricandia moricandioides* lacked efficacy against *G. pallida*. These plants are said to possess large quantities of indole glucosinolates, which are incapable of forming stable ITCs (Halkier & Gershenzon, 2006). Therefore, a comprehensive survey and understanding of the chemical structures of all known isothiocynates-forming-glucosinolates and the plant families and species from which they are found, as well as factors affecting their profile could charecterise their biofumigation potential.

1.3.2 Other glucosinolate breakdown products

Although the enzymatic hydrolysis of glucosinolate at neutral conditions typically results in isothiocyanate formation, the presence of a hydroxyl group at the C-2 of the glucosinolate R-group results into unstable isothiocyanate which cyclizes into oxazolidine-2-thiones. Acidic conditions (pH = 2-5) involving the presence of Fe^{2+} ions favours nitriles formation *in vitro* (Uda *et al*., 1986), whereas in an *in vivo* situation, epithiospecifier proteins are involved (Lambrix *et al*., 2001; Bernardi *et al*., 2003). The presence of epithiospecifier proteins results in the formation of epithionitrile for GSL with an R-group containing a terminal double bond. The formation of thiocyanates is solely from 4-methylsulfinylbutyl-, allyl- and benzyl-GSLs (Hasapis &, Macleod, 1982).

1.4 Factors affecting biofumigation

Factors affecting the biofumigation process can be broadly categorised under agronomic practices and climatic conditions (Ahuja *et al*., 2010b). A better insight into the effects of agronomic practices and climatic factors is needed to manipulate the content of desired compounds from a pest control perspective.

Glucosinolate concentration varies extensively among different plant regions and the developmental stage of a plant (Blazevic & Mastelic, 2009; Van Dam *et al*., 2009). For instance, three-day-old broccoli and cauliflower sprouts were found to possess ten to one hundred times higher levels of glucoraphanin per gram compared to corresponding mature plants (Fahey *et al*., 1997). However, glasshouse trials by Bjorkman *et al*. (2008) showed a 2.6 times higher total glucosinolate level in white cabbage foliage compared to roots when 13-week-old plants were analysed. Bellostas *et al*. (2007) observed a reduction in the concentration of total aliphatic glucosinolates whereas glucobrassicin, an indole GL, increased within a seven day

sprouting period on selected *Brassica* species. Van Dam *et al*. (2009) came to a conclusion that roots contain higher concentrations and a greater diversity of glucosinolates compared to shoots based on their review of 74 studies that concentrated on 29 plant species. Van Dam *et al*. (2009) also found a remarkable variation of glucosinolates content within roots. For instance, in the roots of oilseed rape, the inner periderm was found to possess greater than three folds the level of total GSL of the outer periderm and ten times the level found in the inner pericycle.

Intercropping of white cabbage with *Trifolium pratense* (red clover) was found to generally reduce levels of both root and foliar glucosinolates (Bjorkman *et al*., 2008). It is clear that the effects of plant density on plant secondary metabolites results from a combined effect of many factors involved in plant competition, among which are decreased availability of nutrients, light and water.

1.4.1 Effect of fertilization and soil pH on glucosinolate concentration in the Brassicaceae

The application of sulphur and nitrogen fertilizers to brassica crops and the ratio between them are known to affect the concentration of glucosinolates in the brassica plants tissues. Higher levels of total (Li *et al*., 2007) and individual glucosinolates such as glucoraphanin and glucoraphasatin (Krumbein *et al.*, 2001), glucobrassicanapin, sinigrin, gluconapin and progoitrin (Kaur *et al.*, 1990) have been reported as a result of an increased sulphur supply. However, an increasing nitrogen supply at rates of 80, 160 and 320 kg ha^{-1} at a high sulphur pole (60 kg ha^{-1}) had no significant impact on the total glucosinolate concentration, but resulted in an increase in indole GSL containing nitrogen (Li *et al*., 2007). Therefore, the concentration of glucosinolates varies proportionately with sulphur application. At low sulphur levels (10 – 20 kg ha^{-1}), sulphur containing methionine-derived aliphatic and aromatic

glucosinolates decrease with increasing nitrogen supply (Li *et al*., 2007). Schonhof *et al*. (2007a) reported an increase in total glucosinolate concentration in broccoli plants treated with insufficient nitrogen and an optimum sulphur application. These researchers also found that plants treated with an insufficient sulphur supply and an optimal nitrogen supply had reduced GSL levels. An increase in the susceptibility of oilseed rape (canola) to various fungal pathogens was correlated with sulphur deficiency and this loss of antifungal activity was demonstrated to be related to the reduction of various GSL (Dubuis *et al*., 2005).

The relationship between soil pH and glucosinolate levels in the Brassicaceae has not been investigated. However, glucosinolate degradation products and pH are known to be related. The degradation of glucosinolates mediated by myrosinase enzyme is strongly pH dependent. Isothiocyanate production is favoured by neutral pH whereas nitrile production takes place at a lower pH (Figure 1.7) (Borek *et al*., 1994; Bones & Rossiter, 1996, 2006). Allyl isothiocyanate production was demonstrated to increase from pH 2.5 to approximately pH 5.0 following a non-linear pattern (Borek *et al*., 1994). This study also found allyl isothiocyanates to be the only sinigrin breakdown product at pH 6.0.

1.4.2 Effect of soil structure, soil type and soil organic matter content on biofumigation

Soil structure and composition plays a vital role in the distribution of volatile biofumigant gasses in the soil ecosphere after incorporation. Many fumigants tend to be less effective in soil than in non-soil systems. For instance, Lehman (1942) found that, the toxicity of 2-propenyl ITC to wireworms decreased from approximately 193 times in air to about 29 times in silty loam soil when comparing with carbon disulphide (CS_2). Matthiesson *et al*. (1996) reported similar results with methyl ITC

compared to CS_2. Moreover, sorption of methyl ITC to soil has been found to increase in soil with highr organic matter content (Smelt & Leistra 1974) and this is an important mechanism that decreases ITC effectiveness against soil borne pathogens. Increased 2-propenyl ITC disappearance from soil has been correlated with greater organic carbon contents (Borek *et al*., 1995a) and this is typically true for methyl ITC. A possible mechanism underlying this rapid disappearance is the ability to react with nucleophilic groups such as phenols, amines, alcohols, carboxylic acids, and thiols associated with soil organic matter. It is likely that, the reduced efficiency of volatile ITC released from seed meal tissue compared with other plant tissues as reported by Brown & Morra (1996) were as a result of these types of reactions.

1.4.3 The influence of seasonal variation, temperature, light and water availability on levels of glucosinolates in the Brassicaceae.

The variation in concentrations of glucosinolates with respect to different seasons has been documented for different brassicaceous vegetables which include oilseed rape (Rosa *et al*., 1996; Sarwar & Kirkegaard, 1998), mustard (Sarwar & Kirkegaard, 1998), radish (Ciska *et al*., 2000; Schreiner *et al*., 2002; Ito & Kimura, 2006), turnip (Ciska *et al*., 2000; Padilla *et al*., 2007; Zhang *et al*., 2008) and cabbage crops (Rosa *et al.*, 1996; Ciska *et al*., 2000; Rosa & Rodrigues, 2001; Ito & Kimura, 2006; Cartea *et al*., 2008). Generally, most of these studies have shown that brassica plants sown in spring conditions with high light intensity, medium temperatures, longer photoperiods and drought conditions contain the highest total GSL. On the contrary, plants sown in autumn/winter season with lower temperatures, low intensity of irradiation, shorter photoperiods and maximum soil moisture, have the lowest total glucosinolate concentration.

Growth temperatures have been demonstrated to clearly influence concentrations of glucosinolates in brassicaceous plants. A typical example of this has been shown in broccoli sprouts grown at either high (29 or 33°C) or at low (11 or 16°C) constant temperatures which resulted in higher glucosinolate contents than those grown at an intermediate temperature (21.5°C) (Pereira *et al*., 2002). This study also found that the highest average glucosinolate levels (56.6 µmole g^{-1} of dry weight) occurred when Brussel sprouts were grown at 30/15°C day/night temperatures respectively. These concentrations differed significantly ($P < 0.01$) from the concentrations observed under 22/15°C (47.1 µmol g^{-1} dry weight) and 18/12°C (45.8 µmol g^{-1} dry weight) day/night temperature which did not differ significantly from each other. These studies agreed with earlier studies by Rosa and Rodrigues (1998) and were later confirmed by the findings of Charron & Sams (2004), Engelen-Eigles *et al*. (2006) and Schonhof *et al*. (2007b).

Long photoperiods are said to positively influence the concentrations of glucosinolates in brassicaceous plants. A higher total glucosinolate concentration was reported in stems and roots of *B. oleracea* grown at 18 and 24 h photoperiods compared to a 12 h photoperiod (Charron & Sams, 2004). Similarly, Keskitalo (2001) found a higher glucosinolate concentration in white mustard seeds that had been produced under a 22 h compared to a 14 h photoperiod. Bodnaryk (1992) had earlier reported a significant reduction in the amount of glucobrassicin detected in the cotyledons of a one week old seedlings of oilseed rape cv. Westar when moved from light to darkness for 24 h. Recently, higher total glucosinolate levels in roots and shoots were observed by Pérez *et al*. (2008) in broccoli sprouting in light compared to plants sprouting in darkness. However, in some cases, no effect has been observed as a result of photoperiod, and a typical example for this is the findings by

Charron and Sams (2004) who demonstrated the absence of any effect from photoperiod in leaf glucosinolate levels in *B. oleracea*.

The intensity (irradiation) and quality (wavelength) of light seems to have an effect on specific glucosinolate. A combination of moderate irradiation (116 – 150 µmol m^{-2} s^{-1}) and low temperatures (7 – 13°C) were found to increase the alkyl glucosinolate levels in broccoli heads compared to low irradiation levels (23 – 69 µmol m^{-2} s^{-1}), while increasing the levels of the indole glucosinolates (glucobrassicin) after head induction (Schonhof *et al*., 2007b). An increased level of gluconasturtiin in watercress was observed under long photoperiods characterized by red irradiation, in contrary with far-red light (Engelen-Eigles *et al*., 2006). However, when light-emitting diodes were used in studies of kale shoots grown at wavelengths of 400, 440, 525, 640 and 730 nm, Lefsrud *et al*. (2008) did not notice any significant effect on total GSL content. The aliphatic GSL sinigrin seemed to be the only glucosinolate that responded to the wavelength treatment and it is said to peak in red light at 640 nm (Lefsrud *et al*., 2008).

Higher concentrations of GSL have been reported in many Brassicaceae plants grown under water stress than for plants grown under favourable conditions (Milford & Evans, 1991; Rosa *et al.*, 1997; Radovich *et al*., 2005; Zhang *et al*., 2008). However, these concentrations might be partly associated to an increased concentration per unit of dry weight (Bjorkman *et al*., 2011). Ciska *et al*. (2000) attributed the higher glucosinolate concentrations found in one out of two different years of their studies with cultivars of *B. oleracea*, *B. rapa* and *R. sativus* to low water availability. Sarwar and Kirkegaard (1998) had earlier reported the same effect with mustards, rape and broccoli which was later on confirmed by Vallejo *et al*. (2003) when comparing plants from two different seasons. The period of irrigation can also

be vital to glucosinolate concentrations as higher levels of total and individual glucosinolates have been reported on cabbage plants that did not receive irrigation during head formation compared to irrigated cabbage (Radovich *et al*., 2005).

1.5 Optimising biofumigation on a field scale

Biofumigation with Brassicaceae to control soil-borne pests and diseases has been proven to be effective in several studies. However, most of the results obtained in biofumigation studies have been inconsistent as reviewed by Matthiessen and Kirkegaard (2006). This entails that when developing biofumigation systems factors such as pulverization, incorporation strategy, irrigation, sealing and mulching need careful consideration. A combination of high plant biomass with thorough pulverization in the incorporation process and high soil moisture content are vital for isothiocyanate production (Matthiessen & Kirkegaard, 2006). For instance, approximately 100 nmol g^{-1} soil concentrations of allyl isothiocyanate was achieved by Matthiessen *et al*. (2004) after incorporation following thorough pulverization of mustard in combination with heavy irrigation. By contrast, Gardiner *et al*. (1999) found very low concentrations of isothiocyanates (approximately 1 nmol g^{-1} of soil) following a plough-down of whole winter rapeseed plants, which is below the recommended 260 pound per acre concentration of methyl ITC for pest control. Nevertheless, it is worth noting that low concentrations of isothiocyanates released over a prolonged period, such as in the case with plant tissue plough-down in soils is also likely to reduce damage induced by other soil pathogens (Muelchen *et al*., 1990; Mattner *et al*., 2008). A higher concentration of ITC release following incorporation of biofumigant material has also been demonstrated in waterlogged soils (Morra & Kirkegaard, 2002).

The timing at which the plant tissue should be macerated is vital for biofumigation efficacy to soil borne pests and diseases. This is supported by the fact that greater suppression to pathogens has been demonstrated by macerated roots from mature biofumigant plants as compared with those from immature plants (Mattner *et al*., 2008). Also, the suppression of *Rhizoctonia fragariae* growth with a blend of *Brassica rapa* and *B. napus* has been linked with thorough maceration at anthesis as compared with same degree of maceration at the establishment stage of the plants (Mattner *et al*., 2008).

The types and concentrations of ITCs which are released for biofumigation are predetermined by the glucosinolate content and profile in the plant material. *Brassica* spp. producing a high amount of short-chain aliphatic ITCs has the greatest potential in suppressing soil pathogens (Matthiessen & Kirkegaard, 2006). Roubtsova *et al.* (2007) found that after a thorough and uniform distribution of broccoli plant tissue, there was an improvement in the efficacy of biofumigation against the root-knot nematode *Meloidogyne incognita*.

Concentrations of glucosinolate hydrolysis products in soil may also be limited by the soil microbial population via microbial degradation. This has been confirmed by an accelerated loss of methyl ITC after successive soil applications in field and laboratory studies by Smelt *et al*. (1989). Similarly, sterilized soils have been found to reduce the disappearance of methyl ITC in sealed bottles (Ashley *et al*., 1963). However, these observations have not been consistent. One typical example has been reported by Borek *et al*., (1995a) in attempts to sterilize soils by ethyleneoxide or autoclaving treatments which failed to change the rate of disappearance of 2-propenyl ITC from the sterile soil.

1.6 Other exploitable biochemicals from *Brassica* species

Decomposing brassicaceous tissues have been shown to produce other volatile sulphur-containing toxins in addition to the glucosinolate hydrolysis products. These include methyl sulphide, dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol (Lewis & Papavizas, 1971; Gamliel & Stepleton, 1993; Wang *et al*., 2009) which may be useful in the biofumigation process (Bending & Lincoln, 1999; Wang *et al*., 2009). Although these compounds possess lower toxicities than ITC's (Figure 1.9), they are produced in larger amounts and for a much longer period than allyl ITC's (Walker *et al*., 1937; Virtanen, 1965; Lewis & Papavizas, 1971; Germliel & Stepleton, 1993). A greater total amount of these sulphur containing compounds and longer production periods may compensate for lower toxicities, thereby, increasing the potential importance of these compounds in pest inhibition (Brown & Morra, 1997).

A reduction in population densites of *Verticillium dahliae* has been linked to methyl sulphide and dimethyl disulphide after soil amendment with brassicaceous green manures (Wang *et al*., 2009), while a synergistic interaction has been reported between carbon disulphide and methyl isothiocyanate in toxicity to fungi (Canessa & Morrell, 1995). There are also reports of toxicity on nematodes by carbon disulphide and dimethyl disulphide (Chapman & Parker, 1929; Rosskopf *et al*., 2006; Gu *et al*., 2007).

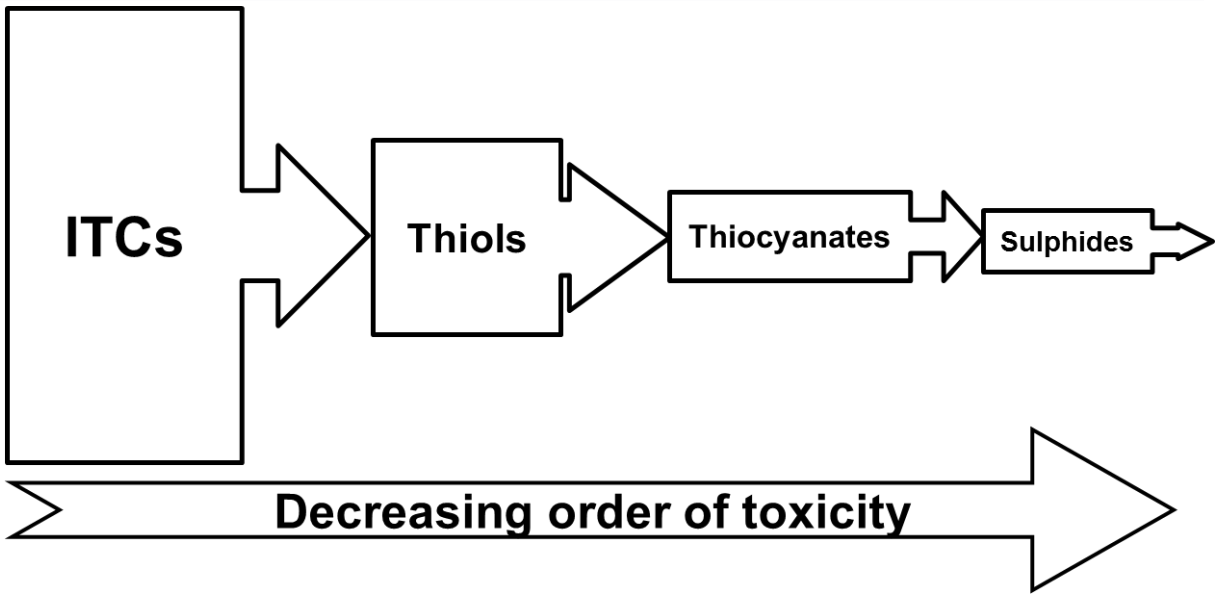


Figure 1.8: An illustration of the decreasing order of toxicity for the hydrolysis products of glucosinolates. Figure adapted from (Walker *et al*., 1937; Virtanen, 1965; Lewis & Papavizas, 1971; Germliel & Stepleton, 1993)

1.7 Effect of biofumigation on soil microbial population

Indirect effects resulting from incorporated brassicaceous residues are possible. This can occur through influencing the indigenous microbial community which may affect pathogen populations through antagonism, competition, predation or parasitism (Wiggins & Kinkel 2005; Raaijmakers *et al*., 2009). For instance, the severity of *V. dahliae* disease on potato has been negatively correlated with the proportion of antagonists to *V. dahliae* within the Streptomycete community upon incorporation of buckwheat or brassicaceous green manures unlike fallow (Wiggins & Kinkel, 2005). These researchers also noted that, initial streptomycete densities influenced the change in streptomycetes pathogen inhibitory activity among green manure treatments. For example, among green manure-treated soils, initial streptomycete densities (estimated 20 days after green manure incorporation) were positively correlated with the change in proportion of streptomycetes antagonistic against *S. scabei* (R=0.39342, *P=0.0075*), *V. dahliae* (R=0.339, *P=0.023*), *R. solani* (R=0.405, *P=0.006*) and *F. oxysporum* (R=0.321, *P=0.031*). The induction of resistance to *Rhizoctonia solani* in apple appeared to be as a result of the stimulation of

Streptomyces spp. after soil amendment with *Brassica napus* seed meal (Cohen & Mazzola, 2006). This has been confirmed by the fact that soil pasteurization prior to *R. solani* infestation eliminated the control of this fungal pathogen on apples by brassica seed meals unlike unpasteurized soil treatments (Mazzola *et al*., 2007).

1.8 Aditional benefits of using brassica crops in agricultural systems

Additional benefits may be obtained by using biofumigant Brassicaceae plants in pest control approaches. Phytoremediation (otherwise referred to as bioremediation) describes the treatment of environmental problems through the use of plants that mitigate the problem on environment thus, saving the time and energy needed to excavate these contaminants. This phenomenon consists of mitigating the concentration of pollutants in contaminated soils, water or air, with plants such as brassicas that are able to hold, degrade, and/or eliminate non-degradable pollutants especially heavy metals and other contaminants. Phytoremediation is considered as the cheapest and the most environmentally friendly technology for cleaning up contaminated soil systems. However, the success of phytoremediation depends principally on the choice of plant species, which should possess the ability to accumulate large amounts of heavy metals. *Brassica juncea* cv Czern has been studied extensively for its ability to accumulate heavy metals (Szczygłowska *et al*., 2011**)**. This species of *Brassica* has been found to exhibit a high capacity of Cadmium accumulation especially in the shoots, where 1450 µg Cd/g dry weight has been recorded, which is three times higher than that reported in *Brassica napus* (555 µg/g dry wt) (Nouairi *et al*., 2006). It is also likely that, accumulation of heavy metals via phytoremediation by brassicas might intensify the synthesis of bioactive compounds, including GSL. Induction of the absorption of sulphate to sustain greater sulphur demand during the biosynthesis of GSL has been reported for Cd, Zn and Cu

(Schiavon & Malagoli, 2008). *Brassica napus* is also known for its efficiency in the up take of phosphorus from phosphorus-deficient soils (Grinsted *et al*., 1982; Hedley *et al*., 1982b).

The fast root development and establishment by biofumigant Brassicaceae such as oilseed radish and mustard, alongside the amendment of the organic matter ensures an improved soil structure and prevention of soil erosion and nitrate leaching. Amendment of the plant tissue can also serve as a source of nutrient, for instance, seed meals of high protein-containing oilseed rape has been used as a source of supplementary nitrogen (Kücke 1993; Johansson & Ascard 1994). This implies that mineralising these meals would release substantial quantities of nitrogen available for plant. Following soil incorporation, biofumigant biomass provides a pole of fresh organic matter (Campbell *et al*., 1991; Shepherd *et al*., 2002), thus providing food to beneficial soil microbes, enhancing an increase in their numbers and activity (Wang *et al*., 2014). Integrating biofumigant compost with Brassicaceae seed meal would therefore simultaneously reduce pest, weeds and diseases while adding nitrogen to the soil.

1.9 Negative effects of biofumigation on the soil microbial community

Although the use of biofumigation is advantageous for soil borne pest control as it is an environmentally sound practice, the fact that beneficial organisms including biocontrol agents are also affected by the hydrolysis products of glucosinolates should be acknowledged. Consequently this may have implications for pest control in an integrated pest management (IPM) agro-ecosystem. For instance, in an *in vitro* experiment, Klingen *et al*. (2002) noticed the inhibition of the insect pathogenic fungi *Metarhizium anisopliae* and *Tolypocladium cylindrosporum* by isothiocyanates. Inyang *et al*., (1999a) also observed the inhibition of germination *in vitro* and the

subsequent growth of *Metarhizium anisopliae* by isothiocyanates and its ability to infect *Phaedon cochleariae*. However, surface leachates and soluble extracts from leaves of different brassicas were found to increase the germination of conidia and the virulence of *M. anisopliae* (Inyang *et al*., 1999b). Larval feeding by *Pieris brassicae* (white cabbage butterfly) has been reported to activate the release of volatiles that attracts *Cotesia glomerata,* a parasitic wasp (Mattiacci *et al*., 1995). Handerson *et al*. (2009) reported an adverse effect on beneficial nematodes such as the insect pathogenic nematodes in the genus *Steinernema*, by isothiocyanates released after soil incorporation with *B. carinata* seed meals.

1.10 Managment of pest nematode species with biofumigation

A cross-section of research has investigated the effect of biofumigation for the management of plant parasitic nematodes (Kruger *et al*., 2013). However, most of these research has been glasshouse and laboratory based and this sometimes does not provide an accurate prediction of natural field conditions.

Pinto *et al*. (1998) found that when active myrosinase enzyme was added to 2-propenyl glucosinolates, there was the release of 2-propenyl isothiocyanates which was nematotoxic to J2's of *G. rostochiensis*. However, the level of toxicity depended on the glucosinolate concentration. For instance, 100% mortality was observed with a 2-propenyl glucosinolates concentration of 1mg ml^{-1} within 24 h of exposure, while at a concentration of 0.05mg ml^{-1}, resulted in 65% mortality after 96 h. These findings led these researchers to a conclusion that the nematotoxic effect of brassicaceous plants derived isothiocyanates depends on concentration and exposure time.

Upon soil amendment with leaf and root tissues of *Brassica* spp., Potter *et al*., (1998) noticed a significant reduction in populations of *Pratylenchus neglectus.* This difference was remarkable (98% mortality) when the soil was amended with leaves of

B. *oxyrrhina* compared to the rest of the *Brassica* spp used in their experiment. During this experiment, Potter *et al.,* (1998) noticed that soil amendment with brassicaceous leaf tissues had a greater suppressive effect compared with amendments with root tissues. They noticed a weak non-significant correlation between total leaf glucosinolate content and the suppression of populations of *P. neglectus* (R^2 = 0.48, *P > 0.05*).

In a dose response experiment, Serra *et al*. (2002) reported a significant difference (*P<0.001*) in the paralysis of *G. rostochiensis* juveniles when exposed to different concentrations (0.05, 0.3, and 1mg ml^{-1}) of the hydrolytic products of 2-phenylethyl glucosinolate at different time intervals (8, 16 and 24 h). These researchers also found that, with longer exposure times (32, 40 and 48 h), there was a reduced effect (*P<0.05*). Upon an extended exposure (56, 64 and 72 h), the nematicidal effects decreased to zero indicating that there is a possibility of a reversal effect. That is, after an extended period of exposure, it is possible that the active biocidal compounds must have volatilized to a non-toxic concentration during which the J2's were able to recover from their paralysis to normal activity. All concentrations used by Serra *et al*. (2002) were shown to be toxic to J2's of *G. rostochiensis* only when active myrosinase enzyme was added to the glucosinolate extract. They found that with 1mg ml^{-1} w/v of 2-phenylethyl glucosinolate, the mortality of *G. rostochiensis* J2 was observed to be 100% within 8 h upon hydrolysis with myrosinase enzyme.

Similar to Serra *et al*. (2002), Buskov *et al*. (2002) noticed that intact glucosinolates were incapable of inducing mortality on the J2's of the potato cyst nematode *G. rostochiensis*. The addition of myrosinase to each of the tested glucosinolates in their studies demonstrated a significant difference (*P* < 0.001) between different glucosinolates as well as between concentrations. They reported that the effect was

dependent on the type and concentration of glucosinolate with the greatest effect resulting from phenethyl and benzyl glucosinolates. These two glucosinolates accounted for 100% mortality to J2's of *G. rostochiensis* within just 16 h of exposure time at a concentration of 1.0 mg ml^{-1} w/v. Buskov *et a*l. (2002) also observed that within the first 8 h of exposure to the test solutions, the hydrolysis of benzyl and phenylethyl GSLs induced the same mortality. However, beyond this time period phenethyl-GSL derived ITC was slightly more effective than benzyl-GSL derived ITC. This may be an indication that the volatilization of benzyl-ITC is faster than that of phenylethyl-ITC provided that both glucosinolates had the same quantities of myrosinase added to them.

Yu *et al*. (2007) reported nematicidal activity by both mustard bran and seed meal against a wide range of nematode spp. (*Heterodera glycines, H. schachtii, Meloidogyne incognita, M. hapla, P. neglectus, P. penetrans* and *Ceanohabditis elegans*) with variable dose responses. The most sensitive nematode species to both treatments was *H. glycines* (LD_{50} 311µg ml^{-1}) while the least was *C. elegans* (*P=0.05*) (LD_{50}, 726µg ml^{-1}). These researchers found a variation in anti-hatching activity which was dependent on the nematode species, with lower concentrations (250 µg ml^{-1}) stimulating hatching in some cases. Yu *et al*. (2007) also reported that, seed meal was more potent on the tested nematodes species than mustard bran in all cases.

Zasada *et al*. (2009) reported that when exposed to different concentrations (0.01, 0.02 and 0.03 mM) of benzyl isothiocyanates (BITC) for different time periods (1, 2, 3, 4 and 5 h), the activities of *Meloidogyne incognita* J2's and the reproductive capacity of adult stages of *M. incognita* was significantly (*P<0.05*) reduced in all cases. When treated with low concentrations (0.01 mM) of BITC over a shorter time period (1 h), they noticed a correlation (R^2 = 0.41) between infectivity of *M. incognita*

J2 and subsequent egg production. This observation is a possible indication that an understanding of the sub-lethal effects of ITCs may play a significant role in developing effective biofumigation strategies for control of plant-parasitic nematodes considering the short half-life of ITCs in soils.

In a glasshouse pot experiment with potato cyst nematodes, Aires *et al*. (2009) in Portugal found that the suppressive effect of brassicaceous green manure to cysts of *G. rostochiensis* was significantly ($P < 0.001$) dependent on the type of brassica plant extract and concentration used. These authors obtained the greatest reduction in newly formed cysts with a total glucosinolate concentration of 0.2 µmol 100 g^{-1} dry weight of *Nasturtium officinalis* (water cress) (27±1 cyst g^{-1}) compared with those recovered after treatment with the same concentration of *Brassica olarecea* var *tronchuda* (66±8 cyst g^{-1}). In either case, they noticed a significant difference ($P < 0.005$) in the number of new cysts between untreated controls compared with treated plants thus confirming the potential role of biofumigant crops in the management of PCN. Aires *et al.* (2009) recommended the incorporation of a larger biomass of the brassicaceous green manure in order to obtain good results.

Lord *et al*. (2011) demonstrated that brassica leaf tissue incorporated into soil can cause high levels of mortality to encysted eggs of *G. pallida*, thus demonstrating the potential of brassica rotation crops to control *G. pallida* in potato production. They observed over 85% and 95% mortality of encysted eggs in uncovered and polyethylene covered soil respectively when treated with selected cultivars of *B. juncea*. This mortality level compares favourably with that of metham sodium or 1, 3-dichloropropene (mean mortality rates of 60 and 64%, respectively) applied in fields in northern England (Storey, 1982) as well as 48 to 72% obtained in The Netherlands by fumigation with 1, 3-dichloropropene in clay soils (Been & Schomaker, 1999).

Based on their glasshouse experiments, Lord *et al*. (2011) anticipated a possible reduction of 50% or more in *G. pallida* infested field population densities by biofumigation with *B. juncea*. Their estimate was based upon their observed mortality induced by the *B. juncea* cultivars used in their experiments, coupled with other findings such as the vertical distribution of PCN cysts in potato fields (Been & Schomaker, 1999) and a typical yield of 4 tonnes dry weight ha^{-1} for the above ground parts of *B. juncea* grown for 8-9 weeks in the field (Larkin & Griffin, 2007; Motisi *et al*., 2009; Friberg *et al*., 2009).

Moura *et al*. (2012) reported synergistic effect of soil solarisation and organic amendments with *B. oleracea* var capitata against *Pyrenochaeta lycopersici Meloidogyne* spp. on *Lycopersicon esculentum* Mill under greenhouse conditions. In carrot fields infested with *Heterodera carotea* (carrot-cyst nematode), the application of Biofence® (dry pellets of *B. juncea*) had no significant effect as observed by Gresen (2012) on the number of live nematode in soil sampled post-carrot harvest.

Valdes *et al*. (2012) reported inconsistency in the ability of developing yellow mustard and its amendment in reducing hatching and infectivity of *G. rostochiensis* juveniles, but noticed a reduction in the population densities of plant-parasitic nematodes. These authurs also observed changes in the abundance of nematode trophic groups in plots amended with yellow mustard, and this effect was reported to be more pronounced when the amended plots were seald with polyethene bags (Valdes *et al*., 2012).

Argento *et al*. (2013) investigated the effectiveness of milled powder of *Brassica juncea, Eruca sativa, Raphanus sativus* and *Brassica macrocarpa* against *Meloidogyne* spp. on tomatoes in a field trial and noticed significant reduction in the level of infestation on tomato roots relative to the untreated control.

Xiao *et al.* (2013) observeved an enhacement in the efficacy of organic materials (chicken manure, pig manure and rice straw) against *Meloidogyne* spp. on tomatoes when these materiale were incorporated alongside the biological control agents *Bacillus cereus* X5 *B*. *thuringiensis* BTG, or *Trichoderma harzianum* SQR-T037 in *in-vitro*, glasshouse and field experiments. *Guerrero-Díaz et al*. (2013) reported reduction in the gall index produced by *M. incognita* on pepper when soil were amended with *Brassica carinata* pellets relative to untreated control. These authours also found significant increase in pepper yield in treatments with *B. carinata* pellets, which was comparable with or higher than yield in treatments with methyl bromide plots during summer cultivation than when cultivated during the autumn season. In a laboratory bioassay, Barros *et al*. (2014) reported nematicidal activity of volatile organic compounds emited from *B. juncea* on *M. incognita.*

The results reported by the above researchers show that brassicaceous green manures have the potential to contribute to the integrated management of potato cyst nematodes and other phyto-parasitic nematodes. These studies were mostly laboratory and glasshouse based. Therefore, it is of utmost importance to investigate and quantify the effect of biofumigant plants on nematodes (especially cyst forming nematodes) under field conditions.

1.11 Future prospects

1.11.1 Review of previous research and selection of the best approach

Recent advances in the strategies to enhance ITC release from brassicaceous biofumigants suggest the need to review previous research and adapt the most appropriate strategies. In most of the previous work on biofumigation, the level of suppression that has been achieved has mostly been limited by lack of knowledge on the key ellements needed to maximise success. Some of these include the selection

of appropriate varieties with high content of toxic ITC-produsing GSL. Also, the growing of a critical amount of biomass, approprite timing and incorporating at stages with maximum glucosinolate contents such as at anthesis as demonstrated in Mattner *et al*. (2008) can improve on the biofumigation effects. Thorough pulverization of the biomass followed by rapid incorporation, sealing and/or waterlogging post-incorporation to minimise the loss of volatile gases from soil is likely to increase the biofumigation effect. Taking into account these elements and implementing as many as they suit the farming practice will inprove the chances of success for biofumigation in pest and pathogen control.

1.11.2 Modification of previous studies and adding new research ideas to increase the efficacy of biofumigation

The modification of previous research alongside addition of new ideas such as focused attention on selective or purposeful breeding for brassicaceous plants with high ITC-releasing glucosinolates are useful approaches to improve upon the biofumigation system. The elucidation of the genes involved in the biosynthesis of glucosinolates may in the future provide oppotunities to engineer specific biofumgant types to raise levels obove those likely to be achieved in conventional crossing programs (Halkier & Du, 1997; Mithen, 2001). Also, innovations in the design of machinery to simultaneously pulverize and incorporate green brassicaceous residues in soils with rapid covering or irrigation to prevent rapid lost of the volatile gases would improve on the biofumigation potential. Intergrated aproaches such as the combination of biofumigation with other control strategies are strongly being encouraged. Wang *et al*. (2014) recently demonstrated an elevation in the population of soil beneficial bacterial following integration of biofumigation with *Bacillus*

amyloliquefaciens, and this was negatively corelated with disease insidence by *Phytophthora* blight on pepper.

1.11.3 Conclusion

The potential of biofumigation with brassicaceous green manure to control soil borne pests and pathogen has been well established, as discussed above. However, from a PCN perspective, experiments investigating the potential of biofumigation to control this group of soil borne pests has mostly been based either *in-vitro* or glasshouse based experiments. Although these are the most common ways of predicting what could eventually take place in the field, they are not a true representative of the field situation. Moreover, previous research examining the biofumigation effect of brassicas on PCN has often overlooked the effect on these nematode pests that could be resulting from the growing crop. Small amounts of glucosinolates have been reported to be exuded into the rhizosphere from the roots of *Brassica* crops during cell turnover (McCully *et al*., 2008). Soil microflora, such as *Aspergillus* spp, which produce myrosinase have been linked with the conversion of the exuded GSL into ITC (Borek *et al*., 1996; Sakorn *et al*., 1999; Gimsing & Kirkegaard, 2006). Capitalising on these observations by manipulating the development of *Brassica* roots and/or canopy with chemical growth regulators could possibly improve their use in pest and disease management.

In Europe, triazoles, such as metconazole and tebuconazole, are now being utilised on brassicas including oilseed rape for their growth regulatory and fungicidal attributes. These triazoles are known to inhibit the early steps of gibberellin biosynthesis (Rademacher, 2000). The inhibition of gibberellic acid in plants has the potential to manipulate many aspects of plant growth, including rooting through an alteration of the partitioning of assimilate between the roots and shoot (Berry &

Spink, 2009). Greater root production as such could possibly lead to an increase in the rhizospheric secretion of biochemicals, such as GSL. To date, there is no published data on the control of field populations of *G. pallida* using brassicaceous green manure prior to incorporation (partial biofumigation) or post-incorporation (complete biofumigation). Therefore, this study was initiated to investigate the effects of selected *Brassica* species (*Brassica juncea, Eruca sativa* and *Raphanus sativus*) on the viability and population densities of *G. pallida* during plant growth and development (partial biofumigation) and upon crushing and incorporation of the crops (complete biofumigation) under field conditions.

1.12 Hypothesises (null):

i. Sowing and incorporation of brassicaceous residues under field conditions does not affect PCN viability and infestations of potatoes.

ii. Sowing and incorporation times do not affect the efficiency in biofumigation for the management of PCN under field conditions.

iii. Under temperate conditions, the profile of GSLs does not vary between different *Brassica* species and within different plant regions.

1.13 Objectives

i. Determine the effect of sowing and incorporating brassicaceous residues on the viability of PCN encysted eggs in field soils

ii. Monitor nematode populations pre-sowing of brassica crops, pre- and post-incorporation of brassicaceous residues, pre-planting and post-harvest of potato crops in the field

iii. Assess the effect of metconazole application on the brassica crop biomass and the suppressive effect of the tested brassicas, and finally

iv. Analyse the types and concentrations of GSLs for the tested brassica species sown at different times of the year.

CHAPTER TWO

2. Chapter 2: General Materials and Methods

2.1 Experiment site selection, setup and sampling for potato cyst nematode (PCN)

2.2 Field selection and setup

Local sites in Shropshire (UK), previously identified with PCN were pre-sampled to identify PCN hot spots with infestation levels matching the requirements for the study (specific details are described for individual sites in Chapter 3). Selected sites were drilled to 30 cm depth and the PCN hot spots were marked (with canes) into individual plots of 8x8 m^2 each with a 2 m buffer between the plots to avoid border effects. Table 2.1 shows the grid references and the time (months/years) of establishment for the different field experiments while Figure 2.1 shows the map with the sites. Soil samples from these sites were further analysed to determine their particle size distribution, organic matter contents and pH (Figure 2.2) (MAFF, 1986). Individual plots at each experimental site were mapped using a GPS system (Leica Viva GS08 NetRover, Atherton, UK) to an accuracy of 20 mm so that they could be relocated at any point in time. Thermochron temperature data loggers (DS1921G-F5#, iButton, Whitewater, US), pre-set to record temperature data every 60 min were buried to approximately 20 cm depth at two locations in each experimental site.

Table 2.1: Grid references for the different experiments and their respective years of establishment

Experiment №.	Grid reference	Locality	Year of initiation
1	SJ 72288 21947	Shropshire, UK	July 2011
2	SJ 78412 15781	Shropshire, UK	September 2011
3	SJ 70854 22651	Shropshire, UK	July 2012
4	SJ 77407 15424	Shropshire, UK	September 2012

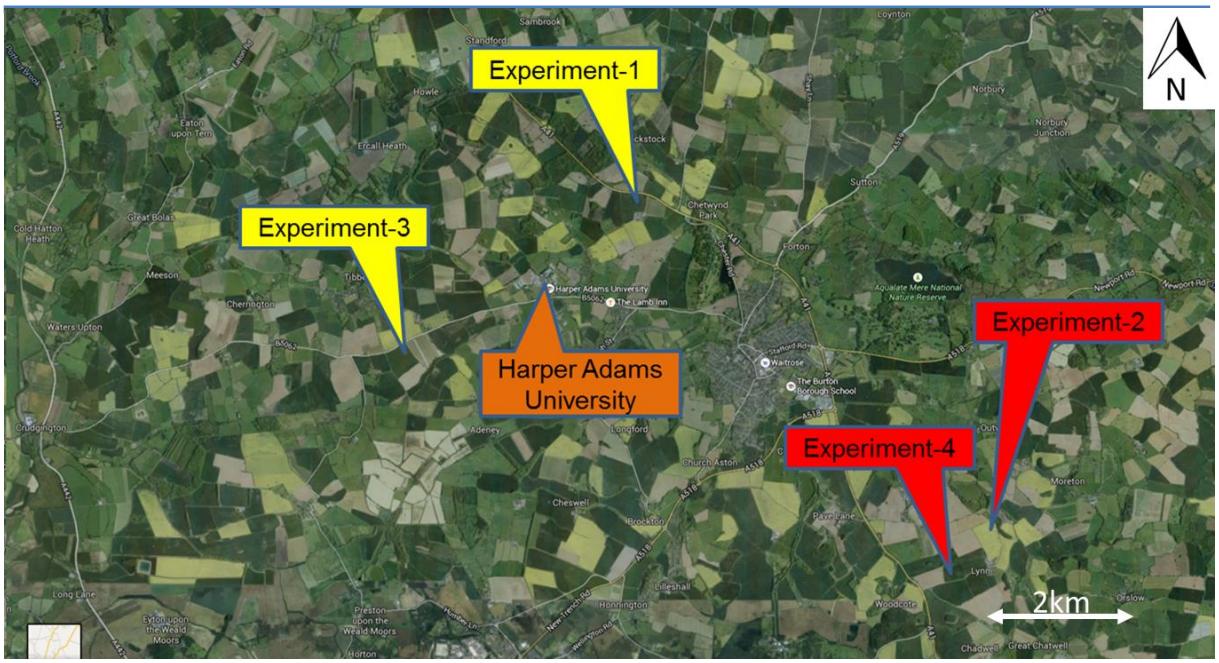
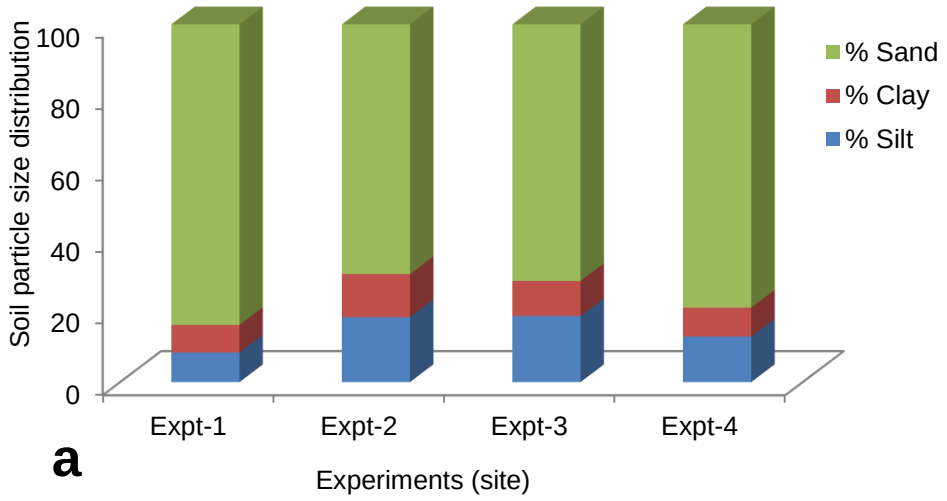
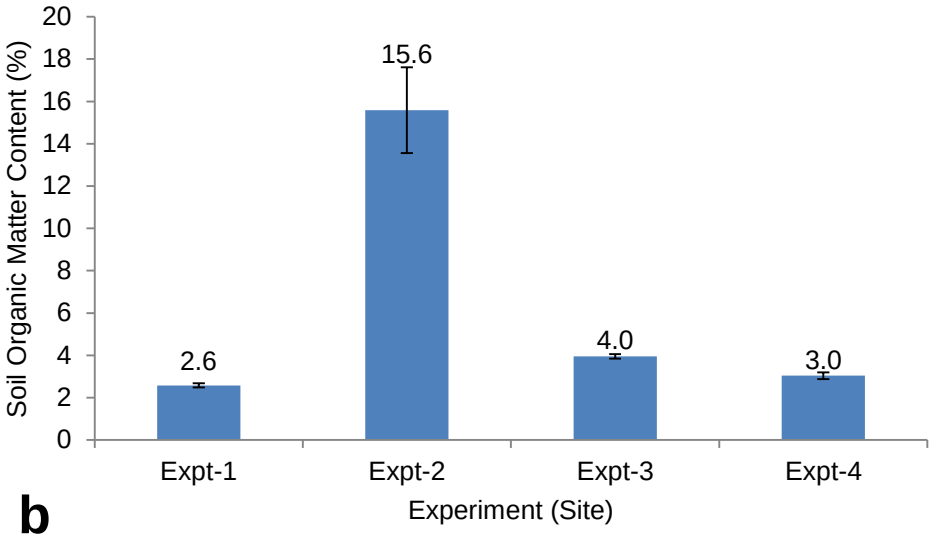


Figure 2.1: Locations of the field sites for the experiments. Experiments 1 & 3 (yellow) were summer-cultivated in 2011 and 2012 respectively and Experiments 2 & 4 (red) were overwintered



a



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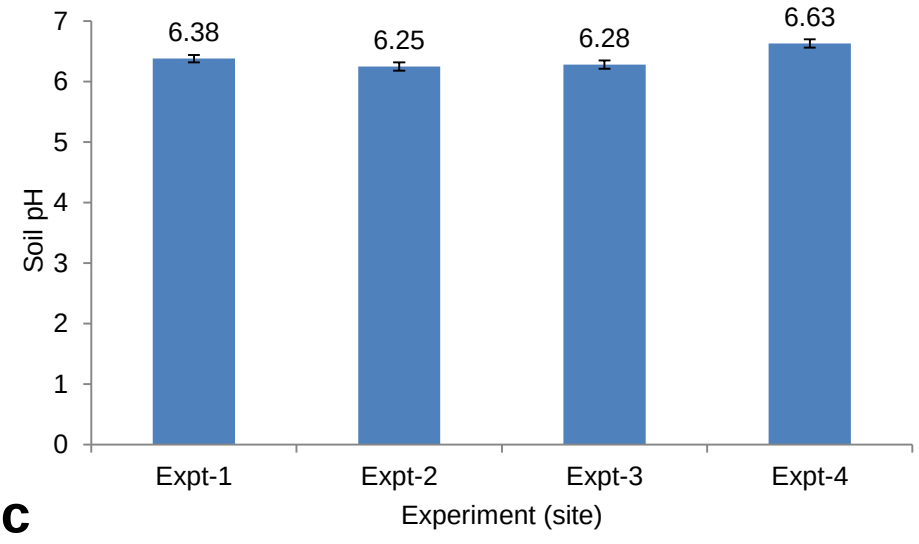


c

Figure 2.2: a) Soil particle size distribution, b) organic matter content and c) soil pH for field experiments 1 (Expt-1), 2 (Expt-2), 3 (Expt-3) and 4 (Expt-4)

2.3 Soil sampling and processing for PCN

Marked plots were sampled to determine the initial and final PCN population densities (P_i and P_f respectively). At each time of sampling, soil samples were taken at regular intervals, in a 'W pattern' across each plot (Figure 2.3). Evans *et al*. (2000) investigated sampling patterns and found the 'W pattern' of sampling to be the most accurate and consistent in terms of results irrespective of orientation. The angles of the 'W' were adjusted to suit the shape of the plots. Twenty cores were taken from each plot at each time of sampling by inserting a semi-cylindrical (15 mm diameter) blade to a depth of 25-30 cm. Soil cores from each plot were placed into appropriately labelled cloth bags and secured to prevent cross contamination between samples. Each sample from individual plots weighed approximately 1.5 – 2 kg. Samples were air-dried for 48-72 h at 25°C in a drying chamber at Harper Adams University. Air-dried samples were passed through a 5 mm mesh to remove stones and large organic debris, while sub-samples of 200g where taken to the nearest ±0.02g for PCN extraction.

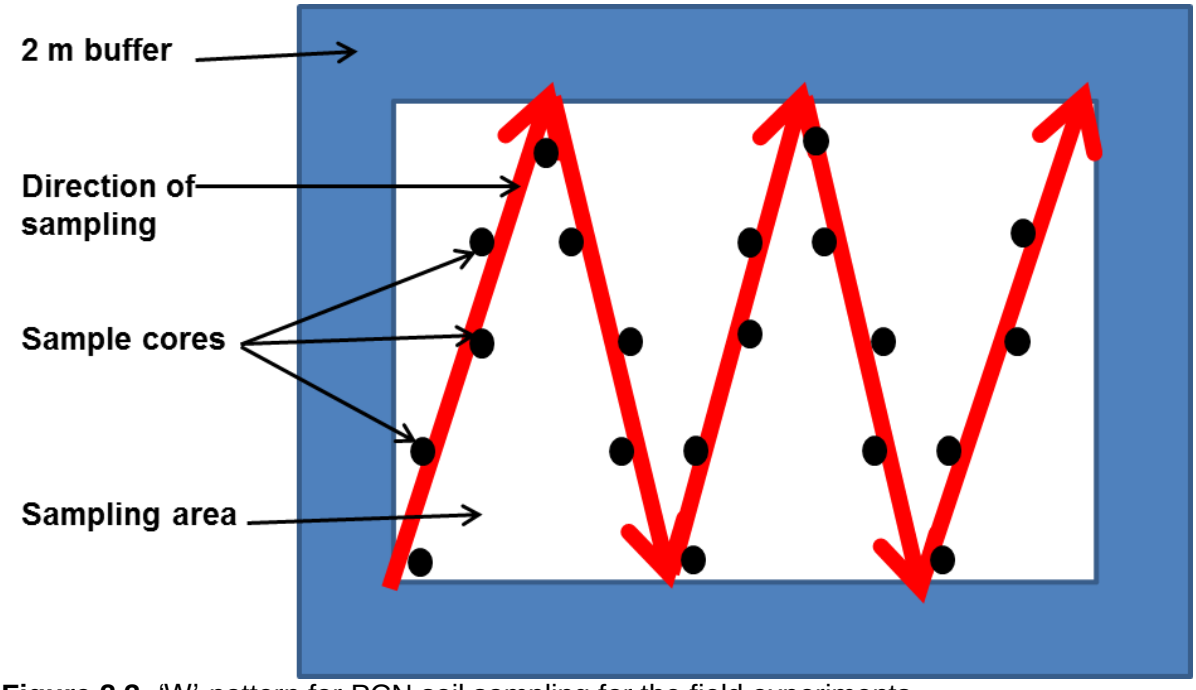


Figure 2.3: 'W'-pattern for PCN soil sampling for the field experiments.

2.4 Cyst extraction, identification, quantification and species determination

Cysts and organic soil debris were extracted from 200g sub-samples using a Fenwick can (Figure 2.4) (Fenwick, 1956) with special care to avoid cross contamination between samples. The cysts and fine organic debris for each sample was raped in a 250 µm mesh, appropriately labled before placing on a tray and air-dried for 48 h at 25°C. Cysts were then separated from organic debris under a dissecting microscope (magnification = ×20) and further assessed for population densities and viability. Fifty cysts taken from each sample were placed in glass blocks half filled with distilled water and soaked for 48 hours, gently crushed and homogenised in 50 ml of distilled water in a 100ml glass tube to form a suspension. Aliquots of 1 ml PCN egg suspension were observed under a dissecting microscope (magnification = ×40) and the number of eggs and juveniles scored. The number of eggs per cyst and the PCN population densities (eggs g^{-1} of soil) was estimated using equations (1) and (2) respectively as outlined by Southey (1970).

$$No.of\ eggs\ cyst^{-1} = \frac{Water\ (ml) in\ egg\ suspention}{No.of\ cyst\ in\ egg\ count\ (50)} \times No.of\ eggs\ ml^{-1} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$No.of\ eggs\ g^{-1}\ soil = \frac{water\ (ml) in\ egg\ suspension\ \times No.of\ eggs\ ml^{-1}}{No.of\ cysts\ used\ in\ egg\ count\ (50)} \times \frac{cysts\ in\ 200g\ soil}{soil\ wt.(200g)} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

PCN species were further determined using quantitative-polymerase chain reaction (qPCR) (Appendix 9.6).

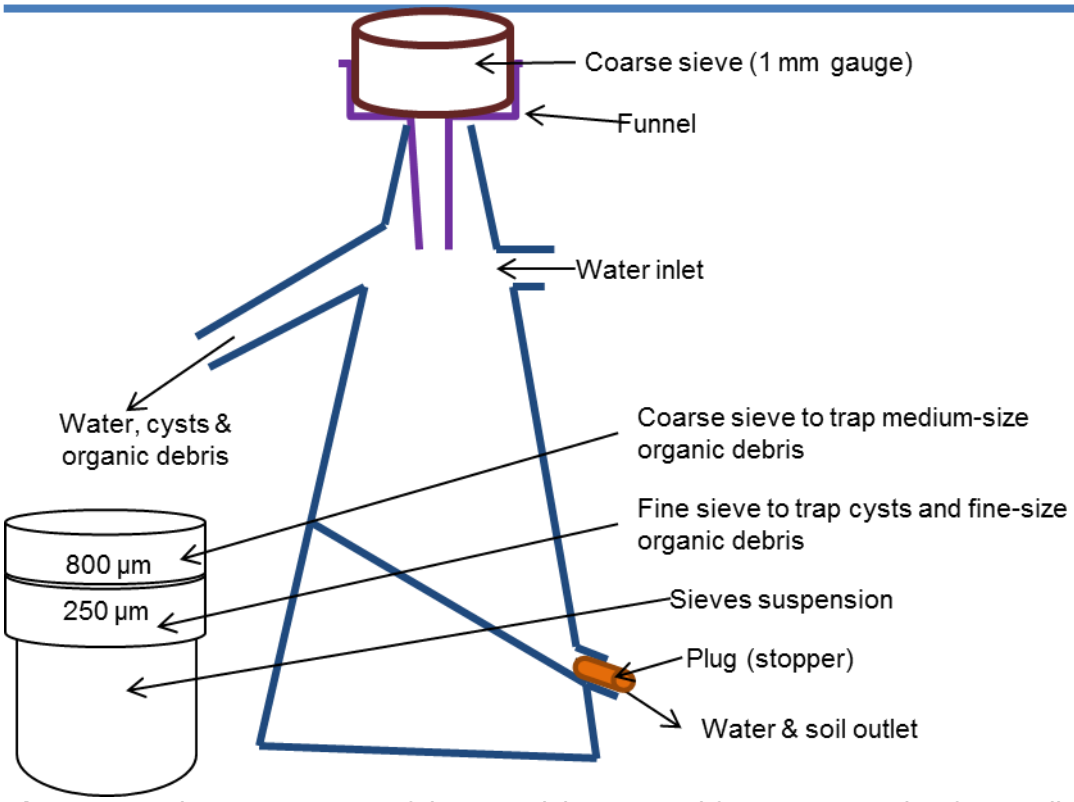


Figure 2.4: The components of the Fenwick can used for cyst extraction from soil

2.5 Estimating viability of encysted eggs

The viability of encysted eggs was estimated by a combination of hatching assays and Meldola's blue staining technique (Shepherd, 1962). For each sample, fifty cysts were soaked in distilled water for 7 days and stained with 0.05% w/v Meldola's blue staining solution (Sigma Aldrich, Poole, UK) for 7 days following the procedure of Shepherd (1962). The blue stain was replaced with distilled water and left for 24 h, gently crushed and vigorously mixed with 50 ml of distilled water. One ml aliquots of PCN egg suspension were observed under a dissecting microscope (magnification = ×40) to distinguish between the number of viable and non-viable eggs. The number of viable eggs (those unable to absorb and retain the blue stain) g^{-1} of soil, was estimated using equation (2). For the hatching assays, a batch of 10 cysts from the same sample were placed into 1.5 ml Eppendorf tubes with the standard lid replaced with a 250 *µ*m aperture mesh and the constricted end cut open to fit into the wells of a 24-well plastic plate previously filled with 1.5 ml of six-week old potato root leachate

(PRL, see section 2.6). The plates were appropriately labelled and sealed with parafilm to minimise evaporation. The set-up was incubated in the dark at 16°C to mimic the situation in the soil during the summer season. The number of hatched J2's was counted at weekly (7 days) intervals and the PRL was replaced at each assessment. Each experiment was monitored for 5-6 weeks after which un-hatched eggs were stained with 0.05% w/v Meldola's blue staining solution (Sigma Aldrich, Poole, UK) following the procedure of Shepherd (1962) to distinguish the number of un-hatched viable eggs from non-viable eggs. The number of viable PCN eggs g^{-1} of soil was estimated using equation (2).

2.6 Collection of potato root leachate (PRL) for hatching experiments

To prepare PRL for hatching experiments, disease-free potato plants cv *Estima* were pre-sprouted for two weeks at 12°C. The pre-sprouted tubers were then transplanted into 12 inch pots three-quarter filled with sterilised silver sand/organic compost (John Innes no 1, John Innes Manufacturers Association, Reading, UK) mixed in the ratio 3:1. Potatoes were grown in the glasshouse at a day/night temperature of 16/5°C respectively with a 14 h photoperiod for 6 weeks and watering with 200 ml tap water every two days. Pots were saturated with tap water at six weeks post-emergence and suspended on a funnel lined with a filter paper (Whatman Nº5) that was inserted into a 1 L glass bottle for the collection of PRL (Plate 2.1). The collected leachate was filter-sterilized through a 0.45 µm sterile filter (SARSTEDT, Germany). The concentrated sterile PRL was preserved at 4°C for up to six months and diluted to 20% v/v (1:4) with sterile distilled water (SDW) when required for hatching assays.

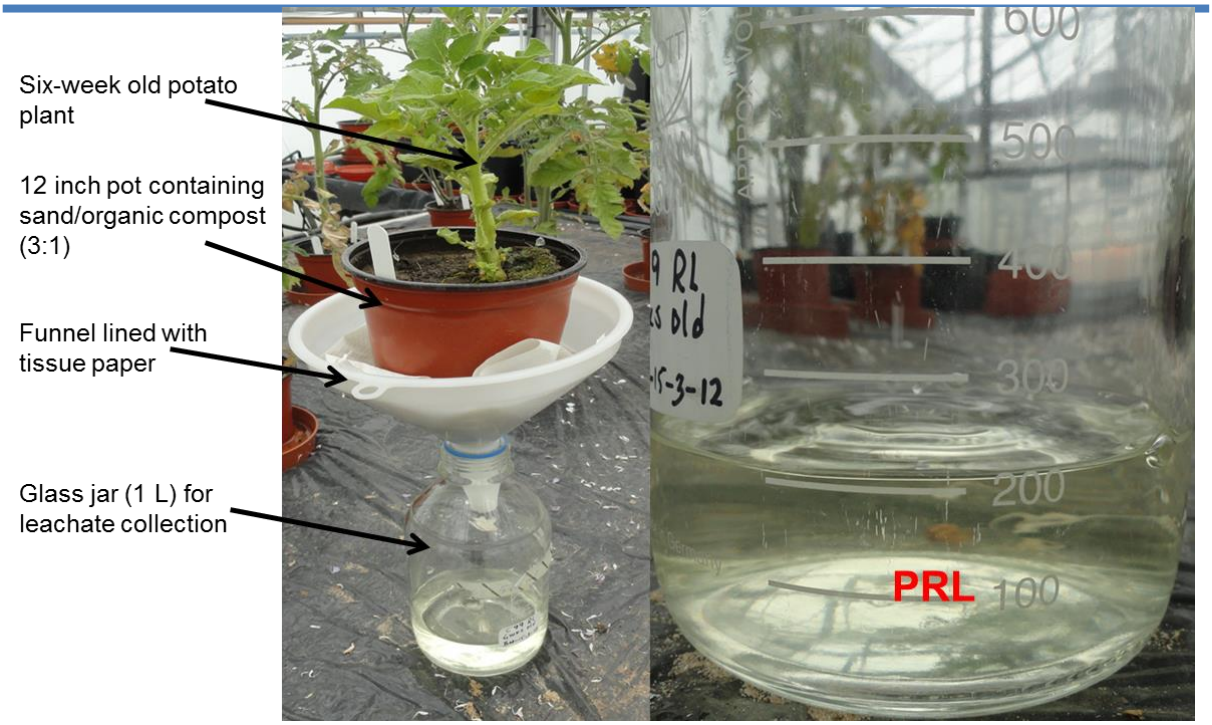


Plate 2.1: Collection of potato root leachates

2.7 Brassica plant material

The selection of *Brassica* species for the experiments was based on a number of factors such as their commercial availability as biofumigant crops, because they are reported to contain high levels of effective isothiocyanate-producing glucosinolates, or that they have been reported elsewhere as effective against other soil borne organisms including PCN. Details of seed samples and their supplier are presented on Table 2.2.

Table 2.2: Details of the *Brassica* varieties used for the field experiments

Species name	Common name	Cultivar	Seed rate	Supplier
Brassica juncea	Indian mustard	Caliente 99	10 kg ha^{-1}	Plant Solutions Ltd, UK
B. juncea	Brown mustard	Etamine	6.5 kg ha^{-1}	Soufflet Agriculture, France
Raphanus sativus	Oilseed radish	Bento	20 kg ha^{-1}	Agrovista® Ltd, UK
R. sativus	Oilseed radish	Teranova	20 kg ha^{-1}	Joordens Zaden, Holland
R. sativus	Oilseed radish	Doublet	20 kg ha^{-1}	Joordens Zaden, Holland
Eruca sativa	Rocket	Nemat	10 kg ha^{-1}	Plant Solutions Ltd, UK

2.8 Application of treatments

For each field experiment, individual plots were either drilled (Plate 2.2) with *Brassica* seeds using the recommended seed rates (Table 2.2), or left fallow as a control. Specific details for each field experiment are detailed in Chapter 3. The incorporation of brassicaceous green material was done at complete flowering (for the summer experiments) or two weeks prior to potato planting (for the overwintered experiments). At incorporation, the green tissue was flailed using a tractor-driven McConnel 2.8 m flail topper (McConnel Ltd**,** Ludlow, England; Plate 2.3) immediately followed by incorporation within the top 30 cm of soil with a Howard 300 rotary tiller (Iowa Farm Equipment, Iowa, USA; Plate 2.4). Following soil incorporation of the green tissue, the soil surface was immediately rolled with a Weston 6 m hydraulic folding Cambridge roll (RES Tractors, Harby, England) to reduce soil porosity (Plate 2.5).

Plate 2.2: Brassica seed drilling with a John Deer tractor driven New Holland seed drill

Plate 2.3: Chopping of brassicaceous green tissues prior to incorporation using a tractor-driven McConnel 2.8 m flail topper

Plate 2.4: Field incorporation of brassicaceous residues into the soil using a Howard 300 rotary tiller

Plate 2.5: Soil sealing with a Weston 6 m hydraulic folding Cambridge roll post-incorporation of brassicaceous residues

2.9 Potato seed planting

The planting of the potato seeds in the field experiment plots was undertaken during the spring season with the aid of a Standen-Pearson SP cup potato planter (Plate 2.6). Details for the timing of planting dates for individual field experiments are discussed further in Chapter 3.

Plate 2.6: Standen-Pearson SP cup potato planter used for the planting of potato seeds in field experiments

2.10 Assessment of potato plant emergence and canopy ground cover

The emergence of potato plants was assessed from two weeks post-planting until complete emergence to investigate any phytotoxic effects from the incorporated green manure. For each individual plot, a central 4x4 m^{-2} sub-plot was assessed for the number of emerged plants and expressed as a percentage of the total planted tubers (Plate 2.7). Each plant was considered to have fully emerged when the shoot was visible above the soil surface (Bastiman *et al.,* 1985).

Measurements of plant development was assessed by mearsuring the percentage of ground covered by the canopy of three plants on the same row, with the aid of a 80 x 60 cm ground cover grid containing 100 squares (Plate 2.8). At each time of measurement, the grid was suspended at approximately 1 m height over three plants on the same harvest row and observed from above. All squares occupied by the

green canopy were counted (Burstall & Harris, 1983) to allow the calculation of percentage ground cover. An average was taken from two readings obtained from the central 4x4 m^{-2} for each plot.

Plate 2.7: An experimental plot from a field experiment showing the central 4x4 m^{-2} sub-plots used for emergence and ground cover assessments

Plate 2.8: Measurement of potato plant canopy via percentage ground cover

2.11 Quantification of PCN invasion of potato roots

Two plant samples were collected from the central 4x4 m^{-2} of each plot at six weeks post-emergence, for the assessment of PCN root invasion. Whole potato plants were carefully collected, by using a garden fork to unearth the plant and the earth was gently detached by hand to reduce root damage. The plants were transported to the laboratory for preparation. The roots were carefully washed to remove soil, and then blotted dry using adsorbent tissue paper (Plate 2.9). The number of juveniles within the potato roots was determined according to the methods described by Hooper (1986). Roots of each experimental plant were carefully pruned from the main stem, cut into approximately 1 - 2 cm pieces and homogenised, before a 2 g sub-sample was taken and preserved in formic acetic alcohol (FAA). The root samples were stained with acid fuchscin and the stained root samples were cut into approximately 3 - 5 mm pieces and homogenised with 200 ml of tap water using a laboratory blender.

Plate 2.9: Potato plant samples prepared for PCN root invasion assessment

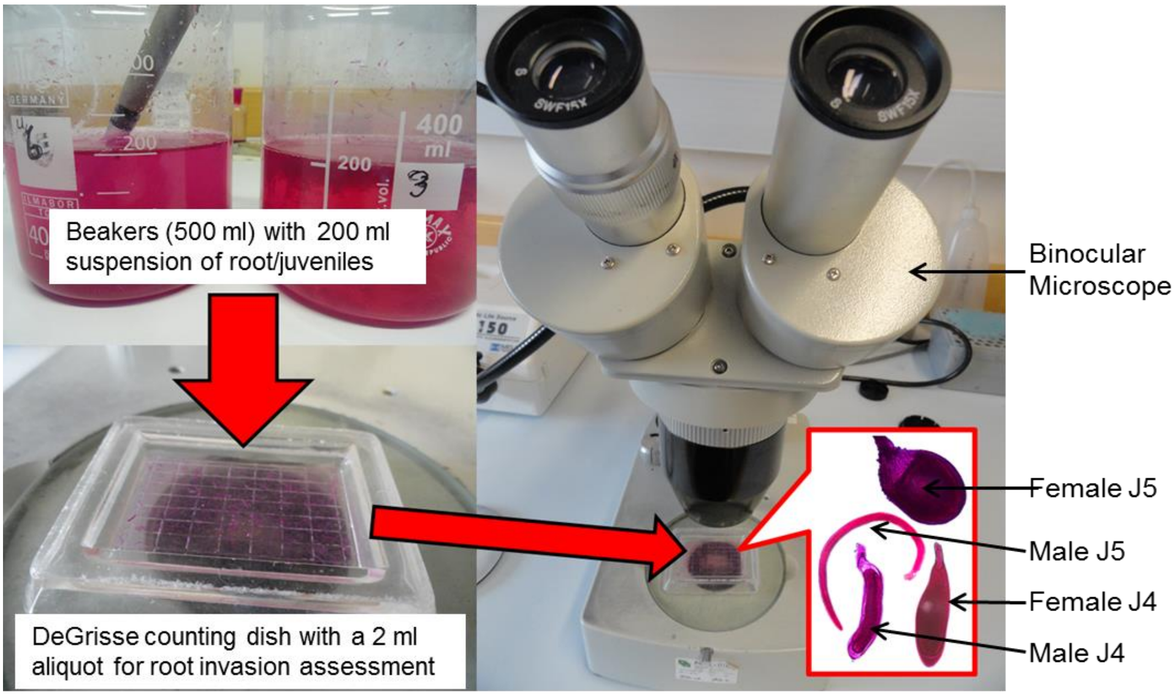


Plate 2.10: The assessment of various juvenile stages of PCN within potato plant roots

A 2 ml sub-sample of the suspension was pipetted onto a DeGrisse counting dish and the different juvenile stages were assessed under a binocular microscope (magnification = x40) (Plate 2.10). The total number of juveniles per gram of root was estimated by combining the data from each juvenile stage and calculated using equation (3).

$$Juveniles\ g^{-1}\ of\ root = \frac{Total\ juveniles\ in\ 2\ ml\ subsample\ \times\ 100}{Weight\ of\ root\ sample\ in\ g\ (2\ g)} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(3)$$

CHAPTER THREE

3. Chapter 3: Field Experiments: The effect of *Brassica juncea*, *Raphanus sativus* and *Eruca sativa* on *Globodera pallida* infestations of potatoes under field conditions

3 The effect of *Brassica juncea*, *Raphanus sativus* and *Eruca sativa* on *Globodera pallida* infestations of potatoes under field conditions

3.1 Introduction

Field experiments were conducted during the period from July 2011 to September 2013 at four sites in Newport, Shropshire, to investigate the effects of selected *Brassica* species on the viability and population densities of *Globodera pallida* in potato fields. As previously discussed in Chapter 1, the use of *Brassica* species to control the potato cyst nematode *G. pallida* has not previously been reported under natural field conditions. However, there have been a number of investigations using biofumigation to control this nematode species under controlled conditions. The need for field investigation on the use of biofumigation to control this potato pest under field conditions has been recommended by a number of researchers (e.g. Lord *et al*., 2011; Valdes *et al.,* 2012). Any reduction in pests or diseases by biofumigant crops grown under natural field conditions will reflect those grown in a commercial situation. Moreover, assessments undertaken throughout the crop growing period may provide more detailed information on the effects of the developmental stage of the crop on the targeted pests.

3.2 General objectives

The objectives of these experiments were to:-

i. Determine the effect of selected *Brassica* species on the population densities of *G. pallida* under commercial field conditions.

ii. Monitor the viability of encysted eggs of *G. pallida* before planting biofumigant *Brassica* crops, following a period of biofumigant crop growth and after incorporation of the brassicaceous crop residues.

iii. Replicate these experiments at different sites, and during different seasons of the year to account for possible variations in the effects of the *Brassica* species on PCN.

iv. Assess the effect of metconazole for any possible additional effect on biofumigation of the selected brassicaceous green manures.

v. Analyse the types and concentrations of GSL of the tested *Brassica* species just before incorporation to account for possible relationships between *G. pallida* control and the incorporated GSL and biomass.

Objectives (i) - (iv) are reported in Chapter 3, whereas objective (v) is reported in Chapter 4.

3.3 General hypothesis (null):

Crushing and incorporation of selected *Brassica* species will have no effect on the viability and population densities of the potato cyst nematode, *G. pallida* under field conditions.

3.4 Materials and methods

3.5 Field Experiment-1

Field experiment 1 (Table 2.1) was conducted from 21st July 2011 to 14th September 2012. The rotation sequence at this site consisted of winter wheat - oilseed rape – biofumigant mustard – potatoes – winter wheat. The site had a historical problem with potato cyst nematodes (PCN), predominantly *Globodera pallida* (Appendix 9.6) due to cultivation of the potato cultivar Maris Piper (Mr Neil Furniss per comm) which confers resistance to *G. rostochiensis* but not to *G. pallida*.

Preliminary analysis of soil samples from the site using a bulk sample of 60 cores taken per four ha area revealed population densities between 0 to 10 eggs g^{-1} of soil (Figure 3.1) prior to the biofumigation experiment (6th June 2011). An area with an average PCN population density of 10 eggs g^{-1} of soil (Furniss 2, Figure 3.1) was chosen for intensive sampling. The distribution of the nematode population densities across the selected area then provided information on the direction of blocking for the experiment. The selected area with statistically insignificant variability ($P > 0.05$) of *G. pallida* eggs g^{-1} of soil was therefore selected and demarcated into individual plots of 8x8 m^2 with a 2 m buffer in-between the plots using a tape measure (Figure 3.2) and sampled for Pi as described previously in section 2.3. The experiment was laid out in a randomised block design (Figure 3.2) such that each block had a similar PCN population within each treatment (as described below) and replicated six times. Plot sizes were made to be sufficiently large (8x8 m^2) to allow for the overlay of potato beds in the following spring. Designated plots were then drilled with the *Brassica* treatments following randomisation in GenStat 15th edition (Figure 3.2).

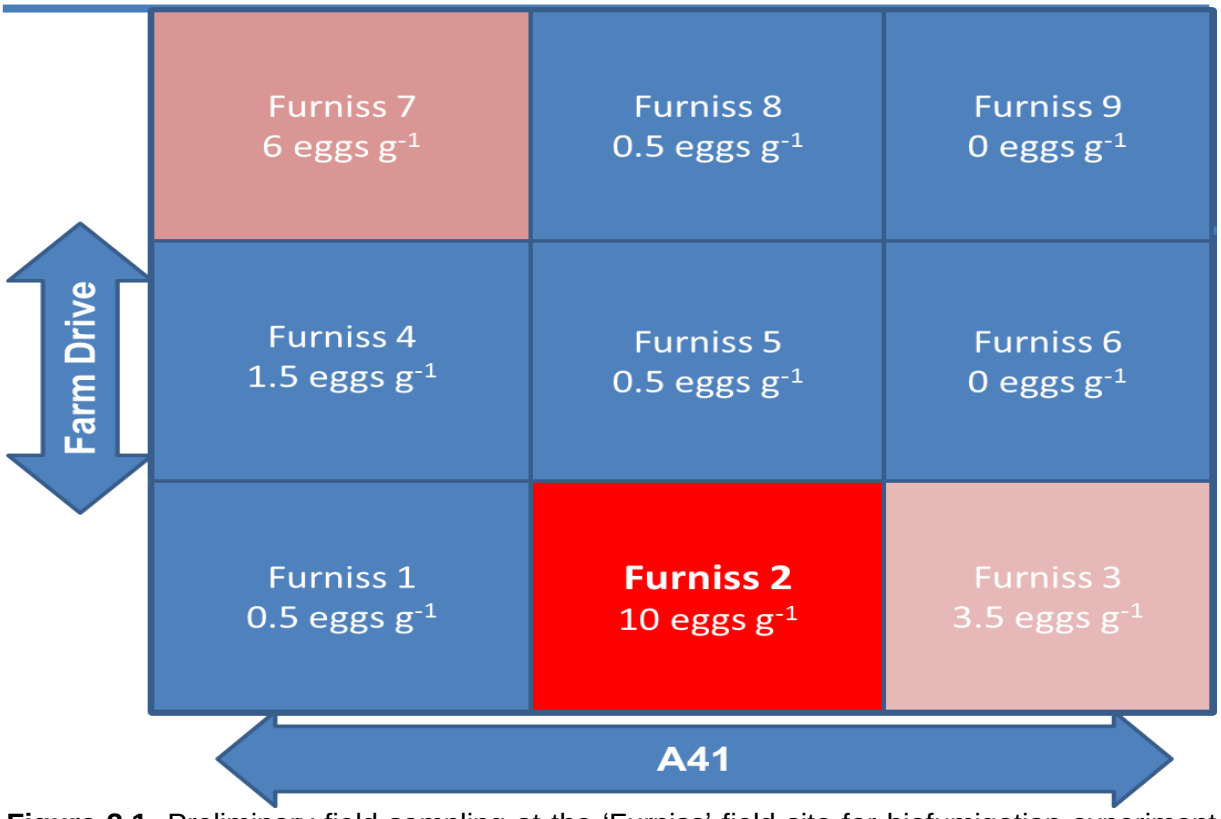


Figure 3.1: Preliminary field sampling at the 'Furniss' field site for biofumigation experiment site selection

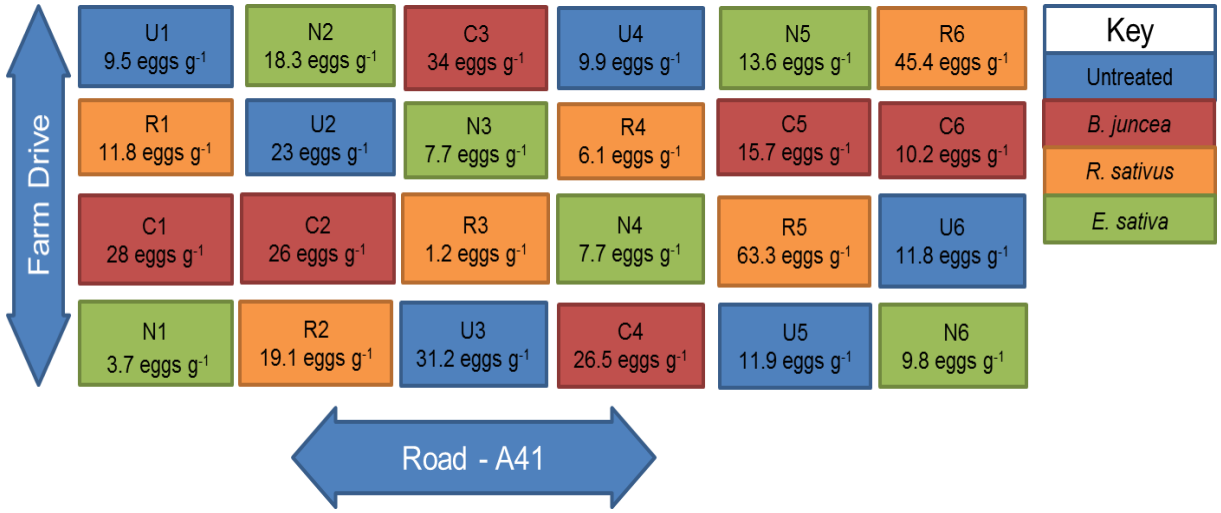


Figure 3.2: Randomized block design layout for field experiment-1 and their respective Pi values (eggs g^{-1} of soil). Different colours/letters represent treatments; *Brassica juncea* cv Caliente 99 (C), *Raphanus sativus* cv Bento (R), *Eruca sativa* cv Nemat (N) and untreated fallow (U). Numbers next to the letters indicate blocks

3.6 Field Experiment-2

This experiment (Chapter 2, Table 2.1) was conducted from 21st September 2011 to 14th September 2012. The farmer (Mr Mark Davies) was interested in autumn season cultivation of biofumigant brassicas and overwintering the crop for spring incorporation prior to a commercial potato crop. The rotation sequence at this site

consisted of potato - wheat – oilseed rape – biofumigant mustard/radish mix – potatoes. The PCN population within the field was relatively high (average of 36 ± 30 eggs g^{-1} soil) and was predominantly *G. pallida* due to the cultivation of Maris Piper (Mr Mark Davies' personal communication).

Routine soil analysis conducted across the field, prior to brassica crop establishment, by an agronomist from Agrovista UK Ltd identified an area with average counts of 36 eggs g^{-1} of soil. This area was marked out into a 60x50 m^2 plot and intensively sampled for PCN distribution, to enable the design and layout of the experimental plots. Twenty four individual plots of 8x8 m^2 were demarcated within the 60x50 m^2 area with a 2 m buffer between them to avoid border effect. The experiment was initially designed as a rendomised block. However, due to a technical error during plot drilling with treatments, the resultant design (Figure 3.3) was adapted with each treatment replicated six times.

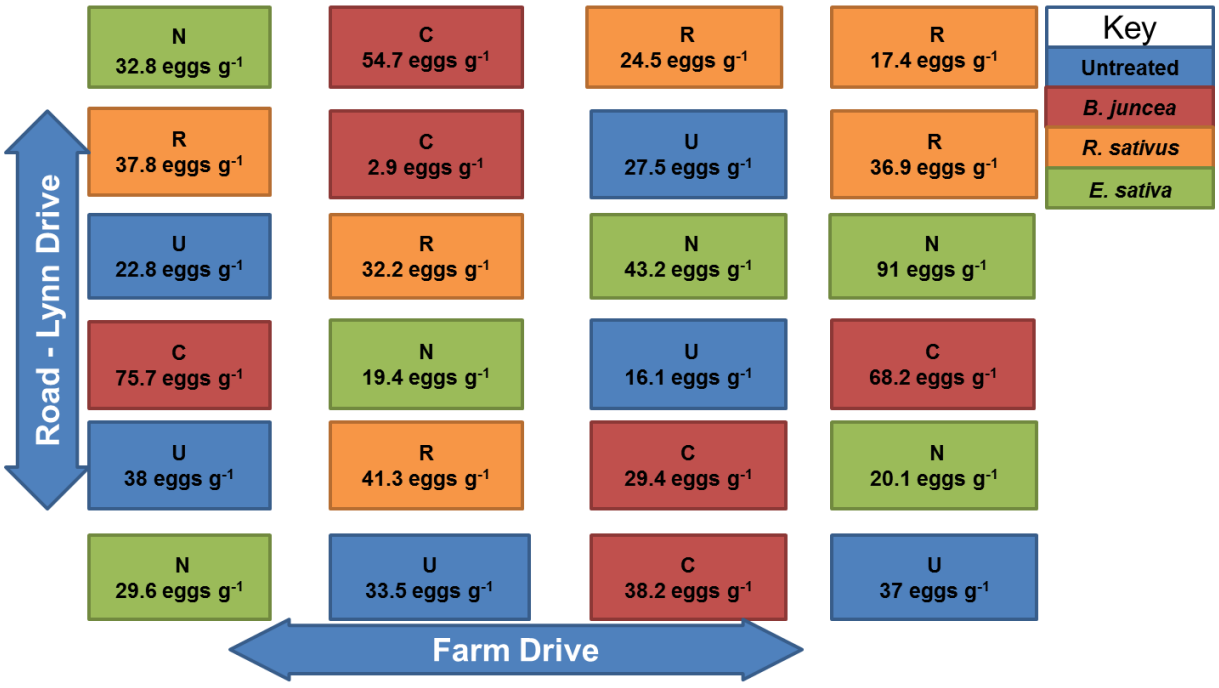


Figure 3.3: Randomised design layout and *G. pallida* distribution (eggs g^{-1} of soil) in field experiment 2

3.6.1 Plant material selection and treatments

The selection of *Brassica* species for the field experiments was based on a number of factors such as their commercial availability as biofumigant crops or because they are known to produce high levels of effective isothiocyanate-producing GSLs (Lord *et al*., 2011). Seeds of *Brassica juncea* cv Caliente 99 and *Eruca sativa* cv Nemat were supplied by Plant Solutions Ltd, Cobham, UK, while *Raphanus sativus* cv Bento was supplied by Agrovista® Ltd, Stapleford, UK.

Experiments-1 and -2 received identical treatments as detailed in Table 3.1. The calculation for the seeding rates was based on the weight per thousand seeds count paying respect to the recommendations by the seed supplying company. *Brassica* seeds were drilled (see section 2.8) into individual plots on 21st of July 2011 (Experiment-1) and 21st September (Experiment-2). Following the establishment of the treatments, two TinnyTagPlus® data loggers were buried to a depth of 20 cm, pre-set to record soil temperature data at an hourly interval. All plots were then mapped using a Leica Viva GS08plus (Leica Geosystems Ltd, Milton Keynes, UK) GPS.

Table 3.1: Treatments for Field Experiments 1 (Chetwynd) and 2 (Lynn)

Species name	Common name	Cultivar	Seed rate	Supplier
Brassica juncea	Yellow mustard	Caliente 99	8kg ha^{-1}	Plant Solutions Ltd, UK
Raphanus sativus	Oil radish	Bento	20kg ha^{-1}	Agrovista® Ltd, UK
Eruca sativa	Rocket	Nemat	8 kg ha^{-1}	Plant Solutions Ltd, UK
Untreated fallow	Control	---------	---------	---------

3.6.2 Assessments

3.6.2.1 Soil sampling and processing

Field sampling for PCN population densities and viability tests in Experiments-1 and Experiment-2 was undertaken at the following times:-

1. Pre-sowing of brassicas
2. Pre-incorporation of brassicas
3. Six weeks post-incorporation of brassicas
4. Post-harvest of the potato crop

However, in Experiment-2, no samples were taken six weeks post-incorporation (iv) because the gap between spring incorporation of the overwinter brassicas and potato establishment was just two weeks. Therefore, the sampling was undertaken prior to potato crop establishment. At each time of sampling, soil samples were taken at regular intervals, in a 'W' pattern (Chapter 2, Figure 2.2) and processed for PCN population densities and viability as previously described in Chapter 2, sections 2.2 – 2.4.

3.6.2.2 Brassica plant density and biomass determination

Prior to incorporation, three 1 m^2 sub-plots were measured at different locations for each plot. Plants in the area were scored for plant density and an average of the total counts from the sub-plots represented the number of plants m^{-2} for the entire plot. Nine whole plants were then randomly sampled from each plot and taken to the laboratory where they were separated into shoots and roots. The shoots or roots (for 9 plants) were weighed and the average fresh weight per individual plant was calculated. The average weight per individual plant was further multiplied by the average number of plants m^{-2} to obtain the biomass that was incorporated per m^{-2}.

3.6.2.3 Potato establishment and assessments

Potato tubers cv Maris Piper were planted in experiments 1 and 2 on 12th March and 10th May 2012 respectively using a Standen-Pearson SP cup potato planter (Chapter two, Plate 2.4). At two weeks post-planting, a central 4x4 m^2 area was measured for all plots for both experiments within which the potato plants were scored for emergence at a weekly interval until complete emergence to investigate any possible

effect on the crop emergence resulting from the incorporated biofumigant crops as compared with the fallow plots. A potato plant, within the area of assessment, was considered to have fully emerged when the shoot was visible from above the ground (Bastiman *et al.,* 1985).

Following complete emergence (at 6 weeks post-planting), potato plant growth was assessed as percentage of ground covered by the canopy of three plants on the same row with the aid of an 80 x 60 cm ground cover grid containing 100 squares (See Chapter 2, section 2.10).

At six weeks post-planting, two plant samples were harvested from the assessment area (4x4 m^{-2}) in each plot for PCN root invasion analysis. Details for these assessments have been discussed previously (Chapter 2, section 2.10). Tuber yield assessments were undertaken on 26 July and 14 September 2012 for experiments 1 and 2 respectively. Potato tubers were hand harvested from the 4x4 m^2 assessment area with the aid of a garden fork and graded into ≤45 cm (non-marketable), 45 ≤ 65 cm and 65 ≤ 85 cm sizes.

3.6.3 Statistical analysis

All data from the experiments were subjected to general analysis of variance (ANOVA) using GenStat® (15th Edition) statistical software. Where necessary, data were log_{10}-transformed to normalise residuals. Significant differences between treatments were determined using Tukey's multiple range tests (5% significance level).

3.6.4 Results

3.6.4.1 Effect of growing brassicaceous plants on the viability of *G. pallida* encysted eggs

The initial *G. pallida* population densities prior to brassica crops establishment did not differ significantly among plots in experiments 1 and 2 (Tables 3.2), with the average viability ranging from ≈ 85-95%. The viability of *G. pallida* encysted eggs assessed just before incorporation of brassicaceous crop residues was significantly reduced (*P = 0.021*) in plots sown with each of the *Brassica* species compared with the fallow plots in Experiment-1 (Table 3.2). In Experiment-2, the assessment of the viability of encysted *G. pallida* eggs g^{-1} of soil just before incorporation revealed a significant reduction (*P = 0.021*) in egg-viability in plots treated with *R. sativus* only (Table 3.2).

3.6.4.2 Effect of soil incorporation of brassicaceous residues on the viability of *G. pallida* encysted eggs

In Experiment-1, assessments undertaken six weeks after incorporation of brassicaceous residues showed significant reductions (*P < 0.001*) in the number of viable eggs g^{-1} of soil for plots treated with *B. juncea* and *R. sativus* compared with the untreated fallow, but not for plots treated with *E. sativa* (Table 3.2). An assessment of the invasion of the potato roots by *G. pallida* juveniles, six weeks post-planting, did not show any statistical significant differences between treatments. Also, at this stage, the predominant developmental stage of *G. pallida* found within the potato roots was J2 (Figure 3.4A). A significant reduction (*P = 0.03*) in the *G. pallida* final population density as well as a reduction in the rate of *G. pallida* multiplication (Pf/Pi = 0.92) was found in plots treated with *B. juncea* (Table 3.2). However, the final population density in plots treated with *R. sativus* and *E. sativa* did not differ significantly from untreated fallow plots.

In Experiment-2, the level of control did not differ statistically in biofumigant treatments and the fallow after incorporation of brassicaceous residues and post-harvest of the commercial potato crop. However, the population densities assessed at these times was non-significantly lower in plots treated with *R. sativus* when compared with the rest of the treatments (Table 3.2). No significant differences in root invasion were found between the treatments (Figure 3.4B). At this stage, all juvenile stages were represented, with the second and third juvenile stages (J2 and J3 respectively) making up more than 75% of all juveniles within the roots (Figure 3.5).

Table 3.2: Viable *Globodera pallida* eggs g^{-1} soil in field Experiments 1 & 2 for different treatments with *Brassica juncea* cv Caliente 99, *Raphanus sativus* cv Bento, *Eruca sativa* cv Nemat or left fallow, assessed at different stages of the plant development (pre-planting, pre-incorporation, post-incorporation and post-harvest of potato crop). Different superscript letters represent significant differences in mean viable eggs g^{-1} soil between treatments for individual experiments according to Tukey's multiple range test (5% significance level)

	Treatments													
	Untreated		***B. juncea***		***R. sativus***		***E. sativa***		***P-value***		***S.E.M.***		***CV%***	
Time of assessment	Exp-1	Exp-2	Exp-1	Exp-2	Exp-1	Exp-2	Exp-1	Exp-2	Exp-1	Exp-2	Exp-1	Exp-2	Exp-1	Exp-2
Initial population densities (P_i)	11.6	29	19.2	46	14.4	32	11.0	40	0.656	0.690	4.4	1.6	47.2	26.6
Pre-incorporation	12.2^{b}	27^{B}	5.2^{ab}	14^{AB}	2.8^{a}	12^{A}	4.1^{a}	14^{AB}	0.021	0.021	0.7	8.6	26.9	51.0
Post-incorporation	7.7^{b}	39	1.0^{a}	39	1.8^{a}	20	4.1_{ab}	38	<0.001	0.247	0.6	10.5	28.9	53.3
Final population densities (P_f)	31.3^{b}	60.1	13.3^{a}	56.9	17.1^{ab}	34.9	19.6^{ab}	67.5	0.03	0.164	4.1	0.3	49.3	14.3

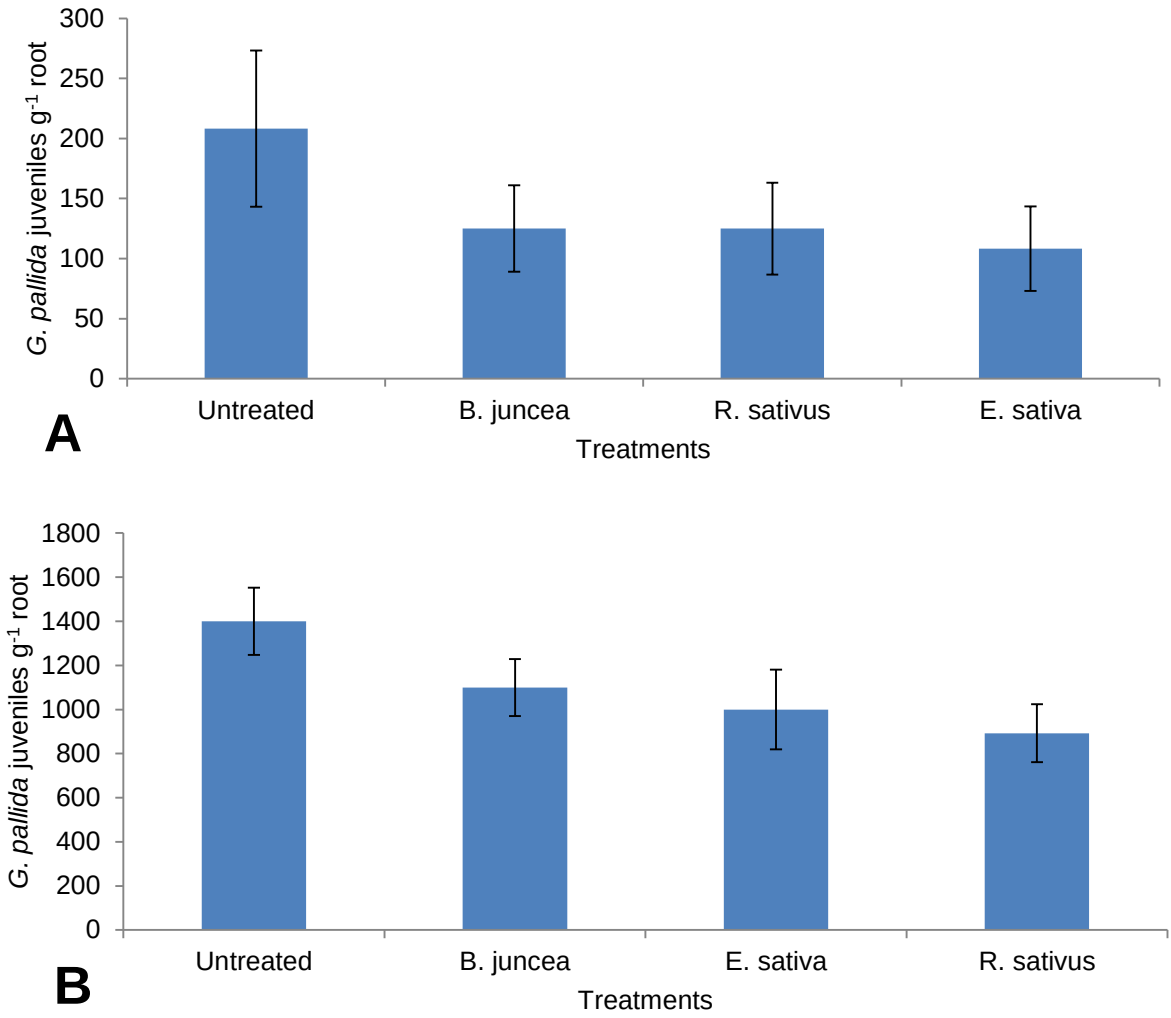


Figure 3.4: Number of second stage juveniles (J2s) of *Globodera pallida* found within potato plant roots assessed six weeks post-planting of potatoes in (A) Experiment-1 and (B) Experiment-2. Plots were previously planted with *Brassica juncea*, *Raphanus sativus*, *Eruca sativa* or left untreated (fallow). Error bars represent standard errors of means

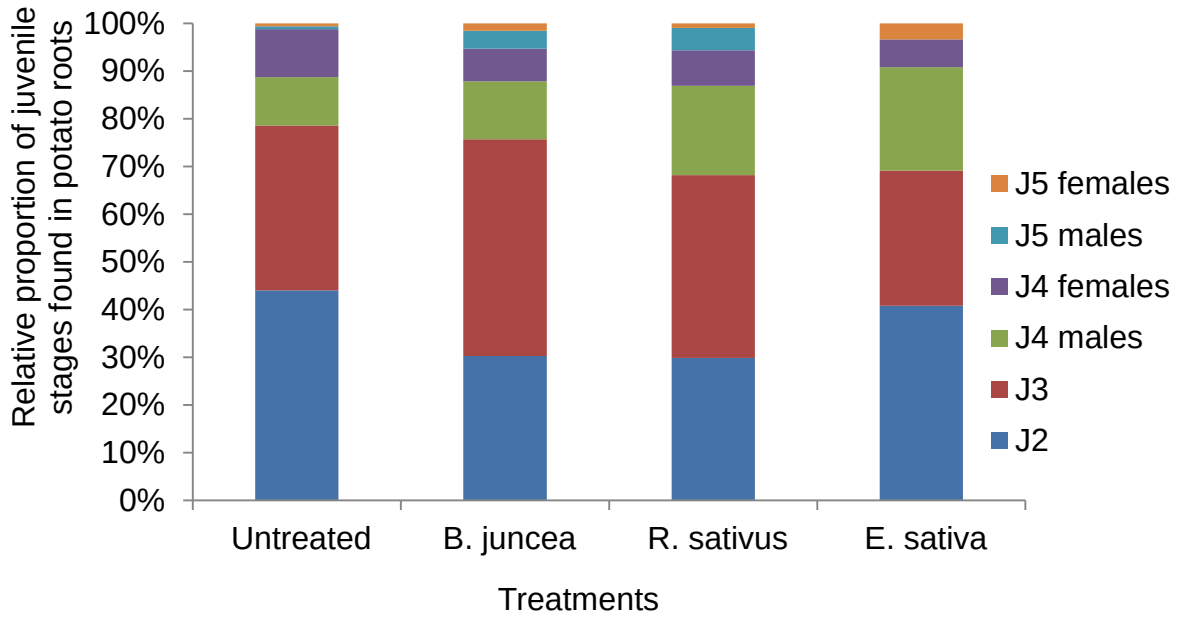


Figure 3.5: Proportions of *Globodera pallida* juvenile stages found within potato plant roots assessed six weeks post-planting of potatoes in Experiment-2. Plots were previously planted with *Brassica juncea*, *Raphanus sativus*, *Eruca sativa* or left untreated (fallow)

3.6.4.3 Effect of brassicaceous residues on the growth and development of potato plants

In experiments 1 and 2, the emergence of the potato plant assessed from planting until complete emergence (5 weeks post-planting) was not significantly affected by the treatments applied prior to the potato crop establishment (Figure 3.6A & B). Also, there was no evidence to suggest any changes in the potato crop development in terms of the percentage ground cover by the canopy as a result of the green manure crop treatments in both experiments 1 and 2 (Figure 3.7A & B).

3.6.4.4 Effect of brassicaceous green manure on the commercial potato crop yield

No significant differences ($P > 0.05$) in the total crop yield (tonnes/ha) assessed post-harvest of the commercial potato crop were found between the treatments in experiments 1 and 2 (Tables 3.3 & 3.4 respectively).

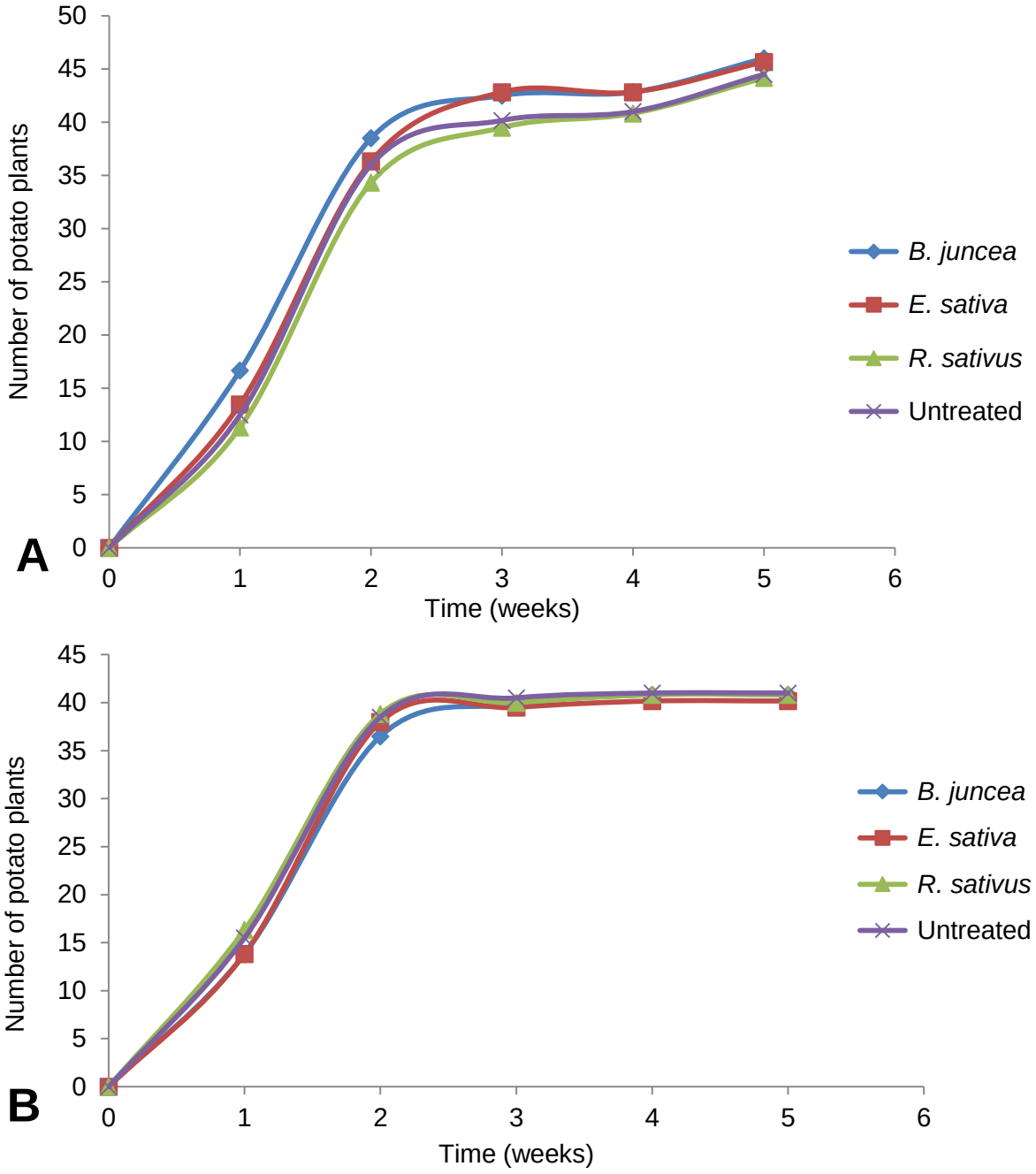


Figure 3.6: Potato plant emergence assessed at weekly intervals (0 – 5 weeks post planting) in (A) Experiment-1 and (B) Experiment-2. Plots were previously planted with *Brassica juncea* cv Caliente 99, *Raphanus sativus* cv Bento, *Eruca sativa* cv Nemat or left untreated (fallow)

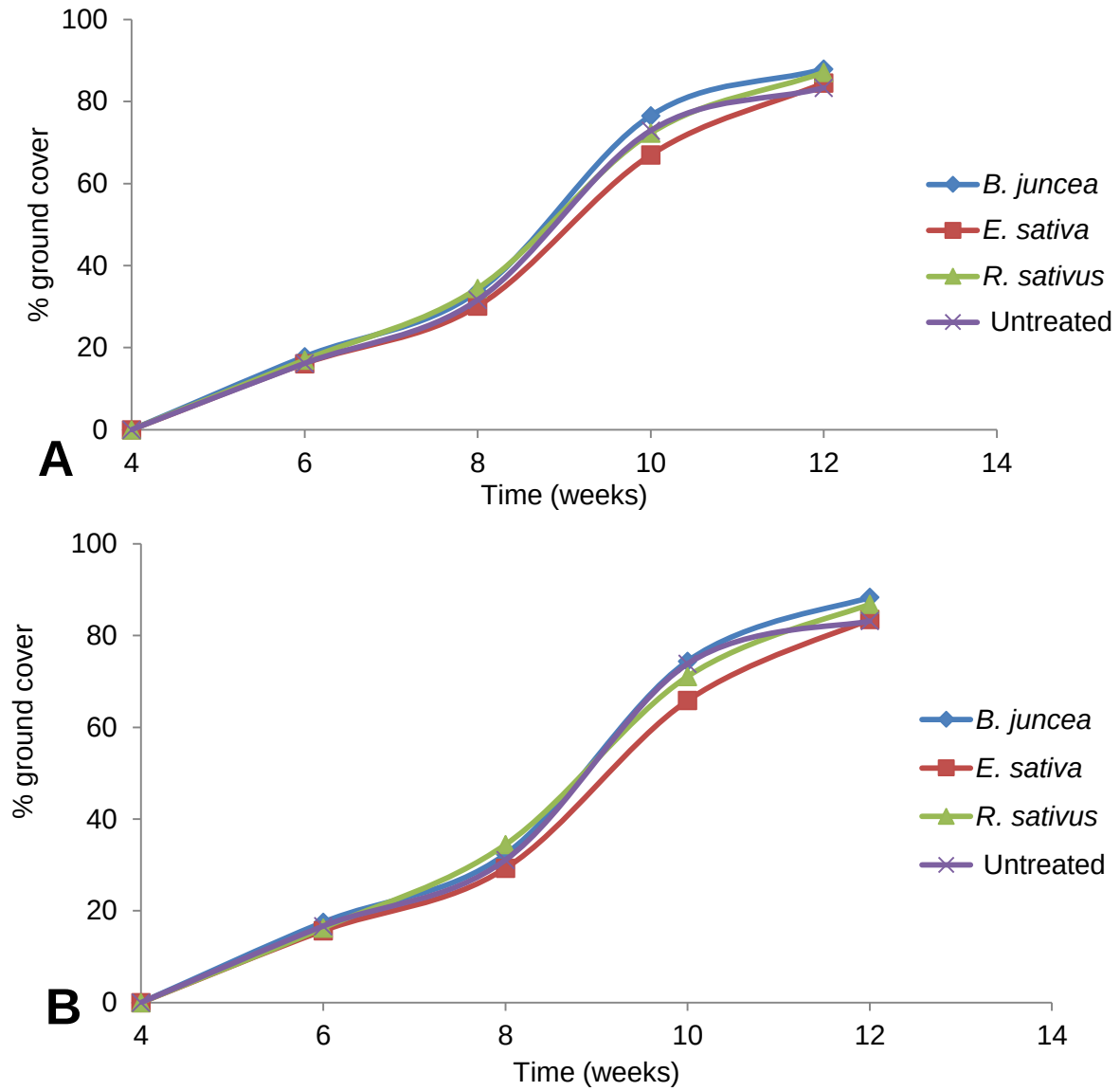


Figure 3.7: Potato plant growth and development assessed as the percentage ground cover by the canopy at two-week intervals (4 – 12 weeks after planting, WAP) in (A) Experiment-1 and (B) Experiment-2. Plots were previously planted with *Brassica juncea* cv Caliente 99, *Raphanus sativus* cv Bento, *Eruca sativa* cv Nemat or left untreated (fallow).

Table 3.3: Potato crop yield assessment (tonnes/ha) in Experiment-1 plots previously treated with *Brassica juncea*, *Raphanus sativus*, *Eruca sativa* or left untreated (fallow). The potato sizes were graded into ≤ 45 mm (non-marketable), 45 ≤ 65 mm and 65 ≤ 85 mm (marketable sizes) and weight measured in kg plot^{-1}

Size range	Treatments				*P-value*	S.E.M.	% CV
	Untreated	***B. juncea***	***R. sativus***	***E. sativa***			
≤45 mm (kg)	0.8	0.6	0.7	0.7	0.821	0.2	46.5
45mm ≤ 65mm (kg)	5.7	6.3	6.4	6.1	0.875	0.9	25
65mm ≤ 85mm (kg)	0.7	0.1	0.4	0.5	0.210	0.3	107.2
Total yield (tonnes/ha)	**44.7**	**45.5**	**48.0**	**46.2**	**0.947**	**4.1**	**21.6**

Table 3.4: Potato crop yield assessment (tonnes/ha) in Experiment-2 plots previously treated with *Brassica juncea*, *Raphanus sativus*, *Eruca sativa* or left untreated (fallow). The potato sizes were graded into ≤ 45 mm (non-marketable), 45 ≤ 65 mm and 65 ≤ 85 mm (marketable sizes) and weight measured in kg plot^{-1}

Size range	Treatments				*P-value*	S.E.M.	% CV
	Untreated	***B. juncea***	***R. sativus***	***E. sativa***			
≤45 mm (kg)	0.3	0.4	0.4	0.3	0.795	0.1	55.1
45mm ≤ 65mm (kg)	5.7	5.5	6.1	5.0	0.488	0.7	22.7
65mm ≤ 85mm (kg)	1.9	2.4	1.9	2.6	0.462	0.5	42
Total yield (tonnes/ha)	**48.6**	**50.7**	**51.4**	**47.3**	**0.814**	**3.4**	**16.3**

3.6.4.5 Effect of different planting seasons (summer and overwintering) on the biomass of *Brassica* species

Generally, all the brassicaceous crops used for these experiments produced more crop biomass when sown in summer. Individual plants differed in the amount of biomass produced during both seasons. For *B. juncea* and *R. sativus,* the average crop biomass produced during the summer season was approximately two-fold that produced after overwintering of these crops (Table 3.5). The overwintered brassicaceous crops in Experiment-2 were severely affected by frost/snow (Plate 3.1), and this effect increased the susceptibility of *R. sativus* to disease, causing curling of the leaves during the spring season (Plate 3.2A).

Eruca sativa appeared to be more suitable for winter conditions, as the average biomass obtained for this crop after overwintering was similar to that obtained during the summer season (Table 3.5).

Eruca sativa was also severely affected by frost/snow, but its recovery was much faster, followed by flowering during early spring of 2012. *Raphanus sativus* had better root and shoot biomass as a summer crop, although the roots were infested by the larvae of *Delia radicum* (cabbage root fly, Plate 3.2B).

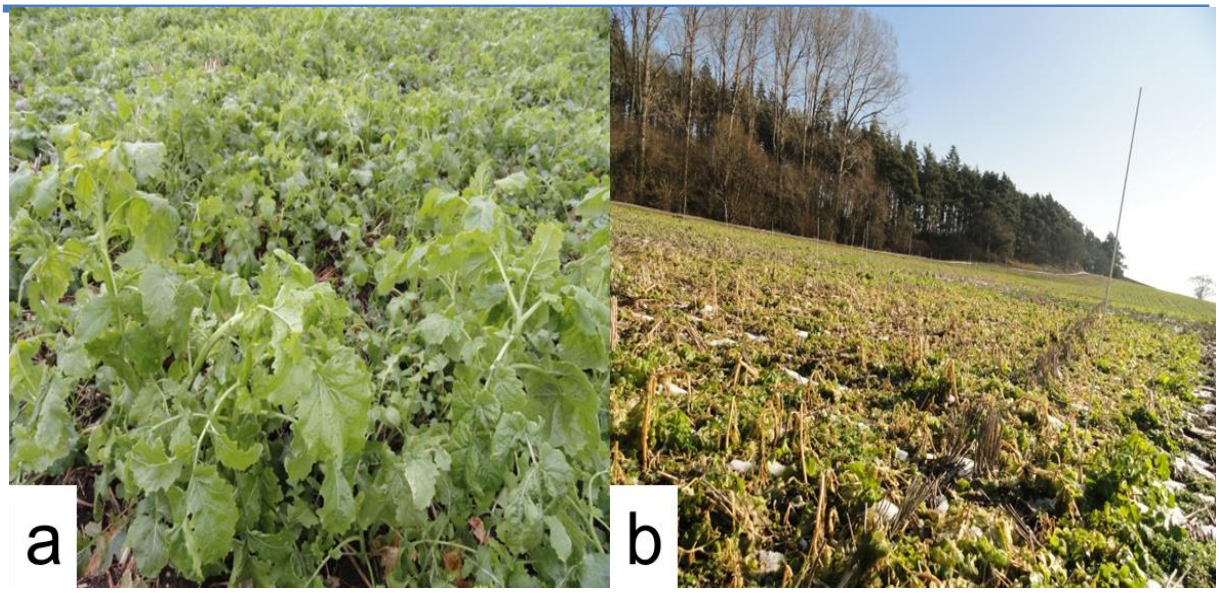


Plate 3.1: Brassicaceous green manure crops cultivated in Experiment-2; (a) before and (b) after frost/snow in the 2011/2012 cropping season.

Plate 3.2: (A) *Raphanus sativus* cv Bento prior to incorporation after overwintering in Experiment-2, (B) extensive root damage caused by the larvae of *Delia radicum* (cabbage root fly) in summer cultivated brassicas

Table 3.5: Fresh weights (kg m^{-2}) of *Brassica juncea*, *Raphanus sativus*, and *Eruca sativa* for Experiment-1 (summer cultivation) and Experiment-2 (overwinter cultivation). Different superscript letters represent significant differences in biomass between treatments of the same experiment according to Tukey's multiple range test (5% significant level)

Experiments	Treatments			*P-value*	SEM	CV%
	Brassica juncea	*Raphanus sativus*	*Eruca sativa*			
Experiment-1	2.1^{a}	3.2^{a}	7.1^{b}	<0.001	0.17	4.9
Experiment-2	1.0^{a}	1.6^{a}	4.7^{b}	<0.001	0.17	5.2

3.7 Field Experiment-3: Effect of metconazole treatment on the biofumigation potential of *Brassica juncea* and *Raphanus sativus* cultivated during summer season of 2012

Based on results and observations from field experiments 1 and 2, *Brassica juncea* and *Raphanus sativus* undoubtedly performed better when used as biofumigant green manure crops in terms of efficacy against PCN under field conditions. The efficacy of these two species was observed during the crop growing period and after incorporation when cultivated in the summer. However, it was noticed that, due to dry conditions during the summer of 2011 (average soil moisture of 11% volumetric water content (vwc)), there were inconsistencies in the plant development for the different biofumigant brassica crops used. Therefore, we hypothesised that the availability of adequate moisture (between 20 - 30% vwc), and the application of soil additives such as sulphur and metconazole (Caramba®) could enhance the production of a better root biomass. A high root biomass as such could improve on the biofumigation potential of these crops against PCN under field conditions.

As discussed previously in Chapter 1, triazoles, such as metconazole and tebuconazole, are now commonly being utilised on brassicas to regulate growth in addition to their fungicidal attributes. An enhanced root-biomass production could possibly lead to an increase in rhizospheric secretion of phytochemicals, such as GSL through leaching or root tissue damage.

Eruca sativa cv Nemat had slow development when cultivated during the summer season (Experiment-1), but appeared to be a potential cold-tolerant variety as it was able to survive snow/frost, followed by a rapid regeneration and flowering in early spring season of 2012 in Experiment-2. Therefore it was omitted from the summer experiment (Experiment-3), but was included for overwintering in Experiment-4.

The principal objective of this study was to assess the effect of the fungicide, metconazole (Caramba®) on the root biomass production of the biofumigants, *B. juncea* and *R. sativus*, their glucosinolate production and subsequent biofumigation effect on *G. pallida* field populations.

3.7.1 Preliminary sampling, design and Treatments

This experiment (Table 2.1) was undertaken on a sandy loam soil (71.7% sand, 18.5% silt, 9.8% clay, pH=6.28) that had initial *G. pallida* population densities ranging from 12 - 23 eggs g^{-1} of soil. An area identified by the potato farmer as a *G. pallida* hot spot was divided into 4 sections of 30 x 20 m^2, intensively sampled and processed to understand the distribution pattern of *G. pallida* in the designated area. Subsequently, the experiment was organised on a 60x50 m^2 area as a randomised block design with 6 replicates of each treatment (Table 3.6, Figure 3.8). The experiment was drilled on 3rd August 2012, chopped and incorporated on 9th November 2012 and followed by a commercial potato crop on 18th April 2013. Plot sizes were designed to be sufficiently large (9x9 m^2) to allow for the ease of allocating treatments and laying of potato beds in the following spring season of 2013, with a 1 m buffer zone between them to minimise cross contact of treatments (Figure 3.8).

Table 3.6: Treatments for Field Experiment-3 (Chetwynd) in 2012

Treatment	Variety	Metconazole (1.2 litre ha^{-1})	Seed rates
Untreated fallow	--	--	--
Brassica juncea	Caliente 99	Yes	8 kg ha^{-1}
Brassica juncea	Caliente 99	No	8 kg ha^{-1}
Raphanus sativus	Bento radish	Yes	20 kg ha^{-1}
Raphanus sativus	Bento radish	No	20 kg ha^{-1}

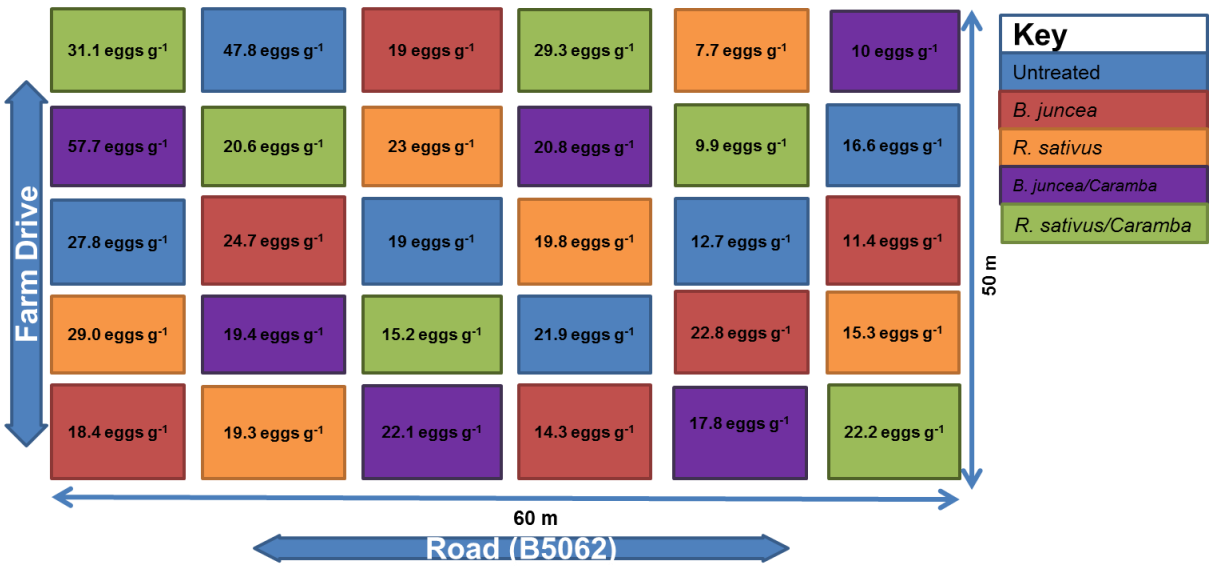


Figure 3.8: Randomised block design in Field Experiment-3 showing treatments for individual plots with their respective Pi values (eggs g^{-1} of soil)

3.7.2 Assessments

The assessments undertaken in this experiment were similar to those for the previous field experiments (Experiments 1 and 2). However, assessments of potato plant emergence, development and *G. pallida* root invasion were not conducted as they were deemed to be of little value following the results of the previous field experiments.

3.7.2.1 Management of biofumigant crops

After drilling the biofumigant brassicaceous green manure seeds, all plots were uniformly treated with granulated sulphur 'N' fertilizer (Sulphur N 26N 35SO_3, Origin Fertilizers UK Ltd) at a rate of 120 kg nitrogen (N) and 64 kg sulphur ha^{-1} (Plate 3.4). Two TinyTagPlus® data loggers were buried to a depth of 20 cm and pre-set to record soil temperature data at an hourly interval. All plots were then mapped using an accurate GPS (Leica Viva GS08plus, Leica Geosystems Ltd, Milton Keynes, UK). Metconazole (Caramba®) was applied at a rate of 1.2 l ha^{-1} during the early flowering stage (seven weeks after planting).

Globodera pallida soil population densities and viability testing were undertaken in each plot at the following times and assessed as previously described in Chapter 2.

1. Pre-sowing of brassicas (P_i) (2nd August 2012)
2. Pre-incorporation of brassicas (partial biofumigation) (9th Novermber 2012)
3. Six weeks post-incorporation of brassicas (complete biofumigation) (18th December 2012)
4. Post-harvest of potatoes (P_f) (24th September 2013)

Brassica samples for GSL analysis were taken prior to their incorporation as described previously. A measurement of the brassicaceous plant biomass as well as brassica plant density counts was undertaken prior to their incorporation to determine the incorporated biomass m^2. The biofumigant crops were incorporated on 9th November 2012 as described in Chapter 2, section 2.7.

Plate 3.3: Sulphur 'N' fertiliser application in Experiment-3 immediately after brassicaceous green manure seed establishment during the summer of 2012

3.7.2.2 Potato establishment and assessments

A commercial potato crop was established on 18 April 2013. The commercial potato crop was harvested on 24 September 2013 and graded in the same manner as described in section 3.6.2.3. Following potato harvest, plots were sampled and assessed for *G. pallida* final population densities.

3.7.3 Statistical analysis

A general analysis of variance (ANOVA) was performed for the data collected using GenStat® (15th Edition) statistical software. Where necessary, data were $\log_{10}$-transformed to normalise residuals. Significant differences between treatments were determined using Tukey's multiple range tests (5% significance level).

3.7.4 Results

3.7.4.1 Effect of growing brassicaceous plants on the viability of *G. pallida* eggs

Before establishing the brassicaceous plant treatments, the initial *G. pallida* population densities were similar among plots within the experiment (Table 3.7) with average viability ranging from ≈ 80 - 95%. However, the viability of *G. pallida* encysted eggs just before incorporation of the brassica plants was significantly reduced in plots sown with brassica species compared with the fallow plots except for *B. juncea* treatments that did not receive metconazole (Table 3.7). Similar to previous experiments, *R. sativus* demonstrated its consistency in affecting the viability of PCN during the plant growth and development stage. Root materials examined in the field were found to have suffered severe damage from root feeding invertebrates (Plate 3.4) and they produced unpleasant odours.

Table 3.7: Viable *Globodera pallida* eggs g^{-1} soil in field Experiment-3 for different treatments with *Brassica juncea* or *Raphanus sativus* either left untreated or treated with metconazole (Caramba), or left untreated (fallow), assessed at different stages of the plant development (pre-planting (P_i), pre- and post-incorporation of plant residues and post-harvest of potatoes (P_f)). Different superscript letters indicate significant differences in mean viable eggs g^{-1} of soil between treatments according to Tukey's multiple range test (5% significant level)

	Treatments							
Time of assessment	**Untreated**	*B. juncea*	*R. sativus*	*B.jl*metconazole	*R.sl* metconazole	***P-values***	***SEMs***	***CV%***
Initial population (P_i)	15.4	16.9	12.5	23.2	19.9	0.338	4.4	46.7
Pre-incorporation	28^{b}	12^{ab}	9^{a}	10^{a}	11^{a}	0.011	0.2	20.3
Post-incorporation	22^{b}	9^{a}	5^{a}	5^{a}	12^{ab}	0.001	2.8	66.8
Final population (P_f)	66.5^{b}	27.4^{a}	31.3^{a}	25.9^{a}	32.5^{a}	< 0.001	10.15	27.7

Table 3.8: Fresh weight g m^{-2} in Experiment-3 for *Brassica juncea* and *Raphanus sativus* either left untreated or treated with metconazole (Caramba®) assessed pre-incorporation

Treatments	*B. juncea*		*R. sativus*		*Bj* / metconazole		*Rs* / metconazole		***P-value***		***SEMs***		***CV%***	
	Shoot	roots	Shoot	roots	Shoot	roots	Shoot	roots	Shoot	roots	Shoot	roots	Shoot	roots
Fresh weight (g m^{-2})	97.8^{a}	11.1^{A}	232.5^{b}	29.6^{B}	100.0^{a}	11.5^{A}	241.8^{b}	31.4^{B}	< 0.001	0.006	23.2	4.7	33.4	55.5

Table 3.9: Potato crop yield assessment (tonnes/ha) in Experiment-3 plots previously treated with *Brassica juncea* or *Raphanus sativus* either left untreated or treated with metconazole (Caramba®) or untreated fallow as control. The potato sizes were graded into ≤45 mm (non-marketable size), 45≤65 mm and 65≤85 mm (marketable sizes)

	Treatments							
Size range	**Untreated**	*B. juncea*	*R. satuvus*	*B.jl*metconazole	*R.sl* metconazole	***P-values***	***SEMs***	***CV%***
≤45 mm (discarded)	7.10	5.34	7.10	5.06	4.9	0.26	0.1	38.2
45mm ≤ 65mm	60.07	53.58	60.96	56.53	60.75	0.539	0.6	17.8
65mm ≤ 85mm	9.14	18.00	18.00	12.09	15.12	0.628	0.674	80.2
Total marketable yield	**75.6**	**75.5**	**87.3**	**74.9**	**85.1**	**0.422**	**5.9**	**18.2**

3.7.4.2 Effect of soil incorporation of brassicaceous residues on the viability of *G. pallida* eggs

Six weeks after incorporation of brassicaceous residues there were significant reductions (*P = 0.001*) in the number of viable eggs g^{-1} soil in all experimental plots as compared with untreated fallow except for *R. sativus* plots that were treated with metconazole (Table 3.7, post-incorporation). However, the final population density of *G. pallida* post-harvest of potatoes was significantly lower (*P < 0.001*) for both *B. juncea* and *R. sativus* either untreated or treated with metconazole when compared with the fallow plots (Table 3.7, P_f). The rate of *G. pallida* multiplication was significantly lower (*P = 0.018*, Pf/Pi = 0.89) for treatments with *B. juncea* when compared with untreated fallow plots.

3.7.4.3 Effect of metconazole application on the biomass of brassica crops

As discussed previously, biomass production by the brassica crops was better when cultivated during the summer season (Plate 3.5). Experiment-3 had adequate moisture conditions throughout the brassica crop development period (average soil moisture of 23% v/v of field capacity). The application of sulphur 'N' fertiliser appeared to have contributed to biomass production (Plate 3.5 and Table 3.8).

The application of metconazole resulted in a non-significant increase in the biomass of *B. juncea and R. sativus* by 3.9% and 1.0% respectively.

3.7.4.4 Effect of brassicaceous green manure on the potato crop yield

The potato crop yield assessed post defoliation of the canopy did not differ significantly (*P > 0.05*) between all brassicaceous green manure plants treatments and untreated plots. However, plots treated with *R. sativus* either untreated or treated with metconazole (Caramba®) produced 11.7 and 9.5 tonnes ha^{-1} respectively more

marketable potatoes than the untreated plots (Table 3.9). Yield in *B. juncea* treated plots was similar to that obtained in the untreated plots.

Plate 3.4: *Raphanus sativus* root examination for infestation by larvae of *Delia radicum* (cabbage root fly) in field Experiment-3 just before incorporation of the residues in November 2013

Plate 3.5: *Brassica juncea* in field Experiment-3 just before incorporation in November 2013

3.8 Field Experiment-4: Cold tolerant attributes of selected *Brassica* species overwintered in 2012/2013 and their biofumigation potential on *G. pallida* encysted eggs

On the basis of the findings from Field Experiment-2, *Eruca sativa* 'Nemat' was selected due of its tolerance to harsh conditions and its rapid regeneration and flowering in early spring season. In addition, *B. juncea* 'Etamine' (brown mustard), and three *R. sativus* lines (Bento, Teranova, and Doublet; Chapter 2, Table 2.1), were also selected because they are marketed as 'winter-hardy' biofumigant varieties.

The principal objective of this experiment was to identify brassica varieties capable of tolerating harsh winter conditions whilst producing sufficient biomass/glucosinolates for incorporation during spring season to control PCN.

3.8.1 Site selection and experimental design

This experiment (Table 2.1) was conducted at Lynn, Newport, Shropshire, in the 2012/2013 cropping season. The soil at the site was a sandy loam soil (79% sand, 13% silt, 8% clay) with a pH of 6.63. The field had the same rotation sequence as in Experiment-2 (potato Maris Piper - wheat – oilseed rape – overwinter biofumigant mustard/radish mix – potatoes Maris Piper). Due to the short rotation sequence (one potato crop in three years), PCN population densities were moderate to high (ca. 28 – 161 eggs g^{-1} soil on average) and were predominantly *G. pallida* as the field had been cultivated with the potato cultivar Maris Piper in previous potato cropping years (Mr Mark Davies' personal communication).

An area within the field was selected due to previous sampling by the grower indicating high population densities of PCN. This area was intensively sampled to further characterise the PCN population density in order to determine the layout of

the experiment. Analysis of variance for the PCN samples from the allocated area revealed no significant difference in PCN distribution at 5% significance level using Tukey's multiple range tests. Although the analysis for soil characteristics (pH, organic matter content and particle size distribution) within the experiment was observed to be fairly uniform across the plots, the area was lying on a gentle slope. Therefore, it was necessary to block the experiment in the direction of the slope in a bid to control possible heterogeneity in nutrients amongst the *Brassica* species treatments (EPPO Standard PP 1/152). The *Brassica* species (Table 3.10) were therefore laid out in a randomised complete block design (6 treatments 5 replicates, Figure 3.9).

The experiment was established on 19 September 2012, chopped and incorporated on 30 April 2013 and was followed by the commercial potato crop cultivar Maris Piper on 3 May 2013. Plot sizes measured 9x9 m, to accommodate mechanical maceration/incorporation of the biofumigant crops and the laying of potato beds in the spring season of 2013. A 1 m buffer zone was left between plots to minimise the possibility of cross contact between treatments during maceration and incorporation.

The assessments undertaken in this field experiment were the same as those undertaken in Experiment-3, othere wise described where applicable. However, following DEFRA restrictions on the field application of manufactured nitrogen fertilisers during certain periods of the year (between 1 September to 15 January) (DEFRA, 2009), sulphur 'N' was avoided as the experiment was established on 19 September 2012 and sampled for PCN Pi. The plots were sampled for PCN viability estimates on 29 April 2013 and incorporated the following day as described in Chapter two, section 2.7. Following field incorporation, a commercial potato crop was established in this field on 7 May 2013 and soil samples were obtained for PCN

viability estimates post-incorporation 14 May 2013. The potato crop was harvested on 30 September 2013, after which the field was sampled for *G. pallida* final population density (P_f).

Table 3.10: Treatments in field Experiment-3 and their respective seed rates as recommended by the seed suppliers

Treatment	Variety	Seeding rates
Untreated fallow	---	---
Brassica juncea	Etamine	6.5 kg ha^{-1}
Eruca sativa	Nemat	8 kg ha^{-1}
Raphanus sativus	Bento	20 kg ha^{-1}
Raphanus sativus	Teranova	20 kg ha^{-1}
Raphanus sativus	Doublet	20 kg ha^{-1}

Figure 3.9: Initial PCN distribution in field Experiment-4. Different colours represent the different treatments (see key) while the numbers represent the PCN eggs g^{-1} soil assessed prior to the establishment of the experiment

3.8.2 Statistical analysis

A general analysis of variance (ANOVA) was performed for the data collected using GenStat® (15th Edition) statistical software. An analysis of repeated measurements was undertaken for different time points for changes in viable *G. pallida* eggs g^{-1} of soil. Where necessary, data were $\log_{10}$-transformed to normalise residuals.

Significant differences between treatments were determined using Tukey's multiple range tests (5% significance level).

3.8.3 Results

3.8.3.1 Effect of growing and incorporating brassicaceous plants on the viability of *G. pallida* eggs

The initial PCN population densities (Pi) were similar among plots within the experiment (Table 3.11) with percentage viability in the range of ≈ 80 - 95%. At the second sampling point prior to *Brassica* incorporation, the viability of PCN was similar between plots for all treatments and the untreated control. However, PCN viability assessed at two weeks post-incorporation of the brassicaceous crop residues was significantly lower ($P = 0.054$) in plots sown with *B. juncea* cv Etamine when compared to plots treated with *R. sativus* 'Teranova', but not with the rest of the treatments and control plots (Table 3.11). After the final assessment conducted post-harvest of the commercial potato crop, there was no difference in PCN viability between any of the treatments and control.

A general analysis of variance using repeated measurements surprisingly revealed a general decline in PCN viability with time for all plots in this experiment. The general decline in PCN egg viability with time was highly significant ($P < 0.001$), but there was no interaction effect between treatments and time. Some of the eggs observed under a binocular microscope had structures similar to fungal hyphae attached to them (Plate 3.6) and these require further investigation.

3.8.3.2 Effect of overwintering on biomass production by the *Brassica* species

A significant difference was found between the biomass of *E. sativa* cv Nemat and *B. juncea* cv Etamine but not between the other *Brassica* species (Table 3.12). Although all the *Brassica* species survived the mild winter of 2012/2013 as immature plants

(Plate 3.7), the post-winter development was slow. The slow development of the crops meant that the biomass available at time of incorporation was very low (Table 3.12) when compared with the biomass obtained in previous experiments when the crops were cultivated during the summer season. At time of incorporation, only *E. sativa* was observed to be flowering while the rest were still at the 5 to 8 leaf stage (Plate 3.8).

3.8.3.3 Potato crop yield following treatments with overwintered brassica species

There was no significant difference ($P > 0.05$) in potato crop yield between treatments (Table 3.13). However, *E. sativa* cv Nemat treated plots produced a relatively low yield when compared with yield in the untreated plots (4.9 tonnes/ha lower, Table 3.13). In this field, there was a high incidence of *Streptomyces scabiei* (common scab) which affected the quality of the potatoes irrespective of the treatments or fallow (Plate 3.9).

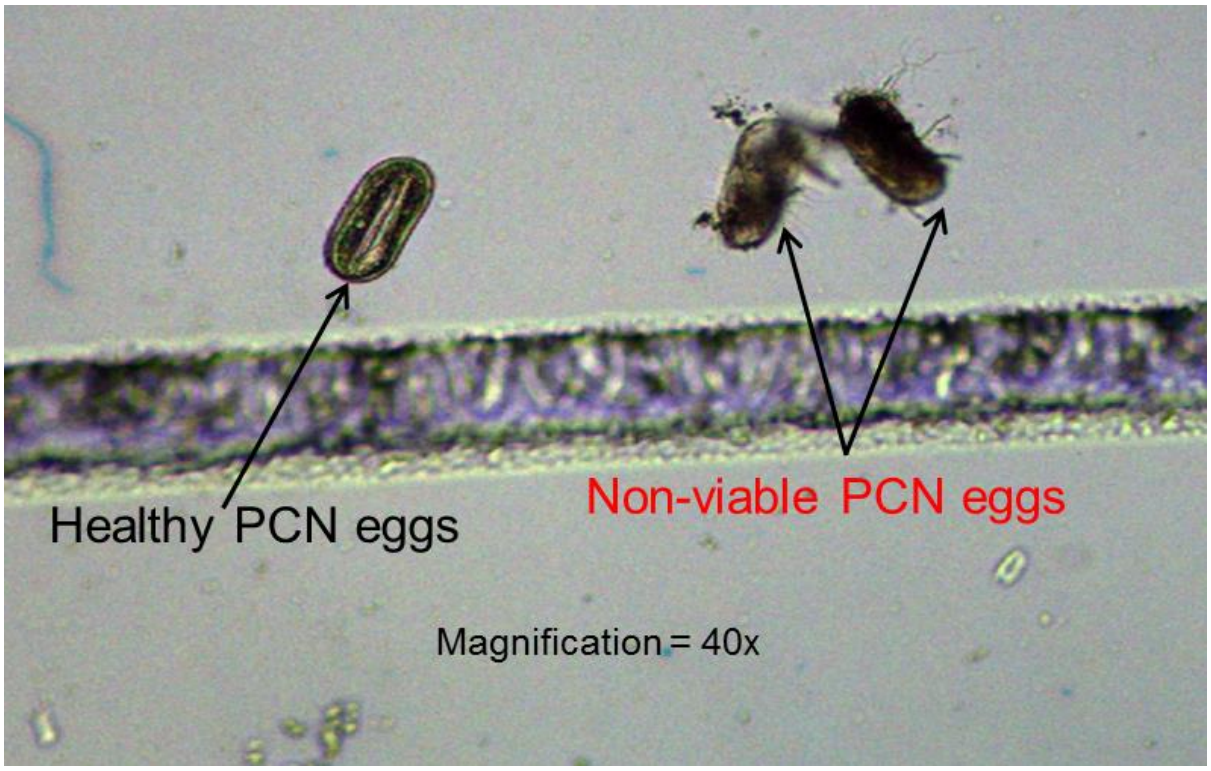


Plate 3.6: Observation of PCN eggs under the binocular microscope (magnification = 40x) for viability in samples collected from Field Experiment-4 in 2013

Table 3.11: Viable *Globodera pallida* eggs g^{-1} of soil recorded in field plots with brassica species (*Brassica juncea* cv Etamine, *Raphanus sativus* cv Bento, *Eruca sativa* cv Nemat, *R. sativus* cv Teranova, *R. sativus* cv Doublet), or left fallow as a control, assessed at different time points;- pre-planting (P_i), pre- and post-incorporation of plant residues and post-harvest of potato crop (P_f) in Field Experiment-4. Different superscript letters indicate significant differences in *G. pallida* viable eggs g^{-1} of soil between treatments according to Tukey's multiple range test (5% significance level)

Time of assessment	Treatments						*P-value*	*SEM*	*CV%*
	Untreated	Etamine	Bento	Nemat	Teranova	Doublet			
Initial population (P_i) PP*	81.2	95.5	79.4	68	63.7	75.2	0.724	14.9	42.8
Pre-incorporation	64.81	43.48	51.72	32.32	53.34	37.38	0.186	9.22	43.7
Post-incorporation	41.15^{ab}	29.43^{a}	34.27^{ab}	42.21^{ab}	48.14^{b}	41.08^{ab}	0.054	4.0	23.3
Final population (P_f)	25.9	28.7	28.2	29.2	29.0	26.8	0.99	6.4	51.1

*PP = pre-planting of brassicas

Table 3.12: Fresh weight (kg m^{-2}) of *Brassica juncea* cv Etamine, *Raphanus sativus* cv's (Bento, Teranova or Doublet respectively) or *Eruca sativa* cv Nemat assessed prior to soil incorporation in Experiment-4. Different superscript letters indicate significant differences in the incorporated fresh weight (kg m^{-2}) between treatments according to Tukey's multiple range test (5% significance level).

Treatment	Etamine	Bento	Nemat	Teranova	Doublet	*P-value*	*SEM*	*CV%*
Biomass (kg m^{-2})	1.7^{a}	2.2^{ab}	3.7^{b}	2.5^{ab}	2.4^{ab}	0.019	0.4	34.0

Table 3.13: Potato crop yield assessment (tonnes/ha) in plots previously treated with *Brassica juncea* cv Etamine, *Raphanus sativus* cv's (Bento, Teranova or Doublet respectively) or *Eruca sativa* cv Nemat in Experiment-4. The potato sizes were graded into ≤ 45 mm (non-marketable size), 45 ≤ 65 mm and 65 ≤ 85 mm (marketable sizes).

Size range	Treatments						*P-value*	*SEM*	*CV%*
	Untreated	Etamine	Bento	Nemat	Teranova	Doublet			
≤45mm (discarded)	4.2	5.9	4.8	4.8	5.3	6.5	0.597	1.0	40.6
45mm≤65mm	36.4	44.5	42.1	37.4	41.3	41.5	0.828	4.7	26.1
65mm≤85mm	20.1	12.3	17.6	13.4	12.7	16.3	0.788	4.5	64.8
Total marketable yield	**60.7**	**62.7**	**65.5**	**55.8**	**59.4**	**64.3**	**0.901**	**5.9**	**21.6**

Plate 3.7: Overwintered brassica species in Field Experiment-4 in February 2013.

Plate 3.8: *Eruca sativa* cv Nemat and *Brassica juncea* cv Etamine in Field Experiment-4 prior to soil incorporation

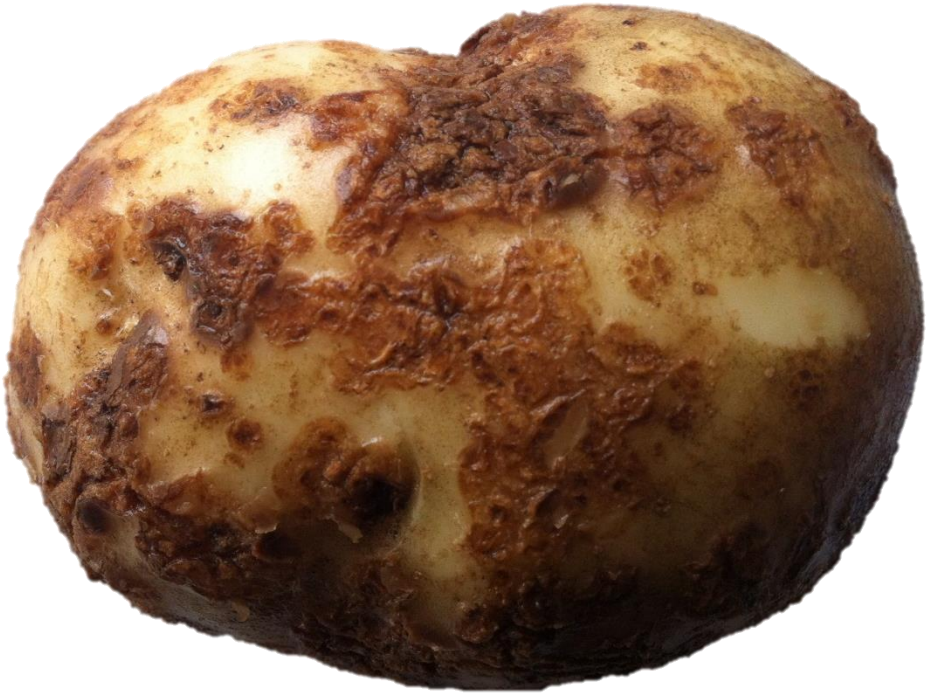


Plate 3.9: Infection of potato tuber by *Streptomyces scabiei* (common scab) as observed in Field Experiment-4 during harvest in 2013

3.9 Discussion

Four experiments conducted between 2011 and 2013 investigated the control of field populations of *G. pallida* following growing and incorporation of different *Brassica* species during different seasons of the year. Experiments 1 and 3 were based on summer cultivation followed by autumn incorporation of the tested species, whereas experiments 2 and 4 involved overwintered *Brassica* species followed by incorporation in the following spring. In addition to summer cultivation, Experiment-3 also evaluated the effect of metconazole on biomass and GSL production by the *Brassica* species and their effect on PCN. Summer cultivated *B. juncea* and *R. sativus* demonstrated their biofumigation potential against *Globodera pallida* encysted eggs in field plots. These species showed consistency in their ability to manage the population of *G. pallida* under field conditions when cultivated during summer and allowed to attain their maximum biomass production potential. When

overwintered, the *Brassica* species produced less biomass and thus, had no significant effect on field population of *G. pallida.*

These findings have not previously been reported elsewhere. The closest report to the findings reported herein is that of Valdes *et al*., (2012) who investigated the biofumigation potential of brassicas against *G. rostochiensis*. However, these authors did not allow their brassicas to attain their full biomass production potential prior to incorporation as they reported that just a few of the plants were flowering at time of incorporation. It would have been useful to compare their results with the summer cultivated brassicas which demonstrated significant effects on PCN. As demonstrated by Malik *et al*. (2010), *R. sativus*, is known to produce maximum levels of glucosinolates at the 50% flowering stage. This means that incorporation of the biofumigant crop at this stage would release a relatively high level of the corresponding ITC's. The results obtained by Valdes *et al*. (2011) are a useful comparison to our overwintered crops as reported for Experiments-2 and -4 of this study.

During biofumigant crop development, *R. sativus* consistently reduced the viability of *G. pallida* encysted eggs for both the summer and the winter cultivations, thus, confirming its potential in rotation to manage *G. pallida* in potato production. In Experiment-1 cultivated during the summer season, *B. juncea* and *E. sativa* also caused a significant reduction in the viability of *G. pallida* encysted eggs during crop development. However, when overwintered in experiments 2 and 4, these two plants did not differ statistically in their effect on the viability of *G. pallida* encysted eggs compared with fallow plots during crop development. The reduction in *G. pallida* viability obtained with *R. sativus* during its developmental phase may be attributed to the higher underground biomass as well as the high concentration of aromatic GSL in

root tissues of this plant. Unlike *B. juncea* and *E. sativa*, *R. sativus* produced a large root biomass, which was found to contain a high concentration of the aromatic GSL, gluconasturtiin (2-phenylethyl-GSL) (Chapter 4), which is hydrolysed enzymatically to produce 2-phenylethyl –ITC.

The ability of biofumigant crops to produce ITC's during crop development has been reported previously (Tang & Takenaka, 1983; Choesin & Boeener, 1991; Yamane *et al*., 1992). However, the quantities produced are often cited as being too small to induce significant reduction in soil-borne pathogens (Tang & Takenaka, 1983; Choesin & Boeener, 1991; Watt *et al*., 2006). Continuous release of GSL by roots of oilseed rape into the rhizosphere during cell turnover has been demonstrated (McCully *et al*., 2008). However, myrosin cells containing thioglucoside glucohydrolase (myrosinase) are found at deeper sites in the root, thus, the release of this enzyme into the soil for the hydrolysis of GSL may be unlikely. Therefore, the presence of ITC in the rhizosphere of brassicaceous crops detected in previous studies may be due to activity of myrosinase-producing soil microbes such as *Aspergillus* spp (Borek *et al*., 1996; Gimsing *et al*., 2007). *Raphanus sativus* roots in summer sown crops in this study were found to be severely damaged by the larvae of cabbage root fly. It is possible that the disruption of the root tissues by root feeding activated the enzymatic hydrolysis of glucosinolates which led to the release of toxic ITC's in the rhizosphere, resulting in toxicity to *G. pallida* encysted eggs. These findings are in line with those reported recently on the reduction of primary infections by *Rhizoctonia* root rot on sugar beet in field experimental plots where *B. juncea* was grown (Motisi *et al*., 2013). Additionally, ITC or other compounds released by brassicaceous plant roots may influence microbial communities present in the rhizosphere. A change in the community of microbes as such would affect the

pathogen populations in one way or another such as via competitiveness or antagonism (Rumberger & Marschner, 2004; Kirkegaard & Matthiessen, 2004). An increase in the population of nematode antagonists is detrimental to the nematode populations in the rhizosphere. Rumberger and Marschner (2004) demonstrated positive relationship between the structure of bacterial communities and the concentration of 2-phenylethyl-ITC in the rhizosphere of canola over time. Although the concentrations of these molecules may be low, their prolonged release over the plant growth period could induce a significant effect on soil borne pests observed in our study.

In Experiments-1 and -3, there were further reduction in *G. pallida* encysted egg viability observed when the residues of *B. juncea* and *R. sativus* were incorporated compared with untreated fallow. However, the incorporation of *E. sativa* residues did not statistically reduce *G. pallida* encysted egg viability when compared with the untreated plots despite producing the highest crop density/biomass. This may be due to the low concentrations of ITC-producing GSL present in *E. sativa* as demonstrated by results from the HPLC analysis (Chapter 4). The observed effect from *B. juncea* and *R. sativus* after soil incorporation with fresh tissue was probably due to the addition of ITC released from the enzymatic hydrolysis of glucosinolates from the crushed and incorporated plant tissue. This is further supported by the close correlation between the percentage mortality of *G. pallida* and the concentration of GSL observed (Chapter 4). *Brassica juncea* foliage was found to produce predominantly 2-propenyl GSL (sinigrin), whereas *R. sativus* foliage produced predominantly 4-methylsulfinylbutyl GSL (glucoraphanin) (Chapter 4). These two aliphatic GSL, when hydrolysed, yield predominantly ITC at pH 6-8 which are

implicated with the toxicity of biofumigant *Brassica* spp. (Borek *et a*l., 1994; Gimsing & Kirkegaard, 2006).

The volatile ITC released when mustard residues are incorporated into the soil are well known to reduce both the viability of potato cyst nematode (PCN) encysted eggs as well as the mortality of PCN hatched juveniles (Pinto *et al*., 1998; Serra *et al*, 2002; Buskov *et al.*, 2002; Aires *et al*., 2009; Lord *et al* 2011). This suggests that the crushing and incorporation of selected brassicaceous crop residues containing active ITC-producing GSL into the soil is an important component for successful PCN control under field conditions.

Nevertheless, there are inconsistencies associated with the level of pest or pathogen suppression achieved with brassicaceous green manures. Some researchers have reported moderate to high levels of soil-borne pathogen control following soil incorporation of brassicaceous residues. For instance, Rahman and Somers, (2005) on the root knot nematode *M. javanica*, Motisi *et al*. (2009; & 2010) on root rot of sugar beet, Lord *et al.* (2011) on the PCN sp. *G. pallida*. By contrast, other researchers have shown little or no effects on target organisms, as was also the case in Experiment-2 and -4 of our study, and in some cases there have been stimulatory effects (Johnson *et al*., 1992; Stephens e*t al*., 1999; Friberg *et al*., 2009; Valdes *et al*., 2011; Valdes *et al*., 2012; Vervoort *et al*., 2014). These inconsistencies can be addressed and improved upon through a comprehensive understanding of important factors that influence biofumigation process and this will be discussed in Chapter 4.

The potential of the tested *Brassica* species to contribute to the integrated management of PCN are evident from the results obtained during our experiments. Crops grown during summer had higher concentrations of GSL than those grown through the winter season. This is in line with previous reports by Rosa *et al*. (1996)

for Portuguese kale as well as kale varieties grown in north-western Spain in different planting seasons (Cartea *et al*., 2008). The soil pH in our experimental plots was approximately 6.3±0.3, which falls within the optimum pH range for ITC production as demonstrated in Borek *et al*. (1994). The soil bulk density and moisture content was fairly uniform across the field after incorporation of brassicaceous residues.

The two brassicaceous species (*B. juncea* and *R. sativus*) that proved to be effective biofumigant varieties could act as a sustainable option for PCN management under field conditions if included in an integrated PCN management strategy. Summer cultivation, thorough shredding at mid-flowering under adequate soil-moisture conditions (17 - 25% of field capacity), while simultaneously incorporating and sealing to minimise soil porosity should be highly encouraged for maximum biofumigation benefits.

Previous work has demonstrated that metconazole was able to significantly increase rooting in a wide range of rapeseed varieties cultivated under different environments (Berry & Spink, 2009). This reported increase in rooting varied depending on the growth stage at which metconazole was applied as well as rate of application, with optimum rooting achieved with a 1.2 L ha^{-1} applied between early stem extensions and flowering (Berry & Spink, 2009). However, there are no previous studies investigating the effect of metconazole on GSL production. Our treatments used a single application of 1.2 L ha^{-1} metconazole at early flowering. It is possible that extended rates as well as different application timings could provide additional benefits to biofumigation. The closest reports to these findings are the reports of enhancement of GSL and the genes responsible for their biosynthesis following foliar application of jasmonic acid or methyl jasmonate on Brassicaceae *Arabidopsis* and cabbage (Mikkelsen *et al*., 2003; Mewis *et al*., 2005; Wielanek & Urbanek, 2006).

During the 2012/2013 cropping season, *R. sativus* treatments increased the commercial potato crop yield by approximately 16%, but yield in *B. juncea* treated plots was similar to fallow plots in Experiment-3. However, in Experiment-4 where potato crops were cultivated during the same cropping season as in Experiment-3, but with the brassicas overwintered prior to incorporation, potato yield was similar for all treatments except for *E. sativa* treated plots where the yields was 4.9 tonnes/ha lower than the fallow plots. The average potato crop yield obtained in Experiment-3 (treated with summer cultivated brassicas) produced approximately 18.9 tonnes higher yield than that in Experiment-4 (treated with autumn cultivated/overwintered brassicas). Therefore, the beneficial effects of biofumigation may not be limited to disease suppression, but also to an improved soil health and crop yield. Enhanced crop yield has also been demonstrated for cucumber in Oman (Deadman *et al*., 2006), chickpea in Ethiopia (Abera *et al*., 2011), *Asclepias tuberosa* in Kenya (Kagai *et al*., 2012), and strawberries in Slovenia (Koron *et al*., 2014). In addition to enhanced fruit production following biofumigation treatments, Koron *et al*. (2014) also reported minimal detrimental effect on strawberry root colonization by arbuscular mycorrhizal fungi (AMF) compared with fumigant treatments, thus demonstrating the soil health benefits of using biofumigation in sustainable strawberry production.

Although there was no observed difference in the PCN population between all brassica treatments and fallow post-harvest of the potato crop in Experiment-4, there was an observed general decline in the PCN population across this experiment from the initial PCN density to the final PCN density post potato crop. The soil at this site may be suppressive to PCN, and this supposition can be supported by our observations of fungal mycelium on the encysted eggs collected from this experiment and the general decline in PCN population during the course of experiment. The

parasitizing of plant-parasitic nematodes via the production of nematicidal metabolites is well documented (Mishra *et al*., 1987; Dicklow *et al*., 1993; Samac & Kindel, 2001; Sun *et al*., 2006; Ruanpanun *et al*., 2011). However, the observations made during our experiment were beyond the scope of the present study and not investigated further due to time factor.

During the present study, *Brassica juncea* proved to be an effective biofumigant crop for *G. pallida* management under field conditions. It had a marked effect on the viability of *G. pallida* during the crop developmental period which added to the suppression of *G. pallida* densities after the biofumigant crop was crushed and incorporated into the soil. Similarly, *R. sativus* effectively reduced the viability of *G. pallida* during the crop development and after incorporation into the soil. Treatment of *B. juncea* with metconazole increased the production of sinigrin in foliage and roots. The present study is the first to show that brassicaceous plants grown under field conditions are capable of reducing the viability of *G. pallida* encysted eggs during biofumigant crop development (partial biofumigation) and after incorporation (complete biofumigation). An attempt to understand the cause of the reduction in PCN viability during the period of brassica growth will be examined in Chapter 5. Analysis of the glucosinolate profiles in the tested brassicaceous green manure crops are presented in the following Chapter.

CHAPTER FOUR

4. Chapter 4: Analysis of glucosinolates using High-Performance Liquid Chromatography (HPLC)

4 Analysis and quantification of glucosinolates in brassica using high-performance liquid chromatography (HPLC)

4.1 Introduction

Glucosinolates (GSL) are a group of plant secondary metabolites characterised by sulphur bonds that are mainly produced by members of the Brassicaceae. As previously discussed (Chapter 1), the intact form of this group of plant secondary metabolites is not toxic, but are capable of releasing toxic products during catalytic hydrolysis by the endogenous enzyme myrosinase (thioglucoside glucohydrolase 3.2.3.1). More than 132 different GSLs have been identified within the Brassicaceae (Fahey *et al*., 2001; Agerbirk & Olsen, 2012) and their occurence within these plants can vary both qualitatively and quantitatively, even among cultivars of the same species (Kirkegaard & Sarwar, 1998; Padilla *et al*., 2007). Furthermore, substantial variation in the toxicity of products released during the enzymatic hydrolysis of different GSLs are well documented (Lazzari *et al*.,1993; Buskove *et al*., 2002; Sera *et al*., 2002; Zasada & Ferris, 2003; Lazzari *et al*., 2004; Lord *et al*., 2011). This implies that the different *Brassica* species are expected to vary substantially in their toxicity to pests and pathogens. This Chapter reports the analysis of GSL in the *Brassica* species used in the field experiments. The objectives were to identify and quantify the GSL profiles presents in the *Brassica* species described previously (Chapter 3), at incorporation and to correlate the GSL profiles for the different plants to their observed toxicity to *G. pallida* encysted eggs in commercial potato field.

The sampling, extraction, analysis and quantification of GSLs in intact freeze-dried plant material were performed using the procedure published in ISO 9167-01-1992 and the guide-lines published by Wathelet *et al*. (2004). The principles of this method are illustrated in Figure 4.1. Some of the steps in this procedure were modified to

maximise the separation of GSLs from freeze-dried vegetative plant parts and the modifications are detailed wherever applicable. The initial phase of the extraction process involved enzymatic deactivation with boiling methanol followed by purification using ion-exchange resin column. This was then followed by enzymatic desulfatation of intact GSLs as all GSLs are characterised by a strongly acidic sulphate group. The separation and identification of the desulfo-GSLs was achieved with the aid of a reverse phase gradient high performance liquid chromatography (RP-HPLC) analysis with a *UV* absorbance at 229 nm for detection of the desulfo-GSLs. Eluted desulfo-glucosinolates were identified according to their order of elution in the chromatography. The desulfo-GSLs were further quantified relative to appropriate IS and expressed in µmol g^{-1} of freeze-dried plant material. Application of relative response factors (RRF, Table 4.3) was necessary to correct for variation in *UV* absorbance between the detected desulfo-GSL and the IS.

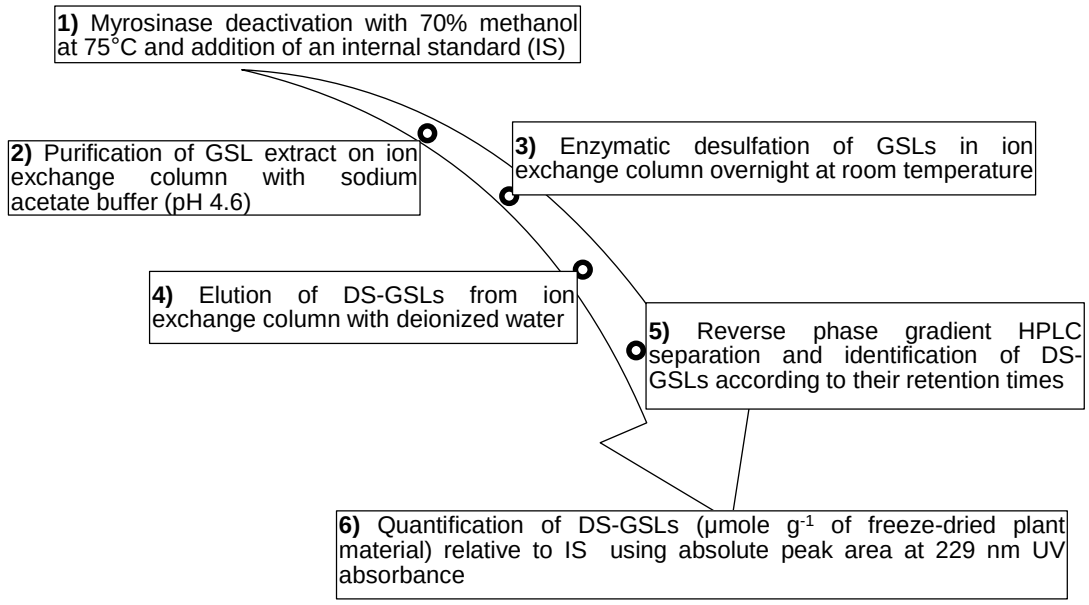


Figure 4.1: Flow chart illustration for glucosinolate (GSL) extraction, ion exchange column desulfatation and reverse phase gradient high performance liquid chromatographic (RP-HPLC) analysis and identification. Method based on ISO 9167-1-1992

4.1.1 The underlying mechanism for the glucosinolates desulfatation reaction

Glucosinolate desulfatation is generally achieved with the aid of sulfatase enzyme type *H-1* from *Hellix pomotia* (Aryl-sulfate sulfohydrolase) available commercially (SIGMA ALDRICH®, CAS Number 9001-45-0, Poole, UK). The sulfatase enzyme normally catalyses the hydrolysis of sulphate esters of a wide variety of aromatic compounds. The desulfatation reaction can be demonstrated by an equilibrium reaction as illustrated in Figure 4.2. The recovery of the desulfated GSLs is highly affected by different experimental parameters. In a bid to optimize GSLs desulfatation prior to HPLC, Quinsac and Ribaillier (1987) noticed that the ratio of the peak area of the IS to that of the desulfo-glucosinolate vary according to the incubation period. More so, the time at which individual GSLs attain equilibrium during the desulfatation reaction is determined by the nature of the glucosinolate. A typical example was demonstrated by Fiebig, (1991) with benzyl glucosinolate which showed a desulfatation rate approximately two-folds that of 2-propenyl glucosinolate using sulfatase enzyme from *Helix pomotia*. This implies that the total desulfo-glucosinolate calculated with reference to benzyl glucosinolate as an internal standard would be greater than that calculated with reference to 2-propenyl glucosinolate as an IS. Therefore, all experiments investigating the profiles of desulfo-glucosinolates should be carefully designed to ensure efficiency, reproducibility and adequate time for enzymatic desulfatation.

Desulfatation of GSLs is normally achieved on an ion exchange resin as this resin has the ability to eliminate unwanted hydrophilic materials in the extract which would otherwise, be eluted with the desulfated GSL, and thus, would interfere with the analytical method. It is necessary to appropriately rinse the ion exchange resin with a buffer to remove unbound compounds prior to sulfatase enzyme application.

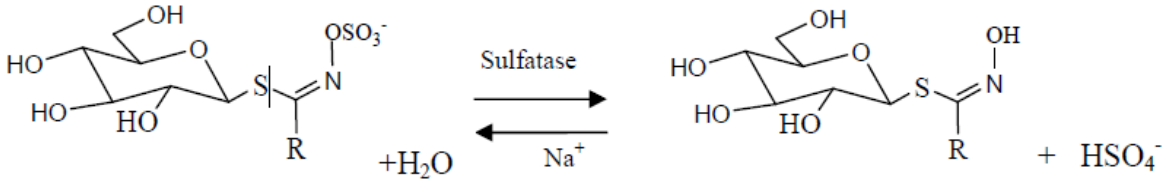


Figure 4.2: General structures of glucosinolate (left) and desulfo-glucosinolate (right) in equilibrium, catalysed by sulfatase enzyme type *H-1* from *Helix pomotia*

4.2 Aims

The aim of this work was to quantify the glucosinolate profiles in the *Brassica* species used in the field experiments described in Chapter Three, in a bid to account for any variation between species and planting seasons.

4.3 Objectives

i. Develop an experimental protocol for the extraction and analysis of desulfo-glucosinolates from the tested brassicaceous green manure plant species

ii. Develop an HPLC method to analyse and separate individual desulfo-glucosinolates

iii. Identify individual desulfo-glucosinolates from the tested brassicaceous green manure plant species with reference to available standards

iv. Quantify the individual desulfo-glucosinolates from the chromatogram based on absolute peak area using *UV* absorbance at 229 nm and relative response factors

4.4 Materials and methods

4.4.1 Reagents

All reagents used (Table 4.1) were of HPLC grade. Glucotropaeolin (benzyl glucosinolate) isolated from *Lepidium sativum* (cress) was used as internal standard two (IS-2) for the quantification of individual glucosinolates in freeze-dried *Brassica juncea* (Indian mustard) plant samples as it is naturally absent in this plant, whereas

sinigrin (2-propenyl glucosinolate) was used as IS-1 for *Raphanus sativus* and *Eruca sativa* as they naturally do not contain this glucosinolate.

Table 4.1: List of reagents used for the extraction and analysis of GSLs and their respective suppliers

Products	Supplier
Glucotropaeolin (from cress seeds) Benzyl glucosinolate, H2O, K+ salt	Wilkinson, Worksop, UK
Sinigrin monohydrate	C_2 Bioengineering, Karlslunde, Denmark
2-propenyl glucosinolates	Sigma Aldrich, Poole, UK
2M Acetic Acid	Sigma Aldrich, Poole, UK
Ethylene Diamine	Sigma Aldrich, Poole, UK
Formic Acid	Sigma Aldrich, Poole, UK
Imidazole	Sigma Aldrich, Poole, UK
Sulfotase type *H-1* from *Helix pomotia* (activity of 10KU)	Sigma Aldrich, Poole, UK
Acetonitrile	Sigma Aldrich, Poole, UK
Absolut Methanol	Sigma Aldrich, Poole, UK
Sephadex A-25	Sigma Aldrich, Poole, UK
Sephadex C-25	Sigma Aldrich, Poole, UK

4.4.2 Brassica plant material

The *Brassica* species used for the analysis were sampled from the field experiments prior to incorporation (see Chapters 2 & 3). A list of these brassicaceous green manure plant species and their respective suppliers as well as their seeding rates have been previously described in Chapter 2.

4.4.3 Brassica plant sampling and processing

Fresh *Brassica* samples were collected and prepared according to the method described by Wathelet *et al*. (2004) with some minor modifications. Three whole plants were randomly collected from each field experimental plot just before the incorporation of the brassica plant residues and quickly taken to the laboratory for processing. Whole plant samples were collected with the aid of a garden fork on a cool day (approximately 8±2°C) and quickly taken to the laboratory in ventilated plastic containers 555 x 355 x 167 mm internal diameter (Plastic Mouldings Northern

Ltd., Durham, UK). Preliminary HPLC analysis of plant samples in triplicate showed no significant difference in GSL concentration between plant samples collected and transported as described above and those preserved in dry ice at -80°C prior to laboratory transportation for freeze-drying. While in the laboratory, impurities were carefully brushed from green tissue with a dry soft brush, and roots were then washed to eliminate soil, blotted with an absorbent tissue paper, carefully separated from foliage using a pair of scissors and placed into separate plastic bags.

Separated batch samples consisting of shoot or leaves from three randomly sampled plants for each plot were appropriately labelled, weighed and flash-frozen in liquid nitrogen and stored at -80°C prior to freeze-drying (Wathelet *et al*., 2004). Frozen batch samples were freeze-dried (GVD6/13 MKI freeze dryer, GIROVAC Ltd, North Walsham, UK) for one week, re-weighed, milled to fine powder in a micro-grinder (Retsch GmbH Cyclone Mill-Twister, Haan, Germany) and stored below -18°C in screw cap tubes prior to glucosinolate extraction and analysis.

4.4.4 Extraction of glucosinolates from plant tissues

The extraction of glucosinolates from freeze-dried *Brassica* tissues was based on the combination of the method described in ISO 9167-1-1992 and the guidelines reported by Wathelet *et al*. (2004). An amount of 0.3±0.01 g of freeze-dried plant tissue was placed in 15 ml polypropylene tubes and extracted using 4 ml of 70% v/v methanol in a boiling water bath set at 75°C to deactivate the myrosinase enzyme. The tubes containing the freeze-dried samples were pre-heated to 75 ˚C for 1 min prior to methanol addition, and then maintained at 75 ˚C and gently agitated for 10 min before being allowed to cool. The tubes were then placed in a centrifuge at 5000 g for 10 min at 4˚C (Beckman Avanti^{TM} 30 High Speed Compact Centrifuge). Each sample was twice extracted and the supernatant was combined in a 15 ml

polypropylene tube while 1µmol (200µl from a 5mM stock solution) of internal standard (IS) was added. Following the addition of the IS, the combined extract was adjusted to 5 ml, securely capped, gently mixed and either stored below -18°C if not used immediately, or subjected to ion exchange purification and enzymatic desulfatation prior to HPLC analysis.

4.4.5 Purification and enzymatic desulfotation of intact glucosinolates

In order to obtain a pure extract of glucosinolates for HPLC analysis, it was necessary to ion-exchange purify the crude methanol extract and subject the extract to an enzymatic desulfotation to break the sulphur bonds.

4.4.5.1 Ion exchange resin column preparation

DEAE Sephadex A-25 (Sigma Aldrich®, UK) is known to be a weak anion exchanger, thus the ion exchange step was necessary to remove contaminating hydrophilic impurities that could interfere with detection and quantification as well as bind to intact glucosinolates. The anion exchange resin column was prepared by adding ca. 0.5 ml of DEAE-Sephadex A-25 resin suspension into a glass pipette lined at the constricted end with a glass wool plug. The column/resin was washed with 2 ml of 6 M imidazole formate and rinsed with 2x1 ml aliquots of deionised water before the methanol extract was gently added to the column. Following the addition of the extract, the column was allowed to drain before the sephadex matrix including the bound intact GSLs was equilibrated in sodium acetate buffer pH 4 in situ. A low pH was necessary to avoid the degradation of indole GSLs associated with higher pH (> 4).

4.4.5.2 Preparation and purification of Sulfotase solution

4.4.5.3 Preparation of Sephadex A-25 (Acetate form)

DEAE Sephadex A-25 (ca. 5g) was suspended in 75ml deionized water in a beaker, sealed with parafilm and stored overnight at 4°C. The settled Sephadex was then re-suspended by stirring and filtered through a Gooch crucible (porosity = 1) using a vacuum pump and Buchner flask to speed filtration.

Five hundred millilitres of 0.5 M sodium hydroxide (NaOH) was then poured through the Sephadex retained on the Gooch crucible and rinsed with 250 ml of deionized water followed by 100 ml of 0.5 M acetic acid. The sephadex was then transfered into a 100ml measuring cylinder containing 50ml deionized water. The suspension was allowed to settle for about 60 min while the volume of the supernatant liquid was adjusted with deionised water to twice as much as that of the settled Sephadex A-25. The cylinder was then sealed with parafilm and appropriately labelled. Sephadex A-25 resin could be stored at 0 ≤ 4°C and used for up to 2 weeks.

4.4.5.4 Preparation of Sephadex C-25 (Sodium form)

Powdered Sephadex C-25 (ca. 1 g) was suspended in 40ml deionized water in a 50ml measuring cylinder, sealed with parafilm and stored overnight at 4°C. The volume of the supernatant liquid was then adjusted with deionized water to twice as much as that of the settled Sephadex C-25. Sephadex C-25 resin was sealed with parafilm, labelled and stored refrigerated at 0 ≤ 4°C, thus could be used for up to 2 weeks.

4.4.5.5 Purification of Sulfotase

To reduce the concentration of ß- glucuronidase contaminants present in the commercial sulfatase, it require purification prior to use. Sulfotase enzyme type *H-1* from *Helix pomotia* with an activity of 10KU g^{-1} solid was commercially available and

was purchased from Sigma Aldrich®, Poole, UK. The purification and dilution of Sulfotase for glucosinolate desulfatation was done as described in ISO 9167-1-1992 with some modifications. Sulfotase (ca. 70 mg) was dissolved in 3 ml deionised water and mixed with 3 ml absolute methanol by gentle shaking (150 rpm for 3 min) in a 20 ml polypropylene screw cap tube. The suspension was centrifuged at 2000 rpm for 10 min at 4 ˚C (Beckman Avanti™ 30 High Speed Compact Centrifuge). The supernatant was transferred to new tubes and re-suspended in 9 ml absolute methanol and re-centrifuged at 2000 rpm for 10 min, at 4˚C. The precipitate from the second centrifugation was dissolved in 2 ml deionised water in a polypropylene tube.

A small portion of glass wool plug was placed at the constricted end of two glass pipettes and the pipettes were labelled ‘A’ and ‘C’. A suspension of Sephadex A-25 acetate was produced by shaking the container on a magnetic stirrer at 200 rpm and adding a 0.5 ml aliquot to Pipette ‘A’. This process was repeated for the glass pipette marked ‘C’ using Sephadex C-25. Both pipettes were allow to drain and the pipette marked ‘A’ was snaped 3cm above the neck. The small piece of glass was discarded and the broken end of pipette ‘A’ was inserted into the top of pipette ‘C’. A vial capable of holding 2ml was then place underneath the pipette ‘C’ and the 2ml of re-dissolved sulfatase was added to the top of pipette ‘A’. The purified product was then collected in the vial, divided into smaller aliquots of 1.5 ml, sealed, labelled and stored below -18°C for use when required.

To purify and enzymatically desulfate the GSLs, the methanol extract (section 4.3.4) was homogenised by shaking and ca. 1 ml was gently transferred onto the Sephadex-resin in the ion exchange column without disturbing the resin surface before being allowed to drain. The extract was carefully washed with two 1 ml aliquots of 0.02 M sodium acetate buffer (pH 4.0), and the buffer was allowed to drain

after each addition. This step was done in order to remove non-charged molecules; GSL and anionic compounds are immediately bound in the top part of the column. The purified samples were then treated with ca. 75 µl of purified diluted sulfatase type *H-1* from *Helix pomotia*, and the columns were sealed with parafilm and incubated overnight at room temperature to allow for the desulfotation reaction to remove the sulphate group of the GSL. Following the desulfotation reaction, the desulfo-glucosinolates (DS-GSLs) were eluted with 3 x 0.33 ml aliquots of deionized water into HPLC vials, allowing the water to drain after each addition. The vials were then capped, labelled, gently mixed and loaded onto an auto-sampler for RP-HPLC analysis.

4.4.5.6 Preparation of internal standard 1 (IS-1)

Sinigrin monohydrate (Table 4.1) was used as an internal standard 1 (IS-1). To prepare a 5 mM IS-1 stock solution, ca. 207.7 mg of potassium allylglucosinolate monohydrate was dissolved in distilled de-ionised water (DDW) in a 100 ml one-mark volumetric flask, and the volume was adjusted to the mark with DDW. The stock solution could be kept below 4°C to be used within one week or below -18°C for a longer duration.

To check for the purity of IS-1, three 1 mL aliquots of the stock solution were ion-exchanged column purified and desulfated before HPLC analysis in quadruplets (Figure 4.3). Desulfated IS-1 solutions were considered as pure if the peak area of the IS-1 was greater than 98% of the total peak area. A 1 mL aliquot of the 5 mM IS-1 stock solution accounted for a peak area of 5000±250 milli-absorbance units (mAU) upon HPLC analysis and detection at a *UV* absorbance of 229 nm with a column temperature of 30°C at 1.5 mL min^{-1}.

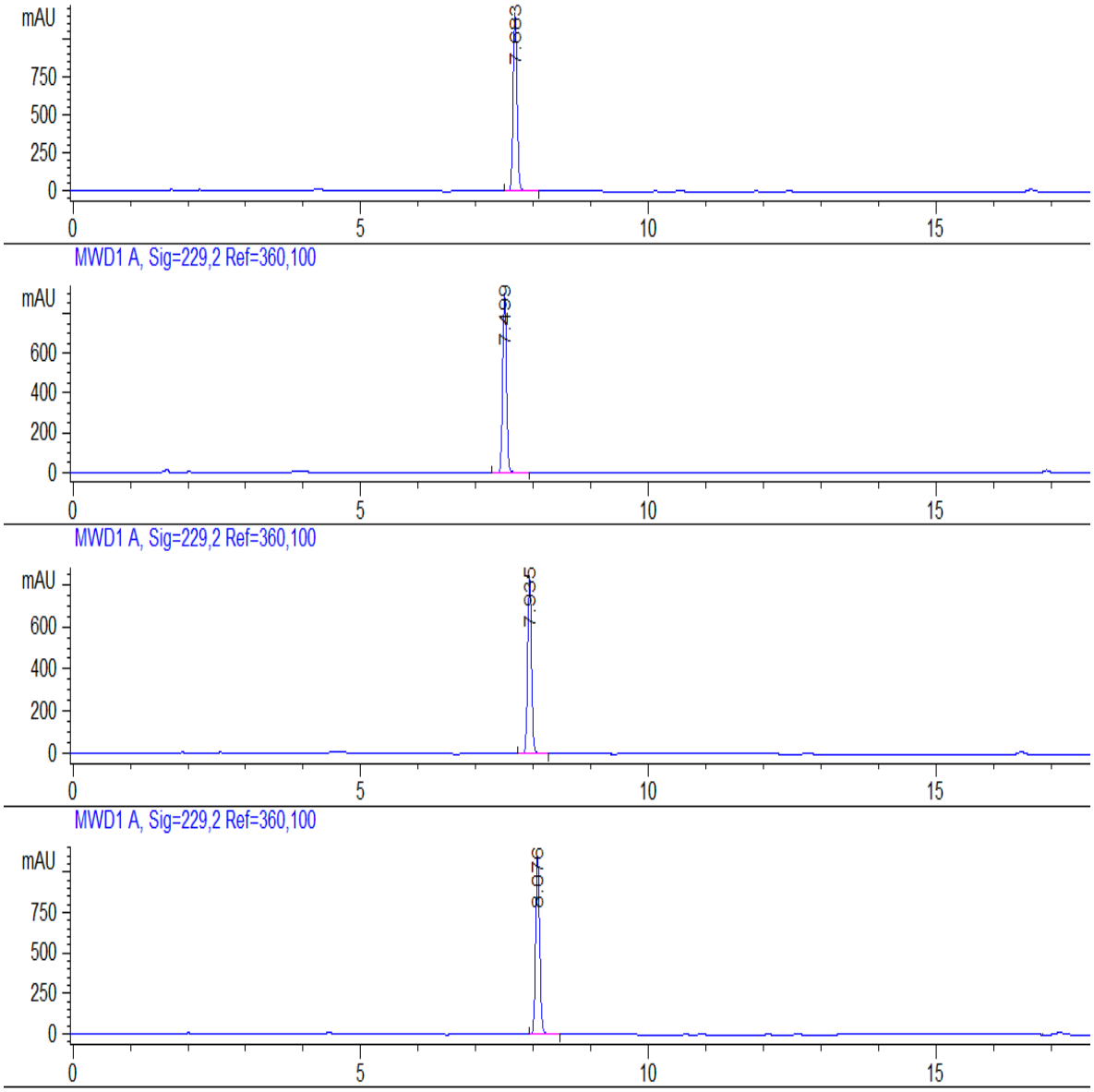


Figure 4.3: Chromatograms of a 1 mL aliquot of sinigrin 5 mM concentration used as internal standard one (IS-1) at a *UV* absorbance of 229 nm with a column temperature of 30°C and flow rate of 1.5 mL min^{-1}. The x-axis represents retention time (min) and the y-axis represents the peak area measured in milli-absorbance units (mAU)

4.4.5.7 Preparation of internal standard 2 (IS-2)

Glucotropaeolin (Table 4.1) was used as internal standard 2 (IS-2) to analyse the GSL profile in *B. juncea* samples as this plant species naturally contains sinigrin (IS-1). Finely milled (Retsch GmbH Cyclone Mill-Twister, Haan, Germany) freeze-dried garden cress seed powder, ca. 20 g, was placed in a 1 L conical flask and pre-heated in a water bath (80°C) for 1 min. A boiling methanol-water solution (70:30% v/v) ca. 500 ml was added to the conical flask containing the powder and shaken at 150 rpm for 10 min in the water bath. The suspension was allowed to cool before

transferring in to 50 mL tubes and centrifuged at 3000 g for 10 min at 30°C (Bjerg & Sørensen, 1987). The supernatant liquid was re-centrifuged and filtered via a 110 mm (Ø) filter paper before the filtrate was freeze-dried to obtain a fine powder. The freeze-dried powder was stored refrigerated at 4°C and used as IS-2 when required.

Glucotropaeolin (IS-2) was prepared by dissolving ca. 250 mg of the freeze-dried powder in a 100 mL one-mark volumetric flask with DDW and making up the volume to the mark with DDW. Three 1 mL aliquots of the IS-2 were then subjected to an ion-exchange column purification/desulfatation and HPLC analysis in quadruplets with reference to IS-1 as the internal standard. The concentration of Glucotropaeolin (IS-2) stock solution (chromatogram on Figure 4.4) was then calculated using equation 4.1.

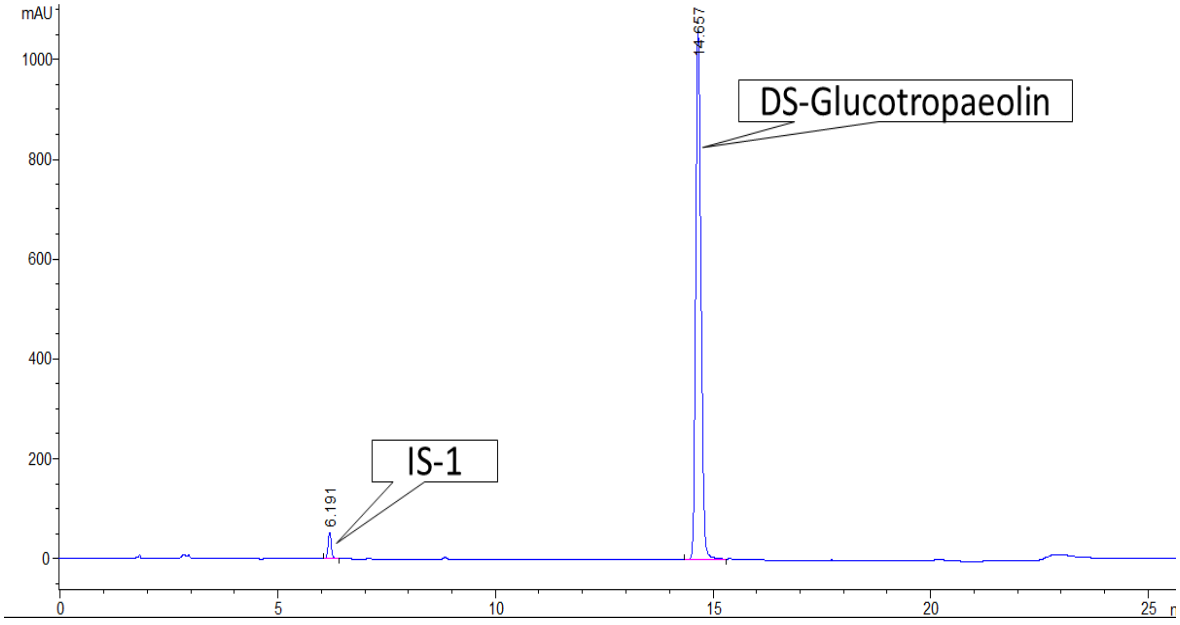


Figure 4.4: Chromatogram of a 1 mL Glucotropaeolin stock solution analysed with sinigrin as internal standard (IS-1)

4.4.5.8 Calibration of the HPLC column

A Reverse-phase gradient HPLC column Spherisop® RP-C_{18} ODS-2 (250 x 4.6 mm) purchased from Phenomenex®, Macclesfield, UK, with a particle size of 5 µm was used for the separation of DS-GSL. In order to calibrate the column for HPLC to

ensure accuracy/reproducibility, three 1 mL aliquots of IS-1 (sinigrin 5 mM) were ion-exchange column purified/desulfated and 10 μL of the eluent were auto-injected into the HPLC column in triplicate for the separation and detection as described under section 4.3.12. The flow rate of the HPLC pump was set at 0.9 mL min^{-1} with a *UV* absorbance at 229 nm and was monitored at a constant temperature of 30°C for each set of injections. The flow rate was then successively increased by 0.2 mL min^{-1} (Table 4.2) after each set of analysis until a stable retention time/peak area was obtained for the replicates. A flow rate of 1.5 mL min^{-1} was adopted and used throughout the analysis for experimental samples.

Table 4.2: Timetable for the reverse-phase gradient HPLC column calibration with acetonitrile-water (70:30% v/v) used as the solvent (Eluent B**)**. Different calibration flow rates are indicated as Cal-1 to Cal-5

Time	Eluent B%	Flow Rates (mL min^{-1})				
		Cal-1	Cal-2	Cal-3	Cal-4	Cal-5
0.0	0	0.9	1.1	1.3	1.4	1.5
18.0	20	0.9	1.1	1.3	1.4	1.5
18.1	30	0.9	1.1	1.3	1.4	1.5
19.1	30	0.9	1.1	1.3	1.4	1.5
20.0	0	0.9	1.1	1.3	1.4	1.5
26.0	0	0.9	1.1	1.3	1.4	1.5
28.0	0	0.9	1.1	1.3	1.4	1.5

4.4.5.9 Optimising the separation and detection of individual glucosinolates peaks

It was necessary to perform a quality control to ensure stability and reproducible for the chromatography prior to the analysis of each batch of samples. Consequently, this was achieved by an auto injection of a 10 μL aliquot of the relevant internal standard used for the said batch of samples previously ion-exchange column purified and desulfated from a 1 mL aliquot of the stock solution. This test was performed until a stable *UV* chromatogram for the relevant IS peak area was obtained at a stable retention time was obtained (6±0.5 min for IS-1 and 15±0.5 for IS-2).

Following the calibrations and quality assessments, an oilseed rape reference sample (with a high GSL profile) extracted, purified and desufated as described

previously, was analysed in triplicates to confirm reproducibility of the method via the chromatograms and retention time of the peaks (Figure 4.5). Two samples each of the brassicas collected from the field experiment were also analysed in triplicates using either IS-2 for *B. juncea* (Figure 4.6) or IS-1 for *R. sativus*, (Figure 4.7) and *E. sativa*. Identification of individual peaks was undertaken with reference to the oilseed rape reference chromatogram (Figure 4.5) and literature (Cataldi *et al.,* 2007).

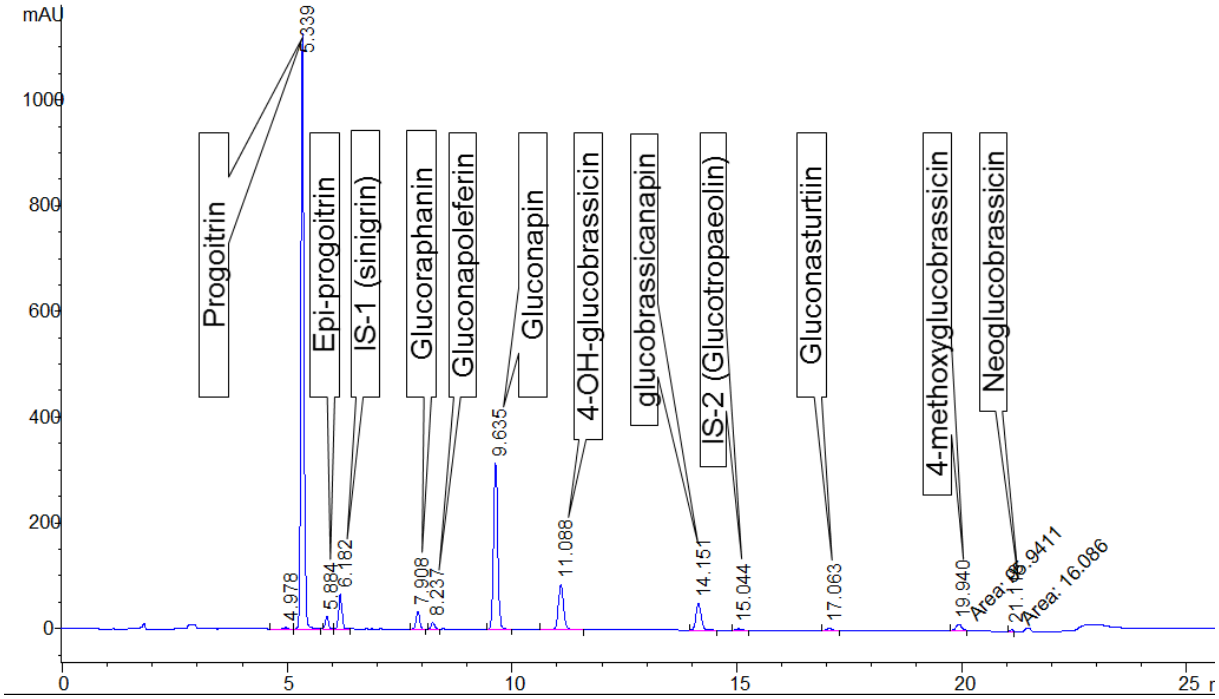


Figure 4.5: Chromatogram of a 1 mL extract prepared from an oilseed rape reference sample analysed using sinigrin as internal standard (IS-1)

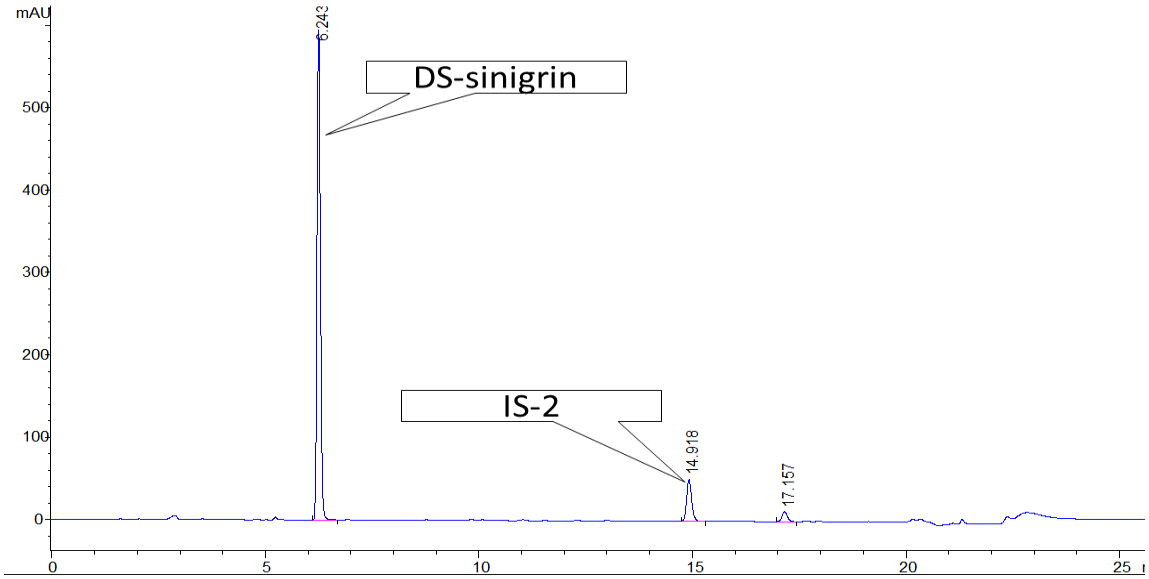


Figure 4.6: Chromatogram of a 1 mL extract prepared from freeze-dried *Brassica juncea* leaves sample analysed using glucotropaeolin as internal standard (IS-2)

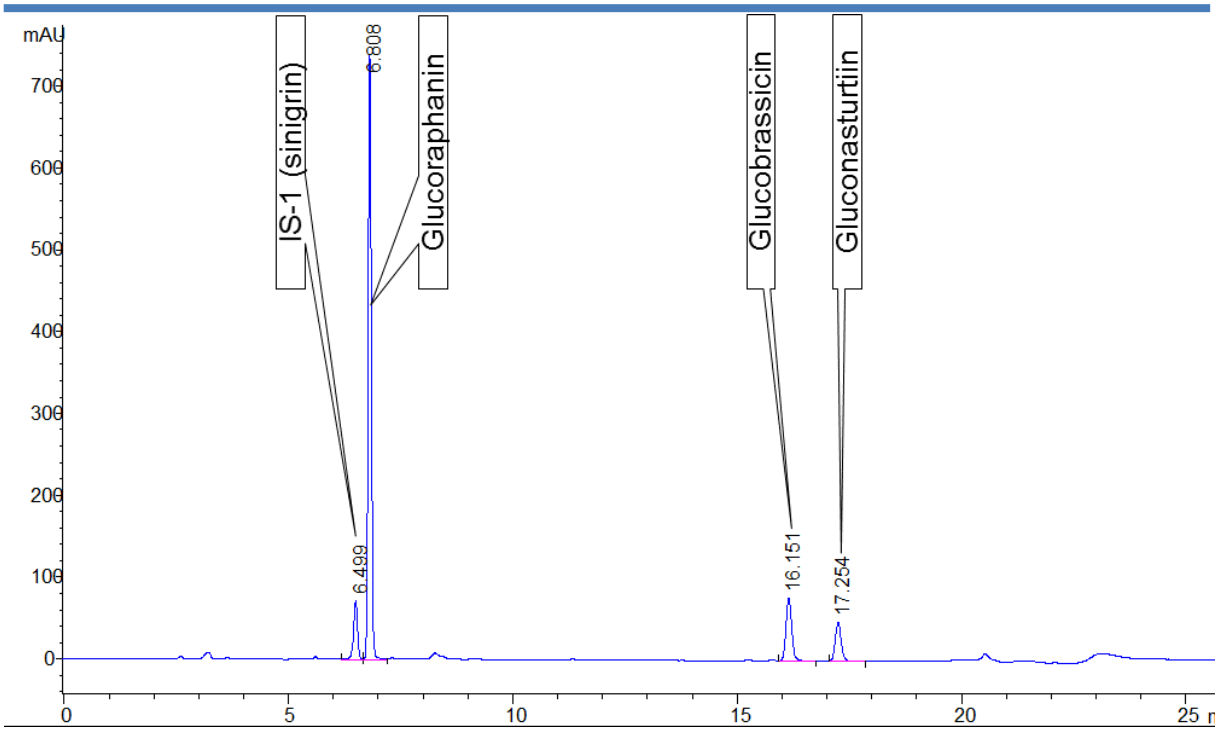


Figure 4.7: Chromatogram of a 1 mL extract prepared from freeze-dried *Raphanus sativus* leaves sample analysed using sinigrin as internal standard (IS-1)

4.4.5.10 HPLC separation and quantification of desulfo-glucosinolates

The HPLC apparatus (Agilent HPLC series 1100, Plate 4.1) equipped with a pump for binary elution gradient was used for the separation and the measurement of DS-GSL. The mobile phase consisted of Eluent-A (deionised water) and Eluent-B (acetonitrile/de-ionised water 70:30% v/v). The column temperature was regulated at 30°C throughout the analysis to avoid possible degradation of DS-GSL associated with higher column temperatures (ISO 9167-1-1992). For each analysis, an aliquot of 10 µl of each sample was auto-injected into the column. A linear gradient was then performed at a flow rate of 1.5 ml min^{-1} from 0-30% Eluent-B over a period of 18 min. The gradient was held at 30% Eluent-B for 1 min before returning to 0% Eluent-B for another 1 min. Following the analysis of each sample, an equilibrium was established over 6 min with a post-run time of 2 min (Table 4.3). The detection of desufated GSL (DS-GSL) was made at a *UV* absorbance of 229 nm. An illustration of the HPLC process is presented in Figure 4.8. All samples were replicated six times with each

replicate analysed in triplicate. Individual GSL concentrations were determined using equation 4.1 where:- *Ag* = peak area of the relative GSL, *As* = peak area of the internal standard (IS), *n* = amount (µmol) of the IS, *m* = mass (g) of the freeze-dried test sample and *RRF* = relative response factor of the GSL (Table 4.4).

$$GSL\ (conc.) = \frac{Ag}{As} \times \frac{n}{m} \times RRF \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4.1)$$

Table 4.3: Timetable for the reverse phase gradient HPLC separation of desulfated glucosinolates from brassica samples with acetonitrile-water (70:30% v/v) used as the solvent (Eluent-B).

Time	Eluent B%	Flow Rate (ml min^{-1})
0.0	0	1.5
18.0	20	1.5
18.1	30	1.5
19.1	30	1.5
20.0	0	1.5
26.0	0	1.5
28.0	0	1.5

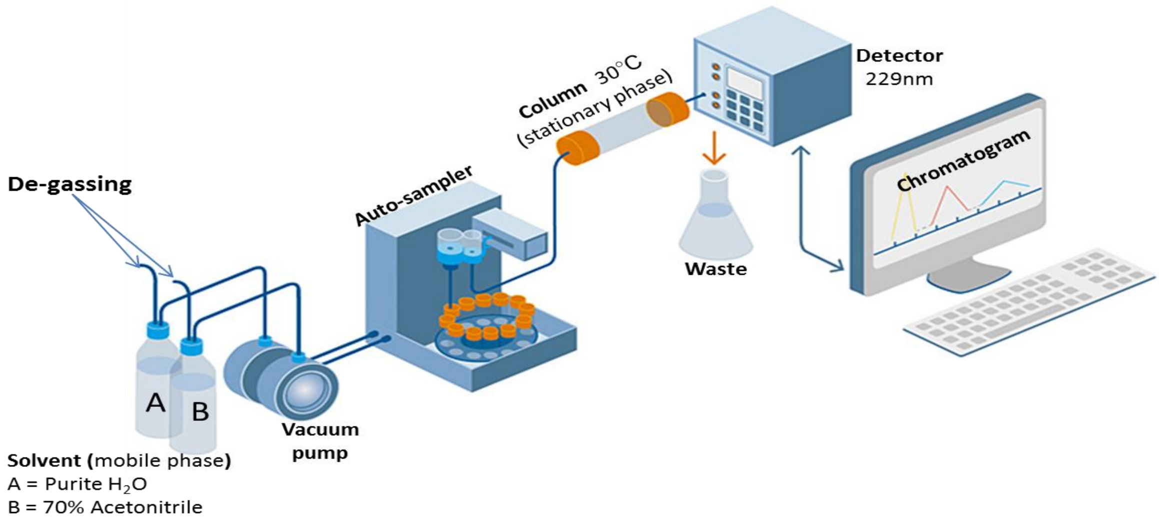


Figure 4.8: An illustration for the reverse phase high performance liquid chromatography (RP-HPLC) analysis of desulfo-glucosinolate from brassica plant extracts

Table 4.4: *UV* relative response factors (RRF) for desulfo-glucosinolates at 229 nm relative to the RRF of desulfo-sinigrin (Brown *et al*., 2003; Clarke, 2010)

Glucosinolate (abbreviation)	Side chain	RRF	Source
2-Propenyl	Sinigrin	1.00	*Iberis umbellata, Brassica juncea*
3-Butenyl	Gluconapin	1.00	*A. thaliana, Alyssum maritimum, B. napus*
Methyl	Glucocapparin	1.00	*Cleome spinosa*
1-Methylethyl	Glucoputranjivin	1.00	*Lunaria annua*
3-Hydroxypropyl (3OHP)	Glucoerysimumhieracifolium	2.10	*A. thaliana*
4-Hydroxybutyl (4OHB)	Glucoarabidopsithalianain	1.40	*A. thaliana*
3-Methylsulfinylpropyl (3MSOP)	Glucoiberin	1.20	*I. umbellata*
4-Methylsulfinylbutyl (4MSOB)	Glucoraphanin	0.90	*Erysimum allioni*
5-Methylsulfinylpentyl (5MSOP)	Glucoalyssin	0.90	*L. annua*
6-Methylsulfinylhexyl (6MSOH)	Glucohesperin	1.00	*Arabis perenans, L. annua*
8-Methylsulfinyloctyl (8MSOO)	Glucohirsutin	1.10	*A. thaliana*
3-Methylsulfonylpropyl	Glucocheirolin	0.90	*Cheiranthus cheiri*
4-Methylsulfinyl-3-butenyl	Glucoraphenin	0.90	*Matthiola incana*
3-Methylthiopropyl (3MTP)	Glucoibervirin	0.80	*I. umbellata*
4-Methylthiobutyl (4MTB)	Glucoerucin	0.90	*A. thaliana*
6-Methylthiohexyl (6MTH)	Glucosquerellin	1.00	*Alyssum maritimum, Arabis perenans*
7-Methylthioheptyl (7MTH)	Glucoarabishirsutain	1.00	*A. thaliana*
8-Methylthiooctyl (8MTO)	Glucoarabishirsuin	1.10	*A. thaliana*
Benzyl	Glucotropaeolin	0.80	*Tropaeolum majus, Lepidium sativum*
2-Phenylethyl (2PE)	Gluconasturtiin	1.00	*Nasturtium officinale*
3-Benzoyloxypropyl (3BzOP)	Glucomalcomiin	0.40	*A. thaliana*
4-Benzoyloxybutyl (4BzOB)	----	0.30	*A. thaliana*
4-Hydroxybenzyl	Sinalbin	0.40	*Sinapis alba*
Indol-3-ylmethyl (I3M)	Glucobrassicin	0.29	*Isatis tinctoria*
4-Methoxyindol-3-ylmethyl (4MOI3M)	4-Methoxyglucobrassicin	0.25	*Eruca sativa*
1-Methoxyindol-3-ylmethyl (1MOI3M)	Neoglucobrassicin	0.20	*E. sativa*
2-hydroxy-1-methylethyl	Glucosisymbrin	1.23	*Sisymbrium loesilii*
(2R)-2-hydroxy-3-butenyl	Progoitrin	1.09	*B. napus*
(2S)-2-hydroxy-3-butenyl	epi-Progoitrin	1.09	*Crambe abyssinica*
4-methylthio-3-butenyl	Glucoraphasatin	0.40	*Raphanus sativus*
2-hydroxy-2-methylpropyl	Glucoconringiin	1.00	*Conringia orientalis*
4-pentenyl	Glucobrassicanapin	1.15	*B. rapa*
(R)-2-hydroxy-3-pentenyl	Gluconapoleiferin	1.00	*E. sativa*
2-hydroxy-2-methylbutyl	Glucocleomin	1.07	*Conringia orientalis*
(R)-2-hydroxy-2-phenylethyl	Glucobarbarin	1.09	*Barbarea vulgaris*

Plate 4.1: Agilent HPLC series 1100 used for the analysis of desulfo-glucosinolates extracted from freeze-dried brassica plant extracts

4.5 Results

4.5.1 Effect of different cropping seasons on the concentration of total and individual GSL

There were differences in the concentration of GSL between the different cropping seasons. Summer sown brassica crops were found to contain a higher concentration of total GSL within the plant tissues than winter sown crops (Figure 4.9). In field experiment one (FE-1) cultivated during the summer season of 2011, there was no significant difference in the concentration of total GSL between the three tested brassicas (Figure 4.9A). However, for the overwinter brassicas in field experiment

two (FE-2), the total GSL concentration in *R. sativus* was significantly higher ($P < 0.001$) than that in *B. juncea* and *E. sativa,* which both did not differ from each other (Figure 4.9B).

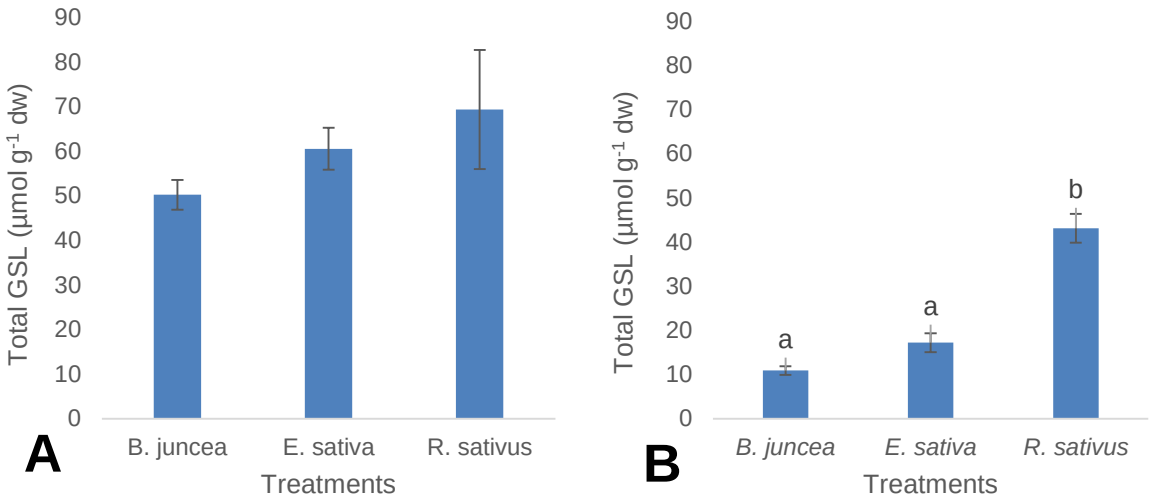


Figure 4.9: Total glucosinolate (GSL) concentrations in *Brassica juncea* cv Caliente, *Eruca sativa* cv Nemat or *Raphanus sativus* cv Bento cultivated during the summer season of 2011 in field experiment-1 (A) or overwintered prior to the GSL analysis in field experiment-2 (B). Different letters represent significant differences ($P < 0.05$) in GSL concentrations (µmol g^{-1} dry weight (dw)). Error bars show the standard error of means (SEM) for the total GSL concentration in the brassica species

Similar to total GSL concentration, individual GSL concentrations were higher for summer cultivated brassicas as compared with the overwintered crops (Figures 4.10 & 4.11), with the exception of gluconasturtiin (2-phenylethyl-GSL) concentration which was higher for overwintered *R. sativus* as compared with the same plant cultivated during summer (Figure 4.10B). Approximately 97% of the GSL found in *B. juncea* leaves was sinigrin (Figure 4.10A), whereas, *R. sativus* leaves produced predominantly glucoraphanin (Figure 4.10B).

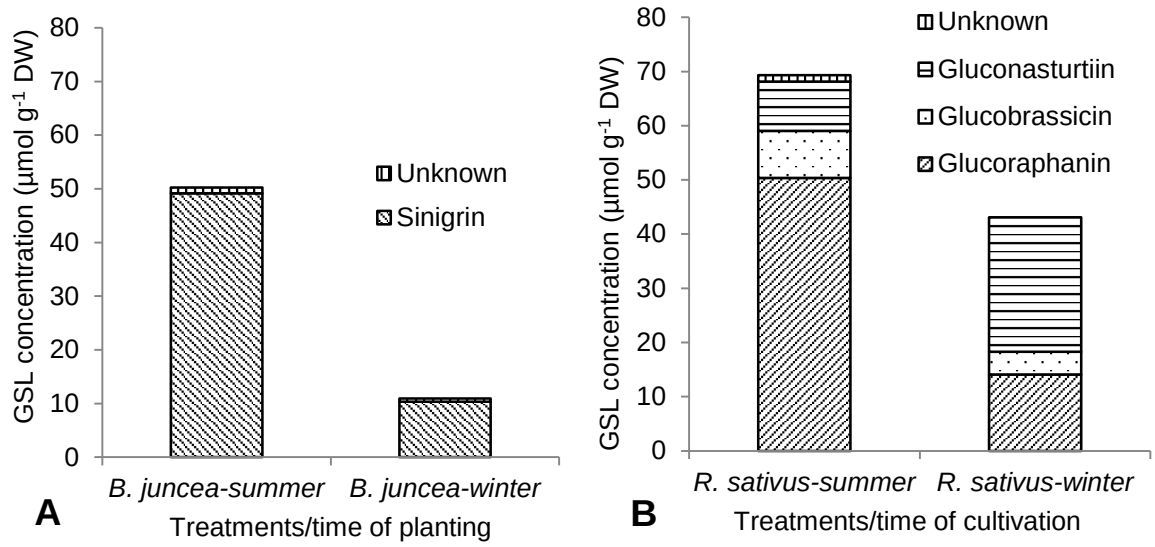


Figure 4.10: Individual glucosinolate concentrations in the tissue of (A) *Brassica juncea* or (B) *Raphanus sativus* cultivated during the summer season of 2011 in field experiment-1 or overwintered in field experiment-2 prior to GSL analysis

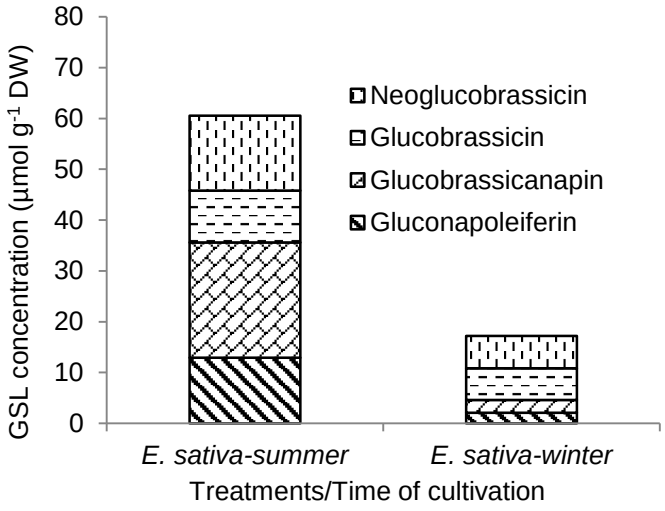


Figure 4.11: Individual glucosinolate concentrations in *Eruca sativa* tissue cultivated during the summer season of 2011 or overwintered in field experiment-1 or overwintered in field experiment-2 prior to GSL analysis

The concentration of total foliar GSL in *B. juncea* was significantly ($P < 0.001$) higher compared with *R. sativus* or *E. sativa*. However, for root tissues GSL concentration, *R. sativus* produced a significantly ($P = 0.006$) higher total root GSL when compared with the root GSL in *B. juncea* or *E. sativa* (Figure 4.12-i). Also, *R. sativus* and *E. sativa* root tissues produced approximately two-fold the total concentration of GSL present in their respective foliage (Figure 4.12-i). Approximately 87% of the GSL detected in *R. sativus* root tissues was the aromatic GSL, gluconasturtiin (Figure

4.12-ii). By contrast, *B. juncea* foliage produced approximately three-fold the concentration of GSL present in it root tissues (Figure 4.12-i) of which approximately 97% was sinigrin (Figure 4.12-ii).

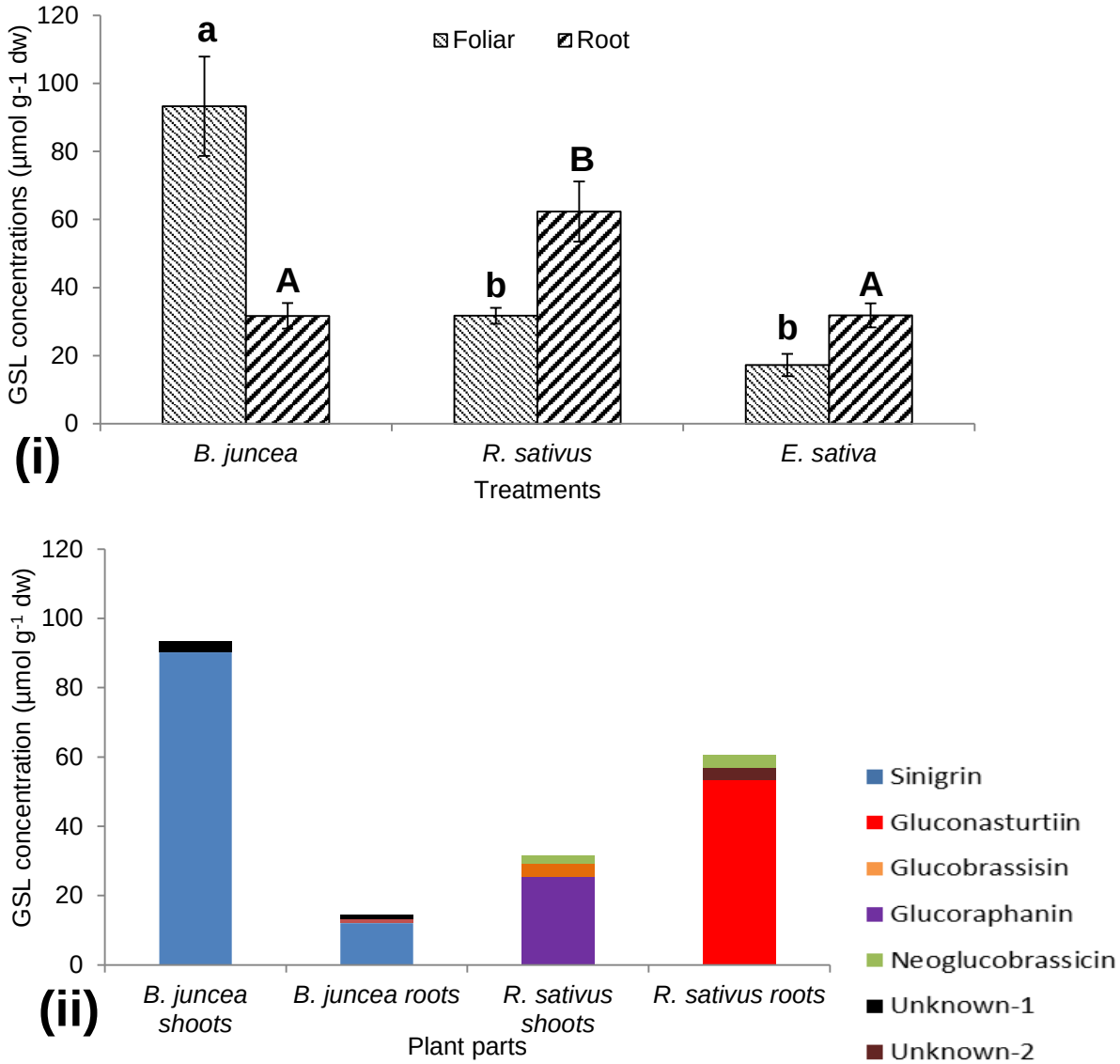


Figure 4.12: Comparison of the total foliar and root glucosinolate (GSL) concentrations in *Brassica juncea, Raphanus sativus* or *Eruca sativa* from field experiment-1 (i) and the individual GSL present in the foliar and root tissue of *B. juncea* and *R. sativus* from field experiment-3 (ii). Lower or upper case letters represent significant differences ($P < 0.05$) in total foliar or root GSL concentrations respectively. Error bars represents standard error of means for the total foliar or root GSL in the *Brassica* species

4.5.2 Effect of metconazole treatments on total and individual Glucosinolate concentrations

The application of metconazole on brassicas cultivated during the summer season in field experiment-3 (FE-3) significantly ($P < 0.001$) increased the total GSL

concentration in *B. juncea* but had no effect on levels in *R. sativus.* However, the total concentration of GSL in untreated *B. juncea* plants was similar to both *R. sativus* and treated *B. juncea* plants (Figure 4.13).

Brassica juncea tissues produced a relatively higher concentration of sinigrin when metconazole was applied to the plants compared with the untreated plants (Figure 4.14A). However, the individual concentration of GSLs in *R. sativus* tissues was unaffected by the metconazole treatment (Figure 4.14B).

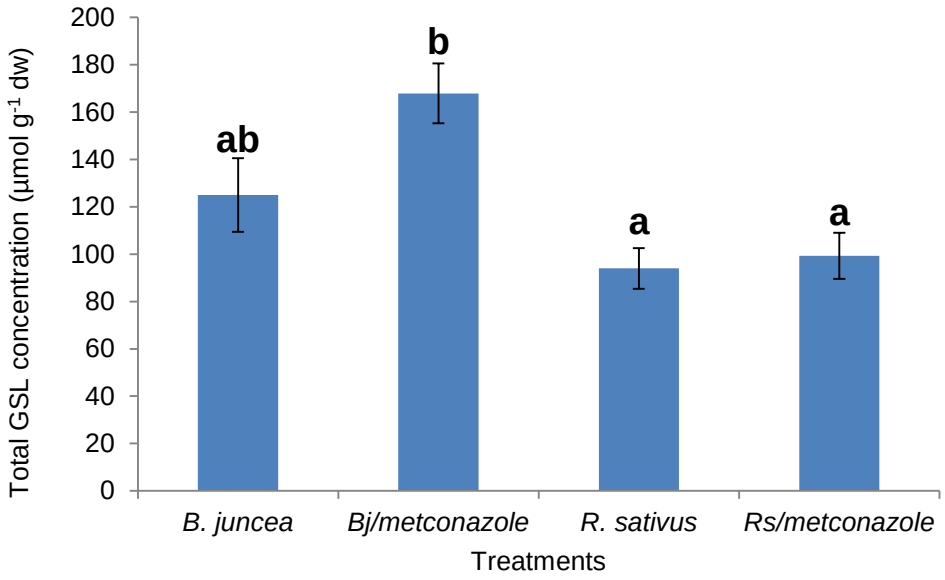


Figure 4.13: Total glucosinolate concentrations in *Brassica juncea* (Bj) or *Raphanus sativus* (Rs) either untreated or treated with metconazole (Caramba) in field experiment-3. Different letters represent significant differences in the mean total GSL between treatments. Error bars represents standard error of means for the total GSL in the brassica species

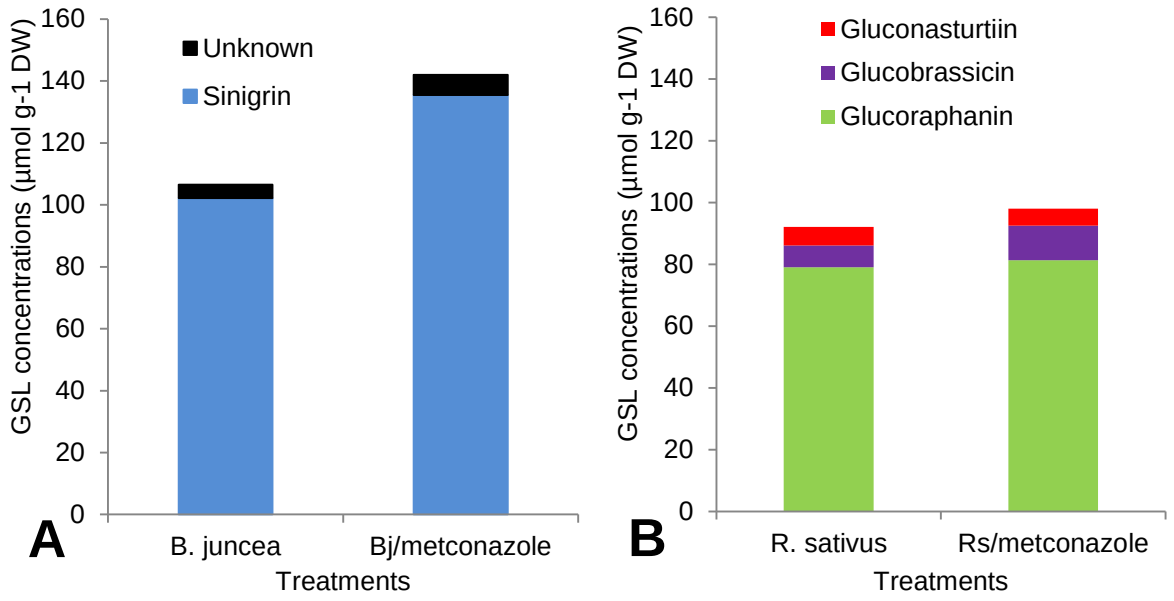


Figure 4.14: Individual glucosinolate concentrations in (A) *Brassica juncea* (Bj) and (B) *Raphanus sativus* (Rs) either untreated or treated with metconazole (Caramba) in field experiment-3

The GSL concentrations in *R. sativus* shoots or root tissues remained unchanged with metconazole treatment (Figure 4.15A). However, sinigrin concentration in the shoots of *B. juncea* was increased by approximately 28% following the application of metconazole compared with the untreated plants (Figure 4.15B). Similarly, the root concentration of sinigrin was also increased by approximately 48%.

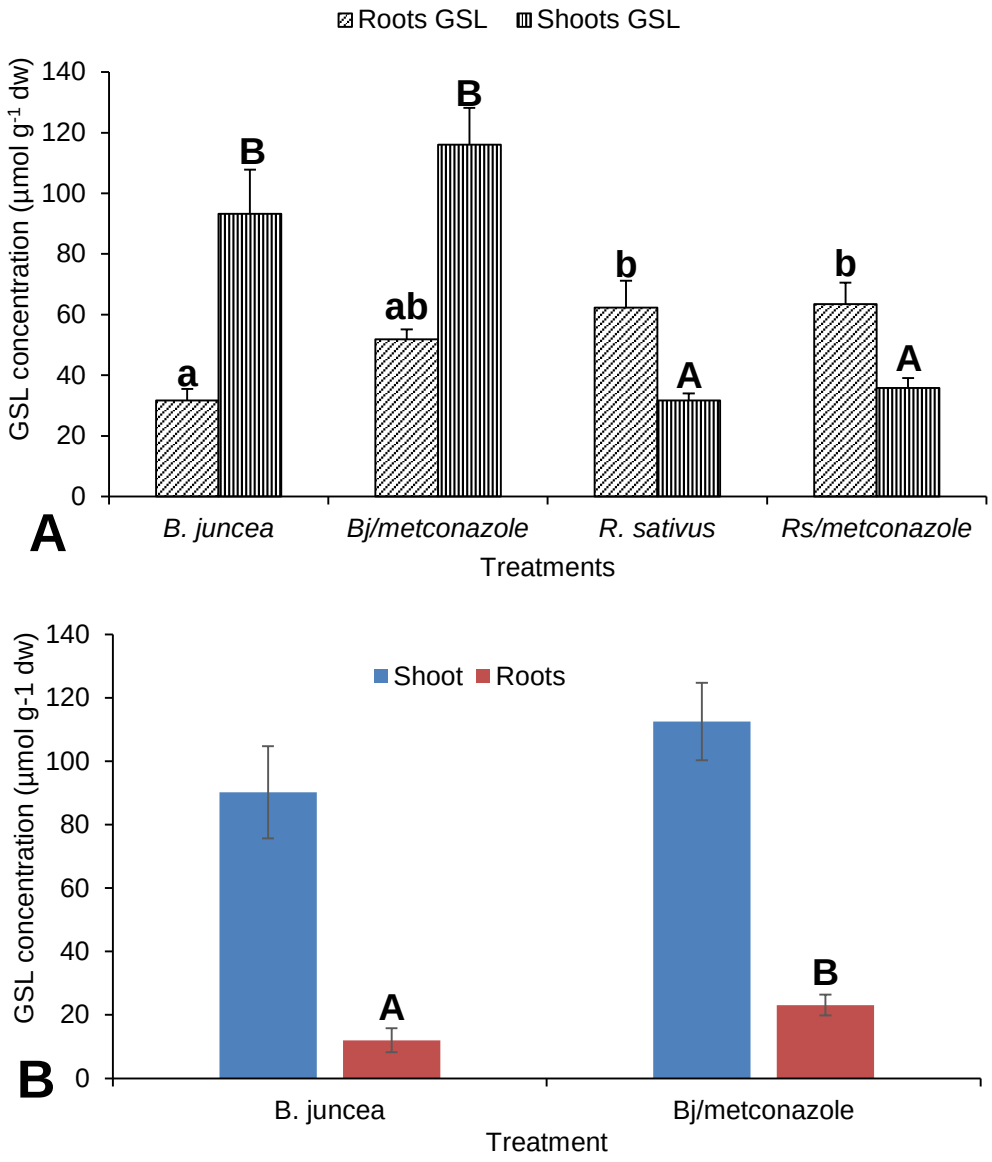


Figure 4.15: (A) Total foliar and root glucosinolates in *Brassica juncea* or *Raphanus sativus* and (B) sinigrin concentration in *B. juncea* root tissues either treated with metconazole or left untreated in field experiment-3. Lower or upper case letters represent significant differences ($P < 0.05$) in foliar or root GSL concentrations respectively. Error bars represent standard error of means (SEM)

4.5.3 Effect of glucosinolate concentrations on the viability of *Globodera pallida* eggs

Positive linear relationships between GSL concentrations in the incorporated biomass and the mortality of *G. pallida* encysted eggs g^{-1} of soil assessed six weeks post-incorporation of brassicaceous residues were determined (Figures 4.16 & 4.17). In field Experiment-3 these relationships were significant except for (Figure 4.16) However, this relationship was weaker and non-significant ($R^2 = 0.42$) for *R. sativus* treatments that did not receive metconazole application (Figure 4.16(ii)).

The relationship was significant for all brassicas cultivated during the summer seasons in field experiment-1 (Figure 4.17(i), (ii) & (iii)); *B. juncea* (*P = 0.029*), *R. sativus* (*P = 0.004*) and *E. sativa* (*P = 0.006*). However, when cultivated during the winter season in field experiment-2, only *R. sativus* and *E. sativa* demonstrated a significant (*P = 0.011*) relationship between the GSL concentration and mortality of *G. pallida* (Figure 4.17(v) & (vi)).

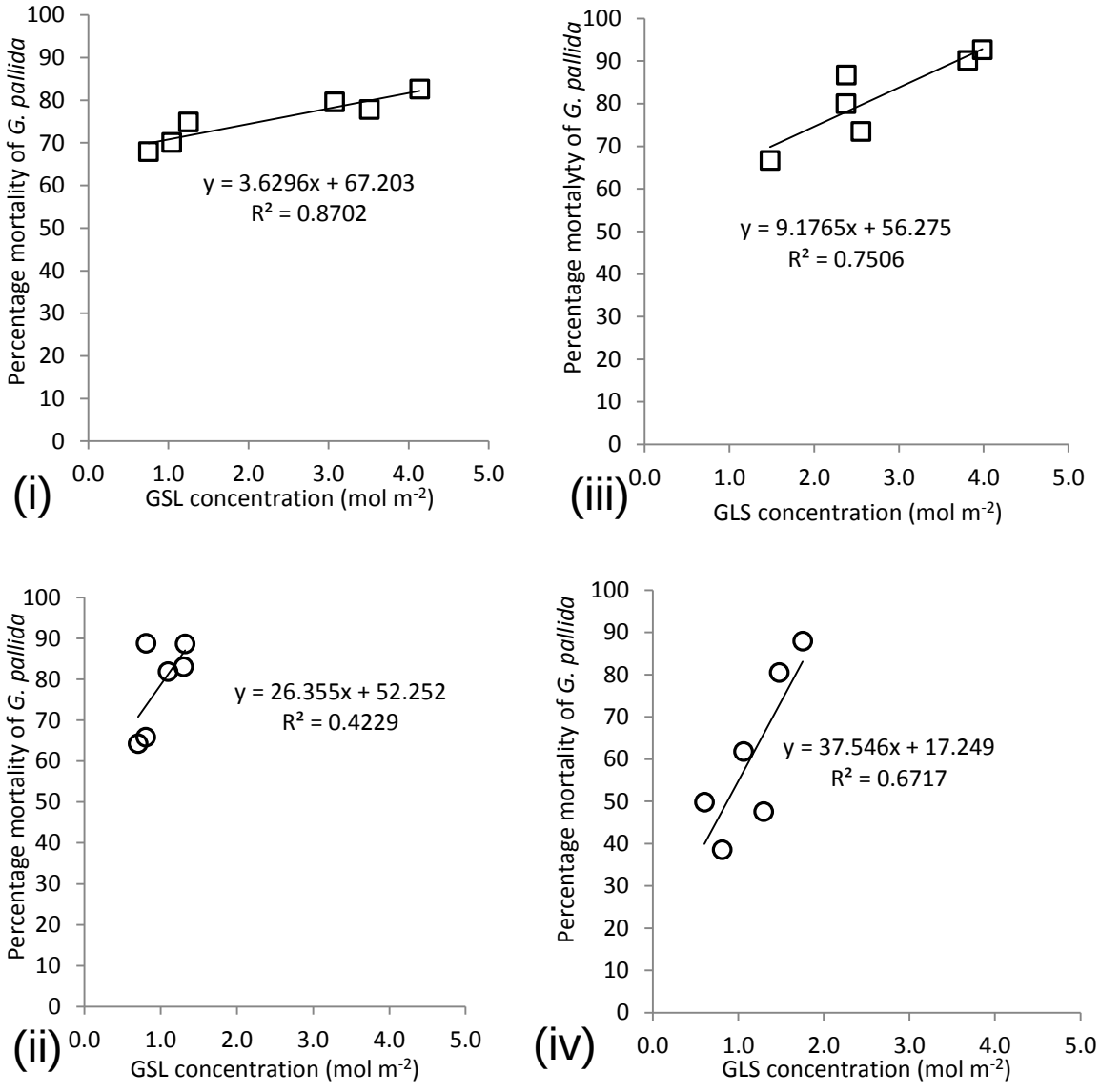


Figure 4.16: Relationships between percentage mortality of *Globodera pallida* eggs g^{-1} of soil and glucosinolate (GSL) concentrations in Experiment-3 plots treated with (i) *Brassica juncea,* (ii) *Raphanus sativus,* (iii) *B. juncea*/metconazole or (iv) *R. sativus*/ metconazole

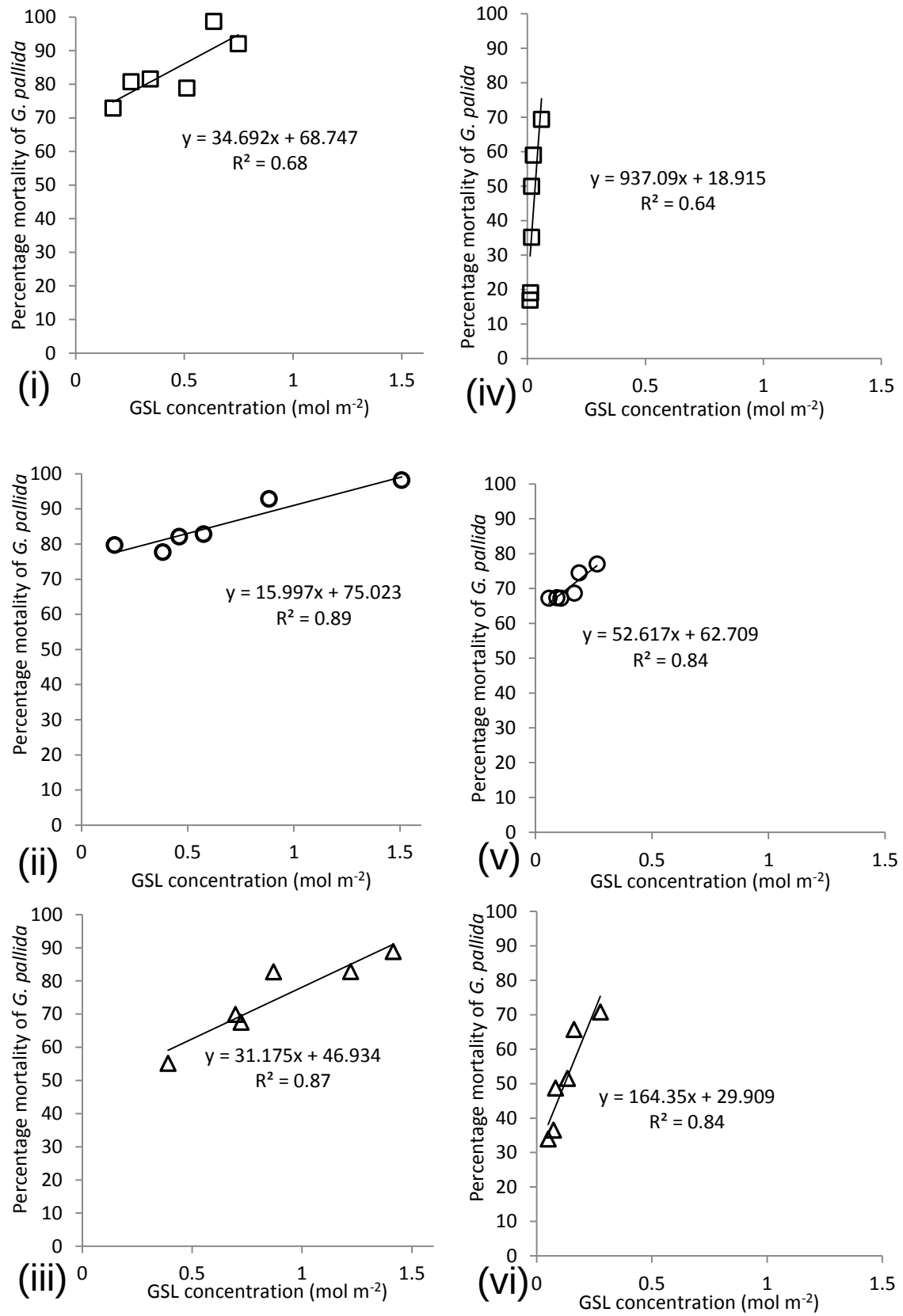


Figure 4.17: Relationships between percentage mortality of *Globodera pallida* eggs g^{-1} of soil and glucosinolate (GSL) concentrations in Experiment-1 plots treated with (i) *Brassica juncea,* (ii) *Raphanus sativus* or (iii) *Eruca sativa*, and Experiment-2 plots treated with (iv) *B. juncea,* (v) *R. sativus* or (vi) *E. sativa*

4.6 Discussion

The three *Brassica* species (*Brassica juncea*, *Raphanus sativus* and *Eruca sativa*) studied demonstrated variability in type and concentration of glucosinolates (GSL) between the species and within regions of the same plant. The total concentration of GSL was also affected by the season in which the brassicas were grown, whereas the type of glucosinolates produced in each species was the same irrespective of planting season. These observations are in line with those reported in literature (Kirkegaard & Sarwear, 1998; Cartea *et al*., 2008). Nine GSL were detected in the brassica species studied, seven of which could be classified as aliphatic, aromatic or indole GSL. Two GSL's could not be characterised. Neoglucobrassicin was the only common GSL detected in the three brassica species.

The major GSL in *B. juncea* cv Caliente 99 foliage was sinigrin, accounting for approximately 97% of the total foliar GSL. This means that the toxic effect observed for this species against *G. pallida* (Chapter 3) in field plots was resulting from the ITC associated with this GSL. This was well supported by *in-vitro* experiments (Chapter 6) in which *B. juncea* foliage were observed to be highly toxic to encysted eggs of *G. pallida*. A similar proportion of sinigrin in *B. juncea* leaves has been reported recently by Malabed *et al.* (2014), and high toxicity to *G. pallida* encysted eggs in glasshouse experiments has also been reported (Lord *et al*., 2010). However, neoglucobrassicin was the dominant GSL found in the root tissues of *B. juncea* followed by sinigrin and an unknown GSL. When *B. juncea* was treated with metconazole, the root concentration of sinigrin increased to concentrations similar with that of neoglucobrassicin indicating that this product may contain growth regulatory attributes. The high concentration of toxic ITC-liberating GSL (sinigrin) as shown for *B. juncea* indicates that there is a significant possibility to increase the biofumigation

potential (Kirkegaard & Sarwar 1998) of this plant without necessarily increasing it total GSL concentration.

Raphanus sativus cv Bento produced five different glucosinolates, four of which could be identified (glucoraphanin, gluconasturtiin, glucobrassicin, neoglucobrassicin). The total root GSL in *R. sativus* was two folds more than the GSL available in its foliage, of which approximately 86% was the aromatic GSL, gluconasturtiin. Glucoraphanin dominated the foliage tissues of *R. sativus*, accounting for approximately 80% of the total foliar GSLs. These results contradict those reported for *R. raphanistrum* (wild radish) in which root tissues produced < 15% of the total GSL (Malik *et al*., 2010). Also, none of the GSL (glucoerucin, glucoraphenin, and glucotropaeolin) that accounted for > 90% of the total as reported by these authors was detectable in the *R. sativus* cv Bento used in our study. Unlike *B. juncea,* the GSL content in *R. sativus* was unaffected by metconazole treatment. The occurrence of gluconasturtiin (2-phenylethyl GSL) in significant concentrations in the roots of *R. sativus* raises questions about its function. *In-vitro* studies reported in Chapter 6 revealed *R. sativus* root extract to be highly toxic to encysted eggs of *G. pallida*. The ITC associated with aromatic GSL such as 2-phenylethyl are known to be less volatile when compared to the aliphatic counterparts (Kirkegaard & Sarwar, 1998), suggesting that they may thus persist for longer durations in the soil. Direct exudation of ITC from actively growing roots has been reported (Elliot & Stowe, 1971). This exudation of root ITC coupled with their soil persistence and that root penetrates the soil in a natural manner may have provided the suppression to *G. pallida* as observed in our field experiments (Chapter 3). There is little knowledge about 2-phenylethyl ITC activity in the soil as previous reports have often focused attention on aliphatic ITC such as 2-propenyl ITC or methyl ITC (the active

compounds in metam sodium). *In-vitro* studies has shown gluconasturtiin derived ITC (2-phenethyl ITC) to be significantly more toxic than the sinigrin derived ITC (2-propenyl ITC) against insects (Borek *et al*., 1995) and fungal pathogen (Drobnica *et al*., 1967a; Sarwar *et al*., 1998). There is therefore a need to optimize root production of these GSL particularly for brassica crops that are grown in rotation with other crops.

In *E. sativa* cv Nemat, glucobrassicanapin (58%), neoglucobrassicin (25%), gluconapoleiferin (21%) and glucobrassicin (16%) were detected. These results are consistent with reports in literature (Cataldi *et al*., 2007).

The concentration of GSL in *B. juncea* and *R. sativus* did not only vary between different seasons, but also varied between the same seasons in different years. For instance, when cultivated during the summer of 2011, *B. juncea* and *R. sativus* produced a total GSL concentration of 50 and 69µmol g^{-1} DW respectively. However, when these two plants were cultivated in the following summer season (2012), they both produced a total GSL concentration of 125 and 94µmol g^{-1} DW respectively. It is likely that this variation in GSL concentrations between the same seasons in different years was probably due to changes in weather conditions during the different years. Unlike the summer season of 2011 which was characterised by an average precipitation of 40 mm during the green manure crop growing period, the summer season of 2012 had an average precipitation of 90 mm, approximately two folds that of the former (Appendix 9.4). However, the soil temperature recorded at a 20 cm depth during the green manure crop development for both years was similar (Appendix 9.1).

Another explanation for the variability in GSL concentration in the brassicas during the different years of cultivation could possibly be as a result of the inconsistency

observed in the plant development. During the summer of 2011, some of the *B. juncea* plants where senescing at time of incorporation when samples were collected for analysis, whereas most of the *R. sativus* had just commenced flowering and almost 70% of *E. sativa* had not commenced flowering. These inconsistencies in plant development were probably due to low soil moisture as a result of the low rate of precipitation observed during this cropping season.

Species and variety selection as well as timing for sowing and incorporation amongst others are vital for the successful application of biofumigation for optimum benefits. This has been demonstrated clearly by the results of studies where the concentration of GSL in brassicaceous tissues was influenced by planting seasons. Although the level of GSL in the species/cultivars in Valdes *et al*. (2011 & 2012) at time of incorporation was not reported, the *B. juncea* cultivar used in our experiments produced between two- to four-fold more sinigrin at incorporation than that for the *B. juncea* cultivars reported in Vervoort *et al*. (2014). Also, considering the fact that ITC are highly volatile/sopped onto organic matter in soil, it is possible that most of the ITC volatilised prior to cyst treatment with the extract as reported in Valdes *et al*. (2011) taking into account the methods used by these authors in preparing the extracts. A similar explanation or difficulty in direct ITC-soil application coupled with sorption onto organic matter (Gimsing *et al*., 2006) could explain the failure observed in Vervoort *et al*. (2014) considering that 2-propenyl ITC treated plots were incorporated with *T. aestivum* (wheat). It would have been useful if these authors allocated fallow plots and ITC-only (without any crop incorporated) to compare/contrast treatments with Brassicaceae.

Brassica juncea cv Caliente 99 has undoubtedly proven to be a good biofumigant variety as seen with the type and concentration of the GSL produced in this plant as

well as it vigorous growing attribute and ease of disruption. This confirms its ability to mitigate PCN populations in field as observed in the field experiments reported in the previous Chapter. The observed production of ITC-producing aromatic GSLs in roots of *R. sativus* has added to the understanding of why these species demonstrated PCN reduction in field during the crop development. Although the foliar concentration of ITC-producing GSLs in *R. sativus* was lower than its observed root concentration, it is apparent that this species is a good biofumigant plant as the combination of root exudates produced during the crop development would add to the benefits from chopping and incorporating the foliage residues in to the soil. *Eruca sativa* on the other hand produced a variety of different GSL, most of which were either indole, or those known to be producing less ITCs upon hydrolysis. The concentration of the individual GSLs in *E. sativa* was also low regardless of the season of cultivation, possibly due to its slow development.

CHAPTER FIVE

5. Chapter 5: Glasshouse Experiments; Elucidation of partial biofumigation from *Brassica juncea* and *Raphanus sativus* on *Globodera pallida*

5 Sinigrin hydrolysis and *G. pallida* mortality in soil cultivated with *Brassica* species under controlled environmental conditions

5.1 Introduction

As demonstrated in the field experiments (Chapter 3), it is clear that *Brassica* species had a significant suppressive effect on field populations of *Globodera pallida* particularly when cultivated in mid-summer for late-autumn incorporation. This effect was recorded not only in response to the crushing and incorporation of the crop residues, but also from the developing biofumigant crop. The mechanism underlying this suppressive effect is not fully understood. As demonstrated by McCully *et al*. (2008), brassica crops do release glucosinolates (GSLs) into the soil during cell replenishment. However, intact GSL occurring on their own are understood to be non-toxic until they become hydrolysed in the presence of myrosinase enzyme. The enzymatic hydrolysis of GSL releases toxic and/or less toxic compounds depending on the reaction conditions as discussed previously in Chapter 1. The exudation of ITC from actively growing roots into the soil has been reported (Elliot & Stowe, 1971) as mentioned before.

The release of myrosinase into the soil by developing brassica root tissues is unknown to date. However, reports of myrosinase producing soil microbes are well documented. Borek *et al*. (1996) demonstrated myrosinase activity in soil extracts from a field cultivated with rapeseed but soil extracts from pasture soil showed no/little activity. These authors also specified that myrosinase activity was highest in the soil sampled directly from rapeseed rows, which was four times greater than the activity observed in the soil sampled in-between the rapeseed rows. Sakorn *et al*. (1999) demonstrated both high myrosinase activity and sinigrin degradation when a strain of *Aspergillus* sp. was incubated in a medium containing sinigrin. Linking these

previous findings to field observations in the reduction of viability of PCN during the brassica crop development, two hypotheses were formulated; (1) glucosinolates may be exuded from root tissues alongside endogenous myrosinase for hydrolysis, or (2) exuded glucosinolates are being hydrolysed by myrosinase released by soil microbes.

5.2 Aim

The aim of these experiments was to understand the underlying cause of reduction in the viability of PCN encysted eggs following the growing of biofumigant brassica species (partial biofumigation) as observed in the field experiments (Chapter 3).

5.3 Objectives

i. To determine the partial biofumigation effect of selected biofumigant brassica species on *G. pallida* under glasshouse controlled conditions
ii. To monitor the hydrolysis of inoculated glucosinolates during the growth and development of selected brassica species under glasshouse controlled conditions
iii. To monitor microbial activity following brassica plant development and after chopping and incorporation.

5.4 Hypotheses (null):

i. The viability of PCN is not affected during the growth and development of biofumigant brassica species under controlled conditions.
ii. Pure glucosinolates are not hydrolysed in soil during the growth and development of biofumigant brassicas
iii. Microbial activity is not affected by the growth and development of biofumigant brassicas.

5.5 Materials and methods

5.5.1 Experimental set-up and treatments

Two glasshouse experiments (GE) were conducted during 2013/2014 to monitor the underlying cause of the reduction in viability observed in the field experiments. The first experiment (GE-1) tested the first two hypotheses and had five treatments each of which was replicated five times (Figure 5.1i) and these are listed in Table 5.1 with their respective seed rates. In the second experiment which was a follow-up of the first experiment, an additional treatment was introduced which included sterilised untreated fallow soil (Figure 5.1ii) alongside the third hypothesis. The soil used for the experiments was collected from the sites where field experiments 3 and 4 had been conducted (UK ordinance survey grid reference: SJ 70386 21266 and SJ 77726 15689, Shropshire, UK respectively) and taken to the glasshouse where it was homogenised, coarsely sieved via a 1 cm^2 sieve and three-quarter filled into 1.7 l pots (Plate 1). Where sterilisation was required, the soil was sterilised for 1.5 h (Camplex Plant-care Soil Steriliser, 50Hz, 3000 Watts, Themoforce Ltd, Cumbria, England), before cooling overnight prior to potting. During the setting up of pots, all treatments were inoculated with 50 cysts of *G. pallida* each of size ≥ 500 µm in diameter, wrapped in a 250 µm nylon mesh (cyst sachets) at a depth of 10 cm. The pots (Plate 5.1) were randomized and blocked in the direction of light source in the glasshouse.

The selected biofumigant *Brassica* species were sown into their designated pots at the supplier's recommended seed rates (Table 5.1) resulting in 17 plants per pot for each species. The experiment was maintained in the glasshouse at a day/night temperature of 15/5°C respectively with a 16 h photoperiod. Application of water to each pot was done twice weekly for the first three weeks post-germination by applying 200 ml to each pot and thereafter, every two days following determination of

the required amount of water to be applied with the aid of a moisture meter (Theta Prop Type HH2, Delta-T Devices Ltd, Burwell, UK, Plate 2). In this way, pots with a soil moisture reading below 32% volumetric water content (vwc) were balanced by adding the required amount of water.

At ten weeks post planting, the cyst sachets were removed for assessments and the brassicaceous tissue was chopped using a plant shredder (Viking GE150 Shredder, Tom's Garden Equipment, Ashburton, UK**)** and homogenised with the soil (Plate 5.3) before re-potting. The soil moisture level was recorded before and after incorporation and the setup was left for six weeks while the soil moisture was maintained at 20±3% vwc before the final soil samples were collected to measure glucosinolate degradation and microbial activity post-incorporation.

Table 5.1: Treatments used in the glasshouse experiments. Glasshouse Experiment-1 had 5 treatments with 5 replicates and Glasshouse Experiment-2 had an additional treatment (Untreated sterilized)

Treatments	**Variety**	**Sterilization**	**Seed rates**	*G. pallida* **pot^{-1}**
Untreated (fallow)	N/A	N/A	N/A	50
B. juncea	Caliente 99	N/A	8 kg ha^{-1}	50
B. juncea	Caliente 99	Yes	8 kg ha^{-1}	50
R. sativus	Bento	N/A	20 kg ha^{-1}	50
R. sativus	Bento	Yes	20 kg ha^{-1}	50

N/A = not applicable

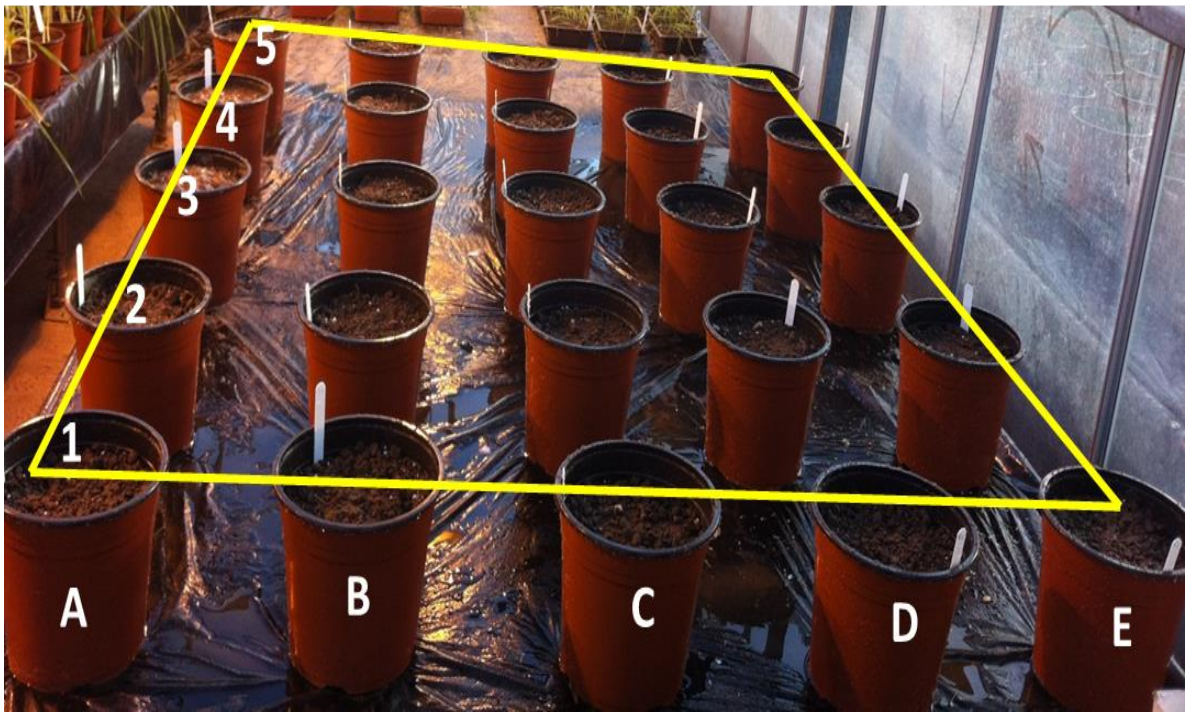


Plate 5.1: Pot arrangement for Glasshouse Experiment-1. Letters represent blocks (replicates) and numbers represent treatments

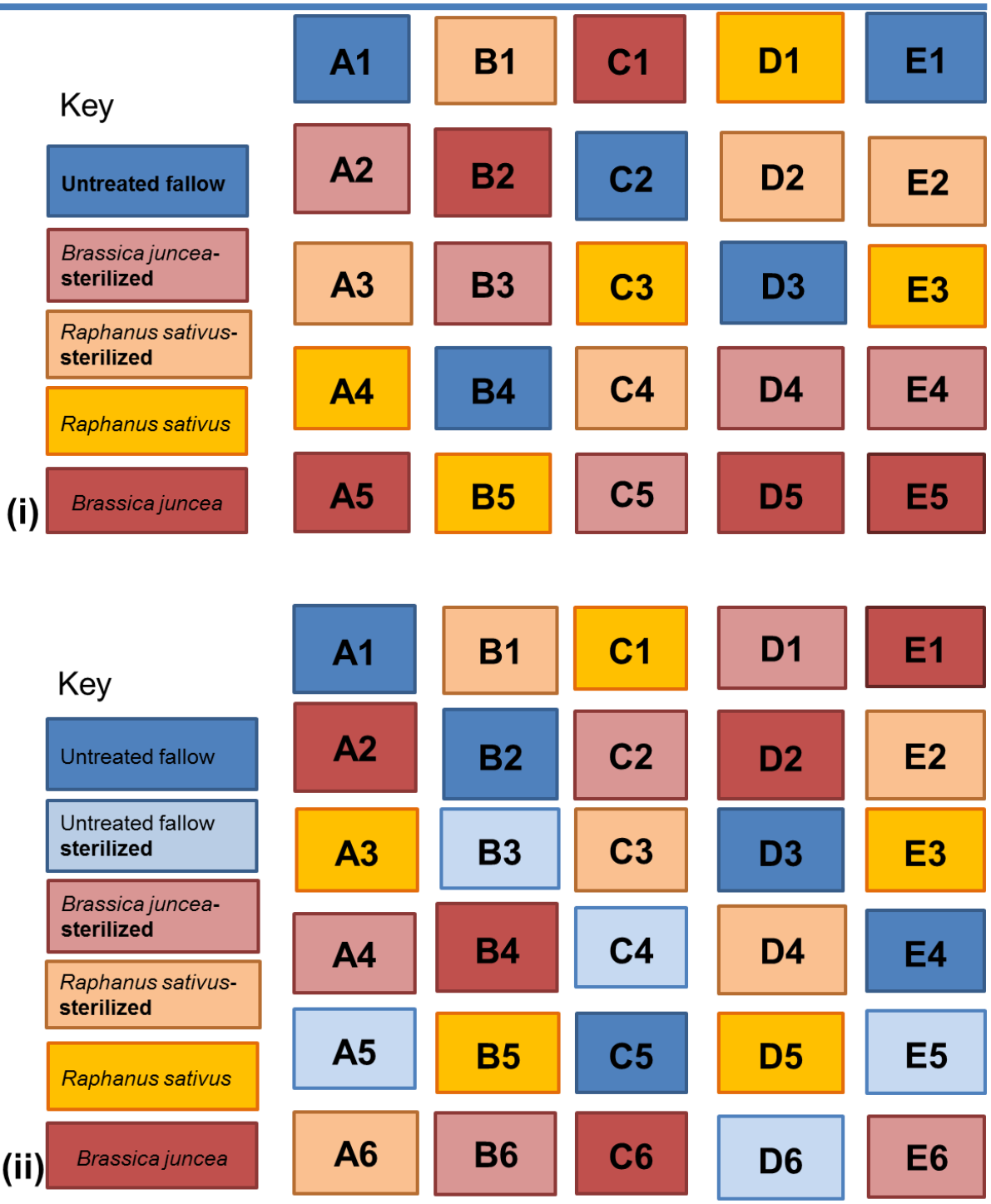


Figure 5.1: Randomised complete block design for (i) Glasshouse Experiment-1 with treatments including untreated (fallow) pots, *Brassica juncea* or *Raphanus sativus* cultivated in sterilised or un-sterilised soil, and (ii) follow-up experiment with the addition of sterilised untreated fallow pots (Untreated-S)

Plate 5.2: Soil moisture reading using a Theta Probe type HH2

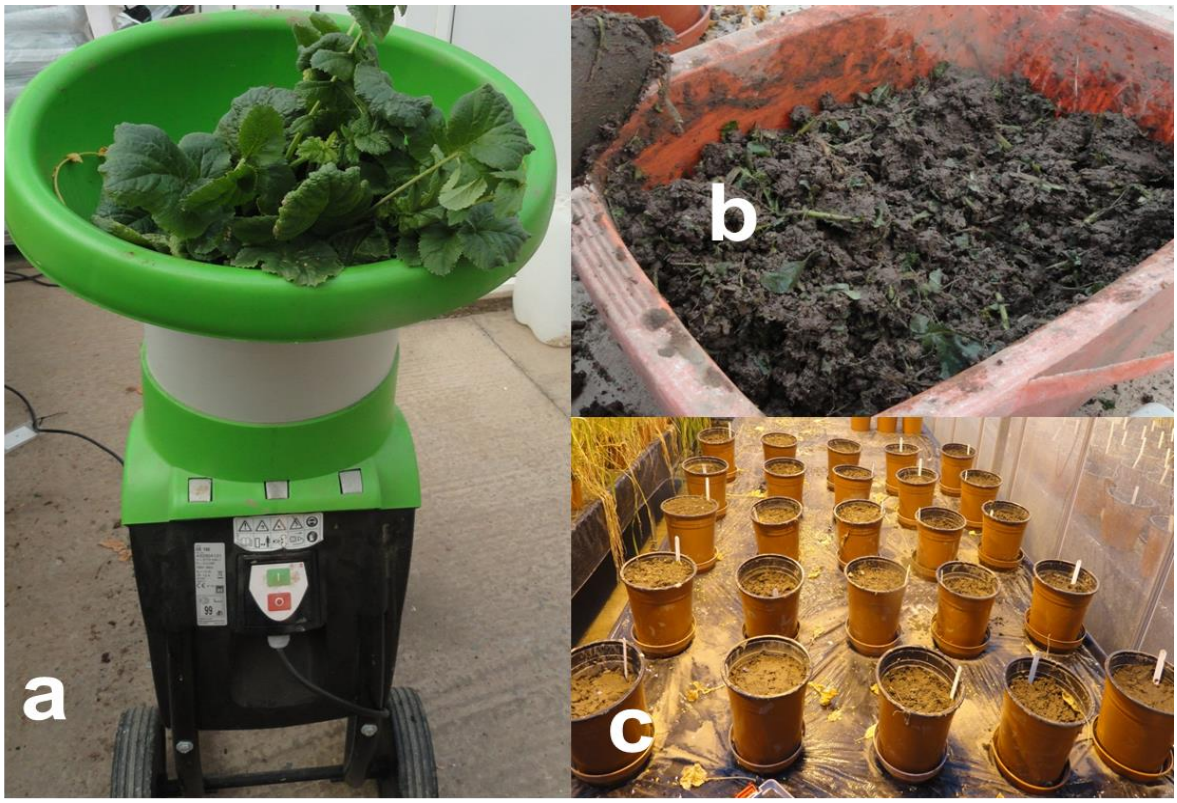


Plate 5.3: (a) Shredding of brassicaceous plants material, (b) homogenising of plant residues in soil and (c) soil/brassicaceous residues re-potting

5.5.2 Assessments

5.5.2.1 *Hydrolysis of glucosinolates in soil*

Soil samples were collected from the experimental pots at the following times to determine the hydrolysis of sinigrin (2-propenyl GSL):

1. before establishing brassicas,
2. before incorporation of brassicas and
3. Six weeks post-incorporation of brassicas.

At each time of sampling, the soil from each pot was homogenised and sub-samples of 50 g were placed into sterile polythene bags and taken to the laboratory where they were either immediately processed or stored below 4°C prior to processing. Each soil sample was passed via a 1 mm sieve and completely homogenised before a 2.5 g sub-sample was placed into a 15 ml polypropylene tube and spiked with a 1 ml aliquot of 5 mM sinigrin solution. The inoculated tubes were securely caped and incubated in darkness at room temperature for 96 h before the sinigrin was re-extracted with 70% Methanol at room temperature following the method described by Gimsing *et al*. (2005). The extract was ion-exchange column purified and desulfated before being subjected to HPLC analysis as described in Ngala *et al*. (2014) to determine the degradation of sinigrin in the individual treatments.

5.5.2.2 *Total microbial enzyme activity analysis via fluorescein di-acetate hydrolysis*

To measure the presence/concentration of GSL degrading soil microbes, fluorescein di-acetate (FDA) hydrolysis assay was conducted to measure microbial enzyme activity. In this assay, the production of a bright yellow colouration indicates microbial activity, and the intensity indicates the level of activity (light glow = least, strongest

glow = greatest enzymatic activity). Therefore, following soil sieving as described above, sub-samples of 2g were processed according to the protocol outlined in Solaiman (2007). The colour intensity was quantified using a spectrophotometer at a *UV* absorbance of 490 nm and results were expressed as µg FDA g^{-1} oven dry soil.

5.5.2.3 *Determination of the viability of* Globodera pallida *encysted eggs induced by partial biofumigation*

To determine the partial biofumigation effect of the brassica species on *G. pallida*, it was necessary to recover and assess the inoculated cysts prior to crushing and incorporation of the brassicaceous residues. Therefore, at 10 weeks post planting, the sachets containing *G. pallida* cyst were recovered from the pots and the cysts were re-isolated. Out of the 50 cysts in each sachet, 40 and 10 cysts were random selected and assessed for viability via Meldola's blue staining and hatching assays respectively as previously described (Chapter 2, section 2.4).

5.6 Data analysis

All data collected was subjected to general analysis of variance (ANOVA) using GenStat 16th edition statistical software to compare treatment effects. Regression analysis were undertaken to determine the relationship between total microbial activity and percentage mortality of *G. pallida* or sinigrin degradation. Significant differences between treatments were determined using Tukey's multiple range tests ($P \leq 0.05$).

5.7 Results

5.7.1 Hydrolysis of glucosinolates in soil

The concentration of sinigrin assessed following 96 h incubation in soil taken prior to brassica plants establishment was similar between sterilized and unsterilized soil in glasshouse experiment-1 (Figure 5.2i). Just before incorporation, the concentration of

sinigrin re-extracted after 96 h inoculation was significantly lower ($P < 0.001$) for all brassica treatments in either sterilized or unsterilized soil as compared with the untreated fallow pots (Figure 5.2ii). At six weeks post-incorporation, there was a significant hydrolysis ($P < 0.001$) of sinigrin in sterilized or unsterilized soil incorporated with *B. juncea* or *R. sativus* as compared with the untreated soils (Figure 5.2iii). Sterilised soil treated with *R. sativus* almost completely exhausted the spiked sinigrin.

In glasshouse experiment-2, unsterilized soil demonstrated sinigrin hydrolysis prior to brassica plant establishment (Figure 5.3i). This hydrolysis was significant ($P = 0.017$) when comparing with the sterilised soil in which *B. juncea* was subsequently planted, but not with the rest of the treatments. Analysis conducted just before incorporation of the brassicas was highly variable as illustrated by the error bars and showed no statistical difference between treatments in sinigrin hydrolysis (Figure 5.3ii). After incorporation, sinigrin was significantly ($P < 0.001$) hydrolysed in all soil samples in which brassicas were incorporated. The hydrolysis of sinigrin was significantly higher ($P < 0.001$) in sterilized fallow soil compared with unsterilized fallow soil (Figure 5.3iii).

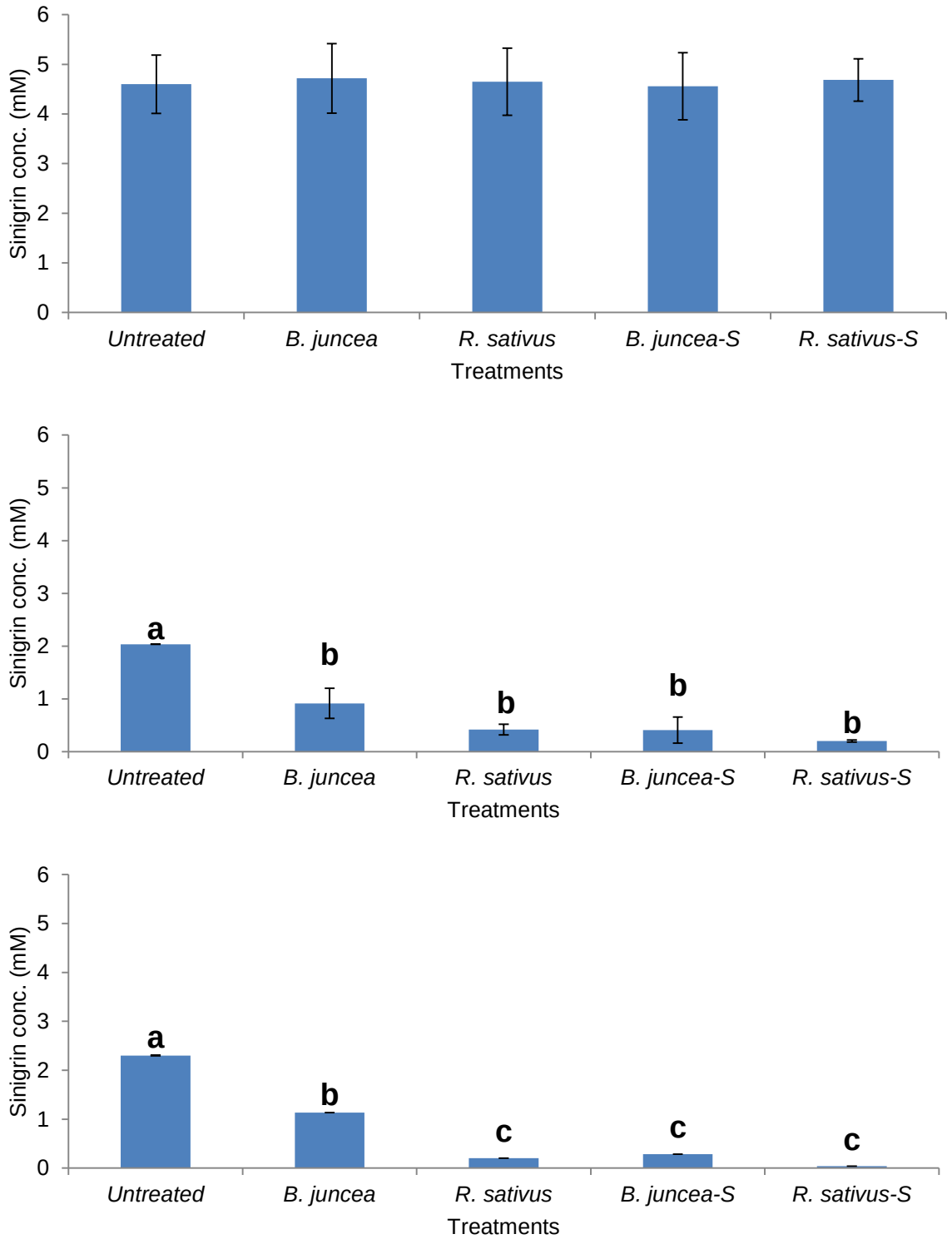


Figure 5.2: Sinigrin concentration (mM) after 96 h inoculation in sterile (S) or unsterile soil previously planted with *Brassica juncea*, *Raphanus sativus* or left fallow in Glasshouse Experiment-1: (i) pre-planting, (ii) pre-incorporation and (iii) post-incorporation of brassicas. Error bars represent standard error of means. Different letters indicate significant differences between treatments (Tukey's test: $P \leq 0.05$)

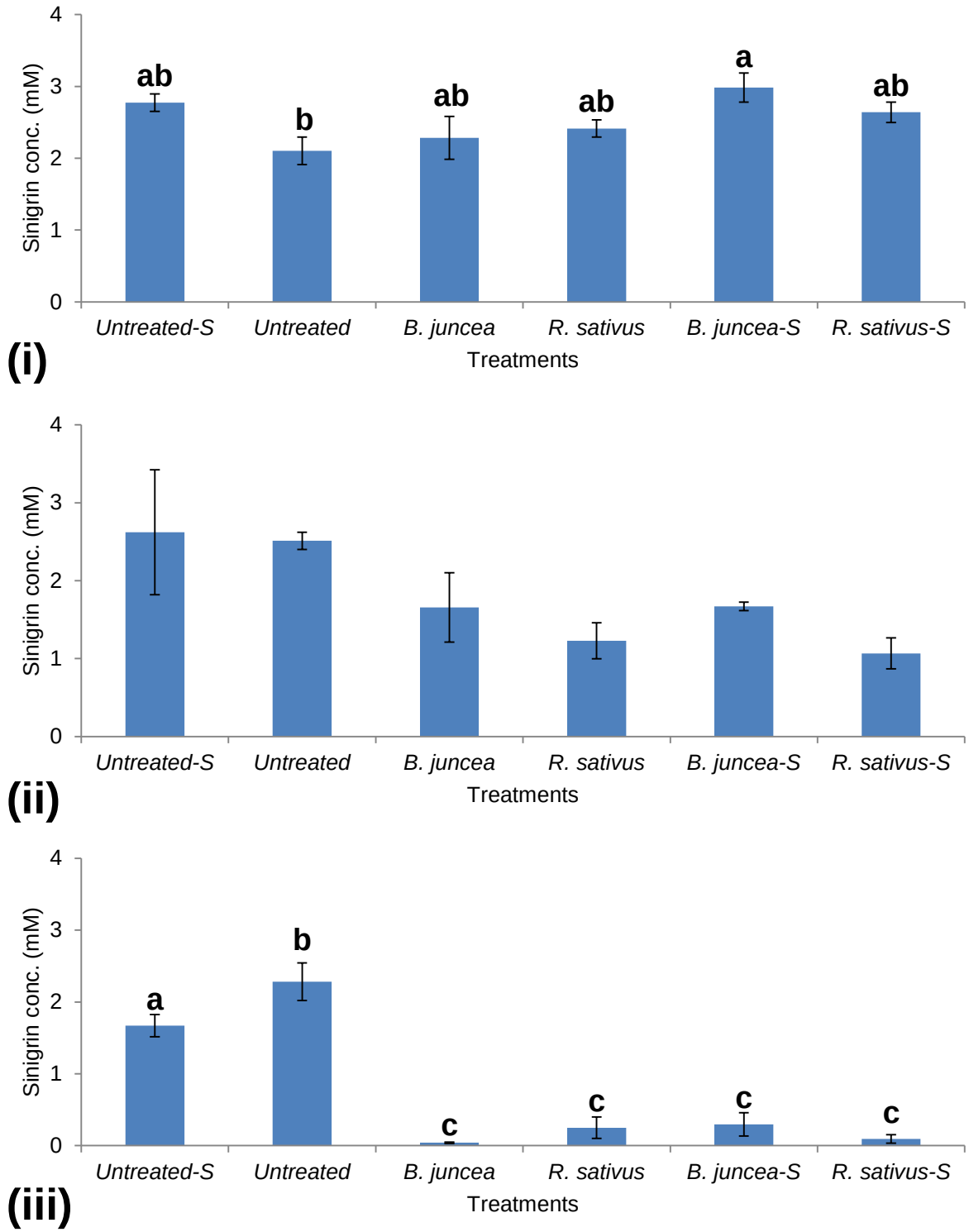
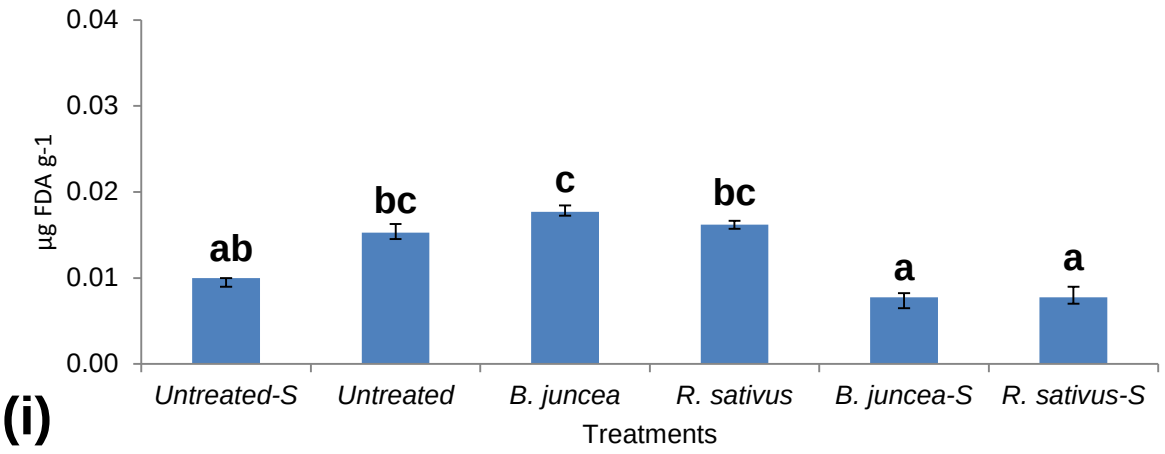
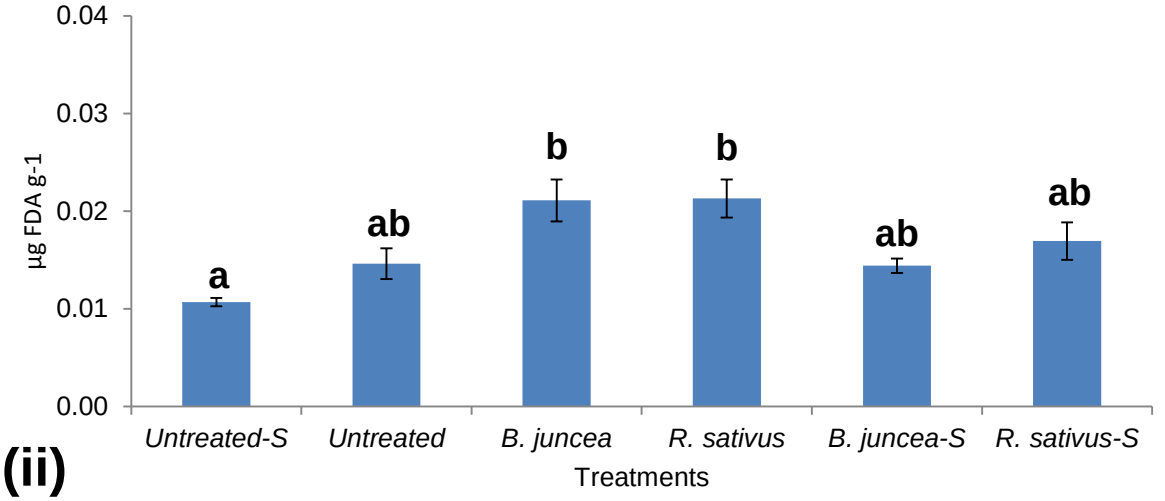


Figure 5.3: Sinigrin concentration (mM) after 96 h inoculation in sterile (S) or unsterile soil previously planted with *Brassica juncea*, *Raphanus sativus* or left fallow in Glasshouse Experiment-2: (i) pre-planting, (ii) pre-incorporation and (iii) post-incorporation of brassicas. Error bars represent standard error of means. Different letters indicate significant differences between treatments (Tukey's test: $P \leq 0.05$)

5.7.2 Total microbial activity

Total microbial activity (TMA) measured as μg FDA g^{-1} soil was observed in all the treatments prior to brassica plants establishment. However, the TMA was significantly lower ($P < 001$) in sterilised soil as compared with unsterilized soil treatments (Figure 5.4i). Prior to incorporation, TMA increased significantly ($P < 0.001$) in unsterilized soil planted with *B. juncea* and *R. sativus* when compared with the sterilised fallow soil only (Figure 5.4ii). A similar pattern in TMA was observed six weeks post-incorporation of brassicaceous residues for unsterile soil cultivated with either *B. juncea* or *R. sativus* as compared with the sterilized fallow soil (Figure 5.4iii). Sterilised soil planted with *B. juncea* also had significantly higher ($P < 0.001$) TMA than sterilised fallow soil at six weeks post incorporation.





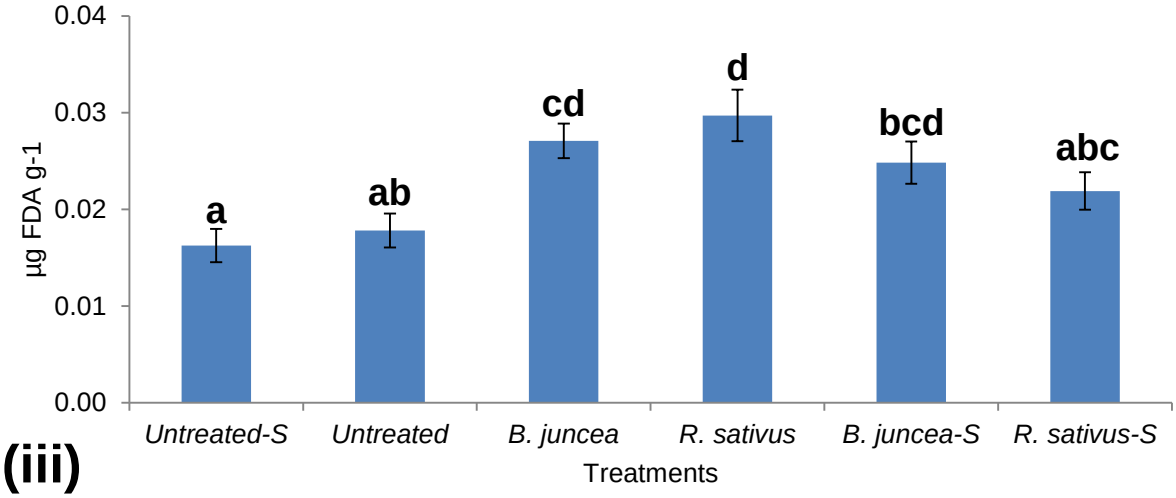


Figure 5.4: Total microbial activity (µg FDA g^{-1}) in sterile (S) or unsterile soil planted with *Brassica juncea*, *Raphanus sativus* or left fallow in Glasshouse Experiment-2, assessed pre-planting (i), pre-incorporation (ii) and six weeks post-incorporation (iii) of brassicas. Error bars represent standard error of means. Letters indicates significant differences (Tukey's test: $P \leq 0.05$)

5.7.3 The mortality of *Globodera pallida* encysted eggs following partial biofumigation

In Glasshouse Experiment-1, the percentage mortality of *G. pallida* encysted eggs cyst^{-1} assessed prior to incorporation of brassicaceous residues revealed a significant increase (*P = 0.027*) in mortality for unsterilized soil planted with *R. sativus* as compared with sterilised soil in which *B. juncea* or *R. sativus* was planted (Figure 5.5i). However, the percentage mortality of *G. pallida* encysted eggs cyst^{-1} was statistically similar between the rest of the treatments. Between 30 - 35% mortality of *G. pallida* encysted eggs cyst^{-1} was induced in unsterilized soil planted with either *B. juncea* or *R. sativus*, which was approximately 15 - 20% more than mortality in untreated fallow soil.

In Glasshouse Experiment-2, a similar pattern was observed as in Glasshouse Experiment-1 with the mortality of *G. pallida* encysted eggs cyst^{-1}. However, unlike Glasshouse Experiment-1, a statistical significant difference (*P = 0.02*) was observed only between sterilised fallow soil and unsterilized *R. sativus* cultivated soil in Glasshouse Experiment-2 (Figure 5.5ii). Unsterilized soil planted with brassicas induced between 8 – 10% mortality over the fallow soil treatments.

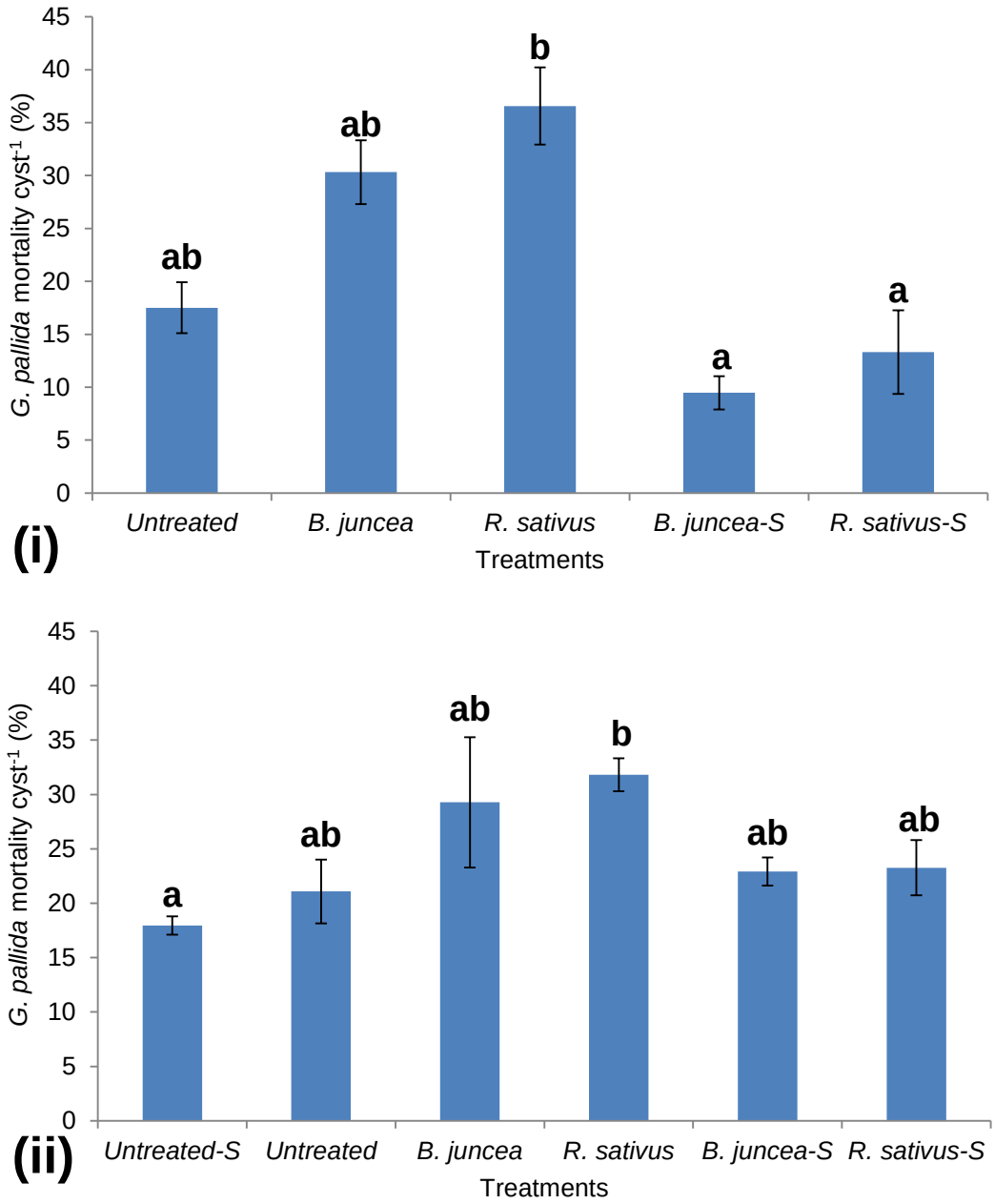


Figure 5.5: Percentage mortality of *Globodera pallida* encysted eggs cyst^{-1} following partial biofumigation with *Brassica juncea* or *Raphanus sativus* cultivated in sterilised (S) or unsterilized soil as compared with sterilised (S) or unsterilized fallow soils in (i) Experiment-1 and (ii) Experiment-2. Different letters represent significant differences ($P \leq 0.05$) between treatments. Error bars represent standard error of means

5.7.4 Relationships between microbial activity, glucosinolate hydrolysis and G. pallida mortality

Regression analysis revealed a strong and highly significant ($R^2 = 0.94$, $P < 0.001$) positive relationship between total microbial activity (TMA) measured as µg FDA g^{-1} of soil and percentage mortality of *G. pallida* encysted eggs cyst^{-1} (Figure 5.6A).

Unsterilized fallow soil and sterilised soil treated with *B. juncea* were similar in microbial activity and % mortality of *G. pallida*. Sterilised fallow soil demonstrated the least microbial activity/*G. pallida* mortality whereas unsterilized soil treated with *R. sativus* demonstrated the highest microbial activity/*G. pallida* mortality. Weak relationships were observed between the concentrations of re-isolated sinigrin and mortality of *G. pallida* eggs cyst^{-1} in soil collected pre-incorporation of brassicaceous residues (Figure 5.6B).

The concentration of inoculated sinigrin decreased with increased soil microbial activity (μg FDA g^{-1}). The coefficient of correlation (R^2 – value) varied from 0.51 to 0.72 depending on the time of assessment (Figure 5.7). After incorporation of the brassicas, sinigrin was almost completely hydrolysed, and this correlated with the level of microbial activity (Figure 5.7C)

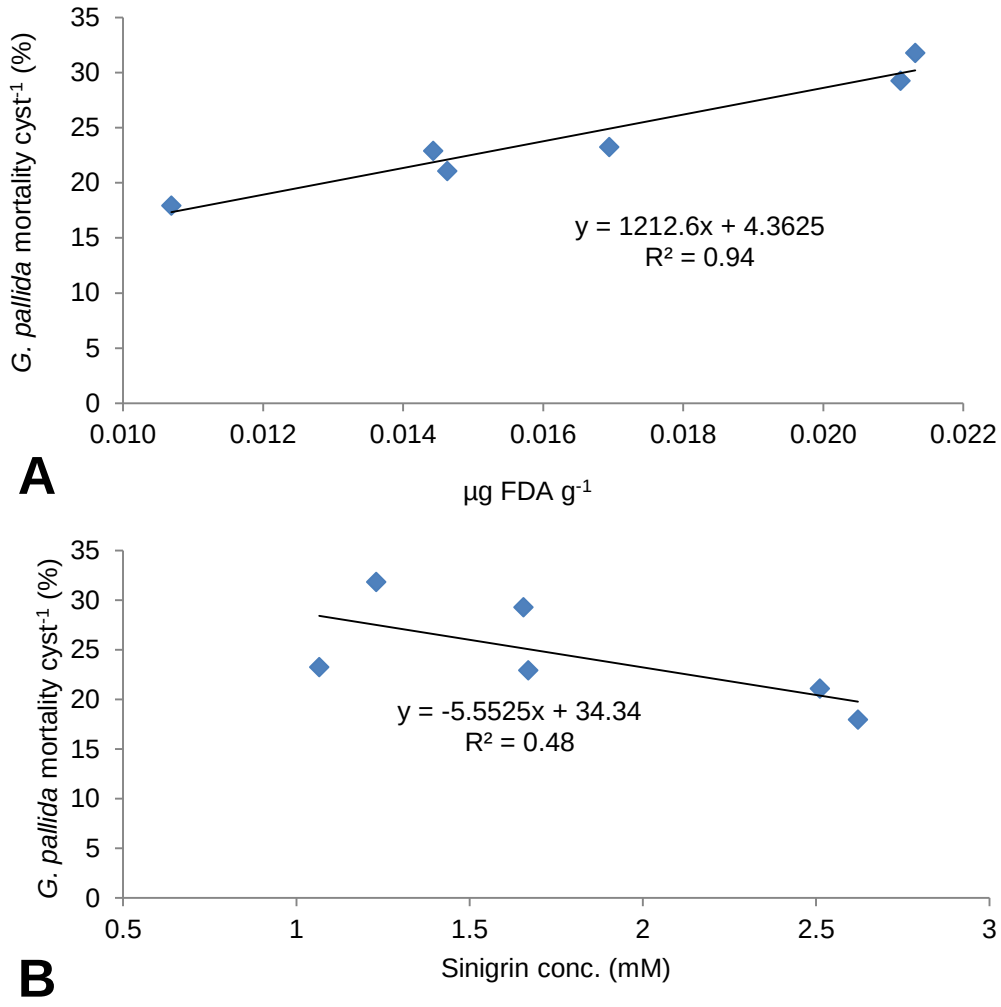


Figure 5.6: Relationships between percentage mortality of *Globodera pallida* encysted eggs cyst^{-1} and µg FDA g^{-1} (A) or concentration of re-isolated sinigrin (B) following treatments with *Brassica juncea* or *Raphanus sativus* cultivated in sterilized or unsterilized soil as compared with untreated soil

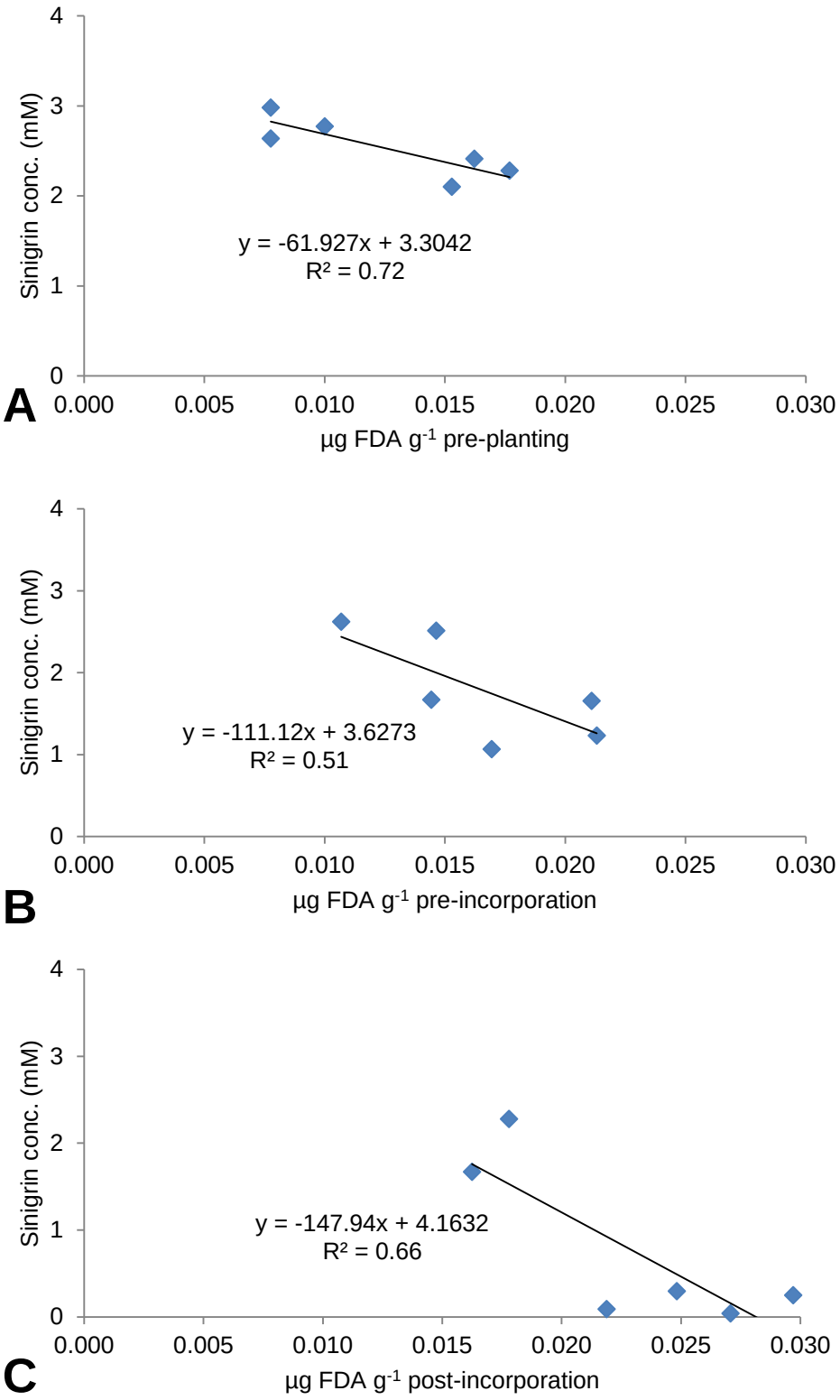


Figure 5.7: Relationships between µg FDA g^{-1} and concentration of re-isolated sinigrin (mM) following treatments with *Brassica juncea* or *Raphanus sativus* cultivated in sterilized or unsterilized soil as compared with fallow soil assessed pre-planting (A), pre-incorporation (B) and six weeks post-incorporation (C) of the brassicas

5.8 Discussion

During the present study, the cultivation and subsequent incorporation of field soil with biofumigant brassica species in the glasshouse enhanced both sinigrin hydrolysis and microbial activity. Partial biofumigation with the brassicas caused mortality of encysted eggs of *G. pallida* in unsterilized soils. The *G. pallida* mortality showed a strong positive correlation with microbial activity, demonstrating that these organisms may play a role in the suppression of *G. pallida* under brassica cover crops.

The degradation of pure sinigrin following incubation in soil previously planted with a biofumigant brassica species during the present experiments is an indication of myrosinase activity in these soils. This is clearly evident from the significant difference between the pots planted with brassicas and the fallow soil. Prior to incorporation, either sterilised or unsterilized soil samples cultivated with *R. sativus* demonstrated the greatest hydrolysis of inoculated sinigrin when compared with the rest of the treatments (Figure 5.2ii). This degradation of sinigrin increased remarkably six weeks after the soil was incorporated with the brassicaceous residues compared with the untreated pots. *Brassica juncea* and *R. sativus* treated soils were statistically similar in the hydrolysis of spiked sinigrin post-incorporation (Figure 5.2iii). These findings are similar to those reported by Rumberger and Marschner (2003) in which 2-phenylethyl GSL (gluconasturtiin) or its derivative, 2-phenylethyl isothiocyanate (PEITC) were detected in the rhizosphere of canola. These authors associated the PEGSL/PEITC production to injured cells in which the separation between GSL and myrosinase were destroyed (Bones & Rossiter, 1996) leading to GSL hydrolysis.

The degradation of sinigrin in soil post-incorporation as observed in this study was probably due to either myrosinase residues from the incorporated biomass, or

myrosinase release as a result of a build-up of myrosinase-producing soil microbes. Sinigrin was detected at very low concentrations when spiked into unsterilized soil incorporated with *B. juncea* as revealed by HPLC analysis of the re-extracted sinigrin six weeks post-incorporation (Figures 5.2iii & 5.3iii).

The total microbial activity (TMA) increased during brassica development. After incorporation, there was an increase in the TMA for all treatments including sterilized fallow soil. However, the increase in TMA post incorporation was significantly higher in unsterilized soils incorporated with either *B. juncea* or *R. sativus* compared with the sterilised fallow soil (Figure 5.4). Sterilised soil treated with *B. juncea* also significantly increased in TMA compared with the sterilised fallow soil, but not with the rest of the treatments indicating that microbial activity was enhanced by the incorporation of *B. juncea* residues.

The mortality of *G. pallida* encysted eggs cyst^{-1} as a result of partial biofumigation was significantly greater in unsterilized soil cultivated with *R. sativus* compared with sterilised fallow soil. The proportion of dead eggs cyst^{-1} strongly correlated positively with FDA concentration, implicating the soil microbial population as being significant in the mortality of *G. pallida* encysted eggs. An increasing microbial activity was inversely related with the hydrolysis of inoculated sinigrin, suggesting the presence of either GSL degrading soil microbes or myrosinase activity during the biofumigant crop growth and development. The highest microbial activity was observed in unsterilized soil cultivated with *R. sativus,* and this treatment had the lowest concentration of re-extracted sinigrin following a 96 h incubation period. Rumberger and Marschner (2003) noticed continuous release of gluconasturtiin derived ITC in the rhizosphere of canola which strongly altered the composition of the soil micro flora in their study. It is worth noting that gluconasturtiin was the dominant GSL

detected in root tissues of the *R. sativus* cultivar used in the present study, and was possibly releasing products which affected *G. pallida* and the microbial population.

Although sterilised soil cultivated with *R. sativus* demonstrated high degradation of GSL both during the crop development and after incorporation, the TMA as well as the partial biofumigation-induced mortality to *G. pallida* encysted eggs in this treatment was similar with the fallow soil treatments. Sterilised soil cultivated with *B. juncea* behaved in a similar manner, but, unlike *R. sativus*-cultivated sterilised soil treatment, *B. juncea*-cultivated sterilised soil had a significant higher microbial activity compared with sterilised fallow soil (Figure 5.4).

During the field experiments reported in Chapter 3, partial biofumigation-induced mortality of encysted eggs of *G. pallida* following *R. sativus* and *B. juncea* crop development was observed. The observations reported herein are therefore a confirmation of the observed effects in the field. The fact that there were strong positive correlation between *G. pallida* mortality and TMA suggest that the growth and development of the biofumigant brassicas may be enhancing *G. pallida* detrimental soil microbes. Although the relationship between TMA and GSL degradation was weak as observed in these experiments, the inverse relationship is an indication of myrosinase activity, thus, suggesting that part of the mortality of *G. pallida* was as a result of GSL hydrolysis in to toxic products, or a direct release of ITC from root tissues. Mortality of up to 30% was observed when *R. sativus* was cultivated in unsterilized field soil, and this percentage mortality is possibly as a result of a combination of both soil microbes and GSL hydrolysis product. However, the specific soil microbes causing mortality of *G. pallida* following brassica green manure crop development are unknown and thus merit further investigation.

CHAPTER SIX

6. Chapter 6: *In-vitro* experiments investigating dose response effect of brassicaceous extracts on encysted eggs of *Globodera pallida*

6 Toxicity of *Brassica juncea* and *Raphanus sativus* root and shoot extracts to the encysted eggs of *Globodera pallida* *in-vitro*

6.1 Introduction

Studies on the utilisation of natural and environmentally friendly options for plant pest and disease control are gaining attention due to concerns about the environmental impact of synthetic chemicals (Bocquene & Franco 2005; Coat *et al*., 2006). *Brassica* species have the ability to synthesise phytochemicals that are detrimental to certain plant pests and pathogens. Determination of effective doses of these brassica plant extracts for pest and disease control are essential for their field implementation.

Noling, (2002) categorised the toxicity induced by a plant protection product (PPP) on plant parasitic nematodes as either reversible or irreversible. In the former case, the effect of the PPP subsides upon the removal of the source of exposure, whereas in the latter case, the effect persists. Reversibility exists for certain PPP irrespective of exposure dosage (Sikora & Hartwig, 1991), whereas for others reversibility is a product of dose (concentration) and exposure time (CT) (Noling, 2002). Glucosinolate breakdown products have been demonstrated to irreversibly inhibit egg hatching of *Heterodera glycines* (Tylka *et al*., 1997). There are also reports of a dose dependent reversibility in potato cyst nematodes activity with plant extracts (Danquah *et al*., 2011). The ability of a pest or pathogen to be able to resume normal activity after previously being inhibited by a PPP should therefore be carefully considered when designing PPP to control individual targets.

Toxicity is normally represented graphically as the change in the effect on the organism caused by the different levels of exposure (dose) to the PPP over a certain exposure time. Here, the acute toxicity depicts the dose at which 50% of the response population is inhibited or killed, generally abbreviated as LD_{50} or EC_{50} to mean lethal dose or effective concentration respectively.

Previous experiments investigating the LD or EC of brassicaceous plant extract on nematodes have often focused on second stage juveniles (J2) or free-living stages (Pinto *et al*., 1998; Serra *et al*., 2002; Buskov *et al*., 2002; Yu *et al*., 2007; Zasada *et al*., 2009). However, effective doses determined using free-living stages of plant parasitic nematodes may not represent similar effectiveness on encysted eggs, as in the case of PCN, the eggs are protected within the cyst. Therefore, separate studies are necessary when determining the LD of brassicaceous plant extracts for both free-living and cyst nematodes.

6.2 Aim:

The aim of the studies reported in this chapter was to investigate the hatching activity of *G. pallida* encysted eggs in PRL following exposure to different concentrations of *B. juncea* or *R. sativus* leaf or root extracts under *in vitro* conditions. The viability of unhatched *G. pallida* eggs cyst^{-1} after six weeks of hatching in PRL was determined using Meldola's Blue to differentiate between viable and non-viable eggs.

6.3 Objectives:

i. To determine effective concentration of biofumigant brassicaceous leaf or root extracts for the management of *G. pallida* encysted eggs *in vitro*

ii. To determine the brassicaceous extracts that are more toxic to *G. pallida* encysted eggs at lower concentrations *in vitro*

iii. To determine the type and concentrations of the GSL responsible for the toxicity in the different extracts

6.4 Hypothesis (null):

The hatching of *G. pallida* in potato root leachates will not be affected by pre-exposure to different doses of *B. juncea* and *R. sativus* leaf and root extracts.

6.5 Materials and methods

6.5.1 Treatments

In order to determine the level of efficacy of *Brassica juncea* and *Raphanus sativus* root and leaf extracts, four experiments were initiated. These experiments involved exposing *G. pallida* cysts to concentrations of either *B. juncea* or *R. sativus* leaf or root extracts ranging from 3.2 – 100%, with sterile distilled water (DW) as a control (Table 6.1). The treatments for these experiments were replicated four times and the experiments were repeated to check for consistency.

Extracts of *B. juncea* and *R. sativus* were selected for these experiments as these were used in the field experiment (Chapter 3) and observed to be effective against *G. pallida.* Additionally, HPLC analysis (Chapter 3) demonstrated a significant variation in the types and concentrations of GSL present in the different parts (root/shoot) of the same *Brassica* species.

Table 6.1: *In vitro* experiments and the respective concentrations (treatments) of the biofumigant brassicaceous extracts measured in mg ml^{-1} weight by volume (w/v)

Experiment	Treatment	Extract	Concentrations (mg ml^{-1} w/v)						
1	*B. juncea*	Shoot	1.0	0.5	0.25	0.125	0.063	0.031	0
2	*B. juncea*	Root	1.0	0.5	0.25	0.125	0.063	0.031	0
3	*R. sativus*	Shoot	1.0	0.5	0.25	0.125	0.063	0.031	0
4	*R. sativus*	Root	1.0	0.5	0.25	0.125	0.063	0.031	0

6.5.2 Preparation of brassica root and leaf extracts

Brassica plants were collected from field experiment 3 just before incorporation and processed as previously described in Chapter 4 (Ngala *et al*., 2014) and stored below -18°C in polypropylene screw cap tubes until required. Extracts were prepared by dissolving the respective quantities (Table 6.1) of the milled freeze-dried powder in

1.5 ml Eppendorf tubes with 1 ml distilled water, caped and homogenised to form a suspension (Plate (6.1).

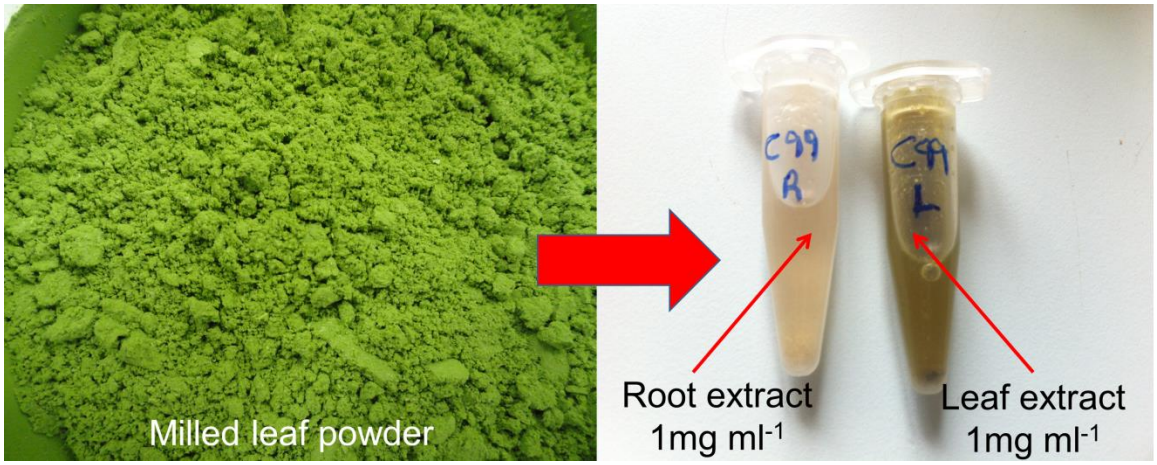


Plate 6.1: Biofumigant brassicaceous plant extract preparation from milled freeze-dried plant material

6.5.3 Preparation of potato root leachates (PRL) for *G. pallida* hatching assays

Leachates collected from disease-free potato tubers (*Solanum tuberosum* cv Estima) were used to activate hatching of *G. pallida in vitro*. In order to obtain leachates from the potato plants, sprouted tubers of Estima were planted in 1.7 l pots three-quarter filled with sterilized silver sand and maintained in the glasshouse at a day/night temperature of 18/5 ±2°C respectively under a 14 h photoperiod. All pots received 200 ml of tap water after every 3 days to maintain moisture. After a period of six weeks, the pots were suspended on 3 l plastic beakers and saturated with 500 ml of tap water to allow for leachate collection in the beakers (Widdowson, 1958). The collected leachates were combined and passed via a doubled Genuine Whatman Nº 5 filter paper (W. & R. Balston LTD., England), stored below 4°C in the dark for up to six months. When required for hatching, the leachate was diluted to 20% (v/v) with sterile distilled water.

6.5.4 Extraction of *G. pallida* from soil

The cysts used in these experiments were from the same population of *G. pallida* collected from a nematicide-free site (Grid reference: SJ 668 186, Shropshire, UK) and extracted from soil with the aid of a Fenwick can (Fenwick, 1940). Quantitative-Polymerase Chain Reaction (q-PCR) analysis (Appendix 9.5) confirmed the cyst population as pure *G. pallida*. The initial viability of the cysts was determined as ≥ 80% via hatching assays and Meldola's blue stain as previously described (see Chapter 2). The number of eggs per cyst was determined by selecting and soaking four batches of 10 cysts of similar sizes (400 ≤ 600 µm) in sterile DW following the procedure outlined in Chapter 2; section 2.4. The cysts were placed in sealed glass jars, appropriately labelled and stored in the dark at 8°C for experimental usage when required.

6.5.5 Hatching activity of *G. pallida* encysted eggs in PRL following exposure to brassicaceous root or leaf extracts

These experiments were carefully planned with special attention to the fact that enzymatic hydrolysis of GSL begins immediately after exposure of the freeze-dried plant material to water. Therefore, in order to determine the toxicity of the brassicaceous roots or leaf extracts on *G. pallida* encysted eggs, ten cysts each were pre-mixed with the different quantities (Table 6.1) of brassicaceous root or leaf powder in 1.5 ml Eppendorf tubes before adding 1 ml of sterile distilled water (DW). Following the addition of DW, the tubes were immediately caped (to trap the ITC released) and homogenised to form a suspension. The setup was incubated at 16±1°C for 96 h before the cysts were collected with the aid of a pair of forceps and rinsed with DW before transferring into 1.5 ml Eppendof tubes with the standard lids replaced with a 250 µm aperture mesh and the top cut open to fit in a 3 ml 24-well plastic plate. The modified tubes containing the re-isolated cysts were placed into

appropriately labelled 24-well plastic plate with wells of 3 ml capacity previously half-filled with 1.5 ml of 20% v/v potato root leachate (PRL). The lid of the 24-well plate was replaced, sealed with parafilm and incubated in the dark at 16±1°C to simulate the field conditions during late spring. The numbers of hatched *G. pallida* juveniles (J2) were scored at weekly (7 days) intervals and the PRL was refreshed at each assessment. Each experiment was monitored for six weeks before the cysts were stained in 1 ml 0.05% w/v Meldola's blue staining solution (Sigma Aldrich, Poole, UK) for 7 days following the procedure of Shepherd (1962) to distinguish the number of un-hatched viable eggs from non-viable eggs at the end of the hatching assays (Figure 6.1).

6.5.6 Glucosinolate extraction and analysis using HPLC

In a bid to determine the profiles of the glucosinolates (GSL) present in the biofumigant brassicaceous plant material used, sub-samples of the milled freeze-dried plant material were extracted and analysed using HPLC technique. The procedures followed were the same as previously described in Chapter 4.

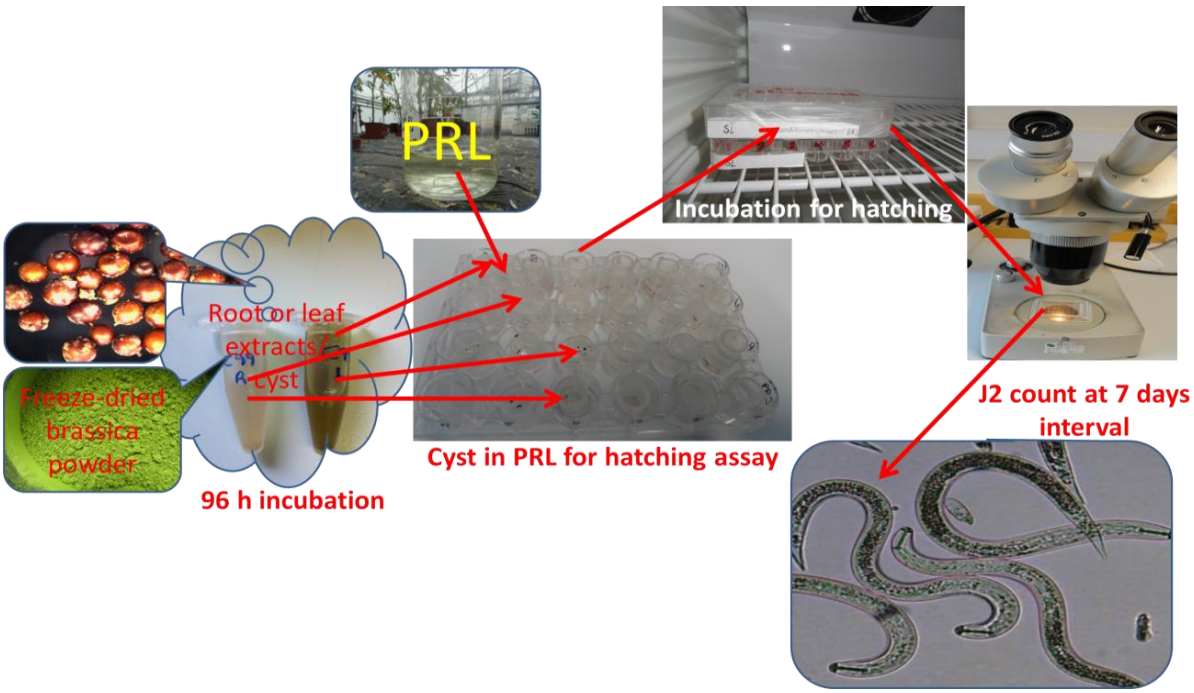


Figure 6.1: An illustration of the hatching assays with *Globodera pallida* encysted eggs in potato root leachates (PRL) following a 96 h exposure to brassicaceous root or leaf extracts

6.6 Statistical analysis

All data were subjected to a general analysis of variance (ANOVA) using GenStat® (15th Edition) statistical software. Probit analysis was performed to determine the LD_{50} for the different brassicaceous extracts. Cumulative percentage hatching in time were calculated and cumulative hatching curves constructed. Where necessary, data were log_{10}-transformed to normalize residuals. Significant differences between treatments were reported at 5% significance level using Tukey's multiple range tests.

6.7 Results

6.7.1 Mortality of *G. pallida* encysted eggs following exposure to brassicaceous leaf or root extracts

The hatching of *G. pallida* encysted eggs in potato root leachates (PRL) was completely prevented after the cysts were previously exposed to 25 - 100% (0.25 - 1.0 mg ml^{-1} w/v) concentrations of leaf or root extracts of *B. juncea* (Figure 6.2). When previously exposed to 50% w/v concentrations of either leaf or root extracts of *B. juncea*, approximately 100% mortality of *G. pallida* encysted eggs was observed, whereas exposure to 100% w/v of these extracts induced 87% mortality while irreversibly paralysing the remaining 13% of the encysted eggs. Concentrations of 12.5% (0.125 mg ml^{-1} w/v) of *B. juncea* leaf extract accounted for more than 85% mortality of *G. pallida* encysted eggs, whereas 3.1 – 6.3% (0.031 - 0.063 mg ml^{-1} w/v) of *B. juncea* root and leaf extracts accounted for approximately 70% mortality of *G. pallida* encysted eggs (Figure 6.2).

Raphanus sativus leaf extract was effective only at higher concentrations of 50 - 100% (0.5 - 1.0 mg ml^{-1} w/v) accounting for approximately 60% reduction in hatching of *G. pallida* encysted eggs, of which 45% were found to be non-viable and 16 % still viable but did not hatch at the end of the sixth week (Figure 6.3i). Root extracts of *R. sativus* however, were observed to be similar with *B. juncea* extracts in toxicity

induction to *G. pallida* encysted eggs with previous exposure to concentrations of 25% w/v and above completely preventing hatching of *G. pallida* (Figure 6.3ii).

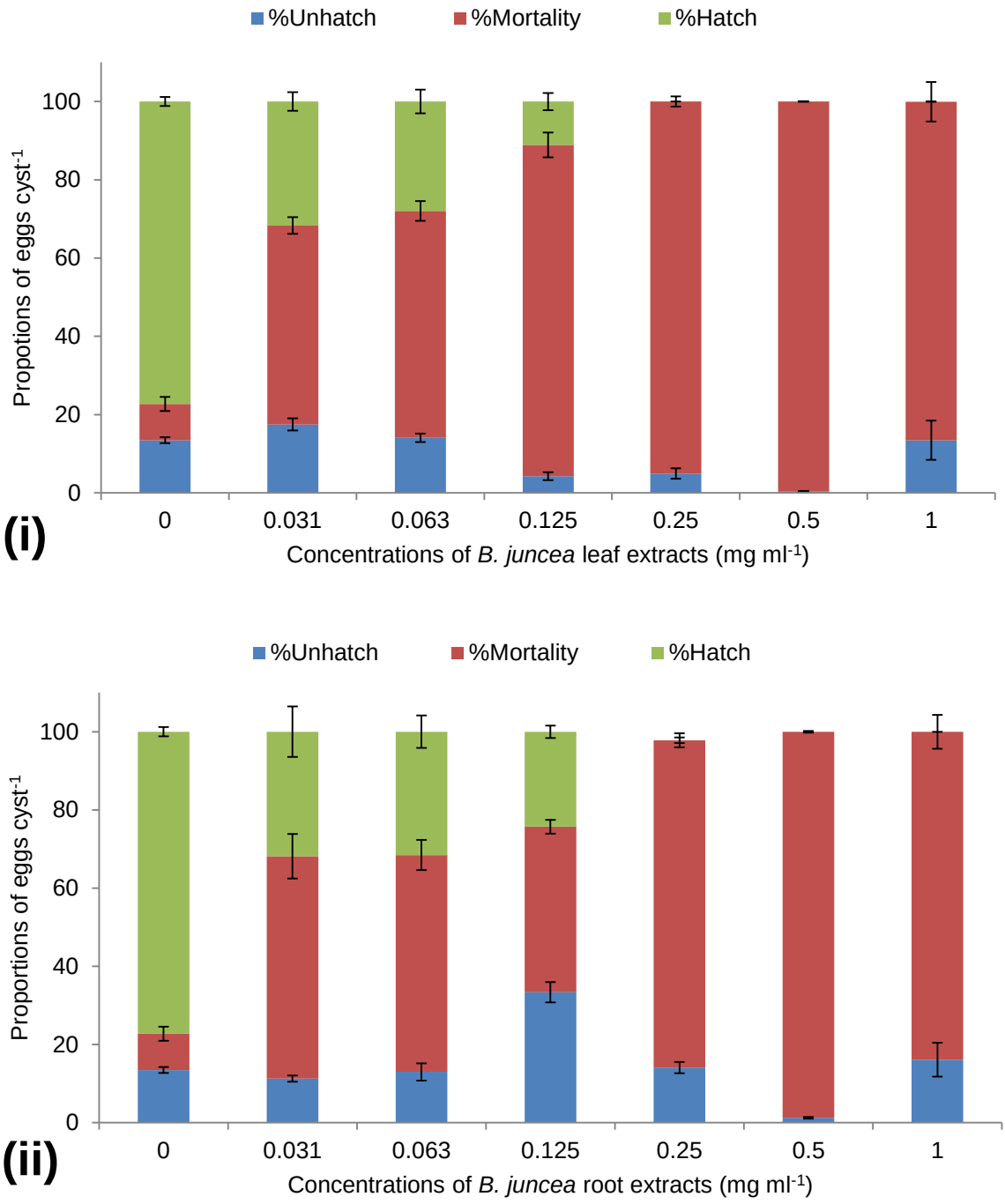


Figure 6.2: Proportions of hatched (green bars), unhatched-viable (blue bars) and dead (red bars) *Globodera pallida* eggs cyst^{-1} in potato root leachates following 96 h exposure to different concentrations (3.1 - 100% w/v) of (i) root or (ii) leaf extracts of *Brassica juncea* as compared with distilled water (DW) control

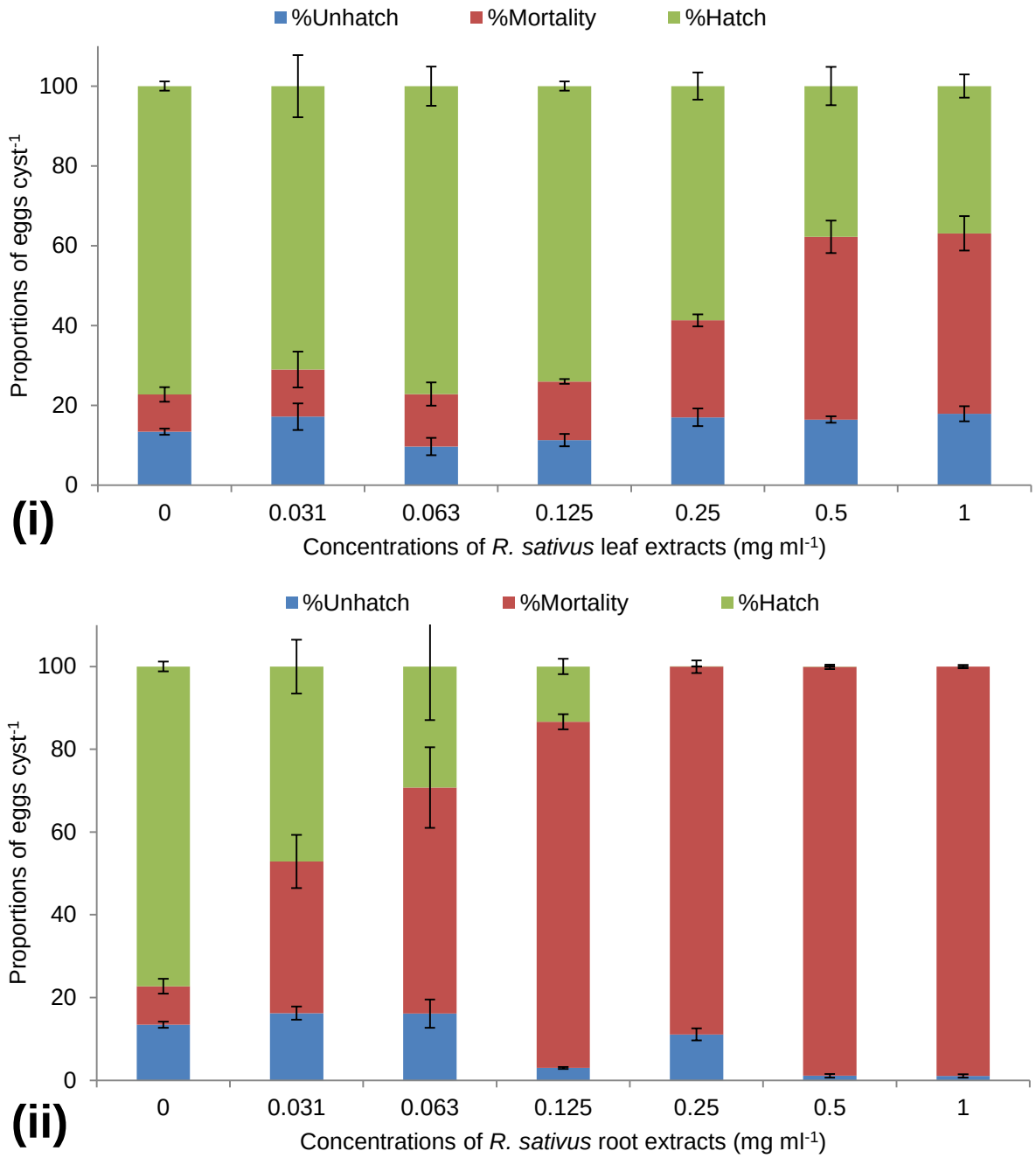


Figure 6.3: Proportions of hatched (green bars), unhatched-viable (blue bars) and dead (red bars) *Globodera pallida* eggs cyst^{-1} in potato root leachates following 96 h exposure to different concentrations (3.1 - 100% w/v) of (i) leaf or (ii) root extracts of *Raphanus sativus* as compared with distilled water (DW) control

6.7.2 Effect of different concentrations of brassicaceous leaf or root extracts on hatching behaviour of *G. pallida* encysted eggs in PRL

After exposing encysted eggs of *G. pallida* to distilled water (DW) for 96 h, more than 75% of eggs cyst^{-1} hatched in PRL during the first 3 weeks, after which hatching

reduced to less than 2% in subsequent assessments undertaken during the following three weeks. However, when exposed to different biofumigant brassicaceous plant extracts, the hatching activity of *G. pallida* differed depending on the extract and concentrations used.

For *B. juncea* leaf extracts, hatching of *G. pallida* was completely prevented in concentrations from 25% w/v (0.25 mg ml^{-1}) and above. When exposed to 12.5% w/v (0.125 mg ml^{-1}), hatching was delayed for four weeks, and thereafter, just 10% of J2 hatched during the final two weeks of assessment. Concentrations of 3.1 – 6.3% w/v (0.031 – 0.063 mg ml^{-1}) delayed hatching for one week, followed by slow hatching during the following five weeks of assessment, accounting for a cumulative hatch of approximately 30% at the end of the sixth week (Figure 6.4A).

Hatching in PRL after previous exposure to *B. juncea* root extracts followed a similar pattern as with *B. juncea* leaf extract. However, unlike the later, hatching activity began slowly from the first week in the former following exposure to concentrations of 3.1, 6.3 and 12.5% w/v, with 12.5% w/v resulting in a cumulative hatch of approximately 25% by the end of the sixth week compared to 10% cumulative hatch in the leaf extract. A concentration of 25% w/v delayed hatch for 4 weeks and the cumulative hatch was approximately 2% by the end of the sixth week (Figure 6.4B).

Pre-exposure to 6.3% w/v (0.063 mg ml^{-1}) *R. sativus* leaf extract resulted in a cumulative hatch of approximately 77% within the first three weeks. Hatching activity of *G. pallida* in PRL following exposure to 3.1 or 12.5% w/v was similar to DW during the first three weeks of assessment. The cumulative hatch in PRL at the end of the sixth week was approximately 5% lower following exposure to 3.1% w/v of *R. sativus* leaf extract as compared with DW control. With pre-exposure to 25% w/v *R. sativus* leaf extract, hatching was delayed for one week, and the cumulative hatch was

approximately 58% by the end of the sixth week. Pre-exposure to 50 or 100% w/v resulted in a cumulative hatch of approximately 38% in PRL at the end of the sixth week (Figure 6.5A).

Unlike the leaf extract, *R. sativus* root extract completely prevented hatching of *G. pallida* at concentrations ≥ 25% w/v. Pre-exposure to concentrations of 3.1, 6.2, or 12.5% w/v of *R. sativus* root extract resulted in a cumulative hatch of 47, 29 and 13% respectively at the end of the sixth week (Figure 6.5B).

A general ANOVA comparing the means of hatched J2 for the different extracts revealed similarity between *B. juncea* leaf/root or *R. sativus* root extracts, resulting in a mean cumulative hatch of approximately 16% J2 cyst^{-1}. Pre-exposure to *R. sativus* leaf extract resulted in a mean cumulative hatch of approximately 59% compared with 78% for DW (Figure 6.6). The different concentrations used followed the same pattern when the experiments were repeated in time (Figure 6.7; Table 6.2). The LD_{50} for *B. juncea* leaf/root and *R. sativus* root extracts were similar unlike for *R. sativus* leaf extract that had a higher LD_{50} value (Table 6.3).

Table 6.2: Summary analysis of variance (ANOVA) for different experiments with different concentrations (mg ml^{-1}) of *Brassica juncea* and *Raphanus sativus* leaf or root extracts each repeated in time (1 & 2). Deferent letters represents significant differences in mean hatched juveniles (J2) cyst^{-1} between concentrations according to Tukey's test ($P < 0.05$)

Dose (mg ml^{-1})	*B. juncea* leaf		*B. juncea* root		*R. sativus* leaf		*R. sativus* root	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
0.0%	a	a	a	a	ab	a	a	a
3.1%	b	b	ab	b	ab	b	b	a
6.3%	bc	b	bc	b	ab	a	ab	b
12.5%	c	c	c	c	b	a	bc	b
25.0%	c	c	c	c	b	ab	cd	b
50.0%	c	c	c	c	ab	b	d	b
100.0%	c	c	c	c	b	b	d	b
***P*-value**	< 0.001	< 0.001	< 0.001	< 0.001	0.011	< 0.001	< 0.001	< 0.001
SEM (*df*=21)	67.3	156.3	89.9	200.5	133.6	326.2	79.7	235.1
CV%	62.5	33.9	65.5	37.5	32	26.1	41.7	41.7

Table 6.3: The lethal doses (LD_{50}) for *Brassica juncea* and *Raphanus sativus* leaf (L) or root (R) extracts (mg ml^{-1}) against encysted eggs of *Globodera pallida in-vitro.* Different superscript letters represent significant differences in LD_{50} for the different extracts according to Tukey's test ($P < 0.05$)

Extracts	LD_{50}	SEM	Lower 95%	Upper 95%
B. juncea-L	0.027[a]	0.0040	0.027	0.028
B. juncea-R	0.032[a]	0.0054	0.031	0.033
R. sativus-L	0.546[b]	0.004	0.538	0.555
R. sativus-R	0.035[a]	0.0037	0.034	0.036

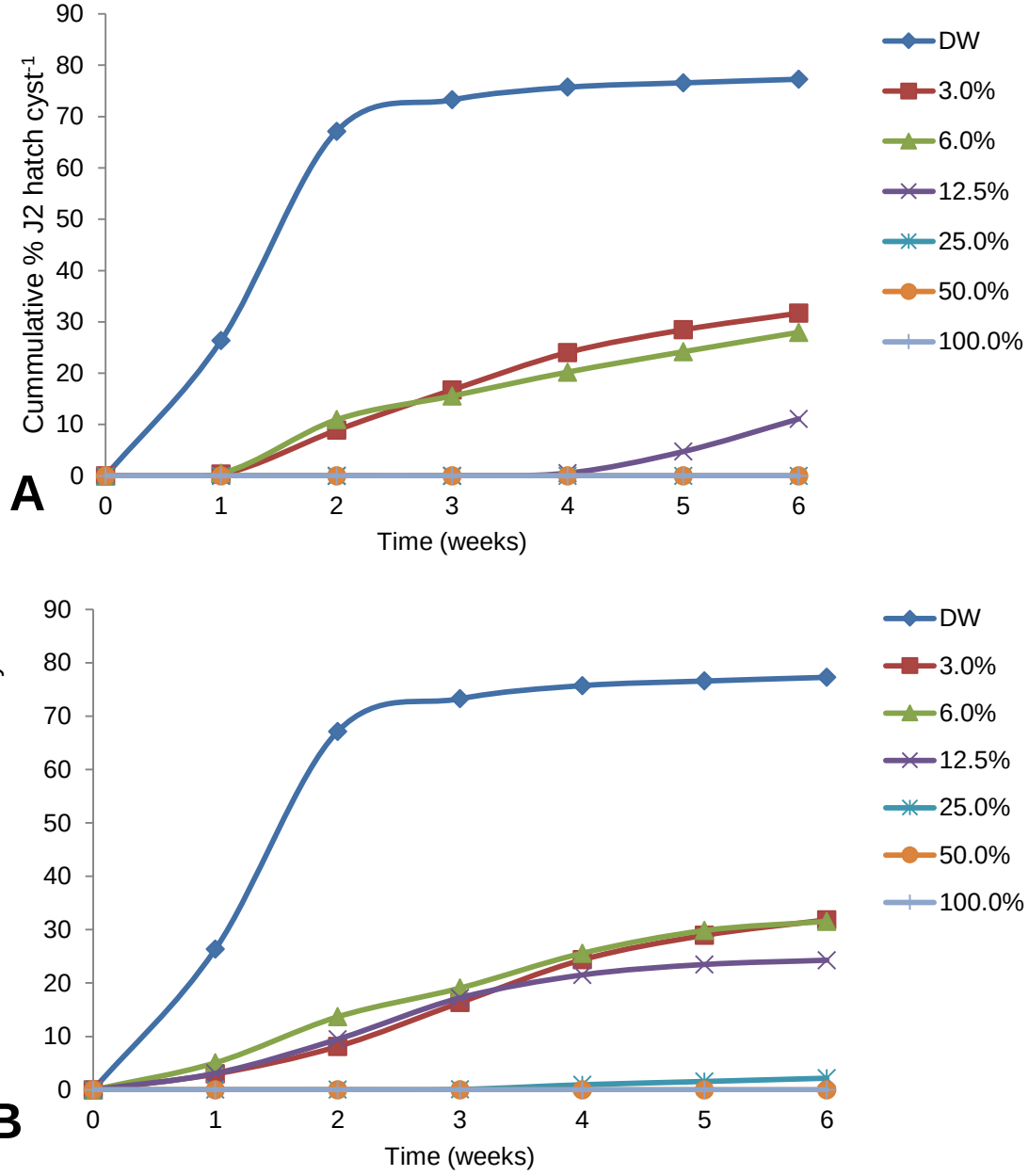


Figure 6.4: Cumulative % hatch *G. pallida* juveniles (J2) in potato root leachate (PRL) following 96 h exposure to different concentrations (3.1 - 100% w/v) of *Brassica juncea* (A) leaf or (B) root extracts as compared with distilled water (DW) control

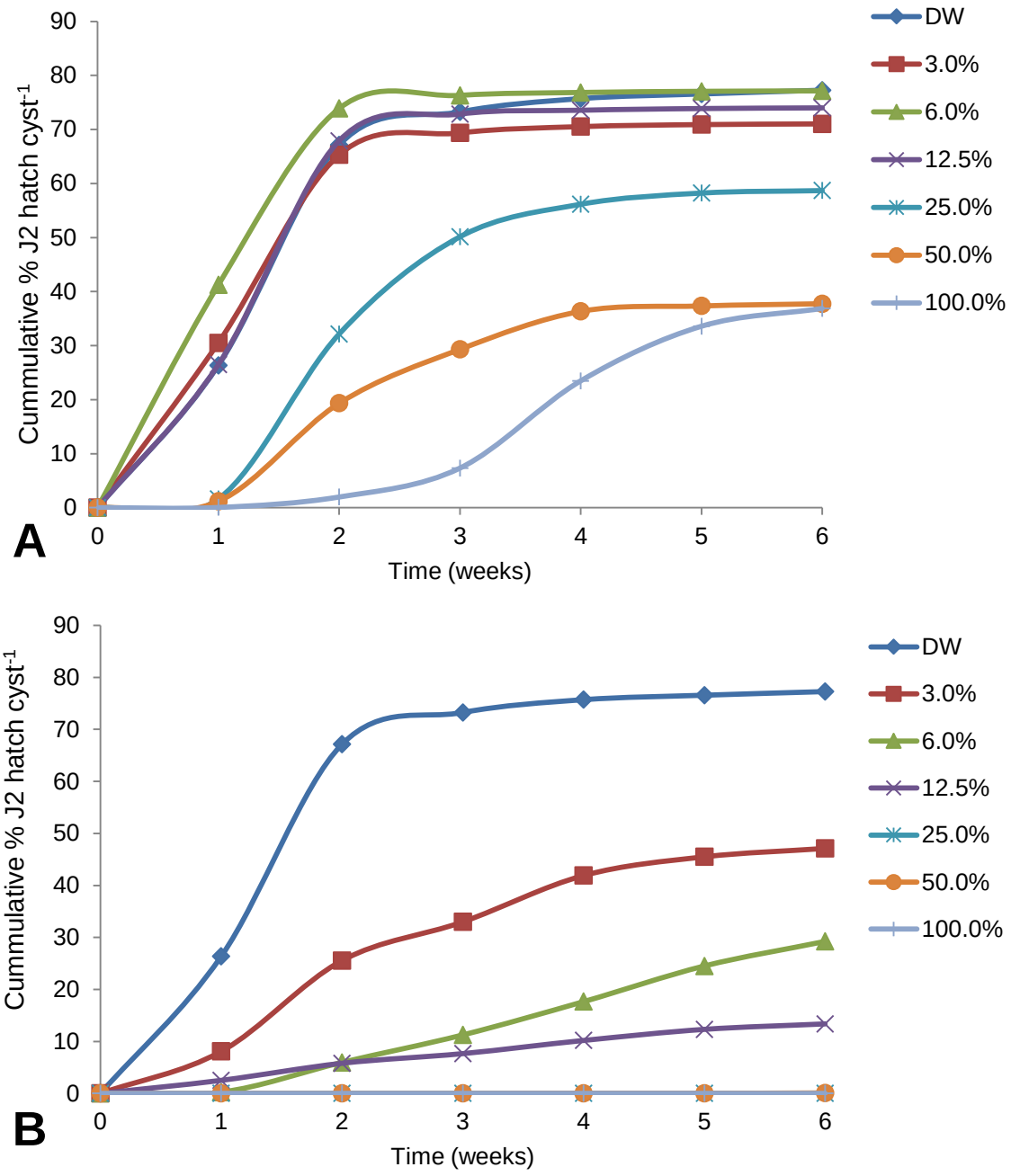


Figure 6.5: Cumulative % hatch of *Globodera pallida* juveniles (J2) in potato root leachate (PRL) following 96 h exposure to different concentrations (3.1 - 100% w/v) of *Raphanus sativus* (A) leaf or (B) root extracts as compared with distilled water (DW) control

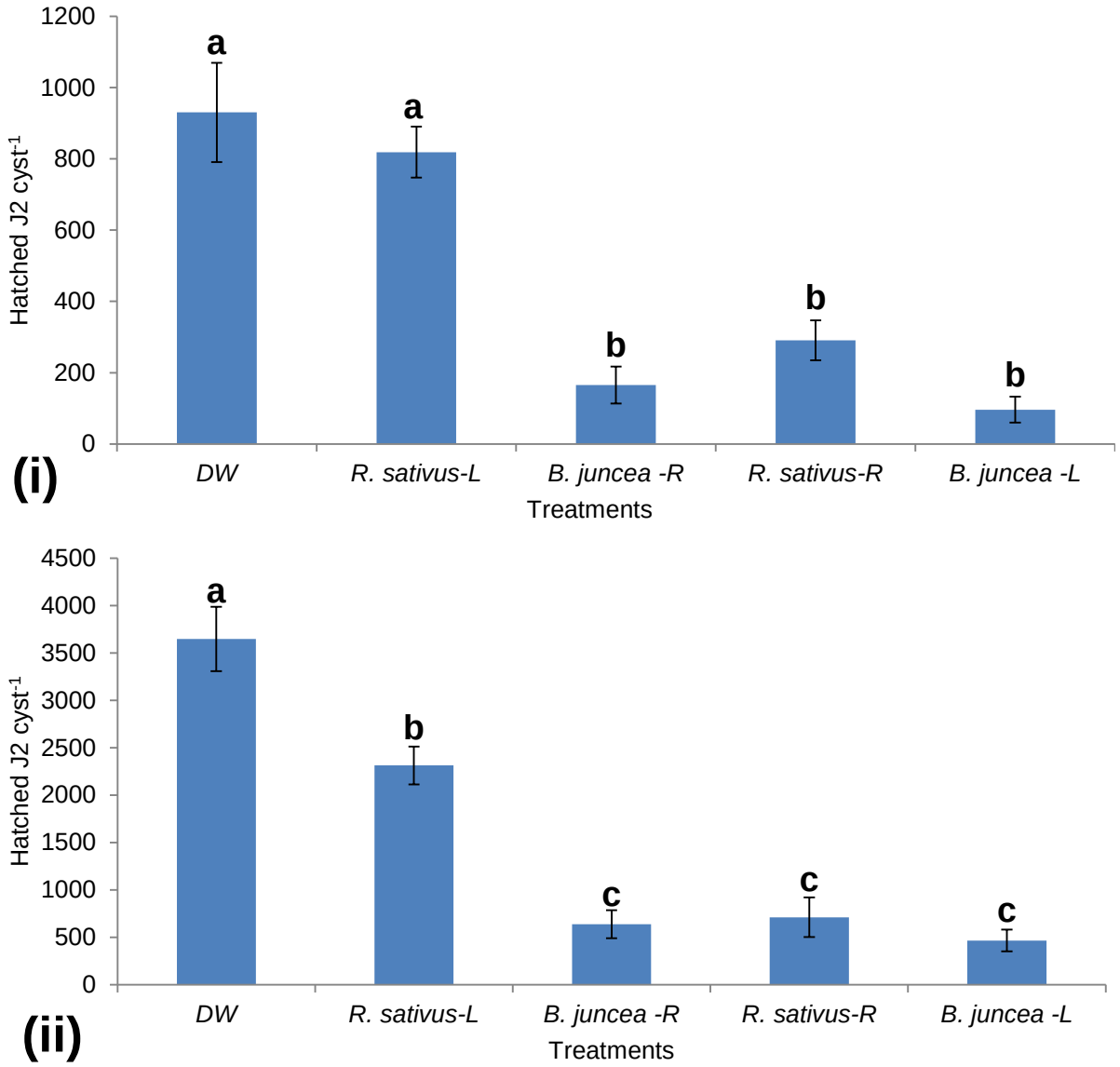


Figure 6.6: Hatched *Globodera pallida* second stage juveniles (J2) cyst^{-1} in potato root leachates (PRL) following 96 h exposure to root (R) or leaf (L) extracts of *Brassica juncea* or *Raphanus sativus* as compared with distilled water (DW) control in (i) Experiment-1 and (ii) Experiment-2. Different letters represent significant differences (Tukey's test: $P < 0.05$) between extracts. Error bars represents standard error of means

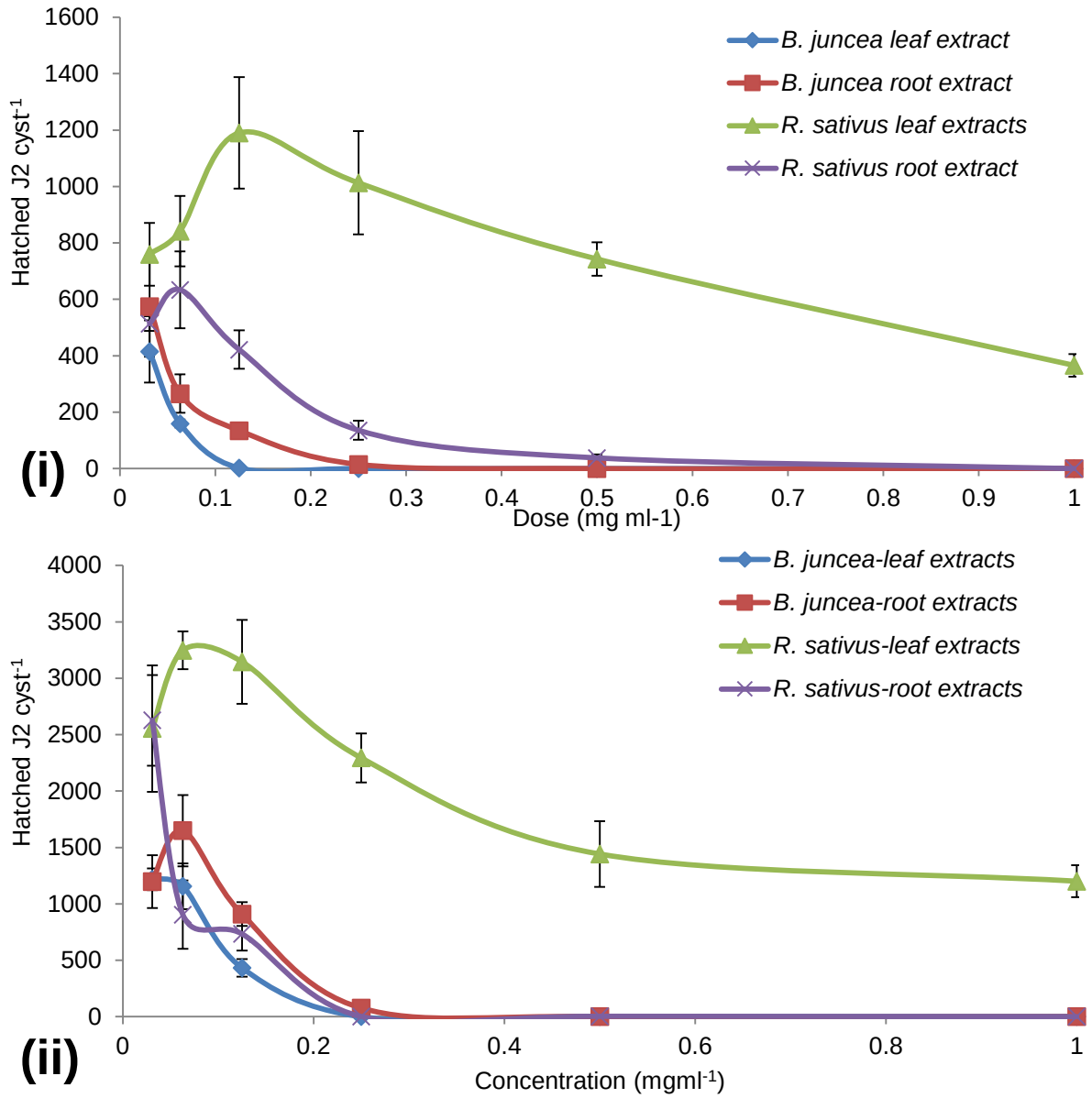


Figure 6.7: Hatched *Globodera pallida* second stage juveniles (J2) cyst^{-1} in potato root leachates (PRL) following 96 h exposure to different doses (0.031 – 1 mg ml^{-1}) of root or leaf extracts of *Brassica juncea* or *Raphanus sativus* in (i) Experiment-1 and (ii) Experiment-2. Error bars represents standard error of means

6.7.3 Glucosinolate profile in the brassicaceous leaf or root extracts

The total GSL concentration in the *B. juncea* leaf tissue used for these experiments was approximately 94 µmol g^{-1} dry weight (dw), with sinigrin alone accounting for 90 µmol g^{-1} dw. The *B. juncea* root tissue was also predominated by sinigrin, but unlike the leaf tissues, root tissues had an approximate total GSL concentration of 14 µmol g^{-1} dw, of which approximately 12 µmol g^{-1} dw was sinigrin. In *R. sativus* leaf tissue glucoraphanin was the abundant GSL, whereas the root tissue was dominated by

gluconasturtiin. The total GSL in *R. sativus* root tissue was two folds that of its leaf tissue (Table 6.4).

Table 6.4: Glucosinolate profiles (µmol g^{-1} dry weight) in *Brassica juncea* and *Raphanus sativus* foliage and root tissues used for the *in-vitro* experiments

Glucosinolates	*B. juncea*		*R. sativus*	
	Leaf	**Root**	**Leaf**	**Root**
Sinigrin[a]	90.2	12	-	-
Glucoraphanin[a]	-	-	25.4	-
Gluconasturtiin[r]	-	-	-	53.6
Neoglucobrassicin[i]	-	1.3	3.9	3.3
Unknown-1	3.1	-	-	-
Unkwon-2	-	-	2.4	3.6
Total	**93.3**	**13.3**	**29.3**	**60.5**

a: aliphatic, r: aromatic, i: indole

6.8 Discussion

The profiles of glucosinolates (GSL) occurring in the different plant tissue and the toxicity of their respective isothiocyanates (ITC) to the target organism provide useful information on which plant regions emphasis should be focused upon when applying the biofumigation system. Nevertheless, knowledge of the amount of fresh or dry matter to be incorporated is equally as important. An *in vitro* assessment of different concentrations of leaf or root extracts of *B. juncea* and *R. sativus* was conducted to determine the effective concentrations conferring toxicity to encysted eggs of *G. pallida*. *Brassica juncea* root or leaf extracts effectively inhibited hatching of *G. pallida* encysted eggs at lower concentrations (Figure 6.2). *Raphanus sativus* root extracts were effective against *G. pallida* encysted eggs, but the leaf extracts were effective to a lesser extent (Figure 6.3). The LD_{50} for *B. juncea* leaf or root extracts were respectively 0.027 and 0.031 mg ml^{-1}, whereas that for *R. sativus* was 0.035 mg ml^{-1} for root and 0.546 mg ml^{-1} for leaf extracts.

The reduction of *G. pallida* hatching following exposure to lower concentrations of root extracts in this study may in part explain field observations (Chapter 3) where a

reduction of PCN viability was observed during the crop growth and development (partial biofumigation). The underlying mechanisms may be the result of root exudation of GSL alongside the myrosinase enzyme, or that root-exuded GSL are possibly being hydrolysed in the rhizosphere by myrosinase producing soil microbes as discussed previously (Chapter 3).

According to Noling (2002), secondary by-products such as volatile ITC which are released upon the hydrolysis of GSL directly penetrate nematode body wall and interfere with vital processes including enzymatic, nervous, and respiratory systems. This normally results into permanent paralysis or rapid death of the nematode within minutes of exposure depending on the dose and exposure time. The high mortality of *G. pallida* encysted eggs observed in this study with the brassicaceous plant extracts can therefore be attributed to the release of ITC upon enzymatic hydrolysis of the GSL found in the plant tissues. Analysis of GSL using HPLC revealed leaf and root tissues of *B. juncea* to be rich in 2-propenyl-GSL (sinigrin, Table 6.4), which is known to release 2-propenyl-ITC upon enzymatic hydrolysis (Kissen & Bones, 2009). *Raphanus sativus* root tissue was rich in 2-phenylethyl-GSL (gluconasturtiin, Table 6.4), which hydrolyses to release 2-phenylethyl-ITC (Kissen & Bones, 2009). These hydrolytic produces are well known for their toxicity to plant parasitic nematodes (Pinto *et al*., 1998; Serra *et al*., 2002; Buskuv *et al*., 2002).

Hatching activity of *G. pallid*a in PRL following exposure to *R. sativus* leaf extract concentrations ≤ 0.125 mg ml^{-1} w/v was comparable with DW control (Figure 6.5A). Previous exposure of *G. pallida* to lower concentrations (0.062 mg ml^{-1} w/v) of *R. sativus* leaf extracts enhanced hatching in PRL within the first three weeks of assessments (Figure 6.5A). These observations are similar to those reported by Yu *et al*. (2005) with ally-ITC which showed that hatching of *H. schachtii* was enhanced

following exposure to a low dose of ally-ITC. With higher concentrations of *R. sativus* leaf extracts (50 – 100% w/v), 45% mortality of *G. pallida* encysted eggs cyst^{-1} was observed (Figure 6.3i), with a reversible effect of up to 55% eggs cyst^{-1} (Figure 6.5A). The leaf tissues of *R. sativus* produced predominantly 4-methylsulfinylbutyl-GSL (glucoraphanin) (Table 6.4), which yields 4-methylsulfinylbutyl-ITC upon hydrolysis. The toxicity of 4-methylsulfinylbutyl-ITC has not previously been reported for pest control, but there is evidence of a reversible decrease in colonization of gastric cells by *Helicobacter pylori* in mice following feeding with glucoraphanin-rich broccoli sprouts (Yanaka *et al*., 2009). This observed reversibility in PCN hatching indicates that, to control PCN, glucoraphanin producing plants such as *R. sativus* leaf tissues must be incorporated into an integrated pest management scheme as an ongoing option. As such, a continuous exposure of PCN to glucoraphanin producing plants may lead to permanent disruption of the nematode's sensory perception to the host plant signals, which would lead to starvation and death.

To enhance the chance of survival and host root finding, hatching in PCN is closely synchronised with the presence of the host plant such that majority of the J2 hatch rapidly when signals from the host plant roots are detected. This is because increased concentrations of sesquiterpenes, particularly solavetivone, are released in potato plant roots elicited by pathogenic infection (Aliferis & Jabaji, 2012). For *G. pallida*, the majority of J2 normally hatch within three weeks of exposure to host root exudate, beyond which, hatching is paused (Woods *et al*., 1999). Any viable eggs left within cysts after this period are said to be in a state of diapause. This simulation of hatch is possibly a mechanism developed by *G. pallida* to maximize the survival and host root location by its J2. Within the cyst, a small proportion of viable PCN eggs remain dormant requiring additional stress factor to induce hatch. Such stress

induction usually increases hatch by inducing hatching in eggs that otherwise would not hatch spontaneously (Hominick *et al*., 1985). The hatch induction of J2 by lower concentration of *R. sativus* leaf extracts (6.3% w/v) over the DW treatments in this study may be due to a response by dormant eggs to stress compounds present in the leaf extract. These observations could be utilise in an advantage as a 'Push' factor in conjunction with recent advances in the chemistry of hatching factors such as the synthesis of the key hatching-stimulant, solanoeclepine A (Tanino *et al*., 2011), as a 'Pull' factor. Utilisation of this 'Push' and 'Pull' system in field as a pre-plant application to stimulate PCN hatching without a suitable host would reduce the initial population.

In conclusion, *B. juncea* extracts provide a possible natural alternative to synthetic soil fumigant nematicides for use in an integrated management scheme for PCN. The low concentrations of the leaf extract (3.1 – 12.5% w/v) accounting for over 70% mortality observed in the present study are feasible concentrations that could be achievable under field conditions. This is considering a typical yield of 23 kg fresh matter m^{-2} for *B. juncea* foliage grown for eight to nine weeks in the field (Chapter 3) and foliar GSL concentration of 90 – 120 μmol g^{-1} (Chapter 4). Yield of four tonnes dry matter ha^{-1} for *B. juncea* foliage in the field has also been reported (Larkin *et al*., 2007; Motisi *et al*., 2009; Friberg *et al*., 2009) as well as yields of around ten tonnes dry matter ha^{-1} (Lazzeri *et al.,* 2009). The high toxicity exhibited by *R. sativus* root extract would suggest that it would be useful to manipulate the proportion of below ground biomass production by this crop. Reducing the seed rate of *R. sativus* would allow for maximum belowground biomass production which when the above ground biomass is treated with a foliar application of herbicide, would enhance the decay of the roots (personal observations). The decay of below ground biomass is often

characterised by unpleasant odours (personal observations), possibly resulting from the activation of the GSL-myrosinase system. The GSL's found in the roots of *R. sativus* appear to be underutilised in current biofumigation systems.

CHAPTER SEVEN

7. Chapter 7: General discussion

7 General discussion

Prior to the present studies, there has been little information on the effectiveness of the biofumigation system to manage potato cyst nematode (PCN) infestations under field conditions. Success stories have been documented in *in-vitro* studies with second stage juveniles (Pinto *et al*., 1998; Serra *et al*., 2002; Buskov *et al*., 2002; Yu *et al*., 2007; Zasada *et al*., 2009) and in glasshouse pot experiments with encysted eggs of PCN (Lord *et al*., 2011). Based on existing literature, the need for a comprehensive study to fully understand the feasibility of the biofumigation system as part of an integrated PCN management scheme was clear (Lord *et al*., 2011). This project was initiated with field experiments in 2011 and 2012 conducted during different seasons in these years to monitor the effect of different brassicas on PCN under field conditions. Glasshouse experiments were conducted to understand field observations and dose response experiments were undertaken *in-vitro* in a bid to understand the effectiveness of brassica leaf and root extracts in PCN management. Finally, the glucosinolate (GSL) content in the brassicas was analysed to see if efficacy could be linked to specific GSLs.

The potential of the tested brassicas to manage PCN in potato field have been evaluated in four field experiments (Chapter 3). Positive results against *G. pallida* were consistently observed with summer cultivated brassicas whereas the effectiveness of overwinter crops was limited by harsh conditions that resulted in either crop damage and/or low biomass production. The control obtained against *G. pallida* with the brassicas was associated with the released of toxic ITC through enzymatic hydrolysis of GSL, as the production of GSL was demonstrated through HPLC analysis to be higher in summer cultivated crops compared with the overwintered crops. Also, positive relationships were demonstrated between GSL present in the incorporated crops and the PCN mortality observed post incorporation.

This concept has been reported previously under controlled conditions where GSL content was positively correlated with PCN mortality when incorporated pots were left uncovered unlike those that were sealed with polythene bags (Lord *et al*., 2011), indicating that other factors other than GSL hydrolysis product were involved in sealed pots. Incorporation of the brassicas was done within the top 30 cm and PCN soil sampling was undertaken to this depth. Schomaker and Been (1999) reported that approximately 84% of PCN are found within the top 21 – 30 cm range of soil. Therefore, a uniform incorporation of a biofumigant crop within this region would affect most of the PCN population.

Developing brassica crops in the field demonstrated suppressive effects on the viability of *G. pallida*. Therefore it was hypothesised that, GSL were either being leached into the soil alongside myrosinase enzymes by the brassica roots, or that, leached GSL were being degraded by myrosinase producing soil microbes present in the rhizosphere of developing brassicas. Pot experiments using sterilised or unsterilized soil sown with brassicas (Chapter 5) demonstrated that indeed, pure GSL were being degraded in the rhizosphere of developing brassicas compared with untreated sterilised soils, thus indicating the presence of GSL degrading soil microbes. However, effort to quantify GSL in brassica root leachates was unsuccessful (preliminary studies). There is evidence in the literature of enhanced root leaching of GSL mediated by salicylic acid and methyl jasmonate (Schreiner *et al*., 2011) and direct release of ITC from developing brassica root tissues (Rumberger & Marschner, 2003) which are in conformity with the observations in field on PCN viability (Chapter 3) and in glasshouse on both PCN viability and GSL degradation (Chapter 5). Valdes *et al*. (2011) found that hatching of *G. rostochiensis* was significantly stimulated in tomato root diffusate (TRD) following exposure to exudates

from brassicas. As discussed in Chapter 6, dormant eggs within a cyst normally require an additional stress factor to induce hatch (Hominick *et al*., 1985). This implies that the stimulation of hatching observed by Valdes *et al*. (2011) by brassica root exudates over the TRD-only treatment was due to a stress factor present in the brassica root exudate. A prolonged exposure of nematode to a stress factor would normally results to permanent disruption of its sensory perception of the host signals causing starvation and death (Noling, 2002). Therefore, the period from brassica establishment to incorporation (10 – 12 weeks) would have been a sufficient exposure time of the leached stress compounds from the roots to PCN thus causing the mortality observed with partial biofumigation. Lord *et al*. (2011) noticed a level of PCN suppression that was greater than the predicted level based on the GSL content present in the *R. sativus* cultivar used in their studies leading them to a conclusion that other factors were involved. These authors overlooked the effect that could have resulted from the developing crop which was possibly responsible for part of the PCN suppression as demonstrated herein. In the present study it was found that the dominant GSL present in *R. sativus* leaves was not the same as that present in its root tissue. Also, the *in-vitro* studies showed that *R. sativus* root extract was more toxic to encysted eggs of PCN than its leaf extract, thus a possible explanation to the observations by Lord *et al*. (2011).

It was clear after two successive years of experiments that overwintering brassicas as an intercrop in potato production to control PCN would not provide adequate control (Chapter 3, Experiments 2 and 4). This is because the development of brassica crops in the field during the spring season following overwintering was slow for the species that demonstrated positive results in summer cultivation. Therefore,

considering the time between incorporation and potato planting in spring, sufficient biomass would not be obtained at time of incorporation.

Hatching assays in potato root leachates following exposure to different concentrations of brassica extracts revealed a high mortality of encysted eggs of *G. pallida*, with an LD_{50} of 0.027 mg ml^{-1} for *B. juncea* leaf extract. This mortality rate could be attributed entirely to 2-propenyl-ITC as the *B. juncea* leaf extract used produced approximately 98% of 2-propenyl-GSL which is known to release 2-propenyl-ITC upon hydrolysis (Kissen & Bones, 2009). This LD_{50} recorded in the hatching studies should be achievable field rates considering the incorporated dry mass in summer field experiments (3384 g m^{-2}) within the top 30 cm of soil, a soil bulk density of 0.7 to 1.2 measured immediately after incorporation (Appendix 9.6) and an average total GSL concentration (shoot + root) of 124 µmol g^{-1} of biomass in *B. juncea* at time of incorporation (Chapter 4). The most interesting finding was the level of mortality caused by the root extract of *B. juncea* and *R. sativus* (Chapter 6, LD_{50} = 0.032 and 0.035 mg ml^{-1} w/v respectively). Relating the level of control obtained with root extracts to the observed partial biofumigation in field (Chapter 3) and glasshouse (Chapter 5) experiments confirm the release of toxic products from roots. This, therefore, implies that, it may be useful to consider options such as enhancing below grown biomass production and GSL leaching for these crops in field, or foliar treatments with a herbicide to enhance root decay in soil as mentioned previously.

Gimsing and Kirkegaard (2006) reported a soil ITC release efficiency of 18.5% for *B. juncea* following tissue pulverisation and incorporation, an efficiency that was 17.5% greater than the release efficiency reported by Morra and Kirkegaard (2002) in the same field following un-pulverised *B. juncea* tissues. This implies that the degree to

which the plant tissue is disrupted would play a vital role in the release of ITC in the soil. In the *in-vitro* experiments (Chapter 6) the freeze-dried plant tissues used for the extracts was milled to a fine powder, thus allowing for a 100% GSL and ITC release efficiency upon hydrolysis. However, the level of tissue disruption obtained in *in-vitro* studies may not be achievable in the field. Nevertheless, biofumigant brassicas cultivated to the recommended levels of maximum biomass and GSL production may be harvested and processed into fine powder before field application and incorporation. Irrigation following soil incorporation of the powder material would ensure a maximum ITC release efficiency, thus making it possible to achieve the level of control similar to that in *in-vitro* experiments. In this situation, water availability following incorporation would be vital for the hydrolysis of GSL to ITC as insufficient water would limit GSL hydrolysis (Lazzeri *et al*., 2004).

The concentration of methyl ITC, the active substance in metam-sodium, required for soil sterilization is between 517 – 1294 nmol g^{-1} (Brown & Morra, 1997). An LD_{90} of 182 nmol methyl-ITC g^{-1} has also been reported for some insect pests (Borek *et al*., 1997). Therefore, considering an ITC soil concentration of 100 nmol g^{-1} (Matthiessen *et al*., 2004) and 91 nmol g^{-1} (Gimsing & Kirkegaard, 2006), significant level of pest control would be possible in field. The total GSL content (shoot + root) in the *B. juncea* reported in Gimsing and Kirkegaard (2006) was 40.4 µmol g^{-1} biomass, producing a maximum soil ITC of 91 nmol g^{-1} following incorporation of a biomass of 568.1 g m^{-2}. A total GSL concentration of up to 124 µmol g^{-1} biomass was detected in the *B. juncea* incorporated in the field experiment (Chapter 4), and a biomass of up to 23 kg m^{-2}. These values are approximately three and six folds (for GSL and biomass respectively) greater than the figures reported in Gimsing and Kirkegaard (2006),

indicating that, with other factors kept constant, an ITC soil concentration of 273 nmol g^{-1} was possible in the field experiments.

7.1 Integrating the biofumigation system into the current PCN management scheme

The Directive, 2007/33/EC, on the management of PCN infested fields came into force from July 1, 2010. The directive set out available options to potato growers with fields officially recorded with PCN. The purpose is to restrict the pest from spreading to un-infested land and to ensure a decline of the population in infested field in time. Although the directive ensures flexibility for potato growers, it requires that any management programme adapted must be consistent with the presented options and in line with the required legal objective of PCN suppression. The effectiveness and reliability of any novel PCN management option would determine the ease of its adoption by potato farmers. This would also depend on the degree of changes to be adapted including knowledge and agronomic input as well as the ratio of the cost to its benefit. Biofumigation would comply with most of these points for the current potato production practices.

Biofumigant brassicas are selected especially for their rapid growth and development (8 – 10 weeks on average) during the growing season, and their ease of disruption for incorporation. Within the UK, overwintered cereals grown in rotation with potatoes are normally harvested in July thus, providing a window of opportunity for the establishment of a biofumigant crop prior to a potato crop in the following spring. A number of commercial potato growers in Shropshire, UK, are now adapting the biofumigation system into their farming practices to manage PCN and fungal pathogens and are being advised by local agronomist and researchers from Harper Adams University. In some cases, agronomists are providing these farmers with mixtures; a typical example is the “Hardy-Mix” provided by local agronomists from

Agrovista® consisting of *R. sativus* and *B. juncea*. These mixtures are especially considered because of their partial and complete biofumigation as discussed previously. With the level of suppression observed during this study, it implies that successive application of the biofumigation system would ensure a steady decline in PCN and would lead to a reduction in the length of rotation (one potato crop in six years) currently recommended for field detected with PCN.

The pulverisation of the biofumigant brassicas in the field is normally done with a rotary flail and incorporated using a rotary tiller and/or disk harrow followed by a roller such as a Cambridge roll. Most UK potato growers would normally possess these types of equipment. As discussed above, the degree of pulverization would play a vital role in the level of control. This aspect and other agronomic practices such as soil moisture, sealing following incorporation and the type of equipment used for incorporation are currently being investigated at Harper Adams University.

As discussed in Chapter 1, an integrated approach for PCN management is considered the most effective. This is achieved through the use of granular nematicides and cultivar resistance in tandem with crop rotation. Philips and Trudgill (1998) estimated that granular nematicides would reduce root invasion by infective juveniles by approximately 90%. Considering the level of suppression observed with summer cultivated brassicas (Chapter 3), an integration of this system with granular nematicide would provide sufficient PCN reduction necessary to reduce the length of rotation to an economically desired three to four years. The use of trap cropping (Scholte, 2000b; Timmermans *et al*., 2006) or synthesised chemicals (Tanino *et al*., 2011), both of which would trigger hatch without further multiplication, in combination with biofumigation system are possible options to be considered to achieve adequate level of control in PCN infested fields.

7.2 Future considerations

The series of experiments conducted during the present studies has demonstrated for the first time that biofumigation has the potential to be an important component of PCN management at field level. The most effective species were *B. juncea* and *R. sativus*, producing high levels of GSL associated with the release of toxic ITC when summer cultivated. While *B. juncea* foliage produced high biomass/GSL, most of which was 2-propanyl-GSL, *R. sativus* roots produced high level of biomass/2-phenylethyl-GSL. Extracts produced from foliage and roots sections of *B. juncea* and *R. sativus* respectively caused high mortality to encysted eggs of *G. pallida in-vitro*. Therefore, it would be of particular interesting to investigate possible synergistic effect resulting from a mixture of these brassicas in terms of partial and complete biofumigation against PCN. This information would be very useful to both seed producing companies and farmers, especially those currently putting this system into practice.

Although breeding for a high level of GSL/biomass has been quite successful, for instance *B. juncea* cv Caliente 99, there are still no varieties that possess these attributes alongside winter hardiness. Future breeding programs should therefore consider winter hardiness and sufficient biomass/GSL production in early spring for incorporation prior to potato planting. These varieties would act as cover crops traping nutrients through the winter in fields that would otherwise be fallowed, thus, making the nutrients to be readily available to the following crop.

Another important aspect to investigate would be seeding rates and enhanced root decay mediated by foliar herbicide treatment. Observations made during the present studies suggest that this could be an important component for pest control in field. Species such as *R. sativus* are capable of producing a massive below grown

biomass. These root are particularly susceptible to infestation by *Delia radicum* (cabbage root fly) which was thought to have been involved in the partial biofumigation effect caused by the brassicas. This supposition therefore needs scientific backing through pot experiment with sterilised and *D. radicum* infested soils cultivated with brassicas and their respective effects on PCN.

7.3 Conclusions

Biofumigation has proved through the present studies to be an important component of PCN management if well utilised as an intercrop in potato production. *Brassica juncea* was particularly consistent in controlling these nematode pests when cultivated as a summer crop, thus has proved to be an effective biofumigant crop for *G. pallida* management under field conditions. It demonstrated a marked effect on *G. pallida* during biofumigant crop development and after incorporation which was further confirmed in pot experiments and *in-vitro* with extracts prepared from this crop prior to incorporation. Foliar treatment of *B. juncea* with metconazole unexpectedly enhanced the production of 2-propanyl GSL in its foliage and roots tissues, suggesting that this fungicide can play an important role in biofumigation with *B. juncea*. Similarly, *R. sativus* effectively suppressed *G. pallida* particularly during the crop development. *In-vitro* experiments with *R. sativus* extracts provided useful information as to which parts of the plant should be given particular attention, when considering this crop in rotation to manage PCN.

This project is the first to show that brassicaceous plants grown under field conditions are capable of reducing *G. pallida* population during biofumigant crop development (partial biofumigation) and after incorporation (complete biofumigation). Also, the aspect of enhanced GSL production by metconazole production as demonstrated in this study has never been reported before, and thus lays a framework for future

biofumigation research. Research is ongoing at Harper Adams University to optimise ways of utilising these crops in potato production to obtained maximum benefits. This together with the present studies would provide useful guidelines on the incorporation of the biofumigation system into an integrated PCN management scheme for potato famers with PCN problems.

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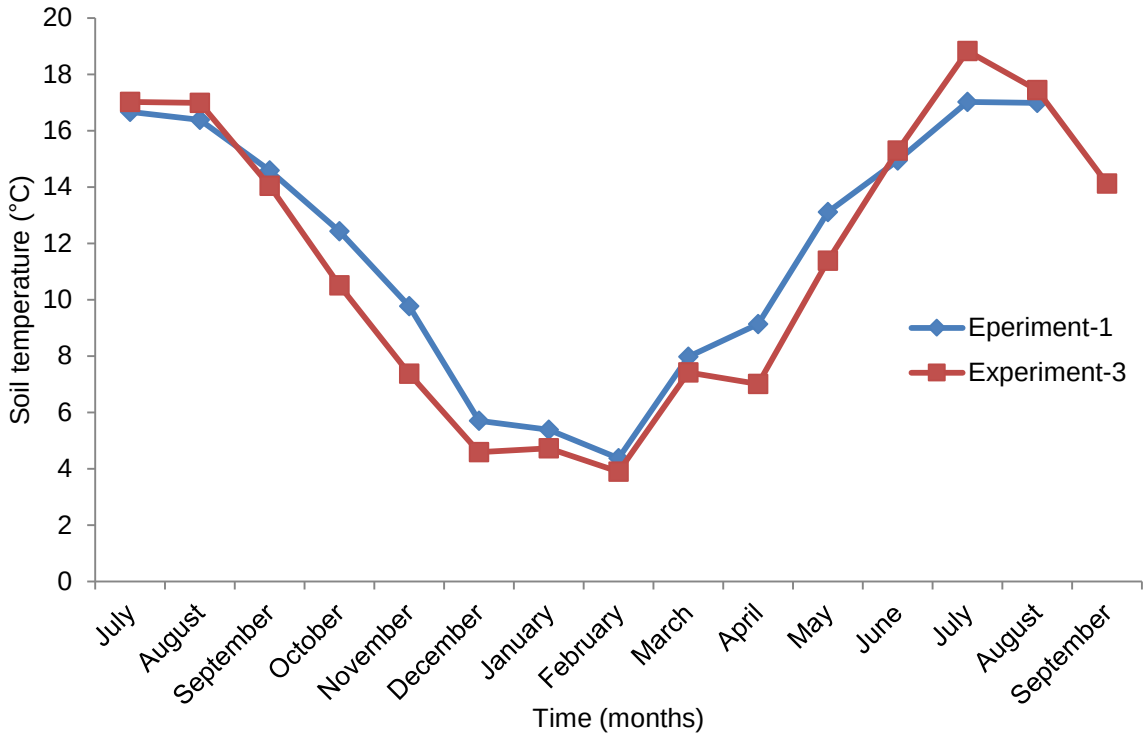
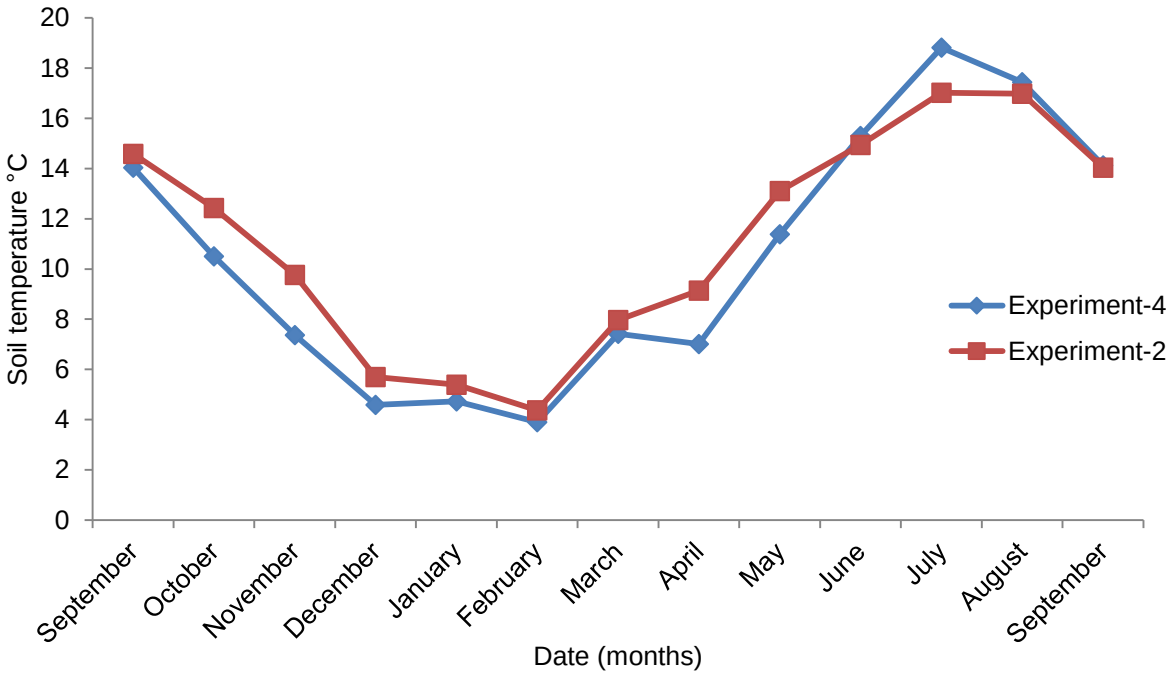
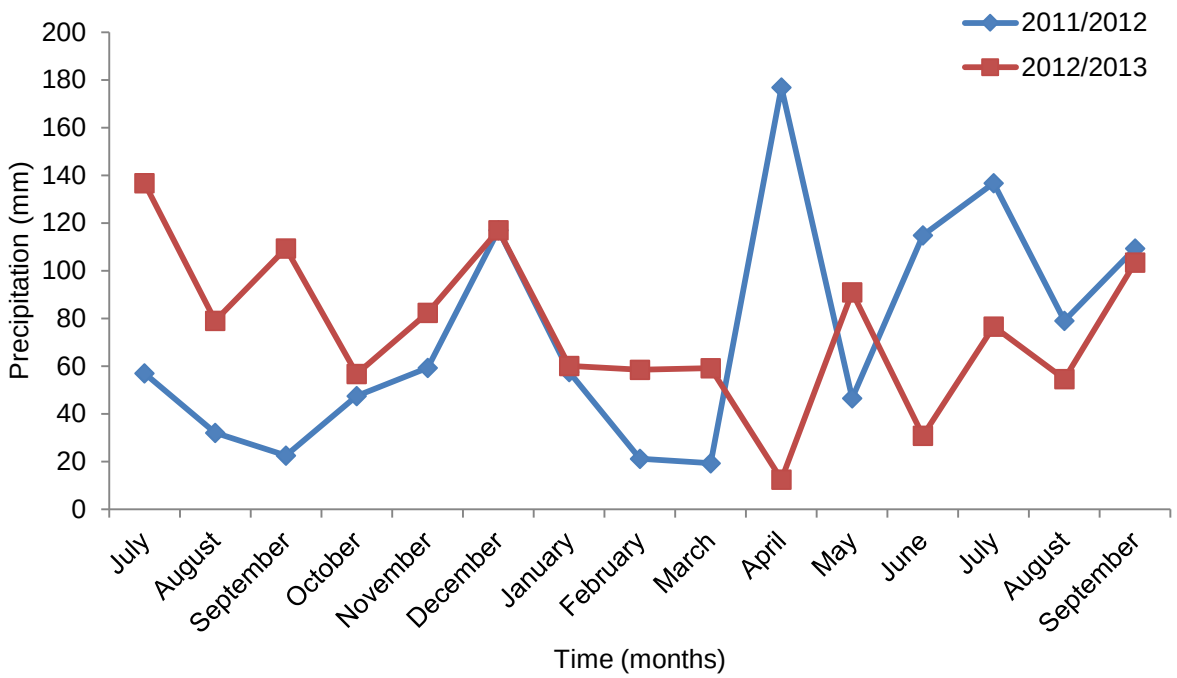
9. Appendices



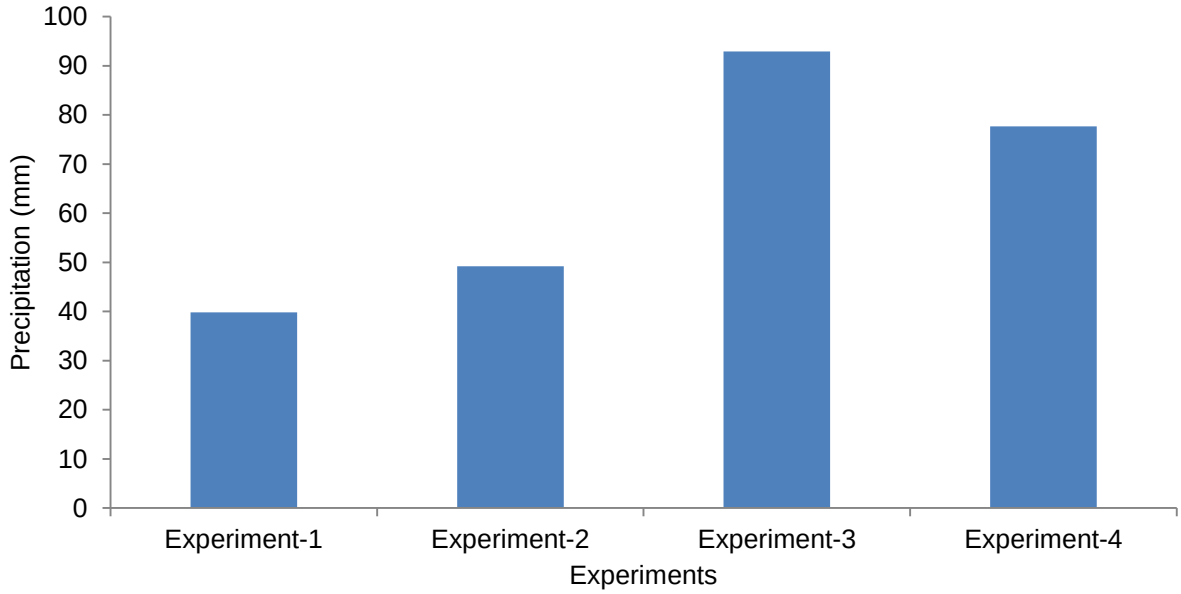
Appendix 9.1: Average monthly soil temperature recorded at a depth of 20 cm during the summer field experiments (Experiments 1 and 3)



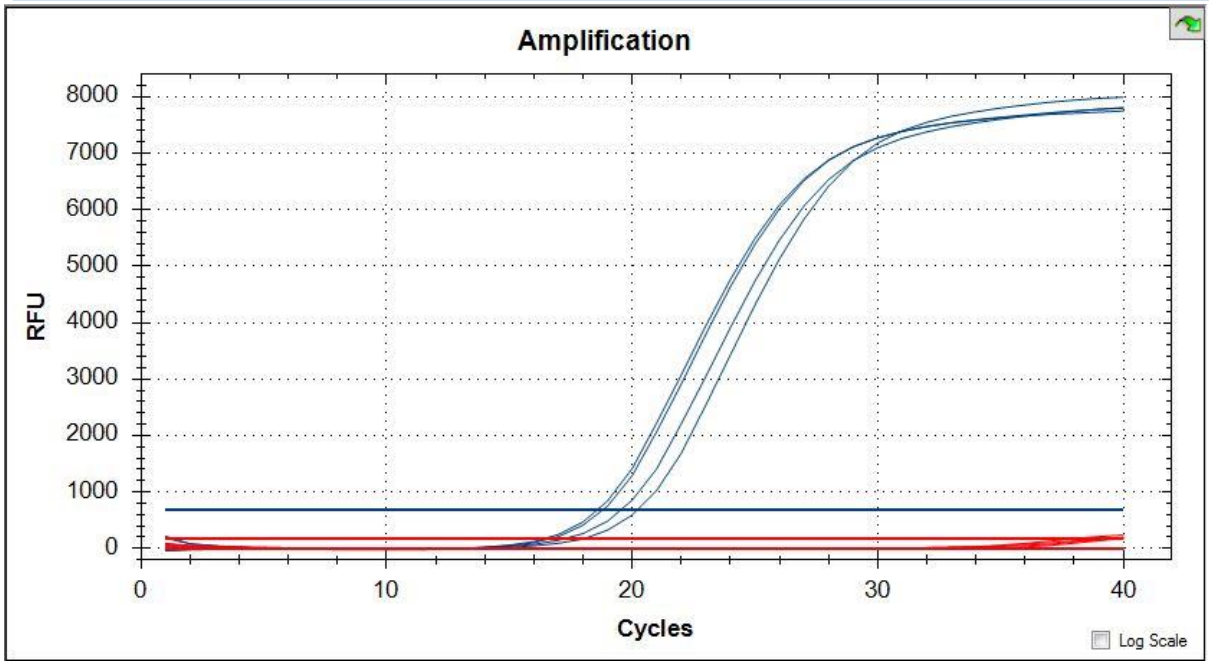
Appendix 9.2: Average monthly soil temperature recorded at a depth of 20 cm during the overwintered field experiments (Experiments 2 and 4)



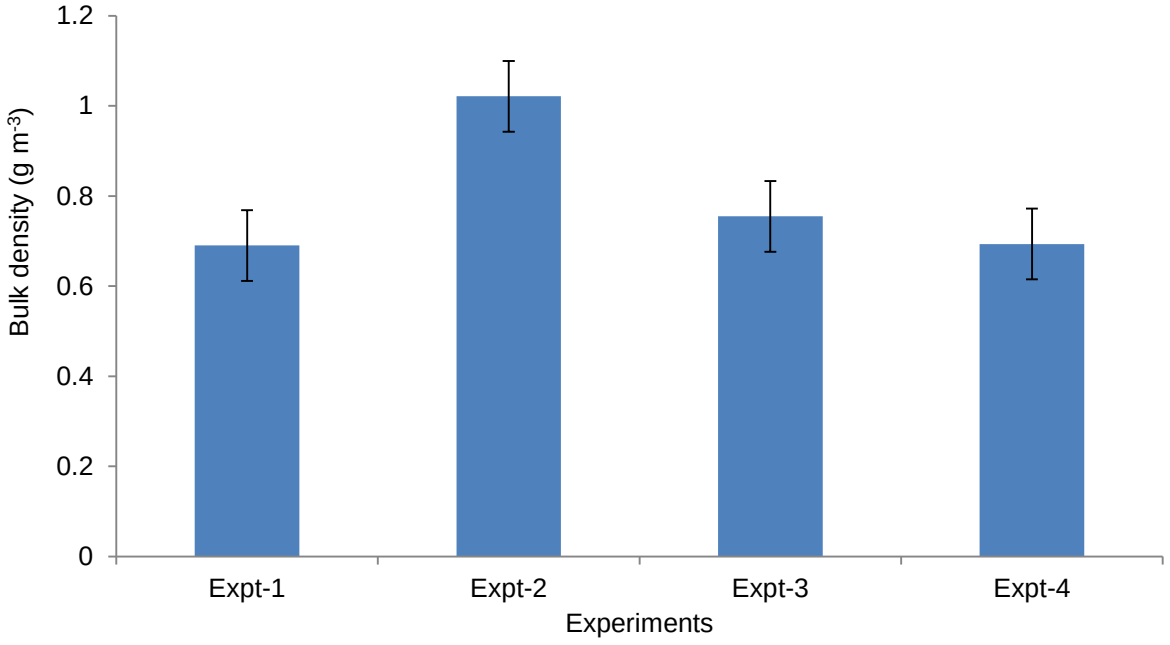
Appendix 9.3: Monthly precipitation during the 2011/2012 and 2012/2013 cropping seasons measured as a sum of the daily (24 h) precipitation (mm). Data obtained from the Harper Adams weather station



Appendix 9.4: Average precipitation (mm) during the biofumigant crop growth and development period for the different experiments



Appendix 9.5: Potato cyst nematode (PCN) species identification from field experiments 1, 2, 3 and 4 using quantitative Polymerase Chain Reaction (qPCR). Blue lines indicates amplified signals for *Globodera pallida* and red lines indicates amplified signals for *G. rostochiensis*



Appendix 9.6: Soil bulk densities (g m^{-3}) for field experiments 1 (Expt-1), 2 (Expt-2), 3 (Expt-3) and 4 (Expt-4) measured after incorporation of brassicas