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Soil-borne pathogens of oilseed rape (*Brassica napus*): assessing their distribution and potential contribution to yield decline

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CONTENTS

1. **ABSTRACT** **1**
2. **INTRODUCTION** **2**
2.1 Oilseed rape production **2**
2.1.1 Yield trends 2
2.1.2 Yield decline in agricultural crops 2
2.1.3 Rotations and yield decline 5
2.1.4 Changes in microbial diversity and pathogen suppression 6
2.1.5 Changes in pathogen inoculum 7
2.2 Soil-borne pathogens of oilseed rape **7**
2.2.1 *Sclerotinia sclerotiorum* 8
2.2.2 *Plasmodiophora brassicae* 9
2.2.3 *Verticillium longisporum* 9
2.2.4 *Rhizoctonia solani* 10
2.2.5 *Pythium* spp. 11
2.3 Conclusions from literature review **12**
2.4 Objectives **12**
3. **MATERIALS AND METHODS** **13**
3.1 General methods – fungal isolates **13**
3.2 Survey **14**
3.2.1 Sampling 14
3.2.2 DNA extraction 14
3.2.3 High throughput sequencing 14
3.2.4 Data analysis and statistics 16
3.3 Characterisation of *Rhizoctonia solani* **17**
3.3.1 Real-time PCR 17
3.3.2 Data analysis and statistics 17
3.4 Yield loss and inoculum threshold (glasshouse experiments) **18**
3.4.1 Inoculum production 18
3.4.2 Preliminary trials 18
3.4.3 Measurements 18
3.4.4 Experimental conditions 19
3.5 Main bioassay using *Brassica oleracea* DHSL150 .... **19**
3.5.1 Inoculum 19
3.5.2 Measurements 19
3.5.3 Experimental conditions 19
3.5.4 Data analysis and statistics 19
3.6 Isolate pathogenicity and variability **20**
3.6.1 Pectin zymograms 20
3.6.2 Genetic variation 21

4. **RESULTS** **22**
4.1 Next Generation Sequencing **22**
4.1.1 Data analysis 22
4.1.2 Community structure 22
4.1.3 Changes in community diversity and structure 22
4.2 *Rhizoctonia solani* survey **25**
4.3 Examining yield loss and inoculum threshold **26**
4.3.1 Preliminary 26
4.3.2 Pathogenicity bioassays 26
4.4 Isolate pathogenicity and variability **31**
4.4.1 Isolate pathogenicity 31
4.4.2 Pectin zymograms 31
4.4.3 Genetic variation 31
5. **DISCUSSION** **33**
6. **FUTURE RECOMMENDATIONS** **38**
7. **REFERENCES** **39**

1. Abstract

With a rising global population it has become ever more important to improve agricultural output in order to meet our societies demand for food, fuel and fibre. Whilst many carbohydrate staples such as wheat, rice and maize are rightly the focus of this effort, other groups such as oilseeds are also important as sources of essential proteins, fats and oils, along with secondary uses such as biofuels and break crops. As an island nation, it is essential that crop yields are maximised in order to meet these increasing demands, as coercing more land area into production is not possible. During the latter half of the 20th century this was achievable as yields rose steadily due to improved breeding, management and technological advances. However, with the dawn of the 21st century this trend has plateaued. Many studies attributed this to market pressures and a grower's need to maximise profitability, resulting in simplified and often shortened arable rotations between wheat and OSR, which as a consequence has led to a build-up of soil-borne pathogens causing an 'unseen' erosion of yield, or a yield decline.

This project aimed to elucidate the relationship between soil-borne pathogens and yield decline in oilseed rape crops, focusing on finding candidate pathogens within commercial crops and examining their occurrence and abundance in relation to agronomic factors such as rotational frequency. Using cutting edge molecular techniques, we were able to examine the fungal microbiome of commercial crops across 50 field sites. A wide range of fungal species were found including many generalists and saprophytes, whilst *Rhizoctonia solani* was the only pathogenic species frequently observed. Real-time PCR was used to quantify its commonality to many field sites irrespective of agronomic or geographical factors, suggesting that factors such as shortened rotations may not be influencing this pathogen. Similarly, through the use of anastomosis group specific primers we were able to demonstrate that AG 2-1 was the most common group occurring in root samples, similar to the findings of other surveys in Canada, Australia and USA. Here we hypothesise that the low influence of agronomic factors is likely due to the survival structures and wide range of host species, particularly wheat, the principal UK arable crop. In glasshouse experiments *R. solani* was found to be an effective and highly virulent pathogen of brassica seedlings. Yield loss appeared to be caused by a reduction in germination and plant stands, resulting from relatively low amounts of inoculum. Although, variability in inoculum production meant the true clinical threshold for disease was not reached, with this similarly demonstrating the limitations of studying this pathogen where artificial inoculation techniques are difficult to standardise.

2. Introduction

2.1 Oilseed rape production

Oilseed rape (*Brassica napus*) (OSR) is a member of the genus *Brassica*, part of the larger *Brassicaceae* family. This group encompasses many brassica vegetables and oilseed crops, with these thought to have been some of the earliest crops to be cultivated, with indications of vegetable brassicas being grown some 10,000 years ago (Snowdon *et al*., 2007).

Today, OSR is a one of the most commonly cultivated oil crops being only surpassed by palm and soybeans (FAO, 2014). Cultivation has also increased with many regions of the globe producing oil from Brassica crops, however the majority of production is centred on North America (in particular Canada), Western Europe and China (Berry and Spink, 2006). Widespread cultivation is a relatively new occurrence with the UK area totalling 600 ha a year from a global total of 6 million in the 1950's and 60's (FAO, 2014). However, today OSR is a common sight within arable areas of the UK with over 750,000 ha planted each year. This stark contrast to the area grown in the 1960's has now resulted in OSR being third most widely grown crop after wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (AHDB, 2013).

Europe is a large world producer of OSR, producing 35% of the world supply from only 26% of the total area grown globally (FAO, 2014). This disproportionate production is possible to due to higher yields of European countries, with countries such as Germany, France and UK achieving >3.0 t ha^{-1}, compared to other regions at <3.0 t ha^{-1} (Berry and Spink, 2006).

2.1.1 Yield trends

Since its reintroduction to the UK in the early 1960/70s oilseed rape yields have grown considerably, doubling from 1.5 t ha^{-1} to 3.0 t ha^{-1} within 20 years (Berry and Spink, 2006). Similar trends can be seen within many countries where OSR is commonly grown with Germany and France showing a similar improvement within 30 years of cultivation. However, despite this period of rapid growth UK yields have now begun to plateau, attaining an average of 3.4 t ha^{-1} between 2010 and 2013 (FAO, 2014), whilst yields in other countries such as Germany averaged 3.6 t ha^{-1} over this period and continue to rise (Figure 1.0).

2.1.2 Yield decline in agricultural crops

Yield decline has become a widely used term to describe the loss of productivity in agricultural crops, although definitions often remain vague and vary between studies. Perhaps the most comprehensive definition is provided by the review from Bennett *et al.* (2012), where the author specifically outlines how yield decline can occur from multiple biotic and abiotic factors linked to the repeated growing of crops both in the short and long term. Historically, yield decline has been associated with monoculture or continuous cropping systems. However, short and limited rotations or crop sequences have also been shown to lead to yield loss (Stobart, 2012). As a result, many commercial rotations whilst seeming to break cyclical factors which contribute to yield decline, may still lead to lower yields within many crops globally (Table 1).

Although yield decline itself is a simple concept, the underlying mechanisms responsible are still not fully understood (Figure 1.1), with many studies often attributing yield decline to a specific situation or cause, often appearing to be simple to overcome (Dobermann *et al.*, 2000). Additionally, difficulty in designing complex experiments, which encompass multiple factors and interactions, has led to a polarisation of opinion with authors often concentrating on a single topic or interaction.

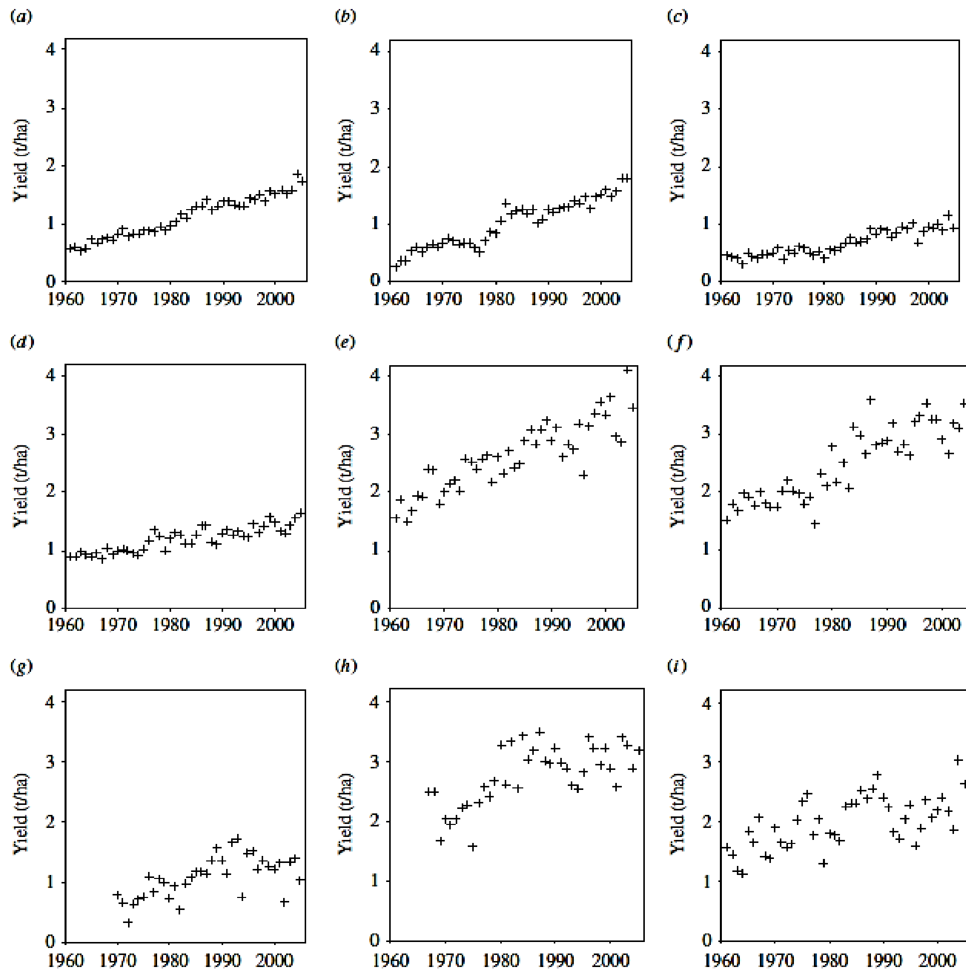

Figure 1.0. Oilseed rape yield trends for *(a)* World, *(b)* China, *(c)* India, *(d)* Canada, *(e)* Germany, *(f)* France, *(g)* Australia, *(h)* UK, *(i)* Poland. Source: Berry and Spink, 2006.

Table 1. Global yield decline in agricultural crops.

Crop	**Yield decline** (range of % losses)
Barley (*Hordeum vulgaris*)	11-19
Wheat (*Triticum aestivum*)	9-20
Rice (*Oryza sativa*)	19-59
Sugarcane (Saccharum spp.)	3-50
Maize *(Zea mays*)	7-36
Oilseed rape (*Brassica napus*)	3-25
Potatoes (*Solanum tuberosum*)	10-30
Rye (*Secale cereale*)	Up to 30%
Soybean (*Glycine max*)	8-20
Sweet potato (*Ipomoea batatas*)	21-57

Adapted from: Bennett *et al.*, 2012.

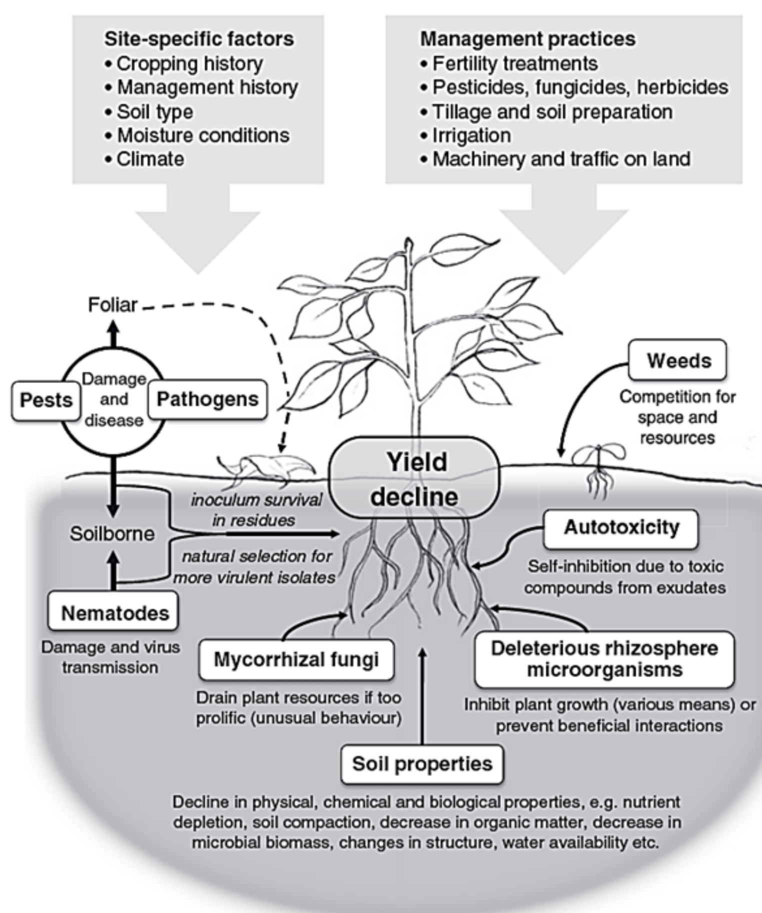

Figure 1.1. Factors associated with yield decline in crop plants. Source: Bennett *et al.* (2012).

2.1.3 Rotations and yield decline

Rotational cultivation of different crop types, in sequence, has long been known to improve yields helping to break cycles of weeds, pests and diseases, whilst improving nutritional and other properties of the soil (Finch *et al.,* 2002). Simple two or three crop rotations were the mainstay of early civilizations with grain crops such as wheat or barley alternating with legumes or fallow land. This practice of rotating crops in sequence has continued through to modern day although rotations often differ between regions as farms have becoming more specialised due to mechanisation and market related factors (Halloran and Archer, 2008). Consequently, rotations maybe shortened in intensive arable areas compared to longer rotations on mixed enterprise holdings. However, growers are increasingly being put under pressure to maximise output and in particular financial returns from individual crops. As a result, rotations have become shortened as the most profitable crops are grown. This has led to a dramatic rise in UK OSR production with the planted area more than doubling within the period 1990-2012 as OSR became the most profitable break crop for a cereal based system (Berry and Spink, 2006; AHDB, 2013).

Yield effects of short rotations

It is widely agreed that constant cultivation of a single crop leads to a drop in productivity. In OSR however, little information is available, with a few studies demonstrating the effect in field conditions. Work by George *et al.* (1985) (in Sieling *et al.*, 1997), based on survey data from eastern Germany reported a 13% decline in yield in OSR grown after OSR, when compared to crops grown with a 4-year break. However, rotational experiments by Polish researchers Gonet and Ploszynska (1987) (in Sieling *et al.,* 1997), observed no yield response to the proportion of OSR grown within the rotation.

In a study conducted on samples from a long term rotational experiment based in Germany, Sieling *et al.,* (1997) observed lower above ground biomass production at maturity in shorter rotations. In contrast, OSR grown after a pea-wheat rotation was found to produce a significantly higher biomass, compared to that grown after 2-years of OSR. Seed yields were also depressed when OSR was grown more intensively.

Recently a long-term rotation experiment was conducted by Stobart (2012) to investigate yield decline in OSR when grown in different rotations. The experiment was established in 2003 and conducted over an eight-year period. Treatments comprised of eight rotations consisting of variations between continuous through to one in six year rotations of OSR with winter wheat. The experiment was performed at a single site in Norfolk, UK on a site that had no previous history of brassica cultivation. Results from this eight-year study showed that yields were depressed as the inclusion of OSR within the rotation increased (Figure 1.2). Crops grown within a continuous rotation averaged 2.7 t ha^{-1} over the eight-years, whilst the four 'virgin' crops resulted in an average yield of 3.9 t ha^{-1}. Although yields in the continuous rotation were, on average, lower than other rotations, a significant decline in yields was not witnessed until the fourth year. In years 4 to 8 of the study an analysis of plot yield components was conducted. Measurements of final plant populations at harvest showed plant numbers were consistently reduced by continuous rotations compared to other treatments (Figure 1.3).

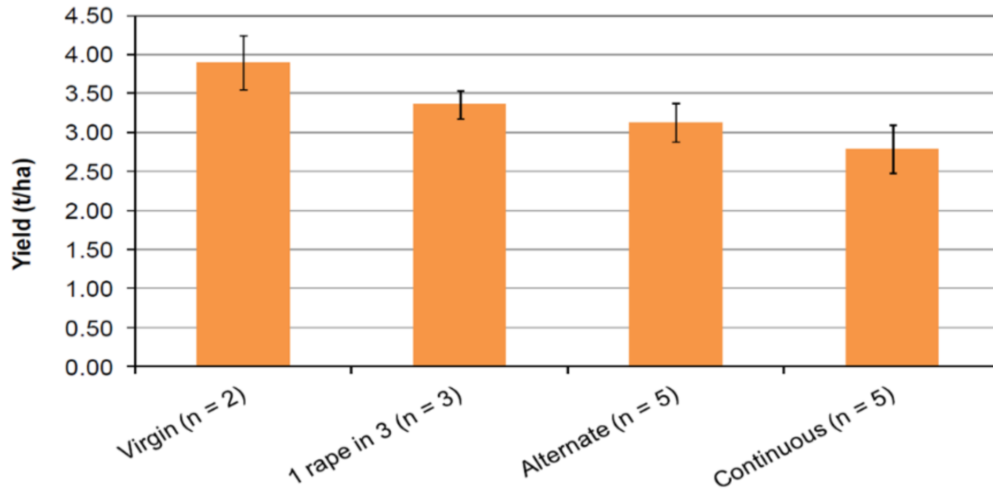

Figure 1.2. Average yield of OSR in different rotations after eight-years. Error bars represent the standard error of the mean, based on individual seasons. Source: Stobart (2012).

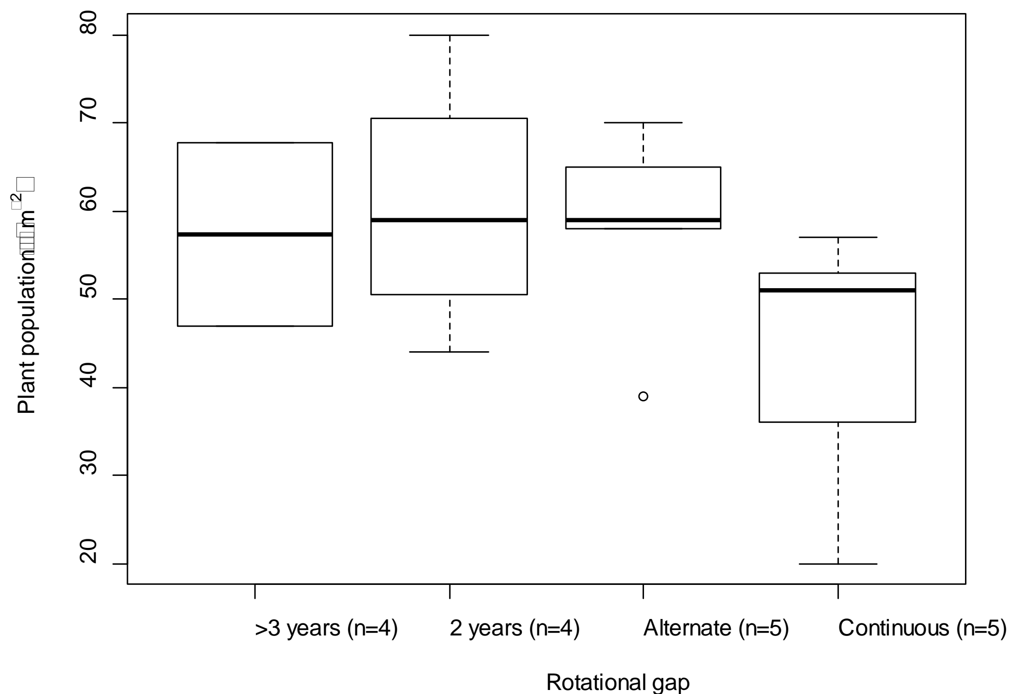

Figure 1.3. Average oilseed rape plant populations, at harvest, from within the rotation experiment from 2007-11. Adapted from: Stobart (2012).

2.1.4 Changes in microbial diversity and pathogen suppression

During the course of a rotation, changes in microbial communities occur due to factors such as the addition of different crop residues and exudates from the roots of growing crops (Bennett *et al.,* 2012). These changes may lead to alterations in the microbial diversity of the soil, affecting plant growth through favouring deleterious rhizosphere microorganisms (DRMOs) or by altering a soils ability to support or suppress pathogens (Peters *et al.,* 2003).

Deleterious rhizosphere microorganisms are organisms which cause alterations in plant growth. Unlike pathogens, which colonise and damage tissues, DRMOs have been suggested to affect plant growth through the production of inhibitory metabolites, phytotoxins and plant hormones, without producing visual symptoms meaning diagnosis is often difficult (Schippers *et al*., 1987). Conversely, an over production of protective antibiotics by these organisms may also depress plant growth (Nehl *et al.,* 1997; Schippers *et al.,* 1987). Because of this DRMOs have been implicated in the yield decline of many crop species, including wheat, barley, potatoes, corn, tobacco, bean and sugarcane (Nehl *et al*., 1997). In particular, deleterious bacteria such as *Pseudomonas spp., Bacillus spp.* and *Desulfovibro desulfuricans* have been shown to cause yield depression in pot studies.

2.1.5 Changes in pathogen inoculum

Along with changes in microbial communities, changes in pathogen inoculum also occur within rotational sequences (Hilton *et al.,* 2013). One of the most documented cases of this is the increase in the disease take-all (*Gaeumannomyces graminis* var. *tritici*) during continuous monoculture of wheat (Soon *et al.,* 2005; Cunfer *et al.,* 2006; Hwang *et al.,* 2009; van Toor *et al.,* 2013). Initially pathogen inoculum is low with the growing of the first wheat crop, with few primary infected (those infected from soil-borne inoculum during the autumn) and few secondary infected plants (those infected from root-root contact between infected plants during the spring). In the second year of wheat, soil inoculum is higher, leading to an increase in primary and secondary infections. This cycle of increasing inoculum results in a higher incidence of infections and continues as long as wheat is grown continuously until the fourth or fifth year of cultivation. During the growth of these crops, antagonistic soil-borne bacteria such as *Pseudomonas spp.* proliferate, producing antibiotic compounds on the surface of the roots leading to lower infection rates within plants, and increased yields. However, yields are often not as high as those of the first wheat.

Similar to *G. graminis var. tritici*, many other soil-borne pathogens are able to increase under short-term rotations, with many pathogens being capable saprophytes or able to produce long lived spores. For instance, known OSR pathogens such as *Verticillium longisporum, Rhizoctonia solani* and *Sclerotinia sclerotiorum* are able to form structures capable of long-term survival allowing the pathogen to survive and persist in the absence of a suitable host. This time period can range from months to years with the literature citing between two to five years in the case of *R. solani* (Carling *et al.,* 1986; Ritchie *et al.,* 2013), being able to persist for up to 8 years in soil (Adams and Ayers, 1979), or in excess of ten-years for micro sclerotia of *V. longisporum* (Heale and Karapapa, 1999). Due to this the rotations commonly practiced within OSR (1 in 2, 3 or 4 years) may not be suitable for the management of these pathogens resulting in an increase in populations over time.

2.2 Soil-borne pathogens of oilseed rape

Many soil borne pathogens have been implicated in the cause of disease and yield loss in oilseed rape. In their book on oilseed rape production Kimber and McGregor (1995) cited the main soil-borne pathogens of concern as being *Sclerotinia sclerotiorum, Plasmodiophora brassicae, Verticillium* wilt, and the damping off complex of *Rhizoctonia solani, Fusarium* spp.*, Pythium* spp. and *Phytophthora megasperma.* This in turn is supported by the work of various authors conducting disease surveys in Canada, where *S. sclerotiorum*, *R. solani*, and *F. roseum* were implicated in causing foot and stem rots of commercial OSR crops (Petrie, 1973; 1985; 1986; Morrall, 2000; 2013).

In the UK, few surveys examining soil borne diseases of oilseed rape have been conducted. In a review of OSR diseases Hardwick *et al.* (1991) suggested that *S. sclerotiorum* was the only soil borne disease of significance, but did discuss other diseases such as *R. solani*, *P. brassicae* and *Verticillium* wilt as being minor diseases. Since then several surveys on individual pathogens have been conducted (Table 1.2), however these largely concentrate on *S. sclerotiorum, P. brassicae* and *Verticillium* wilt. Thus, information on the distribution and infection levels of pathogens such as *R. solani, Pythium* spp. and *P. megasperma* are limited, however a survey of horticultural brassicas by Budge *et al.* (2009b) have shown *R. solani* to be present within the UK.

Table 1.2. Summary of UK OSR soil-borne pathogen surveys.

Pathogen	Reporting Period	No. sites	% Positive[a]	% Individual infection[b]	Reference
Sclerotinia sclerotiorum	1997-2006	-	low	low	cropmonitor.com
Plasmodiophora brassicae	2007-2008	96	48.5	26.6	Burnett *et al.,* 2013
Verticillium longisporum	2009-2011	292	16	3.3	Gladders *et al.,* 2013
Rhizoctonia solani	-	-	-	-	-
Pythium spp.	-	-	-	-	-

a: Percentage occurrence is indicative of the number of positive field sites.

b: Percentage individual infection is representative of the average in field infection across the survey sites.

2.2.1 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum is a widely distributed phytopathogen with a host range of over 400 host species including many agricultural and horticultural crops (Boland and Hall, 1994), belonging to the *Sclerotiniaceae* family within the phylum *Ascomycota*. It is necrotrophic in nature causing a range of soft rots resulting in wilting and premature senescence, before forming simple resting structures.

Lifecycle

Sclerotinia is able to persist between hosts as resting structures (sclerotia) and mycelium within living and dead plant material (Agrios, 1997), although sclerotia are the main component of survival (Bolton *et al.*, 2006). Sclerotia are formed from the dense aggregation of hyphae, with those of *S. sclerotiorum* comprising of a dark melanised outer rind and a non-melanised fibrillar inner portion or medulla. This structure is a capable resting form, being able to persist for up to 8 years in soil (Adams and Ayers, 1979). Under moist temperate conditions the sclerotia undergo carpogenic germination producing a stipe followed by a cup or disc shaped apothecium, from which ascospores are released. The ascospores are produced over the course of a few weeks and discharged upwards to be carried away on air currents. Infections in OSR often coincide with the end of flowering and as such these spores largely land on the dying petals. From here the infected petals fall to the crop canopy below, and the ascospores germinate utilising the petal as an initial food source before infecting the leaf. Infection may also be initiated through direct myceliogenic germination of sclerotia leading to stem

infections, however this mode is not important in *S. sclerotiorum* infection of oilseed rape (Clarkson *et al.*, 2013).

Management

Traditionally management of this pathogen has been difficult due to the sporadic nature of disease outbreaks. Varietal resistance has been partially achieved in other crops, but so far not in OSR. Due to this the main management tool is the use of fungicides, particularly applied during OSR flowering to protect the petals from spore germination. The use of biological agents such as the mycoparasite *Coniothyrium minitans* to degrade in field sclerotia has also been proposed (Bolton *et al.*, 2006).

2.2.2 *Plasmodiophora brassicae*

Plasmodiophora brassicae is the causal agent of club root disease in brassicas. It is an economically important phytopathogen occurring in more than 60 countries worldwide, causing yield losses of between 10-15% (Dixon, 2009). Belonging to the protist supergroup *Rhizaria, P. brassicae* is a phytomyxid and as such a obligate parasite (Hwang *et al.*,2012). Disease symptoms typically manifest as club-shaped galls, which interrupt the vascular tissues leading to wilting, stunting and premature senescence.

Lifecycle

The life cycle is initiated by the production of zoospores from long lived resting structures in the soil. These zoospores are motile and move towards the root hairs before encysting on the surface and infecting the below tissues. Once present in the root plasmodia are produced within the cortex and vascular tissues, here they cause an up regulation in the production of plant auxins and cytokines leaving to hypertrophic growth. This mechanism causes the club-shaped galls, within which the plasmodia become cleaved to produce millions of resting spores. In turn this causes a disintegration in the root material, allowing the spores to be released back into the soil where they may persist for up to 20 years (Wallenhammar, 1996).

Management

Resistant cultivars have been the most recent tool in managing *P. brassicae* infections, particularly with the introduction of the variety 'Mendel' in the early 2000s to European markets (Diederichsen *et al.*, 2014). However, due to the varied and often mixed pathotypes seen within European soils, increased resistance gene inclusion or 'stacking' is required to be totally effective (Hwang *et al.*, 2012), with some break down in 'Mendel' resistance already known in parts of Germany (Diederichsen *et al.*, 2014). Other methods of management aim to prevent inoculum multiplication between host crops by managing brassicaceous weed species and OSR volunteers through the use of herbicides or mechanical means (Diederichsen *et al.*, 2014). Traditional practices such as wide rotations may also prove useful to lower soil inoculum over time.

2.2.3 *Verticillium longisporum*

Verticillium longisporum (previously, *Verticillium dahliae var. longisporum*) is a pathogen of world-wide distribution and importance (Clewes *et al.,* 2008). First isolated from horseradish it was mistakenly identified as a long spored variant of *V. dahliae* (Zeise and Von Tiedemann, 2002). However subsequent DNA analysis has demonstrated it to be a separate species with a specificity

for brassicaceous plants and OSR in particular (Zeise and Von Tiedemann, 2002; Steventon *et al.*, 2002; Clewes *et al.*, 2008).

The first report of the disease within the UK was from infected OSR crops in Kent and Hereford during 2007 (Gladders et al., 2011). Coupled with other reports from continental Europe the disease is now considered endemic within northern Europe and also the UK (Heupel, 2013).

Lifecycle

Initial infection occurs through the roots of young plants, with hyphae migrating towards the root in response to root exudates (Mol and van Riessen, 1995). Upon contact with the root epidermis, hyphae rapidly colonise the surface infecting the root tip. Once the epidermis is penetrated, hyphae grow inter- and intracellularly through the root cortex, colonising the vascular bundle and invading the xylem tissues. In the xylem conidia are produced either directly from hyphae or short phialides (Buckley *et al.,* 1969); these are transported by the transpiration stream forming new infection sites within the plant (Beckman, 1987; Gold *et al.,* 1996). At the onset of plant senescence the pathogen switches to a limited saprophyte, forming microsclerotia in the dying parenchyma (soft non-vascular tissues) of the plant. As the plant material is degraded, and eventually returned to the soil, microsclerotia are released into the environment providing a source of inoculum for new infections to occur (Schnathorst, 1981; Neumann and Dobinson, 2003).

Management

Management of the pathogen post infection is often difficult and uneconomical, as infections occur within the stem, a difficult region to target with fungicides (Inderbitzin *et al.,* 2011; Heupel, 2013). Along with this, symptoms are also difficult to spot as they manifest later in the growing season, coinciding with maturation of the crop. However, even if early symptoms were spotted, or preventative treatment was pursued, then management would not be guaranteed as there are currently no registered products for the management of the disease, and few if any marketed resistant varieties (Heupel, 2013).

2.2.4 *Rhizoctonia solani*

The soil-borne pathogen *Rhizoctonia solani* is a prominent pathogenic species occurring on a wide range of agriculturally important plant species (Ogoshi, 1987). Whilst discussed as a single pathogen *R. solani* constitutes a disease complex currently comprising of 13 current known types classified by their ability to anastomose through hyphal connections (Anderson, 1982; Ogoshi, 1987; Carling *et al.*, 2002). Surveys have highlighted its occurrence within OSR crops and particularly in countries such as Canada and Australia (Khangura *et al.,* 1999; Zhou *et al.,* 2014), where infection by anastomosis groups (AGs) AG 2-1, 2-2, 4 and 8 were prominent. The pathogen possesses both a necrotrophic and saprophytic ability, causing damping off and lesion formation on roots before producing survival structures (sclerotia) and colonising plant material in the soil to perpetuate between hosts.

Lifecycle

Hyphae within the soil, either from decaying plant matter or from germinating sclerotia, make contact with the root system and begin the infection process. From here primitive apressoria or infection cushions are formed and infection pegs enter the root by means of both mechanical (Keijer, 1996 in

Sneh *et al.*, 1996) and enzymatic degradation of cells (Trail and Koller, 1990). Once inside the root material the hyphae grow inter- and intracellularly to the cortex resulting in the formation of a necrotic lesion from which a feeding site is established (Weinhold and Sinclair, 1996 in Sneh *et al.*, 1996). This causes the typical damping off symptoms in young seedlings or necrotic and often girdling lesions in older plants. Once the plant has succumbed to infection sclerotia may be produced, consisting of an undifferentiated aggregation of melanised hyphae, in order to persist between crops (Sumner, 1996 in Sneh *et al.*, 1996). In a similar way, hyphae may remain within the decomposing plant, becoming a saprophyte.

Management

Management of *R. solani* within OSR crops has been limited to date, largely relying on the use of fungicidal seed treatments and cultural methods to promote vigorous seedling establishment (Drizou *et al.*, 2017). Commonly used fungicidal products containing; thiram, metalaxyl-m, fludioxonil, iprodione and difenoconazole, have been shown to improve OSR establishment and root health (Lamprecht *et al.*, 2011).

2.2.5 *Pythium* spp.

Pythium spp. are soil-borne parasites belonging to the class of organisms known as Oomycetes. Although once termed, and often still mistaken as fungi, they are a type of 'water mould' and as such are part of the kingdom *Stramenophila* (Chromista) (Trigiano *et al.*, 2008). *Pythium spp.* are economically important pathogens, with Hawksworth (1995) suggesting there are over 120 different species, of which many cause seed and root rots in field and glasshouse crops. Infection often occurs in one of two stages; pre-emergence infection results in "damping off", and post-emergence wilting/lesions followed by seedling death. Due to these two infection methods, *Pythium spp.* can cause severe losses through the reduction in emergence and establishment rates. Most of the species associated with plants cause diseases such as "damping off", foot rots, seedling blights and fruit-rots of stored crops such as potatoes (Cullen *et al.,* 2007).

Lifecycle

Typically infection is instigated by the contact of zoospores or hyphae on the surface of the seed coat or seedling. Zoospores and hyphae derived from zoospores, oospores or saprophytic mycelium migrate towards the germinating seed/seedling, driven by factors such as root exudates and also to a certain extent by chance (Agrios, 1997). Initial contact with the seed coat results in penetration through the swollen tissues of the germinating seed, or through cracks and fissures. Further penetration of the embryo and seedling tissues occurs through mechanical pressure or via the production of cell degrading enzymes, such as cellulose and pectinolytic enzymes (Agrios, 1988), resulting in macerated tissue and watery lesions resulting in the death of seeds and seedlings.

Management

Typical fungicidal seed treatments are used to protect young seedlings from infection. Other methods such as soil sterilisation using steam, dry heat or chemical fumigation can also be effective, however these are mainly limited to use in greenhouse or specialist crops. Currently no known resistance is available in commercial varieties (Agrios, 1997).

2.3 Conclusions from literature review

Yield decline is witnessed in many crop and plant species worldwide, contributing to a decrease in agricultural productivity. This decline has been linked previously to many different factors both biotic and abiotic in nature, however it would appear that soil is integral to many of these. Whilst it would be naive to assume that one factor may contribute more than any other across all plants and situations, it is important to study them individually in order to inform the bigger picture. Due to this we have endeavoured to study the soil-borne pathogenic component and in particular its relation to oilseed rape production in the UK.

Within recent years the productivity of UK oilseed rape has plateaued with yields averaging *c.*3 t ha^{-1}, however in comparison the yields within regions such as Germany and France continue to rise currently averaging >4 t ha^{-1}. Many suggestions have been offered for this gap in yield, such as better varieties, management practices and environmental conditions. However, there is a lack of evidence to support these. Another opinion is that increased economic and technological pressure has led to OSR being the preferred break crop in shortened wheat rotations. This has resulted in short cropping sequences of 1 in 3, with some practicing even shorter intervals if conditions allow. Whilst this may increase economic output in the short-term, in the long-term these rotations may allow soil-borne pathogens to survive and persist more easily, leading to an erosion in yield if unnoticed. Furthermore, our understanding of what, where and how these pathogenic species cause yield loss is still lacking, confounding the problem.

2.4 Objectives

This project aims to explore some of the knowledge gaps identified in section 2.3, with this being conducted in three stages. Firstly, to identify the pathogenic species present within commercial crops of OSR, utilising molecular methods to highlight their occurrence and potential interactions with agronomic factors. Secondly, to use artificially inoculated compost to examine the impacts of these species on OSR growth and yield in the glasshouse environment, highlighting the threshold levels needed to cause disease and elucidating the mechanism behind yield loss. Finally, to examine the homogeneity of UK isolates of these pathogens, utilising an *in vitro* bioassay to study pathogenicity whilst utilising molecular methods to examine possible genetic variation.

Overall:
1) To understand how soil-borne pathogens contribute to the yield decline of UK OSR crops.
2) To understand how agronomic factors may impact on the occurrence, distribution and persistency of soil-borne pathogens.

Specific:
1) Survey for and elucidate which soil-borne pathogenic species are present within UK crops.
2) Examine how they impact the growth and yield of oilseed rape when grown under glasshouse conditions.
3) Define the inoculum threshold for the chosen pathogenic-species, and examine for any possible sub-clinical effects on plant growth and yield.

Null hypotheses:
1) Soil-borne pathogenic fungi are not present within UK crops of OSR.
2) Soil-borne pathogenic fungi do not cause any decline in the yield of OSR.

3. Materials and methods

3.1 General methods – fungal isolates

A range of fungal isolates were obtained from UK institutions and laboratories (Table 3.1) with the aim to setup a collection of isolates for assay specificity testing and for use in inoculation experiments. The majority of isolates were received on potato dextrose agar (PDA; Oxoid, Loughborough, UK) plates and transferred to PDA slopes and autoclaved barley grain vials for long term storage. Working cultures were maintained in 90 mm Petri dishes containing PDA sealed with parafilm (ThermoFisher Scientific, Loughborough, UK).

Fungal cultures for use in qPCR were grown on autoclaved cellophane discs placed on top of a clean plate of potato dextrose agar (PDA; Oxoid, Loughborough, UK) and inoculated with a 5 mm agar core. After one-week the hyphae were aseptically removed from the cellophane and into a 1.5 ml grinding tube from the FastDNA® Spin Kit for Soil (MP Biomedicals, Cambridge UK). Following homogenisation, DNA was extracted using a Wizard® Magnetic DNA purification kit (Promega, Southampton, UK) as described by Budge *et al.* (2007).

Table 3.1. Isolates collected from other UK institutions and laboratories for use in qPCR assay specificity testing. Key: R, *Rhizoctonia solani*; Py, *Pythium ultimum*. FERA, the Food and Environment Research Agency; HAU, Harper Adams University; RHS, Royal Horticultural Society; SASA, Science and Advice for Scottish Agriculture; UoW, University of Warwick.

HAU Code	Supplier Code	Source	Other
R5	1832	FERA	AG 3
R6	1938	FERA	AG 4II
R7	1835	FERA	AG 4
R8	1986	FERA	*R. zea*
R13	1971	FERA	AG 2-1
Py1	Unknown	SASA	Unknown
Py2	H1C5	RHS	var. ultimum
Py3	Unknown	UoW	Unknown
Py4	PI237	Chemtura AgroSolutions	Unknown

3.2 Survey

Molecular methods have become a standard tool for plant pathologists and diagnosticians in the detection and diagnosis of plant disease in environmental samples. Traditionally such tasks were undertaken through physical identification of pathogens and symptoms, followed by isolation and confirmation through Koch's postulates. Whilst these techniques are still useful today they require a depth of technical knowledge, time and labour in order to effectively deal with large numbers of samples in an efficient time period. As a consequence, this project aims to utilise molecular methods such as; conventional and quantitative polymerase chain reaction (PCR, qPCR), and High Throughput Sequencing (HTS) in order to examine the common soil-borne pathogens present within commercial crops of OSR, relating their occurrence and/or severity to agronomic practices where possible.

3.2.1 Sampling

Fifty individual field sites were sampled in the spring of 2011 and 2012, under a previous Innovate UK project (Innovate UK 100889) with individual samples submitted by participating consortium members. OSR plants were sampled between growth stages 18 (8 fully expanded leaves) and 33 (early stem extension) (BBCH), with sites chosen systematically based on the area of OSR grown within each growing region (Figure 3.0). Secondary to this, samples were also selected to evenly sample a range of high (1-3), medium (4-6) and low (>7) rotational frequency field sites to better study the effect of rotation on plant pathogens. Other factors such as soil type, establishment method and crop variety were also recorded.

Samples comprised of fifteen to twenty-five plant root systems per site collected along a standard 'W' shaped pattern across a 1 ha portion of the field. Plants were removed from the soil, and the above ground portion discarded. Root systems were washed with tap water to remove excess soil and debris before being sealed in plastic bags and frozen at -20°C. Once frozen samples were freeze-dried in a Girovac 6/13 Freeze dryer (Girovac Ltd., Norfolk, UK), sealed within individual plastic bags and stored at room temperature until DNA extraction

3.2.2 DNA extraction

Deoxyribonucleic acid (DNA) extraction was performed using a FastDNA® Spin Kit for Soil (MP Biomedicals, Cambridge UK) according to the manufacture's protocol, however DNA was extracted from 150-160 mg of the freeze-dried OSR roots instead of soil. The resulting DNA was eluted in 100 µl of Tris-EDTA (TE) buffer and assessed for quantity and quality using a NanoDrop 2000c® spectrophotometer (ThermoFisher Scientific, Loughborough, UK), before being diluted to a working concentration of 10 ng μl^{-1}.

3.2.3 High throughput sequencing

Following DNA extraction from OSR roots, initial PCR amplicons were generated using the universal primers of Toju *et al.* (2012), which anneal to the small sub unit (SSU) and the 5.8S region of rRNA, so covering the full ITS 1 region, the recently proposed universal fungal barcoding region (Schoch, 2012). Primers were adapted to Illumina sequencing with the addition of overhang sequences (underlined) as described previously (Illumina, 2013);

ITS1-F_KY02 CGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGGAAGTAAAAGTCGTAA and ITS2_KY02 GTCTCGTGGCTCGGAGATGTGTATAAGAGACAGTTYRCTRCGTTCTTCATC. Individual PCR reactions were performed using a Phusion® High-Fidelity PCR Kit (New England Biolabs Inc., Hitchin, UK). The manufacturer's protocol was followed, with the exception of the reagent quantities. Individual reactions consisted of 1X HF buffer, 200 µM dNTPs, 300 nM of each primer, 1.25 U Phusion® high fidelity DNA polymerase with the remaining amount made up of molecular grade water to a final volume of 24 µl, to this 1 µl of template DNA was added per well. One well was used as a negative control by substituting DNA with molecular grade water. Amplification was carried out on a C1000 thermal cycler (Biorad, Hemel Hempstead, UK) starting with an initial single denaturing step at 98°C for 2 min, followed by 30 cycles of denaturation at 98°C for 20 s, annealing at 54°C for 30 s and extension at 72°C for 90 s, a final extension step at 72°C for 5 min and held at 12°C. PCR products (*c.*300-600 bp) were individually visualised on a 1.2% agarose gel under UV light.

Following the first PCR, samples were cleaned using an Agencourt® AMPure XP PCR purification kit (Beckman Coulter, High Wycombe, UK) with the ratio of DNA: beads altered from 1.0:1.8 to 0.8:1.0 as suggested by Illumina (2013).

Indexing

In order for the samples to be recognised and tracked individual indices were added to the samples during a second PCR reaction. Individual reactions comprised of 1X HF buffer, 300 µM dNTPs, 2 mM $MgCl_2$, 1 U Phusion high fidelity DNA polymerase and 22 µl molecular grade water resulting in a total volume of 35 µl per reaction. To each individual reaction 5 µl of cleaned PCR product, 1 µM of a single index 1 primer and single index 2 primer from Illumina index set C were added, resulting in a final reaction volume of 50 µl. PCR conditions were an initial single denaturing step at 98°C for 3 min, followed by 8 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min and held at 12°C.

Normalisation and sequencing

The indexed PCR product was cleaned using the Agencourt® AMPure XP PCR purification kit, and the resulting product quantified by measuring the dsDNA fluorescence with a Qubit 1.0 Fluorimeter (Invitrogen, Paisley, UK) using the manufacturer's instructions. Three samples were also chosen at random in order to measure the product lengths within the samples using an Agilent 2200 TapeStation (Agilent Technologies Inc., Stockport, UK). Average product length, using the mode, was 430 bp (range: 355-669 bp) and along with the individual Qubit quantifications was used to calculate the molarity of each sample before pooling at equimolar concentration to form a 20 nM pooled library.

From the 20 nM pool a 4 nM stock was made and denatured using an equal volume of 2N NaOH, before diluting to 20 pM using HT1 buffer (Illumina). Phi X (Illumina) and additional HT1 buffer were added, resulting in a 15 pM DNA pool containing 5% Phi X. This was then denatured with heat at 96°C for 2 min before being cooled and added to a reagent cartridge prior to loading into the MiSeq machine (Illumina Inc., San Diego, California, US).

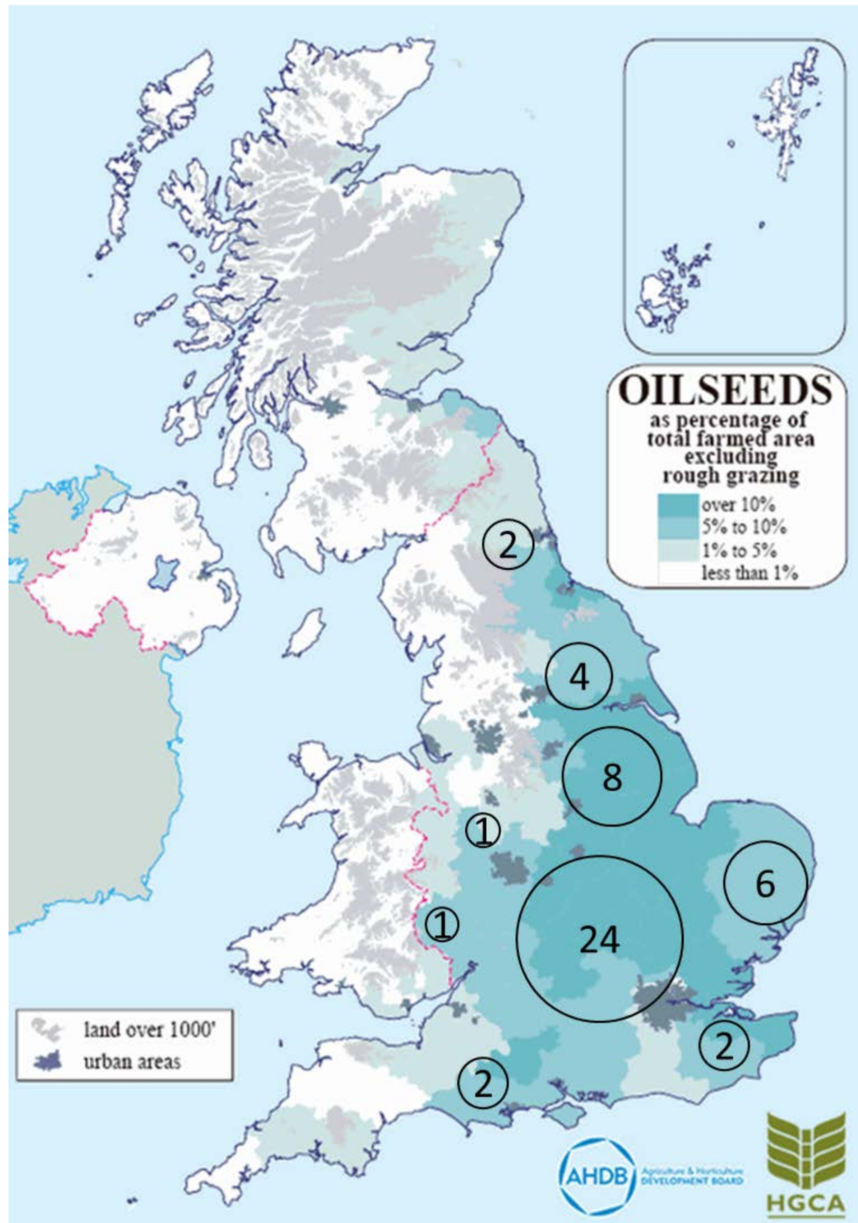

Figure 3.0. Oilseed rape production as a percentage of the farmed area in 2012 across the UK. Circles denote the production areas sampled with the size and number indicating the number of fields sampled. Map source: AHDB, 2012.

3.2.4 Data analysis and statistics

Initial manipulation of the reads was conducted using the Quantitative Insights Into Microbiology (v 1.5.0) software package (Caporaso *et al.*, 2010) before passing to USEARCH (v 9.2)(Edgar, 2010). Firstly, paired end reads were merged using the *multiple_join_paired_end.py* command before using *extract_barcodes.py* to remove amplification primers. After this sequences were passed to USEARCH and quality filtered using the *fastq_filter* command to remove sequences with more than one basepair expected error, and a length <250 bp. Filtered reads were then concatenated and dereplicated using the *fastq_uniques* command. Operational Taxonomic Unit (OTU) picking was performed with *cluster_otus* which utilises the UPARSE algorithm (Edgar, 2013) to form OTU clusters and remove chimeric sequences. Taxonomy was assigned to individual OTUs through use

of UTAX algorithm with comparison to the Ribosomal Database Project (RDP) Warcup ITS training set (v 2) (Deshpande *et al.,* 2016). After assigning taxonomy an OUT table was generated using *usearch_global* and used for statistical analysis in R studio (v 1.0.153) using the package VEGAN (Oksanen *et al.*, 2007).

Similarities between communities at each site were assessed using a Bray-Curtis dissimilarities matrix, constructed from the OTU table, and visualised in a non-metric multidimensional scaling (nMDS) plot. Analysis of similarity (ANOSIM) was used to test for significant differences between explanatory variables and the data set. Information on species diversity, richness and evenness were calculated using Simpson's, Pielous' and Shannon (H') Indices, and compared using a general linear model against individual explanatory variables.

3.3 Characterisation of *Rhizoctonia solani*

Based on the results from section 3.2, it was found that *Rhizoctonia solani* was the most commonly occurring pathogenic species. Whilst commonly discussed as a single pathogen *R. solani* constitutes a disease complex of 13 different strains or anastomosis groups (AG) which often have limited spatial or host ranges. Due to this real-time PCR (qPCR) was used to characterise and quantify individual AG across the survey material.

3.3.1 Real-time PCR

Previously published primers and TaqMan® probes for *R. solani* AG 2-1, 2-2 and 4-HGII, 5 and 8 were used (Budge *et al.,* 2009a) to amplify target DNA from the samples in section 3.2.1. Real-time PCR was carried out in 96-well plates using an ABI 7300 (Applied Biosytems, Warrington, UK) and a Bio-rad CFX96 (Biorad, Hemel Hempstead, UK) in a total reaction volume of 25 µl per well. PCR master mix comprised 1X Environmental Master Mix 2.0 (Applied Biosystems, Warrington, UK), 300 nM of each primers, 100 nM of probe and 50 ng of sample DNA with the remaining volume made up with molecular grade water. Cycling conditions consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold (C_t) value for each reaction was assessed using the manufacture's software, and an average of two replicates taken. A standard curve was constructed using a ten-fold dilution of genomic DNA (gDNA) extracted from pure cultures as described in Budge *et al.* (2007). Samples with C_t values >39 cycles were classed as negative, whilst those which amplified after the last standard (*c.* 35 C_t) but before 39 cycles were assigned a value half that of the last standard, as they constituted a positive result but fell below the limit of detection (LoD).

3.3.2 Data analysis and statistics

Data was analysed in GenStat (version 17) using a generalized linear model, fitting individual explanatory variables in a step-wise fashion.

3.4 Yield loss and inoculum threshold (glasshouse experiments)

From the results of section 3.3 it was found that *R. solani* AG 2-1 was the main AG present across field sites. In addition, individual quantities of pathogen within the root material varied widely. As a consequence, this section aims to explore the relationship between inoculum densities of *R. solani* AG 2-1 and plant growth and yield components to better estimate the threshold levels needed to impact on crop yield.

3.4.1 Inoculum production

Rhizoctonia solani inoculum was prepared using the method of Dhingra and Sinclair (1995). Whole maize (*Zea mays*) plants (stem, leaves and cobs) were dried at 100°C before milling using a mill (Christy Turner Ltd., Suffolk, UK) with a 1 mm sieve. The powdered maize meal was then combined with silver sand at a rate of 3% w/w in autoclave bags and dampened with tap water. Each autoclave bag contained no more than 2 kg of mixture in order to ensure complete sterilisation. The mixture was then autoclaved for 15 min at 121°C on two consecutive days. After cooling, six 5-mm agar plugs from a week-old PDA culture were added to each kilogram of the mix. Bags of autoclaved mixture were partially sealed by forming the bag opening into a Pasteur swan's neck and incubated at room temperature for 6 weeks, agitating weekly to disperse inoculum. After 6 weeks, inoculum was stored at 4°C until used, usually within one week.

3.4.2 Preliminary trials

Experiments utilised the *Rhizoctonia solani* AG 2-1 isolate R13, a known OSR pathogenic isolate, to decipher the optimum dose range needed for infection and to impact on plant yield.

Inoculum was prepared using the method outlined in section 3.4.1. After six weeks the 2kg bags of inoculum were added to uninoculated sterile maize meal-sand in a two-fold dilution series across six orders from 250:0 - 0:250 g of inoculated:uninoculated maize-sand. This was then mixed with 1 kg of John Innes No. 2 before being added to individual experimental units (100 x 115 x 180 mm, square pots). Into each pot four surface sterilised seeds of *B. napus* (*cv.* DK Cabernet) were sown at 10 mm depth in a square formation. Each dose was replicated six times, before being arranged in a 6x6 randomised latin square arrangement designed in GenStat (version 17).

3.4.3 Measurements

Seedling emergence and post emergence damping off were observed daily up to 30 days post inoculation (30 dpi), after which percentage emergence and seedling vigour were calculated. Plants were also removed from pots and the root system gently washed free of soil and debris under a running tap. Plants were separated into root and shoot by cutting the stem where the first root emerged, and the root system assessed for disease symptoms using a 1-5 scale for disease symptoms (Grosch *et al.*, 2004). The root system and the shoot material was placed in separate oven bags before being dried at 100ºC for 72 hours, after which dry matter (DM) was measured by weighing the material.

3.4.4 Experimental conditions

Glasshouse conditions consisted of minimum day and night air temperatures of 15 and 5°C and a photoperiod of 16 h and 8 h respectively. Pots were watered biweekly with an aim to maintain 30% moisture content by measuring volumetric water content with a theta probe (Delta-T, Cambridge, UK) and watering to this level with tap water.

3.5 Main bioassay using *Brassica oleracea* DHSL150

In order to examine the true effect of the pathogen on the growth and yield of OSR a model system was developed from initial trials in the previous section. This utilised *Brassica oleracea* (DHSL150) an experimental strain suited to glasshouse growth in place of OSR, due to its compact growth habit and rapid life cycle (Hilton *et al.*, 2013).

3.5.1 Inoculum

Inoculum was prepared as previously (3.4.1) with the exception that a range of lower doses was used. During experiment 1 and 2, the doses comprised of 80, 40, 20, 10, 5 and 0 g kg^{-1} of inoculated sand maize meal, whilst in experiment 3 they were 20, 10, 5, 2.5, 1 and 0 g kg^{-1}, in a total of 1kg of John Innes No. 2. Into each pot a single surface sterilised seed of *B. oleracea* (DHSL150) was sown at 10 mm depth. Each dose was replicated six times, before being arranged in a 6 x 6 randomised latin square arrangement designed in Genstat (version 17).

3.5.2 Measurements

During the experiment setup triplicate 500 mg soil samples were taken from each treatment and DNA extracted using a FastDNA spin kit for soil (MP Biomedicals, Cambridge, UK) before performing real-time PCR as described in section 3.3.1.

Measurements taken at seedling stage were the same as those in 3.4.3. Prior to maturity the flowering date and number of flowering racemes (stems) were recorded, whilst at maturity yield components such as number of pods, number of seeds per pod, and total seed yield per plant were recorded. Biomass samples were taken as before in order to calculate dry matter production.

3.5.3 Experimental conditions

Conditions remained the same as in 3.4.4, with the exception that plants were fed once at flowering using a liquid general purpose fertilizer (NPK: 25-15-15), and watering was stopped after 112 days (*c.*16 weeks) encouraging seed maturity and natural senescence before hand-harvesting at *c.*150 days.

3.5.4 Data analysis and statistics

Analysis was conducted in R Studio (version 1.0.153). The preliminary experiment was analysed by means of simple linear regression models (y~x) for individual variables.

The bioassay utilising DHSL150 was analysed using the DRC package (Ritz *et al.*, 2015) which comprises of scripts for the analysis of dose response data in agricultural experiments. Simple

models were used to describe the data with single response variables fitted against the quantity of inoculum using a 4 parameter log-logistic model (*drm*) after which the effective dose script (*ED*) was used to define the intercepts for ED_{05}, ED_{10}, and ED_{50}. Following this individual dose response curves were plotted and the ED values transposed onto this where these fell within the boundaries of the plot.

3.6 Isolate pathogenicity and variability

The pathogenicity assay was modified from that described by Budge *et al.* (2007). In brief, 10 ml of molten tap water agar (2% w/v) (TWA; Agar No.2, Lab M, Heywood, UK) were poured into sterile 50 ml screw top centrifuge tubes (Sarstedt UK Ltd., Leicester, UK) and allowed to solidify overnight. To each tube a single 5 mm agar core from a one-week old PDA culture was added, with this done six times for each isolate plus an uninoculated control. Five surface sterilised OSR seeds (cv. DK Cabernet) were then added aseptically to each tube, being placed equidistance from each other around the perimeter of the tube. At the same time, a single 5 mm agar core from each isolates was added to 10 cm petri dishes containing PDA and TWA, being replicated three times in order to record mycelia growth. Centrifuge tubes and agar plates were then sealed with a screw top lid or parafilm (ThermoFisher Scientific, Loughborough, UK) before being placed into a Sanyo MLR-352H-PE (Panasonic Biomedical, Loughborough, UK) environmental cabinet and incubated in the dark at 20°C.

After 5 days the seeds had germinated and the tubes were assessed. Individual seedlings were scored on a 1-5 disease scale (Grosch *et al.*, 2004), 1: no disease symptoms, 2: minor discolouration, 3: small lesions (<1 mm), 4: large lesions (>1 mm) and 5: seedling death. A disease severity index (DSI) was calculated using the following formula:

$$Disease\ Severity\ Index\ (DSI) = \frac{Sum\ of\ scores\ x\ 100}{Total\ number\ of\ ratings\ x\ Max.\ score}$$

After the assessment a single seedling was taken from each tube which displayed symptoms and placed onto fresh PDA before being incubated overnight. Colonies were then observed under a stereomicroscope for *Rhizoctonia* features in order to satisfy Koch's postulates.

The growth of the agar plates was monitored daily with the mycelial matt measured after 3 and 5 days post inoculation along two perpendicular axis. After 7 days the appearance and morphology of each culture was recorded, along with the final colony diameter.

3.6.1 Pectin zymograms

Pectin zymograms were set up using a modified method of Stodart *et al.*, 2007. A 5 mm agar plug from a 7 day old PDA culture was added to 10 ml of induction media (Sweetingham *et al.*, 1986) contained in a sterile 20 ml screw top tube and incubated for 7 days in the dark at 20°C. Afterwards the fluid was filtered through filter paper (Whatman No. 2, ThermoFisher Scientific, Loughborough, UK) before being centrifuged at 5000 g for 5 min to remove debris. One-hundred micro litres of this filtrate was then added to an equal volume of PAGE buffer (20 mM Tris-HCl; 0.0125% Bromophenol Blue; 40% sucrose v/v) before loading 20 µl into the stacking gel. The gel comprised of a stacking

and resolving gel made with 4 % and 12 % (w/v) acrylamide:bis-acrylamide (36.5:1, Bio-rad, Hemel Hempstead) respectively with the addition of 0.2 % (w/v) citrus pectin (Sigma Aldrich, Poole, UK). Gels were cast and run using a Mini Protean II system (Bio-rad, Hemel Hempstead) with running conditions set to 100V (120 mA) for 6 hours. After electrophoresis, gels were incubated in 0.1 M DL-malic acid (Sigma Aldrich, Poole) for 90 min before being stained in a 0.02% (w/v) ruthenium red (Sigma Aldrich, Poole) solution for 4 h followed by de-staining in 3 mM Na_2CO_3 overnight. Bands were then visualised using a light box, with isolate R13 being used on individual gels as a marker for comparison between gels.

3.6.2 Genetic variation

DNA extraction

Prior to DNA extraction individual isolates were grown for 7 days in 10 ml of potato dextrose broth in sterile 20 ml screw top tubes, before storing at 4°C until used. Mycelium was recovered by filtering through filter paper (Whatman No. 2, ThermoFisher Scientific, Loughborough, UK) before being placed into a sterile 2 ml screw cap tube containing a small amount (*c.* 0.1 ml) zircon beads and frozen at -20°C until needed. Once thawed, DNA was extracted using a Wizard® Magnetic DNA Purification System for Food (Promega) with the tissue lysed by vortexing the zircon beads during the first extraction buffer step, from this point the standard protocol was followed ending with the DNA being eluted into 100 µl of molecular grade water. Samples were analysed for quality and DNA concentration using a NanoDrop 2.0 spectrophotometer (ThermoFisher Scientific, Loughborough, UK) before diluting to 10 ng μl^{-1}.

PCR

To examine the genetic variation between different AG and isolates two phylogenetic regions were chosen to study differences in DNA sequences and sequence lengths.

The internal transcribed spacer (ITS) region has long been used to detect inter- and intraspecific variation between species, and has recently been proposed as the universal barcode region for fungi (Schoch *et al.,* 2012). In this study we utilised the well-used primer pair ITS 4 and 5 (White *et al.*, 1990) to amplify the whole ITS1-5.8S-ITS2. In addition to the ITS region we also used the intergenic spacer (IGS) region to examine the variation in our isolate collection as this has been utilised previously to described variation in *R. solani* isolates (Woodhall, 2007). To amplify the IGS 1 region the forward primer (LR12R) of Harrington and Wingfield (1995) and the reverse primer of Woodhall *et al.* (2007) was used, with these being located on the large sub unit (LSU, 28S) and 5S spacer unit, thus amplifying the whole IGS1 region.

Data analysis and statistics

Pathogenicity data was analysed in R Studio (version 1.0.153) by means of a linear model comparing (DSI~Isolate*AG). This model was showed to be the most effective model for our data by conducting stepwise comparisons using Akaike's Information Criterion (AIC).

4. Results

Through a combination of molecular methods, *in vitro* and glasshouse experiments it was found that *Rhizoctonia solani* AG 2-1 was the most common and pathogenic species present in commercial crops, resulting in damping off and reduced plant stands.

4.1 Next Generation Sequencing

The ITS1 region was used as a phylogenetic marker to assess differences in fungal communities associated within different commercial oilseed rape crops in England. In total, over 25 million reads were produced within a single MiSeq run, with 19.7M (78.8%) of these passing the in-built quality filter. Sample reads averaged 385,874 with a range of 176,680 to 757,198 over all 50 samples.

4.1.1 Data analysis

Operational taxonomic unit (OTU) picking resulted in 4,066 OTU clusters at a 97% similarity level. After the removal of plant sequences the number of sequences per sample ranged from 941 to 44,013, with the lowest number being used for rarefaction in order to normalize all samples (Gihring *et al.*, 2012) prior to further statistical analysis.

4.1.2 Community structure

Across the samples there was an almost equal balance of Basidiomycete and Ascomycete fungi accounting for 40% and 36.6% of all OTUs, followed by Zygomycetes (16.8%), Chytridiomycetes (6.5%) and other phyla (<0.1%). Similarly, each phylum was dominated by a few OTUs identified to species or genus level (Table 4.1.2) with many of these matching to known groups of saprophytes (Cadophora, Xenasma, Mortierella, Mucor), fungal parasites (Tremella) and plant pathogens (*Rhizoctonia solani*).

4.1.3 Changes in community diversity and structure

No significant differences were seen between species richness (Simpson), diversity (H' Shannon) or evenness (Pielous' index) when using the explanatory variables (previous year, cropping intensity, soil type, crop variety, region or establishment method). Analysis of OTUs using Bray-Curtis dissimilarity and ANOSIM also demonstrated there were no significant differences between samples, with the low R values similarly indicating that there was no separation between samples (Figure 4.1.3)

Table 4.1.2. Percentage relative abundance (%RA) of fungal genera and species found on the roots of English oilseed rape crops following operational taxonomic unit (OTU) assignment. Individual OTUs present at <1% relative abundance were placed in the category other. Underlined text denotes assignment possible to genus level only, italicised text denotes species level.

Taxonomy	%RA
Ascomycota	**36.6**
Cadophora	9.5
Lewia	1.9
Basidiomycota	**40**
Tremella	43
Xenasma	6.7
Sphacelotheca	6.4
Dichomitus	2
Rhizoctonia solani	1.1
Chytridiomycota	**6.5**
Zygomycota	**16.8**
Mortierella	6.5
Mucor	4.4
Other	**<0.1**
Other	18.5

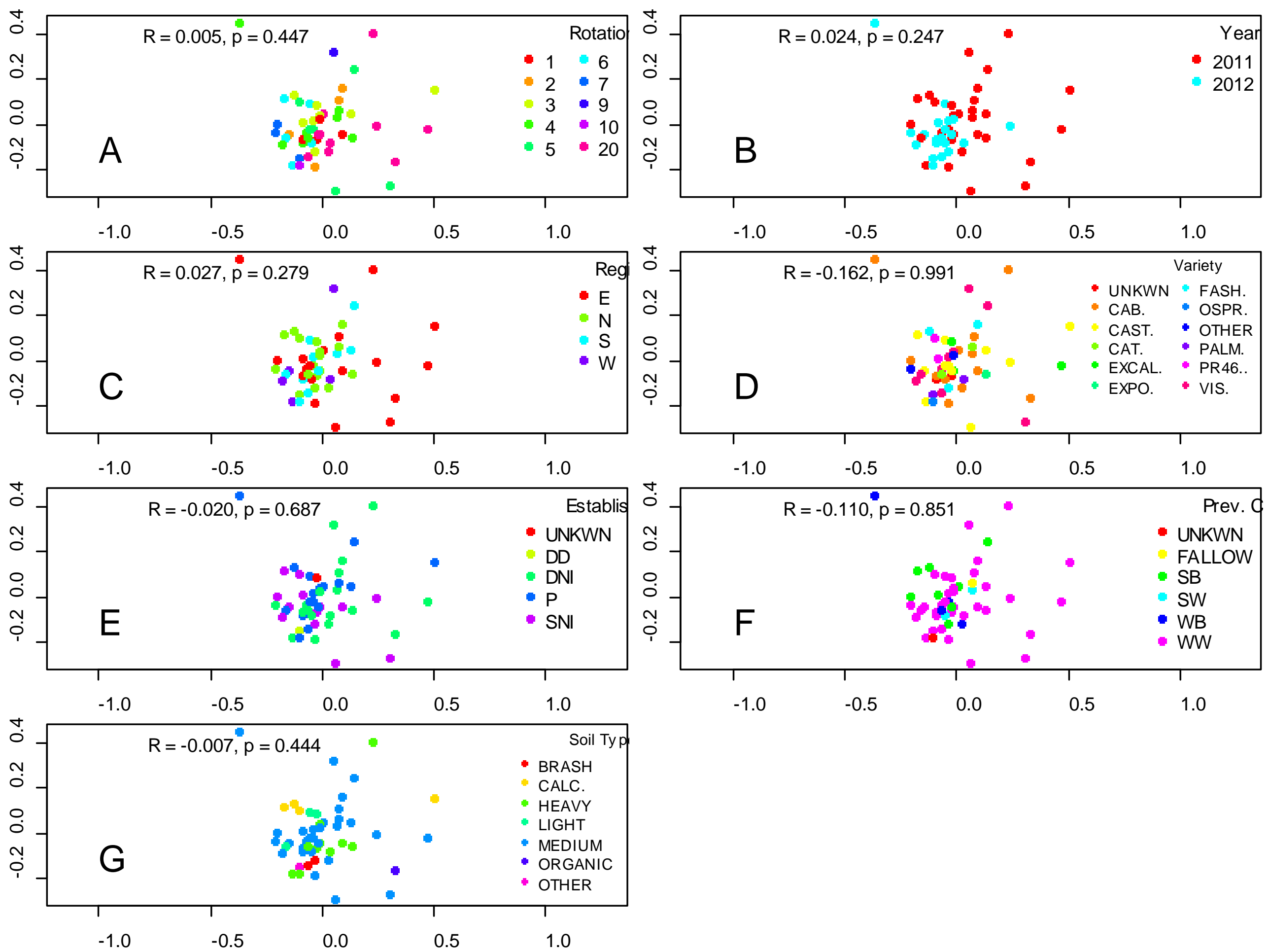

Figure 4.1.3. Bray-Curtis non-metric multidimensional scale (nMDS) plots generated from individual agronomic factors. A: Rotational frequency, B: Sampling year, C: Region, D: Variety (CAB. – DK Cabernet, CAST. – Castille, CAT. – Catana, EXCAL. – DK Excalibur, EXPO. – DK Expower, FASH. – Fashion, OSPR. – Osprey, PALM. – Palmedor, PR46. – PR46W21, VIS. – Vision, UNKWN – Unknown), E: Establishment technique (DD – Direct drilled, DNI – Deep non inversion, P – Plough, SNI – Shallow non inversion), F: Previous crop (SB – Spring barley, SW – spring wheat, WB – winter barley, WW – winter wheat), G: Soil type.

4.2 *Rhizoctonia solani* survey

Detection of individual anastomosis groups varied widely, although AG 2-1 dominated the samples being present in 60% (30/50) of the survey sites followed by AG 8 at 4% (2/50) and AG 5 at 2% (1/50) (Figure 4.2). AG 2-2 and 4HGII were not detected in any of the samples. Individual levels of AG 2-1 DNA ranged from 81.9 to 2.0 x10^{-3} pg μl^{-1} total DNA, with an average of 3.3 pg μl^{-1} total DNA. AG 8 was detected at 4.0 x10^{-2} and 1.0 x10^{-2} pg μl^{-1} total DNA, and AG5 at 9.0 x10^{-2} pg μl^{-1} total DNA. No significant relationship ($p>0.05$) could be drawn between pathogen levels and any agronomic or environmental variables.

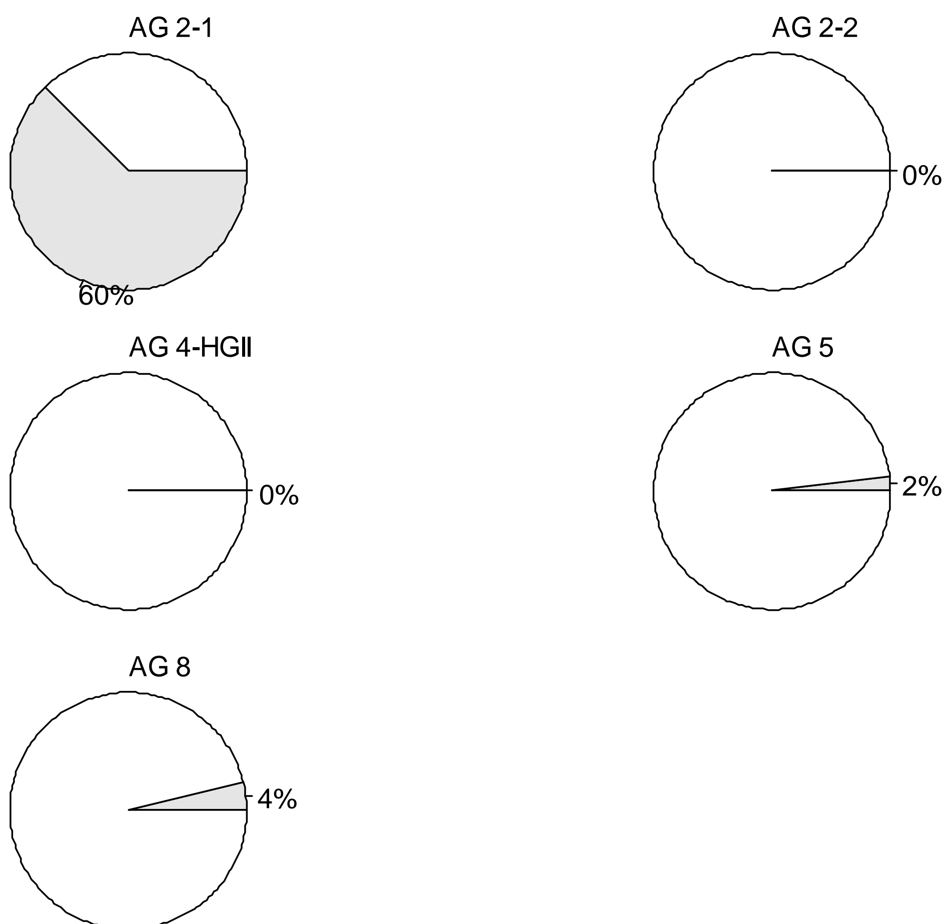

Figure 4.2. Occurrence of individual *Rhizoctonia solani* anastomosis groups (AG) across oilseed rape field sites (n=50) as determined by real-time PCR analysis of the crop root system.

4.3 Examining yield loss and inoculum threshold

4.3.1 Preliminary

Seedling emergence

A significant ($p<0.001$) reduction in OSR seedling emergence was observed as the proportion of *R. solani* (AG 2-1) inoculum increased (Fig 4.3.1). The greatest reduction in emergence was seen at doses of >125 g kg^{-1} where no emergence was observed in any of the replicates. However, between 15.63 – 62.5 g kg^{-1} seedling emergence was significantly reduced ($p=0.034$, 0.0049 and 0.0049). No damping off was observed within the control (0 g kg^{-1}) treatments, with the number of emerged seedlings being close to the expected viability of the seed lot (results not shown).

Biomass production

Biomass production (shoot and root) was significantly ($p = 0.007$; $p < 0.001$) reduced as the proportion of *R. solani* (AG 2-1) inoculum increased (Fig 4.3.1). Both shoot and root biomass was affected the greatest at the lowest dose (15.63 g kg^{-1}), with larger doses showing little to no biomass production as a result of the poor germination.

Disease incidence

Disease incidence was significantly ($p <0.001$) increased as the proportion of *R. solani* (AG 2-1) inoculum increased (Fig 4.3.1). The greatest incidence of disease (5) occurred at rates of between 31.25 and 250 g kg^{-1}, whereby all seedlings were killed at the point of assessment. A reduced score of 2.7 was seen at 15.63 g kg^{-1} whilst no disease symptoms were seen in the control (0 g kg^{-1}).

4.3.2 Pathogenicity bioassays

Real-time PCR and dose correlation

Through the use of real-time PCR it was found that DNA quantity per gram of soil and the actual inoculum quantity per kg of potting mix were often significantly correlated (Fig 4.3.2). Although, this relationship was dependent on the effectiveness of the inoculum production. Real-time PCR successfully amplified the target from both experiment 1 and 2, however in experiment 1 the levels failed to cross our defined threshold of 35 cycles (the lowest standard used) and as such were discounted from further analysis. In experiment 2 the quantity of target DNA was much larger and as such all but the control treatment amplified before the 35 C_t point. The linear model also suggested a strong significant relationship between the inoculum dose (g kg^{-1}) and pathogen quantity (Log+1 ng target DNA g^{-1} of soil). No results from experiment 3 could be presented due to the loss of samples following a mechanical malfunction in storage.

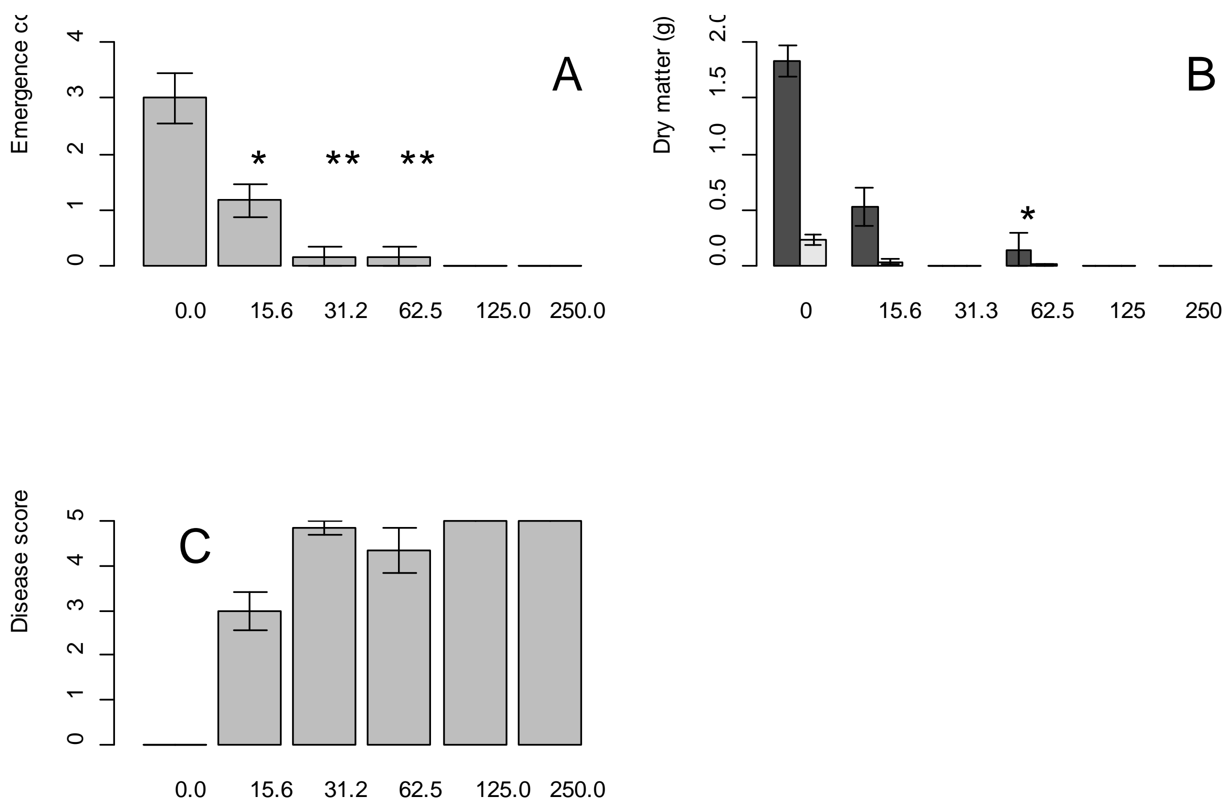

Figure 4.3.1. The response of *Brassica napus* (*cv*. DK Cabernet) seedlings (n=4) at 30 dpi when grown in pots containing increasing doses (g kg^{-1}) of *Rhizoctonia solan*i AG 2-1 inoculum. A: Emergence count, B: Dry matter (g) and C: Disease score. Error bars represent the standard error of the mean.

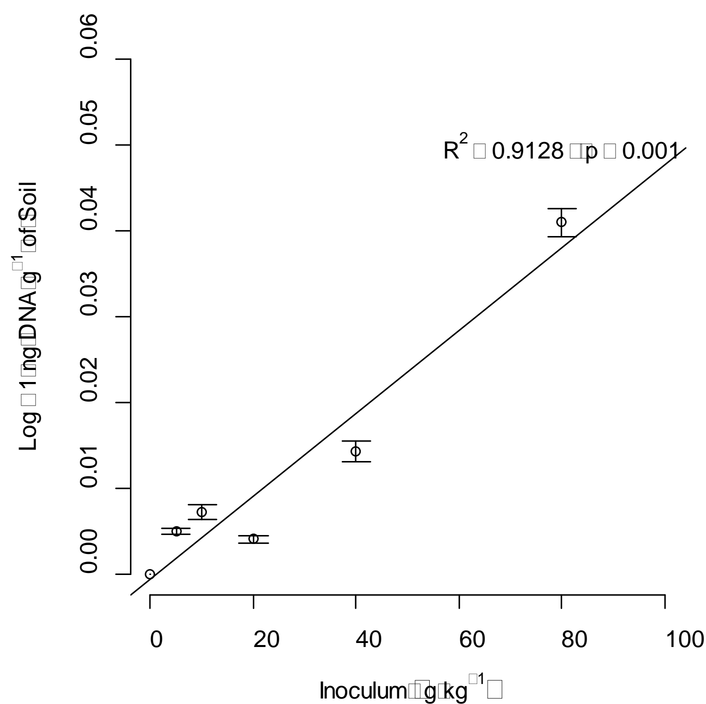

Figure 4.3.2. Relationship between inoculum quantity (g kg^{-1}) and *Rhizoctonia solani* AG 2-1 quantity (Log+1 ng DNA g^{-1}) as measured by real-time PCR at planting in experiment 2. Error bars represent the standard error of the mean.

Experiment 1, Dose response curves

The dose response curves were constructed for the measured variables of emergence (%), raceme number, pod number (total and empty), seeds per pod, root DM, shoot DM, and seed yield (Figure 4.3.3). Across all of these variables, the expected sigmoidal shaped response was not seen. Many of those that did respond, failed to reach the level of LD_{50} with the exception of raceme number, pod number and seeds per pod where the LD_{50} corresponded to 88.5, 19.3 and 44.0 g kg^{-1} respectively. Overall, the combined effect of these was not great enough to limit overall seed yield.

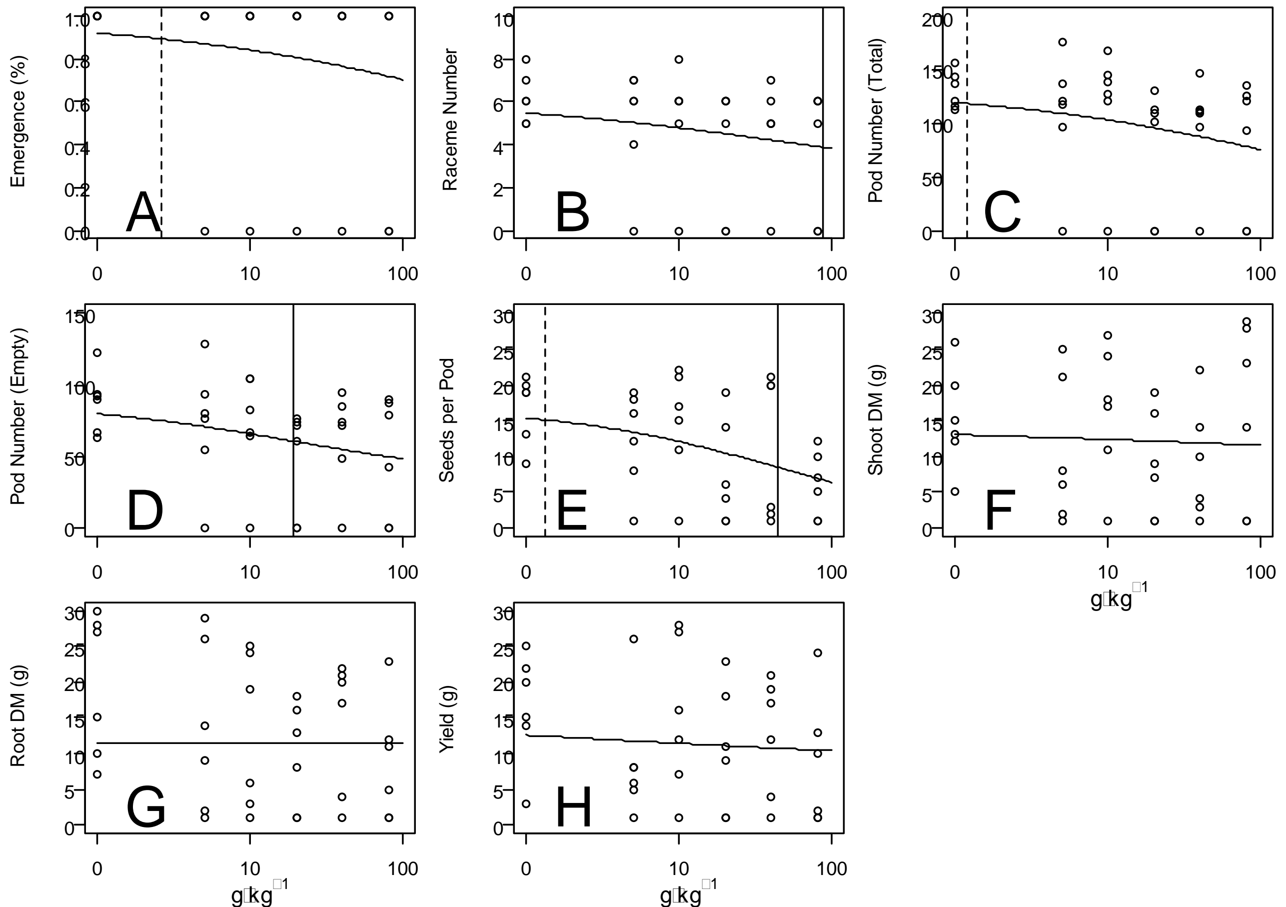

Figure 4.3.3. Experiment 1: Dose response curves fitted from 4 parameter log-logisitic models for multiple response variables at differing doses of pathogen inoculum (g kg^{-1}). A, Percentage emergence; B, Raceme number; C, Pod number (total); D, Pod number (Empty); E, Seeds per pod; F, Shoot dry matter (g); G, Root dry matter (g); H, Seed yield (g). Vertical lines represent LD_{10} (dashed) and LD_{50} (solid) thresholds.

Experiment 2, Dose response curves

The dose response curves were constructed for the measured variables of emergence (%) and emergence (Dpi) (Figure 4.3.4); other variable could not be assessed due to the lack of emergence across the experiment. As a result of the poor emergence the experiment was ended after 30 days. Across both variables, however, the expected sigmoidal response was observed with values attained for LD_{05}, LD_{10} and LD_{50}. At the LD_{50} level a dose of 5.3 and 5.0 g kg^{-1} was needed for each variable respectively.

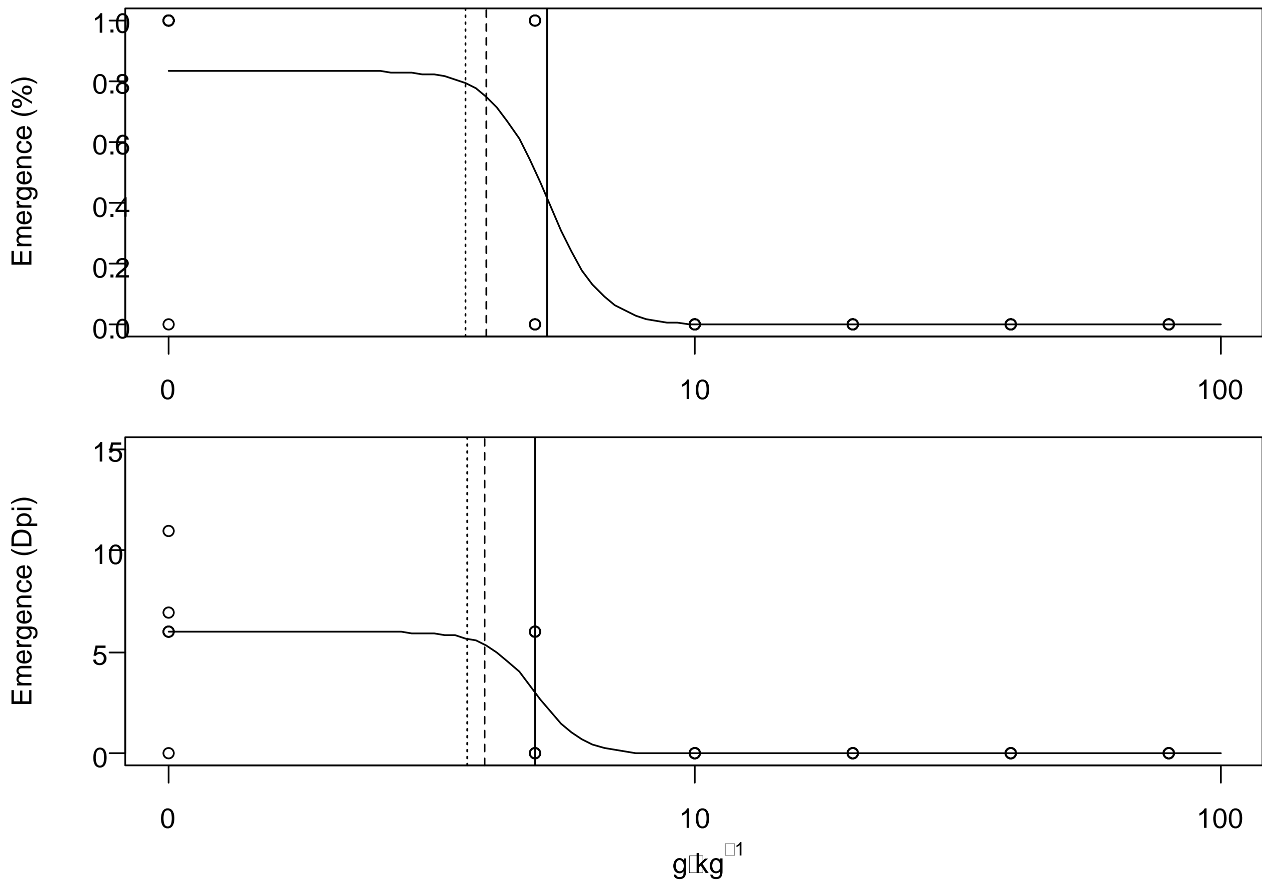

Figure 4.3.4. Experiment 2: Dose response curves fitted from 4 parameter log-logistic models for percentage emergence (T) and days to emergence (B) at differing doses of pathogen inoculum (g kg^{-1}). Vertical lines represent LD_{05} (dotted), LD_{10} (dashed) and LD_{50} (solid) thresholds.

Experiment 3, Dose response curves

Dose response curves were constructed as for experiment 1. Across variables, however, the expected sigmoidal response was not observed although values were attained for LD_{05}, LD_{10} and LD_{50} with many of the variables. Typically the LD_{50} occurred between 2.5 and 5 g kg^{-1} for all of the variables, with the exception of pod number (total) where this level was not reached and pod number (without seed), root dry matter and seed yield where a higher figure of between 5 and 10 g kg^{-1} was required (Figure 4.3.5).

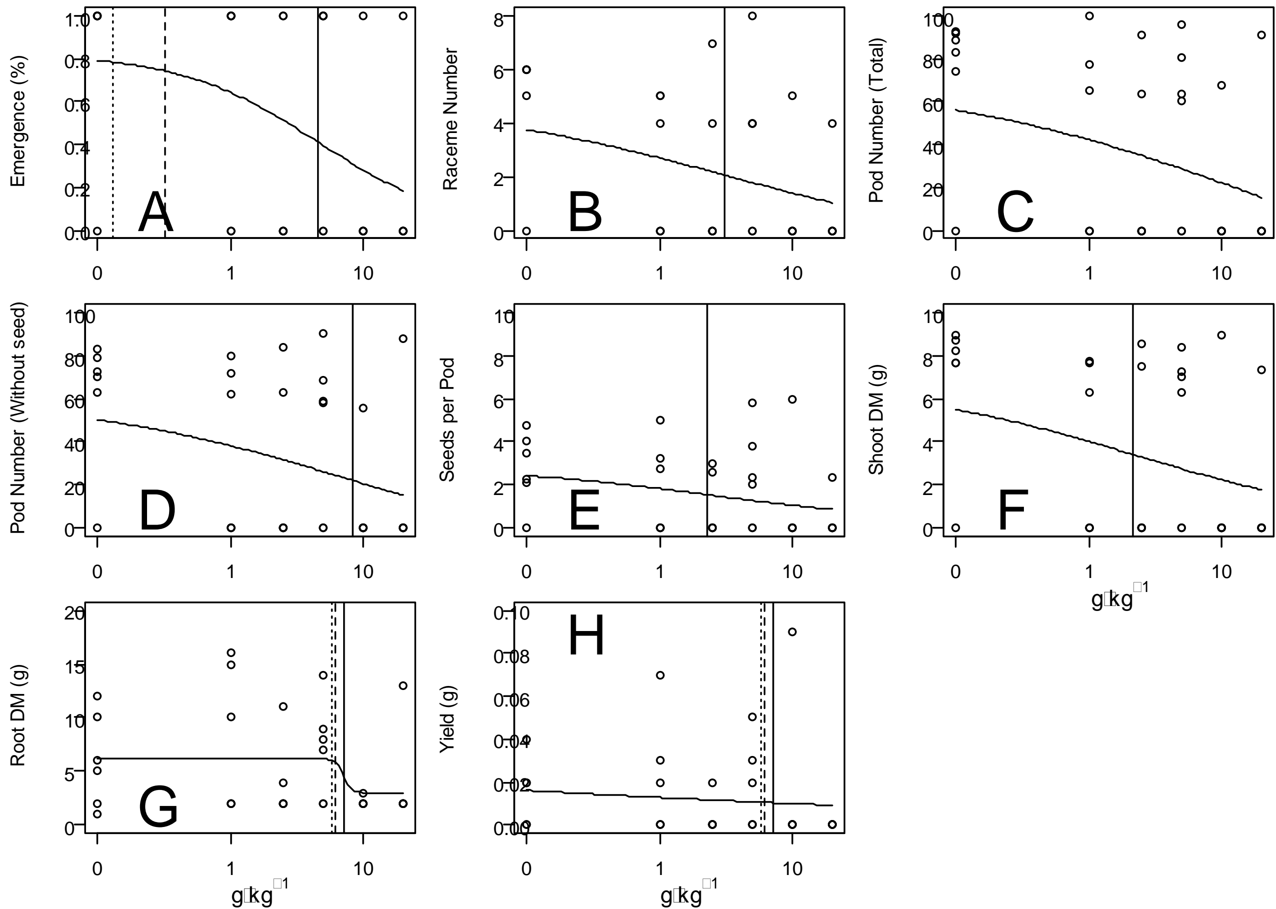

Figure 4.3.5. Experiment 3: Dose response curves fitted from 4 parameter log-logisitic models for multiple response variables at differing doses of pathogen inoculum (g kg^{-1}). A, Percentage emergence; B, Raceme number; C, Pod number (total); D, Pod number (Empty); E, Seeds per pod; F, Shoot dry matter (g); G, Root dry matter (g); H, Seed yield (g). Vertical lines represent LD_{05} (dotted), LD_{10} (dashed) and LD_{50} (solid) thresholds.

4.4 Isolate pathogenicity and variability

4.4.1 Isolate pathogenicity

Disease symptoms were evident on the majority of seedlings after 5 days post infection (dpi) (Figure 4.4.1), with scores ranging across the whole severity scale. Using the DSI a score of ≤ 20 was considered non-pathogenic, 21 – 60; weakly pathogenic and >61 pathogenic. Based on this system isolates represented; *R. zeae*, AG 3PT, AG 9 and AG 4HGII were showed to be weakly pathogenic to young OSR seedlings, whilst the majority of isolated representing AG 2-1 were pathogenic.

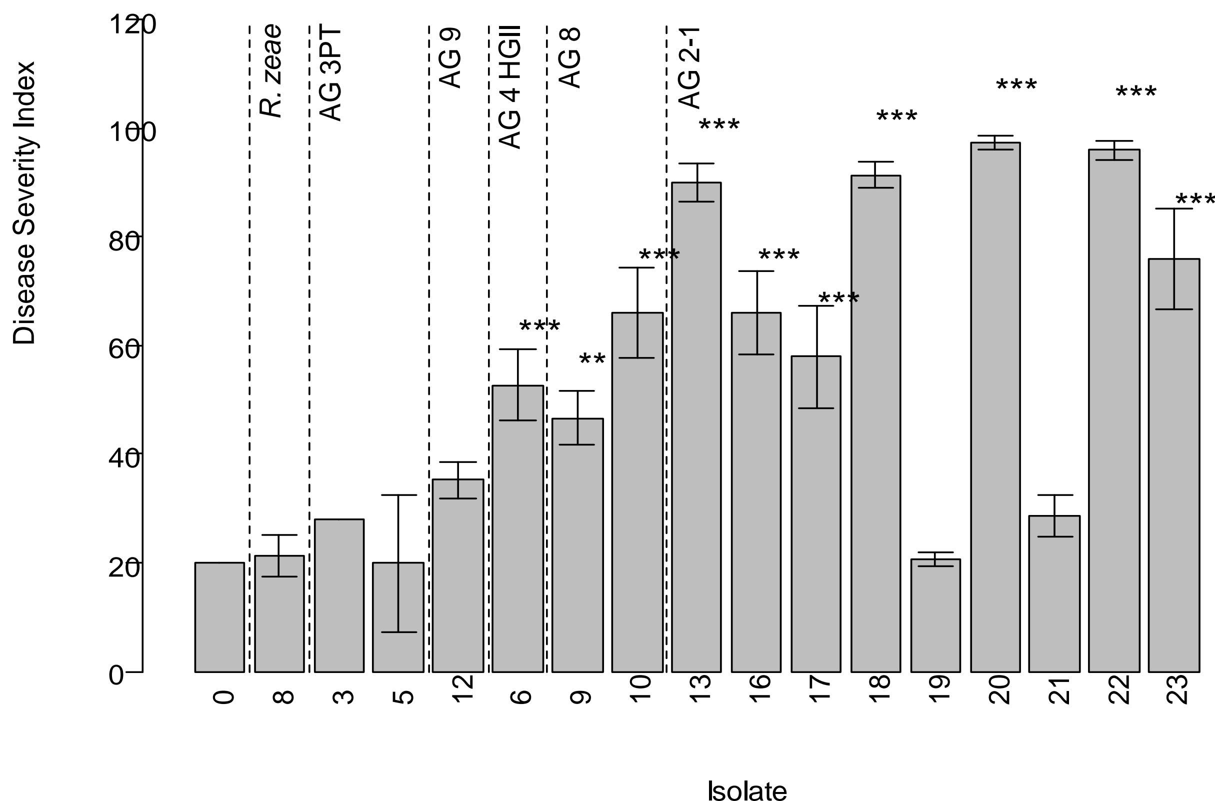

Figure 4.4.1. Disease Severity Index (DSI) of several *Rhizoctonia* isolates when tested on five-day-old oilseed rape seedlings. Error bars represent standard error of the mean. Significantly different means are represented by; ”*”, p<0.05; “**”, p<0.01, “***”, p<0.001.

4.4.2 Pectin zymograms

Pectin zymograms failed to produce any banding on the gels.

4.4.3 Genetic variation

The ITS region was amplified for most of the isolates available (Fig 4.4.2) with gel electrophoresis suggesting a mean product length of 750 bp.

The IGS region showed more variation than that of ITS, with product lengths varying between *c.* 600 – 1k bp (Figure 4.4.3).

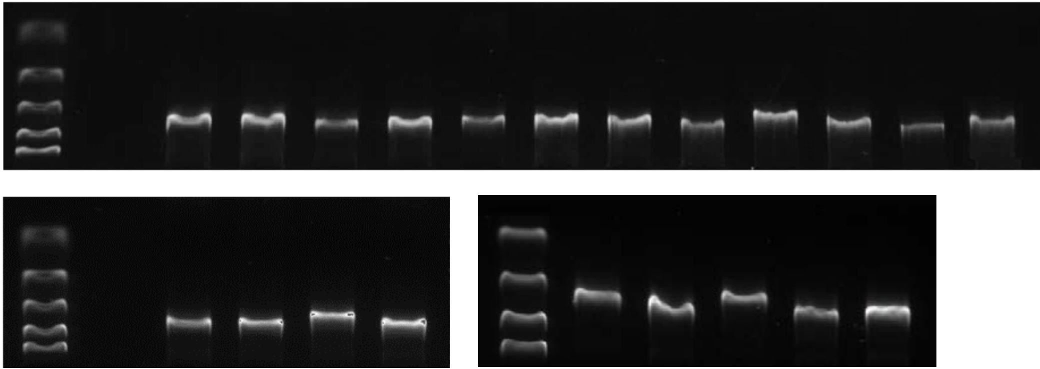

Figure 4.4.2. Gel electrophoresis of the ITS1 amplicons of several *Rhizoctonia solani* isolates. Top; ladder, R3, R5 – R15. Bottom; ladder, R17 – R20, ladder, R21 - 25. Ladder (T-B); 100, 200, 500, 1k and 2k bp.

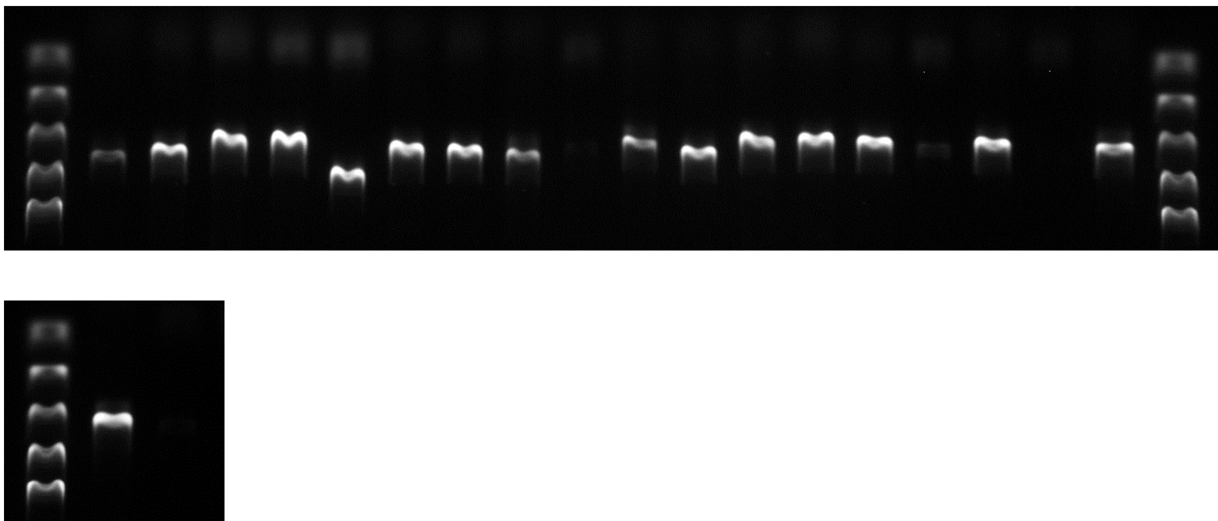

Figure 4.4.3. Gel electrophoresis of the IGS amplicons of several *Rhizoctonia solani* isolates. Top; ladder, R3, R5 – R15, R17-R22. Bottom; R23, R25. Ladder (T-B); 100, 200, 500, 1k and 2k bp.

5. Discussion

It is often perceived that the practice of crop rotation limits the accumulation of soil-borne pests and diseases, whilst improving soil flora and fauna. Our results, however, contradict this suggesting that the root microbiome of OSR may be simpler than first thought, with cropping intensity and other agronomic factors having limited influence on such communities. Many of the fungal species found were not pathogenic in nature but general saprophytes, although the pathogenic species *Rhizoctonia solani* accounted for a high number of sequence reads and was also common to many of the samples, suggesting that cropping intensity may have limited effects on this pathogen.

High throughput sequencing is an increasingly utilised technique for examining environmental samples, although its use within agriculture and the managed environment has been limited to date (Xu *et al.*, 2012; Liu *et al.*, 2014). Here we used the Illumina MiSeq platform to examine fungal communities associated with the roots of commercial OSR crops grown at different cropping intensities across England.

In contrast with other studies using HTS to investigate soil and plant roots (Schmidt *et al.,* 2013; Shakya *et al.,* 2013; Huang *et al.,* 2014) we found an almost equal proportion of Basidiomycetes and Ascomycetes, which together accounted for the largest components of the fungal communities. One possible explanation maybe the use of different sequencing regions or primers. For example, both Schmidt *et al.* (2013) and Huang *et al.* (2014) used the ITS primers of White *et al.* (1990), with Schmidt *et al.* (2013) targeting ITS1 (primers ITS1F12/2) and Huang *et al.* (2014) ITS1 and 2 (primers ITS1/2 and ITS3/4) regions respectively. Whilst targeting the same region as the current study, *in silico* analysis of these primers by both Bellemain *et al.* (2010) and Toju *et al.* (2012) demonstrated how these primers may bias coverage due to preferential amplification of Ascomycetes over Basidiomycetes, whilst the primers we use here (Toju *et al.*, 2012) were selected for their even amplification across different phyla. This may explain the high %RA 78.14 and >80 described by Schmidt *et al.* (2013) and Huang *et al.* (2014) respectively, in comparison to our own study. In addition, the samples of Schmidt *et al.* (2013) comprised of soil cores, which may contain a greater diversity of fungi as they originate from bulk soil (Berendsen *et al.,* 2012).

At genus level, samples were largely dominated by *Tremella* spp. a group of 'jelly fungi' which produce gelatinous basidiocarps and are exclusively parasitic of other fungi. Other groups found comprised of saprophytes (Xenasma, Mortierella, Mucor, Dichomitus, Cadophora) and plant pathogens (Sphacelotheca, Thanatephorus). This is similar to other studies (Buee *et al.*, 2009; Xu *et al.*, 2012; Liu *et al.*, 2013; Shakya *et al.*, 2013) where soil saprophytes compose a large part of the community being implicated in the breakdown of leaf litter and other organic matter (OM). Whilst we did not sample from the soil community directly, these organisms may be present by their sheer abundance in the soil, for example Wallander *et al.* (2001) showed that ectomycorrhizal (EM) fungi were able to produce several hundred kilograms per hectare of hyphal biomass. Alternatively, they may also be feeding directly or indirectly from the root system, through excreted carbon sources (Bonkowski *et al.*, 2009), or indirectly as a consequence of necrosis caused by pathogen, pest or mechanical damage.

Individual samples and associated OTUs showed little variation and no detectable differences. This is contrary to what was expected due to the fact that the samples covered many different sites across

England, encompassing different soil types and agricultural practices, most notably different cropping intensities of OSR. In other work investigating cropping intensity it has often been suggested that soil-borne communities would be altered, and in particular the presence of plant pathogenic species (Hilton *et al.*, 2013; Liu *et al.*, 2014). For instance, Hilton *et al.* (2013) found that increased cropping frequency of OSR significantly altered the fungal community in a long-term field experiment, with this attributed to an increase in pathogens such as *Olpidium brassicae* and *Gibellulopsis nigrescens.* Species-specific quantitative-PCR also demonstrated how levels of *O. brassicae* were approximately tenfold higher in plots where OSR had been grown continuously for 4 years compared to a 'virgin' plot where OSR was grown for the first time. However, the degree of separation, using nMDS, between 'virgin' plots and those of 1 in 2 or 1 in 3 was relatively low when compared to that between 'virgin' and continuous OSR. This potentially supports our results here as the difference between cropping intensities of between 1 in 2 and 1 in 20 years was not significant, suggesting that these effects are only prominent when comparing the 'extremes' of rotational frequency.

This similarity between field sites may be due to the ability of plant species to modify the rhizosphere through chemical processes. In a review on root microbiomes Berendsen *et al.* (2012) showed how, in general, rhizosphere communities were biomass rich but species poor compared to those of bulk soil. This phenomenon termed the 'rhizosphere effect' is primarily mediated by the plant, and in particular through the production of root exudates. One of the main mechanisms for this plant-microbe interaction is the fact that bulk soil is largely carbon poor in comparison to the rhizosphere where a plant roots excrete carbon rich molecules. In fact it has been suggested that up to 40% of a plant photosynthates are excreted into the rhizosphere (Bais *et al.*, 2006) representing a significant increase in available carbon. This mechanism, however, may also be more selective than purely providing a carbon source under carbon starved conditions, with exudates containing compounds such as flavonoids (Bais *et al.*, 2006) and glucosinolates (Kirkegaard and Sarwar, 1998; Rumberger and Marschner, 2003; Bressen *et al.*, 2009) being shown to alter bacterial and fungal communities in crops of soyabean (*Glycines max*) and mustard (*B. juncea* and *B. nigra*).

The species *Rhizoctonia solani* is a well-known pathogen of many plant species worldwide and is found extensively infecting OSR crops in Canada (Zhou *et al*., 2014), Australia (Khangura *et al*., 1999) and other regions, along with other species of brassica (Budge *et al*., 2009b). In these regions it is linked to a reduction in plant yield and health, causing seedling, stem and root diseases (Budge *et al.,* 2009b). Although often referred to as a single species, *R. solani* constitutes a disease complex currently comprised of thirteen different sub-groups or anastomosis groups (AG) with known differences in pathogenicity and host range (Carling *et al*., 2002). In addition survey data from commercial crops have largely been limited to Canada and Australia (Khangura *et al*., 1999; Zhou *et al*., 2014), where foot rots and seedling diseases are shown to be prominent in OSR. These studies have also demonstrated that these types of infection are largely caused by *R. solani* and in particular AG 2-1, 2-2, 4 and 8. However currently limited data exists on the quantities and distribution of *Rhizoctonia solani* within UK crops of OSR.

In the current study AG 2-1 was found to be the predominant group associated with commercial OSR crops, which is in agreement with other studies (Yitbarek *et al.,* 1987; Budge *et al*., 2009b; Brown *et al.,* 2014). The low detection of AG 8 and 5 is also similar to that of Brown *et al.* (2014), but contradictory to that of Budge *et al.* (2009b) where only AG 4-HGII was found in addition to AG 2-1

or Yitbarek *et al.* (1987) where AG 2-2, 3, 4 and 5. Some of this variation however is to be expected as these studies were based on soil samples, as opposed to root material as in our study, with the exception of Yitbarek *et al.* (1987) where both soil and plant samples were taken. Whilst sampling soil offers a means to examine potential inoculum and thus risk pre or post planting it is less effective to study in crop infection, due to the fact that bulk soil contains many organisms and 'candidate pathogens' in a rotational setting. In this instance, not all of the detected propagules may lead to infection, either by means of host specificity, lack of virulence, or their ability to interact with a host (pathozone). For example in the soil samples collected by Yitbarek *et al.* (1987) 6 different AG were found, whilst only 2-3 were found infecting young seedlings and adult plants. This may also be influenced by sampling distance and the pathozone effect (Gilligan, 1985). This zone is an area surrounding either plant or propagule which has the ability to interact with the others zone of influence, which for a model system involving *R. solani* and radish (*Raphanus sativus*) was determined to be 12-16 mm (Gilligan and Simons, 1987). As a consequence our sampling method provides a more realistic overview of the pathogenic organisms present on or within the root system, where they are more likely to be causing disease and impacting on plant health.

In addition, little information is available about the quantity of inoculum present at field sites, along with any defined thresholds for disease symptoms and yield loss. In the current study, pathogen inoculum densities varied by approx. 3000 fold between the highest and lowest sites, whilst mean levels across all sites averaged 2.7 pg ng^{-1} total DNA. This quantity was approx. 3 times higher than that found by Brown *et al.* (2014), although no information on the range of inoculum densities across this study was reported. In contrast, these levels are much lower than those found by Budge *et al.* (2009b) where a mean figure of 73 ng g^{-1} of soil was recorded, some 27,000 times greater the figure from this study, although only a 3 fold difference was seen across these sites. However, two points of conflict arise from these comparisons. Firstly the difference in units between the current study and that of other published work makes direct comparison difficult. Our choice of units was determined by the original samples, which were acquired from a previous project. In this respect it was difficult to attain a more meaningful unit such as ng g^{-1} of plant material. Additionally it is difficult to make the comparison between soil and root extracts. However in general these studies and our own demonstrate the variability in populations of *R. solani* AG 2-1, with a 100-fold difference between the means of Budge *et al*. (2009b) and Brown *et al.* (2010), with ours similarly showing a large variation in pathogen inoculum across samples.

Traditionally agronomic factors have been thought to play an important role in mediating inoculum inoculum survival/quantity. In the current study we found no significant link between pathogen quantity/occurrence on the roots of OSR and agronomic factors (year, region, rotation, previous crop, soil type, variety and establishment technique) when using either the HTS (occurrence) or qPCR data (quantity). This may be due to the biological mechanisms by which *R. solani* AG 2-1 is able to persist in soil, such as the production of resting structures (sclerotia) and an adaptive saprophytic activity. Naiki and Ui (1977) investigated *R. solani* survival and sclerotia formation, and found that Japanese isolates of AG 2-1 produced an abundance of sclerotia in artificially infested soil, considerably more than other AG tested (AG 1, 2-2, 3, 4 and 5). However, many of the sclerotia showed poor persistence with only 30% germinating on agar after 30 days in soil, with many cells showing a lack of cytoplasm indicating that they were unviable. This mechanism of survival may be less important in UK arable rotations where wheat is the rotational partner of OSR, along with the occurrence of *Brassicaceous* weeds, which are susceptible hosts for AG 2-1 (Verma, 1996;

Tewoldemedhin *et al*., 2006; Sturrock *et al*., 2015). In addition, the strong saprophytic ability of *R. solani* (Sumner, 1996 in Sneh *et al*., 1996) may also reflected by our findings with OSR in particular being a high residue crop.

Glasshouse studies conducted in this project highlighted that *R. solani* AG 2-1 is a predominantly pre- and post-damping off pathogen. Other soil-borne pathogens may cause yield loss by other mechanisms such as delayed flowering (Hilton *et al*., 2013), premature ripening (Gladders *et al*., 2009) or reduced numbers of seeds per pod (Strehlow *et al.,* 2015), but this was not seen in our investigation on *R. solani* infected OSR and *B. oleracea*. It has been suggested that some soil-borne pathogens exist at subclinical levels in the soil, until a threshold is reached. In experiments 2 and 3, no threshold effect for clinical disease was seen, with visible disease occurring on all but the control treatments. This was similarly supported by the literature when investigating the link between inoculum quantity and disease severity. In future it may prove useful to identify this at lower levels than tested here.

Yield loss was directly related to a reduction in emergence with the extent of seedling loss mediated by the quality and quantity of inoculum as witnessed in other similar studies (Budge *et al*., 2007). However when inoculum production proved successful, a strong positive correlation was seen between inoculum density and the amount of pathogen DNA per gram of potting mixture, a trend which was not seen by Budge *et al.* (2009b), but has been shown for other pathogens. Similarly, seedling emergence and survival was also reduced by a low density of inoculum, much smaller than previously shown (Budge *et al*., 2007) although our inoculum production method appeared to favour denser inoculum production with DNA data suggesting a factor of 100 difference between the two studies. In contrast, the study by Zhou *et al.* (2014) was able to establish a linear reduction in seed yield and biomass with an increasing dose of *R. solani* AG 2-1 inoculum. However due to the choice of units used in the two studies a direct comparison is not possible.

Within recent years, UK OSR growers have moved towards lower sowing rates and plant populations in an effort to decrease competition and increase individual plant canopy size. Currently the recommendation is to use a sowing rate of 30-40 seeds m^{-2} to achieve a population of 25-35 plants m^{-2} (AHDB, 2014), with this range being shown to be optimum within UK population trials (ADAS). However these recommendations also serve to highlight the risks to growers by assuming a high level of germination and establishment (*c.* 80-90%), demonstrating that even a small reduction in establishment rate would cause a reduction in optimum plant populations and in turn effect the yield. Our work highlights that even small amounts of *R. solani* AG 2-1 inoculum could cause a significant reduction in seed germination and plant establishment leading to suboptimal plant populations. However, it is also true that OSR is well known for its ability to compensate with low populations of 8-10 plants m^{-2} sometimes being able to yield as well as those at the recommended rate in conducive years (Mendham *et al.,* 1981; Leach *et al.,* 1999).

Although considerable variation occurs between and within field sites, there is also variation within *R. solani* and in particular AG 2. This group is the most diverse encompassing the sub-groups; 2-1, 2-2 IIIB, 2-2 IV, 2-2 LP, 2-3, 2-4, 2-t and 2-BI (Carling *et al*., 2002b). Similarly with the advent of DNA sequencing and isozyme patterns further variation has been demonstrated within these sub groupings (Woodhall *et al*., 2007; Stodart *et al*., 2005; Carling *et al*., 2002b; Gonzalez et al., 2001; Salazar *et al.,* 1999).

In our current study we aimed to utilise both molecular and enzymatic techniques to examine any differences between our isolates with a particular focus on factors affecting pathogenicity. Previous studies have demonstrated AG 2-1 to be highly aggressive on *Brassica spp*. along with others to a lesser extent such as AG 2-2, 4, 8 and 10 (Verma, 1996; Khangura *et al.,* 1999; Tewoldemedhin *et al*., 2006; Babiker *et al*., 2013). The results of our pathogenicity testing supported these views with AG 2-1 isolates largely proving to be the most pathogenic followed by 8 and 4HGII, whilst AG 9, 3PT and *R. zeae* showed a low level of pathogenicity largely causing no more than a slight discolouration of the hypocotyl and root. However, within the collection of AG 2-1 isolates a wide variation in pathogenicity was observed, with the majority of isolates causing seedling death apart from two isolates (R19 and 21) which failed to produce any symptoms. Whilst it may be possible that R19 and 21 were not viable, subsequent isolations showed them to be viable, suggesting a low rate of growth and colonisation. However, no differences in growth rate was seen across any of the isolates tested (data not shown) and the short distance between seeds and the initial agar core (10 mm) would negate this effect. In addition, the low number of isolates per AG means that the true variation within each isolate may be restricted.

In conclusion, our results here have suggested that the root systems of commercial OSR crops in the UK contain a diverse range of fungi which maybe recruited from the soil microbiome. In addition a proportion of these are known plant pathogens with *R. solani* being particularly common to many crops, despite intentionally sampling a diverse range of agronomic factors. The use of DNA methods has also shown how widespread this pathogen is and in particular the sub group AG 2-1, a known OSR pathogen but previously under researched in UK OSR. Glasshouse and laboratory studies demonstrated that this strain is highly pathogenic causing pre- and post-emergence damping off leading to reduced plant stands and poor yield production. Although, significant variation between UK isolates was observed particularly with regards to pathogenicity.

This work has allowed us to gain a greater understanding of UK OSR root health with a particular focus on the interactions between root systems and soil-borne pathogens, a hidden and understudied area. Our work sets the scene to increase our understanding of the ‘who’, ’what’ and ‘where’ in terms of OSR soil-borne pathogens, allowing future work to concentrate on elucidating management practices to counter act their effects on plant heath and productivity. It is from this knowledge, combined with better management, newer varieties and a greater agronomic understanding of modern OSR, that on farm yields might be raised thus shortening the yield decline gap between current on farm yields and crop potential.

6. Future recommendations

Based on our findings and the lack of current knowledge on communities of microorganisms in different arable cropping sequences, and the mechanisms involved within the rhizosphere of commercial OSR, further research is required. Fluctuations in communities caused by changes in crop species and sequential or rotational growing of agricultural crops (e.g. OSR_1-Wheat-OSR_2) needs closer examination. In particular focusing on the effects that this may have throughout a rotation on the microbiome causing; i) a defined change with each crop species, or ii) a diffuse effect whereby there may be a long lasting effect of an individual crop species.

In addition, our finding of *R. solani* AG 2-1 as being a pathogen of concern requires further attention, particularly in our understand of its infield distribution and methods to manage disease development such as varietal resistance, biological and synthetic seed treatment products and other cultural methods. The occurrence of this pathogen alongside other pathogenic and non-pathogenic fungi, and organisms also warrants further research, as this pathogen has been previously found to form disease complexes with species of plant parasitic nematodes leading to altered disease development and severity.

7. References

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