Identification of *Avr* alleles in *Leptosphaeria maculans* isolates from different oilseed rape cultivars

Lakshmi Harika Gajula

‘Submitted to the University of Hertfordshire in partial fulfilment of the requirements of the degree of MSc by Research awarded under the Schedule K’

October, 2014.

Abstract

This report describes a survey of the regional distribution of races of *Leptosphaeria maculans* done by characterising isolates from phoma leaf spot samples taken from different oilseed rape cultivars at different sites in the UK. Phoma leaf spot assessment was done on cultivars Drakkar (with no *R* gene), Bilbao (with *Rlm4*), Adriana (with *Rlm4* and quantitative resistance), Roxet and Excel (with *Rlm7*) sampled from three sites (Harpenden, Cranwell & Bainton) results showed that cultivar Drakkar had most phoma leaf spots. The cultivar Adriana had fewer phoma leaf spots than Bilbao or Roxet. The identity of alleles at eight *Avr* loci was determined for *L. maculans* isolates from these cultivars. Additionally some more isolates from Banbury, Morley and Rothwell in UK and Verpillieres in France were also used for the identification of *Avr* alleles. The results of the cotyledon tests confirmed that isolates from cultivars Bilbao and Adriana were virulent against *Rlm4.* An isolate from cultivar Roxet was virulent against *Rlm7.* The isolates from cultivar Drakkar were virulent against *Rlm1, Rlm2, Rlm3, Rlm5* and *Rlm9*. The frequency of the avirulent *AvrLm1* allele was 35% in this study. No isolates of *L. maculans* possessed the *AvrLm2* allele. In this survey it was observed that the frequencies of the *AvrLm3* allele (3.8%) and *AvrLm9* allele (3.8%) were less than those of the *AvrLm4* allele (50%), *AvrLm5* allele (84.6%), *AvrLm6* allele (96.2%) and *AvrLm7* allele (88.5%). This study suggested that *Rlm6* and *Rlm7* are still effective sources of resistance against *L. maculans* in oilseed rape in the UK. Seventy-two isolates obtained from nine different oilseed rape cultivars from the sites Harpenden and Spalding in the UK and from Poland were checked for their identity as *L. maculans* by species-specific PCR and for the presence of the *AvrLm1* allele by allele-specific PCR. Sixty-nine of them were confirmed as *L. maculans* and only four of these sixty-nine isolates (from the cultivar Roxet) had the avirulent *AvrLm1* allele.

Acknowledgements

I am grateful to **Prof. Bruce Fitt** for giving me an opportunity and support to carry out my work successfully.

With profound respect, I wish to express my deep sense of gratitude to **Dr Yongju Huang** for her valuable suggestions and encouragement during the course of my work.

I would also like to thank **Georgia Mitrousia, Dahlia Sidique and Chinthani Karandeni** for their help and cooperation in conducting the experiments. I would like to convey my sincere thanks to other Research Associates for their cooperation during my work.

I give heartfelt thanks to **my parents** for their encouragement, cooperation and support in all my endeavours.

Finally, my biggest heartfelt thanks go to my husband **Kishore Malabhanti** for his moral support, without which I would not have been able to accomplish this journey of learning.

LAKSHMI HARIKA GAJULA

Contents

Page

List of Figures

Page

List of tables

Page

List of Appendices

Page

1. Introduction

1.1 Oilseed rape (*Brassica napus*)

Oilseed rape (*Brassica napus*) is one of the most economically important crops, which is grown world-wide as a source of vegetable oil and biodiesel (Fitt, Brun, Barbetti & Rimmer, 2006a). This crop is grown in temperate climates in the northern hemisphere (China, Canada, Europe and India) and in southern hemisphere (Australia, South Africa and South America). Oilseed rape is the third most important arable crop in the UK, after wheat and barley. In the UK, oilseed rape production has increased both in area and in price. The area of oilseed rape planted has increased over the last decade. Due to the poor weather conditions at sowing in 2013, the area of oilseed rape cultivation was less (690 thousand hectares) than the previous season and the price has decreased to £310 per tonne (Figure 1).

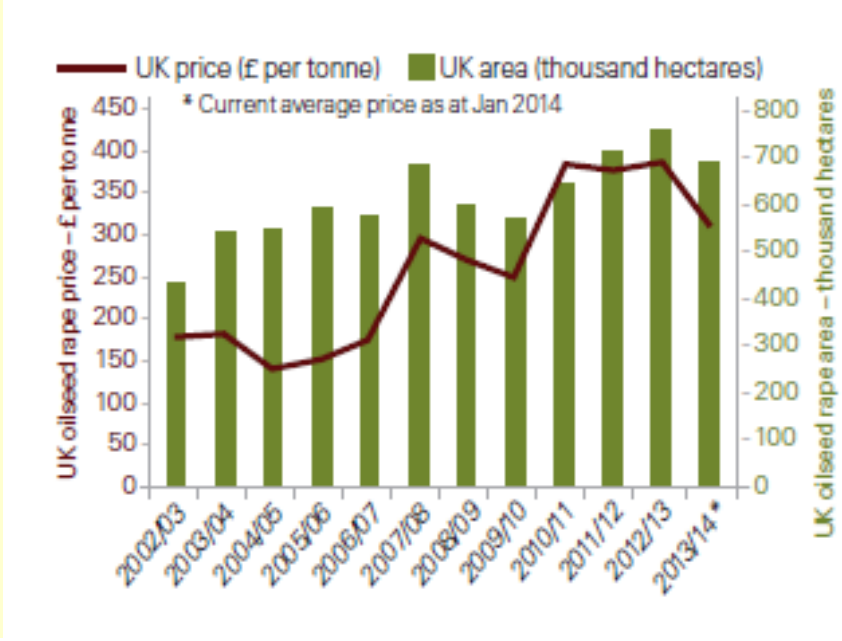

Figure 1: The oilseed rape crop area of cultivation and change in the price in the UK from 2002/03 to 2013/14 (Source: HGCA Recommended lists 2014/15 for cereals and oilseeds)

Most (98%) of the oilseed rape is cultivated for the production of food grade oil and the remaining meal is used in the preparation of animal feed. The oil is usually used for margarines and vegetable oils and the derivatives are used in the manufacturing of cosmetics and detergent products (Kings, 1997). 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. It is responsible for worldwide losses worth more than £1000M in each growing season (at a price of £370 t^{-1}) (Fitt *et al*., 2011). It is caused by two closely related fungal species, *Leptosphaeria maculans* and *L. biglobosa* (Shoemaker & Brun, 2001; Fitt *et al*., 2006b). *L. maculans* is more damaging causing stem base canker, whereas *L. biglobosa* is less damaging causing upper stem lesions (West *et al*., 2002a; Huang *et al*., 2005).

Phoma stem canker is an economically important disease with a nearly world-wide distribution (Fitt *et al*., 2006a). Phoma stem canker is the cause of serious losses on oilseed rape crops in Europe, Australia and North America (Howlett, 2004; West *et al*., 2001). In the UK, despite use of fungicides costing £20M per year, phoma stem canker causes more than £100M losses per year at a price of £370 t^{-1} (Fitt *et al*., 2006a; Stonard *et al*., 2010). These losses will increase further if the most effective fungicides are no longer permitted by EU legislation (Mahmuti *et al*., 2009). 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).

1.3 *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 of oilseed rape, a disease of worldwide importance (Fitt *et al*., 2006a). More damage to oilseed rape is caused by *Leptosphaeria maculans* than by the other members of the *Leptosphaeria* species complex, such as *L. biglobosa* (Shoemaker and Brun, 2001), that are found on species in the Brassicaceae.

The fungus can survive on the infected crop residues for several years and can produce both sexual and asexual fruiting bodies (pseudothecia and pycnidia, respectively) (Figure 2). In the UK in autumn, the ascospores are discharged from pseudothecia formed on residues and spread the pathogen from field to field (West *et al*., 2001; Fitt *et al*., 2006c). *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 stem, *L. maculans* does not cause any symptoms on the adult plant throughout most of its life cycle. These cankers result in the lodging and significant yield loss (Howlett *et al*., 2001).

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

Ascospores, the most common primary inoculum, are released from the pseudothecia formed on woody remains of infected plants over an extended period of time after wetting by rain and dew. Ascospores adhere to the cotyledons and leaves of new crops and germinate in humid or wet conditions to produce hyphae that infect leaves. The hyphae 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*. The first symptomless period (5-15 days in winter oilseed rape in Europe) takes place in leaves after the penetration of stomata by hyphae produced from airborne ascospores before the appearance of phoma leaf spot lesions (Biddulph *et al*., 1999; Toscano-Underwood *et al*., 2001; Huang *et al*., 2003). The second symptomless period (5-6 months) occurs between the appearance of leaf lesions in autumn and appearance of cankers on stems the following spring, in which the pathogen colonises the host stem tissues through the leaf petioles and then causes canker (Hammond *et al*., 1985; West *et al*., 1999; Huang *et al*., 2005).

1.4 Resistance against *L. maculans* in *Brassica napus*

A major contribution to global food security is plant resistance against pathogens of arable crops (Beddington, 2010; Brun *et al.*, 2010), especially in areas of the world where subsistence farmers in marginal environments are threatened by devastating epidemics and do not have the option to use fungicides (Fitt *et al.,* 2011). To exploit such crop resistance effectively, it is important to understand its phenotype in relation to the life cycle of the pathogens. To grow a crop with limited pesticide applications and low production costs, use of resistant cultivars is the best option (Brun *et al*., 2010).

Two types of resistance against *L. maculans* have been identified in *B. napus*. The first type is a qualitative or race-specific (*R* gene-mediated) resistance operating in cotyledons (Figure 2) and leaves during the first symptomless phase that occurs immediately after the penetration of leaves

by hyphae from the ascospores (Ansan-Melayah *et al*., 1998; Balesdent *et al*., 2001). *R* gene-mediated resistance against *L. maculans* is associated with a gene-for-gene interaction in which the product of a pathogen effector (*Avr*) gene is recognized by the product of a host *R* gene so that the pathogen is unable to infect the host (i.e. resistant reaction). For example, the *L. maculans* effector gene *AvrLm1* interacts with the resistance gene *Rlm1* (Gout *et al*., 2006).

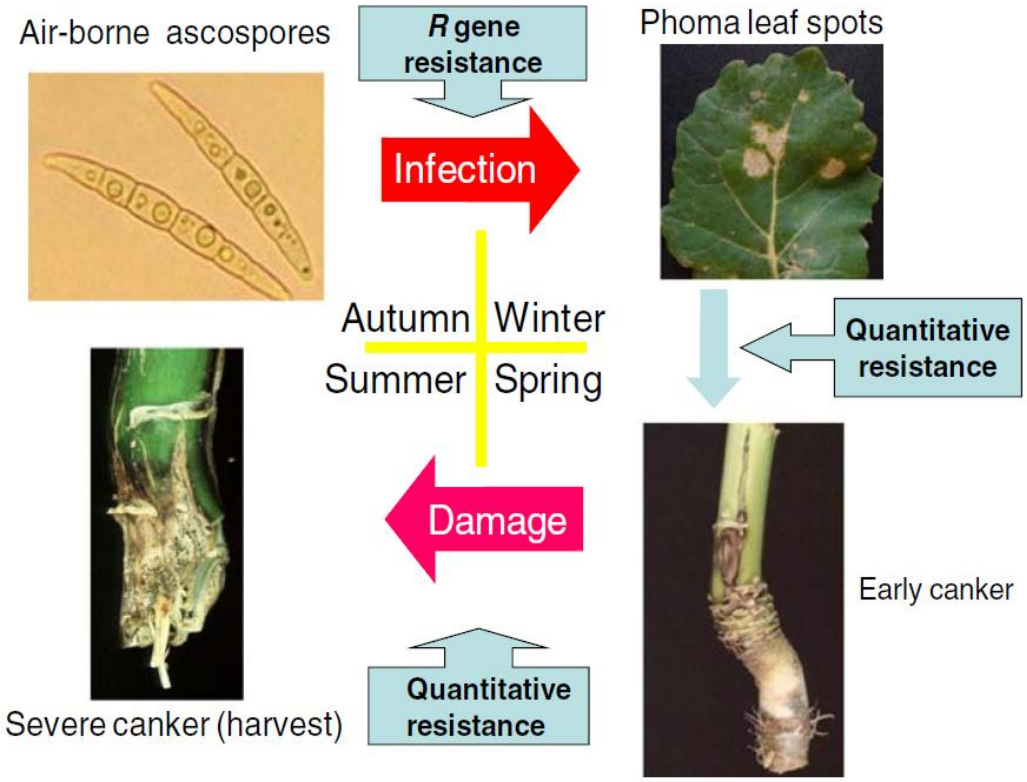

Figure 2: Seasonal cycle of phoma stem canker epidemics in relation to components of oilseed rape (*Brassica napus*) resistance to *Leptosphaeria maculans* (Fitt *et al*., 2006a; Huang *et al*., 2010)

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 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; 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*., 2003a). However, it became

clear that the *L. maculans* populations could change from avirulent to virulent when there was large-scale cropping of cultivars with specific resistance genes (Howlett, 2004).

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 population. Therefore, such *R* genes are often not durable because the pathogen populations change from avirulent to virulent against them. Widespread use of host resistance is the main force 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 3). The ETD operates 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 3). Only after an elapsed period of endophytic pathogen growth does effector-triggered defence trigger host cell death. ETD responses against *L. maculans* are comparatively slow and are not associated with a fast hypersensitive host cell death response (HR) (Stotz *et al*., 2014).

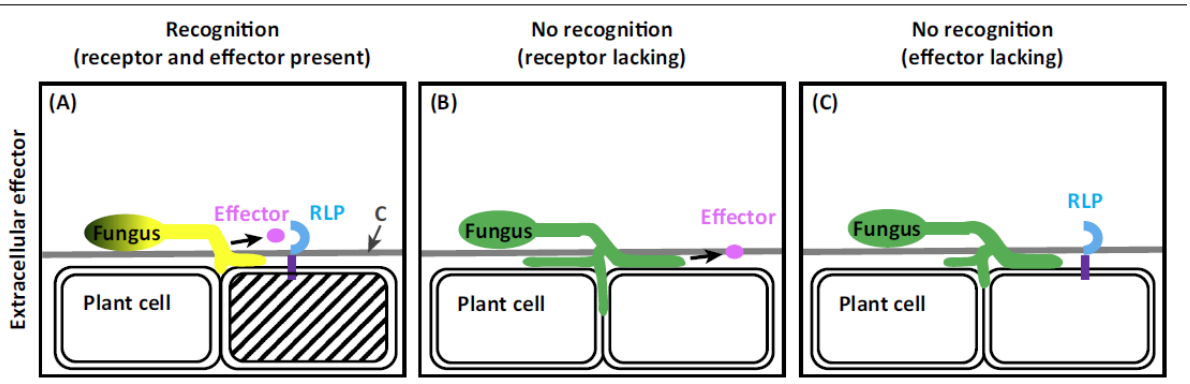

Figure 3: Resistance (*R*) gene-mediated effector-triggered defence (ETD) results in incompatible interactions with hemibiotrophic apoplastic fungal leaf pathogens (Stotz *et al*., 2014)

L. maculans is an apoplastic pathogen. During the early stages of colonisation, *L. maculans* does not trigger an effective host defence response. It slowly colonises the apoplast and gets adapted

to its 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 after the senescence of the host begins (Stotz *et al*., 2014).

The second type of resistance is quantitative resistance that operates during the symptomless pathogen growth in petiole and stem tissues that occurs between initial development of leaf spots and the formation of stem cankers (Figure 2) (Pilet *et al*., 1998; Delourme *et al*., 2006). Moreover, in comparison with the qualitative/*R* gene-mediated resistance, quantitative resistance (often mediated by many genes) is partial, considered to be race non-specific and more durable than qualitative resistance (Boyd, 2006; Delourme *et al*., 2006).

Breeding of resistant cultivars is the most common and effective way to control phoma stem canker disease caused by *Leptosphaeria maculans* in oilseed rape (*Brassica napus*) (Delourme *et al*., 2006; Raymer, 2002). Use of host resistance is also an environmentally friendly method for controlling the disease (Delourme *et al*., 2006). In Europe, Canada and Australia, many resistant cultivars have been registered and cultivated. However, there are some instances of breakdown of race-specific resistance because of rapid evolution in *L. maculans* populations. Therefore, understanding the genetic basis of resistance in oilseed rape is strategically important for management of resistant cultivars.

The interaction between *L. maculans* and oilseed rape is a typical gene-for-gene relationship (Balesdent *et al*., 2005). Numerous effector genes are involved in the interaction between the pathogen and its host, but a subset of these (termed *Avr* genes) renders the pathogen avirulent on host genotypes with the corresponding resistance (*R*) genes (Ellis *et al*., 2009). *Avr* proteins are known to be effectors involved in plant pathogenesis that were recognised by the plant surveillance machinery in the course of plant-pathogen co-evolution (Jones & Dangl, 2006).

At least fourteen *R* genes (*Rlm*1-*Rlm*11, *LepR*1, *LepR*2 and *LepR*3) conferring resistance to *L. maculans* have been identified in *brassicas* and one gene (*LepR*3) has been cloned (Larkan *et al*., 2013). Corresponding to *Rlm*1-*Rlm*11, eleven *L. maculans Avr* genes have been genetically characterised (*AvrLm1—AvrLm1*1) (Balesdent *et al*., 2013). For predicting and managing the risk of severe disease epidemics, monitoring the frequency of avirulent and virulent alleles within fungal populations plays an important role in guiding the effective deployment of *R* genes. *Avr* genotypes are determined by pathogenicity testing on cultivars with corresponding resistance genes and/or using molecular markers for avirulent alleles (Van de Wouw *et al*., 2010).

Deployment of *R* genes is made 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).

In the UK, HGCA does give a phoma stem canker resistance rating to all the recommended list of cultivars every year on the basis of the field experiments (Source: HGCA Recommended lists 2014/15 for cereals and oilseeds) (www.hgca.com). Resistance to lodging, stem stiffness, shortness of stem, earliness of flowering and earliness of maturation were the agronomic features taken into consideration by the HGCA during the selection of a specific cultivar for the recommended list. The year of introduction and the recommended list status of the cultivar should be checked while selecting the cultivar (HGCA Recommended Lists 2014/15 for cereals and oilseeds).

But the recommended list does not give any information on 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 virulence genes by annual monitoring would help for the exploitation of the required *R* genes (Gladders *et al*., 2006). By knowing the frequency of *Avr* alleles in populations, it is possible to select certain oilseed rape cultivars for recommendation each year in France (CETIOM) (www.cetiom.fr) and in Australia (GRDC) (Grains Research and Development Corporation) (www.grdc.com.au). It would be better if such procedures could start in the UK to guide more effective deployment of *R* genes.

1.5 Aim and objectives

This work aims to survey the regional distribution of races of *L. maculans,* using isolates from phoma leaf spot samples from different oilseed rape cultivars in the UK, with the identity of *L. maculans* isolates confirmed by species-specific PCR and races identified by using a cotyledon test method.

These are the following objectives:

1. To assess severity of phoma leaf spots on different cultivars of oilseed rape at different sites in the UK.
2. To confirm the identification of *L. maculans* isolates by species-specific PCR.
3. To determine *Avr* alleles in *L. maculans* isolates by cotyledon inoculation of a differential set of *B. napus* cultivars.
4. To detect the presence of *AvrLm1* allele in the *L. maculans* isolates by allele-specific PCR.

2. Materials and methods

Nine oilseed rape cultivars with different *R* genes operating against *L. maculans* with/without quantitative resistance in the background from various sites in the UK, France and Poland (Appendix 1) were used in this study (Table 1).

Table 1: The different oilseed rape cultivars used for the experiments with details of their types of resistance (Huang *et al*., 2013)

Cultivar	*R* gene[+]	Quantitative resistance background	Current field resistance*
NK Grandia	No	Yes	Resistant
Es-Astrid	No	Yes	Resistant
DK Cabernet	*Rlm1*	Yes	Resistant
Adriana	*Rlm4*	Yes	Resistant
Capitol	*Rlm1*	No	Susceptible
Bilbao	*Rlm4*	No	Susceptible
Excel	*Rlm7*	Not known	Resistant
Roxet	*Rlm7*	Not known	Resistant
Drakkar	No	No	Susceptible

[+]Resistance gene operating against *L. maculans*

*The resistance shown by the cultivar in field experiments

2.1 Assessment of phoma leaf spots on oilseed rape cultivars

Ten leaves with phoma leaf spots were sampled randomly from 3-month-old winter oilseed rape plants in three replicate plots at three different sites in the UK (Cranwell, Harpenden and Bainton) (Figure 4) from five cultivars (Drakkar with no *R* gene, Bilbao with *Rlm4*, Adriana with *Rlm4* and quantitative resistance, Roxet and Excel with *Rlm7*) in January 2013 (Harpenden) or in February 2013 (Cranwell and Bainton) (Appendix 1).

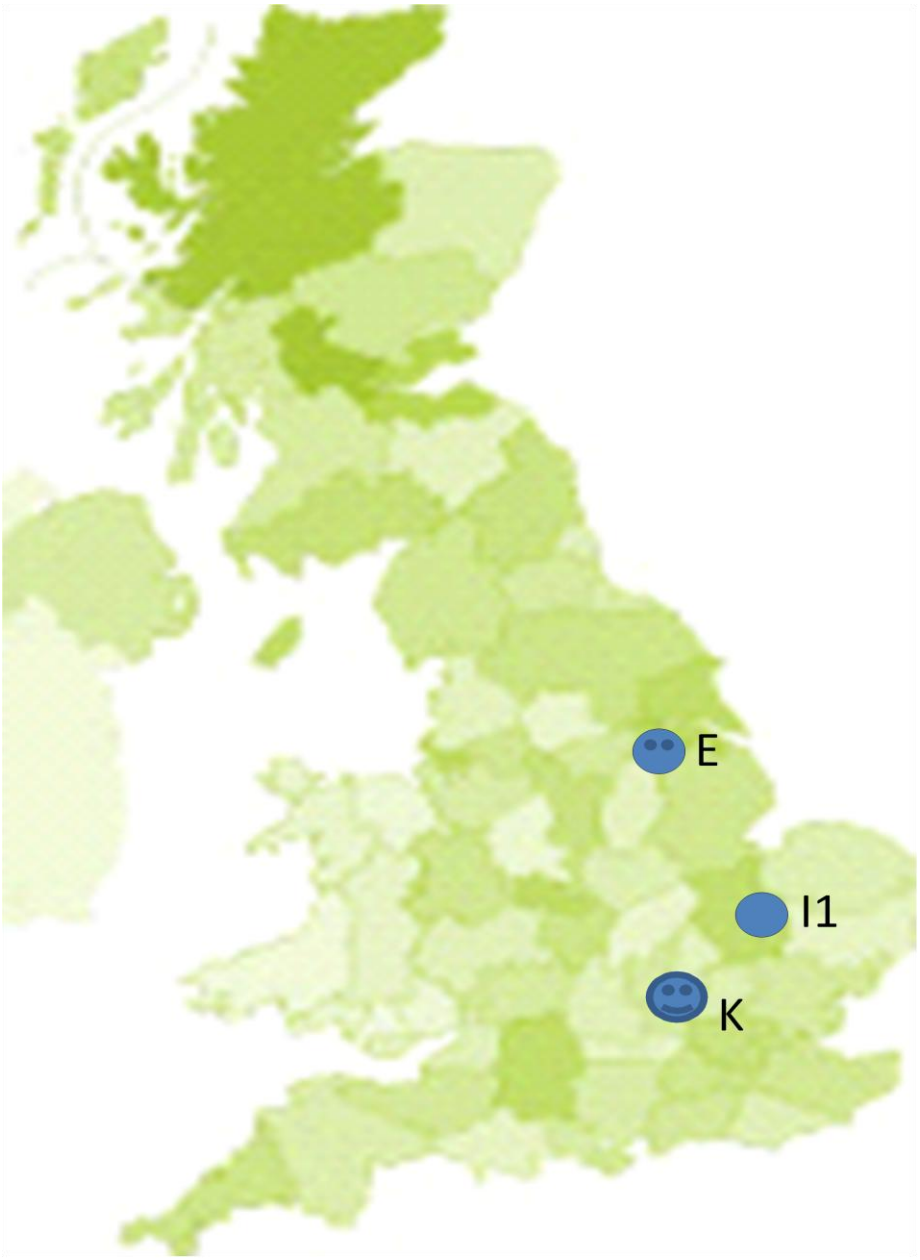

Figure 4: Location of 2012/2013 winter oilseed rape experimental sites sampled in the United Kingdom to assess phoma leaf spotting

(I1- Cranwell, K- Harpenden, E- Bainton)

Disease severity on leaves was assessed by counting the number of spots caused by *L. maculans* per leaf for each cultivar (Figure 5). The disease score was 1 if the number of leaf spots were <5. The score was 2, when the leaf sample had 5-10 phoma leaf spots and it was scored 3 for leaf samples with >10 leaf spots. Initial species identification was based on visual observation

of the leaf spots since *L. maculans* causes large pale lesions with pycnidia and *L. biglobosa* causes smaller, darker lesions (Fitt *et al*., 2006a).

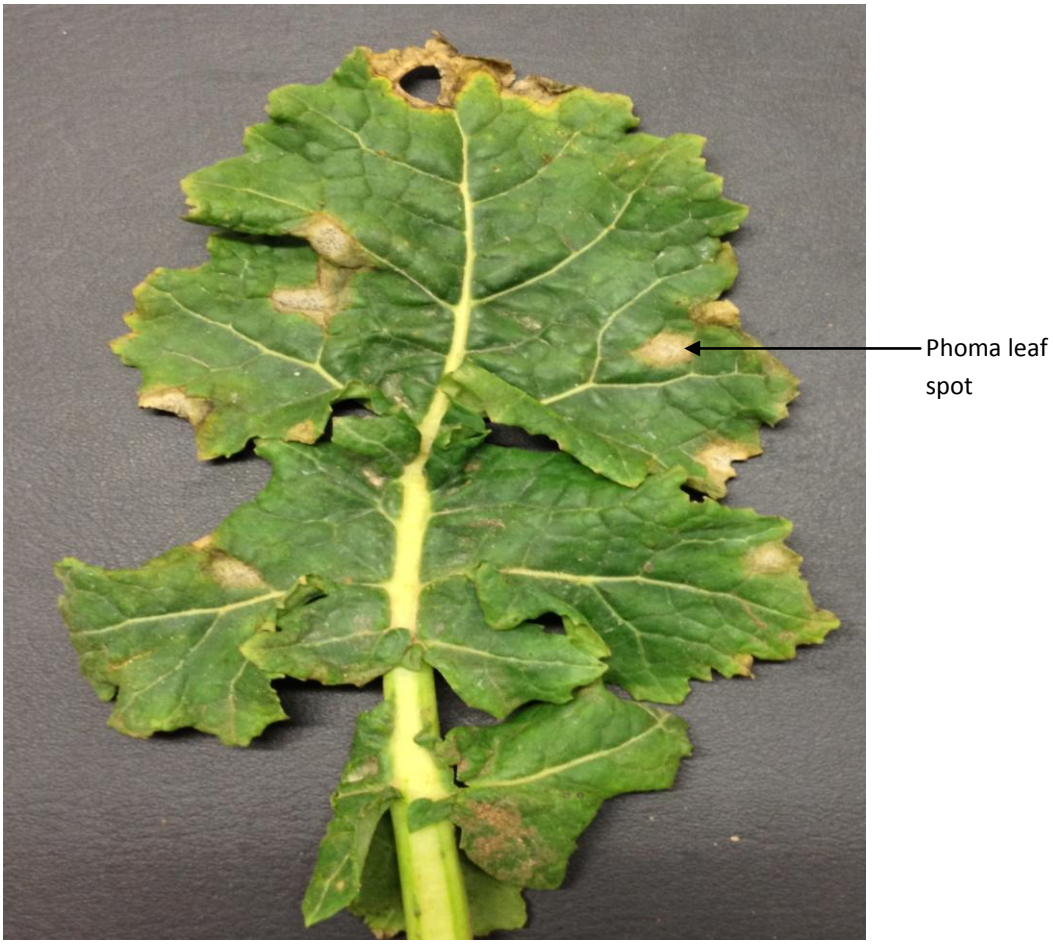

Figure 5: Leaf of the cultivar Excel showing large pale leaf lesions, indicating the presence of *L. maculans*

2.1.1 Isolation of the pathogens

The leaves that had been used to assess disease severity from three sites (Cranwell, Harpenden and Bainton) and thirty leaf samples from three other sites (Morley, Banbury and Rothwell) in the UK and ten leaf samples from Verpillieres in France were used to obtain *Leptosphaeria* isolates (Figure 6) (Appendix 1).

The isolates of *L. maculans* from the phoma leaf spot samples were obtained by the method of West *et al*. (2002). The leaves with phoma leaf spots 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 7a). The leaf pieces were placed in Petri dishes on a

Whatman No.1 filter paper that had been sprayed with distilled water (Figure 7b). The leaves were incubated for 3-5 days at 20°C under 12h light/12h darkness to induce pycnidial production.

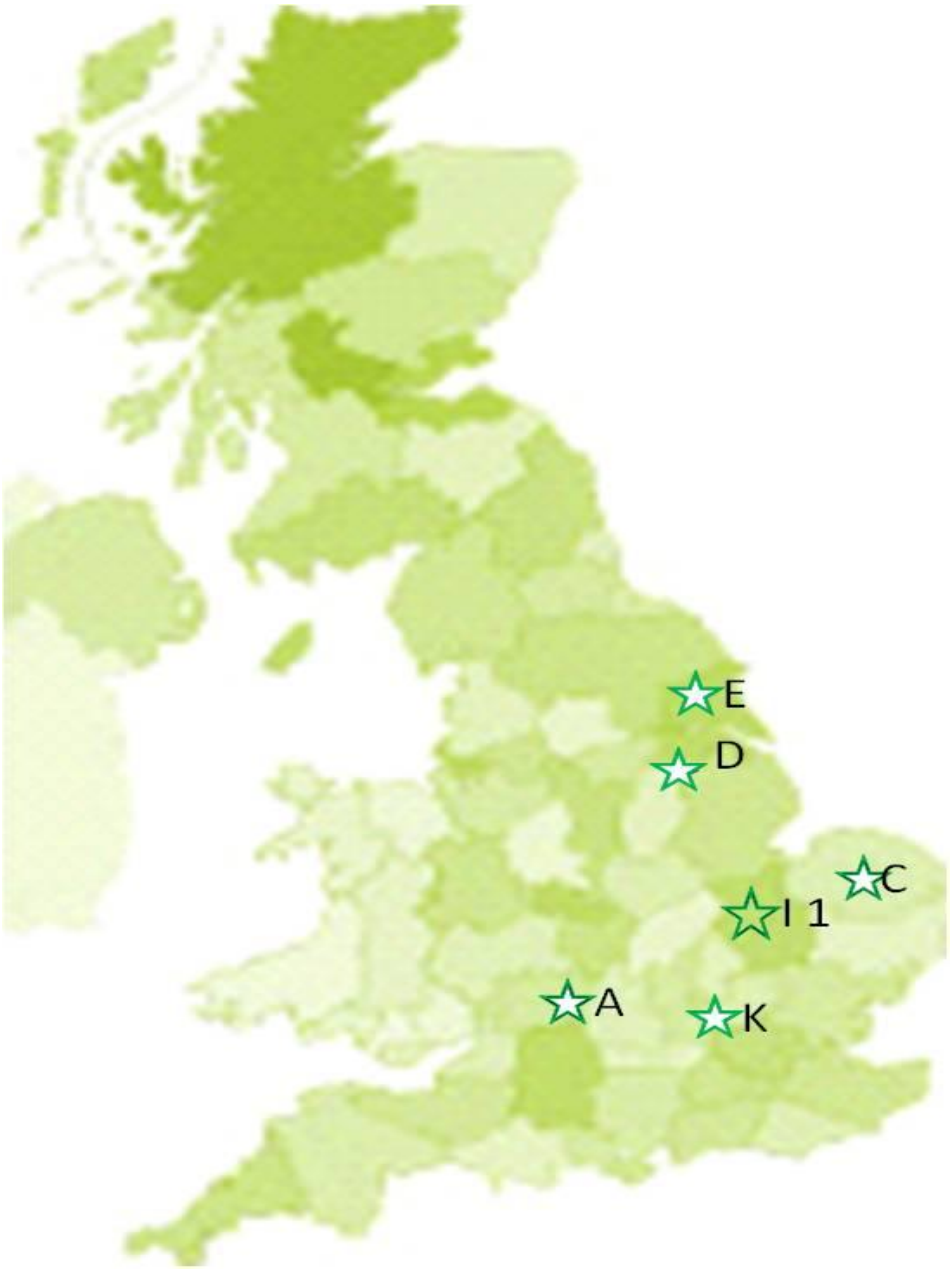

Figure 6: Sites in the United Kingdom used to obtain *Leptosphaeria maculans* isolates
(I1- Cranwell, K- Harpenden, E- Bainton, A- Banbury, C-TAG Morley and D-Rothwell)

A dissection microscope was used to observe the pycnidia and single pycnidial isolates were obtained by sampling the cirrhus from a mature pycnidium by using a fine needle (Figure 7c). This was done under sterile conditions in a laminar air flow chamber. The cirrhus was transferred to a drop of sterilized water to make a spore suspension and then onto PDA (Potato Dextrose Agar) medium (OXOID) (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 pathogens (Figure 7d).

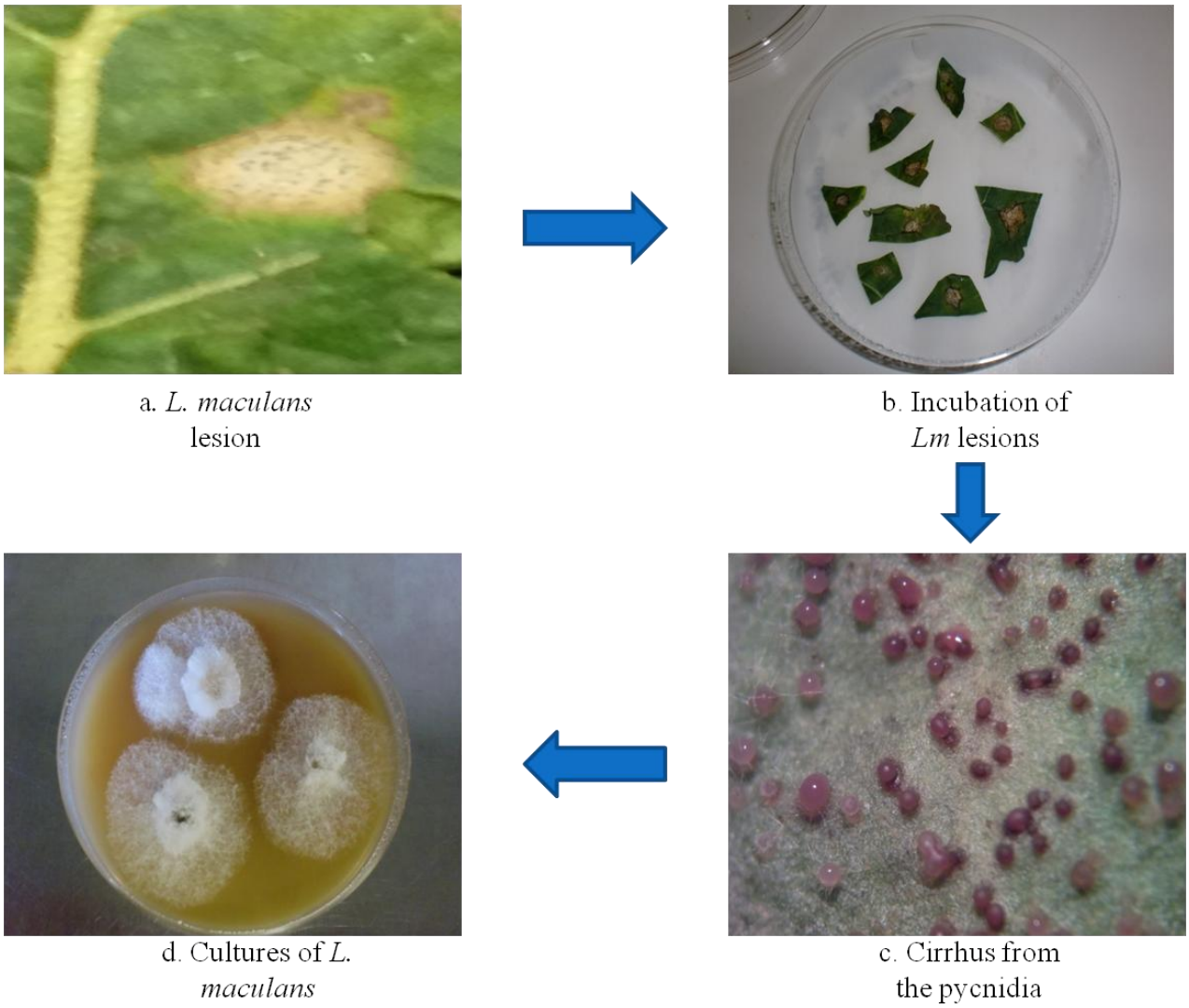

Figure 7: The procedure for the single pycnidial isolation to obtain isolates of *Leptosphaeria* species (West *et al*., 2002)

2.1.2 Identification of *Leptosphaeria maculans* and *L. biglobosa*

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* have no pigment, whereas *L. biglobosa* produces a yellow pigment with many pycnidia on PDA medium (Figure 8).

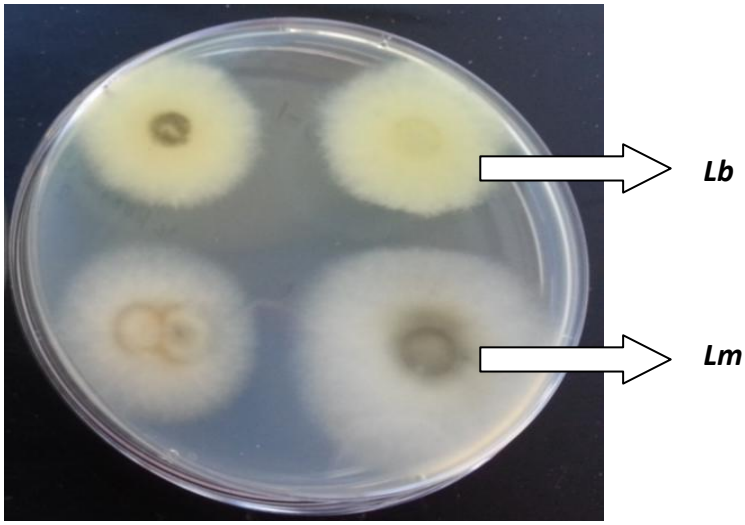

Figure 8: Isolates obtained from leaf lesions grown on PDA for visual identification of *L. maculans* (*Lm*) or *L. biglobosa* (*Lb*)

2.1.3 DNA extraction using DNAMITE plant kit

The pathogen isolates subcultured onto V8 agar plates with cellulose discs were incubated at 20^0C until mycelial growth was observed. Then the plates were opened in a biosafety cabinet and the mycelium was collected into sterile 0.5ml tubes with a sterile scalpel. Then the samples in the tubes were freeze-dried and ground to powder. A small amount of the ground mycelium of each isolate was placed into a 2ml sterile tube. Sterile stainless steel beads (5mm) (QIAGEN) were placed in each tube (3 beads/ tube). 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) for 40 seconds. 100µl of Solution PA (protein denaturation solution) was added and the samples were vortexed briefly. Then the samples were centrifuged at 11,000 rpm for 5 minutes in a micro-centrifuge (Eppendorf).

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 minutes and were spun in a micro-centrifuge at 13,000 rpm for 7 minutes 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 minutes in the biosafety cabinet. Then 100µl of sterile distilled water was added to each of the tubes and the tubes were

left for 30 minutes to allow the DNA to rehydrate. Then the samples were stored at -20^{o}C. The concentration of the DNA was measured by using a nano-drop spectrophotometer (Nano-drop-ND-1000 spectrophotometer). The DNA concentration was measured in ng/µl units and its purity was assessed at 260/280 nm.

2.1.4 PCR

The identity of the *L. maculans* isolates was confirmed by the 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 3).

For *L. maculans*:

LmacF- Forward primer -CTTGCCCACCAATTGGATCCCCTA (24nt)

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

For *L. biglobosa*:

LbigF - Forward primer -ATCAGGGGATTGGTGTCAGCAGTTGA (26nt)

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

All the ingredients were placed at 0^{o}C (Table 3). The reaction mixture was vortexed briefly and then centrifuged for a short time to mix the ingredients well. The required number of 0.2ml PCR tubes were taken, 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. The details of the PCR cycle are given in Table 2.

Table 2: PCR thermal cycle

Denaturation	**95°C**	**2 minutes**	
Denaturation	**95°C**	**30 seconds**	30 cycles
Annealing	**61°C**	**30 seconds (65°C for *Lm*, 63°C for *Lb*)**	
Annealing	**72°C**	**1 minute**	
Extension	**72°C**	**10 minutes**	
Hold	**4°C**		

After the completion of the PCR, the samples were taken and stored at 0^{o}C. The PCR products were viewed on 1.5% agarose gel.

Table 3: Ingredients used in the reaction mixture for species-specific PCR

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

2.2 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. Sterile 15ml tubes were labelled with the names of the isolates and date. 4ml of sterile distilled water was added to the plate and spread around the plate using a sterile Lazy-L spreader to encourage the conidia to be released into the water. A sterile glass funnel was placed on the top of the 15ml tube using sterile forceps. A sterile double layer of sterile Mira-Cloth was placed inside the funnel using 1ml of sterile distilled water to cause it to adhere to the funnel. The conidial suspension was filtered through it into the 15ml tube.

The concentration of the conidial suspension was measured using a haemocytometer slide. 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^{0}C for subsequent use in the cotyledon test.

2.2.1 Inoculation of spore suspensions onto cotyledons of a differential set of cultivars

The seedlings grown in 24 compartment-trays in the glasshouse were placed inside the controlled environment chamber and after 14 days (water sprayed daily to maintain the moisture and humidity) they were taken out for inoculation. The cotyledons were 14 days old at inoculation with the conidial suspensions. 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 the each plant did not touch each other and that the inoculated drops stayed on the cotyledons (Figure 9).

Table 4: List of the oilseed rape cultivars/lines with different *R* genes used in a differential set to identify races of *Leptosphaeria maculans*

***R* gene**	**Cultivar/line**
***Rlm*1-3**	**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**

Figure 9: Trays with seedlings of the *Brassica* cultivars and lines of the differential set before inoculation with the 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 A. A sharp pin was used to gently wound at the centre of each cotyledon lobe without passing through it (Figure 10).

Figure 10: Schematic representation of inoculation plan on the two cotyledons of each *Brassica* seedling. 1-4 represent the four inoculation sites for four different spore suspensions

A Gilson pipette (p20) was used to place 11µl of the conidial suspension directly on the wound site of each lobe of the cotyledon. All the inoculation sites were assigned numbers to identify the type of inoculum. After all the plants were 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 hours. After 72 hours, the lids were removed (Figure 11) 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 humidity and a 12 h day length at 20^0C). The plants were sprayed twice every day with distilled water to encourage symptom development and watered whenever necessary.

Figure 11: Tray with seedlings of the *Brassica* cultivars and lines of the differential set immediately after inoculation with the spore suspensions of *Leptosphaeria maculans*

The disease development in 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. 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 similar to those as score 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 increased diameter with no or few pycnidia. Large grey-green lesions with profuse sporulation were given score 9.

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) (Figure 12). If the 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 (Appendix 5).

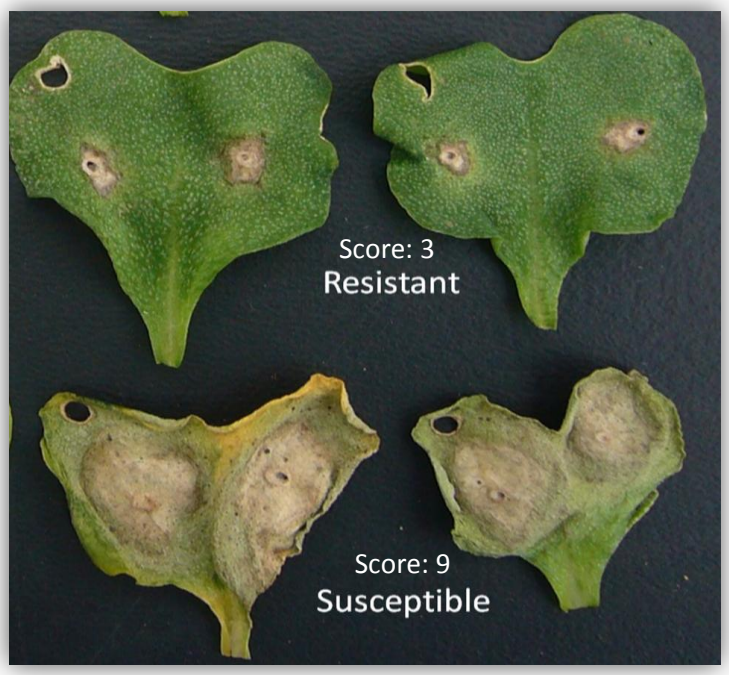

Figure 12: Disease scores on cotyledons of resistant (Adriana with *Rlm4*) and susceptible (Drakkar) cultivars

2.3 PCR

The presence of the avirulent *AvrLm1* alleles in the *L. maculans* isolates was studied by using allele-specific PCR. The major mechanism that confers virulence towards *Rlm1* is the deletion of the entire *Avr* locus. Thus when subjected to PCR with *AvrLm1* primers, only the avirulent allele can be detected as the virulent allele has the deletion of *AvrLm1* and thus only the avirulent allele is amplified (Van de Wouw *et al*., 2012). The primers used for this PCR process were

AvrLm1-F - AATCCATTCCTCACCTCGTG
AvrLm1-R - GCACCAGAGGCAAAGACTTC (Van de Wouw *et al*., 2010)

The procedure followed for this PCR was the same as that for the species-specific PCR (Table 2). The PCR cycle was same as that for the species-specific PCR. After the completion of the PCR cycle, the presence of the *AvrLm1* allele was detected by running the samples in agarose gel. The presence of a band indicated the presence of the avirulent *AvrLm1* allele.

Table 5: Ingredients used in the reaction mixture for allele-specific PCR

Redtag mix	**10µl (×1) (SIGMA Cat. No. R2523, Red Tag reaction mix with $MgCl_2$)**
***AvrLm1*-F**	**0.3 (10µM)**
***AvrLm1*-R**	**0.3 (10µM)**
Sterile distilled H_2O	**8.4**
DNA	**20ng**
Total	**20µl reaction**

2.4 Statistical analysis:

The standard errors of the mean and significant statistical differences in number of phoma leaf spots between the five cultivars at different sites were calculated using analysis of variance (Oneway ANOVA) tests (Appendices 1-4). Multiple comparisons of the number of phoma leaf spots were made between the cultivars and within the cultivars from different sites by using the Oneway ANOVA tests (Appendix 4). The numbers of *Avr* alleles in each isolate at different sites were saved in a Microsoft excel sheet. 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 (Figures 18 & 19).

3. Results

3.1 Winter oilseed rape fields experiments

3.1.1 Phoma leaf spot assessment

Phoma leaf spot assessment was done on the leaf samples of five different cultivars collected from three different sites in the UK (Appendix 1) (Figure 5), on 30 January 2013 in Harpenden, 13 February 2013 in Bainton and 28 February 2013 in Cranwell. There were differences between the different sites in the UK in number of leaf spots per leaf caused by *L. maculans* (Figures 13 & 14). The susceptible cultivar Drakkar (with no *R* gene) had a greater number of phoma leaf spots than all the other cultivars (Figure 13). The number of spots caused by *L. maculans* on Drakkar was greatest at Cranwell (23.7 spots/leaf), followed by Harpenden (14.8 spots/leaf) and least at Bainton (4.9 spots/leaf).

The number of *L. maculans* spots on Excel (with *Rlm7*) was less than on Drakkar (Figure 14) at two sites, with most at Cranwell (12.6 spots /leaf) and Harpenden (3 spots/leaf), and there were more on Excel than on Drakkar at Bainton (6.3 spots/leaf). The number of *L. maculans* spots on Roxet (with *Rlm7)* was greatest at Cranwell (17.4 spots/leaf) and least at Bainton (6.8 spots/leaf) and Harpenden (3.6 spots/leaf). The number of *L. maculans* spots on Adriana (with *Rlm4* and quantitative resistance) was more at Cranwell (11.7 spots /leaf) than at Bainton and Harpenden (both 5 spots/leaf). For the cultivar Bilbao (with *Rlm4)*, the number of *L. maculans* spots was more at Cranwell (11.7 spots /leaf) than at Bainton (10 spots /leaf) and Harpenden (6.1 spots /leaf).

For the mean of the three sites, the greatest number of *L. maculans* leaf spots was observed on cultivar Drakkar (14.4), followed by Bilbao (12.6), Roxet (9.3), Excel (7.3) and the smallest number was on Adriana (7.2) (Figures 13 & 14). More phoma leaf spots were observed at Cranwell than at Harpenden or Bainton (Figure 14). The standard error of the mean (SEM) is the standard deviation of the sample means, an estimate of a population mean. Standard deviation showed the amount of deviation of the number of phoma leaf spots from the average of each cultivar at a particular site. A small standard error of the mean indicated that the number of phoma leaf spots on all the leaves of a particular cultivar tended to be very close to the mean. A large standard error of the mean indicated that the numbers were spread out over a wide range of values (Figure 14).

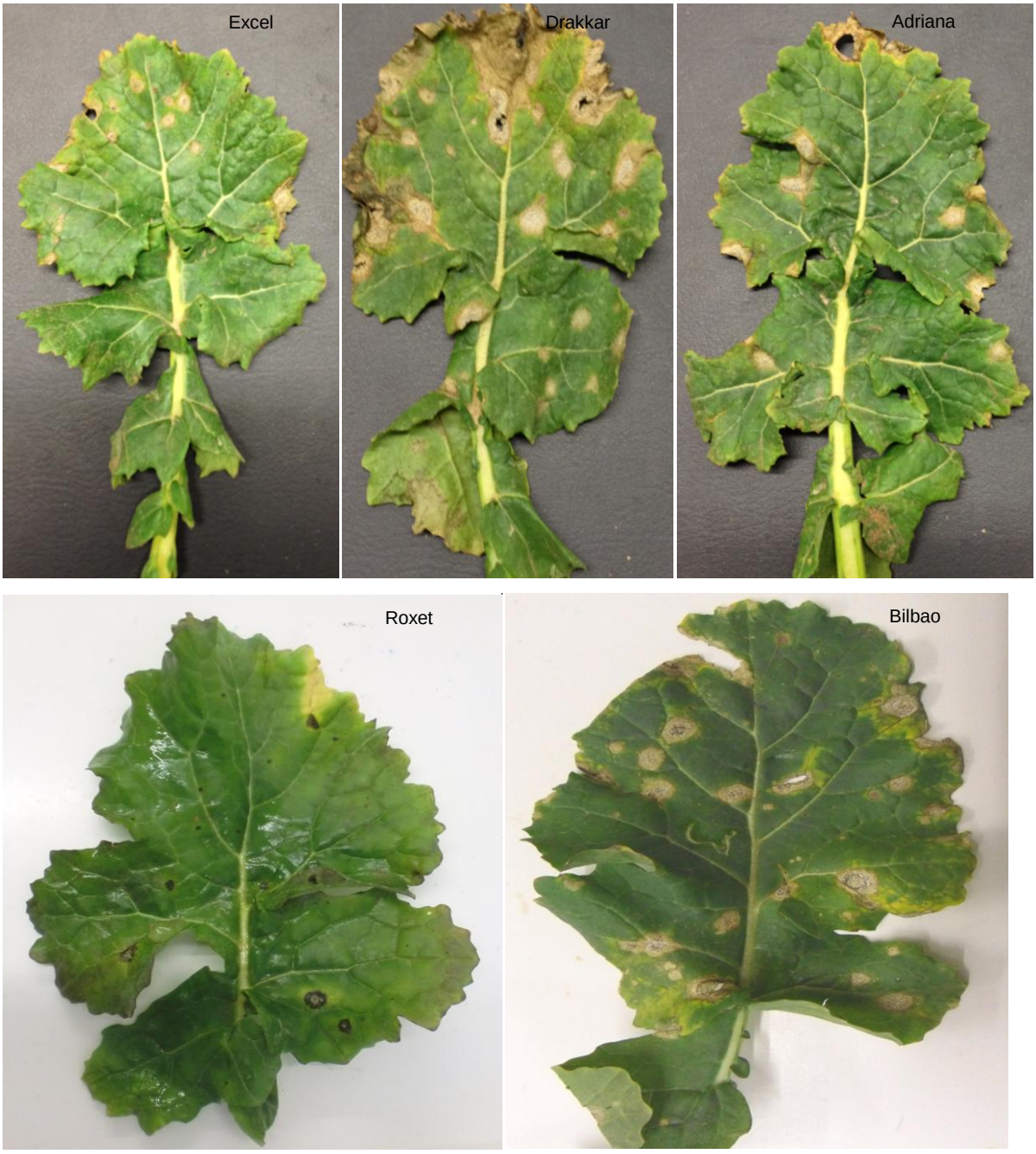

Figure 13: Leaf samples with phoma leaf spots of the cultivars Excel, Drakkar, Adriana, Roxet and Bilbao from Harpenden, 30 January, 2013

Significant statistical differences in the number of phoma leaf spots between the five cultivars at the three sites were also seen (Appendix 5). At Cranwell, the difference in the number of phoma leaf spots between the five cultivars was significant statistically (0.003). It was 0.00 at Harpenden and 0.01 at Bainton (Appendix 5). From the ANOVA test, the observation was that within the Cranwell site the number of leaf spots were significant between Drakkar and Excel cultivars (P=0.031) and (P=0.015) among Drakkar and Adriana cultivars signifying

potential statistical significance (P<0.05). At the Bainton site, there was a statistically significant difference between Drakkar and Bilbao cultivars (P=0.017) and also Adriana versus Bilbao (P=0.016). Interestingly there was no significant difference among all cultivars at the Harpenden site (Appendix 5).

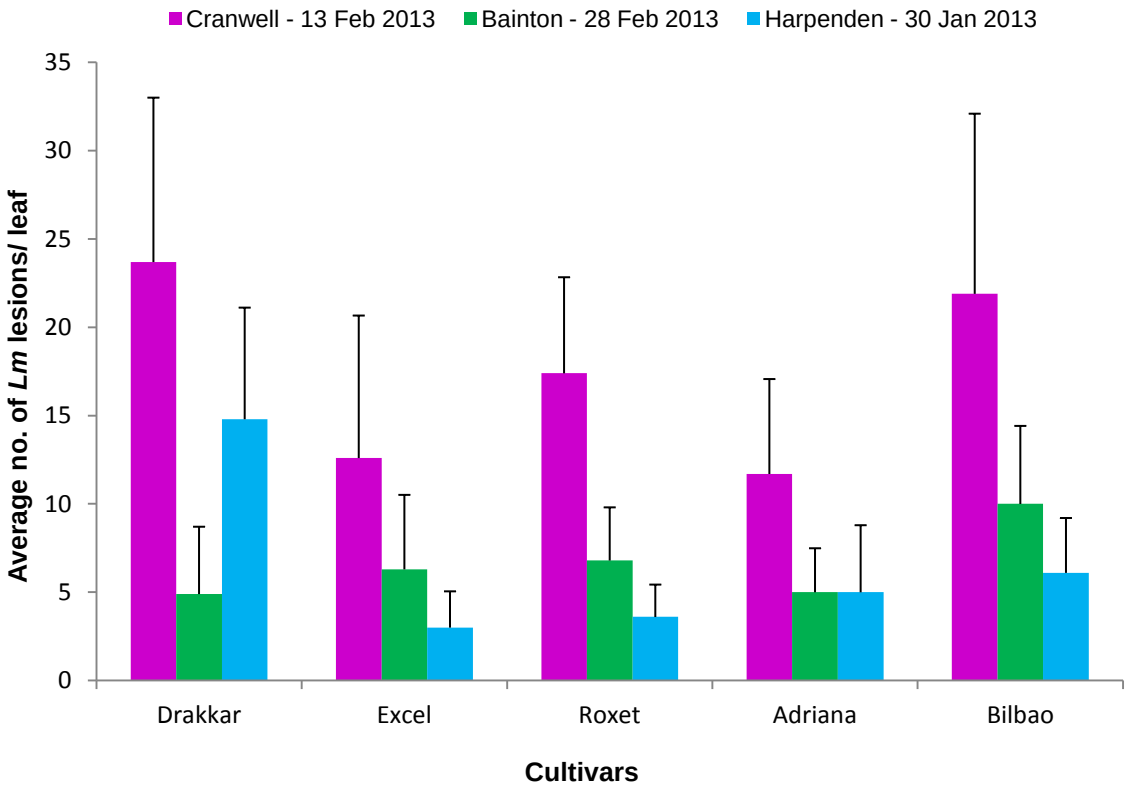

Figure 14: Average no. of *L. maculans* lesions/leaf for five cultivars at the three different sites. Values are means over 10 plants at each of three sites (Cranwell, Bainton and Harpenden) (Figure 2) (Table 1). Vertical lines are the standard errors of the mean (variation in the number of phoma leaf spots amongst the ten leaf samples of a particular cultivar at a particular site).

3.2 Isolation and identification of *Leptosphaeria maculans* from winter oilseed rape

The phoma leaf samples from the five cultivars were collected from winter oilseed rape cultivars from the sites Cranwell, Bainton and Harpenden (phoma leaf spot assessment sites), Morley, Banbury and Rothwell in the UK and Verpillieres in France (Appendix 1). Thirty pathogen isolates were isolated from the leaf samples (Figure 7) and were visually identified by culturing on PDA plates (Figure 15 & 16).

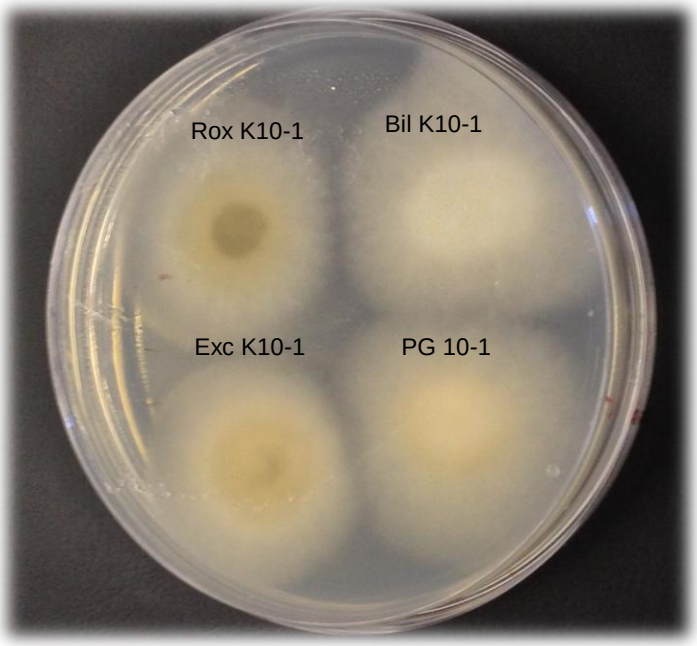

Figure 15: Visual identification of *L. maculans* isolates from the phoma leaf spot samples from Harpenden on a PDA plate (all four isolates were *L. maculans*)
(Rox K10-1- Roxet, Bil K10-1- Bilbao, Exc K10-1-Excel, PG 10-1- Drakkar)

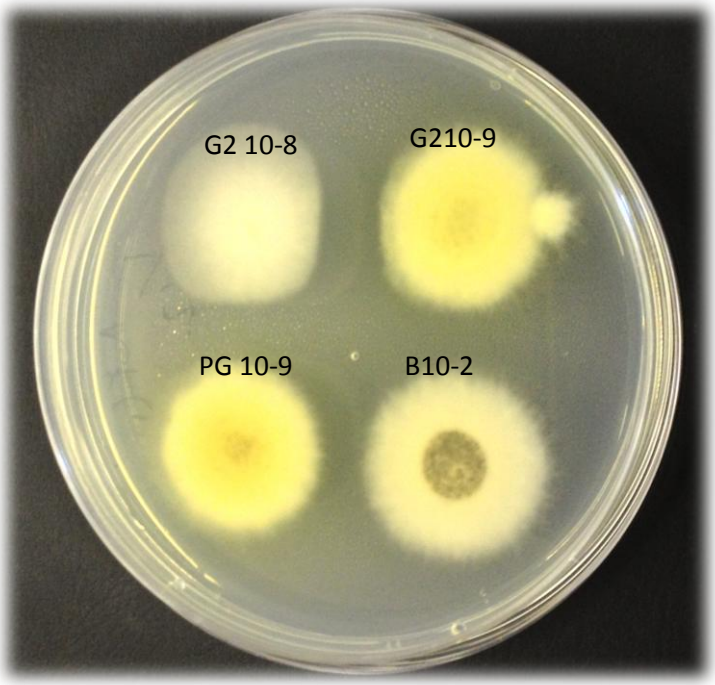

Figure 16: Visual identification of *L. maculans* and *L. biglobosa* isolates from the phoma leaf spot samples from the Spalding site in the UK and Poland on a PDA plate
(G2 10-8-Drakkar (*Lm*); G2 10-9-Drakkar (*Lb*)) - Poland
(PG10-9-Drakkar (*Lb*); B10-2-Drakkar (*Lm*)) - Spalding

Only twenty-six isolates were visually identified as *L. maculans* on PDA plates. The isolates of *L. maculans* were subcultured onto V8 agar media plates with cellulose discs for mycelium production. Then the mycelium was harvested from the Petri plates and stored in 1.5 ml tubes at 0^{o}C for DNA extraction. Twenty-six *L. maculans* isolates were obtained from this procedure and their identity was confirmed by species-specific PCR (Appendix 1).

3.3 Confirmation of the identification of *L. maculans* isolates by species-specific PCR

Mycelial samples harvested from the V8 agar plates with cellulose discs were freeze-dried. Then the samples were ground and DNA was extracted using the DNAMITE Plant Kit. Concentration of the DNA was measured using nano-drop spectrophotometer equipment and then by using species-specific PCR, isolates were confirmed as *L. maculans* or *L. biglobosa*. DNA extraction was done from seventy-two isolates (Appendix 1, 7 & 8).

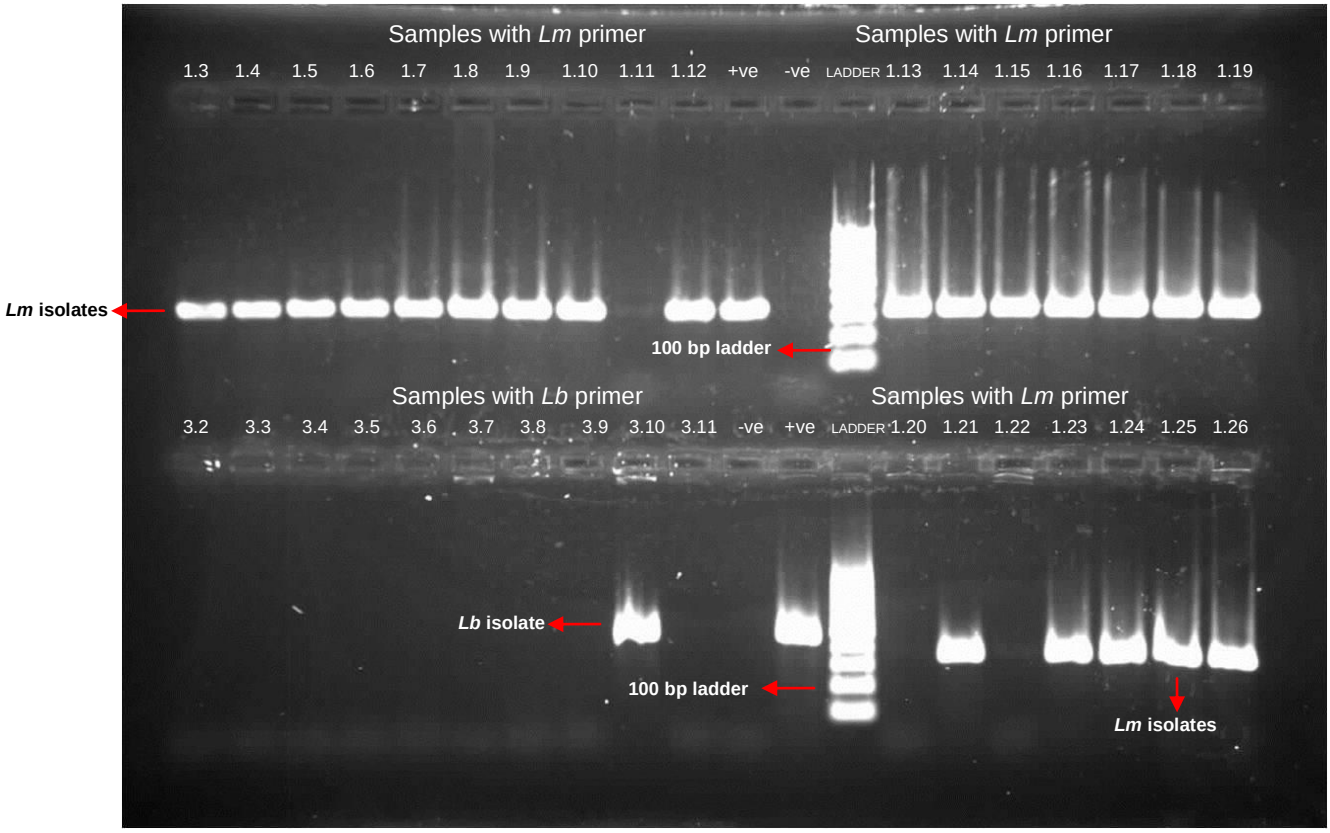

Figure 17: Agarose gel analysis of species-specific PCR products. Samples with *L. maculans* primers were run in the first lane, and the same set of samples with *L. biglobosa* primers were run in the second lane (3.2-3.11)

The isolates run in this agarose gel (Figure 17) are mentioned in Appendices 7 & 8. In this

gel, twenty-four isolates were checked for their identity. Among them, three were *L. biglobosa* isolates and twenty-one were *L. maculans* isolates. The same set of samples were run by using two types of primers, one set with *Lm* primers and the other one with *Lb* primers so as to confirm the identity of the isolates. 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). Out of seventy-two isolates, sixty-nine isolates were *L. maculans*. Rox KB10-1, Ari K10-3, Cab KB10-4 isolates were *L. biglobosa* isolates.

3.4 Detection of *Avr* alleles by cotyledon inoculation test with *L. maculans* isolates obtained from five cultivars

3.4.1 *Leptosphaeria* isolates obtained from leaf samples for cotyledon test

From the thirty isolates obtained from the phoma leaf spot samples from Drakkar, Bilbao, Adriana, Roxet and Excel, twenty-six isolates were visually identified as *L. maculans* and four of them were *L. biglobosa*. Of those four *L. biglobosa* isolates, three were from Drakkar and one was from Excel. These twenty-six *L. maculans* isolates were subcultured onto V8 agar plates for conidial production. The spore suspensions of the twenty-six isolates were prepared for the cotyledon test (Appendix 1).

*L. maculan*s races were identified by inoculating each isolate onto cotyledons of a set of eight differential cultivars/lines (Balesdent *et al*., 2005). The spore suspensions prepared from the twenty-six *L. maculan*s isolates were inoculated on the cotyledons of the differential set of cultivars (Table 4).

Two assessments were made for scoring the disease on the cotyledons. The first one was done 14 days after inoculation and the second one was done 19 days after inoculation (Appendix 6). The name of the isolate, cultivar, location of the site and races of each *L. maculans* are listed in Table 6.

Isolates from the cultivar Drakkar were found to have avirulent *Avr* alleles *AvrLm4, AvrLm5, AvrLm6* and *AvrLm7*. The isolates from the cultivar Adriana mostly had avirulent *Avr* alleles *AvrLm5, AvrLm6* and *AvrLm7*. The isolates from the cultivar Bilbao also had avirulent *Avr* alleles *AvrLm5, AvrLm6* and *AvrLm7* (Table 6). From the results obtained, it can be observed that isolates from cultivars Bilbao and Adriana were virulent against *Rlm4.* The isolate from the cultivar Roxet had avirulent *Avr* alleles *AvrLm4, AvrLm5* and *AvrLm6* and this showed

that isolate from the cultivar Roxet was virulent to *Rlm7.*

All isolates were virulent to *Rlm2* and only 6.25% of isolates were avirulent to *Rlm3* and *Rlm9*. In contrast, all isolates were avirulent to *Rlm6*, except from cultivar Adriana (75%). 37.5% of isolates from Drakkar, 50% of isolates from Adriana and 20% of isolates from Bilbao were avirulent to *Rlm*1. Isolates from Drakkar (81.25%) and Roxet (100%) were avirulent to *Rlm4*. Similarly, the isolate from cultivar Roxet was avirulent to *Rlm5* (100%) and all isolates from cultivar Bilbao possessed avirulence alleles to *Rlm6* and *Rlm7* (100%) and *Rlm5* (80%). The isolates from the cultivar Adriana were avirulent (75%) to *Rlm5*, *Rlm6* and *Rlm7* (Figure 18).

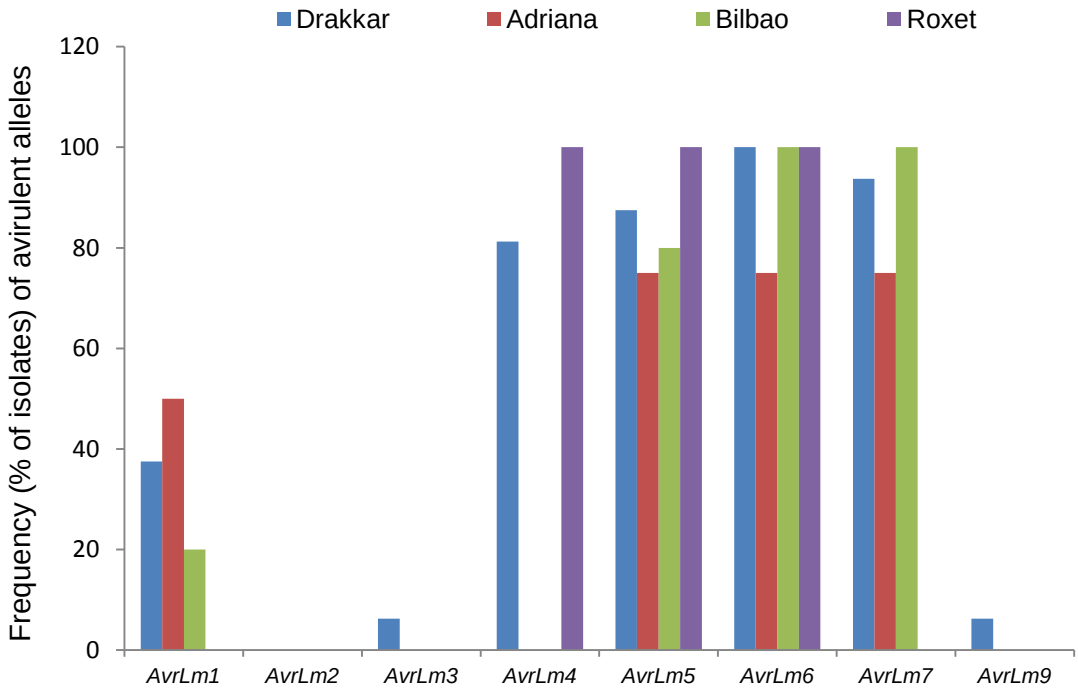

Figure 18: Mean frequencies (%) of avirulent alleles in isolates in *Leptosphaeria maculans* populations isolated from phoma leaf spot samples from cultivars Drakkar, Adriana, Bilbao and Roxet in the UK and France

There were differences in the presence of avirulent alleles at different sites. At Harpenden site, 62.5% isolates were avirulent to *Rlm*1, 37.5% were avirulent to *Rlm4*, 75% were avirulent to *Rlm5* and *Rlm7*, 87.5% were avirulent to *Rlm6* and 12.5% were avirulent to *Rlm9*. All the isolates from Morley site were avirulent to *Rlm4*, *Rlm5*, *Rlm6* and *Rlm7*. At

Cranwell site, 37.5% of isolates were avirulent to *Rlm1* and *Rlm4*, 100% to *Rlm5*, *Rlm6* and *Rlm7*. The isolates from Verpillieres site were avirulent to *Rlm5*, *Rlm6* and *Rlm7*, 75% were avirulent to *Rlm4* and 25% were avirulent to *Rlm*1 and *Rlm3*. All isolates from Banbury site were avirulent to *Rlm4*, *Rlm6* and *Rlm7* and 50% were avirulent to *Rlm5*. Similarly, all isolates from Rothwell site were avirulent to *Rlm5*, *Rlm6* and *Rlm7* (Figure 19).

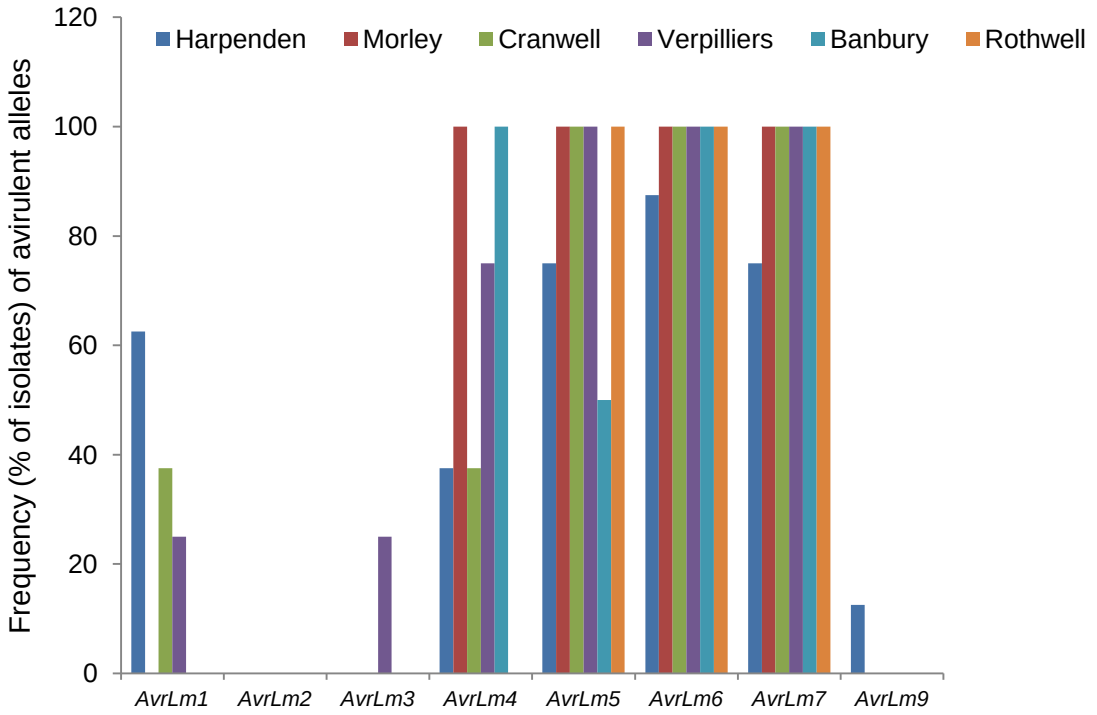

Figure 19: Mean frequencies (%) of avirulent alleles in isolates in *Leptosphaeria maculans* populations isolated from phoma leaf spot samples at various sites in the UK and France

The isolates from the cultivar Adriana mostly had avirulent *Avr* alleles *AvrLm5, AvrLm6* and *AvrLm7* from the Cranwell and Harpenden sites. At Harpenden, Adriana isolates also had avirulent *Avr* allele *AvrLm1* for the isolate K Adr 12-5-21. The isolates from the cultivar Bilbao had avirulent *Avr* alleles *AvrLm5, AvrLm6* and *AvrLm7* at the Cranwell and Rothwell sites. The isolate from the cultivar Roxet had avirulent *Avr* alleles *AvrLm4, AvrLm5* and *AvrLm6* at the Harpenden site (Table 6). The Drakkar isolates obtained from the Cranwell site were mostly avirulent against *Rlm5, Rlm6* and *Rlm7*. The Drakkar isolates obtained from the Harpenden site were avirulent against *Rlm4, Rlm5, Rlm6*, *Rlm7* and *Rlm9*. The isolates of the cultivar Drakkar obtained from France from the Verpillieres site were avirulent against *Rlm4*, *Rlm5*, *Rlm6* and *Rlm7*.

Table 6: *Leptosphaeria maculans* isolates obtained from different sites in the UK and France from cultivars Adriana, Bilbao, Drakkar and Roxet with the detection of avirulent and virulent alleles after cotyledon inoculation

Name of the isolate	Location	Cultivar	Race	
K12-34	Harpenden, UK	Drakkar	*AvrLm*1-4-5-6-7-(9)	*avrLm*2-3
K12-35	Harpenden, UK	Drakkar	*AvrLm*(1)-4-5-6-7	*avrLm*2-3-9
K12-36	Harpenden, UK	Drakkar	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
C12-2	Morley, UK	Drakkar	*AvrLm*(4)-5-6-7	*avrLm*1-2-3-9
I1 12-1	Cranwell, UK	Drakkar	*AvrLm*(1)-4-5-6-7	*avrLm*2-3-9
I1 12-2	Cranwell, UK	Drakkar	*AvrLm*(4)-5-6-7	*avrLm*1-2-3-9
I1 12-3	Cranwell, UK	Drakkar	*AvrLm*(1)-5-6-7	*avrLm*2-3-4-9
I1 12-4	Cranwell, UK	Drakkar	*AvrLm*(1)-4-5-6-7	*avrLm*2-3-9
I2 12-2	Verpillieres, France	Drakkar	*AvrLm*1-3-5-6	*avrLm*2-4-7-9
I2 12-4	Verpillieres, France	Drakkar	*AvrLm*(4)-5-6-7	*avrLm*1-2-3-9
I2 12-5	Verpillieres, France	Drakkar	*AvrLm*(4)-5-6-7	*avrLm*1-2-3-9
I2 12-8	Verpillieres, France	Drakkar	*AvrLm*4-5-6-7	*avrLm*1-2-3-9
A-Dr 12-5	Banbury, UK	Drakkar	*AvrLm*4-5-6-7	*avrLm*1-2-3-9
A-Dr 12-6	Banbury, UK	Drakkar	*AvrLm*4-(5)-6-7	*avrLm*1-2-3-9
A-Dr 12-13	Banbury, UK	Drakkar	*AvrLm*4-6-7	*avrLm*1-2-3-5-9
A-Dr 12-10	Banbury, UK	Drakkar	*AvrLm*4-6-7	*avrLm*1-2-3-5-9
I1 Adr 12-7	Cranwell, UK	Adriana	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
I1 Adr 12-3	Cranwell, UK	Adriana	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
I1 Bilb 12-3	Cranwell, UK	Bilbao	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
I1 Bilb 12-4	Cranwell, UK	Bilbao	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
K Adr 12-7-22	Harpenden, UK	Adriana	*AvrLm*(1)-5-6-7	*avrLm*2-3-4-9
K-Adr 12-5-21	Harpenden, UK	Adriana	*AvrLm*1	*avrLm*2-3-4-5-6-7-9
K Bilb 12-9-21	Harpenden, UK	Bilbao	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
K Bilb 12-11-21	Harpenden, UK	Bilbao	*AvrLm*(1)-6-7	*avrLm*2-3-4-5-9
D Bilb 12-8	Rothwell, UK	Bilbao	*AvrLm*5-6-7	*avrLm*1-2-3-4-9
K Rox 12-5-32	Harpenden, UK	Roxet	*AvrLm*4-5-6	*avrLm*1-2-3-7-9

One isolate (I2 12-2) from Verpillieres was avirulent against *Rlm*1 and *Rlm3* (Table 6). It was observed from the results that no isolates of *L. maculans* had the avirulent alleles *AvrLm2* or *AvrLm3*. Avirulent alleles *AvrLm6* and *AvrLm7* were present in all the isolates from all the sites. From the results, it was clear that the *Rlm6* and *Rlm7* resistances still seem to be effective in the United Kingdom.

3.5 Detection of avirulent *AvrLm1* allele in *L. maculans* isolates by allele-specific PCR

Sixty-nine isolates of *L. maculans* were tested for the presence of the avirulent *AvrLm1* allele by allele-specific PCR (Appendix 1).

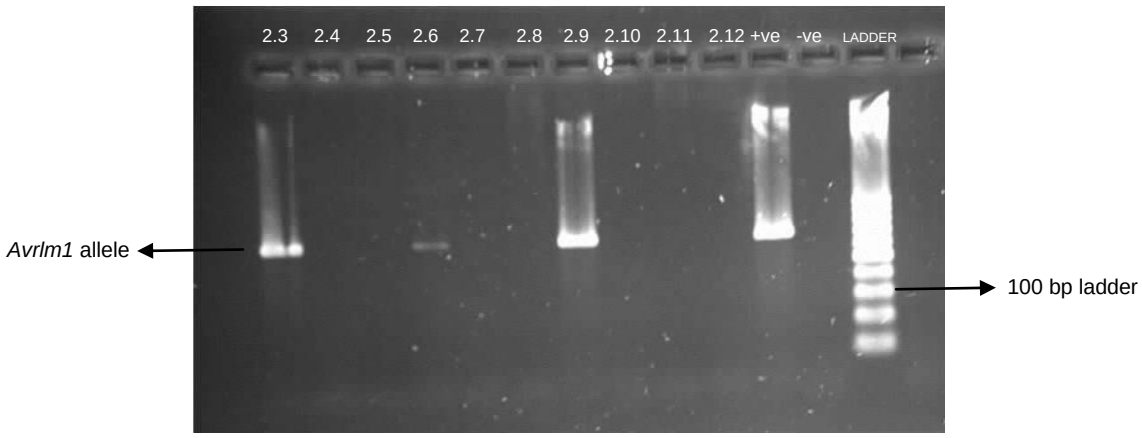

Figure 20: Agarose gel analysis of allele-specific PCR products. Three samples analysed in this gel showed the presence of the avirulent *AvrLm1* allele (samples in 1, 4 and 7 lanes). A 100 bp ladder was used in this gel. The PCR amplicons observed had approximately 500bp.

In this agarose gel (Figure 20), seventeen *L. maculans* isolates were tested for the presence of the avirulent *AvrLm1* allele. Among them, three isolates showed bands confirming its presence. The isolates were Rox K10-1, Rox K10-4 and Rox K10-7. The isolates were from the cultivar Roxet and were from Harpenden site. Twenty-four *L. maculans* isolates were tested in another agarose gel to check the presence of *AvrLm1* allele. Among them, only one isolate showed the presence of *AvrLm1* allele. That isolate was 3.7 from the cultivar Roxet from the Harpenden site (Appendix 10). Out of seventy-two isolates of *L. maculans,* only four isolates had the avirulent *AvrLm1* allele, which were from the cultivar Roxet and from the Harpenden site (Appendix 10).

4 Discussion

This study reveals similarities between various sites of the UK in the presence of *Avr* alleles and populations race structure of *L. maculans*. This survey of the regional distribution of races of *L. maculans* isolates from phoma leaf spot samples from different oilseed rape cultivars in the UK in 2013 was comparable to that of similar surveys in France, 2000/2001 (Balesdent *et al*., 2006) and Europe, 2002/2003 (Stachowiak *et al*., 2006). These surveys used the same methodology and a similar kind of differential set of oilseed rape cultivars, except for *Rlm9*.

The presence/absence of the resistance genes in the oilseed rape cultivars showed a great impact on the severity of the phoma leaf spotting in these field experiments. The absence of resistance genes increased the number of phoma leaf spots on cultivars (e.g. Drakkar) and the presence of resistance genes decreased the number of phoma leaf spots on cultivars (e.g. Adriana). From the data it was evident that Drakkar cultivar without any resistance genes (Balesdent *et al*., 2006) had more phoma leaf spots than the other cultivars with *R* genes with/without quantitative resistance. There were differences in the number of phoma leaf spots on the same cultivars between different sites in the UK. When the sites were compared, there were a greater number of phoma leaf spots at the Cranwell site compared to the Harpenden and Bainton sites.

From the error bars on the graph (Figure 14), the number of leaf spots on cultivar Drakkar varied from leaf to leaf whereas for all other cultivars the error bars were close, suggesting that the number of leaf spots were consistent for all leaf samples. Differences between sites in number of phoma leaf spots may have occurred because of the differences in weather conditions between sites during the season (Toscano-Underwood *et al*., 2001). Previous studies have 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 (Appendices 2, 3 & 4).

There were differences between sites and between cultivars in race composition of *L. maculans* populations. In order to detect the efficiency of the existing cultivar resistance, *Avr* alleles were detected by a cotyledon test by inoculating the spore suspensions onto a

differential set of cultivars. The observation that no isolate of *L. maculans* possessed avirulent alleles of *AvrLm2*, *AvrLm3* and *AvrLm9* is consistent with the French and European surveys. It suggests that virulent alleles of *AvrLm2*, *AvrLm3* and *AvrLm9* are fixed in the *L. maculans* populations (Table 6) (Balesdent *et al*., 2006 & Stachowiak *et al*., 2006). Therefore, from these results it is evident that *Rlm2*, *Rlm3* and *Rlm9* resistances are no longer effective.

A survey in France stated that there was a rapid decrease in the frequency of the virulent *AvrLm1* allele over three seasons of intensive cultivation of cultivars with *Rlm*1 resistance gene (Rouxel *et al*., 2003a). In this study, only two avirulent *AvrLm1* alleles were obtained from the isolates from cultivar Adriana. All these isolates were from the site Harpenden (Table 6). 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). The *AvrLm4* alleles were observed only in the isolates from the cultivars Roxet and Drakkar.

The European survey suggested that the *Rlm6* and *Rlm7* resistant genes were the effective sources of resistance against *L. maculans* (Stachowiak *et al*., 2006). In support of that statement, in this study all the isolates from different sites possessed the *AvrLm6* and *AvrLm7* alleles, except one isolate K-Adr 12-5-21 from the cultivar Adriana from the Harpenden site. The presence of the *AvrLm5* allele was also similar to that in the French and European surveys. Until now, the *Rlm5* resistance gene has not been used in commercial cultivars. However the presence of virulent alleles of *AvrLm5* may have occurred because of the widespread use of *B. juncea* (source of *Rlm5*). 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).

Changes in the frequencies of *Avr* alleles were observed when compared to the European and French surveys. The frequency of *AvrLm1* allele has increased to 35% in this study. It was 8% in the European survey and 20% in the France survey. The observation that no isolates of *L. maculans* possessed *AvrLm2* was consistent with the other two surveys. The frequency of *AvrLm3* allele was 3.8% in this study. But in the previous surveys there were no isolates possessing *AvrLm3* allele. There was a huge increase in the frequency of *AvrLm4* allele (50%) in this study when compared to European survey (2%) and French survey (9%). The frequency of *AvrLm5* allele (84.6%) was mostly consistent with the European survey (86%) and French survey (84%). The frequency of *AvrLm6* allele has decreased to 96.2%

when compared to the European and France surveys (100%). Even the frequency of *AvrLm7* allele (88.5%) has appeared to be changed from the European and France surveys (99%). The frequency of *AvrLm9* allele was observed to be 3.8% in this study which was nil in the European and France surveys.

The difference in the presence of *Avr* alleles in the cultivars between various sites could have been because of widespread use of resistant cultivars or the unregulated introduction of new resistance genes (Rouxel *et al*., 2003a; Howlett, 2004; Sprague *et al*., 2006). The isolates obtained from the Cranwell site mostly had the avirulent *Avr* alleles of *AvrLm5, AvrLm6* and *AvrLm7*. The isolates obtained from the Harpenden site mostly had avirulent *AvrLm4, AvrLm5, AvrLm6*, *AvrLm7* and *AvrLm9* (Table 6). Increased cultivation of cultivars with the *Rlm*1 resistance gene resulted in a rapid decrease in frequency of the avirulent allele *AvrLm1* 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 resistances (Rouxel *et al*., 2003a; Howlett, 2004; Sprague *et al*., 2006).

Species-specific PCR confirmed the identification of most isolates as *L. maculans*. Seventy-two samples were visually differentiated on PDA plates and then later confirmed by the PCR. Sixty-nine of them were confirmed as *L. maculans* isolates. The remaining three might be an experimental error during isolation of pathogen. Among the sixty-nine isolates, isolates from cultivar Roxet were detected with the presence of *AvrLm1* allele (Appendix 10). There are differences between the French-European surveys and this survey. These surveys involved samples taken in 2000, 2001& 2002, whereas the samples used in this survey were collected in 2013. They surveyed 600-800 samples, but due to the time and sample availability only twenty-six samples were used in this survey; They were from the cultivars NK Grandia, Es-Astrid, Adriana, Capitol, Excel, Bilbao and DK Cabernet from two sites in the UK (Harpenden, Spalding) and from Poland (Appendix 1). If a larger number of samples from various sites were surveyed, it would give a clear idea about the race distribution of *L. maculans* isolates in the UK.

The *L. maculans* population was mostly virulent against *Rlm1.* In this study the presence of *AvrLm1* allele was checked in sixty-nine *L. maculans* isolates by using allele-specific PCR. Only four isolates from the cultivar Roxet were detected with the presence of *AvrLm1* allele. In conclusion, the results from this study suggest that the effectiveness of resistance

depends on the type of resistance genes containing 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 for 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) every year might be more useful for the farmers in the UK. 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. So relying on the information of the frequency of *Avr* alleles rather than confirming the resistance of the cultivar from the field experiments may be more useful. Such knowledge will therefore help with deployment of cultivars with different resistance genes.

Strategically planning deployment of specific resistant genes by using the cultivars with known resistance in rotation has also been shown to contribute to the durability of resistance (Aubertot *et al*., 2005). New schemes such as surveying the populations of *Leptosphaeria maculans* in the oilseed rape cultivars for the presence of virulence and avirulence 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). For an effective deployment of *R* genes and to maintain the valuable sources of resistance of the cultivars against the fungal pathogens constantly for a long term use, the *Avr* alleles have to be surveyed over large areas with a huge number of samples every year in the UK as in France and Australia (Gladders *et al*., 2006). But due to the lapse of time only few samples were surveyed in this study. The farmers are to be guided properly by the organizations in selecting the cultivars every year based on their effective resistance through a web based scheme.

References

Aubertot J.N., West J.S., Bousset-Vaslin L., Salam M.U., Barbetti M.J and Diggle A.J. (2006). Improved resistance management for durable disease control: a case study of phoma stem canker of oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114, 91–106.

Andrew F. Bent and David Mackey. (2007). Elicitors, Effectors and *R* Genes: The New Paradigm and a Lifetime Supply of Questions. *Annual Review of Phytopathology* 45, 399–436.

Ansan-Melayah D., Balesdent M.H., Delourme R. (1998). Genes for race-specific resistance against blackleg disease in *Brassica napus* L. *Plant Breeding,* 117, 373–8.

Balesdent M.H., Fudal I., Ollivier B., Bally P., Grandaubert J., Eber F, Chevre A.M., Leflon M and Rouxel T. (2013).The dispensable chromosome of *Leptosphaeria maculans* shelters an effector gene conferring avirulence towards *Brassica rapa*. *New Phytologist* 198*,* 887–898*.*

Balesdent M.H., Barbetti M.J., Li H., Sivasithamparam K., Gout L. and Rouxel T. (2005). Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology* 95, 1061-1071.

Balesdent M. H., Attard A., Ansan-Melayah D., Delourme R., Renard M., and Rouxel T. (2001). Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. *Phytopathology* 91, 70-76.

Beddington J*.* (2010)*.* Food security: contributions from science to a new and greener revolution. *Philosophical Transactions of the Royal Society* B 365, 61–71.

Biddulph J.E., Fitt B.D.L., Leech P.K., Welham S.J., Gladders P. (1999). Effects of temperature and wetness duration on infection of oilseed rape leaves by ascospores of *Leptosphaeria maculans* (stem canker). *European Journal of Plant Pathology* 105, 769–81.

Boyd LA. (2006). Can the durability of resistance be predicted? *Journal of the Science of Food and Agriculture* 86, 2523–2526.

Brun H., Chevre A.M., Fitt B.D.L., Powers S., Besnard A.L., Ermel M., Huteau V., Marquer B., Eber F., Renard M., Andrivon D. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytologist* 185, 285-99.

Butterworth M.H., Semenov M.A., Barnes A., Moran D., West J.S., and Fitt B.D.L. (2010). North–South divide: contrasting impacts of climate change on crop yields in Scotland and England. *Journal of the Royal Society Interface* 7, 123-130.

Catanzariti, A. M., Dodds, P. N., Lawrence, G. J., Ayliffe, M. A., and Ellis, J. G. (2006). Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. *Plant Cell* 18, 243-256.

Delourme R., Chevre A.M., Brun H., Rouxel T., Balesdent M.H., Dias J.S., Salisbury P., Renard M. and Rimmer S.R. (2006). Major gene and polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114, 41-52.

Ellis JG., Rafiqi M., Gan P., Chakrabarti A., Dodds PN. (2009). Recent progress in discovery and functional analysis of effector proteins of fungal and oomycete plant pathogens. *Current Opinion in Plant Biology* 12, 399–405.

Evans N., Baierl A., Semenov M. A., Gladders P. and Fitt B.D.L. (2008). Range and severity of a plant disease increased by global warming. *Journal of the Royal Society Interface* 5, 525-531.

Fitt B.D.L., Brun H., Barbetti M.J. and Rimmer, S.R. (2006a). World-wide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 114, 3-15.

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

Flood J. (2010). The importance of plant health to food security. *Food Security* 2, *215*–31.

Fitt B.D.L., Fraaije B.A., Chandramohan P., Shaw M.W. (2011). Impacts of changing air composition on severity of arable crop disease epidemics. *Plant Pathology* 60, 44–53.

Flor H. (1947). Host-parasite interactions in flax rust – Its genetics and other implications. *Phytopathology* 85, 213-217.

Gladders P., Evans N., Marcroft S. and Pinochet X. (2006). Dissemination of information about management strategies and changes in farming practices for the exploitation of resistance to *Leptosphaeria maculans* (phoma stem canker) in oilseed rape cultivars. *European Journal of Plant Pathology* 114, 117–126.

Greenberg, J.T and Yao, N. (2004). The role and regulation of programmed cell death in plant–pathogen interactions. *Cellular Microbiology* 6, 201–211.

Gout L., Fudal I., Kuhn M.L., Blaise F., Cattolico L., Balesdent M.H. and Rouxel T. (2006). Lost in the middle of nowhere: the *AvrLm1* avirulence gene of *Leptosphaeria maculans*. *Molecular Microbiology* 60, 67–80.

Hall R. (1992). Epidemiology of blackleg of oilseed rape. *Canadian Journal of Plant Pathology* 14, 46 ± 55.

Hammond K.E. and Lewis B.G. (1987). Differential responses of oilseed rape leaves to *Leptosphaeria maculans*. *Transactions of the British Mycological Society* 88, 329-333.

Hammond K.E., Lewis B.G. and Musa T.M. (1985). A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathology* 34, 557-565.

Hammond-Kosack K.E. and Jones J.D.G. (1997). Plant disease resistance genes. *Annual Review of Plant Physiology and Plant Molecular Biology* 48, 575- 607.

Howlett B.J. (2004). Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* 26, 245–252.

Howlett B.J., Idnurm A., Pedras M.S.C. (2001). *Leptosphaeria maculans*, the causal agent of blackleg disease of *Brassicas*. *Fungal Genetics and Biology* 33, 1–14.

Huang, Y., Mitrousia, G. & Fitt, B. (2013). Understanding interactions between quantitative resistance and qualitative resistance for sustainable management of phoma stem canker (*Leptosphaeria maculans*) in oilseed rape (*Brassica napus*). *10th International Congress of Plant Pathology. Chinese Society for Plant Pathology*, Vol. 43,p.

Huang Y.J., Balesdent M.H., Zi-Qin Li., Evans N., Rouxel T and Fitt B.D.L. (2010). Fitness cost of virulence differs between the *AvrLm1*and *AvrLm4* loci in *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *European Journal of Plant Pathology* 126, 279–291.

Huang Y.J., Fitt B.D.L., Jedryczka M., Dakowska S., West J.S., Gladders P., Steed J.M., Li Z.Q. (2005). Patterns of ascospore release in relation to phoma stem canker epidemiology in England (*Leptosphaeria maculans*) and Poland (*L. biglobosa*). *European Journal of Plant Pathology* 111, 263–77.

Huang Y.J., Toscano-Underwood C., Fitt B.D.L., Hu X.J. and Hall A.M. (2003). Effects of temperature on ascospore germination and penetration of oilseed rape (*Brassica napus*) leaves by A- or B-group *Leptosphaeria maculans* (phoma stem canker). *Plant Pathology* 52, 245-255.

Jacobsen B.J. and Williams P.H. (1971). Histology and control of *Brassica oleracea* seed infection by Phoma lingam. *Plant Disease Reporter* 55, 934 ± 8.

Jones J.D. and Dangl J.L. (2006). The plant immune system. *Nature* 444, 323–329.

Keen, N.T. (1990). Gene-for-gene complementarity in plant–pathogen interactions. *Annual Review of Genetics* 24, 447–463.

Kings (1997). http:www.kings.co.uk/oilseed.htm

Larkan N.J., Lydiate D.J., Parkin I. A. P., Nelson M. N., Epp D. J., Cowling W. A., Rimmer S. R. and Borhan M. H. (2013). The *Brassica napus* blackleg resistance gene *LepR*3 encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector *AvrLm1*. *New Phytologist* 197, 595–605.

Liu S.Y., Liu Z., Fitt B.D.L., Evans N., Foster S.J., Huang Y.J., Latunde-Dada A. O and Lucas J.A. (2006). Resistance to *Leptosphaeria maculans* (phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathology* 55, 401-412.

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.

Pilet ML., Delourme R., Foisset N., Renard M. (1998). Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculans* (Desm.) Ces. et de Not., in winter rape seed (*Brassica napus L*.). *Theoretical and Applied Genetics* 96, 23–30.

Rouxel T., Penaud A., Pinochet X., Brun H., Gout L., Delourme R., Schmit J. and Balesdent M.H. (2003a). A ten year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm*1 resistance gene of oilseed rape. *European Journal of Plant Pathology* 109, 871–881.

Shoemaker R.A. and Brun H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. *Canadian Journal of Botany* 79, 412–19.

Sprague S.J., Balesdent M.H., Brun H., Hayden H.L., Marcroft S.J., Pinochet X., Rouxel T. and Howlett B.J. (2006). Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. *European Journal of Plant Pathology* 114, 33–46.

Stachowiak A., Olechnowicz J., Jedryczka M., Rouxel T., Balesdent M.H., Happstadius I., Gladders P., Latunde-Dada A and Evans N. (2006). Frequency of Avirulence Alleles in Field Populations of *Leptosphaeria maculans* in Europe. *European Journal of Plant Pathology* 114, 67-75.

Stergiopoulos I., De Kock M.J., Lindhout P., and De Wit P.J.G.M. (2007). Allelic variation in the effector genes of the tomato pathogen *Cladosporium fulvum* reveals different modes of adaptive evolution. *Molecular Plant-Microbe Interactions* 20, 1271-1283.

Stonard J.F., Latunde-Dada A.O., Huang Y.J., West J.S., Evans N. and Fitt B.D.L. (2010). Geographic variation in severity of phoma stem canker and *Leptosphaeria maculans/ L. biglobosa* populations on UK winter oilseed rape (*Brassica napus*). *European Journal of Plant Pathology* 126, 97-109.

Stotz H.U., Mitrousia G.K., De Wit P.J.G.M. and Fitt B.D.L. (2014). Effector-triggered defence against apoplastic fungal pathogens. *Trends in Plant Science*, Vol. 19, No. 8, 491-500.

Toscano-Underwood C., Huang Y.J., Fitt B.D.L., Hall A.M. (2003). Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathology* 52, 726-36.

Toscano-Underwood C., West J.S., Fitt B.D.L., Todd A.D. and Jedryczkab M. (2001). Development of phoma lesions on oilseed rape leaves inoculated with ascospores of A-group or B-group *Leptosphaeria maculans* (stem canker) at different temperatures and wetness durations. *Plant Pathology 50*, 28- 41.

A.P. Van de Wouw and B.J. Howlett. (2012). Estimating frequencies of virulent isolates in field populations of a plant pathogenic fungus, *Leptosphaeria maculans*, using high-throughput pyrosequencing. *Journal of Applied Microbiology ISSN,* 1364-5072.

Van de Wouw A., Cozijnsen A.J., Hane J.K., Brunner P.C., McDonald B.A. Oliver R.P. and Howlett B.J. (2010). Evolution of linked avirulence effectors in *Leptosphaeria maculans* is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathogens* 6, e1001180.

Van Der Biezen, E.A., and Jones, J.D.G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23, 454–456.

West J.S., Biddulph J.E., Fitt B.D.L. and Gladders P. (1999). Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK. *Annals of Applied Biology* 135, 535–46.

West J.S., Kharbanda P., Barbetti M.J. and Fitt B.D.L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) in Australia, Canada and Europe. *Plant Pathology* 50, 10–27.

West J.S., Balesdent M.H., Rouxel T., Narcy J.P., Huang Y.J., Roux J., Steed J.M., Fitt B.D.L. and Schmit J. (2002a). Colonization of winter oilseed rape tissues by A/Tox+ and B/ Tox0 *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology* 51, 311-321.

West J.S., Jedryczka M., Leech P.K., Dakowska S., Huang Y.J and Fitt B.D.L. (2002b). Biology of *Leptosphaeria maculans* (stem canker) ascospore release in England and Poland. *International Organisation for Biological Control Bulletin* 25, 21–30.

Wood P. McR and Barbetti M.J. (1977a). The role of seed infection in the spread of blackleg of rape in Western Australia. *Australian Journal of Experimental Agriculture and Animal Husbandry* 17, 1040 ± 4.

Appendices

Appendix 1: Summary of experiments done on the isolates of *Leptosphaeria maculans* from different cultivars from various sites

Cultivar	Site	No. of samples /isolates	Used to study
		150 (total number of phoma leaf spot samples)	Phoma leaf spot assessment
Drakkar	Harpenden, Bainton	30	
Bilbao	and Cranwell	30	
Adriana	(10 leaves from each cultivar	30	
Excel	from each of the three sites)	30	
Roxet		30	
		72 (total number of isolates)	Species-specific PCR to Confirm the identity of *L. maculans* isolates
Drakkar	Spalding	18	
Drakkar	Harpenden	4	
Drakkar	Stockbridge	1	
Drakkar	Poland	8	
Roxet	Harpenden	12	
NK Grandia	Harpenden	4	
Es-Astrid	Harpenden	4	
Adriana	Harpenden	4	
Excel	Harpenden	4	
Capitol	Harpenden	4	
Bilbao	Harpenden	3	
DK Cabernet	Harpenden	6	
		26 (total number of isolates)	Cotyledon test method (*Avr* alleles)
Drakkar	Harpenden	3	
Drakkar	Morley	1	
Drakkar	Cranwell	4	
Drakkar	Verpillieres	4	
Drakkar	Banbury	4	
Adriana	Cranwell	2	
Adriana	Harpenden	2	
Bilbao	Cranwell	2	
Bilbao	Harpenden	2	
Bilbao	Rothwell	1	
Roxet	Harpenden	1	

		69 (total number of isolates)	Allele-specific PCR for the detection of the presence of *AvrLm1* allele
Drakkar	Spalding	18	
Drakkar	Harpenden	4	
Drakkar	Stockbridge	1	
Drakkar	Poland	8	
Roxet	Harpenden	11	
NK Grandia	Harpenden	4	
Es-Astrid	Harpenden	4	
Adriana	Harpenden	3	
Excel	Harpenden	4	
Capitol	Harpenden	4	
Bilbao	Harpenden	3	
DK Cabernet	Harpenden	5	

Appendix 2: Phoma leaf spot assessment on cultivars grown at Cranwell (No. of spots/leaf) on 13 February 2013

	Number of leaf spots				
Leaf	**Drakkar**	**Excel**	**Roxet**	**Adriana**	**Bilbao**
1	38	17	16	6	32
2	39	31	12	9	10
3	13	17	18	17	36
4	25	6	17	12	21
5	17	1	27	6	16
6	24	12	11	12	12
7	11	8	19	21	36
8	26	11	10	12	14
9	20	10	23	5	28
10	24	13	21	17	14
Average	23.7	12.6	17.4	11.7	21.9
Standard deviation	9.31	8.07	5.44	5.37	10.20
Standard error mean	2.94	2.55	1.72	1.7	3.22

Appendix 3: Phoma leaf spot assessment on cultivars grown at Bainton (No. of spots/leaf) on 28 February 2013

	Number of leaf spots				
Leaf	**Drakkar**	**Excel**	**Roxet**	**Adriana**	**Bilbao**
1	6	3	9	4	14
2	3	6	2	11	6
3	4	6	6	4	6
4	10	15	4	5	3
5	1	8	7	4	7
6	4	2	8	5	14
7	13	3	11	2	14
8	2	2	10	3	16
9	4	11	8	5	11
10	2	7	3	7	9
Average	4.9	6.3	6.8	5	10
Standard deviation	3.81	4.21	3.01	2.49	4.42
Standard error mean	1.20	1.33	0.95	0.78	1.39

Appendix 4: Phoma leaf spot assessment on cultivars grown at Harpenden (No. of spots/leaf) on 30 January 2013

	Number of leaf spots				
Leaf	**Drakkar**	**Excel**	**Roxet**	**Adriana**	**Bilbao**
1	13	6	3	14	5
2	6	2	4	3	4
3	16	1	5	1	5
4	17	6	3	2	3
5	11	1	2	4	3
6	9	1	3	4	13
7	18	5	6	4	6
8	12	2	0	7	9
9	29	4	4	3	8
10	17	2	6	8	5
Average	14.8	3	3.6	5	6.1
Standard deviation	6.32	2.05	1.83	3.80	3.10
Standard error mean	1.99	0.64	0.58	1.20	0.98

Appendix 5: Significant statistical difference in number of phoma leaf spots between the five cultivars at three sites

Oneway ANOVA

Bainton

	Sum of Squares	DF	Mean Square	F	Significance
Between cultivars	185.59	4	46.39	3.76	0.010
Within cultivars	604.60	49	12.33		
Total	790.19	53			

Multiple Comparisons

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Standard Error	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Drakkar	Excel	-1.40	1.53	1.00	-5.91	3.11
	Roxet	-1.90	1.53	1.00	-6.41	2.61
	Adriana	-0.10	1.53	1.00	-4.61	4.41
	Bilbao	-5.10*	1.53	0.01	-9.61	-0.58
Excel	Drakkar	1.40	1.53	1.00	-3.11	5.91
	Roxet	-0.50	1.49	1.00	-4.90	3.90
	Adriana	1.30	1.49	1.00	-3.10	5.70
	Bilbao	-3.70	1.49	0.17	-8.10	0.70
Roxet	Drakkar	1.90	1.53	1.00	-2.61	6.41
	Excel	0.50	1.49	1.00	-3.90	4.90
	Adriana	1.80	1.49	1.00	-2.60	6.20
	Bilbao	-3.20	1.49	0.37	-7.60	1.20
Adriana	Drakkar	0.10	1.53	1.00	-4.41	4.61
	Excel	-1.30	1.49	1.00	-5.70	3.10
	Roxet	-1.80	1.49	1.00	-6.20	2.60
	Bilbao	-5.00*	1.49	0.01	-9.40	-0.59
Bilbao	Drakkar	5.10*	1.53	0.01	.058	9.61
	Excel	3.70	1.49	0.17	-0.70	8.10
	Roxet	3.20	1.49	0.37	-1.20	7.60
	Adriana	5.00*	1.49	0.01	0.59	9.40

*. The mean difference is significant at the 0.05 level.

Cranwell

	Sum of Squares	DF	Mean Square	F	Significance
Between Groups	1154.52	4	288.63	4.59	0.003
Within Groups	2829.90	45	62.88		
Total	3984.42	49			

Multiple Comparisons

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Standard Error	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Drakkar	Excel	11.10*	3.54	0.03	0.63	21.56
	Roxet	6.30	3.54	0.82	-4.16	16.76
	Adriana	12.00*	3.54	0.01	1.53	22.46
	Bilbao	1.80	3.54	1.00	-8.66	12.26
Excel	Drakkar	-11.10*	3.54	0.03	-21.56	-0.63
	Roxet	-4.80	3.54	1.00	-15.26	5.66
	Adriana	0.90	3.54	1.00	-9.56	11.36
	Bilbao	-9.30	3.54	0.11	-19.76	1.16
Roxet	Drakkar	-6.30	3.54	0.82	-16.76	4.16
	Excel	4.80	3.54	1.00	-5.66	15.26
	Adriana	5.70	3.54	1.00	-4.76	16.16
	Bilbao	-4.50	3.54	1.00	-14.96	5.96
Adriana	Drakkar	-12.00*	3.54	0.01	-22.46	-1.53
	Excel	-0.90	3.54	1.00	-11.36	9.56
	Roxet	-5.70	3.54	1.00	-16.16	4.76
	Bilbao	-10.20	3.54	0.06	-20.66	0.26
Bilbao	Drakkar	-1.80	3.54	1.00	-12.26	8.66
	Excel	9.30	3.54	0.11	-1.16	19.76
	Roxet	4.50	3.54	1.00	-5.96	14.96
	Adriana	10.20	3.54	0.06	-0.26	20.66

*. The mean difference is significant at the 0.05 level.

Harpenden

	Sum of Squares	DF	Mean Square	F	Significance
Between Groups	919.60	4	229.90	16.04	0.000
Within Groups	644.90	45	14.33		
Total	1564.500	49			

Multiple Comparisons

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Standard Error	Significance	95% Confidence Interval	
					Lower Bound	Upper Bound
Drakkar	Excel	11.80*	1.69	0.00	6.80	16.79
	Roxet	11.20*	1.69	0.00	6.20	16.19
	Adriana	9.80*	1.69	0.00	4.80	14.79
	Bilbao	8.70*	1.69	0.00	3.70	13.69
Excel	Drakkar	-11.80*	1.69	0.00	-16.79	-6.80
	Roxet	-0.60	1.69	1.00	-5.59	4.39
	Adriana	-2.00	1.69	1.00	-6.99	2.99
	Bilbao	-3.10	1.69	0.73	-8.09	1.89
Roxet	Drakkar	-11.20*	1.69	0.00	-16.19	-6.20
	Excel	0.60	1.69	1.00	-4.39	5.59
	Adriana	-1.40	1.69	1.00	-6.39	3.59
	Bilbao	-2.50	1.69	1.00	-7.49	2.49
Adriana	Drakkar	-9.80*	1.69	0.00	-14.79	-4.80
	Excel	2.00	1.69	1.00	-2.99	6.99
	Roxet	1.40	1.69	1.00	-3.59	6.39
	Bilbao	-1.10	1.69	1.00	-6.09	3.89
Bilbao	Drakkar	-8.70*	1.69	0.00	-13.69	-3.70
	Excel	3.10	1.69	0.73	-1.89	8.09
	Roxet	2.50	1.69	1.00	-2.49	7.49
	Adriana	1.10	1.69	1.00	-3.89	6.09

*. The mean difference is significant at the 0.05 level.

Appendix 6: Identification of *Avr* alleles in different *L. maculans* isolates by the cotyledon test method (R- resistant, MR- medium resistant and S- susceptible)

	Phenotype of different cultivars/lines								
Name of the Isolate	*Rlm1*	*Rlm2*	*Rlm3*	*Rlm4*	*Rlm5*	*Rlm6*	*Rlm7*	*Rlm9*	Race
K12-34	R	S	S	R	R	R	R	MR	*AvrLm1*-4-5-6-7-(9)
K12-35	MR	S	S	R	R	R	R	S	*AvrLm*(1)-4-5-6-7
K12-36	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
C12-2	S	S	S	MR	R	R	R	S	*AvrLm*(4)-5-6-7
I1 12-1	MR	S	S	R	R	R	R	S	*AvrLm*(1)-4-5-6-7
I1 12-2	S	S	S	MR	R	R	R	S	*AvrLm*(4)-5-6-7
I1 12-3	MR	S	S	S	R	R	R	S	*AvrLm*(1)-5-6-7
I1 12-4	MR	S	S	R	R	R	R	S	*AvrLm*(1)-4-5-6-7
I2 12-2	R	S	R	S	R	R	S	S	*AvrLm1*-3-5-6
I2 12-4	S	S	S	MR	R	R	R	S	*AvrLm*(4)-5-6-7
I2 12-5	S	S	S	MR	R	R	R	S	*AvrLm*(4)-5-6-7
I2 12-8	S	S	S	R	R	R	R	S	*AvrLm4*-5-6-7
A-Dr 12-5	S	S	S	R	R	R	R	S	*AvrLm4*-5-6-7
A-Dr 12-6	S	S	S	R	MR	R	R	S	*AvrLm4*-(5)-6-7
A-Dr 12-13	S	S	S	R	S	R	R	S	*AvrLm4*-6-7
A-Dr 12-10	S	S	S	R	S	R	R	S	*AvrLm4*-6-7
I1 Adr 12-7	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
I1 Adr 12-3	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
I1 Bilb 12-3	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
I1 Bilb 12-4	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
K Adr 12-7-22	MR	S	S	S	R	R	R	S	*AvrLm*(1)-5-6-7
K-Adr 12-5-21	R	S	-	-	-	-	-	-	*AvrLm1*
K Bilb 12-9-21	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
K Bilb 12-11-21	MR	S	S	S	-	R	R	S	*AvrLm*(1)-6-7
D Bilb 12-8	S	S	S	S	R	R	R	S	*AvrLm5*-6-7
KRox 12-5-32	S	S	S	R	R	R	S	S	*AvrLm4*-5-6

Appendix 7: Samples with *Lm* primers run in the agarose gel electrophoresis (Figure 17)

Sample ID	Isolate	Cultivar	Location
1.3	Rox K10-1	Roxet	Harpenden
1.4	Rox K10-2	Roxet	Harpenden
1.5	Rox K10-3	Roxet	Harpenden
1.6	Rox K10-4	Roxet	Harpenden
1.7	Rox K10-5	Roxet	Harpenden
1.8	Rox K10-6	Roxet	Harpenden
1.9	Rox K10-7	Roxet	Harpenden
1.10	Rox K10-8	Roxet	Harpenden
1.11	Rox KB10-1	Roxet	Harpenden
1.12	Rox KB10-2	Roxet	Harpenden
1.13	Rox KB10-5	Roxet	Harpenden
1.14	Exc K10-1	Excel	Harpenden
1.15	Exc K10-2	Excel	Harpenden
1.16	Exc K10-3	Excel	Harpenden
1.17	Exc K10-4	Excel	Harpenden
1.18	Ari K10-1	Adriana	Harpenden
1.19	Ari K10-2	Adriana	Harpenden
1.20	Ari K10-3	Adriana	Harpenden
1.21	Cab K B10-3	DK Cabernet	Harpenden
1.22	Cab K B10-4	DK Cabernet	Harpenden
1.23	Cab K10-1	DK Cabernet	Harpenden
1.24	Cab K10-2	DK Cabernet	Harpenden
1.25	Cab K10-3	DK Cabernet	Harpenden
1.26	Cab K10-4	DK Cabernet	Harpenden

Appendix 8: Samples with *Lb* primers run in the agarose gel electrophoresis (Figure 17)

Sample ID	Isolate	Cultivar	Location
3.2	Rox K10-1	Roxet	Harpenden
3.3	Rox K10-2	Roxet	Harpenden
3.4	Rox K10-3	Roxet	Harpenden
3.5	Rox K10-4	Roxet	Harpenden
3.6	Rox K10-5	Roxet	Harpenden
3.7	Rox K10-6	Roxet	Harpenden
3.8	Rox K10-7	Roxet	Harpenden
3.9	Rox K10-8	Roxet	Harpenden
3.10	Rox KB10-1	Roxet	Harpenden
3.11	Rox KB10-2	Roxet	Harpenden

Appendix 9: *L. maculans* isolates tested for the presence of the *AvrLm1* allele (Figure 20)

Sample ID	Isolate	Cultivar	Location
2.3	Rox K10-1	Roxet	Harpenden
2.4	Rox K10-2	Roxet	Harpenden
2.5	Rox K10-3	Roxet	Harpenden
2.6	Rox K10-4	Roxet	Harpenden
2.7	Rox K10-5	Roxet	Harpenden
2.8	Rox K10-6	Roxet	Harpenden
2.9	Rox K10-7	Roxet	Harpenden
2.10	Rox K10-8	Roxet	Harpenden
2.11	Rox KB10-1	Roxet	Harpenden
2.12	Rox KB10-2	Roxet	Harpenden

Appendix 10: Summary of the results of isolates of *L. maculans*, their DNA concentration, purity and the presence of *AvrLm1* allele

Isolate	DNA Concentration (ng/µl)	Purity (at 260/280 nm)	*Lm/Lb*	*AvrLm1*
Rox K10-1	65.62	1.85	*Lm*	+
Rox K10-2	64.37	1.91	*Lm*	-
Rox K10-3	40.59	1.8	*Lm*	-
Rox K10-4	48.33	1.78	*Lm*	+
Rox K10-5	46.56	1.78	*Lm*	-
Rox K10-6	58.82	1.81	*Lm*	-
Rox K10-7	85.19	1.93	*Lm*	+
Rox K10-8	32.75	1.84	*Lm*	-
Rox K B10-1	42.47	1.75	*Lb*	N/A
Rox K B10-2	119.01	1.84	*Lm*	-
Rox K B10-5	126.52	1.97	*Lm*	-
Exc K10-1	71.35	1.77	*Lm*	-
Exc K10-2	106.38	1.71	*Lm*	-
Exc K10-3	73.06	1.83	*Lm*	-
Exc K10-4	58.27	1.86	*Lm*	-
Ari K10-1	67.8	1.75	*Lm*	-
Ari K10-2	180.88	1.63	*Lm*	-
Ari K10-3	50.3	1.91	*Lb*	N/A
Cab K B10-3	100.08	1.9	*Lm*	-
Cab K B10-4	57.96	1.85	*Lb*	N/A
Cab K10-1	73.84	2.04	*Lm*	-
Cab K10-2	127.55	1.74	*Lm*	-
Cab K10-3	97.57	1.88	*Lm*	-

Cab K10-4	78.93	1.86	*Lm*	-
Ast K10-1	187.48	1.67	*Lm*	-
Ast K10-2	130.59	1.98	*Lm*	-
Ast K10-3	155.09	1.65	*Lm*	-
Ast K10-4	42.34	1.84	*Lm*	-
Bil K10-1	193.33	1.6	*Lm*	-
Bil K10-2	67.25	1.79	*Lm*	-
Bil K10-3	59.02	1.88	*Lm*	-
Gra K10-1	73.93	1.87	*Lm*	-
Gra K10-2	114.95	1.81	*Lm*	-
Gra K10-3	186.94	1.7	*Lm*	-
Gra K10-4	63.64	1.85	*Lm*	-
Cap K10-1	79.75	1.9	*Lm*	-
Cap K10-2	57.02	1.63	*Lm*	-
Cap K10-3	37.53	1.79	*Lm*	-
Cap K10-4	153.8	1.65	*Lm*	-
Ari K B10-3	196.05	1.94	*Lm*	-
G2 10-1	210.93	1.96	*Lm*	-
G2 10-2	182.91	1.96	*Lm*	-
G2 10-3	188.07	1.96	*Lm*	-
G2 10-4	193.12	1.95	*Lm*	-
G2 10-5	211.7	2.01	*Lm*	-
G2 10-6	149.74	1.95	*Lm*	-
G2 10-7	179.42	1.94	*Lm*	-
G2 10-8	189.14	1.93	*Lm*	-

Isolate	DNA Concentration (ng/µl)	Purity (at 260/280 nm)	*Lm/Lb*	*AvrLm1*
F2 B10 2	108.42	1.93	*Lm*	-
PG10-1	194.9	1.98	*Lm*	-
PG10-2	197.35	1.91	*Lm*	-
PG10-4	176.58	1.93	*Lm*	-
PG10-6	146.66	1.88	*Lm*	-
B10-11	208.27	2.03	*Lm*	-
B10-12	193.19	2.04	*Lm*	-
B10-13	158.96	1.76	*Lm*	-
B10-14	150.6	1.84	*Lm*	-
B10-15	161.39	2.04	*Lm*	-
B10-16	139.07	2.03	*Lm*	-
B10-1	178.43	1.78	*Lm*	-
B10-2	224.74	1.94	*Lm*	-
B10-3	224.93	1.97	*Lm*	-
B10-4	226.18	1.98	*Lm*	-
B10-5	213.98	1.89	*Lm*	-
B10-6	207.9	1.91	*Lm*	-
B10-7	222.67	1.95	*Lm*	-
B10-8	218.11	1.94	*Lm*	-
B10-9	223.29	1.87	*Lm*	-
B10-10	196.89	1.95	*Lm*	-
B10-17	206.01	1.97	*Lm*	-
B10-18	212.62	1.91	*Lm*	-
3.7	200.74	1.86	*Lm*	+