"Control of soil borne potato pathogens using *Brassica* spp. mediated biofumigation"

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Abstract

Biofumigation is being increasing used as alternative control method for soil borne pathogens. This method exploits toxic compounds, specifically isothiocyanates (ITCs), which are released during the breakdown of *Brassica* plant tissues. To date field and glasshouse experimentation assessing the potential for using biofumigation to control agricultural pests and pathogens have produced promising results. Yet large gaps still remain in the specifics of the biofumigation process. It is hoped that further research to analyse how specific toxic compounds produced during *Brassica* tissue breakdown, specifically ITCs, affect different pathogens. Additionally analysis of the specific isothiocyanates and concentrations produced by *Brassica* spp. will allow a more pathogen targeted approach to biofumigation to be generated. The importance of assessing the biofumigation process on different scales is also understood, and therefore this study has encompassed work carried out *in vitro* and using glasshouse experimentation to establish a comprehensive overview of the biofumigation process. Assessing the effects different agricultural treatments have on soil microbial communities has also been recognised and therefore was also be investigated in this study.

This study aimed to determine the effects isothiocyanates, produced by *Brassica* spp., have on three economically important soil borne fungal pathogens, *Colletotrichum coccodes, Rhizoctonia solani* and *Helminthosporium solani*. Initial assessment was carried out using *in vitro* bioassays, allowing assessment of the overall toxicity of each ITC. Results identified that the pathogen response was dependent on both the structure of the ITC and the concentration of ITC present. The most significant pathogen suppression was observed with *R. solani* when exposed with benzyl or methyl ITC and *H. solani* when exposed to 2-phenylethyl ITC.

To gain understanding of the naturally produced ITCs Gas-Chromatography Mass-Spectrometry analysis was used to analyse specific isothiocyanates produced by a range of different *Brassica* spp., at different development stages. Results identified Allyl, Benzyl and 2-Phenylthyl ITC as the most commonly produced by the *Brassica* cultivars used within this study. Overall the Allyl was found within the highest concentrations; however the specific ITCs and concentrations produced were dependent on both the development time and cultivar.

Glasshouse experimentation was also carried out to assess both the effects of pure ITCs on *R.* solani and *C. coccodes* fungal inoculum within compost and diversity changes within the soil microbial community, in response to isothiocyanate incorporation and the biofumigation process. In order to examine changes in microbial communities' analysis was carried out using Terminal Restriction Fragment Length Polymorphism, a DNA fingerprinting method which allows bacterial diversity shift to be traced and statistically analysed. Overall incoproation of pure ITCs did not significantly reduce black scurf or black dot disease symptoms on daughter potato tubers. Additionally

after 30 days soil microbial community diversity was not greatly altered by the addition of ITCs. Therefore as it is often suggested that biofumigation is influenced by the soil activity it is thought that this may be due to the addition of *Brassica* tissue. The increase of organic matter into agricultural soils may influence both biological and chemical processes which may in turn aid pathogen suppression.

Overall this study provides a detailed insight into establishing the specific interactions that occur during biofumigation. Results produced findings of ITCs which significantly suppressed the growth of fungal potato pathogens. Development of a novel GC-MS assay revealed previously unknown data of levels and profiles produced by a number of different *Brassica* plants. Additionally study was also carried out to evaluate the effects of biofumigation of soil microbial communities, which is often ignored within other studies. Overall this study aimed to gain an increased level of knowledge of such processes in order for the methods and the results presented, to be used to establish effective, pathogen targeted biofumigation systems.

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Author's Declaration

I hereby declare that the research reported within this thesis is my own work, unless otherwise stated, and that at the time of submission is not being considered elsewhere for any other academic qualification.

Fiona Isabelle Taylor

Abbreviations

AG	Anastomosis Groups
AITC	Allyl Isothiocyanate
ANOVA	Analysis of Variance
ATP	Adenosine-5'-triphosphate
BITC	Benzyl Isothiocyanate
BLAST	Basic Local Alignment Search Tool
CMCA	Carboxymethylcellulose
CT	Critical Thresholg
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EC	European Community
EDTA	Ethylenediaminetetraacetic Acid
ESP	Epithiospecifier protein
GC-MS	Gas-Chromatography Mass-Spectrometry
GPS	Global Positioning System
GSL	Glucosinolate
IITC	Isopropyl Isothiocyanate
IPM	Integrated Pest Management
IS	Internal Standard
ITC	Isothiocyanate
MB	Methyl Bromide
MITC	Methyl Isothiocyanate
MRS	de Man, Rogosa, Sharpe
mSNP	Multiple Single Nucleotide Polymorphisms
N	Nitrogen
NCBI	National Centre for Biotechnology Information
NITC	1-Napthyl Isothiocyanate

PCN	Potato Cyst Nematode
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PED	Potato Early Dying
PEITC	2-Phenylethyl Isothiocyanate
PITC	Propyl Isothiocyanate
PPi	Pyrophosphate
qPCR	Quantitative Polymerase Chain Reaction
R^2	Correlation Coefficient
RFLP	Restriction Fragment Length Polymorphism
S	Sulphur
SASA	Science and Advice for Scottish Agriculture
SDS	Sequence Detection System
SNP	Single Nucleotide Polymorphism
TAE	Tris-acetate-EDTA
TBZ	Thiobendazole
TQ	Triple Quad
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UK	United Kingdom
US	United States of America
USA	United States of America

Chapter 1

1. Introduction

1.1 The economic importance of potatoes

Potatoes are the world's number one non-grain food crop; and the fourth main food crop grown in the world after maize, rice and wheat (Cunnington 2008). In 2007, 325 million tonnes were produced worldwide. Potato production occurs in over 100 countries, the majority in Asia and Europe (Fiers *et al.* 2012). In the UK around six million tonnes of potatoes are produced annually. The majority of production occurs in eastern parts of England, the west Midlands and south east Scotland (Cunnington 2008).

Between 2001 and 2010 the value of potatoes increased by £46 million with most of the increase occurring between 2005 and 2006 when there was both a rise in production (26%) and market price. The 2006 increase can be attributed to the favourable growing and harvesting conditions, which produced high yields (Anon 2011). In the UK approximately 3.5 million tonnes of potatoes each year go into storage. Around half of which are used for the fresh market, the rest of which will go to a range of different processing and food service markets, which includes multi-national processors for French fries and crisps (Cunnington 2008). Although the economic value of potatoes is clear, potato crops are susceptible to more than 40 pests and diseases, caused by insects, nematodes, viruses, bacteria and fungi, diseases produced by such microorganisms can lead to can lead to reduced crop yield, (Fiers *et al.* 2012).

1.2 Potato pathogens and diseases

Generally speaking disease caused by viruses or viroid's will produce foliar symptoms, including; leaf distortion, mosaic, leaf and vein necrosis, dwarfing and leaf rolling. A small number of viruses, such as tobacco rattle virus and potato virus Y can cause tuber symptoms, including rots and blemishes (Fiers *et al.* 2012).

Soil-borne pathogens can be further divided into two groups according to the symptoms they produce: tuber symptoms or those which damage other parts of the plant. Pathogens such as *Sclerotinia sclerotiorum* (Rahmanpour *et al.* 2010) *Pectobacterium atrosepticum* (Cahill *et al.* 2010) and *Dickeya* species (Kelly *et al.* 2012) which lead to disease affecting the stems and roots may disrupt crop development and reduce yield. Other soil-borne pathogens may also cause aerial symptoms such as necrosis or chlorosis (*Phoma* leaf spot, *Verticillium* wilt) and occasionally wilting and rolling (bacterial ring rot). Predominantly root rots are caused by nematodes, such as *Meloidogyne* spp., feeding on the roots which can lead to necrosis or rots (Fiers *et al.* 2012).

Diseases affecting tubers can be divided into three categories: galls, rots and blemishes. Galls are mostly provoked by infection by powdery scab (Merz & Falloon 2009) and root-knot nematodes (Mojtahedi *et al.* 1991). Rots can be further divided into different types including dry, soft and flesh rots, flesh discolouration and vascular ring discolouration. Blemishes affect the tuber skin and are most often caused by fungal pathogen infection (*Rhizoctonia solani, Colletotrichum coccodes* and *Helminthosporium solani)*. Blemish diseases are increasing in economic importance due to the rise in consumer habits which now focus on the want for washed ascetically pleasing tubers (Fiers *et al.* 2012).

With increasing consumer pressure on growers to deliver disease free washed tubers and environmental pressure to reduce pesticide and fumigant use, the use of integrated pest management practices to reduce fungal blemish pathogens of potatoes should be explored. This project will investigate the potential of using an alternative control strategy known as biofumigation system to decrease incidence of three fungal potato pathogens: *Rhizoctonia solani, Colletotrichum coccodes* and *Helminthosporium solani*. Each of these fungal pathogens causes substaintial economic losses to the UK potato industry, it is estimated that collectively they cause losses of £5 to 9 million to the UK ware potato industry annually. As a result effective control strategies are continually welcomed by growers.

1.2.1 Control of potato blemish pathogens

Cultural practices to reduce the incidence of tuber diseases are often found to be effective dependent on the targeted pathogen. Crop rotation with grain crops and the use of methods which reduce contact time between pathogen infected tubers and plants, such as seed tuber planting in dry warmer conditions which will encourage sprouts to emerge quickly followed by swift harvesting of tubers, have all be shown will all help to aid management of blemish diseases (Secor & Gudmestad 1999). Early harvesting after haulm destruction has also been used to prevent the development of black scurf (Tsror *et al.* 2001). Traditionally chemical fungicides have been used to control fungal diseases in potato crops (Brewer & Larkin 2005). Yet fungicidal control is most effective when the inoculum is tuber borne. Whereas seed borne inoculum is not well controlled through fungicide application, particularly when high levels of disease are present (Wilson *et al.*, 2008). Resistant cultivars would be an ideal method to control potato pathogens, however as yet no completely resistant varieties are available (Tsror *et al.* 2001; El Balkali & Martin 2006). In the case of *R. solani,* although some varieties have shown to differ in their susceptibility to *R. solani* infection and some have shown varying levels of resistance towards the formation of sclerotia on tubers. To date no potato variety has displayed resistance to sprout nipping or stem lesions also caused by *R. solani* (El Balkali & Martin 2006). However potato cultivars showing signs of resistance to one disease may be more susceptible to

another, therefore selecting cultivars for soils with multiple pathogens present can be difficult. However if a single disease is dominant targeted pathogen control can prove advantageous.

1.2.2 *Rhizoctonia solani*

Rhizoctonia solani Kühn (telomorph *Thanaterphorus cucumeris)* was originally observed on potato tubers in 1885, (Hide *et al.* 1973; Lees *et al.* 2002; Ritchie *et al.* 2006; Rauf *et al.* 2007; Woodhall *et al.* 2007; Okubara *et al.* 2008; Wilson *et al.* 2008). It is now considered an important fungal pathogen, that causes two diseases of potato – stem canker and black scurf – which may lead to reduced tuber yield and lower quality of tubers respectively (Brewer & Larkin 2005b). *Rhizoctonia solani* is of particular importance within seed potatoes, as it is believed that the majority of inoculum re-enters field soil, through the planting of *R.* solani infected seed potatoes. *Rhizoctonia solani* has been found to be present in all areas of potato production (Hide *et al.* 1973; El Balkali & Martin 2006). Whilst in this review it will be discussed in the context of potato crops, it should be noted that *R. solani* has a wide host range (Yitbarek *et al.* 1987; Lehtonen 2009). In potatoes *R. solani* infection exhibits several symptoms including damping-off, rots on roots, shoots and fruits, canker lesions on sprouts and tuber sclerotial diseases (Lehtonen 2009). The fungus can survive in decomposing plant tissues, on tuber surfaces or within the soil for extended periods of time (El Balkali & Martin 2006).

Rhizoctonia solani is divided into sub-groups, known as anastomosis groups (AGs). Isolates are assigned to an individual AG on the basis that only hyphae from isolates of the same AG will fuse (Anderson 1982; Lees *et al.* 2002; Rauf *et al.* 2007). Although this method for assigning AGs can be time consuming and requires a degree of skill, it remains the most common, and has to date resulted in the recognition of 13 individual AGs (Lees *et al.* 2002; Ritchie *et al.* 2006). Anastomosis groups can be determined by several other methods, including distinguishing between cultural and pathogenic variation. Molecular and biochemical methods including the use of restriction fragment length polymorphism (RFLP), electrophoresis of soluble proteins and pectic zymograms have been successfully used to identify differences between *R. solani* groups (Lees *et al.* 2002). However generally RFLP methods are more revealing of detailed genetic diversity between the different groups.

Of the 13 AGs, AG-3 has been identified as the main *R. solani* group infecting potato crops worldwide (Carling et al. 1989; Lehtonen 2009) additionally it has been shown to be the most virulent of all AG isolates on potato. However other AGs have also been isolated from potatoes (Carling *et al.* 1989; Rauf *et al.* 2007), and AG-4, AG-5 and AG-9 have also been shown to be virulent and cause disease in potato crops (Carling *et al.* 1989; Lees *et al.* 2002; Woodhall *et al.* 2007; Lehtonen 2009). Apart from the

previously mentioned, other AGs have also shown to have the ability to infect potato, but may be unable to infect certain parts of the plant. Additionally it has also been recognised that different AGs will affect different parts of the plant (Woodhall *et al.* 2007).

AG-3 has also been shown to alter in virulence, dependent on the part of the potato plant it is isolated from. Those obtained from stolons, sclerotia and hymenia were shown by Hill & Anderson (1989) to be the most virulent, this was followed by stem isolates. Single basiophore isolates were the least virulent, this differed from previous conclusions that sclerotial isolates from potato tubers were of low virulence.

Disease in potatoes is initiated when the *R. solani* hyphae from germinating sclerotia begin to grow towards a host as a result of chemical cue exudates being released. On reaching the host, hypha will begin to grow over the plant. After a few hours the hypha will flatten and begin to grow over the epidermal cells. Prior to penetration of the host, T-shaped branches of hyphae will form thickened cushions that attach to the epidermis. The fungus will eventually enter the plant when it locates a weakened spot on the plant tissue surface where it will successfully enzymatically break down the outer protective layer. Subsequently the thickened hyphal tips on infection cushions form infection pegs which penetrate the host cuticle and epidermal cell walls into the hosts epidermal tissue and outer layer of the cortex. Penetration is achieved through hydrostatic pressure and is aided by degrading enzymes such as cutinases, pectinases and xylanses. Once successfully inside the host the fungus will grow both inter and intracellularly in turn degrading the tissue which it comes into contact with. This results in visible necrotic lesions on epidermal tissue of shoots (Banville 1989), roots and stolons or as damping-off on young seedlings. In cool and moist soils the hyphae will also attack the developing sprouts producing reddish-brown coloured lesions. Where infection is severe the growing sprout are often killed due to severe infection, the plant will in turn produce new sprouts in order to replace those that have been killed, resulting in depletion of the tuber food reserves required for future growth and development. This 'sprout nipping' leads to delayed emergence and plant maturity and also weakened and uneven plant stands.

As the season continues the potato plant will grow and infection will be identified by the presence of additional reddish-brown sunken cankers forming around the stems, stolons and roots (Carling *et al.* 1989; El Balkali & Martin 2006). This form of disease is known as, stem canker. These cankers cause decreased plant productivity as well as the quality and quantity of tubers the plant will produce due to restriction of the flow of water and nutrient through the plant, patchy tuber emergence may also result (El Bakali & Martin 2006). Above ground, infection is recognisable by aerial potato tubers and purplish coloured leaves – which are particularly visible when levels of infection are severely high or the plant is stressed (El Balkali & Martin 2006).

Infection of tubers with *R. solani* can also cause a disease known as, black scurf. This is recognisable by the presence of black sclerotia masses on the tuber surface and is often referred to as the "dirt that won't wash off" (El Balkali & Martin 2006). Although this form of disease does not affect the interior of the tuber, the presence of tuber borne sclerotia can reduce tuber quality (Anderson 1982; Carling *et al.* 1989; Tsror *et al.* 2001; Wilson *et al.* 2008) thus reducing the overall market value of the crop (El Balkali & Martin 2006).

1.2.3 *Helminthosporium solani*

Silver scurf of potatoes is an important storage blemish disease caused by the fungal pathogen *Helminthosporium solani,* (Ryu *et al.* 2000; Errampalli *et al.* 2001a). It is an imperfect fungus which belongs to the Moniales order, its telomorph is yet to be described and its phylogenetic position is not known. Potato silver scurf was first reported in 1871 in Moscow. Throughout the 20th century it was long thought of as a minor disease of potato, however due to a rise in incidence since 1968, it is now considered a pathogen of major importance throughout Europe (Errampalli *et al.* 2001a), and is known to occur in most potato growing areas (Elson *et al.* 1997; Frazier *et al.* 1998; Errampalli *et al.* 2001a). Over the last 20 years economic losses have become common place within the potato industry due to disease caused by *H. solani*. Prior to 1977, postharvest development of silver scurf was controlled through the application of thiobendazole (TBZ) (Elson *et al.* 1997). However overtime and intensive use of the fungicide, *H. solani* developed TBZ resistance, forcing alternative control methods to be deployed. Crop rotation, bruise prevention and other cultural methods in combination with chemical seed tuber treatments applied at planting and post-harvest as well as efficient management of potato stores have been shown to reduce incidence of silver scurf (Frazier *et al.* 1998; Secor & Gudmestad 1999). Integrated disease management approaches that will combine cultural, biological and chemical control methods are considered to be an effective approach to combat long term control of *H. solani* (Errampalli *et al.* 2001a). TBZ resistance is one reason contributing to the rise in silver scurf incidence, however a lack of resistance in potato cultivars – (there are currently no known resistant potato varieties) (Elson *et al.* 1997) and failure to establish alternative effective control measures, may also be to blame (Errampalli *et al.* 2001a).

Silver scurf is a tuber blemish disease and therefore symptoms are limited to the tuber periderm. This results in disease cost incurred through the downgraded quality caused by the presence of discolouration on the tuber surface, (Elson *et al. 1997;* Errampalli *et al.* 2001a) sloughing of the skin and moisture losses which may have been caused by rupturing of the periderm (Elson *et al.* 1997). However these symptoms can lead to the infected tubers being rejected from the fresh market, especially in current times when there is a trend towards buying washed aesthetically pleasing tubers (Errampalli *et al.*

2001a). Economic losses also occur in the processed market, (Errampalli *et al*. 2001a) as silver scurf makes tuber skins difficult to peel and can cause unwanted burnt edges (Errampalli *et al*. 2001b).

Symptoms of *H. solani* are also confined to the tuber and have to date never been observed on the haulm or root (Errampalli *et al*. 2001a). Initial symptoms can be recognised by light brown rounded spots on the tuber surface or lesions on the stolon end of the tuber, these may turn a darker olive colour as fungal sporulation continues. The lesions will remain small as the tubers remain in the soil, but post-harvest during storage they will enlarge. The individual lesions will initially have definite margins but will merge as the disease develops. The silver discolouration characteristic of the disease is caused through the loss of pigment through cell desiccation (Errampalli *et al*. 2001a). In severe disease incidences 'skin freckling' can be observed which can lead to the disease being known as 'elephant ear' due to the texture observed on the tuber surface (Errampalli *et al*. 2001a). Screening of field cultivars have shown that severe infection levels of *H. solani* do not delay emergence or early plant growth, however it may affect cultivars with a low sprouting vigour (Errampalli *et al*. 2001a). Lesions may lead to an increased level of permeability of the tuber skin which may result in shrinkage/water loss and therefore weight loss, thus having a direct effect on overall potato growth and tuber yield (Errampalli *et al*. 2001a). Periderm rupture has also been shown to make tubers more susceptible to infection from other diseases particularly during storage (Elson *et al*. 1997).

Helminthosporium solani can infect tubers during both the growing season and tuber storage. Although both soil and seed borne infection can occur it is known that the primary source of infection is infected seed tubers, which occurs when seed tubers come into direct contact with progeny tubers (Frazier *et al*. 1998). Infection can also be the result from soil borne inoculum and conidia which have been shown to transfer through irrigation. Most infection occurs prior to harvesting, however some will take place during harvest (Errampalli *et al*. 2001a). Lesions may be visible during harvesting particularly if tubers are wet (Ryu *et al*. 2000; Errampalli *et al*. 2001a). In storage, conidia are airborne and may be dispersed through ventilation systems. Warm and humid conditions favour spore germination resulting in infection of both healthy intact tuber periderm and wounded tubers where more severe infections may result (Frazier *et al*. 1998).

The key steps of infection of *H. solani* have been determined through the use of transmission and scanning electron microscopy by Martinez *et al.*, (2004). Their findings showed that six hours after inoculation unipolar germ tubes appeared. Six to nine hours after inoculation, penetration of the periderm will occur. It is thought that as in the case of most fungal pathogen – host interactions this will be aided by enzymes that will degrade the cell wall. Hyphae found growing over the tuber surface are surrounded in an extracellular sheath, which may be involved in binding of fungal

structures to plant surfaces. The sheath is probably produced in response to the presence of host or other fungal cells, as this phenomenon is not observed under *in vitro* conditions. There have been contrasting reports as to whether the penetration process involves structures, such as appressoria or hyphal enlargement. More recently it has been hypothesised that the appearance of such structures is dependent on the specific *H. solani* strain (Martinez *et al.* 2004). Nine hours after infection, hyphae are shown to be present in large numbers of cells in both the periderm and cortex. Hyphae are largely intracellular and do not possess a surrounding sheath and probably induce cell necrosis by obtaining nutrients from surrounding cells. The breakdown of cells is not solely limited to invaded cells and therefore demonstrating that *H. solani* acts as a necrotrophic fungus through gaining nutrients from dead and moribound cells. It is also thought that the fungus may inhibit the plant cells natural defence mechanism prior to cell wall penetration. After four days condiogenesis occurs, ultimately resulting in air pockets at the periderm level producing the tan to grey colour on the tuber surface (Martinez *et al.* 2004).

Approximately five to 30 conidiophores and subsequently conidia will form at the basal end (Frazier *et al.* 1998; Errampalli *et al.* 2001a), additional large, thick walled, cylindrical, dark coloured conidia will then be produced. The dark colouration of conidia and hyphae can be attributed to melanin which is present within the surrounding sheath. Melanin has been shown to aid survival and longevity of the fungi and in some cases be beneficial during the infection process (Martinez *et al.* 2004). Lesions with high volumes of conidia and conidiophores will be of a darker colour than those with non-sporulative lesions. Eventually mature lesions of a silvery colour are formed at the junctions of dead tissue (Errampalli *et al.* 2001a). Prior to the introduction of TBZ, silver scurf was controlled by soil sterililants and soil treatment fungicides, although this proved effective if used prior to planting it was not effective on subsequent crops (Errampalli *et al.* 2001a).

In 1968 a systemic broad spectrum, post-harvest fungicide was found to be effective in controlling several soil-borne and seed-borne pathogens including *H. solani*. Thiabendazole (TBZ) achieved high levels of silver scurf control when applied to tubers immediately after harvest by preventing the spread of lesions throughout the storage period for several months. By the early 1970s TBZ was used on a large scale in several countries to control storage diseases of potatoes (Errampalli *et al.* 2001a). However since 1977, *H. solani* strains began to exhibit resistance towards TBZ, due to a mutation which prevents TBZ and other benzimidazole fungicides binding to the *H. solani* ß-tubulin (Errampalli *et al.* 2001a).

With the appearance of TBZ resistant phenotypes, several other fungicides have been tested for their potential use in controlling silver scurf. Azoystrobin, imazalil, prochloraz and fenpiclonil, both alone and in combination with other fungicidal treatments have been shown to be effective in controlling silver scurf, (Errampalli *et*

al. 2001a) however overuse of such fungicides may also result in resistance. Therefore the introduction of integrated pest management strategies may be favoured for *H. solani* control. Some results have shown that soil possessing high total bacterial counts have reduced levels of *H. solani* (Elson *et al*. 1997), therefore indicating that the use of soil amendments that may increase soil microbial activity should be further investigated to determine their effectiveness in controlling incidence levels of silver scurf.

1.2.4 *Colletotrichum coccodes*

Colletotrichum coccodes (Wallr) Hughs is responsible for the disease known as potato black dot, so called because of the black microsclerotia that appear on all plant parts after infection (Read & Hide 1995; Lees & Hilton 2003; Ingram & Johnson 2010). Potato black dot disease has been reported in all areas of potato production worldwide (Cullen *et al*. 2002; Lees & Hilton 2003). Although in most cases overall yield is usually unaffected by black dot symptoms, the number of tubers of marketable ware size is often lower, while the number of small tubers is greater (Read & Hide 1995). *Colletotrichum coccodes* has the ability to colonise all underground parts and basal stems (Lees *et al*. 2010) infection leads to silvery/brown coloured lesions and black microsclerotia developing on the tuber surface. Microsclerotia are a mass of melanised hyphae with abundant setae. Such tuber symptoms are usually present on the heel end of the tuber (Lees & Hilton 2003), in the UK microsclerotia are also observed on roots, stems and stolons from June onwards (Lees & Hilton 2003; Glais-Varlet *et al*. 2004). Once infected the host periderm may develop stroma, which in turn develops conidia bearing aceruvulii. Under the right environmental stimulus aceruvuli may also develop from superficial sclerotia if periderm penetration is absent. Acervulus have also been shown to develop from microsclerotia (Ingram 2008). Conidia are straight, hyaline and aseptate and formed in gelatinous matrix, the full functions of this matrix are still unknown, but it is hypothesised conidia are sequestered in the aceruvuli until moisture levels required for germination and successful host penetration are present (Ingram 2008).

Foliage symptoms have also been described in some potato growing areas, although they have not yet been observed in UK crops. It is thought disease on foliage will arise through wounds caused by windblown soil and sand, they usually first appear as water-soaked lesions and then later turn dark brown to black. Plants infected with *C. coccodes* often become wilted, lower and middle leaves often become chlorotic (Lees & Hilton 2003) and sloughing of the root cortex occurs (Ingram & Johnson 2010). Foliar symptoms can regularly be confused with that caused by Verticillium wilt, caused by *Verticillium dahilae* (Ingram & Johnson 2010). *Colletotrichum coccodes* has been shown to be involved in complexes with soil borne pathogens including *V. dahliae* which

can lead to potato early dying (PED) resulting in stunting, wilting, premature senescence and reduced yields (Lees & Hilton 2003).

Colletotrichum coccodes infection can be both seed-borne and soil-borne – the latter has shown to be the most aggressive form (Nitzan *et al.* 2006; Ingram & Johnson 2010). Although studies by Nitzan *et al.* (2006) reported a non-linear relationship between the level of soil borne inoculum present and disease severity. In uncontaminated soils the disease will enter into the soil as seed borne inoculum and will then survive as microsclerotia until it attacks a following crop (Ingram & Johnson 2010; Lees *et al.* 2010).

The host-pathogen interaction between *C. coccodes* and potato has not been well studied, however infection of tomato (*Solanum lycopersicum* L.) is very well documented and therefore certain conclusions about the infection process in potato can be made. In tomato plants it is known that conidia on immature fruits and leaves will form appressoria and using physical force will penetrate the cuticle. Following successful penetration, colonization of neighbouring cells is limited. When fruit have matured, an unknown signal induces latent infection which rapidly spreads and causes dark sunken lesions to appear on the crop. Applications of protectant fungicides throughout fruit development retards latent infection development. In potato plants it must be assumed that penetration is similarly aided by appressoria and physical force. Evidence also exists to suggest that a similar type of latent infection exists (Ingram 2008).

There is limited knowledge about black dot as a tuber storage disease, although it is known that the incidence level and severity of black dot is greater in storage temperatures of 15°C than 5°C, however this increase is only observed on unwashed tubers (Read & Hide 1995). The development of black dot symptoms have also been shown to reduce during storage (5°C) if tubers are dried for 2 weeks after harvesting, compared to those that are not dried (Glais-Varlet *et al.* 2004). Further studies by Glais-Varlet *et al.* (2004) showed that storage temperatures of 5-8°C, the *C. coccodes* mycelium can grow and produce sclerotia, allowing black dot symptoms to establish and increase in size. This suggests that black dot has the ability to spread throughout tubers in commercial storage. Their study also further concluded that a high percentage of seemingly healthy tubers are latently infected at harvest, therefore demonstrating that effective black dot control must begin in the vegetative phase of the crop.

Numerous chemical fungicides and fumigants have been tested for their ability to control black dot, however to date none have been shown to provide adequate control. Control has been attempted both to minimise soil borne and seed borne inoculum. Treating seed tubers with prochloraz has shown to reduce the transmission of *C. coccodes* from seed tubers to progeny tubers if planted in virgin soils. However this was not observed when planted in infested soils. Imazalil produced a decrease of

potato black dot early in the growing season, however the reduction in disease is not passed onto progeny tubers (Cummings & Johnson 2008). Such studies have proved that *C. coccodes* cannot be controlled when soil inoculum is present, therefore it may be suggested that soil fumigation will have a greater success rate of control. However, use of common potato fumigants 1, 2-dichloropropane, 1, 3-dichloropropene or sodium *N*-methyldithiocarbamate are shown to be ineffective in reducing levels of *C. coccodes* in stems, roots and tubers (Cummings & Johnson 2008). The most significant levels of control with a synthetic fumigant have been observed through the application of methyl bromide, however as discussed previously this is no longer an option for controlling soil borne pathogens (Cummings & Johnson 2008).

The use of strobilurin fungicide, azoxystrobin ((Methyl (E)-2-{2-[6-2-cyanophenoxy) pyrimidin-4-yloxy] phenyl -3-mehoxyacrylate) has been shown to decrease *C. coccodes* in potato stems and progeny tubers if multiple foliar applications are made on plants growing in naturally infested soil. Azoxystrobin belongs to a class of chemicals (Qol) which act by blocking fungal respiration by inhibiting electron transport within the mitochondria (Nitzan *et al.* 2005; Cummings & Johnson 2008). The application of azoxystrobin may prove useful in reducing early stages of infection as underground infection in potatoes is known to occur soon after emergence and will develop in stems between 7-11 weeks, therefore its use may be ideal within an integrated management programme (Cummings & Johnson 2008).

1.3 Chemical control of soil borne pathogens

Soil borne pests have been traditionally controlled through the use of soil fumigation using methyl bromide (Poulsen *et al.* 2008) or metam sodium (Dungan *et al.* 2003; Matthiessen & Shackleton 2005). Methyl bromide (MB) is known to be an extremely hazardous fumigant pesticide which is toxic towards a wide range of pests and pathogens, including fungi, nematodes, weeds, insects, mites and rodents, and was commonly used prior to planting. However as environmental awareness rose, the Montreal Protocol was established in 1987, its aim was to phase out the use of all ozone depleting substances. In 1992 MB was identified an ozone depleting substance. This resulted in a phase out programme being initiated, which outlined that MB use would cease in developed countries by 1st January 2005, and undeveloped countries by 2015 (Dungan *et al.* 2003). Ozone depleting substances in the European Community (EC) are governed by regulation (EC) No. 2037/2000, 'Substances that Deplete the Ozone Layer'. This regulates the production, import, export, marketing, use and destruction of ozone depleting substances in the EC, (CABI 2008) Introduction of a ban on the use of MB has spurred on research and use of alternative, more environmentally friendly pathogen control methods, including biofumigation.

Since the 1950s metam-sodium (sodium-*N*-methyldithiocarbamate) has been used on a world wide scale to control most soil borne pathogens (Matthiessen & Shackleton 2005), including nematodes, fungi and insects (Dungan *et al*. 2003) that pose a threat in intensive cropping systems (Matthiessen & Shackleton 2005). It is known to be a less toxic pesticide than the previously used MB which has therefore made it desirable to growers. On contact with moist soil the primary breakdown product of metam sodium is the broad range, toxic compound -methyl isothiocyanate (Dungan *et al*. 2003; Matthiessen & Shackleton 2005).

Due to the diverse range of pathogens that can induce disease within potato crops high numbers of pesticides, soil fumigants and seed treatments have traditionally been used in control programmes. However since the early 1970's, when fungicides were first introduced, the intensive use of fungicides within agricultural practices has resulted in cases of certain pathogenic strains becoming insensitive to fungicide application. Traditionally thiobendazole (TBZ) was used on seed potatoes to control Fusarium dry rot and silver scurf disease, on potatoes (Secor & Gudmestad 1999). Yet overtime silver scurf isolates were shown to become increasingly resistant to the application of TBZ. Several factors have been shown to influence the development of pathogen resistance to pesticides. These include: type of fungicide used, frequency of use, the specific genetic properties of the pathogen, crop rotation practices and climatic conditions, (Wharton 2005). Additionally the mechanisms of resistance spread from one production area to another.

A significant amount of negative publicity surrounds pesticide use due to their hazardous effects upon the environment. Famously the detrimental effects of pesticides were first brought into the public eye by the effects of the application of dichlorodiphenyltrichloroethane (DDT). In the early 1950s it was common to observe dead birds in fields after they were sprayed with DDT or other similar insecticides. Its intensive use has led to 99 % of the US population having some DDT and DDT related metabolites within their body tissues (Beard 2006). Furthermore, to date pesticides still cause damage to aquatic life and they are also being held responsible for declines in insect numbers (van der Werf 1996) including the recent decline in honeybees (Henry *et al*. 2012). It must however be remembered that their use is necessary to produce healthy crop yields that meet public demand. It is this battle between the need to produce large crop yields and increasing government legislation on pesticide use that has left growers searching for an alternative which will provide protection of crops against soil borne pests and diseases, but which will also comply with environmental standards and government regulations.

1.4 Alternative control methods

Increasing research is being carried out into the pathogen suppression properties of bacteria and fungi, many of which are naturally found within soil. Termed biological control, incorporating several different microorganisms into soil has shown to prevent a number of different plant root diseases (Elson *et al*. 1997; Tsror *et al*. 2001; Bafti *et al*. 2005; Henderson *et al*. 2009). The method of disease suppression has been shown to differ depending on the microbial species; the production of antifungal compounds or elicit induced systemic resistance within the host plant, some bacterial species have also been shown to interfere with fungal pathogenicity factors (Bafti *et al*. 2005; Haas & Défago 2005; Al-Mughrabi 2010). One bacterium which has been extensively studied is *Bacillus subtilis,* which has been shown to produce antibiotics which can suppress the growth of several microorganisms and plant pathogens, including *R. solani*. Another group of bio control agents are *Trichoderma* species, which been shown to parasitize a number of fungal plant pathogens, again including *R. solani*. *Trichoderma* spp. also produce antibiotics which may suppress a range of different plant pathogens sites (Bernard *et al*. 2011). Biological control methods must also be assessed for their effects on the natural soil microbial community. Although many of the microorganisms used within biological control are naturally found within soil, they have not been shown to be found at such high levels as required for pathogen control. Little is currently known about the long term implications of using biological control methods; however some have produced significant levels of pathogen reduction.

Studies have shown that the use of canola rapeseed, sweet corn and barley/clover as rotation crops reduced the overall severity of *Rhizoctonia* stem canker disease in comparison to potato crops under monoculture. Similarly the use of canola and rapeseed as rotation crops were shown to be the most effective in reducing the onset of black scurf on tubers (Larkin *et al*. 2010). It has been suggested that the use of rotation crops can lead to a reduction of soil borne pathogens by one (or all) of three methods. Firstly the crop may interrupt or even break the host-pathogen cycle of inoculum production, or its growth or survival, secondly it may change the physical, chemical or biological nature of the soil in turn making it less conducive for pathogen growth and development, and in turn this may also benefit microbial activity, diversity or even plant growth promoting bacteria. Finally crop rotation may lead to direct suppression of pathogens through the inhibition or production of toxic compounds in the roots or plant residues or through the stimulation of microbial antagonists (Larkin *et al*. 2010). Biological control agents are also being increasing studied for use to control soil borne pathogens,

A further alternative control method which has been used over several decades is soil solarisation; this involves heating the soil to a temperature which is lethal to soil borne pathogens (Stapleton & DeVay 1986; Stapleton *et al*. 2000). This method of control has been practiced using glasshouses and steam however it now most commonly practiced

by covering soils with plastic tarps, which under sunlight will heat the soil temperature, and kill soil borne pathogens present within the soil (Fig. 1.1). This method has been shown to be effective in controlling several plant diseases (Stapleton & DeVay 1986; Stapleton *et al.* 2000). It has been suggested that soil solarisation could be used in combination with alternative pathogen control treatments, such as biofumigation. However the effects that soil solarisation may have on natural soil microbial communities must be assessed as it would be expected that such a process would have a negative impact on naturally occurring soil bacterial and fungi which have beneficial effects within the soil.

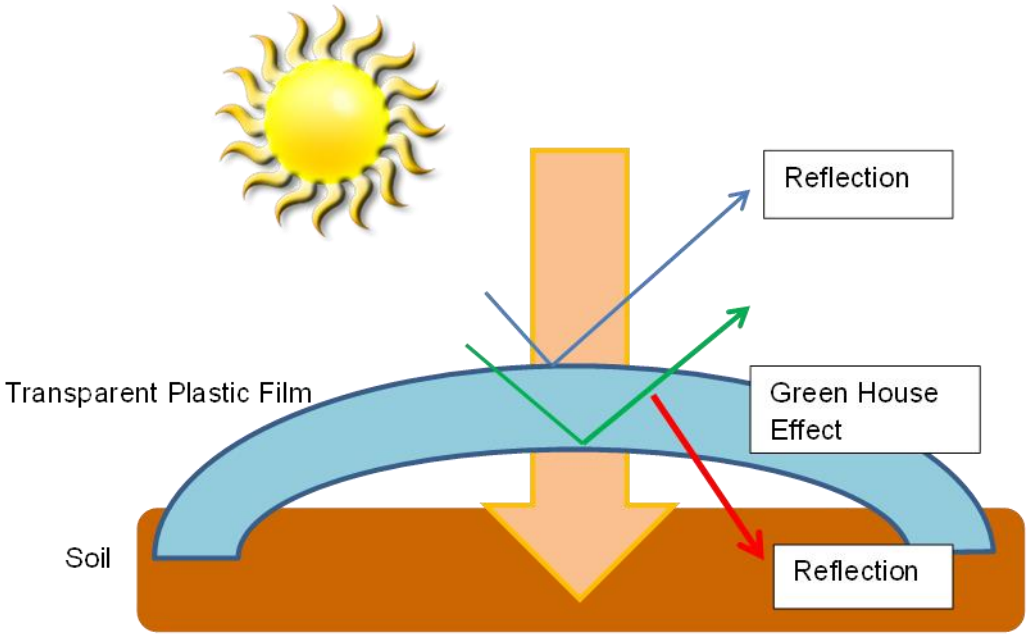


Figure 1.1 Diagram of the soil solarisation process used for pathogen control.

Increasing importance placed on the significance to establish effective alternative measures to reduce soil borne pests and pathogens has spurred large amounts of research within such areas. However there is still much to be discovered about the longevity of such methods and the most effective ways in which they should be deployed. Yet such research is necessary to introduce sustainable methods of crop production with little negative impacts on the environment and human health. To establish such methods within common agricultural practice it is suggested that they should be used in conjunction with pesticides and fumigant applications; however it is possible that application rate could be reduced, whilst maintaining soil borne pathogen control. Careful monitoring of pathogen levels will be required in order for alternative methods to be well established and in ensure growers that crops of high quality and yield are maintained.

1.4.1 Integrated pest management

The use of control strategies in combination with one another is now commonly referred to as integrated pest management, such control strategies in which a number of methods are used together, is being increasingly practiced and generating a number of success stories for pathogen suppression in a number of geographical regions (Oka 1991; Kogan 1998; Ratnadass *et al.* 2012) (Fig. 1.2). In Indonesia, IPM strategies have been implemented by the National Program of IPM which was launched in 1989; overall they decreased pesticide use to 60 %. Farmers also recognised significant savings on pesticides purchased and there was noticeable preservation of life components (Oka 1991). In the USA a considerable emphasis has been placed on adopting IPM strategies within agricultural systems. In September 1993, Clinton's administration aimed to implement IPM practices on 75% of the nation's crop acres by the year 2000 (Kogan 1998). The USA also set up the IPM Collaborative Research Support Program, which initially began in Virginia Polytechnic Institute and State University. Their research has produced several IPM success stories across several continents. They have developed biopesticides (plant extracts with pesticide activity) to control grasshoppers and locusts in Sub-Saharan Africa. They have also implemented IPM strategies to increase olive production in Albania. Both of which are helping farmers in developing countries achieve maximum crop yields, while reducing the cost of traditional pesticides. The use of integrated pest management to control a range of pests and pathogens, not solely soil borne pests and pathogens appears to be a strategy which if used correctly can produced significant reductions in targeted pests. It is hoped that such control methods will be increasingly adopted in agriculture to increasingly establish sustainable farming, without the overuse of synthetic chemical fumigants. It is often suggested and increasingly being practiced, that alternative control methods may be most beneficial in developing countries where access to pesticides is limited and is an undesired expense.

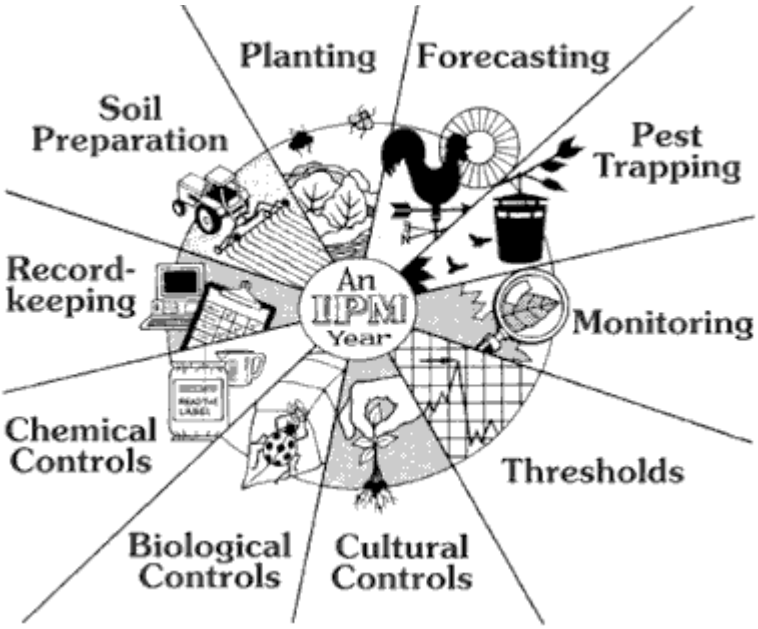


Figure 1.2 Diagram displaying a typical IPM strategy used for pest and pathogen management, Image from http://www.ipminstitute.org/index.htm

1.4.2 Future for alternative control strategies in agriculture

The basis for studying different methods and trying to establish it as an effective and efficient pest management system centres on the need for alternative control measures. Which have been driven forward both by legislation preventing the use of many previously used pesticides and soil fumigants (Regulation (EC) No 1107/2009). Additionally consumer pressure where there is a growing desire to buy produce which has been grown with little or no intervention of synthetic chemicals, however there still remains a demand to buy aesthetically pleasing produce, free from disease or remnants of pests, is pushing forward the need for alternative control.

Many alternative control strategies including biofumigation can be seen to be based on the foundations of once traditional practice of crop rotation, used to break the cycle of soil borne pathogens (Fig. 1.3). However with ever increasing demand on certain valuable crops it is difficult to implement sufficient crop rotation time periods (Larkin & Griffin 2007; Bernard *et al.* 2011).

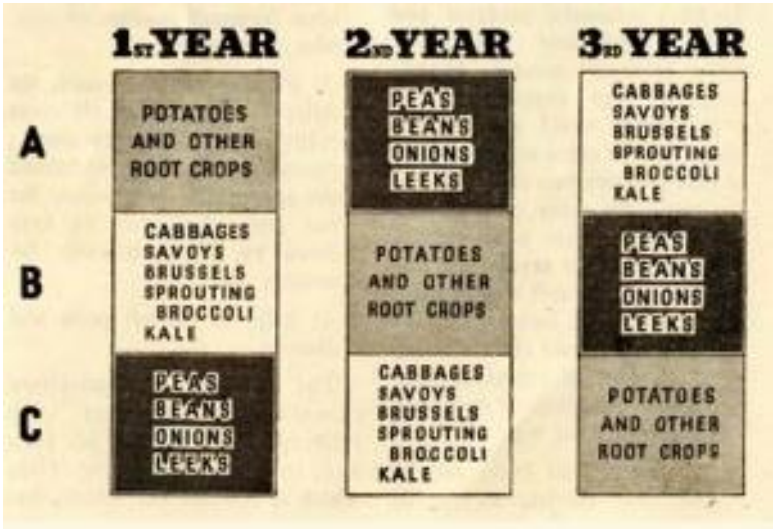


Figure 1.3 1945 Ministry of Agriculture Crop Rotation Guide – Image from (Ministry of Agriculture 1945).

1.5 An introduction to biofumigation

Biofumigation is an alternative control method which works on the principle of exploiting the natural biocide compounds from glucosinolate containing plants (Kirkegaard *et al*. 1998, 1999, 2000; Matthiessen & Shackleton 2005) to suppress soil microorganisms, such as fungal, bacterial pathogens and nematodes, (Angus *et al*. 1994; Brown & Morra 1997; Sarwar *et al*. 1998; Bianco *et al*. 2000; Smolinska *et al*. 2003). The term was first coined by Kirkegaard *et al* (1993) who specifically described using glucosinolate hydrolysis products, notably isothiocyanates, to control soil borne pests and pathogens in horticulture and agriculture. Isothiocyanates are produced during glucosinolate hydrolysis which occurs when *Brassica* plant tissues are broken down, allowing both glucosinolates and a myrosinase to come into contact with each other and hydrolysis to occur. In turn this releases one of several products, including isothiocyanates (Fig. 1.4) a detailed description of glucosinolate hydrolysis can be found in section 1.8.

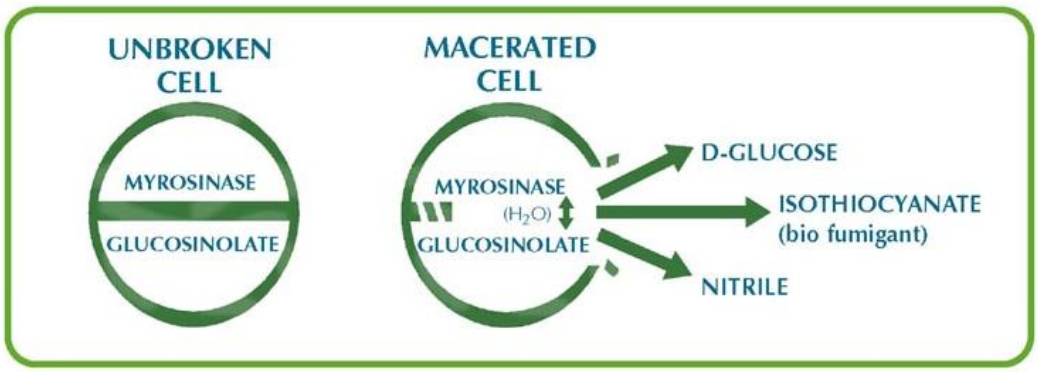


Figure 1.4 Basics of the biofumigation process, glucosinolates and myrosinase are compartmentalised until tissue disruption. When glucosinolates and myrosinase come into contact with each other, glucosinolate hydrolysis occurs, which may produce several products. Isothiocyanates have been shown to have toxic properties towards several microorganisms. Image from http://serve-ag.com.au/services/seed-sales-production/biofumigation-seed/

The problem of managing soil borne pests and diseases presents a number of challenges

- It is hard to predict disease epidemics from one year to the next.
- Problems arise from trying to target specific pathogens within complex soil ecosystems.
- Difficulties exist in detecting and quantifying pathogens and defining pathogen levels which will cause crop damage.

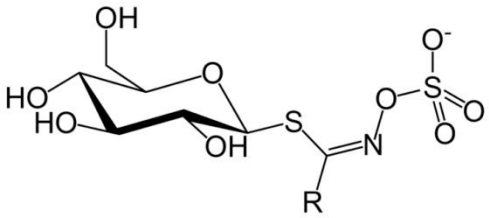
1.6 Details of the biofumigation process

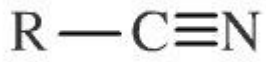
Biofumigation has been proposed as one alternative control method. This method exploits the glucosinolate hydrolysis products produced by *Brassica* plants. It has been well documented that *Brassica* spp. produce organic anion, secondary metabolites called glucosinolates (Brown *et al.* 1991; Bianco *et al.* 2000; Gimsing & Kirkegaard 2006), (sulphur containing glucosides) within their tissues (Fenwick & Heaney 1983; Brown *et al.* 1991; Gardiner *et al.* 1999; Gimsing & Kirkegaard 2009); additionally they also produce myrosinase enzymes intracellularly, which are necessary for glucosinolate hydrolysis.

Glucosinolates and myrosinases remain separated from each other while the plant tissues are intact, as they are compartmentalised within different cells. However upon tissue mastication, the cells are lysed and they will be brought into contact with other and resultantly glucosinolate hydrolysis occurs (Fig 1.6) (Bones & Rossiter 1996; Bianco *et al.* 2000; Gimsing & Kirkegaard 2006, 2009; Fan *et al.* 2008). The enzymatic mechanism of myrosinase involves two steps: The glycosylation step, in which the glycosyl-enzyme is formed and subsequently the aglycone is released. This is followed by the deglycolyation step in which the glycosyl enzyme is hydrolysed by a water

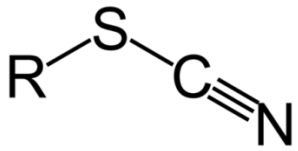
molecule (Burmeister et al. 1997). Glucosinolate hydrolysis can potentially release several different hydrolysis products, including nitriles, thiocyanates, however most commonly isothiocyanates are produced (Fig. 1.5)(Fenwick & Heaney 1983; Brown *et al.* 1991; Bending & Lincoln 2000; Bianco *et al.* 2000; Fahey *et al.* 2001; Gimsing & Kirkegaard 2009).

a) b)





c) d)



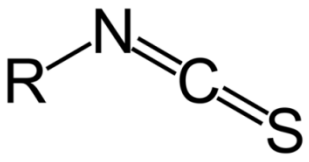


Figure 1.5 General structure of a) glucosinolates b) nitriles c) thiocyanates d) isothiocyanates.

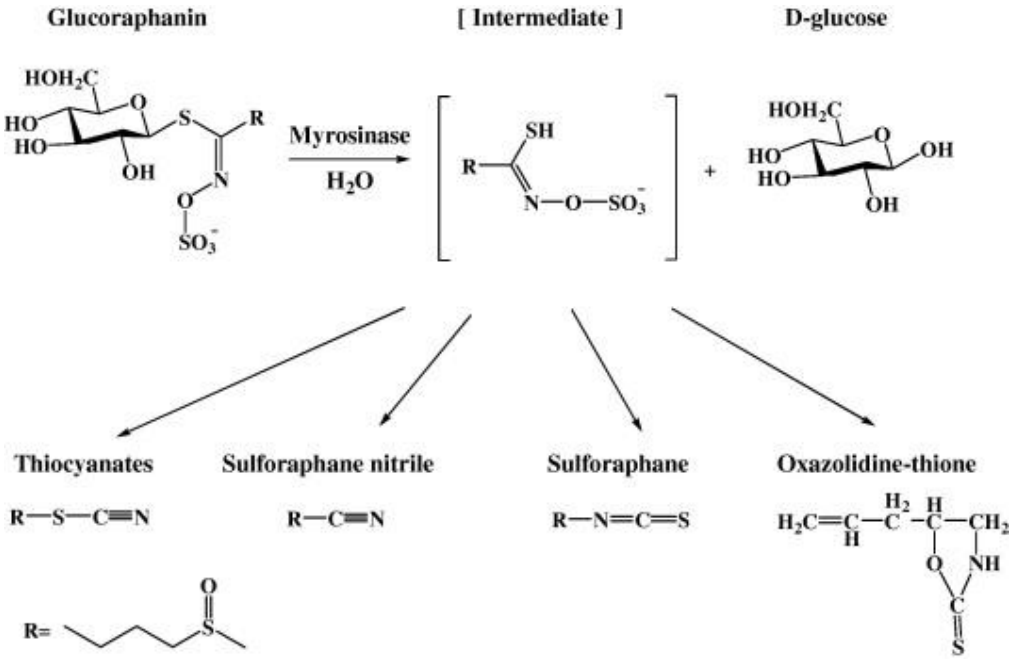


Figure 1.6 Glucosinolate hydrolysis – the diagram shows the different products that can be formed at different stages of the reaction. Image from (Shen *et al.* 2010).

As glucosinolate content and concentration is known to differ between *Brassica* cultivars and throughout development (Al-Gencly & Lockwood 2003; Bellostas *et al.* 2007), it is well accepted that the efficacy of biofumigation is dependent on the specific glucosinolate hydrolysis products formed during tissue breakdown. It is understood that different biofumigant crops used will potentially have different biofumigation potential and produce different levels of pathogen control (Motisi *et al.* 2009). Therefore to achieve the most effective biofumigation results it appears that it is necessary to gain an understanding of glucosinolate hydrolysis products formed by different *Brassica* cultivars, and their interactions with different soil borne pathogens. By gaining a greater understanding of the specific processes occurring during biofumigation, it is hoped that it can be used in a targeted manner to control specific pathogens, and aim to provide of more effective and efficient soil borne pathogen control.

1.7 Previous biofumigation research

The antifungal properties of isothiocyanates were first described in 1937; Walker *et al.* described the toxic effects of 'sulphur oils' produced by *Brassica* plants on a range of microorganisms. They noted that the overall level of inhibition differed greatly depending on the microorganism and the structure of the ITC. They also highlighted that in the parental glucosinolate state, no level of toxicity existed and hydrolysis had to occur to form biotoxic compounds (Walker *et al.*, 1937).

Research into the use of biofumigation has been carried out on a wide range of different pests and pathogens. The majority of this research has focused on *in vitro* methods to establish the fundamentals of the isothiocyanates-pathogen interaction. Previous *in vitro* studies have displayed examples of successful inhibition of pathogenic bacteria, postharvest fruit pathogens, and soil fungi including pathogens and saprophytes, using isothiocyanates (Brown & Morra 1997; Rosa, 1997).

Isothiocyanates have also been shown to exhibit activity against the potato cyst nematode *Globodera rostochiensis* (Pinto *et al.* 1998; Buskov *et al.* 2002; Serra *et al.* 2002). Studies conducted by Buskov *et al.*,(2002) highlighted that the parental glucosinolates alone did not have any effect on *G. rostochiensis* and that myrosinase had to be present, in order for secondary metabolites to be formed, before significant mortality of the nematodes was observed. Studies have also indicated that different microorganisms may vary in response to the same isothiocyanate. Smith and Kirkegaard, (2002) tested the sensitivity of 2-phenylethyl isothiocyanate – the glucosinolate (GSL) hydrolysis product of 2-phenylethyl GSL which has be shown to be a dominant GSL in the roots of canola *(Brassica napus)* (Gardiner *et al.* 1999; Kirkegaard *et al.* 2000) against a range of fungi, oomycetes and bacteria. In addition to *in vitro* methods their results demonstrated good control of several fungal species – including

Macrophomina phaseolina, Fusarium oxysporum, Pythium ultimum, and *Rhizoctonia solani* by incorporating canola leaves or ground seed meals into soil.

1.7.1 Physiological response of fungi to isothiocyanates

The inhibition of fungal pathogens using isothiocyanates has highlighted the different types of responses that can be exhibited by fungi when exposed to various individual isothiocyanates. Predominantly two terms are used to describe fungal responses to toxic compounds: fungistatic and fungitoxic. Fungistatic describes the instance when the initial point of fungal growth is delayed in responses to the presence of the toxic compound. Fungitoxic describes the fungi being killed and therefore unable to grow and develop, in response to the presence of a toxic compound. Studies using *Fusarium oxysporum* by Smolinska *et al.*,(2003) displayed both fungistatic and fungitoxic responses. In this instance fungistatic responses were attributed to the concentration the fungus was exposed to. Inyang *et al.*, (1999) showed using *in vitro* methods that isothiocyanates can also inhibit conidial germination and mycelial growth of the insect-pathogenic fungus *Metarhizium anisopliae.* Fungitoxic effects on the mycelial growth of *Alternaria* spp. through exposure to allyl and benzyl isothiocyanate have also been observed (Sellam *et al.* 2006).

1.8 Biofumigant incorporation methods

As interest in the use of biofumigation as a sustainable agricultural practice increases, several methods to incorporate the biocidal isothiocyanates have been practiced. To date the most common method is the incorporation of green manures; here the *Brassica* crops are grown on the land that is to be fumigated. Prior to planting of the susceptible crop the *Brassicas* are chopped, mulched and pulverised and ploughed into the soil (Matthiessen & Kirkegaard 2002). The green manuring process will disrupt the *Brassica* tissues; allowing glucosinolate hydrolysis to take place, releasing isothiocyanates into the soil. Further options also exist which allow growers to avoid growing the *Brassica* crops. One such method results from a by-product from canola oil production, the process involves the extraction of oil from canola seeds, the seeds are then dried and crushed, and the resultant seed meal can then be ploughed into soil. This method has been seen to be an attractive option, as early studies of glucosinolate profiles indicate that seeds may contain high concentrations of the parental glucosinolates for isothiocyanate formation, (Borek & Morra 2005). Additionally the use of dried *Brassica* plant material has also been described as an option for biofumigation practice. Dried green manures known to contain high concentrations of both glucosinolates and myrosinase can be supplied to growers. The plant material can then be ploughed into the ground, and with the addition of water the toxic alleochemicals

are formed (Lazzeri *et al.* 2004). In order for the most effective biofumigation to occur, research must also be carried out to assess which methods achieve the highest levels of glucosinolate hydrolysis, in turn releasing the highest concentration of isothiocyanates. This is an area of research not covered in this project, but must be considered when developing fully effective biofumigation practices.

1.9 *Brassicas*

Brassica is a genus of plants within the Brassicaceae, commonly known as the mustard family. However the family is comprised of a range of cruciferous vegetables, cabbages and mustards. The genus contains a number of important agricultural and horticultural crops, which includes several common types of *Brassicas* which used as food crops, including cabbage, cauliflower, broccoli and brussel sprouts. *Brassicas* are native in the wild within Western Europe, the Mediterranean and within temperate regions of Asia. Due to their agricultural importance, *Brassica* plants have been the subject of large amounts of scientific interest, six species have arisen as being particularly important (*Brassica carinata*, *B. juncea*, *B. oleracea*, *B. napus*, *B. nigra* and *B. rapa*), which have all been derived through combining the chromosomes from three earlier species, this theory is commonly termed the triangle of U (Janick 2009). The triangle of U theory was first published in 1935 by Jang-choon. The theory states that genomes of three ancestral species of *Brassica* combined in order to create three of the common modern vegetables and oilseed crop species, since its first proposal it has now been confirmed through DNA and protein studies (Nagahara 1935).

In recent times interest in *Brassicas* as important food crops has risen, not only are they established as providing high levels of vitamin C and soluble fibre but they also contain several anticancer properties and compounds including: diindolylmethane, sulforaphane and selenium. Additionally they are also known to contain high concentrations of indole-3-carbinol, which is known to boost DNA repair in cells (Verkerk *et al.* 2009).

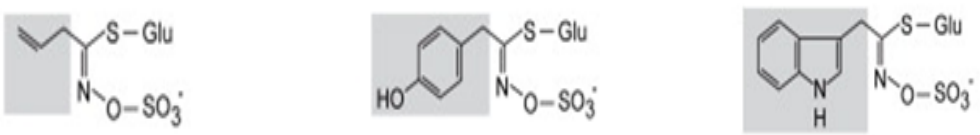
Further to their beneficial effects within the human diet, *Brassicas* have also been put under the spotlight as a useful agricultural crop which may possess pathogen suppression properties. The antimicrobial properties of oils released by *Brassica* tissues has been known for decades (Walker *et al.* 1937). However their use within agricultural systems to exploit such properties is a relatively new practice. It is hoped further understanding of hydrolysis compounds released from *Brassicas* will lead to the most effective methods of pathogen control using biofumigation.

1.10 Glucosinolates

Glucosinolates (GSLs) are present within all parts of the plant, but differ in profiles and concentrations throughout the plants tissues (Velasco *et al*. 2007). To date studies have shown that a single plant will most commonly contain approximately four differently structured GSLs in significant concentrations, however as many as 15 differently structured GSLs have been identified within the same plant (Verkerk *et al*. 2009). GSLs are commonly found to most readily accumulate in all vegetative and reproductive parts throughout plant development (Buskov *et al*. 2002). GSL concentration and composition are primarily affected by the plants genetics, however environmental and physiological factors, such as radiation, temperature and photoperiod, will also influence GSL expression and accumulation. Concentration and composition of GSLs are also known to change significantly throughout the plants development (Leoni *et al*.1997 ; Verkerk *et al*. 2009). GSL content within plants have also been shown to be affected by a number of agronomic factors, including; soil type, moisture and mineral nutrient availability (Velasco *et al*. 2007). Soil health has also been identified as having a significant influence on levels of GSLs in growing plants. Short days, cool temperatures and frost conditions during winter have been demonstrated to have a negative effect on GSL content (Velasco *et al*. 2007).

1.10.1 Glucosinolate structure

GSLs are derived from α-amino acid precursors (Gimsing *et al*. 2005, 2007; Bellostas *et al*. 2007a) that are ß-thioglycoside *N*-hydroxysulphates that possess a side chain 'R' and a sulphur linked ß-D-glucopyranose oxime moiety (Magrath *et al*. 1993; Bourderioux *et al*. 2005; Gimsing *et al*. 2005; Verkerk *et al*. 2009), both the side chain and sulphate group possess an anti stereochemical configuration across the C=N double bond (Holst & Williamson 2004). The sulphate group is normally balanced by a (potassium) cation (Verkerk *et al*. 2009). To date more than 120 side chains have been identified, it is the side chain structure which largely determines the group each glucosinolate is assigned to, however chemical properties and biological activity also play their part. Based on their structure GSLs are divided into three groups – aliphatic, aromatic and indolyl (Fig. 1.7) (Dawson *et al*. 1993; Holst & Williamson 2004; Gimsing *et al*. 2005).



a) b) c)

Figure 1.7 Different classes of glucosinolates a) aliphatic b) aromatic and c) indolyl Image from http://ars.els-cdn.com/content/image/1-s2.0981942808000363-gr1.jpg

The most abundantly produced GSLs in *Brassica* plant tissues are the aliphatic glucosinolates, derived from methionine. It is thought that the side chain elongation that is needed to develop aliphatic glucosinolates occurs early in the biosynthetic pathway (Fig. 1.8). Before development of the glycine moiety through the single or multiple addition of the methyl carbon of acetate to methionine, after glycine moiety formation side chain modification will occur (Magrath *et al.* 1993). Generally GSLs are polar, highly water soluble compounds, but on contact with the enzyme myrosinase they will hydrolyse quickly, particularly if water is present (Gimsing *et al.* 2005).

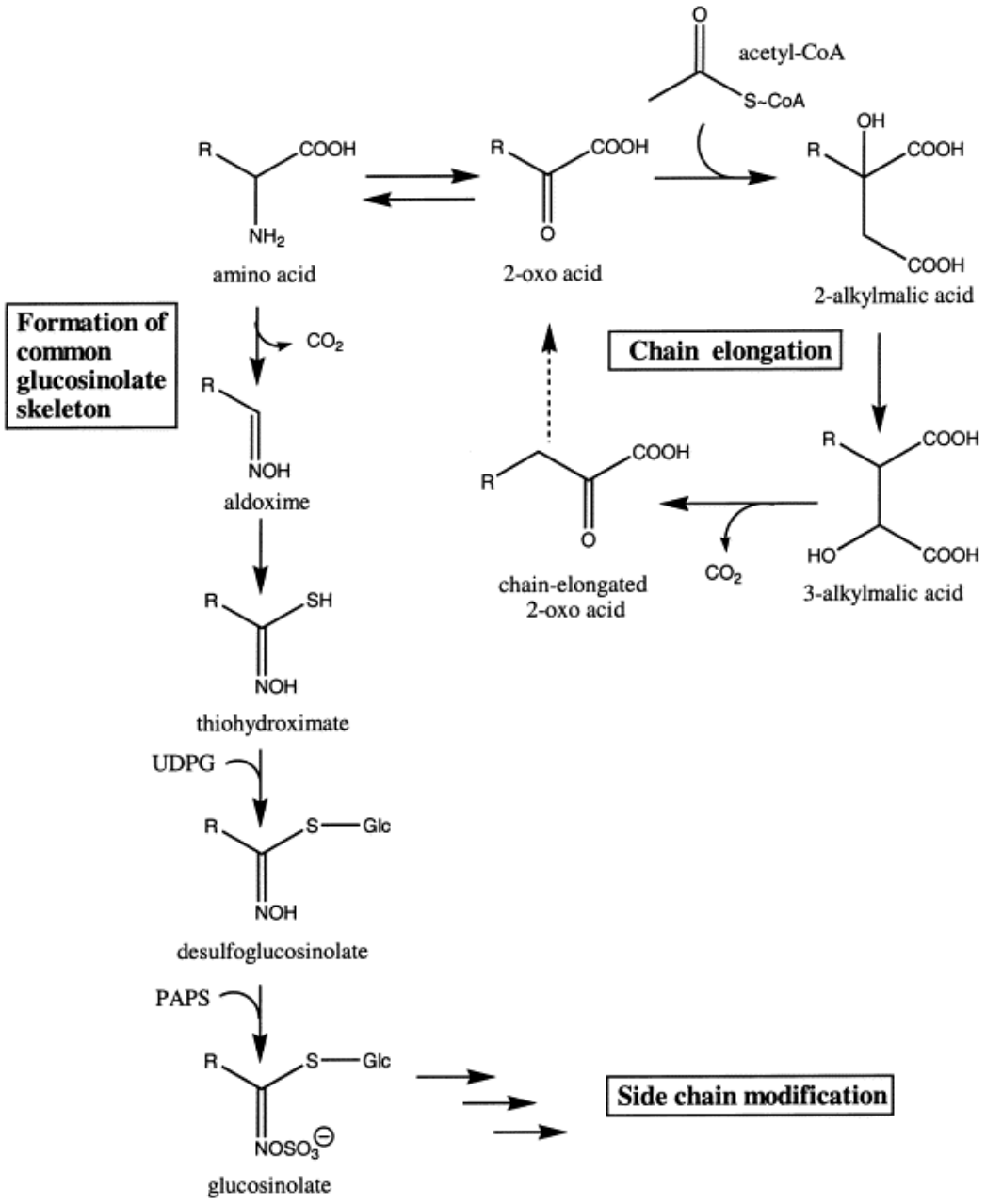


Figure 1.8 Glucosinolate biosynthesis process; image from http://ars.els-cdn.com/content/image/1-s2.0-S003194220000501X-gr1.gif

1.11 Myrosinase

Myrosinase [thioglucoside (glucosinolate) glycohydrolase, EC 3.2.3.1] is understood to exist in all plants and plant organs that contain glucosinolates. Myrosinase activity has also been found in fungi, bacteria, mammals and insects (Tani *et al.* 1974; Rask *et al.* 2000). Myrosinase belongs to the large hydrolytic superfamily of enzymes, the *O*-Glycosyl hydrolases or glycosidases. Due to its large number of members this family has been sub divided into – as it currently stands - 70 families, based on their amino acid sequence similarities. Myrosinase belongs to family 1 along with *O*-ß-glucosidases, 6-phospho- ß-glucosidases, 6-phosopho- ß-galactosidases, ß-galactosidases and lactase/phlorizin hydrolase (Rask *et al.* 2000). Glycosidases can also belong to one of two classes; retaining or inverting, based on the stereochemical outcome of the hydrolysis reaction they catalyse. Myrosinase has been identified as a retaining enzyme, which during hydrolysis undergoes a two-step mechanism, each involving an inversion therefore resulting in a net retention of stereochemistry, which is consistent with its sequence similarity with family 1 *O*-glycosidases (Bourderioux *et al.* 2005). Analysis of the three-dimensional structure of myrosinase identified several amino acid residues that are involved in binding the glucose ring and the aglycone they are also involved in the catalytic mechanism (Fig. 1.9). Within the myrosinase sequences the aglycone-binding residues are conserved, however this is not the case for *O*- ß-glucosidases.

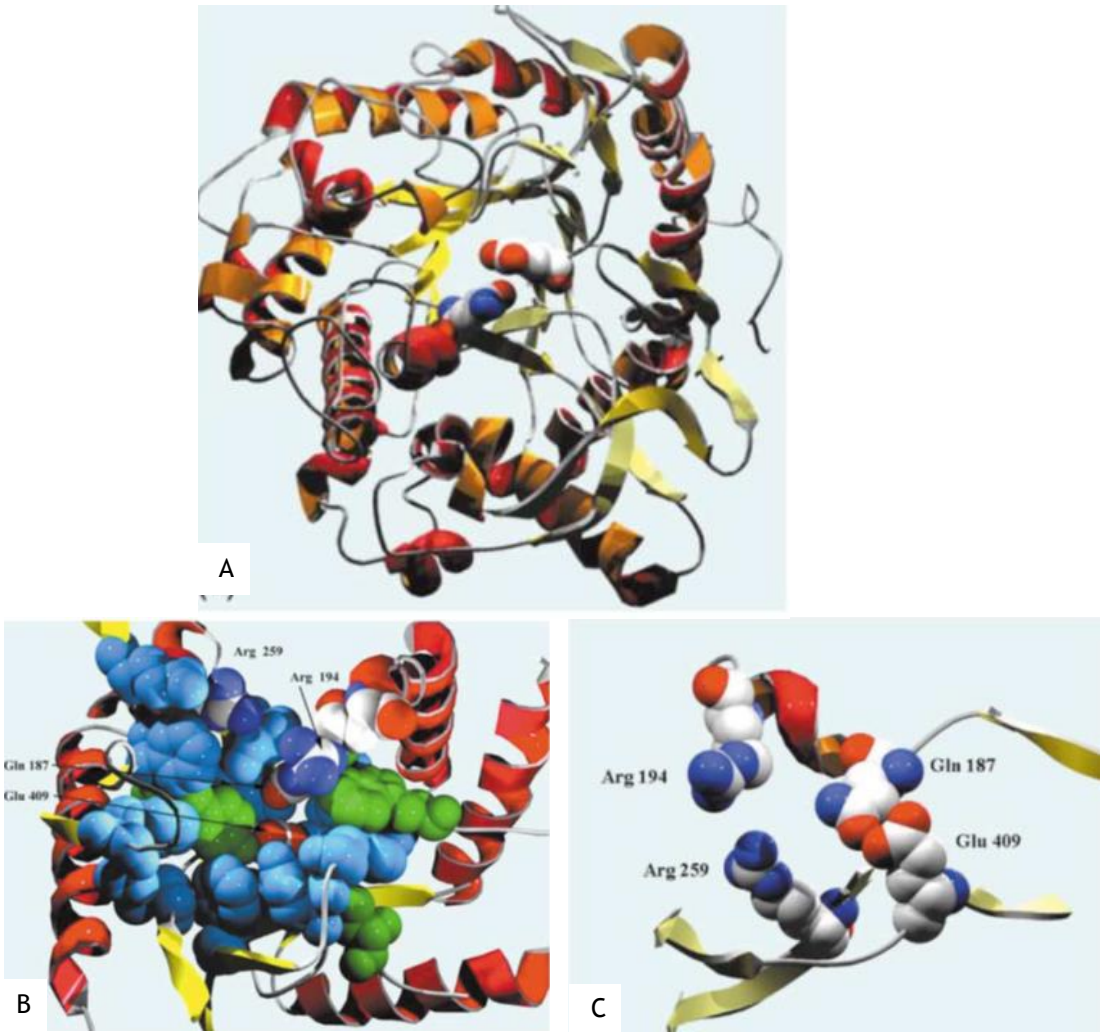


Figure 1.9 Location of residues involved in the active sites of plant myrosinases. A) Shows the ribbon model showing the active site residues. B) Shows the residues involved in substrate recognition, blue hydrophobic pocket, green general glycosyl hydrolase family 1 mechanism; labelled residues are shown in detail in C). Image from Rask *et al*. (2000)

Myrosinase activity within plants is dependent on several factors which include the species, cultivar and the specific plant organ studied. Most previous studies have identified that the highest levels of myrosinase activity occurred in seeds and seedlings. In addition to differences in myrosinase concentration levels different myrosinase isoenzymes have also been identified in different plant organs of the same plant. It should be noted that no direct correlation between levels of myrosinase activity and glucosinolate concentrations in plant tissues have yet been observed (Rask *et al*. 2000).

In 1884, Heinricher identified a special type of cell in Brassicaceae species which differed in both size and morphology from the adjacent cells. These cells have been referred to as 'protein-accumulating idioblasts', 'myrosin tubes' and more recently 'myrosin cells' (Rask *et al*. 2000), it is myrosin cells which have been shown to contain

myrosinase within the plant. Myrosin cells have been observed in seeds, parenchyma tissue, epidermis, and guard cells; the morphology of myrosin cells varies according to both the organ and tissue, and age of tissue in which they are present (Bones & Rossiter 1996) (Fig. 1.10). The primary organelles in the myrosin cells are spherical myrosin grains, which appear to fuse during differentiation of the myrosin cells, the exact intracellular localisation of myrosinase has been greatly debated (Rask *et al.* 2000).

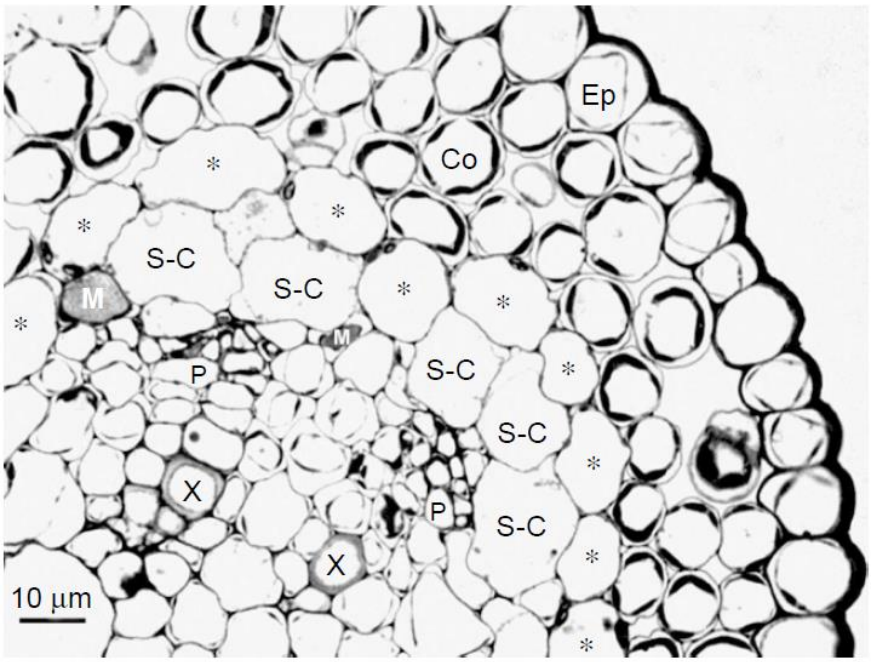


Figure 1.10 Compartmentalisation of glucosinolates and myrosinase within *A. thaliana*. Glucosinolates are thought to be present in sulphur rich cells (S-C) which are localised separated from myrosinase, stored in adjacent cells (M). Image shows a transverse section of a pedicle of *A. thaliana* is shown in which epidermis (Ep), cortex (Co), starch sheath (*), vascular bundles containing xylem (X) and phloem (P) can be seen. Image from Rask *et al.* (2000).

1.12 Glucosinolate-Myrosinase system

The myrosinase-glucosinolate system has long been known to be the defining phytochemical characteristic of the Capparale order (Bones & Rossiter 1996). The majority of glucosinolates are both chemically and thermally stable and as a result hydrolysis has to be enzymatically driven. The process is initiated through the hydrolysis of the thioglucosidic bond which produces a glucose and unstable aglycone – the thiohydoximate-*O*-sulphonate. The thiohydroximate-O-sulphonate then undergoes a spontaneous rearrangement, producing one of several possible products. The resultant product is dependent on the side chain structure, the parent glucosinolate and the reaction conditions. At pH 6 to 7 the most common hydrolysis products formed are stable ITCs, unless the GSL possesses a ß-hydroxylated side chain or an indole moiety; ß-hydroxyl-ITCs are unstable and consequently will cyclise to oxazolidine-2-thiones, whereas indole ITCs will undergo lysis. Which will result in the corresponding alcohol, such as indol-3-carbinol being formed, this will condense into dimers, trimers

or tetramers. At pH 4 to 7 and when ascorbic acid is present during the reaction, thiocyanates and ascorbigen are the major products of indole GSL hydrolysis. Glucosinolates possessing an aliphatic structured side chain are generally hydrolysed producing isothiocyanates at a neutral pH, yet in a more acidic pH or in the presence of Fe^{2+} ions it is more common that nitriles will be yielded (Holst & Williamson 2004). Generally glucosinolates will only yield a specific isothiocyanate, therefore examination of *Brassica* glucosinolate profiles can determine what potential isothiocyanates may be released (Table 1.1).

Table 1.1 Summary of commonly found glucosinolates, their common name and corresponding isothiocyanate

Glucosinolate	Common Name	Isothiocyanate
Benzyl	Glucotropaeolin	Benzyl isothiocyante
Methyl	Glucocapparin	Methyl isothiocyanate
2-Phenylethyl	Gluconasturtiin; phenethyl	2-Phenylethyl isothiocyanate
2-Propenyl	Allyl, Sinigrin	Allyl isothiocyanate
3-Butenyl	Gluconapin	3-Butenyl isothiocyanate
3-Methylthiopropyl	Glucoibervirin	3-Propyl isothiocyanate

1.12.1 Additional protein interaction during glucosinolate hydrolysis

Specific proteins have also been identified in some plants which are involved in glucosinolate hydrolysis, aside from myrosinase. One of which is a protein that promotes a class of nitriles, known as epithiospecifier protein (ESP) (Lambrix *et al.* 2001; Holst & Williamson 2004). When it is present during hydrolysis of alkenyl glucosinolates it will lead to the formation of epithionitriles instead of isothiocyanates, by transferring the sulphur atom from the basic glucosinolate backbone structure to the terminal alkene on the side chain. ESPs are small proteins (30-40 kDa), which were first isolated from *Crambe abyssinica* seeds, although the protein does not have myrosinase activity, it interacts with myrosinase encouraging sulphur transfer from the S-moiety (Bones & Rossiter 1996). ESP has been shown to inhibit myrosinase activity non-competitively, by interacting at a site on the enzyme that is not the substrate binding site (Bones & Rossiter 1996). They require ferrous ions to be present to be active; studies of ESP in *Brassica napus* have shown that the protein is completely inactive in the absence of Fe^{2+} ions (Bones & Rossiter 1996).

ESP is common in plants that contain glucosinolates with a terminal alkene, yet it appears ESP has no catalytic activity without the presence of myrosinase. Under various conditions glucosinolates without terminal alkene functions may yield nitriles, however it is unclear whether ESP or another protein plays a role in this process (Lambrix *et al.* 2001).

As glucosinolates contain both sulphur and nitrogen and are recognised as sinks for these nutrients, studies have shown that an increase in the nitrogen levels available favours the 'hydroxylation' step which converts gluconapin to progoitrin. Aliphatic glucosinolates have been demonstrated to be more sensitive to a deficiency in sulphur in comparison to indoly glucosinolates; this is most probably due the precursor of aliphatic glucosinolates - methionine, which contains sulphur. This overall suggests that the evolution of the glucosinolate myrosinase system was not in order to provide plants with an extra sulphur reservoir (Rask *et al.* 2000).

The glucosinolate-myrosinase system is susceptible to alteration at nearly every stage of the process, including biosynthesis, regulation and breakdown which is largely controlled by gene duplication and subsequent functional diversification (Kliebenstein *et al.* 2005). However, although genetic fluctuations play an important role in determining the glucosinolate profile, they are not the sole driving force. Plant development, and abiotic factors including nitrogen, sulphur or potassium supply have also been shown to have an influential effect (Kliebenstein *et al.* 2005). Glucosinolate biosynthetic pathway gene expression and profiles have also been shown to alter in response to pathogen attack and herbivory. During insect herbivory, jasmonates are released which contribute to signal transduction, this in turn increases total glucosinolate concentration, primarily due to changes in indole glucosinolate concentration. Jasmonates also induce the formation of several aliphatic glucosinolates. A pathogen attack signal – salicylate- has also been shown to stimulate glucosinolate accumulation either alone or in combination with other phytohormones and may counteract the glucosinolate induction mediated by jasmonates (Kliebenstein *et al.* 2005).

1.12.2 Natural role of the glucosinolate-myrosinase system

It is hypothesised that the glucosinolate-myrosinase system evolved for use as a defence mechanism against attacking organisms (Dawson *et al.* 1993). As the associated proteins and hydrolysis products have been shown to repel generalist herbivores, aerial herbivores and pathogens, but may attract certain specialist herbivores (Bones & Rossiter 1996) or act as behaviour modifying chemicals in some insect species (Haughn *et al.* 1991). Herbivorous attack, particularly by chewing insects induces a large amount of glucosinolate hydrolysis, resulting in the variety of toxic products; this is in turn can markedly alter GSL levels within plant leaves. Some

species of insects have adapted to feed on crucifers which have subsequently been shown to use glucosinolates and their catbolites as semiochemical cues to aid them in locating and colonizing plants. Some Coleopeterous pests of *B. napus* are known to be highly adapted to feed on crucifers and utilise specifically alkenyl homologues, 3-butenyl and 4-pentenyl isothiocyanates as attractants (Dawson *et al.* 1993).

There is much evidence to suggest that secondary compounds stimulate insect feeding and ovioposition, they also provide indirect plant defence, by providing cues to natural enemies of herbivores (Rask *et al.* 2000). Resultantly this can drive evolutionary change, as plants in a population containing high concentrations of secondary metabolites may be more resilient to herbivore damage. Therefore they may have a natural advantage over less protected plants. Additionally some insects may adapt to overcome this plant defence system, and will have less competition from other, non-adapted herbivores. In this case the chemical signals released by defensive compounds now in essence become host finding stimulants. Some insects including aphid species may also develop methods to store and detoxify the potent compounds and use them as defensive mechanisms against their own predators (Kliebenstein *et al.* 2005).

Herbivorous insects can be grouped into two classes, generalists and specialists, although these are not definitive groups and a gradient exists between them – some insects will utilise a single plant species, whereas others will exploit plants from many families. Using this classification spectrum it is thought that generally the presence of glucosinolates within plant tissues will be more advantageous for specialists and deleterious for generalist species (Rask *et al.* 2000).

Blau *et al.* (1978), first demonstrated the negative effects that the glucosinolate myrosinase system has on herbivores in 1978. In a series of laboratory experiments they revealed the Brassicaceae specialist *Pieris rapae* was unaffected by exposure to increasing doses of sinigrin (allyl glucosinolate). In contrast two species of non-Brassicaceae specialists showed signs of increasing growth inhibition and even mortality. To date studies have focussed around allyl isothiocyanate as to date it has been identified as the most commonly produced isothiocyanate within *Brassica* plants. Evidence also indicates that Brassicaceae specialists may be more responsive to specific glucosinolates, which suggests that the specific structure of glucosinolates produced by plants may be of greater importance than the total glucosinolate concentration within the plant cells (Rask *et al.* 2000).

The glucosinolate-myrosinase system may also provide an indirect form of defence, as released volatiles have also been shown to attract natural enemies of herbivores, including several species of parisitoids of Brassicaceae specialists (Rask *et al.* 2000). In addition herbivores have also been shown to use secondary metabolites as defence mechanisms. For example, aphids produce olfactory signal, alarm pheromones in order to escape enemies. The alarm pheromone produced by the mustard aphid *Liaphis erysimi* and the cabbage aphid *Bevicoryne brassicae* have been shown to contain

isothiocyanates. It is presumed that their presence is due to catabolism of glucosinolates within the aphid using its own myrosinase enzyme (Rask *et al.* 2000).

1.13 Isothiocyanates

Isothiocyanates have been shown as likely compounds for allelopathic activity as they have been shown to produce adverse effects on growth and survival of bacteria, fungi, mammals and insects (Choesin & Boerner 1991). Their broad range biocidal activity can be attributed to their ability to cause inhibitory effects through interaction with proteins, yet the mechanisms involved are not completely understood (Morra & Kirkegaard 2002). However three main hypotheses have been proposed as an explanation for observed reduction in microbial growth: 1) the intracellular enzymes may be inactivated through oxidative breakdown of the –S-S- bridges. 2) inhibition of metabolic enzymes by a thiocyanate radical 3) uncoupled action of oxidative phosphorylation as indicated by the inhibition of oxygen uptake of plant pathogenic *Pythium* fungal strains by several ITCs (Mancini *et al.* 1997).

As previously stated (section 1.2) isothiocyanates are used in traditional control methods of soil borne pathogens, through the primary breakdown product of metam sodium – methyl isothiocyanate. Methyl isothiocyanate is the simplest structure of a large number of isothiocyanates that may be produced through *Brassica* tissue disruption, however methyl isothiocyanate is not commonly found to be produced in the Brassicaceae (Matthiessen & Shackleton 2005). One of the most ubiquitous and proportionally high identified and studied ITCs is Allyl isothiocyanate (AITC), it's parental glucosinolate, Allyl glucosinolate, has been readily found in *Brassica nigra, Brassica carinata* and *Brassica juncea* all of which have been shown to produce AITC through glucosinolate hydrolysis at a pH of 4.0 or greater (Mayton *et al.* 1996). The high levels of allyl glucosinolate which have been found within plant tissues and the fact that studies have shown AITC to be as toxic to a range of fungi as MITC *(*Mayton *et al.* 1996) have prompted a number of studies for its use a pathogen control agent.

It was originally thought that AITC was the only commonly found ITC to provide a level of disease control or pathogen suppression due to a number of studies investigating its effectiveness. Reports from Dhingra *et al.*, (2004) also show that decreases in fungal growth were directly related to the concentration of AITC the fungal species were exposed to. However with an increasing number of studies on biofumigation, it is now understood that AITC is not the only ITC that has suppressive effects on microorganisms.

1.13.1 Environmental impact of isothiocyanates

With the use of new innovative methods of controlling soil borne pathogens, it is important their effects on the environment are monitored. To date, the majority of studies on ITC levels after their incorporation into soil have centred on AITC. AITC concentrations in soils have been shown to decrease over time, with the ITCs only being active within the soil for relatively short durations of 5 to 7 days (Price *et al.* 2005). Soil texture has also been demonstrated as having an influence on available AITC concentrations. Bending and Lincoln (2000) found that in clay loam soils, gas phase AITC concentrations were lower than in sandy loam soil, perhaps due to the higher organic carbon content within clay loam soil onto which could adsorb AITC and then react with it. Adsorption of MITC into soil has been shown to increase with an increasing organic matter content (Price *et al.* 2005).

ITCs can be portioned into the phases within the three phase soil system (vapour, aqueous and soil), depending on their solubility in water. It is assumed that the rate of AITC volatilisation is dependent on the rate of partitioning of AITC between the vapour and the aqueous phases. Higher soil temperatures may also encourage AITC escape from the aqueous phases, due to higher kinetic energies (Price *et al.* 2005). Price *et al.*, (2005) also showed that higher concentrations of AITC may be released into the soil when the biofumigation process was carried out at higher soil temperatures. As well as monitoring the overall environmental consequences that ITC release may have, research into the volatilisation of ITCs provides important information that will aid efficient use within biofumigation systems.

As MITC is the primary degradation product of metam sodium, information also exists on its behaviour in the environment; such knowledge may also be transposable to other ITCs. MITC is known to possess a high diffusability rate, resulting in gradual penetration of the soil profile. MITC is also known to decompose quickly into inactive harmless compounds, which suggests it does not leave residues within the environment unlike xenobiotic pesticides (Matthiessen & Kirkegaard 2002). Most fumigants have been shown to degrade relatively slowly and in the case of the primary breakdown product of metam sodium, MITC, the degradation half-life has been shown to range from a few days to weeks. Therefore it can be predicted that a significant portion of MITC released into the soil will eventually be volatilised into the atmosphere. Studies have also indicated that applying organic amendments to soil can benefit degradation and therefore decrease fumigant pesticide emissions from soil (Dungan *et al.* 2003). MITC degradation has also been shown to be affected by soil temperature, moisture content, texture and organic carbon content (Dungan *et al.* 2003).

Although metam sodium possesses many desirable qualities for use as a soil fumigant it is too expensive for use in many production systems. This is another factor that has encouraged research in the use of naturally released isothiocyanates for pathogen control.

1.14 Soil microbial communities

1.14.1 Soil health and soil quality

In recent times there has been increasing interest in understanding the effects agricultural practices have on soil microbial communities. This has been somewhat fuelled by increasing knowledge of the links between above and below ground communities. It is now well recognised that the overall 'health' and microorganism structure within soils can have significant effects on above ground crops and vegetation. The link operates as a feedback system in which vegetation is influenced by the soil microbial community and the specific species of vegetation planted in the soil will influence the bacterial species present in the soil microbial community (Korthals *et al.* 2001). Therefore it is important to understand all aspects of agricultural practices, such as biofumigation, and how they may affect soil microbial communities. In order to establish how the process will affect overall soil health and quality.

The Soil Science Society of America Ad Hoc Committee on soil quality originally defined soil quality as 'the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality and support human health and habitation' (Doran & Zeiss 2000). Johnson *et al.* (1997) defined soil quality as a 'measure of the condition of soil relative to the requirements of one or more biological species and/or to any human purpose'. Although both use the term 'soil quality', other authors prefer 'soil health', as they believe it to emphasise the living dynamic nature of soil, where functions are driven by a diversity of living organisms that require management and conservation (Doran & Zeiss 2000). However accurate definitions and terminology are perhaps trivial as long as the message of the overall importance of preserving and maintaining a healthy soil microbial community is understood.

1.14.2 The effect agricultural practices have on soil microbial communities

In recent decades sustainable agricultural practices, such as the incorporation of organic matter amendments, biological control organisms, (pathogen antagonistic bacteria and fungi) and crop rotation, all of which when trialed have displayed potential for long term suppression of soil borne pathogens, have emerged and are becoming more commonly practiced. Yet the impact of such alternative pathogen control strategies have on soil microbial communities is not well studied and understood. Understanding the effects alternative control methods have on soil microbial communities will provide a fuller picture of the capabilities of alternative methods to manage soil borne pathogens (Bernard *et al.* 2011).

Microorganisms, predominantly bacteria, play a major role in important soil processes. However much still remains unknown about soil bacteria diversity and subsequently how this diversity and microorganism community structures are affected by management and land uses (Fierer & Jackson 2006; Acosta-Martinez *et al.* 2008; Griffiths *et al.* 2011). Limited knowledge of the specific bacteria involved in such roles can be attributed to their immense diversity and the vast numbers they are found in.

1.14.3 The importance of soil microorganisms

Certain genera of rhizobacteria have been shown to exhibit close association with plants (Egamberdiyeva & Hoflich 2003; Egamberdieva 2008), including *Pseudomonas, Bacillus, Arthrobacter, Azospirillium, Klebsiella,* and *Enterobacter.* Such bacteria have been shown to promote plant growth, through the production of phytohormones, their roles also include antagonistic defence against pathogens (Egamberdieva 2008) as well as aiding their ability to establish themselves in unfavourable environments (Egamberdiyeva & Hoflich 2003).

The association between soil and plant productivity is one that is well understood at least at a superficial level. Importantly soil condition is also known to have an effect on air and water quality. Intensive land management practices have impacted on the overall quality of surface and sub-surface water in several parts of the world consequently this has resulted in the imbalance of C, N and water cycling within soil (Doran & Zeiss 2000). Soil management practices including tillage, cropping patterns and the use of pesticides and fertilisers are also known to have an effect on atmospheric quality by altering soil's capacity to produce or consume essential atmospheric gases including carbon dioxide, nitrous oxide and methane (Doran & Zeiss 2000).

Advances in soil science have identified that land use and management has a significant effect on soil bacterial biodiversity. Studies have concluded that previous traditional agricultural practices have significantly degraded and reduced the overall quality of many soils worldwide. Specifically the use of mechanical cultivation and the intensive production of row crops has been identified as leading to a physical loss of soil, displacement due to erosion and decreases in organic matter content which have been related to a release of CO_2 in the atmosphere (Doran & Zeiss 2000).

Yet some management practices have been highlighted as having beneficial effects on soil quality. One of which is crop rotation which has been shown to increase soil fertility, tilth and aggregate stability it has also been shown to improve soil water management and can reduce erosion. Crop rotation is an essential practice in maintaining crop productivity and has also been shown to decrease the build-up of soil borne plant pathogens and diseases (Larkin *et al.* 2010). Cover crops which are primarily grown to cover and protect soil from erosion and nutrient losses between crop

production periods, have also been shown to reduce soil borne pathogens levels (Larkin *et al*. 2010). This may be a result of complex changes to soil microbial community composition. It is understood that different crops will have differing effects on soil micro biota; therefore there is a need to understand the effects specific crops have on overall composition and different bacterial groups in order to achieve maximum disease suppression.

1.15 Influence of biofumigation on soil microbial communities

To date little research has been carried out investigating how the practice of biofumigation affects naturally occurring soil microbial communities. It is believed that through the stimulation of microbial growth of antagonistic bacteria most organic matter amendments will achieve inhibition of soil borne pathogens. However in studying the use of a biofumigation system, there is the added component of the release of biotoxic compounds. Although the use of green manures may encourage the development of antagonistic bacteria and aid in microbial suppression, it is unknown what the release of isothiocyanates will have on the naturally existent and beneficial bacterial groups. It could be assumed that if the addition of glucosinolate myrosinase products has a suppressive effect on soil borne pathogens, then the diversity of soil microorganisms may also be altered and again the effects of specific crops should be understood to achieve the maximum efficiency within a biofumigation system (Larkin *et al*. 2010). However until detailed study is carried out such assumptions cannot be confirmed.

1.16 Aims of this study

The main aim of this study was to establish if biofumigation could be successfully used as an alternative agricultural practice to control levels of soil borne potato diseases. Work focussed on three fungal potato diseases, *R. solani, H. solani* and *C. coccodes*. However the study also aimed to develop methods which could be used with additional pathogens to gain an understanding of their potential to be controlled using biofumigation. To date, biofumigation within field and glasshouse experiments has demonstrated successful results in pathogen control; however gaps still remain in understanding the detailed specifics of the process, both in terms of the pathogens and isothiocyanates involved. It was decided that in order to establish an enhanced understanding of the biofumigation process, a range of molecular studies and chemistry analysis should be deployed under *in vitro* bioassay, glasshouse and field experimentation conditions. It was hoped that results from each of these various methods and techniques would further knowledge of all aspects of biofumigation and

combining the results would lead to development of effective, efficient biofumigation strategies for potato soil borne pathogens.

The three key objectives of were as follows:

1) Assess the toxicity levels of differently structured isothiocyanates against different soil borne pathogens, *C. coccodes, H. solani* and *R. solani*.
2) Analyse the specific structures and concentrations isothiocyanates formed, during glucosinolate hydrolysis in a range of *Brassica* cultivars.
3) Establish if the biofumigation process and release of isothiocyanates into soil has lasting effects on the soil microbial community composition.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals used were obtained from Sigma Aldrich, Dorset, UK unless otherwise stated (Table 2.1).

Table 2.1 – Chemicals used throughout the study that were not obtained from Sigma Aldrich Ltd.

Product	Supplier
Ammonium Sulphate $(NH_4)SO_4$	Fisher Scientific
Bacto Agar	Scientific Laboratory Supplies Ltd
Bacto Yeast Agar	Scientific Laboratory Supplies Ltd
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems™
Chelex 100 Resin	BioRad Laboratories
Easy-DNA™ Kit	Invitrogen™
Gel Red	VWR International Ltd
GeneScan Lysis Buffer	Eurofins
GeneScan™ -500 LIZ® Size Standard	Applied Biosystems™
Glucose	VWR International
Glycerol	Scientific and Chemical
Hi-Di™ Formamide	Applied Biosystems™
Magnesium sulphate heptahydrate $(MgSO_47H_2O)$	VWR International
Nutrient Agar	Fisher scientific
Potassium Dihydrogen Phosphate (KH_2PO_4)	Fisher Scientific
Potassium Hydrogen Phosphate (K_2HOP_4)	Fisher Scientific
Potato Dextrose Agar	Fisher Scientific
TaqMan® Environmental Master Mix 2.0	Applied Biosystems ™
Tris-acetate-EDTA	Severn Biotech Ltd
Type-it Microsatellite PCR Kit	Qiagen
UltraClean®-htp 96 Well Soil DNA Isolation Kit	MO BIO Laboratories, Inc
Yeast Extract	Scientific Laboratory Supplies Ltd

2.2 General Laboratory Practice

2.2.1 Autoclave

All sterilisation of equipment and solutions that required heat were carried out in bench top (Swiftlock Secure Touch+, Astell, Kent, UK) or free standing (Swift lock, Secure Touch, Astell, Kent, UK) autoclaves.

2.2.2 pH Meter

The pH measurement of solutions was performed using, pH meter, (Corning 430, Flintshire, UK) as per the manufacturer's instructions.

2.3 Growth Conditions

2.3.1 Fungi

All fungal cultures, provided by Potato Section, SASA (Science Advice for Scottish Agriculture), were incubated on potato dextrose agar (PDA) at 22°C in alternating periods of 12 hours darkness 12 hours light. Plates were periodically sub-cultured for experimental use. Fresh cultures were made by coring 7 mm fungal plugs, using a sterile cork borer from the outer margin of mature colonies and transferring onto fresh PDA.

2.3.3 Plants

Plants grown in glasshouses were grown in glasshouses at SASA; they were watered daily by Horticulture staff, SASA.

2.4 Preparation of Isothiocyanate Solutions

Isothiocyanate solutions for agar plates were made to the desired concentration using 50% ethanol solution. GC-MS standard ITC solutions were made in Ethyl Acetate. All other methodologies using ITC solutions were made up using 1% dimethyl sulfoxide (DMSO) solution.

2.5 Growth Media

All growth media, with the exception of ITC incorporated PDA plates, were made as below, by the Media Preparation Unit, Diagnostics and Molecular Biology Section, SASA.

2.5.1 ITC - Potato Dextrose Agar

500 µl of the isothiocyanate solutions (Allyl (AITC), Benzyl (BITC), Isopropyl (IITC), Methyl (MITC), Naphthyl (NITC), 2-Phenylethyl (PEITC) and Propyl (PITC), (Sigma Aldrich, Dorset, UK) were thoroughly mixed, with 20 ml of molten PDA swirl mixed and poured into a 85 mm diameter Petri dish. Control plates, in which 500 µl of 50 % ethanol solution was added to the molten agar, were also made. Eight replicates were used for each concentration and controls.

2.5.2 Potato Dextrose Agar

39 g of Potato dextrose agar (PDA) was added to every litre of dH_2O and adjusted to pH 5.6, then autoclaved for 15 minutes at 121°C and cooled to 45 °C before dispensing, 20 ml/plate.

2.5.3 Nutrient Media

28 g of Nutrient agar was added to every litre of dH_2O, then autoclaved for 15 minutes at 121 °C and cooled to 45 °C before dispensing, 20 ml/plate.

2.5.3 Carboxymethylcellulose Media

10 g of Carboxymethylcellulose (CMCA) 16 g Bacto Agar, 5 g Bacto Yeast Extract, 2 ml of glycerol, 9 g 50x phosphate (7g K_2HPO_4 + 2 g KH_2PO_4 (pH 6.9-7.1)), 1 g $(NH_4)_2SO_4$ and 0.1 g of $MgSO_4$ was added to 1 litre of dH_2O. Sterilised at 121 °C cooled to 50 °C for dispensing, 20 ml/plate.

2.5.4 Phosphate Media

20 g of glucose, 1 g yeast extract, 0.2 g calcium chloride, 0.5 g $Mg\ SO_{4.7}H_2O$, 5.02 g $CaPO_4$ and 40 g of agar added to 2 litres of dH_2O. The solution was sterilised at 121 °C for 15 minutes, cooled to 50°C, 20 ml/plate.

2.5.5 Starch Media

46 g of nutrient agar and 6 g of starch (Potato – Sigma) was added to 2 litres of dH_2O. Solution was sterilised at 121°C for 15 minutes, cooled to 50°C and dispensed at 20 ml/plate.

2.5.6 MRS Media

62 g of MRS agar was added to 1 litre of dH_2O. The solution was sterilised at 121°C for 15 minutes, and cooled to 50°C before dispensing, 20 ml/plate.

2.6 Molecular Biology

2.6.1 DNA extraction

2.6.1.1 Fungal extraction

Cultures of both *R. solani* and *C. coccodes* were maintained on PDA as described above. Prior to DNA extraction both fungal pathogens were grown in liquid culture to achieve high DNA yields. Five 7 mm cores were taken from the outer margin of a growing culture, and added into 20 ml of nutrient broth, in a 50 ml centrifuge tube. The tubes were incubated at 27°C and mixed constantly for five days. DNA extraction was carried out using a method provided by Dr. A Reid, (SASA, UK): Cultures were centrifuged at 13,000g for 10 minutes, the resultant supernatant was removed and transferred into 5 ml of Eurofins GeneScan lysis buffer and 50 µl of Proteinase K solution (20 mg/ml) was added to the supernatant and vortexed. The solution was incubated for 1 hour at 65 °C then centrifuged for 10 minutes at 13,000 g after which the supernatant was transferred into a new centrifuge tube to which 3250 µl Chloroform/isoamyl alcohol was added and mixed. The upper aqueous phase was transferred into a fresh tube, to which 2,600 µl of propan-2-ol was added and the solution was mixed, this was then incubated on ice. After 30 minutes the solution was centrifuged for 10 minutes at 13,000 g to pellet the DNA, the supernantant was discarded. The resultant DNA pellet was then washed twice with 500 µl of 75 % EtoH, after each wash the DNA was centrifuged for 5 minutes at 13,000 g. The pellet was air dried and resuspended in sterile H_2O.

2.6.2 Fungal extraction from soil

Fungal DNA was extracted from 1 g of soil using MO BIO UltraClean-htp™ 96-well Soil DNA Extraction Kit (CamBio, Cambridge, UK) as per the manufacturer's instructions.

2.6.3 Pure bacterial cultures

DNA from pure bacterial type cultures was extracted using Invitrogen Easy DNA Kit (Life Technologies, Paisley, UK), as per the manufacturer's instructions.

2.6.4 Bacterial DNA extraction from soil

Bacterial genomic DNA was extracted from 1 g of soil using MO BIO UltraClean-htp™ 96-well Soil DNA Extraction Kit (CamBio, Cambridge, UK) (Fitzpatrick *et al.*, 2010; Martin-Laurent *et al.*, 2001).

2.6.5 Nanodrop

DNA concentration and quality was determined using a NanoDrop (NanoDrop Technologies, Inc) as per the manufacturer's instructions.

2.6.6 PCR amplification

2.6.6.1 16s conventional PCR

Bacterial genomic DNA was amplified using 16S-23S region primers, 27f 10 pmol/µl (5' AGAGTTTGATYMTGGCTCAG 3') and 1492r 10 pmol/µl (5' TACGGYTACCTTGTTACGACTT 3'). 20 µl reactions contained 10 µl JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma Aldrich, Gillingham, UK), 7 µl of H_2O, 1 µl of each primer and 1 µl of DNA template. Amplification was carried out on a Veriti Thermal Cycler (Life Technologies, Paisley, UK). The initial denaturation step was 5 minutes at 94°C and the amplification was performed using 35 cycles, consisting of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes. Final elongation was performed at 72°C for 15 minutes (Mullis & Faloona 1987).

2.6.7.2 16s fluorescent PCR

DNA was amplified using fluorescently labelled PCR primers 27f 10 pmol/µl (6-FAM - CCAGAGTTTGATGMTGGCTCAG) and 1492r 10pmol/µl (VIC - ACGGGCGGTGTGTACA) (Life Technologies, Paisley, UK). In addition to 1 µl of DNA each 20 µl reaction contained 10 µl Type-it Multiplex PCR Master Mix, (Qiagen, Crawley, UK), 7 µl of H_2O and 1 µl of each primer. Amplification was carried out on a Veriti Thermal Cycler (Life Technologies, Paisley, UK). The initial denaturation step was 5 minutes at 95°C, amplification was performed using 30 cycles consisting of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute 30 seconds. Final elongation was performed at 72°C for 10 minutes.

2.6.8 Gel electrophoresis of PCR products

80 ml of TAE containing 1% agrose was microwaved until all agrose had dissolved. The gel was cooled and stained with 4 µl of Gel Red. Gels were poured into electrophoresis equipment (BioRad, Hertfordshire, UK) containing a sample comb and allowed to solidify at room temperature. The comb was removed and the tank was flooded with TAE until the gel was covered. PCR products amplified using JumpStart Sigma Ready mix (Sigma Aldrich, Gillingham, UK), did not require the addition of loading dye. PCR products using TypeIt II Ready Mix (Qiagen, Crawley, UK), were mixed with 2 µl loading buffer and pipetted into wells alongside 2 kb PCR marker which was loaded in each outermost well. Electrophoresis was carried at conditions of a constant voltage of 80V,

for at least 30 minutes. The migration of the PCR product was detected by exposure to UV light and visualised using a detection camera.

2.6.9 Restriction Digests

3 µl of fluorescent PCR product was added to 0.25 µl restriction enzyme, 0.15 µl 10x enzyme buffer and 1.6 µl H_2O, which were mixed by centrifugation. In each instance PCR products were individually digested with *Hhal* and *Alu*I which were obtained from Invitrogen™, Life Technologies, Paisley, UK.

Digestion

PCR products were digested at 37°C for 2 hours, and denatured at 65°C for 10 minutes, on a Veriti Thermal Cycler, (Life Technologies, Paisley, UK). 1 µl of the digested product was added to 9 µl of HiDi-Formamide and 1 µl of GeneScan LIZ 500 size standard.

2.6.10 T-RFLP

Digested PCR amplicons are analysed by capillary electrophoresis using 3130xl Genetic Analyser (Life Technologies, Paisley, UK). Analysis was performed using Genemapper software, (Life Technologies, Paisley, UK).

2.7 Chemistry

2.7.1 Gas Chromatography Mass Spectrometry

2.7.1.1 Exogenous hydrolysis

Five, 3 mm tungsten carbide beads (Qiagen, Crawley, UK) were added to a 2 ml centrifuge tube (Eppendorf, Stevenage, UK) to which 0.5 g of defrosted plant material was added on top of beads adapted from Lisec *et al.*, (2006) . Two units of myrosinase and 200 µl H_2O was added to plant material as described in Al-Gendy & Lockwood, (2003). Tubes were secured into tissue lysis plates and bead beaten for 30 minutes at 25 Hz. Samples were removed from tissue lyser plate attachement and mixed in Thermomixer (Eppendorf, Stevenage, UK) for 12-15 hours at 300 rpm at 27 °C.

2.7.1.2 Solvent extraction

The ITC extraction method was adapted from Al-Gendy & Lockwood (2003); 0.5 g sodium sulphate and 1 ml of Ethyl Acetate were added to plant material. Samples were bead beaten for 30 minutes at 25 Hz, using a Tissue Lyser (Qiagen, Crawley, UK) then mixed using a Thermomixer (Eppendorf, Stevenage, UK) for 30 minutes, at 300 rpm at 24 °C. The mixture was then separated into aqueous and organic phases by centrifugation for 5 minutes at 3,500 rpm. Aqueous solution was transferred to 2ml glass volumetric flask.

Each solvent extraction was repeated with the addition of a further 1 ml of ethyl acetate (sodium sulphate was not added) the aqueous phase was added to the original extract. Each flask was made up to exactly 2 ml, using ethyl acetate. 100 ul of Biphenyl (2 ug/ml) internal standard (IS) was added to each individual sample and mixed by inversion. Each sample was cleaned twice by syringe filtering using 0.45 nm nylon syringe filters (Crawford Scientific, Lanarkshire, UK). Samples were transferred into amber vials and sealed with crimp caps.

2.7.1.3 GC-MS blanks

For each set of 21 samples three controls were made i) a matrix, with enzyme – 0.5 g of plant material was hydrolysed and extracted in the same way as all other samples, ii) a matrix blank without enzyme – no hydrolysis was carried out, only solvent extraction on 0.5 g of plant material and iii) Spike – 20 µl of 50 µg/ml ITC mix solution added to plant material samples were left at 4°C overnight, no hydrolysis step was performed. Solvent extraction was carried out as per 2.7.1.2.

2.7.1.4 Instrumentation

GC-MS analysis was performed on a Varian GC MS/MS (Aglient, Berkshire, UK), the Gas Chromatograph 4000 was equipped with a Triple Quad TQ 300MS and a 8400 Autosampler. Separation was carried out in DB-5MS column (30m x 0.25 mm i.d., film thickness 0.25 µm) (Agilent Technologies J&W). Helium was used as the carrier gas at flow rate 1.3 ml/minute. The injector temperature was set at 250°C, the injection volume was 10 µl all injections were splitless. The oven programme was set as shown in Table 2.1.

The SIM mode was used for quantitative analysis. The ions monitored were AITC-99, IITC-101, PITC-101, BITC-149, PEITC-163, NITC-185, Biphenyl (IS)-154. Electron ionisation acquisition occurred between 1.5 – 17.4 min.

Table 2.2 – The oven program, used for GC-MS analysis

Temp (°C)	Rate (C/min)	Hold (min)	Total (min)
50	2	2	
200	25	0	8
250	10	0	13
320	50	3	17.40

2.7.1.5 Quantitative estimation of isothiocyanates

A 50 µg/ml mixed solution of all 6 measurable ITCs (AITC, BITC, IITC, NITC, PEITC, PITC) was produced from which four dilutions were made (2 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.2 µg/ml) which were used to construct a calibration curve, ITC standards were processed with each sample batch. In addition each sample run contained a plant sample that was spiked 0.5 ug/ml of each ITC to determine the recovery rate of the assay. A matrix blank (sample that was not processed in hydrolysis step) and a matrix blank including enzyme (included in hydrolysis step) were also included in sample processing to determine whether the extraction was successful.

2.8 Statistical analysis of data

All *in vitro* bioassay data (Chapter 3) was analysed using ANOVA followed by Fisher's Least Significant Difference, to determine statistical difference between both isothiocyanates and concentrations and their effects on different pathogens used with bioassays.

Pathogen pot trial data was analysed by first converted all tuber disease scores to a score out of 100. Following this transformation data was statistically analysed using standard error of the difference of the mean ANOVAs.

All statistical analysis of T-RFLP data was performed using Principal co-ordinate analysis using Jaccard co-efficient. Data from this analysis was plotted as scatter plots in order for the statistical results to be displayed graphically.

All statistical tests were performed using Genstat V.14 (VSN International Limited), all graphs were made using Microsoft Excel 2010 (Microsoft Office Professional Plus 2010).

Chapter 3

Bioassays assessing the toxicity of isothiocyanates to fungal potato pathogens

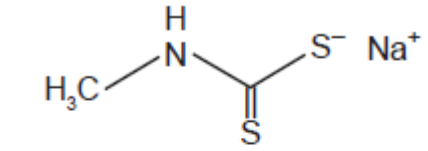
3.1 Introduction

3.1.1 Previous studies on pathogen isothiocyanate interactions

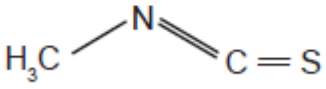
Biofumigation has been shown to suppress pathogen levels, however the level of response observed is dependent on both the ITC and the pathogen involved (Kirkegaard *et al.* 1996; Leoni *et al.* 1997; Mancini *et al.* 1997; Sarwar *et al.* 1998; Sellam *et al.* 2006). It was concluded that the most effective way to assess how differently structured ITCs affected potato fungal pathogens was to develop a bioassay, which would monitor this interaction without being influenced by additional factors. Previously authors have used several different types of bioassay to study this interaction; however it was determined that with respect to the pathogens used within this study, it would be best to use an agar diffusion assay (Finn 1959; Troncoso *et al.* 2005).

3.1.2 Selecting isothiocyanates for bioassay

Although limited study on profiling glucosinolate hydrolysis products from *Brassica* plants has been conducted, a number of naturally occurring ITCs are readily available to purchase from chemical suppliers. The ITCs used in this study were selected according to availability and those which had been identified as readily produced during *Brassica* plant tissue glucosinolate hydrolysis in previous studies. Although MITC has been identified as a hydrolysis product from some plants, it does not appear to be commonly produced by *Brassica* spp. Yet it was included in this study as MITC has been identified as a breakdown product of metam sodium, a commonly used synthetic soil fumigant, further discussed in Chapter 1, (Fig. 3.1).



Sodium N-methyldithiocarbamate (metam sodium)



Methyl isothiocyanate (MITC)

Figure 3.1 The structures of metam sodium and its breakdown product, methyl isothiocyanate.

3.1.3 Mechanism of isothiocyanate toxicity

The specific mechanisms that allow ITCs to induce toxic responses in microorganisms are not yet fully understood, however studies have begun to uncover the cellular responses of microbes when exposed to ITCs. Brown & Hampton (2011), demonstrated that the central electrophilic carbon of the ITC reacts with biological nucleophiles particularly amines and thiols (Fig 3.2). Through the use of ^{14}C-labelled phenethyl ITC it was shown that cells gradually accumulate ITCs within proteins, perhaps suggesting that the longer microbes are exposed to ITCs the greater toxicity responses will be. However their studies did not identify the specific proteins which were being directly altered by ITCs (Brown & Hampton 2011).

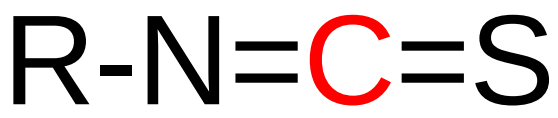


Figure 3.2 General structure of isothiocyanates, central electrophilic carbon shown in red.

Work by Tajima et al. (1998) suggested that the antimicrobial activity of ITCs was caused by their interaction with cellular sulfhydryl groups, which may also be affected by the hydrophilicity (or hydrophobicity) of the ITC by altering cell permeability or uptake. Work on *E. coli* identified the synthesis of ATP to be interrupted by ITC derivatives, suggesting that they may target the sulfydryl groups of enzymes or other proteins required during ATP synthesis (Fig. 3.3). ATP is vital for cell and organism function, as it is required for DNA replication and synthesis of various biomaterials including RNA and protein; thus highlighting the high level of toxicity which certain ITCs may possess when interacting with specific microorganisms.

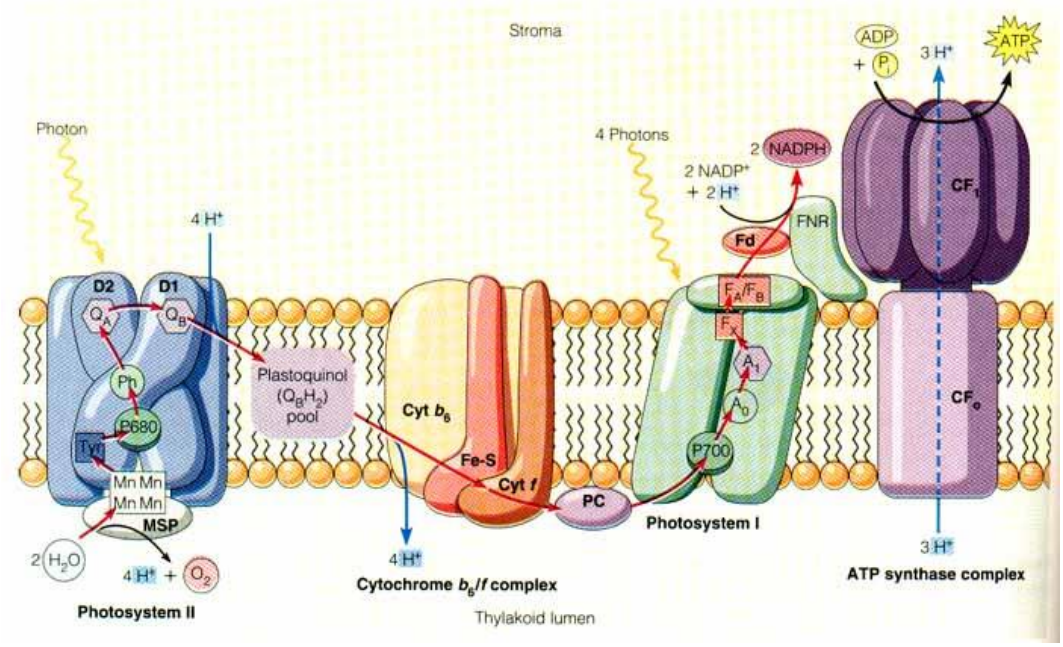


Figure 3.3 Diagram of ATP synthesis, which is interrupted by isothiocyanates. Image provided by www.winona.edu

Although it is important to gain understanding of mechanisms which allow ITCs to induce toxic responses in microbes, this aspect will not be investigated within this study. It was identified that in order to establish an effective biofumigation control system of fungal potato pathogens it should be determined whether differences in antimicrobial properties between ITCs and pathogens are present, thus determining if individual pathogens used within this study would be more susceptible to a biofumigation system and if certain ITCs possessed greater levels of toxicity towards the pathogen species. It was envisaged that results from initial bioassays would provide such information which could subsequently be used to develop glasshouse experiments which will further examine the use of biofumigation to control economically important fungal potato pathogens, *Colletotrichum coccodes, Rhizoctonia solani* and *Helminthosporium solani*.

Null hypothesis

H_0 The *in vitro* bioassay developed for the purpose of this experimentation will show that differently structured isothiocyanates have no effect on different soil borne fungal pathogens.

3.2 Materials and Methods

3.2.1 Fungal bioassays

Using a method adapted from Dhingra *et al*. (2004), seven isothiocyanates Allyl (AITC), Benzyl (BITC), Isopropyl (IITC), Methyl (MITC), Naphthyl (NITC), 2-Phenylethyl (PEITC) and Propyl (PITC), (Sigma Aldrich, Dorset, UK) (Appendix 4) that can be formed naturally by glucosinolate hydrolysis in *Brassica* spp. were studied using an agar diffusion assay to determine the effects they had on the radial growth of *C. coccodes, R. solani* and *H. solani*. The inhibition effects were determined by culturing the individual fungus on Potato Dextrose Agar (PDA) incorporated with each of the isothiocyanates (AITC, BITC, IITC, MITC, NITC, PEITC and PITC) at different concentrations, 3.125, 6.25, 12.5, 25 and 250 ppm, eight replicates were performed for each treatment and concentration, additionally untreated (50 % EtOH) control assay, again consisting of eight replicates, was performed alongside every ITC fungal bioassay. Once inoculated with a 7 mm fungal plug, from a mature pure fungal colony, plates were placed in large growth cabinets, which were maintained at 22 °C, with a light cycle of 12 hours light and 12 hours darkness. Plates were arranged in a randomised block designed, produced by Genstat V.14 (VSN International).

3.2.2 Fungal cultures

All fungal cultures, provided by Potato Branch, SASA (Science Advice for Scottish Agriculture), were incubated on PDA at 22 °C in alternating periods of 12 hours darkness 12 hours light. Plates were periodically sub-cultured for experimental use. Fresh cultures were made by coring 7 mm fungal plugs from the outer margin of mature colonies and transferring onto fresh PDA.

3.2.3 Agar incorporation

Isothiocyanate solutions were made using 50 % ethanol solutions. ITC incorporation agar plates were then made as according to Chapter 2 (2.5.1). Each ITC incorporated culture plate was inoculated with a 7 mm fungal plug taken from the advancing margin of a mature fungal colony on PDA, Petri dishes were sealed with Parafilm 'M' and incubated at 22°C in alternating periods of 12 hours darkness and 12 hours light. Radial colony growth, along 4 axes from the edge of the fungal plug to the outer margin of new growth, was measured daily throughout the duration of the experiment (Fig. 3.4).

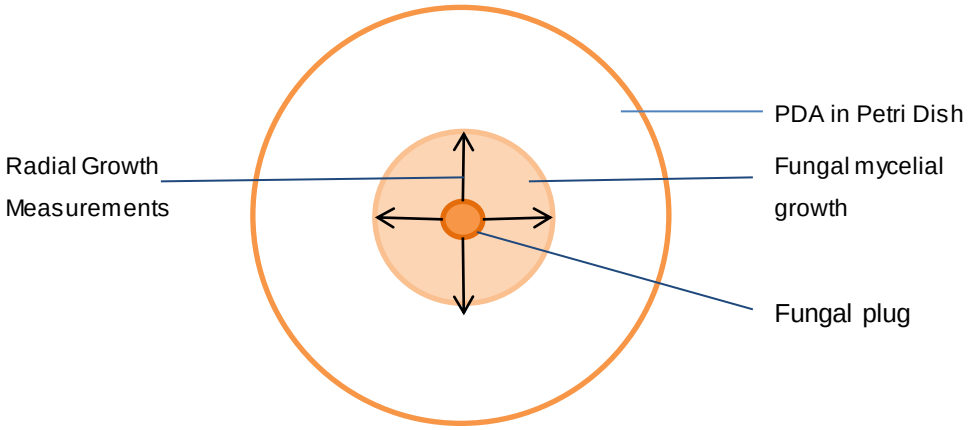


Figure 3.4 Diagram of fungal growth measurements made throughout the bioassay.

3.2.4 Data Analysis

The experiment was completed when the control colonies had reached significant maturity and size. In the case of *C. coccodes* and *R. solani* this was when mycelial growth reached the edge of the Petri dish, as *H. solani* is a slower growing pathogen, experimentation was terminated when mycelial growth reached a diameter over 60 mm. Mean values, from eight replicates, for each time point were calculated and comparisons between treatments over the time course where made through line graphs, which plotted the mean radial growth of the fungal cultures and the time duration of each experiment. Least signicant difference two-way ANOVA tests were also carried out on the data to make comparisons and investigate differences between the specific isothiocyanate treatments and the concentrations at specific time points throughout the duration of the studies (Appendix 1-3).

3.3 Results

Agar diffusion bioassays were carried out using three fungal pathogens and seven differently structured isothiocyanates (Allyl (AITC), Benzyl (BITC), Isopropyl (IITC), 1-Naphthyl (NITC), Methyl (MITC), 2-Phenylethyl (PEITC), and Propyl (PITC)) to determine the different levels of biocidal effects they had on the soil borne potato pathogens, *C. coccodes*, *R. solani* and *H. solani*. For each interaction the concentration of each ITC that would be required to achieve 50 % suppression of colony growth, in comparison to growth on control colonies was calculated (Table 3.3).

Table 3.3 Concentrations of each ITC required to achieve 50 % suppression of colony growth.

Isothiocyanate	*C. coccodes*	*R. solani*	*H. solani*
Allyl	144 ppm	25 ppm	19 ppm
Benzyl	39 ppm	Undetermined	0.5 ppm
Isopropyl	$1x10^{26}$ppm	112 ppm	63630 ppm
Methyl	16 ppm	Undetermined	20 ppm
1-Napthyl	131 ppm	131 ppm	50 ppm
2-Phenylethyl	32 ppm	28 ppm	$1 x10^{9}$ ppm
Propyl	52 ppm	12 ppm	$1 x10^{51}$ ppm

3.3.1 Effect of isothiocyanates on the radial growth of *Colletotrichum coccodes*

There was no significant decrease ($p>0.05$) in colony growth of *C. coccodes* on any agar plates incorporated with AITC at < 250 ppm (Fig.3.5). However in the presence of 250 ppm AITC there was a delay of seven days in the initiation of growth after inoculation after which growth occurs at a similar rate to that on control plates.

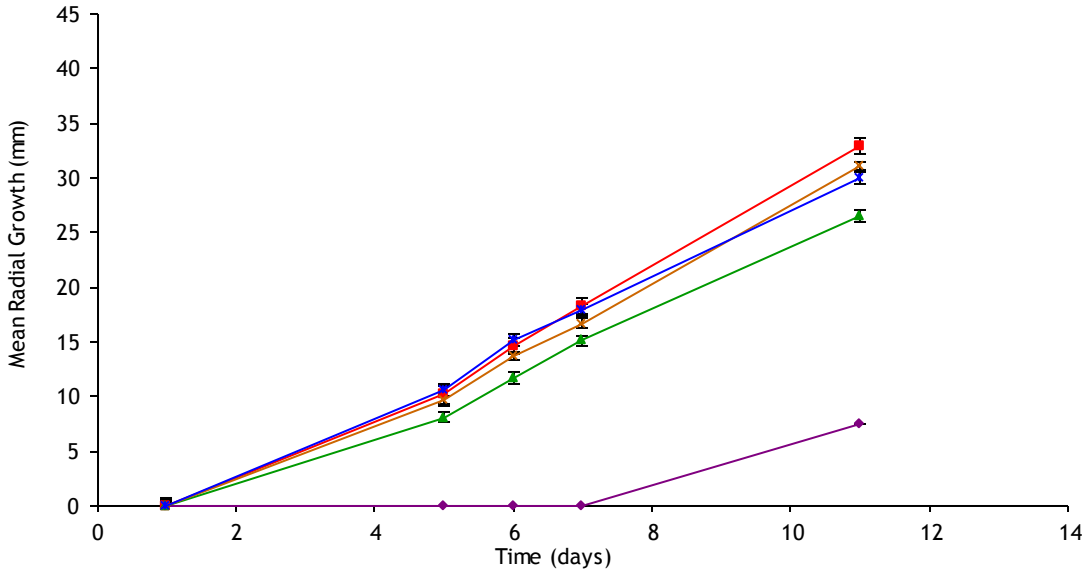


Figure 3.5 The effect of varying concentrations of allyl isothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Agar plates incorporated with BITC showed no decrease in growth compared to that observed on control plates, at a dose of 3.125 and 6.25 ppm (Fig 3.6). However those incorporated with 12.5 and 25 ppm BITC showed that higher concentrations produced decreasing resultant colony sizes, after 15 days of incubation (Fig 3.12b). No growth was observed on plates incorporated with 250 ppm.

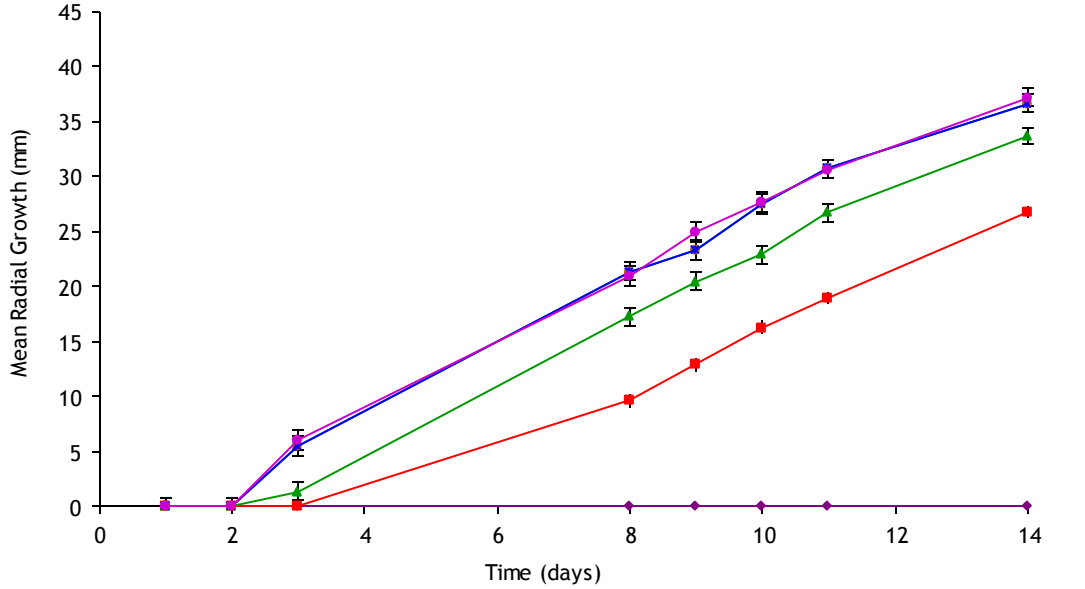


Figure 3.6 The effect of varying concentrations of benzylisothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Incorporation of IITC into growth media resulted in no change in *C. coccodes* colony sizes after 13 days of incubation, regardless of concentration (Fig. 3.7).

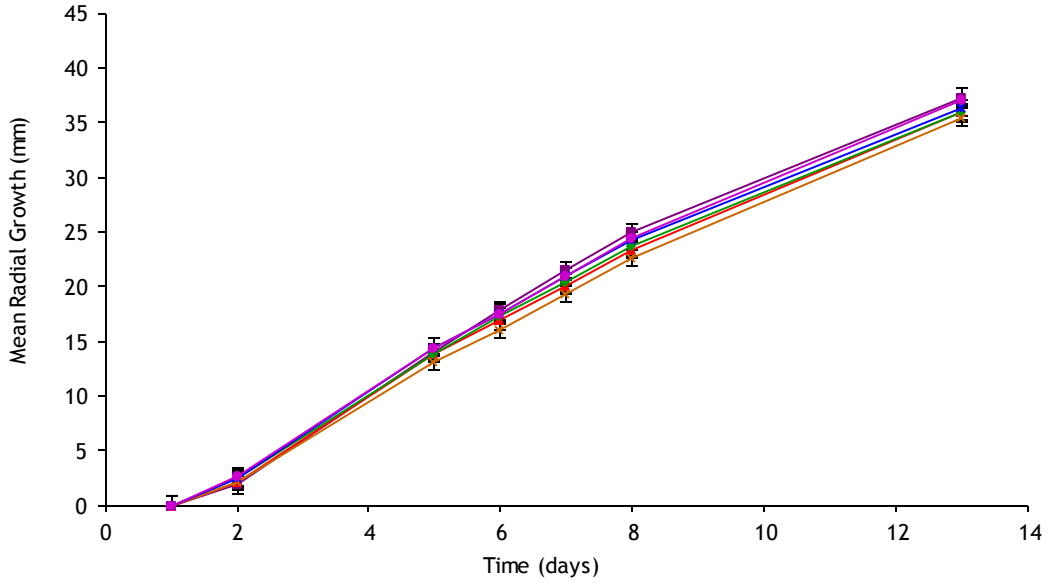


Figure 3.7 The effect of varying concentrations of isopropylisothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Incorporation of MITC 25 ppm into PDA plates resulted in a significant reduction in the overall colony size (Fig 3.8 and 3.12c). No growth was observed on treatment plates incorporated with 250 ppm MITC after 14 days of incubation. All other treatment concentrations did not produce a significant reduction ($p > 0.05$) in colony size.

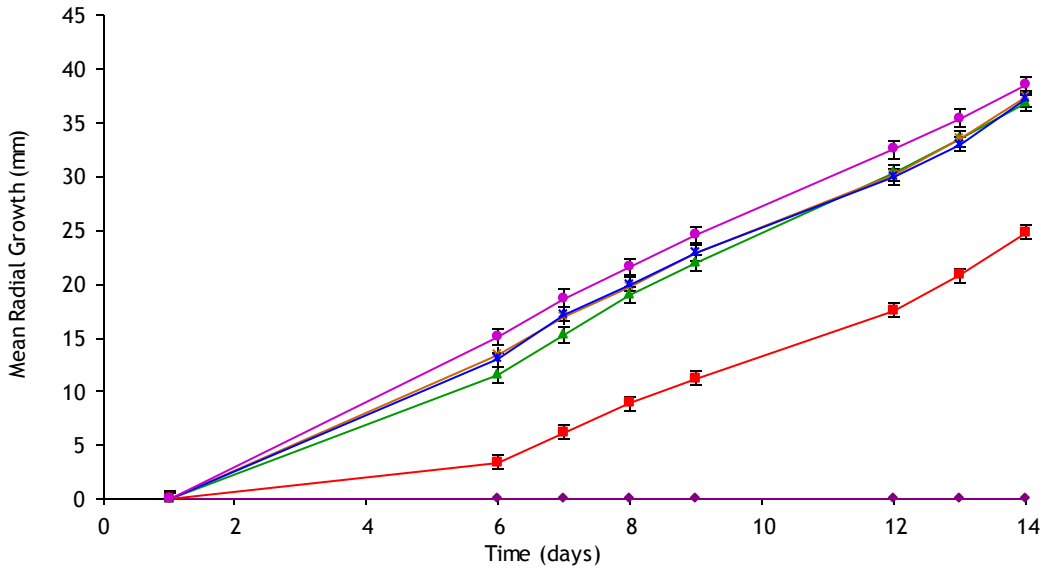


Figure 3.8 The effect of varying concentrations of methylisothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Colletotrichum coccodes growth on agar plates incorporated with NITC at concentrations < 250 ppm did not show any significant difference in growth rate compared to the control ($p > 0.5$) (Fig. 3.9). Growth on 250 ppm treatment plates which started after three days was at a much slower rate than the other treatments and therefore the resultant colony sizes after 13 days of incubation was significantly smaller than that of the control.

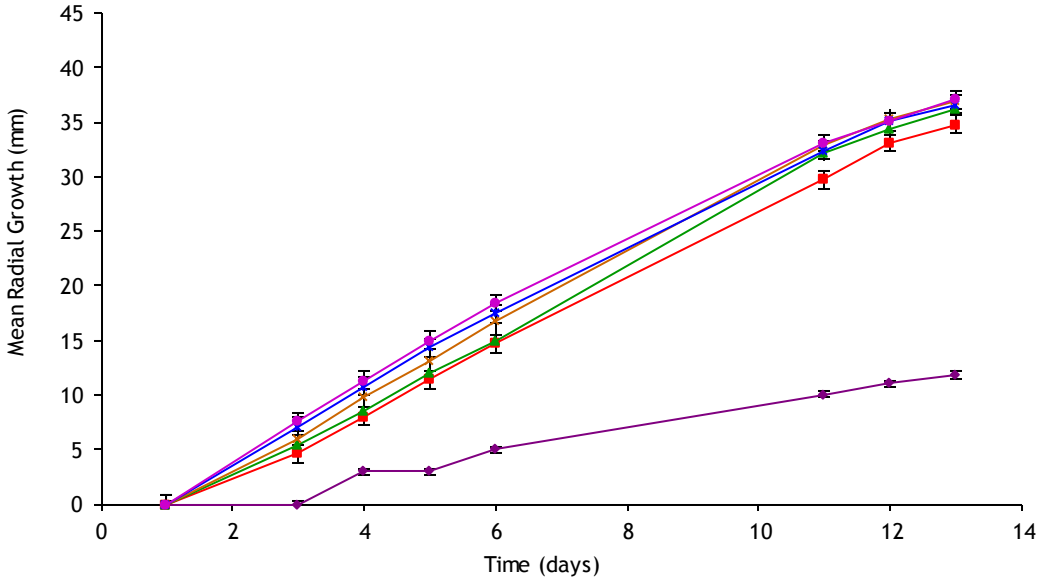


Figure 3.9 The effect of varying concentrations of 1-naphthyl isothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

The incorporation of PEITC into growth media showed that at concentrations < 12.5 ppm growth of *C. coccodes* was unaffected by its presence (Fig 3.10). Colony growth on plates incorporated with 12.5 and 25 ppm was delayed for 7 days before occurring at a similar rate to that observed on control plates (Fig. 3.12c). No growth was observed on plates incorporated with 250 ppm PEITC throughout the duration of the study.

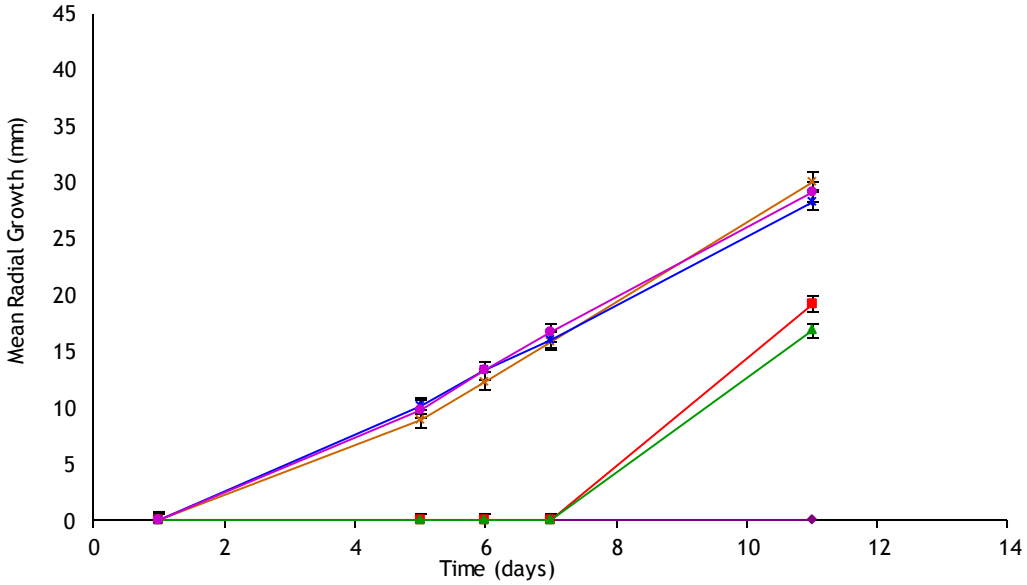


Figure 3.10 The effect of varying concentrations of 2-phenylethyl isothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Growth of C. coccodes was unaffected by the incorporation of PITC 25 ppm and lower into PDA plates at concentrations (Fig. 3.11). No growth was observed on plates incorporated with 250 ppm PITC.

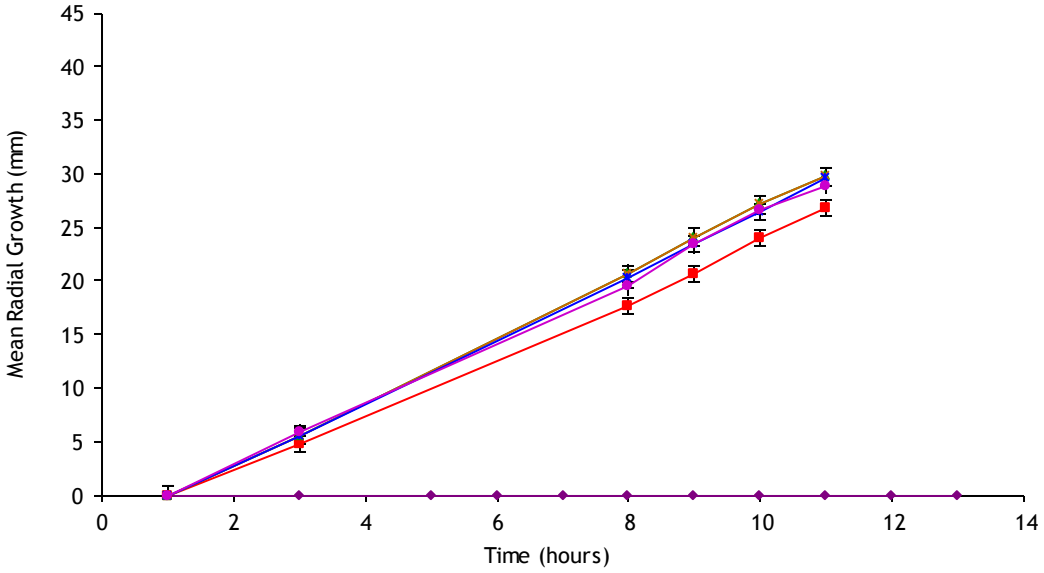


Figure 3.11 The effect of varying concentrations of propyl isothiocyanate on *C. coccodes*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

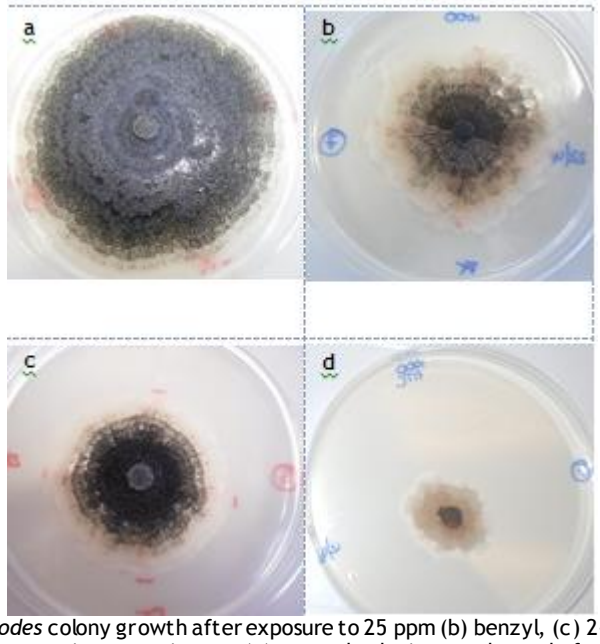


Figure 3.12a-d *C. coccodes* colony growth after exposure to 25 ppm (b) benzyl, (c) 2-phenylethyl, (d) methyl Isothiocyanate, in comparison to (a) control colonies, at the end of each study.

3.3.1.1 Summary of *C. coccodes* bioassay results

Out of the seven isothiocyanates screened against C. coccodes, PEITC was shown to have the most significant suppressive effect ($p<0.05$) on the growing cultures (Fig. 3.10). However an effect on growth was only observed on plates incorporated with ≥12.5 ppm. Growth on 3.125 and 6.25 ppm did not differ from that observed on control plates. On plates incorporated with 12.5 and 25 ppm a fungistatic response was observed during which initial growth was delayed until day seven at which point growth commenced at the same rate as the control treatment. No growth was observed on plates incorporated with 250 ppm PEITC throughout the duration of experimentation. BITC (Fig. 3.6) and MITC (Fig. 3.8) showed only a limited level of control of *C. coccodes*; no growth was observed on plates incorporated with 250ppm - there was a slight decrease in resultant colony size on plates incorporated with 25ppm. Plates incorporated with 250 ppm PITC, showed no growth throughout the duration of the study, also no effect was observed on plates incorporated with ≤25ppm. Plates incorporating either IITC (Fig. 3.7) or NITC (Fig 3.9) showed no effect at any concentration used within this study.

3.3.2 *In vitro* experiments investigating the effect of isothiocyanates on *Rhizoctonia solani*

Incorporating AITC into growth media had a significant effect ($p<0.05$) on the growth rate of R. solani (Fig. 3.13). On plates incorporated with 25 ppm AITC, the initial point of mycelial growth was delayed for approximately 5 days, however afterwards growth occurred at a similar rate to that observed on lower concentration plates, and after 15 days of incubation, colonies reached the same growth diameter. Throughout the duration of the study no *R. solani* growth was observed on the plates incorporated with 250 ppm AITC.

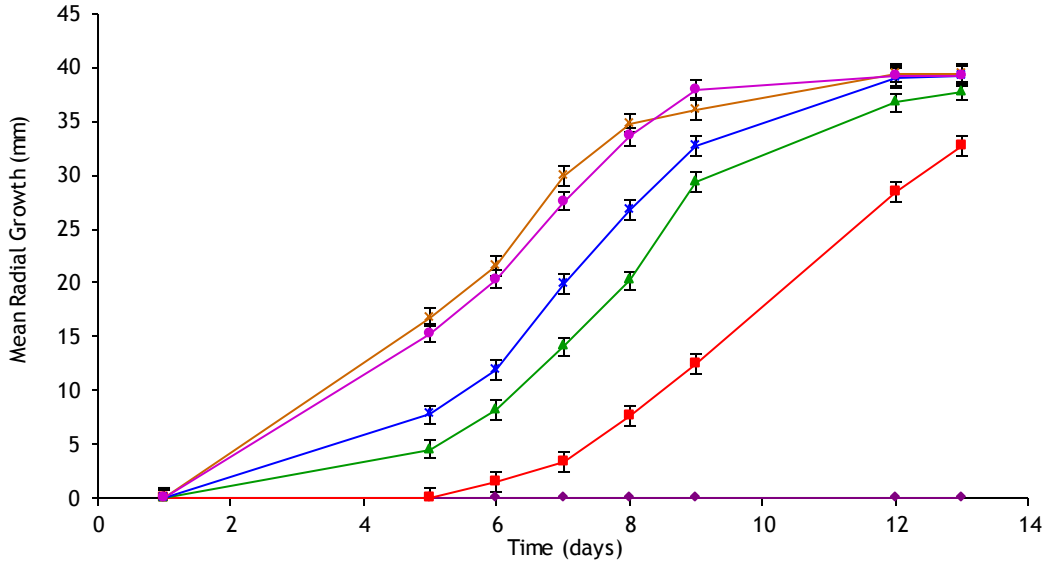


Figure 3.13 The effect of varying concentrations of allyl isothiocyanate on R. solani, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

All concentrations of BITC prevented growth of *R. solani* over an observation period of 14 days (Fig. 3.14).

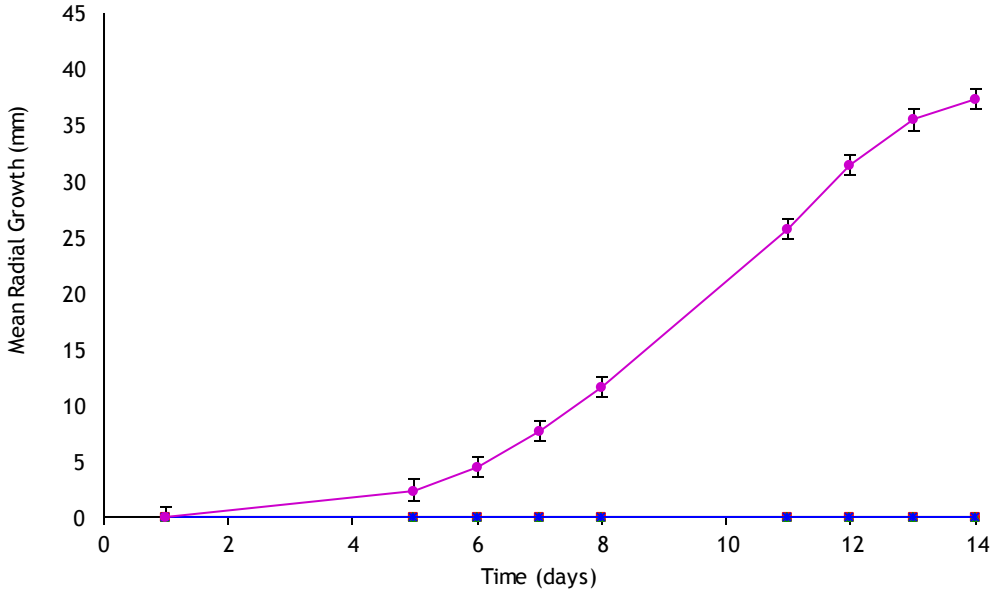


Figure 3.14 The effect of varying concentrations of benzylisothiocyanate on *R.solani*, incorporated into PDA media. Verticalbars show the standard error of the mean. Control (●), 3.125 ppm (✱), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Growth of *R. solani* colonies were somewhat affected by the presence of IITC in the growth media (Fig. 3.15). Growth on plates incorporated with <25 ppm of IITC were unaffected, however plates incorporated with 25 ppm showed a delay in the initial point of growth. Growth of *R. solani* at 250 ppm ITC also showed a delay in the initiating growth, after which colony growth occurred at a slower rate than observed on other treatments.

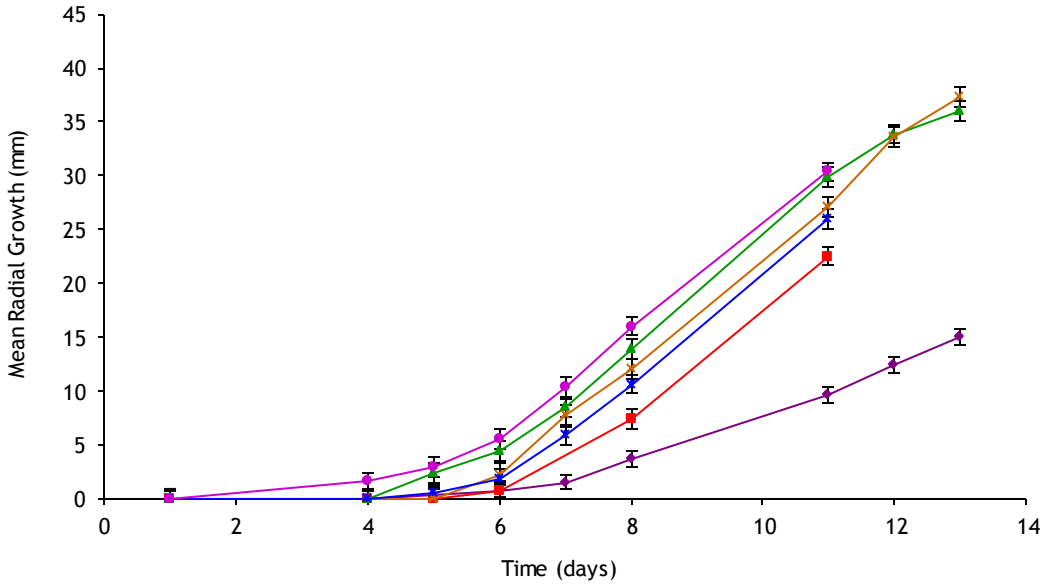


Figure 3.15 The effect of varying concentrations of isopropyl isothiocyanate on *R.solani*, incorporated into PDA media. Verticalbars show the standard error of the mean. Control (●), 3.125 ppm (✱), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Rhizoctonia solani cultures on PDA incorporated with MITC showed no growth throughout the duration of the study of 13 days, as shown in Figure 3.16.

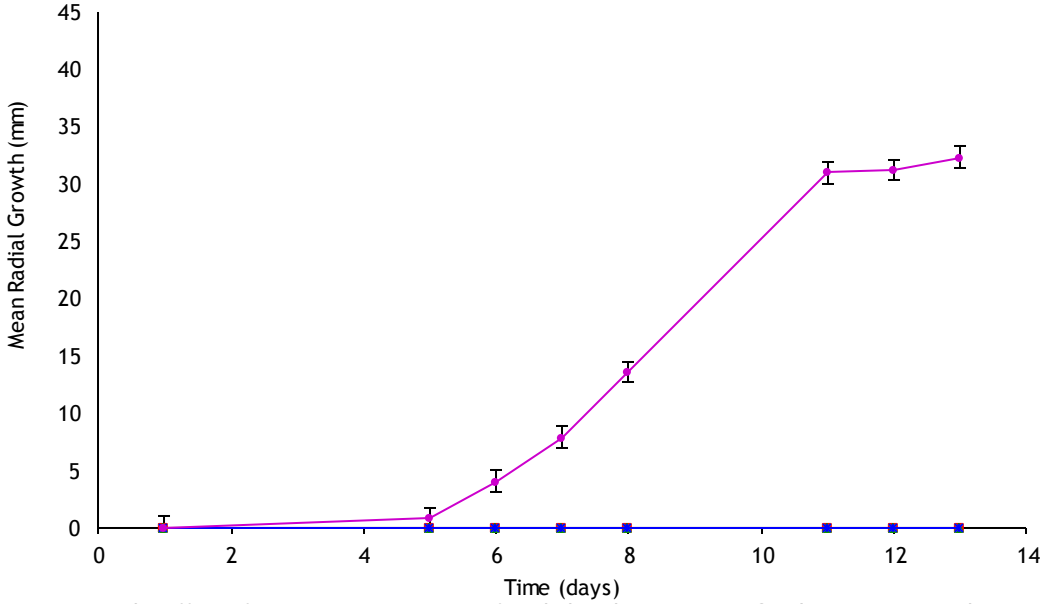


Figure 3.16 The effect of varying concentrations of methylisothiocyanate on *R.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✱), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Incorporation of NITC into PDA showed a slight decrease in resultant colony size and general mycelial growth rate on media incorporated with 6.5, 12.5 and 25 ppm NITC (Fig. 3.17). No growth of *R. solani* was observed at 250 ppm NITC throughout the study.

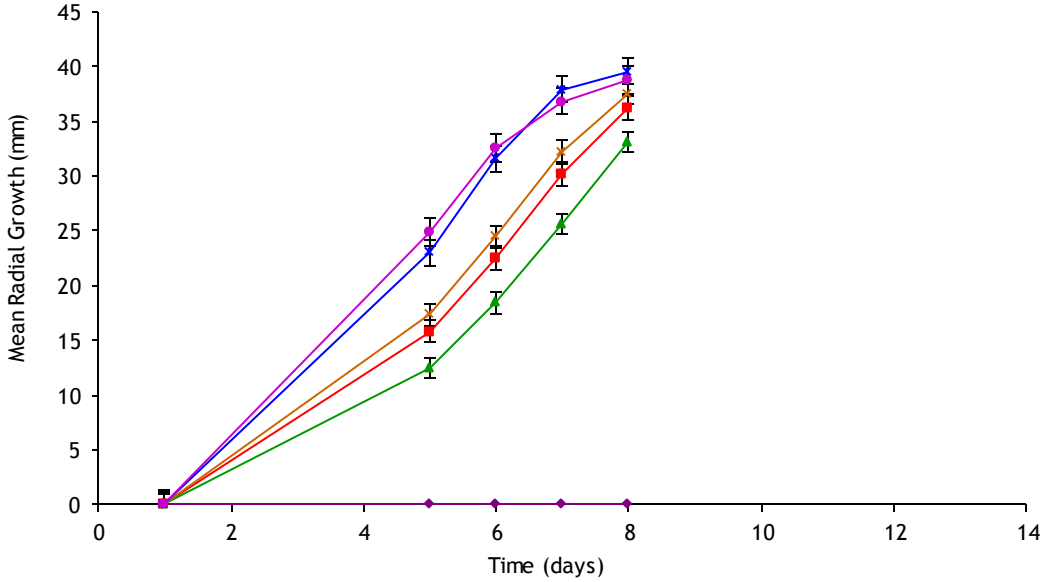


Figure 3.17 The effect of varying concentrations of 1-Napthylisothiocyanate on *R.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✱), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Growth of *R. solani* was relatively unaffected by concentrations of PEITC <12.5 ppm. At 12.5 ppm PEITC the initial point of growth was delayed for 4 days, however growth proceeded at a similar rate to that observed on the control plates. *Rhizoctonia solani* growth was delayed for 6 days on PDA incorporated with 25 ppm PEITC, growth then commenced although at a slower rate to that observed on control plates (Fig 3.18). No growth was observed on PDA incorporated with 250 ppm PEITC.

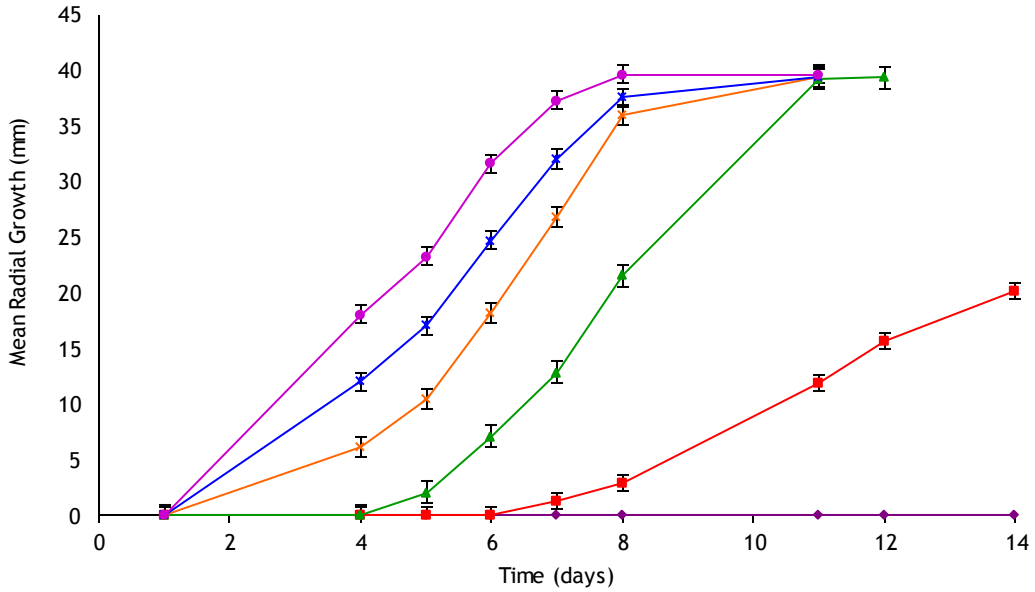


Figure 3.18 The effect of varying concentrations of 2-phenylthyl isothiocyanate on *R.solani*, incorporated into PDA media. Verticalbars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Growth th of *R. solani* colonies was relatively unaffected by the presence of 3.125 ppm PITC, 6.25 and 12.5 ppm produced a delay in the initial point of growth (Fig 3.19). 25 ppm PITC delayed the initial point of mycelial growth until 8 days. However thereafter growth proceeded at the same rate as that of the control. No *R. solani* growth was present on PDA incorporated with 250 ppm PITC throughout the duration of the study.

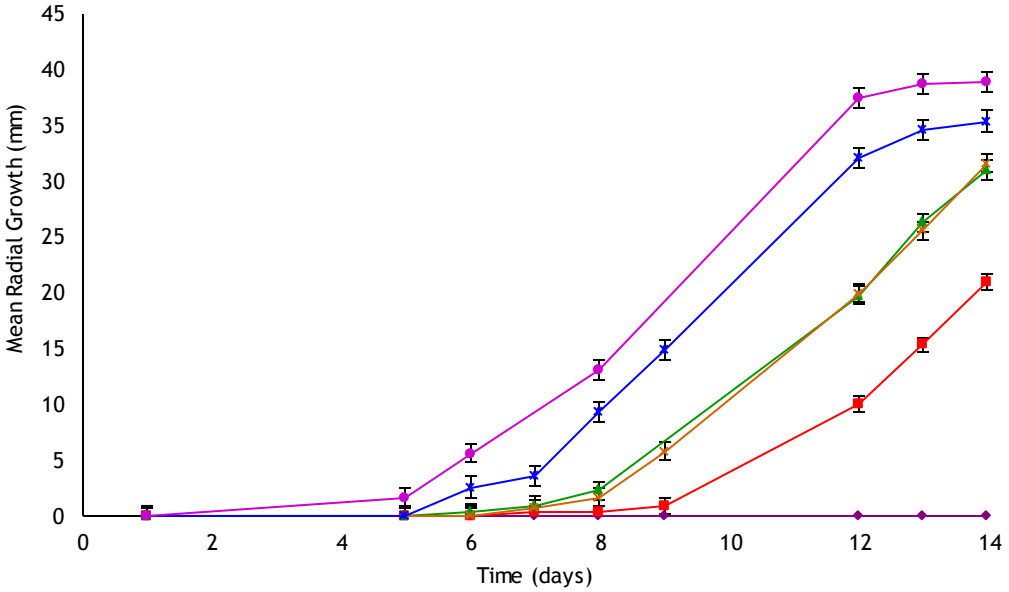


Figure 3.19 The effect of varying concentrations of propylisothiocyanate on *R.solani*, incorporated into PDA media. Verticalbars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

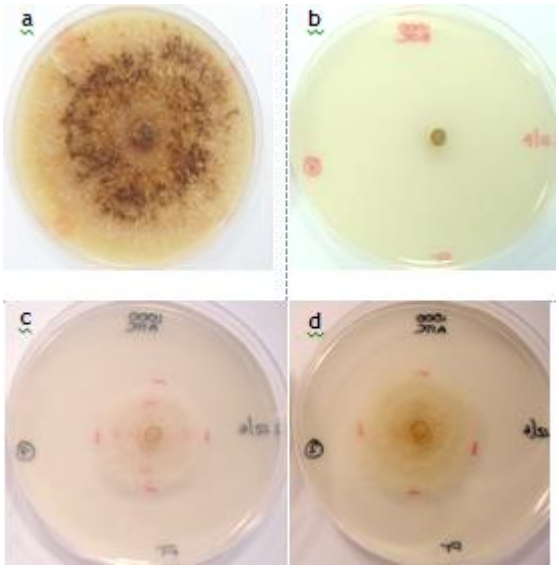


Figure 3.20ac *R. solani* colony growth after exposure to 25 ppm (b) benzyl, (c) 2-phenylethyl, (d) allyl Isothiocyanate, in comparison to (a) control colonies, at the end of each study.

3.3.2.1 Summary of *R. solani* results

The screening of the seven isothiocyanates against *R. solani* cultures showed a wide range of different responses. Growth suppression was visible at the lowest concentration (3.125 ppm) in cultures growing on the media incorporated with BITC or MITC, (Fig. 3.14 and 3.16). In each instance no growth was observed on any of the treatment plates after 14 days incubation. Results from the assay investigating the growth of *R. solani* in the presence of PITC, showed that an increase in PITC concentration delayed the initial growth time for five, six and seven days, at 3.125, 6.25 and 12.5 ppm respectively after which growth occurred at a similar rate to that of the control. While both PITC (Fig. 3.19) and PEITC (Fig. 3.18) treatments still allowed colony growth, both were shown to suppress development and growth rates. After seven days growth a statistically significant difference ($p<0.05$) was observed between growth on PEITC and PITC plates incorporated with 6.25 ppm compared to 25 ppm. After a further day a statistically significant difference ($p<0.001$) between colony growth on PEITC and PITC treatment plates of all concentrations was measured. After a total of 10 and 13 days of incubation there was significant difference ($p<0.001$) in colony growth, on PEITC and PITC treatment plates of all incorporated concentration <250 ppm. PEITC, BITC, MITC, PEITC and PITC were shown to have the most significant levels of control on the growth of *R. solani* colonies in comparison to other ITCs studied. Results from AITC (Fig. 3.13 and 3.20d) assays showed a minor fungistatic response, with growth on 25 ppm treatment plates delayed until 120 hours of incubation (Fig. 3.13). NITC showed no growth on plates incorporated with 250 ppm; however plates incorporated with ≤25 ppm displayed no difference in growth rate or resultant colony size from the control plates, (Fig.3.17). While IITC incorporated plates displayed a slight decrease in the overall radial colony size of 17 mm, in comparison to control radial colony size on 250 ppm, there was no difference in colony size on plates incorporated with lower concentrations.

3.3.3 *In vitro* experiments investigating the effect of isothiocyanates on *Helminthosporium solani*

As the concentration of AITC increased the resultant *H. solani* size decreased (Fig 3.21). In the instance of 25 ppm the initial time for mycelial growth to occur is delayed until 27 days, before occurring at a slower rate than that observed on lower AITC concentrations (Fig 3.21). No growth occurred on PDA incorporated with 250 ppm AITC.

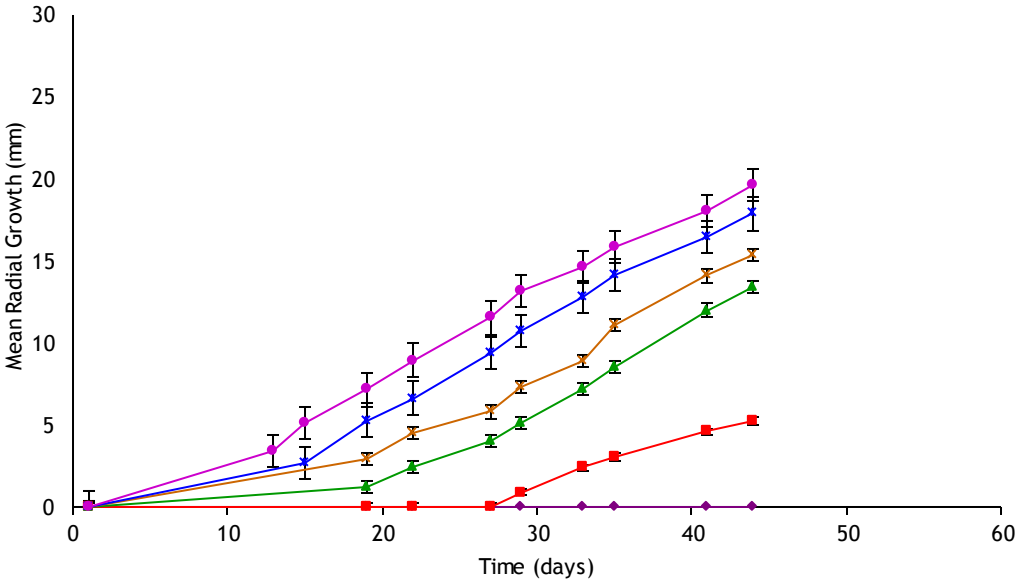


Figure 3.21 The *in vitro* effect of varying concentrations of allyl isothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Helminthosporium solani growth (Fig. 3.22) on PDA incorporated with BITC at concentrations greater than 3.125 ppm throughout the duration of the study does not occur. Growth on PDA incorporated with 3.125 ppm was initially delayed until day 8 after which it proceeded at the same rate as the control treatment.

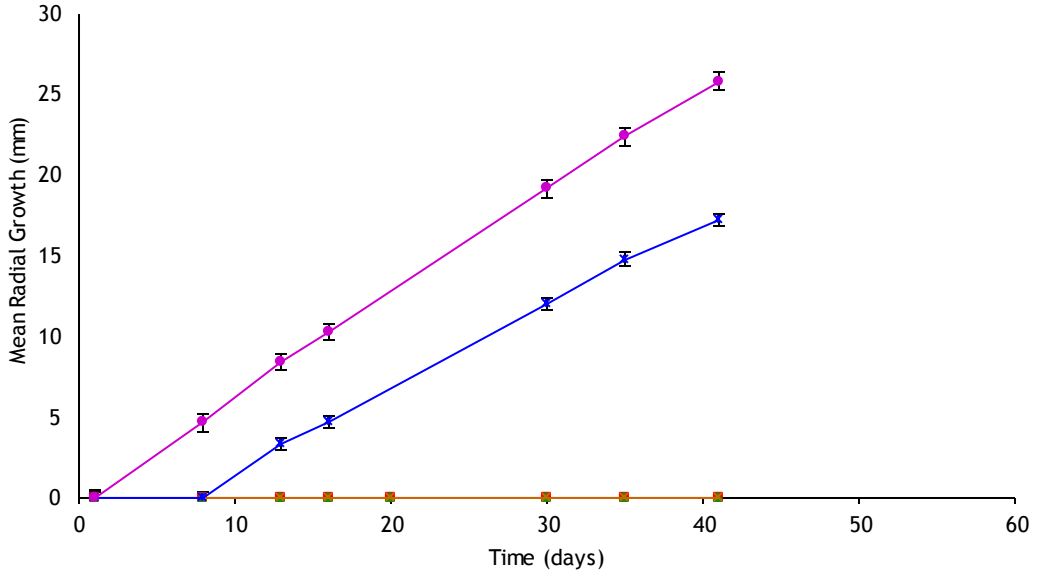


Figure 3.22 The effect of varying concentrations of benzylisothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 65 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Generally the incorporation of IITC into PDA did not produce significant differences ($p>0.05$) in the growth of *H. solani* (Fig. 3.23). At 250 ppm IITC produced a significantly faster rate of colony growth than recorded on lower IITC concentrations and the control was observed.

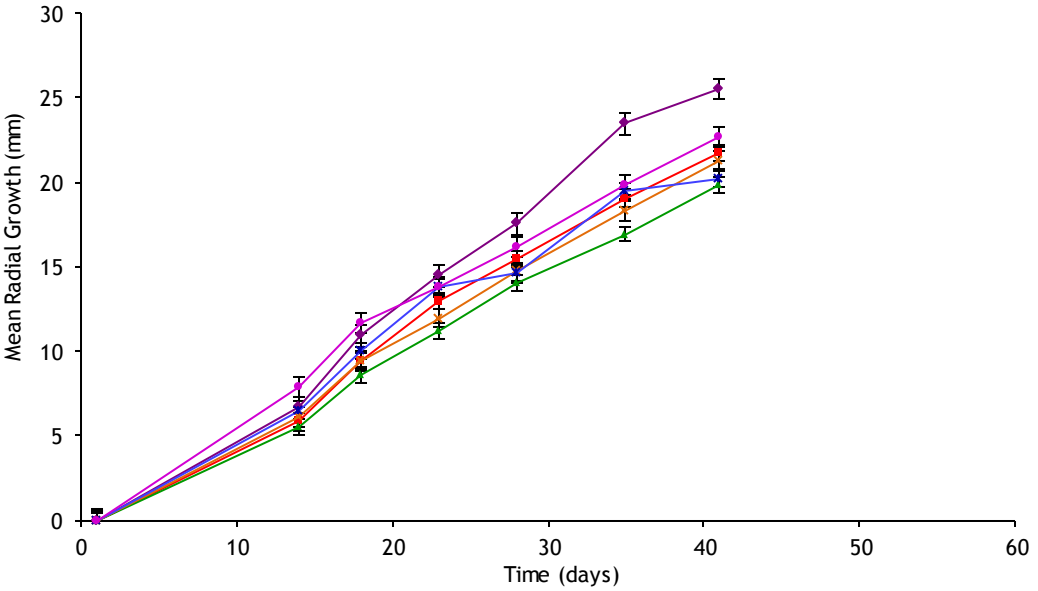


Figure 3.23 The effect of varying concentrations of isopropylisothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

The incorporation of MITC into PDA did not suppress the growth or development of *H. solani* cultures at a concentration of < 25 ppm (Fig. 3.24). At concentrations of 25 ppm the colony growth was delayed until day 21, and then continued at a slower growth rate. No *H. solani* growth occurred on PDA incorporated with 250 ppm MITC.

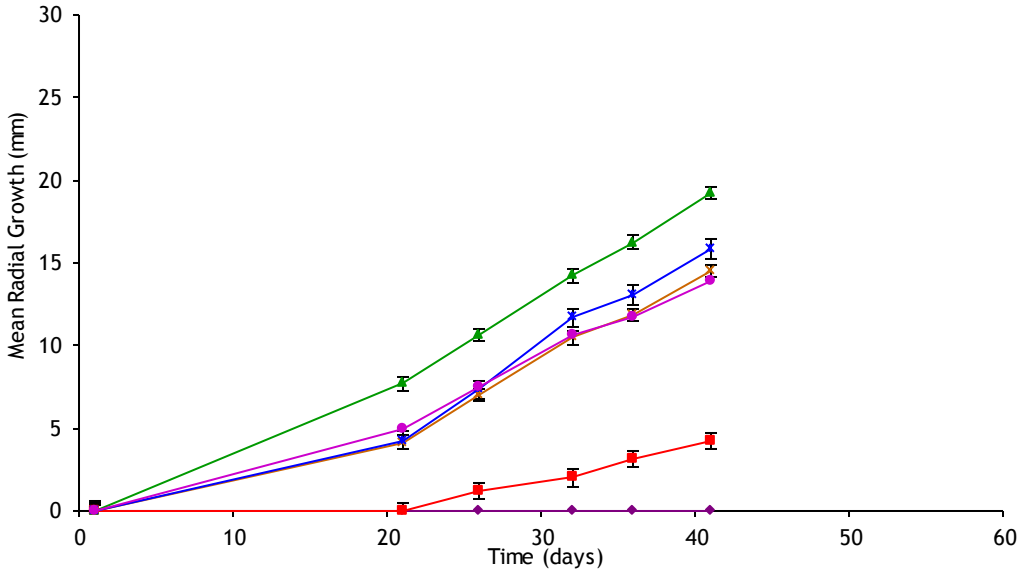


Figure 3.24 The effect of varying concentrations of methyl isothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✱), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

PDA incorporated with NITC at concentration below 250 ppm had no effect on the rate of *H. solani* colony growth. However at 250 ppm no growth occurred on the PDA throughout the duration of the study, (Fig. 3.25).

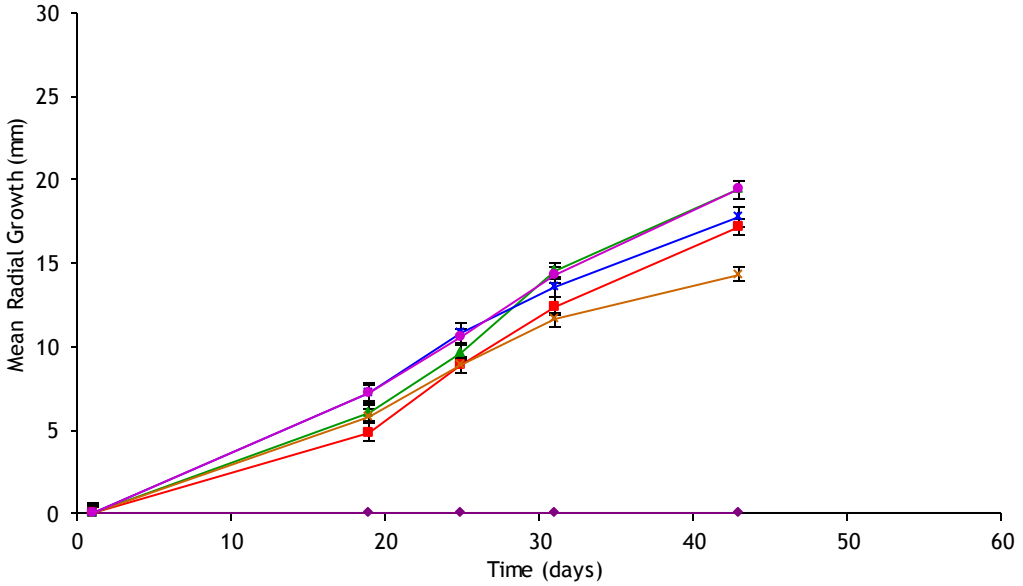


Figure 3.25 The effect of varying concentrations of 1-napthylisothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✱),6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

PDA incorporated with PEITC at concentrations > 3.125 ppm showed no *H. solani* growth throughout the duration of the study (Fig. 3.26). Growth on PDA incorporated with 3.125 ppm was delayed until day 35 thereafter colony growth occurred at the same rate as the control.

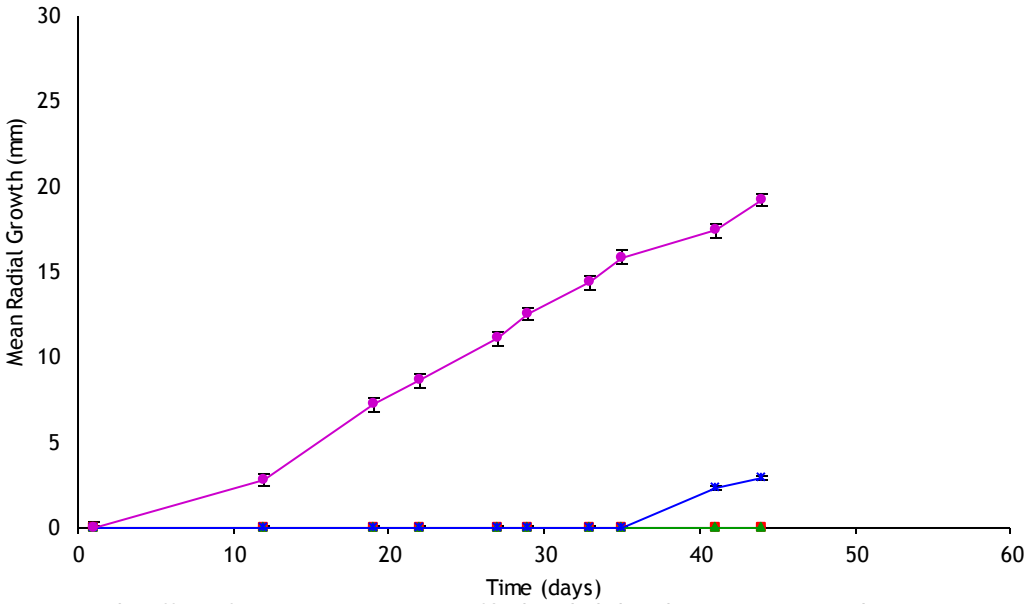


Figure 3.26 The effect of varying concentrations of 2-phenylethyl isothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control (●), 3.125 ppm (✳), 6.5 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

Although *H. solani* colony growth on PDA incorporated with PITC treatments < 250 ppm was delayed until day 22, this did not affect the overall colony sizes at the end of the study, which were of the same size as control colonies (Fig. 3.27). Growth on PDA incorporated with 250 ppm PITC was delayed until day 34, and therefore growth rate of colonies was smaller than the controls and those on lower concentrations of PITC.

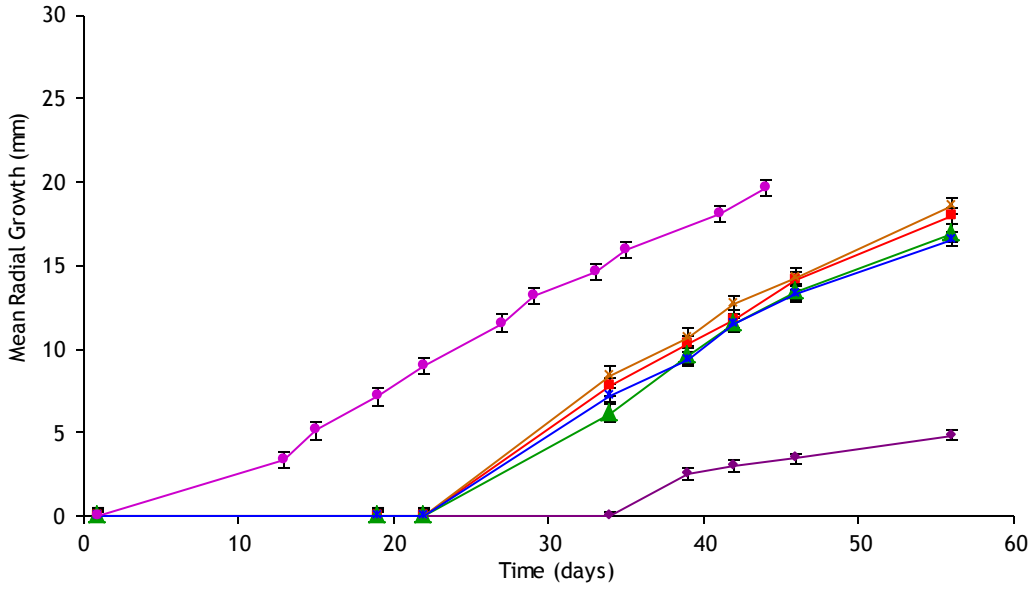


Figure 3.27 The effect of varying concentrations of propylisothiocyanate on *H.solani*, incorporated into PDA media. Vertical bars show the standard error of the mean. Control(●), 3.125 ppm (✳), 6.25 ppm (X), 12.5 ppm (▲), 25 ppm (■), 250 ppm (♦).

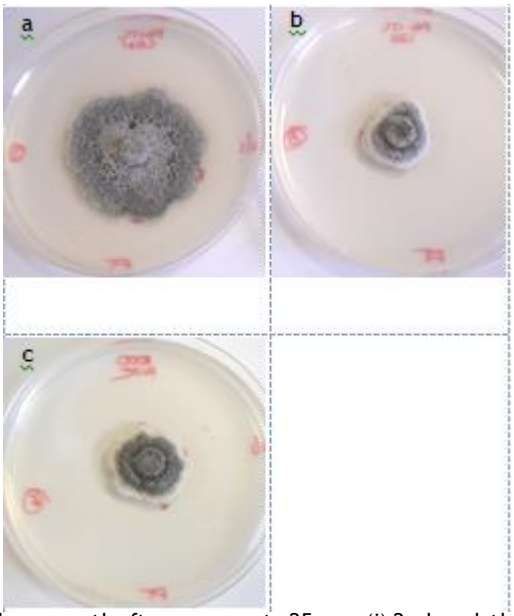


Figure 3.28a-c *H. solani* colony growth after exposure to 25 ppm (i) 2-phenylethyl, (j) allyl, in comparison to (h) control colonies, at the end of each study.

3.3.3.1 Summary of *H. solani* results

Growth of *H. solani* cultures on isothiocyanate media resulted in a range of responses, both dependent on the isothiocyanate present and the dosage incorporated into the growth media. The growth of *H. solani* was controlled at the lowest concentration on media containing PEITC. In this instance growth was only observed on the 3.125 ppm treatment after 35 days.

AITC appears to have only limited effect upon the growth of *H. solani* cultures, with all concentrations >25ppm having no effect on the rate of growth but producing a delay in growth initiation (Fig 3.21). However growth on 25 ppm did not commence until after 27 days of incubation and in this interaction a significantly slower rate of growth is also observed. No growth was observed in the presence of 250 ppm AITC.

The incorporation of BITC >3.125ppm into *H. solani* growth media, had a significant effect on the growth of the fungal colonies (Fig. 3.22). Incorporation of concentrations >3.125ppm in all instances produced no colony growth over the 41 day period. Growth on 3.125ppm plates was inhibited for a short period of time, (8 days) then continued at the same rate observed on control plates.

Incorporation of AITC, BITC and PEITC into growth media produced a suppressive effect on *H. solani* colony growth. *R. solani* cultures were shown to be more tolerant of exposure to AITC, compared to exposure to both BITC and PEITC. Colony growth in the presence of PEITC (Fig. 3.26) was significantly more suppressed at 3.125 and 6.25 ppm compared to that seen with BITC.

AITC, BITC and PEITC incorporation into growth media showed the most significant levels of suppression in comparison to other ITCs studied. No effect on *H. solani* growth was observed through IITC incorporation regardless of the dose, (Fig. 3.23). In

the case of NITC no growth was observed on plates incorporated with 250ppm. However growth rates at doses <250ppm did not differ from that observed on control plates. Again with PITC incorporation, concentrations <250ppm colony growth was not affected. Plates incorporated with 250ppm resulted in colonies 73 % smaller than observed on control plates.

3.4 Discussion

The results from this study clearly indicate that the isothiocyanate – pathogen interaction is one of great specificity. The overall effect that isothiocyanates have on fungal pathogens is not only dependent on the specific structure of isothiocyanate but also the fungal pathogen that it is targeting. The specificity of the interaction has also been observed within previous studies, Yulianti *et al.* (2006) showed that the level of suppression achieved was dependent upon the strain of the fungus and the type of ITC and the growth media.

The results to date confirm the toxic nature and antifungal effects of ITCs towards soil borne potato pathogens that have previously been observed, (Sarwar *et al.* 1998; Smolinska *et al.* 2003; Larkin & Griffin 2007). Specifically this study demonstrates that isothiocyanates can have an inhibitory effect upon the growth of *C. coccodes, R. solani* and *H. solani*, which cause economically important diseases in potato crops, under *in vitro* conditions. This supports previous data which has shown that glucosinolate hydrolysis products, particularly isothiocyanates can suppress the growth of fungal pathogens (Sarwar *et al.* 1998; Morra & Kirkegaard 2002). It also further demonstrates the significant variation of toxicity of different isothiocyanates towards different fungal pathogen species (Sarwar *et al.* 1998; Smolinska *et al.* 2003).

Results from the current study agree with conclusions that have also been made in previous studies, which state that individual glucosinolate profiles of *Brassica* plants and therefore ultimately the individual isothiocyanates that individual *Brassica* spp. isolates can produce, will determine their overall potential to be used as a biofumigant crop. Morra & Kirkegaard (2002) also observed that the suppression of soil-borne pests using *Brassica* spp. will be aided by the use of varieties possessing high glucosinolate content and those which supply sufficient volumes of moisture to promote the release of isothiocyanates and the retention of soil. However, although many other factors must be considered in the fine tuning of the biofumigation system, ultimately the most influential factor determining the level of pest suppression is the specific isothiocyanates released by the incorporated *Brassica* sp. This is clearly highlighted by the results from this study which show that different levels of control are exerted by different isothiocyanate compounds. Furthermore emphasising the important effect that altering the R-group side chain, which is known to alter important physical and chemical properties such as volatility and hydrophobity, (Brown & Morra 1997) has upon the specific toxicity of individual isothiocyanates, (Sarwar *et al.* 1998; Smith & Kirkegaard 2002; Smolinska *et al.* 2003; Gimsing & Kirkegaard 2009).

It has also been shown that a high degree of variation occurs between both the toxicity of different isothiocyanates and the sensitivity of different fungal species. Therefore the different toxicity levels that are evident between different isothiocyanate R-groups and varying pathogens, can be assumed to be an interaction between both the

structure of the R-group and the susceptibility of the fungal pathogen, (Fan *et al.* 2008).

Results from this study back up findings by Borek *et al.* (1998), which do not suggest that any trend exists between the level of toxicity of different isothiocyanates and structure. This is in contradiction to other studies (Carter *et al.* 1963; Sarwar *et al.* 1998; Fan *et al.* 2008) which have suggested that in *in vitro* studies aromatic ITCs show greater toxicity towards fungal pathogens. Previous studies have also shown trends between increasing molecular weight of the individual isothiocyanate and increasing toxicity (Borek *et al.* 1998). However no such trends between molecular structure and toxicity levels are exhibited from the results from this study.

This study has highlighted that PEITC exhibits antifungal properties towards each of the three fungal pathogens examined within this study. Its high level of toxicity has been demonstrated in other studies using a range of different pathogens, (Drobnica *et al.* 1967; Kirkegaard *et al.* 1996; Kirkegaard *et al.* 1998). Kirkegaard & Sarwar (1998) also suggested that this may be the ideal isothiocyanate for biofumigation as its aromatic structure indicates that it is less volatile compared to aliphatic ITCs and therefore may persist for longer periods of time within soil. Identifying PEITC as a highly toxic compound towards soil borne pathogens suggests that incorporation of green manures from *Brassica* species that contain high concentrations of the parental glucosinolate, 2-Phenylethyl glucosinolate, will suppress development of black scurf, silver scurf and black dot within potato crops. Previous studies have shown that phenylethyl glucosinolate is dominant within the roots of oil-seed rape, (Gardiner *et al.* 1999; Kirkegaard *et al.* 2000), therefore it can be suggested that this potentially may be a biofumigant crop to aid the control of all three pathogens examined within this study, further data on PEITC produced by *Brassica* cultivars is presented in Chapter 4.

Significant suppression of the growth of *H. solani* and to a lesser extent *R. solani* was also observed in the presence of AITC. With *H. solani* growth was not observed on plates containing 25 ppm until after 27 days of incubation. Therefore the release of AITC into agricultural soil may play a significant role in the control of soil borne pathogens, and in particular *H. solani*. Research has previously shown that high concentrations of AITC can be found within some mustard, horseradish and wasabi species; however a high degree of variation exists between cultivars of the same species (Dhingra *et al.* 2004). As a result further work has been to be carried out to identify which specific cultivars have the potential to release the highest concentrations of AITC (Chapter 4), to potentially achieve the greatest level of disease control through tissue incorporation. The antimicrobial activity of AITC has been previously documented (Lin *et al.* 2000; Dhingra *et al.* 2004), and it has been suggested that disease control and suppression of pathogens within *Brassica* spp. was due to the production of AITC (Mayton *et al.* 1996; Olivier *et al.* 1999; Dhingra *et al.* 2004).

However as this and additional studies have shown other isothiocyanates, may be involved in microorganism suppression.

Previously only a limited amount of research has been carried out on the effects of isothiocyanates on the growth and development on *C. coccodes*, and therefore it's potential to be controlled through biofumigation. Results produced by this study show that it may respond to control through the incorporation of *Brassica* plant tissue with the potential to produce large concentrations of PEITC. Studies have identified PEITC as being commonly produced by a range of different *Brassica* spp.; this specific ITC has been shown in this study to inhibit the growth of *C. coccodes*. Results show a relationship between the level of control, and the concentration of the treatment, as the resultant colony size after 13 days decreased as the treatment ITC concentration increases. However as the concentration of ITC was increased the lag time prior to growth initiation also increased. This perhaps suggests that here a fungistatic response is taking place, with growth only occurring once the concentration of ITC has declined in the growth medium to a level at which growth may proceed, however further study would have to be carried out to determine this. Although ultimately in terms of a biofumigation system, a fungitoxic response is more desired, a fungistatic response may also be beneficial dependent upon the time scale that growth and development of the pathogen is suspended for.

There has been limited previous work on the use of isothiocyanates to control the growth of *H. solani*. Yet due to the slow growing nature of this fungal pathogen it shows promise to be controlled through biofumigation practice. Olivier *et al.* (1999) showed that in the presence of AITC there was a greater suppressive effect on *H. solani* than fungi with faster growth rates. Their work also concluded that the presence of 3-butenyl, BITC and significant amounts of 2-PEITC released from *Brassica* tissues may also account for the suppressive action observed. Results from the above study again showed the most promising results for control are observed on AITC, BITC and PEITC incorporation studies.

Work by Sarwar *et al.* (1998) found that aromatic ITCs including PEITC and BITC were more toxic towards *R. solani*, than aliphatic ITCs dissolved in agar. Yulianti *et al.* (2006) also concluded that PEITC had a higher toxicity level on *R. solani* cultures growing on agar, than aliphatic AITC. Kirkegaard *et al.* (1996) also showed that the *R. solani* culture growth was inhibited by exposure to volatile ITCs produced through glucosinolate hydrolysis. Their work also demonstrated that the level of suppression was also related to the type of ITC released from the *Brassica* and the concentration they were released in. The above study has produced parallel findings, displaying a range of different levels of control of *R. solani* dependent both upon the concentration of isothiocyanate, incorporated into the agar plate, but probably most significantly is the specific isothiocyanate used within the study. The greatest level of control was observed by BITC and MITC, which inhibited growth at all concentration levels used.

Further work would be required to analyse the lowest levels at which control is achieved and also examine whether the effects observed were due to a fungistatic, in which growth had been delayed by the ITC treatment, or fungitoxic effect was being displayed, where the level of ITC present within the agar had killed the pathogen outright. However the results from the above experimentation greatly highlight the potential for using BITC and MITC to control *R. solani* spread and development.

PEITC also exhibited a level of control over *R. solani*. Results showed that as the concentration was increased between 6.25 – 25 ppm then overall the time for the cultures to reach their maximum size was not significantly altered. Yet at 25 ppm the time taken for the cultures to begin to grow increased, indicating a level of fungistatic control. At 50 ppm after the 15 day incubation period *R. solani* cultures showed 49.2% inhibition in comparison to control plates, the time taken for mycelial growth to first become visible was almost double that of control and lower concentrations. This suggests that the effect observed was of a fungistatic nature with *R. solani* growth only preceded once sufficient breakdown and degradation of the isothiocyanate has occurred.

Although there is still limited information concerning the concentrations of isothiocyanates that are liberated as a result of *Brassica* tissue incorporated into soil, on-going work is focussed on the concentrations of specific parent glucosinolates present within a range of different *Brassica* species. This information allows informed selective decisions to be made when choosing a *Brassica* cultivar to be incorporated into the soil to achieve maximum pathogen suppression. Comparison between the data produced in the above bioassays and concentrations of major glucosinolates present in *Brassica* spp. highlights that the suppressive effects upon fungal pathogens shown above may be achievable.

Overall the results show the need to understand the specific interactions occurring between differently structured isothiocyanates and different pathogens. Simple *in vitro* screening of isothiocyanates and their ability to affect the growth of a range of important soil borne potato pathogens allows study and biofumigant crop breeding to be angled towards producing crops that will produce the most effective isothiocyanates that will lead to the highest possible level of control to be achieved. In order to achieve effective biofumigation in practice it will be necessary to identify crops which contain high levels of parent glucosinolates that will result in the specific isothiocyanates identified here that have been shown to lead suppressing the growth of soil borne pathogens. Further experimentation carried out within this study have been used to identify and quantify isothiocyanates formed by several different *Brassica* cultivars, and is discussed in Chapter 4.

Chapter 4

Analysis of *Brassica* spp. glucosinolate hydrolysis products

4.1 Introduction

Over 140 differently structured glucosinolates have been identified, of which approximately 30 are known to exist within *Brassica* spp. (Fahey *et al.* 2001). Glucosinolate profiles vary between both *Brassica* species and cultivars of a single species, with profiles varying in both the composition of the specific glucosinolates present and the concentration present (Bellostas *et al.*, 2007a). Research has identified that the glucosinolate profile will also alter dependent on which tissue is sampled and with plant development (Bellostas *et al.* 2007a&b). Environmental growth conditions may also attribute to differences occurring in the plant (Bellostas *et al.*, 2007b), yet ultimately it is the plant's genetic background which determines the glucosinolate concentration and composition (Verkerk *et al.*, 2009). Therefore although growth conditions, such as soil, climate and fertilisation may alter the outcome of glucosinolates hydrolysis, the hydrolysis products are understood not to vary greatly if sufficient cell maceration, and therefore glucosinolate hydrolysis has occurred.

Trends in glucosinolate accumulation have been shown in previous studies. Work by Kirkegaard *et al.*, (1998) and Kirkegaard and Sarwar, (1998) showed that aliphatic structured glucosinolates dominated shoot profiles in comparison to roots which were shown to be dominated by aromatic glucosinolates. Such work highlights the complexity of the glucosinolate profile, and that research to assess how it alters throughout plant development is important to achieve effective, pathogen targeted biofumigation. If there is a greater understanding of which parental glucosinolates are present, or furthermore which ITCs are formed through glucosinolate hydrolysis, in both different *Brassica* cultivars and at different stages of their development, then more informed decisions can be made when choosing specific *Brassica* cultivars and an incorporation time. This will aim to achieve maximum suppression of soil borne pests and pathogens.

It is thought that with increased knowledge gained through both work carried out determining the specifics of the isothiocyanate-pathogen interactions and chemical analysis of hydrolysis products, that this will aid breeding programmes to generate more effective biofumigant crops. Breeders have already altered the types and concentrations of glucosinolates through selection for flavour and selection for resistance to herbivores (Fenwick & Heaney 1983) and currently attention is turning to selecting for the potential to produce biocidal products.

In recent years interest in *Brassicas* has also risen due to the interest in their believed benefits in human health. Increasing numbers of studies have concluded that a correlation exists between reduced cancer risk and consumption of dark green vegetables, mainly *Brassicas* (Verkerk *et al.*, 2009). It is thought that this link is largely due to the production of isothiocyanates, which have shown to possess tumour suppressive qualities. Tumour suppression is thought to occur through the modulation of detoxification enzymes which results in the prevention of initiation/DNA damage, and partly by modulation post-initiation events, in particular inhibition of proliferation and induction of apoptosis.

Studies here focussed on analysis of isothiocyanates formed through glucosinolate hydrolysis from *Brassica* spp. leaves, as this will provide the major component of tissue which is incorporated into soil when using green manure incorporation as part of the biofumigation process.

Null hypothesis

H_0 Use of a gas chromatography mass spectrometry assay designed for the purpose of this experiment will show that isothiocyanates, formed during *Brassica* glucosinolate hydrolysis, do not alter in isothiocyanate profile or concentration between species, cultivar and throughout plant development.

4.2 Materials and Methods

4.2.1 Gas Chromatography Mass Spectrometry

The decision was made to use Gas chromatography – Mass Spectrometry (GC-MS) to analyse isothiocyanates produced by *Brassica* spp. Although the advantage offered using liquid chromatography based methods were recognised, such as the ability to identify a broader range of metabolites, liquid chromatography does suffer from lower reproducibility of retention times, which is not observed in gas chromatography methods (Lisec *et al.*, 2006). The method utilises two commonly used chemical analysis methods in one process; gas chromatography separates volatile and semi volatile compounds with great resolution, but is unable to identify them. The use of mass spectrometry in combination with gas chromatography, can provide detailed structural information on the majority of compounds (Hites, 1997), allowing both identification and robust quantification of hundreds of metabolites within only a single plant sample (Lisec *et al.*, 2006).

4.2.2 Method development

Initial extraction of glucosinolate hydrolysis products was attempted using the method described in Lisec *et al.*, (2006). Samples initially showed no recovery of isothiocyanates and it was thought that the samples should be more concentrated. This was first carried out with a rotary evaporator, but again isothiocyanates were not detected when analysed using GC-MS, the method was repeated on this occasion concentrating the samples under nitrogen, again ITCs were not present during analysis. It was decided that a simplified method should be used, and different extraction solvents should be tested. A method was adapted from Al-Gendy and Lockwood, (2003) as described in Chapter 2, (2.7.1.1 and 2.7.1.2), initially chloroform was used as an extraction solvent, however expected results were still not observed. The method was then repeated using ethyl acetate, which when analysed using GC-MS showed the presence of several isothiocyanates.

4.2.3 Method validation

To determine the reproducibility of the extraction method it was validated before carrying out analysis glasshouse produced plant samples.

Validation was carried out by spiking three 1 g *Brassica* plant material (Commence B, mustard) samples with 20 µl of 50 µl/ml ITC mix, of equal volumes of AITC, BITC, IITC, MITC, NITC, PEITC and PITC. Samples were left at 4°C for 15 hours, additionally a matrix blank with and without enzyme were also set up as described in Chapter 2, section 2.7.1.3. After the overnight incubation a solvent extraction was carried out,

followed by GC-MS analysis of spiked samples, matrix blanks, and ITC mix standards (0.2, 0.5, 1, 2 µl/ml). Individual ITC results from each sample were used to form standard curves, allowing concentrations of ITCs in both spike and matrix blank samples to be determined.

4.2.4 *Brassica*s Grown for GC-MS study

Fifteen different *Brassica* spp. (Table 4.1) were grown in four replicates in a controlled glasshouse environment in a randomised block design, created in Genstat v14 (VSN International Limited), according to cultivar number. Seeds were planted in John Innes No. 2 compost in 5 litre pots and watered evenly daily. Plants were not treated with any fertiliser or pesticides throughout the duration of the study. Plants were harvested at their appropriate development times 1-5 (Table 4.2), the leaves were removed from that plant, for analysis. Plant material was stored in sealed freezer bags at -20°C. It was recognised that liquid nitrogen flash freezing may have been a better approach to freezing plant material, however due to limited availability of liquid nitrogen and limited budget, this was not possible.

Table 4.1 Cultivars used in glass house experimentation to generate plant material for GC-MS analysis.

Cultivar	Species	Supplier
1. BRJ CAAA (mustard)	*Brassica juncea*	Barworth Agriculture
2. BRJ CAAB (mustard)	*Brassica juncea*	Barworth Agriculture
3. BRJ CAAC (mustard)	*Brassica juncea*	Barworth Agriculture
4. BRJ CAAD (mustard)	*Brassicajuncea*	Barworth Agriculture
5. Caliente mustard 20	*Brassica juncea*	Barworth Agriculture
6. Caliente mustard 61	*Brassica juncea*	Barworth Agriculture
7. Caliente mustard 99	*Brassica juncea*	Barworth Agriculture
8. Forage Rape	*Brassica* napus	R.M.Welch and Son Ltd.
9. Kale Maris	*Brassica oleracea*	R.M.Welch and Son Ltd
10. Nemat	*Eruca sativa*	Barworth Agriculture
11. New Radish Apoll	*Raphanus sativus*	Barworth Agriculture
12. Old Radish Consul	*Raphanus sativus*	Barworth Agriculture
13. Radish Vienna	*Raphanus sativus*	Thompson and Morgan
14. *Sinapsis Alba* Mirly	*Sinapsis alba*	Barworth Agriculture
15. Turnip Green Massif	*Brassica rapa*	R.M.Welch and Son Ltd.

Table 4.2 Development stage that plant tissue samples were collected. Development times were based around the key growth stages for oil seed rape.

Development Stage	Plant Appearance
1	Appearance of first flower
2	70% of flower buds open
3	Pod development
4	Seeds developing
5	Most seeds brown

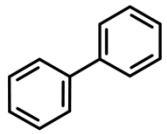
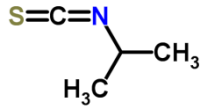
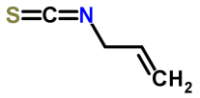
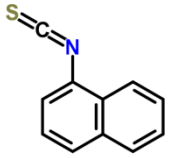
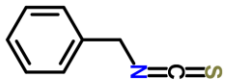
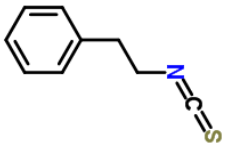
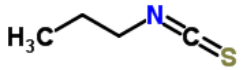
4.2.5 GC-MS analysis of *Brassica* spp.

Once all glasshouse material had been collected, extractions were carried out on 0.5 g leaf material samples as detailed in Chapter 2 (2.7.1.2).

4.2.6 Analysis of results

To determine the presence of the standard isothiocyanates (AITC, BITC, IITC, MITC, NITC, PEITC, PITC), a reference ion and retention time for each isothiocyante was calculated (Table 4.3). The chromatograms of each compound are shown in appendix 5, they also illustrate that MITC was immeasurable using GC-MS, due to its short retention time and therefore removed from the study. The results were used to determine the presence of each of the isothiocyanates within the extracted samples from the glasshouse grown *Brassica* cultivars.

Table 4.3 Ions and retention times used for GC-MS identification of each compound.

Compound	Quan Ions	Retention Time	Compound	Quan Ions	Retention Time
Biphenyl 	154.0	7.225 +/- 0.200	Isopropyl ITC 	101.0	3.521 +/-0.200
Allyl ITC 	99.0	3.895 +/-0.200	1-Napthyl ITC 	185.0	9.272 +/-0.500
Benzyl ITC 	149.0	7.109 +/-0.400	2-Phenylethyl ITC 	163.0	7.648 +/-0.200
Propyl ITC 	101.0	4.108 +/-0.200			

4.3 Results

Using Gas-Chromatography Mass-Spectrometry the ITCs formed during glucosinolate hydrolysis were analysed against six ITC standard solutions (AITC, BITC, IITC, NITC, PEITC and PITC). To quantify, ITC outputs were compared to standard curves produced through standard ITC mix solution dilutions, which were analysed simultaneously with every sample run. Results allow general conclusions to be made about the concentrations of the ITCs produced throughout the five sampled development stages; they also allow comparisons to be made about the patterns of individual ITC levels between different cultivars. Differences in ITC levels at each sampled development stage were also examined.

4.3.1 Total isothiocyanates produced by *Brassica* cultivars

Initially mean values for each ITC produced at the five development stages were calculated. To assess which of the analysed ITCs were dominant, the sum of individual ITCs produced by each cultivar across all development stages was calculated (Fig 4.1). This showed that AITC was the dominant ITC being produced in significantly higher concentrations than the other five ITCs, in several cultivars. Highest levels of AITC were most commonly seen in mustard cultivars, with the exception of *B. juncea* cv. BRJ CAAC, and the *B. rapa* cv. Turnip Green Massif. Much lower levels of AITC were measured in radish, rocket, kale and white mustard cultivars, suggesting that AITC is not the dominant ITC produced by these *Brassica* varieties, and/or parental glucosinolate levels of are much less common than those present in the mustard and turnip cultivars, further analysis using additional ITC standards would be required to establish dominant ITCs produced by these cultivars.

Removal of the dominant AITC levels from the results produced allowed easier analysis of the other ITCs which are produced as a result of glucosinolate hydrolysis of the assessed cultivars (Fig 4.2). This revealed that both BITC and PEITC were both produced at levels of up to 162 ppm and 81 ppm respectively. Interestingly the highest levels of either ITC did not coincide with appreciable levels of the other ITC. The figure also shows that IITC, NITC were rarely detected and concentrations greater than 10 ppm were not produced in any of the cultivars analysed within this study. PITC was not detected in any cultivar used within the study.

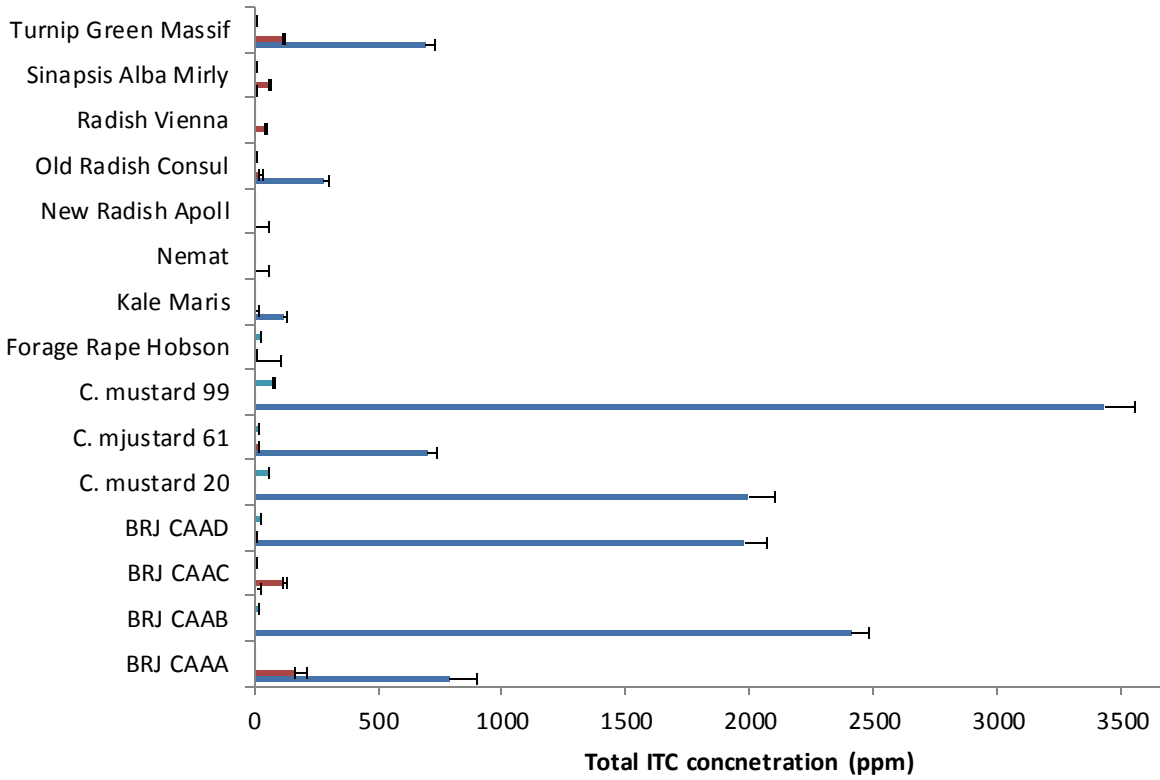


Figure 4.1 Total accumulation of all development stages, of each ITC produced by cultivar analysed by GC-MS. Error bars display the standard error of the mean.

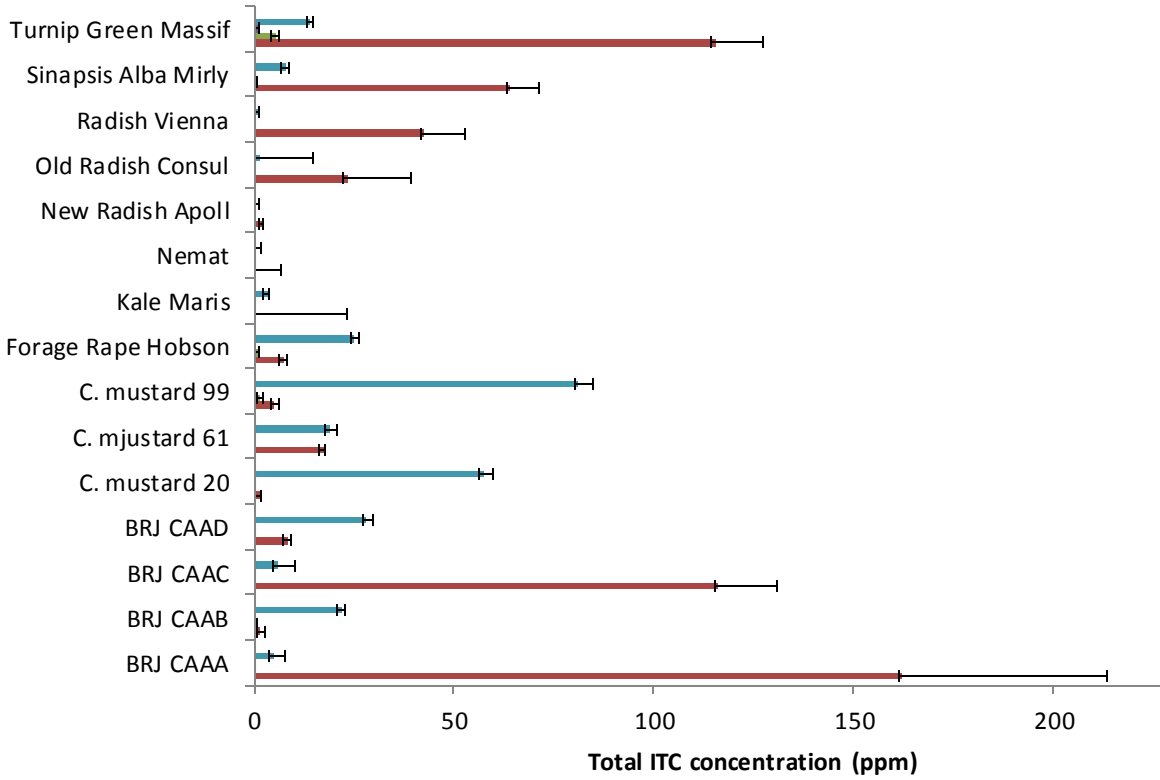


Figure 4.2 Total accumulation of all development stages, of each ITC, except AITC produced by cultivar analysed by GC-MS. Error bars display the standard error of the mean.

4.3.2 Individual ITCs produced throughout plant development

Mean values for the concentration of each standard ITC were also calculated. The values were plotted on line graphs to show the patterns of ITC loss and accumulation throughout each individual cultivars development (Fig. 4.3).

Generally *Brassica* cultivars which overall produce relatively high concentrations of AITC, produce the highest concentrations in development stage 5, this is true for *B. juncea* cvs. BRJ CAAB, BRJ CAAC and C. mustard 20 (Fig. 4.3). *B. juncea* cv. C. mustard 99, is the only cultivar studied which shows a surge in AITC production at development stage 3, when all other cultivars are shown to decrease in AITC levels. *B. juncea* cv. BRJ CAAA, *B. rapa* cv. Turnip Green Massif and *B. juncea* cv. C. mustard 61 produce high concentrations of AITC in their growth cycle at the first stage after which levels decrease rapidly, as by stage 3 only trace levels were observed. The lowest levels of AITC produced were from *B. oleracea* cv. Kale Maris, *Sinapsis alba* cv. Mirly, *R. sativus* cv. Old Radish Consul, *R. sativus* cv. New Radish Apoll in all cases levels were not recorded above 100 ppm. Overall the highest levels of AITC were produced from the hydrolysis of *B. juncea* cv. BRJ CAAA (1320 ppm) at development stage 5.

The highest level of BITC was produced by hydrolysis of *B. rapa* cv. Turnip Green Massif cultivars at development stage 1, (687.0 ppm), this concentration is significantly higher than found in any other cultivars (Fig. 4.4). The second highest concentration of BITC was produced by the mustard cultivar *B. juncea* cv. BRJ CAAA, (156.9 ppm). Other cultivars producing high levels of BITC were *B. juncea* cv. BRJ CAAC and *B. oleracea* cv. Kale Maris when the concentration of BITC was measured at 95.1 and 49.3 ppm respectively, both from samples collected at the first development stage.

In contrast to AITC, levels of BITC were produced at stage 1 or 2 with only low levels observed from development stage 3 onwards. Compared to AITC and BITC, levels of PEITC were overall significantly lower (Fig. 4.5). The highest levels of PEITC were measured in *B. juncea* cv. C. mustard 99 at development stage 1 (28 ppm) and development stage 3 (31 ppm), levels dipped in development stage 2 to 11 ppm, increased in stage 3 then dropped dramatically to 3 ppm at development stage 4. A similar trend was observed in *B. napus* cv. Forage Rape Hobson, where the highest levels of PEITC production during its development were recorded at development stage 1 (5 ppm) and development stage 3 (18 ppm). Relative to concentrations measured from all cultivars, development stage 1 was the most consistent in producing the highest recorded levels of PEITC, *B. rapa* cv. Turnip Green Massif (12 ppm) and *B. juncea* cv. BRJ CAAD (13 ppm) all produced levels of PEITC after hydrolysis.

Analysis of IITC levels produced by glucosinolate hydrolysis showed that IITC was not a dominant ITC formed by the *Brassica* spp. examined within this study (Fig. 4.6). In most instances IITC was observed at trace levels. The highest level of IITC was

produced by *B. rapa* cv. Turnip Green Massif (4.8 ppm) in development stage 5. The second highest level was measured at development stage 2, by *B. juncea* cv. C. mustard 99 (1.7 ppm). All other measurements of IITC were below 1 ppm.

Similarly to IITC, NITC was recorded at trace levels (Fig. 4.7), with the highest concentration recorded in *B. rapa* cv. Turnip Green Massif at development stage 3, however this was still a very low concentration (0.9 ppm). PITC was only measured in trace amounts in four cultivars, *B. rapa* cv. Turnip Green Massif and *B. juncea* cvs C. mustard 99, C. mustard 61, BRJ CAAC (Fig. 4.8). The highest concentration of PITC was produced by *B. oleracea* cv. Kale Maris in development stage 1, (3.1 ppm). However no other cultivars produced concentrations of PITC close to this level.

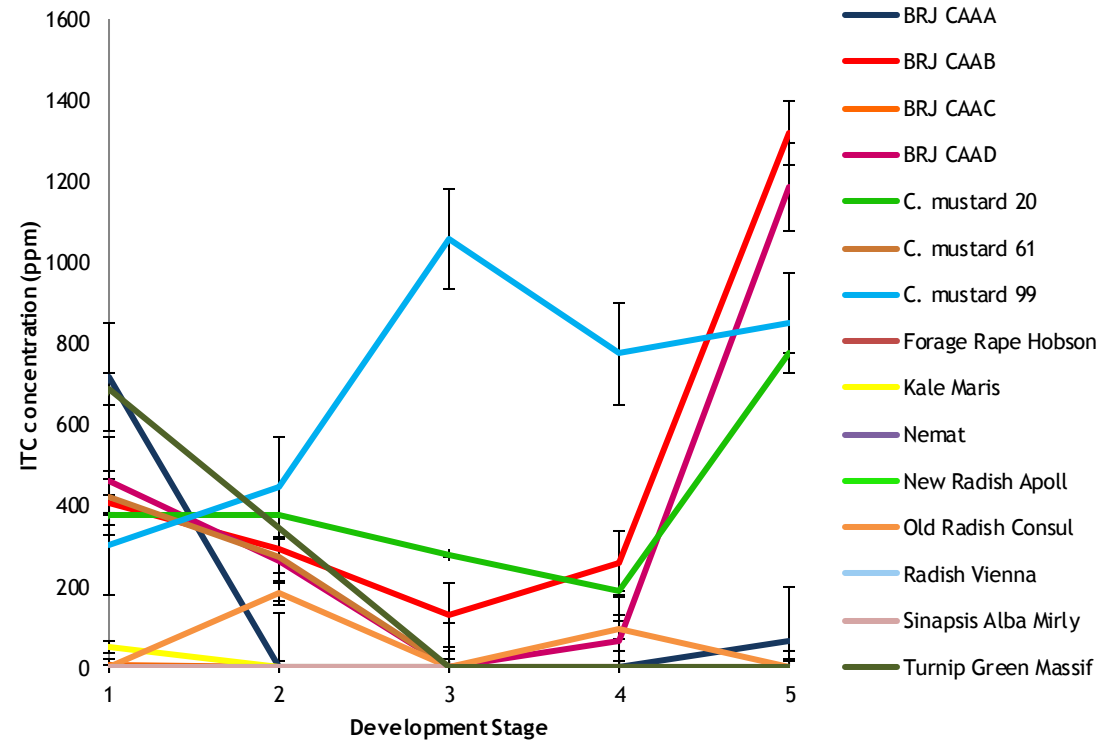


Figure 4.3 Mean concentration of AITC analysed by GC-MS, at the five development stages, for each cultivar. Y error bars show the standard error of the mean.

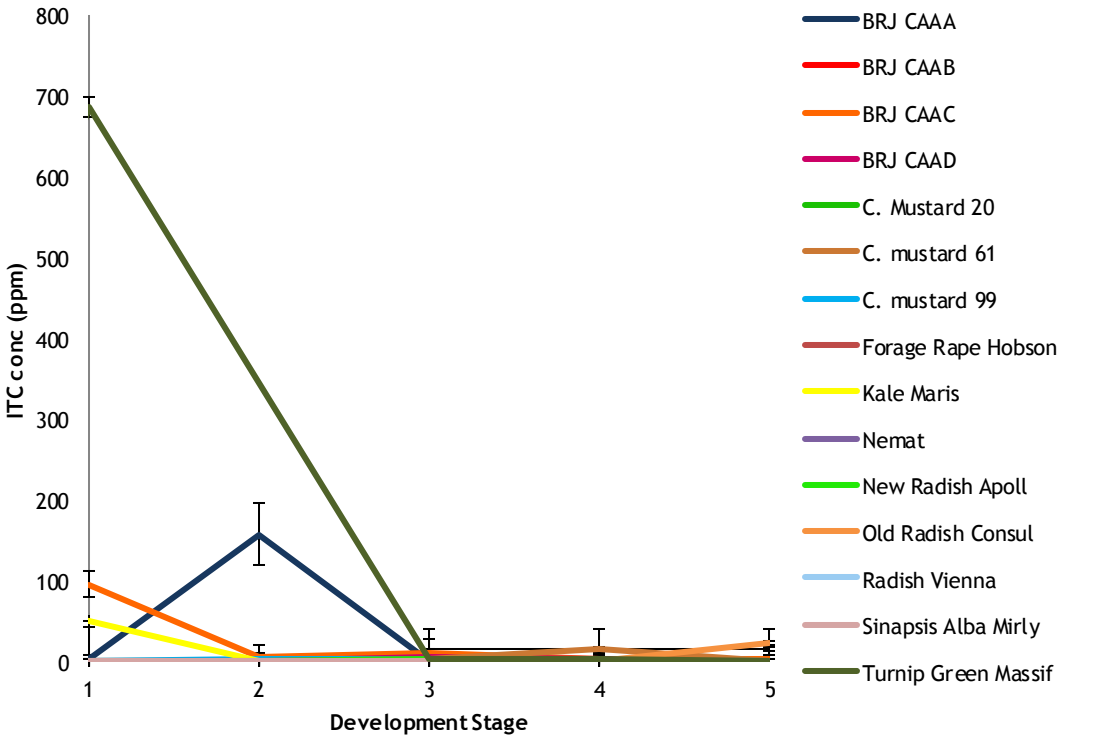


Figure 4.4 Mean concentration of BITC analysed by GC-MS, at the five sampled development stages, for each cultivar. Y error bars show the standard error of the mean.

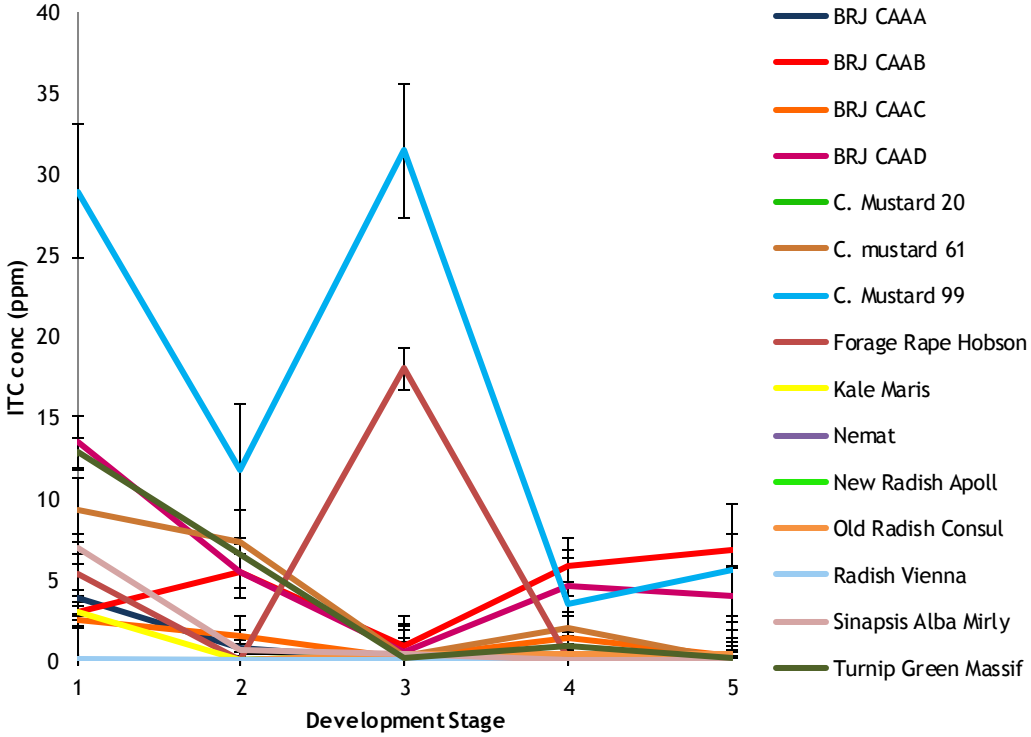


Figure 4.5 Mean concentration of PEITC analysed by GC-MS, during the five sampled development times, for each cultivar. Y error bars show the standard error of the mean.

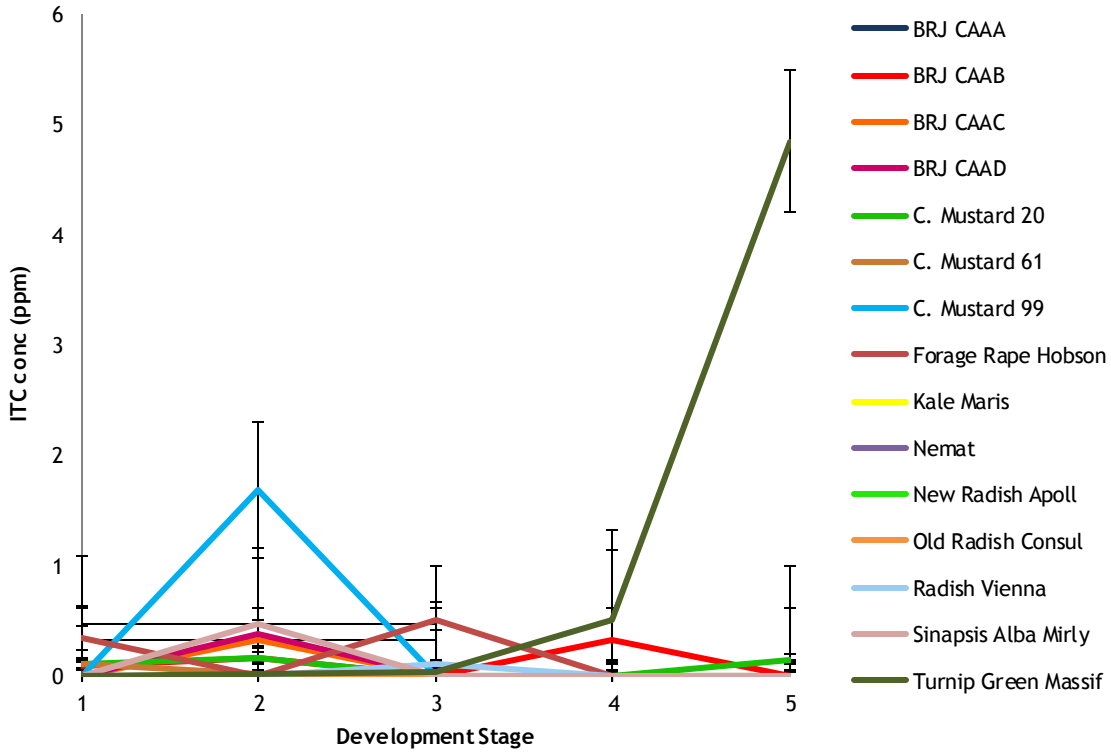


Figure 4.6 Mean concentration of IITC analysed by GC-MS, during the five sampled development times, for each cultivar. Y error bars show the standard error of the mean.

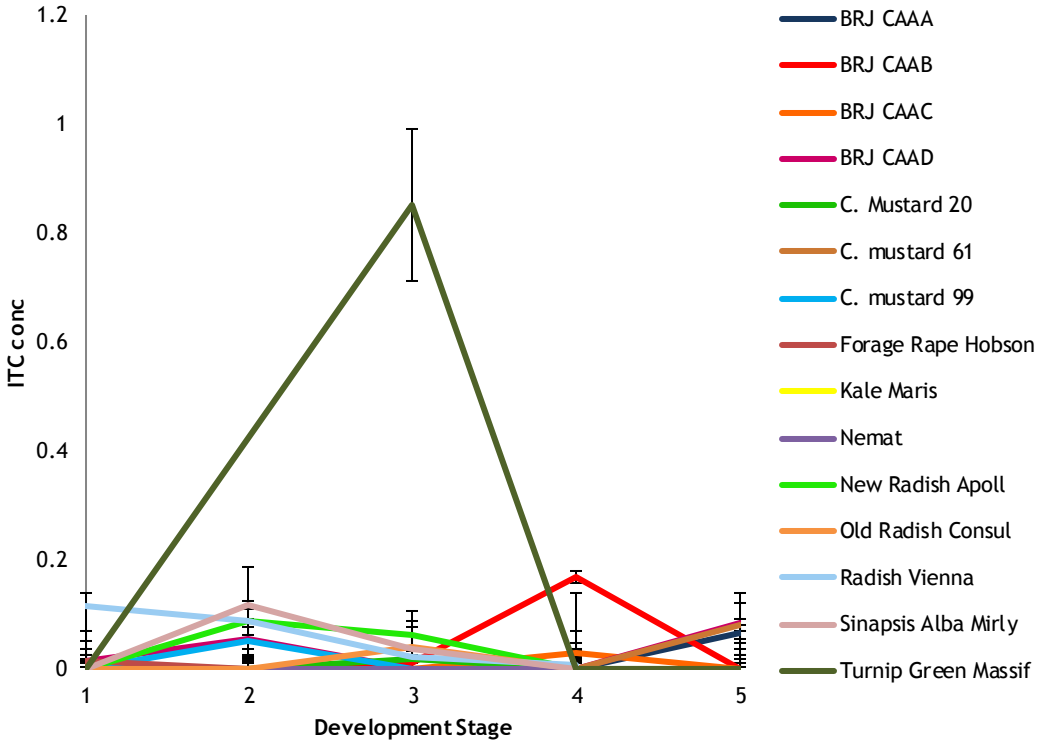


Figure 4.7 Mean concentration of NITC analysed by GC-MS, during the five sampled development times, for each cultivar. Y error bars show the standard error of the mean.

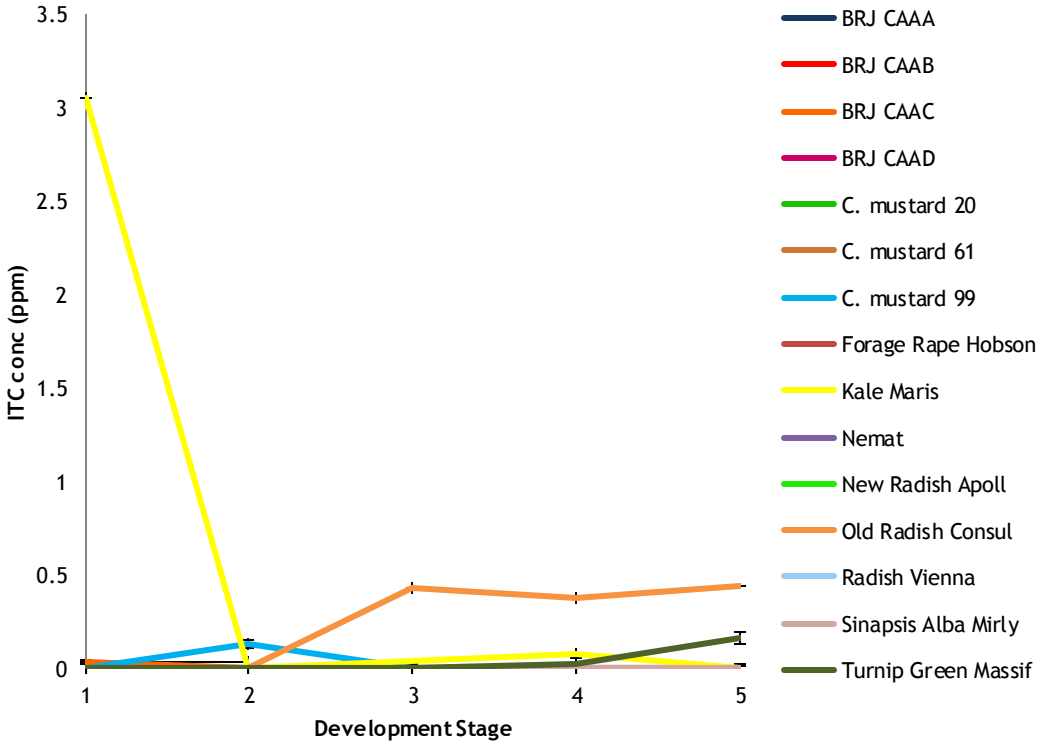


Figure 4.8 Mean concentration of PITC analysed by GC-MS, during the five sampled development times, for each cultivar. Y error bars show the standard error of the mean.

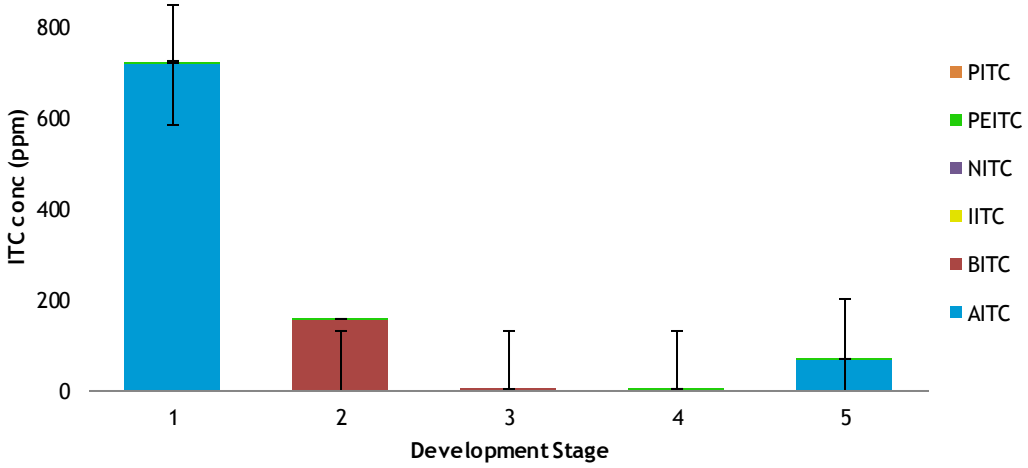
4.3.3 ITC Concentrations in *Brassica* spp. at each development stage

Analysis of ITCs produced by *B. juncea* cv. BRJ CAAA showed that of the six ITCs analysed, AITC was produced in the highest concentration, during the first development stage (717 ppm) (Fig. 4.9a). During the three following development stages AITC was not present. At the fifth sampling stage analysis showed AITC to again occur through glucosinolate hydrolysis, but at a lower concentration than previously measured, (68 ppm). The highest level of BITC was observed at development stage 2 (156 ppm). BITC was also measured at lower concentrations during development stage 1, 3 and 4 with levels of 2.6, 0.7 and 2.1 ppm respectively. PEITC was detected at all development stages except 3 with the highest level being recorded at development stage 1 (3.9 ppm) with levels of <1 ppm at stages 2, 4 and 5. NITC was only recorded at 0.07 ppm in development stage 5 and IITC and PITC were not found in *B. juncea* cv. BRJ CAAA.

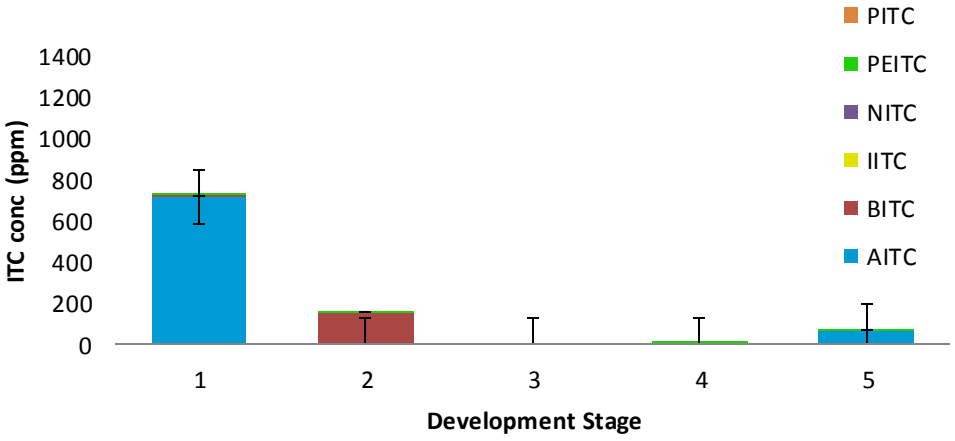
Overall analysis of cultivar *B. juncea* cv. BRJ CAAB showed that high levels of AITC are present in all development stages (Fig. 4.9b). The highest concentration was recorded from hydrolysis products from development stage 5 (1320 ppm). High levels were also observed at development stage 1 (406 ppm), concentrations decreased until development stage 3, which had the lowest levels of AITC (132 ppm). Levels then increased in stage 4 and then again in stage 5. Of all ITCs detected the second highest concentrations were of PEITC, again the highest level was recorded in stage 5 (6.8 ppm) and the lowest in stage 3 (0.8 ppm). BITC was not found in stage 5 and at < 1 ppm in all other sampled stages. IITC was detected in stages 1, 2 and 4 at concentrations below 0.5 ppm. NITC was measured in stages 3 and 4 at <0.5 ppm. PITC was not recorded at any development stage.

Hydrolysis products from *B. juncea* cv. BRJ CAAC showed BITC to be the most abundant compared to the other five analysed ITCs (Fig. 4.9c). Highest levels of BITC were recorded in development stage 1 (95 ppm), they then decreased in stage 2 to 5.2 ppm, a slight increase was found at stage 3 (10.8 ppm), however levels again decreased to 2.4 and 2.7 ppm for stages 4 and 5 respectively. The highest levels of both AITC (6.1 ppm) and PEITC (2.5 ppm) were recorded at development stage 1, in both cases levels dropped in stage 2 (AITC – 1.4 ppm; PEITC – 1.5 ppm). AITC was not recorded at stage 3 and only 0.2 ppm of AITC was recorded at both development stage 4 and 5. Levels of PEITC decreased in development stage 3 (0.1ppm), then increased slightly in stage 4 (1.5 ppm) before decreasing again in stage 5 to 0.2 ppm. A low level of IITC was recorded in stage 2 only, 0.33 ppm; NITC was only detected in stage 4 (0.03ppm); PITC was only recorded in development stage 1 again at 0.03 ppm.

a)



b)



c)

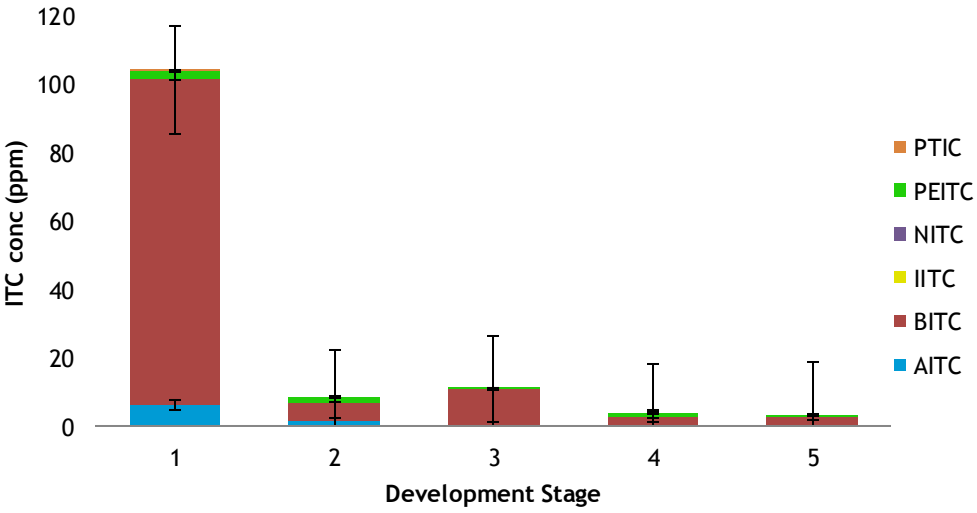


Figure 4.9a-c Concentrations of isothiocyanates measured in cultivars a) BRJ CAAA b) BRJ CAAB c) BRJ CAAC at each development stage. Vertical error bars indicate the standard error of the mean.

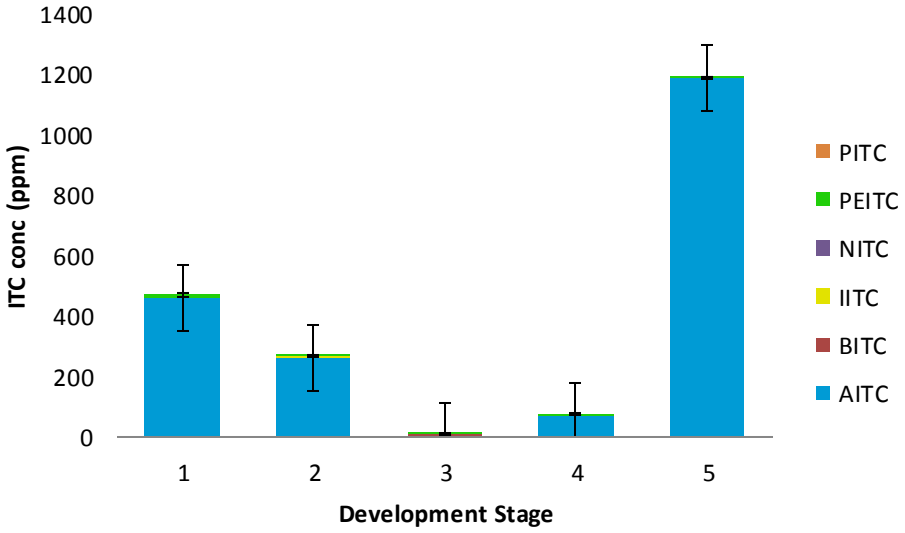
Analysis of *B. juncea* cv. BRJ CAAD hydrolysis products showed that AITC was the most abundant ITC produced (Fig. 4.10a). From a level of 460 ppm in the first development stage, levels then decreased to 262 ppm at development stage 2. AITC levels further decreased in stage 3 (1.1 ppm) before increasing to 67 ppm in stage 4 and reaching their highest level in development stage 5 (1186 ppm). Of all the ITCs analysed the second most abundant was PEITC, with the highest levels being recorded at stage 1 (13 ppm), they decreased in stage 2 (5.5 ppm), then further decreased to the lowest measured level in stage 3 (0.56 ppm) before then increasing to 4 ppm in stages 4 and 5. BITC was recorded at every stage of development with the highest concentrations recorded in samples from development stage 3 and 4, at 6 and 2 ppm respectively; all other development stages produced concentrations of BITC below 0.5 ppm. IITC was only produced in a low level at stage 2 (0.4 ppm), NITC was found in low levels at stages 1,2 and 5 at levels < 0.1 ppm, PITC was not detected at any development stage.

In *B. juncea* cv. C. mustard 20, AITC was found to be the dominant ITC with high concentrations measured in all development stages sampled (Fig. 4.10b). AITC produced from samples from development stage 1 and 2 were of similar concentrations (378.5 and 376.9 ppm). AITC levels were then shown to decrease in stage 3 to 275.6 ppm and then again in development stage 4 to 189.8 ppm. The level of AITC then peaked in the final development stage (775.6 ppm). The second most commonly produced ITC was PEITC, which again was measured in similar concentrations in development stage 1 and 2 (11.0 and 14.7 ppm), again in development stage 3 concentrations decreased to 5.5 ppm. However unlike AITC the highest concentration of PEITC was measured from samples at development stage 4, (24.8 ppm), in the final sampling stage PEITC concentrations decreased to 1.5 ppm. Very low levels of BITC were measured in development stage 1, 2, 4 and 5 and; IITC in development stage 1, 2 and 5 and NITC in development stage 3 all were <0.5 ppm. PITC was not recorded in *B. juncea* cv. C. mustard 20.

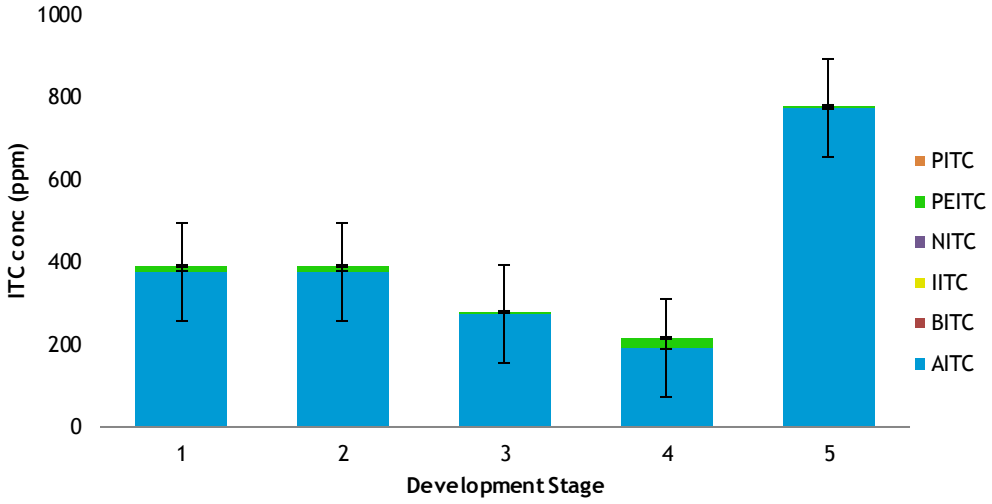
The dominant ITC produced by *B. juncea* cv. C. mustard 61, in this study was AITC, which was produced in high concentrations in both development stages 1 (422.3 ppm) and 2 (274.4 ppm) (Fig. 4.10c). AITC levels dramatically decreased in the final three development stages, with levels of 1 ppm, and 0.2 ppm in development stages 3 - 5 respectively. The highest concentration of BITC was recorded in development stage 4 (14.4 ppm), after which BITC levels were ≤0.5 ppm, with the exception of samples from development stage 3, which showed a slight increase (1.5 ppm). Where high levels of AITC were found in development stages 1 and 2, the highest levels of PEITC were also recorded, yet at much lower concentrations than AITC, 9.3 and 7.3 ppm respectively. A much lower concentration of PEITC was recorded in development stage 3 (0.2 ppm) which slightly increased in development stage 4 (2.0 ppm), no PEITC was detected in the *B. juncea* cv. C. mustard 61 cultivar in development stage 5. Of the remaining ITC

analysed only trace amounts were detected, if at all. IITC concentrations ≤0.1 ppm was measured in development stage 1 and 2.

a)



b)



c)

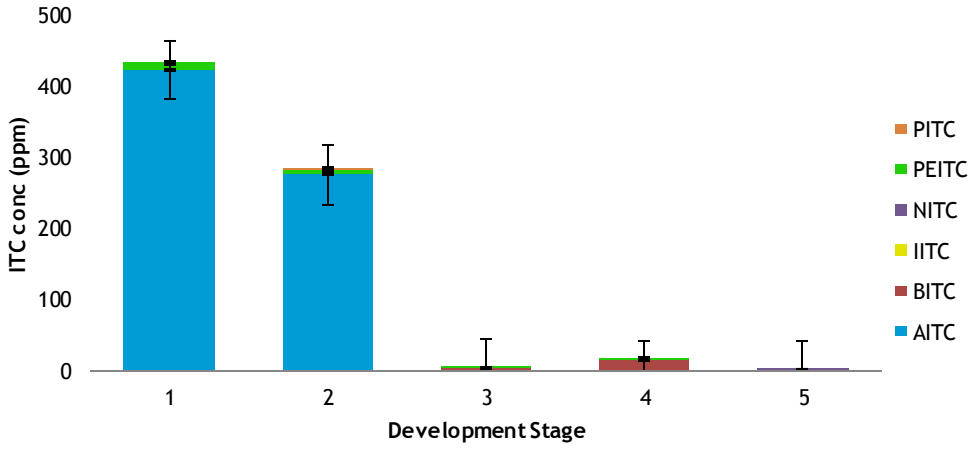


Figure 4.10a-c Concentrations of isothiocyanates measured in cultivars a) BRJ CAAD b) C. mustard 20 c) C. mustard 61 at each development stage. Vertical error bars indicate the standard error of the mean.

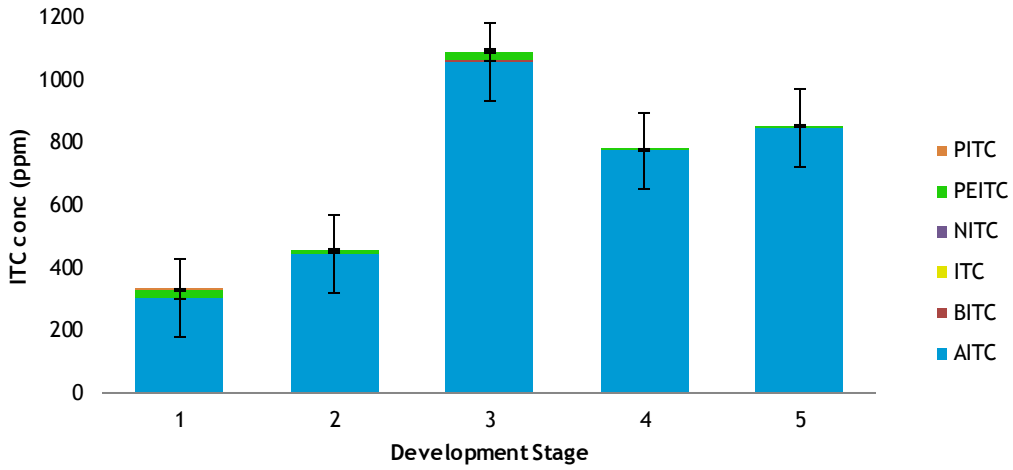
In *B. juncea* cv. C. mustard 99, AITC was found to be the dominant ITC produced during glucosinolate hydrolysis at all development stages sampled (Fig. 4.11a). Initial levels were measured at 303.1 ppm. They then increased in the second stage to 444.4 ppm, before peaking in stage 3 at 1059.7 ppm. The concentration of AITC then decreased slightly to 774.4 ppm before increasing to 849.3 ppm in development stage 5. The second most readily produced ITC by *B. juncea* cv. C. mustard 99 was PEITC. At development stage 1 PEITC levels were found at 28.9 ppm, they decreased in stage 2, to 11.7 ppm and then increased to the highest concentration recorded in development stage 3, 31.5 ppm. A significant decrease was then observed in development stage 4, to 3.5 ppm, concentrations increased slightly in development stage 5 to 5.6 ppm. Low levels of BITC were found at each development stage, the highest of which 2.0 ppm was detected in development stage 2 although it decreased slightly in development stage 3 to 1.8 ppm. All other recorded levels were below 1 ppm. The only appearance of IITC was at development stage 3, where a low concentration of 1.7 ppm was recorded. Development stage 2 showed the only presence of measurable NITC at a very low level of 0.05 ppm. A low level of PITC was detected in development stage 1, of 0.1 ppm, it was not produced by *B. juncea* cv. C. mustard 99 cultivars at any other development stage.

In *B. rapa* cv. Green Massif, the highest ITC concentration was AITC measured in the second development stage (687 ppm) in all other development stages levels of AITC were significantly lower, <1 ppm in the first and last stage and 1.3 ppm in stages 3 and 4 (Fig. 4.11b). In the third sampling stage high levels of BITC were recorded (101 ppm); BITC levels were much lower in the first two stages at levels of 1.6 ppm and 2.0 ppm respectively. After peaking in the third development stage, levels of BITC dropped significantly to 0.7 ppm, an increase was detected in the final sampling stage (10.9 ppm). PEITC was found at each development stage, with the highest levels recorded at stage 2 (12 ppm), increasing from initial levels of 2.6 ppm recorded in the first development stage. In the final three development stages concentrations were below 1 ppm. Levels of IITC were recorded at all development stages except 2, being highest in the final development stage (4.8 ppm). NITC was only found in the first and third development stage at concentrations below 1 ppm. PITC was recorded in low levels <1 ppm in the first, fourth and fifth development stage.

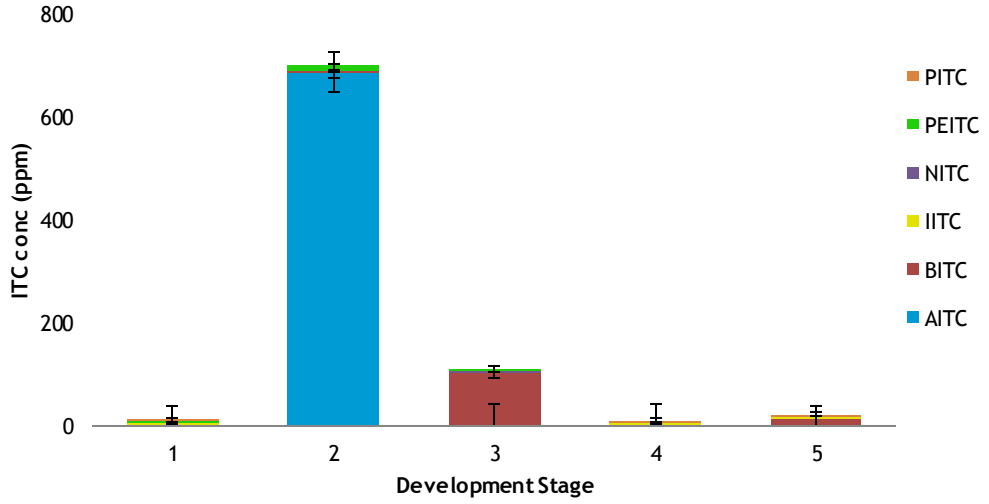
AITC was the dominant ITC produced by the *R. sativus* cv. Old Radish Consul cultivar being found in highest concentrations overall in development stage 2 (185 ppm) followed by development stage 4 (93 ppm) (Fig. 4.11c). In all other development stages AITC was only recorded at low levels < 0.5 ppm. The highest level of BITC was measured in the final development stage (22 ppm); BITC was also recorded in the development stage 2 and 3 however at much lower levels < 1 ppm. NITC was only found at development stage 3 at a very low level (0.04 ppm), PEITC was recorded in

the final three development stages, again at low concentrations <0.5 ppm. IITC and PITC were not detected at any development stage.

a)



b)



c)

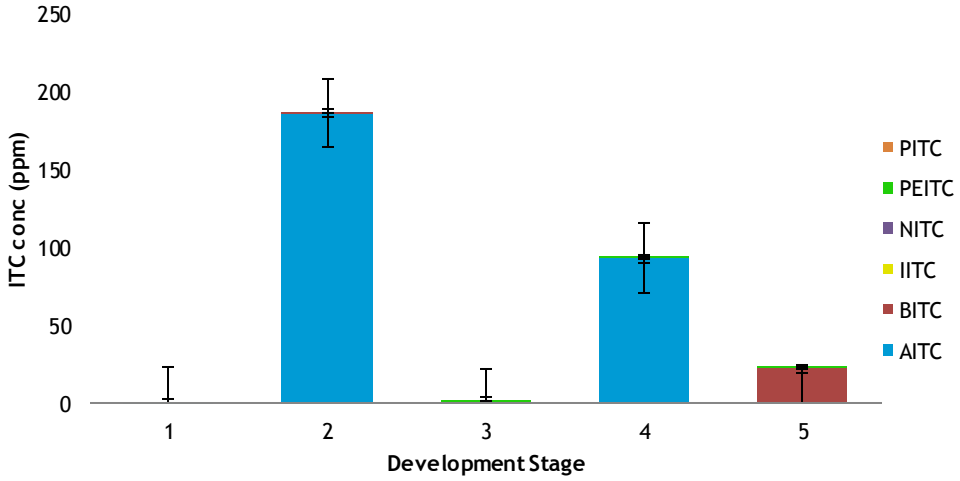


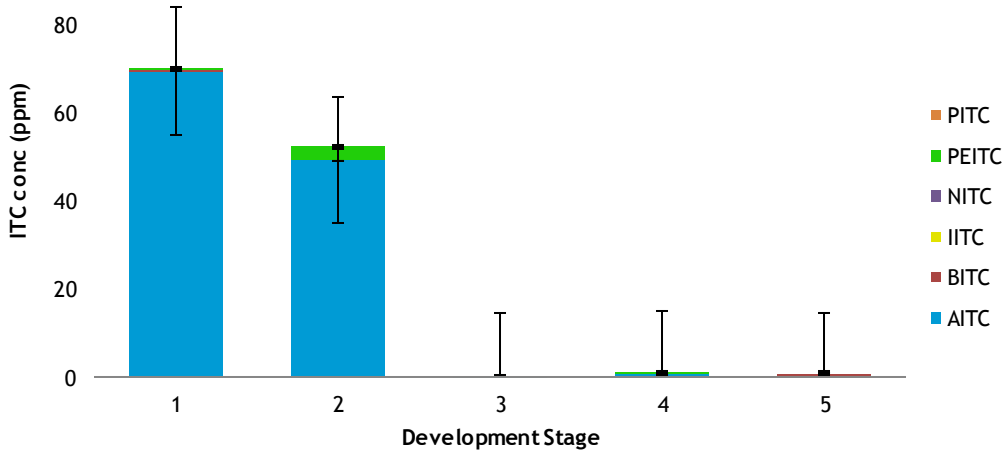
Figure 4.11a-c Concentrations of isothiocyanates measured in cultivars a) C. mustard 99 b) Turnip Green Massif c) Old radish consul at each development stage.

In *B. oleracea* cv. Maris, AITC was more abundant than the other five ITCs analysed (Fig. 4.12a). The highest level was measured in development stage 1 (69 ppm), which decreased in development stage 2 (49 ppm), the final three development stages showed much lower levels of AITC (< 1 ppm). PEITC was the next most abundant ITC and at the second development stages its concentration peaked at 3.1 ppm. At development stage 1 and 4 it was at low levels <0.5 ppm and absent in the third and fifth development stage. Low levels of BITC were recorded in the first and final development stages, at concentrations below 0.5 ppm and absent from stages 2-4. A very low concentration of NITC was recorded in the first development stage (0.06 ppm); however it was not detected at any of the subsequent development stages. IITC and PITC were not present in hydrolysis products from *B. oleracea* cv. Maris.

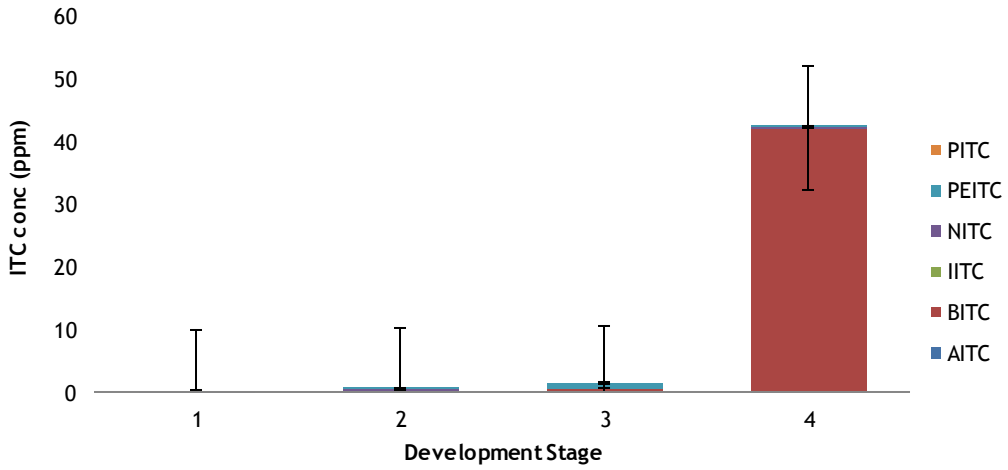
In *R. sativus* cv. Vienna results showed that BITC was produced in the highest concentration, compared to the other ITC standards (Fig, 4,12b), at the fourth development stage (42 ppm). Prior to this stage it was also present in the second and third development stage, however at significantly lower concentrations <0.5 ppm. Low levels of AITC and NITC were present at every stage of development, in all cases these were below 0.5 ppm. PEITC was not measured in the first development time, but produced in low concentrations at all other stages sampled (<0.1 ppm). A low concentration of IITC was measured during the third development stage (0.1 ppm). No PITC was measured in any *R. sativus* cv. Vienna samples. Due to time constraints Radish Vienna cultivars could not to be sampled at the fifth development time as this is a slow growing *Brassica* cultivar.

In the *B. napus* cv. Hobson the most dominant ITC produced was PEITC, the levels of which peak in the fourth development stage (18 ppm) (Fig. 4.12c). In the first and second stage, levels of PEITC were found to increase from 1.5 to 5.3 ppm, but then decreased in the third development stage, as did all ITCs, to 0 ppm. AITC was found in the first four development stages with the highest concentration being recorded at development stage 4 (1.3 ppm). The highest level of BITC was measured in the second development stage (5.8 ppm), low levels of BITC were also measured in the development stages 1, 4 and 5, all of which were below 1 ppm. IITC was measured at very low levels at the first, second and fourth development stage with all concentrations being below 1 ppm. Very low concentrations of NITC were detected in the first and second development stages (<0.1 ppm).

a)



b)



c)

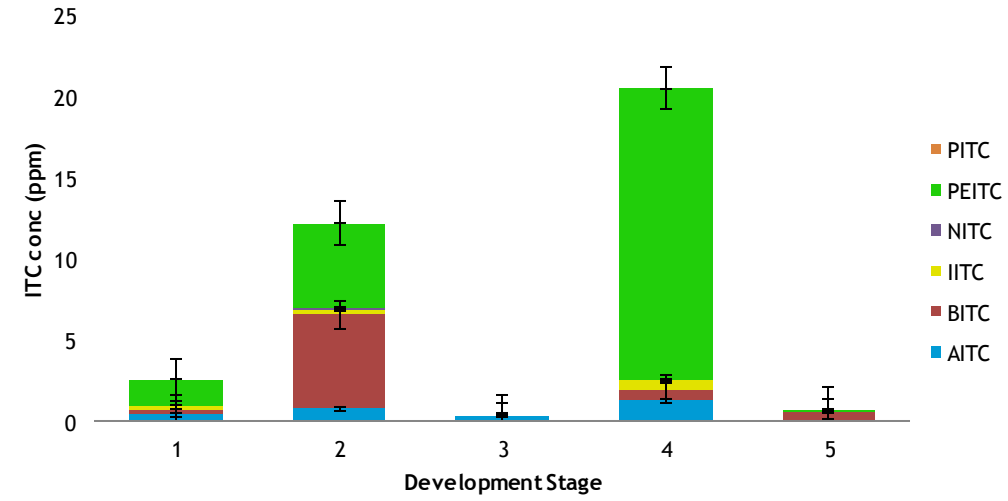


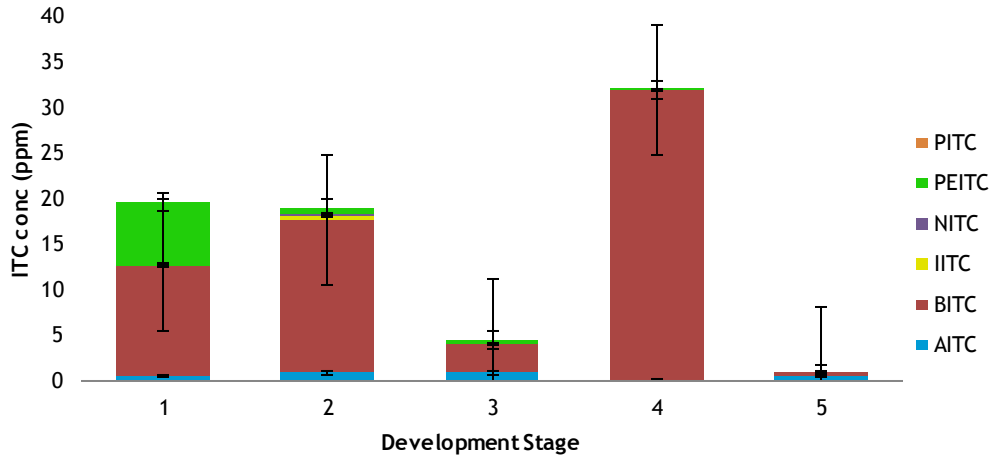
Figure 4.12a-c Concentrations of isothiocyanates measured in cultivars j) Kale Maris k) Radish Vienna l) Forage Rape Hobson at each development stage. Vertical error bars indicate the standard error of the mean.

The dominant ITC found in *Sinapsis alba* cv. Mirly was BITC, this was present at the highest concentrations at the fourth development stage (31.9 ppm) (Fig. 4.13a). In the first and second development stages sampled there was an increase in BITC levels from 12.2 ppm to 16. 8 ppm followed by a decrease in concentration to 3.2 ppm. After peaking in development stage 4, the concentration of BITC fell to 0.3 ppm at stage 5. Of the other ITCs recorded, PEITC was found to be the second most abundant although in relatively low concentrations, with a peak of 6.9 ppm in development stage 1. PEITC was also found in development stage 2, 3 and 4 at levels below 1 ppm. AITC was present at each development stage, but consistently below 1 ppm. IITC was only detected in the second development stage, however at a low level of 0.47 ppm. NITC was only found at concentrations below 0.2 ppm in development stages 2 and 3. PITC was not detected in *Sinapsis alba* cv. Mirly.

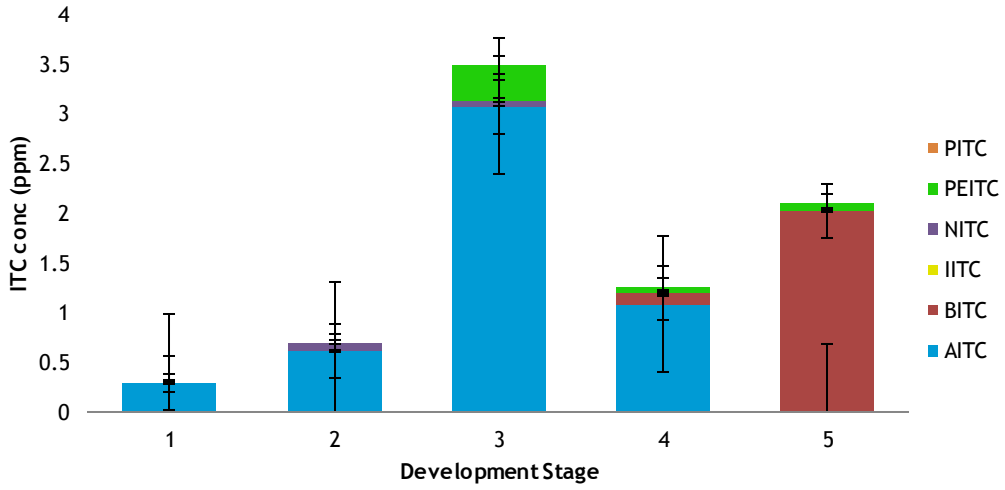
Analysis of the hydrolysis products of *R. sativus* cv. New Radish Apoll, showed that all ITCs analysed were produced in very low concentrations (Fig. 4.13b). The figure shows that AITC was found to be the dominant ITC in the first four development stages, with levels peaking at 3.1 ppm in development stage 3. BITC was present in the final two development stages reaching a level of 2 ppm in stage 5. Low levels of NITC were present in the second and third development stage both being <1 ppm. Low levels of PEITC were recorded in development stages 2, 3, 4 and 5 all of which were below 0.5 ppm. No IITC or PITC was detected at any of the sampled development times.

GC-MS analysis of *E. sativa* cv. Nemat showed that all ITCs assessed, if present, were found in very low concentrations (Fig. 4.13c). Only AITC was found at each sampled development stage, BITC was the only other ITC with measurable concentrations, but was only found in development stage 1 and 5. The highest concentration of AITC was recorded in the first development stage; afterwards all concentrations were measured below 0.5 ppm.

a)



b)



c)

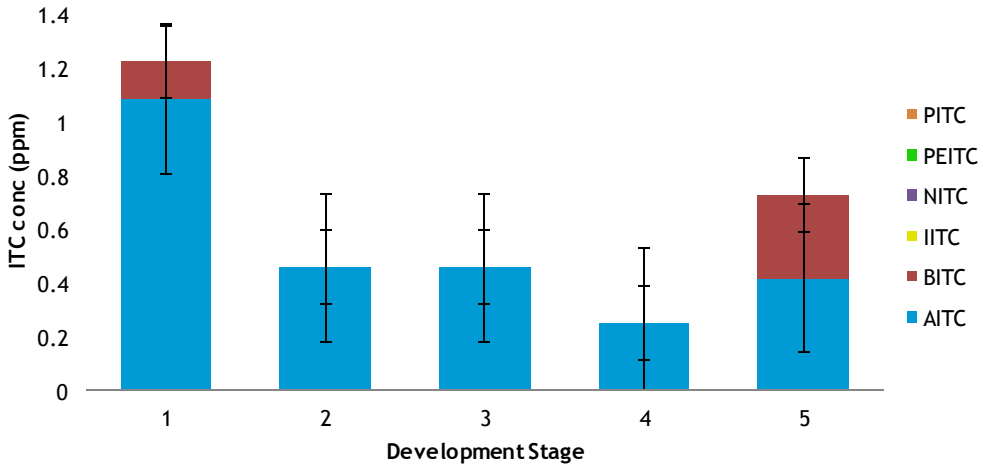


Figure 4.13a-c Concentrations of isothiocyanates measured in cultivars m) Sinapsis Alba Mirly n) Radish Vienna o) Nemat at each development stage. Vertical error bars show the standard error of the mean

4.4. Discussion

4.4.1 Analysis and conclusions of GC-MS analysis

The study presents findings from GC-MS analysis of isothiocyanates (ITCs) produced by a range of different *Brassica* spp. The results, although only analysing a small number of the potential ITCs that may be formed through glucosinolate hydrolysis, are very important in terms of assessing their use as biofumigants. Results can be used in conjunction with *in vitro* studies (Chapter 3) to determine the potential effects the release of the ITCs in the relevant concentrations may have on various soil borne pests and pathogens.

Results show that overall AITC was the most dominant ITC out of all six ITCs analysed using GC-MS. This backs up work carried out Choesin and Boerner, (1991) and Walker *et al.*, (1937) which also suggest that AITC is a commonly produced ITC through glucosinolate hydrolysis. What is notable however is that AITC is not always the dominant ITC produced by *Brassica* spp. although the results above suggest that it is commonly one of the dominant ITCs produced by mustard cultivars. They also provide results which highlight that in several other *Brassica* spp., BITC is dominant and much lower concentrations of AITC are produced if at all. Results from analysis of *Sinapsis alba* cv. Mirly indicate that BITC is the most commonly produced ITC, and in contrast to other mustard cultivars, analysis of *B. juncea* cv. BRJ CAAA hydrolysis products showed BITC to be dominant in development stage 1, and was absent of the commonly found AITC. Of course the standards used in this study are not representative of all possible ITCs which could be produced through glucosinolate hydrolysis. Yet the above data allows interpretation of the results presented in the previous chapter in terms of the possible level of suppression and control of fungal pathogens used within bioassays.

This study only examines a small number of ITCs which could be produced through glucosinolate hydrolysis, it must not be forgotten that the profile of hydrolysis products may also include other products such as thiocyanates and nitriles, (Al-Gendy and Lockwood, 2003; Holst and Williamson, 2004). As the conditions of the reaction are important to determining the product, Gardiner *et al.*, (1999) suggested that nitriles may be formed at the expense of ITCs. Yet studies have shown that nitriles are most commonly formed in acidic conditions, and therefore the loss of ITCs to the formation of nitriles would most likely occur within acidic soils. This once again suggests that environmental conditions are important in determining resultant hydrolysis products and aiding beneficial ITC development.

4.4.2 Alterations of the glucosinolate profile within *Brassica* cultivars

Work by numerous authors, has concluded that both the type and concentration of glucosinolates will vary dependent on both the plant organ and stage of development (Bennett *et al.*, 2003; McCulley *et al.*, 2008; Gardiner *et al.*, 1999; Brown *et al.*, 2003; Malik *et al.*, 2010). Research by McCulley *et al.*, (2008) also further showed that concentrations of glucosinolates may similarly vary between individual cells, as well as between different tissues. They also suggested that variation in data may be caused by the type of root and proportion of root sampled; this could indicate that in the results presented here may have levels of variability, between differences in specific leaves sampled. Doughty *et al.*, (1991) showed that within *B. napus* the overall ability of leaf tissue to synthesise and accumulate glucosinolates, particularly aliphatic glucosinolates declines with plant age. Although this is reflected in the majority of cultivars that were examined in the above study, this is not always shown to be the case within the time scale of this study. With *B. juncea* cvs BRJ CAAB, BRJ CAAD and C. mustard 20, there was a sudden surge of AITC production within the final development stage sampled, in the case of both radish cultivars studied, development stage 5 also showed a large increase in the concentration of BITC produced from leaf tissues.

There have been a number of studies which have examined GSL levels within *Brassica* cultivars, with an additional small number on the specific ITCs produced by glucosinolate hydrolysis. The results presented above do not provide findings of new ITCs but they do indicate the relative abundance of the three main ITCs detected – AITC, BITC and PEITC during glucosinolate hydrolysis of several *Brassica* spp. Previous work found much lower levels of ITCs, in particular work by Al-Gendy and Lockwood, (2003) showed that *Farsetia aegyptia* only produced low levels of benzyl and 2-phenylethyl ITC, at concentrations of 0.29 and 0.5 µmol/g respectively. Higher levels have been recorded by Cole, (1976), who recorded levels of 51 ppm of BITC produced by *Lepidium rederale* and 20 ppm of AITC produced by *Alliana pertiolata*. However these levels are still much lower than those recorded in this study, highlighting the continued need to screen *Brassicas* for their ITC levels, in order to confirm their potential biocidal activity. The difference in the levels observed may also be accountable for changes in both analysis and extraction, which demonstrates the need to develop methodology that will provide full hydrolysis and analysis techniques that allow detailed, accurate analysis. Differences in results may have been encountered through harvesting, which may have resulted in tissue disrupted and therefore release of isothiocyanates prior to extraction, as dicussed earlier slow freezing of plant material in a -20 °C may have also cause this to occur. Differences in isothiocyanate levels between both cultivars and *Brassica* spp. may also be in response to different abiotic factors such as light, temperature and moisture. Plants were grown in close proximity to one another, therefore some plants, and particularly those growing at a faster rate may have been exposed to greater levels of light and moisture. However plants towards the centre of the glasshouse benches may have received light, moisture

and may of also encourtered higher temperatures. It is also important to note, particularly when considering cultivars bred specifically for biofumigation (Caliente, BRJ and Nemat), that specifically Caliente was not bred within the UK, but Italy, therefore it unknown how UK environmental conditions may effect its glucosinolate profile, and therefore its isothiocyanate production.

The results allow conclusions to be made regarding patterns of ITC production that may occur. This may allow growers and plant breeders to make informed decisions concerning targeting combinations of pathogens with single cultivars. GC-MS results from individual cultivars indicate that high levels of BITC and AITC did not occur together within a cultivar at a single developmental sampling point. However, high levels of PEITC, relative to all PEITC levels produced, showed that high levels of BITC and PEITC; and PEITC and AITC would be produced together within one development stage. Knowledge of common patterns of ITCs produced will allow informed decisions to be made concerning future *in vitro* work to test the effects of multiple ITCs on the growth of soil borne pathogens.

The findings also allow conclusions to be made about glucosinolate accumulation that occurs throughout plant development, and therefore the ITCs which are formed during glucosinolate hydrolysis at each development stage. This study identified that there was not an overall trend of glucosinolate accumulation that occurred within each *Brassica* cultivar studied. Isothiocyanate concentrations produced varied between cultivars, at each development stage. Therefore current guidance provided on biofumigant incorporation, which states to incorporate biofumigants at the time of maximum biomass, which in most cases was observed to occur at development stage 3, may not always be the most beneficial in producing the highest concentrations of ITCs. Therefore to achieve the most effective levels of biofumigation, and pathogen suppression it is necessary to understand both the specific ITCs and the potential concentrations they may be released in, throughout the plants development. Variation throughout plant organs must also be considered, although the study presented here solely examined ITC release from leaf material, previous studies have also identified that root and stem tissue have significant amounts of GSLs that would be converted to ITCs under field conditions.

4.4.3 Using isothiocyanate analysis to develop biofumigation strategies

The above results provide evidence that if ITCs produced through glucosinolate hydrolysis are to be used within agriculture to control soil borne pests and pathogens, then there is a need for growers and breeders to move on from the simple model currently employed. Greater emphasis should be placed on understanding that to achieve the most effective results this process is not simply a matter of growing any

Brassica cultivar and ploughing it into soil. Understanding the development of parental glucosinolates, and myrosinase throughout plant development will aid effective biofumigation. Further to understanding the specific ITCs which are formed by each cultivar, it is also beneficial to understand how each crop will respond when incorporated using mechanical methods in the field, as some tissues may be easier to break down than others, resulting in greater efficiency of ITC release. When incorporated material from some cultivars may also be shown to have greater beneficial effects on the soil microclimate than others. Deciding which biofumigant crop to use may also be based on the volume of biomass produced by each crop. In order to produce large amounts of isothiocyanates during incorporation a large amount of *Brassica* tissue may be desired. Although not measured in this study it may be desirable to compare volumes of biomass produced by different *Brassica* cultivars, and additionally compare the concentrations of ITCs produced by individual plants. These are all areas which would require further research.

Previous work assessing glucosinolate content and isothiocyanate formation by *Brassica* spp. has concentrated on products formed by oil seed rape and commonly grown mustards, the results presented here highlight the need to analyse other *Brassica* material. This is evident from results of BITC analysis, which hasn't previously been reported in high concentrations, yet within this study was shown to be present in *B. juncea* cv. BRJ CAAC, *R. sativus* cv. Radish Vienna, *Sinapsis alba* cv. Mirly and *R. sativus* cv. New Radish Apoll.

4.5 Conclusions

From the above results it is clear that great variation occurs between the ITC produced both between cultivar and at different development times. Through further *in vitro* work, conclusions can be made about which ITCs may have the greatest toxic action on specific pathogens. Using chemical analysis to determine when said ITCs can be released will allow growers to make informed decisions when choosing a biofumigant crop. However, the above data also presents further problems, in determining whether it is more beneficial to choose a biofumigant crop that will produce desired ITCs in relatively high concentrations throughout the majority of its development stages. Or alternatively, rely on a single development stage that is shown to produce the highest ITC concentrations, and ensure that incorporation takes place at stage.

Chapter 5

Glasshouse experimentation investigating the effects of commonly produced isothiocyanates on *Colletotrichum coccodes* and *Rhizoctonia solani*

5.1 Introduction

Results from Chapter 3 suggested that soil borne fungal potato pathogen growth and development can be inhibited through exposure to isothiocyanates (ITCs). The results provided clear evidence that both the specific ITC structure and the ITC concentration involved in the interaction were key in determining desired results, in which fungal growth was inhibited by the presence of ITCs. Chapter 4 identified commonly hydrolysed ITCs that were formed from a range of different *Brassica* cultivars and provided data on the ITC concentration levels naturally produced and additionally combinations of ITCs formed during *Brassica* spp. glucosinolate hydrolysis. Results from both experiments were used to design a study which would investigate the use of ITCs to control two economically important fungal potato pathogens, *Colletotrichum coccodes* and *Rhizoctonia solani,* in a glasshouse experimentation, in which compost containing fungal inoculum would be treated with commonly produced ITCs – AITC, BITC and PEITC – prior to planting potato tubers. Assessment of disease symptoms on daughter tubers, would determine if the potential of the biofumigation system, in which ITCs are incorporated into soils to prevent the growth and development of soil borne potato fungal pathogens. Due to its slow growing nature silver scurf was not included in this study, due to time constraints.

5.1.1 Potato tuber blemish diseases

With an increase in consumer desire for washed aesthetically pleasing fruit and vegetables, supermarkets are increasingly putting pressure on growers to supply them with produce that meets such standards. Potatoes are affected by a number of different pathogens that can lead to their periderm becoming blemished or the tuber itself being misshapen, but yet are harmless to the consumer (Cunnington 2008). However demand for 'perfect' potato tubers does not appear to be decreasing whilst at the same time consumers are also becoming more environmentally aware and do not wish pesticides and fumigants to be overused in produce production. Additional changes in European Union Regulation of Pesticides has decreased and banned the use of specific chemical treatments which may have been previously used to control such

pathogens which cause decreased tuber quality. Such pressures result in the value of blemished tubers becoming significantly decreased, or they may be rejected from the fresh market altogether (Cullen *et al*. 2002; Cunnington 2008).

Several fungal potato pathogens can lead to skin blemishes including, *C. coccodes* and *R. solani* which cause skin blemish diseases black dot and black scurf respectively. Both these pathogens occur in high levels throughout all areas of potato production (Lees & Hilton 2003; El Balkali & Martin 2006), in 1989-90 a survey in the UK found that black dot and black scurf were present in 75 and 85 % respectively, of the potato crops surveyed (Lees & Hilton 2003). With the incidence of these diseases being particularly high it is important to seek alternative effective control measures, which will reduce levels of fungal blemishes on tubers, and ultimately increase their market value.

5.1.2 Potato black scurf

Black scurf is the term used to describe the presence of sclerotia of the fungus *R. solani* on a potato tuber surface (Hide *et al*. 1973; Ritchie *et al*. 2006) (Fig. 5.1). Black scurf on the tuber surface downgrades the quality of tubers and may also lead to the development of misshapen tubers in reduced numbers and size (Tsror *et al*. 2001; El Balkali & Martin 2006). Tubers may also suffer from russeting, cracking and malformed tubers all of which reduce the market value (El Balkali & Martin 2006).

The fungal sclerotia is a long term survival structure of the fungus (Brewer & Larkin 2005a; Ritchie *et al*. 2006) and can also be used as inoculum for infection of underground shoots, which may lead to lesions on the stems, known as stem canker (Hide *et al*. 1973) (Fig 5.1). Cultural practices, such as crop rotation and methods to reduce the amount of time potato plants are in contact with the pathogen, such as planting during warmer, drier conditions and prompt harvesting of tubers can be used to control infections by *R. solani*. Chemical fungicides, such as Imazalil and azoxystrobin may also be used in severe cases (Brewer & Larkin 2005a).

Figure 5.1 Potato tuber with a high level of black scurf, caused by *R. solani* infection. Image provided by SASA photography department.

Figure 5.2 Symptoms of stem canker on potato plant stems, caused by *R. solani* infection. Image provided by SASA photography department.

5.1.3 Potato black dot

Currently the majority of potato cultivars are susceptible to infection by *C. coccodes*. Symptoms arise as dark brownish-grey blemishes on the tuber surface, which will turn into brown coloured lesions with poorly defined margins covered in tiny black dots of sclerotia (Cullen *et al.* 2002; Lees & Hilton 2003) (Fig 5.3). Tuber symptoms are most commonly found at the heel end and are often mistaken for potato silver scurf, which may have caused a previous underestimation of the incidence of potato black dot infection (Lees & Hilton 2003). Skin blemishing and lesions can lead to an overall decrease in skin quality. Infection may also cause weight loss during storage, caused by damaged periderm which increases skin permeability (Lees & Hilton 2003).

As discussed in Chapter 1, section 1.2.4, the infection process of *C. coccodes* is initiated by conidia which form appressoria in order to penetrate the cuticle of the host plant. After successful penetration, colonization of neighbouring cells is limited. However, after potatoes reach maturity, an unknown signal causes latent infection to occur, as a result the pathogen rapidly spreads throughout producing dark sunken lesions to appear on the potato tubers (Ingram 2008). *Colletotrichum coccodes* also has the ability to infect all underground plant parts, (stolons, roots and daughter tubers), basal stems and foliage (Read & Hide 1995; Cullen *et al.* 2002; Lees *et al.* 2010). However, the roots have been reported as the most susceptible part of the potato plant with *C. coccodes* infection being shown to potentially infect up to 93 % of the length of the root system (Andrivon *et al.* 1998)

Infection by *C. coccodes* does not only affect the overall quality and yield of potatoes but also additionally provides an important infection source for future crops – through the planting of contaminated seed tubers, or through spores that travel with wind or water movement (Fig 5.4). *C. coccodes* can survive on colonized plant material for up to two years or in field soils it may survive for up to eight years. Therefore to decrease viable inoculum levels lengthy crop rotations are required. Rotation is key for

minimising the incidence level of *C. coccodes*, as to date there are no effective control measures, or specific fungicides to control potato black dot spread and development (Cullen *et al.* 2002; Nitzan *et al.* 2005).

Figure 5.3 Grey coloured lesions on the potato tuber surface caused by infection of *C. coccodes* (black dot). Image from Michigan Potato Diseases, Michigan State University, www.potatodiseases.org

Figure 5.4 *C. coccodes* conidia which are dispersed in air currents, on windblown soil particles, or in irrigation water. Image from Michingan Potato Diseases, Michigan State University, www.potatodiseases.org

This glasshouse experiment studied the effects that incorporation of ITCs has on the development of both *R. solani* and *C. coccodes*. In addition plants and daughter tubers were inspected for possible phytotoxic effects as a result of ITC exposure. This study assessed the effects three different ITCs would have on the fungal pathogens. ITCs and concentrations were chosen based on results from analysis of glucosinolate hydrolysis products of *Brassica* cultivars, observed in Chapter 4.

Overall the results from this chapter will allow understanding of benefits gained by the incorporation of either a single ITC or a combination of ITCs has a greater suppressive

action on soil borne potato pathogens. The use of controlled glasshouse experimentation, will allow further conclusions to be made concerning the potential for using *Brassica* spp. mediated biofumigation. Results will also aim to determine whether ITCs will remain in the soil after the advised fourteen day period, post *Brassica* tissue incorporation, before tuber planting.

Null hypothesis

H_0 Isothiocyanates incorporated into compost will not lower the disease incidence of black dot and black scurf on potato tubers.

H_0 Differently structured isothiocyanates will not have different efficacies of controlling the onset of fungal potato blemish diseases.

H_0 Isothiocyanates applied in combination treatments of different concentrations of isothiocyanates, will not be more effective at controlling potato blemish diseases, than sole isothiocyanates.

5.2 Materials and Methods

A glasshouse experiment was designed to assess the effect three different ITCs have on the levels of *R. solani* and *C. coccodes*. Fungal inoculum (5.2.1.1) was well mixed with compost before adding the mixture to 5 litre pots. Two different rates of fungal inoculum were used (high and low). High levels were incorporated at 20 g per 5 l pot and low at 7 g per 5 litre pot. Pots were labelled to identify if they contained *C. coccodes* or *R. solani* inoculum. ITC treatments (Table 5.1) were applied to pots according to the randomised block design. Treatments were applied by pouring 124 ml of ITC solution into the compost and throughourly mixing into the compost (5.2.1.2). After 14 days, which is the usual time biofumigant crops are left after incorporation into field soil. It was decided that in order to investigate the effect of ITCs on each disease individually, cultivars with the same level of resistance to black scurf and black dot should be used within the experiment. Cultivars selected had a moderate level of natural resistance towards the respective fungal pathogens, a rating of five according to The British Potato Variety Database. *C. coccodes* infected pots were planted with Estima whereas *R. solani* pots were planted with Saxon. Tubers were of elite classification, obtained from SASA potato plots, which are regularly tested to ensure soils are disease free. Prior to planting tubers were inspected to ensure no disease symptoms were visible, tubers selected were of a similar small size (approximately 5 – 10 cm in length) and unchitted. Following planting potato plants were allowed to proceed through their natural life cycle without any further treatments. After 16 weeks plants began to senescence and watering ceased. After 25 weeks daughter tubers were harvested. Daughter tubers were harvested and placed into storage to allow disease symptoms to develop for a period of 8 or 12 weeks respectively the tuber from *R. solani* and *C. coccodes* experiments. Symptoms were then assessed and assigned a number score based on the level of disease according to Figure 5.5 and 5.6. Tuber disease assessment results for each pot were transformed into a score out of 100; the data was plotted graphically in order to identify the overall levels of disease observed with each ITC treatment and to compare to control pots.

5.2.1 Pathogen glasshouse experimentation

5.2.1.1 Fungal Inoculum

Bulk fungal inoculum was produced on a maize flour medium. 1 l vermiculite (B&Q, UK), 500 g maize flour and 1 l H_2O was transferred into an autoclave bag mixed and autoclave sterilised. Petri dishes of individual fungal cultures (*R. solani* and *C. coccodes)* were cut into small cubes (approximately 1 cm^2). Ten cubes were added to the vermiculite mixture once cooled and mixed well. The bags of inoculum were incubated in the dark at room temperature for a total of 14 days. After seven days the inoculum was well mixed and fungal clumps were separated. After 14 days the inoculum was mixed into the compost (John Innes No. 2). Two levels of inoculum were created, high at 20 g per 5 litre pot and low at 7 g per 5 litre pot.

5.2.1.2 ITC incorporation

ITC solutions were produced at two different concentrations high (250 ppm) and low (1 ppm) prior to incorporating into the compost in 124 ml volumes, a volume which equated to 70 tonnes per hectare, as advised by Barworth Agriculture. This is the average amount of *Brassica* tissue typically incorporated into field soils during biofumigation practice. Seven different ITC treatments (Table 5.1) were applied to each of the fungal pathogen species at both levels of inoculum.

Each treatment, pathogen and pathogen level was replicated four times and pots were kept in controlled glasshouse conditions arranged in a randomised block design.

5.2.1.3 Tuber Planting

Fourteen days post ITC treatment a single seed potato tuber was planted in each pot, at a depth of 10-15 cm; pots were watered daily and were not treated with any fertiliser or pesticides throughout the experiment.

5.2.1.4 Tuber Harvesting

After 25 weeks daughter tubers were removed from pots, tranferred into paper bags and placed into cool dark storage conditions for a period of 8 weeks for *R. solani* and 12 weeks for *C. coccodes* infected tubers. After the respective storage time periods tubers were assessed for their level of disease symptoms.

Table 5.1 Shows the ITC treatment concentrations used with their respective abbreviations, which were applied to the soil microbial glasshouse experiment.

Abbreviation Used	Treatment Concentrations
Control	No ITC
AITC	250 ppm Allyl ITC
BITC	250 ppm Benzyl ITC
PEITC	250 ppm 2-phenylethyl ITC
AITC bitc peitc	250 ppm Allyl ITC, 1 ppm Benzyl ITC, 1 ppm 2-Phenylethyl ITC
BITC aitc peitc	250 ppm Benzyl ITC, 1 ppm Allyl ITC, 1 ppm 2-Phenylethyl ITC
PEITC aitc bitc	250 ppm 2-Phenylethyl, 1 ppm Allyl ITC, 1 ppm Benzyl ITC

Figure 5.5 Scale used to assess tubers grown in pots inoculated with *C. coccodes* . 1 is used to describe a minimal amount of black dot symptoms present on the tuber surface, increasing amounts of black dot, determine if tubers are scored 3, 5 or 7. With 9 describing a high level of black dot symptoms on the tuber surface (80-100 %). Scale images used by SASA for routine disease assessment.

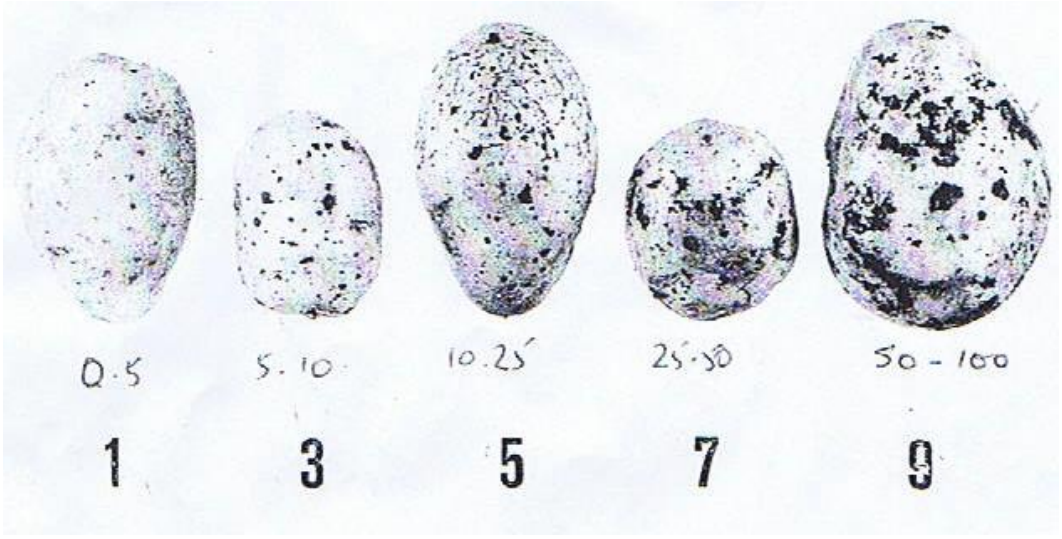


Figure 5.6 Scale used to assess tubers grown in pots inoculated with *R. solani*. The pictures give a rough guide to the symptoms that are expressed on the tuber surface for each number category. Tubers from glasshouse experiments were divided into one of the above numbered groups, depending on their level of symptoms 1 – being minimal amounts of black scurf, through to 9 – describing a high amount of black scurf on the tuber. Scale images used by SASA for routine disease assessment.

5.3 Results

The glasshouse experimentation was used to determine if the incorporation of pure ITC solutions had a significant effect on the levels of potato black dot and black scurf observed on daughter tubers from potato plants grown in compost incorporated with pathogenic fungal inoculum. ITC concentrations used in this study were based on results from GC-MS analysis of various *Brassica* cultivars which indicated that the most commonly liberated ITCs were, AITC, BITC and PEITC. Results also showed that regularly a dominant high concentration ITC was found in combination with other ITCs at a low concentration, as detailed in Chapter 4.

To establish if commonly produced ITCs had an effect on black dot and black scurf symptoms, potato plants were grown in compost incorporated with corresponding fungal inoculum. For each pathogen two levels of inoculum were used; high (20 g per pot) and low (7 g per pot) to demonstrate different levels of inoculum that may be found within field conditions. Box plots (Figs 5.3.1a-b and Figs 5.3.2a-b) show that the overall level of disease may vary even when treated with the same ITC solution. However trends can be observed with indicate that some ITC treatments appear to decrease the level of blemishes on the tuber surface caused by the individual fungal pathogens.

5.3.1 Levels of black dot on daughter potato tubers treated with isothiocyanates

Untreated tubers produced a high level of variability within the data, therefore making it difficult to determine whether clear differences existed between the results produced from treatment pots (Fig. 5.3.1a). However treatment with different ITCs appeared to produce different levels of disease severity on the daughter tubers. It is clear from the box plot that overall the single AITC treatment had the least effect on the level of black dot. Although a reduction in black dot is observed on tuber surfaces from those harvested from pots treated with BITC, PEITC, 'AITC bitc peitc' and 'PEITC aitc bitc' the variability of disease level still remains high. The greatest, and most consistent level of reduction in black dot observed on tuber surfaces was identified on daughter tubers harvested from potato plants grown in 'BITC aitc petic' treated compost, (Fig. 5.3.1c). Such results highlight the importance of low concentration ITCs in combination with high concentration ITCs, as the variability between incidence results is much lower in those treated with the combination 'BITC aitc peitc' treatment than 'BITC' alone. Results from GC-MS analysis in Chapter 4, indicated that *Brassica* plants naturally produce several ITCs, commonly one will be produced at a much higher concentration than the additional ITCs, such results determine the concentrations and solution combinations used in this study.

Daughter tubers harvested from compost incorporated with a low level of *C. coccodes* inoculum indicated a decrease in the level of black dot on tuber surfaces when treated with ITCs. Examination of the box plot initially indicates that this is greater than results from compost incorporated with high level of fungal inoculum (Fig. 5.3.2b). Again the variability of disease level differs between different treatments and is relatively high on control tubers. A decrease in the incidence of black dot on tuber surfaces when compared to control tubers is observed on daughter tubers harvested from pots treated with 'AITC bitc peitc', BITC, 'BITC aitc peitc' PEITC and 'PEITC aitc bitc'. In this instance although a decrease in black dot levels was observed in some tubers harvested from AITC treated compost it was not as great as those harvested from other ITC treatment pots. The greatest reductions in black dot were observed on tubers harvested from pots incorporated with PEITC solution, however a large decrease was also observed in daughter tubers from plants grown in 'BITC aitc peitc' treated compost and in this instance the variability of black dot levels was much smaller. Therefore it may be suggested that this treatment may lead to a reduction in black dot levels and also much more consistent control.

5.3.1.1 Statistical analysis

Black dot tuber symptoms score were analysed using ANOVA in Genstat v14, (VSN International), to determine statistical significance between disease symptoms on tubers from treatment pots and untreated control pots. ANOVA, standard error of differences of means analysis revealed that no statistically significant differences were found between the levels of black dot on control tubers and those from pots treated with ITC solutions with both inoculum levels. Therefore although observation and graphical analysis suggests that a decrease in the levels of black dot occurs due to ITC treatment, no treatment combination used within this study produced a statistically significant decrease in black dot levels (Appendix 6).

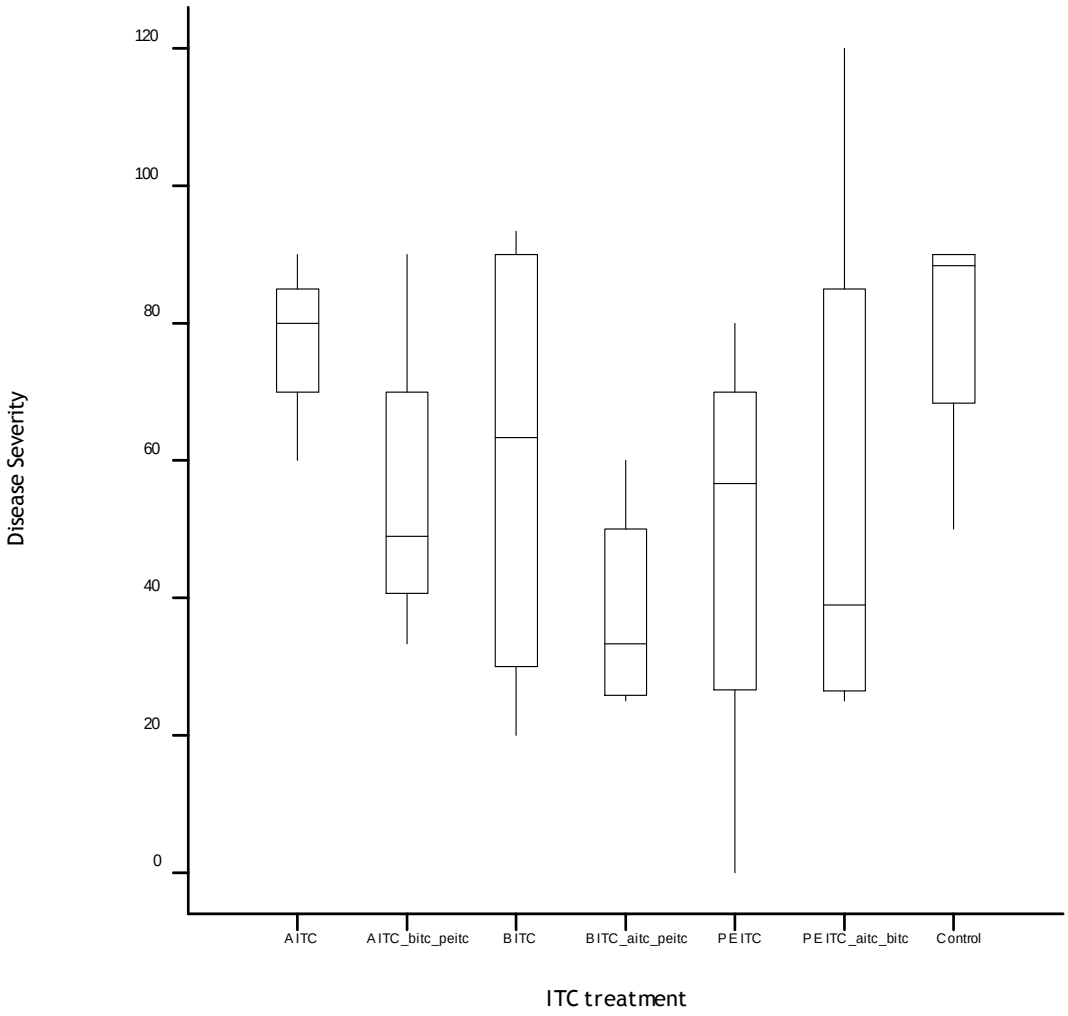


Figure 5.4 Schematic box plot of black dot symptoms on tubers from each ITC treatment of *C. coccodes* after 12 weeks of storage in paper bags at approximately 4-10°C, harvested from pots containing high level inoculum. 8 pot replicates were carried out for each treatment.

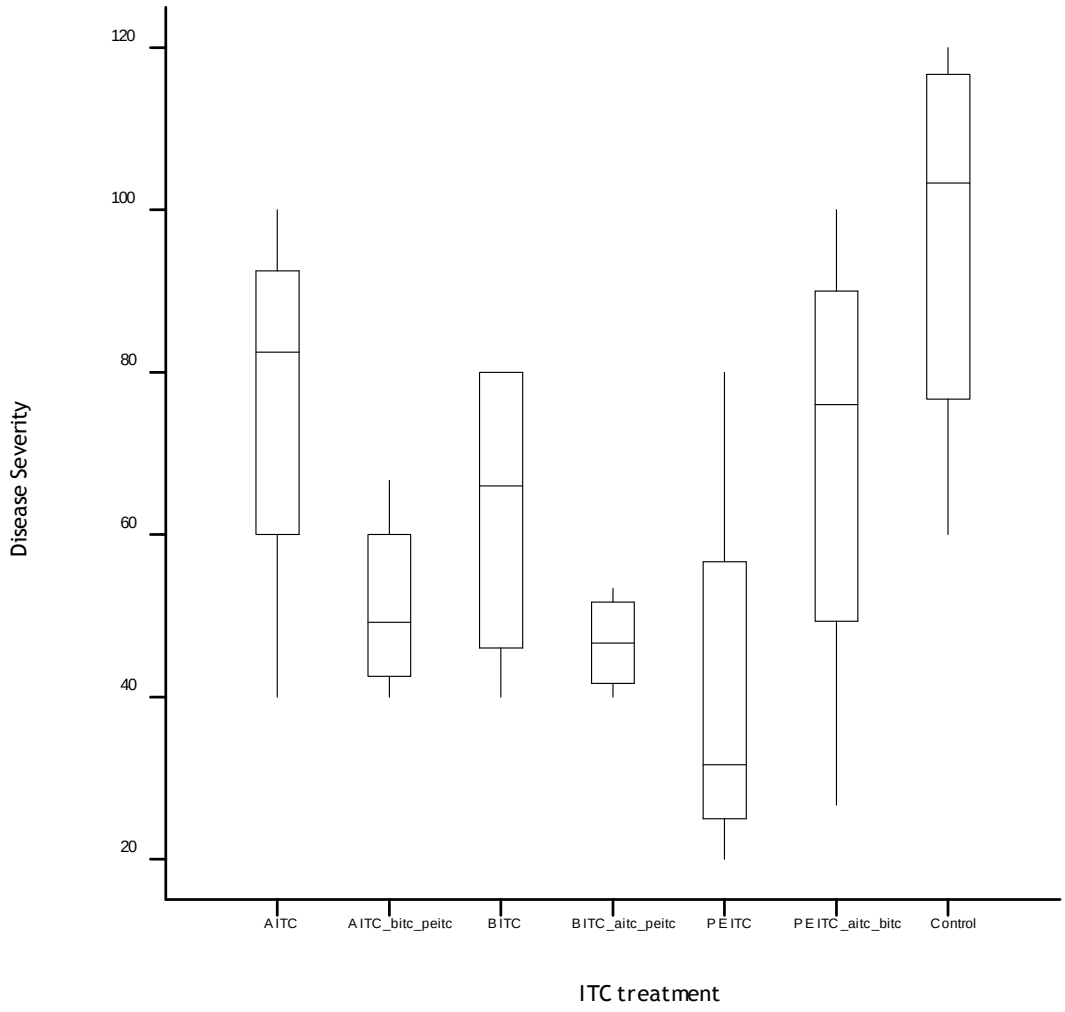


Figure 5.5 Schematic box plot of black dot symptoms on tubers from each ITC treatment after 12 weeks of storage, from low level inoculum pots. 8 replicates were carried out for each treatment.

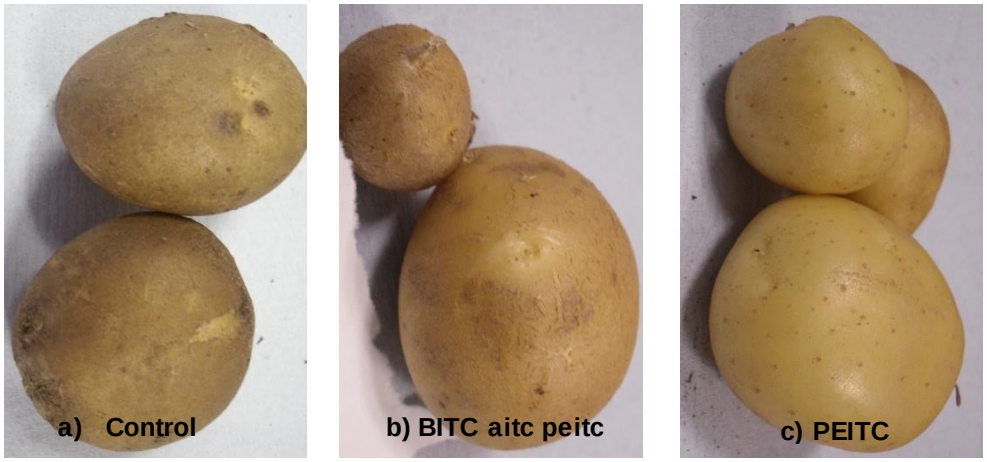


Figure 5.6 The range of black dot symptoms observed on tubers from *C. coccodes* incorporated pots (high inoculum level), that were grown in composts treated with different ITC treatments a) Control – No ITCs b) 250 ppm, BITC 1 ppm, AITC 1 ppm PEITC c) 250 ppm PEITC, 1 ppm AITC BITC 1 ppm

5.3.2 Levels of black scurf on daughter potato tubers treated with isothiocyanates

Overall there was a high level in the variability of black scurf present on tubers harvested from untreated pots. However results show a general trend towards a decrease in disease incidence in those harvested from plants grown in 'AITC bitc peitc', 'BITC', 'BITC aitc peitc' and 'PEITC aitc bitc' treated compost (Fig. 5.7). The greatest decrease in black scurf on tuber surfaces compared to control tubers was observed on those harvested from plants grown in compost treated with 'AITC bitc peitc' and 'BITC'. In both cases a decrease in the variability of disease incidence between tubers and replicate pots was also observed, suggesting that both treatments may be useful to decrease levels of *R. solani*. It is important to note the differences that are present between the similar treatments, for instance AITC alone does not produce a decrease in black scurf on the tuber surface when compared to control. However with the addition of BITC and PEITC at low concentrations (1 ppm), a large decrease in disease incidence was observed. The opposite is true of the BITC treatments, which showed a much lower level of disease when the treatment is applied on its own, without additional ITCs. Such results highlight that the interaction occurring between different ITC structures may be important to achieving effective biofumigation.

The results observed from the experiment conducted using low levels of *R. solani*, are considerably different from those presented from the experiment using higher levels of fungal inoculum (Fig. 5.8 and 5.9). Although ITC treatments indicate that they have reduced the overall level of variability of black scurf incidence that occurs both between individual tubers and pots, when compared to daughter tubers harvested from control (untreated) pots, there are no decreases or increases in levels of black scurf. It could be suggested that in this incidence the pathogen level was too low to be affected by treatment.

5.3.2.1 Statistical Analysis

Scores produced from the analysis of the black scurf tuber symptoms were analysed using ANOVA in Genstat v14, to determine statistical significance between disease symptoms on tubers from treatment pots and untreated control pots. ANOVA, standard error of differences of means analysis did not reveal any statistically significant differences were found between the levels of black scurf on control tubers and those from pots treated with ITC solutions with both inoculum levels. Therefore although observation and graphical analysis suggests that a decrease in the levels of black scurf occurs due to ITC treatment, no treatment combination used within this study produced a statistically significant decrease in black scurf levels (Appendix 7).

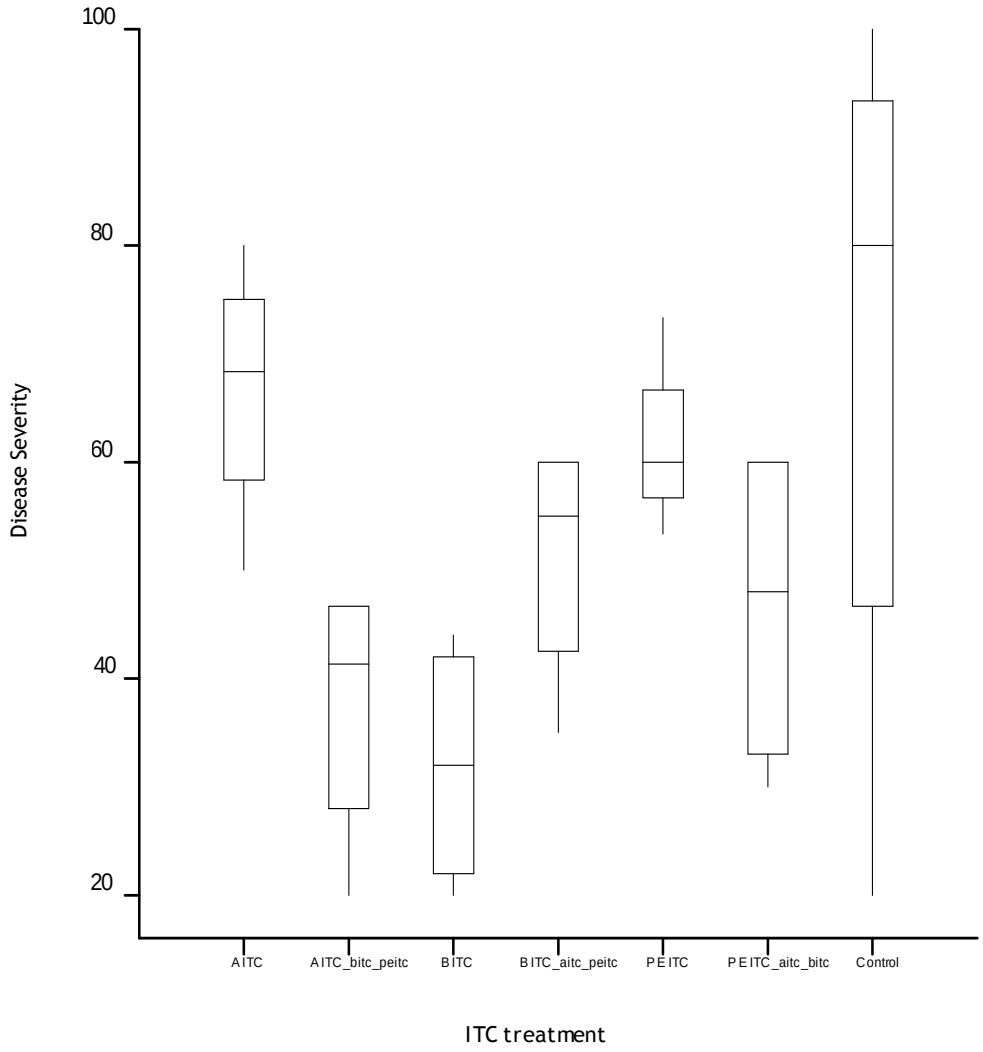


Figure 5.7 Schematic box plot of black scurf symptoms on tubers from each ITC treatment, from high level inoculum pots, after 8 weeks of storage in paper bags at between 4-10 °C. 8 pot replicates were carried out for each treatment.

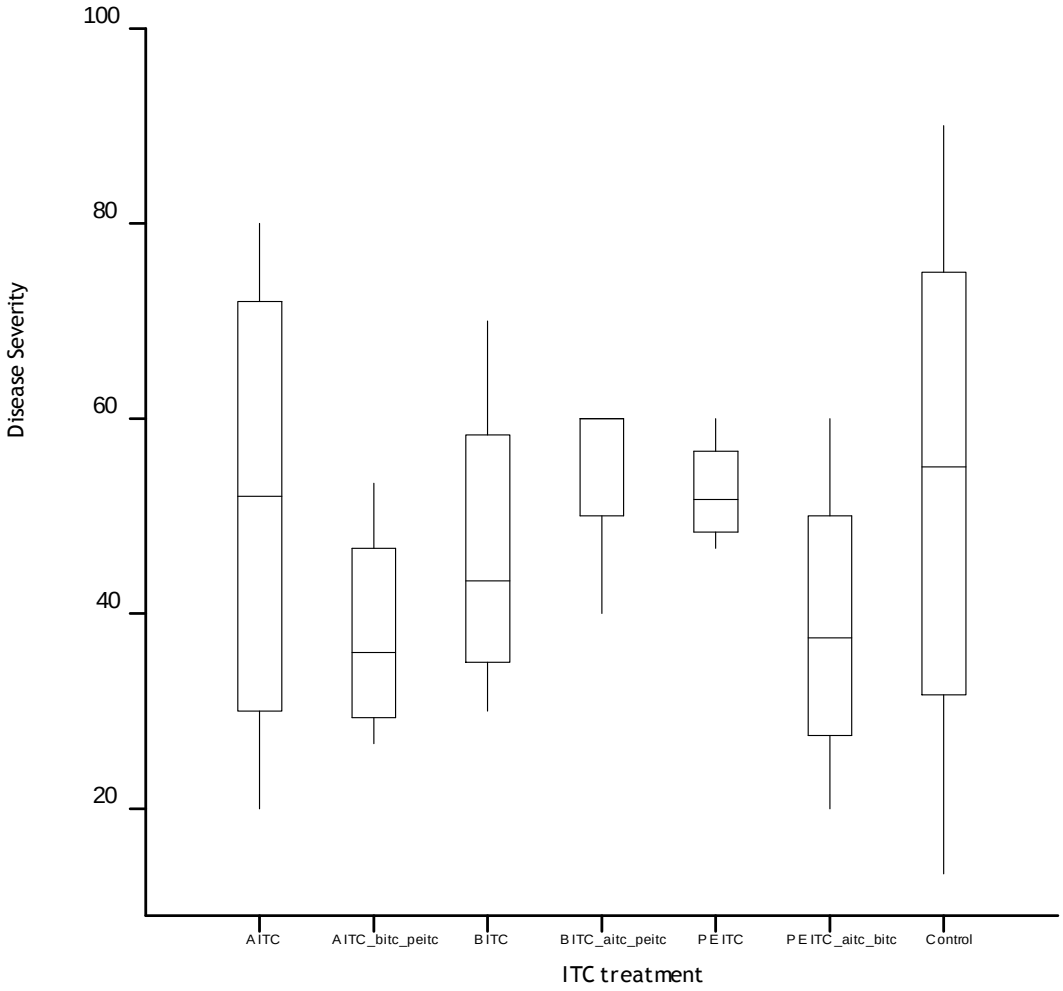


Figure 5.8 Schematic box plot of black scurf symptoms on tubers from each ITC treatment, from high level inoculum pots, after 8 weeks of storage in paper bags, at 4-10 °C. 8 pot replicates were carried out for each treatment.

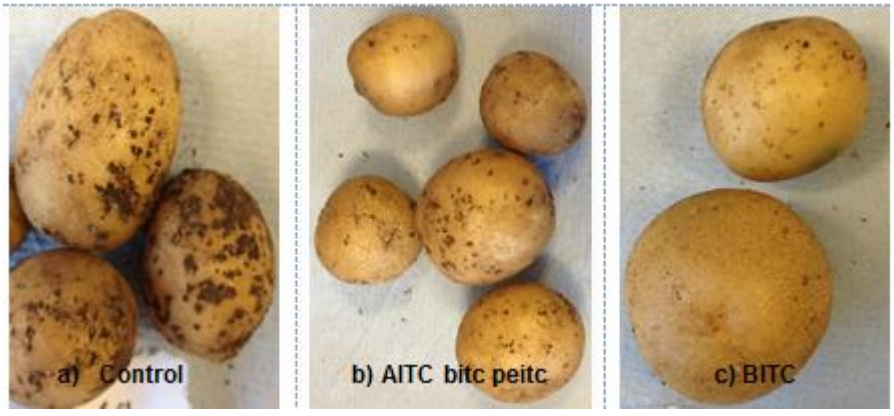


Figure 5.9 – Displays the range of black dot observed on tubers from *R. solani* (low inoculum level) incorporated pots, that were grown in composts treated with different ITC treatments, a) Control – No ITCs b) 250 ppm, AITC 1 ppm BITC 1 ppm, PEITC c) 250 ppm, BITC.

Overall the results produced from this glasshouse experimentation showed that there was a general trend towards a decrease in the levels of black dot and black scurf on the tuber surface when fungal inoculated compost was treated with ITC solution in comparison to control tubers. However in the case of both pathogens used in this study no ITC treatment completely prevented tuber disease symptoms. Therefore to assess the ability of ITCs to control both the onset of black dot and black scurf the overall trends have been examined.

Different results were also observed between the different levels of fungal inoculum (high and low). Although treatment of *C. coccodes* remained unaffected by the amount of inoculum present and decreases in the disease incidence were still observed. *Rhizoctonia solani* results showed that low levels of inoculum were unaffected by any of the ITC treatments used within this study.

5.4 Discussion

In the instance of both *C. coccodes* and *R. solani* the graphical data shows that the incorporation of every ITC treatment generally brings about a decrease in the overall level of disease present on the tuber surface, with the exception of low *R. solani* levels. However the decrease from the level observed on control tubers is largely dependent on the specific ITC treatment. In each treatment a range of different levels of disease on the tuber surfaces were observed. As a result the overall trends and average disease levels were examined to draw conclusions concerning the effects ITCs have on blemishes caused by the fungal pathogens.

5.4.1 Isothiocyanates produced from *Brassica* spp.

This study was based on results obtained from GC-MS assays from glasshouse grown *Brassica* plants, (Chapter 4) which indicated patterns in the production of ITCs from glucosinolate hydrolysis. Data suggested that in most cultivars a dominant ITC was produced with other ITCs produced at much lower concentrations. However the dominant ITC could potentially change throughout plant development. As the results above indicate the three most commonly formed ITCs, from GC-MS data (Chapter 4), do not provide total control of either black scurf or black dot when used either alone or in combination with lower concentration ITCs, suggesting effective biofumigation of soil borne pathogens should involve growing mixed cultivar crops. Growing a variety of different *Brassica* cultivars which when incorporated into the soil as green manures may provide a mix of ITCs at high concentrations in turn providing a greater level of control towards several different soil borne pathogens (Gimsing & Kirkegaard 2006).

Results highlight that the interaction that occurs between ITCs and pathogens is one of great specificity, as different results are observed in response to the incorporation of different ITCs. This study also highlights the influence complementary ITCs that are released at lower concentrations may have on the overall suppression of pathogens. This suggests that ITCs which are produced during glucosinolate hydrolysis in low levels should not be overlooked for their biofumigant properties, particularly if they are found in combination with dominant ITCs produced at relatively high concentrations. This may provide explanation as to differences which are observed between *in vitro* assays, which investigate the suppressive effects on pathogens using a single ITC and results observed in glasshouse and field trials wherein *Brassica* plant tissue is incorporated, which may contain high levels of the ITC used in *in vitro* study, but also produce lower concentrations of complementary ITCs. The above results highlight a need to investigate the interactions that occur between different combinations of ITCs on different soil borne pathogens. It can be suggested that a combination of ITCs provide a greater suppressive effect on the development of both *C. coccodes* and *R. solani*, than a single ITC. As *Brassica* plants have been shown to produce several ITCs during

glucosinolate hydrolysis this highlights their potential for use in a biofumigation system to control different soil borne pathogens (Leoni *et al*. 1997; Bellostas *et al*. 2005; Gardiner *et al*. 1999)

5.4.2 Allyl Isothiocyanate

Results from disease glasshouse experimentation of black dot and black scurf indicate that AITC does not produce a notable decrease in disease symptoms compared to levels observed on control tubers. This also agrees with findings in Chapter 3 which showed AITC was not shown to have a significant effect ($p > 0.05$) on fungal pathogens in *in vitro* assays. Yet AITC has been the most extensively studied ITC in regards to determining its toxic properties towards a range of different microorganisms (Dhingra *et al*. 2004). The level of research carried out on this particular ITC is due to it being commonly produced during glucosinolate hydrolysis by several *Brassica* cultivars; it was also one of the first hydrolysis products that was established to possess antimicrobial properties (Walker *et al*. 1937). The results from this study suggest that attention should be turned to investigate the toxic properties, particularly anti-fungal properties, of other ITCs as they may reduce greater levels of fungal pathogens than AITC. Identification of combinations of specific ITCs may assist in breeding programmes to produce suitable *Brassica* biofumigant crops that can be used to produce ITCs that will reduce levels of soil borne pathogens.

5.4.3 Isothiocyanate movement through soil

The movement of ITCs within soil and potting mixes may also have a large influence on the overall toxic effects and results that are observed in studies of this type. There is still a large amount that is unknown about ITCs behaviour within soil, but preliminary studies have provided results which will aid understanding of this process. Therefore allowing the most effective biofumigation incorporation methods and delivery of isothiocyanates into the soil to be achieved, thus aiding the greatest levels of pathogen suppression. Soil structure and environmental conditions, such as pH and moisture content may provide more favourable conditions for ITCs (Gan *et al*. 1999; Mattner *et al*. 2008; Mercier & Jimenez 2009). The volatile properties of the compounds have often prompted suggestions that they will quickly escape from the soil before they will have an antimicrobial effect on pathogens within in the soil. However in contradiction to this, studies have shown sealing the soil surface using methods such as 'tarping', is not required to achieve sufficient levels of soil borne pathogen reduction (Mercier & Jimenez 2009). Repeating this study using a soil sealing method may determine if this increases pathogen suppression activity of ITCs. Additionally it has been suggested that soil structure has an influence on the rate that volatile compounds, including ITCs, deplete from the soil (Price *et al*. 2005). Mercier & Jimenez (2009) showed that

biofumigant volatile compounds were present in natural field soil for longer periods of time than when they were incorporated into a commercially bought potting mix. The potting mix used in their study was shown to interfere with the movement of volatile compounds. Studies have also shown other soil fumigants including 1,3-dichloropropene, bind to organic matter within soils. Hence the overall composition, structure and environmental conditions can all influence the activity and movement of volatile compounds within soil, in the case of ITCs thus affecting their toxicity towards soil borne pathogen. In relation to this study, this suggests that if an identical study was carried out using different field soils, different results may be observed. Although this study provides an insight into the use of ITC release to control fungal inoculum and its development on daughter tubers, further research would be required to determine how these results would alter in different conditions. With this understanding it may be possible to optimise the use of ITCs as effective biofumigants both in composts and soil.

What may be essential to the interactions that occur between inoculum within soil, or in this case compost and ITCs is the specific structures and survival forms of soil borne pathogens. For instance fungi possess sclerotia to protect them in unfavourable conditions, such as coming into contact with toxic fumigants. This has shown to be particularly true for *R. solani*, which is capable of exerting several survival forms. Yulianti *et al.* (2006) identified that particularly the thick-pigmented cell wall of *R. solani* hyphae may protect it from volatiles released from *Brassica* plants. It is believed that this response is influenced by melanin levels within the hyphal cells, as some strains of *R. solani* that lack melanin, have been shown to has less ability to survive within soil, whereas strains containing melanin survived well within soil conditions. Therefore observed results may differ when using a biofumigation system to control *R. solani* dependent on the particular *R. solani* strain present, (Yulianti *et al.* 2006). In the case of *C. coccodes* survival structures centre on the use of spores, which are used to disperse the fungus when conditions are not favourable, hence why *C. coccodes* has proven to be a difficult pathogen to control through traditionally used synthetic chemicals (Cullen *et al.* 2002; Nitzan *et al.* 2006). Therefore an alternative method of control that shows promise for decreasing the incidence of *C. coccodes* on potato tubers would be welcomed by growers.

5.4.4 Results from glasshouse experimentation investigating the effects of commonly produced ITCs on *C. coccodes* and development of black dot symptoms on daughter tubers

Three ITC solutions (AITC, BITC and PEITC) were incorporated at a high concentration individually and in combination with the remaining two ITCs at a low combination, in order to assess if ITCs worked better individually or in combination. ITCs used in this study were selected following GC-MS the analysis reported in Chapter 4 revealed that they were commonly produced by several difference *Brassica* cultivars. As previous studies (Bones & Rossiter 1996; Bellostas *et al*. 2007) and results in Chapter 4, indicate that *Brassica* species release multiple ITCs during glucosinolate hydrolysis. Examination of *C. coccodes* results show that on average the levels of potato black dot are decreased by a greater amount when compared to control results when ITCs are applied as a high level dominant ITC in combination with the remaining two ITCs at a low concentration (1 ppm). Therefore the data suggests that either low concentration ITCs do have an effect on the development of *C. coccodes* alternatively the majority of the reduced tuber disease symptoms may be attributed to the high concentration ITC, which in terms of *Brassica* plant application would be the dominant ITC produced. Yet results from this study demonstrate that this may not be strictly true as shown in the case of AITC (Fig 5.3.1a). As a sole ITC, the decrease in levels of black dot was very small in comparison to results from control tubers - whereas when applied in addition with low concentrations of BITC and PEITC, a greater decrease in levels of black dot are observed. Such results, in which greater disease suppression occurs with the addition of low concentration ITCs with the high concentration dominant ITC, demonstrate the ITCs at low levels may aid the suppression of black dot, when applied in combination with AITC at a high concentration, in comparison to AITC applied individually. This result indicates the importance that ITCs produced in lower concentrations may have on the overall antimicrobial activity that *Brassica* spp. have towards different soil borne pests and pathogens. Such results emphasise a need to carry out large scale hydrolysis product profiling, to determine the full complement of products that are released through hydrolysis, and test the different released combinations for their antimicrobial levels against different pathogens. The above results indicate that ITCs of lower concentrations may aid disease suppression, when released in turn with a dominant high concentration ITC, suggesting that the release of ITCs in combination will produce the greatest levels of pathogen suppression.

Generally comparison between the results from high and low *C. coccodes* levels exhibited the same trend in reductions in black dot levels on tuber surfaces. Results showed that variability of the levels of black dot was reduced by a greater amount within low fungal inoculum pots. However in both cases it appears that the greatest decrease in black dot is caused by the incorporation of 'BITC aitc peitc' or PEITC. Although some differences are observed between different inoculum levels are observed, this is to be expected, as different pathogens will respond to the presence of

antifungal treatments in different ways. It therefore may be advised to assess the severity and concentration level of *C. coccodes* within the soil before deciding on the best ITC treatment to control the fungal pathogen.

Although graphical data comparing the average levels of black dot on the tuber surface, suggests that ITC treatments are producing a decrease in symptoms. ANOVA analysis revealed that there was no significant difference between the results observed on the treated and non-treated tubers. Therefore it cannot be reported that ITC solutions incorporated into compost prior to tuber planting produce significant differences in the levels of potato black dot observed on daughter tubers. Although the results do not produce any statistically significant findings they do suggest that interactions between levels and *C. coccodes* and different ITCs are occurring. Further study is required to determine the optimum concentrations and combinations of ITCs to achieve reliable and significant potato black dot control.

5.4.2 Results from glasshouse experimentation investigating the effects of commonly produced ITCs on *R. solani* and development of black dot symptoms on daughter tubers

Results from pots containing *R. solani* inoculum from average results indicate that ITC treatment may lower the overall levels of black scurf observed on the tuber surface. However the interaction occurring appears to be affected by the specific ITC, and additional ITCs that may be present at a lower level. Graphically it is suggested that the lowest level of black scurf is observed on tubers that were produced from plants grown in compost treated with 'AITC bitc peitc', BITC and 'PEITC aitc bitc', yet the only statistically significant result was observed on tubers from 'PEITC aitc bitc' treated compost ($p < 0.05$). Suggesting that overall a treatment composing of a high concentration of PEITC with complimentary lower levels of AITC and BITC would provide a degree of control over the growth and development of *R. solani*. Without further study it cannot be determined if increasing the concentration of both complimentary ITCs will decrease black scurf levels further. However it can be hypothesised that this would aid the antimicrobial properties of the ITC treatment.

Further study would be required to determine the optimum concentration and combination of ITCs to achieve the greatest level of control of *R. solani* prior to planting potatoes. However results from this study indicate the importance that the uses of ITCs in combination have on pathogen growth and development. Here it is demonstrated that the incorporation of 250 ppm PEITC solution prior to planting does not produce a significant decrease ($p > 0.05$) in levels of black scurf on daughter tubers. However, incorporating the same concentration of PEITC with the addition of 1 ppm AITC and 1 ppm BITC a significant decrease in black scurf levels was observed. This highlights that a small difference in biofumigant treatments can have large effects on

the results observed. Such results depict the specificity of the pathogen – ITC interaction, and suggest that controlling pathogens through the addition of ITCs may be one of great complexity.

5.4.6 Soil inoculum levels

Different results may also be observed by altering the amount of fungal inoculum present within the compost, as the ITC may have a threshold in its antimicrobial ability. Hence if inoculum levels were lower a larger decrease in disease incidence or even total control of black dot or black scurf symptoms may be observed. Further study would be required to determine the ITC to fungal ratio that would achieve reliable, high levels of control of both pathogens. Different combinations of ITCs may also aid pathogen suppression. This study highlights the complexity of the biofumigation interaction, dependent on pathogen, ITC and environmental and soil conditions. Consequently in the first instance and until understanding and knowledge of such processes is improved on, biofumigation should be perhaps be used as an environmentally conscious alternative control method, for soil borne pathogens in conjunction with lower level use of traditional control methods.

5.4.7 Improvements to study

This study did not produce the expected levels of disease suppression, both within *R. solani* and *C. coccodes* experimentation. It can be suggested that imporvements to the experiment methodology may lead to greater levels of suppression and less variability within the observed results. Firstly ITC contact time with the soil borne pathogens may be increased by using improved sealing methods, once the ITCs have been incorporated into the compost. Compacting top layers of compost or either placing tarpolen sheeting on top of the compost may prevent the release of the volatile ITCs. Addtionally although tubers were selected to be of relatively uniform size, differences were present, therefore disease levels may vary between tubers of different sizes. Repeating the experimentation using further replicates should also help to reduce variability. Incorporation of ITCs may also have been by throughourly mixing ITCs with compost prior to placing in pots. However although such methods may lead to even distribution of ITCs, they may also lead to further loss of ITCs. Future study should also be compared using the same variety for each pathogen in order for results to be compared between the pathogens.

More recently work has been carried out to investigate the phenomena of so called 'partial biofumigation'. This term describes growing a *Brassica* crop and then harvesting the crop without incorporating the residues. Although some ITCs may be released into the rhizopshere during the biofumigant crop growth, it is thought that

they will be produced in too small concentrations to directly affect soilborne pathogens. However it is thought that ITCs and other compounds released from the *Brassica* plant roots may influence and change the structure of microbial communities within the rhizosphere. In turn it is thought that such changes may directly affect pathogen populations through changes in competitiveness of pathogens or increase the number of pathogen antagonists. Therefore it can be considered that if such experimentation was repeated using the addition of *Brassica* tissue, rather than pure ITCs different suppressive may have been observed.

5.5 Conclusions

The above study provides initial results of experiments using isothiocyanates to control the development of potato black dot and black scurf on daughter tubers. Such glasshouse experiments are useful in beginning to transfer *in vitro* investigations into practice and determine their success rates, in a more natural environment. To date similar studies have not been carried out which investigate the effect of pure ITC incorporation has *C. coccodes* and *R. solani* inoculum within compost. From this study it is shown that neither *C. coccodes* nor *R. solani* inoculum were completely controlled by the incorporation of ITCs. Although this novel study investigates the incorporation of pure ITC solutions, it does not investigate what effect the use of green manures releasing ITCs would have on the pathogen levels. It has been suggested that incorporating green manures and thus incorporating a large proportion of organic matter into soil will increase the microbial activity of beneficial soil microorganisms, which may also help to reduce pathogen levels (Bending & Lincoln 2000). What is clear from the results from this study is that ITCs appear to have no negative impact on the daughter tubers. Therefore a biofumigation system can be used in confidence knowing that the release of ITCs will not adversely affect the quality of subsequent potato crop, although no negative impacts caused by ITCs on potato tubers have been reported, it is often a result that is ignored by other authors, however regularly questioned by growers. This original study into the effects of pure ITCs at concentrations naturally hydrolysed by *Brassica* plants (Chapter 4) demonstrates that ITCs as an individual compound will have no negative effect on daughter tubers or plant growth. Although it is recognised that further work is needed to fully establish biofumigation as an effective control measure, results from this study suggest that potentially if the right combination of ITCs released through mixed crops and/or *Brassica* breeding programmes decreased soil borne pathogen levels can also be achieved.

Chapter 6

The effect of isothiocyanates on soil microbial composition

6.1 Introduction

6.1.1 Importance of soil health

Soil can be singled out to be the most important component of the earth's biosphere, not only is it vital for food production but also functions in maintaining local, regional and global environmental quality and as such is the foundation for all agricultural and natural plant communities. The overall health and quality of soil are known to determine agricultural sustainability and environmental quality which in turn can affect plant, animal and human health. In current times it has been recognised that soil degradation is the most destructive force in decreasing the world's soil based resources, (Parr *et al.* 1992). Overall soil quality is determined by complex interactions of physical, chemical and biological processes which ultimately lead to sustained production of food crops. However there are many factors that can reduce soil quality, such as water and wind erosion, nutrient losses caused from runoff and leaching, the depletion of organic matter, additionally the overuse of chemical fertilisers and pesticides can also lead to the accumulation of toxic substances within the soil. Previously the characterisation of soil has centred on soil chemical and physical properties due to the availability of simple standardised methods to measure them. Whereas soil biological properties have been somewhat neglected, yet restoring and maintaining soil quality is highly reliant on organic matter content and a wide range of beneficial macro- and micro- organisms. Soil microorganisms and invertebrates are responsible for the decomposition of organic matter and nutrient cycling and could therefore be important indicators of soil quality.

6.1.2 Microbial composition of soil

It has been well demonstrated that soil bacteria and fungi play vital roles in several biogeochemical cycles and are necessary for the cycling of organic compounds, including carbon, sulphur, phosphorus and nitrogen (Fig. 6.1). However in recent years they have also been shown to interact and influence responses of above ground ecosystems, through contribution to plant nutrition, plant health, soil structure and soil fertility (Kirk *et al.* 2004).

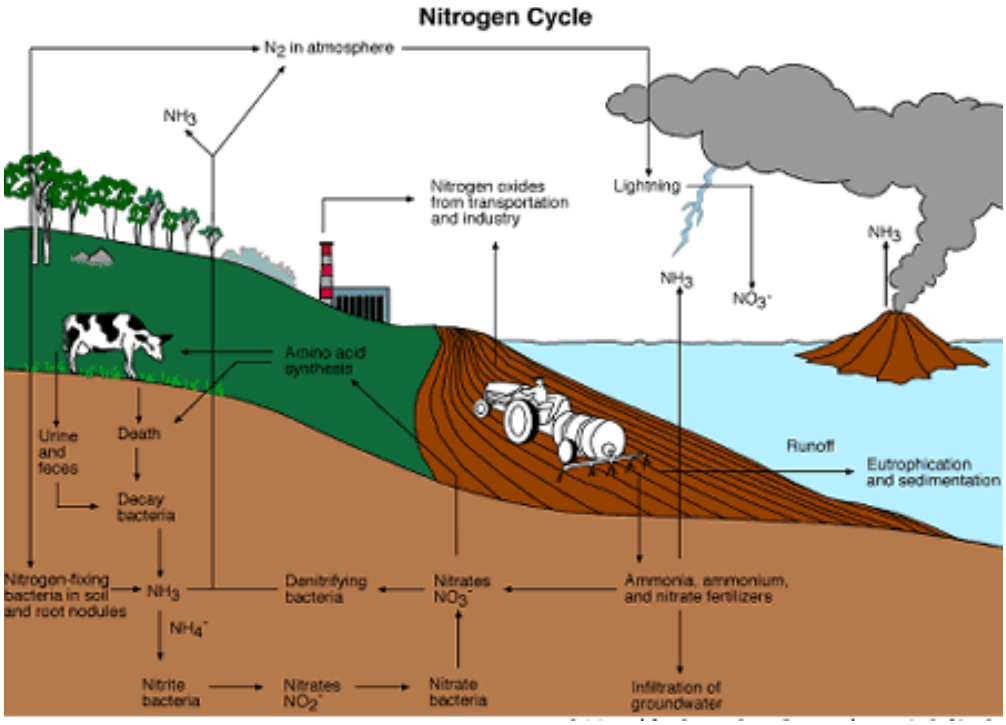


Figure 6.1 The roles of soil microorganisms within the Nitrogen Cycle. Image from Environmental Literacy Council; www.enviroliteracy.org

Bacteria are known to make up a large portion of the overall biodiversity of soil (Griffiths *et al.* 2011), it has been estimated that based on DNA-DNA re-association, there are 4000 bacterial species in 1 g of soil. It is understood that the numbers and functions of these microbial organisms will respond to environmental stresses and changes in soil conditions, which can be attributed to agricultural practices such as tillage and cropping cycles, (Parr *et al.* 1992; Filip 2002; Cohen *et al.* 2005). Gaining an understanding of how different agricultural management practices affect the microbial composition of soil and therefore biological processes, can transpose into effective use of practices that will maintain and even restore the maximum level of soil health. This will ultimately lead to higher levels of crop productivity. Obviously the complexity and diversity of bacteria within soil presents a great challenge in trying to create a profile of all species present within a soil ecosystem. However with advances in molecular ecology methods, researchers can now make assessments of overall changes in diversity or monitoring of key, commonly found species and indicator species, that alert to upsets in the overall ecosystem balance, or indicate soil of maximum health and quality. Several bacterial groups have been recognised to be beneficial microbes, including some species of *Bacillus* spp. which produce antibiotics that suppress the growth of soil borne pathogens and compete with other pathogens for infection sites. Knowledge of the ability to restrict pathogen growth, and particularly decrease incidence of stem canker and black scurf has led to alternative control methods in which *Bacillus subtilis* and *Trichoderma virens* are applied to the soil (Cohen *et al.* 2005). However little work has been carried out to establish what effect this will ultimately have on the natural soil microbial composition.

To date the majority of work on the microbial composition of soil has focussed on the rhizosphere – the soil environment in close contact with root systems. The rhizosphere has been identified as a hot spot of microbial activity, due to the presence of root exudates and rhizodeposits which aid microbial growth. Microorganisms from the rhizosphere have shown a range of different effects on the plants they interact with, but an increasing number are being classed as 'plant growth promoting bacteria' or 'plant growth promoting rhizobacteria. These are bacteria which have been shown to have positive effects on plant growth, increase yield, reduce biotic and abiotic induced plant stress and reduce pathogen infection (Compant *et al*. 2010). They are a key group of bacteria which demonstrate the importance for maintaining microbial balance within soil. Just as bacteria are known to influence plants and the above ground ecosystem, the diversity of soil microorganisms will be indirectly linked to vegetation characteristics, including root excudates, carbon input and litter type. A larger number of different food resources produced by plants will result in a greater diversity within the soil community (Korthals *et al*. 2001).

6.1.3 Agricultural practices affecting bacterial diversity

It has been well documented that agricultural practices, such as tillage and fertilisation will affect soil conditions. In turn affecting ecological processes which are known to alter microbial communities within the soil (Cohen *et al*. 2005). Environmental conditions may also have an indirect effect on the microbial structure, by altering vegetation, in turn influencing soil microclimates, leading to changes in the soil community. Practices known to alter the pH of soil, including fertilisation, may lead to large changes within soil bacteria, additionally it has been shown that the highest bacterial diversity can be found in soils of a higher pH (Griffiths *et al*. 2011). Such changes in diversity are probably connected to the feedback system which occurs between the below and above ground communities. Soil pH will alter carbon and nitrogen availability, which is thought to occur through the changes that take place in the above ground communities. Soil management practices including tillage, cropping patterns and fertiliser use are also known to alter soil by influencing atmospheric quality by changing the soils capacity to produce and use atmospheric gases, including carbon dioxide, nitrous oxide and methane, which will ultimately be controlled by and alter the composition of soil bacteria (Doran and Zeiss, 2000).

6.1.4 The effect of biofumigation on soil microbial community

Previous studies have clearly demonstrated the effect that agricultural management practices can have on soil microbial communities, and in turn the overall health and quality of the soil influencing crop yield and disease incidence. Therefore the importance of assessing the effects new agricultural practices have on the soil microbial composition is being increasingly recognised. To date limited work has been conducted on investigating the effects biofumigation has on soil bacteria with work focussing on assessing the effects the process has on specific bacterial groups. Bending & Lincoln (2000) and Brown & Morra (1996) both demonstrated that ITCs inhibited the nitrification processes by directly changing the size of nitrifying bacteria communities and decreasing their nitrifying activities. They also showed that long term use of ITCs may encourage the mineralisation of soil nitrogen. It was suggested that this might be due to the fumigant effect of the ITCs killing a portion of the microbial biomass, which are subsequently degraded by surviving organisms, causing the mineralisation of nitrogen. Omirou *et al.* (2011) assessed the effects propyl ITC had on soil bacteria, they found this particular ITC to be highly toxic to soil microorganisms in *in vitro* conditions, however the biocidal activity appeared to be deactivated when applied to soil. This was attributed to high levels of adsorption and their low availability to the liquid and air phase of the soil. To date study on the effects biofumigation and consequently the effects ITCs have on soil bacteria, has shown that overall the composition may be altered, in turn affecting microbial processes. However further work is required to investigate the effects a wider range of ITCs have on soil bacteria and the overall microbial diversity. Understanding how biofumigation affects microbial communities will ultimately provide a greater understanding of the mechanics of disease suppression that occur, both through ITC and beneficial pathogen suppressive bacteria interactions.

6.1.5 Terminal Restriction Fragment Length Polymorphism soil bacteria analysis

To assess the overall changes in the composition of the bacterial community, Terminal Restriction Fragment Length Polymorphsim (T-RFLP) methodology was used. T-RFLP is a community profiling method which is commonly used to analyse complex communities (Kirk *et al.* 2004; Blackwood & Buyer 2006). T-RFLP analysis uses fluorescently labelled PCR primers, the resulting amplicons are digested with restriction enzymes, each enzyme produces a different community fingerprint, it is important to use more than one restriction enzyme to provide a complete assessment of diversity changes (Kirk *et al.* 2004). The restriction fragments with fluorescent primer ends, known as T-RFs, are separated by size using capillary electrophoresis, the T-RFs are visualised by peaks in fluorescence on an eletropherogram. Presence, height or area of peaks can be used to compare community profiles (Blackwood 2006).

Null hypothesis

H_0 The application of commonly produced isothiocyanates (determined in Chapter 4) will have no effect on the overall diversity of soil bacteria.

6.2 Materials and Methods

To assess the effects three commonly produced ITCs have on the community composition of soil bacteria, a glasshouse experiment was set up as described in section 6.2.1. ITC solutions were made up in solution using 1 % DMSO. The treatment concentrations and subsequent abbreviations for each treatment are listed in Table 6.1.

6.2.1 Soil microbial glasshouse experimentation

Field soil was collected from Lincolnshire, UK and put into 5 litre pots. Initial soil samples were taken in 2ml Eppendorf tubes and stored at -20°C. King Edward, potato tubers were planted in the pots, which were kept in controlled glasshouse conditions, and watered daily.

After 24 hours four replicates of each of the seven ITC treatments as detailed in 2.9.2 were added according to a random block design formulated in Genstat v14 (VSN International Limited). At assigned time points, 1, 5, 10, 20, 30 days after incorporation, both bulk and rhizosphere soil samples were taken in 2 ml eppendorf tubes and stored at -20 °C. Samples were taken from a depth of approximately 10 cm from the same pots at each time point

Table 6.1 ITC treatment concentrations used with their respective abbreviations, which were applied to the soil microbial glasshouse experiment.

Abbreviation Used	Treatment Concentrations
Control	No ITC
AITC	250 ppm Allyl ITC
BITC	250 ppm Benzyl ITC
PEITC	250 ppm 2-phenylethyl ITC
'AITC bitc peitc'	250 ppm Allyl ITC, 1 ppm Benzyl ITC, 1 ppm 2-Phenylethyl ITC
'BITC aitc peitc'	250 ppm Benzyl ITC, 1 ppm Allyl ITC, 1 ppm 2-Phenylethyl ITC
'PEITC aitc bitc'	250 ppm 2-Phenylethyl, 1 ppm Allyl ITC, 1 ppm Benzyl ITC

Using a ¾" soil corer, approximately 5 g rhizosphere and bulk soil samples were collected just prior to treatment application, then 1, 5, 10, 20 and 30 days after the ITC solutions had been applied. DNA extraction was carried out on each soil sample according to Chapter 2, section 2.6.4. Bacterial DNA was amplified as per Chapter 2, section 2.6.7.2, successful amplification was assessed using gel electrophoresis (Chapter 2, section 2.6.8). Each PCR product was digested using two individual restriction enzymes, *HhaI* and *AluI*, PCR products were digested using conditions

described in Chapter 2, section 2.6.10. The resulting fragments were then separated and analysed using T-RFLP according to Chapter 2, section 2.6.11.

6.2.1 T-RFLP data analysis

Using Microsoft Excel software, T-RFLP peaks were standardised by calculating each peak height and area as a percentage of the total peak height and total area respectively. All peaks less than 1% of the total value were given a value of '0'. The percentage of the total peak height and area was then recalculated for each sample. The data was aligned using a crosstab macro ("treeflap"), which rounded each peak size to the nearest integer and reorganised the data into a crosstab table. The "treeflap" macro was written by Dr. C Walsh and is available from : http://www.wsc.monash.edu.au/~walsh/treeflap.xls Crosstab data was analysed using Genstat v14 (VSN Interntational Limited), using principal coordinate analysis (PCoA), based on Jaccard coefficient, two dimensions were calculated for each sample and plotted on a scatter plot using Microsoft Excel software. Average link hierarchal cluster analysis was also performed using Genstat v14, to display similarities between T-RFLP results taken at the time of sampling.

6.3 Results

T-RFLP results are presented as both scatter plots and cluster diagrams. Scatter plots display the principal co-ordinate statistical analysis results and allow identification of changes in diversity; a greater distance from the origin represents greater bacterial diversity within the samples. Cluster analysis displays the similarities between the bacteria found within the individual soil samples taken at each time point.

6.3.1 Bulk Soil – *AluI*

Control

Overall bacterial diversity remains relatively constant throughout sampling; a slight loss in overall diversity is recognised in bulk soil samples taken at 30 days (Fig. 6.2).

AITC

Initially when the ITC solution was first applied, no large change in bacterial diversity was observed, however after one day AITC appeared to have caused a decrease in diversity (Fig. 6.2a). Diversity was then shown to increase at five days compared to day 0 and 1; bacteria samples were most varied at 10 days. After 20 and 30 days the level of diversity decreases. At the initial time of sampling pots treatment pots indicate a higher level of bacterial diversity than control soil samples. Yet one day after treatment application, AITC treated samples show a reduced level of diversity, much lower than control samples taken at this time point.

Comparison between control and AITC treated pots five days after application showed a higher level of bacterial diversity in treated soil. At both 20 and 30 days after application of ITC treatments levels of bacterial diversity appeared to be of a similar level both within the control and treated soil.

AITC bitc peitc

At the initial point of ITC application the diversity of bacteria decreased within the soil samples (Fig. 6.2b). Diversity increased slightly, one day after application, it increased again five days after application, and again after 10 days. Twenty days after ITC incorporation there was a slight decrease in overall diversity, which remained relatively constant in samples taken after 30 days.

At the initial sampling time bacterial diversity levels were similar in both control and treatment soils, one day after application soil samples showed a greater level of bacterial diversity in control samples compared to 'AITC bitc peitc' treated pots. After five days diversity was slightly greater in control soil samples, compared to treatment

soils. Ten days after ITC application bacterial diversity was greater in control samples compared to treated soil. After 20 days the level of bacterial diversity was similar in both the control and treated soil samples, however 30 days after treatment incorporation, bacterial diversity was lower than control samples.

BITC

Bacterial diversity remains relatively constant after application of BITC in all samples taken. It remained close to the highest level of diversity observed in control soil samples taken throughout the study (Fig. 6.2c).

At the initial time of sampling a high level of diversity was present in both control and treatment pots. The bacterial diversity level remained of a similar level between control and treatment soil samples until 30 days after treatment, when control samples showed a slightly lower level of bacterial diversity than control samples.

BITC aitc peitc

Again with the application of 'BITC aitc peitc', bacterial diversity remained relatively constant throughout the duration of the study (Fig. 6.2d). Although it can be seen that at the initial point of ITC incorporation and after 10 days, an individual sample showed a low level of bacterial diversity, generally it does not alter greatly from the highest levels of diversity that were seen in control pots.

Prior to ITC treatment soil samples were shown to contain similar levels of bacterial diversity. One day after treatment a slightly lower level of diversity was seen in treatment pots compared to controls. At day five after ITC application the diversity levels were similar in both treatment and control soil samples. Ten days after treatment a slightly lower diversity level was observed in 'BITC aitc peitc' treated samples than controls. Twenty and 30 day samples showed a similar level of bacterial diversity both in control and treatment soil samples.

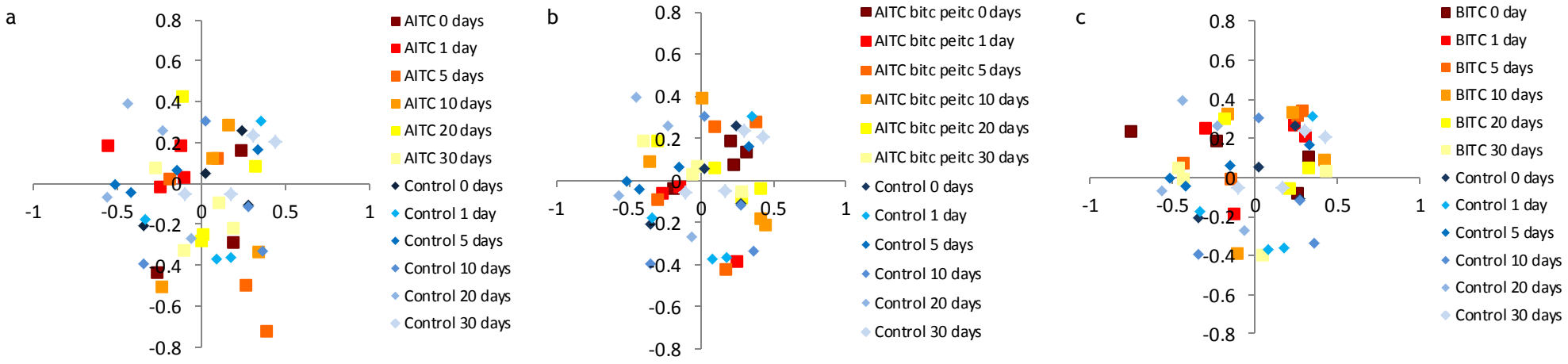
PEITC

The incorporation of PEITC showed a slight decrease in bacterial diversity at the initial point of application (Fig. 6.2e). This increased after one day and remained relatively constant until 10 days after application, a slight decrease in diversity was observed after 20 days, however is it shown to increase 30 days after incorporation. Prior to treatment a similar level of bacterial diversity was observed both in the control and treatment pots, diversity levels remained of similar levels between control and treatment soil samples throughout the duration of the study.

PEITC aitc bitc

Overall bacterial diversity remained relatively high after the application of 'PEITC aitc bitc'. A slight decrease occurred five and 10 days after incorporation, but overall diversity remains constant at similar levels to control samples that exhibited the highest diversity levels (Fig. 6.2f).

Prior to treatment, soil samples in assigned control pots showed a slightly lower level of diversity than in treatment pots. One day after ITC application bacterial diversity was very similar in both control and treatment samples, this was also true in samples taken five days after treatment. Ten and 20 days after treatment lower diversity was present in treated samples than controls. Thirty days after treatment application bacterial diversity levels were similar in both control and treatment samples.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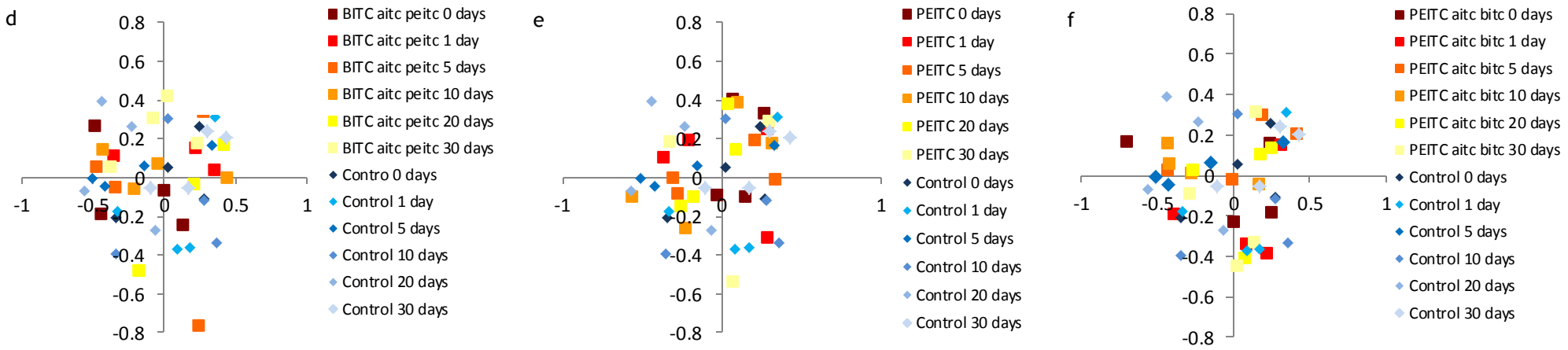

Figure 6.2 Scatter plot showing bacterial diversity in control and a) AITC b) AITC bitc peitc c) BITC d) BITC aitc peitc e) PEITC f) PEITC aitc bitc, treated bulk soil samples at each time point using *Alu*I

6.3.2 Rhizosphere Soil – *AluI*

Control

What is first noticeable from the control samples is that there appears to be a lower level of diversity observed within the control rhizosphere samples (Fig. 6.3) than the bulk soil samples (Fig. 6.2). However diversity in rhizosphere samples remains largely constant throughout the duration of the study. The lowest level of diversity was measured in samples taken 20 days after treatment; however this increased in samples taken 30 days after treatment.

AITC

Prior to treatment bacterial diversity was similar in both control and treatment pots, this remained constant in samples taken one day after treatment. With the application of AITC the overall bacterial diversity did not significantly alter at any sampling point, remaining as diverse as control samples (Fig. 6.3a). For the remainder of the study bacterial diversity was greater in treated soil than control samples.

AITC bitc peitc

The incorporation of 'AITC bitc peitc' caused the largest decrease in bacterial diversity between five and ten days after ITC incorporation, it remained relatively constant within samples taken after 20 and 30 days (Fig. 6.3b). The greatest diversity was observed in samples taken at the time of incorporation and one day after treatment application.

Comparison between control and treatment soils prior to ITC application showed similar levels of bacterial diversity, this is also true in samples taken one day after treatment. Five, 10 and 20 days after treatment a greater level of diversity was observed in 'AITC bitc peitc' treated samples than control soil samples. Samples taken 30 days after treatment showed similar levels of bacterial diversity both in control and treated soil samples.

BITC

Incorporation of BITC produced the highest level of bacterial diversity at the time of application; diversity decreased slightly after one day and remained at a similar level within samples collected five days after treatment (Fig. 6.3c). This decreased within samples taken 10 days after BITC incorporation, after 20 days it was shown to increase again, but then decreased slightly after 30 days, although such changes are not statistically significant.

Samples taken before treatment application showed similar levels of bacterial diversity in both control and treatment soils. This was also true 1, 5, 10 and 30 days after treatment, soil samples taken 20 days after treatment showed a higher level of diversity in those treated with BITC.

PEITC

Bacterial diversity was highest in PEITC treated pots after the initial application (Fig. 6.3e). Diversity decreased marginally at day one, and again at five days, diversity remained relatively constant at 10 and 20 days post 'PEITC' incorporation. Bacterial diversity was shown to increase in samples collected 30 days after incorporation. Overall diversity did not differ significantly from that observed within control rhizosphere soil samples.

Prior to sampling the level of bacterial diversity was high in both control and treatment pots. One day after treatment, bacterial diversity was marginally higher in control samples than those treated with PEITC. Diversity decreased in samples taken five days after treatment application although it was a similar level in both control and treated soil. At day 10 a higher level of bacterial diversity was found in treated samples than the control. In samples taken at both 20 and 30 days after ITC application a similar level of diversity was observed in both the treatment and control samples.

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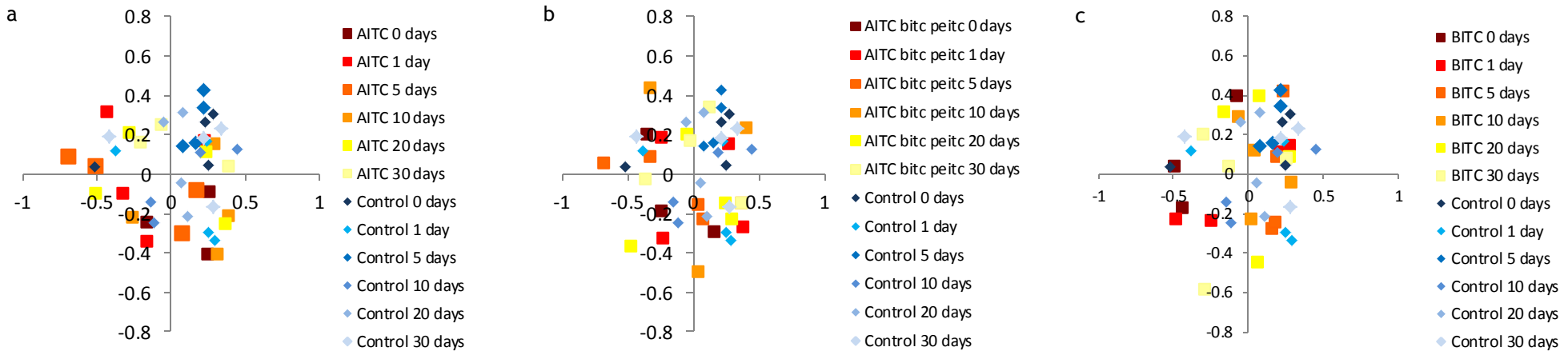
PEITC aitc bitc

The lowest level of bacterial diversity was observed in samples taken one day after 'PEITC aitc bitc' incorporation (Fig. 6.3f). However 10 days after ITC application the diversity level recovered and remained constant in samples taken after 20 and 30 days after incorporation. Overall bacterial diversity at the two final sampling points was greater in samples treated with 'PEITC aitc bitc' compared to control samples.

Before ITC application, diversity in soil samples was similar in both control and treatment pots. One day after treatment was applied, treatment samples showed a lower level of bacterial diversity than seen in control samples. After five days a similar level of diversity was seen in both treatment and control soil, this is also true in samples taken 10 and 20 days after treatment. After 30 days bacterial diversity was marginally greater in control samples than in soil treated with 'PEITC aitc bitc'.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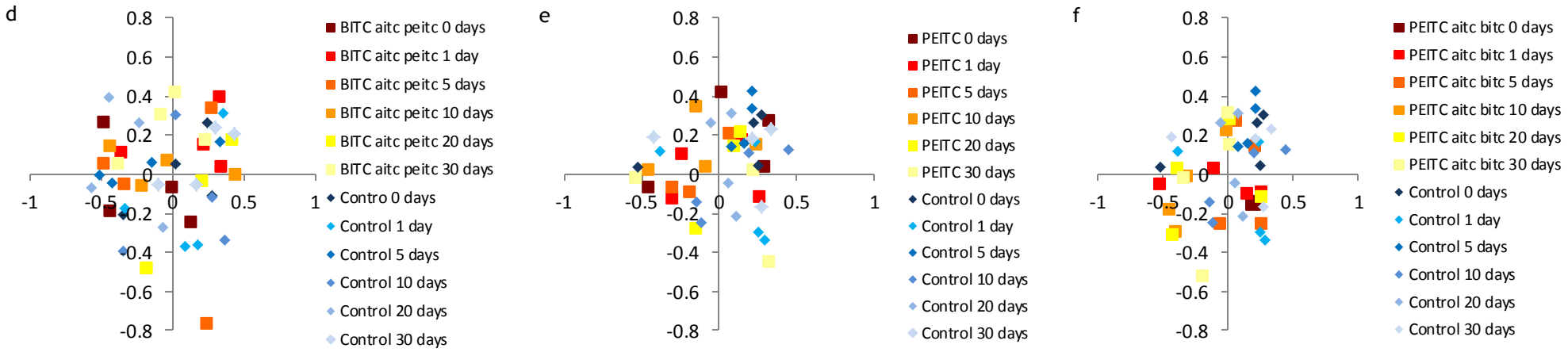


Figure 6.3 Scatter plot showing bacterial diversity in control and a) AITC b) AITC bitc peitc c) BITC d) BITC aitc peitc e) PEITC f) PEITC aitc bitc, treated rhizosphere soil samples at each time point using *Alu*I

6.3.3 Bulk Soil – *HhaI*

Control

Bacterial diversity was at its lowest in samples taken at day 0, a large increase was observed in control samples taken one day after treatment (Fig. 6.4). After five days bacterial diversity decreased, however it then increased in samples taken at 20 days, before then decreasing in soil samples taken 30 days after treatment application.

AITC

A high level of bacterial diversity was observed in bulk soil samples taken at the time of 'AITC' incorporation (Fig. 6.4a). The diversity was shown to decrease one day after ITC treatment, diversity remained relatively constant in samples taken five and 10 days after ITC incorporation. Bacterial diversity then increased slightly in samples taken after 20 days then decreased 30 days after ITC incorporation, although changes were not shown to be significant.

A greater level of bacterial diversity was observed in treated pots. One day after treatment, bacterial diversity was similar in both control and treatment samples. After five and 10 days diversity was lower in control samples than those treated with AITC. Bacterial diversity was similar in samples taken 20 and 30 days after ITC application.

AITC bitc peitc

Results show that overall bacterial diversity was not greatly altered by the incorporation of 'AITC bitc peitc'. Diversity did not alter greatly in any samples taken after incorporation (day one) and was shown to be relatively constant within all samples taken after incorporation as shown (Fig. 6.4b).

Before 'AITC bitc peitc' was applied to soil, bacterial diversity was marginally greater in treatment pots than controls. However in samples taken one day after treatment greater diversity was present in control samples than treated soils. At day five a higher level of bacterial diversity was measured in treated soil samples than untreated controls, in samples taken both 10 and 20 days, after treatment was applied, bacterial diversity was a similar level in both control and treatment samples. After 30 days greater diversity was seen in control samples than those treated with 'AITC bit petic'.

BITC

Overall BITC incorporation tends to show a decrease in the overall bacterial diversity observed within the soil samples (Fig. 6.4c). One day after incorporation of 'BITC' diversity increased from the initial time of treatment. To some extent, bacterial diversity decreased in samples taken five days after incorporation, it increased in samples taken 10 days after treatment, a small decrease was observed in soil sampled at 20 days. The greatest level of bacterial diversity in treatment samples was recorded in samples taken 30 days after incorporation.

Before treatment the level of bacterial diversity was similar in control and treatment pots, this remained true one day after treatment. At day five, diversity was higher in treatment soils than controls, however after 10 days diversity was greater in the control samples, this remained true for the remainder of the study.

BITC aitc peitc

The lowest level of bacterial diversity was observed in samples taken at the time of incorporation of 'BITC aitc peitc', a small increase in samples taken one day after incorporation was recorded (Fig. 6.4d). It increased again in samples taken after five days, and again in samples taken after 10 days. Thereafter there was a mild decrease in bacterial diversity in samples taken after 20 days and 30 days.

A similar level of bacterial diversity was recorded in control and treatment samples prior ITC application. One day after treatment diversity levels remained similar in both control and treated samples. At day five samples indicated a higher level of bacterial diversity in control samples, than those treated with 'BITC aitc petic'. Ten days after treatment was diversity was greater in treatment samples compared to the controls. Soil samples taken 20 and 30 days showed a similar level of diversity in both controls and treatment soils.

PEITC

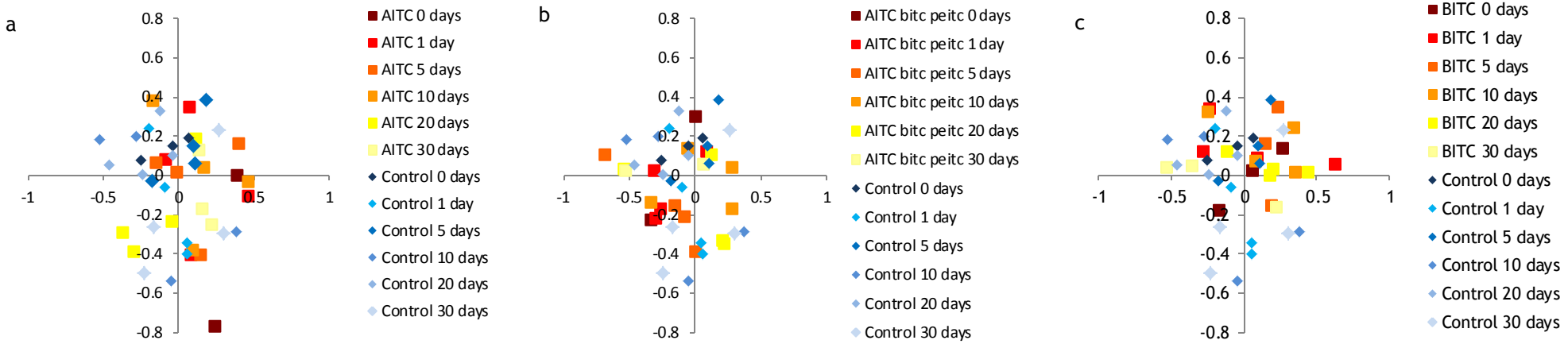
Overall bacterial diversity does not alter significantly from that observed from the data presented from control samples, (Fig 6.4e). Generally diversity increased between one and five days, followed by a decrease at days 10 and 20. In samples taken 30 days after incorporation showed a slight increase in bacterial diversity.

Prior to treatment diversity in both control and treatment pots was of a similar level, this remained true throughout the duration of the study.

PEITC aitc bitc

Bacterial diversity was shown to increase between samples taken at the initial time of 'PEITC aitc bitc' application and one day afterwards (Fig. 6.4f). Diversity decreased slightly in samples taken after five days, but then increased very slightly in samples taken 10 days after treatment. A decrease was observed in samples taken at 20 days after incorporation, another decrease in diversity was observed in samples taken after 30 days (Fig 6.4f).

Before treatment application diversity in control and treatment soils was of a similar level, one day after treatment diversity was seen to be marginally higher in treated soils than controls. 5, 10 and 20 days after 'PEITC aitc bitc' was applied diversity was a similar level in both the control and treated soils. After 30 days bacterial diversity was lower in treated samples than controls.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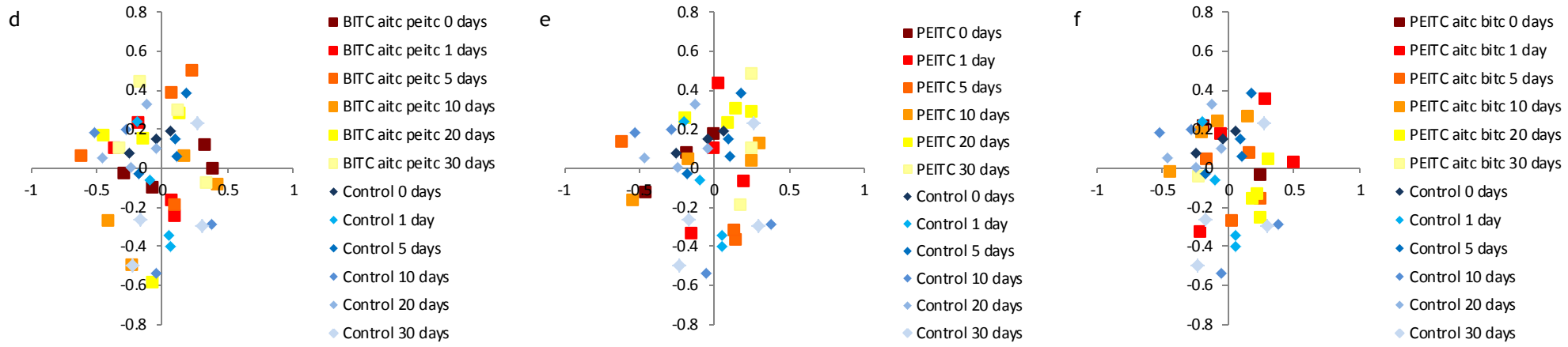


Figure 6.4 Scatter plot showing bacterial diversity in control and a) AITC b) AITC bitc peitc c) BITC d) BITC aitc peitc e) PEITC f) PEITC aitc bitc, treated bulk soil samples at each time point using *Hha*I

6.3.4 Rhizosphere Soil – *HhaI*

Control

From control samples taken over the 30 day time period, it can be seen from the scatter plot that overall the level of diversity doesn't alter greatly. Indicating that in the absence of an ITC treatment levels of bacterial diversity were maintained at relatively constant levels throughout the study.

AITC

Examination of all samples taken from AITC treatment shows that in comparison to control samples overall bacterial diversity levels did not differ greatly (Fig 6.5a). After one day, diversity levels remain similar to those recorded at the time of treatment, the largest decrease in bacterial diversity was observed five days after AITC was applied. Levels of diversity then increased after 10 days, stayed constant until 20 days after application, before showing signs of diversity increasing 30 days after the treatment was applied.

Prior to treating soil with AITC the bacterial soil diversity was similar in both control and treatment pots. One day after treatment was applied, much greater diversity than seen in control samples was recorded in treatment samples; this is also due in soil samples collected five and 10 days after treatment. Twenty days after treatment diversity within the control samples increased to be similar to that in treated soil. After 30 days, diversity within the treated soil samples was greater than in the controls.

AITC bitc peitc

Overall comparison between 'AITC bitc peitc' treatment samples and control samples showed a general trend towards a greater level of bacterial diversity in treatment samples (Fig. 6.5b). Between the time of treatment application and one day afterwards, diversity increased, five days after treatment application diversity was measured at its highest level. After 10 days levels decreased, before increasing again within samples taken at 20 days, a slight diversity decrease was observed in soil sampled 30 days after ITC application.

Before the ITC treatment was applied diversity of soil bacteria was similar in both treated and control pots. Diversity remained of a similar level between control and treated samples one day after treatment application. In samples taken 5, 10 and 20 days after treatment bacterial diversity was greater in treated soil than controls. After 30 days diversity was similar in both control and treated soil samples.

BITC

Overall the level of bacterial diversity in soil samples treated with 'BITC' was not significantly different to control samples. The lowest level of diversity was observed in samples taken at the time of treatment, this increased in samples taken 1 day after ITC treatment, and was seen to remain relatively constant in samples taken five days after the application of treatments, the largest increase in bacteria diversity was recorded 10 days after the treatment was applied. It then decreased slightly within samples taken 20 days after, and remained at a constant level within samples taken 30 days after 'BITC' treatment (Fig.6.5c).

Before treatment was applied a similar level of bacterial diversity was recorded in both treatment and control soil. One day after treatment diversity remained similar in both treated and control soil until 10 days. After 10 days diversity was greater in BITC treated soil, after 20 days diversity increased in samples taken at 20 days and a similar level in both control and treatment samples this was also true in samples taken 30 days after treatment.

BITC aitc peitc

Bacterial diversity within soil samples treated with 'BITC aitc peitc' appeared to be overall lower that recorded in control samples (Fig. 6.5d). The greatest diversity was observed within soil samples taken one day after ITC incorporation. Generally diversity decreased within subsequent samples, but then was shown to increase slightly in T-RFLP analysis conducted on samples taken 30 days after ITC treatment.

On day 0 diversity in control and treatment soils was similar, one day after treatment was applied diversity was marginally greater in treated soil, than controls. However after five days diversity was reduced in treated soils and shown to be the same in both control and treated samples, this was also true in samples taken 10, 20 and 30 days after treatment was applied.

PEITC

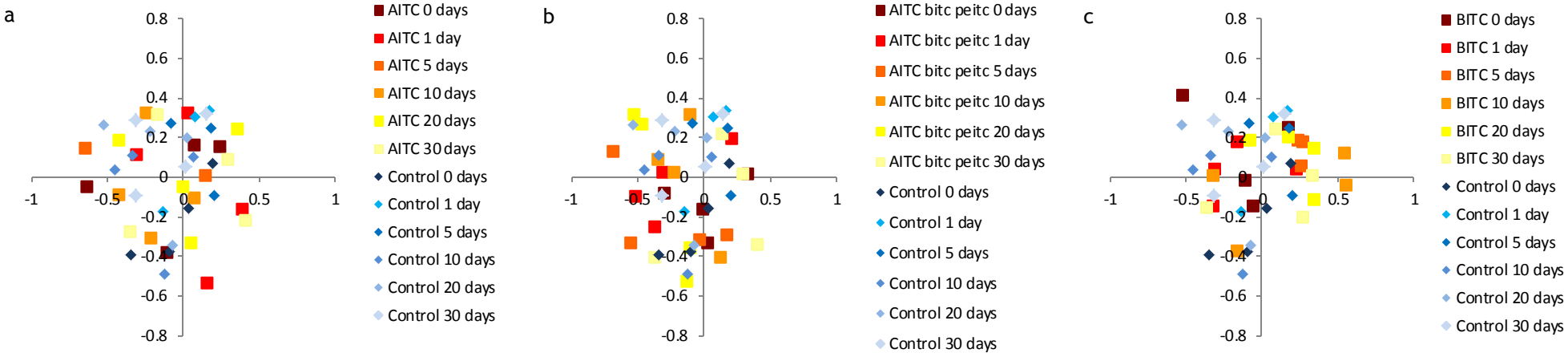
In comparison to control samples taken throughout the duration of the study, PEITC treatment appears to increase the overall level of diversity of bacteria (Fig. 6.5e). A relatively high level of diversity was measured in samples taken at the time of PEITC treatment application. After one day this was shown to decrease, and diversity levels further decreased five days after the ITC was applied. After 10 days the bacterial diversity increased, before decreasing within samples taken after 20 days and then increasingly again within samples taken 30 days after treatment.

Initially soil bacterial diversity was similar in both control and treatment samples; diversity was seen to be marginally greater in treated samples one day after application. Diversity in treated samples continued in increase and was greater when compared to control samples 10 days after treatment. In samples taken 20 and 30 days after treatment diversity was greater in control samples than treatment samples.

PEITC aitc bitc

Overall bacterial diversity appeared to be increased by the application of 'PEITC aitc bitc' when compared to control samples taken throughout the study (Fig 6.5f). Between the time of treatment application and one day after, there was a small decline in bacteria diversity; this level remained largely constant within samples taken five days after the ITC solution was added to soil. Within samples taken 10 days after treatment, there was an increase in diversity, which increased further in samples taken after 20 days. The bacterial diversity level decreased in samples taken 30 days after ITC application.

Before treatment was applied soil bacterial diversity was of a similar level in both the control and treatment samples. This was also true in samples collected between one and 20 days after the ITC treatment was applied. After 30 days diversity was greater in treated samples when compared to control soil.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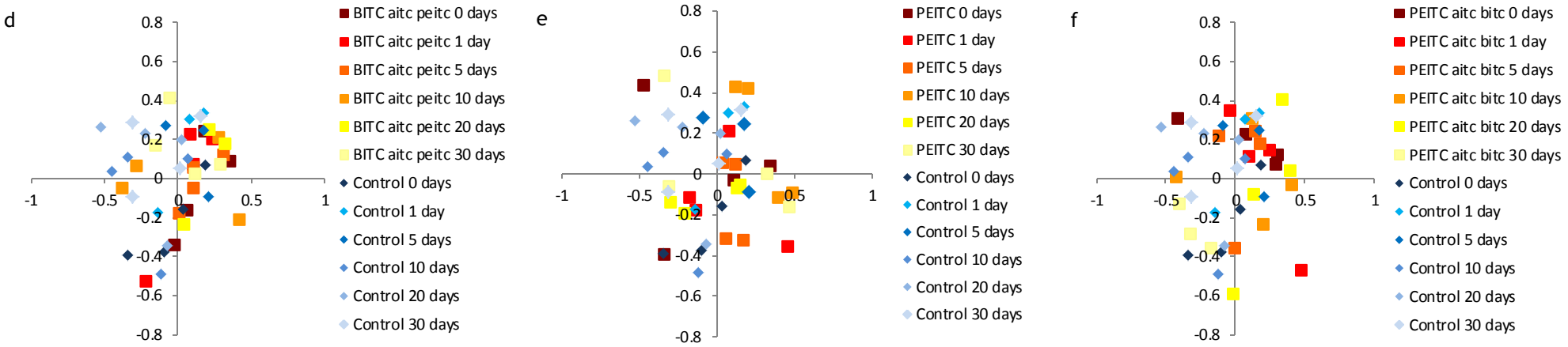


Figure 6.5 Scatter plot showing bacterial diversity in control and a) AITC b) AITC bitc peitc c) BITC d) BITC aitc peitc e) PEITC f) PEITC aitc bitc, treated rhizosphere soil samples at each time point using *HhaI*

6.3.5 Comparison between enzymes

Overall diversity recorded within samples appears to be of a similar level produced by T-RFLP using each restriction enzyme, *HhaI* and *AluI*. As expected differences in individual diversity shifts were recognised, as each enzyme provided a different community profile. By comparing the results from both enzymes more detailed conclusions about the changes in bacterial diversity trends can be made.

6.3.6 Comparison between bulk and rhizosphere soil

HhaI

Profiles produced using *HhaI* restriction enzyme, using data provided at each time point, showed that there was a general increase in the levels of bacteria diversity when compared to that observed within control samples. This was true for all treatments excluding 'BITC aitc peitc', which showed little to no change in diversity levels. In comparison bacterial diversity measured in bulk soil samples, showed no change in bacterial diversity in soil treated with 'AITC', 'AITC bitc peitc' and 'BITC aitc peitc'. A slight decline in the overall diversity was measured in samples taken from 'PEITC' and 'PEITC aitc bitc' treatment soils, and a larger overall decrease in bacterial diversity was measured in pots which were applied with 'BITC' solution.

AluI

Rhizosphere soil samples taken throughout the duration of the study, demonstrated a trend towards an increase in bacterial diversity with all treatments, excluding 'AITC', when compared to the diversity levels observed in control samples taken throughout the study. A different trend was observed in bulk soil samples, taken at each sampling time, in which diversity levels stayed relatively constant. With the exception of 'AITC' treatment soil, which showed an overall increase in bacterial diversity levels, and 'BITC' which showed a decrease in the diversity of bacteria measured within the samples.

6.3.7 Bacterial diversity recovery

To analyse the similarities between samples, hierarchal cluster analysis was carried out, a dendrogram showing the average link between samples at different time points was created, the similarity index was plotted along the x-axis. Results below display dendrograms demonstrating the similarity between T-RFLP samples from bulk and rhizosphere soil, using both *AluI* and *HhaI* restriction enzymes, at 0, 5 and 30 days, to highlight the changes that occurred to the bacterial diversity throughout the study.

6.3.7.1 Bulk Soil – *AluI*

Figures 6.6a-c show the similarity between samples collected at different time points. Comparison between the above dendrograms allows assessment of how bacterial communities within each soil sample have diversified from one another over time and if specific ITC treatments lead to greater differences when compared to untreated control samples.

Five days after the ITC treatments were incorporated into the soil, T-RFLP analysis showed that the differences between samples increased. At this time there appeared to be grouping occurring between two BITC treated samples with two control samples, which have altered to become more similar to one another. Suggesting that in such cases the bacterial communities are responding to applied treatments in a similar way. After 30 days the samples diversified further, and bacterial communities within them became even more dissimilar from one another. Again it can be seen that in some cases soil treatment with the same ITCs become more similar to one another, as is true in some instances for BITC, 'BITC aitc peitc', and control samples, suggesting that the treatments may induce similar responses. However in this instance between five and 30 days the control samples are still shown to increase in differences that occur between their bacterial communities. Therefore suggesting that in the case of bulk soil analysed using *AluI* restriction enzyme, the length of time soil samples are left for has the largest influence on the changes of the bacterial community composition.

Fig. 6.6a

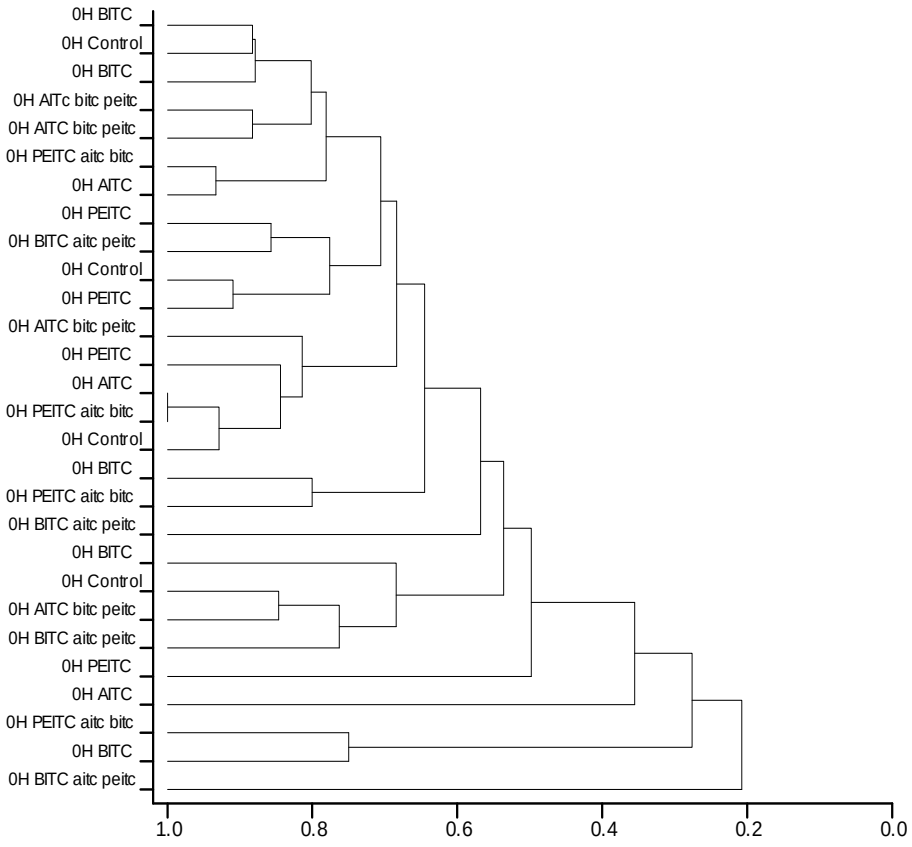
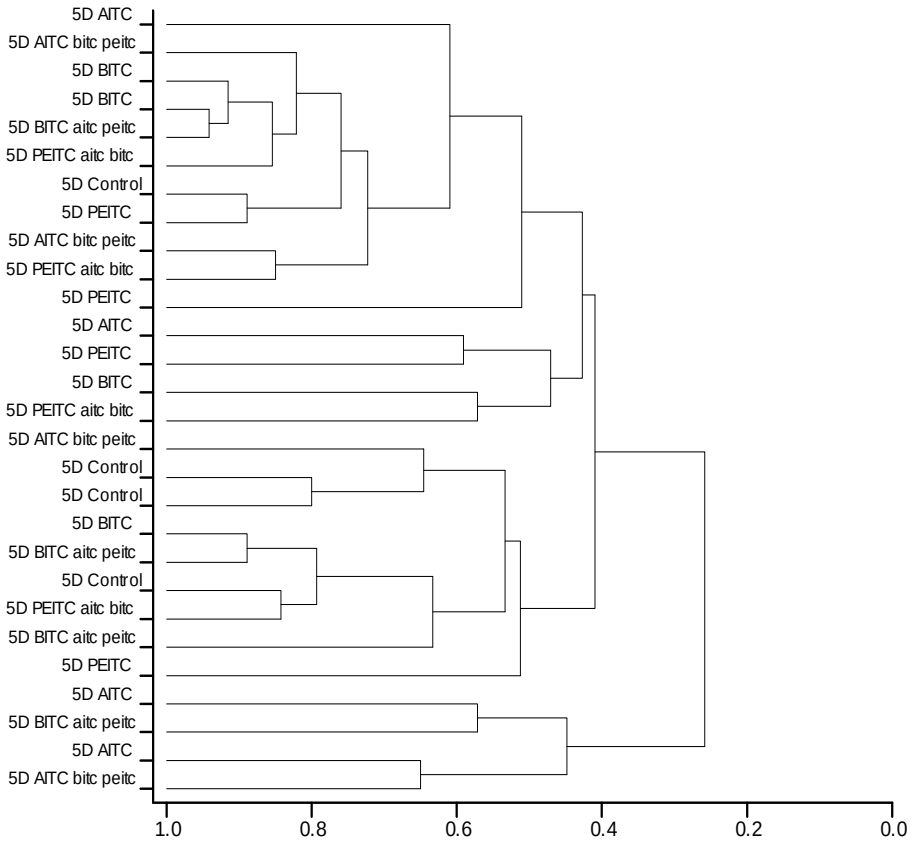
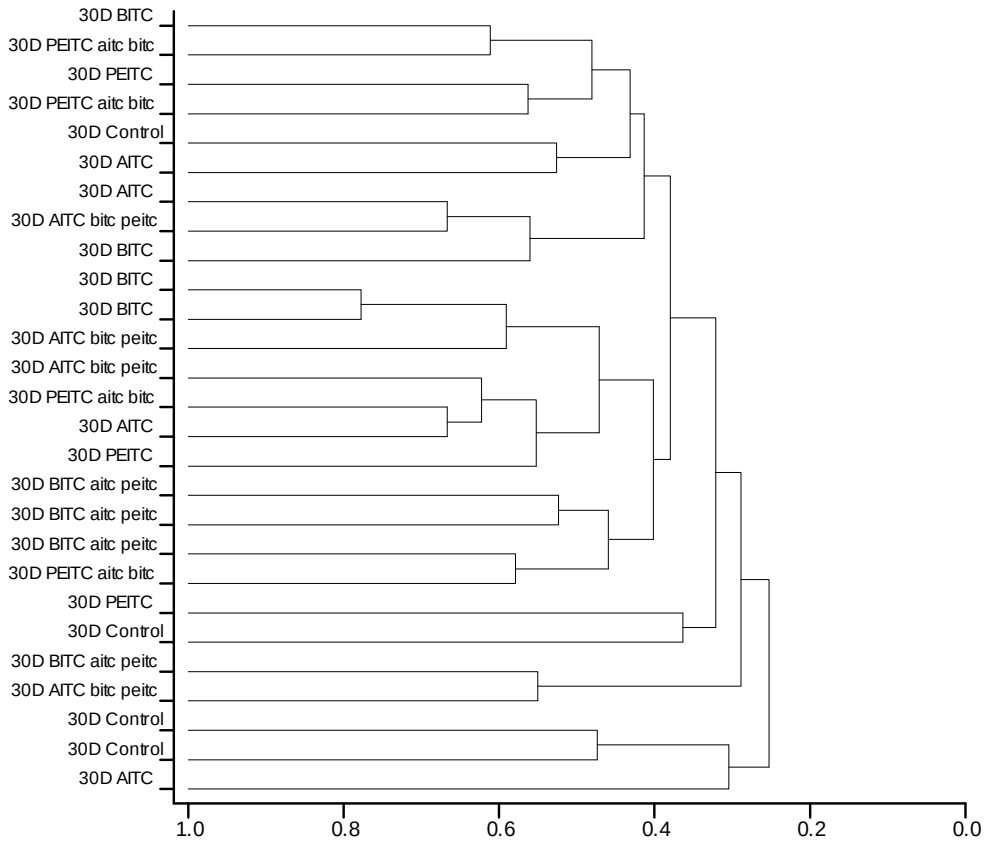


Fig. 6.6b





Figures 6.6a-c Average link hierarchical cluster analysis of bulk soil samples using *Hhal* T-RFLP data from soil samples taken at 0, 5 and 30 days after ITC incorporation

6.3.7.2 Rhizosphere Soil – *AluI*

In soil samples collected from the rhizosphere and analysed with *AluI* restriction enzyme at the first sampling time, a large number of samples were shown to possess large similarities between one another (Fig. 6.7a). However it is still notable that an amount of variation did occur at this time, between the soil samples analysed. Five days after treatments were applied it was clear that the differences in bacterial communities within the soil samples had increased further (Fig. 6.7b). Notably two control samples maintained a similarity index of >0.8, a similarity level that is only seen elsewhere between the same control samples and one 'BITC' treated soil sample. Although this may indicate that ITC treatments are driving the bacterial communities to become dissimilar, as other control samples have diversified, it is difficult to conclude this. Thirty days after ITCs are applied to the soil, the bacterial communities became even more different from one another and similarities that were observed between control samples disappeared (Fig. 6.7c). Thus suggesting the sub sampling of soil into pots has had an impact of changing the microbial communities within the soil.

Fig. 6.7a

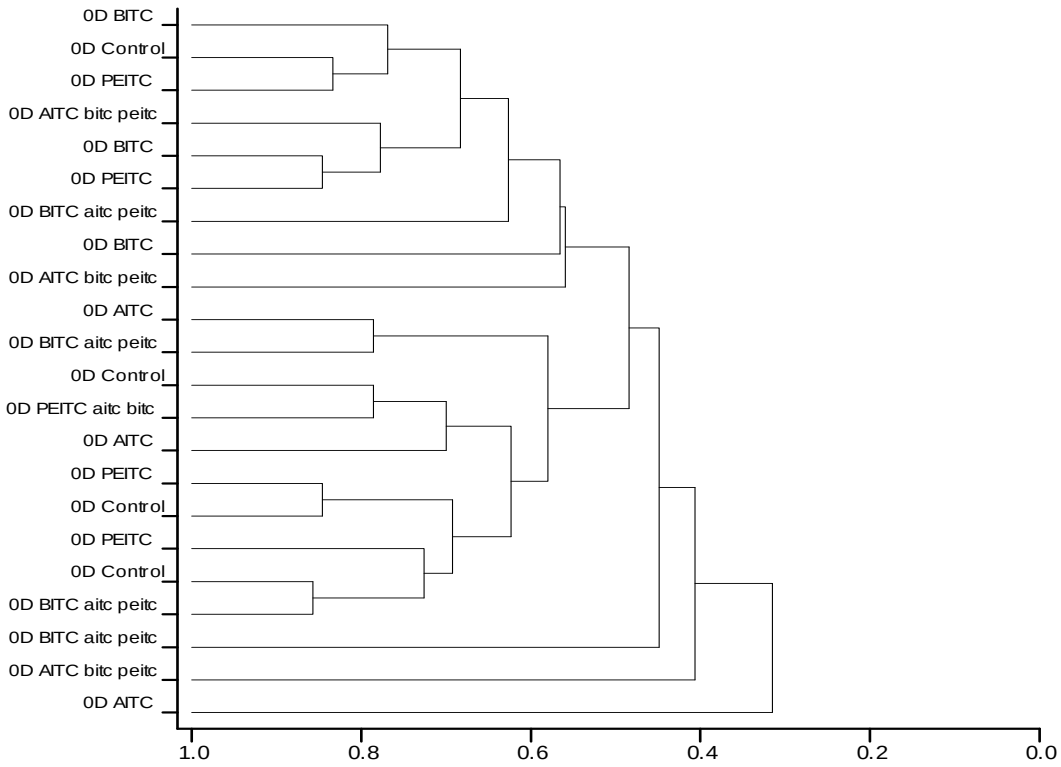
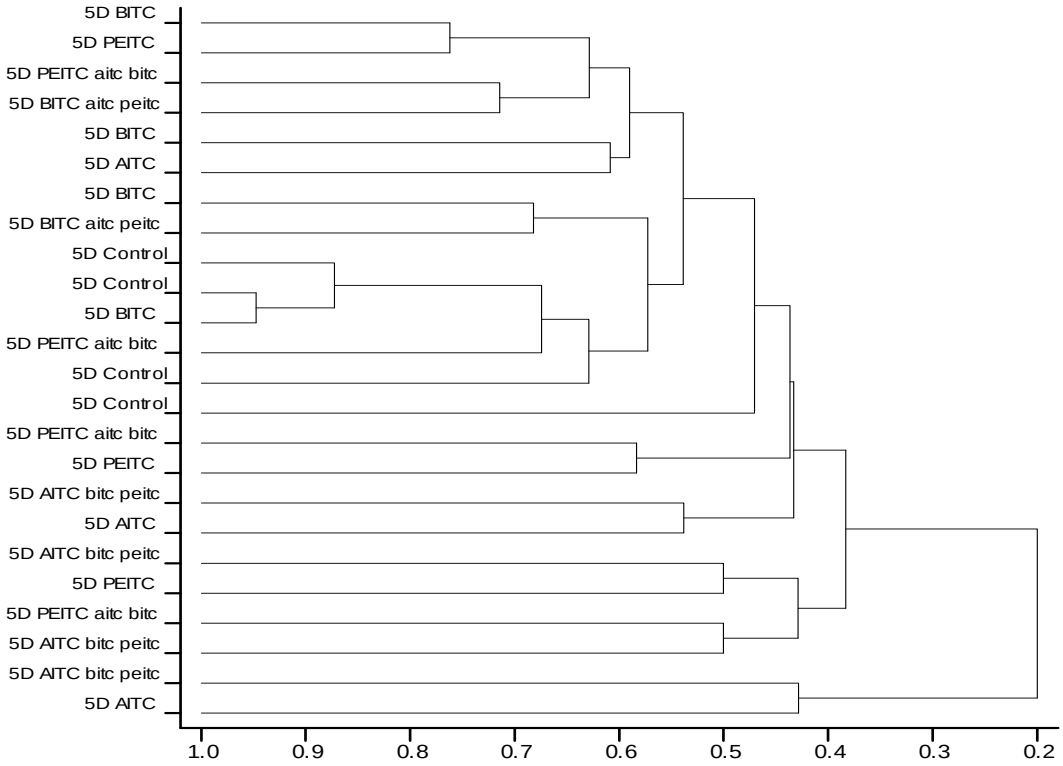
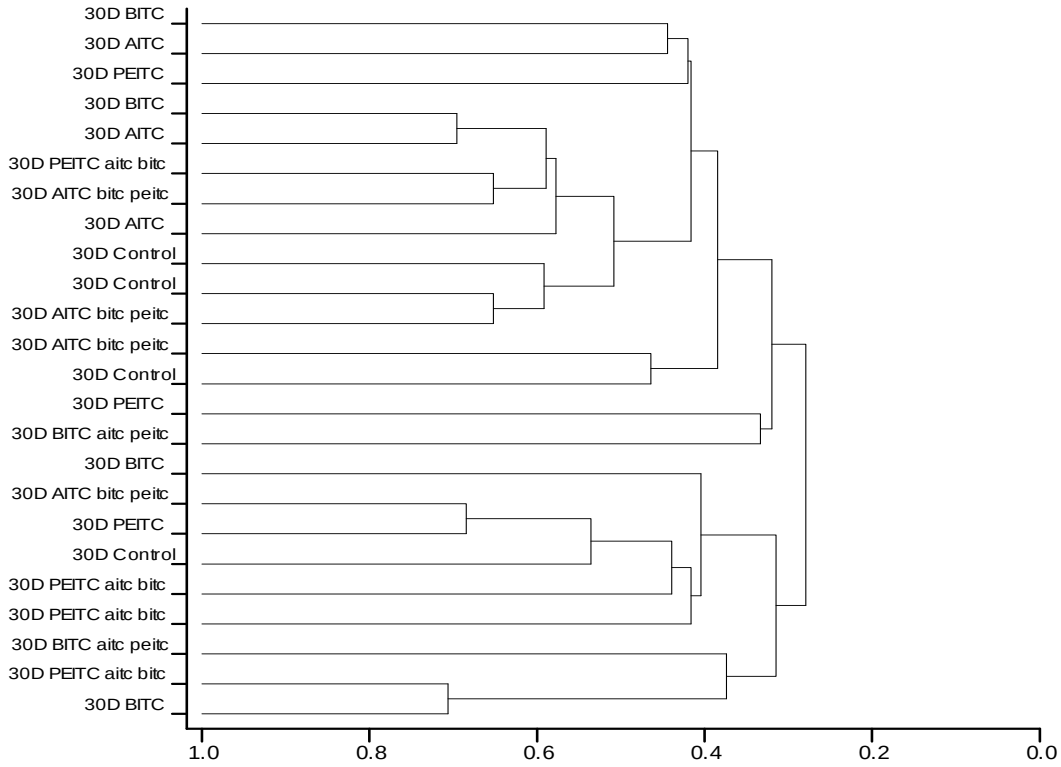


Figure 6.7b





Figures 6.7a-c Average link hierarchial cluster analysis of rhizosphere soil samples using AluI T-RFLP data from soil samples taken 0, 5, 30 days after ITC incorporation.

6.3.7.3 Bulk Soil - *HhaI*

Bulk soil samples analysed using HhaI restriction enzyme indicate that at the first sampling point, several samples were very similar to at least one other sample (Fig. 6.8a). The dengrogram shows that although diversity is present between the bacterial communities, there are several samples that could be clustered to show large similarities between their bacterial community compositions. Five days after treatments were incorporated, into the soil, the overall differences between samples increased. At this time point there appeared to be some grouping occurring between samples that were treated with the same ITC combination, as is true for 'BITC aitc peitc', AITC and 'PEITC aitc bitc' (Fig. 6.8b). It could be suggested that in such instances the ITC treatments caused the bacterial communities to respond in similar ways. After 30 days the overall difference observed between the samples remained largely similar to that observed after five days, however the grouping of similarities between treatment groups disappeared and samples changed in their level of similarity in comparison to one another (Fig. 6.8c).

Fig. 6.8a

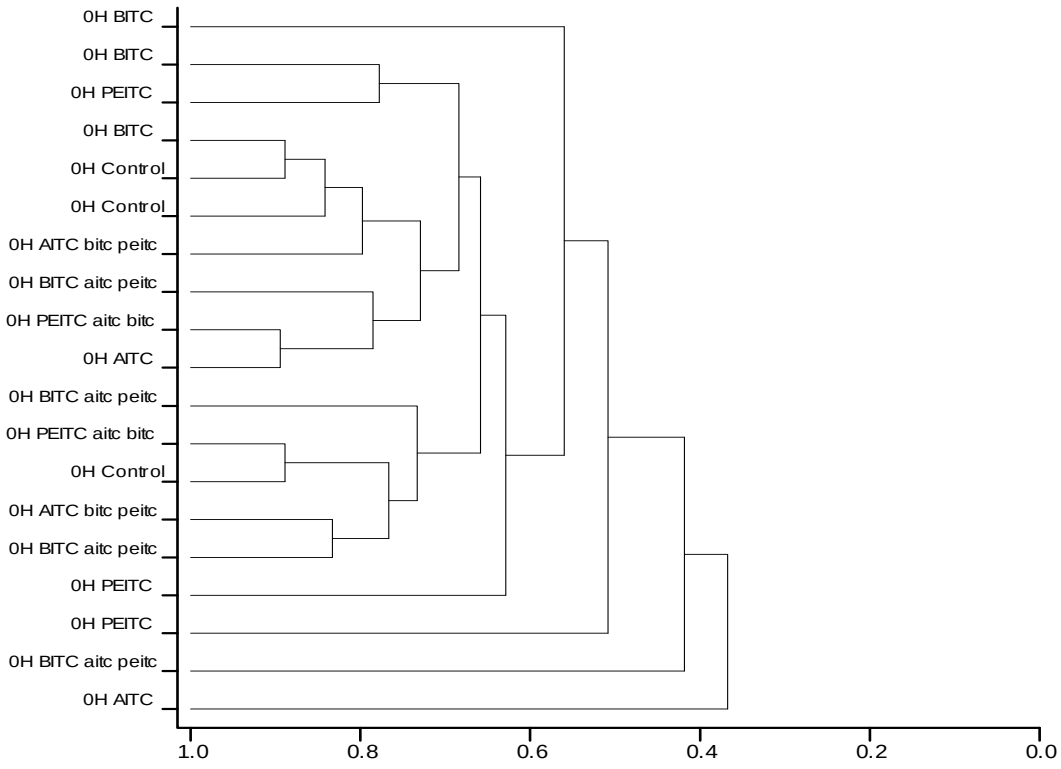


Fig. 6.8b

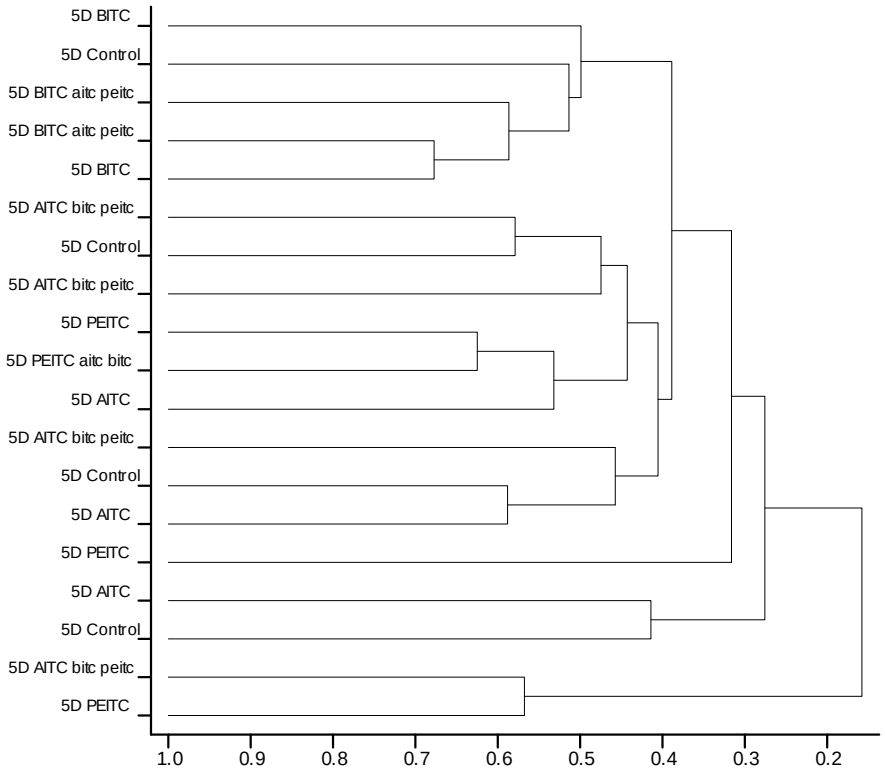
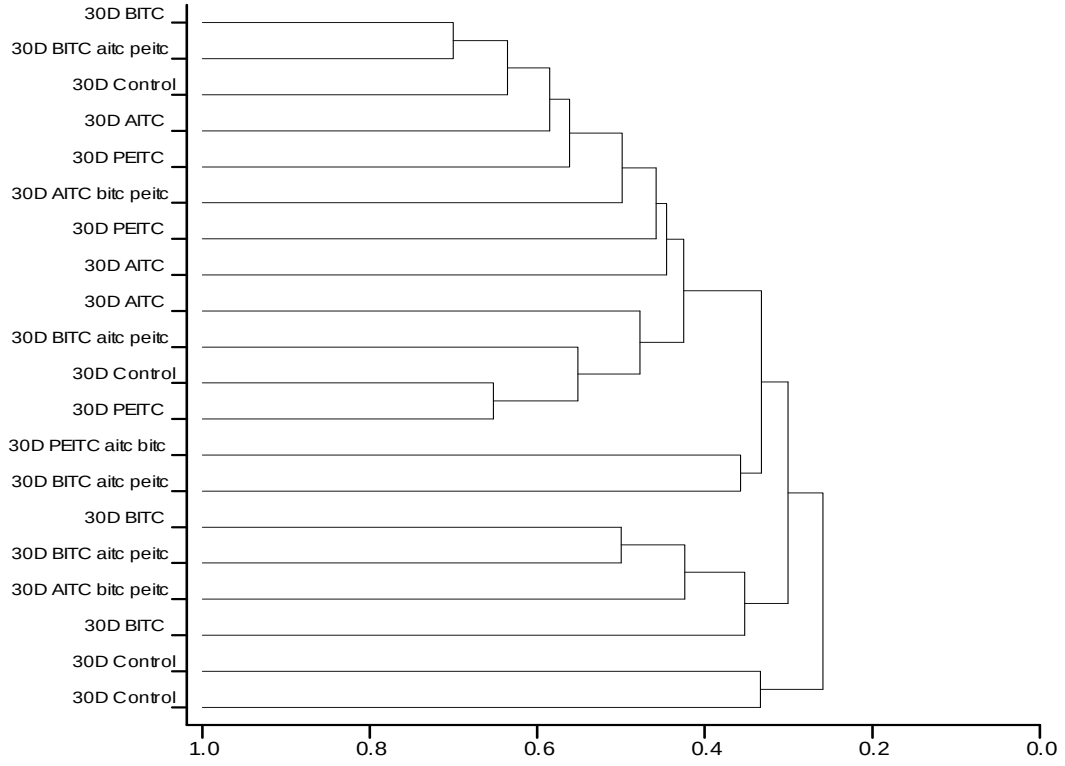


Fig. 6.8c



Figures 6.8a-c Average link hierarchical cluster analysis of bulk soil samples using *HhaI* T-RFLP data from soil samples taken 0, 5, 30 days after ITC incorporation.

6.3.7.4 Rhizosphere *HhaI*

Analysis using *HhaI* restriction enzyme shows that within rhizosphere soil sampled at the beginning of the study, there was already a high level of diversity that occurred between samples a large proportion of samples collected showed a similarity index of >0.6 between one another (Fig. 6.9a). Within samples taken five days after ITC treatments were incorporated into the soil glasshouse experiment, differences between the bacterial community composition increased, again there are a few cases where soil samples treated with the same ITCs become more similar, as is the case for BITC, 'BITC aitc peitc' and AITC (Fig. 6.9b). This may suggest that there is similar microbial activity occurring within these samples, driven by the effect the individual ITCs have had on specific bacterial groups. Control samples appear to become more diverse from one another, which can be believed to be a natural occurrence through the sub sampling process. The dendrogram shows that within samples collected 30 days after treatment, the bacteria became more different from one another, the groupings of similarities between treatments disappeared (Fig. 6.9c). Again in this instance control samples remain very different from one another indicating that after a significant time after treatment, ITCs do not influence the bacterial communities.

All hierarchal cluster analysis revealed that differences between bacterial community composition increased over time. Initial findings show that this does not appear to be largely influenced by the ITC treatment, and is more likely in response to the soil being sub sampled into pots and left to develop its own bacterial community structure.

Fig. 6.9a

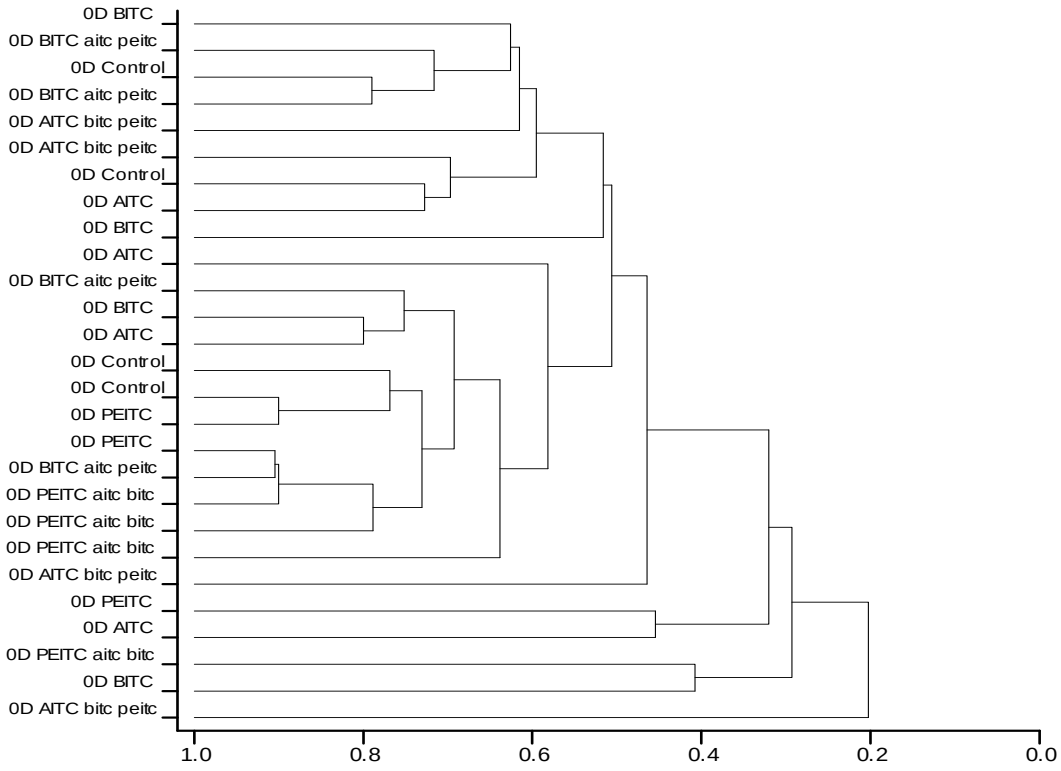
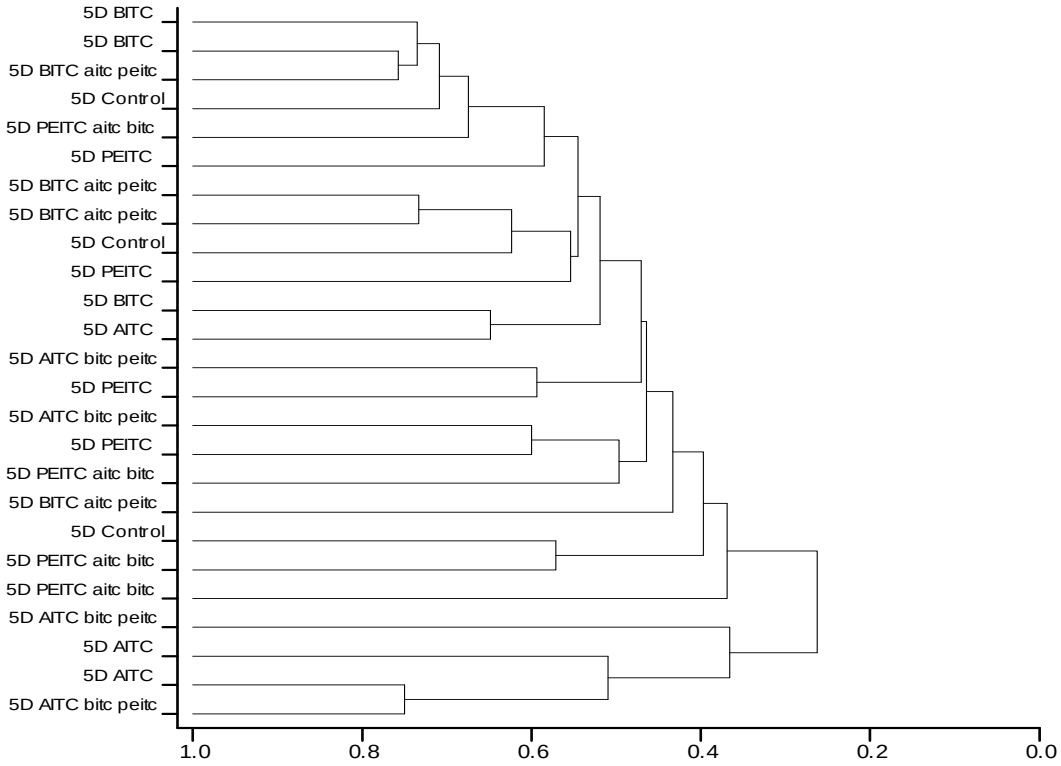
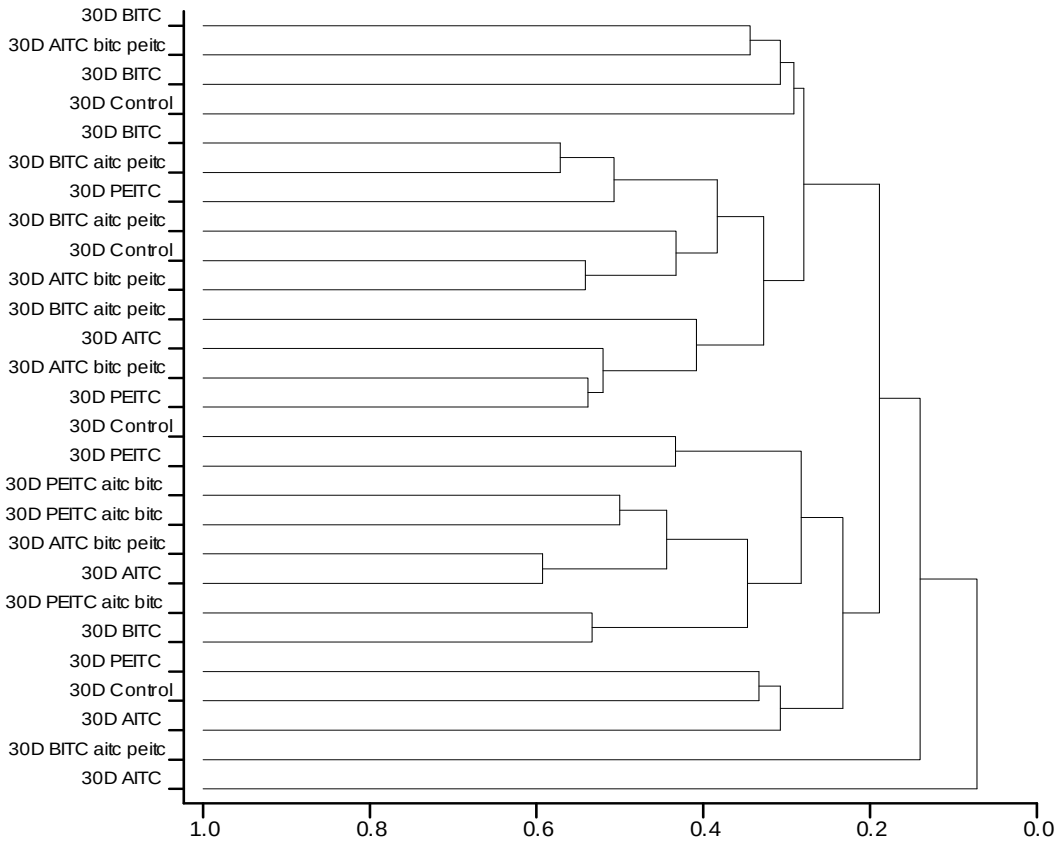


Fig. 6.9b





Figures 6.9a-c Average link hierarchical cluster analysis of rhizosphere soil samples using *HhaI* T-RFLP data from soil samples taken 0, 5 and 30 days after ITC incorporation.

6.3.8 Statistical analysis of T-RFLP

Analysis of Variance (ANOVA) was carried out to determine differences between principal coordinate values for each treatment at each sample taken throughout the duration of the study. The bacterial diversity within each treatment sample was compared to that measured within control samples. All ANOVA tests concluded that there was no overall significant difference ($p > 0.05$) between bacterial diversity observed within control samples and treatment samples. With the exception of 'BITC aitc peitc' and 'PEITC aitc bitc' treatments on bulk soil samples analysed with T-RFLP using *AluI* restriction enzyme, which both indicated significant difference in diversity between the bacterial communities (p value = 0.045 and p value = 0.025 respectively).

6.4 Discussion

Analysis of the soil bacterial communities treated with different ITC solutions, showed that although bacterial diversity changed throughout of the experiment. This was only significant when comparing the control treatments to bulk soil treated with 'BITC aitc peitc' and 'PEITC aitc bitc', however such significant differences were only observed in community fragments digested with *AluI* enzyme. No significant differences were observed in bulk soil, treated with the same ITCs analysed with *HhaI*, this highlights that changes were occurring within the bacterial community. However such changes occurred within different bacterial species and genera and different methods of analysis will present different results dependent on specific bacterial community fingerprints that are analysed. This emphasises the need for the use of more than one restriction enzyme when using T-RFLP for community composition analysis.

T-RFLP analysis suggests shifts occur within the bacterial community composition throughout the study, yet in the majority of cases this does not result in overall significant differences. Therefore the results indicate that although the communities are changing the diversity is not significantly altered, when compared to control samples, suggesting that perhaps individual bacterial groups are altering. Two instances when significant changes in bacterial diversity composition were observed compared to the control were present in bulk soil treated with 'BITC aitc peitc' and 'PEITC aitc bitc'. In both circumstances there were no significant differences observed between control treatment and BITC or PEITC suggesting that the presence of ITCs at the lower concentration had an impact on the bacterial community composition. Without further study, it cannot be determined if this is due to the presence of a single ITC at a lower concentration, or due to the combined effect both lower concentration (1 ppm) ITCs are having on the soil bacteria. Significant differences are only observed within the bulk soil, and not in rhizosphere soil from the same treatment pots, highlighting the differences between bulk soil bacterial communities and those present within the rhizosphere.

Previous studies have shown that large differences in bacterial community composition can occur between bulk and rhizosphere soil (Singh *et al.* 2004; Compant *et al.* 2010). Generally the rhizosphere is described to be a 'hot spot' of bacterial activity, due to the beneficial properties that being in close association to plant roots provide. Generally bacterial colonization of the rhizosphere has been linked to root exudation. Root exudates are known to contain a variety of compounds which will aid bacterial colonization and multiplication, including carbon which is fixed through photosynthesis and translocated to the roots and released within exudates. Several carbohydrates, amino acids, organic acids are also released providing bacteria with nutrients (Compant *et al.*, 2010). Some groups of bacteria have also been shown to be drawn to the rhizosphere by root mucilage (Compant *et al.* 2010). The rhizosphere colonisation process by bacteria is aided by characteristics such as bacterial flagella and quorum

sensing. As T-RFLP analysis revealed changes in the diversity throughout the study, but not overall within any rhizosphere samples, it could be suggested that the greater intensity of bacterial activity known to occur within the rhizosphere compared to the bulk soil provides it with some level of protection from what could potentially be community composition altering treatments. This bacterial intensity will be maintained through the production of root exudates released from the potato plant roots (Tilak *et al*. 2005). From these results it could be suggested that as the community composition is not significantly altered by ITC application within rhizosphere samples that ITCs do not affect the exudates released from the roots.

Hierarchical cluster analysis also suggests that over the duration of the study generally bacterial composition within the soil samples alters, and samples become increasingly different from one another. This is also seen within control samples that were not treated with ITCs. This proposes that sub sampling of soil may lead to the development of different compositions of soil bacteria communities. Such differences occurred within a controlled glasshouse environment in which soil was all taken from the same source, incorporated into the same size pots, kept at the same temperature, and received the same amount of light and watering. It can therefore be suggested that if a large soil sample is sub divided it can lead to differences within the bacterial community structure that alter over time. One explanation for such divergence occurring could be due to the specific bacterial groups and their quantities that are present by chance within soil sample. This again demonstrates the key role that individual bacterial groups have within influencing the overall community composition within soil, as well as suggesting the influence they have within overall soil quality (Filip 2002; Wang *et al*. 2008). Although overall diversity may not be altered through application of ITC treatments, individual soil bacterial species may be changing over time, which may be affecting the quantities of beneficial and deleterious bacterial species to plant growth (Egamberdieva & Hoflich 2003; Egamberdieva 2008).

6.4.1 Overall responses to ITC treatments

From scatter plot analysis it is shown that generally bacterial diversity increases within the rhizosphere after ITC treatment. This is not the same within bulk soil samples in which diversity remain relatively unaltered, except in the case of soil treated with BITC. The shifts and changes that occur within the rhizosphere and the increase in the resultant level of bacterial diversity that occurs after 30 days highlights that the rhizosphere generates a hive of bacterial activity and provides nutrients that aid bacterial growth and development (Compant *et al*. 2010). Scatter plot analysis reveals no loss in overall bacterial diversity which would indicate that ITC treatment would not lead to a negative effect on the overall level of soil quality and health. T-RFLP analysis allows this understanding but further analysis would be required to determine the specific bacterial groups that were being altered, as an increase in diversity may lead

to plant deleterious bacterial species being present. As the complexity of soil microbial systems are so vast it is often suggested that a soil quality profile should be defined allowing quick identification and assessment of nutrient content and specific microbial species that suggest that a soil is of good quality and lead to healthy crop yield (Schloter *et al*. 2003).

Analysis of scatter plots indicates that treatment with BITC leads to an overall decrease of bacterial diversity compared to control samples, it can be suggested that the interaction occurring between soil microbial species and differently structured ITCs is specific. In this instance BITC may have a more broad range level of toxicity towards the bacterial species found within the field soil samples, compared to other ITCs used in this study. What is noticeable is that 'BITC aitc peitc' does not lead to a decrease in overall diversity within the bulk soil, and BITC leads to an increase in overall diversity within rhizosphere soil. Here results can be linked to the information provided by the dendrogram analysis which demonstrates that over time the bacterial communities are becoming more different from each other. Thus suggesting that the bacterial species within the samples are changing and therefore different bacterial groups are likely to be affected by the addition of ITCs in different ways, as other agriculutural practices have been shown to alter bacterial populations (Filip 2002; Widmer *et al*. 2006; Elfstrand *et al*. 2007) .

Results from the above study demonstrate that generally the incorporation of ITCs into soil do not reduce bacterial diversity, both at the immediate time of application and within a 30 day time period after incorporation. Such data indicates that ITCs do not have detrimental effects on bacterial diversity within the soil, and therefore may not lead to alterations in overall soil health and quality. As it is often assumed that the release of ITCs through the practice of biofumigation will have a negative impact on soil quality (Ibekwe *et al*. 2001; Omirou *et al*. 2011). Such results are of great importance as they highlight that pure ITCs do not reduce overall diversity and microbial diversity is not significantly affected by exposure to such compounds.

6.4.3 Field application

This study has assessed the biofumigation process and it's interaction with soil microbial communities in one of its simplest forms, evaluating the effects that pure ITCs produced during glucosinolate hydrolysis have on the diversity of soil microbial communities. This study allows analysis of the possible effects that may be caused through release of these biocidal compounds. However it does not take into account other factors which may influence the bacterial communities when used in field applications, when ITCs are released into soil via other means such as green manures or dried plant material (Elfstrand *et al*. 2007). The addition of organic matter into soil is known to influence the composition of soil bacteria, as its incorporation will provide

different growth resources and change chemistries of the soil environment, such as pH and nutrient levels of nitrogen, phosphorus and iron all of which are known to lead to direct responses in the soil microbiology (Tiedje *et al.* 1999). Incorporation of green manures into soil may also alter microbial organism dispersal by changing the soil structure and routes of dispersal. Therefore although this study suggests that the release of toxic biocidal isothiocyanates into soil may not lead to lasting effects on the overall soil community composition. Yet if biofumigation is to be used within fields through the application of *Brassica* green manures then microbial diversity and changes in communities should be continued to be monitored using similar methods to assess for any negative effects on the soil community which may lead to a decrease in overall soil quality.

Although T-RFLP provides a useful tool in assessing the effects soil fumigants have on the overall soil microbial composition, this study also recognises that there are limitations with this fingerprinting method (Smalla *et al.*, 2007; Thies, 2007). One major drawback is an inability to identify the bacterial groups which are altering in response to treatment

6.5 Conclusions

Evidence from the above study demonstrates that the incorporation of commonly produced ITCs from *Brassica* plants does not lead to significant changes in bacterial diversity; suggesting that the practice of biofumigation will not lead to negative impacts on soil microbial communities and overall soil health. Evidence from previous research additionally indicates that the incorporation of plant tissue into soil may also lead to promoting growth of beneficial bacteria, and improving overall soil quality. Yet although overall diversity is not altered further study is required to establish alterations that may occur within certain bacterial groups in response to ITC incorporation.

Chapter 7

Final discussion, conclusions and future perspectives

7.1 Conclusions and future directions of this study

7.1.1 Findings and conclusions from this study

The principle objective of this study was to evaluate the effectiveness of using biofumigation – the incorporation of *Brassica* tissue into soil – to control fungal potato pathogens. To elucidate this process, a progressive series of different *in vitro,* biochemical, molecular biology, glasshouse experiments were utilised to gain a detailed insight into the processes that occur during biofumigation. Biofumigation is progressively growing in its popularity as a method of pathogen control and increasing numbers of agronomic companies are producing biofumigant crop seeds with claims that they will produce the highest level of control towards a number of different nematode and microbial plant pathogens (Kirkegaard *et al.* 1993; Mari *et al.* 1996; Kirkegaard *et al.* 1998; Sarwar *et al.* 1998). This study aimed to establish the scientific underpinnings of this process, and to evaluate the relevant importance of both the biofumigant crop and the targeted pathogen within the biofumigation process. Emphasis was placed on designing laboratory assays which could be used in the future to quickly establish interactions between pathogens and ITC structure and concentration of ITCs released during glucosinolate hydrolysis.

Initial bioassay studies revealed that the antimicrobial effect of ITCs on pathogens is one of great specificity. The level of suppression observed varied in accordance to ITC, pathogen and concentration present, with no single ITC producing uniform control of the three pathogens studied. The suppression response could be classified into one of three categories; no response, in which the pathogen was unaffected; fungistatic response, in which the time for initial mycelial growth to appear was increased due to the presence of the ITC; and fungitoxic response, in which the inoculum fungal plug appeared to be killed and could no longer grow due to the presence of the ITC. Of the seven ITCs studied the presence of 2-phenylethyl ITC produced a range of different fungistatic responses across all pathogen experiments at different concentrations. Therefore suggesting that its incorporation may produce broad range control of all three fungal potato blemish inducing pathogens. The incorporation of benzyl ITC into agar plates also caused significant suppression of growth of *Rhizoctonia solani* and *Helminthosporium solani.*

A Gas Chromatography – Mass Spectrometry (GC-MS) assay was designed which would rapidly assess plant material for its ability to produce ITCs. The assay was also designed to be quantitative in order to establish the relevant concentration of ITC production. This methodology could be modified to extend the range of hydrolysis ITCs

detected following glucosinolate hydrolysis. It is also possible that the assay could be used to detect nitriles and thiocyanates to build up an increasingly detailed hydrolysis product profile for a range of different *Brassica* cultivars. Experimental and growth conditions could also be altered to determine how the hydrolysis product profile is affected.

Results from GC-MS of *Brassica* plant tissue grown in glasshouses produced results indicating that several different cultivars could produce high concentrations of ITCs. Including allyl, 2-phenylethyl and benzyl, which results in Chapter 3 have indicated would have antifungal effects on potato fungal pathogens. Of the ITCs analysed within this study, GC-MS results indicated that commonly one (either allyl or benzyl) ITC within a cultivar was dominant; however several additional ITCs could also be produced during glucosinolate hydrolysis (Chapter 4). Thus it may be beneficial to assess the effects of ITCs on pathogens in combination. Results from GC-MS studies also conclude that concentrations and specific ITCs produced during glucosinolate hydrolysis alter throughout plant development. Therefore the time of plant incorporation used to achieve maximum pathogen suppression may alter depending on the specific biofumigant cultivar being grown.

Assessing the pathogenic properties of different ITCs in combination was also highlighted to be an important area of biofumigation research from results of Chapter 5. This study assessed the levels of potato black scurf and potato black dot on daughter tubers after the incorporation of ITCs into the soil. Results indicated that the presence of low concentration ITCs had an effect on the observed responses when incorporated in combination with a dominant, high concentration of another ITC. Such results were emphasised by the studied interaction between PEITC and *R*. solani where it was observed that a statistically significant decrease in the levels of *R. solani* were recorded on daughter tubers grown in compost treated with 250 ppm PEITC, 1 ppm BITC and 1 ppm AITC. However no significant decrease was observed when compost was incorporated with 250 ppm PEITC alone. It is unclear at this stage why the addition of alternative ITCs at low concentrations would have such a large effect on pathogen suppression, however it again appears to be dependent both on the specific structure of the ITC and the pathogen involved.

Results from glasshouse experimentation suggested that although decreases in the incidence of *C. coccodes* may be observed through the application of ITCs, such decreases were not statistically significant when compared to daughter tubers from untreated control plots. Yet is has often been hypothesised that green manure application may produce higher levels of pathogen suppression than the sole application of ITCs, due to the incorporation of large amounts of organic matter into the soil, which can lead to increased numbers of beneficial, pathogen suppressing bacteria and fungi (Ibekwe *et al.* 2001; Friberg *et al.* 2009).

Analysis of GC-MS results in light of observations made in Chapter 3 indicates a number of plant cultivars that may provide supportive levels of control of the individual fungal potato pathogens. Overall PEITC was shown to produce a level of control in all three pathogens studied. Results from *C. coccodes* studies showed a fungistatic response lasting seven days when exposed to concentrations of 12.5 and 25 ppm. *R. solani* displayed fungistatic responses lasting six days whereas *H. solani* cultures displayed a fungistatic response lasting 35 days when exposed to 3.125 ppm. No growth was present on plates incorporated with higher concentrations. Analysis of GC-MS assays, suggests that C. mustard 99 and Forage Rape Hobson if incorporated at the correct development stage and in such a way to ensure maximum ITC release would produce fungitoxic response in *R. solani* and *H. solani* and a delayed growth response in *C. coccodes* pathogens. However glasshouse experiment results suggested that pathogens might not be as susceptible to ITCs when incorporated into compost, perhaps due to ITCs binding with organic matter within the compost. Yet results from glasshouse experimentation suggest that *R. solani* levels may be reduced by the application of high concentration of PEITC in combination with lower concentrations of AITC and BITC. Therefore from GC-MS results it may be suggested that the incorporation of Forage Rape Hobson may be most suitable to control *R. solani* levels.

Bioassay results also showed *R. solani* and *H. solani* growth may also be suppressed by exposure to BITC. Results for GC-MS analysis (Chapter 4) revealed that C. mustard 99 had the ability to produce relatively high concentrations of BITC. However here again glasshouse experiments did not show a significant decrease in *R. solani* levels when exposed to BITC solution, suggesting that pathogens are more difficult to control within compost, and additionally within field conditions.

7.1.2 Comparisons between results for *in vitro* and *in vivo* study

The studies presented in previous chapters aim to provide detailed analysis of the different aspects of the biofumigation system. It is recognised that glucosinolate hydrolysis, which occurs during the breakdown of *Brassica* tissues releases several products (Mattner *et al.* 2008). Of these, isothiocyanates have been identified to be the most toxic towards a wide range of different microorganisms (Drobnica *et al.* 1967; Mari *et al.* 1996; Sarwar *et al.* 1998; Sellam *et al.* 2006; Gimsing & Kirkegaard 2009). It is hoped that through understanding of the processes involved in this interaction that the process of biofumigation can be used to effectively control soil borne pests and pathogens. Reports have suggested that the interaction between ITCs and pathogens is specific and largely dependent on the structure of the ITC (Walker *et al.* 1937; Kirkegaard *et al.* 1996; Mancini *et al.* 1997). Therefore to establish the possibility of using such a system for pathogen control, it must be assessed both which ITCs have antifungal effects on fungal pathogens and which ITCs are released from *Brassica* tissues. Using *in vitro* methods, results indicated that indeed antifungal responses

observed in fungal potato pathogens were specific to the ITC (Chapter 3). Results from GC-MS analysis of *Brassica* plants which highlighted that the same ITCs (mainly allyl, 2-phenylethyl and benzyl) were produced through glucosinolate hydrolysis (Chapter 4). Yet it is well understood that *in vitro* results do not always transcribe well within field settings. To initially build on conclusions obtained from *in vitro* work, glasshouse experiments were used to analyse ITC potential to control potato soil borne pathogens, within controlled glasshouse conditions (Chapter 5). Again decreases in disease incidence of potato black dot and black scurf were observed in several treatments, however not to the same extent observed in *in vitro* agar diffusion studies.

Glasshouse experimentation assessing the diversity of soil microbial communities also revealed differences between results produced from field trial studies. Generally no diversity change was observed in results produced in glasshouse experiments; however principal coordinate analysis of data from field trials revealed that there was a trend towards a decrease in the overall bacterial diversity after biofumigation. Importantly it should be noted that post biofumigation field soil samples were only taken at one time point after biofumigation (8 weeks), this time period could unfortunately not be sampled in the glasshouse experimentation, due to availability of glasshouse space. However the early time periods indicated no change in diversity in glasshouse experimentation. As the glasshouse experiment only examined the incorporation of ITCs in solution and field trials assessed bacterial diversity changes after green manure incorporation, it can be suggested that the incorporation of organic matter (plant tissue) has a greater impact on the soil bacterial communities that ITCs alone.

7.1.3 Effectiveness of biofumigation as an alternative control strategy based on results from this study

Results from this study indicate that although ITCs possess antifungal properties towards the fungal potato pathogens included in this study, implementing a biofumigation system in which ITCs are released to suppress fungal growth may not produce the desired magnitude of pathogen suppression. In this study inconsistencies of pathogen suppression may be due to low concentrations of ITCs released during glucosinolate hydrolysis. Such low levels of ITCs which differed significantly from identical cultivars grown under glasshouse conditions (Chapter 4) may be due to abiotic factors encountered during growth within the field. Such abiotic factors may also lead to reduced toxicity of ITCs during the fumigation process. Until the relationship between environmental factors, glucosinolate accumulation and hydrolysis and the resulting soil fumigation is fully understood it will be difficult to predict the full potential of pathogen suppression using biofumigation. As published data has produced evidence that biofumigation can successfully be used to control several fungal pathogens (Petersen *et al.* 2001; Chung *et al.* 2002; Friberg *et al.* 2009), it was expected that results from this experiment would show a reduced level in *C. coccodes*.

However many authors have failed to assess the interaction that may be occurring between the ITCs and the soil microbial communities. It is well understood that some soil bacterial and fungal species are antagonistic towards several pathogens (Ibekwe *et al.* 2001; Friberg *et al.* 2009). Therefore the prospect that ITCs are influencing the soil microbial community allowing more beneficial fungi and bacteria to establish must be considered when assessing the pathogen suppression effects seen to be caused through the biofumigation process. The results from this study indicate that biofumigation using the cultivar treatments from the field trials will not effectively eliminate *C. coccodes* from field soil. However the use of different cultivars which possess glucosinolate profiles more resistant to the effects of abiotic factors, including moisture and temperature, may produce different results. *C. coccodes* is known to be notoriously difficult to control using traditional control methods, and therefore the pathogen itself may possess attributes which prevent effective control.

7.2 Future perspectives in biofumigation research

7.2.1 Future research that continue to assess the effectiveness of biofumigation

Biofumigation is being increasingly researched as an alternative control strategy to manage a range of different soil borne pests and pathogens. With increased research comes growing understanding of the processes involved in such practice. This will hopefully filter down to companies who successfully aim to breed effective biofumigant crops and therefore aid growers to successfully introduce this technology as part of their disease management programme. Such research, which is further emphasised by this study, highlights that the interaction that occurs between pathogens and toxic hydrolysis products is very specific, therefore suggesting that pathogens must be targeted through the release of certain ITCs. Results from this study suggest that in the case of fungal potato pathogens, some ITCs may have a broader antifungal effect than others (Chapter 3). However it is recognised that it is necessary to understand the ITC release potential from *Brassica* cultivars and therefore the biofumigation potential of individual *Brassica* cultivars. Findings from this study (Chapter 3 & 4) also suggest that it may be most effective to grow a mix of different *Brassica* cultivars, which when incorporated into the soil will release high concentrations of a range of different ITCs, therefore increasing the ability to suppress and eliminate growth a range of different soil borne pathogens.

Although findings from this study indicate that ITCs possess antimicrobial properties towards different potato pathogens, it appears that the level of antimicrobial activity may be lost when ITCs are incorporated into the soil matrix. Therefore perhaps while continuing work is carried out to investigate the responses between specific ITCs and pathogens, and determining hydrolysis product profiles of different *Brassica* cultivars is important. In order to achieve effective biofumigation it is equally imperative that

detailed research is carried out on assessing depletion rates of ITCs and the most efficient methods to prolong contact with pathogens. Although some studies investigating ITC degradation within soil have been carried out (Gan *et al.* 1999; Gimsing *et al.* 2007), it appears that no definitive guidelines of how to reduce this have been presented to growers. Research should be carried out at field scale, to determine the most effective methods to seal ITCs into the soil, to aid contact time with pathogens, in a bid to increase the antimicrobial effects.

The high volatility level of ITCs has often been highlighted as an aspect which may limit the efficiency of a biofumigation system (Gimsing & Kirkegaard 2006). However the biofumigation principal works on the 'mustard bomb' effect, releasing a short blast of ITCs at high concentrations which aims to kill soil borne pathogens within the soil. It is also hoped that this approach will limit any adverse effects on non-targeted soil bacteria or fungi. However investigating an incorporation method which will best seal ITCs into the soil, and limit their initial depletion will allow them to come into contact with increased numbers of pathogens within the soil.

It is also important to investigate incorporation methods which will achieve the most efficient tissue breakdown ensuring the maximum release of myrosinase and glucosinolates, in order for them to come into contact with each other, producing the highest concentrations of ITCs possible. As well as evaluating the most effective methods of incorporating green manures to maximise ITC release, other methods which are being used within biofumigation systems should also be studied for their effectiveness in pathogen suppression. The incorporation of seed meals and dried *Brassica* plant material are being increasingly used in the USA for biofumigation, as such products are produced as by-products of Canola oil production (Kumar *et al.* 2011). If such materials can effectively be used to control soil borne pathogens within a biofumigation system then this may assist to minimise the costs of using an alternative control measure for pathogen control. However it is often suggested, and as discussed in Chapter 6 (Gimsing & Kirkegaard 2006), the addition of green manures and organic matter into soil may lead to improved soil health, and thus improve the quality and yield of future crops grown on the land. Therefore the incorporation of green manures into soil may have additional beneficial effects beyond pathogen control. However further study must be carried out to determine this, as results from this study suggested that the incorporation of green manures may lead to a decrease in diversity levels of soil bacteria. Nevertheless detailed analysis is required to assess the particular bacteria groups which are affected by green manure incorporation; in doing so their recovery rates can also be monitored to determine if biofumigation has lasting effects on soil microbial community composition.

7.2.5 Biofumigation may more affected by biology than fumigation

Results from study and previous work carried out by others has often shown that results from field that have shown the incorporation of green manures to have significant effects on soil borne pathogen levels (Price *et al.* 2005; Mattner *et al.* 2008). However when compared to chemical analysis of the ITCs released from the incorporated cultivars, or analysis of ITC concentrations released into the field soil, they often do not compare. In fact they are much lower than those required to achieve the same levels of control, in glasshouse or *in vitro* studies. Such results and high levels of pathogen suppression could be accounted for by the release of other hydrolysis products, such as thiocyanates and nitriles (Buskov *et al.* 2002; Gimsing & Kirkegaard 2006). However several studies have shown that their toxicity towards microorganisms is much lower than ITCs (Petersen *et al.* 2001; Mattner *et al.* 2008). With this in mind, growing emphasis is being placed on understanding the biofumigation process as one that achieves observed results by altering biological activities within soil. Research has shown that levels of pathogen suppression are much greater in soil with an active microbial community, when compared to carrying out biofumigation in a pasteurized soil (Cohen *et al.* 2005; Matthiessen & Shackleton 2005; Friberg *et al.* 2009; Larkin *et al.* 2010). Traditionally the phenomenon of pathogen suppression that is observed when biofumigants, seed meals or dried plant materials are incorporated into soil, are attributed to ITC release may in fact be linked to a number of different factors. Although the dominant functional mechanism will alter between the type of cultivar incorporated and the targeted pathogen. Evidence is mounting that such positive results observed after the practice of biofumigation, may have limited connectivity within the incorporation of ITCs and secondary glucosinolate hydrolysis products. However it is easy to understand why this aspect of the biofumigation process has been largely ignored, due to the complexity of soil and the difficulties encountered when trying to assess processes within it, particularly those associated with microbial communities. However with advances in molecular ecology it is hoped that future techniques will allow further assessment of the soil microbial processes in response to biofumigation. Perhaps emphasis has largely been placed on the toxicity of ITCs, as they are easier entity to study than soil, and within laboratory experiments they showed high levels of toxicity towards pathogens. However if experiments have shown to lose a high proportion of ITCs from the soil, but still produce significant levels of soil pathogen suppression then further processes that are aiding this suppression, which may be indirectly related to ITC release, must be investigated. To evaluate the true potential of a biofumigant the activity spectrum, mode of action and influence to the soil system of ITCs must be understood.

7.3 Concluding remarks

From the data produced by the studies above, it is clear that isothiocyanates do possess antifungal properties towards soil borne potato fungal pathogens. Yet the results also conclude that the interaction between isothiocyanates and pathogens is very specific and small changes to the molecular structure of the isothiocyanate can alter its potential toxicity greatly. Results have also shown the variation in ITC production that occurs between different *Brassica* cultivars, and how this may be affected by growing conditions. In summary although the components for achieving pathogen suppression through ITC release from glucosinolate hydrolysis appear to be present the difficulty of implementing biofumigation within a field setting may lie within the specific nature of this system.

However this study also aims to highlight the importance of examining alternative pathogen control strategies fully and to gain further understanding of all processes and interactions involved. It has identified that the biofumigation process may in fact lead to improved soil health and as a result produce naturally occurring pathogen antagonistic bacteria and fungi. It also shows that some pathogens may be more respondent to certain control methods than others. It is anticipated that this study will lead to future work which may lead to develop biofumigants that can successfully release ITCs which will cause pathogen suppression and studies which will further assess the impact such processes have on the important microbial soil community. Additionally it is hoped that this study will emphasise the need for research into alternative control strategies for pathogen control and push for future work and education into IPM strategies. It is believed that collaboration of research on both synthetic and natural control programmes will lead to less environmentally damaging cropping systems.

To continue to comply with legislation on the use of pesticides and fumigants it will become increasingly important for growers to adopt new strategies for pest and pathogen control. Perhaps the first emphasis should be placed on education both for the grower and the consumer, it appears that there is ever mounting pressure on growers to produce the perfect crop with little application of synthetic pesticides. If growers can be informed of their options and be provided with assistance to implement such strategies, while consumers education is being implemented to allow them not to continually expect perfect produce, then alternative control strategies may be more widely adopted, and we may begin to implement a sustainable farming future.

References

Abdo Z, Schuette UME, Bent SJ, Williams CJ, Forney LJ & Joyce P 2006 Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16s rRNA genes. Environmental Microbiology **8** 929–938.

Acosta-Martinez V, Dowd S, Sun Y & Allen V 2008 Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology & Biochemistry **40** 2762–2770.

Aires A, Carvalho R, Barbosa D, C M & Rosa E 2009 Suppressing potato cyst nematode, *Globodera rostochiensis*, with extracts of Brassicacea plants. American Journal of Potato Research **86** 327–333.

Al-Gendy AA & Lockwood GB 2003 GC-MS analysis of volatile hydrolysis products from glucosinolates in *Farsetia aegyptia* var. *ovalis*. Flavour and Fragrance Journal **18** 148–152.

Al-Mughrabi KI 2010 Biological control of *Fusarium* dry rot and other potato tuber diseases using *Pseudomonas fluorescens* and *Enterobater cloacea*. Biological Control 53 280–284.

Anderson A N 1982 The genetics and pathology of *Rhizoctonia solani*. Annual Review of Phytopathology **20** 329–347.

Andrivon D, Lucas JM, Guerin C & Jouan B 1998 Colonization of roots, stolons, tubers and stems of various potato (*Solanum tuberosum*) cultivars by the black-dot fungus *Colletotrichum coccodes*. Plant Pathology **47** 440–445.

Angus JF, Gardner PA, Kirkegaard JA & Desmarchelier JM 1994 Biofumigation: Isothiocyanates released from *Brassica* roots inhibit growth of the take-all fungus. Plant and Soil **162** 107–112.

Anon 2011 Economic Report on Scottish Agriculture 2011 Edition - A National Statistics Publication for Scotland. Ed TS Government. www.scotland.gov.uk.

Anon 2012 Wisconsin veterinary diagnostics laboratory - Real Time PCR.

Bafti SS, Bonjar GHS, Aghighi S, Biglari S, Farrokhi PRF & Aghelizadeh A 2005 Biological control of *Fusarium oxysporum* f.sp. *melonis*, the causal agent of root rot disease of greenhouse cucurbits in Kerman province of Iran. American Journal of Biochemistry and Biotechnology **1** 22–26.

El Balkali AM & Martin MP 2006 Black scurf of potato. Mycologist **20** 130–132.

Banville GJ 1989 Yield losses and damage to potato plants caused by *Rhizoctonia solani* Kuhn. American Potato Journal **66** 821–834.

Beard J 2006 DDT and human health. *The science of the Total Environment* **355** 78-79.

Bellostas N, Sorensen JC & Sorensen H 2007 Profiling glucosinolates in vegetative and reproductive tissues of four *Brassica* species of the U-triangle for their biofumigation potential. Journal of the Science of Food and Agriculture **87** 1586–1594.

Bellostas N, Kachlicki P, Sorensen C J & Sorensen H 2007 Glucosinolate profiling of seeds and sprouts of *B. oleracea* varieties used for food. Scientia Horticulturae **114** 234–242.

Bending GD & Lincoln SD 2000 Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. Soil Biology and Biochemistry **32** 1261–1269.

Bennett T L, Kasel S & Tibbits J Non-parametric multivariate comparisons of soil fungal composition: sensitivity to thresholds and indications of structural redundancy in t-rflp data. *Soil Biology & Biochemistry* **40** 1601–1611.

Bernard E, Larkin P R, Tavantzis S, Erich S M, Alyokhin A, Sewell G, Lannan A & Gross D S 2011 Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems. Applied Soil Ecology **52** 29–41.

Bianco V, Niholls J, Mattner S, Allen D & Porter I 2000 Biofumigation in Australian horticulture: A integrated approach to MB replacement. Agriculture Victoria 4.

Blackwood CB 2006 Analysing microbial community structure by means of terminal restriction fragment length polymorphism (T-RFLP). In Molecular Approaches to Soil, Rhizosphere and Plant Microorganism Analysis, pp 84–98. Eds JE Cooper & JR Rao. CABI Publishing.

Blackwood CB & Buyer JS 2006 Evaluating the physical capture method of terminal restriction fragment length polymorphism for comparison of soil microbial communities. Soil Biology and Biochemistry **39** 590–599.

Blau PA, Feeny P, Contardo L & Robson DS 1978 Allyl glucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. Science **200** 1296–1298.

Bones AM & Rossiter JT 1996 The myrosinase-glucosinolate system, its organisation and biochemistry. Physiologia Plantarum **97** 194–208.

Borek V & Morra MJ 2005 Ionic thiocyanate (SCN-) production from 4-hydroxybenzyl glucosinolate contained in *Sinapis alba* seed meal. Journal of Agriculture and Food Chemistry **53** 8650–8654.

Borek V, Elberson LR, MaCaffrey JP & Morra MJ 1998 Toxicity of isothiocyanates produced by glucosinolates in *Brassicaceae* species to black vine weevil eggs. Journal of Agriculture and Food Chemistry 46 5318–5323.

Bourderioux A, Lefoix M, Gueyrard D, Tatibouet A, Cottaz S, Artz S, Burmeister WP & Rollin P 2005 The glucosinolate-myrosinase system. New insights into enzyme-substrate interactions by use of simplified inhibitors. Organic Biomolecular Chemistry **3** 1872–1879.

Brewer MT & Larkin RP 2005a Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. Crop Protection **24** 939–950.

Brierley JL, Stewart JA & Lees AK 2009 Quantifying potato pathogen DNA in soil. Applied Soil Ecology **41** 234–238.

Brown KK & Hampton MB 2011 Biological targets of isothiocyanates. Biochimica *et.*Biophysica Acta **1810** 888–894.

Brown PD & Morra MJ 1996 Control of soil-borne plant pests using glucosinolate-containing plants. Advances in Agronomy **61** 167–231.

Brown PD & Morra MJ 1997 Hydrolysis products of glucosinolates in *Brassica napus* tissues as inhibitors of seed germination. Plant and Soil **181** 307–316.

Brown PD, Tokuhisa JG, Reichelt M & Gershenzon J 1991 Variation of glucosinolate accumulation among different organs and development stages of *Arabidopsis thaliana*. Phytochemistry **62** 471–481.

Burmeister P W, Cottaz S, Driguez H, Lori R, Palmieri S & Henrissat B 1997 The crystal structures of S*inapis alba* myrosinase and a covalent glycosyl-enzyme intermediate provide insights into the substrate recognition and active-site machinery of an S-glycosidase. Structure **5** 663–675.

Buskov S, Serra B, Rosa E, Sorensen H & Sorensen JC 2002 Effects of intact glucosinolates and products produced from glucosinolates in myrosinase-catalysed hydrolysis on the potato cyst nematode (G*lobodera rostocheinsis* cv woll). Journal of Agricultural and Food Chemistry **50** 690–695.

CABI 2008 European initiatives - pesticide news **79**. Europe Turns the Tide on Methyl Bromide.

Cahill G, Frazer K, Kowalewska J M, Kenyon M D & Saddler S G 2010 Recent findings from the *Dickeya* survey and monitoring programme. Proceedings Crop Protection in Northern Britain 2010 - The Dundee Conference 171–175.

Carling E D, Leiner H R & Westphale C P 1989 Symptoms, signs and yield reduction associated with *Rhizoctonia* disease of potato induced by tuber-borne inoculum of *Rhizoctonia-solani* AG-3. American Potato Journal **66** 693–701.

Carter GA, Garraway JL, Spencer DM & Wain RL 1963 Investigations on fungicides vi. The antifungal activity of certain dithiocarbamic and hydroxydithioformic acid derivatives. Annals of Applied Biology **51** 135–151.

Choesin DN & Boerner RET 1991 Allyl isothiocyanate release and the alleopathic potential of *Brassica napus* (Brassicaceae). American Journal of Botany **78** 1083–1090.

Chung WC, Huang JW, Huang HC & Jen JF 2002 Effect of ground *Brassica* seed meal on control of *Rhizoctonia* damping-off of cabbage. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie **24** 211–218.

Cohen MF, Yamasaki H & Mazzola M 2005 Brassica napus seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. Soil Biology & Biochemistry **37** 1215–1227.

Cole RA Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in cruciferae. Phytochemistry **15** 759–762.

Compant S, Clement C & Sessitsch A 2010 Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology & Biochemistry **42** 669–678.

"Council Directive 2007/33/EC" 2007. In Official Journal of the European Union.

Cullen DW, Lees AK, Toth IK & Duncan JM 2002 Detection of colletotrichum coccodes from soil and potato tubers by conventional and quantitative real-time PCR. Plant Pathology **51** 281–292.

Cummings TF & Johnson DA 2008 Effectiveness of early-season, single applications of azoxystrobin for the control of potato black dot as evaluated by three assessment methods. American Journal of Potato Research **85** 422–431.

Cunnington AC 2008 Developments in potato storage in Great Britain. Potato Research **51** 403–410.

De Curtis F, Lima G, Vitullo D & De Cicco V 2010 Biocontrol of *Rhizoctonia solani* and *Sclerotium rolfsii* on tomato by delivering antagonistic bacteria through a drip irrigation system. Crop Protection **29** 663–670.

Dawson GW, Hick AJ, Bennett RN, Donald A, Pickett JA & Wallsgrove RM 1993 Synthesis of glucosinolate precursors and investigations into the biosynthesis. The Journal of Biological Chemistry **268** 27154–27159.

Dhingra OD, Costa MLN & J SJG 2004 Potential of allyl iosthiocyanate to control *Rhizoctonia solani* seedling damping off and seedling blight in transplant production. Journal of Phytopathology **152** 352–357.

Doran JW & Zeiss MR 2000 Soil health and sustainability: managing the biotic component of soil quality. Applied Soil Ecology **15** 3–11.

Doughty KJ, Porter AJR, Morton AM, Kiddle G, Bock CH & Wallsgrove R Variation in the glucosinolate content of oilseed rape (*Brassica napus* l.) leaves. ii. Response to infection by *Alternaria brassicae* (berk.) sacc. Annals of Applied Biology **118** 469–477.

Drobnica l, Zemanova M, Nemec P, Antos K, Kristian P, Stullerova A & Knoppova V 1967 Antifungal activity of isothiocyanates and related compounds. Applied Microbiology **15** 701–709.

Dungan RS, Gan J & Yates SR 2003 Accelerated degradation of methyl isothiocyanate in soil. Water, Air and Soil Pollution **142** 299–310.

Egamberdieva D 2008 Plant growth promoting properties of rhizobacteria isolated from wheat and pea grown in loamy sand soil. Turkish Journal of Biology **32** 9–15.

Egamberdiyeva D & Hoflich G 2003 Influence of growth-promoting bacteria on the growth of wheat in different soils and temperatures. Soil Biology & Biochemistry **35** 973–978.

Elfstrand S, Hedlund K & Mårtensson A 2007 Soil enzyme activities, microbial community composition and function after 47 years of continuous green manuring. Applied Soil Ecology **35** 610–621.

Elson MK, Schisler DA & Bothast RJ 1997 Selection of microorganisms for biological control of silver scurf (*Helminthosporium solani*) of potato tubers. Plant Disease **81** 647–652.

Errampalli D, Saunders JM & Holley JD 2001a Emergence of silver scurf (*Helminthosporium solani*) as an economically important disease of potato. Plant Pathology **50** 141–153.

Errampalli D, Saunders J & Cullen D 2001b A PCR based method for detection of potato pathogen, *Helminthosporium solani*, in silver scurf infected tuber tissue and soils. Journal of Microbiological Methods **44** 59–68.

Fahey JW, Zalcmann AT & Talalay P 2001 The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56** 5–51.

Fairfax MR & Salimnia H 2010 Quantitative PCR: An introduction. In Molecular Diagnostics Techniques and Applications for the Clinical Laboratory, pp 3–14. Eds WW Grody, RM Nakamura, MD Strom & FL Kiechie. Detroit: Academic Press.

Fan M C, Xiong R G, Qi P, Jr H G & He Q Y 2008 Potential biofumigation effects of *Brassica oleracea* var. caulorapa on growth of fungi. Journal of Phytopathology **156** 321–325.

Fenwick GR & Heaney RK 1983 Glucosinolates and their breakdown products in Cruciferous crops, foods and feedingstuffs. Food Chemistry **11** 249–271.

Fierer N & Jackson RB 2006 The diversity and biogeography of soil bacterial communities. PNAS **103** 626–631.

Fiers M, Edel-Hermann V, Chatot C, Le Hingrat Y, Alabouvette C & Steinberg C 2012 Potato soil-borne diseases. A review. Agronomy For Sustainable Development **32** 93–132.

Filip Z 2002 International approach to assessing soil quality by ecologically-related biological parameters. Agriculture Ecosystems and Environment **88** 169–174.

Finn RK 1959 Theory of agar diffusion methods for bioassay. Analytical Chemistry **31** 975–977.

Fitzpatrick KA, Kersh GJ & Massung RF 2010 Practical method for extraction of PCR-quality DNA from environmental soil samples. Applied and Environmental Microbiology **76** 4571–4573.

Frazier MJ, Shetty KK, Kleinkopf GE & Nolte P 1998 Management of silver scurf (*Helminthosporium solani*) with fungicide seed treatments and storage pesticides. American Journal of Potato Research **75** 129–135.

Friberg H, Edel-Hermann V, Faivre C, Gautheron N, Fayolle L, Faloya V, Montfort F & Steinberg C 2009 Cause and duration of mustard incorporation effects on soil-borne plant pathogenic fungi. Soil Biology & Biochemistry **41** 2075–2084.

Gan J, Papiernik SK, Yates SR & Jury WA 1999 Temperature and moisture effects on fumigant degradation in soil. Journal of Environmental Quality **28** 1436–1441.

Gardiner JB, Morra MJ, Eberlein C V, Brown PD & Borek V 1999 Alleochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. Journal of Agriculture and Food Chemistry **47** 3837–3842.

Gilligan CA, Simons SA & Hide GA 1996 Inoculum density and spatial pattern of *Rhizoctonia solani* in field plots of *Solanum tuberosum*: Effects of cropping frequency. Plant Pathology **45** 232–244.

Gimsing AL & Kirkegaard JA 2006 Glucosinolate and isothiocyanate concentration in soil following incorporation of *Brassica* biofumigants. Soil Biology and Biochemistry 38 2255–2264.

Gimsing AL & Kirkegaard JA 2009 Glucosinolates and biofumigation: Fate of glucosinolates and their hydrolysis products in soil. Phytochemistry Reviews **8** 299–310.

Gimsing AL, Kirkegaard JA & Hansen HCB 2005 Extraction and determination of glucosinolates from soil. Journal of Agricultural and Food Chemistry **53** 9663–9667.

Gimsing AL, Poulsen AL & Hansen HCB 2007 Formation and degradation kinectics of the biofumigant benzyl isothiocyanate in soil. Environmental Science and Technology **41** 4271–4276.

Glais-Varlet I, Bouchek-Mechiche K & Andrivon D 2004 Growth *In vitro* and infectivity of *Colletotrichum coccodes* on potato tubers at different temperatures. Plant Pathology **53** 398–404.

Griffiths I R, Thomson C B, James P, Bell T, Bailey M & Whiteley S A 2011 The bacterial biogeography of British soils. Environmental Microbiology **13** 1–13.

Haas D & Défago G 2005 Biological control of soil-borne pathogens by fluorescent *Pseudomonads*. Nature Reviews. Microbiology **3** 307–319.

Halkier BA & Du LC 1997 The biosynthesis of glucosinolates. Trends in Plant Science **2** 425–431.

Harvey SG & Sams CE 2000 Allyl isothiocyanate released from *Brassica juncea* suppresses mycelial growth of *Sclerotium rolfsii*. Methyl Bromide Alternative Outreach 3.

Haughn GW, Davin L, Giblin M & Underhill EW 1991 Biochemical genetics of plant secondary metabolites in *Arabidopsis thaliana*. Plant Physiology **97** 217–226.

Heid CA, Stevens J, Livak KJ & Williams PM 1995 Real time quantitative PCR. Genome Research **6** 986–994.

Henderson DR, Riga E, Ramirez RA, Wilson J & Snyder WE 2009 Mustard biofumigation disrupts biological control by *Steinernema* spp. nematodes in the soil. Biological Control **48** 316–322.

Henry M, Béguin M, Requier F, Rollin O, Odoux JF, Aupinel P, Aptel J, Tchamitchian S & Decourtye A 2012 A common pesticide decreases foraging success and survival in honey bees. Science **336** 348–350.

Hide GA, Hirst JM & Stedman OJ 1973 Effects of black scurf (*Rhizoctonia solan*i) on potatoes. Annals of Applied Biology **74** 139–148.

Hill B C & Anderson A N 1989 An evaluation of potato disease caused by isolates of *Rhizoctonia solani* AG-3. American Potato Journal **66** 709–721.

Hites RA Gas chromatography mass spectrometry. In Handbook of Instrumental Techniques for Analytical Chemistry, pp 609–626. Ed F Settle. Indiana University.

Holst B & Williamson G 2004 A critical review of the bioavailability of glucosinolates and related compounds. Natural Product Reports **21** 425–447.

Ibekwe AM, Papiernik SK, Gan J, Yates SR, Yang CH & Crowley DE 2001 Impact of fumigants on soil microbial communities. Applied and Environmental Microbiology **32** 45–3257.

Ingram J & Johnson DA 2010 Colonization of potato roots and stolons by *Colletotrichum coccodes* from tuber borne inoculum. American Journal of Potato Research **87** 382–389.

Ingram T J 2008 Spread of *Colletotrichum coccodes* from infected potato seed tubers and effect of fungicides on stem infection. In Department of Plant Pathology. Washington: Washington State University.

Inyang EN, Butt TM, Doughty KJ, Todd AD & Archer S 1999 The effects of isothiocyanates on the growth of the entomopathogenic fungus *Metahizium anisopliae* and its infection of the mustard beetle. Mycology Research **103** 974–980.

Ji P, Momol MT, Olson SM, Pradhanang PM & Jones JB 2005 Evaluation of thymol as biofumigant for control of bacterial wilt of tomato under field conditions. Plant Disease **89** 497–500.

Johnson DL, Ambrose SH, Bassett TJ, Bowen ML, Crummey DE, Isaacson JS, Johnson DN, Lamb P, Saul M & Winter-Nelson AE 1997 Meanings of environmental terms. Journal of Environment Quality **26** 581.

Kelly RM, Cahill G, Elphinstone JG, Mitchell WJ, Mulholland V, Parkinson NM, Pritchard L, Toth IK & Saddler GS 2012 Development of a real-time PCR assay for the detection of "*Dickeya solani*". Proceedings Crop Protection in Northern Britain 2012 201–206.

Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H & Trevors JT 2004 Method of studying soil microbial diversity. Journal of Microbiological Methods **58** 169–188.

Kirkegaard JA & Sarwar M 1998 Biofumigation potential of *Brassicas* I. variation in glucosinolate profiles of diverse field-grown *Brassicas*. Plant and Soil **201** 71–89.

Kirkegaard JA, Wong PTW & Desmarchelier JM 1996 *In vitro* suppression of fungal root pathogens of cereals by *Brassica* tissues. Plant Pathology **45** 593–603.

Kirkegaard JA, Sarwar M, Wong PTW & Mead A 1998 Biofumigation by *Brassicas* reduces take-all infection. In 9th Austrailian Agronomy Conference, pp 465–468. Eds DL Michalle & JE Pratley. Austrailia: Agronomy - growing a greener future.

Kirkegaard JA, Matthiessen JN, Wong PTW, Mead A, Sarwar M & Smith BJ 1999 Exploiting the biofumigation potential of *Brassicas* in farming systems. 10th International Rapeseed Congress.

Kirkegaard JA, Sarwar M, Wong PTW, Mead A, Howe G & Newell M 2000 Field studies on the biofumigation of take-all by *Brassica* break crops. Australian Journal of Agricultural Research **51** 445–456.

Kirkegaard A J, Gardener A P, Desmarchelier M J & Angus F J 1993 Biofumigation - using *Brassica* species to control pests and diseases in horticulture and agriculture. In 9th Austrailian Research Assembly on Brassicas, pp 77–82. Eds N Wratten & R Mailer. Wagga Wagga.

Kliebenstein DJ, Kroymann J & Mitchell-Olds T 2005 The glucosinolate-myrosinase system in an ecological and evolutionary context. Current Opinion in Plant Biology **8** 264–271.

Koch C A 1994 Growth measurment. In Methods for General and Molecular Bacteriology. Eds P Gerhardt, R Murray G, E, W Wood A & N Krieg R. Washington: ASM Press.

Kogan M 1998 Integrated pest management: Historical perspectives and contemporary developments. Annual Review of Entomology **43** 243–270.

Korthals GW, Smilauer P, Van Dijk C & Van der Putten WH 2001 Linking above- and below-ground biodiversity: Abundance and trophic complexity in soil as a response to experimental plant communities on abandoned arable land. Functional Ecology **15** 506–514.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Stahlberg A, Zoric N 2006 The real-time polymerase chain reaction. Molecular Aspects of Medicine **27** 95–125.

Kumar R, Kumar S, Sangwan S, Yadav IS & Yadav R 2011 Protein modeling and active site binding mode interactions of myrosinase-sinigrin in *Brassica juncea*-an *In silico* approach. Journal of Molecular Graphics and Modelling **29** 740–746.

Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ & Gershenzon J 2001 The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. The Plant Cell **13** 2793–2807.

Larkin RP & Griffin TS 2007 Control of soilborne potato disease using *Brassica* green manures. Crop Protection **26** 1067–1077.

Larkin RP, Griffin TS & Honeycutt CW 2010 Rotation and cover crop effects on soil borne potato diseases, tuber yield, and soil microbial communities. Plant Disease **94** 1491–1502.

Lazzeri L, Curto G, Leoni O & Dallavalle E 2004 Effects of glucosinolates and their enzymatic hydrolysis products via myrosinase on the root-knot nematode *Meloidogyne incognita* (Kofoid et white) chitw. Journal of Agricultural and Food Chemistry **52** 6703–6707.

Lazzeri L, Curto G, Dallavalle E, D'Avino L, Malaguti L, Santi R & Patalano G 2009 Nematicidal efficacy of biofumigation by defatted Brassicaceae meal for control of *Meloidogyne incognita* (Kofoid *et* White) chitw. on a full field zucchini crop. Journal of Sustainable Agriculture **33** 349–358.

Lees AK & Hilton AJ 2003 Black dot (*Colletotrichum coccodes*): an increasingly important disease of potato. Plant Pathology **52** 3–12.

Lees AK, Cullen DW, Sullivan L & Nicolson MJ 2002 Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. Plant Pathology **51** 293–302.

Lees AK, Brierley JL, Stewart JA, Hilton AJ, Wale SJ, Gladders P, Bradshaw NJ & Peters JC 2010 Relative importance of seed-tuber and soil borne inoculum in causing black dot disease of potato. Plant Pathology **59** 693–702.

Lehtonen MJ 2009 *Rhizoctonia solani* as a potato pathogen - variation of isolates in Finland and host response. In Department of Applied Biology, Faculty of Agriculture and Forestry, p 81. Helsinki: University of Helsinki.

Leoni O, Iori R, Palmieri S, Esposito E, Menegatti E, Cortesi R & Nastruzzi C Myrosinase-generated isothiocyanate from glucosinolates: Isolation, characterization and *in vitro* antiproliferative studies. Bioorganic & Medicinal Chemistry **5** 1799–1806.

Leoni O, Lori R, Palmieri S, Esposito E, Menegatti E, Cortesi R & Nastruzzi C 1997 Myrosinase-generated isothiocyanate from glucosinolates: isolation, characterization and *in vitro* antiproliferative studies. Bioorganic and Medicinal Chemistry **5** 1799–1806.

Lin C, Kim J, Du W & Wej C 2000 Bacterial activity of isothiocyanate against pathogens on fresh product. Journal of Food Protection **63** 25–30.

Lisec J, Schauer N, Kopka J, Willmitzer L & Fernie AR 2006 Gas chromatography mass spectrometry-based metabolite profiling in plants. Nature Protocols **1** 387–396.

Magrath R, Herron C, Giamoustaris A & Mithen R 1993 The inheritance of aliphatic glucosinolates in *Brassica napus*. Plant Breeding **111** 55–72.

Malik MS, Riley MB, Norsworthy JK & W BJ 2010 Glucosinolate profile variation of growth stages of wild radish, (raphanus raphanistrum). Journal of Agricultural and Food Chemistry **58** 3309–3315.

Mancini LM, Lazzeri L & Sandro P 1997 *In vitro* fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. Journal of Agriculture and Food Chemistry **45** 2768–2773.

Mari M, Iori R, Leoni O & Marchi A 1996 Bioassays of glucosinolate-derived isothiocyanates against postharvest pear pathogens. Plant Pathology **45** 753–760.

Martinez C, Rioux D & Tweddell RJ 2004 Ultrastructure of the infection process of potato tuber by *Helminthosporium solani*, causal agent of potato silver scurf. Mycological Research **108** 828–836.

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon C J, Soulas G & Catroux G 2001 DNA extraction from soils: Old bias for new microbial diversity analysis methods. Applied and Environmental Microbiology **67** 2354–2359.

Matthiessen JN & Kirkegaard JA 2002 Potato grower's positive experiences with biofumigant green manure. Horticulture Biofumigation Update 2.

Matthiessen JN & Shackleton MA 2005 Biofumigation: environmental impacts on the biological activity of diverse pure and plant-derived isothiocyanates. Pest Management Science **61** 1043–1051.

Mattner SW, Porter IJ, Gounder RK, Shanks AL, Wren DJ & Allen D 2008 Factors that impact on the ability of biofumigants to suppress fungal pathogens and weeds of strawberry. Crop Protection **27** 1165–1173.

Mayton S H, Olivier C, Vaughn F S & Loria R 1996 Correlation of fungicidal activity of brassica species with allyl isothiocyanate production in macerated leaf tissue. The American Phytopathological Society **86** 267–271.

McCulley E M, Miller C, Sprague J S, Huang X C & Kirkegaard A J 2008 Distribution of glucosinolates and sulphur-rich cells in roots of field-grown canola (brassica napus). *New Phytologist* **180** 193–205.

Mercier J & Jimenez JI 2009 Demonstration of the biofumigation activity of *Muscodor albus* against *Rhizoctonia solani* in soil and potting mix. Biocontrol **54** 797–805.

Merz U & Falloon RE 2009 Review: powdery scab of potato - increased knowledge of pathogen biology and disease epidemiology for effective disease management. Potato Research **52** 17–37.

Ministry of Agriculture 1945 Allotment and garden guide. Ministry of Agriculture **1** 1–8.

Mojtahedi H, Santo S G, Hang N A & Wilson H J 1991 Suppression of root-knot nematode populations with selected rapeseed cultivars as green manure. Journal of Nematology **23** 170–174.

Morra MJ & Kirkegaard JA 2002 Isothiocyanate release from soil-incorporated *Brassica* tissues. Soil Biology and Biochemistry **34** 1683–1690.

Motisi N, Montfort F, Dore T, Romillac N & Lucas P 2009 Duration of control of two soil borne pathogens following incorporation of above and below ground residues of *Brassica juncea* into soil. Plant Pathology **58** 470–478.

Mullis KB & Faloona FA 1987 Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. Methods in Enzymology **155** 335–350.

Nitzan N, Cummings TF & Johnson DA 2005 Effect of seed-tuber generation, soil borne inoculum, and azoxystrobin application on development of potato black dot caused by *Colletotrichum coccodes*. Plant Disease **89** 1181–1185.

Nitzan N, Lucas S B & Christ J B 2006 Colonization of rotation crops and weeds by the potato black dot pathogen *Colletotrichum coccodes*. American Journal of Potato Research **83** 503–507.

Oka IN 1991 Success and challenges of the Indonesia national integrated pest management program in the rice-based cropping system. Crop Protection **10** 163–165.

Okubara PA, Schroeder KL & Patilitz TC 2008 Identification and quantification of *Rhizoctonia solani* and *R. oryzae* using real-time polymerase chain reaction. Phytopathology **98** 837–847.

Olivier C, Vaughn F S, Mizubuti S, G E & Loria R 1999 Variation in allyl isothiocyanate production within *Brassica* species and correlation with fungicidal activity. Journal of Chemical Ecology **25** 2687–2701.

Omirou M, Rousidou C, Bekris F, Papadopoulou KK, Menkissoglou-Spiroudi U, Ehaliotis C & Karpouzas DG 2011 The impact of biofumigation and chemical fumigation methods on the structure and function of the soil microbial community. Microbial Ecology **61** 201–213.

Parliament E 2009 Regulation (EC) no 1107/2009 of the European parliament and of the council. pp 1–50.

Parr F J, Papendick I R, Hornick B S & Meyer E R 1992 Soil quality: Attributes and relationship to alternative and sustainable agriculture. American Journal of Alternative Agriculture **7** 5–11.

Petersen J, Belz R, Walker F & Hurle K 2001 Weed suppression by release of isothiocyanates from turnip-rape mulch. Agronomy Journal **93** 37–42.

Philip S & Martin B 2002 PCN control - the alternative way to reduce potato cyst nematodes. Available: http://www.pcncontrol.co.uk/spudguard

Pinto S, Rosa E, Santos S, Thomas G & Monteiro AA 1998 Effect of 2-propenyl glucosinolate and derived isothiocyanate on the activity of the nematodes G*lobodera rostochiensis* (woll.). Brassica **97** 323–327.

Poulsen JL, Gimsing AL, Halkier BA, Bjarnholt N & Hansen HCB 2008 Mineralization of benzyl glucosinolate and its hydrolysis product the biofumigant benzyl isothiocyanate in soil. Soil Biology & Biochemistry **40** 135–141.

Price AJ, Charron CS, Saxton AM & Sams CE 2005 Allyl isothiocyanate and carbon dioxide produced during degradation of *Brassica juncea* tissue in different soil conditions. Horticultural Science **40** 1734–1739.

Rahmanpour S, Backhouse D & Nonhebel HM 2010 Reaction of glucosinolate-myrosinase defence system in *Brassica* plants to pathogenicity factor of *Sclerotinia sclerotiorum*. European Journal of Plant Pathology **128** 429–433.

Rask L, Andreasson E, Ekbom B, Eriksson S, Pontoppidan B & Meijer J 2000 Myrosinase: Gene family evolution and herbivore defence in *Brassicaceae*. Plant Molecular Biology **42** 93–113.

Ratnadass A, Fernandes P, Avelino J & Habib R 2012 Plant species diversity for sustainable management of crop pests and diseases in agroecosystems: a review. Agronomy for Sustainable Development **32** 273–303.

Rauf CA, Ahmad I & Ashravf M 2007 Anastomosis groups of *Rhizoctonia solani* Kuhn isolates from potato in Pakistan. Pakistan Journal of Botany **39** 1335–1340.

Read PJ & Hide GA 1995 Effects of fungicides on the growth and conidial germination of *Colletotrichum coccodes* and on the development of black dot disease in potatoes. Annals of Applied Biology **126** 437–447.

Ritchie F, McQuilken MP & Bain RA 2006 Effects of water potential on mycelial growth, sclerotial production, and germination of *Rhizoctonia solani* from potato. Mycological Research **110** 725–733.

Rosa A, S E 1997 Daily variation in glucosinolate concentrations in the leaves and roots of cabbage seedlings in two constant temperature regimes. Journal of the Science of Food and Agriculture **73** 364–368.

Ryu KY, Hahm Y, Kim JS & Park CS 2000 Silver scurf of potato caused by *Helminthosporium solani*. Plant Pathology **16** 318–320.

Sarwar M, Kirkegaard JA, Wong PTW & Desmarchelier JM 1998 Biofumigation potential of *Brassicas* iii. *In vitro* toxicity of isothiocyanates to soil-borne fungal pathogens. Plant and Soil **201** 103–112.

Schloter M, Dilly O & Munch JC 2003 Indicators for evaluating soil quality. Agriculture, Ecosystems and Environment **98** 255–262.

Secor GA & Gudmestad NC 1999 Managing fungal diseases of potato. Plant Pathology **21** 213–221.

Sellam A, Lacomi-Vasilescu B, Hudhomme P & Simoneau P 2006 *In vitro* antifungal activity of brassinin, camalexin and two isothiocyanates against the crucifer pathogens *Alternaria brassicicola* and *Alternaria brassicae*. Plant Pathology **56** 296–301.

Serra B, Rosa E, Iori R, Barillari J, Cardoso A, Abreu C & Rollin P 2002 *In vitro* activity of 2-phenylethyl glucosinolate, and its hydrolysis derivatives on the root-knot nematode *Globodera rostochiensis* (woll.). Scientia Horticulturae **92** 75–81.

Shen L, Su G, Wang X, Du Q & Wang K 2010 Endogenous and exogenous enzymolysis of vegetable-sourced glucosinolates and influencing factors. Food Chemistry **119** 987–994.

Singh BK, Millard P, Whiteley AS & Murrell JC 2004 Unravelling rhizosphere-microbial interactions: opportunities and limitations. Trends in Microbiology **12** 386–393.

Smalla K, Oros-Sichler M, Milling A, Heuer H, Baumgarte S, Becker R, Neuber G, Kropf S, Ulrich A & Tebbe CC 2007 Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16s rRNA gene fragments: do the different methods provide similar results? Journal of Microbiological Methods **69** 470–479.

Smith BJ & Kirkegaard JA 2002 *In vitro* inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. Plant Pathology **51** 585–593.

Smolinska U, Morra MJ, Knudsen GR & James RL 2003 Isothiocyanates produced by Brassicaceae species as inhibitors of *Fusarium oxysporum*. Plant Disease **87** 407–412.

Stapleton JJ & DeVay JE 1986 Soil solarization: A non-chemical approach for management of plant pathogens and pests. Crop Protection **5** 190–198.

Stapleton JJ, Elmore CL & DeVay JE 2000 Solarization and biofumigation help disinfest soil. California Agriculture **54** 42–45.

Stephens PM, Davoren CW & Wicks T 1999 Effect of methyl bromide, metham sodium and the biofumigants Indian mustard and Canola on the incidence of soil borne fungal pathogens and growth of grapevine nursery stock. Australasian Plant Pathology **28** 187–196.

Tajima H, Kimoto H, Taketo Y & Taketo A 1998 Effects of synthetic hydroxy isothiocyanates on microbial systems. Bioscience, Biotechnology and Biochemistry **62** 491–495.

Tani N, Ohtsuru M & Hata T 1974 Isolation of myrosinase producing microorganism. Agricultural and Biological Chemistry **38** 1617–1622.

Thies E J 2007 Soil microbial community analysis using terminal restriction fragment length polymorphisms. Soil Science Society of America Journal **71** 579–591.

Tiedje JM, Asuming-Brempong S, Nüsslein K, Marsh TL & Flynn SJ 1999 Opening the black box of soil microbial diversity. Applied Soil Ecology **13** 109–122.

Tilak KVBR, Ranganayaki N, Pal KK, De R, Saxena AK, Shekhar Nautiyal C, Mittal S, Tripathi AK & Johri BN 2005 Diversity of plant growth and soil health supporting bacteria. Current Science **89** 136–150.

Troncoso R, Espinoza C, Sanchez-Estrada A, Tiznado E M & Garcia S H 2005 Analysis of the isothiocyanates present i cabbage leaves extract and their potential application to control *Alternaria* rot in bell peppers. Food Research International **38** 701–708.

Tsror L, Barak R & Sneh B 2001 Biological control of black scurf on potato under organic management. Crop Protection **20** 145–150.

Velasco P, Cartea E M, Gonzalez C, Vilar M & Ordas A 2007 Factors affecting the glucosinolate content of kale (*Brassica oleracea* acephala group). Journal of Agricultural and Food Chemistry **55** 955–962.

Verkerk R, Schreiner M, Krumbein A, Ciska E, Holst B, Rowland I, De Schrijver R, Hansen M, Gerhauser C, Mithen R et al. 2009 Glucosinolates in brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. Molecular Nutrition & Food Research **53** 219–265**.**

Walker C J, Morell S & Foster H H 1937 Toxicity of mustard oils and related sulphur compounds to certain fungi. American Journal of Botany **24** 241–536.

Wang Y, Li Q, Shi J, Lin Q, Chen X, Wu W & Chen Y 2008 Assessment of microbial activity and bacterial community composition in the rhizosphere of a copper accumulator and non-accumulator. Soil Biology & Biochemistry **40** 1167–1177.

Watt M, Kirkegaard A J & Passioura B J 2006 Rhizosphere biology and crop productivity - a review. Australian Journal of Soil Research **44** 299-317

Van der Werf HMG 1996 Assessing the impact of pesticides on the environment. Agriculture, Ecosystems & Environment **60** 81–96.

Wharton P 2005 Fungicides - a practical approach to resistance management to potato diseases. Michigan Potato Diseases.

Widmer F, Rasche F, Hartmann M & Fliessbach A 2006 Community structures and substrate utilization of bacteria in soils from organic and conventional farming systems of the DOK long-term field experiment. Applied Soil Ecology **33** 294–307.

Wilson S P, Ahvenniemi M P, Lehtonen J M, Kukkonen M, Rita H & Valkonen P, T J 2008 Biological and chemical control and their combined use to control different stages of the *Rhizoctonia* disease complex on potato through the growing season. Annals of Applied Biology **153** 307–320.

Woodhall JW, Lees AK, Edwards SG & Jenkinson P 2007 Characterization of *Rhizoctonia solani* from potato in Great Britain. Plant Pathology **56** 286–295.

Yitbarek M S, Verma R P & Morrall A, A R 1987 Anastomosis groups, pathogenicity, and specificity of *Rhizoctonia solani* isolates from seedling and adult rapeseed canola plants and soils in Saskatchewan . Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie **9** 6–13.

Yulianti Y, Sivasithamparam K & Turner W D 2006 Response of different forms of propagules of *Rhizoctonia solani* AG2-1 (ZG5) exposed to the volatiles produced in soil amended with green manures. Annals of Applied Biology **148** 105–111.

Yulianti T, Sivasithamparam K & Turner DW 2006 Response of different forms of propagules of *Rhizoctonia solani* AG2-1 (ZG5) exposed to the volatiles produced in soil amended with, green manures. Annals of Applied Biology **148** 105–111.

Zasada IA & Ferris H 2004 Nematode suppression with Brassicaceous amendments: Application based upon glucosinolate profiles. Soil Biology & Biochemistry **36** 1017–1024.

Zasada IA, Masler EP, Rogers ST & Halbrendt JM 2009 Behavioural response of *Meloidogyne incognita* to benzyl isothiocyanate. Nematology **11** 603–610.

Appendices

Appendix 1

C. coccodes Bioassay results – ANOVA Least Significant Difference (1 % level)

1 day

Tables of means

Variate: %24

Grand mean 1.00

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		1.00	1.00	1.00	1.00	1.00
Allyl		1.00	1.00	1.00	1.00	1.00
Benzyl		1.00	1.00	1.00	1.00	1.00
Isopropyl		1.00	1.00	1.00	1.00	1.00
Methyl		1.00	1.00	1.00	1.00	1.00
Naphthyl		1.00	1.00	1.00	1.00	1.00
Propyl		1.00	1.00	1.00	1.00	1.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	*	*	*
s.e.d.	0.000	0.000	0.000

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	*	*	*
l.s.d.	*	*	*

4 days

Tables of means

Variate: %96

Grand mean 64.53

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		102.53	91.14	0.00	0.00	0.00
Allyl		106.75	97.47	82.28	103.48	0.00
Benzyl		95.85	91.71	23.32	0.00	0.00
Isopropyl		100.00	90.93	95.90	96.11	96.98
Methyl		85.57	88.66	76.29	22.68	0.00
Naphthyl		95.30	86.74	75.97	70.99	26.52
Propyl		94.68	93.62	86.70	80.32	0.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	2.633	2.225	5.888

(Not adjusted for missing values)

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	6.837	5.779	15.289

6 days

Tables of means

Variate: %144

Grand mean 68.47

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		100.00	92.71	0.00	0.00	0.00
Allyl		91.20	82.36	70.54	87.99	0.00
Benzyl		99.70	102.09	82.54	46.12	0.00
Isopropyl		99.82	90.94	98.22	96.09	101.42
Methyl		91.99	90.48	81.97	33.22	0.00
Naphthyl		95.22	87.11	79.63	75.88	19.96
Propyl		103.69	106.09	98.72	90.71	0.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	2.155	1.821	4.818

(Not adjusted for missing values)

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	5.595	4.729	12.511

8 days

Tables of means

Variate: %192

Grand mean 70.98

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		96.62	95.50	0.00	0.00	0.00
Allyl		107.78	100.38	90.81	109.94	0.00
Benzyl		96.49	93.61	82.08	51.75	0.00
Isopropyl		98.98	92.59	96.68	95.79	102.04
Methyl		92.35	91.05	87.73	41.13	0.00
Naphthyl		94.58	90.68	81.36	80.00	27.29
Propyl		100.13	102.80	95.86	88.38	0.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	2.173	1.837	4.859

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	5.643	4.769	12.618

12 days

Tables of means

Variate: %288

Grand mean 79.2

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		56.7	86.4	75.7	90.0	2.0
Allyl		53.3	64.5	111.0	40.1	46.1
Benzyl		120.7	103.4	106.6	78.2	2.0
Isopropyl		141.0	128.9	135.6	135.1	47.2
Methyl		112.9	116.4	97.5	70.5	2.0
Naphthyl		86.9	87.2	94.5	83.6	58.5
Propyl		72.1	105.1	77.0	79.6	2.0

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	10.76	9.09	24.06

(Not adjusted for missing values)

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	27.94	23.61	62.47

Appendix 2

R. solani ANOVA Least significant differences (1 % level)

5 days

Tables of means

Variate: %120

Grand mean 20.8

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		73.4	45.0	30.6	0.0	0.0
Allyl		50.7	110.0	29.9	0.0	0.0
Benzyl		0.0	0.0	0.0	0.0	0.0
Isopropyl		16.0	0.0	83.0	0.0	12.8
Methyl		0.0	0.0	0.0	0.0	0.0
Naphthyl		92.8	69.8	50.5	63.9	0.0
Propyl		0.0	0.0	0.0	0.0	0.0

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
s.e.d.	5.15	4.35	11.51

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
l.s.d.	13.37	11.30	29.89

6 days

Tables of means

Variate: %144

Grand mean 26.4

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		78.2	57.8	40.6	0.0	0.0
Allyl		58.9	106.3	40.5	7.1	0.0
Benzyl		0.0	0.0	0.0	0.0	0.0
Isopropyl		33.9	41.8	79.6	12.4	14.1
Methyl		0.0	0.0	0.0	0.0	0.0
Naphthyl		97.3	75.5	56.6	69.4	0.0
Propyl		48.0	0.0	4.7	0.0	0.0

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
s.e.d.	6.00	5.07	13.41

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
l.s.d.	15.58	13.17	34.83

7 days

Tables of means

Variate: %168

Grand mean 32.1

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		86.1	71.9	57.9	3.3	0.0
Allyl		72.1	108.6	51.1	12.5	0.0
Benzyl		0.0	0.0	0.0	0.0	0.0
Isopropyl		56.5	75.0	81.5	29.2	14.7
Methyl		0.0	0.0	0.0	0.0	0.0
Naphthyl		102.9	87.6	69.5	81.7	0.0
Propyl		43.7	7.6	11.0	0.0	0.0

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	4.80	4.06	10.74

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	12.48	10.54	27.90

8 days

Tables of means

Variate: %192

Grand mean 38.0

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		94.8	91.0	99.2	7.2	0.0
Allyl		79.9	103.7	60.1	22.7	0.0
Benzyl		0.0	0.0	0.0	0.0	0.0
Isopropyl		66.6	75.8	86.7	46.5	23.2
Methyl		0.0	0.0	0.0	0.0	0.0
Naphthyl		101.9	96.9	85.5	93.5	0.0
Propyl		62.4	12.7	17.0	1.9	0.0

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
s.e.d.	4.40	3.72	9.84

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	237	237	237
l.s.d.	11.42	9.65	25.54

Appendix 3

H. solani Bioassay results – ANOVA Least Significant Difference (1 % level)

Table of means

1 day

Variate: 1 day

Grand mean 1.00

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		1.00	1.00	1.00	1.00	1.00
Allyl		1.00	1.00	1.00	1.00	1.00
Benzyl		1.00	1.00	1.00	1.00	1.00
Isopropyl		1.00	1.00	1.00	1.00	1.00
Methyl		1.00	1.00	1.00	1.00	1.00
Naphthyl		1.00	1.00	1.00	1.00	1.00
Propyl		1.00	1.00	1.00	1.00	1.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.		40	56	8
d.f.		*	*	*
s.e.d.		0.000	0.000	0.000

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.		40	56	8
d.f.		*	*	*
l.s.d.		*	*	*

Tables of means

28 days

Variate: 28 days

Grand mean 19.52

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		2.00	2.00	2.00	2.00	2.00
Allyl		69.50	39.88	32.62	2.00	2.00
Benzyl		59.12	2.00	2.00	2.00	2.00
Isopropyl		20.25	15.75	18.38	22.88	25.50
Methyl		19.13	22.50	37.00	6.75	2.00
Naphthyl		56.88	70.38	65.38	65.38	2.00
Propyl		2.00	2.00	2.00	2.00	2.00

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
s.e.d.	2.611	2.207	5.838

Least significant differences of means (1% level)

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	238	238	238
l.s.d.	6.779	5.730	15.159

Tables of means

41 days

Variate: 41 days

Grand mean 31.5

ITC	Conc	125.	250.	500.	1000.	10000.
2-Phenylethyl		13.6	0.0	0.0	0.0	0.0
Allyl		91.2	68.2	66.5	25.6	0.0
Benzyl		66.8	0.0	0.0	0.0	0.0
Isopropyl		0.7	1.2	0.9	1.0	1.0
Methyl		0.7	1.0	1.2	0.3	0.0
Naphthyl		92.4	73.9	100.0	88.6	0.0
Propyl		92.9	102.3	93.4	95.5	24.2

Standard errors of differences of means

Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	233	233	233
s.e.d.	4.69	3.97	10.50

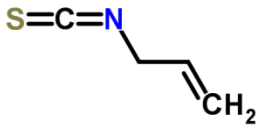
(Not adjusted for missing values)

Least significant differences of means (1% level)

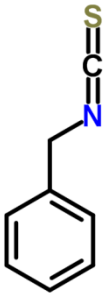
Table	ITC	Conc	ITC Conc
rep.	40	56	8
d.f.	233	233	233
l.s.d.	12.19	10.31	27.26

Appendix 4 Molecular Structures of Isothiocyanates

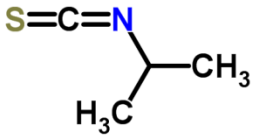
Allyl Isothiocyanate



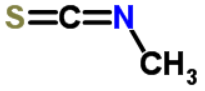
Benzyl Isothiocyanate



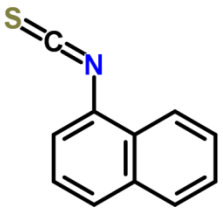
Isopropyl Isothiocyanate



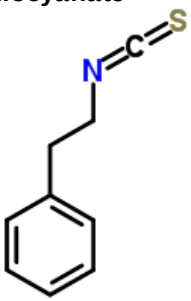
Methyl Isothiocyanate



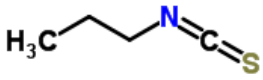
1-Napthyl Isothiocyanate



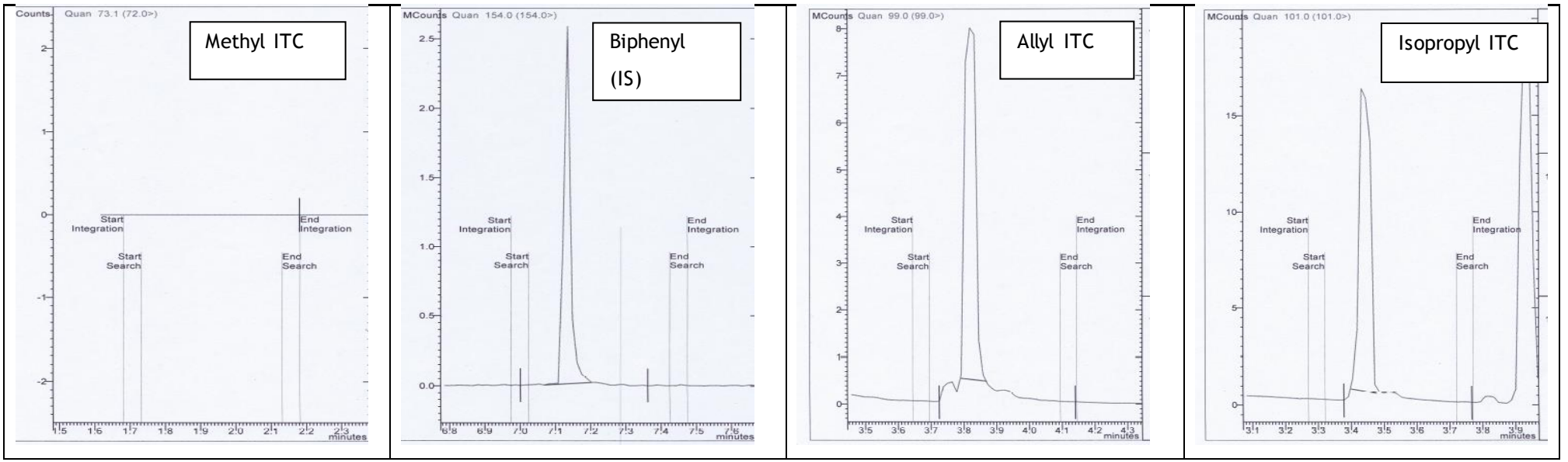
2-Phenylethyl Isothiocyanate

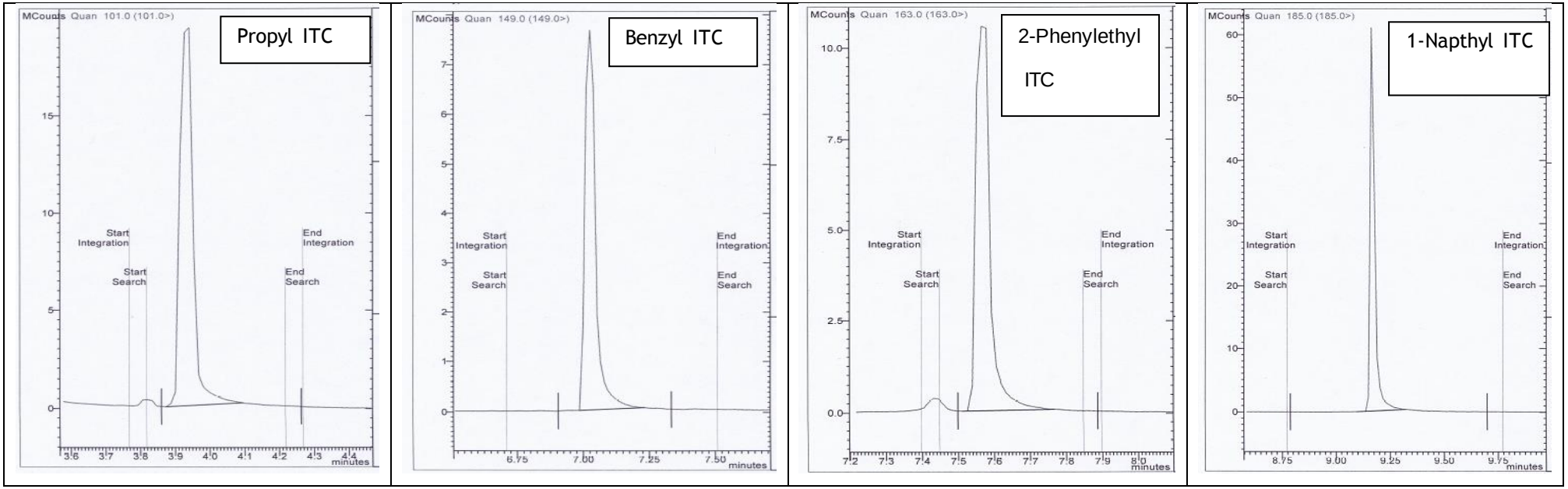


Propyl Isothiocyanate



Appendix 5 Chromatograms of ITCs from GC-MS analysis





NB – Methyl Isothiocyanate was undetectable by GC-MS

Appendix 6

C. coccodess **Chapter 5 ANOVA s.e.d results**

Tables of means

Variate: DI

Grand mean 61.3

Inoculum_Level	C. coccodes High	C. coccodes Low
	59.1	63.5

Treatment	AITC	AITC bitc peitc	BITC	BITC aitc peitc	Control	PEITC	PEITC aitc bitc
	76.9	53.3	61.5	42.3	87.9	44.6	62.7
Innoc level high	77.5	55.3	60.0	37.9	79.2	48.3	55.8
Innoc level low	76.3	51.3	63.0	46.7	96.7	40.8	69.7

Standard errors of differences of means

	Innoc level	Treatment	Innoc level * Treament
Rep	28	8	4
d.f	39	39	39
s.e.d	6.76	12.64	17.87

Least significant differences of means (5% level)

	Innoc level	Treatment	Innoc level * Treament
Rep	28	8	4
d.f	39	39	39
s.e.d	13.44	25.56	36.15

Appendix 7

***R. solani* chapter 5 ANOVA s.e.d results**

Tables of means

Variate: DI

Grand mean 50.0

Inoculum_Level	R. solani High	R. solani Low
	52.2	47.9

Treatment	AITC	AITC bitc peitc	BITC	BITC aitc peitc	Control	PEITC	PEITC aitc bitc
	58.8	37.7	39.3	53.1	61.7	57.1	42.6
Innoc level high	66.7	37.3	32.0	51.2	70.0	61.7	46.5
Innoc level low	51.0	38.0	46.7	55.0	53.3	52.5	38.7

Standard errors of differences of means

	Innoc level	Treatment	Innoc level * Treament
Rep	28	8	4
d.f	39	39	39
s.e.d	2.66	4.98	7.05

Least significant differences of means (5% level)

	Innoc level	Treatment	Innoc level * Treament
Rep	28	8	4
d.f	39	39	39
s.e.d	5.39	10.08	14.26