Understanding *R* gene-mediated resistance against *Leptosphaeria maculans* for effective control of phoma stem canker in oilseed rape

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Abstract

This project aims to improve understanding of *R* gene-mediated resistance against *Leptosphaeria maculans* for effective control of phoma stem canker in oilseed rape. This was achieved through three objectives by (i) monitoring the UK regional distribution of races of *L. maculans*, (ii) investigating molecular mechanisms of mutation to virulence in *L. maculans* populations, (iii) understanding effects of environmental factors on *Brassica napus* resistance against *L. maculans*.

The regional distributions of *L. maculans* races in the UK were monitored in air and crops in 2015/2016, 2016/2017 and 2017/2018 cropping seasons. The release of ascospores in the air was monitored using Burkard spore samplers at four different sites. The frequencies of avirulent alleles of effector genes *AvrLm1* and *AvrLm6* in the *L. maculans* ascospore populations were determined by qPCR. There were differences between the four sites and seasons in patterns of ascospore release and in dates of first major ascospore release. *L. maculans* ascospores with avirulent *AvrLm1* alleles were mostly released earlier than ascospores with *AvrLm6* alleles. *AvrLm6* alleles were detected more frequently than *AvrLm1* alleles in *L. maculans* ascospores sampled at all sites in all seasons.

Changes in frequencies of avirulent alleles of different effector genes were investigated by inoculation of conidial suspensions onto the cotyledons of a set of *Brassica napus* differential cultivars/lines. The *AvrLm7* allele was predominant in the UK *L. maculans* populations until 2015/2016; however, the frequency of isolates virulent towards the corresponding resistance gene *Rlm7* has increased from 6.8% (2016/2017) to 16.3% (2017/2018), suggesting that there is a risk of breakdown of *Rlm7*-mediated resistance in the UK. There were differences in the frequencies of avirulent alleles of different effector genes in *L. maculans* populations from leaf samples (single pycnidial isolates) between sites during three cropping seasons. Overall, the frequencies of *AvrLm5* (85.5%), *AvrLm6* (97.2%) and *AvrLm7* (94.7%) were greater than those of *AvrLm1* (5.7%), *AvrLm2* (3.6%), *AvrLm3* (3.2%), *AvrLm4* (17.7%) and *AvrLm9* (1.2%) in *L. maculans* populations from leaf samples.

Similarly, for *L. maculans* populations from stem samples (single ascospore isolates) from two different sites in 2016/2017, the frequencies of avirulent alleles of *AvrLm5* (100%), *AvrLm6* (94.1%) and *AvrLm7* (75.9%) were greater than those of *AvrLm1* (22.7%), *AvrLm2* (11.9%), *AvrLm3* (23.7%), *AvrLm4* (15.6%) and *AvrLm9* (16.5%). Seven races were identified in the *L. maculans* populations from leaf samples in 2015/2016. In 2016/2017, 15 and 19 races were identified in the *L. maculans* isolates from leaf and stem samples, respectively. A total of 17 races were observed in the UK *L. maculans* populations from leaf samples in 2017/2018, however *Av5-6-7* remained the major race for the three cropping seasons.

Molecular mechanisms of mutation in *L. maculans* populations leading to virulence towards *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes were investigated. Sanger sequencing (55 *L. maculans* isolates) and whole genome sequencing (40 *L. maculans* isolates) were used in this study. For Sanger sequencing, whole gene deletion of the *AvrLm1* gene was observed in 96% of the isolates. For the remaining isolates, partial deletion (1%) and single-base point mutation (70%) were identified as the cause of virulence towards the resistance gene *Rlm1*. For whole genome sequencing, whole gene deletion of the *AvrLm1* gene was identified in all the isolates (100%).

Molecular mechanisms of mutation leading to virulence towards *Rlm4* were identified as whole gene deletion (6.6%) from Sanger sequencing. From whole genome sequences, whole gene deletion (18.2%), partial deletion (3.03%), single-nucleotide (non-synonymous) point mutation (84.8%) and single-nucleotide (synonymous) point mutation (6.1%) were identified. Eighteen different single-nucleotide polymorphisms were identified at thirty-four codon sites; that had led to amino-acid changes or created stop codons. From whole genome sequences, whole gene deletion of the *AvrLm6* gene was identified in one *L. maculans* isolate virulent towards the *Rlm6* resistance gene and no other molecular mechanisms of mutation were identified. From whole genome sequences, various molecular mechanisms of mutation leading to virulence towards the *Rlm7* resistance gene were identified in this study, such as whole gene deletion (27.3%), partial deletion (13.6%), single-nucleotide (non-

synonymous) point mutation (68.2%), single-nucleotide (synonymous) point mutation (9.1%), Nonsense point mutation (50%), single-nucleotide deletion (27.3%), repeat-induced point mutation (RIPs) (68.2%), transition (68.2%) and transversion mutation (13.6%).

Effects of environmental factors on *Brassica napus* resistance against *L. maculans* were investigated in three cropping seasons. Severities of phoma leaf spot and phoma stem canker were assessed on twelve different oilseed rape cultivars at different environments/sites in the UK. There were differences in the phoma leaf spot and phoma stem canker severities between sites and between cropping seasons. Among all the sites, Morley site had the greatest phoma leaf spot and phoma stem canker severities; whereas, Trumpington and Risby sites had the least disease severity. Among the three cropping seasons, less phoma stem canker severity was observed among the sites in 2016/2017 than in other two seasons.

Cultivars Drakkar, Incentive and Mentor (with no *R* gene against *L. maculans*) developed more severe phoma leaf spots and phoma stem cankers than cultivars with *R* genes and background quantitative resistance. Cultivars with the *Rlm7* gene (DK Exalte, Whisky, Amalie, Harper and DK Extrovert) had less severe phoma leaf spotting and less severe phoma stem canker than cultivars DK Cabernet (with the *Rlm1* gene) and Adriana (with the *Rlm4* gene). Cultivar Angus (with the *LepR3* gene) also had less phoma leaf spot and phoma stem canker severity. Cultivars with QR (Es Astrid, Adriana and DK Cabernet) had less severe phoma stem cankers in summer even though they had severe phoma leaf spots in autumn/winter. Analysis of the relationship between disease severity and weather data among the different sites in the three growing seasons showed that increased severity of phoma leaf spots was associated with increased rainfall and moderate mean temperatures during the phoma leaf spot development stage and increased severity of phoma stem canker was associated with increased temperature during the stem canker development stage.

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Publications

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Gajula L.H., Fitt B.D.L., Huang Y.J. (2019). Identification of new virulent races in *Leptosphaeria maculans* populations on oilseed rape in the UK. *LMS Research conference*, 16 April 2019, University of Hertfordshire, UK. **Poster presentation.**

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Gajula L.H., Huang Y.J., Fitt B.D.L. (2019). Identification of new virulent races of *Leptosphaeria maculans* populations on oilseed rape in the UK. *IOBC-WPRS Bulletin* (in press). 7-9 September 2016, Tartu, Estonia. **Oral presentation.**

Gajula L.H., Mitrousia G., Fitt B.D.L., Huang Y.J. (2018). Molecular mechanisms of mutation to virulence in *Leptosphaeria maculans* populations in the UK. *International Congress of Plant Pathology (ICPP)*, 29 July - 3 August 2018, Boston, USA. **Poster presentation.**

Huang Y.J., Javaid A., **Gajula L.H**., Karandeni-Dewage C.S., Mitrousia G., Fitt B.D.L. (2018). Ignored fungal pathogen sibling - *Leptosphaeria biglobosa*. *International Congress of Plant Pathology (ICPP)*, 29 July - 3 August 2018, Boston, USA. **Poster presentation.**

Gajula L.H., Fitt B.D.L., Huang Y.J. (2018). Understanding *R* gene-mediated resistance against *Leptosphaeria maculans* for effective control of phoma stem canker in oilseed rape. *LMS Research conference*, 10 April 2018, University of Hertfordshire, UK. **Poster presentation.**

Gajula L.H., Fitt B.D.L., Huang Y.J. (2017). Identification of new virulent races of *Leptosphaeria maculans* populations on oilseed rape in the UK. *AFCP Forum 2017*, 5 April 2017, University of Hertfordshire, UK. **Oral presentation.**

Gajula L.H., Javaid A., Fitt B.D.L., Huang Y.J. (2017). Monitoring the regionl distribution of races of *Leptosphaeria maculans* populations in the UK. *LMS Research conference*, University of Hertfordshire, UK. **Poster presentation.**

Javaid A., **Gajula L.H**., Fitt B.D.L., Huang Y.J. (2017). Investigating the risk of severe phoma stem canker caused by *Leptosphaeria biglobosa* on winter oilseed rape in the UK. *Crop Production in Southern Britain. Aspects of Applied Biology* 134*,* p. 53-58. **Oral presentation.**

Huang Y.J., Cai X., Karandeni-Dewage C.S., **Gajula L.H**., Javaid A., Li G.Q., Fitt B.D.L. (2016). Understanding phoma stem canker epidemics caused by *Leptosphaeria biglobosa* in the UK and China. *Abstracts. Brassica 2016,* 3-7 October 2016, Melbourne, Australia, p. 43. **Oral presentation.**

Mitrousia G., Huang Y.J., Sidique S., **Gajula L.H**., Fitt B.D.L. (2016). Coexistence of *Leptosphaeria* spp. on oilseed rape crops in the UK. *Abstracts. Brassica 2016,* 3-7 October 2016, Melbourne, Australia, p. 94. **Oral presentation.**

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Chapter 1 General introduction

1.1 Oilseed rape

Oilseed rape (*Brassica napus*) is one of the most economically important crops that is grown world-wide (http://www.fao.org/home/en) (Fitt *et al*., 2006a). This crop is grown in temperate climates in the northern hemisphere (China, Canada, Europe and India) and in the southern hemisphere (Australia, South Africa and South America) and is best adapted to the moist and cooler production areas. *Brassica napus* (genome $A_nA_nC_nC_n$) was formed approximately 7500 years ago by allopolyploidy, i.e. hybridization followed by chromosomal doubling between its ancestors; *Brassica oleracea* (Mediterranean cabbage, genome C_oC_o) and *Brassica rapa* (Asian cabbage or turnip, genome A_rA_r) (Chalhoub *et al*., 2014; Nagaharu, 1935) (Figure 1.1).

Brassica crop species are known to be an important source of oil, condiments, vegetables, forage and green manure worldwide (Boukema & Van Hintum, 1999; Diederichsen, 2001). Oilseed rape (*Brassica napus*) is the third most important arable crop in the UK, after wheat and barley. It is a source of vegetable oil and biodiesel. The oilseed rape seed contains *c*. 40-50% oil (Fortescue & Turner, 2003). The market share of oilseed rape oil is expanding because it is recognised as one of the healthiest vegetable oils since it is rich in mono-unsaturated fatty acids (oleic), a good source of essential omega-3 fatty acids and is low in saturated fatty acids (Knodel & Watrin, 2003).

Most (98%) of the UK oilseed rape is cultivated to produce food grade oil. The oil is usually used for margarines, shortenings, salad dressings and vegetable oils and the derivatives are used in the manufacturing of cosmetics and detergent products (Kings, 1997). Rapeseed oil has been shown to decrease serum total and LDL cholesterol concentrations when replacing saturated fats (Karvonen *et al*., 2002). After extracting oil, the remaining high protein seed residue can be used as an animal feed (Noquet *et al*., 2003).

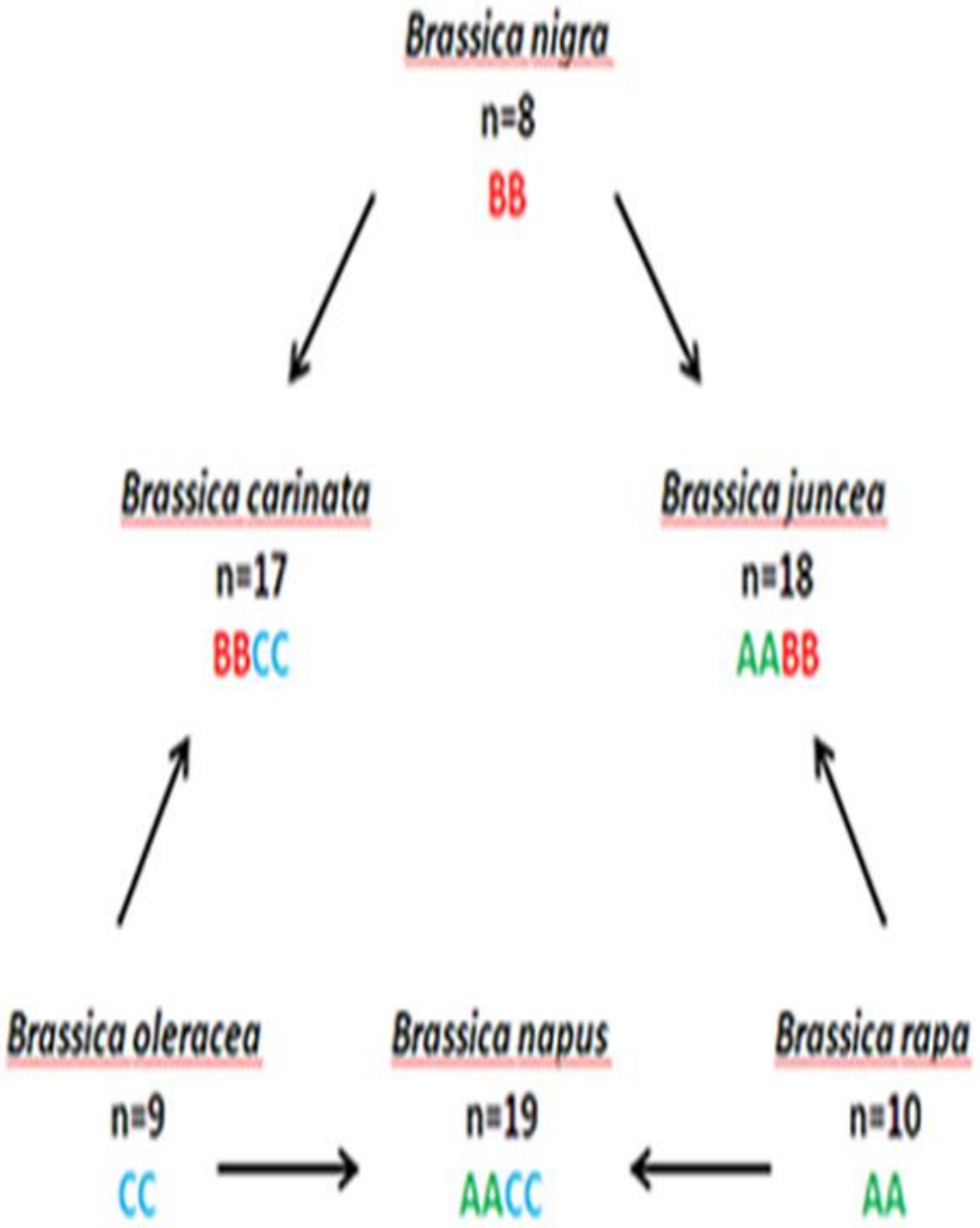


Figure 1.1: Formation of *Brassica napus* from its ancestors (Nagaharu, 1935). This diagram illustrates the genetic relationship between six species of *Brassica* and the evolution of *Brassica napus* (AACC) from *Brassica oleracea* (CC) and *Brassica rapa* (AA). The letter 'n' is the number of chromosomes in each genome.

Being capable of extracting mineral N from the soil, *Brassica napus* can also be grown as a 'catch' crop to reduce nitrate leaching from arable cropping systems (Schjoerring *et al*., 1995). *Brassica napus* flowers are also a source of nectar and pollen, which are of great importance for bee colonies in most parts of Europe (Von der Ohe & Von der Ohe, 2003).

1.1.1 Oilseed rape production in the world

Sustainable growth over the past 20 years has been observed in oilseed rape production worldwide. It is now the second most produced oilseed in the world behind soybeans (Figure 1.2). Rapeseed meal is the second major oilseed meal produced worldwide (after soybean meal). China, India, Canada and the European Union (27) are the major producers of oilseed rape (Carre & Pouzet, 2013). Over time, the area of cultivation has markedly increased in Canada and Europe while it has generally remained unchanged in China and to a lesser extent in India (Figure 1.3).

Since 2002/2003, oilseed rape areas have increased in Europe and Canada. Far behind this group, the oilseed rape area of cultivation has been increasing in Australia compared to Ukraine, Russia and USA (Figure 1.3). So far, Europe is the world's largest producer of oilseed rape with about 20 Mt per year followed by Canada (15 Mt) and China (12 Mt). India with about 6 Mt yearly is well behind although with a comparable acreage (Figure 1.4) (Carre & Pouzet, 2013).

1.1.2 Oilseed rape production and consumption in the UK

In the UK, oilseed rape production has increased. There has been an increase in the oilseed rape area from 269 thousand hectares in 1984 to 756 thousand hectares in 2012. However, the area has decreased from 2013 and was 563 thousand hectares in 2017 (Defra, 2017) (Figure 1.5). Average UK yields for oilseed rape have increased with time. They showed the largest proportional increase from 3.1 tonnes per hectare in 2016 to 3.9 tonnes per hectare in 2017. This was despite some reports of crops struggling in some areas due to flea beetle damage, pigeon damage or poor establishment (Defra, 2017) (Figure 1.6).

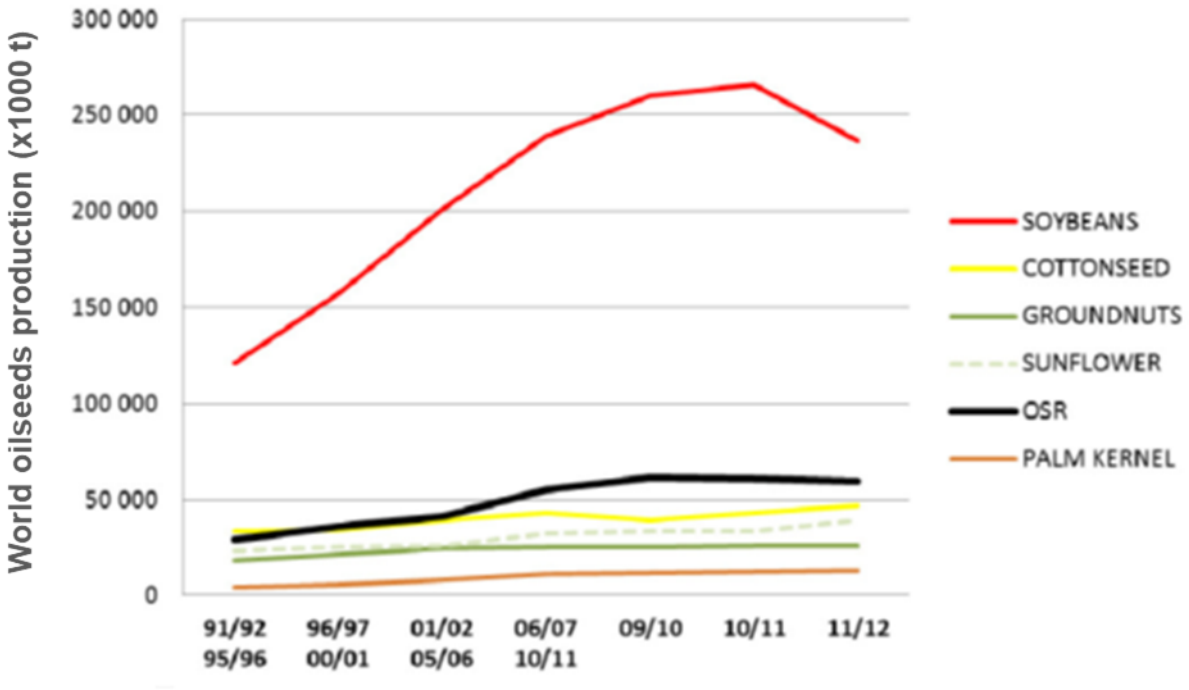


Figure 1.2: World oilseeds production (×1000 t) of soybeans, cottonseeds, groundnuts, sunflower, OSR (oilseed rape) and palm kernel from 1991/1992 to 2011/2012 (Source: Oil World, 2012).

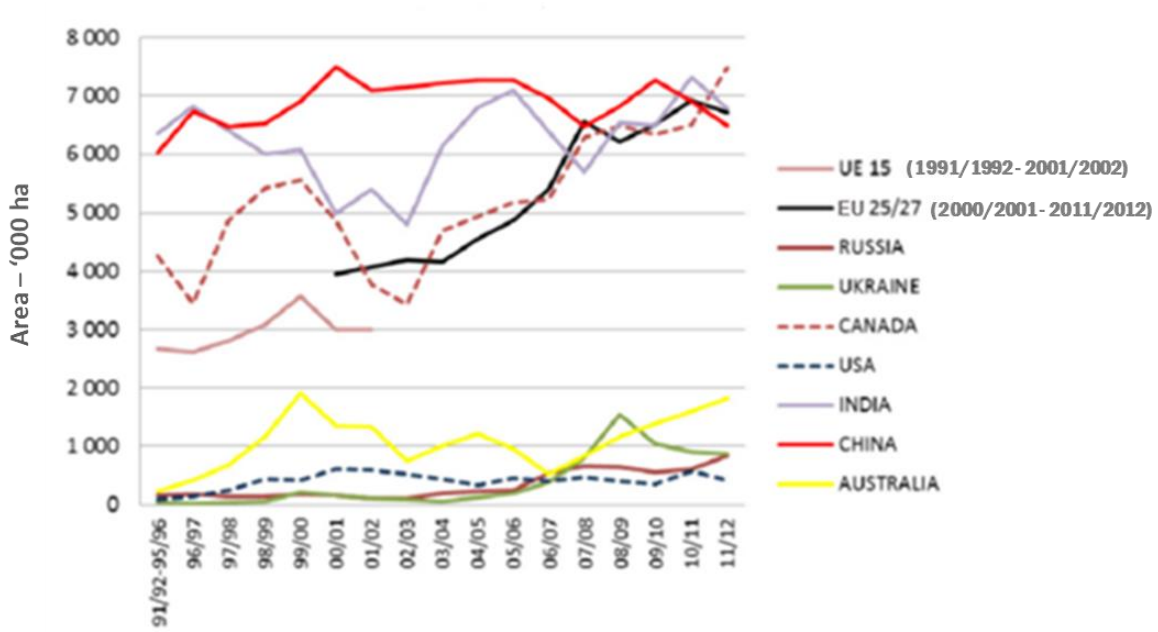


Figure 1.3: World oilseed rape (OSR) area (×1000 ha) in the major areas of production (EU, Russia, Ukraine, Canada, USA, India, China and Australia) from 1991/1992 to 2011/2012 (Source: Oil World, 2012).

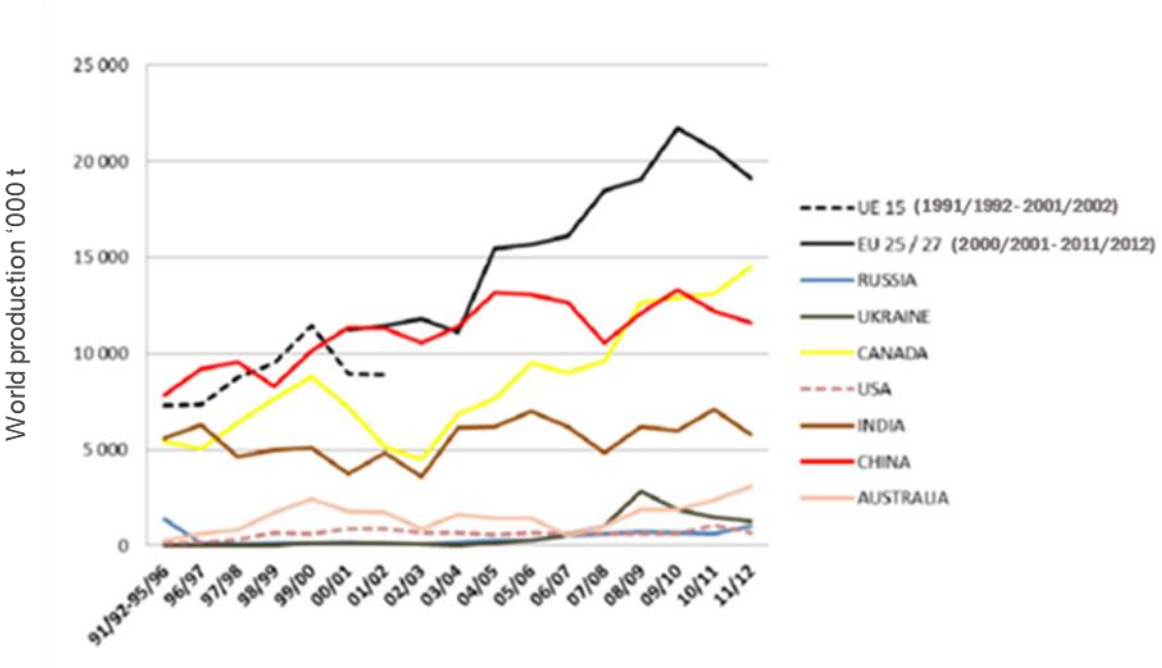


Figure 1.4: World oilseed rape (OSR) production (×1000 t) in the major areas of production (EU, Russia, Ukraine, Canada, USA, India, China and Australia) from 1991/1992 to 2011/2012 (Source: Oil World, 2012).

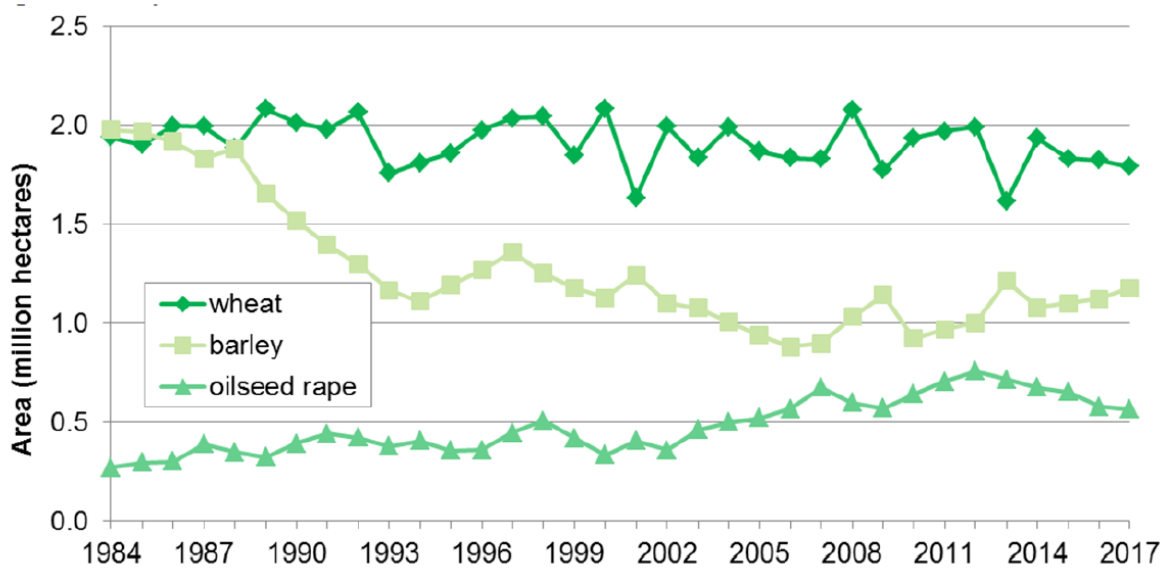


Figure 1.5: Areas of cultivation of oilseed rape, wheat and barley from sowing years 1984 to 2017 in the UK (Source: Defra, 2017).

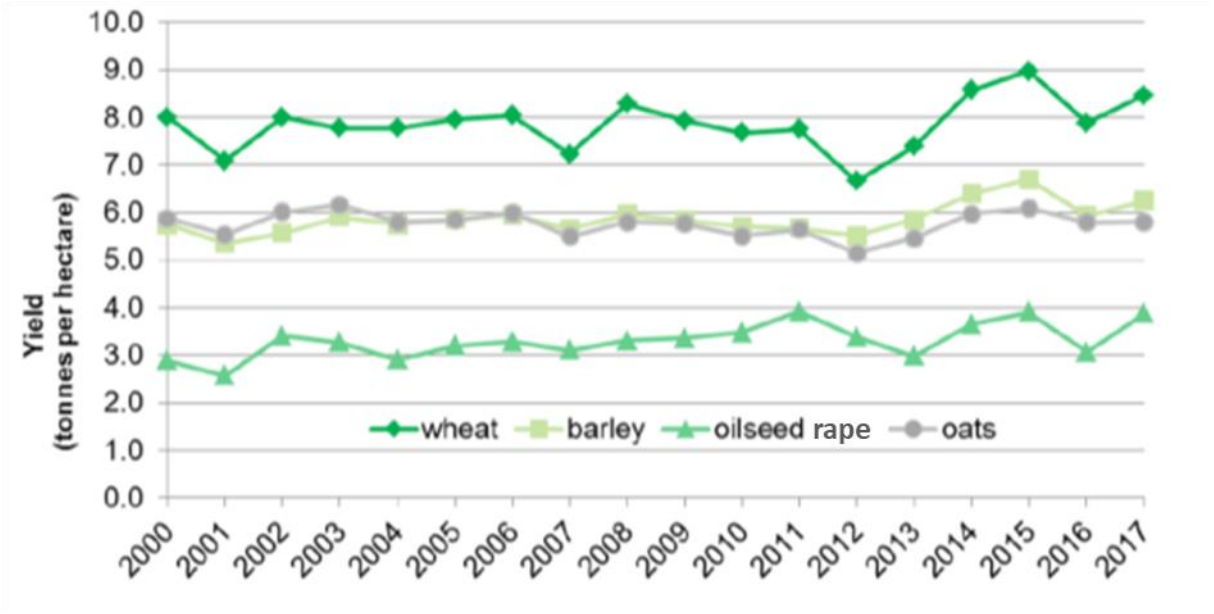


Figure 1.6: Yields of oilseed rape, wheat, barley and oats in the UK from harvest years 2000 to 2017 (Source: Defra, 2017).

The price of oilseed rape has more than doubled since 2002. The price of oilseed rape has steadily increased from 2006 to 2012. The price has decreased from 2013 and now it is £325 per tonne (AHDB, 2018) (Figure 1.7). In 2000, in the UK 1.1M tonnes of oilseed rape seeds were harvested from 716000 ha (Defra, 2013); in 2016, 1.7M tonnes were harvested from 579000 ha (Figure 1.8) (www.fao.org). There has been increase in area of cultivation, production and yield of winter sown oilseed rape. This oilseed rape cropping provides associated markets for seeds for sowing (£40-50M pa), fungicides (£40-50 M pa) and distributors (£110-130 M pa). Over recent years, there has been a large increase in global demand for oilseed rape derived from demand for both protein meal used in animal feed, vegetable oil for human consumption and biodiesel.

Two of the most frequently occurring fungal pathogens on UK oilseed rape are *Leptosphaeria maculans* (*L. maculans*) and *Leptosphaeria biglobosa* (*L. biglobosa*), which cause phoma stem canker (blackleg) disease. Oilseed rape is also attacked by other fungal pathogens, such as *Pyrenopeziza brassicae* cause of light leaf spot, *Sclerotinia sclerotiorum* cause of sclerotinia stem rot and *Alternaria brassicae* cause of dark pod spot (Fitt *et al*., 2006a).

1.2 Phoma stem canker

Phoma stem canker is a disease of worldwide economic importance, especially on oilseed rape and *Brassica* vegetables (Fitt *et al*., 2006a; Van de Wouw *et al*., 2014). It is responsible for worldwide losses worth more than £1000M in each oilseed rape cropping season (at a price of £325 per tonne) (Source: AHDB Recommended Lists 2018/19 for cereals and oilseeds) and can cause yield losses of up to 50% if the disease is not controlled (Fitt *et al*., 2011).

It is caused by two related fungal species, *Leptosphaeria maculans* (*Plenodomus lingam*) and *Leptosphaeria biglobosa* (*Plenodomus biglobosus*) (Shoemaker & Brun, 2001; Fitt *et al*., 2006b; Gruyter *et al*., 2013). *L. maculans* is considered more damaging, often causing stem base canker, whereas *L. biglobosa*, which often causes upper stem lesions, is considered less destructive (West *et al*., 2002a; Huang *et al*., 2005).

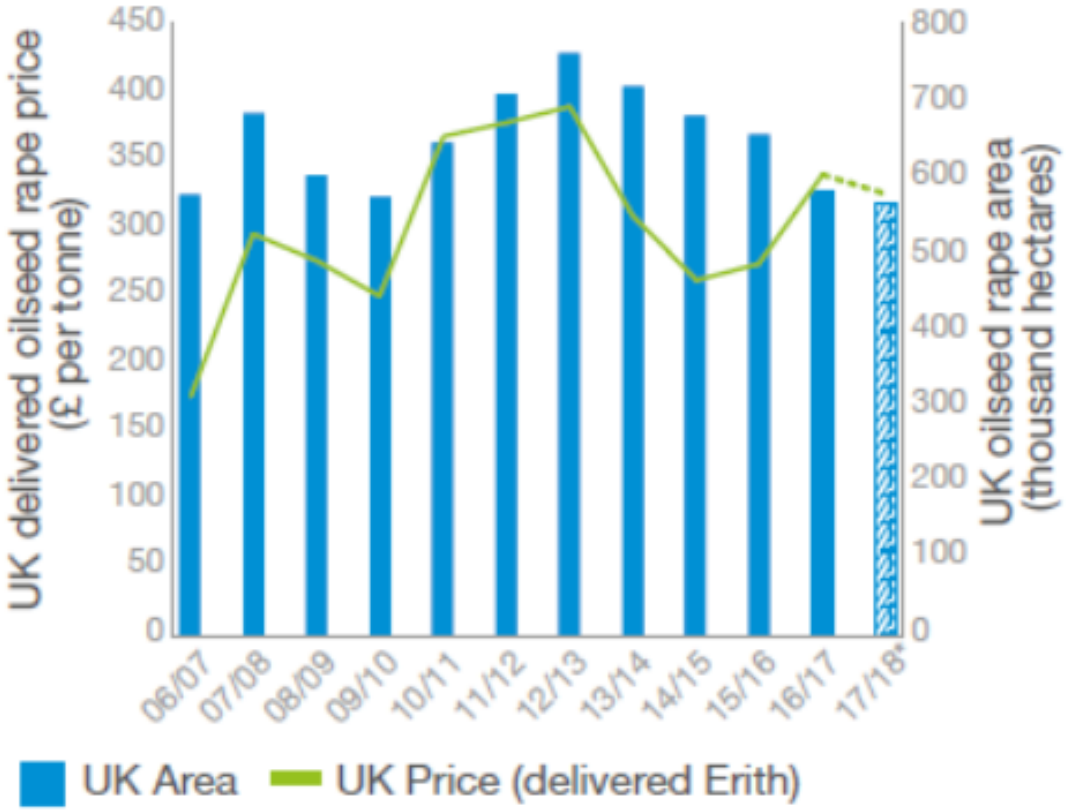


Figure 1.7: Area of oilseed rape cultivation and price from harvest years 2007 to 2018 in the UK (Source: AHDB Recommended Lists 2018/2019 for cereals and oilseeds).

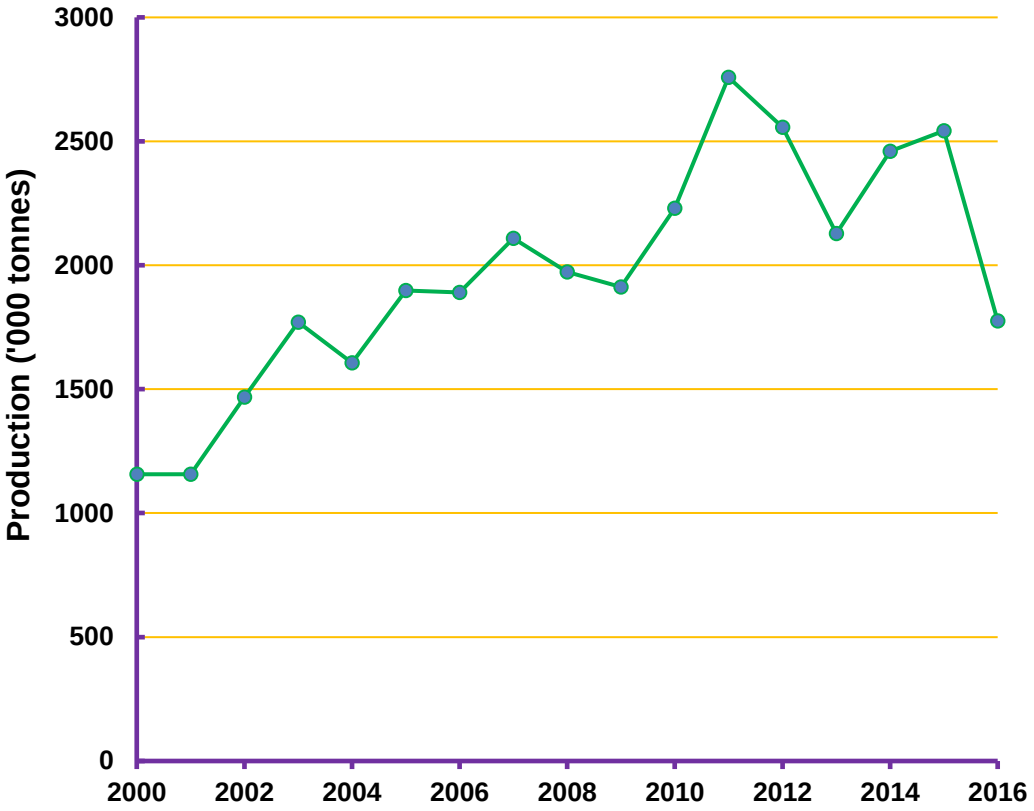


Figure 1.8: Production of oilseed rape in the UK from harvest years 2000 to 2016 (Source: www.fao.org).

Phoma stem canker is the cause of serious losses in oilseed rape crops in Europe, Australia and North America (Howlett, 2004; West *et al*., 2001). In 2010, despite use of fungicides costing £20M per year, phoma stem canker caused losses worth more than £100M per year at a price of £325 per tonne in the UK (Source: AHDB Recommended Lists 2018/19 for cereals and oilseeds) (Fitt *et al*., 2006a; Stonard *et al*., 2010). With recent loss of the most effective fungicides through EU legislation, potential yield losses will increase (Mahmuti *et al*., 2009). Due to the development of fungicide insensitivity in pathogen populations, fungicides can easily lose their effectiveness and that can cause huge losses to the farmers (Huang *et al*., 2018; Carter *et al*., 2014).

Moreover, it is predicted that the severity and range of this disease will continue to increase under climate change (Evans *et al*., 2008; Butterworth *et al*., 2010). Work has estimated that UK oilseed rape losses due to stem canker, at current prices, may increase to £140M p.a. by the 2020s (Evans *et al*., 2010). As suggested by the Defra survey, phoma stem canker caused yield losses up to £90M in 2011. The losses caused by phoma stem canker were comparatively less in the harvest years 2012 and 2013. However, the disease severity increased in 2014 and 2015 harvest years, causing losses worth £79M. The losses caused by phoma stem canker had again increased in the harvest year 2016 (£98.7M). After a decrease of losses in harvest year 2017 (£59.8M), the losses caused by phoma stem canker to the farming industry in England and Wales had again increased in the harvest year 2018 (£86.9M) (http://www.cropmonitor.co.uk) (Figure 1.9).

Control of this disease can be achieved by fungicide treatments, crop management strategies and breeding resistant oilseed rape cultivars (Van de Wouw *et al*., 2010). Phoma stem canker is now the most common disease of winter oilseed rape with greatest risks of disease occurring in the eastern and central counties of England (http://www.cropmonitor.co.uk) (Figure 1.10). The phoma stem canker disease epidemics on winter oilseed rape were more severe in southern England when compared to Scotland, where the temperatures are less (http://www.cropmonitor.co.uk) (Figures 1.10 &1.11).

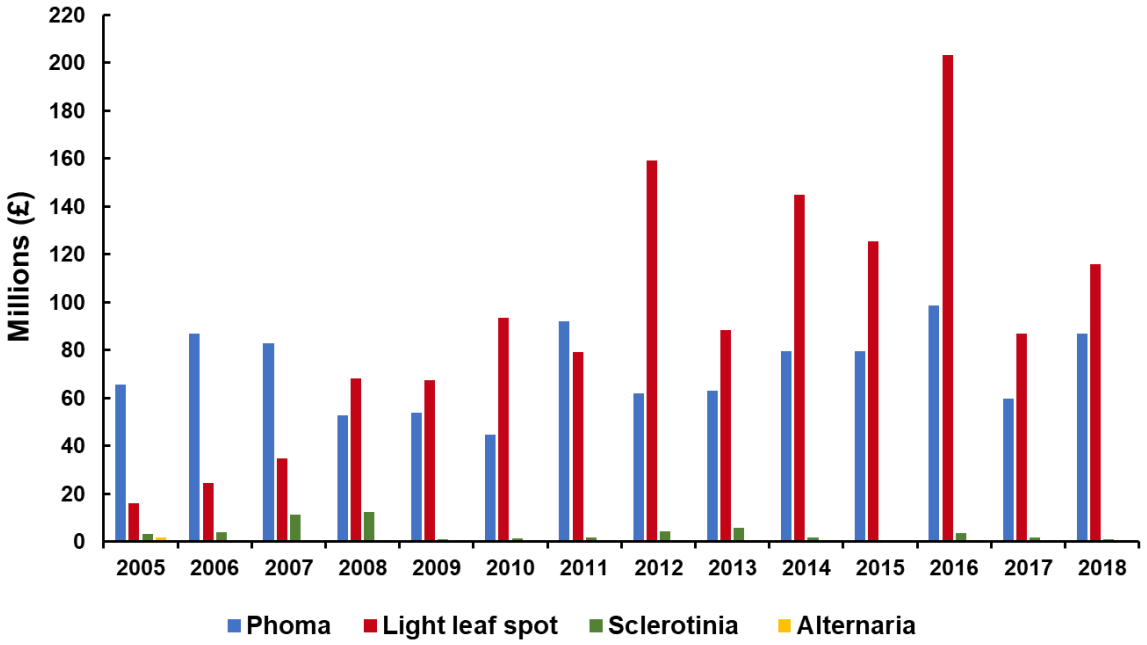


Figure 1.9: Economic losses from four diseases in winter oilseed rape crops from harvest years 2005 to 2018 in England and Wales (www.cropmonitor.co.uk).

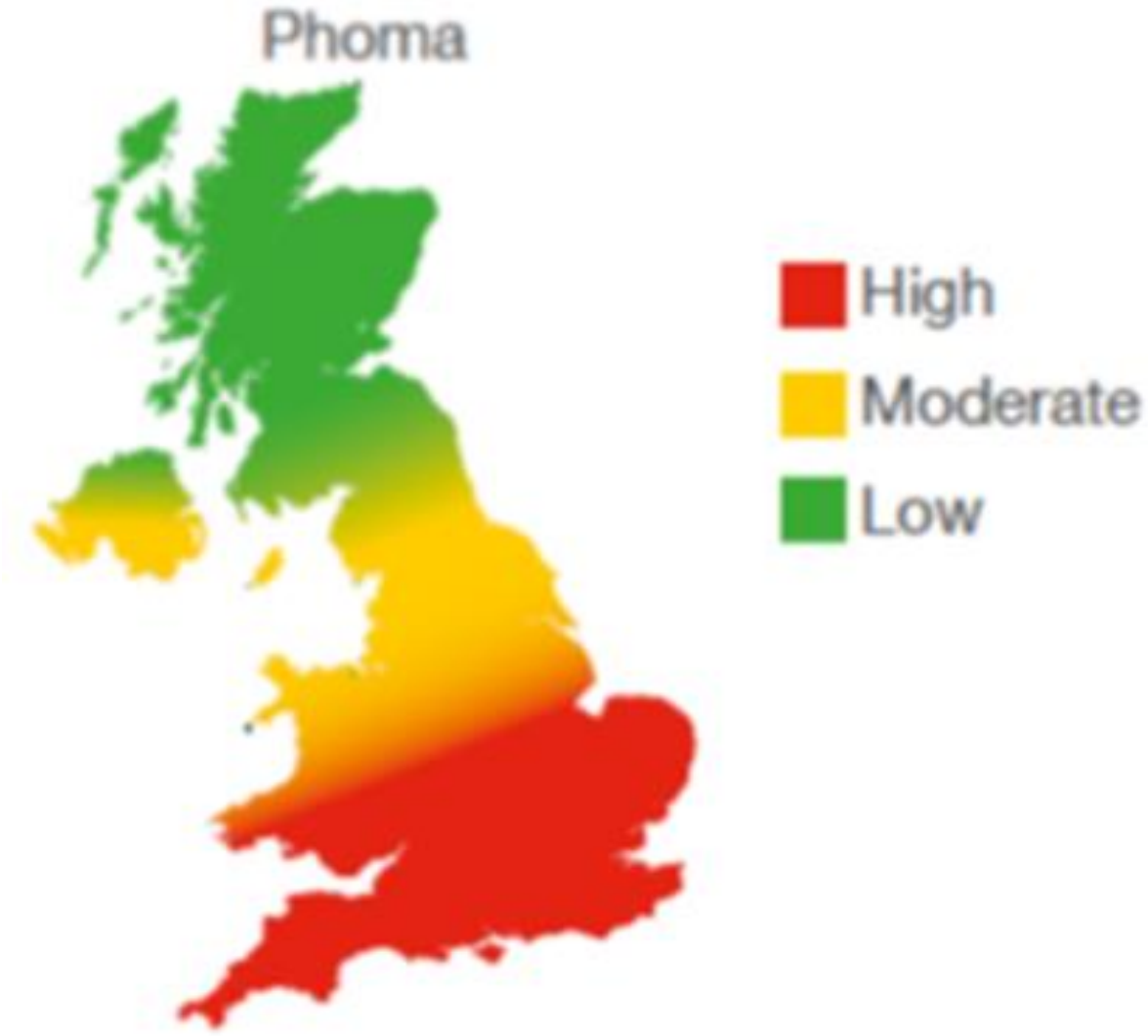


Figure 1.10: Disease risk of phoma stem canker in the UK (Source: AHDB Recommended Lists 2018/19 for cereals and oilseeds).

website: https://cereals.ahdb.org.uk/media/1392721/rl1819-recommended-lists-summer-edition-booklet-.pdf

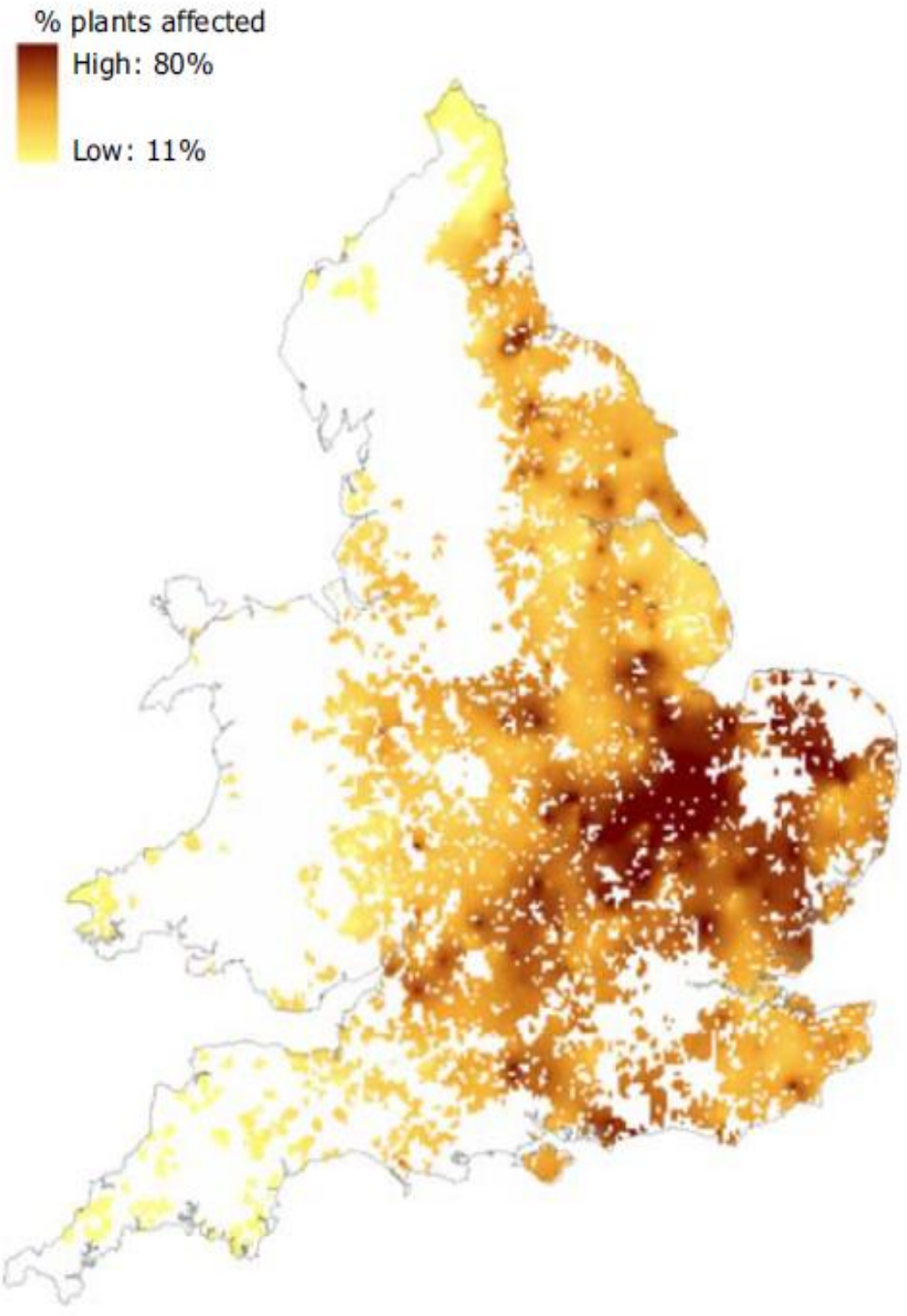


Figure 1.11: Regional variation in phoma stem canker disease incidence on winter oilseed rape crops in England and Wales (based on data from 1997 to 2006) (Stonard *et al*., 2010).

Even though there is phoma leaf spotting in the winter oilseed rape crops in Scotland, there is no development of phoma stem canker due to the low temperatures in winter/spring when compared to southern England (Evans *et al*., 2008). The reason for the low incidence of phoma stem canker in Scotland might also be differences in the pathogen population and other environmental factors (Stonard *et al*., 2010). Now a detailed disease forecast for phoma stem canker for England is available:

(http://resources.rothamsted.ac.uk/phoma-leaf-spot-forecast/phoma-forecast)

1.3 Severity of phoma stem canker caused by *Leptosphaeria maculans*

Leptosphaeria maculans, which especially infects *Brassica* species, is an economically important pathogen of cruciferous crops (Fitt *et al*., 2011). *Leptosphaeria maculans* is a heterothallic ascomycete that causes phoma stem canker disease of oilseed rape (Fitt *et al*., 2006a). In the *Leptosphaeria* species complex, *Leptosphaeria maculans* is the most destructive species, causing more losses in oilseed rape production than *L. biglobosa* (Shoemaker and Brun, 2001).

The *L. maculans* genome has now been published and the genomic size is 45.12 Mb, comprised of AT (Adenine and Thymine) and GC (Guanine and Cytosine) rich blocks. The *L. maculans Avr* (effector) genes encode Small Secreted Proteins (SSPs), which are present in AT-rich blocks containing effector-like proteins (effectors) and Transposable elements (TEs) that favour rapid response to selection (Rouxel *et al*., 2011; Daverdin *et al*., 2012).

The pathogen can survive after harvest for several years on crop debris remains affected by phoma stem canker and can produce both sexual and asexual fruiting bodies (pseudothecia and pycnidia, respectively). In the UK in autumn, the ascospores are discharged from pseudothecia produced on crop debris (Figure 1.12) and transmit the pathogen from one field to another field (West *et al*., 2001; Fitt *et al*., 2006c; Brun *et al*., 2010).

Leptosphaeria maculans causes stem base cankers on the oilseed rape crop (West *et al*., 2002a; Huang *et al*., 2005). Until it forms a canker at the crown of the

Figure 1.12: Pseudothecia (black spots) of *Leptosphaeria* species observed on stem samples from cultivar Incentive (susceptible) grown at Morley, Norfolk in the harvest year 2017. Pseudothecia were present both on stem base cankers and upper stem lesions.

stem, *L. maculans* does not cause any symptoms on the adult plant during the cropping season. The formation of phoma stem canker is caused by the invasion of the pathogen into the stem cells, resulting in host cell tissue death (Stonard *et al*., 2010). These cankers result in lodging and significant yield loss (Howlett *et al*., 2001) (Figure 1.13). Recently, lesions caused by *L. maculans* were observed on the pods in one of the field experiment sites (Figure 1.14).

The senescent stem tissues are colonised rapidly by *L. maculans* after harvest of the oilseed rape crop and initially asexual pycnidia are produced on them. Subsequently *L. maculans* can colonise the stubble saprophytically, thus increasing the amount of inoculum and numbers of sexual pseudothecia. Airborne ascospores are released from these pseudothecia after their development and maturation in the crop residues over an extended period. Infected seeds of oilseed rape may also initiate disease in crops (Jacobsen & Williams, 1971; Wood & Barbetti, 1977a). The other possible sources of inoculum may be the various cruciferous hosts of *L. maculans* (Hall, 1992).

Ascospores, the most common primary inoculum in the UK, are discharged from the pseudothecia that develop on the debris of affected plants over an extended period after wetting by rain and dew. Ascospores attach to the cotyledons and leaves of young oilseed rape crops. Ascospores germinate in wet or humid conditions and produce hyphae. The hyphae or germ tubes from the ascospores infect the cotyledons and leaves through stomatal pores and wounds (West *et al*., 2001).

There are two periods of symptomless growth for the pathogen *Leptosphaeria maculans*. In Europe, the first symptomless phase before the appearance of phoma leaf spots in winter oilseed rape lasts for 5 to 15 days and takes place in leaves following hyphal penetration of stomata. These hyphae are produced from airborne ascospores that infect the leaves causing phoma leaf spot lesions (Figure 1.15) (Biddulph *et al*., 1999; Toscano-Underwood *et al*., 2001; Huang *et al*., 2003; Huang *et al*., 2009).

The second symptomless period (5-6 months) takes place between the appearance of leaf lesions in autumn and appearance of cankers on stems in the

Figure 1.13: Stem lodging due to phoma stem canker disease on oilseed rape cultivar Drakkar at the Woodhall Farm, Hertfordshire in June 2016 (2015/2016 cropping season).

Figure 1.14: Large lesions with dark margins caused by *Leptosphaeria maculans* observed on the oilseed rape pods at Grove farm, Suffolk, UK in June 2018.

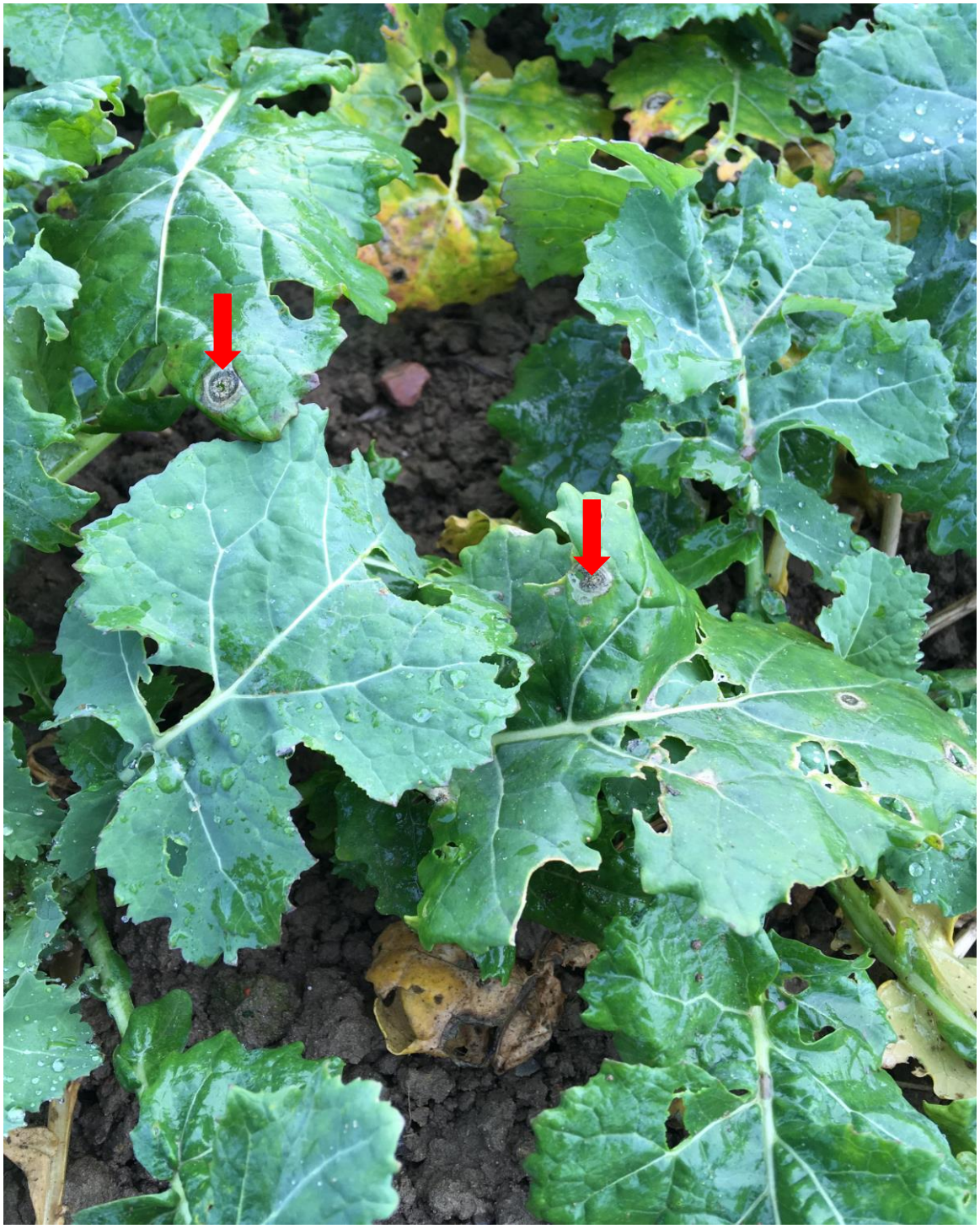


Figure 1.15: Phoma leaf spot lesions (large pale lesions with pycnidia) caused by *Leptosphaeria maculans* on a susceptible oilseed rape cultivar were observed at Stanway farm, Ipswich, UK in December 2018.

following spring, when the pathogen grows along the leaf petioles. It then colonises the host stem cortex, wood and pith tissues and then causes cankers (Hammond *et al*., 1985; West *et al*., 1999; Huang *et al*., 2005). Severe stem canker causes disruption of the free flow of nutrients and water in the plant that can result in premature (Figure 1.16), and often sudden, plant death.

1.4 Resistance against *Leptosphaeria maculans* in *Brassica napus*

A major contribution to global food security is maintaining effective resistance in plants against pathogens of arable crops (Beddington, 2010; Brun *et al*., 2010). There is a need for food security, specifically in areas of world where severe infectious disease losses are threatening production by the farmers (Fitt *et al*., 2011). To accomplish effective crop resistance, it is crucial to know about the host-pathogen relationship.

To cultivate crops with minimal fungicide applications and low production costs, use of host resistance is the best option (Brun *et al*., 2010). Resistance against *L. maculans* found in *B. napus* is of two types. The first type is a qualitative or race-specific (*R* gene-mediated) resistance operating in cotyledons (Figure 1.17) and leaves during the first symptomless phase that occurs as soon as the hyphae from the ascospores penetrate the leaves (Ansan-Melayah *et al*., 1998; Balesdent *et al*., 2001; Huang *et al*., 2009).

1.4.1 *R* gene-mediated (qualitative) resistance

R gene-mediated resistance against *L. maculans* is associated with a gene-for-gene interaction that involves the recognition of a pathogen effector (*Avr*) gene product by the host *R* gene product, thus rendering the pathogen unable to colonise the host. For example, the *L. maculans* effector gene *AvrLm1* interacts with the *B. napus* resistance gene *Rlm1* (Gout *et al*., 2006).

When the *Avr* proteins produced by the pathogen are recognized by the plant *R* proteins directly or indirectly, effectors activate the plant defence systems (Keen, 1990; Van Der Biezen and Jones, 1998). The recognition of the *Avr* protein of the

Figure 1.16: Premature yellowing of pods was observed on cultivar Mentor at Grove farm, Suffolk, UK in June 2018.

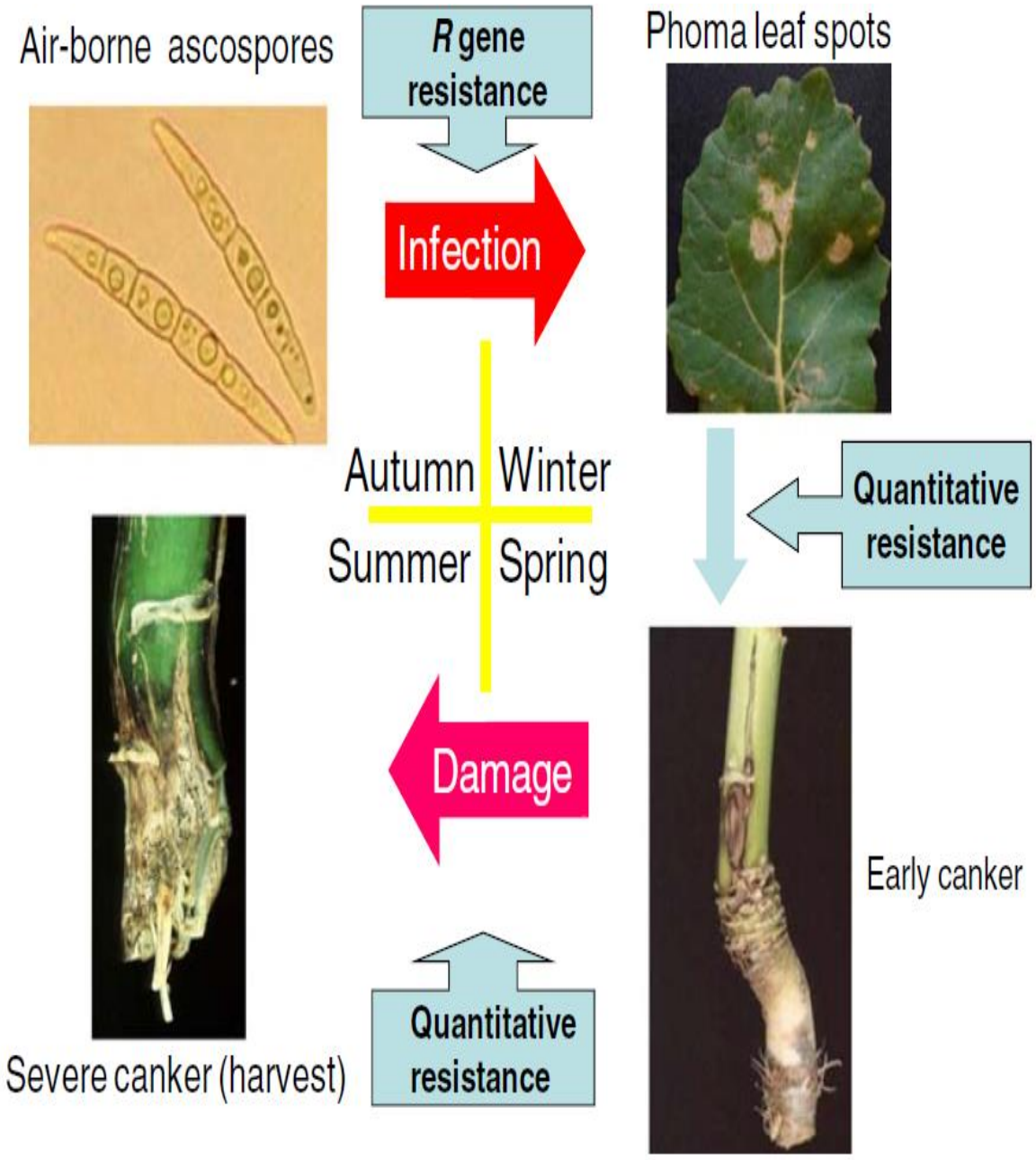


Figure 1.17: Seasonal cycle showing the relationship between phoma stem canker disease epidemics and the oilseed rape host resistance against *Leptosphaeria maculans* in the UK winter oilseed rape (Fitt *et al*., 2006a; Huang *et al*., 2010).

pathogen by the plant host protein starts an array of defence responses that usually result in a localized host cell death (Greenberg and Yao, 2004). By undergoing different molecular changes, such as point mutations, frame-shift mutations or deletion of the entire *Avr* gene, pathogens can escape host *R* gene recognition and attack plants again (Catanzariti *et al*., 2006; Jones and Dangl, 2006; Stergiopoulos *et al*., 2007).

R gene-mediated resistance (race-specific resistance) against *L. maculans* proved to be very effective initially when it was introduced into cultivars (Rouxel *et al*., 2003). However, it became clear that the *L. maculans* populations could change from avirulent to virulent within three cropping seasons when there was large-scale cropping of cultivars with specific resistance genes (Howlett, 2004). *L. maculans* has a high evolutionary potential because of its reproductive system and dispersal ability (Mitrousia *et al*., 2018). Due to this rapid adaptation of *L. maculans* populations by selection, there was a breakdown of *Rlm1* resistance of commercial cultivars in France (Rouxel *et al*., 2003) and a breakdown of Surpass 400 *Brassica sylvestris*-derived resistances in Australia (Li *et al.,* 2003; Sprague *et al*., 2006).

R gene-mediated resistance in crop cultivars is effective only when the avirulent allele of the corresponding pathogen effector gene is predominant in the local pathogen population. Therefore, such *R* genes are often not durable because the pathogen populations change from avirulent to virulent against them. Pathogens have various molecular mechanisms to alter or eliminate their *Avr* genes to avoid recognition by the host resistance genes, such as point mutations, partial or entire gene deletion and transposon insertions (Gout *et al*., 2007).

Widespread use of host resistance is the main factor selecting for pathogen population evolution from avirulent to virulent. There is evidence that introducing an *R* gene into a cultivar with a quantitative resistance background can increase the durability of the *R* gene-mediated resistance by slowing down the change in the pathogen population from avirulent to virulent (Brun *et al*., 2010).

R gene-mediated host resistance against *L. maculans* is an example of effector-triggered defence (ETD) (Figure 1.18) (Stotz *et al*., 2014). The ETD operates

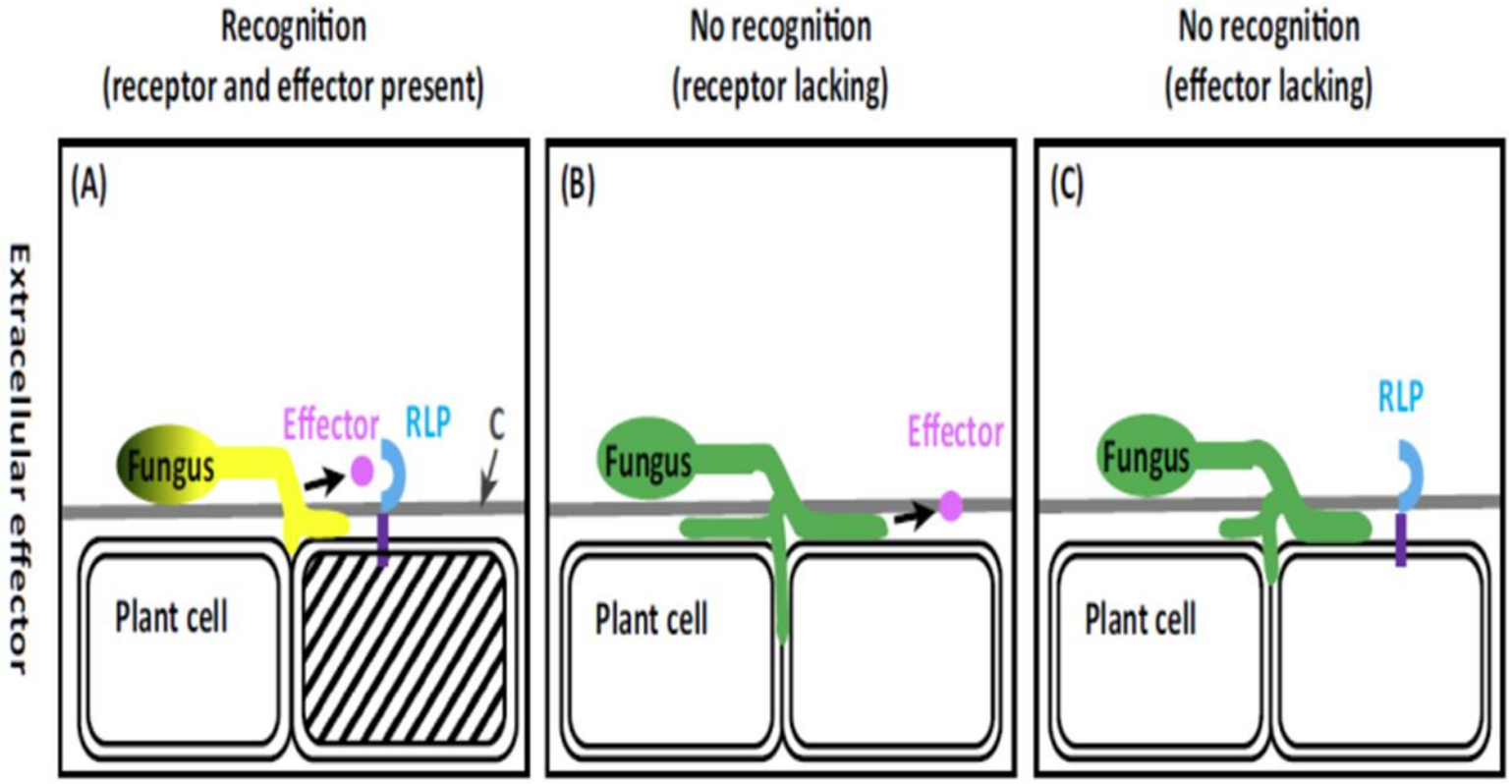


Figure 1.18: Resistance (*R*) gene-mediated effector-triggered defence (ETD) results in incompatible interactions with hemibiotrophic apoplastic fungal leaf pathogens (Stotz *et al*., 2014).

against apoplastic pathogens that are adapted to colonise the intercellular matrix of the host. The hyphae from the ascospores of *L. maculans* enter the oilseed rape leaves through stomata, and then grow between host mesophyll cells. Effectors of apoplastic pathogens are recognized at the cell surface. ETD is mediated by *R* genes, which encode cell surface-localized receptor-like proteins (RLPs) that contain the receptor-like kinase SOBIR1 (Figure 1.18).

Only after an elapsed period of endophytic pathogen growth (often >7 days) does effector-triggered defence trigger host cell death. ETD responses against *L. maculans* are comparatively slow and are not associated with a fast (within 24 hours) hypersensitive host cell death response (HR) (Stotz *et al*., 2014). In contrast, effector-triggered immunity (ETI) is initiated by intracellular recognition of effectors of intracellular obligate biotrophic pathogens by nucleotide-binding site (NBS) leucine-rich repeat (LRR) receptors (NLRs) present in the host cytoplasm (Jones and Dangl, 2006; Stotz *et al*., 2018). This recognition causes a rapid hypersensitive response (<2dpi) resulting in both host and pathogen cell death (Stotz *et al*., 2014).

Eukaryotic plant pathogens can colonise hosts to access nutrients and favour reproduction by developing different strategies. Obligate biotrophic pathogens can attain nutrition, growth and sporulation by directly penetrating and managing to live in the host cells. Necrotrophic pathogens kill the host tissues to feed on the plant dead tissues. However, extracellular hemibiotrophic fungal pathogens colonise the apoplast and grow like endophytes for a long part of their life cycle and then transform to a necrotrophic phase (Stotz *et al*., 2014). Examples of intracellular obligate biotrophic, extracellular hemi-biotrophic and necrotrophic eukaryotic pathogens of oilseed rape (*Brassica napus*) causing significant yield losses include *Plasmodiophora brassicae* (clubroot), *L*. *maculans* (phoma stem canker) and *S*. *sclerotiorum* (stem rot), respectively (Stotz *et al*., 2018).

L. maculans is an apoplastic extracellular pathogen. During the early stages of colonisation, *L. maculans* does not trigger an effective host defence response. It slowly colonises the apoplast and becomes adapted to its host's constitutive antimicrobial compounds. ETD defence responses by oilseed rape (*Brassica napus*) against *L. maculans* colonisation are slow (4-36 days after infection) and

sometimes cause a delayed cell death. These host defence responses against *L. maculans* are not able to eliminate the pathogen. The pathogen does not die but can resume growth later, after the senescence of the host begins (Stotz *et al*., 2014).

1.4.2 Quantitative resistance

Quantitative resistance (QR) is the second type of resistance that operates during the symptomless growth phase of the pathogen in the petiole and stem tissues that occurs between initial development of phoma leaf spots and the subsequent stem canker formation (Figure 1.17) (Pilet *et al*., 1998; Delourme *et al*., 2006). Quantitative resistance is partial and considered to be race non-specific as it is mediated by many minor genes. Furthermore, quantitative resistance is deemed to be more durable than qualitative resistance (Boyd, 2006; Delourme *et al*., 2006).

Quantitative resistance does not prevent infection by the pathogen during the leaf spotting stage but reduces the severity of stem canker before harvest (Huang *et al*., 2009). It is quite difficult to assess quantitative resistance in young plants; hence it is also known as ‘adult plant resistance’ (Delourme *et al*., 2006). This resistance against *L. maculans* could generally be assessed only in winter oilseed rape field experiments just before the harvest (Pilet *et al*., 1998; Fitt *et al*., 2006; Delourme *et al*., 2008). However, it is now possible to detect quantitative resistance against *L. maculans* in young plants by using a technique called ‘leaf petiole inoculation’ (Huang *et al*., 2014). Combining *R* gene and QR can provide effective, stable control of phoma stem canker in different environments (Huang *et al*., 2018).

1.5 Disease control

The main methods to control the disease are deployment of resistance genes in commercial cultivars, use of fungicides and use of cultural methods.

1.5.1 Resistant cultivars

One of the most economical and environmentally friendly methods to control crop diseases is the use of cultivar resistance against pathogens (Huang *et al*., 2018). Major resistance genes have been widely used to protect crops against fungal

plant diseases and many breeding companies have deployed them in their programmes over the last 100 years (Stuthman *et al*., 2007; Stukenbrock & McDonald, 2008). *R* gene-mediated resistance against *L. maculans* operates at the leaf infection stage to prevent phoma leaf spot lesion development, whereas QR operates later in the disease cycle to prevent development of severe phoma stem canker (Fitt *et al*., 2006b).

L. maculans develops gene-for-gene interactions with its host plant resistance (*R*) genes. A given host *R* gene is effective only when the protein coded by the *R* gene recognises an effector produced by the corresponding *L. maculans* effector gene. With both sexual and asexual reproduction, *L. maculans* has a high potential for mutation to overcome recognition by host *R* genes. Hence, combining *R* genes with quantitative resistance (QR) against *L. maculans* has been suggested as a strategy to increase the effectiveness of *R* genes (Brun *et al*., 2010; Zhang *et al*., 2017).

1.5.2 Fungicides

In addition to the use of crop resistance, fungicides are commonly used in the UK to control phoma stem canker on winter oilseed rape (Mitrousia *et al*., 2018; Sewell *et al*., 2016). Stem canker, light leaf spot and sclerotinia are the most serious diseases of oilseed rape against which fungicides are used. Disease development is very variable from year to year and spray timing is critical to ensure effective disease control.

The most effective time for the fungicide spray against phoma stem canker is when the air-borne ascospores have been released from the pseudothecia of the previous year's crop stubble. Disease forecasts, crop monitoring information and thresholds play major roles in deciding the timing of fungicides sprays in the crops. This can be achieved by applying fungicides in response to thresholds (10 to 20% plants affected by phoma leaf spots and further treatment when new phoma leaf spots are observed 4 to 10 weeks later) to decrease the likelihood of the disease spreading to the stems (cereals.ahdb.org.uk/frag).

Different combinations of fungicide seed treatments, soil fungicides or foliar fungicide sprays are used for control of phoma stem canker in different regions,

depending on the disease epidemiology and the crop economics (West *et al*., 2002). The Fungicide Resistance Action Group in the UK (FRAG-UK) has the list of all effective fungicides currently being used on oilseed rape in the UK (Table 1.1). Information about their mode of action and target site is also given (Table 1.2). Typically, azole fungicides are applied because of their effective action against *L. maculans* (Sewell *et al*., 2016) as well as their relatively low cost compared to alternatives. Examples include flusilazole, prothioconazole and tebuconazole (Eckert *et al*., 2010; Huang *et al*., 2011). Other fungicides are available to growers; these included quinone outside inhibitor fungicides (QoI) and succinate dehydrogenase inhibitors fungicides (SDHI), both of which disrupt energy production in the fungal cell (Lucas *et al*., 2015).

Legislation from the European Union has led to the withdrawal of some fungicides used to control fungal plant pathogens (Marx-Stoelting *et al*., 2014). An example is flusilazole, a chemical widely used for phoma stem canker control in the UK until 2014. The reason behind its withdrawal was evidence suggesting that flusilazole had the potential to cause liver, bladder and testicular tumours, affecting reproductive and developmental toxicity in humans (Sewell *et al*., 2016) (European Commission 2007).

1.5.3 Cultural methods

Cultural practices such as stubble management and crop rotation can be used to control disease (West *et al*., 2001). In all regions, stubble management and good crop rotation (4-year breaks between oilseed rape crops are usually recommended) decrease the risks of infection by ascospores released from colonised residue (West *et al*., 2002).

Crop residues are a source of inoculum for phoma leaf spot and stem canker (*Leptosphaeria* spp), light leaf spot (*Pyrenopeziza brassicae*) and dark leaf-spot (*Alternaria* spp). Deep ploughing to bury residues, followed by minimal tillage or direct drilling, is recommended. Direct drilled crops may, therefore, be at greater risk than crops drilled after deep ploughing. Burying crop residues can help to decrease the production of air- or splash-borne spores. It is recommended to avoid planting new crops adjacent to the previous year's stubble. If possible, it is better

Table 1.1: Fungicides applied to winter oilseed rape in the UK, published on August 2017 (FRAG-UK, Fungicide Resistance Action Group).

Fungicide groups available for use on oilseed rape

Fungicide Groups	FRAC Mode of Action Code	Chemical Families	Common name of active substance	Examples of products with active substances	
				Alone	In mixtures
DMI fungicides (DeMethylation Inhibitors) (SBI: Class I)	3	Imidazole	prochloraz	Poraz	Bumper P
		Triazole	cyproconazole	Centaur	Priori Xtra
			difenoconazole	Plover	Toprex
			metconazole	Caramba	Tectura
			propiconazole	Bumper 250 EC	Bumper P
			prothioconazole	Proline	Prosaro
			tebuconazole	Folicur	Agate
SDHI fungicides	7	Pyridine carboxamide	boscalid	Filan	Tectura Pictor
			penthiopyrad	-	Refinzar
			fluopyram	-	Propulse
			isopyrazam	-	Symetra
			bixafen	-	Skyway 285 Xpro
Qol fungicides (Quinone outside Inhibitors)	11	Strobilurin	picoxystrobin	Galileo	Refinzar
			dimoxystrobin	-	Pictor
			azoxystrobin	Amistar	Priori Xtra
Dicarboximides	2	Dicarboximide	iprodione	Rovral WG	Compass
MBC fungicides (Methyl Benzimidazole Carbamates)	1	Thiophanate	thiophanate-methyl	Topsin WG	Compass
					-
Dithiocarbamates and relatives	M3	Dithiocarbamate	thiram	Thiraflo	Hy-Pro Duet

FRAC- Fungicide Resistance Action Commitee

Table 1.2: The details of the target site and mode of action of the FRAC mode of action codes in Table 1.1.

FRAC Code	Target site	Mode of action
1	ß-tubulin assembly in mitosis	Cytoskeleton and motor protein
7	complex II: succinate-dehydrogenase	Respiration (mitochondrial complex III, ATP production, inhibition of spore germination, mycelial growth & sporulation)
11	complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (cyt b gene)	Respiration (mitochondrial complex III, ATP production, inhibition of spore germination, mycelial growth & sporulation)
3	C14-demethylase in sterol biosynthesis (erg11/cyp51)	Sterol biosynthesis in membranes
2	MAP/histidine- kinase in osmotic signal transduction (os-1, Daf1)	Signal transduction
M3	multi-site contact activity	multi-site contact activity

to isolate new crops by 200 to 500m from the previous crop location (Marcroft *et al*., 2012) (cereals.ahdb.org.uk/frag published on August 2017). Crop rotation is one of the most effective cultural practices for disease control. Winter oilseed rape was initially grown in one-in-five season rotations with cereals. Economic pressures have led to shorter rotations of one-in-two or one-in-three seasons becoming more common (AHDB oilseed rape guide, January 2014).

Another cultural practice is to avoid the risk of severe disease epidemics (Gladders and Musa 1980). Early sowing can allow the crop to have produced enough leaves, by the time ascospores are released, to evade infection at the crop's most susceptible stage (LePage & Penaud, 1995). In the UK, sowing oilseed rape seeds by late August is recommended, so that plants are well-grown prior to the onset of phoma leaf spot. The disease is less damaging and easier to manage on plants with large leaves than on plants with small leaves (cereals.ahdb.org.uk/frag published on August 2017).

1.6 Rationale

The use of *R* genes has become ever more important for phoma stem canker disease management. *R* gene-resistance against *L. maculans* is race-specific and is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent. Changes in *L. maculans* populations from avirulent to virulent can render these *R* genes ineffective leading towards severe economic losses. Therefore, information of *L. maculans* is crucial for effective use of *R* gene-mediated resistance to control phoma stem canker.

1.7 Aim and objectives

Aim: To improve understanding of *R* gene-mediated resistance against *Leptosphaeria maculans* for effective control of phoma stem canker in oilseed rape.

To achieve the aim, three aspects of effectiveness of resistance have been examined through testing three hypothesises. **Hypothesis 1**: Extensive use of oilseed rape cultivars with specific *R* genes will lead to increased frequency of the virulent alleles of the corresponding pathogen effector gene. To test this

hypothesis, *L. maculans* populations were sampled using air samplers and crops in three seasons, and the frequencies of virulent alleles of different effector genes were analysed (Objective 1- Chapter 3). **Hypothesis 2**: Molecular mechanisms of mutation towards virulence in different *L. maculans* effector genes are different. This information will help to understand the importance the effector genes for survival of pathogen and the durability of the corresponding host *R* gene. To test this hypothesis, molecular mechanisms of mutation towards virulence in four different *L. maculans* effector genes were investigated (Objective 2 – Chapter 4). **Hypothesis 3**: Host background resistance and environmental factors affect the effectiveness of *R* gene resistance. To test this hypothesis, twelve cultivars with different *R* genes in background with/without quantitative resistance or with no *R* genes were used in field experiments at different sites in three different growing seasons (Objective 3 – Chapter 5).

Objectives:

1. To monitor the UK regional distribution of races of *Leptosphaeria maculans*.
2. To investigate molecular mechanisms of mutation to virulence in *L. maculans* populations.
3. To understand effects of environmental factors on *Brassica napus* resistance against *L. maculans*.

Chapter 2 General materials and methods

2.1 Winter oilseed rape field experiments

Winter oilseed rape field experiments were set up at eight different sites in the UK (Woodhall Farm (Hertfordshire), Morley (Norfolk), Rothwell (Lincolnshire), Impington (Cambridgeshire), Trumpington (Cambridgeshire), Wisbech (Cambridgeshire), Risby (Suffolk) and West Farm Barns (Oxfordshire) from 2015/2016 to 2017/2018 cropping seasons (Figure 2.1). Twelve cultivars with different *R* genes with/without quantitative resistance in their background were selected for the field experiments; Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus (Table 2.1). Cultivar Drakkar (with no *R* gene), used as a trap crop, was also used for sampling *L. maculans* populations.

Each site had three replicate plots of each cultivar and the plots received no fungicide spray. The design was a randomised block design with three replicates. The design and plot layout were different for each site (Appendices 2-A to 2-M). The soil was as uniform as possible within each block (replicate). The size of the plots was 2m×12m. The seed rate for conventional cultivars was 80 seeds/m^2. All hybrids were sown at 70 seeds/m^2. Mean daily temperature and rainfall data were collected from each site.

2.2 Weather monitoring

The temperature and rainfall were recorded daily at all the winter oilseed rape field experiment sites (Morley, Rothwell, Impington, Trumpington, Woodhall Farm, Wisbech and Risby) (Figure 2.1) and at four Burkard spore trap sites (Rothwell, Impington, Eye and Bayfordbury) (Figure 2.2). Weather data were obtained from the nearest synoptic weather stations located nearby all the sites (Morley, Market Stainton, Wilbraham, Rothamsted, Ely, Diss and Framlingham) (Appendix 2-N). The minimum and maximum air temperatures of a day (average temperature calculated) and daily rainfall (mm) were noted by the weather stations throughout the year for three cropping seasons at all the field experiment sites. Weather

Table 2.1: List of different cultivars used in the winter oilseed rape field experiments and controlled environment experiments and details of resistance genes present in them.

S. No	Cultivar	Qualitative Resistance	Quantitative resistance	Variety
1	Drakkar	No	No	Conventional
2	Mentor	No	No	Restored hybid
3	Incentive	No	No	Restored hybid
4	Es Astrid	No	QR	Conventional
5	DK Cabernet	*Rlm1*	QR	Conventional, open-pollinated
6	Adriana	*Rlm4*	QR	Conventional
7	DK Extrovert	*Rlm7*	-	Restored hybid
8	DK Exalte	*Rlm7*	-	Restored hybid
9	Amalie	*Rlm7*	-	Conventional, open-pollinated
10	Harper	*Rlm7*	-	Restored hybid
11	Whisky	*Rlm7*	-	Restored hybid
12	Angus	*LepR3*	-	Restored hybid
13	Roxet	*Rlm7*	-	Information not available
14	Excel	*Rlm7*	QR	Restored hybid
15	Hearty	*Rlm7*	-	Information not available
16	Line 01-23-2-1	*Rlm7*	-	Conventional

- indicates information not known

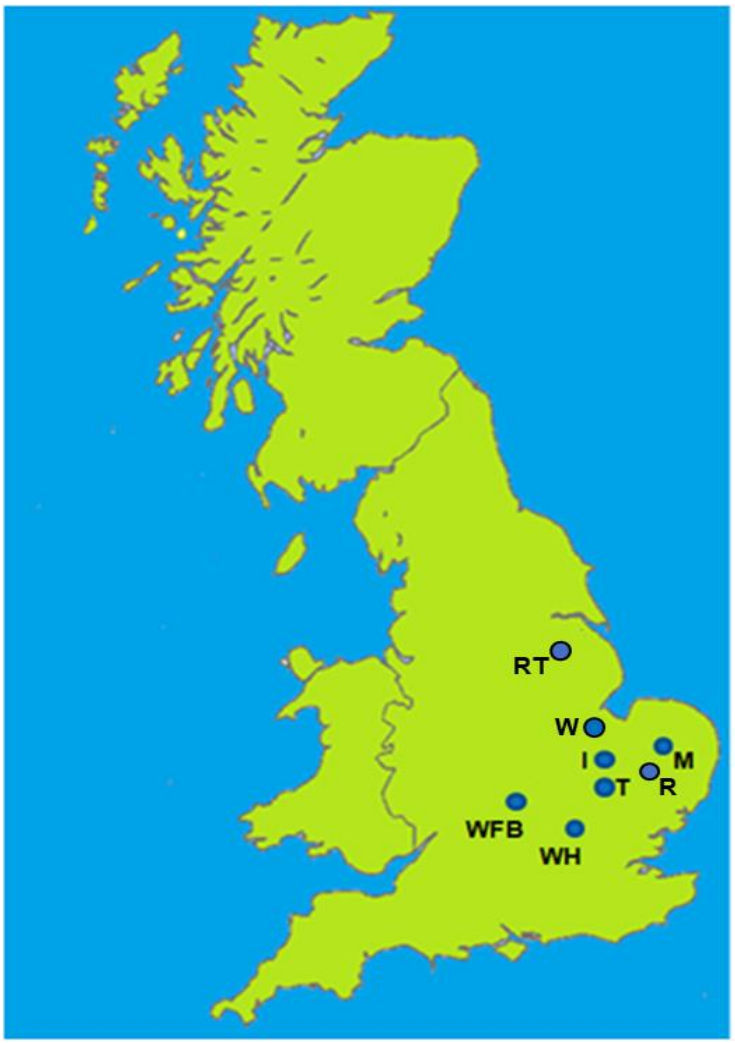


Figure 2.1: Location of winter oilseed rape field experiment sites in the UK. Winter oilseed rape field experiment sites for sampling *L. maculans* isolates, assessing phoma leaf spotting and phoma stem canker in the 2015/2016, 2016/2017 or 2017/2018 cropping seasons. The sites are represented with codes. RT- Rothwell, W- Wisbech, I- Impington, T- Trumpington, R- Risby, M- Morley, WFB- West Farm Barns and WH-Woodhall Farm.

- Morley, Rothwell, Impington, Trumpington, Woodhall Farm and West Farm Barns (field trial with one replicate of cultivars, used only for obtaining isolates) in the 2015/2016 cropping season.
- Morley, Rothwell, Woodhall Farm and Wisbech in the 2016/2017 cropping season.
- Morley, Rothwell, Wisbech, Risby and Woodhall Farm in the 2017/2018 cropping season.

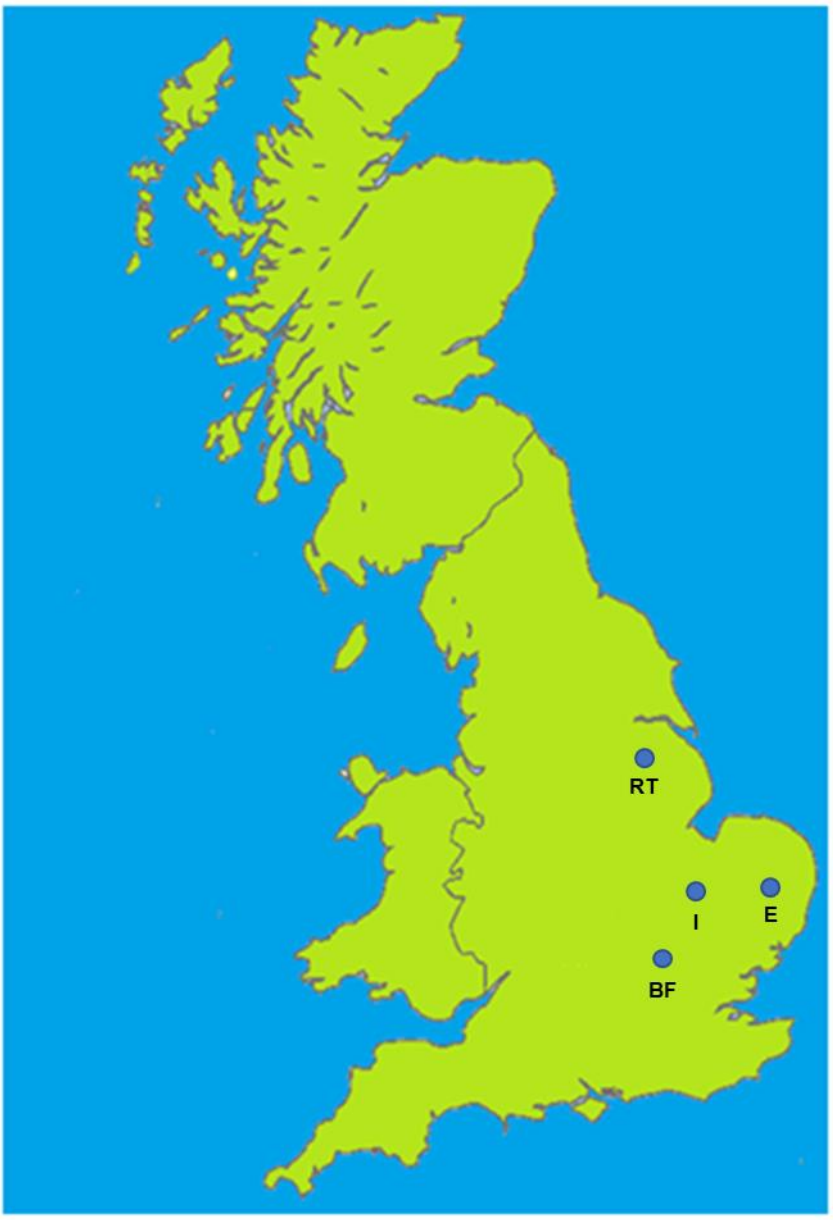


Figure 2.2: Location of the Burkard spore sampler sites in the UK. Air-borne ascospores were monitored at these sites from September to February/March each year (2015/2016, 2016/2017 and 2017/2018 cropping seasons). The sites are represented with codes. RT- Rothwell, I- Impington, E- Eye and BF- Bayfordbury.

monitoring was done from August to March in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons at the four Burkard spore sampler sites.

2.3 Isolation and identification of *Leptosphaeria species* from winter oilseed rape

2.3.1 Media preparation

The morphology and growth of *L. maculans* and *L. biglobosa* were observed on both potato dextrose agar (PDA) and V8 agar (V8A) media.

2.3.1.1 Preparation of PDA

For the preparation of PDA medium (400ml), the below ingredients were weighed.

- Potato dextrose agar (PDA) - 19.5 g
- 500 ml distilled water

The weighed PDA was transferred to a 500ml Duran bottle and 500ml of distilled water was added. The lid of the bottle was screwed on loosely. Autoclave tape was placed around the bottle to ensure autoclaving was done properly by noting its colour change. The bottle was kept in an autoclave at 121^0C for 15 min. The autoclave tape changed colour to confirm that the proper autoclaving process had occurred.

A laminar air flow hood was sterilised with ethanol and continuous air flow was maintained during the process of pouring the medium onto Petri plates. Gloves were worn, and the outside of the bottle was swabbed with ethanol; the Duran bottle containing the medium was allowed to cool after autoclaving. Subsequently, stock solutions of 100mg/ml penicillin and 100mg/ml streptomycin were prepared. The autoclaved medium was allowed to cool enough to hold (42^{o}C) and the penicillin and streptomycin solutions were added to the media with a final concentration of 0.1 mg/ml. Then the mixture was poured into labelled 9 cm diameter Petri plates. The agar was allowed to set and dry for 15 min. The plates were stored at 12-16^{o}C until required.

2.3.1.2 Preparation of V8 agar

The V8 agar medium was used for culturing *Leptosphaeria* species, both for conidial production and for harvesting mycelium. In order to prepare the medium, the ingredients were weighed and transferred to a 500ml Duran bottle.

- V8 juice - 100ml
- Calcium carbonate ($CaCO_3$) - 1g
- Agar - 10g

Four hundred milliliter distilled water was added to make up the volume. The lid of the bottle was screwed on loosely to avoid explosion in the autoclave. The medium prepared was autoclaved at 121^{o}C for 15 min and the tape of the autoclave bottle was checked for a colour change in order to ensure the accuracy of the autoclaving process. The V8 medium prepared was poured into 9 cm diameter Petri plates in the laminar air flow cabinet.

The flow cabinet was swabbed with 70% ethanol (IMS solution) and the air flow was turned on to ensure that there was a sterile environment inside the flow cabinet. Latex gloves were worn and the bottle containing cooled liquid medium was brought inside the flow cabinet. The lid of the autoclaved bottle was removed, and the neck of the bottle was flamed to ensure that no contamination had occurred while pouring into the plates.

In addition, antibiotics streptomycin and penicillin were added to decrease the risk of bacterial contamination. Penicillin (100mg/ml) and streptomycin (100mg/ml) solutions were prepared. Then the penicillin and streptomycin solutions were added to the media with a final concentration of 0.1 mg/ml. Then the bottle containing media was swirled gently to remove the bubbles before the media was poured into 9 cm diameter Petri plates. The plates were labelled and stored at 12-16^{o}C until required.

2.3.1.3 Preparation of 2% water agar media

The water agar medium was used for isolating single ascospores released from the stem samples. For the preparation of the medium, 10g of agar was weighed

and poured into a 500ml Duran bottle. Five hundred milliliter of distilled water was added to the bottle, mixed well and the medium was autoclaved at 121^{o}C for 15 min. The autoclaved medium was allowed to cool enough to hold (42^{o}C), and the penicillin and streptomycin solutions were added to the media with a final concentration of 0.1 mg/ml. Then the mixture was poured into labelled 9 cm diameter Petri plates in a flow cabinet. The agar was allowed to set and dry for 15 min. The plates were stored at 12-16^{o}C until required.

2.3.2 Isolation of *Leptosphaeria maculans* from phoma leaf spot lesions

The *L. maculans* isolates from the phoma leaf spot samples were obtained by the method of West *et al*. (2002). The leaves with phoma leaf spots from cultivar Drakkar from different sites were used for obtaining *L. maculans* isolates. Species identification of phoma leaf spots was done based on visual identification, as *L. maculans* causes large pale leaf lesions with pycnidia and *L. biglobosa* causes smaller, darker lesions (Fitt *et al*., 2006a) (Figure 2.3).

The leaves were washed under running tap water to remove the mud and other particles. An area of leaf with a phoma spot was cut from each of 10 leaves of each of the cultivars (Figure 2.4a). The leaf pieces were placed in Petri dishes on Whatman No.1 filter paper that had been sprayed with distilled water (Figure 2.4b). The leaves were incubated for 3-5 days at 20°C under alternating 12h light/12h darkness to induce pycnidial production.

A dissection microscope was used to observe the *L. maculans* pycnidia and single pycnidial isolates were obtained by sampling the cirrhi from mature pycnidia by using a fine needle (Figure 2.4c). This was done under sterile conditions in a laminar air flow chamber. Each cirrhus was transferred to a drop of sterilized water to make a spore suspension and then onto PDA (Potato Dextrose Agar) medium (OXOID, UK) (amended with 80mg/ml of penicillin and 40mg/ml of streptomycin) with a pipette. Plates with spore suspensions were incubated at 20°C in darkness for 3-5 days to produce colonies of the pathogen (Figure 2.4d).

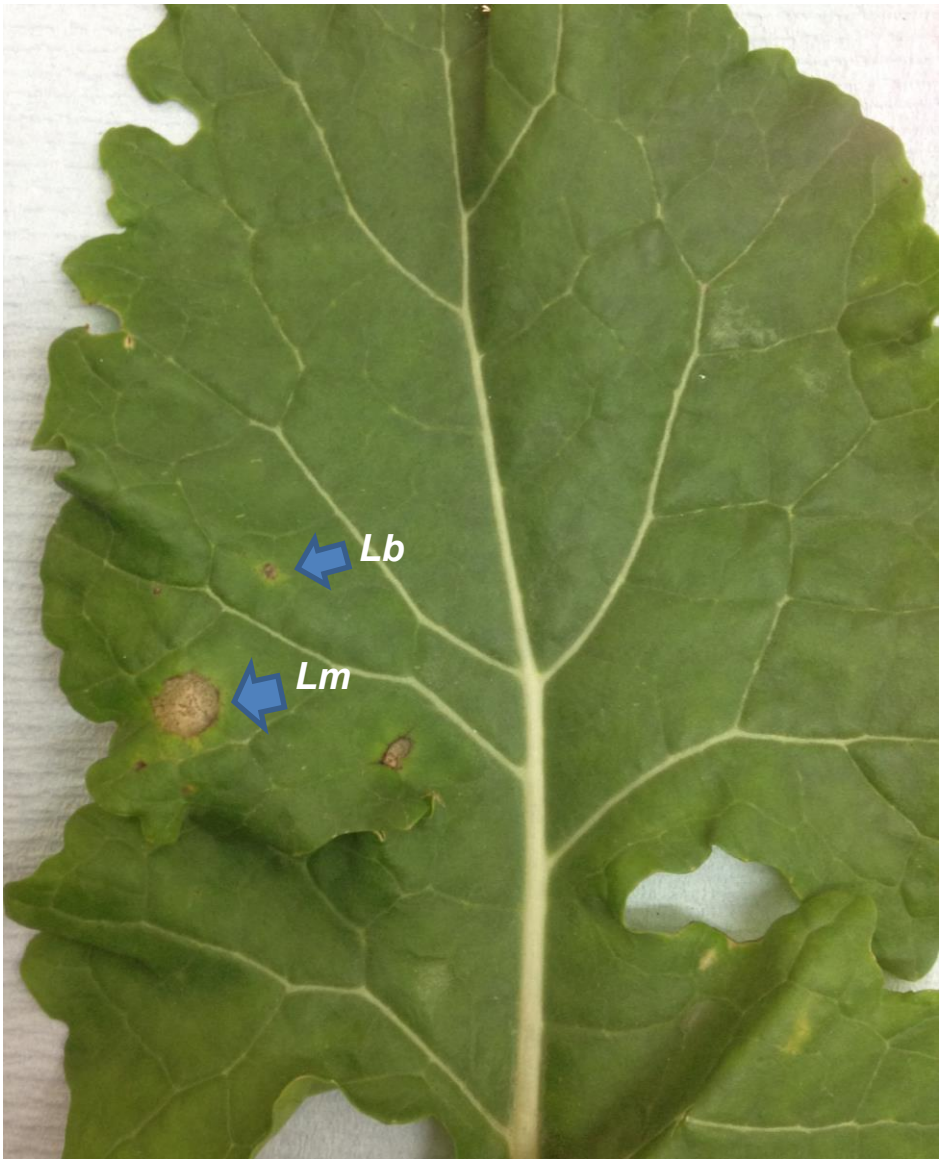


Figure 2.3: Oilseed rape leaf from cultivar Adriana at Woodhall farm site showing phoma leaf spots caused by *Leptosphaeria maculans* and *Leptosphaeria biglobosa* (2015/2016 cropping season; 13 November 2015). *L. maculans* (*Lm*) caused a large pale lesion with pycnidia, whereas *L. biglobosa* (*Lb*) caused a small dark lesion.

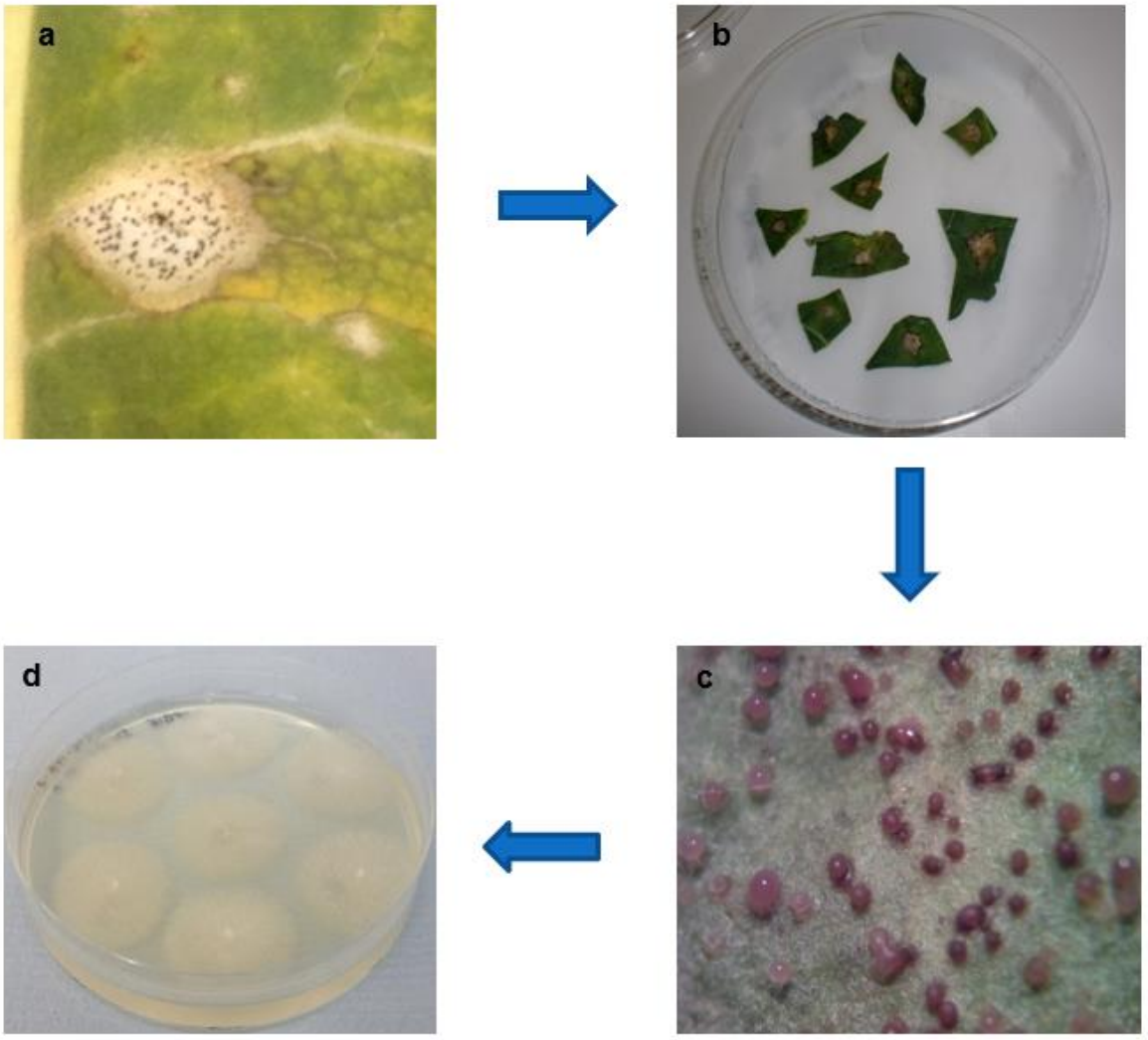


Figure 2.4: The procedure for the single pycnidial isolation to obtain isolates of *Leptosphaeria maculans*. Leaf lesions caused by *L. maculans* (a) were incubated in a Petri dish lined with Whatmann filter paper (b). After 2-3 days of incubation the cirrhus produced from a pycnidium (c) was mixed with sterile distilled water and pipetted on a PDA agar media plate. *L. maculans* cultures were observed after 4-5 days of incubation (d).

Identification of the species of the pathogen isolates was based on observation of morphological characteristics of the colony of the isolate and the pigment production (Fitt *et al*., 2006a). Cultures of *L. maculans* showed no pigmentation, whereas those of *L. biglobosa* produced a yellow pigment with many pycnidia on PDA medium. After identification, *L. maculans* isolates were then subcultured onto V8 agar media plates and incubated for 14 days in a controlled environment (CE) cabinet to induce conidial production (Figure 2.5).

2.3.3 Isolation of *Leptosphaeria maculans* from stem samples

To investigate whether the *L. maculans* ascospores identified from those *Rlm7* cultivars were virulent or avirulent against *Rlm7*, *L. maculans* isolates were obtained from the stem samples of *Rlm7* cultivars. A single ascospore isolation method was used to obtain *L. maculans* isolates from stem samples. Stem samples were collected before harvest from the winter oilseed rape field experiment sites at Wisbech (Cultivars: DK Extrovert, Whisky, Harper and DK Exalte) and Morley (Cultivars: DK Extrovert, Whisky, Harper, DK Exalte, Amalie, Angus, Drakkar and Incentive) in the 2016/2017 cropping season. The stem samples were incubated in natural weather conditions from June 2017 to November 2017.

Then the stems were allowed to dry to remove any moisture and viewed under a dissection microscope to observe the mature pseudothecia (Figure 2.6b). Stem samples with mature pseudothecia on stem base cankers or upper stem lesions were selected from each cultivar. Stem pieces were cut, with dimensions 4cm×0.6cm. The number of mature pseudothecia per square centimetre on each stem piece was subsequently counted under a dissection microscope at x3 magnification. Mature pseudothecia are larger than pycnidia but they are otherwise similar in colour and shape (Figure 2.6b).

Three stem pieces with mature pseudothecia were selected and attached to the lid of a 2% water agar media plate by using vaseline (Figure 2.6a). Water was sprayed on stem pieces and left for 5 min to induce ascospore release (Figure 2.6c). Then water was removed and the lid with stem samples was placed back on the water agar media plate (Figure 2.6d). After 2 h of incubation, single ascospores were

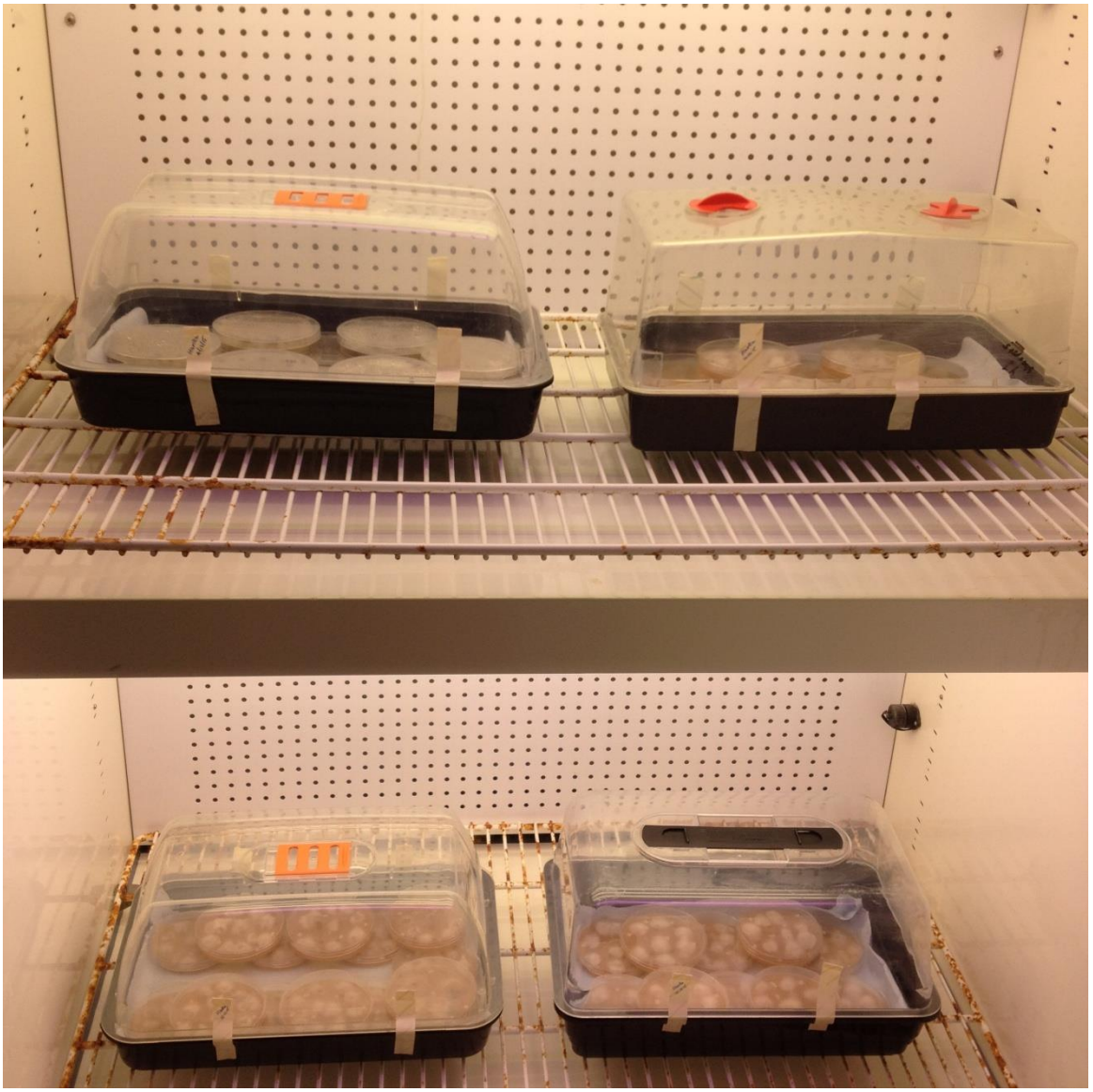


Figure 2.5: Sub-cultured plates incubated in a CE cabinet for conidial production. Isolates were moved from the incubator to the CE cabinet 4 days after sub-culturing. An alternating 12h light/ 12h darkness and a temperature of 20^{o}C were maintained in the CE cabinet to initiate conidial production.

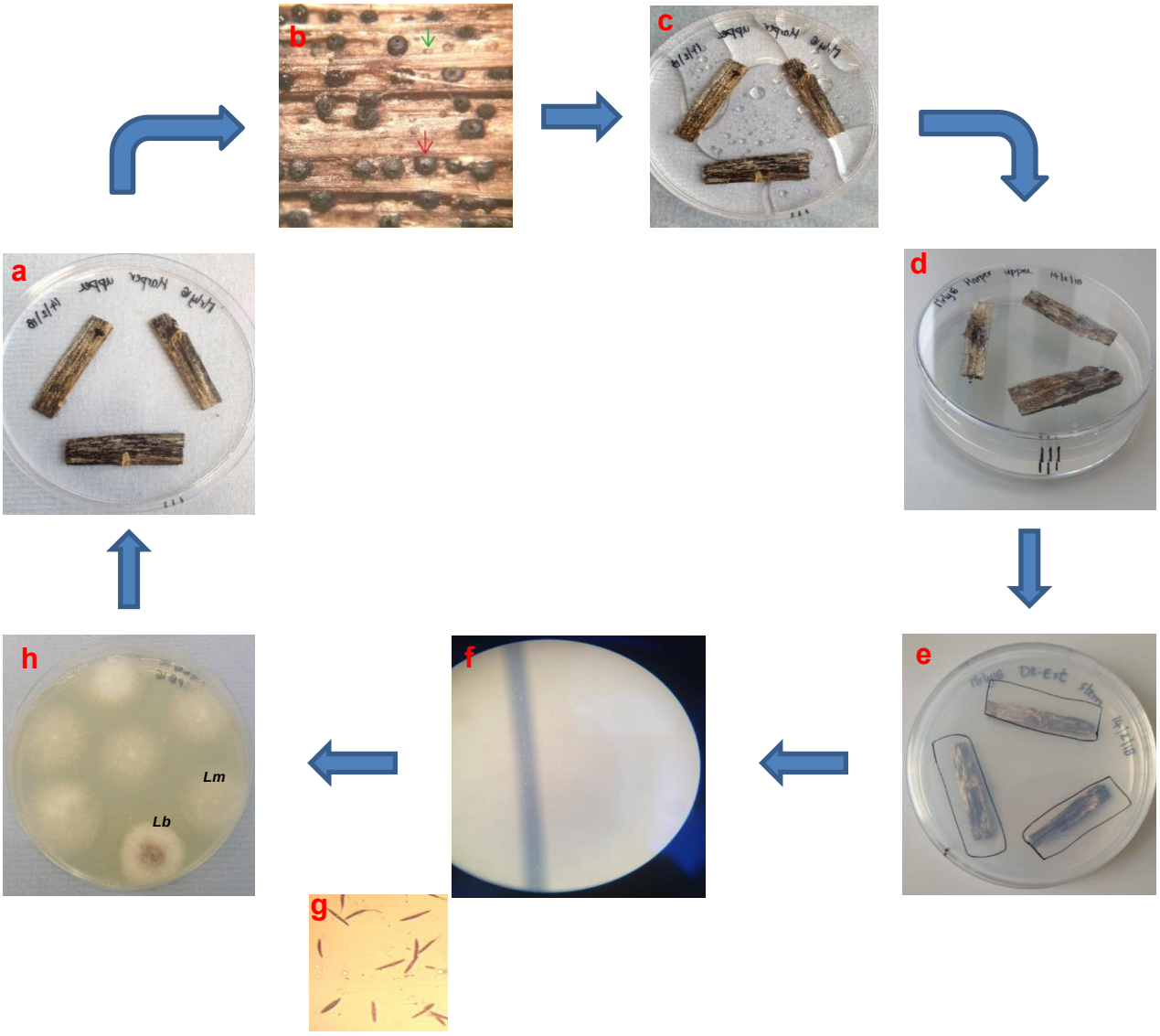


Figure 2.6: Single ascospore isolation of *Leptosphaeria maculans*. Stem pieces (a) with mature pseudothecia (b) attached to lid of Petri-dish. Pseudothecia are highlighted using a red arrow, pycnidia are highlighted using a green arrow (b). Pycnidia present an overall morphology similar to pseudothecia but are flat and smaller in size. Water was sprayed on stem pieces to induce ascospore release (c). After 5 min, water was removed, and lid was placed back on to the water agar plate (d). After 2 h, single ascospores (f) were observed under the microscope on the water agar media and cultured on a PDA plate. *Leptosphaeria* isolates were observed after 7-days post isolation (h). *Leptosphaeria* spores with tryphan blue stain (g).

released onto the water agar media that was observed under a dissection microscope (Figure 2.6f). By using a sterile scalpel, single ascospores were cultured on a PDA plate. *Leptosphaeria* isolates were observed 7-days post isolation (Figure 2.6h). After identification, *L. maculans* isolates were then subcultured onto V8 agar media plates and incubated for 14 days in a controlled environment (CE) cabinet to induce conidial production (Figure 2.5).

2.3.4 Preparation of conidial suspensions of *Leptosphaeria maculans* isolates

This process was done under sterile conditions in the laminar air flow chamber. The V8 plates with fungal sub-cultures for conidial production were collected and placed in the flow chamber (Figure 2.7a). Sterile 15ml tubes were labelled with the names of the isolates and the dates. A sterile glass funnel was placed on the top of the 15ml tube using sterile forceps. A double layer of sterile Mira-Cloth was placed inside the funnel using 1ml of sterile distilled water to make it adhere to the funnel (Figure 2.7b). 4ml of sterile distilled water was added to each plate (Figure 2.7c) and spread around the plate using a sterile Lazy-L spreader to ensure that the conidia were released into the water (Figure 2.7d). The conidial suspension was filtered through it into a 15ml tube (Figure 2.7e, f & g).

The concentration of the conidial suspension was measured using a Bright-Line haemocytometer slide (Sigma Aldrich, USA). After shaking the 15ml tube to mix the conidial suspension, a 7µl sample of the suspension was used to count the number of spores per small square at 40x magnification. The concentration of the conidial suspension was calculated by counting the number of conidia in ten randomly chosen small squares. The data were recorded and calculated based on the formula below:

Number of spores per small square × 4 × 10^6 = Number of spores per ml

Then the conidial suspensions were stored at -20^0C for subsequent use in a cotyledon test.

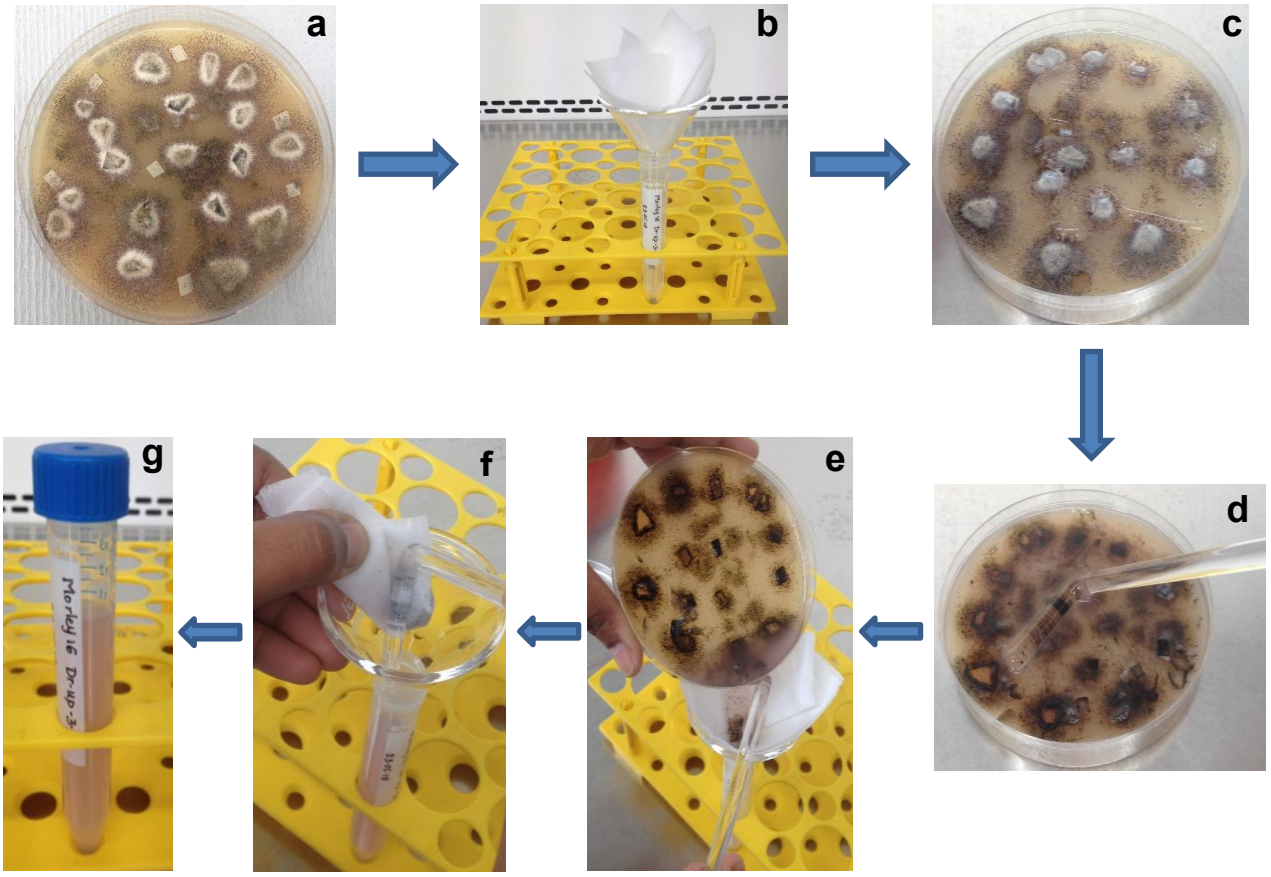


Figure 2.7: Preparation of conidial suspensions of *Leptosphaeria maculans* isolates. The V8 plate with a fungal sub-culture was placed in the flow chamber (a). A sterile glass funnel was placed on the top of the 15ml tube and a double layer of sterile Mira-Cloth was placed inside the funnel (b). Sterile distilled water added to plate (c) and spread around the plate using a sterile Lazy-L spreader to ensure that the conidia were released into the water (d). The conidial suspension was filtered through it into a 15ml tube (e, f, g).

2.4 Controlled environment experiments

2.4.1 Growth of plants

The seeds were pre-germinated by incubating them in a Petri dish lined with Whatman filter paper, sprayed with water. John Innes No.3 and Miracle grow compost were mixed in equal proportions in volume (v/v) and used to fill 40-compartment trays, which were placed in plastic trays layered with capillary matting to retain moisture. The germinated seeds were sown in the 40-compartment trays and watered regularly. The trays were kept in the glasshouse until the plants were ready for inoculation.

2.4.2 Cotyledon inoculation with wounding

Leptosphaeria maculans isolates were used to inoculate cotyledons of 13-day old oilseed rape plants. Point inoculation of cotyledons with wounding was used in this study. Any true leaves produced subsequently were cut off using scissors and sticks were used to hold the plants to make sure that the cotyledons of each plant did not touch each other and that the drops of inoculum stayed on the cotyledons (Figure 2.8).

A small hole was punctured in the edge of one of the cotyledon lobes of each seedling with a Pasteur pipette to mark that cotyledon lobe as 1 (Figure 2.9). A sharp pin was used to gently wound the centre of each cotyledon lobe without passing through it.

A Gilson pipette (2-20µl volume) was used to place 11µl of the conidial suspension directly on the wound site on each lobe of the cotyledon (Figure 2.10). All the inoculation sites were assigned numbers to identify the type of inoculum. After all the plants had been inoculated with different isolates, the leaves were sprayed gently with distilled water. The tray cover was also sprayed with distilled water to maintain high humidity. The trays were covered with lids and transferred into plant growth chambers.

The seedlings were kept under high humidity and darkness for 72 h. After 72 h, the lids were removed, and the seedling trays were transferred into plant growth rooms at Bayfordbury (with a light intensity of 210µe m^{-2} s^{-1}, 80-85% relative

Figure 2.8: Trays with 13-day old seedlings of the *Brassica* cultivars before inoculation with the *Leptosphaeria maculans* spore suspensions.

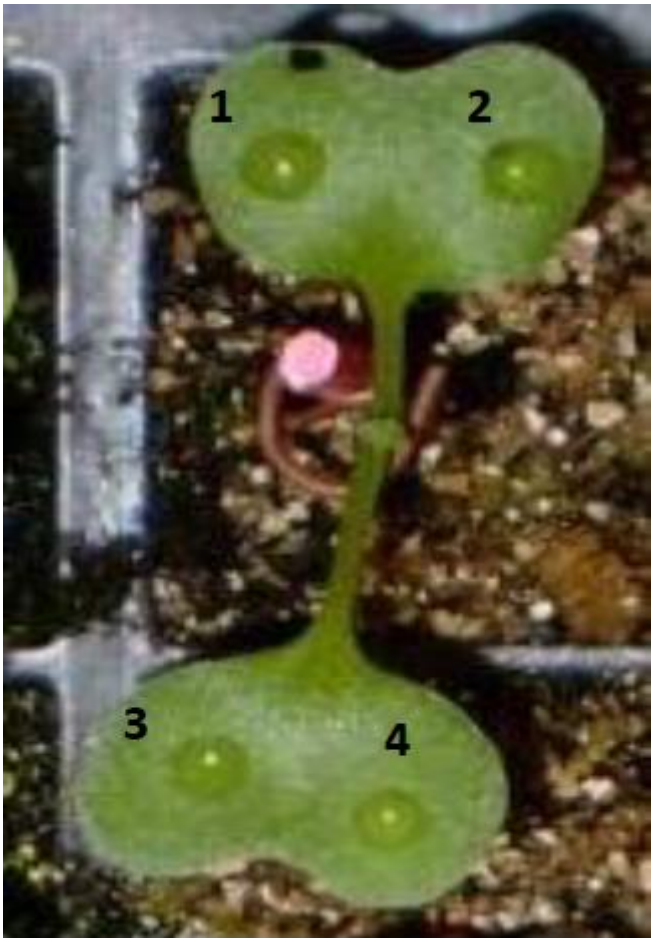


Figure 2.9: Schematic representation of inoculation plan on the two cotyledons of each *Brassica* seedling. 1-4 represent the four inoculation sites for four different *Leptosphaeria maculans* spore suspensions. A small hole was punctured in the edge of one of the cotyledon lobes of each seedling with a Pasteur pipette to mark that cotyledon lobe as 1.

Figure 2.10: Tray with 14-day old seedlings of the *Brassica* cultivars immediately after inoculation with the spore suspensions of *Leptosphaeria maculans*.

humidity and a 12 h day-length at 20^{o}C; optimal conditions for cotyledon-stage growth). The plants were sprayed twice every day with distilled water to encourage symptom development and watered whenever necessary.

The disease development on plants was monitored by observing the lesions produced on each lobe of each of the cotyledons every day until the plants died (Toscano-Underwood *et al*., 2001). The lesions were scored at 17 days-post-inoculation (Figure 2.11). A 0-9 scale (modified from Koch *et al*., 1991) was used to score the disease severity on cotyledons of *B. napus* at 17-days post inoculation (Figure 2.12).

The phenotype of the response to the *L. maculans* isolates was given as resistant when the score was 1-3, intermediate when the score was 4-5 or susceptible when the score was 6-9. If the *L. maculans* isolate developed a resistant/intermediate interaction on a cultivar with a specific *R* gene, then it was considered to have the avirulent allele of the corresponding *Avr* gene. When it developed a susceptible interaction, then the isolate was considered to have the virulent allele of the corresponding *Avr* gene.

2.5 Agarose gel analysis of PCR products

2.5.1 Preparation of a 1.5% agarose gel

Agarose gel was used to view the PCR products. The gel casting tray was rinsed and dried with 70% IMS (Industrial Methylated Spirit). Tape was placed on ends of the casting tray. Then the casting tray was set on a level surface. Combs were placed on the casting tray to form wells in the agarose gel.

Approximately 1.3 g of agarose was weighed and poured in a sterile conical flask. 90ml of TBE buffer was added to the conical flask and heated in a microwave until a clear solution was formed. Then the solution was allowed to cool down and poured into the casting tray. The gel was allowed to solidify for about 10 min. The combs and tape were removed carefully.

Figure 2.11: Phoma leaf spot disease symptoms on cotyledons of *Brassica* cultivars or lines at 17 days post inoculation with *Leptosphaeria maculans* conidial suspensions.

Figure 2.12: The 0-9 scale (modified from Koch *et al*., 1991) used to score the severity of phoma leaf spot disease on cotyledons of *Brassica napus* at 17-days post inoculation. If there was no darkening around the wounds, as in controls, score 0 was given. Score 1 was for limited blackening around wounds (lesion diameter 0.5-1.5 mm). Score 3 for dark necrotic lesions (1.5-3 mm). Score 5 was given if there were dark lesions (3-6 mm) that were brownish on the lower surface. If the lesions were like those that scored 5, but less necrotic, score 6 was given. Grey-green lesions of limited size or large necrotic lesions scored 7. Score 8 was given if the lesions were grey-green in colour with greater diameter (>6 mm) with no or few pycnidia. Large grey-green lesions with profuse sporulation were given score 9.

2.5.2 Loading and running the gel

The PCR samples stored at -20^{o}C were taken out. The casting tray (with the gel on it) was inserted into the electrophoresis chamber with the wells closest to the negative (black) electrode. As the DNA is negatively charged, during electrophoresis it migrates from the negative to the positive electrode. 1xTBE (Tris-Borate-EDTA; electrophoresis buffer) was added to the chamber until the buffer just covered the top of the gel.

A volume of 15µl of the sample was loaded into each well carefully without puncturing the well bottom. Loading dye was not added as the samples already contained it. An aliquot of 5µl of DNA ladder was also added to one of the wells in a row. The power pack was adjusted to 100V for 60 min or 120V for 40 min.

2.6 Statistical analysis

The data obtained from respective experiments were analysed by using GenStat 17th edition statistical software (Payne *et al*., 2011), R software (Source: https://www.r-project.org) and Microsoft Excel. Specific details about the statistical analysis of each experiment are described in relevant chapters.

Chapter 3 Monitoring regional distribution of races of *Leptosphaeria maculans*

3.1 Introduction

Phoma stem canker disease is initiated by the infection of leaves by hyphae from air-borne ascospores released from the mature pseudothecia that develop on the previous season's crop stubble (Figures 1.12 & 1.17). The hyphae penetrate the leaves through stomata and start colonising the leaf tissues, causing phoma leaf spots. Temperature and rainfall are the two main factors that play a major role in pseudothecial maturation during the period between harvest of the previous crop and establishment of the new crop (Evans *et al*., 2008).

They determine the timing of phoma leaf spotting in that cropping season as they influence the maturation of pseudothecia (fruiting bodies), release of ascospores (sexual spores), dispersal of ascospores, germination (development of hyphae) and infection of cotyledons or leaves (through stomata) (Evans *et al*., 2010). Ascospores are more infectious than conidia (asexual spores) (Rouxel and Balesdent *et al*., 2005) and can travel long distances (Travadon *et al*., 2011). Models have predicted that global warming will increase the range and severity of phoma stem canker disease epidemics (Evans *et al*., 2008; Butterworth *et al*., 2010). Therefore, monitoring the ascospore release patterns in that particular area is the first crucial step for phoma stem canker disease management.

In this study, ascospore release patterns were monitored at four different sites in the UK that were nearby the winter oilseed rape field experimental sites. The frequencies of avirulent *AvrLm1* and *AvrLm6* alleles were detected in the UK *L. maculans* spores during 2006/2007, 2007/2008 and 2008/2009 cropping seasons (Van de Wouw *et al*., 2010). It was predicted that both *AvrLm1* and *AvrLm6* genes are expressed during the early plant infection by *L. maculans* (Gout *et al*., 2007; Fudal *et al*., 2009). As both these genes have been cloned and the main event of mutation leading to virulence is deletion of the corresponding *Avr* gene, it is not difficult to detect them by using basic molecular biology techniques (Gout *et al*., 2006). As these two genes might play an important role in the infection of leaves

by ascospores, in this study, their frequencies were monitored in the air samples to identify any differences in frequencies that have occurred in the time-period.

Use of host resistance to control this disease is becoming ever more important (Fitt *et al*., 2011; Delourme et al., 2006). Resistance against *L. maculans* is composed of major (*R*) gene-mediated resistance and quantitative resistance. *R* gene-mediated resistance against *L. maculans* is race-specific and is associated with a gene-for-gene interaction that involves the recognition of the pathogen effector (*Avr*) gene product by the host *R* gene product, thus rendering the pathogen unable to infect the host. *R* gene-mediated resistance operates in cotyledons and leaves during the first symptomless phase that occurs after the hyphae from the ascospores penetrate the leaves.

R gene-mediated resistance is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent (e.g. *Rlm1* in cultivar Capitol in France; Rouxel *et al*., 2003) (e.g. *LepR3* in Surpass 400 in Australia; Sprague *et al*., 2006). In the UK, this causes losses not only to farmers (yield loss >£100M pa) but also to breeders (loss of germplasm when resistance breaks down). Therefore, detection and identification of new virulent races of *L. maculans* is crucial for effective deployment of *R* gene-mediated resistance. Deployment of *R* genes is more effective when they are used in a rotation guided by monitoring the current races of the pathogen *L. maculans* in each region and using only the *R* genes that are effective in that region. Removing *R* genes from use before they become widely ineffective also contributes to increasing the durability of these *R* genes (Bent & Mackey, 2007).

The durability of resistance depends mostly on the pathogen population structure, capability of the pathogen for population evolution, crop rotation strategies and stubble management (McDonald & Linde, 2002; Aubertot *et al*., 2006). For anticipating and managing the risk of severe disease epidemics, observing the frequency of avirulent and virulent alleles within fungal populations plays an important role in guiding the effective deployment of *R* genes. Pathogenicity testing can be used to determine *L. maculans Avr* genotypes on oilseed rape cultivars with corresponding resistance genes (Van de Wouw *et al*., 2010).

As *Leptosphaeria maculans* is the worldwide causal agent of phoma stem canker, monitoring of new races (different combinations of *Avr* alleles) of *L. maculans* has been done in the past in western Canada (Liban *et al*., 2015; Zhang *et al*., 2016), Australia (Balesdent *et al*., 2006), Europe (Stachowiak *et al*., 2006) and UK (Huang *et al*., 2018; Mitrousia *et al*., 2018). By knowing the importance of monitoring the *L. maculans* races every year, some countries have included the regional race structure information in selecting oilseed rape cultivars for recommendation each year e.g. France (Terres Inovia) (www.terresinovia.fr), Australia (GRDC) (Grains Research and Development Corporation) (www.grdc.com.au) and Canada (Canola Council of Canada) (https://www.canolacouncil.org).

In the UK, AHDB gives a phoma stem canker resistance rating to all the 'recommended list' cultivars every year based on data from a regional series of field experiments over several years (Source: AHDB Recommended lists 2015/2016 for cereals and oilseeds) (www.ahdb.com). Resistance to lodging, stem stiffness, shortness of stem, earliness of flowering and earliness of maturation are the agronomic features taken into consideration by the AHDB during the selection of specific cultivars for the recommended list. The year of introduction and the recommended list status of the cultivar should be checked by farmers when selecting a cultivar for growing on their farms (AHDB Recommended Lists 2015/2016 for cereals and oilseeds).

However, the recommended list does not give any information about the pathogen races present in different regions or specific *R* genes in different cultivars. A detailed understanding of the changes in the pathogen populations and effector gene alleles by annual monitoring would help in the exploitation of the required *R* genes (Gladders *et al*., 2006). Previous work done in the UK on *L. maculans* populations has involved a limited number of *R* genes (Stachowiak *et al*., 2006, Huang *et al*., 2018; Mitrousia *et al*., 2018). This study tested four different *R* genes (*Rlm1*, *Rlm4*, *Rlm7* or *LepR3*) used in commercial cultivars (that are currently being used in the UK) in different environments over three cropping seasons. Therefore, this work can provide more useful information for breeders and growers. This study is the first in the UK to suggest including information about *L. maculans* races every year in the recommended list of oilseed rape cultivars by the AHDB. There is a UK

cereal pathogen virulence survey (UKCPVS) (to monitor changes in virulence of rust and mildew populations in cereal crops in the UK) managed by NIAB and funded by AHDB and Defra (https://ahdb.org.uk/ukcpvs). In this study, frequencies of avirulent alleles of *L. maculans* were monitored both in air and in crops.

3.1.1 Objectives

Hypothesis 1: Extensive use of oilseed rape cultivars with specific *R* genes will lead to increased frequency of the virulent alleles of the corresponding pathogen effector gene. To test this hypothesis, there are three objectives.

Objective 1. To monitor the timing of release of *Leptosphaeria* ascospores in the air at different sites in the UK. Phoma stem canker epidemics are initiated from ascospores, the timing of ascospore release is crucial for effective control of the epidemics by fungicide. Previous studies showed that the timing of ascospore release varied between seasons and between regions. Therefore, four air samplers were set up at four different sites over three seasons to investigate whether there are differences between sites and between seasons in timing of ascsopores release. This information is important for development of strategies for effective control of the disease.

Objective 2. To monitor the frequencies of *AvrLm1* and *AvrLm6* alleles in the *L. maculans* ascospore populations. Previous studies showed that the resistance mediated by the resistance gene *Rlm1* is partly breakdown while the *Rlm6* is still effective in the UK. Therefore, the frequencies of avirulent alleles of the corresponding *AvrLm1* and *AvrLm6* effector genes in the released *L. maculans* ascospores are important for control the disease either by deployment of resistant cultivars or by fungicides. The resistance gene *Rlm6* has not been used commercially in the UK, the frequency of *AvrLm6* should be still predominant in the *L. maculans* populations. Results of this Objective will test the Hypothesis 1.

Objective 3. To characterise *L. maculans* isolates from diseased oilseed rape crop leaves and stems at different sites in the UK and identify the regional distribution of *L. maculans* races. *L. maculans* infects the leaves causing phoma leaf spots in the autumn, then grows along the leaf petiole to the stem causing phoma

stem cankers in the summer. Therefore, in addition to characterise *L. maculans* isolates from diseased oilseed rape crop leaves, there is a need to investigate whether *L. maculans* can reach stem base and cause cankers. The *Rlm7* resistance gene is currently the most effective resistance gene used in the UK, thus whether *L. maculans* can cause severe stem base cankers in oilseed rape cultivars containing *Rlm7* resistance gene and subsequently produce pseudothecia on stem debris was investigated in this study. With the wide use of *Rlm7* resistance gene in the UK, the frequency of virulent *L. maculans* isolates from both leaf lesions and stems should be increased. Results of this Objective will test the Hypothesis 1. In addition, previous studies showed that *L. maculans* is mainly associated with stem base cankers whereas, *L. biglobosa* mainly associated with upper stem lesions. Therefore, there is also a need to investigate whether pseudothecia on stem bases and upper stems of different cultivars contain ascospores of *L maculans* or *L. biglobosa*.

3.2 Materials and methods

3.2.1 Weather conditions at the Burkard spore sampler sites

Weather data were collected over the three cropping seasons 2015/2016, 2016/2017 and 2017/2018 at four Burkard spore sampler sites (Bayfordbury, Hertfordshire; Langton Green Eye, Suffolk; Rothwell, Lincolnshire; and Impington, Cambridgeshire) in the UK (Figure 2.2); located near the winter oilseed rape field experiments (Figure 2.1). Air temperature and rainfall data were collected daily using automated weather stations (Campbell Scientific, UK).

3.2.2 Monitoring release of air-borne ascospores

The air-borne ascospores from field experiments at four different sites (Bayfordbury, Hertfordshire; Langton Green Eye, Suffolk; Rothwell, Lincolnshire; and Impington, Cambridgeshire) (Figure 2.2) in the UK were collected for three winter oilseed rape growing seasons (2015/2016, 2016/2017 and 2017/2018). Each year, spore collection was done in autumn-winter, from September until February (Table 3.1). The spores were collected by using Burkard seven-day volumetric spore samplers (Burkard Manufacturing Company Ltd, Rickmansworth, UK) (Figure 3.1).

Table 3.1: The period of collection of air-borne ascospores using Burkard spore samplers at four sites in three growing seasons.

Site	2015/2016 season	2016/2017 season	2017/2018 season
Impington, Cambridgeshire	16 Sept 2015 - 20 Feb 2016	21 Sept 2016 - 07 Mar 2017	21 Sept 2017 - 13 Mar 2018
Bayfordbury, Hertfordshire	09 Sept 2015 - 16 Feb 2016	14 Sept 2016 - 27 Feb 2017	06 Sept 2017 - 13 Mar 2018
Rothwell, Lincolnshire	23 Sept 2015 - 29 Feb 2016	13 Sept 2016 - 27 Feb 2017	12 Sept 2017 - 28 Feb 2018
Eye, Suffolk	14 Sept 2015 - 15 Feb 2016	19 Sept 2016 - 28 Feb 2017	18 Sept 2017 - 11 Mar 2018

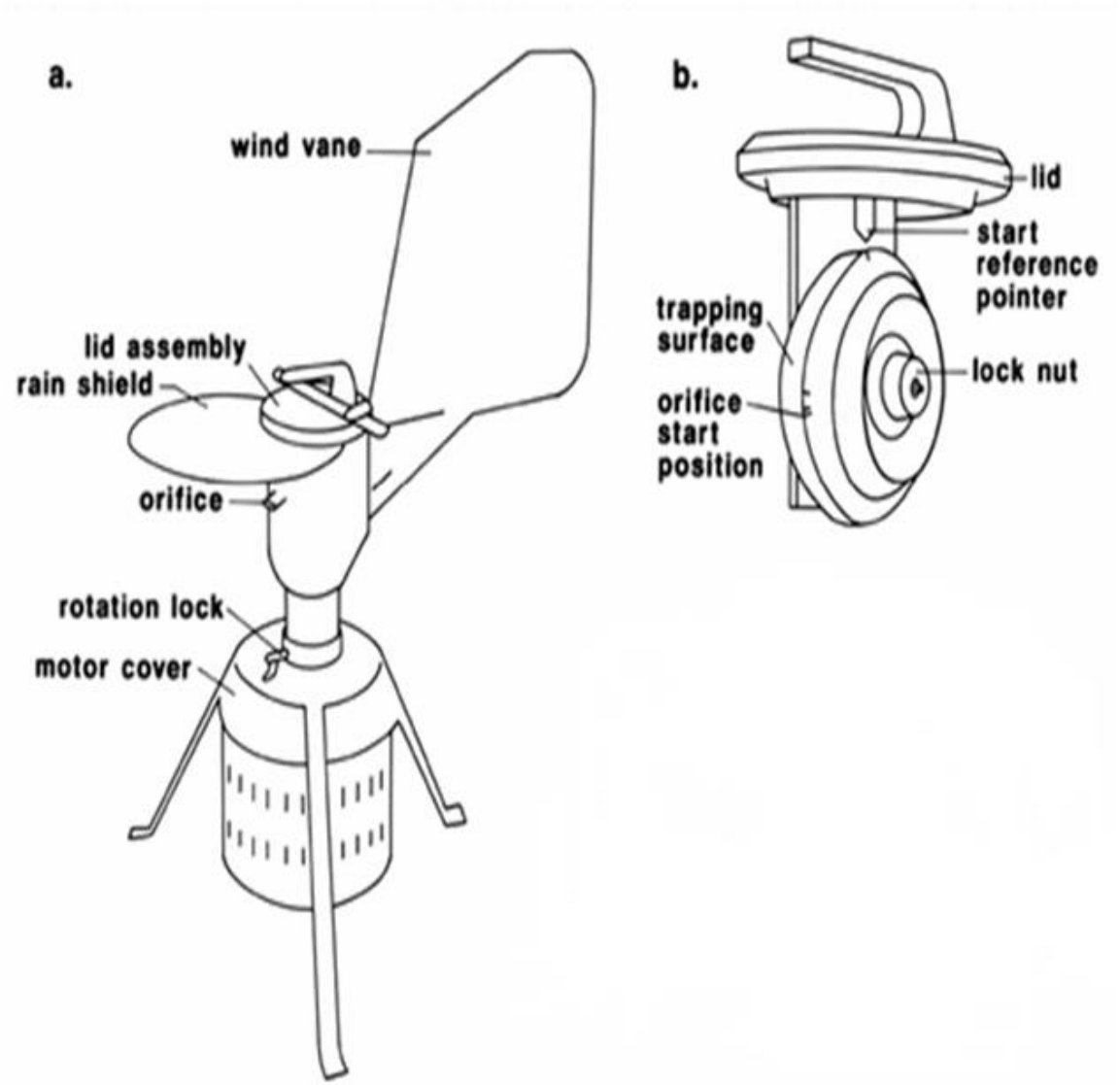


Figure 3.1: Burkard seven-day volumetric spore sampler, showing a). All external parts: The Burkard spore sampler has a wind vane which helps to move the sampler in the direction so that the orifice faces into the wind. There is a rain shield which prevents the entry of water into the orifice inlet. The air is sucked into the sampler through the orifice. b). The lid assembly that allows continuous sampling of air for 1 week, displaying the internal parts of the sampler such as a place to fix the drum, its start position and the lock nut.

3.2.2.1 Burkard spore sampler set up

Winter oilseed rape stems that were affected by phoma stem canker (30 to 50 cm long) were collected in the last week of July each year from replicate plots (that were not treated with fungicides) at field experiment sites such as Woodhall Farm, Hertfordshire; Langton Green, Eye, Suffolk; Rothwell, Lincolnshire; and Impington, Cambridgeshire, respectively. The stem debris were placed directly on a ground cover around a Burkard sampler at a distance of approximately 2 to 3m as well as being placed in free draining plastic trays (45x75cm) in the first week of August. The diseased stems were placed around the sampler in a manner so that they were exposed to all weather conditions such as rainfall, wind and temperature (Figure 3.2). This encourages the maturation of pseudothecia and release of ascospores (Huang *et al*., 2005).

3.2.2.2 Preparation of Burkard drums

The Burkard drums were wiped with 70% IMS. The trapping tape (Melinex tape) was secured to the 7-day drum by applying adhesive to the tape and was coated with Vaseline dissolved in hexane under a fume hood. The coated drums were allowed to dry and labelled with the site name and start date and time. Weekly, a new coated unexposed drum was loaded into each Burkard spore sampler (according to the Burkard company directions) (See Figure 3.1a & Figure 3.1b). The inlet orifice of Burkard sampler and the motor were checked every week while changing the drum. The drum was changed on the same day and at the same time every week.

3.2.2.3 Processing of Burkard drums

The orifice of the Burkard spore sampler, having a dimension of 14×2 mm, sampled 10 L min^{-1} of air (Figure 3.1a). The drum in the Burkard spore sampler rotates at a speed of 2mm per hour. Thus, a whole rotation of the drum takes one week (Figure 3.1b). The Burkard sampler was run continuously to obtain spores that attached to the Vaseline coated Melinex tape on the drum. After one week, the used drum was collected, and a newly prepared drum was placed in the sampler.

Figure 3.2: (a) Burkard spore sampler set up at Bayfordbury site in Hertfordshire (2015/2016 season). Burkard sampler surrounded by stem debris placed in free-draining plastic trays. (b) Weather station located nearby to collect weather data can be seen in background.

The coated tape containing spores was removed and cut into 48 mm pieces. Hence, seven pieces were obtained each week and each piece represented one day. Each piece was also cut into two halves longitudinally; the top half was mounted on a microscope slide with fungal mounting fluid that was stained with 0.1% trypan blue + lactophenol solution and a cover slip was applied (Figure 3.3). Mounted microscope slides were viewed under a light microscope at x100 magnification. The number of *Leptosphaeria* ascospores (Figure 3.4) was counted in two traverses of each slide and an average number of ascospores from the two traverses was calculated (Lacey and West, 2006; Huang *et al*., 2005). The daily average concentration of spores per m^3 was calculated as follows:

$$N = Nc \times (W \div f) \times 10^3 \times (10^3 \div (24 \times 60 \times F)) = Nc \div f \times 972$$

where *Nc* is the total number of ascospores counted in one traverse across the tape; *F* is the flow rate of the sampler (10 L min^{-1}); f is the field width measured in µm; *W* is the width of the trap orifice (14 mm).

The other half of the tape was placed in a 2ml screw top micro-centrifuge tube and stored at -20^0C for extracting DNA and determining the frequencies of virulent/avirulent alleles of *AvrLm1* and *AvrLm6* in the air by quantitative PCR (Van de Wouw *et al*., 2010).

3.2.2.4 Extraction of DNA from ascospores

DNA was extracted from the half-tapes placed in the 2ml screw cap tubes (Figure 3.3) from all four Burkard spore sampler sites by using the CTAB (hexadecyltrimethylammonium bromide) protocol, as described by Kaczmarek *et al*. (2011), with some modification. The 2ml-screw top micro-centrifuge tubes with spore tapes were taken and to each of these tubes one scoop (approx. 150 mg) of sterile acid-washed Sigma grade Ballotini beads (400-455µm diameter) was added. DNA was extracted with 440µl of DNA extraction buffer comprising: 2x TEN (400mM Tris-HCl (Fischer, USA), 50mM EDTA (Fischer, USA), 500mM NaCl (Sigma, USA) pH 8), 2% w/v polyvinylpyrrolidone (Sigma PVP-40T, USA), 5mM phenanthroline (Aldrich 131377, USA) and 450ml of sterile distilled water.

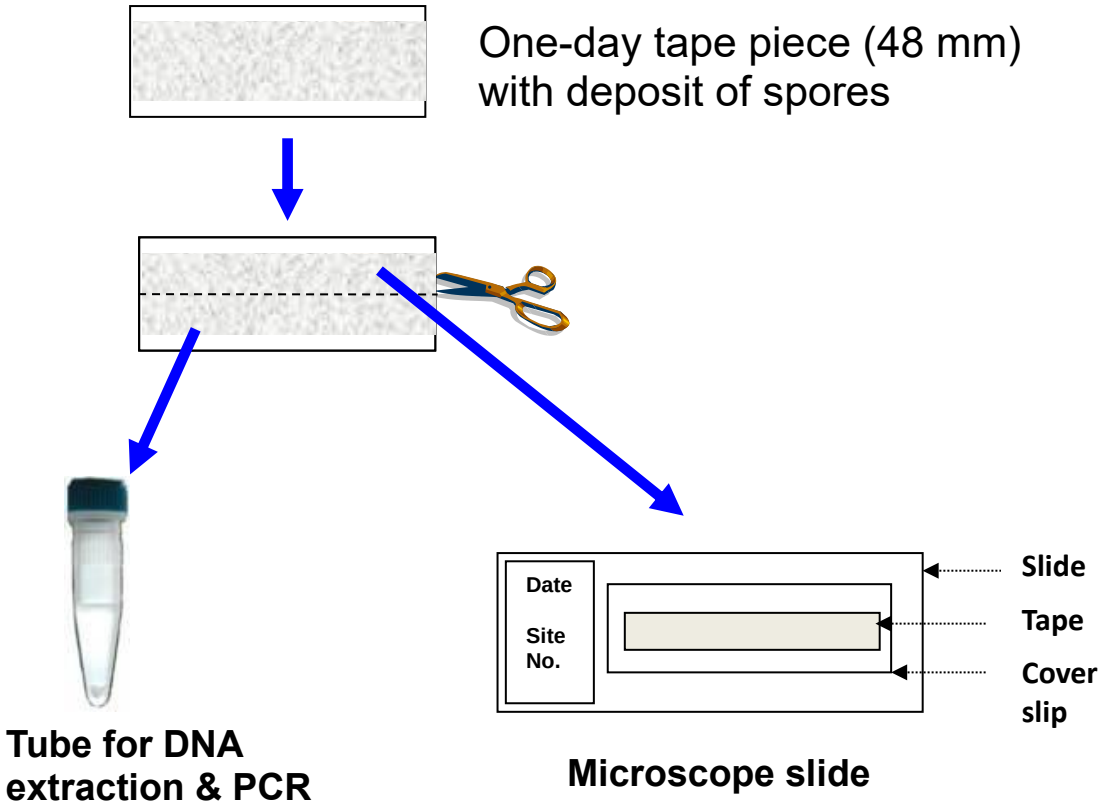


Figure 3.3: Processing of the spore tape from Burkard spore samplers. The 48 mm piece of spore tape was cut longitudinally and the piece on the top was placed on a microscopic slide, mounted with trypan blue and covered with a cover slip for counting the number of *Leptosphaeria* ascospores. The bottom piece was stored in a 2 ml screw cap tube for DNA extraction.

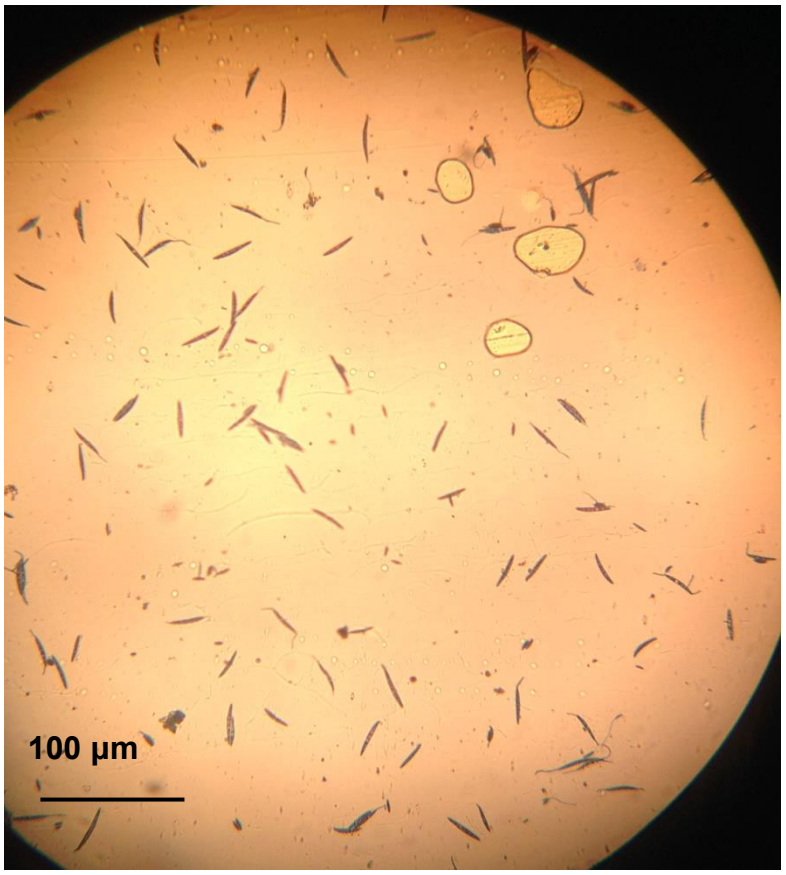


Figure 3.4: Microscopic image of *Leptosphaeria* ascospores from the Rothwell site (2015/2016 season) at x100 magnification.

DNA extraction buffer with 0.1% beta-mercaptoethanol (Sigma M3148, USA) was added at the time of use. Samples were subjected to three Fast prep ($6.0ms^{-1}$, 40s) cycles (MP Biomedicals, USA), and to the extracts 2% Sodium Dodecyl Sulphate (SDS) (Sigma, USA) was added; samples were then vortexed (by using Vortex Genie-2, Scientific industries, USA) or inverted briefly and heated at 65°C for 30 min. The supernatant was extracted against an equal volume of a 25:24:1 phenol:chloroform:isoamyl alcohol mixture (Sigma, USA) by vortexing and centrifugation (Eppendorf centrifuge 5415R, Germany) (15,700 xg, 10 min, 4°C). DNA was precipitated by incubation for 16h with a mixture of 7.5M ammonium acetate (Sigma, USA) + 480µl isopropanol + 1µl glycogen (Roche, Switzerland) at -20°C.

The DNA pellet was obtained by centrifugation (15,700 xg, 30 min at 4°C). DNA pellets were washed with ice-cold 70% (v/v) ethanol (Sigma, USA), dried in a laminar flow cabinet and dissolved in 30µl of sterile distilled water (Sigma molecular biology grade, USA), diluted to a 1:10 concentration in water for PCR and stored at -20°C.

3.2.3 Patterns of *Leptosphaeria maculans AvrLm1* and *AvrLm6* alleles in the ascospore samples

The frequencies of virulent/avirulent alleles of *AvrLm1* and *AvrLm6* (both have been cloned; the dominant virulent allele has the gene deleted) were determined using quantitative PCR. A reaction mixture was prepared using the ingredients shown in Table 3.2. All the reactions in the qPCR were done in duplicate. A volume of 17.5 µl of reaction mixture was added to the wells of a 96×0.2ml PCR plate (SARSTEDT, Germany). 2.5 µl aliquots of serially diluted *L. maculans* standard DNA (25000pg, 2500pg, 250pg, 25pg, 2.5pg) were added in two rows (in duplicate). Non-template controls were also made by adding water instead of DNA.

A PCR cycle was run to amplify the *AvrLm1* and *AvrLm6* regions using a Stratagene Mx3005P PCR machine (Agilent Technologies, USA) as follows: initial denaturation for 2min at 95°C; 40 cycles of 95°C for 15s, annealing for 30s at 60°C, extension time for 45s at 72°C and fluorescence detection for 15s at 83°C. The temperatures and times specific for each primer set are listed in Table 3.3. Finally,

Table 3.2: Ingredients used in the reaction mixture for quantitative PCR for determining frequencies of virulent/avirulent alleles of *Leptosphaeria maculans* effector genes *AvrLm1* and *AvrLm6* in the air.

Super mix	**10 µl (×1) (Agilent 2x Brilliant III SYBR Green QPCR master mix with Rox reference dye)**
***AvrLm1* qF or *AvrLm6* qF**	**0.6 µl (10µM)**
***AvrLm1* qR or *AvrLm6* qR**	**0.6 µl (10µM)**
Sterile distilled H_2O (Sigma)	**6.3 µl**
DNA	**2.5 µl (50 ng)**
Total	**20 µl reaction**

Table 3.3: PCR primers, their sequences and PCR conditions used to quantify *AvrLm1* and *AvrLm6* allele frequencies in *Leptosphaeria maculans* ascospore samples (Van de Wouw *et al*., 2010).

Gene amplified	Primer name	Sequence (5' to 3')	PCR conditions			
			Annealing temp (°C)	Extension time (s)	Fluorescence detection (°C)	Number of cycles
AvrLm1	*AvrLm1* qF	GGGTGTTTACTTCGCCTCAC	58	30	81	40
	AvrLm1 qR	ACGTTGTAATGAGCGGAACC				
AvrLm6	*AvrLm6* qF	TATTGGACAAAAGCCGAAGG	58	30	81	40
	AvrLm6 qR	GCGAGAAGCAAGTGGAATGT				

- R- reverse primer
- F- forward primer

a dissociation (melting) curve was run for 1min at 95^{o}C, 1min at 60^{o}C and 15s at 95^{o}C. A standard curve was generated by plotting quantities of DNA against cycle threshold (C_t) values after the completion of the qPCR run. Analysis of data was done using the Stratagene MxPro QPCR Software.

3.2.4 Isolation of *Leptosphaeria maculans* from leaf and stem samples

Field experiments were set up at different sites in the UK in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons. Leaves with phoma leaf spots were collected from cultivar Drakkar (no *R* gene; used as trap crop) and other cultivars (with *Rlm7* resistance gene) (Table 2.1) from all the sites (Woodhall Farm (Hertfordshire), Morley (Norfolk), Rothwell (Lincolnshire), Impington (Cambridgeshire), Trumpington (Cambridgeshire), Wisbech (Cambridgeshire), Risby (Suffolk) and West Farm Barns (Oxfordshire)).

Ten leaves of cultivar Drakkar with phoma leaf spots were sampled randomly in autumn from each of the sites in the UK for obtaining *L. maculans* isolates. Sampling of Drakkar leaves was done in November/December, at a time when there was a phoma leaf spot disease incidence of 50% plants affected in the field experiments. *L. maculans* isolates were obtained from the leaf lesions for all the three cropping seasons by using the single pycnidial isolation method (Section 2.3.2).

Stem samples (from stem base cankers and upper stem lesions) with pseudothecia were selected randomly from the field experiment sites after harvest and were incubated under natural weather conditions for maturation of pseudothecia. Single ascospore isolation (Figure 2.6) was done to obtain *L. maculans* isolates (single ascospore isolates) from stem samples from two sites (Morley (Norfolk) and Wisbech (Cambridgeshire)) from the 2016/2017 cropping season (Section 2.3.3).

3.2.5 Detection of *Avr* alleles in *Leptosphaeria maculans* isolates

Changes in frequencies of avirulent alleles of different effector genes were investigated by inoculation of conidial suspensions on the cotyledons of a set of *Brassica napus* differential cultivars/lines (Table 3.4). The seedlings of differential

Table 3.4: List of the oilseed rape cultivars/lines with different *R* genes used in a differential set to identify races of *Leptosphaeria maculans* (Balesdent *et al*., 2002).

R gene	Cultivar/line
Rlm1-3	**Columbus**
Rlm2	**Bristol**
Rlm3	**02-22-2-1**
Rlm4	**Jet Neuf**
Rlm5	**99-150-2-1**
Rlm6	**Darmor-MX**
Rlm7	**01-23-2-1**
Rlm9	**01-190-1-1**

set of cultivars/lines were grown in 40-compartment trays in a glasshouse for 13 days. When the cotyledons were 14 days old, they were inoculated with the conidial suspensions following the method of point inoculation with wounding (Section 2.4.2). There were five replicates per isolate tested in a completely randomised design on cotyledons. At 17 days post-inoculation, cotyledons were assessed by scoring the phenotype on a 0-7 scale (Figure 2.12).

3.2.6 Statistical analysis

Analysis of variance (Anova) was used to analyse the differences in the pseudothecial density on upper stem and stem base stem samples between Morley and Wisbech sites (Appendices 3-A & 3-B). Binomial test was used to analyse the differences in the percentages of *L. maculans* and *L. biglobosa* isolates obtained from the upper stem lesions and stem base cankers on stem samples from Morley and Wisbech sites (Appendices 3-C & 3-D). The numbers of *Avr* alleles in each isolate at different sites were recorded in a Microsoft Excel sheet (Appendices 3-E to 3-H). The frequencies and mean frequencies (%) of *Avr* alleles in isolates in *L. maculans* populations isolated from phoma leaf spot samples from all the cultivars and sites were calculated using the Microsoft Excel sheet and graphs were derived from the data.

3.3 Results

3.3.1 Weather conditions at Burkard spore sampler sites

At Impington, Cambridgeshire, differences in the patterns of rainfall were observed between the three cropping seasons. In the 2015/2016 cropping season, a total rainfall of 189 mm was recorded in August, September and October. The greatest amounts of rainfall were recorded at the end of August and in mid-September. Prolonged periods of rainfall were observed from mid-November to mid-January (Figure 3.5b). In the 2016/2017 cropping season, less rainfall was recorded in autumn with a total rainfall of 70 mm from August to October. Prolonged periods of rainfall were recorded in November. The total rainfall recorded from November to February was 168 mm (Figure 3.5d). In the 2017/2018 cropping season in August, September and October, a total rainfall of 181 mm was recorded.

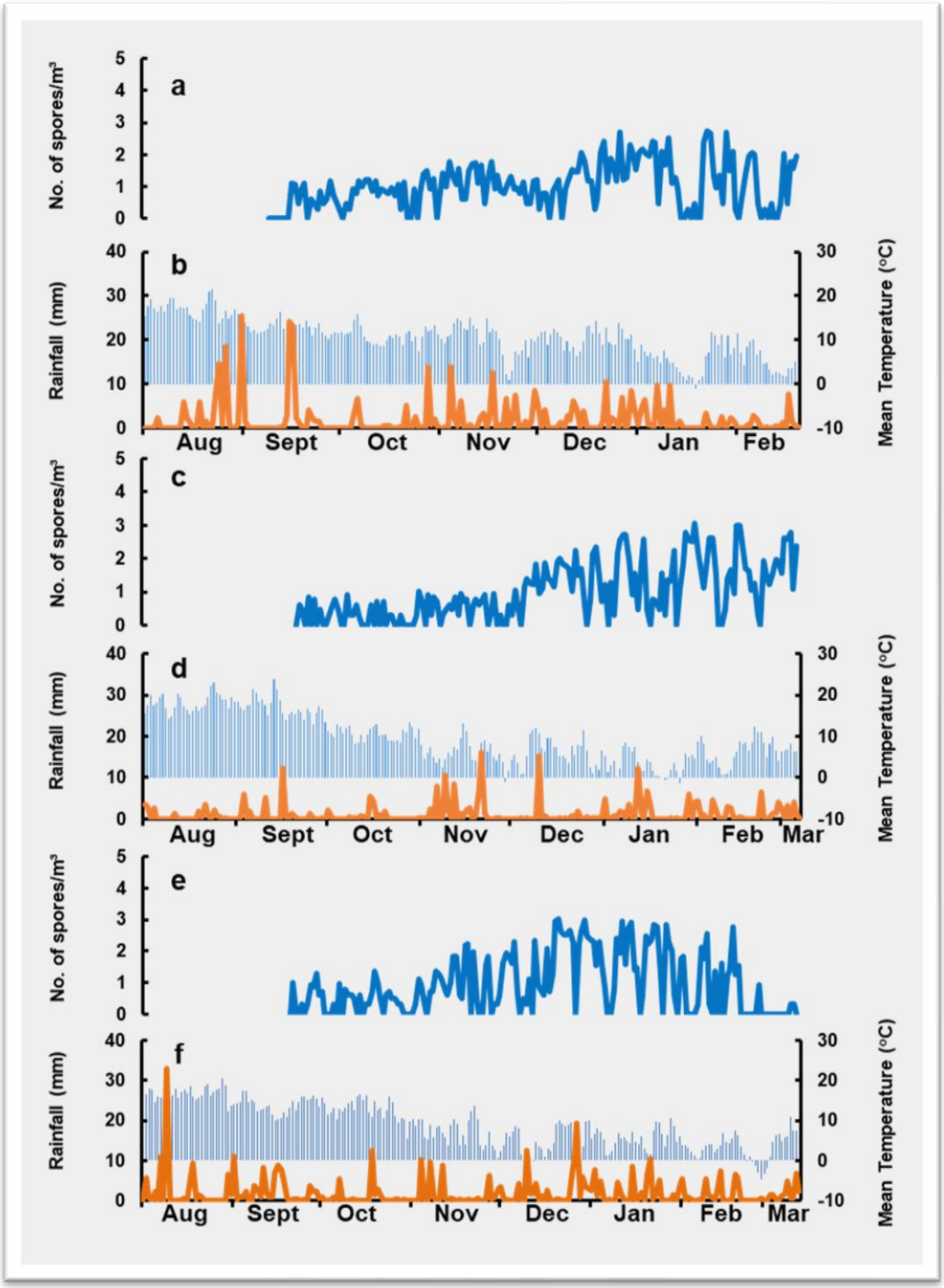


Figure 3.5: Patterns of *Leptosphaeria* ascospore release (log transformed) (a, c, e), mean temperature and daily rainfall (b, d, f) monitored over three cropping seasons at the Impington site; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. Weather data were collected using a day interval automated weather station. The blue lines represent mean temperature (°C) and orange bars represent total daily rainfall (mm). Daily counts of ascospores were collected using a Burkard spore sampler at this site.

Prolonged periods of rainfall were observed in September and December (Figure 3.5f). The mean daily temperature at Impington for the period August to October in the 2015/2016 cropping season fluctuated from a maximum of 21.5^{o}C to a minimum of 7.3^{o}C. Mean temperature between November and December was 10.2^{o}C. Lowest temperatures (≤ 0^{o}C) and snowfall were recorded in January (Figure 3.5b). In November and December, the mean daily temperature was 6^{o}C in the 2016/2017 cropping season. A pattern of daily mean temperatures like the 2015/2016 season was observed in this season. Lowest temperatures (≤ 0^{o}C) were recorded in late-November and late-January. The mean daily temperature for the period August to October fluctuated from a maximum of 23.8^{o}C to a minimum of 8^{o}C (Figure 3.5d). A similar pattern of daily average temperatures was recorded in the 2017/2018 cropping season until November. One notable difference in this cropping season was uncharacteristic periods of cold weather in late-February and late-March. Snowfall and temperatures ≤ 0^{o}C were recorded during these periods (Figure 3.5f).

Rainfall patterns differed between the three cropping seasons at Bayfordbury; Hertfordshire during autumn/winter. In the 2015/2016 cropping season, the autumn and winter months were wet compared with the 2016/2017 cropping season. In August, September and October 193 mm of rainfall was recorded. Periods of prolonged rainfall commenced from the last week of October 2015 until February 2016 with a total rainfall of 258 mm (Figure 3.6b). In the 2016/2017 cropping season, the total rainfall recorded from August-October was 131 mm.

The autumn was dry, and rainfall occurred much later, with periods of substantial rainfall commencing in mid-October and continuing to mid-March with the occasional short dry period. From November-February, 182 mm of rainfall was recorded (Figure 3.6d). In the 2017/2018 cropping season, the rainfall recorded was greater than that of 2015/2016 and 2016/2017 growing seasons. Rainfall started in September, with greatest amounts of rainfall recorded in mid-November, December and January. In August, September and October, 184 mm of rainfall was recorded. A period of prolonged rainfall occurred between October and February (318 mm over 143 days) (Figure 3.6f).

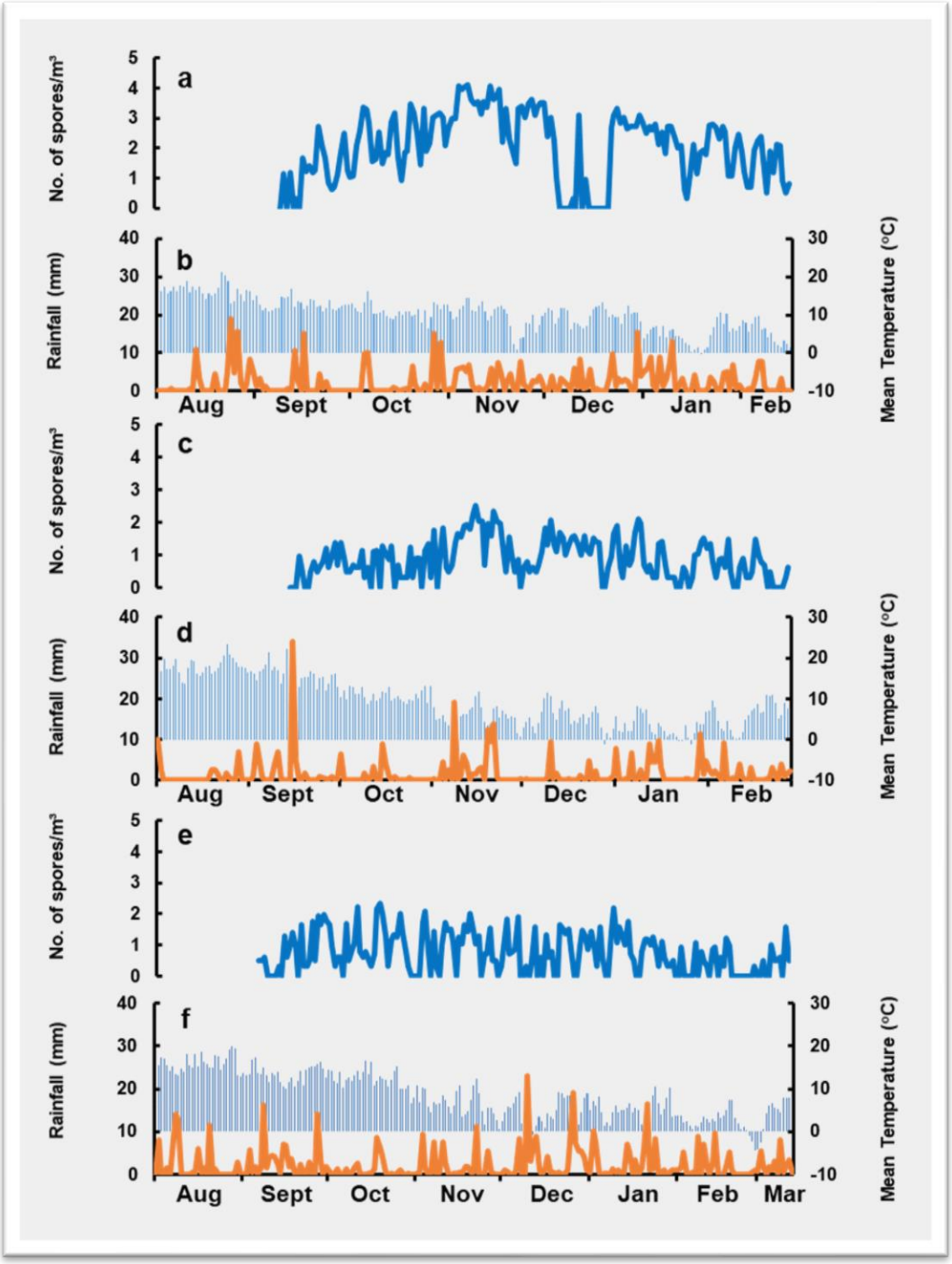


Figure 3.6: Patterns of *Leptosphaeria* ascospore release (log transformed) (a, c, e), mean temperature and daily rainfall (b, d, f) monitored over three cropping seasons at the Bayfordbury site; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. Weather data were collected using a day interval automated weather station. The blue lines represent mean temperature (°C) and orange bars represent total daily rainfall (mm). Daily counts of ascospores were collected using a Burkard spore sampler at this site.

The mean daily temperature at Bayfordbury for the period of August to October in the 2015/2016 cropping season fluctuated from a maximum of 21^{o}C to a minimum of 6^{o}C. Mean temperature between November and December was 9.6^{o}C. Lowest temperatures (≤ 0^{o}C) were recorded in January and February (Figure 3.6b). In the 2016/2017 cropping season, days with lower temperatures (≤ 0^{o}C) occurred for a prolonged period in January and February. Mean temperature in November and December was 5.5^{o}C (Figure 3.6d). In 2017/2018, a similar pattern was observed to that of the 2016/2017 cropping season. The mean daily temperature for the period of August to October fluctuated from a maximum of 23^{o}C to a minimum of 1.9^{o}C. One notable difference in this cropping season was an uncharacteristic period of cold weather in March. Low temperatures (≤ 0^{o}C) were recorded during this period (minimum temperature reached -6.8^{o}C on 01 March 2018). Mean temperature between November and December was 5.6^{o}C (Figure 3.6f).

Rainfall patterns at Rothwell, Lincolnshire differed between the three seasons during autumn/winter. In the 2015/2016 cropping season, the autumn (August, September and October) was dry with a total rainfall of 124 mm. Prolonged periods of rainfall were recorded from November to mid-January (162 mm in 76 days) (Figure 3.7b). In the 2016/2017 cropping season, the total rainfall recorded from August to October was 117 mm. The greatest rainfall (25 mm) was recorded in late-November. No period of prolonged rainfall was recorded in this season (Figure 3.7d). Greater amounts of rainfall were recorded in the 2017/2018 cropping season compared to the other two seasons with a total rainfall of 165 mm in August, September and October. Prolonged periods of rainfall were recorded in early-September and in March (Figure 3.7f).

Across the three seasons, mean temperature followed a similar pattern in August, September and October. In the 2015/2016 cropping season at Rothwell, Lincolnshire, the mean daily temperature for the period of August to October fluctuated from a maximum of 20 ^{o}C to a minimum of 7.7^{o}C. Mean temperature in November and December was 9.1^{o}C. Low temperatures (≤ 0^{o}C) were not recorded in the winter period at this site (Figure 3.7b). In 2016/2017 cropping season, the mean daily temperature for the period of August to October fluctuated from a maximum of 21.6^{o}C to a minimum of 8.7^{o}C. Mean temperature in November and

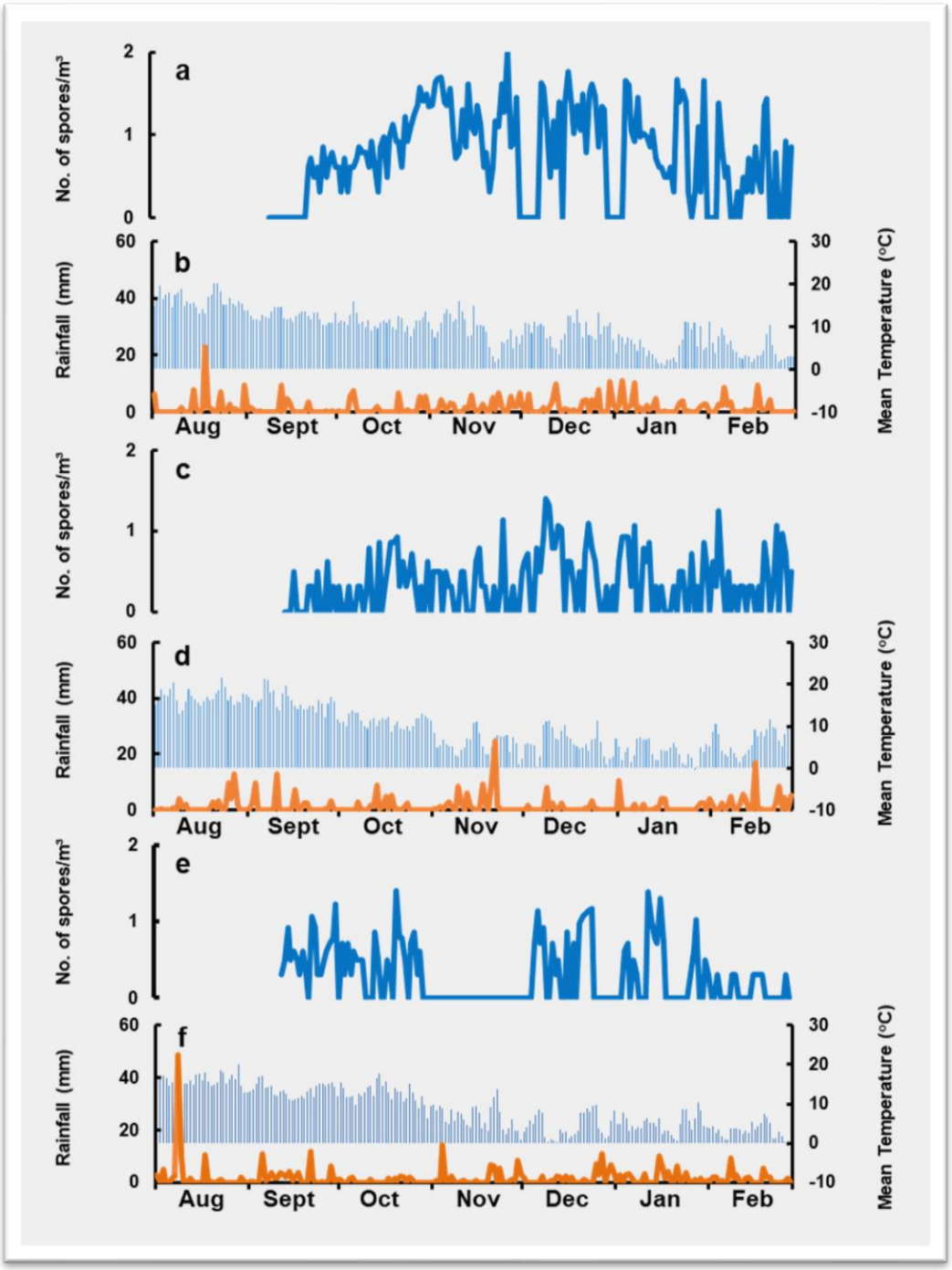


Figure 3.7: Patterns of *Leptosphaeria* ascospore release (log transformed) (a, c, e), mean temperature and daily rainfall (b, d, f) monitored over three cropping seasons at the Rothwell site; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. Weather data were collected using a day interval automated weather station. The blue lines represent mean temperature (°C) and orange bars represent total daily rainfall (mm). Daily counts of ascospores were collected using a Burkard spore sampler at this site.

December was 6ºC. Low temperatures (≤ 0ºC) were recorded in late-January at this site (Figure 3.7d). In the 2017/2018 cropping season, the mean daily temperature for the period August to October fluctuated from a maximum of 20ºC to a minimum of 6.4ºC. Mean temperature in November and December was 5.4ºC. Low temperatures (≤ 0ºC) and snowfall were recorded in late-February and March at this site (Figure 3.7f).

Rainfall patterns differed between the three cropping seasons at Eye, Suffolk. In the 2015/2016 cropping season, a total rainfall of 262 mm was recorded in August, September and October. Prolonged periods of rainfall were recorded from late October to mid January. Greatest amounts of rainfall were recorded in mid-August and mid-September (Figure 3.8b). In the 2016/2017 cropping season, a total rainfall of 112 mm was recorded in August, September and October. The greatest amount of rainfall was recorded in mid-October (22 mm, one day). Prolonged rainfall was recorded only in the first two weeks of November. Compared to the previous season, the autumn/winter were dry in the 2016/2017 season (Figure 3.8d). In the 2017/2018 cropping season, a total rainfall of 142 mm was recorded in August, September and October. Greatest rainfall (29 mm, one day) was recorded on 09 August 2017. Prolonged periods of rainfall were recorded in early/mid-September, mid/late-November and mid-January to late-February (Figure 3.8f).

Mean temperature followed a typical pattern over the three cropping seasons at the Eye site. In the 2015/2016 cropping season, the mean daily temperature for the period August to October fluctuated from a maximum of 21.7ºC to a minimum of 7.7ºC. Mean temperature in November and December was 6.7ºC. Low temperatures (≤ 0ºC) were recorded in mid-January (Figure 3.8b). In the 2016/2017 cropping season, the mean daily temperature for the period August to October fluctuated from a maximum of 23.6ºC to a minimum of 7.7ºC. Mean temperature between November and December was 6ºC. Low temperatures (≤ 0ºC) were recorded in mid/late-January (Figure 3.8d). In the 2017/2018 cropping season, the mean daily temperature for the period of August to October fluctuated from a maximum of 19.6ºC to a minimum of 5.6ºC. Mean temperature in November

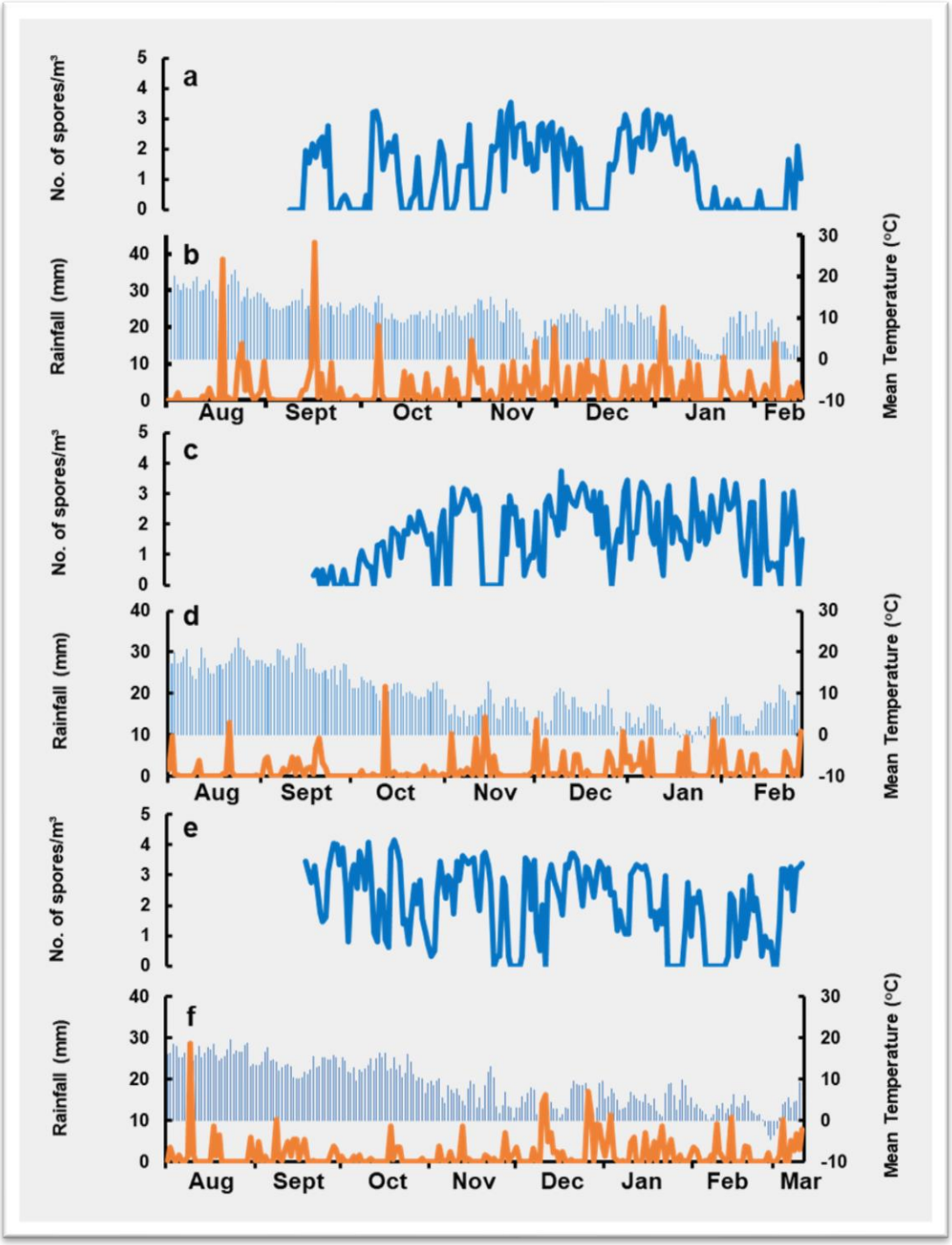


Figure 3.8: Patterns of *Leptosphaeria* ascospore release (log transformed) (a, c, e), mean temperature and daily rainfall (b, d, f) monitored over three cropping seasons at the Eye site; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. Weather data were collected using a day interval automated weather station. The blue lines represent mean temperature (°C) and orange bars represent total daily rainfall (mm). Daily counts of ascospores were collected using a Burkard spore sampler at this site.

and December was 6.6^{o}C. Low temperatures (≤ 0^{o}C) and snowfall were recorded in mid-December, late-February and early/mid-March (Figure 3.8f).

3.3.2 Release of air-borne ascospores

3.3.2.1 Number of ascospores

There were differences in the patterns of ascospore release between the four different sites. There were differences in the patterns of ascospore release between the three cropping seasons among the four sites. A common pattern among all four seasons was the relationship between rainfall and ascospore release. In most seasons, spore release in large numbers commenced after a period of prolonged or heavy rainfall. However, some spores were also released after periods of light rainfall.

At the Impington site, there were differences in the pattern of ascospore release between cropping seasons (Figure 3.5a, c & e). In the 2015/2017 cropping season, the first major ascospore release was observed in mid-December. Not many spores were observed from the mid-September until mid-December. From mid-December to the first week of January 2016, a large number of ascospores were released at this site with a rapid decrease in the second week of January. In the third week of January, large numbers of ascospores were released and in February there were fluctuations in the numbers of ascospores released (Figure 3.5a). In the 2016/2017 cropping season, the ascospore release pattern differed from the previous season. The first ascospore release occurred in early-January. However, the major ascospore release was in February and March (Figure 3.5c). The ascospore release pattern in the 2017/2018 cropping season was similar to that in the 2015/2016 cropping season. The first major ascospore release was observed in mid-December and ascopore release occurred until mid-February with fluctuations (Figure 3.5e).

At the Bayfordbury site, there were differences in the pattern of ascospore release between cropping seasons (Figure 3.6a, c & e). In 2015/2016, the first major ascospore release was observed in late October and early November. There were fluctuations in the number of ascospores released from the first week of October to the second week of November. There was a rapid decrease in the release of

ascospores after the second week of November and then an increase was observed in the third week of November. From early December to the second week of January, there were fluctuations in the release of ascospores at this site. By the first week of February, not many spores were observed at this site (Figure 3.6a). In 2016/2017, the first major ascospore release was observed in early-November until late-November and after that some ascospores were observed in December and January (Figure 3.6c). In 2017/2018, the first ascospore release was observed in late-September. The first major ascospore release was observed in early-October and the ascospore release continued until early-December with some fluctuations. The second major ascospore release was observed in early-January as there had been a prolonged period of rainfall in December (Figure 3.6e).

At the Rothwell site, there were differences in the pattern of ascospore release between cropping seasons (Figure 3.7a, c & e). In 2015/2016, there was a gradual increase in the numbers of ascospores released from early-October to early-November. The first major increase in number of ascospores released was observed in late-October and the maximum number of ascospores released was observed in mid-November at this site. There were fluctuations in the release of ascospores at this site from early December and another maximum was observed in mid-December. Again, fluctuations in ascospore numbers were observed from the last week of December to mid-February (Figure 3.7a).

In 2016/2017, ascospores were first released in early-October. The first major ascospore release was observed in late-November and the second major release was in early-December. There were fluctuations in the release of asospores after that and other major releases were observed in late-January and in late-February (Figure 3.7c). In 2017/2018, ascospore release was observed in September. The first major ascospore release was observed in mid-October. Due to the breakdown of the Burkard spore sampler at this site, ascospore release was not monitored from late-October until early-December. There were fluctuations in the release of ascospores at this site in December. The second major release was in early/mid-January (Figure 3.7e).

At the Eye site, there were differences in the pattern of ascospore release between cropping seasons (Figure 3.8a, c & e). In 2015/2016, the first maximum was

observed in mid-September. After a rapid decrease in the ascospore release, another maximum was observed in early October. The greatest ascospore release was observed in mid-November at this site. There were also fluctuations in the ascospore release from late-November to late-December, when another maximum was observed. From the second week of January, there was little ascospore release observed at this site (Figure 3.8a).

In 2016/2017, the ascospore release was later than in the previous season. The first ascospore release was observed in early-November. The first maximum was observed in mid-December. Many ascopores were released between December and late-February with some fluctuations (Figure 3.8c). In 2017/2018, an early ascospore release was observed by late-September. The first major ascospore release was observed in early/mid-October. There were fluctuations in the release of ascospores from November until mid-January. There was a decrease in the numbers of ascospores released from mid-January to late-February. Ascospore release was again observed in early-March (Figure 3.8e).

3.3.3 Patterns of *Leptosphaeria maculans AvrLm1* and *AvrLm6* alleles in the ascospore samples

The amounts of avirulent *AvrLm1* and *AvrLm6* DNA in the spore samples were detected by qPCR at four different sites. There were differences in the timing of release of *L. maculans* ascospores with *AvrLm1* and *AvrLm6* alleles between sites. *AvrLm6* alleles were detected by qPCR from *L. maculans* spore samples more frequently compared to *AvrLm1* alleles. At the Impington site, more DNA of the *AvrLm6* allele was detected when compared to the *AvrLm1* allele in all the seasons. There were similarities in the pattern of ascospore release for the *AvrLm1* allele and *AvrLm6* allele between three seasons (Figure 3.9).

In 2015/2016, DNA of the *AvrLm1* was mostly detected in ascospores released between mid-September and mid-November. After this, there was a fluctuation in the release of the ascospores with the *AvrLm1* allele (Figure 3.9a). The DNA of *AvrLm6* allele was mostly observed in large amounts from mid-December to early February (Figure 3.9b). In 2016/2017, DNA of the *AvrLm1* was mostly detected in ascospores released between mid-September and late-December (Figure 3.9c).

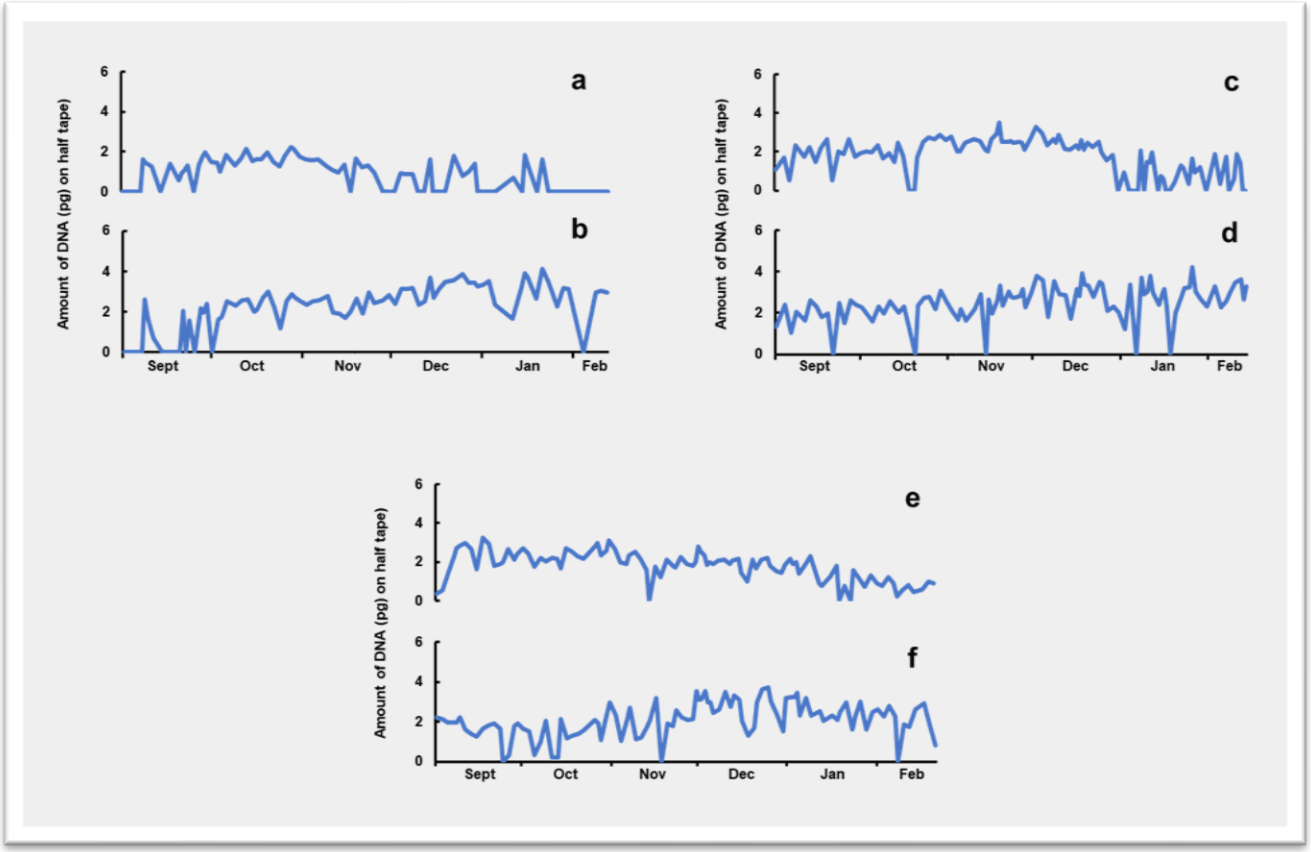


Figure 3.9: Patterns of *AvrLm1* (a, c, e) and *AvrLm6* (b, d, f) alleles in the *Leptosphaeria maculans* ascospore samples at Impington site monitored over three cropping seasons; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. The DNA was extracted on the half tape by CTAB method and qPCR was used for detecting the amounts of *AvrLm1* and *AvrLm6* DNA present on the half-tape. The amounts of DNA were log transformed.

The DNA of the *AvrLm6* allele was mostly observed in large amounts from mid-November to mid-February (Figure 3.9d). In 2017/2018, DNA of *AvrLm1* was mostly detected in ascospores released between early-September and early-December (Figure 3.9e). The DNA of the *AvrLm6* allele was mostly observed in large amounts from early-November to mid-February (Figure 3.9f).

At the Bayfordbury site, more DNA of the *AvrLm6* allele was detected when compared to *AvrLm1* allele in all the seasons. There were also similarities in the pattern of ascospore release for the *AvrLm1* allele and *AvrLm6* allele between the three seasons. In 2015/2016, both *AvrLm1* and *AvrLm6* DNA were found to be observed in large amounts in early November. The patterns of release of *AvrLm1* and *AvrLm6* alleles seemed to be same at this site (Figure 3.10a & b). In 2016/2017, ascospores with *AvrLm1* and *AvrLm6* alleles were detected from early-September until early-February (Figure 3.10c & d). In 2017/2018, ascospores with *AvrLm1* and *AvrLm6* alleles were detected from early-September until early-January (Figure 3.10e & f). More release of ascospores with *AvrLm1* alleles than *AvrLm6* alleles was detected in December (Figure 3.10e & f).

At the Rothwell site, the amount of *AvrLm6* DNA was great when compared to *AvrLm1* DNA in all the seasons. There were differences in the pattern of release of ascospores with the *AvrLm1* allele and *AvrLm6* allele between the three seasons (Figure 3.11). In 2015/2016, the greatest maximum was observed in mid-October for the *AvrLm1* allele and in late-November for the *AvrLm6* allele. Ascospores with *AvrLm1* alleles were also detected in large amounts in late-January and early-February (Figure 3.11a & b). In 2016/2017, the pattern of release of *AvrLm1* and *AvrLm6* alleles was same. Major release of ascospores with *AvrLm1* alleles and *AvrLm6* alleles were detected in mid-December (Figure 3.11c & d). In 2017/2018, DNA of *AvrLm1* was mostly detected in ascospores released between early-September and late-November (Figure 3.11e). The DNA of the *AvrLm6* allele was mostly observed in large amounts from early-December to mid-February (Figure 3.11f).

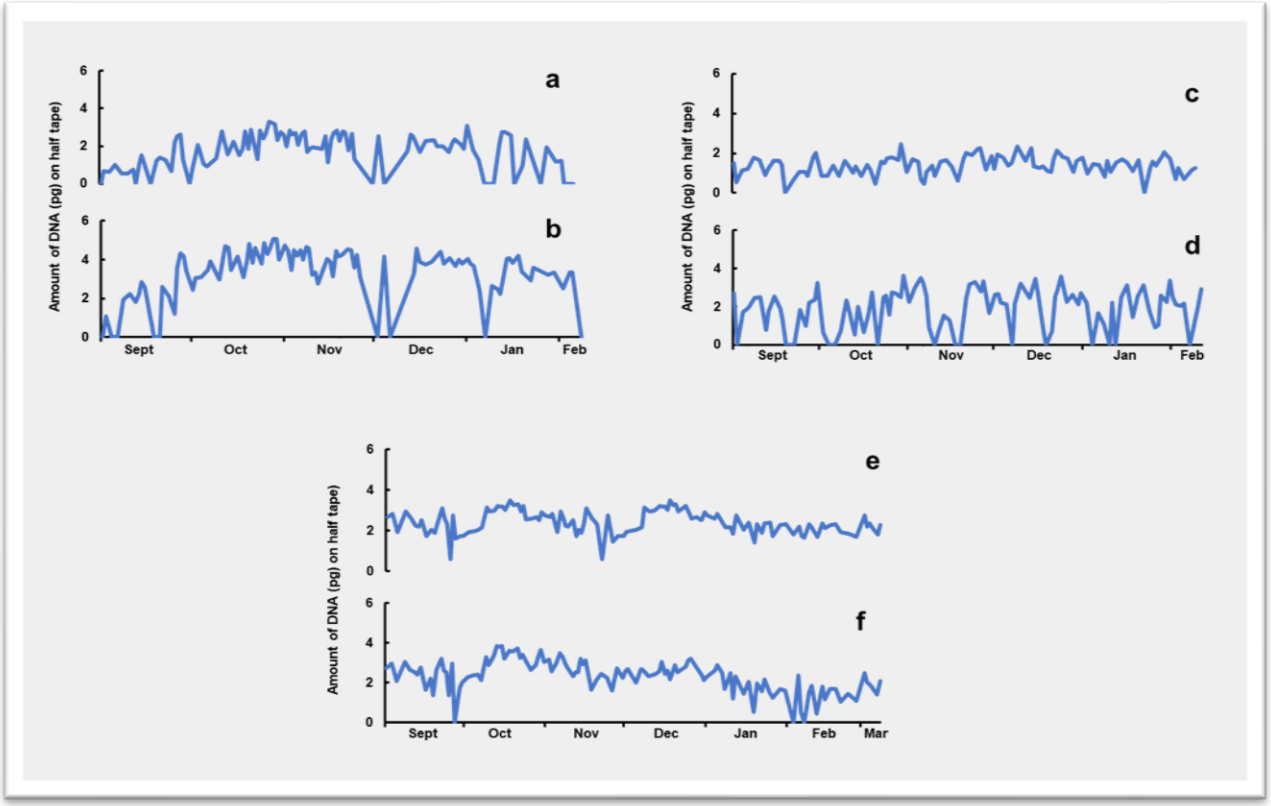


Figure 3.10: Patterns of *AvrLm1* (a, c, e) and *AvrLm6* (b, d, f) alleles in the *Leptosphaeria maculans* ascospore samples at Bayfordbury site monitored over three cropping seasons; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. The DNA was extracted on the half tape by CTAB method and qPCR was used for detecting the amounts of *AvrLm1* and *AvrLm6* DNA present on the half-tape. The amounts of DNA were log transformed.

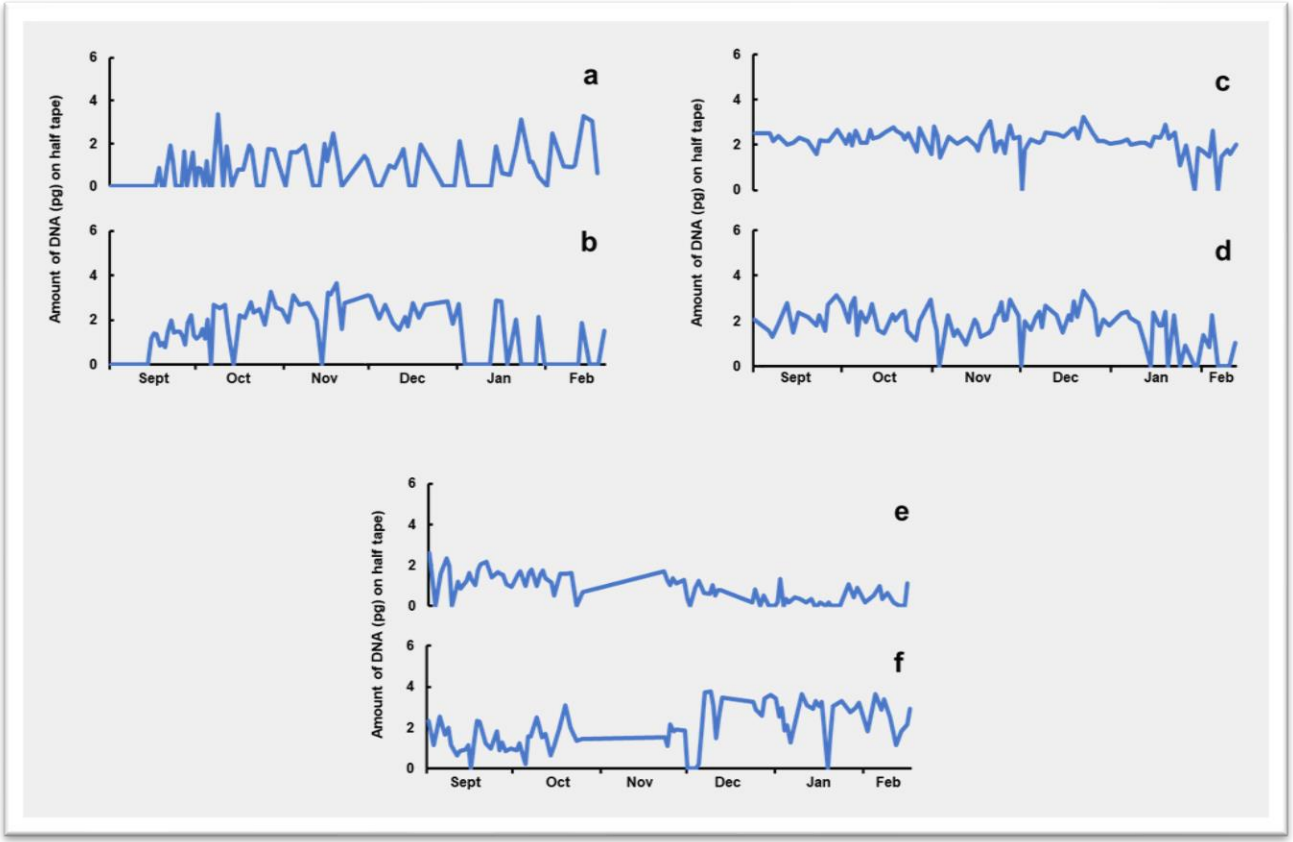


Figure 3.11: Patterns of *AvrLm1* (a, c, e) and *AvrLm6* (b, d, f) alleles in the *Leptosphaeria maculans* ascospore samples at Rothwell site monitored over three cropping seasons; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. The DNA was extracted on the half tape by CTAB method and qPCR was used for detecting the amounts of *AvrLm1* and *AvrLm6* DNA present on the half-tape. The amounts of DNA were log transformed.

At the Eye site, more DNA of the *AvrLm6* allele was detected when compared to *AvrLm1* allele in all the seasons. There were similarities in the pattern of ascospore release for the *AvrLm1* allele and *AvrLm6* allele between the three seasons. In 2015/2016, major release of the *AvrLm1* allele in ascospores was observed in early-November and the next greatest maximum was in mid-January. There were many large maxima observed in the release of *AvrLm6* alleles at this site. There were also differences in the patterns of release of *AvrLm1* and *AvrLm6* alleles. There was a similarity observed at this site in the major release of both *AvrLm1* and *AvrLm6* alleles in early-November (Figure 3.12a & b). In 2016/2017, DNA of *AvrLm1* was detected in ascospores released between early December and early-February (Figure 3.12c). The DNA of the *AvrLm6* allele was detected from late-September until early February. In 2017/2018, the pattern of release of *AvrLm1* and *AvrLm6* alleles was the same. The DNA of *AvrLm1* and *AvrLm6* alleles was detected from mid-September to late-December. Again, it was detected in mid-February (Figure 3.12 e & f).

3.3.4 *Leptosphaeria maculans* isolates from leaf samples

The phoma leaf samples from cultivar Drakkar were collected from winter oilseed rape field experiment sites (Woodhall Farm in Hertfordshire, Morley in Norfolk, Rothwell in Lincolnshire, Impington in Cambridgeshire, Trumpington in Cambridgeshire, Wisbech in Cambridgeshire and West Farm Barns in Oxfordshire) from the 2015/2016 to 2017/2018 cropping seasons (Figure 2.1). Most of the isolations were done from phoma leaf spots on leaves of Drakkar. To obtain isolates virulent against *Rlm7*, isolations were also done from cultivars with the *Rlm7* resistance gene.

A total of 243 *L. maculans* isolates were obtained in 2015/2016 cropping season, by single pycnidial isolation and were confirmed as *L. maculans* by visual identification on PDA media plates (Table 3.5). In the 2016/2017 cropping season, a total of 94 *L. maculans* isolates were obtained from phoma leaf spots by single pycnidial isolation (Table 3.6). In the 2017/2018 cropping season, a total of 94 *L. maculans* isolates were obtained from phoma leaf spots (Table 3.7). Conidial suspensions were prepared for all the *L. maculans* isolates (Figure 3.13) and

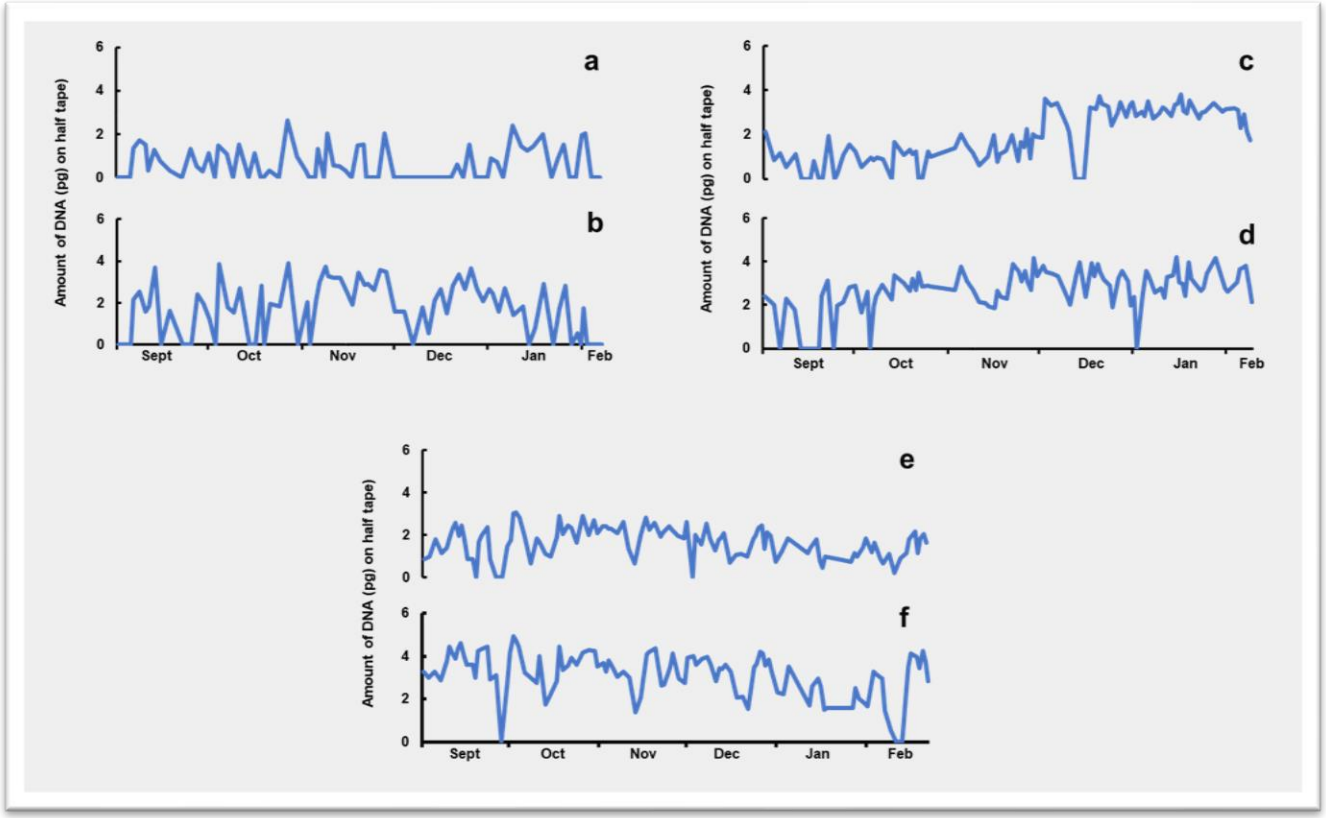


Figure 3.12: Patterns of *AvrLm1* (a, c, e) and *AvrLm6* (b, d, f) alleles in the *Leptosphaeria maculans* ascospore samples at Eye site monitored over three cropping seasons; a-b) 2015/2016; c-d) 2016/2017; e-f) 2017/2018. The DNA was extracted on the half tape by CTAB method and qPCR was used for detecting the amounts of *AvrLm1* and *AvrLm6* DNA present on the half-tape. The amounts of DNA were log transformed.

Table 3.5: Number of *Leptosphaeria maculans* isolates obtained from phoma leaf spot lesions on different cultivars in the 2015/2016 cropping season from winter oilseed rape field experiments at different sites in the UK.

No. of *L. maculans* isolates obtained in the 2015/2016 season

Cultivar	Site*				
	WH	WFB	I	T	M
Es Astrid	12	-	-	-	-
Harper	18	-	11	-	-
Drakkar	13	8	13	18	37
DK Cabernet	9	-	-	-	-
Amalie	12	-	-	-	15
Mentor	7	-	-	-	-
DK Extrovert	9	-	-	-	-
DK Exalte	3	-	11	-	-
Angus	6	-	12	-	-
Whisky	8	-	-	-	-
Adriana	12	-	-	-	-
Incentive	9	-	-	-	-
Total	**118**	**8**	**47**	**18**	**52**

*For location of sites, see Figure 2.1.

Table 3.6: Number of *Leptosphaeria maculans* isolates obtained from phoma leaf spot lesions on different cultivars in the 2016/2017 cropping season from winter oilseed rape field experiments at different sites in the UK.

No. of *L. maculans* isolates obtained in the 2016/2017 season

Cultivar	Site*			
	WH	I	M	W
Es Astrid	-	-	-	-
Harper	-	5	-	-
Drakkar	-	12	12	-
DK Cabernet	-	-	5	-
Amalie	-	5	5	5
Mentor	-	-	-	-
DK Extrovert	5	5	5	-
DK Exalte	-	5	5	-
Angus	-	5	5	-
Whisky	-	5	5	-
Adriana	-	-	-	-
Incentive	-	-	-	-
Total	**5**	**42**	**42**	**5**

*For location of sites, see Figure 2.1.

Table 3.7: Number of *Leptosphaeria maculans* isolates obtained from phoma leaf spot lesions on different cultivars in the 2017/2018 cropping season from winter oilseed rape field experiments at different sites in the UK.

No. of *L. maculans* isolates obtained in the 2017/2018 season

Cultivar	Site*			
	WH	RT	W	M
Es Astrid	-	-	-	-
Harper	4	-	4	2
Drakkar	12	15	12	12
DK Cabernet	-	-	-	-
Amalie	4	-	4	2
Mentor	-	-	-	2
DK Extrovert	4	-	4	2
DK Exalte	4	-	4	2
Angus	4	-	4	-
Whisky	4	-	4	2
Adriana	-	-	-	-
Incentive	-	-	-	-
Total	**36**	**15**	**36**	**24**

*For location of sites, see Figure 2.1.

Figure 3.13: Pycnidial production from a *Leptosphaeria maculans* isolate after 12 days of incubation on a V8 agar medium plate.

stored at -20ºC (Section 2.3.4). Glycerol stocks (20%) were prepared for all the conidial suspensions for long-term storage of the isolates and stored at -80ºC.

3.3.5 *Leptosphaeria maculans* isolates from stem samples

In addition to obtaining *L. maculans* isolates from leaf lesions, single ascospore isolation was done to obtain single ascospore isolates from stem debris of different cultivars. Stems of different cultivars with stem cankers were sampled from the Morley and Wisbech sites. These stems were incubated outdoors to induce pseudothecial production (Figure 1.12). Interestingly, pseudothecia were produced on both stem bases and upper stems of the *Rlm7* cultivars Harper, DK Extrovert, DK Exalte and Whisky. To investigate the differences between different cultivars in production of pseudothecia, the density of pseudothecia on the stem samples from Wisbech and Morley sites was assessed.

There were differences between cultivars, sites, stem bases and upper stems in pseudothecial density (Tables 3.8 & 3.9; Appendices 3-A & 3-B; Figure 3.14). At the Morley site, more pseudothecia were observed on stem pieces from upper stem lesions than on stem pieces from stem base lesions in cultivars DK Extrovert, Whisky, Harper, DK Exalte, Amalie, Angus and Drakkar, whereas greatest numbers of pseudothecia were observed on stem base pieces from cultivar Incentive (Figure 3.14a). Pseudothecial density (no./cm^2) on stem base (SB) and upper stem (US) pieces from different cultivars at the Morley site are given in Appendix 3-A. There was a significant difference (P<0.05) in the stem base pseudothecial density between cultivars but there was not a significant difference (P>0.13) between cultivars in the upper stem pseudothecial density. F-test showed that the effect of cultivar on the upper stem pseudothecial density was not statistically significant (P>0.13). There was also a significant difference (P<0.05) among cultivars in the difference between stem base pseudothecial density and upper stem pseudothecial density (Table 3.8).

At the Wisbech site, more pseudothecia were observed on stem pieces from upper stem lesions than on stem pieces from stem base lesions in cultivar Harper. Pseudothecia were observed only in the upper stem lesion pieces in cultivar DK Extrovert, whereas greatest numbers of pseudothecia were observed on stem

Table 3.8: Pseudothecial density (no. cm^2) on stem base and upper stem pieces from different cultivars at the Morley site in the 2016/2017 cropping season.

Cultivar	Stem base_density*	Upper stem_density**	(Stem base)-(Upper stem) ***
Amalie	26.80a	37.60	-10.80a
Angus	39.45a	44.26	-4.81a
DK Exalte	17.55a	36.08	-18.53a
DK Extrovert	4.90a	36.83	-31.92a
Drakkar	33.02a	43.02	-10.00a
Harper	10.16a	19.41	-9.25a
Incentive	99.19b	13.28	85.91b
Whisky	12.17a	15.85	-3.67a

*Least significant difference (LSD) at 5% was 47.5(*df*=24).

**F-test showed that the effect of cultivar was not statistically significant (*P*>0.13).

***Least significant difference (LSD) at 5% was 50.3 (*df*=24).

Multiple comparisons between cultivars were done by F-test to see the statistically significant difference between cultivars. If F-test is not significant at *P*≤5%, then the cultivars do not statistically differ from one another. This was why there are no alphabets in upper stem_density row in the Table 3.8.

Table 3.9: Pseudothecial density (no. cm^2) on stem base and upper stem pieces from different cultivars at the Wisbech site in the 2016/2017 cropping season.

Cultivar	Stem base_density*	Upper stem_density**	(Stem base)-(Upper stem)***
DK Exalte	26.46	9.48	16.98
DK Extrovert	0.12	34.15	-34.03
Harper	22.66	56.07	-33.40
Whisky	34.46	17.23	17.23

*F-test showed that there was no statistically significant difference (P>0.36) between cultivars.

**F-test showed that there was no statistically significant difference (P>0.11) between cultivars.

***F-test showed that there was no statistically significant difference (P>0.08) between cultivars.

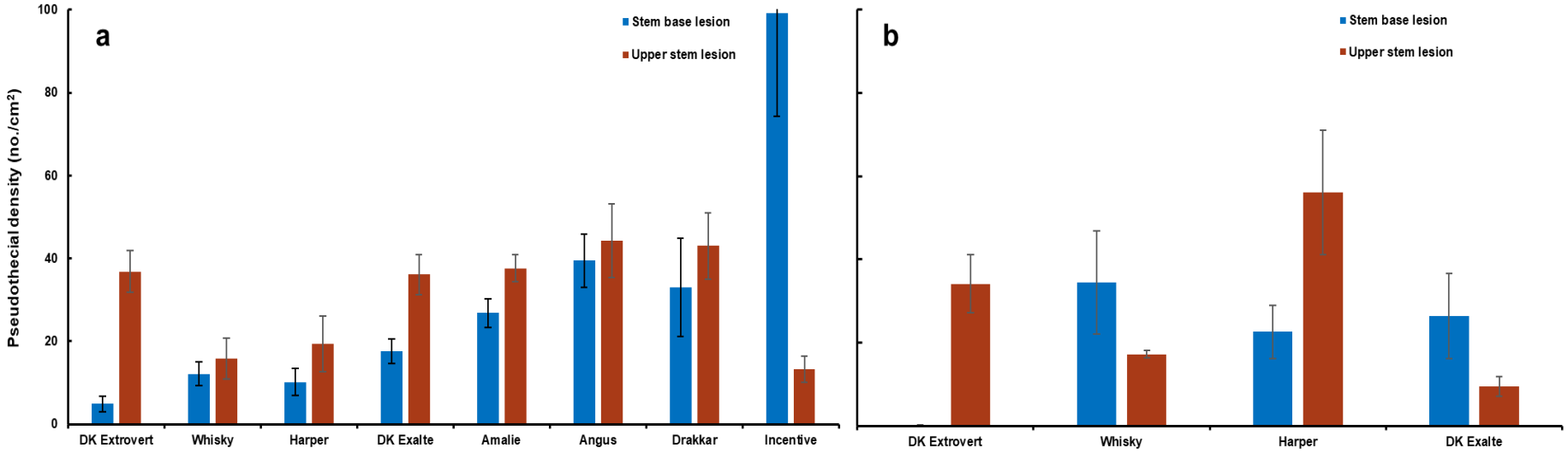


Figure 3.14: Pseudothecial density (no./cm^2) was calculated by counting no. of pseudothecia present on five stem pieces (each with dimensions 4cm×0.6cm) taken from stem bases (blue bars) or upper stems (red bars) on stem samples of different cultivars from two different sites; Morley (a) and Wisbech (b) in the 2016/2017 cropping season. Error bars mean standard error of mean.

base pieces from cultivars Whisky and DK Exalte (Figure 3.14b). Pseudothecial density (no./cm^2) for stem base (SB) and upper stem (US) pieces from different cultivars at the Wisbech site are given in Appendix 3-B. There was no significant difference (P>0.36) in stem base pseudothecial density between cultivars and there was no significant difference (P>0.11) in upper stem pseudothecial density between cultivars. There was no significant difference (P>0.08) among cultivars in the difference between stem base pseudothecial density and upper stem pseudothecial density (Table 3.9).

To investigate whether the pseudothecia on stem bases and upper stems of different cultivars contained ascospores of *L. maculans* or *L. biglobosa,* a total of 294 single ascospore isolates were obtained from those stem samples. Surprisingly, *L. maculans* ascospores were identified from both stem bases and upper stems of all cultivars. There were differences between cultivars in proportions of *L. maculans* and *L. biglobosa* ascospore isolates obtained from stem bases or from upper stems (Figure 3.15).

A total of 256 *L. maculans* isolates were obtained by the single ascospore isolation method. Among them, 151 *L. maculans* isolates were obtained from upper stem lesions and 105 *L. maculans* isolates were obtained from stem base lesions. The percentages of *L. maculans* and *L. biglobosa* isolates obtained from single ascospore isolation from upper stem (US) or stem base lesions (SB) from different cultivars at the Morley (Figure 3.15a) and Wisbech (Figure 3.15b) sites are given in Appendices 3-C & 3-D.

At the Morley site, there were no statistically significant differences in the numbers of *L. maculans* and *L. biglobosa* isolates obtained from stem base samples between cultivars Angus, DK Exalte and Incentive. Whereas, there were significant differences (P<0.01) in the percentages of *L. maculans* and *L. biglobosa* isolates obtained from stem samples of cultivars Amalie, DK Extrovert, Drakkar and Whisky (Table 3.10).

At the Morley site, there were no significant differences in the numbers of *L. maculans* and *L. biglobosa* isolates obtained from upper stem samples of cultivars Angus, DK Exalte, Harper and Whisky, whereas, there were significant differences

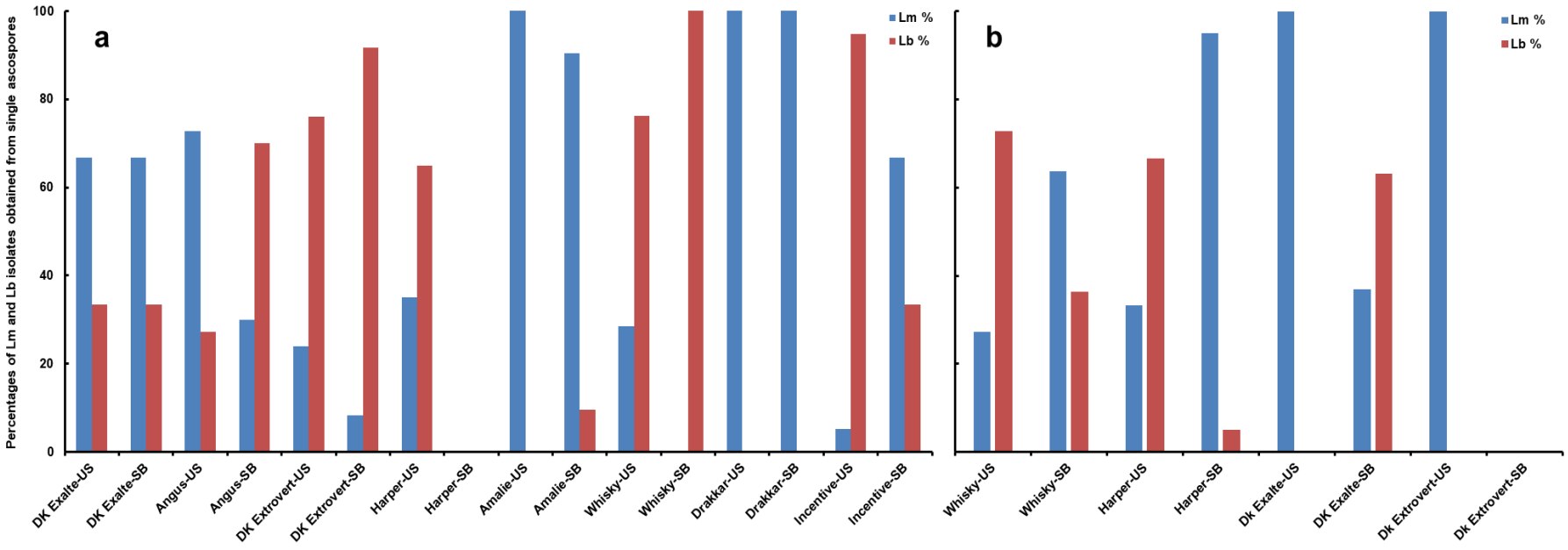


Figure 3.15: Percentages of *Leptosphaeria maculans* (blue bars) and *Leptosphaeria biglobosa* (red bars) isolates obtained from single ascospore isolations from stem pieces taken from stem base or upper stem lesions of different cultivars from two different sites; Morley (a) and Wisbech (b) in the 2016/2017 cropping season.

Table 3.10: Binomial test analysis on the numbers of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates obtained from single ascospore isolations from stem pieces obtained from stem bases and upper stems of different cultivars from the Morley site in the 2016/2017 cropping season.

Cultivar	Stem base			
	Lm	***Lb***	**Total**	**Significance for *Lm* being half**
Amalie	19	2	21	*P*<0.001
Angus	6	14	20	*P*=0.12
DK Exalte	12	6	18	*P*=0.23
DK Extrovert	1	11	12	*P*=0.006
Drakkar	20	0	20	*P*<0.001
Harper	0	0	0	N/A
Incentive	14	7	21	*P*=0.18
Whisky	0	21	21	*P*<0.001
	Upper stem			
Cultivar	***Lm***	***Lb***	**Total**	**Significance for *Lm* being half**
Amalie	20	0	20	*P*<0.001
Angus	16	6	22	*P*=0.05
DK Exalte	14	7	21	*P*=0.18
DK Extrovert	6	19	25	*P*=0.02
Drakkar	20	0	20	*P*<0.001
Harper	7	13	20	*P*=0.26
Incentive	1	18	19	*P*<0.001
Whisky	14	7	21	*P*=0.18

(*P*<0.05) in the percentages of *L. maculans* and *L. biglobosa* isolates obtained from stem samples of cultivars Amalie, DK Extrovert, Drakkar and Incentive (Table 3.11).

At the Wisbech site, there were no statistically significant differences in the percentages of *L. maculans* and *L. biglobosa* isolates obtained from stem samples from stem base cankers of cultivars DK Exalte and Whisky, wheras cultivar Harper had significant difference (*P*<0.001). There were no significant differences in the percentages of *L. maculans* and *L. biglobosa* isolates obtained from stem samples from upper stem lesions of cultivars Harper and Whisky, whereas cultivars DK Exalte and DK Extrovert differed significantly (*P*<0.001) (Appendix 3-D).

3.3.6 *Avr* alleles in *Leptosphaeria maculans* isolates

Changes in the frequencies of avirulent *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7* or *AvrLm9* alleles in *L. maculans* populations at different sites in the UK for the 2015/2016, 2016/2017 and 2017/2018 cropping seasons were investigated by inoculation of conidial suspensions onto the cotyledons of a differential set of cultivars (Balesdent *et al*., 2005) (Figures 3.16 & 3.17). In the 2015/2016 cropping season, sixty-four *L. maculans* isolates were tested on cotyledons, among which twenty-eight isolates were from cultivar Drakkar from five different sites and thirty-six isolates were from cultivars with the *Rlm7* resistance gene (Appendix 3-E).

All the isolates tested from different sites were avirulent against *Rlm7* and *Rlm6*. There were differences between sites in the frequencies of alleles that were avirulent against *Rlm1* (*AvrLm1*) or *Rlm4* (*AvrLm4*). None of the isolates tested from different sites were avirulent against *Rlm3* (*AvrLm3*) or *Rlm9* (*AvrLm9*) at all sites. A total of 9.1% at the Impington site and 16.7% of isolates were avirulent against *Rlm1* at the Trumpington site. None of the isolates at Morley, West Farm Barns and Woodhall farm were avirulent against *Rlm1* (Figure 3.17a).

A total of 4.8% of isolates were avirulent against *Rlm2* at the Morley site and 16.7% of isolates were avirulent against *Rlm2* at the Trumpington site. None of the isolates at Impington, West Farm Barns and Woodhall farm were avirulent against *Rlm2*. A total of 14.3% of isolates were avirulent against *Rlm4* at the Morley site,

Table 3.11: Binomial test analysis on the numbers of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates obtained from single ascospore isolations from stem pieces obtained from stem bases and upper stems of different cultivars from Wisbech site in the 2016/2017 cropping season.

Cultivar				Stem base
	Lm	***Lb***	**Total**	**Significance for *Lm* being half**
DK Exalte	7	12	19	*P*=0.35
DK Extrovert	0	0	0	N/A
Harper	19	1	20	*P*<0.001
Whisky	14	7	21	*P*=0.18
				Upper stem
Cultivar	***Lm***	***Lb***	**Total**	**Significance for *Lm* being half**
DK Exalte	21	0	21	*P*<0.001
DK Extrovert	20	0	20	*P*<0.001
Harper	6	12	18	*P*=0.23
Whisky	6	14	20	*P*=0.12

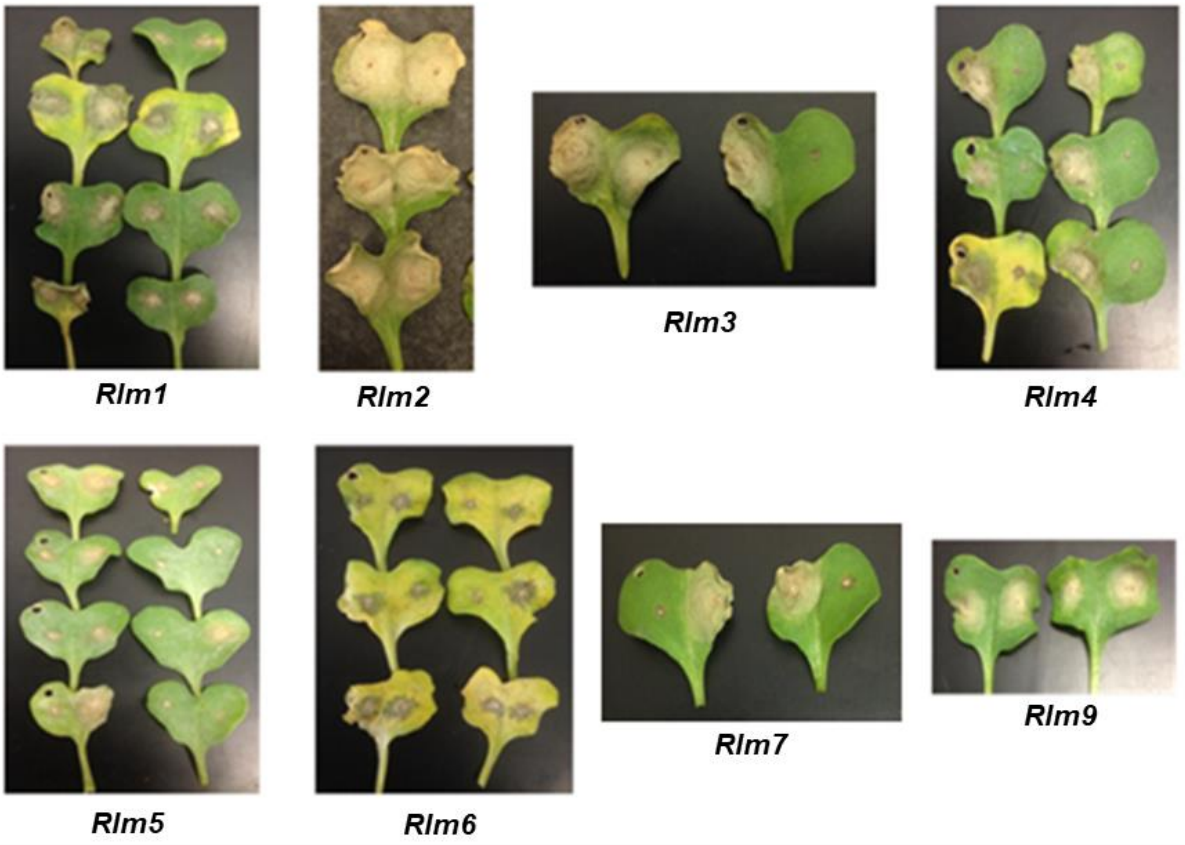


Figure 3.16: Phoma leaf spot disease symptoms on a differential set of cultivars/lines with resistance genes *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7* or *Rlm9* at 17-days post-inoculation with *Leptosphaeria maculans* isolates obtained in the 2017/2018 cropping season. If large pale lesions with pycnidia and no dark margin, showing susceptible phenotypes are observed on cotyledons of a differential set of cultivar/line (e.g. *Rlm2*), it indicates the isolate carry the virulent allele of the corresponding *Avr* gene (*avrLm2*). If small lesions with dark margins showing resistant phenotypes are observed on cotyledons of a differential set of cultivar/line (e.g. *Rlm7*), it indicates the isolate carry the avirulent allele of the corresponding *Avr* gene (*AvrLm7*).

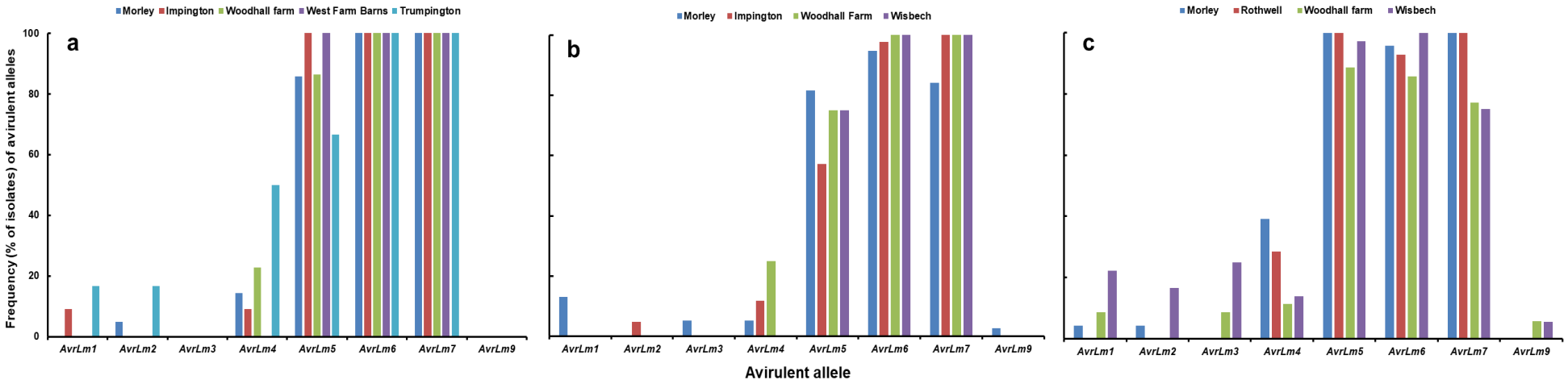


Figure 3.17: Mean frequencies (%) of avirulent alleles in *Leptosphaeria maculans* populations from leaves at different UK sites in 2015/2016 (a), 2016/2017 (b) and 2017/2018 (c) cropping seasons (see Appendix 3-E, 3-F & 3-G for details of isolates from each site).

9.1% at the Impington site, 22.7% at the Woodhall farm and 50% at the Trumpington site. None of the isolates were avirulent against *Rlm4* at West Farm Barns (Figure 3.17a). A total of 85.7% of isolates from the Morley site, 86.4% from the Woodhall Farm site, 66.7% from the Trumpington site were avirulent against *Rlm5* and all isolates were avirulent at Impington and West Farm Barns sites (Figure 3.17a).

In the 2016/2017 cropping season, 88 *L. maculans* isolates were tested on cotyledons, among which 22 isolates were from cultivar Drakkar from two different sites, three isolates from cultivar DK Cabernet, nine isolates from cultivar Angus and 54 isolates were from cultivars with the *Rlm7* resistance gene (Appendix 3-F). All the isolates tested from Impington, Wisbech and Woodhall Farm were 100% avirulent against *Rlm7*. A total of 84.2% of isolates were avirulent against *Rlm7* at the Morley site. All the isolates tested from Wisbech and Woodhall Farm were 100% avirulent against *Rlm6*. A total of 94.7% of isolates were avirulent against *Rlm6* at the Morley site and 97.6% of isolates were avirulent against *Rlm6* at the Impington site. Only 13.2% of isolates were avirulent against *Rlm1* (*AvrLm1*) at the Morley site. None of the isolates tested from Impington, Wisbech and Woodhall Farm were avirulent against *Rlm1* (*AvrLm1*). Only 4.8% of isolates were avirulent against *Rlm2* (*AvrLm2*) at the Impington site. None of the isolates tested from Morley, Wisbech and Woodhall Farm were avirulent against *Rlm2* (*AvrLm2*) (Figure 3.17b).

Only 5.3% of isolates were avirulent against *Rlm3* (*AvrLm3*) at the Morley site. None of the isolates tested from Impington, Wisbech and Woodhall Farm were avirulent against *Rlm3*. Only 2.6% of isolates were avirulent against *Rlm9* (*AvrLm9*) at the Morley site. None of the isolates tested from Impington, Wisbech and Woodhall Farm were avirulent against *Rlm9*. A total of 5.3% of isolates were avirulent against *Rlm4* at the Morley site, 11.9% at the Impington site, 25% at the Woodhall Farm site and none of the isolates were avirulent against *Rlm4* at Wisbech. A total of 81.6% of isolates from the Morley site, 57% from the Impington site, 75% from the Wisbech and Woodhall farm sites were avirulent against *Rlm5* (*AvrLm5*) (Figure 3.17b).

In the 2017/2018 cropping season, 109 *L. maculans* isolates were tested on cotyledons, among which 49 isolates were from cultivar Drakkar from four different sites, eight isolates from cultivar Angus, two isolates from cultivar Mentor and 50 isolates were from cultivars with the *Rlm7* resistance gene (Appendix 3-G). All the isolates tested from Morley and Rothwell sites were avirulent against *Rlm7*. A total of 77.1% and 75% of isolates were avirulent against *Rlm7* at the Woodhall Farm and Wisbech sites. All the isolates tested from Wisbech were 100% avirulent against *Rlm6*. A total of 95.7% of isolates were avirulent against *Rlm6* at Morley, 85.7% at Woodhall Farm and 92.9% at Rothwell. A total of 4.3% of isolates were avirulent against *Rlm1* (*AvrLm1*) at Morley, 8.6% at Woodhall Farm, 22.2% at Wisbech. None of the isolates were avirulent towards *Rlm1* at the Rothwell site (Figure 3.17c).

A total of 4.3% of isolates were avirulent against *Rlm2* (*AvrLm2*) at Morley, 16.7% at Wisbech. None of the isolates tested from Rothwell and Woodhall farm sites were avirulent against *Rlm2* (*AvrLm2*). A total of 8.6% of isolates were avirulent against *Rlm3* (*AvrLm3*) at Woodhall Farm, 25% at Wisbech. None of the isolates tested from Morley and Rothwell sites were avirulent against *Rlm3*. A total of 39.1% of isolates were avirulent against *Rlm4* at Morley, 28.6% at Rothwell, 11.4% at Woodhall Farm and 13.9% at Wisbech were avirulent against *Rlm4*. All isolates from Morley and Rothwell sites, 85.7% from Woodhall Farm and 97.2% from Wisbech were avirulent against *Rlm5* (*AvrLm5*). A total of 8.3% at Woodhall Farm site and 5.5% at Wisbech site were avirulent against *Rlm9* (*AvrLm9*) and none of the isolates were avirulent against *Rlm9* at Morley and Rothwell sites (Figure 3.17c).

A total of 92 single ascospore isolates obtained from the Wisbech and Morley sites for the 2016/2017 cropping season were screened for avirulent alleles of eight different effector genes (Appendix 3-H). Among 36 isolates from Wisbech site, 22.2% of isolates were avirulent against *Rlm1* (*AvrLm1*), *Rlm4* (*AvrLm4*) and *Rlm9* (*AvrLm9*). A total of 16.7% were avirulent against *Rlm2* (*AvrLm2*), 27.8% were avirulent against *Rlm3* (*AvrLm3*), 100% were avirulent against *Rlm5* (*AvrLm5*), 97.2% were avirulent against *Rlm6* (*AvrLm6*) and 80.6% were avirulent against *Rlm7* (*AvrLm7*) (Figure 3.18).

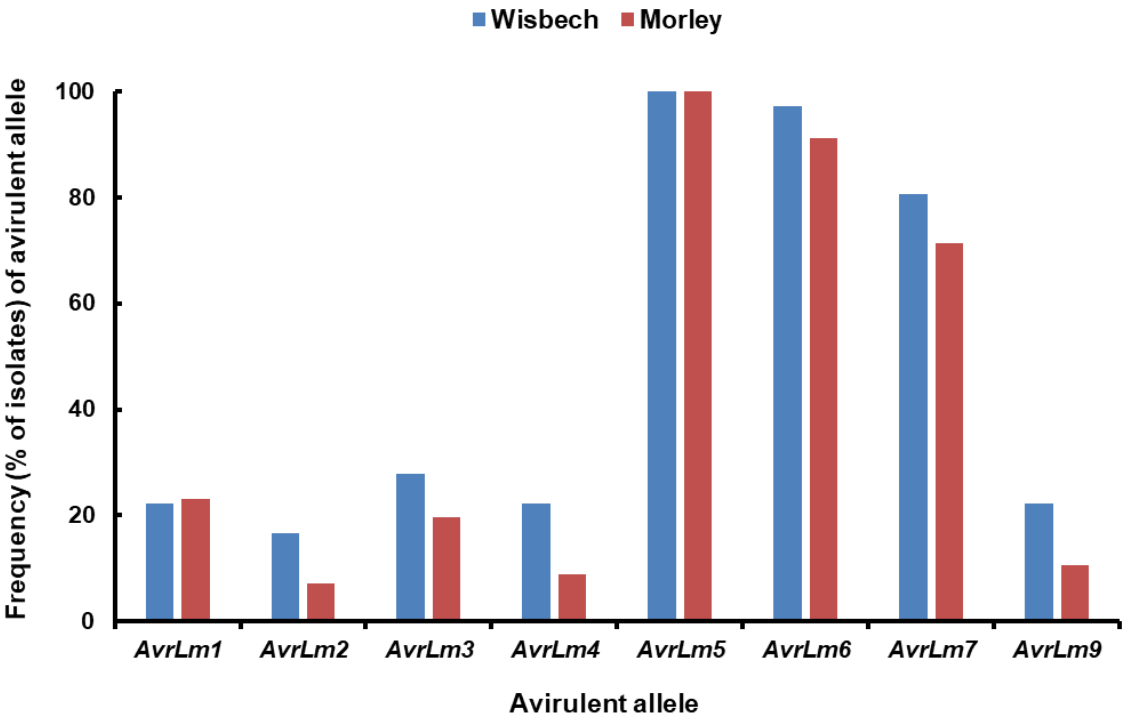


Figure 3.18: Mean frequencies (%) of avirulent alleles in *Leptosphaeria maculans* single ascospore isolates from stem samples at Wisbech and Morley sites in the UK in the 2016/2017 cropping season (see Appendix 3-H for details of isolates from each site).

Among 56 isolates tested from the Morley site, 23.2% of isolates were avirulent against *Rlm1* (*AvrLm1*), 7.1% were avirulent against *Rlm2* (*AvrLm2*), 19.6% were avirulent against *Rlm3* (*AvrLm3*), 8.9% were avirulent against *Rlm4* (*AvrLm4*), 100% were avirulent against *Rlm5* (*AvrLm5*), 91.1% were avirulent against *Rlm6* (*AvrLm6*) and 71.4% were avirulent against *Rlm7* (*AvrLm7*) and 10.7% were avirulent against *Rlm9* (*AvrLm9*) (Figure 3.18). The frequency of single ascospore isolates virulent against those *Rlm7* was 30%, which was greater than that detected in isolates from leaf lesions (16%).

3.3.7 Changes in the race structure of *Leptosphaeria maculans* populations

From the *L. maculans* isolates obtained from the 2015/2016 cropping season, seven races were identified with *AvrLm5-6-7* being the most frequent race (67.2%) (Table 3.12; Figure 3.19). *AvrLm5-6-7* was followed by races *AvrLm4-5-6-7* (12.5%), *AvrLm4-6-7* (6.3%). Fifteen *L. maculans* races were identified from the 2016/2017 cropping season. Among the fifteen races, *AvrLm5-6-7* is the most frequent race (52.3%), followed by *AvrLm6-7* (20.5%) and *AvrLm4-5-6-7* (9.1%) (Table 3.12; Figure 3.19). However, a slight decrease in the frequencies of *AvrLm5-6-7* and *AvrLm4-5-6-7* races was observed from 2015/2016 cropping season to 2016/2017 cropping season. An emergence of new races was observed in the 2016/2017 cropping season. An increase to seventeen *L. maculans* races was observed in the 2017/2018 cropping season with *AvrLm5-6-7* being the major race (56.5%), followed by *AvrLm4-5-6-7* (14.8%) (Table 3.12; Figure 3.19).

A total of nineteen different *L. maculans* races were identified from the stem samples collected from the 2016/2017 cropping season (Figure 3.20). Among them, *AvrLm5-6-7* is the major one (48.9%), followed by *AvrLm4-5-6-7*, *AvrLm1-3-5-6*, *AvrLm5-6* and *AvrLm1-2-3-4-5-6-7-9* that were accounted for 5.4% of all isolates (Figure 3.20).

Table 3.12: The frequencies of different races identified in the *L. maculans* isolates obtained from phoma leaf spot samples from three cropping seasons (2015/2016, 2016/2017 and 2017/2018).

S. No	(2015/2016) Race	Frequency (%)
1	*AvrLm 5-6-7*	67.2
2	*AvrLm 6-7*	7.8
3	*AvrLm 4-5-6-7*	12.5
4	*AvrLm 4-6-7*	6.25
5	*AvrLm 2-5-6-7*	1.6
6	*AvrLm 1-5-6-7*	3.1
7	*AvrLm 2-4-5-6-7*	1.6

S. No	(2016/2017) Race	Frequency (%)
1	*AvrLm 1-5-6*	1.1
2	*AvrLm 5-6-7*	52.3
3	*AvrLm 1-5-6-7*	1.1
4	*AvrLm 5-7*	1.1
5	*AvrLm 4-5-6-7*	9.1
6	*AvrLm 5-6*	1.1
7	*AvrLm 6-7*	20.5
8	*AvrLm 1-3-6*	1.1
9	*AvrLm 1-6*	1.1
10	*AvrLm 7*	2.3
11	*AvrLm 6*	1.1
12	*AvrLm 5-6-7-9*	1.1
13	*AvrLm 1-3-5-6*	1.1
14	*AvrLm 2-6-7*	1.1
15	*AvrLm 2-5-6-7*	1.1

S. No	(2017/2018) Race	Frequency (%)
1	*AvrLm 5-6-7*	56.5
2	*AvrLm 1-4-5-6-7*	0.9
3	*AvrLm 4-5-6-7*	14.8
4	*AvrLm 2-5-6-7*	3.7
5	*AvrLm 4-5-7*	1.9
6	*AvrLm 1-3-5-6*	4.6
7	*AvrLm 5-6*	2.7
8	*AvrLm 6-7*	0.9
9	*AvrLm 1*	0.9
10	*AvrLm 3-5-6-7-9*	0.9
11	*AvrLm 1-5-6-7*	0.9
12	*AvrLm 5-7*	1.8
13	*AvrLm 3-5-6*	1.8
14	*AvrLm 4-9*	0.9
15	*AvrLm 1-6*	0.9
16	*AvrLm 1-2-4-5-6-7-9*	0.9
17	*AvrLm 1-2-3-5-6*	0.9

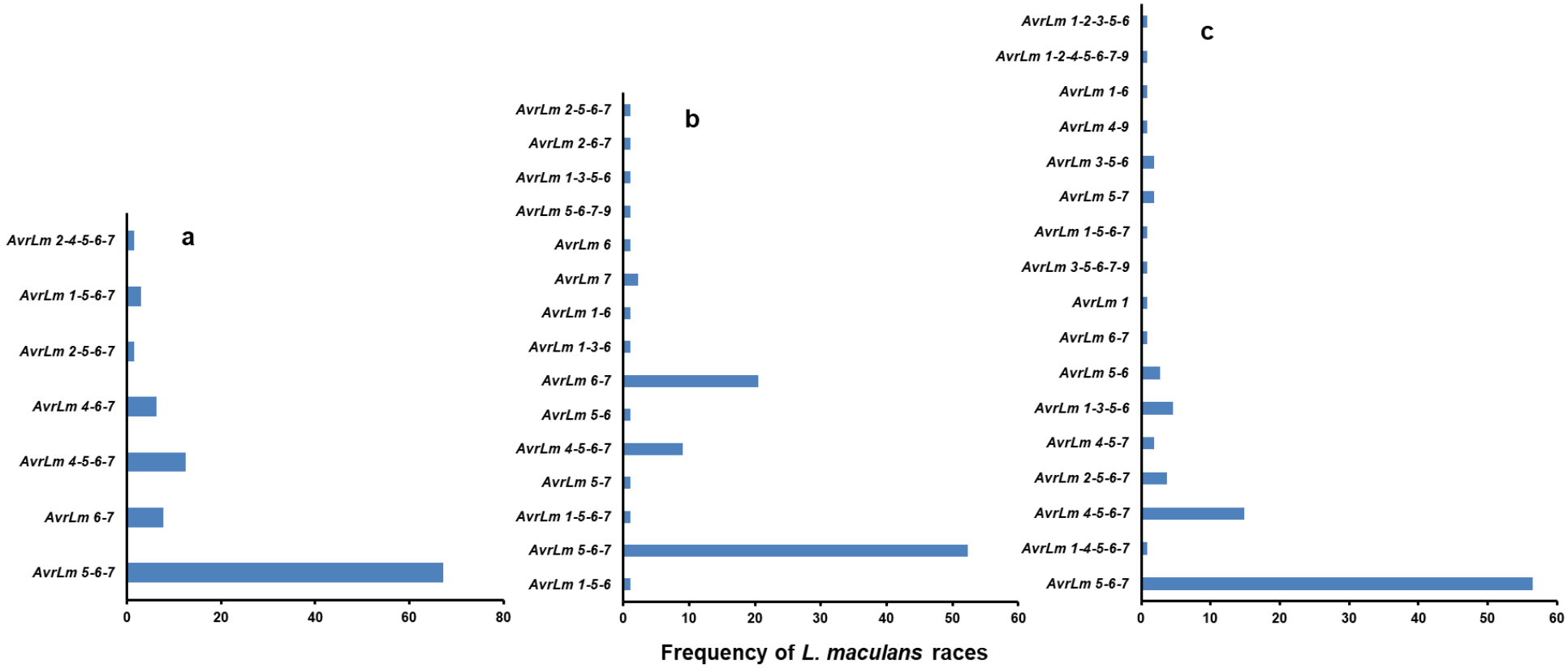
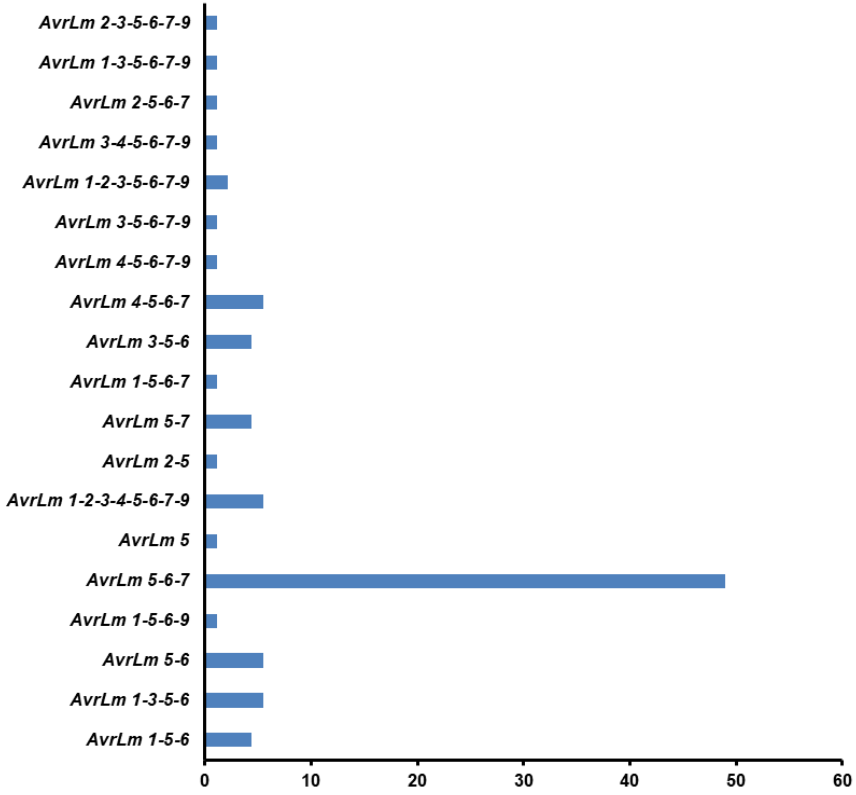


Figure 3.19: Frequencies of *L. maculans* races in isolates from leaf samples collected from the 2015/2016 (a), 2016/2017 (b) and 2017/2018 (c) cropping seasons. A total of seven races were identified in 2015/2016, fifteen races were identified in 2016/2017 and seventeen races were identified in 2017/2018. However, *AvrLm5-6-7* race was predominant among three cropping seasons.



S. No	(2016/2017) Race	Frequency (%)
1	*AvrLm 1-5-6*	4.3
2	*AvrLm 1-3-5-6*	5.4
3	*AvrLm 5-6*	5.4
4	*AvrLm 1-5-6-9*	1.1
5	*AvrLm 5-6-7*	48.9
6	*AvrLm 5*	1.1
7	*AvrLm 1-2-3-4-5-6-7-9*	5.4
8	*AvrLm 2-5*	1.1
9	*AvrLm 5-7*	4.3
10	*AvrLm 1-5-6-7*	1.1
11	*AvrLm 3-5-6*	4.3
12	*AvrLm 4-5-6-7*	5.4
13	*AvrLm 4-5-6-7-9*	1.1
14	*AvrLm 3-5-6-7-9*	1.1
15	*AvrLm 1-2-3-5-6-7-9*	2.2
16	*AvrLm 3-4-5-6-7-9*	1.1
17	*AvrLm 2-5-6-7*	1.1
18	*AvrLm 1-3-5-6-7-9*	1.1
19	*AvrLm 2-3-5-6-7-9*	1.1

Figure 3.19: Frequencies of *L. maculans* races in isolates from stem samples collected from the 2016/2017 cropping season. A total of 19 races were identified and *AvrLm5-6-7* was the major race.

3.4 Discussion

The most important findings of this chapter are the changes in the *L. maculans* race structure in the UK and increase in the frequency of virulent alleles towards *Rlm7* resistance gene. Surprisingly, *L. maculans* caused both upper stem and stem base cankers in oilseed rape cultivars containing *Rlm7* resistance gene indicating the breakdown of this effective reistance gene.

This work suggests that the differences in the timing of ascospore release observed between sites and cropping seasons were related to the differences in the weather factors like temperature and rainfall. As described in the previous studies, temperature and wetness (rainfall) affect the rate of maturation of pseudothecia and the timing of ascospores release (Toscano-Underwood *et al*., 2003; Huang *et al*., 2005; Huang *et al*., 2007). In agreement to the information provided in the weather-based forecasting model (Huang *et al*., 2007), the ascospore release data in this study confirmed that the release of ascospores of *Leptosphaeria* species is stimulated by wetness as the release of ascospores was observed on days after heavy or prolonged periods of rainfall.

In addition to that, in this study major ascospore release occurred after rainfall and when the temperature was >10^{o}C at a site. Hence, weather-based forecasting models based on temperature and rainfall are being used in the UK to predict the timing of ascospore release (Huang *et al*., 2007) and timing of appearance of phoma leaf spots (Evans *et al*., 2008) so as to guide the fungicide sprays to minimise the severity of disease epidemics (http://resources.rothamsted.ac.uk/phoma-leaf-spot-forecast/phoma-forecast). Weather-based forecasting models are also used in France and Australia (Peres *et al*., 1999; Salam *et al*., 2003).

These results suggest that there were differences in the patterns of *Leptosphaeria* ascospore release and in the dates of first major ascospore release between sites and cropping seasons (Figures 3.5-3.8). These Burkard spore sampler sites were located near the field experiment sites. If there is an early ascospore release at a site, the ascospores can easily infect young plants in oilseed rape crops located nearby and can cause severe disease epidemics

(Huang *et al*., 2007). Woodhall Farm (field experiment site) located near Bayfordbury (Burkard spore sampler site) had severe phoma leaf spots and phoma stem canker as there was an early release of ascospores when compared to the other three sites in the 2015/2016 season. Deployment of spore sampers near a network of field sites (e.g. AHDB Recommended list trial sites) could provide such guidance nationally to predict severity of epidemics.

In the 2016/2017 cropping season, major ascospore release was observed (in early-December) 15 days after a heavy rainfall (in mid-November) at the Rothwell site. In 2015/2016 cropping season, maximum mumber of ascsopores were released at the Eye site in November as there was a prolonged period of rainfall at that time. There were also a few instances where major ascospore release was observed one month after a prolonged period of rainfall. The numbers of ascospores released also depend on the numbers of pseudothecia that developed on the previous season's crop debris and on the *R* genes used in the oilseed rape cultivars at that site. Compared to all the other Burkard spore sampler sites, a late ascospore release was observed at the Impington site in all the three cropping seasons, where less severe phoma stem canker severity was observed.

The results from this study on the frequencies of *AvrLm1* and *AvrLm6* alleles detected in the spore samples by quantitative PCR (Van de Wouw *et al*., 2010), suggested that there were differences in the timing of release of *Leptosphaeria maculans* ascospores with *AvrLm1* and *AvrLm6* alleles between sites and cropping seasons (Figures 3.9-3.12). At the Bayfordbury site, there were similarities in the patterns of release of *L. maculans* ascospores with *AvrLm1* and *AvrLm6* alleles between the three cropping seasons. The *AvrLm1* and *AvrLm6* alleles were detected at the same time in this site in all the seasons. Similar to a survey done in the UK in 2006-2009 cropping seasons (Van de Wouw *et al*., 2010), the frequency of *AvrLm6* was greater than that of *AvrLm1* (Figures 3.17 & 3.18).

The decrease in the *AvrLm1* alleles in the UK *L. maculans* isolates has been due to the use of the *Rlm1* resistance gene in the oilseed rape cultivars. The technique of determining frequencies of avirulent alleles from spore samples is

less biased than that used for estimating allele frequencies by culturing single pycnidial isolates or single ascospore isolates from crop samples. This is because the *R* genes present in the cultivars can influence the frequency of alleles. A problem with detecting frequencies of avirulent alleles by qPCR is that it can only detect specific mutations (such as deletion) that lead to virulence. Among the avirulent alleles of *L. maculans*, only *AvrLm1* or *AvrLm6* have the deletion mechanism conferring virulence towards *Rlm1* or *Rlm6* (Gout *et al*., 2006). There are various other molecular mechanisms of mutations causing virulence towards *R* genes in other avirulent alleles of *L. maculans* (Daverdin *et al*., 2012). Therefore, this technique cannot be applicable for avirulent alleles of other effector genes (Van de Wouw *et al*., 2010).

The results from the *Avr* screening suggest that there were differences in the regional distribution of *L. maculans* races at different sites in the UK between the three cropping seasons. There were differences in the frequencies of avirulent alleles of different effector genes in *L. maculans* populations from leaf samples (single pycnidial isolates) between sites during three cropping seasons. The results from this study showed that the frequencies of avirulent alleles of *AvrLm5* (85.5%), *AvrLm6* (97.2%) and *AvrLm7* (94.7%) were greater than those of *AvrLm1* (5.7%), *AvrLm2* (3.8%), *AvrLm3* (3.2%), *AvrLm4* (17.7%) and *AvrLm9* (1.2%) effector genes in *L. maculans* populations from leaf samples. Similarly, for *L. maculans* populations from stem samples (single ascospore isolates) from two different sites in the 2016/2017, the frequencies of avirulent alleles of *AvrLm5* (100%), *AvrLm6* (94.1%) and *AvrLm7* (75.9%) were greater than those of *AvrLm1* (22.7%), *AvrLm2* (11.9%), *AvrLm3* (23.7%), *AvrLm4* (15.6%) and *AvrLm9* (16.5%) effector genes.

This survey was comparable to that of similar surveys in France, 2000/2001 (Balesdent *et al*., 2006), Europe, 2002/2003 (Stachowiak *et al*., 2006) and Canada, 2010/2011 (Liban *et al*., 2016). As it was believed that the deployment of specific *R* genes in cultivars can influence the predominance of specific races in *L. maculans* populations (Van de Wouw *et al*., 2010), in this study in addition to obtaining *L. maculans* isolates from phoma leaf spot lesions (single pycnidial isolates), isolates were also obtained from stem samples (i.e. single ascospore

isolates). These surveys used the same methodology (France, Europe and Canada) and a similar differential set of oilseed rape cultivars/lines (France and Europe), except for *Rlm9*. The observation that no *L. maculans* populations possessed avirulent alleles of the *AvrLm3* and *AvrLm9* effector genes in the 2015/2016 cropping season was consistent with the French and European surveys. It suggests that virulent alleles of the *AvrLm3* and *AvrLm9* effector genes may be fixed in the *L. maculans* populations (Figure 3.17a; Appendix 3-E) (Balesdent *et al*., 2006; Stachowiak *et al*., 2006).

Therefore, from these results it is evident that *Rlm3* and *Rlm9* resistances were no longer effective in the UK upto the 2015/2016 cropping season. However, 8% of isolates had avirulent alleles of the *AvrLm3* effector gene and 1.5% had avirulent alleles of the *AvrLm9* effector gene in a Canadian survey (Liban *et al*., 2016). In agreement to this evidence, an increase in the frequency of avirulent alleles of the *AvrLm3* and *AvrLm9* effector genes was observed in the UK in the 2016/2017 and 2017/2018 cropping seasons (Figures 3.17b, 3.17c & 3.18). This information agrees to the statement that *L. maculans* populations can adapt to selection pressure and the host-pathogen interactions are like an arms-race.

In both French and European surveys no avirulent alleles of the *AvrLm2* effector gene were detected. However, in the Canadian survey 80.6% of isolates had avirulent alleles of the *AvrLm2* effector gene (Liban *et al*., 2016). In this UK study, an increase in the frequency of isolates with avirulent alleles of the *AvrLm2* effector gene was observed in the 2017/2018 cropping season. There were variations in the frequencies of avirulent alleles against *Rlm1* and *Rlm4* between sites and cropping seasons. A survey in France stated that there was a rapid decrease in the frequency of the avirulent *AvrLm1* allele over three seasons of intensive cultivation of cultivars with *Rlm*1 resistance gene (Rouxel *et al*., 2003a). In agreement to this statement, 13.7% of isolates with avirulent alleles of the *AvrLm1* effector gene were detected in the Canadian survey (Liban *et al*., 2016).

Previous studies stated that even though the cultivars with *R* genes *Rlm*1 and *Rlm4* have been used commercially for more than 10 years, the *AvrLm1* and *AvrLm4* alleles are still present in the *L. maculans* populations (Huang *et al*., 2010). In the 2015/2016 cropping season, the frequency of isolates with avirulent

alleles of the *AvrLm1* effector gene was less. An increase in the frequency of avirulent alleles of the *AvrLm1* effector gene was observed by the 2017/2018 cropping season as there had been a decrease in the usage of *Rlm1* resistance gene in the UK oilseed rape cultivars. Whereas, a decrease in the frequency of avirulent alleles of the *AvrLm4* effector gene was observed by the 2017/2018 cropping season. This is because *Rlm4* resistance gene is still being used in the UK oilseed rape cultivars and the *L. maculans* populations had changed from avirulent to virulent.

The European and Canadian surveys suggested that the *Rlm6* and *Rlm7* resistance genes were the effective sources of resistance against *L. maculans* (Stachowiak *et al*., 2006; Liban *et al*., 2016). The UK *L. maculans* isolates collected were 100% avirulent against *Rlm7* in 2002 (Stachowiak *et al*., 2006) and in 2010 (Huang *et al*., 2018), suggesting that *Rlm7* was effective for controlling phoma stem canker in the UK even though this resistance gene has been used commercially for more than 15 years (Rouxel *et al*., 2017). The first report of *L. maculans* populations (3%) virulent against the *Rlm7* gene in the UK was in the 2012/2013 cropping season (Mitrousia *et al*., 2018).

In this study all the isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season. In the 2016/2017 cropping season, 6.8% of isolates were virulent towards *Rlm7*, whereas the frequency of isolates virulent towards *Rlm7* had increased to 16.3% in the 2017/2018 season. For single ascospore isolates from the 2016/2017 season, 25% of them were virulent towards *Rlm7*. So far, *Rlm7* resistance gene has been considered to be reliable in the UK. From the results of this study, it is evident that the *Rlm7* resistance gene is at risk as it is being widely used in the UK oilseed rape cultivars. Breakdown of this important source of resistance against *L. maculans* would cause substantial losses to the oilseed rape breeding industry and to farmers.

In a French survey (2000-2001), only one isolate virulent towards *Rlm7* was detected and that isolate showed an avirulent phenotype towards *Rlm3* (Plissonneau *et al*., 2016). However, in this study there were isolates that were avirulent towards *Rlm7* that showed a virulent phenotype towards *Rlm3*, virulent towards *Rlm7* and avirulent towards *Rlm3* and virulent towards both *Rlm3* and

Rlm7. To investigate whether the *L. maculans* ascospores identified from those *Rlm7* cultivars were virulent or avirulent against *Rlm7*, *L. maculans* populations from stem samples at Wisbech and Morley sites in the UK for 2016/2017 cropping season were investigated by inoculation of conidial suspensions onto the cotyledons of a differential set of cultivars. The results showed that 43.2% of the *L. maculans* isolates obtained from the single ascospores on *Rlm7* cultivars at the Morley site were virulent against *Rlm7* and 19.4% of *L. maculans* isolates obtained from the single ascospores on *Rlm7* cultivars at the Wisbech site were virulent against *Rlm7* (Figure 3.18) (Appendix 3-H).

So far, it has been assumed that *L. maculans* was associated with stem base cankers and *L. biglobosa* was associated with upper stem lesions (Toscano-Underwood *et al*., 2003). The results in this study showed that both *L. maculans* and *L. biglobosa* can cause both stem base cankers and upper stem lesions (Figure 3.15; Tables 3.10 & 3.11; Appendices 3-C & 3-D).

In this study, all the isolates from different sites were avirulent against *Rlm6* in the 2015/2016 season. There had been a slight decrease in the frequency of the isolates with avirulent alleles of the *AvrLm6* effector gene by the 2017/2018 cropping season. The presence of the *avrLm5* alleles in this survey in 2015/2016 cropping season was similar to that in the French and European surveys. There was an increase in the frequency of isolates with the avirulent alleles of the *AvrLm5* effector gene by the 2017/2018 cropping season (Figures 3.17 & 3.18). Until now, the *Rlm5* resistance gene has not been used in commercial cultivars. However, the presence of virulent alleles of the *AvrLm5* effector gene may have occurred because of the widespread use of *B. juncea* (source of *Rlm5*) for the production of mustard in Europe and the UK. Thus, the isolates with this virulent allele were observed before the use of this resistance gene in commercial oilseed rape cultivars (Stachowiak *et al*., 2006).

Seven races were identified in the UK *L. maculans* populations from 2015/2016 cropping season and *Av5-6-7* was the major race followed by race *Av4-5-6-7*. This was similar to work done by Huang *et al*. (2018). From the 2016/2017 cropping season, 15 and 19 races were identified in the *L. maculans* isolates from leaves and stem samples, respectively. A total of 19 races were identified in the

2017/2018 cropping season (Appendices 3-E to 3-H). Interestingly, the race *Av5-6-7* remained the major race compared with the *L. maculans* populations in 2000-2001 in France (Baledent *et al*., 2006) and 2002 in the UK (Stachowiak *et al*., 2006). The number of UK *L. maculans* races identified in 2015/2016 was similar to that in other European countries (France, Germany, Poland and Sweden) [Baledent *et al*., 2006; Stachowiak *et al*., 2006). The number of UK *L. maculans* races identified in 2017/2018 was similar to that in the Americas (Canada, USA and Chile) (35 races) (Zhang *et al*., 2016; Rouxel *et al*., 2017).

Recently, 150 races were identified in western Canada based on characterisation of *L. maculans* isolates at 12 *Avr* loci (Zhang *et al*., 2016) and a total of 55 races were detected in *L. maculans* populations at 10 *Avr* loci in western Canada (Liban *et al*., 2016). There might be an increase in the races in thuis study, if *L. maculans* isolates were characterized at 12 Avr loci. One of the reasons for the smaller number of races in Europe may be due to continuous use of *R* genes leading to the fixing of virulent alleles in the *L. maculans* populations (Rouxel *et al*., 2017). Another reason may be due to the interactions between different effector genes. Recent studies showed that host recognition of the *AvrLm3* and *AvrLm9* effector genes was masked by the presence of the *AvrLm4-7* effector gene (Plissonneau *et al*., 2016; Ghanbarnia *et al*., 2018).

Selection from the race-specific resistance genes in oilseed rape cultivars has probably played a significant role in the current *L. maculans Avr* profile (Liban *et al*., 2016). Increased cultivation of cultivars with the *Rlm1* resistance gene resulted in a rapid decrease in frequency of avirulent allele of the *AvrLm1* effector gene after three seasons in a survey in France (Rouxel *et al*., 2003a). The factors that are influencing the rapid adaptation of the *L. maculans* isolates to the resistance genes are the widespread use of resistant cultivars or the unregulated introduction of new resistance genes. This decreases the efficiency of new resistance genes (Rouxel *et al*., 2003a; Howlett, 2004; Sprague *et al*., 2006).

In comparison to the frequency of *AvrLm1* and *AvrLm6* alleles monitored, the amounts of *AvrLm6* alleles were detected in greater numbers than *AvrLm1* alleles both in ascospores and in crops. In conclusion, the results from this study suggest that the effectiveness of resistance depends on the type of resistance genes

contained within the cultivars. The most important thing observed in this study was that there might be changes in the frequencies of *Avr* alleles from one cropping season to the other. So, continuous monitoring of the frequency of *Avr* alleles should be done to guide the effective use of resistant cultivars.

Then publishing that information in the form of recommended list of cultivars by Agriculture and Horticulture Development Board (AHDB) (AHDB Recommended Lists 2018/2019 for cereals and oilseeds) every year might be useful for the farmers in the UK. This procedure was already in use in France (Terres Inovia) (www.terresinovia.fr), in Australia (GRDC) (Grains Research and Development Corporation) (www.grdc.com.au) and Canada (Canola Council of Canada) (https://www.canolacouncil.org). Hence, by knowing the presence of *Avr* alleles in the isolates, it is easy to determine the effectiveness of the resistance gene in the cultivar each year. So, relying on the information of the frequency of *Avr* alleles in *L. maculans* populations rather than confirming the resistance of the cultivar from the field experiments may be more useful.

Farmers could be guided properly by the organizations in selecting the cultivars every year based on their effective resistance through a web-based scheme. It would be better if such procedures could start in the UK to guide more effective deployment of *R* genes. New schemes such as surveying the populations of *L. maculans* in the oilseed rape cultivars for the presence of virulent and avirulent alleles for checking the durability of resistance of the specific cultivar provides opportunities for plant breeders, specialist technical organisations, cooperatives, advisory services and farmers to collaborate and exploit the cultivar resistance (Stotz *et al*., 2014).

Chapter 4 Investigation of molecular mechanisms of mutation to virulence in *Leptosphaeria maculans* populations

4.1 Introduction

Microorganisms such as bacteria and fungi secrete proteins that often act to suppress the host immune defence responses. These proteins are known as effectors or avirulence (*Avr*) proteins. They can cause disease if they are not recognised by the host resistance (*R*) genes. In the same manner, plants also have a defence system which consists of disease resistance (*R*) proteins encoded by the host resistance (*R*) genes that recognise the pathogen effector proteins upon infection and activate defence responses (Chisholm *et al*., 2006). If the pathogen is able to escape the recognition of host defence *R* genes and cause disease, then it is virulent. The interaction between the host and pathogen can also be described as an arms-race, where pathogens develop new effector proteins to overcome recognition by the host and plants evolve new recognition (*R*) proteins to target novel *Avr* proteins (Gout *et al*., 2007).

4.1.1 *R* genes and *Avr* genes

The genes conferring resistance against *L. maculans* in *Brassica napus* are called *R* genes (Delourme *et al.,* 2006). They can directly or indirectly recognise the pathogenicity factors (effectors) entering the host and activate the defence responses after infection (Gout *et al*., 2007). They are also known as receptors that can detect and activate strong defence responses. The interaction between *L. maculans* and oilseed rape is a typical gene-for-gene relationship (Flor, 1971; Balesdent *et al*., 2005).

The host-pathogen interactions involve several effector genes but a group of these (named *Avr* genes) undergoes mutations due to the adaptation of pathogen population to selection associated with the presence of the corresponding resistance (*R*) genes (Ellis *et al*., 2009; Van de Wouw *et al*., 2010). *Avr* proteins are known to be effectors involved in plant pathogenesis that have been

recognised by the plant surveillance machinery in the course of plant-pathogen co-evolution (Jones & Dangl, 2006).

At least eighteen *R* genes (*Rlm1-Rlm11*, *RlmS*, *LepR1-LepR4*, *BLMR1* and *BLMR2*) conferring resistance against *L. maculans* have been identified in *Brassicas* and two genes (*LepR3* and *Rlm2*) have been cloned (Yu *et al*., 2005, 2008; Delourme *et al*., 2006; Van de Wouw *et al*., 2009; Long *et al*., 2011; Balesdent *et al*., 2013; Larkan *et al*., 2013; Van de Wouw *et al*., 2014; Larkan *et al*., 2014; Zhang *et al*., 2017) (Table 4.1).

At least twelve *L. maculans Avr* genes have been identified (*AvrLm1-AvrLm11*, *AvrLmS & AvrLepR1*) (Van de Wouw *et al*., 2010; Balesdent *et al*., 2013); among which two genetic clusters of Avr genes were identified; *AvrLm1-2-6* and *AvrLm3-4-7-9* (Rouxel and Balesdent, 2005). The *Avr* genes are known to be located in the AT-rich, TE (transposable element)-rich blocks of the genome. Seven *Avr* genes (*AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4-7*, *AvrLm6*, *AvrLm11* and *AvrLmJ1*) have been cloned (Gout *et al*., 2006; Fudal *et al*., 2007; Parlange *et al*., 2009; Daverdin *et al*., 2012; Balesdent *et al*., 2013; Van de Wouw *et al*., 2013; Ghanbarnia *et al*., 2015; Zhang *et al*., 2017) (Table 4.1). *AvrLm1*, *AvrLm6* and *AvrLm4-7* encode Small Secreted Proteins (SSPs) (Daverdin *et al*., 2012). In this study, the molecular mechanisms of mutation towards virulence against *Rlm1*, *Rlm4, Rlm6* and *Rlm7* were investigated.

4.1.1.1 *AvrLm1*

AvrLm1 was the *Avr* gene in *L. maculans* that was first identified. The *AvrLm1* gene is embedded in 269kb of a non-coding sequence, heterochromatin-like region (Figure 4.1). This region mainly consists of retrotransposons (Gout *et al*., 2007). Retrotransposons play a vital role in the genomic evolution and in the adaptation of *L. maculans* to selection (Fudal *et al*., 2009). Retrotransposons are are genetic elements that can amplify themselves in a genome. They can be truncated and degenerated by repeat induced point mutations (RIPs). *AvrLm1* is stongly expressed during the infection of the plant (Gout *et al*., 2006). The molecular mechanism of mutation in *AvrLm1* towards virulence against *Rlm1* was found to be linked with the large deletion of a 260kb chromosomal segment (Gout

Table 4.1: Different *R* genes in *Brassica napus* and their corresponding *Avr* genes in *Leptosphaeria maculans* identified to date. Information about the sources of the *R* genes and *Avr* genes that were cloned so far were also given in this table (Zhang *et al*., 2017).

S. No	*R* gene identified	Source of *R* gene	Corresponding *Avr* gene identified
1	*Rlm1*	*Brassica napus*	*AvrLm1* (cloned)
2	*Rlm2* (cloned)	*Brassica napus*	*AvrLm2* (cloned)
3	*Rlm3*	*Brassica napus*	*AvrLm3* (cloned)
4	*Rlm4*	*Brassica napus*	*AvrLm4-7* (cloned)
5	*Rlm5*	*Brassica juncea*	*AvrLm5/AvrLmJ1* (cloned)
6	*Rlm6*	*Brassica juncea*	*AvrLm6* (cloned)
7	*Rlm7*	*Brassica napus*	*AvrLm4-7* (cloned)
8	*Rlm8*	*Brassica rapa*	*AvrLm8*
9	*Rlm9*	*Brassica napus*	*AvrLm9*
10	*Rlm10*	*Brassica nigra*	-
11	*Rlm11*	*Brassica rapa*	*AvrLm11* (cloned)
12	*LepR1*	*Brassica rapa subsp. Sylvestris*	*AvrLepR1*
13	*LepR2*	*Brassica rapa subsp. Sylvestris*	*AvrLepR2*
14	*LepR3* (cloned)	*Brassica rapa subsp. Sylvestris*	-
15	*LepR4*	*Brassica rapa subsp. Sylvestris*	-
16	*RlmS*	*Brassica rapa subsp. Sylvestris*	*AvrLmS*
17	*BLMR1*	Surpass 400	-
18	*BLMR2*	Surpass 400	-

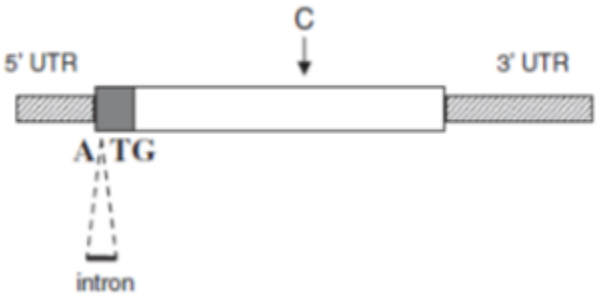


Figure 4.1: Schematic representation of the *Leptosphaeria maculans AvrLm1* effector gene (Gout *et al*., 2006). In the figure, 5' and 3' untranslated regions are indicated by hashed boxes; the white box indicates the ORF (Open Reading Frame) region; the intron is indicated by the grey box.

et al., 2007). Solo-ORF U1/L1 primers were used to amplify (1124bp) the whole open reading frame (ORF) region of the *AvrLm1* gene (Figure 4.1).

4.1.1.2 *AvrLm6*

AvrLm6 is also a gene present in a large non-coding, heterochromatin-like region of 133kb made up of retrotransposons and it encodes a cysteine-rich protein. Retrotransposons can be degenerated by repeat-induced point mutations (RIPs). They play an important role in genome evolution and adaptation to selection in *L. maculans*. Like *AvrLm1*, *AvrLm6* is over-expressed during leaf infection by *L. maculans*. It is located in 520kb GC-rich isochores. The *AvrLm6* gene is 817bp in length consisting of a 435bp ORF, 58bp of 5' untranslated UTR regions and 178bp of 3' UTR regions (Figure 4.2). The main molecular mechanism of mutation in *AvrLm6* towards virulence is deletion of the *AvrLm6* gene and others are insertions that occur at this locus or multiple events of deletion or Repeat-induced point mutation (RIPs) that cause modifications in bases or generate 2 to 4 stop codons (Fudal *et al*., 2007 & 2009). By using AvrLm6extU/L primers, the promoter region and 3' UTR region of *AvrLm6* can be amplified.

4.1.1.3 *AvrLm4-7*

The *AvrLm4-7* gene is located within a 96kb region comprised of AT-rich isochores, in which *AvrLm7* is located within a 60kb region (Figure 4.3). *AvrLm4* and *AvrLm7* are two distinct alleles of a single gene. They are recognised by two different *R* genes, *Rlm4* and *Rlm7*, respectively. This *AvrLm4-7* gene encodes cysteine-rich proteins that are expressed during initial stages of plant infection by *L. maculans*. The molecular mechanism of mutation towards virulence against *Rlm4* in *AvrLm4* is due to a single-base mutation leading to a change of glycine to arginine residue in the protein. There are various molecular mechanisms of mutation towards virulence against *Rlm7* in *AvrLm7*, such as single point mutations leading to changes in amino-acid sequence, single/dinucleotide deletions, insertion of repeated sequences in the coding sequence, partial or whole deletion of the *AvrLm4-7* gene and repeat-induced point mutations producing stop codons in the genome. AvrLm4-7ext-F & R primers were used to amplify (1434bp fragment) the part of the promoter region and part of the 3' UTR

Figure 4.2: Schematic representation of the *Leptosphaeria maculans AvrLm6* effector gene (Fudal *et al*., 2009). The 5' and 3' regions are indicated by grey boxes, introns are indicated by black boxes, exons by white boxes. Sites specific for the AvrLm6extU/L primers are also shown.

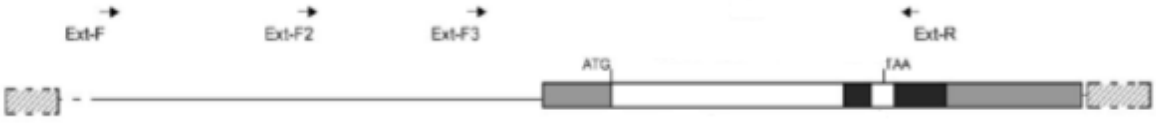


Figure 4.3: Schematic representation of the *Leptosphaeria maculans AvrLm4-7* effector gene (Daverdin *et al*., 2012). The *AvrLm4-7* gene is comprised of transposable elements (hashed boxes), 550bp of promoter region, ORF region and introns (black boxes). The sites of AvrLm4-7ext-F & R primers are also shown.

region of *AvrLm4-7* gene (Figure 4.3) (Parlange *et al*., 2009; Daverdin *et al*., 2012).

Breeding of resistant cultivars is the most accepted and efficient way to control phoma stem canker disease in oilseed rape (Delourme *et al*., 2006; Raymer, 2002). Use of host resistance is also an environmentally friendly method for controlling the disease (Delourme *et al*., 2006; Daverdin *et al*., 2012). However, there are some instances of *R* gene resistance breakdown due to rapid transformations in *L. maculans* populations (Delourme *et al*., 2006). Therefore, for managing cultivars with good resistance, gaining knowledge about the genetic basis of the resistance plays an important role. Phoma stem canker disease management can be achieved by breeding for crop resistance, strategies for crop management and by guided applications of fungicides (Van de Wouw *et al*., 2010).

The molecular mechanisms of mutation towards virulence in the *AvrLm1* gene was observed to be whole gene deletion in *L. maculans* isolates analysed from France, Australia and Mexico (Gout *et al*., 2007). In the *AvrLm6* gene, the molecular mechanisms of mutation towards virulence were observed to be gene deletion and Repeat induced point mutations (RIPs) in *L. maculans* isolates analysed from France (Fudal *et al*., 2009). Single base mutation was the observed molecular mechanism of mutation towards virulence in the *AvrLm4* gene in *L. maculans* isolates analysed from France (Parlange *et al*., 2009). An extreme diversity of independent molecular mechanisms of mutations leading to virulence in the *AvrLm7* gene were observed in *L. maculans* isolates analysed from France (Daverdin *et al*., 2012).

There have not been such studies to identify the molecular mechanisms of mutations towards virulence against the *R* genes that are currently being used in the UK. Hence, in this study, the molecular mechanisms of mutations leading towards virulence against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes in the UK were identified. This study will improve understanding of the operation of *R* gene-mediated resistance against *L. maculans* in oilseed rape. New knowledge about factors affecting operation of resistance can be used to improve breeding programmes. Information about the molecular mechanisms of mutation to

virulence in *L. maculans* can be used to optimise the use of novel resistance so that it does not break down quickly. This can be used to develop new strategies to increase the durability of host resistance against the phoma stem canker pathogen *L. maculans*.

4.1.2 Objectives

Hypothesis 2: Molecular mechanisms of mutation towards virulence in different *L. maculans* effector genes are different. To test this hypothesis, molecular mechanisms of mutation towards virulence in four different *L. maculans* effector genes (*AvrLm1, AvrLm4, AvrLm6* or *AvrLm7* corresponding to resistance genes *Rlm1, Rlm4*, *Rlm6* or *Rlm7*) were investigated. To test this hypothesis, there are four objectives.

Objective 1. To obtain *Leptosphaeria maculans* isolates virulent against *B. napus* resistance genes *Rlm1, Rlm4*, *Rlm6* or *Rlm7*. All the isolates were tested on a differential set of cultivars with known *R* genes to identify isolates virulent against *Rlm1, Rlm4*, *Rlm6* or *Rlm7* so that mechanisms of mutation to virulence can be analysed in objective 2.

Objective 2. To amplify the regions of the *L. maculans* genome corresponding to the relevant *Avr* genes in these isolates. For the isolates identified in the objective 1, the genome region corresponding to the relevant *Avr* genes in these isolates will be amplified and compared between virulent and avirulent isolates.

Objective 3. To obtain the whole genome sequences of *L. maculans* isolates that were phenotyped for virulence against *Rlm1, Rlm4*, *Rlm6* or *Rlm7* resistance genes. For the isolates identified in the objective 1, some of the isolates were selected for whole genome sequencing to compare a large region corresponding to the relevant *Avr* genes between virulent and avirulent isolates.

Objective 4. To analyse molecular events in *L. maculans* leading to virulence against these *R* genes. Different mechanisms, such as whole gene deletion, part deletion, repeat induced point mutation, were observed in fungal

pathogen for mutation from avirulence to virulence. These mechanisms were investigated for these identified virulent isolates in this chapter.

4.2 Materials and methods

4.2.1 Detection of virulent isolates against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes

L. maculans isolates virulent against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes were identified by testing the conidial suspensions on cotyledons of a differential set of cultivars/lines (Section 2.4.2).

4.2.1.1 Long-term storage of *Leptosphaeria maculans* isolates

So as to store the *L. maculans* isolates that were tested on cotyledons (Appendices 3-E to 3-H) for a long time, glycerol stocks were made. A sterile 250ml Duran bottle was taken. A volume of 40ml of glycerol was added to it and 60ml of distilled water was added and allowed to mix well by placing it on a magnetic stirrer for 30 min. Then the solution was autoclaved and stored at 20^{o}C. Approximately, 1ml aliquots of conidial suspensions of each isolate were placed in 2.5ml Eppendorf tubes and centrifuged at 11,200 xg for 10 min. The supernatant was removed and 1ml of sterile distilled water was added to each of the pellets in their respective tubes and the pellets were resuspended. Then 1ml of 40% glycerol was added to each tube and mixed thoroughly. Then the glycerol stock tubes were stored at -80^{o}C.

4.2.2 DNA extraction from *Leptosphaeria maculans* isolates using different methods

4.2.2.1 DNA extraction from conidial suspensions by using DNAMITE Plant kit

A volume of 2ml of an undiluted conidial suspension of each of these isolates was placed into a 2ml screw capped tube (Star lab, UK) for extracting DNA by using a DNAMITE plant kit (Microzone, UK). The number of cells/ml in all the samples was measured by using the haemocytometer slide and the score was noted. The

samples were centrifuged at 11,200 xg for 5 min and the supernatant was removed.

The DNA was extracted from the conidial pellet. Sterile stainless-steel beads were placed in each tube (3 beads/ tube). A volume of 1ml of Solution LA (cell lysis solution) was added to each tube and the samples were vortexed briefly. The samples were placed in a fast prep homogeniser (MP Biomedicals, USA) for 40 sec. 100µl of Solution PA (protein denaturation solution) was added and the samples were vortexed briefly. Then the samples were centrifuged at 11,200 xg for 5 min in a micro-centrifuge (Eppendorf, Gemany).

A volume of 600µl of the supernatant was carefully transferred into a new tube containing 500µl of CA solution (capture solution). The samples were mixed carefully and left on the bench for 5 min and spun in a micro-centrifuge at 15,700 xg for 7 min to pellet the DNA. Then the supernatant was poured out and the tubes were re-spun briefly and the remaining supernatant was removed using a pipette. The pellets were allowed to dry for 30 min in the biosafety cabinet. Then 100µl of sterile distilled water was added to each of the tubes and the tubes were left for 30 min to allow the DNA to rehydrate. Then the samples were stored at -20^{o}C. The concentration of the DNA was assessed by Qubit fluorometer (Invitrogen life technologies, Malaysia) and purity of the DNA was assessed by ND-1000 spectrophotometer (Nanodrop technologies, USA).

4.2.2.2 DNA extraction from mycelial samples

L. maculans isolates tested on cotyledons (Appendices 3-E to 3-H) were subcultured onto V8 agar media plates with cellulose discs by placing 100µl of conidial suspension onto the plate and spreading it over the plate by using a sterile L-shaped glass rod. The plates were incubated at 20^{o}C until mycelial growth was observed. Then the plates were opened in a biosafety cabinet and the mycelium was collected into sterile 2ml screw cap tubes with sterile forceps. Then the samples in the tubes were freeze-dried and ground to powder by adding three sterile stainless-steel beads (5mm) (Qiagen, Germany) and placing them in a fast prep homogeniser for 40s.

DNA was extracted from the samples by using a DNAMITE plant kit. A small amount of the ground mycelium of each isolate was placed into a 2ml sterile tube. The procedure followed is described in section 4.2.2.1 and the samples were stored at -20^{o}C. The concentration of the DNA was measured in ng/µl units by Qubit fluorometer and and its purity was assessed at 260/280 nm by a ND-1000 spectrophotometer.

4.2.3 Species-specific PCR

The identification of the *L. maculans* isolates was confirmed by species-specific PCR. A sterile 1.5ml tube was taken and placed at 0^{o}C and all the ingredients were added to prepare the reaction mixture (Table 4.2).

For *L. maculans*:

LmacF- Forward primer -CTTGCCCACCAATTGGATCCCCTA (24nt)

LmacR-Reverse primer -GCAAAATGTGCTGCGCTCCAGG (22nt) (Liu *et al*., 2006)

All the ingredients were placed at 0^{o}C (Table 4.2). The reaction mixture was vortexed briefly and then centrifuged for a short time to mix the ingredients well. The required numbers of 0.2ml PCR tubes (StarLabs, UK) were labelled and placed at 0^{o}C.19µl of the reaction mixture was aliquoted into each tube.

Then 1µl of the DNA was added to each of the tubes from the respective DNA sample. Positive and negative controls were also prepared. Then the PCR tubes were checked for the presence of any bubbles, centrifuged briefly and placed in the PCR machine (Eppendorf mastercycler, Germany). The details of the PCR cycle are given in Table 4.3. After the completion of the PCR, the samples were taken and stored at 0^{o}C. The PCR products were viewed on a 1.5% agarose gel.

Table 4.2: Ingredients used in the reaction mixture for species-specific PCR for the identification of both *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates. Forward and reverse primers for *L. maculans* (Lmac F/R) and *L. biglobosa* (Lbig F/LmacR) were used to obtain the respective isolate identifications.

Redtag mix	10 µl (×1) (SIGMA Cat. No. R2523, Red Tag reaction mix with $MgCl_2$)
LmacF or LbigF	0.3 µl (10µM)
LmacR	0.3 µl (10µM)
Sterile distilled H_2O	8.4 µl
DNA	20 ng
Total	20 µl reaction

4.2.4 Amplifying the corresponding region of the *Leptosphaeria maculans Avr* genes for Sanger sequencing

4.2.4.1 *AvrLm1*

The *AvrLm1* region was amplified from thirty *L. maculans* isolates containing the *avrLm1* allele and four isolates containing the *AvrLm1* allele by using Solo-ORFcU1/Cl1 primers (Appendices 4-A & 4-B). A positive control for *AvrLm1*, a positive control for *avrLm1* and a negative control (H_2O) were also included in the PCR. These primers amplify the complete open reading frame region generating an 1124bp fragment (Gout *et al*., 2006). An annealing temperature of 54^{o}C and elongation time of 60s were used for amplifying the *AvrLm1* region with Solo-ORFcU1/Cl1 primers (Appendix 4-A). The samples mentioned above (Appendix 4-B) were also amplified using internal primers AvrLm1 IntU/F (Dilmaghani *et al*., 2009) (Appendix 4-A).

4.2.4.2 *AvrLm4*

The *AvrLm4-7* gene (approximately 800bp) was amplified using external AvrLm4-7ext-F-3 & R primers (Appendix 4-C) with thirty *L. maculans* isolates containing the *avrLm4* allele, five isolates containing the *AvrLm4* allele, an *AvrLm4* positive control, an *avrLm4* positive control and a negative control (Appendix 4-C). An annealing temperature of 60^{o}C and 90s elongation time were used in the PCR programme (Appendix 4-A).

4.2.4.3 *AvrLm6*

The *AvrLm6* gene (816bp) was amplified using external AvrLm6extU/L primers (Appendices 4-A & 4-D) in five *L. maculans* isolates containing the *AvrLm6* allele, eight isolates containing the *avrLm6* allele, an *AvrLm6* positive control, an *avrLm6* positive control and a negative control (Appendix 4-D). An annealing temperature of 58^{o}C and elongation time of 60s was used in the PCR programme to amplify this gene (Appendix 4-A).

4.2.4.4 *AvrLm7*

The *AvrLm4-7* gene (800bp) was amplified using external AvrLm4-7ext-F-3 & R primers (Appendices 4-A & 4-E) with five *L. maculans* isolates containing the *AvrLm7* allele, six isolates containing the *avrLm7* allele, an *AvrLm7* positive control, an *avrLm7* positive control and a negative control (Appendix 4-E). An annealing temperature of 60ºC and 90s elongation time were used in the PCR programme (Appendix 4-A).

4.2.5 Whole genome sequencing

The genomic DNA from the *L. maculans* isolates that were phenotyped to be virulent against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* was obtained by the procedure mentioned in section 4.2.2.2.

4.2.5.1 RNAse treatment

The DNA samples were treated with RNAse A (Ribonuclease A, 10mg/ml solution, VWR AMRESCO Life Science, Ireland) to remove RNA. RNAse A working solution (1μg/μl; 1:10 dilution with TE buffer) was prepared and vortexed. A volume of 100μl of DNA samples was aliquoted in 0.5 ml Eppendorf tubes. Then 4μl of RNase working solution was added to each of the DNA samples and they were incubated at 37°C for 30 min. A volume of 10μl of 3M sodium acetate (NaOAc, pH 5.2; Sigma, USA) was added to each sample and the samples very gently mixed. Two volumes of absolute ethanol were added to each sample and inverted to mix, and the samples were stored overnight at -20°C.

The samples were centrifuged at 15,700 xg for 30 min at 4°C and the supernatant was removed carefully. Then 200μl of 70% ethanol was added to each sample and centrifuged at 15,700 xg for 5 min at 4°C. The supernatant was removed using a pipette and the pellet was dried for 20 min. Then the DNA pellet was re-suspended using 90μl of nuclease-free H_2O. The concentration of the DNA was measured in ng/μl units by a Qubit fluorometer and and its purity was assessed at 260/280 nm by a ND-1000 spectrophotometer.

4.2.5.2 Quality and concentration of *Leptosphaeria maculans* DNA

The DNA samples were loaded in a 1% agarose gel to view the bands before sending them for whole genome sequencing. DNA samples that showed clear bands without any smear were selected for whole genome sequencing. A species-specific PCR was done to confirm the identity of the *L. maculans* samples. Then the samples (5µl of each sample) were sent for sample quality control by the DNA Tapestation technique to Imperial College, London. DNA Tapestation was performed using an Agilent 2200 Tapestation system and Agilent genomic DNA screen tape assay (Agilent Technologies, USA). This technique is used to assess the quantity and integrity of the genomic DNA (gDNA) material to determine DIN (DNA Integrity Number), a measure of gDNA integrity. Samples with highly intact gDNA were given a DIN-10 score; much degraded gDNA were given a DIN-1 score.

Based on the results of the quality controls, *L. maculans* DNA samples were selected for whole genome sequencing. The sample requirements for whole genome sequencing are given in Table 4.4. Samples were sent for whole genome sequencing to Edinburgh Genomics, UK. Automated TruSeq DNA PCR free gel free libraries (350 bp insert) were prepared for sequencing genomic DNA samples. Illumina sequencing was used to sequence the whole genome of the gDNA samples and NovaSeq S1 150PE data were generated.

4.2.6 Analysis of molecular events leading to virulence in *Leptosphaeria maculans*

The PCR products of the *L. maculans* isolates were purified using the QIAGEN PCR purification kit, Germany. The manufacturer's instructions were followed, and the PCR products were sequenced at GATC Biotech Ltd (SUPREMERUN, Sanger sequencing, GATC, Germany). Translation of the nucleotide sequence to an amino acid sequence was done using Geneious software. Nucleotide and amino acid sequences were compared to the sequence of reference avirulent allele (*AvrLm4-7* (GenBank: AM998638.1); *AvrLm1* (GenBank: AM084345.1); *AvrLm6* (GenBank: AM259336.1)). Sequence alignment was done using Clustal W and Geneious software.

Table 4.3: Sample requirements for library preparation for whole genome sequencing of genomic DNA samples.

Library Prep	Minimum Quantity (µg)	Recommended Quantity (µg)	Minimum Concentration (ng/µL)
TruSeq DNA PCR free 350 bp	1.2	2.2	20

The whole genome sequences were obtained in FASTQ format via Aspera software by Edinburgh Genomics. Raw sequencing reads were first cleaned of adapter sequences and quality trimmed using cutadapt v1.10 (DOI:10.14806/ej.17.1.200; Martin, 2011). Reads were mapped to the *Leptosphaeria maculans* reference genome (Taxonomy ID: 225342; https://www.ncbi.nlm.nih.gov/taxonomy/?term=Leptosphaeria+maculans+brassicae+group&report=info) using Burrows-Wheeler Aligner (BWA) v0.7.8 (Li & Durbin, 2009).

The resulting sequence alignment map (SAM) files were processed using SAMtools v1.3.1 with the "fixmate" and "sort" programs to prepare the files for variant discovery. The variant discovery was prepared in a two-step process using freebayes version dbb6160 (Garrison & Marth, 2012). BAM files were sorted for each of the isolates and were independently called to find variant positions (Valenzuela-Sanchez *et al*., 2018). Sequences covering corresponding genomic region of *AvrLm1*, *AvrLm4-7* or *AvrLm6* genes were obtained by using Bedtools intersect intervals, Galaxy (https://usegalaxy.org/) (Table 4.5). Consensus sequence for each isolate from intersected files from Galaxy were generated by using Geneious R9.1.8 software (Biomatters Limited, NewZealand).

4.3 Results

4.3.1 Isolates virulent against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes

Among the 64 *L. maculans* isolates (2015/2016 cropping season) obtained from phoma leaf spot lesions and tested on cotyledons of a differential set of cultivars, 61 isolates were virulent against *Rlm1*, 51 isolates were virulent against *Rlm4*. No isolates virulent against *Rlm6* or *Rlm7* were detected (Appendix 3-E). Among the 88 *L. maculans* isolates (2016/2017 cropping season) obtained from phoma leaf spot lesions and tested on cotyledons of a differential set of cultivars, 83 isolates were virulent against *Rlm1*, 79 isolates were virulent against *Rlm4*, 3 and 6 isolates were virulent against *Rlm6* or *Rlm7*, respectively (Appendix 3-F).

Table 4.4: Details of the three *Avr* genes used in this study that were obtained from the whole genome sequences of *Leptosphaeria maculans* isolates.

***Avr* gene**	**Supercontig (sequence ID)**	**Genomic position**	**Length (nucleotide)**	**Size of the protein**	***Avr* sequence**
AvrLm4-7	FP929135.1	(JN3-assembly ASM23037v1) Complement (1,374,587:1,375,064)	478 nt (aligned length-432 nt)	143 aa	TTAGTCGCAACCACGAGTCCTTGCTTTCATCTATTTGTAGTTAGTCTTAGGTTCCTAGTAGAAT ATTTAATCTTACGTTAACAACCCATTCAGGGTCATGGTCTAAACCAGTCTCCTGGCCAAATAT AACTCCAGGTGCTGAGCTAAAGCTGCTATAGGGAATTTTGGGAACGAATTCTCGATATCTAT GCGCCGTGATCTTGCAAGTGCAAAGAACTACATCCCAAGGGTCGGTAGTTATGCAGGGCGC ACATTGGACTTTGGCCGTCATATCTATACCCCAGGCACGTTGATGCGATTGACTAAAGTCAAT TAAGCCCTTCGTTACTTTGTTACAATCATTCAAAGCTTCAGTCTTTGTGGGACAAGTGCCTTG AGGATAGCGAATTTCTCCAGATATTGAGGCCTCTCTACAAGCTGTAATTGTAGGGATAGAGA GAGCGAGTAGCGTTAAGATTATCTCGAGGGATAGTGGCAT
AvrLm6	FP929083	(JN3-assembly ASM23037v1) Complement (1,839,097:1,839,677)	432 nt	143 aa	CTATTGGATTTGTCCTTCCCAGTTAACAGATAACCAAGCGCTGACGCACCCATATTTCCCGTC TGGACCGCAGGAGTGGGTCGATTTGGCCTTAAGATTAGGCGAGAAGCAAGTGGAATGTTCA GGACATAAACTGTTCATCTCCTCTCCGCCGATGAGACCATCGTCATGGGCTTGGATGTTAGT CCCTTTTTTCGTAATAGTGCCGTTTATAGCATGAGCCTAGAACGCGTTAAAAAGTGAATATAT TAAAGGAAGCAGATCTAGTACATAGTTGCCTTCGTGCGGAGCGCCTTCGGCTTTTGTCCAAT ATCTGCTGCCTTAGAGTGTTAGCAAATTTTAGGATAGGTTGTATCTAGTAACATACTAAAAACA AATCTGCCTCCAGTGCCTTTGACAACTTTCAAAGTTCGGGTATCGTCAACTCCATCACGTCTA CCGCTCTCGCAGGCGCACAACAAATGAGGCTGACTAATCGTGGTAGTCCTAGATTGGGTTTA AAAGTGCGTTTAGTGTAGCGTTAAGAGAGACACGTACGCTATTCCTAAGACGAGAAGGTATA GGGGTAGGTAAATCACCAT
AvrLm1	AM084345.1	*AvrLm1* gene, exon 1	1053 nt	205 aa	ATCTCCTAATCCATTCCTCACCTCGTGGTCACCTAGTTTAAATAGTCATATTTCTCTATATCTT ACACAACGCACTCTATACACACTCTACACACCACCTTAGCAATCTCCTTGTTAAAGACAATTA CCAACAGTATGTTATATTTTTCTCCCATCCTTCGTAAGTACTAATGTACATCTAGTGGTTCAAT TCAAGACTATCTTTCTATCAACAGCTCTTGCAGCTCTCTTTTCAACAGGTTCTAGCTCCCCAG CTACCAAGAACAATGTGAATCAACCTCTGGACAATATTTCCAGACGTTCCGAGTGGAAATCC GTCCAGATAAGCCCTGTAAAAGAACACTCGGCCAAAACAGCAGATAATACTGAAAATAACCA CAACCTGGAGAAGCGGGTGTTTACTTCGCCTCACATGAAACGGACCTTCACACTAGCGCTG GAGAATACCTTCTACGCTATGGCCTGGCTGATAGACTTCAGCTTTAGCGATGATGGCGAACC CCACTTCAGCTATAAACTGCAGTCGTTTAATCACGAAGACAACCCACCTAAAATATTAGCCGA TGTCGTGGTTCCGCTCATTACAACGTCCTACAACAGCGCAAATCAGTATCGCGGCAAAGCGA ATGTTGAACTTCTTTGTCATCTAGCAAAGGAATACGTCCATGTCTATTTTAGTGTGGAAGTCTT TGCCTCTGGTGCATCATTCGTGATTGGCAAAATCATTGATTATCCTACAGTTTACGTTAATAAT CAATTTAGGAAGGTTGTAAAATTTGATATTGCGGGTGCAATATAAAAACTTGGACTTGTCCAA GTTTTTAGAAAGAAGAACAGAAGTTCAATCTCTTTATGCTTTTCTGTATCTTGGTTGCTTTGTT TATCTTTGTTGCCTTATCTATCTTTGTTGAGTGTTTTATCTTTGTTGGTTTGTTTGTCTAGGTTG ATTTGATTCTCGTTATTGCTTTGTTGTGTTTATTGCTTCTTTTTGATTATTGAATTAATAGCCAT ATCAATTAGTAGCTAGTTAACGTACAATGAAGCCTACAGCGC

A total of 110 *L. maculans* isolates obtained from phoma leaf spot lesions were tested on the cotyledons of a differential set of cultivars in the 2017/2018 cropping season. Among them, 99 isolates virulent against *Rlm1*, 87 isolates virulent against *Rlm4*, 7 and 19 isolates virulent against *Rlm6* or *Rlm7*, respectively were detected (Appendix 3-G). Among the 92 *L. maculans* isolates (2016/2017 cropping season) obtained from stem samples, tested on cotyledons of a differential set of cultivars, 71 isolates were virulent against *Rlm1*, 79 isolates were virulent against *Rlm4*, 6 and 23 isolates were virulent against *Rlm6* or *Rlm7*, respectively (Appendix 3-H).

4.3.2 Quality and concentration of *Leptosphaeria maculans* DNA

4.3.2.1 From conidial suspensions

Concentrations of DNA extracted from 40 conidial suspensions from the 2015/2016 cropping season tested on cotyledons (Appendix 3-E) were measured by using a nano-drop spectrophotometer. Only five samples had good DNA concentration and purity. All others had very low concentrations. This technique needs conidial suspensions with a large number of spores. Thus, this technique was not used further for DNA extraction in this study.

4.3.2.2 From mycelial samples

DNA extracted from the mycelial samples of all the *L. maculans* isolates from the three cropping seasons that were tested on cotyledons (Appendices 3-E to 3-H) had good concentration (ng/µl) and purity at 260/280 nm. DNA samples were stored at -20^{o}C. Therefore, this method was used for extracting DNA from all the *L. maculans* isolates in this study.

4.3.3 Species-specific PCR

Species-specific PCR was done to confirm the identity of the *L. maculans* isolates with both *L. maculans* and *L. biglobosa* primers. The PCR amplicons of *L. maculans* isolates were found to have 330 base pairs and those of *L. biglobosa* isolates had 444 base pairs (Liu *et al*., 2006). All the isolates tested on cotyledons were confirmed to be *L. maculans* (Figure 4.4).

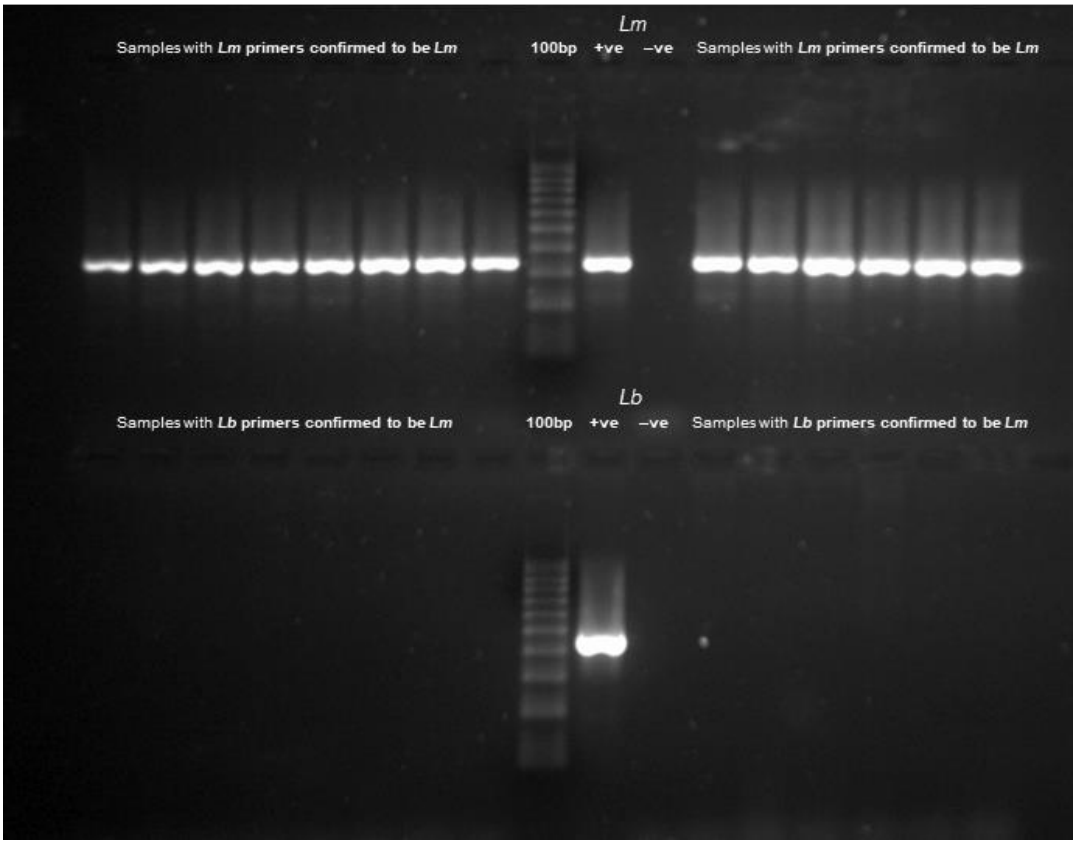


Figure 4.4: Agarose gel analysis of species-specific PCR products. Samples with *L. maculans* primers were run in the first lane, and the same set of samples with *L. biglobosa* primers were run in the second lane. A 100bp ladder was used to confirm the *L. maculans* (330bp) and *L. biglobosa* isolates (444bp). *L. maculans* positive control (ME24); *L. biglobosa* positive control (2003.2.8); negative control (H_2O).

4.3.4 Amplification of the corresponding region of the *Leptosphaeria maculans Avr* genes for Sanger sequencing

4.3.4.1 *AvrLm1*

Amplification of the *AvrLm1* region was observed in all the samples containing the *AvrLm1* allele, positive control for *AvrLm1* and in four samples (Imp16 DK Ext-9, MS15 Dr-1-6, WH15 Dr-2-3 and Imp15 DK-Exl-3) detected with the *avrLm1* allele in the cotyledon test method (Figure 4.5). To confirm that no amplification in the other samples was due to whole *AvrLm1* gene deletion, the samples mentioned above (Appendix 4-B) were amplified using internal primers AvrLm1 IntU/F (Appendix 4-A). Amplification was observed in all the samples containing the *AvrLm1* allele, positive control for *AvrLm1* and in those four samples with the *avrLm1* allele amplified earlier with Solo-ORFcU1/Cl1 primers (Figures 4.5 & 4.6). Hence, it might have been due to whole *AvrLm1* gene deletion in the samples with no amplification (Figure 4.6).

4.3.4.2 *AvrLm4*

Amplification of the *AvrLm4-7* gene (800bp) was observed in all the samples containing the *AvrLm4* allele, positive controls for *AvrLm4* and *avrLm4*, and in twenty-eight samples detected with the *avrLm4* allele in the cotyledon test method (Figure 4.7). No amplification was observed in the negative control and in two samples detected with the *avrLm4* allele (Sf16 DK Ext-2 & Sf16 Why-5; Appendix 4-C) in the cotyledon test method. It might have been due to whole *AvrLm4* gene deletion in the samples with no amplification (Figure 4.7).

4.3.4.3 *AvrLm6*

Amplification of the *AvrLm6* gene (816bp) was observed in all the samples and positive control for *AvrLm6* (Figure 4.8). No amplification was observed in the positive control for *avrLm6* and in the negative control (Figure 4.8).

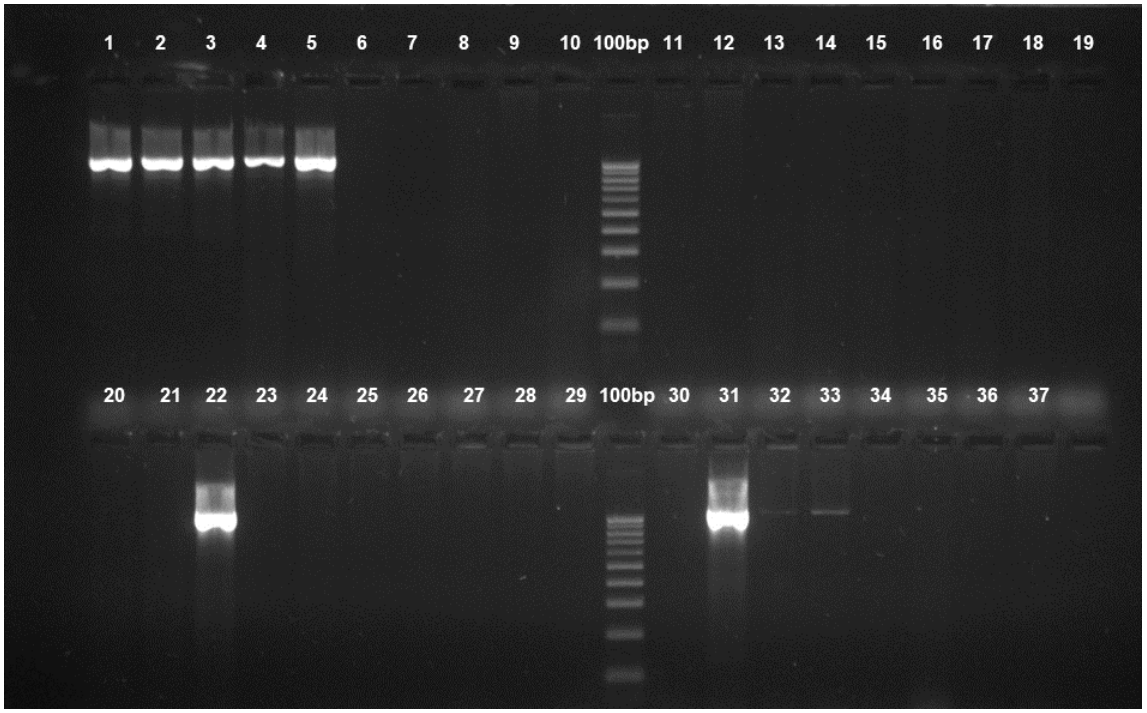


Figure 4.5: PCR products amplified by using *AvrLm1* primers Solo-ORFcU1/Cl1 primers. Amplification was observed at 1124bp position in four *L. maculans* isolates with the *AvrLm1* allele, positive *AvrLm1* control and four *L. maculans* isolates with the *avrLm1* allele. For details of isolates please refer to Appendix 4-B.

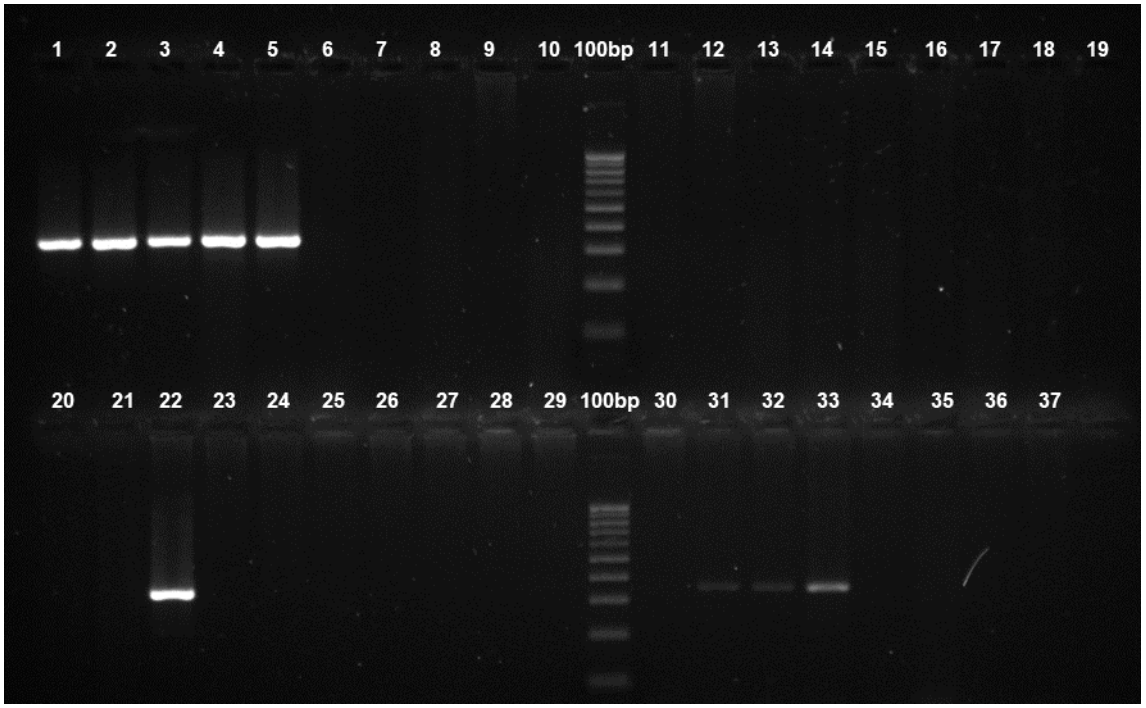


Figure 4.6: PCR products amplified by using the internal primers AvrLm1IntU/F. Amplification at approximately the 330bp position was observed in four *L. maculans* isolates with the *AvrLm1* allele, positive *AvrLm1* control and four *L. maculans* isolates with the *avrLm1* allele. For details of isolates please refer to Appendix 4-B.

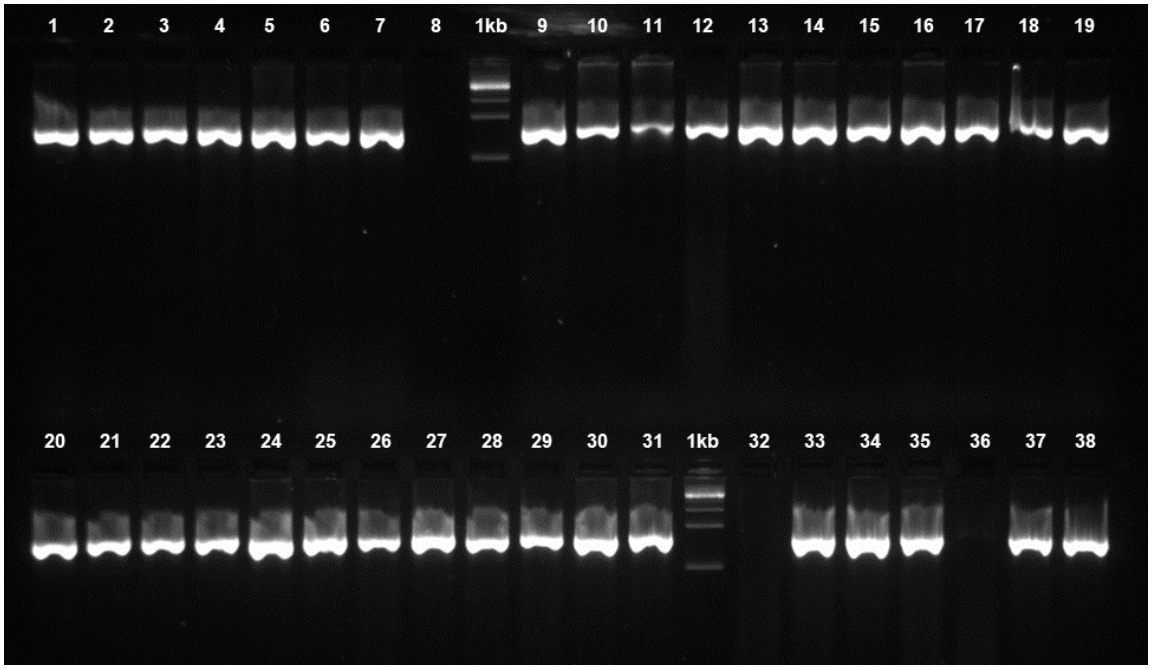


Figure 4.7: Amplification of the *AvrLm4-7* region (approximately 800bp) in the *Leptosphaeria maculans* samples containing avirulent and virulent alleles of *AvrLm4* by using AvrLm4-7ext-F-3 & R primers. Amplification was observed in all the samples, except in two *L. maculans* isolates (Sf16 DK Ext-2 & Sf16 Why-5) with the *avrLm4* allele. For details of isolates please refer to Appendix 4-C.

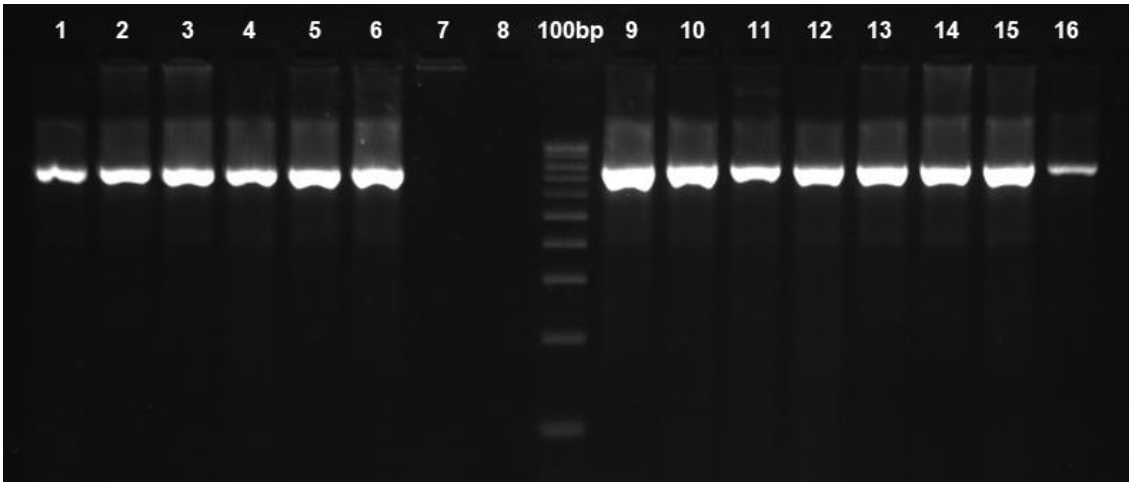


Figure 4.8: Amplification of the *AvrLm6* region (816bp) *Leptosphaeria maculans* samples containing avirulent and virulent alleles of *AvrLm6* by using AvrLm6extU/L primers. Amplification was observed in all the samples, except in positive *avrLm4* controls and in negative control. For details of isolates please refer to Appendix 4-D.

4.3.4.4 *AvrLm7*

Amplification of the *AvrLm4-7* gene (approximately 800bp) was observed was observed in all the samples containing the *AvrLm7* allele, positive control for *AvrLm7*, and in three samples detected with the *avrLm7* allele in the cotyledon test method (Figure 4.9). No amplification was observed in the negative control, positive control for *avrLm7* and in three samples detected with the *avrLm7* allele (Sf16 Why-5, Sf16 Why-11 & Sf16 DK Ext-2; Appendix 4-E) in the cotyledon test method (Figure 4.9). Lack of amplification might have been due to whole gene deletion of the *AvrLm4-7* region.

4.3.5 Analysis of molecular events leading to virulence in *Leptosphaeria maculans* by Sanger sequencing

The PCR products after amplification with Solo-ORFcU1/Cl1 primers, AvrLm4-7ext-F & R primers and AvrLm6extU/L primers were purified using the Qiagen purification kit following the manufacturer's instructions. Before sending the samples for sequencing, the concentration and purity of the PCR products were assessed by Qubit fluorometer and ND-1000 spectrophotometer, respectively. Then 5µl of each PCR product were mixed with 1µl of loading dye (Qiagen, Germany) and loaded in 2% agarose gel to identify if there was any problem occurred in the samples through the purification method (Appendices 4-B to 4-E).

4.3.5.1 *AvrLm1*

The ten samples (four *AvrLm1*, four *avrLm1* (amplified with Solo-ORFcU1/Cl1 primers; Figure 4.5), one *AvrLm1* positive control and one *avrLm1* positive control) sent for sequencing were observed and aligned using Geneious software (Figure 4.10). The low-quality ends were trimmed in the sequences. There was no coverage of the *AvrLm1* region in most of the samples, except for three samples with the *AvrLm1* allele and two samples with the *avrLm1* allele (Imp16 DK-Ext-9 & MS15 Dr-1-6; Appendix 4-B). Following generation of consensus sequences after sequencing with the forward and reverse primers and alignment to the sequence of the *AvrLm1* reference genome (GenBank: AM084345.1), single-base (non-synonymous) point mutation (70%) and partial deletion (1%)

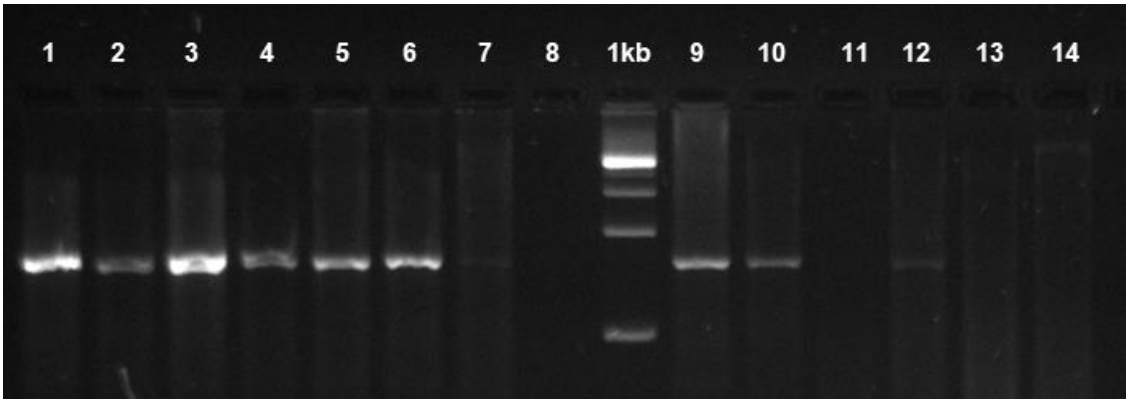


Figure 4.9: Amplification of the *AvrLm4-7* region (approximately 800bp) in the *Leptosphaeria maculans* samples containing avirulent and virulent alleles of *AvrLm7* by using AvrLm4-7ext-F-3 & R primers. Amplification was observed in five *L. maculans* isolates with the *AvrLm7* allele, positive *AvrLm7* control and three *L. maculans* isolates with the *avrLm7* allele. For details of isolates please refer to Appendix 4-E.

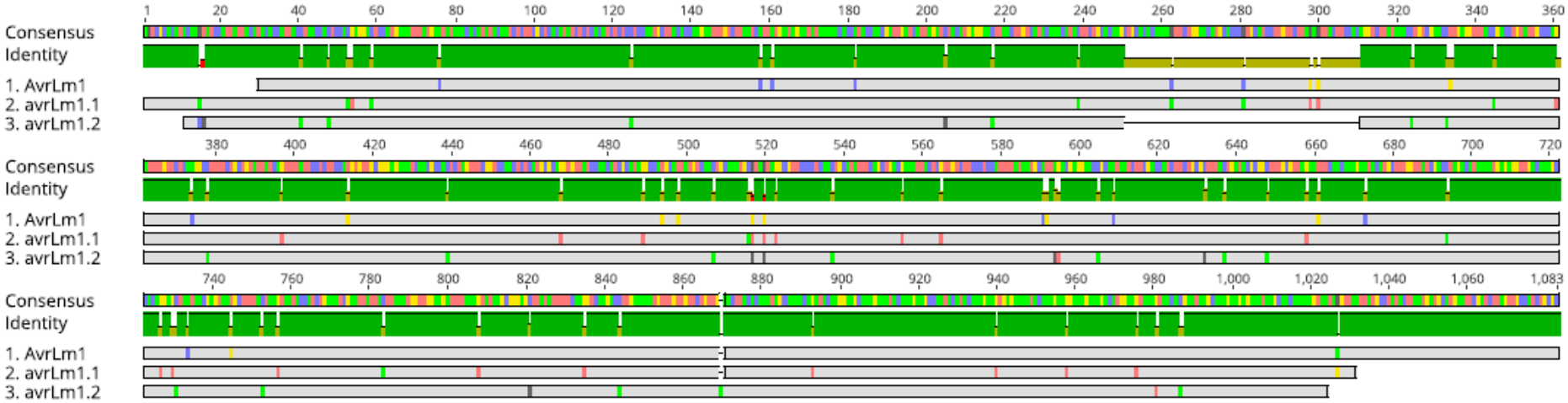


Figure 4.10: Alignment of genomic regions of two sequences of *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm1* effector gene with the sequence of the *AvrLm1* reference genome (GenBank: AM084345.1) using Geneious software. Different colours represent different mutations, Green colour represents 100% identity; Greeny-brown represents 30<100% identity; Red colour represents <30% identity. The lines in grey colour represent deletion of gene at that region. For details of isolates refer to Appendix 4-J.

were observed as the molecular mechanisms of mutation towards virulence in those two samples (Figures 4.10 & 4.11; Appendices 4-K & 4-L).

4.3.5.2 *AvrLm4*

The thirty-five samples (five *AvrLm4*, twenty-eight *avrLm4* (that were amplified using AvrLm4-7ext-F-3 & R primers; Figure 4.7), one *AvrLm4* positive control and one *avrLm4* positive control; Appendix 4-C) sent for sequencing were observed and aligned using Clustal W and Geneious software (Figures 4.12 & 4.13). The low-quality ends were trimmed in the sequences. The sequencing was not good for most of the samples. Following comparison to the sequence of the reference avirulent allele (*AvrLm4-7*; GenBank: AM998638.1), it was observed that the sequence data did not cover the polymorphic site. Hence, no molecular mechanisms of mutation were observed (Figures 4.12 & 4.13).

4.3.5.3 *AvrLm6*

The fifteen samples (five *AvrLm6*, eight *avrLm6*, an *AvrLm6* positive control and an *avrLm6* positive control; Appendix 4-D) sent for sequencing were observed and aligned using Clustal W software. The low-quality ends were trimmed in the sequences. Good coverage of the *AvrLm6* region was observed in only two samples. Following comparison to the sequence of the reference avirulent allele (*AvrLm6*; GenBank: AM259336.1), no mutation events leading towards virulence were observed (Figure 4.14).

4.3.5.4 *AvrLm7*

The ten samples (five *AvrLm7*, three *avrLm7* (amplified using AvrLm4-7ext-F-3 & R primers; Figure 4.9), an *AvrLm7* positive control and an *avrLm7* positive control; Appendix 4-E) PCR products DNA concentration did not meet the sample requirement concentration for Sanger sequencing. Several attempts were made (increasing concentration of template DNA, increasing amounts of PR reaction mixtures) to increase the concentration of the PCR products after purification; however, they were not successful. There is a need to optimise the methods to obtain a high concentration of the purified PCR products.

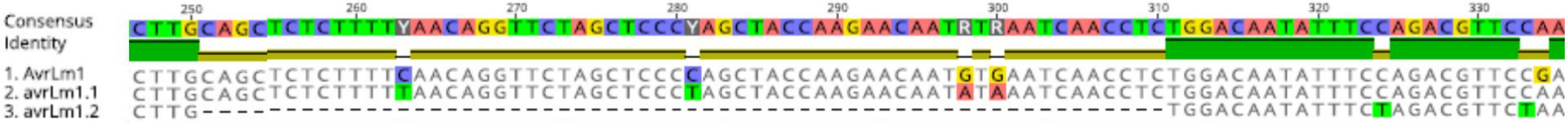


Figure 4.11: Single-base (non-synonymous) point mutations (highlighted in colours) and partial deletion (---) observed in the two *L. maculans* isolates containing virulent allele of the *AvrLm1* effector gene. For whole sequence alignment refer to Appendix 4-K.

Figure 4.12: Nucleotide sequence alignment of the *AvrLm4-7* gene amplified in a virulent *Leptosphaeria maculans* isolate and virulent positive control of *Avrlm4-7* allele using Clustal W software. The sequence of the reference avirulent allele was used to compare the nucleotide sequence following sequencing (SUPREMERUN, Sanger sequencing, GATC, Germany). Dashes indicate data not available; * indicates conservation score; n indicates no base pair score; a indicates weak read by the sequencing.

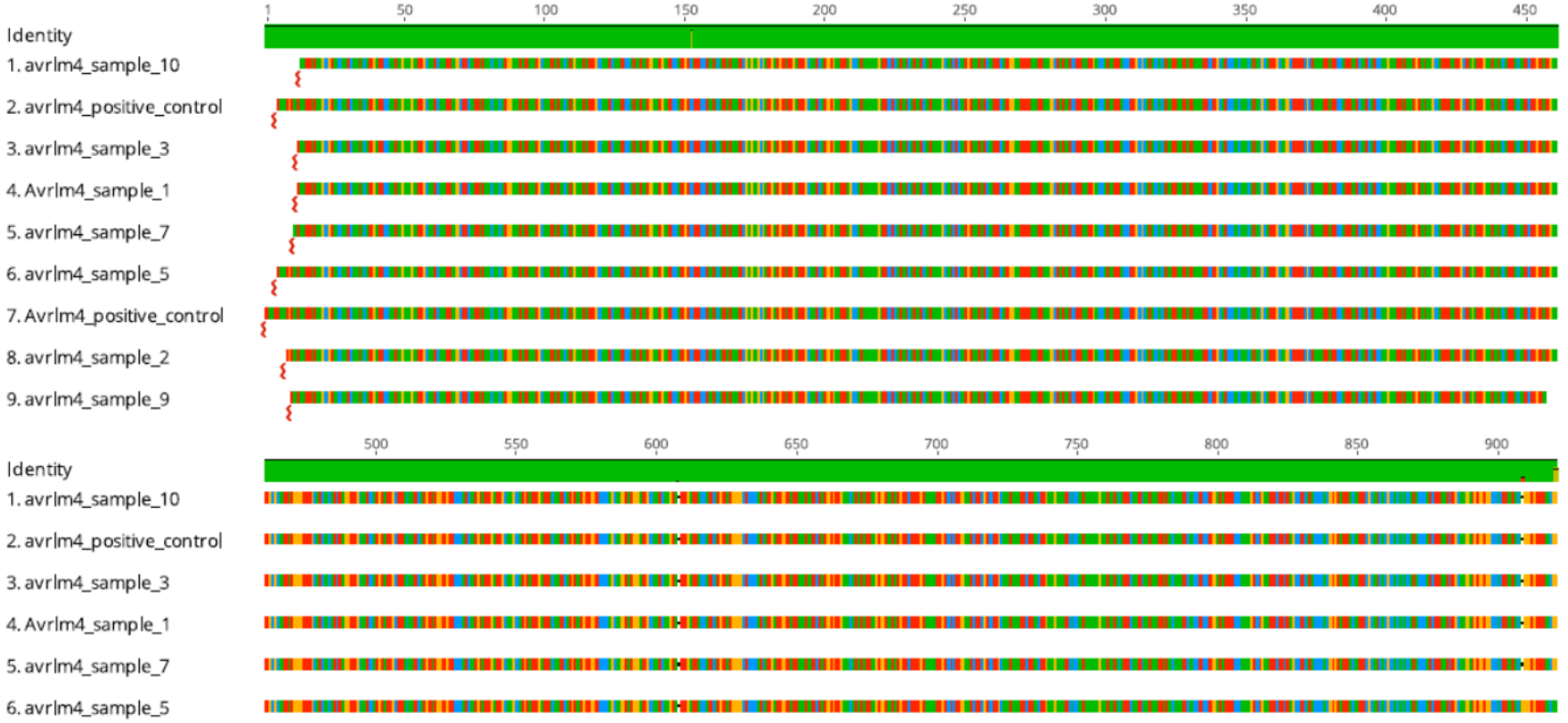


Figure 4.13: Nucleotide sequence alignment of the *AvrLm4-7* gene amplified in an avirulent *Leptosphaeria maculans* isolate (positive control), a virulent isolate (positive control), an avirulent isolate and six isolates virulent towards *Rlm4* using Geneious software.

```
AM259336.1      --------------ATTCTTAACAATCTATAAACAATCTTCGCACGTTCCAAAGCCCTAT
6               caTaTTcnCTTcncaTTCTtAncAnTCTATAAaCAATCTTCGCACGTTCCAAAGCCCTAT
3               --------CTTcncnTTCTTAAcaATCTATAAACAATCTTCGCACGTTCCAAAGCCCTAT
8               --------CTTcncaTTCTTAacAaTCTATAAaCAATCTTCGCACGTTCCAAAGCCCTAT
1               --------CTTcncaTTCTTAacAaTCTATAAaCAATCTTCGCACGTTCCAAAGCCCTAT
4               caTaTTcnCTTcacaTTCTTAacAnTCTATAAaCAATCTTCGCACGTTCCAAAGCCCTAT
7               --------CTTcncaTTCTTAacAnTCTATAAaCAATCTTCGCACGTTCCAAAGCCCTAT
                              ****** ** **********************************

AM259336.1      TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
6               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
3               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
8               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
1               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
4               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
7               TTATAACCTAGCATGGTGATTTACCTACCCCTATACCTTCTCGTCTTAGGAATAGCGTAC
                ************************************************************

AM259336.1      GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
6               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
3               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
8               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
1               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
4               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
7               GTGTCTCTCTTAACGCTACACTAAACGCACTTTTAAACCCAATCTAGGACTACCACGATT
                ************************************************************

AM259336.1      AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
6               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
3               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
8               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
1               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
4               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
7               AGTCAGCCTCATTTGTTGTGCGCCTGCGAGAGCGGTAGACGTGATGGAGTTGACGATACC
                ************************************************************

AM259336.1      CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
6               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
3               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
8               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
1               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
4               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
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7               CGAACTTTGAAAGTTGTCAAAGGCACTGGAGGCAGATTTGTTTTTAGTATGTTACTAGAT
                ************************************************************

AM259336.1      ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
6               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
3               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
8               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
1               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
4               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
7               ACAACCTATCCTAAAATTTGCTAACACTCTAAGGCAGCAGATATTGGACAAAAGCCGAAG
                ************************************************************

AM259336.1      GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
6               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
3               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
8               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
1               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
4               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
7               GCGCTCCGCACGAAGGCAACTATGTACTAGATCTGCTTCCTTTAATATATTCACTTTTTA
                ************************************************************

AM259336.1      ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
6               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
3               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
8               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
1               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
4               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
7               ACGCGTTCTAGGCTCATGCTATAAACGGCACTATTACGAAAAAAGGGACTAACATCCAAG
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AM259336.1      CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
6               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
3               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
8               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
1               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
4               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
7               CCCATGACGATGGTCTCATCGGCGGAGAGGAGATGAACAGTTTATGTCCTGAACATTCCA
                ************************************************************

AM259336.1      CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
6               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
3               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
8               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
1               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
4               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
7               CTTGCTTCTCGCCTAATCTTAAGGCCAAATCGACCCACTCCTGCGGTCCAGACGGGAAAT
```

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                ************************************************************

AM259336.1      ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
6               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
3               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
8               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
1               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
4               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
7               ATGGGTGCGTCAGCGCTTGGTTATCTGTTAACTGGGAAGGACAAATCCAATAGCCATGAC
                ************************************************************

AM259336.1      CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCACTACGGTT
6               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTACnttc
3               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTACGnnt
8               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTACggtt
1               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTAcgt--
4               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTAcgtnC
7               CAACTCCAAAAGCCAACTAATCTGGGCAGGCGCCTTGGCTGTATTGCTACCTCTACgtnC
                ************************************************** ****

AM259336.1      CAAAACTGGTTTACAGTTGCCATATTTATCTAAAGTAGACTAAGAAGTAGCTTAGGTAGA
6               cAAAAaCTGGAa--------------------------------------------------
3               --------------------------------------------------------------
8               nAAAaaCTGGaa--------------------------------------------------
1               --------------------------------------------------------------
4               CAAAAACTGgaa--------------------------------------------------
7               CAAAAACTGGaa--------------------------------------------------


AM259336.1      AGAAGTAAACCTAAATAGGGTTTACTATACTATGTATTCTACTTTTACTTC
6               ---------------------------------------------------
3               ---------------------------------------------------
8               ---------------------------------------------------
1               ---------------------------------------------------
4               ---------------------------------------------------
7               ---------------------------------------------------

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Figure 4.14: Nucleotide sequence alignment of the *AvrLm6* gene amplified in six *avrLm6* samples using Clustal W software. The sequence of the reference avirulent allele of *AvrLm6* gene (Genbank: AM259336.1) was used to compare the nucleotide sequences following sequencing. Dashes indicate data not available; * indicates conservation score; n indicates no base pair score; a indicates weak read by the sequencing. No molecular mechanisms of mutation towards virulence in *Rlm6* were observed.

4.3.6 Whole genome sequencing

L. maculans isolates collected from the diseased leaves and stems from the three cropping seasons that were phenotyped to be virulent towards *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* were selected for whole genome sequencing. Genomic DNA was extracted from those samples and were treated with RNAse A to remove RNA from the DNA samples. After RNAse treatment the concentration and purity of the DNA samples were checked to make sure that they meet the requirements for whole genome sequencing.

4.3.6.1 Quality of DNA samples for whole genome sequencing

Out of sixty-one DNA samples, fifty samples showed clear bands without any smear (Figures 4.15 & 4.16) (Appendices 4-F & 4-G). Out of sixty-one DNA samples, forty samples had highly intact genomic DNA (<DIN-6 score) (Appendix 4-H). Hence, forty *L. maculans* gDNA samples were selected for sending for whole genome sequencing (Appendix 4-I).

4.3.7 Analysis of molecular events leading to virulence in *Leptosphaeria maculans* by whole genome sequencing

Whole genome sequencing was done for forty *L. maculans* isolates virulent towards *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* that were obtained from different sites from the three cropping seasons (Appendix 4-I).

4.3.7.1 *AvrLm1*

Twenty-seven genome sequences of *L. maculans* isolates containing virulent alleles of the *avrlm1* gene were obtained (Appendices 4-I & 4-J). There was a problem in uploading bam files of three sample sequences. There was no sequence coverage for the *Avrlm1* region for the remaining twenty-four sample sequences. This might have been due to whole gene deletion of the *AvrLm1* region.

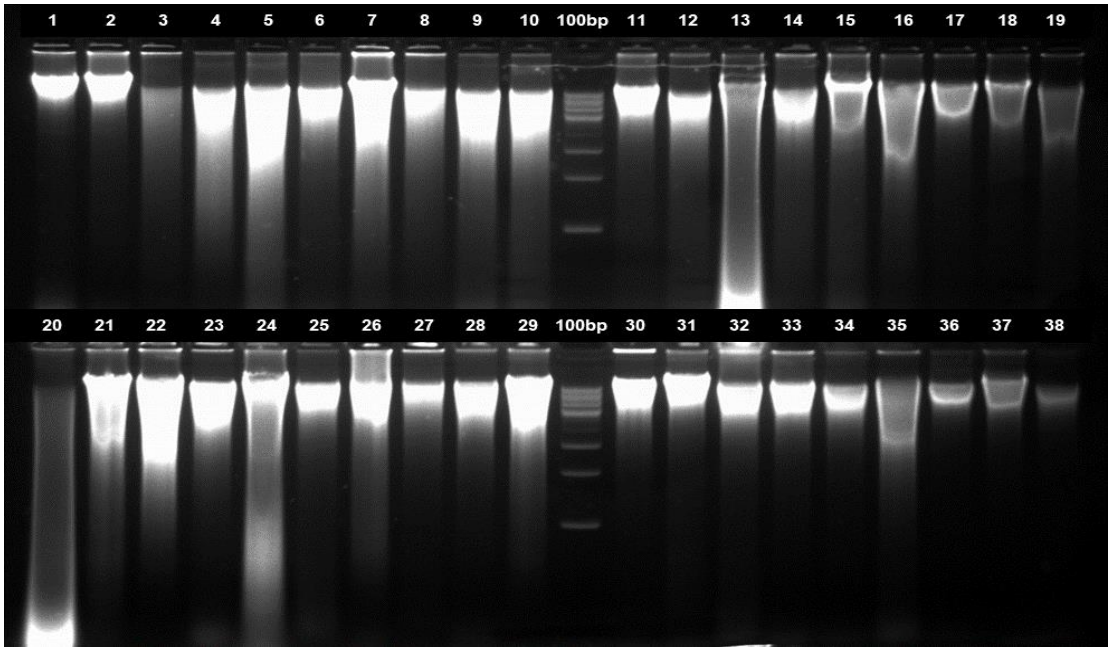

Figure 4.15: Genomic DNA of *Leptosphaeria maculans* isolates run on a 1% agarose gel for selecting them for whole genome sequencing (details of the samples are given in Appendix 4-F).

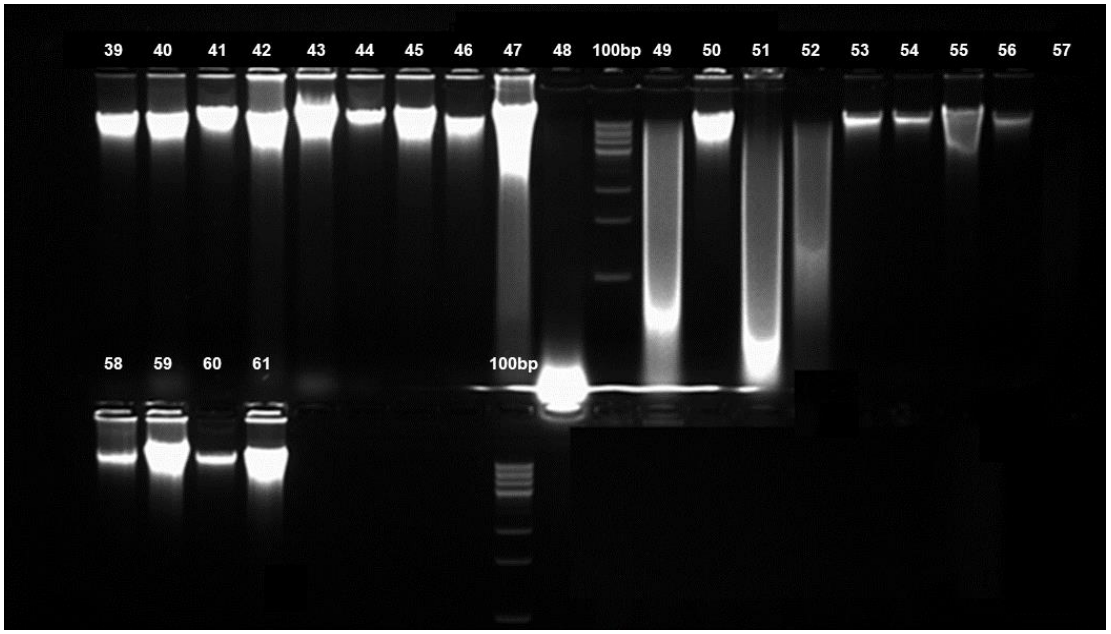

Figure 4.16: Genomic DNA of *Leptosphaeria maculans* isolates run on a 1% agarose gel for selecting them for whole genome sequencing (details of the samples are given in Appendix 4-G).

4.3.7.2 *AvrLm4*

Thirty-seven genome sequences of *L. maculans* isolates containing avirulent (one) or virulent alleles (thirty-six) of the *AvrLm4* effector gene were obtained (Appendices 4-I & 4-J). The main molecular events causing mutations towards *Rlm4* resistance gene are identified to be whole gene deletion (18.2%), partial deletion (3.03%), single-nucleotide (non-synonymous) point mutation (84.8%) and single-nucleotide (synonymous) point mutation (6.1%) in the *L. maculans* isolates containing virulent alleles of the *AvrLm4* effector gene (Figure 4.17). The consensus sequences were aligned with the *AvrLm4-7* published genome (Genbank: ASM23037v1; v23.2.3) by using Geneious software (Figure 4.18; Appendices 4-M & 4-N). There was a problem in uploading bam files of four sample sequences.

There was no sequence coverage for the *AvrLm4-7* region for six sample sequences (18.2%) (WH17 Ang-7, WB17 Har-6, WH17 Why-1, Sf16 Dk-Ext-2, LSPB16 Har-Sb-3-7 and WH17 Dk-Exl-2) (Appendix 4-J). This was due to complete deletion of the *AvrLm4-7* region as there was good coverage of sequence before and after the *AvrLm4-7* region. Single-nucleotide (non-synonymous) point mutations were identified in twenty-eight genome sequences (84.8%) leading to an amino acid change (Figure 4.17). Eighteen different single-nucleotide (non-synonymous) point mutations (SNPs) were identified at thirty-four codon sites; that had led to amino-acid changes or created stop codons (Table 4.6).

Seven single-nucleotide changes were at multiple codon sites. A (C -> T) nucleotide change at different codons (91, 109, 121, 133, 310, 352 and 394) led to amino acid change to a stop codon (Gln -> stop) at various codons (31, 37, 41, 45, 104, 118 and 132) in protein sequences. An (A -> T) nucleotide change at four codons (232, 233, 436 and 464) led to amino acid change to a stop codon (Arg -> stop) at three codons (78, 146 and 148) in protein sequences. A (G -> A) nucleotide change at three codons (181, 292 and 301) led to amino acid change (Asp -> Asn) at three codons (61, 98 and 101) in protein sequences. A (G -> A) nucleotide change at two codons (100 and 118) led to amino acid change (Gly -> Ser) at two codons (34 and 40) in protein sequences.

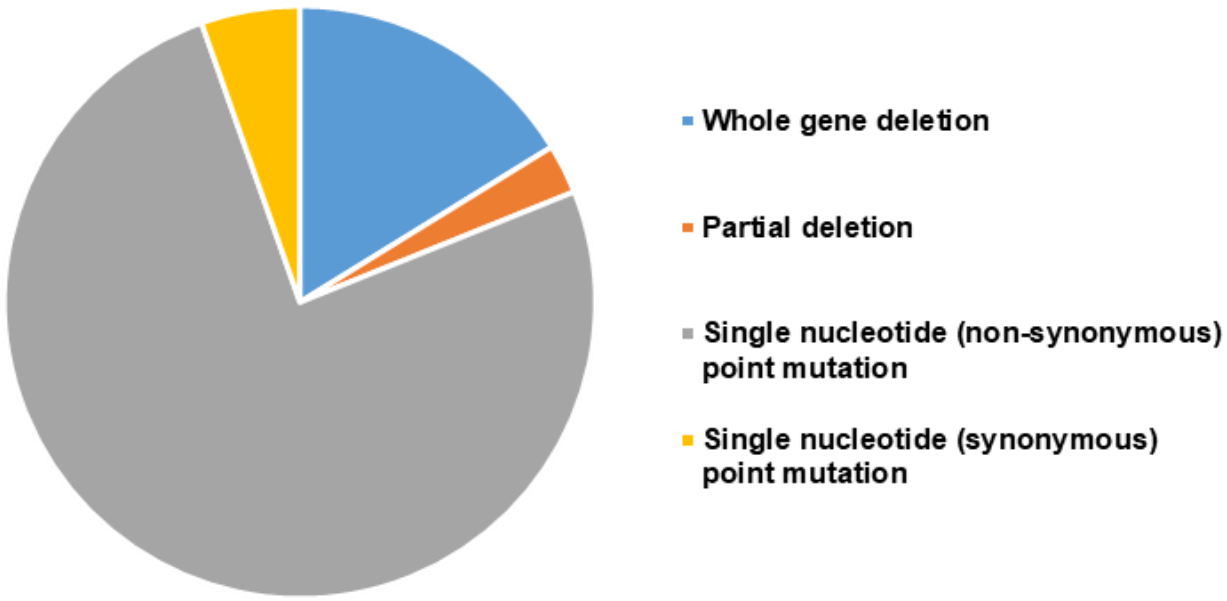

Figure 4.17: Different types of mutations observed in thirty-three genome sequences containing virulent alleles of the *AvrLm4* effector gene. For details of isolates please refer to Appendices 4-I & 4-J.

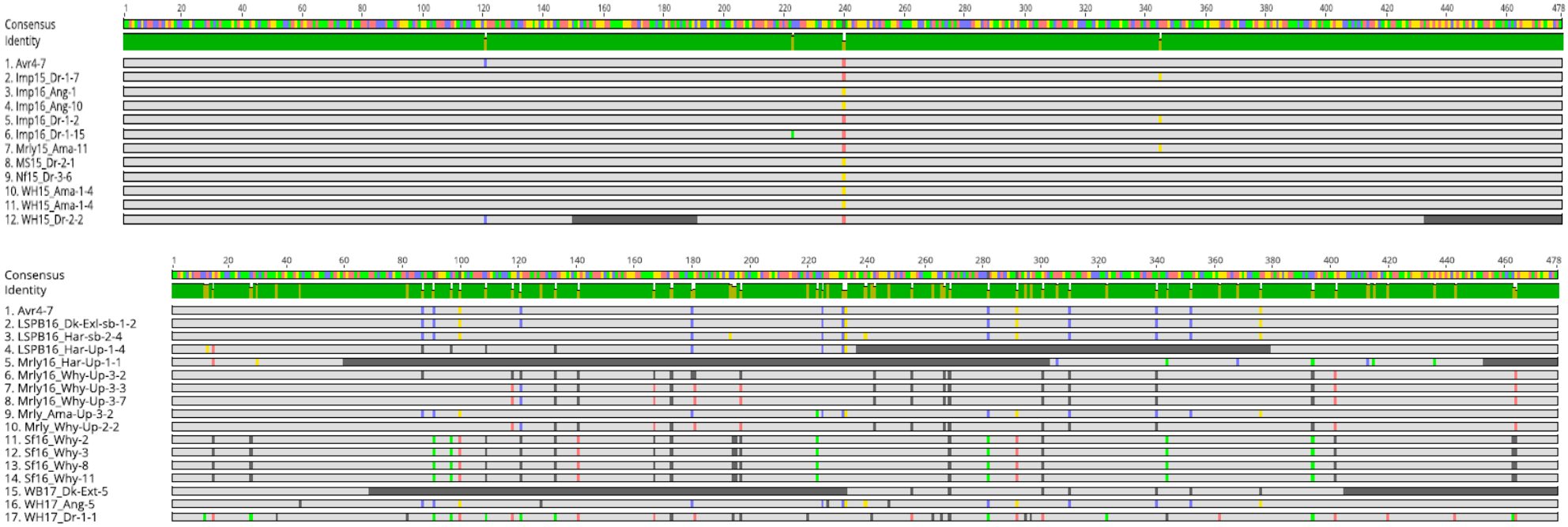

Figure 4.18: Alignment of genomic regions of twenty-seven sequences of *Leptosphaeria maculans* isolates containing avirulent or virulent alleles of the *AvrLm4* or *AvrLm7* effector genes with the sequence of the reference *AvrLm4-7* gene (isolate v23.2.3) using Geneious software. Different colours represent different mutations, Green colour represents 100% identity; Greeny-brown represents 30<100% identity; Red colour represents <30% identity. The shaded areas in grey colour represent no coverage. For details of isolates refer to Appendix 4-J.

Table 4.5: Single-nucleotide polymorphisms (SNPs) identified in the nucleotide genome sequences of *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm4* effector gene leading to an amino acid change in the protein sequences (Appendices 4-M, 4-N, 4-Q & 4-R).

S. No	Nucleic acid change	Amino acid change	No. of isolates
1	A^{13} -> G^{13}	Thr^{4} -> Ala^{4}	1
2	C^{28} -> T^{28}	His^{10} -> Tyr^{10}	1
3	T^{30} -> G^{30}	His^{10} -> Gln^{10}	1
4	C^{91} -> T^{91}	Gln^{31} -> stop	5
5	G^{100} -> A^{100}	Gly^{34} -> Ser^{34}	5
6	C^{109} -> T^{109}	Gln^{37} -> stop	1
7	G^{118} -> A^{118}	Gly^{40} -> Ser^{40}	4
8	C^{121} -> G^{121}	Gln^{41} -> Glu^{41}	5
9	C^{121} -> T^{121}	Gln^{41} -> stop	1
10	C^{133} -> Y^{133}	Gln^{45} -> stop	1
11	C^{133} -> T^{133}	Gln^{45} -> stop	1
12	G^{167} -> A^{167}	Trp^{56} -> stop	4
13	G^{181} -> A^{181}	Asp^{61} -> Asn^{61}	4
14	C^{193} -> G^{193}	Pro^{65} -> Ala^{65}	1
15	C^{223} -> T^{223}	Pro^{75} -> Ser^{75}	6
16	C^{232} -> T^{232}	Arg^{78} -> stop	9
17	G^{233} -> A^{233}	Arg^{78} -> stop	1
18	G^{256} -> A^{256}	Gly^{85} -> Arg^{85}	1
19	G^{292} -> A^{292}	Asp^{98} -> Asn^{98}	5
20	G^{301} -> A^{301}	Asp^{101} -> Asn^{101}	1
21	A^{306} -> C^{306}	Stop -> Tyr^{102}	1
22	C^{310} -> T^{310}	Gln^{104} -> stop	6
23	C^{323} -> T^{323}	Ser^{108} -> Leu^{108}	1
24	C^{340} -> T^{340}	His^{114} -> Tyr^{114}	6
25	C^{344} -> T^{344}	Ser^{115} -> Leu^{115}	5
26	C^{352} -> T^{352}	Gln^{118} -> stop	10
27	G^{362} -> A^{362}	Trp^{121} -> stop	1
28	A^{368} -> C^{368}	Lys^{123} -> Thr^{123}	1
29	G^{377} -> A^{377}	Glu^{126} -> Lys^{126}	10
30	C^{394} -> T^{394}	Gln^{132} -> stop	6
31	A^{413} -> C^{413}	Tyr^{138} -> Ser	1
32	A^{415} -> T^{415}	Lys^{139} -> stop	1
33	A^{436} -> T^{436}	Arg^{146} -> stop	1
34	G^{464} -> A^{464}	Arg^{148} -> stop	1

A (G -> A) nucleotide change at two codons (167 and 362) led to amino acid change to a stop codon (Trp -> stop) at two codons (56 and 121) in protein sequences. A (C -> T) nucleotide change at two codons (323 and 344) led to amino acid change (Ser -> Leu) at two codons (108 and 115) in protein sequences. A (C -> T) nucleotide change at two codons (28 and 340) has led to change of amino acid change (His -> Tyr) at two codons (10 and 114) in protein sequences. Other single-nucleotide changes were at single codons sites (Table 4.6) (Appendices 4-M, 4-N, 4-Q & 4-R).

Location of the single-nucleotide poylmorphisms (SNPs) leading to an amino acid change and number of isolates in which the mutations are identified were given in Table 4.6. Partial deletion was observed in the genome sequence (3%) of WH15 Dr-2-2 isolate that is virulent to *Rlm4* and avirulent towards *Rlm7* (Figure 4.19). Partial deletions were also observed in another three genome sequences (Mrly16 Har-up-1-1, LSPB16 Har-up-1-4 and WB17 Dk-Ext-5) (Appendix 4-Q); this might have been due to virulence towards the *Rlm7* gene, as they have virulent alleles of both *avrLm4* and *avrLm7*. Two synonymous substitutions were observed (6.1%) in the isolates virulent towards *Rlm4* and avirulent towards *Rlm7*. They are at A^{240}-> G^{240} and A^{345}-> G^{345} leading to no change in amino acids Cys^{81} and Ser^{115}, respectively (Figure 4.19; Appendices 4-M & 4-N).

4.3.7.3 *AvrLm6*

Nine genome sequences of *L. maculans* isolates containing virulent alleles of the *avrlm6* gene were obtained (Appendices 4-I & 4-J) and the consensus sequences were aligned with the *AvrLm6* published genome (Genbank: ASM23037v1) by using Geneious software (Figure 4.20). There was problem in uploading bam files of two sample sequences. Complete deletion of the *AvrLm6* gene was observed in one genome sequence (WH17 Dk-Exl-2) (Appendix 4-J). No other molecular mechanisms of mutations leading to virulence against *Rlm6* were observed in the genome sequences (Figure 4.20; Appendix 4-O).

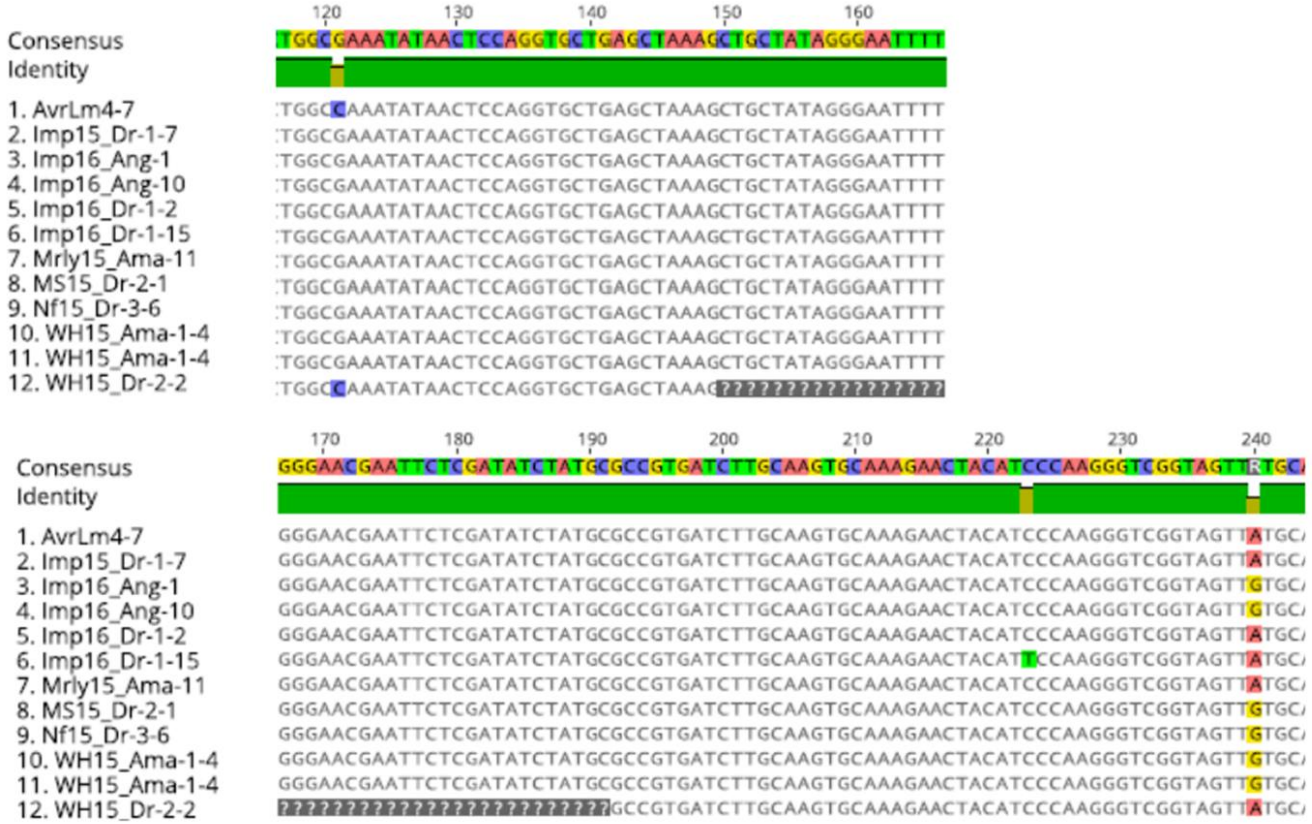

Figure 4.19: Partial deletion (????), non-synonymous substitution (C^{121} -> G^{121}) and synonymous substitution (A^{240}-> G^{240}) observed in *L. maculans* isolates containing virulent allele of the *AvrLm4* effector gene. For whole sequence alignment refer to Appendix 4-M.

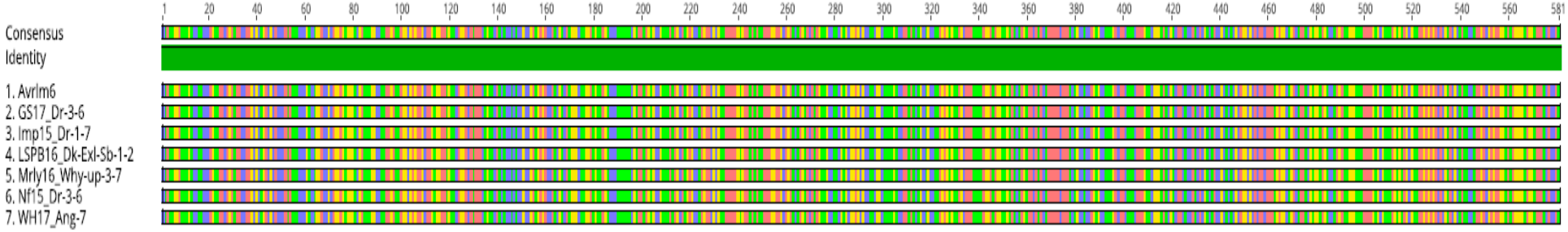

Figure 4.20: Alignment of genomic regions of six sequences of *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm6* effector gene with the sequence of the reference *AvrLm6* gene (v23.2.3) using Geneious software. Green colour on the identity bar represents 100% identity of all sequences with the reference gene sequence. For details of isolates refer to Appendix 4-J.

4.3.7.4 *AvrLm7*

Twenty-six genome sequences of *L. maculans* isolates containing avirulent (one) and virulent alleles (twenty-five) of the *AvrLm7* effector gene were obtained (Appendices 4-I & 4-J). There was problem in uploading bam files of four sample sequences. There was no sequence coverage for the *AvrLm4-7* region for six sample sequences (WB17 Har-6, WH17 Ang-7, WH17 Why-1, Sf16 Dk-Ext-2, LSPB16 Har-sb-3-7 and WH17 Dk-Exl-2) of *L. maculans* isolates containing virulent alleles of *avrLm7* (Appendix 4-J). This was due to whole gene deletion of the *AvrLm4-7* region as there was good coverage of sequence before and after the *AvrLm4-7* region.

Genome sequences (consensus sequences) of the remaining sixteen *L. maculans* isolates containing avirulent (one) or virulent (fifteen) alleles of the *AvrLm7* effector gene (Appendix 4-Q) were aligned with the *AvrLm4-7* published genome (Genbank: ASM23037v1; v23.2.3) by using Geneious software (Figure 4.18). Various molecular mechanisms of mutation leading to virulence towards the *Rlm7* resistance gene were identified in these isolates, such as whole gene deletion (27.3%), partial deletion (13.6%), single-nucleotide (non-synonymous) point mutation (SNPs) (68.2%), single-nucleotide (synonymous) point mutation (9.1%), Nonsense point mutation (50%), repeat-induced point mutation (RIPs) (68.2%), single-nucleotide deletion (27.3%), transition (68.2%) and transversion mutation (13.6%) (Figure 4.21; Appendices 4-Q & 4-R).

Partial deletions were observed in three genome sequences (Mrly16 Har-up-1-1, LSPB16 Har-up-1-4 and WB17 Dk-Ext-5) (Figure 4.22; Appendix 4-Q). Single-nucleotide (non-synonymous) point mutations (SNPs) (Table 4.6) were observed in fifteen genome sequences. Transition mutations in fifteen genome sequences and transversion mutations in three genome sequences. Repeat-induced point mutations were identified in fifteen genome sequences. Synonymous substitution was identified in the genome sequences at the 240 codon (A->G) (Leucine) that did not lead to an amino acid change (LSPB16 Har-Sb-2-4 and WH17 Ang-5). Single-nucleotide deletions were identified in six genome sequences. Nonsense point mutations (stop-gain and start-loss) were identified in eleven genome sequences (Table 4.6) (Figure 4.22) (Appendices 4-Q & 4-R).

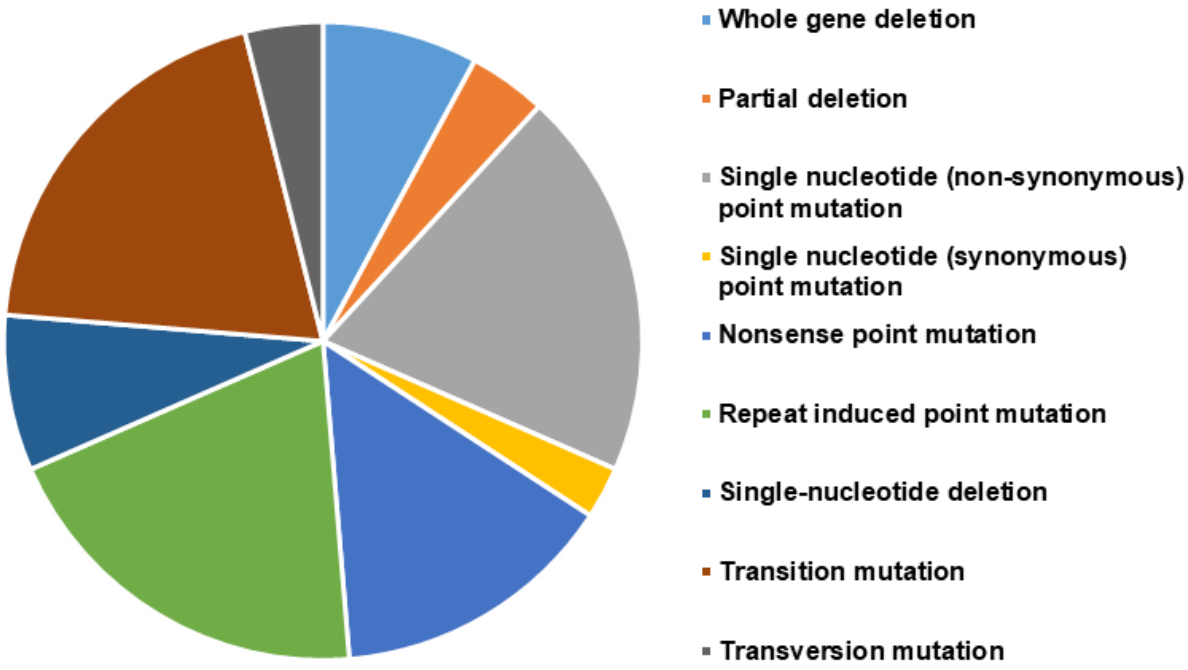

Figure 4.21: Different types of mutations observed in twenty-two genome sequences of isolates containing virulent alleles of the *AvrLm7* effector gene. For details of isolates please refer to Appendices 4-I & 4-J.

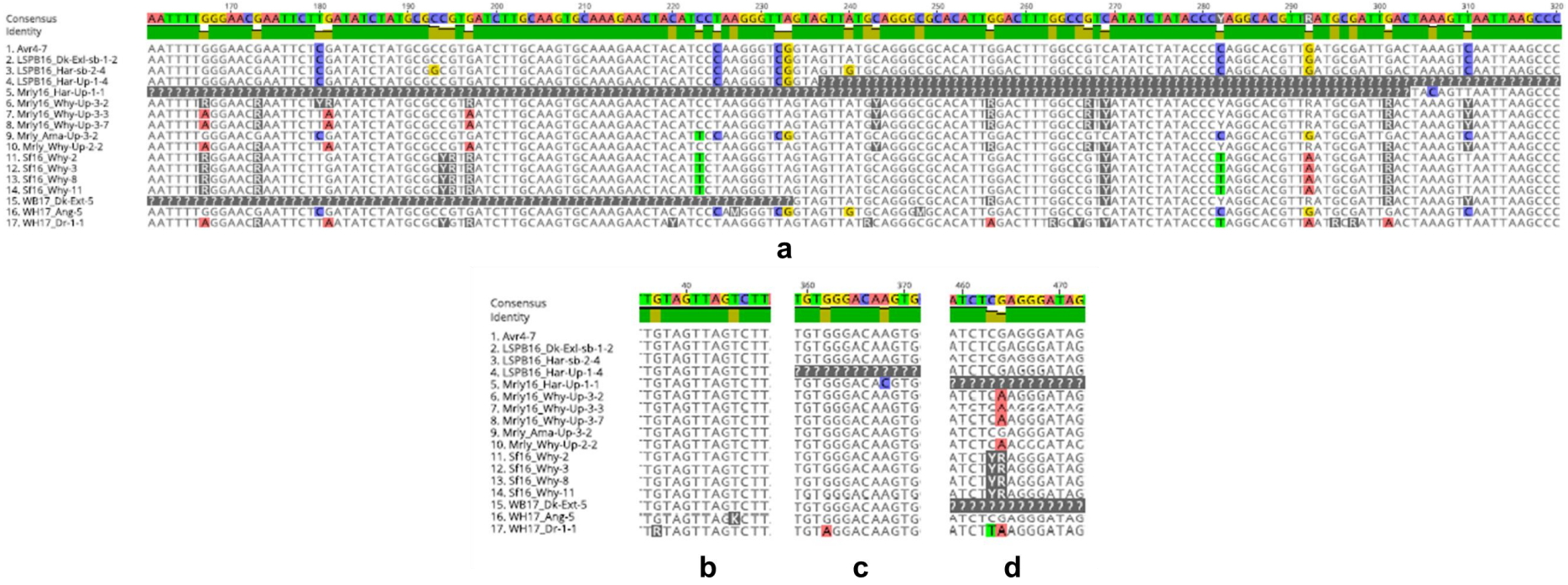

Figure 4.22: Partial deletions (????) (a), single-nucleotide polymorphisms (a), single-nucleotide deletions (b), Transition/Transversion mutations (c) and Repeat-induced point mutations (d) observed in *L. maculans* isolates containing virulent allele of the *AvrLm7* effector gene. For whole sequence alignment refer to Appendix 4-Q.

4.4 Discussion

The most important finding of this chapter is that *Rlm7* resistance gene is an important *R* gene and the corresponding *AvrLm7* gene is crucial either for the survival of *L. maculans* or for its pathogenicity. This is because the pathogen has undergone various molecular mechanisms of mutations towards virulence in the *AvrLm7* gene so as to avoid the host recognition.

In this work, 243 *L. maculans* isolates obtained from diseased leaves were virulent against *Rlm1*, 217 *L. maculans* isolates virulent against *Rlm4*, 10 *L. maculans* isolates virulent against *Rlm6* and 25 *L. maculans* isolates virulent against *Rlm7* were obtained by an established cotyledon test method (Balesdent *et al*., 2002) for the three cropping seasons (2015/2016 to 2017/2018). From diseased stems, 71 *L. maculans* isolates virulent against *Rlm1*, 79 *L. maculans* isolates virulent against *Rlm4*, 6 and 23 *L. maculans* isolates virulent against *Rlm6* or *Rlm7* were identified by testing the isolates on a set of diferential set of cultivars/lines. DNA was obtained from the mycelial samples of all the *L. maculans* isolates and their identity was confirmed by species-specific PCR.

From the results obtained with virulent isolates, it is evident that the percentage of isolates containing virulent alleles of the *AvrLm7* effector gene was increasing and this novel resistance gene is at risk in the UK. Greater numbers of isolates virulent towards *Rlm7* were obtained from stem samples (single ascospore isolations) than from leaf samples. This work confirms that the virulent alleles of *AvrLm7* effector gene were able to reproduce as pseudothecia on the stem debris and infect the cotyledons and leaves of the next crop. There are chances of increase in the percentages of virulent alleles of *AvrLm7* effector gene, if the isolates obtained from stem samples of 2017/2018 harvest year are screened. The *Rlm6* resistance gene is still effective in the UK, as there were not many virulent isolates obtained. However, this resistance gene is not currently being used in UK oilseed rape commercial cultivars. In comparison to the numbers of isolates obtained containing virulent alleles of the *AvrLm6* or *AvrLm7* effector genes, to that of the numbers of isolates containing virulent alleles of the *AvrLm1* or *AvrLm4* effector genes suggests that the respective *R* genes were no longer effective in the UK. For the investigated three years, more avirulent alleles of

different effector genes were detected suggesting the increased diversity of races (Table 3.12 & Figure 3.19).

The *AvrLm1*, *AvrLm4*-7 and *AvrLm6* regions were amplified in the *L. maculans* isolates, respectively (Figures 4.5 to 4.9) by using Solo-ORFcU1/Cl1, AvrLm1IntU/F, AvrLm4-7ext-F-3 & R and AvrLm6extU/L primers (Appendix 4-A). However, the concentrations of the PCR products after purification were not great enough to send them for Sanger sequencing to identify the molecular mechanisms of virulence towards *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes. Despite making several attempts to optimise the technique to improve the concentration of PCR products, this was not successful. Few PCR products with good concentrations were sent for Sanger sequencing and the sequencing quality was not good for most of the samples. Not many sequences were obtained to analyse and identify the molecular mechanisms of mutations leading towards virulence against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes with this method (Figures 4.10 to 4.14).

Whole genome sequences of *L. maculans* isolates that were phenotyped for virulence against *Rlm1, Rlm4*, *Rlm6* or *Rlm7* resistance genes were obtained (Appendices 4-I & 4-J). However, there were few problems such as loading bam files for five isolates (Appendix 4-J). There was no output from bam files for the corresponding *AvrLm1* region for all the genome sequences of *L. maculans* isolates containing virulent alleles of the *AvrLm1* effector gene. This might have been due to whole gene deletion of the *AvrLm1* gene. No amplification of the *AvrLm1* region (1124bp) was observed in twenty-six *L. maculans* isolates containing virulent alleles of the *AvrLm1* effector gene amplified using Solo-ORFcU1/Cl1 primers (Figure 4.5). This might have been due to whole gene deletion. However, it must be confirmed by Southern blotting.

It was mentioned earlier in a French study that the molecular mechanism of mutation leading towards virulence in *Rlm1* was due to whole gene deletion (Gout *et al*., 2007). Single-base point mutations (70%) were identified to be another molecular mechanism leading to virulence against *Rlm1* gene (Figures 4.11 & 4.12), which was also identified in the French study (Gout *et al*., 2007). In addition to them, partial deletion (1%) was also identified in one of the two sequences of

L. maculans isolates containing virulent alleles of the *AvrLm1* effector gene (Figures 4.11 & 4.12). There is need to optimize the methods to identify the molecular mechanism of mutation leading towards virulence against *Rlm1*. One problem may have been the DNA extraction method; that might be splicing the DNA and not making it appropriate for sequencing studies.

Events leading to mutations leading to virulence towards *Rlm6* were observed in the *L. maculans* isolates from both Sanger sequencing results and whole genome sequences. Even though the sequence data obtained from Sanger sequencing covered the *AvrLm6* coding region, no mutations were observed in any of the isolates (Figure 4.14). Deletion of the *AvrLm6* region was observed in one of the isolates (WH17 Dk-Exl-2) sent for whole genome sequencing, as there was good coverage of sequencing before and after the *AvrLm6* region (Appendix 4-J). In France, mutations like deletion of the *AvrLm6* gene, point mutations and RIP inactivation were observed to be leading to virulence towards *Rlm6* (Fudal *et al*., 2009). In this study, the nucleotide sequences of the isolates thought to be containing virulent alleles of the *AvrLm6* effector gene were similar to the nucleotide sequence of isolates thought to be containing the avirulent alleles of *AvrLm6* effector gene (Figures 4.14 & 4.20; Appendix 4-O). However, these isolates caused disease symptoms (large lesions with dark margins) on the cotyledons of the *Rlm6* cultivar in the cotyledon test method (Figure 3.16).

Three molecular mechanisms of mutations leading towards virulence in *Rlm4* resistance gene were identified in this study. The major one was single-nucleotide (non-synonymous) point mutation or single-nucleotide polymorphism (SNPs) (84.8%), followed by whole gene deletion (18.2%) and partial deletion (3.03%) (Figure 4.17). In a French study, it was observed that the single-nucleotide polymorphism (G^{358} -> C^{358}) leading to an amino acid change (G^{120} -> R^{120}) was the main cause of virulence towards the *Rlm4* resistance gene (Parlange *et al*., 2009). In this study, eighteen different single-nucleotide polymorphisms were identified at thirty-four codon sites, that led to amino-acid changes or created stop codons (Table 4.6) (Appendices 4-M, 4-N, 4-Q & 4-R).

Whole gene deletion was observed in (18.2%) six *L. maculans* isolates containing virulent alleles of the *AvrLm4* and *AvrLm7* effector genes in this study. Similarly,

in the French study, whole gene deletion of the *AvrLm4-7* region was observed in the *L. maculans* isolates containing virulent alleles of *avrLm4* or *avrLm7* (Parlange *et al*., 2009). The whole gene deletion might have been the molecular mechanism of virulence towards the *Rlm7* gene. As the *Rlm4* and *Rlm7* genes are closely linked to each other and all the *L. maculans* isolates contained virulent alleles of the *AvrLm4* and *AvrLm7* effector genes, it is difficult to predict the causes of whole gene deletion of the *AvrLm4-7* region.

This was also confirmed by the lack of amplification observed in two isolates (Sf16 Why-5 & Sf16 DK Ext-2) that were virulent towards both *Rlm4* and *Rlm7* resistance genes (Figures 4.7 & 4.9). Partial deletion was identified in one of the isolates that is virulent towards *Rlm4* and avirulent towards *Rlm7*, which is in support to the French study (Parlange *et al*., 2009). Synonymous substitutions were identified at two different codons (Figure 4.18; Appendix 4-M) in isolates virulent towards *Rlm4* and avirulent towards *Rlm7*. These were not identified in the French study (Parlange *et al*., 2009).

In this study, nine different molecular mechanisms of mutation leading to virulence towards the *Rlm7* resistance gene, such as whole gene deletion, partial deletion, single-nucleotide (non-synonymous) point mutation, single-nucleotide (synonymous; silent mutations that does not cause amino acid change in the protein sequence) point mutation, Nonsense (stop-gain and start-loss) point mutation, Repeat induced point mutation, single-nucleotide deletion, transition (T<->C or A<->G) and transversion (G<->C, G<->T, C<->A and T<->A) were identified (Figure 4.21). This is the first report of various molecular mechanisms of mutations of virulence towards the *Rlm7* resistance gene in the UK. These results are in agreement of French studies (Daverdin *et al*., 2012; Carpezat *et al*., 2014) that stated that there are a large diversity of molecular events leading to virulence towards the *Rlm7* resistance gene.

However, in those studies they have amplified the *AvrLm4-7* gene by using different primers. In this study, whole genome sequencing was used and a large fragment (493 bp) containing the whole coding sequence of the *AvrLm4-7* gene was used to identify molecular mechanisms of mutation leading towards virulence in *L. maculans* isolates containing virulent alleles of the *AvrLm4* effector gene

and avirulent/virulent alleles of the *AvrLm7* effector gene (Figure 4.18; Appendices 4-M & 4-Q). In agreement with the French study (Daverdin *et al*., 2012), RIP mutations were identified as one of the molecular mechanisms of mutations leading towards virulence towards *Rlm7* resistance gene. Di-nucleotide (AA) deletions were not observed in any of the *L. maculans* isolates genome sequences.

One of the main reasons for the various molecular mechanisms of mutation leading towards virulence towards the *Rlm7* resistance gene in the UK is due to the selection pressure (Daverdin *et al*., 2012). Another main reason is that the corresponding *AvrLm7* gene seems to contribute a lot for the survival of the pathogen or for its pathogenicity (Rouxel *et al*., 2017). Other reasons could be lack of rotation of *R* genes in oilseed rape cultivars (the *Rlm7* resistance gene has been widely used in commercial UK oilseed rape cultivars for more than 15 years), increased rate of sexual reproduction due to unburied crop debris from previous seasons (it was proved in this study that *L. maculans* single ascospore isolates from pseudothecia on stem samples from the previous year's crops had virulent alleles of the *AvrLm7* effector gene).

The change of *L. maculans* races from avirulent to virulent was due to various molecular mechanisms of mutation with the most common being whole gene deletion (Ghanbarnia *et al*., 2015). In agreement to this statement, whole gene deletion was observed as a common molecular mechanism of mutation leading to virulence towards *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes in this study. However, it must be confirmed in isolates virulent towards *Rlm1* resistance gene by Southern blotting analysis. Early detection of virulent isolates of the *AvrLm7* effector gene and molecular mechanisms of mutations towards virulence towards *Rlm7* resistance gene in the UK was done in this study, which will be valuable information for breeders and farmers in the UK.

Chapter 5 Understanding effects of environmental factors on *Brassica napus* resistance against *Leptosphaeria maculans* in field experiments

5.1 Introduction

Environmental factors such as temperature and rainfall have considerable influence on crop disease epidemics (Coakley *et al.,* 1999; Chakraborty, 2005), which threaten food security (Evans *et al*., 2008; Fisher *et al*., 2012). Effects of environmental factors might be on the pathogen, the host or on the host-pathogen interaction (Huang *et al*., 2005). There are regional differences in climatic conditions in the UK, where temperature is often greater in the south and relatively lower in the north.

An increase in temperature may have an impact not only on the pathogen development but also on the host resistance response (Huang *et al*., 2006). Worldwide, the oilseed rape crop faces a great threat from phoma stem canker disease, especially in Australia, where the temperatures are greater during the growing season than in Europe (Howlett *et al*., 2001; Sprague *et al*., 2006). In the UK, severe phoma stem canker disease epidemics were observed in the south where the temperature is greater compared to Scotland where the temperature is less (Evans *et al*., 2008) (Figure 1.5). Due to the lower temperatures in Scotland, the phoma leaf spots observed on the oilseed rape crops are unable to cause severe phoma stem cankers. This variation is directly related to differences in climatic conditions, since weather-based models show that stem canker severity increases with increasing winter/spring temperature and temperatures are greater in the south of the UK (Stonard *et al*., 2010).

Temperature and rainfall affect the release of ascospores from the pseudothecia in the autumn (Toscano-Underwood *et al*., 2003). High temperatures and continuous rainfall in summer/autumn favour the maturation of pseudothecia and the early release of ascospores (Huang *et al*., 2007). If the phoma leaf spots are observed on the oilseed rape crops in early autumn, it results in severe phoma stem cankers before harvest (Sun *et al*., 2001). This is because the plants are

very small in autumn and it does not take much time for the pathogen to grow from the leaves to the stem tissues along the petioles and cause cankers (Huang *et al*., 2006). If the leaf spotting does not appear until late November or December, then the phoma stem cankers are less likely to be severe the next summer (Huang *et al*., 2007).

Environmental factors like temperature and rainfall play a major role in the maturation of pseudothecia and release of ascospores during autumn (Evans *et al*., 2006; Fitt *et al*., 2006). Temperature and *R* gene-mediated resistance play an important role during the appearance of phoma leaf spots and colonization of leaf tissues by the pathogen during winter. Temperature and quantitative resistance play a vital role during colonization of petioles and stem tissues by the pathogen during spring before the appearance of early canker. Temperature plays an important role in increasing the severity of phoma stem canker in summer (Evans *et al*., 2008).

The severity of stem cankers also depends on the temperature during the winter and early spring when the pathogen is in its second symptomless phase, i.e. colonizing stem tissues (Stonard *et al*., 2010). There is evidence that temperature affects both *R* gene-mediated resistance (Huang *et al*., 2006) and quantitative resistance (Huang *et al*., 2009) against *L. maculans*. *Rlm6* resistance is rendered ineffective when there is an increase in the temperature from 15^{o}C to 25^{o}C (Huang *et al*., 2006). Previous studies revealed that *Rlm1* resistance to *L. maculans* is not effective at a temperature of 27^{o}C (Badawy *et al*., 1992). By this, it is evident that ineffective host resistance at higher temperatures can result in severe phoma stem cankers (Stonard *et al*., 2010).

To control crop diseases, two types of resistances (quantitative resistance (QR) and *R* gene-mediated qualitative resistance) play a major role in any plant host. QR is usually controlled by several minor genes (quantitative trait loci; QTL), whereas qualitative resistance is usually controlled by single dominant *R* genes (Stuthman *et al*., 2007; Delourme *et al*., 2006). As *R* gene-mediated resistance is race-specific, it is effective only when the avirulent allele of the corresponding effector gene is predominant in the pathogen population.

Hence, it is not effective in protecting the crop when new pathogen races evolve, resulting in severe disease epidemics (Rouxel *et al*., 2017; Huang *et al*., 2018). In addition to that, effectiveness of *R* gene-mediated resistance may also be influenced by the environmental factors (MacQueen *et al*., 2016), such as temperature (Huang *et al*., 2006). QR is race non-specific and more durable (Brun *et al*., 2010). However, in the presence of large amounts of inoculum of different pathogen races in an environment favourable for disease development, QR cannot provide effective protection (Huang *et al*., 2018).

It has been hypothesised that combining *R* gene-mediated resistance and quantitative resistance in one crop cultivar will provide more effective resistance across a range of environments than use of either type of resistance on its own (Brun *et al*., 2010). Evidence to support this hypothesis has been provided by Huang *et al*. (2006) and Huang *et al*. (2018). However, this work was done using a limited numbers of *R* genes. Since there are different types of *R* genes (Stotz *et al*., 2014) and different types of quantitative resistance (Poland *et al*., 2009), there is a need to test this hypothesis with cultivars carrying different *R* genes that are currently being used in the UK.

An aim of this study is to combine quantitative resistance with *R* genes that are currently effective, rather than combining it with *R* genes that are no longer effective in the UK and render effective disease management by providing more temperature-resilient oilseed rape cultivars. Therefore, in this study, there is a need to test this hypothesis with different *R* genes (*Rlm1*, *Rlm4*, *Rlm7* or *LepR3*) and with different background resistance (QR) used in commercial cultivars currently used in the UK in different environments over three growing seasons (2015/2016, 2016/2017 and 2017/2018). Therefore, this work can provide more useful information for breeders and growers in the UK.

Oilseed rape cultivars with such temperature-sensitive *R* genes can be deployed in regions where the temperatures are lower to minimize the risk of severe epidemics (Huang *et al*., 2006). Hence in this study, the effect of environmental factors on the effectiveness of resistance in eleven cultivars with different *R* genes with/without quantitative resistance in their background is investigated in natural conditions. This can guide the deployment of cultivars with specific *R*

genes (resistant at increased temperature) at different locations in the UK based on the predicted seasonal weather conditions.

5.1.1 Objectives

Hypothesis 3: Host background resistance and environmental factors affect the effectiveness of *R* gene resistance. To test this hypothesis, there are four objectives related to field experiments (with 12 cultivars carrying different *R* genes in background with/without quantitative resistance or with no *R* genes) at different sites in three different growing seasons and controlled environment experiments (with 8 cultivars carrying *Rlm7*).

Objective 1. To investigate effectiveness of *R* genes in different cultivars in control of phoma leaf spots at different sites in the UK. Previous studies showed that combining *R* gene and quantitative resistance increasing effectiveness of cultivar resistance against *L. maculans.* In this study, 8 cultivars with different *R* genes in genetic background with/without quantitative resistance were selected, for comparison, 3 cultivars with no *R* genes and one cultivar with only quantitative resistance were selected for field experiments. In total, there were 12 cultivars.

Objective 2. To investigate effectiveness of different cultivars in control of phoma stem canker at different sites in the UK. Since *L. maculans* initially infects the leaves causing phoma leaf spots, then grows along the leaf petiole to the stem causing phoma stem cankers before harvest, to investigate whether *L. maculans* can grow along the leaf petiole to the stem causing phoma stem cankers on cultivars with different *R* genes, the severity of phoma stem canker were compared between different cultivars.

Objective 3. To analyse the relationship between weather conditions and disease severity in the field experiments. The relationship between weather conditions (temperature and rainfall) and disease severity in the field experiments were investigated by correlation analysis between severities of phoma leaf spot or phoma stem canker and various weather variables at all the sites and in different seasons.

Objective 4. To investigate effectiveness of *Rlm7* resistance gene in different *Brassica napus* lines/cultivars in controlled environment conditions. The *Rlm7* resistance gene is currently the most effective gene used in UK oilseed rape cultivars, to investigate whether the background quantitative resistance affects the effectiveness of *Rlm7* on control of *L. maculans* and whether there are any differences between *L. maculans* isolates in pathogenicity on different cultivars carrying *Rlm7,* 9 cultivars/lines carrying *Rlm7* were selected for controlled environment experiments.

5.2 Materials and methods

5.2.1 Winter oilseed rape field experiments for assessing phoma leaf spot and phoma stem canker severity

Winter oilseed rape field experiments were established at different sites in the UK (Woodhall Farm in Hertfordshire, Morley in Norfolk, Rothwell in Lincolnshire, Impington in Cambridgeshire, Trumpington in Cambridgeshire, Wisbech in Cambridgeshire, Risby in Suffolk and West Farm Barns in Oxfordshire) (Figure 2.1 & Appendix 2-N) for assessing effects of environmental factors on phoma leaf spot and phoma stem canker severities on cultivars with different resistance genes in three cropping seasons (2015/2016, 2016/2017 and 2017/2018) (Table 5.1). The layout or field design for all the field experiments for the three cropping seasons are shown in Appendices 2-A to 2-M.

Twelve cultivars with different *R* genes with/without quantitative resistance in their background were selected for the field experiments; Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus (Table 2.1). To assess the resistance of cultivars against the pathogen in natural environmental conditions, fungicides were not sprayed on the field experiments.

5.2.2 Weather conditions at the field experiment sites

Weather data (daily mean air temperature and rainfall) were collected from all the winter oilseed rape field experiment sites throughout the year for the three cropping seasons (2015/2016, 2016/2017 and 2017/2018) in the UK (Sections

Table 5.1: Summary of data obtained from different sites in the UK for three cropping seasons (2015/2016, 2016/2017 and 2017/2018).

2015/2016	Phoma leaf spotting	Phoma stem canker	Yield
Morley	✓	✓	✓
Impington	✓	✓	✓
Trumpington	✓	✓	Assessment not done by consortium partner
Woodhall Farm	✓	✓	✓
2016/2017			
Morley	✓	✓	Assessment not done by consortium partner
Rothwell	✓	Field trial damaged by pigeons	Field trial damaged by pigeons
Wisbech	✓	✓	Assessment not done by consortium partner
Woodhall Farm	Field trial damaged by cabbage stem flea beetle	Field trial damaged by cabbage stem flea beetle	Field trial damaged by cabbage stem flea beetle
2017/2018			
Morley	✓	✓	✓
Rothwell	✓	✓	✓
Wisbech	Assessment not done by consortium partner	✓	✓
Risby	Assessment not done by consortium partner	✓	✓
Woodhall Farm	✓	Field trial damaged by pigeons	Field trial damaged by pigeons

2.1 and 2.2). Daily rainfall, air maximum and minimum temperature data were obtained from the closest weather station for each experimental site (Appendix 2-A). Mean daily air temperature was calculated as mean of minimum and maximum temperatures. Then the monthly mean air temperature and monthly total rainfall were calculated.

The mean air temperature and total rainfall were calculated for combined months of August to September (Aug-Sept), October to November (Oct-Nov), December to March (Dec-Mar) and April to June (Apr-Jun). Weather in Aug-Sept represented environmental conditions in summer for pseudothecial maturation (i.e. the development of sexual ascospores in pseudothecia), whereas Oct-Nov & Dec-Mar represented environmental conditions in autumn/winter for the phoma leaf spot development stage, and Apr-Jun represented environmental conditions in spring/summer for the phoma stem canker development stage (Huang *et al*., 2018). This information was used in assessing the effects of temperature on resistance in cultivars with different *R* genes with/without quantitative resistance under natural conditions.

5.2.3 Severity of phoma leaf spot caused by *Leptosphaeria maculans*

Disease severity on leaves of the twelve different cultivars was assessed by counting the number of spots caused by *L. maculans* per leaf for each cultivar at different sites in the UK (Figure 2.1) (Table 5.1). Phoma leaf spot assessment was done in October/November, when there was a phoma leaf spot incidence of 50% plants affected at each of the field experiment sites. Disease severity was assessed by counting the number of phoma leaf spots caused by *L. maculans* for each cultivar (10 plants/plot). The disease score was set at 1 if the number of leaf spots was <5 per leaf. The score was 2 when the leaf sample had 5-10 phoma leaf spots/leaf and 3 for leaf samples with >10 leaf spots per leaf.

A detailed phoma leaf spot assessment was done at the Woodhall Farm site for the 2015/2016 and 2017/2018 cropping seasons (Table 5.1). Ten plants were pulled out from each replicate plot for each cultivar (360 plants in total/site). The numbers of leaves on the plant, number of leaves with phoma leaf spots caused by *L. maculans* and number of leaves with phoma leaf spots caused by *L.*

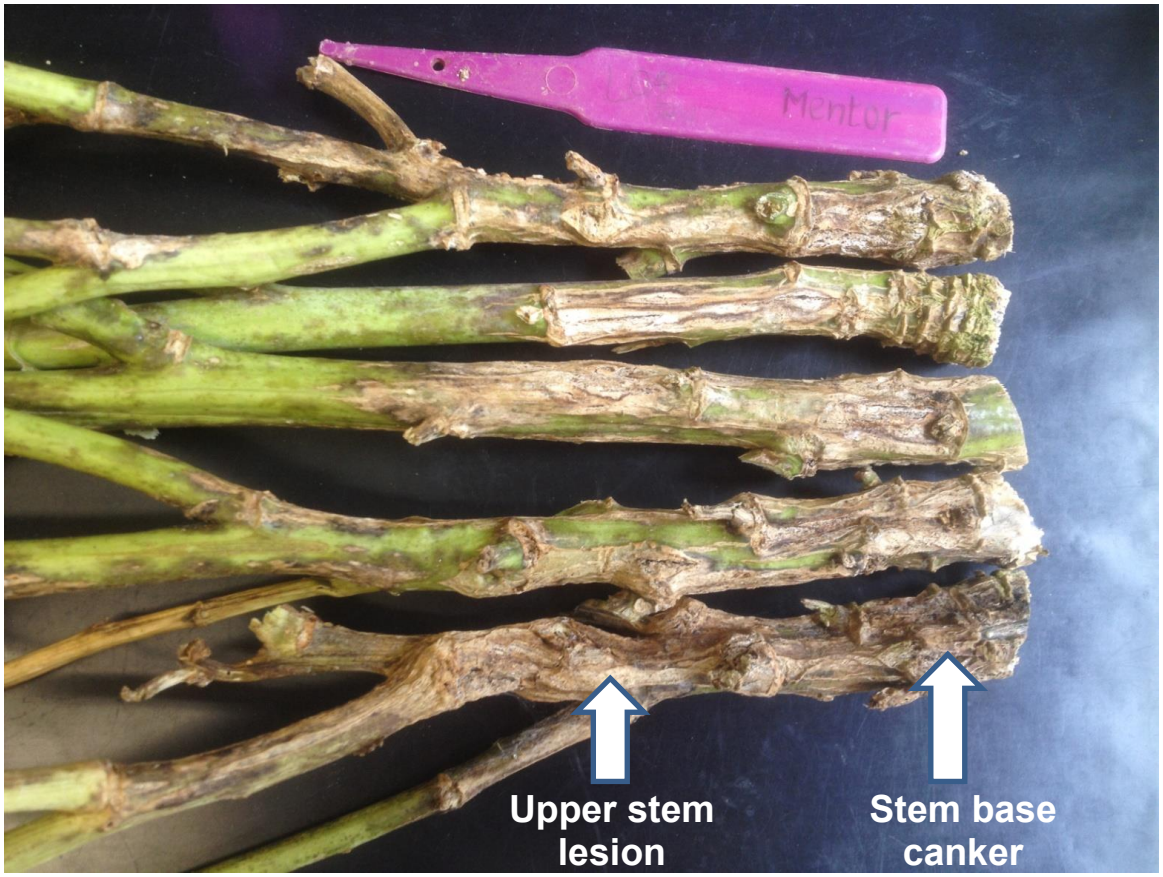

Figure 5.1: Stem samples from cultivar Mentor at Woodhall Farm in June 2016 (2015/2016 cropping season) showing both stem base cankers and upper stem lesions.

biglobosa were assessed. The numbers of phoma leaf spots/plant caused by both *L. maculans* and *L. biglobosa* were also noted. Photos were taken of at least 4 out of the 10 leaves with leaf lesions for future reference.

5.2.4 Severity of phoma stem canker caused by *Leptosphaeria maculans*

Severity of phoma stem canker was assessed on the twelve cultivars at different sites (Figure 2.1) in June from 2015/2016 to 2017/2018 cropping seasons (Table 5.1). Disease severity was assessed in all three replicates of each cultivar at each site for three cropping seasons. Assessment was done by pulling up 20-25 stems per plot in late June just before swathing or desiccation. Stem base canker severity assessment was done by cutting the stem base and upper stem lesion severity assessment was done by cutting the upper stem at the site of lesions. Basal cankers and upper stem lesions were assessed by observing the specific symptoms. Symptoms were considered to be upper stem lesions if they were observed >5 cm above the root crown and basal cankers if they were observed at the root crown or <5 cm above it (Figure 5.1).

The disease scores were done using a 0-7 score (modified from the 1- 6 scale of Lo-Pelzer *et al*., 2009) (Figure 5.2) individually on each of 20 plants per plot. If there were no observable symptoms, score ‘0’ was given. Score ‘1’ was given if the cross-sectional area of girdling of the stem was ≤5%. If the girdling was 6-25%, score ‘2’ was given. Score ‘3’ for 26-50% girdling; score ‘4’ for 51-75% girdling; score ’5’ for 76-99% girdling; score ’6’ was given for 100% girdling + stem weak and score ’7’ was given for 100% girdling + stem dead or lodged (Figure 5.2). The modified G2 disease index was then used to assess the severity of phoma stem canker on cultivars from different sites for the three cropping seasons (2015/2016 to 2017/2018). The modified G2 disease index was calculated by integrating the distribution of the proportions of plants observed within seven canker severity categories into a single index. The G2 disease index was found to be a good indicator of observed distribution of canker severities (Lo-Pelzer *et al*., 2009).

Figure 5.2: Image showing the 0-7 scale used to assess severity of phoma stem canker on oilseed rape stem samples (June 2017).

Modified G2 DI used in this study= ((no.stems with score 1+ no.stems with score 2)+ no.stems with score 3*3+ no.stems with score 4*5+ no.stems with score 5*7+(no.stems with score 6+ no.stems with score 7)*9)/ total no. stems assessed

5.2.5 Effectiveness of *Rlm7* resistance gene in different *Brassica napus* lines/cultivars in controlled environment conditions

The effectiveness of cultivars containing *Rlm7* resistance gene (Roxet, Excel, Hearty, 01-23-2-1, DK Extrovert, DK Exalte, Harper, Amalie and Whisky) (Table 2.1) were tested in controlled environment conditions by inoculating the cotyledons of the cultivars with eight *L. maculans* isolates. The seedlings of nine cultivars were grown in 40-compartment trays in the glasshouse for 13 days (Figure 5.3). The cotyledons were 14 days old at inoculation with the conidial suspensions. The procedure of cotyledon inoculation was similar to that mentioned in section 2.4.2. Among the eight isolates, isolate V23.11.9 was the positive control for avirulent *AvrLm7*; isolate HRox-12-2-1 was the positive control for virulent *avrLm7*; Sf16 Why-2; Sf16 Why-3; Sf16 Why-5; Sf16 Why-8; Sf16 Why-11 and Sf16 DK Ext-2 were isolates found to have a virulent allele of *avrLm7*.

Disease assessment was done 14 days, 17 days and 20 days post-inoculation (dpi). Along with scoring lesions, lesion size was also measured (length and width) on the three assessment days. Then the cotyledons of the nine cultivars inoculated with Sf16 Why-2; Sf16 Why-5; Sf16 Why-5 and Sf16 Why-8 were incubated in a Petri dish lined with a wet Whatmann No.1 filter paper for three days. The numbers of mature pycnidia (i.e. pycnidia producing a cirrhus) on the incubated cotyledons were recorded to determine the differences in pycnidial production between different *Brassica napus* cultivars/lines containing the *Rlm7* resistance gene.

5.2.6 Statistical analysis

1. **Effectiveness of *R* genes in different cultivars in control of phoma leaf spots at different sites in the UK:**

For field experiments at different sites over the three cropping seasons, analysis

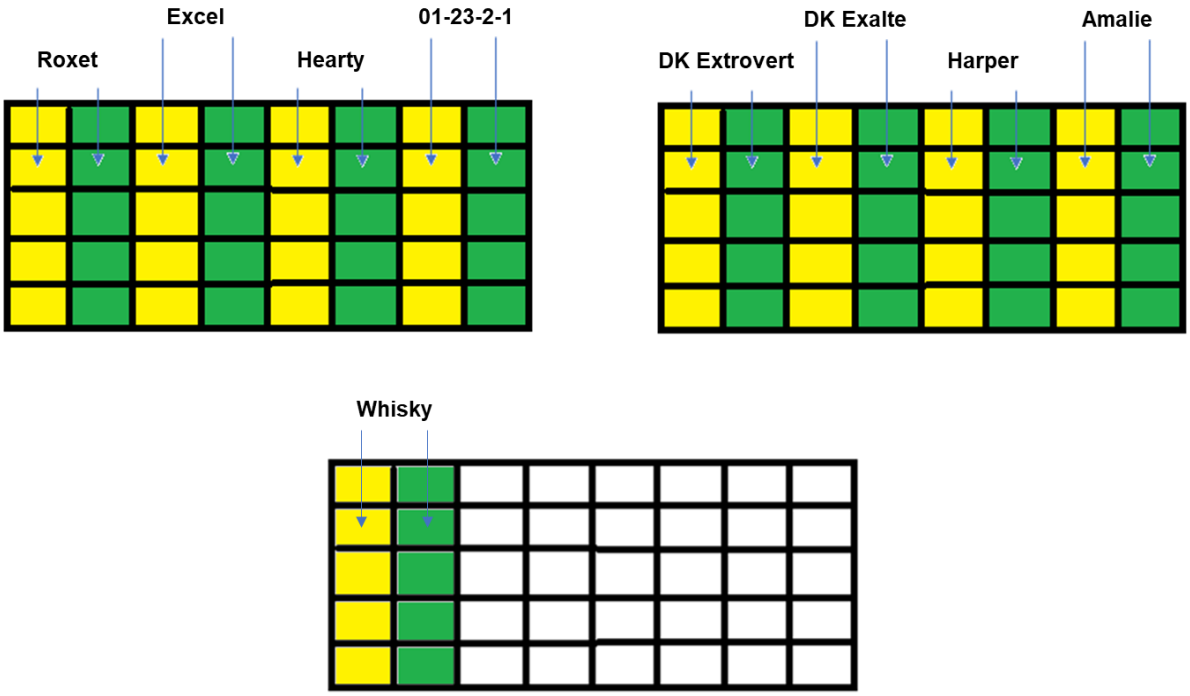

Figure 5.3: Schematic representation of 40-compartment trays sowed with seeds of *Brassica napus* cultivars/lines carrying the *Rlm7* resistance gene. Seeds of the same cultivar/line were sowed in two rows of five compartments (five seeds per row). V23.11.9 (*AvrLm7* +ve control), HRox-12-2-1 (a*vrLm7* +ve control), Sf16 Why-2 and Sf16 Why-5 isolates containing virulent allele of *avrLm7*, were inoculated on each part of each lobe of cotyledons in rows highlighted in yellow (for inoculation scheme refer to Figure 2.9). Sf16 Why-5, Sf16 Why-8, Sf16 Why-11 and Sf16 DK Ext-2 isolates containing virulent allele of *avrLm7*, were inoculated on each part of each lobe of cotyledons in rows highlighted in green (for inoculation scheme refer to Figure 2.9). The inoculation scheme was similar to that shown in Figures 2.9 & 2.10.

of variance was done with the data of phoma leaf spot severity to assess the differences between cultivars, between sites and between seasons. For each cropping season, the cultivar mean phoma leaf spot severity score was calculated from the three replicates and the site mean phoma leaf spot severity score was calculated for each of the twelve cultivars at each site. Analysis of variance was done to assess relative differences between different cultivars in phoma leaf spot severity. To assess cultivar response to environmental factors at different experimental sites, the relationships between cultivar phoma leaf spot severity score and site mean phoma leaf spot severity score were analysed using linear regression.

1. **Effectiveness of different cultivars in control of phoma stem canker at different sites in the UK:**

Similar analysis was done with data for phoma stem canker severity and yield of different cultivars.

2. **Analyse the relationship between weather conditions and disease severity in the field experiments:**

To analyse the relationship between weather conditions (temperature and rainfall) and disease severity in the field experiments, correlation analysis was done between phoma leaf spot and phoma stem canker severities of all the twelve cultivars and various weather variables at all the sites and seasons.

3. **Effectiveness of *Rlm7* resistance gene in different *Brassica napus* lines/cultivars in controlled environment conditions:**

Analysis of variance (ANOVA) was used to analyse the measurements of lesion length, width and the lesion area to assess the effectiveness of *Brassica napus* lines/cultivars carrying *Rlm7* resistance gene in controlled environment conditions. Analysis of variance was used to analyse the numbers of mature pycnidia for different cultivars/lines and different isolates. Since there were no replicates in this experiment, the data were analysed using error variance component in the interaction of cultivar with isolate. All the analyses were done using Microsoft Excel, SigmaPlot or GENSTAT statistical software.

5.3 Results

5.3.1 Weather conditions at the field experiment sites

Rainfall patterns differed between the field experiment sites and three cropping seasons during autumn/winter (phoma leaf spot development stage) and summer (phoma stem canker development stage). In the 2015/2016 cropping season, larger amounts of rainfall in the autumn and winter months were recorded at all the sites compared with the 2016/2017 cropping season. The greatest amounts of rainfall were recorded at the Morley site, especially in mid-August, mid-September, late-May and in June. Similar patterns of weather conditions were observed at Impington and Trumpington sites, as they were close to each other. There were prolonged periods of rainfall at all the sites in August and September and again from November until January. The spring and summer were dry with occasional periods of short-term rainfall. Greatest amounts of rainfall were observed at three sites from late-May till late-June except at the Woodhall Farm site, where large amounts of rainfall were recorded from early-March until late-June (Figure 5.4).

In the 2015/2016 cropping season, temperature followed a similar pattern at Morley, Impington, Trumpington and Woodhall Farm sites, with temperatures decreasing to ≤ 0^{o}C in January. Average temperature between 1 October and 31 May was 8.3^{o}C at Morley, 8.5^{o}C at Impington and Trumpington and 8.2^{o}C at Woodhall Farm. Higher temperatures were recorded from May till late-July at all the sites, Morley (23.6^{o}C); Impington and Trumpington (23.9^{o}C) and Woodhall Farm (24.2^{o}C) (Figure 5.4).

In the 2016/2017 cropping season, rainfall followed a typical pattern at three sites: Morley, Rothwell and Wisbech. The amounts of rainfall recorded were less compared to the 2015/2016 season. There were periods of substantial rainfall commencing from early-August and continuing to mid-March at Morley and Rothwell sites. The amounts of rainfall recorded at Wisbech site were relatively low compared to the other two sites. The autumn and winter were dry with occasional periods of short-term rainfall in November and mid-December until mid-January. Large amounts of rainfall were observed at all the three sites in

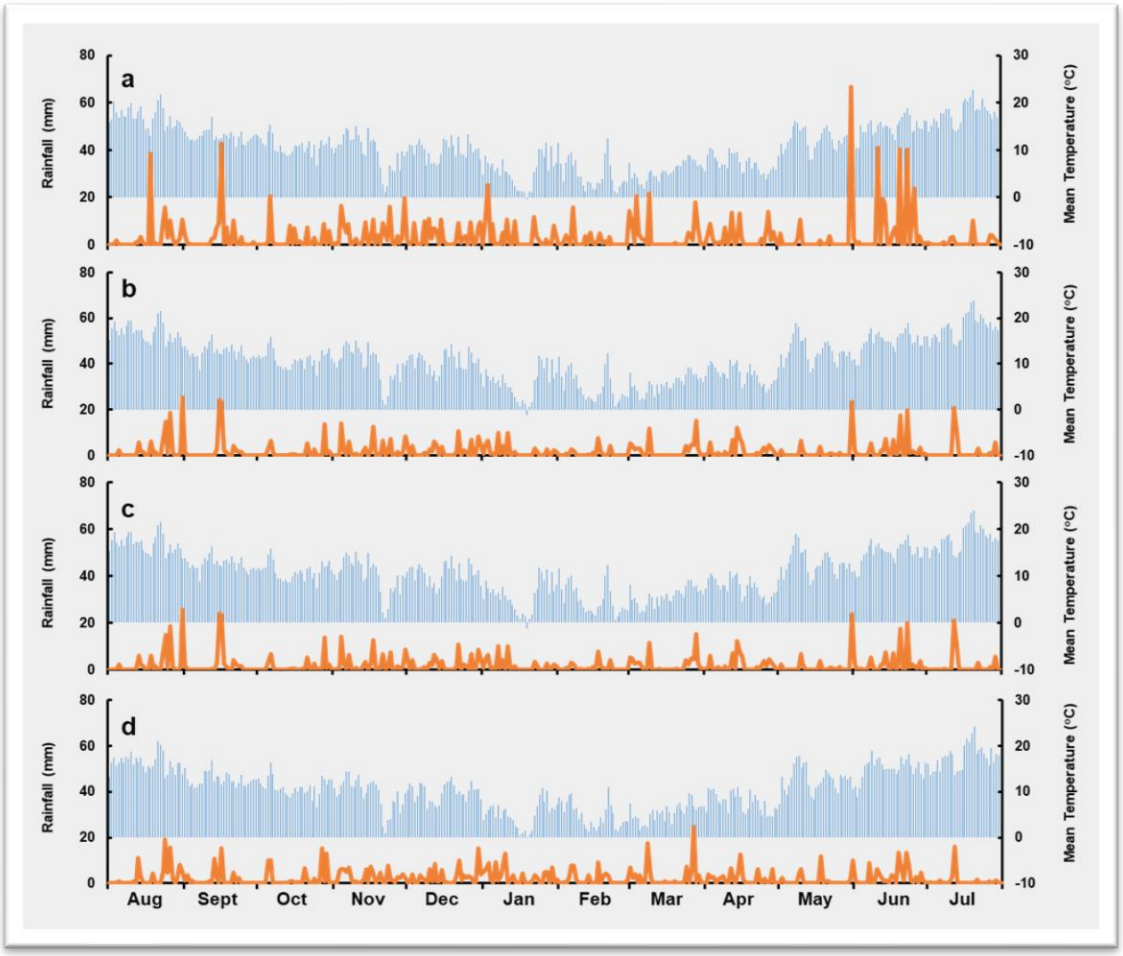

Figure 5.4: Weather data were collected at Morley (Norfolk) (a), Impington (Cambridgeshire) (b), Trumpington (Cambridgeshire) (c) and Woodhall Farm (Hertfordshire) (d) sites using day interval automated weather stations in the 2015/2016 cropping season. The blue bars represent mean temperature (°C) and orange lines represent total daily rainfall (mm).

summer. There were prolonged periods of heavy rainfall at the Morley site in July (Figure 5.5).

In the 2016/2017 cropping season, there were similarities in the pattern of daily mean temperature at the three sites. Lower temperatures (≤ 0^{o}C) were recorded from mid-January until late-January at all the three sites. Average temperature between 1 October and 31 May was 7.8^{o}C at Morley, 7.7^{o}C at Rothwell and 7.9^{o}C at Wisbech. Daily mean temperatures were high from May until late-July at all the sites, with a highest 23.2^{o}C at Morley; 21.2^{o}C at Rothwell and 23.4^{o}C at Wisbech (Figure 5.5).

In 2017/2018 cropping season, the patterns of rainfall differed between the five sites: Morley, Rothwell, Wisbech, Risby and Woodhall Farm. Among the five sites, largest amounts of rainfall were recorded at the Rothwell site. In comparison to the previous cropping seasons, the autumn and winter months were predominantly wet in the 2017/2018 cropping season. Prolonged periods of rainfall were recorded from August till late-Febraury at the Morley site. The spring and summer were dry with occasional periods of short-term rainfall at all the sites (Figure 5.6).

In the 2017/2018 cropping season, daily mean temperatures followed a similar pattern at all five sites. Lower temperatures (≤ 0^{o}C) occurred in late-February at all sites. One notable difference in this cropping season was an uncharacteristic period of cold weather in early/mid-March at all sites. Average temperatures between 1 October and 31 May was 6.6^{o}C at the Morley site, 7.1^{o}C at the Rothwell site, 7.7^{o}C at the Wisbech site, 7.4^{o}C at the Risby site and 7.5^{o}C at the Woodhall farm site (Figure 5.6).

5.3.2 Phoma leaf spot severity caused by *Leptosphaeria maculans*

In the 2015/2016 cropping season, phoma leaf spot severity was scored by using the disease severity score (0-3 scale) at three sites (Morley, Impington and Trumpington). A detailed phoma leaf spot assessment was done at the Woodhall Farm site (no. phoma leaf spots/plant). At the Morley site, DK Exalte (with *Rlm7*) had the smallest disease score (score-1.5) and Mentor had greatest disease score (score-2.8) amongst all the cultivars. At the Impington site, Harper (*Rlm7*)

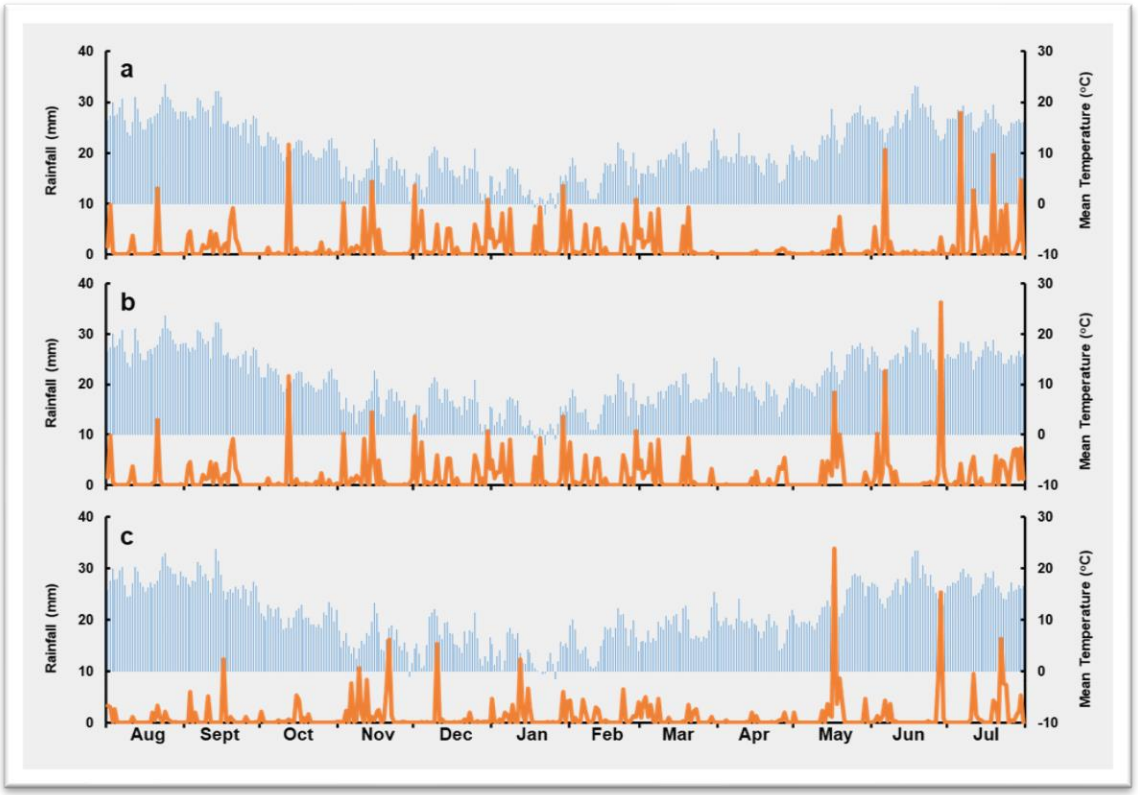

Figure 5.5: Weather data were collected at Morley (Norfolk) (a), Rothwell (Lincolnshire) (b) and Wisbech (Cambridgeshire) (c) sites using day interval automated weather stations in the 2016/2017 cropping season. The blue bars represent mean temperature (°C) and orange lines represent total daily rainfall (mm).

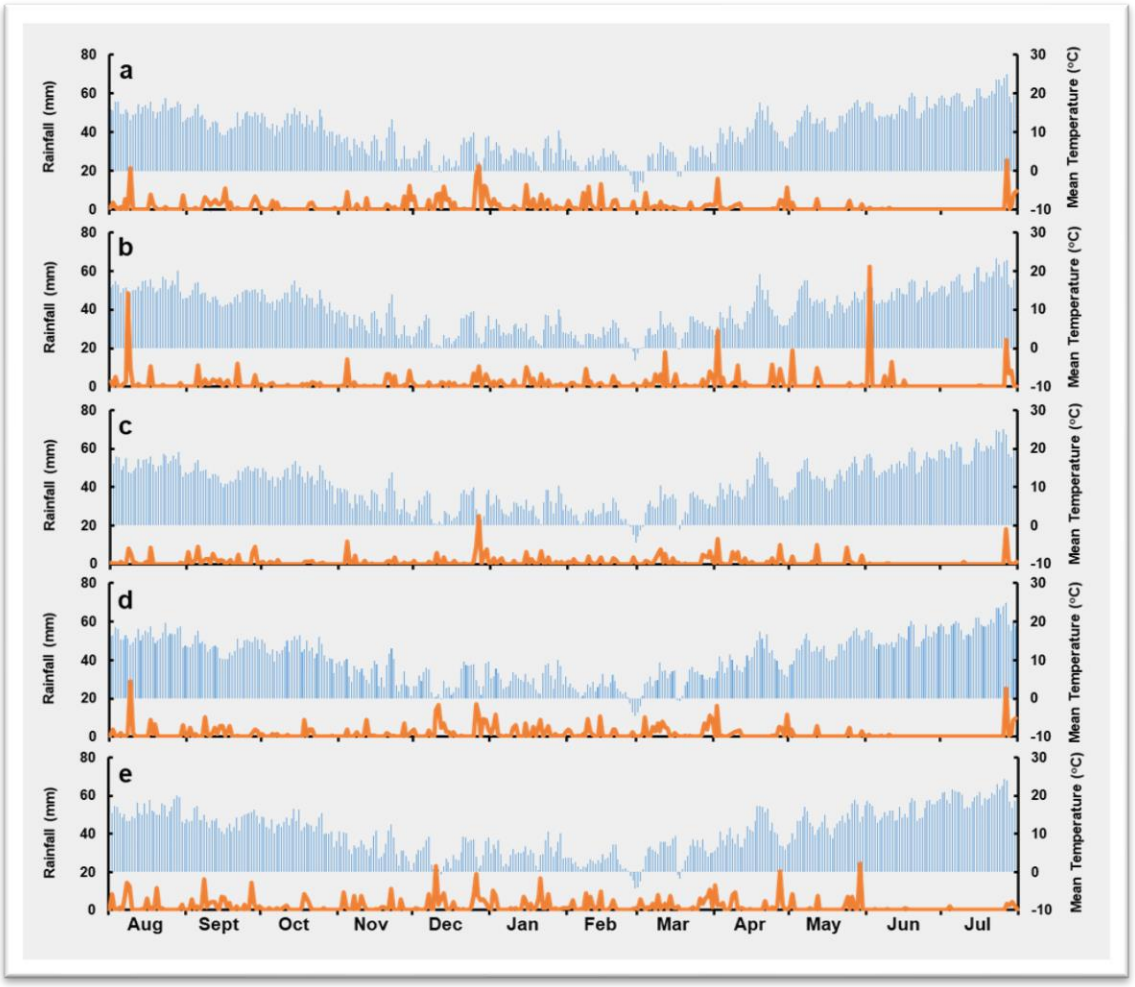

Figure 5.6: Weather data were collected at Morley (Norfolk) (a), Rothwell (Lincolnshire) (b), Wisbech (Cambridgeshire) (c), Risby (Suffolk) (d) and Woodhall Farm (Hertfordshire) (e) sites using day interval automated weather stations in the 2017/2018 cropping season. The blue bars represent mean temperature (°C) and orange lines represent total daily rainfall (mm).

had fewer phoma leaf spots (score-1) when compared to all other cultivars and cultivars Mentor and Incentive (with no *R* genes) had the greatest numbers of phoma leaf spots (score-2.9) (Figure 5.7a). At the Trumpington site, cultivar DK Exalte had the smallest disease score (score-0.1) and cultivar Drakkar had the greatest disease score (score-2.6). Comparing the three sites, the Impington site had more phoma leaf spot severity except on cultivars Harper (score-1), Es Astrid (score-1.6) and Whisky (score-1.4) for which the Morley site had more phoma leaf spot severity. The Trumpington site had less phoma leaf spot severity when compared to other two sites (Figure 5.7a).

In the 2016/2017 cropping season, phoma leaf spot severity was assessed at three different sites Morley, Rothwell and Wisbech. In this season, fewer phoma leaf spots were observed on cultivar Angus at Wisbech (score-0.5) and no phoma leaf spots at Rothwell (score-0). At the Morley site, cultivar Mentor had the greatest disease score (score-1.8) and cultivar DK Exalte and Harper had the smallest disease scores (score-0.7). At the Wisbech site, Angus (score-0.4) and Whisky (score-0.5) had the smallest disease score and Mentor had greatest disease score (score-3) amongst all the cultivars (Figure 5.7b).

At Rothwell site, cultivars Whisky (0.1), Es Astrid (score-0.1), Amalie (score-0.2), Harper (score-0.2), DK Exalte (score-0.3), DK Cabernet (score-0.3) and Incentive (score-0.3) had less phoma leaf spot severity and cultivars Mentor (score-2.1) and Drakkar (score-2.6) had the greatest numbers of phoma leaf spots. Amongst all the three sites, the Rothwell site had less incidence of phoma leaf spotting for all cultivars, except for cultivars DK Extrovert (score- 2) and Mentor (score-2.1); whereas Wisbech site had more severe phoma leaf spot severity (Figure 5.7b).

In the 2017/2018 cropping season, phoma leaf spot severity was assessed at three different sites Morley, Rothwell and Woodhall Farm. Phoma leaf spot severity was scored by using the disease severity score (0-3 scale) at two sites(Morley and Rothwell). In this season, less severe phoma leaf spotting was observed in cultivar DK Exalte at both sites (disease score at Morley-0.16 & Rothwell-0.2); whereas more severe phoma leaf spotting was observed on Mentor (score-1.4) at the Morley site and on Drakkar (score-1) at the Rothwell site (Figure 5.7c).

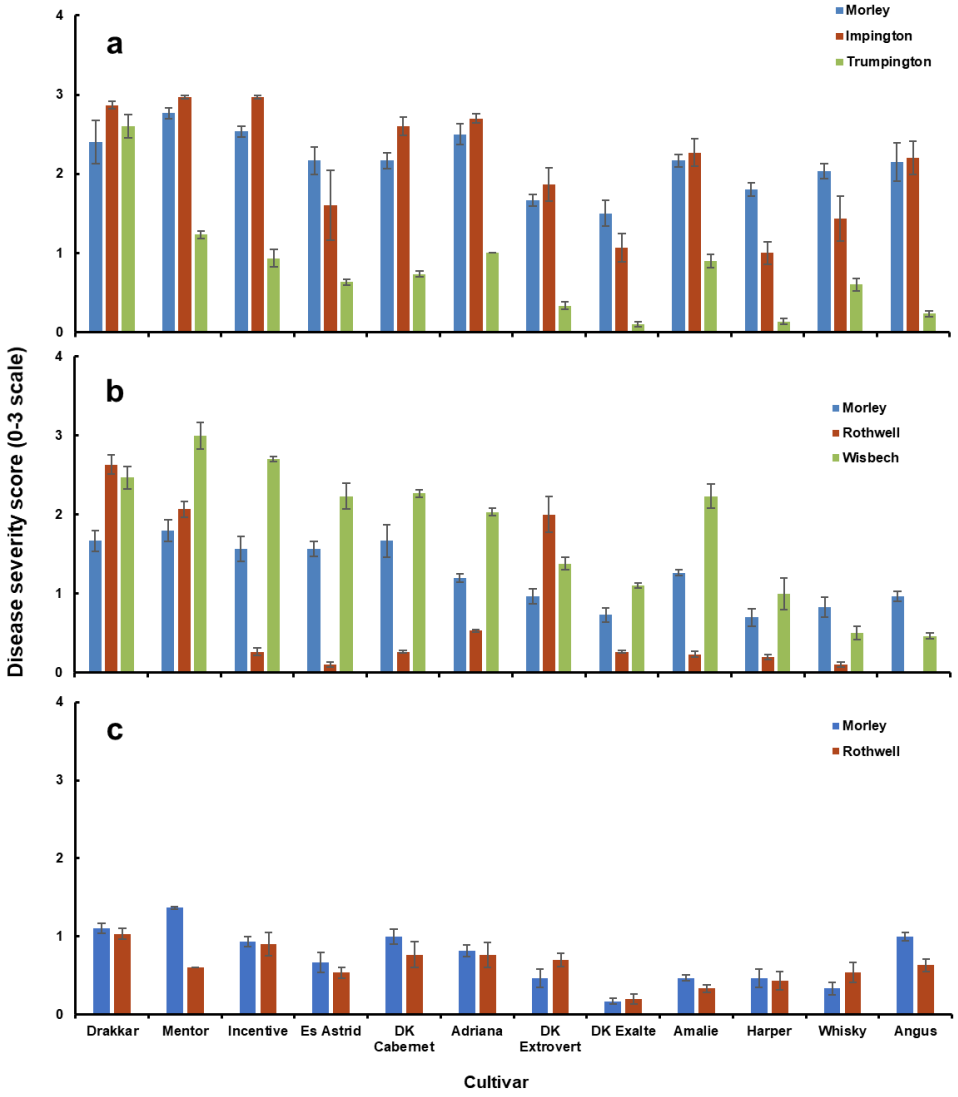

Figure 5.7: Phoma leaf spot severity (*Leptosphaeria maculans*) on leaves of different cultivars (Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus) at three UK sites Morley, Impington and Trumpington in the 2015/2016 cropping season(a); three different UK sites Morley, Rothwell and Wisbech in the 2016/2017 cropping season (b) and two different UK sites Morley and Rothwell in the 2017/2018 cropping season (c). Phoma leaf spot severity was assessed by using a 0-3 scale (0: 0 leaf spots; 1:1-4 leaf spots per plant, 2: 5-9 leaf spots per plant; 3: more than 10 leaf spots per plant). The mean score was calculated from scores on 10 plants from three replicate plots. The error bars represent standard error of the mean.

A detailed phoma leaf spot assessment was done at the Woodhall Farm site (no. of phoma leaf spots/plant) in 2015/2016 (Figure 5.8a) and 2017/2018 (Figure 5.8b) cropping seasons. In the 2015/2016 cropping season, cultivar Drakkar hadthe greatest numbers of phoma leaf spots/plant (37.8) and cultivar DK Exalte had the smallest numbers of phoma leaf spots/plant (8.6) when compared to other cultivars (Figure 5.8a). In the 2017/2018 cropping season, smaller numbers of phoma leaf spots were observed on cultivars Amalie (1.8), Harper (1.9), DK Exalte (2) and DK Extrovert (2.5) at the Woodhall Farm site; whereas cultivars Drakkar (8.9) and Incentive (5.9) had greatest numbers of phoma leaf spots. Amongst all sites, cultivar DK Exalte with the *Rlm7* gene had less severe phoma leaf spotting when compared to Adriana (with *Rlm4* gene) and DK Cabernet (with *Rlm1* gene) over the three seasons (Figures 5.7 & 5.8).

Analysis of variance of phoma leaf spot severity on twelve cultivars in the experimental plots at Morley, Impington, Trumpington, Rothwell and Wisbech in three consecutive cropping seasons (2015/2016, 2016/2017 and 2017/2018) showed that phoma leaf spot severity differed significantly ($P<0.01$) between cropping seasons. There were also significant differences ($P<0.01$) between cultivars and sites in phoma leaf spot severity. Analysis of variance showed that there were significant differences in mean phoma leaf spot severity between the twelve cultivars ($P<0.05$) across sites and cropping seasons (Table 5.2).

The mean phoma leaf spot severity score was greatest on susceptible cultivars Drakkar, Mentor and Incentive at all sites and in all seasons; however, there was no significant difference ($P>0.05$) between them (Table 5.2). The mean phoma leaf spot severity score was significantly less on DK Exalte, Harper, Whisky and Angus and there was no significant difference ($P>0.05$) between them. Analysis of variance showed that cultivars DK Extrovert, Amalie and Es Astrid had significantly greater phoma leaf spot severity than DK Exalte and Harper. Analysis of variance showed that cultivars DK Cabernet and Adriana had significantly less phoma leaf spot severity than Drakkar and Mentor (Table 5.2).

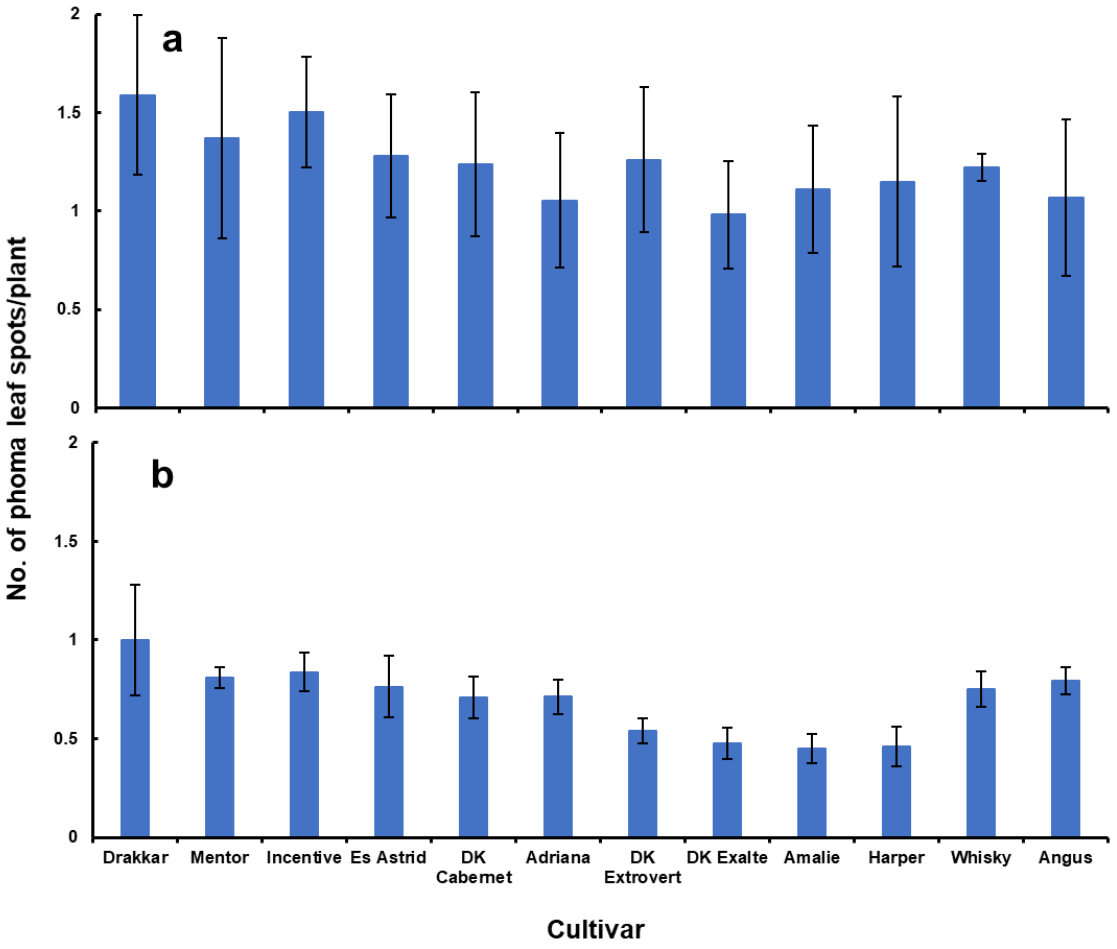

Figure 5.8: Phoma leaf spot severity (2015/2016 (a) and 2017/2018 (b) cropping seasons) on leaves of different cultivars Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus at Woodhall Farm, Hertfordshire in the UK. At this site, no. of phoma leaf spots/plant were counted. The mean was calculated from scores on 10 plants from three replicate plots. The error bars represent the standard error of the mean. The numbers of phoma leaf spots were log transformed so as to adjust the scales.

Table 5.2: The mean phoma leaf spot severity for each cultivar across sites in the 2015/2016, 2016/2017, 2017/2018 cropping seasons. Phoma leaf spot severity values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	Phoma leaf spot severity*
Adriana	1.44c
Amalie	1.23bc
Angus	0.96ab
DK Cabernet	1.43c
DK Exalte	0.64a
DK Extrovert	1.17bc
Drakkar	2.04d
Es-Astrid	1.19bc
Harper	0.73a
Incentive	1.60cd
Mentor	1.95d
Whisky	0.80ab

*Least significant difference (LSD) at 5% was 0.44 (*df*=76).

Two-way Anova analysis for the phoma leaf spot severity at the Woodhall Farm site for the 2015/2016 and 2017/2018 cropping seasons showed that DK Exalte, Amalie, Angus, Whisky, Harper and Adriana had significantly fewer phoma leaf spots than Incentive and Drakkar in the 2015/2016 cropping season (Table 5.3). In the 2017/2018 cropping season, cultivars DK Exalte, Amalie and had significantly fewer phoma leaf spots than all other cultivars (Table 5.4). Mean phoma leaf spot severity for each cultivar was plotted against mean seasonal site phoma leaf spot severity and the relationship between them were linear (Figure 5.9).

A simple linear relationship (y=a+b*x) (Figure 5.10) was fitted to describe the cultivar phoma leaf spot severity by the seasonal site mean phoma leaf spot severity by using estimated intercept (a) and slope (b) (Appendix 5-A); where y is cultivar and x is seasonal site mean of phoma leaf spot severity. This showed that cultivars DK Exalte, Harper and Whisky had least phoma leaf spot severity in all seasons and sites; whereas, cultivars Drakkar and Incentive had greatest phoma leaf spot severity in all seasons and sites (Figure 5.10).

5.3.3 Severity of phoma stem canker caused by *Leptosphaeria maculans*

There were significant differences in the phoma stem canker severity between cultivars and sites (P<0.01) (Figures 5.10-5.12). The interaction between sites and cultivars was also statistically significant (P<0.01). Cultivars with the *Rlm7* resistance gene had less severe phoma stem canker compared to cultivars with *Rlm1* and *Rlm4* resistance genes.

In the 2015/2016 cropping season, there were differences in the phoma stem canker severity between cultivars and sites (Figures 5.11 & 5.12a). Phoma stem canker severity was greatest on cultivar Drakkar (susceptible) in June 2015 at all the four different field experiment sites; Morley (score-8.7), Impington (score-7.7), Trumpington (score-9) and Woodhall Farm (score-8.2). At the Morley site, cultivar cultivar DK Extrovert (score-1.35) had the least severe phoma stem canker. At the Impington site, cultivars Angus (0.8) and Whisky (0.8) had least severe phoma stem canker (Figure 5.12a).

Table 5.3: ANOVA results for no. of phoma leaf spots/plant at the Woodhall Farm site in the 2015/2016 cropping season. Phoma leaf spot numbers that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	No. of phoma leaf spots/plant*
Adriana	10.33ab
Amalie	11.90abc
Angus	10.73ab
DK Cabernet	16.27bcd
DK Exalte	8.59a
DK Extrovert	17.20bcd
Drakkar	37.77e
Es Astrid	18.13cd
Harper	13.13abc
Incentive	30.85e
Mentor	22.40d
Whisky	15.70abcd

*Least significant difference (LSD) at 5% was 7.12 (*df*=24).

Table 5.4: ANOVA results for no. of phoma leaf spots/plant at the Woodhall Farm site in the 2017/2018 cropping season. Phoma leaf spot numbers that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	No. of phoma leaf spots/plant*
Amalie	1.83a
Angus	5.20c
DK Cabernet	4.13bc
DK Exalte	2.00a
DK Extrovert	2.47ab
Drakkar	8.97d
Es Astrid	4.80c
Harper	1.90a
Incentive	5.87c
Mentor	5.43c
Whisky	4.63c
Adriana	4.17bc

*Least significant difference (LSD) at 5% was 1.85 (*df*=24).

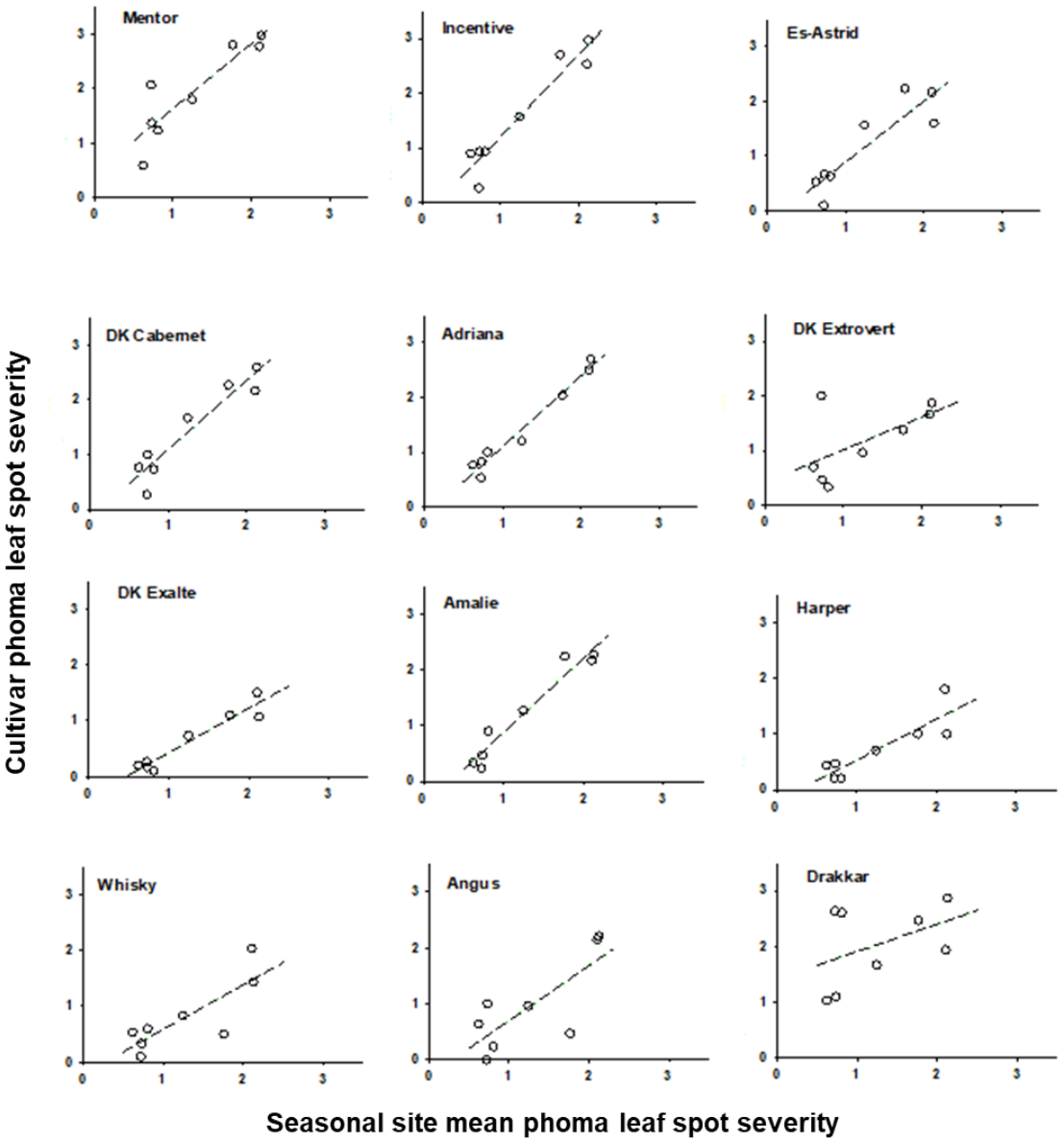

Figure 5.9: Relationships between mean phoma leaf spot severity for each of the twelve cultivars and seasonal site mean phoma leaf spot severity for the three cropping seasons (2015/2016, 2016/2017 and 2017/2018). The relationships are shown for each of the cultivars Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky, Angus and Drakkar and each of the different sites Morley, Impington, Trumpington, Rothwell, Wisbech or Woodhall Farm. For equations refer to Appendix 5-A.

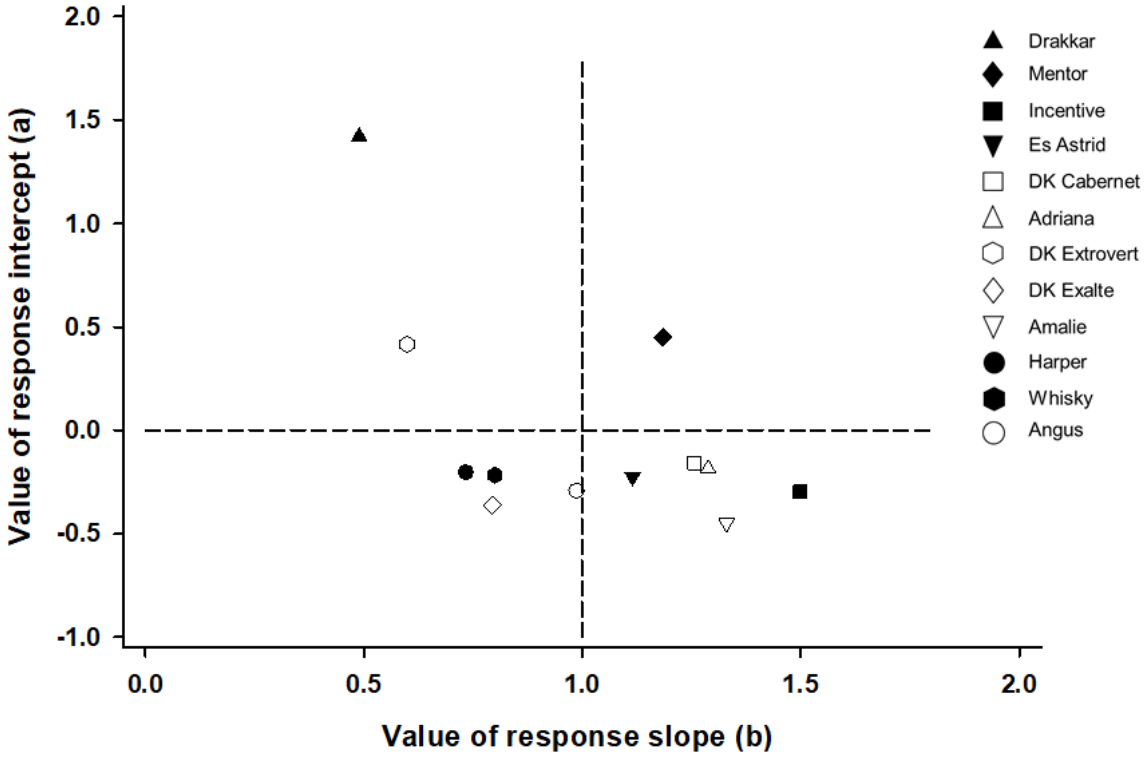

Figure 5.10: Discrimination of cultivar in the values of estimated slope (b) and intercept (a) when a simple linear relation was fitted to describe the cultivar phoma leaf spot severity by the seasonal site mean phoma leaf spot severity.

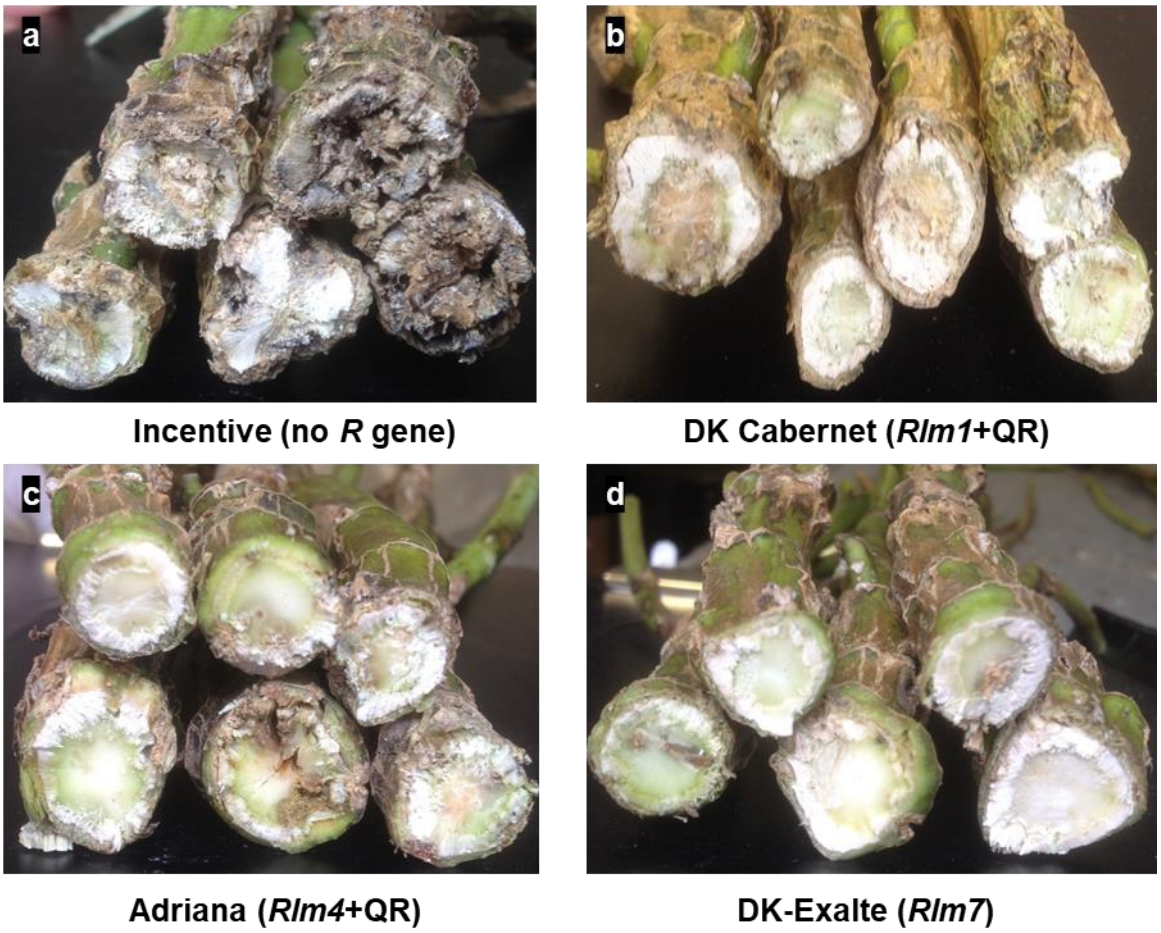

Figure 5.11: Symptoms of phoma stem canker on different cultivars (2015/2016 season). a: Incentive (no *R* gene) had severe stem base canker; b: DK Cabernet (*Rlm1*+QR) and c: Adriana (*Rlm4*+QR) also had stem base cankers; d: DK-Exalte (*Rlm7*) showed good resistance against the phoma stem canker pathogen.

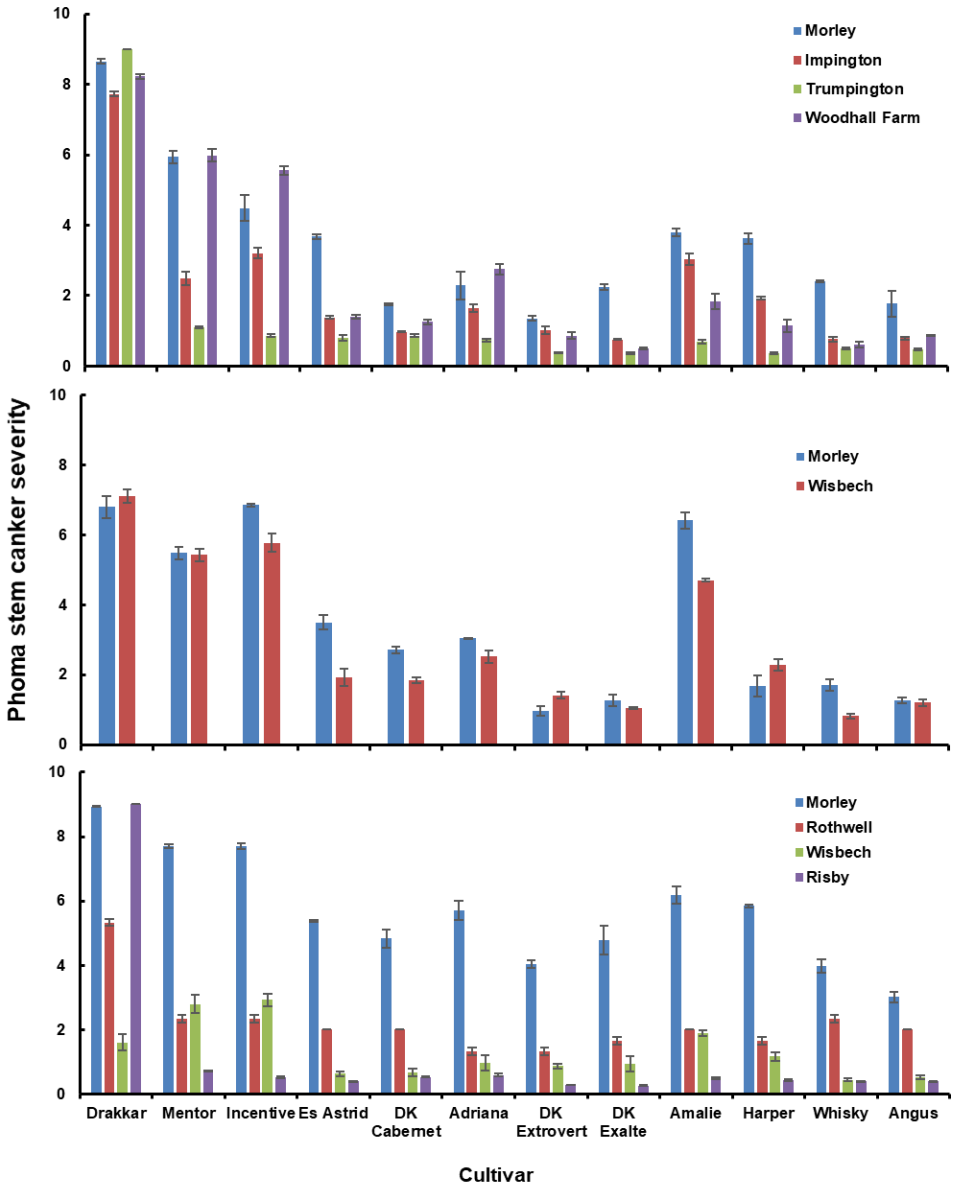

Figure 5.12: Severity (modified G2 disease index) of phoma stem canker on cultivars Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus in the UK at four different sites (Morley, Impington, Trumpington and Woodhall Farm) in the 2015/2016 cropping season (a); two different sites (Morley and Wisbech) in the 2016/2017 cropping season (b) and four different sites (Morley, Rothwell, Wisbech and Risby) in the 2017/2018 cropping season (c). A modified G2 disease index was used to assess the severity of phoma stem canker on cultivars. The error bars represent the standard error of the mean (24 degrees of freedom) between three replicate plots at each site.

At the Trumpington site, cultivars DK Exalte (score-0.4), DK Extrovert (0.4) and Harper (0.4) had least severe phoma stem canker. At the Woodhall Farm site, cultivar DK Exalte (score-0.5) had the least severity of phoma stem canker (Figure 5.12a). The Trumpington site had less severe phoma stem canker on all cultivars, except on Drakkar when compared to other three sites in the 2015/2016 cropping season (Figure 5.12a).

In the 2016/2017 cropping season, phoma stem canker severity was assessed at two different sites (Morley and Wisbech) (Figure 5.12b). At the Morley site, cultivar Incentive (score-6.9) had greatest phoma stem canker severity and cultivars DK Extrovert (score-1), DK Exalte (score-1.3) and Angus (1.3) had least phoma stem canker severity. At the Wisbech site, cultivar Drakkar (score-7.1) had the greatest severity of phoma stem canker and cultivars Whisky (score-0.8), DK Exalte (score-1), Angus (score-1.2) and DK Extrovert (score-1.4) had least phoma stem canker severity (Figure 5.12b).

In the 2017/2018 cropping season, phoma stem canker severity was assessed at four different sites (Morley, Rothwell, Wisbech and Risby) (Figure 5.12c). Amongst all the sites, the Morley site had greatest phoma stem canker severity on all cultivars and Risby site had least phoma stem canker severity on all cultivars, except on Drakkar. Amongst all the sites, cultivar Drakkar had the greatest phoma stem canker severity at three sites; Morley (score-8.9), Rothwell (score-5.3) and Risby (score-9). At the Morley site, cultivar Angus (score-3) had the least severe phoma stem canker. At the Rothwell site, cultivars DK Extrovert (score-1.3) and Adriana (score-1.3) had least severe phoma stem canker. At the Wisbech site, cultivars Incentive (score-2.9) and Mentor (score-2.8) had the greatest phoma canker severity and cultivars Whisky (score-0.4) and Angus (score-0.5) had least severe phoma stem canker. At the Risby site, cultivars DK Exalte (score-0.3) and DK Extrovert (score-0.3) had least severe phoma stem canker (Figure 5.12c).

Analysis of variance of phoma stem canker severity on twelve cultivars in the experimental plots at Morley, Impington, Trumpington, Woodhall Farm, Wisbech, Rothwell and Risby in three consecutive cropping seasons (2015/2016, 2016/2017 and 2017/2018) showed that phoma stem canker severity differed

significantly (P<0.01) between cropping seasons. There were also significant differences (P<0.01) between cultivars and sites in phoma stem canker severity. Analysis of variance showed that there were significant differences in mean phoma stem canker severity between the twelve cultivars (P<0.05) across sites and cropping seasons (Table 5.5).

The mean phoma stem canker severity score was greatest on susceptible cultivars Drakkar, Incentive and Mentor at all sites and in all seasons and significantly differ (P>0.05) from each other (Table 5.5). The mean phoma stem canker severity score was significantly less on Angus, DK Exalte, Whisky, DK Extrovert, Harper, Es Astrid and DK Cabernet with no statistically significant difference between them (P>0.05). Analysis of variance showed that cultivars Adriana and Amalie had significantly greater phoma stem canker severity than Angus, DK Exalte, DK Extrovert, Whisky, Harper, Es Astrid and DK Cabernet (Table 5.5).

Mean phoma stem canker severity for each cultivar was plotted against seasonal site mean phoma stem canker severity and the relationship between them were linear (Figure 5.13). A simple linear relationship (y=a+b*x) (Figure 5.14) was fitted to describe the cultivar phoma stem canker severity by the seasonal site mean phoma stem canker severity by using estimated intercept (a) and slope (b) (Appendix 5-B); where y is cultivar and x is seasonal site mean of phoma stem canker severity. This showed that cultivars DK Exalte, Whisky, DK Extrovert, Angus and DK Cabernet had least phoma stem canker severity in all seasons and sites; whereas, cultivars Drakkar, Incentive and Mentor had greatest phoma stem canker severity in all seasons and sites (Figure 5.14).

There was an effect of phoma stem canker severity on the yield at all the sites. In the 2015/2016 cropping season, there was a greatest yield at the Impington site for all cultivars except for Drakkar (no yield) (Figure 5.15a) and this site had less severe phoma stem canker when compared to Morley and Woodhall Farm sites (Figure 5.12a). Among the twelve cultivars, yield of the susceptible cultivar Drakkar was smallest at all three sites; Morley (mean yield-0.54 t ha^{-1}), no yield obtained at Impington and Woodhall Farm sites. In association with less severe

Table 5.5: The mean phoma stem canker severity for each cultivar across sites in the harvesting years of 2016, 2017 and 2018. Phoma stem canker severity that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	Phoma stem canker severity*
Adriana	2.25bc
Amalie	3.23cd
Angus	1.14a
DK Cabernet	1.72ab
DK Exalte	1.25ab
DK Extrovert	1.34ab
Drakkar	7.45e
Es-Astrid	2.12ab
Harper	2.05ab
Incentive	4.21d
Mentor	4.18d
Whisky	1.30ab

*Least significant difference (LSD) at 5% was 1.06 (*df*=85).

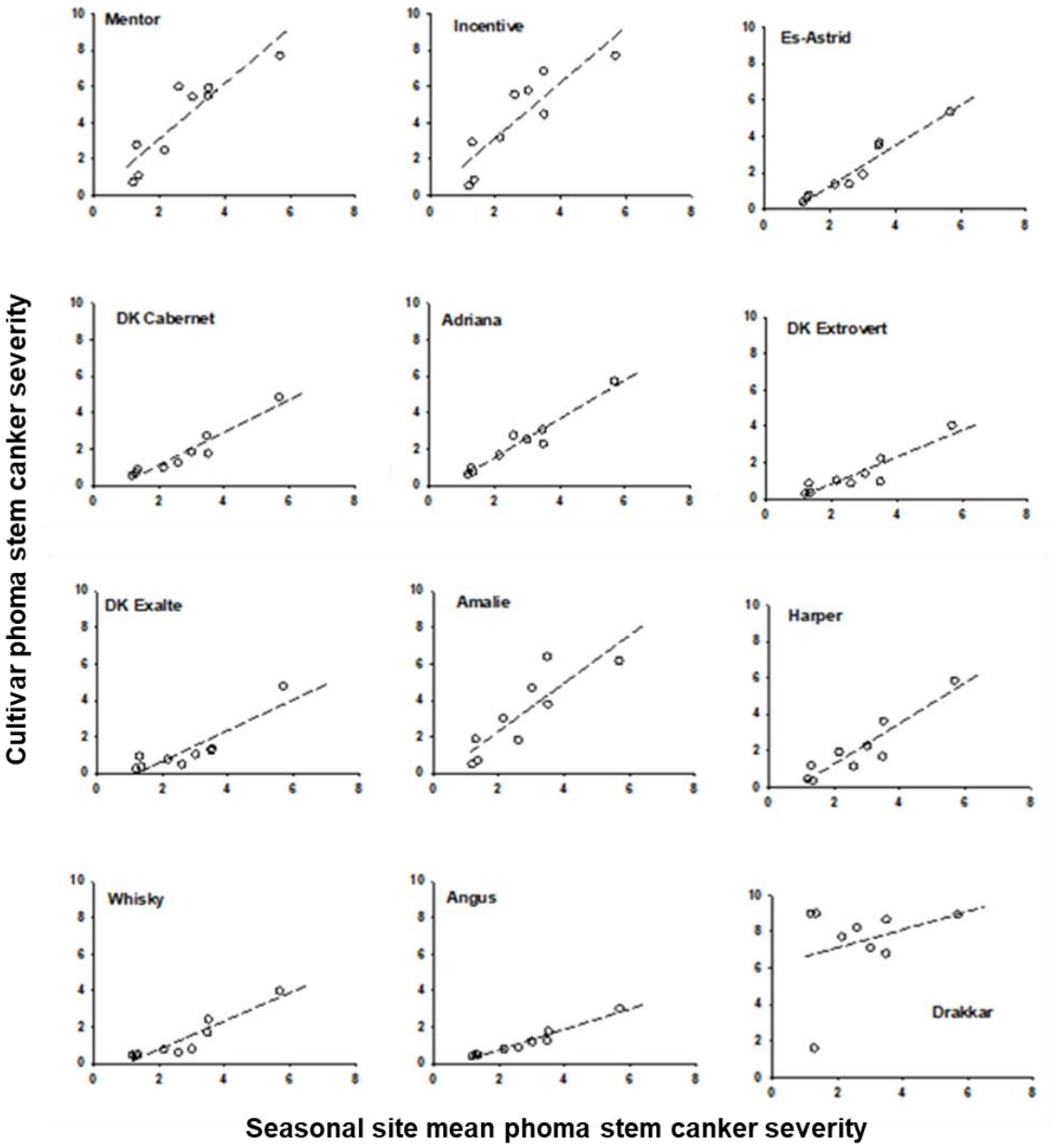

Figure 5.13: Relationships between mean phoma stem canker severity for each of the twelve cultivars and seasonal site mean phoma stem canker severity for the three cropping seasons (2015/2016, 2016/2017 and 2017/2018). The relationships are shown for each of the cultivars Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky, Angus and Drakkar and each of the different sites Morley, Impington, Trumpington, Woodhall Farm, Rothwell, Wisbech or Risby. For equations refer to Appendix 5-B.

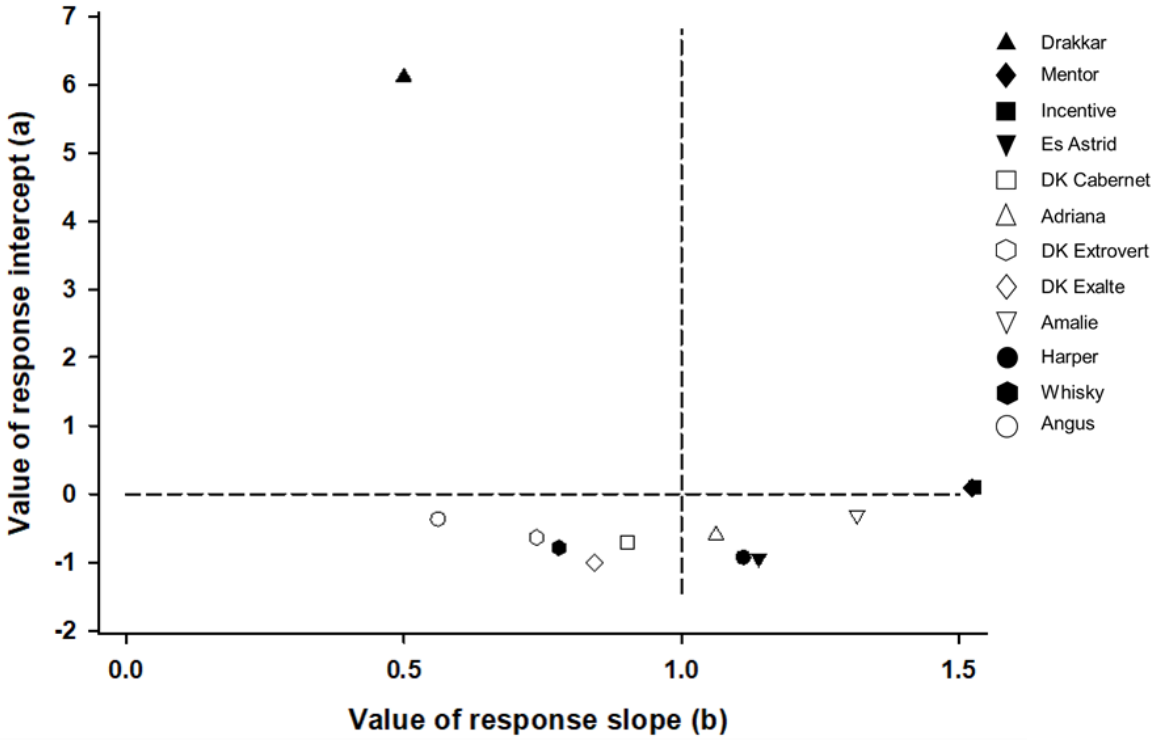

Figure 5.14: Discrimination of cultivar in the values of estimated slope (b) and intercept (a) when a simple linear relation was fitted to describe the cultivar phoma stem canker severity by the seasonal site mean phoma stem canker severity.

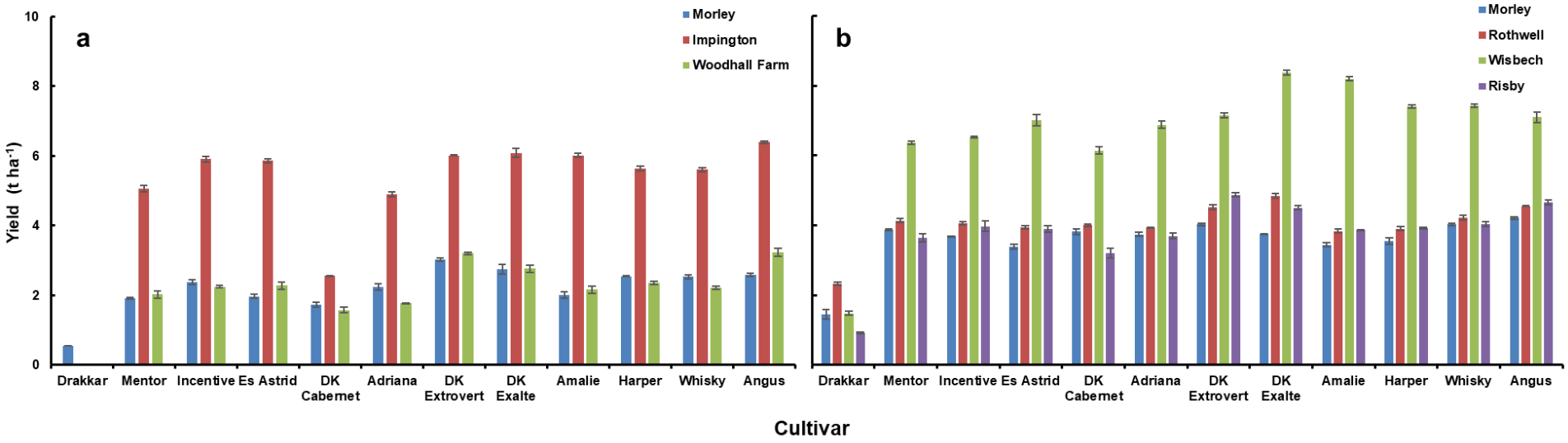

Figure 5.15: Mean yield (t ha^{-1}) of cultivars Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus at three different sites (Morley, Impington and Woodhall Farm) in the 2015/2016 (a) cropping season. Mean yield (t ha^{-1}) of cultivars Drakkar, Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky and Angus at three different sites (Morley, Rothwell, Wisbech and Risby) in the 2017/2018 (b) cropping season. The error bars represent the standard error of mean. There was no yield data for the 2016/2017 cropping season.

phoma stem canker, larger yields were obtained from cultivar DK Extrovert (mean yield-3 t ha^{-1}) at the Morley site, cultivar Angus (mean yield-6.4 t ha^{-1}) at the Impington site and cultivars Angus (mean yield-3.2 t ha^{-1}) and DK Extrovert (mean yield-3.2 t ha^{-1}) at the Woodhall Farm site (Figure 5.15a).

In the 2017/2018 cropping season, the greatest yields were obtained at the Wisbech site for all cultivars (except for Drakkar) and the smallest yields were at the Morley site (except for Drakkar and Mentor), which had the greatest phoma stem canker severity (Figure 5.15b). Among the twelve cultivars, yield of the susceptible cultivar Drakkar was smallest at all four sites; Morley (mean yield-1.43 t ha^{-1}), Rothwell (mean yield-2.32 t ha^{-1}), Wisbech (mean yield-1.48 t ha^{-1}) and Risby (mean yield-0.92 t ha^{-1}). Larger yields were obtained from cultivar DK Extrovert and Whisky (mean yield-4.03 t ha^{-1}) at the Morley site, cultivar DK Exalte (mean yield-4.8 t ha^{-1}) at the Rothwell site, cultivar DK Exalte (mean yield-8.4 t ha^{-1}) at the Wisbech site and cultivar DK Extrovert (mean yield-4.9 t ha^{-1}) at the Risby site (Figure 5.15b).

Analysis of variance of mean yield (t ha^{-1}) on twelve (including cultivar Drakkar) cultivars in the experimental plots of Morley, Impington, Woodhall Farm, Rothwell, Wisbech and Risby in two cropping seasons (2015/2016 and 2017/2018) showed that mean yield differed significantly ($P<0.01$) between cropping seasons (Table 5.6). There were also significant differences ($P<0.01$) between cultivars and sites. Analysis of variance showed that there were significant differences in mean yield between the twelve cultivars ($P<0.05$) across sites and cropping seasons (Table 5.6). The mean yield was greatest on cultivars DK Exalte, DK Extrovert, Angus, Whisky, Amalie, Harper and Incentive and there was no significant difference ($P>0.05$) between them. The mean yield was significantly less on cultivar Drakkar. Analysis of variance showed that cultivars Adriana, DK Cabernet and Mentor had significantly ($P<0.05$) less mean yield than cultivars DK Exalte, DK Extrovert and Angus (Table 5.6). Mean yield for each cultivar was plotted against mean seasonal site phoma leaf spotting severity and the relationship between them were linear (Figure 5.16).

Table 5.6: The mean yield (t ha $^{-1}$) for each cultivar across sites in the harvesting years of 2016 and 2018. Yield values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	Mean yield (t ha $^{-1}$)*
Adriana	3.88bc
Amalie	4.22cd
Angus	4.68d
DK Cabernet	3.29b
DK Exalte	4.72d
DK Extrovert	4.69d
Drakkar	1.34a
Es-Astrid	4.05c
Harper	4.19cd
Incentive	4.11cd
Mentor	3.86bc
Whisky	4.29cd

*Least significant difference (LSD) at 5% was 0.63 (*df*=63).

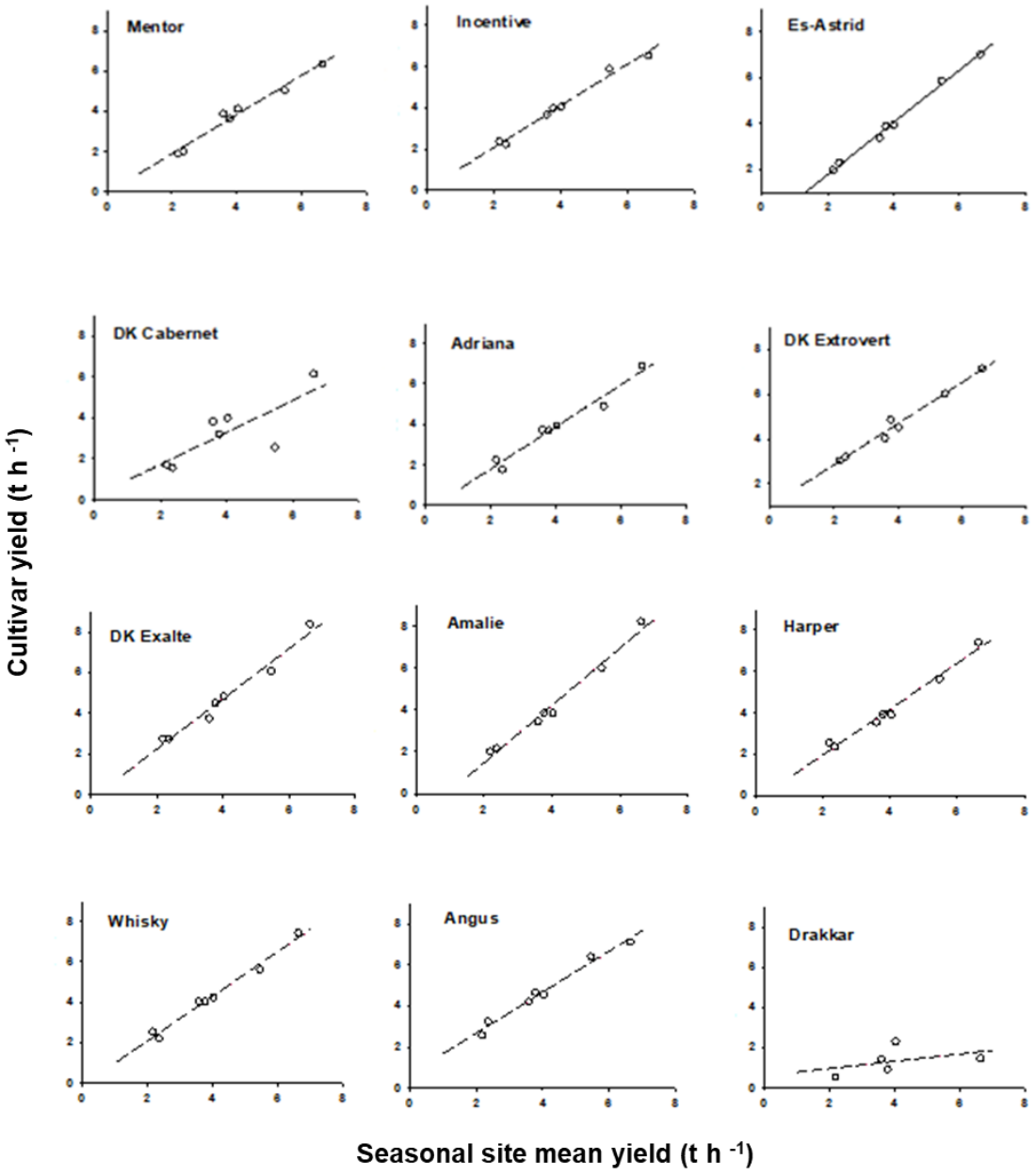

Figure 5.16: Relationships between cultivar mean yield (t h^{-1}) for each of the twelve cultivars and seasonal site mean yield (t h^{-1}) for the two cropping seasons (2015/2016 and 2017/2018). The relationships are shown for each of the cultivars Mentor, Incentive, Es Astrid, DK Cabernet, Adriana, DK Extrovert, DK Exalte, Amalie, Harper, Whisky, Angus and Drakkar and each of the different sites Morley, Impington, Trumpington, Woodhall Farm, Rothwell, Wisbech or Risby. For details of equations refer to Appendix 5-C.

A simple linear relationship (y=a+b*x) (Figure 5.17) was fitted to describe the cultivar mean yield by the seasonal site mean yield by using estimated intercept (a) and slope (b) (Appendix 5-C); where y is cultivar and x is seasonal site mean yield. This showed that cultivars DK Extrovert, Angus, DK Exalte, Whisky, and DK Cabernet had greatest yield in all seasons and sites; whereas, cultivar Drakkar had least yield in all seasons and sites (Figure 5.17).

5.3.4 Relationship between weather conditions (temperature and rainfall) and disease severity in the field experiments

Monthly mean temperature and monthly total rainfall differed between growing seasons at a given site and differed between sites within a given season (Tables 5.7 & 5.8). From the calculated standard deviations and coefficient of variations, the monthly mean temperature varied most in the winter months, followed by autumn months, but varied least in summer months (Table 5.7). However, the coefficient of variation in the monthly total rainfall was within a narrow range of *c*. 50% between months during the cropping season, except in October, April, May and June (Table 5.8). There was a large coefficient of variation (*c*. 55.7%) in the combined months total rainfall during the period April to June and the smallest coefficient of variation in December to March (*c*. 26.2%). However, the coefficient of variation in the combined months mean temperature was within a range of *c*. 10% between months, except during December to March (*c*. 23.2%) (Table 5.9).

A simple correlation coefficient was calculated for each cultivar to examine the relationship between the phoma leaf spot severity score and each of the mean temperature and total rainfall in August, September, August to September, October, November and October to November (Table 5.10). If the correlation coefficient was negative, it meant that the disease severity was not increased by that weather variable. If the correlation coefficient was positive, it meant that the disease severity increased with the increase in that weather variable. Phoma leaf spot severity was related to both rainfall and temperature for all cultivars, except to mean temperature in September and October in some cultivars (Table 5.10).

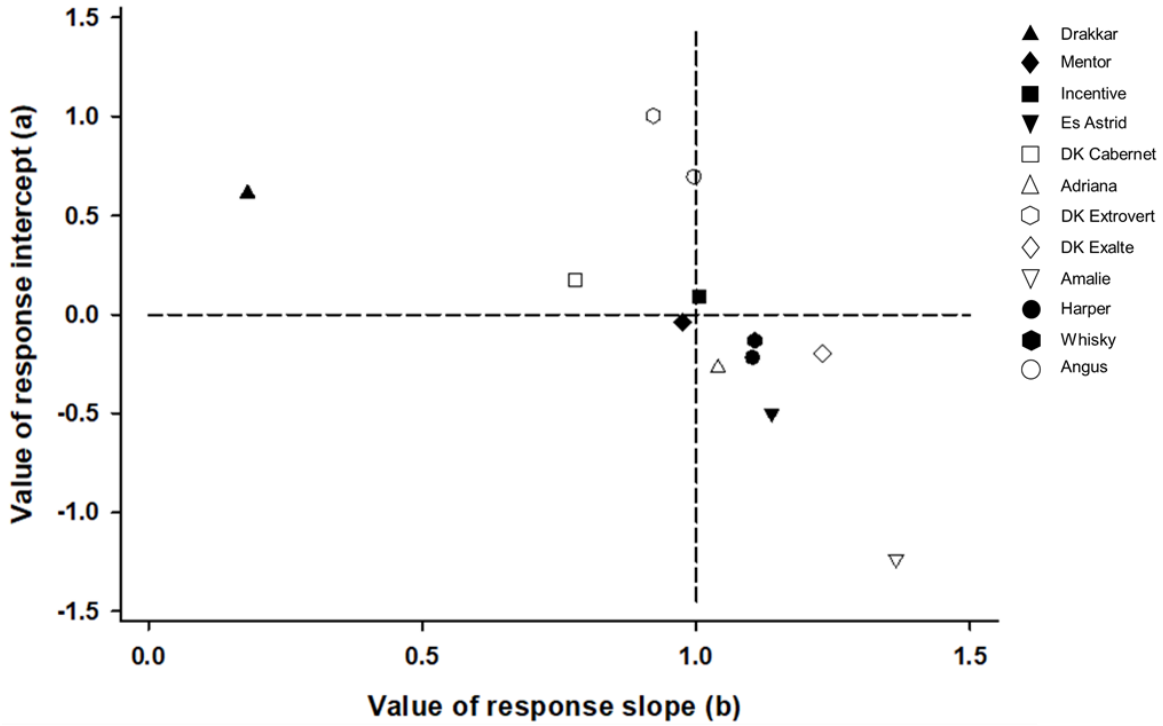

Figure 5.17: Discrimination of cultivar in the values of estimated slope (b) and intercept (a) when a simple linear relation was fitted to describe the cultivar mean yield (t ha $^{-1}$) by the seasonal site mean yield.

Table 5.7: Monthly mean temperature (°C) during the three cropping seasons (2015/2016, 2016/2017, 2017/2018) in winter oilseed rape field experiments at different sites.

Location	Cropping season	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Morley	2015/2016	17.02	12.88	10.90	9.68	9.97	5.49	4.87	5.66	7.11	12.21	14.74
Morley	2016/2017	17.89	17.10	11.00	6.10	5.96	2.90	6.11	8.52	8.53	13.16	16.69
Morley	2017/2018	15.78	12.92	12.09	6.15	4.29	4.82	1.99	2.98	9.99	13.35	15.86
Impington	2015/2016	16.89	12.49	10.96	9.99	10.47	5.70	5.05	5.64	7.63	12.73	14.99
Trumpington	2015/2016	16.89	12.49	10.96	9.99	10.47	5.70	5.05	5.64	7.63	12.73	14.99
Woodhall Farm	2015/2016	16.30	12.68	11.02	9.44	9.88	4.94	4.80	5.50	7.70	12.64	15.12
Woodhall Farm	2017/2018	16.00	13.55	12.27	6.58	4.53	5.16	2.27	4.91	10.32	13.27	16.21
Rothwell	2016/2017	17.89	17.10	11.00	6.10	5.96	2.90	6.11	8.57	8.50	12.58	15.76
Rothwell	2017/2018	15.87	13.50	12.54	6.51	4.42	4.91	2.90	4.61	8.93	12.00	14.25
Wisbech	2016/2017	18.15	17.07	10.69	5.97	6.24	3.16	6.39	8.59	9.02	13.10	16.77
Wisbech	2017/2018	16.15	13.34	12.59	6.71	4.71	5.15	2.50	4.92	10.40	13.22	16.24
Risby	2017/2018	16.38	13.57	12.63	6.64	4.67	5.24	2.18	4.59	10.09	13.35	15.86
Mean		16.77	14.06	11.55	7.49	6.80	4.67	4.18	5.84	8.82	12.86	15.62
SD		0.83	1.87	0.78	1.71	2.60	1.06	1.70	1.79	1.17	0.45	0.80
CV%		4.97	13.30	6.79	22.88	38.22	22.62	40.55	30.70	13.22	3.50	5.11

The mean, standard deviation (SD) and coefficient of variation (CV %) were calculated across sites/cropping years.

Table 5.8: Monthly total rainfall (mm) during the three cropping seasons (2015/2016, 2016/2017, 2017/2018) in winter oilseed rape field experiments at different sites.

Location	Cropping season	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Morley	2015/2016	103.60	92.40	66.20	130.80	106.60	104.40	44.20	113.40	90.40	90.60	214.60
Morley	2016/2017	32.20	49.80	30.40	52.00	72.00	64.60	55.00	47.00	6.20	19.10	39.40
Morley	2017/2018	55.80	66.80	20.40	50.20	125.60	58.20	61.20	37.20	53.00	18.40	2.80
Impington	2015/2016	87.80	64.80	36.80	72.00	65.60	51.00	22.00	65.20	60.40	41.20	78.00
Trumpington	2015/2016	87.80	64.80	36.80	72.00	65.60	51.00	22.00	65.20	60.40	41.20	78.00
Woodhall Farm	2015/2016	83.22	45.45	64.64	84.03	81.81	92.31	46.87	84.31	62.02	39.39	84.84
Woodhall Farm	2017/2018	66.50	86.80	31.10	53.20	110.70	76.10	48.50	78.30	75.00	61.90	3.50
Rothwell	2016/2017	32.20	49.80	30.40	52.00	72.00	64.60	55.00	49.40	18.80	56.00	93.00
Rothwell	2017/2018	87.40	61.20	16.60	52.80	49.60	49.00	33.80	70.20	82.60	37.80	84.80
Wisbech	2016/2017	21.20	30.40	18.00	64.60	21.20	48.20	34.40	31.60	8.20	66.40	48.60
Wisbech	2017/2018	27.20	61.80	11.20	26.40	64.60	35.00	24.20	50.60	52.80	30.00	1.00
Risby	2017/2018	62.80	56.60	23.00	32.60	107.40	70.40	43.40	84.80	50.60	18.40	2.80
Mean		62.31	60.89	32.13	61.89	78.56	63.73	40.88	64.77	51.70	43.37	60.95
SD		28.39	16.96	17.50	27.07	29.57	19.79	13.50	23.30	27.47	22.02	60.78
CV%		45.57	27.85	54.47	43.74	37.64	31.05	33.03	35.98	53.14	50.78	99.72

The mean, standard deviation (SD) and coefficient of variation (CV %) were calculated across sites/cropping years.

Table 5.9: Mean temperature (°C) and total rainfall (mm) during August to September (Aug-Sept), October to November (Oct-Nov), December to March (Dec-Mar) and April to June (Apr-Jun) during the three cropping seasons (2015/2016, 2016/2017, 2017/2018) in winter oilseed rape field experiments at different sites.

Site	Cropping season	Mean Temperature (°C)				Total Rainfall (mm)			
		Aug-Sept	Oct-Nov	Dec-Mar	Apr-Jun	Aug-Sept	Oct-Nov	Dec-Mar	Apr-Jun
Morley	2015/2016	14.98	10.30	6.53	11.36	196.00	197.00	368.60	395.60
Morley	2016/2017	17.50	8.59	5.87	12.80	82.00	82.40	238.60	64.70
Morley	2017/2018	14.37	9.17	2.80	13.07	122.60	70.60	253.80	74.20
Impington	2015/2016	14.73	10.49	6.74	11.80	152.60	108.80	203.80	179.60
Trumpington	2015/2016	14.73	10.49	6.74	11.80	152.60	108.80	203.80	179.60
Woodhall Farm	2015/2016	14.52	10.24	6.31	11.83	128.68	148.68	305.30	186.25
Woodhall Farm	2017/2018	14.80	9.47	4.27	13.26	153.30	84.30	313.60	140.40
Rothwell	2016/2017	17.50	8.59	5.88	12.28	82.00	82.40	241.00	167.80
Rothwell	2017/2018	14.70	9.57	4.24	11.73	148.60	69.40	202.60	205.20
Wisbech	2016/2017	17.62	8.37	6.09	12.96	51.60	82.60	135.40	123.20
Wisbech	2017/2018	14.77	9.69	4.36	13.29	89.00	37.60	174.40	83.80
Risby	2017/2018	15.00	9.69	4.22	13.10	119.40	55.60	306.00	71.80
Mean		15.44	9.55	5.34	12.44	123.20	94.01	245.58	156.01
SD		1.23	0.72	1.24	0.68	39.07	41.17	64.33	86.91
CV%		7.95	7.55	23.22	5.45	31.71	43.79	26.20	55.71

The mean, standard deviation (SD) and coefficient of variation (CV %) were calculated across sites/cropping years.

Table 5.10: The simple correlation coefficients calculated between phoma leaf spot severity score and the total rainfall (mm) or the mean temperature (ºC) at different sites over three cropping seasons (2015/2016, 2016/2017 and 2017/2018).

Weather variable	Months	Mentor	Incentive	Es Astrid	DK Cabernet	Adriana	DK Extrovert	DK Exalte	Amalie	Harper	Whisky	Angus	Drakkar	Across all cultivars
Rainfall	**Aug**	-0.10	0.20	-0.04	0.15	0.34	-0.10	0.06	0.14	0.21	0.57	0.56	0.02	0.14
	Sept	0.03	0.17	-0.13	0.18	0.31	0.04	0.01	0.09	0.10	0.47	0.65	0.07	0.14
	Aug-Sept	-0.09	0.14	0.02	0.10	0.31	-0.09	0.15	0.12	0.37	0.68	0.61	-0.13	0.15
	Oct	0.53	0.47	0.21	0.47	0.61	0.47	0.38	0.49	0.28	0.58	0.62	0.63	0.41
	Nov	0.58	0.67	0.36	0.64	0.75	0.42	0.46	0.64	0.36	0.57	0.65	0.60	0.49
	Oct-Nov	0.50	0.48	0.52	0.44	0.64	0.36	0.68	0.57	0.79	0.87	0.64	0.23	0.46
Temperature	**Aug**	0.59	0.26	0.38	0.27	0.22	0.55	0.40	0.40	0.12	-0.04	-0.22	0.65	0.25
	Sept	0.19	-0.11	0.06	-0.07	-0.24	0.33	0.04	-0.05	-0.19	-0.47	-0.50	0.17	-0.06
	Aug-Sept	0.32	-0.01	0.18	0.02	-0.11	0.41	0.17	0.08	-0.06	-0.33	-0.44	0.30	0.03
	Oct	-0.77	-0.49	-0.52	-0.47	-0.52	-0.54	-0.56	-0.65	-0.35	-0.31	-0.11	-0.84	-0.44
	Nov	0.29	0.40	0.25	0.34	0.57	0.10	0.34	0.46	0.41	0.72	0.56	0.44	0.34
	Oct-Nov	0.02	0.25	0.05	0.20	0.41	-0.09	0.13	0.25	0.26	0.63	0.55	0.19	0.20

A simple correlation coefficient was calculated for each cultivar to examine the relationship between the phoma stem canker severity score and each of the mean temperature and total rainfall in combined months, August to September, October to November, December to March and April to June (Table 5.11). From the results, phoma stem canker severity was related to both rainfall and temperature. However, the mean temperatures during October to November and December to March showed no effect on increase in severity of phoma stem canker (Table 5.11).

5.3.5 Effectiveness of *Rlm7* resistance gene in *Brassica napus* lines/cultivars in controlled environment conditions

The effectiveness of the cultivars containing the *Rlm7* resistance gene (Roxet, Excel, Hearty, 01-23-2-1, DK Extrovert, DK Exalte, Harper, Amalie and Whisky) was tested in controlled environment conditions by scoring and measuring the lesions produced 17 dpi on the cotyledons of the cultivars. All isolates tested (except isolate V23.11.9 - positive control for avirulent *AvrLm7*) produced typical large/grey lesions on cotyledons (Figures 5.18 & 5.19). Analysis of variance was used to analyse the measurements of lesion length, width and area. The lesion area values were square root transformed to make the residual errors homogeneous between cultivars and *L. maculans* isolates (Tables 5.12 & 5.13).

Lesion length differed significantly between cultivars (P<0.01) and between isolates (P<0.01) (Tables 5.12 & 5.13). The interaction between cultivar and isolate was also significant (P<0.05). Lesion width differed significantly between cultivars (P<0.01) and between isolates (P<0.01). The interaction between cultivar and isolate was also significant (P<0.05). Square root of lesion area differed significantly between cultivars (P<0.01) and between isolates (P<0.01). The interaction between cultivar and isolate was also significant (P<0.05) (Tables 5.12 & 5.13).

Analysis of variance was done on the lesion length, width and square root of lesion area in each cultivar (Table 5.12). Amongst all the cultivars, Whisky, Harper and Amalie had signicantly (P<0.05) smaller lesion areas compared to other cultivars. 01-23-2-1, Excel and DK Extrovert had signicantly (P<0.05)

Table 5.11: The simple correlation coefficients calculated between phoma stem canker severity score and the total rainfall (mm) or the mean temperature (ºC) at different sites over three cropping seasons (2015/2016, 2016/2017 and 2017/2018).

Weather variable	Months	Mentor	Incentive	Es Astrid	DK Cabernet	Adriana	DK Extrovert	DK Exalte	Amalie	Harper	Whisky	Angus	Drakkar	Across all cultivars
Rainfall	**Aug-Sept**	-0.10	-0.30	0.11	-0.13	-0.11	0.13	-0.02	-0.30	0.15	0.21	0.10	0.48	0.00
	Oct-Nov	0.32	0.12	0.23	-0.05	0.07	0.12	-0.14	0.00	0.16	0.16	0.16	0.48	0.10
	Dec-Mar	0.21	0.00	0.28	0.06	0.12	0.18	0.05	-0.10	0.19	0.33	0.23	0.50	0.11
	Apr-Jun	0.15	-0.08	0.12	-0.19	-0.12	0.10	-0.18	-0.12	0.15	0.11	0.08	0.30	0.02
Temperature	**Aug-Sept**	0.20	0.37	0.12	0.13	0.07	-0.15	-0.14	0.52	-0.09	-0.09	-0.02	-0.13	0.07
	Oct-Nov	-0.42	-0.58	-0.37	-0.50	-0.43	-0.24	-0.34	-0.67	-0.25	-0.25	-0.33	0.20	-0.23
	Dec-Mar	-0.18	-0.19	-0.32	-0.50	-0.43	-0.51	-0.67	-0.20	-0.44	-0.48	-0.45	0.17	-0.20
	Apr-Jun	-0.01	0.14	0.01	0.25	0.15	0.10	0.30	0.21	0.05	0.05	0.08	-0.46	0.03

Figure 5.18: Lesions at 17 dpi on representative cotyledons of the *Brassica napus* cultivars/lines carrying the *Rlm7* resistance gene inoculated with *Leptosphaeria maculans* isolates carrying the avirulent or virulent allele of the *AvrLm7* effector gene. Isolate 1 was avirulent towards *Rlm7*; isolates 2-8 were virulent towards *Rlm7*. 1-4 and 5-8 represent isolates used at each point of inoculation during this experiment. Cotyledons of 17-day old plants were inoculated by wound-inoculation with 10µl of conidial suspension (10^7 spores/ml) of *Leptosphaeria maculans* isolates.

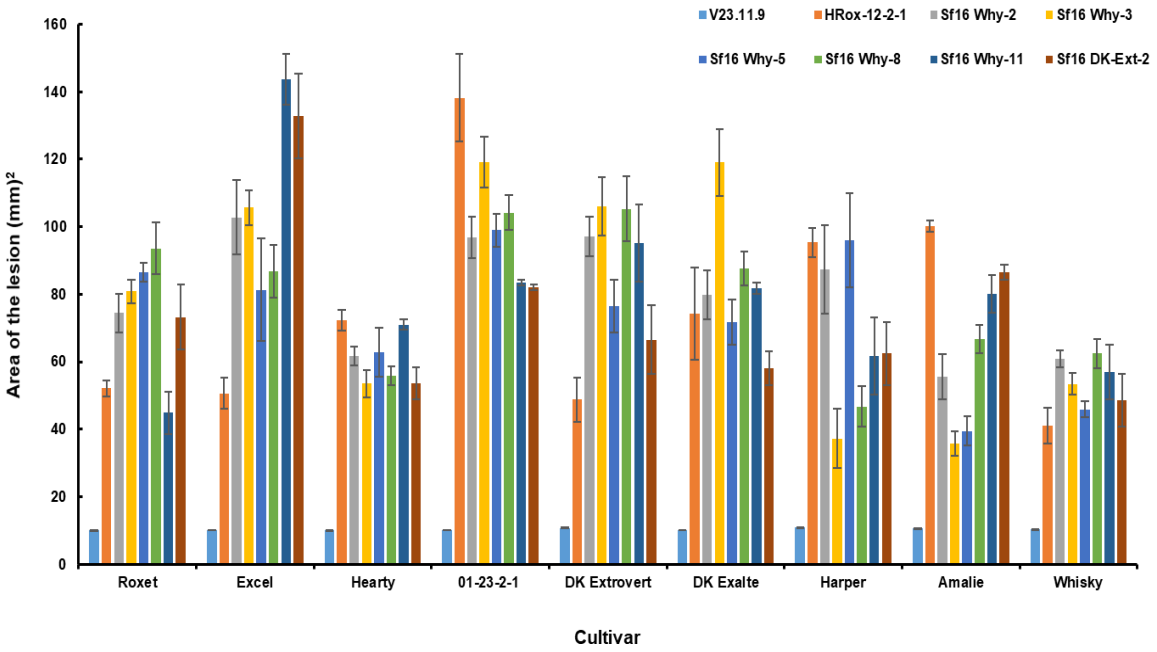

Figure 5.19: Phenotype scores (lesion area, mm²) caused by the *Leptosphaeria maculans* isolates inoculated on cotyledons of different *Brassica napus* cultivars/lines. Cotyledons were inoculated by wound inoculation on cultivars Roxet, Excel, Hearty, 01-23-2-1, DK Extrovert, DK Exalte, Harper, Amalie and Whisky. Conidial suspensions (10^7 spores/ml) of *Leptosphaeria maculans* isolates V23.11.9, HRox-12-2-1, Sf16 Why-2, Sf16 Why-3, Sf16 Why-5, Sf16 Why-8, Sf16 Why-11 or Sf16 DK-Ext-2 were used in this experiment. Error bars represent the standard error of mean.

Table 5.12: Table showing lesion length, width and square root of lesion area in each cultivar. Values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	Length*	Width**	Square root Area***
Amalie	9.79ab	7.03a	7.31ab
DK Exalte	11.04cd	7.84bc	8.21cd
DK Extrovert	11.43d	8.28cd	8.59de
Excel	11.15cd	8.81d	8.76de
Harper	9.88bc	7.08ab	7.38abc
Hearty	9.11ab	7.25ab	7.17ab
01-23-2-1	12.16d	8.87d	9.18e
Roxet	10.14cd	7.91bc	7.91bcd
Whisky	8.61a	6.84a	6.77a

*Least significant difference (LSD) at 5% was 1.22 (*df*=278).

**Least significant difference (LSD) at 5% was 0.84 (*df*=278).

***Least significant difference (LSD) at 5% was 0.85 (*df*=278).

Table 5.13: Table showing lesion length, width and square root of lesion area caused by each isolate. Values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the isolate	Length*	Width**	Square root Area***
HRox12-2-1	11.04bc	8.52b	8.53bc
Sf16 DK-Ext-2	11.70bc	8.39b	8.76bc
Sf16 Why-11	12.21c	8.84b	9.18c
Sf16 Why-2	11.49bc	8.35b	8.64bc
Sf16 Why-3	11.23bc	8.24b	8.49bc
Sf16 Why-5	10.54b	8.05b	8.14b
Sf16 Why-8	11.22bc	8.52b	8.63bc
V23.11.9	3.84a	3.44a	3.22a

*Least significant difference (LSD) at 5% was 1.15 (*df*=278).

**Least significant difference (LSD) at 5% was 0.79 (*df*=278).

***Least significant difference (LSD) at 5% was 0.80 (*df*=278).

greater square root of lesion area values than other cultivars. Cultivars Roxet and DK Exalte had signicantly (P<0.05) greater square root of lesion area values than cultivars Whisky, Harper and Amalie. The results showed that cultivar Hearty had a signicantly (P<0.05) smaller lesion area (Table 5.12); however, the cotyledon size of this cultivar was very less compared to other cultivars (Figure 5.18).

Analysis of variance was also done on the lesion length, width and square root of lesion area caused by each *L. maculans* isolate (Table 5.13). Amonst all the isolates V23.11.9 (containing avirulent allele of *AvrLm7* effector gene) had produced signicantly (P<0.05) smaller lesions on all the cultivars containing the *Rlm7* resistance gene. Sf16 Why-11 had produced significantly (P<0.05) greater lesions when compared to other isolates. Isolates HRox12-2-1, Sf16 Why- 2, Sf16 Why-3, Sf16 Why-5, Sf16 Why-8 and Sf16 DK-Ext-2 had produced significantly (P<0.05) smaller lesions than isolate Sf16 Why-11; however, there were no significant differences (P>0.05) between them (Table 5.13).

No. of mature pycnidia (i.e. pycnidia producing cirrhus) on the incubated cotyledons inoculated with *L. maculans* isolates Sf16 Why-2, Sf16 Why-3, Sf16 Why-5 and Sf16 Why-8 were recorded to determine the differences in pycnidial production between different *Brassica napus* cultivars/lines containing the *Rlm7* resistance gene (Figures 5.20 & 5.21). Since there were no replicates, the data were analysed using the error variance component in the interaction of cultivar with isolate (Tables 5.14 & 5.15).

Analysis of variance indicates that the formation of pycnidia varied significantly between cultivars and between isolates (P<0.05). Cultivars Amalie, Whisky, DK Exalte and Roxet had significantly (P<0.05) fewer mature pycnidia; however, there were no significant differences (P<0.05) between them. Whereas, cultivar Excel had significantly (P<0.05) greatest numbers of mature pycnidia than all other cultivars (Table 5.14). Number of pycnidia produced per cultivar by each isolate was also analysed by Analysis of variance (Table 5.15). Isolate Sf16 Why-3 was found to be significantly (P<0.05) more virulent compared to isolates Sf16 Why-2, Sf16 Why-5 and Sf16 Why-8 (Table 5.15).

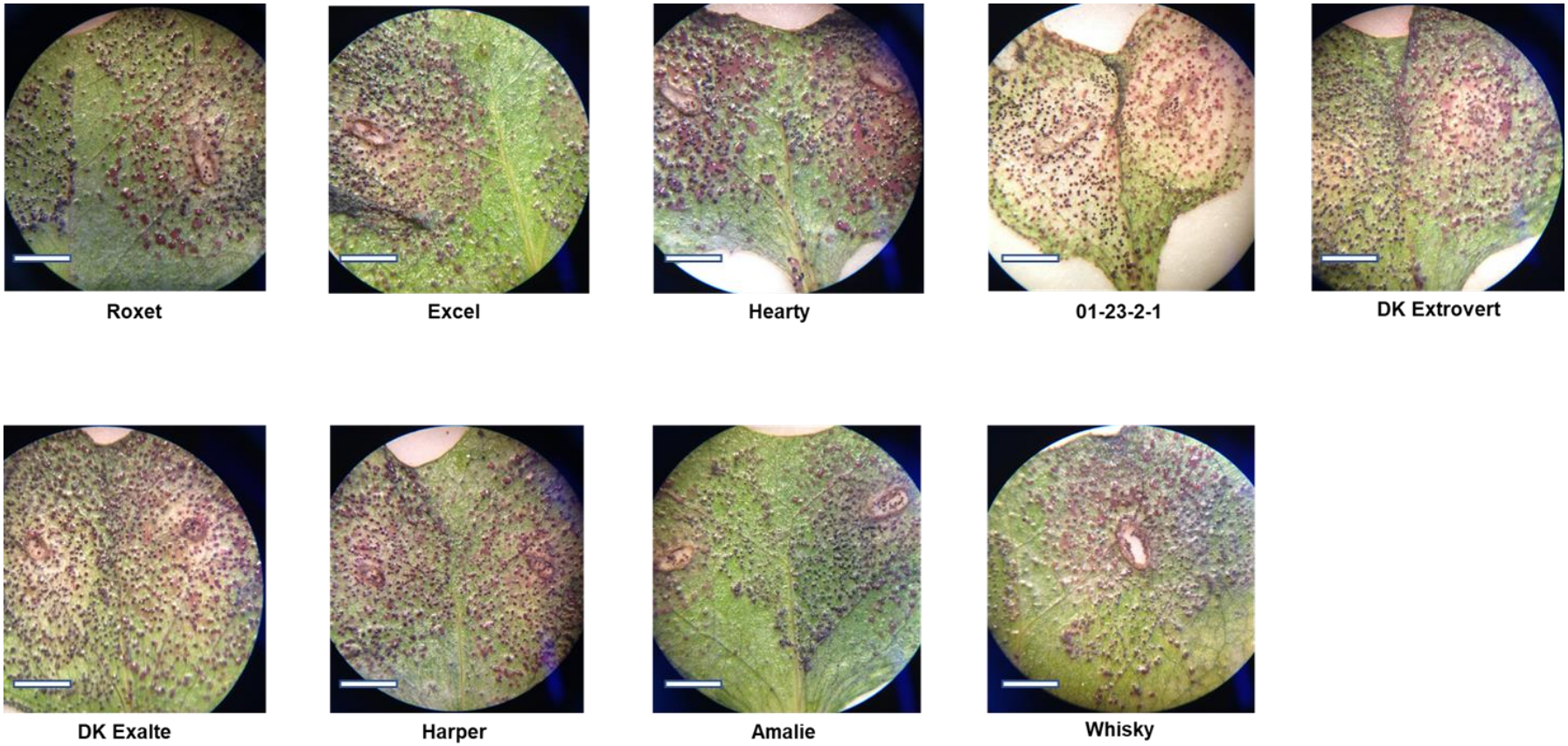

Figure 5.20: Pycnidial production on representative cotyledons of *Brassica napus* cultivars/lines (inoculated with *Leptosphaeria maculans* isolates Sf16 Why-2 and Sf16 Why-3). Cotyledons of cultivars Roxet, Excel, Hearty, 01-23-2-1, DK Extrovert, DK Exalte, Harper, Amalie and Whisky inoculated with the *Leptosphaeria maculans* isolates were detached from the plants at 17-dpi, incubated for 5 days at 20^{o}C and were photographed under a stereomicroscope (scale bar: 0.5 cm).

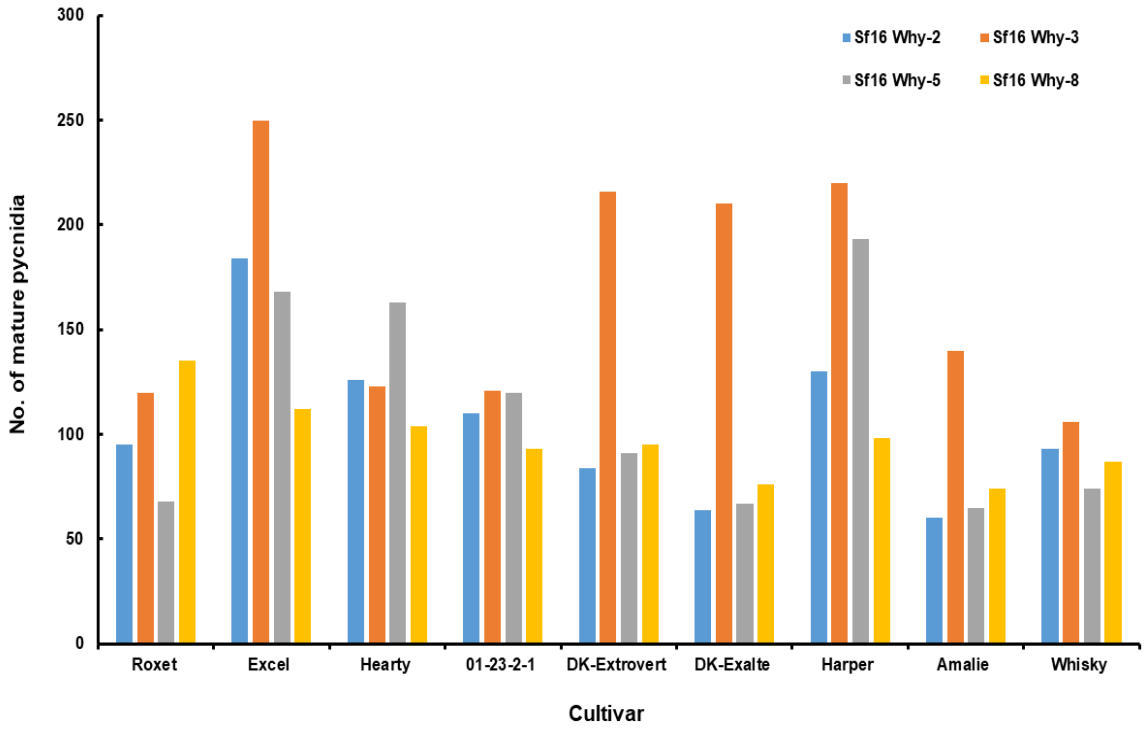

Figure 5.21: Differences in the no. of mature pycnidia (pycnidia that produced cirrhus) between *Brassica napus* cultivars/lines. Cotyledons of cultivars Roxet, Excel, Hearty, 01-23-2-1, DK Extrovert, DK Exalte, Harper, Amalie and Whisky inoculated with *Leptosphaeria maculans* isolates Sf16 Why-2, Sf16 Why-3, Sf16 Why-5 and Sf16 Why-8 after 17 dpi were detached from plant and incubated in Petri dishes at 20^{o}C. There are no error bars as the numbers of mature pycnidia were counted on one cotyledon from each cultivar inoculated with isolates.

Table 5.14: Table showing the number of mature pycnidia produced per isolate in each cultivar. Values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the cultivar	No. of mature pycnidia*
Amalie	84.75a
Whisky	90.00a
DK Exalte	104.25a
Roxet	104.50a
01-23-2-1	111.00ab
DK-Extrovert	121.50ab
Hearty	129.00abc
Harper	160.25bc
Excel	178.50c

*Least significant difference (LSD) at 5% was 24.26 (*df*=24).

Table 5.15: Table showing the number of mature pycnidia produced per cultivar by each isolate. Values that have a common letter mean that they were not significantly different when a least significant difference (LSD) was calculated using a probability of 5%.

Name of the Isolate	No. of mature pycnidia
Sf16 Why-8	97.11a
Sf16 Why-2	105.11a
Sf16 Why-5	112.11a
Sf16 Why-3	167.33b

*Least significant difference (LSD) at 5% was 16.17 (*df*=24).

5.4 Discussion

The main findings of this chapter are the effectiveness of *R* gene-mediated resistance is affected by the host background resistance and environmental factors. Cultivars with *Rlm7* or *LepR3* resistance genes showed less severe phoma leaf spots and phoma stem canker in the UK. Increased phoma leaf spot severity was associated with increased rainfall and moderate mean temperatures in the autumn months, August to November. Increased severity of phoma stem canker was associated with increased temperatures in April to June. There were differences in the effectiveness of *Rlm7* resistance gene in different *Brassica napus* lines/cultivars in controlled environment conditions.

The results from this study suggest that there were differences in the phoma leaf spot severity between cultivars and between sites in the UK. The presence/absence of the resistance genes in the oilseed rape cultivars (Table 2.1) had a great impact on the phoma leaf spot severity in the field experiments (Figures 5.7 & 5.8). In cultivars with no resistance genes, the number of phoma leaf spots were greater (e.g. Drakkar) and the presence of resistance genes decreased the number of phoma leaf spots on cultivars (e.g. DK Exalte) (Figures 5.7 & 5.8) (Tables 5.2, 5.3 & 5.4).

Information about phoma leaf spot severity on a susceptible cultivar can provide evidence about the inoculum concentration at that site in that cropping season, in relation to seasonal and regional differences in severity of disease epidemics. Information about phoma leaf spot severity on a resistant cultivar can provide evidence about the resistance breakdown and current effectiveness of that cultivar or *R* gene. The results from this study showed that *Rlm7* resistance gene that was considered to be effective in the UK until the 2015/2016 season, has started to lose its effectiveness (cultivars Amalie and Harper). This may have occurred because of the extensive use of this resistance gene in many cultivars in the UK (Mitrousia *et al*., 2018). However, there are cultivars containing the *Rlm7* resistance gene that are still performing well (e.g. DK Exalte).

These results also suggest that there were differences in the severity of phoma stem canker between cultivars and cropping seasons in the UK (Figure 5.12;

Table 5.5). From the data, it was evident that cultivars Drakkar, Mentor and Incentive without any resistance genes (Table 2.1) (Balesdent *et al*., 2006) had more phoma leaf spots and more severe phoma stem canker than the other cultivars with *R* genes with/without quantitative resistance in their background (Table 2.1). The cultivars DK Exalte, DK Extrovert and Whisky with the *Rlm7* resistance gene (Table 2.1) had fewer leaf spots and less severe phoma stem canker when compared to cultivars Adriana and DK Cabernet with the *Rlm1* and *Rlm4* resistance genes, respectively.

Severe phoma leaf spots in winter on susceptible cultivars Incentive and Mentor was associated with severe phoma stem canker severity in summer, supporting previous studies showing that phoma stem canker is influenced by the incidence of phoma leaf spotting (Sun *et al*., 2001). However, in this study, there were cases where less phoma stem canker severity in summer were observed in cultivars with severe phoma leaf spots in the previous autumn/winter (e.g. cultivars Es Astrid and DK Cabernet at different sites in the three cropping seasons). There were differences in the phoma leaf spot severity and phoma stem canker severity on the same cultivars between different sites in the UK. Among all the sites, Morley site had the greatest phoma leaf spot and phoma stem canker severities; whereas, Trumpington and Risby sites had the least disease severity. Among the three cropping seasons, less phoma stem canker severity was observed among the sites in 2016/2017 than in other two seasons (Figures 5.7, 5.8 & 5.12).

Cultivars could be classified into three groups based on the severity of phoma stem canker disease severity [Mentor, Incentive and Drakkar, with no *R* gene and QR (most susceptible) (Line E); Es Astrid, Adriana and DK Cabernet (intermediate) (Line C); Amalie, Harper (above intermediate) DK Exalte, DK Extrovert, Whisky and Angus (least susceptible) (Line B)] (Figure 5.22). In conclusion, incorporating both qualitative (*R* gene) resistance and quantitative (QR) resistance makes the cultivar resistance more durable and temperature-resilient (Line A; no cultivar in this group was observed).

Therefore, combining *R* gene and QR can provide effective, stable control of phoma stem canker in different environments (Huang *et al*., 2018). It was also observed that there was a decrease in the effectiveness of resistance towards

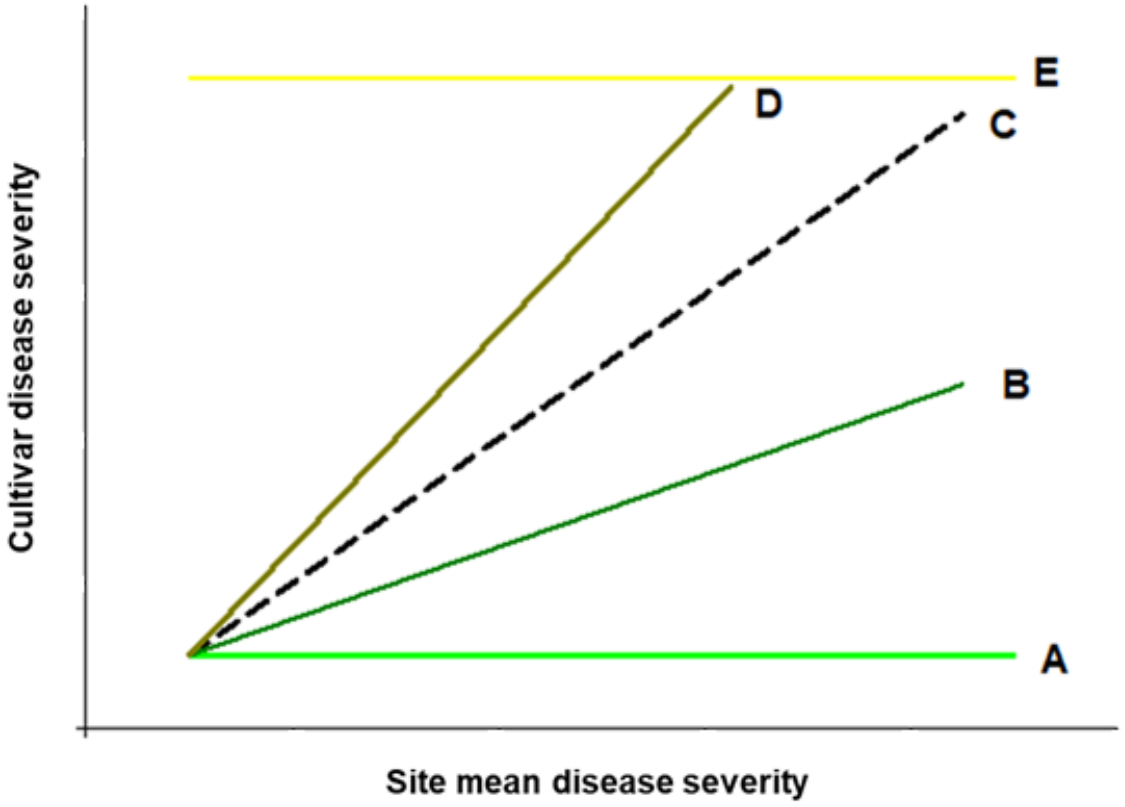

Figure 5.22: Graph showing response types of cultivar disease severity scores in relation to the seasonal site mean disease severity scores when different cultivars were grown and evaluated for phoma stem canker at different sites in different cropping seasons.

Theoretically, there are at least five apparent response types. One response type can represent cultivars that are qualitatively resistant to the pathogens responsible for the disease symptoms (Line A). One response type can represent cultivars that are qualitatively susceptible to the pathogens responsible for the disease symptoms (Line E). One response type can represent cultivars that are quantitatively resistant to the pathogens responsible for the disease symptoms and show above average adaptation (Line D) while one response type can represent cultivars that are quantitatively resistant to the pathogens responsible for the disease symptoms but show below average adaptation (Line B). Line C shows the response type for quantitatively resistant cultivars that show an average adaptation to the pathogen inoculum at multiple seasonal locations.

the phoma stem canker pathogen in cultivars (e.g Adriana) that were been used for years probably due to the changes in pathogen populations due to adaptation

(Sprague *et al*., 2006). Hence, there is a need to combine QR with *R* genes for effective disease management.

There is also an effect of phoma stem canker disease on the yield (t/ha) of winter oilseed rape crops. There were differences in the yield between cultivars, sites and cropping seasons (Figure 5.15; Table 5.6). The results in this study showed that cultivar Drakkar, a susceptible cultivar, had less yield than other cultivars at all the sites in the UK in different cropping seasons (Figure 5.15). This was partially due to the damage to the crop by phoma stem canker disease. As Drakkar is a spring cultivar, it is not suitable for growing as winter oilseed rape crop.

In contrast to that, cultivars Mentor and Incentive (susceptible cultivars) still had good mean yields in the two cropping seasons. The Impington site where there was less phoma stem canker incidence had more yield when compared to other two sites in the 2015/2016 cropping season and the Morley site that had high disease incidence in both 2015/2016 and 2017/2018 cropping sesons had comparitively less yield than other sites. Wisbech site that had less disease incidence had more yield when compared to other two sites in the 2017/2018 cropping season, whereas Risby site that had even less disease incidence than Wisbech site had less yield than Wisbech site in the 2017/2018 cropping season (Figures 5.12 & 5.15). This might be because of the soil fertility at that site.

Previous studies showed that the differences between sites in phoma leaf spot severity and phoma stem canker severity may have occurred because of the differences in weather conditions between sites during the season (Toscano-Underwood *et al*., 2001). Previous studies have also shown that phoma stem canker is influenced by the timing and incidence of phoma leaf spot in winter/autumn (Sun *et al*., 2001). Phoma leaf spot development also depends on the maturation of pseudothecia, which was dependent on both temperature and wetness; dry weather delays the maturation and release of spores. However,

differences between sites in this study might also have been due to the difference in the time of sampling between these sites.

From the results of this study, it is evident that there is a positive relationship between weather conditions and disease severity in the field experiments (Tables 5.10 & 5.11). It was stated that temperature and rainfall are the two major environmental factors affecting severity of phoma stem canker (Huang *et al*., 2018). Analysis of the relationship between phoma leaf spot severity and weather data between different sites in the three growing seasons showed that increased phoma leaf spot severity was associated with increased rainfall and moderate mean temperatures in the autumn months, August to November (during the phoma leaf spot development stage) (Table 5.10).

Analysis of the relationship between severity of phoma stem canker and weather data among the different sites in the three growing seasons showed that increased severity of phoma stem canker was associated with increased temperatures in April to June (during the stem canker development stage) (Table 5.11). This is in agreement with the previous work done by Huang *et al*. (2018). Phoma stem canker disease epidemics were initiated by the asospores released from pseudothecia on stem debris and it occurs when heavy rainfall occurs that favours ascospore maturation (West *et al*., 2001; Huang *et al*., 2005).

Increased rainfall in August and September favours ascospore maturation and increased rainfall from October to March favours ascospore release and phoma leaf spot development (the first visible symptom of phoma stem canker disease) (Huang *et al*., 2018). Temperature plays a major role (when *L. maculans* reaches leaf petiole and stem tissues) for the development of phoma stem canker, since previous work showed that increased temperature caused increase in the severity of phoma stem canker (Huang *et al*., 2009).

The results of this study showed that there were differences in the effectiveness of *Rlm7* resistance gene in different *Brassica napus* lines/cultivars in controlled environment conditions. There were also differences between *L. maculans* isolates in pathogenicity on different cultivars carrying *Rlm7* resistance gene. From the results of this preliminary experiment it is evident that the background

quantitative resistance affects the effectiveness of *Rlm7* on control of *L. maculans* by delaying its growth at the cotyledon or leaf stage. However, further work needs to be done to get a conclusion.

Chapter 6 General discussion

The overall aim of this project was to improve the understanding of *R* gene-mediated resistance against *Leptosphaeria maculans* for effective control of phoma stem canker in oilseed rape in the UK.

The overall aim of this project has been achieved through improved understanding of changes in *L. maculans* race structure over seasons, mechanisms of mutation from avirulence to virulence in different *L. maculans* effector genes and effects of background quantitative resistance and environmental factors on effectiveness of *R* gene-mediated resistance against *L. maculans.* Results from Chapter 3 showed that *L. maculans* race structure changed over the three growing seasons, with the extensive use of the resistance gene *Rlm7*, the frequency of isolates virulent against *Rlm7* increased from 0% in 2015/2016 to 16.3% in 2017/2018 for isolates collected from leaf lesions. This suggests that there is a risk of breakdown of the currently widely used resistance gene *Rlm7*.

Results from Chapter 4 showed that there are differences between the four *L. maculans* effector genes *AvrLm1, AvrLm4, AvrLm6* or *AvrLm7* (corresponding to resistance genes *Rlm1, Rlm4*, *Rlm6* or *Rlm7*) in mechanisms of mutation from avirulence to virulence in the UK *L. maculans* populations. More molecular events leading to virulence in *AvrLm7* than in *AvrLm1, AvrLm4* or *AvrLm6* were identified suggesting that the *AvrLm7* avirulence function is more important for the survival of *L. maculans* than *AvrLm1, AvrLm4* or *AvrLm6* avirulence function. This suggests that the corresponding resistance *Rlm7* is more durable than *Rlm1*, *Rlm4* or *Rlm6.*

Results from Chapter 5 showed that there were differences in severity of phoma leaf spot or phoma stem canker between cultivars carrying the same *R* gene in field experiments or between sites/seasons for the same cultivar. This suggests that background quantitative resistance and environmental factors affect the effectiveness of *R* gene resistance. This is further approved by the results from controlled environment experiments that were done with 9 cultivars/lines (all carrying the same resistance *Rlm7*) inoculated with 8 different *L. maculans*

isolates. Differences between the nine cultivars/lines or between the eight isolates in leaf lesion area or number of mature pycnidia produced on leaf lesions suggest that the background quantitative resistance affects the effectiveness of *Rlm7* mediated resistance against *L. maculans.* There is a need to combine *Rlm7* and quantitative resistance to increase the effectiveness of cultivar resistance for the effective control of phoma stem canker.

The most important findings of this study are that the pathogen race structure will change from season to season. Hence, they need to be monitored every year for the effective deployment of *R* genes. *Rlm7* resistance gene was thought to be still effective in the UK has started to breakdown. In addition to that various molecular mechanisms of mutation leading towards virulence against *Rlm7* resistance gene were identified which confirms that this gene is an importance source of resistance of *Brassica napus*. There were differences in the effectiveness of *R* genes in different cultivars in control of phoma leaf spots and phoma stem canker at different sites in the UK. From the results of this study, it is clear that background resistance and environmental factors such as temperature and rainfall have positive effect on the phoma stem canker disease severity.

The results of each experiment in all the chapters were discussed below in a detailed manner. The results of the monitoring of ascospores by air samplers showed that there were differences in the patterns of ascospore release between the three cropping seasons among the four sites between sites and cropping seasons. At Impington site, the ascopore release pattern was similar in the 2015/2016 and 2016/2017 seasons. An early ascospore release was observed in the 2017/2018 season (Figure 3.5). Similar patterns of ascospore release to the Impington site were observed at the Bayfordbury site (Figure 3.6). Differences in the ascospore release patterns and the first major ascospore release were observed at the Rothwell and Eye sites (Figures 3.7 & 3.8).

A common pattern among all four seasons was the relationship between rainfall and ascospore release. In most seasons, spore release in large numbers commenced after a period of prolonged or heavy rainfall. These differences were directly related to the differences in the weather factors like temperature and

rainfall. The results of this study were in agreement to the statement that temperature and wetness (rainfall) affect the rate of maturation of pseudothecia and the timing of ascospores release (Toscano-Underwood *et al*., 2003; Huang *et al*., 2005; Huang *et al*., 2007).

The release of ascospores of *Leptosphaeria* species were observed on days after heavy or prolonged periods of rainfall due to stimulation by wetness and major ascospore release occurred when the temperature was >10^{o}C at a site. Models predicting timing of ascospore release and models describing phases of phoma stem canker development can be combined to guide the timing of fungicide sprays each year to minimise severe disease epidemics (Evans *et al*., 2006; Evans *et al*., 2008). To predict the dates of ascospore release, weather factors (i.e. rainfall and temperature) must be used as a parameter to forecast phoma stem canker severity, as they influence both growth of the crop and the pathogen infection.

This work showed that among the UK *Leptosphaeria maculans* races, the avirulent alleles of the *AvrLm6* effector gene are still predominant in *L. maculans* populations sampled from ascospores and crops. This is because *Rlm6* resistance gene has never been used in the UK oilseed rape commercial cultivars (Huang *et al*., 2018). The regional distribution of *Leptosphaeria maculans* races were monitored for three consecutive cropping seasons in ascospores (air) and in crops. Changes in the race structure of *L. maculans* populations were observed on oilseed rape in the UK. As phoma stem canker is initiated by the ascospore release from the pseudothecia in autumn, the frequency of avirulent alleles of the *AvrLm1* or *AvrLm6* effector genes in the *Leptosphaeria* ascospores was monitored.

As these two genes have been cloned and the major molecular mechanism of mutation leading to virulence to the corresponding *R* genes, *Rlm1* or *Rlm6*, is whole gene deletion, quantitative PCR was used to detect the avirulent alleles of the *AvrLm1* or *AvrLm6* effector genes. It was also proved from previous studies that *AvrLm1* or *AvrLm6* genes play a major role at the leaf infection stage (Rouxel *et al*., 2011). The results showed that there were differences in the timing of release of *Leptosphaeria maculans* ascospores with *AvrLm1* and *AvrLm6* alleles

between sites and cropping seasons. *AvrLm6* alleles were detected more frequently from *Leptosphaeria maculans* ascospores compared to *AvrLm1* alleles at all sites in all seasons (Figures 3.9-3.12). This is in agreement to the previous UK study Van de Wouw *et al*. (2010).

At the Impington site, there were similarities in the pattern of ascospore release for the *AvrLm1* allele and *AvrLm6* allele between three seasons, where DNA of the *AvrLm1* was mostly detected in ascospores released between mid-September and mid-November and DNA of *AvrLm6* allele was mostly observed in large amounts from mid-December to early February (Figure 3.9). At the Bayfordbury site, the patterns of release of *AvrLm1* and *AvrLm6* alleles were same in the three years (Figure 3.10). At the Rothwell site, the ascospore release for the *AvrLm1* allele and *AvrLm6* allele followed a typical pattern between three seasons (Figure 3.11). At the Eye site, there were similarities in the pattern of ascospore release for the *AvrLm1* allele and *AvrLm6* allele between the three seasons (Figure 3.12). The frequency of avirulent alleles of the *AvrLm1* effector gene is still less as the *Rlm1* resistance gene has been in use in the commercial UK oilseed rape cultivars.

L. maculans isolates were obtained from phoma leaf spot lesions, the first visible symptom of phoma stem canker disease on oilseed rape, from different field experimental sites for the three cropping seasons (2015/2016, 2016/2017 and 2017/2018) and the regional distribution of *L. maculans* races were monitored. *L. maculans* isolates were also obtained from diseased stem samples, the second visible symptom of phoma stem canker disease on oilseed rape, from different field experimental sites for the 2016/2017 cropping season. Thus, in this study, *L. maculans* isolates screened were from different sources. An established cotyledon test method (Balesdent *et al*., 2001) was used to characterise the *L. maculans* isolates obtained from diseased leaves and stems. However, this method is time-consuming and requires much inplanta work. There is a need to develop molecular tools for characterising *L. maculans* other effector genes. Recently, an advanced method of high-resolution melting PCR has been developed (Carpezat *et al*., 2014) to characterise different *avrLm7* profiles.

From the results of this work, seven races were identified in the UK *L. maculans* populations from 2015/2016 cropping season. From the 2016/2017 cropping season, 15 and 19 races were identified in the *L. maculans* isolates from leaves and stem samples, respectively. A total of 17 races were observed in the UK *L. maculans* populations from the 2017/2018 cropping season (Appendices 3-E to 3-H). Interestingly, the race *Av5-6-7* remained the major race for the three cropping seasons.

There were differences in the frequencies of avirulent alleles of different effector genes in *L. maculans* populations from leaf samples (single pycnidial isolates) between sites during three cropping seasons. The results from this study showed that the frequencies of avirulent alleles of *AvrLm6* (97.2%), *AvrLm7* (94.7%) and *AvrLm5* (85.5%) were greater than those of *AvrLm4* (17.7%), *AvrLm1* (5.7%), *AvrLm2* (3.6%), *AvrLm3* (3.2%) and *AvrLm9* (1.2%) effector genes in *L. maculans* populations from leaf samples. Similarly, for *L. maculans* populations from stem samples (single ascospore isolates) from two different sites in 2016/2017, the frequencies of avirulent alleles of *AvrLm5* (100%), *AvrLm6* (94.1%) and *AvrLm7* (75.9%) were greater than those of *AvrLm3* (23.7%), *AvrLm1* (22.7%), *AvrLm9* (16.5%), *AvrLm4* (15.6%), and *AvrLm2* (11.9%) effector genes. From the results of this study, it is evident that the *R* genes that were used on a large scale for many years are likely to lose their efficiency due to the adaptation of the pathogen to selection pressure.

There is an evidence of breakdown of major resistance genes (e.g. *LepR3* in Australia and *Rlm1* in France) (Sprague *et al*., 2006; Rouxel *et al*., 2003), which caused severe losses to farmers. Thus, there is a need to optimise the use of novel sources of resistance so that it does not break down quickly. This project has helped in improving the understanding of factors that affect *L. maculans* population changes from avirulent to virulent. Improved understanding of changes in pathogen populations can enable development of new disease management strategies to deploy cultivars with effective resistance and reduce the risk of resistance breakdown. Using host resistance for effective control of the disease will not only reduce yield losses to address the challenge of food security but also benefit the environment by reducing the use of fungicides.

The results from this study also showed that the frequency of avirulent *Avr* genes has increased in the local populations where the corresponding *R* genes had not been in use for a few years. An example is the increase in the frequency of avirulent *AvrLm9* alleles in the 2017/2018 *L. maculans* populations (Figure 3.17). Hence, for the effective deployment of *R* genes, there is a need to monitor the pathogen races in the local populations. Preventing the use of resistance *R* genes where the frequency of virulent alleles is predominant in the local population helps in preventing the *R* gene from complete breakdown. This saves farmers from severe crop losses and plant breeders from loss of germplasm. From the results of this study, it is evident that there might be changes in the frequencies of *Avr* alleles from one cropping season to the other. Hence continuous monitoring of the frequency of *Avr* alleles guides the effective use of resistant cultivars and this practice should be introduced in the UK oilseed crops, such as UK cereal pathogen virulence survey (UKCPVS) managed by NIAB and funded by AHDB and Defra (https://ahdb.org.uk/ukcpvs).

Schemes to advise farmers on deployment of oilseed rape cultivars with *R* genes where the corresponding avirulent alleles of *L. maculans* effector genes are predominant in the local population by continuous monitoring of the *L. maculans* races in the UK populations should be started. Information about *L. maculans* regional races for the recommended list of cultivars by Agriculture and Horticulture Development Board (AHDB) (AHDB Recommended Lists 2018/2019 for cereals and oilseeds) might be useful for the farmers in the UK. Deployment of such scheme in the UK to provide advice in selecting appropriate cultivars with specific resistance genes to avoid severe disease epidemics due to resistance breakdown is needed. Such schemes are already in use in France (Terres Inovia) (www.terresinovia.fr), in Australia (GRDC) (Grains Research and Development Corporation) (www.grdc.com.au) and Canada (Canola Council of Canada) (https://www.canolacouncil.org).

In this study, in addition to detecting frequency of avirulent alleles of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm5*, *AvrLm6*, *AvrLm7* or *AvrLm9* effector genes at different sites in the UK, the molecular mechanisms of mutations leading towards virulence against resistance genes were also identified. *Rlm1*, *Rlm4* and

Rlm7 are currently and previously used resistance genes in the UK. Monitoring the frequency of avirulent alleles of *AvrLm1*, *AvrLm4*, *AvrLm6* or *AvrLm7* effector genes has provided the information about how efficient these *R* genes are in the UK and guides in crop rotation strategies. Forty isolates virulent against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* were selected for whole genome sequencing to investigate molecular mechanisms of mutations to virulence (Appendix 4-I). Identifying the molecular mechanisms of mutations leading towards virulence against these *R* genes also provides new information about how the pathogen escaped the host recognition.

From the results of this study, it is proved that whole gene deletion is a common molecular mechanism of mutation leading towards virulence against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes. The information about the previously identified molecular mechanism of mutation leading towards virulence against *Rlm4* resistance gene (change of single-base non-synonymous mutation G^{358} -> C^{358} leading to a G^{120} -> R^{120} change) (Parlange *et al*., 2009) was not identified in the UK *L. maculans* populations used in this study. Instead of it, eighteen different SNPs were identified in the *L. maculans* genome sequences containing virulent alleles of *avrLm4* (Table 4.6) (Appendices 4-M & 4-Q). This is an indication of how a pathogen changes from avirulent to virulent under selection.

In agreement with the previous studies, various molecular mechanisms of mutation leading to virulence against the *Rlm7* resistance gene were identified in the UK *L. maculans* populations (Daverdin *et al*., 2012; Carpezat *et al*., 2014) (Figures 4.21 & 4.22; Appendix 4-Q). There is a risk of losing this important resistance gene, since the frequency of the virulent alleles of the *AvrLm7* effector gene increased from 3% to 16.3% (from 2016/2017 to 2017/2018 cropping seasons). *Rlm7* resistance gene is now widely used in the UK oilseed rape commercial cultivars representing >15% of the UK oilseed rape area in 2015/2016 cropping season (Mitrousia *et al*., 2018). This proportion might have increased by now and loss of this effective resistance gene would cause substantial losses to farmers and plant breeders in the UK.

Single-base point mutation and partial deletion were also identified in the *L. maculans* isolates containing virulent alleles of the *AvrLm1* effector gene (Figures

4.10 & 4.11) and no other mutations were identified in *L. maculans* isolates containing virulent alleles of *avrLm6* (Figures 4.14 to 4.20). This new information about the molecular mechanisms of mutation to virulence in the UK *L. maculans* populations can be used to optimise the use of novel resistance so that it does not break down quickly. This can be used to develop new strategies to increase the durability of host resistance against the phoma stem canker pathogen *L. maculans*. The results of this study have justified the high evolutionary potential of *L. maculans* as a pathogen combining a large population size, mixed reproduction regime and high dispersal ability (Daverdin *et al*., 2012). As the location of effector genes of *Leptosphaeria maculans* is in AT-rich, transposable element (TE)-rich blocks of the genome that has major implications for gene mutability, which results in either allelic diversification or gene inactivation under *R* gene selection (Rouxel *et al*., 2011).

The three *L. maculans Avr* genes, *AvrLm1*, *AvrLm6* and *AvrLm4-7*, considered in this study encode Small Secreted Proteins (SSPs) embedded in large TE-rich blocks of the genome termed AT-isochores (Daverdin *et al*., 2012). These three Avr genes are predominantly expressed early in infection (Rouxel *et al*., 2011). Even though, the *Rlm7* resistance gene has been a source of resistance in the UK commercial oilseed rape cultivars for more than 15 years (Huang *et al*., 2018), in a previous study, 3% of isolates were virulent towards *Rlm7* in the 2012/2013 cropping season (Mitrousia *et al*., 2018). In this study, all the isolates from different sites were avirulent against *Rlm7* in the first season. The frequency of isolates virulent towards *Rlm7* was 6.8% in 2016/2017 and increased to 16.3% in 2017/2018 showing that the *Rlm7* resistance gene is at risk. Of single ascospore isolates from the 2016/2017 season, 25% were virulent towards *Rlm7* (Figures 3.17 & 3.18). The results of this study suggest that there is a need to continue monitoring of the *L. maculans* populations in the UK.

It has been proposed in previous studies that combing both *R* genes and QR can provide more durable resistance (Huang *et al*., 2018). This study proposed to combine quantitative resistance with *R* genes that are currently effective, rather than combining it with *R* genes that are no longer effective in the UK to render effective phoma stem canker disease management. This hypothesis was tested

on different *R* genes (*Rlm1*, *Rlm4*, *Rlm7* or *LepR3*) currently used in UK commercial cultivars with different background resistance (QR) in different environments over three growing seasons (2015/2016, 2016/2017 and 2017/2018).

This study has provided evidence that cultivars with *Rlm7* (DK Exalte, DK Extrovert, Whisky, Harper and Amalie) or *LepR3* (Angus) resistance genes showed less phoma leaf spot (Figures 5.7, 5.8; Tables 5.2, 5.3 & 5.4) and phoma stem canker severity (Figure 5.12; Table 5.5) compared to other cultivars with *Rlm1* (DK Cabernet) or *Rlm4* (Adriana) resistance genes and cultivars without any *R* genes (Drakkar, Incentive and Mentor) (Table 2.1). This work also showed that cultivars with QR (Es Astrid, DK Cabernet and Adriana) showed less severe phoma stem canker in summer even if severe phoma leaf spots were observed in the winter of that cropping season (Figures 5.7, 5.8 & 5.12). The results from this study also showed that there were differences in the phoma leaf spot and phoma stem canker severities between different sites and cropping seasons (Figures 5.7, 5.8 & 5.12).

A positive relationship between weather conditions and disease severity in the field experiments was observed. Temperature and rainfall are the two major environmental factors affecting severity of phoma stem canker (Huang *et al*., 2018). Analysis of the relationship between phoma leaf spot severity and weather data among the different sites in the three growing seasons showed that increased severity of phoma leaf spots was associated with increased rainfall and moderate mean temperature in the autumn months, August to November (during the phoma leaf spot development stage) (Table 5.10). This is in agreement with the previous work Huang *et al*. (2018). Analysis of the relationship between severity of phoma stem canker and weather data among the different sites in the three growing seasons showed that increased severity of phoma stem canker was associated with increased temperature in April to June (during the stem canker development stage) (Table 5.11).

The severity of phoma stem canker disease epidemics is influenced by the timing of formation of phoma leaf spot lesions. If the phoma leaf spots are formed on young plants, the pathogen can easily colonise the leaf petioles and stems and

cause severe stem base cankers. *R* gene mediated resistance operates during the penetration of hyphae from the ascospores into the cotyledons and leaves. If the *R* gene in the cultivar is no longer effective, then the pathogen can easily enter the leaves through the stomata.

As high temperature favours the proliferation of the pathogen, it increases the phoma stem canker disease severity. It is important to know the race structure of pathogen populations and deploy *R* genes in cultivars that are effective in the local population. As quantitative resistance can impede the growth of pathogen in leaf petioles and stems (Haung *et al*., 2018), more durable crop resistance can be achieved by combining both QR and *R* genes that are still effective in oilseed rape cultivars. In addition to identifying and breeding for temperature-resilient *R* genes, there is a need to consider cultivars whose genetic background remains effective at increased temperature.

This study has improved our understanding of the effectiveness of *R* gene-mediated resistance in oilseed rape for control of phoma stem canker. Results of this study showed that adaptation to selection has resulted in emergence of virulent *L. maculans* isolates. This study also provided evidence that effective deployment of cultivars with *R* genes should be done in the UK in regions where corresponding avirulent alleles are predominant in the local population for effective phoma stem canker disease management. A scheme to provide information about *L. maculans* pathogen population races should be introduced in the UK. New information about various molecular mechanisms of mutation leading towards virulence towards *R* genes in the UK can be useful in breeding more durable oilseed rape cultivars. Information about the effectiveness of currently used *R* genes can guide the rotation of *R* genes rather than leading to complete resistance breakdown that causes severe losses to farmers and breeders in the UK.

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Appendices

Chapter 2

Appendix 2-A: Layout for winter oilseed rape field experiment plots at the Morley site in the 2015/2016 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Amalie 11 6	DK Cabernet 2 12	Incentive 9 18	Drakkar 1 24	Angus 5 30	Mentor 12 36
Drakkar 1 5	Mentor 12 11	DK Cabernet 2 17	Mentor 12 23	Adriana 6 29	Drakkar 1 35
Angus 8 4	Harper 10 10	Whisky 4 16	Es-Astrid 3 22	DK Exalte 8 28	DK Cabernet 2 34
DK Exalte 5 3	Whisky 4 9	Amalie 11 15	DK Extrovert 7 21	Harper 10 27	Amalie 11 33
Adriana 6 2	DK Extrovert 7 8	Harper 10 14	DK Exalte 8 20	DK Extrovert 7 26	Incentive 9 32
Incentive 9 1	Es-Astrid 3 7	 5 MP	 6 MP	Es-Astrid 3 25	Whisky 4 31

MP- missing plots

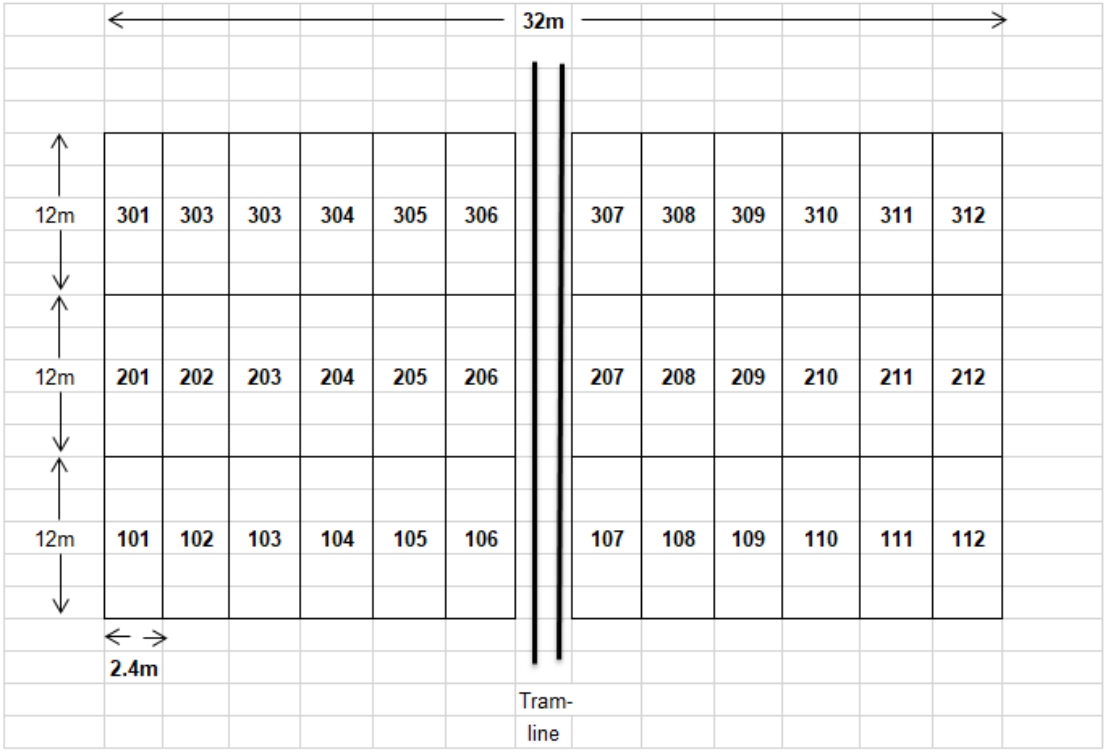
Appendix 2-B: Layout for winter oilseed rape field experiment plots at the Morley site in the 2016/2017 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

		Rep 1			Rep 2			Rep 3	
Block	1	1	1	2	2	2	3	3	3
Plot	10	11	12	22	23	24	34	35	36
Treatment_No	6	11	8	5	4	1	10	12	7
Treatment	**Adriana**	**Amalie**	**DK Exalte**	**Angus**	**Whisky**	**Drakkar**	**Harper**	**Mentor**	**DK Extrovert**
Block	1	1	1	2	2	2	3	3	3
Plot	7	8	9	19	20	21	31	32	33
Treatment_No	9	7	4	2	11	10	6	1	8
Treatment	**Incentive**	**DK Extrovert**	**Whisky**	**DK Cabernet**	**Amalie**	**Harper**	**Adriana**	**Drakkar**	**DK Exalte**
Block	1	1	1	2	2	2	3	3	3
Plot	4	5	6	16	17	18	28	29	30
Treatment_No	5	12	10	7	8	9	3	2	4
Treatment	**Angus**	**Mentor**	**Harper**	**DK Extrovert**	**DK Exalte**	**Incentive**	**Es Astrid**	**DK Cabernet**	**Whisky**
Block	1	1	1	2	2	2	3	3	3
Plot	1	2	3	13	14	15	25	26	27
Treatment_No	3	1	2	3	12	6	9	5	11
Treatment	**Es Astrid**	**Drakkar**	**DK Cabernet**	**Es Astrid**	**Mentor**	**Adriana**	**Incentive**	**Angus**	**Amalie**

Appendix 2-C: Layout for winter oilseed rape field experiment plots at the Morley site in the 2017/2018 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Mentor	**DK Exalte**	**Angus**	**Adriana**	**DK Cabernet**	**Whisky**	**Harper**	**Incentive**	**Drakkar**	**DK Extrovert**	**Es Astrid**	**Amalie**
12	8	5	6	2	4	10	9	1	7	3	11
25	26	27	28	29	30	31	32	33	34	35	36
Adriana	**Incentive**	**Mentor**	**DK Extrovert**	**DK Exalte**	**Angus**	**DK Cabernet**	**Drakkar**	**Harper**	**Es Astrid**	**Amalie**	**Whisky**
6	9	12	7	8	5	2	1	10	3	11	4
13	14	15	16	17	18	19	20	21	22	23	24
Es Astrid	**Adriana**	**Amalie**	**Angus**	**DK Exalte**	**Whisky**	**Incentive**	**DK Cabernet**	**Harper**	**DK Extrovert**	**Drakkar**	**Mentor**
3	6	11	5	8	4	9	2	10	7	1	12
1	2	3	4	5	6	7	8	9	10	11	12

Appendix 2-D: Layout for winter oilseed rape field experiment plots at the Woodhall Farm site for three cropping seasons (2015/2016, 2016/2017 amd 2017/2018). Twelve different cultivars were sown in the field experiment plots in replicates of three.



Appendix 2-E: Field design for winter oilseed rape field experiment plots at the Woodhall Farm site for the 2015/2016 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	101	10	Harper
1	102	11	Amalie
1	103	1	Drakkar
1	104	12	Mentor
1	105	5	Angus
1	106	3	Es Astrid
1	107	7	DK Extrovert
1	108	8	DK Exalte
1	109	9	Incentive
1	110	4	Whisky
1	111	2	DK Cabernet
1	112	6	Adriana
2	201	9	Incentive
2	202	8	DK Exalte
2	203	10	Harper
2	204	11	Amalie
2	205	3	Es Astrid
2	206	5	Angus
2	207	2	DK Cabernet
2	208	1	Drakkar
2	209	7	DK Extrovert
2	210	4	Whisky
2	211	12	Mentor
2	212	6	Adriana
3	301	9	Incentive
3	302	7	DK Extrovert
3	303	1	Drakkar
3	304	6	Adriana
3	305	10	Harper
3	306	5	Angus
3	307	12	Mentor
3	308	8	DK Exalte
3	309	4	Whisky
3	310	2	DK Cabernet
3	311	11	Amalie
3	312	3	Es Astrid

Appendix 2-E: Field design for winter oilseed rape field experiment plots at the Woodhall Farm site for the 2016/2017 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	101	2	DK Cabernet
1	102	8	DK Exalte
1	103	11	Amalie
1	104	12	Mentor
1	105	3	Es Astrid
1	106	10	Harper
1	107	6	Adriana
1	108	5	Angus
1	109	4	Whisky
1	110	7	DK Extrovert
1	111	9	Incentive
1	112	1	Drakkar
2	201	2	DK Cabernet
2	202	9	Incentive
2	203	7	DK Extrovert
2	204	4	Whisky
2	205	6	Adriana
2	206	12	Mentor
2	207	3	Es Astrid
2	208	10	Harper
2	209	8	DK Exalte
2	210	11	Amalie
2	211	1	Drakkar
2	212	5	Angus
3	301	11	Amalie
3	302	9	Incentive
3	303	1	Drakkar
3	304	12	Mentor
3	305	5	Angus
3	306	4	Whisky
3	307	8	DK Exalte
3	308	3	Es Astrid
3	309	7	DK Extrovert
3	310	10	Harper
3	311	2	DK Cabernet
3	312	6	Adriana

Appendix 2-F: Field design for winter oilseed rape field experiment plots at the Woodhall Farm site for the 2017/2018 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	101	2	DK Cabernet
1	102	8	DK Exalte
1	103	11	Amalie
1	104	12	Mentor
1	105	3	Es Astrid
1	106	10	Harper
1	107	6	Adriana
1	108	5	Angus
1	109	4	Whisky
1	110	7	DK Extrovert
1	111	9	Incentive
1	112	1	Drakkar
2	201	2	DK Cabernet
2	202	9	Incentive
2	203	7	DK Extrovert
2	204	4	Whisky
2	205	6	Adriana
2	206	12	Mentor
2	207	3	Es Astrid
2	208	10	Harper
2	209	8	DK Exalte
2	210	11	Amalie
2	211	1	Drakkar
2	212	5	Angus
3	301	11	Amalie
3	302	9	Incentive
3	303	1	Drakkar
3	304	12	Mentor
3	305	5	Angus
3	306	4	Whisky
3	307	8	DK Exalte
3	308	3	Es Astrid
3	309	7	DK Extrovert
3	310	10	Harper
3	11	2	DK Cabernet
3	312	6	Adriana

Appendix 2-G: Field design for winter oilseed rape field experiment plots at the Impington site for the 2015/2016 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	10	Harper
1	2	11	Amalie
1	3	1	Drakkar
1	4	12	Mentor
1	5	5	Angus
1	6	3	Es Astrid
1	7	7	DK Extrovert
1	8	8	DK Exalte
1	9	9	Incentive
1	10	4	Whisky
1	11	2	DK Cabernet
1	12	6	Adriana
2	1	9	Incentive
2	2	8	DK Exalte
2	3	10	Harper
2	4	11	Amalie
2	5	3	Es Astrid
2	6	5	Angus
2	7	2	DK Cabernet
2	8	1	Drakkar
2	9	7	DK Extrovert
2	10	4	Whisky
2	11	12	Mentor
2	12	6	Adriana
3	1	9	Incentive
3	2	7	DK Extrovert
3	3	1	Drakkar
3	4	6	Adriana
3	5	10	Harper
3	6	5	Angus
3	7	12	Mentor
3	8	8	DK Exalte
3	9	4	Whisky
3	10	2	DK Cabernet
3	11	11	Amalie
3	12	3	Es Astrid

Appendix 2-H: Field design for winter oilseed rape field experiment plots at the Trumpington site for the 2015/2016 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	10	Harper
1	2	11	Amalie
1	3	1	Drakkar
1	4	12	Mentor
1	5	5	Angus
1	6	3	Es Astrid
1	7	7	DK Extrovert
1	8	8	DK Exalte
1	9	9	Incentive
1	10	4	Whisky
1	11	2	DK Cabernet
1	12	6	Adriana
2	1	9	Incentive
2	2	8	DK Exalte
2	3	10	Harper
2	4	11	Amalie
2	5	3	Es Astrid
2	6	5	Angus
2	7	2	DK Cabernet
2	8	1	Drakkar
2	9	7	DK Extrovert
2	10	4	Whisky
2	11	12	Mentor
2	12	6	Adriana
3	1	9	Incentive
3	2	7	DK Extrovert
3	3	1	Drakkar
3	4	6	Adriana
3	5	10	Harper
3	6	5	Angus
3	7	12	Mentor
3	8	8	DK Exalte
3	9	4	Whisky
3	10	2	DK Cabernet
3	11	11	Amalie
3	12	3	Es Astrid

Appendix 2-I: Field design for winter oilseed rape field experiment plots at the Wisbech site for the 2016/2017 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	2	DK Cabernet
1	2	7	DK Extrovert
1	3	1	Drakkar
1	4	4	Whisky
1	5	5	Angus
1	6	10	Harper
1	7	6	Adriana
1	8	3	Es Astrid
1	9	11	Amalie
1	10	12	Mentor
1	11	8	DK Exalte
1	12	9	Incentive
2	13	5	Angus
2	14	3	Es Astrid
2	15	7	DK Extrovert
2	16	10	Harper
2	17	2	DK Cabernet
2	18	9	Incentive
2	19	11	Amalie
2	20	8	DK Exalte
2	21	12	Mentor
2	22	6	Adriana
2	23	1	Drakkar
2	24	4	Whisky
3	25	9	Incentive
3	26	11	Amalie
3	27	2	DK Cabernet
3	28	1	Drakkar
3	29	10	Harper
3	30	12	Mentor
3	31	3	Es Astrid
3	32	8	DK Exalte
3	33	7	DK Extrovert
3	34	4	Whisky
3	35	6	Adriana
3	36	5	Angus

Appendix 2-J: Field design for winter oilseed rape field experiment plots at the Wisbech site for the 2017/2018 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	5	Angus
1	2	2	DK Cabernet
1	3	11	Amalie
1	4	6	Adriana
1	5	3	Es Astrid
1	6	10	Harper
1	7	8	DK Exalte
1	8	12	Mentor
1	9	4	Whisky
1	10	9	Incentive
1	11	1	Drakkar
1	12	7	DK Extrovert
2	1	9	Incentive
2	2	6	Adriana
2	3	5	Angus
2	4	12	Mentor
2	5	1	Drakkar
2	6	8	DK Exalte
2	7	11	Amalie
2	8	2	DK Cabernet
2	9	7	DK Extrovert
2	10	10	Harper
2	11	4	Whisky
2	12	3	Es Astrid
3	1	8	DK Exalte
3	2	9	Incentive
3	3	2	DK Cabernet
3	4	6	Adriana
3	5	3	Es Astrid
3	6	11	Amalie
3	7	7	DK Extrovert
3	8	12	Mentor
3	9	5	Angus
3	10	10	Harper
3	11	4	Whisky
3	12	1	Drakkar

Appendix 2-K: Field design for winter oilseed rape field experiment plots at the Rothwell site for the 2016/2017 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	12	Mentor
1	2	8	DK Exalte
1	3	11	Amalie
1	4	3	Es Astrid
1	5	9	Incentive
1	6	5	Angus
1	7	6	Adriana
1	8	2	DK Cabernet
1	9	10	Harper
1	10	4	Whisky
1	11	1	Drakkar
1	12	7	DK Extrovert
2	13	9	Incentive
2	14	11	Amalie
2	15	7	DK Extrovert
2	16	2	DK Cabernet
2	17	12	Mentor
2	18	8	DK Exalte
2	19	3	Es Astrid
2	20	5	Angus
2	21	6	Adriana
2	22	4	Whisky
2	23	10	Harper
2	24	1	Drakkar
3	25	4	Whisky
3	26	7	DK Extrovert
3	27	11	Amalie
3	28	1	Drakkar
3	29	12	Mentor
3	30	10	Harper
3	31	6	Adriana
3	32	5	Angus
3	33	9	Incentive
3	34	8	DK Exalte
3	35	2	DK Cabernet
3	36	3	Es Astrid

Appendix 2-L: Field design for winter oilseed rape field experiment plots at the Rothwell site for the 2017/2018 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	1	Drakkar
1	2	5	Angus
1	3	9	Incentive
1	4	11	Amalie
1	5	4	Whisky
1	6	3	Es Astrid
1	7	2	DK Cabernet
1	8	10	Harper
1	9	8	DK Exalte
1	10	6	Adriana
1	11	7	DK Extrovert
1	12	12	Mentor
2	1	4	Whisky
2	2	8	DK Exalte
2	3	7	DK Extrovert
2	4	11	Amalie
2	5	1	Drakkar
2	6	3	Es Astrid
2	7	2	DK Cabernet
2	8	9	Incentive
2	9	10	Harper
2	10	12	Mentor
2	11	5	Angus
2	12	6	Adriana
3	1	9	Incentive
3	2	11	Amalie
3	3	10	Harper
3	4	5	Angus
3	5	1	Drakkar
3	6	8	DK Exalte
3	7	4	Whisky
3	8	6	Adriana
3	9	12	Mentor
3	10	7	DK Extrovert
3	11	3	Es Astrid
3	12	2	DK Cabernet

Appendix 2-M: Field design for winter oilseed rape field experiment plots at the Risby site for the 2017/2018 cropping season. Twelve different cultivars were sown in the field experiment plots in replicates of three.

Block	Plot	Treatment_No	Cultivar
1	1	3	Es Astrid
1	2	8	DK Exalte
1	3	12	Mentor
1	4	1	Drakkar
1	5	9	Incentive
1	6	11	Amalie
1	7	5	Angus
1	8	10	Harper
1	9	6	Adriana
1	10	4	Whisky
1	11	2	DK Cabernet
1	12	7	DK Extrovert
2	1	12	Mentor
2	2	10	Harper
2	3	9	Incentive
2	4	8	DK Exalte
2	5	1	Drakkar
2	6	3	Es Astrid
2	7	2	DK Cabernet
2	8	7	DK Extrovert
2	9	11	Amalie
2	10	4	Whisky
2	11	5	Angus
2	12	6	Adriana
3	1	5	Angus
3	2	1	Drakkar
3	3	3	Es Astrid
3	4	8	DK Exalte
3	5	6	Adriana
3	6	4	Whisky
3	7	11	Amalie
3	8	2	DK Cabernet
3	9	10	Harper
3	10	9	Incentive
3	11	7	DK Extrovert
3	12	12	Mentor

Appendix 2-N: The latitude and longitude for Burkard spore sampler sites and field experiment sites and the associated weather stations over three growing seasons (2015/2016, 2016/2017 and 2017/2018).

Field experiment			Weather		
Site	**Latitude (º)**	**Longitude (º)**	**Station**	**Latitude (º)**	**Longitude (º)**
Rothwell, UK	53.7555 N	1.4735 W	Market Stainton	53.3995 N	-0.2574 W
Impington, UK	52.2513 N	0.1185 E	Wilbraham	52.1852 N	0.3013 W
Eye, UK	52.3196 N	1.1462 E	Diss	52.2895 N	1.1251 E
Bayfordbury, UK	51.7784 N	0.1033 W	Rothamsted	51.8066 N	-0.3602 E
Woodhall Farm, UK	51.7798 N	0.2110 W	Rothamsted	51.8066 N	-0.3602 E
Wisbech, UK	52.6663 N	0.1588 E	Ely	52.4428 N	0.2993 E
Trumpington, UK	52.1737 N	0.1103 E	Wilbraham	52.1852 N	0.3013 W
Morley, UK	53.7445 N	1.5980 W	Morley	52.5469 N	1.0258 W
Risby, UK	52.2668 N	0.6312 E	Framlingham	52.2605 N	1.2707 E

See Figure 2.1 for map

Chapter 3

Appendix 3-A: Pseudothecial density (no./cm^2) on stem base (SB) and upper stem (US) pieces from different cultivars from the Morley site in the 2016/2017 cropping season.

S. No	Cultivar	SB density (pseudothecia/cm^2)	US density (pseudothecia/cm^2)
1	DK Extrovert	4.91	36.83
2	Whisky	12.17	15.85
3	Harper	10.16	19.41
4	DK Exalte	17.55	36.08
5	Amalie	26.8	37.6
6	Angus	39.46	44.26
7	Drakkar	33.02	43.02
8	Incentive	99.19	13.27

*Statistical analysis for pseudothecial density is shown in Table 3.8.

Appendix 3-B: Pseudothecial density (no./cm^2) on stem base (SB) and upper stem (US) pieces from different cultivars from the Wisbech site in the 2016/2017 cropping season.

S. No	Cultivar	SB density (pseudothecia/cm^2)	US density (pseudothecia/cm^2)
1	DK Extrovert	0.12	34.15
2	Whisky	34.46	17.23
3	Harper	22.66	56.07
4	DK Exalte	26.46	9.48

*Statistical analysis for pseudothecial density is shown in Table 3.9.

Appendix 3-C: Percentages of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* found amongst single ascospore isolates from upper stem (US) or stem base lesions (SB) from different cultivars at the Morley site in 2016/2017 cropping season.

Cultivar	% *Lm* obtained	% *Lb* obtained
DK Exalte-US	66.67 (14)	33.33 (7)
DK Exalte-SB	66.67 (12)	33.33 (6)
Angus- US	72.73 (16)	27.27 (6)
Angus-SB	30 (6)	70 (14)
DK Extrovert- US	24 (6)	76 (19)
DK Extrovert-SB	8.33 (1)	91.67 (11)
Harper- US	35 (7)	65 (13)
Harper-SB	0 (0)	0 (0)
Amalie- US	100 (20)	0 (0)
Amalie-SB	90.48 (19)	9.52 (2)
Whisky- US	28.57 (14)	76.19 (7)
Whisky-SB	0 (0)	100 (21)
Drakkar- US	100 (20)	0 (0)
Drakkar-SB	100 (20)	0 (0)
Incentive- US	5.26 (1)	94.74 (18)
Incentive-SB	66.67 (14)	33.33 (7)

*The values in the brackets ( ) are the numbers of *L. maculans* and *L. biglobosa* isolates obtained. Statistical analysis of the data is shown in Table 3.10.

Appendix 3-D: Percentages of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* found amongst single ascospore isolates from upper stem (US) and stem base lesions (SB) from different cultivars at the Wisbech site in 2016/2017 cropping season.

Cultivar	% *Lm* obtained	% *Lb* obtained
Whisky- US	27.27 (6)	72.73 (14)
Whisky-SB	63.64 (14)	36.36 (7)
Harper- US	33.33 (6)	66.67 (12)
Harper-SB	95 (19)	5 (1)
DK Exalte- US	100 (21)	0 (0)
DK Exalte-SB	36.84 (7)	63.16 (12)
DK Extrovert- US	100 (20)	0 (0)
DK Extrovert-SB	0 (0)	0 (0)

*The values in the brackets ( ) are the numbers of *L. maculans* and *L. biglobosa* isolates obtained. Statistical analysis of the data is shown in Table 3.11.

Appendix 3-E: Details of the isolates from 2015/2016 cropping season tested on cotyledons of a differential set of cultivars.

S. No	Name of the isolate	Cultivar	Site	Race	
1	WH15 Ama-1-1	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
2	WH15 Ama-1-3	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
3	WH15 Ama-1-4	Amalie	Woodhall Farm	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
4	WH15 Ama-1-9	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
5	WH15 Ama-1-10	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
6	WH15 Ama-1-11	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
7	WH15 Ama-1-12	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
8	WH15 Ama-2-1	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
9	WH15 Ama-2-2	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
10	WH15 Ama-2-3	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
11	WH15 Ama-2-13	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
12	WH15 Ama-2-14	Amalie	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
13	WH15 Har-10	Harper	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
14	WH15 Har-15	Harper	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
15	WH15 DK-Ext-1-11	DK Extrovert	Woodhall Farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
16	WH15 DK-Ext-2-16	DK Extrovert	Woodhall Farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
17	WH15 Dr-1-3	Drakkar	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
18	WH15 Dr-2-1	Drakkar	Woodhall Farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
19	WH15 Dr-2-2	Drakkar	Woodhall Farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
20	WH15 Dr-2-3	Drakkar	Woodhall Farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
21	WH15 Dr-2-8	Drakkar	Woodhall Farm	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
22	WH15 Dr-3-8	Drakkar	Woodhall Farm	*AvrLm 4-6-7*	*avrLm 1-2-3-5-9*
23	Morley15 Ama-1	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
24	Morley15 Ama-2	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
25	Morley15 Ama-5	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
26	Morley15 Ama-6	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
27	Morley15 Ama-7	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
28	Morley15 Ama-8	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
29	Morley15 Ama-9	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
30	Morley15 Ama-10	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
31	Morley15 Ama-11	Amalie	Morley	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*
32	Morley15 Ama-12	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
33	Morley15 Ama-14	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
34	Morley15 Ama-15	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
35	Morley15 Ama-17	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
36	Morley15 Ama-18	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
37	Morley15 Ama-19	Amalie	Morley	*AvrLm 1-2-3-4-5-6-7-9*	-
38	Nf15 Dr-1-3	Drakkar	Morley	*AvrLm 4-6-7*	*avrLm 1-2-3-5-9*
39	Nf15 Dr-2-2	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
40	Nf15 Dr-3-5	Drakkar	Morley	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*

S. No	Name of the isolate	Cultivar	Site	Race	
41	Nf15 Dr-3-7	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
42	NF15 Dr-2-6	Drakkar	Morley	*AvrLm 4-6-7*	*avrLm 1-2-3-5-9*
43	NF15 Dr-3-6	Drakkar	Morley	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
44	Imp15 DK-Exl-3	DK Exalte	Impington	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
45	Imp15 Har-3	Harper	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
46	Imp15 Har-5	Harper	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
47	Imp15 Har-6	Harper	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
48	Imp15 Har-7	Harper	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
49	Imp15 Dr-2-4	Drakkar	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
50	Imp15 Dr-2-9	Drakkar	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
51	Imp15 Dr-3-15	Drakkar	Impington	*AvrLm 1-5-6-7*	*avrLm 2-3-4-9*
52	Imp15 Dr-3-21	Drakkar	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
53	Imp15 Dr-1-7	Drakkar	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
54	Imp15 Dr-2-8	Drakkar	Impington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
55	HLH15 Dr-5	Drakkar	West Farm Barns	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
56	HLH15 Dr-7	Drakkar	West Farm Barns	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
57	HLH15 Dr-10	Drakkar	West Farm Barns	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
58	HLH15 Dr-11	Drakkar	West Farm Barns	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
59	MS15 Dr-1-5	Drakkar	Trumpington	*AvrLm 1-5-6-7*	*avrLm 2-3-4-9*
60	MS15 Dr-1-6	Drakkar	Trumpington	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
61	MS15 Dr-1-10	Drakkar	Trumpington	*AvrLm 2-4-5-6-7*	*avrLm 1-3-9*
62	MS15 Dr-1-12	Drakkar	Trumpington	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
63	MS15 Dr-2-1	Drakkar	Trumpington	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
64	MS15 Dr-2-9	Drakkar	Trumpington	*AvrLm 4-6-7*	*avrLm 1-2-3-5-9*

Appendix 3-F: Details of the isolates from 2016/2017 cropping season tested on cotyledons of a differential set of cultivars.

S. No	Name of the isolate	Cultivar	Site	Race	
1	Sf16 Why-8	Whisky	Morley	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
2	Sf16 Dr-2-4	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
3	Sf16 Dr-2-14	Drakkar	Morley	*AvrLm 1-5-6-7*	*avrLm 2-3-4-9*
4	Sf16 Dkc-12	DK Cabernet	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
5	Sf16 Dkc-11	DK Cabernet	Morley	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
6	Sf16 Dr-1-3	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
7	Sf16 Dr-2-7	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
8	Sf16 Dr-1-8	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
9	Sf16 Dr-2-11	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
10	Sf16 Dr-2-6	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
11	Sf16 Why-11	Whisky	Morley	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
12	Sf16 Ama-9	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
13	Sf16 Ama-11	Amalie	Morley	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
14	Sf16 Why-3	Whisky	Morley	*AvrLm 1-3-6*	*avrLm 2-4-5-7-9*
15	Sf16 Why-5	Whisky	Morley	*AvrLm 1-6*	*avrLm 2-3-4-5-7-9*
16	Sf16 Ama-2	Amalie	Morley	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
17	Sf16 Ama-4	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
18	Sf16 Ama-8	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
19	Sf16 Dk-Exl-8	DK Exalte	Morley	*AvrLm 7*	*avrLm 1-2-3-4-5-6-9*
20	Sf16 Dk-Exl-3	DK Exalte	Morley	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
21	SF16 Why-2	Whisky	Morley	*AvrLm 6*	*avrLm1-2-3-4-5-7-9*
22	Sf16 Dk-Ext-4	DK Extrovert	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
23	Sf16 Dr-1-1	Drakkar	Morley	*AvrLm 5-6-7-9*	*avrLm 1-2-3-4*
24	Sf16 Dr-2-9	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
25	Sf16 Dr-1-5	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
26	Sf16 Dk-Exl-3	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
27	Sf16 Dk-Exl-11	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
28	Sf16 Dk-Ext-6	DK Extrovert	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
29	Sf16 Ang-6	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
30	Sf16 Dk-Exl-1	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
31	Sf16 Ang-9	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
32	Sf16 Dk-Ext-7	DK Extrovert	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
33	Sf16 Ang-8	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
34	Sf16 Dk-Exl-6	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm1-2-3-4-9*
35	Sf16 Dkc-3	DK Cabernet	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
36	Sf16 Dk-Ext-2	DK Extrovert	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
37	Sf16 Ang-10	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
38	Sf16 Dk-Ext-11	DK Extrovert	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*

S. No	Name of the isolate	Cultivar	Site	Race	
39	Imp16 Dr-2-5	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
40	Imp16 Dr-2-9	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm1-2-3-4-9*
41	Imp16 Dr-2-11	Drakkar	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
42	Imp16 Dr-1-7	Drakkar	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
43	Imp16 Dr-1-1	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
44	Imp16 Dk-Ext-4	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
45	Imp16 Ang-8	Angus	Wisbech	*AvrLm 7*	*avrLm 1-2-3-4-5-6-9*
46	Imp16 Dr-1-9	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
47	Imp16 Dr-1-15	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
48	Imp16 Ang-5	Angus	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
49	Imp16 Dr-1-2	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
50	Imp16 Dr-1-10	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
51	Imp16 Ang-11	Angus	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
52	Imp16 Ang-1	Angus	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
53	Imp16 Dk-Ext-2	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
54	Imp16 Dk-Ext-9	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
55	Imp16 Dr-2-4	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
56	Imp16 Dr-2-2	Drakkar	Wisbech	*AvrLm 2-6-7*	*avrLm 1-3-4-5-9*
57	Imp16 Dk-Ext-5	DK Extrovert	Wisbech	*AvrLm 4-6-7*	*avrLm 1-2-3-5-9*
58	Imp16 Ang-10	Angus	Wisbech	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*
59	Imp16 Har-3	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
60	Imp16 Dr-2-8	Drakkar	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
61	Imp16 Dk-Ext-11	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
62	Imp16 Ama-1	Amalie	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
63	Imp16 Ama-3	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
64	Imp16 Ama-5	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
65	Imp16 DK-Exl-2	DK Exalte	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
66	Imp16 DK-Exl-1-3	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
67	Imp16 Har-5	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
68	Imp16 Ama-5	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
69	Imp16 Why-1	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
70	Imp16 Why-2	Whisky	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
71	Imp16 Why-6	Whisky	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
72	Imp16 Why-8	Whisky	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
73	Imp16 Why-12	Whisky	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
74	Imp16 Ama-8	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
75	Imp16 Har-7	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
76	Imp Dk-Exl-10	DK Exalte	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
77	Imp16 Dr-Exl-8	DK Exalte	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
78	Imp16 Dk-Exl-16	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
79	Imp16 Ama-11	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*

S. No	Name of the isolate	Cultivar	Site	Race	
80	Imp16 Har-2	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
81	WH16 Dk-Ext-6	DK Extrovert	Woodhall farm	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
82	WH16 Dk-Ext-8	DK Extrovert	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
83	WH16 DK-Ext-1	DK Extrovert	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
84	WH16 Dk-Ext-2	DK Extrovert	Woodhall farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
85	WB16 Ama-5	Amalie	Wisbech	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
86	WB16 Ama-9	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
87	WB16 Ama-1	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
88	WB16 Ama-11	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*

Appendix 3-G: Details of the isolates from 2017/2018 cropping season tested on cotyledons of a differential set of cultivars.

S. No	Name of the isolate	Cultivar	Site	Race	
1	GS17 Why-4	Whisky	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
2	GS17 Har-2	Harper	Morley	*AvrLm 1-4-5-6-7*	*avrLm 2-3-9*
3	GS17 DK-Ext-1	DK Exalte	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
4	GS17 Ama-1	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
5	GS17 Har-3	Harper	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
6	GS17 DK-Exl-3	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
7	GS17 Ama-3	Amalie	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
8	GS17 Why-1	Whisky	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
9	GS17 DK-Exl-2	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
10	GS17 Men-3	Mentor	Morley	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*
11	GS17 DK-Ext-2	DK Extrovert	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
12	GS17 Men-6	Mentor	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
13	GS17 Dr-1-2	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
14	GS17 Dr-3-6	Drakkar	Morley	*AvrLm 4-5-7*	*avrLm 1-2-3-6-9*
15	GS17 Dr-2-7	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
16	GS17 Dr-1-5	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
17	GS17 Dr-2-2	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
18	GS17 Dr-3-4	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
19	GS17 Dr-1-8	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
20	GS17 Dr-2-6	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
21	GS17 Dr-2-3	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
22	GS17 Dr-3-2	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
23	GS17 Dr-3-7	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
24	WH17 Dr-2-2	Drakkar	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
25	WH17 Ang-5	Angus	Woodhall farm	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
26	WH17 Dr-1-1	Drakkar	Woodhall farm	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
27	WH17 Ama-5	Amalie	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
28	WH17 Dr-2-1	Drakkar	Woodhall farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
29	WH17 Ama-3	Amalie	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
30	WH17 Dr-2-6	Drakkar	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
31	WH17 Ang-2	Angus	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
32	WH17 Dr-1-6	Drakkar	Woodhall farm	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
33	WH17 Ang-4	Angus	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
34	WH17 Dr-1-7	Drakkar	Woodhall farm	*AvrLm 6-7*	*avrLm 1-2-3-4-5-9*
35	WH17 Ang-7	Angus	Woodhall farm	-	*avrLm 1-2-3-4-5-6-7-9*
36	WH17 Dr-1-4	Drakkar	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
37	WH17 Dr-3-7	Drakkar	Woodhall farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
38	WH17 Dr-2-8	Drakkar	Woodhall farm	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
39	WH17 Dr-3-1	Drakkar	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
40	WH17 Dr-3-3	Drakkar	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*

S. No	Name of the isolate	Cultivar	Site	Race	
41	WH17 Har 5	Harper	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
42	WH17 Dr-3-5	Drakkar	Woodhall farm	*AvrLm 3-5-6-7-9*	*avrLm 1-2-4*
43	WH17 Why-6	Whisky	Woodhall farm	*AvrLm 1-5-6-7*	*avrLm 2-3-4-9*
44	WH17 DK-Exl-1	DK Exalte	Woodhall farm	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
45	WH17 DK-Ext-2	DK Extrovert	Woodhall farm	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
46	WH17 Ama-2	Amalie	Woodhall farm	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
47	WH17 DK-Exl-2	DK-Exalte	Woodhall farm	*AvrLm 9*	*avrLm 1-2-3-4-5-6-7*
48	WH17 Har-2	Harper	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
49	WH17 DK-Ext-5	DK Extrovert	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
50	WH17 Ama-1	Amalie	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
51	WH17 Why-3	Whisky	Woodhall farm	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
52	WH17 Why-5	Whisky	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
53	WH17 Har-7	Harper	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
54	WH17 DK-Ext-6	DK Extrovert	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
55	WH17 DK-Exl-3	DK Exalte	Woodhall farm	*AvrLm 4-5-7*	*avrLm 1-2-3-6-9*
56	WH17 Har-1	Harper	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
57	WH17 Why-1	Whisky	Woodhall farm	*AvrLm 1-6*	*avrLm 2-3-4-5-7-9*
58	WH17 DK-Exl-8	DK Exalte	Woodhall farm	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
59	WB17 Dr-1-1	Drakkar	Wisbech	*AvrLm 1-2-3-4-5-6-7-9*	-
60	WB17 Ang-6	Angus	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
61	WB17 Dr-2-8	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
62	WB17 DK-Ext-7	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
63	WB17 Dr-2-4	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
64	WB17 Ang-8	Angus	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
65	WB17 DK-Ext-6	DK Extrovert	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
66	WB17 Dr-3-5	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
67	WB17 Dr-3-1	Drakkar	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
68	WB17 DK-Ext-1	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
69	WB17 Dr-3-6	Drakkar	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
70	WB17 DK-Ext-5	DK Extrovert	Wisbech	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
71	WB17 Dr-1-8	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
72	WB17 Dr-1-5	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
73	WB17 Dr-2-1	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
74	WB17 Dr-3-3	Drakkar	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
75	WB17 Dr-1-4	Drakkar	Wisbech	*AvrLm 1-2-4-5-6-7-9*	*avrLm 3*
76	WB17 Ama-6	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
77	WB17 Dr-2-6	Drakkar	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
78	WB17 Har-3	Harper	Wisbech	*AvrLm 2-5-6-7*	*avrLm1-3-4-9*
79	WB17 Ang-1	Angus	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
80	WB17 Why-6	Whisky	Wisbech	*AvrLm 1-2-3-5-6*	*avrLm 4-7-9*
81	WB17 DK-Exl-8	DK Exalte	Wisbech	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
82	WB17 Ama-4	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
83	WB17 DK-Exl-1	DK Exalte	Wisbech	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*

S. No	Name of the isolate	Cultivar	Site	Race	
84	WB17 Ama-8	Amalie	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
85	WB17 Why-1	Whisky	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
86	WB17 DK-Exl-4	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
87	WB17 Ang-2	Angus	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
88	WB17 Why-8	Whisky	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
89	WB17 Har-4	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
90	WB17 Ama-3	Amalie	Wisbech	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*
91	WB17 Why-7	Whisky	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
92	WB17 Har-1	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
93	WB17 Har-6	Harper	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
94	WB17 DK-Exl-5	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
95	RT17 Dr-1-8	Drakkar	Rothwell	*AvrLm 1-2-3-4-5-6-7-9*	-
96	RT17 Dr-3-8	Drakkar	Rothwell	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
97	RT17 Dr-2-8	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
98	RT17 Dr-2-5	Drakkar	Rothwell	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
99	RT17 Dr-1-7	Drakkar	Rothwell	*AvrLm 4-5-7*	*avrLm 1-2-3-6-9*
100	RT17 Dr-1-1	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
101	RT17 Dr-3-5	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
102	RT17 Dr-1-3	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
103	RT17 Dr-2-1	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
104	RT17 Dr-2-3	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
105	RT17 Dr-1-5	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
106	RT17 Dr-2-2	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
107	RT17 Dr-3-3	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
108	RT17 Dr-3-1	Drakkar	Rothwell	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
109	RT17 Dr-2-6	Drakkar	Rothwell	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*

Appendix 3-H: Details of the isolates from stem samples from 2016/2017 cropping season tested on cotyledons of a differential set of cultivars.

S. No	Name of the isolate	Cultivar	Site	Race	
1	Mrly16 Why-Up-2-4	Whisky	Morley	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
2	Mrly16 Why-Up-3-3	Whisky	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
3	Mrly16 Ama-Up-3-2	Amaile	Morley	*AvrLm 5-6*	*avrlm 1-2-3-4-7-9*
4	Mrly16 Why-Up-2-3	Whisky	Morley	*AvrLm 1-5-6-9*	*avrLm 2-3-4-7*
5	Mrly16 Why-Up-2-8	Whisky	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
6	Mrly16 Why-Up-3-2	Whisky	Morley	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
7	Mrly16 Har-Up-1-1	Harper	Morley	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
8	Mrly Why-Up-2-7	Whisky	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
9	Mrly Ama-Up-2-1	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
10	Mrly Why-Up-3-7	Whisky	Morley	*AvrLm 5*	*avrLm 1-2-3-4-6-7-9*
11	Mrly16 Har-Up-3-2	Harper	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
12	Mrly16 Why-Up-3-1	Whisky	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
13	Mrly16 Ama-Up-3-1	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
14	Mrly16 Har-Up-2-6	Harper	Morley	*AvrLm 1-2-3-4-5-6-7-9*	-
15	Mrly16 Har-Up-2-7	Harper	Morley	*AvrLm 1-2-3-4-5-6-7-9*	-
16	Mrly16 Why-Up-2-2	Whisky	Morley	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
17	Mrly16 Har-Up-1-4	Harper	Morley	*AvrLm 2-5*	*avrLm 1-3-4-6-7-9*
18	Mrly16 DK-Exl-Sb-3-5	DK Exalte	Morley	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
19	Mrly16 Ang-Sb-3-5	Angus	Morley	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
20	Mrly16 Ama-Sb-3-5	Amaile	Morley	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
21	Mrly16 Ama-Sb-3-1	Amaile	Morley	*AvrLm 1-5-6-7*	*avrLm 2-3-4-9*
22	Mrly16 Har-Up-3-3	Harper	Morley	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
23	Mrly16 DK-Exl-Up-3-7	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
24	Mrly16 Ama-Sb-2-5	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
25	Mrly16 DK-Exl-Up-2-2	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
26	Mrly16 Ang-Up-1-5	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
27	Mrly16 DK-Exl-Up-3-6	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
28	Mrly16 Ama-Up-2-2	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
29	Mrly16 Ang-Sb-3-2	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
30	Mrly16 DK-Exl-Sb-1-2	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
31	Mrly16 Ang-Up-3-4	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
32	Mrly16 Har-Up-1-8	Harper	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
33	Mrly16 DK-Exl-Sb-3-2	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
34	Mrly16 Ang-Up-3-2	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
35	Mrly16 Ama-Sb-1-2	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
36	Mrly16 Ang-Up-1-1	Angus	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
37	Mrly16 DK-Ext-Up-2-5	DK Extrovert	Morley	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
38	Mrly16 Har-Up-1-9	Harper	Morley	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
39	Mrly16 DK-Exl-Up-2-1	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
40	Mrly16 Ama-Sb-1-7	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*

S. No	Name of the isolate	Cultivar	Site	Race	
41	Mrly16 Dk-Exl-Sb-3-5	DK Exalte	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
42	Mrly16 Dr-Up-2-1	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
43	Mrly Inc-Up-3-1	Incentive	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
44	Mrly16 Inc-Sb-1-6	Incentive	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
45	Mrly16 Dr-Sb-3-4	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
46	Mrly16 Dr-Sb-3-5	Drakkar	Morley	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
47	Mrly 16 Dr-Up-3-1	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
48	Mrly16 Dr-Up-1-5	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
49	Mrly16 Inc-Sb-1-3	Incentive	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
50	Mrly16 Dr-Sb-2-1	Drakkar	Morley	*AvrLm 4-5-6-7-9*	*avrLm 1-2-3*
51	Mrly16 Inc-Sb-3-6	Incentive	Morley	*AvrLm 3-5-6-7-9*	*avrLm 1-2-4*
52	Mrly16 Why-Up-3-6	Whisky	Morley	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
53	Mrly16 Dr-Up-2-3	Drakkar	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
54	Mrly16 Inc-Sb-3-1	Incentive	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
55	Mrly16 Ama-Up-1-7	Amaile	Morley	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
56	Mrly16 Dr-Sb-2-7	Drakkar	Morley	*AvrLm 1-2-3-5-6-7-9*	*avrLm 4*
57	LSPB16 Har-Up-1-2	Harper	Wisbech	*AvrLm 5-7*	*avrLm 1-2-3-4-6-9*
58	LSPB16 Why-Up-1-3	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
59	LSPB16 Why-Up-1-2	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
60	LSPB16 Why-Sb-2-5	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
61	LSPB16 Why-Sb-3-1	Whisky	Wisbech	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
62	LSPB16 Why-Sb-3-7	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
63	LSPB16 Why-Sb-2-2	Whisky	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
64	LSPB16 Har-Sb-1-3	Harper	Wisbech	*AvrLm 5-6*	*avrLm 1-2-3-4-7-9*
65	LSPB16 DK-Ext-Up-2-4	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
66	LSPB16 Har-Up-1-7	Harper	Wisbech	*AvrLm 2-5-6-7*	*avrLm 1-3-4-9*
67	LSPB16 DK-Exl-Sb-1-7	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
68	LSPB16 Har-Up-1-5	Harper	Wisbech	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*
69	LSPB16 DK-Ext-Up-3-6	DK Extrovert	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
70	LSPB16 DK-Exl-Up-1-1	DK Exalte	Wisbech	*AvrLm 3-4-5-6-7-9*	*avrLm 1-2*
71	LSPB16 Har-Up-1-1	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
72	LSPB Dk-Exl-Up-3-3	DK Exalte	Wisbech	*AvrLm 1-2-3-4-5-6-7-9*	-
73	LSPB16 DK-Ext-Up-2-5	DK Extrovert	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
74	LSPB16 Har-Sb-2-1	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
75	LSPB16 Har-Sb-3-3	Harper	Wisbech	*AvrLm 1-3-5-6*	*avrLm 2-4-7-9*
76	LSPB16 DK-Exl-Up-2-3	DK Exalte	Wisbech	*AvrLm 1-3-5-6-7-9*	*avrLm 2-4*
77	LSPB16 DKExt-Up-1-5	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
78	LSPB16 Har-Sb-2-4	Harper	Wisbech	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
79	LSPB16 DK-Exl-Up-2-7	DK Exalte	Wisbech	*AvrLm 1-2-3-4-5-6-7-9*	-
80	LSPB16 DK-Ext-Up-3-5	DK Extrovert	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
81	LSPB16 DK-Ext-Up-1-6	DK Extrovert	Wisbech	*AvrLm 4-5-6-7*	*avrLm 1-2-3-9*
82	LSPB16 DK-Exl-Up-3-4	DK Exalte	Wisbech	*AvrLm 2-3-5-6-7-9*	*avrLm 1-4*
83	LSPB16 Har-Up-1-4	Harper	Wisbech	*AvrLm 3-5-6*	*avrLm 1-2-4-7-9*

S. No	Name of the isolate	Cultivar	Site	Race	
84	LSPB16 DK-Exl-Up-1-3	DK Exalte	Wisbech	*AvrLm 4-5-6-7-9*	*avrLm 1-2-3*
85	LSPB16 Har-Sb-1-4	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
86	LSPB16 Dk-Exl-Sb-1-3	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
87	LSPB16 Har-Up-1-6	Harper	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
88	LSPB16 DK-Exl-Sb-1-5	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
89	LSPB16 DK-Exl-Sb-1-2	DK Exalte	Wisbech	*AvrLm 1-2-3-4-5-6-7-9*	-
90	LSPB16 DK-Exl-Sb-2-3	DK Exalte	Wisbech	*AvrLm 5-6-7*	*avrLm 1-2-3-4-9*
91	LSPB16 Har-Sb-3-7	Harper	Wisbech	*AvrLm 1-5-6*	*avrLm 2-3-4-7-9*
92	LSPB16 DK-Exl-Sb-1-1	DK Exalte	Wisbech	*AvrLm 1-2-3-5-6-7-9*	*avrLm 4*

Chapter 4

Appendix 4-A: Primers and PCR conditions used in this study (Gout *et al*., 2006; Fudal *et al*., 2009 and Daverdin *et al*., 2012).

Markers	Primers (5'-3')		Hybridization temperature (°C)	Elongation time (s)
	Forward (F)	Reverse (R)		
AvrLm4-7 amplification and sequencing				
AvrLm4-7ext-F & -R	TATCGCATACCAAACATTAGGC	GATGGATCAACCGCTAACAA	60	90
AvrLm4-7Int-F & -R	ATATCTGGAGAAATTCGCTATC	CCAAGGGTCGGTAGTTATGC	60	30
AvrLm4-7ext-F2	ATTTGCTACACTAGATTATAC	-	60	-
AvrLm4-7ext-F3	AACCCTGCTAGATAGGTAAGCT	-	60	-
AvrLm6 amplification and sequencing				
A6OrfF	TCAATTTGTCTGTTCAAGTTATGGA	-	58	60
A6OrfR	-	CCAGTTTTGAACCGTAGAGGTAGCA	58	60
AvrLm1 amplification and sequencing				
Solo-ORFcU1	CTATTTAGGCTAAGCGTATTCATAAG	-	54	60
Solo-ORFcL1	-	GCGCTGTAGGCTTCATTGTAC	54	60
AvrLm1 IntU	TAGCTCCCCAGCTACCAAGA	-	60	60
AvrLm1 IntF	-	ACGTTGTAATTGAGCGGAACC	60	60

Appendix 4-B: Details of *Leptosphaeria maculans* isolates that have avirulent and virulent alleles against *Rlm1*, amplified using Solo-ORFcU1/L1 primers (Figure 4.5) and AvrLm1IntU/F primers (Figure 4.6).

Sample ID	Isolate	Cultivar	Site
1	JN3	In vitro cross isolate from France, INRA	N/A
2	AU74	No information	Isolate from Australia, Victoria, in 1988
3	AU75	No information	Isolate from Western Australia in 1987
4	ME24	Apex	Darrington, West Yorkshire, in 2002
5	*AvrLm1* +ve control (V23.11.9)	In vitro cross isolate from France, INRA	N/A
6	*avrLm1* +ve control (V23.2.1)	In vitro cross isolate from France, INRA	N/A
7	-ve control (H2O)	N/A	N/A
8	Imp16 Har-5	Harper	Impington
9	Imp16 Ama-5	Amalie	Impington
10	Imp16 Ang-1	Angus	Impington
11	Sf16 DK-Exl-8	DK-Exalte	Morley
12	Sf16 Dr-1-5	Drakkar	Morley
13	Sf16 Ang-6	Angus	Morley
14	Imp16 Dr-2-5	Drakkar	Impington
15	Imp16 Dr-1-10	Drakkar	Impington
16	WH16 DK-Ext-8	DK-Extrovert	Woodhall Farm
17	Imp16 Why-12	Whisky	Impington
18	Imp16 Ama-8	Amalie	Impington
19	WH16 DK-Ext-1	DK-Extrovert	Woodhall Farm
20	Sf16 DK-Ext-6	DK-Extrovert	Morley

Sample ID	Isolate	Cultivar	Site
21	WB16 Ama-1	Amalie	Wisbech
22	Imp16 DK-Ext-9	DK-Extrovert	Impington
23	Imp16 Dr-1-15	Drakkar	Impington
24	Sf16 DKC-3	DK-Cabernet	Morley
25	WH15 Ama-2-2	Amalie	Woodhall Farm
26	WH15 Har-15	Harper	Woodhall Farm
27	Nf15 Dr-3-5	Drakkar	Morley
28	Imp15 Dr-2-4	Drakkar	Impington
29	WH15 DK-Ext-1-11	DK-Extrovert	Woodhall Farm
30	Imp15 Har-3	Harper	Impington
31	MS15 Dr-1-6	Drakkar	Trumpington
32	WH15 Dr-2-3	Drakkar	Woodhall Farm
33	Imp15 DK-Exl-3	DK-Exalte	Impington
34	Mrly15 Ama-5	Amalie	Morley
35	WH16 DK-Ext-1	DK-Extrovert	Woodhall Farm
36	MS15 Dr-2-1	Drakkar	Trumpington
37	Imp15 Har-7	Harper	Impington

Appendix 4-C: Details of *Leptosphaeria maculans* isolates that have avirulent and virulent alleles against *Rlm4*, amplified using AvrLm4-7ext-F3/R primers (Figure 4.7).

Sample ID	Isolate	Cultivar	Site
1	WH15 Dr-2-3	Drakkar	Woodhall Farm
2	Mrly15 Ama-8	Amalie	Morley
3	Mrly15 Ama-19	Amalie	Morley
4	Sf16 Dr-2-9	Drakkar	Morley
5	Imp16 DK-Exl-10	DK-Exalte	Impington
6	*AvrLm4* +ve control (V23.2.1)	In vitro cross isolate from France INRA	N/A
7	*avrLm4* +ve control (V23.11.9)	In vitro cross isolate from France INRA	N/A
8	-ve control (H2O)	N/A	N/A
9	Imp16 Ang-10	Angus	Impington
10	HLH15 Dr-5	Drakkar	West Farm Barns
11	Sf16 DK-Ext-4	DK-Extrovert	Morley
12	Sf16 Ama-11	Amalie	Morley
13	Imp16 Dr-1-15	Drakkar	Impington
14	WH15 Har-15	Harper	Woodhall Farm
15	Nf15 Dr-3-5	Drakkar	Morley
16	Imp15 Dr-2-4	Drakkar	Impington
17	Sf16 DK-Ext-7	DK-Extrovert	Morley
18	WB16 Ama-5	Amalie	Wisbech
19	Mrly15 Ama-6	Amalie	Morley

Sample ID	Isolate	Cultivar	Site
20	Mrly15 Ama-10	Amalie	Morley
21	MS15 Dr-1-5	Drakkar	Trumpington
22	Imp15 Dr-1-7	Drakkar	Impington
23	MS15 Dr-2-1	Drakkar	Trumpington
24	WH15 Ama-1-4	Amalie	Woodhall Farm
25	Imp15 Har-7	Harper	Impington
26	WB16 Ama-1	Amalie	Wisbech
27	Sf16 DKC-11	DK-Cabernet	Morley
28	WH16 DK-Ext-1	DK-Extrovert	Woodhall Farm
29	Imp16 Why-2	Whisky	Impington
30	WH15 Ama-1-11	Amalie	Woodhall Farm
31	Sf16 Why-2	Whisky	Morley
32	Sf16 DK-Ext-2	DK-Extrovert	Morley
33	Imp16 Dr-2-5	Drakkar	Impington
34	Imp16 Ang-8	Angus	Impington
35	Imp16 Dr-1-2	Drakkar	Impington
36	Sf16 Why-5	Whisky	Morley
37	Sf16 Ama-9	Amalie	Morley
38	WH15 Ama-2-2	Amalie	Woodhall Farm

Appendix 4-D: Details of *Leptosphaeria maculans* isolates that have avirulent and virulent alleles against *Rlm6*, amplified using A6OrfF/R primers (Figure 4.8).

Sample ID	Isolate	Cultivar	Site
1	Sf16 DK-Ext-7	DK-Extrovert	Morley
2	Sf16 Ang-8	Angus	Morley
3	Imp16 Har-2	Harper	Impington
4	Imp16 Dr-2-5	Drakkar	Impington
5	WH15 Ama-1-11	Amalie	Woodhall Farm
6	*AvrLm6* +ve control (ME24)	Apex	Darrington, West Yorkshire, in 2002
7	*avrLm6* +ve control (V29.3.1)	In vitro cross isolate from France INRA	N/A
8	-ve control (H2O)	N/A	N/A
9	Imp16 Ang-8	Angus	Impington
10	Imp16 Ang-5	Angus	Impington
11	Imp16 Dr-1-2	Drakkar	Impington
12	Imp16 Dr-1-10	Drakkar	Impington
13	Imp16 Ang-11	Angus	Impington
14	Sf16 Why-2	Whisky	Morley
15	Sf16 DK-Exl-8	DK-Exalte	Morley
16	Sf16 Dr-2-11	Drakkar	Morley

Appendix 4-E: Details of *Leptosphaeria maculans* isolates that have avirulent and virulent alleles against *Rlm7*, amplified using AvrLm4-7ext-F3/R primers (Figure 4.9).

Sample ID	Isolate	Cultivar	Site
1	WB16 Ama-1	Amalie	Wisbech
2	WH16 DK-Ext-1	DK-Extrovert	Woodhall Farm
3	Sf16 Dr-1-5	Drakkar	Morley
4	Imp16 Why-2	Whisky	Impington
5	WH15 Ama-1-11	Amalie	Woodhall Farm
6	*AvrLm7* +ve control (V23.11.9)	In vitro cross isolate from France INRA	N/A
7	*avrLm7* +ve control (HRox12-2-1)	Roxet	Harpenden
8	-ve Control (H2O)	N/A	N/A
9	Sf16 Why-2	Whisky	Morley
10	Sf16 Why-3	Whisky	Morley
11	Sf16 Why-5	Whisky	Morley
12	Sf16 Why-8	Whisky	Morley
13	Sf16 Why-11	Whisky	Morley
14	Sf16 DK-Ext-2	DK-Extrovert	Morley

Appendix 4-F: Details of *Leptosphaeria maculans* isolates whose genomic DNA was run on a 1% agarose gel for selecting for whole genome sequencing (Figure 4.14).

Sample ID	Isolate	Cultivar	Site
1	WH17 Dk-Ext-3	DK- Extrovert	Woodhall Farm
2	Mrly16 Har-up-1-4	Harper	Morley
3	Mrly16 Why-Up-2-4	Whisky	Morley
4	WB17 Dk-Ext-6	DK- Extrovert	Wisbech
5	WH17 Dr-1-6	Drakkar	Woodhall Farm
6	WH17 Why-3	Whisky	Woodhall Farm
7	WH17 Ang-5	Angus	Woodhall Farm
8	WB17 Why-1	Whisky	Wisbech
9	WB17 Why-8	Whisky	Wisbech
10	WB17 Dk-Exl-8	DK-Exalte	Wisbech
11	Mrly16 Why-Up-2-2	Whisky	Morley
12	WB17 Why-6	Whisky	Wisbech
13	Mrly16 Har-Up-3-2	Harper	Morley
14	WH17 Ang-7	Angus	Woodhall Farm
15	Mrly16 Har-Up-1-1	Harper	Morley
16	Imp16 Ang-8	Angus	Impington
17	WB17 Har-6	Harper	Wisbech
18	WH17 Why-1	Whisky	Woodhall Farm
19	Mrly16 Ama-Up-3-2	Amalie	Morley
20	WH17 Ama-2	Amalie	Woodhall Farm

Sample ID	Isolate	Cultivar	Site
21	Nf15 Dr-3-6	Drakkar	Morley
22	Imp15 Dr-1-7	Drakkar	Impington
23	Imp16 Ang-1	Angus	Impington
24	WH17 Dk-Exl-2	DK-Exalte	Woodhall Farm
25	Mrly16 Why-Up-2-8	Whisky	Morley
26	Mrly16 Why-Up-2-3	Whisky	Morley
27	GS17 Dr-3-6	Drakkar	Morley
28	Sf16 Why-5	Whisky	Morley
29	Imp16 Dr-1-15	Drakkar	Impington
30	Sf16 Why-8	Whisky	Morley
31	Sf16 Why-11	Whisky	Morley
32	Sf16 Dk-Ext-2	DK- Extrovert	Morley
33	Sf16 Why-2	Whisky	Morley
34	Sf16 Why-3	Whisky	Morley
35	Sf16 Dk-Exl-8	DK-Exalte	Morley
36	Mrly15 Ama-11	Amalie	Morley
37	Imp16 Ang-10	Angus	Impington
38	MS15 Dr-2-1	Drakkar	Trumpington

Appendix 4-G: Details of *Leptosphaeria maculans* isolates whose genomic DNA was run on a 1% agarose gel for selecting for whole genome sequencing (Figure 4.15).

Sample ID	Isolate	Cultivar	Site
39	WH15 Ama-1-4	Amalie	Woodhall Farm
40	WB17 Dk-Ext-5	DK- Extrovert	Wisbech
41	Mrly16 Why-up-3-7	Whisky	Morley
42	Mrly16 Why-Up-3-2	Whisky	Morley
43	Mrly16 Why-Up-3-3	Whisky	Morley
44	WH17 Dr-1-1	Drakkar	Woodhall Farm
45	WB17 Why-7	Whisky	Wisbech
46	Mrly16 Har-Up-3-3	Harper	Morley
47	LSPB16 Why-Sb-3-1	Whisky	Wisbech
48	Mrly16 Dk-Exl-Up-2-5	DK-Exalte	Morley
49	Mrly16 Har-Up-1-9	Harper	Morley
50	LSPB16 Har-Sb-1-3	Harper	Wisbech
51	LSPB16 Har-Up-1-5	Harper	Wisbech
52	LSPB16 Har-Sb-3-3	Harper	Wisbech
53	LSPB16 Har-Sb-2-4	Harper	Wisbech
54	LSPB16 Har-Sb-3-7	Harper	Wisbech
55	LSPB16 Dk-Exl-Sb-1-2	DK-Exalte	Wisbech
56	LSPB16 Dk-Exl-Up-1-3	DK-Exalte	Wisbech
57	LSPB16 Har-Up-1-7	Harper	Wisbech
58	Mrly16 Ama-Sb-3-1	Amalie	Morley
59	Imp16 Dr-1-2	Drakkar	Impington
60	Mrly16 Dr-Sb-2-7	Drakkar	Morley
61	LSPB16 Har-Up-1-4	Harper	Wisbech

Appendix 4-H: DNA Tapestation results of samples selected for whole genome sequencing. For details of genomic DNA samples of *Leptosphaeria maculans* isolates refer to Appendix 4-I.

Gel Images

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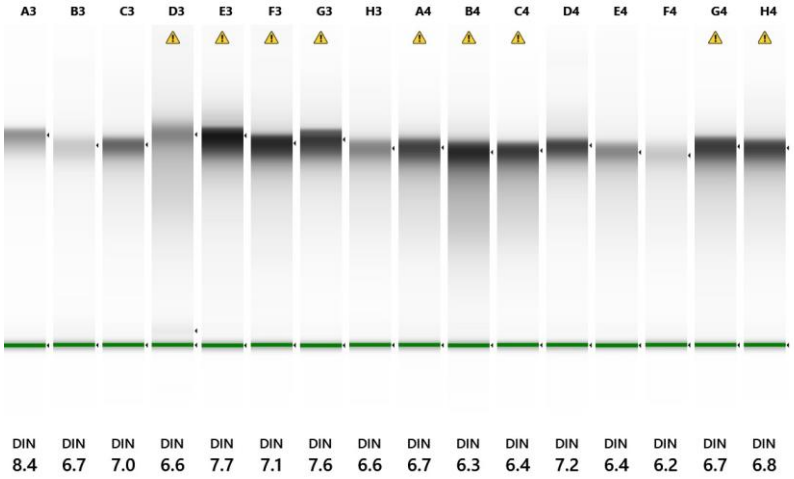

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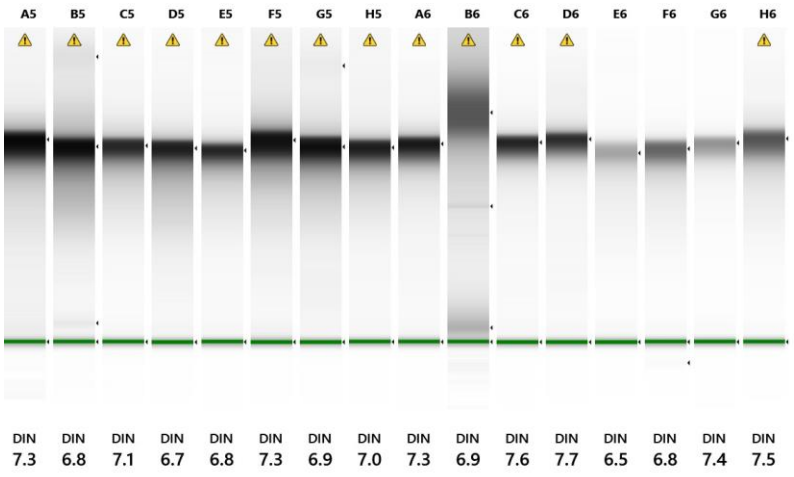

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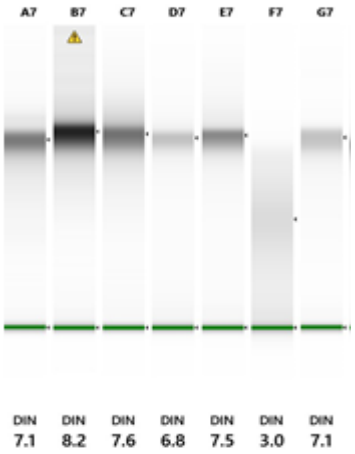

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Sample Info

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
A1	-	24.7	Ladder		Ladder
H2	8.0	73.3	11355FB0016		
A3	8.4	32.8	11355FB0017		
B3	6.7	26.1	11355FB0018		
C3	7.0	66.2	11355FB0019		
D3	6.6	101	11355FB0020	⚠	Sample concentration outside recommended range
E3	7.7	164	11355FB0021	⚠	Sample concentration outside recommended range
F3	7.1	136	11355FB0022	⚠	Sample concentration outside recommended range
G3	7.6	118	11355FB0023	⚠	Sample concentration outside recommended range
H3	6.6	65.5	11355FB0024		
A4	6.7	111	11355FB0025	⚠	Sample concentration outside recommended range
B4	6.3	175	11355FB0026	⚠	Sample concentration outside recommended range
C4	6.4	127	11355FB0027	⚠	Sample concentration outside recommended range
D4	7.2	91.6	11355FB0028		

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
E4	6.4	62.6	11355FB0029		
F4	6.2	30.8	11355FB0030		
G4	6.7	120	11355FB0031	⚠	Sample concentration outside recommended range
H4	6.8	108	11355FB0032	⚠	Sample concentration outside recommended range
A5	7.3	225	11355FB0033	⚠	Sample concentration outside recommended range
B5	6.8	257	11355FB0034	⚠	Sample concentration outside recommended range
C5	7.1	138	11355FB0035	⚠	Sample concentration outside recommended range
D5	6.7	165	11355FB0036	⚠	Sample concentration outside recommended range
E5	6.8	124	11355FB0037	⚠	Sample concentration outside recommended range
F5	7.3	225	11355FB0038	⚠	Sample concentration outside recommended range
G5	6.9	222	11355FB0039	⚠	Sample concentration outside recommended range
H5	7.0	154	11355FB0040	⚠	Sample concentration outside recommended range
A6	7.3	136	11355FB0041	⚠	Sample concentration outside recommended range
B6	6.9	289	11355FB0042	⚠	Sample concentration outside recommended range
C6	7.6	106	11355FB0043	⚠	Sample concentration outside recommended range
D6	7.7	111	11355FB0044	⚠	Sample concentration outside recommended range
E6	6.5	44.2	11355FB0045		
F6	6.8	71.7	11355FB0046		DIN Edited (Marker position changed)

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
G6	7.4	37.2	11355FB0047		
H6	7.5	101	11355FB0048	⚠	Sample concentration outside recommended range
A7	7.1	57.9	11355FB0049		
B7	8.2	135	11355FB0050	⚠	Sample concentration outside recommended range
C7	7.6	74.6	11355FB0051		
D7	6.8	21.6	11355FB0052		
E7	7.5	43.5	11355FB0053		
F7	3.0	34.7	11355FB0054		
G7	7.1	24.2	11355FB0055		
H7	6.8	52.0	11355FB0056		

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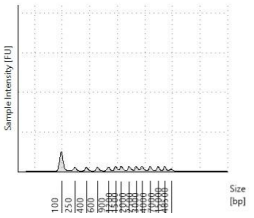

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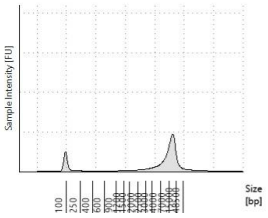

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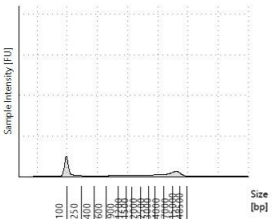

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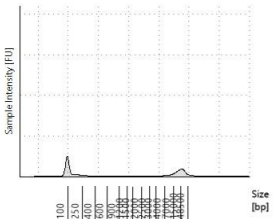

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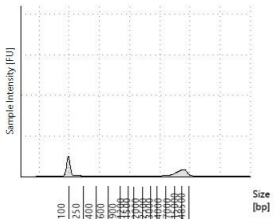

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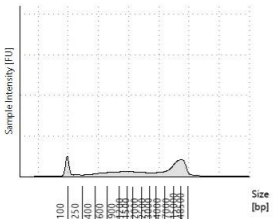

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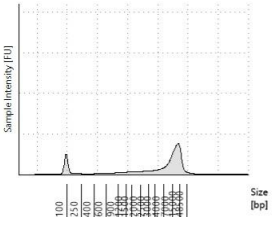

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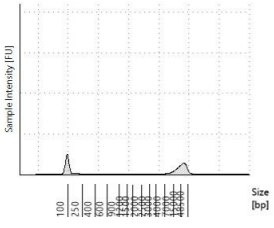

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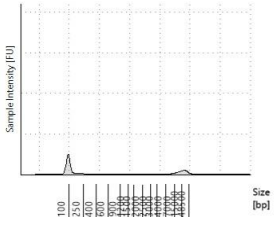

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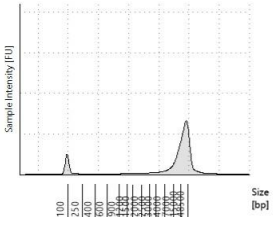

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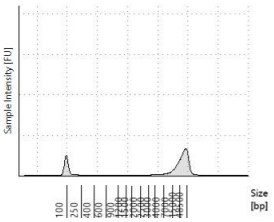

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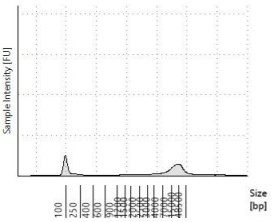

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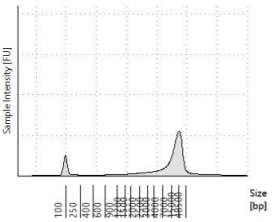

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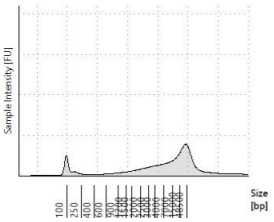

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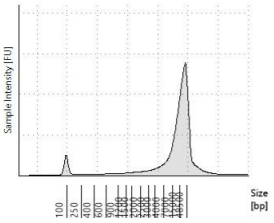

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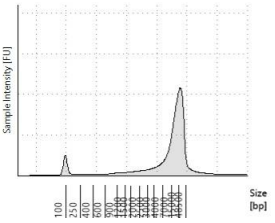

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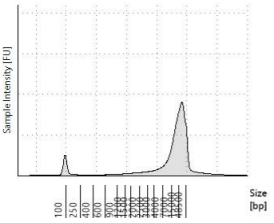

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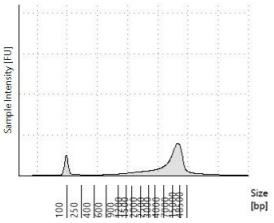

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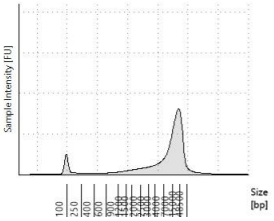

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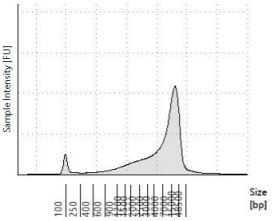

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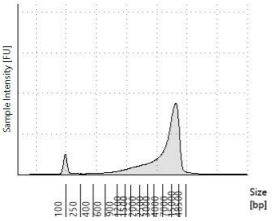

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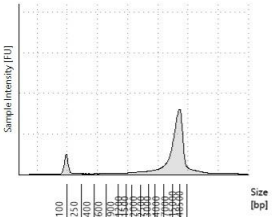

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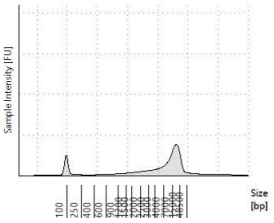

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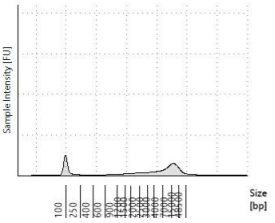

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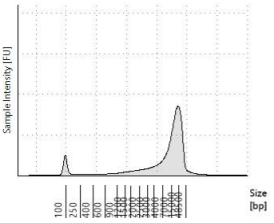

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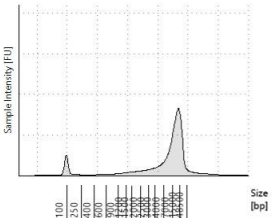

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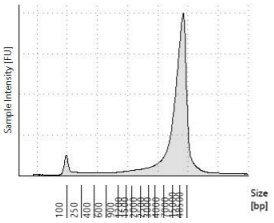

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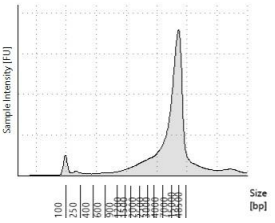

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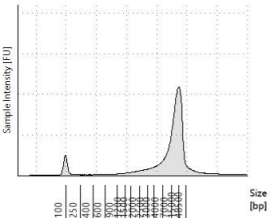

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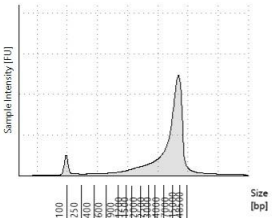

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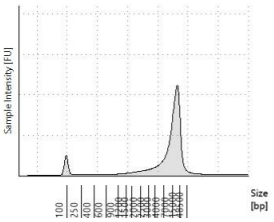

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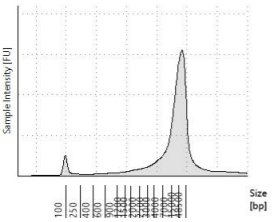

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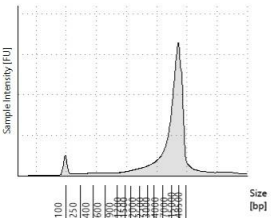

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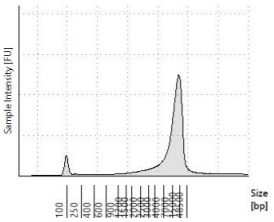

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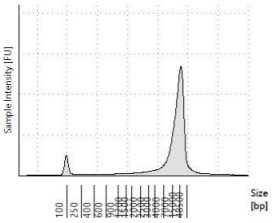

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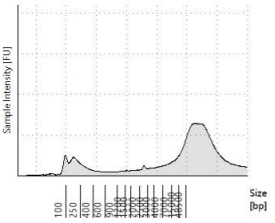

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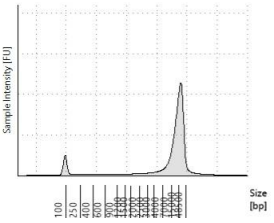

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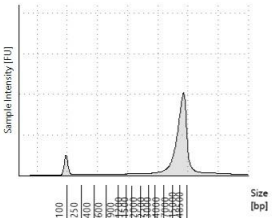

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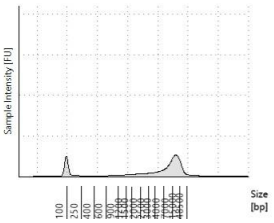

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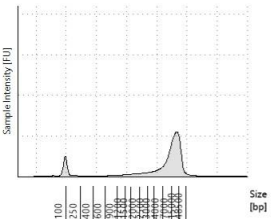

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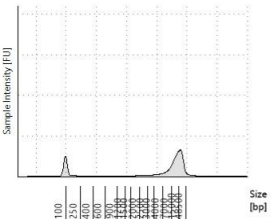

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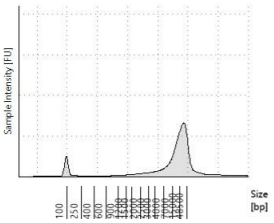

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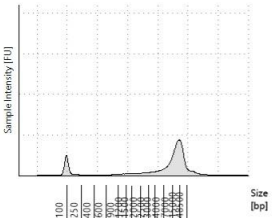

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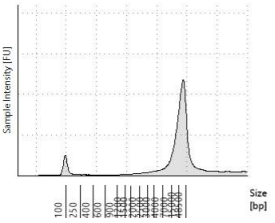

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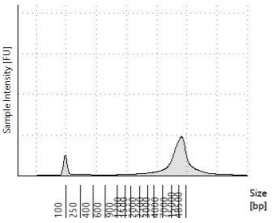

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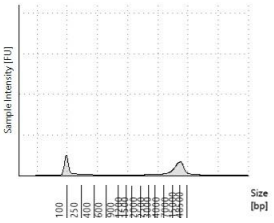

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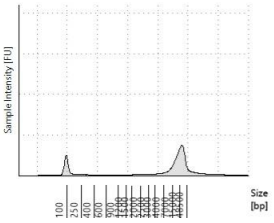
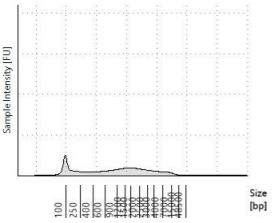
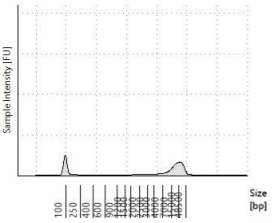
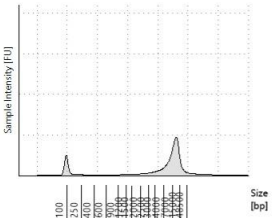








Appendix 4-I: Details of genomic DNA samples of *Leptosphaeria maculans* isolates for whole genome sequencing.

S. No	Well	Name of the isolate	Cultivar	Race	Type of isolate	Type of isolation	Site	Year of Collection	Conc (ng/µL)	Purity (260/280 nm)	DIN value	1% agarose gel	Sp-spec PCR
1	H:02	WH17 Dk-Ext-3	DK Extrovert	*Av 5-9*	*L. maculans*	SPI	Woodhall Farm	2017-2018	33.7	1.84	6.8	√	√
2	A:03	Mrly16 Har-up-1-4	Harper	*Av 2-5*	*L. maculans*	SAI	Morley	2016-2017	45	1.9	7.3	√	√
3	B:03	WH17 Ang-5	Angus	*Av 1-3-5-6*	*L. maculans*	SPI	Woodhall Farm	2017-2018	44.5	1.78	6.4	√	√
4	C:03	Mrly16 Why-Up-2-2	Whisky	*Av 1-3-5-6*	*L. maculans*	SAI	Morley	2016-2017	23.7	1.98	6.4	√	√
5	D:03	WH17 Ang-7	Angus	-	*L. maculans*	SPI	Woodhall Farm	2017-2018	41.6	1.85	6.5	√	√
6	E:03	Mrly16 Har-Up-1-1	Harper	*Av 1-5-6*	*L. maculans*	SAI	Morley	2016-2017	49	1.89	6.8	√	√
7	F:03	WB17 Har-6	Harper	*Av 1-3-5-6*	*L. maculans*	SPI	Wisbech	2017-2018	44.8	1.81	7.1	√	√
8	G:03	WH17 Why-1	Whisky	*Av 1-6*	*L. maculans*	SPI	Woodhall Farm	2017-2018	29.4	1.86	7.5	√	√
9	H:03	Mrly16 Ama-Up-3-2	Amalie	*Av 5-6*	*L. maculans*	SAI	Morley	2016-2017	47.3	1.94	6.6	√	√
10	A:04	Nf15 Dr-3-6	Drakkar	*Av 7*	*L. maculans*	SPI	Morley	2015-2016	26.3	1.86	7.2	√	√
11	B:04	Imp15 Dr-1-7	Drakkar	*Av 5-7*	*L. maculans*	SPI	Impington	2015-2016	25.6	1.81	6.3	√	√
12	C:04	Imp16 Ang-1	Angus	*Av6-7*	*L. maculans*	SPI	Impington	2016-2017	36.3	1.92	6.2	√	√
13	D:04	WH17 Dk-Exl-2	DK Exalte	*Av 9*	*L. maculans*	SPI	Woodhall Farm	2017-2018	45.2	1.94	6.9	√	√
14	E:04	Mrly16 Why-Up-2-8	Whisky	*Av 1-3-5-6*	*L. maculans*	SAI	Morley	2016-2017	23	1.89	6.5	√	√
15	F:04	GS17 Dr-3-6	Drakkar	*Av 4-5-7*	*L. maculans*	SPI	Morley	2017-2018	42.4	1.84	6.3	√	√
16	G:04	Imp16 Dr-1-15	Drakkar	*Av6-7*	*L. maculans*	SPI	Impington	2016-2017	21.5	1.83	6.6	√	√
17	H:04	Sf16 Why-8	Whisky	*Av1-5-6*	*L. maculans*	SPI	Morley	2016-2017	47.2	1.86	6.6	√	√
18	A:05	Sf16 Why-11	Whisky	*Av5-6*	*L. maculans*	SPI	Morley	2016-2017	36.4	1.81	6.5	√	√
19	B:05	Sf16 Dk-Ext-2	DK Extrovert	*Av1-3-5-6*	*L. maculans*	SPI	Morley	2016-2017	35.9	1.93	6.4	√	√
20	C:05	Sf16 Why-2	Whisky	*Av6*	*L. maculans*	SPI	Morley	2016-2017	30	1.96	6.3	√	√
21	D:05	Sf16 Why-3	Whisky	*Av1-3-6*	*L. maculans*	SPI	Morley	2016-2017	21.3	1.89	6	√	√

S. No	Well	Name of the isolate	Cultivar	Race	Type of isolate	Type of isolation	Site	Year of Collection	Conc (ng/µL)	Purity (260/280 nm)	DIN value	1% agarose gel	Sp-spec PCR
22	E:05	Mrly15 Ama-11	Amalie	*Av 2-5-6-7*	*L. maculans*	SPI	Morley	2015-2016	24	1.85	6.2	√	√
23	F:05	Imp16 Ang-10	Angus	*Av2-5-6-7*	*L. maculans*	SPI	Impington	2016-2017	48.7	1.79	6.5	√	√
24	G:05	WH15 Ama-1-4	Amalie	*Av 6-7*	*L. maculans*	SPI	Woodhall Farm	2015-2016	24.8	1.93	6.2	√	√
25	H:05	WB17 Dk-Ext-5	DK Extrovert	*Av 3-5-6*	*L. maculans*	SPI	Wisbech	2017-2018	30.9	1.87	6.3	√	√
26	A:06	Mrly16 Why-up-3-7	Whisky	*Av 5*	*L. maculans*	SAI	Morley	2016-2017	38	1.93	6.6	√	√
27	B:06	Mrly16 Why-Up-3-2	Whisky	*Av 5-6*	*L. maculans*	SAI	Morley	2016-2017	31.3	1.88	6.7	√	√
28	C:06	Mrly16 Why-Up-3-3	Whisky	*Av 1-3-5-6*	*L. maculans*	SAI	Morley	2016-2017	31.9	1.86	6.1	√	√
29	D:06	WH17 Dr-1-1	Drakkar	*Av 5-6*	*L. maculans*	SPI	Woodhall Farm	2017-2018	33	1.83	6	√	√
30	E:06	Mrly16 Har-Up-3-3	Harper	*Av 1-5-6*	*L. maculans*	SAI	Morley	2016-2017	42.6	1.83	8.4	√	√
31	F:06	LSPB16 Har-Sb-2-4	Harper	*Av 1-5-6*	*L. maculans*	SAI	Wisbech	2016-2017	34.4	1.89	6.8	√	√
32	G:06	LSPB16 Har-Sb-3-7	Harper	*Av 1-5-6*	*L. maculans*	SAI	Wisbech	2016-2017	31.2	1.84	7	√	√
33	H:06	LSPB16 Dk-Exl-Sb-1-2	DK Exalte	*Av 1-2-3-4-5-6-7-9*	*L. maculans*	SAI	Wisbech	2016-2017	38.7	1.81	7.2	√	√
34	A:07	LSPB16 Dk-Exl-Up-1-3	DK Exalte	*Av 4-5-6-7-9*	*L. maculans*	SAI	Wisbech	2016-2017	38	1.9	6.8	√	√
35	B:07	Imp16 Dr-1-2	Drakkar	*Av6-7*	*L. maculans*	SPI	Impington	2016-2017	42	1.76	7.9	√	√
36	C:07	LSPB16 Har-Up-1-4	Harper	*Av 3-5-6*	*L. maculans*	SAI	Wisbech	2016-2017	32.1	1.87	7.4	√	√
37	D:07	MS15 Dr-2-1	Drakkar	*Av 6-7*	*L. maculans*	SPI	Trumpington	2015-2016	25.4	1.96	7.4	√	√
38	E:07	WH15 Ama-1-4	Amalie	*Av 6-7*	*L. maculans*	SPI	Woodhall Farm	2015-2016	36.8	1.9	7.6	√	√
39	F:07	SF16 Dr-1-8	Drakkar	*Av 4-5-6-7*	*L. maculans*	SPI	Morley	2016-2017	32.6	1.89	7.1	√	√
40	G:07	WH15 Dr-2-2	Drakkar	*Av 5-6-7*	*L. maculans*	SPI	Woodhall Farm	2015-2016	32.4	1.87	7.1	√	√

SPI- Single pycnidial isolation

SAI- Single ascospore isolation

Appendix 4-J: Details of genome sequences of *Leptosphaeria maculans* isolates analysed for molecular mechanisms of mutation towards virulence against *Rlm1*, *Rlm4*, *Rlm6* or *Rlm7* resistance genes in this study.

S.No	Lm isolates (*AvrLm1*)	Race
1	WH17 Dk-Ext-3	*Av 5-9*
2	Mrly16 Har-up-1-4	*Av 2-5*
3	WH17 Ang-7	_
4	Mrly16 Ama-Up-3-2	*Av 5-6*
5	Nl15 Dr-3-6	*Av 7*
6	Imp15 Dr-1-7	*Av 5-7*
7	Imp16 Ang-1	*Av6-7*
8	WH17 Dk-Exl-2	*Av 4-9*
9	GS17 Dr-3-6	*Av 4-5-7*
10	Imp16 Dr-1-15	*Av6-7*
11	Sf16 Why-11	*Av5-6*
12	Sf16 Why-2	*Av6*
13	Mrly15 Ama-11	*Av 2-5-6-7*
14	Imp16 Ang-10	*Av2-5-6-7*
15	WH15 Ama-1-4	*Av 6-7*
16	WB17 Dk-Ext-5	*Av 3-5-6*
17	Mrly16 Why-up-3-7	*Av 5*
18	Mrly16 Why-Up-3-2	*Av 5-6*
19	WH17 Dr-1-1	*Av 5-6*
20	LSPB16 Dk-Exl-Up-1-3	*Av 4-5-6-7-9*
21	Imp16 Dr-1-2	*Av6-7*
22	LSPB16 Har-Up-1-4	*Av 3-5-6*
23	MS15 Dr-2-1	*Av 6-7*
24	WH15 Ama-1-4	*Av 6-7*
25	SF16 Dr-1-8	*Av 4-5-6-7*
26	WH15 Dr-2-2	*Av 5-6-7*
27	LSPB16 Dk-Exl-Sb-1-2	*Av 1-2-3-4-5-6-7-9*

S.No	Lm isolates (*AvrLm4*)	Race
1	WH17 Dk-Ext-3	*Av 5-9*
2	Mrly16 Har-up-1-4	*Av 2-5*
3	WH17 Ang-5	*Av 1-3-5-6*
4	Mrly16 Why-Up-2-2	*Av 1-3-5-6*
5	WH17 Ang-7	_
6	Mrly16 Har-Up-1-1	*Av 1-5-6*
7	WB17 Har-6	*Av 1-3-5-6*
8	WH17 Why-1	*Av 1-6*
9	Mrly16 Ama-Up-3-2	*Av 5-6*
10	Nl15 Dr-3-6	*Av 7*
11	Imp15 Dr-1-7	*Av 5-7*
12	Imp16 Ang-1	*Av6-7*
13	Mrly16 Why-Up-2-8	*Av 1-3-5-6*
14	Imp16 Dr-1-15	*Av6-7*
15	Sf16 Why-8	*Av1-5-6*
16	Sf16 Why-11	*Av5-6*
17	Sf16 Dk-Ext-2	*Av1-3-5-6*
18	Sf16 Why-2	*Av6*
19	Sf16 Why-3	*Av1-3-6*
20	Mrly15 Ama-11	*Av 2-5-6-7*
21	Imp16 Ang-10	*Av2-5-6-7*
22	WH15 Ama-1-4	*Av 6-7*
23	WB17 Dk-Ext-5	*Av 3-5-6*
24	Mrly16 Why-up-3-7	*Av 5*
25	Mrly16 Why-Up-3-2	*Av 5-6*
26	Mrly16 Why-Up-3-3	*Av 1-3-5-6*
27	WH17 Dr-1-1	*Av 5-6*
28	Mrly16 Har-Up-3-3	*Av 1-5-6*
29	LSPB16 Har-Sb-2-4	*Av 1-5-6*
30	LSPB16 Har-Sb-3-7	*Av 1-5-6*
31	Imp16 Dr-1-2	*Av6-7*
32	LSPB16 Har-Up-1-4	*Av 3-5-6*
33	MS15 Dr-2-1	*Av 6-7*
34	WH15 Ama-1-4	*Av 6-7*
35	WH15 Dr-2-2	*Av 5-6-7*
36	LSPB16 Dk-Exl-Sb-1-2	*Av 1-2-3-4-5-6-7-9*
37	WH17 Dk-Exl-2	*Av 9*

S.No	Lm isolates (*AvrLm6*)	Race
1	WH17 Dk-Ext-3	*Av 5-9*
2	Mrly16 Har-up-1-4	*Av 2-5*
3	WH17 Ang-7	_
4	Nl15 Dr-3-6	*Av 7*
5	Imp15 Dr-1-7	*Av 5-7*
6	WH17 Dk-Exl-2	*Av 9*
7	GS17 Dr-3-6	*Av 4-5-7*
8	Mrly16 Why-up-3-7	*Av 5*
9	LSPB16 Dk-Exl-Sb-1-2	*Av 1-2-3-4-5-6-7-9*

S.No	Lm isolates (*AvrLm7*)	Race
1	WH17 Dk-Ext-3	*Av 5-9*
2	Mrly16 Har-up-1-4	*Av 2-5*
3	WH17 Ang-5	*Av 1-3-5-6*
4	Mrly16 Why-Up-2-2	*Av 1-3-5-6*
5	Mrly16 Har-Up-1-1	*Av 1-5-6*
6	WB17 Har-6	*Av 1-3-5-6*
7	WH17 Why-1	*Av 1-6*
8	Mrly16 Ama-Up-3-2	*Av 5-6*
9	WH17 Dk-Exl-2	*Av 9*
10	Mrly16 Why-Up-2-8	*Av 1-3-5-6*
11	Sf16 Why-8	*Av1-5-6*
12	Sf16 Why-11	*Av5-6*
13	Sf16 Dk-Ext-2	*Av1-3-5-6*
14	Sf16 Why-2	*Av6*
15	Sf16 Why-3	*Av1-3-6*
16	WB17 Dk-Ext-5	*Av 3-5-6*
17	Mrly16 Why-up-3-7	*Av 5*
18	Mrly16 Why-Up-3-2	*Av 5-6*
19	Mrly16 Why-Up-3-3	*Av 1-3-5-6*
20	WH17 Dr-1-1	*Av 5-6*
21	Mrly16 Har-Up-3-3	*Av 1-5-6*
22	LSPB16 Har-Sb-2-4	*Av 1-5-6*
23	LSPB16 Har-Sb-3-7	*Av 1-5-6*
24	LSPB16 Har-Up-1-4	*Av 3-5-6*
25	LSPB16 Dk-Exl-Sb-1-2	*Av 1-2-3-4-5-6-7-9*
26	WH17 Ang-7	_

No sequence file found

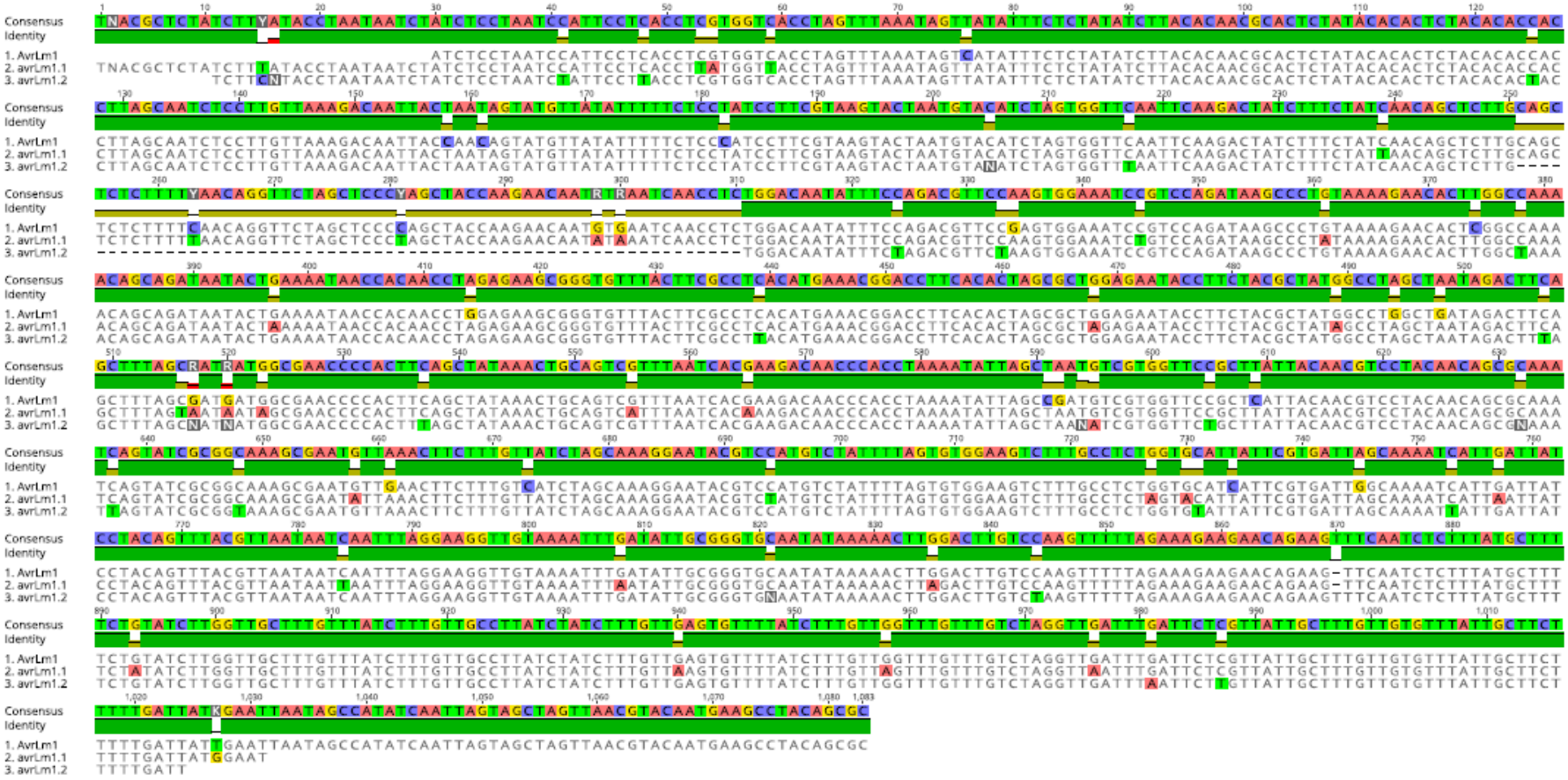
Bam files uploaded successfully

Problem uploading the bam file

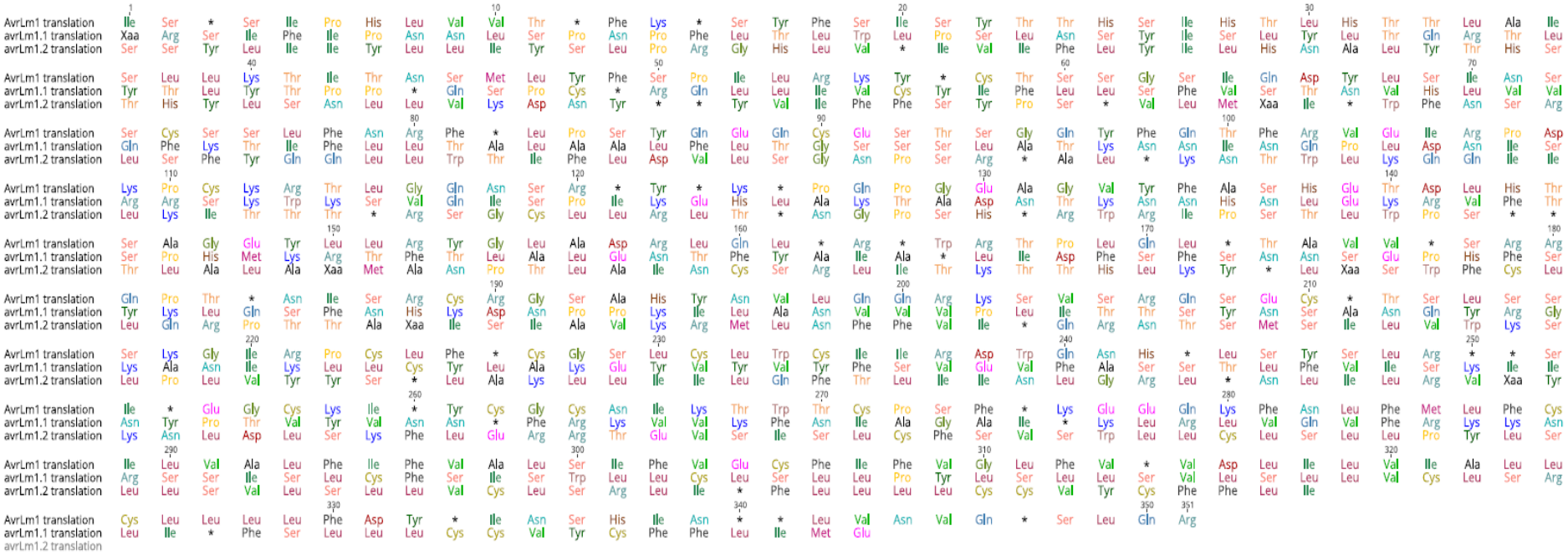
Zero output from bam files for the corresponding Avrlm region

Considered as whole gene deletion

Appendix 4-K: Nucleotide sequence alignment of the *AvrLm1* gene amplified in two *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm1* effector gene and compared to the sequence of the *AvrLm1* reference genome (GenBank: AM084345.1) using Geneious software. Dashes indicate data not available; stop codon is represented by *; changes in colours in codons is due to point mutations.

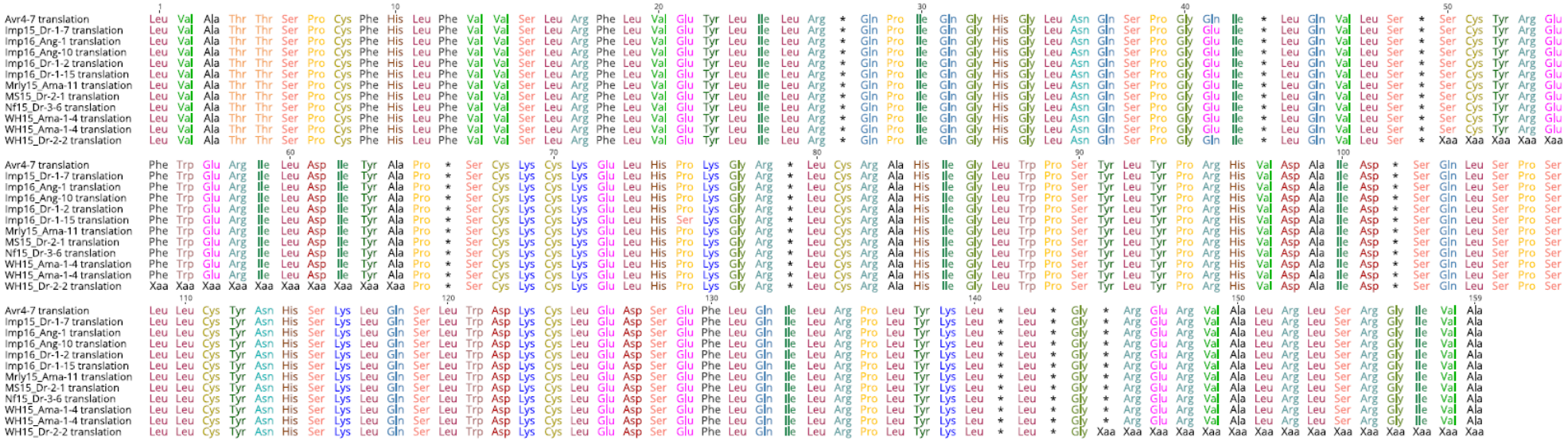


Appendix 4-L: Alignment of translated sequences of two *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm1* effector gene with the reference sequence of the *AvrLm1* gene (GenBank: AM084345.1) using Geneious software. Full forms of the amino acids were given in this image. Stop codon is represented by *; changes in colours is due to amino acid change.



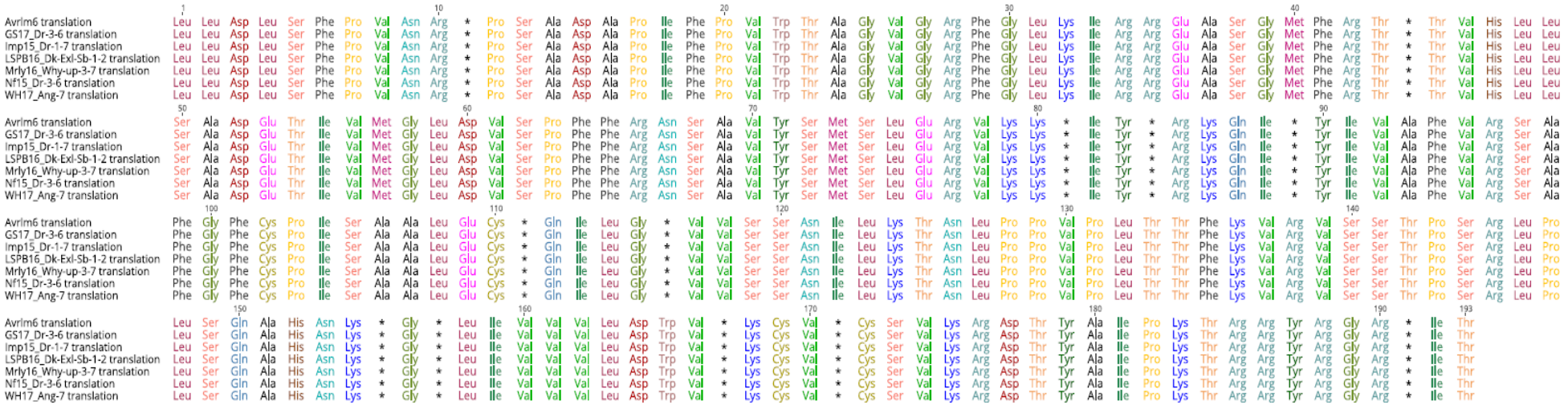
Appendix 4-M: Nucleotide alignment of eleven genome sequences of *Leptosphaeria maculans* isolates containing avirulent or virulent alleles of the *AvrLm4* effector gene and avirulent alleles of the *AvrLm7* effector gene with the sequence of the reference *AvrLm4-7* gene (v23.2.3) using Geneious software showing mutations in nucleotide sequences. For details of isolates refer to Appendix 4-J. Data not available is represented by ?.

Appendix 4-N: Alignment of translated sequences of twelve *Leptosphaeria maculans* isolates containing avirulent or virulent alleles of the *AvrLm4* or *AvrLm7* effector genes with the reference sequence of the *AvrLm4-7* gene (isolate v23.2.3) using Geneious software. Full forms of the amino acids were given in this image. Stop codon is represented by *; changes in colours is due to amino acid change.

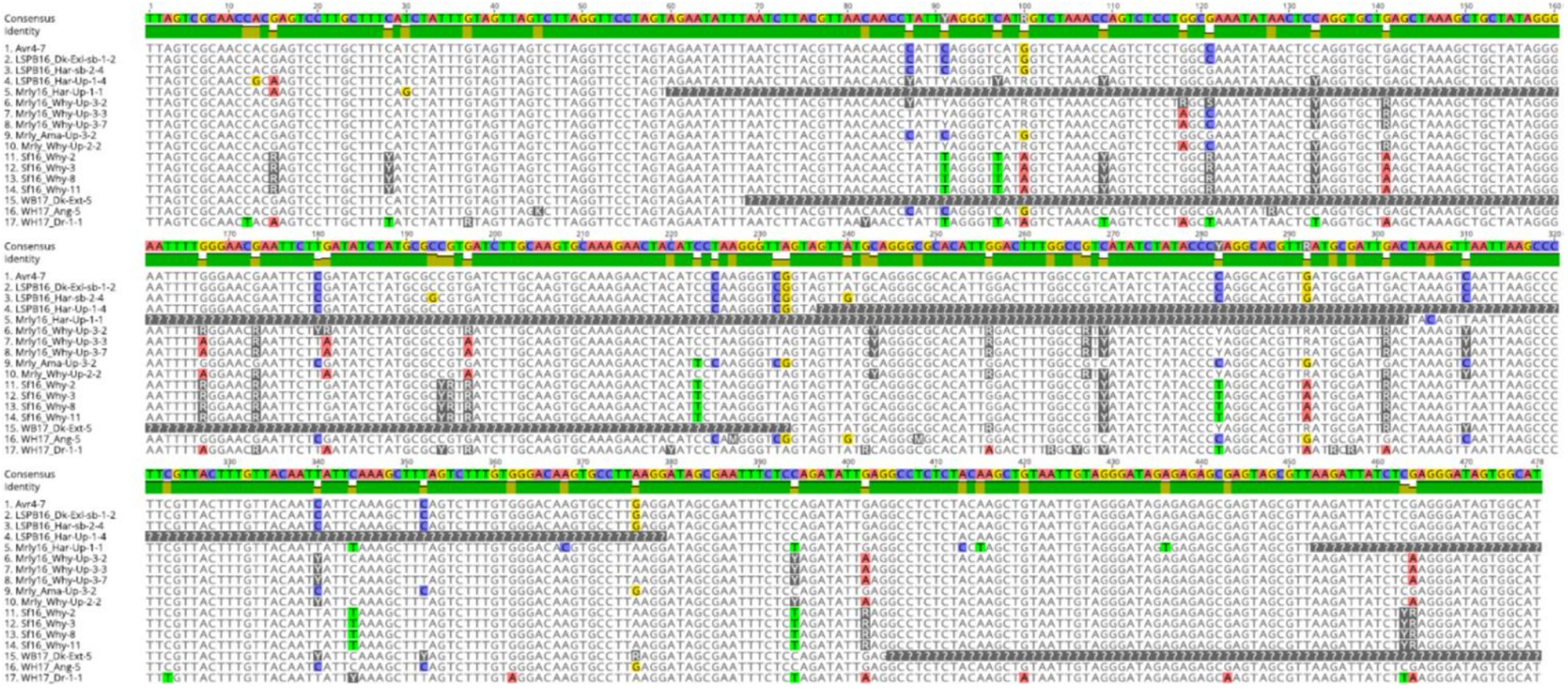


Appendix 4-O: Nucleotide alignment of six genome sequences of *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm6* effector gene with the sequence of the reference *AvrLm6* gene (isolate v23.2.3) using Geneious software to identify mutations in nucleotide sequences. For details of isolates refer to Appendix 4-J. No molecular mechanisms of mutations leading towards virulence against *Rlm6* resistance gene were identified.

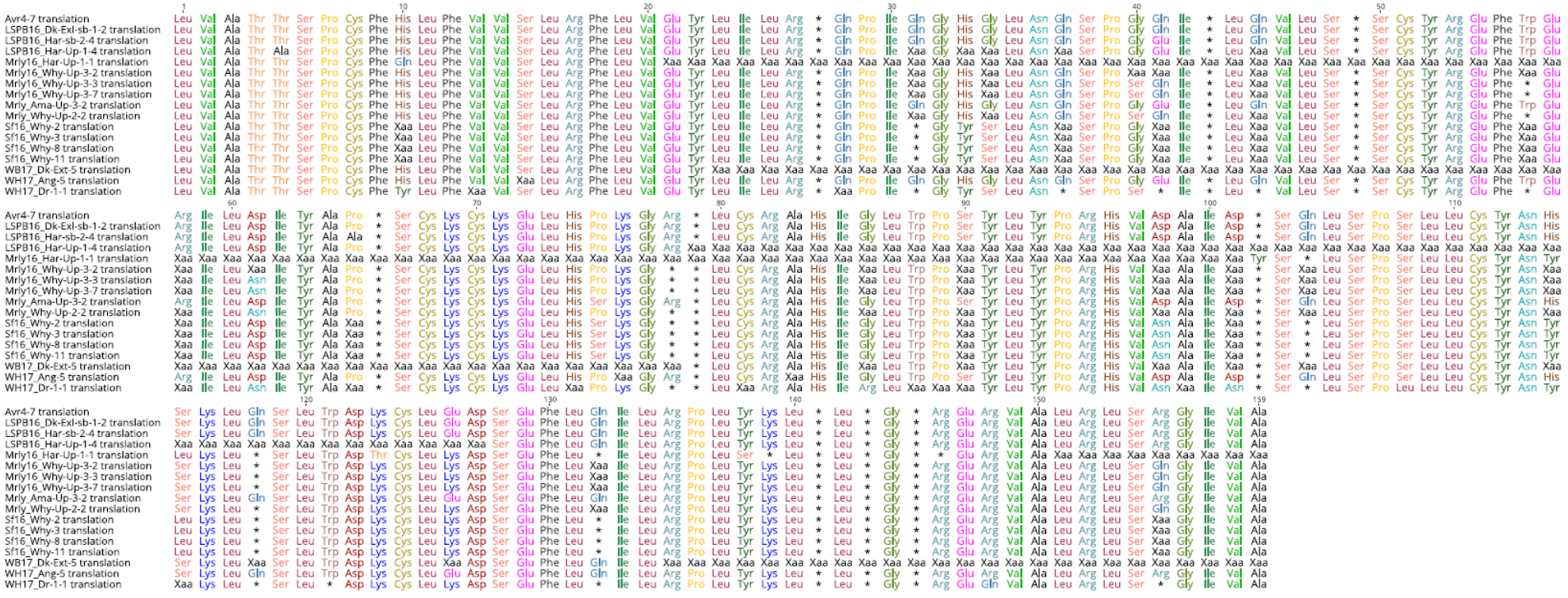
Appendix 4-P: Alignment of translated sequences of six *Leptosphaeria maculans* isolates containing virulent alleles of the *AvrLm6* effector gene with the reference sequence of the *AvrLm6* gene (isolate v23.2.3) using Geneious software. Full forms of the amino acids were given in this image.



Appendix 4-Q: Nucleotide alignment of sixteen genome sequences of *Leptosphaeria maculans* isolates containing avirulent or virulent alleles of the *AvrLm4* or *AvrLm7* effector genes with the sequence of the reference *AvrLm4-7* gene (v23.2.3) using Geneious software showing mutations in nucleotide sequences. For details of isolates refer to Appendix 4-J; data not available is represented by ?; mutations are highlighted in colours.



Appendix 4-R: Alignment of translated sequences of sixteen *Leptosphaeria maculans* isolates containing avirulent or virulent alleles of the *AvrLm4* or *AvrLm7* effector genes with the reference sequence of the *AvrLm4-7* gene (v23.2.3) using Geneious software. Full forms of the amino acids were given in this image. Stop codon is represented by *; changes in colours is due to amino acid change.



Chapter 5

Appendix 5-A: The estimated parameters “a” and “b” in the simple linear relationship (i.e. y=a+b*x) between cultivar phoma leaf spot severity and the seasonal site mean phoma leaf spot severity. Where y= cultivar; x= seasonal site mean of phoma leaf spot severity.

Name of the cultivar	a	b
Adriana	-0.1860	1.2880
Amalie	-0.4500	1.3300
Angus	-0.2930	0.9870
DK Cabernet	-0.1580	1.2570
DK Exalte	-0.3630	0.7940
DK Extrovert	0.4150	0.5990
Drakkar	1.4190	0.4890
Es Astrid	-0.2240	1.1150
Harper	-0.2030	0.7330
Incentive	-0.2970	1.4990
Mentor	0.4510	1.1850
Whisky	-0.2170	0.8000

Appendix 5-B: The estimated parameters "a" and "b" in the simple linear relationship (i.e. y=a+b*x) between cultivar phoma stem canker severity and the seasonal site mean phoma stem canker severity. Where y= cultivar; x= seasonal site mean of phoma stem canker severity.

Name of the cultivar	a	b
Adriana	-0.6000	1.0620
Amalie	-0.3100	1.3160
Angus	-0.3700	0.5610
DK Cabernet	-0.7100	0.9020
DK Exalte	-1.0100	0.8430
DK Extrovert	-0.6400	0.7390
Drakkar	6.1100	0.5000
Es Astrid	-0.9400	1.1390
Harper	-0.9300	1.1110
Incentive	0.1000	1.5280
Mentor	0.0900	1.5230
Whisky	-0.7900	0.7790

Appendix 5-C: The estimated parameters "a" and "b" in the simple linear relationship (i.e. y=a+b*x) between cultivar yield and the seasonal site mean yield. Where y= cultivar; x= seasonal site mean yield.

Name of the cultivar	a	b
Adriana	-0.2720	1.0400
Amalie	-1.2350	1.3650
Angus	0.6960	0.9960
DK Cabernet	0.1740	0.7800
DK Exalte	-0.1970	1.2310
DK Extrovert	1.0050	0.9220
Drakkar	0.6120	0.1810
Es Astrid	-0.4990	1.1380
Harper	-0.2180	1.1030
Incentive	0.0910	1.0060
Mentor	-0.0390	0.9750
Whisky	-0.1320	1.1070

Publications

Identification of new virulent races of *Leptosphaeria maculans* populations on oilseed rape in the UK

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The IOBC/WPRS Working Group "Integrated Control in Oilseed Crops "entitled "Prospects and progress for sustainable oilseed crop protection "Institute of Agricultural and Environmental Sciences of the Estonian University of Life Sciences, Tartu, Estonia, September 7-9, 2016.

Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled (Fitt *et al*., 2011). Currently, this disease causes UK annual yield losses >£100M despite use of fungicides (http://www.cropmonitor.co.uk). With recent loss of the most effective fungicides through EU legislation, potential yield losses will increase (Mahmuti *et al*., 2009). Use of host resistance to control this disease is becoming ever more important. However, new sources of resistance are often rendered ineffective due to pathogen population changes from avirulent to virulent. There is a need to monitor regional distribution of new virulent races of *L. maculans* and prevent them from spreading into new regions and to investigate molecular mechanisms of mutation from avirulent to virulent in *L. maculans* populations.

Phoma leaf spot assessment was done on eleven different oilseed rape cultivars with different resistance (*R*) genes with/without background quantitative resistance (Drakkar, DK Cabernet, Es Astrid, Whisky, Angus, Adriana, DK Extrovert, DK Exalte, Incentive, Harper, Amalie and Mentor) at four different sites (Woodhall Farm, Hertfordshire; Morley, Norfolk; Impington, Cambridgeshire and Trumpington, Cambridgeshire) in the UK (2015/16 cropping season). Cultivars with no *R* gene against *L. maculans* developed severe phoma leaf spotting compared to cultivars with *R* genes and background quantitative resistance. Cultivars with *Rlm7* gene had less phoma leaf spotting compared to cultivars with *Rlm1*or *Rlm4* resistance genes.

Leaves with phoma leaf spots were collected from cultivar Drakkar (no *R* gene) from five different sites (Woodhall Farm, Hertfordshire; Morley, Norfolk; Impington, Cambridgeshire; Trumpington, Cambridgeshire and West Farm Barns, Oxfordshire) and *L. maculans* isolates were obtained from the leaf lesions. Single pycnidial isolates (243) were obtained from leaf lesions and pathogen identification was done by morphology on PDA and will be confirmed by species-specific PCR. Changes in the frequencies of avirulent *AvrLm1*, *AvrLm4* and *AvrLm7* alleles in *L. maculans* populations at different sites in the UK are being investigated by inoculation of conidial suspensions on the cotyledons of a differential set of cultivars (Balesdent *et al*., 2005). The molecular events leading to virulence against *R* genes that are currently used (*Rlm1*, *Rlm4* and *Rlm7*) will be analysed by exploiting the *L. maculans* genome sequence (Rouxel *et al*., 2011) and *Brassica napus* genome sequence (Chalhoub *et al*., 2014) data.

The release of ascospores in the air was monitored by using Burkard spore samplers at four different sites (Bayfordbury, Hertfordshire; Langton Green Eye, Suffolk; Rothwell, Lincolnshire; Impington, Cambridgeshire) in the UK (2015/16 season) and the frequencies of *AvrLm1* and *AvrLm6* in the *L. maculans* ascospore populations will be identified by qPCR. Weather conditions such as rainfall, wind speed and temperature influence the maturation of pseudothecia and release of ascospores (Huang *et al*., 2005).

The temperatures and rainfall were recorded daily at all the Burkard spore sampler sites by weather stations located near the sites. The minimum and maximum temperatures each day (average temperature was calculated) and daily rainfall (mm) were noted in the weather stations from September to February (2015/16 season). The pattern of major ascospore release differed between sites. The first major ascospore release was observed in November at the Bayfordbury site and in October at the Eye site. At the Impington site the first major ascospore release was in December and at the Rothwell site it was observed during November.

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Mahmuti M., West J.S., Watts J., Gladders P. and Fitt B.D.L. (2009). Controlling crop disease contributes to both food security and climate change mitigation. *International Journal of Agricultural Sustainability* 7: 189-202.

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Theme- **Blackleg**, **Oral** presentation.

Brassica 2016, page 43, Melbourne, Australia; 3-7 October 2016.

Understanding phoma stem canker epidemics caused by *Leptosphaeria biglobosa* in the UK and China

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Phoma stem canker is a globally important disease of oilseed rape, caused by two closely related species, *Leptosphaeria maculans* and *L. biglobosa.* Since *L. maculans* generally causes stem base canker while *L. biglobosa* causes upper stem lesions, *L. maculans* is considered more damaging than *L. biglobosa*. Therefore, previous work has mainly focused on *L. maculans* and the importance of *L. biglobosa* in phoma stem canker epidemics has been ignored. However, results of our recent work show that *L. biglobosa* can cause severe phoma stem canker, leading to substantial yield losses, in the UK and China. In the UK, nine cultivars were used in field experiments in 2010/2011 and 2011/2012. Severity of phoma stem canker was assessed and diseased stems were collected for detection of *L. maculans* and *L. biglobosa* by quantitative PCR. In 2010/2011, the amounts of *L. biglobosa* DNA were greater than those of *L. maculans* DNA in upper stem samples but were similar to those of *L. maculans* DNA in stem base samples. However, in 2011/2012 the amounts of *L. biglobosa* DNA were greater than those of *L. maculans* DNA in both upper stem and stem base samples. In China, incidence of phoma stem canker was assessed in May 2012 (17 crops) and May 2013 (13 crops) in Hubei Province. The causal pathogens were identified by species-specific PCR. In the same seasons, plants of three cultivars were grown in pots and stems were inoculated with *L. biglobosa* conidia at the green bud stage. The field disease survey showed that only *L. biglobosa* was detected and 40% of the crops had 10-75% plants affected. Yield losses caused by *L. biglobosa* ranged from 10 to 37%. For

inoculated plants, *L. biglobosa* caused yield losses ranging from 28.8 to 56.4%. These results suggest that *L. biglobosa* can cause severe upper stem lesions and stem base cankers leading to yield losses in the UK and China.

Coexistence of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape crops

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Session: 2; Oral presentation

Leptosphaeria maculans and *L. biglobosa* are associated with phoma stem canker of oilseed rape. In many countries, *L. maculans* is considered to be more damaging than *L. biglobosa*, which is generally associated with upper stem lesions. However, *L. biglobosa* is an important pathogen in Poland and in China the disease is associated only with *L. biglobosa*. This work studied the coexistence of *Leptosphaeria* spp. on winter oilseed rape crops over three to five cropping seasons in Germany, the UK and Poland. The relative contribution of the two *Leptosphaeria* spp. to phoma leaf spot and phoma stem canker severity was examined on cultivars differing in their resistance against *L. maculans*, including cultivars with the *Rlm7* resistance gene. There was extensive colonisation by *L. biglobosa* on cultivars with the *Rlm7* gene. Effective control of *L. maculans*, by using cultivars resistant against this pathogen, may increase the possibility of severe epidemics caused by *L. biglobosa* in the future. Combined data for the abundance of the two *Leptosphaeria* spp. in air-borne inoculum and their contribution to disease severity showed that *L. biglobosa* has an increasingly important role in development of disease epidemics.

Keywords: phoma stem canker, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, oilseed rape**.**

Aspects of Applied Biology 134, 2017
Crop Production in Southern Britain

Investigating the risk of severe phoma stem canker caused by *Leptosphaeria biglobosa* on winter oilseed rape in UK

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Summary

Phoma stem canker is a damaging disease of oilseed rape (*Brassica napus*). This disease is caused by two pathogens; *Leptosphaeria maculans* and *L. biglobosa*. Air sampler data collected from four sites to monitor release of ascospores of *Leptosphaeria* spp. indicated that ascospores were released from September 2015 to February 2016 at the sites. The timings of first major ascospore and maximum ascospore released differed between the four sites. The qPCR analysis of the air samples indicated that there was more *L. maculans* DNA observed than *L. biglobosa* DNA at three sites whereas one site had more *L. biglobosa* DNA than *L. maculans* DNA. Field experiment results for the effects of fungicides on control of phoma leaf spot and phoma stem canker indicated that neither prothioconazole nor penthiopyrad plus picoxystrobin reduced the severity of *L. maculans* and *L. biglobosa* phoma leaf spots on most cultivars. However, both fungicides reduced the severity of phoma stem canker, especially on susceptible cultivars.

Key words: Phoma stem canker, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, ascospores, prothioconazole, penthiopyrad plus picoxystrobin, phoma leaf spots

Introduction

Oilseed rape (*Brassica napus*) is the third most important arable crop in the UK, after barley and wheat. Phoma stem canker is a damaging disease of this crop that has led to £100M crop losses per growing season (Stonard *et al*., 2010; Huang *et al*., 2011). This disease is caused by two closely related fungal pathogens; *Leptosphaeria maculans* and *L. biglobosa*. *L. maculans* is generally considered more damaging, causing stem base cankers, whereas *L. biglobosa* has been associated with the less damaging, superficial upper stem lesions (Toscano-Underwood *et al*., 2003; Fitt *et al*., 2006). However, recent studies suggest that *L. biglobosa* can cause both stem base cankers and upper stem lesions, leading to severe yield losses (Huang *et al*., 2014; Liu *et al*., 2014). Furthermore, *L. biglobosa* is less sensitive to some triazole fungicides than *L. maculans* (Eckert *et al*., 2010; Huang *et al*., 2011) and no resistance against *L. biglobosa* has been bred into cultivars (Fitt *et al*., 2006). This work aimed to understand the importance of *L. biglobosa* in causing phoma stem canker and to improve phoma stem canker control by targeting both fungal pathogens.

Materials and Methods

To determine the prevalence of the two *Leptosphaeria* spp. in the air, ascospore release was monitored from September 2015 to March 2016, using Burkard spore samplers set up at four sites

in the UK (Bayfordbury, Impington, Rothwell and Eye). The tapes from the spore samplers were cut into two equal halves; one half was used for microscopic spore counting whereas the other half was used for DNA extraction and qPCR to determine the proportions of *L. maculans* and *L. biglobosa* in the air samples. DNA extraction and qPCR were done using previous published methods (Huang *et al*., 2011).

To investigate the effects of fungicides on control of phoma stem canker, a field experiment was set up at Boxworth UK. Six cultivars with differing 'field' resistance to phoma stem canker pathogens were selected (Table 1) and sown in a randomised block design with three replicates. The cultivars were treated with two fungicides; Proline (prothioconazole) and Refinzar (penthiopyrad plus picoxystrobin) and assessed for phoma leaf spotting and phoma stem canker.

Table 1. *Cultivars with different AHDB (Agriculture and Horticulture Development Board) recommended resistance ratings used in field experiment. Resistance rating range 1–9, where 9 is highly resistant*

Cultivars	DK-Cabernet (DKC)	Fencer	Harper	Incentive	PR46W21	Quartz
AHDB resistance rating	6	8	8	4	3	9

Results

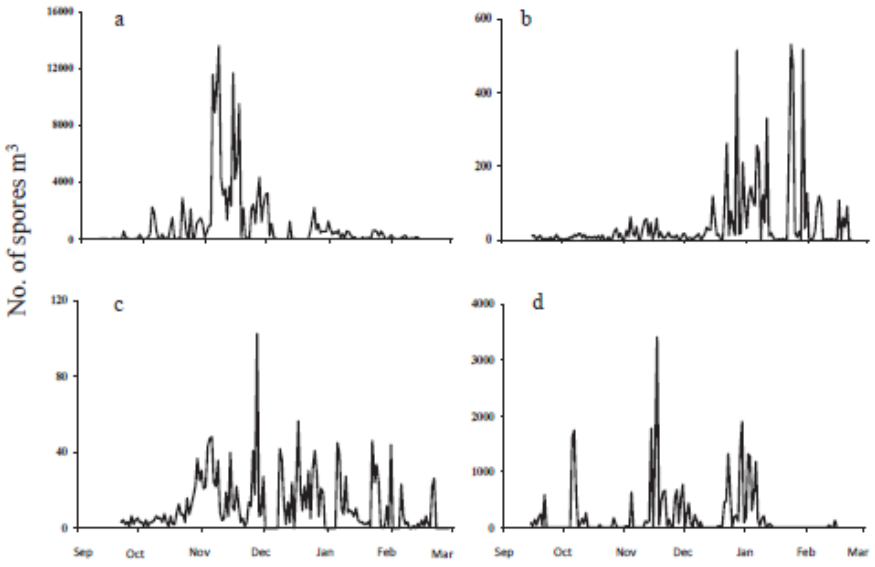

Fig. 1. Patterns of *Leptosphaeria* spp. ascospore release at (a) Bayfordbury (b) Impington (c) Rothwell and (d) Eye from September 2015 to March 2016. Spores were counted on the spore tapes at 100× magnification using a compound microscope.

Data from the microscopic analysis of spore tapes at the four sites in the UK showed that there were differences between the sites in the timing of first major ascospore release and the timing of maximum ascospore release (Fig. 1). Ascospores were released from September until February at all four sites. After February, there was little or no ascospore release. The qPCR analysis of spore tapes indicated that there was a correlation between the number of ascospores released and the

amount of *Leptosphaeria* DNA detected; large amounts of DNA were detected when there were more ascospores released and less DNA was detected when there were fewer ascospores. It was also observed that there was more *L. maculans* DNA than *L. biglobosa* detected at three sites (Impington, Rothwell and Eye) whereas there was more *L. biglobosa* DNA than *L. maculans* DNA detected at one site (Bayfordbury).

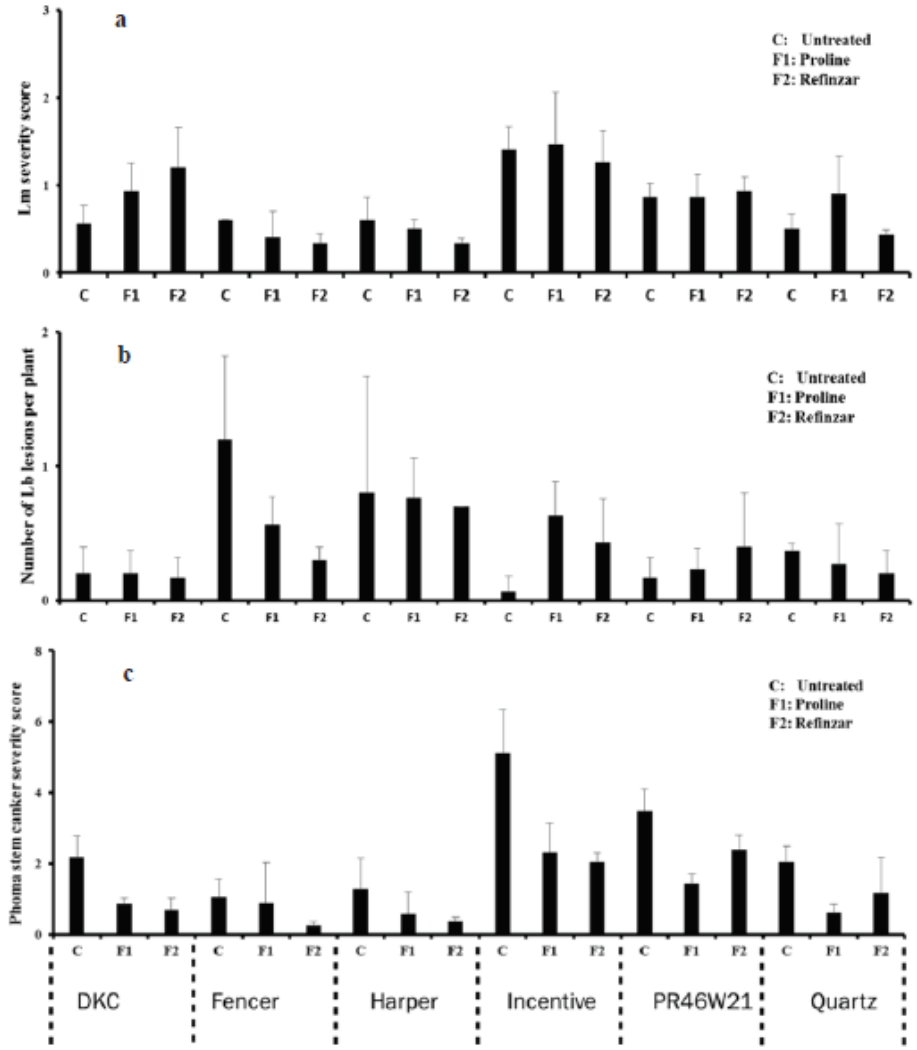

Fig. 2. Effects of fungicides on (a) *L. maculans* (Lm) phoma leaf spot severity [severity score range 0–3; 0, 0 lesions on plant; 1, 1–5 lesions on plant; 2, 6–10 lesions on plant; 3, >10 lesions on plant] (b) number of *L. biglobosa* (Lb) phoma leaf spots and (c) phoma stem canker severity [stem base cut and cross section assessed from phoma stem canker; severity score range 0–7; 0, no canker; 1, <5% stem affected; 2, 6–25% stem affected; 3, 26–50% stem affected; 4, 51–75% stem affected; 5, 76–99% stem affected; 6, 100% stem affected; 7, plant dead] on (i) untreated cultivars (control; C), (ii) cultivars treated with Proline (F1) or (iii) cultivars treated with Refinzar (F2). Error bars represent standard deviations from mean.

In the field experiment at Boxworth, both Proline (prothioconazole) and Refinzar (penthiopyrad plus picoxystrobin) had similar effects on control of phoma leaf spotting and phoma stem canker on most cultivars (Fig. 2). Neither fungicide reduced the severity of *L. maculans* phoma leaf

spotting (Fig. 2a) or the number of *L. biglobosa* phoma leaf spots (Fig. 2b) on most cultivars but both fungicides reduced the severity of phoma stem canker caused by both *L. maculans* and *L. biglobosa*, especially on susceptible cultivars (e.g. Incentive and PR46W21) (Fig. 2c).

Discussion

The pattern of ascospore release indicated that the timing of the first major ascospore release and maximum ascospore release differed between sites. The timing of the first major ascospore release in an area can be used to guide the optimal timing of fungicide sprays (Gladders *et al*., 1998; Kaczmarek *et al*., 2009; Huang *et al*., 2011). The qPCR analysis of spore tapes indicated that the amounts of *L. maculans*/*L. biglobosa* DNA detected at the four sites was different for each site with three sites having more *L. maculans* DNA and one site having more *L. biglobosa* DNA. Information on the prevalence of each *Leptosphaeria* species can help in the choice of fungicides for that particular area.

Results from the field experiment designed to study the effects of fungicides on the control of phoma stem canker indicated that both fungicides were effective in reducing the severity of phoma stem canker, especially on susceptible cultivars. As *L. maculans* and *L. biglobosa* cannot be distinguished phenotypically on stems, further qPCR analysis will be done to determine the proportions of *L. maculans* and *L. biglobosa* in untreated and fungicide treated stem samples of different cultivars. These experimental data can help in the choice of cultivars and effective fungicides in relation to the predominant *Leptosphaeria* spp. in a particular locality. Further controlled environment experiments and fungicide sensitivity tests will be done to investigate the importance of both fungal pathogens in causing phoma stem canker.

Acknowledgements

We thank the funders, Felix Thornley Cobbold Agricultural Trust, University of Hertfordshire, AHDB Cereals and Oilseeds, Biotechnology and Biological Sciences Research Council (BBSRC) UK and Innovate UK, for their financial contributions to this work.

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Poster presentation at LMS Research conference 2017, University of Hertfordshire.

MONITORING THE REGIONAL DISTRIBUTION OF RACES OF *LEPTOSPHAERIA MACULANS* POPULATIONS IN THE UK.

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Introduction: Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Currently, this disease causes UK annual yield losses >£100M despite use of fungicides. With the recent loss of some effective fungicides (e.g. Punch C) and likely loss of more fungicides through EU legislation, and predicted global warming, yield losses are likely to increase. Use of durable host resistance to control this disease is becoming ever more important. Resistance against *L. maculans* relies on major resistance (*R*) gene-mediated resistance and quantitative resistance. *R* gene-resistance against *L. maculans* is race-specific and is associated with a gene-for-gene interaction. *R* gene-mediated resistance is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent. This causes losses not only to UK farmers but also to UK breeders (loss of germplasm when resistance breaks down). Therefore, detection and identification of virulent races of *L. maculans* is crucial for effective deployment of *R* gene-mediated resistance.

Methods: The release of ascospores in the air was monitored by using Burkard spore samplers at four different sites in the UK and the frequencies of avirulent *AvrLm1* and *AvrLm6* in the *L. maculans* ascospore populations were identified by qPCR. Winter oilseed rape field experiments were set up at four different sites in the UK. Single pycnidial isolates were obtained from leaf lesions on cultivar Drakkar from all the sites and pathogen identification was done by morphology on PDA and confirmed by species-specific PCR. Changes in the frequencies of avirulent *AvrLm1*, *AvrLm4*, *AvrLm6* or *AvrLm7* alleles in *L. maculans* populations at different sites in the UK were investigated by

inoculation of conidial suspensions on the cotyledons of a differential set of cultivars.

Results: There were differences between the four sites in patterns of ascospore release and in dates of first major ascospore release. There were also differences between sites in the timing of release of ascospores with avirulent *AvrLm1* and *AvrLm6* alleles. *AvrLm6* alleles were detected more frequently from spore samples compared to *AvrLm1* alleles. All the isolates tested from different sites were avirulent against *Rlm7*. There were variations between sites in the frequencies of avirulent *AvrLm1* and *AvrLm4* alleles. None of the isolates from different sites were avirulent against *Rlm3* (*AvrLm3*) or *Rlm9* (*AvrLm9*).

Conclusion: The *AvrLm7* allele is predominant in the UK *L. maculans* populations suggesting that the corresponding *Rlm7* resistance gene is still effective. Virulent *avrLm3* and *avrLm9* alleles are predominant in the UK *L. maculans* populations suggesting that *Rlm3* and *Rlm9* resistance genes are no longer effective in the UK. There is a need to continue the monitoring the regional distribution of *L. maculans* populations in the UK for the effective deployment of *R* genes.

Poster presentation at AFCP forum 2017, 5 April 2017, University of Hertfordshire.

Identification of new virulent races of *Leptosphaeria maculans* populations on oilseed rape in the UK

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Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Currently, this disease causes UK annual yield losses >£100M despite use of fungicides. With recent loss of the most effective fungicides through EU legislation, potential yield losses will increase (Mahmuti *et al*., 2009). Use of host resistance to control this disease is becoming ever more important. However, new sources of resistance are often rendered ineffective due to pathogen population changes from avirulent to virulent. There is a need to monitor emergence of new virulent races of *L. maculans* and prevent them from spreading into new regions and to investigate molecular mechanisms of mutation from avirulent to virulent in *L. maculans* populations.

Leptosphaeria maculans, the cause of phoma stem canker disease of oilseed rape, develops gene-for-gene interactions with its host plant resistance genes. Pathogens develop new effector proteins to overcome recognition by the host and plants evolve new recognition proteins to target novel *Avr* proteins. In this study, the regional distribution of the *L. maculans* races in the UK was monitored and the molecular mechanisms of mutation to virulence were investigated. Field experiment sites were set up at different sites in the UK: from leaf spot lesions on Drakkar (susceptible cultivar, trap crop) and other cultivars (with *Rlm7* resistance gene), 64, 88 and 111 *L. maculans* isolates, were obtained in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons, respectively. Ninety-two single ascospore isolates were obtained from stem samples from two sites from 2016/2017 cropping season. Changes in frequencies of avirulent *AvrLm1*,

AvrLm4 or *AvrLm7* alleles were investigated by testing isolates on cotyledons of a differential set of cultivars. Isolates virulent towards *Rlm1*, *Rlm4* or *Rlm7* were investigated for molecular events. There were variations in the frequencies of avirulent *AvrLm1* and *AvrLm4* alleles between cropping seasons. All the isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season. In the 2016/2017 season, 6.8% of isolates were virulent towards *Rlm7*, whereas the percentage of virulent isolates towards *Rlm7* has increased to 16.3% in 2017/2018 season. The molecular mechanism of mutation to virulence in *AvrLm1* was observed to be whole gene deletion in 86% of isolates. The other 13% were sequenced to investigate events leading to virulence. Whole gene deletion was observed in 6% or 50% of isolates carrying the virulent alleles of *AvrLm4* or *AvrLm7*, respectively. The others need to be sequenced for further investigation.

Poster presentation at LMS Research conference 2018, 10 April 2018, University of Hertfordshire.

UNDERSTANDING *R* GENE-MEDIATED RESISTANCE AGAINST *LEPTOSPHAERIA MACULANS* FOR EFFECTIVE CONTROL OF PHOMA STEM CANKER IN OILSEED RAPE

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Introduction: Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Use of host resistance to control this disease is becoming ever more important. *R* gene-mediated resistance against *L. maculans* is associated with a gene-for-gene interaction that involves the recognition of a pathogen effector (*Avr*) gene product by the host *R* gene product, thus rendering the pathogen unable to colonise the host. However, new sources of resistance are often rendered ineffective due to pathogen population changes from avirulent to virulent. There is a need to understand the *R* gene-mediated resistance against *L. maculans* for effective control of Phoma stem canker.

Methods: Severity of phoma leaf spotting and phoma stem canker were done on eleven different oilseed rape cultivars with different resistance (*R*) genes with/without background quantitative resistance (DK Cabernet, Es Astrid, Whisky, Angus, Adriana, DK Extrovert, DK Exalte, Incentive, Harper, Amalie and Mentor) at different sites (Woodhall Farm, Hertfordshire; Morley, Norfolk; Rothwell, Lincolnshire; Impington, Cambridgeshire and Trumpington, Cambridgeshire) in the UK (2015/2016 to 2017/2018 cropping seasons). Leaves with phoma leaf spots were collected from cultivar Drakkar (no *R* gene; used as trap crop) from all the five sites and *L. maculans* isolates were obtained from the leaf lesions for all the three cropping seasons. Changes in the frequencies of avirulent *AvrLm1*, *AvrLm4*, *AvrLm6* and *AvrLm7* alleles in *L. maculans*

populations at different sites in the UK were investigated by inoculation of conidial suspensions on the cotyledons of a differential set of cultivars.

Results: There were differences in the severity of phoma leaf spotting and phoma stem canker between cultivars and sites. Cultivars with *Rlm*7 resistance gene showed less phoma leaf spotting and phoma stem canker compared to other cultivars with other resistance genes and cultivars without resistance genes. There were variations in the frequencies of avirulent *AvrLm1* and *AvrLm4* alleles between cropping seasons and sites. All the isolates from different sites were avirulent against *Rlm7* and *Rlm6* in the 2015/2016 season. In the 2016/2017 season, 6.8% of isolates were virulent towards *Rlm7*.

Conclusion: The *AvrLm6* and *AvrLm*7 alleles are predominant in the UK *L. maculans* populations suggesting that the corresponding *Rlm6* and *Rlm7* resistance genes are still effective. Virulent *avrLm3* and *avrLm9* alleles are predominant in the UK *L. maculans* populations suggesting that *Rlm3* and *Rlm9* resistance genes are no longer effective in the UK. There is a need to continue the monitoring of the regional distribution of *L. maculans* populations in the UK for the effective deployment of *R* genes.

Acknowledgements: Innovate UK consortium, Perry Foundation, Chadacre Agriculture Trust, Department of Biological and Environmental Sciences; University of Hertfordshire.

Poster presentation at International Congress of Plant Pathology (ICPP) 2018, 29 July- 3 August 2018, Boston, USA.

Molecular mechanisms of mutation to virulence in *Leptosphaeria maculans* populations in the UK.

Lakshmi Harika Gajula, Georgia K. Mitrousia, Bruce D. L. Fitt and Yongju Huang

Leptosphaeria maculans, the cause of phoma stem canker disease of oilseed rape, develops gene-for-gene interactions with its host plant resistance genes. Pathogens develop new effector proteins to overcome recognition by the host and plants evolve new recognition proteins to target novel *Avr* proteins. In this study, the regional distribution of the *L. maculans* races in the UK was monitored and the molecular mechanisms of mutation to virulence were investigated. Field experiment sites were set up at different sites in the UK: from leaf spot lesions on Drakkar (susceptible cultivar, trap crop) and other cultivars (with *Rlm7* resistance gene), 64 and 88 *L. maculans* isolates, were obtained in the 2015/2016 and 2016/2017 cropping seasons, respectively. Changes in frequencies of avirulent *AvrLm1*, *AvrLm4* or *AvrLm7* alleles were investigated by testing isolates on cotyledons of a differential set of cultivars. Isolates virulent towards *Rlm1*, *Rlm4* or *Rlm7* were investigated for molecular events. There were variations in the frequencies of avirulent *AvrLm1* and *AvrLm4* alleles between cropping seasons. All the isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season. In the 2016/2017 season, 6.8% of isolates were virulent towards *Rlm7*. The molecular mechanism of mutation to virulence in *AvrLm1* was observed to be whole gene deletion in 86% of isolates. The other 13% were sequenced and the events will be investigated. Whole gene deletion was observed in 6% or 50% of isolates carrying the virulent alleles of *AvrLm4* or *AvrLm7* respectively. The others need to be sequenced for further investigation.

ICPP Boston 29 July- 3 August 2018; proposed abstract; short talk

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Ignored fungal pathogen sibling – *Leptosphaeria biglobosa*

In nature, plants are often infected by more than one pathogen and there are many factors affecting their co-existence on their host which lead to changes in their predominance. Phoma stem canker is a damaging disease of oilseed rape (*Brassica napus*) and Brassica vegetables. This disease is caused by two closely related sibling pathogens, *Leptosphaeria maculans* and *L. biglobosa.* Since *L. maculans* generally causes stem base canker while *L. biglobosa* causes upper stem lesions, *L. maculans* is considered more damaging than *L. biglobosa*. Therefore, previous work has mainly focused on *L. maculans* and the importance of *L. biglobosa* in phoma stem canker epidemics has been ignored. However, results of our recent work show that *L. biglobosa* can cause both damaging upper stem lesions and stem base cankers. Furthermore, *L. biglobosa* is less sensitive to some triazole fungicides than *L. maculans.* The need for effective host resistance to control this disease is greater than ever. However, previous breeding for cultivar resistance has targeted only *L. maculans*; there is no information about cultivar resistance against *L. biglobosa*. Recent studies have shown that cultivars resistant against *L. maculans* are often more susceptible to *L. biglobosa*. For effective control of phoma stem canker, there is a need to target both *L. maculans* and *L. biglobosa.* In this talk, the reasons why *L. biglobosa* has recently increased in importance in phoma stem canker epidemics in the UK will be discussed, using results from field experiments over three seasons and controlled environment experiments.

Poster presentation at AFCP forum 2019, 11 April 2019, University of Warwick, UK.

Identification of new virulent races in *Leptosphaeria maculans* populations on oilseed rape in the UK

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Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Currently, this disease causes UK annual yield losses >£100M despite use of fungicides. With recent loss of the most effective fungicides through EU legislation, potential yield losses will increase. Use of host resistance to control this disease is becoming ever more important. However, new sources of resistance are often rendered ineffective due to pathogen population changes from avirulent to virulent.

L. maculans develops gene-for-gene interactions with its host plant resistance (*R*) genes. A given host *R* gene is effective only when the protein coded by the *R* gene recognises an effector produced by the corresponding *L. maculans* effector gene. With both sexual and asexual reproduction, *L. maculans* has a high potential for mutation to overcome recognition by host *R* genes. For effective use of host *R* genes, there is a need to monitor emergence of new virulent races of *L. maculans* and prevent them from spreading into new regions.

Field experiments were set up at different sites in the UK; from leaf spot lesions on Drakkar (susceptible cultivar, trap crop) and other cultivars (with *Rlm7* resistance gene), 64, 88 and 111 *L. maculans* isolates, were obtained in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons, respectively. Ninety-two single ascospore isolates were also obtained from stem samples from two sites from the 2016/2017 cropping season. Changes in frequencies of avirulent alleles of *AvrLm1*, *AvrLm4* or *AvrLm7* effector genes were investigated by

testing isolates on cotyledons of a differential set of cultivars. Isolates virulent towards resistance genes *Rlm1*, *Rlm4* or *Rlm7* were investigated for molecular events leading to virulence.

There were variations in the frequencies of avirulent alleles of *AvrLm1* and *AvrLm4* between sites and cropping seasons. All the isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season. In the 2016/2017 season, 6.8% of isolates were virulent towards *Rlm7*, whereas the frequency of isolates virulent towards *Rlm7* had increased to 16.3% in the 2017/2018 season. For single ascospore isolates from the 2016/2017 season, 25% of them were virulent towards *Rlm7*. Forty important isolates were selected on the basis of their race structure for whole genome sequencing to investigate molecular mechanisms of mutations to virulence.

Poster presentation at LMS Research conference 2019, 16 April 2019, University of Hertfordshire.

Identification of new virulent races in *Leptosphaeria maculans* populations on oilseed rape in the UK

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Introduction: Phoma stem canker, caused by the fungal pathogen *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Currently, this disease causes UK annual yield losses >£80M despite use of fungicides. With recent loss of the most effective fungicides (e.g. Refinzar) and predicted climate change, potential yield losses will increase. Use of host resistance to control this disease is becoming ever more important. However, new sources of resistance are often rendered ineffective due to pathogen population changes from avirulent to virulent. There is a need to monitor emergence of new virulent races of *L. maculans* for the effective deployment of *R* genes. In the UK, *Rlm7* is currently the most effective gene used for control of phoma stem canker. For effective use of this resistance, changes in *L. maculans* populations over three growing seasons were monitored.

Methods: Field experiment sites were set up at different sites in the UK in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons. Leaves with phoma leaf spots were collected from cultivar Drakkar (no *R* gene; used as trap crop) and other cultivars (with *Rlm7* resistance gene) from all the sites and *L. maculans* isolates were obtained from the leaf lesions for all the three cropping seasons. *L. maculans* isolates (single ascospore isolates) were also obtained from stem samples from two sites from 2016/2017 cropping season. Changes in frequencies of avirulent alleles of different effector genes were investigated by inoculation of conidial suspensions on the cotyledons of a set of *Brassica napus* differential cultivars/lines.

Results: From leaf spot lesions on Drakkar and other cultivars with *Rlm7* resistance gene, 64, 88 and 111 *L. maculans* isolates were obtained in the 2015/2016, 2016/2017 and 2017/2018 cropping seasons, respectively. Ninety-two single ascospore isolates were obtained from stem samples from the 2016/2017 cropping season. There were variations in frequencies of avirulent alleles of *AvrLm1, AvrLm3, AvrLm4, AvrLm5* and *AvrLm6* between sites and cropping seasons. All the isolates from different sites were avirulent against *Rlm7* in 2015/2016. The frequency of isolates virulent towards *Rlm7* was 6.8% in 2016/2017 and increased to 16.3% in 2017/2018. Of single ascospore isolates from the 2016/2017 season, 25% were virulent towards *Rlm7*.

Conclusion: The *AvrLm7* allele was predominant in the UK *L. maculans* populations until 2015/2016; however, the frequency of isolates virulent towards the corresponding resistance gene *Rlm7* has increased since 2016/2017 suggesting that there is a risk of breakdown of *Rlm7* mediated resistance in the UK.

Acknowledgements: BBSRC Innovate UK consortium, Perry Foundation, Chadacre Agriculture Trust, Department of Biological and Environmental Sciences; University of Hertfordshire.

Oral presentation at 15th International Rapeseed Congress, 16-19 June 2019, Berlin

Changes in race structure of *Leptosphaeria maculans* populations on oilseed rape in the UK

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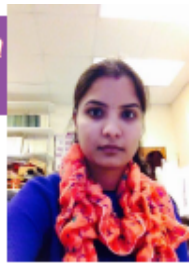
Phoma stem canker, caused by *Leptosphaeria maculans*, is a damaging disease on oilseed rape in the UK and can cause yield losses up to 50% if the disease is not controlled. Currently, this disease causes UK annual yield losses >£100M despite use of fungicides. With recent loss of the most effective fungicides, potential yield losses will increase. Use of host resistance to control this disease is becoming ever more important. Resistance against *L. maculans* relies on a combination of major resistance (*R*) gene-mediated resistance and quantitative resistance. *R* gene-resistance is race-specific and is often rendered ineffective when pathogen populations evolve from avirulent to virulent against the *R* genes. Therefore, detection and identification of new virulent pathogen races is crucial for effective deployment of *R* gene-mediated resistance.

In the UK, *Rlm7* is currently the most effective gene used for control of phoma stem canker. For effective use of this resistance, changes in *L. maculans* populations over three growing seasons were monitored. Field experiments were set up at different sites in the UK; *L. maculans* isolates were obtained from leaf spot lesions on Drakkar (susceptible cultivar, trap crop) and other cultivars carrying the *Rlm7* resistance gene in the 2015/2016, 2016/2017 and 2017/2018 seasons. In addition, single ascospore isolates were obtained from phoma stem canker samples from 2016/2017. Changes in frequencies of avirulent alleles of different effector genes were investigated by testing isolates on cotyledons of a differential set of cultivars. Isolates virulent towards resistance genes *Rlm1*, *Rlm4* or *Rlm7* were investigated for molecular events leading to virulence.

There were variations in frequencies of avirulent alleles of *AvrLm1, AvrLm3, AvrLm4, AvrLm5* and *AvrLm6* between sites and cropping seasons. All the

isolates from different sites were avirulent against *Rlm7* in the first season. The frequency of isolates virulent towards *Rlm7* was 6.8% in 2016/2017 and increased to 16.3% in 2017/2018. Of single ascospore isolates from the 2016/2017 season, 25% were virulent towards *Rlm7*. Forty isolates were selected on the basis of combination of effector genes for whole genome sequencing to investigate molecular mechanisms of mutations to virulence.

Monitoring the regional distribution of races of *Leptosphaeria maculans* populations in the UK



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INTRODUCTION

- Phoma stem canker, caused by the fungus *Leptosphaeria maculans*, is an important disease of oilseed rape.
- Use of host resistance is the most important and effective way to control phoma stem canker.
- Two types of resistance to *L. maculans* are qualitative resistance (*R*-gene resistance) and quantitative resistance (QR).
- *R* gene-resistance against *L. maculans* is race-specific and is associated with a gene-for-gene interaction.
- *R* gene-mediated resistance is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent.
- This work aims to monitor virulent races of *L. maculans* both in air (ascospores) and in winter oilseed rape crops as a basis for the effective deployment of *R* gene-mediated resistance in the UK.

MATERIALS & METHODS

Release of ascospores in air was monitored by using Burkard spore samplers at four sites in the UK and frequencies of avirulent *AvrLm1* and *AvrLm6* in the *L. maculans* ascospore populations were identified by qPCR.

Figure 1: Burkard spore sampler at Bayfordbury in Hertfordshire (2015/16 season). Burkard sampler surrounded by stem debris in free-draining plastic trays. Weather station in background.

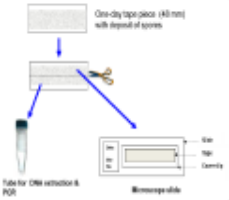

Figure 2: Processing of the spore tape from Burkard samplers. 48 mm piece of tape cut longitudinally and piece on top placed on a microscopic slide, for counting number of *Leptosphaeria* ascospores. The bottom piece stored in a 2 ml tube for DNA extraction.

Winter oilseed rape field experiments were set up at five sites. Single pycnidial isolates were obtained from leaf lesions on cultivar Drakkar from all sites and pathogen identification was done by morphology on PDA and confirmed by species-specific PCR. Changes in frequencies of avirulent *AvrLm1*, *AvrLm4*, *AvrLm6* or *AvrLm7* alleles in *L. maculans* populations at different sites were investigated by inoculation of conidial suspensions on cotyledons of a differential set of cultivars.

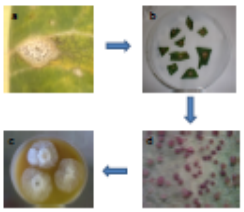

Figure 3: Procedure for single pycnidial isolation to obtain isolates of *Leptosphaeria* species. Leaf lesions (a) were incubated in a Petri dish lined with filter paper (b). After 2-3 days of incubation the cirrhus produced from a pycnidium (c) was mixed with sterile water and pipetted on a PDA agar media plate. *L. maculans* cultures were observed after 4-5 days of incubation (d).

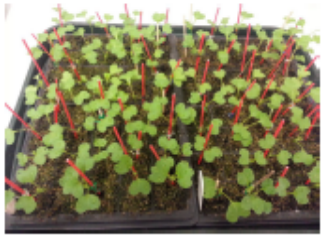

Figure 4: Tray with 14 day old seedlings of the *Brassica* cultivars or lines from the differential set immediately after inoculation with the spore suspensions of *Leptosphaeria maculans*

RESULTS

- Differences between the four sites in patterns of ascospore release and in dates of first major ascospore release.
- Differences between sites in the timing of release of ascospores with avirulent *AvrLm1* and *AvrLm6* alleles.
- *AvrLm6* alleles were detected more frequently from spore samples compared to *AvrLm1* alleles.
- All the isolates tested were avirulent against *Rlm6* or *Rlm7*. There were variations between sites in the frequencies of avirulent *AvrLm1* and *AvrLm4* alleles.
- None of the isolates were avirulent against *Rlm2* (*AvrLm2*), *Rlm3* (*AvrLm3*) or *Rlm9* (*AvrLm9*) at four sites.

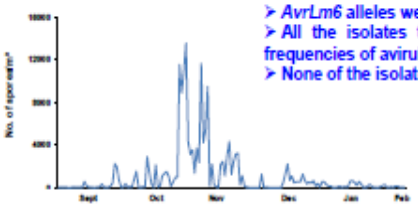

Figure 5: Patterns of *Leptosphaeria* ascospore release at Bayfordbury (2015/16 season). Daily counts of ascospores released from 9 September 2015 to 16 February 2016 from winter oilseed rape debris placed around a Burkard spore sampler.

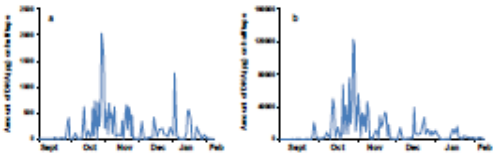

Figure 6: Patterns of *AvrLm1* (a) and *AvrLm6* (b) alleles in the spore samples at Bayfordbury (2015/16 season). The DNA was extracted on half tape by CTAB method and qPCR was used for detecting the amounts of *AvrLm1* and *AvrLm6* DNA.

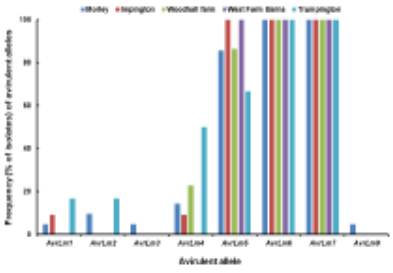

Figure 7: Mean frequencies (%) of avirulent alleles in *Leptosphaeria maculans* populations at different sites in the UK.

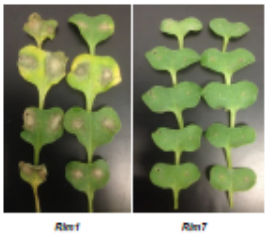

Figure 8: Phoma leaf spot disease symptoms on differential set of cultivars/lines with resistance genes *Rlm1* or *Rlm7* at 17-days post-inoculation with *L. maculans* isolates.

CONCLUSION

The *AvrLm6* or *AvrLm7* alleles are predominant in the UK *L. maculans* populations suggesting that the corresponding *Rlm6* or *Rlm7* resistance genes are still effective. Virulent *avrLm2*, *avrLm3* and *avrLm9* alleles are predominant in the UK *L. maculans* populations suggesting that *Rlm2*, *Rlm3* or *Rlm9* resistance genes are no longer effective in the UK. There is a need to continue the monitoring of the regional distribution of *L. maculans* populations in the UK for the effective deployment of *R* genes.

ACKNOWLEDGEMENTS

Molecular mechanisms of mutation to virulence in *Leptosphaeria maculans* populations in the UK

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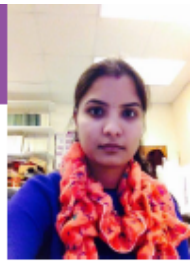


INTRODUCTION

- Phoma stem canker disease of oilseed rape caused by the fungus *Leptosphaeria maculans* is often controlled by deploying race-specific *R* genes in the UK.
- However, *R* gene-mediated resistance is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent.
- Due to this rapid adaptation of *L. maculans* populations to selection, new virulent races through mutation or deletion of effector genes rise.
- In this study, the regional distribution of the *L. maculans* races in the UK and the molecular mechanisms of mutation to virulence are being investigated to provide effective management of the disease.

MATERIALS & METHODS

- Winter oilseed rape field experiments at different sites in 2015/2016 & 2016/2017 cropping seasons with twelve different cultivars with different *R* genes with/without quantitative resistance in background.
- Single pycnidial isolates obtained from leaf lesions on cultivar Drakkar (susceptible, trap crop) and other cultivars (with *Rlm7* resistance gene)
- Changes in frequencies of avirulent *AvrLm1*, *AvrLm4* or *AvrLm7* alleles in *L. maculans* populations at different sites investigated by inoculation of conidial suspensions on cotyledons of a differential set of cultivars.

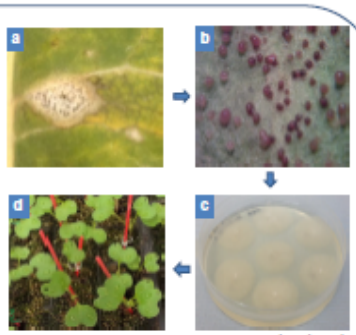
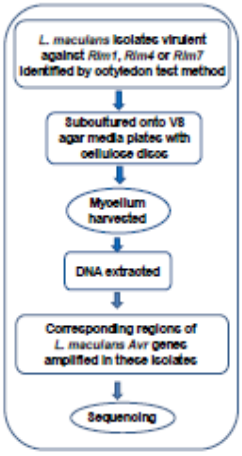


Figure 1: Procedure for single pycnidial isolation to obtain isolates of *Leptosphaeria* species. Leaf lesions (a) incubated in a Petri dish lined with filter paper. After 2-3 days of incubation, the cirrhus produced from a pycnidium (b) was mixed with sterile water and pipetted on a PDA plate. *L. maculans* cultures observed after 4-5 days incubation (c). Conidial suspensions made from the isolates inoculated onto a differential set of cultivars (d).



RESULTS

- Differences in frequencies of avirulent *AvrLm1* and *AvrLm4* alleles between sites and cropping seasons.
- All isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season.
- In the 2016/2017 season, 6.8% of isolates were virulent towards *Rlm7*.

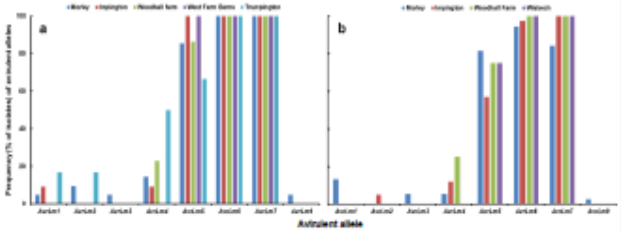


Figure 2: Mean frequencies (%) of avirulent alleles in *Leptosphaeria maculans* populations at different sites in the UK in 2015/2016 (a) and 2016/2017 (b) cropping seasons.

- No amplification with the *AvrLm1* external primers was observed in 86% of isolates carrying the virulent alleles of *AvrLm1*, that indicates whole gene deletion. However, this needs to be confirmed by Southern blotting.
- The other 13% were sequenced to investigate events leading to virulence in *AvrLm1*, found to be point mutations.
- No amplification with the *AvrLm4-7* external primers was observed in 6% or 50% of isolates carrying the virulent alleles of *AvrLm4* or *AvrLm7*, respectively. This is an indication of whole gene deletion.

RESULTS

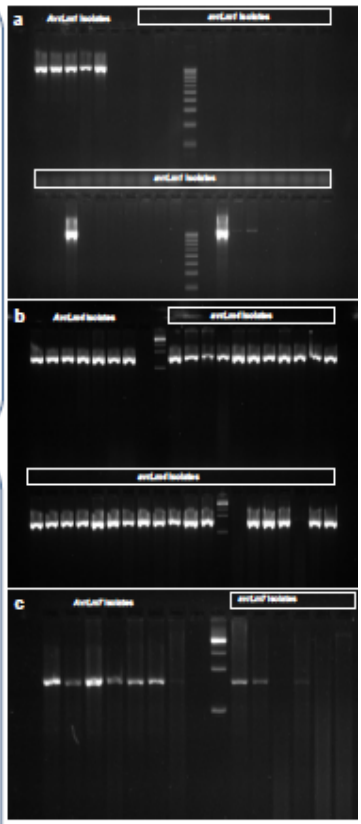


Figure 3: Gel images showing amplification of *AvrLm1* (a) and *AvrLm4-7* (b)(c) regions in *L. maculans* isolates containing avirulent and virulent alleles against *Rlm1* (a), *Rlm4* (b) and *Rlm7* (c) resistance genes.

DISCUSSION

- The predominance of the avirulent *AvrLm7* allele in UK *L. maculans* populations suggests that the corresponding *Rlm7* resistance gene is still effective. There is a need to continue monitoring of regional distribution of *L. maculans* races in the UK for effective deployment of *R* genes.
- Whole gene deletion is likely to be the major molecular mechanism of mutation towards virulence in the *AvrLm1* gene. Some isolates carried the virulent alleles of *AvrLm4* or *AvrLm7*. However, there is a need to investigate other molecular mechanisms of virulence in *AvrLm4* or *AvrLm7* genes in the UK.
- New information about molecular mechanisms of mutation to virulence in *L. maculans* can be used to optimise the use of novel resistances so that they do not break down quickly. This can be used to develop new strategies to increase the durability of resistance against the phoma stem canker pathogen *L. maculans*.

ACKNOWLEDGEMENTS

University of Hertfordshire · Perry Foundation · Chadacre Agricultural Trust · BBSRC bioscience for the future · Innovate UK · LS Plant Breeding Combining Excellence · Limagrain United Kingdom · Grainseed ltd · MONSANTO · WOODHALL ESTATE HERTFORDSHIRE · HUTCHINSONS

Identification of new virulent races in *Leptosphaeria maculans* populations on oilseed rape in the UK

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INTRODUCTION

- Phoma stem canker disease of oilseed rape caused by *Leptosphaeria maculans* is often controlled in the UK by deploying race-specific *R* genes.
- However, *R* gene-mediated resistance is often rendered ineffective in 2-3 years due to *L. maculans* population changes from avirulent to virulent.
- For effective use of host *R* genes, there is a need to monitor emergence of new virulent races of *L. maculans* and prevent them from spreading into new regions.

MATERIALS & METHODS

- Winter oilseed rape field experiments at different sites in 2015/2016, 2016/2017 & 2017/2018 cropping seasons with twelve cultivars.
- Single pycnidial (from leaf lesions) (Fig 1) and single ascospore isolates (from stem samples) (Fig 2) were obtained.
- Changes in frequencies of avirulent alleles of different effector genes in *L. maculans* populations investigated by inoculation of conidial suspensions on cotyledons of a differential set of cultivars.

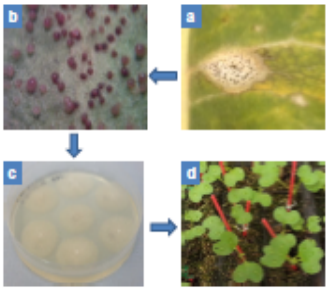

Fig 1: Single pycnidial isolates of *Leptosphaeria maculans*. Leaf lesions (a) incubated for pycnidial production (b). Cirrhus from a single pycnidium cultured on a PDA plate (c). Conidial suspensions made from isolates inoculated onto a differential set of cultivars (d).

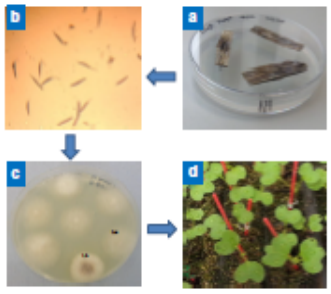

Fig 2: Single ascospore isolation of *Leptosphaeria maculans*. Stem pieces with mature pseudothecia (a) attached to lid of Petri-dish. Water sprayed on stem pieces to induce ascospore release. Single ascospores (b) cultured on PDA plates. *Leptosphaeria* isolates observed at 7-days post isolation (c). Conidial suspensions made from the isolates inoculated onto a differential set of cultivars (d).

Fig 3: The 0-9 scale used to score the disease on cotyledons of *B. napus* at 17-days post inoculation.

RESULTS

➢ There were variations in frequencies of avirulent alleles of different effector genes in *L. maculans* populations between sites and cropping seasons (Fig 4). All the isolates from different sites were avirulent against *Rlm7* in the 2015/2016 season (a). In the 2016/2017 season (b) 6.8% of isolates were virulent towards *Rlm7*, whereas the frequency of isolates virulent towards *Rlm7* had increased to 16.3% in the 2017/2018 season (c). For single ascospore isolates from the 2016/2017 season (d), 25% of them were virulent towards *Rlm7*.

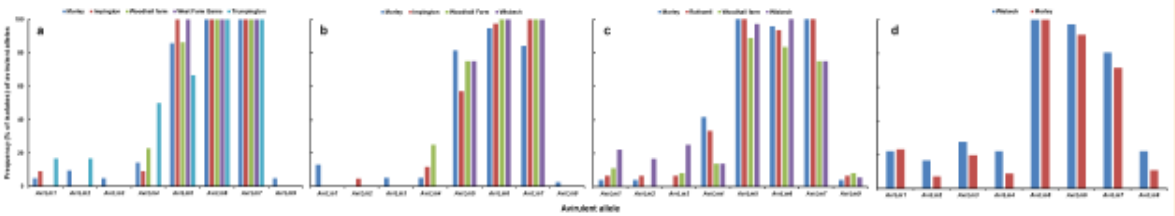

Fig 4: Frequencies (%) of avirulent alleles in *Leptosphaeria maculans* populations from leaves at different UK sites in 2015/2016 (a), 2016/2017 (b) and 2017/2018 (c) cropping seasons and from stem samples at two UK sites in the 2016/2017 cropping season (d).

CONCLUSIONS

✓ The *AvrLm7* allele was predominant in the UK *L. maculans* populations until 2015/2016; however, the frequency of isolates virulent towards *Rlm7* has increased in 2016/2017 and 2017/2018. This suggests that the *Rlm7* resistance gene that has been widely used in the UK oilseed rape cultivars is at risk.

✓ There is a need to continue monitoring regional distribution of *L. maculans* populations in UK to guide effective deployment of *R* genes.

ACKNOWLEDGEMENTS

University of Hertfordshire · UH · Perry Foundation · Chadacre Agricultural Trust · BBSRC bioscience for the future · Innovate UK · LS Plant Breeding Combining Excellence · Limagrain United Kingdom · Grainseed ltd · MONSANTO · WOODHALL ESTATE HERTFORDSHIRE · HUTCHINSONS