Harper Adams University

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Factors affecting biofumigation success against potato cyst nematodes

A thesis submitted to Harper Adams University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida,* cause in excess of £25M in losses to the UK potato industry per annum. Nematicides are used by potato growers to manage PCN, and protect yield. However, increasingly restrictive legislation within the European Union (EU), could instigate the retraction of nematicides from the region in the near future. Alternative management strategies, such as biofumigation, are therefore being evaluated for their potential to replace nematicides. Biofumigation involves the incorporation of glucosinolate (GSL) rich plant residues into soil for pest management. Upon tissue disruption, GSL's are liberated from biofumigant plant cells, and are subsequently enzymatically hydrolysed to form toxic volatile organic compounds (VOC's). The VOC's are chemically similar to the synthetic fumigant nematicide currently used for PCN management within the EU, and have been found to consistently reduce field populations of PCN by *c.*40-50% in previous studies. However, biofumigation efficacy is dependent on a number of agronomic factors, many of which are poorly understood. This project investigated the effect of soil moisture conditions at biofumigant incorporation on biofumigation efficacy against PCN. Commercially available maceration and incorporation implements were also sourced and evaluated for their effectiveness in generating, and placing PCN suppressive biofumigant material into soil.

Soil moisture investigations in the glasshouse found *Brassica juncea* biofumigation to reduce *Globodera pallida* viability by between 17-43%, and that efficacy could be improved by up to 14.3 % under certain soil moisture conditions. Soil moisture of 50% of field capacity was found to be more effective for biofumigation than 0, 25, 75 and 100% of field capacity, and water saturated soil. This could be due to an optimal water to air ratio within soil pores for VOC diffusion, and retention within soil. However, evidence is also presented which could suggest, that the optimal soil moisture condition for biofumigation, may be dependent upon the quantity of biofumigant biomass incorporated

into soil, and the depth of biofumigant incorporation. Further work is required. *In-vitro* studies were then carried out which investigated the effect of water films surrounding PCN cysts, on the efficacy of biofumigant VOC's. There was no evidence that water films might act as a barrier to biofumigant VOC contact with encysted PCN eggs.

Maceration and incorporation implements, and combinations of implements, were investigated in field experiments for their effect on biofumigation efficacy against PCN. Biofumigation efficacy ranged between 27-34% for three geographically separated sites, over two years. A flail topper was found to improve biofumigation efficacy against *G. pallida* by 7% when compared to a roll conditioner. However, further investigation of haulm topper tines and shear-plate positioning provided no evidence that the implement set-up of topping implements, might influence biofumigation efficacy against PCN. Incorporation implements; spader, plough and rotavator, facilitated no extra efficacy against PCN when analysed in isolation to the effects of maceration implement, despite incorporating biofumigant material to diverse working depths, and despite producing contrasting soil structure. However, a separate analysis showed a flail-spader implement combination to produce a *c.*8% more effective biofumigation of *G. pallida* than a roll conditioner-rotavator combination. Further work is therefore required. Biofumigant biomass ranged between 14-48 t ha^{-1} fresh weight for field studies, and equivalent to 0-100 t ha^{-1} for glasshouse studies. Glucosinolate contents ranged between 3-12 µmol g^{-1} dry weight between studies, with 2-propenyl (sinigrin) being the dominant GSL. Clearly, biofumigation could have an important role to play in the future management of PCN.

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Common Abbreviations, Units & Symbols

‘A’	assessment biofumigant pots in glasshouse experiment 1
AChE	acetyl cholinesterase enzyme
AHDB	Agricultural and Horticultural Development Board
AITC	allyl-isothiocyanate
ANOVA	analysis of variance
BCN	beet cyst nematode
bsp	British standard pipe unit of measurement
c.	circa
Cq	quantification cycle
CV	coefficient of variation
cv.	cultivar or variety
‘D’	discard biofumigant pots in glasshouse experiment 2
DMDS	dimethyl disulphide
DMS	dimethyl sulphide
DNA	deoxyribonucleic acid
EU	European Union
EV	electron volt
g	unit related to gravitational force
g	grams
GB	Great Britain
GC-MS	gas chromatograph-mass spectrometer
GSL	glucosinolate
h	hour
H_2	hydrogen carrier gas
ha	hectare
HAU	Harper Adams University
HPLC	high performance liquid chromatography
‘I/A’	incorporation/assessment biofumigant pots in glasshouse experiment 2
IS	glucosinolate internal standard
ITC	isothiocyanate
J1-5	potato cyst nematode juvenile moult stages 1-5
kg	kilogram
km	kilometre
L	litre
LSD	least significant difference
m	metre

M	million
mg	milligram
mm	millimetre
mM	millimolar
min	minute
mL	millilitre
MITC	methyl-isothiocyanate
mol	unit for quantification of a substance relative to carbon -12
M/Z	unit for mass and charge of an ion
n	nano
N	nitrogen
OM	organic-matter
P	probability value in statistics
Pa	pathotype group nomenclature for *Globodera pallida*
PCN	potato cyst nematode
PCR	polymerase chain reaction
P_f	nematode population final sample date
pH	power of hydrogen (measure of acidity/alkalinity)
P_i	nematode population initial sample date
PRD	potato root diffusate
P_s	nematode population secondary sample date
PTFE	polytetrafluoroethylene
PTO	power take-off
R	denotes a glucosinolate sidechain of variable length
RDF	residual degrees of freedom
Ro	pathotype group nomenclature for *Globodera rostochiensis*
rpm	revolutions min^{-1}
s	seconds
SEM	standard error of the mean
t	metric tonne
TDR	time domain reflectometer
TE	a chelating agent in PCR
thsds	thousands
USA	United States of America
UK	United Kingdom
UV	ultraviolet radiation
v/v	volume per volume
VOC	volatile organic compound

W	watt
w/v	weight per volume

°C	temperature in centigrade
°	degree symbol
µ	micro
%	percent
£	pounds sterling
$	United States of America dollars
®	registered trademark
©	copyright
™	unregistered trademark
$^{-1}$	representative of a unit within another unit
$^{-2}$	squared
$^{-3}$	cubed
♂	male
♀	female
≤	less than or equal to

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A review of literature relevant to the potato cyst nematodes and their management in potato production systems in the United Kingdom

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A review of literature relevant to the potato cyst nematodes and their management in potato production systems in the United Kingdom

1.1 The European potato

1.1.1 *Ancestry*

Potato plants belong to the Solanaceae plant family and originate from South America where they have been cultivated as a food crop for over 8,000 years (Lutaladio & Castaldi, 2009). During that time, indigenous people frequently cross pollinated successful landraces for improved agricultural traits such as edible tuber yield and palatability (Hawkes, 1978; Spooner *et al*., 2005). It is believed that all domesticated potatoes today descend from four wild relatives within the *Solanum brevicaule* complex although the most widely accepted immediate relative is the Andean potato *Solanum tuberosum* ssp. *andigena* (Jones, 1970; Spooner *et al*., 2005). Subspecies *andigena* was first imported into Europe as a botanical curiosity following colonial exploration and settlement of South America in the 16th and 17th centuries (Jones, 1970; Spooner *et al*., 2005). Subsequent selection for early formation of tubers under temperate conditions resulted in sufficient genetic shift for the classification of European subspecies, *tuberosum* (syn. Irish Potato) (Jones, 1970; Hawkes, 1978; Haydock, 1990). The domesticated European potato is better suited to long summer photoperiods than subspecies *andigena* and has comparatively high and uniform tuber yield (Hawkes, 1978). These characteristics, as well as the long storage potential and self-propagating qualities of the tubers, hereafter referred to as 'seed' when relating to self-propagation, have given rise to the global distribution and cultivation of the potato (Hawkes, 1978).

1.1.2 *Physiology*

Potato plants are dicotyledonous perennial angiosperms easily identified by a paired arrangement of pinnately compound leaves on stems, of which there are several per

plant. Their canopies can grow to between 300-1000 mm in height, and roots typically between 400-500 mm depth (Cutter, 1992). Flowers form near the end of branches in a five-point star arrangement with pigmentation dependent on variety. True botanical seeds form after flowering within tomato-like fruits of *c.*15-20 mm diameter (Cutter, 1992). The rooting system is composed of fine fibrous and adventitious roots, whilst stolon's form before canopy emergence at basal nodes of the hypocotyl (Cutter 1992). Stolon's generate the edible tubers which are composed of *c.*70-80% water and 20-30% solids. The solid fraction of tuber yield typically comprises 16-20% carbohydrates (95% starch), 0.1-0.2% fats, 2.5-3.2% nitrogenous compounds (including proteins), 0.8-2% minerals, and a remaining 0.6% fibre (Bajaj, 1987). Potato tubers are highly nutritious, globally distributed, and are the primary non-cereal source of carbohydrate worldwide (FAO, 2015). Figure 1.1 illustrates a pinnately compound potato leaf (A), potato fruits containing true botanical seed (B) and a potato tuber (C) from the variety Desiree. Figure 1.2 shows a commercial potato crop prior to canopy closure. Differences in biomass can be observed between plants, which could be due to variability of seed size and spacing at planting, in addition to other seed and field factors (Bremner & Taha, 1966).

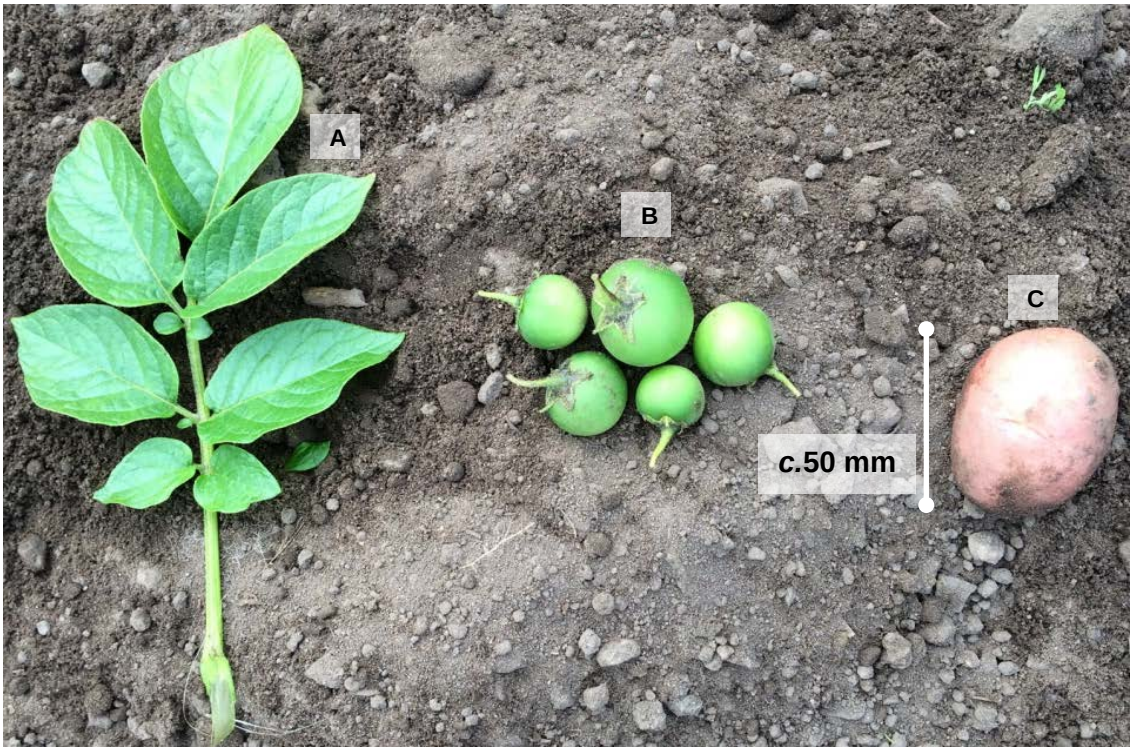


Figure 1.1: Potato leaf (A), fruits (B) and tuber (C) from the potato variety Desiree. The scale relates approximately to the length of the potato tuber.

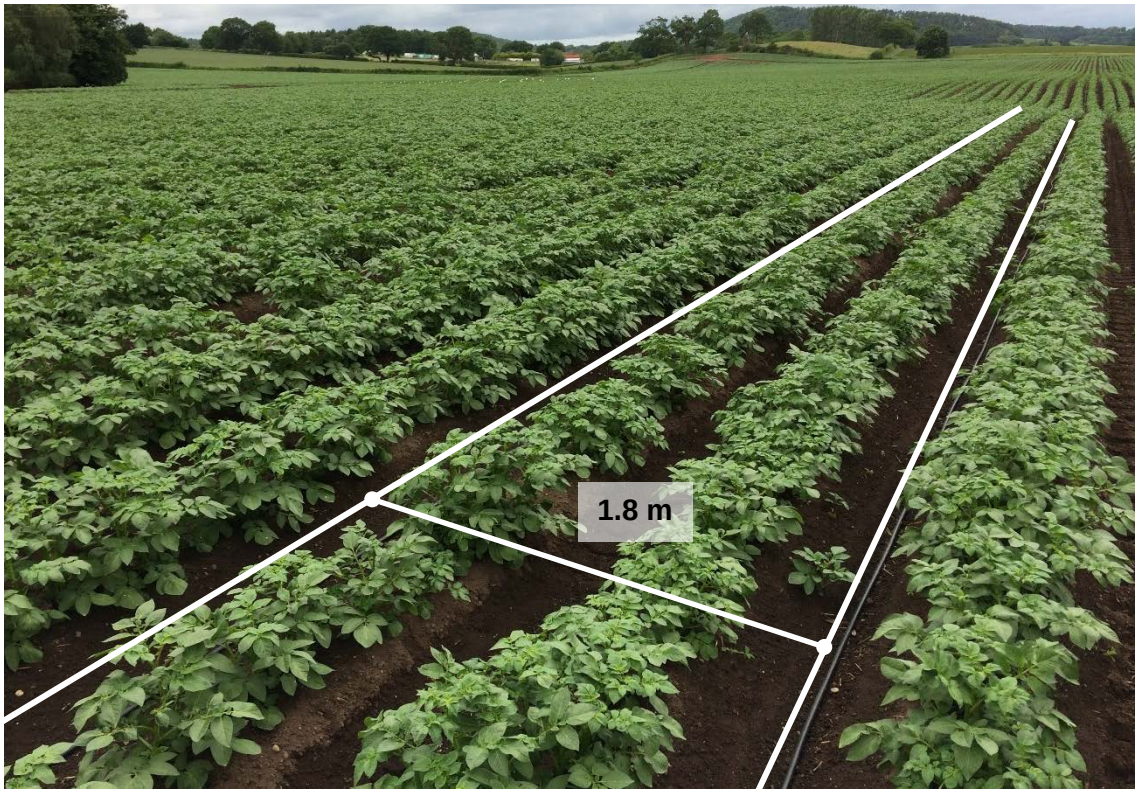


Figure 1.2: Commercial potato crop (variety: Arsenal) prior to canopy closure at *c.*60 days after planting, highlighting a two ridged bed of 1.8 m width.

1.1.3 *Production and demand*

Potatoes are grown in at least 150 countries worldwide, and are the seventh most economically important world crop (FAO, 2015). The United Kingdom (UK) is the 16th most economically important potato crop producer worldwide at a gross market value of *c.*US$664M per annum, and the eighth most economically important potato producing nation (FAO, 2015). Given a meagre 0.71% share of the total world potato cropped land, but impressive economic standing amongst potato producing nations, the UK is considered one of the world's most effective producers of the crop (AHDB, 2017). Average ware (potatoes for human consumption) yield in the UK is *c.*2.5 times (*c.*47 t ha^{-1}) greater than the world average which is notably due to high fertiliser and water inputs, efficiency and effectiveness of mechanised production strategies and compound approaches to pest and disease management (FAO, 2015; AHDB, 2017). Mechanisation, has led to larger potato farming enterprises in the UK, which through

economies of scale, have been able to invest heavily into the newest and most efficient and effective technologies, thereby maximising yields (AHDB, 2017). Potatoes in the UK are typically established into stone-free beds which are conventionally generated by autumn ploughing followed by spring time light cultivations, bed-tilling and stone separating. Mechanisation has changed the face of UK potato production over the last 50 years, resulting in an approximate 97% decline in Great Britain (GB) in the number of growers specifically, and a 60% decline in the planted area for only a 10% decline in total yield per annum (AHDB, 2017). Figure 1.3 shows lightly cultivated land being bed-tilled. Figure 1.4 shows tilled beds being stone separated prior to potato planting which is illustrated in Figure 1.5.

Figure 1.3: Tilling (A) of shakerator cultivated ground (B) using a 5.4 m wide Grimme bed-tiller. The working width of the tiller, which generates 3 beds during cultivations, is highlighted.

Figure 1.4: De-stoning (A) of tilled-beds (B) using a ScanStone 1.8 m width stone separator.

Figure 1.5: Potato planting into single 1.8 m wide destoned beds using a Standen belt planter.

The potato industry in GB is currently accounted for by approximately 2000 growers who collectively use 116,000 ha of land to grow the national crop each year, of which *c.*18% constitutes fresh ware crop, *c.*30% processing crop, *c.*37% pre-pack fresh and *c.*15% seed (AHDB, 2017). Approximately 55% of the total area is accounted for by East Anglia and Yorkshire, which are predominantly ware producing regions, whilst Scotland accounts for 23% of the total land and is the UK's primary seed producing region due to its cool seasons and subsequent unsuitability for aphid virus vectors (AHDB, 2017). A further 11% is accounted for by the West Midlands in England, and the remainder distributed across England and Wales (AHDB, 2017). Great Britain produced 5.22M tonnes of potatoes in 2016, of which *c.*15% was exported (AHDB, 2017). When combined with import potatoes this equates to *c.*100 kg of potatoes consumed per head of the UK population per annum, providing *c.*7% of the energy intake of a UK citizen each year (AHDB, 2017; FAO, 2015; Bates *et al*., 2010). High domestic demand has led to a near mono-varietal cropping pattern of the nation's favourite varieties; Maris Piper, M. Peer, Melody, Lady Rosetta and Markies being particularly well established (30% of the planted area in 2016) (AHDB, 2017). Rigid consumer demand has, however, also selected for pest and disease pressures of which the potato cyst nematodes (PCN) *Globodera rostochiensis* (Wollenweber, 1923 [Behrens, 1975]) and *Globodera pallida* (Stone, 1973) are the most economically important pests to the UK crop; attributable to *c.*9% of the total annual UK potato crop loss (Haydock & Evans, 1998a; Evans & Brodie, 1980).

1.2 Potato cyst nematodes

1.2.1 *Taxonomy*

Potato cyst nematodes *Globodera rostochiensis* and *G. pallida* are bilaterally symmetrical worm-like invertebrates belonging to the cyst nematode subfamily Heteroderinae of the Hoplolaimidae family of the Nematoda (Decraemer & Hunt, 2013). Initially believed to be a strain of the beet cyst nematode (BCN) *Heterodera schactii*, PCN have undergone several taxonomic reclassifications in recent history worth noting

when reviewing the available literature. Wollenweber (1923) recognised a single species he named *Heterodera rostochiensis* 'the potato strain of beet cyst nematode' after the Rostock region of Germany he originally isolated his population from. Skarbilovich (1959) later proposed a reclassification to *Heterodera* (*Globodera*) *rostochiensis* after the characteristic globose shape of mature females. In 1973, Stone identified *Heterodera pallida* as a separate species to *Heterodera rostochiensis*, and two years later Behrens (1975) elevated the subgenus *Globodera* to genus. Our taxonomic understanding of these nematodes remains unchanged since 1975, although some researchers question whether newly discovered species, such as *Globodera ellingtonae,* might also be considered PCN (Handoo *et al*., 2012).

1.2.2 *Morphology and physiology*

There are few morphological differences between the two PCN species. Cysts, which are the globose body remains of fertilised females (Figure 1.6), range between *c*.250-1000 µm diameter, and the vermiform bodies of invasive juveniles (Figure 1.7) between *c.*350-500 µm length. Qualitative differences in colour can be made between cysts. Mature *G. pallida* females are white/yellow and later tan light brown, whereas *G. rostochiensis* females are golden in their mature stage and later tan dark brown as cysts (Turner & Subbotin, 2013). Quantitative differences are more difficult to make, although the Granek's ratio, which is the distance between the anus and vulva divided by the diameter of the vulva, is a good morphological diagnostic for differentiating species from cyst examinations. Additionally, the number of cuticular ridges between anus and vulva can be used for species determination. Granek's ratio values above three are broadly associated with *G. rostochiensis,* and below three with *G. pallida* (Turner & Subbotin, 2013). Stylet length and knob shape are good diagnostics for differentiating invasive juveniles, although these methods are now secondary to techniques such as isoelectric focussing, enzyme-linked immunosorbent assay, or more commonly, polymerase chain reaction (PCR) (Fleming & Marks, 1982; Schots *et al*., 1992; Nakhla *et al*., 2010).

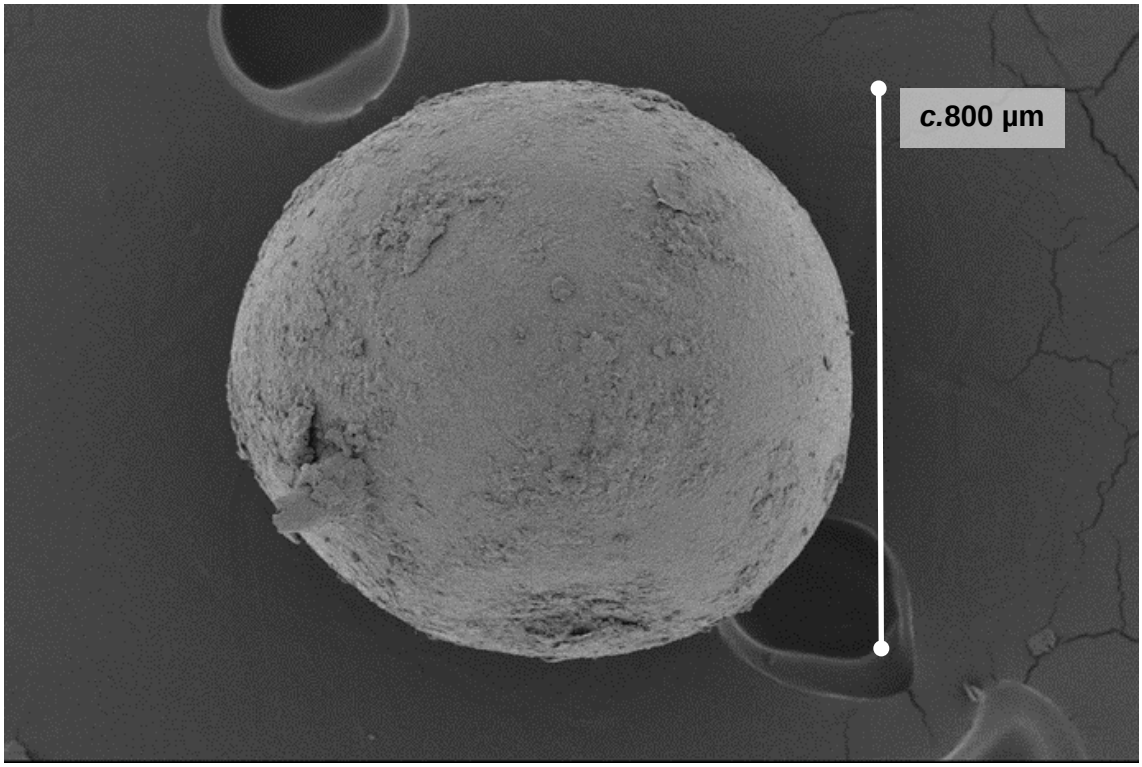


Figure 1.6: *Globodera pallida* potato cyst nematode cyst (Source: Victoria Taylor, Arcis Bio. Ltd.).

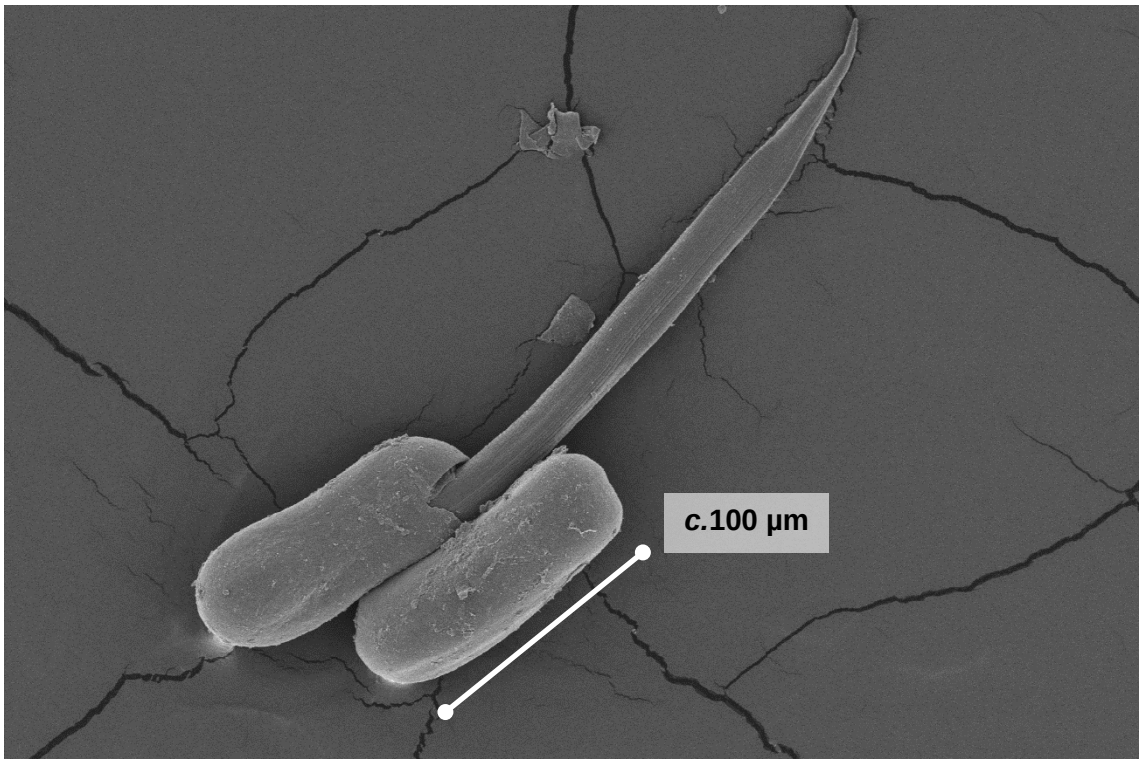


Figure 1.7: *Globodera pallida* potato cyst nematode eggs and juvenile (Source: V. Taylor, Arcis Bio. Ltd.).

The physiology between PCN species differ subtly, as discussed later in the embryo development and hatching and chemotaxis divisions of this chapter. *Globodera pallida* hatch over a lengthier period than *G. rostochiensis*, are more persistent in soil, hatch at lower soil temperatures and metabolise lipids more slowly (Lane & Trudgill, 1999; Robinson *et al*., 1987). Consequently, *G. pallida* is the more successful PCN species in temperate regions, as discussed later in the management sections of this chapter. Physiological differences are also notifiable within species. Historically PCN have been separated into pathotypes by schemes proposed by Canto-Saenz & de Scurrah (1977) and by Kort *et al.* (1977), of which the latter is used in the UK. The Kort *et al.* (1977) scheme uses a number of *Solanum* clones to subdivide PCN into pathotypes based upon their virulence to the clones; Ro1-Ro5 for *G. rostochiensis*, and Pa1 and Pa2/3 for *G. pallida* respectively. However, the pathotyping scheme has been criticised, notably by Trudgill (1985) who recognised that the authors had used an arbitrary methodology for determining the resistance of *Solanum* clones which were later used to determine PCN pathotypes. Kort *et al.* (1977) proposed that a P_f/P_i value (which is the ratio of cysts after potatoes compared to before potatoes) of ≤1 indicated varietal resistance to PCN. However, as Trudgill (1985) suggests, P_f/P_i measurements can be too readily influenced by nematode and environmental factors to be used as a measure of nematode virulence or plant resistance, for example; variability in the initial PCN population density, variability in the vigour of plant growth, and variability of the nematode in response to different environmental conditions. Despite these weaknesses, the scheme has been relatively unchallenged until recently. Eves-van den Akker *et al*. (2015) believe that PCN could be more effectively separated into virulence groups by assessment of mitochondrial deoxyribonucleic acid (DNA), however, this new approach is still being validated.

1.2.3 *Origins and distribution*

Potato cyst nematodes are indigenous to South America where they coevolved with their potato hosts (Turner & Evans, 1998). *Globodera pallida* originates north of Lake Titicaca along the Peruvian/Bolivian border, and *G. rostochiensis* originates further to the south

below 15.6° latitude where temperatures are warmer (Jones & Jones, 1984; Turner & Evans, 1998). Potato cyst nematodes are, however, now globally distributed over at least 65 countries following their introduction into Europe in the late 1840's and subsequent export to colonies and allied countries worldwide (Turner & Evans, 1998). It is widely accepted that the search for late blight (*Phytophthora infestans*) resistant potatoes following the Irish potato famine (1845-1846), as well as the use of Peruvian guano as fertiliser in the 19th century, are the primary vehicles through which PCN migrated to Europe, after which tuber exports and unchecked soil contaminated cargoes, such as military equipment used in World Wars I and II are suggested to have further distributed both species (Inagaki & Kegasawa, 1973; Jones & Jones, 1984; Jones, 1970). Cysts could also have been distributed locally by wind under dry field conditions, water under flooded field conditions, and during soil cultivations (Decker, 1981; Turner & Subbotin, 2013). Today, cysts are most commonly transported by farm machinery, which easily facilitates the spread of PCN within fields, and between fields, if not properly cleaned. Clods of PCN infested soil can cling to tractors and implements only to fall off during work, leading to localised PCN hotspots in fields.

The first report of PCN in the UK was made in Lincolnshire in 1924, however, PCN have been suggested to be in the UK since at least 1900 (Morgan, 1925; Jones, 1970). The last published PCN survey (limited to just England and Wales) found that PCN were present in 64% of potato cropped fields, of which 67% of the populations were *G. pallida*, 8% were *G. rostochiensis*, and the remainder were mixed populations (Minnis *et al*, 2002). The higher presence of *G. pallida* populations in the UK today has been suggested to be a result of the nation's preference for Maris Piper potatoes which confer resistance to *G. rostochiensis* but not *G. pallida* (Minnis *et al*, 2002). A more recent, but as yet unpublished survey, suggests the total PCN infested area in England and Wales to have declined to 48% (Pers. Comm. Katarzyna Dybal; PhD Research Student at Harper Adams University). The decline could indicate improved management, but could also be a consequence of different field sites being sampled between surveys. There is

also a possibility that as the national potato growing area has declined between surveys, growers have selected PCN free land to continue growing on as opposed to fields with high PCN populations. Only pathotypes Ro1, Pa1 and Pa2/3 are currently present in the UK for *G. rostochiensis* (Ro) and *G. pallida* (Pa) (AHDB, 2015). See Marks & Brodie (1998), and Minnis *et al*. (2002) for global and UK PCN distribution maps respectively.

1.2.4 *Lifecycle*

Potato cyst nematodes are specialised sedentary endoparasites with several survival strategies. Some populations can persist for 30 years between host crops as encysted juveniles, and then at low-moderate population levels (*c.*1-50 eggs g^{-1} soil), multiply by up to 50 times in a lifecycle (Haydock & Evans, 1998b; Wale *et al*., 2011).

1.2.4.1 *Embryo development and hatching*

The PCN lifecycle commences when a free-living adult male (fifth juvenile moult: J5) mates with a J5 female (Figure 1.8: A, B). Embryos known as first stage juveniles (J1) develop in the female to form egg encased vermiform second stage juveniles (J2), of which there can be up to 600 per female adult (Figure 1.8: B) (Wale *et al*., 2011; Jones & Jones, 1984). The female subsequently dies and detaches from the root system to surrounding soil (Figure 1.8: B,C), however, during the terminal stages of the J5 female's life, signals may be transmitted to developing J1/J2 juveniles to influence juvenile hatching behaviour in response to a declining host plant photoperiod (Perry *et al*., 2013). The signals which are indicative of unfavourable conditions for further potato parasitism halt juvenile activity through an arrested developmental mechanism known as obligate diapause which is not broken until an extended period of chilling and reheating has been experienced over several weeks, such as a winter period going into the spring season (Perry *et al*., 2013). These temperature fluctuations are experienced whilst eggs are still in the body remains of their dead female parent (cyst) (Figure 1.8: B, C) (Duncan, 1995). Cysts protect PCN eggs and are formed following polyphenol oxidase tanning of the cuticular tissues of a recently dead female.

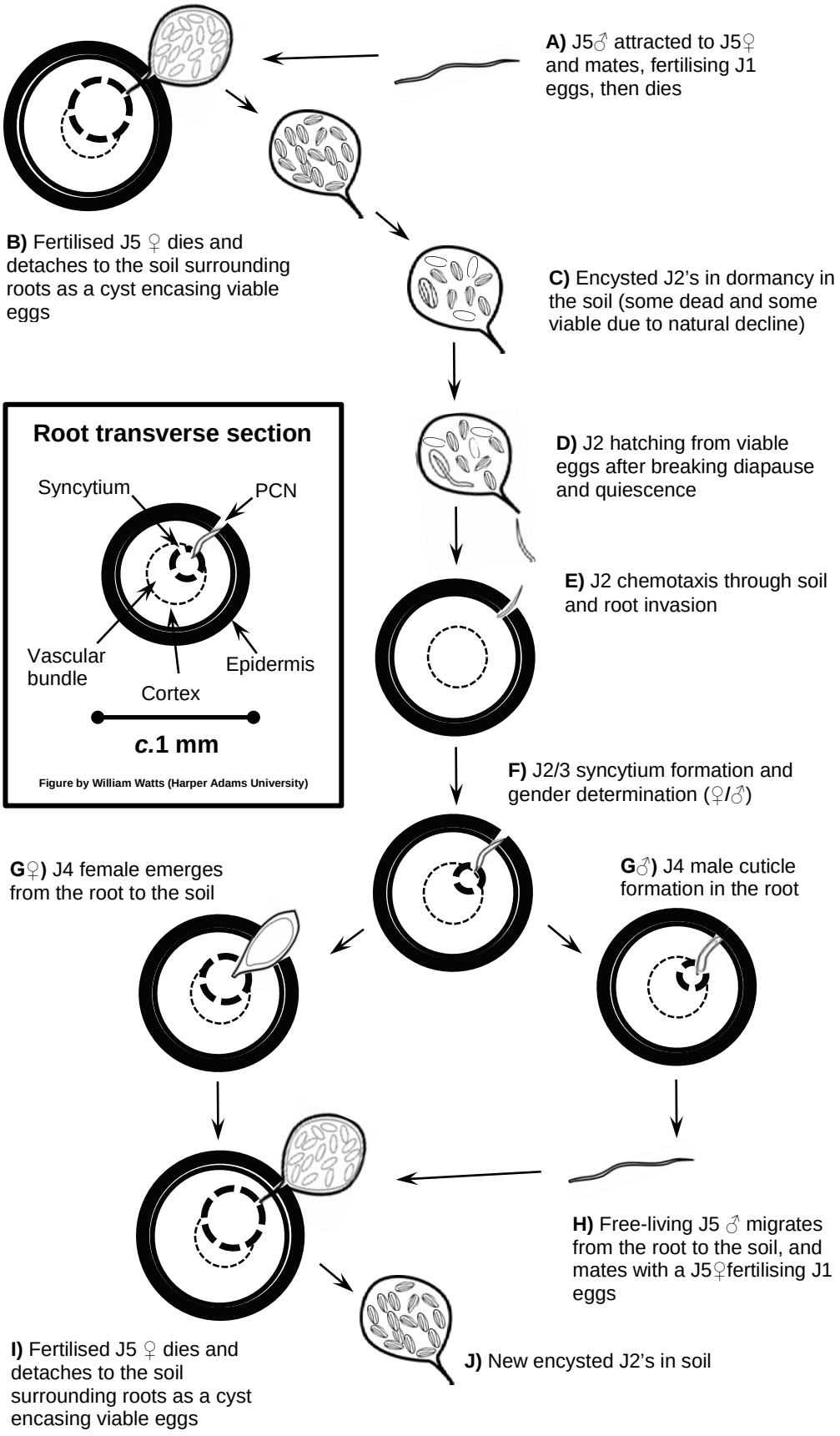


Figure 1.8: Generic potato cyst nematode life-cycle. Note/ nematode moults within this figure are only approximately to scale.

Juveniles do not hatch continuously from eggs. Obligate diapause is the first mechanism which prevents PCN hatching in the absence of a host (Turner & Subbotin, 2013). The second mechanism is obligate quiescence, which is an arrested developmental state only broken by favourable edaphic conditions such as soil temperature (hatching initiates between *c*. 5-11°C for both species although *c.*16°C is optimal for mass *G. pallida* hatch and *c*.20°C for *G. rostochiensis*) and by stimulation from host potato root diffusates (PRD) after obligate diapause has broken (Kaczmarek, 2014; Turner & Subbotin, 2013). Potato root diffusate permeates the egg shell of J2 PCN which mediates loss of Ca^{2+} from the egg shell, which in turn allows the release of trehalose from the perivitelline fluid surrounding the egg encased juvenile nematode in exchange for water (Turner & Subbotin, 2013; Perry *et al*., 2013). Water hydrates the juvenile which initiates metabolic activity. The juvenile then either commences stylet probing and cutting of the egg shell to begin hatching, or undergoes a further quiescent stage known as facultative quiescence (Figure 1.8: C, D) (Perry *et al*., 2013).

Facultative quiescence is the third dormancy stage and is less stable than those previously mentioned. Facultative quiescence is induced when conditions suddenly become unfavourable after breaking obligate quiescence, which could be due to changing environmental conditions and/or the absence of suitable host cues during or after breaking obligate quiescence (Perry *et al*., 2013). Potato cyst nematodes in this stage of dormancy are partially hydrated and metabolically active which leads to spontaneous hatching thereafter. Spontaneous hatching in the absence of PRD along with predation losses and physical damage to encysted juveniles is included in the 'natural decline' of PCN populations between host crops (most often 10-30% per annum depending on season, population, soil biota and PCN species; *G. pallida* typically declining more slowly than *G. rostochiensis,* although up to 50% decline per annum has been recorded) (Devine *et al*., 1999; Winslow & Willis, 1972; Whitehead, 2002). A final dormancy strategy, facultative diapause, is initiated in unhatched juveniles when environmental cues indicate the onset of a winter period, and therefore, poor conditions

for parasitism. This stage is again terminated by increasing soil temperatures in the spring (Turner & Subbotin, 2013; Perry *et al*., 2013). When environmental conditions and host cues are suitable, between 70-80% of encysted juveniles can hatch (Figure 1.8: D) (Rawsthorne & Brodie, 1986; Kaczmarek, 2014). The remaining 20-30% ensure that a PCN population persists even in a poor season when a host might die prematurely. Typically, *G. pallida* eggs hatch more uniformly in the presence of a host than *G. rostochiensis* (Kaczmarek, 2014). It is possible that this characteristic is a factor which leads to higher natural decline in *G. rostochiensis* than *G. pallida,* as encysted juveniles frequently break diapause but not quiescence.

1.2.4.2 *Chemotaxis and root invasion*

After hatching, a J2 nematode initiates host location using its amphids; a pair of chemoreceptive organs located on either side of the nematode head next to the mouth (Duncan, 1995). The J2 follows gradients of PRD in soil water and from soil air by swimming along water channels between soil colloids to the potato host roots (Figure 1.8: E) (Duncan, 1995; Reynolds *et al*, 2011). This is known as long-distance chemotaxis which describes general J2 movement towards higher PRD semiochemical concentrations (Reynolds *et al*, 2011). Long distance chemotaxis can initiate from up to *c.*800 mm away from the host roots (Turner & Rowe, 2006). The J2 then follows short-distance attractants to locate potato roots, and then local attractants to orientate itself to a preferred invasion site near the point of emergence of lateral roots from primary roots or at root tips (Reynolds *et al*, 2011; Jones & Jones, 1984). Second stage juveniles proceed to probe and pierce the root epidermis with their stylet, using subventral oesophageal proteins known as Egase enzymes (*β*-1, 4-endoglucanase) to soften the root tissues, initially by cleaving cellulose bonds in the epidermis (Figure 1.8: E) (Perry *et al*, 2013; Smant *et al*, 1997; Smant *et al*, 1998). This enables the J2 to enter the root cortex through which it migrates within one to five minutes to the vascular bundle (Duncan, 1995; von Mende *et al.*, 1998). The J2 makes a series of precise stylet thrusts at the vascular bundle to break through the pericycle and establishes a feeding tube

through which it draws nutrition during parasitism (Duncan, 1995; Turner & Subbotin, 2013). This exploratory stage within the PCN lifecycle persists for approximately 11 days in *G. rostochiensis* before lipid reserves in the J2 are exhausted and the juvenile dies (Perry, 1998). *Globodera pallida*, with higher lipid content and lower lipid metabolism could potentially survive for longer during this 'free-living' period (Haydock & Evans, 1998a; von Mende *et al*., 1998).

1.2.4.3 *Moult development and mating*

Once a feeding tube has been established, the J2 nematode injects saliva from its pharyngeal glands into the chosen plant cell and then the contents are withdrawn into the J2 (Turner & Subbotin, 2013). This action enlarges roots cells within the affected area by breaking down the cell walls to form a single cavernous nutrient transfer cell known as a syncytium (Figure 1.8: F) (von Mende *et al*., 1998). The size of the syncytium is critical in determining the gender to which the J2 becomes; a large syncytium with greater nutrient transfer potential leads to the development of a female nematode whilst a smaller syncytium results in the development of a male nematode (Grundler *et al*., 1991). The development from J2 to J3 moult typically takes around seven days after which the J3 becomes distinguishable from J2 by its newly developed genital primordia and rectum. The male J3 has a single testis and the female has paired ovaries (Turner & Subbotin, 2013). However, J3 moults are very alike in appearance when viewed using a dissection microscope and so researchers do not routinely distinguish between the genders (Figure 1.8: F).

During the J3 moult stage, females form a globose shape and develop a reproductive system and eggs, after which they are considered a J4 moult (Figure 1.8: G♀). The J4 female body subsequently ruptures the root cortex and epidermis to become exposed to the rhizosphere for mating (Figure 1.8: H) (Turner & Subbotin, 2013). The J3 male moult stage develops at the same rate as the female, however, only requiring *c.*1% of the nutrition of a developing female, the male remains comparatively small and saccate

(Turner & Subbotin, 2013; Evans & Trudgill, 1992). During the fourth moult, males reform their body to a vermiform J5 male approximately three times the length of a J2, within the cuticle of their J3 body (Figure 1.8: G♂, H) (von Mende *et al*., 1998). The adult J5 male subsequently emerges and migrates through the rhizosphere, following pheromones to root exposed J5 females with which it mates and completes its generations life-cycle (Figure 1.8: H, I, J). The composition of the female sex pheromones of PCN is still unknown. They are, however, known to be the sole attractant of males to females for copulation (Riga *et al*., 1996). Potato root exudates have been shown to be ineffective in stimulating mature males (Riga *et al*., 1996). Furthermore, the pheromone attractants are known to be secreted from the whole body of the mature female PCN, and to be required at an accumulated concentration for mature males to be attracted into mating (Green & Greet, 1972). The concentration of sex pheromone required for mating is unknown. Each male can fertilise several females, however, they only survive for around 10 days after emergence (Evans, 1970). Developmental time from J3 moult to mature J5 moults can be as short as 20 days depending upon environmental conditions (Turner & Subbotin, 2013; von Mende *et al*., 1998). A PCN lifecycle can take between 30-50 days to complete, although will often extend up to 100 days (Turner & Subbotin, 2013).

1.2.5 *Crop damage*

Intracellular migration of J2 PCN through the roots of potato causes localised root damage which generates root galleries. More extensive root damage occurs when PCN advance to J3 moults and establish syncytium (Schmitt & Ferris, 1998). Phloem are damaged through feeding and photosynthate drained from the vascular system to the syncytium. This can reduce leaf size, internode length and the numbers of stems, although the root system biomass is most notably reduced (Schmitt & Ferris, 1998; Schans & Arntzen, 1991). Xylem are also damaged during nematode exploration of the vascular bundle, which in combination with reduced root biomass can lead to poor water and nutrient uptake, particularly of nitrogen, phosphorus and potassium (Figure 1.9). This

can cause moisture stress and stunting in the plant, and sometimes plant death (Figure 1.10) in intolerant varieties (Trudgill, 1986). In peat soils such as the Fenland of East Anglia, potato plants can withstand far greater PCN parasitism without a yield reduction due to the fertility and moisture retentive capacity of the high organic-matter (OM) peat. Light sandy soils which are common throughout the remainder of East Anglia and the English Midlands, can be prone to severe PCN damage at low population densities due to poorer fertility and low water retention properties (Schmitt & Ferris, 1998).

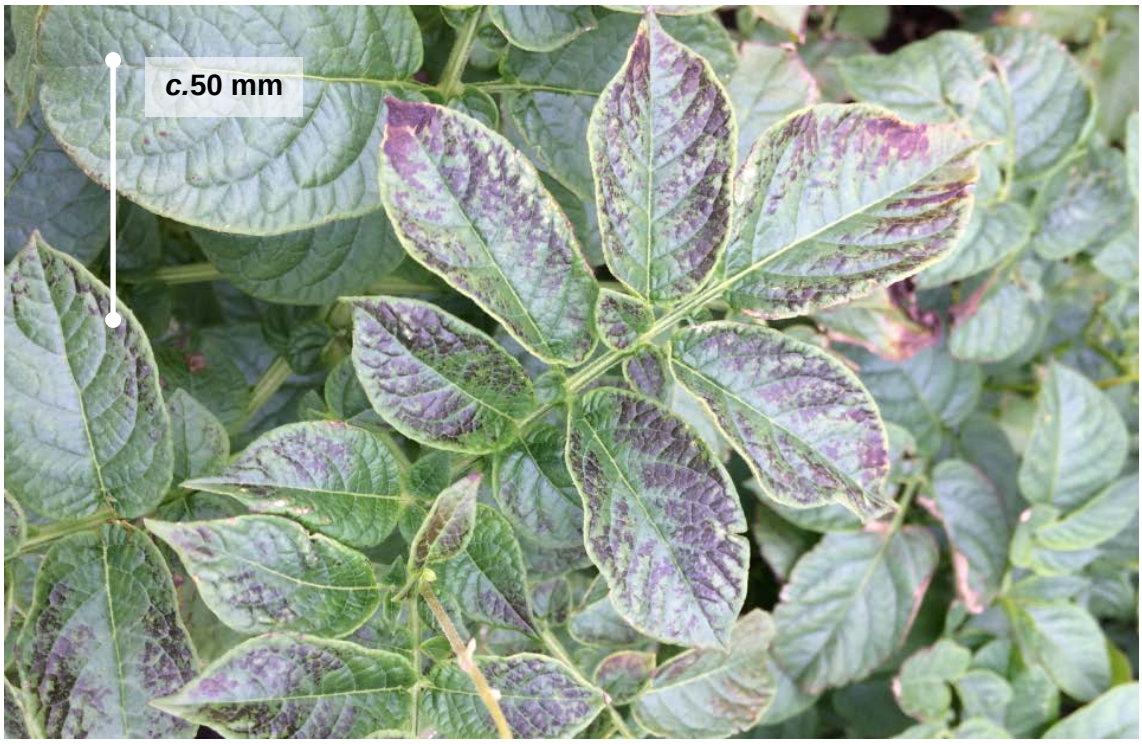


Figure 1.9: Potato cyst nematode damage induced phosphorous deficiency (purple leaf coloration) of the potato variety Taurus.

Figure 1.10: Severe potato cyst nematode damage causing stunting and plant death of the intolerant potato variety Marfona. The scale indicates a single ridge width from a 1.8 m wide two ridged bed.

During J4 and J5 female moults, PCN rupture the root cortex as they emerge from roots which causes extensive physical damage to roots, in addition to the nutrient sink they maintain on the infested plant (Turner & Subbotin, 2013; Schmitt & Ferris, 1998). The J5 male moult also damages roots as it emerges but due to its much smaller and vermiform shape, damage is localised and low level. It is estimated that PCN population densities as low as 5-10 eggs g^{-1} soil can inflict economic damage in untreated intolerant potato varieties and that PCN are accountable for *c.*£25-50M in losses to the UK crop per annum (Twinning *et al*., 2009; Wale *et al*., 2011; Winslow & Willis, 1972). Potato cyst nematode damage is easily identifiable in the field as circular or elliptical patches of dead or stunted plants. Infested plants can also be lifted and cysts observed on their roots by eye after tuber initiation (Figure 1.11). To prevent PCN population increases and damage it has been estimated that management strategies need to be at least 94% effective (Wale *et al*., 2011). Achieving this high level of control is a challenge for growers and so

many use several strategies between potato crops. Care should also be taken to manage fungal pathogens such as *Rhizoctonia solani*, which interact synergistically with PCN and enhance yield losses (Back *et al*., 2006).

Figure 1.11: *Globodera pallida* potato cyst nematode cysts (marked *) on roots.

1.3 Potato cyst nematode management

Potato cyst nematode management practices are used by growers to try and achieve economically viable yields in both the short and long term. Emphasis is placed on keeping PCN populations below threshold levels (Whitehead & Turner, 1998). This can be achieved using cultural, biological and chemical management methods. Historically, cultural control methods have been defined as practices used to prevent the distribution and proliferation of PCN through simple field and potato variety selections, and sanitary techniques (Brown, 1978). Biological control involves the application of predatory or parasitic organisms to soil to reduce PCN populations (Brown, 1978; Stirling 1991). Chemical management relies on the application of toxic compounds to soil used to kill or paralyse encysted and migratory juveniles (Haydock *et al*., 2013). However, before a PCN management strategy can be implemented, the scale of PCN infestation should be

identified. Appropriate management techniques can then be selected to reduce PCN to sub-threshold population levels on an individual field basis.

1.3.1 *Sanitation and potato cyst nematode sampling*

Sanitation includes any method used to prevent PCN movement between fields and farms or to prevent population increases on self-set 'volunteer' plants from previous cropping (Turner & Subbotin, 2013). European Council Directive 69/465/EEC (OJEC, 1969) first recognised the need for sanitary procedures for PCN management in Europe. However, poor knowledge of factors influencing PCN movement and population dynamics, in addition to overestimating the effectiveness of resistant potato varieties and synthetic management tools, led to increasing PCN populations. New European legislation; Directive 2007/33/EC, addresses some of those weaknesses (OJEU, 2007). Ware potatoes can be produced on infested land, however, an extensive management programme should be followed in compliance with other pesticide approvals and agronomic practice legislation (Regulation EC/1107/2009 and Directive 2009/128/EEC). Additionally, designated areas for 'cull soil' and cleaning of potato equipment between operations is now required. Dr Andy Evans recently suggested at the 2017 Potatoes in Practice industry event (Dundee, UK) that where practical, fields with high PCN populations should be cultivated or harvested after other potato fields as an extra measure used to limit PCN distribution between fields (Pers. Comm. Dr Andy Evans: Nematologist at Scotlands Rural College). Control of volunteer plants is less well defined in the legislation but now common practice in the UK regardless. Cereal crops typically follow potato in the UK so that a broad-leaf herbicide such as Butoxone DB Extra®, Nufarm UK (2, 4-DB + MCPA) or Hiker®, Dow AgroSciences (Florasulam + Fluroxypyr) can be used for volunteer management (Lainsbury, 2016). In high PCN risk situations, planting of natural windbreaks (hedges and small wooded areas) can be adopted to prevent wind dispersal of PCN in small soil aggregates (Turner & Subbotin, 2013). The new European Union (EU) directive also sets a harmonized soil sampling system for determining PCN presence in fields, which requires 0.5% of the total national ware crop,

and 100% of the national seed crop to be sampled each year for PCN. This sampling system is useful for surveying PCN occurrence and movement in the UK in addition to limiting the opportunity for PCN distribution on seed stocks (OJEU, 2007).

1.3.2 *Crop rotation*

Crop rotation describes a cyclic order of cropping which begins and ends with a like crop; for instance, potato-wheat-barley-oilseed rape-wheat-potato. A good rotation should be sufficiently complex to limit the build-up of crop diseases and pests by staggering like crops which would have similar pest and disease susceptibilities. Long rotations allow time for natural viability decline of an organism which in PCN is mostly influenced by spontaneous hatching, predation by natural enemies and physical damage to cysts and eggs by cultivations (Lane & Trudgill, 1999; Stirling, 1991; Haley, 2004). Rotation can be an effective PCN management strategy, however, potato production economics typically require potatoes be grown every 4-6 years which is too short to benefit from the 10-30% natural decline expected per annum (Lane & Trudgill, 1999). Furthermore, *G. pallida* decline more slowly than *G. rostochiensis* between potato crops, so additional management practices are often required to achieve economic potato yields where this species is present (Turner & Subbotin, 2013). Hancock (1988) suggests rotation lengths of 7-8 years for effective PCN management, which corroborates with historical records describing a seven-course potato rotation used in South America by the Incas (Haydock & Evans, 1998a). Table 1.1 has been adapted from Haydock & Evans (1998b) and illustrates the expected number of viable PCN eggs in soil over a ten year period for an initial population of 100 viable eggs g^{-1} soil declining naturally between 10-50% per annum. Population density categories; low, medium and high, have been assigned to the expected viable eggs g^{-1} soil figures for each year at each decline rate whereby 0-10 eggs is considered low, 11-59 eggs is considered medium, and 60-100 eggs is considered a high PCN population density.

Table 1.1: Natural rotational decline of a 'high' 100 eggs g^{-1} soil PCN population over ten years at decline rates between 10-50% (Adapted from Haydock & Evans, 1998b).

Years since last potato crop	Natural decline rate (%) 10	20	30	40	50	Population density category
0	100	100	100	100	100	HIGH
1	90	80	70	60	50	
2	81	64	49	36	25	MEDIUM
3	73	51	34	22	13	
4	66	41	24	13	6	
5	59	33	17	8	3	
6	53	26	12	5	2	
7	48	21	8	3	1	LOW
8	43	17	6	2	0	
9	39	13	4	1	0	
10	35	11	3	1	0	

1.3.3 *Resistant and tolerant potato varieties*

The ability of a plant to restrict or prevent a parasite from multiplying is known as resistance (Nijboer & Parleviet, 1990). Mechanisms of PCN resistance in potato include vacuolation of syncytial cytoplasm, necrosis around an invasion site and enclosure of syncytium (Hoopes *et al*., 1978). All restrict or degenerate feeding sites sufficiently to inhibit juvenile growth or cause death. Giebel (1982) suggested that Egases used by PCN during root invasion can hydrolyse glycosides found in some resistant potato plant roots which causes a release of phenolic aglycones, subsequently causing the necrosis of invasion sites as mentioned. The *H1* gene, common to the variety Maris Piper, confers a robust monogenic resistance to *G. rostochiensis* pathotypes Ro1 and Ro4 which causes juvenile death by syncytium degradation (Huijsman, 1955; Hoopes *et al*., 1978). Due to the robustness and widespread inclusion of this gene in breeding programmes, *G. rostochiensis* now poses a reduced threat to UK crops, however, only polygenic resistance is available for *G. pallida*. Moreover, use of *H1* varieties has selected for *G. pallida* in the UK so that this species now poses a greater threat to potato production (Minnis *et al*., 2002). Using a scale of 1-9 where 1 is equivalent to no resistance and 9 is

less than 1% susceptible, only Panther scores an 8 for *G. pallida* resistance in Agricultural and Horticultural Development Board (AHDB) Potatoes Division validated experiments (OJEU, 2007; AHDB, 2015). A further 3 varieties score 6 whilst all other varieties are at least 15% susceptible (AHDB, 2015). The varieties Arsenal, Innovator, Eurostar and Performer are suggested as having a score of 9, however, the AHDB have not validated these varieties (AHDB, 2015). Although *G. pallida* resistant varieties are limited, tolerant varieties can be used to achieve economic yields in the presence of PCN. Tolerance describes the ability of a potato plant to withstand or recover from PCN parasitism (Trudgill, 1991). Tolerant varieties usually have large root systems which compensate for the nutrient sink effects of PCN parasitism. However, tolerance of damage is independent of resistance so that where a PCN tolerant but non-resistant potato variety is grown, PCN populations can increase to levels which would greatly reduce yield in a subsequent intolerant crop (Trudgill, 1991). Ideally a potato variety should be resistant and tolerant of PCN. Whilst some new varieties offer resistance and tolerance to PCN, they tend to be only suitable for the processing industry. Figure 1.12 shows the resistant and tolerant variety Arsenal and Figure 1.13 the resistant but intolerant variety Innovator at *c.*90 days after planting on a field site with over 100 PCN eggs g^{-1} soil.

Figure 1.12: Resistant tolerant potato cv. Arsenal ground cover at *c.*90 days after planting, on a site with *c.*100 potato cyst nematode eggs g^{-1} soil. The scale indicates a single ridge width from a 1.8 m wide two ridged bed.

Figure 1.13: Resistant intolerant potato cv. Innovator ground cover at *c.*90 days after planting, on a site with *c.*100 potato cyst nematode eggs g^{-1} soil. The scale indicates a single ridge width from a 1.8 m wide two ridged bed.

1.3.4 *Trap cropping*

Trap crops stimulate PCN hatch and root invasion, but either do not support PCN development, or are terminated before new cysts are formed (Scholte, 2000a). A close relative of potato, *Solanum sisymbriifolium* (Figure 1.14), the Sticky Nightshade*,* is resistant to both PCN species and has been recorded to reduce PCN populations by up to 80% when grown for 150 days under field conditions (Timmermans *et al*., 2006; Scholte, 2000a). However, some field populations of PCN appear to hatch less readily than others when in the presence of *S. sisymbriifolium*, which is a potential limitation to the technique (Scholte, 2000a). *Solanum sisymbriifolium* can also be difficult to establish and requires a full growing season be set aside in the UK for PCN to be reduced effectively. *Solanum sisymbriifolium* seed and establishment are also costly at *c.*£285 ha^{-1} and *c.*£70-100 ha^{-1} respectively (Sparkes, 2013). *Solanum sisymbriifolium* is therefore only justifiable for fields with high populations of susceptible PCN. Furthermore, current advice suggests the crop be planted between May and July in Northern Europe which poses a workload clash with other on farm operations such as blight spraying, harvest of early potato crops and second planting of seed crops (Timmermans *et al*., 2007). Black Nightshade, *Solanum nigrum* (Figure 1.15)*,* is fully resistant to *G. rostochiensis* but only partially resistant to *G. pallida* (Scholte, 2000a). It is also suggested to be less tolerant of PCN than *S. sisymbriifolium* (Scholte, 2000b), however, Figure 1.15 presents some anecdotal evidence that the tolerance of the trap crop is quite high. *Solanum nigrum* is commonly considered a weed species and so perhaps less likely to be used for PCN reduction in commercial systems than *S. sisymbriifolium*. However, the trap crop has an emerging interest in the industry and has recently been showcased at the 2017 AHDB strategic potato farm demonstrations for the West at Shawbury, Shropshire (UK). Alternatively, partially resistant and tolerant discard potatoes known as ‘chats’ could be planted later in the year between cash crops and grown for approximately 6-8 weeks to stimulate hatching and invasion. This technique would produce a market for the waste potatoes which are less expensive to purchase and easier to establish than *S. sisymbriifolium,* however, management of volunteers

would be crucial to prevent virulent *G. pallida* strains overcoming the currently available partial resistance in these potato varieties (Pers. Comm. Dr Ivan Grove: Principal Lecturer and Nematologist at Harper Adams University & Peter Blaylock: Independent Potato Agronomist). Additionally, care to ensure cysts did not form and detach from roots to soil whilst growing the crop would have to be taken (Scholte, 2000a). Trap cropping maintains a following in the UK with *S. sisymbriifolium* products DeCyst and Foil-sis promoted by UK agronomy companies Greenvale AP and Branston Ltd respectively, and Barworth Agriculture promoting *S. nigrum* products.

Figure 1.14: Potato cyst nematode trap crop *Solanum sisymbriifolium,* at *c.*50 days after planting on a site with *c.*100 PCN eggs g^{-1} soil.

Figure 1.15: Potato cyst nematode trap crop *Solanum nigrum,* at *c.*50 days after planting on a site with *c.*100 PCN eggs g^{-1} soil.

1.3.5 *Biological control*

Biological control is the term used to describe methods of reducing pest populations using their natural enemies. For PCN management these currently include nematophagous fungi and bacteria (Stirling, 1991; Kerry, 1988). Nematophagous fungi parasitise nematodes, or capture nematodes using specialised hyphal nooses (Stirling, 1991). Most are obligate parasites although some also have facultative saprophytic survival strategies such as *Purpureocillium lilacinus* and *Pochonia chlamydosporia* (Karssen *et al*., 2013; Kerry, 1988). Whilst facultative species tend to be less efficacious than obligate parasites, they represent the greatest opportunity for long term nematode suppression on account of their persistence in soil (Kerry, 1988). Nematophagous fungi with endoparasitic lifecycles colonise the body of their host with assimilative hyphae, zoosporgania, resting spores or conidiophores (Stirling, 1991). Infection of the nematode host is achieved either through successful chemo-location by motile flagellated zoospores which encyst near the anus of a parasitized nematode through successful

adhesion of spores to nematodes and subsequent body assimilation by hyphae, or by ingestion of conidia which germinate in the gut of a nematode (Stirling, 1991). Ectoparasitic nematophagous fungi are perhaps better recognised and include the trapping fungi which form hyphal nooses to ensnare motile nematodes (Stirling, 1991). The facultative endoparasitic fungi *P. chlamydosporia* has been found 70% effective in reducing multiplication of *G. pallida* in the glasshouse, and 51% effective in the field when granular applied (Crump, 1998; Tobin *et al*., 2008). *Purpureocillium lilacinus* is another promising facultative endoparsitic fungus which has been found 48-70% effective in countering PCN damage in potato when applied as a tuber dip at planting (Davide and Zorilla, 1983). Other PCN parasitic nematophagous fungi include *Trichoderma harzianum* and *Plectospaerella cucumerina,* however, results remain inconsistent and field scale validation and commercial production of these biocontrol products is currently limited (Dandurand & Knudsen, 2016). The endoparasitc bacterium *Pasteuria penetrans* offers another option which could potentially be used for PCN management but is currently unavailable commercially (Pers. Comm. Dr Keith Davies: Senior Lecturer and Nematologist investigating *P. penetrans* for management of potato cyst nematodes at the University of Hertfordshire). *Pasteuria penetrans* is persistent in soil and resilient to nematicides and so has development potential (Karssen *et al*., 2013).

1.3.6 *Nematicides and nematostats*

Nematicides are chemical compounds which are lethal to nematodes whilst nematistats, or nematistatics, are chemical compounds which provide sub-lethal effects used to disrupt nematode behaviour (Haydock *et al*., 2013; Hague & Gowen, 1987). Many nematicidal substances are available worldwide although only metam-sodium (example Metham 510®, Certis) and dazomet (Basamid®, BASF) are currently registered for use in the UK (Lainsbury, 2016). Both liberate biocidal methyl-isothiocyanate (MITC) gas upon contact with soil and so are typically autumn applied to allow the toxicant to decompose before planting of a potato crop (Lainsbury, 2016; Cremlyn, 1991). Methyl-isothiocyanate is liberated from metam-sodium in response to a decline from normal

atmospheric pressure and contact with soil water after liquid injection, and from dazomet after granules breakdown in a hydrolysis reaction with soil water (Figure 1.16) (Cremlyn, 1991; Hague & Gowen, 1987). Isothiocyanates are strong electrophilic reagents which are suggested to move through soil water and air by diffusion, permeating cysts and degrading proteins and essential enzymes in encysted juveniles (Hague & Gowen, 1987; Kawakishi & Kaneko, 1985). Soil moisture should range between 30-70% of field capacity at application for optimal PCN reduction, and soil temperature be above 15°C for optimal MITC release, movement and retention in soil (Hague & Gowen, 1987; Haydock *et al*., 2013). Methyl-isothiocyanate tends to be more efficacious against PCN than nematistat compounds. Up to 80% reduction in PCN egg viability can be achieved when applying fumigants such as metam-sodium to light land. However, efficacy is greatly reduced on organic soils where the fumigant is adsorped by OM (Haydock & Evans, 1998a; Hague & Gowen, 1987; Cremlyn, 1991). Application costs can also be prohibitive so that fumigation is only used for management of high PCN infestations under certain environmental conditions.

Nematistats are granular products applied to depths of 100-200 mm from specified incorporation equipment during the crop establishment stage i.e. bed-tiller, stone separator, other rotary based tillers (Figure 1.17), whereupon the active substance diffuses into soil water (Woods & Haydock, 2000; Woods *et al*., 1999; Lainsbury, 2016; Hague & Gowen, 1987). Figure 1.18 indicates the damage that could be expected in PCN infested fields when granular nematistats are poorly, or intermittently applied. Approved compounds for UK use include organophosphates fosthiazate (Nemathorin®, Syngenta) and ethoprophos (Mocap®, Certis) and the oxime-carbamate oxamyl (Vydate®, DuPont), however, ethoprophos is currently suggested to offer only a useful reduction of PCN rather than control (Lainsbury, 2016). All inhibit acetylcholine esterase (AChE) enzyme which impairs movement in invasive PCN leading to lipid depletion and often death by starvation (Haydock *et al*., 2013; Hague & Gowen, 1987). Potato cyst nematodes can recover from AChE inhibition, however, root invasion is sufficiently

delayed in many instances so that early damage to potato plants is greatly reduced (Hague, 1979; Haydock & Evans, 1998a). Additionally, fosthiazate and oxamyl provide good systemic movement in potato xylem and phloem and so have some action against PCN *in-vivo* (Hague & Gowen, 1987). Granular nematistats offer in excess of 59% control of PCN, but are less costly than fumigants in the UK and therefore more routinely relied upon (Tobin *et al*., 2008). Moisture guidelines for application mention only that soil should be damp and that soil temperature should be in excess of 15°C for optimal PCN suppression (Hague & Gowen, 1987; Lainsbury, 2016). However, soil temperatures are often much lower than 15°C at planting in the UK and so these guidelines are impractical for the UK situation.

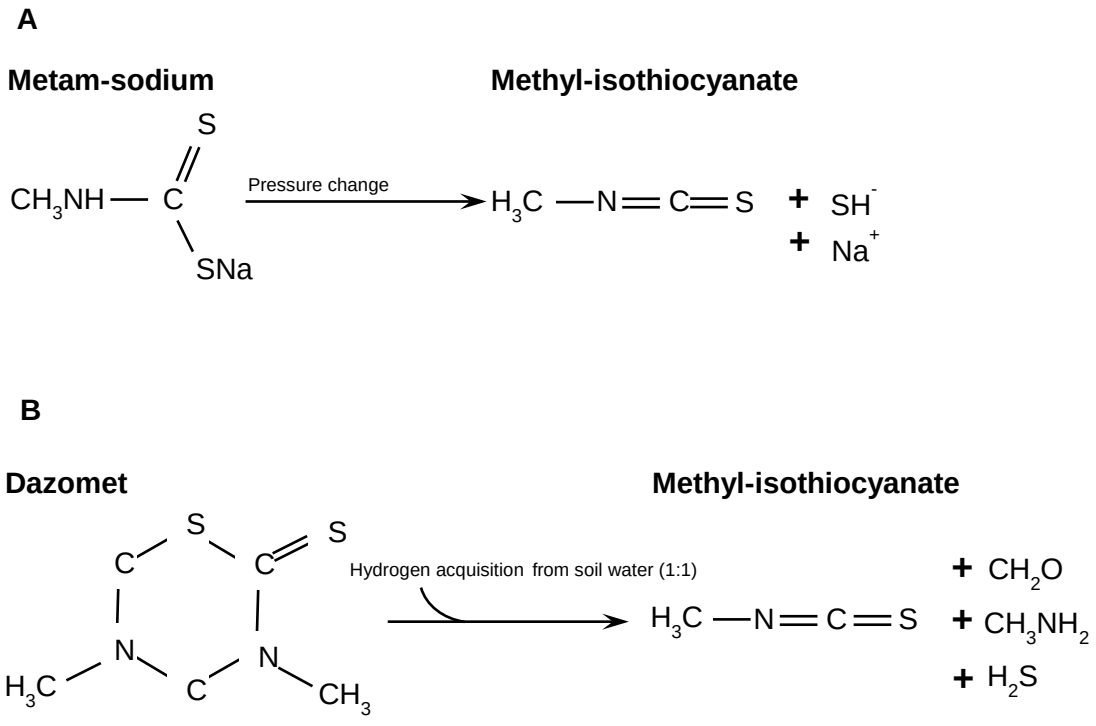


Figure 1.16: Metam-sodium (A) and dazoment (B) hydrolysis and rearrangement to methyl-isothiocyanate (Source: Adapted from Cremlyn, 1991).

Figure 1.17: Refilling the hoppers of a 1.8 m wide Jones Engineering bed-tiller with oxamyl nematostat.

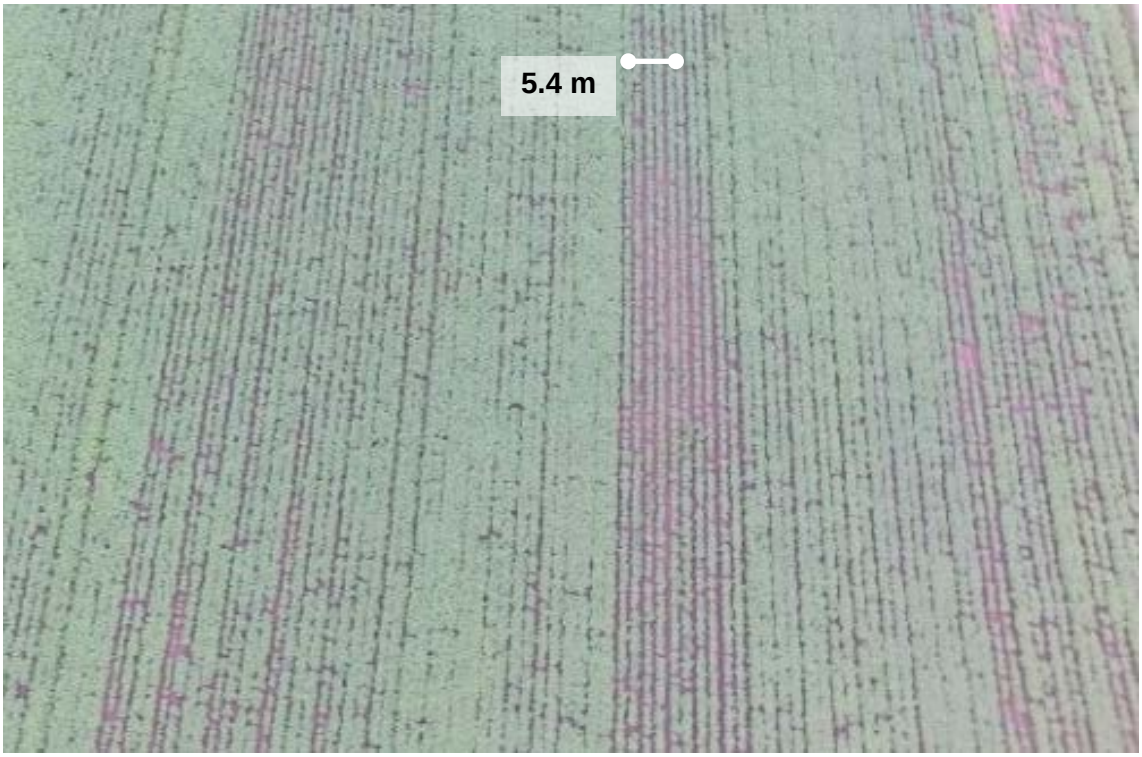


Figure 1.18: Potato cyst nematode damage patches (dark areas) in a potato field with poor and intermittent nematistat incorporation (Source: Dr Ivan Grove, Harper Adams University). Scale indicates the width of three 1.8 m wide potato beds.

A major limitation to the continued use of nematicides and nematistats in the UK could be the enforcement of EU Regulation (EC)1107/2009 which threatens to initiate a phase out of pesticides on a hazard basis (OJEU, 2009; Hillocks, 2012). The regulation is also currently limiting new products coming to the market. Alternative management options are therefore required. Several 'natural' nematicides reliant on aldehydes, ketones, linolenic acids and chitin based products are available and believed to be more acceptable to legislators although the efficacy of these products remains questionable in addition to their commercial availability, longevity and classification as nematicides (Haydock *et al*., 2013; OJEU, 2009). Well established products which are reliant on sulphides found in garlic (*Allium sativum*), such as the dimethyl disulphide (DMDS) fumigant Paladain® (Arkema) or granular NEMguard® (Certis), could also offer potential as PCN management tools. However, neither are currently registered for management of PCN in the UK. Another alternative is the use of brassica green manure plant residues for the biological fumigation (biofumigation) of PCN (Ngala *et al*., 2014). Biofumigation currently falls outside of European pesticide legislation but utilises similar compounds to MITC and so poses the potential to replace nematicides and nematostats should they be retracted from industry (OJEU, 2009; Haydock *et al*., 2013).

1.4 Biofumigation

1.4.1 *Introduction to biofumigation*

The term 'Biofumigation' was coined in the early 1990's to describe the suppression of soil-borne weeds, pests and pathogens following exposure to toxic volatile gases, principally isothiocyanates (ITC), liberated from brassica root and leaf tissues after mechanical maceration (Kirkegaard *et al*., 1993; Angus *et al*., 1994) (Figure 1.19). The volatile organic compound (VOC) emitting tissues are best incorporated into soil (Figure 1.20), and where possible, the soil surface sealed using either a mechanical smear roller, plastic film or irrigation to improve VOC retention and the overall efficacy of the technique (Matthiessen *et al*., 2004; Lord *et al*., 2011). The most common biofumigant species used for PCN management in the UK today include *Brassica juncea* (syn. Indian mustard),

Eruca sativa (syn. rocket/arugala) and *Raphanus sativus* (syn. oil-raddish) (Figures 1.21-3), however, many other brassica species are also available (Ngala *et al*., 2014; Lord *et al*., 2011).

Figure 1.19: Rear mounted 2 m wide hammer-tine flail-topper used to macerate a *Brassica juncea* biofumigant crop.

Figure 1.20: A 2 m wide rotary-tiller (rotavator) used to incorporate a *Brassica juncea* biofumigant crop.

Figure 1.21: *Brassica juncea* biofumigant crop at early-flowering.

Figure 1.22: *Eruca sativa* biofumigant crop at mid-flowering.

Figure 1.23: *Raphanus sativus* biofumigant crop pre-flowering.

1.4.2 *Background to biofumigation of potato cyst nematodes*

Morgan (1925) first documented pest suppression by brassica VOC's when she observed reduced PCN (identified as *H. rostochiensis* at this time) incidence on the roots of potato plants grown in close proximity to white mustard *Sinapis alba*. Triffitt (1930) repeated the work and identified PCN suppression was achieved through a biochemical mechanism, determined that the mechanism could be activated in both root and plant tissues in white mustard specifically, and also proved that white mustard biochemistry was implicated in reducing the size of surviving PCN cysts on potato roots. Ellenby (1945a) discovered that ITC's were responsible for brassica mediated PCN suppression, and also found that ITC's could have sub-lethal effects on PCN, resulting in reversible paralysis where exposure time and/or ITC concentrations were insufficient to kill the nematodes. These observations developed into further experiments aimed at improving the efficacy of ITC's against PCN, primarily by investigation of carrier materials for concentrated mustard oil extracts (Ellenby 1945b; Ellenby, 1951). The research was probably inspired by Smedly (1939) who had researched talcum powder as a carrier for

synthetic ITC oils to be worked into soil in a similar manner to modern granular nematostats. However, the research was eventually stifled following the introduction and widespread uptake of new, economical, highly efficacious and reliable fumigant nematicides (Cremlyn, 1991). Novel synthetic nematicides now rarely enter the industry due to increasingly restrictive legislation. The application of ITC liberating compounds to soil on talcum powder granules specifically, is so similar to other scrutinised pesticides that product registration is unlikely (Hillocks, 2012). Only green manure residue applications appear to be feasible for management of PCN at the current time. There are, however, many factors which influence the efficacy of this technique which need consideration, principally the occurrence and successful enzymatic hydrolysis of glucosinolates (GSL's) which liberate biofumigant VOC's such as ITC's upon degradation.

1.4.3 *Glucosinolate-myrosinase system*

Glucosinolates are secondary plant metabolites which are chemically diverse of a single side-chain (designated 'R'). They have a characteristic core which includes a thiohydroximate anion attached to a sulphate residue (referred to as the aglycone moiety) and a glucose moiety (Figure 1.24) (Agerbirk & Olsen, 2012; Ettlinger & Lundeen, 1956). The GSL class of glucosides is currently known to include 132 variants which can be classified according to their precursor amino acids such as methionine, tryptophan or tyrosine, or more routinely by the structure of their side-chain; aliphatic (alkyl or alkenyl), aromatic or indolyl (Figure 1.24) (Agerbirk & Olsen, 2012; Fenwick *et al*., 1983; Ettlinger & Lundeen, 1956). Glucosinolates are themselves biologically inactive (Buskov *et al*., 2002) and thermally and chemically stable secondary plant metabolites, however, GSL degradation products such as ITC's are unstable, reactive and toxic (Holst & Williamson, 2004; Agerbirk & Olsen, 2012). It is the diversity and structure of side-chain most specifically which dictates the possible range of biocidal VOC's a GSL might degrade into following tissue disruption. Thioglucoside glucohydrolase enzymes known

as myrosinases then catalyse the degradation via enzymatic hydrolysis (Agerbirk & Olsen, 2012; Andréasson *et al*., 2001; Bones & Rossiter, 1996).

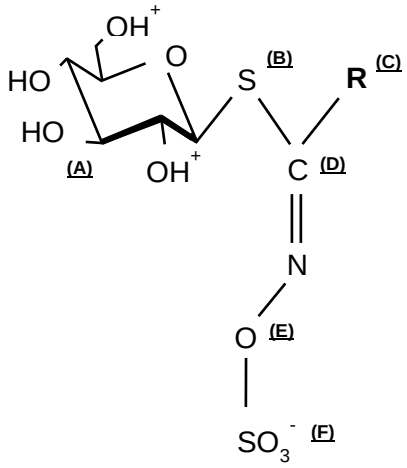


Figure 1.24: Generic glucosinolate described by Ettlinger & Lundeen (1956) detailing (A) glucose moiety, (B) thioglucoside linkage, (C) variable 'R' chain, (D) thiohydroximate anion core structure, (E) O-link, (F) sulphate residue.

Unlike GSL's, which are stored within protein storage vacuoles of non-specific plant cells, myrosinases are located in specialised GSL lacking idioblasts commonly referred to as myrosin cells (Andréasson *et al*., 2001). Glucosinolates and myrosinases remain compartmentalised in intact tissues, however, damaged tissues liberate cellular contents which enables GSL-myrosinase interaction. In the presence of water, myrosinase cleaves the thioglucoside linkage between glucose and aglycone moieties (Figure 1.25: B, C, D) (Bones & Rossiter, 1996; Holst & Williamson, 2004). Following liberation of a sulphate, the GSL aglycone rearranges to one of several VOC's including the biocidal ITC's and nitriles (Figure 1.25: E, F, G) (Andréasson *et al*., 2001; Holst & Williamson, 2004). The presence of ferrous ions, myrosinase-interacting proteins and the pH of hydrolysis are all factors known to influence the profile of hydrolysis products, however, under neutral pH conditions, ITC's are commonly formed from the degradation of most GSL's (Figure 1.25: F) (Holst & Williamson, 2004; Grubb & Abel, 2006).

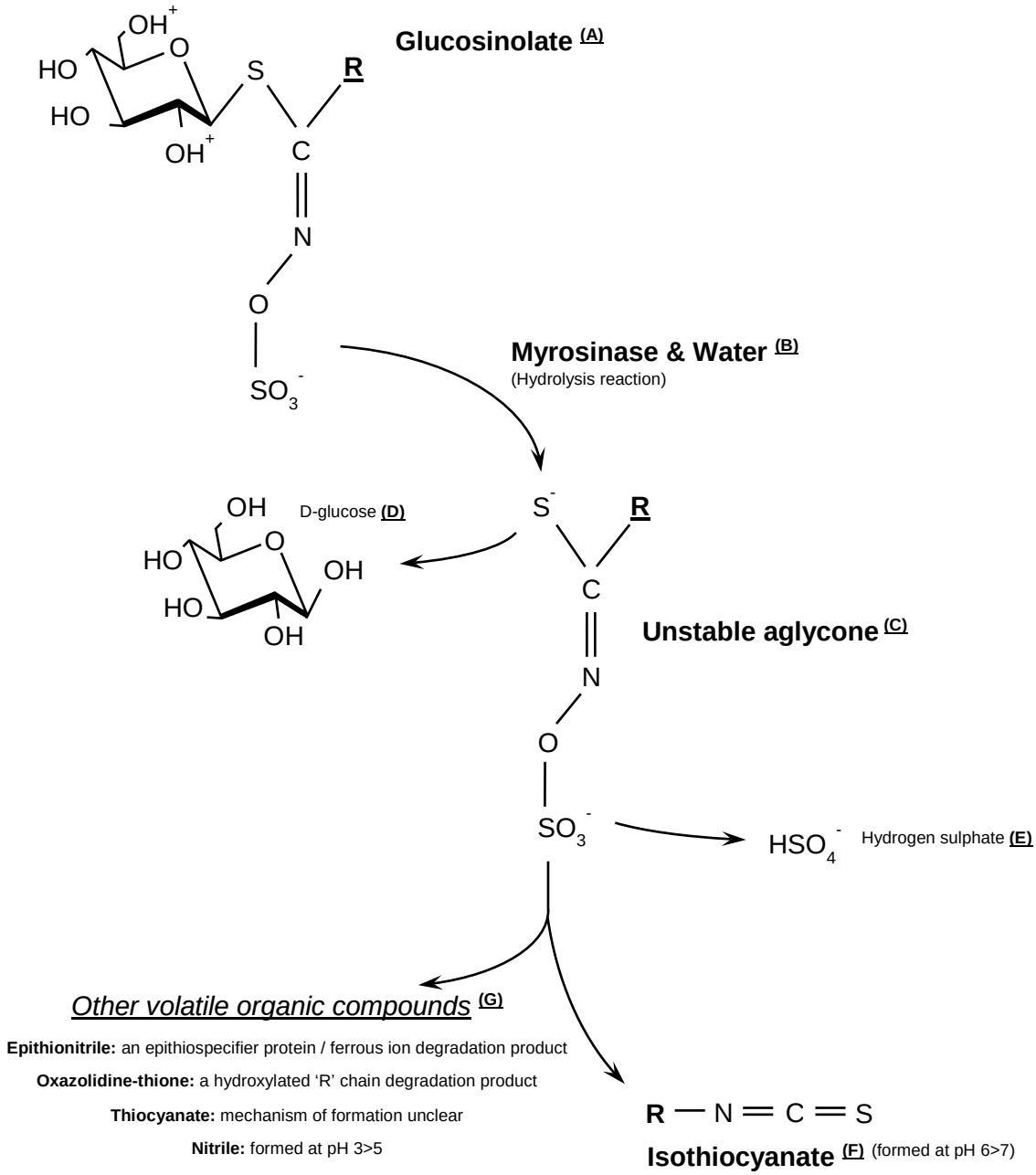


Figure 1.25: Enzymatic hydrolysis of glucosinolate to isothiocyanate and other volatile organic compounds detailing (A) a generic glucosinolate structure, (B) myrosinase and water used in glucosinolate hydrolysis, (C) a generic unstable aglycone structure following cleaving of the thioglucoside linkage during hydrolysis, (D) free glucose moiety, (E) hydrogen sulphate, (F) a generic isothiocyanate structure, and (G) other volatile organic compounds which could be produced by glucosinolate hydrolysis (Adapted from Bones & Rossiter, 1996; Holst & Williamson, 2004; Andréasson *et al*., 2001).

1.4.3.1 *Factors affecting glucosinolate hydrolysis product formation*

All ITC's arrange within a pH range of *c.*5-8 although more consistently between pH 6-7 (Figure 1.25: F). The β-hydroxy and indolylmethyl ITC's are the most volatile, rapidly cyclising to form oxazolidin-2-thiones and alcohols, or ascorbigen when in the presence of ascorbic acid at hydrolysis (Holst & Williamson, 2004). Other 'stable' ITC's have been identified as sulphide precursors in some instances; a chemical group most commonly associated with the pungency of alliums which also exhibit nematicidal properties (Pecháček *et al*., 1997; Bending & Lincoln, 1999). Nitriles are more commonly produced at pH 5 and below, whilst thiocyanate formation conditions are still unclear (Holst & Williamson, 2004). Epithionitriles, however, are common where hydrolysis occurs in the presence of myrosinase-interacting epithiospecifier protein (Figure 1.25: G) (Bones & Rossiter, 1996). Non-volatile hydrolysis products are associated with indolyl GSL's, whilst aromatic and aliphatic GSL's form ITC's and other biocidal VOC's (Bones & Rossiter, 1996). Selection of plants abundant in GSL's belonging to these two groups is therefore essential for pest management (Kirkegaard & Sarwar, 1998; Mithen, 1992).

1.4.4 *Mode of action and characteristics of isothiocyanates*

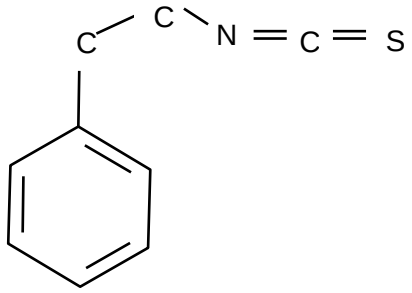
Isothiocyanates are highly reactive electrophiles which interact at their 'R' side-chains with nucleophilic thiol, sulphide and amino groups of amino acids and proteins (Kawakishi & Kaneko, 1985; Romanowski & Klenk, 2000). The interactions are non-specific which explains their biocidal properties. The toxicity of aromatic ITC's to a species of free-living ciliate (*Tetrahymena pyriformis*) correlates strongly with the reactivity of aromatic ITC's to the thiol group of the cysteine residue of glutathione which is essential in respiration (Schultz *et al*., 2005). Isothiocyanate interaction, particularly of highly reactive ITC's, prevents the utilisation of oxygen by the ciliate for metabolic activity. Provided that the concentration and exposure duration of ITC's is sufficient, respiratory disruption will cause death of the exposed organism (Miller *et al*., 2000). Respiratory inhibition has also been recorded against plant parasitic nematodes in MITC investigations (Chitwood & Perry, 2009). The efficacy of ITC's against nematodes is also

known to be influenced by factors including ITC-lipid solubility, ITC volatility and ITC hydrophobicity. Lipid-soluble ITC's (e.g. 2-phenethyl) are able to permeate phospholipid membranes to interact more widely with intracellular functions which kill nematodes, whilst more volatile ITC's (e.g. 2-propenyl) disperse evenly when in gaseous form which increases ITC-target organism exposure under suitable conditions (Holst & Williamson, 2004; Sarwar *et al.*, 1998). Similarly, hydrophobicity of ITC's could be influential of ITC movement and contact with PCN in soil. Water could limit ITC exposure to PCN in wetter soils where ITC hydrophobicity is high, but could also act as a carrier of ITC to PCN where ITC hydrophobicity is low. To the author's knowledge, this research area has not yet been investigated.

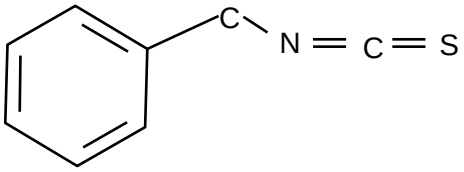
1.4.5 *Toxicity of glucosinolate hydrolysis products to potato cyst nematodes*

At least eight *in-vitro* studies have recorded mortality of PCN following exposure to GSL hydrolysis products (Buskov *et al*., 2002; Ngala *et al*., 2015a; Brolsma *et al*., 2014; Lord *et al*., 2011; Wood *et al*., 2017; Ellenby, 1945a; Serra *et al*., 2002; Ellenby, 1951). Toxicity of VOC's, principally ITC's, arising from the hydrolysis of 2-propenyl (syn. sinigrin), 3-butenyl (syn. gluconapin), benzyl (syn. glucotropaeolin), 2-phenylethyl (syn. gluconasturtiin), 4-methylsulfinyl-3-butenyl (syn. glucoraphenin), 4-hydroxybenzyl (syn. glucosinalbin), 2-hydroxy-3-butenyl (syn. progoitrin), ethyl (syn. glucolepidiin), 4-(methylsulfinyl)butyl (syn. glucoraphanin), methyl (syn. glucocapparin) and 2-hydroxy-2-phenylethyl (syn. glucobarbarin) GSL's have been reported. The formulation type for aqueous solutions used in the experiments, GSL or ITC concentrations in solution and exposure times vary between studies, however, the hydrolysis products of 2-propenyl, benzyl and 2-phenylethyl GSL's are consistently more toxic to PCN than other alternatives. Biofumigants rich in these GSL's could be preferable for PCN management. Figure 1.26 illustrates the chemical structure of 2-propenyl, benzyl and 2-phenylethyl isothiocyanates in comparison to MITC. As discussed, larger ITC's such as 2-phenylethyl are more reactive than smaller ITC's but also less volatile and more hydrophobic.

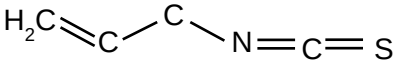
2-phenylethyl isothiocyanate



benzyl isothiocyanate



2-propenyl isothiocyanate



methyl isothiocyanate

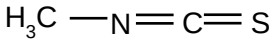


Figure 1.26: Chemical structure of 2-phenylethyl, benzyl, 2-propenyl and methyl isothiocyanates (Adapted from Cremlyn, 1991).

Tables 1.2-1.4 summarise all of the known *in-vitro* literature reporting PCN mortality after exposure to GSL hydrolysis products specifically arising from 2-propenyl, benzyl and 2-phenylethyl GSL's. Tables 1.2-1.4 clearly demonstrate the purity of GSL and ITC aqueous solutions to influence efficacy. Plant extract solutions are less efficacious than pure extracts at comparable concentrations of compounds in solution, and at comparable exposure times. This could indicate that achieving effective biofumigation in a field situation might be increasingly challenging. Hatching suppression and hatching stimulation of encysted PCN eggs has also been documented after exposure to GSL hydrolysis products *in-vitro*, notably by Ellenby (1945a & 1951), Valdes *et al.* (2011), Brolsma *et al*. (2014), Ngala *et al*. (2015a) and Wood *et al*. (2017).

Table 1.2: Potato cyst nematode (*Globodera* spp.) mortality after exposure to 2-propenyl glucosinolate salt and ground tissue aqueous solutions, and 2-propenyl isothiocyanate oil in *in-vitro* investigations

Glucosinolate [Class *] [*MW*]	**Hydrolysis product** [Hydrophobic value **]	**Nematode species**	**Compound formulation**	**Solution pH**	**Toxicity assessment**	**Compound concentration in solution [mM]**	**Exposure time [h]**	**Mortality [%]**	**Reference**
2-Propenyl	Allyl/Propenyl	*G. rostochiensis*	Purified glucosinolate salt	6.5	A	0.13	32	3	Buskov *et al* ., 2002
Syn. Sinigrin	Isothiocyanate					0.13	72	43	
[D] [*397.45*]	[1.17]					0.75	8	1	
						0.75	72	65	
						2.52	8	7	
						2.52	40	100	
		G. pallida	Freeze-dried and ground brassica tissue [2-Propenyl 97% of the glucosinolate profile]	7.0	B	2.80	96	*c* . 44	Ngala *et al* ., 2015a
						5.68	96	*c* . 56	
						11.28	96	*c* . 72	
						22.55	96	*c* . 83	
						45.10	96	100	
						90.20	96	*c* . 86	
		G. pallida	Freeze-dried and ground brassica tissue [2-Propenyl 100% of the glucosinolate profile]	Not described	C	74.00	24	*c* . 90	Lord *et al* ., 2011
		G. pallida	Isothiocyanate oil [assumed 100% purity]	7.0	A	0.13	24	*c* . 19	Wood *et al* ., 2017
						0.13	72	*c* . 78	
						0.26	24	*c* . 59	
						0.26	72	100	
						0.51	24	100	

***** As defined by Fahey *et al*. (2001): D = olefin ****** As defined by Borek *et al*. (1998): high hydrophobicity values indicate greater hydrophobicity than low hydrophobicity values ******* A = J2 exposure to treatment with motility assessment by pricking and heating to determine mortality, B = cyst exposure to treatment with hatching and Meldolas Blue stain assay to determine mortality, C = J2 exposure to treatment with motility assessment using sand columns to determine mortality.

Table 1.3: Potato cyst nematode (*Globodera* spp.) mortality after exposure to 2-phenylethyl glucosinolate salt and ground tissue aqueous solutions, and 2-phenylethyl isothiocyanate oil in *in-vitro* investigations

Glucosinolate [Class *] [*MW*]	**Hydrolysis product** [Hydrophobic value **]	**Nematode species**	**Compound formulation**	**Solution pH**	**Toxicity assessment**	**Compound concentration in solution [mM]**	**Exposure time [h]**	**Mortality [%]**	**Reference**
2-Phenylethyl Syn. Gluconasturtiin [G] [*461.54*]	Phenylethyl Isothiocyanate [2.46]	*G. rostochiensis*	Purified glucosinolate salt	6.5	A	0.11	16	8	Buskov *et al* ., 2002
						0.11	72	57	
						0.65	8	1	
						0.65	72	71	
						2.17	8	83	
						2.17	16	100	
		G. rostochiensis	Purified glucosinolate salt	6.5	A	0.11	8	3	Serra *et al* ., 2002
						0.11	72	8	
						0.65	8	9	
						0.65	72	91	
						2.17	8	100	
		G. pallida	Freeze-dried and ground brassica tissue [2-Phenylethyl 89% of the glucosinolate profile]	7.0	B	1.66	96	*c* . 30	Ngala *et al* ., 2015a
						3.38	96	*c* . 47	
						6.70	96	*c* . 83	
						13.40	96	*c* . 84	
						26.80	96	*c* . 97	
						53.60	96	*c* . 98	
		G. pallida	Freeze-dried and ground brassica tissue [2-Phenylethyl 93% of the glucosinolate profile]	Not described	C	78.00	24	*c* . 95	Lord *et al* ., 2011
		G. pallida	Isothiocyanate oil [assumed 100% purity]	7.0	A	0.08	24	*c* . 26	Wood *et al* ., 2017
						0.08	72	*c*. 75	
						0.17	24	*c* . 11	
						0.17	72	*c*. 59	
						0.34	24	*c*. 12	
						0.34	72	*c* . 71	

***** As defined by Fahey *et al*. (2001): G = aromatic. ****** As defined by Borek *et al*. (1998): high hydrophobicity values indicate greater hydrophobicity than low hydrophobicity values ******* A = J2 exposure to treatment with motility assessment by pricking and heating to determine mortality, B = cyst exposure to treatment with hatching and Meldolas Blue stain assay to determine mortality, C = J2 exposure to treatment with motility assessment using sand columns to determine mortality

Table 1.4: Potato cyst nematode (*Globodera* spp.) mortality after exposure to benzyl glucosinolate salt and benzyl isothiocyanate oil in *in-vitro* investigations

Glucosinolate [Class *] [*MW*]	**Hydrolysis product** [Hydrophobic value **]	**Nematode species**	**Compound formulation**	**Solution pH**	**Toxicity assessment**	**Compound concentration in solution [mM]**	**Exposure time [h]**	**Mortality [%]**	**Reference**
Benzyl	Benzyl	*G. rostochiensis*	Purified glucosinolate salt	6.5	A	0.11	24	5	Buskov *et al*., 2002
Syn. Glucotropaeolin	Isothiocyanate					0.11	72	25	
[G] [*447.51*]	[2.21]					0.67	8	21	
						0.67	72	91	
						2.23	8	83	
						2.23	24	100	
		G. pallida	Isothiocyanate oil [assumed 100% purity]	7.0	A	0. 09	24	*c*. 1	Wood *et al*., 2017
						0.09	72	*c*. 16	
						0.19	24	*c*. 15	
						0.19	72	*c*. 43	
						0.38	24	*c. 23*	
						0.38	72	*c*. 93	

***** As defined by Fahey *et al*. (2001): G = aromatic. ****** As defined by Borek *et al*. (1998): high hydrophobicity values indicate greater hydrophobicity than low hydrophobicity values ******* A = J2 exposure to treatment with motility assessment by pricking and heating to determine mortality

1.4.6 *Biofumigation potential*

To be effective, brassica green manures need to successfully fumigate soil. This is influenced by several factors, principally the profile and concentration of GSL's, and the quantity of plant tissue incorporated into soil (Kirkegaard & Sarwar, 1998). Although the toxicity of ITC's can be used in the selection of biofumigants, it is important to consider the variation in GSL profile and the concentration of GSL's in different plant organs, at different developmental stages, within and between brassica species, and between plants grown under winter or summer seasons (Booth *et al*., 1991; Kiregaard & Sarwar, 1998; Rosa *et al*., 1996; Charron *et al*., 2005).

In general, high biofumigation potential crops can achieve greater than 40 µmol GSL g^{-1} dry-weight tissue (Kirkegaard & Sarwar, 1998; Lord *et al*., 2011). Glucosinolate concentration and biomass peak at around mid-flowering in most brassica species with GSL concentrations having been recorded in excess of 100 µmol GSL g^{-1} dry-weight tissue in field grown brassicas in the summer e.g. *B. juncea* cv. ISCI 99 (Ngala *et al*., 2014). Biomass can also vary greatly between and within species. Fresh biomass of *c.*70 t ha^{-1} has been recorded in field grown *B. juncea* biofumigants drilled at seed-rates of *c.*10 kg ha^{-1} in the UK, and biomass of *B. juncea* found to be approximately double that of *E. sativa* (Ngala *et al*., 2014; Watts *et al*., 2014). Day length, intensity of solar radiation and temperature are also factors known to influence GSL accrual in brassica tissues (Engelen-Eigles *et al*., 2006). Light is essential for photosynthesis which yields glucose, an essential component of all GSL's (Agerbirk & Olsen, 2012). Glucosinolate concentration in tissues is therefore often higher in summer-grown biofumigants when day lengths are longer (Kirkegaard & Sarwar, 1998; Rosa *et al*., 1996; Charron *et al*., 2005). Sulphur and nitrogen availability to biofumigant plants is also known to influence GSL accrual in tissues as both represent essential elements in GSL biosynthesis (Booth *et al*., 1991). Nitrogen is also essential in the biosynthesis of proteins which influences the quantity of biofumigant biomass produced. Typically, rates of *c.*60-100 kg ha^{-1} of nitrogen are applied to biofumigants with sulphur (Lazzeri *et al.,* 2004) applied as

sulphate at a ratio of 5:1 (Pers. Comm. Dr Matthew Back: Reader in Nematology at Harper Adams University investigating nutrient applications to biofumigant crops for AHDB Potatoes). Other general agronomic considerations, such as plant available water in soil, and pest pressure, will also affect biofumigation potential in some situations, so the agronomy of these crops is as important as for a cash-crop if optimal biofumigation potential is to be achieved.

1.4.7 *Brassica biofumigant green manures*

Several seed companies now supply brassica species which they report to have biofumigation properties. The most widely recorded variety in the literature is *B. juncea* cv. ISCI 99 which has high concentrations of 2-propenyl GSL in its tissues (Ngala *et al*., 2014), and can achieve high fresh biomass under summer growing conditions (*c.*40-70 t ha^{-1}) (Figure 1.27) (Lazzeri *et al*., 2004). *Brassica juncea* cv. ISCI 99 supplied by High Performance Seeds, Washington State, United States of America (USA), was used in approximately one third of all papers presented at the 5th International Symposium of Biofumigation held at Harper Adams University in Shropshire (UK) in 2014. Other seed suppliers include Joordens Zaden, Kessel (Holland), and PH Petersen, Lundsgaard (Germany). Many studies have evaluated biofumigant varieties for their biofumigation potential (Bellostas *et al.,* 2007; Ngala *et al*., 2014; Watts *et al*., 2014), however, none as comprehensively as a seminal study completed by Kirkegaard & Sarwar (1998).

Kirkegaard & Sarwar (1998) screened 76 diverse brassica accessions from 13 brassica plant species in their 'biofumigation potential' field studies near Canberra, Australia in 1998. Biofumigants were grown to mid-flowering, then assessed for biomass and GSL content. The most widely screened species included *Brassica napus* (syn. oilseed rape), *Brassica campestris* (syn. field mustard), and *B. juncea* which accounted for 47 of the 76 accessions. *Brassica nigra* (syn. black mustard), *Brassica carinata* (syn. Ethiopian mustard) and *B. juncea* were found to have the highest ITC-liberating GSL concentrations in root and shoot tissues of 13 species under investigation but were

shown to vary greatly in fresh biomass (2.38-28.97 t ha^{-1}). In their work, mid-flowering date ranged from 106-190 days between *Sinapis arvensis* and *B. oleracea* respectively. *Brasica juncea* and *B. nigra* varieties were characterised by having a GSL profile dominated by 2-propenyl, moderate biomass and a maturation period of *c.*116-148 days. Other species were shown to have highly variable GSL profiles depending upon the variety tested. *Brassica juncea* is suitable for a wide range of cropping situations and is a potentially useful biofumigant species on account of its biofumigation properties. This goes some way to explaining why *B. juncea* cv. ISCI 99 is a popular biofumigant variety today. Other popular varieties in the UK include *E. sativa* cv. Trio produced by Joordens Zaden, and *R. sativus* cv. Bento produced by PH Petersen, which each produce high concentrations of 2-phenylethyl GSL's in tissues (Watts *et al*., 2014; Ngala *et al*., 2014).

Figure 1.27: Typical *Brassica juncea* cv. ISCI 99 crop of *c.*50 t ha^{-1} fresh-weight.

1.4.8 *Optimising biofumigation*

Aside from variety selection and agronomy, several factors have been suggested which could be manipulated to enhance biofumigation success. The literature base is, however,

poorly populated and in need of expansion (Mattner *et al*., 2008). Morra & Kirkegaard (2002) suggest only 1% of hydrolysable GSL is normally activated during biofumigation due to difficulties in breaking down plant tissues at the maceration stage (often mid-flowering) (Figure 1.28), whilst Gimsing & Kirkegaard (2006) report the highest concentration of ITC's in soil treated with biofumigation occurs within 30 min of plant maceration and incorporation. It is therefore essential that plants have high biofumigation potential to compensate for low GSL-ITC conversion and persistence in soil, but also important that maceration and incorporation effectiveness be improved to better utilise the potential biofumigants have to reduce pests.

Figure 1.28: Poor to moderate quality of biofumigant residue expected when macerating a *Brassica juncea* crop with most flail-toppers.

1.4.8.1 *Biofumigant maceration and incorporation technique*

Lazzeri *et al*. (2004) suggest that biofumigant crops should be ground and ploughed into soil to maximise ITC release from tissues. The most appropriate mechanical implements to achieve effective grinding of biofumigant tissues is not discussed, and no evidence is presented to support ploughing residues in place of other tillage methods. Matthiessen

et al. (2004) observed that a bladed mulcher (which probably refers to a grass mowing implement) only facilitated *c.*5% of the ITC output from brassica tissues as those macerated with a hammer implement (which probably refers to a flail-topper), indicating that decisions can be made regarding maceration implement selection which could optimise biofumigation. Matthiessen *et al*. (2004) suggest the bladed implement was ineffective because it cut biofumigant tissues, whereas the hammer implement caused widespread tissue damage by liquefying the tissues, subsequently leading to greater GSL hydrolysis. A bladed implement is more likely to cut plant material by acute bending force, as opposed to a blunt implement which is likely to macerate plant material through a combination of torsion and bending forces which cause more widespread tissue damage (Persson, 1987). However, Mathiessen *et al*. (2004) did not quantify the different maceration qualities between maceration treatments, and so their treatments have not yet been fully evaluated. Neither did they statistically analyse their data.

Morra & Kirkegaard (2002) first illustrated the principal of enhanced ITC release from blunt macerated tissues in *in-vitro* work comparing fresh *B. juncea* tissues versus freeze-treated tissues. The frozen tissues released approximately twenty-six times the level of ITC of that released from fresh tissues due to more widespread cellular damage, however, no study has yet linked higher ITC output from a maceration implement to enhanced pest suppression. Moreover, no study has yet compared biofumigant incorporation techniques to determine whether it is also an important factor influencing pest suppression. The only work to have investigated incorporation implements to date, merely compared the effect of incorporated and non-incorporated residues on the concentration of ITC's in soil, whereby the incorporation of tissues into soil increased soil concentrations of ITC's (Matthiessen *et al*., 2004).

1.4.8.2 *Soil conditions for successful biofumigation*

Soil moisture has also been investigated as a factor influencing biofumigation success. Initial *in-vitro* work by Morra & Kirkegaard (2002) found a trend (although not supported

statistically) which suggested ITC release from tissue could be approximately doubled by manipulating soil moisture regime. It was proposed that the trend for enhanced ITC release from tissues under higher soil moistures could have been due to more effective GSL hydrolysis in soil. However, further investigation is required to validate the current thinking. Matthiessen *et al*. (2004) expanded the work of Morra & Kirkegaard (2002) in their field work, and found up to *a c.*ten-fold increase in ITC's measured in soil irrigated with 42 mm of water immediately after tissue incorporation (water was manually applied to pipes embedded in field soil). The authors mentioned that soil moisture prior to the additional application was approximately field capacity, so that after irrigation, soil would have been waterlogged.

Whilst enhanced GSL hydrolysis could explain the higher concentrations of ITC in soil in the Matthiessen *et al*. (2004) study, it is also possible that soil moisture aided the retention of ITC's in soil by blocking ITC movement and forcing ITC's into soil solution; hence the higher soil concentrations of ITC (Simpson *et al*., 2010; Lembright, 1990). If this is true, the diffusion potential of the ITC's, which is critical to efficacy, may have been greatly reduced. Lembright (1990) reports that fumigant diffusion is 10-30,000 times greater through soil pore airspace than soil pore water. More work is therefore needed to understand the role of soil moisture for biofumigation of PCN. Past work investigating the efficacy of synthetic fumigants against soil borne pests found that approximately 42% of field capacity was optimal for fumigation (Lembright, 1990). This lends support to the hypothesis that ITC's may have been concentrated in soil water in the Mathiessen *et al*. (2004) study where they would have been less effective against soil borne targets such as PCN. Soil temperatures are also advised to be no lower than 12°C to ensure effective volatilisation of liquid fumigants, and ensure effective diffusion of a fumigant through soil air (Lembright, 1990; Lane & Trudgill, 1999). Soil temperatures below the threshold level can lead to low fumigant volatility and high fumigant solubility, detrimentally influencing efficacy (Lembright, 1990). It is therefore probable that this is the case for biofumigant VOC's too.

It should also be noted that some soils are less suitable for biofumigation. High OM soils have reduced free MITC concentrations in fumigant experiments, after sorption of ITC's to soil humus (Matthiessen *et al*., 1996). The ITC's 2-propenyl and benzyl have also been shown to readily sorb to soil OM in *in-vitro* work, where the larger benzyl ITC was shown to be more strongly sorbed to OM than 2-propenyl ITC; probably a result of its higher reactivity and lower volatility (Gimsing *et al*., 2009). Work by Price *et al*. (2005) has also shown ITC concentrations in headspace above soil to be higher when *B. juncea* tissues were incorporated into sandy soil compared to a high OM clay in soil column experiments. Gimsing & Kirkegaard (2009) suggest this could be explained by the generally higher sorbtive properties of clays. Otherwise, the clay soil could have had a higher volumetric water content which may have aided retention of ITC's better than the sand (Simpson *et al*., 2010). In general, damp sandy soils or sands covered in plastic film are likely to be the most receptive to biofumigation due to their larger pore spaces and lower water holding capacities (Lembright, 1990). Price *et al*. (2005) found a *c.* three-fold increase in brassica derived ITC retention in soils covered with plastic film compared to a bare surface. There are therefore clearly opportunities to optimise biofumigation for enhanced pest suppression in the field. Blunt maceration techniques which fully incorporate biofumigant material into soil are likely to be most appropriate for biofumigation. Sandy soils of low OM, at soil moisture contents of approximately 50% of field capacity, at temperatures in excess of 12°C, are also likely to be the most receptive to biofumigation.

1.4.9 *Management of potato cyst nematodes using biofumigation*

At least 8 studies have now measured and reported PCN mortality following biofumigation in the glasshouse and the field, and investigated at least 9 different brassica species (Table 1.5). Efficacy has ranged between 0-95%, between and within species, and between studies investigating the same variety; notably *B. juncea* cv. ISCI 99 (Brolsma *et al*., 2014; Ngala *et al*., 2014). Clearly, disparities are likely to be

explainable by some of the previously mentioned agronomic factors, such as GSL profile, GSL concentration in tissues, the quantity of biomass incorporated into soil, the expediency and method of tissue maceration and incorporation, the soil condition and possibly the hydrolysis reaction conditions. It is therefore worth considering the studies which record negative results first, to determine whether the poor efficacy recorded in these studies can be explained.

Valdes *et al*. (2011) investigated the effect of root diffusates and plant extracts of *S. alba*, *R. sativus* and *B. napus* on the hatching of *G. rostochiensis in-vitro*, and the effect of incorporated tissues from the same brassicaceous species on *G. rostochiensis* viability in pot tests. Valdes *et al*. (2012) then expanded upon the earlier work by investigating the effect of incorporated *S. alba* tissues on the viability of *G. rostochiensis* in field soils. No effect of biofumigation on PCN viability was recorded in either study, although enhanced hatching was recorded. Neither study reported the GSL content of biofumigant plants used in experimental work which casts some doubt on the quality of the work, and only Valdes *et al*. (2012) provides details of the biofumigant species and variety used. In this case, *S. alba* cv. Zlata was investigated, which has been documented to be lacking in 2-propenyl, benzyl or 2-phenylethyl GSL's (Bohinc *et al*., 2013). Furthermore, the maceration and incorporation technique adopted by Valdes *et al.* (2012) would appear to be non-conventional and probably sub-optimal. A grass-mower was used as opposed a flail-topper, which as previously reviewed, is a superior maceration implement (Matthiessen *et al*., 2004). The biofumigant residue was then manually incorporated into soil using a spade. This overall methodology is unlikely to have been conducive for generating sufficient quantities of biofumigant VOC's for PCN suppression, or have incorporated tissues expediently enough to make best use of biofumigant VOC's (Matthiessen *et al*., 2004; Gimsing & Kirkegaard, 2006). Overall, the choice of variety and incorporation strategy may have influenced the findings in this study.

Table 1.5: Potato cyst nematode (*Globodera* spp.) mortality after exposure to brassica biofumigant green manures in the field and glasshouse

Species	Variety	Glucosinolate profile	Study type	Nematode species	Toxicity assessment	Mortality [%]	Country	Reference
Barbarea vulgaris		1-methoxyindol-3-ylmethyl (Syn. 1-methoxyglucobrassicin); 2-hydroxy-2-phenylethyl (Syn. Glucobarbarin)	Glasshouse	*Globodera pallida*	E	*c.* 5-10	UK	Lord *et al* ., 2011
Brassica juncea	Arid	3-butenyl (Syn. Gluconapin)	Glasshouse	*Globodera pallida*	E	*c.* 20-55	UK	Lord *et al* ., 2011
	Caliente (ISCI) 99	2-propenyl (Syn. Sinigrin)	Glasshouse	*Globodera pallida*	E	*c.* 85-95	UK	Lord *et al* ., 2011
		2-propenyl (Syn. Sinigrin)	Field	*Globodera pallida*	B	*c.* 15-95	UK	Ngala *et al* ., 2014
		2-propenyl (Syn. Sinigrin)	Glasshouse	*Globodera pallida*	A	0	Holland	Brolsma *et al* ., 2014
	Nemfix	2-propenyl (Syn. Sinigrin)	Glasshouse	*Globodera pallida*	E	*c.* 70-95	UK	Lord *et al* ., 2011
	Fumus	2-propenyl (Syn. Sinigrin); 3-butenyl (Syn. Gluconapin)	Glasshouse	*Globodera pallida*	E	*c.* 85-95	UK	Lord *et al* ., 2011
	Variety #2		Field	*Globodera* spp.	D	45	UK	Watts *et al.,* 2014
	IPK CR 2451	2-propenyl (Syn. Sinigrin)	Glasshouse	*Globodera pallida*	A	0	Holland	Brolsma *et al* ., 2014
Brassica napus	Bn		Glasshouse	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2011
Brassica rapa		3-butenyl (Syn. Gluconapin); 2-hydroxy-3-butenyl (Syn. Progoitrin); 4-pentenyl (Syn. Glucobrassicanapin); 4-hydroxyindol-3-ylmethyl (Syn. Unknown); indolyl (Syn. Unknown)	Glasshouse	*Globodera pallida*	E	*c.* 70-90	UK	Lord *et al* ., 2011
Eruca sativa	Variety #8	4-methylsulfinyl-3-butenyl (Syn. Glucoraphenin); 4-(methylthio)butenyl (Syn. Unknown); 4-mercaptobutyl (Syn. Unknown)	Glasshouse	*Globodera pallida*	E	*c.* 30-75	UK	Lord *et al* ., 2011
	Nemat	4-methylsulfinyl-3-butenyl (Syn. Glucoraphenin); 4-(methylthio)butenyl (Syn. Unknown); 4-mercaptobutyl (Syn. Unknown)	Glasshouse	*Globodera pallida*	E	*c.* 20-65	UK	Lord *et al* ., 2011
		4-pentenyl (Syn. Glucobrassicanapin); 2-hydroxy-4-pentenyl (Syn. Gluconapoleiferin); N-Methoxy-3-indolylmethyl (Syn. Neoglucobrassicin); 3-indolymethyl (Syn. Glucobrassicin)	Field	*Globodera pallida*	B	*c.* 30-90	UK	Ngala *et al* ., 2014
	Trio		Field	*Globodera* spp.	D	46	UK	Watts *et al.,* 2014

A = motility assessment by pricking and heating to determine mortality, B = hatching and Meldolas Blue stain assay to determine mortality, D = Meldolas Blue stain assay to determine mortality, E = RT-qPCR methodology for cDNA measurement which correlates with viable eggs, F = standard egg counting methodology. (Toxicity assessment 'C' was not used in the studies reported in this table. Toxicity assessment 'C' is described in Tables 1.1 and 1.2).

Table 1.5: continued

Species	Variety	Glucosinolate profile	Environment	Nematode species	Toxicity assessment	Mortality [%]	Country	Reference
Moricandia moricandioides		3-butenyl (Syn. Gluconapin); 2-hydroxy-3-butenyl (Syn. Progoitrin); 4-methoxyindol-3-ylmethyl (Syn. 4-methoxyglucobrassicin	Glasshouse	*Globodera pallida*	E	*c.* 10	UK	Lord *et al* ., 2011
Nasturtium officinale		1-methoxyindol-3-ylmethyl (Syn. 1-methoxyglucobrassicin); 2-phenylethyl (Syn. Gluconasturtiin)	Glasshouse	*Globodera pallida*	E	*c.* 50-70	UK	Lord *et al* ., 2011
Raphanus sativus	Weedcheck	4-hydroxyindol-3-ylmethyl (Syn. Unknown); 1-methoxyindol-3-ylmethyl (Syn. 1-methoxyglucobrassicin); 4-methylsulfinyl-3-butenyl (Syn. Glucoraphenin); 4-methylthio-3-butenyl (Syn. Glucoraphasatin)	Glasshouse	*Globodera pallida*	E	*c.* 35-75	UK	Lord *et al* ., 2011
	Variety Rs1		Glasshouse	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2011
	Variety Rs2		Glasshouse	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2011
	Bento	4-(methylsulfinyl)butyl (Syn. Glucoraphanin); 2-phenylethyl (Syn. Gluconasturtiin); 3-indolymethyl (Syn. Glucobrassicin)	Field	*Globodera pallida*	B	*c.* 65-95	UK	Ngala *et al* ., 2014
			Glasshouse	*Globodera pallida*	B	30-35	UK	Ngala *et al* ., 2015
Sinapis alba	Architect		Field	*Globodera* spp.	D	41	UK	Watts *et al*., 2014
	Variety Sa1		Glasshouse	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2011
	Variety Sa3		Glasshouse	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2011
	Zlata		Field	*Globodera rostochiensis*	A	0	Belgium	Valdes *et al* ., 2012
	Metex		Field	*Globodera* spp.	F	16	Holland	Scholte & Vos, 2000

A = motility assessment by pricking and heating to determine mortality, B = hatching and Meldolas Blue stain assay to determine mortality, D = Meldolas Blue stain assay to determine mortality, E = RT-qPCR methodology for cDNA measurement which correlates with viable eggs, F = standard egg counting methodology. (Toxicity assessment 'C' was not used in the studies reported in this table. Toxicity assessment 'C' is described in Tables 1.1 and 1.2).

The glasshouse work reported by Valdes *et al*. (2011) is even more questionable of methodology. Here, the authors report that PCN were exposed to a ratio of 25 g of biofumigant residue to 1 cm^3 of soil. If assumed at the field level where approximately 300 mm depth of soil would be worked during biofumigant incorporation, this quantity of biomass equates to *c.*75,000 t ha^{-1} of material. The calculation used to generate this figure is as follows;

∑ *100 * 100 = 10^3 [cm^2 per 1 m^2], * 10^3 = 10^7 [cm^2 per 1 ha], * 30 [incorporation depth in cm] = 30^8 [cm^3 of soil to be biofumigated per 1 ha], * 25 [g of biofumigant material per cm^3 of soil] = 75^9 [g biofumigant material per 1ha of soil biofumigated], / 10^2 = 75^6 [kg biofumigant material per 1 ha of soil biofumigated], / 10^2 = 75^3 t ha^{-1} biofumigant material per 1 ha of soil biofumigated.*

This can only be seen as a very large addition of plant material to a small proportion of soil. Logically, the figures suggested by Valdes *et al*. (2011) must be incorrect, or the experiment performed under conditions unrepresentative of any field situation and therefore the results be questionable. In addition to the weaknesses mentioned above, only Valdes *et al*. (2012) reported the soil pH that biofumigant residues were incorporated into (pH 5.2 and 6.7 for two experiments respectively), which as reviewed earlier, could have a strong impact on the arrangement of biofumigant VOC's produced. It is possible that the biofumigation carried out on the site with a soil of pH 5.2 might have been predominantly nitrile based rather than ITC (Figure 1.25). Furthermore, neither study provides details of fertiliser inputs or the soil moisture during plant growth or at biofumigant incorporation. In summary, it appears that these studies have some important ommissions which might have negatively influenced biofumigation efficacy.

A similar study carried out by Brolsma *et al.* (2014) investigated the efficacy of 2-propenyl ITC against *G. pallida in-vitro*, and the incorporated tissues of *B. juncea* on *G. pallida* viability in a pot test. Brolsma *et al.* (2014) also reported no effect of biofumigation against

PCN. Furthermore, their study appears to be more rigorous than those previously mentioned and used known biofumigant varieties, grown under appropriate conditions. PCN were also exposed to residue levels in soil which would equate to *c.*60 t ha^{-1} of material, which is far more representative of a real field situation. The calculation used to generate this figure is as follows;

∑ *100 * 100 = 10^3 [cm^2 per 1 m^2], * 10^3 = 10^7 [cm^2 per 1 ha], * 30 [incorporation depth in cm] = 30^8 [cm^3 of soil to be biofumigated per 1 ha], * 1.6 [typical bulk density of soil g cm^3] = 48^8 [weight of soil to be biofumigated in g], / 10^2 = 48^5 [weight of soil to be biofumigated in kg], / 4 [kg weight of soil used in reported experimental work] = 12^5, * 50 [g weight of biofumigant material used in reported experimental work] = 60^6 [equivalent of biofumigant material used in experimental work, on the hectare basis in g], / 10^5 = 60 t ha^{-1} biofumigant material.*

However, critical mistakes may have been made which could explain the poor biofumigation recorded in this study. Biofumigant material was poorly macerated using hand-pruning shears, and then weighed before being introduced to soil. This methodology would probably be slow and offer a potentially poor disruption of biofumigant tissues when compared to blunt methodologies, such as using a garden shredder (Lazzeri *et al*., 2004). The authors do not record the pH of soil into which residues were incorporated, which could as previously mentioned be influential of the arrangement of hydrolysis products produced after maceration. Furthermore, the authors did not record soil moisture at incorporation. These omissions cast doubt on the results of this study. Moreover, the GSL concentrations in plant tissues are *c.*20% of that recorded by Lord *et al*. (2011), and *c.*10% of that recorded by Ngala *et al*. (2014). The crop reported by Brolsma *et al.* (2014) is therefore likely to have had much lower biofumigation potential than the other studies.

Several other studies have reported positive results despite some weaknesses. A study reported by Lord *et al*. (2011) investigated a range of 22 brassicaceous accessions from 16 species for biofumigation efficacy against *G. pallida*. Experiments used leaf extracts *in-vitro* and incorporated biofumigant residues in soil columns. A high-powered blender was used to macerate biofumigant material before introducing it to soil columns. This methodology would have achieved much greater maceration of plant material than that of the previous studies. Lord *et al*. (2011) report up to 95% efficacy against *G. pallida* when using *B. juncea* varieties specifically. A study reported by Ngala *et al*. (2014) investigated biofumigation of *G. pallida* in the field, using *R. sativus*, *B. juncea* and *E. sativa* and commercially available tractor mounted implements; a flail-topper immediately followed by a rotavator (as illustrated in Figures 1.19 and 1.20), for biofumigant maceration and incorporation. This methodology would have achieved a much finer and expediently incorporated biofumigant residue than that achieved by Valdes *et al*. (2012). Neither Lord *et al*. (2011) or Ngala *et al*. (2014) report the soil moisture at incorporation or the fertiliser rates used in their experiments. Lord *et al*. (2011) also omit soil pH data, however, both studies reported biofumigation effects up to 95% efficacy.

A single experiment study reported by Watts *et al*. (2014) investigated several commercially available varieties and biofumigant blends for management of PCN, and also found positive results with *c.*42% efficacy as an average. Watts *et al*. (2014) do not report the GSL profile or concentration of GSL's in biofumigant tissues, or the soil pH or moisture at incorporation. Furthermore, their biomass figures are questionable after a non-conventional biomass assessment methodology, however, appropriate fertiliser applications were made and the crops grown at a suitable time of year. Furthermore, the maceration and incorporation methodology involving a flail topper and terradisc-plough combination were expedient, and in line with the current best practice suggested by Lazzeri *et al*. (2004) and Matthiessen *et al.* (2004). Table 1.5 shows some of the core material discussed from each study surrounding biofumigants species and variety, GSL profiles, the type of study undertaken and the mortality recorded. Biomass and GSL

concentrations are omitted but have been discussed here sufficiently. Other authors have recorded reduced PCN incidence on potatoes following biofumigation, however, these are not considered mortality data specifically (Morgan, 1925; Triffitt, 1930; Aires *et al*., 2009; Fatemy & Sepideh, 2016). Similarly, authors have recorded hatching suppression and stimulation in some instances (Ellenby, 1945a; Ellenby, 1945b; Ellenby, 1951; Brolsma *et al.*, 2014).

1.4.10 *Alternative biofumigation strategies*

Alternative biofumigation strategies include partial biofumigation, which involves the growing of a biofumigant crop but not its incorporation into soil. Glucosinolates are released from the roots of the biofumigant during growth and enzymatically hydrolysed by soil microbes (Ngala *et al*., 2015b). This technique has been found up to 30-35% efficacious in reducing *Globodera pallida* in a recent study and is well suited to winter hardy biofumigant species with large GSL containing tap roots, such as *R. sativus* (Ngala *et al*., 2015b). Its major benefit over conventional biofumigation is that it is low intensity and in the UK, growers could also receive payments for such crops under environmental 'greening schemes' whilst reducing PCN populations. The technique is currently in need of further research to determine whether it is more suitable for biofumigation of PCN than the more established classical approach. Defatted seed meals offer another alternative, are high in GSL content and have been found effective in reducing the root knot nematode *Meloidogyne incognita* on zucchini in Italy (Lazzeri *et al*., 2009). Defatted seed meals have also been used to suppress weed seed germination and so pose the potential to be the second generation of biofumigant products. Research is also on-going in Italy to register biofumigant liquids which use water and oil as carriers of defatted seed meals (Pers. Comm. Dr Luca Lazzeri: Biofumigation Researcher at the Centre for Industrial Crops, Rome).

1.5 Conclusion and identification of the research gap

In conclusion, biofumigation poses good potential as an alternative or complimentary method for PCN management, however, it lacks consistency in many instances. Biofumigation is a highly complex technique reliant on maximising GSL content and biofumigant biomass, then accessing that potential effectively and delivering ITC at sufficient concentration in soil for an adequate duration to achieve maximum PCN mortality. Of all the known biofumigation factors, those surrounding hydrolysis appear to be least understood. The literature documents the type and concentration of GSL's required to reduce PCN in the field. However, there is little data on maceration and incorporation of biofumigant residues into soil for PCN management, despite some encouraging initial studies documenting differences in ITC emission from tissues treated with different maceration implements. Furthermore, there is currently no understanding of whether soil moisture enhances or negatively influences biofumigation efficacy against PCN. Studies have suggested ITC concentrations may be enhanced in soils at high soil moisture contents, however, further work is required. Mechanisms for enhanced ITC release from biofumigant tissues as affected by type of maceration implement, or the soil conditions at incorporation, need to be investigated.

1.5.1 *General PhD aims*

i) Determine whether soil moisture conditions at biofumigant incorporation are influential of biofumigation efficacy against PCN.

ii) Determine whether cutting implement selection/set-up for biofumigant maceration, influences biofumigation efficacy against PCN.

iii) Determine whether tillage implement selection/set-up for biofumigant incorporation, influences biofumigation efficacy against PCN.

iv) Investigate mechanisms for enhanced PCN suppression for soil moisture, maceration and/or incorporation factors, subject to positive results.

2.1 Introduction

This chapter describes the general materials and methods which are common to most or all of the experiments reported in this thesis. Where changes in materials or methods were made in an experiment, description and justification of those changes are reported in the relevant chapter. This chapter is structured into three sections; the first describes general methods used in experimental design and data analysis, the second describes common soil sampling and quantification methods, and the third describes the biofumigant material used in experiments and glucosinolate (GSL) and volatile organic compound (VOC) quantification.

2.2 Experimental design and analysis

In all instances, experimental design was initiated with the calculation of the replicates required to effectively determine differences between selected treatments. This was based upon the projected variation of the test material, often potato cyst nematode (PCN) egg viability, and the expected difference to be found between treatments after the method of Berndston (1991). Variation in natural PCN viability was expected to be around 3-15% (Danquah, 2012), whilst treatment differences were determined in a more arbitrary manner due to lack of supporting literature. Often, the number of projected replicates would be impractical for experimental work and so the maximum realistic number of replicates would be selected for further experimental design. Skeleton analysis of variance (ANOVA) were created for each potential experiment to ascertain whether the minimum requirement of 15 residual degrees of freedom (RDF) could be achieved (Mead *et al*., 1993). Treatment spatial allocation within experiments were then randomly allocated using the random numbers function in Microsoft Excel 2013® to generate a fully randomised block design. Efforts were made to follow the same experimental design methodologies for pilot studies as used for full experimental work,

although due to low replication this could not always be achieved. Where practical, factorial experiments were designed so that treatments could be analysed individually and by shared characteristics. Statistical analyses were performed using GenStat® (15th Edn.). Common analyses included ANOVA with multiple comparisons (Tukeys test at 95% confidence), repeated measures ANOVA using least significant difference (LSD) at 99% confidence for multiple comparisons, and linear regression.

2.3 Soil sampling and quantification

2.3.1 *Soil sampling for potato cyst nematode quantification*

Prior to any experimental work, contact was made with local growers to Harper Adams University (Shropshire, United Kingdom [UK]) and Frontier Ag Ltd. (Norfolk, UK) to locate PCN infested sites. The identified PCN infested field sites were then sampled using a gridded sampling approach to generate a crude PCN population density estimate for areas of each field. A semi-circular soil corer of 20 mm diameter was used to collect approximately 20 x 1 kg samples for areas ranging between *c.*1-2 ha, at 0-300 mm depth per coring. Sampling sites were marked either using plastic canes, or by DGPS using a Garmin eTrex 20® (*c.*3 m accuracy, 95% typical) system when sampling in standing crops prior to harvest. Typically, tramlines were followed during sampling, with samples taken approximately 2 m from a tramline at regular intervals to ensure samples were collected from uncompacted field soil. Soil was then air-dried in cloth bags for *c.*72 h at 25°C before being sieved using a 10 mm aperture sieve to remove large stones and break soil clods for PCN extraction. After sampling and PCN quantification, experiments could be positioned in areas of acceptably high PCN presence and uniformity. Experimental plots were then sampled by taking approximately 25 cores per plot at each sampling time; before biofumigant planting as a population initial (P_i) and eight-weeks post biofumigant incorporation, termed population secondary (P_s) rather than population final (P_f) which normally describes samples taken after potato cropping. Potato cyst nematodes were also collected in bulk from field sites to produce cyst cultures for glasshouse and *in-vitro* experiments.

2.3.2 *Potato cyst nematode extraction and quantification*

Potato cyst nematode cysts were extracted from 200 g of air-dried soil from a collected soil sample using the established Fenwick can method (Fenwick, 1940) in combination with a secondary flotation method using a conical flask to further separate cysts from organic-matter (OM) (Morgan, 1925). Samples of extracted material were dried in silk sachets at 25°C for 24 h, then transferred to a gridded aluminium counting slide for cyst counting using a binocular microscope at x20 magnification (Shepherd, 1986). Potato cyst nematodes were differentiated from other cysts morphologically, then fifty of the cysts counted from each sample were hand extracted using forceps and soaked in 1 mL of water for seven days, followed by seven days in a 0.05% w/v Meldola's blue staining solution, and finally a 24 h period in distilled water. Processed cysts were crushed using an aluminium crushing block and glass slide after the method of Reid (1955), washed into a 100 mL boiling tube using distilled water, and made up to 50 mL. The 50 mL egg suspension was then agitated and 2 mL extracted and pipetted into a 2 mL capacity counting slide. Egg number was determined by counting full eggs and free juveniles within 1 mL of egg suspension on the counting slide grid, using a binocular microscope at x40 magnification. Eggs were considered dead if stained, and viable if unstained after the method of Ogiga & Estey (1975). Using the data collected, PCN were quantified as total and viable PCN eggs g^{-1} soil, total and viable eggs $cyst^{-1}$, and as an overall viability percentage of the total present PCN eggs using Equations 2.1-3. Figure 2.1 shows the Fenwick can and conical flask set-up, and Figure 2.2 shows laboratory tools used in the extraction and quantification of PCN from float material. Figure 2.3 and 2.4 show a gridded aluminium counting slide with float material containing PCN cysts. Figures 2.5 and 2.6 show viable and dead eggs after Meldolas Blue staining.

Equation 2.1

$$\text{PCN eggs g}^{-1}\text{ soil} = \frac{\text{Water volume of the egg suspension in mL}}{\text{Cysts used to generate the egg suspension}} \times \frac{\text{Number of cysts counted in 200 g air-dry soil}}{\text{Weight of extraction soil in g}}$$

Equation 2.2

$$\text{PCN eggs cyst}^{-1} = \frac{\text{Water volume of the egg suspension in mL}}{\text{Cysts used to generate the egg suspension}} \times \text{Number of eggs counted in 1 mL of egg suspension}$$

Equation 2.3

$$\text{PCN egg viability \%} = \frac{100}{\text{Total number of eggs counted in 1 mL of egg suspension}} \times \text{Viable number of eggs counted in 1 mL of egg suspension}$$

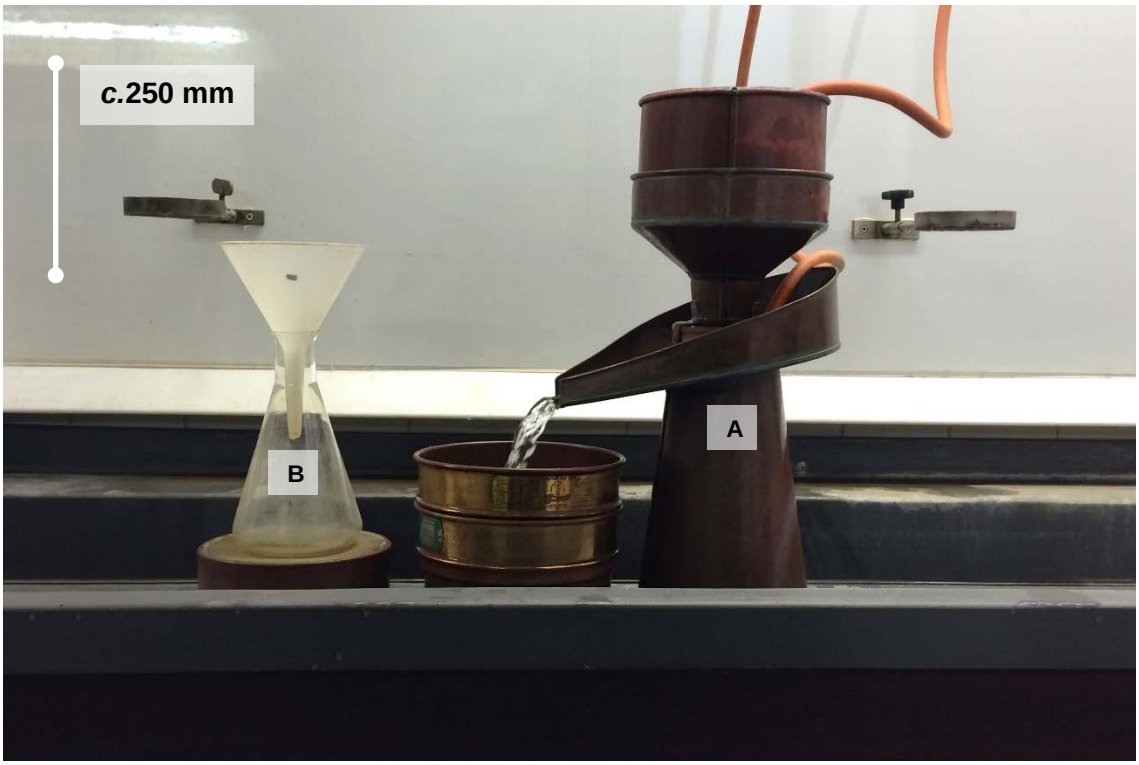


Figure 2.1: Fenwick can (A) used for potato cyst nematode cyst flotation from soil, and conical flask (B) used to reduce organic-matter in the float. The scale is relative to the height of the conical flask.

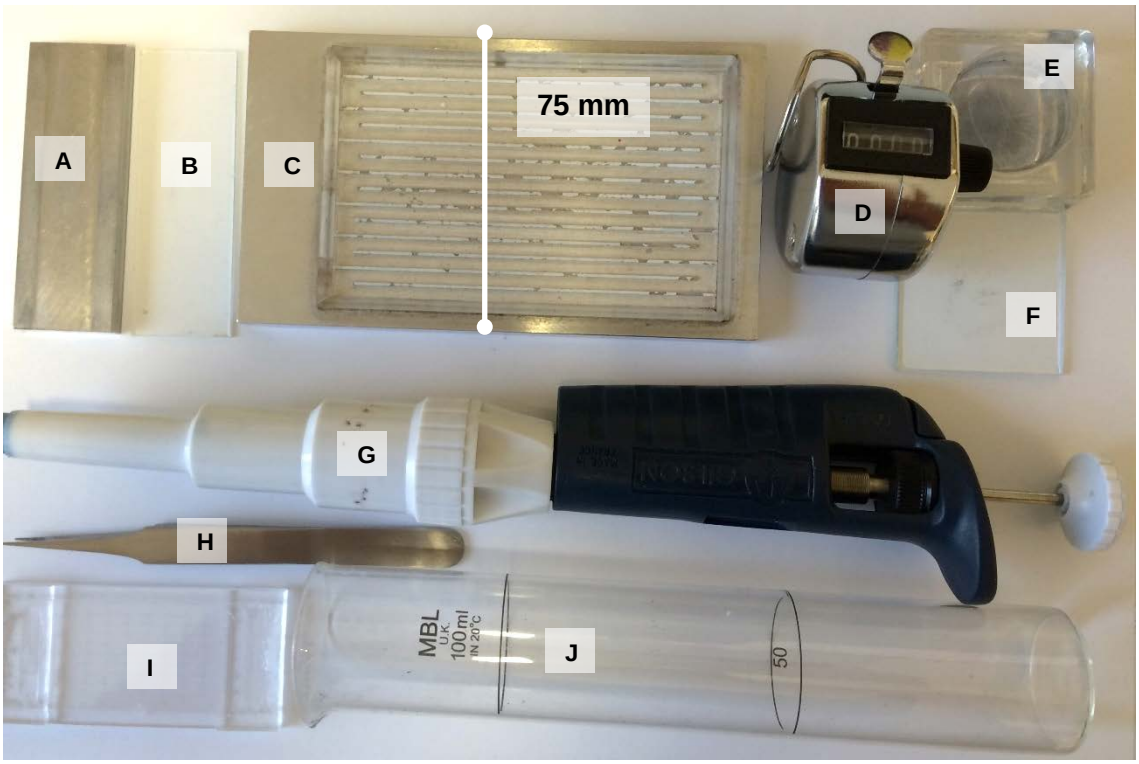


Figure 2.2: Laboratory tools: aluminium crushing block (A); glass slide (B); gridded counting slide (C); counter (D); watch-glass (E); watch-glass slide (F); 5 mL pipette (G); forceps (H); 2 mL counting slide (I); 100 mL boiling tube (J). The scale indicates the width of the counting slide.

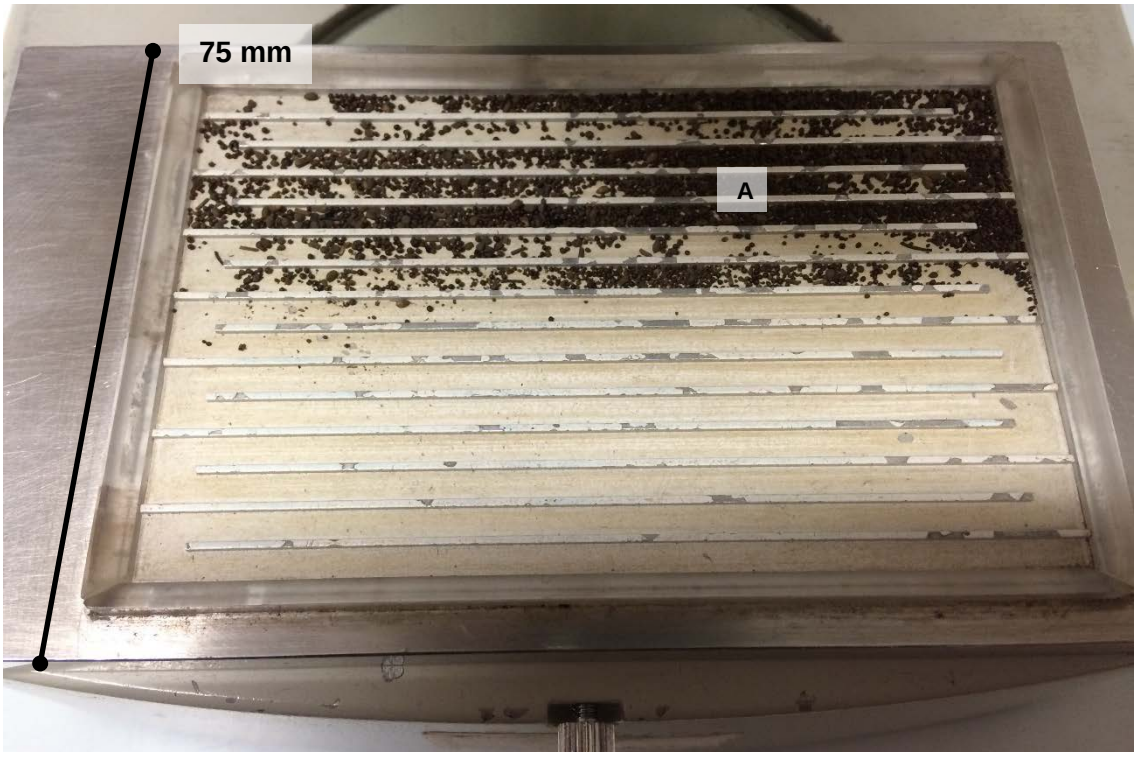


Figure 2.3: Float material containing potato cyst nematode cysts (A), scattered on a gridded aluminium counting slide.The scale indicates the width of the counting slide.

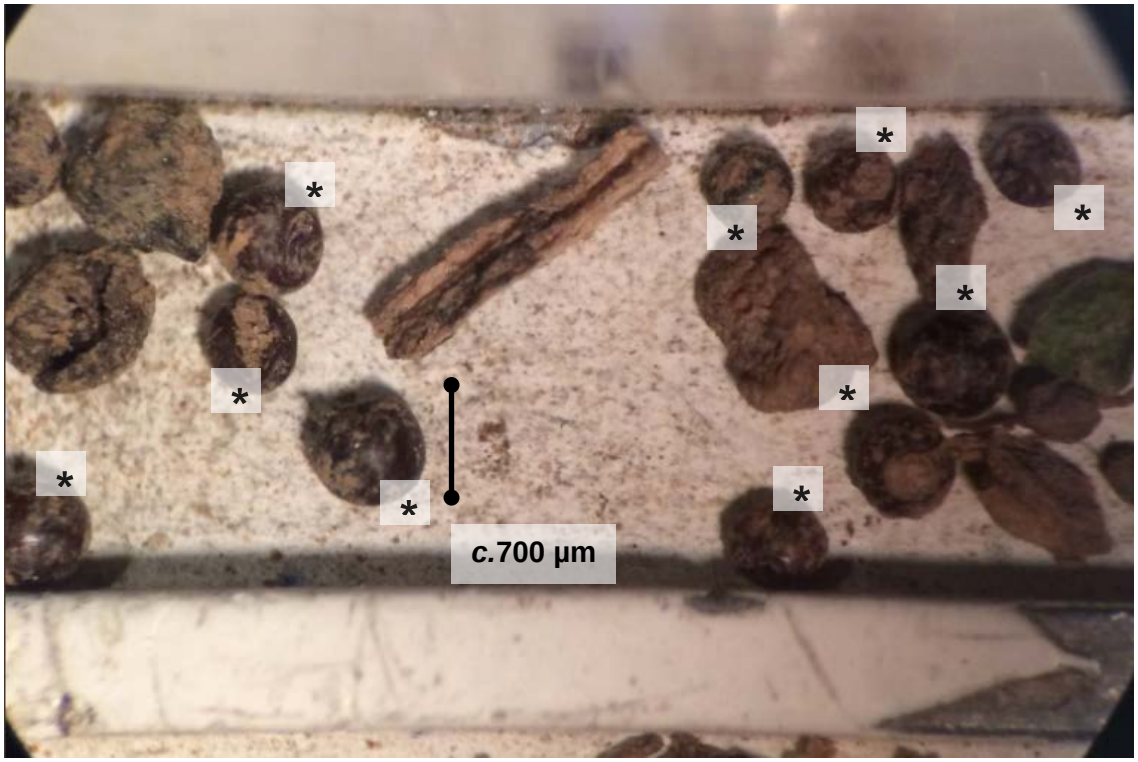


Figure 2.4: *Globodera pallida* potato cyst nematode cysts (marked *) in float material scattered on a gridded aluminium counting slide.The scale indicates the diameter of a single cyst.

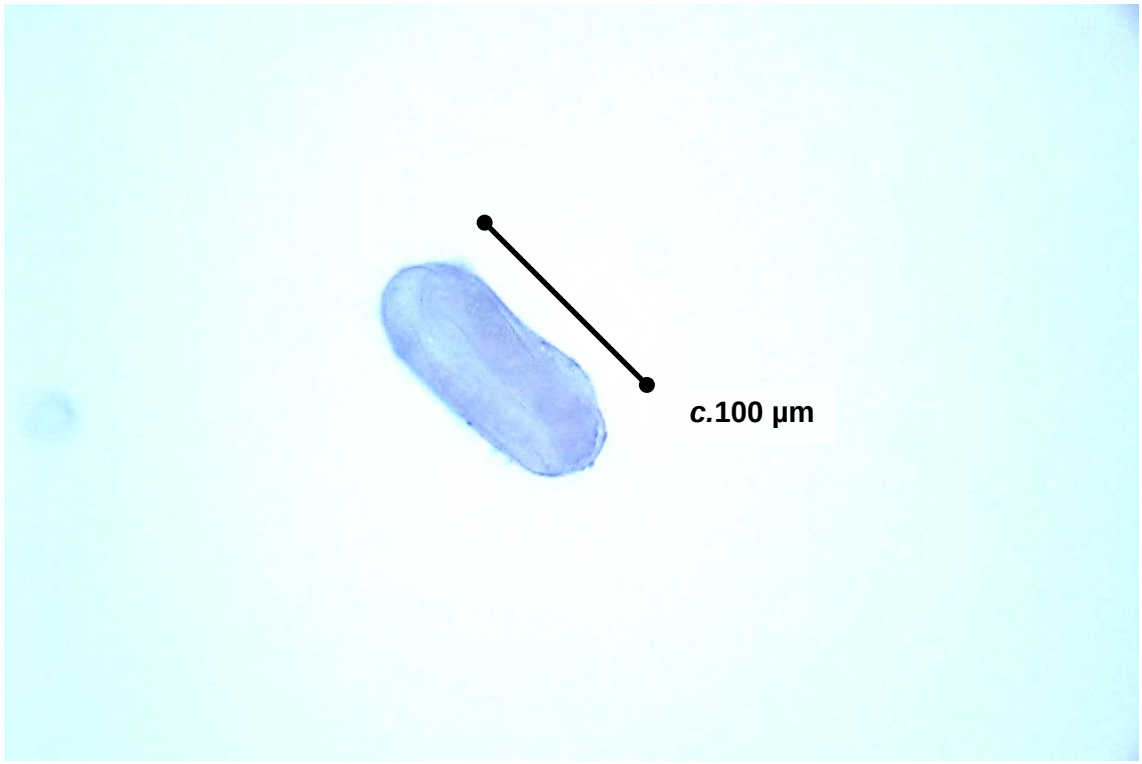


Figure 2.5: Viable *Globodera pallida* potato cyst nematode egg determined using Meldolas Blue stain (Source: Katarzyna Dybal, Harper Adams University).The scale indicates the length of a egg.

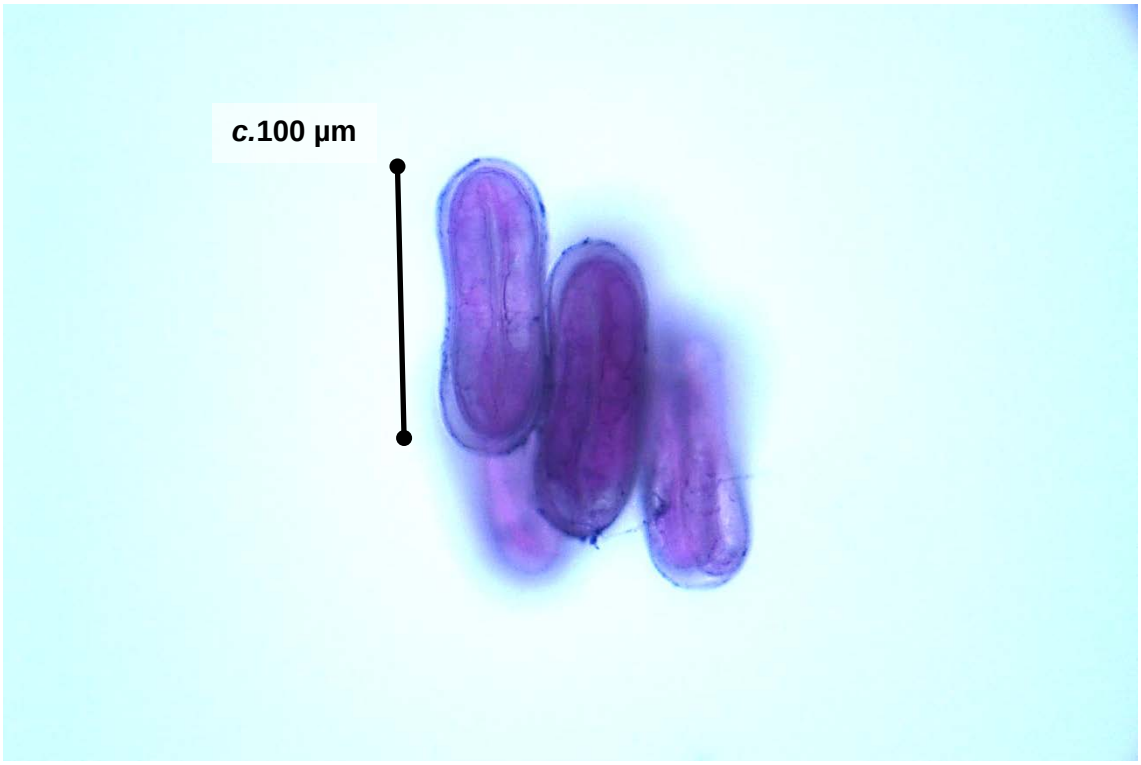


Figure 2.6: Dead *Globodera pallida* potato cyst nematode eggs determined using Meldolas Blue stain (Source: Katarzyna Dybal, Harper Adams University). The scale indicates the length of individual eggs.

Cysts were mass extracted from damp soil for glasshouse and *in-vitro* work using the previously described method. However, larger samples of *c.*1 kg were used to make cyst extraction more time efficient and float dried as previously described. Float material was then sprinkled onto the top of the long edge of a smooth paper-board (*c.*100 x 200 mm), and cysts rolled from the float by gentle tapping of the paper board. All but spherical debris was separated from cysts in this way which enabled faster hand extraction of cysts for experimental work. Viability quantification of encysted eggs used in glasshouse and *in-vitro* work was performed using the Meldolas blue method as used for field work. Figure 2.7 shows the rolling technique using a paper-board. Shepherd (1986) provides comprehensive detail of most of the methods described here.

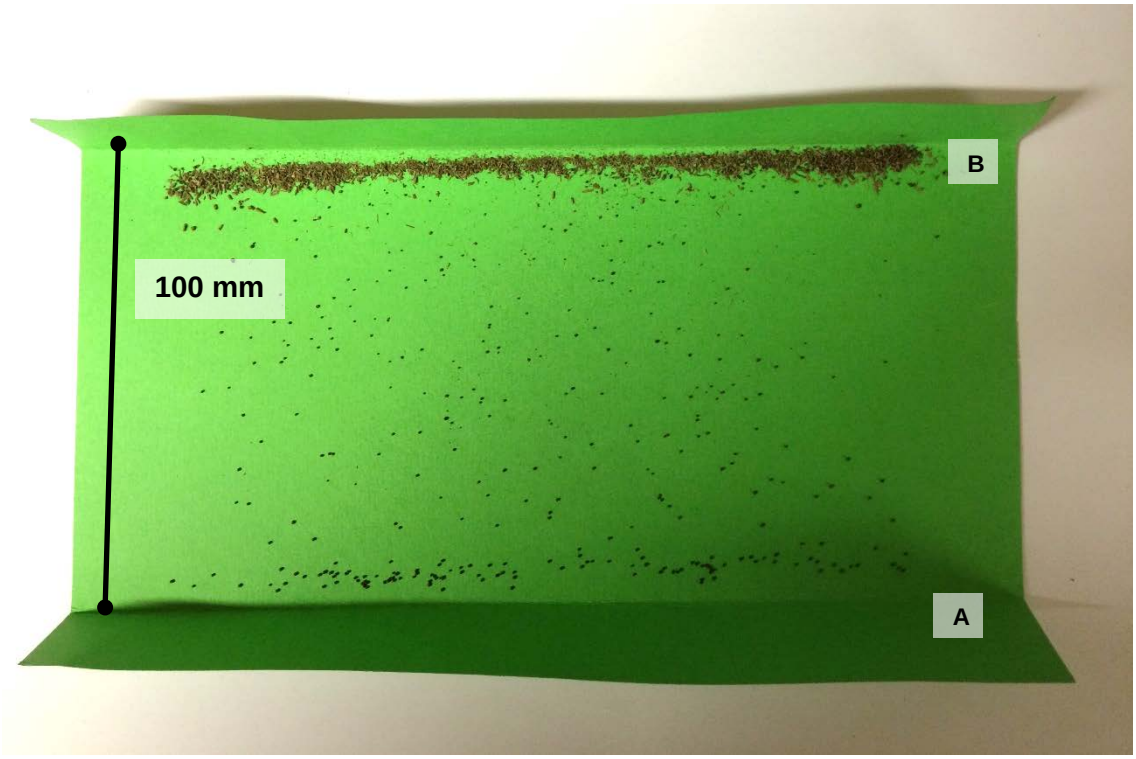


Figure 2.7: *Globodera pallida* cyst extraction (A) from float material (B) using a paper-board and 'rolling' technique. The scale indicates the width of the board.

2.3.3 *Potato cyst nematode species determination*

Potato cyst nematode species was determined for each population using a real time polymerase chain reaction (PCR) assay adapted from those published by Nakhla et al. (2010) and Bulman & Marshall (1997). Twenty-five cysts per sample, of which there were three per PCN population, were washed in 1000 µL of Tris-EDTA (TE) buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0) using a vortex for *c.*15 s per sample, before the buffer was removed and replaced with 150 µL of fresh TE. Cysts were crushed in the TE using a micropestle and *c.*10 mg of activated carbon applied to the TE mix. Samples were vortexed for a further 15 s and then heated to 100°C for 5 min, vortexed for a further 30 s, then centrifuged at 12,000 rpm for 5 min to produce a purified deoxyribonucleic acid (DNA) supernatant. A 100 µL sample of supernatant was then used for PCR. The master mix was Takyon™ No Rox Probe Master Mix dTTP Blue. The PCR primers and probes for *G. pallida* detection were as follows; PITSpf (5'-ACGGACACATGCCCGCTA-3'), PITSp4 (5'-ACAACAGCAATCGTCGAG-3') and TaqMan probe GFAMp (5'-

ACATGAGTGTTGGGGTGTAAC-3') labelled FAM. *Globodera rostochiensis* detection relied upon the primers PGrtf (5'-TCTGTGCGTCGTTGAGC-3'), Prostor (5'-CGCAGACATGCCGCAA-3') and a TaqMan probe GYYp (5'-CGCAGATATGCTAACATGGAGTGTAG-3') labelled Yakima Yellow. The cycling parameters (Takyon™ activation: 95°C/180 s, denaturation 95°C/10 s, annealing/extension 58°C/60 s) used in the method were altered to those previously published to optimise the assay for the equipment available (Bio-Rad CFX96™). Species were determined present for Cq values below 30 in accordance with standard procedure to prevent false positives. *Globodera pallida* and *G. rostochiensis* DNA extracts were provided by the Food and Environment Research Agency and included in each test as a positive controls. Appendix 8.1.4 shows PCR analysis for all cyst populations used in experimental work where all PCN were identified to be *G. pallida*.

2.3.4 *Soil moisture and temperature*

Determination of the capillary water/ field capacity range of soil and compost was common to experimental work. Field capacity was determined by saturating soils and measuring moisture decline from saturation using an IMKO® HD2 model Time Domain Reflectrometer (TDR) and TRIME-PICO 64 sensor to generate a water retention curve. Field capacity expressed as moisture by volume was determined for each soil, and individual units of field capacity converted to a volume of water which could then be used to maintain soil moistures for growing crops within the easily available range and to maintain soil moisture treatments in relevant experimental work (Bailey, 1990; Saeed, 2008). Figure 2.8 shows the TDR being used to measure the moisture of soil in a pot. The TDR was also used to measure soil temperature for field experiments.

Figure 2.8: IMKO® HD2 model Time Domain Reflectrometer (A) and PICO-TRIME 64 sensor (B) being used to measure soil moisture.

2.3.5 *Other common soil methods*

Soil texture was classified for all experiments by NRM Laboratories using a sedimentation methodology and the UK soil texture triangle. Soil pH and OM was also determined by NRM Laboratories for all soils using standard methods detailed by Jackson *et al*. (1986). See Appendices 8.1.1-8.1.3 for results.

2.4 Biofumigant materials and methods

2.4.1 *Biofumigant selection*

Brassica juncea cv. ISCI 99 produced by High Performance Seed Inc. (Washington State, USA) was selected as the biofumigant to be used for all experiments reported in this thesis on account of its commercial availability worldwide and widely documented 2-propenyl (sinigrin) GSL content, as previously reviewed in Chapter 1 (Table 1.5). Tozers Seeds Ltd. (Surrey, UK) supplied all the biofumigant seed used in this research. The

seed resembles condiment mustard seed and is approximately 1.5 mm in diameter as shown in Figure 2.9.

Figure 2.9: *Brassica juncea* cv. ISCI 99 biofumigant seed.

2.4.2 *Quantifying glucosinolate profile and concentration of* Brassica juncea *plants*

Plant samples were taken from both field and glasshouse crops for GSL quantification after the methods of Ngala *et al*. (2014). Three plants per plot were collected from the field, whilst in the glasshouse one plant per pot was taken as standard. The roots of these samples were washed, samples bagged (not sealed) and labelled, and then samples taken to the laboratory for freezing with liquid nitrogen within 2 h of sampling. Where samples were transported over long distances, plants were propped upright and a small amount of water introduced to sample bags to cover roots. This maintained plant turgor and reduced stress during transportation to the laboratory for processing. Flash frozen samples were stored at -80°C prior to freeze-drying which was performed using a Girovac Ltd. GVD6/13 NKI dryer. Freeze-dried samples were then milled using a Retsch GmbH cyclone mill-twister© and powder samples stored at -18°C prior to GSL extraction and analysis. Figures 2.10-2.12 show example plant samples immediately

after harvest, liquid nitrogen freezing of plant material and freeze-dried and milled samples prepared for crude GSL extraction.

Figure 2.10: Harvested *Brassica juncea* biofumigant plants ready for biomass assessment and freezing for glucosinolate assessment.

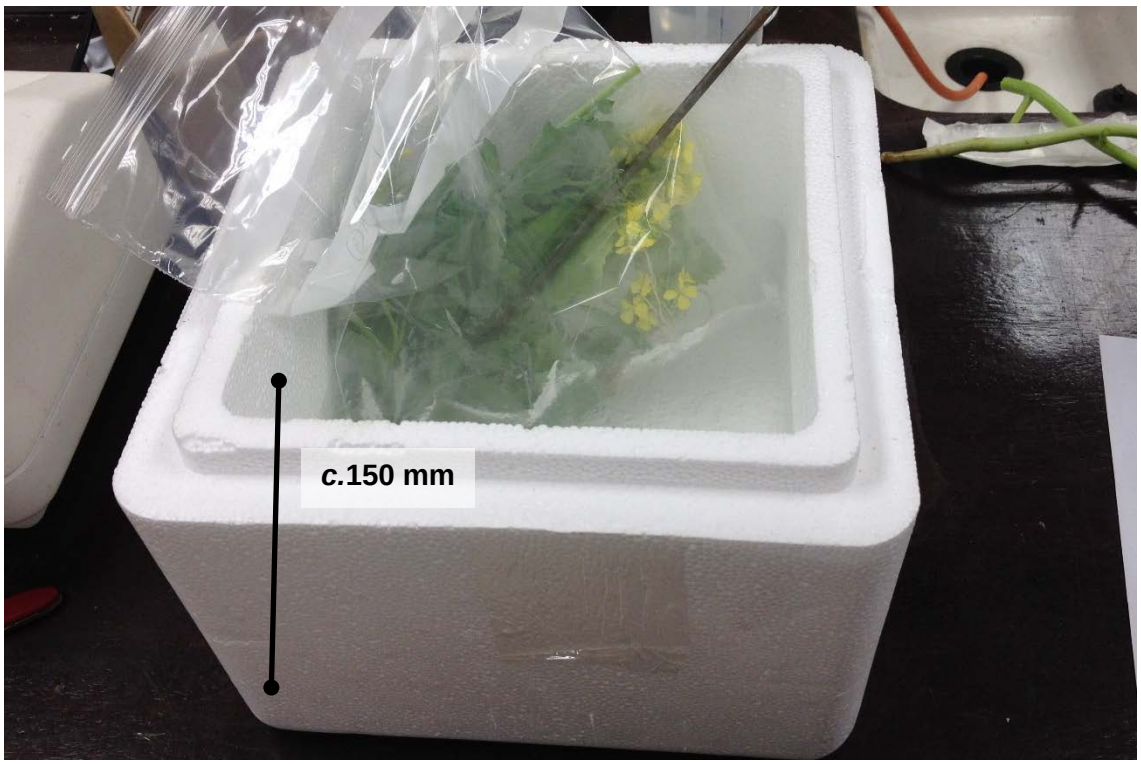


Figure 2.11: Liquid nitrogen freezing of *Brassica juncea* samples. The scale is approximately equal to the width of the bag containing *B. juncea* samples.

Figure 2.12: Freeze-dried and milled *Brassica juncea* biofumigant plant material ready for crude glucosinolate extraction.

Extraction and analysis of GSL's by High Performance Liquid Chromatography (HPLC) was performed according to the method of Ngala *et al*. (2014). Samples of freeze-dried and milled *B. juncea* powder (0.3 g) were introduced to 15 mL polypropylene tubes, before GSL's were extracted using 4 mL of 70% v/v HPLC grade methanol in a water bath at 80°C for 10 min. Polypropylene tubes were preheated in the bath for 1 min prior to hot methanol introduction, and then agitated at 2 min intervals during the methanol phase. After boiling, the samples were centrifuged at 5000 x *g* using a Beckman Avanti™ 30 high-speed compact centrifuge for 3 min at 4°C. Each sample was extracted twice and the supernatant combined in a 15 mL polypropylene tube, followed by the addition of a 200 µL glucotropaeolin (5 mM) internal standard (IS) isolated from cress (*Lepidium sativum*). The IS was generated using the described extraction procedure, followed by freeze-drying of the cress seed supernatant to generate a crude GSL powder extract. This was subsequently quantified using a GSL standard by HPLC. The combined extracts were then adjusted to 5 mL using cold 70% v/v methanol and capped. Samples

were either stored at -18°C, or processed by ion-exchange purification and enzymatic desulfatation (Wathelet *et al*., 2004).

An Agilent series 1100 HPLC© system equipped with a reverse-phase gradient HPLC column (Spherisop® RP-C18 ODS-2, 250 x 4.6 mm) of 5 µm particle size was used for the separation and measurement of desulphoglucosinolates. The mobile phase consisted of eluent A (deionised water) and eluent B (70% v/v acetonitrile). Column temperature was regulated to 30°C, then 10 µL of each sample auto-injected into the column for analysis. A linear gradient was performed for each sample at a flow rate of 1.5 mL min^{-1} from 0-30% eluent B over 18 min, held at 30% eluent B for 1 min, then returned to 0% eluent B for 1min. A further 6 min per sample was used to establish equilibrium within the column, followed by a 2 min post-run duration. Ultraviolet (UV) detection of desulphoglucosinolates was determined at 229 nm. Individual GSL concentrations (µmol g^{-1}) were determined using Equation 2.4. The relative response factors of glucosinolates were obtained from ISO (1992).

Equation 2.4

$$\text{Glucosinolate concentration } (\mu\text{mol g}^{-1}) = \frac{\text{Peak area of glucosinolate of interest}}{\text{Peak area of the internal standard}} \times \frac{\text{Quantity of the internal standard } (\mu\text{mol})}{\text{Mass of the freeze-dried test powder g}} \times \text{Relative response factor of the glucosinolate of interest}$$

Figure 2.13 shows the boiling methanol crude extraction of glucosinolates from freeze-dried *B. juncea* powder, Figure 2.14 shows the purification step using ion-exchange columns, and Figure 2.15 shows the HPLC instrument used in the analysis of the GSL content of *B. juncea* material.

Figure 2.13: Crude extraction of glucosinolates from freeze-dried *Brassica juncea* material (A) using boiling methanol (B).

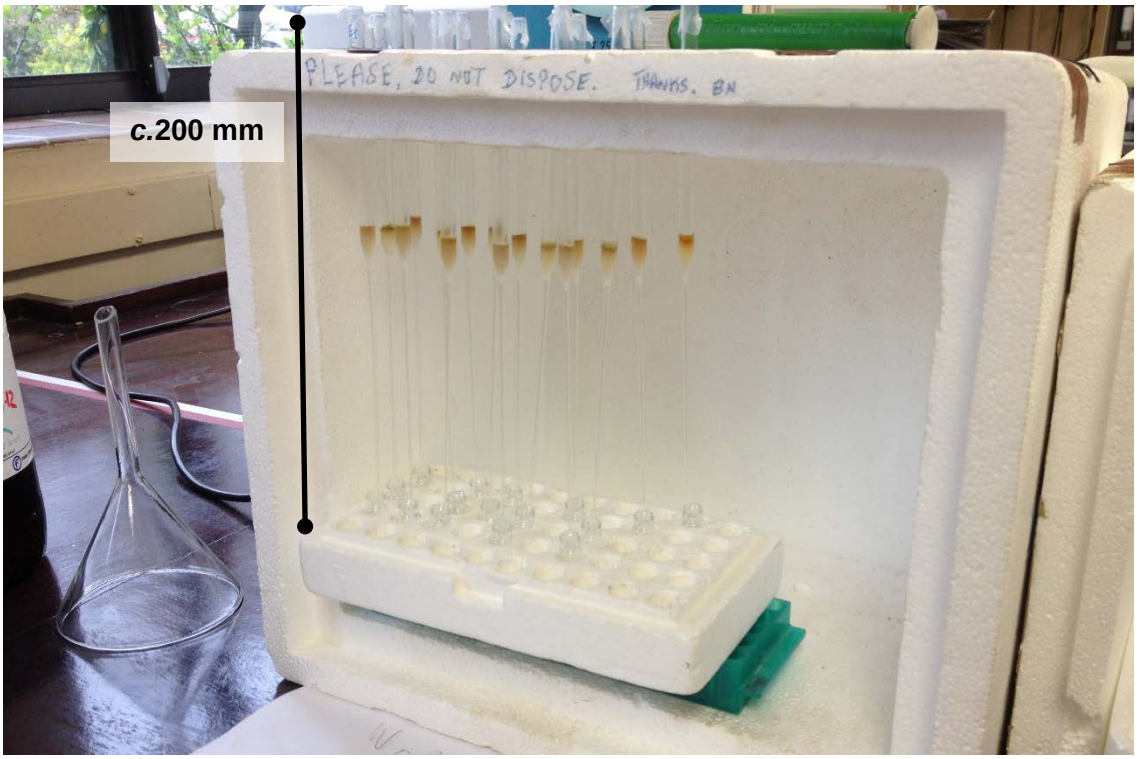


Figure 2.14: Purification and enzymatic desulfatation of glucosinolates from crude *Brassica juncea* glucosinolate extracts. The scale is approximately equal to the length of the glass pipettes.

Figure 2.15: Agilent series 1100 HPLC© system used in the glucosinolate analysis of *Brassica juncea* biofumigant extracts.

2.4.3 *Quantifying volatile organic compounds from macerated* Brassica juncea *plant material*

Volatile organic compounds released from macerated *B. juncea* tissues within glassware or in soil were collected using Tenax-TA sorbent tubes. Tenax-TA sorbent was selected for experimental work due to its long storage potential after VOC sampling, and for the ease with which Tenax-TA sorbent tubes can be used in the field versus other techniques. Papadopoulos & Alderson (2007) have previously demonstrated that Tenax-TA is a suitable sorbent material for biofumigant VOC entrainment. All Tenax-TA VOC entrainment was passive. In laboratory work, *B. juncea* material and *G. pallida* cysts were introduced into glassware with sorbent tubes attached, whilst in field work sorbent tubes were fitted to custom made steel sheaths knows as VOC MOLE's and then introduced to soil for VOC entrainment. Tenax-TA entrained *B. juncea* VOC's were then desorbed from sorbent tubes using a UNITY series 2 thermal desorption unit (Markes International, Llantrisant, UK) by heating the sorbent tube to 250°C for 10 min under a

H_2 flow rate of 20 mL min^{-1}. The desorbed compounds were collected in a general-purpose C_4-C_{32} carbon cold trap (Markes International) at -10°C before ballistic heating to 300°C to ensure a sharp injection of VOC's into the capillary column of the gas chromatograph-mass spectrometer (GC-MS). The temperature programme of the GC-MS was from 40°C (held for 5 min) to 280°C (held for 2 min) at 15°C min^{-1} (total run time: 23 min). The GC instrument was an Agilent 7890B with a HP-5MS column: 30 m x 0.32 mm x 0.25 µm, injection temperature: 250°C, splitless injection). The MS instrument was an Agilent 5977A mass selective detector, 70EV, scan range: 50-500M/Z, source temperature: 230°C, quadrupole temperature: 150°C, solvent delay: 0 min). *Brassica juncea* VOC detection was achieved by comparing spectra with a mass spectra database (NIST MS search 2.2; National Institute of Standards and Technology, USA). Tenax-TA sorbent tubes were reconditioned in the thermal desorption unit at 335°C for 15 min after each desorption to prevent carry-over contamination between samples. Figure 2.16 shows the GC, MS and thermal desorption units.

Figure 2.16: UNITY series 2 thermal desorption unit (A) Agilent 7890B gas chromatography instrument (B) and Agilent 5977A mass spectrometer (C) used in *Brassica juncea* volatile analysis.

Investigation of soil moisture as a factor influencing the efficacy of biofumigation against potato cyst nematodes

3.1 Introduction

Previous studies have suggested that volatile organic compound (VOC) concentrations might be increased in biofumigant treated soils by manipulation of soil moisture prior to, or immediately after incorporation. Morra & Kirkegaard (2002) recorded a doubling of isothiocyanate (ITC) concentrations in pot soils where moisture conditions approached saturation, in comparison to drier soils of approximately 80% of field capacity. Field work by Matthiessen *et al*. (2004) showed up to a ten-fold increase in ITC concentrations in biofumigant treated soil where irrigation was applied, even up to 24 h after incorporation. In both studies, soil moisture was suggested to have a role in reactivating or enhancing the glucosinolate (GSL) hydrolysis reaction. However, the increase in ITC concentrations recorded in soil could instead be explained by enhanced retention of ITCs in soil, by soil moisture impeded off-gassing (Simpson *et al*., 2010; Lord *et al*., 2011). The mechanism for high ITC concentrations in biofumigant treated soils at high soil moisture, is therefore unknown. Furthermore, no study is known to have related the moisture content of soil to biofumigation efficacy against a soil borne pest, or to have investigated soil moisture as a barrier between biofumigant VOC's and a soil borne pest. This chapter aims to uncover whether soil moisture has a role in biofumigation by measuring the viability of PCN eggs, exposed to a range of soil moisture conditions, with and without biofumigation treatment, over a 'realistic to the field' range of incorporated biomass quantities.

3.1.1 *Chapter aim*

i) Determine whether soil moisture conditions at biofumigant incorporation influence the efficacy of *Brassica juncea* biofumigation against PCN.

3.1.2 *Chapter null hypothesis*

i) Soil moisture content does not influence the efficacy of *B. juncea* biofumigation against PCN.

3.2 Materials and methods: glasshouse experiment 1

3.2.1 *Experiment objectives*

i) Quantify the effect of *B. juncea* biofumigation on PCN egg viability over a range of soil moisture conditions.

ii) Quantify the biomass and GSL content of the *B. juncea* biofumigant material used in experimental work.

3.2.2 *Experimental design*

Glasshouse experiment 1 was carried out between February and July 2014 at Harper Adams University (HAU) (Newport, Shropshire [UK]) to address whether soil moisture conditions at biofumigant incorporation can be influential of biofumigation efficacy against PCN. The experiment consisted of two factors; 'soil moisture', for which four levels were selected (25, 50, 75 and 100% of field capacity), and 'biofumigant', for which two levels were selected (with and without the addition of *B. juncea* material into pots). The soil moisture treatment range of 25-100% of field capacity was deemed to represent the full range expected in the field at biofumigant incorporation (Environment Agency, 2018). *Brassica juncea* material was grown in pots of compost using methodologies which will be described in Section 3.2.4.1, and then incorporated into pots containing medium textured sandy clay loam soil at the relevant soil moisture treatment conditions. The medium textured soil was representative of many potato producing soils in the UK. As illustrated in Figures 3.1 and 3.2, treatments were arranged in a randomised block design and replicated seven times, providing 56 experimental units (pots). Replication was sufficient to enable treatment differences of 5-15% in PCN egg viability to be determined for background variation levels in viability of 2-7% between replicates of like treatments (Berndtson, 1991). The design had 42 residual degrees of freedom (RDF).

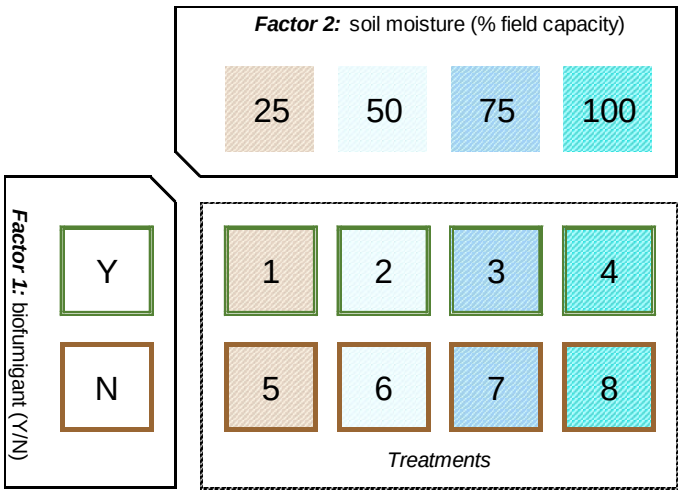


Figure 3.1: Treatment key for glasshouse experiment 1. Border colour is an indicator of *B. juncea* biofumigant treatment, and fill colour is an indicator of soil moisture treatment.

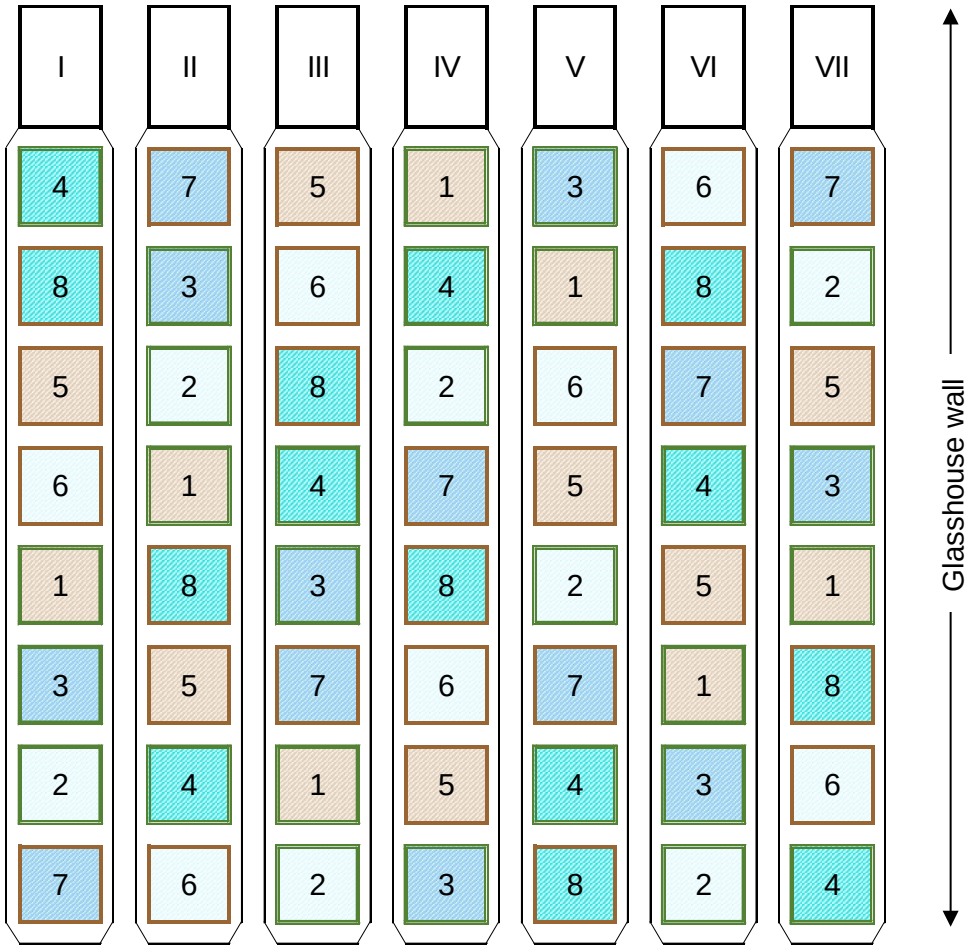


Figure 3.2: Glasshouse experiment 1 design showing the allocation of treatments 1-8 to PCN containing pots of field soil. Blocks are represented using Roman numerals (I-VII).

3.2.3 *Experimental set-up*

Three core materials were required to carry out glasshouse experiment 1; *B. juncea* biomass (i), silk sachets containing PCN cysts (ii), and pots of field soil stabilised at treatment soil moisture conditions (iii). The set-up of this experiment was therefore split into preliminary stages relevant to the production of the three primary materials before the experiment could be properly initiated. Table 3.1 shows the months leading up to the initiation of glasshouse experiment 1 and the time allocated to producing the three core materials.

Table 3.1: Table illustrating the time allocated to producing *B. juncea* biomass, PCN cyst sachets and pots of field soil stabilised at soil moisture conditions for glasshouse experiment 1.

Production of materials	Feb	Mar	Apr	May	Jun	Jul
i: *Brassica juncea* biomass					Glasshouse experiment 1 set-up and in progress	
ii: Cysts and cyst sachets						
iii: Field soil stabilised at treatment soil moisture conditions						

3.2.4 *Core experimental materials*

3.2.4.1 Brassica juncea *biomass production*

Brassica juncea seed was sown into John Innes no.2 compost in trays in February 2014 (Table 3.1), then healthy and uniformly sized seedlings selected prior to the formation of true-leaves for transplantation into pots also containing John Innes no.2 compost. A total of 90 pots were planted with *B. juncea* plants so that there were 56 pots for experimental use, surrounded by 34 guards. Figure 3.3 shows the growing arrangement for the 56 pots described (seven blocks of eight pots). Pots marked 'I' were used for incorporation into *B. juncea* biofumigation treated pots after moisture stabilisation of field soil. Pots

marked ‘A’ were spares from which two pots were randomly selected per block for crop assessments to produce supporting data sets. The position of ‘I’ and ‘A’ biofumitgant pots was randomly assigned. See Appendices 8.1.1-8.1.3 and 8.1.5 for John Innes no.2 compost analysis and field capacity curve.

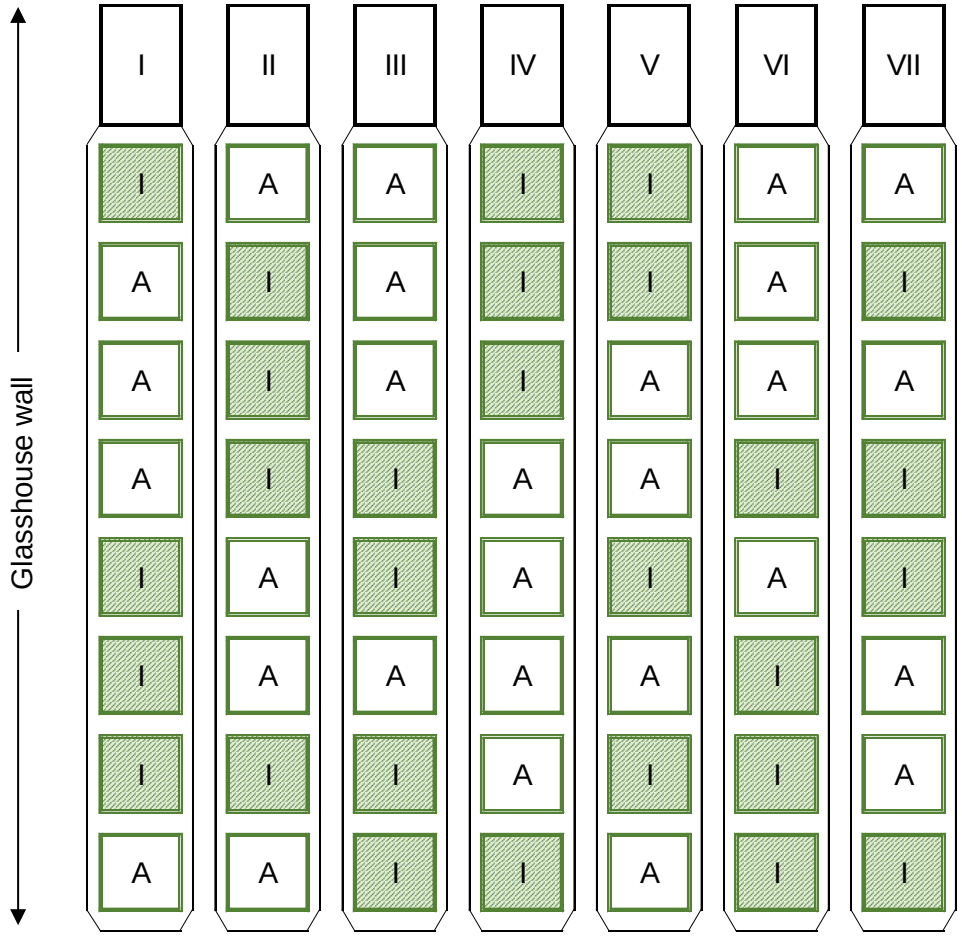


Figure 3.3: *Brassica juncea* growing arrangement for glasshouse experiment 1. Blocks are represented using Roman numerals (I-VII). Units marked ‘I’ indicate *B. juncea* pots which were used for incorporation as treatments. Units marked ‘A’ indicate *B. juncea* pots which were used for crop assessments to generate supporting data, such as dry-matter.

The planting density in *B. juncea* pots (200 mm diameter) was equivalent to an 8 kg ha^{-1} seed rate (17 seedlings per pot). Seedlings were placed equidistantly within pots, split between three rows, using a bespoke planting plate to mark transplant positions (Figure 3.4). Each pot was maintained at between *c.*65-95% of field capacity for the duration of plant growth (see Appendix 8.1.5 for the John Innes no.2 field capacity curve). Pots were measured twice weekly to maintain soil moisture conditions. The glasshouse day/night temperatures were set at 15-5°C respectively, with a 16 h photoperiod using 400W SON-T light bulbs. Figure 3.5 shows *B. juncea* plants two weeks after transplanting, and Figure 3.6 shows plants approximately three weeks before incorporation in May 2014 (Table 3.1). The plants were harvested at 12 weeks, during mid-flowering.

Figure 3.4: Bespoke *B. juncea* transplant plate for a 200 mm diameter pot (equivalent to 8 kg ha^{-1} seed rate).

Figure 3.5: *Brassica juncea* plants at approximately two weeks after transplantation of seedlings. Outer pots were guards. Roman numerals and arrows indicate blocking.

Figure 3.6: *Brassica juncea* plants at the budding stage, approximately three weeks prior to incorporation into pots. Blocks are indicated by Roman numerals.

3.2.4.2 *Potato cyst nematode cyst sachets*

Fifty-six bespoke 250 µm nylon mesh parcels known as 'cyst sachets' were made to hold 50 individual PCN cysts each, using a 200 mm Packer® heat sealer. The population used was identified as *Globodera pallida* (see Appendix 8.1.4 for PCR analysis). Cysts were obtained from a field named Larkshall 10a near Wretham, Thetford (UK) (grid reference: TL 91650 89375) in October 2013 using the soil sampling and PCN quantification techniques described in Section 2.3.1 and 2.3.2. The cysts used in this experiment were only preliminary graded during the extraction procedure and so ranged between 250-1000 µm in diameter. The sachets, which resembled tea bags, were square and approximately 900 mm^2 in size. Figure 3.7 shows cyst sachets and size graded *G. pallida* cysts. Soil texture, pH and organic matter for the Larkshall 10a site can be found in Appendices 8.1.1-8.1.3. The site was not unusual in any of these parameters.

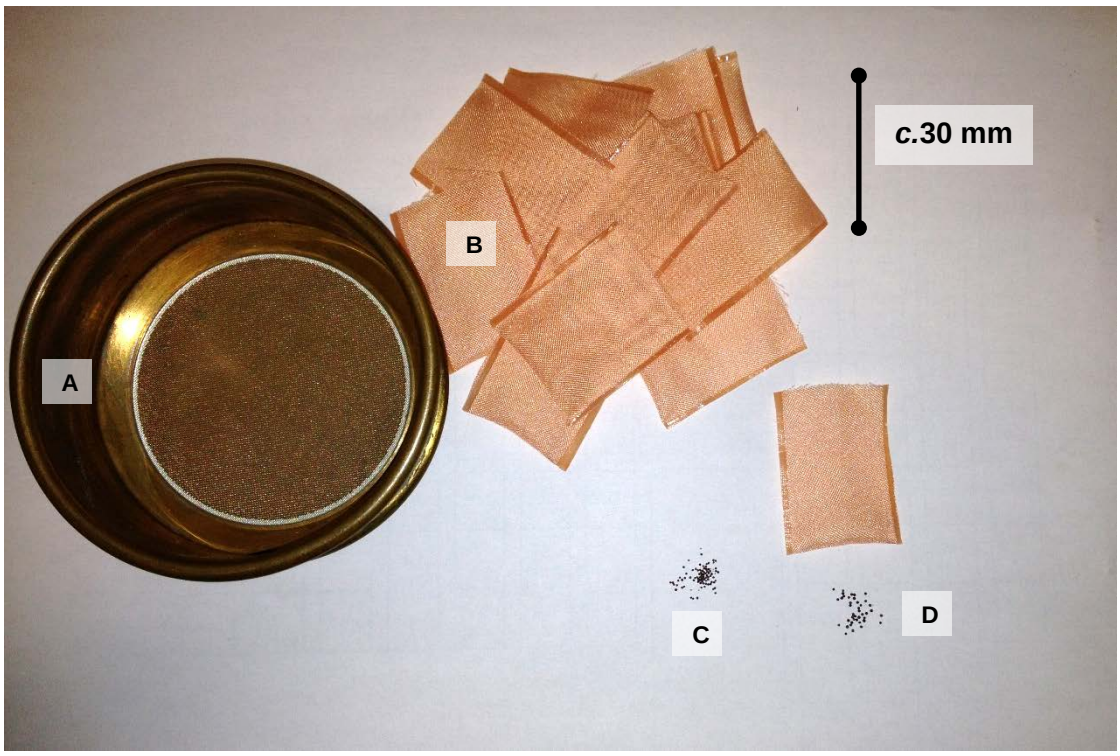


Figure 3.7: 500 µm test sieve (A), 250 µm nylon mesh cyst sachets (B), and graded *G. pallida* cysts below 500 µm in size (C) and above 500 µm in size (D). The scale indicates the approximate length of a cyst sachet.

3.2.4.3 *Field soil and stabilisation of moisture conditions*

Sandy clay loam soil was collected from Black Brook Meadow field (Beard, 1988) at HAU (grid reference: SJ 71003 20717) in March 2014 (Table 3.1). The soil was selected for its medium texture, and pH which was within the acceptable range for biofumigation (see Appendices 8.1.1 – 8.1.3). The soil was air-dried until it could be graded using a 20 mm diameter sieve. Fifty-six 200 mm diameter pots were filled with the soil approximately one month before *B. juncea* incorporation, and then soil moisture stabilised according to treatments using the methodology described in Section 2.3.4 (see Appendix 8.1.5 for the Black Brook Meadow field capacity curve).

3.2.5 *Experiment initiation*

Once *B. juncea* plants reached maturity, pots of field soil were arranged according to the experimental design shown in Figures 3.1 and 3.2. *Brassica juncea* plants destined for incorporation as outlined in Figure 3.3, were then weighed, to quantify biomass, whilst standing in their pots. Whole plants from a single pot were macerated using a Viking® GE150 garden shredder, then the root and foliar residues placed into a bag (Figure 3.8) into which soil from a relevant field soil pot was introduced. The bag was shaken for 30 s until *B. juncea* material was well mixed into the soil. The bag contents were then emptied into the original field soil pot until half full (*c.*100 mm depth). A *G. pallida* cyst sachet was then introduced to the pot where it was placed centrally (Figure 3.9) before the remaining soil and *B. juncea* mix was used to fill the pot. Soil from non-*B. juncea* treatments was also shaken and cyst sachets placed into pots in the same manner as for *B. juncea* treatments. This methodology enabled a rapid maceration and incorporation of *B. juncea* material so that ITC losses could be minimised. The soil moisture of individual pots was measured after incorporation. Water (*c.*100-200 mL) was then applied to raise moisture to treatment levels and seal the soil surface of pots. Lids were placed over the top of pots to reduce moisture evaporation. Figure 3.10 shows the experiment after incorporation of *B. juncea* into relevant pots. Pots were left for eight-

weeks (July 2014), then *G. pallida* sachets extracted and nematode mortality quantified using methods described in Section 2.3.2.

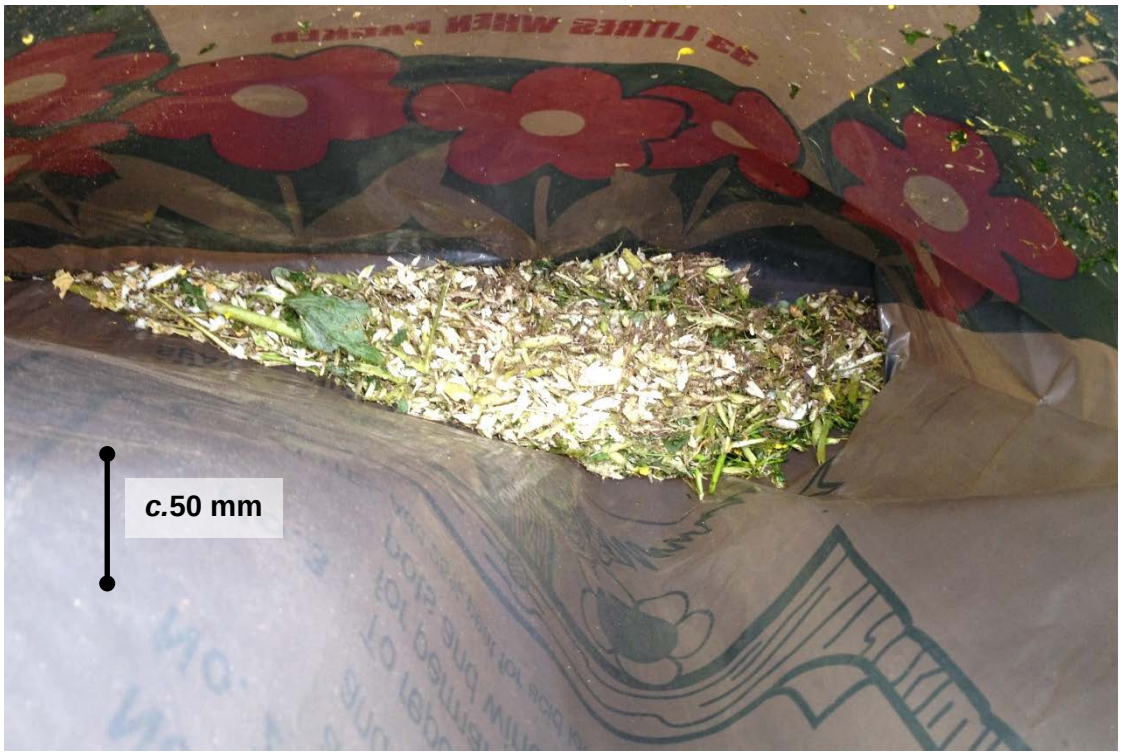


Figure 3.8: *Brassica juncea* residue prior to homogenisation with soil. The scale indicates the approximate, average chop length of biofumigant material.

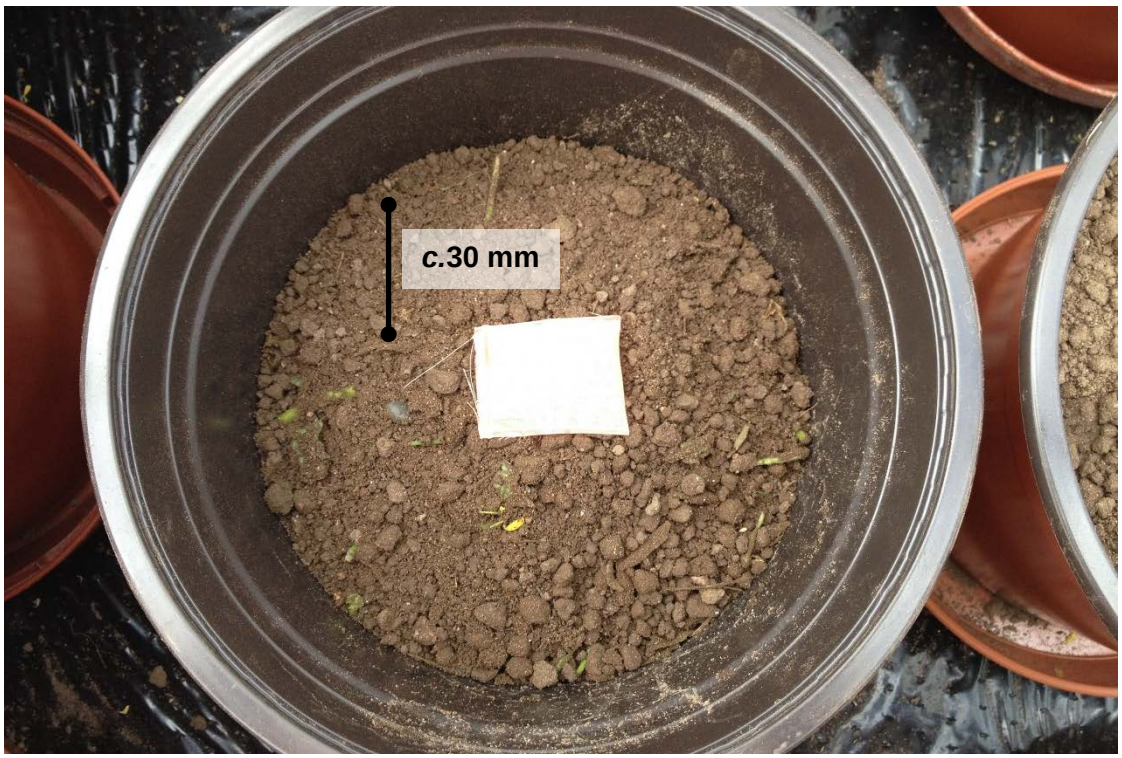


Figure 3.9: Central placement of a cyst sachet in a field soil pot. The scale indicates the approximate length of a cyst sachet.

Figure 3.10: Glasshouse experiment 1 showing field soil pots after *B. juncea* incorporation. Blocks I-VII run numerically from left to right, and are represented using Roman numerals and arrows.

3.3 Materials and methods: glasshouse experiment 2

3.3.1 *Experiment objectives*

i) Quantify PCN cyst contents and viability following treatment with a range of incorporated *B. juncea* biomass quantities over a specified range of soil moisture conditions.

ii) Quantify the biomass and GSL content of *B. juncea* material used in experimental work.

3.3.2 *Experimental design*

Glasshouse experiment 2 was carried out between January and June 2016 at HAU (Table 3.2). The experiment consisted of the same two factors as glasshouse experiment 1; 'soil moisture', of which the level was increased to six (0, 25, 50, 75 and 100% of field capacity, and water saturated soil), and 'biofumigant', which was expanded to include five levels (0, 25, 50, 75 and 100 t ha^{-1} equivalent fresh biomass). The general material production stages remained similar to glasshouse experiment 1 but with modifications to accommodate the increased number of experimental units (pots). As illustrated in Figures 3.11 and 3.12, treatments were arranged within the experiment in a randomised block design and replicated four times. The design was sufficiently replicated to enable treatment differences of 10-15% in PCN egg viability to be determined for background variation levels in egg viability of between 3-4% between replicates of like treatments (Berndston, 1991). The design had 87 RDF.

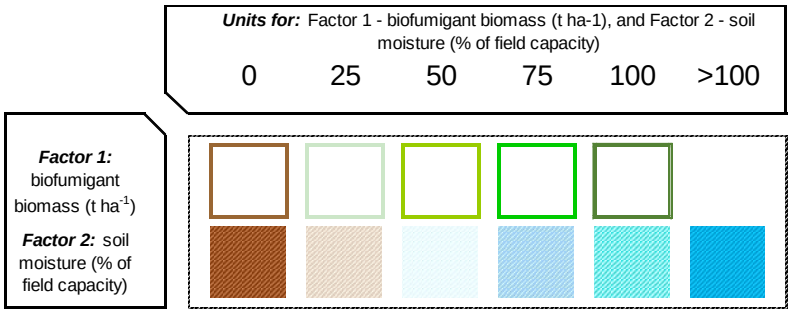


Figure 3.11: Treatment key for glasshouse experiment 2. Border colour indicates *B. juncea* biofumigant treatment, fill colour indicates soil moisture treatment.

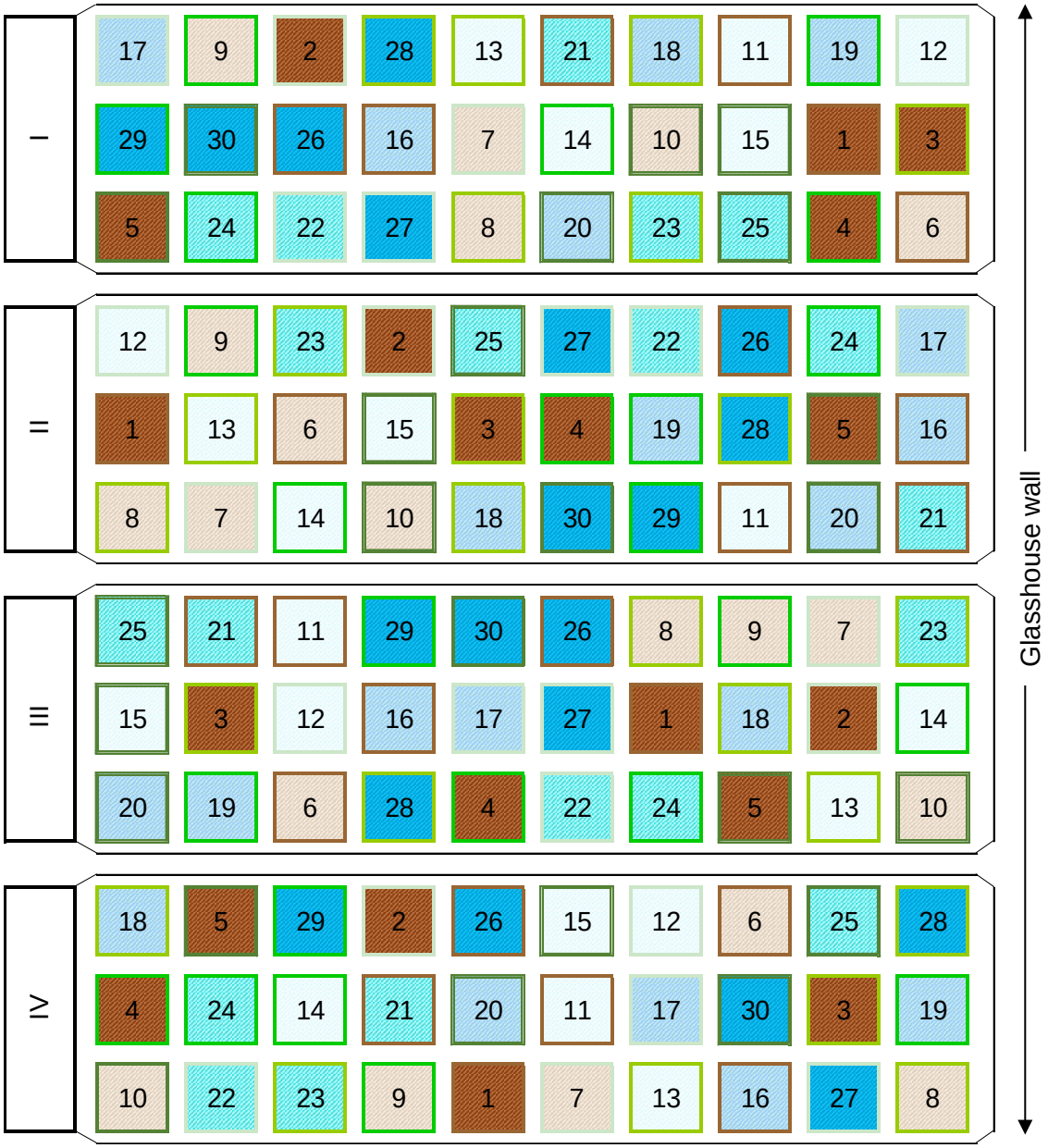


Figure 3.12: Glasshouse experiment 2 design showing allocation of treatments 1-30. Blocks are represented using Roman numerals (I-IV).

3.3.3 *Experimental set-up*

The same three core materials required to carry out glasshouse experiment 1 were required for glasshouse experiment 2; *B. juncea* biomass (i), silk sachets containing PCN cysts (ii), and pots of field soil stabilised at treatment soil moisture conditions (iii). The set-up of this experiment was therefore dominated by the production of those three primary materials. Table 3.2 shows the months leading up to the initiation of glasshouse experiment 2 and the time allocated to the production of the three core materials.

Table 3.2: Table illustrating the time allocated to producing *B. juncea* biomass, PCN cyst sachets and pots of field soil stabilised at soil moisture conditions for glasshouse experiment 2.

	Year: 2016					
Production of materials	Jan	Feb	Mar	Apr	May	Jun
i: *Brassica juncea* biomass					Glasshouse experiment 2 set-up and in progress	
ii: Cysts and cyst sachets						
iii: Field soil stabilised at treatment soil moisture conditions						

3.3.4 *Core experimental materials*

3.3.4.1 Brassica juncea *biomass production*

Brassica juncea seed was sown into John Innes no.2 compost in trays in January 2016 (Table 3.2) and transplanted to pots of compost using the same methodology as the first glasshouse experiment. Pot size was increased to 250 mm diameter to try and reduce the watering frequency required to maintain plants within a 65-95% of field capacity range throughout the growing period. The planting density in *B. juncea* pots remained equivalent to an 8 kg ha^{-1} seed rate. However, the number of transplanted seedlings increased to 26 plants per pot. All other materials and methods for growing *B. juncea*

remained consistent with the first glasshouse experiment. Figure 3.13 shows the growing arrangement for *B. juncea* plants (seven blocks of six pots). The number of *B. juncea* pots was reduced from that of the first glasshouse experiment due to the use of smaller pots in the main experiment and therefore a reduced biomass requirement. Additionally, plant material for assessment was taken from the same pots used for biofumigation treatment in the main experiment. Biomass from pots marked 'I/A' was used for incorporation into biofumigation treated pots after moisture stabilisation of field soil, and for crop assessments. Biomass from pots marked 'D' was discarded. The selection of biomass from 'I/A' pots versus 'D' pots was made on the day of incorporation, and was based upon the uniformity of plant physiology, which was assessed visually. Figure 3.14 shows *B. juncea* plants approximately one week before incorporation in April 2016.

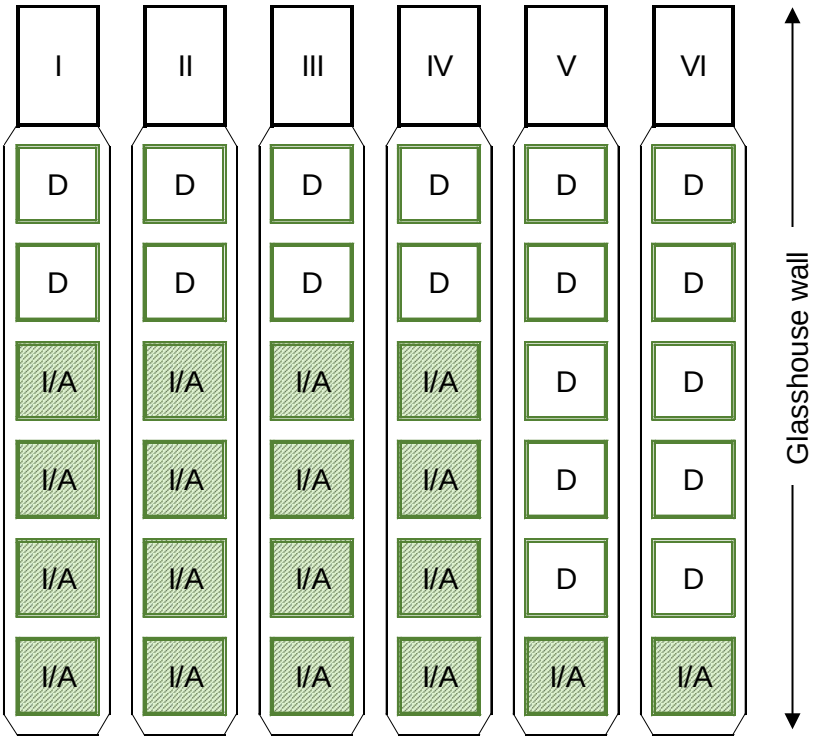


Figure 3.13: *Brassica juncea* pot arrangement for glasshouse experiment 2. Blocks are represented using Roman numerals (I-VI). Units marked 'I/A' indicate *B. juncea* pots which were used for incorporation as treatments and for crop assessments to generate supporting data. Units marked 'D' indicate *B. juncea* pots which were surplus to requirements and therefore discarded.

Figure 3.14: *Brassica juncea* plants at budding to early flowering, approximately one week prior to incorporation into pots, viewed side on to block I.

3.3.4.2 *Potato cyst nematode cyst sachets*

Fifty *G. pallida* cysts, obtained from the same field as for glasshouse experiment 1, were graded to 500-1000 µm in size using the sieve illustrated in Figure 3.7, and then placed into cyst sachets. A total of 120 cyst sachets were produced for glasshouse experiment 2. Cysts were graded in an attempt to reduce background variation in the experiment (Twomey *et al*., 1995) in response to having fewer replicates of treatments than in glasshouse experiment 1. Due to the high number of cysts required, the production of cysts sachets took from January to March 2016 (Table 3.2).

3.3.4.3 *Field soil and stabilisation of moisture conditions*

Sandy clay loam soil was again collected from Black Brook Meadow field at HAU for use in glasshouse experiment 2, and processed in the same manner as for glasshouse experiment 1.

3.3.5 *Experiment initiation*

Brassica juncea plants were macerated using the Viking® GE 150 garden shredder in April 2016, and the residues weighed into 30.7, 61.4, 92.0 and 122.7 g biomass quantities used to represent 25, 50, 75 and 100 t ha^{-1} respectively. The residues were mixed into soil and *G. pallida* sachets placed into field soil pots in the same manner as for the first glasshouse experiment. The 200 mm diameter pots used in glasshouse experiment 1 were replaced with smaller 125 mm diameter pots due to the greater number of experimental units in glasshouse experiment 2, and glasshouse space restrictions. The pots were arranged in the HAU glasshouse according to the design shown in Figure 3.12. Soil moisture within pots was stabilised before biofumigation as for glasshouse experiment 1. Figure 3.15 shows the experiment after incorporation of *B. juncea* material into relevant pots.

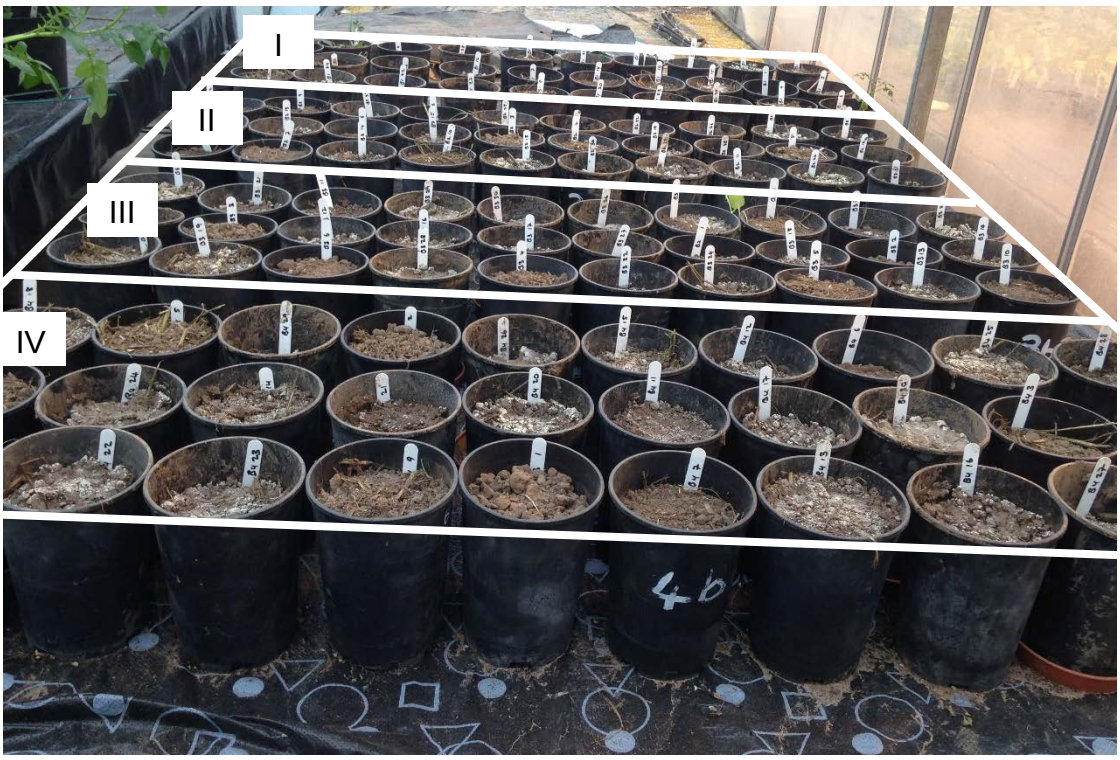


Figure 3.15: Glasshouse experiment 2 showing field soil pots after *B. juncea* incorporation. Blocks I-IV run numerically from back to front.

3.4 Assessments

3.4.1 *Potato cyst nematode*

Globodera pallida egg viability and eggs cyst^{-1} were quantified for glasshouse experiments 1 and 2 as described in Section 2.3.2 using Equations 2.2 and 2.3.

3.4.2 *Biofumigant*

3.4.2.1 *Biomass (fresh and dry-weight)*

In glasshouse experiment 1, pots were weighed prior to plant maceration whilst plants were *in-situ*. Plants were then removed from compost for maceration and the compost filled pots reweighed so that the reduction in weight represented the fresh *B. juncea* plant weight per pot. This methodology enabled an expedient incorporation of *B. juncea* material, although introduced some variability into the first glasshouse experiment in terms of the quantity of biomass used per experimental pot. In glasshouse experiment 2, whole *B. juncea* pots were macerated, and predetermined fractions of fresh biomass used for incorporation into field soil filled experimental pots. This methodology enabled uniform treatment levels of biomass to be used in the experiment. From the total macerated biomass from a single pot in glasshouse experiment 2, a 30 g subsample was taken for biomass dry-matter assessment. A single plant from two randomly selected assessment ('A') *B. juncea* pots per block (Figure 3.3) was collected, dried, and used to assess dry-matter for glasshouse experiment 1. Plant samples for both experiments were dried using a forced air oven at 105°C for 48-72 h and the percentage dry-matter calculated using standard methods (Jackson *et al*.,1986). Fresh and dry-weight biomass figures were then converted to t ha^{-1} equivalent units for ease of comparison between glasshouse and field work using Equation 3.1.

Equation 3.1

$$\text{Biomass t ha}^{-1} = \frac{\text{The number of pots per hectare calculated using pot surface area} \times \text{Biomass (fresh or dry) calculated per pot in g}}{1{,}000{,}000}$$

3.4.2.2 *Glucosinolate content of tissues*

Plant samples were collected from glasshouse experiment 1 for glucosinolate analysis. However, the samples were lost during storage due to a freezer malfunction. A single plant from each assessment ('I/A') pot (Figure 3.13) was collected and processed for glasshouse experiment 2 using the methodologies described in Section 2.4.2.

3.5 Data analysis

Data were analysed by analysis of variance (ANOVA) and regression with groups using GenStat® 16th Edn. (VSN International). Treatment effects for ANOVA analysed data were compared using Tukey's multiple range test at 95% confidence. Regression with groups analysis generated three linear and three polynomial models for each data set, from which the model which accounted for the greatest percentage of variance was used. Models varied in complexity. Coefficient of variation (CV) values were calculated using Microsoft® Excel 2016. Figures were generated using Microsoft® Excel 2016.

3.6 Results: glasshouse experiment 1

3.6.1 Brassica juncea *biomass and dry-matter*

The dry-matter of plants at incorporation was 13.9%. The standard error of the mean (SEM) was 0.85%. No difference was found in the mean quantity of fresh-weight *B. juncea* biomass (P = 0.054) produced for each block of *B. juncea* pots (Figure 3.16 i). However, fresh-weight *B. juncea* biomass ranged between equivalent to 58.1-78.5 t ha^{-1} between blocks I-VII, which represents a large degree of variation (Figure 3.16 i). No difference was found in the mean quantity of fresh-weight biomass incorporated into different *B. juncea* treatment pots (P = 0.859) (Figure 3.16 ii). Mean fresh-weight biomass ranged between equivalent to 69.6-72.4 t ha^{-1} between treatments 1, 2, 3 and 4 (equivalent to 25, 50, 75 and 100% of field capacity soil moisture treatment respectively) indicating the randomisation of *B. juncea* pots across treatments was effective (Figure 3.16 ii).

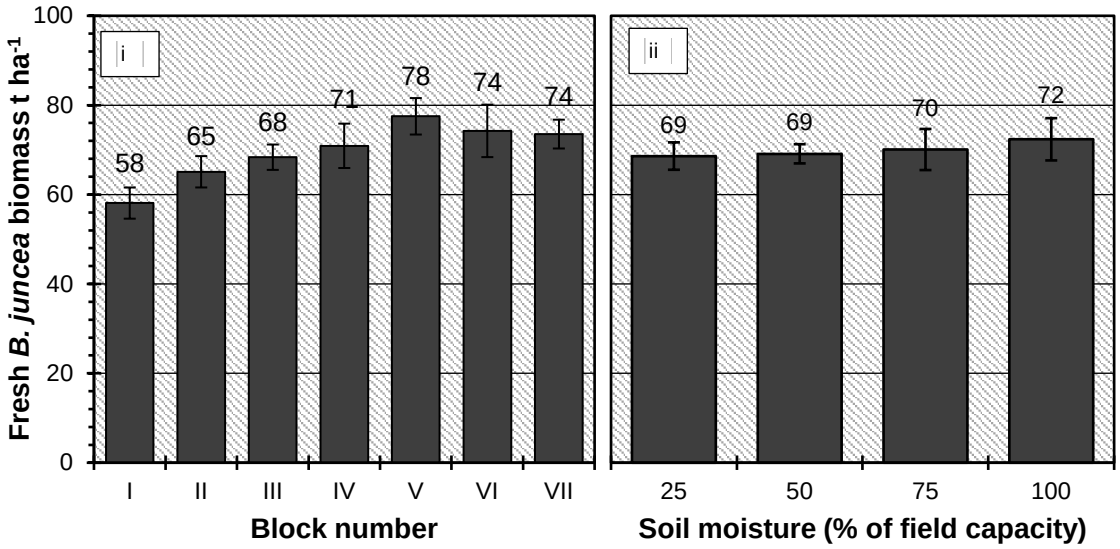


Figure 3.16: Mean fresh-weight *B. juncea* biomass (converted to t ha^{-1}) analysed by block (i) and soil moisture treatment (ii) factors using one-way ANOVA. Error bars represent the SEM.

3.6.2 Globodera pallida *egg viability % following treatment with and without* Brassica juncea *biofumigation at different soil moisture levels*

The viability of encysted *G. pallida* eggs measured eight weeks after biofumigation indicated a negative effect of biofumigation on *G. pallida* viability ($P < 0.001$) (Figure 3.17 i and iii). However, soil moisture treatment had no effect on the viability of *G. pallida* ($P = 0.100$) and there was no interaction between soil moisture and biofumigation on *G. pallida* viability ($P = 0.682$) (Figure 3.17 i and ii). Biofumigation was found to cause a 40.6% mean reduction in *G. pallida* egg viability. Biofumigation efficacy ranged between 35.0-43.1% for soil moisture treatments between 25-100% of field capacity compared to pots where no *B. juncea* material was incorporated. Figure 3.17 i shows a one-way ANOVA analysis of *G. pallida* viability as affected by treatments 1-8 (25, 50, 75 and 100% of field capacity with and without biofumigation) which illustrates the effect of biofumigation and absence of any soil moisture effect on *G. pallida* viability. This is further supported by general ANOVA analyses in plates ii and iii where data was analysed at the factor level. The CV of *G. pallida* egg viability for the *B. juncea* treatments in Figure 3.17 i and iii was 18.8%, whilst for the untreated treatments the CV was 6.4%.

The CV's of egg viability for soil moisture levels in Figure 3.17 ii were; 24.3% for 25% of field capacity, 29.8% for 50% of field capacity, 32.0 for 75% of field capacity and 30.1% for 100% of field capacity.

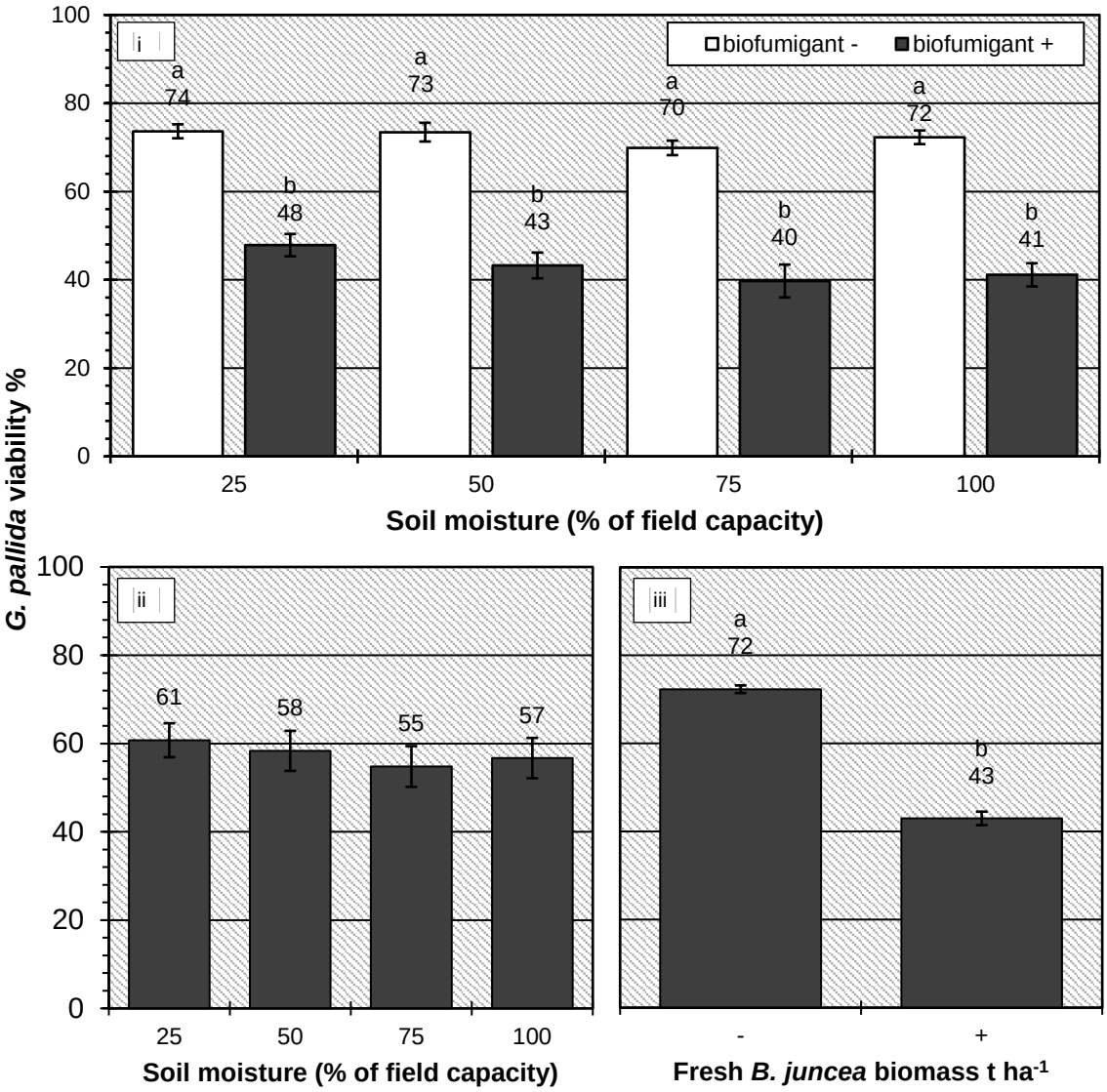


Figure 3.17: *Globodera pallida* viability after exposure to *B. juncea* biofumigation treatment over a range of soil moisture conditions. Plate i shows a one-way ANOVA analysis whilst plates ii and iii were produced after a general ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

3.6.3 *The influence of* Brassica juncea *biomass quantity on* Globodera pallida *viability % at different soil moisture levels*

Figure 3.18 shows how *G. pallida* viability is influenced by increasing *B. juncea* biomass in soil with different soil moisture conditions (25-100% of field capacity, plates i-iv). Only 15.9% of the variance in *G. pallida* viability could be explained overall, by variation in the quantity of *B. juncea* biomass incorporated into pots. No grand regression could be fitted to the complete data set due to the large scatter of data points (P = 0.158), and no significantly different relationships between biomass and *G. pallida* viability were observable between regressions (P = 0.104). However, a moderate-strong positive correlation existed between biomass and *G. pallida* viability for 25% of field capacity, and a moderate-strong negative correlation relationship existed for 100% of field capacity.

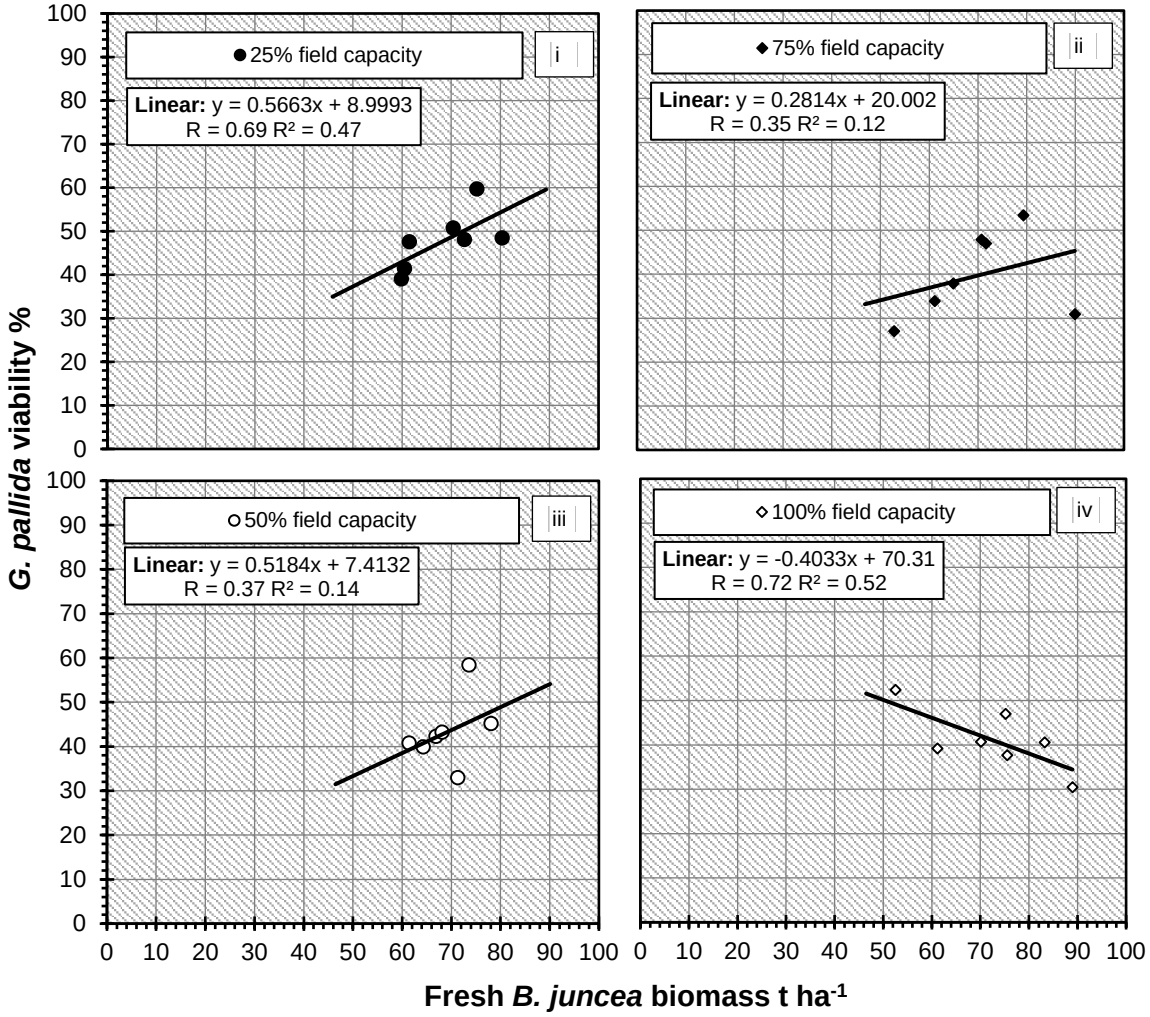


Figure 3.18: *Globodera pallida* viability as influenced by different quantities of *B. juncea* biomass and levels of soil moisture. Soil moisture groups have been presented as individual plates (i-iv) for ease of comparison.

3.6.4 *Effect of soil moisture and* Brassica juncea *biofumigation on the number of* Globodera pallida *eggs cyst^{-1}*

Figure 3.19 shows the total number of *G. pallida* eggs cyst^{-1} for treatments and for *B. juncea* biomass and soil moisture in factor based analyses. No effect of *B. juncea* on *G. pallida* eggs cyst^{-1} was observed between treatments when measured 8 weeks after biofumigation ($P < 0.228$) (Figure 3.19 i). Neither soil moisture or *B. juncea* biomass factors were found to effect *G. pallida* eggs cyst^{-1} either ($P = 0.656$ and $P = 0.109$ respectively), and there was no interaction between soil moisture and *B. juncea* biofumigation on *G. pallida* eggs cyst^{-1} ($P = 0.153$) (Figures 3.19 ii-iii). Eggs cyst^{-1} ranged between 135-213 for treatments 1-8 (Figure 3.19 i). The CV of *G. pallida* eggs cysts^{-1} for the *B. juncea* treatments in Figure 3.19 i and iii was 37.6%, whilst for the untreated treatments the CV was 32.5%. The CV's of eggs cyst^{-1} for soil moisture levels in Figure 3.19 ii were; 38.9% for 25% of field capacity, 32.8% for 50% of field capacity, 32.9 for 75% of field capacity and 38.4% for 100% of field capacity.

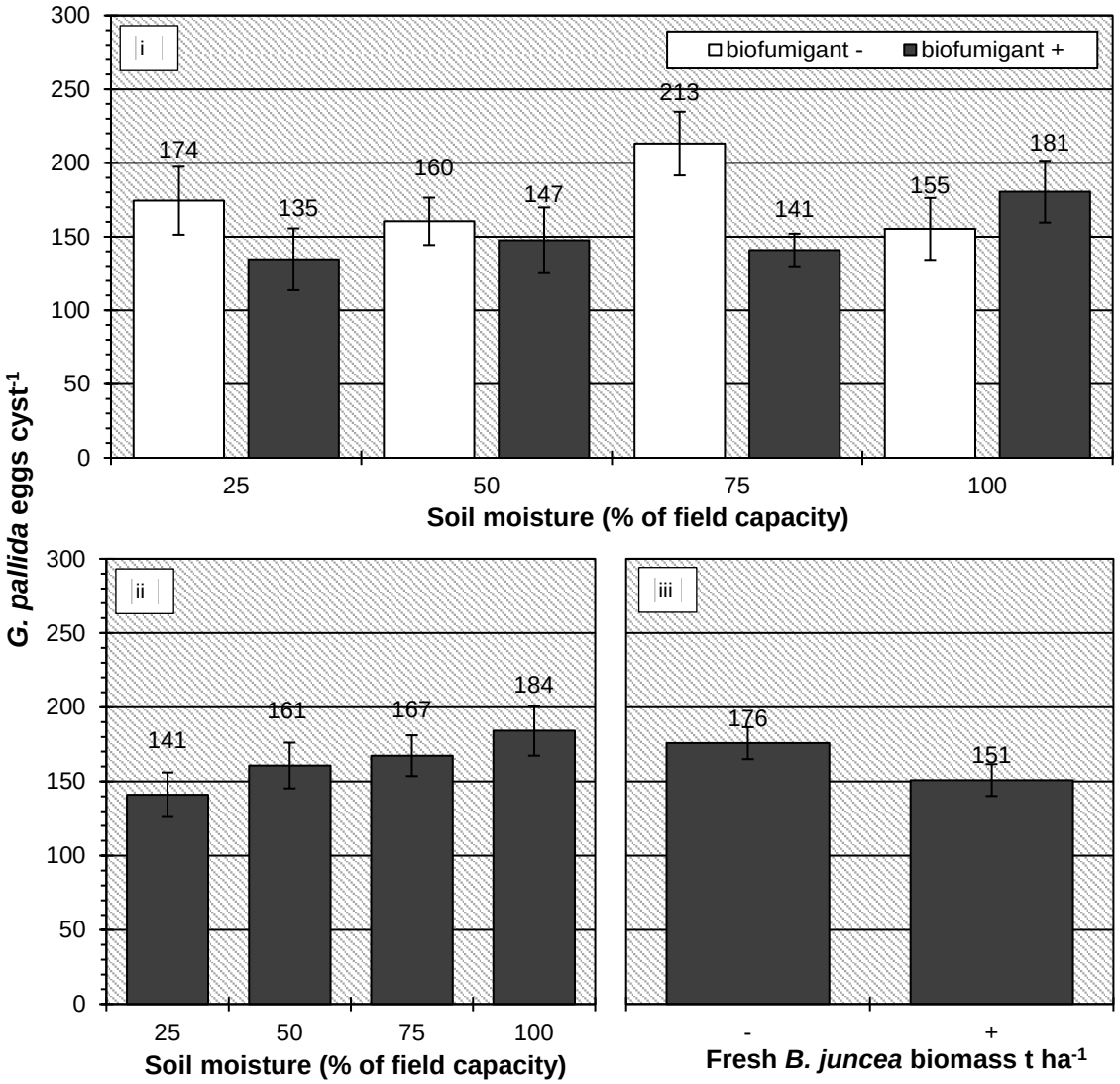


Figure 3.19: *Globodera pallida* eggs cyst^{-1} after exposure to *B. juncea* biofumigation over a range of soil moisture conditions. Plate i shows a one-way ANOVA analysis whilst plates ii-iii were produced using general ANOVA analysis. Error bars represent the SEM.

3.7 Results: glasshouse experiment 2

3.7.1 Brassica juncea *dry-matter and glucosinolate content at incorporation*

The dry-matter of plants at incorporation was 11.4%. The SEM was 0.03%. 2-propenyl GSL (sinigrin) content of combined leaf and stem tissues varied between 3.7-5.0 µmol g^{-1} dry tissue between biofumigant rows. Mean 2-propenyl GSL content was 4.2 µmol g^{-1} dry tissue and the SEM 0.30 µmol g^{-1} dry tissue (Appendix 8.2.4). 2(S)-hydroxy-3-butenyl (epiprogoitrin) and 2(R)-2-hydroxy-3-butenyl (progoitrin) were also observed in *B. juncea* tissues at *c.*0.9 and 1.8 µmol g^{-1} dry tissue respectively.

3.7.2 Globodera pallida *egg viability % following treatment with different quantities of* Brassica juncea *material and different levels of soil moisture*

A negative effect of *B. juncea* biofumigation on *G. pallida* viability was observable eight weeks after *B. juncea* incorporation ($P < 0.001$) (Figure 3.20). Up to a 51.2% difference in efficacy was observed against *G. pallida* between extreme biomass/soil moisture treatments. However, neither biomass or soil moisture effects on *B. juncea* efficacy against *G. pallida* were easily separable with a one-way ANOVA analysis (Figure 3.20). Simple general ANOVA analyses at the *B. juncea* biomass and soil moisture factor levels were more revealing (Figure 3.21 i and ii). Overall, biofumigation was found to cause a 16.5-31.1% reduction in *G. pallida* viability between extremes of incorporated fresh-weight *B. juncea* biomass (0-100 t ha^{-1}), with notable differences in efficacy between 0, 25 and 75-100 t ha^{-1} fresh-weight biomass (Figure 3.21 i). Analysis of soil moisture effects on *G. pallida* viability found a negative impact of 50% of field capacity moisture treatment at *B. juncea* incorporation on the viability of *G. pallida* eggs compared to 0% of field capacity ($P = 0.010$) (Figure 3.21 ii). No other moisture levels could be separated. An extra 14.3% efficacy was achieved when performing biofumigation at 50% of field capacity for the full range of incorporated biomass quantities compared to 0% of field capacity (Figure 3.21 ii). No interaction was observed between the quantity of *B. juncea* biomass incorporated into soil and the moisture level of soil on the efficacy of biofumigation against *G. pallida* in this instance ($P = 0.127$). Treatment CV's from Figure 3.20 ranged between 3.7 and 41.2%. The egg viability CV's for *B. juncea* biomass in Figure 3.21 i were; 12.4% for 0 t ha^{-1}, 20.2% for 25 t ha^{-1}, 22.8% for 50 t ha^{-1}, 30.4% for 75 t ha^{-1} and 28.0% for 100 t ha^{-1}. The egg viability CV's for soil moisture levels in Figure 3.21 ii were; 24.7% for 0% of field capacity, 28.9% for 25% of field capacity, 26.0% for 50% of field capacity, 26.9% for 75% of field capacity, 22.0% for 100% of field capacity and 28.1% for saturated soil. The 25, 50 and 75% of field capacity moisture levels appeared optimal compared to 25 and 100% of field capacity and saturated moisture levels overall (Figure 3.21 ii).

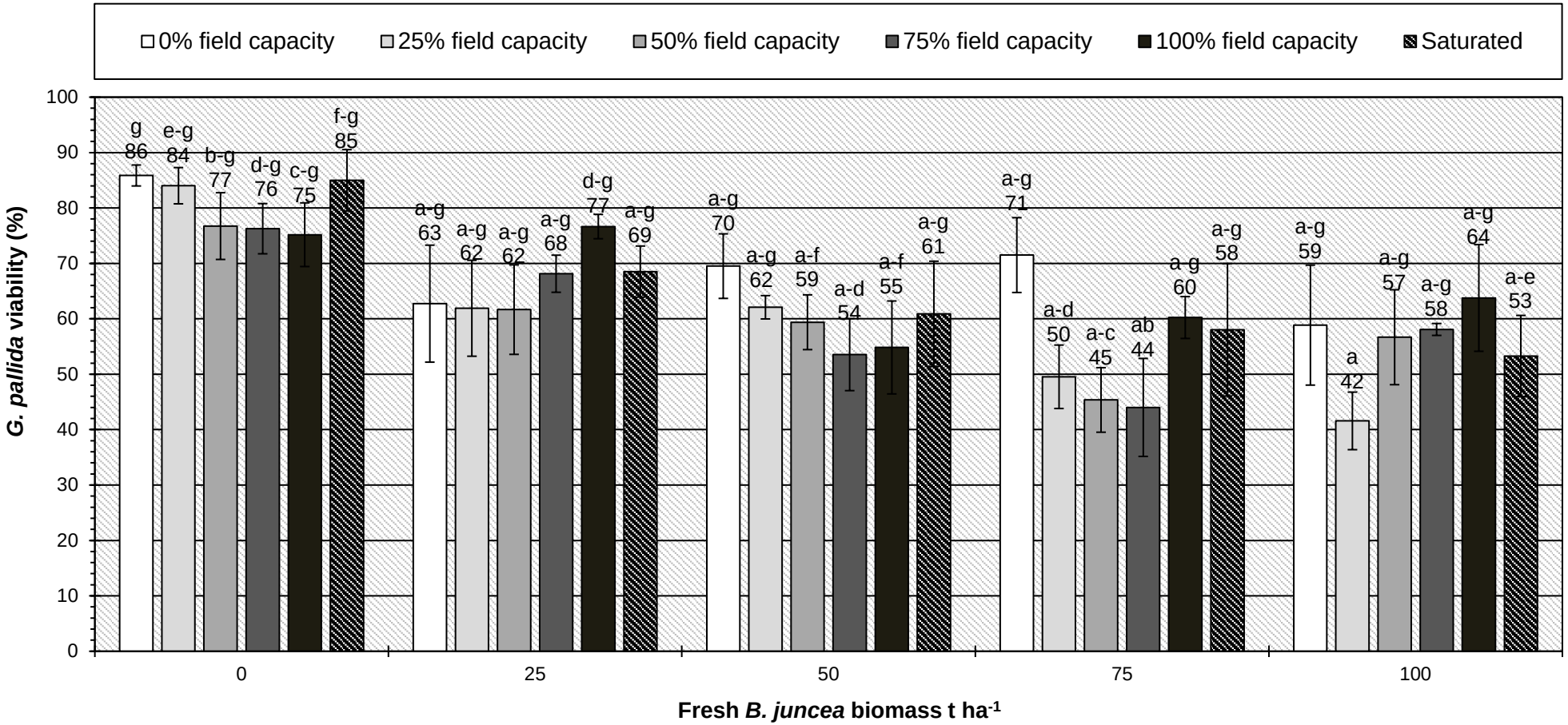


Figure 3.20: *Globodera pallida* viability after exposure to quantities of *B. juncea* material ranging between the equivalent of 0-100 t ha^{-1} fresh-weight biomass and soil moisture ranging between 0% of field capacity and water saturated soil. One-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

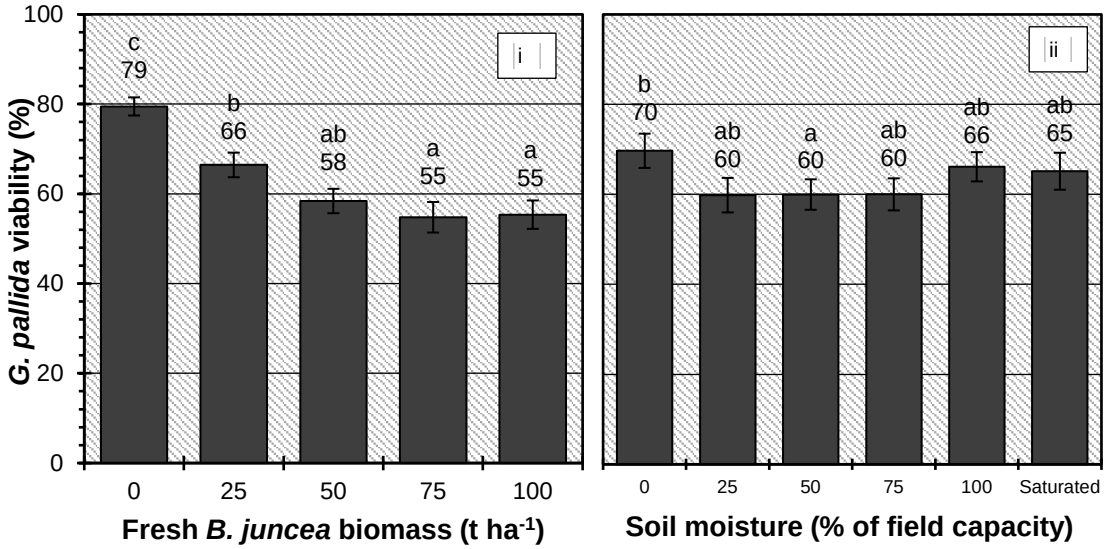


Figure 3.21: *Globodera pallida* viability after exposure to quantities of *B. juncea* material ranging between the equivalent of 0-100 t ha^{-1} fresh-weight biomass (i), and soil moisture ranging between 0% of field capacity and water saturated soil (ii). General ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

3.7.3 *The influence of* Brassica juncea *biomass quantity on* Globodera pallida *viability % for grouped soil moisture levels; 25, 50 and 75% of field capacity, and 0 and 100% of field capacity combined with saturated soil*

The 25-75% of field capacity bars from Figure 3.21 ii appear to be very similar, whilst also appearing to be different to the 0 and 100% of field capacity, and saturated soil bars. Similarly to the first grouping, the 0 and 100% of field capacity, and saturated soil bars appear to be closely aligned. Given that the 0 and 50% of field capacity bars can be separated statistically, it appears logical to carry out further group based correlation and regression analyses, taking into account the clear biomass effects from Figure 3.21 i. Figures 3.22 and 3.23 show linear and polynomial models respectively. They show how *G. pallida* viability is influenced by different quantities of *B. juncea* biomass for grouped soil moistures determined from Figures 3.21 ii. The polynomial models shown in Figure 3.23 appear to be more explanatory of the relationship between the quantity of incorporated *B. juncea* biomass and *G. pallida* viability than the linear models from Figure

3.22 when comparing R and R^2 values. Using the linear models in Figure 3.22, biofumigation efficacy against *G. pallida* can be shown to be improved under optimal soil moisture conditions by 6.0, 8.6, 11.7, 15.3 and 19.5% for inclusions of fresh-weight *B. juncea* material equivalent to 0, 25, 50, 75 and 100 t ha^{-1} respectively compared to the same inclusions of material into soil under sub-optimal moisture conditions. Using the polynomial models in Figure 3.23, biofumigation efficacy against *G. pallida* can be shown to be improved under optimal soil moisture conditions by 3.9, 9.9, 14.8, 17.0, and 15.8% for inclusions of fresh-weight *B. juncea* material equivalent to 0, 25, 50, 75 and 100 t ha^{-1} respectively compared to the same inclusions of material into soil under sub-optimal moisture conditions. Both linear and polynomial models show strong negative relationships between the quantity of *B. juncea* material incorporated into soil and *G. pallida* viability.

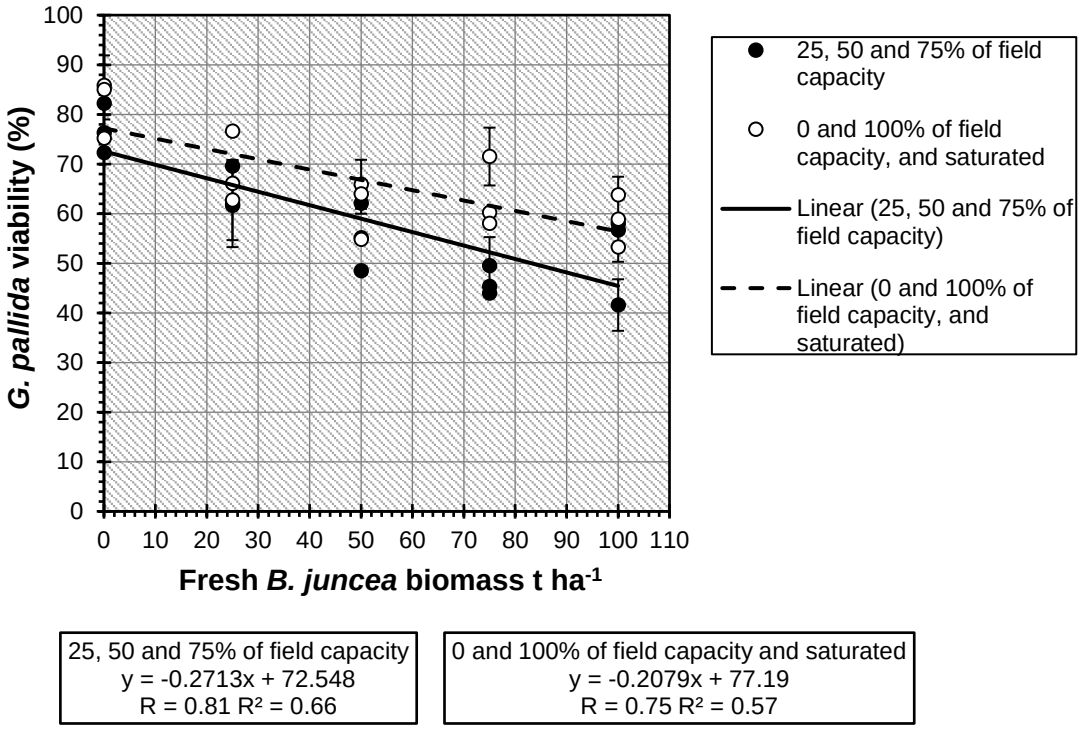


Figure 3.22: *Globodera pallida* viability as influenced by different quantities of *B. juncea* biomass and grouped levels of soil moisture. Error bars represent the SEM.

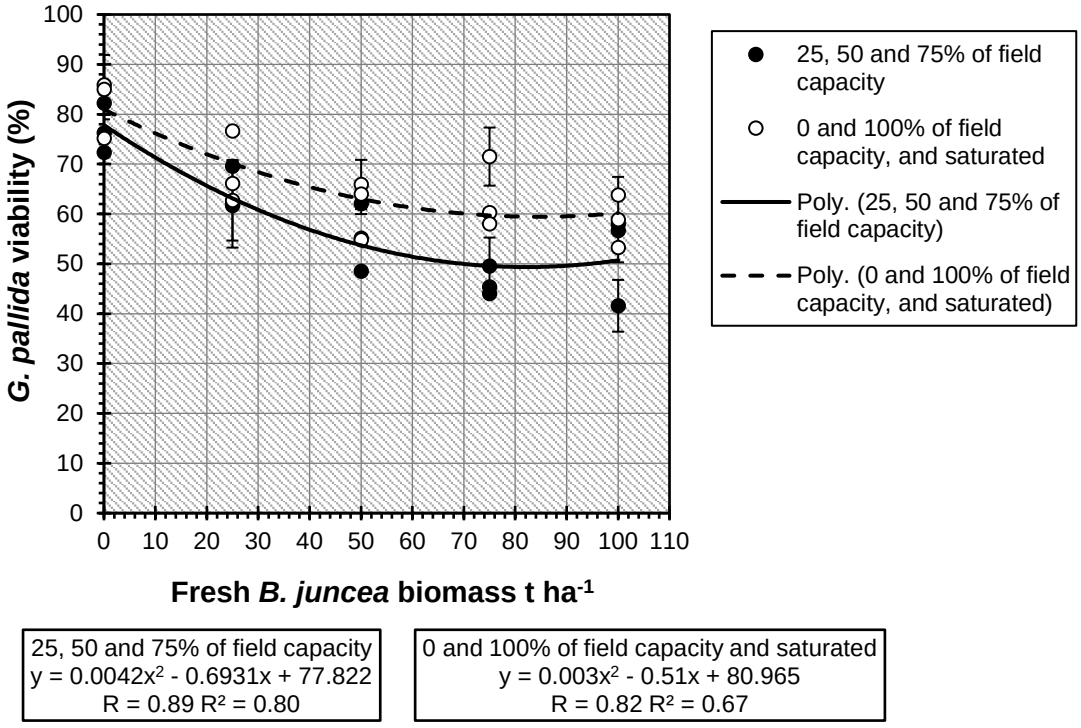


Figure 3.23: *Globodera pallida* viability as influenced by different quantities of *B. juncea* biomass and grouped levels of soil moisture. Error bars represent the SEM.

3.7.4 Globodera pallida *eggs cyst^{-1} following treatment with different quantities of* Brassica juncea *material and different levels of soil moisture*

Differences in *G. pallida* eggs cyst^{-1} were noticed between individual soil moisture/*B. juncea* biomass treatments (P = 0.021) when using a one-way ANOVA analysis. Differences lay between the saturated treatment at 0 t ha^{-1} *B. juncea* biomass in comparison to 50% field capacity treatment at 0 t ha^{-1} *B. juncea* biomass and 25% field capacity treatment at 75 t ha^{-1} *B. juncea* biomass inclusion into soil (Figure 3.24). Eggs cyst^{-1} ranged between 36-198 for treatments 1-30 (Figure 3.24). However, no overall *B. juncea* biomass or soil moisture effects against *G. pallida* eggs cyst^{-1} were easily separable with a one-way ANOVA analysis (Figure 3.24). Simple general ANOVA analyses at the *B. juncea* biomass and soil moisture factor levels were therefore investigated (Figure 3.25 i and ii). No effect of *B. juncea* biomass or soil moisture level on *G. pallida* eggs cyst^{-1} was observed (P = 0.140 and P = 0.56 respectively) (Figure

3.25 i and ii). No interaction was observed between the quantity of *B. juncea* biomass incorporated into soil and the moisture level of soil on *G. pallida* eggs cyst^{-1} in this instance, although *P* was approaching significance (*P* = 0.057). Treatment CV's from Figure 3.24 ranged between 14.1 and 80.8%. The egg cyst^{-1} CV's for *B. juncea* biomass in Figure 3.25 i were; 56.7% for 0 t ha^{-1}, 52.3% for 25 t ha^{-1}, 40.9% for 50 t ha^{-1}, 55.0% for 75 t ha^{-1} and 58.1% for 100 t ha^{-1}. The eggs cyst^{-1} CV's for soil moisture levels in Figure 3.25 ii were; 51.2% for 0% of field capacity, 47.9% for 25% of field capacity, 57.8% for 50% of field capacity, 61.9% for 75% of field capacity, 41.1% for 100% of field capacity and 67.5% for saturated soil.

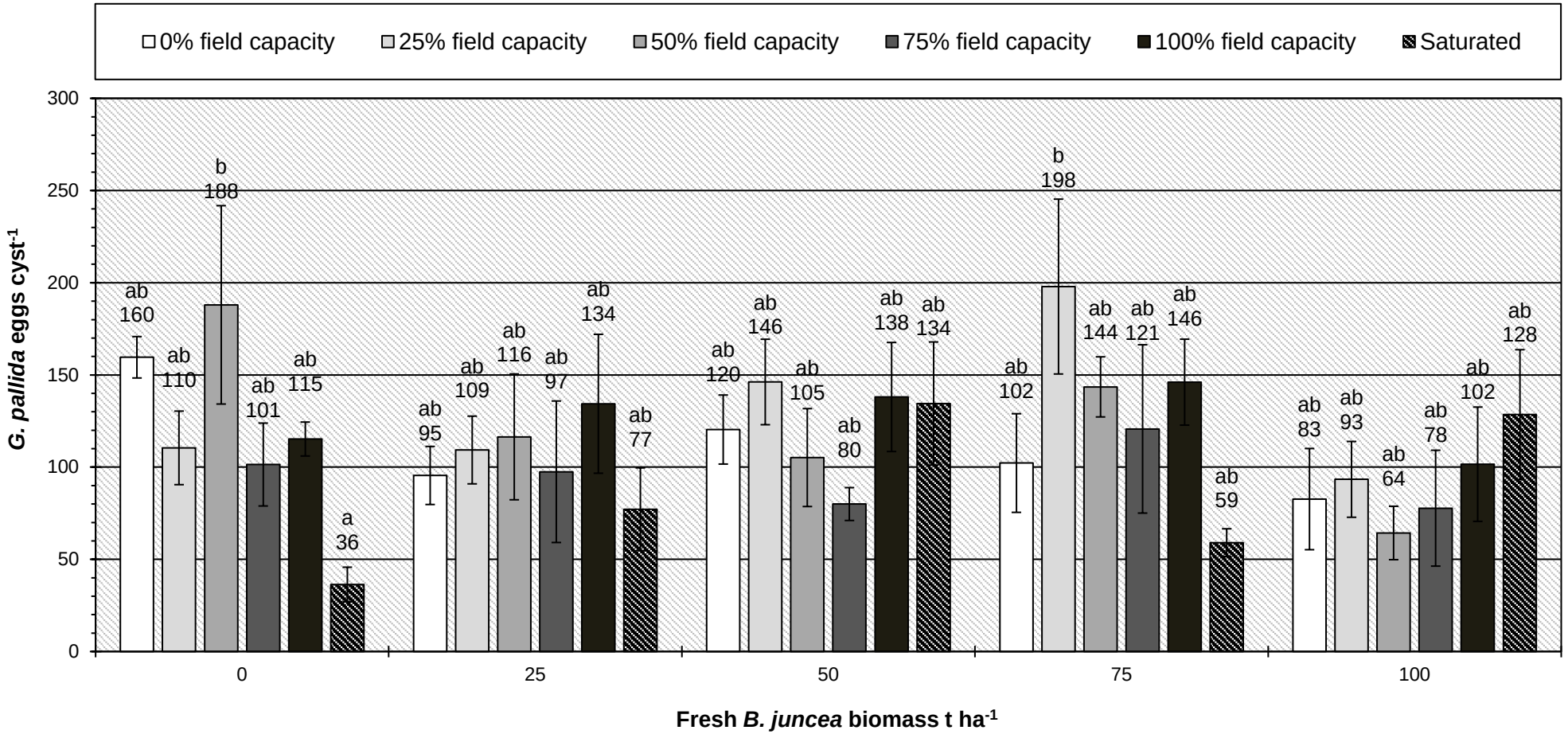


Figure 3.24: *Globodera pallida* eggs cyst^{-1} after exposure to quantities of *B. juncea* material ranging between the equivalent of 0-100 t ha^{-1} fresh-weight biomass and soil moisture ranging between 0% of field capacity and water saturated soil. One-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test

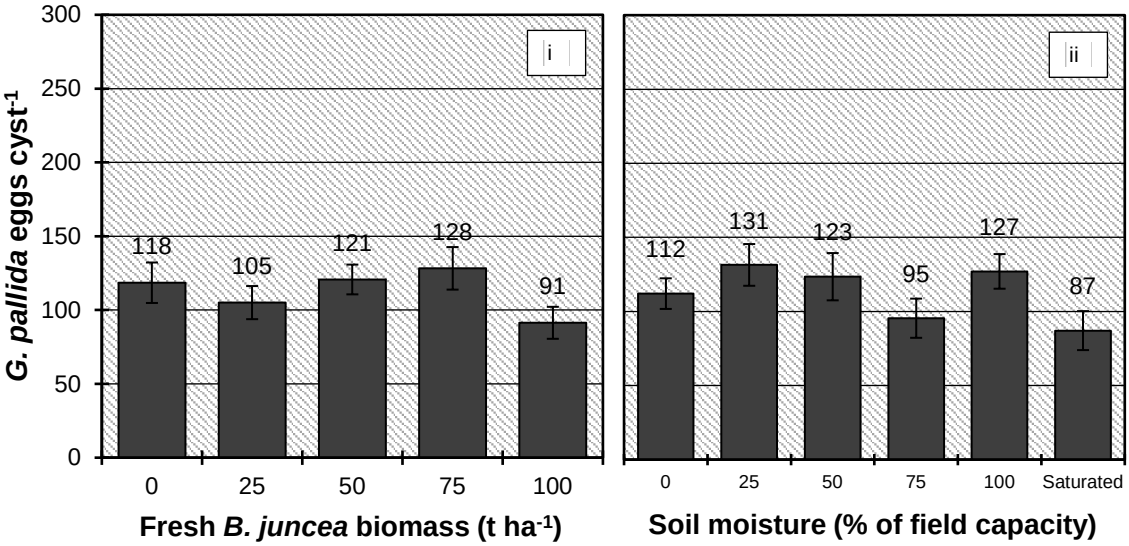


Figure 3.25: *Globodera pallida* eggs cyst^{-1} after exposure to quantities of *B. juncea* material ranging between the equivalent of 0-100 t ha^{-1} fresh-weight biomass (i), and soil moisture ranging between 0% of field capacity and water saturated soil (ii). General ANOVA analysis. Error bars represent the SEM.

3.8 Discussion

The primary aim of the work described in this chapter was to determine whether soil moisture conditions at biofumigant incorporation are influential of efficacy against PCN. The chapter null hypothesis stated that soil moisture level is not influential of *B. juncea* biofumigant efficacy against PCN, after reviewing literature surrounding the subject, most notably presented by Morra & Kirkegaard (2002) and Matthiessen *et al*. (2004). Overall, soil moisture was found to influence biofumigation efficacy against *G. pallida*, with 25-75% of field capacity generally appearing to be an optimal soil moisture range.

3.8.1 Globodera pallida *viability (glasshouse experiment 1)*

3.8.1.1 *Discussion of results analysed by analysis of variance*

Glasshouse experiment 1 investigated four levels of soil moisture; 25, 50, 75 and 100% of field capacity, with and without the addition of *B. juncea* biofumigant material into soil for effects on the viability of *G. pallida*. The headline results for glasshouse experiment 1 surrounded the observable effects of treatments on *G. pallida* egg viability shown in

Figure 3.17. Biofumigation effects ranged between a 35.0 and 43.1% viability reduction between 25-100% of field capacity soil moisture extremes, with a mean reduction in egg viability in the region of 40.6%. These figures are around half that recorded in previous studies investigating *B. juncea* cv. ISCI 99 for management of *G. pallida*, and so efficacy was considered to be moderate/low (Ngala *et al*., 2014; Lord *et al*., 2011). This could be due to the methodology employed, whereby PCN were not exposed to biofumigant VOC's during plant growth as partial biofumigation, but merely at the incorporation stage. Whilst a clear biofumigation effect on *G. pallida* viability was observable in glasshouse experiment 1, no such effect was observable relative to the moisture condition of soil, neither was an interaction between biofumigation and soil moisture condition found in terms of *G. pallida* egg viability. The absence of a soil moisture effect on biofumigation efficacy against *G. pallida* was a little unexpected in light of the robust experimental methodology employed and indications from separate studies that VOC levels should have been substantially higher in the wetter soil (Morra & Kirkegaard., 2002; Matthiessen *et al*., 2004). However, the VOC concentration in soils was not quantified in this experiment.

The experimental design for glasshouse experiment 1 was replicated sufficiently well to enable treatment differences of 5-15% in *G. pallida* egg viability to be determined for background variation levels in egg viability of between 2-7% as previously described (Berndtson, 1991). In a one-way ANOVA analysis there appeared to be an 8% difference in mean PCN viability between soil moisture extremes for *B. juncea* biofumigant treated soils, and a 6% difference for a simplified general ANOVA analysis (Figure 3.17 i and ii). The CV of *G. pallida* egg viability was therefore investigated to check whether the experiment had the power to determine differences of 6-8% in egg viability. The CV of *G. pallida* egg viability for *B. juncea* treatments in Figure 3.17 i and iii was 18.8%, whilst for the untreated treatments the CV was 6.4%. Using a replicate calculator provided by Berndston (1991) it was determined that seven replicates was insufficient to determine a 6-8% difference in *B. juncea* efficacy against *G. pallida*. In excess of 100 replicates

would have been necessary given the variation in egg viability and so no conclusions could be made regarding the importance or role of soil moisture in biofumigation of PCN.

However, untreated *G. pallida* were observed to have far less variation in egg viability than biofumigation treated. This indicated that biofumigation treatment had introduced a high level of variation into glasshouse experiment 1 which could have been masking soil moisture treatment effects, particularly in light of a potential trend for increasing efficacy against *G. pallida* with increasing soil moisture (Figure 3.17 i). It is probable that variation in the quantity of *B. juncea* biomass incorporated into soil was responsible for the variation in suppression of *G. pallida*. Although biomass did not differ significantly between blocks and so could not be used as a covariate in viability analyses, fresh-weight *B. juncea* biomass ranged between equivalent to 58.1-78.5 t ha^{-1} between blocks I-VII which represented a substantial level of variation when trying to determine potentially small soil moisture effects on biofumigation efficacy (Figure 3.16).

In an attempt to increase replication in the analysis of data, soil moisture effects on *G. pallida* viability was analysed at the factor level (Figure 3.17 ii and iii). This meant that *B. juncea* biofumigant treated and untreated treatments for like soil moisture conditions were amalgamated for analysis, providing 14 replicates for each soil moisture level under analysis in place of seven in a one-way ANOVA analysis. However, egg viability CV's also rose in this analysis to 24.3% for 25% of field capacity, 29.8% for 50% of field capacity, 32.0 for 75% of field capacity and 30.1% for 100% of field capacity. Using the Berdston (1991) replicate calculator, several hundreds of replicates would have been required to prove a 6% difference between soil moisture treatments given the background variation in egg viability. This type of analysis was therefore ineffective in this instance in determining whether soil moisture has a role in biofumigation of *G. pallida.*

3.8.1.2 *Discussion of results analysed by regression with groups*

Further viability analysis investigated potential relationships between *G. pallida* viability and the quantity of *B. juncea* biomass incorporated into soil, grouped by soil moistures 25, 50, 75 and 100% of field capacity (Figure 3.18). This was carried out in response to the large range in biofumigant biomass produced in glasshouse experiment 1. No grand regression could be fitted to the data, and no difference was found between individual regressions for 25, 50, 75 and 100% of field capacity groups. However, only 28 data points were analysed in total, so proving statistical significance was challenging. A reasonably convincing trend for decreasing biofumigation efficacy at 25% of field capacity as biomass inclusion into soil increased from *c.*60 to 80 t ha^{-1} was shown, whilst increasing efficacy was true for 100% of field capacity soil moisture conditions for biomass quantities between equivalent to *c.*50-90 t ha^{-1}. Both regressions showed moderate to strong relationships between biomass and *G. pallida* viability indicating that biomass quantity in conjunction with soil moisture could have some importance in influencing biofumigation efficacy against *G. pallida.* However, only seven data points were used to generate each regression, which suggests caution should be taken in data interpretation.

It is possible that where low quantities of *B. juncea* biomass were incorporated into soil (*c.*50 t ha^{-1}) lower soil moisture conditions (25-50% of field capacity) were preferable because those moisture conditions enabled volatile movement whilst also ensuring volatile retention in soil, similarly to methyl-ITC studies carried out by Simpson *et al*. (2010). Where the quantity of *B. juncea* biomass being incorporated into soil was high (*c.*90 t ha^{-1}) it is possible that biomass had a structural effect on soil whereby biofumigant residues created a more open soil structure with a greater number of macropores and therefore a greater potential for loss of VOC's to the atmosphere. Sultani *et al*. (2007) present evidence to suggest a linear relationship between increasing the number of soil macropores in soil as the quantity of green manure amendment to soil is increased. In their work, a 23 t ha^{-1} fresh weight legume amendment to soil increased the number of

soil macropores by 41% compared to an unamended control supporting the hypothesis that at low soil moisture conditions, biofumigant VOC's could be lost to the atmosphere where high biomass quantities are incorporated into soil. Under such a situation, higher soil moisture conditions could help retain VOC's in soil by limiting off-gassing (Simpson *et al*., 2010). Where soil moisture conditions are high, incorporation of low quantities of *B. juncea* biomass into soil could cause biofumigant VOC's to become compartmentalised in soil, or to be absorbed by soil water, reducing the diffusion potential of the VOC's and negatively influencing efficacy (Lembright, 1990). Further work is required to investigate these hypotheses. Figure 3.18 could be alluding to the importance of biofumigant VOC's being in a gaseous phase in soil for maximum efficacy, rather than when being held in soil moisture as the work of Matthiessen *et al*. (2004) might suggest.

3.8.2 Globodera pallida *viability (glasshouse experiment 2)*

3.8.2.1 *Discussion of results analysed by analysis of variance*

Glasshouse experiment 2 investigated six levels of soil moisture; 0, 25, 50, 75, 100% of field capacity and saturated soil, with the addition of *B. juncea* biofumigant material into soil at five levels equivalent to 0, 25, 50, 75 and 100 t ha^{-1} for suppression of *G. pallida*. Biomass quantity was controlled in glasshouse 2 to try to reduce the variation in *G. pallida* egg viability observed in the first glasshouse experiment following biofumigation treatment. A wide range of incorporated biomass quantities were investigated given that biomass would appear to be a primary factor influential of biofumigation success against *G. pallida* in glasshouse experiment 1. The large number of soil moisture and *B. juncea* biomass permutations in glasshouse experiment 2 resulted in 30 treatments. Only four replicates of each treatment could be feasibly achieved in glasshouse experiment 2, so other methods of reducing background variation in *G. pallida* egg viability were investigated and adopted. Cysts were graded to 500-1000 µm to increase the number of eggs used in egg counts and to stabilise the number of eggs used in counts owing to lower variation of eggs $cyst^{-1}$ in larger cysts than in smaller cysts (Twomey *et al*., 1995).

It was anticipated that grading cysts and controlling biomass might be sufficient improvements to the experimental methodology to reduce *G. pallida* egg viability CV's post biofumigation treatment, which might enable soil moisture effects to be observed if present. The design was sufficiently replicated to enable treatment differences of 10-15% in PCN egg viability to be determined for background variation levels in egg viability of between 3-4% between replicates of like treatments (Berndston, 1991)

The headline results for glasshouse experiment 2 surrounded the effects of treatments on *G. pallida* egg viability shown in Figures 3.20-21. One-way ANOVA analysis shown in Figure 3.20 was not very revealing as the variation in egg viability between four replicates of each treatment masked potential effects on *G. pallida* viability between treatments. Coefficient of variation values ranged between 3.7 and 41.2% between treatments which resulted in only a few treatment differences in *G. pallida* viability being separable for just four replications of each treatment. However, general ANOVA analyses shown in Figure 3.21 were revealing.

General ANOVA biomass assessment amalgamated all soil moisture levels sharing the same level of biomass (Figure 3.21 i), and all biomass levels sharing the same levels of soil moisture (Figure 3.21 ii). In the first instance this increased replication from four to 24 for each biomass treatment analysed, and in the second instance 20 replications of each soil moisture treatment were achieved. Using this methodology, differences in *B. juncea* biomass quantity on biofumigation efficacy against *G. pallida* in terms of viability were seen between equivalent to 0-25 t ha^{-1} fresh biomass, and then between 25 t ha^{-1} fresh biomass and 75-100 t ha^{-1} fresh biomass. The effect of 50 t ha^{-1} fresh biomass on *G. pallida* viability could not be separated from either 25 or 50 t ha^{-1} fresh biomass. Efficacy ranged between 16.5-31.1% when carrying out a general ANOVA biomass analysis, with a mean reduction in egg viability in the region of 26.0%. These figures are quite low compared to most of the literature, but still generally in line with previous studies investigating *B. juncea* cv. ISCI 99 for management of *G. pallida* (Ngala *et al*., 2014; Lord

et al., 2011). It is possible that the reduction in size of incorporation pots in glasshouse experiment 2 may be responsible for reduced biofumigation efficacy in the experiment due to an increased soil-pot surface area, leading to greater loss/off-gassing of VOC's from pots. Alternatively, the GSL content of *B. juncea* tissues in glasshouse experiment 2 might have been very low, or as described for glasshouse experiment 1, partial biofumigation effects could be missing as a result of the incorporation methodology used. This is unknown due to the loss of GSL samples from glasshouse experiment 1, although the GSL concentrations recorded for glasshouse experiment 2 (see Section 3.7.1 and Appendix 8.2.4) can be considered very low at *c.*4.2 µmol 2-propenyl GSL g^{-1} dry weight compared to other studies investigating *B. juncea* cv. ISCI 99 for management of PCN (Lord *et al*., 2011; Ngala *et al*., 2014). The low GSL concentrations in biofumigant material could be due to biofumigant plants not receiving fertiliser, due to plants being free from herbivory or water stress, or could be due to the set-up of the HPLC instrument used to quantify GSL's (Hopkins *et al*., 1999; Textor & Gershenzon, 2009). As a secondary observation, the increase in efficacy observed with increasing inclusion of *B. juncea* biomass into soil is in line with Lord *et al*. (2011) who recorded increasing mortality of *G. pallida* in soil in conjunction with increased GSL concentrations in soil. Because different quantities of the same plant material were used in this experiment, the same explanation is probable in this instance.

Differences in efficacy against *G. pallida* were also observed in general ANOVA analyses for different soil moisture conditions. An extra 14.3% efficacy was achieved when performing biofumigation at 50% of field capacity for the full range of incorporated biomass quantities compared to 0% of field capacity (Figure 3.21 ii). This is the first known instance where soil moisture has been shown to influence biofumigation efficacy against a soilborne pest. Generally, 25-75% of field capacity appeared to be the optimal range of soil moisture for *B. juncea* biofumigation of *G. pallida*. If the regression analyses observed for *B. juncea* biomass and *G. pallida* viability from glasshouse experiment 1 are accurate then the interpretation of the soil moisture results from glasshouse

experiment 2 could be more complex than simply maintaining soil moisture conditions between 25-75% for biofumigation of *G. pallida*. Given the data presented for glasshouse experiment 1, it is possible that 25% of field capacity is preferable for inclusions of *c.*25 t ha^{-1} fresh biomass into soil, that 50% of field capacity is preferable for inclusions of *c.*50 t ha^{-1} fresh biomass into soil, and that 75% of field capacity is preferable for inclusions of *c.*75 t ha^{-1} fresh biomass into soil. However, clearly more research is required to support these hypotheses and to understand biomass/soil moisture interactions.

Egg viability CV's ranged between 12.4-30.4% when data was analysed by biomass factor, and by 22.0-28.9% when analysed by soil moisture factor (Figures 3.21 i and ii). Using the Berdston (1991) replicate calculator, it would appear that biomass related effects on *G. pallida* viability were easily proven due to large effect sizes. However, soil moisture treatment effects on *G. pallida* viability were near the point of being undetectable due to background variation in *G. pallida* viability and a small effect size of 14.3% efficacy between extremes of moisture. Berndston (1991) suggest that for a background CV of 20% in egg viability, an effect size no less than *c.*20% should be detectable for *c.*20 replications. Clearly future work investigating the role of soil moisture in biofumigation of PCN would be well advised to increase replication from that used here to nearer 30 replications.

3.8.2.2 *Discussion of results analysed by regression with groups*

Further analysis of the soil moisture and *B. juncea* biomass data discussed, was presented in Figures 3.22 and 3.23. The effect of *B. juncea* fresh biomass on *G. pallida* viability was assessed with soil moisture data from 25, 50 and 75% of field capacity grouped as optimal moisture conditions, and 0 and 25 % of field capacity data combined with saturated data grouped as suboptimal soil moistures. Figure 3.22 showed a linear model whilst Figure 3.23 showed a polynomial model which was generally more explanatory than the linear model. Using the linear models, biofumigation efficacy against *G. pallida* was shown to be improved under optimal soil moisture conditions by

6.0, 8.6, 11.7, 15.3 and 19.5% for inclusions of fresh-weight *B. juncea* material equivalent to 0, 25, 50, 75 and 100 t ha^{-1} respectively, compared to the same inclusions of material into soil under sub-optimal moisture conditions. The primary point to draw from this analysis is to reiterate that *B. juncea* biofumigation efficacy against *G. pallida* increased in line with the quantity of biomass incorporated into soil, similarly to the work of Lord *et al*. (2011) with GSL regressions. Also, soil moistures of 25-75% of field capacity were consistently more effective for biofumigation of *G. pallida* across a full 25-100 t ha^{-1} range of fresh biomass inclusions into soil, than 0 and 100% of field capacity and saturated soil grouped. Using the polynomial models in Figure 3.23, biofumigation efficacy against *G. pallida* can be shown to be improved under optimal soil moisture conditions by 3.9, 9.9, 14.8, 17.0, and 15.8% for inclusions of fresh-weight *B. juncea* material equivalent to 0, 25, 50, 75 and 100 t ha^{-1} respectively, compared to the same inclusions of material into soil under sub-optimal moisture conditions. Generally the polynomial model shows an increase in efficacy for the optimal soil moisture range as biomass increases to 100 t ha^{-1}. However, efficacy does reduce marginally for the final 100 t ha^{-1} fresh biomass level. This might be due to variation in the model. Both optimal and sub-optimal soil moisture regressions are very strong. The flattening of efficacy between 50-100 t ha^{-1} fresh biomass could be evidence that biomass has a structural effect on soil, and that at fresh biomass equivalent to 100 t ha^{-1} incorporated into a pot of 200 mm depth, higher soil moisture conditions are required to retain VOC's in soil. In this instance efficacy may have dropped slightly in response to loss of biofumigant VOC's to headspace above pots (Sultani *et al*., 2007; Simpson *et al*.,2010; Gao & Trout, 2006). Again, this hypothesis needs further investigation.

3.8.3 Globodera pallida *eggs cyst^{-1} (glasshouse experiments 1 and 2)*

For the results discussed so far, nematode data has been expressed as percentage *G. pallida* viability. However, percentages do not take account of the range over which data points spread, which can sometimes lead to inaccurate conclusions if treatments influence the total number of nematodes in an analysis rather than, or in addition to their

viability. The number of *G. pallida* eggs^{-1} cyst was therefore investigated and presented in Figures 3.19, 3.24 and 3.25 as a means of quantifying both cyst uniformity for viability analysis (Twomey *et al*., 1995), and as a control analysis for any potential *B. juncea* biofumigation effect on eggs cyst^{-1}. No study is known to have investigated biofumigation treatment effects on PCN eggs cyst^{-1}. However, work presented by Ngala *et al*. (2014) reported a reduction in eggs g^{-1} soil following biofumigation treatment of *G. pallida* in the field. This reduction could be explained by a loss of eggs from cysts due to biofumigation, by natural decline, or by predation of *G. pallida* by antagonists (Sections 1.3.2 and 1.3.5).

Glasshouse experiment 1 found no effect of biofumigation or soil moisture on eggs cyst^{-1} (Figure 3.19 i-iii). However, CV's ranged between 32.8 and 38.4% between analyses, which represents a high level of variation. Berndston (1991) suggests that for the level of replication used in glasshouse experiment 1, only effect sizes in excess of 30-65% would be determinable for the analyses used in Figure 3.19. Therefore, no effect of biofumigation or soil moisture condition at *B. juncea* biofumigant incorporation on eggs cyst^{-1} were observed, but neither were those two factors shown to not be influential of *G. pallida* eggs cyst^{-1}. No effect of biofumigation or soil moisture on *G. pallida* eggs cyst was observed in glasshouse experiment 2 either (Figures 3.24 and 3.25 i-ii). However, *P* values were near the significance threshold for biomass and soil moisture factors. The 100 t ha^{-1} biomass treatment had *c.*23% fewer *G. pallida* eggs cyst^{-1} than other biomass treatments which, given the greater quantity of toxic material incorporated into soil, appears to be a feasible result. Biofumigant VOC's such as allyl-ITC are known to degrade proteins, as discussed in Section 1.3.6 (Kawakishi & Kaneko, 1985; Romanowski & Klenk, 2000). It is therefore plausible that eggs cyst^{-1} could have been reduced following biofumigation due to chemical degradation of egg shells leading to decomposition. Alternatively, *G. pallida* juveniles could have hatched prematurely in response to biofumigation; also discussed in Chapter 1, Section 1.4.5 (Ellenby, 1951). However, the CV's of different biomass treatments ranged between 40.9-58.1% which is very high, and higher than in glasshouse experiment 1. A more controlled experiment

investigating the effects of biofumigant biomass on eggs cyst^{-1} is required to come to any meaningful conclusion. Soil moisture analysis was similar. The 75% of field capacity and saturated treatments were *c*.26% lower than other soil moisture treatments, but CV's were very high at 41.1-61.9%. Clearly, grading cysts to improve the uniformity of numbers of eggs cyst^{-1} in glasshouse experiment 2 did not sufficiently counter the effects of reducing replications between experiments. It is possible that the 75% of field capacity and saturated treatments did enable a reduction in *G. pallida* eggs cyst^{-1} compared to the other moisture conditions. If so, immediate areas for further investigation should focus on the importance of soil pore air space in relation to soil water, and the effect of this on biofumigant VOC diffusion through soil and subsequent contact with PCN (Lembright, 1990).

3.8.4 *Conclusions*

In conclusion, the null hypothesis for this chapter that 'soil moisture level is not influential of *B. juncea* biofumigation efficacy against PCN' has been disproved and the chapter aim to 'determine whether soil moisture conditions at biofumigant incorporation are influential of biofumigation efficacy against PCN' can be considered answered. Soil moisture is an important factor in biofumigation of *G. pallida*, although, the mechanisms for enhanced biofumigation under certain soil moisture conditions remains obscure.

Manipulation of soil moisture has been shown to improve biofumigation efficacy against *G. pallida* in pots by up to 14.2% when analysing mean soil moisture treatment levels equivalent to 25, 50, 75, 100 % of field capacity and saturated soil. Approximately 50% of field capacity appears to be most appropriate for biofumigation of *G. pallida*, although results may indicate that for fully optimised biofumigation of *G. pallida*, soil moisture should be manipulated between 25-75% of field capacity, depending on the fresh weight of the biofumigant crop being incorporated into soil. In general, it would appear that 25% of field capacity would be appropriate for incorporation of *c.*25 t ha^{-1} fresh weight *B. juncea* crops, whilst for *c.*50 t ha^{-1} crops, 50% of field capacity would be preferable, and

for crops in excess of *c.*75 t ha^{-1}, 75% of field capacity conditions would be preferable. However, further study is required to support the observations of glasshouse experiment 1. Furthermore, whilst increasing biofumigant biomass from equivalent to 25-75 t ha^{-1} appeared to improve biofumigation efficacy against *G. pallida* in general, no improvement in biofumigation efficacy was observed for crops in excess of 75 t ha^{-1}. It is possible that at high inclusions of biomass into soil, off-gassing of biofumigant VOC's increases, which could reduce exposure of PCN to biofumigant VOC's. It would therefore appear that soil moisture may have an important role in retaining biofumigant VOC's in soil. However, the role of soil moisture in aiding or limiting VOC movement in soil is unknown. Two immediate areas for further research appear clear i) investigation of the concentration of VOC's in soil and in headspace above soil treated with different quantities of biofumigant material and different soil moisture conditions, and ii) investigation of the role of moisture films surrounding cysts as barriers to biofumigant VOC's reaching encysted eggs. Whilst the potential for moisture films to influence *G. pallida* exposure to biofumigant VOC's has not generally been discussed, it is clearly an area in need of investigation in light of the known hydrophobic qualities of biofumigant VOC's, and the clear evidence that moderately high soil moistures are beneficial for biofumigation efficacy. If moisture films surrounding cysts are found to limit VOC exposure to PCN, the timing of biofumigant incorporation could be dictated by soil moisture conditions to ensure both VOC retention in soil, and exposure to PCN. In other analyses, biofumigation effects on eggs $cyst^{-1}$ was quantified, but no evidence of a reduction in *G. pallida* eggs $cyst^{-1}$ was found. All project objectives, save for completing GSL analysis for glasshouse experiment 1, were achieved.

3.8.4.1 *Recommendations*

1 Practice biofumigation for *G. pallida* management under soil moisture conditions within a 25-75% of field capacity range.

2 For low biomass crops (*c.*25 t ha^{-1} fresh-weight) aim to incorporate into soils of 25-50% field capacity, for moderate biomass crops (*c.*50 t fresh-weight) ha^{-1} aim to

incorporate into soils of 50% field capacity, and for high biomass crops (*c.*75 t fresh-weight) aim to incorporate into soils of *c.*50-75% field capacity where practical.

3 Aim to produce *B. juncea* crops between 50 and 75 t ha^{-1} fresh-weight.

Investigation of commercially available tractor mounted maceration and incorporation implements and their set-up, for improved biofumigation of potato cyst nematodes

4.1 Introduction

Plant tissues are made up of parenchyma, collenchyma and sclerenchyma cells (Hopkins, 1999). Each cell type has unique structural properties which can influence the rigidity or plasticity of the plant tissues they form (Hopkins, 1999; Brett & Waldron, 1996). The strength of plant tissue is therefore dependent on the composition and arrangement of cells within tissues (Persson, 1987). To achieve effective tissue damage, which is critical to the release of volatile organic compounds (VOC's) from biofumigants, maceration techniques should apply a range of stresses to plant tissues, such as bending, shear, torsional, tensional and compressional stresses (Morra & Kirkegaard, 2002; Persson, 1987). Cutting techniques are generally less effective in causing tissue damage than blunt maceration techniques, because they predominantly rely on shear stress for maceration, which only inflicts localised cellular damage to tissues (Persson, 1987). Blunt techniques apply several stresses to plant tissues in unison, which generally causes more widespread damage (Persson, 1987). Field and *in-vitro* work has shown blunt 'mulching' techniques to increase isothiocyanate (ITC) release from biofumigant tissues by more than two orders of magnitude compared to cutting techniques (Morra & Kirkegaard, 2002; Matthiessen *et al*., 2004). The results suggest greater liberation of myrosinase and glucosinolates (GSL) from blunt macerated biofumigant plant tissues (Holst & Williamson, 2004). However, no study is known to have quantified the effect of maceration technique on biofumigation success against a soil borne pest.

In field work, the incorporation of macerated biofumigant tissues into soil has been found to influence soil concentrations of ITC's. Matthiesen *et al.* (2004) recorded a three-fold increase in ITC concentrations in soil where tissues were incorporated, rather than left

at the soil surface. In a separate glasshouse study, thorough mixing of biofumigant tissues into soil was shown to enhance biofumigation efficacy against the root knot nematode *Meloidogyne incognita* by between 57-80% compared to nematodes exposed to concentrated layers of biofumigant tissues in soil (Roubtsova *et al*., 2007). These studies would suggest that mixing of biofumigant material in soil is important in aiding the distribution of biofumigant VOC's through soil, which could increase nematode exposure to VOC's, subsequently influencing efficacy. However, no field work is known to have investigated incorporation technique as a method of improving biofumigation efficacy against soil borne pests directly. In other research, Woods & Haydock (2000) found that the incorporation depth of granular nematostats into field soil was important for management of potato cyst nematodes (PCN), and that nematostats could be worked too deeply into soil, leading to a dilution effect of the toxicant and a reduction in efficacy. This could also be true for the incorporation of biofumigant material. It is therefore important to investigate a range of primary tillage implements, diverse working depths and mechanisms of tilling, to identify whether the placement of biofumigant material in soil, and the structure of soil after biofumigant incorporation, are important for management of soilborne pests such as the PCN *Globodera rostochiensis* and *Globodera pallida*. This chapter therefore, investigates methods of macerating and incorporating the tissues of *Brassica juncea* biofumigant plants into soil for improved biofumigation of field populations of PCN.

4.1.1 *Chapter aim*

i) Determine if maceration and/or incorporation implement selection and set-up can be influential of *B. juncea* biofumigation efficacy against PCN.

4.1.2 *Chapter null hypothesis*

i) Neither maceration or incorporation implement selection or set-up will affect *B. juncea* biofumigation efficacy against PCN.

4.2 Materials and methods: field experiments 1 and 2 (Hungry Hill, Norfolk and Crossroads, Shropshire)

4.2.1 *Experiment objectives*

i) Quantify PCN cyst contents and viability following *B. juncea* biofumigation treatment achieved with several diverse maceration and incorporation implement combinations, and compare to appropriate controls.

ii) Quantify the biomass and GSL content of *B. juncea* biofumigant material used in experimental work.

iv) Quantify soil penetrometer resistance, temperature and moisture at *B. juncea* incorporation.

4.2.2 *Experimental design*

Field experiments 1 and 2 were carried out in tandem between 2014 and 2015 at two UK locations, one in Norfolk and another in Shropshire respectively. The two experiments were identical in design and only differed in a few supporting data assessment methodologies, which will be discussed later in Section 4.4. The shared design of the experiments consisted of two factors; the 'maceration implement' used to break down *B. juncea* material, of which there were two levels under investigation; a 3 m John Deere® 131 mower roll conditioner, and a 3 m reversible Kuhn® flail topper, and the 'incorporation implement' used to work *B. juncea* material into soil, of which there were three levels under investigation; a 3 m Imants® series 47 SX rotary spader with counter-rotating smear roll, a 3 m Krone® s-bladed rotary tiller or rotavator, and a Kverneland® reversible two-furrow mouldboard plough with no. 8 plough bodies. Maceration implements were selected, based upon their diverse mechanisms of conditioning plant material, and incorporation implements for their diverse working depths and mechanisms of incorporating material into soil by either inversion or rotary action. Figures 4.1-4.4 and Figures 4.5-4.7 show the maceration and incorporation implements used in this work.

Figure 4.1: Reversible Kuhn® flail topper maceration implement (A), and direction of implement travel (B).

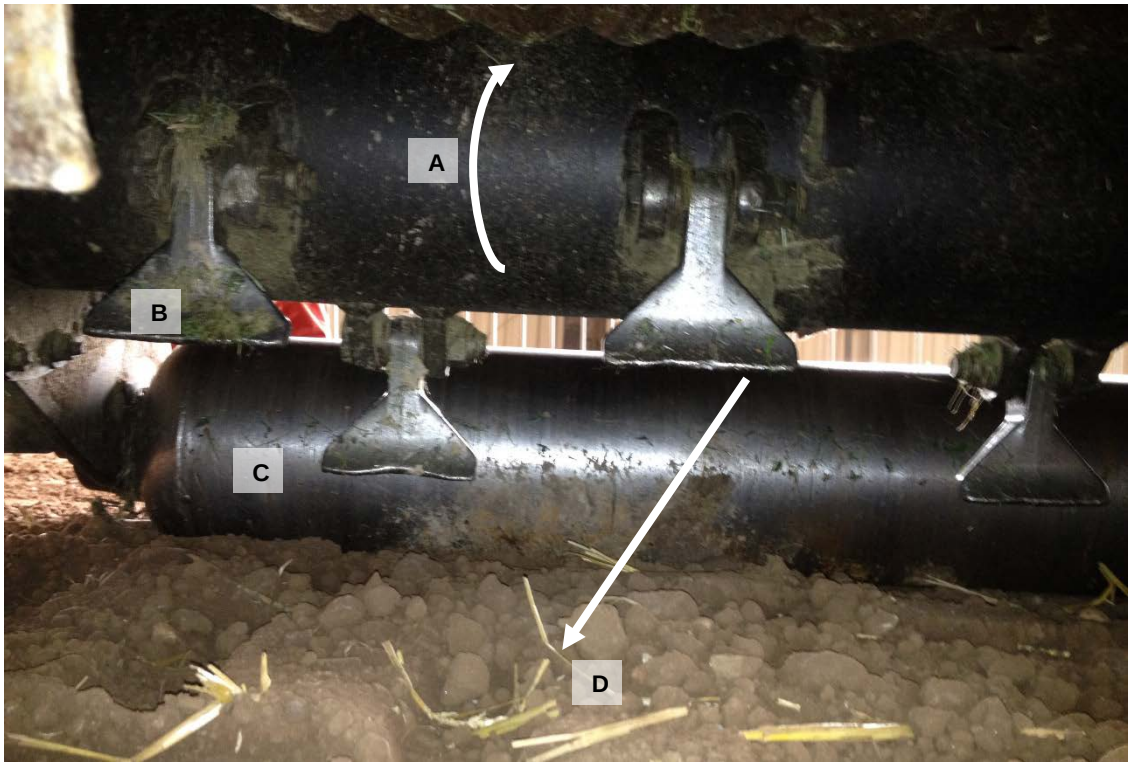


Figure 4.2: Rotor and direction of rotation (A), curved solid body v-tines (Kuhn 6061900) (B) and flail roll (C) within flail topper maceration implement. The direction of implement travel is also shown (D).

Figure 4.3: John Deere® 131 mower roll conditioner maceration implement (A), and direction of implement travel (B).

Figure 4.4: John Deere® 131 mower roll conditioner with mower discs (A) and their direction of rotation, and rolls and their direction of rotation (B). The direction of implement travel is also shown (C).

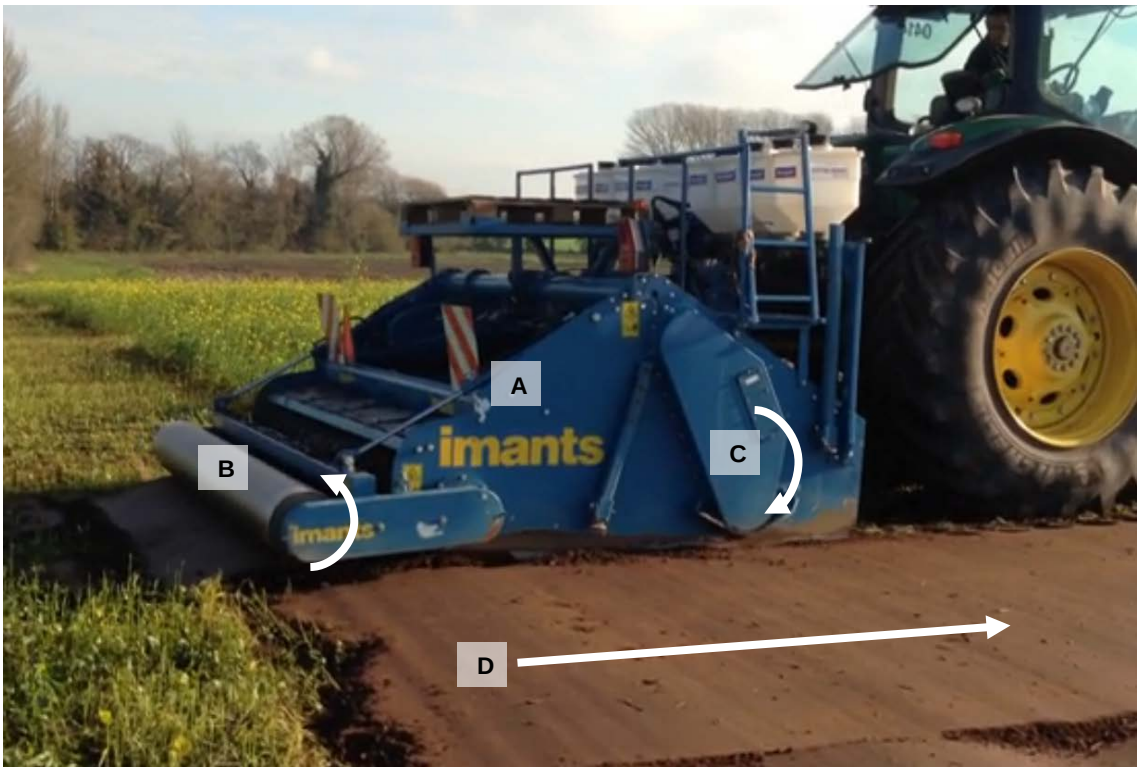


Figure 4.5: Imants® series 47 SX rotary spader incorporation implement (A) with counter-rotating smear roll (B). Also shows rotor position (C). The direction of implement travel is shown (D), and the direction of rotatin for the smear roll and spader rotor.

Figure 4.6: Krone® s-bladed rotary tiller or rotavator incorporation implement, showing the rotor position and direction of tine rotation (A), and the direction of implement travel (B).

Figure 4.7: Kverneland® reversible two-furrow mouldboard plough incorporation implement with no. 8 plough bodies (A).

Flail toppers, such as the 3 m reversible Kuhn® used in this work, are characterised by a rotating shaft, also known as a rotor, which is orientated at 90° to the direction of travel (Persson, 1987). The rotor often rotates in the opposite direction as the forward travel of the tractor so that material is cut in an upwards direction (Persson, 1987). Tines are attached to the rotor which cut standing plant material by shear stress, the bite or residue length of the plant material being determined by the forward speed of the tractor, implement rotor speed, and the distance of the tine from the rotor at the point of plant impact (Persson, 1987; Srivastava *et al*., 2006). In contrast, roll conditioners such as the John Deere® 131, cut standing plants near the base of the stem using a disc set-up, then feed the cut plants through a set of intermeshing crimping rolls which apply a range of blunt maceration forces to plant material (Persson, 1987). These two implement types were therefore found to be sufficiently contrasting in their mechanisms of plant conditioning, to be investigated. Furthermore, both implements could be front mounted to enable a rear mounted incorporation implement to be used for a single pass system.

Mouldboard ploughs such as the Kverneland® reversible two-furrow used in this work, are drawn implements which cut soil at depths of approximately 250 mm. The soil then moves from the share, which is the cutting component of the plough, to the mouldboard, which is the characteristic curved plate section of the plough. The mouldboard turns the soil so that it is completely inverted (Srivastava *et al*., 2006). Rotary tillers or spaders such as the 3 m Krone® or Imants® series 47 SX used in this work, are powered implements. They each have a powered rotor orientated at 90° to the direction of travel, which rotates in the same direction as the forward travel of the tractor. Tines are attached to the rotor which then work soil by shattering it and mixing it, rather than by inversion (Srivastava *et al*., 2006). The working depth of these implements is determined by the power of the tractor, physical strength of the implement, and the type of tines attached to the rotor. Most rotary tillers typically work to *c.*200 mm, whilst spaders often work to *c.*400 mm in depth (Srivastava *et al*., 2006). The three implements therefore offered contrasting working depths, methods of biofumigant residue placement, and tilth quality with which to assess the effect of incorporation technique on biofumigation of PCN.

The effect of plant maceration and incorporation implement treatment combinations on biofumigation efficacy against PCN was compared to a non-*B. juncea* treated control treatment, and a partial biofumigation control where *B. juncea* plants were grown but not macerated or incorporated into soil. The partial biofumigation control enabled the full effects of different maceration and incorporation implement combinations on biofumigation efficacy to be calculated, omitting biofumigation efficacy during plant growth. As illustrated using Table 4.1 and Figure 4.8, treatments were arranged in a randomised block design and replicated six times each providing a total of 48 experimental units (field plots) in each experiment. The shared experimental design was replicated sufficiently to enable treatment differences of 5-20% in PCN egg viability to be determined for background coefficient of variation (CV) values in egg viability of between 2-8% between replicates of like treatments (Berndston, 1991). The design had 35 residual degrees of freedom.

Table 4.1: Treatment codes for field experiments 1 and 2 showing levels for maceration and incorporation factors. Font colour indicates maceration implement and fill colour indicates incorporation implement.

Treatment code	Biofumigant	Factor 1: Maceration implement	Factor 2: Incorporation implement
R/s	Grown, macerated and then incorporated	Roll conditioner	Spader
F/s	Grown, macerated and then incorporated	Flail topper	Spader
R/r	Grown, macerated and then incorporated	Roll conditioner	Rotivator
F/r	Grown, macerated and then incorporated	Flail topper	Rotivator
R/p	Grown, macerated and then incorporated	Roll conditioner	Plough
F/p	Grown, macerated and then incorporated	Flail topper	Plough
P BIO	Partial biofumigant control (biofumigant grown but left standing)		
UNT	Untreated control (no biofumigant grown)		

Figure 4.8: Field experiments 1-2 design showing the allocation of treatments to potato cyst nematode infested plots. Blocks are represented using Roman numerals (I-VI).

4.2.3 *Experimental set-up*

4.2.3.1 *Experimental sites*

Field sites in Norfolk and Shropshire were selected for initial assessment of PCN for experimental work. From initial inspection, two field sites were carried forward, one for each county, based upon the population density and uniformity of PCN in fields. High PCN densities of high uniformity were most desirable. A site near Cromer known as 'Hungry Hill' (UK ordinance survey grid reference: TG 24572 39281, Norfolk, UK) was found to have PCN densities of 10–200 eggs g^{-1} soil across the site, from which an area of 1 ha typically ranging between 50–150 viable eggs g^{-1} soil was selected for extensive sampling. A site near Shrewsbury known as 'Crossroads' (UK ordinance survey grid reference: SJ 62604 17602, Shropshire, UK) was also selected. This site was found to have PCN densities ranging between 10-500 eggs g^{-1} soil from which a 1 ha block ranging between 120-500 eggs g^{-1} soil was selected. Extracted PCN were further quantified as the species *Globodera pallida* in both instances using real time polymerase chain reaction (PCR) (Appendix 8.1.4). Table 4.2 shows the duration of the Hungry Hill and Crossroads experiments between PCN sample collection stages.

Table 4.2: Table illustrating the duration of field experiments 1 and 2 (Hungry Hill, Norfolk and Crossroads, Shropshire respectively).

	Year: 2014-2015						
Experimental stage	Jul	Aug	Sept	Oct	Nov	Dec	Jan
i: Soil sampling for potato cyst nematodes							
ii: *Brassica juncea* growing term							

4.2.3.2 *Experiment construction and maintenance*

Brassica juncea was direct drilled at the Hungry Hill site in the first week of August 2014 using a Vaderstad® Rapid A 600S seed drill, and canes erected to mark experimental

dimensions. The site was drilled with *B. juncea* seed over two separate days, two days apart, due to a calibration error by the drill operator, and the need to collect extra seed to establish the experimental area. In total, approximately 10 kg ha^{-1} seed was established, with the over drilled area receiving a seed-rate of approximately 12 kg ha^{-1}, and the remainder of the experiment receiving an approximate 8 kg ha^{-1} seed-rate. Plant counts were recorded approximately 6 weeks after drilling to take account of this variation. No difference in plant counts were observed between blocks or treatments at 6 weeks post drilling, or between treatments at incorporation. However, differences in plant counts were observable between blocks at incorporation. The results are shown in Appendices 8.2.1 – 8.2.2

Plot dimensions were 9 by 9 m, with inter-block and plot buffers of 5 m width to enable machinery movement at incorporation without damage to neighbouring plots. However approximately 1 ha^{-1} of *B. juncea* was drilled and then inter-block and plot traffic lanes were established using a flail topper only on the day of incorporation. This methodology ensured *B. juncea* material in plots was as representative of a field grown *B. juncea* biofumigant crop as possible on the day of incorporation by helping to prevent easy entry of pests such as pigeons, rabbits or deer into the experiment which might damage individual plots. *Brassica juncea* plants growing in untreated control plots were desiccated from the whole hectare crop at second true leaf stage using Diquat (as REGLONE®, Syngenta) contact herbicide two weeks after planting. Figure 4.9 shows a Diquat treated control plot surrounded by standing mustard before establishment of inter-block and plot traffic lanes. Nitrogen (N) fertiliser (as NITRAM® 34.5% N, CF Fertilisers) was applied to each site using a 12 m Kuhn fertiliser applicator at a rate equivalent to 40 kg N ha^{-1} to enhance biomass and GSL production (Agerbirk & Olsen, 2012). The Crossroads site was established using a 3 m modified pneumatic plot drill (believed to be constructed from Lemken® and Accord® parts) with shoe type coulters and press wheels. Figure 4.10 shows the Hungry Hill experimental site immediately after traffic lanes had been established on the day of incorporation. The Hungry Hill experiment

received two 25 mm irrigation applications using a rain gun in late August and early September due to dry weather conditions. The Crossroads site did not receive any irrigation. All other methodologies remained consitent between the two experiments. The Crossroads experiment was, however, noticeably infested with cabbage root fly larvae (*Delia radicum*) which was subsequently scored approximately seven weeks after drilling (see Appendix 8.2.3). No difference in cabbage root fly damage was observed between treatments, but blocks were shown to have variable damage symptoms.

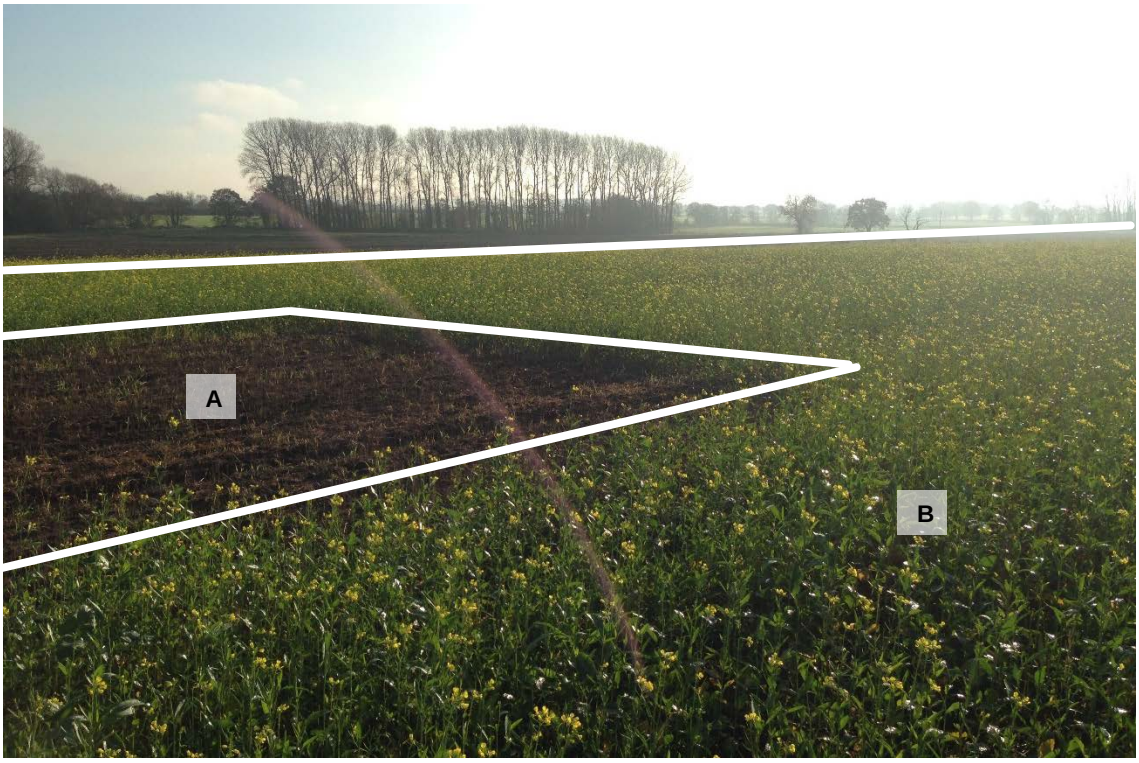


Figure 4.9: Diquat treated control plot (A) in standing mustard (B) prior to inter-block and plot traffic lane establishment.

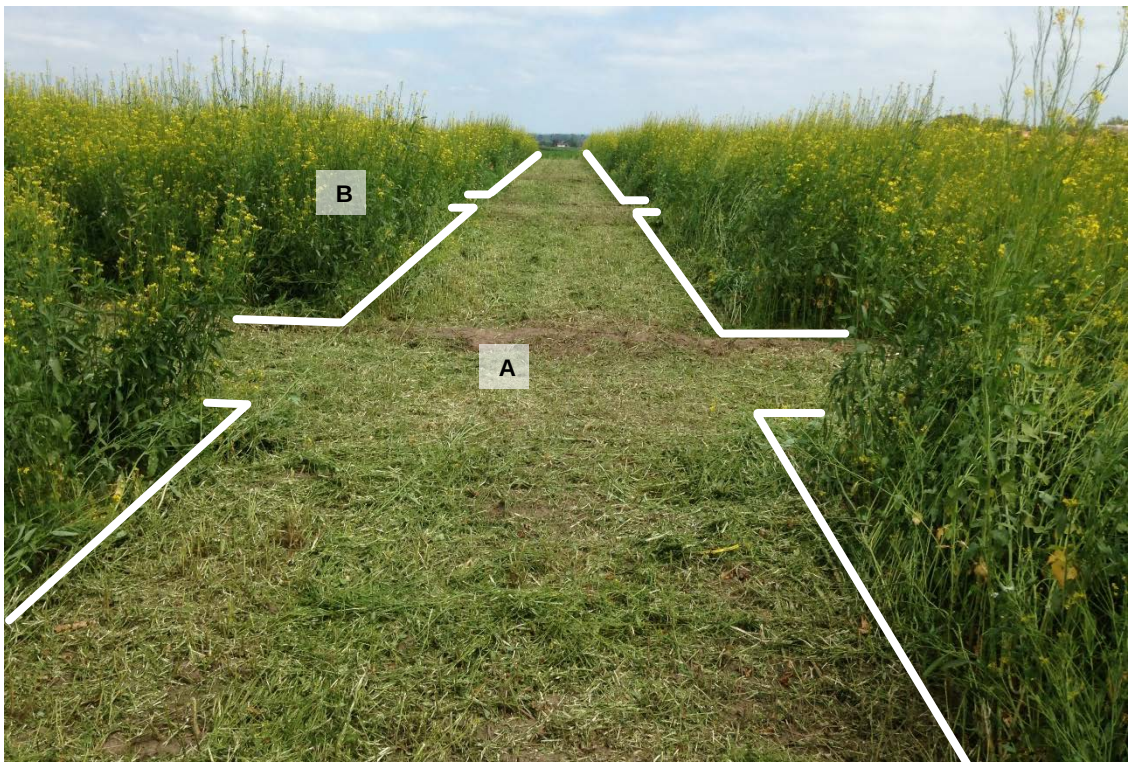


Figure 4.10: Traffic lanes (A) established in *B. juncea* crop on the day of incorporation to generate individual plots (B).

4.2.4 Brassica juncea *biofumigant incorporation procedure*

Once inter-block and plot traffic lanes had been established, incorporation could be undertaken. A John Deere® 6810 was used to mount the maceration implements, the roll conditioner being front mounted and the flail rear mounted. A John Deere® 7280R was required to mount and power the spading implement due to its size, whilst a John Deere 6400 was used to mount the plough, and then the rotavator. The John Deere® 6810 was used to macerate an entire plot which required three passes, whereby on the third pass, either John Deere® 7280R or 6400 tractors could begin their first pass for respective incorporation implements. This methodology enabled an expedient incorporation (*c.*2 min maceration to incorporation) whilst also limiting damage to neighbouring plots. Forward speed during implement operations was undertaken at *c*.3 km h^{-1}. Figure 4.11 shows a biofumigation treated plot as 'A', a partial biofumigation plot 'B' and an untreated plot 'C' once all plots receiving biofumigation had been treated.

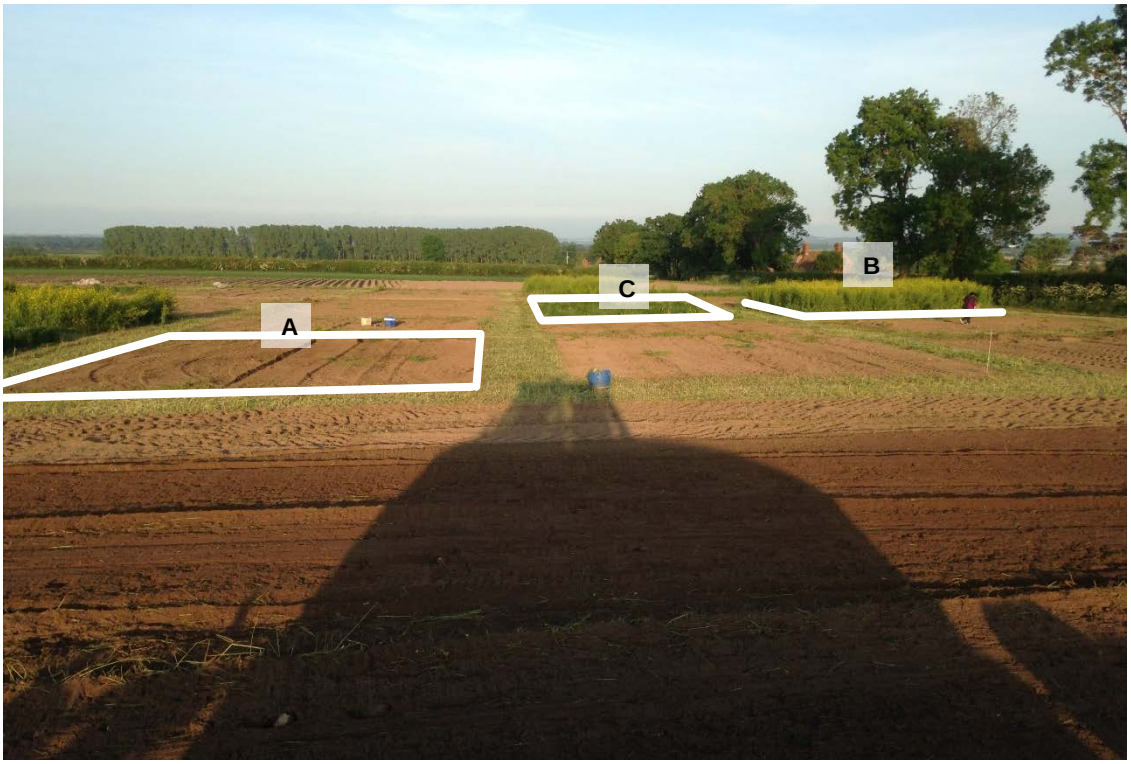


Figure 4.11: *Brassica juncea* biofumigant treated (A), partial biofumigation treated (B) and untreated plots (C).

4.3 Materials and methods: field experiment 3 (Roys Corner, Shropshire)

4.3.1 *Experiment objectives*

i) Measure PCN egg viability and eggs cyst^{-1} following *B. juncea* biofumigation with several diverse flail tine and shear-plate maceration implement treatments compared to appropriate untreated controls.

ii) Quantify the biomass and GSL content of *B. juncea* biofumigant material.

iv) Develop and apply a methodology for collecting *B. juncea* biofumigant volatile organic compounds from soil for quantification.

4.3.2 *Experimental design*

Field experiment 3 was carried out between September 2015 and June 2016 near Shrewsbury in Shropshire. The experiment, which was the second Shropshire experiment, was structurally identical to the previous 2 experiments, but varied in the compliment of treatments under investigation. Field experiment 3 focussed on

maceration implement set-up. The design had two factors; the type of 'flail tine' fitted to a 5.4 m wide Grimme® KS5400 haulm/flail topper, and the 'shear-plate' position on a KS5400 topper. The tine factor consisted of three levels; pig-tail tine (Grimme® KS5400 rotor shaft item number 089.00399), knife tine (rotor shaft item number 089.00409) and a combination of the two tines. The shear-plate factor had two levels, termed open and closed shear-plate (distance between tine and shear-plate: *c.*100 and 10 mm respectively).The two types of tine were selected for their differences in area of cutting face, which in the pig-tail type tine was approximately 7-8 times greater than that of the knife tine. Similarly, the diversity of shear-plate set-up was of interest in offering a counter-shear setting for the tines, and an almost absent counter-shear setting. All *B. juncea* treatments were incorporated using a Grimme® GF600 rotavator with two bed loosening tines fitted to increase working depth (Grimme® GF600 loosening tine item number 200.71602). Figures 4.12-4.18 show the haulm topper and rotavator mounting, which in this instance enabled a single pass operation, bed-loosening tines, and then flail tines and combinations of tines, and shear-plate positioning.

Figure 4.12: Deutz-Fahr® 9 series tractor with front mounted Grimme® KS5400 haulm topper (A), and rear mounted Grimme® GS600 rotavator (B).

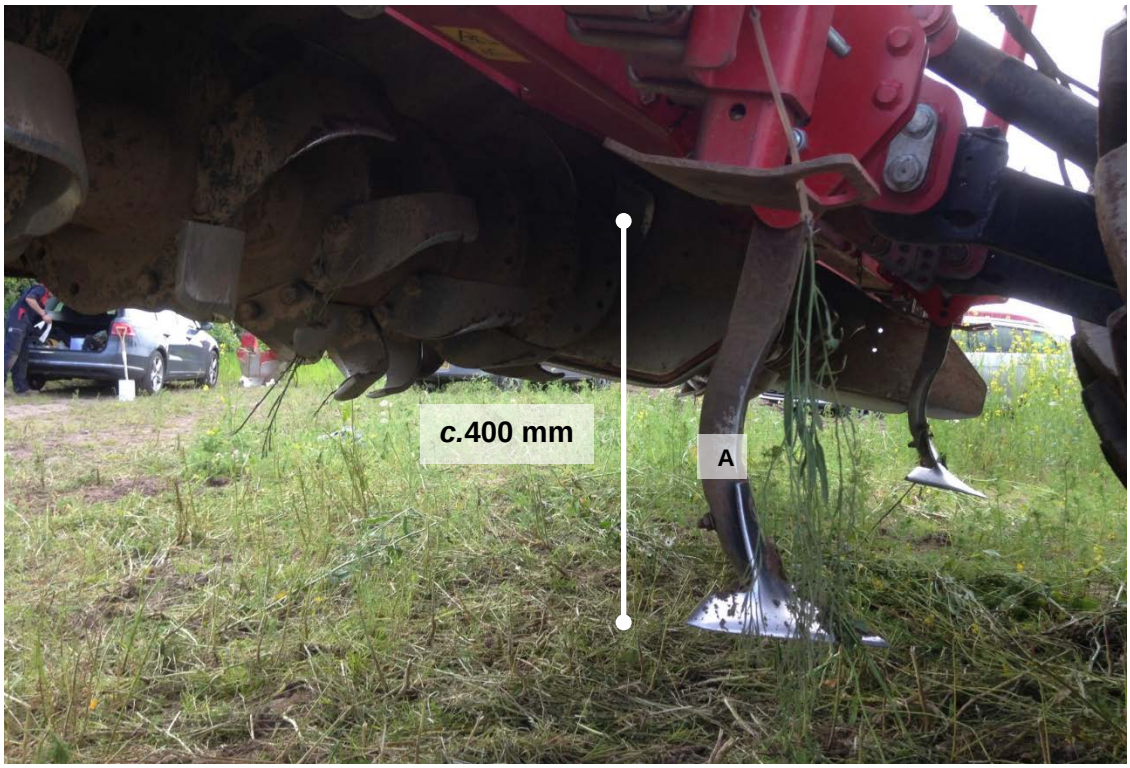


Figure 4.13: Grimme® GS600 rotavator with bed-loosening tines (A). The scale relates approximately to the length of the bed-loosening tines.

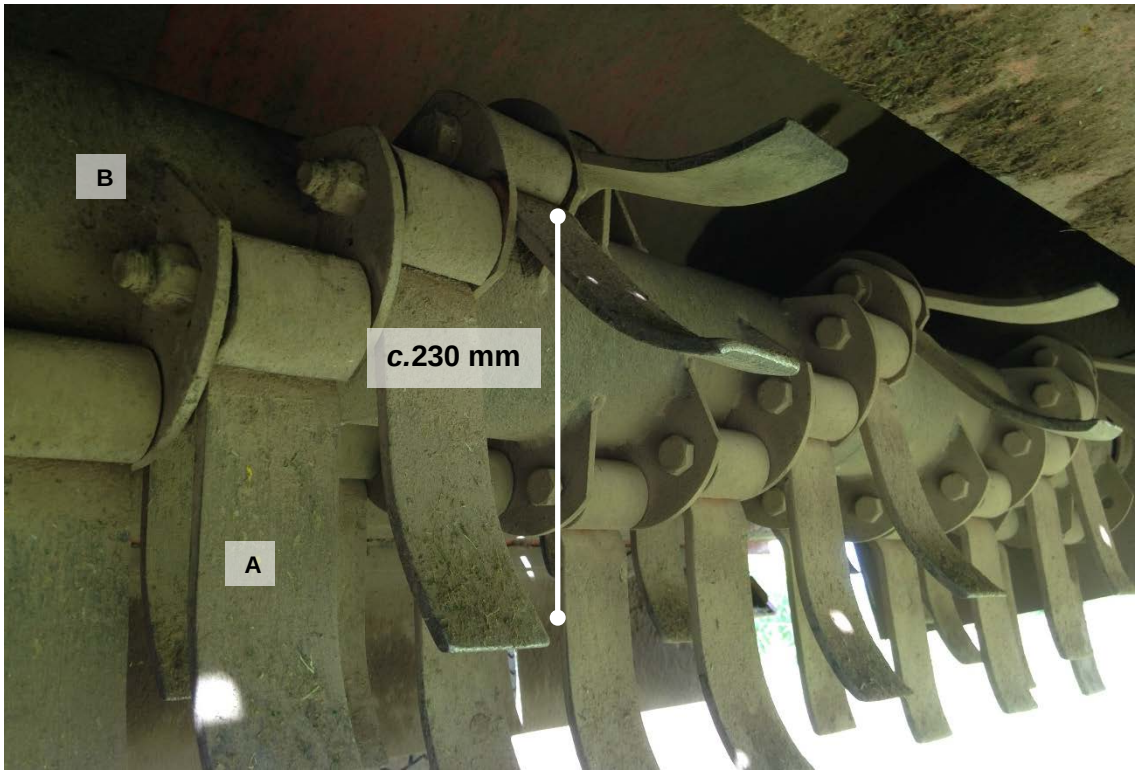


Figure 4.14: Pig-tail tines (A) arranged on a Grimme® KS5400 haulm topper rotor (B). The scale relates approximately to the length of a pig-tail tine.

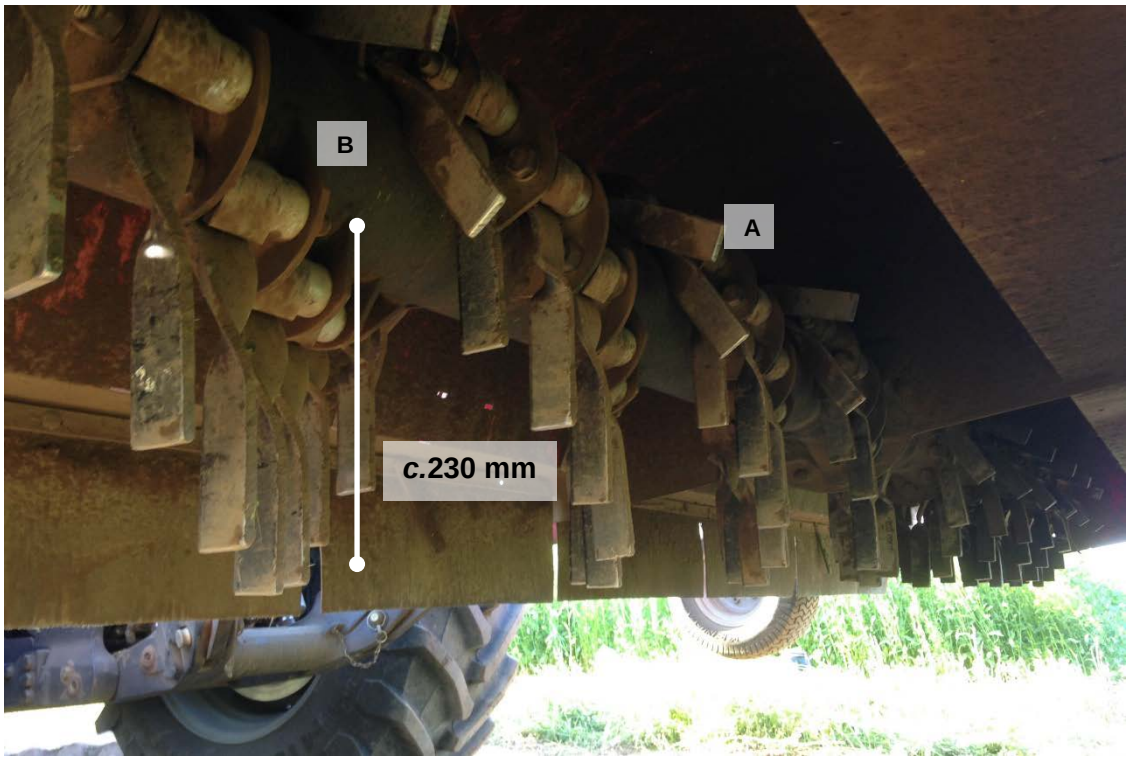


Figure 4.15: Knife tines (A) arranged on a Grimme® KS5400 haulm topper rotor (B). The scale relates approximately to the length of a knife tine.

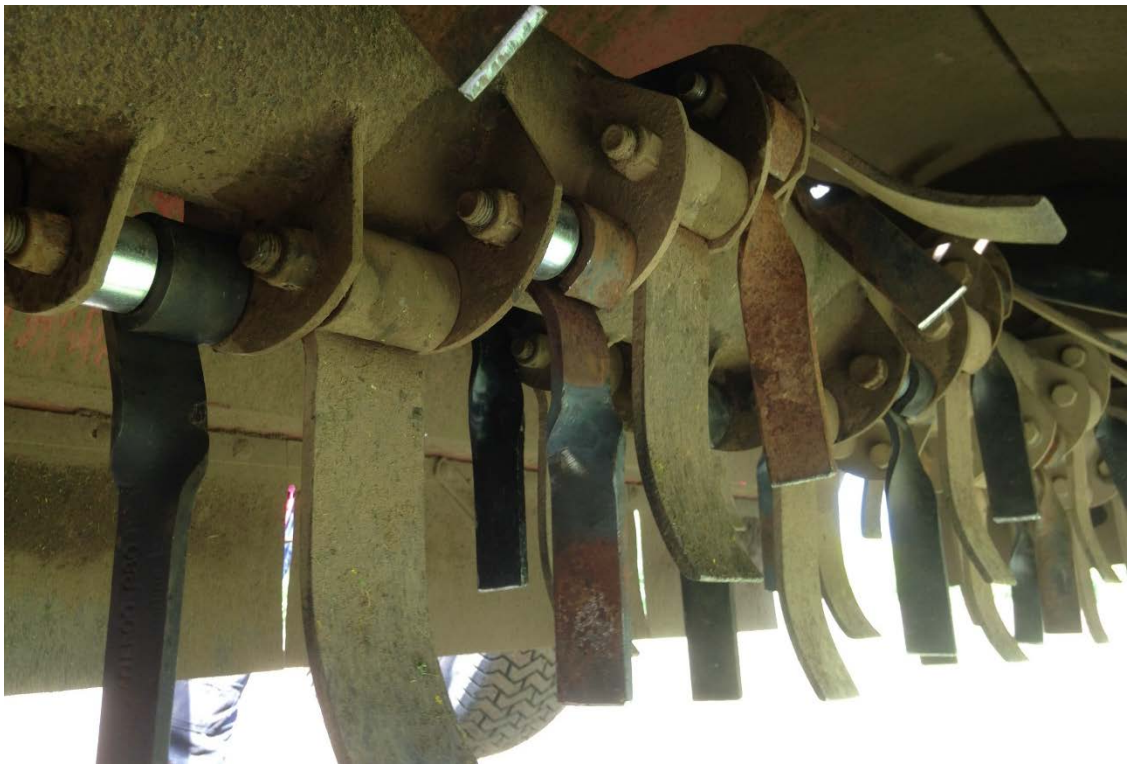


Figure 4.16: Pig-tail tine/knife tine combination arranged on a Grimme® KS5400 haulm topper rotor.

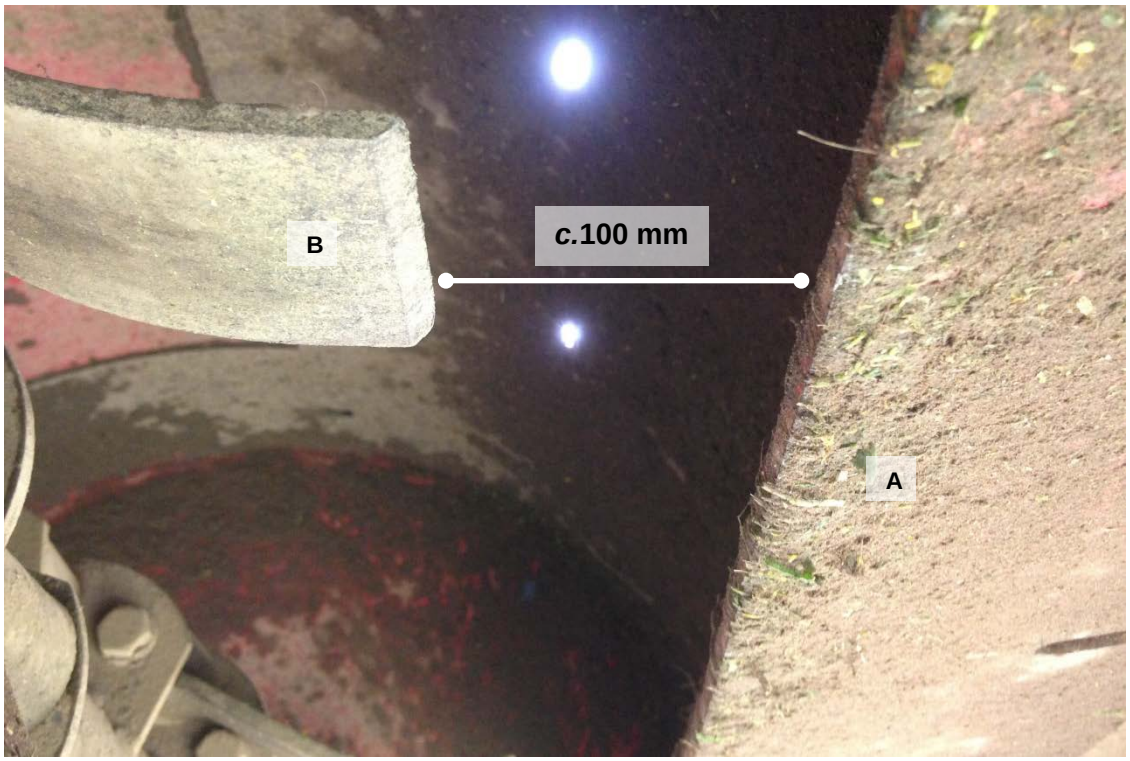


Figure 4.17: Open shear-plate position (A) on a Grimme® KS5400 haulm topper with pig-tail tines (B). The scale shows the approximate distance between tine and shear-plate.

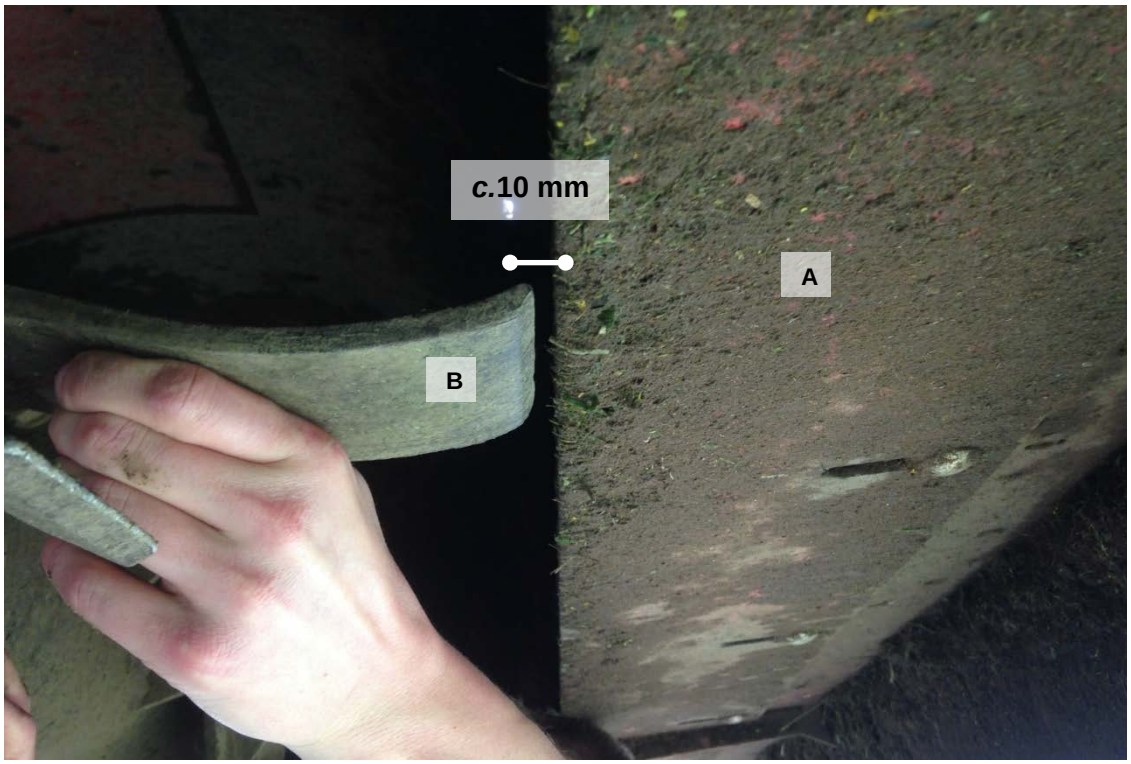


Figure 4.18: Closed shear-plate position (A) on a Grimme® KS5400 haulm topper with pig-tail tines (B). The scale shows the approximate distance between tine and shear-plate.

The effect of maceration implement set-up on biofumigation efficacy against PCN was compared to a non-*B. juncea,* untreated control treatment, and a 'partial biofumigation' control as in the previous field experiments. Maceration implement tines and shear-plate setting were manipulated to produce different quality biofumigant residues for incorporation into soil. The partial biofumigation treatment consisted of *B. juncea* plants which were grown but not macerated or incorporated into soil. As illustrated using Table 4.3 and Figure 4.19, treatments were arranged in a randomised block design and replicated six times each providing a total of 48 experimental units (field plots), the same as the previous two field experiments.

Table 4.3: Treatment codes for field experiment 3 showing levels for maceration implement, tine and shear-plate factors. Font colour is an indicator of shear-plate setting and fill colour is an indicator of tine selection.

Treatment code	Biofumigant	Factor 1: Shear plate setting	Factor 2: Tines
P/O	Grown, macerated and then incorporated	Open	Pig-tail
P/C	Grown, macerated and then incorporated	Closed	Pig-tail
K/O	Grown, macerated and then incorporated	Open	Knife
K/C	Grown, macerated and then incorporated	Closed	Knife
PK/O	Grown, macerated and then incorporated	Open	Pig-tail and knife combination
PK/C	Grown, macerated and then incorporated	Closed	Pig-tail and knife combination
P BIO	Partial biofumigant control (biofumigant grown but left standing)		
UNT	Untreated control (no biofumigant grown)		

Figure 4.19: Field experiment 3 design showing the allocation of treatments to potato cyst nematode infested plots. Blocks are represented using Roman numerals (I - VI).

4.3.3 *Experimental set-up*

4.3.3.1 *Experimental sites*

The second Shropshire site was sampled for initial assessment of PCN for experimental work, and found suitable to be carried forward given time constraints. The site known as 'Roys Corner' (UK ordinance survey grid reference: SJ 62018 17108, Shropshire, UK) was found to have PCN densities of 2–50 eggs g^{-1} soil across the site, from which an area of 1 ha typically ranging between 5–30 viable eggs g^{-1} soil was selected for extensive sampling. Extracted PCN were further quantified as the species *Globodera pallida* using PCR (Appendix 8.1.4). Table 4.4 shows the duration of field experiment 3 between PCN sample collection stages.

Table 4.4: Table illustrating the duration of field experiment 3 (Roys Corner, Shropshire).

	Year: 2015-16									
Experimental stage	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
i: Soil sampling for potato cyst nematodes										
ii: *Brassica juncea* growing term										

4.3.3.2 *Experiment construction and maintenance*

Brassica juncea was direct drilled at the third field experiment site in the first week of September 2015 using the modified Lemken®/Accord® plot drill, and canes erected to mark experimental dimensions. The site was drilled with *B. juncea* at a 10 kg ha^{-1} seed-rate. Plot dimensions were consistent with the first two experiments at 9 by 9 m, with inter-block and plot buffers of 5 m width to enable machinery movement at incorporation without damage to neighbouring plots. A full 1 ha^{-1} block of *B. juncea* was drilled and inter-block and plot traffic lanes established using the same methodologies as for the previous field experiments. *Brassica juncea* plants growing in untreated control plots were also desiccated using Diquat (as REGLONE®, Syngenta) contact herbicide two weeks after planting, in keeping with the previous field experiments, and fertiliser applied

using the same methodologies as for the previous experiments. The fertiliser rate was increased to equivalent of 100 kg N ha^{-1} in an attempt to force rapid growth due to the lateness of drilling. This was only partially successful and so blocks were fleeced to ensure the plants survived from winter 2015 to spring 2016 when they were incorporated. A double layer of 18 g m^2 fleece (Brinkman UK Ltd.) was applied to the site in December 2015, and removed in March 2016.

4.3.4 Brassica juncea *biofumigant incorporation procedure*

Once inter-block and plot traffic lanes had been established, incorporation could be undertaken. A series 9 Deutz-Fahr® tractor was used to mount both the Grimme® KS5400 maceration implement and Grimme®GS600 rotavator. This methodology enabled an expedient incorporation. Care had to be taken to avoid damaging or trafficking partial biofumigation or untreated plots, particularly in light of the large size and wide working width of the implements. Forward speed during implement operations was undertaken at *c*.3 km h^{-1}. Plants were incorporated in mid-April 2016.

4.3.5 *Development of a methodology for collecting* Brassica juncea *biofumigant volatile organic compounds from soil*

A secondary line of experimentation was carried out during the experiment construction and maintenance period, to develop a method of capturing biofumigant VOC's from plots at field experiment 3, immediately after incorporation. A thermal desorption gas chromatography-mass spectrometry (GC-MS) method was available at Harper Adams University (HAU) for the analysis of Tenax-TA entrained *B. juncea* VOC's. Additionally, Markes International were known to produce stainless steel and aluminium sheaths for Tenax-TA tubes which could be introduced to soil for VOC collection. These 'VOC-MOLE's' had been notably shown to effective for enabling biofumigant VOC's to be captured from pot soil in work carried out by Papadopoulos & Alderson (2007). However, modified steel tubes with 28, rather than the commercially available 14 hole MOLEs, were shown most effective in capturing ITC's. A commercially available MOLE was

therefore bought and a mild-steel replica manufactured, in addition to a replica with the addition of 16 further smaller holes (See Figures 4.20-4.22). A small pilot field experiment was then undertaken at HAU which evaluated the three 'MOLEs'. A *B. juncea* biofumigant crop of approximately 30 t ha^{-1} fresh-weight was flail-topped and the macerated tissues rotivated into soil using the Krone® rotavator from the Hungry Hill and Crossroads experiments. Each MOLE was then immediately introduced to the freshly cultivated soil and Tenax-TA tubes collected and replaced from each tube, whilst *in-situ*, at 6, 12, 24, 48 and 72 h after *B. juncea* incorporation. Two replicates of each MOLE treatment for each time point were taken. There were two incorporation events to generate the two replicates, four days apart. The custom made MOLE with 32 holes along its primary body section was found to be most effective for capturing *B. juncea* biofumigant VOC's in the pilot study and so was manufactured and used in the third field experiment for VOC capture. All final modified MOLEs were manufactured from stainless steel (Figure 4.23). The dimensions of each tube can be found in Appendix 8.3.1, and the results from this method development be found in Appendix 8.2.5.

Figure 4.20: Commercially available stainless steel and aluminium 16 hole Markes International VOC-MOLE™.

Figure 4.21: Harper Adams University mild steel 16 hole replica MOLE.

Figure 4.22: Harper Adams University mild steel 32 hole custom MOLE.

Figure 4.23: Harper Adams University stainless steel 32 hole custom MOLE fitted with a Tenax-TA tube.

4.4 Assessments

4.4.1 *Potato cyst nematode sampling and quantification*

Globodera pallida eggs g^{-1} soil, egg viability and eggs $cyst^{-1}$ were quantified for experiments as described in Section 2.3.2 using Equations 2.1 to 2.3.

4.4.2 *Quantifying* Brassica juncea *biofumigant biomass and glucosinolate content in the field*

Prior to *B. juncea* maceration, three 0.33 by 0.33 m areas of crop were randomly selected for each plot of each experiment, and plants counted to determine plants m^2. A total of ten plants were taken from the final area and weighed both fresh and after forced air oven drying at 105°C for *c*.48-72 h to generate a fresh and dry mean plant weight. This weight was used in conjunction with plant counts to project dry and fresh-weight biomass in t ha^{-1} as illustrated in Equation (4.1). This methodology is similar to that used by Ngala *et al.* (2014). A further three plants were also collected from each plot and stored for later

GSL assessment as described in Section 2.4.2. From these samples, one sample was analysed per block of each field experiment as a reference measurement. Samples from 'partial biofumigation' plots were processed for GSL analysis.

Equation 4.1

$$\text{Biomass t ha}^{-1} = \text{Weight in g of a single biofumigant plant (fresh or dry)} \times \frac{\text{Number of plants counted (0.33 * 0.33 m)} \times 40{,}000}{1{,}000{,}000}$$

4.4.3 *Quantification of soil conditions*

Temperature and moisture conditions were determined at incorporation for the Hungry Hill and Crossroads experiments as detailed in Appendices 8.1.6 – 8.1.7 using the methodologies described in Section 2.3.4 and field capacity curves shown in Appendix 8.1.5. Soil texture, pH and organic-matter (OM) were also determined for each site from soil samples taken prior to *B. juncea* planting (Appendix 8.1.1 – 8.1.3).

4.4.4 *Soil penetration resistance*

Soil penetrometer resistance was measured at the Crossroads site on each replicated plot to determine the working depth of implements and general soil structural properties following treatment. A MEXE soil assessment cone penetrometer was used; a model A2451 fitted with a 12.8 mm diameter 30° cone. Penetrometer resistance data were collected at three points per plot, between depths of 0-400 mm, at depth increments of 100 mm. Penetration resistance was not recorded at any other site.

4.4.5 *Quantification of* Brassica juncea *biofumigant bite/section length*

Macerated *B. juncea* tissue samples (*c.*100 g fresh-weight) were collected from each plot of the Roys Corner site. The samples were collected immediately after maceration of *B. juncea* plants but before incorporation of the tissues into soil. The samples were transported to the laboratory and ten tissue sections selected randomly from each sample bag to be measured lengthways. The mean tissue section length for each

treatment was then calculated. The effect of maceration implement on the bruising of *B. juncea* tissues was not quantified. Figure 4.24 shows the approximate range in *B. juncea* tissue bite/section length found in the field at the Roys Corner experiment.

Figure 4.24: Typical range in *B. juncea* tissue bite/section length observed after maceration at field experiment 3 (Roys Corner, Shropshire). The scale shows approximately, the average length of biofumigant residue post maceration.

4.4.6 *Quantification of volatile organic compound release into soil from* Brassica juncea *biofumigant material after incorporation*

Twenty-four custom made 32 hole MOLEs complete with Tenax-TA tubes were placed at the centre of each plot for blocks II, III, VI at the Roys Corner site. The tubes were introduced to soil immediately after *B. juncea* incorporation and left *in-situ* for 72 h to enable passive entrainment of allyl-isothiocyanate (AITC) and other VOC's. Tenax-TA tubes were then taken to the laboratory and samples analysed using the thermal desorption GC-MS methodology previously described in Section 2.4.3. Figures 4.25-4.27 show custom MOLEs in *B. juncea* treated, partial biofumigation treated and untreated plots.

Figure 4.25: Custom made 32 hole MOLE placed in a biofumigation treated plot at field experiment 3 (Roys Corner, Shropshire).

Figure 4.26: Custom made 32 hole MOLE placed in a partial biofumigation treated plot at field experiment 3 (Roys Corner, Shropshire).

Figure 4.27: Custom made 32 hole MOLE placed in an untreated plot at field experiment 3 (Roys Corner, Shropshire).

4.5 Data analysis

Data were analysed by general and repeated measures analysis of variance (ANOVA), by T-test, and where data sets were combined, by probability calculation using a cumulative upper F-variance ratio test. All data was analysed using GenStat® 16th Edn. (VSN International). Treatment effects for ANOVA analysed data were compared using Tukey's test at 95% confidence or Fischer's protected least significant deifference (LSD) at 99% confidence. Figures were generated using Microsoft® Excel 2016.

4.6 Results: field experiment 1 (Hungry Hill, Norfolk)

4.6.1 Brassica juncea *biofumigant biomass and glucosinolate content*

Mean plant dry-matter at incorporation was 13.6% when analysed by block, and 13.5% when analysed by treatment. The standard errors of the mean (SEM) for dry-matter were 0.43% and 0.31% respectively. No difference was found in the quantity of fresh or dry-weight *B. juncea* biomass between treatments (P = 0.795 and P = 0.470 respectively)

(Figure 4.28 ii). However, fresh-weight *B. juncea* biomass did vary between blocks (*P* = 0.027). Multiple comparisons using Tukeys multiple range test, Fischer's protected LSD and the Bonferroni technique at 95% confidence were unable to indicate where differences occurred (Figure 4.28 i). Dry-weight *B. juncea* biomass did not vary between blocks (*P* = 0.186) (Figure 4.28 i). Fresh-weight biomass ranged between equivalent to 20-31 t ha^{-1} between blocks, and 22-27 t ha^{-1} between treatments. Dry-weight biomass ranged between equivalent to 3-4 t ha^{-1} between blocks and between treatments. 2-propenyl GSL (sinigrin) content of combined leaf and stem tissues varied between 2.5 and 3.7 µmol g^{-1} dry tissue between blocks I-VI. Mean 2-propenyl GSL content was 3.0 µmol g^{-1} dry tissue and the SEM 0.15 µmol g^{-1} dry tissue (see Appendix 8.2.4). 2(S)-hydroxy-3-butenyl (epiprogoitrin) and 2(R)-2-hydroxy-3-butenyl (progoitrin) were also observed in *B. juncea* tissues at *c.*0.7 and 0.7 µmol g^{-1} dry tissue respectively.

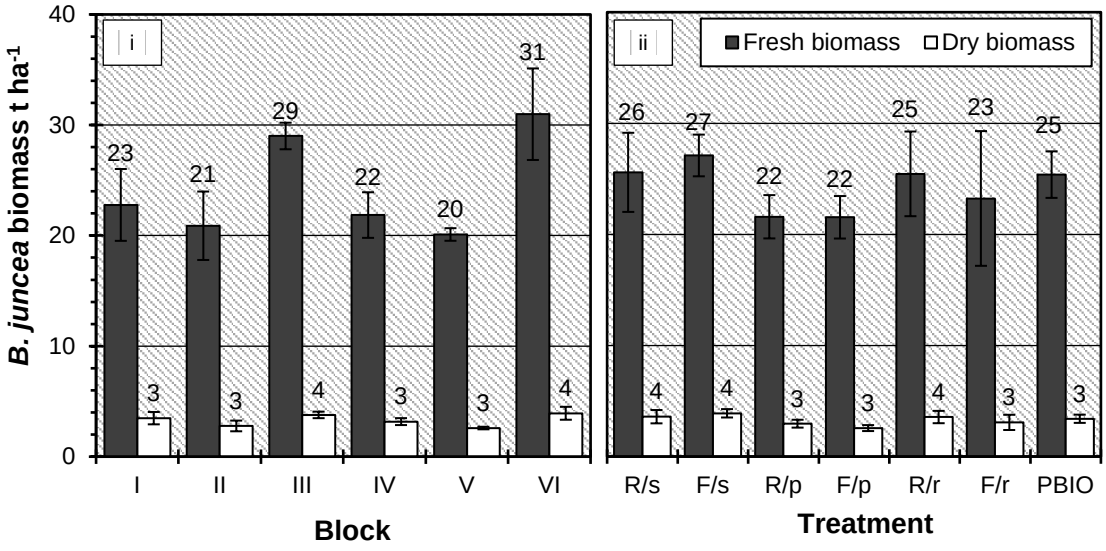


Figure 4.28: *Brassica juncea* biofumigant biomass t ha^{-1} for field experiment 1 (Hungry Hill, Norfolk), analysed by block (i) and treatment (ii) factors using one-way ANOVA. Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

4.6.2 Globodera pallida *viability at* P_i *and* P_s

The Hungry Hill site had a mean *G. pallida* population of 20 total eggs g^{-1} soil before drilling *B. juncea* (SEM of 1.5 eggs g^{-1} soil). No difference was observed in *G. pallida*

viability between treatments when assessed prior to *B. juncea* drilling (P = 0.996); hereafter termed the initial (P_i) sample date (Figure 4.29 i). The Hungry Hill population was 93.6% viable at P_i overall. Differences were observed in *G. pallida* viability between treatments at the population secondary (P_s) sample date, eight-weeks after biofumigation (P < 0.001) (Figure 4.29 ii). Partial biofumigation was ineffective in reducing *G. pallida* viability, but biofumigation treatments were found to reduce *G. pallida* viability by *c.*34% overall when compared to the untreated control. No difference in biofumigation efficacy was observed between maceration and incorporation implement combination treatments using a one-way ANOVA (Figure 4.29 ii). A factor based general ANOVA was used to further investigate the importance of maceration and incorporation implement selections for biofumigation of *G. pallida* (Figures 4.29 iii and Figure 4.29 iv). No difference in biofumigation efficacy against *G. pallida* was observable between incorporation implements; spader, plough and rotavator (P = 0. 637) (Figure 4.29 iii). However, maceration implement selection was found to be influential of biofumigation efficacy against *G. pallida* (P < 0.001) (Figure 4.29 iv). *Globodera pallida* viability after biofumigation with flail treatment was 12.5% lower than *G. pallida* treated with biofumigation using the roll conditioner, where the flail was compared directly to the roll conditioner treatment (Figure 4.29 iv). Overall, biofumigation with the flail was 39% effective, whilst biofumigation with roll conditioner was 30% effective when compared to the control from Figure 4.29 ii, with a clear 7% difference in viability between implements overall. No factor interaction was observed (P = 0.492). Soil moisture was investigated for integration into the P_s analysis as a covariate data set (Figure 4.29 ii) due to differences being found between treatments in general ANOVA analyses (Appendix 8.1.6). However, the data set was not found useful as a covariate (P = 0.917) and so was discarded from the analysis. The crop fresh and dry weight biomass, soil temperature conditions at incorporation and plant density at incorporation were measured, but not investigated as covariates due to no differences being found between treatments in general ANOVA analyses. See Sections 4.6.1 and Appendices 8.1.7 – 8.2.2 for data sets. Egg viability CV's for treatments 1-8 in Figure 4.29 i ranged between

2.3-4.2% with a general CV of 3.0%. Egg viability CV's for untreated and partial biofumigation treatments in Figure 4.29 ii were 6.8 and 7.7% respectively, whilst combined biofumigation treatments in Figure 4.29 ii ranged between 6.0 and 19.9% with a general CV of 15.3%. Egg viability CV's analysed at the factor levels in Figures 4.29 iii and iv were 13.6 and 15.1% for roll conditioner and flail topper treatments respectively, and 15.3, 14.3 and 17.1% for spader, plough and rotavator treatments respectively.

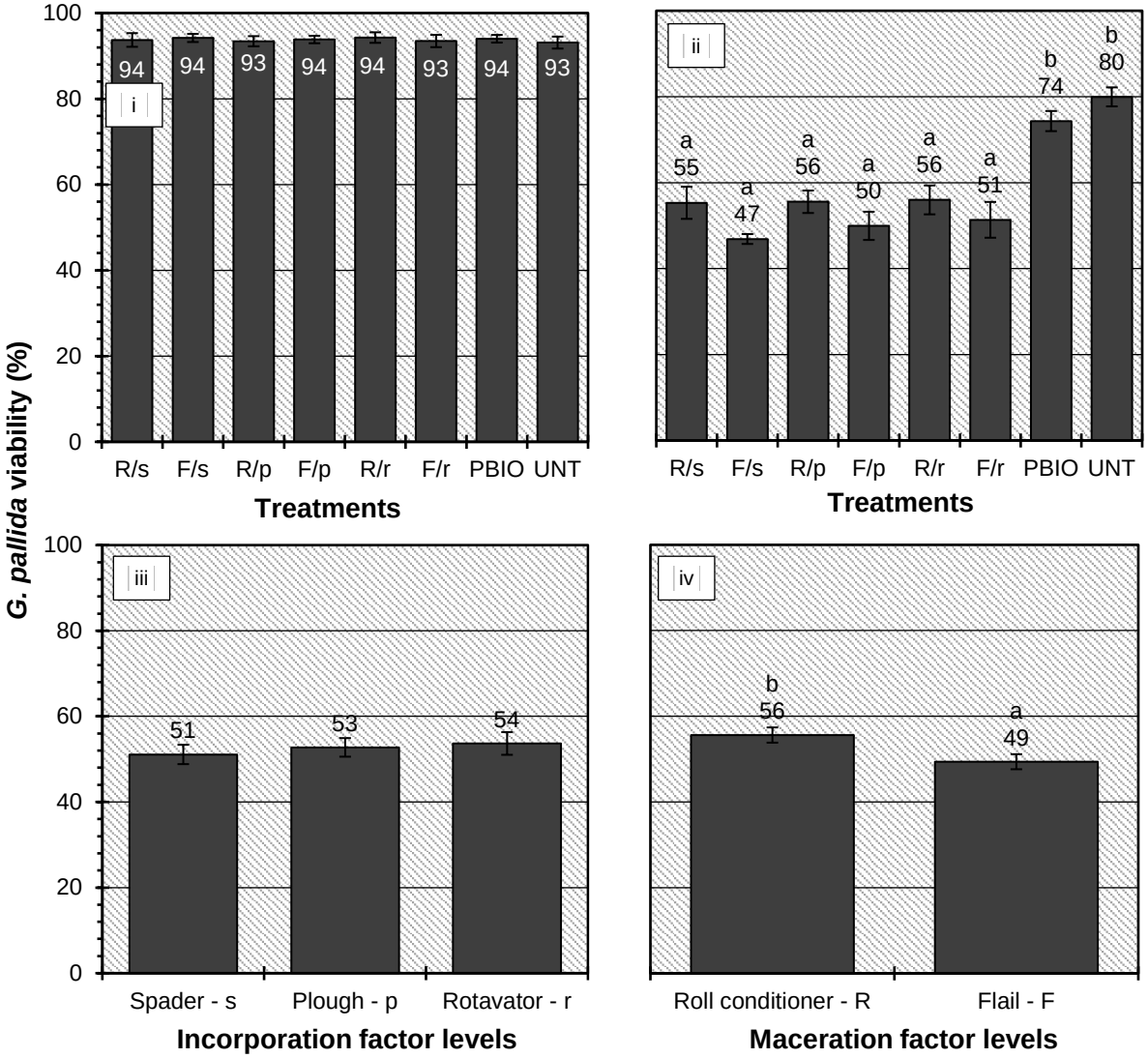


Figure 4.29: *Globodera pallida* viability before and after biofumigation at field experiment 1 (Hungry Hill, Norfolk), analysed by treatment, incorporation and maceration factors using ANOVA. *Globodera pallida* viability for all treatments at P_i, is illustrated in plate i, whilst plate ii shows *G. pallida* viability for all treatments at P_s. Plates iii and iv show *G. pallida* viability analysed by maceration and incorporation factors at P_s. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates Tukey's test groupings.

4.6.3 Globodera pallida *cysts 200 g^{-1} soil and eggs cyst^{-1} at* P_i *and* P_s

Figure 4.30 i shows the mean number of *G. pallida* cysts extracted from plots at Hungry Hill at P_i and P_s sample times. Approximately 56-68 *G. pallida* cysts 200 g^{-1} soil were retrieved for P_i and P_s samples respectively. There was no difference in the number of cysts retrieved between sampling times (P = 0.309). However, assessment of eggs cyst^{-1} between sample times showed a reduction in eggs at P_s compared to at P_i (P < 0.001) (Figure 4.30 ii). Mean *G. pallida* eggs cyst^{-1} were reduced from 168 to 94 between sample times. More detailed analysis of treatments at P_i showed no difference in *G. pallida* eggs cyst^{-1} between treatments (Figure 4.30 iii) (P = 0.732). The same analysis for P_s also found no difference in *G. pallida* eggs cyst^{-1} between treatments (Figure 4.30 iv) (P = 0.106). Factor based eggs cyst^{-1} analysis showed no difference between incorporation implements; spader, plough and rotavator (P = 0.399) (Figure 4.30 v). No difference in eggs cyst^{-1} was observed for roll conditioner and flail topper maceration implements either (P = 0.755) (Figure 4.30 vi), and no interaction was observed between biofumigant incorporation and maceration technique on *G. pallida* eggs cyst^{-1} (P = 0.087). Soil moisture data was investigated for integration into the P_s analysis as a covariate data set due to differences being found between treatments in one-way ANOVA analyses (Appendix 8.1.6). However, soil moisture data were not useful as covariates (P = 0.400) and so were discarded from further analyses. *Brassica juncea* fresh and dry weight biomass, soil temperature and plant density at incorporation were measured, but data were not investigated as covariates due to no differences having been found between treatments in one-way ANOVA analyses. See Sections 4.6.1 and Appendices 8.1.7 – 8.2.2 for data sets. Coefficient of variation values for cysts 200 g^{-1} soil at P_i and P_s sample times were 83.2 and 91.1% respectively (Figure 4.30 i), whilst eggs cyst^{-1} CV's for P_i and P_s samples were 28.2 and 55.8% respectively (Figure 4.30 ii). Eggs cyst^{-1} CV's for treatments 1-8 ranged between 6.1 and 36.4% at P_i (Figure 4.30 iii), and between 31.0 to 76.1% at P_s (Figure 4.30 iv). Eggs cyst^{-1} CV's analysed at factor levels in Figures 4.30 v and vi were 55.5 and 59.0% for roll conditioner and flail topper treatments, and 38.8, 72.5 and 62.1% for spader, plough and rotavator treatments respectively.

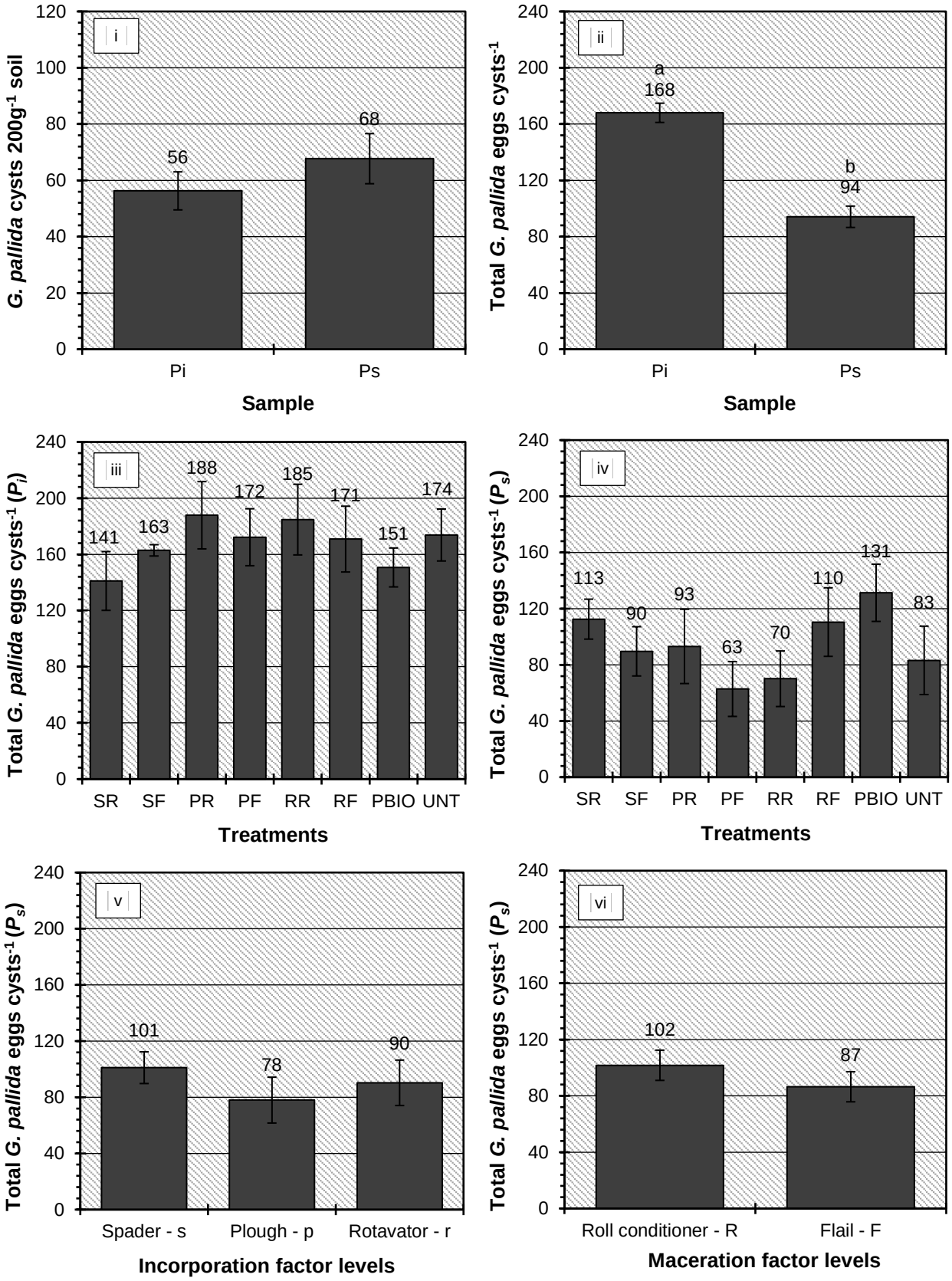


Figure 4.30: *Globodera pallida* cysts 200 g^{-1} soil and eggs $cyst^{-1}$ before and after biofumigation at field experiment 1 (Hungry Hill, Norfolk), analysed by sample date, treatment and incorporation and maceration factors using two sided T-tests, one-way and general ANOVA. *Globodera pallida* cysts 200 g^{-1} soil is illustrated for both P_i and P_s

sample dates in plate i, whilst plate ii shows total eggs cyst^{-1} for P_i and P_s sample dates. Plates iii and iv show *G. pallida* eggs cyst^{-1} analysed by treatment factor for both P_i and P_s samples. Plates v and vi show eggs cyst^{-1} for the P_s data set analysed by maceration and incorporation factor levels. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.7 Results: field experiment 2 (Crossroads, Shropshire)

4.7.1 Brassica juncea *biofumigant biomass and glucosinolate content*

Mean plant dry-matter at incorporation was 11.8% when analysed by block and by treatment. The SEM's for dry-matter were 0.28% and 0.27% for block and treatment respectively. No difference was found in the quantity of fresh or dry-weight *B. juncea* biomass between blocks (P = 0.926 and P = 0.941 respectively) or treatments (P = 0.407 and P = 0.639 respectively) (Figure 4.31 i and ii). Fresh-weight biomass ranged between equivalent to 12-15 t ha^{-1} between blocks I-VI overall, and between 10-17 t ha^{-1} between treatments, indicating large variation in fresh biomass within blocks. Dry-weight biomass ranged between equivalent to 1-2 t ha^{-1} between blocks and between treatments. 2-propenyl GSL (sinigrin) content of combined leaf and stem tissues was found to vary between 3.2 and 26.4 µmol g^{-1} dry tissue between blocks I-VI. Mean 2-propenyl GSL content was 7.9 µmol g^{-1} dry tissue and the SEM 3.30 µmol g^{-1} dry tissue (See Appendix 8.2.4). 2(S)-hydroxy-3-butenyl (epiprogoitrin) and 2(R)-2-hydroxy-3-butenyl (progoitrin) were also observed in *B. juncea* tissues at *c.*1.8 and 2.4 µmol g^{-1} dry tissue respectively.

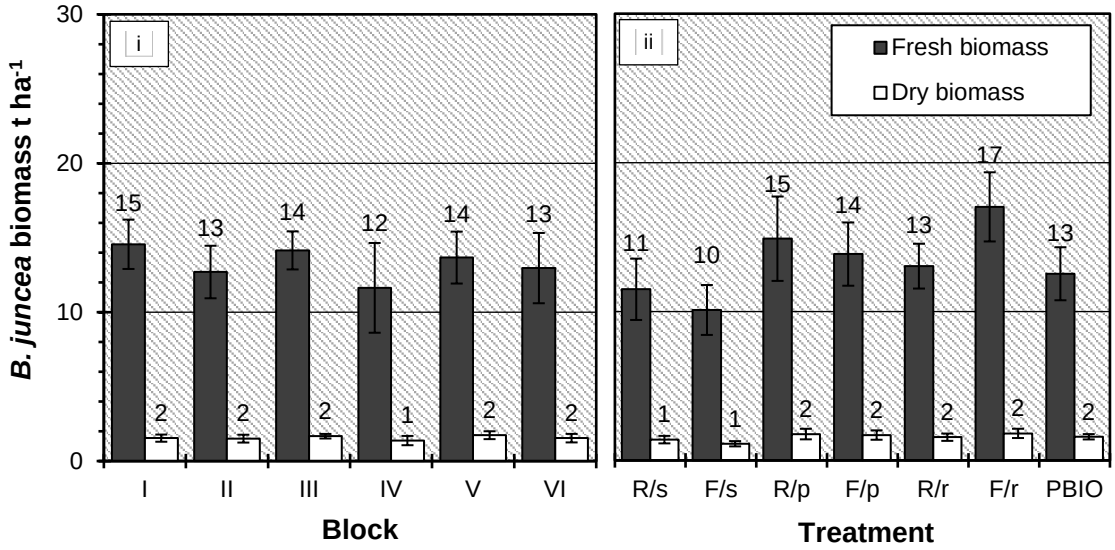


Figure 4.31: *Brassica juncea* biofumigant biomass t ha^{-1} for field experiment 2 (Crossroads, Shropshire), analysed by block (i) and treatment (ii) factors using one-way ANOVA. Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

4.7.2 Globodera pallida *viability at P_i and P_s*

The Crossroads site had a mean *G. pallida* population of 262 total eggs g^{-1} soil at P_i (SEM of 12.2 eggs g^{-1} soil). No difference was observed in *G. pallida* viability between treatments when assessed at P_i (P = 0.983) (Figure 4.32 i). The Crossroads population was 88.3% viable overall. Differences were observed in *G. pallida* viability between treatments at the P_s sample date (P < 0.001) (Figure 4.32 ii). Partial biofumigation was effective in reducing *G. pallida* viability by *c.*23%, whilst full biofumigation treatments were *c.*27% effective when compared to the untreated control. Partial biofumigation and full biofumigation effects were, however, inseparable (Figure 4.32 ii). No difference in biofumigation efficacy was observed between maceration and incorporation implement combination treatments when using a one-way ANOVA (Figure 4.32 ii). However, general ANOVA analyses were revealing (Figures 4.32 iii and Figure 4.32 iv). No difference in biofumigation efficacy against *G. pallida* was observable between incorporation implements; spader, plough and rotavator (P = 0.466) (Figure 4.32 iii), however, maceration implements could be separated (P = 0.003) (Figure 4.32 iv).

Biofumigation with flail treatment was 4% more effective in reducing *G. pallida* viability than biofumigation using the roll conditioner (Figure 4.32 iv). Overall, biofumigation with the flail was 29% effective, whilst biofumigation with roll conditioner was 25% effective in reducing *G. pallida* viability when compared to the control from Figure 4.32 ii. No factor interaction was observed (P = 0.214). Soil moisture (Appendix 8.1.6) and soil penetrometer resistance (Section 4.7.4) were investigated for integration into the P_s analysis as covariates (Figure 4.32 ii) due to differences being found between treatments in one-way and repeated meaures ANOVA analyses. Neither were found useful as covariates (P = 0.966 and P = 0.459 respectively) and so were discarded from the analysis. Cabbage root fly damage, soil temperature conditions at incorporation, the crop fresh and dry weight biomass and plant density at incorporation were also measured, but not investigated as covariates due to no differences being found between treatments in one-way ANOVA analyses. See Sections 4.7.1, and Appendices 8.1.7 – 8.2.3 for discarded data sets. Egg viability CV's for treatments 1-8 in Figure 4.32 i ranged between 2.9-6.5% with a general CV of 4.8%. Egg viability CV's for untreated and partial biofumigation treatments in Figure 4.32 ii were 6.2 and 18.6% respectively, whilst combined biofumigation treatments in Figure 4.32 ii ranged between 8.0 and 12.5% with a general CV of 11.0%. Egg viability CV's analysed at the factor levels in Figures 4.32 iii and iv were 9.7 and 11.5% for roll conditioner and flail topper treatments respectively, and 10.3, 11.1 and 11.8% for spader, plough and rotavator treatments respectively.

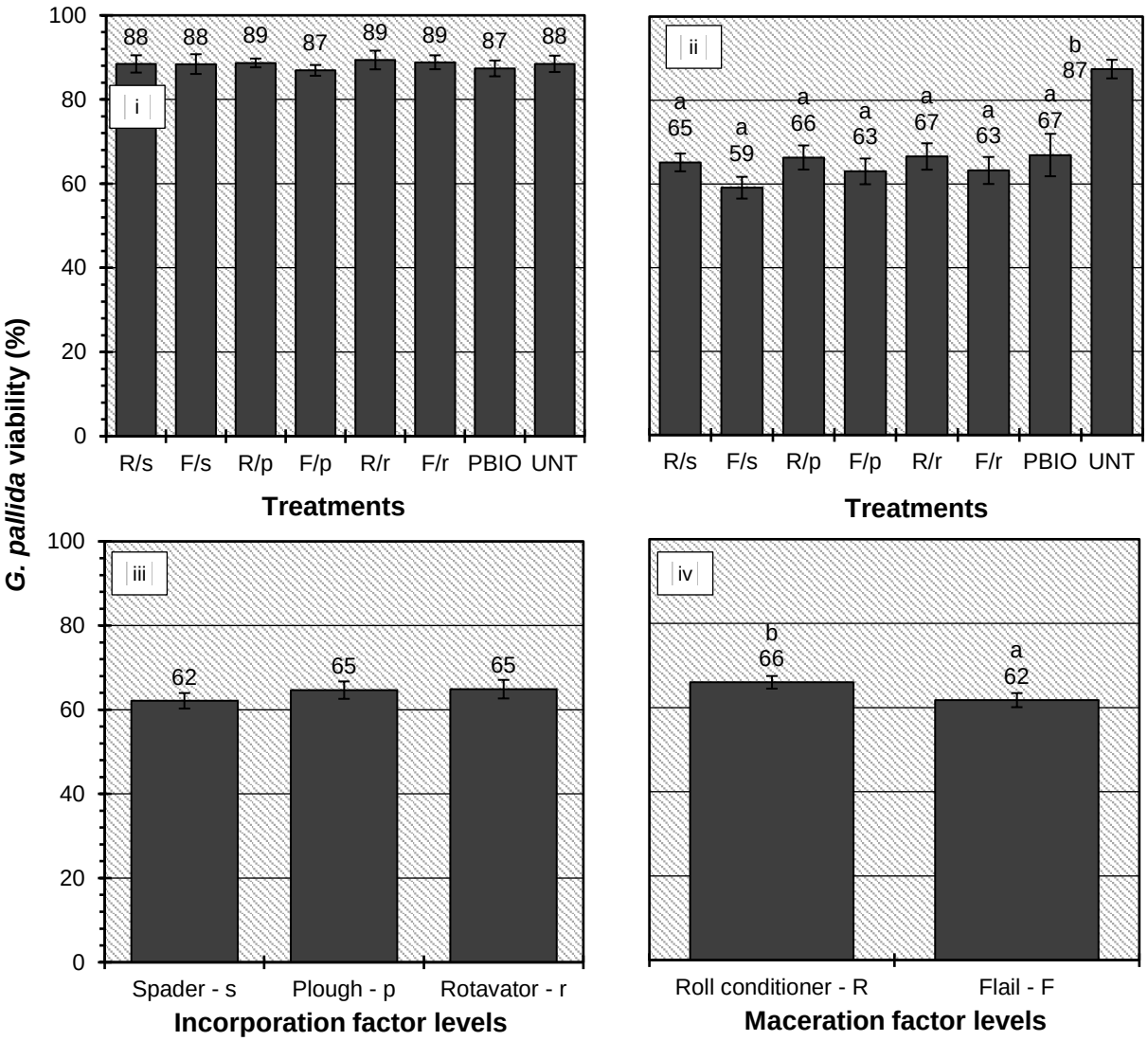


Figure 4.32: *Globodera pallida* viability before and after biofumigation at field experiment 2 (Crossroads, Shropshire), analysed by treatment, incorporation and maceration factors using one-way and general ANOVA. *Globodera pallida* viability before biofumigation for all treatments at P_i, is illustrated in plate i, whilst plate ii shows *G. pallida* viability for all treatments at P_s. Plates iii and iv show *G. pallida* viability analysed by maceration and incorporation factors at P_s. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.7.3 Globodera pallida *cysts 200 g^{-1} soil and eggs cyst^{-1} at* P_i *and* P_s

Figure 4.33 i shows the mean number of *G. pallida* cysts extracted from plots at the Crossroads site, at P_i and P_s sample times. Approximately 201-210 *G. pallida* cysts 200

g^{-1} soil were retrieved for P_i and P_s samples respectively. There was no difference in the number of cysts retrieved between sampling times (P = 0.270). No differences were found in eggs $cyst^{-1}$ between P_i and P_s sample times (P = 0.793) (Figure 4.33 ii). Mean *G. pallida* eggs $cyst^{-1}$ were 249 and 253 for P_i and P_s sample times respectively. More detailed analysis of treatments at P_i showed no difference in *G. pallida* eggs $cyst^{-1}$ between treatments (Figure 4.33 iii) (P = 0.915). The same analysis for P_s also found no difference in *G. pallida* eggs $cyst^{-1}$ between treatments (Figure 4.33 iv) (P = 0.943). Factor based eggs $cyst^{-1}$ analysis showed no difference between incorporation implements; spader, plough and rotavator (P = 0.541) (Figure 4.33 v). No difference in eggs $cyst^{-1}$ was observed for roll conditioner and flail topper maceration implements either (P = 0.653) (Figure 4.33 vi), and no interaction was observed between biofumigant incorporation and maceration technique on *G. pallida* eggs $cyst^{-1}$ (P = 0.792). Soil moisture and penetrometer resistance data were investigated for integration into the P_s analysis as covariates (Figure 4.33 ii) due to differences being found between treatments in one-way ANOVA analyses (Section 4.7.4 and Appendix 8.1.6). However, data were not useful as covariates to primary analyses (P = 0.242 and P = 0.616 respectively) and so were discarded from further analyses. *Brassica juncea* fresh and dry weight biomass, soil temperature, plant density at incorporation and cabbage root fly damage was measured, but data were not investigated as covariates due to no differences found between treatments in one-way ANOVA analyses. See Sections 4.7.1 and Appendices 8.1.7 – 8.2.3 for data sets. Coefficient of variation values for cysts 200 g^{-1} soil at P_i and P_s sample times were 20.1 and 19.2% respectively (Figure 4.33 i), whilst eggs $cyst^{-1}$ CV's for P_i and P_s samples were 28.7 and 30.0% respectively (Figure 4.33 ii). Eggs $cyst^{-1}$ CV's for treatments 1-8 ranged between 18.4 and 41.8% at P_i (Figure 4.33 iii), and between 15.4 and 50.6% at P_s (Figure 4.33 iv). Eggs $cyst^{-1}$ CV's analysed at factor levels in Figures 4.33 v and vi were 38.8 and 22.5% for roll conditioner and flail topper treatments, and 29.8, 25.3 and 40.1% for spader, plough and rotavator treatments respectively.

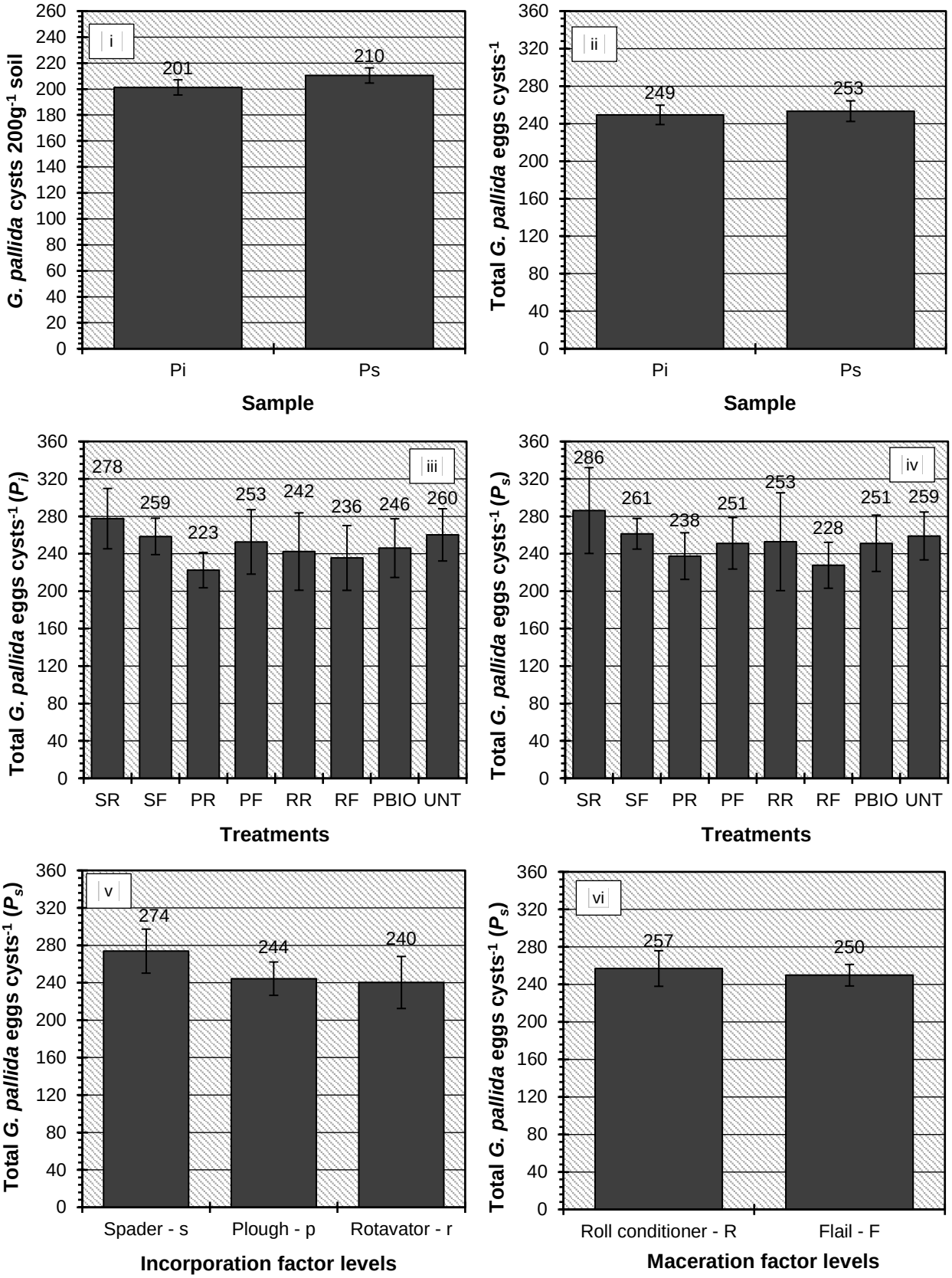


Figure 4.33: *Globodera pallida* cysts 200 g^{-1} soil and eggs $cyst^{-1}$ before and after biofumigation at field experiment 2 (Crossroads, Shropshire), analysed by sample date, treatment and incorporation and maceration factors using two sided T-tests, one-way

and general ANOVA. *Globodera pallida* cysts 200 g^{-1} soil is illustrated for both P_i and P_s sample dates in plate i, whilst plate ii shows total eggs $cyst^{-1}$ for both P_i and P_s sample dates. Plates iii and iv show *G. pallida* eggs $cyst^{-1}$ analysed by treatment factor for both P_i and P_s samples. Plates v and vi show eggs $cyst^{-1}$ for the P_s data set analysed by maceration and incorporation factor levels. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM.

4.7.4 *Soil penetrometer resistance following biofumigation*

Figure 4.34 (i-iv) illustrates trends in soil penetrometer resistance following incorporation whereby spader treatments appeared to generate a looser soil structure at depth (200-400 mm) than plough treatments, which produced a looser structure than the rotavator overall, and compared to untreated and partial biofumigation controls. Table 4.5 supports these observations whereby the spader is shown to produce a looser mean soil structure for depths 100-400 mm than the plough, which was shown to produce a looser mean soil resistance than the rotavator which was shown to not alter soil penetrometer resistance from the partial biofumigation or untreated control plot soils ($P < 0.001$). Table 4.6 shows in more detail where differences in soil penetrometer resistance occured at individual 100 mm depth increments from 100-400 mm depth using repeated measures ANOVA. Significant differences from the untreated control were observed for spader treated plots at 200, 300 and 400 mm depth, and for plough at 200 and 300 mm depth ($P < 0.001$). Partial biofumigation plots appeared to have the same penetrometer resistance as the untreated. Interactions between selected incorporation and maceration implement combinations were shown to influence both mean soil resistance ($P = 0.027$) (Tables 4.5 and 4.6), and soil resistance at different depths ($P = 0.021$) although this effect could not be attributed to a single implement combination or depth (Table 4.6). Soil penetrometer resistance was shown to increase in tandem with the depth of measurement, as might be expected (Table 4.6). However, no effect of maceration implement selection alone, or effect of simply growing a *B. juncea* crop on mean soil penetrometer resistance was observed ($P = 0.993$ and $P = 0.502$ respectively) (Table 4.5), or at different soil depths

(P = 0.647 and P = 0.086 respectively) (Table 4.6). Mean penetrometer resistance ranged between 0.65 and 1.18 Mpa for incorporation implements at 0-400 mm depth.

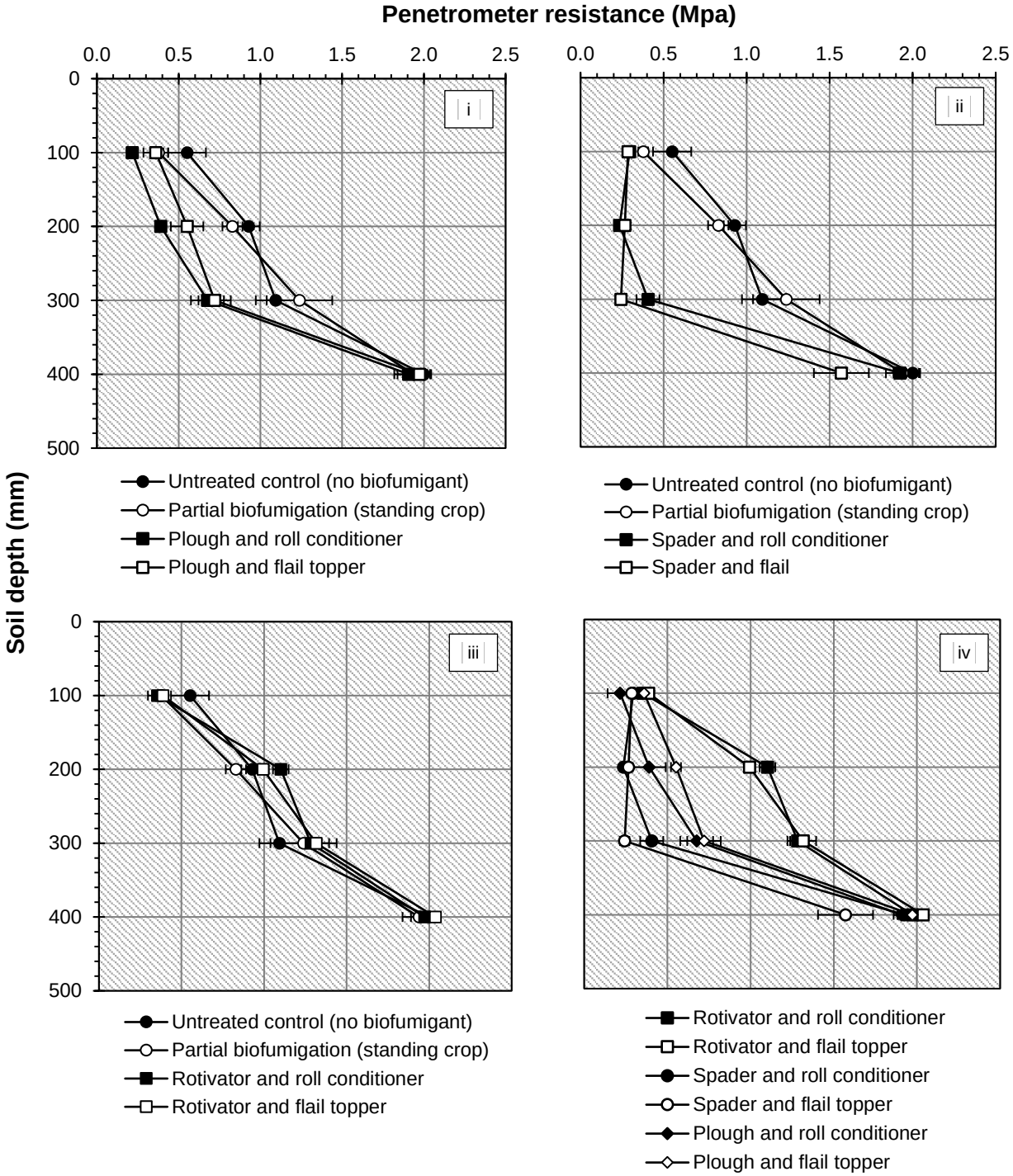


Figure 4.34: Soil penetrometer resistance of plots at field experiment 2 (Crossroads, Shropshire), treated with (i) plough combinations, (ii) spader combinations, (iii) and rotavator combinations in comparison to untreated controls and partial biofumigation plots. Plate iv shows all plough, spader and rotavator implement combinations in comparison. Error bars represent the SEM.

Table 4.5: Mean penetrometer resistance (MPa) of *B. juncea* treated plots analysed by factors incorporation, maceration and crop using general ANOVA. Lower-case lettering indicates differences in mean penetrometer resistance which is attributable to incorporation implements according to Tukeys multiple range test (95% confindence).

Factors & Interactions	Mean soil resistance (Mpa)	SEM	P-value	CV% (35 df)
Incorporation	0.89	0.03	<0.001	8.6
Maceration	0.95	0.03	0.993	
Crop	0.95	0.07	0.502	
Inc.*Mac.	0.92	0.05	0.027	
Inc. implements				
Spader	0.65 a	0.03	<0.001	12.7
Plough	0.85 b	0.04		
Rotavator	1.18 c	0.03		
Untreated	1.12 c	0.07		

Table 4.6: Penetrometer resistance (MPa) of *B. juncea* treated plots analysed by factors soil depth, incorporation, maceration and crop using repeated measures ANOVA. Differences in penetrometer resistance at depth between incorporation implements and reference depths according to the untreated control, as determined using Fishers LSD, are signified by an asterisk (*).

Factors & Interactions		Mean soil resistance (Mpa)	SEM	P-value	CV% (120 df)
Depth		0.95	0.02	<0.001	16
Depth*Inc.		0.89	0.05	<0.001	
Depth*Mac.		0.95	0.05	0.647	
Depth*Crop		0.95	0.09	0.086	
Depth*Inc.*Mac.		0.89	0.07	0.021	
Inc. implement (depth mm)				**Significance (*) compared to 'UNT' reference level**	
Spader	100	0.29	0.01		
	200	0.25	0.01	*	
	300	0.32	0.04	*	
	400	1.75	0.10	*	
Plough	100	0.29	0.04		
	200	0.47	0.06	*	
	300	0.70	0.07	*	
	400	1.94	0.05		
Rotavator	100	0.37	0.03		
	200	1.05	0.03		
	300	1.30	0.05		
	400	2.01	0.04		
Untreated (UNT)	100	0.46	0.12		
	200	0.88	0.07		
	300	1.17	0.12		
	400	1.97	0.05		

4.8 Results: field experiments 1 and 2 combined (Hungry Hill, Norfolk and Crossroads, Shropshire)

4.8.1 Globodera pallida *viability at* P_s

F-variance ratio calculations were carried out to assess whether primary *G. pallida* data sets from the first two field experiments could be combined in an effort to improve viability analyses. The P_i data sets could not be combined as the cumulative upper probability F-variance ratio test employed found the variation between data sets was not comparable (P = 0.038). However, the same assessment of P_s data sets, allowed data between experiments to be combined (P = 0.193), as shown in Figure 4.35 i. The combined site analysis produced a mean *G. pallida* population of 141 total eggs g^{-1} soil at P_s (SEM of 13.8 eggs g^{-1} soil). One-way ANOVA of the combined data sets showed an effect of partial biofumigation on *G. pallida* viability in the region of *c.* 16% efficacy compared to the untreated control, an effect of plough and rotavator combinations with the roll conditioner in the region of *c.* 27% efficacy, and an effect of spader and flail combination in the region of *c.* 37% efficacy when compared to the untreated control (P < 0.001). All other implement combinations were inseparable from the implement combinations just mentioned (Figure 4.35 i). There is clearly an effect of partial biofumigation on *G. pallida* viability, and clearly differences in full biofumigation efficacy between implement combinations, with the spader and flail combination reducing *G. pallida* viability by up to 8% more effectively than other common maceration and incorporation implement combinations. Again, no difference in biofumigation efficacy against *G. pallida* was observable in factor based analyses between incorporation implements alone; spader, plough and rotavator (P = 0.673) (Figure 4.35 ii). Maceration implement selection was again found to be influential of biofumigation efficacy against *G. pallida* in general ANOVA analysis (P = 0.006) (Figure 4.35 iii). No factor interaction was observed (P = 0.500).

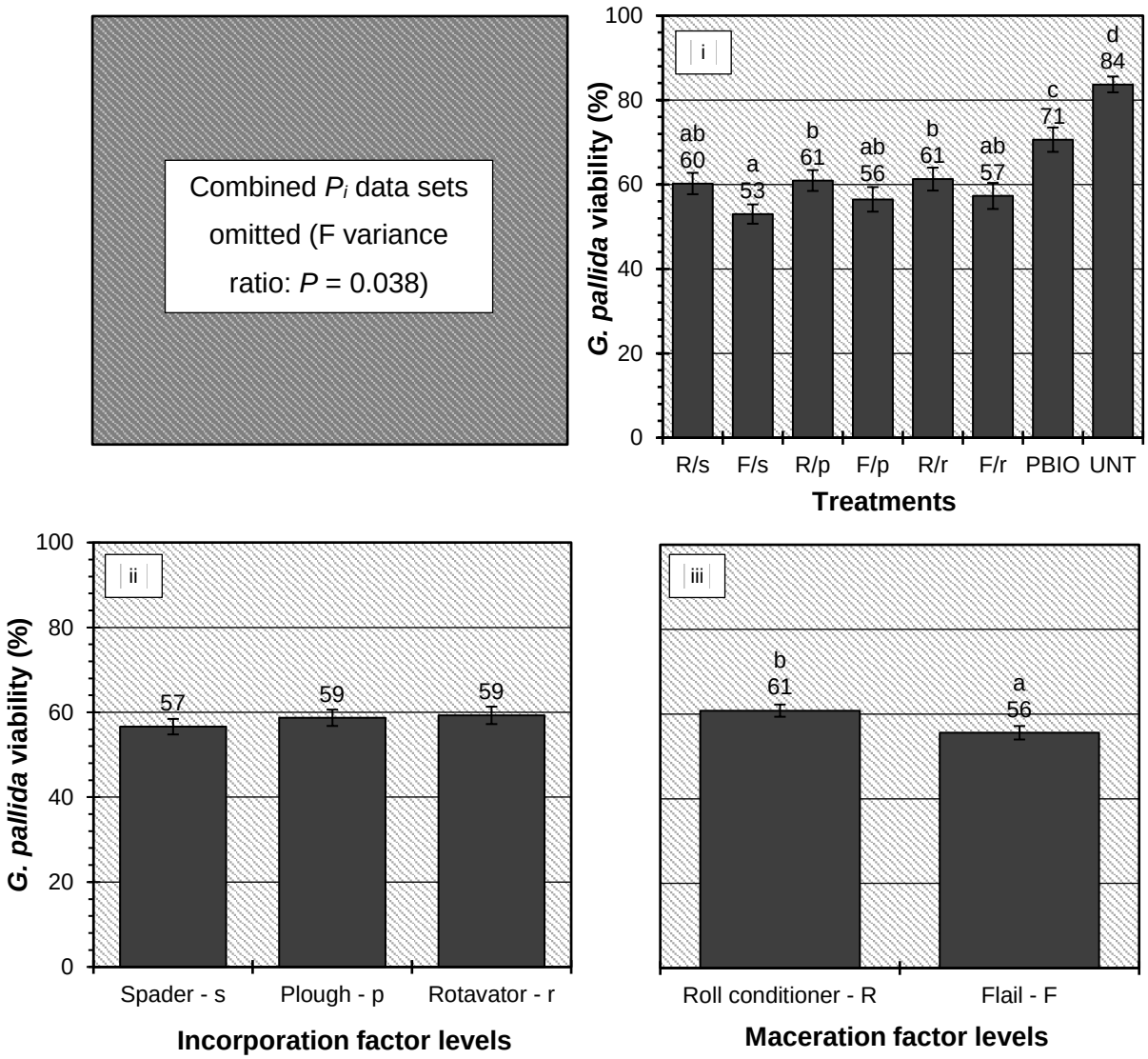


Figure 4.35: *Globodera pallida* viability after biofumigation for both field experiment 1 and 2 (Hungry Hill, Norfolk and Crossroads, Shropshire respectively) as a combined analysis. Data was analysed by treatment, incorporation and maceration factors using one-way and general ANOVA. *Globodera pallida* viability after biofumigation for all treatments is illustrated in plate i, whilst plates ii and iii show *G. pallida* viability analysed by maceration and incorporation factor levels respectively. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.9 Results: field experiment 3 (Roys Corner, Shropshire)

4.9.1 Brassica juncea *biofumigant biomass and glucosinolate content*

Mean plant dry-matter at incorporation was 12.6% when analysed by block, and by treatment. The SEM's for dry-matter were 0.17% and 0.20% respectively. No difference was found in the quantity of fresh or dry-weight *B. juncea* biomass between blocks (*P* = 0.821 and *P* = 0.794 respectively) or treatments (*P* = 0.701 and *P* = 0.420 respectively) (Figure 4.36 i and ii). Mean fresh-weight biomass ranged between 44 and 52 t ha^{-1} when analysed by block and by treatment. Dry-weight biomass ranged between equivalent to 4 and 6 t ha^{-1} between blocks and between treatments. 2-propenyl GSL (sinigrin) content of combined leaf and stem tissues was found to vary between 2.4 and 4.2 µmol g^{-1} dry tissue between blocks I-VI. Mean 2-propenyl GSL content was 3.5 µmol g^{-1} dry tissue and the SEM 0.27 µmol g^{-1} dry tissue (See Appendix 8.2.4). 2(S)-hydroxy-3-butenyl (epiprogoitrin) and 2(R)-2-hydroxy-3-butenyl (progoitrin) were also observed in *B. juncea* tissues at *c.*1.0 and 1.7 µmol g^{-1} dry tissue respectively.

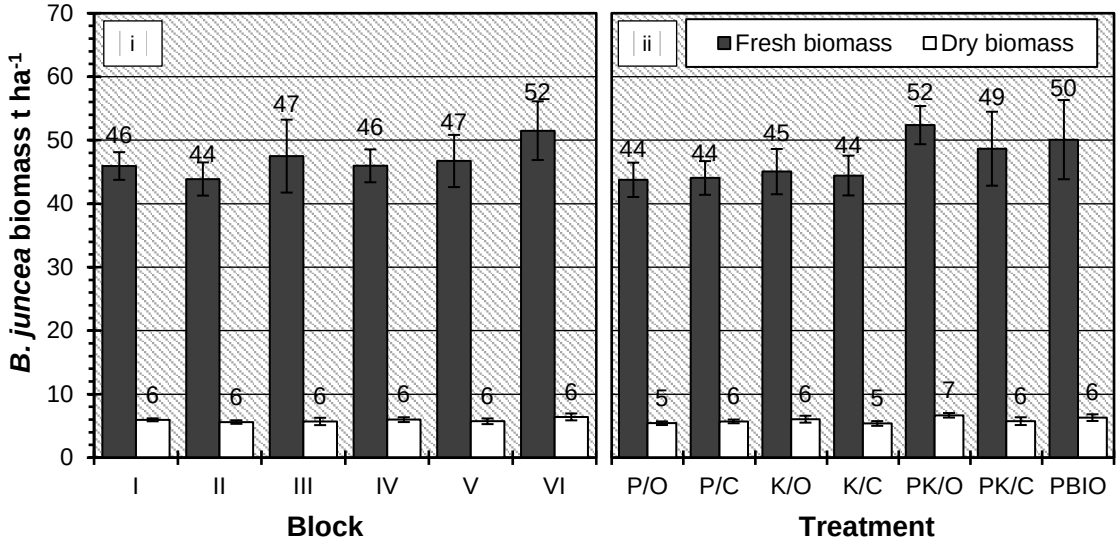


Figure 4.36: *Brassica juncea* biofumigant biomass t ha^{-1} for field experiment 3 (Roys Corner, Shropshire), analysed by block (i) and treatment (ii) factors using one-way ANOVA. Treatment key: pig-tail tine – P; knife tine – K; pig-tail/knife tine – PK; open shear-plate – O; closed shear-plate – C; partial biofumigant – PBIO. Error bars represent the SEM.

4.9.2 Globodera pallida *viability at* P_i *and* P_s

The Roys corner site had a mean *G. pallida* population of 24 total eggs g^{-1} soil at P_i (SEM of 2.8 eggs g^{-1} soil). No difference was observed in *G. pallida* viability between treatments when assessed at P_i (P = 0.886) (Figure 4.37 i). The Roys Corner population was 75.0% viable at P_i overall. Differences were observed in *G. pallida* viability between treatments at the P_s sample date (P = 0.020) (Figure 4.37 ii). Partial biofumigation was ineffective in reducing *G. pallida* viability, but all biofumigation treatments except for the knife-tine and closed shear-plate maceration set-up were found to reduce *G. pallida* viability when compared to the untreated control, by *c.*31% overall. No difference in biofumigation efficacy was observed between any other tine and shear-plate set-up combinations using a one-way ANOVA (Figure 4.37 ii). A factor based general ANOVA analysis was used to further investigate the importance of maceration implement tine and shear-plate selections and set-up for biofumigation of *G. pallida* (Figures 4.37 iii and iv). No difference in biofumigation efficacy against *G. pallida* was observable between tines; knife, pig-tail or the combination of both tine types (P = 0.791) (Figure 4.37 iii). Similarly, shear-plate set-up was not found to influence biofumigation efficacy against *G. pallida* (P = 0.874) (Figure 4.37 iv). *Brassica juncea* fresh and dry weight biomass, and plant density at incorporation were measured, but not investigated as covariates due to no differences being found between treatments in one-way ANOVA analyses. See Section 4.9.1 for biomass and Appendix 8.2.2 for plant counts. Egg viability CV's for treatments 1-8 in Figure 4.37 i ranged between 5.8-11.4% with a general CV of 8.7%. Egg viability CV's for untreated and partial biofumigation treatments in Figure 4.37 ii were 33.2 and 19.5% respectively, whilst combined biofumigation treatments in Figure 4.37 ii ranged between 9.2 and 36.6% with a general CV of 23.1%. Egg viability CV's analysed at factor levels in Figures 4.37 iii and iv were 20.6 and 26.1% for tine and shear-plate treatments respectively, and 17.0, 27.1 and 25.1% for pig-tail, knife and pig-tail/knife tine combination treatments respectively.

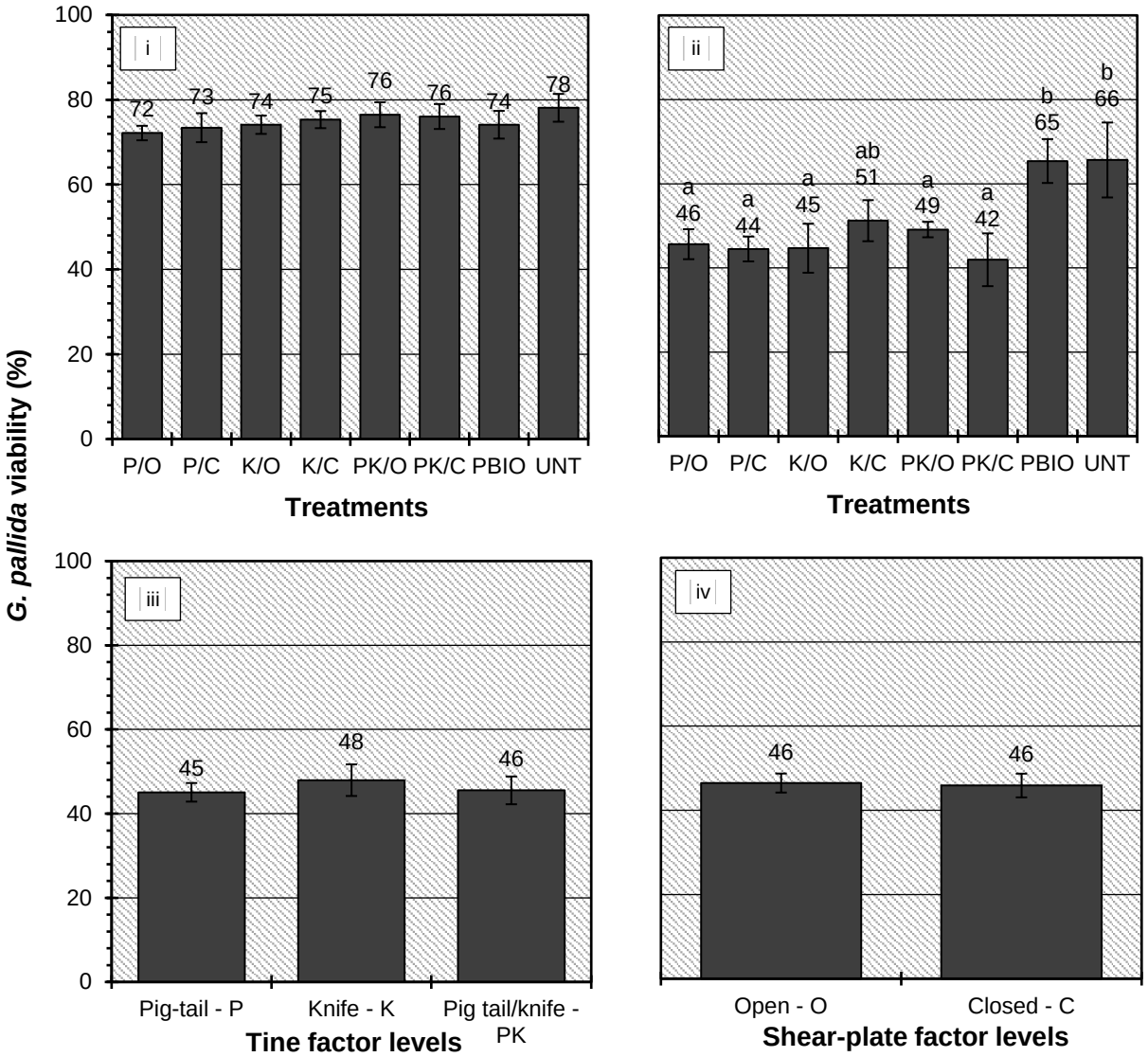


Figure 4.37: *Globodera pallida* viability before and after biofumigation at field experiment 3 (Roys Corner, Shropshire), analysed by treatment, tine and shear-plate factors using one-way and general ANOVA. *Globodera pallida* viability for all treatments at P_i, is illustrated in plate i, whilst plate ii shows *G. pallida* viability for all treatments at P_s. Plates iii and iv show *G. pallida* viability analysed by tine and shear-plate factor levels at P_s. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.9.3 Globodera pallida *cysts 200 g^{-1} soil and eggs cyst^{-1} at* P_i *and* P_s

Figure 4.38 i shows the mean number of *G. pallida* cysts extracted from plots at Roys Corner, at P_i and P_s sample times. Approximately 93-68 *G. pallida* cysts 200 g^{-1} soil were retrieved for P_i and P_s samples respectively. The number of cysts retrieved from soil was lower at P_s than at P_i (P = 0.037). However, no differences were found in eggs cyst^{-1} between P_s and P_i sample times (P = 0.149) (Figure 4.38 ii). Mean *G. pallida* eggs cyst^{-1} were 54 and 41 for P_i and P_s sample times respectively. More detailed analysis of treatments at P_i showed no difference in *G. pallida* eggs cyst^{-1} between treatments (Figure 4.38 iii) (P = 0.253). The same analysis for P_s also found no difference in *G. pallida* eggs cyst^{-1} between treatments (Figure 4.38 iv) (P = 0.224). Factor based eggs cyst^{-1} analysis showed no difference between tine treatments; knife, pig-tail or balanced combination (P = 0.486) (Figure 4.38 v). No difference in eggs cyst^{-1} was observed for open and closed shear-plate position treatments either (P = 0.372) (Figure 4.38 vi), and no interaction was observed between tine and shear-plate set-ups on *G. pallida* eggs cyst^{-1} (P = 0.816). *Brassica juncea* fresh and dry weight biomass, and plant density at incorporation were measured, but data were not investigated as covariates due to no differences being found between treatments in one-way ANOVA analyses. See Sections 4.9.1 and Appendix 8.2.2 for discarded data sets. Coefficient of variation values for cysts 200 g^{-1} soil at P_i and P_s sample times were 57.2 and 91.1% respectively (Figure 4.38 i), whilst eggs cyst^{-1} CV's for P_i and P_s samples were 60.0 and 126.7% respectively (Figure 4.38 ii). Eggs cyst^{-1} CV's for treatments 1-8 ranged between 40.0 and 65.7 at P_i (Figure 4.38 iii), and between 53.7 and 168.3% at P_s (Figure 4.38 iv). Eggs cyst^{-1} CV's analysed at factor levels in Figures 4.38 v and vi were 142.4 and 75.5% for open and closed shear-plate position treatments, and 155.3, 52.9 and 76.7% for knife, pig-tail or combination tine treatments respectively.

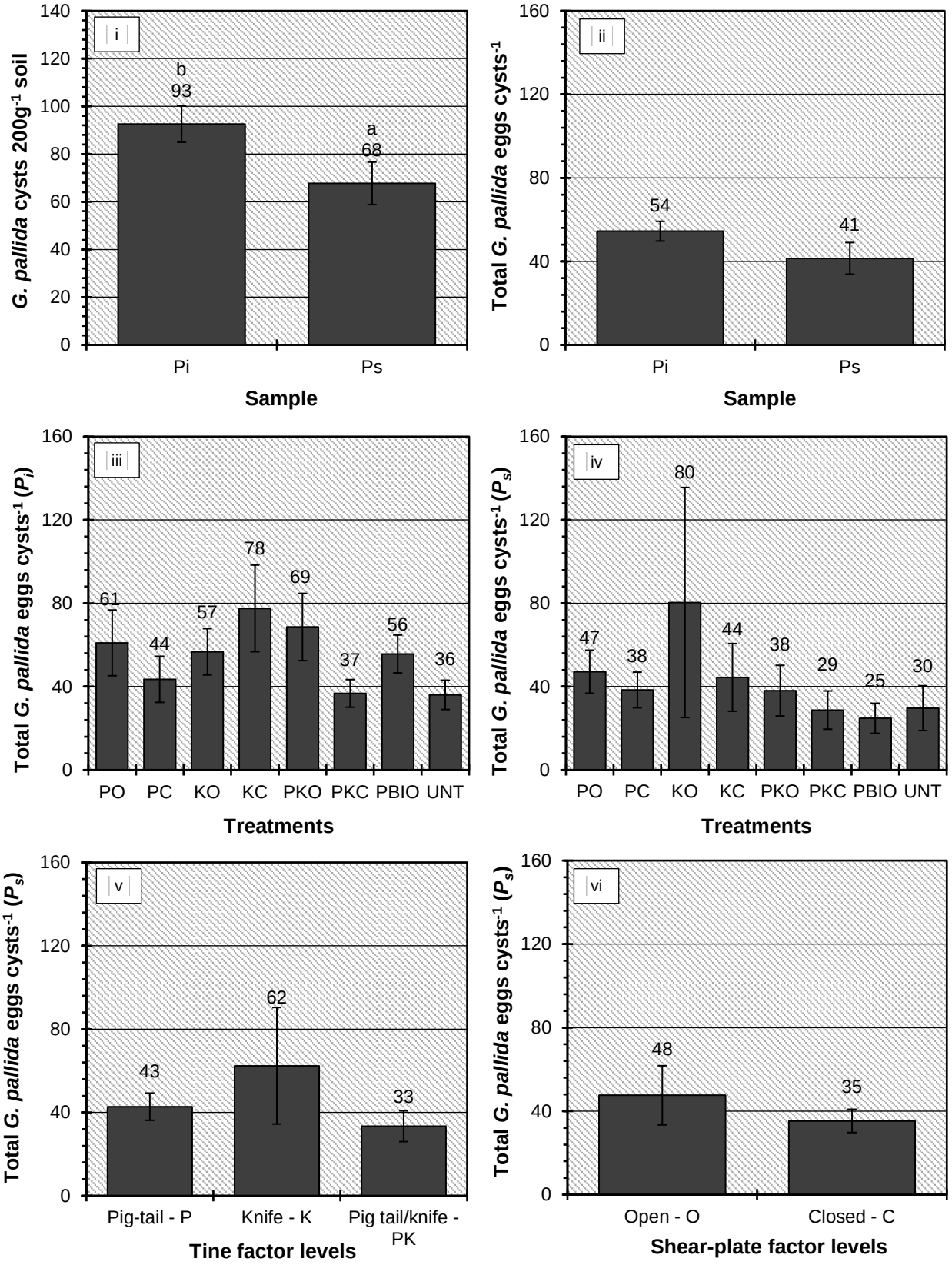


Figure 4.38: *Globodera pallida* cysts 200 g^{-1} soil and eggs $cyst^{-1}$ before and after biofumigation at field experiment 3 (Roys Corner, Shropshire), analysed by sample date, treatment and tine and shear-plate factors using two sided T-tests, one-way and general ANOVA. *Globodera pallida* cysts 200 g^{-1} soil is illustrated for both P_i and P_s sample dates

in plate i, whilst plate ii shows total eggs cyst^{-1} for both P_i and P_s sample dates. Plates iii and iv show *G. pallida* eggs cyst^{-1} analysed by treatment factor for both P_i and P_s samples. Plates v and vi show eggs cyst^{-1} for the P_s data set analysed by tine and shear-plate factor levels. PBIO and UNT represent partial biofumigation and untreated treatments respectively. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.9.4 Brassica juncea *bite/section length*

Figure 4.39 i shows one-way ANOVA analysis of the section length of *B. juncea* biofumigant tissues after maceration with different tine and shear-plate set-ups. Open shear-plate positioning produced a tissue section length of *c.*300 mm, irrespective of flail tine; knife, pig-tail or balanced combination. The pig-tail tine combined with a closed shear-plate position produced a finer chop than the open shear plate positions at approximately *c.*180 mm long tissue section lengths ($P < 0.001$). The remaining closed shear-plate treatments were inseparable from all other treatments. General ANOVA analyses revealed shear-plate set-up to be influential of *B. juncea* tissue section lengths after maceration ($P < 0.001$) (Figure 4.39 ii) but that tine type was not influential of tissue section length ($P = 0.416$) (Figure 4.39 iii). Tine and shear-plate set-up was not found to interact in a way which would influence *B. juncea* tissue section length ($P = 0.240$).

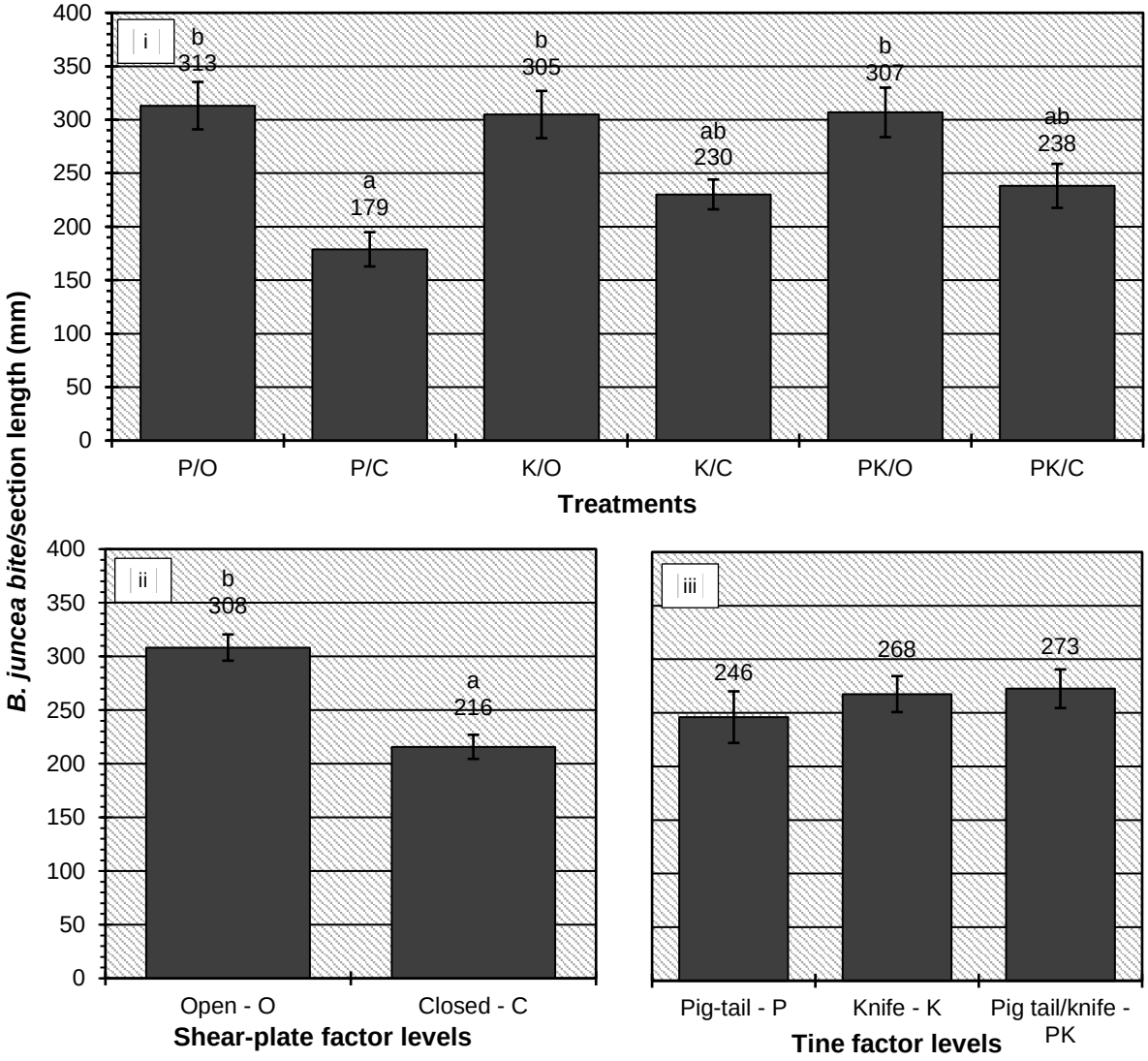


Figure 4.39: *Brassica juncea* biofumigant tissue section length at field experiment 3 (Roys Corner, Shropshire), analysed by one-way (i) and general ANOVA (ii and iii). Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.9.5 Brassica juncea *biofumigant volatile organic compounds measured in soil*

Three primary *B. juncea* VOC's were observed in soil at the Roys Corner site; dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and AITC. Samples were collected 72 h after biofumigation. No difference in VOC peak areas could be determined between any of the treatments for DMS, DMDS or total VOC outputs analyses when using a one-way ANOVA (*P*'s 0.297, 0.583 and 0.277 respectively) (Figures 4.40 i, ii, iv). Differences in VOC output between treatments were present for AITC (*P* = 0.048), however, neither

Tukey or Fischers protected LSD multiple comparisons were able to indicate where treatment differences occurred (Figure 4.40 iii).

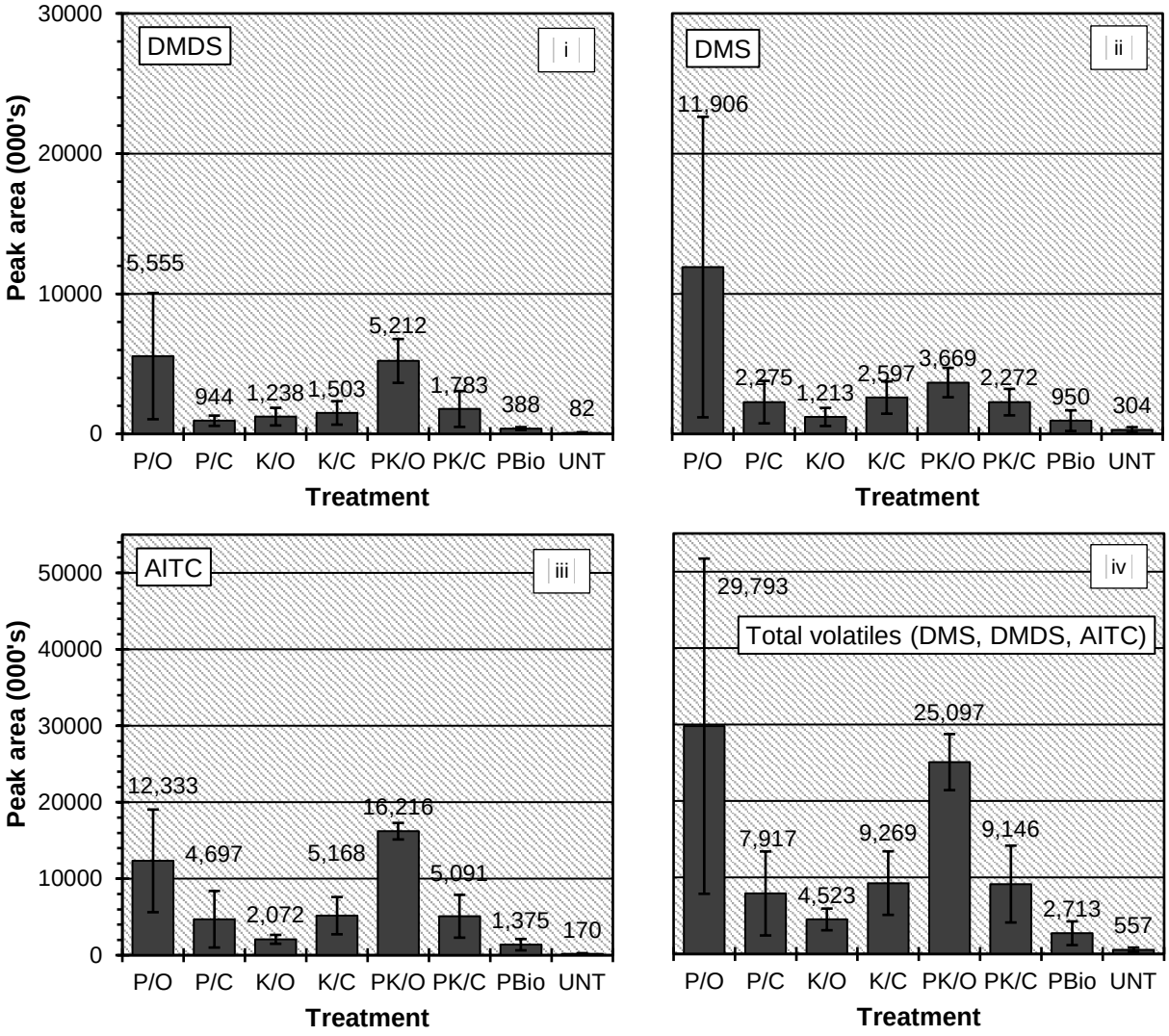


Figure 4.40: Tenax-TA entrained *B. juncea* volatile organic compounds from soil at field experiment 3 (Roys Corner, Shropshire), collected 72 h after incorporation, analysed by one-way ANOVA. Plate i shows DMDS, plate ii shows DMS, plate iii shows AITC and plate iv shows the total combined VOC concentrations observed in *B. juncea* treated soil. Treatment key: pig-tail tine – P; knife tine – K; pig-tail/knife tine – PK; open shear-plate – O; closed shear-plate – C; partial biofumigant – PBIO; untreated - UNT. Error bars represent the SEM.

General ANOVA analyses were also carried out to investigate mean AITC VOC output for tine and shear-plate factors. No differences were observed (*P*'s = 0.208 and 0.120

respectively) (Figures 4.41 i and ii). No interaction was observed between tine and shear-plate selection and set-up on VOC release from *B. juncea* tissues (*P* = 0.193). Further one-way ANOVA analysis was carried out to determine whether the quantities of each VOC captured from soil were comparable. Initial analysis found the peak area data set had a positive skew. The data set was square-root transformed which normalised data. Differences were subsequently observed between the peak areas of DMS, DMDS and AITC (*P* = 0.034) (Figure 4.42). Allyl ITC was present in soil at a higher concentration than DMS, but could not be separated from DMDS. Overall, AITC accounted for 44% of the total VOC peak area in soil, composed of DMS, DMDS and AITC. The remaining 56% was made up of DMS at 25% and DMDS at 31%.

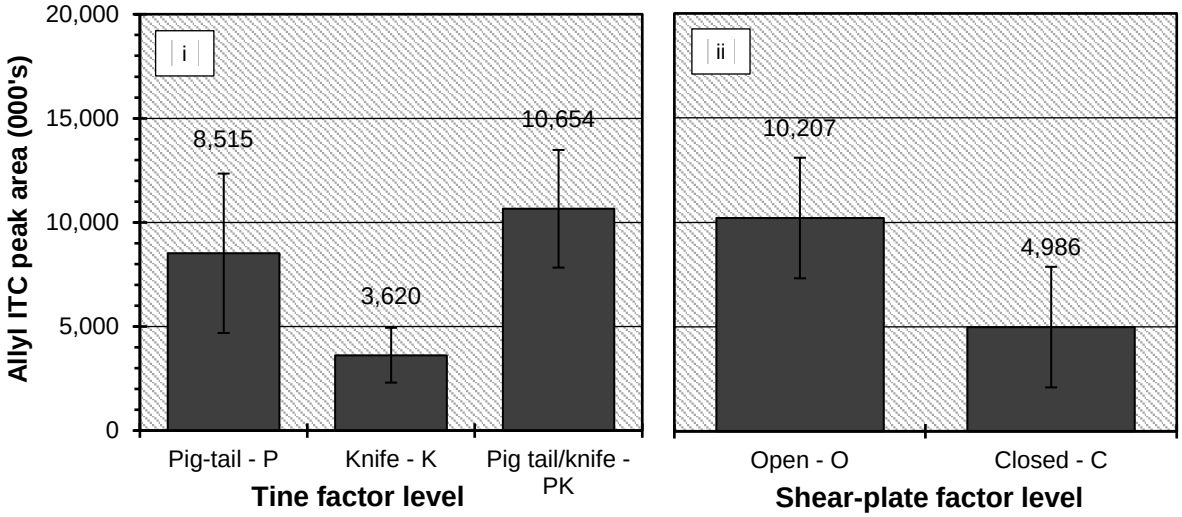


Figure 4.41: Factor based analysis of Tenax-TA entrained *B. juncea* volatile organic compounds from soil at field experiment 3 (Roys Corner, Shropshire), collected 72 h after incorporation, analysed by general ANOVA. Plate i shows AITC peak areas for tine factor levels, plate ii shows AITC peak areas for shear-plate factor levels. Error bars represent the SEM.

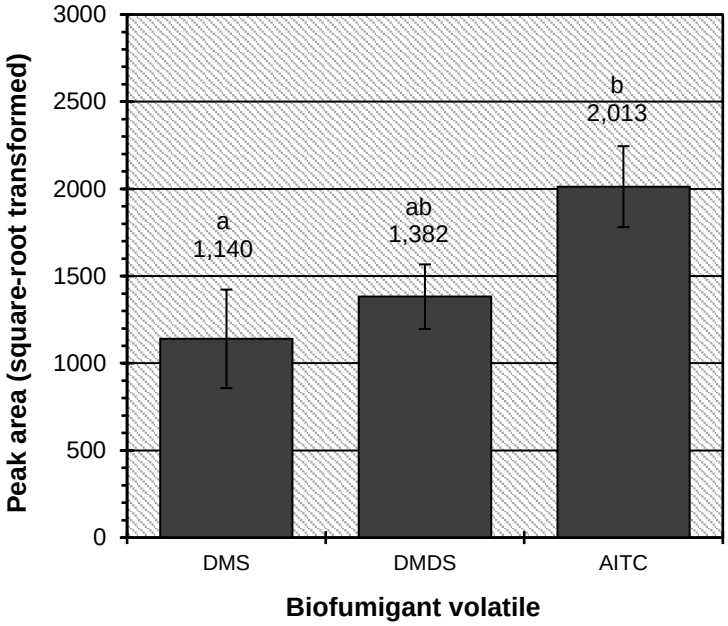


Figure 4.42: Analysis of Tenax-TA entrained *B. juncea* volatile organic compound peak areas for dimethyl sulphide, dimethyl disulphide and allyl isothiocyanate collected from soil at field experiment 3 (Roys Corner, Shropshire) at 72 h after incorporation, analysed by one-way ANOVA. Treatment key: dimethyl sulphide – DMS; dimethyl disulphide – DMDS; allyl isothiocyanate – AITC. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

4.10 Discussion

The primary aim of the work described in this chapter was to determine whether maceration and/or incorporation implement selection and set-up can be influential of *B. juncea* biofumigation efficacy against PCN. The chapter null hypothesis stated that neither maceration or incorporation implement selection or set-up has an effect on *B. juncea* biofumigation efficacy against PCN. Overall, maceration implement selection was found important in influencing biofumigation efficacy against *G. pallida*, with a flail implement being more effective for *B. juncea* biofumigant maceration than a roll conditioner when assessing efficacy by analysis of *G. pallida* viability. However, no evidence could be found that incorporation implement was influential of biofumigation

efficacy. Similarly, maceration implement set-up was not found to influence biofumigation efficacy against *G. pallida* in this instance.

4.10.1 *Field experiment 1 (Hungry Hill, Norfolk)*

4.10.1.1 Globodera pallida *viability*

The Hungry Hill experiment investigated two maceration implement levels; roll conditioner and flail topper, in combination with three incorporation implement levels; spader, plough and rotavator, compared to an untreated control, and a partial biofumigation control, where *B. juncea* plants were grown but not incorporated into soil for suppression of *G. pallida*. The partial biofumigation control was included following recent observations by Ngala *et al.* (2014), where *G. pallida* populations were found to decline in the field in response to just growing *B. juncea* plants. The partial biofumigation control was therefore essential in separating the effects of maceration and incorporation away from any other brassica mediated efficacy. As reviewed in Chapter 1, similar observations for other brassica plants have been made by other authors (see Sections 1.4.2 and 1.4.10). The headline results for the Hungry Hill experiment, and other field experiments discussed hereafter, surrounded the observable effects of treatments on *G. pallida* egg viability, as shown in Figure 4.29.

Figure 4.29 i showed no difference in egg viability between treatments at P_i, and only low levels of variation in egg viability between treatments. Egg viability was therefore highly uniform before biofumigation, which presented a good opportunity to observe biofumigation treatment effects at P_s, if they were present. Figure 4.29 ii shows a clear biofumigation effect, with *G. pallida* viability being reduced by *c.*34% compared to the untreated control using full biofumigation. However, no differences in efficacy were observable between biofumigation treatments using different maceration-incorporation implement combinations. Similar to the glasshouse experiments of Chapter 3, biofumigation treatment was shown to increase variation in *G. pallida* egg viability. This can be detrimental to statistical analyses when trying to determine the significance of

small numerical differences between treatment means. Egg viability CV values increased from *c.*3% at P_i to 15% at P_s for biofumigation treatments, whilst egg viability CV values only increased from *c.*3% at P_i to 7% at P_s for untreated and partial biofumigation treatments. Using a replicate calculator provided by Berndston *et al*. (1991), it can be shown that to prove a 10% difference between treatments, as found between the roll conditioner-rotavator implement combination treatment and the flail topper-spader implement combination treatment (Figure 4.29 ii), an egg viability CV of no greater than *c.*4% would be acceptable for 6 replicates of each treatment. Therefore, the increase in egg viability variation in response to biofumigation may have masked differences between treatments, owing to potentially small effect sizes between treatments. Certainly, the level of replication for the Hungry Hill experiment was too low to determine any probable difference between maceration and incorporation implement combinations. However, Figure 4.29 ii shows each treatment using a roll conditioner, to compare less favourably in terms of efficacy against *G. pallida* than flail topper treatments, when simply observing means. Therefore, factor based analyses were applied to data, whereby data was amalgamated according to shared incorporation and maceration implements (Figures 4.29 iii and iv).

In factor based analyses, egg viability CV's were shown to remain stable whilst replication was effectively increased through a simplification of analysis. Incorporation implement was not found to influence efficacy against *G. pallida* in this instance (4.29 iii), which was in contrast to the work of Roubtsova *et al*. (2007). It is possible that incorporation implements were not diverse enough in their placement of *B. juncea* material in soil, for differences in biofumigation efficacy against *G. pallida* to be observed between them. Alternatively, selection of incorporation implement type is not important for field scale biofumigation. Maceration implement, however, was found to be influential of biofumigation efficacy against *G. pallida* (4.29 iv). An extra 7% efficacy was achieved when practising biofumigation with the flail topper in comparison to a roll conditioner. Whilst the quality of macerated *B. juncea* biofumigant tissues produced by each

implement was not assessed in this experiment, it was visually clear that the roll conditioner treated *B. juncea* material had a much longer section length than that of the flail topper; *c.*400 mm compared to *c.*200 mm, and that flail topper treated *B. juncea* sections showed signs of greater tissue trauma. It is possible that the quantity of *B. juncea* material fed into roll conditioner rolls was not conducive to effective bruising of tissues and that the rolls should have been adjusted from the factory set-up to facilitate bruising. The roll conditioner, which was sourced from the HAU farm, was typically used to mow lucerne (*Medicago sativa*) for ensiling or hay, and so was set-up to process crops of *c.*40 t ha^{-1} fresh weight. The *B. juncea* crop at Hungry Hill achieved an average fresh weight biomass in the region of just 25 t ha^{-1} (Figure 4.28 i and ii), probably due to the moderate quantity of fertiliser applied and the dryness of the season during early plant growth. The biomass represented only *c.*63% of the quantity of material usually fed through the roll conditioner implement which could have led to suboptimal biofumigant tissue damage by the roll conditioner. Whilst it is clear that the flail topper maceration implement is superior to the roll conditioner here, resulting in higher biofumigation efficacy against *G. pallida*, the roll conditioner implement may need further investigation in future. It is improbable that the shorter chop length of flail treated *B. juncea* material was the primary determining factor between maceration implements. As described in the introduction to this chapter, widespread tissue damage is caused by the application of several stresses to plant tissues, which in biofumigation results in a greater release of VOC's from biofumigant tissues (Matthiessen *et al*., 2004; Morra & Kirkegaard., 2002; Persson, 1987). Therefore, the roll conditioner is unlikely to have been as effective as the flail topper in causing widespread tissue damage in this instance, as visually apparent. However, this hypothesis needs further investigation.

Partial biofumigation was not proven to reduce *G. pallida* viability in the Hungry Hill experiment (Figure 4.29 ii). This could be due to a low effect size as there does seem to be some drop in viability in the partial biofumigation treatment compared to the untreated, which is in line with the results of Ngala *et al*. (2014). However, biofumigation effects for

Hungry Hill can only be attributed to the maceration and incorporation of *B. juncea* plant tissues into soil. No interaction was found between maceration and incorporation implement on biofumigation efficacy against *G. pallida* in factor based analyses either. Provided that material is incorporated into soil with some expediency (Matthiessen *et al*. 2004; Gimsing & Kirkegaard, 2006), it would appear that incorporation implement may be an unimportant biofumigation factor. However, it should be noted that the *B. juncea* biofumigant material incorporated in the Hungry Hill experiment had a lower GSL content than biofumigant crops used in other studies investigating *B. juncea* cv. ISCI 99 for management of *G. pallida*; *c.*4.4 µmol g^{-1} dry tissue versus *c.*10-120 µmol g^{-1} dry tissue (Ngala, 2015; Lord *et al*., 2011). This explains the poorer efficacy recorded for the Hungry Hill site than in other published work, which could potentially lead to an underestimation of the role of incorporation implement in biofumigation of *G. pallida* here. Some caution should therefore be taken in these results (see Section 4.6.1 and Appendix 8.2.4 for GSL data).

Glucosinolate concentrations are likely to have been lower than Ngala (2015) specifically, who developed the methodology used in this study, due to several factors. Factor i) lateness of GSL sample collection; GSL concentrations decline in tissues in response to a decline in ultraviolet (UV) radiation, ii) due to a conservative fertiliser regime, lacking in sulphur, which may have limited GSL biosythesis, and iii) due to the use of different tissue samples for high performance chromatography (HPLC) (Björkman *et al*., 2011; Ngala, 2015). Ngala (2015) used only foliar tissues for GSL assessment in his field work, whereas the GSL results recorded here were composed of both leaves and stems. It is understood that plant organs differ in GSL concentrations, and that leaves are amongst the most GSL rich plant organs (Kirkegaard & Sarwar, 1998). Glucosinolate concentrations could therefore be expected to be lower here than in the work recorded by Ngala (2015). However, GSL concentrations fall comfortably within the range expected of *B. juncea* recorded by Kirkegaard & Sarwar (1998), who found

concentrations to typically vary between 0.1-18.7 µmol g^{-1} dry tissue as an average of whole top growth for 14 different accessions.

4.10.1.2 *Further* Globodera pallida *quantification: cysts, eggs cyst^{-1}, and eggs g^{-1} soil*

As discussed in Chapter 3, interpretation of treatment effects on *G. pallida* egg viability could be distorted if treatments influence the total number of nematodes in an analysis rather than, or in addition to their viability. Furthermore, the wide variability in natural PCN population densities in soil, which could range anywhere between 1 and several hundred eggs g^{-1} soil, could be influential of the accuracy of viability percentage analyses in field experiments, and therefore influence the quality of analyses and the fairness of comparison between experiments (Turner & Subbotin, 2013; Lane & Trudgill, 1999). It is therefore important to quantify the number of nematodes used in viability analyses.

Figure 4.30 i shows the mean number of *G. pallida* cysts retrieved from plots at P_i and P_s sample times, using 200 g of air-dried soil. No difference was observed in numbers of cysts extracted from soil, which was an indication that the sampling and extraction methodology was consistent between sample times for the Hungry Hill experiment. However, *G. pallida* eggs cyst were found to decline between the two sample times from 168 eggs cyst^{-1} to 94 eggs cyst^{-1} (Figure 4.30 ii). This meant that the *G. pallida* population density declined from *c.*47 eggs g^{-1} soil to 31 eggs g^{-1} soil, a decline of 34% over 6-7 months. No evidence of any individual treatment effects on *G. pallida* eggs cyst^{-1} were observable at P_i or P_s sample times compared to the untreated control (Figure 4.30 iii and iv), indicating that the decline could have been due to in-field factors. The consistency in numbers of cysts retrieved from soil between sample times would suggest that the same population hotspots were being sampled between sample times, and therefore cysts size should have been similarly uniform. It would appear to be logical that the decline in *G. pallida* eggs cyst^{-1} could be due to natural decline, and potentially in-field antagonists such as *Purpureocillium lilacinum* and *Pochonia chlamydosporium*, as reviewed in Chapter 1 (Sections 1.3.2 and 1.3.5).

Factor based analysis of biofumigation using incorporation and maceration implements revealed no effect of implement selection on *G. pallida* eggs cyst^{-1}, indicating no biofumigation induced decline in cyst contents related to individual implements (Figure 4.30 v and vi). The observation that biofumigation does not influence egg numbers specifically, is in line with glasshouse experiments discussed in Chapter 3, but is in contradiction to the findings of Ngala *et al*. (2014) where *G. pallida* population densities were shown to decline in the field, in response to biofumigation. It could be, as supported by GSL data, that the biofumigation achieved by Ngala *et al*. (2014) was superior to that achieved at Hungry Hill. Alternatively, quantifying biofumigation effects only a few months after biofumigation at Hungry Hill, may not have allowed time for eggs to die or degrade. Given the uniformity between *G. pallida* eggs cyst at each sample time, rather than between them, the viability analyses previously discussed can be considered fair and robust in comparison of treatment effects. Additionally, moderately high number of nematodes used to calculate viability percentages can be considered as adding strength to viability analyses for the Hungry Hill experiment.

4.10.2 *Field experiment 2 (Crossroads, Shropshire)*

4.10.2.1 Globodera pallida *viability*

The Crossroads experiment was a repeat of the Hungry Hill experiment, running in tandem although geographically separated. Figure 4.32 shows *G. pallida* egg viability at Crossroads field at P_i and P_s sample dates, in single and multiple factor analyses.

Figure 4.32 i showed no difference in egg viability between treatments at P_i, at similar levels of variation in egg viability between treatments to that of the Hungry Hill experiment. Egg viability was therefore highly uniform before biofumigation, which, similarly to the Hungry Hill experiment, presented a good opportunity to observe biofumigation treatment effects at P_s if they were present. Figure 4.32 ii shows a clear biofumigation effect compared to the untreated control, with *G. pallida* viability being reduced by *c.*27% compared to the untreated control using full biofumigation. However,

no differences in efficacy were observable between biofumigation treatments using different maceration-incorporation implement combinations, or between full biofumigation treatments and the partial biofumigation control, which could be separated from the untreated control. This analysis therefore indicated no effect of macerating and incorporating *B. juncea* biofumigant tissues into soil on *G. pallida* viability above that of growing a *B. juncea* crop. The results of this experiment support the premise of reducing *G. pallida* populations by partial biofumigation, as reported by Ngala *et al*. (2014).

However, the Crossroads crop was poor on account of a cabbage root fly infestation (see Appendix 8.2.3). On a scale of 0-5, where 0 represented an undamaged *B. juncea* plant, and 5 represented a *B. juncea* plant where the primary root had been sheared off through damage, plants at the site had an average index of 2.5 by block and by treatment analyses, indicating partial root shearing. Consequently, fresh weight biomass was extremely low at *c.*14 t ha^{-1} (Figure 4.31 i and ii). This quantity represents approximately 35% of the standard weight of material fed through the roll conditioner implement per hectare. The roll conditioner therefore induced very little tissue damage above that achieved by mower discs. A further effect of the cabbage root fly damage, was a thin and lignified crop, which did not break down easily with the flail topper. Glucosinolate concentrations were recorded to be higher than the Hungry Hill experiment at 12.1 µmol g^{-1} dry tissue total, probably in response to the cabbage root fly damage, although a lack of irrigation at the Crossroads site may have also contributed to higher GSL levels in tissues (See section 4.7.1 and Appendix 8.2.4 for GSL data) (Textor & Gershenzon, 2009; Björkman *et al*., 2011). Whilst root GSL's were not quantified, it is probable that these were also present in tissues at high concentrations. The work of Ngala *et al*. (2015b) and Textor & Gershenzon (2009) would suggest that loss of GSL's from roots to soil may have been enhanced by cabbage root fly damage. This could have led to microbial breakdown of 2-popenyl GSL in soil and an enhanced partial biofumigation effect in the Crossroads experiment compared to the Hungry Hill experiment. This could explain why partial biofumigation was as effective as full biofumigation in this instance.

Analysis of treatment CV's again indicated that replication was insufficient to prove small differences between biofumigation implement combinations given the variation in egg viability within treatments at P_s (Figure 4.32 ii) (Berndston *et al*., 1991). However, Figure 4.32 ii showed a similar pattern to that observed at Hungry Hill, whereby treatments using a roll conditioner compared less favourably in terms of efficacy against *G. pallida* than flail topper treatments when simply observing means. Therefore, factor based analyses were applied to data, whereby data was amalgamated according to shared incorporation and maceration implements (Figures 4.32 iii and iv).

In factor based analyses, egg viability CV's were shown to remain stable whilst replication was effectively increased through a simplification of analysis, the same observation as were made for Hungry Hill. Incorporation implement was not found to influence efficacy against *G. pallida* in this instance (4.32 iii), which was again in contrast to the work of Roubtsova *et al*. (2007) but in support of the first field experiment. This observation adds weight to the premise that incorporation implement may be of low importance in field scale biofumigation, provided incorporation is swift. For instance, a spader or rotavator which could be mounted to the same tractor as a flail, may only be more appropriate for biofumigation than a plough in practical terms. Maceration implement was again found to be influential of biofumigation efficacy against *G. pallida* (4.32 iv). An extra 4% efficacy was achieved when practising biofumigation with the flail topper than with a roll conditioner. Roll conditioner treated *B. juncea* material had a longer section length than that of the flail topper in the Crossroads experiment; *c*.400 mm compared to *c.*200 mm. Similarly to the Hungry Hill experiment, flail topper treated *B. juncea* sections showed visual signs of greater tissue trauma than roll conditioner treated. The flail topper appeared to again be the superior maceration implement for biofumigation. No interaction was found between maceration and incorporation implement on biofumigation efficacy against *G. pallida* overall.

4.10.2.2 *Further* Globodera pallida *quantification: cysts, eggs cyst^{-1}, and eggs g^{-1} soil*

Figure 4.33 i shows the mean number of *G. pallida* cysts retrieved from plots at P_i and P_s sample times, using 200 g of air-dried soil. No difference was observed in numbers of cysts extracted from soil, which was an indication that the sampling and extraction methodology was consistent between sample times in this experiment, similarly to the Hungry Hill experiment. *Globodera pallida* eggs cyst^{-1} were found to remain consistent between the two sample times, at 249 and 253 eggs cyst^{-1} for P_i and P_s sample times respectively (Figure 4.33 ii).The mean *G. pallida* population density at the Crossroads experiment remained consistent between sample times at 250-265 eggs g^{-1} soil between sample times. No evidence of any individual treatment effects on *G. pallida* eggs cyst^{-1} were observable at P_i or P_s sample times either (Figure 4.33 iii and iv). Factor based analysis of biofumigation treatments using diverse incorporation and maceration implements, also showed no effect of implement selection on *G. pallida* eggs cyst^{-1} (Figure 4.33 v and vi). The conclusion from these analyses in combination, indicates no decline in cyst contents related to biofumigation, or biofumigation carried out using different incorporation and maceration implements. The observation that biofumigation does not influence egg numbers specifically, is again in line with glasshouse experiments discussed in Chapter 3, and with the results for the Hungry Hill experiment. Viability analyses can again be considered fair and robust in comparison of treatment effects due to high uniformity in *G. pallida* counts between sample times and treatments. Although the *B. juncea* crop grown for the Crossroads experiment was poor, the high *G. pallida* counts at the field site, and their high uniformity, would have been beneficial for viability analyses in providing high resolution, fair and comparable viability data.

4.10.2.3 *Penetrometer resistance*

The working depth of incorporation implements and general structural properties of soil was assessed at the Crossroads experiment following biofumigation, using a cone penetrometer. Figure 4.34 visually depicts the working depths of each implement, and general penetrometer resistance at 100 mm depth increments, from 0-400 mm depth.

Figure 4.34 is supported statistically by Tables 4.5 and 4.6 where the spader is shown to work to approximately 400 mm depth, the plough to 300 mm depth and the rotavator to *c.*150 mm depth, although penetrometer resistance did not differ from untreated or partial biofumigation controls in the case of the rotavator. Penetrometer resistance readings did, however, show both the spader and the plough to produce a looser soil structure than the rotavator, and the spader a looser soil structure than the plough. It was therefore surprising that the spader implement, which produced a soil condition more conducive for fumigation and for retention of biofumigant VOC's in soil, should not have facilitated a higher biofumigation efficacy than other incorporation implements in both Hungry Hill and Crossroads field experiments (Whitehead, 1998; Cremlyn, 1991; Lord *et al*., 2011). It is possible that the spader may have worked the *B. juncea* tissues too deep into soil, diluting the material which was already low of GSL concentration, negatively influencing efficacy in a similar way to when granular nematicides are applied too deep (Woods & Haydock, 2000). Alternatively, a looser soil structure may have facilitated greater sorption of biofumigant VOC's to OM, which again, could have negatively influenced efficacy. Gimsing *et al*. (2009) have shown AITC readily sorps to soil OM which supports this hypothesis. Assessment of soil bulk density could have given more detail on soil pore space after spader treatment than penetrometer resistance alone, and so should be considered in future studies.

4.10.3 *Combined analysis for field experiments 1 and 2 (Hungry Hill, Norfolk and Crossroads, Shropshire)*

4.10.3.1 Globodera pallida *viability*

Due to a seemingly consistent pattern of biofumigation effects between Hungry Hill and Crossroads experiments, methods of assessing variance between experiments were investigated. An F-variance ratio test was used to determine whether core viability data sets could be combined between field experiments, to increase replication and tease out potentially small treatment effects obscured by background levels of variation (Mead *et al*., 1993). The P_i data sets could not be combined, although P_s data could be.

Figure 4.35 i shows a partial biofumigation effect on *G. pallida* viability, which is separable from the untreated control, full biofumigation effects which are separable from the partial biofumigation control, and differences in efficacy between full biofumigation treatments using different implement combinations. The most effective implement combination was a flail topper-spader combination, which was 8% more effective in reducing *G. pallida* viability than the roll conditioner-rotavator treatment. It would appear that the spader could have been responsible for some of the biofumigation efficacy observed in this analysis, although still not to any level which would justify the expense of running such an implement versus other implements already widely available on farms. Given that penetrometer results from the Crossroads experiment showed the spader to have the greatest working depth and to produce the loosest soil structure, it is possible that *B. juncea* biofumigant material was worked into soil in a slightly more effective way with the spader, and that encysted *G. pallida* eggs were better exposed to VOC's than with other implement combinations (Whitehead, 1998). It is likely that the spader was more effective in retaining *B. juncea* biofumigant VOC's in soil where they could be effective against *G. pallida*. Both the depth of incorporation and the use of a powered smear roller could have limited VOC off-gassing for the flail topper-spader treatment in this instance (Cremlyn, 1991). Roubtsova *et al*. (2007) found in their soil column work, that mixing biofumigant material into soil improved biofumigation efficacy against root knot nematodes, whilst Lord *et al.* (2011) have shown the importance of sealing biofuimigant VOC's in soil for biofumigation efficacy against *G. pallida*, supporting the hypotheses presented here. Further work should be carried out to investigate placement of biofumigant material in the field and the importance of sealing soil after biofumigation. It is possible that a repeat of the work carried out here with a higher biomass crop might find larger treatment effects and be able to cast further light on the importance of incorporation implements in biofumigation of PCN generally. It is quite possible that incorporation is an important biofumigation factor, but that its importance in biofumigation is second to the quality of biofumigant crop and the effectiveness of maceration strategy. Therefore, incorporation implement might only become important

in influencing biofumigation efficacy against PCN when incorporating high biomass, high GSL crops. A further point for consideration; Chapter 3 indicated that high biomass *B. juncea* crops could be implicated in influencing soil structure after incorporation. Therefore, the spader, which is likely to be involved in diluting biofumigant material within soil as previously discussed, particularly where high biomass crops are incorporated, could mitigate the potential effects of biofumigant biomass on soil structure. This could then reduce the risk of VOC loss to headspace above soil by off-gassing, by presenting a more effective physical barrier to VOC movement, in a similar way to soil moisture or plastic films (Simpson *et al*., 2010; Lord *et al*., 2011). If true, the spader could be a far superior implement for incorporation of high biomass biofumigant crops.

Factor based analyses (Figure 4.35 ii and iii) again showed no overall effect of incorporation implement on biofumigation efficacy, and no interaction between incorporation and maceration implement effects on *G. pallida* viability. Maceration implement was again found to influence biofumigation efficacy against *G. pallida* as would be expected given the results of single experiment analyses (Figure 4.35 iii).

4.10.4 *Field experiment 3 (Roys Corner, Shropshire)*

4.10.4.1 Globodera pallida *viability*

Following Hungry Hill and Crossroads field experiments, it appeared apparent that maceration implement had the potential to markedly influence *B. juncea* biofumigation efficacy against *G. pallida*. The importance of incorporation implement in biofumigation of *G. pallida* appeared to be of lower importance. Field experiment 3 was therefore designed to investigate the set-up of a haulm topper, which is a flail type implement, to determine whether implement set-up, as well as selection, could be influential of efficacy. Field experiment 3 investigated two shear-plate levels; open and closed, in combination with three flail tine levels; pig-tail tine, knife tine, and a balanced combination of pig-tail and knife tines, compared to an untreated control, and a partial biofumigation control.

No difference was observed in egg viability between treatments at P_i (Figure 4.37 i), and only moderately low levels of variation were found in egg viability between treatments. Egg viability was therefore considered moderately uniform before biofumigation, although not so uniform as in the previous two field experiments. Figure 4.37 ii showed a clear biofumigation effect, with *G. pallida* viability being reduced by *c.*31% compared to the untreated control. However, no differences in efficacy were observable between biofumigation treatments using different tine and shear-plate implement combinations. The knife tine with closed shear-plate treatment was inseparable from both the untreated and partial biofumigation treatments. Generally, biofumigation treatment was shown to increase variation in *G. pallida* egg viability in field experiment 3 which could have been detrimental to viability analyses. Egg viability CV values increased from *c.*9% at P_i, to 23% at P_s for biofumigation treatments. Using the replicate calculator provided by Berndston *et al*. (1991), it can be shown that to prove a 9% difference between treatments, as found between the knife tine with closed shear-plate treatment, and the combined tine with closed shear-plate treatment (Figure 4.37 ii), an egg viability CV of no greater than *c.*4% would be acceptable for 6 replicates of each treatment. Therefore, the variation in egg viability between treatments was too high to enable treatment differences to be determined at the P_s timing in field experiment 3. As with Hungry Hill and Crossroads experiments, a factor based analysis was applied to data, whereby data was amalgamated according to shared tine and shear-plate levels, in an attempt to increase replication by simplifying the analysis (Figures 4.37 iii and iv).

In factor based analyses, egg viability CV's were shown to remain stable whilst replication was effectively increased through a simplification of analysis. Neither tine or shear-plate settings were found to influence efficacy against *G. pallida* (Figure 4.37 iii and iv). However, field experiment 3 was found to have weaknesses which were not present in the first two field experiments, which could have negatively influenced results. Fresh biomass was the highest of all three field experiments at *c.*48 t ha^{-1} fresh weight (Figure 4.36) and GSL concentrations in tissues slightly higher than the Hungry Hill

experiment at 6.2 µmol g^{-1} dry tissue. However, the numbers of nematodes used to generate viability data was poor. In general, *G. pallida* counts were considered in the moderate category (*c.*24 eggs g^{-1} soil) described by Haydock & Evans (1998b) as described in Section 1.3.2, which was considerably lower than in other field experiments, and may have negatively influenced the resolution of viability data in this instance. Furthermore, soil moisture conditions were visibly drier in the Roys Corner experiment than the Hungry Hill and Crossroads experiments, due to a spring rather than an autumn incorporation. Soil was probably in the region of 25-50% of field capacity as soil dust could be seen surrounding the rotavator during work, although soil moisture was not recorded in this instance. Given the high level of biomass and moderate incorporation depth of *c.*300 mm, soil moisture conditions could have been insufficient to prevent VOC off-gassing in this instance, and negatively influenced efficacy. Similarly, soil temperatures may have been too low at that time of the year for effective fumigation. No real conclusions could be determined as to the importance of tine and shear-plate set-up on a haulm topper from this analysis, although the combined tine-closed shear-plate haulm topper set-up showed the lowest mean *G. pallida* viability in Figure 4.37 ii.

4.10.4.2 *Further* Globodera pallida *quantification: cysts, eggs cyst^{-1}, and eggs g^{-1} soil*

Figure 4.38 i shows the mean number of *G. pallida* cysts retrieved from plots at P_i and P_s sample times, using 200 g of air-dried soil. The number of cysts retrieved at P_s was lower than at P_i (Figure 4.38 i), 68 versus 93 cysts 200g^{-1} soil respectively. However, no difference was found in eggs $cyst^{-1}$ between sample times (Figure 4.38 ii), which indicated that the difference in cyst counts between sample times was simply due to operator inconsistencies in cyst extraction. Further treatment analyses showed no difference in eggs $cyst^{-1}$ between treatments at P_i or at P_s sample times, indicating no effect of biofumigation on eggs $cyst^{-1}$ (Figure 4.38 iii and iv). Furthermore, factor based analyses revealed no effect of tine or shear-plate biofumigation treatments on *G. pallida* eggs $cyst^{-1}$ (Figure 4.38 v and vi). The consistency of egg numbers was again considered good for fair comparison of treatments, particularly in viability analyses. However, the

low *G. pallida* population density at field experiment 3 may have influenced the resolution of viability data as previously mentioned. The mean *G. pallida* population density at field experiment 3 was found to range between 25 and 13 eggs g^{-1} soil between sample times, which was lower than in other field experiments. It is probable that these low counts may be responsible for the high *G. pallida* CV's observed in field experiment 3, and subsequently, poor results. Future experiments would benefit from *G. pallida* population densities near those recorded for the Crossroads experiment, *c.*250 eggs g^{-1} soil.

In future, a stratified randomised technique might be used to reduce variation in field experiments such as the Roys Corner experiment (Mead *et al*., 1993). The approach allocates treatments within blocks, and blocks themselves, within an experiment according to nematode densities. Appendix 8.4.1 shows how blocks and treatments might have been applied to field experiment 3 using this approach. Analysis of the P_i data set using the fully randomised approach showed the general block CV to be 48%, and treatment CV to be 76% (Figure 4.8). Using the stratified randomised approach shown in Appendix 8.4.1, block CV increased to 85% whilst treatment CV reduced to 40%, indicating an increased power to determine treatment differences from a more uniform P_i treatment data set. Future work should adopt this strategy as a powerful means of reducing the effects of natural variation on field experiment results.

4.10.4.3 Brassica juncea *bite/section length*

The quality of macerated *B. juncea* tissue was quantified in field experiment 3 in terms of the section length of tissues. This assessment methodology did not provide any information on the levels of bruising of *B. juncea* tissues following maceration, however, it was deemed a simple and time efficient assessment technique which might be used in combination with VOC data to assess maceration differences between treatments. A clear difference in *B. juncea* section length was observed following maceration of *B. juncea* plants with the pig-tail tine with closed shear-plate, compared to all open shear-plate treatments regardless of tine set-up (Figures 4.39 i). All other closed shear-plate

treatments were inseparable from the previous treatments. *Brassica juncea* section lengths ranged between *c.*180-300 mm following maceration, with low levels of variance in section length when simply viewing SEM's. It generally appeared that a closed shear-plate position caused a finer chop due to a closer gap between tines and shear-plate for *B. juncea* material to pass through, as anticipated (Persson, 1987). When factor based analysis was carried out, it was found that tine did not influence *B. juncea* section length, but that shear-plate position did (Figures 4.39 ii and iii). However, it was still unclear whether tine type might have influenced the level of bruising of tissues, as influenced by stresses other than shear, such as bending, torsional, tensional or compressional stresses (Persson, 1987). Tine dimensions were diverse and so could have been expected to inflict different levels of damage to tissues; pig-tail tine – 65 x 230 x 8 mm, knife – 40 x 230 x 8 mm, where the pig-tail tine cutting face was 65 mm, and the knife tine cutting face was 8 mm wide. However, no differences were observed.

Tine speed in the Roys Corner experiment was *c.*32 m s^{-1} due to a flail rotor speed of 1250 revolutions min^{-1} (rpm), and a distance between the cutting edge of each tine from the centre of the rotor of *c.*242 mm. Chancellor (1988) and McRandal & McNulty (1978) suggest that tine speeds in excess of 10-20 m s^{-1} are desirable for efficient impact cutting of forage crops because at these speeds, tines efficiently transfer cutting forces through plant material with low material acceleration. However, for the Roys Corner experiment, tine speeds in excess of 10-20 m s^{-1} could be considered detrimental to biofumigation. Lower tine speeds which are less efficient in cutting should accelerate plant material by widely distributing cutting forces through tissues, which could lead to much greater cellular damage in biofumigation. Future experiments should consider tine speed and numbers of tines used on macerators to achieve maximum tissue bruising and cutting, perhaps instead of investigating tine selection as investigated here. The haulm topper used in the Roys Corner experiment made *c.*77 cuts m^{-1} travelled when travelling at 3 km h^{-1}. Due to such high tine speeds, this implement was probably sub optimal for maceration of biofumigant material, although the number of cuts made per meter was

high. Running the implement at a power take-off (PTO) speed of 540 rpm instead of 1000 rpm would half tine speed, potentially improving the implement for biofumigation. However, cuts m^{-1} travelled could also be expected to reduce to 38.

4.10.4.4 Brassica juncea *volatile organic compounds in soil*

Analysis of VOC's collected from soil using the custom made VOC moles described earlier, found no difference in DMS, DMDS or the combination of DMS, DMDS and AITC for different treatments compared to the untreated treatment (Figure 4.40). However, AITC concentration in soil was found to vary between treatments (Figure 4.40 iii). Multiple comparisons could not detect where differences occured. However, it would appear that the pig-tail and knife tine combination with open shear-plate might have been implicated in releasing AITC into soil at levels which could be separated from the untreated control, given that its mean was most distant from the control. This observation is interesting given that the combined tine with open shear-plate treatment produced one of the longest bite/section lengths at maceration, indicating that this combination could have applied a more damaging range of stresses to plant material than some of the other treatments, which were probably more reliant on shear stress and therefore produced a shorter section length, such as the pig-tail with closed shear-plate treatment (Persson, 1987). Factor based general ANOVA analyses were ineffective in determining differences in AITC output between tines or shear-plate settings, probably due to the low number of replications of each treatment and high background variation, despite the simplified analysis (Figure 4.41) (Mead *et al*. 1993). It is possible that the open shear-plate position, which produced a longer section length *B. juncea* residue, might have also enabled material to pass over the haulm topper rotor more effectively, resulting in more blunt maceration than when the shear-plate was closed. Other analyses investigated the composition of the *B. juncea* VOC profile, and found AITC to be more abundant than DMS, but not DMDS. However, the biofumigation here had perhaps a greater quantity of DMS and DMDS than was expected, at a combined contribution of 56% to the total VOC composition (Figure 4.42). Bending & Lincoln (1999) proposed that DMDS most

specifically, could have an important and understated role in biofumigation using *B. juncea*. Perhaps this area needs revisiting in light of these findings.

4.10.5 *General considerations*

The importance of soil moisture and temperature has not been discussed here in relation to biofumigation success against *G. pallida* because neither factor was found to influence *G. pallida* results when used as covariates. It is probable, given the results of Chapter 3, that soil moisture conditions in the field might have influenced efficacy if the differences between plots were greater. Similarly for temperature. However, plots were consistent for these measurements. Other considerations to mention include soil texture, OM and pH (see Appendices 8.1.1 – 8.1.3). Each site was considered a loamy sand which is representative of many UK potato fields, however, it may have been beneficial to investigate biofumigation over a wider range of soil types. Similarly, OM was found to be consistent between sites with a range of 1.6-2.4%. Again, this narrow range of OM does not reflect the full range of OM contents of UK potato growing soils which could be considered a weakness in this work. However, all field sites were found to have acceptable pH for biofumigation which ranged between 6.6-7.2 between sites, which can be considered positive, and conducive to ITC generation during GSL hydrolysis. Also, the geographical separation between Hungry Hill and the two Shropshire experiments can be considered beneficial in testing biofumigation in different climatic conditions, as can the separation of the two Shropshire sites by growing season.

4.10.6 *Conclusions*

In conclusion, the null hypothesis for this chapter that 'neither maceration or incorporation implement selection or set-up has an effect on *B. juncea* biofumigation efficacy against PCN' can be considered disproven in relation to maceration implement selection, where biofumigation using a flail topper was found to be more effective in suppressing *G. pallida* than biofumigation using a roll conditioner. However, the null hypothesis can be broadly accepted for incorporation implement. No difference in *G.*

pallida viability was found between biofumigation treatments analysed by incorporation factor for individual experiments. The spader combination with flail was shown to be the most effective implement combination in a combined analysis of the Hungry Hill experiment and the Crossroads experiment, but further work is required to determine the importance of the spader in this implement combination. Selection of the spader cannot currently be supported for most growers due to expensive running costs for a small return in efficacy, however, where easily available and for high biomass crops, its use may be justified. Similarly, no effect of maceration set-up in terms of tines or shear-plate position was observed on *B*. *juncea* biofumigation efficacy against *G. pallida*, however, results may have been negatively influenced by low *G. pallida* population densities in the field. The null hypothesis that maceration set-up is not influential of efficacy can be broadly accepted in this instance, with regard to tine and shear-plate factors, although future investigations might consider tine speed, numbers of tines per flail rotor and implement forward speed as factors influencing biofumigation efficacy against PCN. The chapter aim to 'determine whether maceration and/or incorporation implement selection and set-up can be influential of *B. juncea* biofumigation efficacy against PCN' can be considered answered. Maceration implement selection is important in biofumigation of *G. pallida*. However, the set-up of topping implement by tine and shear-plate positioning would appear to be less important. Similarly, the selection of incorporation implement would appear to be unimportant in biofumigation of PCN. Further research could involve screening different *B. juncea* maceration qualities against PCN *in-vitro*, to determine the potential for improving biofumigation of PCN in the field by manipulation of maceration factors, such as tine speed. All experimental objectives were achieved.

4.10.6.1 *Recommendations*

1 Use a front mounted flail type implement to macerate *B. juncea* biofumigant crops for maximum suppression of *G. pallida*.

2 For high biofumigation potential crops, and where readily available, consider a spader for incorporation of *B. juncea* biofumigant crops for suppression of *G. pallida.*

Investigation of cyst moisture as a factor influencing biofumigation success against potato cyst nematodes

5.1 Introduction

Glasshouse experiments described in Chapter 3, suggested that soil moisture could be influential of biofumigation efficacy against the potato cyst nematode (PCN) *Globodera pallida*. *Brassica juncea* biofumigation efficacy was found to be *c.*14.3% higher where biofumigation was carried out at 50% of field capacity, compared to 0 and 100% of field capacity, and water saturated soil. However, mechanisms for the higher efficacy were not investigated. As reviewed in Chapter 1 (Section 1.4.8), and generally discussed in Chapter 3, soil moisture could have a role in enhancing glucosinolate (GSL) hydrolysis from incorporated biofumigant tissues, to generate volatile organic compounds (VOC) such as allyl-isothiocyate (AITC), and could influence VOC movement and retention in soil, and contact with encysted PCN eggs (Morra & Kirkegaard, 2002; Matthiessen *et al*., 2004; Simpson *et al*., 2010; Borek *et al*., 1998). Of these areas, the role of soil moisture in influencing VOC contact with PCN cysts is perhaps the least well understood. However, evidence has been presented by Borek *et al*. (1998) which would suggest biofumigant VOC's to be generally more hydrophobic than hydrophilic.

A study investigating the antibacterial mechanism of action of AITC found that AITC was more effective in degrading the cell membranes of *Salmonella*, *Escherichia* and *Listeria* spp. bacteria when in a vapour phase, rather than in a liquid solution (Lin *et al*., 2000), which also supports Borek *et al*. (1998). The chemical composition of a PCN egg shell is similar to many bacteria (Clarke *et al*., 1967; Cummins, 1956), comprising of many similar or the same amino acids, such as glycine. It is therefore possible that encysted PCN are also more susceptible to ITC's when cysts are dry. If proven to be true, this could have important consequences for field scale biofumigation and could influence

decision making around the time of biofumigant incorporation according to soil moisture conditions. This chapter therefore, investigates the role of cyst moisture as a factor which could influence *B. juncea* biofumigation efficacy against PCN.

5.1.1 *Chapter aim*

i) Determine whether *B. juncea* biofumigation efficacy against PCN is affected by the moisture condition of cysts.

5.1.2 *Chapter null hypothesis*

i) Cyst moisture condition does not influence efficacy of *B. juncea* biofumigation against PCN.

5.2 Materials and methods: *in-vitro* experiment 1

5.2.1 *Experiment objectives*

i) Develop experimental capsules for exposing PCN to *B. juncea* biofumigant VOC's, and for quantifying VOC compounds using Tenax-TA sorbent tubes.

ii) Determine the quantity of *B. juncea* biofumigant material required for addition into experimental capsules, to induce approximately 50% mortality of PCN.

iii) Quantify *B. juncea* biofumigation effects on PCN cyst contents.

iv) Quantify *B. juncea* biofumigant VOC's within experimental capsules during experimentation.

5.2.2 *Experimental capsule development*

Custom made experimental capsules were required to accurately determine whether cyst moisture could influence *B. juncea* biofumigation efficacy against PCN. The capsules needed to be i) reusable, and therefore able to be cleaned between experiments without material degradation or carryover of contaminants, ii) uniform of internal volume and sealable, and iii) self-contained; capsules needed to house biofumigant material, PCN cysts exposed to relevant moisture conditions, and Tenax-TA

sorbent tubes for assessment of biofumigant VOC's. To meet these criteria, the capsules were constructed from glass, polytetrafluoroethylene (PTFE) and silicon laboratory consumables, which would be uniform of dimensions, and enable cleaning and drying of materials between experiments; ambient air drying for PTFE and silicon components, and forced air drying at 105°C for glassware. Figure 5.1 shows the capsule design, providing a description of core components. Capsules are hereafter referred to as volatile organic compound exposure capsules (VOCEC's).

Figure 5.1: Custom-made VOCEC, composed of a 40 mL glass vial (A), custom made Tygon® R-3603 plastic tubing/ 400 µm diameter PTFE mesh, detachable 'cyst basket' (B), 70 mm cut section of a glass Pasteur pipette (C), polypropylene cap fitted with a 3 mm thick PTFE-silicone septa (D), Tygon® R-3603 plastic tubing connecting glass

pipette and Swagelok® fitting components (E), and Swagelok® fitting for Tenax-TA sorbent tube attachment (F).

5.2.2.1 *Construction procedure for volatile organic compound exposure capsules*

Each VOCEC was made from a 40 mL glass vial fitted with a 3 mm diameter septum cap with composite PTFE-silicone septa (Sigma-Aldrich®) (Figure 5.1 A and D). Septum caps were each pierced using forceps, and then a shortened glass Pasteur pipette (ISO 7712) (Fisherbrand®) inserted into each cap; reduced from 150 to 70 mm in length using a hardened steel needle file (Carl Kammerling int.) (Figure 5.1 C). The junction between the cut pipette and the septum cap was glued using adhesive (Super Glu, Bostik). Sections of Tygon® R-3603 plastic tubing (part number: AAC1S1504) were then cut to 10 and 20 mm length sections. The 10 mm sections were glued to a PTFE mesh sheet (*c.*400 µm diameter) using the adhesive, and left to dry. Once dried, the Tygon® R-3603 plastic tubing sections were cut from the mesh to produce 'cyst baskets' for suspending *G. pallida* cysts within individual VOCEC's, above *B. juncea* biofumigant material (Figure 5.1 B). The 20 mm Tygon® R-3603 plastic tubing sections were used to attach Swagelok® fittings to cut glass pipettes, and glued in place using the adhesive (Figure 5.1 E and F).

5.2.3 *Experimental design*

In-vitro experiment 1 was carried out between September and October 2017 at the Harper Adams University (HAU) nematology laboratory facility. The experiment consisted of one factor; 'number of *B. juncea* leaf discs' introduced into each VOCEC for exposure to PCN. Figure 5.2 shows the experimental design, for which five levels were selected; 0, 1, 2, 4, and 8 *B. juncea* leaf discs. Treatments were arranged in a randomised block design and replicated four times each. Replication was sufficient to enable treatment differences of 15% in PCN egg viability to be determined for background variation levels in egg viability of between *c.*4% between replicates of like treatments (Berndston, 1991). The design had 12 residual degrees of freedom (RDF).

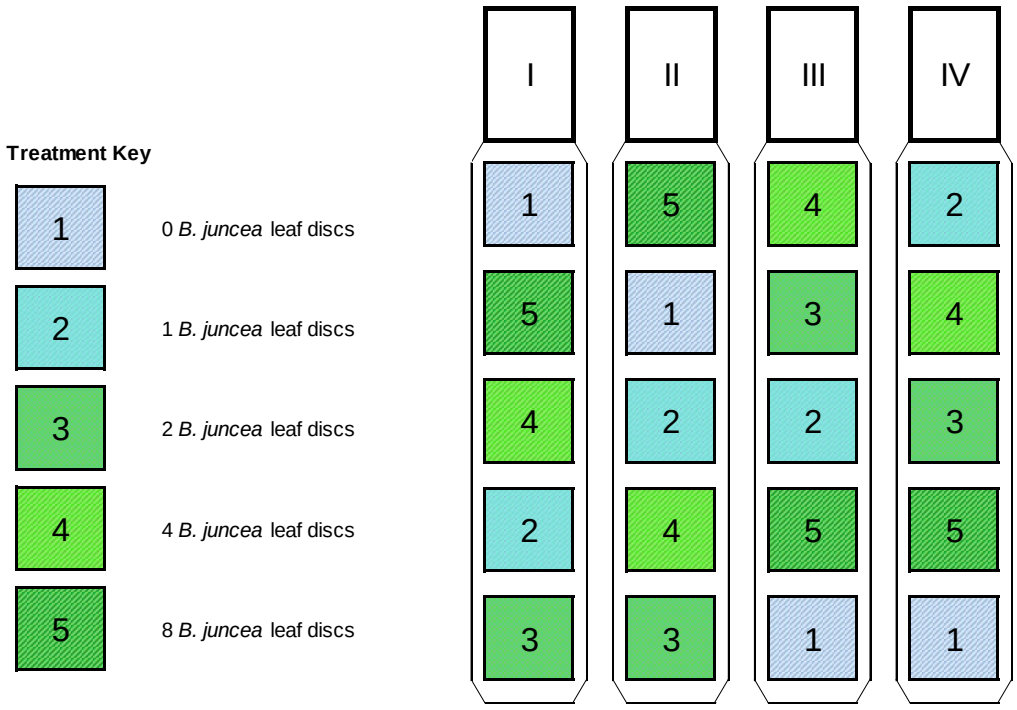


Figure 5.2: *In-vitro* experiment 1 design showing the spatial allocation of treatments 1-5 to VOCEC's. Blocks are represented using Roman numerals (I-IV).

5.2.4 *Core experimental materials*

5.2.4.1 Brassica juncea *leaf discs*

Brassica juncea leaves were collected from discard 'D' pots from glasshouse experiment 2 (see Figure 3.13) in April 2016. Leaf discs were cut from leaves using a stainless-steel 20 mm diameter cork-borer (Figure 5.3), and then discs stacked between layers of 250 µm nylon mesh (Figure 5.4) before being flash frozen with liquid nitrogen, and stored at -20°C. See Section 3.7.1 and Appendix 8.2.4 for the dry-matter and GSL content of plants from glasshouse experiment 2. Note, only leaves were used in this experimental work whereas leaves and stems were combined in dry-matter and GSL analysis in glasshouse experiment 2.

5.2.4.2 *Potato cyst nematodes*

Cysts were obtained from Lodge 1 field near Shawbury, Shropshire (UK) (grid reference: SJ 58768 25337) in April 2017, before potato planting. The PCN were identified as *G. pallida* by polymerase chain reaction (PCR) (see Appendix 8.1.4 for PCR and

Appendices 8.1.1-8.1.3 for site specific details). Cysts were graded to between 500-1000 µm in size, as per glasshouse experiment 2 (see Section 3.3.4.2), in an attempt to reduce variation in egg contents (Twomey *et al*., 1995).

Figure 5.3: *Brassica juncea* leaf (A), 20 mm diameter leaf discs (B), and a stainless-steel cork borer (C). Scale indicates the diameter of leaf discs.

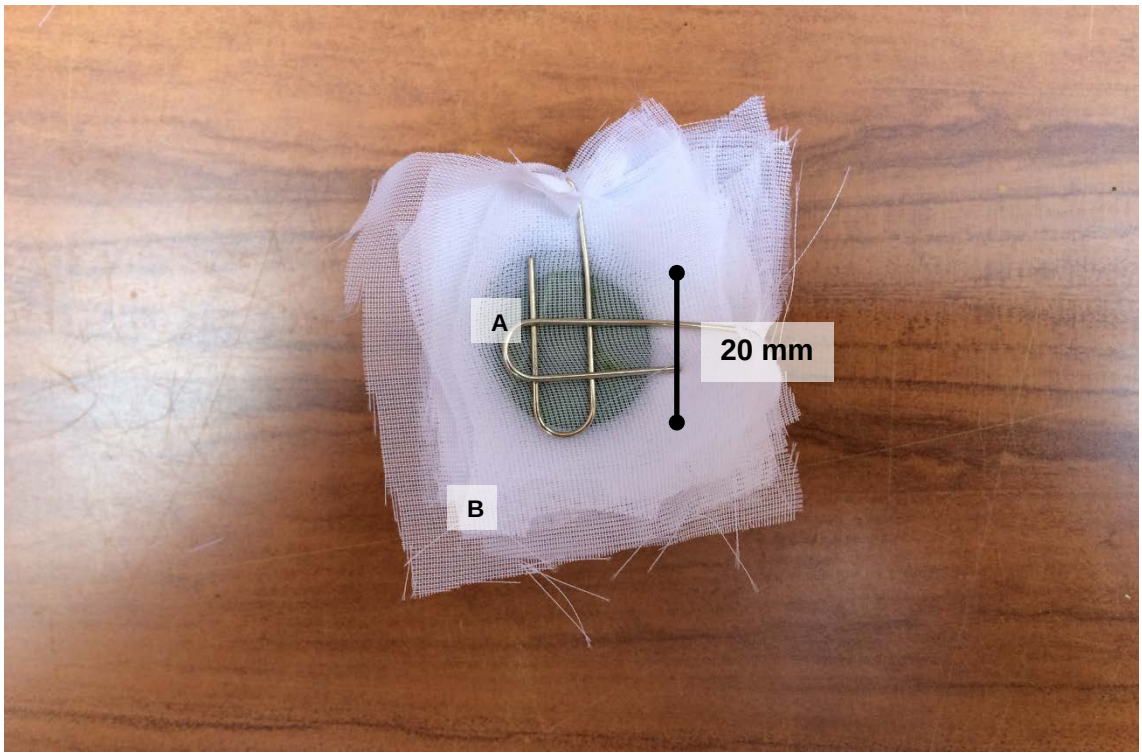


Figure 5.4: *Brassica juncea* leaf disc (A) separated by layers of 250 µm nylon mesh (B). Scale indicates the diameter of leaf discs.

5.2.5 *Experiment initiation*

A total of 50 *G. pallida* cysts were counted for each VOCEC, then introduced into cyst baskets. *Brassica juncea* leaf discs were taken from the freezer and transported to the laboratory on dry ice to prevent discs defrosting prematurely. Leaf discs were then individually peeled from nylon mesh using forceps to generate treatment quantities. Discs were submerged into distilled water for approximately 1 s to thaw (Figure 5.5), then introduced to VOCEC's according to the design shown in Figure 5.2. Individual VOCEC caps were fitted and tightened immediately after introduction of *B. juncea* material, suspending *G. pallida* cysts in cyst baskets above *B. juncea* leaf disc material. Blank Tenax-TA sorbent tubes were than attached to VOCEC Swagelok® fittings for passive entrainment of biofumigant VOC's (Figure 5.6). Tenax-TA sorbent tubes were removed after 72 h for GC-MS analysis (see Section 2.4.3). Cysts and *B. juncea* were left in VOCEC's for a further 11 days, after which *G. pallida* were processed using the Melodolas blue staining technique as per Section 2.3.2. Laboratory conditions were maintained at 20°C temperature for the duration of experimental work. Figure 5.6 shows a single block from *in-vitro* experiment I.

Figure 5.5: Procedure for submerging *B. juncea* leaf discs (A) in distilled water, before introduction into VOCEC's containing *G. pallida* cysts (B).

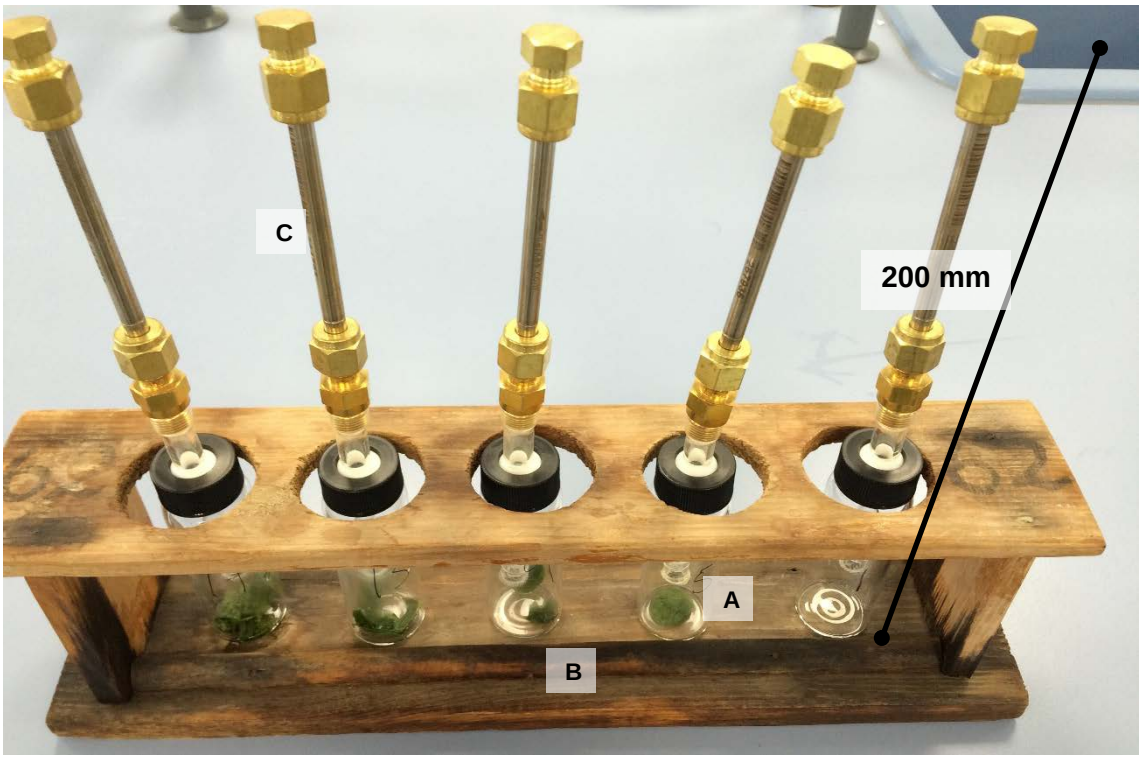


Figure 5.6: VOCEC arrangement for block III from *in-vitro* experiment 1, showing *G. pallida* cysts (A) suspended above different quantities of *B. juncea* leaf discs (B), with Tenax-TA sorbent tubes fitted for VOC entrainment (C). Scale indicates the length of a VOCEC fitted with a Tenax-TA sorbent tube.

5.3 Materials and methods: *in-vitro* experiment 2 and 3

5.3.1 *Experiment objectives*

i) Quantify the contents and viability of eggs in PCN cysts after exposure to a range of cyst moisture conditions in the presence of *B. juncea* leaf disc VOC's.

5.3.2 *Experimental design*

In-vitro experiments 2 and 3 were carried out between November 2017 and January 2018 at the HAU nematology laboratory facility. The experiments were identical of design, and investigated only one factor; the 'moisture condition of cysts' in VOCEC's during biofumigation with *B. juncea* leaf discs. Figure 5.7 shows the experimental design, for which four levels were selected; soaked cysts, water film covered cysts and air-dried cysts, all exposed to *B. juncea* leaf discs, and an air-dried untreated treatment receiving

no. *B. juncea* leaf discs. Treatments were arranged in a randomised block design and replicated five times each. Replication was sufficient to enable treatment differences of 10% in PCN egg viability to be determined for background variation levels in egg viability of between *c.*4% between replicates of like treatments (Berndston, 1991). The design had 12 residual degrees of freedom (RDF).

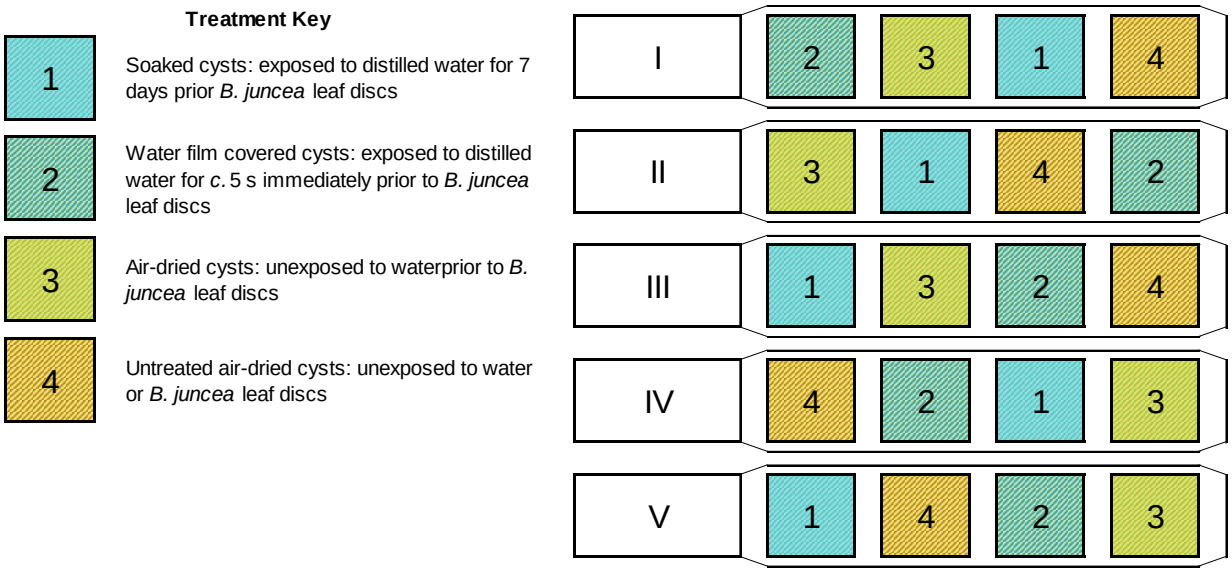


Figure 5.7: *In-vitro* experiments 2 and 3 design, showing the spatial allocation of treatments 1-4 to VOCEC's. Blocks are represented using Roman numerals (I-IV).

5.3.3 *Core experimental materials:* Brassica juncea *leaf discs and potato cyst nematodes*

In-vitro experiments 2 and 3 used the same *B. juncea* leaf discs and *G. pallida* population as *in-vitro* experiment 1, and processed materials using the same methodologies (see Sections 5.2.3.1 and 5.2.3.1).

5.3.4 *Experiment initiation*

A total of 50 *G. pallida* cysts were counted into cyst baskets for each VOCEC. Cyst baskets were soaked in distilled water in well plates for 7 days to generate the soaked cyst moisture treatment. Cysts treated with a water film were only submerged in distilled water immediately prior to exposure to *B. juncea* leaf discs. The remaining air-dried cysts

were only suspended above *B. juncea* leaf discs in VOCEC's. Four *B. juncea* leaf discs were used per VOCEC after the results shown in Figure 5.8 where four *B. juncea* leaf discs were found to be effective in causing *c.*50% mortality of *G. pallida* eggs. The remaining experiment initiation methodologies and conditions remained consistent with *in-vitro* experiment 1 with the exception of Tenax-TA tubes, which were not fitted to VOCEC's in either *in-vitro* experiment 2 or 3 due to a malfunction with GC-MS equipment.

5.4 Assessments

5.4.1 *Potato cyst nematode*

Globodera pallida egg viability and eggs cyst^{-1} were quantified for *in-vitro* experiments 1 and 2 as described in Section 2.3.2 using Equations 2.2 and 2.3.

5.4.2 *Quantification of volatile organic compounds released from B. juncea leaf discs*

Twenty Tenax-TA tubes were collected from VOCEC's in *in-vitro* experiment 1, at 72 hours after initiating the experiment. However, Tenax-TA sorbent tubes were not processed due to a primary malfunction with GC-MS equipment following a power outage at the University. The computer component of the GC-MS was irreparably damaged, and its replacement was too expensive to be purchased immediately after the malfunction. Samples are currently in storage.

5.5 Data analysis

Data were analysed by analysis of variance (ANOVA) using GenStat® 16th Edn. (VSN International). Treatment effects for ANOVA were compared using Tukey's multiple range test at 95% certainty. A cumulative upper F-variance ratio test was used to justify combining *in-vitro* experiment 2 and 3 data sets. Coefficient of variation (CV) values were calculated using Microsoft® Excel 2016. Figures were generated using Microsoft® Excel 2016.

5.6 Results

5.6.1 In-vitro *experiment 1:* Globodera pallida *viability and eggs cyst^{-1} after exposure to different quantities of* Brassica juncea *leaf discs*

The viability of *G. pallida* eggs measured approximately 14 days after exposure to *B. juncea* leaf discs in *in-vitro* 1 indicated a decline in viability in biofumigant treated *G. pallida* ($P < 0.001$) (Figure 5.8 i). Efficacy ranged between 28.4 and 50.2% between 1-4 leaf discs. No difference was observed between *B. juncea* leaf disc treatments in eggs cyst^{-1} ($P = 0.317$) (Figure 5.8 ii). Eggs cyst^{-1} ranged between 152-206, for 0 to 8 *B. juncea* leaf disc treatments. Egg viability CV's for leaf disc treatments ranged between 8.0 and 25.5% (Figure 5.8 i). Egg cyst^{-1} CV's for leaf disc treatments ranged between 11.8 and 36.5% (Figure 5.8 ii).

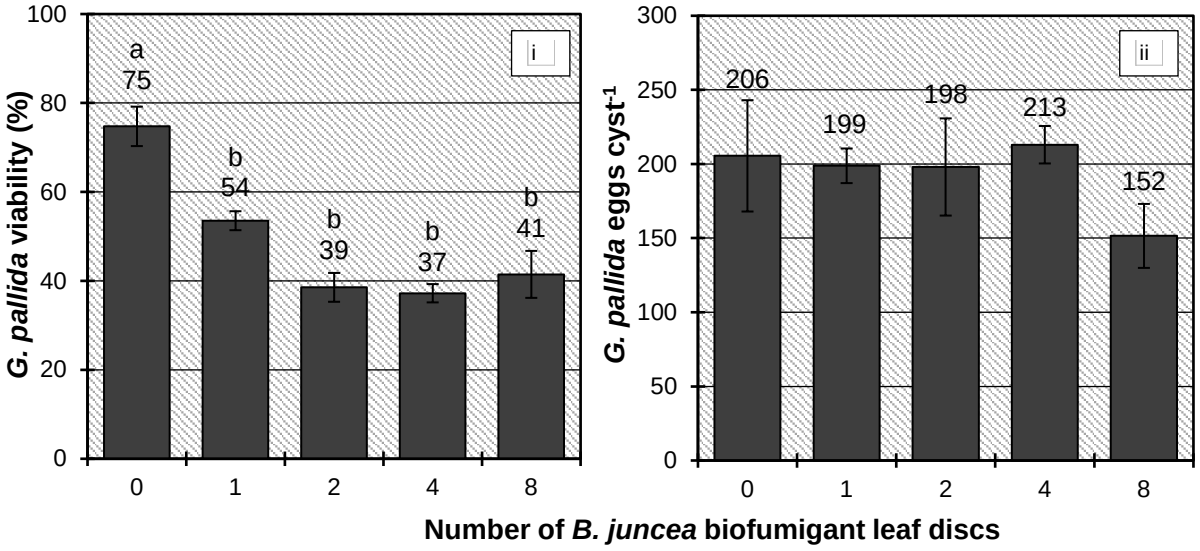


Figure 5.8: *Globodera pallida* viability (i) and eggs cyst^{-1} (ii) after exposure to increasing quantities of *B. juncea* leaf discs in *in-vitro* experiment 1. Plates i and ii show one-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

5.6.2 In-vitro *experiment 2:* Globodera pallida *viability and eggs cyst^{-1} after exposure to different cyst moisture conditions and biofumigation with* Brassica juncea *leaf discs*

The viability of *G. pallida* eggs measured approximately 14 days after exposure to *B. juncea* leaf discs in *in-vitro* experiment 2, indicated a decline in viability caused by biofumigation, but no difference in viability between different cyst moisture treatments (*P* = 0.006) (Figure 5.9 i). Efficacy ranged between 23.7 and 30.0% between cyst moisture treatments treated with *B. juncea* leaf discs. No difference was observed between cyst moisture treatments in eggs cyst^{-1} analysis (*P* = 0.564) (Figure 5.9 ii). Eggs cyst^{-1} ranged between 137-177 different *G. pallida* cyst moisture treatments. Egg viability CV's for *G. pallida* cyst moisture treatments ranged between 5.3 and 25.3% (Figure 5.9 i). Egg cyst^{-1} CV's for cyst moisture treatments ranged between 23.6 and 50.7% (Figure 5.9 ii).

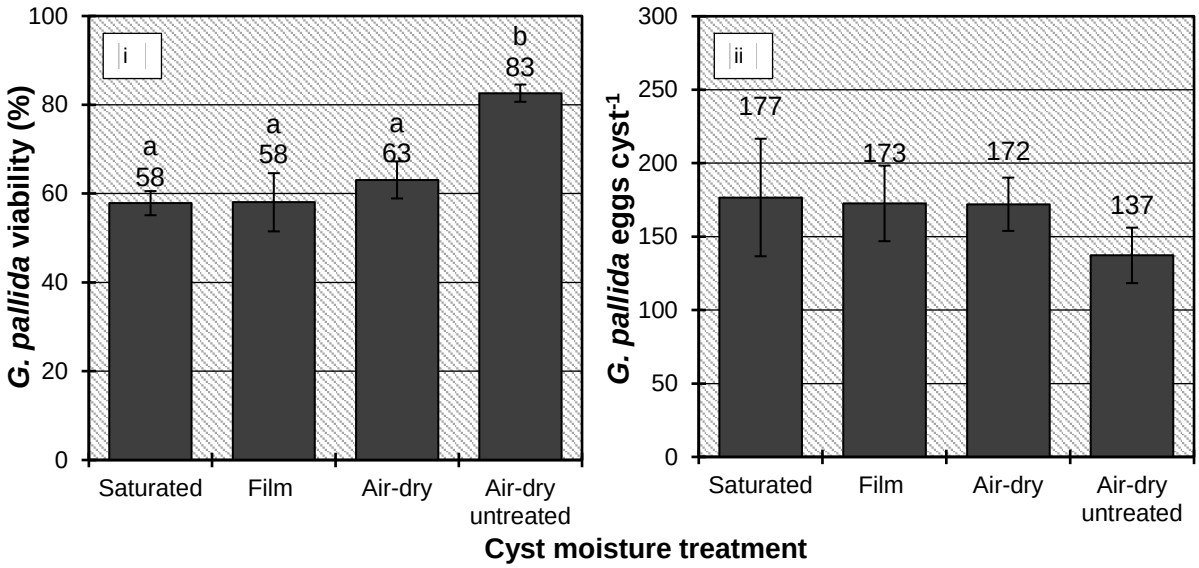


Figure 5.9: *Globodera pallida* viability (i) and eggs cyst^{-1} (ii) after exposure to increasing cyst moisture conditions and biofumigation with *Brassica juncea* leaf discs in *in-vitro* experiment 2. Plates i and ii show one-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

5.6.3 In-vitro *experiment 3:* Globodera pallida *viability and eggs cyst^{-1} after exposure to different cyst moisture conditions and biofumigation with* Brassica juncea *leaf discs*

The viability of *G. pallida* eggs measured approximately 14 days after exposure to *B. juncea* leaf discs in *in-vitro* 3, indicated a decline in viability between air-dried untreated *G. pallida* cysts and air-dried cysts treated with *B. juncea* leaf discs. The other cyst moisture treatments were inseparable from other treatments (P = 0.047) (Figure 5.10 i). Efficacy ranged between 13.6 and 20.4% between cyst moisture treatments treated with *B. juncea* leaf discs. No difference was observed between cyst moisture treatments in eggs cyst^{-1} analysis (P = 0.468) (Figure 5.10 ii). Eggs cyst^{-1} ranged between 120-155 different *G. pallida* cyst moisture treatments. Egg viability CV's for *G. pallida* cyst moisture treatments ranged between 5.5 and 14.6% (Figure 5.10 i). Egg cyst^{-1} CV's for cyst moisture treatments ranged between 18.8 and 35.3% (Figure 5.10 ii).

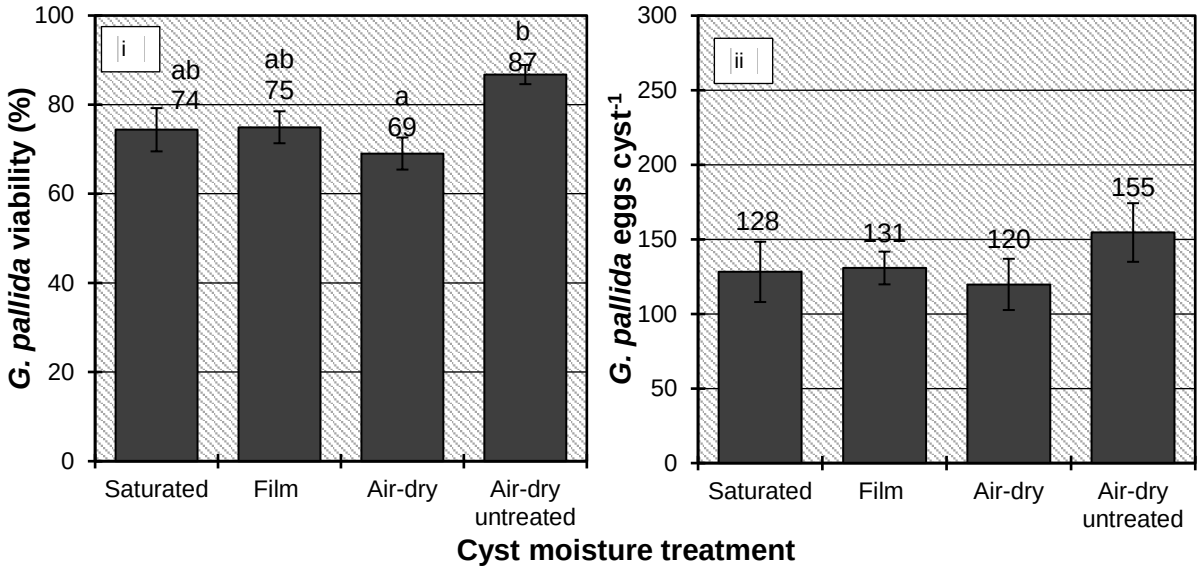


Figure 5.10: *Globodera pallida* viability (i) and eggs cyst^{-1} (ii) after exposure to increasing cyst moisture conditions and biofumigation with *Brassica juncea* leaf discs in *in-vitro* experiment 3. Plates i and ii show one-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

5.6.4 In-vitro *experiment 2 and 3 combined analysis:* Globodera pallida *viability and eggs cyst^{-1} after exposure to different cyst moisture conditions and biofumigation with* Brassica juncea *leaf discs*

An F-variance ratio calculation was made to assess whether data from *in-vitro* experiments 2 and 3 could be combined. In both viability and eggs cyst^{-1} assessment, data could be combined ($P = 0.318$ and $P = 0.179$ respectively). The viability of *G. pallida* eggs measured approximately four weeks after exposure to *B. juncea* leaf discs indicated a decline in viability caused by biofumigation, but no difference in viability between different cyst moisture treatments ($P < 0.001$) (Figure 5.11 i). Combined efficacy ranged between 21.5 and 22.0% between cyst moisture treatments treated with *B. juncea* leaf discs. No difference was observed between cyst moisture treatments in eggs cyst^{-1} analysis ($P = 0.985$) (Figure 5.11 ii). Combined eggs cyst^{-1} ranged between 146-152 for different *G. pallida* cyst moisture treatments. Egg viability CV's for *G. pallida* cyst moisture treatments ranged between 5.7 and 21.4% (Figure 5.11 i). Egg cyst^{-1} CV's for cyst moisture treatments ranged between 28.5 and 46.9% (Figure 5.11 ii).

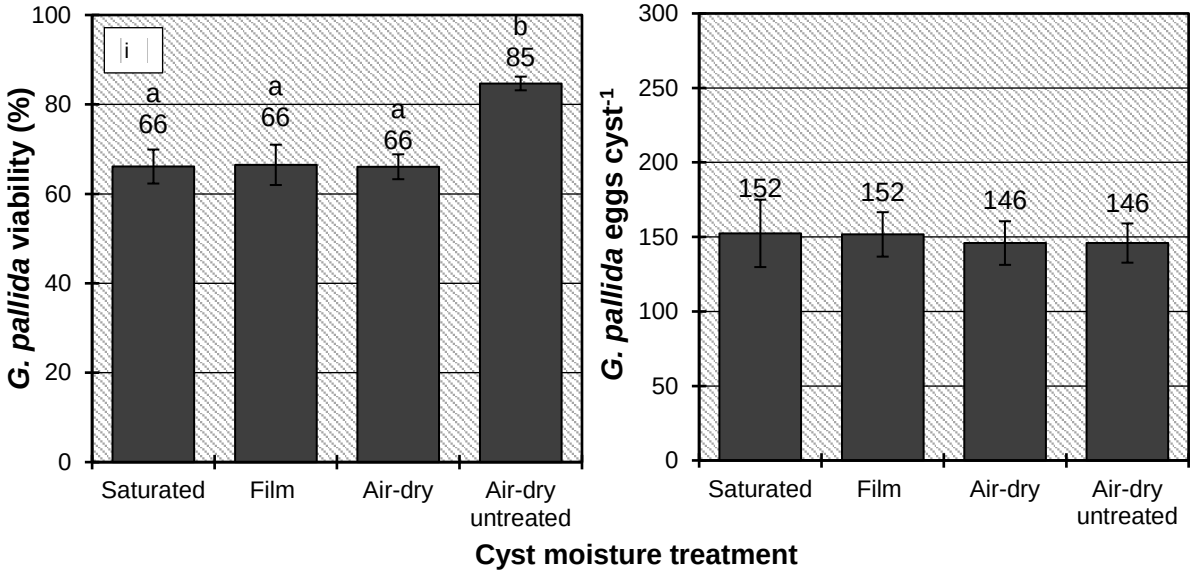


Figure 5.11: *Globodera pallida* viability (i) and eggs cyst^{-1} (ii) after exposure to increasing cyst moisture conditions and biofumigation with *Brassica juncea* leaf discs in a combined analysis of *in-vitro* experiments 2 and 3. Plates i and ii show one-way ANOVA analysis. Error bars represent the SEM. Lettering above data labels indicates differences as determined using Tukey's test.

5.7 Discussion

The primary aim of the work described in this chapter was to determine whether *B. juncea* biofumigation efficacy against PCN can be affected by the moisture condition of PCN cysts. The chapter null hypothesis stated that cyst moisture condition does not influence efficacy of *B. juncea* biofumigation against PCN. Overall, the moisture condition of PCN cysts, *G. pallida* in this instance, was not found to influence biofumigation.

5.7.1 In-vitro *experiment 1:* Globodera pallida *viability and eggs cyst*$^{-}$

In-vitro experiment 1 investigated five levels of *B. juncea* leaf disc inclusion. The primary objective of the work was to determine the number of *B. juncea* leaf discs required to kill approximately 50% of encysted *G. pallida*. This was deemed to be an average efficacy from the literature and so representative of the real field situation. The 50% threshold viability was also used because it would allow treatment effects to be separable in subsequent cyst moisture experimentation, if cyst moisture was found to be influential in *B. juncea* biofumigation of PCN. In this respect the experiment was successful. Four *B. juncea* leaf discs were found to cause *c.*50% mortality and so were taken forward as the leaf disc dose for other experiments. It was also observed that mean efficacy appeared to increase in tandem with increasing leaf disc number (Figure 5.8 i), which is a similar trend to that observed for increased *B. juncea* biomass inclusion into pot soil in glasshouse experiment 2 (see Figure 3.21 i). The frozen leaf disc method was adopted from Morra & Kirkegaard (2002) as a means of ensuring maximum cellular damage and release of VOC's from *B. juncea* tissues and increasing the uniformity of biofumigation treatment between replicates.

Figure 5.8 ii showed no effect of number of *B. juncea* leaf discs on *G. pallida* eggs cyst^{-1}, which is in support of all previous eggs cyst^{-1} analyses in Chapters 3 and 4. However, as in previous chapters, CV's were high for eggs cyst^{-1} which may have obscured the interpretation of results; CV range of 11.8 and 36.5%. It would appear consistently throughout this project that biofumigation does not readily influence eggs cyst^{-1},

indicating that biofumigation efficacy is probably related only to viability, and not egg numbers. Viability CV's were higher than in other studies, possibly because of the low number of replicates, or possibly because the Lodge 1 *G. pallida* population was generally more variable in viability than other populations. The Lodge 1 site is known to be in a five year potato rotation (Pers. Comm. Matthew Wallace: Farm Manager, Lodge 1 field), which suggests a regularity of heavy cultivations and PCN lifecycles, which is known to cause variability in viability of a PCN population (Turner, 1996). In future, populations grown in wider rotation might be preferable for this type of study.

5.7.2 In-vitro *experiments 2 and 3:* Globodera pallida *viability and eggs cyst^{-1}*

The aim of *in-vitro* experiments 2 and 3 was to determine whether cyst moisture has a role in biofumigation PCN, by either blocking or facilitating the entry of biofumigant VOC's into cysts. Neither *in-vitro* experiments 2 or 3 found evidence of biofumigation efficacy against *G. pallida* being influenced by cyst moisture (Figures 5.9 i and 5.10 i). Neither were *G. pallida* eggs cyst^{-1} affected by the moisture condition of cysts during biofumigation with *B. juncea* leaf discs. It would therefore appear than cyst moisture need not be considered in biofumigation of PCN, and that soil moisture should be manipulated simply for mazimum efficacy of biofumigant VOC's in soil, and potentially for an enhanced GSL hydrolysis, rather than manipulation of biofumigant VOC entry into cysts (Simpson *et al*., 2010; Mattheissen *et al*., 2004). However, egg viability and eggs cyst^{-1} CV's were similarly high for *in-vitro* experiments 2 and 3 to *in-vitro* experiment 1. CV's ranged between *c.*5.3 to 25.3% for viability analyses and 18.8 to 50.7% for eggs cyst^{-1} analyses. Given the low replication in these experiments and the CV's recorded, effect size differences between treatments of *c.*70% would be required to prove differences in some cases. Therefore the importance of cyst moisture could not really be fully quantified in this work. Data from *in-vitro* experiments 2 and 3 were combined after F-test analysis to try and reduce variation, and increase replication (Figure 5.11). However CV's were still too high to determine differences between treatments for egg viability or eggs cyst^{-1}. It would appear from simply looking at mean viability and eggs cyst^{-1} data, however, that

cyst moisture did not influence *B. juncea* VOC entry/ exposure to *G. pallida* eggs in the experiments carried out here.

5.7.3 *General points and considerations*

It was unfortunate that Tenax-TA sorbent tubes could not be desorbed using the GC-MS as this data could have provided more evidence for or against cyst moisture as a factor influencing biofumigation success against PCN. The VOCEC's were generally successful and will be useful apparatus for other *in-vitro* studies. An obvious expansion of the work carried out here would be to repeat the work with a more uniform PCN population and more replication. Further studies might also consider screening benzyl or 2-phenylethyl rich biofumigant leaf discs as VOC's arising from these GSL's are known to be less volatile and more hydrophobic than AITC, which was probably the primary biofumigant VOC responsible for biofumigation efficacy in this work (Borek *et al*., 1998). Therefore, cyst moisture might be influential of the efficacy of VOC's arising from these GSL's specifically. All objectives save for quantifying *B. juncea* VOC's, were achieved. The chapter aim can largely be considered answered, and the null hypothesis accepted.

6.1 Introduction

In recent years, pesticide regulatory legislation has become increasingly restrictive, with notable pesticides and classes of pesticides being recalled from commercial use in the European Union (EU) specifically, in response to enforcement of 'Regulation (EC)1107/2009'. The legislation has been instrumental in phasing out the use of pesticides in the EU which are deemed to be hazardous to humans or to threaten biodiversity (OJEU, 2009; Hillocks, 2012). It is in this climate of uncertainty that potato growers require sustainable, and effective alternatives to nematicides and nematostats, for security of potato cyst nematode (PCN) management for the future.

A wealth of literature has now been published surrounding the biofumigation technique, which relies on the hydrolysis of glucosinolates (GSL's) released from macerated brassica tissues, to generate biocidal volatile organic compounds (VOC's) which can be worked into soil for pest suppression (Bones & Rossiter, 1996; Holst & Williamson, 2004). The GSL profiles and the concentrations of GSL's found in brassicaceous plant species are known for many varieties (Kirkegaard & Sarwar, 1998; Lord *et al*., 2011). It is also understood that the GSL content of brassica plants can vary between plant organs, and between plants grown under diverse environmental conditions (Björkman *et al*. 2011). Several studies have now also investigated the macerated tissues of brassica plants for management of PCN in glasshouse and field experiments (Lord *et al*., 2011; Ngala *et al*., 2015b; Ngala *et al*., 2014), and have exposed PCN to concentrated ITC solutions in laboratory studies, with some success (Buskov *et al*., 2002; Wood *et al*., 2017). However, very little research has investigated factors surrounding GSL hydrolysis for maximum release of VOC's from macerated tissues (Lazzeri et al., 2004; Mattner *et al*., 2008). Very little research has investigated methods for incorporating biofumigant tissues into soil, or the soil conditions most conducive to release and retention of

biofumigant VOC's into soil from macerated biofumigant plant tissues (Morra & Kirkegaard, 2002; Matthiessen *et al*., 2004). Furthermore, no study is known to have investigated these biofumigation factors for management of PCN, *Globodera rostochiensis* and *G. pallida*. The need for experiments to investigate these areas and deliver practical recommendations for potato growers was therefore clear at the beginning of this project, in autumn 2013. The project initiated with glasshouse experiments aimed at determining 'whether soil moisture conditions at biofumigant incorporation might influence the efficacy of *Brassica juncea* biofumigation against PCN', followed by three field experiments between 2014 and 2016 (Hungry Hill, Norfolk; Crossroads, Shropshire; Roys Corner, Shropshire) which aimed to determine 'whether maceration and/or incorporation implement selection and set-up might be influential of *B. juncea* biofumigation efficacy against PCN'. *In-vitro* experiments were then designed to investigate moisture films as barriers or vehicles for biofumigant VOC exposure to encysted PCN following, the results of glasshouse work.

6.2 Discussion of chapters

Glasshouse experiments (Chapter 3) revealed that soil moisture is important in influencing *B. juncea* biofumigation efficacy against *G. pallida*, with up to a 14.3% increase in efficacy when practising biofumigation at 50% of field capacity compared to drier and wetter extremes (0 and 100% of field capacity, and water saturated soil), for a mean biomass quantity of *c.*50 t ha^{-1} fresh weight. This extra level of efficacy represents equivalent to 6-12 months of natural viability decline of *G. pallida* (Devine *et al*., 1999; Winslow & Willis, 1972), simply by incorporating biofumigant material into soil of an optimum soil moisture condition. These results could have important implications for potato growers, who in the Shropshire area (UK), tend to over-winter biofumigants to fit between cereal harvest and spring cropping (Pers. Comm. Andrew Wade: Senior Agronomist at Agrovista UK Ltd.). Over-wintering is known to reduce GSL content of biofumigant tissues (Ngala *et al.*, 2014), whilst spring time biofumigant incorporations might generally result in drier soil moisture conditions than autumn incorporation. Under

these circumstances, irrigation could be required to achieve maximum biofumigation suppression of PCN. Generally, a range of 25-75% of field capacity was observed to be optimal for incorporation of *B. juncea* biomass quantities into soil in the region of 25-75 t ha^{-1} fresh weight, which is also in line with past work on synthetic fumigant nematicides (Lembright, 1990). Efficacy ranged between *c.*16-40% in soil moisture experiments. It was hypothesised that drier conditions might not sufficiently retain biofumigant VOC's in soil, whilst wetter soil conditions might impede VOC movement and exposure to PCN. It was also suggested that efficacy may have been suboptimal due to the methodological approach used, whereby PCN were not exposed to partial biofumigation, but only fumigation arising from the incorporation of biofumigant tissues. It was specifically the hypothesis that soil moisture might act as a barrier or vehicle to PCN exposure to biofumigant VOCs depending upon the hydrophobicity of VOC's, which lead to *in-vitro* experiments (Chapter 5) investigating different levels of cyst moisture condition as a factor influencing biofumigation efficacy against *G. pallida*. However, no evidence was observed to suggest cyst moisture was influential of biofumigation efficacy against *G. pallida* in Chapter 5. This could indicate that the soil moisture enhanced biofumigation efficacy observed in Chapter 3, might be related to retention of biofumigant VOC's in soil and/or, enhanced GSL hydrolysis from macerated tissues (Simpson *et al*., 2010, Matthiessen *et al*., 2004).

In other analyses of glasshouse experiment data (Chapter 3), the quantity of *B. juncea* biomass incorporated into pots was generally shown to influence *G. pallida* mortality. Evidence was presented which suggested that incorporation of high biomass quantities into soil might have a structural effect on soil, potentially leading to loss of VOC's from soil by off-gassing where soil moisture conditions are low (*c.* 25% of field capacity), and the quantity of biomass incorporated into soil is high. Further research is required here to understand the interactions between biofumigant biomass quantity, level of tissue damage, soil moisture condition and depth of incorporation, which should have a role in diluting biofumigant material into soil. Ideally, biofumigant material would be pulverised

to release maximum quantities of biofumigant VOC's, whilst also having a minimal effect on soil structure after incorporation (Sultani *et al*. (2007).

Hungry Hill and Crossroads experiments carried out in autumn 2014 in Norfolk and Shropshire (Chapter 4), showed the first recording of enhanced biofumigation efficacy against PCN related to the implement used to macerate *B. juncea* biofumigant material. Efficacy was improved by up to 7%, again, representing *c.*6 months of natural decline in PCN by a simple selection of maceration implement (Devine *et al*., 1999; Winslow & Willis, 1972). A flail topper was found to generate a more effective biofumigation of *G. pallida* than a roll conditioner, probably due to greater levels of tissue trauma following cutting, but potentially due to the flailed biofumigant residue having a reduced structural property in soil compared to material macerated with the roll conditioner, leading to better retention of biofumigant VOC's in soil as alluded to in Chapter 3.

Incorporation implement was not found to influence biofumigation efficacy against *G. pallida* in Hungry Hill or Crossroads field experiments directly, although a spader-flail combination was *c.*8% more effective in reducing *G. pallida* viability than a roll-conditioner-rotivator combination when data from the two sites were combined. This could suggest that incorporation implement selection is secondary to maceration implement selection, but that where a high biofumigation potential crop is grown (Kirkegaard & Sarwar, 1998), and an effective maceration implement is used, incorporation implement might enable a more effective biofumigation by generating a soil environment more conducive to fumigation. However, the cost of buying or hiring such a specialist implement is probably not justifiable given the low level efficacy increase spading appears to offer here compared to plough and rotavator implements. It is possible that the spader was too effective in working biofumigant material into soil for the poor to moderate level of biomass and GSL content of the field crops reported here; ranging between *c.*14-48 t ha^{-1} fresh weight crops with a range between *c.*4.4-12.1 µmol g^{-1} GSL contents in top growth. It is possible the spader diluted biofumigant tissues in

soil and that the looser soil structure enabled greater sorption of VOC's to organic-matter. The low GSL concentrations in tissues, which are *c.*10 to 20 times lower than some other studies (Ngala *et al*., 2014; Kirekgaard & Sarwar, 1998) could be related to sampling time, which in the Crossroads and Hungry Hill experiments was in November, and in the Roys Corner experiment was after winter in April. It is likely that due to low ultraviolet levels and low plant stress, GSL concentrations were low in plant tissues (Björkman *et al*. 2011). Differences in methodology may also be responsible for differences between studies. Ngala *et al*. (2014) measured foliar GSL's whereas all GSL analysis in this project assessed a composite of leaf and stem material. Leaves are generally known to be higher in GSL concentration than other organs in brassica plants, lending some weight to this hypothesis (Björkman *et al*. 2011)

Following Hungry Hill and Crossroads experiments, it appeared clear that implement selection for maceration of biofumigant crops in the field might have important implications for biofumigation success against *G. pallida* (Chapter 4), particularly in light of the moderate levels of damage observed for even flail treated plant material in the field. Implement set-up was therefore investigated in the subsequent Roys Corner experiment where tine and shear-plate set-ups were manipulated. No effect on *G. pallida* viability was observed for biofumigation treatment using diverse tine and shear-plate set-up combinations, indicating flail/haulm topper maceration implement set-ups may not be important for biofumigation of PCN. However, the Roys Corner site had a low PCN population, and the experiment was over wintered which was likely to be suboptimal (Björkman *et al*. 2011). Further consideration of the haulm topper implement found tine speed to be *c.*32 m s^{-1} at incorporation, which may have been too high to cause plant acceleration resulting in widespread tissue damage. Speeds below 15 m s^{-1} would be preferable and could improve maceration procedure in future (McRandal & McNulty, 1978). Clearly this is an area for further investigation. It would have been beneficial to assess plant tissue damage more thoroughly in this work, perhaps using a microscopy approach. Efficacy ranged between 23-39% in field experiments overall.

6.3 Future work

It is possible that maceration implements are at, or near their limit in terms of the quality of biofumigant residue they can produce from an engineering standing. However, other methods of improving biofumigation could be considered. The ‘acid growth theory’ refers to an auxin induced process in plants whereby the apoplast becomes acidic causing irreparable cellular damage within plants, and rapid enlarging of cells with water (Hopkins, 1999). The most common example of this is use of the herbicide 2,4-D which kills plants by forcing them to grow out of control by auxin induced apoplastic damage. Therefore, a timely application of an auxin product such as 2,4-D could have the potential to condition *B. juncea* plant before incorporation, leading to a more effective maceration. A further idea for future work could be to fit a water tank and nozzle set-up to the haulm topper used in the Roys Corner experiment. If nozzles were positioned under the haulm topper hood, they could mist biofumigant residues immediately before incorporation using the rotavator set-up. This could greatly enhance GSL hydrolysis leading to a peak in biofumigant VOC production, whilst aiding soil sealing post incorporation also. This line of enquiry would marry well with work on flail tine speed to make a logical continuation of this PhD. Further research might also investigate the structure of PCN cysts as a factor influencing biofumigation success. New cysts may be more fortified and therefore resilient to biofumigant VOC’s than older cysts. The VOCEC’s from Chapter 5 could be used in this work. If such work were carried out, it may be beneficial to calculate the volume of air within each VOCEC and use a quantity of biofumigant material which would be representative of the field situation in terms of soil pore air space and incorporated biofumigant biomass. Such investigations could also include a range of temperatures and relative humidity conditions to give a more detailed view of optimal soil conditions for biofumigation of PCN. The implications of the ‘cyst age’ line of investigation could be that biofumigation be found to be best placed immediately preceding a potato crop, rather than at other points in the rotation.

6.4 Recommendations

Immediate recommendations for growers attempting to manage PCN with biofumigation, would be to grow crops in excess of 50 t ha^{-1} fresh weight, macerate and incorporate using a haulm topper/flail implement combined with a rotavator or spader, and aim to incorporate into soils of approximately 75% of field capacity. Where a low biomass crop is to be incorporated, soil moisture conditions of around 50% of field capacity would be beneficial.

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8.1 Supplementary soil and potato cyst nematode results

8.1.1 *Soil pH*

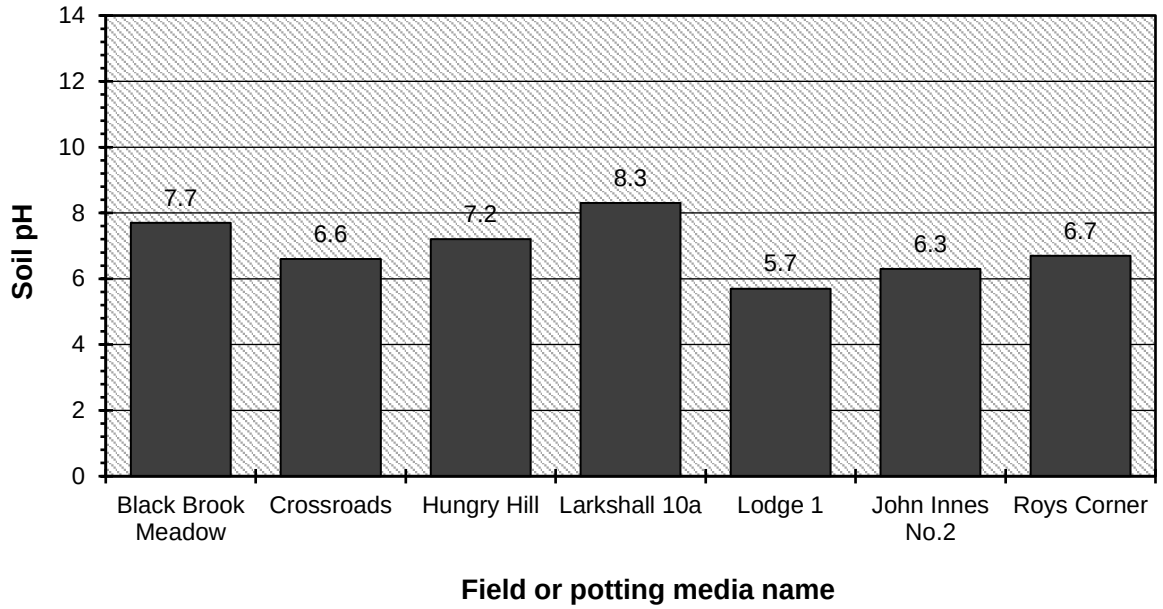


Figure 8.1: Soil pH of the seven soils or potting medias used in experimental work.

8.1.2 *Soil organic-matter*

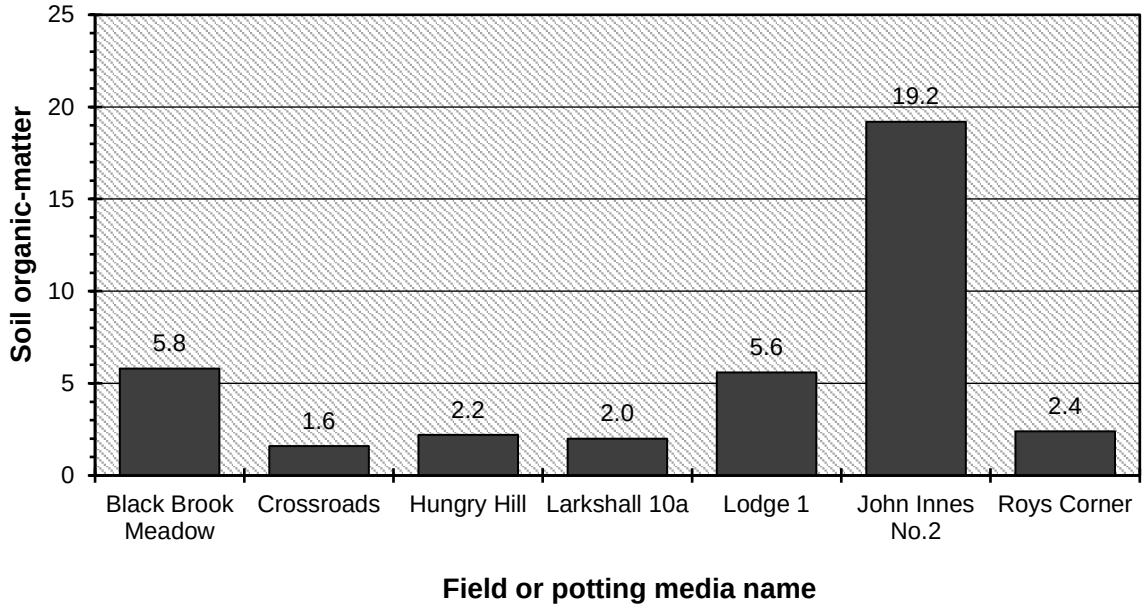


Figure 8.2: Organic-matter of the seven soils or potting medias used in experimental work.

8.1.3 *Soil texture (UK texture triangle)*

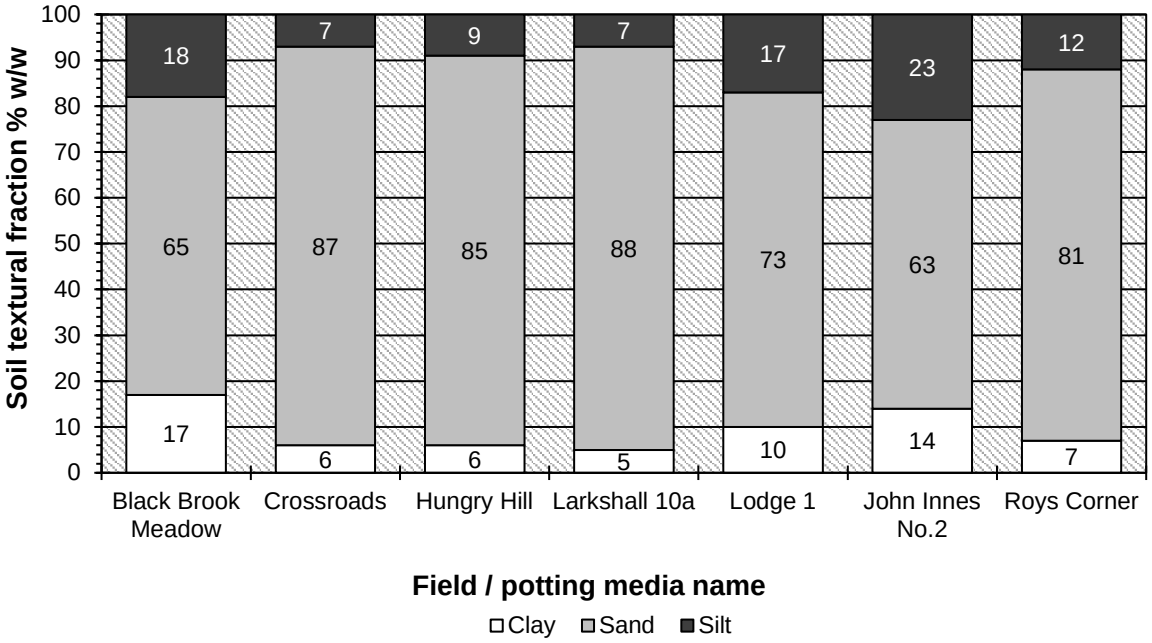


Figure 8.3: Soil textural classification by sedimentation of the seven soils or potting medias used in experimental work. Black Brook Meadow: sandy loam, Crossroads: loamy sand, Hungry Hill: loamy sand, Larkshall 10a: sand, Lodge 1: sandy loam, John Innes No. 2: organic sandy loam, Roys Corner: loamy sand.

8.1.4 *Potato cyst nematode polymerase chain reaction results*

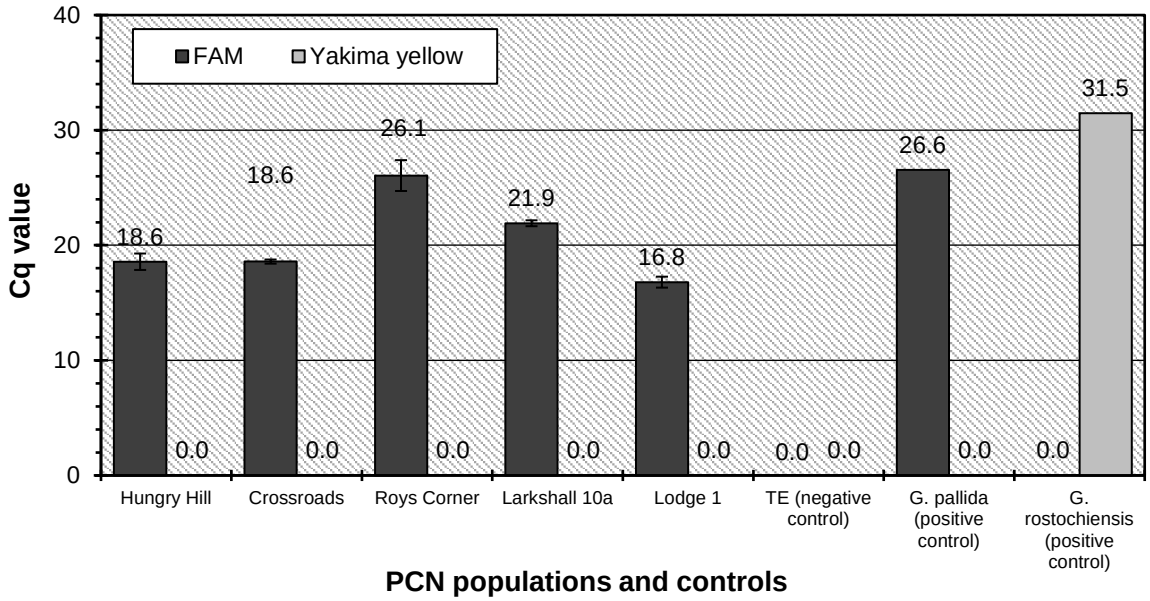


Figure 8.4: Polymerase chain reaction analysis of potato cyst nematodes from each population used in experiments, showing Cq values for amplified *Globodera rostochiensis* and *G. pallida* DNA compared to controls using probes FAM and Yakima yellow.

8.1.5 *Soil moisture: water retention/field capacity curves*

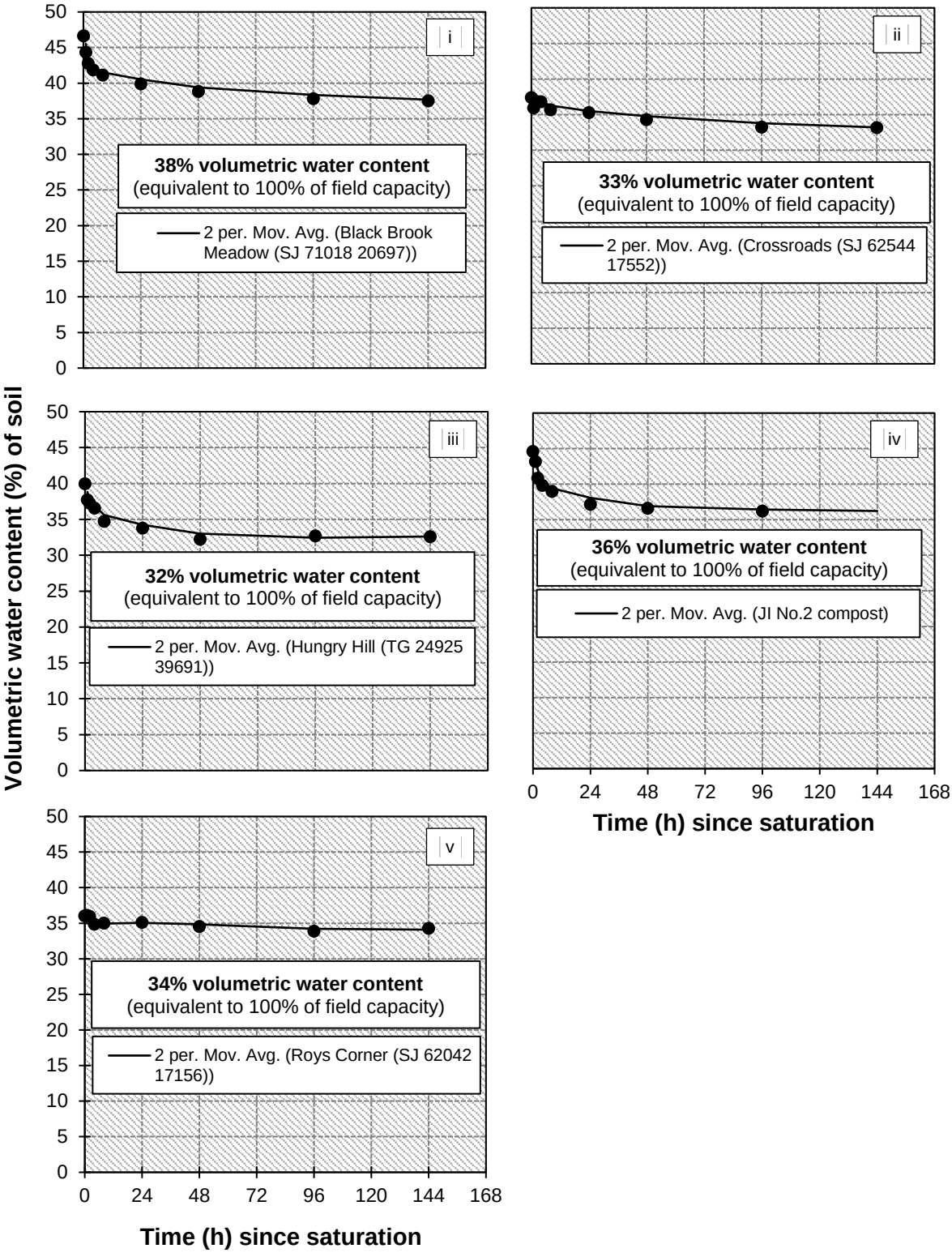


Figure 8.5: Water retention curves (field capacity) for Black Brook Meadow, Crossroads, Hungry Hill, John Innes No.2 compost and Roys Corner field (plates i-v respectively). Data points represent the mean of three replicates.

8.1.6 *Soil moisture: field experiment conditions at* Brassica juncea *incorporation*

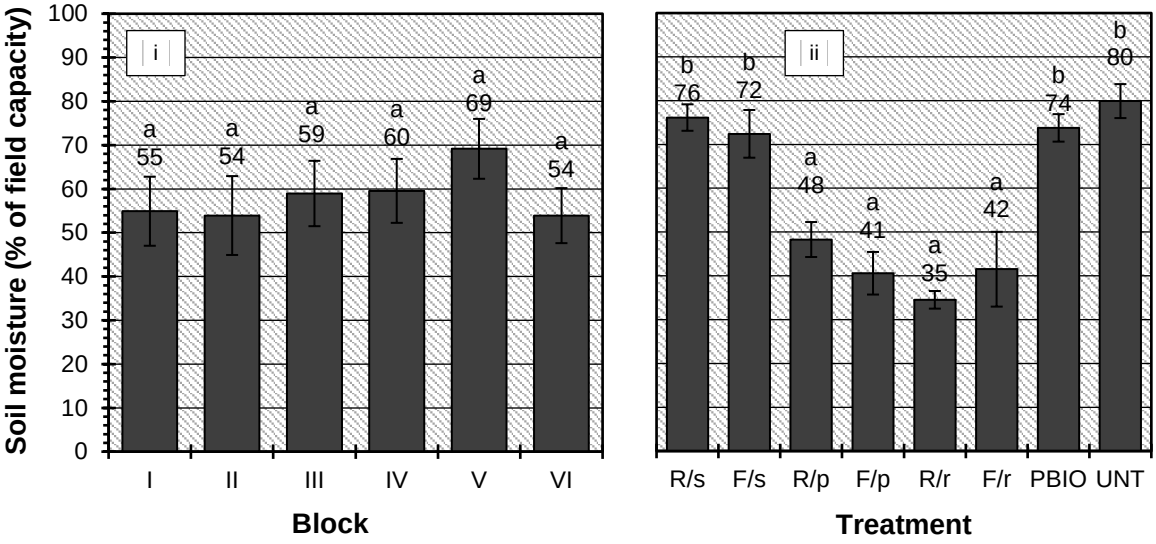


Figure 8.6: Soil moisture conditions immediately after *Brassica juncea* incorporation at field experiment 1 (Hungry Hill, Norfolk), analysed by block (i) (P = 0.699) and by treatment (ii) using one-way ANOVA (P < 0.001). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO; untreated - UNT. Error bars represent the SEM.

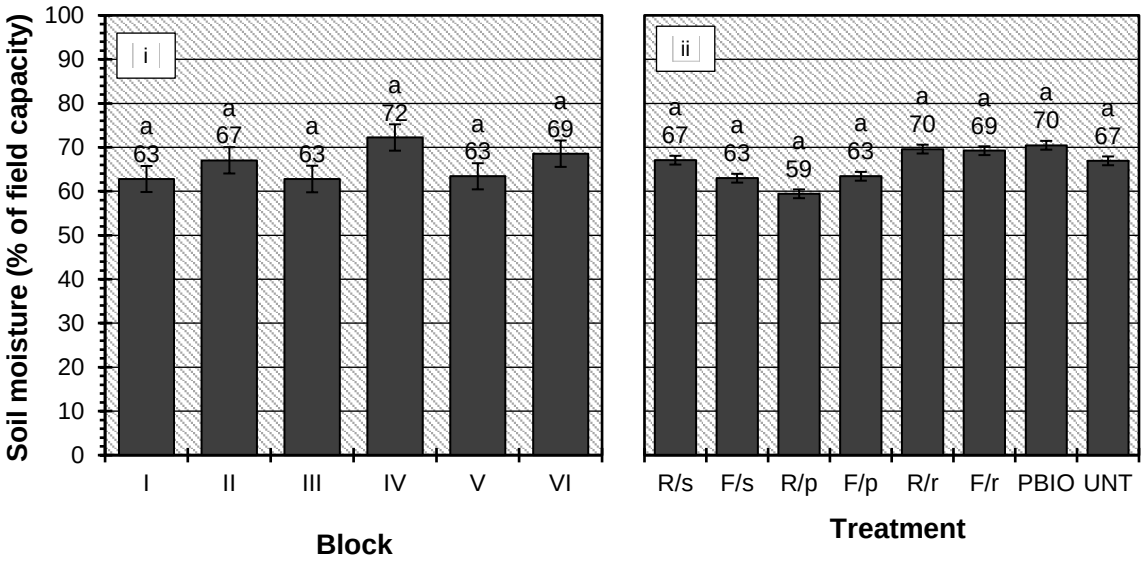


Figure 8.7: Soil moisture conditions immediately after *Brassica juncea* incorporation at field experiment 2 (Crossroads, Shropshire), analysed by block (i) (P = 0.037) and by treatment (ii) using one-way ANOVA (P = 0.035). Neither Tukey or Fischer's protected LSD were able to determine differences between factor levels. Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO; untreated - UNT. Error bars represent the SEM.

8.1.7 *Soil temperature: field experiment conditions at* Brassica juncea *incorporation*

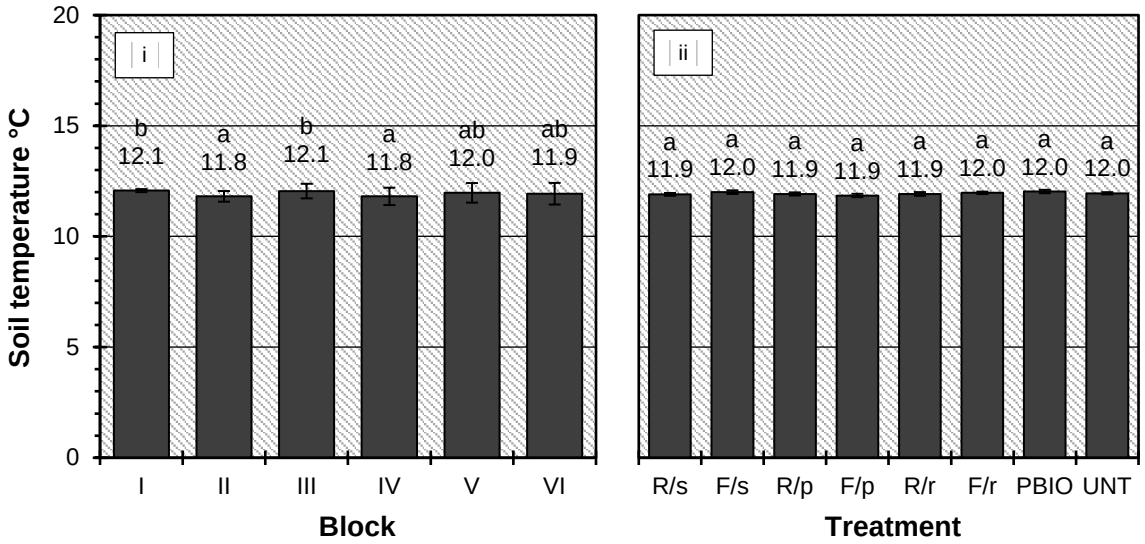


Figure 8.8: Soil temperature conditions immediately after *Brassica juncea* incorporation at field experiment 1 (Hungry Hill, Norfolk), analysed by block (i) ($P < 0.001$) and by treatment (ii) using one-way ANOVA (P 0.412). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO; untreated - UNT. Error bars represent the SEM.

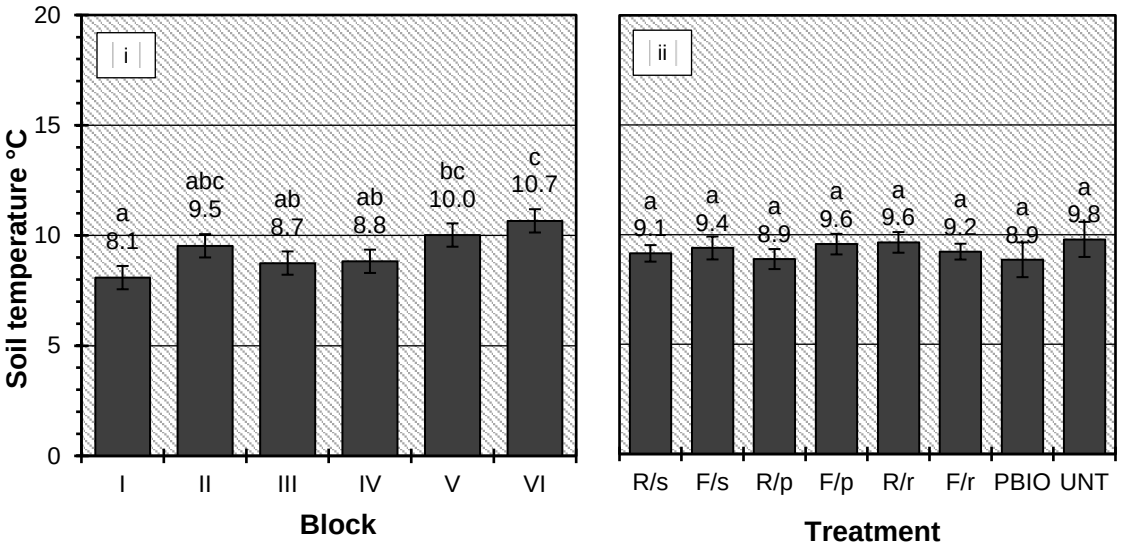


Figure 8.9: Soil temperature conditions immediately after *Brassica juncea* incorporation at field experiment 2 (Crossroads, Shropshire), analysed by block (i) ($P < 0.001$) and by treatment (ii) using one-way ANOVA ($P = 0.715$). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO; untreated - UNT. Error bars represent the SEM.

8.2 Supplementary biofumigant results

8.2.1 Brassica juncea *biofumigant plant counts at field experiment 1 (Hungry Hill, Norfolk), at 3 weeks post drilling*

Figure 8.10: Typical *Brassica juncea* establishment for experimental blocks I and II at field experiment 1 (Hungry Hill, Norfolk).

Figure 8.11: Typical *Brassica juncea* establishment for experimental blocks III and IV field experiment 1 (Hungry Hill, Norfolk).

Figure 8.12: Typical *Brassica juncea* establishment for experimental blocks V and VI field experiment 1 (Hungry Hill, Norfolk).

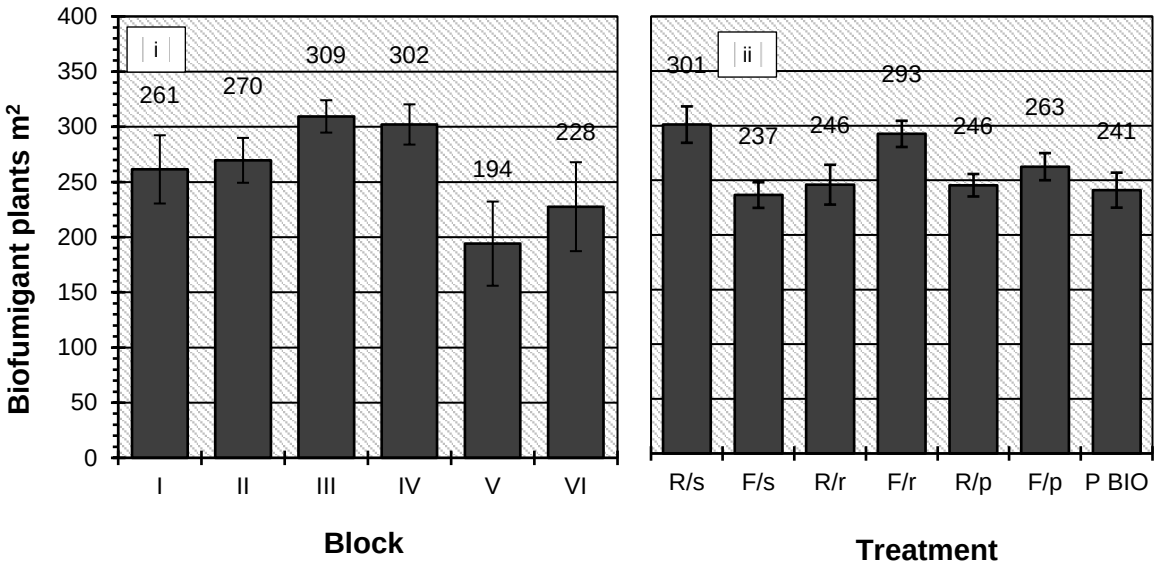


Figure 8.13: *Brassica juncea* plant counts m^2 at 3 weeks post drilling at field experiment 1 (Hungry Hill, Norfolk), analysed by block (i) ($P = 0.062$) and by treatment (ii) ($P = 0.685$). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

8.2.2 Brassica juncea *plant counts at field experiments 1, 2 and 3 (Hungry Hill, Norfolk and Crossroads and Roys Corner, Shropshire) on the day of incorporation*

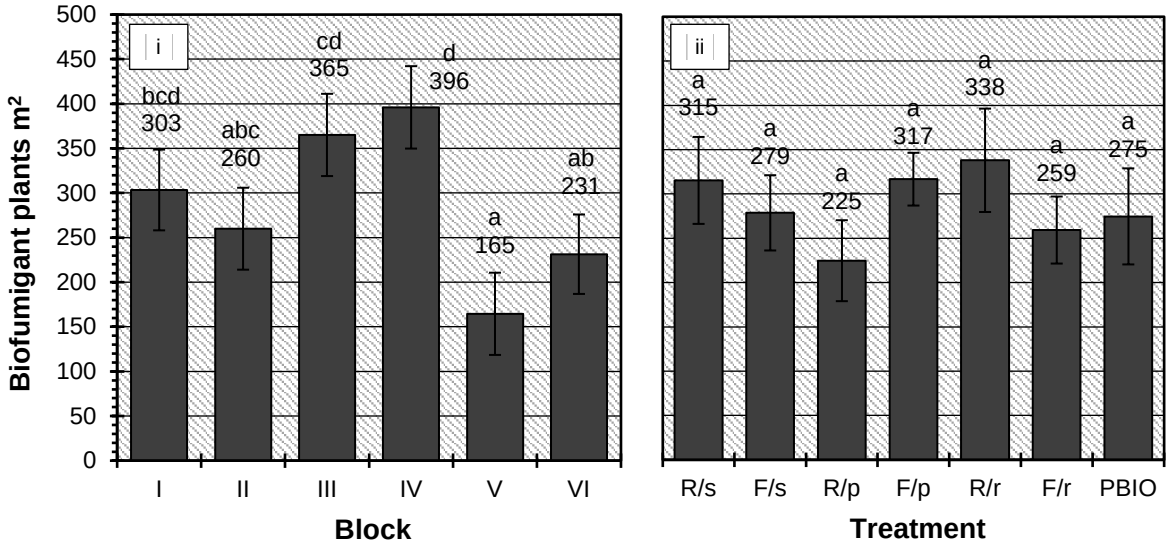


Figure 8.14: *Brassica juncea* plant counts m^2 on the day of biofumigant incorporation field experiment 1 (Hungry Hill, Norfolk), analysed by block (i) ($P < 0.001$) and by treatment (ii) using one-way ANOVA ($P = 0.228$). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

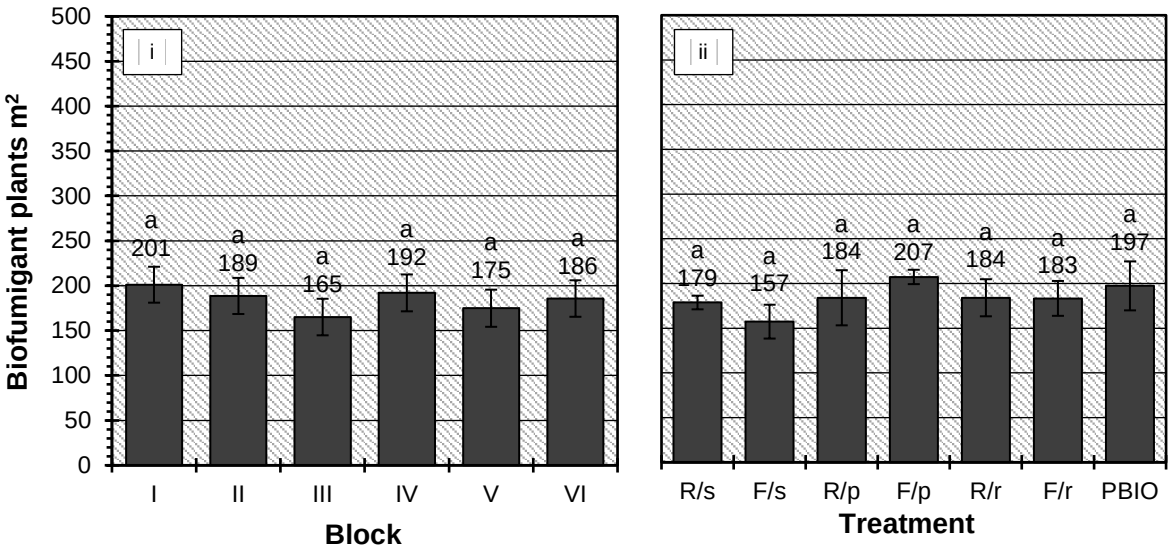


Figure 8.15: *Brassica juncea* plant counts m^2 on the day of biofumigant incorporation field experiment 2 (Crossroads, Shropshire), analysed by block (i) ($P = 0.814$) and analysed by treatment (ii) using one-way ANOVA ($P = 0.787$). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

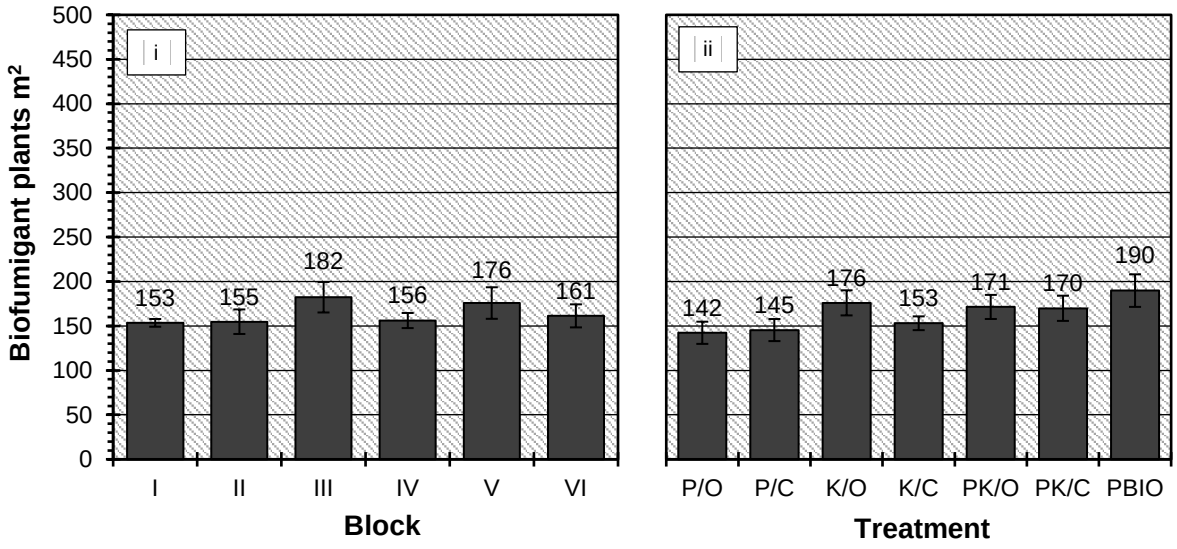


Figure 8.16: *Brassica juncea* plant counts m^2 on the day of biofumigant incorporation field experiment 3 (Roys Corner, Shropshire), analysed by block (i) (*P* = 0.532) and by treatment (ii) using one-way ANOVA (*P* = 0.170). Treatment key: pig-tail tine – P; knife tine – K; pig-tail/knife tine – PK; open shear-plate – O; closed shear-plate – C; partial biofumigant – PBIO. Error bars represent the SEM.

8.2.3 *Cabbage root fly (*Delia radicum*) damage to* Brassica juncea *plants at field experiment 2 (Crossroads, Shropshire), at 7 weeks post drilling*

Figure 8.17: Cabbage root fly (*Delia radicum*) damage index system for biofumigant plants at field experiment 2 (Crossroads, Shropshire).

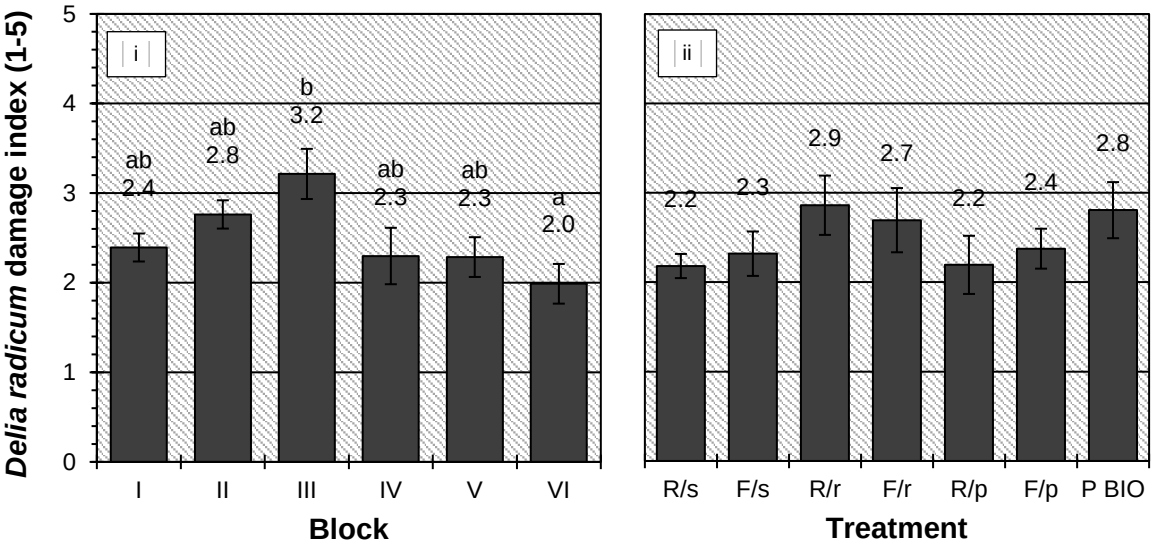


Figure 8.18: Cabbage root fly (*Delia radicum*) damage of *Brassica juncea* plants at field experiment 2 (Crossroads, Shropshire), analysed by block (i) (P = 0.012) and treatment (ii) (P = 0.238) using one-way ANOVA, and expressed on a 1-5 scale (Figure 8.17). Treatment key: roll conditioner - R; flail - F; spader - s; plough - p; rotavator - r; partial biofumigant – PBIO. Error bars represent the SEM.

8.2.4 Brassica juncea *glucosinolate analysis for glasshouse experiment 2 and field experiments 1, 2 and 3 (Hungry Hill, Norfolk, and Crossroads and Roys Corner, Shropshire)*

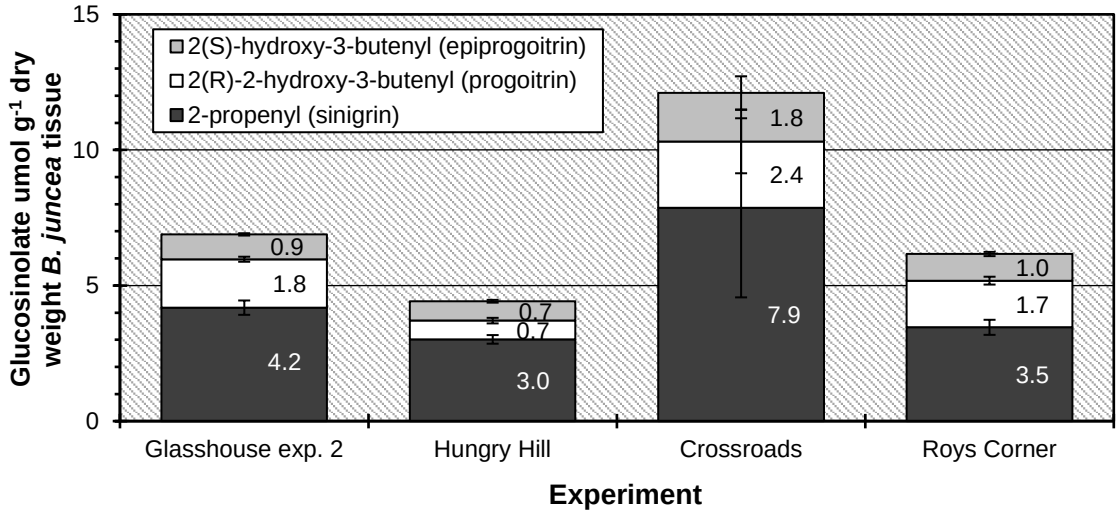


Figure 8.19: Glucosinolate content of *Brassica juncea* plants used in experimental work.

8.2.5 *Pilot study results for development of custom volatile organic compound sampling MOLE's for field experiment 3 (Roys Corner, Shropshire)*

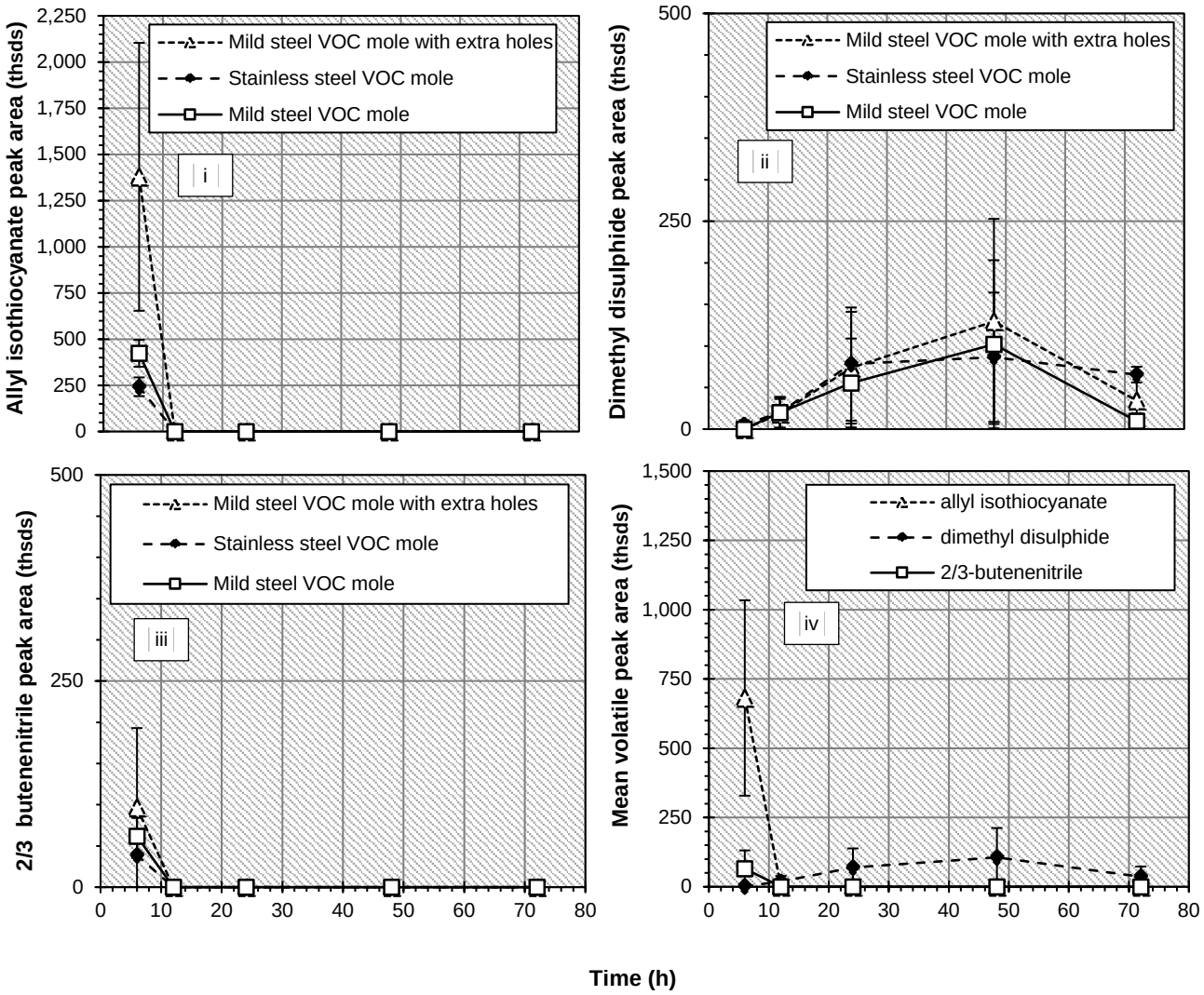


Figure 8.20: Passive entrainment of *Brassica juncea* biofumigant volatile organic compounds from field soil using a Markes International VOC MOLE^{TM} in comparison to two custom made MOLE's. Plates i-iii show entrainment of allyl isothiocyanate, dimethyl disulphide and 2/3 butenenitrile for each MOLE respectively. Plate iv shows the mean quantity of the three recorded volatiles entrained from soil overall. Mole specific data points represent the mean of two replicates.

8.3 Manufactured tools for experimental work

8.3.1 *Custom made volatile organic compound MOLE drawings*

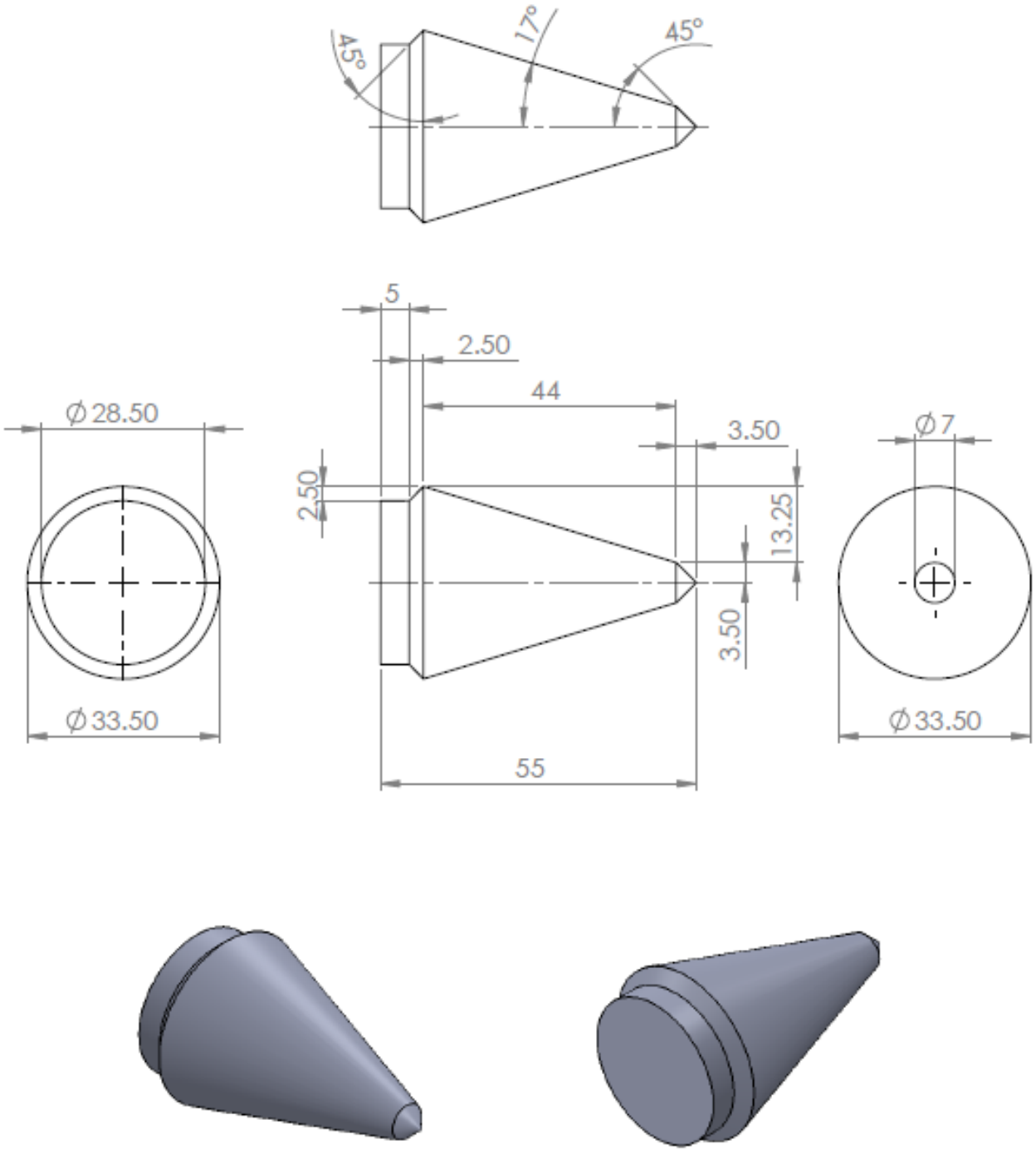


Figure 8.21: Part: volatile organic compound mole tip

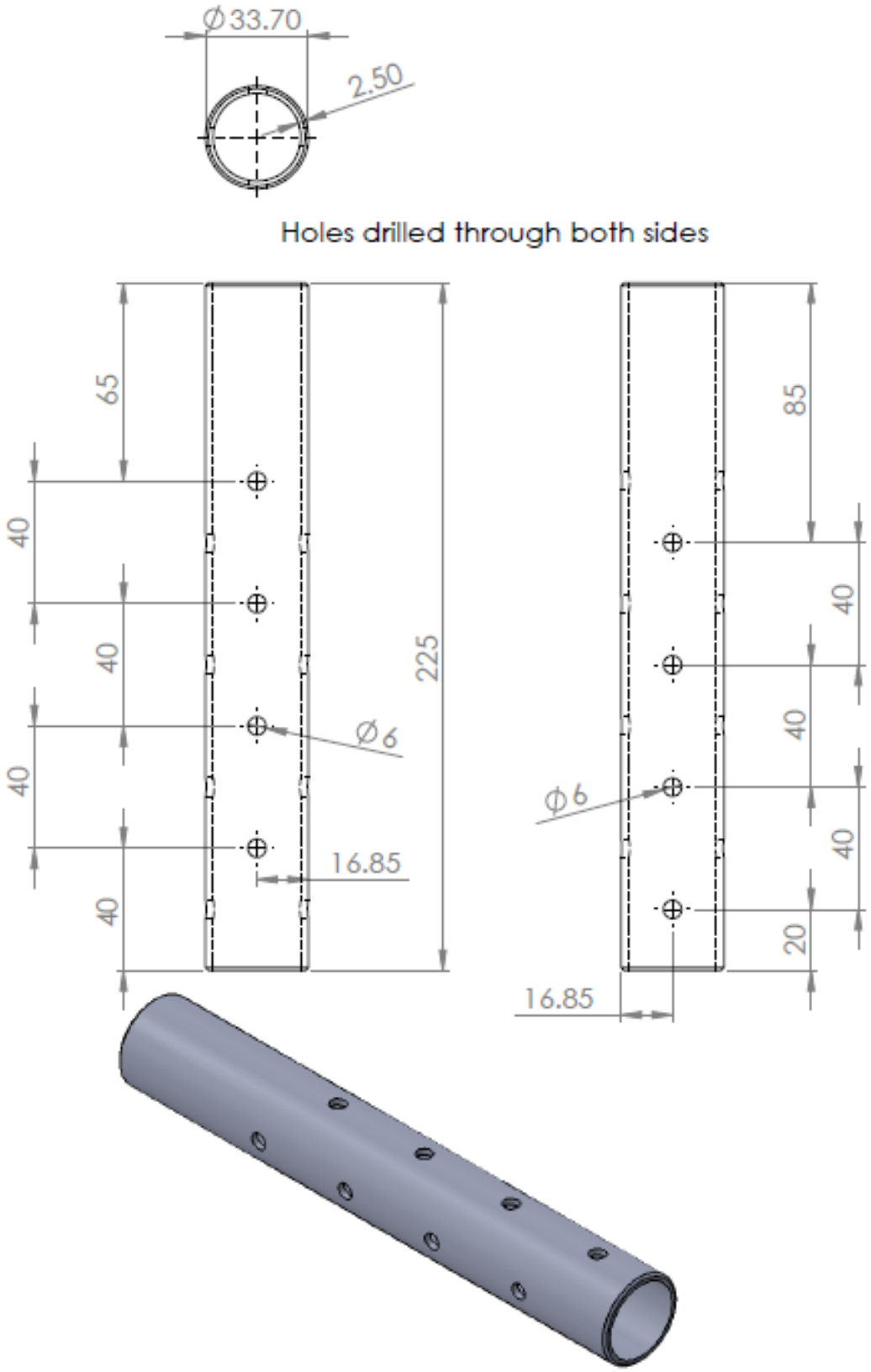


Figure 8.22: Part: replica volatile organic compound mole pipe

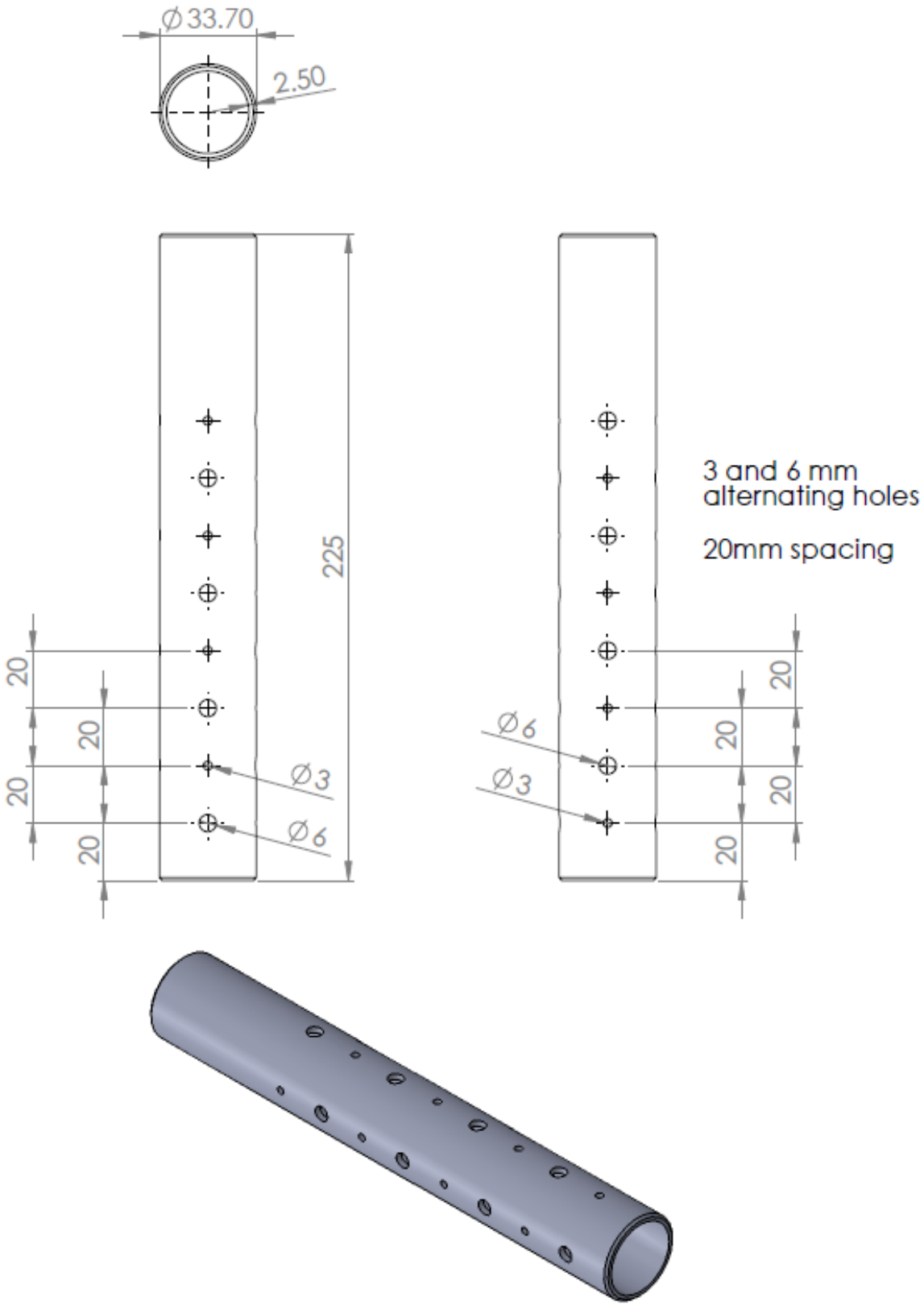


Figure 8.23: Part: improved volatile organic compound mole pipe

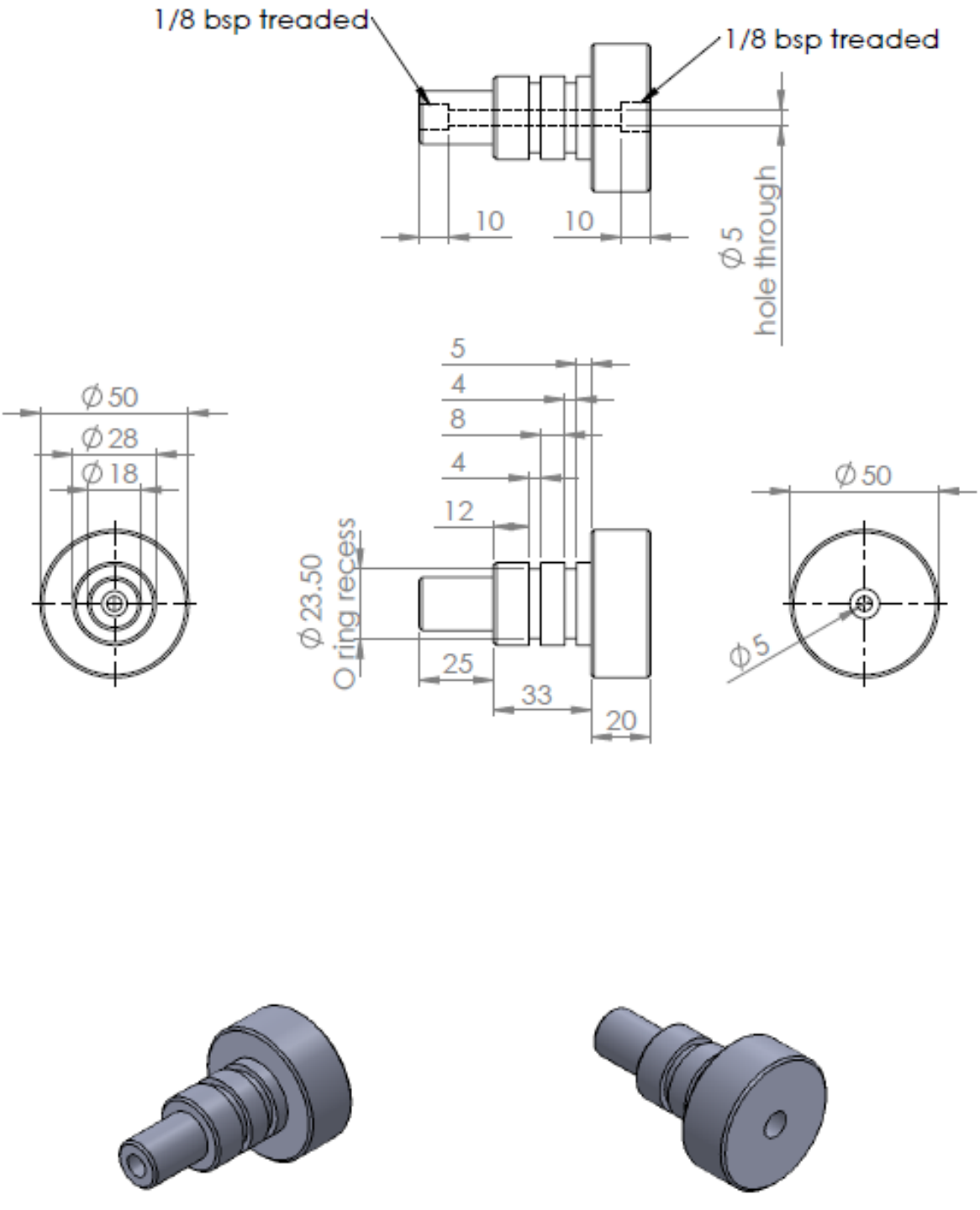
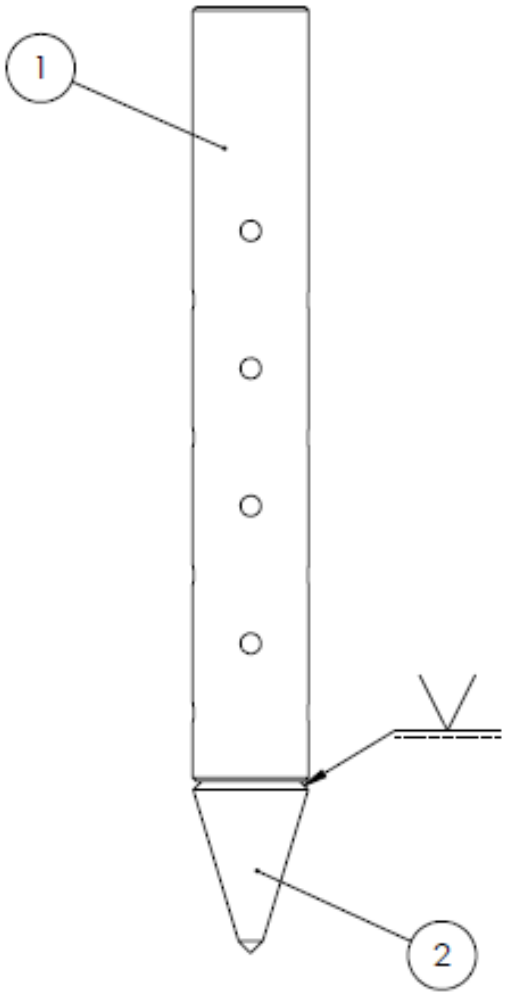


Figure 8.24: Part: volatile organic compound mole top



ITEM NO.	PART NUMBER	QTY.
1	pipe	1
2	tip	1

Figure 8.25: Volatile organic compound mole tip and pipe assembly

8.4 Miscellaneous supplements

8.4.1 *Alternative stratified randomised complete block design for field experiment 3 (Roys Corner, Shropshire): block allocation by viable potato cyst nematode eggs g^{-1} soil.*

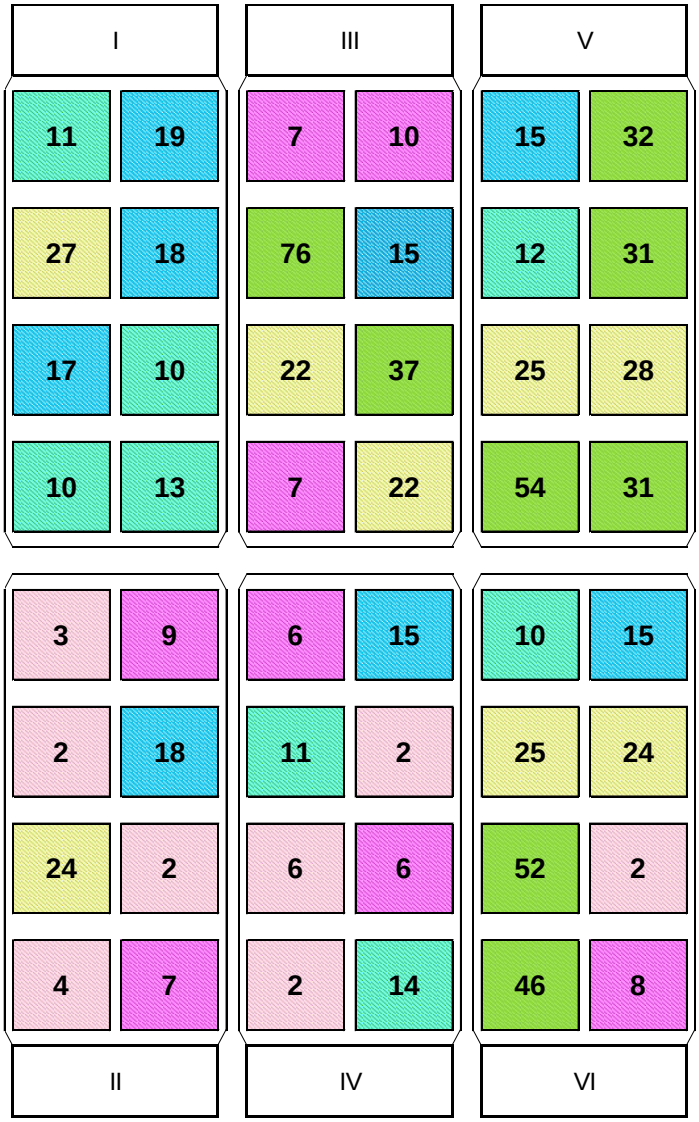


Figure 8.26: Revised field experiment 3 design showing the allocation of blocks to plots using a stratified approach according to the numbers of viable eggs g^{-1} soil, represented here using the numbers within plots. Plots sharing like colours belong to the same block. The original block structure using Roman numerals has been left in place to illustrate the different structural organisation of the experiment between the original randomised complete block approach, and the proposed stratified randomised block approach.

Table 8.1: Treatment codes for field experiment 3 (Roys Corner, Shropshire) showing levels for maceration implement tine and shear-plate factors. Font colour is an indicator of shear-plate setting and fill colour is an indicator of tine selection.

Treatment code	Biofumigant	Factor 1: Shear plate setting	Factor 2: Tines
P/O	Grown, macerated and then incorporated	Open	Pig-tail
P/C	Grown, macerated and then incorporated	Closed	Pig-tail
K/O	Grown, macerated and then incorporated	Open	Knife
K/C	Grown, macerated and then incorporated	Closed	Knife
PK/O	Grown, macerated and then incorporated	Open	Pig-tail and knife combination
PK/C	Grown, macerated and then incorporated	Closed	Pig-tail and knife combination
P BIO	Partial biofumigant control (biofumigant grown but left standing)		
UNT	Untreated control (no biofumigant grown)		

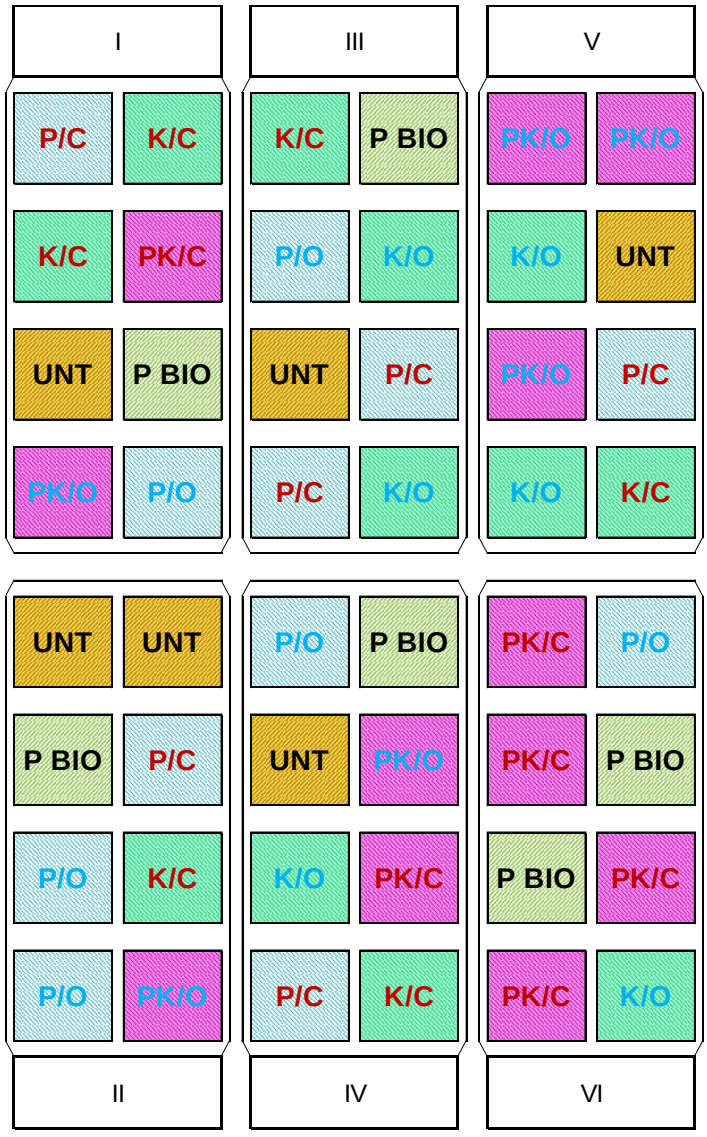


Figure 8.27: Revised experiment 3 design showing the random allocation of treatments to potato cyst nematode infested plots within stratified blocks from Figure 8.26. The

original block structure using Roman numerals has been left in place to illustrate the different structural organisation of treatments across the experimental area i.e. the clustering of replicates of PK/C treatment in the original block VI.