Harper Adams University

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# SOIL-BORNE PATHOGENS OF OILSEED RAPE: ASSESSING THEIR DISTRIBUTION AND POTENTIAL CONTRIBUTION TO YIELD DECLINE.

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# DECLARATION

The work presented in this thesis is an original compilation of the author and is in line with the registered title of the research programme. All the relevant sources of information referred to in this thesis are citied within the text and the sources appropriately referenced. None of the findings reported herein have been previously presented for award of a degree or other qualification in another institution.

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# ABSTRACT

Yield decline of oilseed rape (OSR) is a complex and multifaceted problem, with many contributing components. This is further compounded by soil-borne pathogens which are becoming an increasing concern, with shortened and species poor rotations implicated in their increase in recent decades. As a consequence, this thesis aimed to utilise molecular techniques to observe for pathogenic species within commercial crops of OSR, relating their occurrence to possible agronomic factors, whilst elucidating their role in yield decline through glasshouse experiments. Chapter 3 utilised real-time PCR (qPCR) to examine the occurrence of *Rhizoctonia solani*, *Pythium ultimum* and *Gibellulopsis nigrescens*. However this proved difficult as many of the assays available were not well designed or validated. Instead high throughput sequencing was utilised in Chapter 4 to examine the fungal communities within OSR roots, aiming to elucidate any pathogenic species present, along with their relation to potential agronomic factors. From this it was found that fungal communities were relatively simple, comprising of a few main phyla, genera and species; although this varied drastically with analysis technique used. Similarly, no relationship was observed between these species and the agronomic factors associated with the samples. However, both techniques used suggested that *R. solani* was a prominent pathogen within all of the samples, and as such warranted further study. *Rhizoctonia solani* is a diverse and variable pathogen comprising of 13 known sub-groups or anastomosis groups (AG), with Chapter 5 aiming to characterise these utilising AG specific qPCR assays. From this it was found that AG 2-1 was the main AG present within the samples, followed by AG 8 and 5. This was also confirmed by pathogenicity testing of UK isolates in chapter 6, where only AG 2-1 caused significant disease on OSR seedlings. Glasshouse experiments examined the role of pathogen inoculum in causing disease, and to clarify the mechanism by which yield loss occurred. Whilst the results varied between experiments as a consequence of inoculum preparation, they suggested that no sub-clinical disease symptoms occurred at the doses tested. In addition to this, the mechanism of yield loss appeared to be restricted to pre- and post-emergence damping off of seedlings which impacted on plant emergence and stand evenness. Further experiments in Chapter 6 examined the variability in a collection of UK isolates comprising primarily of AG 2-1 and other OSR non-pathogenic AG. Utilising an *in vitro* pathogenicity test it was found that AG 2-1 resulted in the most severe disease followed by AG 8 and 4 further supporting the qPCR results in Chapter 5 and the literature; although this was limited by the number of isolates. Interestingly, variation also existed within the AG 2-1 isolates with some being non-pathogenic in this system.

In conclusion, from this work it is apparent that soil-borne pathogens are present within UK crops of OSR, being present at levels which may impact on plant emergence and thus yield. However there is still a considerable way to go before effective management practices can be implemented by growers, with this being apparent from the lack of correlation between

agronomic factors and the presence of *R. solani* AG 2-1. In addition this may also be further compounded by genetic and pathogenic variation in UK populations of AG 2-1, but this requires further study.

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# RESEARCH OUTPUT

Poster Presentations

1. Molecular Biology of Plant Pathogens, University of Reading, UK 25th – 27th March 2014.
2. Annual Postgraduate Colloquium, Harper Adams University, UK 27th November 2014.
3. Annual HGCA/Potato Council PhD Symposium, Throws Farm Technology Centre, UK 21st April 2015
4. AHDB Smart Agrii Conference, Birmingham, UK 8th September 2015.
5. BSPP Presidential Meeting, University of Bristol, UK 13-15th September 2015.

Oral Platform Presentations

1. Annual Postgraduate Colloquium, Harper Adams University, UK 27th November 2013.
2. AgriiFocus (Guest Speaker) – OSR Event, Swindon, UK 6th November 2014.
3. Annual HGCA/Potato Council PhD Symposium, Harper Adams University, UK 8th April 2015.
4. Lunchtime research seminar, Harper Adams University, UK 29th October 2015.
5. Annual Postgraduate Colloquium, Harper Adams University, UK 6th December 2015.
6. 9th Australasian Soil-borne Diseases Symposium, Canterbury, New Zealand 14-18 November 2016.

Other

*Crops.* 2014. Research in focus: Scientists dig deep to resolve soil-borne oilseed rape disease dilemma. April, p. 30.

*Crop Production Magazine.* 2017. Theory to field: Out of sight, out of mind. June, p. 36-39.

*Farmers Guardian*. 2018. Soil-borne pathogens exposed. June, Online.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AG | Anastomosis group |
| AHDB | Agricultural and Horticultural Development Board |
| ATP | Adenosine Triphosphate |
| DEFRA | Department for the Environment, Food and Rural Affairs |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotide triphosphate |
| ddNTP | Di-deoxyribonucleotide triphosphate |
| F-dNTP | Fluorescently labelled dNTP |
| F-ddNTP | Fluorescently labelled ddNTP |
| DRMOs | Deleterious Rhizosphere Microorganisms |
| dsDNA | Double stranded DNA |
| gDNA | Genomic Deoxyribonucleic Acid |
| HGCA | Home-Grown Cereals Authority, *syn.* AHDB Cereals and Oilseeds |
| HTS | High Throughput Sequencing *syn.* NGS |
| ITS | Internal Transcribed Spacer |
| MAFT | Morley Agricultural Foundation Trust |
| NGS | Next Generation Sequencing |
| NIAB | National Institute of Agricultural Botany |
| OSR | Oilseed rape |
| PCR | Polymerase Chain Reaction |
| qPCR | Quantitative Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |
| rRNA | Ribosomal RNA |
| ssDNA | Single stranded DNA |
| TAG | The Arable Group |
| TRFLP | Terminal Restriction Fragment Length Polymorphism |
| TSB | Technology Strategy Board |
| UK | United Kingdom of Great Britain and Northern Ireland |

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# 1

# Literature Review

Oilseed rape (OSR, *Brassica napus*) is the third most important oil and protein crop globally after palm and soybean, and is utilised for industrial and culinary purposes. Europe is a large producer of OSR, with Germany, France, Poland, the UK and the Czech Republic accounting for *c.* 80% of the OSR produced within the EU. Similarly, yields within these countries are also some of the highest globally averaging over 3 t $ha^{-1}$. Alongside this, OSR also provides an economic and agronomic benefit within European and UK arable rotations, being the second most profitable crop after wheat, and providing a 'break' in cereal based rotations leading to improved rotational production. However, since the early 2000s UK yields have become limited to 3.4 t $ha^{-1}$ on average, whilst those of other European producers continue to increase beyond this. This thesis aims to examine this phenomenon in relation to agronomic factors, such as rotation frequency, and in particular the influence on soil-borne diseases and subsequent yield loss in UK commercial crops.

## 1.1 General overview of oilseed rape production

### 1.1.1 Classification and origin of oilseed rape

Oilseed rape (*Brassica napus*) (OSR) is a member of the genus *Brassica*, part of the larger *Brassicaceae* family. The term oilseed rape, is derived from the Latin word *rapum* meaning turnip, but is also known by different names including Swede rape, Rape, Rapa, Colza, Raps, and Canola. Many of these names also refer to the genealogy of the crop, with turnip and swede rape being a different species to that of true OSR.

Although described as a single species, modern cultivated OSR is an amphidiploid hybrid (AACC, 2*n* = 38), resulting from the spontaneous interspecific hybridisation between wild turnip rape (*B. rapa, 2n* = 20) and cabbage (*B. oleracea, 2n* = 18) (Figure 1.0) (Kimber and McGregor, 1995; Allender and King, 2010; Kole, 2011). This hybridization, followed by chromosome doubling, resulted in the species now commonly grown worldwide and is thought to have occurred in the Mediterranean region of Europe. However, as no wild forms of *B. napus* can be found it is thought to be a recent hybrid event compared to other crop species influenced by widespread cultivation (Kimber and McGregor, 1995; Snowdon *et al.,* 2007; Allender and King, 2010).

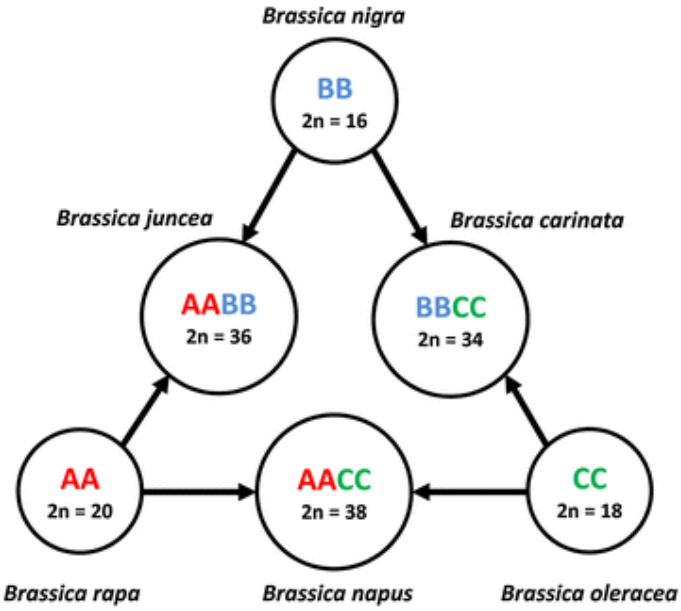


Figure 1.0. Triangle of 'U', depicting the interrelatedness of several major *Brassica* spp. and their hybrid species. Source: Koh *et al.*(2017).

### 1.1.2 History and crop use

Brassica vegetables and oilseed crops are thought to be some of the earliest crops to be cultivated, with indications of vegetable Brassicas being grown some 10,000 years ago (Snowdon *et al*., 2007). In his work on the centres of diversity for crop plants, Vavilov (1951) suggested oilseed Brassicas originated from the Mediterranean and neighbouring Asia-minor regions, where the wild ancestors of *B. napus* were largely confined, with records suggesting they were being cultivated around 4000 BC in India (Snowdon *et al*., 2007) and 1100 BC in China (Li, 1980 in Kimber and McGregor, 1995). In Europe, there is documentation suggesting that the Romans cultivated swedes (*B. napoBrassica*) and that by the 14$^{th}$ and 16$^{th}$ century these were being utilised for oil production (Colton and Sykes, 1992; Kimber and McGregor, 1995; Snowdon *et al*., 2007). From the 18$^{th}$ century, significant cultivation areas were being recorded as rapeseed oil gained in popularity as a high quality lubricant for machinery (Kimber and McGregor, 1995).

It was only recently that Brassica oils were used for culinary purposes, with the seed meal by-product utilised as an animal feed. This was due to the high concentrations of erucic acid (cis-13-docosenoic acid, 22:1n-9) and glucosinolates which gave the oil and seed meal a displeasing taste and in high doses caused health problems in humans and livestock (Snowdon *et al*., 2007). However, by the mid-20$^{th}$ century breeders in North America, and Canada in particular, began breeding varieties low in erucic acid and glucosinolates. These

'double low' varieties were then safe and palatable for use in both human and livestock feeds (Kimber and McGregor, 1995; 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). In the UK, it is an economically important food and industrial crop, despite being regarded as a recent addition, largely occurring after the Second World War in the 1950s and 60s. At this point, the average UK area totalled 600 ha a year, out of 6 million ha grown worldwide (FAO, 2013). 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 1960s has now resulted in OSR being the third most widely grown crop after wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (HGCA, 2013).

### 1.1.4 Oilseed rape production

Production of OSR is mainly based upon the cultivation of four *Brassica* species, with the species grown within a region differing based upon location/climate (Berry and Spink, 2006). The main producers of oil from oilseed rape are Canada and Europe with these regions also pertaining to some of the best yields of the crop (FAO, 2012) (Table 1.1).

Table 1.1. Overview of oilseed production, 2010 - 2013, from different *Brassica* spp. and geographic locations.

| **Region** | **Preferred Species** | **Ave. yield** ($t\ ha^{-1}$) | **Ave. production** ($1 \times 10^6$ Tonnes) |
|---|---|---|---|
| Europe | *B. napus* (Winter) | 2.6 | 23.4 |
| Canada | *B. napus* / *B. rapa* (Spring) | 2.0 | 15.1 |
| China | *B. napus* (Winter) | 1.9 | 13.7 |
| India | *B. juncea* | 1.2 | 7.3 |
| N. Europe | *B. rapa* (Spring) | 2.8 | 4.4 |
| Australia | *B. napus* (Spring/Summer) | 1.2 | 3.0 |
| Africa | *B. carinata* | 1.6 | 0.2 |

Adapted from: FAO (2013) and Berry and Spink (2006).

Europe is a major world producer of OSR, producing some 35% of the world supply from only 26% of the total area grown globally. This disproportionate production is as a consequence of higher yields attained in European countries, with countries such as Germany, France and UK achieving in excess of 3.0 t $ha^{-1}$, compared to other regions at less than 3.0 t $ha^{-1}$ (Figure 1.1).

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 across 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, with average yields being in the region of 3.3 t $ha^{-1}$ (FAO, 2014), whilst yields in other countries such as Germany continue to increase, averaging 4.0 t $ha^{-1}$ between 2009-2014 (Figure 1.2).

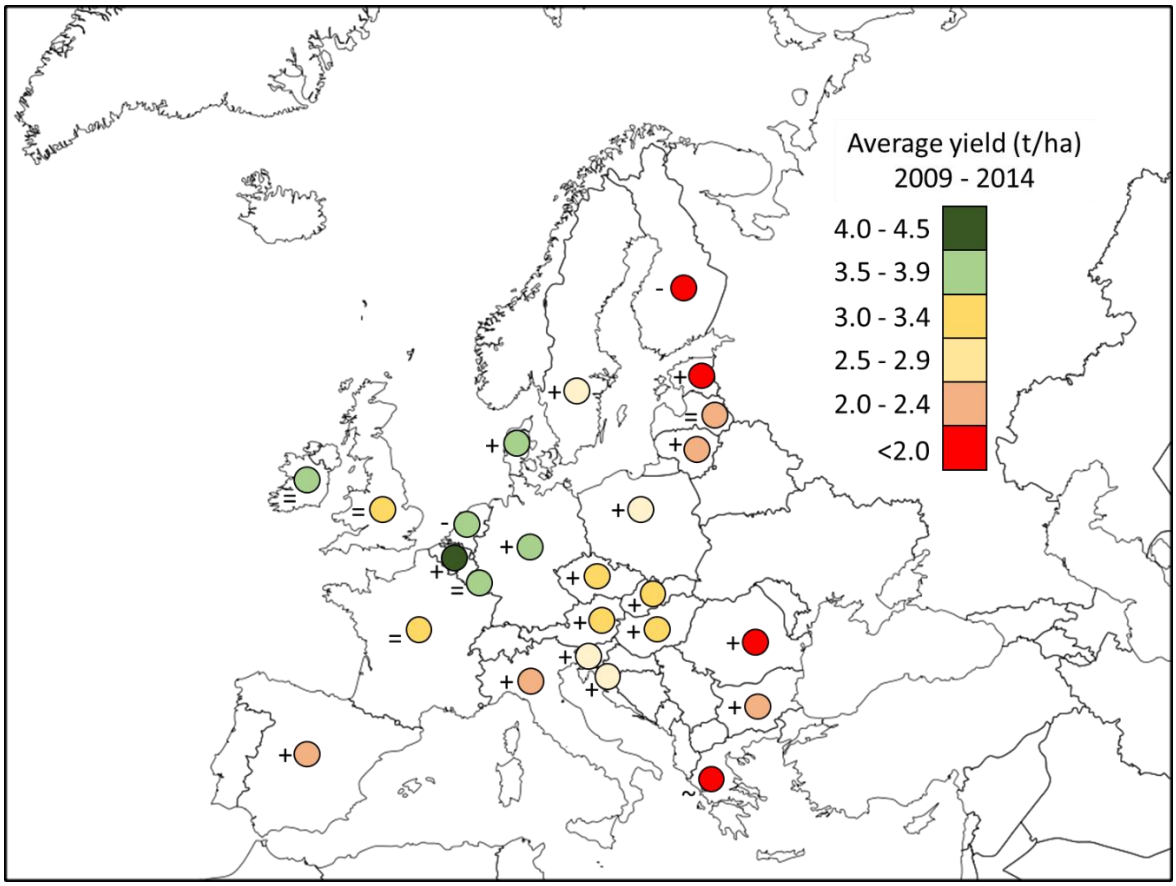


Figure 1.1. Average oilseed rape yields (t $ha^{-1}$) for individual EU countries between 2009 and 2014. Symbols represent the general yield trend during this period (+, increased; -, decreased; = no change,~ no clear trend). Sources; yield data – FAOStat.com; map - Wikipedia.com.

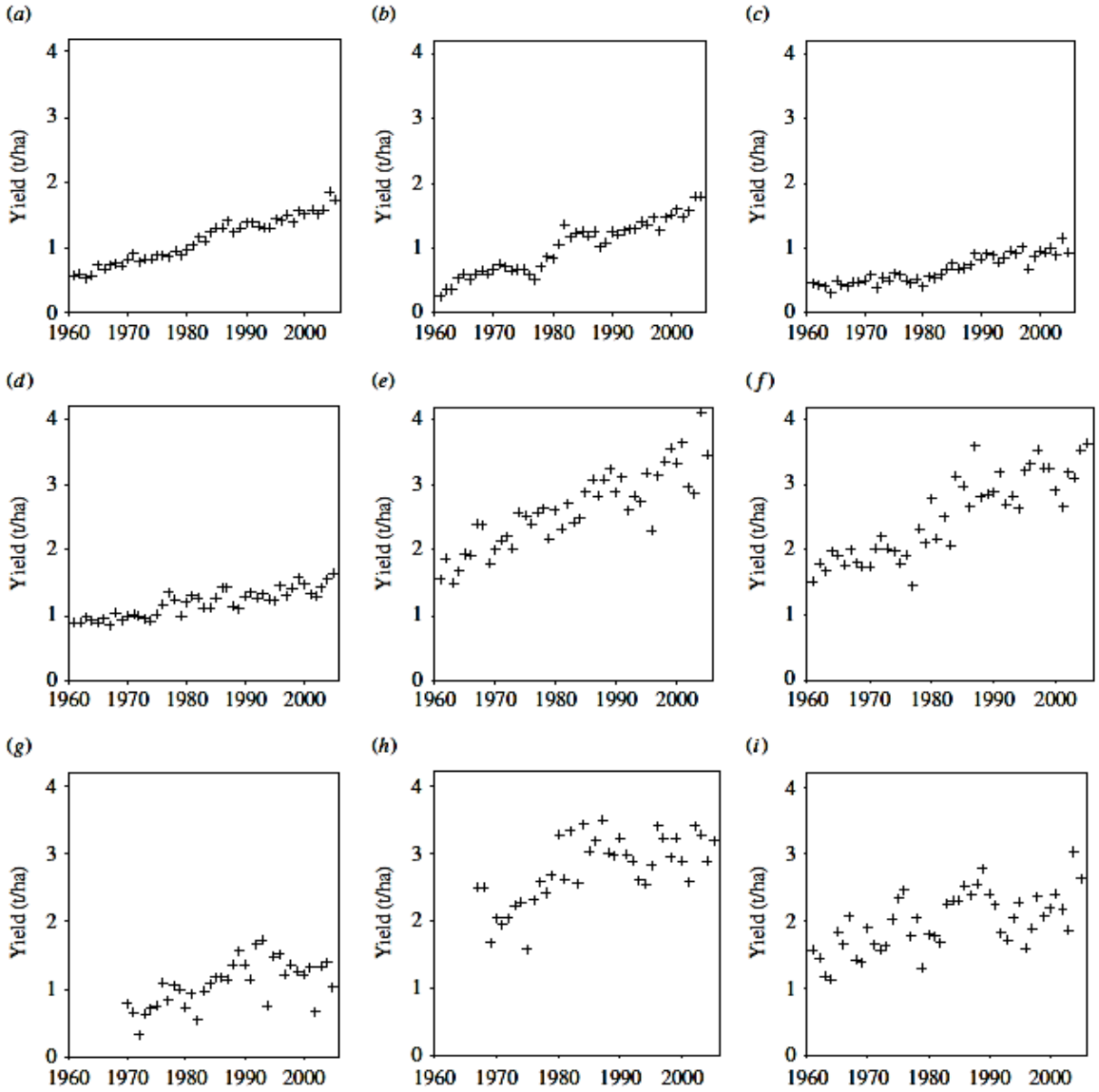


Figure 1.2. Oilseed rape yield trends for *(a)* World, *(b)* China, *(c)* India, *(d)* Canada, *(e)* Germany, *(f)* France, *(g)* Australia, *(h)* UK, *(i)* Poland. Source: Berry and Spink (2006).

### 1.1.5 Growth and physiology

*1.1.5.1 General growth and development stages*

Oilseed rape can be grown as either an annual (spring) or biennial (winter) plant from dormant seeds which are placed directly into the soil. Seeds are typically dark in colour being black or dark brown and, similar to all dicotyledonous crops, comprise of several differentiated tissues. Such tissues include two small seed leaves (cotyledons) which act also as storage organs containing fats and lipids, a hypocotyl (stem) and a radicle or primordial root which is surrounded by a seed coat or testa.

The physiological process of germination is initiated by a complex mixture of temperature and moisture, although it is generally more influenced by moisture, with development and

germination time influenced by temperature. In warmer autumn drilling climates, such as Europe, sufficient soil moisture is more of a concern, with temperature being more important in spring sowing regions such as Canada. Germination is initiated with the imbibition of water across the seed coat, once 40% moisture (w/w) is achieved the seed begins to synthesise storage molecules into DNA, RNA and proteins, before imbibing further and advancing development (Blake *et al*., 2004). In work by Kondra *et al.* (1983) soil temperature had limited effects on the percentage germination, with *B. napus* showing >90% between 2-25°C. However, lower temperatures limited the development time, taking 11-14 days at 2°C compared to 1 day at 21-25°C. Once fully imbibed the seed produces a radicle or root after which the cotyledons contained within the seed expand and elongate before emerging at soil level producing two typically kidney shaped leaves.

True leaves are produced post emergence from the shoot apex radiating outwards in a helical formation or phyllotaxy of approximately 130°. The rate at which leaves are initiated (plastochron) is generally faster than the rate at which they appear (phyllochron), which when coupled with the high leaf production (up to 10-15 leaves), leads to the formation of a closely packed rosette (Mendham and Salisbury, 1995). Depending on genotype and a minimum leaf number, the shoot apex becomes sensitive to photoperiod and vernalisation cues causing morphological changes to the meristem. This stage defines the switch from vegetative growth and leaf production to stem extension and reproductive growth.

Stem elongation involves the extension of the internodes between each leaf which are typically 5-10 cm in length, with *B. napus* capable of up to 15-20 internodes (Hayward, 2011). Flower buds also form in the centre of the elongating rosette, or primary raceme, remaining green until the oldest (basal) of these become yellow and open. Secondary raceme also develop in the leaf axials. During the green bud stage the sepals, stamens, petals and gynoecium develop, with meiosis occurring in the sexual organs producing microspores or pollen and ovules (Mendham and Salisbury, 1995). Flowering continues until all floral nodes have fully developed, flowered and become fertilised, commencing the formation of seed.

Post flowering the fertilised seeds (ovules) begin to develop within the ovaries causing an elongation of the two fused cepals (stigma, style and ovary tissues) forming the bivalved silique or seed pod. The seeds develop and grow to full size before ripening, progressing from soft and translucent to green, brown and finally black and firm (Hayward, 2011).

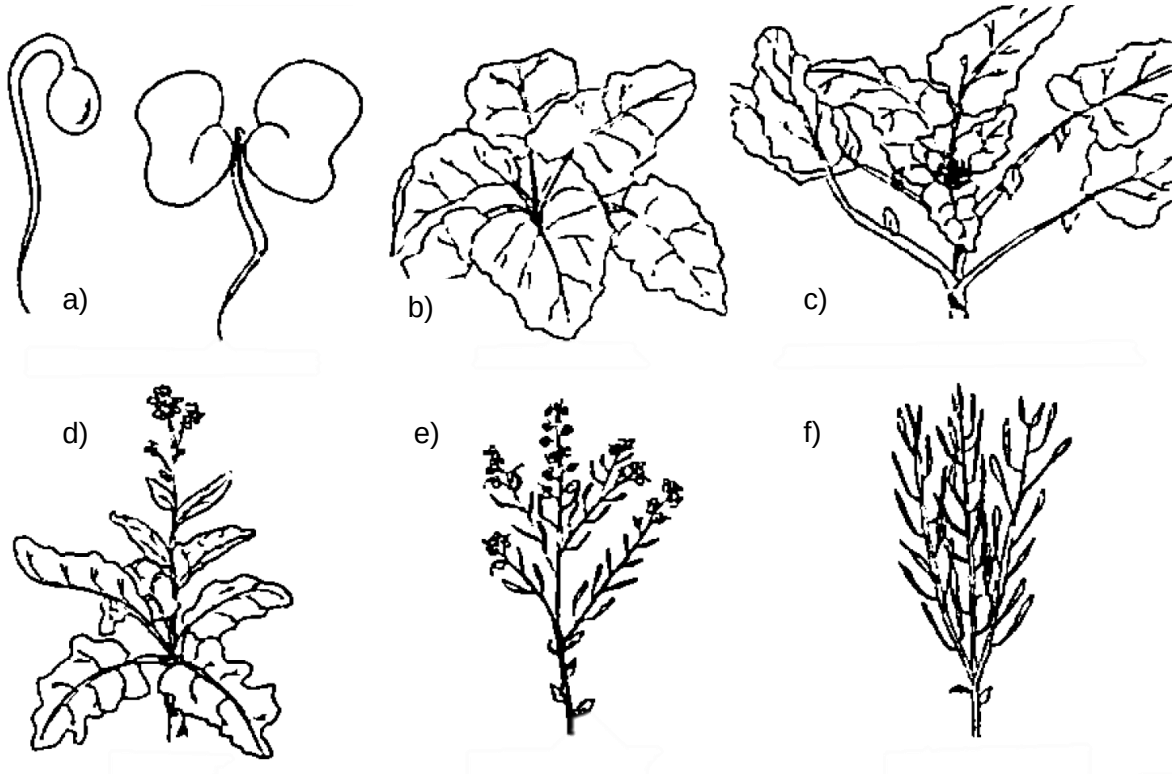


Figure 1.3. Life cycle of oilseed rape. a) Radicle and cotyledon emergence, b) rosette formation, c) stem elongation, d) Inflorescence, e) mid-flowering/beginning of pod formation, f) seed development and maturity. Adapted from: Hayward (2011).

*1.1.5.2 Root development*

Following emergence from the testa, the radicle or primordial tap root grows rapidly extending vertically downwards into the soil triggered by gravitropism. This is followed by a proliferation in secondary roots, which are produced laterally increasing the root surface area and ability to acquire nutrients and moisture (Kimber and McGregor, 1995).

Following the initial establishment of the radicle and lateral root system, biomass continues to increase under favourable conditions such as sufficient moisture, temperature and in the absence of limiting factors such as soil compaction and pest or disease damage. Work by Mendham *et al.* (1981) demonstrated how root biomass development in UK OSR was gradual in the autumn, with this almost ceasing in the winter months, until spring when the most marked increase in growth was seen. Sowing date also played a crucial part in this development with late August sowings favouring increased autumn root mass (c.50 g $m^{-2}$) compared to those sown in September and early October (c.3 g $m^{-2}$). This delay in biomass acquisition also affected the spring root growth and inflorescence date, with the August sown crops reaching mid-flowering some 2 weeks ahead of the latest sowing, whilst root biomass peaked at c.100 g $m^{-2}$ compared to others at 60, 50 and 30 g $m^{-2}$ respectively before ripening.

*1.1.5.3 Yield formation and potential*

The formation of OSR yield and quality is governed by a complex range of biological and environmental factors, although these can broadly be described in terms of the two yield components; seeds $m^{-2}$ and seed weight. These two components are reviewed in detail by Berry and Spink (2006), and discussed in relation to improving UK OSR yields. Furthermore, they suggest that seeds $m^{-2}$ forms the main component, as it determines the sink potential and thus maximum yield, with this supported by the results of Mendham *et al.* (1981) where 85% of yield variation was attributable to this. Using data from Lunn *et al.* (2001) they demonstrate that the optimum number of seeds $m^{-2}$ is currently *c.* 93,000, with this achieved at a canopy size of 6,000 - 8,000 pods $m^{-2}$. Below this the canopy inefficiently intercepts solar radiation, whilst above this, excessive flower production causes absorption and reflection of light prior to reaching the leaf canopy. The second component, seed weight, is largely determined post flowering, with stem reserves of carbohydrate contributing a negligible amount to the total yield. As a consequence, radiation interception by the canopy, and in particular the growing pods, contributes the majority (*c*. 90%) of yield. Here the limitations to yield are; radiation interception and water uptake, which occur through both physiological and environmental limitations. Examples of physiological limitations include; high stem to leaf ratio (1:3 ideal, Lunn *et al.*, 2003), leaf survival, lodging and poor root systems, whilst environmental limitations are largely; low solar radiation and low rainfall. As a consequence of these effects, suboptimal production of either or both components can occur within any individual year or crop to limit OSR yields.

In comparison to commercial crops the UK recommended list (RL) variety trials has continued to see an increase in yield, with a general increase of 0.5 t $ha^{-1}$ between 1980 and the early 2000s. In another article on the subject, Spink and Berry (2010) outlined how commercial crops tended to be, grown in short rotations (<1 in 3), are sown earlier and at higher seed rates, using minimal tillage techniques, whilst applying less inputs such as fungicides, and nitrogen and sulphur fertilisers compared to RL trials. In addition, many of these practices, whilst being time and cost effective, have also been linked to lower OSR yields. Shorter rotations have been shown to reduce yields (Mendham *et al.*, 1981; Christen and Seiling, 1995; Stobart *et al.*, 2012), whilst earlier sowing could contribute an additional yield loss of 0.1 to 0.2 t $ha^{-1}$ (Baer and Frauen, 2003) linked to thicker canopies. Tillage technique was considered to be of little importance alone, but may cause suboptimal establishment and growth by poor residue burial and increased pathogen and pest survival therein. However, despite the discussion of these factors, and the increasing yield of RL trials, no UK crops of OSR are consistently yielding near 6.5 t $ha^{-1}$, the calculated potential yield of current varieties under normal management. Although, some commercial crops do

achieve higher yields, with the current UK record being 6.7 t $ha^{-1}$ across a 17ha field (Farmers Weekly, 2015). In addition, when all conditions are considered optimal the 'ultimate' theoretical yield may be as much as 9 t $ha^{-1}$ (Berry and Spink, 2006).

### 1.2.1 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 (Table 1.1). Many studies also use differing terminology such as "soil sickness" in place of yield decline leading to further confusion.

As seen from Table 1.2, the most comprehensive definition is provided by the review from Bennett *et al.* (2012). This definition 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 with other crops have also been shown to lead to yield loss (Stobart *et al.,* 2012). As a result, many commercial rotations whilst seeming to break cyclical factors which contribute to yield decline, still lead to lower yields within many crops globally (Table 1.3).

### 1.2.2 General factors attributed to cause yield decline

Although yield decline itself is a simple concept to understand, the underlying mechanisms responsible are still not fully understood (Figure 1.4). Many studies have attributed yield decline in a specific crop or situation to one cause, often appearing to be simple to overcome. For instance, in the work of Dobermann *et al.* (2000), yield decline of rice in a continuous cropping system appears to be resolved through adjustments in nitrogen fertiliser usage. However, the authors acknowledged that other factors may also have influenced the results with up to nine factors such as nematode damage, disease pressure or changes in soil organic matter being suggested. 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.

Table 1.2. Definitions of yield decline from previous studies.

| Definition of yield decline | Reference |
|---|---|
| "A decrease in grain yields over a period of at least several years" | Dawe & Dobermann (1999) |
| "The loss of productive capacity of soil under long-term monoculture" | Pankhurst *et al.* (2003) |
| "The loss of productive capacity in soil caused by biotic and abiotic factors when crops are grown repeatedly on the same land, in short rotation or monoculture, resulting in poor plant growth and development, delayed crop production and reduced yields" | Bennett *et al.* (2012) |

Adapted from: Bennett *et al*. (2012).

Table 1.3. Global yield decline in agricultural crops.

| Crop | Yield decline (%) |
|---|---|
| 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).

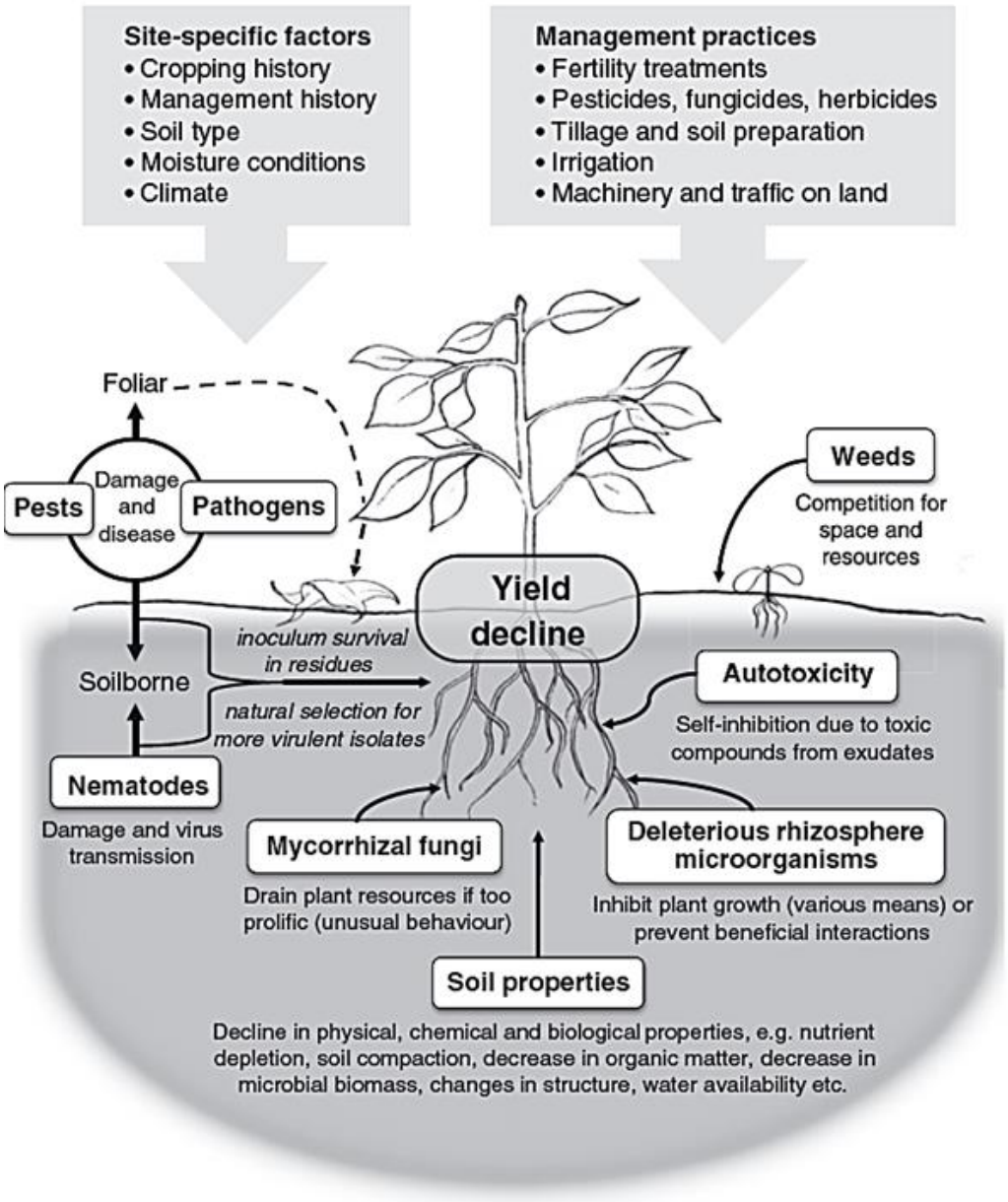


Figure 1.4. Factors associated with yield decline in crop plants. Source: Bennett *et al*. (2012).

### 1.2.3 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 (McNeill and Winiwarter, 2004). 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).

*1.2.3.1 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 with 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. Whilst 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 *et al.* (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 seven 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 1.5). Crops grown within a continuous rotation averaged 2.7 t $ha^{-1}$ over the eight-years, whilst the two '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 1.6).

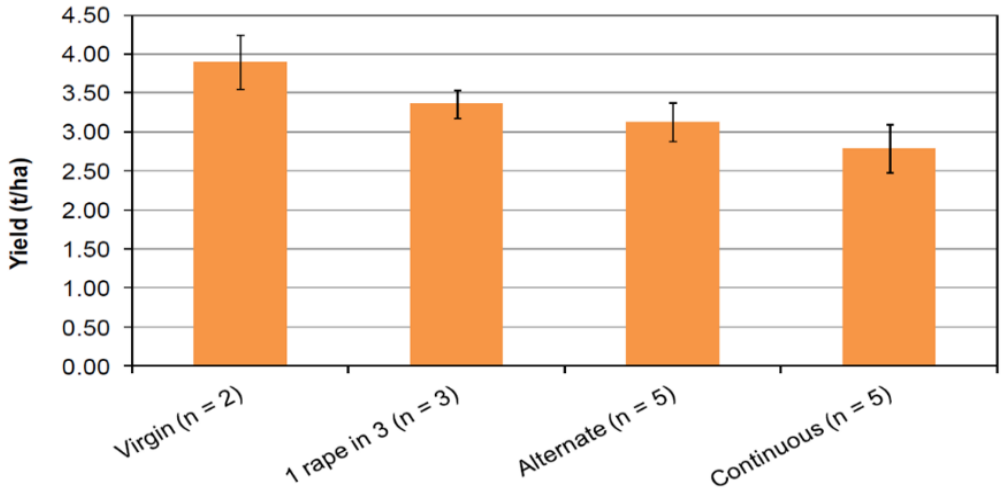


Figure 1.5. Average yield of OSR in different rotations after eight-years. Error bars represent the standard error of the mean, based on individual seasons. Source: Stobart *et al.* (2012).

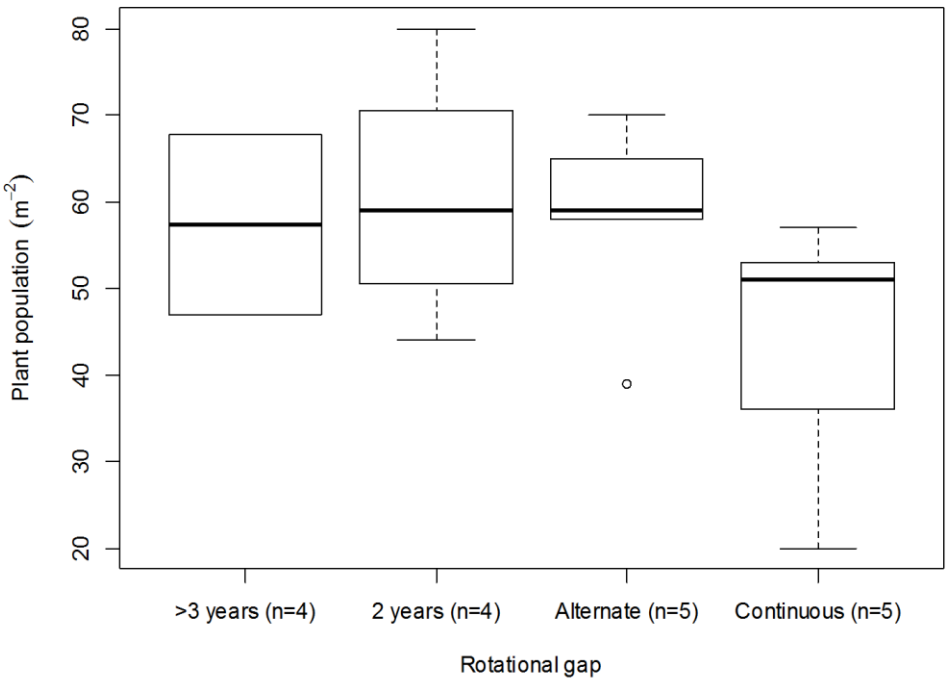


Figure 1.6. Average oilseed rape plant populations, at harvest, within a replicated rotational experiment. Adapted from: Stobart *et al.* (2012).

### 1.2.4 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 (Whipps and Lynch, 1986; Peters *et al.,* 2003).

Deleterious rhizosphere microorganisms are often referred to as pseudo pathogens as they lack the direct influence of pathogens *senso stricto*. Lacking the direct colonisation and damage caused by true pathogens, 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 (Whipps and Lynch, 1986; 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 glasshouse based studies.

### 1.2.5 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.,* 2008, 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 (Raaijmakers and Weller, 1998). However, yields are often not as high as those of the first wheat (Figure 1.7).

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 survival structures (sclerotia) 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), up to 8 years for *S. sclerotiorum* (Adams and Ayers, 1979), or in excess of ten years for microsclerotia 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.

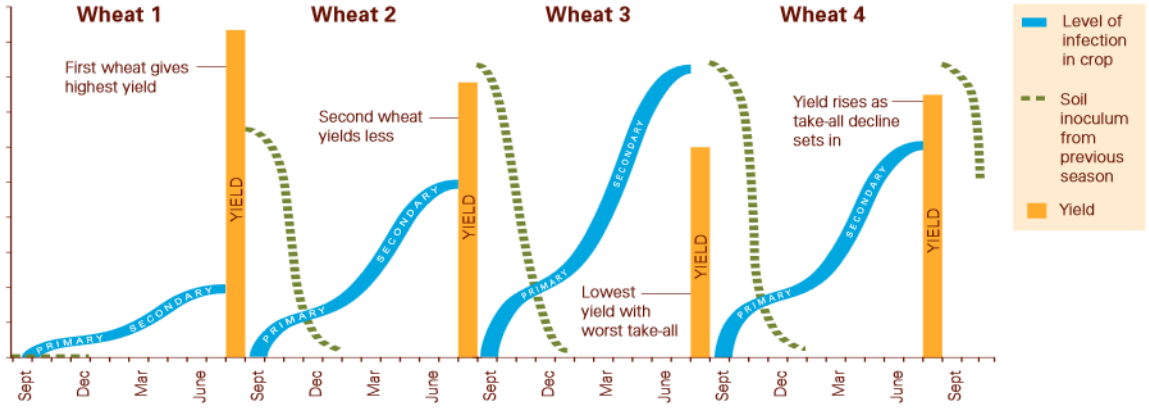


Figure 1.7. Take-all (*Gaeumannomyces graminis var. tritici)* infection levels and wheat yield during consecutive years of wheat monoculture. Source: AHDB (2006).

### 1.4 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, 1985; 1986; Morrall, 2000; 2011; 2013). Similarly, in a survey of crops grown in Finland between 1981 and 1987, Hannukkala (1988) found that damping off caused by *Rhizoctonia solani* and infections by *S. sclerotiorum* and *P. Brassicae* were common.

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 1.3), however these largely concentrated

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 by Budge *et al.* (2009a) showed *R. solani* to be present within UK horticultural Brassicas.

Table 1.3. 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 (2015) |
| *Plasmodiophora Brassicae* | 2007-2008 | 96 | 48.5 | 26.6 | Burrnett *et al.* (2013) |
| *Verticillium* wilt | 2009-2011 | 292 | 16 | 3.3 | Gladders *et al.* (2013) |
| *Rhizoctonia solani* | - | - | - | - | - |
| *Pythium* spp. | - | - | - | - | - |

a: Percentage positive 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.

### 1.4.1 *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. First reported in Berkshire in 1973, it has subsequently spread to much of the UK causing yield losses of in excess of 20% in extreme years (Fitt *et al.*, 1992).

#### *1.4.1.1 Lifecycle*

*Sclerotinia* spp. are 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) (Figure 1.8). Sclerotia are formed from the dense aggregation of hyphae, with those of *S. sclerotinia* comprising of a dark melanised outer rind and a non-melanised fibrillar inner portion or medulla. This structure is a long-term 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, although earlier autumn based infection of damaged leaves has been reported (McQuilken *et al.*, 1994). 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 of infection is not important in *S. sclerotiorum* (Clarkson *et al.*, 2013).

*1.4.1.2 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).

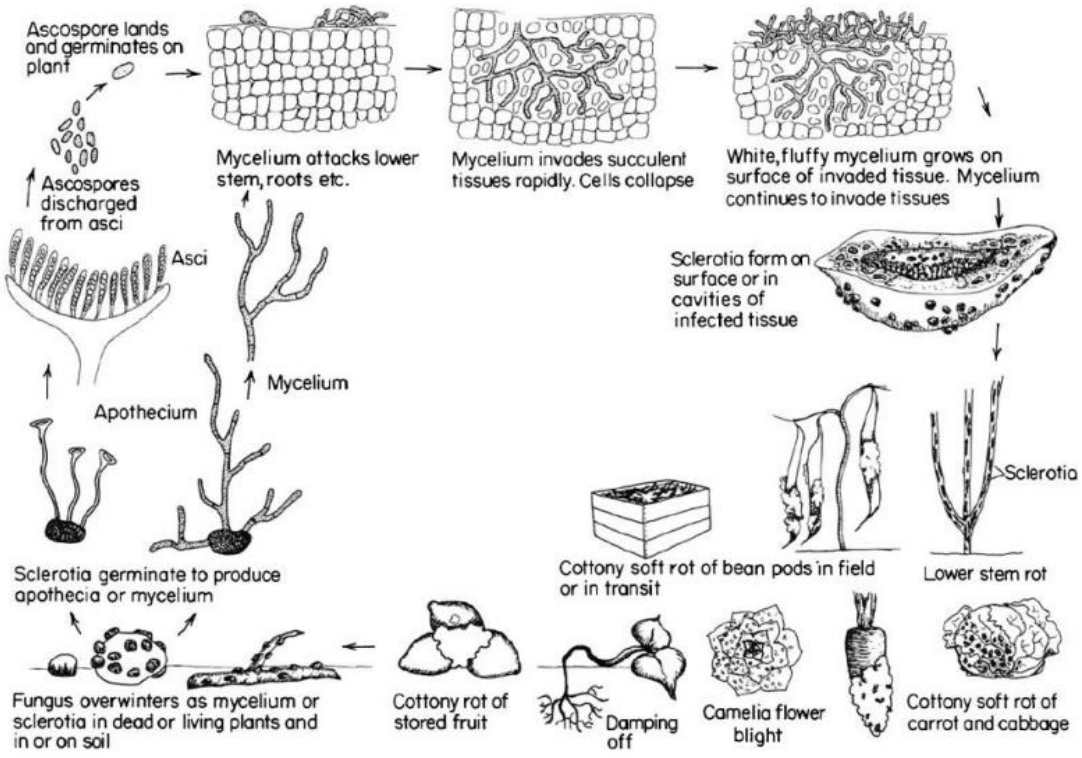


Figure 1.8. Lifecycle of *Sclerotinia sclerotiorum* and disease symptoms on different host species. Infection is initiated when dormant sclerotia germinate carpogernically to produce apthoecium, or less so in OSR, by directly produce mycelium within soil. In the case of apothecium, ascospores are produced when mature which are carried by air currents onto the leaves and stems of nearby plants. From here infection is initiated with the ascospores forming mycelium which invade the plant tissues, before forming sclerotia on or within the

tissues. After the plant senesces the sclerotia are returned to the soil and remain dormant until conditions are conducive for spore production and infection. Source: Agrios, 1997.

### 1.4.2 *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 an 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 senesces.

#### *1.4.2.1 Lifecycle*

The life cycle is initiated by the production of zoospores from long lived resting spores in the soil (Figure 1.9). 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, where they cause an up regulation in the production of plant auxins and cytokinins leading 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 (Wallenhammer, 1996).

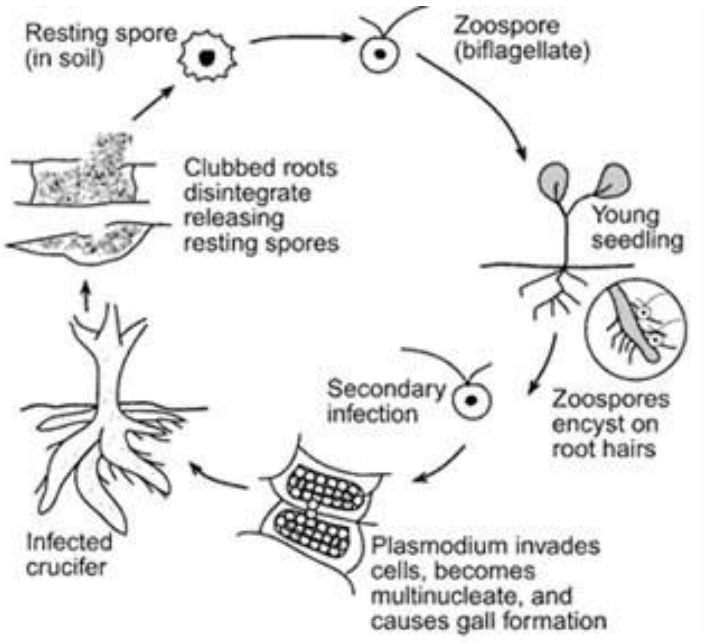


Figure 1.9. Lifecycle of *Plasmodiophora brassicae*, demonstrating the initial infection of young seedlings by motile zoospores, which encyst and infect the root tissues. Afterwards plasmodium form and proliferate within the root tissue leading to hypertrophy and eventual

degradation of the tissue, resulting in the dispersal of resting spores back to the soil.. Source: Canola Council of Canada, 2017.

*1.4.2. 2 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 breakdown in 'Medel' 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). Whilst traditional practices such as wide rotations, improved drainage and liming to increase pH may also prove useful to lower soil inoculum over time.

### 1.4.3 *Verticillium* wilt

*Verticillium* species are endemic and economically important pathogens on a wide range of dicotyledonous crops (Clewes *et al*., 2008) including many agricultural and horticultural crops (Inderbitzin and Subbarao, 2014). Formally classified by Nees von Esenbeck in 1816 (Isaac, 1967) the genus now comprises of ten known species with the most prominent being; *V. dahliae*, *V. albo-atrum* and *V. longisporum*. *Verticillium longisporum* is of greatest concern to OSR and Brassica crops as it is specifically pathogenic to these, compared to other *Verticillium* spp. which have a wide host range of non-Brassica crops (Zeise and Tiedemann, 2002). In the UK, it was first reported in 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, including the UK (Heupel, 2013).

A soil-borne hemibiotroph in nature, initial symptoms comprise of colonisation of the roots and vascular tissues by hyphae resulting in tracheomycosis and wilting. Following this the pathogen becomes necrotrophic producing long lived resting structures in the form of microsclerotia or dark resting mycelium (Clewes *et al.*, 2008).

*1.4.3.1 Lifecycle*

Initial infection occurs through the roots of young plants, with hyphae migrating towards the root in response to root exudates (Mol and Scholte, 1995) (Figure 1.10). 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).

*1.4.3.2 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).

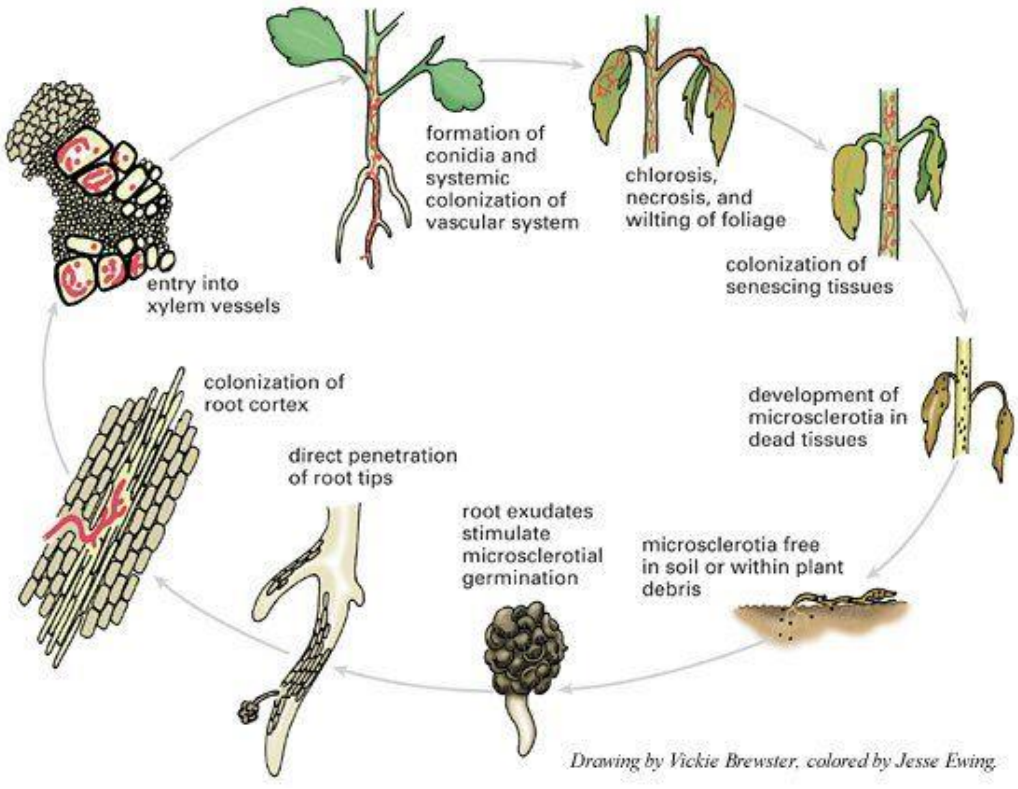


Figure 1.10. Lifecycle of *Verticillium longisporum* demonstrating the initial infection process by microsclerotial which germinate and invade plant roots. Afterwards mycelium colonise

the root cortex resulting in xylem blockage and tracheomycosis, leading to chlorosis and senescence of the plant tissues. Upon senescence microslcerotia are produced within the tissues being returned to the soil. Source: Berlanger and Powelson (2000).

### 1.4.4 *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 made of 13 known groups of individuals classified by their ability to anastomose through hyphal connections (anastomosis groups or AGs) (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 AG 2-1, 2-2, 4 and 8 are prominent. The pathogen has 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.

#### *1.4.4.1 Lifecycle*

Hyphae within the soil, either from decaying plant matter or from germinating sclerotia, make contact with the root system of a plant and begin the infection process. From here primitive appressoria 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 on 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.

#### *1.4.4.2 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).

### 1.4.5 *Pythium* spp.

*Pythium* spp. are soil-borne parasites belonging to the class of organisms known as the Oomyceta. Although once termed, and often still mistaken as fungi, they are actually a type of 'water mould' and as such are part of the kingdom *Stramenophila* (Chromista) (Trigiano *et al*., 2004). The genus was established in 1858 by Pringsheim, after observing cultures produced 'swarms of spore' which were morphologically similar to those of the genera *Saprolengiales* (Ward, 1883; Rao, 1963). Later on in the 19$^{th}$ century it was reallocated to the order *Peronosporales*, which also contain the genus *Phytophthora*, by Fischer in 1892 (Rao, 1963), with *Pythium monospermum* being the type species.

*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" symptoms where the germinating seedling is fully colonised and killed before emerging from the soil surface. The second form of infection occurs post-emergence, whereby the seedling is colonized and infected, resulting in wilting and lesions on the stem bases. After this seedlings will often succumb to infection and die. Due to these two infection methods, *Pythium* spp. can cause severe losses through the reduction in emergence and establishment rates.

#### *1.4.5.1 Lifecycle*

As mentioned previously, *Pythium* spp. are soil-borne parasites, but they are also present within a range of ecological niches from soil to aquatic habitats. Many of the species possess different infection processes ranging from saprophytes to obligate parasites (Trigiano *et al*., 2008). With most species associated with plants causing disease such as "damping off", foot rots, seedling blights and fruit-rots of stored crops such as potatoes (Cullen *et al.,* 2007).

*Pythium* spp. follow the basic life cycle of other oomycetes, and can be described as mainly diploid (2n) for the majority of its lifecycle (Figure 1.11). 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, 1997), resulting in macerated tissue and watery lesions. The macerated tissue

serves as a feeding site for the pathogen, allowing it to absorb energy for propagation and other metabolic activities. Thus, resulting in the death of seeds and seedlings, with tissue finally comprising of degraded tissue and fungal mycelium.

The above infection process remains the same for seedlings and older plants, with the exception being that mature plants are able to better resist and cope with infection; due to an increased cell wall thickness and differences in chemical composition. Infection can also occur on the rootlets of plant, with the pathogen proliferating within the young tissue at its tip. Mycelium may try to invade older tissues from this, however is usually suppressed by the morphology/composition of older tissues (Agrios, 1997).

*1.4.5.2 Management*

Typically, 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).

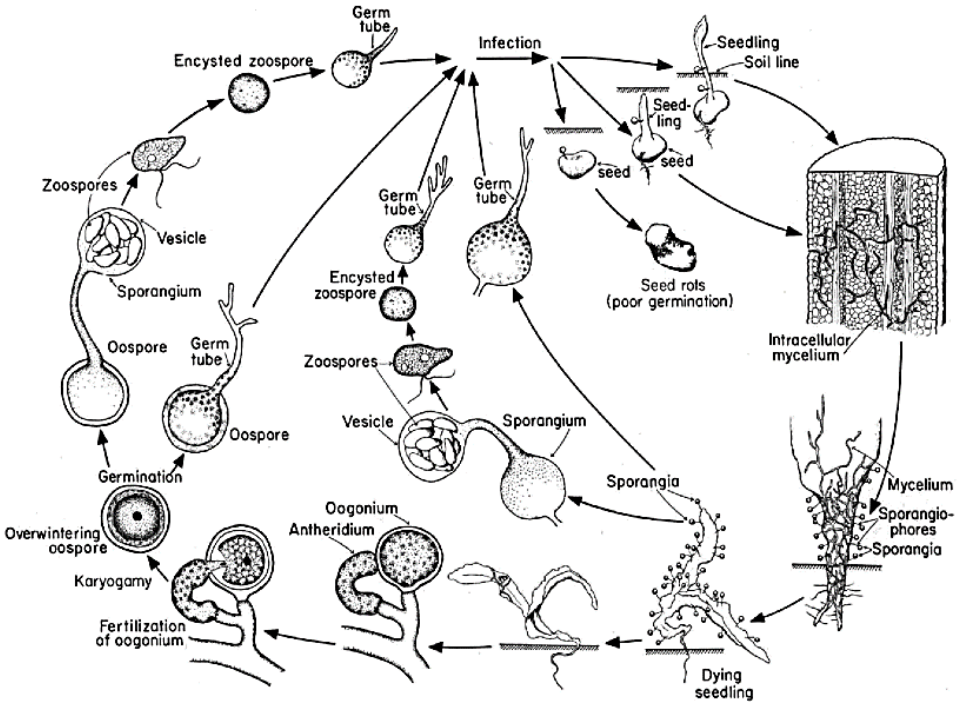


Figure 1.11. Lifecycle of *Pythium* spp., demonstrating 'damping off' and seedling infection. Infection of the plant root is initiated from mycelium which maybe produced from encysted zoospores or directly from sporangia. Infection results in the degredation of the plant tissue

often resulting in water soaked lesions and ‘soft rots’. Once the plant senesces sporangia, oogonium and oospores maybe produced, from which both zoospores and mycelium maybe produced when conditioned are conducive to infection. Source: Agrios (1997).

## 1.5 Molecular methods utilised to study soil-borne pathogens

The term 'Molecular biology' was coined by Weaver in 1938, to describe the perceived future study of molecular level interactions within cells which was gaining popularity at the time. Before this the basis of the field had already been established, with the idea of genetic inheritance and the isolation and chemical composition of DNA examined. Afterwards further advances would complete the final links in the puzzle, elucidating the double helix structure of DNA and its role in biosynthesis through the central dogma concept. Although, it wouldn't be until much later in the 20$^{th}$ century until this field became more widely recognised.

Today, in 2018, 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. Through the development of specific, sensitive and high throughput methods these problems can in part be overcome, with an ever-increasing number of published diagnostic assays for phytopathogens promoting the growth and use of these tools.

### 1.5.1 Polymerase Chain Reaction

#### *1.5.1.1 Conventional PCR*

One of the first tools to become ubiquitous was that of the polymerase chain reaction (PCR). First demonstrated by Mullis *et al.* (1986) this technique became widely available with the advent of thermostable polymerases (Saiki *et al.*, 1988) and automated thermocyclers, becoming the basis of many molecular tools. In principle, PCR uses the biosynthesis mechanisms utilised by cells to translate and replicate DNA, using this to construct multiple copies over the course of a reaction. In the laboratory, a reaction mixture comprising of primers (sense and anti-sense or forward and reverse, oligonucleotides), DNA polymerase, dNTPs, $MgCl_2$ and buffer is prepared within a reaction vessel (microtitre plate or individual 0.2 ml tubes), after which the dsDNA sample is added. The vessel is sealed and placed within an automated thermocycler, where it undergoes several cycles of differing temperatures. These are separated into; initial denaturation, followed by cycles of [denaturation, annealing and extension/elongation], before a final elongation step (Fig 1.12). Initial denaturation aims to convert dsDNA to ssDNA by raising the reaction temperature to *c*. 95°C causing the double helix structure to unravel and separate. Annealing then takes place with the reaction cooled (<70°C, actual temperature used is

dependent on primer melting temperature) resulting in the primers annealing to the target sites, forming the starting point for extension. The temperature of the reaction mixture is then raised (*c*. 72°C) and the DNA polymerase begins to extend or elongate the sequence from the 3' end of the primer, utilising dNTPs to form the complementary strand in the presence of $MgCl_2$ which acts as a catalyst. After sufficient time has lapsed the cycle of denaturation, annealing and extension/elongation is begun again, with this typically being done for 30-40 cycles in total. After the last cycle, a final extension/elongation is performed to ensure amplified DNA products (amplicons) are fully extended. Once complete the process results in multiple copies of the target region, resulting in a theoretical 1.1 x $10^{12}$ ($2^{40}$) copies from a single DNA molecule after 40 cycles. Direct observation of this result is difficult, however gel electrophoresis is commonly used to visualise the presence/absence and size differences of amplicons, whilst DNA sequencing determines the sequence of nucleotides present.

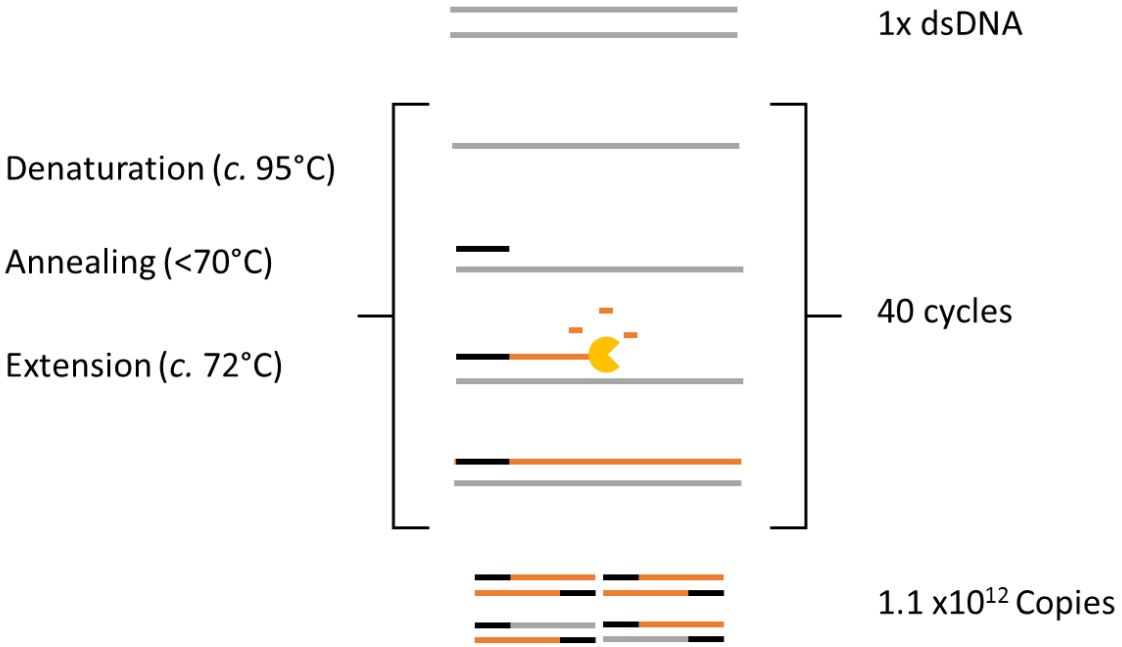


Figure 1.12. A diagrammatical representation of the polymerase chain reaction process. Double standed DNA (dsDNA) is first denatured by the application of heat (c. 95°C). Once denatured small oligonucliotides (primers) anneal to the complimentary sequence under cooler conditions (<70°C), forming a starting position from which DNA Polymerase extends the sequence using free dNTPs which are present in the reaction mix. Once complete the new strands are once again denatured and the process conducted again. This typically occurs over 40 cycles resulting in a large number of dsDNA amplicons, which maybe visualised by gel-electrophoresis and with the aid of UV florescence dyes. Authors own.

*1.5.1.3 Quantitative PCR*

Quantitative real-time PCR (qPCR), not to be confused with reverse-transcription PCR (RTPCR), utilises the concept of conventional PCR whilst utilising fluorescent dyes to quantify the PCR reaction in real-time. Whilst there are many methods which are currently available, two of these; SYBR Green and TaqMan® have become the most widely used.

SYBR Green is an intercalating asymmetrical cyanine dye which has a strong affinity for dsDNA, forming a DNA-Dye complex (Fig 1.13). It is added as part of the PCR mastermix where it intercalates with the dsDNA as it is constructed. At the end of each PCR cycle the reaction is exposed to blue light ($\lambda_{max}$ = 497 nm) exciting the DNA-dye complex which emits green light ($\lambda_{max}$ = 520 nm) that is then measured.

The TaqMan system uses a similar concept to that of SYBR Green, with the exception that the dye is not intercalary but bound to a DNA probe (Fig 1.13). The probe is comprised of a short oligonucleotide, similar to a primer, with the addition of a reporter dye at the 5' and a quencher molecule at the 3'. In this state any light emitted from the reporter dye is absorbed by the quencher through a process known as Forster resonance energy transfer (FRET). In addition, the probe is designed to a complimentary sequence between the locations of the two PCR primers, resulting in the mechanism of quantification. During the PCR cycle both the primers and probe anneal to the ssDNA, with the primers forming the site of extension. As the extension step is performed the DNA polymerase extends the complimentary strand towards the probe, before removing and cleaving the probe in order to continue synthesising the complimentary strand. As the probe is released, the reporter dye and quencher are separated allowing the light emitted from the reporter dye after excitation to be measured. If the probe is not removed by the polymerase, or remains in solution, then the quencher absorbs the energy required for the reporter dye to fluoresce, giving no signal.

Assuming that both techniques amplify at 100% efficiency this results in an increasing signal over time, resulting in a sigmoidal curve by the end of the reaction. Quantification is achieved by establishing a cycle threshold ($C_t$) across the samples, and is usually done automatically by the instrument. The resulting $C_t$ value can then be derived into a meaningful unit for quantification by comparison to a serial dilution of pure DNA (calibration curve) which are amplified alongside the samples.

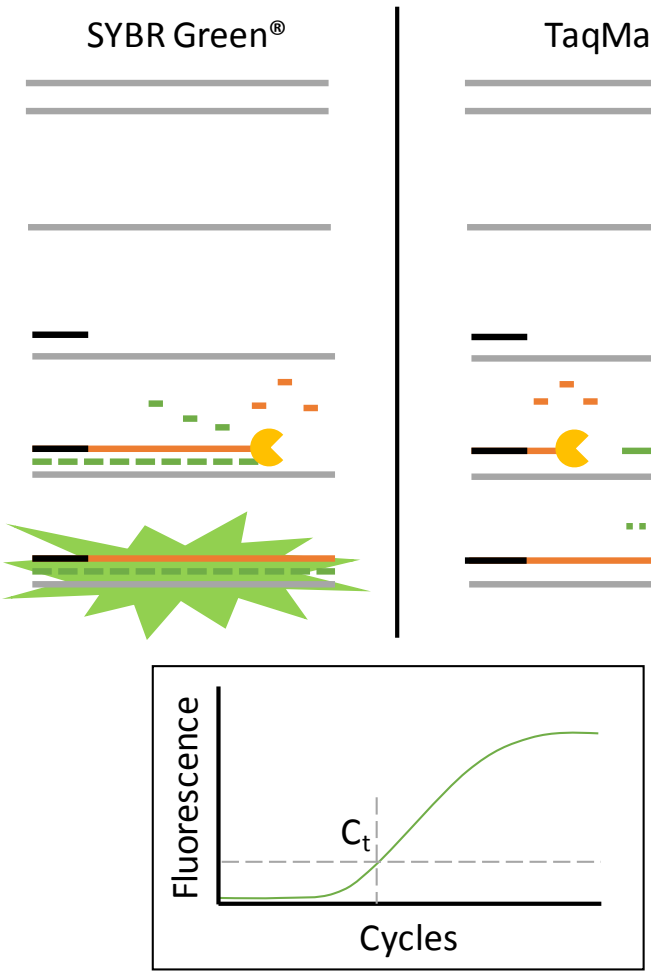


Figure 1.13. Comparison of the SYBR Green and TaqMan systems. The SYBR Green system (Left), utilises an intercalating dye which has a high affinity for dsDNA and becomes bound to the dsDNA during each PCR cycle. After each cycle the dye is excited causing the dye to fluoresce which is then measured. In comparison, the TaqMan system employs an additional oligonucleotide (probe) comprised of a fluorophore and quencher molecule. When attached together on the probe molecule the quencher prevents the fluorophore from fluorescing through FRET. However, during the extension process the DNA polymerase causes the bound probe to become cleaved separating the fluorophore from the quencher, and thus allowing it to fluoresce. As with the SYBR method, the amount of fluorescence is measured after each cycle and is proportional to the starting quantity of the target DNA sequence. Authors own.

### 1.5.2 High throughput sequencing (HTS)

Alongside PCR similar advancements were also made in reading DNA/RNA through the establishment of sequencing methods. Beginning with the pioneering use of di-deoxynucleotide triphosphates (ddNTPs) to terminate DNA strands by Sanger *et al.* (1977), with the later modification of fluorescently labelled ddNTPs (F-ddNTPs) (Smith *et al.*, 1986)

allowing for automation and increased uptake. Together both PCR and DNA sequencing techniques formed the basis of molecular biology until the advent of the 21st century.

During the 1990s interest in the use of molecular biology within life and medical sciences increased, with one of the beacon projects being that of the Human Genome Project (HGP). Established in 1990 by the United States government, HGP aimed to sequence the complete human genome by 2005, but was completed in 2003 ahead of schedule. As a consequence, high throughput sequencing became more popular, efficient and cost effective leading to the development of newer sequencing technologies with Roche 454 launching the GS FLX platform in 2005. The Roche 454 GS FLX was the first departure from the Sanger method, utilising a new generation of sequencing based on the detection of the pyrophosphate generated from dNTP incorporation, as opposed to the direct observation of F-ddNTPs, leading to the colloquial term 'Next generations sequencing' (NGS) which is used synonymously with the term HTS.

To date (June 2018) four key companies (Roche, Illumina, ThermoFisher Scientific [previously Applied Biosystems and Ion PGM Inc.] and Pacific Biosciences) develop and supply these platforms, which cover four main techniques or sequencing technologies. Each platform and technology has its own advantages and dis-advantages, particularly with reference to read length, accuracy and intended function. However, those which utilise the sequencing by synthesis method (Roche, Illumina and Ion PGM-ThermoFisher Sci.) are the most common. As a consequence of this, and in order to prevent this becoming an NGS review, only this technique and its use in amplicon sequencing will be described further.

*1.5.2.1 Pre-sequencing*

In brief, amplicons of the target region are constructed by PCR, with either biotin (Roche 454) or adapter sequences (Illumina and ThermoFisher Sci.) added before denaturing to form single strands. Two methods are then utilised to capture the strands for sequencing using either magnetic beads or a glass flow cell. The Roche 454 system utilises the strong covalent bonds produced between biotin and streptavidin to secure the strands to streptavidin-coated magnetic beads (Fig 1.14, A), whilst in the Ion torrent system the biotin-streptavidin complex is replaced with embedded adapters which complement those found on the amplicon (Fig 1.14, B). The Illumina system (Fig 1.14, C) utilises a glass flow cell with an embedded lawn of two (A and B) adapter sequences, but only one is available for binding at the beginning of the reaction.

Once adhered to the sequencing surface both the bead and flow cell methods utilise variants of PCR to generate clonal clusters of the adhered amplicon strand. The simplest

technique is the use magnetic beads (Fig 1.14. A and B) and emulsion PCR (emPCR). The beads become individually encapsulated within a hydrophilic droplet, containing polymerase, dNTPs and other reagents needed for PCR, within a hydrophobic matrix (emulsion). This isolates the beads and their amplicon strand separating them from other beads preventing sequence contamination. Multiple copies of the strand are then produced by PCR which become bound to the bead due to the encapsulation process, forming a clonal cluster. This process also forms the basis for the Illumina technique (Fig 1.14, C) with the exception that bridge PCR (bPCR) is used rather than emPCR. In brief, the compliment of the secured strand is generated onto the embedded A adapter (Fig 1.14, C2), before cleaving the initial strand. This bound strand now comprises of the embedded A adapter at the 3' end, the sequence of interest, and a 5' A adapter sequence. The 5' A adapter sequence then binds to a neighbouring embedded B adapter resulting in a 'bridge' (Fig 1.14, C3). Following this, PCR generates the complimentary strand which becomes attached to the B adapter (Fig 1.14, C4) at the 3'. The products are then denatured to release any unattached 3' and 5' ends (Fig 1.14, C5), before repeating Steps 3-5. This results in a cluster of clonal sequences in both forward and reverse positions. Due to the need to sequence individual clusters the reverse strands are cleaved leaving the forward strand to be sequenced (Fig 1.14, C6).

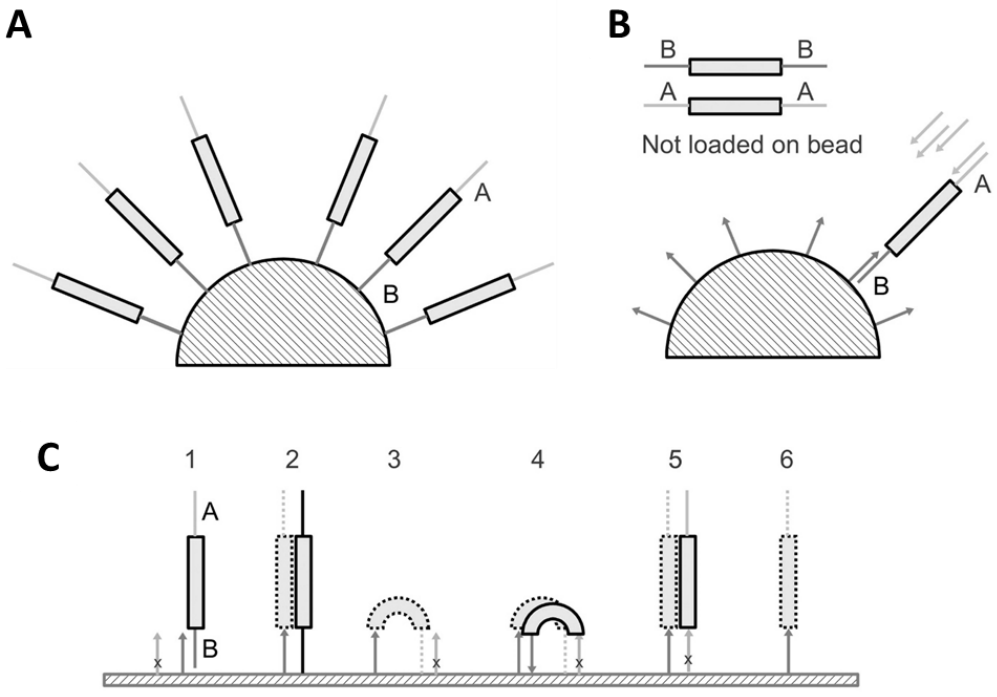


Figure 1.14. A, emulsion amplification (biotin- streptavidin); B, emulsion amplification (adapter sequences); C, bridge amplification. Adapted from: Buermans and den Dunnen (2014).

*1.5.2.1 Sequencing by synthesis*

Sequencing by synthesis (SbS) was a departure from the two-step PCR-Read process of the Sanger method, combining the two into a single real-time process. Initially pyrosequencing (Roche 454) was used as the method to recognise and call bases, whilst later platforms utilised fluorescently labelled dNTPs (F-dNTPs) (Illumina) and pH changes occurring from the release of $H^+$ during dNTP incorporation (ThermoFisher-Ion PGM). Whilst the principle remains the same across platforms, to call bases as they are synthesised into the DNA strand in real-time, the individual chemistries/mechanisms used differ.

Pyrosequencing (Roche 454) was the first type of SbS, and utilises a chemical cascade to derive a fluorescent signal from the incorporation of a dNTP by DNA polymerase. In brief, the magnetic beads (section 1.5.2.1) are precipitated into individual hexagonal cells within a microtiter plate, with the space surrounding the bead and the cell wall filled with smaller beads coated with the enzyme Sulfurylase and Luciferase. From here SbS is initiated with the addition of sequencing primers and polymerase to begin the synthesis of dsDNA, before successive cycles of; dNTP (A, T, C or G), adenosine 5' phosphosulfate (APS) and luciferin are added followed by plate washing. If complimentary the nucleotide is incorporated by DNA polymerase releasing a pyrophosphate molecule (PPi) which begins the cascade effect, the result of which is a fluorescent signal (Fig 1.15) that is read by a charge coupled device (CCD) registering the base call. In addition to this the amount of light produced is proportional to the number of PPi molecules released allowing for homopolymers to be detected. At the end of each cycle a wash solution containing apyrase is added removing any unincorporated dNTPS and breaking down any excess ATP and oxyluciferin produced during that cycle, thus resetting the reaction.

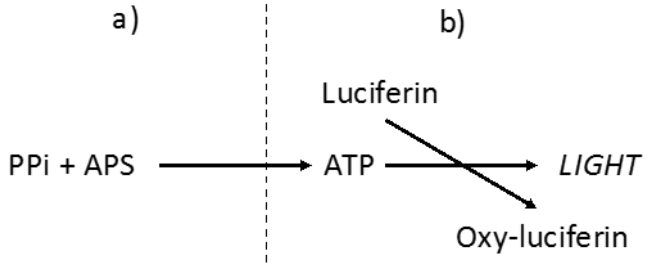


Figure 1.15. An overview of the pyrosequencing principle. Enzymes: a) Sulfurylase and b) Luciferase. Authors own.

Unlike pyrosequencing the Illumina technique is simpler, relying on four independently labelled fluorescent dNTPS (F-dNTPs) similar to those utilised in the Sanger method (F-

ddNTPs), although these do not permanently terminate the reaction. In brief, sequencing primers are added which anneal to the adapter sequence on the free 5' of the forward strand, thus forming an extension site for DNA polymerase. During each sequencing cycle all four of the F-dNTPs are added, becoming incorporated by the polymerase onto the first free base only. Due to a modification, the F-dNTPs temporarily terminate the chain, preventing other F-dNTPs from being incorporated. The attached F-dNTPs are then excited using light, and due to the formation of clonal clusters each sequence within a cluster responds by fluorescing at the same wavelength, allowing the clusters and base calls to be identified by a CCD. The fluorophore is then cleaved and the 5' un-blocked to allow elongation within the next cycle. After fully sequencing the forward strand a second bridge amplification step is performed, generating the reverse strand attached to the embedded adapters so that the amplicon maybe read in the opposite direction. The forward strand is then released from the adapter and removed from the reaction. The process then begins again, resulting in the reverse read.

*1.5.2.2 Metabarcoding, Data analysis methods and pipelines*

DNA barcoding is a technique used to describe and classify organisms based on their DNA profiles alone (Herbert *et al.*, 2003; Taberlet *et al.*, 2012). Conserved primer sites are used to amplify DNA from across multiple taxa, whilst flanking regions of suitable variation to allow for greater taxonomic resolution. For example, in fungi the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) has been proposed by Schoch *et al.* (2012) as the barcoding region, whilst 16S is used for bacteria (Tanabe and Toju, 2016) and *CO1* for higher eukaryotes and insects (Herbet *et al.*, 2003). Afterwards sequences are compared to curated sequence depositories, where both morphological and DNA sequence information is deposited from voucher/reference species, thus allowing for classification and identification. The most recent improvement on this technique is that of metabarcoding, whereby DNA barcoding is combined with HTS technology to simultaneously sequence multiple organisms across both an individual sample and a group of samples (Fig 1.16). However, this increased information also leads to an increase in the amount of data generated, necessitating the use of computer software in order to effectively interpret datasets.

As with HTS platforms, multiple software packages exist to process the large amounts of data generated. Many of these are complete packages, taking raw sequencing data and processing through to statistical analysis and graphical interpretation, leading to the term 'pipeline'. The varied nature of HTS experiments and the expected outcomes has also resulted in different pipelines being developed, but some of the most ubiquitous include; QIIME (Caporaso *et al.*, 2010), Mothur (Schloss *et al.*, 2009) and USEARCH (Edgar, 2010),

with citations numbering several thousand for each. In general, these pipelines can be separated into their individual components comprised of; primer removal, quality filtering, merging (contigs), operational taxonomic unit (OTU) picking, taxonomic assignment, and community measures/analysis. In some cases however, pipelines or individual packages are merged with the main pipeline to perform specialist tasks, for example in the detection of chimeric sequences (UChime – Edgar *et al.*, 2011) or sequence extraction (ITSx – Bengtsson-Palme *et al.*, 2013). Combining these packages allow the user to easily process data, abstracting patterns and relationships.

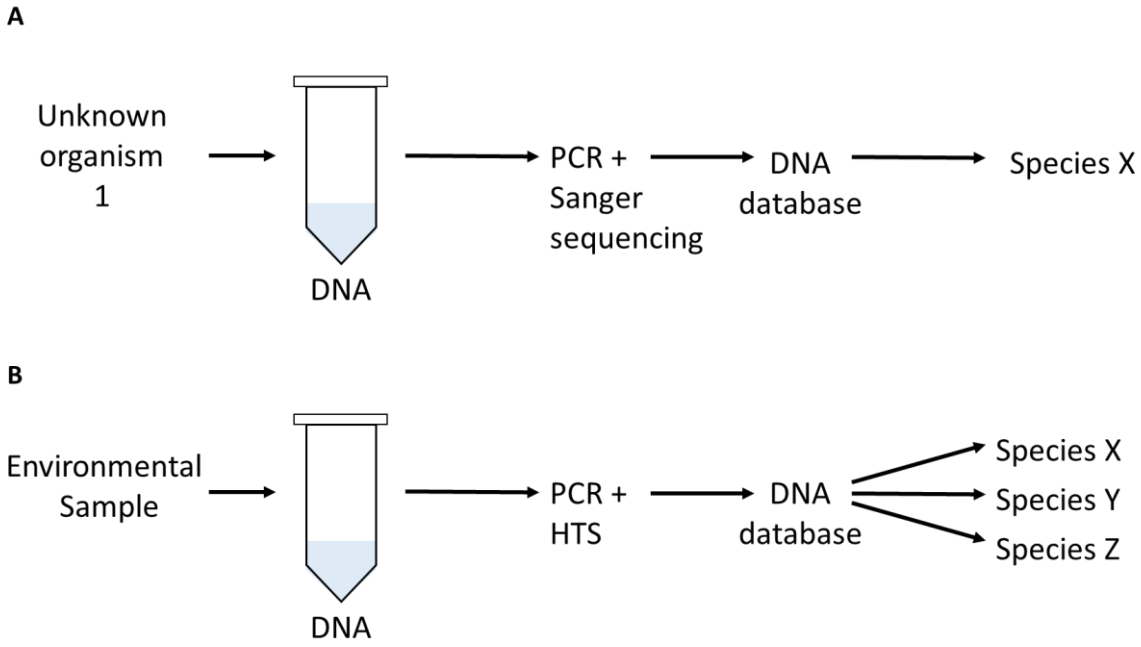


Figure 1.16. An overview of DNA barcoding techniques. A, conventional DNA barcoding; B, metabarcoding. Authors own.

*1.5.2.3 Use of high throughput sequencing in environmental studies*

Increasing availability along with decreasing costs and improved analysis methods has led to a greater uptake of HTS technology, particularly in the areas of biomedical research, but also more widely in the study of complex environmental samples such as soil (Shokralla *et al.,* 2012). Consequently, large scale examination of the microbial communities of fields, forests and even whole landscapes have been conducted, something which would have been otherwise difficult to conduct without HTS (Smith and Peay, 2014; Schmidt *et al.,* 2013; Buée *et al.,* 2009). Alongside this, HTS has also become widely used to study plant pathogens within agricultural crops. For example, Liu *et al.* (2014) used HTS to observe how *Fusarium* spp. changed during the course of different potato (*Solanum tuberosum*) rotations, with these shown to correlate with decreased yield. Similarly, Xu *et al*., (2012)

also showed how soils conducive to pea (*Pisum sativum*) footrot demonstrated higher levels of the pathogen *Didymella pinodella* (syn. *Phoma medicaginis* var. *pinodella*) suggesting this was implicated in causing disease and subsequent yield decline.

### 1.5 Conclusions from literature review

Oilseed rape production has increased dramatically within recent years as a consequence of increased demand for both food and industrial uses. This has in turn resulted in OSR being the second most profitable combinable crop within the UK after winter wheat. However, as production has grown the amount of land available to growers has remained the same, leading growers to shorten rotations to increase production. As a result, OSR is popularly grown 1 year in 2, 3 or 4 years on many UK holdings, in conjunction with cereals such as winter wheat and winter/spring barley. However, at the same time UK OSR yields have plateaued currently averaging *c.*3 t $ha^{-1}$, whilst countries such as Germany and France continue to witness increasing OSR yields averaging more than 4 t $ha^{-1}$; although both of these are lower than the theoretical yield potential of 6.5 t $ha^{-1}$ (Berry and Spink, 2006). Many suggestions have been offered for this decline in yield such as better varieties, management practices and environmental conditions, but more recently shortened crop rotations have become implicated. In a longterm field experiment Stobart *et al.* (2011) demonstrated a yield depression of up to 20% from intense rotations (continuous), with a lower depression of *c.* 13% seen within those rotations which are commonly practiced (1 in 3). In addition, the role of soil-borne pathogens in this decline was examined by Hilton *et al.,* (2013), finding that two pathogens, *Olpidium brassicae* and a *Pyrenochaeta* sp*.* occurred within shortened rotations leading to reduced yield. This link between yield decline, pathogens and shortened rotations has been witnessed in many crops worldwide (Bennett *et al.*, 2012), however the role of soil-borne fungi and in particular pathogenic fungi is not yet fully understood, particularly within UK OSR crops. Consequently, this thesis aims to examine the occurrence of soil-borne pathogens in commercial crops of OSR, and examine their effect on OSR growth and yield under controlled conditions.

## 1.6 Objectives

### 1.6.1 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.

### 1.6.2 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.

### 1.6.2 Null hypothesises

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.

# 2

# General Materials and Methods

## 2.1 Laboratory

All chemicals were sourced from Sigma Aldrich (Poole, UK) unless specified otherwise.

### 2.1.1 Media preparation

#### *2.1.1.1 Overview of culture maintenance*

Fungal cultures of *R. solani*, *V. longisporum*, *P. ultimum* and *S. sclerotiorum* were maintained on either; potato dextrose agar (PDA) or autoclaved barley grains. Working cultures were maintained in 90 mm Petri dishes containing PDA sealed with parafilm (ThermoFisher Scientific, Loughborough, UK). When not in use cultures were stored on either PDA slopes store in the dark at 4°C and/or in small bijous containing autoclaved barley grains stored at -20°C.

#### *2.1.1.2 Water agar*

Water agar was made by adding 19 g $L^{-1}$ of Agar No.2 Bacteriological (Lab M Ltd., Heywood, UK) to either 250, 500 ml or 1 L of deionised water. This was followed by autoclaving at 121°C for 15 min to sterilise. After cooling to *c*. 45°C, filtered sterilised penicillin and streptomycin were added at a rate of 100 mg $L^{-1}$.

#### *2.1.1.3 Potato dextrose agar*

Potato dextrose agar (PDA) was made by adding 39 g $L^{-1}$ of potato dextrose agar (Merck, Watford, UK) to either 250, 500 ml or 1 L of deionised water. This was followed by autoclaving at 121°C for 15 min to sterilise. Antibiotic amendments were added as described previously.

#### *2.1.1.4 Barley grain cultures*

Small glass universals *c*. 30 ml were three-quarters filled with barley grains and half filled with sterile distilled water (SDW). Each universal was then capped loosely before autoclaving at 121°C for 15 min. Once cooled a single 5 mm agar core from a one-week old culture was placed on top of the grains and the bottle sealed with a screw cap. These were then left on the laboratory bench for one week to allow the fungus to colonise the grains

after which they were stored at -20 °C until needed. When recovering isolates a single grain was aseptically picked from a bottle and placed onto a fresh PDA plate.

### 2.1.3 DNA extraction

*2.1.3.1 Roots*

DNA extraction was performed using a FastDNA® Spin Kit for Soil (MP Biomedicals, Cambridge UK) according to the manufacturer'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 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 $\mu l^{-1}$ or 1 ng $\mu l^{-1}$.

*2.1.3.2 Fungal culture*

Fungal cultures for use in qPCR were grown on an autoclaved cellophane disc placed on top of a clean plate of PDA and inoculated with a 5 mm agar plug. After one-week the hyphae were aseptically removed from the cellophane and placed into a 1.5 ml grinding tube from the FastDNA® Spin Kit for Soil (MP Biomedicals, Cambridge UK). DNA was extracted using a Wizard® Magnetic DNA purification kit (Promega, Southampton, UK) as described by Budge *et al.* (2009b).

### 2.1.4 Real-time PCR

*2.1.4.1 Primers/Probes*

Primer pairs and probes were manufactured by Eurogentec (Southampton, UK) and Life Technologies (Paisley, UK) and purified by the manufacturer using the selective precipitation optimized process (SePOP) prior to being lyophilized. Upon arrival primers were rehydrated in a buffer containing 50% glycerol in PCR grade water to a defined stock concentration and stored at -20°C until use. Probes were manufactured with 5' 6-Carboxyflourescein (FAM) and 3' tetramethylrhodamine (TAMRA) quencher or 3' minor groove binder (MGB) depending on assay requirements. Probes were purified by the manufacturer using Reverse Phased High Performance Liquid Chromatography (HPLC-RP) and sent in TE buffer at 100 µM.

2.1.4.2 *Real-time PCR setup*

Individual reactions were setup under sterile conditions with all reagents, plates and equipment remaining within a UV sterilised PCR cabinet prior to sealing.

For each plate a master mix was prepared in a 1.5, 2 or 10 ml sterile Eppendorf or vial. This comprised of 0.5 µl of each primer (final concentration dependent upon assay), 0.25 µl

probe (probe assays only) (final concentration dependent upon assay), 12.5 µl 2X Environmental Master Mix 2.0 (Life Technologies, Paisley, UK) (Probe assays) or 2X Power SYBR Green (Life Technologies, Paisley, UK) (Non-probe assays) and molecular grade water to a total reaction of 20 µl per well. This mixture was vortexed to mix before pipetting. Reactions were setup in Hard-Shell® 96-well plates (Biorad, Hemel-Hempstead, UK) with each well containing 20 µl of the prepared master mix. Each well was then spiked with 50 ng of template DNA or positive/negative standard with each sample conducted in duplicate. Plates were sealed with a Microseal® 'B' adhesive seal ensuring all wells were sealed and then spun in a MPS 1000 mini PCR plate spinner (Labnet, Edison, USA) followed by vortexing on an MixMate plate vortex mixer (Eppendorf, Stevenage, UK).

Individual plates were placed into a CFX 96 Touch™ Real-time PCR detection system (Biorad, Hemel-Hempstead, UK) immediately, or were wrapped in aluminium foil and stored at 4°C for no more than 24 hours prior to use.

2.1.4.3 *Real-time PCR conditions*

Conditions varied depending upon the assay in use but comprised of an initial denaturing step of 95°C for 10 min, followed by 40 cycles of denaturation, annealing and extension, followed by cooling to 4°C once finished.

## 2.2 Glasshouse work

### 2.2.1 Seed

Oilseed rape seed used in the experiments were sourced from Agrii Ltd. through the Crop and Environment Research Centre (CERC) at Harper Adams University. All experiments conducted with OSR comprised of untreated seed of the variety DK Cabernet, which was chosen based on its commercial popularity at the beginning of the project. Experiments which utilised the model plant *Brassica oleracea* (DHSL150) were sourced from Dr Graham Teakle at the University of Warwick.

Before use in experiments batches of seed was surface sterilised in sodium hypochlorite solution (1% available chlorine) for 1 min before being rinsed in two changes of distilled water and blotted dry on clean paper towels.

A continuing culture of DHSL150 was setup from initial seed stocks and maintained in the glasshouse, ensuring that only self-pollination took place. After harvest it was noted that differences occurred in the size and shape of seed. Following experimentation it was found that most seeds produced varied in size (<1.7 – 3.0 mm), but that the best germination and seedling vigour was seen at sizes between 1.7 - 1.9 mm diameter (75% germination after

7 days). Those which were less than 1.7mm were often shrivelled and misshapen resulting in a low germination rate and poor seedling vigour (35% germination after 7 days), whilst the production of seeds above 2.0 mm diameter was low. Due to this only seed between 1.7-1.9 mm in size was used in experiments.

### 2.2.2 Assessments

Assessments were carried out at defined physiological stages in the crops growth. Emergence was defined as being the point at which both cotyledons were present above soil and fully expanded. Whilst flowering, was defined as when the first flower bud was fully open. Biomass was assessed by drying the plant material at 100°C for 72 hours.

Where plants were taken to yield they were prepared as following. Watering was stopped after approximately 120 days (4 months) and plants were allowed to naturally desiccate over the next 3 weeks. After this individual pods were striped from each stem and counted, before liberating the seed by hand. Seeds were then allowed to dry for a further week in plant pot saucers on the glasshouse bench, after which the seed was weighed as a measure of seed yield.

In some instances where full maturity was not possible, or could not be achieved, biomass (DM) was taken as a proxy for yield.

# 3

# Utilising real-time PCR to elucidate the soil-borne pathogens associated with commercial crops of Oilseed rape.

## 3.1 Introduction

In order to understand the role that soil-borne pathogens play in the yield decline of UK OSR, it is crucial to understand their distribution and occurrence within commercial crops. Currently there have been several individual surveys for single pathogens such as *Verticillium longisporum* (Gladders *et al*., 2013) and *Plasmodiophora Brassicae* (Burnett *et al.*, 2013), however information on other possible pathogens such as *Rhizoctonia solani* and *Pythium ultimum* remains limited, despite these being commonly found in other OSR growing regions such as Canada and Australia (Morrall, 2011; Khangura *et al.*, 1999). In addition to these 'old adversaries', the advent of molecular technology has uncovered previously unknown pathogens in OSR, such as *Olpidium brassicae* and *Pyrenochaeta* sp. (Hilton *et al*., 2013), alongside evidence of *Gibellulopsis nigrescens* (Mills, 2013, Pers. comm.).

Along with pathogen detection, it is also important to consider the agronomic factors currently practiced within the UK, and in particular how these may impact on profile of soil - borne pathogens of OSR. Advancements in technology and agronomy have resulted in a variety of different cropping systems, techniques and management practices which are employed across the UK. However, in general, a reductionist view has been taken, with rotations simplified to the most profitable crops such as wheat and oilseed rape, with minimal and zero tillage systems becoming more prevalent (Halloran and Archer, 2008). Whilst this has led to a reduction in time, labour and most importantly costs for growers, it may also be inadvertently influencing soil-borne pathogens through shortened periods of non-host plants, less disturbance of hyphal networks and exposure of resting bodies to environmental factors, leading to an increase in their survival and proliferation.

### 3.2 Objectives

The objective of this Chapter was to identify the distribution and occurrence of potential soil-borne pathogens of oilseed rape (*S. sclerotiorum*, *P. brassicae*, *R. solani*, *P. ultimum* and *Gibellulopsis nigrescens*), by utilising species specific real-time polymerase chain reaction (qPCR) to examine the presence and quantity of pathogens on the roots of commercial OSR crops. These inoculum densities will also be compared to agronomic management practices associated with the samples to elucidate the relationship, if any, between pathogen levels and management practices.

### 3.3 Null Hypotheses

1) Soil-borne plant pathogenic species are not associated with UK crops of OSR.

2) The occurrence and/or quantity of pathogenic species are not related to agronomic practices or management.

### 3.4 Materials and methods

#### 3.4.1 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. The majority of isolates were received on PDA plates and transferred to PDA slopes and autoclaved barley grain vials for long term storage. Once sufficient isolates had been obtained DNA was extracted from pure cultures as described in Section 2.1.3.2. The resulting DNA extracts were then used to test for assay specificity. UK isolates of *G. nigrescens* were only available from the University of Warwick, although these were not successfully recovered from long term storage (S. Hilton,, Pers. comms) and so could not be utilised to design a new assay. As a consequence, no further work was conducted on *G. nigrescens*.

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, Fera Science Ltd.; 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 | James Woodhall, Fera | AG 3 |
| R6 | 1938 | James Woodhall, Fera | AG 4II |
| R7 | 1835 | James Woodhall, Fera | AG 4 |
| R8 | 1986 | James Woodhall, Fera | *R. zea* |
| R13 | 1971 | James Woodhall, Fera | AG 2-1 |
| | | | |
| Py1 | Unknown | David Kenyon, SASA | Unknown |
| Py2 | H1C5 | Jenny Denton, RHS | *var.* ultimum |
| Py3 | Unknown | John Clarkson, UoW | Unknown |
| Py4 | Pl237 | Chemtura AgroSolutions | Unknown |

#### 3.4.2 Sample acquisition

One hundred individual field sites from across England were previously sampled under an Innovate UK project (Innovate UK 100889) with individual samples submitted by participating consortium members. Sites were sampled in the spring of 2011 and 2012, with plants sampled before growth stages 51-57 (flowering, BBCH) in both years. Samples comprised of fifteen to twenty-five plant root systems in total per site, made upcomprising of 3 or 5 subsamples collected along a standard ‘W’ shaped pattern across a 1 ha portion

of the field. Individual growers were also asked to supply information such as; rotational frequency, establishment method, variety and other agronomic factors (Appendix Table A1).

### 3.4.3 Sample processing

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.

#### *3.4.3.1 Root DNA extraction*

DNA extraction was performed using a FastDNA® Spin Kit for Soil (MP Biomedicals, Cambridge UK) using a modification of the manufacturer's protocol. In place of soil, DNA was extracted from 150-160 mg of fine (<2 mm) freeze dried OSR roots per individual subsample, which were then processed according to the manufacturer's instructions. The resulting DNA was eluted in 100 µl of TE buffer, and assessed for quantity and quality using a NanoDrop 2000c™ (ThermoFisher Scientific, Loughborough, UK) spectrophotometer, before being diluted to a final working concentration of 1 ng $\mu l^{-1}$ and individual site subsamples pooled to form one DNA sample per site.

### 3.4.4 Stratification of samples

Whilst the initial samples present a general overview of grower practices at the time of sampling, they may not fully be representative due the lack of systematic sampling. As a consequence of this, a subset of 50 samples were selected based on the proportion of OSR grown per AHDB region, using information collected from the Defra OSR surveys (1999 - 2010) to define the relative area grown per AHDB region (Appendix Table A2). 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.

### 3.4.5 Real-time PCR

Published species-specific real-time PCR assays were chosen from the literature (Table 3.2), where available, to detect individual pathogen levels within samples.

Real-time PCR was carried out in 96-well plates using a Bio-Rad CFX96 (Biorad, Hemel Hempstead, UK) in a total reaction volume of 25 µl per well. Full details of the reaction setup can be found in Section 2.1.4.2.

Cycling conditions differed with assay; *R. solani* AG 2-1 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 for *R. solani* AG 2-1, whilst

those for *P. ultimum* consisted of 95°C for 10 min, and 40 cycles of 95°C for 10 s, 70°C for 5 s and 72°C for 10 s, followed by a melt curve from 65°C to 95°C at a rate of 0.1°C $s^{-1}$.

The cycle threshold ($C_t$) value for each reaction was assessed using the manufacturer's software, and an average of two replicates taken. A standard curve was constructed using a ten-fold dilution across six orders with genomic DNA (gDNA) extracted from pure cultures as described in Budge *et al.* (2002). Samples which amplified between the last standard ($C_t$ = *c.* 35) but before the end of the programme ($C_t$ = 40) were classed as below the limit of quantification (LoQ) and assigned a value half that of the last standard for statistical purposes. Whilst those which showed no amplification prior to cycle 40 were classed as below the limit of detection and assigned no value.

Table 3.2. Table of primers (p) and probes (pr) used for the detection of *Rhizoctonia solani*, *Pythium ultimum* and *Gibellulopsis nigrescens*. Dual labelled probes were labelled using 6-carboxyfluroscein (FAM) 5' with a minor groove binder (MGB) and tetramethylrhodamine (TAMRA) 3' modification. The *Pythium ultimum* assay was amplified using SYBR green II

| Primer/Probe | Sequence (5'→3') | Position (size) | Source |
|---|---|---|---|
| <u>*R. solani* AG 2-1</u> | | | a |
| AG-2-1_F$^{p}$ | CTT CCT CTT TCA TCC CAC ACA | 56-76 | |
| AG-2-1_R$^{p}$ | TGA GTA GAC AGA GGG TCC AAT AAC CTA | 139-165 | |
| AG-2-1_P$^{pr,a}$ | AAG TAA ATT CCC CAT CTG T | 95-113 | |
| | | | |
| <u>*P. ultimum*</u> | | (414bp) | b |
| ULT1F$^{p}$ | GAC ACT GGA ACG GGA GTC AGC | | |
| ULT4R$^{p}$ | AAA GGA CTC GAC AGA TTC TCG ATC | | |
| | | | |
| <u>*G. nigrescens*</u> | | | |
| No assay currently developed | | | |

a, Budge *et al.* (2009a); b, Schroeder *et al.* (2006)

## 3.5 Results

### 3.5.1 Background information from Survey

Due to the limitations of the original survey sites, a complete stratified survey could not be conducted. However, in general the regions with the greatest area of OSR were better represented than before, whilst all of the rotational intensities were represented equally.

#### *3.5.1.1 OSR production by county*

Originally, 27 counties from across England were sampled (Figure 3.0, A), with the majority of samples (37%) originating from the Eastern region (Fig 3.1, A). However, the county of Gloucestershire was over represented, whilst those within the North Western or North Eastern regions were unrepresented or absent.

In comparison, the subset of samples covered fewer counties (20) (Figure 3.0, B), but was more representative of the production regions, with the majority of samples (42%) originating from the Eastern regions of England (Figure 3.1, B). However due to the limitations of the original samples, the Northern and Western regions were still somewhat under represented.

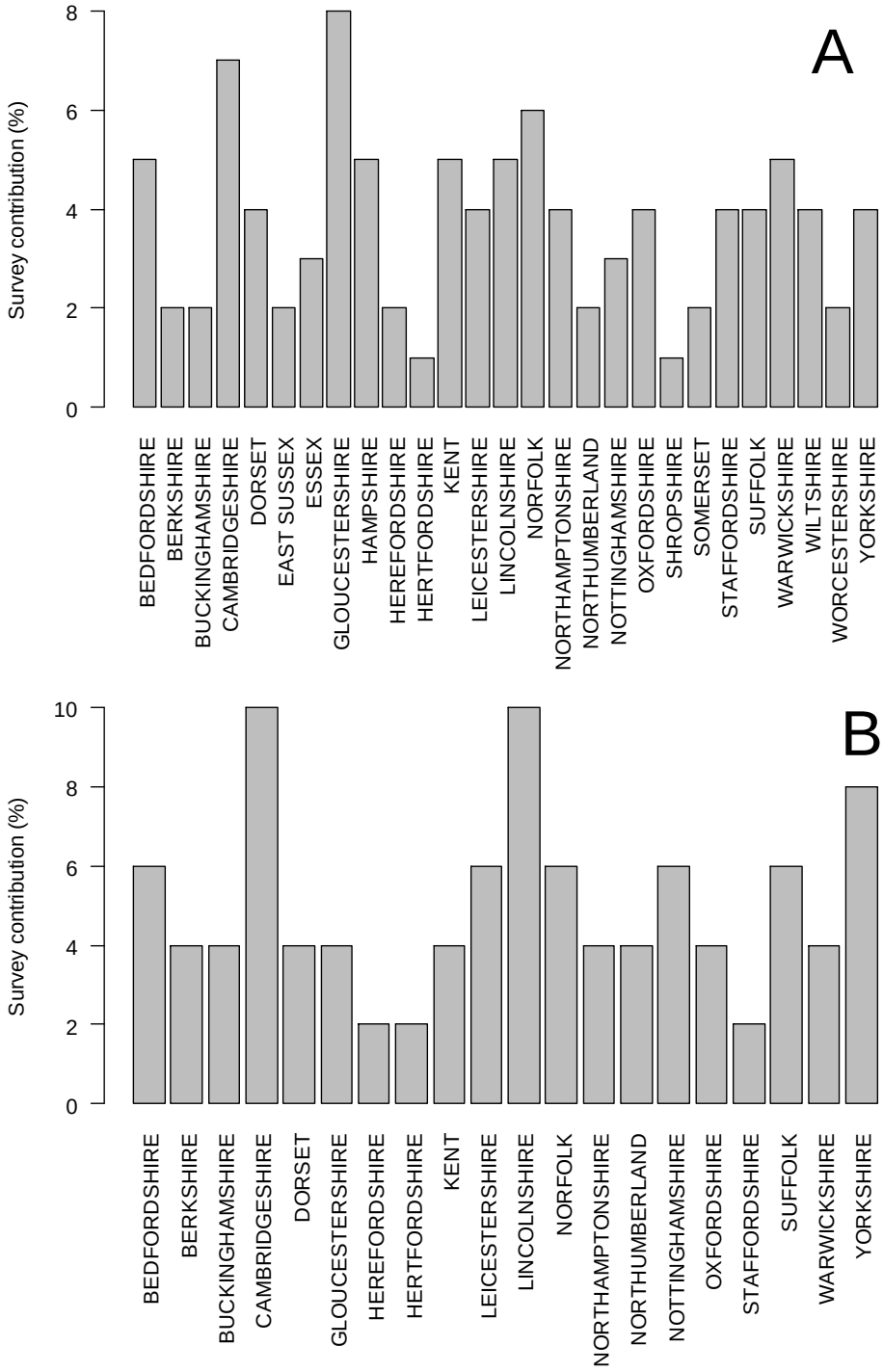


Figure 3.0. Summary of the counties sampled as a whole (A, n=100) and stratified (B, n=50).

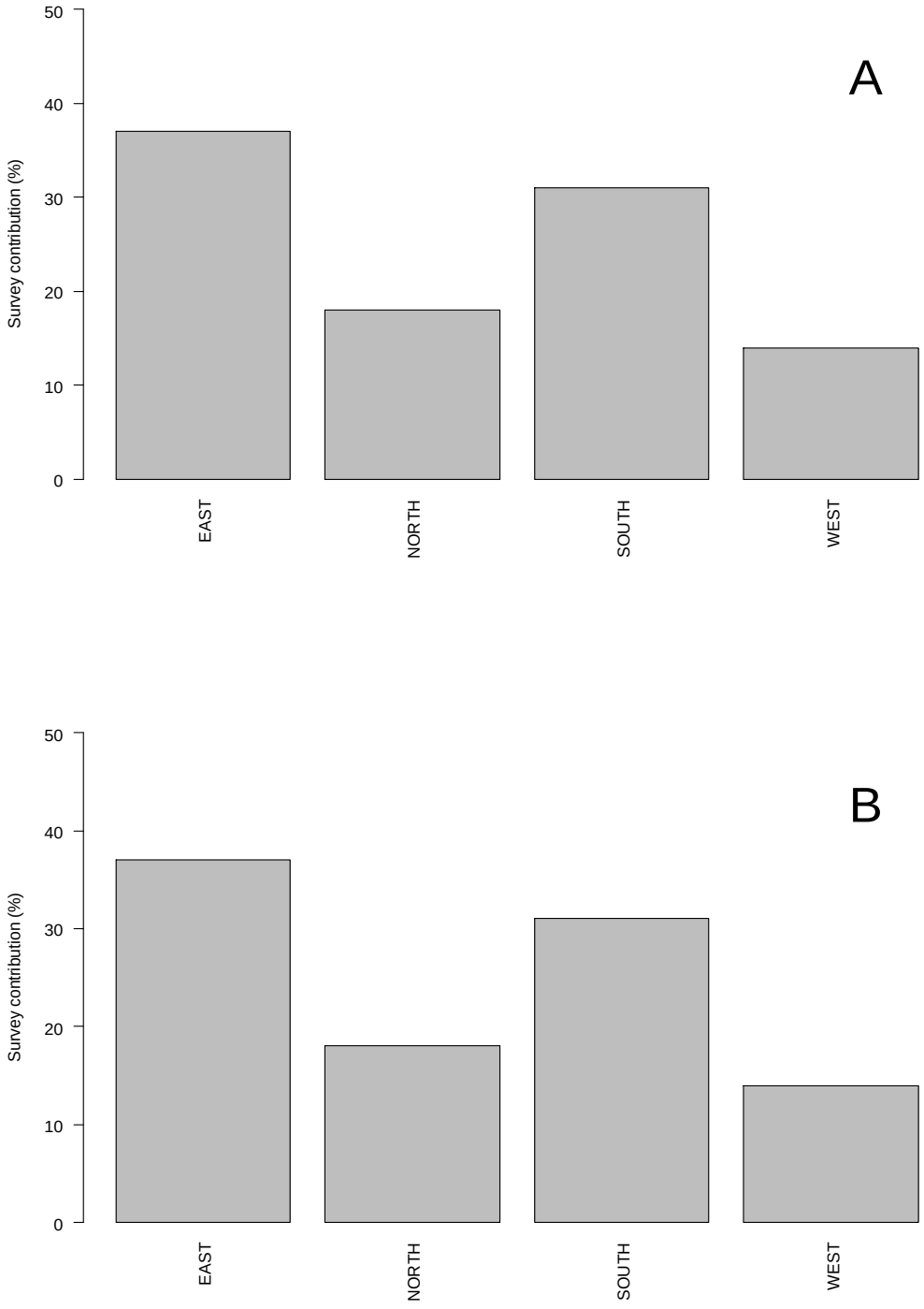


Figure 3.1. Summary of the regions sampled as a whole (A, n=100) and stratified (B, n=50).

*3.5.1.2 Varieties*

Originally the samples represented 13 main varieties with Castille (22%), DK Cabernet (16%) and Vision (16%) being the most popular (Figure 3.2, A). In comparison, the subset covered 11 main varieties, with DK Cabernet (24%), Castille (22%) and Vision (18%) remaining popular with some adjustments in their overall ranking (Figure 3.2, B).

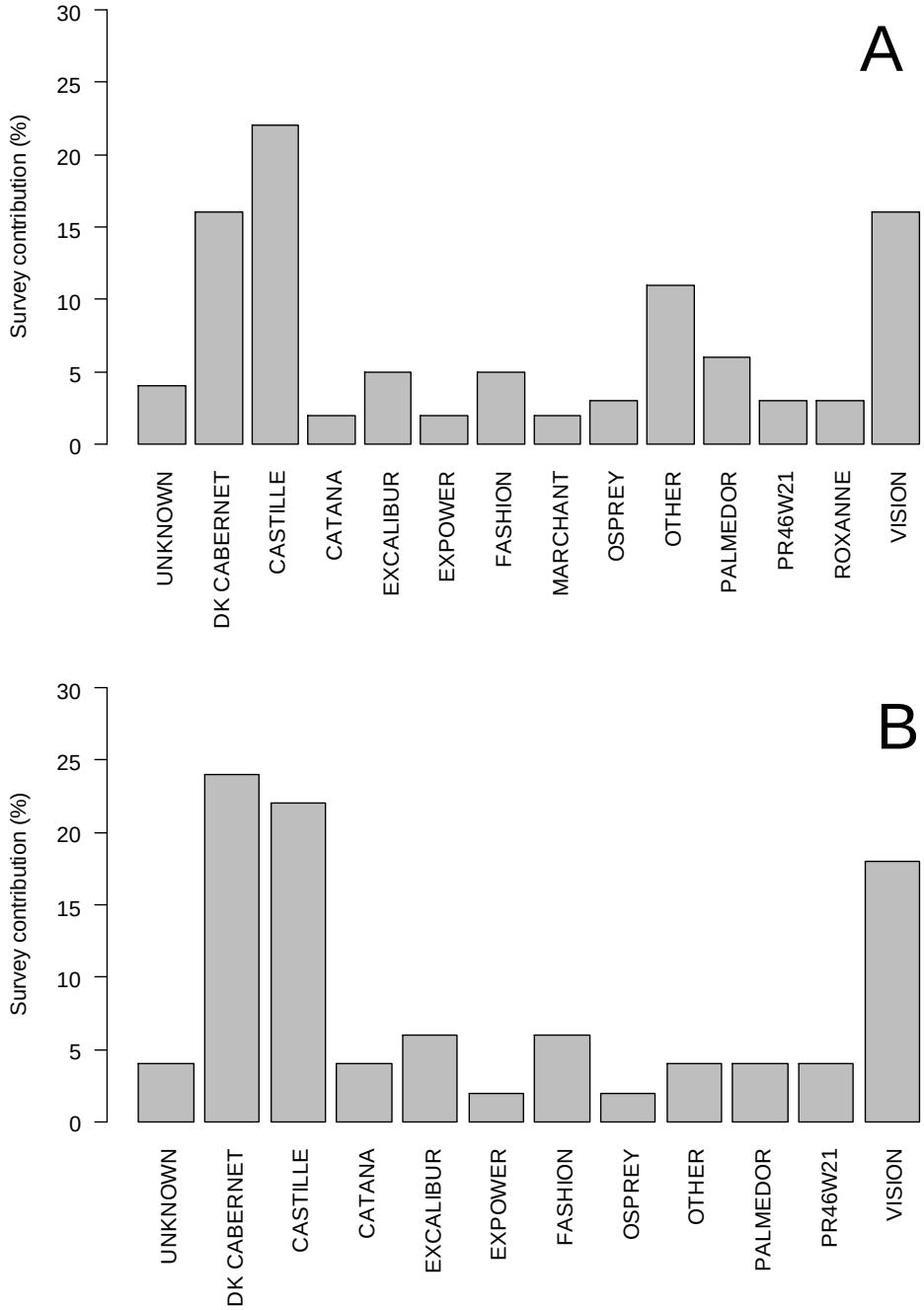


Figure 3.2. Summary of the varieties sampled as a whole (A, n=100) and stratified (B, n=50).

*3.5.1.3 Establishment technique*

Of the original set of samples, 5 establishment techniques were represented with the majority (43%) being represented by deep non-inversion tillage (DNI), followed by ploughing (27%) and shallow non-inversion tillage (SNI) (23%) (Figure 3.3, A). This picture was similar for the stratified samples, with the exception that broadcast techniques were absent, and a decrease in DNI (36%) and subsequent increase in ploughing (34%) and SNI (26%) (Figure 3.3, B).

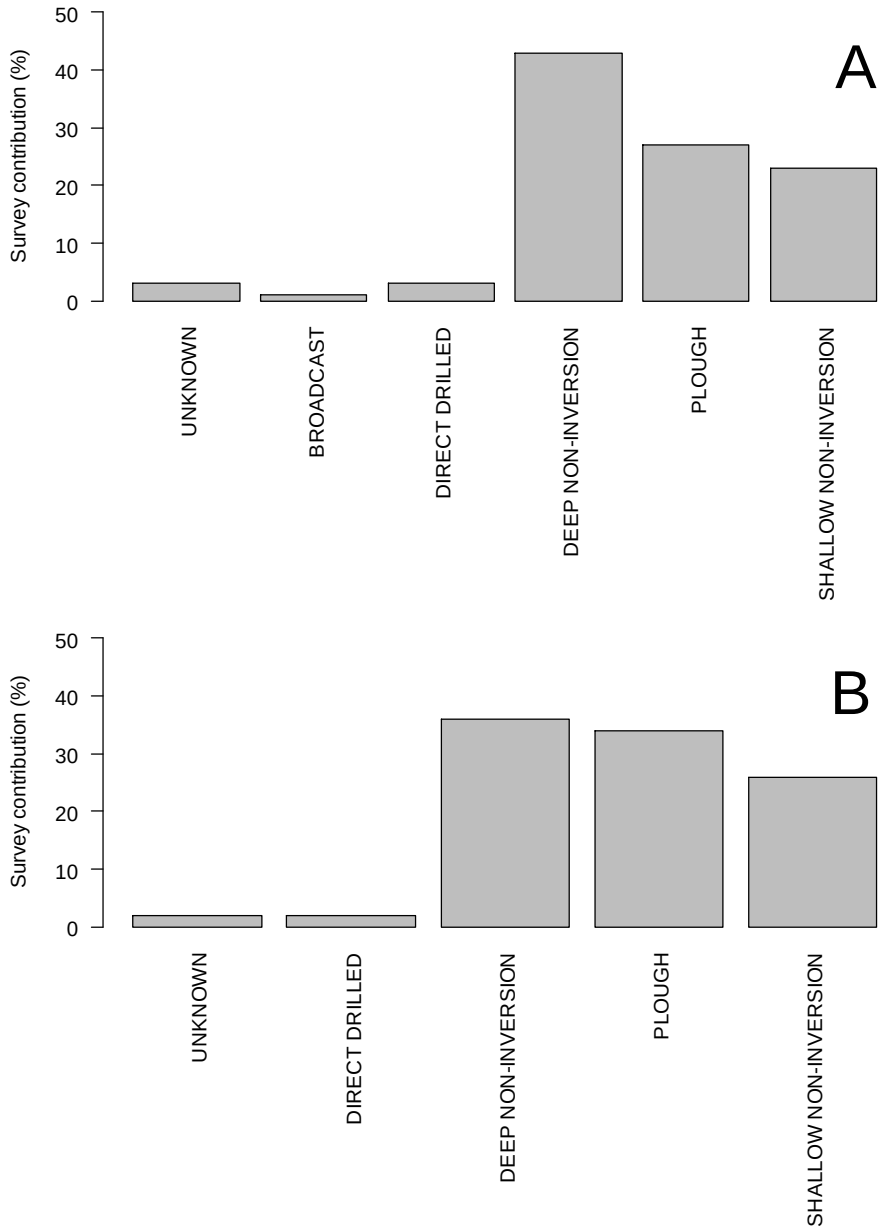


Figure 3.3. Summary of the establishment techniques utilised, sampled as a whole (A, n=100) and stratified (B, n=50).

*3.5.1.4 Rotational intensity and placement*

Rotational intensity showed a near Poisson distribution in the original samples, encompassing high through to low intensity rotations from continuous (0) to 1 in >10 year rotations (Figure 3.4, A). The largest proportion of samples occurred at 3 years (20%) and >10 years (20%), whilst continuous cultivation was the least represented (1%). This distribution of samples altered for the subset, due to the aim to evenly sample all rotational intensities. As a consequence, High (32%), Medium (38%) and Low (30%) intensities were almost equally represented, whilst the largest proportion of samples was still represented by 3 years (16%) and >10 years (22%), with non-from continuous cultivation (Fig 3.4, B). Previous cropping was severely skewed by the popularity of winter wheat in both the original (77%) and subset (68%) of samples (Figure 3.5, A and B). Whilst the number of individual crop species decreased from 8 in the original samples to 5 in the subset.

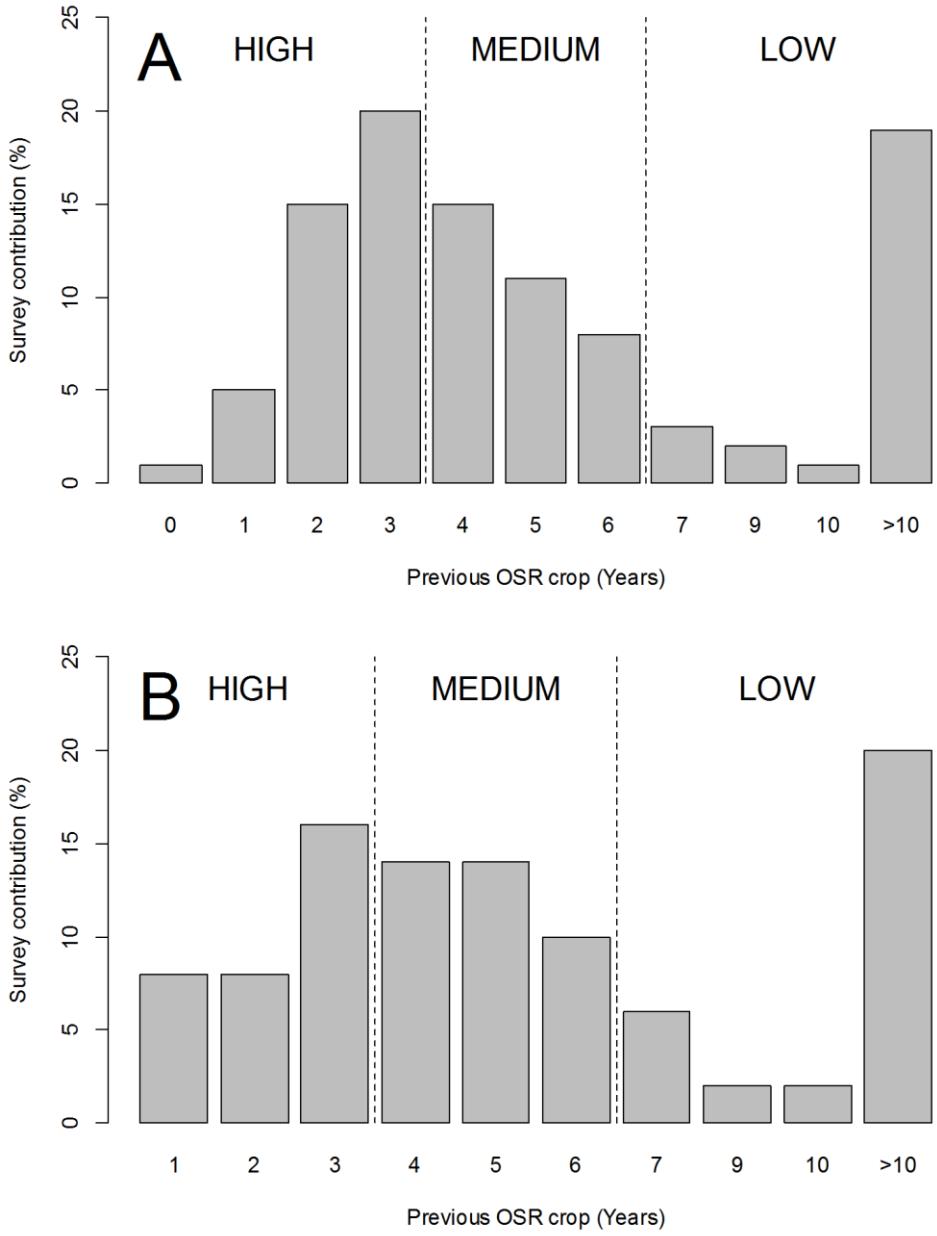


Figure 3.4. Summary of oilseed rape cropping frequency, as a whole (A, n=100) and stratified (B, n=50).

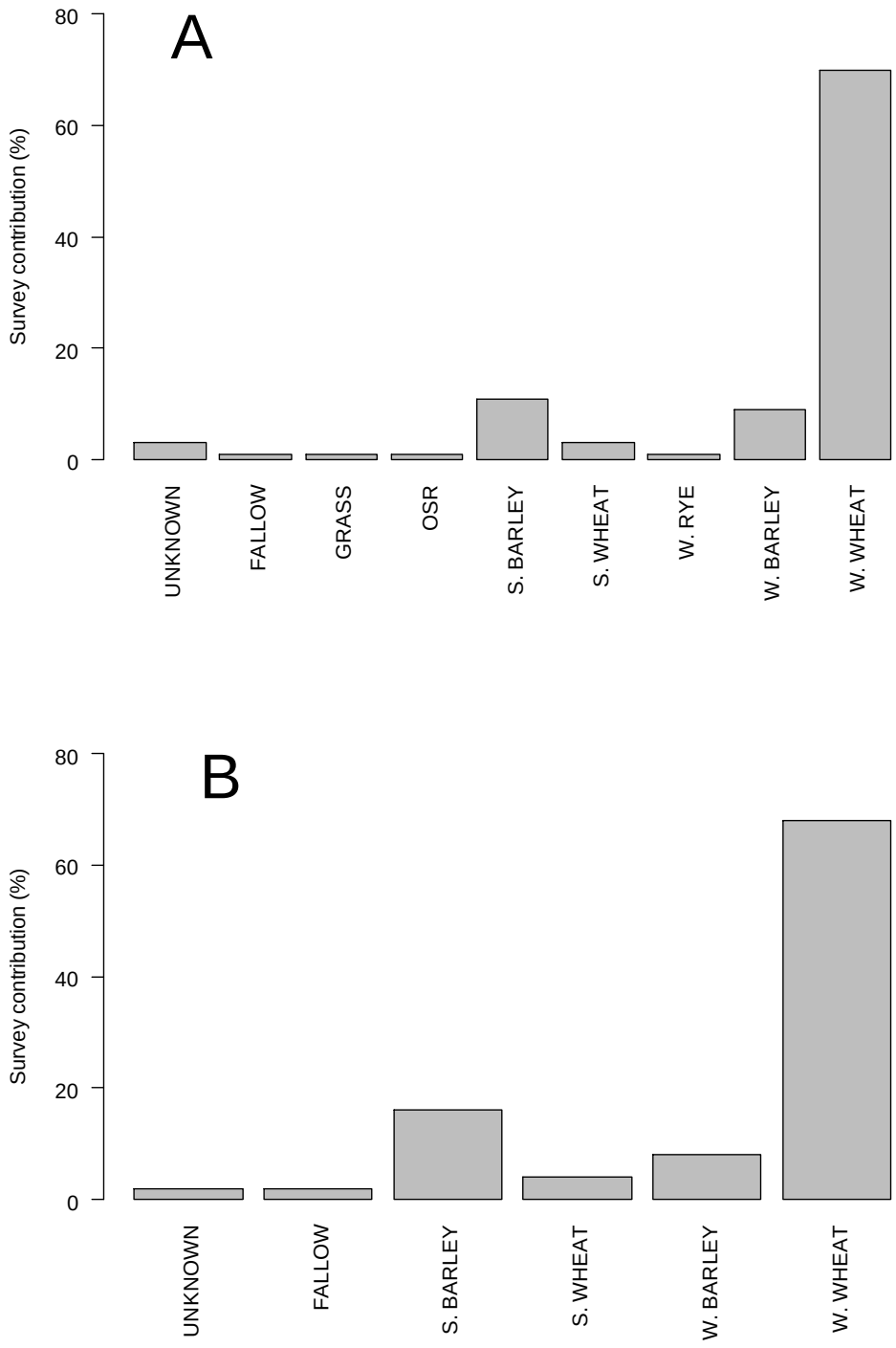


Figure 3.5. Summary of the previous crop, sampled as a whole (A, n=100) and stratified (B, n=50).

### 3.5.1 *Rhizoctonia solani* (AG 2-1)

The assay amplified well over a range of DNA concentrations from 10 ng $\mu l^{-1}$ to 100 fg $\mu l^{-1}$, although the first standard (10 ng) showed an early plateau phase compared to the others (Figure 3.6). The dilution series was amplified with an efficiency of 96.9% and showed an $R^2$ value of 0.997, suggesting that the assay had been well optimized, was robust and repeatable between individual reactions. When compared with other anastomosis groups of *Rhizoctonia solani*, AG2-1 was the only isolate which amplified, supporting the specificity of this assay (Table 3.2).

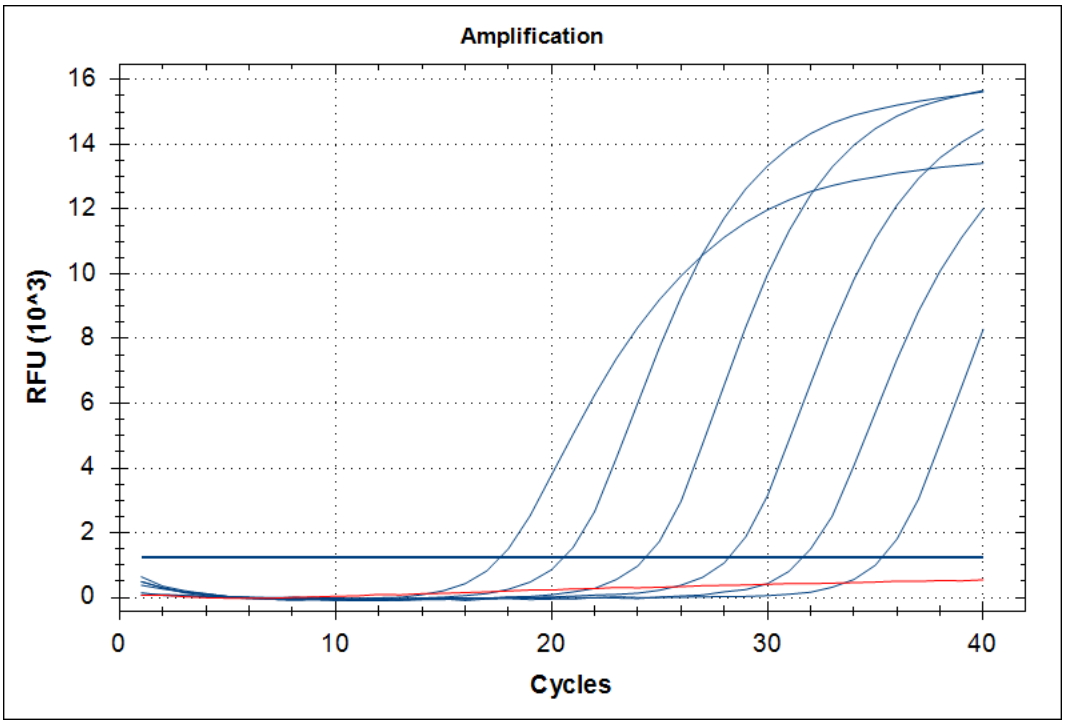


Figure 3.6. Real-time amplification plot showing a 10-fold dilution series of *Rhizoctonia solani* AG2-1 gDNA. L-R, 10 ng $\mu l^{-1}$ to 100 fg $\mu l^{-1}$, negative control. The horizontal line represents the software defined threshold ($C_t$).

Table 3.2. Comparison of *Rhizoctonia solani* (AG 2-1) qPCR assay specific against a number of different anastomosis groups.

| Isolate (AG) | $C_t$ value |
|---|---|
| R5 (3PT) | >40 |
| R6 (4HGII) | >40 |
| R7 (4) | >40 |
| R8 (*R. zea*) | >40 |
| R13 (2-1) | 17 |

Both the complete (n=100) and subset (n=50) were examined using qPCR, with both providing similar results in terms of positive samples and the range of DNA concentrations (Table 3.3, Figure 3.7). Across the samples 69 and 68% were positive respectively, whilst the range of DNA concentrations varied slightly with 6.0 $x10^{-5}$ – 0.0165 and 8.5 $x10^{-5}$ - 0.0165 ng DNA $ng^{-1}$ Total DNA between the whole set and subset respectively.

Table 3.3. Summary of real-time PCR data attained from both the whole (n=100) and subset (n=50*) survey samples.

| | **Whole** | **Subset** |
|---|---|---|
| No. of samples | 99* | 49* |
| No. Positive (%) | 69 (69) | 34 (68) |
| Max. (ng/ng Total DNA) | 0.0165 | 0.0165 |
| Min. (ng/ng Total DNA) | 6.0 $x10^{-5}$ | 8.5 $x10^{-5}$ |

**One sample (G52) was removed due to poor amplification across both PCR replicates.*

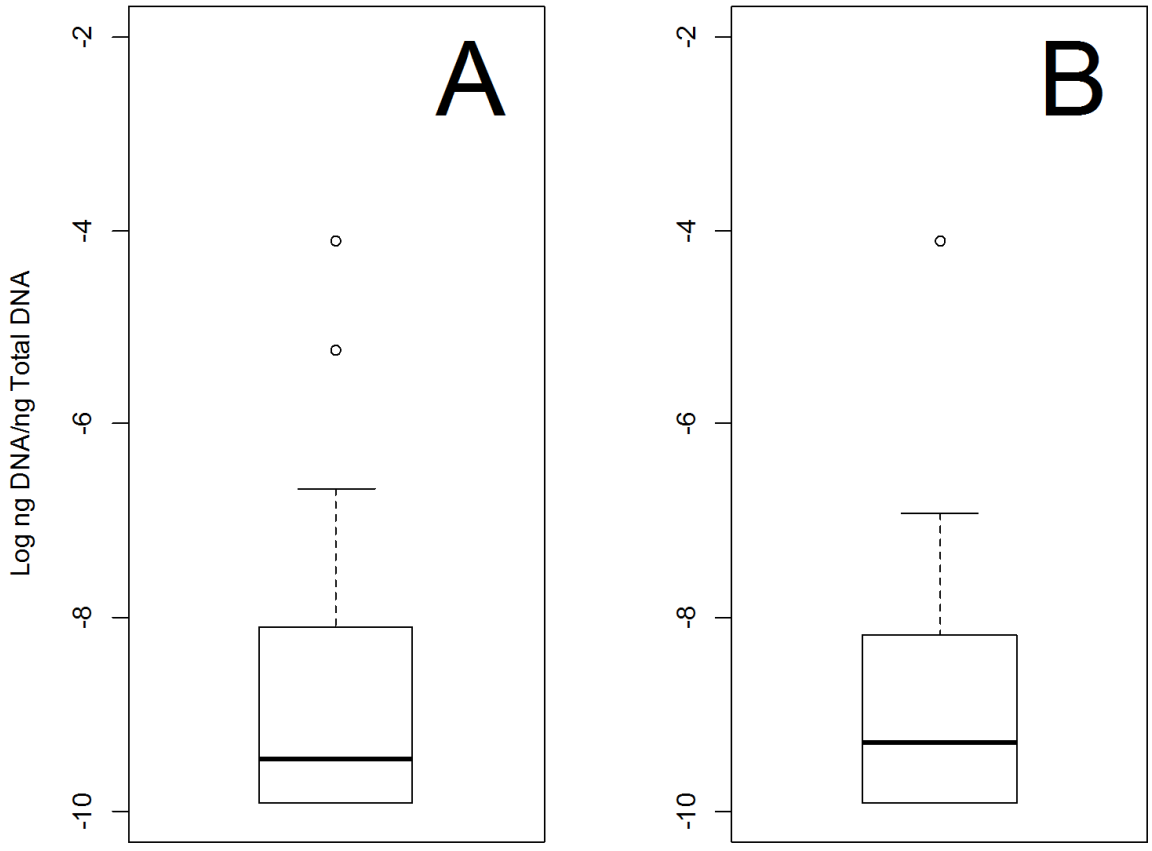


Figure 3.7. The range of DNA concentrations (log ng DNA $ng^{-1}$ Total DNA) as determined by real-time PCR from; A, whole set (n=100) and B, subset (n=49), of survey samples.

### 3.5.2 *Pythium ultimum*

The assay failed to amplify any of the pure or diluted gDNA extracted from isolates of *P. ultimum* (Py1 and Py4). Isolate Py1 was used to construct a dilution series for quantification covering a range of DNA concentrations from 10 ng $\mu l^{-1}$ to 100 fg $\mu l^{-1}$ but no amplification was observed (Fig 3.8; Table 3.4).

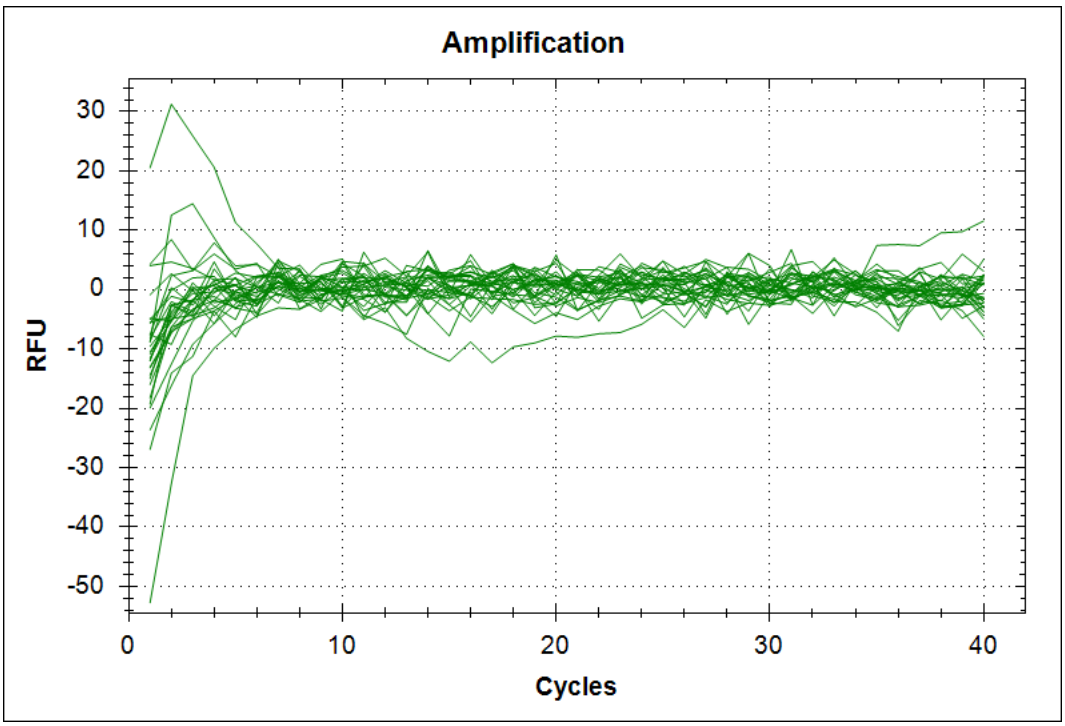


Figure 3.8. Real-time amplification plot showing a 10-fold dilution series of *Pythium ultimum* gDNA.

Table 3.4. Comparison of *Pythium ultimum* qPCR assay specific against a number of different fungal species and *P. ultimum* isolates.

| Isolate | $C_t$ value |
|---|---|
| Py 1 | >40 |
| Py 4 | >40 |
| Scl 2 | >40 |
| Scl 4 | >40 |
| VD 1 | >40 |
| VL 1 | >40 |
| R 3 | >40 |
| R 4 | >40 |

Using spectrophotometry (NanoDrop 2000c™, ThermoFisher Scientific, Loughborough, UK) DNA concentration and purity appeared satisfactory (Table 3.5), showing between 25.7 and 41.4 ng $\mu l^{-1}$ with an A260/280 ratio of 1.70 and 1.72 for the two *P. ultimum* isolates. These figures were suitable for use in validating the assay which required a minimum DNA concentration of 10 ng $\mu l^{-1}$, with an A260/280 ratio of ~1.8. However later amplification with the universal primers ITS 4 and 5 (White *et al.,* 1990) at an annealing temperature of 50°C similarly failed to produce amplicons for either Py1 or Py4 gDNA extracts, despite amplification of other isolates (data not shown). However, when examining the UV-Vis spectra in detail (Figure 3.9) it became apparent that the absorbance values alone were misleading in these assumptions, with high levels of contamination absorbing at ~230 nm.

Table 3.5. Genomic DNA concentration (ng $\mu l^{-1}$) and purity (A260/230, A260/280) from TE buffer blank and *Pythium ultimum* cultures (Py1 and Py4) used in qPCR, measurements conducted using UV-Vis spectrophotometry.

| Isolate | Conc. (ng $\mu l^{-1}$) | A260/280 | A260/230 |
|---|---|---|---|
| *TE Buffer* | *0.1* | *-0.52* | *0.10* |
| Py 1 | 41.4 | 1.72 | 0.05 |
| Py 4 | 25.7 | 1.70 | 0.03 |

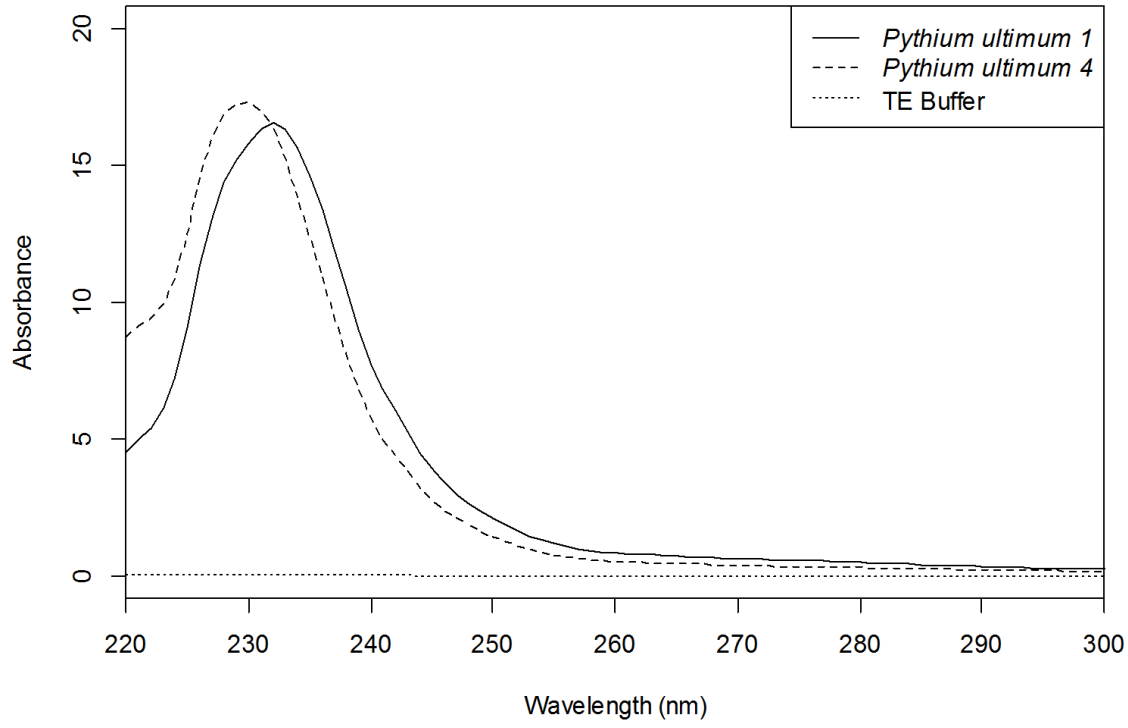


Figure 3.9. UV-Vis absorbance of two DNA extracts (*Pythium ultimum* 1 and 2) and TE Buffer, as recorded using a NanoDrop 2000c.

### 3.6 Discussion

One of the first steps in the study of plant pathology and its relation to yield decline is to establish which plant pathogenic organisms, if any, may be found in relation to commercial agricultural crops. Whilst previous studies have already identified and observed several pathogenic species within UK OSR crops (Gladders *et al*., 2013; Burnett *et al*., 2013), several potential pathogens such as *Rhizoctonia solani, Pythium ultimum* and *Gibellulopsis nigrescens* have still yet to be explored. Traditionally such surveys were conducted 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 survey a large number of sites. Due to this it was deemed essential to use molecular techniques, drastically cutting the reliance on these resources; whilst negating the effects of isolating from environmental material where mixed infections, obligate pathogens and asymptomatic/non-descript lesions make isolation, identification and quantification difficult. However, despite advancements in these techniques and the large amount of published species-specific PCR and qPCR assays these techniques are still not without their drawbacks.

One of the first drawbacks came as a consequence of the survey samples, with site selection, sampling and DNA extraction being conducted as part of a previous Innovate UK project. Whilst these provided a readily accessible source of DNA extracts, covering a number of commercial sites and agronomic variables, they also restricted the scope and results possible due to collection and processing methods used. For example, DNA was extracted from individual subsamples before being quantified and pooled to form a single extract, meaning that absolute quantification was difficult to achieve; particularly due to a lack of dilution and individual sample weight information. Other methods could have been utilised such as the selection of an endogenous or 'housekeeping' gene against which to compare samples, as has been done before in Brassicas (Mazzara *et al.*, 2007), however this would not have aided in the comparison to other studies. Consequently, the results were limited to relative quantification and comparison between samples. In addition to DNA extraction, the sampling time was also restrictive. Taken primarily before flowering, this restricted the 'window of observation' to one time point. Whilst this was the optimum sample timing for *Olpidium brassicae* and *Pyrenochaeta* spp. (P. R. Mills, Pers. comm.), the targets of the previous project, it limited the temporal observation of pathogen levels before, during and after the growing crop, in turn also potentially affecting the species observed at a given time point.

Along with these methodological issues site selection was also a concern, particularly because of the non-systematic method used, with the primary basis being to sample high and low intensity rotations of OSR. In order to be more representative of commercial OSR production a systematic subset of 50 samples was taken from the original 100 samples providing a more accurately representation of the main production areas, whilst equally sampling high, medium and low intensity OSR rotations. In addition, it also provided background information highlighting the most common agronomic decisions made by growers. Of particular interest was the prevalence of three main varieties (DK Cabernet, Castille and Vision) which was supported by the independent AHDB planting survey (AHDB, 2012). The preference of minimum tillage (DNI and SNI) compared to ploughing supported the suggestions of simpler, more specialised farming systems as proposed by Halloran and Archer (2008). That said, the result may have been affected by the lack of samples originating from the North Western and Western regions, where conditions are potentially less conducive to minimum tillage.

In addition to sample selection and extraction, the availability of qPCR assays, and how well these were developed and suited to the platforms and resources utilised within our laboratory were also challenging. Whilst assays were available for *R. solani* and *P. ultimum* none had been published for *G. nigrescens*, a newly highlighted candidate pathogen of OSR. However, the time limitations for this part of the thesis, coupled with the lack of UK isolates negated the ability to design and validate an assay to explore this pathogen further. In comparison, there was a number of available assays for both *R. solani* and *P. ultimum*, with those of Budge *et al.* (2009a) and Schroeder *et al.* (2006) chosen respectively, due to their validation and use in samples originating from soil. In addition, it was decided to concentrate on the 2-1 anastomosis group (AG) of *R. solani* due to the prevalence of this sub- group in other OSR growing regions (Verma, 1996).

The qPCR assay developed by Budge *et al*. (2009a) proved to be specific, effective and reliable, being able to discriminate between AG 2-1 and the other different but closely related anastomosis groups present within *R. solani*. Similarly, the assay worked well over a range of DNA concentrations between 100 fg $\mu l^{-1}$ - 10 ng $\mu l^{-1}$ ($R^2$=0.997), providing good amplification with an efficiency of 97% without any modification or optimisation, despite using a different qPCR platform.

However, this was not the case for the *P. ultimum* assay, which was taken from the published paper of Schroeder *et al.* (2006). In their study the assay showed specificity against other closely related *Pythium* spp. and good amplification over a range of 10 fg to 10 ng ($R^2$=0.999) at an efficiency of 85% from soil samples. However, in the current study

no amplification of the UK isolates of the *P. ultimum* received was possible. This may be due to poor primer design with the original paper sequencing the ITS region of one isolate in order to design primers, and using this same isolate for specificity confirmation and performance. Although an *in silico* analysis of the primers, conducted using NCBI BLAST, showed a 100% match to 100 different sequences of *P. ultimum* (results not shown). As a consequence of further experimentation, the likelihood of contaminants within the DNA extracts seemed plausible. When measured by UV-Vis, the DNA concentration and purity appeared to be acceptable, showing a concentration of between 25.7 and 41.4 ng $\mu l^{-1}$ with an A260/280 of 1.70 and1.72 and an A260/230 of 0.03 and 0.05 respectively. These figures are within the expected concentration attained from pure cultures within this thesis, whilst the A260/280 is not far removed from the 'ideal' A260/280 of ~1.8. In contrast, the A260/230 observed is somewhat lower than the 'ideal' of ~2.0, but some DNA extraction kit manufacturers also state that there is no consensus on the lower limit for what is an acceptable A260/230 (Qiagen, 2010). However, after observing the UV-Vis spectra in detail it became apparent that whilst the curve was similar in shape to that of high purity dsDNA, it was in fact that of contamination as its peak absorbance was at ~230 nm and not the expected ~260 nm. Based on technical notes produced by Qiagen (2010) absorbance at this wavelength is not uncommon when using commercial silica column kits (e.g FastDNA® Spin Kit for Soil) or performing DNA extraction from plant material, with contaminants such as guanidine thiocyanate (GITC) (A230), carbohydrates (A210-270) and peptides (A205) likely being implicated. This view was also similarly supported by the lack of amplification with the universal ITS 4 and 5 primers of White *et al.* (1990), although even the dilution of gDNA several times over did not improve amplification.

The aim of this chapter was to establish the limited information currently available on the distribution and occurrence of soil-borne pathogens within crops of OSR, and also to highlight some of the difficulties in advancing this knowledge. Principle amongst these issues is the scope of such studies to examine a suitably representative numbers of samples using conventional methods, and whereby the use of molecular methods becomes necessary. However, even with the use of molecular methods, few assays are suitably developed for all of the candidate pathogens, and in fact may not take into account others that are previously not known or well-studied as in the case of Hilton *et al.* (2013). In light of this, other technologies such as high throughput sequencing (HTS) may serve as a better investigative tool, being able to observe the occurrence and importance of known and cryptic pathogenic species within complex and varied environmental samples. In chapter 4, HTS was utilized to examine the fungal communities associated with the subset of samples described here. Highlighting the occurrence of pathogenic fungi, along with any influence

agronomic practices may have on these species and the fungal communities in general, leading to further areas of study

# 4

# Metabarcoding to describe the fungal communities of OSR roots, with a focus on pathogenic species.

## 4.1 Introduction

Recently, plant roots have been regarded as the 'second green revolution' in agriculture, with plant breeders aiming to increase plant growth, yield and nutrient acquisition through improving root growth and architecture (Lynch, 2007; Gewin, 2010). Alongside this, newer molecular methods have revolutionised the study of complex, but often hidden, microbial communities associated with plants (Shokralla *et al.*, 2012) and in particular, those which inhabit the 'hinterland' between soil and root tissue known as the rhizosphere. The term rhizosphere was first used to describe the area surrounding plant roots (McNear, 2013), but has since been refined to encompass three distinct zones (endorhizosphere, rhizoplane and ectorhizosphere) that exist between the inner root tissues and bulk soil. Together these form a variety of different habitats and points of interaction between microorganisms and plants; as illustrated in the paper title "The rhizosphere; a playground and battlefield…." by Raaijmakers *et al.* (2009), capturing the complex and juxtaposed positions of these communities. The fact that soil-borne pathogens are part of a larger community of other organisms, both antagonistic and synergistic, is also an important point to consider; especially as many are studied and discussed almost in isolation of this fact. For example, disease conducive and suppressive interactions have been elucidated between soil-borne fungi and other organisms such as nematodes (Back *et al.*, 2002), bacteria, protozoans and other fungi (Whipps, 2001). As a consequence, newer techniques allow for greater information to be collected, allowing researchers to examine their roles and interactions in causing/suppressing disease.

High-throughput sequencing (HTS) is a technique increasingly utilised in the study of soil communities and their ecology (Shokralla *et al.,* 2012) and has been used extensively to compare different environments such as meadows (Schmidt *et al.,* 2013), forests (Smith and Peay, 2014; Buée *et al.,* 2009) and even wine and beer production (Bokulich and Mills, 2013). Whilst other molecular methods such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) have been used

previously to outline the effects of cropping practices and rotations (Viebahn *et al.*, 2005; Hilton *et al.,* 2013), they often lack the capability to perform species determination, or to deal with large numbers of samples compared to HTS platforms. However, despite the previous use of these practices to examine agricultural samples, few studies have been published on the use of HTS on agricultural soils and plants, despite these being physically diverse and variable habitats (Garbeva, 2004).

More recently using one such HTS platform, Liu *et al.* (2014) demonstrated how several years of intensive potato monoculture resulted in few differences between fungi associated with different cropping intensities, with the exception of increasing levels of *Fusarium* spp. resulting in decreased crop yield. Similarly, Xu *et al*.(2012) examined the fungi associated with diseased and healthy pea crops grown in Denmark. From this they were able to demonstrate that the pathogen *Didymella pinodella* (syn. *Phoma medicaginis* var. *pinodella*) was abundant within the disease soils, with glasshouse experiments showing a significant increase in disease symptoms with these soils. Whilst both these studies were limited in their scope, i.e one field experiment and a few pea field sites, they do demonstrate how NGS maybe a useful tool to examine the link between agronomic practices and microbial communities, particularly when components such as pathogens are unknown or not well studied.

## 4.2 Objectives

The aim of this Chapter was to examine the fungal microbiome of oilseed rape (*Brassica napus*) roots with reference to the influence of cropping intensity and other agronomic factors on community composition and plant pathogenic species.

## 4.3 Null Hypothesis

1) The fungal communities and species, present on OSR roots, do not differ between field sites.

2) Agronomic factors have no significant effect on the composition of fungal communities on the roots systems of OSR crops grown at different field sites.

## 4.4 Materials and methods

### 4.4.1 Sampling and DNA extraction

Samples and DNA extracts comprised of the 50 subsamples selected from those attained in section 3.4.2. These were selected based on the proportion of OSR grown per AHDB region, using information collected from the Defra OSR surveys (1999 - 2010) to define the relative area grown per AHDB region (Appendix Table A2). 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.

### 4.4.2 Library preparation

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 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGGAAGTAAAAGTCGTAA 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, 1.25U 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 for 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).

### 4.4.3 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 $MgCl_2$, 0.5 µl 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, 5 µl of a single index 1 primer and 5 µl of a single index 2 primer from Illumina index set C were added. 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.

### 4.4.4 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 adding ssDNA from the bacteriophage ϕX174 (Illumina) resulting in a final sample containing 15 pM of DNA and 5% ϕX174.

This was then heated 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). Sequence data was deposited in the European Nucleotide Archive (ENA) as PRJEB12290.

### 4.4.5 Data Analysis and Statistics

Two forms of data analysis was conducted on the data as a result of changing methods and pipelines since the initial sequencing was conducted (Table 4.0). Initial analysis was conducted using the Quantitative Insights Into Microbiology (QIIME, v 1.5.0) software package (Caporaso *et al.*, 2010) (Method A), whilst the later analysis utilised QIIME initially, followed by USEARCH (v 9.2) (Edgar, 2010) (Method B). From herein these will be referred to as method A and method B.

Table 4.0. An overview of the two methods used to process high throughput sequence data.

| Method A | | Method B | |
|---|---|---|---|
| **Process** | **Software** | **Process** | **Software** |
| *Primer removal* | Cutadapt (v.1.4.2)<br>Martin (2011) | *Paired read merging* | QIIME (v.1.5.0) |
| *Quality filtering* | Sickle (v.1.21)<br>Joshi and Fass (2011) | *Primer removal* | QIIME |
| *Paired read merging* | Pear (v.0.9.6)<br>Zhang (2014) | *Quality filtering* | USEARCH (v.9.2)<br>Edgar (2010) |
| *Concatenation* | QIIME (v.1.5.0)<br>Caporaso *et al*. (2010) | *Concatenation* | USEARCH |
| *OTU picking* | USEARCH method<br>Edgar (2010) | *OTU picking* + *Chimera filtering* | UPARSE method<br>Edgar (2013) |
| *OTU classification* | BLAST<br>Altschul *et al*. (1990) | *OTU classification* | UTAX |
| *Reference database* | UNITE (v.7, r.31.1.16)*<br>Kõljalg *et al.* (2005) | *Reference database* | RDP Warcup ITS v.2<br>Deshpande *et al.* (2016) |
| *Beta diversity* | VEGAN (v.2.4-4)<br>Oksanen *et al*. (2017) | *Beta diversity* | VEGAN |
| *Alpha diversity* | VEGAN | *Alpha diversity* | VEGAN |

*Additional sequences were added from NCBI to cover pathogens of interest.

*4.4.5.1 Method A*

Quality control on the demultiplexed data was initially carried out using Cutadapt v1.4.2 (Martin, 2011) to remove amplification primers with relaxed parameters to account for errors caused by PCR primer degeneracies. Low quality 3' ends were removed from all sequences using Sickle v1.210 (Joshi & Fass, 2011) paired end. Paired-end reads were merged by Pear v0.9.6 (Zhang, 2014) with default settings followed by applying sickle single end, removing any sequences containing ambiguous bases (N) and with a quality score less than 30. Following the addition of unique identifiers all sequence reads were concatenated prior to analysis with Quantitative Insights Into Microbiology (QIIME, v.1.5.0) software package (Caporaso *et al.*, 2010).

Operational Taxonomic Unit (OTU) picking was applied using the *pick_otus.py* command (Edgar, 2010). From each OTU bin the first sequence was used as the representative sequence using the *pick_rep_set.py* command. Taxonomy was assigned to representative sequences by comparing to the UNITE reference database (Kõljalg *et al.,* 2005) using the *assign_taxonomy.py –m blast* command (Altschul *et al*., 1990). Due to the lack of plant pathogens present within UNITE, additional sequences were taken from the National Centre for Biotechnology Information (NCBI) database for inclusion in assigning taxonomy (Appendix Table S1). After assigning OTUs to taxonomic levels *per_library_stats.py* was used to assess the number of OTUs per sample. The *beta_diversity_through_plots.py* workflow was utilized to rarefy samples to the lowest number of OTUs (5572) per sample ensuring equal sequencing depth and to generate a Bray-Curtis distance matrix. Singletons and doubletons were removed prior to further data analysis.

*4.4.5.2 Method B*

Initial manipulation of the reads was conducted using QIIME (v.1.5.0) (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 the use of the UTAX algorithm with comparison to the Ribosomal Database Project (RDP) Warcup ITS training set (v 2) (Deshpande *et al.,* 2016). After assigning taxonomy an OTU table was generated using *usearch_global.*

*4.4.5.3 Statistical analysis*

Following the construction of OTU tables, both methods A and B were analysed in R studio (v.1.0.153) using the package VEGAN (Oksanen *et al*., 2017). Similarities between communities at each site were assessed using a Bray-Curtis dissimilarities matrix, constructed from the individual OTU tables, 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.

## 4.5 Results

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.

### 4.5.1 Data Analysis

Operational taxonomic unit (OTU) picking resulted in 10,246 and 4,066 OTU clusters at a 97% similarity level for method A and B respectively. After the removal of contaminating sequences (plant and chimeric) the number of sequences per sample ranged from 5,527 to 84,716 for method A, and 941 to 44,013 for B, with the lowest number being used for rarefaction within each method in order to normalize all samples (Gihring *et al.*, 2012) prior to further statistical analysis.

### 4.5.2 Community structure

Sequence assignment showed a marked difference in taxonomic structure (Table 4.1), with method A assigning 26.6% of sequences to phylum level only, 48.7% to 12 individual genera, 21.7% to 5 species with an additional 2.5% being grouped as other due to low relative abundance (<1%). In comparison, Method B assigned 0% to phylum level only, 80.4% to 8 genera, 1.1% to 1 species and 18.5% grouped as other. Overall, method A, showed an almost equal balance of Basidiomycete and Ascomycete fungi accounting for 43.5% and 32% of all sequences, with the remainder being placed into Zygomycetes (24.3%). Similarly, each phylum was dominated by a few OTUs identified to species or genus level (Table 4.1) with many of these matching to known groups of saprophytes such a *Mortierella* and *Mucor* and plant pathogens *(Ilyonectria, Leptosphaeria, Pyrenochaeta, Gibellulopsis, Microdochium, Fusarium, Botrytis* and *Rhizoctonia).* However, despite using a well curated database, 26.8% of the sequences could not be identified to either species or genus level. In comparison, the community structure of method B was markedly different to that of A, with 59.2% being assigned to Basidiomycota, whilst 11.4% could be assigned to Ascomycota, 10.9% to Zygomycota and 18.5% to other phyla. Similarly, very few genera or species were described compared to method A, although some were shared such as *Mortierella*, *Mucor* and *Rhizoctonia solani*.

Table 4.1. Percentage relative abundance (%RA) of fungal genera and species found on the roots of English oilseed rape crops following operational taxonomic unit (OTU)

assignment using methods A and B. 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.

| Fungal Taxonomy | % RA | |
|---|---|---|
| | A | B |
| **Ascomycota** | **43.5** | **11.4** |
| Pyrenopeziza | 4.0 | - |
| Ilyonectria | 2.7 | - |
| Monographella | 2.6 | - |
| *Leptosphaeria maculans* | 2.5 | - |
| *Leptosphaeria biglobosa* | 2.1 | - |
| *Pyrenochaeta lycopersici* | 2.0 | - |
| Davidiella | 1.8 | - |
| *Gibellulopsis nigrescens* | 1.6 | - |
| Gibberella | 1.5 | - |
| Microdochium | 1.4 | - |
| Fusarium | 1.3 | - |
| Botrytis | 1.1 | - |
| *Cadophora* | - | 9.5 |
| *Lewia* | - | 1.9 |
| Unidentified | 16.4 | - |
| Other | 2.5 | - |

| | % RA | |
|---|---|---|
| | A | B |
| **Basidiomycota** | **32.0** | **59.2** |
| *Rhizoctonia solani* | 13.8 | 1.1 |
| Itersonilia | 5.1 | - |
| Leucosporidium | 2.9 | - |
| Tremella | - | 43.0 |
| Xenasma | - | 6.7 |
| Sphacelotheca | - | 6.4 |
| Dichomitus | - | 2.0 |
| Unidentified | 10.2 | - |
| **Zygomycota** | **24.3** | **10.9** |
| Mortierella | 22.4 | 6.5 |
| Mucor | 1.9 | 4.4 |
| **Other** | **-** | **18.5** |
| Other | - | 18.5 |

| **Summary of Taxons** | | |
|---|---|---|
| Phylum | 26.6 | 0.0 |
| Genus | 48.7 | 80.4 |
| Species | 22.0 | 1.1 |
| Other | 2.5 | 18.5 |

### 4.5.3 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), within either method. Similarly, 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 (Fig 4.0 and 4.1).

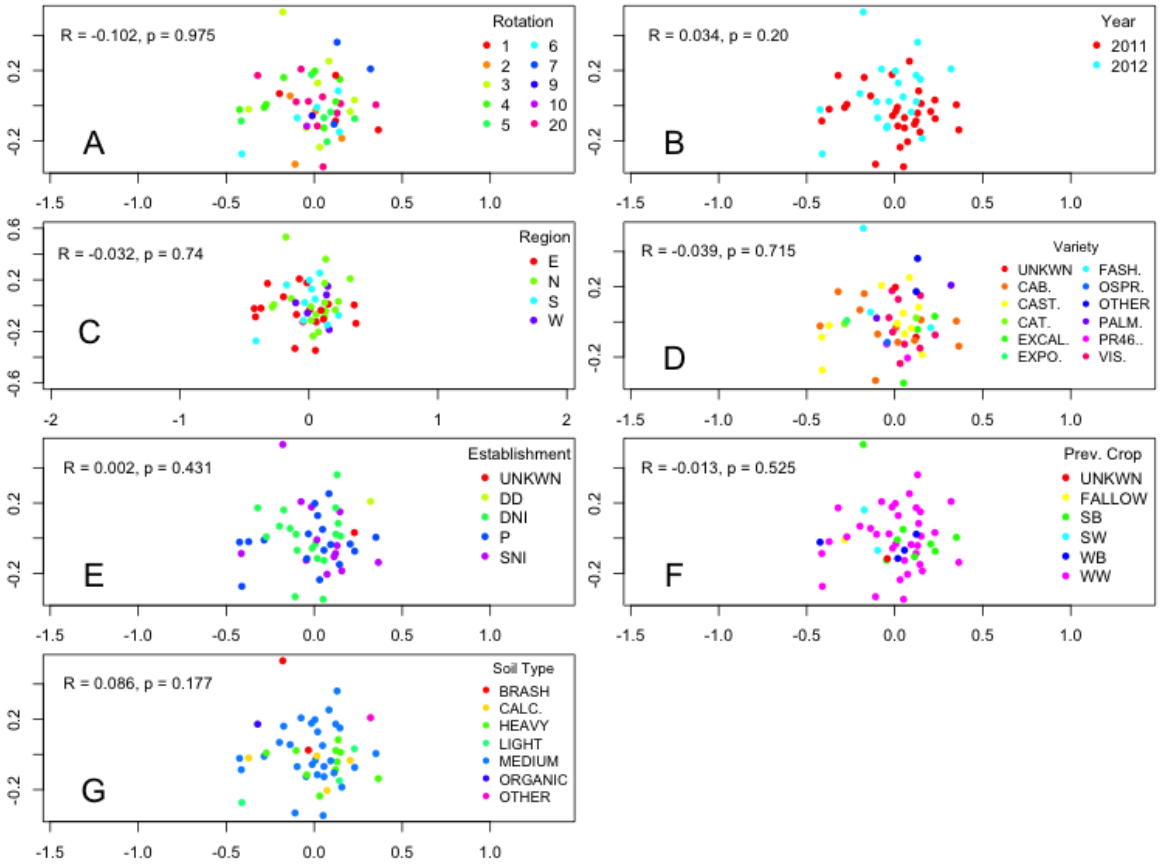


Figure 4.0. Method A, Bray-Curtis non-metric multidimensional scale (nMDS) plots generated from individual agronomic factors. A: Rotational frequency, B: Sampling year, C: AHDB Region, D: Variety, E: Establishment technique (DD – Direct drilled, DNI – Deep non inversion, P – Plough, SNI – Shallow non inversion), F: Previous crop (SB – Spring barley, SW – spring wheat, WB – winter barley, WW – winter wheat), G: Soil type.

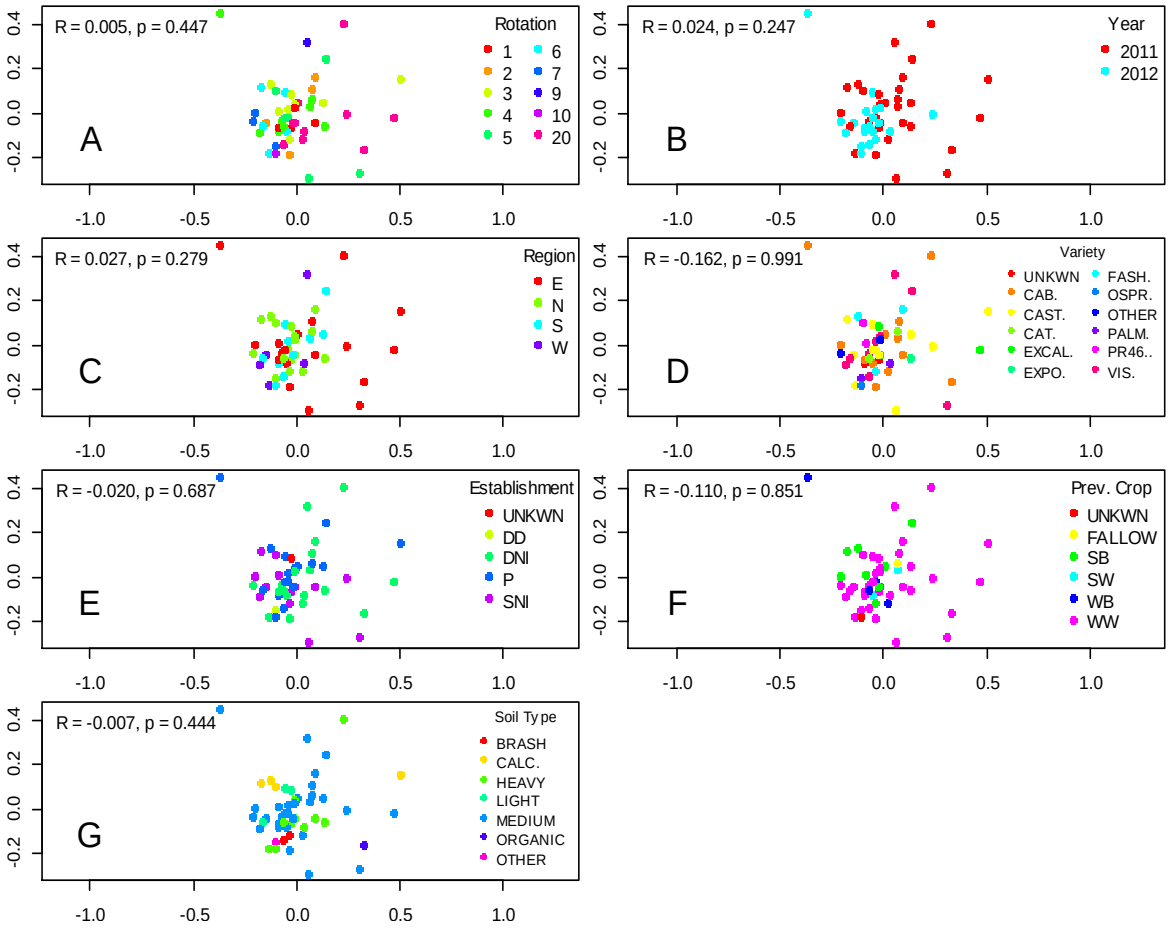


Figure 4.1. Method B, Bray-Curtis non-metric multidimensional scale (nMDS) plots generated from individual agronomic factors. A: Rotational frequency, B: Sampling year, C: AHDB Region, D: Variety, E: Establishment technique (DD – Direct drilled, DNI – Deep non inversion, P – Plough, SNI – Shallow non inversion), F: Previous crop (SB – Spring barley, SW – spring wheat, WB – winter barley, WW – winter wheat), G: Soil type.

## 4.6 Discussion

In recent years, plant roots have become the centre of a second 'green revolution' allowing for improved growth and yield through a better understanding and manipulation of root architecture and other traits (Gewin, 2010). Due to their unique position, they form a vital function to the plant providing anchorage, storage, and nutrient and water acquisition. However, despite these simple roles plant roots are not inert one-sided structures. For example, through the production of exudates and leachates they are able to recruit microorganisms from the bulk soil (Berendsen *et al*., 2012), forming neutral, beneficial or deleterious relationships with plants (Raaijmakers *et al*., 2009). Previously, these interactions and relationships were difficult to examine and elucidate, however with the advent of improved molecular methods such as high throughput sequencing, researchers are now able to study these in more detail than previously possible. As a result of this, high throughput sequencing is becoming 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).

This chapter aimed to use an Illumina MiSeq platform to examine the effects of agronomic factors, and in particular rotational frequency, on the microbiome of commercial OSR roots with a particular focus on elucidating plant pathogens. As a result of the fast-paced development of analysis tools and pipelines, two pipelines were used to compare the same data set. This provided an overview of their effectiveness and how such choices may effect the final results.

Initial data analysis and sequencing showed no significant divergence from what was expected, with the large number of reads (19.7 M) being typical for this type of study using Illumina technology. However, the two analysis methods used, showed a marked difference in overall analysis and taxonomic classification of sequences. During the initial data analysis method A resulted in 10,246 OTUs compared to method B at 4,066, with this marked difference likely due to the added step of chimera detection; as both USEARCH and UPARSE use a similar heuristic 'greedy' algorithm. This algorithm is designed to produce more biologically similar results (i.e 1 OTU = 1 species) by picking OTU clusters in order of decreasing sequence abundance, thus giving preference to the most abundant (and thus more likely real) sequence reads. However, by adding in the additional chimera detection step UPARSE is able to dramatically reduce the number of final OTUs. In a similar manner, the number of sequences per sample also varied between A and B, with this also likely being a consequence of removing sequences believed to be chimeric. Although, it is

surprising the magnitude of the reduction resulting in 5,527 sequences across all samples in A and 941 for B after rarefication.

Similar to OTU picking, differences in community structure and taxonomic assignment were also seen between methods. Method A, showed an almost equal balance of Basidiomycetes and Ascomycetes accounting for 43.5% and 32% of all OTUs, with the remainder being placed into Zygomycota (24.3%). In comparison, method B resulted in 59.2% being assigned to Basidiomycota, whilst 11.4% could be assigned to Ascomycota, 10.9% to Zygomycota and 18.5% to other phyla (grouped together as they accounted for <1% RA individually). Whilst some factors such as primer and PCR bias have been shown to affect community recovery (Bellemain *et al.*, 2010; Toju *et al.*, 2012), these are unlikely here as both methods utilise the same sequence data, and thus the same biases. Therefore, taxonomy assignment method, reference database and previous analysis seem more plausible explanations. Taxonomy was assigned to each OTU using either; A, BLAST (Altschul et al., 1990) or B, UTAX. The Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) is an alignment based tool which uses sequence base scoring to search for the 'best matching' sequence. In addition, it also seeks to classify to the lowest taxonomic level possible, however this is often at the expense of increased computational needs and limited consideration of matches other than the top result. Due to this, more recent studies have turned to composition and probabilistic methods such as the RDP classifier and the UTAX algorithm, which are also computationally less demanding (Lindahl *et al*., 2013). As a consequence, the use of UTAX in method B should lead to a more accurate taxonomic assignment, although this seemed to be at the expense of taxonomic level with many sequences delimitated to genus only and not the desired species; a problem encountered elsewhere when using this method (Richardson *et al.,* 2017). In addition to this, the lack of peer-reviewed information about the mechanisms behind UTAX make comparisons to other methods more difficult, something found here when the UTAX algorithm information was removed from the USEARCH manual by the author, in favour of newer methods.

In order for taxonomy to be assigned a reference database is required for comparison and thus sequence annotation. Method A utilised the UNITE database (Kõljalg *et al.,* 2005) with some additional sequences added from the NCBI database. UNITE is a widely used and curated reference database, and as such overcomes the problems of other widely used databases which contain low quality, poorly annotated and potentially chimeric sequences (Kõljalg *et al.,* 2013). In addition, it also utilises a two stage clustering process to better classify sequences to genus and subgenus level, forming a 'species hypothesis' (sh) database for taxonomic assignment. As of the v.7 release (31.01.2016), UNITE contained

37,020 representative sequences (computer generated sh based on 97% similarity clustering) and 6,273 reference sequences (sequences confirmed by users who specialise in a particular taxon), resulting in 43,293 sequences. However despite this large number of sequence very few, if any, sequences of common OSR pathogens were present, as a result 23 additional sequences covering the genera; Plasmodiophora, Pyhtophthora, Gibellulopsis, Verticillium, Olpidium, Pyrenochaeta, Pythium, Rhizoctonia and Leptosphaeria, were added from high quality NCBI GenBank accessions. In comparison, the RDP Warcup ITS v.2 set was used in method B, built on a similar 97% similarity clustering principle to that of UNITE, it contains sequences which have a high confidence of assignment to genus and species level. Additionally, it also contained accessions of the main pathogenic genera of interest meaning that no additional sequences were required. When examining the results of these two methods it was surprising just how different the taxonomic assignment was. Both methods assigned all of the sequences to phylum level or lower, although method B was able to assign a higher proportion to genus or lower taxonomic classification (100%) compared to method A (73.2%). In contrast though, method A assigned a higher proportion of the sequences to species level (A, 22%; B, 1.1%), whilst method B produced a higher proportion of other sequences (18.5%) compared to method A (2.5%), with these mostly being attributed to other less abundant genera. This comparison highlights some of the differences between the two databases, which may be compounded by biases from the assignment method. Method A was able to assign more sequences to lower taxonomic levels as a consequence of BLAST seeking to describe the lowest taxon possible. The limitations of the database appear to be related to the lack of genera and species information, due to the greater number of sequences that were unassignable to lower than phylum. Method B, in comparison, was able to assign a higher proportion of sequences to genera but not species, possibly due to the UTAX algorithm which has been shown to result in greater genus assignment than species (Richardson *et al*., 2017). Despite containing fewer sequences than UNITE it was able to describe more sequences, perhaps pointing to a more diverse set of references, which describe rarer or less well-known taxa. Ultimately these differences are most likely caused by the large discrepancy in OTU numbers between methods due to the conservative nature of USEARCH and the additional chimera detection, with this in turn impacting on the number of sequences available for assignment (A, 5,527 compared to B, 941). After this point, differences are likely to be seen between taxonomy assignment method and database used, although the varied and unstructured way in which this comparison is formed makes this difficult, if not impossible, to elucidate effectively. However, what was interesting was the shared taxa between the two, and in particular the pathogenic species *Rhizoctonia solani*.

Despite the differences between the two methods, both showed that agronomic factors appeared to have limited influence on the communities of fungi. Individual samples and associated OTUs showed little variation and no detectable differences. This is contrary to what was expected as 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 (Xu *et al*., 2012; 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.

This similarity between field sites may be due to the ability of plant species to modify the rhizosphere through chemical processes (Whipps and Lynch, 1986). In a review on root microbiomes Berendsen *et al.* (2014) 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 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 (Whipps and Lynch, 1986; 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 soybean (*Glycines max*) and mustard (*B. juncea* and *B. nigra*) respectively.

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.,* 2009). 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). Survey data from commercial crops have largely been limited to Canada and Australia, but have demonstrated that *R. solani* AG 2-1, 2-2, 4 and 8 are present within crops. However currently limited data exists on the quantities and distribution of *Rhizoctonia solani* within UK crops of OSR. As a consequence, Chapter 5 aims to quantify and characterise the individual AG of *R. solani* across the 50 field sites through the use of previously published qPCR assays.

# 5

# Characterization of *Rhizoctonia solani* within OSR crops, and its contribution to yield decline

## 5.1 introduction

The soil-borne pathogen *Rhizoctonia solani* (teleomorph, *Thanatephorus cucumeris*) 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 made of 13 groups classified by their ability to anastomose through hyphal connections (Anderson, 1982; Ogoshi, 1987; Carling *et al*., 2002). These individual anastomosis groups (AG) often represent groups of isolates which are adapted to a specific environment or host range.

Surveys highlighting soil-borne pathogens in OSR have been conducted widely in countries such as Canada and Australia (Khangura *et al.,* 1999; Zhou *et al.,* 2014), where foot rots and seedling diseases are prominent in OSR. These surveys 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 distribution of *Rhizoctonia solani* within UK crops. In a survey of 90 UK wheat fields Brown *et al.* (2014) found AG2-1 to be present at 64% of sites sampled. The quantity of pathogen DNA present was also found to correlate with previous cropping and particularly OSR, after which DNA levels were significantly higher. Other surveys have also been conducted, with Budge *et al.* (2009) finding AG 2-1 to be the prominent group associated with field grown cabbage (*B. oleracea*) in Lancashire, Lincolnshire and Yorkshire, with low levels of AG 4 found. Whilst various *in vivo* experiments have shown AG 2-1 to be highly virulent on *Brassica* spp. and to a lesser extent AG 2-2, 4, 8 and 10 (Verma, 1996; Khangura *et al.,* 1999; Tewoldemedhin *et al*., 2006; Budge, 2007; Babiker *et al*., 2013).

Studies have also attempted to describe the relationship between *R. solani* AG 2-1 inoculum and yield loss in OSR (Zhou *et al*., 2016; Budge, 2007). Zhou *et al*. (2016) was able to demonstrate a decline in OSR emergence, plant height, biomass and seed weight with increasing pathogen inoculum, although interestingly the effect on damping-off symptoms showed a ‘bell shaped’ curve. In comparison, Budge (2007) suggested that damping-off

occurred only at lower inoculum levels, above which no appreciable increase was seen; but at which wire-stem symptoms became more prevalent. In addition they also found a poor correlation between the percentage inoculum incorporated into pots, disease symptoms and pathogen DNA. These findings are supported by Zhou *et al.* (2016), who observed an exponential relationship between pathogen DNA and inoculum, although a strong linear trend was also seen between inoculum and $C_t$ values.

### 5.2 Objectives

The current study aims to elucidate the occurrence and distribution of *R. solani* within UK crops of OSR using AG species real-time PCR assays to detect the occurrence of AG 2-1, 2-2, 4 HGII, 5 and 8. Secondly, glasshouse experiments will utilise a dilution series of artificially infested soil to determine the threshold required for disease symptoms to occur and the effect on plant growth and yield. Direct and indirect mechanisms of yield loss will also be determined.

### 5.3 Null hypotheses

1) *Rhizoctonia solani* is not present in UK crops of OSR.

2) *Rhizoctonia solani* does not infect OSR or the model plant *B. oleracea* (DHSL150), nor does it cause any negative effects to plant health and yield of these plant species.

## 5.4 Materials and methods

### 5.4.1 Survey

#### *5.4.1.1 Sample acquisition and DNA extraction*

Fifty samples were selected from those described in section 3.4.2. Samples were chosen on a stratified basis based on the area of OSR grown within each growing region, and the period between the last OSR crop aiming to evenly sample both intensive (1-3 years), moderate (4-10) and low intensity (10-20) rotations with wheat or barley. Other factors such as soil type, establishment method and crop variety were also recorded. DNA was extracted as previously described in section 3.4.3.

#### *5.4.1.2 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.,* 2009). 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 12.5 µl 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 determined using the manufacture's software, and an average of two replicates taken. A standard curve was constructed using a ten-fold dilution using 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 quantification (LoQ).

### 5.4.2 Pathogenicity bioassay (OSR)

#### *5.4.2.1 Inoculum production*

*Rhizoctonia solani* inoculum was prepared using the method of Sinclair and Dhingra (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 did not contain 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 20°C for 6 weeks, agitating weekly to disperse inoculum. After 6 weeks, inoculum was stored at 4°C until used, usually within one week.

*5.4.2.2 Preliminary trials*

Inoculum was prepared as described above, utilising isolate R13 (section 4.2.1, table 3.1), a known OSR pathogenic isolate. After six weeks the batches of inoculum were added to uninoculated sand-maize meal in a two-fold dilution series. This comprised of 5 doses from 250-15.25 g of inoculated maize-sand mixed with uninoculated maize-sand to a total weight of 250 g, plus an uninoculated control (0 g). 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 (section 2.2.1) 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).

*5.4.2.3 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 (Table A2).

*5.4.2.4 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 0-5 scale (Table 5.0). From each pot, 1 g of root material and 10 g of soil was taken and stored at -20°C, before DNA extraction and qPCR. The remainder of the root system and the shoot material was placed in separate oven bags before being dried at 100$^{o}$C for 72 hours, after which dry matter (DM) was weighed.

Table 5.0 Disease score system used. Authors own.

| Score | Symptoms |
|---|---|
| 0 | No visible damage (0%) |
| 1 | <10% of root area infected |
| 2 | 11 – 20% of root area infected |
| 3 | 21 – 40% of root area infected |
| 4 | 41 – 80% of root area infected |
| 5 | >81% of root area infected OR seedling death |

### 5.4.3 Virulence bioassay (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).

#### *5.4.3.1 Inoculum*

Inoculum was prepared as previous (5.4.2.2) 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 $kg^{-1}$ (Figure 5.0), whilst in experiment 3 they were 20, 10, 5, 2.5, 1 and 0 g $kg^{-1}$ of total potting media (Figure 5.0).

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) (Figure 5.1 and 5.2).

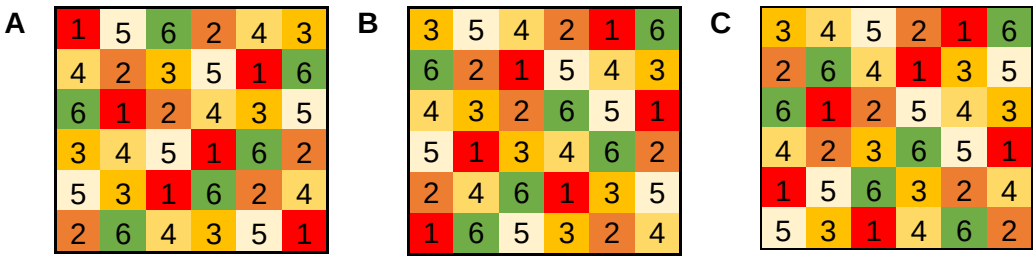


Figure 5.0. Experiment 1 (A) and 2 (B): design/layout. 1, 80 g $kg^{-1}$; 2, 40 g $kg^{-1}$; 3, 20 g $kg^{-1}$; 4, 10 g $kg^{-1}$; 5, 5 g $kg^{-1}$; 6, 0 g $kg^{-1}$. Experiment 3 (C): design/layout. 1, 20 g $kg^{-1}$; 2, 10 g $kg^{-1}$; 3, 5 g $kg^{-1}$; 4, 2.5 g $kg^{-1}$; 5, 1.25 g $kg^{-1}$; 6, 0 g $kg^{-1}$.

Figure 5.1. Photograph of experiment 2 in glasshouse bay. Plant showing 2 true leaves.

Figure 5.2. Photograph of experiment 2 in glasshouse bay. Plants showing 4-5 true leaves.

*5.4.3.2 Experimental conditions*

Conditions remained the same as in section 5.4.2.2, 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 (Figure 5.3).

Figure 5.3. Mature plants of DHSL150 prior to the harvest of experiment 2.

*5.4.3.3 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 5.4.1.2.

Measurements taken at seedling stage were the same as those in 5.4.2.3. 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.

*5.4.3.4 Statistical Analysis*

Survey data was analysed in GenStat (v17) using a generalized linear model, fitting individual explanatory variables in a step-wise fashion.

Glasshouse data was analysed in RStudio (v1.0.153), using the core functions and the additional package DRC (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.

## 5.5 Results

### 5.5.1 Survey

Detection of individual anastomosis groups varied across the samples with AG 2-1 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 5.4). 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 $ng^{-1}$ total DNA, with an average of 3.3 pg $ng^{-1}$ total DNA. Whilst AG 8 was detected at 4.0 $x10^{-2}$ and 1.0 $x10^{-2}$ pg $ng^{-1}$ total DNA, and AG5 at 9.0 $x10^{-2}$ pg $ng^{-1}$ total DNA. No significant relationship ($p>0.05$) could be drawn between pathogen DNA concentration and any of the agronomic or environmental variables.

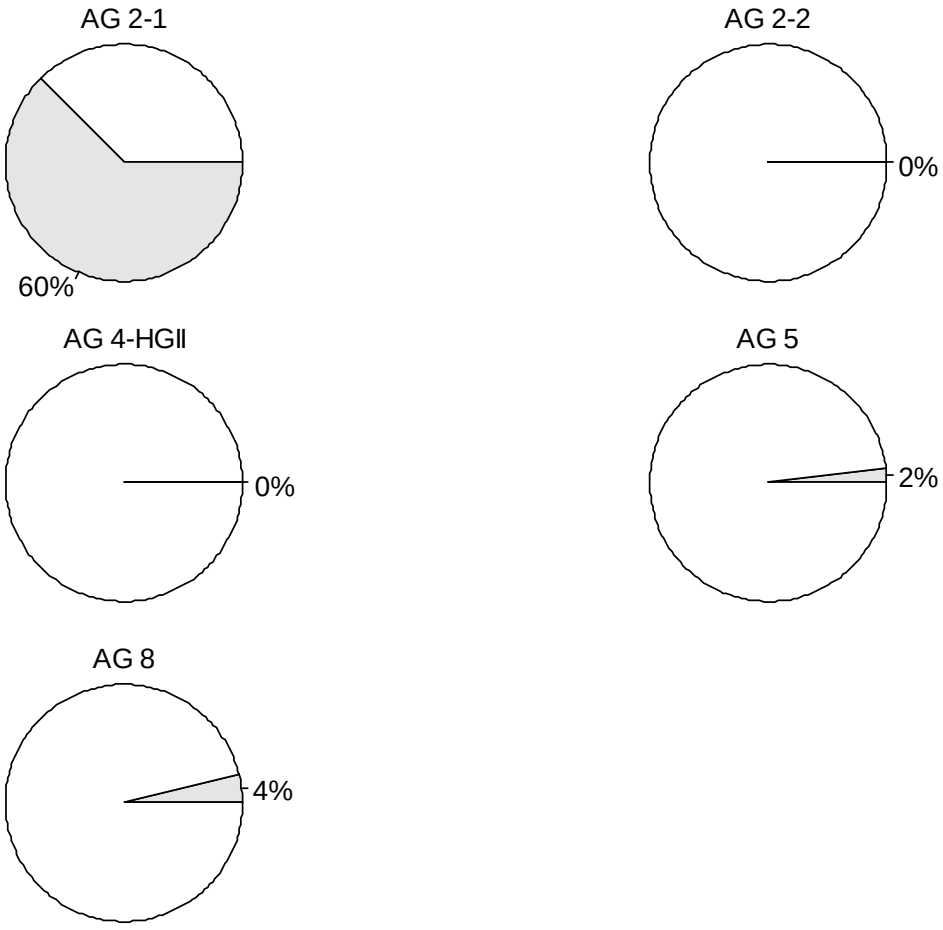


Figure 5.4. Occurrence of individual *Rhizoctonia solani* anastomosis groups (AG) across oilseed rape field sites (n=50) as determined by real-time PCR analysis of DNA extracted from root samples.

### 5.5.4 Virulence bioassay (OSR)

*5.5.4.1 Preliminary experiments*

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 (Figure 5.5). 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, 31.3 and 62.5 g $kg^{-1}$ seedling emergence was significantly reduced ($p=0.034$, 0.0049 and 0.0049) when compared to the un-inoculated pots. 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.

Biomass production

Root and shoot biomass production was significantly reduced ($p = 0.007$; $p < 0.001$ respectively) as the proportion of *R. solani* (AG 2-1) inoculum increased (Figure 5.6). The greatest reduction in shoot and root biomass occurred 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 score

Disease score significantly increased ($p <0.001$) as the proportion of *R. solani* (AG 2-1) inoculum increased (Figure 5.7). The greatest severity (5) occurring at rates of between 31.25 and 250 g $kg^{-1}$, whereby all seedlings were either killed (Figure 5.8) or showed severe wire stem (Figure 5.8, 5.9 and 5.10) at the point of assessment. A reduced score of 2.7 was seen at 15.63 g $kg^{-1}$ (Figure 5.11) whilst no disease symptoms were seen in the control (0 g $kg^{-1}$).

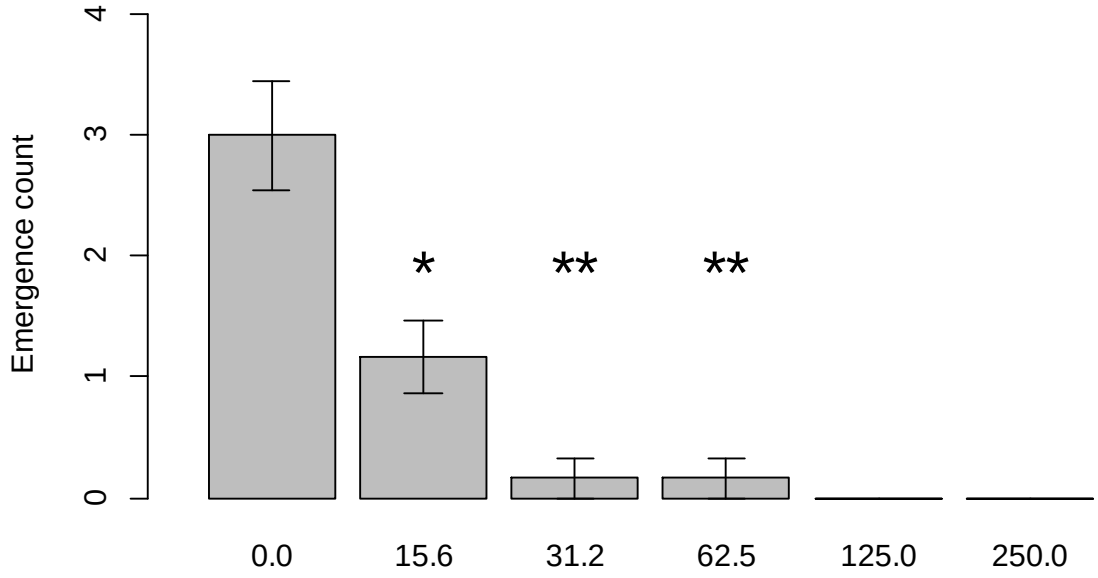


Figure 5.5. Emergence of *Brassica napus* (*cv*. DK Cabernet) seedlings (3 seeds per pot, 6 replicated pots, n=24) at 30 dpi when grown in pots containing increasing doses (g $kg^{-1}$) of *Rhizoctonia solani* AG 2-1 inoculum. Error bars represent the standard error of the mean. Asterisks denote a significant difference from the uninoculated control; *, $p < 0.01$ and **, $p < 0.001$.

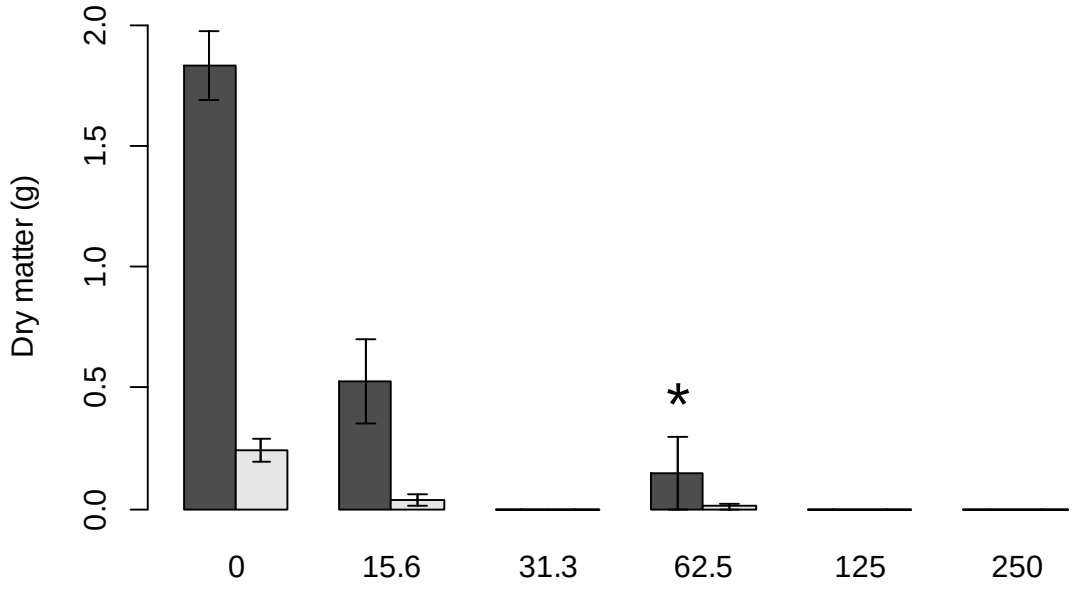


Figure 5.6. Dry matter production of *Brassica napus* (*cv*. DK Cabernet) seedlings (n=24) at 30 dpi when grown in pots containing increasing doses (g $kg^{-1}$) of *Rhizoctonia solani* AG 2-1 inoculum. Dark bars = shoot DM, light bars = root DM. Error bars represent the standard error of the mean. Asterisks denote a significant difference from the uninoculated control; *, $p < 0.01$ and **, $p < 0.001$.

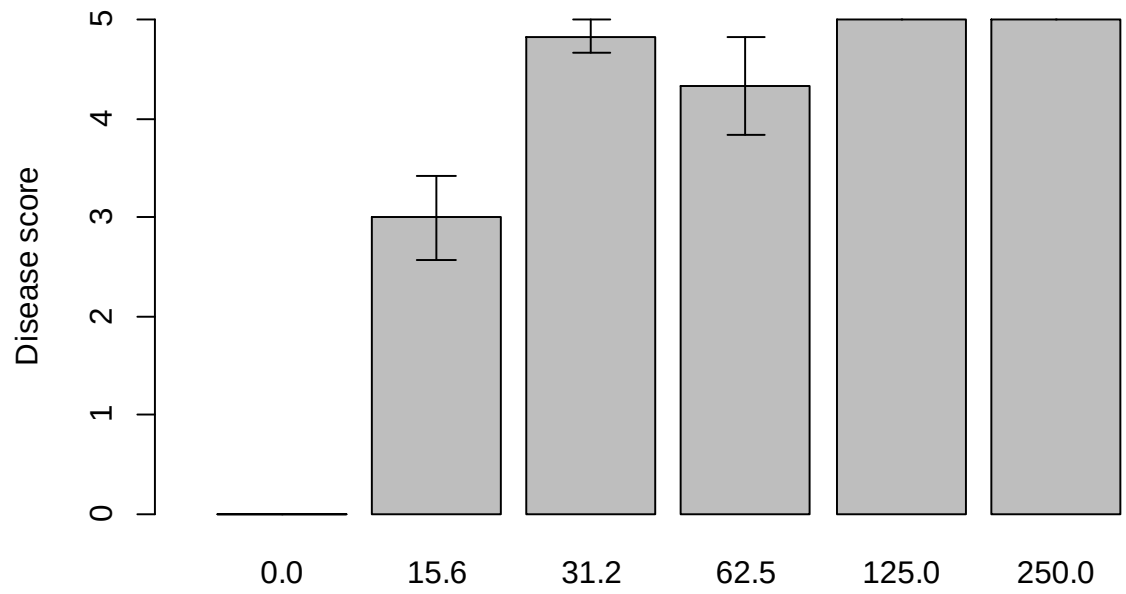


Figure 5.7. Disease score of *Brassica napus* (*cv*. DK Cabernet) seedlings (n=24) at 30 dpi when grown in pots containing increasing doses (g $kg^{-1}$) of *Rhizoctonia solani* AG 2-1 inoculum. Error bars represent the standard error of the mean.

Figure 5.8. Preliminary OSR experiment before harvest, demonstrating the variation in emergence and plant growth.

Figure 5.9. Girdling lesion of *Rhizoctonia solani* AG 2-1, causing ‘wire stem’ of oilseed rape.

Figure 5.10. Stem discolouration caused by *Rhizoctonia solani* AG 2-1.

Figure 5.11. Surface discolouration and runner hyphae of *Rhizoctonia solani* AG 2-1.

### 5.5.5 Virulence bioassays (DHSL150)

*Real-time PCR and Dose correlation*

Through the use of real-time PCR, it was found that DNA quantity per gram of soil and inoculum quantity per kg of potting mix were often significantly correlated (Figure 5.12). However, this relationship appeared to be dependent on the effectiveness of the inoculum preparation, with variation in aggressiveness occurring between experiments 1, 2 and 3 and this being similarly reflected in the quantity of DNA detectable. Real-time PCR successfully amplified the target from both experiments 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 greater, with all but the control treatment amplifying before the 35 $C_t$ point. The linear model showed a strong significant relationship between the inoculum dose (g $kg^{-1}$) and pathogen quantity (Log ng target DNA $g^{-1}$ of soil +1). No results from experiment 3 could be presented due to the loss of samples following a mechanical malfunction in storage.

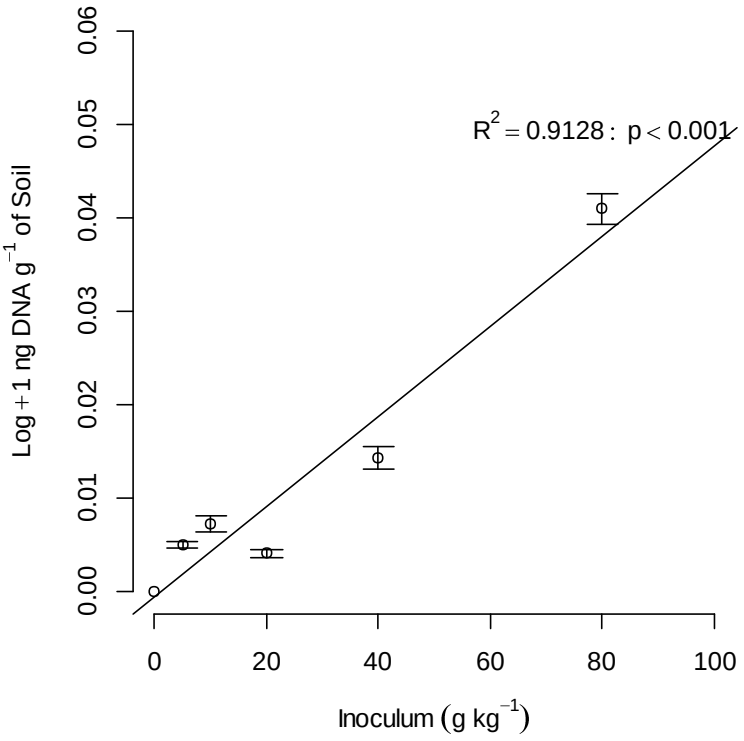


Figure 5.12. Relationship between inoculum quantity (g $kg^{-1}$) and *Rhizoctonia solani* AG 2-1 quantity (Log ng DNA $g^{-1}$ soil +1) as measured by real-time PCR at planting in experiment 2. Error bars represent the standard error of the mean.

Figure 5.13. DHSL150 plant at late flowering (L), demonstrating its compact growth habit and suitability for glasshouse use, compared to oilseed rape at a similar stage (R). Scale bar indicates 30 cm.

*Experiment 1, Dose response curves*

In experiment 1, dose response curves were constructed for the measured variables of emergence (%), emergence (Dpi), raceme number, pod number (total and empty), seeds

per pod, root DM, shoot DM, and seed yield (Figure 5.14). Across all of these variables, the expected sigmoidal shaped response was not seen except for root DM, shoot DM and yield which showed a decline in response as dose was increased. Many of those that did respond, however, failed to reach the level of $ED_{50}$ with the exception of raceme number, pod number and seeds per pod where the $ED_{50}$ corresponded to 88.5, 19.3 and 44.0 g $kg^{-1}$ respectively. Overall, the combined effect of these was not sufficient to limit overall seed yield.

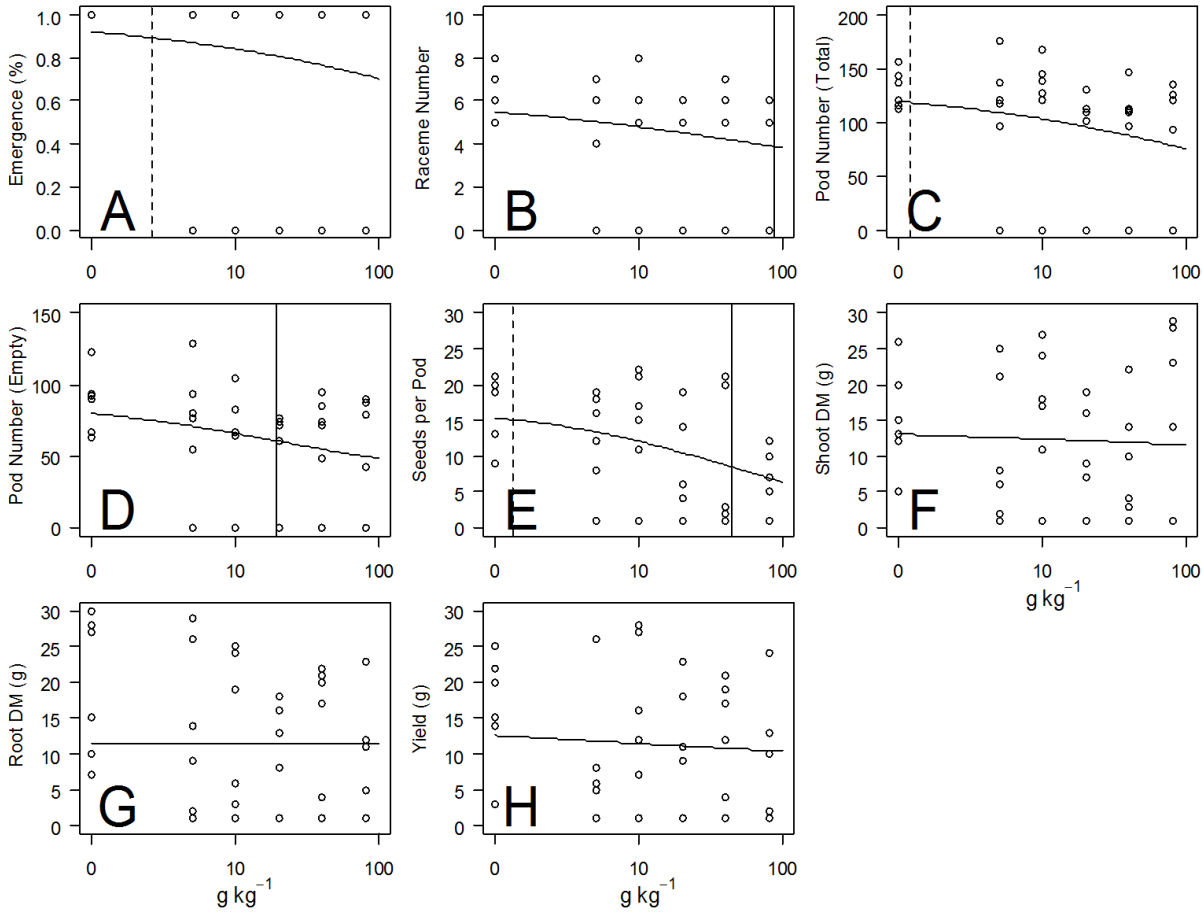


Figure 5.14. Experiment 1: Dose response curves fitted from 4 parameter log-logistic models for multiple response variables at differing doses of *Rhizoctonia solani* AG 2-1 inoculum (g $kg^{-1}$). A, Percentage emergence; B, Raceme number; C, Pod number (total); D, Pod number (Empty); E, Seeds per pod; F, Shoot dry matter (g); G, Root dry matter (g); H, Seed yield (g). Vertical lines represent $ED_{10}$ (dashed) and $ED_{50}$ (solid) thresholds.

*Experiment 2, Dose response curves*

Experiment 2, dose response curves were constructed for the measured variables of percentage emergence and days to emergence (Dpi) (Figure 5.15); 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 $ED_{05}$, $ED_{10}$ and $ED_{50}$. At the $ED_{50}$ level a dose of 5.3 and 5.0 g $kg^{-1}$ was needed for each variable respectively.

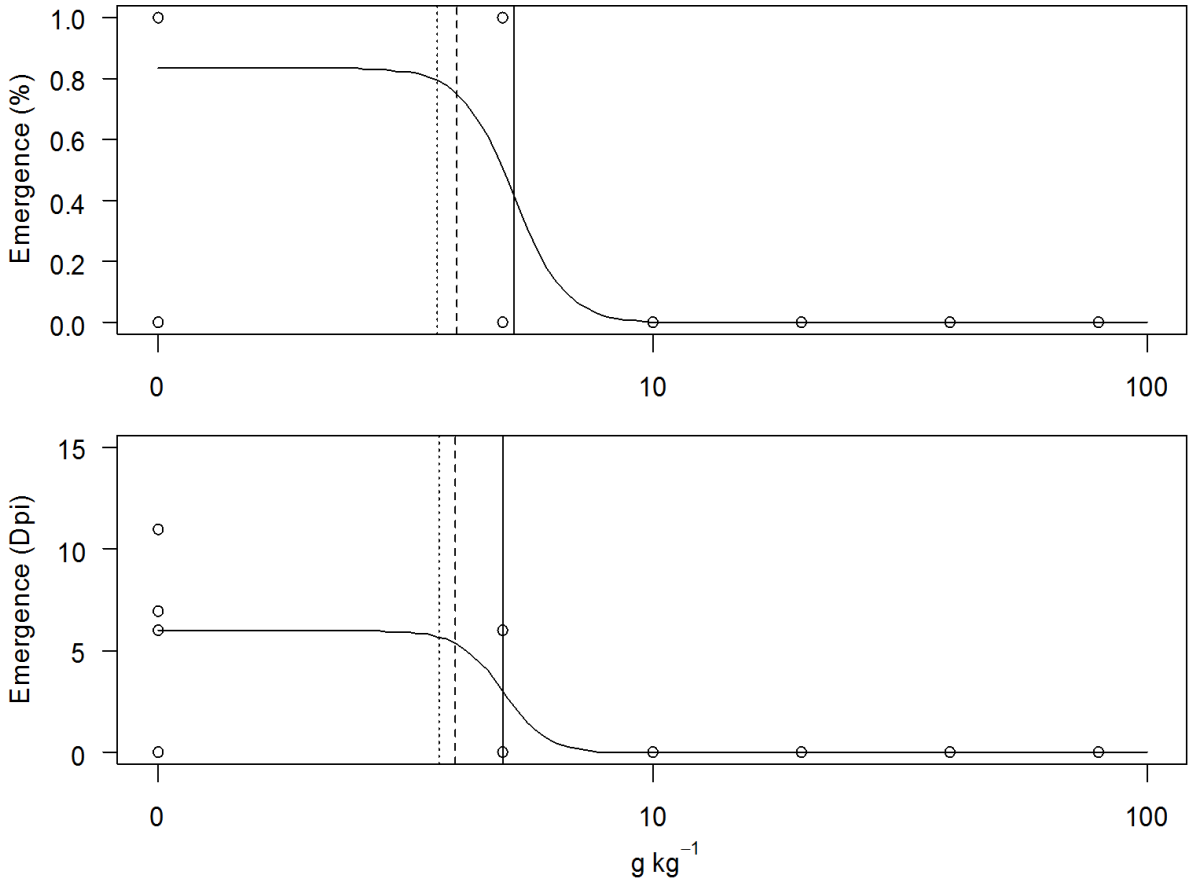


Figure 5.15. Experiment 2: Dose response curves fitted from 4 parameter log-logistic models for percentage emergence (Top) and days to emergence (Bottom) at differing doses of *Rhizoctonia solani* AG 2-1 inoculum (g $kg^{-1}$). Vertical lines represent $ED_{05}$ (dotted), $ED_{10}$ (dashed) and $ED_{50}$ (solid) thresholds.

*Experiment 3, Dose response curves*

In experiment 3, dose response curves were constructed as for experiment 1. Across all variables the expected sigmoidal response was not observed (Figure 5.16) although values were attained for $ED_{05}$, $ED_{10}$ and $ED_{50}$ with many of the variables. Typically the $ED_{50}$ 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 the $ED_{50}$ was between 5 and 10 g $kg^{-1}$.

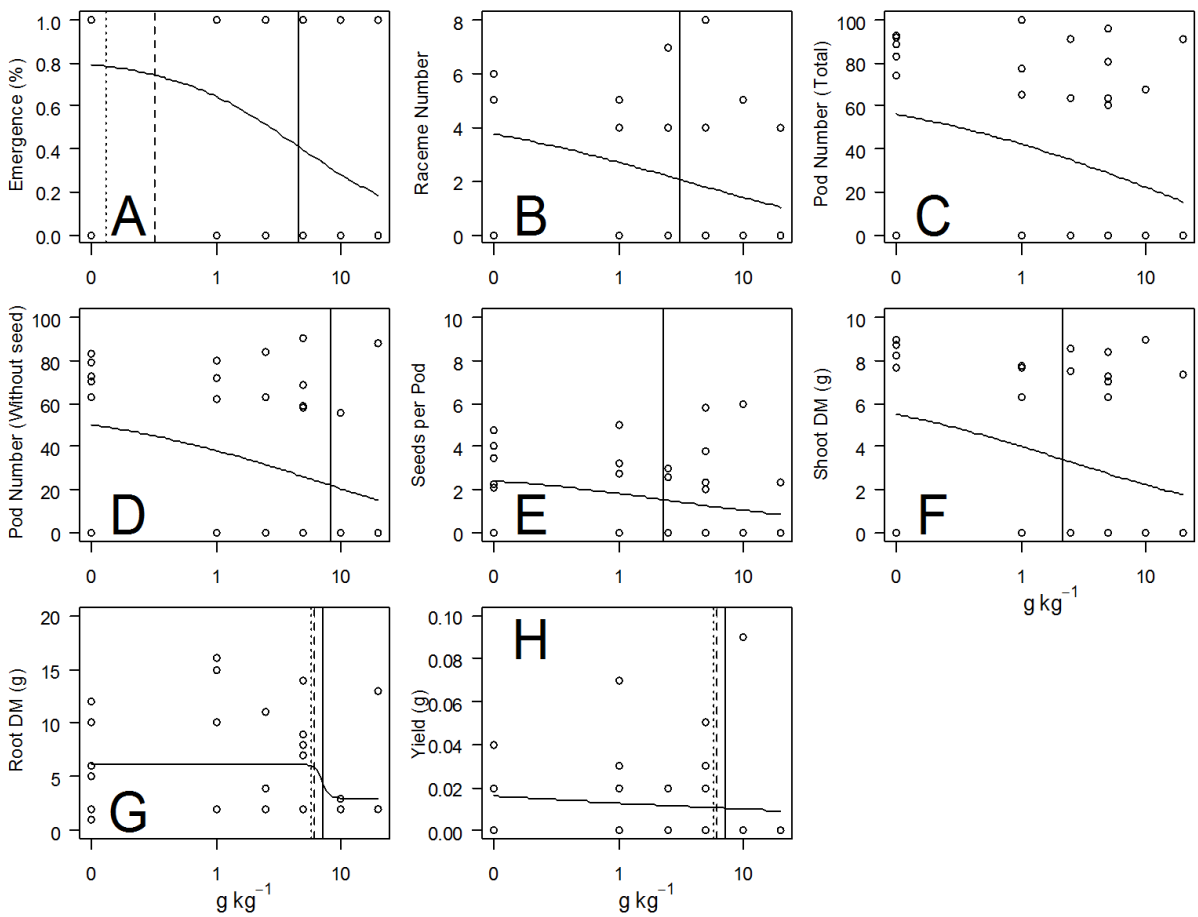


Figure 5.16. Experiment 3: Dose response curves fitted from 4 parameter log-logistic models for multiple response variables at differing doses of *Rhizoctonia solani* AG 2-1 inoculum (g $kg^{-1}$). A, Percentage emergence; B, Raceme number; C, Pod number (total); D, Pod number (Without Seed); E, Seeds per pod; F, Shoot dry matter (g); G, Root dry matter (g); H, Seed yield (g). Vertical lines represent $ED_{05}$ (dotted), $ED_{10}$ (dashed) and $ED_{50}$ (solid) thresholds.

## 5.6 Discussion

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.,* 2009). 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 and caused by *R. solani* 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 AG 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. Whilst Yitbarek *et al.* (1987) found a much larger number of AG 2-2, 3, 4 and 5 in soil cultivated with OSR. Some variation between this study and other studies might be expected as the surveys conducted by Budge *et al.* (2009b) and Brown *et al.* (2014) were based on soil samples, while the survey by Yitbarek *et al.* (1987) included both soil and plant samples. Often soil samples are used to examine the levels of inoculum and the potential for disease prior to planting, or similarly after a crop as an indication of how inoculum levels may have been affected by that crop. However, as many crops are grown in a rotational setting it also contains a wide range of organisms with differing host requirements, virulence levels and dormant or unviable propagules. In addition to this, some propagules may be incapable of causing infection due to spatial separation from plant roots in the soil profile (Gilligan, 1985), with 12-16 mm being suggested as the required zone for infection for *R. solani* infecting radish (*Raphanus sativus*) (Gilligan and Simons, 1987). The work of Yitbarek *et al.* (1987) demonstrates how any of these factors might influence our interpretation of soil samples in isolation as they found six different AG present in soil, but only two to three of these were present on infected seedlings and adult plants. Consequently, sampling plant roots provides a more realistic overview of the pathogens present, negating these issues as those on or within the root system are more likely to be causing disease and impacting on plant health.

Whilst some surveys have been conducted to examine the pathogens present in the soil and roots of OSR, few have used quantitative tools such as real-time PCR to examine inoculum levels. In addition to this, there has been little research examining the link between

inoculum quantity on infection and disease symptoms in OSR, or examining the relationship between disease symptoms, severity or yield loss at different inoculum quantities. In the current study, *R. solani* inoculum densities varied greatly between sites averaging 2.7 pg $ng^{-1}$ total DNA. This quantity was approx. three times higher than that found by Brown *et al.* (2014) (1.08 pg $g^{-1}$ of soil), although no information on the range of inoculum densities across this study was reported. In contrast, the DNA quantities found here and by Brown *et al.* (2014) are much lower than those found by Budge *et al.* (2009b) where a mean figure of 73 ng $g^{-1}$ of soil was recorded, although only a three-fold difference was seen across the sites in their study. 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. The choice of units in the current study 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. In general however, the current findings and those of Budge *et al.* (2009b) and Brown *et al.* (2014) demonstrate the variability seen in populations of *R. solani* AG 2-1 and inoculum levels between field sites.

Traditionally agronomic factors have been thought to play an important role in mediating inoculum survival/quantity. In the current study, no significant link was found between pathogen quantity/occurrence on the roots of OSR and agronomic factors (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) or 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 *R. solani* cells showing a lack of cytoplasm indicating that they were unviable. However, 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, both of which are susceptible hosts for AG 2-1 (Verma, 1996; Tewoldemedhin *et al*., 2006; Sturrock *et al.*, 2016). In addition, the strong saprophytic ability of *R. solani* (Sumner, 1996 in Sneh *et al.*, 1996) may also be reflected in the current findings, with OSR in particular, being a high residue crop.

Glasshouse studies 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 observed here. Some authors have also suggested that soil-borne pathogens exist at subclinical levels in the soil, until a threshold is reached. For example, Johansson et al. (2006) showed how >1 cfu $g^{-1}$ soil was required for *V. longisporum* infection in OSR, despite finding much lower levels in naturally infested fields. In experiments 2 and 3, a threshold effect for clinical disease was undetermined, with visible disease occurring on all but the control treatments. This was similarly supported by the findings of Budge(2007) where all inoculum levels (5-75% w/w) resulted in disease symptoms. Consequently, future research may prove useful to identify this at lower levels than tested here. Whilst a threshold for clinical disease was undetermined, a link between yield loss and a reduction in emergence was seen, with this in turn mediated by the quality and quantity of inoculum as found in other similar studies (Budge, 2007). However, when inoculum production was successful, a strong positive correlation was found between inoculum density and the amount of pathogen DNA per gram of potting mixture, a trend that was not seen by either Budge (2007) or Zhou *et al.* (2016). Similarly, it was also demonstrated how seedling emergence and survival was reduced at inoculum quantities which were lower than those examined previously (Budge, 2007). However this trend was not seen in seed yield as suggested by the results of Zhou *et al.* (2016) were a linear reduction in seed yield and biomass with increasing inoculum of *R. solani* AG 2-1. However due to the choice of units used, direct comparison with the results of this study was 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. However, these recommendations also serve to highlight the risks to growers by assuming a high level of germination and establishment (*c.* 80-90%). The findings here highlight that even small amounts of *R. solani* AG 2-1 inoculum could thus impact on this, causing a significant reduction in seed germination and plant establishment leading to suboptimal plant populations and thus lower yields. Whilst it is also true that OSR is well known for its ability to compensate at low populations (8-10 plants $m^{-2}$) and in some instances is able to yield as well as those at the recommended rate (Mendham *et al.,* 1981; Leach *et al.,* 1999), this is greatly influenced by the growing season and other external pressures such as pest damage or other agronomic influences.

In conclusion, this chapter identifies that *Rhizoctonia solani* is a prominent pathogen within UK crops of OSR, being present at 60% of the 50 sites examined. In addition, the main anastomosis group present was identified as AG 2-1 along with AG 5 and 8 to a lesser

extent, similar to other studies in OSR globally. Glasshouse experiments showed that relatively small quantities of inoculum resulted in poor seedling emergence and establishment, resulting in a reduction in yield. Furthermore, no threshold for disease was observed using log-logistic based models, suggesting that the threshold was below that of the lowest doses used; although this was hindered by the inconsistency of inoculum batches. Whilst these findings prove useful in our understanding of the distribution and occurrence of *R. solani*, and in particular AG 2-1, it may also be limited in its extrapolation to field level due to significant variation in isolates of AG 2-1. Anastomosis group 2 and in turn AG 2-1 is one of the most variable of the 13 recognised AG (Carling *et al*., 2002b), as demonstrated by various serological and DNA based studies (Sweetingham *et al.,* 1986; Liu and Sinclair, 1992). Consequently, chapter 6 will aim to examine the genetic and pathogenic variation within a collection of UK AG 2-1 and other *R. solani* isolates, to better explore their variation in the UK compared to other regions globally.

# 6

# Examining variation in *Rhizoctonia solani* AG 2-1 isolates

## 6.1 introduction

While commonly discussed as an individual species, *Rhizoctonia solani* represents a species complex currently comprised of 13 genetically diverse groups, defined through hyphal fusion, giving rise to their grouping term of anastomosis groups (AG) (Carling, 2002a). Through the use of molecular and serological techniques these AG may be sub-divided with this being most evident within AG 2 which encompasses 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). Methods such as DNA sequencing and isozyme patterns have also indicated that further variation exists within these sub-groupings (Woodhall *et al*., 2007; Stodart *et al.*, 2007; Carling *et al.*, 2002b; González et al., 2001; Salazar *et al.,* 1999).

Pectin isozyme patterns (zymograms) were one of the earliest techniques used to measure variation within pathogen species (Sweetingham *et al.,* 1986; MacNish *et al*., 1994; Stodart *et al*., 2007). Zymograms are derived from the electrophoresis patterns of different polygalacturonases, pectinesterases and pectolyases produced by individual pathogen isolates, allowing them to be grouped into zymogram groups (ZG). The introduction of this system enabled greater resolution in studies on *R. solani* populations. For example, Sweetingham *et al.* (1986) described 11 ZG associated within bare patch disease of wheat in Australia, with further research suggesting some of these represented further subgroupings within AG such as AG 8 (ZG 1-1, 1-2, 1-3 and 1-4) (MacNish and Sweetingham, 1993) and AG 2-1 (ZG 5 and 6) (Schneider *et al*., 1997; Stodart *et al*., 2007). Consequently, ZG are regarded as a useful tool for defining differences within *R. solani* AGs (Schneider et al., 1997; Cuberta and Vilgalys, 1997).

Molecular methods have concentrated on the use of non-coding regions of ribosomal DNA (rDNA) (Liu and Sinclair, 1992; Salazar et al., 1999, Woodhall *et al*., 2007; Woodhall, 2004), randomly amplified polymorphic DNA (RAPD) or terminal restriction fragment length polymorphisms (T-RFLP) (Duncan *et al.,* 1993) to elucidate the genetic relatedness of the main AG and their sub-groups. However, studies conducted in recent years have employed

DNA sequencing using widely accepted DNA barcode regions as opposed to RAPD and T-RFLP. The most common of these regions has been the internal transcribed spacer regions (ITS 1 and 2), located between the ribosomal small sub unit (SSU, 18S) and large sub unit (LSU, 28S). This region exhibits good amplification across a wide range of fungi, whilst being variable enough to denote species or lower and has thus been proposed as the international barcode region for fungi (Schoch *et al*., 2012). Other regions used have also included the LSU, and the intergenic spacers (IGS) located between the repeat units of the LSU and SSU (Woodhall, 2004). Some coding regions have also been utilised such as β-tubulin and elongation factor 1-*α* (EF1-*α*) (Budge *et al*., 2007), although these showed poor resolution. Utilising the ITS1 region, Budge *et al*. (2007) was able to separate 102 isolates of AG 2 into three distinct clades related to their subgroupings of AG 2-1, 2-2 and 2-3. Furthermore, it was also possible to further classify these into sub-clades with AG 2-1 separating into three further groups, although the usefulness of this maybe questionable as no differences in pathogenicity was seen between these groups. Similarly, Liu and Sinclair (1992) described two variations in AG 2-1 within their study, whilst Salazar *et al.* (1999) showed that there was differing levels of homology within AG 2-1 ITS1 sequences.

### 6.2 Objectives

To observe for differences in growth, morphology, pathogenicity and genetic variability within several *Rhizoctonia solani* isolates and in particular AG 2-1.

### 6.3 Null Hypothesis

No differences are observed in the growth, morphology, pathogenicity or genetic variation within several *Rhizoctonia solani* isolates including AG 2-1.

## 6.4 Materials and methods

### 6.4.1 Virulence

The virulence assay was designed by adapting methodology 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) was 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 repeated six times for each isolate plus an uninoculated control. At the same time, a single 5 mm agar core from each isolate (Table 6.1) 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 before being placed into a Sanyo MLR-350 (Panasonic Biomedical, Europe) and incubated in the dark at 20°C.

After 5 days the tubes were assessed with individual seedlings 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. The resultant colonies were then inspected under a stereomicroscope for *Rhizoctonia* features, such as straight hyphae with perpendicular hyphal branches with septa, in order to satisfy Koch's postulates.

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

Table 6.1. Isolates collected from other UK institutions and laboratories. Isolate sources: 1, Harper Adams University; 2, Science and Advice for Scottish Agriculture; 3, the University of Warwick; 4, The Food and Environment Research Agency; 5, Arysta Life Science, UK; 6, the University of Nottingham. *Denotes isolates of unproven AG.

| HAU Code | Supplier Code | Source | Anastomosis Group | Plant isolated from |
|---|---|---|---|---|
| R3 | None | 2 | 3 PT* | Unknown |
| R5 | 1832 | 4 | 3 PT | Unknown |
| R6 | 1938 | 4 | 4 HGII | *Iris germanica* |
| R7 | 1835 | 4 | 4 | Unknown |
| R8 | 1986 | 4 | *R. zea* | Unknown |
| R9 | 1947 | 4 | 8 | *Brassica oleracea* var. *botrytis* |
| R10 | 1841 | 4 | 8 | *Triticum aestivum* |
| R11 | 1846 | 4 | 11 | Unknown |
| R12 | 1843 | 4 | 9 | Unknown |
| R13 | 1971 | 4 | 2-1 | *Brassica oleracea* var. *botrytis* |
| R14 | Pl240 | 5 | 2-1 | Unknown |
| R15 | Pl245 | 5 | 4* | Unknown |
| R17 | 1977 | 4 | 2-1 | *Brassica oleracea* var. *germmifera* |
| R18 | 1955 | 4 | 2-1 | *Brassica oleracea* var. *botrytis* |
| R19 | 1942 | 6 | 2-1 | Unknown |
| R20 | 1926/2332 | 6 | 2-1 | Unknown |
| R21 | 1917 | 6 | 2-1 | Unknown |
| R22 | 1935 | 6 | 2-1 | Unknown |
| R23 | 1934 | 6 | 2-1 | Unknown |
| R24 | 1933 | 6 | 2-1 | Unknown |
| R25 | 1927/2023 | 6 | 2-1 | Unknown |

### 6.4.3 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) of 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 $\mu l^{-1}$.

*ITS and IGS PCR*

To examine the genetic variation between different AG and *R. solani* isolates two phylogenetic regions were chosen to study differences in DNA sequences and sequence lengths. The internal transcribed spacer region was amplified using the primer pair ITS 4 and 5 (White *et al.*, 1990), amplifying the ITS 1 and 2 regions in a single amplicon (Table 6.2). In addition, to this the inter genic spacer (IGS) region was also studied by amplifying with the forward primer (LR12R) of Harrington and Wingfield (1995) and the reverse primer of Woodhall *et al.* (2007) (Table 6.2). These primers anneal to the large sub unit (LSU, 28S) and the 5S spacer unit, thus amplifying the whole IGS1 region.

PCR reactions were setup in a total volume of 25 µl comprising of; 1x PCR Buffer (Invitrogen, Paisley), 0.5 U Taq polymerase (Invitrogen, Paisley), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 nM of each primer and a 5 µl of sample DNA. PCR conditions comprised of; 94°C for 75 s, and 40 cycles of 94°C for 15 s, annealing temperature for 15s and 72°C for 45 s, with a final extension of 72°C for 4 min 15 s. Annealing temperatures were 50°C for ITS and 60°C for IGS. PCR products were visualised under UV light on a 2% agarose gel stained with GelRed™ Stain (Biotium, Cambridge).

Table 6.1. Table of conventional PCR primers used to examine variation in *Rhizoctonia solani*.

| Primer/Probe | Sequence (5'→3') | Source |
|---|---|---|
| ITS | | |
| ITS 4 | TCC TCC GCT TTA TTG ATA TGC | White *et al*. (1990) |
| ITS 5 | GGA AGT AAA AGT CGT AAC AAG G | |
| *IGS* | | |
| LR12R | GAA CGC CTC TAA GTC AGA ATC C | Harrington and Wingfield (1995) |
| Seq5S | CAG ATC AGA CGG GAT GCG GT | Woodhall *et al*. (2007) |

## 6.5 Results

### 6.5.1 Virulence

Disease symptoms were evident on the majority of seedlings after 5 days post infection (dpi) (Fig 6.1), with scores ranging across the whole severity scale. Using the DSI a score of ≤ 20 was considered non-aggresive, 21 – 60; moderately aggresive and >61 highly aggressive. Based on this system isolates represented; *R. zeae*, AG 3PT, AG 9 and AG 4HGII were shown to be weakly pathogenic to young OSR seedlings, whilst the majority of isolates representing AG 2-1 were pathogenic.

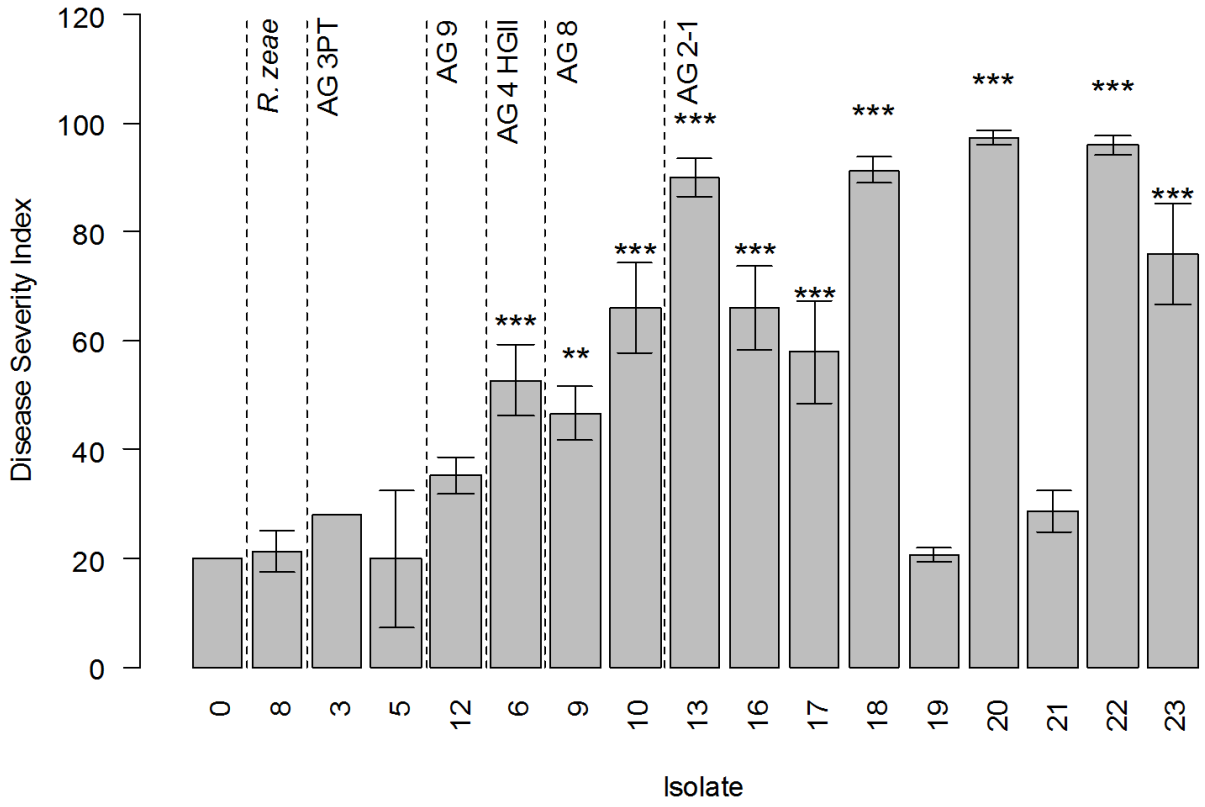


Figure 6.1. Disease Severity Index (DSI) of several *Rhizoctonia* isolates when tested on five-day-old oilseed rape seedlings. Error bars represent standard error of the mean. Means which were significantly different from the uninoculated control (0) are represented by; ”*”, p<0.05; “**”, p<0.01, “***”, p<0.001.

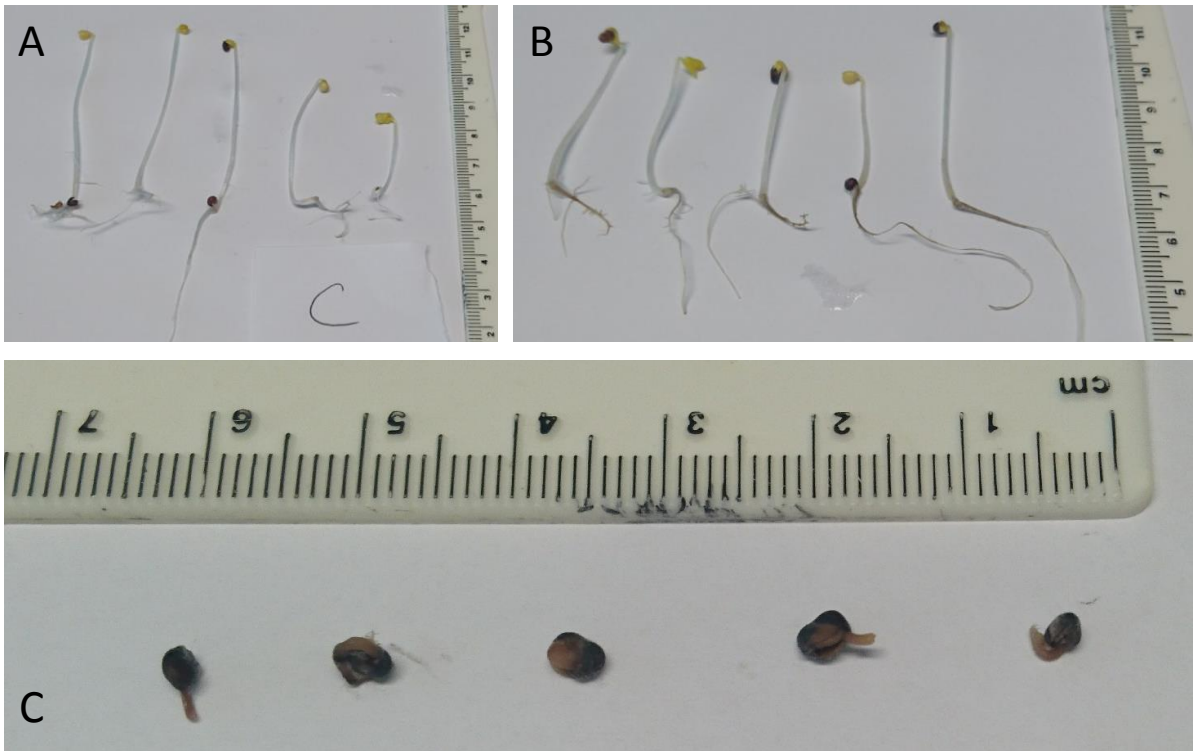


Figure 6.2. Five day-old seedlings taken from the virulence test. A, Control; B, AG 2-1 (R19); C, AG 2-1 (R20).

### 6.5.2 ITS and IGS amplification

The ITS region was amplified for most of the isolates available (Fig 6.3) with gel electrophoresis showing isolates had a similar product length of *c.*750 bp. The IGS region showed more variation than that of ITS, with product lengths varying between *c.* 600 – 1k bp (Fig 6.4).

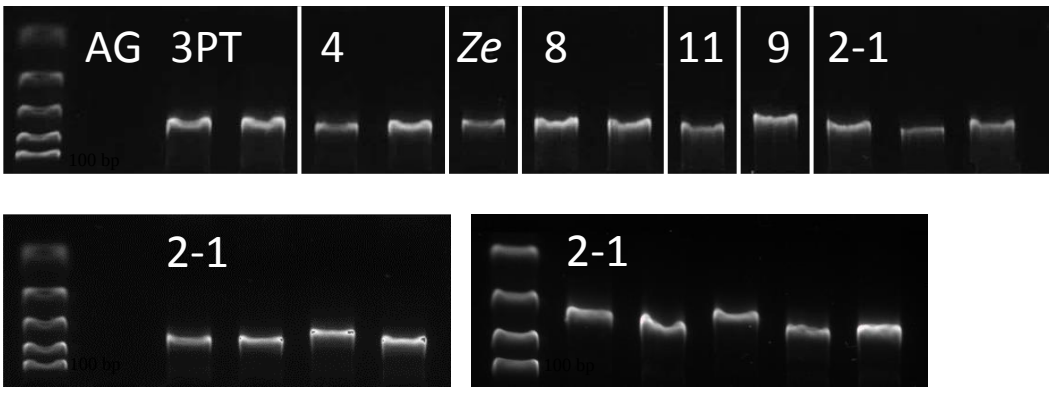


Figure 6.3. Gel electrophoresis of the ITS1-5.8S-ITS2 amplicons of several *Rhizoctonia solani* isolates. Text denotes the anastomosis group of those isolates, with exception of *Ze* which corresponds to the species *Rhizoctonia zeae*. Top; ladder, R3, R5 – R15. Bottom; ladder, R17 – R20, ladder, R21 - 25. Ladder (T-B); 100, 200, 500, 1k and 2k bp.

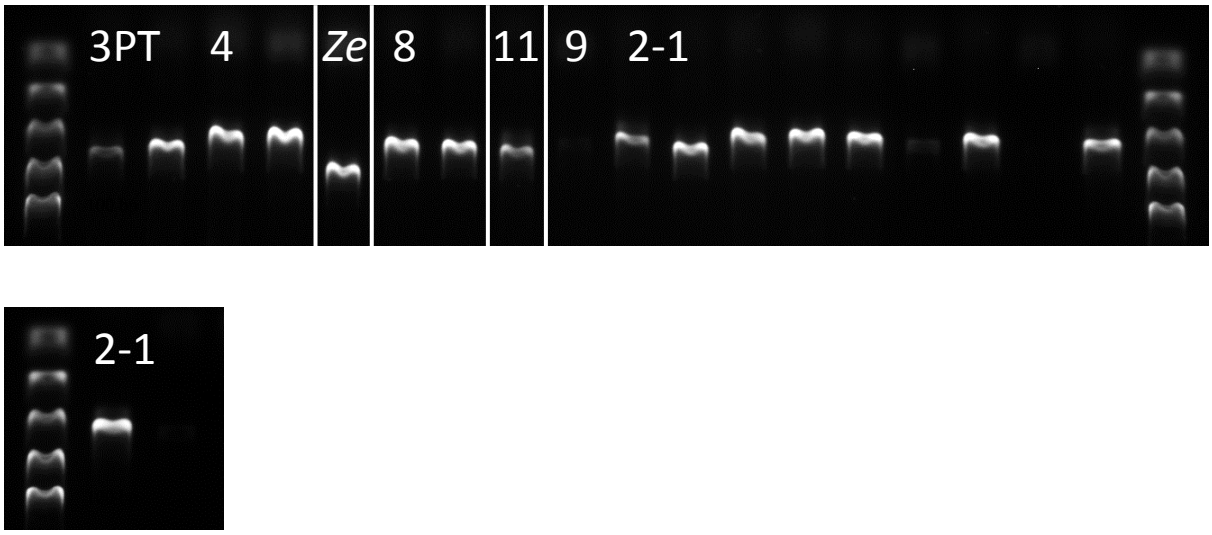


Figure 6.4. Gel electrophoresis of the IGS amplicons of several *Rhizoctonia solani* isolates. Text denotes the anastomosis group of those isolates, with exception of *Ze* which corresponds to the species *Rhizoctonia zeae*. Top; ladder, R3, R5 – R15, R17-R22. Bottom; R23, R25. Ladder (T-B); 100, 200, 500, 1k and 2k bp.

## 6.6 Discussion

Whilst variation in individual *R. solani* AG can occur both within and between field sites, there is also variation within many AG and in particular AG 2. This is the most diverse AG within *R. solani* 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 (Salazar *et al.,* 1999; González et al., 2001; Carling *et al.*, 2002b; Stodart *et al.*, 2005; Woodhall *et al*., 2007), although how useful this maybe is debatable.

In the current chapter the aim was to utilise both molecular and enzymatic techniques to examine any differences between 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). Verma (1996) found that AG 2-1 was the main cause of damping-off and brown girdling root rot in canola grown in Canada and the United States, whilst AG 4 was more prevalent on adult plants resulting in basal rots. They also found that AG 2-1 infection was favoured by cool moist conditions at planting, a result which was supported by Tewoldemehin *et al.* (2006) in their work. Similarly, Khangura *et al.* (1999) found AG 2-1 to be prevalent in Western Australian crops of canola, with isolates proving to be high pathogenic causing pre- and post-emergence damping off. Other isolates, such as AG 8, led to little disease on canola, but were able to infect a wide range of rotational hosts such as lupin and wheat. In comparison, Tewoldemehin *et al.* (2006) found AG 4 HGII (69%) was the most prevalent across their cropping systems in South Africa, with AG 2-1 (19%) being present to a lesser extent. This was thought to be linked to the wide host range of AG 4 HGII which was found to be virulent on a wide range of crops including; canola, medic, lupin, lucerne and barley, whilst AG 2-1 was highly virulent to canola and other crops to a much lesser extent. Utilising *in vitro* methods Babiker *et al.* (2013) found that many different genotypes of *B. napus* and other *Brassica* spp. were highly susceptible to damping-off caused by AG 2-1, with some showing moderate to total resistance to infection by AG 8, 10 and binucleate *Rhizoctonia* spp. Consequently, the pathogenicity testing reported here supports the findings of these studies with AG 2-1 isolates largely proving to be the most aggressive followed by 8 and 4HGII, whilst AG 9, 3PT and *R. zeae* showed a lower level of aggression largely causing no more than a slight discolouration of the hypocotyl and root. However, within the collection of AG 2-1 isolates a wide range of aggressiveness 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 these isolates were not viable, subsequent

isolations from the same stock culture showed successful growth and plate colonisation. In a similar manner, a lack of growth may also have be attributed to this; 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 to pathogenicity testing, molecular and enzymatic techniques were used to further examine the variation within the collection of isolates and in particular within those belonging to AG 2-1. However, the number of isolates tested along with the lack of DNA sequencing and phylogenetic inference, and successful pectin zymograms (isozymes) limits the scope of this chapter. Initially the aim was to use isozyme patterns along with DNA sequencing and phylogenetic analysis to examine the variation within the set of UK isolates, as suggested by other researchers (Budge *et al*., 2007). Following the methods of Sweetingham *et al.* (1986) and Stodart *et al*. (2007), no isozyme patterns could be discerned from the polyacrylamide-pectin gels. This may be attributed to a number of discrepancies between the methods described and those used, as a consequence of differences in equipment available. For example, Stodart *et al*. (2007) performed electrophoresis at 250 V for 4 hrs utilising cooling equipment to maintain the equipment at 5°C. In comparison, the current study did not have access to such equipment, and as a result electrophoresis was conducted at 100 V for 6 hrs, with frozen icepacks used to chill the buffer tank. However, despite this the temperature of the buffer tank was not maintained consistently, reaching above 50°C on several occasions leading to partial evaporation of the buffer. Consequently, the heating may also have detrimentally affected the pectinase enzymes, which are required to be active post electrophoresis in order to produce bands. Whilst the partial evaporation of the buffer, and subsequent addition of new buffer may have altered the composition of the solution, similarly affecting enzymatic activity post electrophoresis.

Following the use of pectin zymograms, molecular techniques and in particular examination of the ITS and IGS regions of rDNA was used to discern differences between isolates as had been previously described (Budge *et al*., 2007; Woodhall *et al*., 2007). End point PCR was used to amplify the ITS and IGS regions of each isolate, whilst gel electrophoresis was used to visualise product size. When examined the ITS region produced an amplicon of *c*. 750 bp in size, although slight variation in size occurred across the AG 2-1 isolates, with this being consistent with Budge *et al.* (2007). In comparison, the IGS region showed increased variation producing amplicons of 600 – 1K bp in size, something which was witnessed by Woodhall *et al*. (2007) although their AG 2-1 amplicons varied into three shorter groups; 510, 550 and 570 bp in length. In addition to this, variation in length was also shown to correlate with isolate from different sources of inoculum, such as sclerotia and stem or stolon cankers. Similar to both Budge *et al*. (2007) and Woodhall *et al*. (2007)

the aim of the current study was to proceed with DNA sequencing using the sanger di-deoxy method, before using phylogenetic methods to elucidate relatedness between sequences and thus isolates. However, due to the time constraints of the project this was not possible, and consequently full examination of the isolates genetic variation and comparison with other studies is limited. However, based on amplicon sizes, as visualised by gel electrophoresis, and the findings of other researchers it is likely that similar variation exists in the isolate collection. Thus in combination with the results of the pathogenicity assay, it is not unrealistic to assume that a diverse number of AG 2-1 isolates exist within OSR. Consequently, this may impact on the findings of the previous chapters, in that qPCR is unable to separate pathogenic and non-pathogenic isolates. Based on these findings, this chapter aims to suggest that whilst variation does exist in the aggressiveness and genetic make up of AG2-1 this may have limited impact on field isolates in the UK, which were generally highly pathogenic towards young OSR seedlings.

# 7

# General Discussion

Oilseed rape has has been transformed from a minor crop to the most popular break crop in UK cereal rotations as a consequence of production changes within the last 60 years or so. This rise in popularity has largely been driven by market and technological factors, as growers became more specialised moving away from mixed enterprises to purely arable/combinable crops. At the same time, improved techniques enabled fast and cost effective establishment, whilst an increasing demand for home produced vegetable oils and protein sources have provided growers with a lucrative break crop. However, within recent years the popularity of OSR has come under threat with stagnating yields, lower prices and regulatory changes around pesticide usage. The combination of these factors has caused many growers to re-examine their cropping systems and reduce the amount of OSR grown. This trend towards lower production of OSR can be seen in the market information attained by DEFRA, whereby the area committed to OSR production has fallen from its peak of 700,000 ha in 2012 to *c.* 510,000 ha currently (October, 2017). Similarly, the value of the crop has also decreased falling from £387 $t^{-1}$ in 2012 to £272 $t^{-1}$ in 2015, with current prices (May, 2018) remaining close to this level at £285 $t^{-1}$. Combined with stagnant yields and increasing production pressures from the restricted use of pesticides such as neonicotinoids, grower confidence in the crop has reduced as has and its role as the most popular cereal break crop. In order for this trend to be reversed, then significant improvements in the future production and profitability of OSR are required, principly amongst which is the improvement of crop yields to counteract decreased prices and yield losses caused by increased pest pressure as a result of pesticide losses. Consequently, this thesis aimed to establish how soil-borne pathogens maybe implicated in stagnating or declining yields, along with other agronomic factors which may influence their occurrence and survival in commercial crops. In this situation, the loss in productivity can be related to the term 'yield decline', whereby the loss in yield occurs compared to the potential of current biological and technological maxima. In the case of UK OSR this is thought to currently be 6.5 t $ha^{-1}$ (Berry and Spink, 2006) with modern germplasm and production techniques. However, a review by Berry and Spink (2006) also established how optimum crop characteristics are already achievable, with the hindrance being to combine these in a single

crop and in balance with wider inputs and techniques. In particular they highlight the role of rotational frequency, establishment techniques, and fertiliser and pesticide regimes in holding back the potential of on farm yield increases. Other studies have similarly supported this view with many implicating rotational frequency as a key factor (Sieling and Christen, 1997; Stobart *et al.*, 2012; Hilton *et al.*, 2013), which is especially pertinent as this may also impact on other factors of concern such as soil fertility and pests and diseases which affect crop establishment. Whilst many of these topics have been discussed before, limited information has been established so far on the relationships between soil-borne pathogens, rotational frequency and yield decline in OSR (Sieling and Christen, 1997; Stobart *et al.*, 2012; Hilton *et al*., 2013). Consequently, the first aim of this work was to establish the likely pathogen 'candidates' in commercial crops, before elucidating their link to agronomic factors and yield decline.

In chapter 3, a list of candidate pathogens including; *Plasmodiophora Brassicae*, *Pythium ultimum*, *Rhizoctonia solani*, *Gibellulopsis nigrescens*, *Verticillium longisporum* and *Sclerotinia sclerotiorum* were selected based upon findings of a literature search. The candidate list was further refined by selecting those with limited UK distribution data or knowledge gaps. For example, the distribution and effect on plant yield is already well documented for *V. longisporum*, *S. sclerotiorum* and *P. Brassicae* (Gladders *et al*., 2007; Burnett *et al.*, 2013). As a result of this *R. solani*, *P. ultimum* and *G. nigrescens* became the research focus in an attempt to identify their distribution and contribution to yield decline in OSR.

Molecular methods have become the 'gold standard' tool for plant pathologists and diagnosticians in the detection and diagnosis of plant disease in environmental samples. Through the development of specific, sensitive and high throughput methods such as real-time PCR a large number of samples may be examined in a short period of time. These techniques also negate the need for complex isolation techniques and specialist skills in taxonomic identification. As such, real-time PCR was considered the tool of choice, being a readily available laboratory technique, supported by an ever-increasing number of published diagnostic assays for phytopathogens. However, it was apparent that not all of the target pathogens had published assays available, and of those that did, many were not immediately suitable for use. For example, little information is available for *G. nigrescens* with reports suggesting it occurs only as a minor pathogen, with little research conducted thus far and no molecular tools developed. In contrast, a large number of *R. solani* assays were available with some capable of amplifying specific anastomosis groups. Two assays were available for *P. ultimum*; one used for detection in field soil (Schroeder *et al.*, 2006) and another for storage rots in potato tubers (Cullen *et al.*, 2007).

For example, little information is available for *G. nigrescens* with reports suggesting it occurs only as a minor pathogen, with little research conducted thus far and no molecular tools developed. In contrast, there was a large number of *R. solani* assays available with this linked to the different 'strains' or anastomosis groups present. Whilst two assay were found for *P. ultimum*, one used for detection in field soil (Schroeder *et al.*, 2006) and a second for storage rots in potato tubers (Cullen *et al.*, 2007).

Due to the shortage of time, and a lack of UK isolates for *G. nigrescens* and related *Verticillium* spp., no attempt was made to design and validate a qPCR assay for this pathogen. In addition, a review of the literature suggested that this species was a minor pathogen, which was mostly saprophytic/opportunistic in nature (Vesper *et al.*, 1983; Zare *et al*., 2007). In contrast, a greater number of assays were available for *R. solani* AG s (Lees *et al.*, 2002; Woodhall *et al.*, 2013) due to its widespread occurrence and known pathogenicity to a range of crop species. For instance, Budge *et al.* (2009b) designed and validated 11 different qPCR assays for the detection of 11 of the 13 currently known AG within field soil. However, from the literature it was apparent that only a few of the known *R. solani* AGs were pathogenic towards *Brassica* spp. and would occur within the climatic conditions of the UK, with AG 2-1 being the most likely. In this respect, the AG 2-1 TaqMan™ assay developed by Budge *et al.* (2009b) was selected. Whilst other assays were available for the detection of *R. solani* AG 2-1, many were regarded as being problematic. For example, the assay designed by Okubara *et al.* (2008) was reported to lack specificity, detecting AG 2-1, 8 and 10 in root and soil samples. Zhou *et al.* (2014) claimed to have produced the most sensitive qPCR for detecting AG 2-1, but failed to consider that developed by Budge *et al.* (2009b) or to compare the two assays. In addition, the assay of Budge *et al.* (2009b) is well validated, widely utilised and trusted, being the assay of choice for studies originating from the former UK Food and Environment Research Agency (FERA, York). The current project also found that this assay was reliable, whereby only AG 2-1 was amplified from a range of non-target *R. solani* AG and other soil-borne pathogens, whilst consistent amplification occurred across six orders of DNA quantity, with all of this occurring on the first attempt.

Unfortunately, the assay for *P. ultimum* detection was problematic. The assay of Schroeder *et al.* (2006) was selected in preference to that of Cullen *et al.* (2007) due to its validation for use in soil samples, showing good amplification across a range of DNA concentrations. However, the assay failed to amplify DNA from the pure cultures of *P. ultimum* attained, despite method refinements and iterations. These included using different methods of liquid and solid culturing media; with the hyphal mat found to collapse on solid media within a few days. UV-Vis spectrometry suggested that the extract contained DNA but of low yield and

quality, with this similarly supported by the lack of amplification using universal primers (White *et al.*, 1990). Consequently, improvements to culturing methods and DNA extraction procedures to favour oomycetes, organisms not routinely studied in at Harper Adams University, should be considered in the future.

Although qPCR provided a useful tool to explore the occurrence and quantity of individual pathogens, it also restricted the scope of the work. Many of the candidate pathogens were based on previous reports in the literature, which may inadvertently be biased by 'traditional' and easily identified or cultured pathogens. As a result, minor, previously unknown or 'cryptic' species such as obligate biotrophs would not be fully accounted for, as found by Hilton *et al.* (2013) in their finding of *Olpidium brassicae* and an unknown *Pyrenochaeta* sp. in UK OSR crops for the first time. Similarly, qPCR is also a discrete technique on its own, examining one component of a much greater picture. Whilst this is not an issue with samples which harbour a relatively simple community, it does not allow for the exploration of whole communities of organisms, or more importantly their interactions, as occurs in complex environments such as soil and the rhizosphere. Previously this area was difficult to study directly, but with the advent of high throughput sequencing techniques and metagenomics it has become a less laborious and more informative field of study.

In chapter 4, an Illumina MiSeq HTS platform was utilised to study the fungal communities associated with the roots of commercial OSR crops. From this it was found that fungal communities showed limited variation between field sites and agronomic factors. In addition, these communities were largely comprised of saprophytes and generalists, although the pathogenic species *Rhizoctonia solani* was also prevalent at many sites. Whilst the survey samples allowed the root and in particular the rhizosphere microbiome to be examined, they restricted the potential outcomes as observation of the surrounding soil was not possible. This was one of the many limitations of using samples collected from another study, which were not initially intended for this purpose. Sampling soil and roots separately may have provided a greater insight not just of each community but also their movement from soil to the rhizosphere, and how this may have influenced agronomic factors. In addition, multiple sampling times would have allowed for any temporal differences in community composition to be observed and how this changes during crop growth within a rotational setting. This is particularly important from the point of bulk soil, which by its very nature is species rich but biomass poor, primarily through a lack of nutrient sources. However, when a plant root enters into this system, it changes the environment, altering the communities from which a plant can 'select' specific species, either by design as with symbionts or by coincidence as with pathogenic species (Berendsen *et al*., 2014). Known as the 'rhizosphere effect', it is a

complex driving force and can have profound impacts on plant health and productivity. The results reported within this thesis support this view of roots recruiting selected species from those of bulk soil; with the individual fungal communities showing a high level of similarity across sites, despite the wide range of environmental and agronomic factors associated with the samples. The choice to use two bioinformatic pipelines was driven by the complex and fast-paced improvement in these techniques within recent years, which pertain to constantly advance and improve community description and composition. However, the results generated here also highlight how this may not always be true, with some methods generating poorer quality datasets through overzealous selection criteria. This served two purposes; 1. To highlight the importance of clear, simple and understandable bioinformatics programmes and coding and; 2. To emphasise the importance of comparing methods to ensure that newer techniques offer improvements over those which came before. These points in particular were considered important for plant scientists who are often unfamiliar or not trained in bioinformatics, ecology, molecular biology and computational methods. Whilst direct comparison between the two pipelines used here is difficult, they did highlight the limited role of agronomic practices in altering community composition, and also identified several common fungal species, one of which (*R. solani*) is a known OSR pathogen.

Chapter 5, served to further investigate the results generated from HTS in the previous chapter, and to build upon the initial *Rhizoctonia solani* data generated in chapter 3. *Rhizoctonia solani* is a species complex comprised of 13 different individuals, which cause a variety of diseases on a range of plant species. Chapter 3, highlighted the occurrence of *R. solani* AG 2-1, but other AGs such as 2-2, 4 HGII, 5 and 8 are known to occur in other *Brassica* spp. and OSR growing areas. Results from chapter 5 highlighted that AG 2-1 (60%) was the dominant AG, followed by 8 (4%) and 5 (2%), with this being the first time such a wide scale characterisation of this pathogen has been conducted in a UK or even a European crop of OSR. Whilst some widespread soil surveys e.g. such as that by Goll *et al*. (2014) serve to highlight the 'potential' *R. solani* AGs found within European soils, the results here demonstrate that AG 2-1 is the most widespread within UK OSR, and thus potentially other European regions.

Experiments conducted within chapter 5 aimed to examine the effect of differing inoculum doses of *R. solani* AG 2-1 on plant growth, health and yield. Such assessments are often not considered, and as such limited information exists as regards to pathogen 'thresholds' in causing physical disease symptoms and economic yield loss. In particular, the aim was to determine whether lower degrees of infection, which were potentially non-symptomatic, affected plant health and yield. During the process of conducting three glasshouse

experiments, it was apparent that inoculum production and method standardisation was challenging, as reported in similar studies with *R. solani* (Budge, 2007). For example, high inoculum colonisation resulted in significant seedling death and thus difficulty in assessing yield in Experiment 2. Whilst the inoculum in Experiments 1 and 3 had lower colonisation, and therefore, no disease symptoms or yield reduction were observed. These experiments suggested that subclinical levels of infection, were unlikely to exist at the range of inoculum doses tested. However, when inoculum colonised to a suitable level, relatively little was required to cause pre- or post-emergence damping off. Pre- and post-emergence damping off also appeared to be the main cause of yield loss, with no changes in other yield parameters such as plant biomass, flowering date or seed parameters, as has been seen for other pathogens. Taken in the context of OSR cultivation, whereby lower seed rates, additional pest damage and suboptimal establishment techniques are also common, this forms part of a larger contribution to yield loss through poor plant emergence and stand evenness, which can also result in other problems later in the production cycle.

The final chapter aimed to examine the variability in UK isolates of AG 2-1, and in particular the pathogenic and genetic variation, which may further impact upon the occurrence and severity of disease symptoms in the field. Previously, other studies have shown AG 2 to be one of the most diverse AG of *R. solani*, showing genetic variation through enzymatic production/patterns and DNA sequencing. However, it is unknown how significant this variation is to the pathogenicity under field conditions. Within this chapter, AG 2-1 was shown to be the most pathogenic towards OSR seedlings, followed by AG 8 and 4. Similarly, within the isolates of AG 2-1 variation between highly pathogenic and non-pathogenic isolates occurred. However, further work is required as this assay used a relatively small number of isolates. Similarly, little variation as witnessed in ITS and IGS length between isolates of AG 2-1, although this is not representative of sequence differences. In addition, other phylogenetic regions such as *tub2*, *tef1* and *rpb2* may offer better phylogenetic resolution.

In conclusion, the results of this study have suggested that the root systems of commercial OSR crops in the UK contain a diverse range of fungal species, many are non-pathogenic saprophytes or generalists although a small proportion of the fungi detected are known plant pathogens. One such pathogen *R. solani* was particularly common amongst samples being an important pathogen on a range of crops including *Brassica* spp. The use of DNA based diagnostic methods confirmed the widespread nature of *R. solani* AG 2-1, a known OSR pathogen, in OSR crops grown under different agronomic regimes and environmental conditions. Glasshouse and laboratory studies demonstrated that *R. solani* AG 2-1 was highly pathogenic causing pre- and post-emergence damping off, which may cause

reduced plant stands and poor yield production; although a small number of weak and non-pathogenic isolates was also seen. This suggested that whilst the majority of UK fields sites are infected by virulent and pathogenic isolates of AG 2-1, there are also non-pathogenic isolates present and that this should be considered when interpreting DNA based survey data, where this information is not inferred.

# Future perspectives/recommendations

Based on the findings described in this thesis a number of areas have been identified as gaps or under examined areas of current knowledge surrounding soil-borne pathogens of OSR, and the use of DNA based techniques to examine environmental samples.

**Molecular diagnostics and pathogen viability**

Both PCR and qPCR have led to improved detection and management of plant pathogens, through being able to characterise their occurrence in environmental samples such as soil. However, these techniques are based on the detection of pathogen DNA which may persist in such environments after infective propagules have become unviable, deteriorated or broken down. Consequently, this may lead to an inflation of the pathogen risk as detected by DNA based PCR methods. Thus methods which utilise robust but ephemeral molecules might be more suited to perform combined detection and viability of pathogens and their propagules in environmental samples.

***Rhizoctonia solani* distribution and management**

Whilst the current study has outlined the occurrence and effects of *R. solani*, and in particular AG 2-1 in UK OSR crops additional knowledge is required to form effective management strategies. As a soil-borne pathogen, *R. solani* often occurs as patches wihin fields and as such additional information on patch formation and spread within fields is required. This might then lead to more targeted management practices as seen in other 'patchy' organisms such as some weeds and foliar pathogens. In addition, more information surrounding management practices is also required, such as the examination of varietal resistance, biological and synthetic seed or soil-applied fungicidal treatments and other cultural methods which promote crop health and growth.

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# APPENDICES

## 9.1 General Information

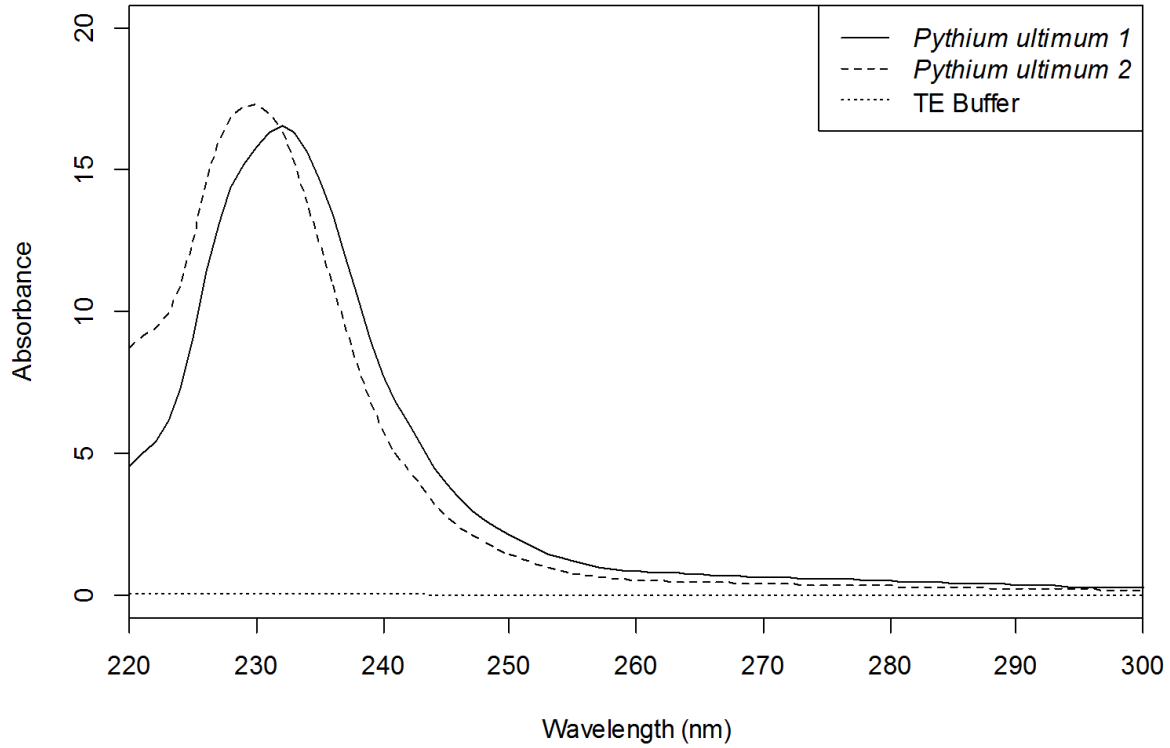


Figure A1. UV-Vis absorbance of two DNA extracts (*Pythium ultimum* 1 and 2) and TE Buffer, as recorded using a NanoDrop 200c.

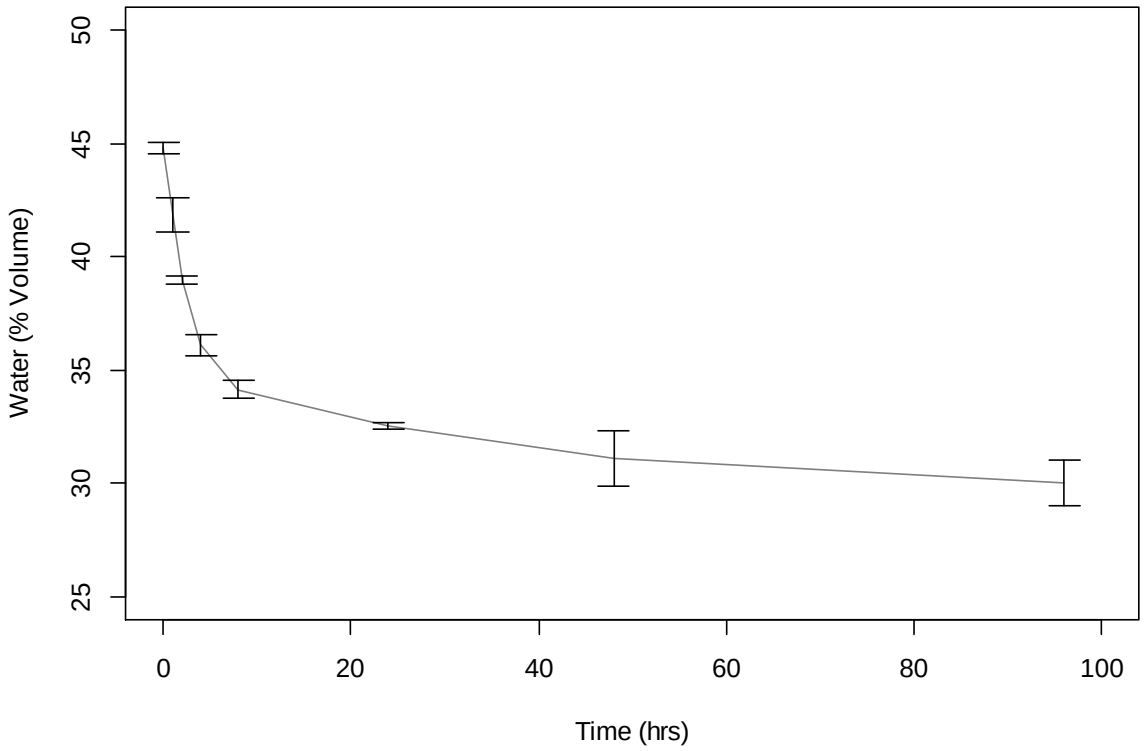


Figure A2. Soil-moisture curve for the John Innes No. 2 and sand-maize meal mix, as used in glasshouse experiments.

### 9.2 Tables

Table A1. Table of agronomic and environmental explanatory variables associated with the individual survey samples (n=100). Key: *Denotes missing information. Region: N, North; S, South; E, East; W, West. Establishment method: P, Plough; SNI, Shallow Non Inversion; DNI, Deep Non Inversion; DD, Direct Drilled. Previous crop: WW, Winter Wheat; WB, Winter Barley; SB, Spring Barley; F, Fallow. Soil type: Calc., Calcareous.

| Sample No. | Year | Region | Variety | Establishment method | Years previous OSR | Previous crop | Soil Type |
|---|---|---|---|---|---|---|---|
| 102 | 2012 | S | CASTILLE | P | 6 | WW | LIGHT |
| 101 | 2012 | W | CASTILLE | SNI | 2 | WW | MEDIUM |
| 99 | 2011 | E | CABERNET | DNI | 2 | WW | MEDIUM |
| 95 | 2011 | S | CABERNET | DNI | 4 | SW | MEDIUM |
| 94 | 2011 | N | CASTILLE | P | 5 | WB | MEDIUM |
| 92 | 2011 | N | * | SNI | 1 | WW | HEAVY |
| 91 | 2011 | E | CABERNET | P | 20 | SB | MEDIUM |
| 90 | 2011 | S | CASTILLE | P | 3 | WW | MEDIUM |
| 89 | 2011 | E | CABERNET | SNI | 7 | SB | MEDIUM |
| 88 | 2011 | E | VISION | SNI | 5 | WW | MEDIUM |
| 85 | 2011 | E | CASTILLE | SNI | 5 | WW | MEDIUM |
| 84 | 2011 | N | FASHION | DNI | 2 | WW | MEDIUM |
| 81 | 2011 | W | CASTILLE | DNI | 6 | WW | HEAVY |
| 78 | 2011 | N | EXCALIBUR | SNI | 20 | WW | HEAVY |
| 77 | 2011 | N | CATANA | P | 4 | F | MEDIUM |
| 73 | 2011 | E | CABERNET | DNI | 2 | WW | MEDIUM |
| 72 | 2011 | E | CABERNET | DNI | 20 | WW | HEAVY |
| 65 | 2011 | N | EXCALIBUR | * | 3 | WW | LIGHT |
| 64 | 2011 | N | CASTILLE | SNI | 6 | SB | CALC. |
| 61 | 2011 | S | VISION | P | 5 | SB | MEDIUM |
| 60 | 2011 | S | VISION | P | 6 | WW | LIGHT |
| 59 | 2011 | E | VISION | DNI | 4 | WW | MEDIUM |
| 57 | 2011 | W | VISION | DNI | 9 | WW | MEDIUM |
| 56 | 2011 | E | CASTILLE | P | 3 | WW | CALC. |
| 55 | 2011 | N | EXPOWER | DNI | 4 | WW | HEAVY |
| 54 | 2011 | N | CABERNET | DNI | 20 | WB | MEDIUM |
| 53 | 2011 | E | CABERNET | DNI | 20 | WW | ORGANIC |
| 50 | 2011 | N | VISION | P | 3 | WW | HEAVY |
| 49 | 2011 | E | EXCALIBUR | DNI | 20 | WW | MEDIUM |
| 47 | 2011 | N | FASHION | P | 3 | SB | CALC. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 44 | 2011 | N | PR46W21 | SNI | 5 | WW | CALC. |
| 39 | 2012 | E | CABERNET | P | 4 | WB | MEDIUM |
| 38 | 2012 | E | CABERNET | DNI | 6 | SW | MEDIUM |
| 37 | 2012 | S | VISION | P | 3 | WW | MEDIUM |
| 35 | 2012 | N | PALMEDOR | DD | 7 | WW | OTHER |
| 34 | 2012 | W | PALMEDOR | DNI | 20 | WW | HEAVY |
| 33 | 2012 | S | CASTILLE | P | 20 | SB | MEDIUM |
| 32 | 2012 | E | PR46W21 | SNI | 3 | SB | MEDIUM |
| 31 | 2012 | W | VISION | SNI | 4 | WW | MEDIUM |
| 27 | 2012 | S | OSPREY | P | 10 | * | HEAVY |
| 26 | 2012 | S | VISION | P | 20 | WW | CALC. |
| 25 | 2012 | S | * | P | 4 | WW | MEDIUM |
| 24 | 2012 | E | CASTILLE | SNI | 20 | WW | MEDIUM |
| 22 | 2012 | E | CABERNET | DNI | 1 | WW | MEDIUM |
| 17 | 2012 | E | CASTILLE | P | 5 | WW | MEDIUM |
| 15 | 2012 | N | FASHION | SNI | 3 | SB | CALC. |
| 14 | 2012 | N | CATANA | DNI | 4 | WB | HEAVY |
| 13 | 2012 | N | OTHER | DNI | 7 | WW | MEDIUM |
| 12 | 2012 | N | OTHER | DNI | 1 | WW | MEDIUM |

Table A2. UK Oilseed rape production area by region (2005-2010), adapted from Defra OSR survey data.

| **Area** | **2005** | **2006** | **2007** | **2008** | **2009** | **2010** | **Mean** | **%** |
|---|---|---|---|---|---|---|---|---|
| North East | 23363 | 22309 | 25916 | 26317 | 18613 | 24396 | 23486 | 4 |
| North West & Merseyside | 3692 | 3297 | 3885 | 3841 | 3774 | 4630 | 3853 | 1 |
| Yorkshire & Humber | 62494 | 59718 | 74894 | 76692 | 69210 | 84102 | 71185 | 13 |
| East Midlands | 111945 | 110658 | 136393 | 134820 | 124274 | 147941 | 127672 | 23 |
| West Midlands | 35373 | 32467 | 41294 | 41335 | 38451 | 46951 | 39312 | 7 |
| Eastern | 97475 | 101545 | 127822 | 127978 | 122985 | 138864 | 119445 | 22 |
| South East and London | 77705 | 75493 | 89407 | 85484 | 71865 | 82991 | 80491 | 15 |
| South West | 43126 | 41330 | 50086 | 48467 | 43607 | 51928 | 46424 | 8 |
| **Scotland** | 35591 | 33742 | 36334 | 33623 | 29043 | 36002 | 34056 | 6 |
| **Wales and Northern Ireland** | 3304 | 3085 | 3218 | 3591 | 4617 | 5885 | 3950 | 1 |
| | | | | | | Total | 549873 | 100 |

Table A3. Table of agronomic and environmental explanatory variables associated with the survey sample subset (n=50). Key: *Denotes missing information. Region: N, North; S, South; E, East; W, West. Establishment method: P, Plough; SNI, Shallow Non Inversion; DNI, Deep Non Inversion; DD, Direct Drilled. Previous crop: WW, Winter Wheat; WB, Winter Barley; SB, Spring Barley; F, Fallow. Soil type: Calc., Calcareous.

| Sample No. | Year | Region | Variety | Establishment method | Years previous OSR | Previous crop | Soil Type |
|---|---|---|---|---|---|---|---|
| **12** | 2012 | S | CASTILLE | P | 6 | WW | LIGHT |
| **13** | 2012 | W | CASTILLE | SNI | 2 | WW | MEDIUM |
| **14** | 2011 | E | CABERNET | DNI | 2 | WW | MEDIUM |
| **15** | 2011 | S | CABERNET | DNI | 4 | SW | MEDIUM |
| **17** | 2011 | N | CASTILLE | P | 5 | WB | MEDIUM |
| **22** | 2011 | N | * | SNI | 1 | WW | HEAVY |
| **24** | 2011 | E | CABERNET | P | 20 | SB | MEDIUM |
| **25** | 2011 | S | CASTILLE | P | 3 | WW | MEDIUM |
| **26** | 2011 | E | CABERNET | SNI | 7 | SB | MEDIUM |
| **27** | 2011 | E | VISION | SNI | 5 | WW | MEDIUM |
| **31** | 2011 | E | CASTILLE | SNI | 5 | WW | MEDIUM |
| **32** | 2011 | N | FASHION | DNI | 2 | WW | MEDIUM |
| **33** | 2011 | W | CASTILLE | DNI | 6 | WW | HEAVY |
| **34** | 2011 | N | EXCALIBUR | SNI | 20 | WW | HEAVY |
| **35** | 2011 | N | CATANA | P | 4 | F | MEDIUM |
| **37** | 2011 | E | CABERNET | DNI | 2 | WW | MEDIUM |
| **38** | 2011 | E | CABERNET | DNI | 20 | WW | HEAVY |
| **39** | 2011 | N | EXCALIBUR | * | 3 | WW | LIGHT |
| **44** | 2011 | N | CASTILLE | SNI | 6 | SB | CALC. |
| **47** | 2011 | S | VISION | P | 5 | SB | MEDIUM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **49** | 2011 | S | VISION | P | 6 | WW | LIGHT |
| **50** | 2011 | E | VISION | DNI | 4 | WW | MEDIUM |
| **52** | 2011 | W | VISION | DNI | 9 | WW | MEDIUM |
| **53** | 2011 | E | CASTILLE | P | 3 | WW | CALC. |
| **54** | 2011 | N | EXPOWER | DNI | 4 | WW | HEAVY |
| **55** | 2011 | N | CABERNET | DNI | 20 | WB | MEDIUM |
| **56** | 2011 | E | CABERNET | DNI | 20 | WW | ORGANIC |
| **57** | 2011 | E | CABERNET | SNI | 1 | WW | HEAVY |
| **59** | 2011 | N | VISION | P | 3 | WW | HEAVY |
| **60** | 2011 | E | EXCALIBUR | DNI | 20 | WW | MEDIUM |
| **61** | 2011 | N | FASHION | P | 3 | SB | CALC. |
| **64** | 2011 | N | PR46W21 | SNI | 5 | WW | CALC. |
| **65** | 2012 | E | CABERNET | P | 4 | WB | MEDIUM |
| **72** | 2012 | E | CABERNET | DNI | 6 | SW | MEDIUM |
| **73** | 2012 | S | VISION | P | 3 | WW | MEDIUM |
| **77** | 2012 | N | PALMEDOR | DD | 7 | WW | OTHER |
| **78** | 2012 | W | PALMEDOR | DNI | 20 | WW | HEAVY |
| **81** | 2012 | S | CASTILLE | P | 20 | SB | MEDIUM |
| **84** | 2012 | E | PR46W21 | SNI | 3 | SB | MEDIUM |
| **85** | 2012 | W | VISION | SNI | 4 | WW | MEDIUM |
| **88** | 2012 | S | OSPREY | P | 10 | * | HEAVY |
| **89** | 2012 | S | VISION | P | 20 | WW | BRASH |
| **90** | 2012 | S | * | P | 4 | WW | MEDIUM |
| **91** | 2012 | E | CASTILLE | SNI | 20 | WW | MEDIUM |
| **92** | 2012 | E | CABERNET | DNI | 1 | WW | MEDIUM |
| **94** | 2012 | E | CASTILLE | P | 5 | WW | MEDIUM |
| **95** | 2012 | N | FASHION | SNI | 3 | SB | BRASH |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **99** | 2012 | N | CATANA | DNI | 4 | WB | HEAVY |
| **101** | 2012 | N | OTHER | DNI | 7 | WW | MEDIUM |
| **102** | 2012 | N | OTHER | DNI | 1 | WW | MEDIUM |

Table A4. Table for the adjustment of potting mix moisture content (MC), to achieve 30% by volume.

| Measured MC (%) | Water addition (ml) |
|---|---|
| 30 | 0 |
| 29 | 17 |
| 28 | 34 |
| 27 | 52 |
| 26 | 69 |
| 25 | 86 |
| 24 | 103 |
| 23 | 120 |
| 22 | 138 |
| 21 | 155 |
| 20 | 172 |
| 19 | 189 |
| 18 | 206 |
| 17 | 223 |
| 16 | 241 |
| 15 | 258 |
| 14 | 275 |
| 13 | 292 |
| 12 | 309 |
| 11 | 327 |
| 10 | 344 |

Table A5. Table of Rhizoctonia solani isolates attained from other institutions. Sources: 1, James Woodhall, FERA, York. 2, David Kenyon, SASA, Edingburgh. 3, John Clarkson, University of Warwick, Warwick. 4, Shuvash Bhattarai, Chemtura AgroSolutions Ltd. 5, Rumiana Ray, Univeristy of Nottingham, Sutton Bonnington.

| HAU assigned code | Original code | Source | Host Crop | AG |
|---|---|---|---|---|
| R1 | ZJ | 1 | Potato | 3PT |
| R2 | Ct Mz | 1 | Maize | 3PT |
| R3 | None | 2 | Unknown | 3PT |
| R4 | None | 3 | Unknown | 3PT |
| R5 | 1832 | 1 | Unknown | 3 |
| R6 | 1938 | 1 | Unknown | 4HGII |
| R7 | 1835 | 1 | Unknown | 4 |
| R8 | 1986 | 1 | Unknown | *R. zeae* |
| R9 | 1947 | 1 | Unknown | 8 |
| R10 | 1841 | 1 | Unknown | 8 |
| R11 | 1846 | 1 | Unknown | 11 |
| R12 | 1843 | 1 | Unknown | 9 |
| R13 | 1971 | 1 | Unknown | 2-1 |
| R14 | Pl240 | 4 | Unknown | 2-1 |
| R15 | Pl245 | 4 | Unknown | 4 |
| R16 | 1971 | 1 | Unknown | 2-1 |
| R17 | 1977 | 1 | Unknown | 2-1 |
| R18 | 1955 | 1 | Unknown | 2-1 |
| R19 | 1942 | 5 | Unknown | 2-1 |
| R20 | 1926/2332 | 5 | Unknown | 2-1 |
| R21 | 1917 | 5 | Unknown | 2-1 |
| R22 | 1935 | 5 | Unknown | 2-1 |
| R23 | 1934 | 5 | Unknown | 2-1 |
| R24 | 1933 | 5 | Unknown | 2-1 |
| R25 | 1927/2023 | 5 | Unknown | 2-1 |

Supplementary Table S1. Internal transcribed spacer (ITS) 1 sequences, taken from the National Centre for Biotechnology Information (NCBI) nucleotide database for additional comparison to those contained within UNITE.

| **Ascension No.** | **Species** | **Source** |
|---|---|---|
| AF231027.1 | *Plasmodiophora brassicae* | Faggian *et al.,* Unpublished |
| AF266801.1 | *Phytophthora brassicae* | Cooke *et al.,* 2000 |
| HQ643153.1 | *Phytophthora brassicae* | Robideau *et al.,* 2011 |
| JQ647441.1 | *Gibellulopsis nigrescens* | Cannon *et al.,* 2012 |
| JQ647440.1 | *Gibellulopsis nigrescens* | Cannon *et al.,* 2012 |
| JN187977.1 | *Gibellulopsis nigrescens* | Inderbitzin *et al.,* 2011a |
| HQ206916.1 | *Verticillium longisporum* | Inderbitzin *et al.,* unpublished |
| HQ206914.1 | *Verticillium longisporum* | Inderbitzin *et al.,* 2011b |
| HQ206863.1 | *Verticillium longisporum* | Inderbitzin *et al.,* 2011b |
| AY373015.1 | *Olpidium brassicae* | Hartwright *et al.,* 2010 |
| AY997067.1 | *Olpidium brassicae* | James *et al.,* 2006 |
| GQ304519.1 | *Olpidium brassicae* | Maccarone *et al.,* Unpublished |
| AY649597.1 | *Pyrenochaeta lycopersici* | Infantino *et al.,* 2005 |
| AY649596.1 | *Pyrenochaeta lycopersici* | Infantino *et al.,* 2005 |
| AY649595.1 | *Pyrenochaeta lycopersici* | Infantino *et al.,* 2005 |
| HQ643867.1 | *Pythium ultimum* | Robideau *et al.,* Unpublished |
| KJ545577.1 | *Rhizoctonia solani (AG2-1)* | Caesar *et al.,* 2014 |
| KF719318.1 | *Rhizoctonia solani (AG2-2IIIB)* | Hua *et al.,* Unpublished |
| KF907732.1 | *Rhizoctonia solani (AG4)* | Hua *et al.,* Unpublished |
| AJ000197.1 | *Rhizoctonia solani (AG Unknown)* | Johanson *et al.,* 1998 |
| JX648199.1 | *Leptosphaeria maculans* | Naumann & Wicklow, 2013 |
| JF740234.1 | *Leptosphaeria maculans* | de Gruyter *et al.,* 2013 |
| DQ133893.1 | *Leptosphaeria biglobosa* | Liu *et al.,* 2006 |