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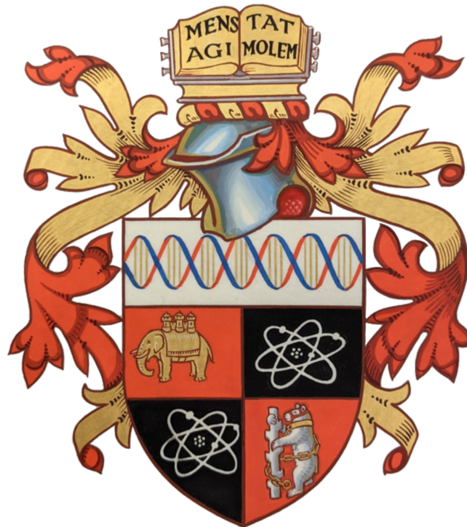

Investigating the phylogeny, interactions, and management of pathogens within the pea foot rot complex

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Life Sciences

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Abbreviations

AE	*Aphanomyces euteiches*
AG	Anastomosis group
ANOVA	Analysis of variance
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CBS	Convention of Biological Diversity
CFU	Colony forming unit
cv.	Cultivar
d.w.	Dry weight
DNA	Deoxyribonucleic acid
DP	*Didymella pinodella*
f. sp.	*Forma specialis*
ff. spp.	*Formae speciales*
fg	Femtogram
FO	*Fusarium oxysporum*
FOSC	*Fusarium oxysporum* species complex
FS	*Fusarium solani*
FSP	*Fusarium solani* f. sp. *pisi*
FSSC	*Fusarium solani* species complex
GLM	Generalised linear model
GSL	Glucosinolate
ha	Hectares
HPLC	High performance liquid chromatography
IGS	*Intergenic spacer*
ITC	Isothiocyanate
ITS	*Internal transcribed spacer*
log	Logarithm

LSU	*Large subunit*
MEGA	Molecular Evolutionary Genetics Analysis
N_2	Dinitrogen
NB	No biofumigant material
NCBI	National Centre of Biotechnology Information
NH_3	Ammonia
p	Probability
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PFR	Pea foot rot
PFRC	Pea foot rot complex
pg	picogram
PGRO	Processors and Growers Research Organisation
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
R-group	Radical group
RPB2	*Second largest subunit of RNA polymerase II*
SCN^-	Ionic thiocyanate
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
sp.	Species
spp.	Subspecies
SRO water	Sterile reverse osmosis water
SSU	Small subunit
TEF1-α	*Translation elongation factor 1-alpha*
TUB2	*Beta-tubulin*
UK	United Kingdom
UoW	University of Warwick
USA	United States of America
var.	Variety

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented was carried out by the author except where otherwise stated and any adaptation of published work has been acknowledged and referenced.

Abstract

The field pea (*Pisum sativum*) is an important legume grown for human and animal consumption. In the UK, growth is restricted to eastern England and Scotland, which has led to intensive production and yield declines of up to 40 %. This is attributed to a build-up of fungal and oomycete pathogens involved in the pea foot rot complex (PFRC); *Fusarium solani forma speciales* (f. sp.) *pisi* (FSP), *F. oxysporum* (FO), *Didymella pinodella* (DP) and *Aphanomyces euteiches*. Knowledge regarding the genetics, dynamics, and control of these pathogens, in particular for DP, is limited. Therefore, the main aims of this project were to characterise selected PFRC pathogen isolates through multilocus sequencing, better understand PFRC pathogen virulence, dynamics and interactions, and identify biofumigant crops that can suppress PFRC pathogens.

Sequencing and multilocus phylogenetic analysis of both DP and *F. solani* (FS) isolates revealed the lack of diversity within DP isolates from the UK and other countries, while UK FS isolates clustered within a previously published clade of FS isolates from a variety of legume hosts, which challenges the notion that FS *formae speciales* are specific to single plant species.

Positive relationships between pathogen inoculum concentration, pea foot rot disease development and plant mortality were established for FO, FSP and DP using a test-tube based assay, and for DP a glasshouse-based assay. A further test tube assay revealed the additive nature of interactions between FO, FSP and DP when co-inoculated in terms of disease development in pea. Preliminary qPCR analysis of root colonisation in this experiment successfully quantified DNA for each of the three pathogens.

Biofumigant crop varieties, particularly species of *Brassica juncea* and *Eruca sativa*, significantly reduced and inhibited mycelial growth of FO, FSP, DP and AE *in-vitro* and significantly supressed DP foot rot in a pot-based glasshouse experiment.

Overall, this research has contributed substantial knowledge regarding the phylogeny, disease development and interactions of PFRC pathogens, and the potential of biofumigation as a management strategy.

1. Introduction

1.1 The domestication of pea and human nutritional benefits

The pea (*Pisum sativum* L.) is a cool season annual crop and a member of the Fabaceae family of plants, which also includes beans (*Phaseolus* and *Vicia* spp.), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and peanuts (*Arachis hypogaea*) (Elzebroek, 2008). The genus *Pisum* itself contains two recognised species, *P. sativum,* which comprises *P. sativum* subsp. *sativum*, the domesticated pea, and *P. sativum* subsp. *elatius*, a wild form, while *P. fulvum* is known as the tawny pea (Elzebroek, 2008; Trněný *et al.*, 2018).

Evidence suggests that pea was first domesticated for consumption around 11,000-9000 years ago in a region known as the Eastern centre of domestication, which now spans modern-day Turkey, Egypt, and parts of West Asia, which was also the region of domestication for several other legume species. Archaeological findings support that domestication of pea then spread westwards following areas around the Danube River, ancient Greece and Rome and eventually the rest of Europe (Kraft & Pfleger, 2001; Trněný *et al.*, 2018).

Pea is an important crop grown for both human and animal consumption and provides a wide range of nutritional benefits. One type of edible pea is vining pea, also commonly known as garden pea, or fresh pea, which in the UK is grown for the frozen, canned and fresh markets. The crop is harvested prior to full maturity (Elzebroek, 2008; Pavek, 2012; PGRO, 2017), with the specific time determined by machine called a tenderometer, which determines the quality of the peas and hence the market it is suitable for, while also determining the payment rate the grower will receive (PGRO, 2017). Other metrics such as size, colour, shape, uniformity, and soaking quality will also determine the overall quality of a harvest (Tulbek *et al.*, 2017). Higher quality harvests, which will be destined for the fresh and frozen markets, will be frozen in just under two and half hours, to maintain quality (PGRO, 2017). Other types for consumption include combining pea, also commonly known as field pea, which is harvested when the seed is dry at full maturity. These are primarily grown for animal feed, as a protein-rich component in addition to grains. Another type is the sugar pea, where the pods, suitable for eating and containing the immature peas, are sold fresh for consumption (Elzebroek, 2008; PGRO, 2017).

Peas provide a good source of a variety of macro- and micronutrients. They are considered an important and abundant source of plant-based protein and are also high in total dietary fibre; pea contains higher levels of protein and fibre compared to wheat (Tulbek, 2014). A benefit of pea protein over animal protein sources is that the essential amino acid composition in pea is better suited to human nutritional needs. Peas are also a good source of several vitamins and minerals, particularly calcium, iron and zinc (Tulbek *et al.*, 2017).

1.2 Pea developmental stages and nitrogen fixation

Prior to germination, pea seed comprises two cotyledons that contain the nutrients required to sustain early seedling growth (Kraft & Pfleger, 2001; Flynn & Idowu, 2015). Upon germination, the plumular hook, or apical hook, a curved section of epicotyl shielding the apical meristem, penetrates and grows upwards through the topsoil (Taiz and Zeiger, 2010; Miyamoto *et al.*, 2014). True leaves develop from the third node, and their form is described as 'pinnately compound', where the leaf is formed by pairs of leaflets along the vein. At each node up the stem a leaf forms, consisting of two stipules that surround the stem, followed by pairs of leaflets, the number of which generally increases as the plant grows, and then between two to five tendrils which seek out and wrap around other plants or support structures to help the stem remain upright. Some varieties are described as semi-leafless, where additional tendrils develop instead of pairs of leaflets. Peas are indeterminate, so will produce flowers and eventual pods whilst new nodes and leaves develop. The number of flowers which can be produced is determined by variety type. Pea flowers are bilaterally symmetrical, consist of five petals and are self-pollinating. The pod begins to develop quickly after fertilisation and the ovules within do not develop until the pod itself is fully grown in length and width, which takes around a week. The pod will reach maturity in around four weeks. The roots of pea plants consist of a main taproot and lateral roots which are able to form nodules, which are an important structure in the process of biological nitrogen fixation, a process undertaken by many legume species (Kraft & Pfleger, 2001; Flynn & Idowu, 2015). Within the nodules are bacteria, often *Rhizobia* spp., which can fix atmospheric nitrogen (N_2, Sørensen & Sessitsch, 2007) into ammonia (NH_3), a form that the plant can utilise. NH_3 is used by the plant to produce amino acids and other key biological compounds. It is a symbiotic relationship, where the plant directly benefits from the ammonia produced by the bacteria, and the bacteria is supplied with nutrients by the plant. During fixation, the

nodules turn red-pink in colour and as long as conditions are suitable, the bacteria will fix nitrogen for the majority of the growing season. However, once pod set occurs, less nutrients are directed towards the bacteria for nitrogen fixation. The majority of the nitrogen is used by the plant and held within the harvestable mass. Therefore, legumes are most effective in enriching soils with nitrogen for other crops when the entirety of the plant is incorporated into the soil (Kraft & Pfleger, 2001; Flynn & Idowu, 2015).

1.3 Production and value of pea in the UK and worldwide

In the UK, both vining (fresh) and combining (dry) peas are grown (PGRO, 2017b). In total, peas are grown by around 700 different farms in the UK, part of 18 farming groups (Yes Peas, 2021), but the area used for growing peas has been in decline since the start of the millennium (PGRO, 2017). A recommended rotation includes planting peas prior to a cereal crop such as wheat or barley which may reap the nitrogen released by the previous crop (Stevenson & Kessel, 1996).

Dry pea is grown in 98 countries worldwide, with Canada, Russia and China the leading producers, with 4,236,500, 2,369,479 and 1,711,000 tonnes produced respectively. These three countries also lead in terms of area harvested. Lebanon, Columbia and the Republic of Ireland are the leading countries in terms of yield for dry pea (FAOSTAT, 2021). In the UK in 2019, 159,000 tonnes of dry peas were produced over an area of 41,000 ha. The average yield of dry pea was 3.90 tonnes ha^{-1}.

Vining pea is grown in 80 countries worldwide, with China, India and France the top three countries in terms of production, producing 13,399,958, 5,562,000 and 282,190 tonnes respectively (FAOSTAT, 2021). China, India and the USA are the leading countries in terms of area harvested, and interestingly, Cyprus, Thailand and India as the top three countries in terms of yield. In Europe, France, the UK and Spain are the leading producers of vining pea. In 2019, the UK produced 153,115 tonnes of fresh peas harvested from an area of 39,039 ha with an average yield of 3.93 tonnes ha^{-1}. In 2009, this value was 10.70 tonnes ha^{-1}. The year 2012 represented a steep decline in yield for vining pea in the UK. On average, annual production between 1961 and 2011 was 10.60 tonnes ha^{-1}. This declined to an average of 4.11 tonnes ha^{-1} between 2012 and 2019, a reduction of 61.2%. The vast majority of vining peas produced in the UK are destined for the frozen market, around 135,000 tonnes, with approximately 3000 tonnes per annum are sold for the canning market. The UK grows

between 85 % - 90 % of its own peas each year, with the rest imported (British Growers, 2020; Yes Peas, 2021) and the UK vining pea market is worth around £50 million per year (AHDB, 2017).

Vining peas are grown and processed along the Eastern seaboard of the UK, from as far south as Essex in England, to Montrose, north of Dundee in Scotland, as this area provides the right climate for growth. This is also because many of the processing plants were converted for use from previously freezing fish. This has led to high demand for land close to the processing plants to ensure quick freezing after harvest, as peas ideally need to be frozen within two and half hours to maintain quality and nutritional value (PGRO, 2017; British Growers, 2020; Yes Peas, 2021). Naturally, restriction of land available has led to intensive production in these areas which has resulted in subsequent pea yield decline in recent years (LEGVALUE, 2017). This is mostly attributed to fungal and oomycete pathogens (Chatterton *et al.*, 2015) causing various diseases.

1.4 Pests and diseases of pea

There are various common pests and diseases of pea of significant economic importance. These include viruses such as pea enation- and pea seedborne mosaic virus, pests such as pea miner, weevil and aphid, and seedling pathogens such as *Rhizoctonia solani* and various *Pythium* spp. (Kraft & Pfleger, 2001). Foliar diseases of pea include downy mildew, caused by the oomycete *Peronospora viciae*, which either affects much of the shoot tissue, resulting in symptoms such as stunted growth and lesions with visible sporulation and chlorosis of leaves, or is localised to a specific area (Stegmark, 1994; Kraft & Pfleger, 2001). Similarly, powdery mildew caused by the fungus *Erysiphe pisi*, also affects the shoot tissue, the most obvious symptoms being small powdery grey colonies and wilt of infection sites (Smith *et al.*, 1996; Kraft & Pfleger, 2001). The fungal pathogen *Sclerotinia sclerotiorum* causes a soft rot of pea shoot tissue in later developmental stages, particularly after flowering begins. The disease is prevalent in wetter climates and high-altitude areas, with symptoms including rotting tissue which is slimy to the touch, accompanied by watery lesions and white fluffy mycelium covering affected areas containing black sclerotia (Kraft & Pfleger, 2001). Soilborne diseases of pea include Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum forma specialis* (f. sp.) *pisi*. One of the main symptoms of this disease is chlorosis of the leaves, which become paper-like and wilt, eventually the whole plant wilts and dies. The disease is also characterised by browning of the

vascular tissues (Figure 1.1) (Kraft & Pfleger, 2001). Complexes of either soil-borne or foliar pathogens can also co-infect pea. For instance, the Ascochyta blight complex (Le May *et al.*, 2009), includes pathogens such as *Didymella pinodella* (DP), *Didymella pinodes*, and *Ascochyta koolunga* (Davidson *et al.*, 2011). Whilst DP has been primarily studied as part of the Ascochyta blight complex, it is also part of a complex of soilborne pathogens known as the pea foot rot complex (PFRC), thought and suggested to be responsible for yield losses of up to 40 % in the UK and up to 75 % in other parts of the world (Biddle & Cattlin, 2007).

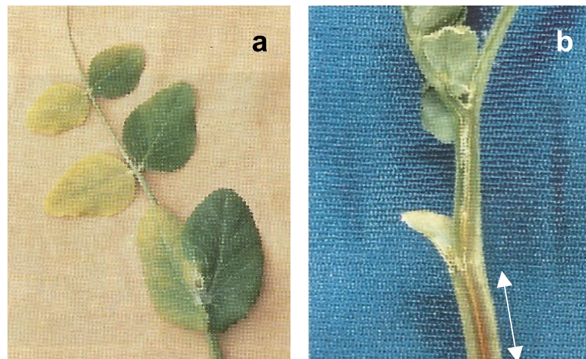


Figure 1.1. a) Symptoms of leaf chlorosis and b) red discolouration of vascular tissues (indicated by arrow) caused by Fusarium wilt (Adapted from Kraft & Pfleger, 2001).

1.5 The pea foot rot complex and key pathogens

1.5.1 Introduction to the pea foot rot complex and its incidence in the UK

The PFRC is a group of soilborne fungal and oomycete pathogens, that primarily cause rot and discoloration of the roots and the hypocotyl (the ‘foot’) in pea plants. The first documented report of foot rot in pea was in the USA, which detailed Fusarium foot and root of pea (Jones, 1923 cited by Jones & Drechsler, 1925). Factors such as increased soil moisture during the early growing season, which promotes spore germination, and a high number of resting spores in the soil are conducive to higher levels of disease. Compacted soils, where pea roots may struggle to develop properly also promote higher levels of pea foot rot (PFR; Jelden & Herold, 2020). Certain species of the PFRC can cause characteristic symptoms, although it is hard to distinguish between them when many PFRC pathogens are present (Porter *et al.*,

2015). The species present within the complex can vary depending on location, and over the years, numerous field surveys, particularly in North America and Europe have been conducted to examine the prevalence, severity and identity of the pathogens involved using both plant and soil samples. These studies have revealed that certain species are consistently present regardless of area or climate, including the oomycete pathogen *Aphanomyces euteiches* (AE), and the fungal pathogens *Fusarium oxysporum* (FO), *F. solani* f. sp. *pisi* (FSP) and *F. avenaceum* (e.g. Persson *et al.*, 1997; Chatterton *et al.*, 2015; Esmaeili Taheri *et al.*, 2017b; Baćanović-Šišić *et al.*, 2018).

The PFRC present in pea growing fields in the UK was first described in 1929, with *Fusarium*-infected roots of pea plants found in Evesham, Worcestershire (Ogilvie, 1930). Overall, in comparison to other countries affected by the PFRC, such as Canada, little published literature exists on the PFRC in the UK. The fungal pathogens FO, FSP and DP have been identified as major pathogen components of the PFRC in UK infected fields (Etebu & Osborn, 2009; Salt & Delaney, 2013; Jelden & Herold, 2020), alongside another fungal soil-borne pathogen, *Thielaviopsis basicola* (Salt & Delaney, 2013). Historically, DP has been underestimated as a PFRC pathogen in the UK, but pathogenicity tests of recovered isolates has revealed an ability to cause severe foot rot and was the pathogen was frequently isolated in surveys of infected pea fields (Biddle 1983, cited by Salt & Delaney, 2013; Salt & Delaney, 2013). The oomycete pathogen AE is also a main pathogen of the UK PFRC (Salt & Delaney, 2013) and was identified in Scottish soils just over 30 years ago. In UK pea crops where foot rot symptoms have been observed, AE was identified in around 70 %, while 50 % of the fields contained pathogen levels so high that pea should not be grown in them for 10 years due to disease risk (Syngenta, 2018). The following sections focus on PFRC pathogens of particular importance in the UK.

1.5.2 *Fusarium* species

1.5.2.1 Species involved in the pea foot rot complex and taxonomy

The fungal genus *Fusarium* is distributed globally (Porter *et al*., 2015), and is found in a wide range of climates, environmental conditions (Nelson *et al.*, 1994) and substrates, including plant material, soil, water (Rana *et al.*, 2017) and air (O'Donnell *et al.*, 2004). The genus includes around 300 different species, most of which have now been grouped into 20 monophyletic species complexes (Aoki *et al.*, 2014; Rana

et al., 2017) following phylogenetic analyses. Prior to phylogenetics, *Fusarium* taxonomy was based on morphological features, including microscopic observations of conidia, with isolates taken from a wide variety of sources (Aoki *et al*., 2014). Many *Fusarium* species are pathogenic to both plants and animals, with certain isolates of both *Fusarium solani* (FS) and FO known to cause fusariosis in immunocompromised individuals (Anaissie *et al.*, 2001).

The *Fusarium* genus includes several species complexes, which comprise of multiple distinct sub-species defined by molecular phylogenetics and/or host range. These complexes include important plant pathogens, such as the FO, *F. solani*, *F. graminearum* and *F. fujikuroi* species complexes (Aoki *et al*., 2014). There are several *Fusarium* spp. involved in the PFRC (Hwang & Chang, 1989; Chatterton *et al*., 2015; Chittem *et al*., 2015; Esmaeili Taheri *et al*., 2017a; Šišić *et al*., 2017), as described later in section 1.6. *Fusarium* species complexes can often be further subclassified into *formae speciales* (ff. spp.) which are defined by pathogenicity to a specific host. FO for example contains over 120 ff. spp., some of which can be further classified into races, which have evolved to break resistance of certain host cultivars. Classification of isolates into ff. spp. or races can sometimes be determined using vegetative compatibility tests, which is advantageous as this is quicker than pathogenicity assays (Correll, 1991).

As aforementioned, two of the major plant pathogenic species complexes within the *Fusarium* genus are the FO species complex (FOSC) and the *F. solani* species complex (FSSC). Members of the FOSC are responsible for a wide range of diseases on plants, such as damping off, vascular wilts, root, foot and crown rots (Aoki *et al*., 2014), and includes the FO responsible for causing foot and root rot in pea, which is different from *F. oxysporum* f. sp. *pisi*, the causal agent of Fusarium wilt in pea. There are 50 *formae speciales* in the FOSC (Baayen *et al*., 2001) while the FSSC is said to comprise of around 60 distinct species (Aoki *et al*., 2014) with 12 *formae speciales* and two races (Suga *et al*., 2000). Both complexes contain species and *forma speciales* capable of infecting several legume hosts, including bean, pea, chickpea, clover (*Trifolium* spp.), soybean (*Glycine max*), lentil, alfalfa (*Medicago* spp.), lupin (*Lupinis* spp.) and pigeon pea (*Cajanus cajan*) (Suga *et al*., 2000; Aoki *et al*., 2014; Williams *et al*., 2016).

1.5.2.2 *Fusarium* *pea foot rot complex pathogen life cycle and epidemiology*

Infection generally initiates from thick-walled resting chlamydospores for both FO and FSP, which are formed at the end of the previous infection cycle and can survive in soil or on rotting plant material for long periods of time (Nelson *et al*., 1994; Biddle & Cattlin, 2007). For FO in particular, infection is favoured by a ground temperature of 25 °C (Kraft, 1994). The chlamydospores germinate in response to pea root exudates (Biddle and Cattlin, 2007) and can develop into various structures including macro- and microconidia (Nelson *et al*., 1994), new chlamydospores (Schippers & Voetberg, 1969) and hyphae (Stover, 1970; Biddle and Cattlin, 2007). The hyphae from chlamydospores or macro- and microconidia grow towards and colonise the root tissue and/or the stem base, either through direct penetration or through wounds (Nelson *et al*., 1994; Porter *et al*., 2015). Mycelium then grow throughout the root system and can also enter the pits of xylem, moving upwards throughout the vascular tissues, although this is limited. During this growth, microconidiophores are formed which produce microconidia, thin-walled spores which are responsible for the majority of the spread of infection within the plant, as they can break away and travel through the vascular tissues (Nelson *et al*., 1994; Agrios, 2005).

Symptoms of FSP (Figure 1.2) infection include small lesions at the base of the stem, brown to black in colour. This can progress up the stem to just above the soil line, where individual lesions become single, larger black legions, and throughout the root system, where more major rot occurs, and lateral root growth can be severely stunted. These lesions do not penetrate the vascular tissue in most cases but can expand when the pea plant develops flowers causing the vascular tissue to turn brick red in colour, due to the release of fusaric acid. Above ground symptoms include chlorosis and wilt of the lower leaves, which increases upwards as the infection progresses (Tu, 1987; Biddle and Cattlin, 2007; Porter *et al*., 2015). Symptoms of FO infection include yellowing of the vascular tissue of the taproot which extends up the stem, with chlorosis of the leaves progressing in a similar fashion (Tu, 1987).

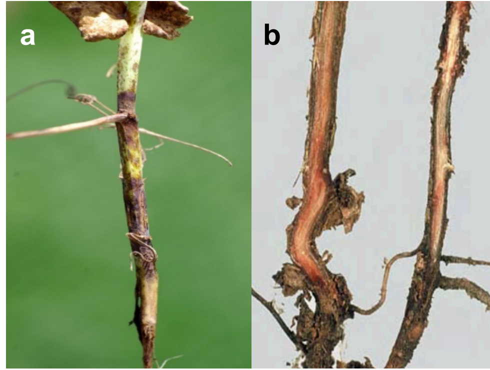


Figure 1.2. a) Lesions and darkening of the stem (Legume Matrix, 2018) and b) reddening of the vasculature, specifically caused by *F. solani* f. sp. *pisi* (adapted from Biddle and Cattlin, 2007).

1.5.3 *Aphanomyces euteiches*

1.5.3.1 Taxonomy and distribution

Another important PFRC pathogen is the oomycete AE, which affects several legumes, including *Medicago* spp. such as alfalfa, common bean *(Phaseolus vulgaris*) and clover (Gaulin *et al.*, 2007). It belongs to the *Aphanomyces* genus which consists of around 45 species, including other plant pathogens such as *A. cochloides*, the causal agent of black root rot on sugar beet and *A. raphani*, the causal agent of black root of radish (Grünwald & Coyne, 2003). It has been noted that a phylogenetic study of the genus which takes into consideration host ranges is overdue (Gaulin *et al.*, 2007).

AE was first identified as a pathogen of pea over 90 years ago, when the cause of infection and subsequent yield decline of pea in Wisconsin, USA was first investigated (Jones and Linford, 1925 cited by Gaulin *et al.*, 2007). It is regarded as one of the major pathogens of the PFRC where it occurs (Grünwald & Coyne, 2003), with isolates often being the most virulent in pathogenicity tests (Persson *et al.*, 1997). Like other PFRC pathogens, it has a cosmopolitan distribution, having been identified in countries such as the USA (Gaulin *et al.*, 2007), Canada (Wu *et al.*, 2018), Sweden (Persson *et al.*, 1997), France (Wicker *et al.*, 2003), the Netherlands (Oyarzun *et al.*, 1997), Australia (Gangneux *et al.*, 2014), New Zealand and Japan (Hughes & Grau, 2007).

1.5.3.2 *Life cycle and epidemiology*

AE oospores, present in plant debris and soil, germinate in response to pea root exudates, particularly during early pea development. The oospores can either germinate and produce a germ tube, which then develops into hyphae, or produce sporangia, which within them develops zoospores which can be released. The hyphae then penetrate the root tips of the pea plant and/or the motile zoospores move towards the root tissue and encyst. Infection is favoured by moist conditions which allow the zoospores the greatest motility, with a temperature range between 22 °C - 28 °C. More oospores develop within the infected root tissue and hyphae grow both between and within the cells. Epicotyl cells collapse and above soil-line infection symptoms become apparent. In the later stages of infection once root invasion is successful, warmer and dryer conditions are more favourable for severe infection. Oospores formed within the tissue can remain as the plant decomposes or are expelled into the surrounding soil, and then remain dormant until the next cropping cycle (Figure 1.3) (Hughes & Grau, 2007; Gaulin *et al*., 2007). There is a chance that if infection is severe enough, post emergence damping-off can occur (Tu, 1987).

Symptoms of AE root rot generally begin to show around ten days post infection (Hughes & Grau, 2007). Typical disease symptoms include softened honey, grey or dark discolouration of the root tissues (Figure 1.4), as well as destruction of root nodules, with an eventual complete loss of function. Stem symptoms include yellowing and browning of the tissues, wilting of the leaves and stem, and a decrease in both formation and successful development of the pods (Hughes & Grau, 2007; Gangneux *et al*., 2014).

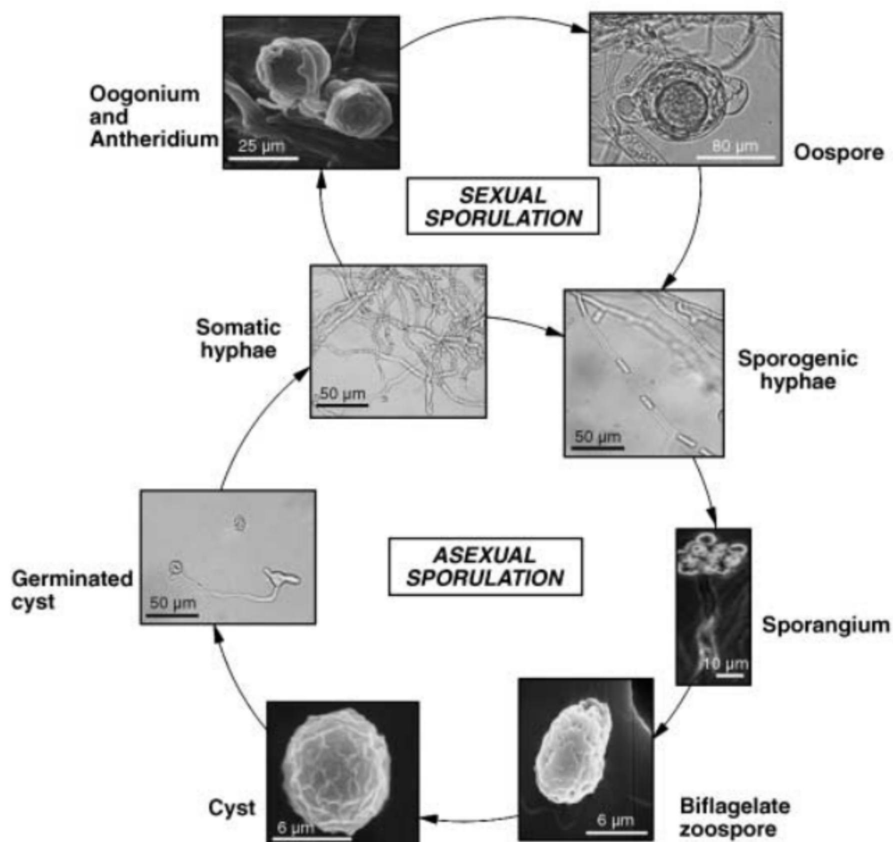


Figure 1.3 Life cycle of *Aphanomyces euteiches* (adapted from Gaulin *et al*., 2007).

Figure 1.4. Typical symptoms of *Aphanomyces euteiches* foot rot, with honey-coloured roots and stunted growth (Adapted from Kraft & Pfleger, 2001).

1.5.4 *Didymella pinodella*

1.5.4.1 Taxonomy and distribution

The fungal pathogen DP is comparatively understudied as part of the PFRC despite being identified several decades ago (Wallen *et al.*, 1967; Wallen, 1974). It has undergone many changes in both name and taxonomy since its discovery, more recently due to advances in molecular phylogeny. It was first identified in 1927 as *Ascochyta pinodella*, in a study to differentiate between different pathogens causing Aschochyta blight of pea and was recognised as the causal agent of pea foot rot (Jones, 1927). Further studies 40 years later showed that the causal agent of black stem of lucerne, then known as *Phoma trifolii*, was morphologically identical to *A. pinodella*, and therefore these isolates were merged and reclassified under a new name, *Phoma medicaginis* var. *pinodella* (Boerema *et al.*, 1965). The current *Phoma* genus, a member of the Didymellaeae family, is polyphyletic and was first described over 140 years ago. It contains around 220 species which occupy a diverse range of habitats including soil and water. The genus *Phoma* is mostly known for its range of plant pathogens but also contains many isolates that can infect immunocompromised individuals (Bennett *et al.*, 2018). In 1987, *A. pinodella* was reclassified as *Phoma pinodella* but following advances in molecular phylogenetics, studies to more accurately classify members of the Didymellaceae resulted in *P. pinodella* being reclassified within the *Peyronellaea* group of the *Phoma* genus as *Peyronellaea pinodella*. Even as recently as 2015, the taxonomy of members of the Didymellaceae, including the *Phoma*, *Ascochyta* and *Didymella* genera remain contested, and it was noted that the *Didymella* genus remained relatively understudied with many species phylogenetically unresolved. Subsequently, phylogenetic analysis of the Didymellaceae was undertaken using a multi-locus sequencing approach and as a result, around 20 isolates of the *Peyronellaea* group formed a subclade within the *Didymella* group in the phylogenetic analyses, including *P. pinodella*. Therefore, *P. pinodella* was reclassified as *D. pinodella* and this is its currently accepted name (Chen *et al.*, 2015). The Didymellaceae family currently includes 36 distinct genera and research is now examining the phylogeny of certain family members in context with their host range (Chen *et al.*, 2017; Hou *et al.*, 2020).

DP has a worldwide distribution, having been identified in several countries in Europe (Persson *et al.*, 1997), including Hungary, the Netherlands, Poland (Zhao *et al.*, 2021) and Germany (Baćanović Šišić *et al.*, 2017); Japan, Canada, the USA (*Zhao et al.*, 2021) and Australia (Davidson *et al.*, 2011). It is particularly prevalent in cooler

climates with high soil moisture (Persson *et al*., 1997; Biddle & Cattlin, 2007). Evidence from diseased pea plant samples analysed by the Processors and Growers Research Organisation (PGRO) suggests that DP may be a major component of the PFRC in certain pea fields in the UK (Dr Lea Herold, personal communication).

1.5.4.2 Life cycle and epidemiology

DP infection of peas can be initiated from infested soil, plant debris and seed (Biddle & Cattlin, 2007; Marcinkowska, 2008). It has various hosts in addition to field pea, including soybean, red clover (*Trifolium pratense*) (Zhao *et al*., 2021), chickpea, alfalfa, sweet pea (*Lathyrus odoratus*) and lupin (Deb *et al.*, 2020).

DP survives both on previous crop debris and within the soil as pycnidiospores, chlamydospores and sclerotia which germinate in response to pea root exudates (Figure 1.5), with the resulting mycelium penetrating through the foot of the stem and the root, by surrounding and invading the epidermis (Biddle & Cattlin, 2007; Deb *et al*., 2020). Within the tissue, pycnidia begin to form, containing pycnidiospores (chlamydospores) which can serve as a source of secondary infection which can also be disseminated to higher aerial parts of the plant causing foliar symptoms (Deb *et al*., 2020). The tissue at the base of the stem then breaks down, causing a characteristic browned, twisted and girdled appearance (Figure 1.6) caused by necrotic lesions (Biddle & Cattlin, 2007; Deb *et al*., 2020).

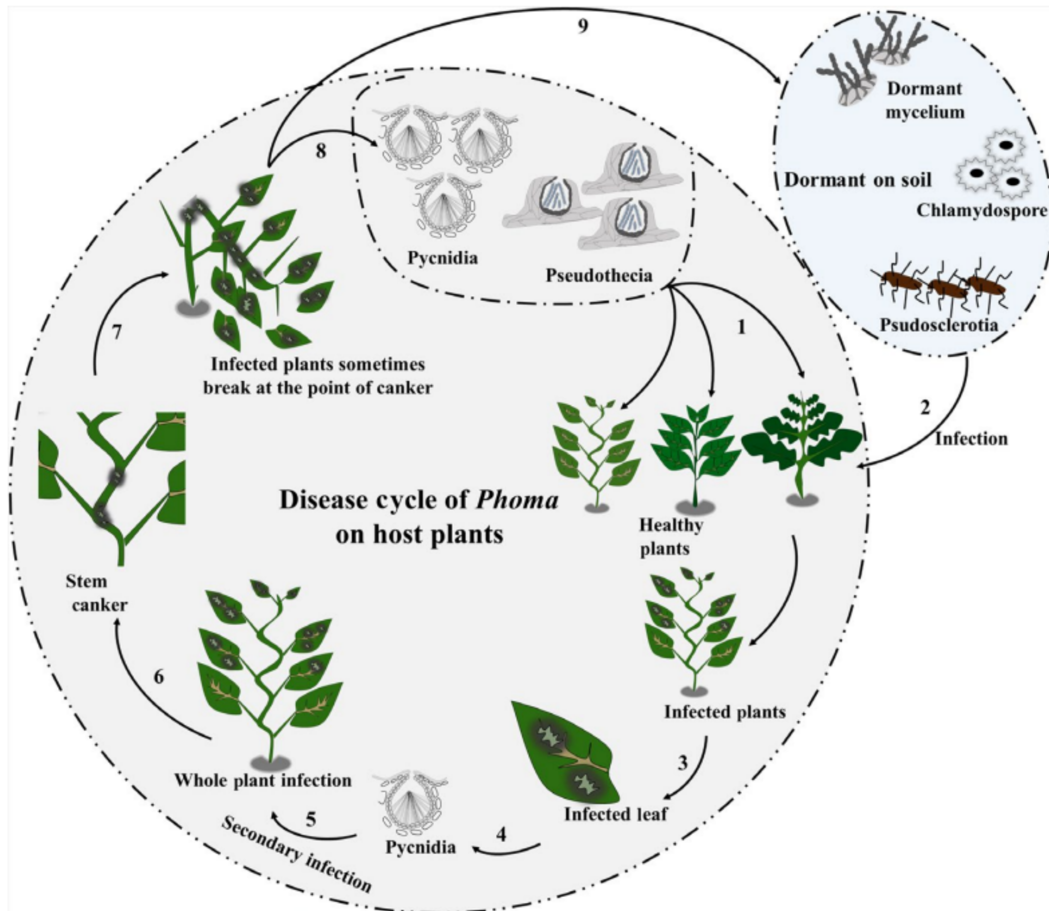


Figure 1.5. Life cycle of species of the *Phoma* genus, which resides in the same family, the Didymellaceae, as *Didymella pinodella* (adapted from Deb *et al*., 2020).

Figure 1.6. Foot rot infection by *Didymella pinodella*, with obvious root discolouration and black lesions.

1.6 Other species involved in the pea foot rot complex

In addition to the primary pathogens of the PFRC in the UK described above, there are also other reported species within the disease complex depending on the study location. Factors such as interactions with other PFRC pathogens and disease severity potential also differ between studies, and this has been partly attributed to differing soil types, climates, and disease management practices (Burke & Kraft, 1974; Persson *et al*., 1997). Other *Fusarium* spp. reported to be part of the PFRC include *F. avenaceum* (Šišić *et al*., 2017), and *F. redolens* (Esmaeili Taheri *et al*., 2017a). The generalist pathogen *Thielaviopsis basicola* also causes black root rot of pea (Bodah, 2017) and approximately 170 other distinct plant species. In pea, key symptoms include black rot of the tap and lateral root tissues and the epicotyl (Soylu & Dervis, 2011; Bodah, 2017), but studies have found that *T. basicola* infection does not occur or extend past the cotyledon (Oyarzun *et al.*, 1993; Bødker *et al.*, 1993) and that the pathogen does not generally inhibit germination of pea (Blume & Harman, 1979). *T. basicola* is a major pathogen in certain regions of Western Europe, including the UK (Salt & Delaney, 2013), the Netherlands (Oyarzun *et al*., 1993) and Denmark (Bødker *et al*., 1993). It is also a constituent of the PFRC in Turkey (Soylu & Dervis, 2011), regions of Northeast USA (Lockwood, 1961) and the Great Lake regions of Michigan, USA (Blume & Harman, 1979) and Ontario, Canada (Tu, 1987). *Pythium* spp. are also known to be involved in the PFRC but often have not been identified to species levels in some studies; however, *P. ultimum* and *P. irregulare* are reported to

be particularly problematic (Blume & Harman, 1979; Kerr, 1963; Tran *et al.*, 2016). Symptoms of Pythium root rot include reduced seedling vigour and stunted growth at the early stages with subsequent chlorosis and yield reduction. Damping-off can also occur as a soft root rot, light in colour (Kerr, 1963; Tu, 1987). *Pythium* spp. have been found in PFRCs in areas around the world such as Michigan, USA (Blume & Harman, 1979), the Canadian Prairies (Gossen *et al.*, 2016), Southern Australia (Kerr, 1963) and the Netherlands (Oyarzun *et al.*, 1993). *Rhizoctonia solani* has also been identified as a component of the PFRC, in areas of the Great Plains regions such as Alberta (Hwang & Chang, 1989) and North Dakota (Mathew *et al.*, 2012), as well as regions of Australia including New South Wales, Queensland and Tasmania (Watson, 2013). *R. solani* as a species can be subclassified further into anastomosis groups (Hwang *et al.*, 2007) with pathogenic isolates of pea belonging to AG-4 and some to AG-5 in studies (Hwang *et al.*, 2007; Mathew *et al.*, 2012). However, it has been noted as being infrequently isolated compared to other PFRC pathogens in surveys, or not to be a key pathogen of the complex in terms of disease severity (Kraft & Roberts, 1970; Hwang & Chang, 1989; Mathew *et al.*, 2012).

There are also reports of pests contributing indirectly to PFR infection, through lesions caused roots and nodules through feeding (Riga *et al.*, 2008; Porter *et al.*, 2015; Willsey *et al.*, 2021). FSP exploits the root lesions caused by the nematode *Pratylenchus penetrans* (Riga *et al.*, 2008), commonly known as the root lesion nematode, to cause infection (Porter *et al.*, 2015). Similarly, *F. avenaceum* exploits root lesions for easy entry caused by the pea leaf weevil, *Sitona lineatus* during its larval life stage where it feeds on root nodules (Willsey *et al.*, 2021).

1.7 Management of pea foot rot complex pathogens

1.7.1 Partial genetic resistance in pea and resistant pea varieties

Currently, there are no commercial varieties entirely resistant to all PFRC pathogens (Grünwald & Coyne, 2003) but general improvement of resistance in pea varieties is considered one of the primary control methods (Gaulin *et al.*, 2007). PFRC resistance research has faced multiple challenges, such as identification of only partial resistance, the complex nature of root and foot rot infection where multiple pathogens are present, and a lack of consistency in pathogenicity testing (Papavizas & Ayers, 1974; Pilet-Nayel *et al.*, 2002). Breeding for resistance against AE in pea has been

undertaken for over 70 years (Pilet-Nayel *et al*., 2002) although research to determine the underlying genetic resistance in pea varieties has only been developed more recently (Gaulin *et al*., 2007). Hosts of AE such as pea and alfalfa also have complicated genomes, so the closely related model plant *Medicago truncula*ta, which is self-fertile and has a smaller genome is now used in resistance research (Cook, 1999; Gaulin *et al*., 2007). Studies have also been undertaken to identify quantitative trait loci (QTLs) responsible for partial resistance against AE root rot and to assess resistance performance in different pea growing environments. Seven genomic regions over six linkage groups have been identified which were associated with multiple disease assessors (root rot disease severity, wilt severity and plant any weight), including the QTL *Aph1*, which at one field site was responsible for up to 49 % variation in resistance (Pilet-Nayel *et al*., 2002). Some studies have also examined how well AE resistance QTLs perform against a variety of AE isolates in addition to different growth conditions of pea (Pilet-Nayel *et al.*, 2005). A decade-long French-American collaboration examined four pea lines known for AE resistance and conducted studies to further understand genetic resistance used in breeding programs (Hamon *et al.*, 2013). Other approaches have attempted to identify plant development genes which are unfavourable for pathogen colonisation and integrating these into breeding programs alongside AE resistance. One study identified a single nucleotide polymorphism (SNP) mapped to a known resistance QTL that was associated with both AE resistance and root development, specifically with increasing root area coverage (Desgroux *et al.*, 2018). This could be beneficial for reducing AE root rot severity if roots could grow further away from a concentrated area of infection (Desgroux *et al.*, 2018).

Partial resistance in pea has also been investigated for the *Fusarium* spp. components of the PFRC, particularly more for FSP than FO. Experimental breeding for resistance has been ongoing for almost fifty years, with early research establishing consistent genetic resistance to FSP and *P. ultimum* in pea lines derived from five commercial cultivars with known resistance to root rot (Muehlbauer & Kraft, 1973; Abstract only). The *Pisum* core collection (387 pea lines from 58 countries) has also been screened in glasshouse experiments both for resistance to Fusarium root rot, and AE, which is important given that FSP and AE infection often occurs together. This study found 44 lines with appropriate levels of partial resistance to FSP, but a weak correlation with resistance to AE (Grünwald & Coyne, 2003). Initially, just two QTLs have been associated with FSP resistance in pea following a study which utilised microsatellite markers to identify resistance QTL's in a recombinant inbred line between markers AA160 and AD53 on chromosome 8 which explained 39 % of

the root rot score variation over two field trials (Feng *et al.*, 2011). However, more recently Coyne *et al*., (2019) identified a QTL, *FSP-Ps2.1*, which explained up to 53 % of resistance variation for metrics such as root disease severity and plant weight and height loss (Coyne *et al.*, 2019).

Genetics approaches to identify resistance to both AE and FSP have also been undertaken in common bean, by identifying whether any QTLs were responsible for partial resistance against both pathogens. Three QTLs were associated with AE and two with FSP, although these mapped to different areas of the genome (Hagerty *et al.*, 2015) and like the study by Grünwald & Coyne, (2003), indicates that genetic resistance to different pathogens are likely controlled by different genetic mechanisms. This indicates the importance and challenges of a multi-pathogen approach for resistance studies.

1.7.2 Cultural practices

The lack of completely effective resistance in pea for the management of the PFRC has resulted in an emphasis on other control strategies such as crop rotation (Skoglund *et al.*, 2011) and cultural practices with an emphasis on employing multiple approaches for efficacy (Hagerty *et al*., 2015). Soil management is particularly important, especially the reduction of soil compaction. There is also evidence that reduced- or zero-till farming may contribute to PFRC pathogen inoculum for the subsequent growing season in infected fields and that multiple PFRC pathogens overwinter on pea debris of the previous season (Esmaeili Taheri *et al.*, 2021). Other practices include raised seedbeds which may reduce root rot severity through better drainage (Tu, 1987). In terms of crop rotation, pea and other host legumes should not be included for up to 10 years if AE has caused a previous foot rot infection (Papavizas & Ayers, 1974), and more than five years for *Fusarium* infected fields as the infective propagules can survive for at least this period in the soil (Haware *et al.*, 1996).

1.7.3 Biological control agents

Research has also examined the potential of biological control agents (BCAs) for control of PFRC pathogens, with much of the research focussing on AE foot and root rot, although control of FO and FSP has also been investigated. BCAs are

beneficial microorganisms which antagonize or suppress pathogenic species (Eilenberg *et al.*, 2001) and their effectiveness against PFRC pathogens (often as seed treatments) has been evaluated through *in-vitro*, glasshouse and field experiments. In field trials with a moderate-severe risk of AE root rot, the BCAs *Pseudomonas cepacia* and *P. fluorescens* applied as seed treatments significantly increased pea seed germination and yield compared to untreated seed (Parke *et al.*, 1991) while *P. cepacia* and *Corynebacterium* spp. were also shown to supress AE root rot incidence at harvest (Bowers & Parke, 1993). Another study on AE identified several *Bacillus* and *Paenibacillus* species that inhibited AE germ tube growth by more than 50 % and these were subsequently used as seed treatments to examine their effects on PFR in a pot-based glasshouse trial. *B. cereus* 15.80 was found to significantly reduce root rot symptoms in one trial, and *P. polyxma* 18.25 reduced both root rot symptoms and number of oospores per gram of root tissue (Wakelin *et al.*, 2002). The BCA *Clonostachys rosea* ACM941 was identified as an antagonist of multiple PFRC pathogens, including AE, FO, and FSP. In a controlled environment experiment, where substrate was inoculated with individual PFRC pathogens, *C. rosea* ACM941 applied as a seed treatment significantly increased germination by 38%, and 26% in FSP and FO inoculated substrate respectively, and by 23% and 17% respectively in field trials (Xue, 2003). Another study identified two bacterial species antagonistic to FSP from the rhizosphere of pea (*B. subtilis*) and okra (*B. halotolerans*) through agar plate based dual culture experiments and these significantly decreased mean pea germination time and increased germination level (Riaz *et al.*, 2021). Combining multiple BCAs as a seed treatment has also been examined for control of FO foot rot, through dual culture assays, and in both glasshouse experiments and field trials. Agar plate tests demonstrated the ability of various *Trichoderma viride* and *B. subtilis* strains to significantly inhibit FO mycelial growth. In the glasshouse, a combination of a mix of arbuscular mycorrhizal fungi, *T. harzianum* HL9 and *P. fluroscens* HE21 were the most effective against pre-emergence, post-emergence damping off and root rot severity (75 %, 75 % and 80 % respectively) compared to the control and performed better than any other combination of the three BCAs and was close in performance to the fungicide Topsin (thiophanate methyl). This effect with the triple BCA combination treatment was also replicated in subsequent field trials (El-Sharkawy *et al.*, 2021).

1.7.4 Biofumigation

Biofumigation is another potentially effective control method for PFRC pathogens. This can be defined as the use of certain plants of the Brassicaceae family which when grown on and/or incorporated into soil, release volatiles (such as isothiocyanates; ITCs) which result in disease suppression (Kirkegaard & Matthiessen, 2004; Gimsing & Kirkegaard, 2009). Biofumigation was one of the alternative control options researched in response to the ban of methyl bromide in the Montreal Protocol of 2005 following confirmation that it was a class 1 ozone depleting substance (Gimsing & Kirkegaard, 2009). Biofumigant crops contain glucosinolates (GSLs), which are stored in cellular vacuole. Upon plant tissue damage, through mechanical means or from pests and pathogens, GSLs come into contact with the enzyme myrosinase, which is found in all plants containing GSLs and stored in a separate myrosinase cell. In brief, myrosinase then catalyses the breakdown of GSLs in the presence of water, most commonly to form isothiocyanates. Other GSL breakdown products include thiocyanates, nitriles, and oxazolidines (Bones & Rossiter, 1996; Mithen, 2001). There is a lack of research into biofumigation strategies against the PFRC pathogens DP, FO and FSP, with almost no published literature regarding the control of DP with biofumigant crops. This area will be covered more extensively later in the thesis.

1.7.5 Cover crops and green manures

The benefits of cover crops and other forms of soil management have become increasingly apparent over the last century. They help protect from soil erosion by providing a barrier between direct rainfall and the soil (Hartwig & Ammon, 2002) while also preventing the establishment of weeds (Dabney *et al.*, 2001), improving soil structure and increasing nutrient availability for future crops (Newman *et al.*, 2007). Cover crops are used as green manures when they are incorporated into the soil to provide further benefits, mostly the addition of organic matter, which enhances the carrying capacity of available nutrients water and in the soil. Therefore, a cover crop can be selected based on the additional nutrients they can provide upon incorporation, as well as their benefits to other soil properties (Newman *et al.*, 2007). There is very little published research available on the benefits of incorporating cover crops into a rotation where pea is the major crop, or to alleviate soil borne diseases of pea although it has been shown that establishing winter/spring oat cover crops prior

to a pea crop can reduce disease severity due to AE (McKay *et al.*, 2003). In another study, oats incorporated as a green manure has also been shown to significantly reduce AE disease severity in comparison with a non-treated control (Williams-Woodward *et al.*, 1997). In contrast, leguminous cover crops, such as vetch and clover, have been found to be hosts for various *Fusarium* spp. which in tests have been found to be highly pathogenic on pea, such as FO and *F. avenaceum* (Šišić *et al.*, 2018).

Another study aimed to examine the effects of cover crops in vining pea rotations in the UK, with an emphasis on soil health and PFRC pathogens. Field trials were conducted over three years from 2017. Part of these trials involved cover crops sown in the autumn preceding pea in early spring, with assessment of various soil health indices, foot rot severity and incidence for FSP, DP and AE. Summary data of the trials indicated that different cover crops performed different roles in reducing soil compaction which has been linked to an increase in PFR disease development and DP spore abundance; phacelia (*Phacelia tanacetifolia*) for example was more effective at reducing topsoil compaction with its shallower but denser root structure, whereas the large tap root of oil radish (*Raphanus sativus*) was able to break up deeper soil. Soil structure was assessed using the visual evaluation of soil scoring matrix and this determined that phacelia and black oat (*Avena stringiosa*) cover crops were the most effective. Oil radish appeared to increase the risk of DP PFR due to an increase in inoculum concentration. Overall, the use of cover crops to improve soil compaction was strongly linked to a reduction in PFR infection (Jelden & Herold, 2020).

1.8 Aims and Objectives

The overall aims of this research are to better understand pathogen dynamics of the PFRC in the UK and to identify biofumigant crops that can suppress the PFRC. The specific objectives are:

1) To identify and characterise isolates of *Fusarium* and *Didymella* from infested pea samples and fields in the UK using molecular methods and phylogenetics.
2) To examine the effects of different spore concentrations of single PFRC pathogens and different combinations of PFRC pathogens on disease development and mortality of pea using *in vitro* and glasshouse-based assays.

3) To identify biofumigant crop species and varieties that can suppress individual PFRC pathogens using *in vitro* assays and glasshouse experiments.

2. Characterisation and phylogenetic analysis of *Didymella* and *Fusarium* isolates

2.1 Introduction

2.1.1 Surveying and characterisation of isolates involved in the pea foot rot complex

The identity and prevalence of pathogens of the pea foot rot complex (PFRC) have been examined through detailed field surveys in different countries and continents around the world (e.g., Persson *et al*., 1997; Chittem *et al*., 2015; Baćanović-Šišić *et al*., 2018; Chatterton *et al.*, 2019). Methods partly involve isolations from root tissue for culturing, for both morphological identification (including microscopy) but also for DNA extraction and polymerase chain reaction (PCR) for molecular identification. Such isolates may then be used in pathogenicity testing to confirm virulence. The study by Chatterton *et al*., (2019) confirmed the importance of molecular identification. They noted that *Aphanomyces euteiches* (AE) was more widespread than suspected previously throughout the Canadian Prairie region (consisting of the Great Plains and the provinces of Saskatchewan, Alberta and Manitoba), due to the more accurate molecular approach taken in identifying causal agents of pea foot rot (PFR) in infected root tissue compared to previous culture-only approaches (Chatterton *et al*., 2019).

2.1.2 Morphology and molecular techniques for the characterisation of fungi

Correct identification of fungi and an understanding of taxonomy is of great importance for understanding basic fungal biology, such as development and diversity, to identifying pathogenic fungi responsible for plant and mammalian diseases (McNeil *et al.*, 2004; Rana *et al*., 2017; Badotti *et al.*, 2018). Prior to advances in molecular biology around 30 years ago, culture morphology was the basis of fungal species identification and phylogeny (Young & Gillung, 2020). This included microscopic assessment of mycelium, spores and other structures. Pathogenicity testing and the observation of disease symptoms and progression was also employed to identify or confirm host specificity (Badotti *et al*., 2018). However, there are several problems associated with the use of morphology, including the influence of phenotypic plasticity, life cycle stage and gender of the organism. Differences in experimental conditions can alter the outcome of pathogenicity testing.

Furthermore, the number of scientists with the necessary skill sets required for this work is also in sharp decline (Hebert *et al.*, 2003; Šišić *et al.*, 2018). Culture based identification of PFRC pathogens has also been shown to be limited for identifying the number of species in infected root tissue compared to molecular methods and can also severely underestimate the frequency of certain pathogens, such as AE (Chatterton *et al.*, 2019). Therefore, molecular approaches have been the primary method employed for accurate identification of fungi since the early 1990's (White *et al.*, 1990). This involves the sequencing of specific regions (loci) of DNA (e.g., partial gene or non-coding sequences including introns), often referred to as 'barcodes', which are amplified by universal primers. The resulting sequences then generally allow sufficient discrimination between different taxa, species, subspecies or *formae speciales* (ff. spp.) (Hebert *et al.*, 2003; Schoch *et al.*, 2012; Tekpinar & Kalmer, 2019). It is recommended that such barcodes are between 500-800 base pairs (bp) in length and that any primers designed can amplify as broad a range of taxa as possible while retaining good species discrimination (Hebert *et al.*, 2003; Schoch *et al.*, 2012).

Several DNA loci have been exploited as barcodes for fungal identification, classification and phylogenetics, with aligned DNA or protein sequences of species of interest enabling examination of evolutionary histories and relationships between species (Yang & Rannala, 2012). These include but are not limited to the *internal transcribed spacer* regions (*ITS*), *intergenic spacer* regions (*IGS*) and *large subunit* (28S; *LSU*) of the rRNA gene, the *second largest subunit of the RNA polymerase II* gene (*RPB2*), the *beta-tubulin* (*TUB2*) gene, and the *translation elongation factor 1-alpha* gene (*TEF1-α*) (Tekpinar & Kalmer, 2019). The *ITS* region consists of the internal transcribed spacer 1 DNA, the *5.8S rRNA* gene, and the internal transcribed spacer 2 DNA and is considered as the primary barcode for fungi (Begerow *et al.*, 2010). It contains a relatively large number of base insertions and deletions which are ideal for taxonomic resolution of most fungi (Seifert, 2009). However, some universal *ITS* primers are unable to amplify DNA from certain taxa, and therefore critical species in survey-type studies may be missed. In addition, there are also issues with discrimination when using *ITS* to identify and resolve species within the *Fusarium* genus (O'Donnell & Cigelnik, 1997) where the *TEF1-α* gene is the barcode of choice. This is due to excellent resolution within the genus, with the *TEF1-α* sequence comparatively diverse between species compared to other barcode regions, such as *TUB2* (Geiser *et al.*, 2004). However, *TEF1-α* sequences do not generally distinguish between ff. spp. within *Fusarium oxysporum* (FO) or *F. solani forma specialis* (f. sp.) *pisi* (FSP).

2.1.3 Characterisation and phylogenetic analysis of the *Fusarium solani* species complex

The phylogeny of the *Fusarium solani* species complex (FSSC) has been investigated in the context of both mammalian and plant pathology. The complex contains numerous plant pathogens, including FSP, *F. solani* f. sp. *eumartii*, the causal agent of Eumartii wilt of potato (Romberg & Davis, 2007) and *F. solani* f. sp. *phaseoli*, the causal agent of root rot of bean (Chen *et al.*, 2020).

Early studies of FSSC molecular phylogeny using the *ITS* and *LSU* regions aimed to test the polytypic species concept (where all taxa can be further classified into sub-species) derived from the non-molecular taxonomic classification of *F. solani* (FS), by studying the pathogens responsible for sudden death syndrome and root rot in soybean. Certain root rot isolates were re-classified as non-*Fusarium* species with isolates responsible for sudden death syndrome identified as *F. solani* f. sp. *phaseoli*, a f. sp. which formed a distinct species as part of the FSSC (O'Donnell & Gray, 1995). Other early molecular phylogenetics studies of FSSC aimed to resolve different *F. solani* ff. spp., with their analyses based on both individual and concatenated sequence alignments of *LSU*, *TEF1-α* and *ITS*. The phylogeny revealed nine FS ff. spp., including FSP and 26 other distinct FSSC species. Some FS isolates also clustered into distinct clades based on their location (O'Donnell, 2000). Another study found that some FS ff. spp. did not form distinct clades, as was the case for FSP and *F. solani* f. sp. *curcubitae* in a phylogenetic analysis based solely on *ITS* sequences, while mating type tests with tester strains confirmed specific f. sp. identity (Suga *et al.*, 2000). This further illustrates that *ITS* does not sufficiently resolve *Fusarium* species.

More recent studies have examined the host specificity and phylogeny of *F. solani* f. sp. *eumartii* isolates (Romberg & Davis, 2007) and FS isolates from legume crops, particularly from pea (Šišić *et al.*, 2018) in the context of other FSSC isolates. The study by Šišić *et al.*, (2018) sampled isolates from several European countries and examined whether location and related climatic conditions were associated with differences in phylogeny, pathogenicity, or host specificity. Phylogenetic analyses of the sampled isolates were conducted using *TEF1-α* and *RPB2* loci alongside other isolates of confirmed monophyletic species of the FSSC. The *TEF1-α* based phylogeny resulted in clades within the already-established 'FSSC clade 3', with most isolates forming two subclades within one major clade and clustering with a pre-confirmed FSP isolate; The authors suggested a new taxonomic identify for the isolates clustered within this major clade; *Fusarium pisi* (Šišić *et al.*, 2018). This was

further informed by pathogenicity tests where isolates from different legume hosts, including species of vetch, clover and *Medicago* were also found to be pathogenic on pea, while a representative isolate causing pea foot rot (PFR) from the same clade was pathogenic on 20 different species of legumes. The same FS isolates in the *RPB2* based and concatenated phylogeny clustered within the *F. pisi* major clade. It was also noted by the authors that the majority of isolates located within this clade were of German and Swiss origin, and isolates samples from Italy formed the majority that clustered in the FSSC 5 clade, which has been designated as *Fusisporum solani* (Schroers *et al.*, 2016; Šišić *et al.*, 2018).

2.1.4 Characterisation and phylogenetic analysis of the Didymellaceae family

The Didymellaceae family is one of the largest and most diverse within the fungal kingdom, with recent studies isolating members from over 50 different biotic and abiotic substrates (Chen *et al.*, 2017; Hou *et al.*, 2020). The family contains the *Phoma* and *Ascochyta* genera and several important plant pathogens, including *A. fabae*, a causal agent of Ascochyta blight on bean (Faridi *et al.*, 2021) and *Epicoccum nigrum*, a more generalist plant pathogen known to cause disease on several crops, including rice, tomato and corn (Taguiam *et al.*, 2021). The Didymellaceae family and its member taxa have undergone several reclassifications. The classification of *Phoma* species was initially based on morphological characteristics and a classification guide split the *Phoma* genus into nine sections (Boerema, 2004). However, the genus has since been noted to show great phenotypic plasticity on agar plate cultures (Aveskamp *et al.*, 2009; Chen *et al.*, 2015), highlighting the importance of robust molecular characterisation.

The Didymellaceae family was first established following a molecular phylogenetics study in 2009 (de Gruyter *et al.*, 2009), which aimed to resolve issues of the *Phoma* classification and its polyphyletic origins. Selected isolates of the nine *Phoma* sections designated by Boerema (2004) and other suborder members of the Phialopycnidiinea were characterised based on *small subunit rRNA* (*SSU*) and *LSU* sequence alignments, and phylogenetic analysis revealed that representative isolates of five of the nine *Phoma* sections clustered within a subclade designated under the Pleosporales, alongside other species including those of *Didymella* and *Ascochyta*; this was designated as the Didymellaceae (de Gruyter *et al.*, 2009). A larger scale phylogenetics study with a multilocus approach published two years later aimed to further resolve the Didymellaceae, with an interest in establishing genera within the

family in addition to *Phoma*. The inconsistency of the former nine sections of *Phoma* was confirmed, with most *Phoma* species resolving into clades designated as part of the Didymellaceae (Aveskamp *et al.*, 2010); those which didn't were the subject of further characterisation (de Gruyter *et al.*, 2012). The genus however still appeared to be polyphyletic, resolving into six different families (Aveskamp *et al*., 2010).

Later studies recognised the polyphyletic nature of *Phoma*, *Ascochyta* and *Didymella* and the lack of research and number of unresolved species in the latter genus. A multilocus phylogenetic approach using concatenating alignments of *ITS*, *RPB2*, *TUB2* and *LSU* sequences resolved 17 genera within the Didymellaceae, nine of which were new. *Didymella*, *Phoma* and *Ascochyta* were at last then defined as separate clades of monophyletic origin (Chen *et al*., 2015). Further phylogenetics studies have subsequently incorporated new taxa into the Didymellaceae (Chen *et al*., 2017). One of these characterised over 1000 isolates from 92 countries collected from more than 50 different substrates, including human, animal and plant tissue, soil, air, and water. This study resolved seven new genera and 40 new species and suggested *RPB2* as a secondary barcode for phylogenetic studies of the Didymellaceae due to good species resolution (Hou *et al*., 2020). However, there are still questions regarding the nature of the species within the Didymellaceae, such as whether there is any evidence of host specificity (Chen *et al*., 2017).

2.1.5 Aims and objectives

The aim of the work in this chapter was to characterise and examine the phylogeny of *Didymella pinodella* (DP) and FS isolates sampled in the UK. The specific objectives were to:

1. Identify and generate multilocus sequence data for potential DP isolates from infected soil and plant samples in the UK through DNA extraction, PCR and sequencing of the *ITS*, *RPB2*, *TUB2* and *LSU* barcoding regions.
2. Identify and generate multilocus sequence data for potential FSP isolates from infected soil and plant samples in the UK through DNA extraction, PCR and sequencing of the *RPB2* and *TEF1-α* barcoding regions.
3. Conduct phylogenetic analyses of UK DP isolate sequences from objective 1 in the context of other species of the Didymellaceae implicated in the Ascochyta blight complex to examine both within-species and geographical diversity.

4. Conduct phylogenetic analyses of UK FSP isolate sequences from objective 2 in the context of other European FSP isolates (Šišić *et al.*, 2018) to examine both within-species and geographical diversity.

2.2 Materials and methods

2.2.1 Collection and maintenance of fungal isolates

Potential *Didymella* and *Fusarium* isolates from pea were supplied on agar plates by the Processors and Growers Research organisation (PGRO) (Table A 1, Table A 2) for species identification and subsequent phylogenetic studies. Isolates previously identified as FS collected from diseased pea plants in fields located across Yorkshire, Lincolnshire and Suffolk by Sascha Jenkins were also obtained from an established culture collection at the University of Warwick (UoW; Table A 2; Jenkins, 2018). For long term storage, all isolates were cultured on potato dextrose agar (PDA; Merck, Germany) in 9 cm Petri dishes for one to two weeks at 20 °C in the dark. A 5 mm plug from the actively growing edge of the culture was placed onto PDA at a slant in a sterile 20 mL screw top Sterilin tubes (Thermo Fisher Scientific, USA) tubes and stored at 4 °C.

2.2.2 Preparation of liquid culture and DNA extraction of fungal isolates

Agar plugs of mycelium from each fungal isolate (3 x 5 mm from the growing edge of a culture) were placed into a Falcon tube containing 20 mL of 50 % potato dextrose broth (PDB; Oxoid, UK) and grown for seven to 10 days at 20 °C depending on the isolate. Liquid cultures were centrifuged at 4200 RPM for five minutes and residual broth removed. Cultures were then washed in sterile reverse osmosis (SRO) water and centrifuged twice, before being frozen at – 80 °C and subsequently freeze-dried for at 48 hours.

To extract DNA, freeze-dried mycelium was placed into tubes (lysing matrix A, MP Biomedicals, USA) and lysed at 6 ms^{-1} for 40 seconds using a FastPrep-24 sample preparation system (MP Biomedicals, USA) and DNA extracted using the DNeasy Plant Mini Kit (Quiagen, Netherlands) with modifications to the manufacturer's instructions as follows. After the addition of buffer AP1 and RNAse stock A, the tube was centrifuged at 13,000 RPM and the supernatant transferred to a new tube. For elution, 50 µL of buffer AE was applied to the membrane for a five-minute incubation period prior to centrifugation and this step was repeated twice for a final elution volume of 100 µL. DNA concentration and quality was assessed using

a Denovix DS-11+ Spectrophotometer (Denovix, USA) and samples stored at -20 °C until use.

2.2.3 Polymerase chain reaction, gel electrophoresis and sequencing of fungal isolates

To characterise potential *Didymella* isolates, four loci and regions were selected for amplification and subsequent sequencing: *ITS*, *RPB2*, *TUB2* and *LSU* (Table 2.1).

Individual 20 µL PCR reactions consisted of; 10 µL REDTaq Readymix (Sigma-Aldrich, USA), 2 µL each of forward and reverse primer (10 mM), 6 µL of sterile PCR-grade water and 2 µL of template DNA. Tubes were briefly centrifuged prior to undergoing PCR and thermocycling conditions for each primer pair are described in Table 2.1.

PCR products (4 µL) were visualised on 1.2 % agarose (Sigma-Aldrich, USA) gels containing 1 µL mL^{-1} GelRed Nucleic Acid Stain (Millipore, USA) with 4 µL 1 kb DNA ladder (Sigma-Aldrich, USA) and photographed using a UV transilluminator. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Netherlands) according to manufacturer's instructions and 5 µL of purified product was added to 5 µL of either the forward or reverse primer (5 mM) and submitted for sequencing (Eurofins, Luxembourg).

Table 2.1. Primer pairs and PCR conditions used to identify *Didymella* and *Fusarium* isolates.

Gene	Primers	Sequences (5'-3')	PCR conditions	References
ITS	ITS1/ITS4	TCCGTAGGTGAACCTGCGG /TCCTCCGCTTATTGATATGC	94 °C 2 min; 35 cycles (94 °C 45 sec, 55 °C 30 sec, 72 °C 1 min); 72 °C 10 min	White *et al*., (1990)
	VG9/ITS4	TTACGTCCCTGCCCTTTGTA /TCCGTAGGTGAACCTGCGG	95 °C 5 min; 35 cycles (95 °C 30 sec, 48 °C 30 sec, 72 °C 1 min 20 sec); 72 °C 10 min	de Hoog & van den Ende, (1998)/White *et al*., (1990)
RPB2	RPB2-5F2/FRPB2-7cR	GGGGWGAYCAGAAGAAGGC /CCCATRGCTTGYTTRCCCAT	94 °C 5 min; 40 cycles (95 °C 45 sec, 60 °C (5 cycles)/58 °C (5 cycles)/ 54 °C (30 cycles) 45 sec, 72 °C 2 min); 72 °C 2 min	Sung *et al*., (2007)/Liu *et al*., (1999)
TUB2	Btub2Fd/BTub4Rd	GTBCACCTYCARACCGGYCARTG /CCRGAYTGRCCRAARACRAAGTTGTC	95 °C 5 min; 35 cycles (95 °C 30 sec, 48 °C 30 sec, 72 °C 1 min 20 sec); 72 °C 10 min	Woudenberg *et al*., (2009)
LSU	LR0R/LR7	ACCCGCTGAACTTAAGC /TACTACCACCAAGATCT	94 °C 5 min; 35 cycles (94 °C 45 sec, 48 °C 45 sec, 72 °C 2 min); 72 °C 7 min	Vilgalys & Hester, (1990)
TEF-1α	EF1/EF2	ATGGGTAAGGARGACAAGAC /GGARGTACCAGTSATCATGTT	95 °C 3 min; 30 cycles (95 °C 30 sec, 53 °C 30 sec, 72 °C 45 sec); 72 °C 7 min	O'Donnell *et al*., (1998)

2.2.4 Identification and phylogenetic analysis of *Didymella* and *Fusarium* isolates

Identity of all fungal isolates was confirmed by subjecting sequences to Basic Local Alignment Search Tool (BLAST) searches using the National Centre of Biotechnology Information (NCBI) website, confirming species with the match of the highest sequence percentage identity.

Phylogenetics analysis of DP isolates was conducted using sequences for the *ITS*, *RPB2*, *TUB2* and *LSU* regions and compared with sequences from the Convention of Biological Diversity (CBS) collection isolates of DP*, Didymella pinodes*, *D. glomerata*, *Ascochyta koolunga, A. pisi* and *Phoma herbarum* downloaded from NCBI (Table A 1). Phylogenetics analysis of FSP isolates was conducted using the FSP population sets for the *TEF-1α* (NCBI ID: 1167505175) and *RPB2* (NCBI ID: 1167505341) genes (Table A 2) generated by Šišić *et al*., (2018).

For each region/locus, sequences were imported into Molecular Evolutionary Genetics Analysis (MEGA; version 10; Stecher *et al.*, 2020), aligned with Clustal W and trimmed after visual inspection. Phylogenetic trees for each locus were constructed using the maximum likelihood method with the Tamura-Nei and nearest neighbourhood interchange models and 1000 bootstrap replications. Phylogenetic trees containing Didymellaceae isolates were rooted using *Leptosphaeria maculans* isolate CBS 275.63, which resides in the Pleosporales order, containing both the Leptosphaeriacea and Didymellaceae families. Trees containing FSP were rooted with *Fusarium redolens*.

2.3 Results

2.3.1 Identification and phylogenetic analysis of Didymellaceae isolates

Potential *Didymella* isolates provided by PGRO were identified and characterised through mycelial DNA extraction, PCR and sequencing of the *ITS*, *RPB2*, *TUB2* and *LSU* barcoding regions (Table 2.2). Of the 19 isolates provided, 14 were confirmed as DP, including a CBS culture collection isolate (CBS 107.46). Other identified species included *F. solani*, *Didymella pinodes* (CBS culture collection isolate 249.47), *Collectotrichum coccodes*, *Ascobolus crenulatus* and *Juxtiphoma eupyrena*.

DNA sequences of all the identified UK DP isolates, alongside those from CBS culture collection isolates of DP, *D. pinodes, A. pisi, A. koolunga* and *P. herbarum,* which are causal agents of the Ascochyta blight complex of pea were subject to phylogenetic analysis to explore any genetic diversity within DP and their relationship to other Ascochyta blight species. Sequences available for *ITS*, *RPB2*, *TUB2* and *LSU* were used to construct maximum likelihood phylogenetic trees.

None of the four phylogenetic trees constructed shared identical tree topology, but sequences of all loci separated DP and *D. pinodes* from all other *Ascochyta* species into different clades (Figures 2.1, 2.2, 2.3, 2.4). When *ITS* (Figure 2.1) and *LSU* (Figure 2.4) based trees were constructed, there was no discrimination between DP and *D. pinodes* isolates, as these species did not form separate clades. In contrast *RPB2* (Figure 2.2) and *TUB2* (Figure 2.3) resolved DP and *D. pinodes* as well as the other related species.

Overall, there was very little diversity within isolates identified as DP for any of the loci, with no variation at all found for *RPB2, TUB2*, and *LSU* sequences. For *ITS*, DP 'Cockie E' showed an increase in the mean nucleotide substitutions per site compared to other DP isolates. *LSU* had the lowest resolution of the four barcodes examined, with no within-species variation detected. In the *TUB2-* and *RPB2-* based trees, isolates of *P. herbarum* were distributed into subclades, as were *D. glomerata* for the *ITS* based phylogeny but bootstrap support for this was weak. There was little genetic variation within isolates of either *A. koolunga* or *A. pisi* in any of the phylogenetic analyses undertaken, apart from variation found within *A. pisi* isolate CBS 122750 in the *TUB2* based tree.

Table 2.2. Identity and field site information for potential *Didymella pinodella* isolates provided by PGRO, identified by sequencing the *β-tubulin* (*TUB2*) gene, *RNA polymerase II* (*RPB2*) gene, *internal transcribed spacer* (*ITS*) region and the *large subunit* (*LSU*, 28S) of the nuclear ribosomal RNA.

Isolate	Identity	Source	Soil type	Location
CBS 107.46	*Didymella pinodella*	Pea	n/a	The Netherlands
CBS 249.47	*Didymella pinodes*	Pea	n/a	Scotland, UK
61B	*Didymella pinodella*	Soil	n/a	Molescroft, England, UK
7838	*Collectotrichum coccodes*	n/a	n/a	Unknown
Cockie A	*Didymella pinodella*	Soil	n/a	Spalding, England, UK
Cockie E	*Didymella pinodella*	Soil	n/a	Spalding, England, UK
Holbeach	*Ascobolus crenulatus*	Soil	Deep Silt	Holbeach Marsh, England, UK
Kincreich	*Juxtiphoma eupyrena*	Soil	Loam	Scotland, UK
Majors South A	*Didymella pinodella*	Soil	Deep silt	Spalding Marsh, England, UK
Majors South B	*Didymella pinodella*	Soil	Deep silt	Spalding Marsh, England, UK
Phoma L	*Fusarium solani*	Root	n/a	Unknown
Ralston A	*Didymella pinodella*	Soil	Clay	Perth, Scotland, UK
Ralston B	*Didymella pinodella*	Soil	Clay	Perth, Scotland, UK
Silt Pits A	*Didymella pinodella*	Soil	Deep silt	Boston, England, UK
Silt Pits B	*Didymella pinodella*	Soil	Deep silt	Boston, England, UK
Telegraph A	*Didymella pinodella*	Soil	Clay-loam (high organic matter content)	Thorney, England, UK
Telegraph B	*Didymella pinodella*	Soil	Clay-loam (high organic matter content)	Thorney, England, UK
VH1	*Didymella pinodella*	Soil	Sandy loam	Barmby-on-the-Marsh, England, UK
VH3	*Didymella pinodella*	Soil	Sandy loam	Barmby-on-the-Marsh, England, UK

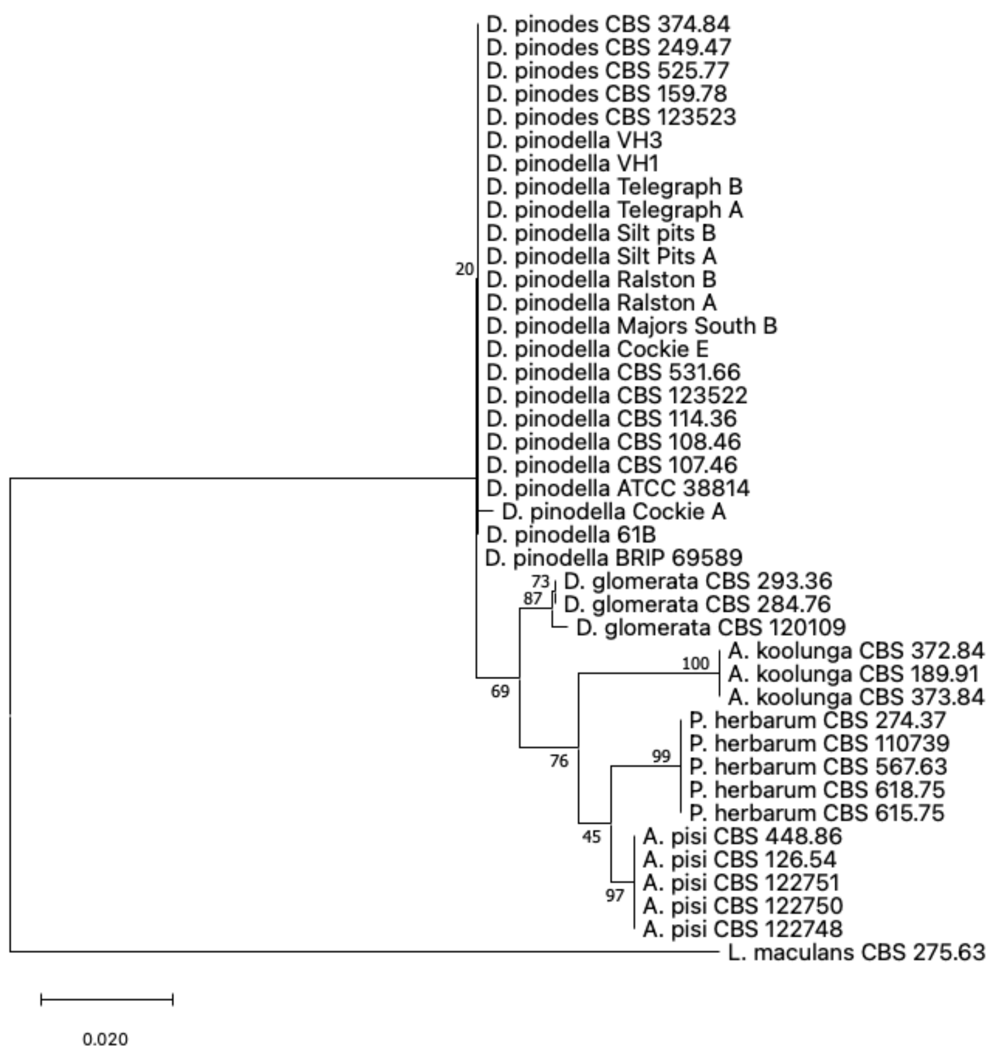


Figure 2.1. Phylogenetic tree constructed using a maximum likelihood method based on *ITS* sequences of UK *Didymella pinodella* isolates and Didymellaceae species associated with the Ascochyta blight and pea foot rot complexes. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of the mean number of base substitutions per site. The tree is rooted to *Leptosphaeria maculans* CBS 275.63.

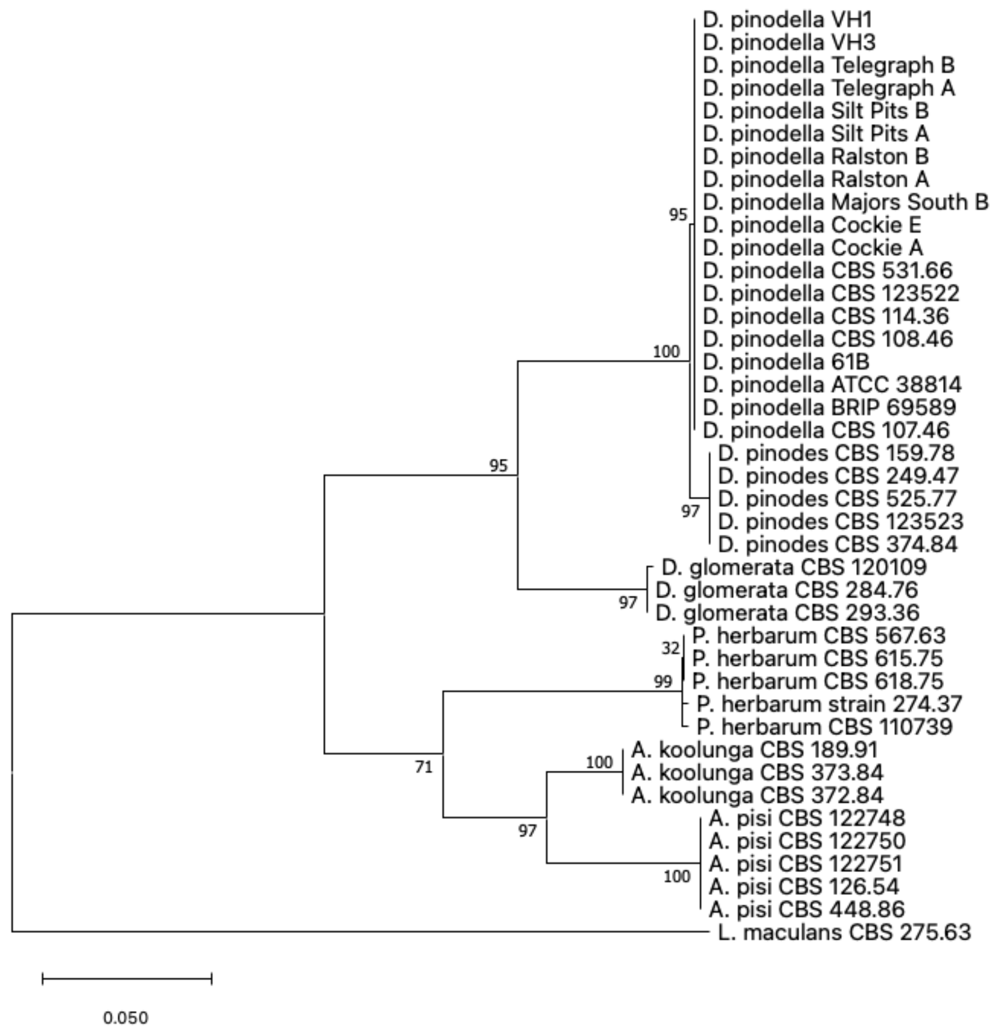


Figure 2.2. Phylogenetic tree constructed using a maximum likelihood method based on *RBP2* sequences of UK *Didymella pinodella* isolates and Didymellaceae species associated with the Ascochyta blight and pea foot rot complexes. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of the mean number of base substitutions per site. The tree is rooted to *Leptosphaeria maculans* CBS 275.63.

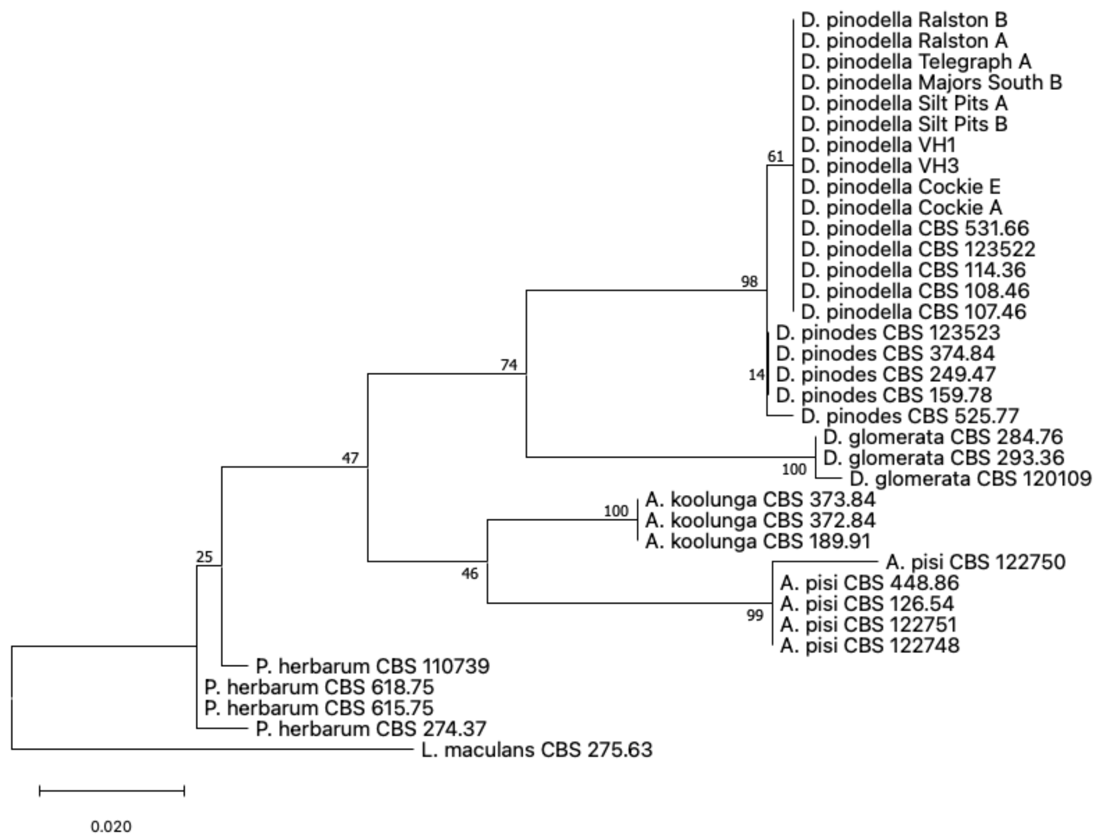


Figure 2.3. Phylogenetic tree constructed using a maximum likelihood method based on *TUB2* sequences of isolates of UK *Didymella pinodella* isolates and Didymellaceae species associated with the Ascochyta blight and pea foot rot complexes. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of the mean number of base substitutions per site. The tree is rooted to *Leptosphaeria maculans* CBS 275.63

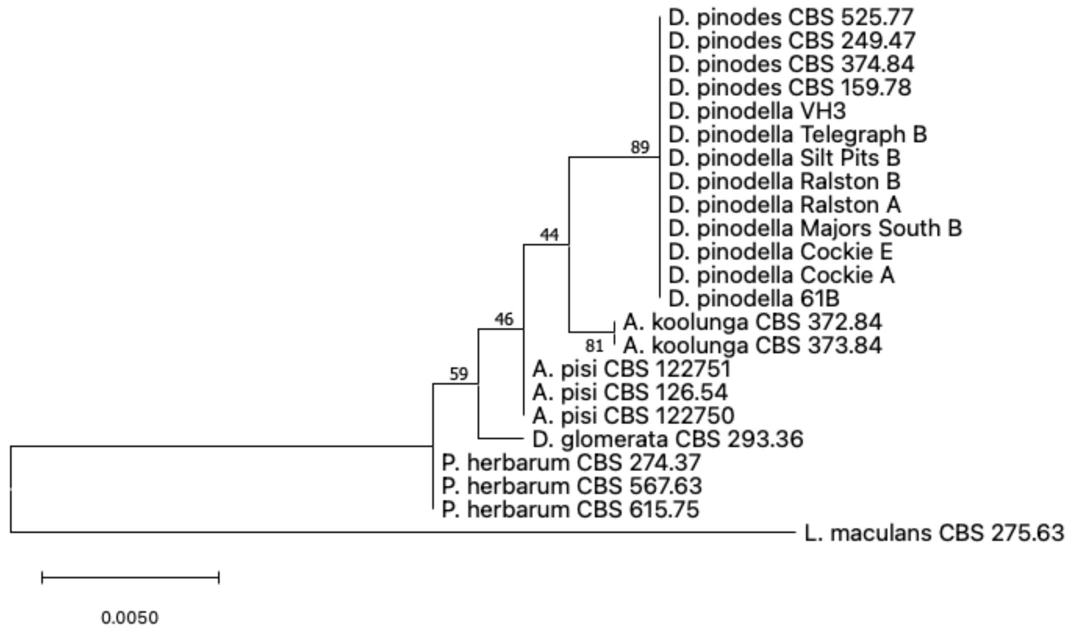


Figure 2.4. Phylogenetic tree constructed using a maximum likelihood method based on *LSU* sequences of isolates of UK *Didymella pinodella* isolates and Didymellaceae species associated with the Ascochyta blight and pea foot rot complexes. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of the mean number of base substitutions per site. The tree is rooted to *Leptosphaeria maculans* CBS 275.63.

2.3.2 Identification and phylogenetic analysis of *Fusarium solani* isolates

Potential FS isolates provided by PGRO and UoW were characterised through sequencing of the *TEF-1α* and *RPB2* genes (Table 2.3). Of the 16 isolates provided by PGRO, 10 were confirmed as FS. Other identified species were *F. oxysporum*, *F. redolens, F. venenatum* and *F. equiseti.*

FS isolates from PGRO/UoW and the population sets of FS isolates established by Šišić *et al*., (2018) were subject to phylogenetic analysis to explore genetic diversity of FS isolates and the relationship between UK FS isolates from pea and those examined by Šišić *et al*., (2018). Sequences available for *TEF-1α* and *RPB2* were used to construct maximum likelihood phylogenetic trees.

There was some genetic diversity among the PGRO and UoW FS isolates examined. Phylogenetic analyses for both *TEF-1α* and *RPB2* genes were able to reconstruct the three major FS clades designated in the analysis by Šišić *et al*., (2018) which are: *Fusarium pisi* comb. nov. (FSSC clade 3), *Fusarium solani* sensu stricto (FSSC clade 5) and Lineage (1) novel species. All the FS isolates from PGRO and UoW were located in either of the two former major clades.

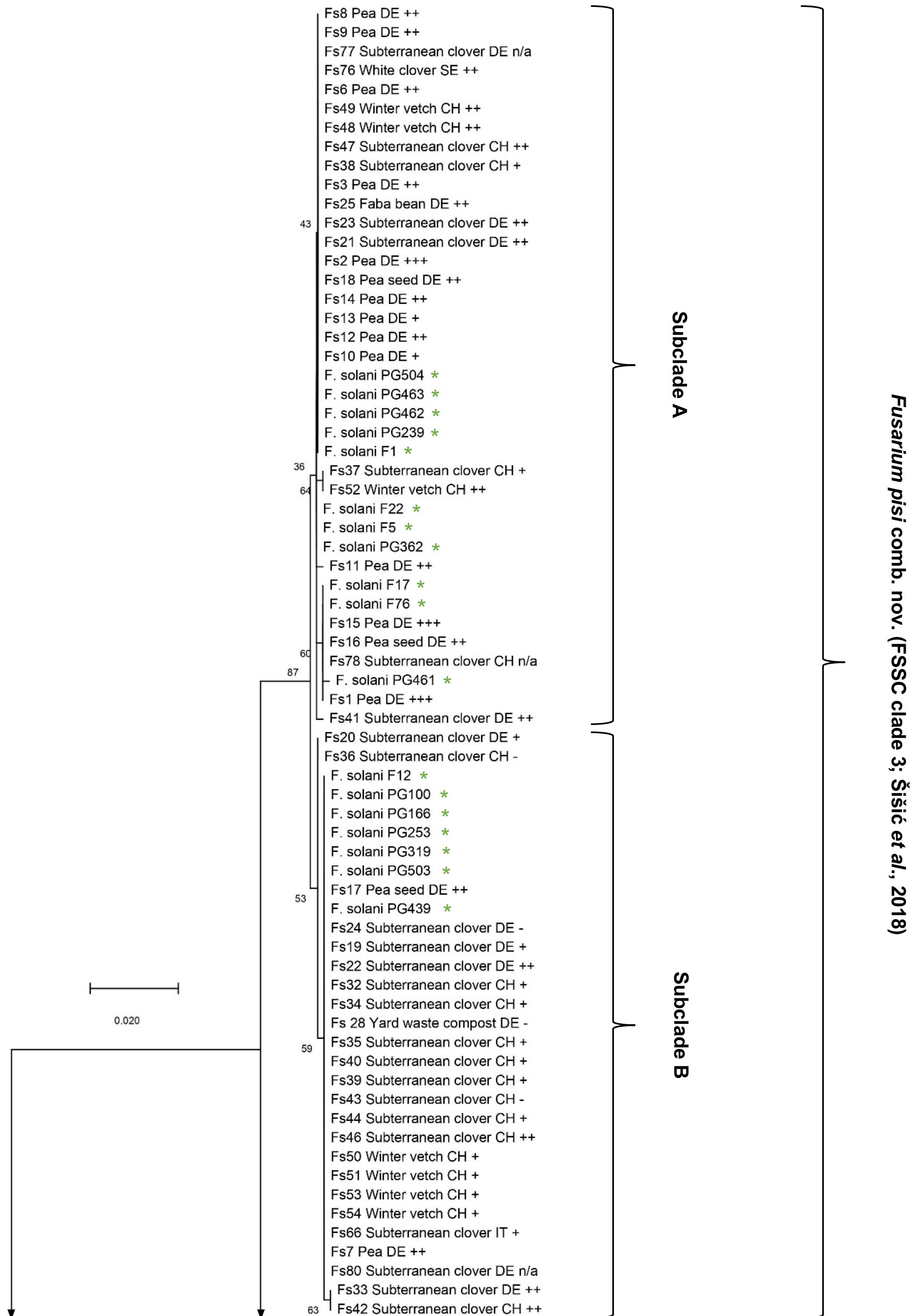
The *TEF-1α* based phylogenetic tree indicated that all but one of the PGRO/UoW isolates originating from the UK were located within the *Fusarium pisi* comb. nov. major clade, which with two exceptions was comprised of isolates from mostly leguminous hosts from Germany and Switzerland (Figure 2.5). The *F. pisi* comb. nov. clade (FSSC clade 3) was divided into two subclades as in the original tree of Šišić *et al*., (2018); the first comprised isolates identical to an established *F. pisi* comb. nov isolate (referred to here as subclade A), while the other comprised isolates which were identical to an established FS isolate (subclade B). However, subclade A was not as well supported in this analysis with a bootstrap value of 36 and there were several additional clades formed within these two subclades. Five of the seven PGRO isolates and six of the 12 UoW isolates examined were within subclade A, while only one of the seven PGRO isolates and six of the UoW isolates examined were in subclade B. Only one of the PGRO isolates, FS F42 was located in the second major clade, *Fusarium solani* sensu stricto (FSSC 5). This was mostly comprised of isolates which grouped with an isolate which in a previous study was reclassified as *Fusisporium solani* (Schroers *et al*., 2016). The isolates in this clade were mostly from vetch and clover in Italy, alongside a few isolates of German origin.

The *RPB2* based phylogeny consisted of selected isolates used in the *TEF-1α* based analysis by Šišić *et al*., (2018) alongside isolates from the UoW/PGRO collection. The PGRO/UoW isolates were located in the same major clades as

identified used in the *TEF-1α* analysis (Figure 2.6), but with a few additional isolates in the *F. pisi* comb. nov. major clade. This major clade did not share the same topology as the *TEF-1α* based tree but did divide into two subclades supported by bootstrap values of 60 and 63, which also did not occur in the *RBP2* based analysis by Šišić *et al.*, (2018).

Table 2.3. Identity of and information regarding potential *Fusarium* isolates provided by PGRO, identified by sequencing the *translation elongation factor 1-α* (*TEF1-α*) gene and RNA polymerase II (*RPB2*) gene.

Isolate	Identity
F1	*Fusarium solani*
F5	*Fusarium solani*
F7	*Fusarium venenatum*
F8	*Fusarium venenatum*
F9	*Fusarium solani*
F12	*Fusarium solani*
F17	*Fusarium solani*
F22	*Fusarium solani*
F42	*Fusarium solani*
F55	*Fusarium oxysporum*
F60	*Fusarium solani*
F61	*Fusarium solani*
F76	*Fusarium solani*
F105	*Fusarium equiseti*
F109	*Fusarium equiseti*
F112	*Fusarium redolens*



Fs8 Pea DE ++
Fs9 Pea DE ++
Fs77 Subterranean clover DE n/a
Fs76 White clover SE ++
Fs6 Pea DE ++
Fs49 Winter vetch CH ++
Fs48 Winter vetch CH ++
Fs47 Subterranean clover CH ++
Fs38 Subterranean clover CH +
Fs3 Pea DE ++
Fs25 Faba bean DE ++
Fs23 Subterranean clover DE ++
Fs21 Subterranean clover DE ++
Fs2 Pea DE +++
Fs18 Pea seed DE ++
Fs14 Pea DE ++
Fs13 Pea DE +
Fs12 Pea DE ++
Fs10 Pea DE +
F. solani PG504 *
F. solani PG463 *
F. solani PG462 *
F. solani PG239 *
F. solani F1 *
Fs37 Subterranean clover CH +
Fs52 Winter vetch CH ++
F. solani F22 *
F. solani F5 *
F. solani PG362 *
Fs11 Pea DE ++
F. solani F17 *
F. solani F76 *
Fs15 Pea DE +++
Fs16 Pea seed DE ++
Fs78 Subterranean clover CH n/a
F. solani PG461 *
Fs1 Pea DE +++
Fs41 Subterranean clover DE ++
Fs20 Subterranean clover DE +
Fs36 Subterranean clover CH -
F. solani F12 *
F. solani PG100 *
F. solani PG166 *
F. solani PG253 *
F. solani PG319 *
F. solani PG503 *
Fs17 Pea seed DE ++
F. solani PG439 *
Fs24 Subterranean clover DE -
Fs19 Subterranean clover DE +
Fs22 Subterranean clover DE ++
Fs32 Subterranean clover CH +
Fs34 Subterranean clover CH +
Fs 28 Yard waste compost DE -
Fs35 Subterranean clover CH +
Fs40 Subterranean clover CH +
Fs39 Subterranean clover CH +
Fs43 Subterranean clover CH -
Fs44 Subterranean clover CH +
Fs46 Subterranean clover CH ++
Fs50 Winter vetch CH +
Fs51 Winter vetch CH +
Fs53 Winter vetch CH +
Fs54 Winter vetch CH +
Fs66 Subterranean clover IT +
Fs7 Pea DE ++
Fs80 Subterranean clover DE n/a
Fs33 Subterranean clover DE ++
Fs42 Subterranean clover CH ++
43
36
64
60
87
53
59
63
0.020
Subclade A
Subclade B
Fusarium pisi comb. nov. (FSSC clade 3; Šišić *et al.*, 2018)

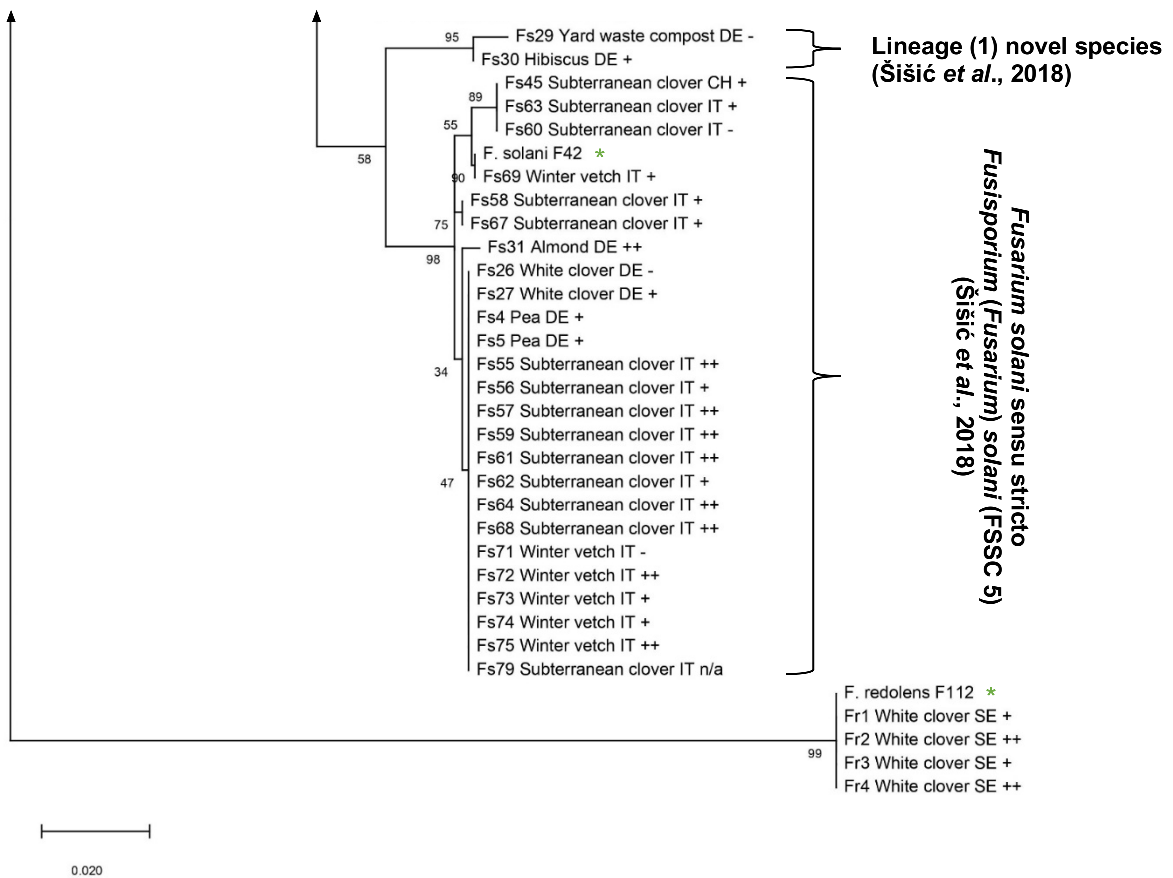


Figure 2.5. Phylogenetic tree constructed using a maximum likelihood method based on *TEF-1α* sequences of isolates of the FSSC. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of mean number of base substitutions per site. The tree is rooted to *F. redolens*. Some isolate sequences are from the population set of Šišić *et al*., (2018; NCBI ID: 1167505175). * = UK *Fusarium solani* isolates.

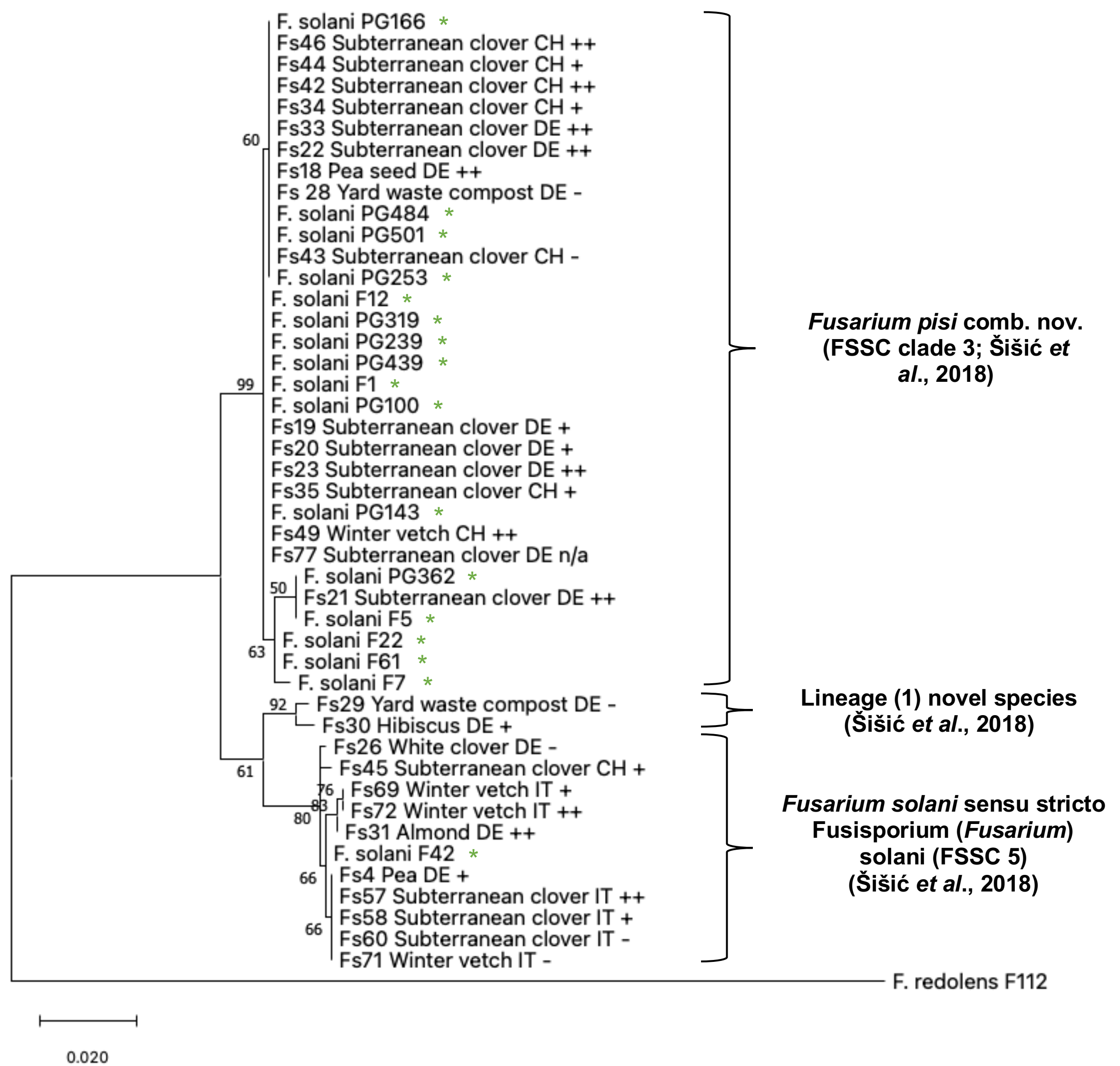


Figure 2.6. Phylogenetic tree constructed using a maximum likelihood method based on *RPB2* sequences of isolates of the FSSC. Bootstrap values of 1000 replications are indicated next to branches. The scale bar is indicative of the mean number of base substitutions per site. The tree is rooted to *F. redolens*. Some isolate sequences are from the population set of Šišić *et al*., (2018; NCBI ID: 1167505341). * = UK *Fusarium solani* isolates.

2.4 Discussion

The characterisation of individual species, phylogeny, and recent resolution of the paraphyletic origins of the genus *Didymella* has been little investigated. Similarly, an understanding of FSSC phylogeny still remains under investigation today, with revisions of taxonomy suggested for certain clades of FSP. The research described in this chapter examined the diversity of UK DP and FSP isolates and for the first time, defined their relationship with other related isolates and species from different geographical origins.

Potential UK *Didymella* and *Fusarium* isolates from both infected soil and plant samples were supplied by PGRO alongside confirmed FS isolates sampled from diseased pea plants from an established collection at UoW (Jenkins, 2018). *Didymella* spp. isolates were identified and characterised through sequencing of *ITS*, *RPB2*, *TUB2* and *LSU*, while potential FS isolates were identified through sequencing of the *TEF-1α* and *RPB2* genes. Of the 19 potential *Didymella* isolates and 16 potential FS isolates, 14 and 10 were identified as DP and FS respectively; this supports the use of molecular methods for identification in addition to, or instead of, culture morphology. Other identified species from the *Didymella* isolate collection included *D. pinodes*, *Collectotrichum coccodes*, *Ascobolus crenulatus* and *Juxtiphoma eupyrena*. As previously discussed, *D. pinodes* is a key pathogen of the Ascochyta blight complex of pea. Records first indicated *D. pinodes* being present in the UK in pea samples from North Yorkshire in 1964 (British Mycological Society, 2018). *Collectotrichum coccodes* is a fungal pathogen with a broad host range but is mostly associated with anthracnose of tomato and black dot disease of potato (Chesters & Hornby, 1965). It has been found to infect alfalfa, a rotation crop with potato (Nitzan *et al.*, 2006) The *Juxtiphoma* genus has recently been created as part of the Didymellaceae family, with *J. eupyrena* previously known as *Phoma eupyrena* (Valenzuela-Lopez *et al.*, 2018). *J. eupyrena* is a widespread soil borne pathogen (Morgan-Jones & Burch, 1988) and has been associated with dry rot of potato tubers (Choiseul *et al.*, 2006) and leaf spot of aloe vera (Avasthi *et al.*, 2017). Other identified species from the *Fusarium* isolate collection included *FO*, *F. redolens*, *F. venenatum* and *F. equiseti*. *F. redolens* has also been implicated as a PFRC pathogen (Zitnick-Anderson *et al.*, 2018) but isolates are also responsible for wilt and pre-emergence damping off diseases of a variety of crops, including banana, asparagus and melon (Baayen *et al*., 2001). *F. venenatum* has been identified in several countries in Europe and also has a wide host range including maize, wheat, hops and potato (Nirenberg,

1995). It has been noted that *F. venenatum* may not be pathogenic to these hosts, but recent studies have shown it to be a causal agent of foot and root rot of wheat in Germany (Farr & Rossman, 2021; Rigorth *et al.*, 2021). *F. venenatum has* also been developed as a source of mycoprotein for consumption (Wiebe, 2002). *F. equiseti* is a known plant endophyte but has been identified as a minor component of the Fusarium head blight complex of wheat (Marin *et al.*, 2012). A study in relation to the PFRC investigated whether the colonisation of *F. equiseti* on pea decreased the severity of foot rot symptoms caused by *F. avenaceum* and DP. Pre-colonisation of pea plants by *F. equiseti* five days prior to inoculation of DP significantly reduced foot rot disease severity while pre- or concurrent inoculation of *F. equiseti* and *F. avenaceum* lowered foot rot disease severity (Šišić *et al.*, 2017). The *Fusarium* species identified from the PGRO collection in this study were the same as identified in a previous study involving the characterisation of UK isolates from infected pea plants and pea seeds (Jenkins, 2018), with the exception of *F. venenatum*.

All the identified DP isolates, alongside CBS culture collection isolates of other species which are causal agents of the Ascochyta blight complex of pea were subject to phylogenetic analysis to explore genetic diversity of DP isolates and their relationship to other Ascochyta bight species. Sequences for *ITS*, *RPB2*, *TUB2* and *LSU* were used to construct maximum likelihood phylogenetic trees. Overall, there was very little diversity evident within UK DP and other DP isolates in any of the phylogenetic trees constructed, with all isolates located in the same clade and representing six different countries (UK, former Czechoslovakia, the Netherlands, Canada, USA and Australia). This supports other phylogenetic analyses of the Didymellaceae and the Ascochyta blight complex, which have mostly either sought to clarify the taxonomic divisions within the Didymellaceae, or have investigated the species diversity and subsequent phylogenetics of Didymellaceae species isolated from field and legume samples, particularly those belonging to the Ascochyta blight disease complex (Davidson *et al.*, 2009; Ahmed *et al.*, 2015; Chen *et al.*, 2015; Chen *et al.*, 2017; Hou *et al.*, 2020; Keirnan *et al.*, 2021). In these studies, DP isolates were located in the same clade and showed no variation; however less DP isolates were used in these studies compared to the research of this chapter. It is also important to note that some of these other studies made use of concatenated alignments of *ITS* and *RBP2* (Keirnan *et al.*, 2021), and *ITS*, *RPB2*, *TUB2* and *LSU* (Chen *et al.*, 2017; Hou *et al.*, 2020) showing a high level of conservation across multiple barcoding regions for DP. Therefore, a concatenation of the sequences used in this work may not result in any further discrimination of DP isolates. DP isolate sequences also appeared conserved despite geographical differences, in contrast to FS isolates

examined as part of this work and that of Šišić *et al*., (2018). For all the loci examined, there was separation of all Didymellaceae spp. into separate clades except for DP and *D. pinodes* for the *ITS* and *LSU* based phylogenetic trees. This is consistent with another study which created an *ITS* based phylogenetic tree of isolates sampled from pea plants in Southern Australia displaying Ascochyta blight symptoms (Davidson *et al*., 2009). Phylogenetic analyses of *RPB2* and *TUB2* sequences described here were able to distinguish between DP and *D. pinodes* as has other research utilising a concatenated alignment approach for several loci (Chen *et al*., 2015; Chen *et al*., 2017; Hou *et al*., 2020; Keirnan *et al*., 2021). Therefore, *RPB2* and *TUB2* are appropriate barcodes for DP identification, especially in the context of identifying the causal agents of PFRC disease in the field. Moreover, *TUB2* was also the most successful barcode for identifying within species genetic variation of the Didymellaceae isolates used in this study. Other studies of Didymellaceae phylogeny have also found *ITS* and *LSU* unsuitable for resolving different species. In the study by Chen *et al*., (2015), *ITS* and *LSU* based sequence alignments did not resolve all taxa even at genus level while *RPB2* was the most successful barcode, resolving all 17 genera. *TUB2* was the second most successful, resolving 13 of the 17 genera. In another study, Chen *et al*., (2017) found that *ITS* and *LSU* successfully resolved only 30 % and 54 % percent of species, compared to at least 90 % for *TUB2* and *RPB2*. Kiernan *et al*., (2021) found that *ITS* alone, even with adjustments made to the method for phylogenetic analysis did not resolve some taxa even at the genus level. It is therefore recommended that *RPB2* and *TUB2* are used for single locus phylogenetic analyses of DP and related species, or together in concatenated alignments. Identifying a consistent and appropriate barcode(s) for studies of Didymellaceae species will be useful in future phylogenetic analyses with more isolates from a wider geographical range.

All the identified *Fusarium solani* isolates from the PGRO/UoW collections, alongside population sets of FS isolates established by Šišić *et al*., (2018) were subject to phylogenetic analysis to characterise the UK isolates in the context of the established FS phylogeny, explore genetic diversity and any potential geographical variation. Sequences for *TEF-1α* and *RPB2* were used to construct maximum likelihood phylogenetic trees. Unlike for DP, there was diversity within the PGRO and UoW FS isolates, with some subclades containing these isolates within the three major clades identified previously: The *Fusarium pisi* comb. nov. clade (FSSC clade 3), Lineage (1) novel species clade and the *Fusarium solani* sensu stricto/*Fusisporium* (*Fusarium*) *solani* (FSSC 5) clade (Šišić *et al*., 2018).

This within-species diversity has also been observed in other phylogenetics studies regarding the FSSC, albeit with far fewer FSP isolates examined (O'Donnell, 2000). This level of discrimination also confirms the suitability of *TEF-1α* as the gold standard barcode for *Fusarium* molecular phylogenetics. *RPB2* also resulted in good species resolution, with the three same major clades defined, but with less within-species diversity defined. Therefore, *TEF-1α* alone or a concatenation of *TEF-1α* and *RPB2* sequence alignments will provide the best resolution of FSSC species.

All but one of the PGRO/UoW FS isolates was located within the *Fusarium pisi* comb. nov. clade, designated by Šišić *et al*., (2018) which comprised isolates mostly of German and Swiss origin isolated from pea plant tissue and seed, faba bean, subterranean clover, white clover, and winter vetch. There was therefore no apparent separation of UK isolates into separate clade(s) by geography. FS isolates also did not group according to their specific host (Šišić *et al*., 2018) and pathogenicity tests of isolates from the *Fusarium pisi* comb. nov. clade from multiple legume species showed they were all pathogenic on pea which suggested a broader host range than expected for FSP isolates which are traditionally associated only with pathogenicity on pea. This suggests a potential *Fusarium solani* 'legume clade', where isolates from different legumes could potentially infect other legume hosts. The idea of host specificity of ff. spp. has been challenged previously; Romberg and Davis (2007) found that *Fusarium solani* f. sp. *eumartii* was not just specific to potato, but also caused foot rot of tomato, and isolates sampled from both potato and tomato were pathogenic on the opposite plant species, as well as pepper and aubergine. This further supports an idea that FS isolates could be specific to multiple hosts from the same family rather than just one species; in this case, isolates of *F. solani* f. sp. *eumartii* could infect species from the Solanaceae family of which potato, tomato, peppers and aubergine are members and isolates of FSP are in fact capable of infecting multiple hosts of the Fabaceae family. Wide scale pathogenicity testing and further phylogenetic studies would need to be carried out to investigate this. However, there are FO f. sp. that also infect multiple hosts from the same family, such as *F. oxysporum* f. sp. *radicis-cucumerinum*, which can infect watermelon, melon, cucumber and bottle and luffa gourds (Vakalounakis, 1996).

In conclusion, the research described in this chapter successfully identified and characterised UK DP and FS isolates and through phylogenetic analyses, placed them in the context of other isolates and related species of diverse geographical origin. The phylogenetic analysis of UK DP isolates and selected isolates from other countries revealed the conserved nature of DP for each of the barcoding regions examined with little diversity and may also be the largest study of DP isolates to date.

The FS isolates characterised as part of this work were aligned with those in the established *Fusarium pisi* nov. comb. clade, a collection of mostly Central European isolates infecting a range of legumes. The use of *RPB2* and *TUB2* for DP phylogenies and *TEF1-α* and *RPB2* for FS phylogenies provided sufficient resolution for distinguishing species different species or within species diversity, although a concatenated alignment approach of *ITS*, *RPB2* and *TUB2* for DP phylogenies and *TEF1-α* and *RPB2* for FS phylogenies may be an improved approach for future studies especially as *ITS* for DP was able to resolve some within-species variation. It will also be important to establish the host range and pathogenicity of these isolates, especially for the FS isolates where the current taxonomic classification of *formae speciales* is debated, and whether these isolates have a larger host range within the Fabaceae.

3. Elucidating the interactions of pea foot rot complex pathogens

3.1 Introduction

3.1.1 Plant pathogen disease complexes, types of pathogen interactions, and the factors that affect them

The idea of single pathogens as the causal agent of plant disease is one that has been central in plant pathology for many years, in contrast to mammalian pathology. More recently, advances in DNA-based molecular methods such as quantitative polymerase chain reaction (qPCR) and next generation amplicon sequencing have allowed components of entire microbial communities to be defined to subspecies level (Lamichhane & Venturi, 2015). This has further promoted the idea that many plant diseases are the result of plant pathogen disease complexes, defined as 'a group of pathogens of different species that cause similar symptoms on a single host plant species' (Le May *et al*., 2009). Establishing the epidemiology and interactions of individual pathogens within these disease complexes is very desirable, as it can help inform the choice of management strategies (Le May *et al*., 2009). Pathogen interactions are affected by numerous factors but primarily thought to be majorly influenced by the competitive exclusion theory, whereby pathogens can co-exist in different niches to ensure their own survival (Fitt *et al.*, 2006). One factor is spatial, where pathogens may infect different parts of the plant and compete for nutrition (Fitt *et al*., 2006; Le May *et al*., 2009). Another factor is temporal, where pathogens cause infection at different times during the growing season, or have differences in infection strategy, including entry route into plant tissues and spore type (Fitt *et al*., 2006). Other factors include production of secondary metabolites that may affect other pathogens in the complex and the effects of pathogen entry into the plant before another (Le May *et al*., 2009).

Different types of pathogen interactions can occur depending on their development and pathogenicity. The presence of two or more pathogens that are independent of one another in the complex are described as additive. Pathogens that exist in a mutually beneficial relationship, or where one pathogen can enhance the pathogenicity of another beyond that of an additive effective is described as synergistic. In contrast, an antagonistic interaction is where one pathogen decreases

the pathogenicity of another below that of the additive effect (Koppenhöfer & Kaya, 1997; Syller, 2012). The interactions of the pathogens within the Ascochyta blight complex of pea overall are considered synergistic (Le May *et al*., 2009; Lamichhane & Venturi, 2015).

Plant pathogen disease complexes examined tend to be all-fungal (Lamichhane & Venturi, 2015), such as Fusarium head blight of cereal crops (Karlsson *et al.*, 2021), all-bacterial (Lamichhane & Venturi, 2015), such as pith necrosis of tomato caused by several *Pseudomonas* sp. (Trantas *et al.*, 2013), or all-viral (Lamichhane & Venturi, 2015), such as maize lethal necrosis caused by maize chlorotic mottle virus in combination with one or more viruses of the Potyviridae family (Redinbaugh & Stewart, 2018). A few disease complexes have been described that involve both fungi and bacteria, such as potato early dying disease, caused by the fungus *Verticillium dahliae* in combination with one or more *Pectobacterium* spp. (Dung *et al.*, 2014; Lamichhane & Venturi, 2015). In legumes, several disease complexes have been described, including the sudden death syndrome in soybean (*Heterodera glycines* and *Fusarium virguliforme*) (Westphal *et al.*, 2014), cowpea stunt (cucumber mosaic virus and blackeye cowpea mosaic virus) (Gillaspie Jr *et al.*, 1998) and in pea, the pea foot rot complex (PFRC) and the Ascochyta blight complex as previously mentioned. Interestingly, *Didymella pinodella* (DP) was for a long time considered part of this complex, and its interactions with other Ascochyta blight pathogens, such as *D. pinodes* have been studied (Le May *et al*., 2009). In one report it was concluded that there was an antagonistic relationship between DP and *D. pinodes* when co-inoculations were made on the same leaf, but there was an increase in pathogenicity when plants were sequentially inoculated with both species (Le May *et al*., 2009).

3.1.2 Elucidation of interactions between pathogens within the pea foot rot complex using non-molecular methods

Despite the many field studies elucidating the identities of pathogens within the PFRC in several regions around the world, the interactions between these pathogens are not well understood, even though initial research was conducted over 60 years ago (Buxton & Perry, 1959). Most studies tested pathogenicity of individual isolates only (e.g., Baćanović-Šišić *et al*., 2018) or focused on interactions between two, rather than multiple pathogens of the PFRC (Peters & Grau, 2002). An initial study of PFRC pathogen interactions involved plating out sections of stem and root tissue onto agar

from UK pea plants with wilt and foot rot symptoms (Buxton & Perry, 1959). Here it was reported that *F. solani* (FS) was more frequently isolated from root tissue samples than from the stem but was often isolated alongside DP. The researchers also noted that FS colonised root tissue at an earlier time than *Fusarium oxysporum* (FO) (Buxton & Perry, 1959). Another study aimed to discover and examine PFRC pathogens that were responsible for yield loss in pea growing areas of Southern Australia. Isolates of FO, FS and *Pythium* spp. were identified in naturally infested soils that had been cropped with peas for several consecutive years and were each determined to be pathogenic on a susceptible pea variety. During initial experiments, it was noted that FO was often co-isolated from tissue alongside *Pythium* spp. A glasshouse experiment was also set up to determine disease development on two cultivars of pea, using artificially inoculated field soils. Treatments included individual pathogen inoculum for FO, FSP and *P. ultimum* and different combinations of multiple pathogen inoculum, with ratios of pathogens in combination determined from relative amounts in naturally infested soils collected and all combinations of pathogens apart from FS with *P. ultimum* were examined. Plants inoculated with *P. ultimum* only resulted in restricted plant growth in comparison to an untreated control for both pea cultivars, but eventually growth recovered. Plants inoculated only with FO showed symptoms only at the onset of flowering in one cultivar. However, plants of one variety died and plants of another were stunted when grown in soil inoculated with both FO and *P. ultimum*. Plants in soil inoculated with FS all showed typical foot rot symptoms at the soil line (Kerr, 1963). Much more recent experiments have examined different combinations of the PFRC pathogens *Aphanomyces euteiches* (AE), FS, *F. redolens* and *F. avenaceum* in a glasshouse experiment. Overall, several combinations showed significant differences in disease severity compared to respective individual pathogens and/or untreated controls. (Willsey *et al.*, 2018). It is clear from these studies that there are interactions between pathogens of the PFRC, however their exact nature (e.g., additive, synergistic, antagonistic) are yet to be fully explored, particularly for the main pathogen species within the UK PFRC.

3.1.3 Elucidation of interactions between pathogens within the pea foot rot complex using molecular methods

Whilst culture-based isolations and pathogenicity testing has been informative for determining the major components of the PFRC and to study interactions, it does not provide quantitative data of the abundance of species in samples, lacks sensitivity

and is time consuming (Zitnick-Anderson *et al*., 2018). However more recently, DNA-based molecular methods such as qPCR assays using PFRC pathogen-specific primers have been developed to address this. One study developed a set of multiplex hybridization probe-based qPCR assays based on amplification of the *TEF1-α* gene for several *Fusarium* spp. of the PFRC which was tested on both artificially inoculated plants and those displaying symptoms recovered from the field (Zitnick-Anderson *et al*., 2018). Three qPCR assays were developed for seven pathogen species (FS, *F. redolens*, *F. avenaceum*, *F. graminearum*, *F. sporotrichioides*, *F. culmorum* and *F. acuminatum*), but not FO. To test these assays, peas were grown in sterile perlite and inoculated with 1 mL of $1x10^5$ spores mL^{-1} spore suspension. The treatments consisted of single pathogens or combinations, interestingly with each pathogen represented by six isolates. DNA quantity decreased for pathogen combinations of *F. avenaceum* and *F. culmorum*, and FS with *F. redolens* and *F. acuminatum* compared to respective DNA quantities for single pathogen inoculations, indicating possible antagonistic relationships within pathogen combinations (Zitnick-Anderson *et al*., 2018). In another study, a pot-based glasshouse assay was employed to examine PFRC pathogen interactions and harvested roots of the plants were used to quantify pathogen colonisation and interactions using multiplex qPCR assays (Willsey *et al*., 2018). Two assays were designed, with the first using primers targeting the *β-TUB1* gene of *P. sativum*, the *ITS* region for AE and the *TEF1-α* gene for *F. redolens*. The second targeted *P. sativum*, alongside FS and *F. avenaceum,* with primers targeting the *TEF1-α* for the latter two species. It was found that for *F. redolens*, there was a significant increase in DNA quantity when co-inoculated alongside AE or any of the other *Fusarium* spp. examined compared to individual inoculation. These interactions were synergistic. For FS, there was a significant increase in DNA quantity when co-inoculated alongside *F. avenaceum*, and *F. redolens* with AE, again these interactions were synergistic. For *F. avenaceum*, DNA quantity was significantly reduced when co-inoculated alongside *F. redolens*, and FS. These interactions were antagonistic. For AE, there was no significant change in DNA quantity when co-inoculated alongside any combination tested. There were also background levels of non-target pathogens found in numerous treatments. qPCR primers used to amplify DP and FSP from soil sampled were designed and tested as part of an Innovate UK project (Clarkson, 2019) to develop a more rapid approach to assessing PFR risk. The development of these qPCR assays has allowed the dynamics of individual PFRC components to be examined over time either in controlled experimental conditions or in the field and this approach could eventually be applied as an infield diagnostic tool.

3.1.4 Aims and objectives

The main aim of the research in this chapter was to establish the relationship between pathogen inoculum concentration and disease development in pea for each UK PFRC pathogen to select suitable inoculum concentrations for interaction and biofumigation experiments. The main objectives were to:

1. Examine the effect of individual UK PFRC pathogen inoculum concentration on disease development in pea, using both an *in vitro* based assay and glasshouse-based experiments.
2. Investigate the interactions of UK PFRC pathogens in terms of disease development in pea using the developed test-tube based assay
3. Quantify root colonisation of pea plants from the experiment of objective 2 using qPCR assays.

3.2 Materials and methods

3.2.1 Determining the effect of pea foot rot complex pathogen inoculum concentration on disease development in a test tube-based system

A test-tube based method was used to examine the effects of different spore concentrations of individual PFRC pathogens FO, FSP and DP (Table 3.1), on disease development and mortality of young pea plants. This was first optimised over a series of several preliminary experiments, which examined plant spacing, spore concentrations and watering methods (selected data shown in Appendix, Figure A 1) and was based on an assay used by PGRO for PFRC pathogen pathogenicity testing. Full experiments for each individual pathogen were then undertaken.

Pea seeds (cv. 'Avola', Kings Seeds/Thompson & Morgan, UK) were pre-germinated by first surface sterilising in 10 % sodium hypochlorite, after which they were washed, placed onto 1 % technical agar (Oxoid, UK) and incubated at 20 °C in a 16-hour light, 8-hour dark cycle for five to seven days. Spore suspensions of each of the PFRC pathogens FO, FSP and DP were prepared first by culturing each pathogen on potato dextrose agar (PDA) from storage (as in section 2.2.1). After two weeks (FO, FSP) and four weeks (DP), agar plates were scraped using sterile plastic spreader and sterile reverse osmosis (SRO) water and the solution filtered through three layers of sterile miracloth (Merck, Germany). The concentration determined using a Fuchs-Rosenthal haemocytometer. Final concentrations for each PFRC pathogen used in experiments were adjusted to 10, $1x10^2$, $10x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ spores mL^{-1} in SRO water.

Experiments were conducted in glass test tubes (150 mm x 25 mm, Fisherbrand, USA) which were washed with 70 % ethanol, filled two-thirds with a non-sterile 50:50 vermiculite (medium grade) perlite mix, and double spaced in racks (Nalgene, Thermo Fisher Scientific, USA, Figure 3.1). The vermiculite perlite mix in each tube was inoculated with 1 mL of spore suspension of each PFRC pathogen at the different concentrations (1 mL SRO for the untreated control), then 9 mL SRO water to promote an even distribution of spores throughout the substrate. A pre-germinated pea seedling was then placed into each tube, covered with non-sterile 50:50 vermiculite/perlite mix and watered with a further 7 mL SRO water.

Experiments were run for three weeks in a growth room under a 16-hour light, 8-hour dark cycle at 20 °C and were watered twice a week, once with Hoagland's solution (1.6 g L^{-1}, Sigma-Aldrich, UK) and once with SRO water according to a

gravimetric method. This consisted of setting up additional tubes to calculate water loss. Four of these tubes contained a pea seed and four contained the vermiculite/perlite substrate only. Prior to watering, the weight of each set of tubes (those with pea and those without) was measured, averaged and the difference in weight to the previous measurement calculated to water the experimental tubes. Tubes where the pea seedling had not grown above the top of the tube were watered with the lower amount calculated from the vermiculite/perlite only tubes to avoid water logging. Tubes where the pea seedling had grown above the top of the tube were watered with the higher amount calculated from the tubes containing pea. At the end of each experiment, plants were gently removed from the tubes using tweezers above the base of the stem, and roots washed in water to remove excess substrate. After blotting dry on paper towels, plants were assessed for disease development using both a root rot scoring index based on percentage root browning, developed by PGRO (Table 3.2) and the level of pea seedling mortality.

The experiment for each PFRC pathogen consisted of two biological repeats, which comprised of 12 replicate tubes per spore concentration treatment. Tubes, including those used for the gravimetric watering method, were arranged in a complete randomized design across the racks (Genstat 19, VSN International 2021). Mean root rot scores and angular-transformed percentage pea plant mortalities for each treatment were subject to a one-way analysis of variance (ANOVA) to determine significance ($p < 0.05$), with the standard error difference of the mean multiplied by the *t*-critical value of the residual degrees of freedom within each analysis used to calculate significant differences between treatment means. The experiment design was designed with support from Andrew Mead (Rothamstead Research, UK). Analyses were carried out with the support of James Lynn (Applied Statistical Solutions Ltd., UK), using Genstat 19 (VSN International, 2021).

Table 3.1. List of fungal isolates used in experiments.

Species	Isolate	Information
Fusarium oxysporum	PG18	Isolated from field Molescroft 61B, Molescroft, England, via PGRO crop clinic in 2012
Fusarium solani f. sp. *pisi*	PG13	Isolated from field 32a, UK, via PGRO crop clinic in 2012
Didymella pinodella	61B	Isolated from field Molescroft 61B, Molescroft, England, via PGRO

Table 3.2. Disease scoring system (designed by PGRO) used for assessing root rot severity due to PFRC pathogens test tube experiments.

Symptom Score	Root browning (%)	Disease severity
0	0	None
1	<10	Slight
2	11-25	Slight-moderate
3	25-50	Moderate
4	51-90	Moderate-severe, no stem girdling
5	91-100	Severe, stem girdling
6	100	Plant dead

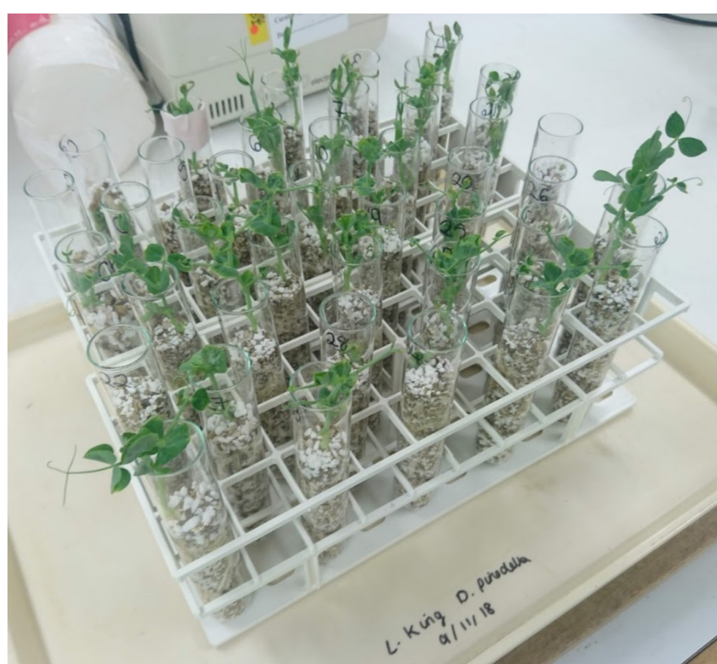


Figure 3.1. Pea plants in test tube assay inoculated with *Didymella pinodella* ‘61B’.

3.2.2 Investigating pea foot rot complex pathogen interactions in a test-tube based system

To examine the effects of both single and combinations of PFRC pathogens on disease development in young pea plants and deduce interactions between them, the test tube system was used with a spore concentration of 10 spores mL^{-1} for each pathogen, informed by the results of the previous experiments in section 3.2.1. These experiments were carried out to determine the inoculum concentration for each pathogen, which caused low levels of pea plant mortality but significantly greater

disease levels than the control, yet still low enough for possible synergistic interactions to be assessed. The same pea cultivar, substrate, watering methods, growth conditions, experiment length and assessment parameters were used as described in section 3.2.1. Treatments in these experiments were the control (uninoculated), FO, FSP, DP, FO + FSP, FO + DP, FSP + DP and FO + FSP + DP. The vermiculite perlite mix in each tube was inoculated with 1 mL of spore suspension of each PFRC pathogen within the treatment at a concentration of 10 spores mL^{-1} (1 mL SRO water for the untreated control), and then further SRO water added to achieve a total of 10 mL inclusive of the volume of inoculum, to promote an even distribution of spores throughout the substrate. Subsequent tube preparation was as described in section 3.2.1. Two biological repeats were conducted for this experiment each comprising 12 technical replicate tubes per treatment. Tubes, including those used for the gravimetric watering method, were arranged in randomized block design using Genstat 19 (VSN International, 2021). Each biological repeat consisted of four blocks (racks), with treatments represented three times in each block. Mean root rot scores of each treatment were subject to an ANOVA to determine significance ($p < 0.05$). Pea plant mortality was analysed using a generalised linear model with a Bernoulli distribution and logit link function. The experiment design was designed with support from Andrew Mead. Analyses were carried out with the support of James Lynn of Applied Statistical Solutions Ltd., using Genstat 19 (VSN International, 2021).

3.2.3 Examining pea root rot complex pathogen root colonisation using quantitative polymerase chain reaction

Pea plant root tissue from each of the single and double pathogen combination treatments of the experiment carried out in section 3.2.2 was harvested after 10 and 21 days for DNA extraction. Subsequent quantitative polymerase chain reactions (qPCR) were then carried out to determine the DNA concentration of FO, FSP and DP within the root tissue for each treatment.

Three plants each from the uninoculated control, FO, FSP, DP, FO + FSP, FO + DP and FSP + DP treatments were harvested from the second biological repeat of the experiment undertaken in section 3.2.2, at days 10 and 21 (final assessment day). Roots were washed and then flash frozen in liquid nitrogen and stored at -80 °C. Root tissue samples were then freeze dried for 48 hours and ground in liquid nitrogen to a powder using a pestle and mortar. Due to the small quantity of root tissue for the day 10 samples, the three root samples of each treatment were pooled into one tube

(lysing matrix A, MP Biomedicals, USA) for DNA extraction (20 mg of root tissue). To extract DNA, samples were lysed at 6 ms^{-1} for 40 seconds using a FastPrep-24 sample preparation system (MP Biomedicals, USA) and DNA extracted using the DNeasy Plant Mini Kit (Quiagen, Netherlands) with modifications to the manufacturer's instructions as follows. After the addition of buffer AP1 and RNAse stock A, the tube was centrifuged at 13,000 RPM and the supernatant transferred to a new tube. For elution, 100 µL of buffer AE was applied to the membrane for a one-minute incubation period prior to centrifugation. DNA concentration and quality was assessed using a Denovix DS-11+ Spectrophotometer (Denovix, USA) and samples stored at -20 °C until use.

Primers used to amplify DP, FO, and FSP are listed in Table 3.3. Individual 19 µL qPCR reactions consisted of; 10 µL PowerUp SYBR Green Master Mix (Applied Biosystems, USA), 1 µL each of forward and reverse primer (10 mM; final concentration 0.5 mM), 7 µL of sterile PCR-grade water and 2 µL of template DNA (sterile PCR-grade water for the non-template control). Samples were examined in triplicate reactions. Quantitative PCR was carried out using a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) using the following conditions: one cycle of 95 °C for 1 minute 20 seconds, then 45 cycles of 95 °C for 30 seconds followed by 60 °C for 30 seconds. The melt curve stage conditions were one cycle of 95 °C for 15 seconds, followed by 60 °C increasing by 0.075 °C $seconds^{-1}$ to 95 °C held for a further 15 seconds. Standard curves for each pathogen were created by amplifying a 10-fold serial dilution of relative pathogen genomic DNA from 10 ng to 1 fg. Melt curve analysis was carried after amplification to confirm a single PCR product melting temperature for each primer pair; any samples with inconsistencies, Ct values below the Ct value of limit of detection for the primer pair and only single values from triplicates for day 21 samples were omitted. The picograms of DNA per microgram of dry root (pg mg^{-1} root d.w.) was calculated for each triplicate, and then averaged for the day 21 samples to give a mean DNA concentration of a pathogen within a given treatment. All pathogens were examined for all treatments to determine potential cross contamination between treatments, which has been an issue in previous studies (Willsey *et al*., 2018).

Table 3.3. Primer pairs used to amplify *Fusarium oxysporum*, *Fusarium solani* f. sp. *pisi* and *Didymella pinodella* in the qPCR assay.

Target	Primers	Sequences (5'-3')	References
FO	Fef1F Fef2R	TAGTCACTTTCCCTTCAATCGC CTCAAGTGGCGGGGTAAGT	Haegi *et al.*, (2013)
FSP	G7048_3F G7048_5R	TGTCCTCCTGGGTTTGATGT GAGCTCATGGGGACTAAGC	Clarkson, (2019)
DP	G10861_F1 G10861_R1	CAATTTCCCACTCATGCGGAAG CGGACACGGTACATTTTGCTC	Clarkson, (2019)

3.2.4 Determining the effect of pea foot rot complex pathogen inoculum concentration on disease development in a pot-based system

Pot-based glasshouse experiments were set up to examine the effects of different spore concentrations of the PFRC pathogens FSP and DP on disease development and mortality of pea plants in the glasshouse. This was carried out to examine the differences in disease development compared to the test tube assay (section 3.2.1) and to select a suitable inoculum concentration for subsequent experiments examining the effects of biofumigants on DP foot rot disease suppression (section 4.2.5). FSP biological repeat 1 was harvested and assessed by Nicole Pereira and Professor John Clarkson due to COVID-19 isolation.

Inoculum of FSP and DP was grown in flasks containing a sterile compost-bran mix. This was prepared by mixing compost (Levington M2, sieved to 4 mm, 100g) and milled wheat bran (Charlecote Mill, UK, 148.4 g) and adjusting moisture content to 78.9 %. The bran compost mix (300 g) was dispensed into 1 L conical flasks sealed with a tight cotton wool bung and two layers of tin foil. Flasks were autoclaved three times at 121 °C for 15 minutes and after cooling, each was inoculated with five 5 mm^2 agar plugs taken from the growing edge of an actively growing culture of either FSP or DP. Flasks were incubated in the dark at room temperature for four (FSP) or six (DP) weeks and were shaken twice a week.

The inoculum concentration for each flask was determined by adding 1 g to 10 mL sterile RO water and vortexing. Serial dilutions were then made in 10 mL SRO water down to 10^{-7} and 100 µL of the dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} plated in triplicate onto PDA agar containing 20 mg L^{-1} chlorotetracyline (Sigma-Aldrich, UK). Plates were incubated at 25 ºC (FSP) or 20 ºC (DP) for two days after which colony forming units (CFUs) were counted. The highest serial dilution which produced CFUs

of 50 or less per plate were used to calculate the mean colony forming unit concentration g^{-1} for each flask.

A pea growing compost/sand mix for an uninoculated control treatment was prepared by mixing horticultural sand (Westland Horticulture, UK) with compost (Levington M2, ICL, UK) in the ratio 70:30 by weight. The compost/sand mix containing FSP or DP inoculum was prepared in the same way, but the equivalent volume of compost substituted for inoculum to give final concentrations of 10, $1x10^2$, $1x10^3$, $1x10^4$ and $1x10^5$ spores g^{-1} substrate (FSP) and $1x10^2$, $1x10^3$, $1x10^4$ and $1x10^5$ and $1x10^6$ spores g^{-1} substrate (DP). The pea growing substrate was then dispensed into 7 cm FP7 pots, pea seeds sown, and reverse/bottom watering carried out throughout the experiment as required. Experiments were carried out in a temperature-controlled glasshouse compartment with supplementary lighting to extend to a 16-hour day light cycle, with recommended 20 °C day and 18 °C night temperatures. For the experiment with DP, the first biological repeat was sown on 11/09/2020, and the second on 09/10/2020. For the experiment with FSP, the first biological repeat was sown on 25/09/2020, and the second on 02/10/2020.

Pea sticks were staked into the corner of each pot two weeks after sowing to support plants. Plants were scored daily for the first two weeks for emergence (defined as reaching growth stage 09 of the BBCH scale; shoot breaking through the surface) and also twice-weekly for disease development using a scoring index based on percentage of leaves displaying wilt symptoms for the duration of the experiment (Table 3.4).

After six weeks (DP biological repeats 1 and 2, FSP biological repeat 1) and eight weeks (FSP biological repeat 2), plants were harvested by first removing excess substrate from the root through disturbance, then washed in tap water to remove as much substrate as possible. Each plant root was photographed and scored for percentage root browning using a scoring system devised by the Processors and Growers Research Organisation (Table 3.5). Plants were also assessed for flower and pod number and presence/absence of pathogen stem girdling. Root and shoot tissue were separated for each plant and both wet and dry weight measured, the latter after tissue was dried at 80 °C for 48 hours.

In total there were 12 replicate pots per FSP/DP spore concentration treatment within each of the two biological repeats which were defined by use of separately prepared DP/FSP inoculum. Pots were arranged in a randomised block design across the glasshouse bench, with treatments represented once per block, designed using Genstat 24 (VSN International, 2019). For analyses, final leaf wilt scores were transformed using log(score +1) for FSP and a square root transformation for DP.

Root rot scores were transformed using log(score +1). Pea plant mortality data was angular transformed and multiplied by 100. Dry shoot and root weights were square root transformed. Plant girdle data was angular transformed and multiplied by 100 for FSP. Transformed final leaf wilt and root rot scores, dry shoot and root weights, pea plant mortality and plant girdle data for FSP were subject to a one-away analysis of variance, with the standard error difference of the mean multiplied by the *t*-critical value of the residual degrees of freedom within each analysis used to calculate significant differences between treatment means. For final emergence, presence/absence of nodules and stem girdling for DP, values were analysed using a generalised linear model with a Bernoulli distribution and logit link function. Statistics were carried out with the support of James Lynn (Applied Statistical Solutions Ltd., UK), using Genstat 19 (VSN International, 2021) (Table A 3, Table A 4, Table A 5).

Table 3.4. Disease scoring system used for assessing leaf wilt severity due to DP in pot experiments.

Symptom score	Leaves displaying wilt symptoms (%)	Disease severity
0	0	None
1	1-25	Slight
2	26-50	Slight-moderate
3	51-75	Moderate
4	75-99	Moderate-severe
5	100	Severe

Table 3.5. Disease scoring system used for assessing root rot severity due to DP in pot experiments.

Symptom score	Root browning (%)	Disease severity
0	0	None
1	<10	Slight
2	11-25	Slight-moderate
3	26-50	Moderate
4	51-90	Moderate-severe, no stem girdling
5	91-100	Severe, stem girdling
6	100	Plant dead

3.3 Results

3.3.1 Determining the effect of pea foot rot complex pathogen inoculum concentration on disease development in a test tube-based system

In order to determine a suitable concentration of PFRC pathogen spores to use in future combination experiments to examine pathogen interactions, pea seedlings were inoculated with different concentrations of spores of individual pathogens in test tube-based experiments, and root rot disease severity and plant mortality assessed (Figure 3.2). For all three PFRC pathogens, the results indicated a positive relationship between spore concentration and both root rot disease severity and pea seedling mortality.

In the experiment with FO, the root rot disease score at harvest increased with spore concentration, with mean scores ranging from 0.4 for the uninoculated control to 6.0 for the highest spore concentration of 1×10^6 spores mL^{-1} ($p < 0.001$, Figure 3.3a). All treatments resulted in a significantly higher root rot score compared to the uninoculated control. Pea plant mortality ranged from 0 % for the uninoculated control to 100 % for the highest concentration of 1×10^6 spores mL^{-1} ($p < 0.001$) but was variable at the middle concentrations tested (Figure 3.4a). All inoculated treatments resulted in a significant increase in pea plant mortality compared to the uninoculated control with the exception of the lowest concentration of 10 spores mL^{-1}.

In the experiment with FSP, the root rot disease score reached a maximum at 1×10^4 spores per ml^{-1} and was variable at the higher spore concentrations tested, with mean scores ranging from 0.7 for the uninoculated control to 4.8 for the concentration of 1×10^4 spores mL^{-1} ($p < 0.001$, Figure 3.3b). Mortality increased with spore concentration, ranging from 9.1 % for the uninoculated control to 62.5 % for 1×10^6 spores mL^{-1} ($p < 0.01$, Figure 3.4b). All inoculated treatments resulted in a significantly higher root rot score compared to the uninoculated control ($p < 0.001$) and concentrations between 1×10^3 spores mL^{-1} and 1×10^6 spores mL^{-1} resulted in significantly increased mortality ($p < 0.01$).

In the experiment with DP, both root rot disease score and pea plant mortality increased with spore concentration. Root rot scores and mortality ranged from 0.3 and 0 % respectively for the uninoculated control to 6.0 ($p < 0.001$) and 100 % ($p < 0.001$) respectively for the highest concentration of 1×10^6 spores mL^{-1} (Figure 3.3c; Figure 3.4c). All treatments resulted in a significantly higher root rot score compared

to the uninoculated control ($p < 0.001$) with the exception of the lowest concentration of 10 spores mL^{-1}.

One aim of these experiments was to determine a single suitable concentration of spores for all PFRC pathogens to use in interaction studies. The lowest concentration examined of 10 spores mL^{-1} was selected as this resulted in low but consistent levels of root rot disease for all three pathogens, which would potentially allow for additive and synergistic effects to be observed when examining effects of two or three pathogens combined. It was also the only concentration tested which resulted in less than 50 % pea plant mortality for all three pathogens.

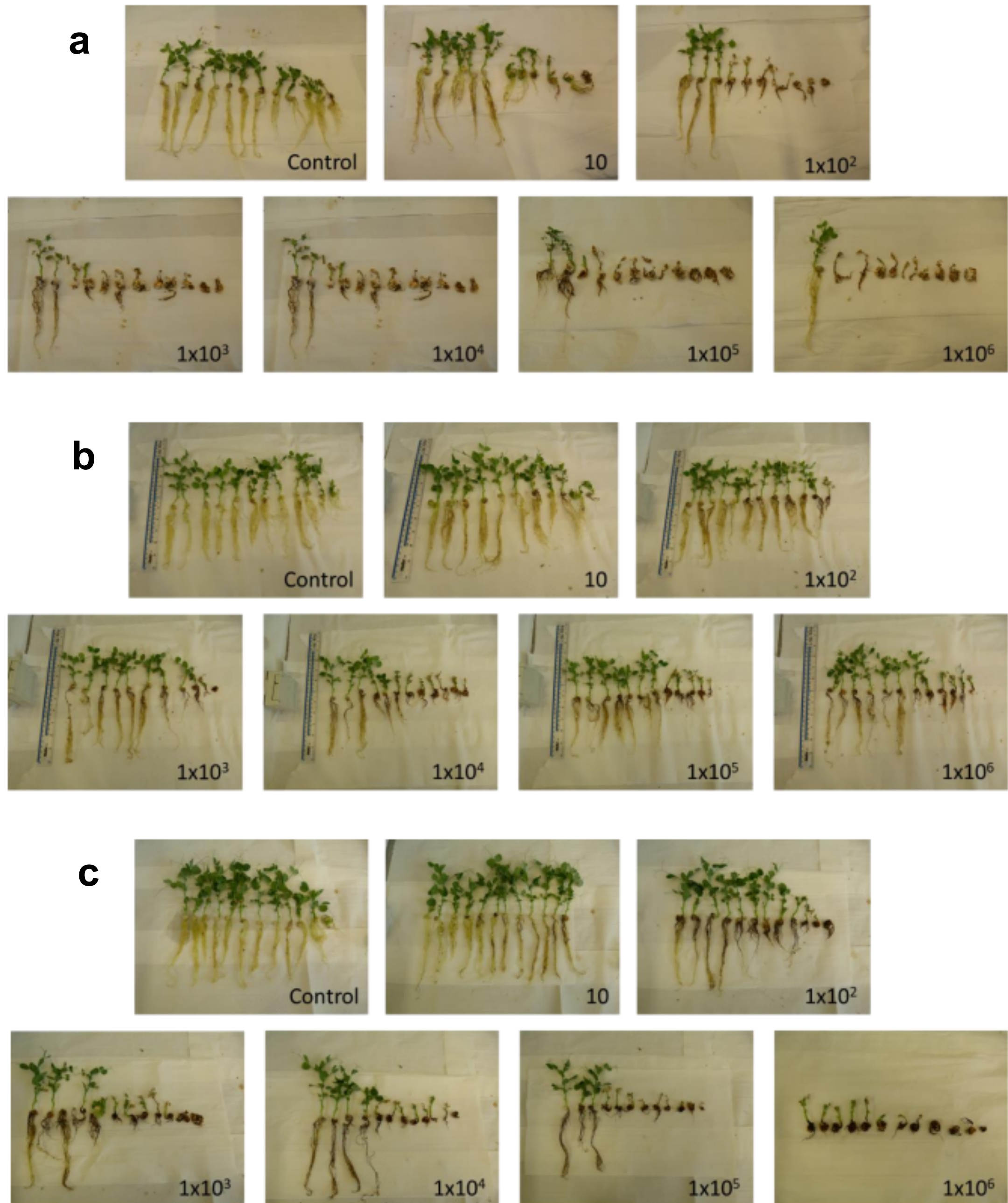


Figure 3.2. Effect of different spore concentrations on foot rot disease symptoms for pea plants inoculated with a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13' and c) *Didymella pinodella* '61B' in test tube assays at 21 days post inoculation.

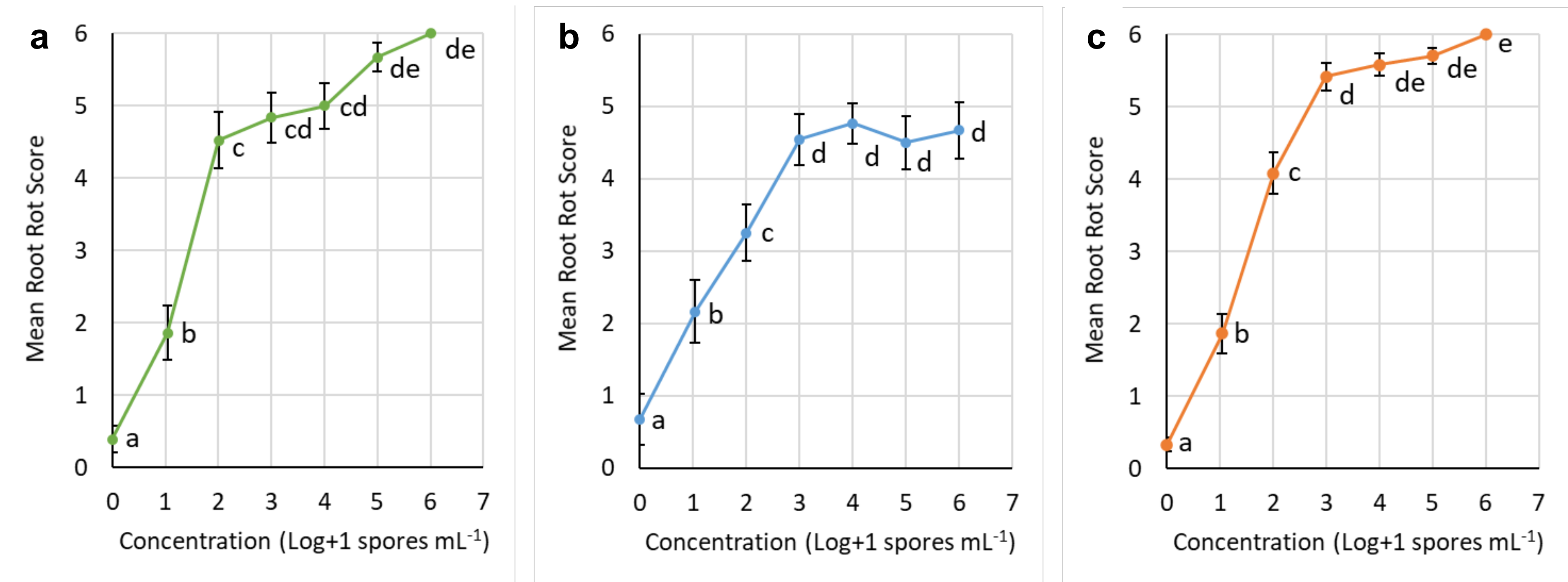
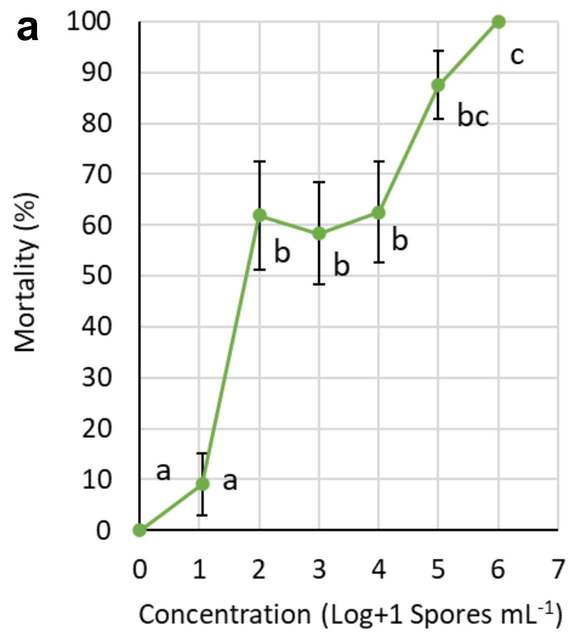
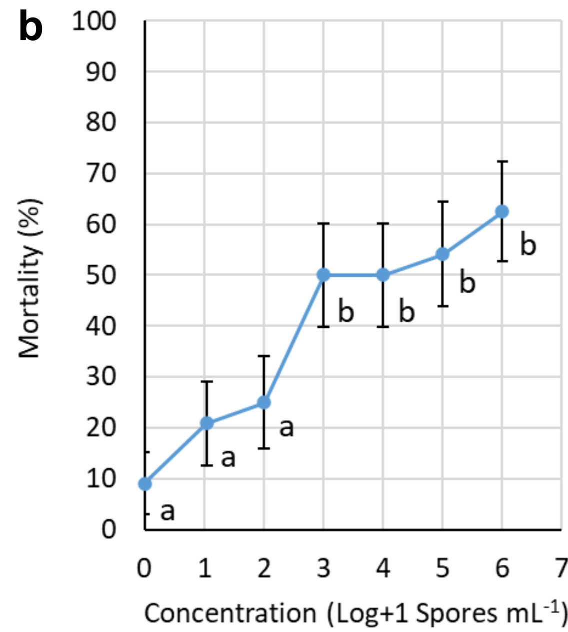


Figure 3.3. Effect of spore concentration on mean root rot disease severity score for pea plants inoculated with a) *Fusarium oxysporum* ‘PG18’, b) *Fusarium solani* f. sp. *pisi* ‘PG13’ and c) *Didymella pinodella* ‘61B’ in test tube assays. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.





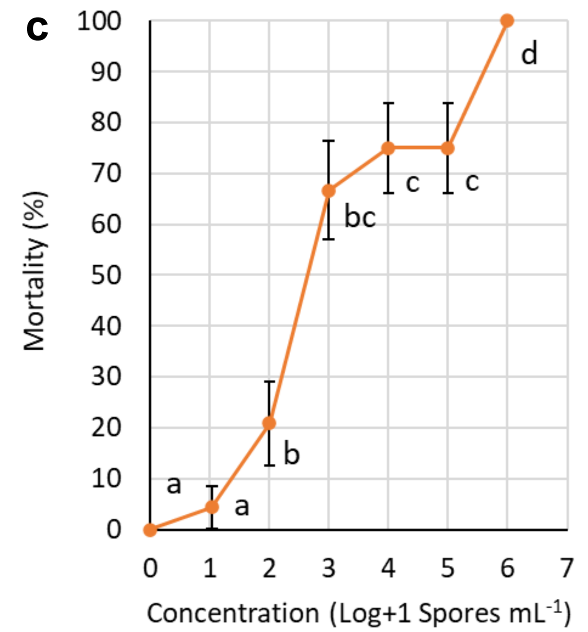


Figure 3.4. Effect of spore concentration on mean percentage pea plant mortality when inoculated with a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13' and c) *Didymella pinodella* '61B' in test tube assays. Statistics were performed on the angular transformation of values. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

3.3.2 Investigating pea foot rot complex pathogen interactions in a test-tube based system

In order to examine potential interactions between PFRC pathogens, pea seedlings were inoculated with different combinations of pathogens in test-tube based experiments. The concentration of pathogen inoculum (10 spores mL^{-1}) used was determined from the results of the experiments described in section 3.3.1. Disease severity and pea plant mortality were assessed but overall, there were no significant synergistic interactions between any combination of PFRC pathogens in relation to mean root rot severity score however there was evidence of additive interactions.

Mean root rot score was lowest for the uninoculated control (0.55), with mean scores of 1.83 for single pathogen inoculations, 2.48 for double pathogen inoculations and 4.21 for the triple pathogen inoculation (Figure 3.5). FO resulted in the highest root rot score of 2.00 for a single pathogen inoculation while the combination of FSP and DP resulted in the highest score of 3.00 for a double pathogen inoculation. ANOVAs of mean root rot scores revealed that all pathogens in their respective two- and three-way interactions increased the mean root rot score, with DP increasing the score most overall, and FSP increasing the score the least. All one-way interactions (FO, FSP and DP) were significant (FSP, $p = 0.004$; FO and DP, $p < 0.001$), meaning that inoculated plants of each single pathogen treatment had a significantly higher mean root rot score compared to that of the uninoculated control. However, at the two- and three-way interaction levels, no combinations resulted in a significantly greater mean root rot score than the sum of the individual pathogen effects. Therefore, these effects were additive rather than synergistic.

Mean pea plant mortality was 4.55 % for the uninoculated control, with means of 10.10 % for single pathogen inoculations, 15.89 % for double pathogen inoculations and 45.83 % for the triple pathogen inoculation (Figure 3.6). The single pathogen treatment of FSP resulted in a slightly lower pea plant mortality (4.17 %) compared to the uninoculated control. The double pathogen treatment of FO and FSP had a lower mortality (9.09 %) compared to the single pathogen treatment of FO alone (12.5 %), while the double pathogen treatment of FO and DP had a lower mortality compared to the single pathogen treatment of DP (13.64 %). The GLM revealed that no interactions at any level were significant, so none of the single pathogen inoculation treatments had a significantly higher pea plant mortality compared to that of the control and the effect of combining pathogens did not result in a significantly greater mean pea plant mortality than the sum of the individual pathogen effects.

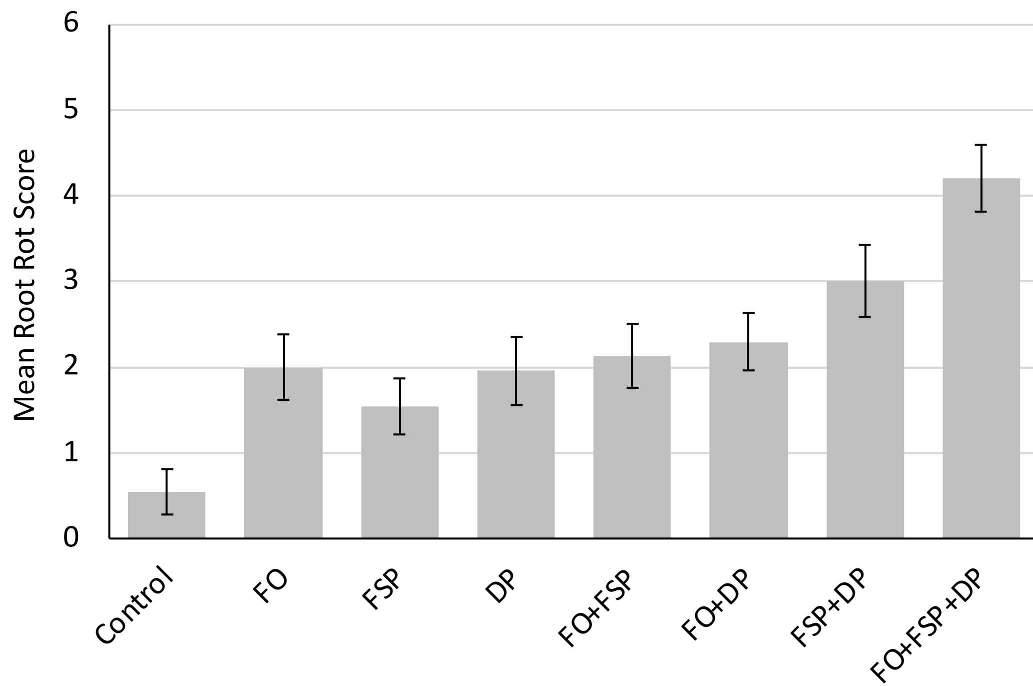


Figure 3.5. Effect of single, double, and triple pathogen combination inoculation on mean pea root rot score. FO = *Fusarium oxysporum* 'PG18', FSP = *Fusarium solani* f. sp. *pisi* 'PG13' and DP = *Didymella pinodella* '61B'. Error bars = ± SEM.

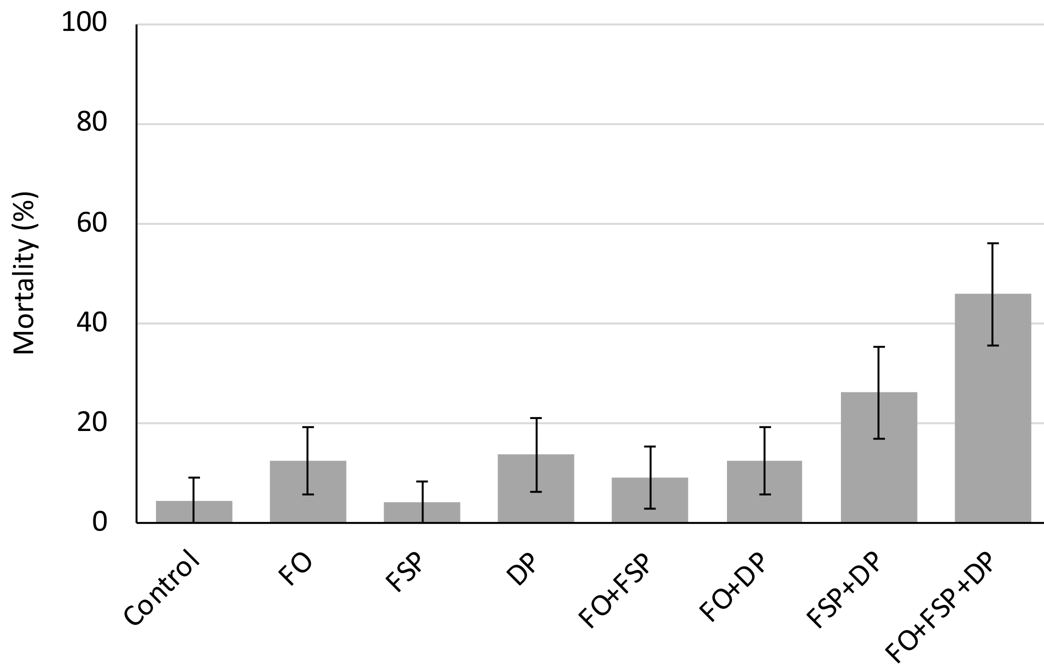


Figure 3.6. Effect of single, double and triple pathogen combination inoculation on mean percentage pea plant mortality. FO = *Fusarium oxysporum* 'PG18', FSP = *Fusarium solani* f. sp. *pisi* 'PG13' and DP = *Didymella pinodella* '61B'. Error bars = ± SEM.

3.3.3 Examining pea foot rot complex pathogen root colonisation using quantitative polymerase chain reaction

Three pea plants of each single and double pathogen combination treatment of the second biological repeat of the experiment carried out in section 3.3.2 were harvested at days 10 and 21. The three samples taken for each treatment were eventually pooled into one sample per treatment. Pea plant roots were processed, and DNA extractions carried out. Subsequent quantitative polymerase chain reaction was undertaken to determine DNA concentration of the pathogens FO, FSP and DP within the root tissue for each treatment at day 10 (Table 3.6a) and day 21 (Table 3.6b), to examine colonisation and possible interactions within double pathogen combinations.

DNA concentration for each pathogen in the uninoculated control samples was undetermined or below the limit of detection at both timepoints. All pathogens were examined for all treatments to determine potential cross contamination between treatments. This was only detected in the FSP + DP treatment at day 21 where FO was identified (2.14 pg mg^{-1} root d.w.), however, this was only found in one of the

three root samples. The qPCR assays successfully quantified the DNA concentration of each pathogen within respective treatments at both time points examined although there was large variation between some of the samples, particularly between individual roots sampled at day 21.

At day 10, DNA concentrations of FO, FSP and DP in their respective single pathogen treatments was 3.76 pg mg^{-1} root d.w., 23.29 pg mg^{-1} root d.w. and 553.68 pg mg^{-1} root d.w. respectively. In the FO + FSP treatment, this increased to 307.57 pg mg^{-1} root d.w. for FO and decreased to 7.50 pg mg^{-1} root d.w. for FSP. In the FO + DP treatment, there was a smaller increase in FO from 3.76 pg mg^{-1} root d.w. to 6.10 pg mg^{-1} root d.w., and for DP, a reduction compared to the DP only treatment from 553.68 pg mg^{-1} root d.w. to 35.92 pg mg^{-1} root d.w. For FSP + DP, there was an increase in DNA concentration for FSP compared to the FSP only treatment, from 23.29 pg mg^{-1} root d.w. to 52.42 pg mg^{-1} root d.w., and a decrease for DP, from 553.68 pg mg^{-1} root d.w. to 78.19 pg mg^{-1} root d.w. In both FO double pathogen treatments at day 10, there was an increase in FO DNA concentration compared to the FO only treatment, a decrease in FSP DNA concentration in the FO + FSP treatment and an increase in concentration in the FSP + DP treatment compared to the FSP only treatment, and a decrease in DNA concentration for DP in both the FO + DP and FSP + DP treatments compared to the DP only treatment.

At day 21, the DNA concentrations detected for FO and DP in their respective single pathogen treatments increased compared to day 10, with values of 80.31 pg mg^{-1} root d.w. and 654.98 pg mg^{-1} root d.w. respectively. The DNA concentration of FSP detected was decreased compared to the day 10 samples, at 15.46 pg mg^{-1} root d.w. As with the day 10 samples, DNA concentration of FO in samples was higher compared to FSP in the FO + FSP treatment, at 5516.41 pg mg^{-1} root d.w. and 1110.66 pg mg^{-1} root d.w. respectively. However, DNA concentration of FSP in the FO + FSP treatment increased compared to the FSP only treatment in contrast to day 10, from 15.46 pg mg^{-1} root d.w. to 1110.66 pg mg^{-1} root d.w. The DNA concentration of FSP in the FO + FSP treatment for day 21 was increased compared to the FSP only treatment, whereas it was decreased at day 10. The DNA concentration for FO was similar in the FO + DP treatment as the FO only treatment, at 80.06. pg mg^{-1} root d.w. For FO + DP, there was a reduction in DP DNA concentration detected in the samples compared to the DP only treatment, from 654.98 pg mg^{-1} root d.w. to 191.89 pg mg^{-1} root d.w. FO DNA was quantified in one of the day 21 samples for the FSP + DP treatment, at 2.14 pg mg^{-1} root d.w. For both FSP and DP, there was a decrease in DNA concentration detected in the samples compared to respective single pathogen treatments. For FSP, this was from 15.46 pg mg^{-1} root d.w. to 1.27 pg mg^{-1}

root d.w. and for DP, this was from 654.98 pg mg^{-1} root d.w. to 204.35 pg mg^{-1} root d.w.

Table 3.6. DNA concentrations of individual PFRC pathogens *F. oxysporum*, *F. solani* f. sp. *pisi* and *D. pinodella* at a) day 10 and b) day 21 of the interactions experiment as measured by qPCR assays. U = undetermined/Ct value below the detection limit. * = only one qPCR triplicate of pooled root material of the treatment (day 10) or set of triplicates of one root sample out of three (day 21) of the treatment was quantified.

a)

Treatment	FO		FSP		DP	
	DNA concentration (pg mg^{-1} root d.w.)	SD	DNA concentration (pg mg^{-1} root d.w.)	SD	DNA concentration (pg mg^{-1} root d.w.)	SD
Control	U		U		U	
FO	3.76	0.06	U		U	
FSP	U		23.29	1.97	U	
DP	U		U		553.68	27.32
FO+FSP	307.57	13.76	7.50	0.55	U	
FO+DP	6.10	0.88	U		35.92	9.64
FSP+DP	U		52.42	28.75	78.19	8.77

b)

Treatment	FO		FSP		DP	
	DNA concentration (pg mg^{-1} root d.w.)	SD	DNA concentration (pg mg^{-1} root d.w.)	SD	DNA concentration (pg mg^{-1} root d.w.)	SD
Control	U		U		U	
FO	80.31	54.32	U		U	
FSP	U		15.46	20.54	U	
DP	U		U		654.98	226.52
FO+FSP	5516.41	7727.60	1110.66	1065.78	U	
FO+DP	80.06	29.26	U		191.89	213.72
FSP+DP	2.14*		1.27	0.35	204.35*	

3.3.4 Determining the effect of pea foot rot complex pathogen inoculum concentration on disease development in a pot-based system

In these experiments, peas were grown in compost/sand-based growing substrate containing different spore concentrations of the PFRC pathogens FSP and DP in pots in the glasshouse. This was to determine a suitable concentration of pathogen inoculum for use in future experiments to examine the effect of biofumigants on PFRC pathogen disease development (section 4.2.5) and to compare the levels of disease development of each pathogen to the results of the test tube assay (section 3.3.2). Several indices of foot rot severity and plant health were examined including emergence, leaf wilt, root rot, flower and pod number and presence/absence of pathogen stem girdling and nodules. Fresh and dry weights of shoot and root material was also measured. Analyses using ANOVA and GLM were undertaken to calculate significant differences between treatments.

3.3.4.1 Fusarium solani f. sp. *pisi*

Overall, there was low foot rot disease incidence in the experiment with FSP. Percentage emergence of plants was similar across all treatments (Figure 3.7) with 87.5 % for the uninoculated control. All FSP inoculated treatments resulted in either similar or slightly increased (but not significant) emergence 15 days after planting with values of 87.5 % for concentrations of both $1x10^4$ spores g^{-1} and $1x10^6$ spores g^{-1} increasing to 95.8 % for concentrations of both 10 spores g^{-1} and $1x10^2$ spores g^{-1}.

All FSP spore concentration treatments resulted in an increase in leaf wilt score throughout the duration of the experiment (Figure 3.8) and as for emergence, final leaf wilt scores were similar across all treatments. There were no significant differences were between any of the treatments and the uninoculated control, which resulted in the lowest final mean leaf wilt score of 0.79. The $1x10^2$ spores g^{-1} treatment had the highest final score of 1.45.

Mean root rot score for the uninoculated control treatment assessed at harvest (for plants that had emerged) was 0.24 (Figure 3.9a) and this increased to 1.58 with FSP spore concentrations up to $1x10^4$ spores g^{-1} but then decreased to 1.25 for $1x10^5$ spores g^{-1}. Root rot scores for all inoculated treatments were significantly increased compared to the uninoculated control ($p < 0.001$).

There was no pea plant mortality for the uninoculated control treatment at harvest (Figure 3.9b). Mortality was variable with increasing FSP spore

concentrations up to $1x10^5$ spores g^{-1} and was highest at $1x10^5$ spores g^{-1} at 10.00 %. None of the percentage mortality values for any inoculated treatments were significantly different to the control.

The mean values for combined flower and pod number were lower for all FSP inoculated treatments compared with the uninoculated control but did not decrease consistently with increasing FSP inoculum concentration (Figure 3.9c). Mean combined flower and pod number was 3.33 for the uninoculated control but the treatment of $1x10^5$ spores g^{-1} resulted in the lowest mean value of 2.23 per plant; this was the only treatment that significantly reduced flower and pod number compared to the uninoculated control ($p < 0.01$).

All FSP-inoculated treatments reduced mean dry root weight compared to the uninoculated control, but this did not decrease consistently with increasing inoculum concentrations (Figure 3.9d). The control had a mean root weight of 1.60 g, which decreased to 1.55 g for $1x10^2$ spores g^{-1} and 1.40 for $1x10^5$ spores g^{-1}. None of the treatments significantly reduced root dry weight compared to the control.

Mean dry shoot weight was 2.48 g for the uninoculated control and 2.81 g for the 10 spores g^{-1} treatment (Figure 3.9e). All other inoculated treatments resulted in a decrease in shoot weight, with values for both the $1x10^4$ spores g^{-1} and $1x10^5$ spores g^{-1} treatments of 2.14 g ($p < 0.05$) and 1.80 g ($p < 0.01$) respectively being significant.

The percentage of plants with FSP stem girdling symptoms for the uninoculated control treatment assessed at harvest was 23.81 % (Figure 3.9f). All treatments resulted in a significant increase in percentage of plants with FSP stem girdling symptoms ($p < 0.001$), with FSP spore concentrations 10 spores g^{-1}, $1x10^4$ spores g^{-1} and $1x10^5$ and spores g^{-1} with 100 % of plants with stem girdling symptoms.

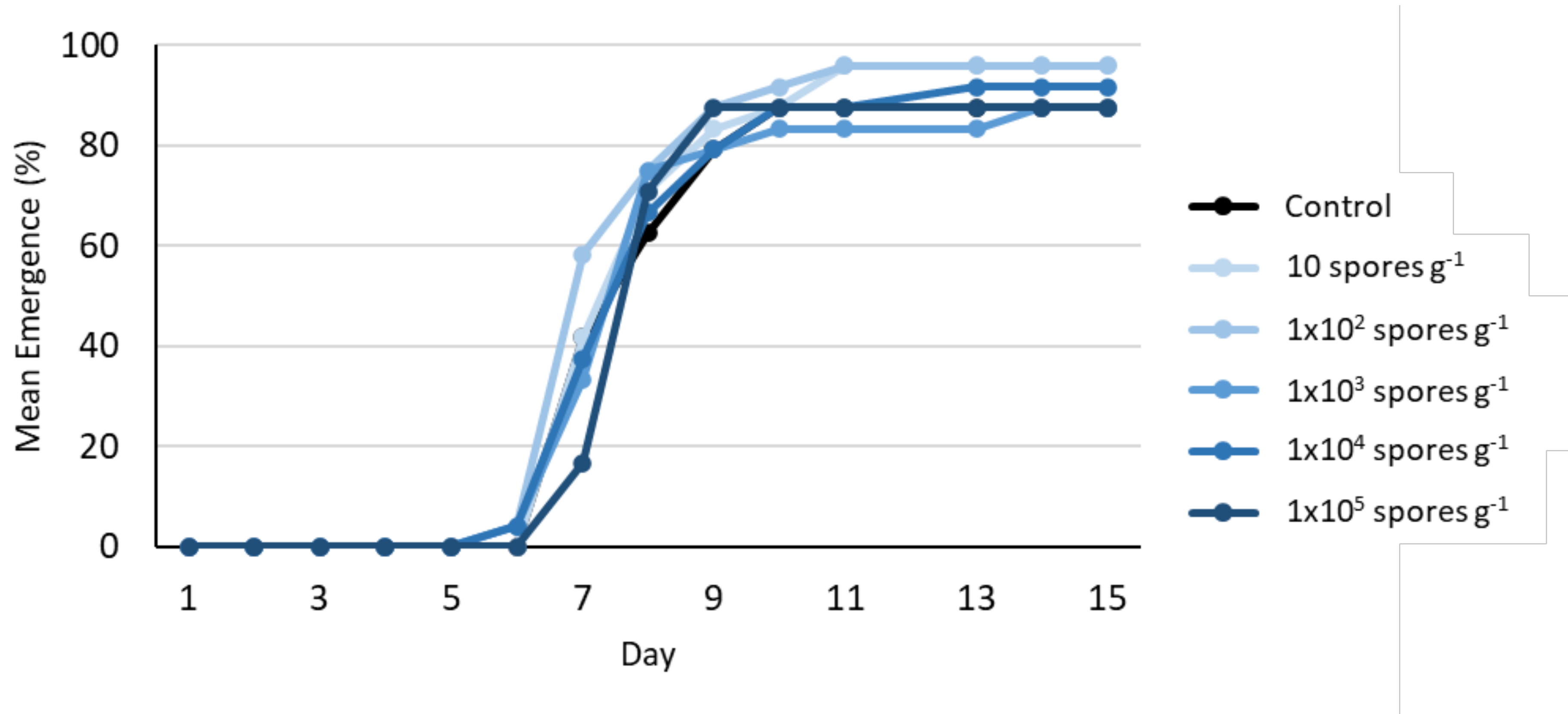


Figure 3.7. Effect of inoculum concentration on mean emergence of pea plants grown in substrate inoculated with *Fusarium solani* f. sp. *pisi* 'PG13' in the glasshouse.

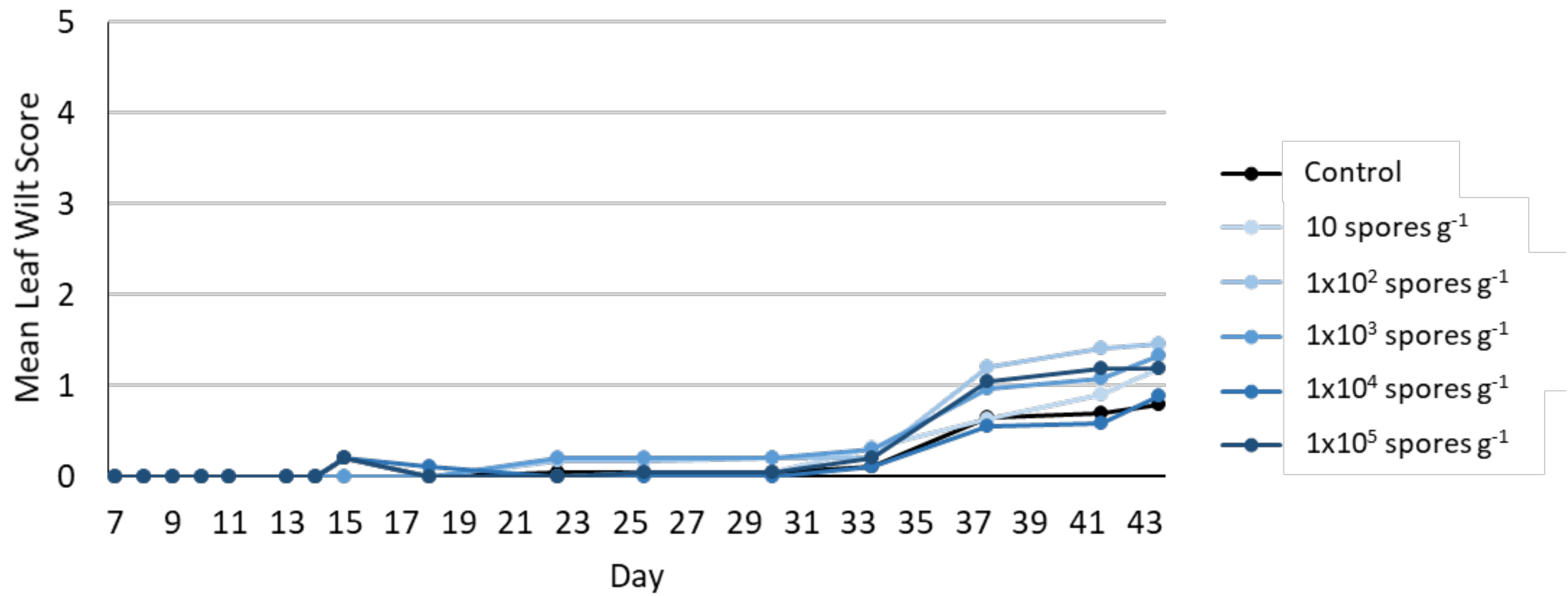


Figure 3.8. Effect of inoculum concentration on mean leaf wilt score of plants grown in substrate inoculated with *Fusarium solani* f. sp. *pisi* ‘PG13’ in the glasshouse.

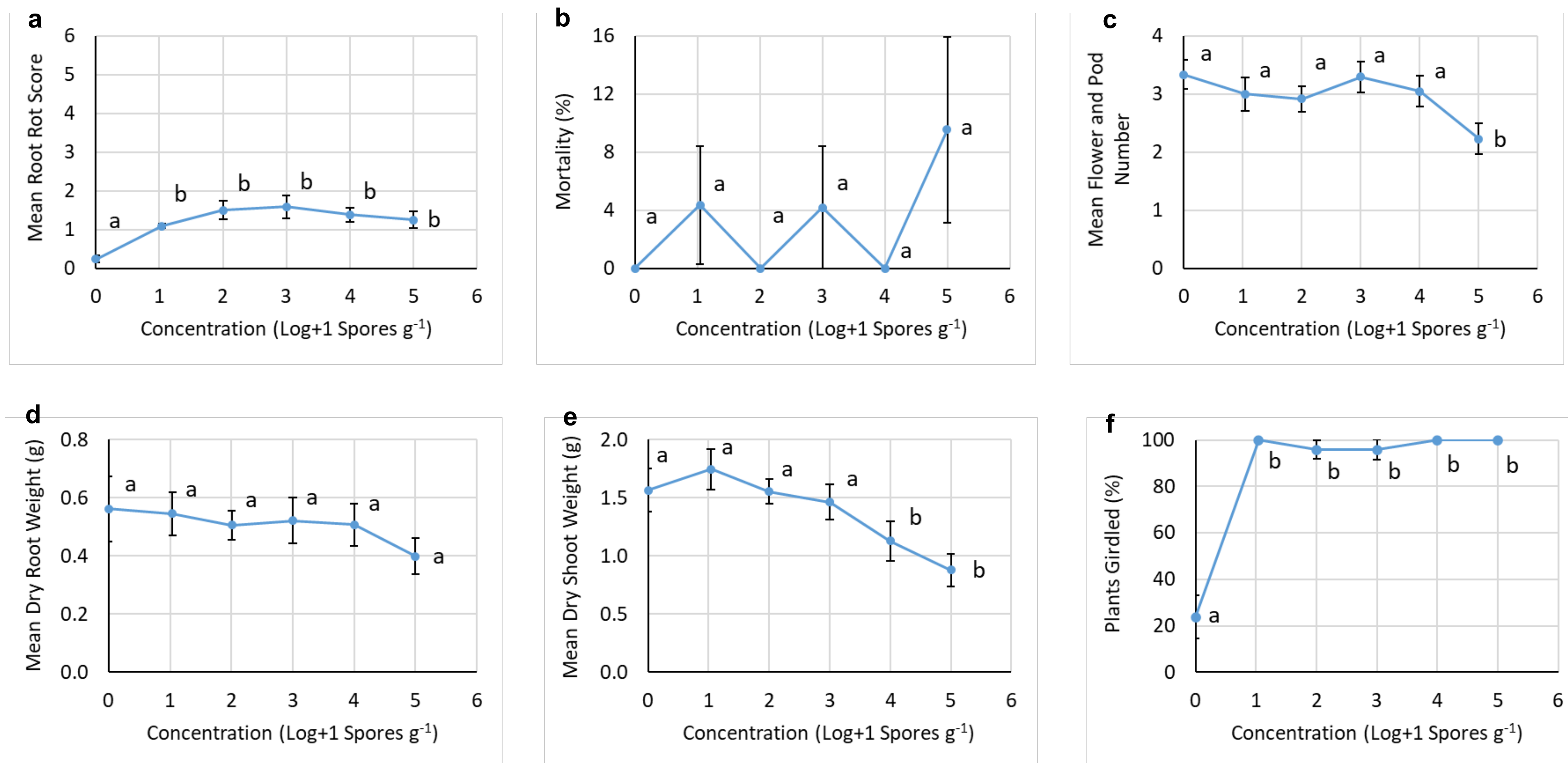


Figure 3.9. Effect of inoculum concentration on a) mean root rot score, b) pea plant mortality, c) mean combined flower and pod number, d) mean dry root weight, e) mean dry shoot weight and f) FSP stem girdling for pea plants grown in substrate inoculated with *Fusarium solani* f. sp. *pisi* 'PG13' in the glasshouse. Statistics on mean root rot scores were performed on the log (score +1), percentage plant mortality and girdling on angular transformation (data*100), and square root of values for both mean root and shoot dry weight. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

3.3.4.2 *Didymella pinodella*

Overall, the results of the experiment with DP indicated a positive relationship between inoculum concentration and the various metrics indicative of disease development, particularly at the three higher spore concentrations tested. Final percentage pea plant emergence 15 days after planting was significantly reduced at the higher concentrations of $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} to 62.5 % (p = 0.03) and 8.3 % ($p < 0.001$) respectively compared to the uninoculated control (88.1 % (Figure 3.10). The remaining spore concentration treatments ranged from 83.3 % to 91.3 % emergence.

All treatments resulted in an increase in leaf wilt score throughout the duration of the experiment and there was a positive relationship between DP inoculum concentration and leaf wilt score apart from at the two lowest spore concentrations. Final leaf wilt scores were significantly greater ($p < 0.001$) for concentrations of $1x10^4$ spores g^{-1}, $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} compared to the uninoculated control (1.42), with values of at 2.90, 4.67 and 5.00 respectively (Figure 3.11).

A similar relationship was observed for mean root rot score Figure 3.12a), with scores increasing above $1x10^3$ spores g^{-1}, although there was a slight decrease at $1x10^6$ spores g^{-1}. Mean DP root score was 1.14 for the uninoculated control treatment while concentrations of $1x10^4$ spores g^{-1}, $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} resulted in significantly increased scores ($p < 0.001$) with values of 3.62, 4.36 and 4.00 respectively.

The percentage pea plant mortality of the uninoculated control treatment was 4.76 %. Percentage plant mortality was significantly greater for concentrations of $1x10^4$ spores g^{-1}, $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} compared to the uninoculated control ($p < 0.001$), with values of at 42.86 %, 71.43 % and 66.67 % respectively (Figure 3.12b).

The mean values for combined flower and pod number were reduced consistently with increasing DP spore concentration in inoculated treatments except for the for the $1x10^2$ spores g^{-1} treatment (Figure 3.12c). Values ranged from 0 for $1x10^6$ spores g^{-1} to 2.29 for the uninoculated control. Concentrations of $1x10^4$ spores g^{-1}, $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} resulted in a significantly lower number of flower and pods compared to the uninoculated control ($p < 0.001$) with values of 1.08, 0.16 and 0 respectively.

The four highest inoculum DP concentrations resulted in a reduction in dry root weight compared to the uninoculated control, but values for the intermediate

concentrations varied (Figure 3.12d). Mean dry root weight was significantly reduced from 0.24 g in the uninoculated control to 0.19 g for $1x10^3$ spores g^{-1} ($p < 0.05$) and 0.22 g ($p < 0.01$), 0.06 g ($p < 0.001$) and 0.00 g ($p < 0.001$) for $1x10^4$ spores g^{-1}, $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} respectively.

Values for mean shoot dry weight followed a similar trend as for root weight (Figure 3.12e). The uninoculated control had a mean of 0.73 g, while the $1x10^2$ spores g^{-1} treatment resulted in the greatest dry weight of 1.14 g. Treatments with $1x10^5$ spores g^{-1} and $1x10^6$ spores g^{-1} resulted in significantly reduced mean shoot dry weight compared to the uninoculated control, with values of 0.11 g ($p < 0.001$) and 0.00 g ($p < 0.001$) respectively.

There was no clear relationship between DP stem girdling and inoculum concentration (Figure 3.12f). Plants with stem girdling ranged from 33.30 % for the uninoculated control, to 76.20 % for the $1x10^4$ spores g^{-1} concentration, a significant increase ($p = 0.007$). The only other significant increase in stem girdling compared to the control was for the $1x10^3$ spores g^{-1} treatment, at 73.70 % ($p = 0.013$).

The original aims of the experiments with FSP and DP was to determine the relationship between inoculum concentration and disease development for both pathogens, to select a suitable inoculum concentration to use in future experiments to examine the effects of biofumigants on DP foot rot disease development (section 4.2.5), and to compare these glasshouse assays with the test tube assay. For DP, two concentrations were selected for future experiments to examine the effects of biofumigants: $1x10^4$ spores g^{-1} and $5x10^5$ spores g^{-1}. The former was chosen due to high levels of emergence, a mean percentage plant mortality below 50.00 %, but a significant difference to the uninoculated control in several metrics of disease development. The latter concentration was chosen to examine the effects of biofumigant treatments on improving pea plant emergence.

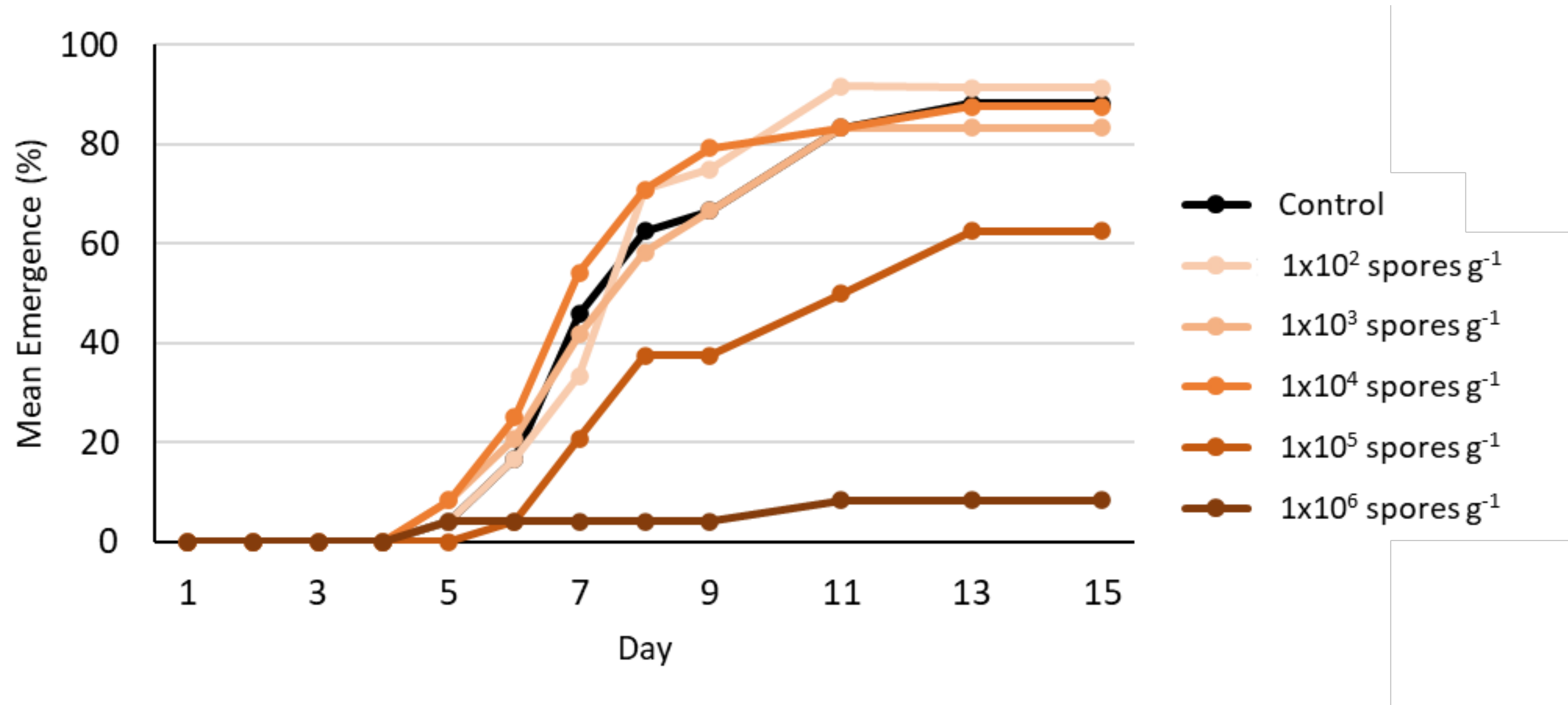


Figure 3.10. Effect of inoculum concentration on mean emergence over time of pea plants grown in substrate inoculated with *Didymella pinodella* '61B' in the glasshouse.

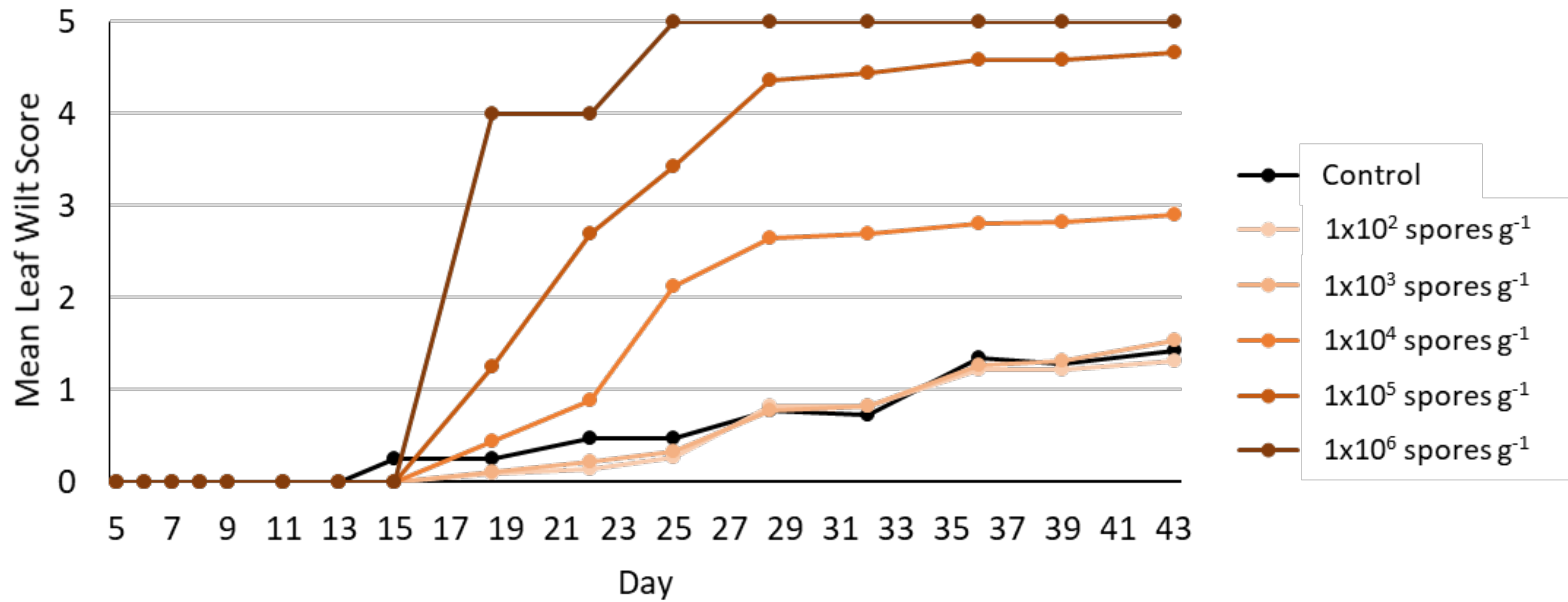


Figure 3.11. Effect of inoculum concentration on mean leaf wilt score over time for pea plants grown in substrate inoculated with *Didymella pinodella* '61B' in the glasshouse.

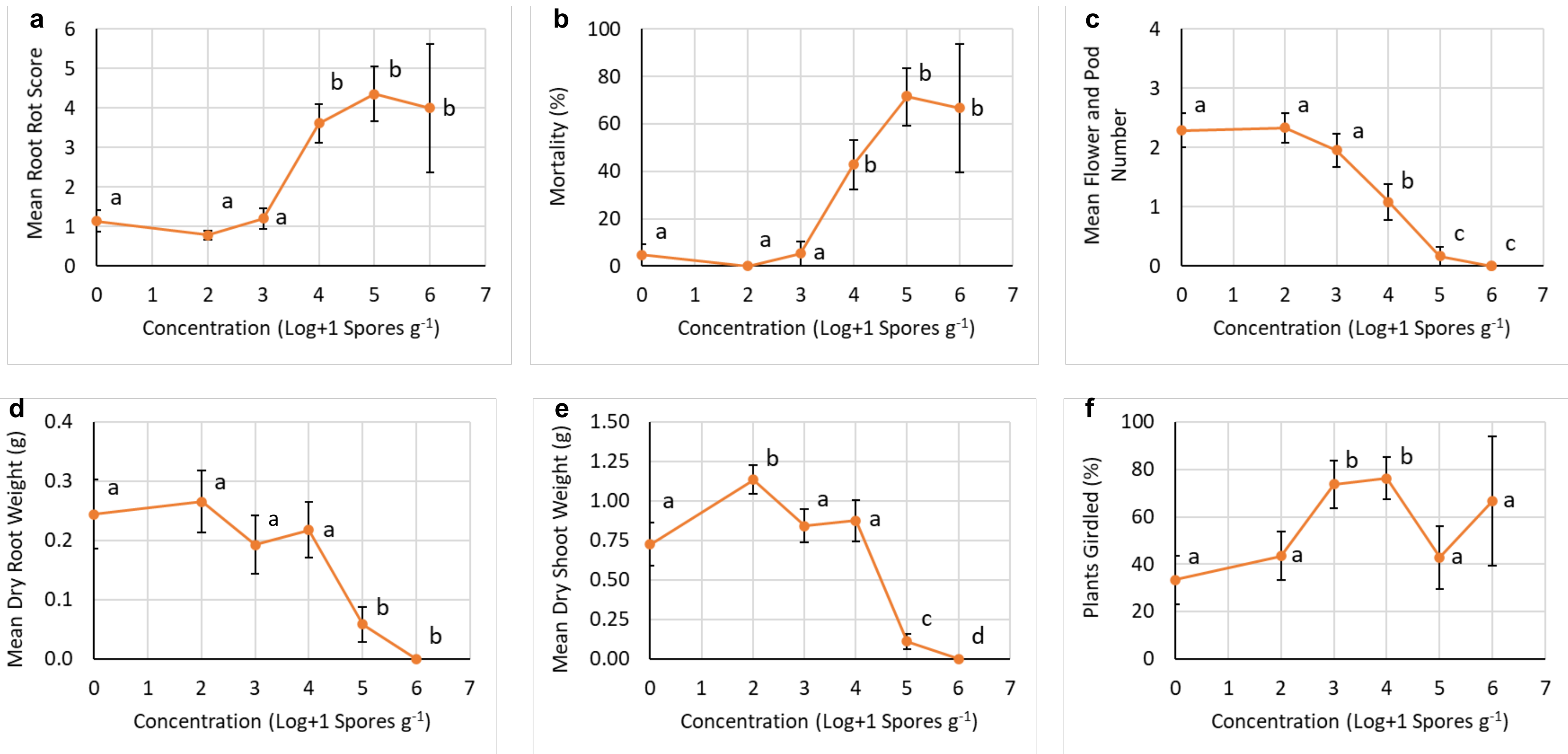


Figure 3.12. Effect of inoculum concentration on a) mean root rot score, b) pea plant mortality, c) mean combined flower and pod number, d) mean dry root weight, e) mean dry shoot weight and f) stem girdling for pea plants grown in substrate inoculated with *Didymella pinodella* '61B' in the glasshouse. Statistics on mean root rot scores were performed on the log (score +1), square root of values for both mean root and shoot dry weight, and angular transformation (data*100) for mortality. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

3.4 Discussion

Though there have been many studies concerning identity of PFRC pathogens through field surveys and molecular studies (e.g., Chittem *et al*., 2015; Chatterton *et al*., 2015; Baćanović-Šišić *et al*., 2018), interactions between pathogens within the complex have remained largely unexplored. The research described in this chapter examined the relationship between inoculum concentration and disease development for individual PFRC pathogens in test-tube and glasshouse experiments. The results of the test-tube experiments established the inoculum concentration required for subsequent interaction experiments where combinations of the PFRC pathogens FO, FSP and DP were assessed for their effect on foot rot development, while qPCR assays were employed to explore root colonisation. While the glasshouse experiments with DP and FSP further established effects of inoculum concentration, the equivalent glasshouse experiment for FO had to be stopped due to the COVID-19 pandemic and lockdown in March 2020. The DP experiment also informed the inoculum concentration employed to assess the potential effects of biofumigants in Chapter 4. The work of this chapter is the first to characterise the disease development and interactions of UK PFRC pathogens in different experimental systems and this is particularly important for DP, which is thought to be the main pathogen of the UK PFRC and relatively understudied.

The importance of conducting such pathogen inoculum dose response experiments has been highlighted previously and establishing the relationship between inoculum level and disease development, aids development of quantitative tests to determine pathogen risk. It has also been shown in previous studies that disease development within the plant and the inoculum level required to cause disease varies according to the experimental system used, such as the choice of growing substrate (Murakami *et al.*, 2002; Jenkins, 2018). Therefore, pathogen dose response experiments are also important to determine the optimum level of inoculum for testing control methods such as genetic resistance, chemical and biological control in artificially inoculated systems and for interaction studies.

The results of the test-tube based experiments indicated a positive relationship between spore concentration and both root rot disease severity and pea seedling mortality for all three PFRC pathogens. Generally, FO 'PG18' and DP '61B' were more virulent in these assays than FSP 'PG13' with 100.00 % pea mortality and a mean root rot score of 6.00 for plants inoculated either with FO 'PG18' and DP '61B' at a concentration of $1x10^6$ spores g^{-1}, compared with a score of 4.67 and a mortality

of 62.50 % at the same concentration for FSP 'PG13'. The glasshouse experiment with DP also indicated a positive relationship between the higher spore concentrations and several other measures of disease development (see below). In contrast, the same glasshouse experiment for FSP did not show such a relationship with lower levels of disease overall, which was in line with the results of the test tube assays. However, the highest level of FSP inoculum that could be obtained was $1x10^5$ spores g^{-1} and it could be that greater effects may have been observed at the higher concentration of $1x10^6$ spores g^{-1} as tested in the experiment with DP. The inoculum concentration required to cause PFR disease varied between pathogens and hence the inoculum concentrations chosen for subsequent interaction experiments were based on minimising pea plant mortality and inducing significantly greater disease levels than the uninoculated control, while still allowing detection of possible synergistic interactions (Willsey *et al*., 2018).

When comparing the test tube and glasshouse assays for DP, the lowest inoculum concentration that caused a significant increase in plant mortality compared to the uninoculated control in the test tube assay was $1x10^2$ spores mL^{-1} while in the glasshouse assay, the equivalent inoculum level was higher, at $1x10^4$ spores g^{-1}. An inoculum concentration of 10 spores mL^{-1} was sufficient to cause a significantly greater DP root browning score compared to the control in the test tube assay, whereas the equivalent inoculum concentration for the glasshouse assay was higher, at $1x10^4$ spores g^{-1}. There are several factors as to why a lower spore concentration sufficient to cause disease development was found in the test tube assay. Firstly, the test-tube system is semi-sterile with no or few other microorganisms to buffer against the inoculated PFRC pathogen. Secondly, the system is enclosed and whilst initial amendments to the method included adjusting watering to ensure no waterlogging, a small number of plants in some assays were beginning to flower at assessment, a possible indicator of stress (Wada and Takeno, 2010), which could have induced more disease.

One potential shortcoming with the glasshouse experiments was the addition of seed directly to the pots rather than transplanting seedlings. Whilst this method allowed effects on emergence to be assessed, only 2/24 plants emerged at the greatest concentration for the DP assay ($1x10^6$ spores g^{-1}), meaning that subsequent assessments were based on only two plants, which explains the larger standard error in some of the analyses. Transplanting of seedlings would be an option, but this is generally not recommended for peas and this consequently hampers any efforts to pre-germinate seeds, if issues in seedling health result from the transplantation itself. The observation of reduced emergence at higher inoculum concentrations also

importantly suggests that if DP inoculum levels in the field are high enough, pre-emergence damping off of pea could occur although has this not been reported previously. Damping off is more commonly associated with other PFRC pathogens such as *P. ultimum* and *P. irregulare* (Kerr, 1963; Tu, 1987), for which chemical and biological control agent (BCA) based seed treatments have been shown to be effective (Gossen *et al*., 2016). However, as the seeds were not treated prior to the experiment and sown directly into the substrate, additional seed-borne DP inoculum and resulting may be a possibility.

It was important that PFRC inoculum level was low enough to potentially identify any synergistic effects in the interaction studies. Kerr, (1963) examined interactions of PFRC pathogens of pea from Southern Australia and based these on the CFUs g^{-1} of FO, FS and *P. ultimum* found in infected field soils and the proportion of pathogenic to non-pathogenic isolates for FO and FS. Therefore, in contrast to the work in this chapter, the interactions studied were based on pathogen concentrations that were representative of levels found in some naturally infested soils. However, this approach resulted in extensive pea plant mortality and the nature of these interactions could not therefore be fully explored.

The test-tube assay developed here is a quick and easy method for establishing the pathogenicity of PFRC or other fungal isolates recovered from the field and is therefore of interest for development commercially (PGRO, personal communication). Previously, a fully sterile test-tube assay was used for testing pathogenicity testing of different FO isolates, including the isolate ‘PG18’. This method involved agar as the growing medium, adding 1 mL of inoculum at a concentration of $5x10^5$ spores mL^{-1} and assessing root rot using the same scoring index used in this study (Table 3.2) after two weeks. Similar to this study, pea seedlings infected with FO ‘PG18’ were found to have significantly higher mean root rot scores compared to the uninoculated control, with a score of ~4.85 (Jenkins, 2018). In comparison, a concentration of $1x10^3$ spores mL^{-1} for FO ‘PG18’ in this study resulted in a score of 4.83, which demonstrates how differences in pea growing medium can affect disease development. The sterile agar test tube system, whilst able to successfully test pathogenicity would not be suitable for the interactions assays carried out in this chapter due to restriction of root growth and the quantity of root material required for DNA extraction for qPCR.

Other research on pathogenicity of the same PFRC species investigated here has also demonstrated differences dependent on both the experiment method and pathogen isolate. Most published research suggests that FSP is the principal PFRC pathogen, especially in the UK (Clarkson, 1978). The study by Clarkson, (1978) of

Fusarium PFRC species in the UK isolated from peas and beans, demonstrated variation in virulence even between isolates of the same species. FS isolates originally isolated from pea were found to cause slightly greater levels of foot rot compared with an isolate of FO from pea. FS isolates from broad bean were also found to be able to infect deeper layers of pea root tissue compared to bean (Clarkson, 1978). Another study conducted pathogenicity testing of PFRC pathogens including isolates of DP, FO, FSP and AE from Sweden and Denmark. A pea root-dip assay was used for FSP and FO, alongside other *Fusarium* PFRC pathogens including *F. avenaceum*, *F. culmorum* and *F. redolens*, with an inoculum concentration of 1×10^6 spores mL^{-1}. Only isolates of FO and *F. avenaceum* caused plant death, although overall, isolates of FS resulted in a slightly higher mean disease score (59.5/100) than the FO isolates (53.8/100). Isolates of *F. redolens* were the least virulent (25.6/100). Another pathogenicity assay, the 'inoculum layer assay', was used to examine isolates of DP only, although the inoculum concentration was not stated. DP isolates were found to be far less pathogenic than both *Fusarium* spp., with a disease score of 19.5/100. However, AE isolates which were used to inoculate individual pea plants ($1\text{x}10^4$ zoospores) were found to be the most pathogenic of all species examined, with a mean disease score of 88.4/100 (Persson *et al*., 1997). However, as different pathogenicity tests were used between DP, AE and the *Fusarium* spp., direct comparison of disease index scores between these species is problematic. In another study of *Fusarium* spp. identified as being responsible for pea foot rot in North Dakota, USA, FSP isolates were also found to be more pathogenic than FO isolates. Here, a vermiculite - inoculum layer method was used but again there was no estimation of inoculum concentration. Mean percentage root rot severity of FSP and FO was 21.3% and 12.9% respectively, a significant difference (Chittem *et al*., 2015). As evident from the results of the PFRC dose response experiments in this chapter, and that of the wider literature, the virulence of pathogenic isolates is highly dependent on the concentration of inoculum, and therefore studies where this is not quantified are very difficult to interpret.

Virulence of pathogenic isolates of the same species can vary considerably (Jenkins, 2018) and this has also been demonstrated for PFRC pathogens. The work of this chapter only examined one isolate for each species, but it may be important in future studies to examine multiple isolates. As noted in Chapter 2, Šišić *et al*., (2018) characterised both virulence and phylogenetic relationships of FSP isolates collected from several legume species in several European counties. They found significant variation in virulence of 48 FSP isolates in pathogenicity tests on pea, with isolates sampled from pea, subterranean and white clover, winter vetch and faba bean. These

isolates all clustered into a major clade in the phylogenetic analysis. Based on a disease scoring system of lesion formation on pea root tissue, 8 % of the FSP isolates were non-virulent, 35 % were weakly virulent, 50 % were moderately virulent and 6 % were highly virulent. Similarly, the study by Chittem *et al*., (2015) recognised *F. avenaceum* as the most prevalent *Fusarium* sp. in field surveys carried out between 2004 and 2009 in North Dakota. Testing of 17 *F. avenaceum* isolates using a growth chamber experiment demonstrated that all isolates were pathogenic but there was significant variation in virulence. The mean disease severity score of these isolates was 44.8 %, the lowest score was 6.3 % and the highest was 88.7 % with most of the isolates classified as moderately virulent. Variation in AE virulence has also been investigated in relation to pea varietal susceptibility and geographical distribution in France. Although there was no geographical effect on virulence, AE isolates fell into different groups according to virulence on six pea genotypes. A total of 101 AE isolates were examined for their virulence on one pea cultivar, Baccara, using a vermiculite-based growth chamber assay where each week-old pea plant was inoculated with a suspension containing 1×10^3 zoospores. When root symptoms were assessed using a scoring index between zero and five, the average score was 3.69. Four isolates had scores between one and two, eight isolates had scores between two and three, 41 isolates had scores between three and four and 29 isolates had scores between four and five (Wicker & Rouxel, 2001).

The PFRC interaction experiment using the optimised test tube-based system is the first to investigate interactions of UK isolates of FO, FSP and DP. Interestingly, results suggested there were no synergistic interactions between any combination of PFRC pathogens with respect to mean root rot severity score. Although disease levels for individual pathogens were all significantly greater than the control, at the two-way interaction level, the effect of combining pathogens in any combination did not result in a greater mean root rot score than the sum of the individual pathogen effects and therefore, the effects were additive rather than synergistic. At the three-way interaction level, the mean root rot score was greater than the sum of individual effects, but this was not significant. Few studies have similarly investigated the interactions of PFRC pathogens and only identified synergistic effects (Willsey *et al*., 2018). Here, a glasshouse assay examined the effect of different inoculation combinations of AE, FS, *F. redolens* and *F. avenaceum* isolates on foot rot development in pea. A seed soaking method was used for the *Fusarium* isolates, using a spore concentration of $4x10^4$ spores mL^{-1} and where all three *Fusarium* spp. were combined, the total concentration remained at $4x10^4$ spores mL^{-1} with each *Fusarium* isolate contributing equally. This is different to the approach in this study

where the inoculum was not diluted for each pathogen in combination treatments. Treatments in the Willsey *et al*., (2018) study also included AE, where *Fusarium* inoculated seeds were planted into soil containing 500 AE oospores g^{-1}. Although all possible combinations were not examined, several combinations of pathogens showed significantly greater effects on disease development compared to the sum of individual pathogens effects and therefore, these interactions were considered synergistic. These included *F. redolens* + AE, FS + AE, *F. redolens* + *F. avenaceum* + FS and all four pathogens combined.

In addition to investigating PFRC pathogen interactions in terms of foot rot disease development and pea plant mortality, root tissue of each single and double pathogen combination treatment was harvested at days 10 and 21 for DNA extraction and qPCR analyses to investigate colonisation and interactions within double pathogen combinations. The qPCR assays successfully quantified the DNA concentration of each pathogen within respective treatments for both time points examined, although there was some variation between some of the samples, particularly between individual roots sampled at day 21. However, this experiment only used one biological repeat of the interactions experiment (section 3.3.2) and it is recommended for future qPCR assays to increase the number of plant samples taken for each treatment (e.g. five) and to use all biological repeats for the qPCR, to allow better comparisons to be made between the DNA concentrations quantified for each pathogen, and disease scores. The study by Willsey *et al*., (2018) used pea plant roots from a pot-based glasshouse experiment to examine PFRC pathogen colonisation and interactions for different combinations of the PFRC pathogens AE, FS, *F. redolens* and *F. avenaceum* using multiplex qPCR assays. Background levels of contaminating non-target pathogens were found in some samples, but in the work of this chapter, only one plant sample from one treatment had a detectable level of a contaminating pathogen. This may be an advantage of the test-tube based system, as it is a more enclosed environment for the growth substrate compared to a pot-based experiment. The authors however did attribute this in part to possible seed contamination (Willsey *et al*., 2018) and as such, this is something to consider for the future.

As observed in the study of Willsey *et al*., (2018), the work of this chapter demonstrated both increases and decreases in individual pathogen DNA concentration in co-inoculated treatments compared to the respective single pathogen treatments. Whilst these changes weren't consistent for FO and FSP when co-inoculated, DNA concentration for DP co-inoculated with FO or FSP were lower compared to the DP only treatment suggesting an antagonistic interaction between

DP and both FO and FSP separately. This could relate to the competitive exclusion theory, as DP occupies a very similar spatial niche to FO and FSP although the entry route into the root tissue and the timing of infection might differ (Fitt *et al*., 2006; Le May *et al*., 2009).

In conclusion, the research of this chapter successfully investigated the relationship between inoculum concentration and disease development for individual PFRC pathogens in test-tube and glasshouse experiments. This may serve as a basis for future work, such as the development of tools to assess disease risk through implementation of molecular diagnostics and development of pathogen management strategies. The work involving DP in this chapter provides novel and fundamental information about the pathogen, which is thought to be the primary cause of PFR in the UK. Furthermore, the interaction studies have identified additive effects in terms of disease development when combining major pathogens of the PFRC while qPCR analyses have started to elucidate the nature of these interactions for the first time.

4. Identifying biofumigant crops for the management of pea foot rot complex pathogens

4.1 Introduction

4.1.1 The concept of biofumigation

Biofumigation can be defined as the use of certain plants of the Brassicaceae family which when grown on and/or incorporated into soil, release volatiles, including isothiocyanates (ITCs) which aid in disease suppression (Matthiessen & Kirkegaard, 2006; Gimsing & Kirkegaard, 2009). This concept was one of the alternative options researched in response to the ban of methyl bromide in the Montreal Protocol of 2005 following confirmation that it was a class 1 ozone depleting substance (Gimsing & Kirkegaard, 2009). Methyl bromide was a popular volatile chemical used from the 1960's for soil fumigation against a range of soilborne pathogens, but also had effects on many non-target soil fungal, bacterial, insect, nematode and weed species. Due to toxicity against non-target species, legislation in the USA attempted to ensure that the amounts and how the agent was applied limited these undesirable effects (Duniway, 2002). Biofumigant plant species may be used in several ways. Firstly, as a rotation crop, where the biofumigant crop(s) and cash crops are rotated seasonally, or at a schedule required for sufficient reduction of the inoculum in the soil causing disease. Secondly, they can be planted alongside the cash crop itself. They can also be directly added to the soil as a 'green manure' (essentially when the plant is still fresh) (Larkin & Griffin, 2007). Thirdly, they can be added as a preserved material, where the biofumigant crop is harvested and then freeze or oven dried to minimise any GSL catalysis to ITCs until use and rehydration with water. Different organs of the biofumigant plant may contain different concentrations and types of glucosinolates (GSLs) and subsequent ITCs so this can aid a more targeted approach (Larkin & Griffin, 2007). This is particularly useful in the context of multi-pathogen disease complexes, such as the pea foot rot complex (PFRC), where different pathogens may be inhibited by different types of ITCs.

4.1.2 Glucosinolates and their hydrolysis

Biofumigant crops are effective in controlling soilborne pathogens and pests due to hydrolysis products of GSLs (such as ITCs), compounds found in relatively high concentrations in certain Brassicaceae species (Matthiessen & Kirkegaard, 2006; Sarwar & Kirkegaard, 1998). Certain hydrolysis products have activity against soilborne plant pathogens and pests. GSLs are a group of around 120 secondary metabolites (Halkier & Gershenzon, 2006) and are generally classed into three groups, aliphatic, aromatic and indole (Fahey *et al.*, 2001) based on the precursor amino acid they are formed from and type of R-group modification (Halkier & Gershenzon, 2006). However, only aromatic and aliphatic GSLs result in production of ITCs (Matthiessen & Kirkegaard, 2006). Glucosinolates are formed in three main stages; in brief, particular amino acids are deaminated and then undergo up to several repeated cycles to elongate the deaminated amino acid with a methylene group. Once elongated with the desired number of methylene groups, the molecule rearranges and is converted into several intermediate compounds during the second main stage and eventually the GSL molecule is formed. In the third stage, the GSL molecule can then undergo various modifications to its R-group, which dictates the identity of the ITC and other breakdown products (Halkier & Gershenzon, 2006).

GSL breakdown occurs when plant tissue is broken down, through mechanical damage or pest and pathogen activity (Figure 4.1). Upon damage, GSLs stored in the vacuole come into contact with the enzyme myrosinase, which is found in all plants containing GSLs and stored in a separate myrosinase cell. In the presence of water, myrosinase then catalyses the breakdown of GSLs, first of all by cleaving off the glucose component which forms an unstable intermediate compound known as an aglycone. The aglycone will undergo different re-arrangements depending on environmental conditions, but most commonly undergoes the Lossen rearrangement to form isothiocyanates. Other GSL breakdown products include thiocyanates, nitriles, and oxazolidines (Bones & Rossiter, 1996; Mithen, 2001).

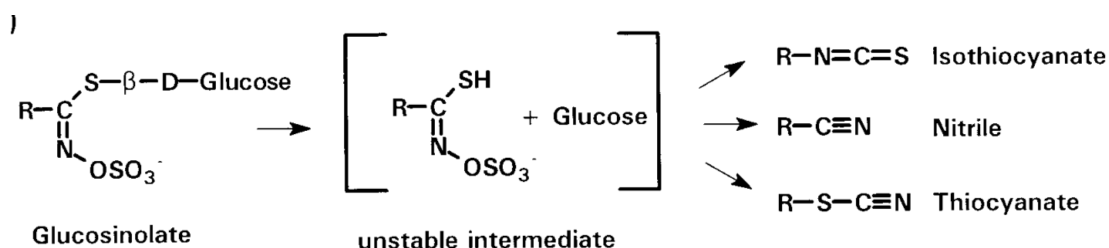


Figure 4.1. Simplified schematic of gluconsinolate hydrolysis to form isothiocyanate and other products (adapted from Mithen, 2001).

4.1.3 Biofumigant plant species, their primary glucosinolates and hydrolysis products

Brassicaceae species have been developed commercially for high GSL content for the purpose of biofumigation (Santos *et al.*, 2020). There are several species of the *Brassica* genus utilised as biofumigants, including *Brassica juncea* (Indian mustard), *Brassica carinata* (Ethiopian mustard), *Brassica nigra* (black mustard), *Brassica rapa* (field mustard) and *Brassica napus* (oilseed rape) (Sarwar & Kirkegaard, 1998). The primary GSL of *B. juncea* and *B. carinata* is sinigrin (2-propenyl GSL), with allyl ITC (2-propenyl ITC) the primary hydrolysis product (Gil & MacLeod, 1980; Tsao *et al.*, 2002). Other species utilised as biofumigants include *Raphanus sativus* (radish), *Sinapis alba* (white mustard) (Kirkegaard & Matthiessen, 2004) and *Eruca sativa* (rocket; Lucarini *et al.*, 2019). The primary GSL found in radish is glucoraphanin (4-methylsulfinylbutyl GSL), with two major hydrolysis products being sulforaphane (4-methylsulfinylbutyl ITC) and sulforaphane nitrile (5-methylsulfinylpentane nitrile) (Matusheski & Jeffery, 2001; Force *et al.*, 2007). The primary GSL of *S. alba* is sinalbin (4-hydroxybenzyl GSL) with 4-hydroxybenzyl ITC the hydrolysis product; however, this is unstable and will degrade to form ionic thiocyanate (SCN^-) (Borek & Morra, 2005). The primary glucosinolate found in *E. sativa* is glucoerucin (4-methylthiobutyl GSL) (Lucarini *et al.*, 2019), with erucin (4-methylsulfanylbutyl ITC) a major hydrolysis product (Wagner *et al.*, 2015).

4.1.4 Biofumigation strategies against soil borne plant pathogens and pests

Biofumigation through *in-vitro*, glasshouse and field experiments have been shown to be effective against various soil borne fungal plant pathogens and pests (Brown & Morra, 1997). One study carried out multiple field experiments to examine the effect of commercial biofumigant varieties on *Globodera pallida*, the potato cyst nematode.

In one field experiment, biofumigant varieties *B. juncea* ‘Caliente 99’, *E. sativa* ‘Nemat’ and *R. sativus* ‘Bento’ were sown in the summer and incorporated in the autumn prior to a potato crop the following spring. At six weeks post biofumigant incorporation, treatments of ‘Caliente 99’ and ‘Bento’ resulted in a significant reduction in *G. pallida* egg viability compared to a fallow control. This reduction remained significant for ‘Caliente 99’ post-harvest of the potato crop (Ngala *et al.*, 2015).

B. juncea plant material (variety not stated) used freshly chopped in an *in-vitro* assay resulted in significantly reduced mycelial growth of several fungal and oomycete soil borne pathogens of potato, including FO, *F. sambicinum*, *Phytophthora erythroseptica*, *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. The same variety also significantly reduced both viable inoculum concentration and disease score of root and stem canker caused by *R. solani* in a glasshouse assay, where the biofumigant was grown in the pot as a cover crop and then incorporated after five weeks, prior to potato planting (Larkin & Griffin, 2007).

Another study examined the effects of *B. juncea*, *B. napus* and *S. alba* seed meal on an isolate of *R. solani* AG8, responsible for root rot of various cereal and legume crops in a controlled environment experiment (Handiseni *et al.*, 2013). For each seed meal variety, both autoclaved and non-autoclaved treatments (to inactivate myrosinase) were included, with the researchers proposing that simply amending the growing medium with the seed meal (organic matter) incited changes in the microbial community which were less conducive to *R. solani* root rot disease. Wheat seedlings were grown post amendment and disease assessments revealed that all treatments, including *B. juncea* ‘Pacific Gold’, resulted in a significant reduction of root rot disease ratings for both autoclaved and non-autoclaved seed meal. However, non-autoclaved treatments of *B. juncea* ‘Pacific Gold’ and *S. alba* resulted in a further significant reduction in root rot disease rating compared to the respective autoclaved treatment, highlighting the importance of GSL breakdown products of these varieties on *R. solani* root rot disease suppression (Handiseni *et al*., 2013).

There is a lack of research into biofumigation strategies against the PFRC pathogens *Didymella pinodella* (DP), *Fusarium oxysporum* (FO) and *F. solani forma specialis* (f.sp.) *pisi* (FSP), however biofumigation strategies against *Aphanomyces euteiches* (AE) are more researched. The following sections will examine biofumigation strategies against these species and members of the Didymellaceae.

4.1.4.1 Biofumigation strategies against isolates of F. oxysporum *and* F. solani

Research into biofumigation as a control method has been conducted on FO *formae speciales*, such as *F. oxysporum* f. sp. *ciceris*, the causal agent of Fusarium wilt in chickpea. One study showed that *S. alba*, *B. nigra* and *B. juncea* all had significant effects on this pathogen in both *in-vitro* and glasshouse systems. *In-vitro* studies utilised a plate-based system with hydrated powdered biofumigant material of each species and examined the effect on *F. oxysporum* f. sp. *ciceris* mycelial growth, which was reduced or inhibited by all three aforementioned species at a rate of 10 mg powder to 10 mL water. In pot-based trials with a *F. oxysporum* f. sp. *ciceris* inoculated soil-manure substrate of known concentration (10^8 spores g^{-1}), freshly macerated tissues of each biofumigant species at a rate 100 g per 2 kg substrate were found to significantly reduce pathogen CFUs compared to an inoculated substrate containing no biofumigant material (Prasad & Kumar, 2017). Another glasshouse study examined the effectiveness of *Brassica carinata* (Ethiopian mustard) cultivars on *F. oxysporum* f. sp. *ciceris* disease development, utilising different rates of seed meal incorporated into an inoculated substrate. Two cultivars (Holleta-1 and S-67) significantly reduced chickpea wilt symptoms and significantly increased fresh and dry pod mass at application rates of 10 g per kg substrate (Abera *et al.*, 2011).

There is very little published research concerning biofumigation strategies against FSP or *F. solani* (FS) in legumes. One study utilising an *in-vitro* plate-based system aimed to examine the effect of different amounts of biofumigant crops on a range of plant pathogens, including an FS isolate responsible for root rot in Himalayan ginseng (*Panax pseudoginseng*). A kohlrabi (*Brassica oleracea* var. *gongylodes* (Gongylodes group)) treatment resulted in 78.99 % inhibition at seven days of the FS isolate, using 10 g of flash frozen ground tissue per plate (Fan *et al.*, 2008).

Another study examined the effects of seed meal, dry and fresh amendments of *B. juncea*, *B. alba* and *B. nigra* on the viability of FS and FO chlamydospores in artificially inoculated soil. Both isolates are casual agents of basal rot in onion. In both experiments carried out for FO and FS, none of the biofumigant treatments resulted in a significant decrease in viable CFUs compared to the non-amended control (Smolinska & Kowalczyk, 2014).

4.1.4.2 *Biofumigation strategies against soil-borne pathogens of the Didymellaceae*

Studies regarding biofumigation as a disease management approach have been undertaken regarding some species residing within the Didymellaceae (Geary *et al.*, 2008; Finckh *et al.*, 2013), however there is no published research regarding biofumigant strategies against DP. One study examined the use of *S. alba* 'Idagold' and *R. sativus* 'Colonel' as alternative control strategies to chemical fumigants for supressing *Phoma terrestris*, the causal agent of pink root of onion through field trials. Overall, neither of the biofumigant treatments significantly reduced pink root disease score compared to a fallow control (Geary *et al.*, 2008). Another study as part of their aims examined the use of *B. juncea*, *R. sativus* and *S. alba* cover crops on Ascochyta blight disease suppression in pea in two field trials over different years, by examining the severity of foot and root rot symptoms by scoring internal and external lesion development, as well as the percentage of plants infected with *Ascochyta medicaginicola* (synonym: *Phoma medicaginis*), *Didymella pinodes* and *Ascochyta pisi* (Finckh *et al.*, 2013). Cover crops were sown in August, incorporated into the topsoil in October prior to sowing of the pea crop. The research found no significant impact of the over crops on foot and root rot disease development, but noted that over the two trials, *A. medicaginicola* was isolated from over 70% of symptomatic plants. For *D. pinodes* and *A. pisi*, incidence was very low in one trial, but in the other, *D. pinodes* and *A. pisi* were isolated from 50 % and 20 % of symptomatic plants respectively (Finckh *et al.*, 2013).

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4.1.4.3 *Biofumigation strategies against* Aphanomyces euteiches *causing pea foot rot*

Biofumigation strategies against the PFRC pathogen *Aphanomyces euteiches* (AE) are much better researched by comparison to other pathogens of the complex. Initial studies involved incorporating dried cabbage (*B. oleracea*) leaves into growing medium artificially inoculated with AE, where the biofumigant treatment resulted in a significant reduction in AE foot rot infection (Papavizas, 1966 cited by Hossain *et al.*, 2012). Whilst ITC's were not identified following analysis of compounds present that might have resulted in the observed pathogen inhibition, further experiments showed that thiomethane, dimethyl sulphide and dimethyl disulphide gases in particular affected AE (Lewis & Papavizas, 1970). Volatile ITCs, powdered aromatic and aliphatic ITCs and the glucosinolate breakdown product 5-vinyloxazolidine-2-thione

isolated from *B. juncea* have also been shown to be effective against AE zoospores and mycelium respectively (Lewis & Papavizas, 1970; Smolinska *et al.*, 1997; Hossain *et al.*, 2015). In field trials held over consecutive growing seasons, the use of *S. alba* as a green manure prior to the main pea crop supressed AE root rot (Muehlchen *et al.*, 1990). Watson, (2013) undertook glasshouse tests to examine the use of onion, canola and Bioqure Mulch (*B. rapa* and *B. napus* mix) amendments against *A. euteiches* in bean. Test crops were grown to flowering/bulbing in pots and then for onion, either root and stem, or just root, was mixed into the soil and left for three weeks. Beans were planted into the soils and the disease severity index measured for AE pathogenicity of both the hypocotyl and roots. The use of both onion roots alone, and onion roots and shoots incorporated into infected soils both significantly reduced disease severity in comparison to the use of other crops and the non-treated control. However, in further experiments which used the bulb, shoot and root material of onion as both wet and dry green manures added to the soils, the reduction of disease severity could not be replicated. It was concluded that the growth of the biofumigant crop in the same growing medium it will then be used in may be a crucial factor in this study (possible due to release of AE-suppressive root exudates and glucosinolates through partial biofumigation); the timing of the application or growth of the green manure was concluded to be critical in this case.

4.1.5 Aims and objectives

The main aim of this chapter was to identify biofumigant crop varieties that could inhibit PFRC pathogens both *in-vitro* and in glasshouse experiments. The objectives were to:

1. Grow and process different biofumigant crop species and varieties and analyse their primary glucosinolate concentration using high performance liquid chromatography (HPLC).
2. Examine the effect of different biofumigants crops on mycelial growth and spore germination of PFRC pathogens *in-vitro*.
3. Examine the effect of different biofumigants crops on DP pea foot rot disease development under glasshouse conditions.

4.2 Materials and methods

4.2.1 Growth and processing of biofumigant crops

Biofumigant plants for experimental use (varieties and batch dates listed in Table 4.1) were grown in 7.5 L pots containing Levington M2 compost at various locations on the Wellesbourne Campus, University of Warwick (Figure 4.2), with help from the Horticultural services team and Dr Alex McCormack. The number of seeds sown per pot was variety dependent, with 14 seeds sown per pot for biofumigant plant mixtures, thinned down to five as standard once the desired plants from the mix could be identified. Plants were grown in five batches on different sowing dates, with different varieties and growth conditions associated with each.

Biofumigant plant batch 1 was sown on 11/08/2017 and was grown in a temperature-controlled glasshouse compartment set at 17 °C day and 15 °C night with natural lighting. Some plants developed symptoms of powdery mildew and were therefore treated once with Thiovit Jet (Syngenta, Switzerland) at a concentration of 2 g L^{-1} with a sulphur burner. Batches 2 and 3 were sown on 12/04/2019 and 11/07/2019 respectively and were grown in a temperature-controlled glasshouse compartment set at 18 °C day and 20 °C night with a daylight extension to 16 hours. Additional top dressings of nitrogen and sulphur at the equivalent rate of 100 kg ha^{-1} and 30 kg ha^{-1} respectively were applied to each pot. Batch 4 was sown on 07/08/20 and was grown in an open-sided polytunnel under natural light. Some plants were colonised by aphids and were therefore treated once with Movento (Bayer Crop Science, Germany) at a concentration of 1 mL L^{-1}. Batch 5 was drilled on 11/08/20 in the field. Prior to drilling, nitrogen was applied at a rate of 100 kg ha^{-1}. Emerging seedlings were protected by netting and removed once established. To control weeds, Sultan (Adama, Israel) was applied at a rate of 1.5 L ha^{-1}.

Each biofumigant plant variety was harvested when the majority had reached mid-flowering. For pots in the glasshouse and polytunnel, shoot, leaf and flower tissue were removed at soil level before being placed into individual paper bags, with fresh weight recorded. Harvested plant material was then dried at 80 °C for 48-72 hours depending on stem thickness, with dry weights from each pot recorded. Plants grown in the field were cut at soil level using a hedge trimmer and plants of each variety placed directly into potato sacks. Harvested plant material was then dried at 80 °C for one week. Following drying, all plant material for each biofumigant plant variety was pooled and milled to produce a fine powder.

Figure 4.2. Biofumigant varieties grown in a) temperature-controlled glasshouse compartment in 2019 and an b) open-sided polytunnel in 2020.

Table 4.1. Biofumigant crop varieties used in experiments. NS = variety not sown, D = died post emergence.

Variety	Supplier	Main glucosinolate	Batch 1		Batch 2		Batch 3	
			Sowing date	**Harvest date**	**Sowing date**	**Harvest date**	**Sowing date**	**Harvest date**
Brassica juncea 'Pacific Gold'	Northwald Agronomy	Sinigrin	11/08/2017	18/10/2017	NS	NS	NS	NS
Brassica juncea 'Caliente 99'	Tozer Seeds	Sinigrin	11/08/2017	18/10/2017	NS	NS	NS	NS
Brassica juncea 'Caliente 199'	Tozer Seeds	Sinigrin	NS	NS	12/04/2019	25/05/2019	11/07/2019	23/08/2019
Brassica juncea 'Caliente Rojo'	Tozer Seeds	Sinigrin	NS	NS	12/04/2019	25/05/2019	11/07/2019	30/08/2019
Brassica carinata 'Cappuchino'	RAGT Seeds	Sinigrin	NS	NS	12/04/2019	17/06/2019	11/07/2019	26/09/2019
Brassica napus 'Windozz'	RAGT Seeds	Sinigrin	NS	NS	NS	NS	NS	NS
Raphanus sativus 'Terranova'	Tozer Seeds	Glucoraphanin	11/08/2017	15/02/2018	12/04/2019	11/07/2019	11/07/2019	04/12/2019
Raphanus sativus 'Contra'	Elsoms Seeds	Glucoraphanin	NS	NS	12/04/2019	08/07/2019	11/07/2019	29/10/2019
Raphanus sativus 'Bento'	Senova	Glucoraphanin	NS	NS	NS	NS	NS	NS
Raphanus sativus 'Triangel'	RAGT Seeds	Glucoraphanin	NS	NS	NS	NS	NS	NS
Eruca sativa 'Trio'	RAGT Seeds	Glucoerucin	NS	NS	12/04/2019	17/06/2019	11/07/2019	14/10/2019
Eruca sativa 'Nemat'	Tozer Seeds	Glucoerucin	11/08/2017	16/11/2017	12/04/2019	07/06/2019	11/07/2019	26/09/2019
Sinapsis alba 'Brisant'	RAGT Seeds	Sinalbin	11/08/2017	18/10/2017	12/04/2019	25/05/2019	11/07/2019	23/08/2019

Table 4.2. Biofumigant crop varieties used in experiments. NS = variety not sown, D = died post emergence.

Variety	Supplier	Main glucosinolate	Batch 4		Batch 5	
			Sowing date	Harvest date	Sowing date	Harvest date
Brassica juncea 'Pacific Gold'	Northwald Agronomy	Sinigrin	NS	NS	NS	NS
Brassica juncea 'Caliente 99'	Tozer Seeds	Sinigrin	NS	NS	NS	NS
Brassica juncea 'Caliente 199'	Tozer Seeds	Sinigrin	07/08/2020	22/09/2020	11/08/2020	02/11/2020
Brassica juncea 'Caliente Rojo'	Tozer Seeds	Sinigrin	07/08/2020	23/09/2020	11/08/2020	02/11/2020
Brassica carinata 'Cappuchino'	RAGT Seeds	Sinigrin	07/08/2020	19/10/2020	11/08/2020	02/11/2020
Brassica napus 'Windozz'	RAGT Seeds	Sinigrin	07/08/2020	19/10/2020	11/08/2020	02/11/2020
Raphanus sativus 'Terranova'	Tozer Seeds	Glucoraphanin	NS	NS	NS	NS
Raphanus sativus 'Contra'	Elsoms Seeds	Glucoraphanin	NS	NS	NS	NS
Raphanus sativus 'Bento'	Senova	Glucoraphanin	07/08/2020	19/10/2020	11/08/2020	02/11/2020
Raphanus sativus 'Triangel'	RAGT Seeds	Glucoraphanin	07/08/2020	19/10/2020	11/08/2020	02/11/2020
Eruca sativa 'Trio'	RAGT Seeds	Glucoerucin	07/08/2020	19/10/2020	11/08/2020	D
Eruca sativa 'Nemat'	Tozer Seeds	Glucoerucin	NS	NS	NS	NS
Sinapsis alba 'Brisant'	RAGT Seeds	Sinalbin	07/08/2020	19/10/2020	11/08/2020	02/11/2020

4.2.2 Analysis of glucosinolate contents of biofumigant plants using high performance liquid chromatography

To determine the primary GSL concentration of the milled biofumigants, a modified protocol (Warmington, 2014), which was adapted from an original method by Tsao *et al.*, (2002) was employed to extract and quantify GSLs from the milled biofumigants using a hot water extraction and high-performance liquid chromatography (HPLC). Hot water extraction and HPLC analysis was undertaken with assistance from Andrew Jukes. To extract the GSLs, each biofumigant plant sample was thoroughly mixed, before 100 mL of boiling RO water was added to 1 g of powder in a round bottom flask. A small quantity of anti-bumping granules (VWR International Ltd., UK) were added to the flask, which was then attached to a reflux condenser to allow continuous boiling for at least 30 minutes. The solution was cooled to room temperature before filtering through filter paper (No. 1, Fischerbrand, UK) to remove plant material.

HPLC analysis was undertaken as described by Warmington, (2014) with a few modifications. Analysis was undertaken on a HP Agilent 110 system, equipped with a UV diode array detector. Sample injection was 20 µL, using a HP110 autosampler. A reverse-phased Zorbax SB-Aq column (5 µm, 4.6 mm x 250 mm, Agilent Technologies, USA) was used, run at 24 °C at a pressure of 43 bar. Detection was set at 228 nm to detect GSLs and 242 nm for ITCs to detect any GSL breakdown. The flow rate was 1 mL min^{-1} for a total run time of 26 minutes. An eluent of ammonium acetate (pH 6.75) was used, the gradient was increased from 99% ammonium acetate to 50% at six minutes and back to 99% at 21 minutes. Retention times of GSLs were between three and eight minutes. Standards of concentrations between 80-100 ppm (Phytolab GmbH & Co, Germany) were run after each group of samples with the same primary GSL, and sinigrin was used in place of glucoerucin, with respective peak area compared to a previous run using the same conditions where both sinigrin and glucoerucin standards were used. No pure allyl ITC standard was run.

4.2.3 Effect of biofumigants on mycelial growth of pea foot rot complex pathogens

A modified version of a method (Sexton *et al.*, 1999) was used to examine the effects of different varieties of biofumigants on mycelial growth of PFRC pathogens (Table 4.3.) Two separate experiments were carried out (Experiments 1 and 2) using biofumigant varieties from batches 1 and 2 respectively (Table 4.1). A 5 mm agar plug from the growing edge of a PFRC pathogen culture was placed in the centre of a 9 cm Petri dish base containing PDA. In a second Petri dish base, 2 g biofumigant powder was hydrated with 20 mL SRO water and mixed. The equivalent volume of SRO water was used as the untreated control. The Petri dish base containing PDA and pathogen plug was placed on top of the Petri dish base containing the hydrated biofumigant, held in place with two strips of tape and immediately sealed with a layer of parafilm (Figure 4.3). Cultures were incubated at 18 °C (Experiment 1) or 20 °C (Experiment 2) in the dark and growth assessed on at least five occasions during the experiment, by measuring the colony diameter along the x and y axis, until approximately seven days (FO, FSP and DP) or the growth of control plates had reached the edge of the plate (AE). For each pathogen, mean growth rates were calculated from each plate of the treatment, by calculating a mean of the five measurements for each time point from growth along the x and y axis. Mean percentage inhibition of each treatment compared to the control (no biofumigant) was calculated by obtaining the mean of the percentage inhibition of each plate within the treatment using; $((C-T)/C) \times 100$, where C is the final growth of the control in cm and T is the final growth of the treatment plate in cm.

For Experiment 1, there were three biological repeats each for FO, FSP and DP, which consisted of four replicate plates for each biofumigant variety arranged in a complete randomised design across two trays. For Experiment 2, there were three biological repeats each for FO, FSP and DP, which consisted of four replicate plates for each biofumigant variety arranged in a randomised block design across four trays. For each pathogen within each experiment, mean growth rates for each treatment were subject to a one-way ANOVA to determine significance ($p < 0.05$), with Tukey's HSD post-hoc analysis used to determine differences between treatment means. Statistical analysis was carried out using SPSS 25 (IBM Corperation, 2021).

Table 4.3. List of fungal isolates used in experiments.

Species	Isolate	Information
Fusarium oxysporum	PG18	Isolated from field Molescroft 61B, Molescroft, England, via PGRO crop clinic in 2012
Fusarium solani f. sp. *pisi*	PG13	Isolated from field 32a, UK, via PGRO crop clinic in 2012
Didymella pinodella	61B	Isolated from field Molescroft 61B, Molescroft, England, via PGRO
Aphanomyces euteiches	Burton	Obtained from the University of Nottingham, October 2018

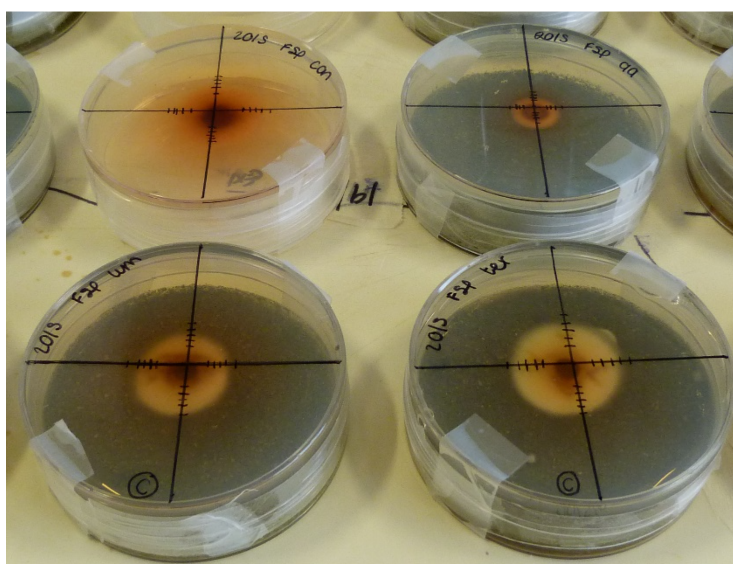

Figure 4.3. Sealed double Petri-dish base system used to test biofumigants for effect on mycelial growth of PFRC pathogens. Base containing PDA agar and a 5 mm plug of pathogen inoculated agar, placed on top of hydrated biofumigant material.

4.2.4 Effect of biofumigants on spore germination of pea foot rot complex pathogens

To examine the effect of biofumigants on PFRC pathogen spore germination, a similar double Petri dish-based system was used as in section 4.2.3; however 100 µL of a $5x10^2$ spores mL^{-1}, made in a similar fashion to the suspensions described in section 3.2.1 were pipetted and spread onto the 9 cm Petri dish base containing PDA. Cultures were incubated at 20 °C in the dark for one week and assessed by counting the number of colony forming units (CFUs) on the plate to examine inhibition of spore

germination in the presence of the biofumigant. The Petri dish base containing PDA and pathogen plug was removed from the biofumigant, sealed with a new sterile lid, and incubated for a further 48 hours where further CFU counts were undertaken to examine whether biofumigants killed spores or only suppressed germination. Mean percentage inhibition for each treatment (as for mycelial growth assay, section 4.2.3) compared to the control was calculated using; ((C-T)/C) x 100, where C is the mean CFU count of the control plates and T is the mean CFU count of the plates from a specific treatment.

Within a biological repeat, there were four (FO, FSP) replicate plates for each biofumigant variety arranged in a complete randomised design across trays, with two biological repeats for each pathogen. For each pathogen, mean colony forming unit (CFU) counts were subject to a one-way analysis of variance to determine significance ($p < 0.05$), with Tukey's HSD post-hoc analysis used to determine differences between treatment means. Statistical analysis was carried out using SPSS 25 (IBM Corporation, 2021).

4.2.5 Determining the effect of biofumigants on suppression of foot rot disease caused by *Didymella pinodella* in a pot-based system

A pot-based experiment was designed to examine the effects of different varieties of biofumigant on DP foot rot disease development and mortality of pea plants in the glasshouse (Figure 4.4).

DP inoculum was grown in flasks containing a sterile compost-bran mix. Inoculum was prepared by mixing compost (Levington M2, sieved to 4 mm, 100g) and milled wheat bran (Charlecote Mill, UK, 148.4 g) and adjusting moisture content to 78.9 %. The bran compost mix (300 g) was dispensed into 1 L conical flasks sealed with a tight cotton wool bung and two layers of tin foil. Flasks were autoclaved three times at 121 °C for 15 minutes and after cooling each was inoculated with five 5 mm^2 agar plugs taken from the growing edge of an actively growing culture. Flasks were incubated in the dark at room temperature for six weeks, each flask shaken twice a week.

DP inoculum concentration for each flask was determined by adding 1 g to 10 mL sterile RO water and vortexing. Serial dilutions were then made in 10 mL SRO water down to 10^{-6} and 100 µL of the dilutions of 10^{-4}, 10^{-5} and 10^{-6} and were plated in triplicate onto PDA agar containing 20 mg L^{-1} chlorotetracyline (Sigma-Aldrich, Gillingham, UK). Plates were incubated at 20 ºC for two days after which colony

forming units (CFUs) were counted. The highest serial dilution which produced CFUs of 50 or less per plate were used to calculate the mean colony forming unit concentration g^{-1} for each flask.

A pea growing compost/sand mix for an uninoculated control treatment was prepared by mixing horticultural sand (Westland Horticulture, UK) with compost (Levington M2; New formulation) in the ratio 70:30 by weight. The compost/sand mix containing DP inoculum was prepared in the same way, but the equivalent volume of compost substituted for inoculum to give final concentrations of $5x10^5$ spores g^{-1} substrate and $1x10^4$ spores g^{-1} substrate. All substrates were weighed into plastic ziplock bags (700 g each) with 32.2 g of one variety of milled biofumigant added and thoroughly mixed (based on double the recommended field rate due to low GSL concentration). There were two bags per treatment as follows:

1) Control (no biofumigant)
2) Control + Caliente 199
3) Control + Trio
4) Control + Cappuchino
5) $1x10^4$ spores g substrate^{-1} (no biofumigant)
6) $1x10^4$ spores g substrate^{-1} + Caliente 199
7) $1x10^4$ spores g substrate^{-1} + Trio
8) $1x10^4$ spores g substrate^{-1} + Cappuchino
9) $5x10^5$ spores g substrate^{-1} (no biofumigant)
10) $5x10^5$ spores g substrate^{-1} + Caliente 199
11) $5x10^5$ spores g substrate^{-1} + Trio
12) $5x10^5$ spores g substrate^{-1} + Cappuchino

The moisture content of the mix was calculated and adjusted to 66 % with tap water. The substrate was thoroughly mixed again and the bag immediately sealed to contain any volatiles. Bags were incubated at room temperature in the dark for four weeks, after which they were opened, placed into a fume cupboard and vented for a further week. The pea growing substrate was then dispensed into 7 cm FP7 pots, pea seeds sown directly and watering carried out throughout the experiment as required. Experiments were carried out in a temperature-controlled glasshouse compartment with supplementary lighting to extend to a 16-hour day light cycle, with 20 °C day and 18 °C night temperatures. The first biological replicate was sown on 30/07/2021, and the second on 06/08/2021. Pea sticks were staked into the corner of each pot two weeks after sowing to support plants. Plants were scored daily for the first two weeks and twice-weekly thereafter for emergence (defined as reaching growth stage 09 of

the BBCH scale; shoot breaking through the surface) and also twice-weekly for disease development using a scoring index based on percentage of leaves displaying wilt symptoms for the duration of the experiment (Table 4.3).

After six weeks, pea plants were harvested by first removing excess substrate from the root through disturbance, then washed in tap water to remove as much substrate as possible. Each plant root was photographed and scored for percentage root browning (Table 4.4). Plants were also assessed for flower and pod number and presence/absence of DP stem girdling and nodulation. Root and shoot tissue was separated for each plant and both wet and dry weight measured, the latter after tissue was dried at 80 °C for 48 hours.

There were two biological repeats which consisted of 12 replicate pots containing one pea plant per treatment and defined by using separately prepared inoculated substrates. Pots were arranged on each bench in a Latin square design, with 12 pairs of rows each treatment occurring twice in each column, once in an even numbered row and once in an odd numbered row. The design was generated using Genstat 24 (VSN International, 2021) by James Lynn of Applied Statistical Solutions Ltd. For analyses, pea plant mortality and DP stem girdling data were angular transformed and multiplied by 100. For final leaf wilt disease score, percentage DP girdling, pea plant mortality, root rot score and dry shoot and root weights, values were subject to a one-away analysis of variance considering the Latin square blocking structure, with the standard error difference of the mean of the biofumigant/inoculum interaction multiplied by the *t*-critical value of the residual degrees of freedom of each analysis used to calculate significant differences between treatment means. For final emergence and presence/absence of nodules, values were analysed using a generalised linear model with a Bernoulli distribution and logit link function. Statistics were carried out with the support of James Lynn of Applied Statistical Solutions Ltd., using Genstat 19 (VSN International, 2021).

Table 4.4. Disease scoring system used for assessing leaf wilt severity due to DP in pot experiments.

Symptom score	Leaves displaying wilt symptoms (%)	Disease severity
0	0	None
1	1-25	Slight
2	26-50	Slight-moderate
3	51-75	Moderate
4	75-99	Moderate-severe
5	100	Severe

Table 4.5. Disease scoring system (designed by PGRO) used for assessing root rot severity due to DP in pot experiments.

Symptom score	Root browning (%)	Disease severity
0	0	None
1	<10	Slight
2	11-25	Slight-moderate
3	26-50	Moderate
4	51-90	Moderate-severe, no stem girdling
5	91-100	Severe, stem girdling
6	100	Plant dead

Figure 4.4 Glasshouse compartment containing the experiment examining the effect of biofumigant plant varieties tested at different inoculum concentrations of *Didymella pinodella* '61B'.

4.3 Results

4.3.1 Analysis of glucosinolate contents of biofumigant plants using high performance liquid chromatography

All biofumigant plant samples contained detectable levels of the primary GSL of interest at the expected retention time, with no significant amounts of other GSLs present (Figure 4.5). Chromatograms of detection at 242 nm showed no obvious peaks representing ITCs, indicating little or no detectable breakdown of GSLs to ITCs during the hot water extraction process.

The mean level of GSLs in biofumigant samples from batch 1 grown in the glasshouse (sown 11/08/2017) was 16.1 µmol g^{-1} dry weight (d.w). The highest primary GSL level was found in *R. sativus* 'Terranova' at 23.2 µmol g^{-1} d.w. and the lowest in *S. alba* 'Brisant' at 8.4 µmol g^{-1} d.w. Overall, mean levels of GSLs were lower in all other batches. For batches 2 (sown 12/04/2019) and 3 (sown 11/07/19), both grown in the glasshouse, the mean primary GSL levels were 4.4 µmol g^{-1} d.w. and 9.1 µmol g^{-1} d.w. respectively. For batch 2, The highest GSL level was found in *R. sativus* 'Contra' at 8.8 µmol g^{-1} d.w. and the lowest in *E. sativa* 'Nemat' at 1.1 µmol g^{-1} d.w. For batch 3, the highest GSL level was found in 'Terranova' at 28.1 µmol g^{-1} d.w. and the lowest in 'Nemat' at 1.3 µmol g^{-1} d.w. The mean level of GSLs in biofumigant samples from batch 4 grown in the polytunnel (sown 06/08/2020) was 5.9 µmol g^{-1} d.w. The highest primary GSL level was found in *R. sativus* 'Bento' at 9.62 µmol g^{-1} d.w. and the lowest in *B. napus* 'Windozz' at 1.9 µmol g^{-1} d.w. Overall, batch 5 grown in the field (sown 11/08/20) had the lowest mean level of primary GSLs of all batches grown at 1.0 µmol g^{-1} d.w., and this batch had the lowest primary GSL levels for varieties grown of all batches. The highest primary GSL level was found in 'Brisant' at 1.95 µmol g^{-1} d.w. and the lowest in *B. carinata* 'Cappuchino' at 0.6 µmol g^{-1} d.w.

In terms of individual GSL concentrations, the highest levels of sinigrin (in *B. juncea* 'Pacific Gold'), glucoerucin (in 'Nemat') and sinalbin (in 'Brisant)' were detected in batch 1 (sown 11/08/17), grown in the glasshouse, with levels of 20.9 µmol g^{-1} d.w., 11.8 µmol g^{-1} d.w. and 8.4 µmol g^{-1} d.w. respectively. The highest levels of glucoraphanin were detected in batch 5 (sown 11/07/19) grown in the glasshouse in 'Terranova', with a level of 28.1 µmol g^{-1} d.w.

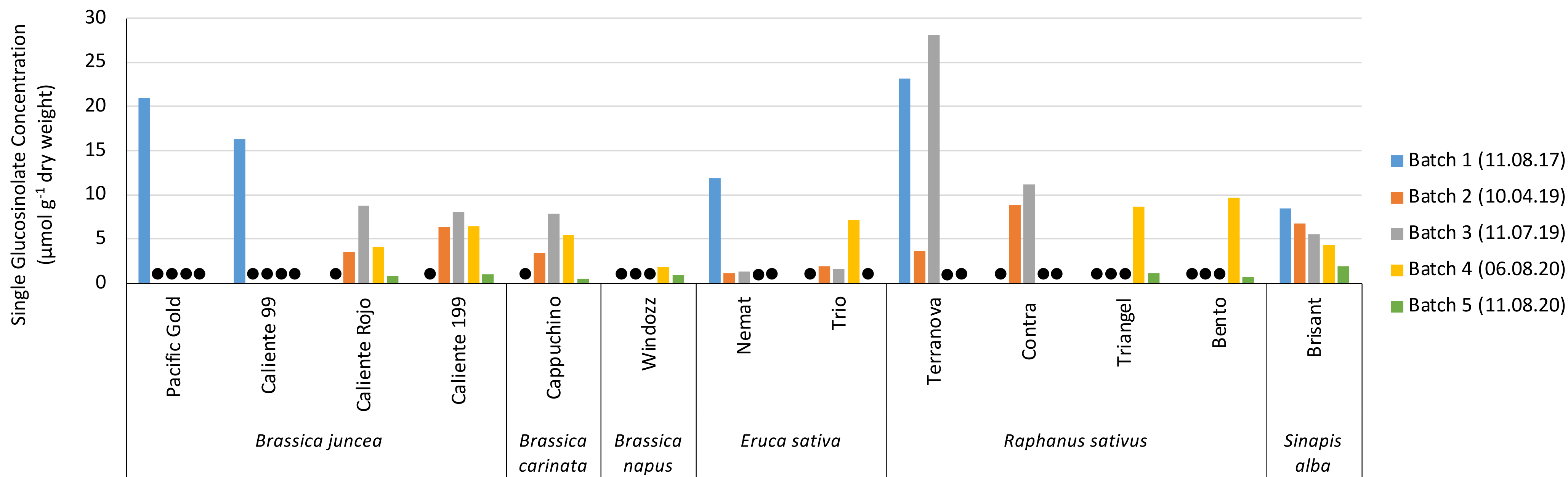

Figure 4.5. Glucosinolate concentrations for different varieties and batches of biofumigant crops, determined by HPLC, grown in the glasshouse (batches 1, 2 and 3), polytunnel (batch 4) and field (batch 5). Black circles indicate the variety was not grown in a particular batch.

4.3.2 Effect of biofumigants on mycelial growth of pea foot rot complex pathogens

4.3.2.1 Experiment 1

The effects of different milled oven-dried biofumigant varieties (Batch 1, Table 4.1) on mycelial growth of FO, FSP, DP and AE were examined in an *in-vitro* system.

For FO, the mean growth rate of untreated control colonies was 5.4 mm day^{-1}. Only colonies treated with the two *B. juncea* varieties 'Pacific Gold' and 'Caliente 99' had significantly lower mean growth rates, at 0.1 mm day^{-1} ($p < 0.001$) and no growth ($p < 0.001$) respectively (Figure 4.6a). The corresponding mean percentage inhibition compared to the control were 99.9 % and 100 % respectively (Figure 4.7a). Colonies treated with *R. sativus* 'Terranova' had a greater mean growth rate compared to the control, at 5.8 mm day^{-1}, although this was not significant. Both 'Terranova' and *E. sativa* 'Nemat' had negative percentage inhibition values of -8.3 % and -0.6 % respectively.

For FSP, the mean growth rate of untreated control colonies was 3.8 mm day^{-1}. Four of the five biofumigant treatments resulted in significantly lower mean growth rates compared to the control (Figure 4.6b; Figure 4.7b); 'Nemat' at 2.9 mm day^{-1} ($p = 0.014$) with an inhibition of 23.3 %, *S. alba* 'Brisant' at 2.7 mm day^{-1} ($p = 0.001$) with an inhibition of 30.6 %, 'Pacific Gold' at 0.1 mm day^{-1} ($p < 0.001$) with an inhibition of 96.2 % and 'Caliente 99', which resulted in no growth of colonies ($p < 0.001$) and 100 % inhibition compared to the control.

For DP, the mean growth rate of untreated control colonies was 6.0 mm day^{-1}. The same four biofumigant treatments as FSP resulted in significantly lower mean growth rates compared to the control; 'Nemat' at 0.2 mm day^{-1} ($p < 0.001$) with an inhibition of 96.6 %, 'Brisant' at 0.7 mm day^{-1} ($p < 0.001$) with an inhibition of 90.0 %, with both 'Pacific Gold' and 'Caliente 99' resulting in no growth of colonies ($p < 0.001$) and 100 % inhibition compared to the control (Figure 4.6c, Figure 4.7c).

For AE, the mean growth rate of untreated control colonies was 16.1 mm day^{-1}. All five biofumigant treatments resulted in significantly lower mean growth rates compared to the control ($p < 0.001$). 'Terranova' had a mean growth rate of 2.2 mm day^{-1} with an inhibition of 86.6 %. All other treatments resulted in no growth of colonies and 100 % inhibition compared to the control (Figure 4.6d, Figure 4.7d).

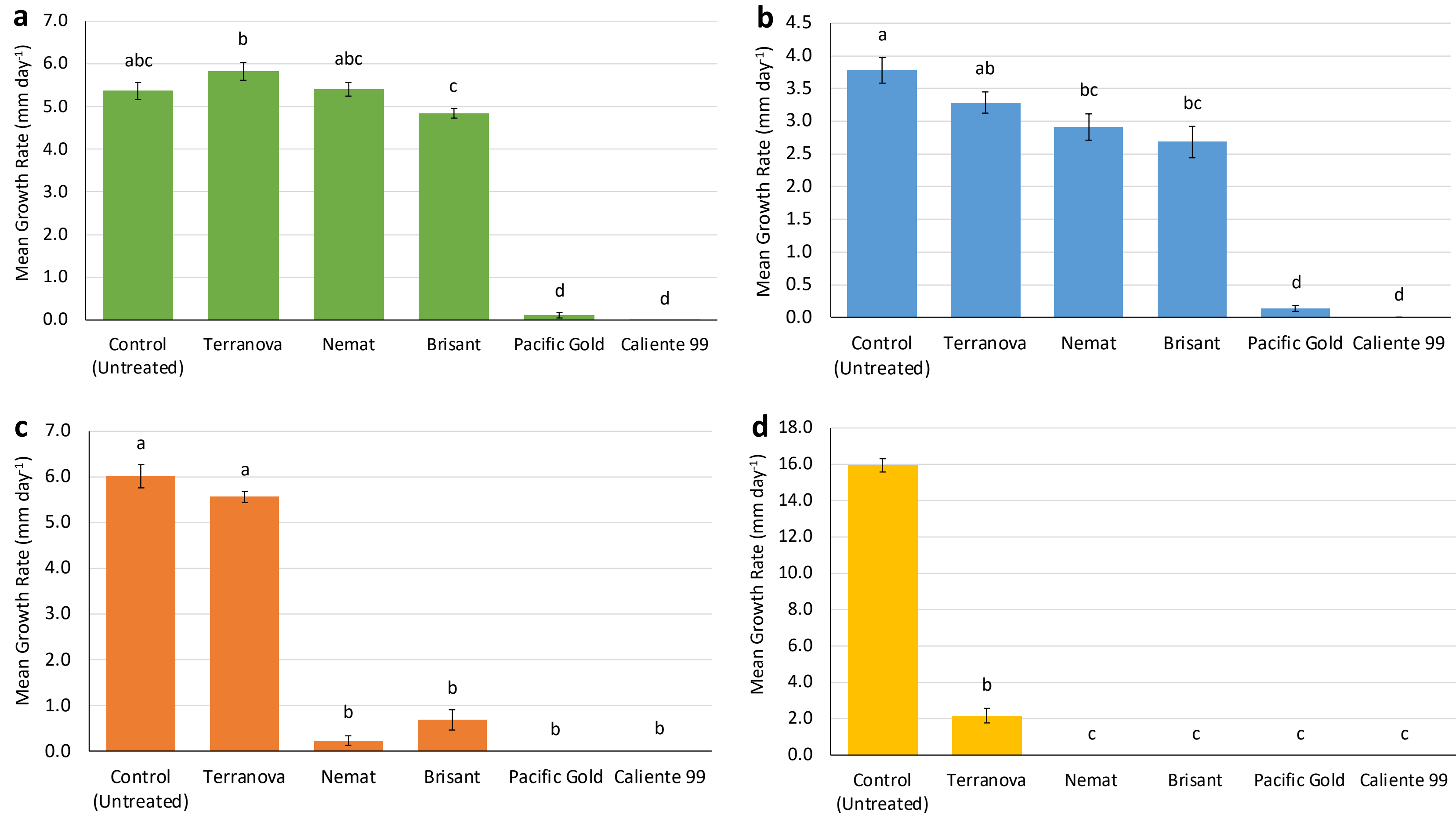

Figure 4.6. Effect of different biofumigant plant varieties on mean growth rates of a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13', c) *Didymella pinodella* '61B' and d) *Aphanomyces euteiches* 'Burton' colonies grown on PDA in Experiment 1. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

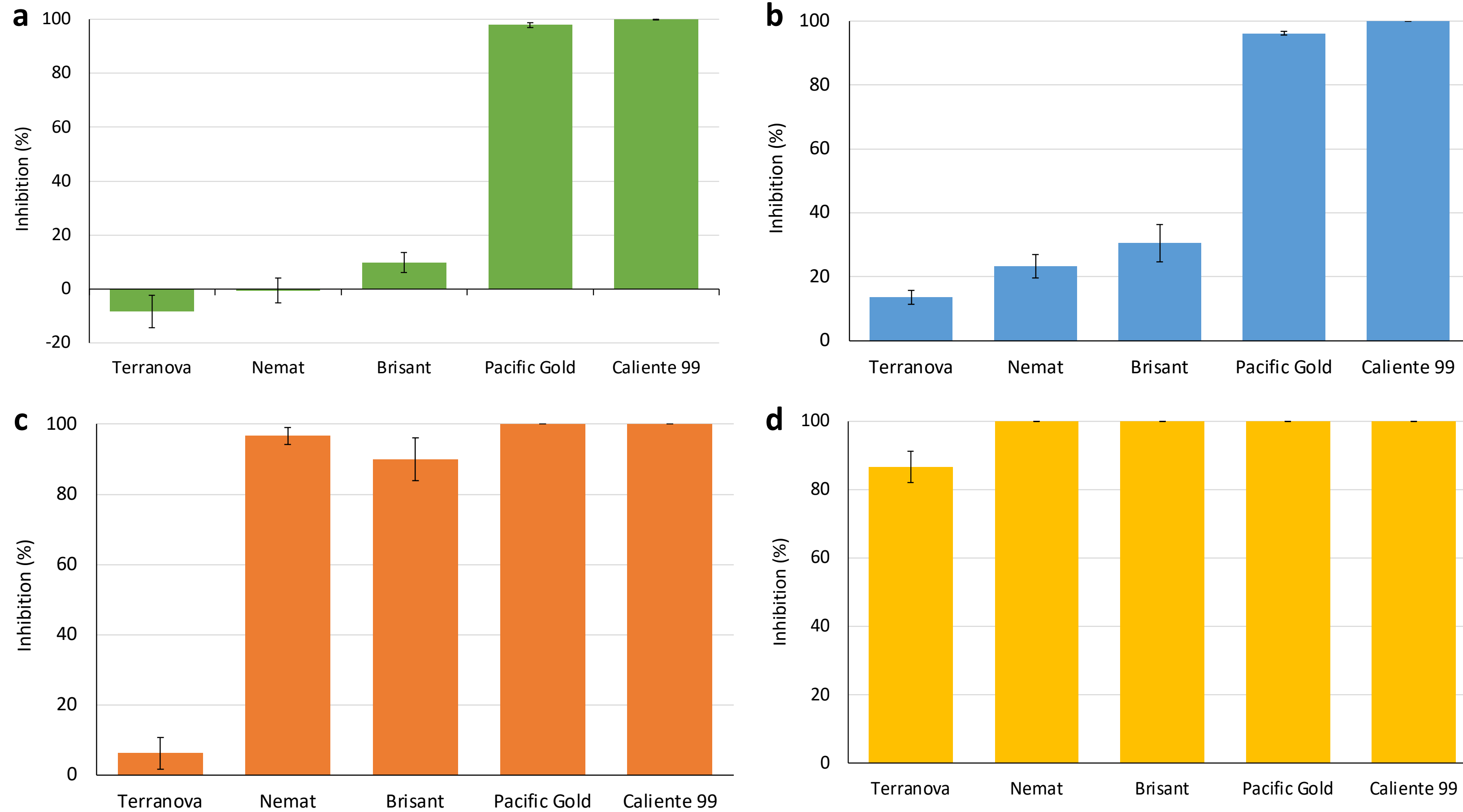

Figure 4.7. Effect of different biofumigant plant varieties on percentage inhibition compared to the control of a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13', c) *Didymella pinodella* '61B' and d) *Aphanomyces euteiches* 'Burton' colonies grown on PDA in Experiment 1. Error bars = ± SEM.

4.3.2.2 Experiment 2

The effects of different milled oven-dried biofumigant varieties of batch 2 (Table 4.1) on mycelial growth of FO, FSP and DP were examined *in-vitro*.

For FO, the mean growth rate of untreated control colonies was 9.0 mm day^{-1}. All eight treatments tested resulted in significantly lower mean colony growth rates compared to the control. Colonies treated with *B. juncea* varieties 'Caliente 199' and 'Caliente Rojo' resulted in the lowest mean growth rates at 6.6 mm day^{-1} ($p < 0.001$) and 6.7 mm day^{-1} ($p < 0.001$) respectively. The corresponding mean percentage inhibitions compared to the control were 26.1 % and 25.7 % respectively. *E. sativa* 'Trio' had the third lowest mean growth rate, at 7.1 mm day^{-1} ($p < 0.001$) with an inhibition of 20.4% (Figure 4.8a, Figure 4.9a).

For FSP, the mean growth rate of untreated control colonies was 4.9 mm day-1. Only two of the eight biofumigant treatments resulted in significantly lower mean growth rates compared to the control; 'Caliente 199' at 3.5 mm day^{-1} ($p = 0.002$) with an inhibition of 27.1 % and 'Trio' at 3.5 mm day^{-1} ($p = 0.002$) with an inhibition of 28.1 % (Figure 4.8b, Figure 4.9b).

For DP, the mean growth rate of untreated control colonies was 7.0 mm day^{-1}. Like FO, all eight treatments tested resulted in significantly lower mean colony growth rates compared to the control. 'Trio' had the lowest mean growth rate at 2.4 mm day^{-1} ($p < 0.001$) with an inhibition of 65.5 %. 'Caliente 199' and 'Caliente Rojo' had second and third lowest mean growth rates at 2.4 mm day^{-1} ($p < 0.001$) and 2.6 mm day^{-1} ($p < 0.001$) respectively. The corresponding inhibitions were 65.5 % and 63.6 % respectively (Figure 4.8c, Figure 4.9c).

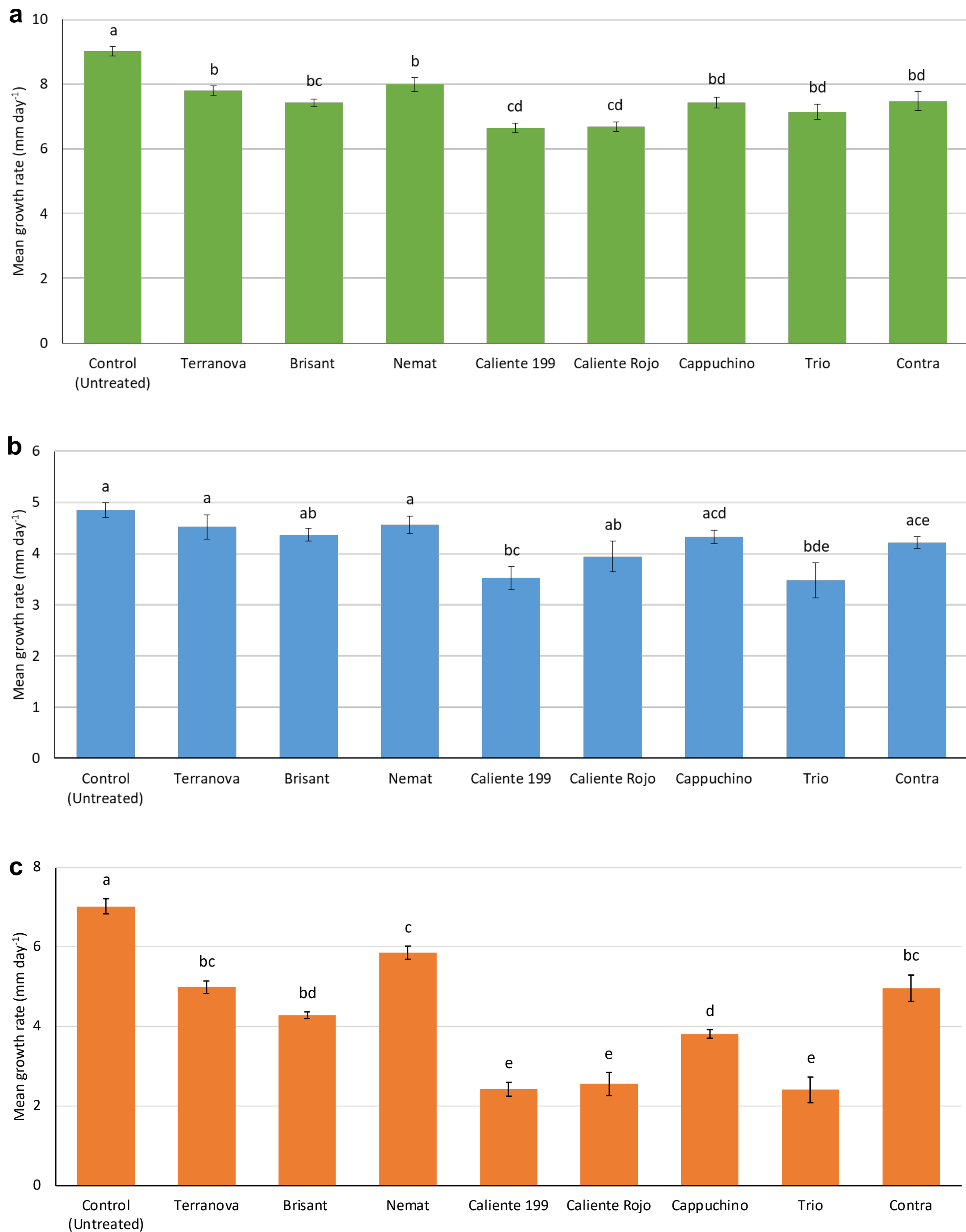

Figure 4.8. Effect of different biofumigant plant varieties on mean growth rates of a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13' and c) *Didymella pinodella* '61B' colonies grown on PDA in Experiment 2. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

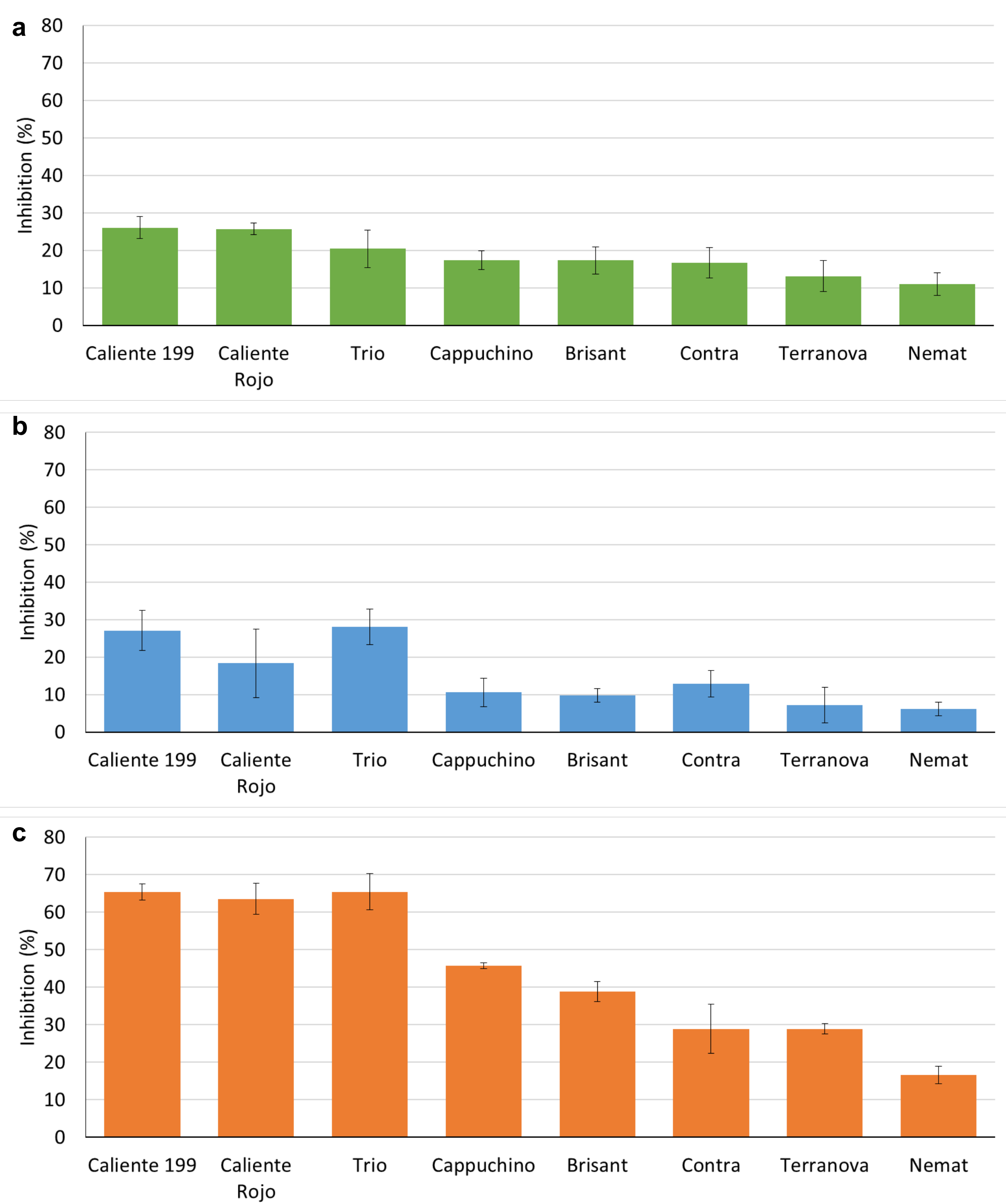

Figure 4.9. Effect of different biofumigant plant varieties on percentage inhibition of mycelial growth compared to the control of a) *Fusarium oxysporum* 'PG18', b) *Fusarium solani* f. sp. *pisi* 'PG13' and c) *Didymella pinodella* '61B' colonies grown on PDA in Experiment 2. Error bars = ± SEM.

4.3.3 Effect of biofumigants on spore germination of pea foot rot complex pathogens

The effects of different milled oven-dried biofumigant crop varieties of batch 2 (Table 4.1) on spore germination of FO and FSP was examined *in vitro*. Pathogen CFUs were quantified both immediately after a one-week exposure to the biofumigant and a further 48 hours after removal of the biofumigant, to examine whether biofumigants had killed spores or only suppressed germination. Experiments with DP were not carried out due to inconsistent spore germination from spore suspensions made from different DP plates (data not shown).

For FO, the mean CFU count of plates of the untreated control was 66 after the 48-hour exposure. *S. alba* ‘Brisant’ resulted in significantly lower mean CFU counts after the 48-hour exposure with a count of 46 (p = 0.021) and a corresponding mean percentage inhibition compared to the control of 30.0 %. The mean CFU count of plates of the untreated control 48-hours after removal of biofumigant material was 65. None of the five other treatments tested resulted in significantly lower mean CFU counts 48-hours after removal of the biofumigant material, with plates initially exposed to ‘Brisant’ with the lowest mean CFU count of 50, with a corresponding mean percentage inhibition compared to the control of 23.5 % (Figure 4.10a, Figure 4.11a).

For FSP, the mean CFU count of plates of the untreated control was 47 after the 48-hour exposure. *E. sativa* ‘Trio’ resulted in significantly lower mean CFU counts after the 48-hour exposure, with a count of 21 (p = 0.016) and a corresponding mean percentage inhibition compared to the control of 58.9 %. The mean CFU count of plates of the untreated control 48-hours after removal of biofumigant material was 48. None of the other five treatments tested resulted in significantly lower mean CFU counts 48-hours after removal of the biofumigant material, with plates initially exposed to *B. juncea* ‘Caliente Rojo’ with the lowest mean CFU count of 37, with a corresponding mean percentage inhibition compared to the control of 31.1 %. Plates initially exposed to ‘Trio’ had a mean CFU count of 40, with a corresponding mean percentage inhibition compared to the control of 15.3 % (Figure 4.10b, Figure 4.11b).

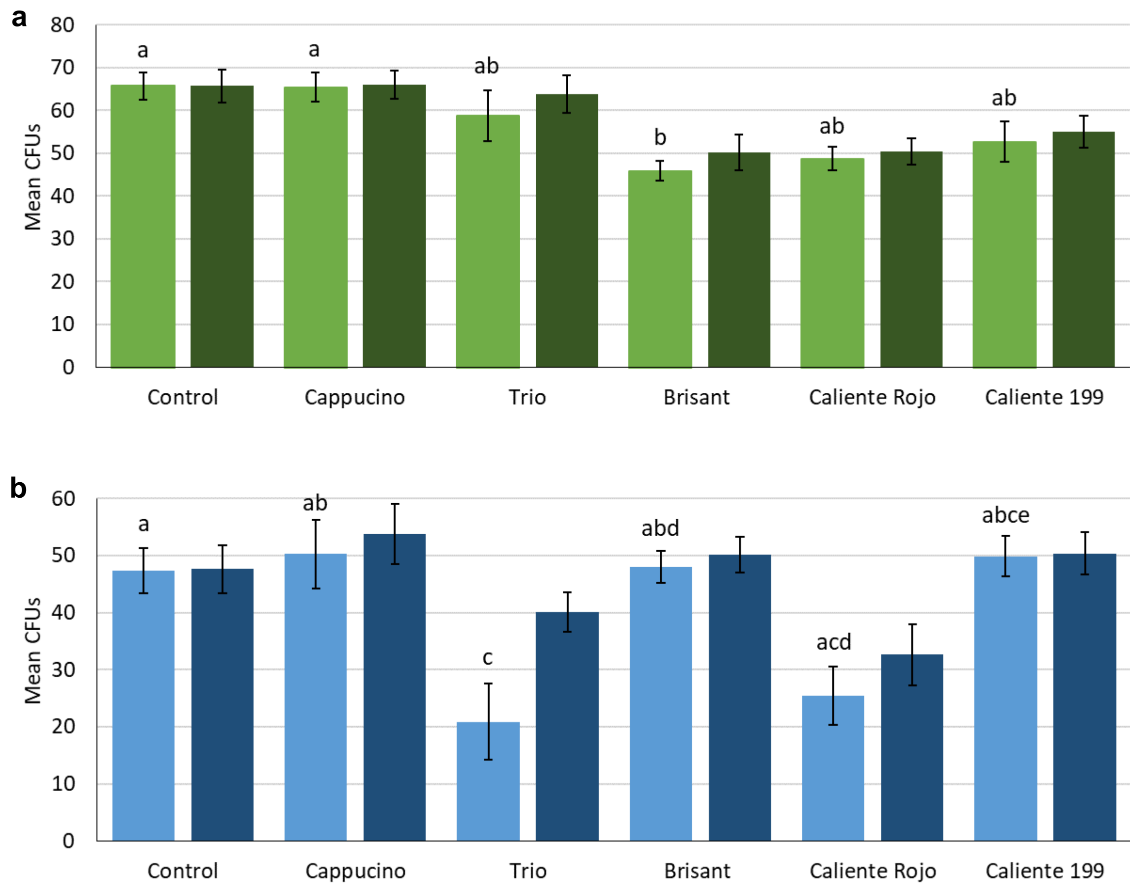

Figure 4.10. Effect of different biofumigant plant varieties on spore germination of a) *Fusarium oxysporum* 'PG18' and b) *Fusarium solani* f. sp. *pisi* 'PG13' colony forming units on PDA. Lighter bars represent inhibition immediately after a one-week exposure to the biofumigant. Darker bars represent inhibition at 48 hours after removal of the biofumigant. Differing letters between bars indicate significant differences ($p < 0.05$). Error bars = ± SEM.

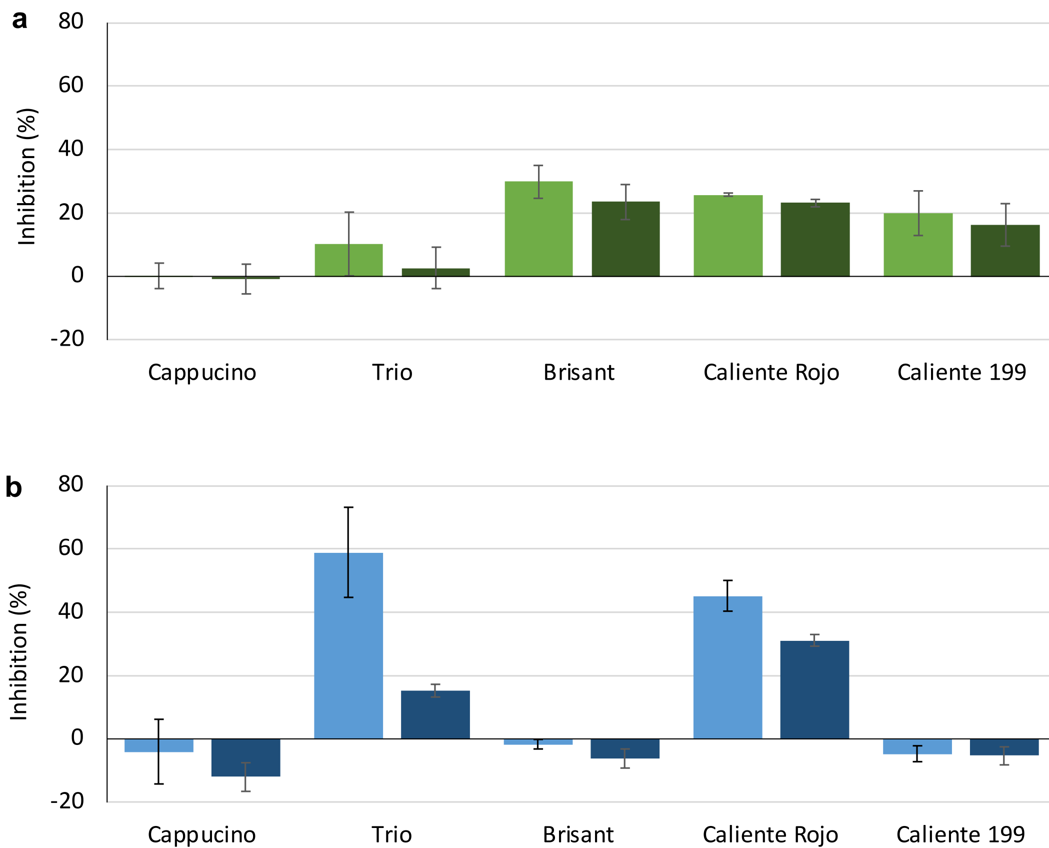

Figure 4.11. Effect of different biofumigant plant varieties on percentage inhibition of spore germination compared to the control of a) *Fusarium oxysporum* 'PG18' and b) *Fusarium solani* f. sp. *pisi* 'PG13' colony forming units on PDA. Lighter bars represent inhibition immediately after a one-week exposure to the biofumigant. Darker bars represent inhibition at 48 hours after removal of the biofumigant. Error bars = ± SEM.

4.3.4 Determining the effect of biofumigants on suppression of pea foot rot disease caused by *Didymella pinodella* in a pot-based system

A pot-based experiment was designed to examine the effects of different varieties of biofumigant plants on DP foot rot disease development and mortality of pea plants in the glasshouse. Several indices of foot rot severity and plant health were examined; emergence, leaf wilt (during experiment), root browning, flower and pod number, presence/absence of DP stem girdling and presence of nodules (at harvest). Fresh and dry weights of shoot and root material was also measured. ANOVA and GLM based analyses were undertaken to calculate significant differences between certain pairs of treatments (Table A 6, Table A 7, Table A 8). The GLM for percentage of pea

roots with nodules determined that there was a significant position effect for 'row' (Table A 8), despite randomisation to avoid effects of being closer to light sources or air conditioning within the glasshouse compartment for example. Therefore, further statistical analysis may have been confounded.

4.3.4.1 Plant emergence

The mean percentage of pea plants that emerged for the control treatment (no biofumigant, no DP inoculum) was 87.5 % (Figure 4.13a). None of the uninoculated treatments amended with biofumigant resulted in significantly different pea plant emergences to the non-inoculated control containing no biofumigant (NB). For substrates inoculated with $1x10^4$ DP spores g^{-1} (Figure 4.13b), the NB treatment had an emergence of 75.0 %, which was lower than the uninoculated control treatment containing no biofumigant, but this was not significantly different. Plants grown in substrate with the addition of *B. juncea* 'Caliente 199' had significantly increased emergence compared to the NB treatment of 95.8 % ($p = 0.019$). For substrates inoculated with $5x10^5$ DP spores g^{-1} (Figure 4.13c), plants of the NB treatment had an emergence of 70.8 %, lower than the uninoculated NB control treatment, but this difference was not significant. All treatments where the substrate had an addition of a biofumigant resulted in an increased emergence compared to the NB treatment, with the treatment *E. sativa* 'Trio' having a mean emergence of 91.7 %, which was the only significantly different treatment compared to the NB treatment ($p = 0.020$).

4.3.4.2 Leaf wilt

All inoculated treatments resulted in a general increase in mean DP leaf wilt score over the duration of the experiment (Figure 4.12). At the end of the experiment, the uninoculated NB control treatment had a mean leaf wilt score of 1.68. Plants grown in non-inoculated substrate with the addition of 'Trio' had a significantly decreased mean wilt score compared to the control of 1.02 ($p < 0.05$, Figure 4.14a). For substrates inoculated with $1x10^4$ DP spores g^{-1} (Figure 4.14b), the NB treatment had a score of 1.60, a slight decrease compared to the uninoculated NB control treatment which was not significantly different. Treatment with 'Trio' resulted in a significantly decreased wilt score compared to the NB treatment of 1.08 ($p < 0.05$). For substrates inoculated with $5x10^5$ DP spores g^{-1} (Figure 4.14c), plants of the NB treatment had a

mean leaf wilt score of 2.75, a significant increase compared to the uninoculated NB control ($p < 0.01$). All treatments where the substrate had an addition of a biofumigant had a lower DP leaf wilt score compared to the NB treatment, however only 'Trio' was significantly different with a score of 0.93 ($p < 0.001$).

Figure 4.12. Different levels of leaf wilt of pea plants seen in the glasshouse experiment examining the effect of biofumigant plant varieties tested at different inoculum concentrations of *Didymella pinodella* '61B'. Leaf wilt score of each plant (left to right): 0, 1, 2, 4.

4.3.4.3 Root rot

The mean DP root rot score for the uninoculated NB control treatment assessed at harvest was 0.50 (Figure 4.15). Plants of all three biofumigant treatments grown in non-inoculated substrate showed an increase in root discolouration compared to the control, however only 'Cappuchino' resulted in a significant increase, having a score of 1.67 ($p < 0.01$). For substrates inoculated with 1×10^4 DP spores g^{-1}, the NB treatment had a score of 1.35, an increase compared to the uninoculated NB control treatment. Plants grown in substrate with the addition of 'Trio' had the lowest root rot

score compared to the NB treatment of the three biofumigant amended treatments at 0.9; however, this was not significant. For substrates inoculated with $5x10^5$ DP spores g^{-1}, plants of the NB treatment had a significantly increased mean root rot score of 2.81 compared to the uninoculated NB control ($p < 0.001$). All treatments where the substrate had an addition of a biofumigant had a significantly lower root rot score compared to the NB treatment, with treatments containing 'Caliente 199', 'Cappuchino' and 'Trio' having scores of 1.75 ($p < 0.05$), 0.75 ($p < 0.001$) and 0.70 ($p < 0.001$) respectively.

4.3.4.4 Plant mortality

There was no mortality for plants within the uninoculated NB control treatment (Figure 4.16). Plant mortality occurred in all treatments where the non-inoculated substrate had an addition of a biofumigant, however none of these values were significantly different to the control. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment had a plant mortality of 11.76 %, an increase compared to the uninoculated control treatment although this was not significant. All treatments where the substrate had an addition of a biofumigant had lower plant mortality rate compared to the NB treatment, with 'Trio' and 'Cappuchino' with no pea plant mortality, although these were not significantly different. For substrates inoculated with $5x10^5$ DP spores g^{-1}, plants of the NB treatment had a mortality of 37.5 %, a significant increase compared to the uninoculated NB control ($p < 0.001$). All treatments where the substrate had an addition of a biofumigant had significantly reduced plant mortality compared to the NB treatment, with 'Caliente 199' resulting in a plant mortality of 15.00 % ($p < 0.01$) while there was no plant mortality for 'Trio' and 'Cappuchino' ($p < 0.001$).

4.3.4.5 Combined pod and flower number

Mean pod and flower number (Figure 4.17) for pea plants of the uninoculated NB control treatment was 1.88. Plants grown in non-inoculated substrate with the addition of 'Trio' and 'Cappuchino' had decreased pod and flower numbers compared to the control of 1.79 and 1.22 respectively; however, these differences were not significant. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment had a mean pod and flower number of 1.29, a decrease compared to the uninoculated NB control treatment, but this was not significantly different. All treatments where the substrate

had an addition of a biofumigant had a higher mean flower and pod number compared to the NB treatment, with treatments containing 'Trio', 'Caliente 199' and 'Cappuchino' having mean numbers of 1.88, 1.77 and 1.46 respectively but none of these values were significantly different to the NB treatment. For substrates inoculated with $5x10^5$ DP spores g^{-1}, plants of the NB treatment had a mean number of 1.13, significantly lower than the uninoculated NB control ($p < 0.05$). All treatments where the substrate had an addition of a biofumigant resulted in a higher flower and pod number compared to the NB treatment, with treatments containing 'Trio' and 'Cappuchino' having significantly greater numbers at 2.48 ($p < 0.001$) and 2.00 ($p < 0.01$).

4.3.4.6 Dry root weight

Mean pea dry root weight for the uninoculated NB control treatment was 0.26 g (Figure 4.18). Non-inoculated substrate treatments with the addition of biofumigant material all had significantly reduced root weights compared to the uninoculated control, with 'Trio', 'Caliente 199' and 'Cappuchino' having mean dry root weights of 0.17 g ($p < 0.01$), 0.15 g ($p < 0.001$) and 0.13 g ($p < 0.001$) respectively. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment had a mean weight of 0.17 g, significantly lower than the uninoculated NB control ($p < 0.01$). 'Caliente 199' had a slightly higher mean root weight at 0.18 g but this was not significant. For substrates inoculated with $5x10^5$ DP spores g^{-1}, plants of the NB treatment had a mean weight of 0.12 g, significantly lower than the uninoculated NB control ($p < 0.001$). All treatments where the inoculated substrate had an addition of a biofumigant had a higher root weight compared to the NB treatment, with treatments containing 'Trio' and 'Cappuchino' significantly different at 0.21 g ($p < 0.01$) and 0.19 g ($p < 0.01$) respectively.

4.3.4.7 Dry shoot weight

Mean pea dry shoot weight for the uninoculated NB control treatment was 1.07 g (Figure 4.19). Non-inoculated substrate treatments with the addition of biofumigant material all had reduced dry shoot weight compared to the uninoculated NB control treatment and this was significant for 'Caliente 199' and 'Cappuchino' with values of 0.67 g ($p < 0.01$) and 0.42 g ($p < 0.001$) respectively. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment had a mean dry shoot weight of 0.66 g, significantly lower than the uninoculated NB control ($p < 0.01$). Both treatments with

additional biofumigant material, ‘Caliente 199’ and ‘Trio’ had greater mean weights than the NB treatment at 0.76 g and 0.72 g respectively, but these values were not significant. For substrates inoculated with $5x10^5$ DP spores g^{-1}, plants of the NB treatment had a mean shoot weight of 0.46 g, significantly lower than the uninoculated NB control ($p < 0.001$). All treatments with addition of a biofumigant had a greater mean dry shoot weight compared to the NB treatment, with the treatment ‘Trio’ significantly different at 1.16 g ($p < 0.001$).

4.3.4.8 DP stem girdling

No pea plants pea displaying DP stem girdling symptoms in the uninoculated NB control treatment (Figure 4.20). All non-inoculated substrate treatments with the addition of biofumigant material resulted in a significantly higher percentage of plants displaying DP girdling symptoms, with ‘Cappuchino’, ‘Caliente 199’ and ‘Trio’ having a percentage DP girdling of 73.33 % ($p < 0.001$), 26.32 % ($p < 0.05$), and 23.53 % ($p < 0.05$) respectively. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment had a mean percentage DP girdling of 76.47 %, a significant increase compared to the uninoculated NB control ($p < 0.001$). All treatments with additional biofumigant material resulted in reduced percentage DP girdling compared to the NB treatment, with ‘Caliente 199’ and ‘Cappuchino’ significantly different at 33.33 % ($p < 0.01$) and 29.41 % ($p < 0.01$) respectively. For substrates inoculated with $5x10^5$ DP spores g^{-1}, 100 % of plants of the NB treatment displayed DP girdling symptoms, significantly higher than the uninoculated NB control ($p < 0.001$). All biofumigant treatments resulted in a lower percentage of plants displaying DP girdling, with treatments containing ‘Trio’ and ‘Cappuchino’ significantly lower at 60.00 % ($p < 0.001$) and 50.00 % ($p < 0.001$) respectively.

4.3.4.9 Nodule formation

The percentage of pea roots with percentage of pea plants whose roots developed nodules was 40.00 % for the uninoculated NB control treatment (Figure 4.21). Plants grown in the non-inoculated substrate with the addition of biofumigant material all showed a reduction in nodulation compared to the control, with pea plants within the ‘Trio and ‘Cappuchino’ treatments having no nodules. For substrates inoculated with $1x10^4$ DP spores g^{-1}, the NB treatment resulted in 47.06 % of plants forming nodules,

an increase compared to the uninoculated NB control. Plants grown in substrate with the addition of biofumigant material all showed a reduction in nodulation compared to the NB treatment, with no plants developing nodules in the ‘Cappuchino’ treatment. For substrates inoculated with $5x10^5$ DP spores g^{-1}, 31.25 % of plants of the NB treatment had formed nodules, a decrease compared to the uninoculated NB control. Plants grown in substrate with the addition of biofumigant material all showed a reduction in nodulation compared to the NB treatment, with no plants developing nodules in the ‘Cappuchino’ and ‘Caliente 199’ treatments.

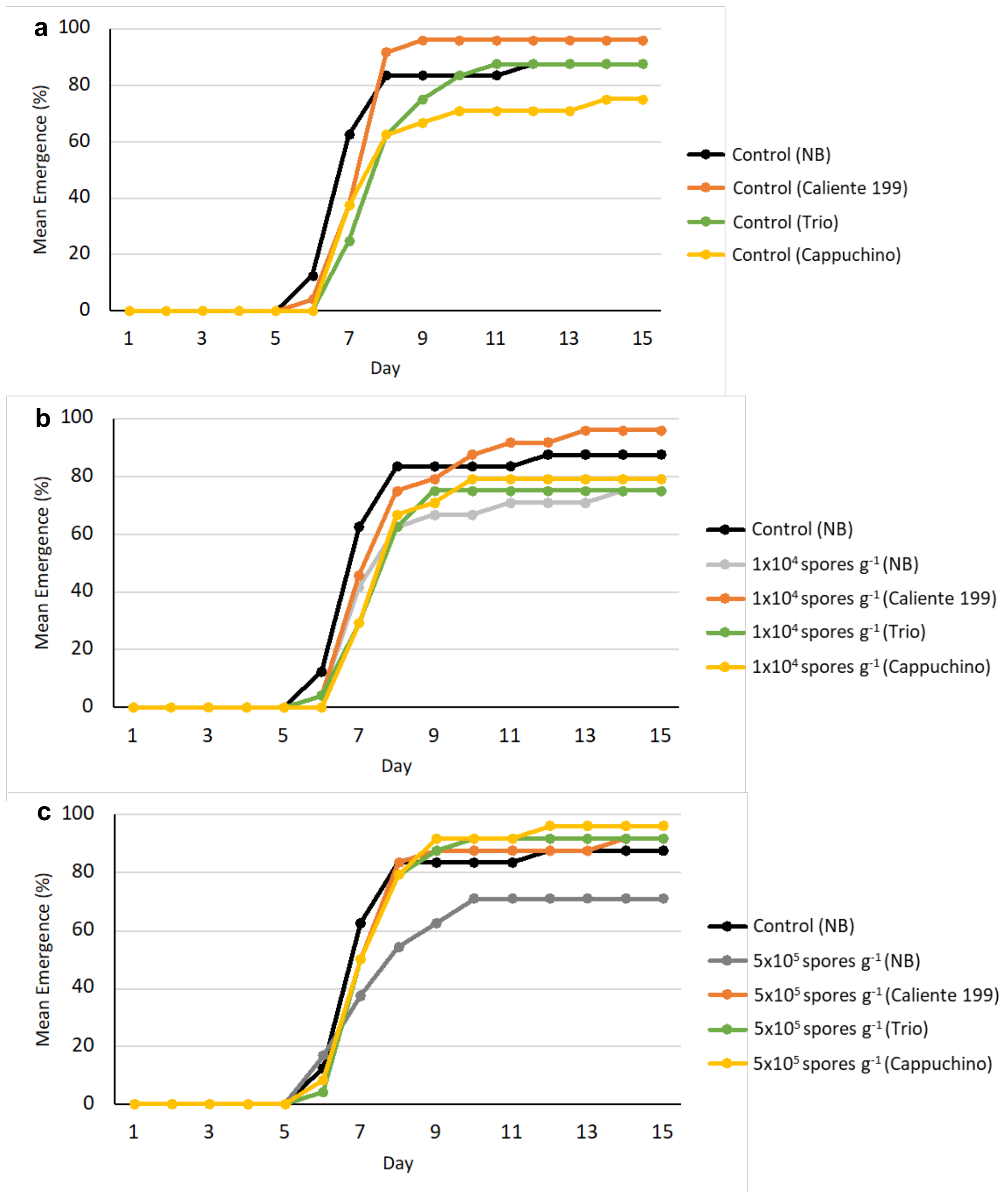

Figure 4.13. Effect of biofumigant plant species and varieties tested at different inoculum concentrations of *Didymella pinodella* '61B' on emergence of pea plants. a) All treatments with uninoculated substrate, b) Uninoculated control treatment (no biofumigant) and all treatments with substrate inoculated at $1x10^4$ spores g^{-1} and c) Uninoculated control treatment (no incorporated biofumigant) and all treatments with substrate inoculated at $5x10^5$ spores g^{-1}. NB = No biofumigant treatment.

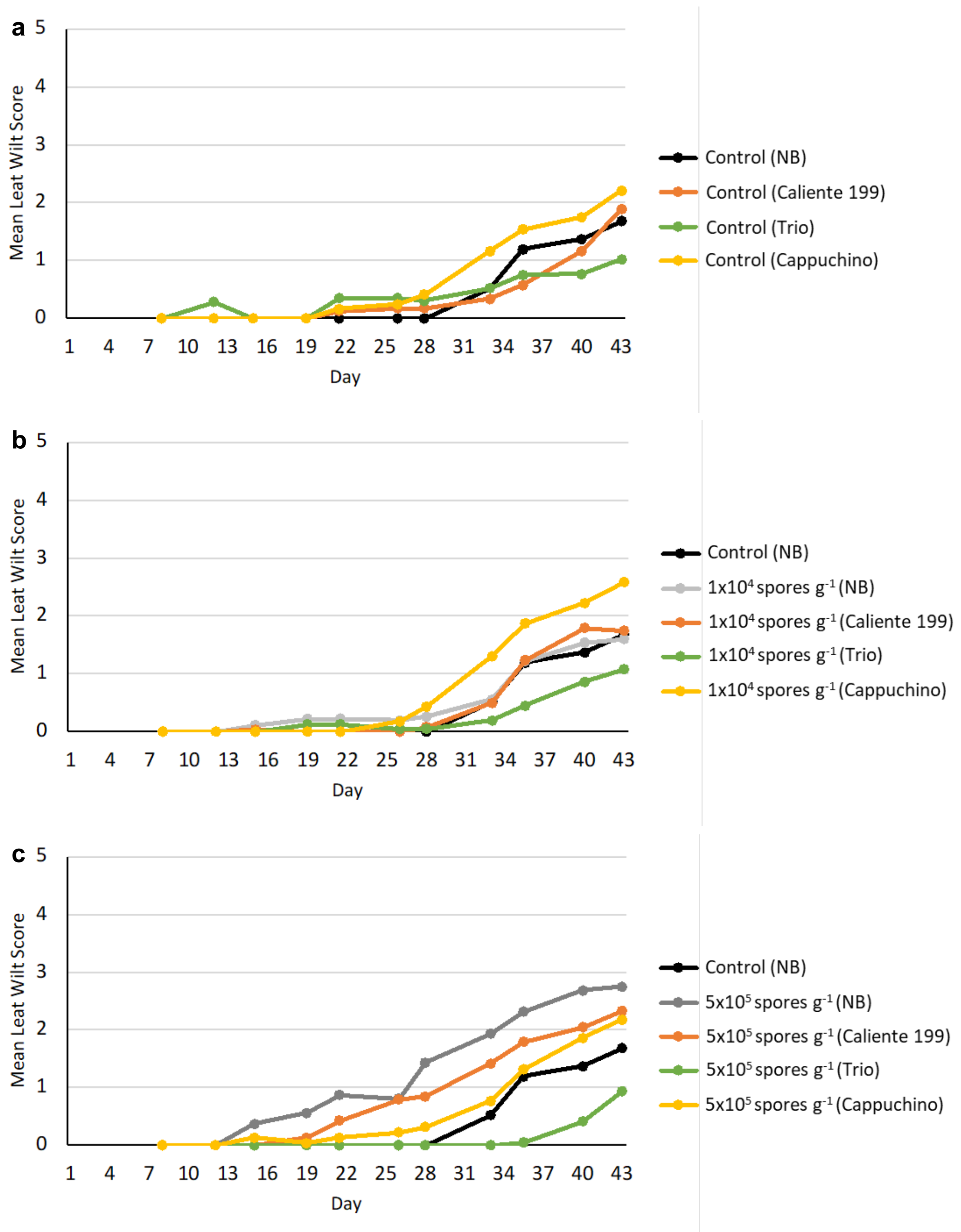

Figure 4.14. Effect of biofumigant plant species and varieties tested at different inoculum concentrations of *Didymella pinodella* '61B' on leaf wilt of pea plants over time. a) All treatments with uninoculated substrate, b) Uninoculated control treatment (no biofumigant) and all treatments with substrate inoculated at $1x10^4$ spores g^{-1} and c) Uninoculated control treatment (no incorporated biofumigant) and all treatments with substrate inoculated at $5x10^5$ spores g^{-1}. NB = No biofumigant treatment.

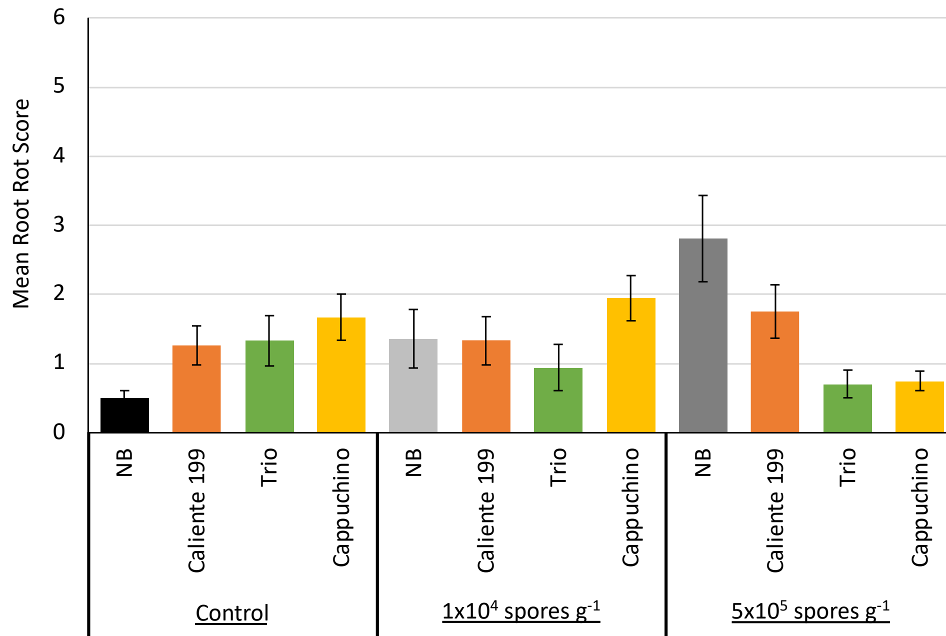

Figure 4.15. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* ‘61B’ on root rot of pea plants over time. NB = No biofumigant treatment. Error bars = ± SEM.

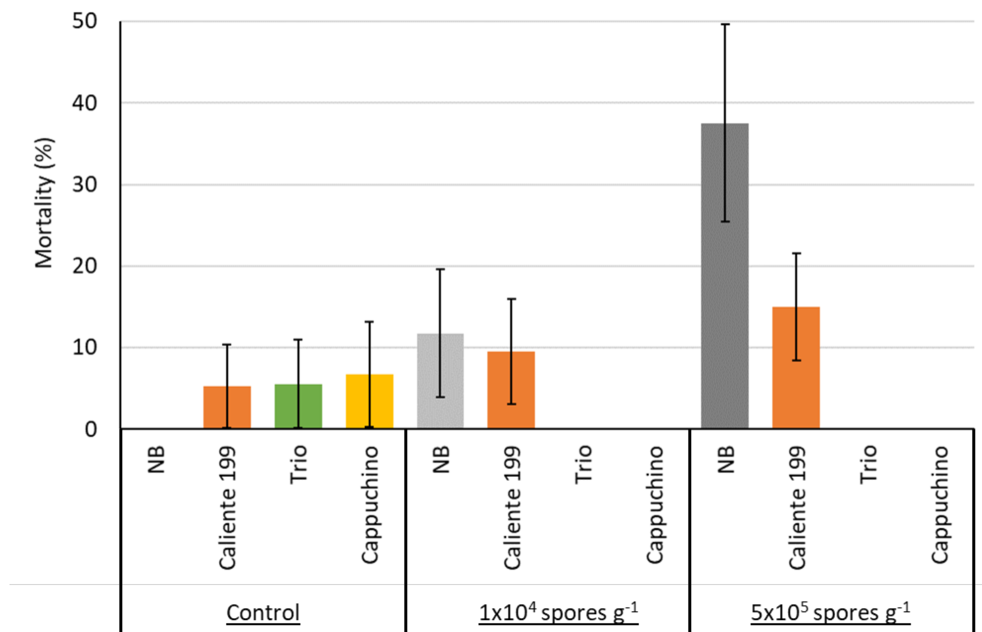

Figure 4.16. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* ‘61B’ on pea plant mortality. NB = No biofumigant treatment. Error bars = ± SEM.

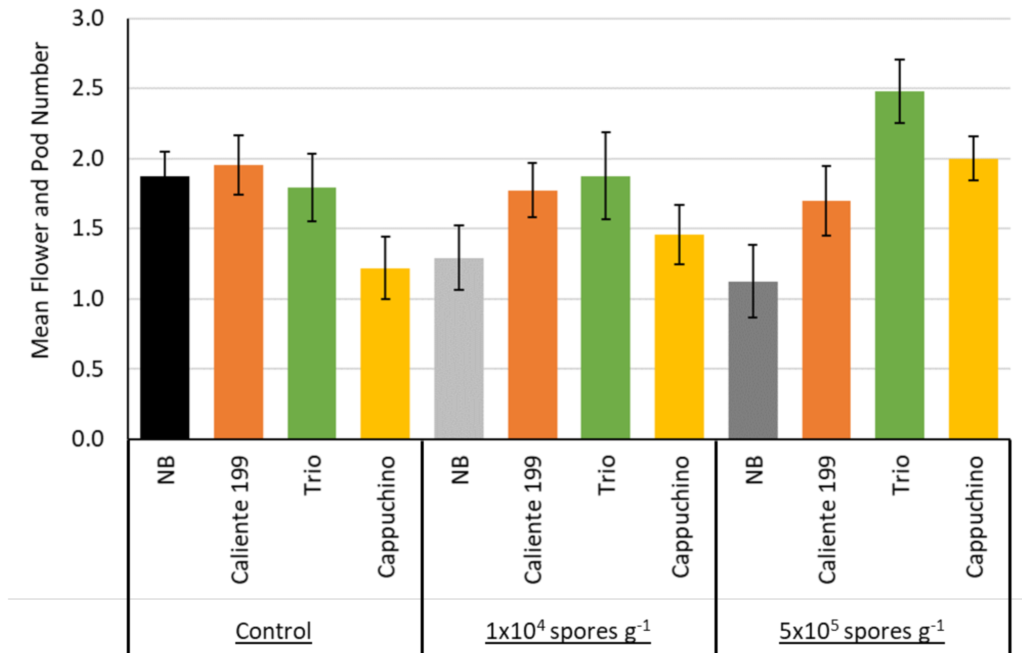

Figure 4.17. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* '61B' on combined flower and pod number of pea plants. NB = No biofumigant treatment. Error bars = ± SEM.

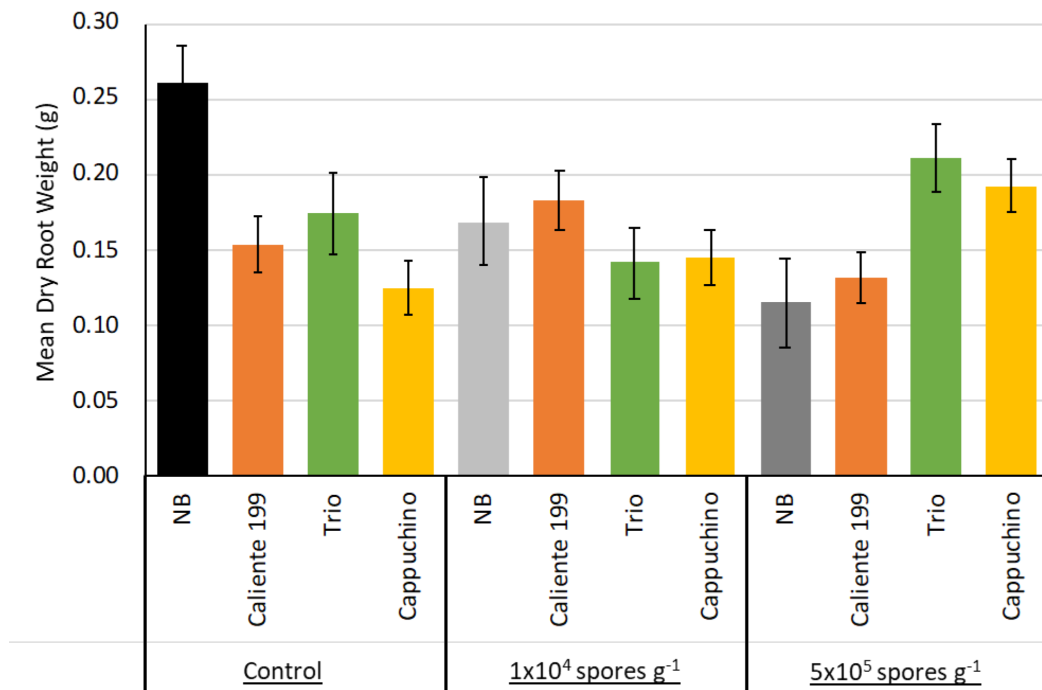

Figure 4.18. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* '61B' on mean dry shoot yield weight. NB = No biofumigant treatment. Error bars = ± SEM.

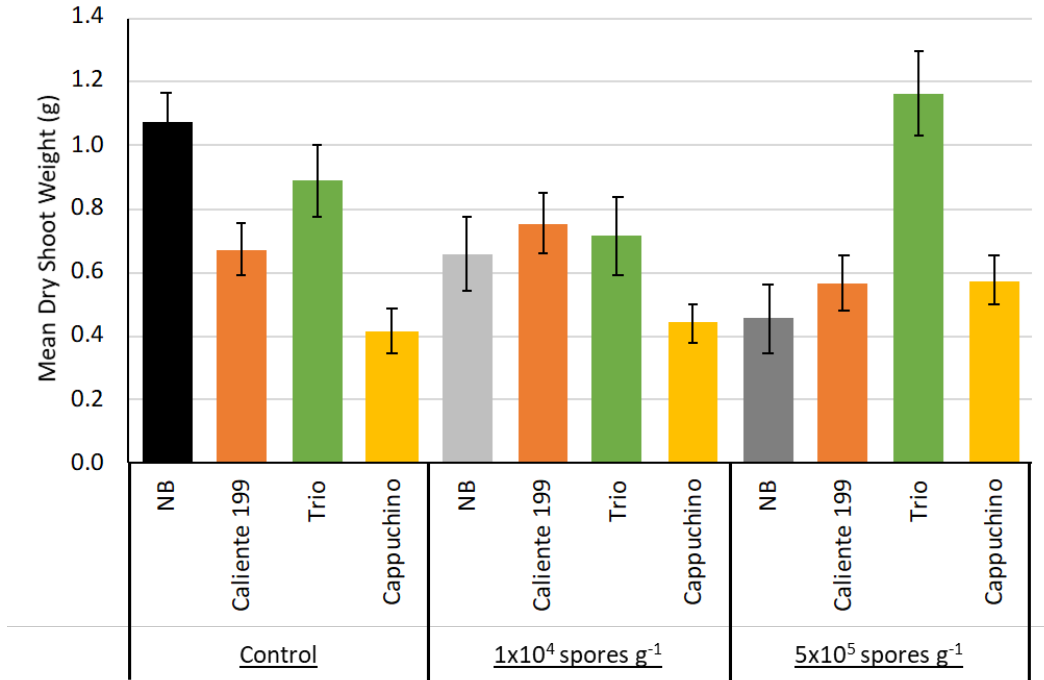

Figure 4.19. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* ‘61B’ on mean dry root yield mass. NB = No biofumigant treatment. Error bars = ± SEM.

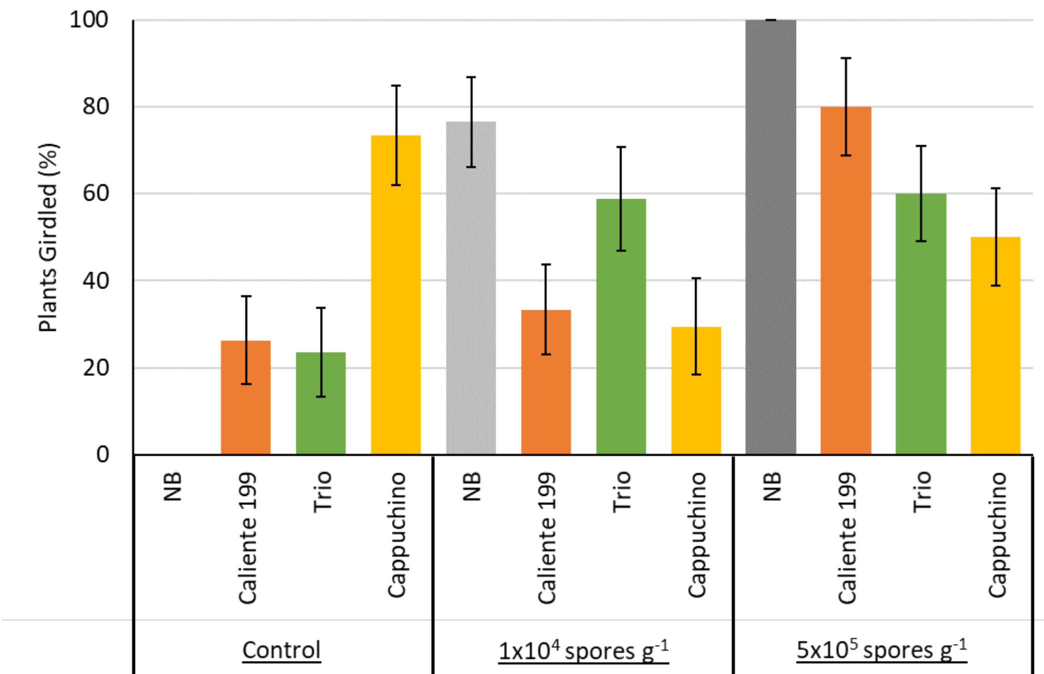

Figure 4.20. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* ‘61B’ on pea plant stem girdling. NB = No biofumigant treatment. Error bars = ± SEM.

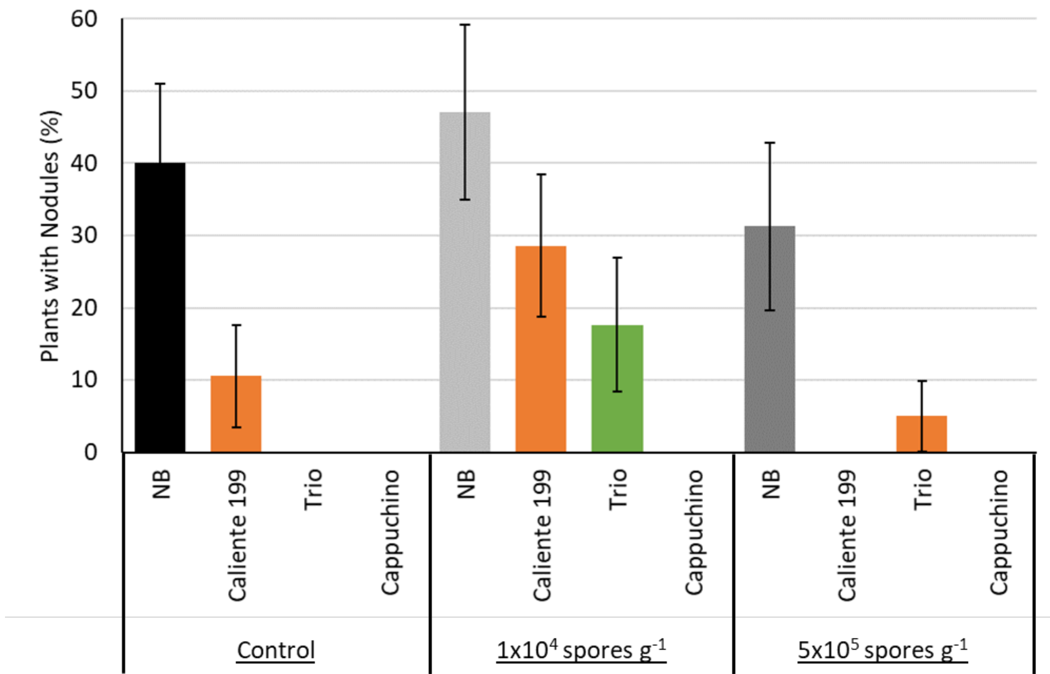

Figure 4.21. Effect of different biofumigant plant species and varieties tested at multiple inoculum concentrations of *Didymella pinodella* '61B' on pea plant root nodulation. NB = No biofumigant treatment. Error bars = ± SEM.

4.4 Discussion

There is a lack of research concerning the potential of biofumigation against the causal agents of the PFRC, particularly FO, FSP and DP. However, the research of this chapter has for the first time identified biofumigant crop species and varieties effective against multiple PFRC pathogens of interest in the UK, particularly against mycelial growth *in-vitro*. The glasshouse experiment identified biofumigant varieties that were significantly effective in reducing root rot score and increasing dry shoot and root weights compared to the non-biofumigant control at the higher DP inoculum concentration ($5x10^5$ spores g^{-1}). These results therefore enhance the knowledge of potential management strategies, particularly against DP, a relatively understudied pathogen of the PFRC and thought to be the main causal agent of PFR in the UK.

A number of different biofumigant crops were grown, including varieties of *B. juncea*, *B. carinata*, *S. alba, R. sativus* and *E. sativa* in different batches over different years and locations which included glasshouse, polytunnel and field. Whilst all biofumigant samples from these different growing conditions had detectable levels of primary GSLs following HPLC analyses, these were lower than expected for batches 2, 3, 4 and 5. Although several of the varieties in these batches were the same species as those grown in batch 1, some were different and therefore cannot be compared directly. For instance, some previous varieties had been updated (e.g., *B. juncea* 'Caliente 199') or they were no longer commercially available. In addition, the chromatograms did not detect significant peaks for any other GSLs that may have been present. A glucoerucin standard was not used due to issues with breakdown during HPLC in previous analyses (Andrew Jukes, personal communication). The reason for this variation is that some growth conditions are more favourable for biomass and GSL production. Generally, higher plant biomass is achieved when biofumigants are sown during the summer (Ngala *et al*., 2015) and GSL concentrations are also highest in plants grown during the summer months, with longer day lengths, warmer temperatures and higher radiation levels being beneficial (Rosa, 1997). There is also some evidence that there can be a difference in GSL concentration between shoot and root material (Sarwar & Kirkegaard, 1998). Research has also shown the importance on the availability of sulphur in the growing substrate for efficient production of GSLs by biofumigant crops, and this has been related to higher levels of indole GSLs and those GSLs which breakdown to isothiocyanates (Rosa, 1997). In contrast, several studies have shown that nitrogen can somewhat inhibit glucosinolate production (Rosa, 1997). In this study, plants were

grown in three different environments. In the glasshouse batches, batches 2 and 3 were potentially grown at a more favourable time of year (April 2019 and July 2019 respectively) compared to batch 1 (August 2017). However, batches 2 and 3 had a top dressing of nitrogen, which may have limited GSL production, plants of batch 1 had a mean GSL concentration of 16.1 μmol g^{-1} d.w. and the concentration for batches 2 and 3 was 4.4 μmol g^{-1} d.w. and 9.1 μmol g^{-1} d.w. respectively. Batches 4 and 5 were sown in mid-August 2020, which may have been rather late in the year for optimum GSL production, however, the timing of sowing was impacted by the COVID-19 pandemic and thus unavoidable. The decision to grow batch 4 in an open-sided polytunnel rather than the glasshouse was based on the fact that slightly more challenging environmental conditions, such as mild pest damage, may promote GSL synthesis (Sarwar & Kirkegaard, 1998). It is worth noting that batch 5 grown in the field was harvested at the beginning of November with none of the varieties achieving mid flowering. Batch 5 had the lowest mean GSL concentration at 1.0 μmol g^{-1} d.w.

One study of multiple *Brassica* spp. suggests that flower bud formation is the growth stage most closely associated with highest GSL concentration, which then declines at flowering with a steep decline at full maturity. However, in a controlled glasshouse compartment, there was also a GSL concentration increase between bud formation and flowering (Sarwar & Kirkegaard, 1998). In commercial use, farmers are more likely to incorporate biofumigants as flowering begins to avoid seed dispersal and due to the higher amount of biomass that would be incorporated (Mattner *et al.*, 2008). Sarwar & Kirkegaard (1998) also concluded that of four different environments tested, including plants sown in the field in Spring and Autumn, and in glasshouse compartments at both ambient and controlled (20 °C/12 °C) temperature, plants grown in the temperature-controlled compartment had the lowest GSL concentrations overall. Therefore, when growing batches of biofumigants in the future, a more tailored approach for individual varieties should be used if possible, with the polytunnel a suitable and cost-effective environment. Sowing should occur late/spring early summer dependent on how long it takes for individual varieties to flower, rather than sowing all varieties at the same time; for example, *B. juncea* varieties reached mid-flowering around six weeks in both the temperature-controlled glasshouse and polytunnel, with *R. sativus* varieties taking around 11-13 weeks. In line with commercial use, the time point of harvesting could also be changed to the beginning of flowering. However, it is important to consider that adjustments made in one environment will not necessarily translate to a different one (Sarwar & Kirkegaard, 1998).

Overall, the results of the *in-vitro* experiments indicated that several biofumigant crop varieties had significant effects on PFRC pathogens by reducing and inhibiting mycelial growth. In the experiments utilising varieties from batch 1, both *B. juncea* varieties ‘Caliente 99’ and ‘Pacific Gold’ resulted in more than 50 % inhibition of all PFRC pathogens examined while *E. sativa* ‘Nemat’ and *S. alba* ‘Brisant’ both achieved 50 % inhibition of DP and AE. *R. sativus* ‘Terranova’ only achieved 50 % inhibition for AE. In the experiment using varieties grown in batch 2, the two *B. juncea* varieties ‘Caliente 199 and ‘Caliente Rojo’ and *E. sativa* ‘Trio’ resulted in 50 % inhibition of DP only, with no varieties effective against FO and FSP. This is the first time biofumigants have been tested and identified to be effective against PFRC specific isolates of FO, FSP and DP.

Both *B. juncea* and *S. alba* varieties and their respective GSL breakdown products have previously been identified to be effective against *Fusarium* spp. and AE (Muehlchen *et al*., 1990; Hossain *et al*., 2015; Prasad & Kumar, 2017). Studies have shown that as little as 100 mg of *S. alba* and *B. juncea* freeze-dried powder hydrated with 0.1 mL water inhibited mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* by as much as 57.4 % and 43.3 % respectively. This is 95 % lower than the amount used in this study and suggests a potentially higher GSL concentration in the plants although this was not reported by the authors. The assay was also run at 27 °C, more suitable for optimal growth conditions for the pathogen but also may be better for ITC release and activity, whereas the temperature selected for this study (20 °C) better reflected glasshouse and field conditions (Prasad & Kumar, 2017). Another study utilising a similar double Petri dish based system examined the effects of a commercial biofumigant mix of *B. rapa* and *B. napus*, BQ-Mulch^{TM} on several soil-borne pathogens of strawberry, including FO. Plant material was freshly chopped, macerated, and used in the *in-vitro* experiments without the addition of water, at a rate of 2.5 g per plate. FO colony growth was reduced by 60 % but all other pathogens were completely inhibited, which included *P. ultimum*, *P. cactorum*, *Colletotrichum dematium*, *Alternaria alternata*, *Rhizoctonia fragariae* and *Cylindrocarpon destructans*. The study also found a higher diversity and concentration of GSLs was present in biofumigant roots. However, shoot material makes up the vast bulk of biofumigant plant material that is generally incorporated into soil (Mattner *et al*., 2008).

Another observation made during the *in-vitro* assays here was the differences in mycelium density of PFRC pathogen colonies for different biofumigant treatments. Whilst growth of two pathogen colonies could be similar, some colonies would only have one or two individual hyphae growing from the plug, whereas other colonies would be much denser. Whilst the mycelial density of the PFRC pathogen colonies

was not quantitatively assessed, the volatiles released by the biofumigant material did appear to lead to a reduction in density in some treatments with certain pathogens, and therefore, if these effects were replicated in a glasshouse or field environment, this could result in decreased foot rot disease. A method to quantify mycelial density would involve measuring the distance between two hyphal branch junctions close to the growing edge of the colony using a microscope eyepiece, after staining the fungal colony with lactophenol blue (Aspray *et al.*, 2013).

It is possible that for the *B. juncea* varieties, the lower GSL concentrations and in turn the lower production of volatile ITCs upon hydration may have been the reason for the decreased mycelial growth inhibition observed in Experiment 2 compared to Experiment 1. However, this did not quite apply for *E. sativa* 'Nemat', especially for FO; here, batch 1 used in Experiment 1 and batch 2 used in Experiment 2 had mean single GSL concentrations of 11.84 µmol g^{-1} d.w. and 1.06 µmol g^{-1} d.w. respectively. Correspondingly in Experiment 1, the percentage inhibition of mycelial growth was 0.6 %, 23.3 % and 96.6 % for FO, FSP and DP respectively, while in Experiment 2, the percentage inhibition of mycelial growth was 11.1 %, 6.1 % and 16.6 % for the same pathogens. These differences in GSL concentrations and/or amounts of biofumigant powder used between experiments described in this chapter and other published *in-vitro* studies make direct comparison of results difficult. However, results could be standardised by calculating inhibition of pathogen growth per µmol GSL g^{-1} d.w. which would then allow comparisons to be made. This would be done by dividing the mean pathogen growth rate by the GSL concentration of the biofumigant and then using this adjusted mean growth value to calculate an adjusted percentage inhibition. However, this method assumes a linear relationship between GSL concentration, release of ITC and effect on the pathogen.

The effect of biofumigants on PFRC pathogen spore germination was also examined in this study. However, only the biofumigants *S. alba* 'Brisant' and *E. sativa* 'Trio' significantly inhibited spore germination for FO and FSP respectively compared to the untreated control. However, when the same plates were examined two days later after the removal of the biofumigant, these differences were no longer significant. This study was therefore unable to identify biofumigant varieties that killed spores of FO and FSP. Similarly, results of the mycelial growth assays in Experiment 2, indicated that although some biofumigant varieties significantly reduced mycelial growth, none resulted in complete inhibition. Comparable results have been observed in related published research. For instance, a study examining the effects of several ITCs on various developmental stages of FO isolates responsible for root rot and damping-off in conifers found that although propenyl ITC was effective at restricting

FO mycelial growth compared to the untreated control after two days, there was no significant difference at day seven (Smolinska *et al.*, 2003). Propenyl ITC was therefore fungistatic, as colony growth resumed a few days after removal of the ITC source. Moreover, in a separate experiment analysing conidial formation from a growing culture exposed to different ITCs, propenyl ITC had no effect. When FO conidial germination was examined however, propenyl ITC completely inhibited germination after a 24-hour exposure period and after a further 24 hours without the presence of the ITC, germination remained completely inhibited, which contrasts with the results reported in this chapter. Similarly, complete inhibition of germination by propenyl ITC was also found in a chlamydospore assay after five days, but any inhibition after removal of the ITC was not examined in this experiment (Smolinska *et al.*, 2003).

As in the above examples, many published studies utilise ITCs directly in *in-vitro* assays, either suspended on a solid surface within the system, such as a filter (Smolinska *et al.*, 2003) or incorporated directly into the agar (Taylor *et al.*, 2014). The advantage of this approach is that it is specific and quantifiable, can screen for a range of ITCs, and may also identify other candidate GSLs and subsequent biofumigant varieties for future work. However, a disadvantage of this approach is that other breakdown products of GSLs apart from the primary ITC formed, or other minor GSLs in addition to the primary GSL of a species may also have activity against pathogens. The work in this chapter screened commercially available biofumigants *in-vitro*, which better inform variety choice for use in *in-planta* experiments in glasshouse and field settings. The use of single-variety or mixed varieties of biofumigant plant material, with a broader range of active compounds might be a better choice given that there is some evidence that individual isolates of the same species such as FO are sensitive to different types of ITCs (Fan *et al.*, 2008).

The results of the *in-vitro* experiments with DP informed variety choice for the glasshouse experiment which was the first time the effects of biofumigation on DP foot rot disease development has been examined and provides information on effective varieties which may be useful for use in the field. Overall, few pot-based studies have examined the effects of biofumigants on PFRC pathogens, but some comparable work has been done with other *Fusarium* ff. spp. and AE in controlled environments. One glasshouse study examined the effects of *Brassica* plant material of different GSL concentrations on suppression of *F. oxysporum f. sp. cucumerinum*, the causal agent of cucumber wilt (Meng *et al.*, 2018). Although the authors did not describe the exact species/varieties of the *Brassica* biofumigant material they did undertake HPLC analysis of several major GSLs. Their approach used both a pea

radical dipping method with a *F. oxysporum* f. sp. *cucumerinum* spore suspension at a concentration of 2x10^{6} spores mL^{-1} as well as a growing substrate naturally infested with *F. oxysporum* f. sp. *cucumerinum*. In both cases, *Brassica* biofumigant material was freshly chopped and incorporated at a rate of 3.5 kg m^{-2} into the growing medium. Pathogen CFU counts of the soil and height of the cucumber seedlings from all biofumigant treatments was recorded, and untreated and hymexazol (systemic fungicide) controls were also set up. Biofumigant treatments with both low and high concentrations of GSLs all resulted in cucumber seedlings with significantly increased seedling height and dry weight, which was at least three times greater than that of the untreated control. By comparison, the biofumigants effective against DP (at the 5x10^{5} spores g^{-1} inoculum concentration) also resulted in increased pea shoot and root dry weights compared to plants grown in the inoculated substrate with no biofumigant; the highest increase in dry shoot weight resulted from *E. sativa* 'Trio' at 1.16 g, which was more than double that of the plants grown in the inoculated substrate with no biofumigant (0.46 g). While the study of Meng *et al*., (2018) used fresh biofumigant material, this work used dried powdered biofumigants incorporated into a DP inoculated compost/sand-based substrate incubated for four weeks prior to the start of the experiment. This prior incubation may have reduced viable DP inoculum before planting, but where the biofumigant was not effective or for the control treatment, inoculum concentration may have increased. Like the Meng *et al*., (2018) study, it may also have been interesting to examine the concentration of viable inoculum of each treatment at the end of the glasshouse experiment. The researchers found that compared to the non-amended treatment (6.94x10^{4} CFUs g^{-1} d.w. soil), both the high glucosinolate and hymexazol treatments resulted in a significant decrease in CFU number, at 2.59x10^{4} CFUs g^{-1} d.w. soil and 1.63x10^{4} CFUs g^{-1} d.w. soil respectively. FO and DP are different pathogens and the concentration of the FO spore suspension used in the Meng *et al*., (2018) study was four times higher than the greatest concentration of DP used in the work here, which demonstrates the potential differences in virulence between these two soil-borne pathogens.

Another published pot-based glasshouse study examined the effectiveness of three *Brassica carinata* cultivar seed meals (yellow dodola, Holleta-1 and S-67) on *F. oxysporum* f. sp. *ciceris* disease development in chickpea (Abera *et al.*, 2011). Treatments applied at 10 g kg^{-1} soil and above resulted in significant reductions in Fusarium wilt after eight weeks compared with the plants of the untreated control which had a wilt score of 100 % by the third week. For both mean plant dry weight and mean pod number per plant, all three *B. carinata* cultivars at rates of 10 g kg^{-1} soil and above resulted in a significant increase in mean plant dry weight and mean

pod number per plant at eight weeks (Abera *et al*., 2011). The work of this chapter also found a similar effect where at the 5×10^5 DP spores g^{-1} concentration, the biofumigant treatments *B. carinata* 'Cappuchino' and *E. sativa* 'Trio' resulted in a significant increase in combined pea pod and flower number.

Interestingly, all the biofumigant treatments tested in the glasshouse here resulted in a decrease in the percentage of pea plants with nodules compared with those in the no biofumigant treatment at both DP inoculum levels. It has been found previously that isothiocyanates can negatively impact Rhizobiaceae bacterial communities (Bressan *et al.*, 2009), many species of which are involved in nodulation and nitrogen fixation. A study examined the effect of different chemicals, including allyl and benzyl isothiocyanate on nodulation in the legume *Amphicarpaea bracteate* (American hog peanut) in a glasshouse assay (Portales-Reyes *et al.*, 2015). Treatments included both isothiocyanates individually and in combination, where they were added to the substrate containing ten-day old *A. bracteate* seedlings and after eleven days, plants were inoculated with *Bradyrhizobium* isolates. The individual allyl isothiocyanate and combined allyl and benzyl isothiocyanate treatments resulted in a significant reduction in *A. bracteate* nodule formation compared to the untreated control. This therefore supports the observation in this study that plants grown in substrate amended with *B. carinata* 'Cappuchino' (principal ITC allyl isothiocyanate) did not form nodules. In another study involving pea, a glasshouse assay examined the effects of *B. juncea* and *S. alba* as cover crops on AE foot rot and pea root nodule formation in a pot-based system. AE root rot disease was significantly lower for pea plants grown following *B. juncea* and *S. alba* treatments where a high level of nitrogen was applied. However, the plants of these treatments also showed a reduction in nodule formation, particularly for *S. alba*, but this was not significant (Hossain *et al*., 2015). In future studies it may be important to consider the potential negative impact of *B. juncea*, *B. carinata* and *S. alba* varieties on nodule formation in pea, however this must be considered alongside other metrics of pea development, such as pod formation and plant weight to see whether the effects of the ITC products resulting from these biofumigants outweigh the effects of the biofumigant on PFRC pathogens.

In the glasshouse experiment, it was also found that for the uninoculated treatments amended with biofumigant material, plants developed apparent DP stem girdling symptoms while none of the plants in the treatment with no biofumigant developed stem girdling symptoms. However, for both DP inoculum concentrations, all treatments amended with biofumigants resulted in a reduction of plants with DP stem girdling compared to inoculated treatments without biofumigant. The apparent increase in girdling symptoms in pea plants of the non-inoculated treatments

amended with the three biofumigants could have been the result of other microorganisms colonising the biofumigant material which were also able to colonise the pea plant and cause similar stem girdling symptoms to DP. Additional work would need to be carried out to explore this and could include plating out sections of plants of both the uninoculated treatments amended with biofumigants, and inoculated treatments to see if there is any difference in the organisms from the stem girdling symptoms.

It is assumed in this study that the GSL concentration within the plant directly affects the concentrations of ITCs released and therefore the amount of inhibition of the pathogen. However, the profile of ITC breakdown products effective against target pathogens and the efficacy of the hydrolysis reaction may differ between biofumigant varieties, experimental methods, biofumigant variety and both biotic and abiotic growth conditions (Bell & Wagstaff, 2014; Hanlon *et al.*, 2009). The hydrolysis efficiency of GSLs has often been found to be lower than expected from calculated values, for instance, one study reported that actual ITC concentrations yielded were as low as 1 % of the expected value (Price *et al.*, 2005). The characterisation of ITCs resulting from glucosinolate hydrolysis can be undertaken in a sealed system, such as the double Petri-dish system utilised in this chapter, through solid phase microextraction and subsequent gas chromatography-mass spectrometry (Price *et al.*, 2005; Mattner *et al.*, 2008). In addition, the method of biofumigant plant material processing prior to use in experiments and HPLC analysis can also be optimised. Whilst the oven-drying technique used in this chapter which removes water prior to milling to powder to stop the hydrolysis reaction has also been used in other studies (Neupane, 2013; Warmington & Clarkson, 2016) this could have affected the effectiveness of myrosinase in the hydrolysis of GSLs (Al-Turki & Dick, 2003). Significant loss of myrosinase activity has been shown at temperatures above 60 °C and total loss at 80 °C in myrosinase extracted from *S. alba*, *B. juncea* and *B. nigra* seed (Okunade *et al.*, 2015). Therefore, an alternative processing approach such as freeze drying (e.g. Price *et al.*, 2005; Gimsing & Kirkegaard, 2006; Neubauer *et al.*, 2014) or incorporated fresh into the growing substrate for glasshouse experiments (e.g. Ngala *et al.*, 2015) would be recommended in future. Freeze drying has also been found to be comparable to fresh tissue in terms of isothiocyanate ratios (Price *et al.*, 2005). However, the quantity of biofumigant plant material required for both the *in vitro* and glasshouse experiments may be impractical for use of freeze-dried material.

The research described in this chapter successfully identified biofumigant plant varieties that were effective against PFRC pathogens both *in vitro* and in

glasshouse experiments, particularly for DP, a relatively understudied pathogen of the PFRC and thought to be the main causal agent of PFR in the UK. The results of the *in vitro* experiments for mycelial growth inhibition indicated the effectiveness of *B. juncea* varieties in particular against all PFRC pathogens examined in Experiment 1 and DP in Experiment 2. The pot-based glasshouse experiment, which examined the effects of different biofumigant plants varieties on the suppression of DP foot rot disease development showed that all three varieties significantly reduced root rot score and increased root and shoot dry weights compared to the non-biofumigant treatment at $5x10^5$ spores g^{-1}. Recommendations have been made for the growth of future biofumigant plant batches to enhance GSL content and their use in subsequent control of PFRC pathogens. Recommendations and possible changes have also been suggested for future glasshouse studies, such as additional disease assessments such as plant height, and establishing the effects of biofumigants directly on viable spore concentrations.

5. Discussion

The pea foot rot disease complex (PFRC) is a group of fungal and oomycete pathogens responsible for root and stem base rot of pea plants. It has been identified in most pea-growing regions around the world, including the UK (Etebu and Osbourne, 2009), Denmark, Sweden (Persson *et al*., 1997), Germany (Baćanović-Šišić *et al*., 2018), the Netherlands (Oyarzun *et al*., 1993), Canada (Chatterton *et al*., 2015), the USA (Chittem *et al*., 2015) and Australia (Kerr, 1963). It has been attributed to yield losses of up to 30 % in the US (Kraft and Pfleger, 2001), 40 % in the UK, and up to 75 % in other pea growing regions (Biddle and Caitlin, 2001). The species which make up the PFRC vary by region, but in the UK, the primary pathogens of the PFRC include *Fusarium oxysporum* (FO), *Fusarium solani forma specialis* (f.sp.) *pisi* (FSP), *Didymella pinodella* (DP) and *Aphanomyces euteiches* (AE) (Salt and Delaney, 2013; Jelden and Herold, 2020). Current management strategies for the PFRC include crop rotation and soil management (Skoglund *et al*., 2011; Esmaeili Taheri *et al*., 2021), biological control agents (often employed as seed treatments e.g., against AE), chemical control and (partial) genetic resistance (e.g., against AE and FSP) (Pilet-Nayel *et al*., 2002; Coyne *et al*., 2019). Research has evaluated other strategies such as cover cropping in the field (Jelden and Herold, 2020) and biofumigation, particularly regarding AE (Muehlchen *et al*., 1990). However, management strategies often remain limited or targeted to one pathogen within the complex, with almost no research undertaken on the PFRC as a whole in terms of disease management. Despite DP being implicated as a key pathogen of the PFRC in the UK (Dr Lea Herold, personal communication), very little is known about its diversity and interactions with other pathogens of the UK PFRC. Therefore, the aim of this thesis was to better understand pathogen dynamics of the PFRC in the UK and to identify biofumigant crops that can suppress the PFRC. The specific objectives were:

1) To identify and characterise isolates of *Fusarium* and *Didymella* from infested pea samples and fields in the UK using molecular methods and phylogenetics.
2) To examine the effects of different spore concentrations of single PFRC pathogens and different combinations of PFRC pathogens on disease development and mortality of pea using test-tube and glasshouse-based assays.

3) To identify biofumigant crop varieties that can suppress individual PFRC pathogens using *in-vitro* assays and glasshouse experiments.

Sequencing of the *ITS*, *RPB2*, *TUB2* and *LSU* barcoding regions of possible UK DP isolates resulted in the identification 14 DP isolates from regions of both England and Scotland. The phylogenetic analysis using all four barcodes individually revealed the lack of diversity within DP isolates from the UK and other countries, as supported by other more limited studies (Davidson *et al*., 2009; Ahmed *et al.*, 2015; Chen *et al*., 2015; Chen *et al*., 2017; Hou *et al*., 2020; Keirnan *et al*., 2021). It was established that *RPB2* and *TUB2* distinguished DP from the closely related pathogen *D. pinodes*, a component of the Ascochyta blight complex. The work of this chapter is the first to investigate the phylogeny and diversity of UK DP isolates; an understudied but important pathogen thought to be the major component of the PFRC causing pea foot rot in the UK. However, future work should seek to undertake a formal field survey of the prevalence and relative virulence of DP and other PFRC species in the major pea growing regions of the UK, as carried out in other countries (e.g. Persson *et al*., 1997; Chittem *et al*., 2015; Baćanović-Šišić *et al*., 2018; Chatterton *et al*., 2019). This could involve systematic sampling, isolation, and molecular identification of pathogens from both field soil and diseased pea plants from different growing regions in the UK. Similar field surveys have been undertaken in the UK to examine *Fusarium* species responsible for root rot and wilt of pea (Jenkins, 2018). Specific quantitative polymerase chain reaction (qPCR) assays for PFRC pathogens as employed in this study could be employed to determine relative prevalence in field soil samples while a next generation amplicon sequencing approach would be an informative way of not only detecting PFRC pathogens but also exploring the associated microbial community. Such molecular approaches have been used to investigate the oomycete pathogens of the PFRC in the Canadian Prairie regions (Esmaeili Taheri *et al*., 2017b). This study used the Illumina MiSeq Platform and primers designed to amplify the ITS1 region in oomycetes and examine the microbial community in pea roots, rhizosphere and soil. However, there are still concerns over the sensitivity of these primers to species such as *Aphanomyces* and therefore further research has been carried out to optimise primers and further investigate choice of barcoding region for amplicon sequencing of the oomycete community (Esmaeili Taheri *et al.*, 2017c)

Sequencing of the *TEF1-*α and *RPB2* barcoding regions of possible UK *F. solani* (FS) isolates resulted in the identification 10 FS isolates alongside other *Fusarium* spp. *TEF1-*α and *RPB2* sequences of the identified FS isolates alongside those from previous studies (Jenkins, 2018; Šišić *et al.*, 2018) were subject to phylogenetic

analysis to examine the diversity and phylogeny of UK FS isolates in the context of those from Central Europe. The majority of the UK isolates were clustered within a previously established clade consisting of FS isolates isolated from a number of legume species, including clover, vetch, faba bean and pea (Šišić *et al.*, 2018). The majority of these 'legume' isolates were also confirmed as pathogenic on pea, but due to the wider range of host species within the Fabaceae family, they were not designated as FSP, but rather as *Fusarium pisi* (Šišić *et al.*, 2018). This is not the first time the taxonomy of the *Fusarium solani* species complex has been challenged (Romberg and Davis, 2007) and there is also a possibility that host specificity may apply to families rather than individual species. It would therefore be interesting to test the pathogenicity the UK FS isolates on a range of legume species with the hypothesis that they should cause disease on multiple species as suggested by Šišić *et al.*, (2018). Such tests could be quickly carried out using the test-tube assay developed in this study (Chapter 3).

The effect of inoculum concentration and potential interactions between PFRC pathogens in relation to disease is poorly understood and this is confounded further by the different species present within the complex in different regions of the world. The research carried out in Chapter 3 for the first time successfully established the relationship between inoculum concentration and PFR disease for UK isolates of FO, FSP and DP in a test-tube based assay, and for FSP and DP a glasshouse-based assay. Such information is of importance for developing disease risk tools for the industry based on quantifying inoculum while also establishing appropriate levels of inoculum to assess control methods against DP in glasshouse or controlled environment experiments (Chapter 4). These experiments also led to the discovery that DP could cause pre-emergence damping off which has previously not been considered a symptom of DP infection. Examining this further in the field would be an important area of future work. The subsequent interactions experiments using the test tube assay with the FO, FSP and DP isolates revealed the additive nature of different combinations of these three pathogens. The original experimental plan, prior to modification due to the COVID-19 pandemic, was to undertake dose response glasshouse assays for all three PFRC pathogens and carry out targeted interaction experiments based on the results of the test tube assays. Hence, undertaking pot-based experiments for FO to examine the relationship between inoculum concentration and disease development would be of interest. The very low disease levels observed in the glasshouse inoculum dose experiment with FSP was unexpected given that this pathogen is widely recognised as a major component of the PFRC, but further work testing different isolate(s) should be considered, and

disease development also examined as part of interaction experiments using the pot-based system. It would also be interesting to develop methods for inoculum production for AE and include this pathogen as part of future dose response and interactions experiments alongside the pathogens examined already and determine whether results corroborate the results of previous work (Willsey *et al*., 2018). Another area of future investigation would be to investigate whether pathogen interactions are affected by differences in time of inoculation (infection). It has been shown previously in co-inoculation experiments with DP and *D. pinodes* (both components of the Ascochyta blight complex), that disease severity increased when the other pathogen was pre-inoculated on pea plant leaves, while co-inoculation resulted in less disease and an antagonistic interaction (Le May *et al*., 2009). Although the work in this thesis only examined one isolate per PFRC pathogen, it has been found previously that virulence can differ between isolates of the same species (Wicker & Rouxel, 2001; Chittem *et al*., 2015; Šišić *et al.*, 2018) and therefore, different dose responses and interactions may have occurred if different representative PFRC isolates were used; this is another area that merits investigation.

The use of qPCR assays was demonstrated to be a successful approach with which to investigate PFRC pathogen colonisation of pea plant roots in the interactions experiments. This is a new area of research that potentially provides new knowledge on the dynamics of PFRC pathogens and is the first time that DP dynamics and interactions have been explored using molecular methods. The primers used to amplify DP and FSP were designed and tested as part of an Innovate UK project between UoW and PGRO (Clarkson, 2019) to develop a more rapid approach to assessing PFR risk, however testing on plant root sampled in this project was limited. With further development of these qPCR assays, there is a potential use for them as a commercially applied disease risk assessment tool. This could be deployed through strategic soil and pea plant sampling of potential PFR infected fields, with a more high-throughput approach such as multiplexed qPCR assays. Problems with assay sensitivity could be managed by using soil-baiting experiments, involving the growth of pea seedlings in the sampled soil to increase pathogen inoculum concentration for DNA extraction. These molecular assays to determine the presence and risk of PFR would be a far more efficient approach than current industry methods, which include plating soils onto amended agar to determine FS and DP colony forming units, or examining the presence/severity of PFR symptoms when peas are grown in sampled soils.

The work carried out in Chapter 4 successfully identified biofumigants which significantly reduced or inhibited mycelial growth of FO, FSP, DP and AE *in-vitro* and

supressed DP induced foot rot in a pot-based glasshouse experiment. This work is the first to identify biofumigant varieties effective against DP both *in-vitro* and *in-planta* and provides the foundations for further research into biofumigation as a practical control option for pathogens of the UK PFRC, which could be highly beneficial for growers. The biofumigant *E. sativa* 'Trio' was the most effective of the treatments tested for DP disease suppression *in-planta* across the different metrics assessed. The next stage in determining if this variety would be of interest for pea growers as a control option would be to examine the effects of this biofumigant in-field where DP is the predominant pathogen. However, future research would need to be undertaken to determine if this and other biofumigants were effective against FO, FSP and AE *in-planta*, with the established pot-based method a good choice for this, making results comparable with the DP experiment. Due to the multiple pathogen nature of the PFRC, it will be important that biofumigation is effective against multiple pathogens and therefore if no single biofumigant is effective then mixtures could be considered. An experiment using an inoculum containing multiple PFRC pathogens would also be of interest for testing this approach.

Overall, the research of this thesis has contributed substantial knowledge regarding the phylogeny, interactions, and management of UK PFRC pathogens. The research has established the relationship between inoculum concentration and disease development of these pathogens, the consequences of different pathogen combinations in terms of disease development and has begun to investigate biofumigation as a new management approach in the absence of any effective control measures of the PFRC except for crop rotation. This can form the basis of an integrated control strategy; using developed molecular diagnostics to determine identity and abundance of PFRC pathogens present in soil and root tissue samples to determine PFR risk, and subsequent management with rotation or biofumigation tailored for identity and abundance of PFRC pathogens present. Further research therefore is recommended on the effects of biofumigants on FO, FSP and AE PFR disease suppression *in-planta* and subsequent field trials to be able to implement a control strategy successfully. The research of this thesis has also contributed new knowledge for DP, a poorly studied pathogen despite it being a major component of the PFRC in the UK. The work of this thesis therefore has made a substantial contribution towards developing a sustainable management strategy of UK PFRC pathogens.

References

Abera, M., Ahmed, S., Fininsa, C., Sakhuja, P. K. and Alemayehu, G. (2011) Effect of Brassica carinata (L.) biofumigants (seed meal) on chickpea wilt (Fusarium oxysporum f. sp. ciceris), growth, yield and yield component in Ethiopia. *Archives of Phytopathology and Plant Protection,* **44,** 1785-1795.

Abera, M., Sakhuja, P. K., Fininsa, C. and Ahmed, S. (2013) Status of chickpea fusarium wilt (Fusarium oxysporum f. sp. ciceris) in northwestern Ethiopia. *Archives of Phytopathology and Plant Protection,* **44,** 1261-1272.

Agrios, G. N. (2005) *Plant Pathology*. Academic Press.

AHDB (2017) *Vining pea growers guide – 2017*. AHDB.

Ahmed, H., Chang, K.-F., Hwang, S.-F., Fu, H., Zhou, Q., Strelkov, S., Conner, R. and Gossen, B. (2015) Morphological characterization of fungi associated with the ascochyta blight complex and pathogenic variability of Mycosphaerella pinodes on field pea crops in central Alberta. *The Crop Journal,* **3,** 10-18.

Al-Turki, A. I. and Dick, W. A. (2003) Myrosinase activity in soil. *Soil Science Society of America Journal,* **67,** 139-145.

Anaissie, E. J., Kuchar, R. T., Rex, J. H., Francesconi, A., Kasai, M., Müller, F.-M. C., Mario, L.-C., Summerbell, R. C., Dignani, M. C. and Chanock, S. J. (2001) Fusariosis associated with pathogenic Fusarium species colonization of a hospital water system: a new paradigm for the epidemiology of opportunistic mold infections. *Clinical Infectious Diseases,* **33,** 1871-1878.

Aoki, T., O’Donnell, K. and Geiser, D. M. (2014) Systematics of key phytopathogenic Fusarium species: current status and future challenges. *Journal of General Plant Pathology,* **80,** 189-201.

Aspray, T. J., Jones, E. E., Davies, M. W., Shipman, M. and Bending, G. D. (2013) Increased hyphal branching and growth of ectomycorrhizal fungus Lactarius rufus by the helper bacterium Paenibacillus sp. *Mycorrhiza,* **23,** 403-410.

Avasthi, S., Gautam, A. K. and Bhadauria, R. (2017) First report of leaf spot disease caused by Phoma eupyrena Sacc. on Aloe vera from Madhya Pradesh, India. *Archives of Phytopathology and Plant Protection,* **50,** 62-69.

Aveskamp, M. M., de Gruyter, J., Woudenberg, J. H., Verkley, G. J. and Crous, P. W. (2010) Highlights of the Didymellaceae: A polyphasic approach to characterise Phoma and related pleosporalean genera. *Studies in Mycology,* **65,** 1-60.

Aveskamp, M. M., Verkley, G. J., de Gruyter, J., Murace, M. A., Perello, A., Woudenberg, J. H., Groenewald, J. Z. and Crous, P. W. (2009) DNA phylogeny reveals polyphyly of Phoma section Peyronellaea and multiple taxonomic novelties. *Mycologia,* **101,** 363-382.

Baayen, R. P., O'Donnell, K., Breeuwsma, S., Geiser, D. M. and Waalwijk, C. (2001) Molecular relationships of fungi within the Fusarium redolens-F. hostae clade. *Phytopathology,* **91,** 1037-1044.

Baćanović-Šišić, J., Šišić, A., Schmidt, J. H. and Finckh, M. R. (2018) Identification and characterization of pathogens associated with root rot of winter peas grown under organic management in Germany. *European Journal of Plant Pathology,* **151,** 745-755.

Badotti, F., Fonseca, P. L. C., Tomé, L. M. R., Nunes, D. T. and Góes-Neto, A. (2018) ITS and secondary biomarkers in fungi: review on the evolution of their use based on scientific publications. *Brazilian Journal of Botany,* **41,** 471-479.

Begerow, D., Nilsson, H., Unterseher, M. and Maier, W. (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied Microbiology and Biotechnology,* **87,** 99-108.

Bell, L. and Wagstaff, C. (2014) Glucosinolates, myrosinase hydrolysis products, and flavonols found in rocket (Eruca sativa and Diplotaxis tenuifolia). *Journal of Agricultural and Food Chemistry,* **62,** 4481-4492.

Bennett, A., Ponder, M. M. and Garcia-Diaz, J. (2018) Phoma infections: classification, potential food sources, and their clinical impact. *Microorganisms,* **6,** 58.

Biddle, A. J. and Cattlin, N. (2007) *Pests, diseases and disorders of peas and beans: A colour handbook*. CRC Press.

Blume, M. and Harman, G. (1979) Thielaviopsis basicola: A component of the pea root rot complex in New York State. *Phytopathology,* **69,** 785-788.

Bodah, E. T. (2017) Root rot diseases in plants: a review of common causal agents and management strategies. *Agricultural Research and Technology,* **5,** 555661.

Bødker, L., Leroul, N. and Smedegaard-Petersen, V. (1993) The occurrence in Denmark of black root rot of pea caused by Thielaviopsis basicola. *Plant Pathology,* **42,** 820-823.

Boerema, G. H. (2004) *Phoma identification manual: differentiation of specific and infra-specific taxa in culture*. CABI.

Boerema, G. H., Dorenbosch, M. M. and Leffring, L. (1965) A comparative study of the black stem fungi on lucerne and red clover and the footrot fungus on pea. *Netherlands Journal of Plant Pathology,* **71,** 79-89.

Bones, A. M. and Rossiter, J. T. (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum,* **97,** 194-208.

Borek, V. and Morra, M. J. (2005) Ionic thiocyanate (SCN-) production from 4-hydroxybenzyl glucosinolate contained in Sinapis alba seed meal. *Journal of Agricultural and Food Chemistry,* **53,** 8650-8654.

Bowers, J. and Parke, J. (1993) Epidemiology of Pythium damping-off and Aphanomyces root rot of peas after seed treatment with bacterial agents for biological control. *Phytopathology,* **83,** 1466-1473.

Bressan, M., Roncato, M.-A., Bellvert, F., Comte, G., el Zahar Haichar, F., Achouak, W. and Berge, O. (2009) Exogenous glucosinolate produced by Arabidopsis thaliana has an impact on microbes in the rhizosphere and plant roots. *The ISME Journal,* **3,** 1243-1257.

British Growers (2020) The importance of British growing; vining peas. Available from: https://britishgrowers.org/importance-of-british-growing/ (Accessed: 2020)

British Mycological Society (2018) Fungal records database of Britain and Ireland. Available from: https://doi.org/10.15468/ietmop (Accessed: 2022).

Brown, P. and Morra, M. (1997) *Control of soil-borne plant pests using glucosinolate-containing plants*. Moscow, Idaho.

Burke, D. and Kraft, J. (1974) Responses of beans and peas to root pathogens accumulated during monoculture of each crop species. *Phytopathology,* **64**.

Buxton, E. and Perry, D. (1959) Pathogenic interactions between Fusarium oxysporum and Fusarium solani on peas. *Transactions of the British Mycological Society,* **42,** 378-387.

Chatterton, S., Bowness, R. and Harding, M. W. (2015) First report of root rot of field pea caused by Aphanomyces euteiches in Alberta, Canada. *Plant Disease,* **99,** 288.

Chatterton, S., Harding, M. W., Bowness, R., Mclaren, D. L., Banniza, S. and Gossen, B. D. (2019) Importance and causal agents of root rot on field pea and lentil on the Canadian prairies, 2014–2017. *Canadian Journal of Plant Pathology,* **41,** 98-114.

Chen, L., Wu, Q., He, T., Lan, J., Ding, L., Liu, T., Wu, Q., Pan, Y. and Chen, T. (2020) Transcriptomic and metabolomic changes triggered by Fusarium solani in common bean (Phaseolus vulgaris L.). *Genes,* **11,** 177.

Chen, Q., Hou, L. W., Duan, W. J., Crous, P. W. and Cai, L. (2017) Didymellaceae revisited. *Studies in Mycology,* **87,** 105-159.

Chen, Q., Jiang, J. R., Zhang, G. Z., Cai, L. and Crous, P. W. (2015) Resolving the Phoma enigma. *Studies in Mycology,* **82,** 137-217.

Chesters, C. and Hornby, D. (1965) Studies on Colletotrichum coccodes: II. Alternative host tests and tomato fruit inoculations using a typical tomato root isolate. *Transactions of the British Mycological Society,* **48,** 583.

Chittem, K., Mathew, F. M., Gregoire, M., Lamppa, R. S., Chang, Y. W., Markell, S. G., Bradley, C. A., Barasubiye, T. and Goswami, R. S. (2015) Identification and characterization of Fusarium spp. associated with root rots of field pea in North Dakota. *European Journal of Plant Pathology,* **143,** 641-649.

Choiseul, J., Allen, L. and Carnegie, S. (2006) Fungi causing dry tuber rots of seed potatoes in storage in Scotland. *Potato Research,* **49,** 241-253.

Clarkson, J. (1978) Pathogenicity of Fusarium spp. associated with foot-rots of peas and beans. *Plant Pathology,* **27,** 110-117.

Clarkson, J. P. (2019) Development of a diagnostic tool to assess risk levels of pea foot rot pathogens in soils. University of Warwick; PGRO.

Cook, D. R. (1999) Medicago truncatula - a model in the making! *Current Opinion in Plant Biology,* **2,** 301-304.

Correll, J. C. (1991) The relationship between formae speciales, races, and vegetative compatibility groups in Fusarium oxysporum. *Phytopathology,* **81,** 1061-1064.

Coyne, C. J., Porter, L. D., Boutet, G., Ma, Y., McGee, R. J., Lesné, A., Baranger, A. and Pilet-Nayel, M.-L. (2019) Confirmation of Fusarium root rot resistance QTL Fsp-Ps 2.1 of pea under controlled conditions. *BMC Plant Biology,* **19,** 1-8.

Dabney, S. M., Delgado, J. A. and Reeves, D. W. (2001) Using winter cover crops to improve soil and water quality. *Communications in Soil Science and Plant Analysis,* **32,** 1221-1250.

Davidson, J., Krysinska-Kaczmarek, M., Wilmshurst, C., McKay, A. and Scott, E. (2011) Distribution and survival of ascochyta blight pathogens in field-pea-cropping soils of Australia. *Plant Disease,* **95,** 1217-1223.

Davidson, J. A., Hartley, D., Priest, M., Herdina, M. K., McKay, A. and Scott, E. S. (2009) A new species of Phoma causes ascochyta blight symptoms on field peas (Pisum sativum) in South Australia. *Mycologia,* **101,** 120-128.

de Gruyter, J., Aveskamp, M. M., Woudenberg, J. H., Verkley, G. J., Groenewald, J. Z. and Crous, P. W. (2009) Molecular phylogeny of Phoma and allied anamorph genera: towards a reclassification of the Phoma complex. *Mycological Research,* **113,** 508-519.

de Gruyter, J., Woudenberg, J., Aveskamp, M., Verkley, G., Groenewald, J. and Crous, P. (2012) Redisposition of Phoma-like anamorphs in Pleosporales. *Studies in Mycology,* **54,** 114-114.

de Hoog, G. and van den Ende, A. G. (1998) Molecular diagnostics of clinical strains of filamentous Basidiomycetes. *Mycoses,* **41,** 183-189.

Deb, D., Khan, A. and Dey, N. (2020) Phoma diseases: Epidemiology and control. *Plant Pathology,* **69,** 1203-1217.

Desgroux, A., Baudais, V. N., Aubert, V., Le Roy, G., de Larambergue, H., Miteul, H., Aubert, G., Boutet, G., Duc, G. and Baranger, A. (2018) Comparative genome-wide-association mapping identifies common loci controlling root system architecture and resistance to Aphanomyces euteiches in pea. *Frontiers in Plant Science,* **8,** 2195.

Dung, J., Johnson, D. and Schroeder, B. (2014) Role of co-infection by Pectobacterium and Verticillium dahliae in the development of early dying and aerial stem rot of Russet Burbank potato. *Plant Pathology,* **63,** 299-307.

Duniway, J. M. (2002) Status of chemical alternatives to methyl bromide for pre-plant fumigation of soil. *Phytopathology,* **92,** 1337-1343.

Eilenberg, J., Hajek, A. and Lomer, C. (2001) Suggestions for unifying the terminology in biological control. *BioControl,* **46,** 387-400.

El-Sharkawy, H. H., Abbas, M. S., Soliman, A. S., Ibrahim, S. A. and El-Nady, I. A. (2021) Synergistic effect of growth-promoting microorganisms on bio-control of Fusarium oxysporum f. sp. pisi, growth, yield, physiological and anatomical characteristics of pea plants. *Pesticide Biochemistry and Physiology,* **178,** 104939.

Elzebroek, A. T. G. (2008) *Guide to cultivated plants*. CABI.

Esmaeili Taheri, A., Chatterton, S., Foroud, N., Gossen, B. and McLaren, D. (2017a) Identification and community dynamics of fungi associated with root, crown, and foot rot of field pea in western Canada. *European Journal of Plant Pathology,* **147,** 489-500.

Esmaeili Taheri, A., Chatterton, S., Gossen, B. and McLaren, D. (2017b) Metagenomic analysis of oomycete communities from the rhizosphere of field pea on the Canadian prairies. *Canadian Journal of Microbiology,* **63,** 758-768.

Esmaeili Taheri, A., Chatterton, S., Gossen, B. D. and McLaren, D. L. (2017c) Degenerate ITS7 primer enhances oomycete community coverage and PCR sensitivity to Aphanomyces species, economically important plant pathogens. *Canadian Journal of Microbiology,* **63,** 769-779.

Esmaeili Taheri, A., Gossen, B. D., Mclaren, D. L. and Chatterton, S. (2021) Overwinter survival and alternative crop hosts of fungi and oomycetes present in field pea residue on the Canadian prairies. *Canadian Journal of Plant Pathology,* **43,** 455-462.

Etebu, E. and Osborn, A. (2009) Molecular assays reveal the presence and diversity of genes encoding pea footrot pathogenicity determinants in Nectria haematococca and in agricultural soils. *Journal of Applied Microbiology,* **106,** 1629-1639.

Fahey, J. W., Zalcmann, A. T. and Talalay, P. (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry,* **56,** 5-51.

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

FAOSTAT (2021) Food and agricultural data. Available from: http://www.fao.org/faostat/en/#home (Accessed: 2021).

Faridi, R., Koopman, B., Schierholt, A., Ali, M. B., Apel, S. and Link, W. (2021) Genetic study of the resistance of faba bean (Vicia faba) against the fungus Ascochyta fabae through a genome-wide association analysis. *Plant Breeding,* **140,** 442-452.

Farr, D. F. and Rossman, A. Y. (2021) Fungal Databases. Available from: https://nt.ars-grin.gov/fungaldatabases/. (Accessed: 2022).

Feng, J., Hwang, R., Chang, K., Conner, R., Hwang, S., Strelkov, S., Gossen, B., McLaren, D. and Xue, A. (2011) Identification of microsatellite markers linked to quantitative trait loci controlling resistance to Fusarium root rot in field pea. *Canadian Journal of Plant Science,* **91,** 199-204.

Finckh, M. R., Saeed, M., Butz, A. F. and Bruns, C. (2013) Einfluss von mischanbau mit hafer, flacher bodenbearbeitung und biofumigations brassicaceen auf beikrautbesatz, erbsenkrankheiten und erträge in der fruchtfolge mit winterweizen. Rheinische Friedrich-Wilhelms-Universität.

Fitt, B. D., Huang, Y. J., van den Bosch, F. and West, J. S. (2006) Coexistence of related pathogen species on arable crops in space and time. *Annual Reviews in Phytopathology,* **44,** 163-182.

Flynn, R. and Idowu, J. (2015) Nitrogen Fixation by Legumes. Available from: www.aces.nmsu.edu/pubs/_a/A129/ (Accessed: 2018)

Force, L. E., O'Hare, T. J., Wong, L. S. and Irving, D. E. (2007) Impact of cold storage on glucosinolate levels in seed-sprouts of broccoli, rocket, white radish and kohl-rabi. *Postharvest Biology and Technology,* **44,** 175-178.

Gangneux, C., Cannesan, M.-A., Bressan, M., Castel, L., Moussart, A., Vicré-Gibouin, M., Driouich, A., Trinsoutrot-Gattin, I. and Laval, K. (2014) A sensitive assay for rapid detection and quantification of Aphanomyces euteiches in soil. *Phytopathology,* **104,** 1138-1147.

Gaulin, E., Jacquet, C., Bottin, A. and Dumas, B. (2007) Root rot disease of legumes caused by Aphanomyces euteiches. *Molecular Plant Pathology,* **8,** 539-548.

Geary, B., Ransom, C., Brown, B., Atkinson, D. and Hafez, S. (2008) Weed, disease, and nematode management in onions with biofumigants and metam sodium. *Horticultural Technology,* **18,** 569-574.

Geiser, D. M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T. J., Zhang, N., Kuldau, G. A. and O'donnell, K. (2004) FUSARIUM-ID v. 1.0: a DNA sequence database for identifying Fusarium. *European Journal of Plant Pathology,* **110,** 473-479.

Gil, V. and MacLeod, A. (1980) The effects of pH on glucosinolate degradation by a thioglucoside glucohydrolase preparation. *Phytochemistry,* **19,** 2547-2551.

Gillaspie Jr, A., Hajimorad, M. and Ghabrial, S. (1998) Characterization of a severe strain of cucumber mosaic cucumovirus seedborne in cowpea. *Plant Disease,* **82,** 419-422.

Gimsing, A. and Kirkegaard, J. (2006) Glucosinolate and isothiocyanate concentration in soil following incorporation of Brassica biofumigants. *Soil Biology and Biochemistry,* **38,** 2255-2264.

Gimsing, A. L. and Kirkegaard, J. A. (2009) Glucosinolates and biofumigation: fate of glucosinolates and their hydrolysis products in soil. *Phytochemistry Reviews,* **8,** 299-310.

Gossen, B. D., Conner, R. L., Chang, K.-F., Pasche, J. S., McLaren, D. L., Henriquez, M. A., Chatterton, S. and Hwang, S.-F. (2016) Identifying and managing root rot of pulses on the northern great plains. *Plant Disease,* **100,** 1965-1978.

Grünwald, N. J. and Coyne, C. (2003) Second international Aphanomyces workshop. In: *International Aphanomyces Workshop 2003,* US Dept. of Agriculture, Agricultural Research Service.

Haegi, A., Catalano, V., Luongo, L., Vitale, S., Scotton, M., Ficcadenti, N. and Belisario, A. (2013) A newly developed real-time PCR assay for detection and quantification of Fusarium oxysporum and its use in compatible and incompatible interactions with grafted melon genotypes. *Phytopathology,* **103,** 802-810.

Hagerty, C., Cuesta-Marcos, A., Cregan, P., Song, Q., McClean, P., Noffsinger, S. and Myers, J. (2015) Mapping Fusarium solani and Aphanomyces euteiches root rot resistance and root architecture quantitative trait loci in common bean. *Crop Science,* **55,** 1969-1977.

Halkier, B. A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annual Reviews of Plant Biology,* **57,** 303-333.

Hamon, C., Coyne, C. J., McGee, R. J., Lesné, A., Esnault, R., Mangin, P., Hervé, M., Le Goff, I., Deniot, G. and Roux-Duparque, M. (2013) QTL meta-analysis provides a comprehensive view of loci controlling partial resistance to Aphanomyces euteiches in four sources of resistance in pea. *BMC Plant Biology,* **13,** 1-19.

Handiseni, M., Brown, J., Zemetra, R. and Mazzola, M. (2013) Effect of Brassicaceae seed meals with different glucosinolate profiles on Rhizoctonia root rot in wheat. *Crop Protection,* **48,** 1-5.

Hanlon, N., Coldham, N., Sauer, M. J. and Ioannides, C. (2009) Modulation of rat pulmonary carcinogen-metabolising enzyme systems by the isothiocyanates erucin and sulforaphane. *Chemico-Biological Interactions,* **177,** 115-120.

Hartwig, N. L. and Ammon, H. U. (2002) Cover crops and living mulches. *Weed Science,* **50,** 688-699.

Haware, M., Nene, Y. and Natarajan, M. (1996) The survival of Fusarium oxysporum f. sp. ciceri in the soil in the absence of chickpea. *Phytopathologia Mediterranea***,** 9-12.

Hebert, P. D., Cywinska, A., Ball, S. L. and DeWaard, J. R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences,* **270,** 313-321.

Hossain, S., Bergkvist, G., Berglund, K., Mårtensson, A. and Persson, P. (2012) Aphanomyces pea root rot disease and control with special reference to impact of Brassicaceae cover crops. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science,* **62,** 477-487.

Hossain, S., Bergkvist, G., Glinwood, R., Berglund, K., Mårtensson, A., Hallin, S. and Persson, P. (2015) Brassicaceae cover crops reduce Aphanomyces pea root

rot without suppressing genetic potential of microbial nitrogen cycling. *Plant and Soil,* **392,** 227-238.

Hou, L. W., Groenewald, J. Z., Pfenning, L. H., Yarden, O., Crous, P. W. and Cai, L. (2020) The Phoma-like dilemma. *Studies in Mycology,* **96,** 309-396.

Hughes, T. and Grau, C. (2007) Aphanomyces root rot (common root rot) of legumes. The Plant Health Instructor, Available from: https://www.apsnet.org/edcenter/disandpath/oomycete/pdlessons/Pages/Aphanomyces.aspx (Accessed: 2021).

Hwang, S. and Chang, K. (1989) Incidence and severity of root rot disease complex of field pea in northeastern Alberta in 1988. *Canadian Plant Disease Survey,* **69,** 139-141.

Hwang, S., Gossen, B., Conner, R., Chang, K., Turnbull, G., Lopetinsky, K. and Howard, R. (2007) Management strategies to reduce losses caused by Rhizoctonia seedling blight of field pea. *Canadian Journal of Plant Science,* **87,** 145-155.

IBM Corporation (2021) IBM SPSS Statistics for Windows. Armonk, NY: IBM Corporation.

Jelden, T. and Herold, L. (2020) Investigating the integration of cover cropping into vining pea rotations. PGRO, Available from: https://www.pgro.org/downloads/Investigatingcovercropsinviningpea-rotations3rdreport.pdf (Accessed: 2020).

Jenkins, S. (2018) Examining identity, phylogeny and pathogenicity factors in Fusarium species affecting pea. University of Warwick.

Jones, F. R. and Drechsler, C. (1925) Root rot of peas in the United States caused by Aphanomyces euteiches. *Journal of Agricultural Research,* **30,** 293.

Jones, L. K. (1927) Studies of the nature and control of blight, leaf and pod spot, and foot-rot of Peas caused by species of Aschochyta. Agricultural Experiment Station, Available from: https://ecommons.cornell.edu/handle/1813/4616 (Accessed: 2020).

Karlsson, I., Persson, P. and Friberg, H. (2021) Fusarium head blight from a microbiome perspective. *Frontiers in Microbiology,* **12,** 371.

Keirnan, E. C., Tan, Y. P., Laurence, M. H., Mertin, A. A., Liew, E. C., Summerell, B. A. and Shivas, R. G. (2021) Cryptic diversity found in Didymellaceae from Australian native legumes. *MycoKeys,* **78,** 1.

Kerr, A. (1963) The root rot-Fusarium wilt complex of peas. *Australian Journal of Biological Sciences,* **16,** 55-69.

Kirkegaard, J. and Matthiessen, J. (2004) Developing and refining the biofumigation concept. *Agroindustria,* **3,** 233-239.

Koppenhöfer, A. and Kaya, H. (1997) Additive and synergistic interaction between entomopathogenic nematodes and Bacillus thuringiensis for scarab grub control. *Biological Control,* **8,** 131-137.

Kraft, J.M. (1994) Fusarium wilt of peas (a review). *Agronomie*, **14**, 561-567.

Kraft, J. and Roberts, D. (1970) Resistance in peas to Fusarium and Pythium root rot. *Phytopathology,* **60,** 1814-1817.

Kraft, J. M. and Pfleger, F. L. (2001) *Compendium of pea diseases and pests*. American Phytopathological Society (APS Press).

Lamichhane, J. R. and Venturi, V. (2015) Synergisms between microbial pathogens in plant disease complexes: a growing trend. *Frontiers in Plant Science,* **6,** 385.

Larkin, R. P. and Griffin, T. S. (2007) Control of soilborne potato diseases using Brassica green manures. *Crop Protection,* **26,** 1067-1077.

Le May, C., Potage, G., Andrivon, D., Tivoli, B. and Outreman, Y. (2009) Plant disease complex: Antagonism and synergism between pathogens of the Ascochyta blight complex on pea. *Journal of Phytopathology,* **157,** 715-721.

LEGVALUE (2017) Vining pea value chain case study and farm network (UK). Available from: http://www.legvalue.eu/industry-studies/vining-pea-for-frozenprocessed-market-eastern-england-united-kingdom/abstract/ (Accessed: 2018).

Lewis, J. and Papavizas, G. (1970) Evolution of volatile sulfur-containing compounds from decomposition of crucifers in soil. *Soil Biology and Biochemistry,* **2,** 239-246.

Liu, Y. J., Whelen, S. and Hall, B. D. (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution,* **16,** 1799-1808.

Lockwood, J. L. (1961) Soil fungicides for control of pea root rot in greenhouse tests. *The Plant Disease Reporter,* **45,** 569.

Lucarini, E., Pagnotta, E., Micheli, L., Parisio, C., Testai, L., Martelli, A., Calderone, V., Matteo, R., Lazzeri, L. and Di Cesare Mannelli, L. (2019) Eruca sativa meal against diabetic neuropathic pain: An H2S-mediated effect of glucoerucin. *Molecules,* **24,** 3006.

Marcinkowska, J. (2008) Fungi occurrence on seeds of field pea. *Acta Mycologica,* **43**.

Marin, P., Moretti, A., Ritieni, A., Jurado, M., Vazquez, C. and Gonzalez-Jaen, M. T. (2012) Phylogenetic analyses and toxigenic profiles of Fusarium equiseti and Fusarium acuminatum isolated from cereals from Southern Europe. *Food Microbiology,* **31,** 229-237.

Mathew, F., Lamppa, R., Chittem, K., Chang, Y., Botschner, M., Kinzer, K., Goswami, R. and Markell, S. (2012) Characterization and pathogenicity of Rhizoctonia solani isolates affecting Pisum sativum in North Dakota. *Plant Disease,* **96,** 666-672.

Matthiessen, J. N. and Kirkegaard, J. A. (2006) Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. *Critical Reviews in Plant Sciences,* **25,** 235-265.

Mattner, S., Porter, I., Gounder, R., Shanks, A., Wren, D. and Allen, D. (2008) Factors that impact on the ability of biofumigants to suppress fungal pathogens and weeds of strawberry. *Crop Protection,* **27,** 1165-1173.

Matusheski, N. V. and Jeffery, E. H. (2001) Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *Journal of Agricultural and Food Chemistry,* **49,** 5743-5749.

McKay, K., Schatz, B. G. and Endres, G. (2003) *Field pea production*. NDSU Extension Service.

McNeil, M., Roberts, A., Cockerell, V. and Mulholland, V. (2004) Real-time PCR assay for quantification of Tilletia caries contamination of UK wheat seed. *Plant Pathology,* **53,** 741-750.

Meng, L., Yao, X., Yang, Z., Zhang, R., Zhang, C., Wang, X., Xu, N., Li, S., Liu, T. and Zheng, C. (2018) Changes in soil microbial diversity and control of Fusarium oxysporum in continuous cropping cucumber greenhouses following biofumigation. *Emirates Journal of Food and Agriculture***,** 644-653.

Mithen, R. (2001) Glucosinolates–biochemistry, genetics and biological activity. *Plant Growth Regulation,* **34,** 91-103.

Miyamoto, K., Yamasaki, T., Uheda, E. and Ueda, J. (2014) Analysis of apical hook formation in Alaska pea with a 3-D clinostat and agravitropic mutant ageotropum. *Frontiers in Plant Science*, **5**, 137.

Morgan-Jones, G. and Burch, K. (1988) Studies in the genus Phoma. X: Concerning Phoma eupyrena, an ubiquitous, soil-borne species. *Mycotaxon,* **31,** 427-434.

Muehlbauer, F. and Kraft, J. (1973) Evidence of heritable resistance to Fusarium solani f. sp. pisi and Pythium ultimum in peas 1. *Crop Science,* **13,** 34-36.

Muehlchen, A., Rand, R. and Parke, J. (1990) Evaluation of crucifer green manures for controlling Aphanomyces root rot of peas. *Plant Disease,* **74,** 651-654.

Murakami, H., Tsushima, S. and Shishido, Y. (2002) Factors affecting the pattern of the dose response curve of clubroot disease caused by Plasmodiophora brassicae. *Soil Science and Plant Nutrition,* **48,** 421-427.

Nel, W.J., Duong, T.A., De Beer, Z.W. and Wingfield, M.J. (2019) Black root rot: a long known but little understood disease. *Plant Pathology*, **68**, 834-842.

Nelson, P. E., Dignani, M. C. and Anaissie, E. J. (1994) Taxonomy, biology, and clinical aspects of Fusarium species. *Clinical Microbiology Reviews,* **7,** 479-504.

Neubauer, C., Heitmann, B. and Müller, C. (2014) Biofumigation potential of Brassicaceae cultivars to Verticillium dahliae. *European Journal of Plant Pathology,* **140,** 341-352.

Neupane, S. (2013) Elucidating the potential of biocontrol of stem canker in potato by Salvia hispanica L. Biofumigation and Arbuscular Mycorrhizal Fungus. Thesis. Aarhus University. Dinamarca.

Newman, Y. C., Wright, D., Mackowiak, C., Scholberg, J. and Cherr, C. (2007) Benefits of cover crops for soil health. *EDIS.*

Ngala, B. M., Haydock, P. P., Woods, S. and Back, M. A. (2015) Biofumigation with Brassica juncea, Raphanus sativus and Eruca sativa for the management of field populations of the potato cyst nematode Globodera pallida. *Pest Management Science,* **71,** 759-769.

Nirenberg, H. I. (1995) Morphological differentiation of Fusarium sambucinum Fuckel sensu stricto, F. torulosum (Berk. & Curt.) Nirenberg comb. nov. and F. venenatum Nirenberg sp. nov. *Mycopathologia,* **129,** 131-141.

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

O'Donnell, K. (2000) Molecular phylogeny of the Nectria haematococca-Fusarium solani species complex. *Mycologia,* **92,** 919-938.

O'Donnell, K. and Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium. *Molecular Phylogenetics and Evolution,* **7,** 103-116.

O'Donnell, K., Cigelnik, E. and Nirenberg, H. I. (1998) Molecular systematics and phylogeography of the Gibberella fujikuroi species complex. *Mycologia,* **90,** 465-493.

O'Donnell, K. and Gray, L. E. (1995) Phylogenetic relationships of the soybean sudden death syndrome pathogen Fusarium solani f. sp. phaseoli inferred from rDNA sequence data and PCR primers for its identification. *Molecular Plant-Microbe Interactions,* **8,** 709-716.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Magnon, K. C., Cox, P. A., Revankar, S. G., Sanche, S., Geiser, D. M., Juba, J. H., van Burik, J. A., Padhye, A., Anaissie, E. J., Francesconi, A., Walsh, T. J. and Robinson, J. S. (2004) Genetic diversity of human pathogenic members of the Fusarium oxysporum complex inferred from multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. *Journal of Clinical Microbiology,* **42,** 5109-5120.

Ogilvie, L. (1930) Observations on the diseases of market-garden crops in the Yale of Evesham.

Okunade, O. A., Ghawi, S. K., Methven, L. and Niranjan, K. (2015) Thermal and pressure stability of myrosinase enzymes from black mustard (Brassica nigra LWDJ Koch. var. nigra), brown mustard (Brassica juncea L. Czern. var. juncea) and yellow mustard (Sinapsis alba L. subsp. maire) seeds. *Food Chemistry,* **187,** 485-490.

Oyarzun, P., Dijst, G., Zoon, F. and Maas, P. T. (1997) Comparison of soil receptivity to Thielaviopsis basicola, Aphanomyces euteiches, and Fusarium solani f. sp. pisi causing root rot in pea. *Phytopathology,* **87,** 534-541.

Oyarzun, P., Gerlagh, M. and Hoogland, A. (1993) Pathogenic fungi involved in root rot of peas in the Netherlands and their physiological specialization. *Netherlands Journal of Plant Pathology,* **99,** 23-33.

Papavizas, G. C. and Ayers, W. A. (1974) Aphanomyces species and their root diseases in pea and sugarbeet. US Department of Agriculture.

Parke, J., Rand, R., Joy, A. and King, E. (1991) Biological control of Pythium damping-off and Aphanomyces root rot of peas by application of Pseudomonas cepacia or P. fluorescens to seed. *Plant Disease,* **75,** 987-992.

Pavek, P. L. S. (2012) Plant guide for pea (Pisum sativum L.). USDA Natural Resources Conservation Service.

Persson, L., Bodker, L. and Larsson-Wikstrom, M. (1997) Prevalence and pathogenicity of foot and root rot pathogens of pea in Southern Scandinavia. *Plant Disease,* **81,** 171-174.

Peters, R. D. and Grau, C. R. (2002) Inoculation with nonpathogenic Fusarium solani increases severity of pea root rot caused by Aphanomyces euteiches. *Plant Disease,* **86,** 411-414.

PGRO (2017) PGRO vining pea growers guide. Available from: http://www.pgro.org/downloads/PGRO-VINING-GUIDE-2016.pdf (Accessed: 2020).

Pilet-Nayel, L., Muehlbauer, F. J., McGee, R. J., Kraft, J. M., Baranger, A. and Coyne, C. J. (2002) Quantitative trait loci for partial resistance to Aphanomyces root rot in pea. *Theoretical Applied Genetics,* **106,** 28-39.

Pilet-Nayel, M.-L., Muehlbauer, F., McGee, R., Kraft, J., Baranger, A. and Coyne, C. (2005) Consistent quantitative trait loci in pea for partial resistance to Aphanomyces euteiches isolates from the United States and France. *Phytopathology,* **95,** 1287-1293.

Portales-Reyes, C., Van Doornik, T., Schultheis, E. H. and Suwa, T. (2015) A novel impact of a novel weapon: allelochemicals in Alliaria petiolata disrupt the legume-rhizobia mutualism. *Biological Invasions,* **17,** 2779-2791.

Porter, L. D., Pasche, J. S., Chen, W. and Harveson, R. M. (2015) Isolation, identification, storage, pathogenicity tests, hosts, and geographic range of Fusarium solani f. sp. pisi causing Fusarium root rot of pea. *Plant Health Progress,* **16,** 136-145.

Prasad, P. and Kumar, J. (2017) Management of Fusarium wilt of chickpea using brassicas as biofumigants. *Legume Research,* **40,** 178-182.

Price, A. J., Charron, C. S., Saxton, A. M. and Sams, C. E. (2005) Allyl isothiocyanate and carbon dioxide produced during degradation of Brassica juncea tissue in different soil conditions. *HortScience,* **40,** 1734-1739.

Rana, A., Sahgal, M. and Johri, B. (2017) Fusarium oxysporum: genomics, diversity and plant–host interaction. In: *Developments in fungal biology and applied mycology.* Springer, pp. 159-199.

Redinbaugh, M. G. and Stewart, L. R. (2018) Maize lethal necrosis: An emerging, synergistic viral disease. *Annual Review of Virology,* **5,** 301-322.

Riaz, R., Khan, A., Khan, W. J., Jabeen, Z., Yasmin, H., Naz, R., Nosheen, A. and Hassan, M. N. (2021) Vegetable associated Bacillus spp. suppress the pea (Pisum sativum L.) root rot caused by Fusarium solani. *Biological Control,* **158,** 104610.

Riga, E., Porter, L.D., Mojtahedi, H. and Erickson, D. (2008) Pratylenchus neglectus, P. thornei, and Paratylenchus hamatus nematodes causing yield reduction to dryland peas and lentils in Idaho. *Plant Disease*, **92**, 979-979.

Rigorth, K. S., Finckh, M. and Sisic, A. (2021) First Report of Fusarium venenatum causing foot and root rot of wheat (Triticum aestivum) in Germany. *Plant Disease*.

Romberg, M. K. and Davis, R. M. (2007) Host Range and phylogeny of Fusarium solani f. sp. eumartii from potato and tomato in California. *Plant Disease,* **91,** 585-592.

Rosa, E. (1997) Glucosinolates in crop plants. *Horticulture Reviews,* **19,** 99-215.

Salt, G. A. and Delaney, K. D. (2013) Infuence of previous legume crops on root diseases in peas and beans. In: *The pea crop: a basis for improvement.* (Hebblethwaite, P. D., Heath, M. C. and Dawkins, T. C., eds.). Butterworth-Heinemann.

Santos, C. A. D., Abboud, A. C. D. S. and Carmo, M. G. F. D. (2020) Biofumigation with species of the Brassicaceae family: a review. *Ciência Rural,* **51**.

Sarwar, M. and Kirkegaard, J. (1998) Biofumigation potential of brassicas: II. Effect of environment and ontogeny on glucosinolate production and implications for screening. *Plant and Soil***,** 91-101.

Schippers, B. and Voetberg, J. S. (1969) Germination of chlamydospores of Fusarium oxysporum f. sp. pisi race 1 in the rhizosphere, and penetration of the pathogen into roots of a susceptible and a resistant pea cultivar. *Netherlands Journal of Plant Pathology,* **75,** 241-258.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. and Consortium, F. B. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences,* **109,** 6241-6246.

Schroers, H. J., Samuels, G. J., Zhang, N., Short, D. P., Juba, J. and Geiser, D. M. (2016) Epitypification of Fusisporium (Fusarium) solani and its assignment to a common phylogenetic species in the Fusarium solani species complex. *Mycologia,* **108,** 806-819.

Seifert, K. A. (2009) Progress towards DNA barcoding of fungi. *Molecular Ecology Resources,* **9,** 83-89.

Sexton, A., Kirkegaard, J. and Howlett, B. (1999) Glucosinolates in Brassica juncea and resistance to Australian isolates of Leptosphaeria maculans, the blackleg fungus. *Australasian Plant Pathology,* **28,** 95-102.

Šišić, A., Baćanović-Šišić, J., Al-Hatmi, A. M., Karlovsky, P., Ahmed, S. A., Maier, W., De Hoog, G. S. and Finckh, M. R. (2018) The 'forma specialis' issue in Fusarium: a case study in Fusarium solani f. sp. pisi. *Scientific Reports,* **8,** 1-17.

Šišić, A., Baćanović, J. and Finckh, M. R. (2017) Endophytic Fusarium equiseti stimulates plant growth and reduces root rot disease of pea (Pisum sativum L.) caused by Fusarium avenaceum and Peyronellaea pinodella. *European Journal of Plant Pathology,* **148,** 271-282.

Skoglund, L. G., Harveson, R. M., Chen, W., Dugan, F., Schwartz, H. F., Markell, S. G., Porter, L., Burrows, M. L. and Goswami, R. (2011) Ascochyta blight of peas. *Plant Health Progress,* **12,** 29.

Smith, P., Foster, E., Boyd, L. and Brown, J. (1996) The early development of Erysiphe pisi on Pisum sativum L. *Plant Pathology,* **45,** 302-309.

Smolinska, U., Knudsen, G. R., Morra, M. J. and Borek, V. (1997) Inhibition of Aphanomyces euteiches f. sp. pisi by volatiles produced by hydrolysis of Brassica napus seed meal. *Plant Disease,* **81,** 288-292.

Smolinska, U. and Kowalczyk, W. (2014) The impact of the Brassicaceae plant materials added to the soil on the population of Fusarium solani (Mart.) Sacc. and Fusarium oxysporum Schlecht. *Journal of Horticultural Research,* **22**.

Smolinska, U., Morra, M., Knudsen, G. and James, R. (2003) Isothiocyanates produced by Brassicaceae species as inhibitors of Fusarium oxysporum. *Plant Disease,* **87,** 407-412.

Sørensen, J. and Sessitsch, A. (2007) Plant-associated bacteria lifestyle and molecular interactions. *Modern Soil Microbiology,* **2,** 211-236.

Soylu, S. and Dervis, S. (2011) Determination of prevalence and incidence of fungal disease agents of pea (Pisum sativum L.) plants growing in Amik plain of Turkey. *Research on Crops,* **12,** 588-592.

Stecher, G., Tamura, K. and Kumar, S. (2020) Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Molecular Biology and Evolution,* **37,** 1237-1239.

Stegmark, R. (1994) Downy mildew on peas (Peronospora viciae f. sp. pisi). *Agronomie,* **14,** 641-647.

Stevenson, F. and Kessel, C. v. (1996) The nitrogen and non-nitrogen rotation benefits of pea to succeeding crops. *Canadian Journal of Plant Science,* **76,** 735-745.

Stover, R. (1970) Banana root diseases caused by Fusarium oxysporum f. sp. cubense, Pseudomonas solanacearum, and Radopholus similis: A comparative study of life cycles in relation to control. In: *Root diseases and soil-borne pathogens.* University of California Press, pp. 197-200.

Suga, H., Hasegawa, T., Mitsui, H., Kageyama, K. and Hyakumachi, M. (2000) Phylogenetic analysis of the phytopathogenic fungus Fusarium solani based on the rDNA-ITS region. *Mycological Research,* **104,** 1175-1183.

Sung, G.-H., Sung, J.-M., Hywel-Jones, N. L. and Spatafora, J. W. (2007) A multi-gene phylogeny of Clavicipitaceae (Ascomycota, fungi): Identification of localized incongruence using a combinational bootstrap approach. *Molecular Phylogenetics and Evolution,* **44,** 1204-1223.

Syller, J. (2012) Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology,* **13,** 204-216.

Syngenta (2018) Emerging soil-borne problems for pea growers. Available from: https://www.syngenta.co.uk/news/product-update/emerging-soil-borne-problems-pea-growers (Accessed: 2021).

Taguiam, J. D., Evallo, E. and Balendres, M. A. (2021) Epicoccum species: ubiquitous plant pathogens and effective biological control agents. *European Journal of Plant Pathology,* **159,** 713-725.

Taiz, L., and Zeiger, E. (2010) *Plant Physiology*. 5th edition. Sinauer Associates, Inc., Sunderland.

Taylor, F. I., Kenyon, D. and Rosser, S. (2014) Isothiocyanates inhibit fungal pathogens of potato in in vitro assays. *Plant and Soil,* **382,** 281-289.

Tekpinar, A. D. and Kalmer, A. (2019) Utility of various molecular markers in fungal identification and phylogeny. *Nova Hedwigia,* **109,** 187-224.

Tran, H. S., You, M. P., Khan, T. N. and Barbetti, M. J. (2016) Pea black spot disease complex on field pea: dissecting the roles of the different pathogens in causing epicotyl and root disease. *European Journal of Plant Pathology,* **144,** 595-605.

Trantas, E. A., Sarris, P. F., Mpalantinaki, E. E., Pentari, M. G., Ververidis, F. N. and Goumas, D. E. (2013) A new genomovar of Pseudomonas cichorii, a causal agent of tomato pith necrosis. *European Journal of Plant Pathology,* **137,** 477-493.

Trněný, O., Brus, J., Hradilová, I., Rathore, A., Das, R. R., Kopecký, P., Coyne, C. J., Reeves, P., Richards, C. and Smýkal, P. (2018) Molecular evidence for two domestication events in the pea crop. *Genes,* **9,** 535.

Tsao, R., Yu, Q., Potter, J. and Chiba, M. (2002) Direct and simultaneous analysis of sinigrin and allyl isothiocyanate in mustard samples by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry,* **50,** 4749-4753.

Tu, J. (1987) Integrated control of the pea root rot disease complex in Ontario. *Plant Disease,* **71,** 9-13.

Tulbek, M. (2014) Pulse flours as functional ingredients. In: *IUFOST Annual Conference.* Montreal, QC, Canada.

Tulbek, M., Lam, R., Asavajaru, P. and Lam, A. (2017) Pea: A sustainable vegetable protein crop. In: *Sustainable Protein Sources.* Elsevier, pp. 145-164.

Vakalounakis, D. J. (1996) Root and stem rot of cumcumber caused by Fusarium oxysporum f. sp. radicis-cucumerinum f. sp. nov. *Plant Disease,* **80,** 313-316.

Valenzuela-Lopez, N., Cano-Lira, J. F., Guarro, J., Sutton, D. A., Wiederhold, N., Crous, P. W. and Stchigel, A. M. (2018) Coelomycetous Dothideomycetes with emphasis on the families Cucurbitariaceae and Didymellaceae. *Studies in Mycology,* **90,** 1-69.

Vilgalys, R. and Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. *Journal of Bacteriology,* **172,** 4238-4246.

VSN International (2021) Genstat for Windows. Hemel Hempstead, UK: VSN International. Wada, K.C. and Takeno, K., 2010. Stress-induced flowering. Plant signaling & behavior, 5(8), pp.944-947.

Wada, K.C. and Takeno, K. (2010) Stress-induced flowering. *Plant Signaling and Behavior*, **5**, 944-947.

Wagner, A. E., Sturm, C., Piegholdt, S., Wolf, I. M., Esatbeyoglu, T., De Nicola, G. R., Iori, R. and Rimbach, G. (2015) Myrosinase-treated glucoerucin is a potent inducer of the Nrf2 target gene heme oxygenase 1—Studies in cultured HT-29 cells and mice. *The Journal of Nutritional Biochemistry,* **26,** 661-666.

Wakelin, S., Walter, M., Jaspers, M. and Stewart, A. (2002) Biological control of Aphanomyces euteiches root-rot of pea with spore-forming bacteria. *Australasian Plant Pathology,* **31,** 401-407.

Wallen, V. (1974) Influence of three Ascochyta diseases of peas on plant development and yield. *Canadian Plant Disease Survey,* **54,** 86-90.

Wallen, V., Wong, S. and Jeun, J. (1967) Isolation, incidence, and virulence of Ascochyta spp. of peas from the soil. *Canadian Journal of Botany,* **45,** 2243-2247.

Warmington, R. and Clarkson, J. P. (2016) Volatiles from biofumigant plants have a direct effect on carpogenic germination of sclerotia and mycelial growth of Sclerotinia sclerotiorum. *Plant and Soil,* **401,** 213-229.

Warmington, R. J. (2014) Pathogen diversity, epidemiology and control of Sclerotinia disease in vegetable crops. University of Warwick. PhD Thesis.

Watson, A. (2013) Development of methods to monitor and control Aphanomyces root rot and black root rot of beans. NSW Department of Primary Industries.

Westphal, A., Li, C., Xing, L., McKay, A. and Malvick, D. (2014) Contributions of Fusarium virguliforme and Heterodera glycines to the disease complex of sudden death syndrome of soybean. *PLoS One,* **9,** e99529.

White, T. J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a Guide to Methods and Applications,* **18,** 315-322.

Wicker, E., Moussart, A., Duparque, M. and Rouxel, F. (2003) Further contributions to the development of a differential set of pea cultivars (Pisum sativum) to investigate the virulence of isolates of Aphanomyces euteiches. *European Journal of Plant Pathology,* **109,** 47-60.

Wicker, E. and Rouxel, F. (2001) Specific behaviour of French Aphanomyces euteiches Drechs. populations for virulence and aggressiveness on pea,

related to isolates from Europe, America and New Zealand. *European Journal of Plant Pathology,* **107,** 919-929.

Wiebe, M. (2002) Myco-protein from Fusarium venenatum: a well-established product for human consumption. *Applied Microbiology and Biotechnology,* **58,** 421-427.

Williams-Woodward, J. L., Pfleger, F., Fritz, V. A. and Allmaras, R. (1997) Green manures of oat, rape and sweet corn for reducing common root rot in pea (Pisum sativum) caused by Aphanomyces euteiches. *Plant and Soil,* **188,** 43-48.

Williams, A. H., Sharma, M., Thatcher, L. F., Azam, S., Hane, J. K., Sperschneider, J., Kidd, B. N., Anderson, J. P., Ghosh, R. and Garg, G. (2016) Comparative genomics and prediction of conditionally dispensable sequences in legume–infecting Fusarium oxysporum formae speciales facilitates identification of candidate effectors. *BMC Genomics,* **17,** 1-24.

Willsey, T., Chatterton, S. and Cárcamo, H. (2019) Interactions between the root rot pathogen Fusarium avenaceum and the pea leaf weevil (Sitona lineatus) in field pea. *Crop Protection*, **116**, 108-114.

Willsey, T., Chatterton, S., Heynen, M. and Erickson, A. (2018) Detection of interactions between the pea root rot pathogens Aphanomyces euteiches and Fusarium spp. using a multiplex qPCR assay. *Plant Pathology,* **67,** 1912-1923.

Woudenberg, J. H., Aveskamp, M. M., de Gruyter, J., Spiers, A. G. and Crous, P. W. (2009) Multiple Didymella teleomorphs are linked to the Phoma clematidina morphotype. *Persoonia,* **22,** 56-62.

Wu, L., Chang, K.-F., Conner, R. L., Strelkov, S., Fredua-Agyeman, R., Hwang, S.-F. and Feindel, D. (2018) Aphanomyces euteiches: a threat to Canadian field pea production. *Engineering,* **4,** 542-551.

Xue, A. G. (2003) Biological control of pathogens causing root rot complex in field pea using Clonostachys rosea strain ACM941. *Phytopathology,* **93,** 329-335.

Yang, Z. and Rannala, B. (2012) Molecular phylogenetics: principles and practice. *Nature Reviews Genetics,* **13,** 303-314.

Yes Peas (2021) Pea Facts Available from: https://peas.org/pea-facts/ (Accessed: 2021).

Young, A. D. and Gillung, J. P. (2020) Phylogenomics - principles, opportunities and pitfalls of big-data phylogenetics. *Systematic Entomology*.

Zhao, P., Crous, P., Hou, L., Duan, W., Cai, L., Ma, Z. and Liu, F. (2021) Fungi of quarantine concern for China I: Dothideomycetes. *Persoonia - Molecular Phylogeny and Evolution of Fungi*.

Zitnick-Anderson, K., Simons, K. and Pasche, J. S. (2018) Detection and qPCR quantification of seven Fusarium species associated with the root rot complex in field pea. *Canadian Journal of Plant Pathology,* **40,** 261-271.

Appendix

Table A 1. Isolates characterised as part of the *Didymella* phylogenetic analysis with the relevant GenBank accession number for sequence data.

Species	Isolate name	Substrate/host	Country	GenBank accession number			
				ITS	***RPB2***	***TUB2***	***LSU***
Ascobolus crenulatus	Holbeach	Soil	UK	n/a	n/a	n/a	n/a
Ascochyta koolunga	CBS 189.91	Pea	Italy	MN972694	MN983286	MN983711	n/a
Ascochyta koolunga	CBS 373.84	Pea	Australia	KT389481	KT389560	KT389775	KT389698
Ascochyta koolunga	CBS 372.84	Pea	Australia	KT389480	MT018254	KT389774	KT389697
Collectotrichum coccodes	7838	Soil	UK	n/a	n/a	n/a	n/a
Didymella glomerata	CBS 120109	*Juniperus* sp.	USA	FJ427015	MN983455	FJ427126	MN973216
Didymella glomerata	CBS 284.76	Black poplar	Russia	FJ427005	MN983459	FJ427116	FJ427005
Didymella glomerata	CBS 293.36	Potato	Germany	FJ427010	MN983466	FJ427121	MN973226
Didymella pinodella	61B	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	VH1	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	VH3	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Telegraph A	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Telegraph B	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Silt Pits A	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Silt Pits B	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Ralston A	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Ralston B	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Cockie A	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Cockie E	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Majors South A	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	Majors South B	Soil	UK	n/a	n/a	n/a	n/a
Didymella pinodella	ATCC 38814	Pea	Canada	MW945409	MZ073894	n/a	n/a
Didymella pinodella	BRIP 69589	Pea	Australia	MN567674	MN604925	n/a	n/a
Didymella pinodella	CBS 531.66	Red clover	USA	FJ427052	KT389613	FJ427162	n/a
Didymella pinodella	CBS 123522	Pea	USA	MN972896	MN983527	MN983911	n/a

Species	Isolate name	Substrate/host	Country	GenBank accession number			
				ITS	***RPB2***	***TUB2***	***LSU***
Didymella pinodella	CBS 114.36	Faba bean	Czechoslovakia	MN972899	MN983530	MN983914	n/a
Didymella pinodella	CBS 108.46	Pea	The Netherlands	MN972894	MN983525	MN983909	n/a
Didymella pinodella	CBS 107.46	Pea	The Netherlands	MN972895	MN983526	MN983910	n/a
Didymella pinodes	CBS 159.78	Pea	Iraq	MN972910	MN983542	MN983925	MN973297
Didymella pinodes	CBS 249.47	Pea	UK	MN972907	MN983539	MN983922	MN973294
Didymella pinodes	CBS 525.77	Pea	Belgium	GU237883	KT389614	GU237572	GU238023
Didymella pinodes	CBS 123523	Pea	USA	MN972908	MN983540	MN983923	n/a
Didymella pinodes	CBS 374.84	Pea	The Netherlands	MN972909	MN983541	MN983924	MN973296
Ascochyta pisi	CBS 122748	Pea	Bulgaria	MN972702	MN983296	MN983718	n/a
Ascochyta pisi	CBS 122750	Pea	USA	KT389477	MN983298	KT389771	n/a
Ascochyta pisi	CBS 122751	Pea	Canada	KP330432	EU874867	KP330388	KP330444
Ascochyta pisi	CBS 126.54	Pea	The Netherlands	GU237772	DQ677967	GU237531	EU754137
Ascochyta pisi	CBS 448.86	Pea	Egypt	MN972704	MN983299	MN983720	n/a
Fusarium solani	Phoma L	Unknown root	Unknown	n/a	n/a	n/a	n/a
Juxtiphoma eupyrena	Kincreich	Soil	UK	n/a	n/a	n/a	n/a
Leptosphaeria maculans	CBS 275.63	*Brassica* sp.	UK	MH858282	KT389669	KT389841	JF740306
Phoma herbarum	CBS 567.63	*European crab apple*	USA	JF810528	MT018261	MT005699	MN943799
Phoma herbarum	CBS 615.75	Multiflora rose	The Netherlands	FJ427022	KP330420	FJ427133	EU754186
Phoma herbarum	CBS 618.75	Common Ivy	Italy	MN973010	MN983648	MN984022	n/a
Phoma herbarum	CBS 274.37	Norway Spruce	UK	KT389537	KT389662	KT389835	KT389754
Phoma herbarum	CBS 110739	*Eucalyptus nitens*	South Africa	MN973008	MN983646	MN984020	MN973397

Table A 2. Isolates characterised as part of the *Fusarium* phylogenetic analysis with the relevant GenBank accession number for sequence data.

Species	Isolate name	Substrate/host	Country	GenBank accession number	
				TEF1-α	***RPB2***
Fusarium solani	PG504	Pea	UK	n/a	n/a
Fusarium solani	PG463	Pea	UK	n/a	n/a
Fusarium solani	PG462	Pea	UK	n/a	n/a
Fusarium solani	PG239	Pea	UK	n/a	n/a
Fusarium solani	PG362	Pea	UK	n/a	n/a
Fusarium solani	PG461	Pea	UK	n/a	n/a
Fusarium solani	PG100	Pea	UK	n/a	n/a
Fusarium solani	PG166	Pea	UK	n/a	n/a
Fusarium solani	PG253	Pea	UK	n/a	n/a
Fusarium solani	PG319	Pea	UK	n/a	n/a
Fusarium solani	PG503	Pea	UK	n/a	n/a
Fusarium solani	PG439	Pea	UK	n/a	n/a
Fusarium solani	PG484	Pea	UK	n/a	n/a
Fusarium solani	PG501	Pea	UK	n/a	n/a
Fusarium solani	PG143	Pea	UK	n/a	n/a
Fusarium solani	F1	n/a	UK	n/a	n/a
Fusarium solani	F5	n/a	UK	n/a	n/a
Fusarium venenatum	F7	n/a	UK	n/a	n/a
Fusarium venenatum	F8	n/a	UK	n/a	n/a
Fusarium solani	F9	n/a	UK	n/a	n/a
Fusarium solani	F12	n/a	UK	n/a	n/a
Fusarium solani	F17	n/a	UK	n/a	n/a
Fusarium solani	F22	n/a	UK	n/a	n/a
Fusarium solani	F42	n/a	UK	n/a	n/a
Fusarium oxysporum	F55	n/a	UK	n/a	n/a
Fusarium oxysporum	F55	n/a	UK	n/a	n/a

Species	Isolate name	Substrate/host	Country	GenBank accession number	
				TEF1-α	***RPB2***
Fusarium solani	F60	n/a	UK	n/a	n/a
Fusarium solani	F61	n/a	UK	n/a	n/a
Fusarium solani	F76	n/a	UK	n/a	n/a
Fusarium equiseti	F105	n/a	UK	n/a	n/a
Fusarium equiseti	F109	n/a	UK	n/a	n/a
Fusarium redolens	F112	n/a	UK	n/a	n/a
Fusisporium solani	FS5	Pea	Germany	KY556511	n/a
Fusarium solani	FS6	Pea	Germany	KY556459	n/a
Fusarium solani	FS7	Pea	Germany	KY556466	n/a
Fusarium solani	FS8	Pea	Germany	KY556450	n/a
Fusisporium solani	FS4	Pea	Germany	KY556500	KY556500
Fusarium solani	FS1	Pea	Germany	KY556491	n/a
Fusarium solani	FS2	Pea	Germany	KY556463	n/a
Fusarium sterciola	FS29	Subterranean clover	Germany	KY556524	n/a
Fusarium solani	FS3	Pea	Germany	KY556448	n/a
Fusarium witzenhausenense	FS30	Subterranean clover	Germany	KY556525	n/a
Fusarium solani	FS31	Subterranean clover	Germany	KY556520	n/a
Fusarium solani	FS45	Subterranean clover	Switzerland	KY556521	n/a
Fusarium solani	FS60	White clover	Italy	KY556518	n/a
Fusarium solani	FS63	Subterranean clover	Italy	KY556519	n/a
Fusarium solani	FS69	Pea	Italy	KY556522	n/a
Fusisporium solani	FS79	Subterranean clover	Italy	KY556516	KY556516
Fusarium solani	FS9	Pea	Germany	KY556451	n/a
Fusarium solani	FS10	Pea	Germany	KY556452	n/a
Fusarium solani	FS11	Pea	Germany	KY556497	n/a
Fusarium solani	FS12	Pea	Germany	KY556447	n/a
Fusarium solani	FS13	Pea	Germany	KY556453	n/a
Fusarium solani	FS14	Pea	Germany	KY556449	n/a
Fusarium solani	FS15	Pea seed	Germany	KY556492	n/a

Species	Isolate name	Substrate/host	Country	GenBank accession number	
				TEF1-α	***RPB2***
Fusarium solani	FS16	Pea seed	Germany	KY556493	n/a
Fusarium solani	FS17	Pea seed	Germany	KY556471	n/a
Fusarium solani	FS18	Pea seed	Germany	KY556458	KY556526
Fusarium solani	FS19	Pea seed	Germany	KY556472	KY556535
Fusarium solani	FS20	Subterranean clover	Germany	KY556488	KY556536
Fusarium solani	FS21	Subterranean clover	Germany	KY556454	KY556537
Fusarium solani	FS22	Subterranean clover	Germany	KY556473	KY556527
Fusarium solani	FS23	Subterranean clover	Germany	KY556455	KY556538
Fusarium solani	FS24	Subterranean clover	Germany	KY556474	n/a
Fusarium solani	FS25	Faba bean	Germany	KY556460	n/a
Fusisporium solani	FS26	White clover	Germany	KY556517	KY556542
Fusisporium solani	FS27	White clover	Germany	KY556501	n/a
Fusarium solani	FS28	Compost	Germany	KY556475	KY556528
Fusarium solani	FS29	Compost	Germany	KY556524	KY556552
Fusarium solani	FS30	Hibiscus	Germany	KY556525	KY556553
Fusisporium solani	FS31	Cherry	Germany	KY556520	KY556549
Fusarium solani	FS32	Subterranean clover	Switzerland	KY556476	n/a
Fusarium solani	FS33	Subterranean clover	Switzerland	KY556486	KY556529
Fusarium solani	FS34	Subterranean clover	Switzerland	KY556484	KY556530
Fusarium solani	FS35	Subterranean clover	Switzerland	KY556482	KY556539
Fusarium solani	FS36	Subterranean clover	Switzerland	KY556487	n/a
Fusarium solani	FS37	Subterranean clover	Switzerland	KY556495	n/a
Fusarium solani	FS38	Subterranean clover	Switzerland	KY556456	n/a
Fusarium solani	FS39	Subterranean clover	Switzerland	KY556477	n/a
Fusarium solani	FS40	Subterranean clover	Switzerland	KY556478	n/a
Fusarium solani	FS41	Subterranean clover	Switzerland	KY556464	n/a
Fusarium solani	FS42	Subterranean clover	Switzerland	KY556485	KY556531
Fusarium solani	FS43	Subterranean clover	Switzerland	KY556479	KY556532
Fusarium solani	FS44	Subterranean clover	Switzerland	KY556470	KY556533

Species	Isolate name	Substrate/host	Country	GenBank accession number	
				TEF1-α	***RPB2***
Fusisporium solani	FS45	Subterranean clover	Switzerland	KY556521	KY556543
Fusarium solani	FS46	Subterranean clover	Switzerland	KY556467	KY556534
Fusarium solani	FS47	Subterranean clover	Switzerland	KY556461	n/a
Fusarium solani	FS48	Winter vetch	Switzerland	KY556489	n/a
Fusarium solani	FS49	Winter vetch	Switzerland	KY556490	KY556540
Fusarium solani	FS50	Winter vetch	Switzerland	KY556480	n/a
Fusarium solani	FS51	Winter vetch	Switzerland	KY556468	n/a
Fusarium solani	FS52	Winter vetch	Switzerland	KY556496	n/a
Fusarium solani	FS53	Winter vetch	Switzerland	KY556465	n/a
Fusarium solani	FS54	Winter vetch	Switzerland	KY556483	n/a
Fusisporium solani	FS55	Subterranean clover	Italy	KY556515	n/a
Fusisporium solani	FS56	Subterranean clover	Italy	KY556502	n/a
Fusisporium solani	FS57	Subterranean clover	Italy	KY556503	KY556545
Fusisporium solani	FS58	Subterranean clover	Italy	KY556498	KY556546
Fusisporium solani	FS59	Subterranean clover	Italy	KY556504	n/a
Fusisporium solani	FS60	Subterranean clover	Italy	KY556518	KY556547
Fusisporium solani	FS61	Subterranean clover	Italy	KY556505	n/a
Fusisporium solani	FS62	Subterranean clover	Italy	KY556509	n/a
Fusisporium solani	FS63	Subterranean clover	Italy	KY556519	n/a
Fusisporium solani	FS64	Subterranean clover	Italy	KY556510	n/a
Fusarium solani	FS66	Subterranean clover	Italy	KY556509	n/a
Fusisporium solani	FS67	Subterranean clover	Italy	KY556519	n/a
Fusisporium solani	FS68	Subterranean clover	Italy	KY556510	n/a
Fusisporium solani	FS69	Winter vetch	Italy	KY556522	KY556550
Fusisporium solani	FS71	Winter vetch	Italy	KY556507	KY556548
Fusisporium solani	FS72	Winter vetch	Italy	KY556512	KY556551
Fusisporium solani	FS73	Winter vetch	Italy	KY556508	n/a
Fusisporium solani	FS74	Winter vetch	Italy	KY556514	n/a
Fusisporium solani	FS75	Winter vetch	Italy	KY556513	n/a

Species	Isolate name	Substrate/host	Country	GenBank accession number	
				TEF1-α	***RPB2***
Fusarium solani	FS76	White clover	Sweden	KY556462	n/a
Fusarium solani	FS77	Subterranean clover	Germany	KY556457	KY556541
Fusarium solani	FS78	Subterranean clover	Switzerland	KY556494	n/a
Fusisporium solani	FS79	Subterranean clover	Italy	KY556516	n/a
Fusarium solani	FS80	Subterranean clover	Germany	KY556481	n/a
Fusarium redolens	FR1	White clover	Sweden	KY556443	n/a
Fusarium redolens	FR2	White clover	Sweden	KY556444	n/a
Fusarium redolens	FR3	White clover	Sweden	KY556445	n/a
Fusarium redolens	FR4	White clover	Sweden	KY556446	n/a

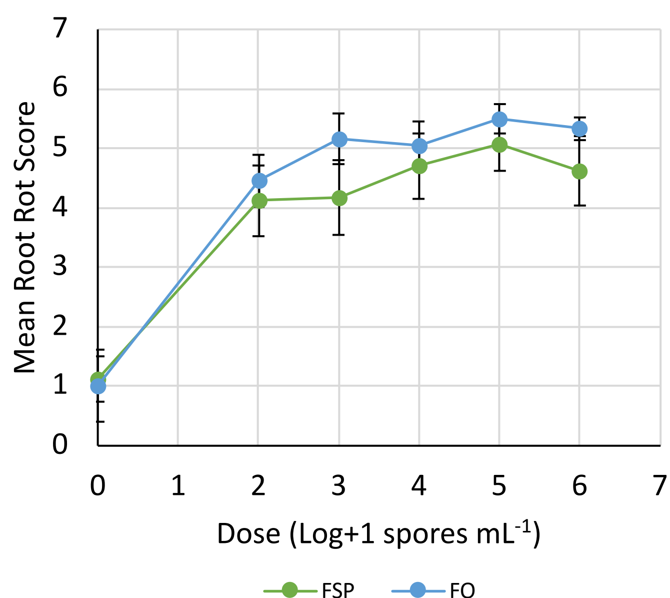

Figure A 2. An initial experiment to examine the effect of spore concentration on mean root rot disease severity score for pea plants inoculated with *Fusarium oxysporum* 'PG18' and *Fusarium solani* f. sp. *pisi* 'PG13' in test tube assays. These experiments were undertaken with the same experimental design as the ones described in Chapter 3, however here it was noted that future experiments should also include the concentration of 10 spores mL^{-1} which was initially considered too low, but eventually became the concentration of choice for the test tube assays examining interactions in Chapter 3 due to the concentration resulting in root rot scores and mortalities which were significantly higher than the uninoculated control, but low enough for any synergistic effects to be explored. Differing letters between bars indicate significant differences (p < 0.05). Error bars = ± SEM.

Table A 3. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of different *D. pinodella* inoculum concentrations on pea plant emergence. Analysis undertaken in Genstat 19.

Change	**Degrees of freedom**	**Deviance**	**Mean deviance**	**Deviance ratio**	**Approx. χ^2 probability**
+ Repeat	1	0.2986	0.2986	0.30	0.585
+ Repeat.Block	22	15.4161	0.7007	0.70	0.844
+ Concentration	5	75.0628	15.0126	15.01	<.001
Residual	115	84.8112	0.7375		

Table A 4. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of different *F. solani* f. sp. *pisi* inoculum concentrations on pea plant emergence. Analysis undertaken in Genstat 19.

Change	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	Approx. χ^2 probability
+ Repeat	1	0.2986	0.2986	0.30	0.585
+ Repeat.Block	22	15.4161	0.7007	0.70	0.844
+ Concentration	5	75.9623	15.1925	15.19	<.001
Residual	115	83.9116	0.7297		

Table A 5. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of different *D. pinodella* inoculum concentrations on pathogen stem girdling. Analysis undertaken in Genstat 19.

Change	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	Approx. χ^2 probability
+ Repeat	1	2.868	2.868	2.87	0.090
+ Repeat.Block	22	20.997	0.954	0.95	0.521
+ Concentration	5	14.157	2.831	2.83	0.015
Residual	72	101.191	1.405		

Table A 6. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of biofumigant plant varieties tested at different inoculum concentrations of *D. pinodella* on pea plant emergence. The GLM compared all treatments to the control treatment (no biofumigant, no DP inoculum). Analysis undertaken in Genstat 19.

Change	Degrees of freedom	Deviance	Mean deviance	Deviance ratio	Approx. χ^2 probability
+ Bench	1	0.0273	0.0273	0.03	0.869
+ Bench.Row	46	81.0015	1.7609	1.76	0.001
+ Bench.Column	10	13.5605	1.3561	1.36	0.194
+ Treatment	11	30.5986	2.7817	2.78	0.001
Residual	219	117.5974	0.5370		

Table A 7. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of biofumigant plant varieties tested at different inoculum concentrations of *D. pinodella* on pea plant emergence. Analysis undertaken in Genstat 19.

Change	**Degrees of freedom**	**Deviance**	**Mean deviance**	**Deviance ratio**	**Approx. χ^2 probability**
+ Bench	1	0.0273	0.0273	0.03	0.869
+ Bench.Row	46	81.0015	1.7609	1.76	0.001
+ Bench.Column	10	13.5605	1.3561	1.36	0.194
+ Treatment	11	30.5986	2.7817	2.78	0.001
Residual	219	117.5974	0.5370		

Table A 8. Accumulated analysis of deviance for the generalised linear model (GLM) examining the effect of biofumigant plant varieties tested at different inoculum concentrations of *D. pinodella* on pea root nodulation. Analysis undertaken in Genstat 19.

Change	**Degrees of freedom**	**Deviance**	**Mean deviance**	**Deviance ratio**	**Approx. χ^2 probability**
+ Bench	1	0.0548	0.0548	0.05	0.815
+ Bench.Row	45	76.6897	1.7042	1.70	0.002
+ Bench.Column	10	5.8630	0.5863	0.59	0.827
+ Rest_one_two	10	82.2336	8.2234	8.22	<0.001
+ Test_one_two	1	2.5954	2.5954	2.60	0.107
Residual	152	92.3214	0.6074		