Effects of host resistance on colonisation of *Brassica napus* (oilseed rape) by *Leptosphaeria maculans* and *L. biglobosa* (phoma stem canker)

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Abstract

Leptosphaeria maculans and *L. biglobosa* co-infect winter oilseed rape plants to cause phoma stem canker disease. The sexual spores of both species are produced in pseudothecia on infected winter oilseed rape stem debris after harvest and this is the most important source of inoculum for infection of newly-emerged plants in autumn. Field experiments investigated the effects of host resistance on proportions of pathogens *Leptosphaeria maculans* and *L. biglobosa* in most leaf and stem tissues during 2011/2012, 2012/2013 and 2013/2014 growing seasons and on the pseudothecial development on crop debris on nine winter oilseed rape cultivars; cvs Adriana (*Rlm4* + quantitative resistance (QR)), Bilbao (*Rlm4*), Capitol (*Rlm1*), Drakkar (no *R* gene against *L. maculans*), DK Cabernet (*Rlm1* + (QR), Es-Astrid (QR), Excel (*Rlm7*), Roxet (*Rlm7*) and NK Grandia (QR). Cultivars with a combination of *R*-gene resistance and QR [Adriana (*Rlm4* + QR), DK Cabernet (*Rlm1* + QR)] or cultivars with only QR [(Es-Astrid and NK Grandia)] had more numbers of *L. maculans* leaf spots than other cultivars in autumn but less stem canker damage. There was greater number of *L. biglobosa* leaf spots on leaves of cvs Roxet and Excel with resistance gene *Rlm7* than those of other cultivars and later more *L. biglobosa* DNA was detected in their stems than in those of other cultivars. In all cultivars in the three growing seasons, there was a greater amount of *L. biglobosa* DNA than *L. maculans* DNA in basal stem canker and upper stem lesions. The cv. Drakkar (no *R* gene against *L. maculans*) was susceptible in all three growing seasons, with a great number of *L. maculans* and *L. biglobosa* leaf spots and severe stem cankers.

There were four cultivars (Adriana, Bilbao, Drakkar and NK Grandia) selected for the study of pseudothecial development under natural conditions with different times of exposure and in controlled environment conditions (20^{o}C, continuous wetness). The fastest development was on the susceptible cv. Drakkar (no *R* gene against *L. maculans*), followed by Bilbao (*Rlm4*), Adriana (*Rlm4* + QR) and NK Grandia (QR) for stem base cankers and upper stem lesions in controlled conditions. Results for pseudothecial development on stems of the nine winter oilseed rape cultivars that were exposed in natural conditions at Bayfordbury support the controlled environment results, with pseudothecia on stems of cultivars with a combination *R*-gene and QR consistently maturing later than those on other cultivars, regardless of the weather conditions in three growing seasons. Ascospores produced in pseudothecia are the primary inoculum that initiate phoma stem canker epidemics in autumn. Ascospore release was later in autumn 2011 than in autumn/winter 2012/2013 or 2013/2014 because of dry weather. The pattern of ascospore release had a peak, or maximum in autumn/winter 2011/2012 (4958 spores/m^{-3} on 22 Jan 2012) and several maxima in autumn/winter 2012/2013 (1307 spores/m^{-3} on 5 Nov 2012, 1291 spores/m^{-3} on 15 Nov 2012, 1306 spores/m^{-3} on 25 Dec 2012) and 2013/2014 (4575

spores/m^{-3} on 27 Oct 2013, 4619 spores/m^{-3} on 3 Nov 2013, 3674 spores/m^{-3} on 9 Nov 2013, 3521 spores/m^{-3} on 12 Dec 2013). Results from the qPCR showed that ascospores of *L. maculans* were released earlier than ascospores of *L. biglobosa* at Bayfordbury in the 2013/2014 growing season.

There were differences in phenotype of isolates amongst ninety-five isolates of *L. maculans* and forty-eight isolates of *L. biglobosa* obtained from different sources (phoma leaf spots, upper stem lesions or basal stem cankers) on different cultivars. Cotyledon tests showed that the resistance genes *Rlm4, Rlm5, Rlm6* and *Rlm7* are still effective in England. Most isolates from phoma leaf spots carried avirulent *AvrLm4* (39 isolates; 97.5%), *AvrLm5* (39 isolates; 97.5%) and *AvrLm6* alleles (36 isolates; 90%) and all 40 isolates carried the avirulent allele *AvrLm7* (100%). Fewer isolates from basal stem cankers carried avirulent *AvrLm4* (4 isolates, 16.7%) or *AvrLm6* alleles (16.7%) but all 24 isolates carried the avirulent *AvrLm7* (100%). Fewer isolates from upper stem lesions carried the avirulent *AvrLm4* allele (5 isolates; 16.1%), but 15 isolates carried avirulent *AvrLm5* (48.4%), 21 isolates carried *AvrLm6* (67.7%) and all 31 isolates carried *AvrLm7* (100%). By contrast, all isolates were virulent against *Rlm1, Rlm2, Rlm3* and *Rlm9*. This knowledge, together with knowledge about *R* genes present in current winter oilseed rape cultivars, should be useful to provide recommendations on cultivar selection to growers based on regional frequencies of avirulent alleles of *Avr* allele genes in the *L. maculans* populations (races) and improved understanding of the race structure of *L. maculans*. There is a need to further investigate any *R* genes that operate against *L. biglobosa* (possibly from wild brassicas) and to study if any *R* genes or QR can provide resistance against both *L. maculans* and *L. biglobosa.*

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"My Lord! Increase me in knowledge" [Quran, 20:114]

In loving memory of my father, Mohamed Sidique (1946–2008)

Ayah thank you for not sending me to boarding school

and gave me the freedom to explore and learn.

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Abbreviations

cm	centimeter
mya	million years ago
mm	millimetre
mL	mililitre
PDA	potato dextrose agar
V8A	V8 juice agar
DWA	distilled water agar
g	gram
min	minutes
mg	milligram
ng	nanogram
pg	picogram
µm	micrometre
µl	microlitre
hr	hours
RH	relative humidity
TBE	a mixture of Tris base, boric acid and EDTA

Chapter 1

General introduction

1.1 Oilseed rape (*Brassica napus* L. var. *oleifera*)

Brassica napus is a plant species in the Brassicaceae (= Cruciferae) family, which is grown for the production of vegetable oil, animal feed and also as a potential source of biodiesel (Raymer, 2002; Branca & Cartea, 2011). The common types of brassica used for food include cabbage, cauliflower, broccoli, Brussels sprouts (*Brassica oleracea*), and oil-producing rapeseed (*Brassica napus*). Brassicas are important crops dating back to 5000 BCE, based on archaeological evidence (Yan, 1990), and are among the oldest cultivated plants known to humans with written records dating back to 1500 BC (Prakash 1980).

Due to their agricultural importance, *Brassica* plants have been the subject of much scientific interest. The relationship among the different Brassica species started to be explained by Morinaga (1934) and U (1935) with the U-triangle (Fig. 1.1). The six particularly important species are *Brassica carinata*, *B. juncea*, *B. oleracea*, *B. napus*, *B. nigra* and *B. rapa*. *B. napus, B. juncea* and *B. carinata* are derived by combining the chromosomes from the three earlier species, *B. rapa, B. oleracea* and *B. nigra,* as described by the 'Triangle of U'. U (1935) demonstrated the evolution and relationships between these members of the plant genus *Brassica*, showing how genomes of three ancestral *Brassica* species have combined to form three of the common contemporary vegetable oilseed crop species (Fig. 1.1). Based on chromosome pairing in *Brassica*

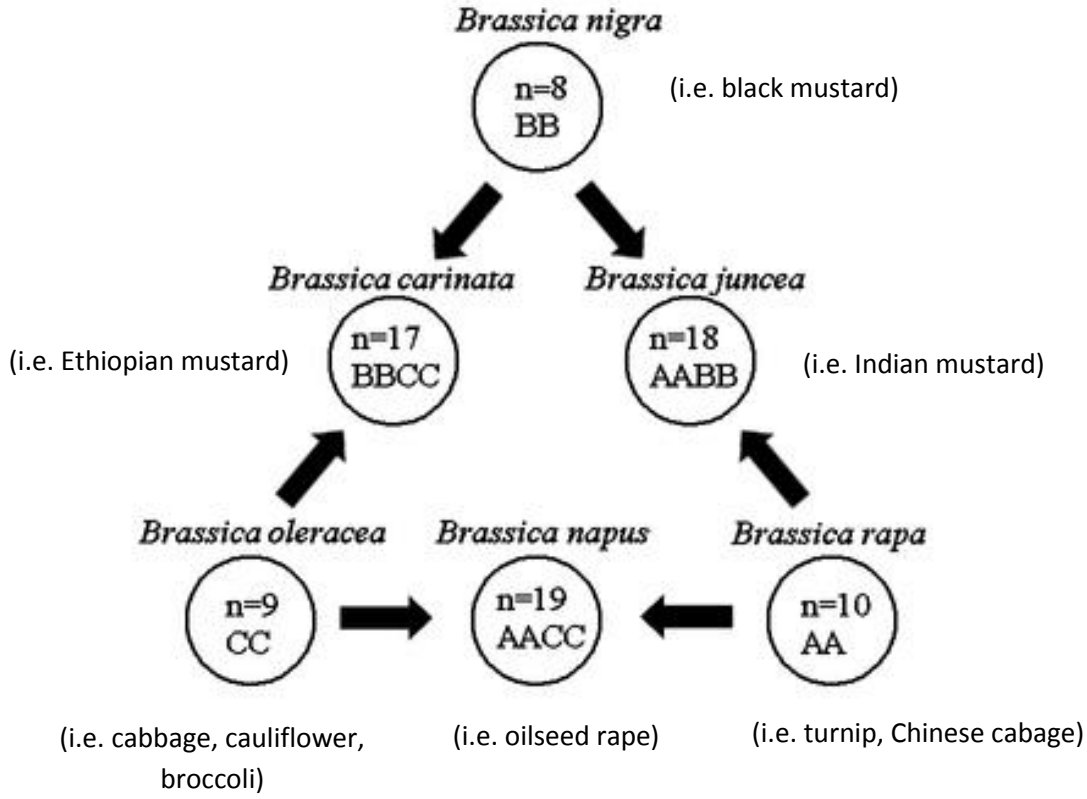


Fig. 1.1: The “Triangle of U” representing the genetic relationships between the six *Brassica* species. Originally established by U (1935). (Source: Kimber and McGregor, 1995).

inter-specific hybrids, Morinaga (1934) hypothesized that *B. napus* (2n=38, AACC), *B. juncea* (2n=36, AABB) and *B. carinata* (2n=34, BBCC) are the amphidiploids of the diploid species *B. nigra* (2n=16, BB), *B. oleracea* (2n=18, CC) and *B. rapa* (2n=20, AA). U (1935) successfully synthesized these amphidiploids species from inter-specific crosses between the diploid species and proposed the genome relationship between the *Brassica* species. The winter type *B. napus* is the main oilseed rape crop in most of Europe and in central parts of China, whereas the spring type *B. napus* is grown in Canada, northern Europe and northern China (Sovero, 1993). It provides the world's third most important source of vegetable oil, after soybean and palm oil, and was 13-16 percent of world vegetable oil production in terms of volume between 1999/2000 – 2008/2009, (USDA, 2012).

There is often confusion in the use by different people of the terms "oilseed rape", "rapeseed" and "canola." A factor distinguishing different types of oilseed rape is their individual chemical or fatty acid profile. Generally, "industrial rapeseed" refers to any oilseed rape that produces oil with a high content (at least 45 percent) of erucic acid and it is primarily used to produce machine lubricants, inks and slip agents in the production of polyethylene (Vollman and Rajcan, 2009; HGCA, 2012). The high levels of erucic acid, which is damaging to cardiac muscle of animals, and glucosinolates, made it less nutritious in animal feed (rapeseed press cakes) (O'Brien, 2008). The term "canola" is a registered trademark of the Canadian Canola Association and refers to the edible oil crop that is characterized by low erucic acid (less than 2 percent by weight in the USA) (CFR, 1977) and low glucosinolate content (Raymer, 2002). In 1977, cultivation of food cultivars low in erucic acid content became mandatory; to meet EU quality standards

only cultivars with less than 5% erucic acid in the seed oil were allowed in food crops (EEC, 1980) with special regulations for infant food. The concentrations of erucic acid in human foods are restricted because it may adversely affect heart tissue although low concentrations of erucic acid are not believed to cause harm in humans (FSA, 2004). The term “canola” is used in Canada and Australia for indicating specific double low types of oilseed rape and the term oilseed rape is used worldwide.

The first double low oilseed rape genotypes were mainly susceptible to phoma stem canker pathogens; after the introduction of the double-low quality standard in 1984, UK winter oilseed rape yields decreased until 1994 (Knight *et al.,* 2012). There followed a period of great variation in yields until 2004, after which yields increased (Kightley *et al.,* 2012). Little phoma stem canker was found on winter oilseed rape in the UK before 1977 but a substantial increase in incidence was noted in 1977 and 1978 (Gladders & Musa, 1979; Humpherson-Jones, 1983; Rawlinson & Muthyalu, 1979). This occurred in conjunction with an increase in the intensity of winter oilseed rape cultivation (Cook & Evans, 1978); the area grown is now more than 700,000 hectares (Kightley *et al.*, 2012).

World oilseed rape production is growing rapidly, with 61 million metric tonnes (MT) produced in the 2010/2011 cropping season (FAO, 2012). Canada was the country that produced the most oilseed rape, producing 14.2 M tonnes p.a., and China was second, producing about 13.4 M tonnes (FAO, 2012). The 27 countries of the European Union (EU) together produced 23 M tonnes (Source: http:// faostat.fao.org). In the UK, winter oilseed rape has a relatively low yield compared to those of wheat and barley but it is still the third most important arable crop due to its high price (£342 per tonne in 2013,

www.hgca.com). Over the twenty years from 1992, the UK area of production of oilseed rape has expanded from 421,000 hectares in 1992 to 756,000 hectares in 2012 (Defra, 2013) (Fig. 1.2). This crop is valued by farmers, especially in Europe, because it is also used to make bio-diesel. Over one quarter of global oilseed rape production is used as a renewable source and the official mandates for biodiesel blending in fuels are currently legislated until 2020 (HGCA, 2012). After crushing the crop, the waste product provides a valuable, protein-rich animal feed. The crop also helps to maintain soil fertility and improve drainage and soil structure (HGCA, 2007).

1.2 Diseases of oilseed rape

The production of winter oilseed rape can be limited by the numerous diseases of oilseed rape. This affects the potential of oilseed rape to increase the yield and quality of its products to meet increasing global demand. There are four important diseases of oilseed rape in the UK; phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*), light leaf spot (*Pyrenopeziza brassicae*), sclerotinia stem rot (*Sclerotinia sclerotiorum*) and alternaria dark pod spot (*Alternaria brassicae*) (Fig. 1.3) (HGCA, 2012).

Phoma stem canker and light leaf spot are the most important foliar diseases of winter oilseed rape. Light leaf spot is the most serious disease in Scotland but phoma stem canker is often the most important disease of winter oilseed rape in southern and eastern England (HGCA, 2012). In recent years, light leaf spot has severely affected crops in the UK; it may have increased in importance because of its insensitivity to fungicides (HGCA, 2012).

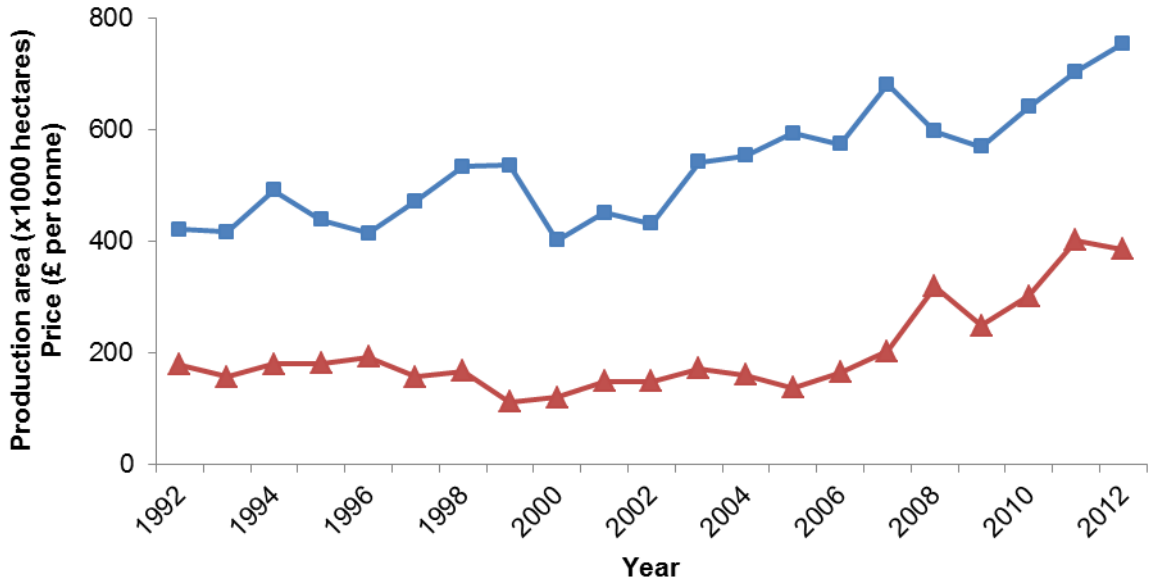


Fig. 1.2: Total production area (hectares) at harvest of the winter (autumn-sown) oilseed rape crop in the UK (■) and price (£ per tonne) (▲) from 1992 until 2012 (Defra, 2013).

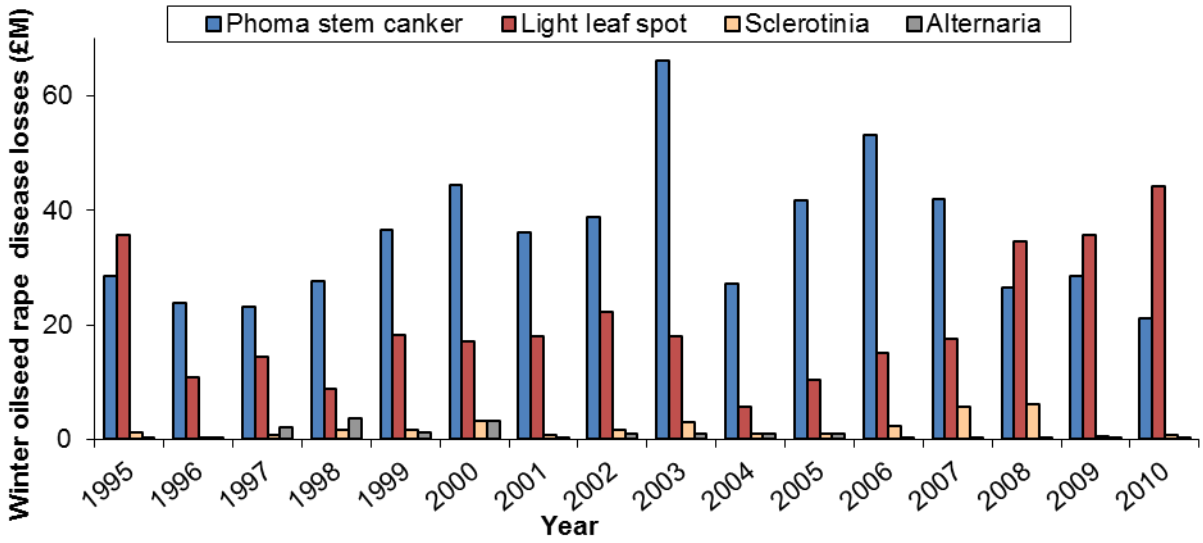


Fig. 1.3: Major crop diseases losses (£M) of winter oilseed rape in England and Wales caused by fungi such as *Leptosphaeria maculans*/*L. biglobosa* (phoma stem canker), *Pyrenopeziza brassicae* (light leaf spot), *Sclerotinia sclerotiorum* (sclerotinia stem rot) and *Alternaria brassicae* (alternaria dark pod spot) from 1995 until 2010 (http://www.cropmonitor.co.uk)

Sclerotinia stem rot and alternaria dark pod spot generally cause relatively small losses in the UK, when compared with phoma stem canker and light leaf spot, which are of concern (Fitt *et al.,* 2006a). Yields of the crop can be halved by sclerotinia stem rot due to premature ripening and *Alternaria brassicae* is capable of infecting any part of the plant, resulting in yield losses of 36% (Sharma *et al.,* 2006; BASF, 2012).

There are also some soil-borne diseases that have increased in importance in the UK in recent years because of the shorter crop rotations; these include clubroot (*Plasmodiophora brassicae*), verticillium wilt (*Verticillium longisporum*) and sclerotinia stem rot (*Sclerotinia sclerotiorum*) (HGCA, 2012). Oilseed rape is usually grown in a 4 year rotation with cereals (West *et al.,* 2001; UK Agriculture, 2011). It is grown as part of a crop rotation system to facilitate the control of cereal crop pathogens (West *et al.,* 2001; UK Agriculture, 2011). Due to economic pressures, the rotations have been shortened; with winter oilseed rape grown in one in two or one in three year rotations; that may increase severity of both soil-borne and foliar diseases of winter oilseed rape (HGCA, 2012). Other minor diseases at early crop growth stages (seedlings) are damping off caused by various soil-borne fungi and downy mildew caused by *Hyaloperonospora parasitica*.

1.3 Phoma stem canker disease of winter oilseed rape

One of the most economically and an internationally important diseases of winter oilseed rape is phoma stem canker (Fitt *et al.,* 2006a). It is now considered to be caused by two pathogens, *L. maculans* and *L. biglobosa* (Grandaubert *et al.,* 2014). Phoma stem

canker has been found on cruciferous crops for over a century (Gugel & Petrie, 1992; Rouxel & Balesdent, 2005). In the UK, the disease is monocyclic (one disease cycle per growing season), starting from a long period of ascospore release over the autumn and winter (West *et al.,* 2002a; Fitt *et al.,* 2006a). There is little evidence for secondary spread from leaf to leaf by splash-dispersed conidia (Fitt *et al.,* 2006a). Phoma stem canker is also known as 'blackleg' disease in some other countries, such as the USA and Australia, because of the blackened stem base associated with girdling symptoms (Fig. 1.4). Other vegetable brassicas recorded with the disease include *B. oleracea* and *B. rapa* (Rimmer & Van den Berg, 2007).

Phoma stem canker causes serious losses internationally in Europe, North America and Australia, with annual worldwide yield losses valued at more than £500 million in 2008 at a price of £150 per tonne (Fitt *et al*., 2008). The severity of the disease is much greater in southern England than in northern England, whereas in Scotland there is no development of phoma stem canker. The disease occurs in Scotland but the stem canker does not develop because of the lower temperatures in winter/spring seasons (Evans *et al.,* 2008; Stonard *et al.,* 2010). In 1977 and 1978, severe epidemics of the disease were first identified in England and they almost prevented the production of the crop (West *et al.,* 2001; Rouxel & Balesdent, 2005). Later, the introduction of resistant cultivars such as Jet Neuf and Rafal maintained relatively good control of the disease until the late 1980s, when a change in European Union policy resulted in the introduction of 'double low' cultivars with low levels of erucic acid and glucosinolates in the seed (Hardwick *et al.,* 1989). The disease remained common and damaging in the 1980s and

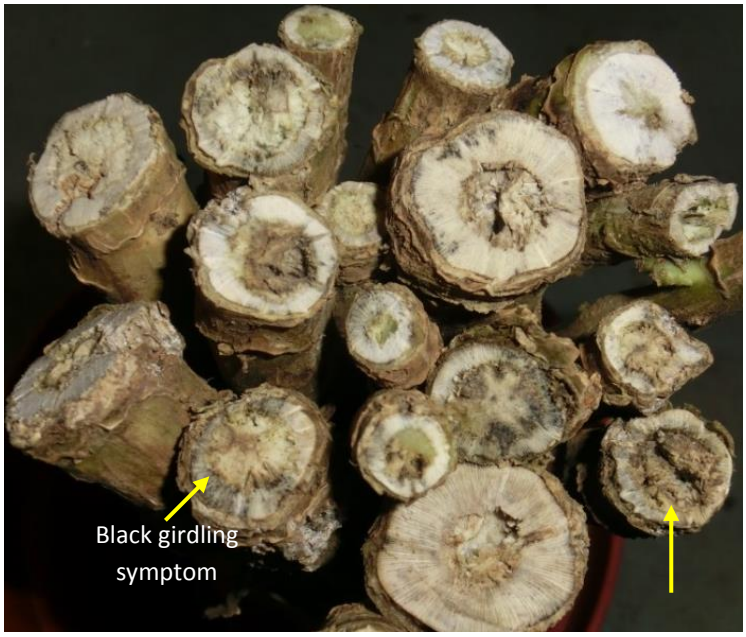


Fig. 1.4: Cross-sections of oilseed cv. Bilbao (*Rlm 4*) stem bases with necrosis (arrows) and girdling symptoms of phoma stem canker, sampled from a Rothamsted experimental plot in the 2013/2014 growing season.

until the mid-1990s, with widespread severe epidemics of phoma stem canker in England occurring, with 84% plants diseased recorded in 1995 (Gladders *et al.,* 1998).

Other European countries are affected by this disease on oilseed rape, including France (West *et al*., 2002b), Germany (Kuswinanti *et al*., 1999), Scandinavia (Kuusk *et al*., 2002), Poland (Pedras *et al*., 2007) and Hungary (Magyar *et al*., 2006). Southern states of the U.S.A, such as Oklahoma, have recently started to grow spring oilseed rape as a crop in rotation with wheat; in 2010, the first report of *L. maculans* as a pathogen that caused phoma stem canker in these crops occurred (del Rio Mendoza *et al.,* 2011). There are mixed populations of *L. maculans* and *L. biglobosa* in several countries that produce oilseed rape, such as the UK, France, Germany, Scandinavia, Australia, Canada, Brazil and Iran but *L. biglobosa* is usually the less aggressive pathogen (Fitt *et al.,* 2006a; West *et al*., 2002b; Kuswinanti *et al*., 1999; Kuusk *et al*., 2002; Pedras *et al*., 2007; Fernando & Chen*,* 2003; Khangura & Barbetti, 2001). In 2013, *L. biglobosa* was reported as present in New Zealand's oilseed rape (*Brassica napus*) cropping areas, whereas before, only *L. maculans* was identified as the cause of phoma stem canker there (Lob *et al.,* 2013). However, in China and Poland, only *L. biglobosa* was known to cause yield losses in oilseed rape (Liu *et al.,* 2014; Kaczmarek *et al.,* 2009). After 2002, in Poland, *L. maculans* has become widespread on oilseed rape, firstly in western regions and later in eastern Poland (Karolewski *et al.,* 2002; Jedryczka & Lewartowska, 2006). A similar trend of introduction of *L. maculans* was observed in Canada, Mexico, Hungary, Sweden and the Czech Republic (Fitt *et al.,* 2006a; Moreno-Rico *et al.,* 2002; Szlávik *et al.,* 2003). The global spread of *L. maculans* is now a threat to oilseed rape production in China, the second largest producer of oilseed rape worldwide, where

currently only the less damaging *L. biglobosa* is present (Liu *et al.,* 2006; Fitt *et al.,* 2008; Zhang *et al.,* 2014).

There are several quarantine regulations and inspections by plant health professionals to control and to prevent spread of pathogens causing phoma stem canker. In Canada, where only the less damaging *L. biglobosa* was present, under the Agricultural Pest Act, 1984, the Alberta provincial government declared *L. maculans* as a pest to prevent severe phoma stem canker epidemics like those that had occurred in Saskatchewan (Fitt *et al.,* 2008). Therefore, China started to act since they have imported several millions tons of oilseeds from Canada, Australia or the Ukraine since 1998 that potentially may have introduced *L. maculans* on the seed or in associated oilseed rape debris (a potent source of initial inoculum) (Zhang *et al.,* 2014). Now, *L. maculans* causing phoma stem canker is listed as a quarantine pathogen in China. Imports of oilseed rape seed from countries where *L. maculans* is present has been restricted until further safeguards are put in place to protect the oilseed rape crop in China from *L. maculans* (http://www.canola- council.org/) (Chen *et al.,* 2010; Zhang *et al.,* 2014).

1.3.1 *Leptosphaeria maculans* and *L. biglobosa*

These pathogens are the causal agents of phoma stem canker for both spring and winter oilseed rape and there is evidence that *Leptosphaeria maculans* and *L. biglobosa* are adaptable to a wide range of climates (West *et al.,* 2001). These pathogens are able to colonise the tap root, stem base, upper stem parts, cotyledons, leaves and seeds of the susceptible oilseed rape host (Paul & Rawlinson, 1992; Huang *et al.,* 2002).

In Europe, appearance of phoma stem canker epidemics on winter oilseed rape first starts in autumn with greyish or yellow-brown phoma leaf spot lesions that vary in size and shape (West *et al*., 2001). There are often numerous black, pinhead-sized pycnidia observed in the dead tissues at the centre of the phoma leaf spot lesions (Fig. 1.5a). Later in the cropping season, the typical stem canker symptoms develop in the period before harvest (Fig. 1. 5c).

Leptosphaeria maculans (Desm.) Ces. & de Not. [anamorph *Phoma lingam* (Tode ex Fr.) Desm.] was considered as a single species before 2001, but it was divided into two groups, A group or A-type (highly virulent/aggressive) and B group or B-type (weakly virulent/non-aggressive) that could be differentiated by pigmentation in culture, colony growth rate, molecular characters and disease reactions on specific lines of *Brassica napus* ssp. *oleifera* (Williams & Fitt, 1999; Howlett, 2004; Gout *et al.,* 2006b).

Shoemaker and Brun (2001) proposed that these pathogens be named *L. maculans* (A group or A-type) and *L. biglobosa* (B group or B-type), based on the morphology of their pseudothecia. Both pathogens belong to the phylum Ascomycota, class Dothideomycetes (Loculoascomycetes) and order Pleosporales (Rouxel & Balesdent, 2005). They reproduce asexually through mechanisms such as budding, a fragmentation of mycelium and from pycnidia that contain vegetative spores (conidia) (Kaczmarek & Jedryczka, 2011). They produce resting spores such as chlamydospores (Kaczmarek & Jedryczka, 2011). However, they mainly reproduce using sexual reproduction (pseudothecia producing ascospores) (Kaczmarek & Jedryczka, 2011). *L. maculans* is haploid and outcrossing; thus both mating types (MAT1-1 and MAT1-2

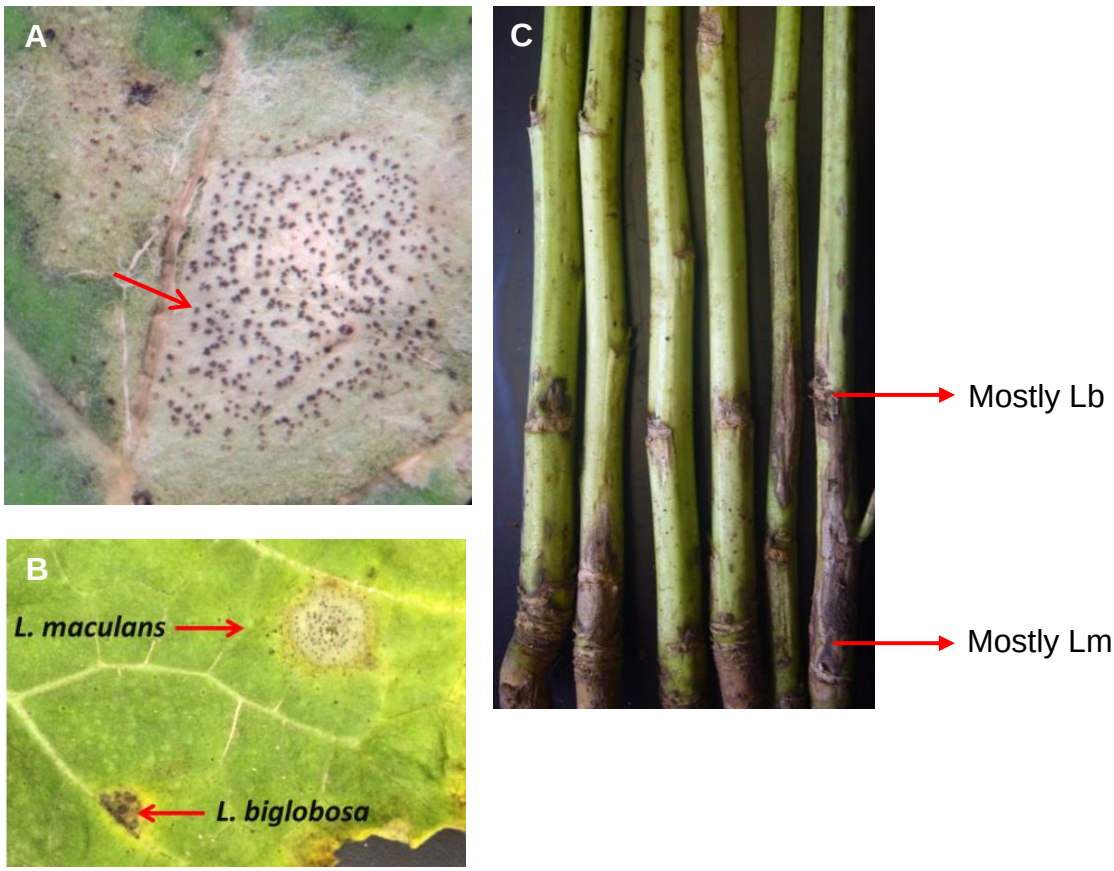


Fig. 1.5: (a) phoma leaf spot lesion caused by *Leptosphaeria maculans* (Lm) with black, pinhead-sized pycnidia (arrow), (b) differences between *L. biglobosa* (Lb) and Lm leaf lesion assessed on 25 January 2012 and (c) basal stem cankers and upper stem lesions before harvest on cv. Excel (*Rlm 7*) assessed on 13 July 2012.

alleles) must be present for sexual reproduction (Cozijnsen & Howlett, 2003). In the UK, pseudothecia (sexual fruiting bodies) are formed in diseased stems of *B. napus* over the summer, releasing ascospores (sexual spores), which are air-borne and infect newly sown crops in autumn. Rapid pathogen population changes are attributed to sexual reproduction and dispersal of air-borne spores by wind. There are several reports on genetic variation in field populations of *L. maculans* that show the importance of sexual reproduction as part of the life cycle of *L. maculans* (Mahuku *et al.,* 1997; Barrins *et al.,* 2002, 2004; Gout *et al.,* 2006b).

The pseudothecial morphology of *L. maculans* and *L. biglobosa* is similar, except that the pseudothecia of *L. biglobosa* have a more prominent enlarged beak (200-400 µm long by 200-300 µm wide) at the apex of the pseudothecium (plural pseudothecia) (Shoemaker & Brun, 2001). Pseudothecia of *L. biglobosa* are usually located under the epidermis of the stem tissue while those of *L. maculans* form on the surface of the epidermis of the stem tissue (Toscano-Underwood *et al.,* 2003). The diameter of the pseudothecia (fruiting bodies) of *L. maculans* ranges from 300 to 400 µm and the diameter of the pseudothecia of *L. biglobosa* ranges from 280 to 350 µm (Toscano-Underwood *et al.,* 2003).

In Europe, *L. maculans* often co-exists with *L. biglobosa* and both have similar life cycles. However, the growth rate of *L. maculans* is 2-3 times slower than that of *L. biglobosa* in winter oilseed rape tissues (Kaczmarek & Jedryczka, 2011). In culture, the slow-growing isolates of *L. maculans* can form abundant pycnidia, while isolates of *L. biglobosa* form more aerial mycelia and fewer pycnidia (Williams & Fitt, 1999). *L.*

maculans invades the stem and causes severe stem cankers at root collars and stem bases, while *L. biglobosa* tends to be more common in upper stem lesions (Fitt *et al*., 2006a; Kaczmarek & Jedryczka, 2011).

L. maculans and *L. biglobosa* were morphologically considered as two phenotypes of the same species for more than 70 years (Pound, 1947) and are known as a pair of sibling pathogens (may have evolved from a common ancestor) that co-exist on the same host (Gudelj *et al.,* 2004). In plant pathology, 'sibling' species are closely related but non-interbreeding organisms that are difficult to differentiate by classical mycological methods based on morphology, life-cycle or life history parameters (Gudelj *et al.,* 2004; Fitt *et al.,* 2006b). Sibling species often occupy slightly different niches. For example, biological and epidemiological differences between *L. maculans* and *L. biglobosa* allow the two species to coexist through occupation of different niches related to differences between them in time, space and resource use (Fitt *et al.,* 2006b). Niche differences between *L. maculans* and *L. biglobosa* are discussed in chapter 1 (refer to 1.3.2).

Recently, divergence time between *L. maculans* and *L. biglobosa* was determined and estimated as *ca.* 22 million years ago (MYA) (Grandaubert *et al.,* 2014), which strongly indicates that *L. maculans* and *L. biglobosa* are different species (Grandaubert *et al.,* 2014). *L. maculans* may have become a very successful pathogen because of its evolution from a less aggressive pathogen.

Populations of the phoma stem canker pathogens were first divided into several pathogenicity groups (PGs) on the basis of phenotypic interaction (IP) of isolates on the

differential cultivars Westar, Glacier and Quinta. Four PGs were recognised as PG-1, PG-2, PG-3 and PG-4 (Keri, 1999; Mengistu *et al.,* 1991). Isolates in groups PG-2, PG-3 and PG-4 were classified as highly virulent (*L. maculans*) but isolates in PG-1 were weakly virulent (*L. biglobosa*). Now, the *L. maculans* pathogenicity groups have been more precisely classified as *Avr* (avirulent) and *avr* (virulent) alleles at a range of *Avr* (effector) loci that correspond to a range of resistance (*R*) genes in oilseed rape cultivars (Balesdent *et al.,* 2006, 2013; Daverdin *et al.,* 2012) (refer to 1.5 for cultivar resistance).

Now, *L. biglobosa* isolates have been clustered into six subclades related to their host specificity and geographic origin, i.e. *L. biglobosa* 'brassicae' (from various *Brassica* species, mostly in Europe), *L. biglobosa* 'canadensis' (mostly found in central Canada), *L*. *biglobosa* 'thlaspii' (from *Thlaspi arvense*), *L. biglobosa* 'erysimii' (from *Erysimum sp.*), *L. biglobosa* 'australensis' (from Australia) and *L. biglobosa* 'occiaustralensis' (has been identified in western Australia) (Mendes-Pereira *et al.,* 2003; Voigt *et al.,* 2005; Vincenot *et al.,* 2008; Delourme *et al.,* 2006). However, there is little known about the host resistance mechanisms operating against *L. biglobosa* and no gene-for-gene relationships have been determined (Fitt *et al.,* 2006a; Delourme *et al.,* 2006).

L. maculans can produce phytotoxic secondary metabolites in the sirodesmin family, such as sirodesmin PL; this metabolite is not produced by *L. biglobosa*. Therefore *L. maculans* isolates can be referred to as Tox^{+} (producing sirodesmin PL, highly virulent) and *L. biglobosa* isolates as Tox^{o} (not producing sirodesmin PL, weakly virulent) (Fitt *et al*., 2006a). Although *L. biglobosa* does not produce sirodesmins, it does produce other secondary metabolites that have some phytotoxic properties (Pedras *et al.,* 1995;

Pedras & Biesenthal, 2001). Polish isolates of *L. biglobosa* produce polanrazines and phomapyrones; this suggests that they may be more virulent than other *L. biglobosa* isolates from Canada, France or Germany (Pedras & Biesenthal, 2000; Pedras *et al.,* 2007).

There are differences in the *Leptosphaeria* pathogen population structure between countries, regions and seasons that have been observed. For example, in the UK both pathogens co-exist, with *L. maculans* the dominant species (West *et al.,* 2001; Stonard *et al.,* 2010), whereas in Poland until the late 1990s, *L. biglobosa* was dominant over *L. maculans* (Jedryczka *et al.,* 1994; Toscano-Underwood *et al.,* 2001). In France, 100% of the isolates from southern regions were identified as *L. maculans,* whereas in central regions and western regions only 70% of isolates were *L. maculans* and 30% were *L. biglobosa* (Ansan-Melayah *et al.,* 1997). Jedryczka & Lewartowska (2006) reported the differences between seasons in Poland from 2000 to 2004, where in autumn 71.9% of isolates were *L. maculans* and by spring only 55.7% of isolates were *L. maculans*. Furthermore, isolates collected before harvest included more *L. biglobosa* (78.6%) than *L. maculans* (21.4%) isolates (Jedryczka & Lewartowska 2006). This work from different countries could be evidence that the two pathogens have differences in their environmental requirements.

Currently differences in genome size between the two species have been identified; the genome of *L. maculans* is significantly larger (45 Mb) than that of *L. biglobosa* (30-40 Mb) (Rouxel *et al.,* 2011). This difference in genome size is due to the presence of non-coding repetitive DNA in the *L. maculans* genome that has given the *L. maculans*

genome a unique structure. Rouxel *et al.,* (2011) have reported that the *L. maculans* genome has an unusual bipartite structure, with alternating distinct guanine and cytosine-equilibrated and adenine and thymine (AT)-rich blocks of homogeneous nucleotide composition. The larger size is consistent with the genome having been extensively invaded by transposable elements (TEs) and the AT-rich blocks that comprise one-third of the genome containing effector genes and families of TEs (Rouxel *et al.* 2011). The *Leptosphaeria maculans* genome is rich in transposable elements (TEs) affected by repeat-induced point mutation (RIP), a fungal-specific genome defence mechanism (Gout *et al.,* 2007; Fudal *et al.,* 2009; Rouxel *et al.,* 2011) with 32.5% of the *L. maculans* genome TE-invaded compared to 3.2% of the genome of *L. biglobosa*. Although both species have similar amounts of non-repetitive DNA (*ca.* 28-29 Mb) (Grandaubert *et al.,* 2014), recent work indicates that differences in amounts of TE contribute to the differences in size between the genomes of *L. maculans* and *L. biglobosa* (Grandaubert *et al.,* 2014). It has been confirmed that *L. maculans* and *L. biglobosa* are different species (Grandaubert *et al.,* 2014).

1.3.2 Life cycles of the causal organisms

Both *L. maculans* and *L. biglobosa* produce ascospores in pseudothecia. These sexually-produced spores produced in asci in pseudothecia on diseased winter oilseed rape stubble in autumn are released after moistening of the stubble by rain or dew (McGee, 1977) and germinate on the cotyledons and young leaves of oilseed rape plants over a temperature range of 5^{o}C to 20^{o}C (Huang *et al.*, 2003b). In the initial

colonisation of leaves in autumn, the pathogens have an endophytic life style without producing visual symptoms (Stotz *et al.,* 2014).

After infection by germinating ascospores, leaf lesions that are either large and pale (Lm) or small and dark (Lb) (Fig. 1.5b) appear a few days later (Sexton & Howlett, 2001). The small dark spots formed within leaf lesions are the fruiting bodies of the asexual stage of the pathogens (pycnidia). They contain conidia which are transmitted by rain-splash over short distances, usually from 2 to 40 cm (Travadon *et al.,* 2007). This secondary inoculum of the pathogen may infect oilseed rape leaves in some circumstances (Hall, 1992; Canola Council, 2014). The pathogens grow from the first leaf lesions along the leaf petiole (Fig. 1.6) in the xylem vessels or between cells of the xylem parenchyma and cortex to reach stem tissues (Huang *et al.,* 2006b; Stonard *et al.,* 2010). *Leptospheria maculans* is more aggresive than *L. biglobosa* and continues to colonise the pith tissues of the stem base, whereas *L. biglobosa* is mainly confined to the pith in upper stems and occurs only in the cortex of the stem base before plants senesce (Stonard *et al*., 2010). The necrotrophic phase when the stem cortex is killed results in black and/or brown necrotic discoloration in the interior of the stems of plants affected by the virulent pathogen (Fig. 1.4). Both pathogens (*L. maculans* and *L. biglobosa*) produce sexual fruiting bodies (pseudothecia) that lead to production of primary inoculum (ascospores) of these pathogens the following growing season (Canola Council, 2014). There may be some disease spread to pods, which develop brown lesions with black margins, potentially leading to premature ripening and seed infection which can be a secondary source of inoculum that spreads to new crops (Kaczmarek & Jedryczka, 2011). In Australia, the transmission of the pathogen by

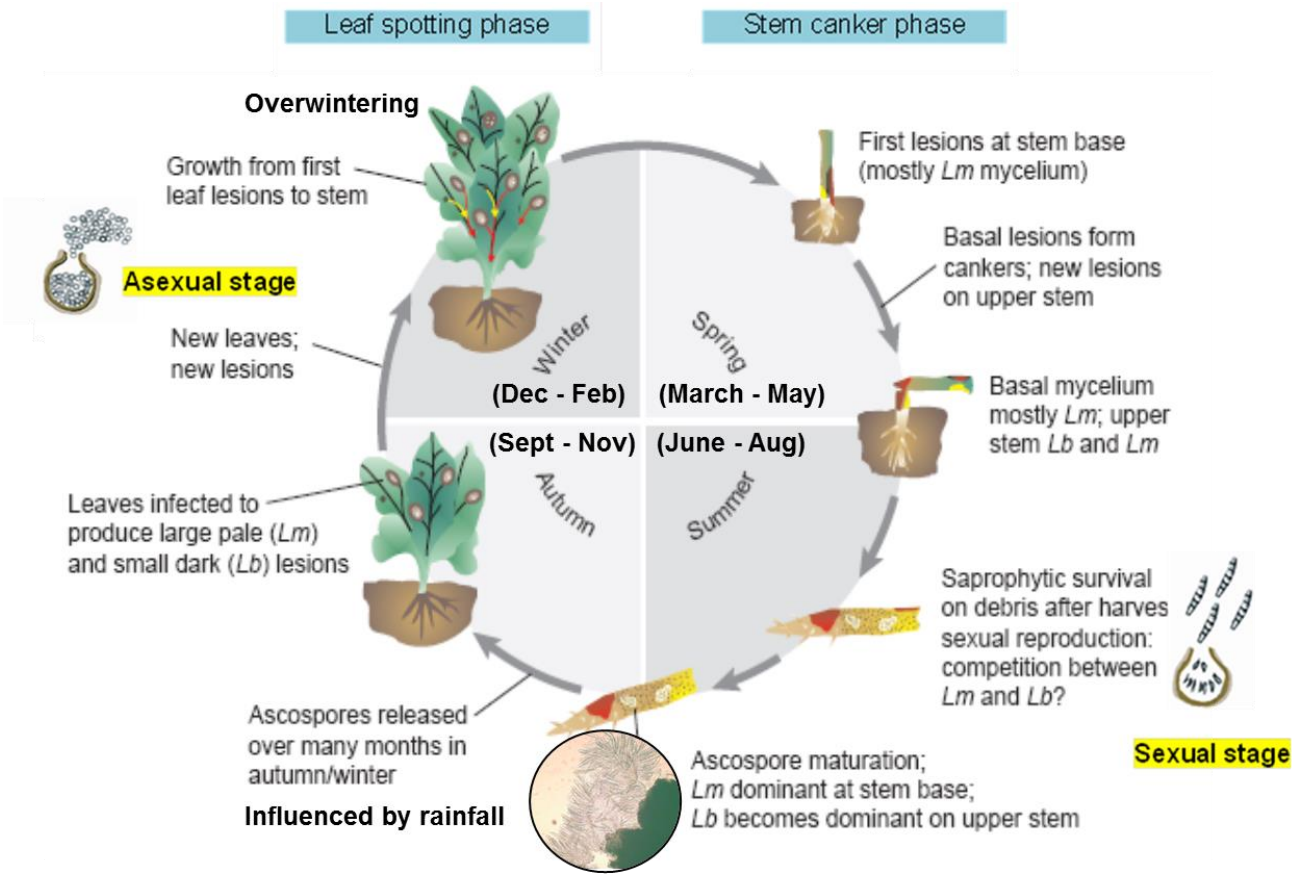


Fig. 1.6: Life cycles of *L. maculans* and *L. biglobosa*, causing phoma stem canker of winter oilseed rape, showing two symptoms, leaf spotting and stem canker (Modified from figure in Fitt *et al.,* 2006b).

infected seeds is regarded as an important source of crop infection (Salisbury *et al.,* 1995; Li *et al.,* 2003). However, in China, the potential long distance transport of infected crop debris (stem and pod debris) carries a much higher risk than transport of infected seeds and there is little evidence of pathogen transmission from infected seeds to seedlings (Zhang *et al.,* 2014). Lodging caused by stem canker is the most serious effect of the disease because it disrupts water transport (Davies, 1986). Severe cankers weaken the stems, causing lodging and death of the plant (West *et al.,* 2001). In France, 5 to 20% of the average oilseed rape yield losses are due to lodging of the crop (Pinochet *et al.,* 2003). The severely affected plants die without producing any seed (Howlett *et al.,* 2001).

There are niche differences between the two species in the stages of their life cycles (summarised in Table 1.1). They coexist in winter oilseed rape by separation in space (*L. maculans* mostly on stem bases and *L. biglobosa* on upper stems), time (differences in timing of ascospore release) and separation in use of resources (ability to colonise living or dead plant tissues) (Fitt *et al.,* 2006b). There are also differences in their infection strategy that enable the two species to co-exist on winter oilseed rape in the UK (Fitt *et al.,* 2006b). The life cycle is summarised in Fig. 1.6. Previous studies have provided evidence that there are biological and epidemiological differences between *L. maculans* and *L. biglobosa* (Table 1.1). It is essential to understand the life cycles of *L. maculans* and *L. biglobosa* to allow growers to manage winter oilseed rape to minimize the damaging effects of the pathogens.

Table 1.1: Differences between *L. maculans* and *L. biglobosa* in their life-cycles on winter oilseed rape, enabling them to co-exist.

Experiment	**Parameter**	***L. maculans***	***L. biglobosa***
Pseudothecial maturation (Toscano-Underwood *et al*., 2003)	Maturation (at 5-10^{o}C)	Occurred earlier	Occurred later
	Cultivar Lipton (*Rlm3*) and position on debris	Mature faster on stem base	Mature slower on upper stem
Ascospore release (Huang *et al*., 2005; West *et al.,* 2002a)	Period of release	Early autumn/winter	Late autumn/winter/spring when pseudothecia mature (Class D)
Ascospore germination (40 h incubation) (Huang *et al*., 2001, 2003a)	Start of germination (at 5-20^{o}C)	Slow germination	Fast germination
	Germ tube length (µm)	Shorter	Longer
	Hyphal growth	Curved	Almost linear
Penetration of leaf tissues (Huang *et al*., 2003b)	Mode of entry (ascospore)	Through stomata	Through stomata
Symptoms (Fitt *et al*., 2006b; Toscano-Underwood *et al*., 2003; West *et al*., 2002b; Brun *et al*., 1997)	Leaf lesion, time of appearance and number of leaf lesions	Large beige lesions with many pycnidia. Symptoms and number of leaf lesions more in autumn	Small dark lesions with few/no pycnidia. Symptoms in late autumn/spring and more leaf lesions in spring
	Growth along petiole	Slower (0.7 mm day^{-1} at 15^{o}C)	Faster (1 mm day^{-1} at 15^{o}C)
	Position of lesions on stem	Mainly stem base	Mainly upper stem
	Internal growth in stem tissues	Extensively colonises all stem base (cortex/pith) and tap root tissues	Mostly restricted to cortex of stem base, occurs in pith in upper stems
Survival (natural conditions) (Huang *et al.*, 2003b; West *et al.*, 2002a)	Buried debris in sand	Survives longer (1 year) on stem bases	Survives <2 months on upper stems
	Exposed on sand	Survives 1 year	Survives 1 year

[a]Modified and extended from Table 1 in Stonard *et al*., 2010.

1.4 Inoculum production, dissemination and infection

The primary inoculum is mainly in the form of air-borne ascospores produced in pseudothecia, which develop in affected winter oilseed rape debris (Toscano-Underwood *et al*., 2001). In Canada, *L. maculans* can survive for at least 5 years on stubble and ascospores can continue to be released from the stubble (McGee, 1977; Petrie, 1995; Canola Council, 2014). The ascospores from the mature pseudothecia are released mainly from affected stubble of previous crops (McGee, 1977). Previous reports showed that stubble infected by *L. maculans* can serve as an inoculum source in USA for a period of 3 years (Baird *et al*., 1999). In the UK, after harvest the pseudothecia may already be visible at the stem base in crops with severe canker and debris degrade within 2 years (West *et al*., 1999a). Huang *et al*. (2003a) demonstrated that *L. maculans* survives longer than *L. biglobosa* on buried debris and that both pathogens survive longer on unburied debris than buried debris.

In the 1970s, oilseed rape seed was tested for infection by *L. maculans* as a short-term strategy to minimise spread of *L. maculans* across Alberta in Canada but seed is not generally an important source of inoculum because there is a relatively low incidence of seed transmission of the pathogen. It has been estimated that about 2% of harvested Canadian oilseed rape seed is infected by *L. maculans* and it is possible that the pathogen will enter China through seeds since China is importing oilseed rape from Canada for crushing (Fitt *et al.,* 2008). Although the risk is low from the imported seed, problems may occur because of transport spillage or from the cleaning of ship loads (Fitt

et al., 2008; Zhang *et al.,* 2014). However, the main method of dispersal is as wind-borne ascospores produced on crop debris (Fitt *et al.,* 2006a, 2008).

Pycnidia of *L. maculans* develop rapidly on diseased plant leaves in crops and in laboratory conditions. Although of relatively minor importance for disease development in the UK, conidia in pycnidia produced on primary leaf lesions may initiate phoma leaf spots in suitable environmental conditions (Barbetti, 1976). In contrast, even small amounts of debris affected by stem canker can produce sufficient numbers of ascospores to spread the disease throughout a crop (Petrie, 1995) because in optimal conditions only one or two ascospores are sufficient to cause leaf spots on the leaf surface and later cause stem canker (Wood & Barbetti, 1977).

The maturation of ascospores in pseudothecia is related to the weather conditions, especially temperature and wetness. It has been reported that the optimum temperature for pseudothecial maturation is 14 – 15^{o}C. Earlier work showed that the number of ascospores released was influenced by rain (qualitatively) and temperature (quantitatively) (Huang *et al*., 2007).

It had been reported that *L. maculans* ascospores can be dispersed over several kilometres (Gladders & Musa, 1980), although many ascospores are deposited within a few hundred metres from the source (Marcroft *et al.*, 2004a). In the UK, rainfall and temperature are the most important weather factors that affect the release of ascospores (Huang *et al*., 2005). Previous work by Huang *et al.* (2007) in the UK produced a relationship that involved summing the daily temperature-dependent rate of

pseudothecial maturation for days after 1 August with rainfall >0.5 mm, to predict the dates when 30% or 50% of pseudothecia were mature and forecast timing of ascospore release.

In Australia, Salam *et al.,* (2007) developed forecasting models (Improved 'Blackleg Sporacle' model and 'SporacleEzy' model) based on temperature and rainfall parameters. The models predicted that there is a risk of *L. maculans* ascospore release in Australia when 43 days with temperature <22^{o}C and weekly rainfall ≥4mm have elapsed since harvest (Salam *et al.* 2003).

A large number of plants with leaf lesions have often been observed in autumn before the maximum ascospore discharge in the UK and France (West *et al*., 1999a), confirming the ability of the first ascospores released to produce a high incidence of leaf spotting due to the susceptibility of the crop to infection in early growth stages. Gradients of ascospore dissemination depend on the wind-speed, topography of the area and type of crop canopy which filters ascospores from the air (West and Fitt, 2005).

In crops, wind-borne ascospores are deposited onto the cotyledon and leaf surfaces of new crops and germinate in humid or wet conditions to produce germ tubes, which penetrate the leaf through stomata (Huang *et al.,* 2003b). In the laboratory, both *L. maculans* and *L. biglobosa* ascospores germinated at 5 to 20^{o}C on distilled water agar or detached oilseed rape leaves and the percentage of spores germinating increased with temperature from 5 to 20^{o}C (Huang *et al*., 2001, 2003b). The two pathogens differ in ascospore germination; after 40 h of incubation, the percentage of germinated

ascospores that penetrated stomata was greater for *L. maculans* than for *L. biglobosa* (Huang *et al.,* 2003b).

In the UK, the first symptoms of phoma stem canker disease on winter oilseed rape occur in the autumn with the occurrence of phoma leaf spot lesions. The number of lesions was greatest following 48 h of leaf wetness at 20^{o}C (Biddulph *et al*., 1999). Toscano-Underwood *et al.* (2001) found that the greatest number of leaf lesions was produced following 48 h of leaf wetness at 15 – 20^{o}C for both *L. maculans* and *L. biglobosa*. Subsequently, the pathogens grow through the leaf lamina and along the leaf petiole to the stem where they finally cause stem canker in the following spring/summer.

1.5 Disease control; cultivar resistance

An increase in incidence and severity of phoma stem canker occurred as a result of the introduction of the 'double low' (low erucic acid/low glucosinolate content) winter oilseed rape cultivars (Scarisbrick & Daniels, 1986; Kightley *et al.,* 2012), together with an increase in area of oilseed rape grown in the UK in the 1970's (Cook & Evans, 1978). An important method for control of stem canker disease is to breed oilseed rape cultivars for resistance against *L. maculans* that operates at both seedling and adult plant stages. There are two types of resistance against *L. maculans* that have been identified; major resistance (*R*) gene-mediated qualitative resistance and quantitative resistance (QR) (Fitt *et al.,* 2006a; Balesdent *et al.,* 2002).

Oilseed rape has been identified as having both major gene resistance and polygenic resistance against *L. maculans* (Ansan-Melayah *et al.,* 1997; Sprague *et al.,* 2006a). Major gene resistance is usually governed by single genes (*Rlm*) (i.e race-specific qualitative resistance) that operate in the seedling to young plant growth stages and confer resistance against races of *L. maculans* with the corresponding effector *Avr* gene (*AvrLm*), whereas quantitative resistance (QR) is partial resistance that is governed by multiple genes that operate after the leaf infection stages and do not prevent pathogens from colonisation of plants but decrease symptom severity and/or epidemic progress over time (Balesdent *et al.,* 2001; Delourme *et al.*, 2006; Liu *et al.,* 2007; Huang *et al.,* 2009; Hayward *et al.,* 2012) (Fig. 1.7).

The interaction between *L. maculans* and *B. napus* at the seedling stage follows Flor's "gene-for-gene" interaction hypothesis, where host and pathogen genes, determine if recognition takes place between them (Ansan-Melayah *et al.,* 1995). The effector (*Avr*) allele gene of the pathogen always matches the resistance gene of the host, satisfying the gene-for-gene interaction hypothesis (Table 1.2), which helps in analysis of the host defence response by *Brassica napus* (Howlett, 2004). This concept was explained by Van der Plank (1963) as 'vertical resistance' that in the host (cultivar) shows a strong interaction with the pathogen race (race-specific) whereas the 'horizontal resistance' or quantitative resistance (QR) is not race-specific and mostly inherited quantitatively (Keane, 2012). Since (*R*) gene-mediated qualitative resistance is race-specific resistance, the effectiveness of resistance depends on the races of *L. maculans* in local populations. More than 18 different major resistance genes (*Rlm1-9*, *RlmS*, *LepR1-3* and *BLMR1-2*) have been identified (Larkan *et al.,* 2014). *LepR3* was the first one of

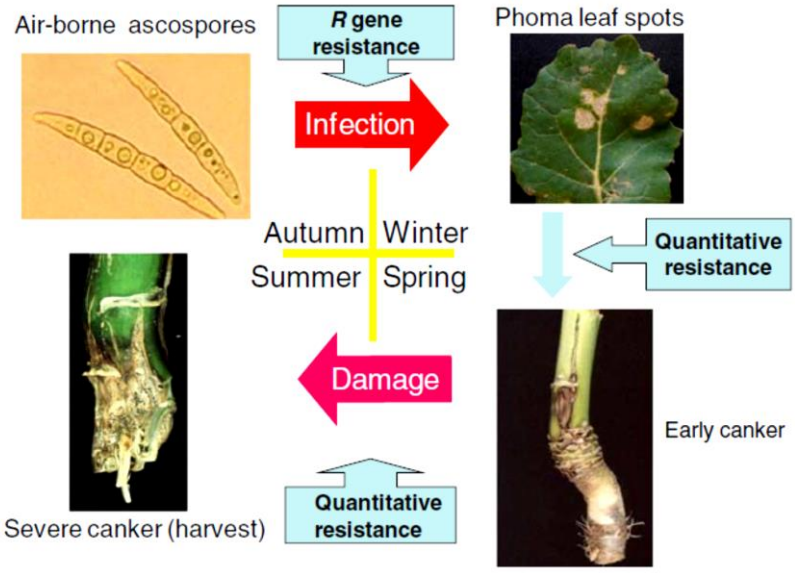


Fig. 1.7: Epidemiology and components of resistance against *L. maculans*; *R* gene resistance operates on the leaf and quantitative resistance (QR) operates during its symptomless growth phase after initial leaf infection before formation of stem cankers. Source: Fitt *et al.,* 2006a.

Table 1.2: The gene-for-gene interactions for *Brassica napus* (oilseed rape) major resistance (*R*) gene (*Rlm1* recognizing effector gene *AvrLm1* of *Leptosphaeria maculans*), the *AvrLm1-Rlm1* interactions as characterised in cotyledons of oilseed rape.

Pathogen *Avr* - gene	**Host *R*-gene interaction**	
	Rlm1	***rlm1***
AvrLm1	Resistant	Susceptible
avrLm1	Susceptible	Susceptible

Avr = Avr gene, *Lm* = *Leptosphaeria maculans*, *Rlm* = resistance gene

(Source:http://www.genoscope.cns.fr/spip/-Leptosphaeria-maculans-.html)

these *R*-genes to be cloned (Marcroft *et al.,* 2012; Larkan *et al.,* 2013) and recently *Rlm2* (Larkan *et. al.,* 2015) has been cloned. The pathogen isolates with the corresponding *L. maculans Avr* genes (*AvrLm*) have been identified and four from thirteen of the *Avr* genes have been cloned, namely *AvrLm1*, *AvrLm6*, *AvrLm4-7* and *AvrLm11* (Gout *et al*., 2006a; Fudal *et al*., 2007; Parlange *et al*., 2009; Balesdent *et al.,* 2013).

Such strict host specificities led breeders to widely breed for and deploy the corresponding resistances (i.e. major resistance genes) in crops. This has resulted in the unfortunate neglect of quantitative resistance (QR). This is because major (*R*) gene resistance is much easier to work with than QR, which often exhibits a large genotype-environment interaction (Brun *et al.,* 2010; McDonald, 2010). The plants harbouring these *R* genes are fully immune to the pathogen and such resistance genes exert extremely strong selection pressure on pathogen populations that usually can easily develop single mutations in their effector genes. There is evidence that *Avr* genes differ in their interaction with the host in relation to selection of virulent isolates. The mutation (point mutation) at the *AvrLm4-7* locus of *L. maculans* leading to virulence against *Rlm 4* results in ineffectiveness of the host resistance (Parlange *et al.,* 2009). This is because the host *R* (resistance) gene could not then recognise the product of the *Avr* gene, resulting in a susceptible interaction (Huang *et al.,* 2010). These mutational changes influencing qualitative resistance are due to selection against avirulent isolates in the *L. maculans* population (Gout *et al*., 2006a; Huang *et al.,* 2009). This will help to explain the phenomenon of ‘weak’ and ‘strong’ vertical resistance genes proposed by Van der Plank (1968), who suggests that some vertical resistance genes break down much more

rapidly than others if the mutations to virulence have little cost in terms of fitness of the pathogen (Keane, 2012). It differs from the change to virulence against *Rlm 7* because this involves deletion of the effector (*Avr*) gene. This may explain why *Rlm 7* resistance is more durable than *Rlm 4* resistance (Parlange *et al.,* 2009; Clarke*,* 2014).

Quantitative resistance (QR), often mediated by many genes, usually does not operate by the gene-for-gene recognition system of Flor (1956) involving a hypersensitive necrotic response in the plant, and on initial evidence, a particular molecular expression of some of the genes involved (Ellis *et al.*, 2007). Lacking this gene-for-gene interaction, quantitative resistance (QR) is more stable than the *R* - gene resistance (Pilet *et al*., 2001), which is its big advantage. Experimental studies with *Brassica napus* have also revealed that the polygenic quantitative resistance (QR) operates at a time between the infection of leaves and the formation of cankers during the asymptomatic phase of *L. maculans* and, therefore, reduces growth of the pathogen within the plant (Delourme *et al.,* 2006; Huang *et al.,* 2009). There is less known about quantitative resistance and its operation during the symptomless phase of the pathogen (Huang *et al.,* 2009). However, Lowe *et al.* (2014) discovered that during early infection *L. maculans* highly expresses CBM50 (LysM) gene (small-secreted-protein-encoding gene), which suppresses effector-triggered defence (ETD) to evade detection of the pathogen by the plant at 7 days post-inoculation.

Traditionally, plant pathogen defence has been divided into two broad forms; pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006). The PTI is the first action the plant takes against a pathogen and is triggered when the

pathogen lands on the plant. Subsequently the ETI is triggered when the pathogen releases molecules called effectors into the plant cells, which the plant recognises and reacts against. If the effectors are not recognised, the pathogen can spread with little resistance. However, a new concept called effector-triggered defence (ETD) is proposed to explain how plants protect themselves against the pathogens that grow in the space outside plant cells (the apoplast), such as *L. maculans* (Stotz *et al.,* 2014; Fitt, 2014). Effector-triggered defence (ETD) is mediated by *R* genes encoding cell surface-bound receptor-like proteins (LRR-RLPs) that engage the receptor-like kinase LRR-RLK SUPPRESSOR OF BIR1-1 (SOBIR1) with an extracellular recognition (Kruijt *et al.,* 2005; Yang *et al.,* 2012 & Stotz *et al.,* 2014). The response is host cell death after an extended period of endophytic pathogen growth and this is in contrast to ETI, in which detection of intracellular pathogens (e.g. rusts and mildews) occurs within cells and usually triggers fast host cell death (Stotz *et al.,* 2014).

The problems with major gene resistance are associated with both the ability of the pathogen population to change rapidly to render the resistance ineffective and the influence of the weather conditions on the effectiveness of resistance (e.g. when it operates well only at a certain range of temperatures) (Huang *et al.,* 2006b; Rouxel *et al*., 2003).

In Australia, the introduction of new cultivars with major gene resistance (vertical resistance) against *L. maculans* without a background of quantitative resistance (horizontal resistance) resulted in the resistance rapidly being rendered ineffective (Sprague *et al.,* 2006a, b). It appears that severity of stem cankers in the ‘vertical

resistance' cultivars was very high compared with severity in nearby older cultivars with only 'horizontal resistance' (Sprague *et al.,* 2006b). This also occurred in France where new cultivars with *R* – gene (*Rlm 1*) resistance were grown in uniform monoculture over large areas. This caused an increase in severity of epidemics because *L. maculans* (*AvrLm1*) populations were changing to become virulent (*avrLm1*) against the *Rlm 1* resistance gene and this led to breakdown of resistance (Rouxel *et al*., 2003). Yield losses in Australia in 2003 were 90% because of the breakdown of the resistance gene (named *LepR3*) after two years of use in commercial cultivars (Sprague *et al.,* 2006a; Van de Wouw *et al.,* 2010) in response to selection pressure of the crop monoculture on *L. maculans* populations (Balesdent *et al.,* 2006, 2013).

Recently, Larkan *et al.* (2013) showed that the *L. maculans* effector *AvrLm1* confers avirulence to both *LepR3* and *Rlm1* and this may explain the loss of effectiveness of *LepR3* in Australia. There is a possibility that in Australia some populations of *L. maculans* had been selected towards a high proportion of virulent *avrLm1* pathotypes through previous exposure to cultivars with *Rlm1;* this had effectively enriched the entire population of the pathogen for virulence against *LepR3* so that rapid breakdown of resistance occurred when *LepR3* was introduced (Larkan *et al.*, 2013). Thus, it greatly reduced the effective life-span of the *R* gene in Australia (Larkan *et al.*, 2013).

However, field experiments in Europe have shown that Chinese oilseed rape cultivars are very susceptible to *L. maculans* (Fitt *et al.,* 2008; Zhang *et al.,* 2014). Therefore, it is advisable to introduce into Chinese cultivars the polygenic 'quantitative' resistance against *L. maculans* that is present in European winter oilseed rape cultivars rather than

major gene resistance that will not provide durable resistance, based on the experience in Australia and France (Rouxel & Balesdent, 2005; Sprague *et al.,* 2006b; Fitt *et al.,* 2006a; Zhang *et al.,* 2014).

General strategies, such as rotation of different resistance genes in space and time and use of mixtures of cultivars with different resistance genes, could extend the durability of resistance genes (Gladders *et al.,* 2006). It has been suggested that a combination of polygenic resistance (QR) and major (*R*) gene resistance can be used to increase the durability of plant resistance against *L. maculans* (Sprague *et al.,* 2006b; Brun *et al.,* 2010). Although resistance against *L. maculans* has been described, little is known about resistance against *L. biglobosa*. Therefore, breeding programmes must include both *L. maculans* and *L. biglobosa* as a key requirement for sustainable management of phoma stem canker (West *et al.,* 2001).

1.6 Disease control; cultural practices

Cultural practices such as stubble management, crop rotation and disease escape can be used for controlling the disease. In Europe, it is recommended that deep ploughing to bury stubble may reduce the risk of phoma stem canker, if followed by minimal tillage or direct seeding to prevent the stubble from being brought back to the soil surface. This is difficult to achieve on heavy soils (Gladders & Musa, 1980; West *et al.,* 2001), where cultivation, chopping, slashing and harrowing are often used (Gladders *et al*., 2006). Burying infected stubble can prevent ascospores from being released into the air and will also enhance the rate of break-down of debris (Kharbanda & Otashewski, 1997;

Turkington *et al*., 2000a, b). In China, cultural practices, such as removal of crop residues for fuel and rotating oilseed rape with paddy rice, which involves flooding the field within a single growing season, help to reduce the amount of inoculum (West *et al.,* 2000; Eckert, 2005; Fitt et. al, 2006a).

Crop rotation is one of the most effective cultural practices for control of the disease. In Canada, Australia and Europe, there are usually 4-year breaks between oilseed rape crops (West *et al.,* 2001; Canola Council, 2014), whereas in China and India there are only 1- or 2-year breaks between oilseed rape crops because more labour is required to manage the crop. In the UK, winter oilseed rape is now grown in one year in two or one year in three rotations that may increase severity of both soil-borne and foliar diseases (HGCA, 2012). Crop rotations in south-eastern Australia are considered to be effective if the crops are sown at least 200 m from oilseed rape stubble from the previous cropping season (separation of 400 m is much more preferable) with a 4 year or shorter rotation to minimise risk of inoculum survival (Marcroft *et al.,* 2003).

In the UK, growers generally sow winter oilseed rape early in autumn (in late August/early September) so that the plants are well established before maximum ascospore release occurs (Sept/Oct) (Gladders & Musa, 1980; West *et al*., 2001). This type of cultural practice is known as ‘disease escape’ to avoid risk of severe disease epidemics (Gladders & Musa, 1980; Khangura & Barbetti, 2004). In contrast, no correlation was observed between sowing date and disease severity in Western Australia (Khangura & Barbetti, 2001). The differences between these findings may be

due to the differences in weather conditions between countries, which influence the timing of ascospore release and infection of leaves. In addition, there is a difference in the oilseed rape type grown; in Australia more than 95% of the crop is spring-type oilseed rape grown over winter whereas in the UK there is mainly winter-type oilseed rape (West *et al.,* 2001).

1.7 Disease control; fungicides and forecasting disease risk

Fungicide applications are effective for protecting oilseed rape against *L. maculans* for only a limited period due to degradation of active ingredients, leaf expansion and the production of new untreated leaves (West *et al*., 2000). The value of different combinations of fungicides (seed treatments or foliar fungicide sprays) for control of the disease in different areas depends upon the economics of the crop production and the epidemiology of phoma stem canker. In the UK, when 10-20% of plants show phoma leaf spot symptoms in autumn then a fungicide spray is generally applied (Eckert *et al.,* 2010; HGCA, 2012). Potential activity of fungicides as growth regulators also influences the choice of fungicide product for application. For example, there is use of flusilazole or prothioconazole when plants require growth regulators to prevent premature extension growth and use of metconazole on larger plants later in the growing season (HGCA, 2012). In the UK, more than 90% of winter oilseed rape crops were treated with foliar fungicides from two groups; triazoles (flusilazole, difenoconazole, tebuconazole, prothioconazole) and the methyl benzimidazole carbamates (carbendazim, benomyl) (Eckert *et al.,* 2010). During the period October to March in the UK, fungicide combinations, such as flusilazole + carbendazim (e.g. Punch C) or difenoconazole +

carbendazim, can give good control of the disease (West *et al.,* 2002a; Steed *et al.,* 2007). The carbendazim combines with the azole fungicides to provide effective control of both phoma stem canker and light leaf spot (*Pyrenopeziza brassicae*) (Boys *et al.,* 2007; Huang *et al.,* 2011).

Differences in fungicide use may also influence pathogen populations, since *L. maculans* and *L. biglobosa* differ in their sensitivities to different azole fungicides and methyl benzamidazole carbamates (MBCs), where *L. biglobosa* is often less sensitive to fungicides than *L. maculans* (Stonard *et al.,* 2010; Eckert *et al.,* 2010). The MBCs group includes benomyl and carbendazim (Tarlochan, 2011). Steed *et al.* (2007) reported that the winter oilseed rape that was treated with flusilazole + carbendazim (i.e. Punch C) showed less severe phoma stem canker than untreated crops. However, all plant protection products containing flusilazole have been withdrawn by the European Commission (EC) from the market, after 12 October 2013. The main reason is because those agrochemicals have the potential to be 'endocrine disrupters' that cause adverse health effects in an organism, its progeny or (sub) populations (http://ec.europa.eu/environment/chemicals/endocrine). This has had a large impact in plant protection; the azole active substances are used for controlling a large number of diseases for many crops and this will result in 18 products being withdrawn from the market including Punch C (http://ec.europa.eu/food/plant/standing). It has been estimated that there will be a 9.8% production loss to UK growers (70 million euro) because of the effect of losing all azoles and the consequent loss of control of the main fungal diseases phoma stem canker (*L. maculans*) and light leaf spot (*P. brassicae*) (ADAS-European Crop Protection Association, 2012). In countries such as Australia and

Canada where yields are lower, seed treatments and soil fungicides are often used. However, 90% of winter oilseed rape in the UK, where yields are great enough to justify the extra cost, receive foliar fungicides (West *et al.,* 2001; Eckert *et al.,* 2010). Foliar fungicides do not control the disease once the pathogen has reached the stem or after a certain growth stage (Gladders *et al.,* 1998). Fungicide use is economic and effective only when applications are correctly timed, so there is a need for a forecasting scheme to predict the severity of epidemics at the time when chemical control could be effective (Gladders *et al.,* 1998).

Forecasting risk of severe stem canker disease epidemics is important for improving disease control and can reduce the number of fungicide applications required. The use of weather parameters that influence stages in the life cycle, such as the maturation of ascospores in pseudothecia, the release of ascospores and the infection of leaves, improves the accuracy of forecasting. Forecasting models to predict the timing of ascospore release that are based on rainfall and temperature have been used in France and Australia (Pérès *et al.,* 1999a; Salam *et al.,* 2003).

A model was developed for predicting pseudothecial maturity and seasonal ascospore discharge in Western Australia (Salam *et al*., 2003). The model considered a combination of two weather parameters (daily average temperature and daily total rainfall) to determine the development of pseudothecial maturation on stubble left from previous growing seasons. The pseudothecia were mature after approximately 43 favourable days with temperature $<22^{o}$C and rainfall ≥4mm had elapsed since harvest

(Salam *et al.,* 2003). This Australian model has been tested with data from other different countries to assess whether it can be applied worldwide (Salam *et al*., 2007).

In eastern England, the relationships between the incidence of stem canker before harvest in summer and rainfall in the previous August and September were used as the basis of an annual forecast (Gladders & Symonds, 1995). This relationship provided indirect evidence that factors affecting pseudothecial maturation on stubble are crucial in determining the subsequent severity of stem canker disease (West *et al*., 1999a). The timing and incidence of leaf spotting in autumn/winter is important for defining severity of canker the following spring (Sun *et al*., 2000). Work at two sites over five seasons in England (Huang *et al*., 2005) also suggested that the timing of ascospore release in autumn is related to the amount of rainfall in August and September. There was a suggestion to combine the empirical models used in the UK developed by Evans *et al.* (2006, 2008) and Huang *et al*. (2007) with the SporacleEzy model (Australia) (Salam *et al.,* 2007) to predict the date of ascospore release and later to determine phases of stem canker development in the autumn/winter/spring and the severity of disease in summer.

Currently, in the UK the model published by Evans *et al.* (2008) that is used for weather-based disease forecasting demonstrates the effect of regional variation in summer temperature and rainfall on the date of onset of phoma leaf spotting in autumn (www.rothamsted.ac.uk/phoma-leaf-spot-forecast/). The model predicts an earlier onset of symptoms in southern England than in northern England. In the south, warm weather in spring while cankers are developing results in earlier appearance of canker in spring and more severe stem cankers at harvest (Fig. 1.8) (Stonard *et al.,* 2010; Evans *et al.,* 2008). The forecast is issued in late September to guide fungicide spray decisions in the

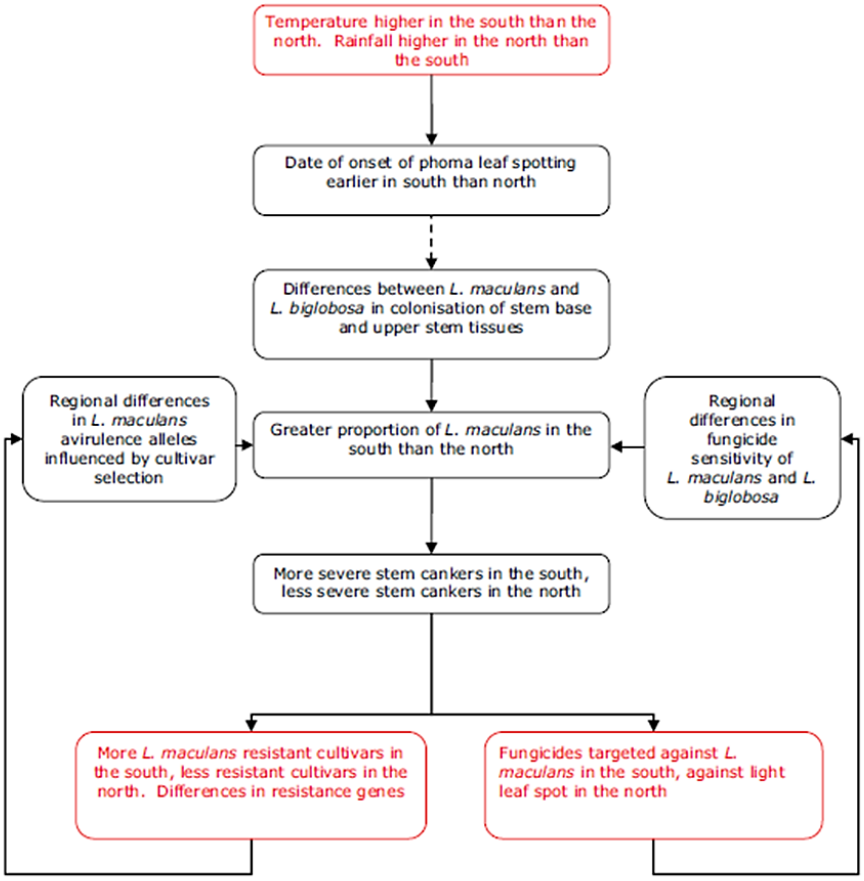


Fig. 1.8: This illustrates the direct effect of differences in weather (e.g. temperature and rainfall) and agronomic (e.g. cultivar choice and fungicide use) factors (shown in red) on both populations of *L. maculans* and *L. biglobosa* and severity of epidemics between the north and south of the UK (in black) (Stonard *et al.,* 2010).

autumn (West *et al.,* 2002a). The recommended threshold for fungicide application in autumn to control this disease is 10% of oilseed rape plants with symptoms of phoma leaf spot (Fig. 1.9). Fungicide applications are important for effective control of the disease and therefore the forecast is updated in February/March to help decisions about a second fungicide treatment when further leaf spots occur before early stem extension (Gladders *et al.,* 1998). West *et al.* (1999b) suggested that monitoring pseudothecial maturation, timing of ascospore release and conditions favourable for infection could be used to optimise fungicide applications to control stem canker in the UK as part of a disease forecasting scheme. Now, that the most effective fungicides (e.g. Punch C) are no longer permitted by EU legislation, the use of host resistance even more important and is the most effective way to control phoma stem canker.

1.8 Rationale of study

Phoma stem canker is an important disease in most countries growing oilseed rape. Understanding the epidemiology of the disease helps development of strategies to manage it effectively. In the UK, *L. maculans* and *L. biglobosa* co-exist but *L. maculans* is more aggressive in causing phoma stem canker. Therefore, *L. maculans* has been studied more and resistance genes operating against it have been described. Previous work has shown that *L. maculans* mostly occurs on stem bases and *L. biglobosa* mostly occurs on upper stems (Fitt *et al.,* 2006b). This study will evaluate the colonisation of different cultivars by *L. biglobosa* and *L. maculans* and discuss how this may contribute to new strategies for managing phoma stem canker disease. This is an especially important issue, since the withdrawal of flusilazole will lead to higher input costs for

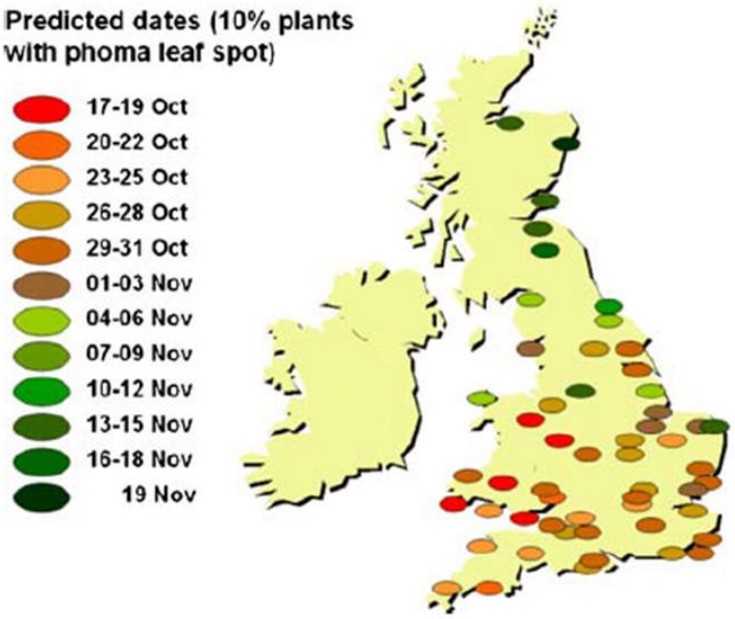


Fig. 1.9: The predicted dates when incidence of phoma leaf spot reaches 10% at different sites for the 2006/2007 winter oilseed rape growing season that were obtained with a regression model (Evans *et al.,* 2008) and are based on the weather (temperature and rainfall). The prediction dates are available online for each growing season (www.rothamsted.ac.uk/phoma-leaf-spot-forecast/).

growers associated with use of less effective fungicides and lower yields at the end of the growing season. Therefore, it is better to have a long-term plant disease control strategy considering resistance against the pathogen. Resistant cultivars may have a potential to slow the development of the sexual stage (pseudothecia) of the pathogens that produces ascospores as initial inoculum.

1.9 Aim and objectives

The aim of this project is to investigate the influence of cultivar resistance (*R*-gene/quantitative resistance) and weather on the colonisation of oilseed rape by *L. maculans* and *L. biglobosa* in natural and controlled environment conditions to optimise control of the disease.

Objectives:

1. To investigate influence of cultivar resistance (*R*-gene/quantitative resistance) on the proportions of *L. maculans* and *L. biglobosa* at the phoma leaf spot and stem canker stages in different growing seasons.

2. To determine effect of cultivar resistance on maturation of pseudothecia of these *Leptosphaeria* species in natural and controlled environment conditions.

3. To detect phenotypic responses of different cultivars to *L. maculans* and *L. biglobosa* in cotyledons inoculated with isolates obtained from phoma leaf spots or stem cankers.

Chapter 2

General materials and methods

2.1 Winter oilseed rape field experiments; 2010/2011, 2011/2012 and 2012/2013

The winter oilseed rape field experiments were situated at Rothamsted Research, Harpenden for the 2010/2011, 2011/2012 and 2012/2013 growing seasons. This study started with the assessment of phoma leaf spot in 2010/2011 (on 9 December 2010), 2011/2012 (25 January 2012) and 2012/2013 (24 October 2012, 21 November 2012, 11 December 2012 and 30 January 2013) growing seasons. This was followed by observation of phoma stem canker in each growing season, on 30 June 2011, 13 July 2012 and 26 July 2013. After harvest in summer 2012, the winter oilseed rape crop was sown in August 2012 for the 2012/2013 growing season.

There were nine cultivars of winter oilseed rape planted in the field experiments. The cultivars had different combinations of resistance (*R*) - genes or were without *R*-genes against *Leptosphaeria maculans*; some had quantitative resistance (QR) (Delourme *et al.*, 2006) (Table 2.1). In all growing seasons, the field experiment designs were randomised blocks with three replicate blocks, each with nine plots (Fig. 2.1).

Table 2.1: The nine cultivars of winter oilseed rape planted in the field experiments at Rothamsted Research, Harpenden for the 2010/2011, 2011/2012 and 2012/2013 growing seasons with different combinations of resistance (*R*) - genes or without *R*-genes against *Leptosphaeria maculans*; some had quantitative resistance (QR).

Cultivar	(*R*) – genes and/or quantitative resistance (QR)[1]
Adriana	*Rlm 4* + QR
Bilbao	*Rlm 4*
Capitol	*Rlm 1*
Drakkar	no *R* genes
DK Cabernet	*Rlm 1* + QR
Es-Astrid	QR
Excel	*Rlm 7*
NK Grandia	QR
Roxet	*Rlm 7*

[1] Further information about these *R*-genes and quantitative resistance is given in Delourme *et al.* (2006).

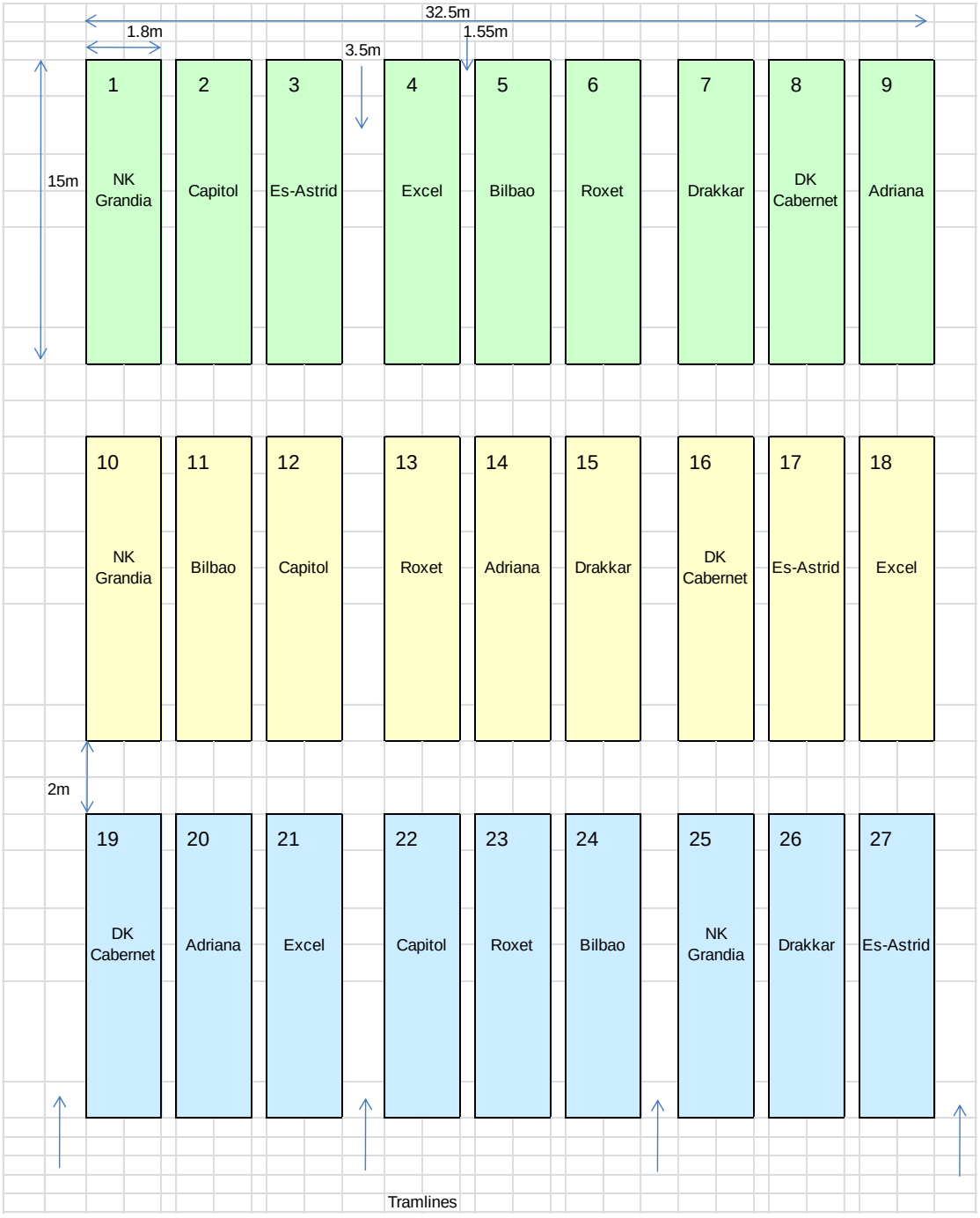


Fig. 2.1: The randomised block design of the winter oilseed rape experiments at Rothamsted Research, Harpenden for the 2012/2013 growing season. (Refer to Appendix 2.1 for the 2010/2011 and 2011/2012 experimental design).

2.2 Collection of winter oilseed rape stem debris

Pieces of winter oilseed rape stem (30-50 cm long) from all nine cultivars with phoma stem canker were randomly collected before harvest from all 27 plots (three replicates per cultivar) at Rothamsted Research, Harpenden. After assessment of phoma stem canker, the samples collected were taken to the laboratory for *Leptosphaeria* species isolation and identification. The basal part with typical phoma stem base canker and upper stem lesions were both sampled by cutting out small pieces about 2 x 2 x 2 mm in size. These pieces were kept in 1.5 mL micro tubes for DNA extraction and PCR-based identification of *Leptosphaeria* species.

Approximately 2 weeks after harvest, stem debris of winter oilseed rape for all nine cultivars was collected by pulling up the whole stem including the tap root. All stem debris collected was dried at 20^{o}C and used for pseudothecial development experiments at Bayfordbury in August/September under natural conditions and also for controlled environment condition experiments.

2.3 Preparation of sterile culture media

The standard media that were regularly used in this research were distilled water agar, potato dextrose agar (PDA) and V8 juice agar (V8A). The ingredients used and the procedures for preparation were:

Distilled water agar (DWA)

Agar (Oxoid No. 3)	15 g

Distilled water	1 L

Potato dextrose agar (PDA)

Potato dextrose agar (Oxoid)	39 g
Distilled water	1L

400ml V8 juice agar (V8A)

V8 juice (Campbell)	80 mL
$CaCO_3$	0.8 g
Agar	8g
Distilled water	320ml
To give pH 6.6	

The sterilization by moist heat at 121°C for 15 minutes dissolved and dispersed the ingredients. To prevent boiling over in the autoclave, the flasks or bottles that were used were no more than half full and the lids were untightened to avoid explosion in the autoclave. An antibiotics solution was prepared for adding to the agar medium; penicillin (100 mg/ml) plus streptomycin (50 mg/ml) sterilized by filtration. Membrane filters with pore size 0.45 µm were used as medium filters; 0.22 µm sterile millipore express® PES membrane (Millex®GP, Carringtwahill, Co. Cork, Ireland). After autoclaving, the medium was allowed to cool until it was cool enough to touch it by hand (about 40°C) and 200 µl streptomycin and 200µl penicillin were added to 400ml of media. This was to decrease potential bacterial contamination of PDA and V8A. The agar medium with antibiotics was then poured into 9 cm diameter plastic Petri dishes to a depth of about 5 mm. The

medium was then allowed to cool until it solidified and the plates were left for a day or two to ensure that none had been contaminated.

2.4 Sterile transfer

All activities involving transferring of pathogens were done in a laminar flow cabinet. The transfer needles and scalpel were dipped into 70% IMS (industrial methylated spirits, which comprises 95% ethanol and 5% methanol) (Waller *et al.,* 2002), and flame sterilized along their entire lengths before they were in contact with cultures to avoid cross-contamination. The transfer needles were cooled by touching them briefly onto the sterile medium to ensure that residual heat in the flamed needle did not kill the sample being transferred. The caps from bottles containing medium were removed and bottles were sterilized by lightly flaming their mouths to kill any propagules of microorganisms that were in contact with the glass.

2.5 Surface sterilization

Surface sterilization is important to ensure a clean laminar flow cabinet where all culturing work with isolates was done. It was done by swabbing the surface area with a liquid disinfectant such as 70% IMS before work started. Trays, benches and other surfaces were sterilized too.

2.6 Isolation of *L. maculans* and *L. biglobosa* from phoma leaf spots and phoma stem cankers

For isolation from phoma leaf spots, ten large pale lesions and ten small dark lesions per cultivar were selected from winter oilseed rape leaves sampled in February 2012. The leaves were surface-sterilised to remove saprophytic organisms (Waller *et al.,* 2002) and small pieces with lesions (0.3 x 0.3 cm) were cut out. All the small pieces of leaves were placed in Petri dishes on wet filter paper for 5 days to enhance the production of ooze (conidia) from pycnidia (Fig. 2.2).

For isolation from diseased stem cortex tissues, stem bases or upper stems, small pieces of lesions (0.5 x 0.5 cm) were cut out. The stem cortex pieces were surface-sterilised by immersion of the pieces in sodium hypochlorite solution (NaOCl) (1% available chlorine) for 3 min and subsequent rinsing in sterile distilled water (Waller *et al.,* 2002). Then they were dried on sterile paper tissues, under a filtered air flow to inhibit the growth of bacteria on the tissue. Four small pieces from each cultivar were placed on PDA medium amended with 100 mg/ml of penicillin and 50 mg/ml of streptomycin and the cultures were incubated at 20°C in darkness. After 5 days, the colonies which developed from diseased tissue were subcultured onto V8 medium amended with 20 units/ml of penicillin and 40 units/ml of streptomycin and incubated at 20 °C for 3 days in darkness. Then they were transfered to an incubator with alternating 12hr light and 12hr darkness for 14 days to induce pycnidial production.

Fig. 2.2: The deep pink ooze exuding from mature pycnidia (arrows) of *Leptosphaeria maculans*.

2.7 Purification of *Leptosphaeria* cultures (single pycnidial isolates)

Pure culture is the priority for the identification of microorganisms and there are a number of techniques used. For *Leptosphaeria* species, a single pycnidial technique was employed. Throughout the procedure for obtaining the single-pycnidial isolates, 70% IMS was used to clean the working surface and a Bunsen burner was used for sterilizing utensils. The pycnidia produced on the small pieces of leaves and on the V8 medium plates after subculture from diseased stem cortex tissues were observed by using a dissection microscope that was kept in a laminar flow cabinet.

One mature pycnidium with a cirrhus (deep pink conidial mass) was chosen from each leaf piece or plate. The cirrhus from each of these selected pycnida was sampled by using a fine needle and was transferred to a drop of sterilized water in order to provide a spore suspension**.** Then the spore suspensions were pipetted onto V8A amended with antibiotics and plates were incubated 20°C in darkness for 5 days. Finally, cultures were used for pycnidial production and also for harvesting mycelium.

L. maculans and *L. biglobosa* were identified visually based on the pigmentation produced on PDA (potato dextrose agar), where *L. biglobosa* produces a yellowish pigmentation in the PDA but *L. maculans* does not (William & Fitt, 1999) (Fig. 2.3).

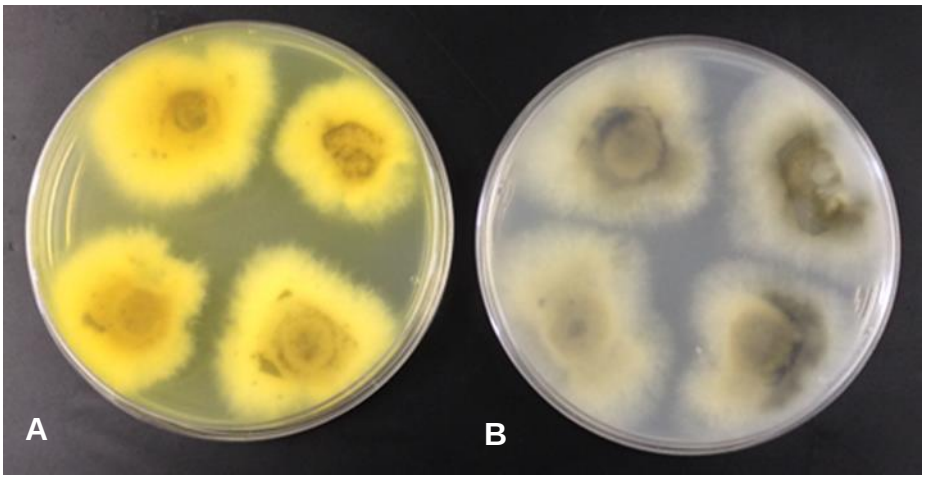


Fig. 2.3: Pigmentation produced on PDA (potato dextrose agar); (A) yellowish pigmentation of *L. biglobosa* and (B) *L. maculans* (no yellow pigment) after growing for 5 days at 20°C in a controlled environment cabinet.

2.8 Preparation of *Leptosphaeria* inoculum

All cultures of *Leptosphaeria maculans* and *L. biglobosa* obtained from phoma leaf spots and phoma stem cankers that produced large numbers of pycnidia were used for making conidial suspensions. The sterile Lazy-L spreader was used to spread sterile distilled water across the plate to allow more conidia to be released from pycnidia. Later, the conidial suspension was filtered through a sterile filter paper in a sterile glass funnel into a 15ml tube. It was done inside the laminar air flow cabinet.

Conidial concentrations were estimated by direct counting techniques, using a Neubauer haemocytometer slide. The concentrations of the conidial suspensions were calculated from ten randomly chosen small squares (at 40x magnification) based on the formula;

$$\text{number of spores/ml} = \text{number of spores /small square} \times 4 \times 10^6$$

The conidial suspensions were stored at -20^0C.

2.9 Preservation of cultures

Both temporary and permanent preservations of *Leptosphaeria* isolates were made. Both are necessary applications for working cultures and for further studies, where it is possible to retain isolates in the condition in which they were at the time of isolation. Isolates in this study were preserved on PDA discs in water, as spore suspensions in water and on paper discs covered with pycnidia for temporary stock cultures. For long-term storage, agar plugs from actively growing cultures were placed in sterile glass tubes, covered with sterile mineral oil and maintained at 4^oC.

2.10 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. All data collected from the study were analysed statistically by analysis of variance (ANOVA) using SPSS version 21.0.

Chapter 3

Proportions of *L. maculans* and *L. biglobosa* in phoma leaf spots and stem cankers on nine cultivars with different types of resistance

3.1 Introduction

Both *L. maculans* and *L. biglobosa* have a worldwide distribution (Fig. 3.1). In some countries, such as France (Penaud *et al.,* 1999), Germany (Kuswinanti *et al.,* 1999), Poland (Karolewski *et al.,* 2002; Piliponyte-Dzikiene *et al.,* 2014), Canada (Gugel and Petrie, 1992), USA (Fitt *et al.,* 2006a) and the UK (Humpherson-Jones, 1983; Stonard *et al.,* 2010), there are mixed populations of *L. maculans* and *L. biglobosa*. However, in Australia, the population was once considered to be entirely *L. maculans* A-group (Ballinger & Salisbury, 1996; Chen *et al.,* 1996). More recently, *L. biglobosa* has been identified in Australia; in Western Australia as *L. biglobosa* 'australensis' or 'occiaustralensis' (Plummer *et al.,* 1994; Vincenot *et al.,* 2008; Liu *et al.,* 2014) and in northern New South Wales as *L. biglobosa* 'canadensis' (Van de Wouw *et al.,* 2008). In New Zealand, *L. maculans* was first reported as 'highly virulent' and 'weakly virulent' by Cunningham (1927) and recently 'weakly virulent' *L. biglobosa* has been recognised (Lob *et al.,* 2013). There are some regions that were first dominated by *L. biglobosa* and later invaded by *L. maculans*. This trend occurred in Eastern Europe [Poland (Fitt *et al.,* 2006a), Hungary (Szlávik *et al.,* 2003; Magyar *et al.,* 2006) and the Czech Republic (Szlávik *et al.,* 2003)] and Canada (Fitt *et al.,* 2006a). In Russia, China and Iran, only *L. biglobosa* has been reported as causing phoma stem canker (Fitt *et al.,* 2006a, 2008). It has been proven that Chinese *B. napus* cultivars are highly susceptible to *L. maculans* when grown in the UK (Fitt *et al.,* 2008) and

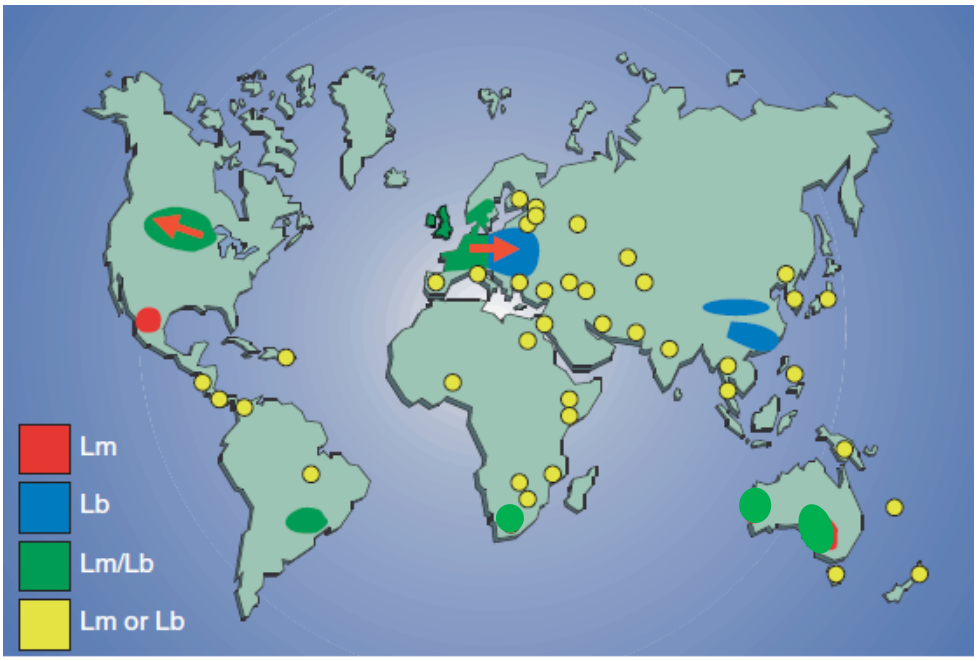


Fig. 3.1: Worlwide distribution of *Leptosphaeria maculans* and *L. biglobosa*. The arrows show the spread of *L. maculans* into areas (in Canada and Poland) where only *L. biglobosa* was predominant. Areas where populations have been characterised as predominantly *L. maculans* (red), *L. biglobosa* (blue) or a mixture of the two species (green) are indicated by patches. Areas where there have been reports of the pathogens (sometimes only a single report) but the species has not been identified are shown by yellow dots (modified from Fitt *et al.*, 2006a).

France (Fitt *et al.,* 2006a). Therefore, *L. maculans* may cause considerable damage if it was to be introduced to China (Zhang *et al.,* 2014).

The invasion of *L. maculans* into areas (e.g. Eastern Europe, Canada and Sweden) where only *L. biglobosa* was previously present (Fig. 3.1) has led to the suggestion that *L. biglobosa* may have evolved earlier than *L. maculans* (Fitt *et al.,* 2006a; Fitt *et al.,* 2006b; Fitt *et al.,* 2008). Later, the species *L. biglobosa* may have diverged into different subclades, possibly related to the region where the pathogen first invaded and the host species (Mendes-Pereira *et al.,* 2003) (refer to Chapter 1.3.1). They are considered to be sibling species (Fitt *et al.,* 2006b). Recently genomic evidence has been used to confirm that *L. maculans* and *L. biglobosa* are different species (Grandaubert *et al.,* 2014) (refer to Chapter 1.3.1).

In the UK, the regional distribution of *L. maculans* and *L. biglobosa* was surveyed in 1982 and 1983 by Humpherson-Jones (Humpherson-Jones, 1983). The survey identified 41% of isolates as *L. maculans* (then classified as *L. maculans* A-group) and 59% as *L. biglobosa* (then classified as *L. maculans* B-group). He also suggested that areas with a longer history of oilseed rape cropping would have a greater proportion of *L. maculans*. Later, Liu (2007) did a survey by using cultural methods as well as PCR (uniplex and multiplex) and recorded an increase of proportion of *L. maculans* by 2001. After the 1980's survey, regional variation in the distribution of *L. maculans* and *L. biglobosa* has been observed, with a greater incidence of *L. biglobosa* in northern-eastern England (Liu, 2007; Stonard *et al.,* 2010). Temperature affects the development of disease caused by *L. maculans* and *L. biglobosa* in *brassicas*. For example, in Scotland, although phoma leaf spotting occurs, stem cankers do not develop (Evans *et al.,* 2008). This might be related to

differences in climate, since disease incidence is greater in south-east England with warmer weather in spring than in northern England (Stonard *et al.,* 2010).

The effects of climate change on phoma stem canker epidemiology have been determined for the 2020s and 2050s by using a model combining weather-based disease forecasting with climate change predictions under high- and low-carbon emissions scenario (Evans *et al.,* 2008). Predicted global warming can increase the range and severity of plant diseases due to changes in the pathogen, the host or the host–pathogen interaction in response to weather changes (Coakley *et al.* 1999; Huang *et al.* 2005, 2006b; Garrett *et al.* 2006).

In crops, the timing of phoma leaf spot development in the autumn/winter affects the incidence and severity of phoma stem cankers/upper stem lesions in the spring/summer. The incubation period (time from inoculation to symptom development, including germination, colonisation and production of lesions) of *L. biglobosa* is shorter than that of *L. maculans* (Huang *et al.,* 2003b, Toscano-Underwood *et al.,* 2001). Co-inoculation experiments, at 15-20^{o}C, using *L. maculans* and *L. biglobosa* isolates transformed with the GFP or DsRed reporter genes, respectively, have shown that *L. maculans* growth from leaf lesions to the petiole is faster than that of *L. biglobosa* (Eckert *et al.,* 2005). However, the growth of *L. biglobosa* was faster than that of *L. maculans* along the petiole to the stem (Fitt *et al.,* 2006b). Under controlled environment conditions, the incubation period until formation of necrotic leaf lesions was dependent on temperature, being 5 days at 20^{o}C and 14 days at 8^{o}C after inoculation with *L. maculans* (Biddulph *et al.,* 1999). The time between appearance of leaf spot lesions and the development of stem cankers is affected by the temperature because the pathogens grow systemically

along the leaf petiole from leaf spots to infect the stem to cause basal canker or upper stem lesions. In controlled environment conditions, Hammond *et al.* (1985) found that the time between the development of phoma leaf spots and development of phoma stem cankers was 77 days at 18/12^{o}C (day/night temperature) and 175 days at 3^{o}C for cv. Primor inoculated with A group (*L. maculans*). In crops, Sun *et al.* (2001) suggested that the rate of *L. maculans* growth along the petiole to the stem may be a function of accumulated degree-days for a given cultivar. Sun *et al.* (2001) showed that the thermal time (^{o}C-days) between occurrence of 10% incidence (% plants affected) of phoma leaf lesions in the autumn and 10% incidence of phoma stem cankers in the spring was consistent between the three seasons of their study for cvs. Lipton, Capitol and Apex.

The asexual fruiting bodies (pycnidia) play an important role in producing conidia that may serve as a secondary inoculum that is dispersed by rain-splash to neighbouring oilseed rape leaves (West *et al.,* 2001; Travadon *et al.,* 2007; Canola Council, 2014). McGee and Emmett (1977) reported that the frequency of infection by conidia is increased by long periods of high humidity in Australia. Work has shown that there are differences between conidial inoculum and ascospore inoculum in incubation period; in controlled environment conditions, phoma leaf spots were observed on susceptible plants and those with quantitative resistance at 7 days post inoculation (dpi) for plants inoculated with ascospores and at 10 dpi for plants inoculated with conidia (Huang *et al.,* 2014a).

Cultivation of resistant cultivars has been identified as the best option to prevent phoma stem canker. In the UK, a combination of this strategy with cultural practices has been identified as a major strategy for controlling phoma stem canker disease

(Fitt *et al.,* 2006a). However, the continuous cultivation of resistant oilseed rape cultivars with major gene resistance against *L. maculans* induces the adaptation of *L. maculans* populations to cause the disease on resistant cultivars (refer to Chapter 1.5). The virulent pathogen races are able to render host resistance ineffective after 2 to 3 years of cultivation of cultivars with this resistance because fungal populations are capable of changing in response to the selection pressure exhibited by resistance genes (Sprague *et al.,* 2006b; Keane, 2012). Hence, major gene resistance of oilseed rape has been associated with the issue of short durability of resistance (Fitt *et al.,* 2006a). Quantitative or polygenic resistance (QR) can be used to extend the life of *R*-genes when they are combined in the same cultivar (Sprague *et al.,* 2006b; Brun *et al.,* 2010; McDonald, 2010).

Previous work had stated that resistance against fungal pathogens involves two general categories, based on the pathogen molecules that trigger the responses; 'pattern-triggered immunity' (PTI), where slowly evolving pathogen-associated molecular patterns (PAMPS) trigger basal defense responses, or 'effector-triggered immunity' (ETI), in which specific pathogen effectors, targeted to disrupt PTI either directly or indirectly, trigger specific *R*-genes (Jones & Dangl, 2006). The pathogen recognition by the plant through effector-triggered immunity (ETI) occurred if an *Avr* effector was recognized by the corresponding NB-LRR (nucleotide binding – leucine rich repeat) protein (protein–protein interactions) encoded by the host *R* gene (Jones & Dangl, 2006; Bent & Mackey, 2007). More recently, Stotz *et al.* (2014) suggested that, whilst ETI that often results in hypersensitive cell death applies to intracellular pathogens such as those that cause mildews and rusts, ETD (effector-triggered defence) applies to apoplastic extracellular pathogens such as *L. maculans;* these pathogens do not produce haustoria but adapt to colonise the intercellular matrix and

rely on effectors secreted into the apoplast (Stergiopoulos & de Wit, 2009). However, little is known about the mechanisms of *B. napus* resistance to *L. biglobosa* (Fitt *et al.,* 2006a) and it is believed that the qualitative *Rlm* genes that operate against *L. maculans* are not effective against *L. biglobosa* (Brun *et al.,* 1997; Somda *et al.,* 1998; Fitt *et al.,* 2006a). Recently, the *LepR3* resistance gene has been cloned (Larkan *et al.,* 2013).

Previous work has suggested that there may be induced resistance interactions between *L. maculans* and *L. biglobosa* (Mahuku *et al.,* 1996; Liu *et al.,* 2006; 2007). In controlled environment experiments with co-inoculation or pre-treatment (24 – 48 hr before) with *L. biglobosa* before inoculation with *L. maculans,* there was a decrease in *L. maculans* leaf lesion size (Liu *et al.,* 2006; 2007). The inoculation of *L. biglobosa* onto the second true leaf was also shown to reduce *L. maculans* lesion size on the same leaf, on neighbouring leaves and on the stem (Mahuku *et al.,* 1996; Liu *et al.,* 2006; 2007). In UK winter oilseed rape crops, pre-treatment with *L. biglobosa* in October resulted in a decrease in phoma leaf spot incidence in autumn and in stem canker severity the following summer (Liu *et al.,* 2006).

Another method of controlling phoma stem canker is use of foliar fungicide sprays. However, these fungicides need to be applied at the correct time to achieve yield response because these fungicides are generally protectant not curative compounds and have only a limited period of action, due to degradation, leaf expansion and the production of new untreated leaves (Aubertot *et al.,* 2006; West *et al.,* 2001). Previous work has showed that *L. biglobosa* is less sensitive to some fungicides than *L. maculans* (Eckert *et al.,* 2010; Huang *et al.,* 2011). In both field experiments and controlled environment experiments, Punch C (containing flusilazole plus MBC, now

withdrawn from the market) decreased the growth of *L. maculans* more than that of *L. biglobosa* (Huang *et al.,* 2011). It is important that fungicides are applied at only the correct dose and at the optimal time to prevent the emergence and development of fungicide insensitivity and increased costs for the grower. For a long-term forecast of the risk of severe stem canker epidemics, monitoring the population distribution and fluctuations in proportions of the two pathogen species is important (West *et al.,* 2001).

Aim: To investigate influence of cultivar resistance (*R*-gene against *L. maculans*/quantitative resistance) on the relative proportions of *L. maculans* and *L. biglobosa* at the phoma leaf spot and stem canker stages of disease development. This was done in three growing seasons through the following objectives:

Objectives:

1. To determine the proportions of *L. maculans*/*L. biglobosa* in phoma leaf spots on winter oilseed rape cultivars with different types of resistance in autumn/winter (2011/2012 and 2012/2013).

2. To determine the proportions of *L. maculans*/*L. biglobosa* in phoma stem cankers on winter oilseed rape cultivars with different types of resistance before harvest in summer (2011, 2012 and 2013).

3. To assess the relationships between amounts of *L. maculans* DNA or *L. biglobosa* DNA and lesion severity in stem base cankers and upper stem lesions in different growing seasons and different cultivars.

4. To examine the seasonal differences in disease progression (phoma leaf spotting and stem cankers) on crops of nine winter oilseed rape cultivars with different types of resistance.

5. To assess the relationships between phoma leaf spot symptoms and phoma stem canker symptoms in crops of nine cultivars with different types of resistance.

6. To analyse the seasonal differences in weather in the 2010/2011, 2011/2012 and 2012/2013 growing seasons.

3.2 Materials and methods

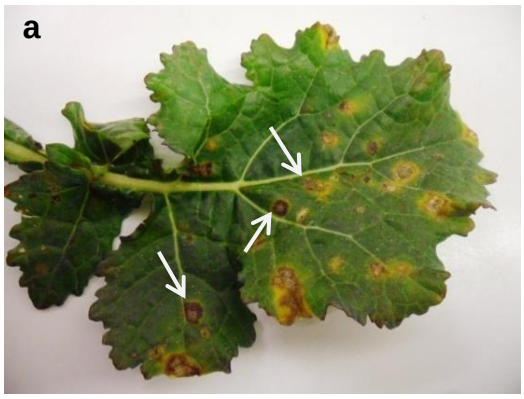
3.2.1 Cultivar and seasonal differences in the proportions of *L. maculans* (Lm) and *L. biglobosa* (Lb) phoma leaf spots

3.2.1.1 Isolation of *L. maculans* and *L. biglobosa* from phoma leaf spots

Samples of leaves with pycnidial production on phoma leaf spots were selected for isolation of the causal pathogen using the method in Chapter 2 (2.6). The isolation procedures are as shown in Fig. 3.2a.

3.2.1.2 Visual identification of *L. maculans* and *L. biglobosa* isolates

Colony morphology and pigmentation were observed on potato dextrose agar (PDA) by sub-culturing from the original stock culture for visual identification of *L. maculans* and *L. biglobosa. L. maculans* colonies usually grew with flat white mycelium, sometimes with brown patches of mycelium and few pycnidia produced. *L. biglobosa* colonies grew and produced more mycelium with a yellowish pigmentation in the

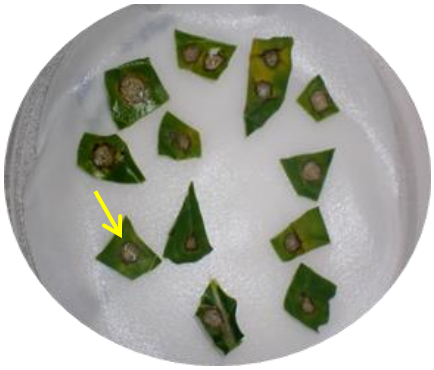


Phoma leaf spot asessment (arrow); sampling in autumn/winter

Phoma stem canker asessment; sampling in summer

10-15 lesions each of Lm or Lb per cultivar were selected

Small pieces of diseased stem cortex tissues were cut from the base and upper part of stems.



Leaf fragment with pycnidia (arrow)

After 5 days growing in PDA medium, the colonies that developed from diseased tissue were subcultured onto V8 medium until pycnidia formed on agar (refer Fig. 2.2 in Chapter 2).

Isolation from single pycnidium to obtain pure culture of *L. maculans* or *L. biglobosa* (refer to section 2.7)

Fig. 3.2: Procedures for isolation of *L. maculans* (Lm) and *L. biglobosa* (Lb) from phoma leaf spots (a) and phoma stem cankers (b).

medium, and sometimes produced many pycnidia in the centre of the colony (refer Fig. 2.2 in Chapter 2).

3.2.1.3 Extraction of genomic DNA of the two *Leptosphaeria* species from freeze-dried mycelium

A cellulose disc was placed on each V8 plate using sterilised forceps and all the isolates were each inoculated onto separate plates. The plates were incubated at 20^{o}C for about 2 weeks until mycelium covered the whole of the V8 medium. Later, all the mycelium of each isolate was harvested and transferred to a 1.5 ml tube for 24 hr of freeze-drying. Freeze-dried mycelium was stored at 4^{o}C in the dark.

The freeze-dried mycelium of *L. maculans* or *L. biglobosa* was used for DNA extraction using a DNAMITE® plant kit (Microzone Limited, UK). Firstly, the freeze-dried mycelium from all samples was ground to powder using a plastic pestle. Only 0.02 g of ground sample was transferred into the 2 ml tube, along with three metal beads (2.38 mm diameter). Then, 1ml of solution LA (cell lysis solution) was added to each tube and samples were vortexed briefly before homogenizing for 40 sec in the fast prep machine with cycles at speed 4.0 M/s (FastPrep®-24, MP Biomedicals, Santa Ana, California, USA). Next, 100 µl of PA solution (protein denaturation solution) was added to each sample and briefly vortexed. The samples were centrifuged at 1100 rpm for 5 min in a microcentrifuge and the clear supernatant (500 µl) produced was transferred to a new 2 ml tube containing 500 µl of CA solution (capture solution). Later, samples were briefly vortexed and left on the bench for 5 min before being spun in a microcentrifuge at 13000 rpm for 7 min to pellet the DNA. The supernatant was decanted, the tubes were briefly re-spun and the dregs were removed with a pipette. This was followed by addition of sterile distilled water (SDW;

usually 50 to 80µl depending on the amount of DNA in the pellet collected). Then, to allow the DNA to rehydrate, tubes with the DNA were left in a flow cabinet for 30 min. After the addition of SDW to all the tubes, the DNA was allowed to rehydrate for 30 min. Then the purity of DNA and the amount of DNA was quantified using a nano-drop spectrophotometer (Thermo Scientific NanoDrop(TM) 1000 UV/VIS Spectrophotometer, Labtech International, Ringmer, UK). A 1.5 – 2 µL aliquot of undiluted extracted genomic DNA was applied to the measurement pedestal of the spectrophotometer and the concentration was automatically calculated from the OD at 260 nm.

3.2.1.4 Confirmation of identification of *Leptosphaeria* species by using species-specific PCR (polymerase chain reaction)

PCR amplifications were done in 20-µl reaction volumes, using *L. maculans*- or *L. biglobosa*-specific primers (Table 3.1). For the detection of either *L. maculans* or *L. biglobosa*, each 20 µl PCR mix comprised 0.3 µl forward primer (10 µm primer), 0.3 µl reverse primer (10 µm primer), 8.4 µl sterile distilled water), 10 µL RED Taq® Ready Mix™ PCR reaction mix (20 mM Tris-HCL, pH 8.3, with 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatine, 0.4 mM dNTP mix, stabilisers, 0.06 unit/µl of *Taq* polymerase; Sigma UK) and 1 µL target DNA template. All the samples were placed in the thermo-cycler (Master cycler gradient-Eppendorf and PCR sprint thermo cycler, Eppendorf UK Limited**)** for the amplification of the genomic DNA. Thermo-cycling parameters for the detection of *L. maculans* or *L. biglobosa* were an initial 95^{o}C for 2 min followed by 30 cycles of further denaturation at 95^{o}C for 30 sec, annealing for 30 sec (at 63^{o}C for *L. maculans* and 61^{o}C for *L. biglobosa*) and elongation at 72^{o}C for 1 min. A final elongation step of 72^{o}C for 10 min was added before cooling to 4^{o}C. For each PCR run, sterile water was used as a negative control.

Table 3.1: Species-specific primers for the identification of *L. maculans* and *L. biglobosa*. Primers were designed by Liu *et al.* (2006).

Primer	*Leptosphaeria* spp.
	L. maculans
Forward	LmacF 5' –CTTGCCCACCAATTGGATCCCCTA-3'
Reverse	LmacR 5' –GCAAAATGTGCTGCGCTCCAGG- 3'
	L. biglobosa
Forward	LbigF 5' –ATCAGGGGATTGGTGTCAGCAGTTGA- 3'
Reverse	LbigR 5' –GCAAAATGTGCTGCGCTCCAGG- 3'

The PCR products (10 µl was loaded into the well) were separated by electrophoresis in 1xTBE buffer (diluted from 10x concentrate) on 1.2% agarose gel stained with ethidium bromide (0.5 µg/ml). Amplicons were viewed under ultraviolet light using a trans-illuminator (Versa Doc™ Imaging System Model 3000, Bio-Rad laboratories, USA) and images were photographed digitally and saved as JPEG files (Fig. 3.3). A PCR product of 331 bp indicated the presence of *L. maculans* (Fig. 3.3a) and a product of 444 bp indicated the presence of *L. biglobosa* (Fig. 3.3b).

3.2.1.5 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. All data collected from the study were analysed statistically by analysis of variance (ANOVA) using SPSS version 21.0. To compare between successful identifications of *L. maculans* or *L. biglobosa* by morphology and identification by species-specific PCR, two-sample binomial tests were done using GenStat (https://www.vsni.co.uk/). The first sample was from results by species-specific PCR and the second sample was from results by morphological identification. The number of *L. maculans* identifications was treated as number of successes while the numbers of *L. maculans* plus *L. biglobosa* identifications was the number of samples.

3.2.2 Cultivar and seasonal differences in the proportions of *Leptosphaeria* species (*L. maculans* (Lm) and *L. biglobosa* (Lb)) in stems

Before harvest, stems were collected and the typical phoma stem base canker lesions and upper stem lessions were cut out from the edges of the stems sampled. Each sample was divided into two pieces where one half was used for isolation of

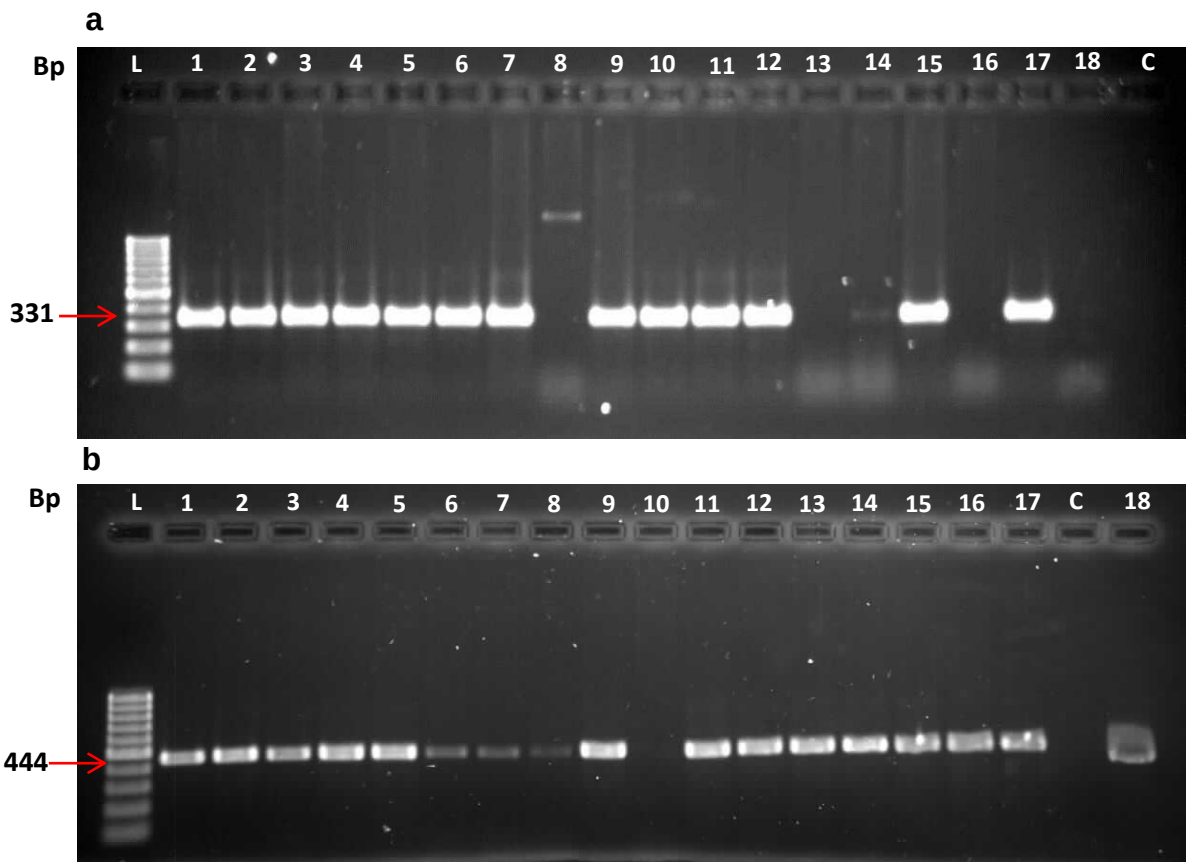


Fig. 3.3: Detection of *L. maculans* and *L. biglobosa* by agarose gel electrophoresis of PCR amplified DNA extracted from mycelia of isolates cultured (phoma leaf spot and stem canker samples), using species-specific primers a) L*mac*F, L*mac*R; L: 100 bp standard marker; C: non-fungal DNA as negative control; Lane 1: *L. maculans* positive control; Lanes 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 15 and 17 show bands for *L. maculans* and b) L*big*F, L*big*R; L: 100 bp standard marker; C: non-fungal DNA as negative control; Lane 18: *L. biglobosa* positive control; Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14,15, 16 and 17 show bands for *L. biglobosa.*

Leptosphaeria species (Fig. 3.2b) and the other half was used for pathogen DNA extraction.

3.2.2.1 Phoma stem canker assessments in spring/summer

The presence of phoma stem canker disease on oilseed rape was determined, based on the observation of stem canker lesions on the upper stem and the basal part of the stem. On 30 June 2011, 13 July 2012 and 26 July 2013, about 2 weeks before harvest, each plot was assessed for phoma stem canker (Fig. 3.4). The assessment was done by pulling up 15 stems selected at random per plot for each cultivar. The assessment was done for stem base cankers and upper stem lesions by using the 0-6 scale; 0 (no symptom), 1 (severity ≤25% girdling of the stem), 2 (severity 26-50% girdling of the stem), 3 (severity 51-75% girdling of the stem), 4 (76-100% girdling of the stem), 5 (100% girdling of the stem and stem weak), 6 (100% girdling of the stem, stem dead or lodged), modified from the 1–6 scale of Lô-Pelzer *et al.* (2009b) (Huang *et al.,* 2011) (Fig. 3.5). Other data recorded were the diameters of ten stems that were randomly selected from 45 stems per cultivar.

3.2.2.2 Extraction of genomic DNA of the two *Leptosphaeria* species from freeze-dried stem samples

All the stem pieces (about 0.3 x 1.0 cm in size) collected from each cultivar from each growing season (June 2011, 13 July 2012 and 26 July 2013) were kept in 1.5 mL tubes at -20^{o}C and then freeze-dried for 48 hours. Later, stem pieces were ground to powder by using a mortar and pestle. The procedure for extraction of fungal DNA from the powder sample was based on that described in section 3.2.1.3.

Fig. 3.4: Phoma stem canker symptoms before harvest on winter oilseed rape cv. Drakkar (susceptible to *L. maculans*), assessed on 30 June 2011 at Rothamsted Research.

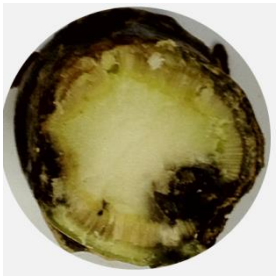
			
0 = no symptoms observable	1 = ≤25% girdling of the stem	2 = 26-50% girdling	3 = 51-75% girdling
			
4 = 76 -100% girdling	5 = 100% girdling and stem weak	6 = stem dead	

Fig. 3.5: Phoma stem canker assessment (stem base cankers and upper stem lesions), scored using a 0-6 scale (Lô-Pelzer *et al.* 2009b; Huang *et al.,* 2011) individually on cross-sections of each of 15 stems per winter oilseed rape plot sampled on 30 June 2011, 13 July 2012 or 26 July 2013 at Rothamsted Research.

3.2.2.3 Isolation of *L. maculans* and *L. biglobosa* from phoma stem cankers

All samples of stems with phoma stem canker were used to obtain isolates using the method described in Chapter 2 (2.6). The isolation procedures were as shown in Fig. 3.2b.

3.2.2.4 Visual identification of *L. maculans* and *L. biglobosa* isolates

The isolates obtained from phoma stem cankers were visually identified, based on the method described in section 3.2.1.2.

3.2.2.5 Extraction of DNA of the two *Leptosphaeria* species from freeze-dried mycelium

The procedure for extraction of fungal DNA from the powdered mycelium sample was based that described in section 3.2.1.3.

3.2.2.6 Confirmation of identification of *Leptosphaeria* species by using species-specific PCR

Please refer to 3.2.1.4 for the procedure.

3.2.2.7 Assessment of proportions of *L. maculans* and *L. biglobosa* in phoma stem cankers by using qPCR

The amounts of DNA extracted from freeze-dried basal stem cankers and upper stem lesions were based on the method in 3.2.2.3. The relative amounts of *L. maculans* and *L. biglobosa* DNA in samples were determined by quantitative PCR (qPCR) using the Sigma SYBR® Green JumpStart™ Taq ReadyMix™ qPCR kit. ROX was adopted as the internal reference dye. Total PCR reaction volume was 20 µL in a 96-well plate (Table 3.2). The default thermocycler programme reaction was 2 min of pre-incubation at 95^{o}C followed by 40 cycles of 95^{o}C (denaturation) for 15 sec; 60^{o}C

Table 3.2: Reaction mixture (volume per sample) for quantitative PCR on a quantitative real-time PCR by Mx3005p (Agilent Technologies).

Item	Volume (in µL)
Forward primer (10 µM)[a]	0.6
Reverse primer (10 µM)[a]	0.6
SIGMA®SYBR Green Mix	10
ROX	0.08
Sd H_2O	6.22
DNA template	2.5 (25 ng/ µL, total 50 ng)
Total volume	20

[a]See table 3.1 for details.

(annealing) for 30 sec; 72^{o}C (elongation) for 45 sec. A dissociation curve was drawn after the reaction (Fig. 3.6). In quantitative PCR, the accumulation of the amplicon is monitored by the emission of fluorescence (at 520 nm), which is detected at the end of the elongation phase for each cycle. At the end of the reaction, the threshold was manually set at the level that reflected the best kinetic PCR parameters (Fig. 3.7). The threshold cycle (Ct) for each sample was automatically determined from this line. Standard curves were generated by plotting the Ct (threshold cycle) values for the dilution against DNA concentration (Fig. 3.7b, c). The resulting regression equations were used to quantify the amount of DNA in "unknown" samples. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng DNA from diseased plant tissue (basal stem cankers or upper stem lesions). To determine the specificity of the procedure, a dissociation (melting) curve was done after the final amplification cycle by heating samples at 95^{o}C for 60 sec; cooling to 60^{o}C for 60 sec and then heating to 95^{o}C for 15 sec; fluorescence was measured continuously. Each DNA sample had two replicates and the final concentration was calculated as the mean of the two replicates.

3.2.2.8 Statistical analysis

Data were saved as Microsoft Excel files. Preliminary analysis, calculation of means etc. was done by Microsoft Excel. Analysis of variance (ANOVA), regression and graphs were done using SPSS version 21.0. In three growing seasons (2010/2011, 2011/2012 and 2012/2013), the distributions of amounts of *L. maculans* and *L. biglobosa* DNA determined by qPCR in basal stem cankers and upper stem lesions were skewed. Therefore, a $\log_{10}$-transformation was applied before analysis for the standardization of distributions. Principal Component Analysis (PCA) is a simple

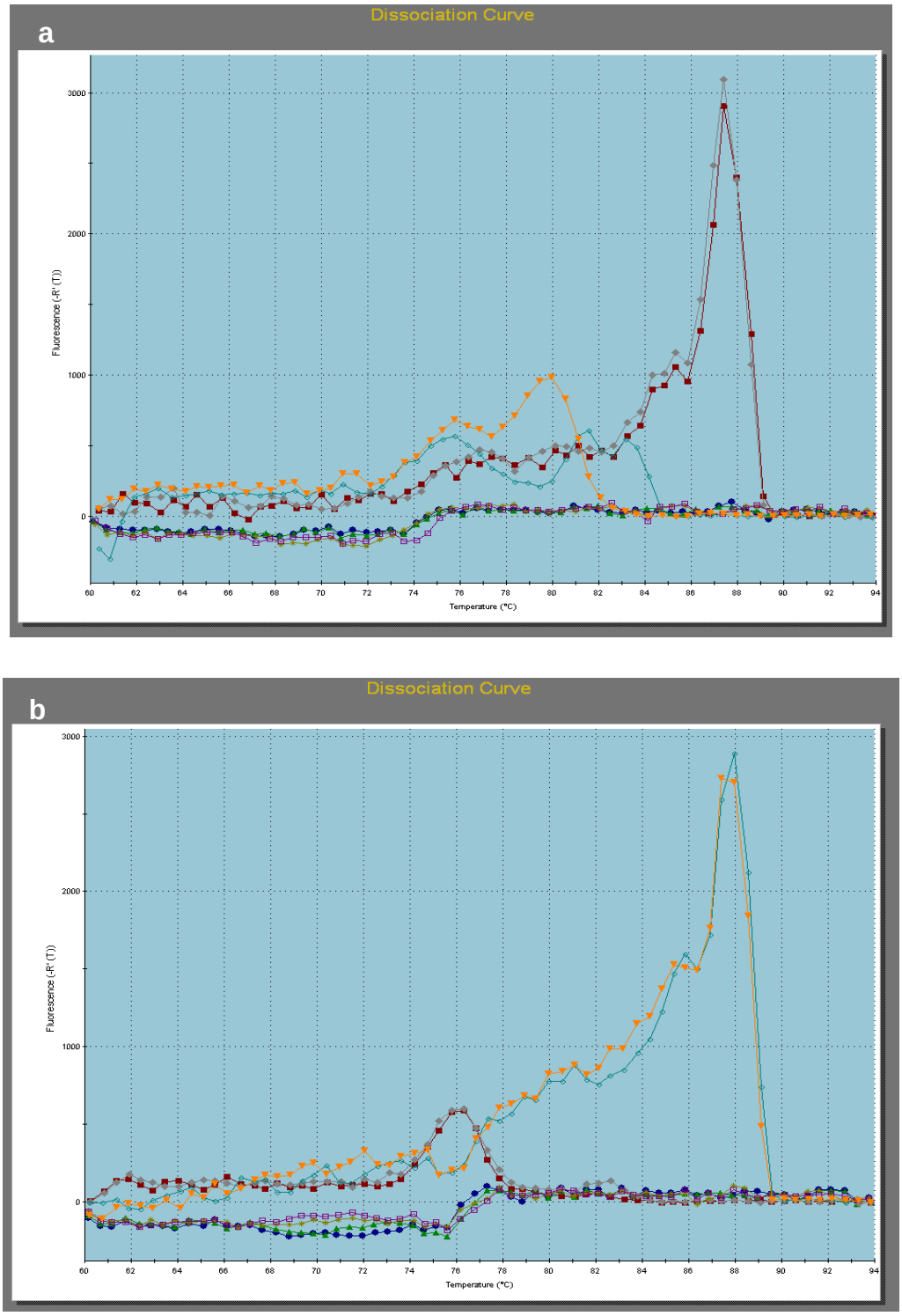


Fig. 3.6: Quantitative PCR dissociation curves for (a) *L. maculans* and (b) *L. biglobosa* detection in the quantitative PCR reaction. The large maximum suggests a single size product at a similar melting temperature and the small maximum indicates primer-dimer artifacts that have a much lower melting temperature because they are short lengths of DNA compared with the target DNA.

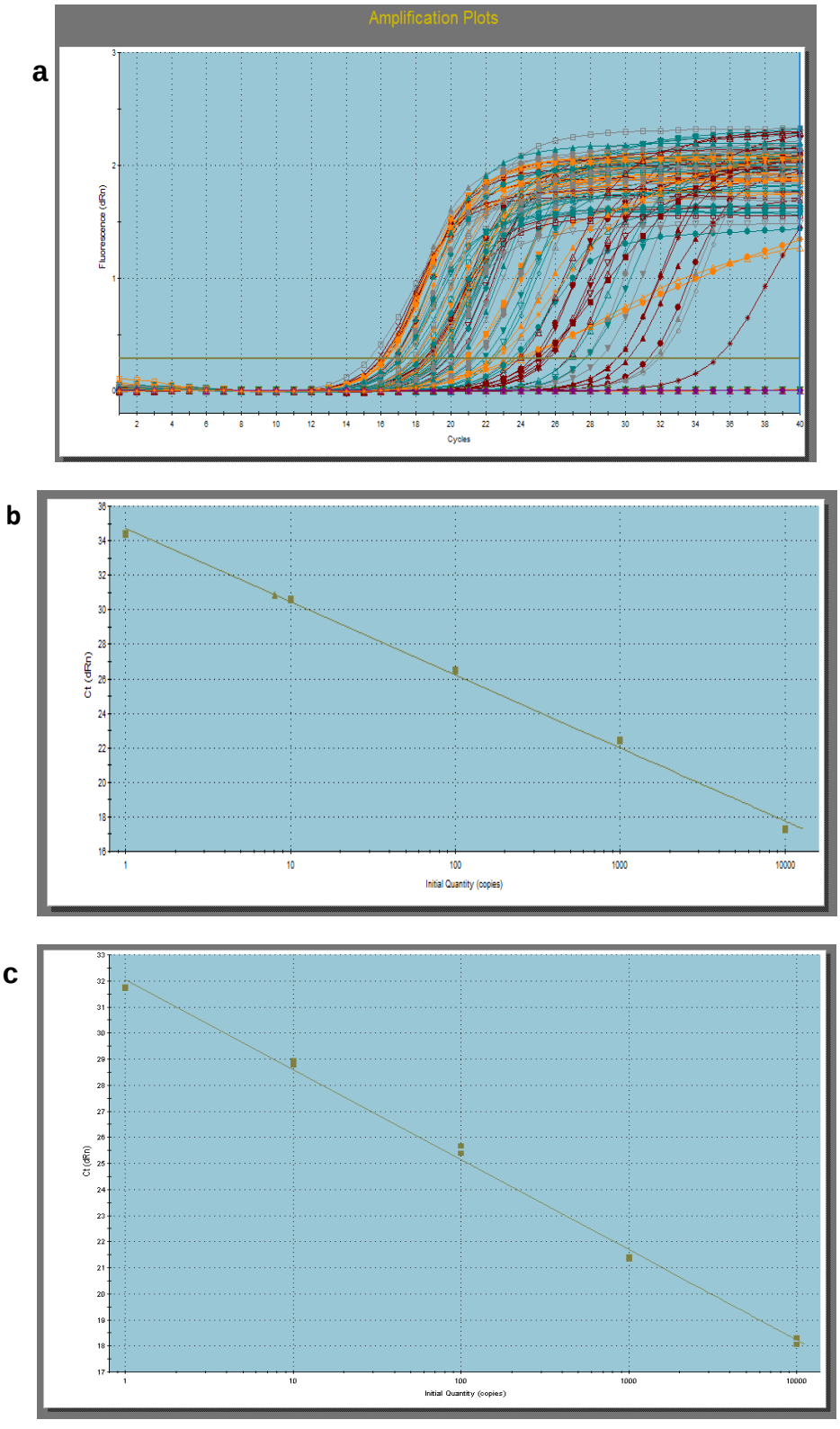


Fig. 3.7: Quantitative PCR (a) Amplification plot; the magnitude of fluorescence signal (Delta *Rn*) was plotted against the PCR cycle number and standard curves of qPCR on DNA samples or (b) *L. maculans* or (c) *L. biglobosa*. The standard curves were drawn by plotting the Ct (threshold cycle) value for the dilution against DNA concentration.

multivariate method. PCA was used to indicate features and clusters in the data. To test the difference between morphological and species-specific PCR identifications of *L. maculans* and *L. biglobosa*, two sample binomial tests were done using GenStat. The first sample was from results by species-specific PCR and the second sample was from results by morphological identification. The number of *L. maculans* identifications was treated as number of successes while the total number of *L. maculans* plus *L. biglobosa* identifications comprised the sample size.

3.2.3 Cultivar and seasonal differences in development of phoma leaf spotting and stem cankers in the 2010/2011, 2011/2012 and 2012/2013 growing seasons

There were nine cultivars of winter oilseed rape planted in the field experiments at Rothamsted Research, Harpenden for the 2010/2011, 2011/2012 and 2012/2013 growing seasons; four with *R*-gene resistance against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia); two with *R*-gene and QR: (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR); and Drakkar (susceptible to *L. maculans*). Details of field experiments are given in section 2.1 and more details of cultivars are given in section 2.2.

3.2.3.1 Phoma leaf spot assessments in autumn/winter

Phoma leaf spotting was observed on winter oilseed rape plots (different cultivars with or without *R* genes against *L. maculans*). Ten plants selected at random were collected from each plot at Rothamsted on 9 December 2010 for the 2010/2011 growing season, 25 January 2012 for the 2011/2012 growing season and on 24 October 2012, 21 November 2012, 11 December 2012 and 30 January 2013 for the 2012/2013 growing season. Data recorded for each cultivar from each plot included

phoma leaf spot incidence (% plants affected), number of leaves, number of leaves with *L. maculans*, number of leaves with *L. biglobosa*, numbers of leaf spots caused by *L. maculans* or *L. biglobosa* (Fig. 3.8), respectively and number of leaf spots with pycnidial production from 30 plants per cultivar (10 plants per plot x three replicates).

The first symptoms, which are the phoma leaf spots that lead to early stem cankers, were identified visually as caused by *L. maculans* or *L. biglobosa*. In general, *L. biglobosa* causes small darker leaf lesions than *L. maculans,* which produces large pale grey lesions with many pycnidia (Fig. 3.8).

3.2.3.2 Phoma stem canker assessment in spring/summer

Please refer to 3.2.2.1 for the procedure.

3.2.3.3 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. Statistical analysis of observations on selected traits was done by means of analysis of variance (ANOVA) and separation of means of treatments such as cultivars was done using the SPSS version 21 statistical packages. The F value (mean square/residual mean square) was examined to indicate the significance or non-significance of a treatment effect. The method of the least significant differences at 5% level (LSD) was used to separate the means of the treatment effects where significance of a treatment effect was indicated by the F-value.

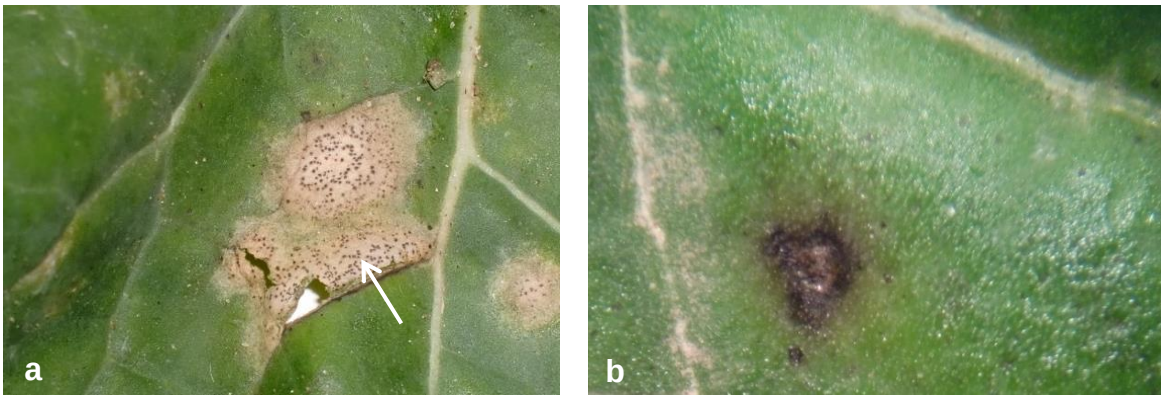


Fig. 3.8: Leaf spotting in autumn with a clear difference in symptoms on leaves between the two *Leptosphaeria* species; (a) large pale lesion (*L. maculans*) with pycnidia (arrow) on cv. Drakkar and (b) small dark lesion (*L. biglobosa*) on cv. NK Grandia, assessed on 25 January 2012 at Rothamsted Research.

3.2.4 Relationships between phoma leaf spotting and stem canker in the 2010/2011, 2011/2012 and 2012/2013 growing seasons

3.2.4.1 Phoma leaf spot assessments in autumn/winter

The data recorded for each cultivar from each plot (10 plants per plot x three replicates); number of leaves with *L. maculans*, number of leaves with *L. biglobosa*, numbers of leaf spots caused by *L. maculans* or *L. biglobosa* from section 3.2.1.2 from three growing seasons were used to analyse relationships between phoma leaf spotting and stem canker. In 2010/2011, only number of leaves with *L. maculans* was assessed.

3.2.4.2 Phoma stem canker assessments in spring/summer

The data for stem canker lesions on the upper stems and basal parts of the stem of 45 stems (15 stems per plot x 3 replicates) were used to analyse relationships but there were no data recorded for the upper stem lesions on 30 June 2011 (2010/2011 growing season).

3.2.4.3 Statistical analysis

Simple linear relationships between variables at the two stages in epidemic development, such as number of leaf spots per plant affected by *L. maculans* or *L. biglobosa* in autumn and phoma stem canker score in spring/summer, were analyzed by means of correlation and regression. The paired data are known as bivariate data and the product moment correlation coefficient (or simply correlation coefficient) (*r*) was calculated for each relationship. The correlation coefficient is a measure of how well the paired data fit a simple line (i.e. a degree of linear association) and it has a numerical value which is between -1 and +1. The greater the correlation coefficient,

either positive or negative, the closer the relationship. However, to assess how much one variable changes for a given change in another variable, a simple linear regression line was estimated. In this case, the square of correlation coefficient (r^2) can be an approximate measure for the proportion of variance of one variable that can be accounted for by its linear regression against another variable.

For testing agreement (i.e. concordance) between cultivar resistance to *L. maculans* at the phoma leaf spotting stage in autumn and at the phoma stem canker stage in spring/summer, the Spearman's rank correlation coefficient (r_S) was calculated. It is the ordinary correlation coefficient (r) between the ranked values of two variables. The value of the Spearman's rank correlation coefficient ranges from a minimum of -1 (complete discordance) to +1 (complete concordance).

When measurements of different traits were done on the same cultivar, these measurements for nine different cultivars formed a multivariate data set. An example was the observations on numbers of leaves per plant with either *L. maculans* or *L. biglobosa*, numbers of phoma leaf spots per plant with either *L. maculans* or *L. biglobosa*, basal phoma stem canker and upper lesion severity scores on each of nine cultivars. When measurements are correlated between traits, multivariate analyses can be used to gain some insights into the relative importance of these different traits. Principal Component Analysis (PCA) is a simple multivariate method.

The fundamental principle of PCA is that the cultivars, which are usually correlated with one another, are converted into an equal number of uncorrelated components. The ultimate objective is then to have fewer principal components (usually two or three) that account for most of the variation (i.e. they contain most of the information) present in the data. Each of the uncorrelated principal components is made up of a

linear combination of the variates. The contribution of each variate to the information in each of the principal components is measured by the loading of the variate. Unlike the situation in univariate statistical methods where a precise statement can be made at a specified level of probability, in the situation of multivariate analysis by PCA it is not possible to make such definite statements. PCA is used to indicate features and clusters in the data.

A number of graphical methods were used to display the results. They include tables, scatter plots, bar charts and column charts.

3.2.5. Rothamsted weather data

Daily meteorological data (temperature and rainfall) at Rothamsted from 0900 h GMT to 0900 h GMT on the following day were obtained from the electronic Rothamsted archive. Temperature was recorded by a 107 thermistor probe (Campbell Scientific, Loughborough, UK) and rainfall was measured using a 0.2 ARG100 tipping bucket rain gauge (Campbell Scientific, Loughborough, UK).

3.3 Results

3.3.1 Cultivar and seasonal differences in the proportions of *L. maculans* (Lm) and *L. biglobosa* (Lb) phoma leaf spots

There were significant differences (P<0.05) between cultivars in mean number of *L. maculans* leaf spots on leaves of 30 plants (10 plants per plot x 3 replicates) in the 2010/2011 growing season (Fig. 3.9). On 25 January 2012 (2011/2012 growing season), there was more leaf spotting caused by *L. maculans* and there were

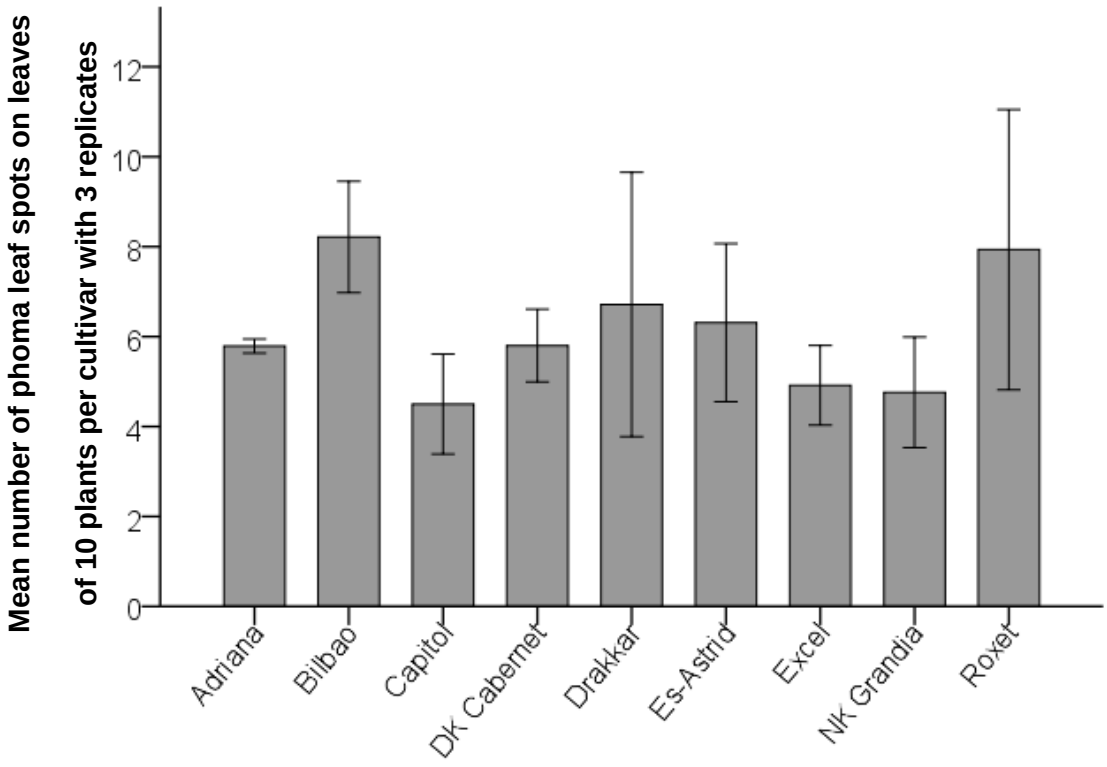


Fig. 3.9: Mean numbers of *L. maculans* (Lm) phoma leaf spots on leaves from 10 plants per cultivar (from each of 3 replicates plots, i.e. 30 plants) of winter oilseed rape (with *R*-genes against *L. maculans* or no *R*-genes or with quantitative resistance (QR)) sampled from an experiment at Rothamsted on 9 December 2010. Average numbers of Lm phoma leaf spots on leaves was determined from 30 plants (Appendix 3.1). Vertical error bars represent standard error.

significant differences (P<0.05) between the nine cultivars in mean number of *L. maculans* phoma leaf spots on leaves of 30 plants (Fig. 3.10). Cultivar Drakkar had the greatest number of leaf spots caused by *L. maculans* with a ratio of Lm:Lb of 6:1 from 30 plants (10 plants per plot x three replicates) (Table 3.3). There was no significant difference (P>0.05) between cultivars in mean number of *L. biglobosa* leaf spots on leaves of 30 plants per cultivar (Fig. 3.10). From all the nine cultivars, only Excel (*Rlm7*) and Roxet (*Rlm7*) had more leaf spots caused by *L. biglobosa* than by *L. maculans* (Fig. 3.10) with a ratio Lm:Lb of 1:2 from 30 plants (10 plants per plot x three replicates) assessed in the 2011/2012 growing season (Table 3.3).

The mean numbers of *L. maculans* and *L. biglobosa* leaf spots on leaves of 30 plants (10 plants per plot x three replicates) were small in October 2012 (Fig. 3.11a) but the number of *L. maculans* leaf spots started to increase from November 2012 to January 2013 (2012/2013 growing season) (Fig. 3.11b-d). The greatest mean numbers of leaf spots caused by *L. maculans* and *L. biglobosa* were observed on 11 December 2012 (Fig. 3.11c). There was no significant difference (P>0.05) between cultivars in mean numbers of *L. maculans* leaf spots on leaves from October to December 2012 and in January 2013 (Fig. 3.11a-d). However, there were significant differences (P<0.05) between cultivars in mean numbers of *L. biglobosa* leaf spots on leaves of 30 plants (10 plants per plot x three replicates) when plants were sampled in October and November 2012, with cv. Roxet (*Rlm*7) having the greatest number of *L. biglobosa* spots in October 2012 (Fig. 3.11a) and Excel (*Rlm*7) having the greatest number in November 2012 (Fig. 3.11b). In December 2012, all cultivars were observed to have more *L. biglobosa* leaf spots, except for cv. Drakkar (Fig. 3.11c, Table 3.3). All cultivars had fewer *L. biglobosa* leaf spots on leaves of 30 plants per cultivar on 30 January 2013 (Fig. 3.11d).

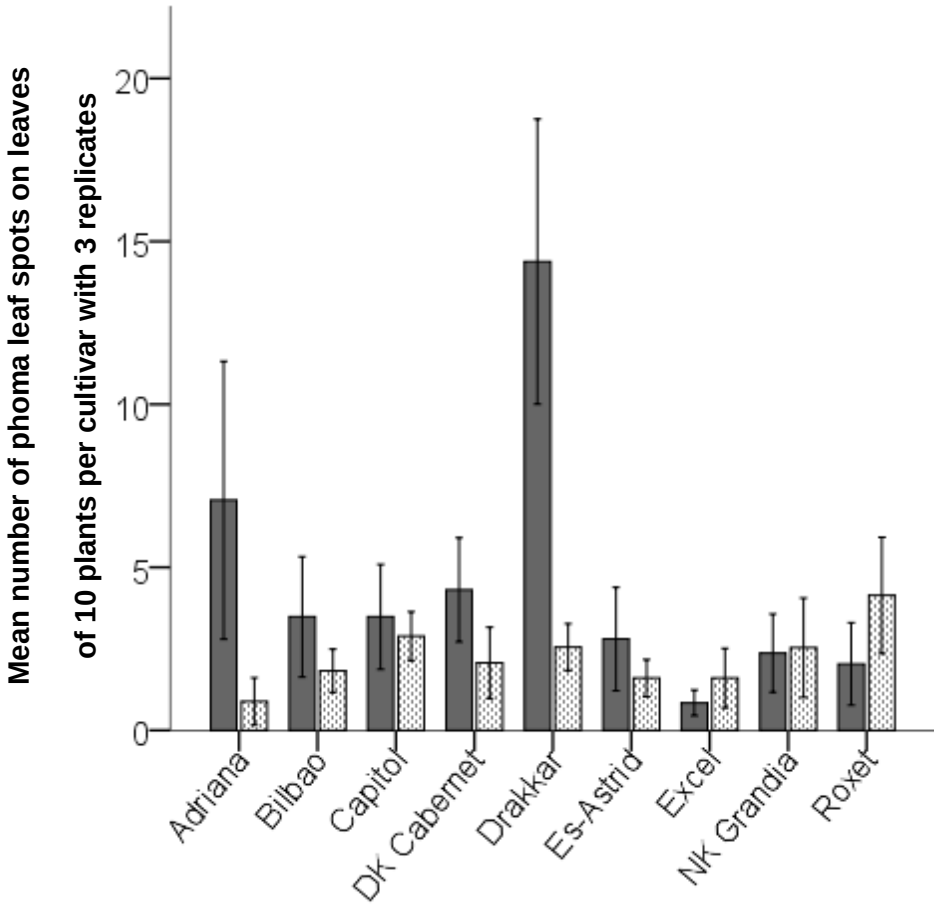


Fig. 3.10: Mean numbers of *L. maculans* (Lm) and *L. biglobosa* (Lb) phoma leaf spots on leaves from 30 plants (10 plants per plot x three replicates) of winter oilseed rape (with *R*-genes or no *R*-genes against *L. maculans* or with quantitative resistance (QR)) sampled from an experiment at Rothamsted on 25 January 2012. Average numbers of Lm and Lb phoma leaf spots were determined from 30 plants (Appendix 3.2). Vertical error bars represent standard error.

Table 3.3: Ratios of number of phoma leaf spots caused by *Leptosphaeria maculans* to number caused by *L. biglobosa* on leaves of 30 plants per cultivar of winter oilseed rape sampled in the 2011/2012 growing season (25 January 2012) and 2012/2013 growing season (21 November 2012, 11 December 2012 and 30 January 2013) and 15 plants per cultivar of winter oilseed rape sampled on 24 October 2012.

Cultivar	Total numbers of phoma leaf spots caused by *L. maculans* (Lm) or *L. biglobosa* (Lb) (ratio Lm:Lb)				
	2011/2012 growing season	2012/2013 growing season			
Sowing	30 Aug 2011	5 Sept 2012			
Sampling date	25 Jan 2012	*24 Oct 2012	21 Nov 2012	11 Dec 2012	+30 Jan 2013
Adriana (*Rlm 4* + QR)	146:31 (5:1)	8:28 (1:3)	93:74 (1:1)	224:427 (1:2)	175:27 (6:1)
Bilbao (*Rlm 4*)	102:54 (2:1)	18:13 (1:1)	111:34 (3:1)	182:411 (1:2)	241:67 (4:1)
Capitol (*Rlm 1*)	99:84 (1:1)	6:6 (1:1)	122:40 (3:1)	212:305 (1:1)	221:44 (5:1)
DK Cabernet (*Rlm 1*+ QR)	127:60 (2:1)	14:12 (1:1)	129:58 (2:1)	221:319 (1:1)	207:40 (5:1)
Drakkar (no R gene)	433:76 (6:1)	11:1 (11:1)	200:58 (3:1)	309:181 (2:1)	263:23 (11:1)
Es-Astrid (QR)	84:48 (2:1)	11:5 (2:1)	130:124 (1:1)	263:413 (1:2)	236:33 (7:1)
Excel (*Rlm 7*)	24:45 (1:2)	20:6 (3:1)	130:269 (1:2)	171:535 (1:3)	186:32 (6:1)
NK Grandia (QR)	75:71 (1:1)	7:6 (1:1)	142:95 (2:1)	172:329 (1:2)	214:39 (5:1)
Roxet (*Rlm 7*)	67:120 (1:2)	17:48 (1:2)	219:367 (1:2)	247:515 (1:2)	219:55 (4:1)

* Only 15 plants per cultivar of winter oilseed rape sampled on 24 October 2012 because most plants had few leaves with phoma leaf spots.

+ Number of spots can decrease because affected leaves senesce and abscise from plants before the next assessment.

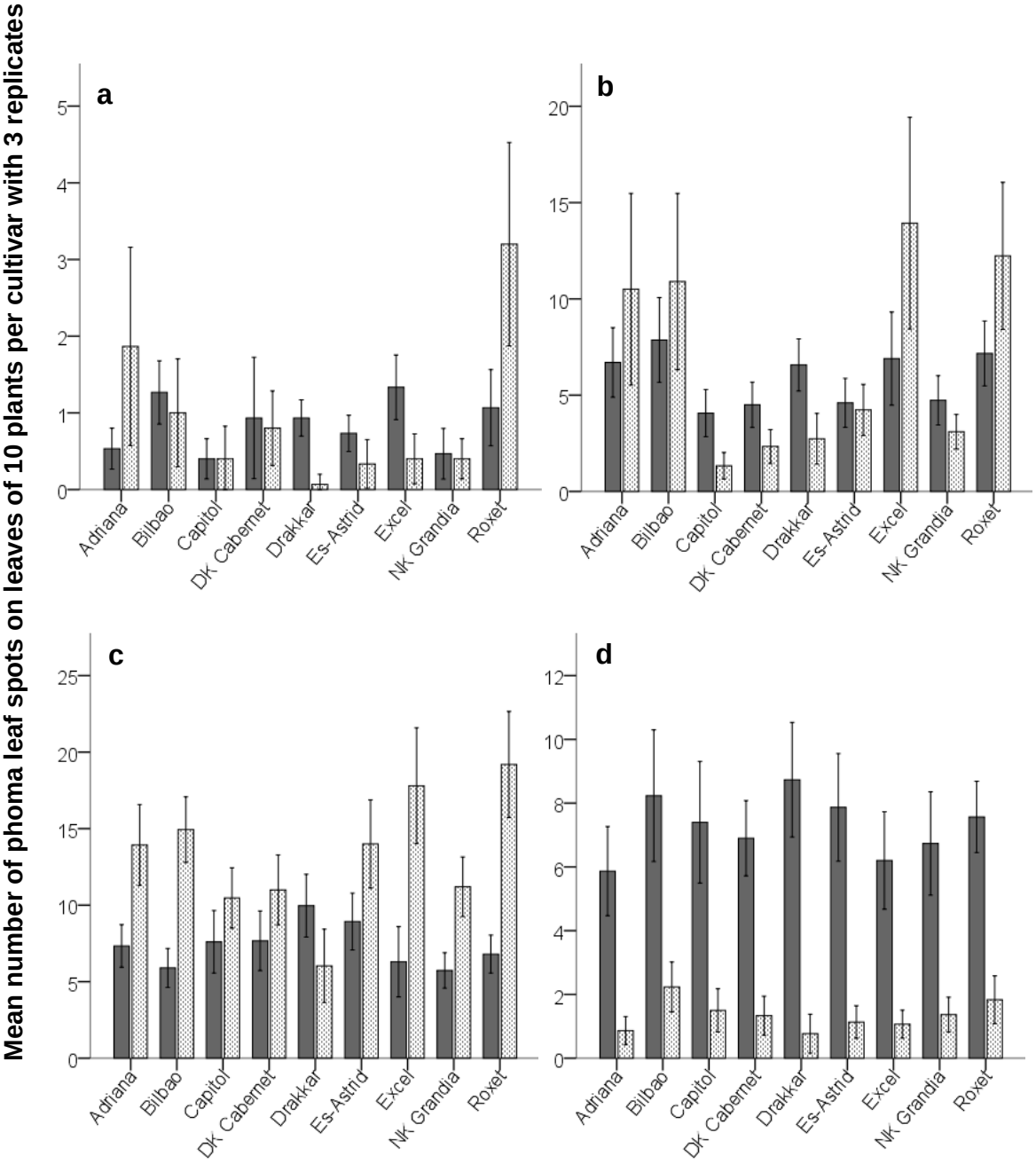


Fig. 3.11: Mean numbers of *L. maculans* (Lm) and *L. biglobosa* (Lb) phoma leaf spots on leaves from 30 plants (10 plants per plot x three replicates) of winter oilseed rape with (*R*-genes against *L. maculans* or no *R*-genes or with quantitative resistance (QR)) sampled from an experiment at Rothamsted on (a) 24 October 2012, (b) 21 November 2012, (c) 11 December 2012 and (d) 30 January 2013. Average numbers of Lm and Lb phoma leaf spots were determined from 30 plants (Appendix 3.3). Vertical error bars represent standard errors of means (8 df).

The cultivar Drakkar had a greater number of *L. maculans* leaf spots than *L. biglobosa* leaf spots in the 2012/2013 growing season (Fig. 3.11a-d), with the ratios of Lm:Lb 11:1, 3:1, 2:1 and 11:1 in October 2012, November 2012, December 2012 and January 2013, respectively (Table 3.3). There was a significant difference ($P<0.05$) between cv. Roxet (*Rlm7*) and other cultivars in mean number of *L. biglobosa* leaf spots on leaves of 30 plants in 24 October 2012 (Fig. 3.11a and Appendix 3.3). All samples from the 2011/2012 and 2012/2013 growing seasons had fewer *L. biglobosa* leaf spots than *L. maculans* leaf spots except on 11 Dec 2012 when there were more *L. biglobosa* leaf spots assessed, with the ratio of Lm:Lb generally 1:2 except for cv. Excel (*Rlm7*, ratio 1:3), cvs Capitol (*Rlm1*) and DK Cabernet (*Rlm1* + QR, ratio 1:1) and Drakkar (ratio 2:1) (Table 3.3). There was no significant difference ($P>0.05$) between cultivars in mean number of *L. biglobosa* leaf spots on leaves of 30 plants in 11 December 2012 except for cv. Drakkar and Roxet (*Rlm7*) ($P<0.05$) (Fig. 3.11c and Appendix 3.3). The cultivars Excel (*Rlm7*) and Roxet (*Rlm7*) had more *L. biglobosa* leaf spots on their leaves than *L. maculans* leaf spots, with a ratio Lm:Lb of 1:2 for both cultivars assessed on 25 January 2012 and 21 November 2012 whereas they had Lm:Lb ratios of 1:3 and 1:2, respectively, on 11 December 2012 (Table 3.3). For samples taken over the two growing seasons, cv. Roxet (*Rlm7*) generally had more *L. biglobosa* leaf spots on the leaves than *L. maculans* leaf spots, except on 30 January 2013 (Table 3.3).

There were six cultivars with an equal number of *L. maculans* and *L. biglobosa* leaf spots (ratio Lm:Lb, 1:1); Adriana (observed on 21 November 2012), Bilbao (observed on 24 October 2012), Capitol (observed on 25 January 2012, 24 October 2012 and 11 December 2012), DK Cabernet (24 October 2012 and 11 December 2012), Es-Astrid (observed on 21 November 2012) and NK Grandia (observed on 25 January

2012 and 24 October 2012) (Table 3.3), whereas cv. Drakkar always had more *L. maculans* spots than *L. biglobosa* spots in all three growing seasons (Fig. 3.10, 3.11, Table 3.3). On 30 January 2013, all cultivars assessed had more *L. maculans* leaf spots than *L. biglobosa* leaf spots on leaves of 30 plants (10 plants per plot x three replicates) (Table 3.3) (Fig. 3.11d). Pycnidial production was observed on the *L. maculans* leaf spots in all three growing seasons and there were significant differences (P<0.05) in pycnidial production between the three growing seasons (Fig. 3.12). In December 2010 (2010/2011) (Fig. 3.12a) and January 2012 (2011/2012) (Fig. 3.12b), there was a significant difference between cultivars (P<0.05) in pycnidial production. However, in the 2012/2013 growing season, there were no significant differences (P>0.05) between cultivars in December 2012, November 2012 and January 2013, except in November 2012 between cvs Adriana and Roxet (P<0.05) (Fig. 3.12c).

A total of 82 isolates were obtained from 175 phoma leaf spots sampled on 25 January 2012, with 54.9% of isolates being *L. maculans* and 45.1% of isolates being *L. biglobosa,* when they were identified morphologically (Table 3.4). In the 2012/2013 growing season (21 November and 11 December 2012), a total of 142 isolates of *L. maculans* or *L. biglobosa* were obtained from all cultivars. Morphological identification suggested that 57.7% of isolates were *L. maculans* and 42.3% were *L. biglobosa* (Table 3.4). Species-specific PCR results suggested that, on 25 January 2012, 37.5% of isolates were *L. maculans* and 62.5% were *L. biglobosa* whereas in the 2012/2013 growing season, 58.5% were *L. maculans* and 41.5% were *L. biglobosa*. A total of 10 isolates obtained from 25 January 2012 samples and 19 isolates from 21 November and 11 December 2012 samples were highly contaminated with bacteria and were discarded. There was no significant difference

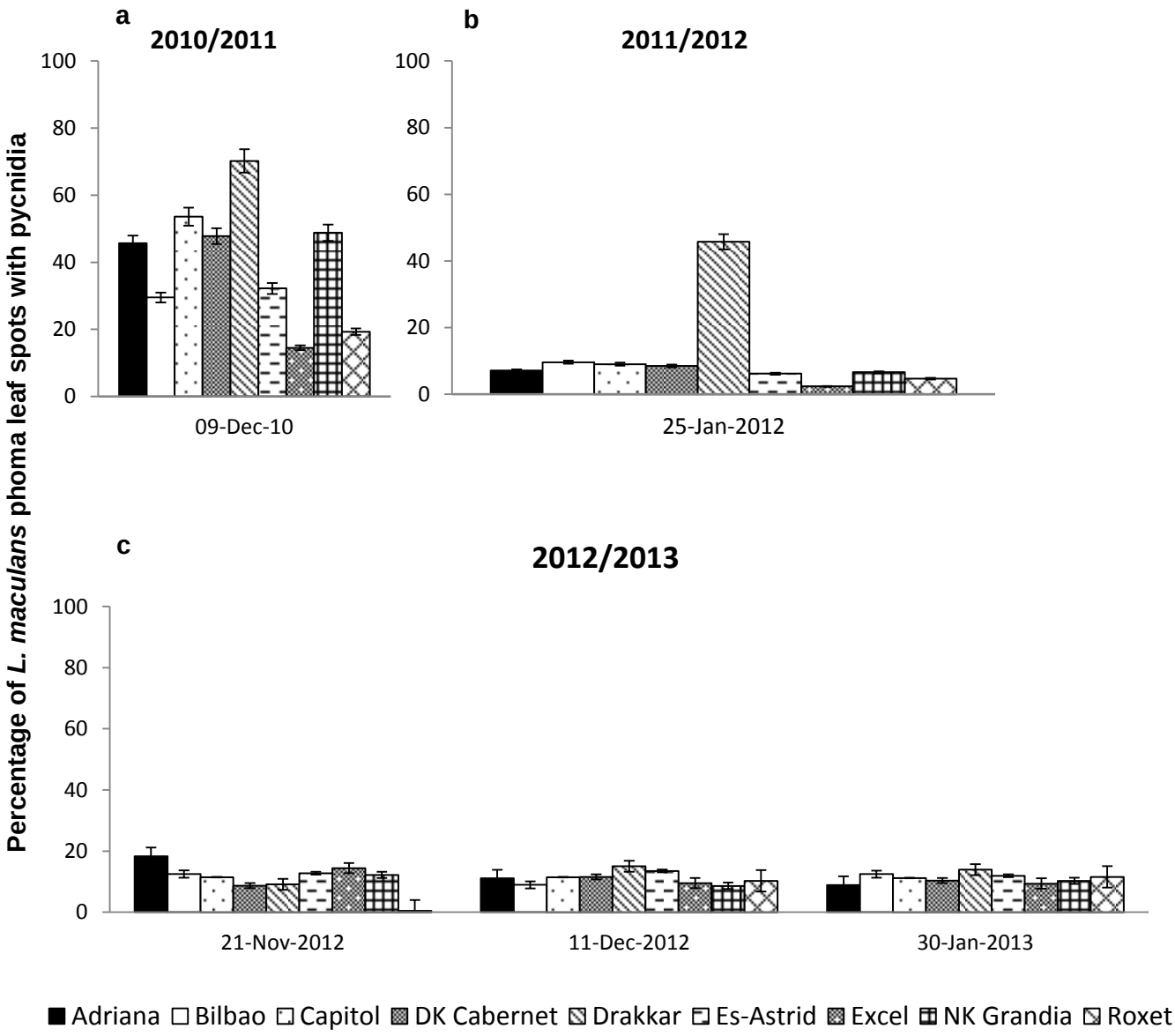


Fig. 3.12: Percentage of *L. maculans* (Lm) phoma leaf spots with pycnidia from 30 plants per cultivar (10 plants per plot x three replicates) of nine winter oilseed rape cultivars (with *R*-genes or no *R*-genes against *L. maculans* or with quantitative resistance (QR)) sampled from an experiment at Rothamsted in the three growing seasons, observed on 9 December 2010 (a, 2010/2011), on 25 January 2012 (b, 2011/2012) and on 21 November 2012, 11 December 2012 and 30 January 2013 (c, 2012/2013).

Table 3.4: Morphological identification and species-specific PCR confirmation of *Leptosphaeria maculans* (Lm) and *L. biglobosa* (Lb) isolated from phoma leaf spot samples from nine cultivars, assessed in the 2011/2012 and 2012/2013 growing seasons. There were no isolates from samples collected in the 2010/2011 growing season.

Cultivar	Morphological identification	Species-specific PCR	*P -values
2011/2012 growing season (assessed on 25 January 2012)			
Adriana (*Rlm*4+QR)	0 Lm, 6 Lb	1 Lm, 4 Lb	0.25
Bilbao (*Rlm*4)	4 Lm, 4 Lb	4 Lm, 4 Lb	1.00
Capitol (*Rlm*1)	7 Lm, 4 Lb	3 Lm, 7 Lb	0.12
DK Cabernet (*Rlm*1+QR)	6 Lm, 5 Lb	5 Lm, 6 Lb	0.67
Drakkar (No *R* gene)	10 Lm, 1 Lb	6 Lm, 3 Lb	0.18
Es-Astrid (QR)	6 Lm, 4 Lb	1 Lm, 6 Lb	0.33
Excel (*Rlm*7)	4 Lm, 4 Lb	0 Lm,8 Lb	0.02
NK Grandia (QR)	8 Lm, 3 Lb	7 Lm, 2 Lb	0.79
Roxet (*Rlm*7)	0 Lm, 6 Lb	0 Lm, 5 Lb	1.00
Total	45 Lm, 37 Lb	27 Lm, 45 Lb	
Percentage (%)	54.9 Lm, 45.1 Lb	37.5 Lm, 62.5 Lb	
2012/2013 growing season (assessed on 21 November and 11 December 2012)			
Adriana (*Rlm*4+QR)	1 Lm, 1 Lb	0 Lm, 2 Lb	0.25
Bilbao (*Rlm*4)	1 Lm, 1 Lb	1 Lm, 1 Lb	1.00
Capitol (*Rlm*1)	17 Lm, 16 Lb	18 Lm, 7 Lb	0.11
DK Cabernet (*Rlm*1+QR)	4 Lm, 19 Lb	6 Lm, 14 Lb	0.33
Drakkar (No *R* gene)	8 Lm, 0 Lb	6 Lm, 0 Lb	1.00
Es-Astrid (QR)	27 Lm, 10 Lb	23 Lm, 9 Lb	0.92
Excel (*Rlm*7)	0 Lm, 5 Lb	1 Lm, 2 Lb	0.17
NK Grandia (QR)	22 Lm, 8 Lb	16 Lm, 15 Lb	0.08
Roxet (*Rlm*7)	2 Lm, 0 Lb	1 Lm, 1 Lb	0.23
Total	82 Lm, 60 Lb	72 Lm, 51 Lb	
Percentage (%)	57.7 Lm, 42.3 Lb	58.5 Lm, 41.5 Lb	

*Two-sample, binomial test, *P*= significance level

(*P*>0.05) between morphological identification and species-specific PCR identification of both *L. maculans* and *L. biglobosa* for most isolates except isolates from cv. Excel (assessed on 25 January 2012) (Table 3.4).

3.3.2 Cultivar and seasonal differences in the proportions of *Leptosphaeria* species (*L. maculans* (Lm) and *L. biglobosa* (Lb)) in stems

In the 2010/2011 growing season, 64 isolates were obtained from the basal part of the stems and 36 isolates from the upper part of the stems of all nine cultivars assessed on 30 June 2011 (Table 3.5). On the basis of morphological identification, 54 *L. maculans* and 10 *L. biglobosa* isolates were obtained from basal stem cankers, whereas 26 *L. maculans* and 10 *L. biglobosa* isolates were obtained from upper lesions. The species-specific PCR confirmed that both basal stem cankers and upper stem lesions had a greater proportion of *L. maculans* isolates, 90.6% and 91.7%, respectively, than *L. biglobosa* isolates (Table 3.5). There were no isolates from cv. Adriana. The stem sample from the 2011/2012 growing season (assessed on 13 July 2012) produced 45 isolates from basal stem cankers and upper stem lesions, with 69% of isolates classified as *L. maculans* and 30.7% of isolates as *L. biglobosa* by morphological identification (Table 3.6). The species-specific PCR confirmed that the basal stem canker isolates were predominantly *L. maculans* (84.6%) rather than *L. biglobosa* (15.4%), whereas for the upper stem lesions, 6.3% were identified as *L. maculans* and 93.7% as *L. biglobosa* (Table 3.6).

In the 2012/2013 growing season, stem canker was assessed on 26 July 2013 and 83 isolates were obtained from the basal stem cankers and upper stem lesions (Table 3.7). According to morphological identification, the basal stem cankers

Table 3.5: Identification of *Leptosphaeria* isolates by morphological identification or species-specific PCR as *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) for isolates from phoma stem canker samples of nine cultivars collected on 30 June 2011. The *P*-values show if differences between morphological identification results and species-specific PCR were significant.

	Basal part			Upper part		
Cultivar	**Morphological identification**	**Species-specific PCR**	***P* -value**	**Morphological identification**	**Species-specific PCR**	***P* -value**
Adriana (*Rlm*4+QR)	2 Lm, 1 Lb	3 Lm	0.27	0	0	1.00
Bilbao (*Rlm*4)	7 Lm, 0 Lb	6 Lm, 1 Lb	0.29	5 Lm,1 Lb	5 Lm, 1 Lb	1.00
Capitol (*Rlm*1)	11 Lm, 0 Lb	10 Lm, 1 Lb	0.31	3 Lm, 2Lb	5Lm, 0 Lb	0.11
DK Cabernet (*Rlm*1+QR)	5 Lm, 0 Lb	5 Lm, 0 Lb	1.00	7 Lm, 1 Lb	7 Lm, 1 Lb	1.00
Drakkar (No *R* gene)	10 Lm, 2 Lb	11 Lm, 1 Lb	0.54	6 Lm, 2 Lb	7 Lm, 1 Lb	0.52
Es-Astrid (QR)	5 Lm, 3 Lb	7 Lm, 1 Lb	0.25	0 Lm, 2 Lb	2 Lm, 0 Lb	0.05
Excel (*Rlm*7)	1 Lm, 2 Lb	3 Lm, 0 Lb	0.08	3 Lm, 0 Lb	3 Lm, 0 Lb	1.00
NK Grandia (QR)	6 Lm, 1 Lb	6 Lm, 1 Lb	1.00	1 Lm, 2 Lb	3 Lm, 0 Lb	0.08
Roxet (*Rlm*7)	7 Lm, 1 Lb	7 Lm, 1 Lb	1.00	1 Lm, 0 Lb	1 Lm, 0 Lb	1.00
Total	54 Lm, 10 Lb	58 Lm, 6 Lb (6.25%)[#]		26 Lm, 10 Lb	33 Lm, 3 Lb (1.94%)[#]	
Percentage (%)	84.4 Lm,15.6 Lb	90.6 Lm, 9.4 Lb		72.2 Lm, 27.8 Lb	91.7 Lm, 8.3 Lb	

*Two-sample, binomial test, *P*= significance level

[#]Percentage (%) of wrongly classified by morphological identification

Table 3.6: Identification of *Leptosphaeria* isolates by morphological identification or species-specific PCR as *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) for isolates from phoma stem canker samples of nine cultivars collected on 13 July 2012. The *P*-values show if differences between morphological identification results and species-specific PCR were significant.

	Basal part			Upper part		
Cultivar	**Morphological identification**	**Species-specific PCR**	***P* -value**	**Morphological identification**	**Species-specific PCR**	***P* -value**
Adriana (*Rlm*4+QR)	1 Lm,0 Lb	1 Lm, 0 Lb	1.00	6 Lm, 5 Lb	1 Lm, 10 Lb	0.02
Bilbao (*Rlm*4)	1 Lm, 0 Lb	1 Lm, 0 Lb	1.00	0 Lm, 1 Lb	0 Lm, 1 Lb	1.00
Capitol (*Rlm*1)	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00	0 Lm, 1 Lb	0 Lm, 1 Lb	1.00
DK Cabernet (*Rlm*1+QR)	2 Lm, 1 Lb	2 Lm, 1 Lb	1.00	1 Lm, 0 Lb	0 Lm, 1 Lb	0.16
Drakkar (No *R* gene)	1 Lm, 1 Lb	2 Lm, 0 Lb	0.23	2 Lm, 0 Lm	0 Lm, 2 Lb	0.05
Es-Astrid (QR)	4 Lm, 2 Lb	5 Lm, 1 Lb	0.51	10 Lm, 2 Lb	1 Lm, 11 Lb	0.01
Excel (*Rlm*7)	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00
NK Grandia (QR)	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00
Roxet (*Rlm*7)	0 Lm, 0 Lb	0 Lm, 0 Lb	1.00	2 Lm, 1 Lb	0 Lm, 3 Lb	0.08
Total	9 Lm, 4 Lb	11 Lm, 2 Lb (15.4%)[#]		21 Lm, 11 Lb	2 Lm, 30 Lb (59.4%)[#]	
Percentage (%)	69.2 Lm, 30.8 Lb	84.6 Lm, 15.4 Lb		65.6 Lm, 34.4 Lb	6.3 Lm, 93.7 Lb	

*Two-sample, binomial test, *P*= significance level

[#]Percentage (%) of wrongly classified by morphological identification

Table 3.7: Identification of *Leptosphaeria* isolates by morphological identification or species-specific PCR as *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) for isolates from phoma stem canker samples of nine cultivars collected on 26 July 2013. The *P*-values show if differences between morphological identification results and species-specific PCR were significant.

	Basal part			Upper part		
Cultivar	**Morphological identification**	**Species-specific PCR**	***P* -value**	**Morphological identification**	**Species-specific PCR**	***P* -value**
Adriana (*Rlm*4+QR)	6 Lm, 2 Lb	6 Lm,2 Lb	1.00	1 Lm, 2 Lb	0 Lm, 3 Lb	0.27
Bilbao (*Rlm*4)	3 Lm, 2 Lb	3 Lm, 2 Lb	1.00	4 Lm, 0 Lb	2 Lm, 2 Lb	0.10
Capitol (*Rlm*1)	4 Lm, 1 Lb	4 Lm, 1 Lb	1.00	1 Lm, 2 Lb	1 Lm, 2 Lb	1.00
DK Cabernet (*Rlm*1+QR)	5 Lm, 1 Lb	4 Lm, 2 Lb	0.51	6 Lm, 2 Lb	5 Lm, 3 Lb	0.59
Drakkar (No *R* gene)	6 Lm, 1 Lb	5 Lm, 2 Lb	0.52	0 Lm, 4 Lb	0 Lm, 4 Lb	1.00
Es-Astrid (QR)	3 Lm, 0 Lb	1 Lm, 2 Lb	0.08	0 Lm, 3 Lb	0 Lm, 3 Lb	1.00
Excel (*Rlm*7)	4 Lm, 3 Lb	5 Lm, 2 Lb	0.58	0 Lm, 3 Lb	2 Lm, 1 Lb	0.08
NK Grandia (QR)	4 Lm, 1 Lb	2 Lm, 3 Lb	0.19	0 Lm, 3 Lb	0 Lm, 3 Lb	1.00
Roxet (*Rlm*7)	0 Lm, 3 Lb	0 Lm, 3 Lb	1.00	1 Lm, 2 Lb	0 Lm, 3 Lb	0.27
Total	35 Lm, 14 Lb	30 Lm, 19 Lb (10.2%)[#]		13 Lm, 21 Lb	10 Lm, 24 Lb (8.8%)[#]	
Percentage (%)	71.4 Lm, 28.6 Lb	61.2 Lm, 38.8 Lb		38.2 Lm, 61.8 Lb	29.4 Lm, 70.6 Lb	

*Two-sample, binomial test, *P*= significance level

[#]Percentage (%) of wrongly classified by morphological identification

produced more *L. maculans* isolates than *L. biglobosa* isolates, whereas the upper stem lesions produced more *L. biglobosa* than *L. maculans* isolates (Table 3.7). The species-specific PCR confirmed that 61.2% of isolates from the basal stem cankers were *L. maculans* and 38.8% were *L. biglobosa*, whereas for isolates from the upper stem lesions 29.4% were *L. maculans* and 70.6% were *L. biglobosa* (Table 3.7). There were no significant differences ($P>0.05$) between the morphological identification and species-specific PCR identification of *L. maculans* and *L. biglobosa* for all isolates (Table 3.5, 3.6 and 3.7, two sample, binomial test). However, there were isolates that had been wrongly identified by visual colony characteristics, with 6.25% in 2011, 15.4% in 2012 and 10.2% in 2013 isolated from basal stem cankers whereas 19.4%, 59.4% and 8.8% were isolated from the upper stem lesions sampled in 2011, 2012 and 2013, respectively (Table 3.5, 3.6 and 3.7).

The amounts of *L. maculans* and *L. biglobosa* DNA extracted were determined by quantitative PCR (Appendix 3.7). The mean amounts of *L. maculans* DNA and *L. biglobosa* DNA in basal stem cankers and upper stem lesions from nine different cultivars are shown in Fig. 3.13 and Fig. 3.14, respectively. There were significant differences ($P<0.05$) between cultivars in amounts of *L. maculans* DNA in basal stem cankers (Fig. 3.13a-b) and in upper stem lesions (Fig. 3.14a-b) in all three growing seasons, but there was no significant difference between cultivars ($P>0.05$) in *L. maculans* DNA in the upper stem lesions in the 26 July 2013 sample (Fig. 3.14c).

The greatest proportions of *L. maculans* DNA detected in the basal stem cankers were in NK Grandia (QR) sampled in June 2011 (3.89 ng *L maculans* DNA out of 50 ng of DNA extract) (Fig. 3.13a), Drakkar sampled in July 2012 (6.56 ng *L. maculans*

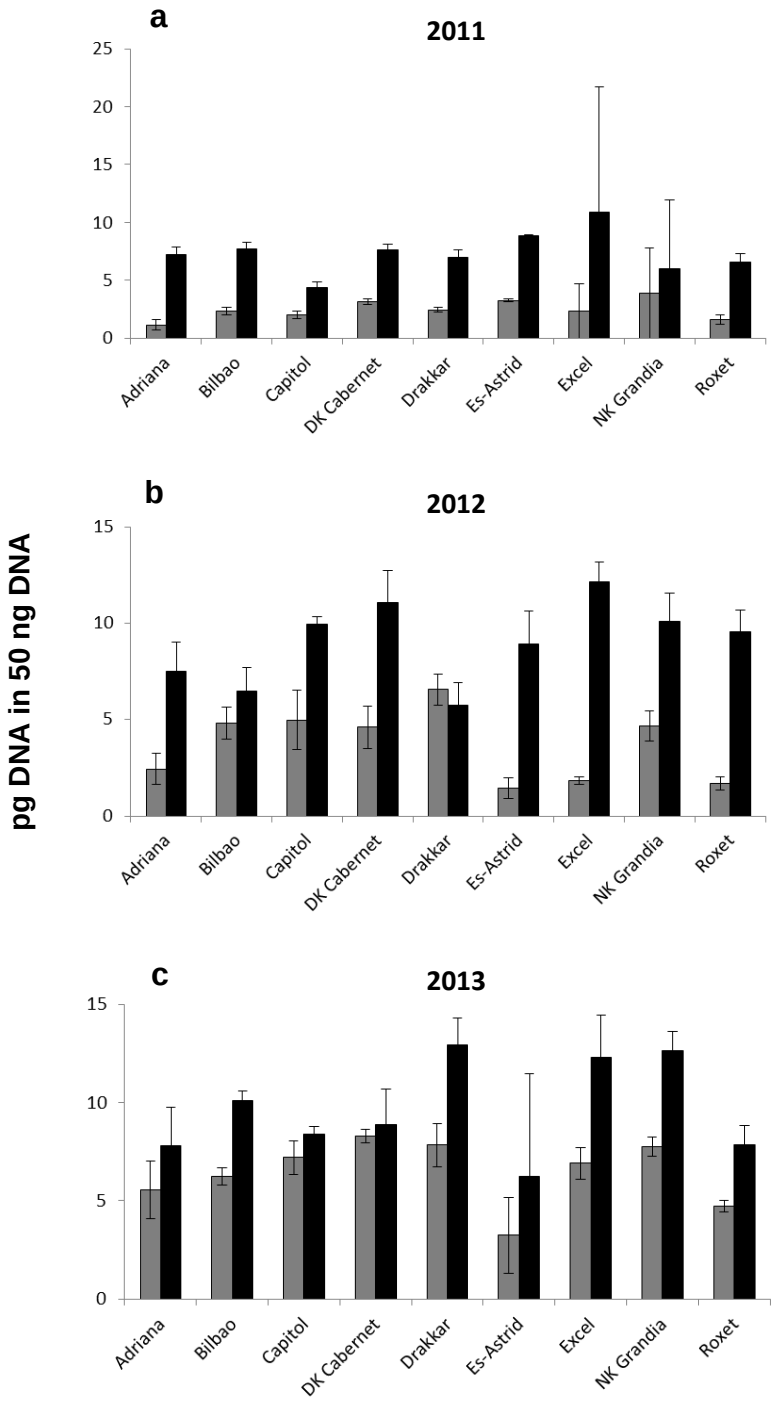


Fig. 3.13: Amounts of *L. maculans* or *L. biglobosa* DNA in basal stem cankers obtained from nine oilseed rape cultivars (with *R*-genes against *L. maculans* or no *R*-genes or quantitative resistance (QR)) from three growing seasons (a) 30 June 2011, (b) 13 July 2012 and (c) 26 July 2013. The amounts of DNA were determined by qPCR. The error bars show the standard errors of means; cvs Adriana in 2011(df 5), 2012 (df 10), 2013 (df 3); Bilbao in 2011 (df 10), 2012 (df 9), 2013 (df 2); Capitol in 2011 (df 11), 2012 (df 3), 2013 (df 5); DK Cabernet in 2011 (df 7), 2012 (df 8), 2013 (df 4); Drakkar in 2011 (df 13), 2012 (df 8), 2013 (df 3); Es-Astrid in 2011 (df 1), 2012 (df 4), 2013 (df 1); Excel in 2011 (df 0), 2012 (df 14), 2013 (df 4); NK Grandia in 2011 (df 0), 2012 (df 11), 2013 (df 1) and Roxet in 2011 (df 7), 2012 (df 8), 2013 (df 5).

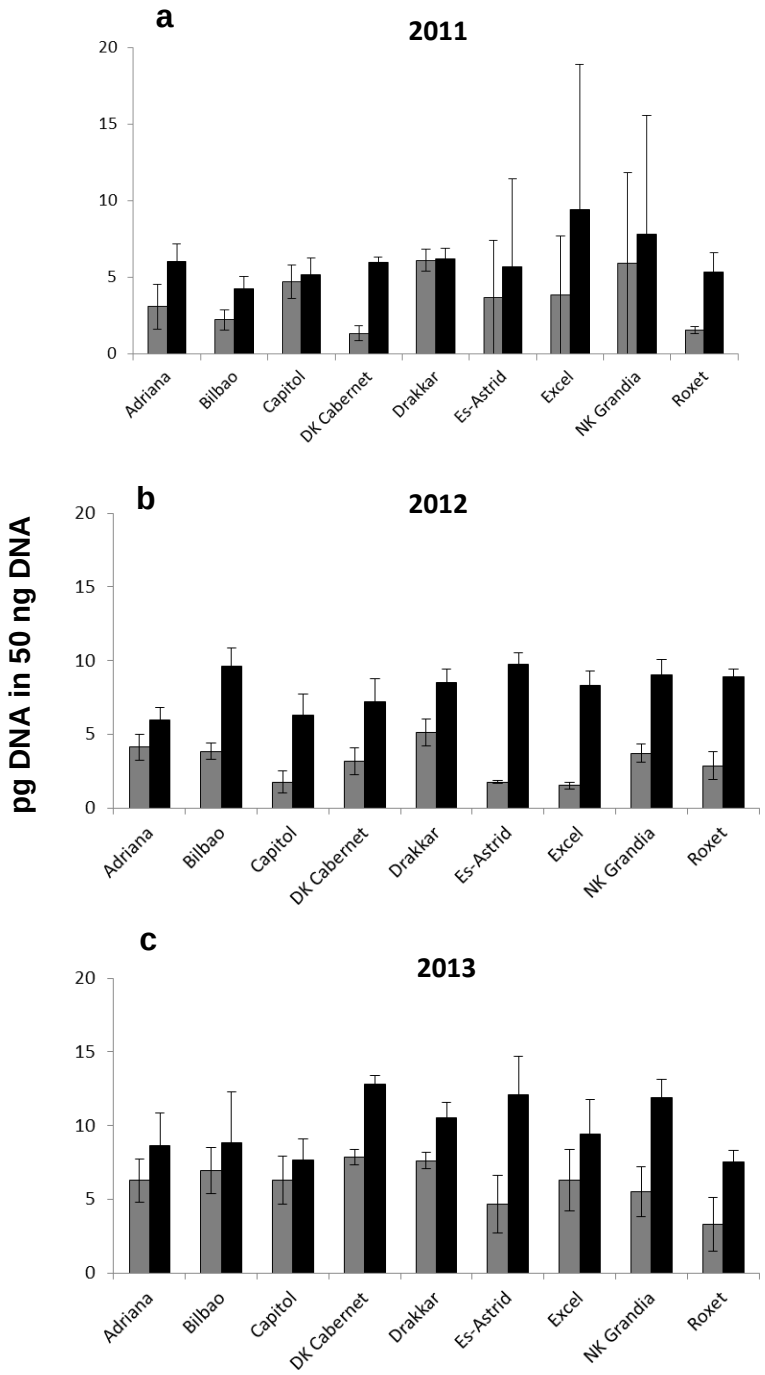


Fig. 3.14: Amounts of *L. maculans* or *L. biglobosa* DNA in upper stem lesions obtain from nine oilseed rape cultivars (with *R*-genes against *L. maculans* or no *R*-genes or quantitative resistance (QR)) from three growing seasons (a) 30 June 2011, (b) 13 July 2012 and (c) 26 July 2013. The amounts of DNA were determined by qPCR. The error bars show the standard errors of means; cvs Adriana in 2011(df 5), 2012 (df 10), 2013 (df 3); Bilbao in 2011 (df 10), 2012 (df 9), 2013 (df 2); Capitol in 2011 (df 11), 2012 (df 3), 2013 (df 5); DK Cabernet in 2011 (df 7), 2012 (df 8), 2013 (df 4); Drakkar in 2011 (df 13), 2012 (df 8), 2013 (df 3); Es-Astrid in 2011 (df 1), 2012 (df 4), 2013 (df 1); Excel in 2011 (df 0), 2012 (df 14), 2013 (df 4); NK Grandia in 2011 (df 0), 2012 (df 11), 2013 (df 1) and Roxet in 2011 (df 7), 2012 (df 8), 2013 (df 5).

DNA out of 50 ng of DNA extract) (Fig. 3.13b) and DK Cabernet (*Rlm1* + QR) sampled in July 2013 (8.30 ng *L. maculans* DNA out of 50 ng of DNA extract) (Fig. 3.13c). For the upper stem lesions, the greatest proportion of *L. maculans* DNA was in cv. Drakkar sampled in June 2011 and in July 2012 (6.11 ng and 5.12 ng *L. maculans* DNA, respectively). In 2013, cv. DK Cabernet had the greatest amount of *L. maculans* DNA (7.84 ng out of 50 ng of DNA extract) (Fig. 3.14a-c).

In all cultivars in all three growing seasons, there was a greater amount of *L. biglobosa* DNA in the basal stem canker than in upper stem lesion samples. For all three growing seasons, the maximum proportion of *L. biglobosa* DNA detected in the basal stem cankers was in cv. Excel (*Rlm7*); 10.87 ng *L. biglobosa* DNA in 2011, 12.15 ng in 2012 and was in cv. Drakkar in 2013 (12.91 ng out of 50 ng of DNA extract (Fig. 3.13a-c). In the upper stem lesions, the greatest proportions of *L. biglobosa* DNA were in Excel (9.44 ng *L. biglobosa* DNA out of 50 ng of DNA extract) in 2011, Es-Astrid (9.77 ng *L. biglobosa* DNA) in 2012 and DK Cabernet (12.83 ng *L. biglobosa* DNA) in 2013 (Fig. 3.14a-c).

3.3.3 Relationships between amounts of *L. maculans* DNA and *L. biglobosa* DNA and lesion severity in stem base cankers and upper stem lesions in different growing seasons and different cultivars

Amounts of *L. biglobosa* DNA differed between growing seasons for cv. NK Grandia (QR); in the 2010/2011 growing season the amount was small in the basal stem cankers but more *L. biglobosa* DNA was detected in basal stem samples collected on 13 July 2012 and 26 July 2013 (Fig. 3.15a-c). The amount of *L. biglobosa* DNA in basal stem cankers was small for cultivar Drakkar in the 2011/2012 growing season

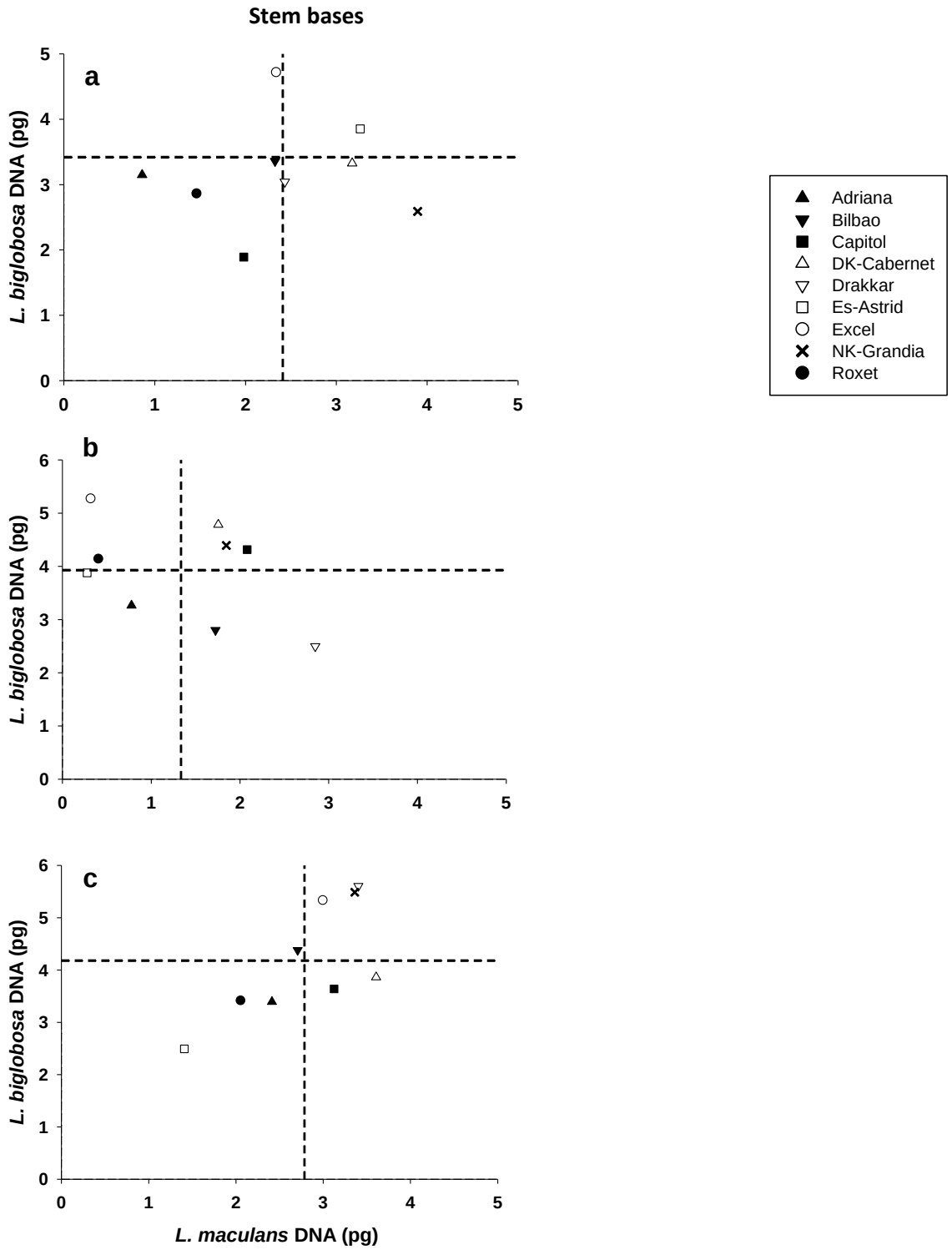


Fig. 3.15: The relationships between the amount of *L. maculans* DNA and that of *L. biglobosa* DNA among nine oilseed rape cultivars. The amount of *L. maculans* DNA and that of *L. biglobosa* DNA were log-transformed (Log_{10}). The x-axis shows the amount of *L. maculans* DNA and the y-axis shows the amount of *L. biglobosa* DNA in the stem base cankers sampled on (a) 30 June 2011, (b) 13 July 2012 and (c) 26 July 2013. The amounts of DNA were determined by qPCR from nine oilseed rape cultivars with *R* genes against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel, (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia), two with *R*-gene and QR (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR)) and cv. Drakkar (susceptible to *L. maculans*).

samples (Fig. 3.15b) but more *L. biglobosa* DNA was detected in the 2012/2013 growing season (26 July 2013 samples) (Fig. 3.15c).

These results shows an increasing amount of *L. biglobosa* DNA in stem samples (stem base canker and upper stem lesions) for the three growing seasons (2010/2011, 2011/2012 and 2012/2013). The amount of *L. biglobosa* DNA in stem samples (stem base canker and upper stem lesions) was greater than the amount of *L. maculans* DNA for samples obtained in 2010/2011 and 2011/2012 growing seasons (Fig. 3.15a-b, 3.16a-b). However, greater amounts of both *L. maculans* DNA and *L. biglobosa* DNA were detected in stem samples (stem base canker and upper stem lesions) for samples obtained in 2012/2013 growing season for all cultivars except Es-Astrid (QR) in the basal stem cankers and Roxet (*Rlm7*) in the upper stem lesions (Fig. 3.15c, 3.16c).

There were no significant relationships ($P>0.05$) between amount of *L. maculans* DNA and amount of *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) from basal stem canker in 2010/2011 and 2011/2012 growing seasons (Appendix 3.8 and 3.9) and for all three growing seasons from upper stem lesions (Appendix 3.8 and 3.10), except in the 2012/2013 growing season between the amount of *L. maculans* DNA and amount of *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) from basal stem cankers ($P<0.05$) (Appendix 3.9c).

In July 2012 (2011/2012), there was a significant relationship ($P<0.05$) between basal stem canker severity and amount of *L. maculans* DNA (pg DNA out of 50 ng of DNA), where 55% of the variance in basal stem canker severity was explained by the change in amount of *L. maculans* DNA (Appendix 3.8 and 3.5b). There were no

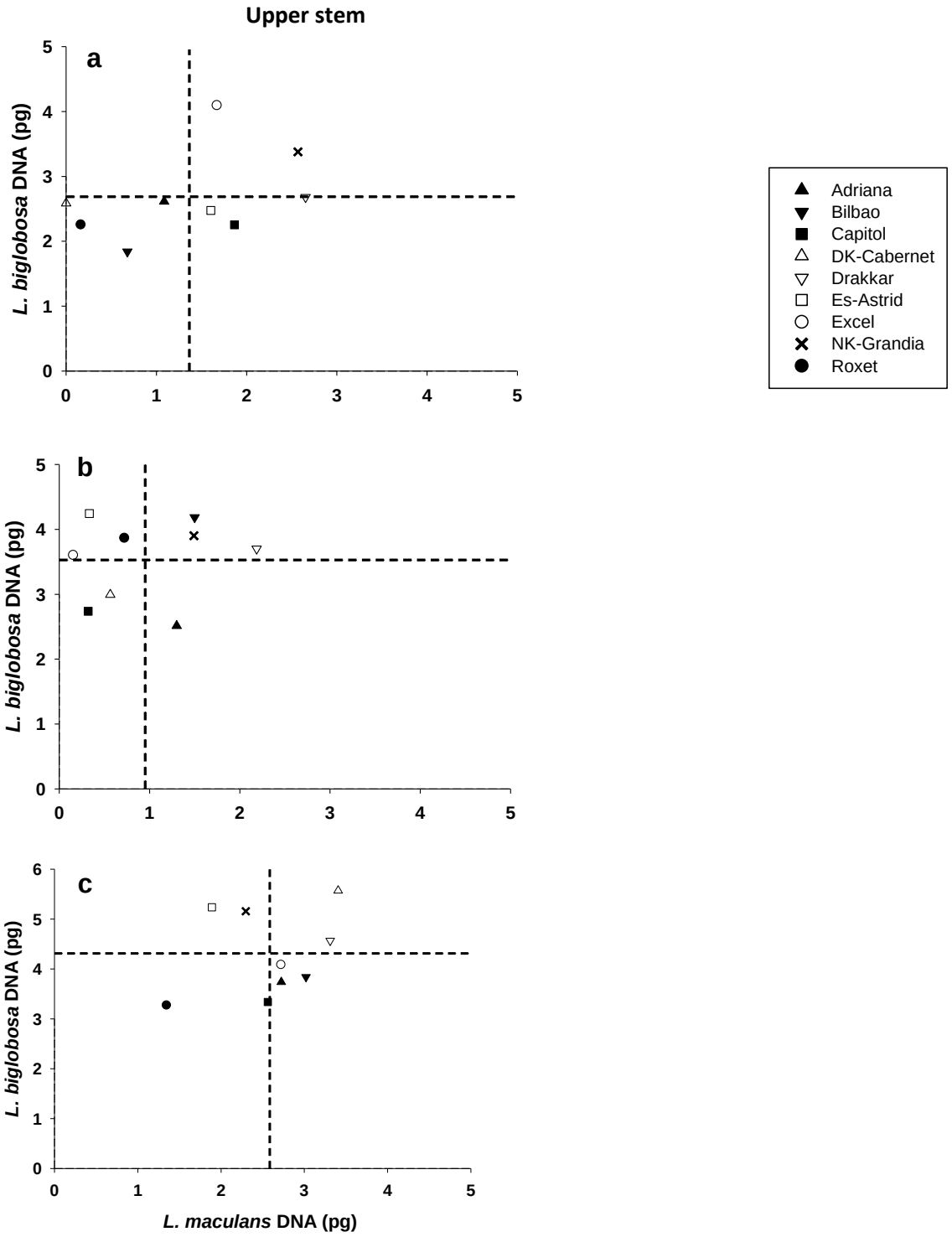


Fig. 3.16: The relationships between the amount of *L. maculans* DNA and that of *L. biglobosa* DNA among nine oilseed rape cultivars. The amount of *L. maculans* DNA and that of *L. biglobosa* DNA were log-transformed (Log_{10}). The x-axis shows the amount of *L. maculans* DNA and the y-axis shows the amount of *L. biglobosa* DNA in the upper stem lesions sampled on (a) 30 June 2011, (b) 13 July 2012 and (c) 26 July 2013. The amounts of DNA were determined by qPCR from nine oilseed rape cultivars with *R* gene against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel, (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia), two with *R*-gene and QR (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR)) and cv. Drakkar (susceptible to *L. maculans*).

significant relationships (*P*>0.05) between basal stem canker severity and amount of *L. maculans* DNA (Appendix 3.11a, b), amount of *L. biglobosa* DNA (Appendix 3.12) or combined amounts of *L. maculans* and *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) (Appendix 3.7) for all three growing seasons (Appendix 3.8). There was a significant relationship (*P*<0.05) between upper stem lesion severity and the combined amounts of *L. maculans* and *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) where 46% of the variance in upper stem lesion severity was explained by the change in combination of amounts of *L. maculans* and *L. biglobosa* DNA in 2011/2012 (Appendix 3.14 and 3.14a) but there were no significant relationships (*P*>0.05) in 2012/2013 (Appendix 3.14 and 3.14b). There were no significant relationships (*P*>0.05) between upper stem lesion severity and amount of *L. maculans* DNA (Appendix 3.15) or amount of *L. biglobosa* DNA (Appendix 3.16) (pg DNA out of 50 ng of DNA) for 2011/2012 and 2012/2013 growing seasons, respectively (Appendix 3.14). There were no upper stem lesions samples collected for extraction of DNA of *Leptosphaeria* species in the 2010/2011 growing season.

3.3.4 Cultivar and seasonal differences in numbers of leaves with *Leptosphaeria maculans* or *L. biglobosa* phoma leaf spots

Over the three growing seasons, there were significant differences (*P*<0.05) between cultivars in incidence of phoma leaf spotting (Fig. 3.17). The phoma leaf spotting incidence was less on 25 January 2012 (Fig. 3.17) because of late ascospore release in that season (refer to chapter 4) that was due to the dry weather in autumn 2011. The greatest incidence of phoma leaf spotting was on 9 December 2010 (Fig. 3.17), with more than 70% plants affected for all cultivars except DK Cabernet (*Rlm1* + QR) and Excel (*Rlm*7). For cv. Roxet (*Rlm*7), the greatest percentage of plants

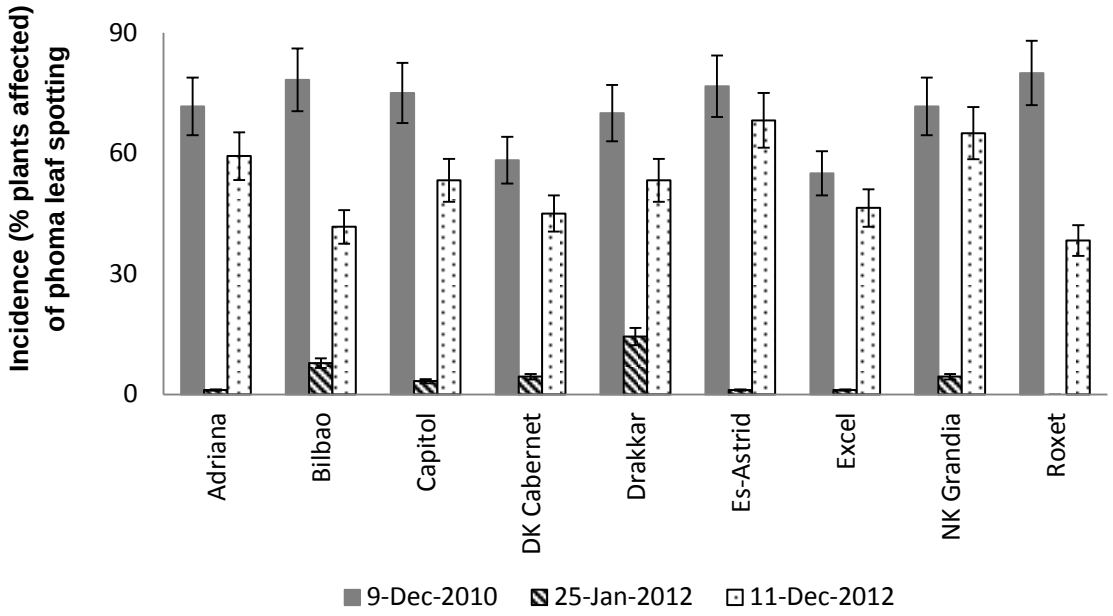


Fig. 3.17: Incidence of phoma leaf spotting (% plants affected) caused by either *L. maculans* or *L. biglobosa* on nine cultivars for three growing seasons observed on 9 December 2010, 25 January 2012 and 11 December 2012.

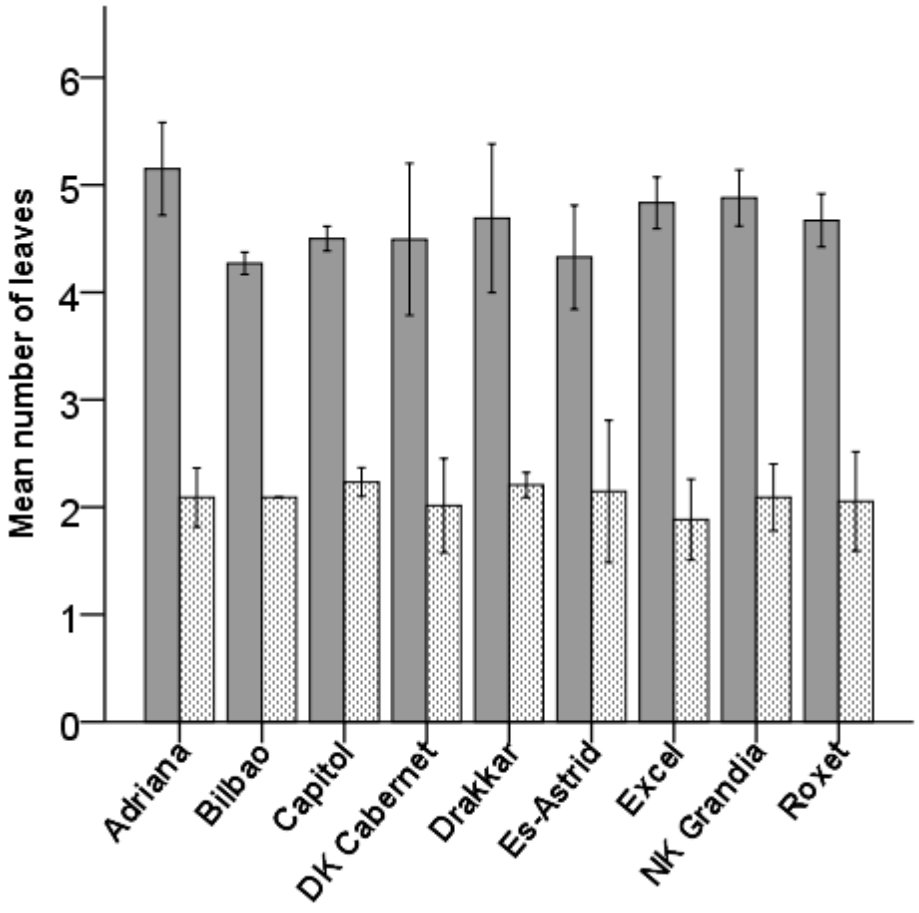


Fig. 3.18: Mean number of leaves per plant and mean number of leaves per plant with *L. maculans* spots . Data are means from 30 plants per cultivar (10 plants per plot x three replicates) of winter oilseed rape sampled in the 2010/2011 growing season (9 December 2010) at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)). Average number of leaves per plant and average number of leaves per plant with Lm spots were determined from 30 plants (Appendix 3.1). Vertical error bars represent standard error.

affected was on 9 December 2010 and incidence was smallest on 25 January 2012 (dry season) and 11 December 2012 (wet season) (Fig. 3.17).

In the 2010/2011 growing season, phoma leaf spotting was first observed on 9 December 2010 (Fig. 3.18) in the Rothamsted field experiment on leaves of all nine cultivars with or without different combinations of resistance (*R*) genes and/or quantitative resistance (QR) (refer to Table 3 in Chapter 2). Most cultivars had less than five leaves per plant and the greatest numbers of leaves per plant were on Adriana (*Rlm4* + QR), Excel (*Rlm7*) and NK Grandia (QR) (Fig. 3.18). There were no significant differences ($P<0.05$) between the cv. Drakkar (susceptible to *L. maculans*) and the cultivars with *R* genes or QR in number of *L. maculans* spots per plant (Fig. 3.18). For the 2010/2011 growing season, there were no data for *L. biglobosa* spots.

In the 2011/2012 growing season, phoma leaf spotting was first observed on 25 January 2012. Results from the assessment of plants showed that most cultivars had seven to ten leaves per plant (Fig. 3.19). The cultivars Adriana (*Rlm4* + QR), Drakkar (no *R* gene), Es-Astrid (QR), NK Grandia (QR) and Roxet (*Rlm7*) had more leaves and there was no significant difference ($P>0.05$) between them (Fig. 3.19). The greatest number of leaves with *L. maculans* spots was on cv. Drakkar. There was a significant difference ($P<0.05$) in number of leaves with *L. maculans* spots between cv. Drakkar and other cultivars with *R* genes and QR (Fig. 3.19). Cultivar Excel (*Rlm 7*) had the smallest number of leaves per plant with *L. maculans* spots (Fig. 3.19). There was no significant difference ($P>0.05$) in number of leaves per plant with *L. maculans* spots between Es-Astrid (QR), NK Grandia (QR), Adriana (*Rlm4* + QR) and DK Cabernet (*Rlm1* + QR) (Fig. 3.19). Cultivar Adriana had the smallest number of leaves per plant with *L. biglobosa* spots (Fig. 3.19) and there was no significant

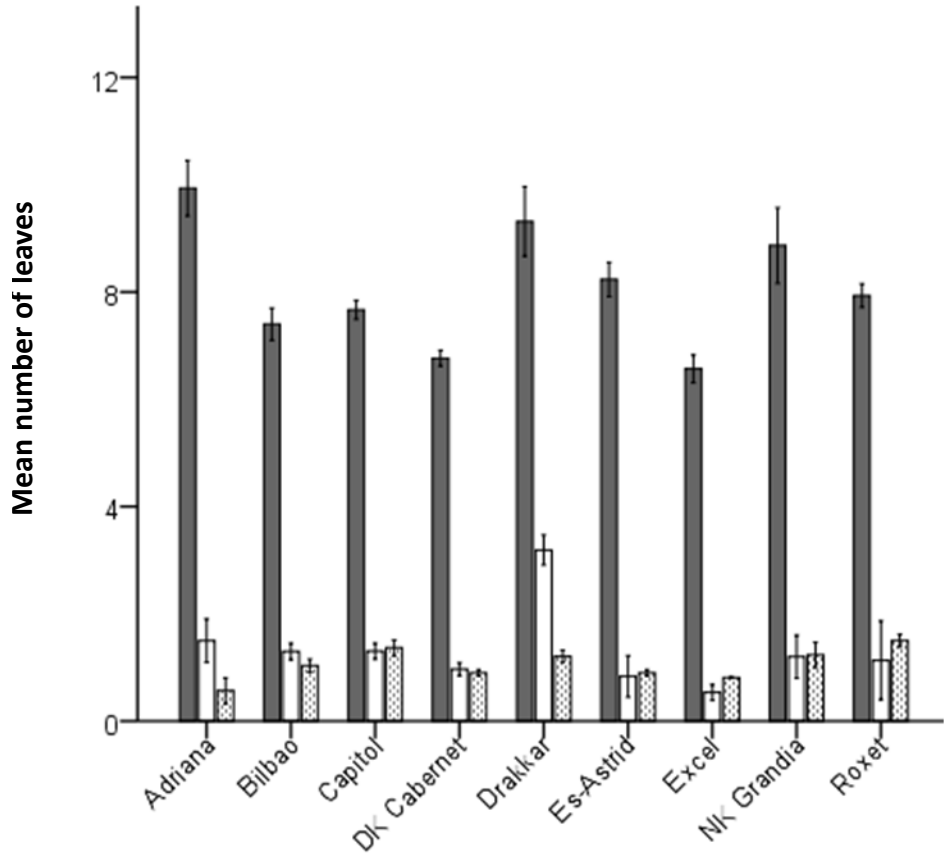


Fig. 3.19: Mean number of leaves per plant ■, mean number of leaves per plant with *L. maculans* spots □ and mean number of leaves per plant with *L. biglobosa* spots ▩. Data are means from 30 plants per cultivar (10 plants per plot x three replicates) of winter oilseed rape sampled in the 2011/2012 growing season (25 January 2012) at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)). Average number of leaves per plant and average number of leaves per plant with Lm spots and Lb spots were determined from 30 plants (Appendix 3.8). Vertical error bars represent standard error.

difference (*P*>0.05) between all nine cultivars in number of leaves per plant with *L. biglobosa* spots except between cvs Adriana and Roxet (P<0.05) (Appendix 3.8).

In the 2012/2013 growing season, the phoma leaf spotting started earlier than in the previous season and was first observed on 24 October 2012, with a small number of leaves with phoma leaf spotting (Fig. 3.20a). Therefore, the phoma leaf spotting was assessed again on 21 November 2012, 11 December 2012 and 30 January 2013 in the 2012/2013 growing season. There was a period with frost that affected the experimental plots, especially the plant growth. The number of leaves present for each cultivar was mostly less than six leaves from October until November 2012 (Fig. 3.20a, b) and some cultivars had more leaves in December 2012 and January 2013 (Fig. 3.20c, d). For plants sampled on 24 October 2012, there was no significant difference (*P*>0.05) in number of leaves per plant with *L. maculans* spots between cultivars, whereas cv. Roxet had significantly greater numbers of leaves per plant with *L. biglobosa* than cv. Drakkar or cv. Es-Astrid (Fig. 3.20a). There were no significant differences (*P*>0.05) between cultivars in numbers of leaves per plant with *L. maculans* or *L. biglobosa* on plants for samples collected on 21 November 2012 (Fig. 3.20b), 11 December 2012 (Fig. 3.20c) and 30 January 2013 (Fig. 3.20d). Although there were more leaves per plant after December 2012, there were no significant differences (*P*>0.05) between cultivars in numbers of leaves per plant with *L. maculans* or *L. biglobosa*. There were also symptoms of cell death observed in the form of a small, dark lesion on some of the cultivars (Fig. 3.21). These may have been the result of recognition of the product of the pathogen *Avr* gene by the product of the host resistance gene through ETD (effector-triggered defence) against *L. maculans* (refer to 3.1).

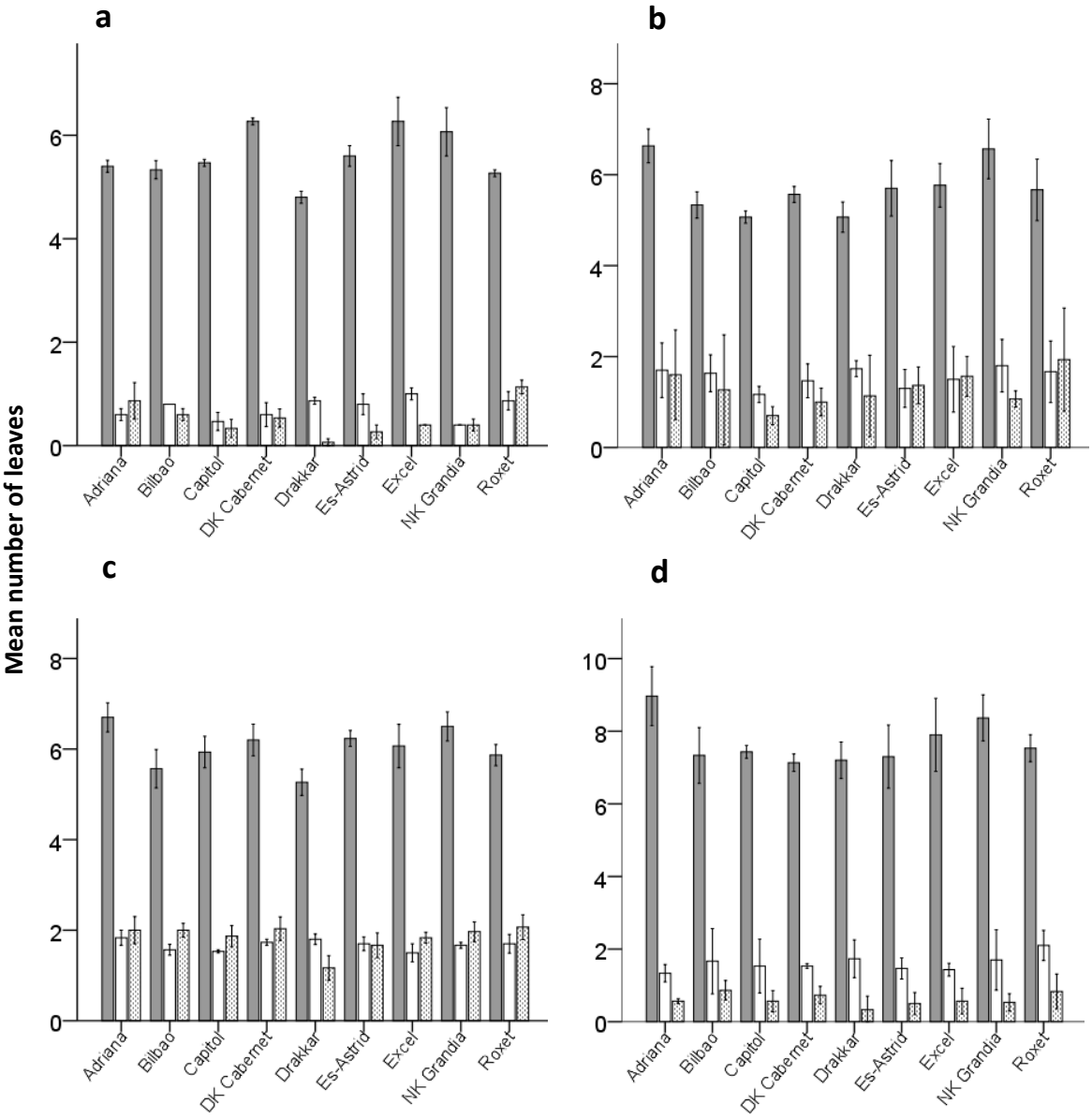


Fig. 3.20: Mean number of leaves per plant ■, mean number of leaves per plant with *L. maculans* spots □ and mean number of leaves per plant with *L. biglobosa* spots ▩. Data are means from 30 plants per cultivar (10 plants per plot x three replicates) of winter oilseed rape sampled in the 2012/2013 growing season (a) on 24 October 2012, (b) 21 November 2012, (c) 11 December 2012 and (d) 30 January 2013 at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)). Average number of leaves per plant and average number of leaves per plant with Lm spots and Lb spots were determined from 30 plants (Appendix 3.3). Vertical error bars represent standard error.

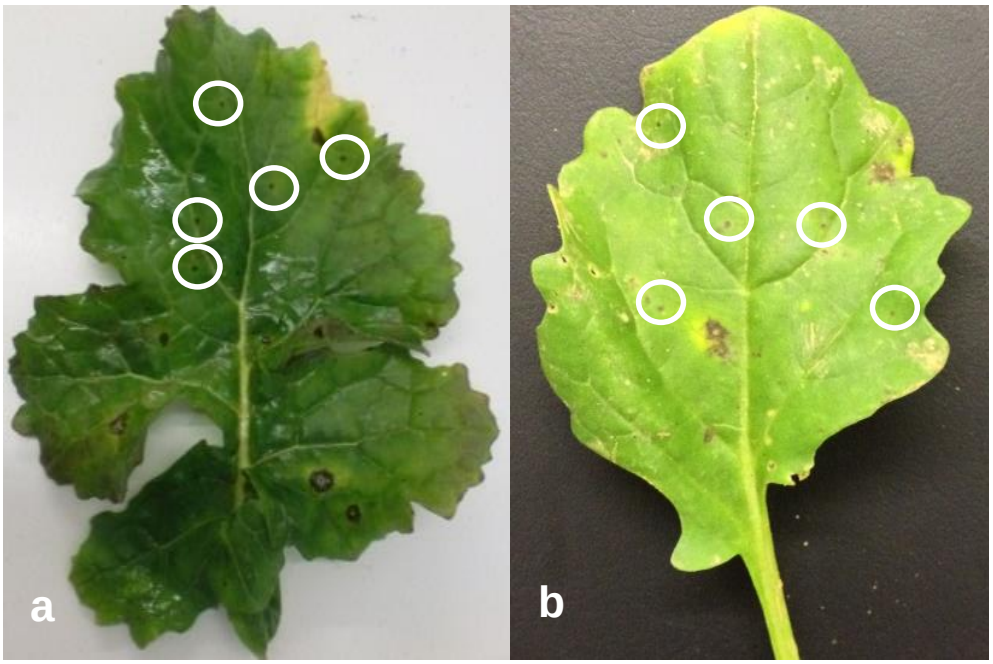


Fig. 3.21: Phoma leaf spotting caused by *L. maculans* on cv. Drakkar (susceptible) (a) and cell death resulting from recognition of the product of the *Avr* gene of the pathogen by the product of the resistance gene of the host (gene-for-gene interaction) on Roxet (*Rlm 7*) (b) sampled on 25 January 2012 at Rothamsted Research. Responses are circled either ETI (effector-triggered immunity) or ETD (effector-triggered defence) (Jones & Dangl, 2006; Stotz *et al.* 2014).

3.3.5 Cultivar and seasonal differences in severity of stem canker in the 2010/2011, 2011/2012 and 2012/2013 growing seasons

There were significant differences (P<0.05) in stem diameter (cm) between the 2010/2011 growing season (30 June 2011) and the two other growing seasons (13 July 2012 and 26 July 2013) (Fig. 3.22) but there were no significant differences (*P*>0.05) between cultivars in the 2012/2013 growing season (26 July 2013). The mean severity of phoma stem canker for all nine cultivars differed between the 2010/2011, 2012/2013 and 2011/2012 growing seasons (Fig. 3.23).

In the summer, the mean phoma stem canker scores from the stem canker assessments before harvest on 30 June 2011, 13 July 2012 or 26 July 2013 differed between the nine cultivars (Fig. 3.24). The most susceptible cultivar Drakkar had a weak stem and the most severe basal stem cankers, with 100% girdling in all three growing seasons (Fig. 3.24). In June 2011, cvs Bilbao (*Rlm 4*) and Capitol (*Rlm1*) had 76 -100% girdling, whereas cvs Adriana (*Rlm4* + QR), Excel (*Rlm7*) and NK Grandia (QR) had 51 -75% girdling. Only cvs DK Cabernet (*Rlm1* + QR), Es-Astrid (QR) and Roxet (*Rlm7*) had less severe phoma stem canker (26-50% girdling) (Fig. 3.24a).

In the 2011/2012 growing season, phoma stem canker was assessed in July 2012, with a smaller severity of phoma stem canker (Fig. 3.24b) than in 2010/2011 and 2012/2013 (Fig. 3.24c) on all cultivars except Drakkar (with 100% girdling; Fig. 3.24a). Cultivars NK Grandia (QR), Adriana (*Rlm4* + QR), DK Cabernet (*Rlm1* + QR), Es-Astrid (QR), Excel (*Rlm7*) and Roxet (*Rlm7*) had less severe phoma stem canker, with a severity score < 2 (≤25% girdling of the stem) (Fig. 3.24b), whereas cvs Bilbao (*Rlm4*) and Capitol (*Rlm1*) had 26-50% girdling (Fig. 3.24b). In the 2012/2013

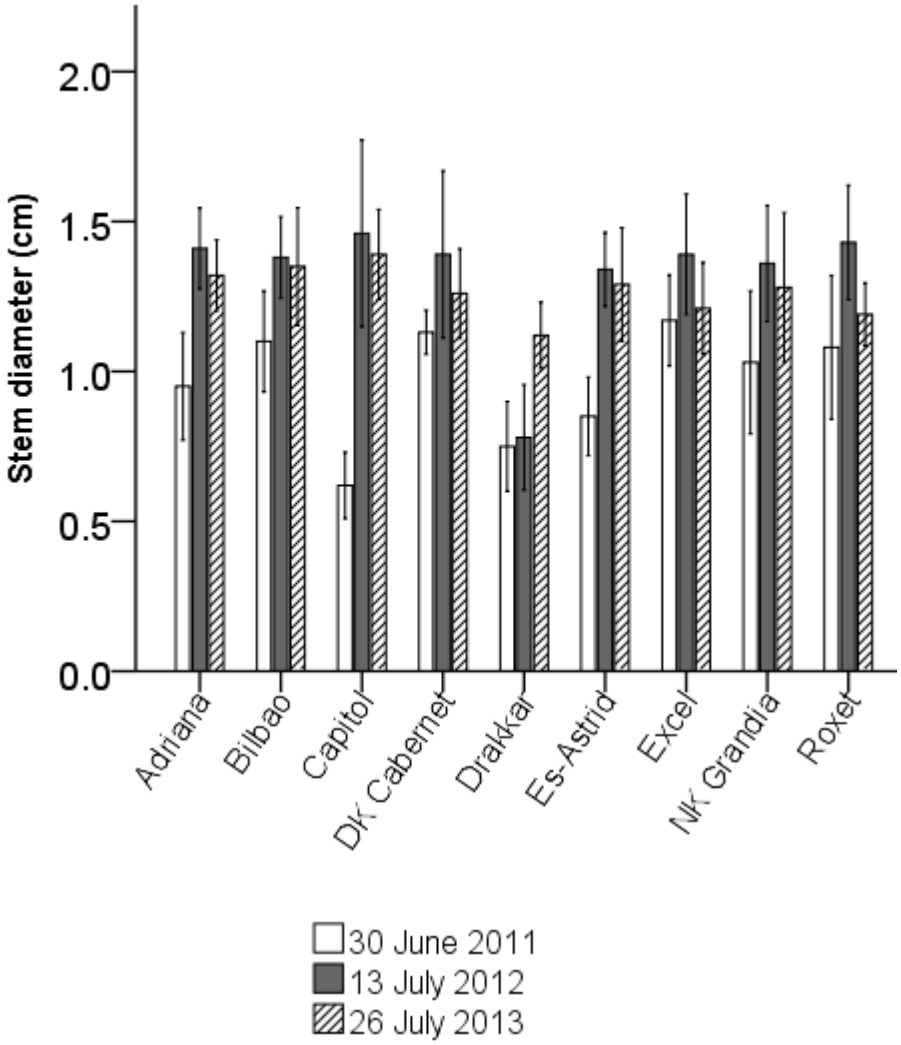


Fig. 3.22: Stem diameter (cm) of nine winter oilseed rape cultivars assessed in summer before harvest in each of the three growing seasons. Data are means from 10 stems per cultivar of winter oilseed rape for each growing season (Appendix 3.4). Vertical error bars represent standard error.

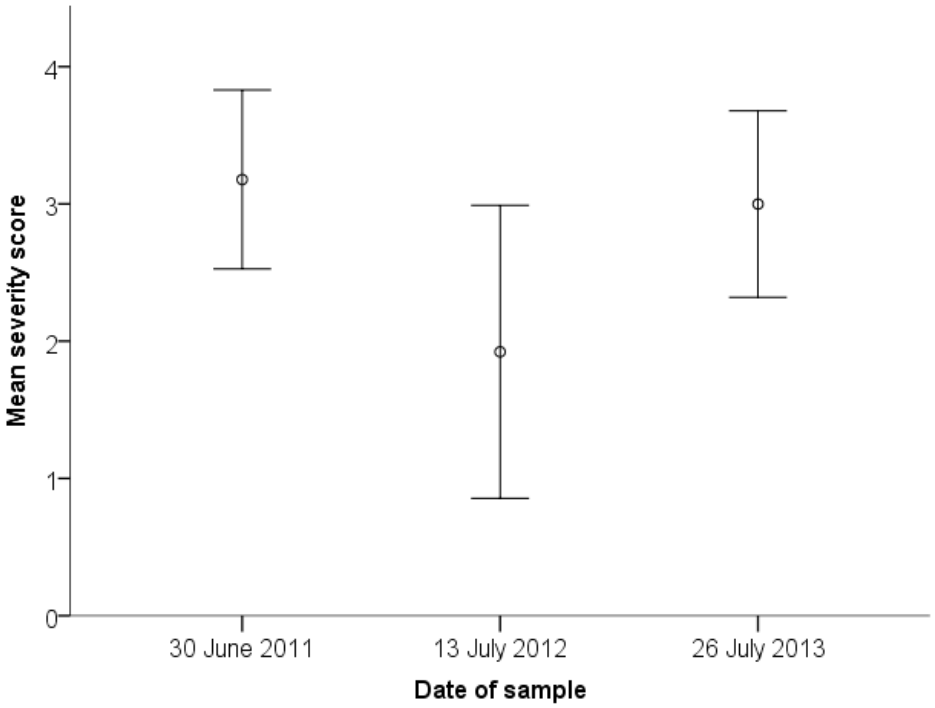


Fig. 3.23: Mean phoma stem canker severity (interval plot) observed in each of the three growing seasons, observed on 30 June 2011, 13 July 2012 or 26 July 2013, respectively. Data are means from 45 stems per cultivar (15 stems per plot x 3 replicates) of nine winter oilseed rape for each growing season.

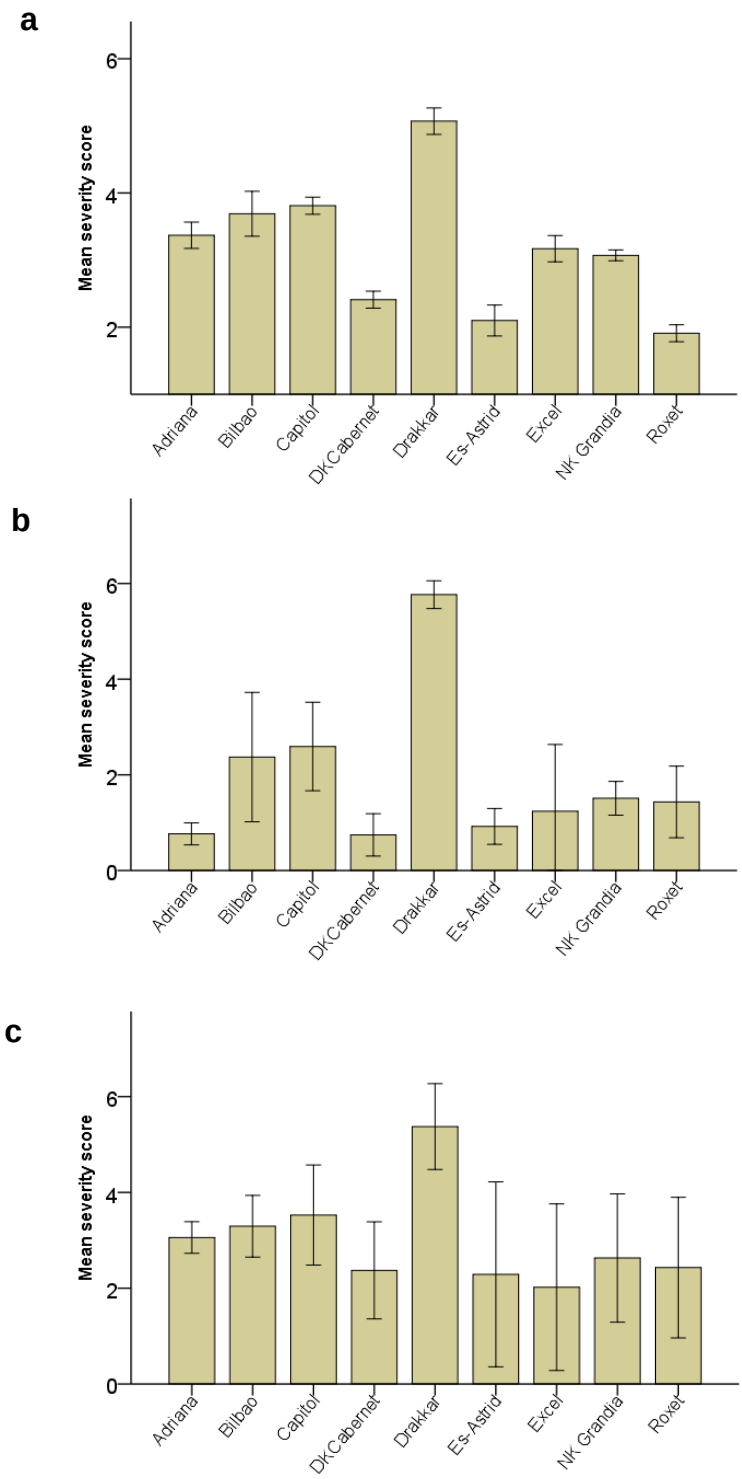


Fig. 3.24: Stem canker severity score (0-6 scale) on basal part of the stem for three growing seasons observed in summer; (a) 30 June 2011, (b) 13 July 2012 and (c) 26 July 2013 at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)). Average number of stem canker severity was determined from 15 plants (Appendix 3.5). Vertical error bars represent standard error.

growing season, phoma stem canker was assessed in July 2013, with severity greater than in July 2012 (Fig. 3.24c). Only cvs NK Grandia (QR), Roxet (*Rlm*7), DK Cabernet (*Rlm1* + QR), Es-Astrid (QR) and Excel (*Rlm7*) had severity scores < 3 (26 – 50% girdling) (Fig. 3.24c, Fig. 3.25c). The cvs Roxet and Excel (both with *Rlm7*) generally had less severe canker in all seasons, with a severity score < 2 (≤25 - 25% girdling) in June 2011(Fig. 3.24a) and July 2012 (Fig. 3.24b, Fig. 3.25b) and <3 (<50%) in July 2013 (Fig. 3.24c).

When severity score was compared between the cultivars, canker was more severe in June 2011 and July 2013 on Adriana (*Rlm4* + QR) than DK Cabernet (*Rlm1* + QR). Canker was more severe on Capitol (*Rlm1*) in all three growing seasons but less severe on both cvs NK Grandia and Es-Astrid with QR. The ranking of cultivars, according to severity of the internal (girdling) stem canker pre-harvest from those with most severe canker to those with less severe canker on 30 June 2011 was: Drakkar > Capitol > Bilbao > Adriana > Excel > NK Grandia > DK Cabernet > Es-Astrid >Roxet; on 13 July 2012 it was: Drakkar > Capitol > Bilbao > NK Grandia > Roxet > Excel > Es-Astrid > Adriana > DK Cabernet and on 26 July 2013 it was: Drakkar > Capitol > Bilbao > Adriana > NK Grandia > Roxet > DK Cabernet > Es-Astrid > Excel.

In the 2011/2012 growing season, most of the upper stem lesions on cv. Drakkar had 100% girdling and those on cv. Bilbao (*Rlm4*) had 51 -75% girdling (Fig. 3.26a). The other cultivars Adriana (*Rlm4* + QR), Capitol (*Rlm1*), DK Cabernet (*Rlm1* + QR), Roxet (*Rlm*7), NK Grandia (QR), Es-Astrid (QR) and Excel (*Rlm*7) had upper stem lesions with ≤25% girdling of the upper stem (Fig. 3.26a). In the 2012/2013 growing season, the severity scores of upper stem lesions on all cultivars were <1. The

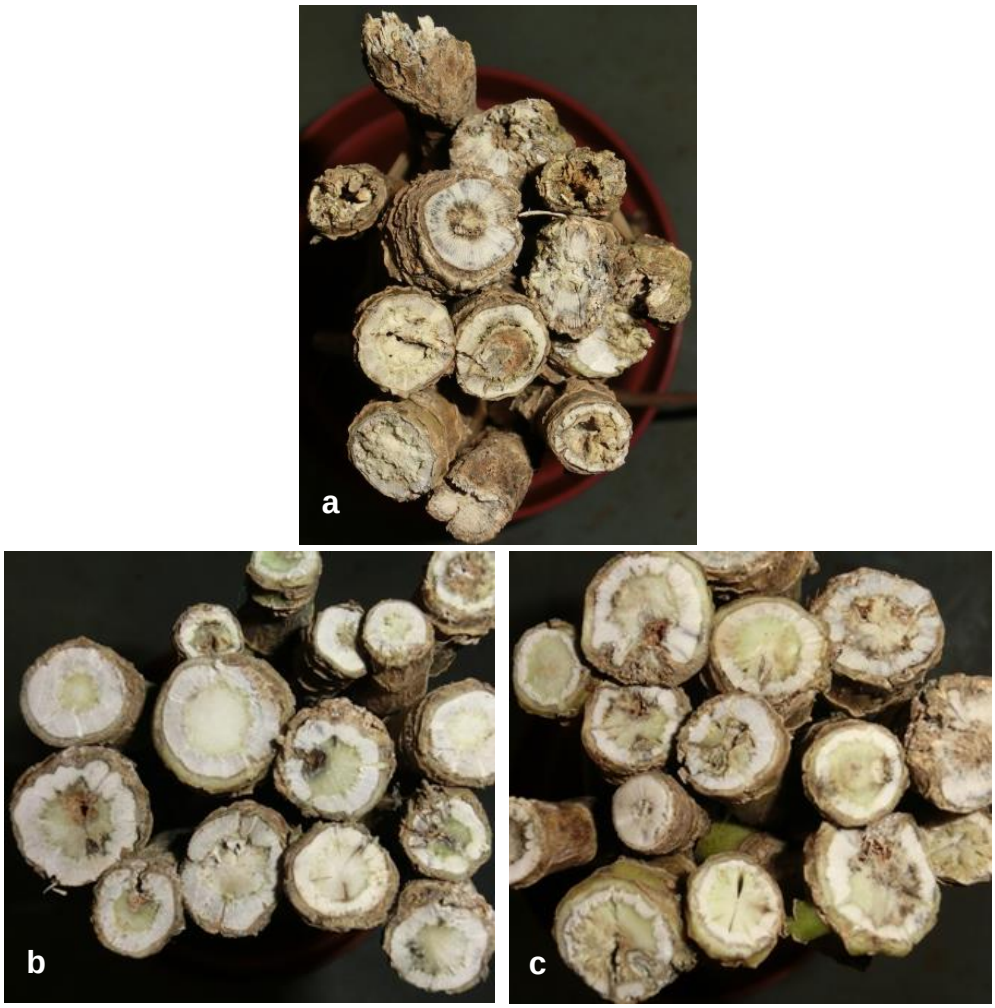


Fig. 3.25: Stem canker girdling at the stem base on winter oilseed rape; (a) 100 % girdling of cv. Drakkar, (b) ≤25 - 25% girdling of cv. Roxet (*Rlm*7) and (c) 26 – 50% girdling of cv. NK Grandia (QR), observed on 23 July 2013 at Rothamsted.

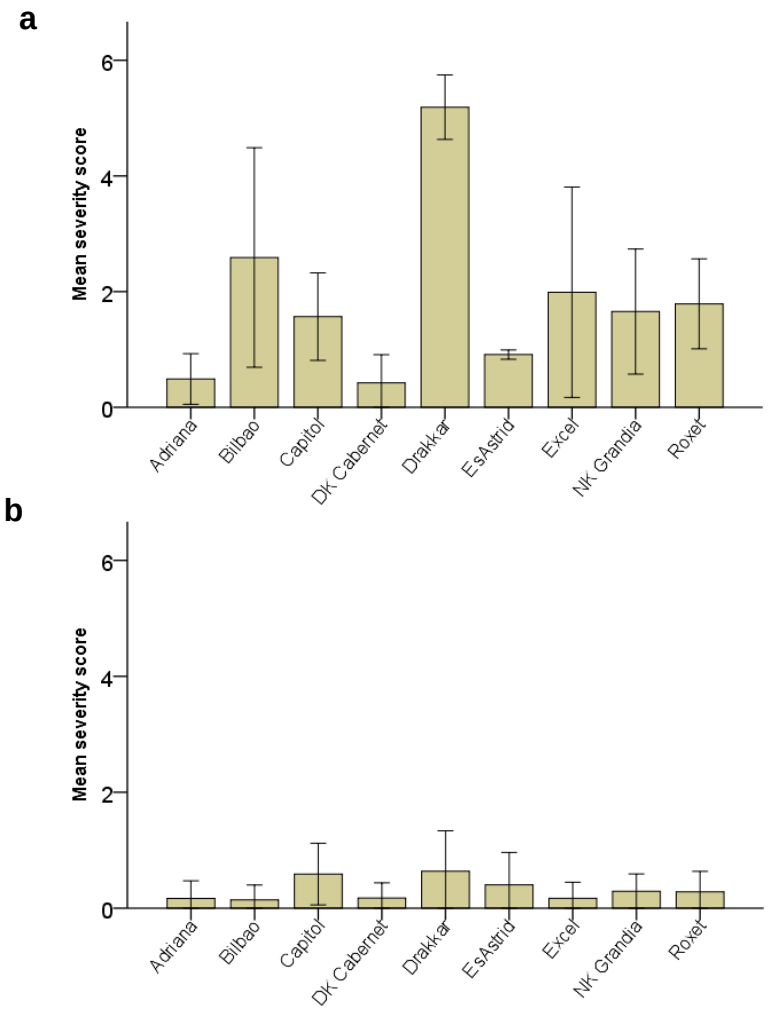


Fig. 3.26: Phoma stem canker severity score for upper part of the stem for two growing seasons observed in summer on samples collected on (a) 13 July 2012 or (b) 26 July 2013 at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)). The upper stem lesion severity was scored using the HGCA 0-6 score shown in Fig. 3.5 (method 3.2.2.1). Average number of upper stem canker severity was determined from 15 plants (Appendix 3.6). Vertical error bars represent standard error.

greatest severity score was on cv. Drakkar (0.96) and the smallest score was on cv. Bilbao (*Rlm4*) (0.14) (Fig. 3.26b). There was no significant difference (P>0.05) in the upper stem lesions severity, whereas cv. Drakkar had significantly severe than Adriana (*Rlm4* + QR) in 13 July 2012 (Appendix 3.6).

3.3.6 Relationships between phoma leaf spotting and stem canker for different cultivars in the 2010/2011, 2011/2012 and 2012/2013 growing seasons

There were no significant relationships (*P*>0.05) between mean number of leaves per plant with *L. maculans* phoma spots and severity of basal stem canker in 2010/2011 (Fig. 3.27) and in 2012/2013 (Table 3.8). However, regressions of stem canker severity at harvest against mean number of leaves per plant with *L. maculans* phoma spots accounted for 80.5% of the variation in 2011/2012 (*P*<0.05) (Fig. 3.28a) for all nine cultivars. A regression of upper stem lesion severity at harvest against mean number of leaves per plant with *L. maculans* phoma spots accounted for 61.9% of the variation in 2011/2012 (*P<0.05*) (Fig. 3.28b) and in 2012/2013 there were no significant differences (Fig. 3.29b). There were no data collected on upper stem lesions in 2010/2011. In 2011/2012, there were significant relationships (*P<0.05*) between mean number of *L. maculans* phoma spots per plant and stem canker severity at harvest or upper stem lesion severity at harvest (Table 3.8) that accounted for 73% (Fig. 3.28c) or 55% (Fig. 3.29d) of the variance, respectively. It was only the upper stem lesion severity that showed a significant (*P*<0.05) relationship with mean number of *L. maculans* phoma spots per plant in 2012/2013 growing season (Table 3.8).

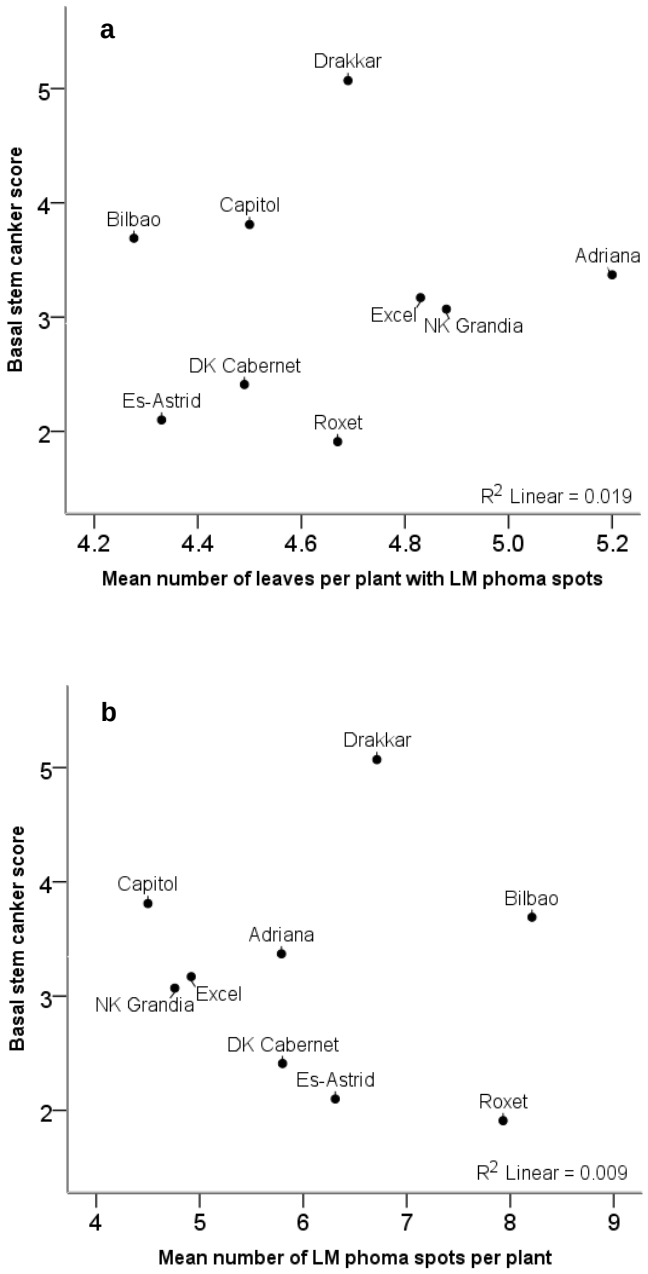


Fig. 3.27: Relationships between the mean number of leaves per plant with *L. maculans* phoma spots or mean number of *L. maculans* phoma spots per plant (assessed on 9 December 2010) and severity (mean score on 0-6 scale) of basal stem canker before harvest in the 2010/2011 growing season; (a) Mean number of leaves per plant with *L. maculans* phoma spots/mean severity; *P*= 0.72, y= 1.05+0.46x and (b) Mean number of *L. maculans* phoma spots per plant/mean severity; *P*= 0.81, y= 3.61-0.07x.

Table 3.8: Relationships between phoma leaf spotting (*L. maculans;* (Lm) and *L. biglobosa;* (Lb)) in autumn/winter and severity of basal stem canker or upper stem lesions in the 2010/2011, 2011/2012 and 2012/2013 growing seasons, assessed by linear regression.

	Regression parameters			
Growing season	Mean number of leaves with Lm	Mean number of leaves with Lb	Mean number of Lm leaf spots	Mean number of Lb leaf spots
2010/2011[a]	r = 0.137	+	r = -0.09	+
Stem canker severity	P = 0.72		P = 0.81	
			r_s = 0.70	
Upper stem lesion severity	+	+	+	+
2011/2012[b]	r = 0.89	r = 0.41	r = 0.85	r = 0.28
Stem canker severity	P =0.001	P = 0.27	P = 0.003	P = 0.45
			r_s = 0.83	r_s = 0.01**
Upper stem lesion severity	r = 0.78	r = 0.39	r = 0.73	r = 0.29
	P = 0.012	P = 0.29	P = 0.023	P = 0.45
			r_s = 0.83	r_s = 0.09
2012/2013[c]	r = 0.32	r = -0.73	r = 0.57	r = -0.74
Stem canker severity	P = 0.39	P = 0.024	P = 0.11	P = 0.022
			r_s = 0.70	r_s = 0.08
Upper stem lesion severity	r = 0.23	r = -0.87	r = 0.76	r = -0.72
	P = 0.55	P = 0.002	P = 0.015	P = 0.028
			r_s = 0.09	r_s = 0.08

[a], Sowing: 13 Sept 2010, phoma leaf spot assessment: 9 Dec 2010, stem canker assessment:30 June 2011

[b], Sowing: 30 Aug 2011, phoma leaf spot assessment: 6 Feb 2012, stem canker assessment: 13 July 2012

[c], Sowing: 5 Sept 2012, phoma leaf spot assessment:11 December 2012, stem canker assessment: 26 July 2013

r_s, Spearmann ranking test for relationship between early stage of disease (mean numbers of *L. maculans*/*L. biglobosa* leaf spots per plant) and final stage of disease (stem cankers/upper stem lesions).

r= Pearson's r correlation test for relationship between early stage of disease (mean numbers of *L. maculans*/*L. biglobosa* leaf spots per plant) and final stage of disease (stem cankers/upper stem lesions).

P= significance level

*Stem canker assessment 2 weeks before harvest at Rothamsted Research, Harpenden.

**Correlation is significant at the 0.01 level (2-tailed).

+ Not assessed in the experiment.

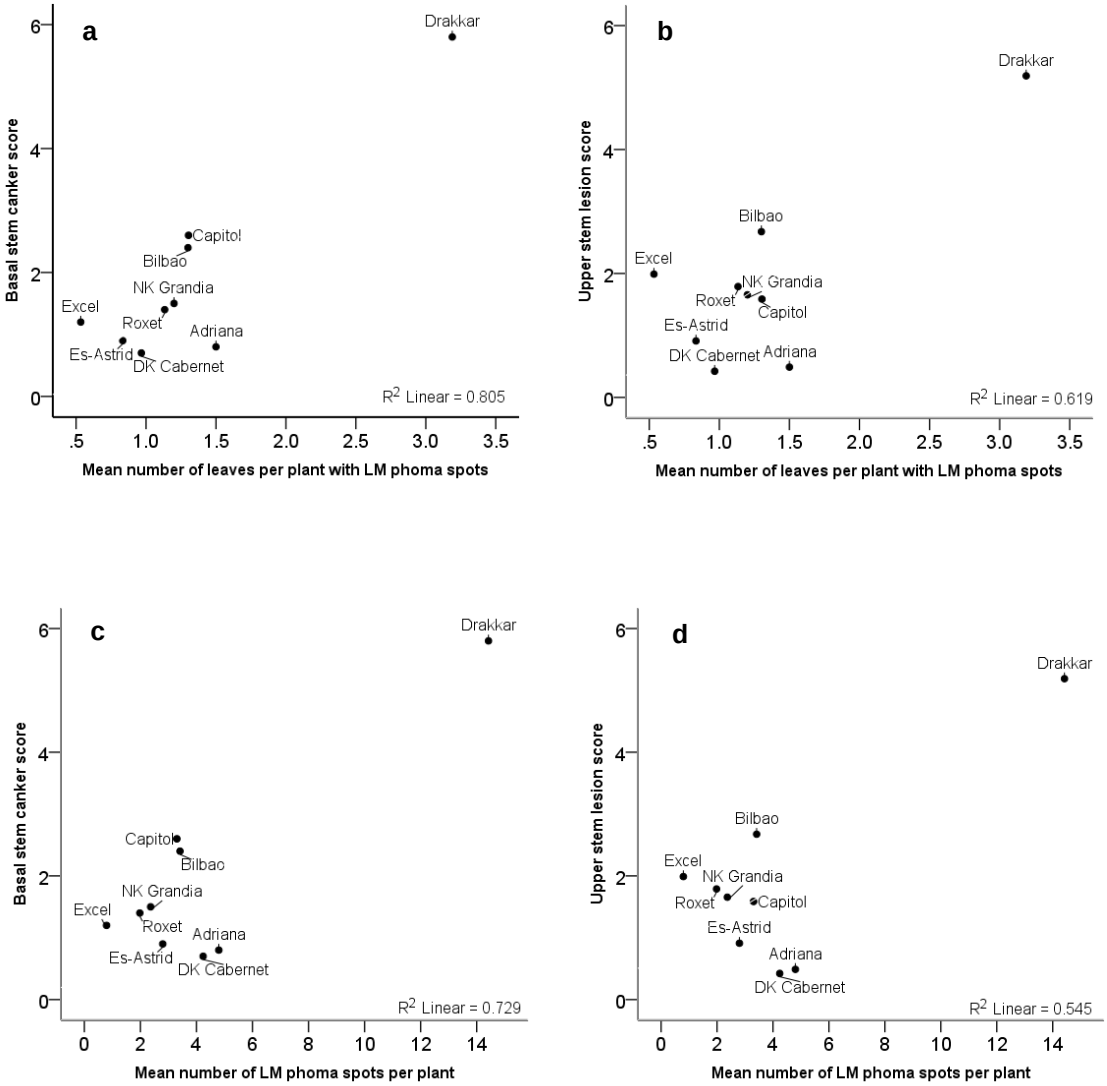


Fig. 3.28: Relationships between the mean number of leaves per plant with *L. maculans* phoma spots or mean number of *L. maculans* phoma spots per plant (assessed on 25 January 2012) and severity (mean score on 0-6 scale) of basal stem cankers or upper stem lesions in the 2011/2012 growing season; mean number of leaves per plant with *L. maculans* phoma spots/mean severity for (a) basal stem canker; *P*= 0.001, y= -0.61+1.9x and (b) upper stem lesion; *P*= 0.012, y= -0.15+1.51x. Mean number of *L. maculans* phoma spots per plant/mean severity for (c) basal stem canker; *P*= 0.003, y= 0.47+0.34x and (d) upper stem lesion; *P*= 0.023, y= 0.73+0.27x.

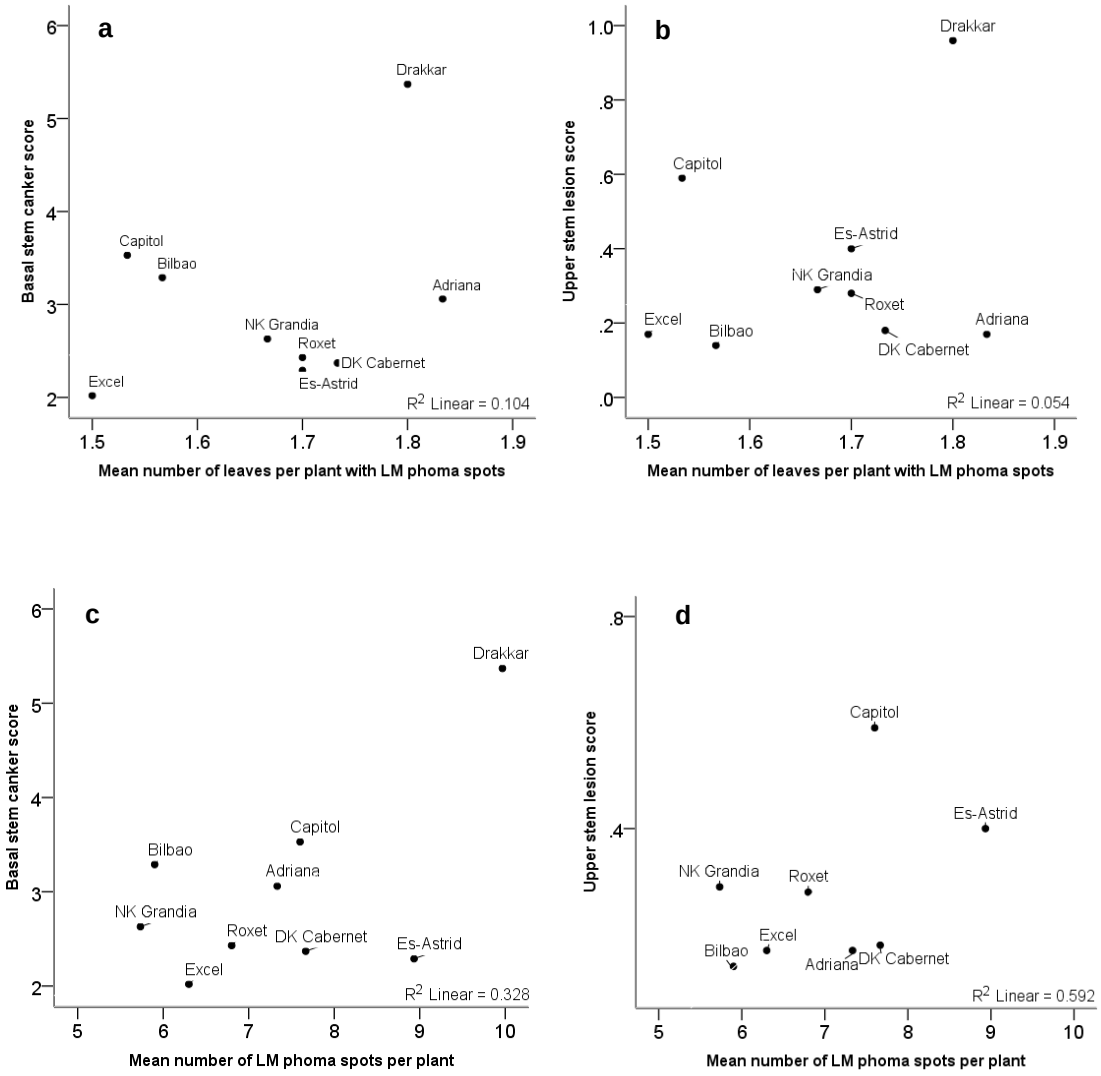


Fig. 3.29: Relationships between the mean number of leaves per plant with *L. maculans* phoma spots or mean number of *L. maculans* phoma spots per plant (assessed on 11 December 2012) and severity (mean score on 0-6 scale) of basal stem cankers or upper stem lesions in the 2012/2013 growing season; mean number of leaves per plant with *L. maculans* phoma spots/mean severity for (a) basal stem canker; *P*= 0.39, y= -1.73+2.83x and (b) upper stem lesion; *P*= 0.55, y= -0.55+0.54x. Mean number of *L. maculans* phoma spots per plant/mean severity for (c) basal stem canker; *P*= 0.11, y= -0.07+0.42x and (d) upper stem lesion; *P*= 0.015, y= 0.74+0.15x.

In the 2011/2012 growing season, both the mean number of leaves per plant with *L. biglobosa* spots and the mean number of *L. biglobosa* leaf spots per plant were not significantly (*P*>0.05) related to the basal stem canker severity (Fig. 3.30a, c) or upper stem lesion severity (Fig. 3.30b, d). In 2012/2013, there were significant relationships (*P*<0.05) between mean number of leaves per plant with *L. biglobosa* spots and stem canker severity at harvest or upper stem lesion severity at harvest (Table 3.8) that accounted for 54% (Fig. 3.31a) or 76.1% (Fig. 3.31b) of the variance, respectively. In 2012/2013, there were a significant (*P*<0.05) relationships between the mean number of *L. biglobosa* leaf spots per plant and stem canker severity at harvest or upper stem lesion severity at harvest (Table 3.8) that accounted for 54.9% (Fig. 3.31c) and 52.1% (Fig. 3.31d) of the variance, respectively. There were no data collected on number of leaves with *L. biglobosa* and number of *L. biglobosa* leaf spots in 2010/2011.

There was no significant relationship (*P<0.05*) between phoma leaf spotting in autumn and the stem canker severity in summer over all three growing seasons but there was a significant relationship (*P<0.01*, r_s = 0.01) between mean number of *L. biglobosa* leaf spots per plant and basal stem canker in 2011/2012 growing seasons (Table 3.8). Relationships between phoma leaf spotting and stem canker severity or upper lesion severity in the 2010/2011, 2011/2012 and 2012/2013 growing seasons are summarized in Table 3.8.

The first two principal components (PC) accounted for 99.7% of the variation in the data for the 2010/2011 growing season (Fig. 3.32). The main effect of the first principal component (PC1) was mean number of *L. maculans* phoma leaf spots per plant (positive). However, the factor that contributed the most to the second principal

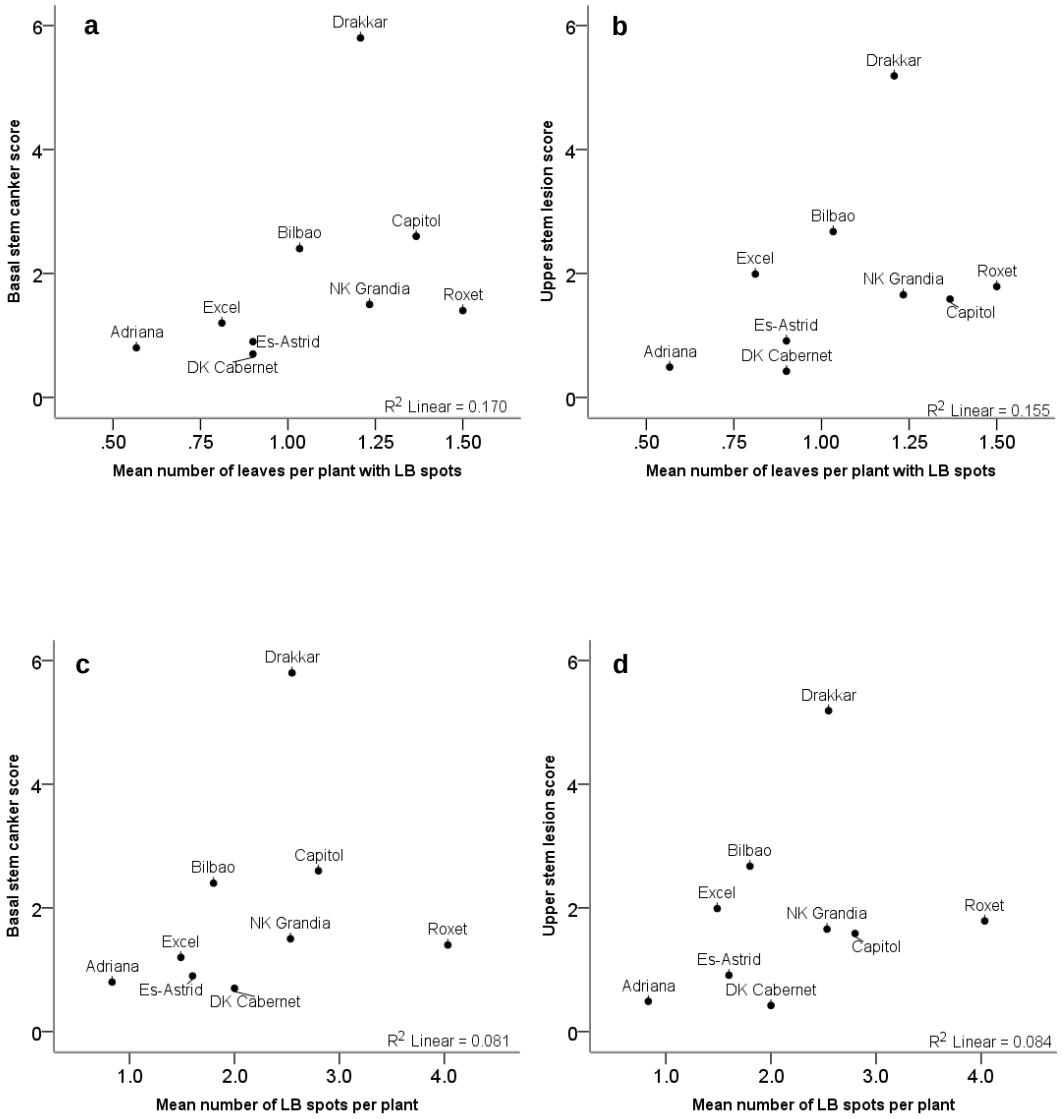


Fig. 3.30: Relationships between the mean number of leaves per plant with *L. biglobosa* phoma spots or mean number of *L. biglobosa* phoma spots per plant (assessed on 25 January 2012) and severity (mean score on 0-6 scale) of basal stem cankers or upper stem lesions in the 2011/2012 growing season; mean number of leaves per plant with *L. biglobosa* phoma spots/mean severity for (a) basal stem canker; *P*= 0.27, y= 0.44+2.23x and (b) upper stem lesion; *P*= 0.39, y=1.93 - 0.19x. Mean number of *L. biglobosa* phoma spots per plant/mean severity for (c) basal stem canker; *P*= 0.45, y= 1.5x-0.75 and (d) upper stem lesion; *P*= 0.45, y= 0.87+0.45x.

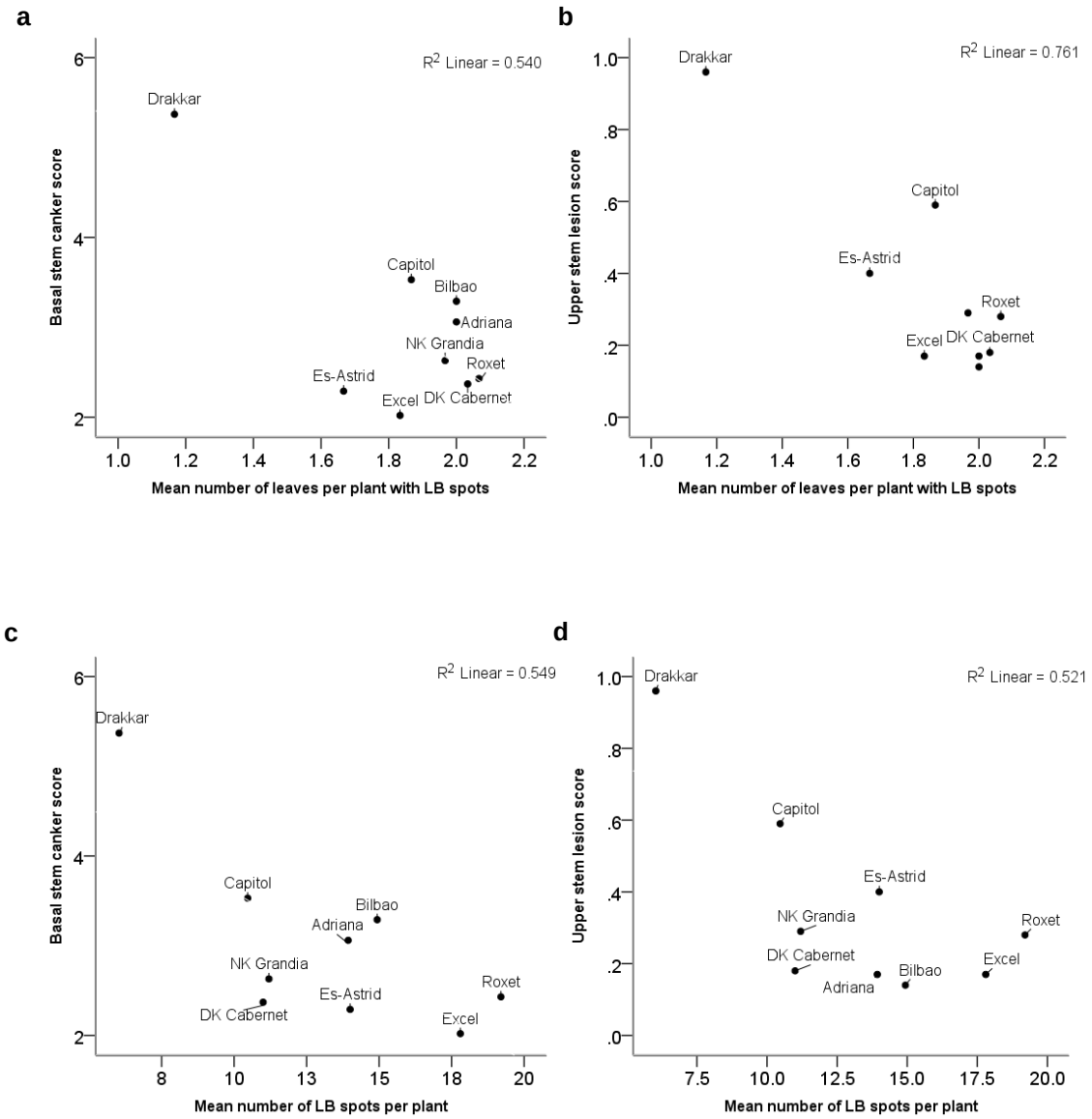


Fig. 3.31: Relationships between the mean number of leaves per plant with *L. biglobosa* phoma spots or mean number of *L. biglobosa* phoma spots per plant (assessed on 11 December 2012) and severity (mean score on 0-6 scale) of basal stem cankers or upper stem lesions in the 2012/2013 growing season; mean number of leaves per plant with *L. biglobosa* phoma spots/mean severity for (a) basal stem canker; *P*= 0.024, y= 7.88+2.65x and (b) upper stem lesion; *P*= 0.002, y= 1.88+0.83x. Mean number of *L. biglobosa* phoma spots per plant/mean severity for (c) basal stem canker; *P*= 0.022, y= 5.48+0.19x and (d) upper stem lesion; *P*= 0.028, y= 0.99+0.05x.

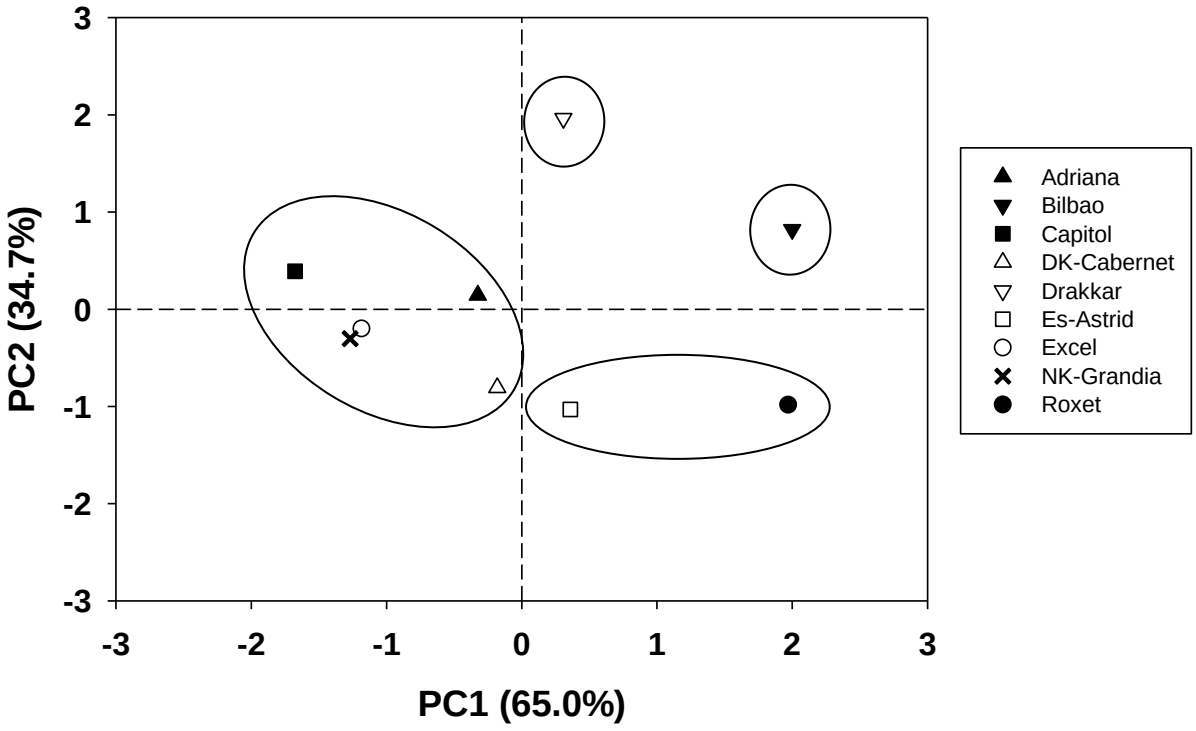


Fig. 3.32: Principal component (PC) analysis showing relative position of nine cultivars in relation to principal component one (PC1 positive; mean number of *L. maculans* phoma leaf spots per plant) and principal component two (PC2 positive; stem canker severity). Data are means from 30 plants per cultivar of winter oilseed rape in the 2010/2011 growing season. When combined, the two principal components accounted for 99.7% of the variation in the data. There were no data for number of *L. biglobosa* leaf spots per plant or upper lesions collected in this season. There were nine cultivars, four with *R*-gene resistance against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel, (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia), two with *R*-gene and QR (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR)) and cv. Drakkar (susceptible to *L. maculans*).

component (PC2) was basal stem canker severity. It was apparent that the nine cultivars could be divided into four distinct groups (Fig. 3.32). The first group consisted of only Drakkar (susceptible cultivar) with a small number of *L. maculans* phoma leaf spots but a severe canker development. The second group consisted only of Bilbao (*Rlm 4*), with more *L. maculans* phoma leaf spots per plant and severe stem canker development. The third group consisted of Es-Astrid (QR) and Roxet (*Rlm7*). These cultivars both had a large number of *L. maculans* phoma leaf spots per plant but less severe canker development. The fourth group included Adriana (*Rlm4* + QR), Capitol (*Rlm1*), DK Cabernet (*Rlm1* + QR), Excel (*Rlm7*) and NK Grandia (QR) where there was a small number of *L. maculans* phoma leaf spots per plant and less severe basal stem canker development. There were no data collected on number of *L. biglobosa* phoma leaf spots per plant or severity of upper stem lesions in the 2010/2011 growing season.

In the 2011/2012 growing season, the two principal components combined accounted for 96.7% of the variation in the data (Fig. 3.33). The main effects of the first principal component (PC1) were mean number of *L. maculans* phoma leaf spots per plant, basal stem canker severity and upper stem lesion severity. The PC1 was strongly correlated with three of the variables and it increased with increasing number of *L. maculans* phoma leaf spots per plant, stem canker severity and upper lesion severity. The main effect of the second principal component (PC2) was mean number of *L. biglobosa* phoma leaf spots per plant. The nine cultivars could be divided into three distinct groups (Fig. 3.33). The first group consisted of only Drakkar with the greatest number of *L. maculans* phoma leaf spots per plant, severe stem cankers and severe upper lesions. All other cultivars had fewer *L. maculans* phoma leaf spots per plant and less severe basal stem cankers and upper stem lesions.

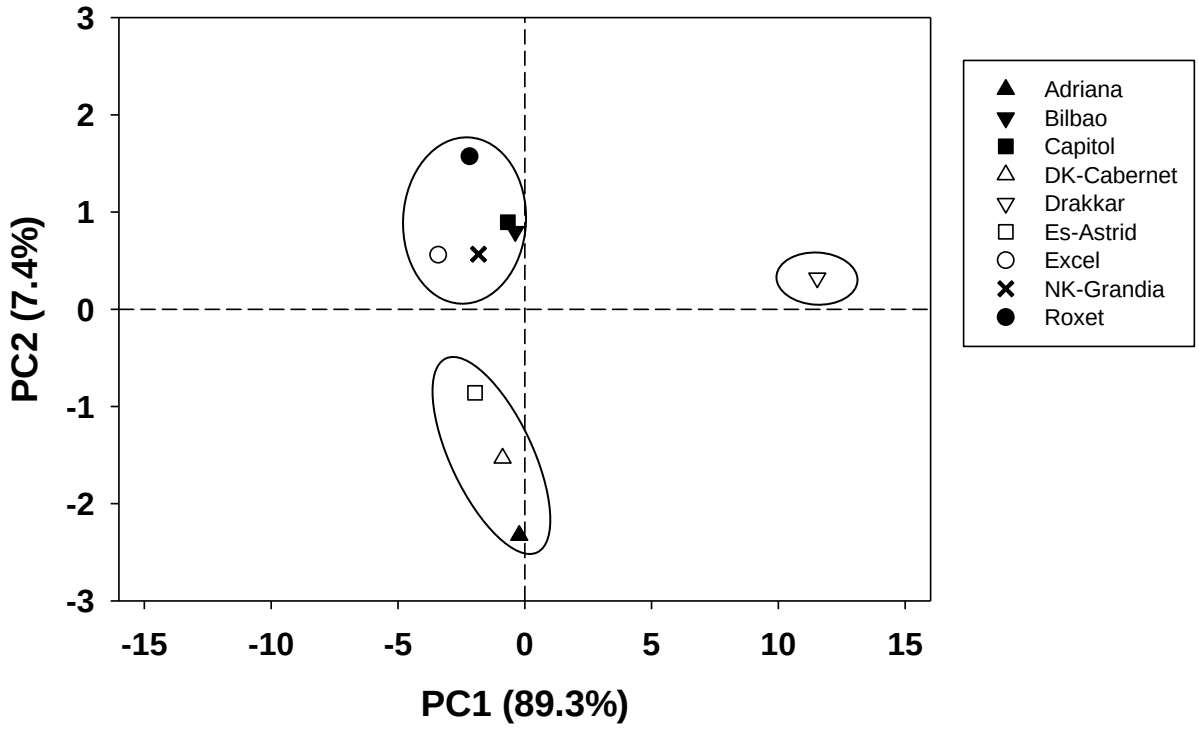


Fig. 3.33: Principal component (PC) analysis showing relative position of nine cultivars in relation to principal component one (PC1 positive; mean number of *L. maculans* phoma leaf spots per plant, stem canker severity and upper lesion severity) and principal component two (PC2 positive; mean number of *L. biglobosa* phoma leaf spots per plant). Data are means from 30 plants per cultivar of winter oilseed rape in 2011/2012 growing season. The two principal components combined accounted for 96.7% of the variation in the data. There were nine cultivars, four with *R*-gene resistance against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel, (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia), two with *R*-gene and QR (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR)) and cv. Drakkar (susceptible to *L. maculans*).

However, the group could be sub-divided, separating cvs Adriana (*Rlm4* + QR), DK Cabernet (*Rlm1* + QR) and Es-Astrid (QR) that had a smaller number of *L. biglobosa* phoma leaf spots per plant. All other cultivars with *R* genes and QR (Bilbao, Capitol, Excel, NK Grandia and Roxet) had a large number of *L. biglobosa* leaf spots per plant (Fig. 3.33).

In the 2012/2013 growing season, the PC1 and PC2 accounted for 97.6% of the variation in the data (Fig. 3.34). The main effects of the first principal component (PC1) were strongly correlated with two of the variables and increased with increasing stem canker severity and decreasing number of *L. biglobosa* phoma leaf spots per plant. However, the largest contributing factor to the second principal component (PC2) was mean number of *L. maculans* phoma leaf spots per plant. The nine cultivars were divided into three distinct groups (Fig. 3.34). The first group included only cv. Drakkar, which had the greatest number of *L. maculans* leaf spots per plant, very severe stem cankers and the smallest number of *L. biglobosa* phoma leaf spots per plant.

Other cultivars had more *L. biglobosa* phoma leaf spots per plant and less severe stem cankers. They were divided into two groups with a greater number of *L. maculans* leaf spots per plant (Adriana, Es-Astrid, Excel and Roxet) or fewer *L. maculans* leaf spots (Bilbao, DK Cabernet, Capitol and NK Grandia) (Fig. 3.34). The cultivars Capitol, DK Cabernet and NK Grandia had more severe stem cankers than cv. Bilbao although the number of *L. maculans* leaf spots was small.

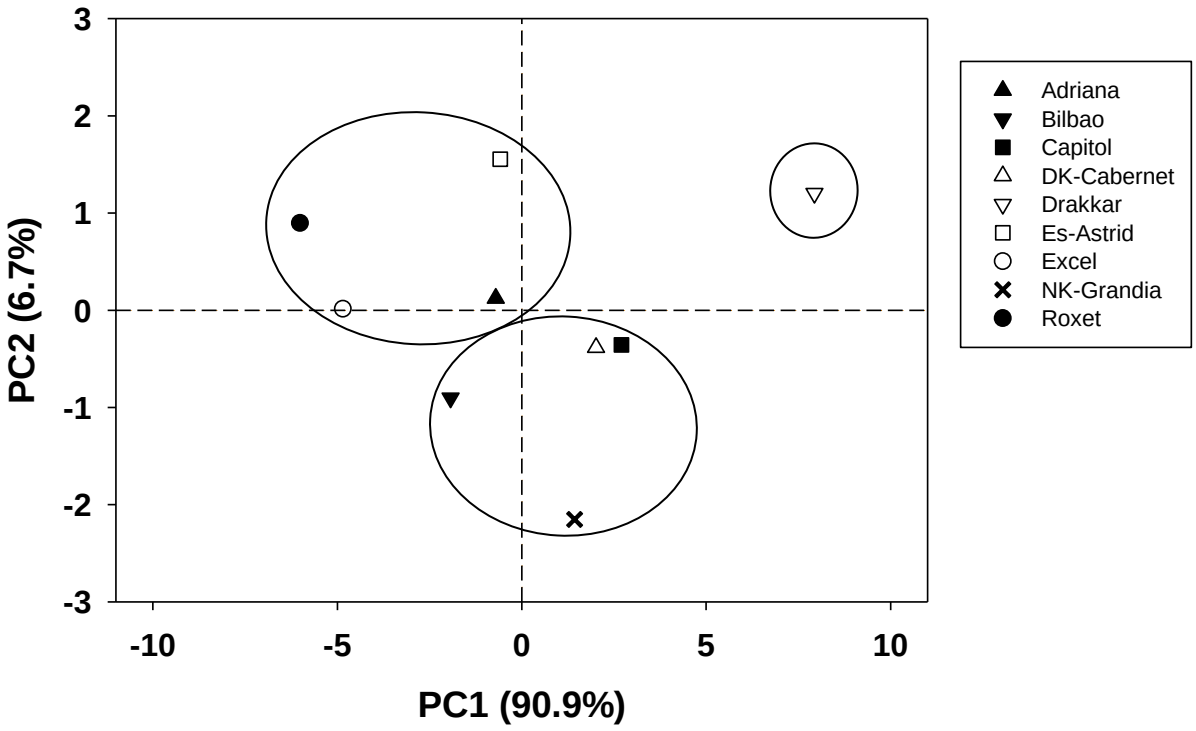


Fig. 3.34: Principal component (PC) analysis showing relative position of nine cultivars in relation to principal component one (PC1 positive; stem canker severity and negative; mean number of *L. biglobosa* phoma leaf spots per plant and principal component two (PC2 positive; mean number of *L. maculans* phoma leaf spots per plant). Data are means from 30 plants per cultivar of winter oilseed rape in 2012/2013 growing season. The two principal components combined accounted for 97.6% of the variation in the data. There were nine cultivars, four with *R*-gene resistance against *L. maculans* only: (Bilbao (*Rlm4*), Capitol (*Rlm1*), Excel, (*Rlm7*) and Roxet (*Rlm7*)); two with QR only: (Es-Astrid and NK Grandia), two with *R*-gene and QR (Adriana (*Rlm4* +QR), DK Cabernet (*Rlm1* + QR)) and cv. Drakkar (susceptible to *L. maculans*).

3.3.7 Seasonal differences in weather in the 2010/2011, 2011/2012 and 2012/2013 growing seasons

In the autumn when leaves of winter oilseed rape start to grow, the weather conditions (rainfall and temperature) are crucial for *Leptosphaeria* ascospores to be released from pseudothecia on stem debris and to germinate once they are attached onto the leaf surfaces to initiate the development of phoma leaf spotting. Therefore, rainfall and temperature data at Rothamsted Research were obtained in this study for three growing seasons. In the 2010/2011 growing season, the weather was normal (Fig. 3.35a) (Table 3.9) and from August until December 2010 the total amount of rainfall was 362.26 mm and average temperature was 8.9^{o}C (Fig. 3.35a). In the 2011/2012 growing season, there was a period of dry weather from August until November (Fig. 3.35b) and only in December 2011 was there more rainfall, which lasted until early January 2012 (Fig. 3.36b). The total amount of rainfall was 263.67 mm and average temperature was 11.8^{o}C from August until December in the 2011/2012 growing season (Fig. 3.35b). However, there was a wet autumn in the 2012/2013 growing season and from August until December the total amount of rainfall was 425.69 mm and average temperature was 10.3^{o}C (Fig. 3.35c). The greatest amount of rainfall in autumn 2012 was in October and December, when average temperatures were 9.7^{o}C and 4.5^{o}C, respectively. In December 2012, there was a period when temperature was < 0^{o}C causing frost on the experimental field plot (Fig. 3.37). Frost was observed from 5 to 7 December 2012 (average temperature 0.37^{o}C) and 11 to 13 December 2012 (average temperature -1.49^{o}C) (Fig. 3.35c).

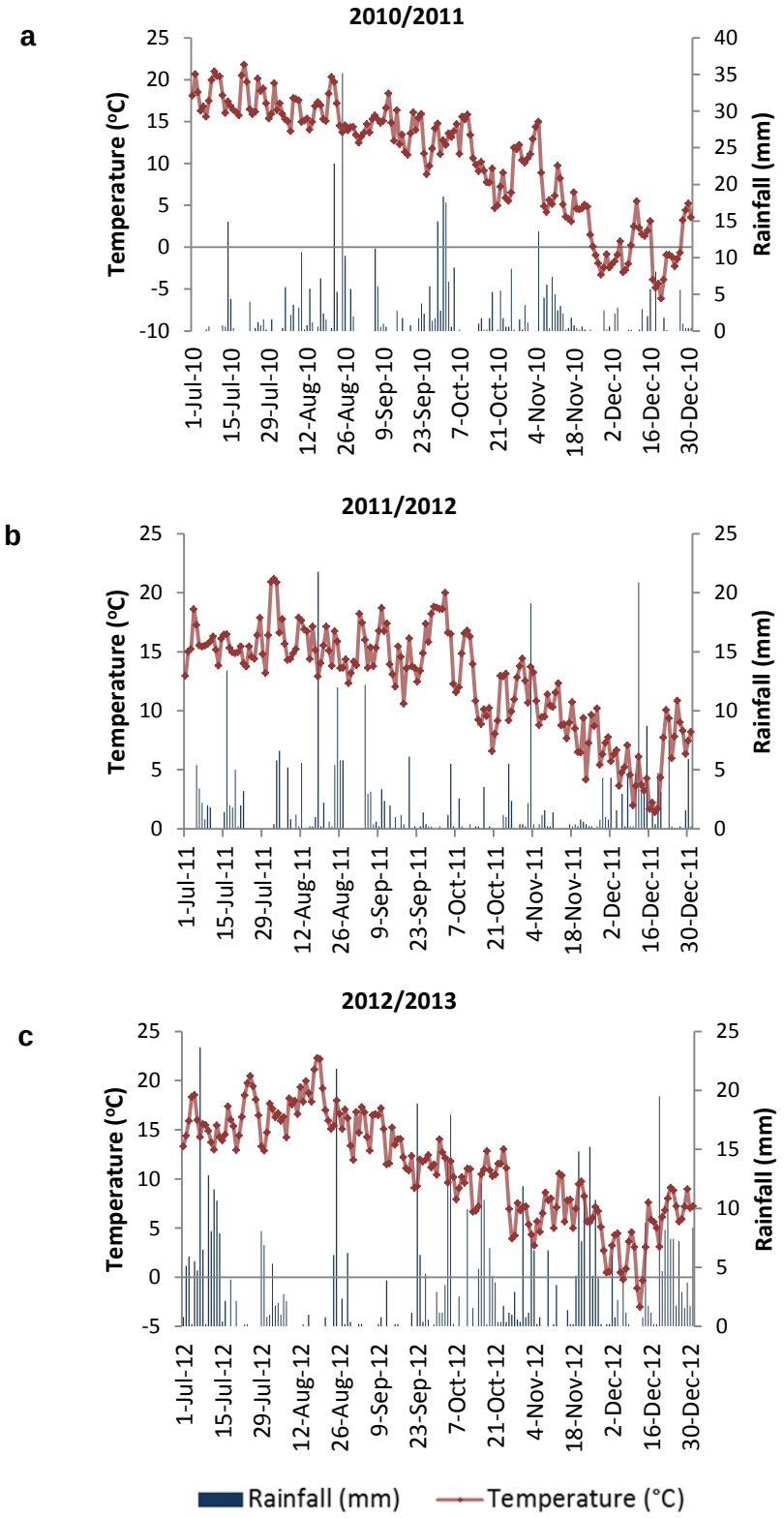


Fig. 3.35: Weather parameters recorded by Rothamsted weather station; total daily rainfall (mm) and average temperature (^{o}C) from 1 July 2010 until end of December 2010 (a) from from 1 July 2011 until end of December 2011 (b) from 1 July 2012 until end of December 2012 (c) at Rothamsted Research, Harpenden.

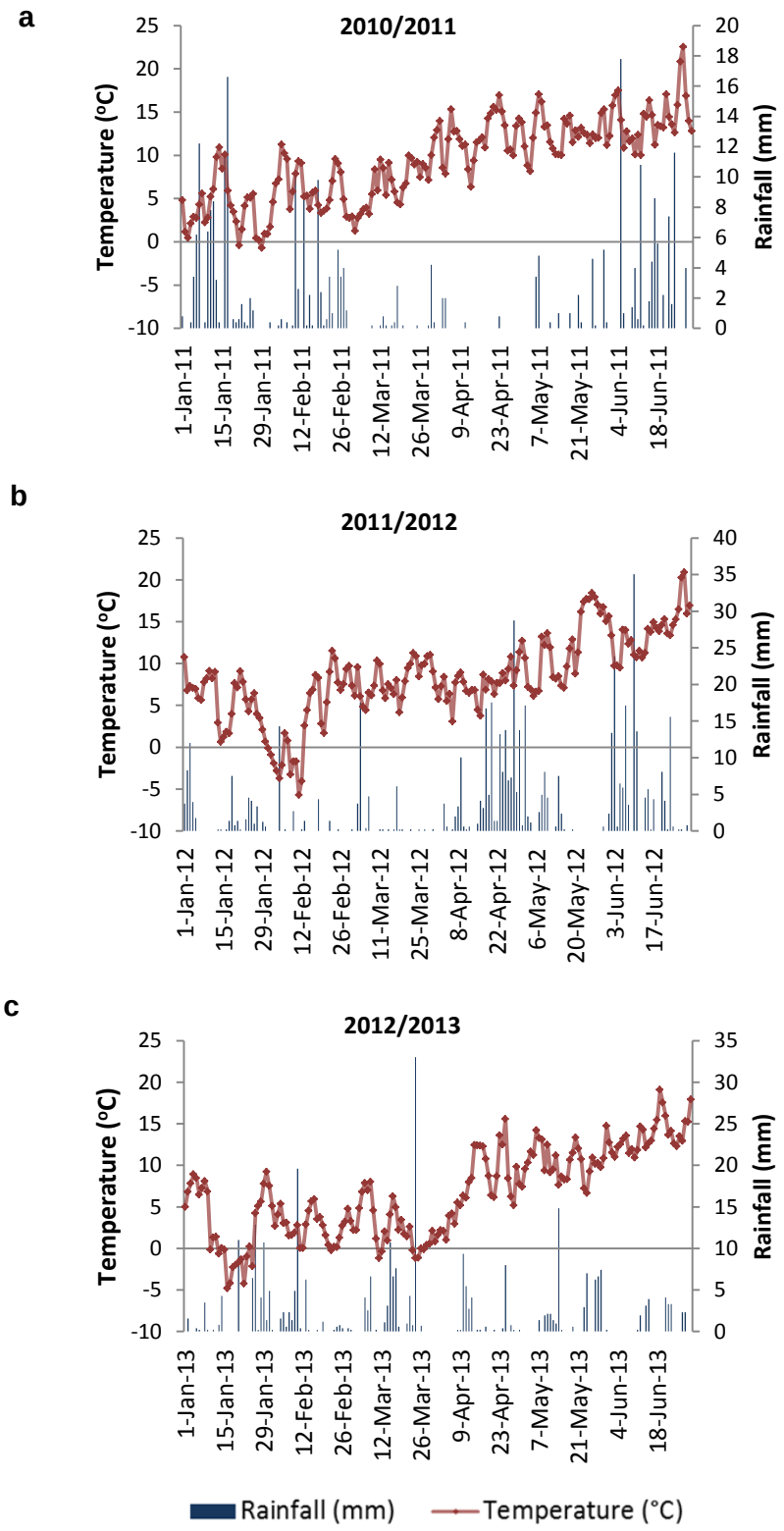


Fig. 3.36: Weather parameters recorded by Rothamsted weather station; total daily rainfall (mm) and average temperature (^{o}C) from 1 January 2011 until end of June 2011 (a) from 1 January 2012 until end of June 2012 (b) from 1 January 2013 until end of June 2013 (c) at Rothamsted Research, Harpenden.

Growth stage		Summer	Autumn			Winter			Spring			Summer	
		Aug	**Sept**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**July**
Cultivation and sowing													
Leaf production													
Stem extension													
Flower bud development													
Flowering													
Pod development													
Seed development													
Senescence													
Harvest													
2010/2011													
mean temperature	°C	15.8	13.8	10.5	5.1	0.3	3.8	6.2	6.5	12.2	12.5	14.3	15.4
rainfall	mm	4.11	1.99	2.74	1.83	1.14	2.73	2.03	0.32	0.17	0.76	2.77	1.44
2011/2012													
mean temperature	°C	15.8	15.3	12.8	9.3	5.7	5.5	3.2	7.9	7.4	12.0	13.8	15.7
rainfall	mm	2.62	1.28	0.81	1.21	2.66	1.87	0.85	1.12	5.62	1.70	5.55	4.14
2012/2013													
mean temperature	°C	17.2	13.3	9.7	6.5	4.5	2.7	2.7	2.5	7.6	10.5	13.7	18.7
rainfall	mm	1.77	1.35	3.74	3.35	3.68	2.02	1.55	2.68	1.09	1.81	0.82	1.53

Table 3.9: Growth stage of winter oilseed rape (autumn sown) in the UK and weather parameters recorded by the Rothamsted weather station; mean temperature (°C) and rainfall (mm) for each month of three growing seasons (2010/2011, 2011/2012 and 2012/2013). Monitoring of phoma leaf spotting was from September until early February for spray thresholds and generally phoma leaf spot sprays were first applied between October and December when 10% of oilseed rape plants had symptoms of phoma leaf spot (Source: HGCA, 2012 and http://www.rothamsted.ac.uk/Content.Section=Leafspot).

Fig. 3.37: Plants of each cultivar covered with frost at Rothamsted Research experimental plots; (a) cv. Drakkar (no *R* gene) plot with few plants, (b) cv. Roxet (*Rlm*7) plot with more plants, (c) frost on plants of cv. Roxet (*Rlm*7) and (d) experimental plot for 2012/2013 growing season (21 November 2012) (refer Fig. 3.26 for average temperature and total daily rainfall in autumn/winter 2012/2013).

3.4 Discussion

The results showed an effect of cultivar (with different types of resistance) on the proportions of *L. maculans* and *L. biglobosa* in phoma leaf spots and phoma stem cankers. Cultivar Drakkar had more *L. maculans* leaf spotting than other cultivars with or without *R* genes or QR (Table 3.3). Cultivars with QR, such as Es-Astrid and NK Grandia had more *L. maculans* leaf spotting when compared with resistant cultivars, such as Capitol (*Rlm1*), Excel (*Rlm7*) and Roxet (*Rlm7*) (Fig. 3.10, 3.11c-d, Table 3.3). Even though more of phoma leaf spotting was observed on cultivar with QR but the stem cankers were less severe (Fig. 3.24) considering that QR operates later in the leaf petiole and stem tissues (Fitt *et al.,* 2006a; Huang *et al.,* 2009).The cultivar Drakkar with no *Rlm* gene or QR had more *L. maculans* leaf spotting than *L. biglobosa* leaf spotting in the 2011/2012 and 2012/2013 growing seasons (Fig. 3.10, 3.11). In 2011/2012 and 2012/2013 growing seasons, results showed that cultivars with the resistance gene *Rlm7* (cvs Roxet and Excel) are still effective; they both had less *L. maculans* phoma leaf spotting than cv. Drakkar (Fig. 3.10, 3.11). Latest field experiments suggest that cultivars with *Rlm7* are still resistant even though the cultivars carrying this gene have been commercially available for more than seven years (Clarke, 2014). Nevertheless, in this study, there was an increasing of number of *L. biglobosa* leaf spots on leaves of cultivars Roxet and Excel (with resistance gene *Rlm7*) in two growing seasons (Fig. 3.10, 3.11). This may have been because the resistance gene (*R*) only recognised the *L. maculans* effector (*Avr*) gene (Rouxel *et al.,* 2003) but did not respond to *L. biglobosa* or because the resistant cultivar was more susceptible to *L. biglobosa* than to *L. maculans*.

A 1980s survey (Humpherson-Jones, 1986) showed that half of isolates collected from brassica leaves and stems were identified as *L. maculans*. The 2010 survey

results indicate that proportion of *L. maculans* in stem cankers was greatest in southern England and that *L. biglobosa* was mostly in northern England (Stonard *et al.,* 2010). *Leptosphaeria maculans* has been the predominant species in England for some decades. However, in this study, the results show that there was an increasing proportion of *L. biglobosa,* when isolate identity was confirmed by species-specific PCR. In the 2011/2012 growing season, 62.5% of isolates from phoma leaf spots were *L. biglobosa* and in the 2012/2013 growing season 41.5% were *L. biglobosa* (Table 3.4).

Generally, typical *L. maculans* leaf spotting is observed first and *L. biglobosa* leaf spotting is seen later (West *et al.,* 2002b, Toscano-Underwood *et al.,* 2003). However, in 2012/2013 growing season, results showed more *L. biglobosa* leaf spotting on leaves sampled from October to December 2012 (Fig. 3.11a-c). In December 2012, there was a period of frost that made affected leaves drop off. By January 2013, there was more *L. maculans* leaf spotting on all cultivars (Fig. 3.11d).

This results (Fig. 3.13 and 3.14) suggest that there was more of *L. biglobosa* DNA in both basal stem cankers and upper stem lesions of nine winter oilseed rape cultivars in all three growing seasons, based on the quantitative PCR results. The results also contradict previous reports that *L. biglobosa* is more predominant in upper stem lessions than basal stem cankers (Fitt *et al.,* 2006a). The cultivars that were most resistant to *L. maculans* were not resistant to *L. biglobosa*, with cvs Excel and Roxet (*Rlm7*) having the greatest proportion of *L. biglobosa* (Fig. 3.13, 3.14).

All cultivars were divided into different groups based on the principal component (PC) analysis (Fig. 3.22, 3.23, 3.24). The cv. Drakkar, susceptible in all three growing

seasons, was isolated from other groups that consisted of cultivars with *R* genes and QR (Fig. 3.22, 3.23, 3.24). Although cvs Es-Astrid (QR) and Roxet (*Rlm7*) were in the same group with more *L. maculans* phoma leaf spots per plant in 2010/2011 growing season (Fig. 3.22) and in 2013/2014, cvs Adriana (*Rlm4* + QR), Es-Astrid (QR), Roxet (*Rlm7*) and Excel (*Rlm7*) were grouped as having more *L. maculans* phoma leaf spots per plant but less stem canker development (Fig. 3.24). This study showed that cultivars with *Rlm7* (Excel and Roxet) were effective for three growing seasons because the *L. maculans* population was not virulent against *Rlm7*. However, when cultivars with a single resistance gene bred into oilseed rape from a related species *B. rapa* ssp. *sylvestris* (cv. Surpass 400) were released, the resistance broke down within three years (Sprague *et al.,* 2006a). There was no background quantitative resistance (QR). A less dramatic breakdown in resistance of oilseed rape occurred in France where over a 10 year period the resistance gene (*Rlm1*) became ineffective (Rouxel *et al.,* 2003, Sprague *et al.,* 2006a). Therefore, combinations of *R* gene and QR should be accumulated in oilseed rape as a primary objective and as the foundation for disease management. There was more *L. biglobosa* on leaves of other cultivars with *R* genes and QR. The PC analysis results separated cvs Roxet, Bilbao, Capitol, Excel, NK Grandia into groups with more *L. biglobosa* phoma leaf spots per plant in the 2011/2012 growing season (Fig. 3.23) and cvs Roxet, Es-Astrid, Excel, Adriana, Bilbao (Fig. 3.24) in the 2012/2013 growing season.

This study suggests that the assessment of early symptoms (phoma leaf spots) is not consistent with the final assessment of stem canker severity or upper stem lesion severity (Table 3.9). Therefore, when screening cultivars for phoma stem canker resistance, early symptoms like number of leaves or numbers of phoma leaf spots

with *L. maculans* or *L. biglobosa* are not reliable to indicate the cultivar resistance in terms of stem canker severity. It has been suggested that the measurement of areas of phoma leaf spot might provide a more accurate assessment of quantitative 'field' resistance than assessing the numbers of phoma leaf spots in field experiments in autumn (Powers *et al.,* 2010). Huang *et al.* (2014) showed that there is a good positive relationship between leaf lesion area and distance grown by *L. maculans* along the petiole towards the stem with large leaf spots causing more severe stem cankers. Although, Hood *et al.* (2007) observed a poor correlation between severity of phoma leaf spots and severity of stem base canker, there are also results which confirm a correlation between early occurrence of leaf spots in autumn and severe basal stem canker (Steed *et al.,* 2007). There were also several leaves observed with small necrotic spots as if cell death had occurred (Fig. 3.32). However, Stotz *et al.* (2014) proposed that *L. maculans* colonises the apoplast slowly and gets adapted to its constitutive antimicrobial compounds. Therefore, the early stages of colonisation growth slower (4-36 days after infection), so that resistance responses are sometimes delayed. These host defense responses (ETI) against *L. maculans* are not able to eliminate the pathogen like ETI (effector-triggered immunity) (Jones & Dangl, 2006) and the pathogen does not die but it can resume growth when host senescence occurs (Stotz *et al.,* 2014).

The results of this study show that temperature and rainfall affect the onset of phoma leaf spotting and subsequent development of phoma stem canker. In the 2011/2012 growing season, the onset of phoma leaf spotting was later (25 January 2012) than in the 2010/2011 (9 December 2010) and 2012/2013 growing seasons (24 October 2012). As the primary inoculum for phoma stem canker epidemics is ascospores produced from the pseudothecia on the crop debris from the previous season and

ascospore discharged is affected by temperature and rainfall (refer Chapter 4), the difference between the date of onset of phoma leaf spotting in the 2011/2012 growing season and the dates of onset in the other two seasons was due to dry weather. The date of first major ascospore release is when >10 ascospores m^{-3} air are sampled on tapes collected from a Burkard spore sampler (Huang *et al.,* 2005). The 2012/2013 field experiment was sown in early September 2012, a month before the start of ascospore release (>10 ascospores m^{-3} air) and ascospore continued until mid-February 2013 (refer Chapter 4). In the 2011/2012 growing season, the ascospore release was very late, starting in November but the greatest ascospore release was in mid-January 2012 (refer Chapter 4). This might be the reason why the incidence of phoma leaf spotting and subsequent phoma stem cankers was less in this season than in the 2010/2011 and 2012/2013 growing seasons.

The results of this study show that the phoma stem canker severity for all nine cultivars differed between the 2010/2011, 2011/2012 and 2012/2013 growing seasons. Generally stem canker severity was less in 2012 (dry autumn) because of late ascospore release, than in 2011 and 2013 (wet autumn). Cultivar Drakkar had the greatest canker severity in all three growing seasons (Fig. 3.35). It was susceptible to *L. maculans* and had weak stems with a smaller diameter than those of other cultivars (Fig. 3.33). Invasion of a weak stem may result in lodging and death of the plant (Hammond & Lewis, 1986a; West *et al.,* 2001). Once the hypocotyls become lignified they are resistant and cultivars in which lignification is delayed could have less resistance than cultivars that are lignified earlier (Bruno *et al.,* 1990).

This study showed that cultivars with *R* genes such as cvs Capitol (*Rlm1*) was not effective with more *L. maculans* phoma leaf spotting and greater stem cankers severity than those with combination with QR (cvs Adriana and DK Cabernet) (Fig. 3.9, 3.10, 3.11 and 3.24). The *Rlm1* gene is fully overcome under European conditions (Balesdent *et al.,* 2005) and is no longer effective. However, there were effects of background quantitative resistance on the effectiveness of *R* gene resistance for cvs Adriana (*Rlm4* + QR) and DK Cabernet (*Rlm1* + QR) (Fig. 3.24). It shows that the resistance genes *Rlm1* and *Rlm4* were more effective when they were in a background with quantitative resistance than cultivars without quantitative resistance such as cvs Bilbao (*Rlm4*) and Capitol (*Rlm1*) (Fig. 3.24). The resistance gene *Rlm7* (presents in cvs Excel and Roxet) is still effective against *L. maculans* and it has been commercially available for more than seven years (Clarke, 2014). In this study, over the three growing seasons, it shows that resistance gene *Rlm7* (cvs Roxet and Excel) and *Rlm4* (cv. Bilbao) had less severe phoma leaf spotting than other cultivars in autumn (Table 3.4). The *Rlm7* (cvs Roxet and Excel) also had less phoma stem canker than other cultivars (Fig. 3.24). These results showed that the *Rlm7* is more effective than other *R* genes commercially available.

Chapter 4

Effects of cultivar resistance and weather on pseudothecial development of *Leptosphaeria* spp. on stems of nine cultivars

4.1 Introduction

In the UK, stem canker epidemics are initiated in autumn by air-borne ascospores produced in pseudothecia on infected crop debris from previous crops (West *et al*., 1999a; Huang *et al.,* 2005). It is in the necrotrophic phase in the dead plant tissues where *L. maculans* and *L. biglobosa* produce the pseudothecia (sexual fruiting bodies) that will go through the process of sexual recombination to produce widely dispersed wind-borne ascospores (Fitt *et al.,* 2006a; Sprague *et al.,* 2006b; Gout *et al.,* 2006b). Pseudothecia must reach complete maturity for each ascus to produce and release its eight fully developed ascospores, each consisting of six cells. Ascospores land on the leaf surfaces, germinate and infect the leaf under favourable conditions to cause leaf lesions (phoma leaf spots) (Toscano-Underwood *et al*., 2001). The pathogen can then grow along the petiole to reach the stem, causing stem base canker (often *L. maculans*) and upper stem lesions (often *L. biglobosa*) (West *et al*., 2002b; Huang *et al.*, 2006b; Fitt *et al.,* 2006a). Therefore, the proportions of the two species in the UK populations have been shown to affect the severity of stem canker epidemics (Stonard *et al.,* 2010). Early infection in autumn (September or October) can often lead to development of severe stem base canker before harvest, resulting in substantial yield loss (Zhou *et al*., 1999; Sun *et al.,* 2001).

Temperature and wetness are the two main factors affecting rate of maturation of ascospores of *L. maculans* and *L. biglobosa* and thus the timing of their release (Toscano-Underwood *et al.,* 2003; Aubertot *et al.,* 2006; Kaczmarek *et al.,* 2010). In

the UK, the period of ascospore release continues for several months from autumn to spring and it has been proven that the concentration of ascospores is much greater in autumn than in the spring (Gladders & Musa, 1980; West *et al.,* 2002a; Huang *et al.,* 2005; Kaczmarek *et al.,* 2010). Kaczmarek *et al.* (2010) reported that in Poland more *L. maculans* ascospores than *L. biglobosa* ascospores were released in the autumn. Results from four years of UK field experiments showed that *L. maculans* ascospores were released before *L. biglobosa* ascospores (West *et al.,* 2002b; Huang *et al.,* 2011). In central Canada, Petrie (1994) showed that the optimum temperature for ascospore production by *L. maculans* was 15^{o}C, whereas in western Canada ascospore dispersal was maximal several hours after rainfall of ≥2mm (Guo & Fernando, 2005). The same weather conditions were observed in France, where the first release of ascospores usually occurred after rainfall at ≤15°C in a period of decreasing temperature at the end of summer (Pérès *et al.,* 1999a). In Australia, ascospores were released in late autumn or early winter to infect winter oilseed rape seedlings (McGee, 1977). Additionally, Marcroft *et al.* (2003) reported that in all Australian regions, fields with 6 month-old stem debris released 30 times more ascospores than fields with older stem debris (18-42 month old). In the UK, where the climate differs from that of France, Canada and Australia, with its relatively mild wet weather, mature pseudothecia can be observed at sites of severe cankers on stem bases shortly after harvest (West *et al.,* 1999a). Under continuous wetness, pseudothecia of both species matured faster at 20^{o}C than at 5^{o}C in controlled environment conditions (Toscano-Underwood *et al.,* 2003).

Another factor to consider is the structure of pseudothecia, where those of *L. biglobosa* have a longer neck than those of *L. maculans* (Shoemaker & Brun, 2001). Therefore, Kaczmarek *et al.* (2010) suggested that the longer necks of pseudothecia

formed by *L. biglobosa* might result in slower release of ascospores over time whereas ascospores of *L. maculans* are released more easily and uniformly over time from pseudothecia of *L. maculans* with shorter necks (Kaczmarek *et al.,* 2010).

Previous work on pseudothecial development was based on four classes and only differentiation of ascospores at Class C and Class D stages was described, whereas the differentiation of pseudothecia and asci was not included (Bernard *et al.,* 1999). Toscano-Underwood *et al.* (2003) had modified the classification of Bernard *et al.* (1999) and included descriptions for asci and ascospores at all classes of maturity (Classes A, B, C, D and E).

Weather-based models for forecasting the timing of ascospore release can be used to guide the timing of fungicide applications (Huang *et al.,* 2007; Salam *et al.,* 2007). This will help to manage epidemics at the phoma leaf spot stage in autumn/winter and currently phoma leaf spot forecasts operated by Rothamsted use a threshold of 10% plants affected by phoma leaf spotting for guiding fungicide spray decisions (http://cereals.ahdb.org.uk/monitoring.aspx and http://rothamsted.ac.uk/tools). However, this threshold does not distinguish *L. maculans* leaf spots from those of *L. biglobosa* and its emphasis is more on controlling *L. maculans* leaf spots that are believed to be associated with more severe basal cankers and greater yield loss in the following summer. Therefore, foliar fungicides must be applied in time to prevent the pathogens reaching the stems to cause stem cankers (Gladders *et al.,* 2006). However, the phoma stem canker pathogens *L. maculans* and *L. biglobosa* differ in their sensitivity to triazole fungicides (Eckert *et al.,* 2010; Huang *et al.,* 2011). Thus, to optimise the use of fungicide for successful management of phoma stem canker in

winter oilseed rape, information on relative proportions of the two species is essential.

It is difficult to distinguish between ascospores of *L. maculans* and those of *L. biglobosa* in samples collected from air by visual methods but they can be distinguished after they germinate (Huang *et al.,* 2001; Huang *et al.,* 2011). However, this technique is time-consuming and it is not practical because the density of ascospores on the spore sampler tape is usually great and spores will overlap when they germinate. However, by using quantitative PCR (qPCR) it is possible to investigate the relative proportions of the two species in air samples (Kaczmarek *et al.,* 2009; Huang *et al.,* 2011). Analysis of air samples to distinguish the timing of *L. maculans* and *L. biglobosa* ascospore release is important for finding effective fungicide applications.

There has been work on survival of *L. maculans* pseudothecia on stem debris over time and effect of weather conditions on pseudothecial development (Petrie, 1995; Pérès *et al.,* 1999a; Huang *et al.,* 2003a; Toscano-Underwood *et al.,* 2003; Huang *et al.,* 2007; Lô-Pelzer *et al.,* 2009a) but there has been little work on effects of cultivar resistance on pseudothecial development. In the case of stem cankers, Hammond and Lewis (1986a), Petrie (1995) and Lô-Pelzer *et al.* (2009a) showed that the quantity of primary inoculum on stubble was greater when the canker on the plant was more severe. Previous work in Australia showed that the quantity of primary inoculum produced was greater in fields where the disease severity was greater (McGee, 1977). This was shown for cultivars with specific resistance (Marcroft *et al.,* 2004a) but the effect of quantitative resistance (QR) on the production of ascospores has not yet been studied (Poisson & Pérès, 1999; Marcroft *et al.,* 2004a). There is a

potential for host resistance to reduce the production of pseudothecia and ascospores on stubble of oilseed rape (Marcroft *et al.,* 2004a). The effect of cultivar on pseudothecial development of *L. maculans* and *L. biglobosa* is not clearly understood. Therefore, this study examined the effect of cultivar resistance (with or without *R*-genes or QR) on pseudothecial development, based on the hypothesis that a combination of *R*-gene and QR may delay the pseudothecial development.

Objectives

1. To determine effects of cultivar resistance on maturation of pseudothecia of *L. maculans* or *L*. *biglobosa* on stems exposed in a controlled environment (20^{o}C) or exposed at different time points in natural conditions during the autumn/winter in 2012/2013 and 2013/2014.

2. To determine effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia of *L. maculans* and *L*. *biglobosa* on stems in the 2011/2012, 2012/2013 and 2013/2014 growing seasons.

3. To investigate effects of weather conditions (temperature and rainfall) on ascospore discharge from pseudothecia in three growing seasons (autumn/winter 2011/2012, 2012/2013 and 2013/2014).

4.2 Materials and methods

4.2.1 Effects of cultivar resistance on pseudothecial maturation on stems in a controlled environment (20^{o}C) or exposed at different times in natural conditions during the autumn/winter in 2012/2013 and 2013/2014.

There were four cultivars, with or without *R*-genes and QR, that were selected [cvs Drakkar (susceptible to *L. maculans*), Adriana (*Rlm4* + QR), Bilbao (*Rlm4*) and NK Grandia (QR)] to observe the maturation of pseudothecia on stems in controlled conditions (20^{o}C) (two experiments were done, in 2013 and 2014, respectively) and in natural conditions at different times during the autumn/winter 2012/2013 (September 2012 and December 2012) and autumn/winter 2013/2014 (October 2013 and January 2014) at the Bayfordbury experimental plot.

4.2.1.1 Preparation of infected stems

For this experiment, winter oilseed rape stems (cvs Drakkar, Adriana, Bilbao and NK Grandia) were collected after harvest from the field experimental plot at Rothamsted, Harpenden in early August 2012 and August 2013. Stems 20-25 cm in length, including the tap roots and crowns, were selected. A total of 80 stems for each cultivar were collected and dried at 20^{o}C. Only 20 pieces of stem per cultivar were used after being kept in a freezer (-15^{o}C) until required.

4.2.1.2 Incubation of infected stems exposed in a controlled environment or exposed at different times in natural conditions during the autumn/winter in 2012/2013 and 2013/2014

The 20 pieces of stem for each cultivar were placed in a tray containing wet sand (2 cm deep) for exposure in a controlled environment (20^{o}C, continuous wetness) (Fig. 4.1). Stems were kept continuously wet by spraying them with rain water (pH: 5.7) twice a day (morning and afternoon) in the controlled environment cabinets with a fluorescent light intensity of 200-210 μem^{-2}s^{-1}, 70-85% relative humidity and 12h day-length. Table 4.1 shows details of stems being exposed at different times in natural conditions at the Bayfordbury experimental plot and in controlled conditions in the

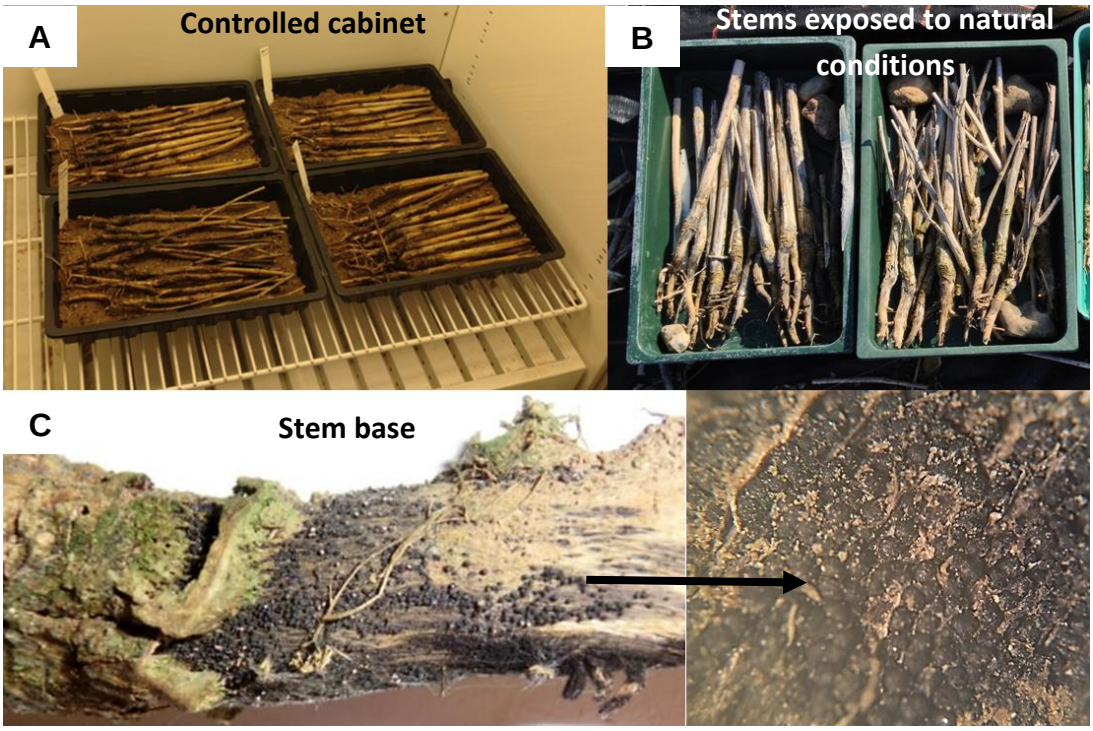


Fig. 4.1: Stems from selected cultivars; Drakkar (susceptible to *L. maculans*), Adriana (*Rlm4* + QR), Bilbao (*Rlm4*) and NK Grandia (QR) (A) in a controlled environment cabinet (temperature: 20^{o}C, 75 – 80% RH, continuous wetness by spraying twice a day with rain water pH 5.7, 12h day length, 205µem^{-2}s^{-1}), (B) in natural conditions at Bayfordbury, Hertfordshire, autumn/winter 2012/2013 (stems were exposed on 5 September 2012 and 28 November 2012) and 2013/2014 (stems were exposed on 9 October 2013 and 8 January 2014), and 2 weekly assessments of (C) pseudothecial (arrow) development on stems (stem base and upper stem) over a period of 70 days.

Table 4.1: Stems (stem bases and upper stems) were exposed at different times in natural conditions at the Bayfordbury experimental plot during the autumn/winter in 2012/2013 and 2013/2014. In the controlled environment cabinet, stems were incubated at 20^{o}C with continuous wetness and a 12h day-length. There were two experiments in controlled environment conditions, completed in 2013 and in 2014. The observation of pseudothecial development was over a period of 70 days (Classes A to E).

Different time points	Date	
[a]In natural conditions	Day 1 (Date experiment started)	Day 70 (Date experiment ended)
Autumn/winter 2012/2013	5 September 2012	14 November 2012
	28 November 2012	6 February 2013
Autumn/winter 2013/2014	9 October 2013	18 December 2013
	8 January 2014	19 March 2014
In controlled conditions (Controlled cabinet 20^{o}C, continuous wetness, sprayed with rain water pH 5.7, 70-80% RH, 12h day length, 205µem^{-2}s^{-1}).		
2013[b] 2014[b]	70 days observation of pseudothecial development	

[a]Refer to Appendix 4.1 for weather conditions at the Bayfordbury experimental plot.
[b]Results were average of two experiments on pseudothecial development, completed in 2013 and in 2014, respectively.

controlled environment cabinet at College Lane, University of Hertfordshire. The experiment in the controlled environment cabinet was done twice (in 2013 and 2014).

4.2.1.3 Assessment of pseudothecial maturation, density and proportions of *Leptosphaeria* species on stems (stem bases and upper stems)

Stems were sampled once every two weeks to assess the maturation stage of pseudothecia (Classes A – D) until pseudothecia released ascospores (Class E) (Fig. 4.2). Five pseudothecia were randomly excised from each stem by using forceps and placed on a glass slide in a drop of distilled water. Then a cover slip was placed on the slide and pressure was applied to force the asci out. The pseudothecial maturation Classes A–D were based on the stage of development of asci and ascospores; Class A: asci and ascospores undifferentiated (early stage of maturation), Class B: asci differentiated and ascospores undifferentiated, Class C: asci and ascospores differentiated and Class D: asci mature with 8 spores/ascus (Fig. 4.2) (Toscano-Underwood *et al.*, 2003). In this study, Class E was included and considered as empty pseudothecia with ascospores released (as described in Toscano-Underwood *et al.*, 2003). The numbers of pseudothecia in each maturity class were recorded for all nine cultivars.

At the end of the experiments (when all the pseudothecia were mature), three stems with abundant pseudothecia were chosen per cultivar. All stems were cut into small pieces (0.8 × 5 cm) at the stem bases (< 5 cm above ground level) and upper stems (> 10 cm above ground level). The number of pseudothecia on each piece was counted under a binocular stereo-microscope (GX microscopes model XTC3A1) (at ×600 magnification). The density (number per cm^2) of pseudothecia on each stem was calculated by taking the mean from all the stem pieces (stem bases and upper

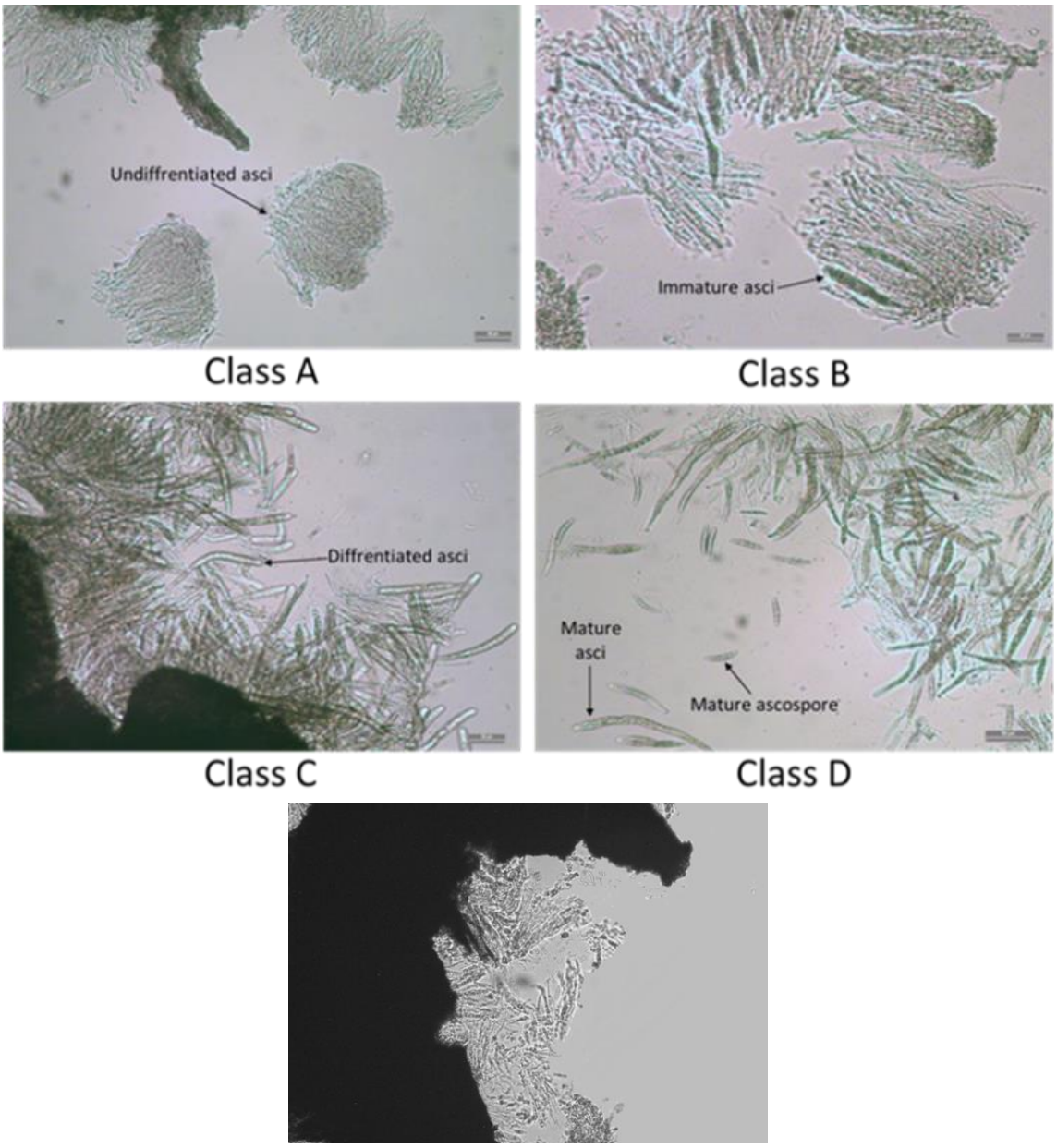


Fig. 4.2: Pseudothecial maturation; Classes A – D were based on the stage of development of asci and ascospores. Class A: asci and ascospores undifferentiated, Class B: asci differentiated and ascospores undifferentiated, Class C: asci and ascospores differentiated, Class D: asci mature with 8 spores/ascus and Class E: empty pseudothecium and no ascospores observed under x400 magnification. (Toscano-Underwood *et al.,* 2003).

stems) from each of the three stems per cultivar. Finally, the pseudothecia on stem base and upper stem pieces (0.8 × 5 cm) were moistened to release ascospores onto distilled water agar (DWA) plates by sticking the stem pieces on the Petri dish lids with petroleum jelly. Fifty ascospores that were discharged from mature pseudothecia (Class D) were isolated using a fine needle and transferred to new PDA plates for identification as *L. maculans* or *L. biglobosa* (please refer Chapter 2, Fig. 2.3).

4.2.1.4 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. The T-test and analysis of variance (ANOVA) were done using the SPSS version 21 statistical package. Results for controlled environment conditions were the average of two experiments completed in 2013 and 2014 over 70 days of pseudothecial development on stems.

4.2.2 Effects of cultivar resistance and weather on maturation of pseudothecia of *Leptosphaeria* species under natural conditions in the 2011/2012, 2012/2013 and 2013/2014 growing seasons

There were nine cultivars of winter oilseed rape planted in the field experiments at Rothamsted Research, Harpenden, Hertfordshire, in the 2010/2011, 2011/2012 and 2012/2013 growing seasons. Stems were collected after harvest for observation of pseudothecial development. The cultivars had different combinations of resistance (*R*) genes or were without *R* genes; some had quantitative resistance (QR) (Delourme *et al.*, 2006). The cultivars were Adriana (*Rlm 4* + QR), Bilbao (*Rlm 4*), Capitol (*Rlm 1*), Drakkar (no *R* gene against *L. maculans*), DK Cabernet (*Rlm 1* + QR), Es-Astrid (QR), Excel (*Rlm 7*), NK Grandia (QR) and Roxet (*Rlm 7*).

4.2.2.1 Preparation of infected stems

Stems of the nine winter oilseed rape cultivars that were collected after harvest in early August 2011, August 2012 and August 2013 from the Rothamsted field experiment were incubated under natural conditions at the Bayfordbury experimental plot at the University of Hertfordshire field station. A total of 150 stems (Fig. 4.3) were collected per cultivar (9 cultivars x 150 = 1350 stems) and dried at 20^{o}C (method described in 4.2.1.1).

4.2.2.2 Infected stems exposed to natural conditions

The experimental plot situated at Bayfordbury, Hertfordshire was used for the observation of pseudothecial maturation. Stems of the nine oilseed rape cultivars that were collected after harvest were exposed to natural conditions in autumn/winter 2011/2012, 2012/2013 and 2013/2014. The exposure and observation of pseudothecial development started on 20 September 2011 and continued until 20 December 2012 (stems from the 2010/2011 growing season), from 5 September 2012 until 28 November 2012 (stems from the 2011/2012 growing season) and was from 18 September 2013 until 11 Decemeber 2013 (stems from the 2012/2013 growing season).

4.2.2.3 Bayfordbury weather data

Daily meteorological data (temperature and rainfall) at Bayfordbury from 0900 h GMT to 0900 h GMT on the following day were obtained from daily monitoring by a Davis weather station for every hour (Davis Instruments, San Francisco Bay Area, USA). A day with >0.2mm rain between 0900 h GMT and 0900 h GMT the next day was counted as one rain-day.

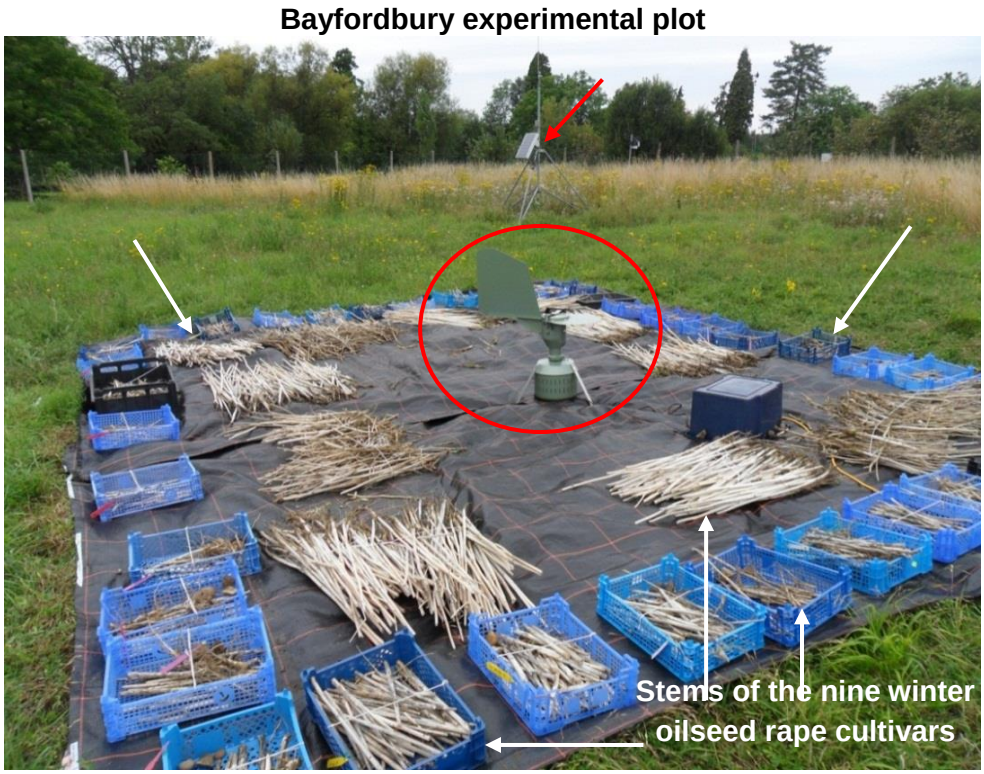


Fig. 4.3: A total of 150 stems per cultivar of Adriana (*Rlm 4* + QR), Bilbao (*Rlm 4*), Capitol (*Rlm 1*), Drakkar (no *R* genes), DK Cabernet (*Rlm 1* + QR), Es-Astrid (QR), Excel (*Rlm 7*), NK Grandia (QR) and Roxet (*Rlm 7*) (white arrows) were placed around a Burkard spore sampler (in circle) at Bayfordbury. The Burkard sampler was used to collect ascospores released from pseudothecia on the stems, with daily monitoring of the weather parameters done by a Davis weather station (red arrow).

4.2.2.4 Assessment of pseudothecial maturation, density and proportions of *Leptosphaeria* species on stems (stem bases and upper stems)

From each of the nine cultivars, five stems were sampled weekly at random to monitor the pseudothecial maturation and development of the ascospores by microscopic examination. Five pseudothecia were randomly excised from each stem to observe pseudothecial development (25 pseudothecia per cultivar).

The pseudothecial maturation was assessed in Classes A–E as described in 4.2.1.3 (Fig. 4.2). The number of pseudothecia in each maturity class was recorded for all nine cultivars. At the end of the experiments (when all the pseudothecia were mature), three stems with abundant pseudothecia were chosen per cultivar for assessment of pseudothecial density and proportions of *L. maculans* and *L. biglobosa*. These proportions were estimated by discharge of ascospores from mature pseudothecia (Class D) and growth of colonies on PDA for morphological identification (method described in 4.2.1.3).

4.2.2.5 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. The T-test and analysis of variance (ANOVA) were done using the SPSS version 21 statistical package. The total daily rainfall (mm) and rain-days were summed and the daily mean temperature was calculated from the temperature data. For testing the relationship between the days required for 50% of pseudothecia to reach Class D (mature) and weather conditions (temperature and rainfall), the Pearson's product – moment correlation coefficient was calculated. It is a measure of the linear correlation (dependence) between two variables X and Y, giving a value

between +1 and −1 inclusive, where +1 is total positive correlation, 0 is no correlation and −1 is total negative correlation.

4.2.3 Assessment of ascospore release under natural conditions with proportions of *Leptosphaeria maculans* and *L. biglobosa* determined by using qPCR

All stems of nine cutivars collected [Adriana (*Rlm 4* + QR), Bilbao (*Rlm 4*), Capitol (*Rlm 1*), Drakkar (no *R* gene against *L. maculans*), DK Cabernet (*Rlm 1* + QR), Es-Astrid (QR), Excel (*Rlm 7*), NK Grandia (QR) and Roxet (*Rlm 7*)] with mature pseudothecia (Class D) were exposed to natural conditions until pseudothecia discharged ascospores at the Bayfordbury experimental plot.

4.2.3.1 Preparation of infected stems

The stems collected from Rothamsted experimental plots were placed around a Burkard spore sampler (Fig. 4.3) at the Bayfordbury experimental plot that was used to collect ascospores released from pseudothecia that developed on the stems.

4.2.3.2 Infected stems exposed to natural conditions

The stem debris was exposed to natural conditions until the majority of pseudothecia were empty (Class E), when all ascospores were discharged. The experiment started on 20 September 2011 and continued until 12 March 2012 (stems from the 2010/2011 growing season), from 5 September 2012 until 12 March 2013 (stems from the 2011/2012 growing season) and was from 18 September 2013 until 18 March 2014 (stems from the 2012/2013 growing season).

4.2.3.3 Bayfordbury weather data

Daily meteorological data (temperature and rainfall) at Bayfordbury from 0900 h GMT to 0900 h GMT on the following day were obtained from daily monitoring done by a Davis weather station for every hour (Davis Instruments, San Francisco Bay Area, USA).

4.2.3.4 Burkard spore sampler and ascospore counting

A Burkard volumetric spore sampler (Burkard Manufacturing Company Ltd.) (Fig. 4.3 and Fig. 4.4) was set up at Bayfordbury to monitor numbers of air-borne ascospores of *Leptosphaeria* species (*L. maculans* and *L. biglobosa*) released from pseudothecia on stems of nine winter oilseed rape cultivars. All stems were from the previous year's harvest during the 2010/2011, 2011/2012 and 2012/2013 growing seasons. Ascospores were sampled from an air intake at a rate of 10L minute^{-1} and deposited onto an exposed tape in the drum over a period of 7 days (Fig. 4.4). At the end of the 7 day period, the exposed tapes were replaced with new tapes each week throughout the sampling period until no more ascospores were collected. The sampling months were were typically September to March (2011/2012, 2012/2013 and 2013/2014).

The exposed tape was removed from the sampler drum at 7-day intervals. Each tape was cut on a perspex cutting block into seven separate 48 mm long pieces that each represented a 24-h period (Fig. 4.5). Then each of the seven 48mm long daily strips were cut in half along the length of the strip, with the upper halves being carefully coiled and inserted into date- and location-labelled, sterile Eppendorf tubes and the lower halves being retained and mounted onto date- and location-labelled

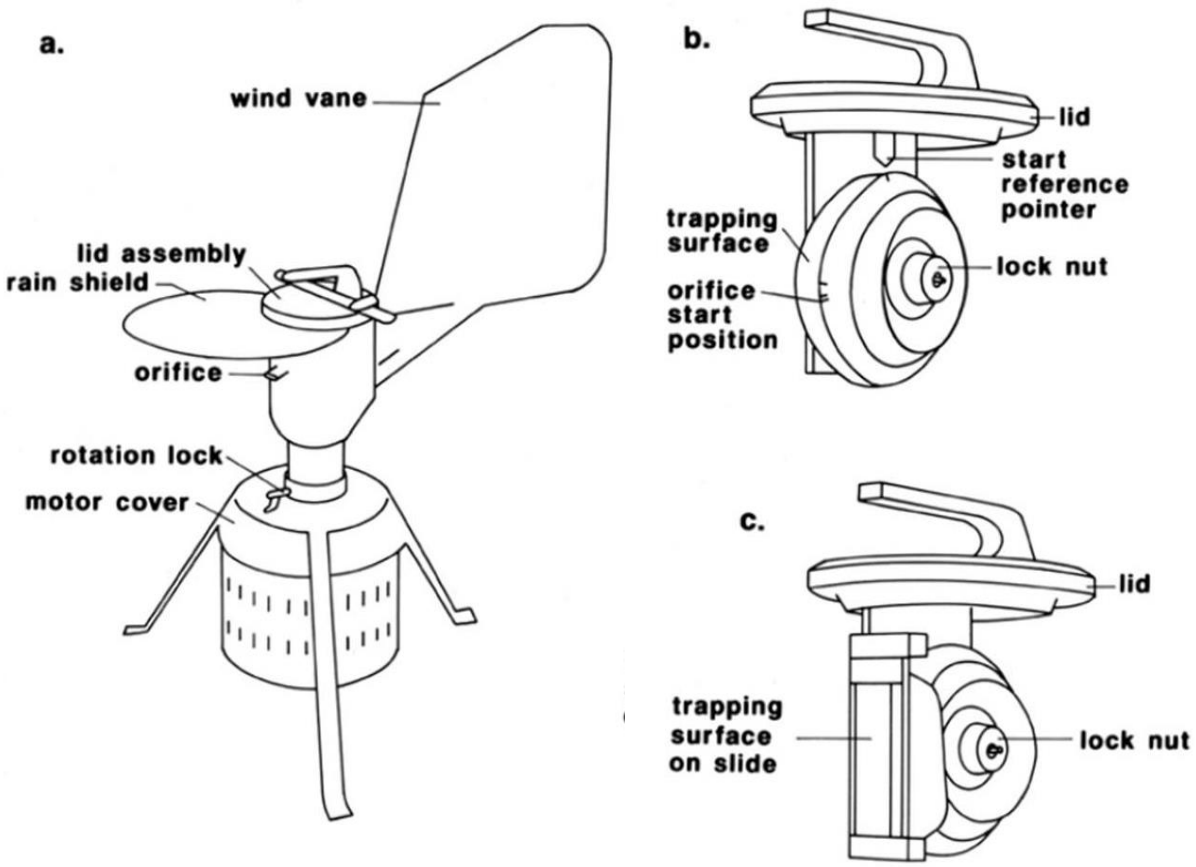


Fig. 4.4: A Burkard spore sampler and its components (a) with a choice of trapping systems on the drum (Lacey and West, 2006) (b) 7-day lid assembly with drum on clockwise movement and (c) lid assembly for 24hr sampling directly onto glass slide. For this work the Burkard sampler used parts a and b.

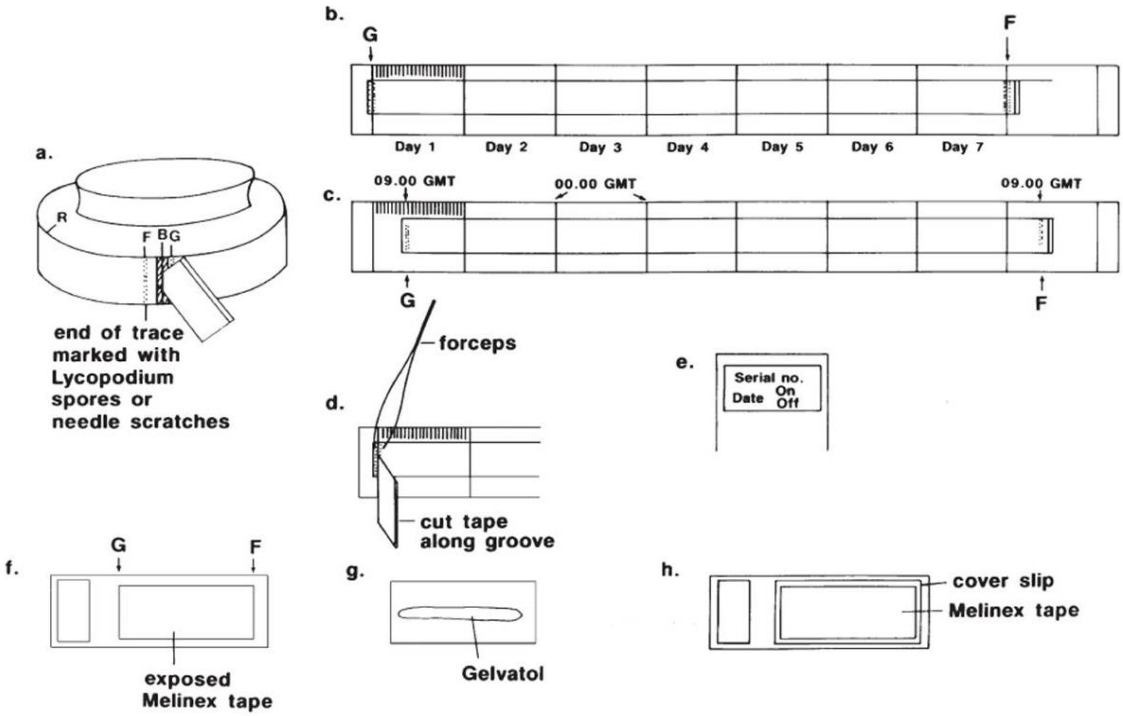


Fig. 4.5: Preparing the spore tape from the Burkard sampler drum onto microscope slides at 7-day intervals (Lacey and West, 2006).

microscope slides. The upper halves in Eppendorf tubes were used for identification of the *Leptosphaeria* species using species-specific PCR (Liu *et al.* 2006). Then the lower halves of the tape pieces were mounted on microscope slides and stained with a trypan blue solution and cover slips were applied. The daily release of ascospores was calculated from the average numbers of ascospores counted in two longitudinal traverses of each piece of tape under a light microscope (x 100 magnification) (Huang *et al.,* 2005; Lacey & West, 2006). The daily mean concentration of ascopores (Nm) per cubic metre of air was calculated by multiplying the number of ascospores counted in one day (Nd) by the correct conversion factor given below, using the figure 0.28 when viewing at x100 magnification (Lacey and West, 2006):

$$N_{mean}\,(Nm) = \frac{Nd \times 0.9722}{\text{width of one traverse (mm)}}$$

4.2.3.5 Assessment of proportions of *L. maculans* and *L. biglobosa* ascospores by using quantitative PCR (qPCR)

DNA was extracted from the tape pieces using a CTAB (hexadecyltrimethylammonium bromide) protocol, as described by Kaczmarek *et al.* (2009), with some modification. One scoop (c. 150mg) of acid-washed glass beads (Ballotini Sigma, 400-455 µm diameter) was added to a 2 ml screw top tube containing one half of the spore tape. 2% CTAB buffer was added and the tubes were processed in a Fast-Prep machine (MP Biomedicals) twice to lyse the cells for 40 sec each time. The samples were then incubated for 30 min at 65°C, centrifuged for 10 min at 4°C and an equal volume of a chloroform:isoamyl alcohol mixture (24:1) was added. DNA was precipitated with ammonium acetate (7.5 M, 30µl), isopropanol (480µl) and 1 µl of glycogen. The DNA pellets were washed with 70% ethanol, dried and re-suspended in 30 µl of sterile distilled water (Sigma molecular biology grade).

DNA concentration and quality were measured using a Nanodrop ND-1000 spectrophotometer (Labtech, International, UK). The ratio of absorbance at 260 nm to absorbance at 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as pure for DNA and, if the ratio is less, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

The amounts of *L. maculans* or *L. biglobosa* DNA present in each spore tape sample were quantified by SYBR Green qPCR with species-specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*) (Liu *et al.,* 2006; Huang *et al.,* 2010) (Table 3.1). PCR reaction mixtures were prepared to a total volume of 20 µl, containing 10 µl of supermix (Sigma, SYBR green jumpstart Tag ready mix, S9194 containing reference dye Rox, lot SLBD6104), 6.3 µl of sterile distilled water, 0.6 µl (10 µM) of forward primer and 0.6 µl (10 µM) of reverse primer and 2.5 µl of DNA.

All reactions were done in 96 × 0.2 ml PCR plates (ABgene) covered with cap strips, using a Stratagene Mx3000P quantitative PCR machine thermocycler (Mx3005P QPCR system, Agilent Technologies, Inc., Santa Clara, CA, USA). Nuclease-free water (Sigma, UK) was used as the no-template control. The thermocycling profile consisted of an initial cycle of 95°C for 2 min followed by 40 cycles of 30 sec at 60˚C, 45 sec at 72˚C and 15 sec at 83˚C. An additional melting curve was added to the end of the reactions with a thermal profile consisting of 1 min at 95˚C, 1 min at 60˚C and 15 sec at 95˚C. In each qPCR run, a standard dilution series consisting of 10000, 1000, 100, 10 or 1 pg of *L. maculans* or *L. biglobosa* DNA was included to produce a standard curve. The amounts of *L. maculans* or *L. biglobosa* DNA for each unknown sample were estimated from the *Ct* value and the value was obtained from the

standard curve using Stratagene MxPro-Mx3000 P v3.2. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in the 2.5 µL DNA sample. The data were then converted to absolute amounts of *L. biglobosa* or *L. maculans* DNA present on one half of 48 mm spore tape pieces, each corresponding to one day.

4.2.3.6 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. The T-test and analysis of variance (ANOVA) were done using the SPSS version 21 statistical package. The total daily rainfall (mm) and number of rain-days were summed and the daily mean temperature was calculated from the maximum and minimum temperature data.

4.3 Results

4.3.1 Effects of cultivar resistance on pseudothecial maturation on stems in a controlled environment (20^{o}C) or exposed at different times in natural conditions during the autumn/winter in 2012/2013 and 2013/2014.

The observation of pseudothecial development on stem bases and upper stems of four selected cultivars [Adriana (*Rlm4* + QR), Bilbao (*Rlm4*), NK Grandia (QR) and Drakkar (no *R* genes against *L. maculans*)] were done in a controlled environment experiment (20^{o}C, 75-80% RH) with continuous wetness (rain water pH 5.7) and in the natural conditions by exposing stems at different times during the autumn/winter in 2012/2013 (5 September and 28 November) and 2013/2014 (9 October and 8 January) for a period of 70 days. All stems were kept in the freezer (-15^{o}C) before being exposed to outside conditions at the Bayfordbury experimental plot.

Pseudothecia on cultivar Drakkar (susceptible) matured faster than those on other

cultivars after being exposed at different times from 5 September 2012 to 14 November 2012, from 28 November 2012 to 6 February 2013, from 9 October 2013 to 18 December 2013, from 8 January 2014 to 19 March 2014 and in controlled conditions (20^{o}C and continuous wetness, rain water pH5.7, 75-80% RH, 12h day-length). After 14 days, stems of cv. Drakkar had a greater number of mature pseudothecia (Class D) than those of other cultivars (Table 4.2). On the stem bases of cv. Drakkar, there were the greatest numbers of mature pseudothecia (Class D) after 28 days in controlled environment conditions, whereas stems being exposed at different times were exposed for 42 days (after being exposed on 5 Sept 2012), 70 days (after being exposed on 28 Nov 2012), 28 days (after being exposed on 9 Oct 2013) and 70 days (after being exposed on 8 Jan 2014) (Table 4.2).

For pseudothecial development on stem bases, the temperature ranged from 10.6^{o}C to 14.4^{o}C, and there were 46 rain-days (74.94mm) from 5 September 2012 to 14 November 2012 (Table 4.2) (Appendix 4.1 and 4.2). The pseudothecia on cvs Drakkar and Bilbao (*Rlm4*) started to release ascospores and empty pseudothecia (Class E) were observed after 42 days of exposure to natural conditions, whereas pseudothecia on cvs Adriana (*Rlm4* + QR) and NK Grandia (QR) were observed with empty pseudothecia (Class E) after 56 days of exposure to natural conditons (Table 4.2, Appendix 4.4).

In autumn/winter 2012/2013, stems were exposed to temperature ranging from 2.0 ^{o}C to 4.8 ^{o}C, with 40 rain-days (146.96 mm) from 28 November 2012 to 6 February 2013 (Table 4.2, Appendix 4.1). The maturation was slower and the temperature was less than in the previous year (5 Sept 2012 to 14 Nov 2012). There were fewer rain-days

Table 4.2: Number of mature (Class D) pseudothecia observed on stem bases of each of four winter oilseed rape cultivars exposed at Bayfordbury in autumn/winter 2012/2013 (5 Sept 2012 and 28 Nov 2012), autumn/winter 2013/2014 (9 Oct 2013 and 8 Jan 2014) and in controlled conditions. Every two weeks, for a period of 70 days, samples of 25 pseudothecia were obtained and Class D was identified.

				Stem base						
Autumn/winter	Day 1 (date)	Observation day	Date	Adriana (*Rlm4* + QR)	Bilbao (*Rlm4*)	Drakkar (No R gene)	NK Grandia (QR)	[a]Temperature (^{o}C)	[b]Total rain (mm)	[c]Rain days
Stems exposed to natural conditions				**Number of mature (Class D) pseudothecia**						
2012/2013	5 Sept 2012	14 days	19 Sept 2012	2	6	8	3	14.4	2.28	3
		28 days	3 Oct 2012	10	15	19	7	13.0	25.09	16
		42 days	17 Oct 2012	17	23	25	13	11.9	50.68	29
		56 days	31 Oct 2012	20	15	12	19	11.4	57.98	40
		70 days	14 Nov 2012	15	9	4	12	10.6	74.94	46
	28 Nov 2012	14 days	12 Dec 2012	0	0	0	0	2.0	10.67	6
		28 days	26 Dec 2012	0	0	4	0	4.2	46.16	14
		42 days	9 Jan 2013	3	4	9	2	4.8	59.32	22
		56 days	23 Jan 2013	8	11	13	6	4.1	102.58	29
		70 days	6 Feb 2013	11	16	19	12	3.0	146.96	40
2013/2014	9 Oct 2013	14 days	23 Oct 2013	3	8	10	5	12.0	66.43	12
		28 days	6 Nov 2013	14	18	24	15	11.5	166.64	22
		42 days	20 Nov 2013	17	21	16	18	7.9	192.57	35

Table continued

		Stem base								
		Observation day	Date	Adriana (*Rlm4* + QR)	Bilbao (*Rlm4*)	Drakkar (No R gene)	NK Grandia (QR)	[a]Temperature (^{o}C)	[b]Total rain (mm)	[c]Rain days
2013/2014	8 Jan 2014	56 days	4 Dec 2013	20	15	10	22	7.1	195.85	39
		70 days	18 Dec 2013	13	9	3	15	6.9	216.67	51
		14 days	22 Jan 2014	0	0	0	0	6.1	30	14
		28 days	5 Feb 2014	3	5	8	2	5.9	105.2	25
		42 days	19 Feb 2014	8	11	13	7	6.1	211.8	34
		56 days	5 Mac 2014	11	17	18	10	6.3	215.8	40
		70 days	19 Mac 2014	16	21	23	18	6.9	251.6	41
Stems in controlled environment conditions (20^{o}C, continuous wetness, rain water pH 5.7 and 70-80% RH)										
2013 and 2014 (results were average of two experiments)		14 days		6	8	12	8			
		28 days		12	18	21	13			
		42 days		18	22	14	19			
		56 days		23	11	7	20			
		70 days		6	2	0	8			

[a]Average temperature (^{o}C) from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

[b]Total rainfall (mm) from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

[c]Number of days with rainfall >0.2mm from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

but dew may have enhanced the maturation. The pseudothecia on cv. Drakkar started to release ascospores and empty pseudothecia (Class E) were observed after 70 days of exposure to natural conditions. No empty pseudothecia were observed on cvs Adriana (*Rlm4* + QR), Bilbao (*Rlm4*) or NK Grandia (QR) but there were more pseudothecia in Class C and Class D on the stem bases (Table 4.2, Appendix 4.5).

The temperature ranged from 6.9 ^{o}C to 12.0 ^{o}C from 9 October 2013 to 18 December 2013 and there were 51 rain-days (216.67mm) (Appendix 4.1). There were more rain-days in autumn/winter 2013/2014 than in autumn/winter 2012/2013. In 2013, it was very wet and pseudothecia on cvs Drakkar and Bilbao (*Rlm4*) started to release ascospores; empty pseudothecia (Class E) were observed after 28 days of exposure to natural conditions (Table 4.2, Appendix 4.6). For cvs Adriana (*Rlm4* + QR) and NK Grandia (QR), the empty pseudothecia were observed after 56 days of exposure to natural conditions (Table 4.2, Appendix 4.6).

In autumn/winter 2013/2014, temperature ranged from 5.9 ^{o}C to 6.9 ^{o}C and there were 41 rain-days (251.6mm) from 8 Jan 2013 to 19 March 2014 (Table 4.2, Appendix 4.1). Therefore, pseudothecial development was slower and cv. Drakkar started to release ascospores later; empty pseudothecia (Class E) were observed after 56 days of exposure to natural conditions, whereas pseudothecia on cvs Adriana (*Rlm4* + QR), Bilbao (*Rlm4*) and NK Grandia (QR) were empty (Class E) after 70 days of exposure to natural conditons (Table 4.2, Appendix 4.7).

In controlled conditions, at 20^{o}C (rain water pH 5.7, 75-80% RH and 12h day length), pseudothecia on cvs Drakkar and Bilbao (*Rlm4*) started to release ascospores and

empty pseudothecia (Class E) were observed after 28 days in continuous wet conditions (Table 4.2, Appendix 4.8). After 42 days, some pseudothecia were empty (Class E) on stem bases of cvs Adriana (*Rlm4* + QR) and NK Grandia (QR) (Table 4.2, Appendix 4.8).

On the upper stems, cv. Drakkar had a greater number of pseudothecia mature (Class D) than other cultivars after 14 days (Appendix 4.9 & 4.11) but upper stems that were exposed from 28 Nov 2012 to 6 Feb 2013 and from 8 Jan 2014 to 19 March 2014 started having mature pseudothecia after 28 days (Table 4.3, Appendix 4.10 & 4.12).

In the autumn/winter 2012/2013, for stems exposed to natural conditions from 5 Sept 2012 to 14 Nov 2012, empty pseudothecia (Class E) were observed on upper stems of cv. Drakkar after 42 days of exposure to natural conditions whereas pseudothecia on cvs Adriana, Bilbao and NK Grandia with empty pseudothecia (Class E) were observed after 56 days of exposure to natural conditons (Table 4.3, Appendix 4.9).

The pseudothecia on cv. Drakkar started to release ascospores and empty pseudothecia (Class E) were observed after 70 days of exposure (from 28 Nov 2012 to 6 Feb 2013) to natural conditions (Appendix 4.10). No empty pseudothecia were observed on cvs Adriana, Bilbao and NK Grandia but there were more pseudothecia in Class B, Class C and Class D on the upper stems (Table 4.3, Appendix 4.10).

In the autumn/winter in 2013/2014, the pseudothecia on cvs Drakkar and Bilbao started to release ascospores after 42 days of exposure to natural conditions (from 9 October 2013 to 18 Dec 2013) and empty pseudothecia (Class E) were observed

Table 4.3: Number of mature (Class D) pseudothecia observed on upper stem of each of four winter oilseed rape cultivars exposed at Bayfordbury in autumn/winter 2012/2013 (5 Sept 2012 and 28 Nov 2012), autumn/winter 2013/2014 (9 Oct 2013 and 8 Jan 2014) and in controlled conditions. Every two weeks, for a period of 70 days, samples of 25 pseudothecia were obtained and Class D was identified.

Upper stem										
Autumn/winter	Day 1 (date)	Observation day	Date	Adriana (*Rlm4* + QR)	Bilbao (*Rlm4*)	Drakkar (No R gene)	NK Grandia (QR)	[a]Temperature (^{o}C)	[b]Total rain (mm)	[c]Rain days
Stems exposed to natural conditions				**Number of mature (Class D) pseudothecia**						
2012/2013	5 Sept 2012	14 days	19 Sept 2012	0	1	4	0	14.4	2.28	3
		28 days	3 Oct 2012	2	7	12	4	13.0	25.09	16
		42 days	17 Oct 2012	13	16	21	12	11.9	50.68	29
		56 days	31 Oct 2012	20	22	13	20	11.4	57.98	40
		70 days	14 Nov 2012	15	9	4	13	10.6	74.94	46
	28 Nov 2012	14 days	12 Dec 2012	0	0	0	0	2.0	10.67	6
		28 days	26 Dec 2012	0	0	3	0	4.2	46.16	14
		42 days	9 Jan 2013	1	4	6	0	4.8	59.32	22
		56 days	23 Jan 2013	8	12	14	9	4.1	102.58	29
		70 days	6 Feb 2013	10	16	18	12	3.0	146.96	40
2013/2014	9 Oct 2013	14 days	23 Oct 2013	2	6	8	2	12.0	66.43	12
		28 days	6 Nov 2013	11	16	19	13	11.5	166.64	22
		42 days	20 Nov 2013	16	20	22	15	7.9	192.57	35

Table continued

2013/2014

			Upper stem						
	Observation day	Date	Adriana (*Rlm4* + QR)	Bilbao (*Rlm4*)	Drakkar (No R gene)	NK Grandia (QR)	[a]Temperature (^{o}C)	[b]Total rain (mm)	[c]Rain days
	56 days	4 Dec 2013	19	15	11	21	7.1	195.85	39
	70 days	18 Dec 2013	14	10	6	16	6.9	216.67	51
8 Jan 2014	14 days	22 Jan 2014	0	0	0	0	6.1	30	14
	28 days	5 Feb 2014	0	0	4	0	5.9	105.2	25
	42 days	19 Feb 2014	4	8	11	5	6.1	211.8	34
	56 days	5 Mac 2014	12	17	20	13	6.3	215.8	40
	70 days	19/3/2014	19	21	13	20	6.9	251.6	41
Stems in controlled environment conditions (20^{o}C, continuous wetness, rain water pH 5.7 and 70-80% RH)									
2013 and 2014 (results were average of two experiments)		14 days	5	8	11	6			
		28 days	10	14	19	10			
		42 days	14	19	21	13			
		56 days	21	21	16	20			
		70 days	13	8	4	10			

[a]Average temperature (^{o}C) from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

[b]Total rainfall (mm) from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

[c]Number of days with rainfall >0.2mm from the date when the stem base was put outside to the date when number of pseudothecia at mature stage (Class D) were observed.

(Table 4.3, Appendix 4.11), whereas for pseudothecia on cvs Adriana and NK Grandia, the empty pseudothecia were observed after 56 days of exposure to natural conditions (Table 4.3, Appendix 4.11).

For stem debris exposed later, the pseudothecial development was slower and pseudothecia on cv. Drakkar started to release ascospores later; empty pseudothecia (Class E) were observed after 56 days of exposure to natural conditions (from 8 Jan 2014 to 19 March 2014) (Appendix 4.12), whereas pseudothecia on cvs Adriana, Bilbao and NK Grandia with empty pseudothecia (Class E) were observed after 70 days of exposure to natural conditons (Table 4.3, Appendix 4.12).

In controlled conditions, at 20^{o}C (rain water pH 5.7 and 75-80% RH), pseudothecia on cv Drakkar started to release ascospores earlier and empty pseudothecia (Class E) were observed after 28 days in continuous wet conditions, followed by pseudothecia on cv. Bilbao after 42 days and pseudothecia on cvs Adriana and NK Grandia after 56 days (Table 4.3, Appendix 4.13).

The pseudothecia were slowest to mature (Class D) on cvs. NK Grandia (QR) and Adriana (*Rlm4* + QR) for stems that were being exposed at different times in natural conditions and in controlled conditions (20^{o}C, continuous wetness, rain water pH 5.7, 75-80% RH and 12h day length, 205 µem^{-2}s^{-1}), for both stem bases and upper stems (Table 4.2, Table 4.3 and Appendix 4.9 – 5.3).

The estimation of days taken to reach 50% of pseudothecia in Class D showed that pseudothecia on cv. Drakkar matured much faster than those on other cultivars

(Adriana, Bilbao and NK Grandia) on both stem bases and upper stems (Table 4.4, Fig. 4.6). On stem bases, pseudothecia on cv. Drakkar reached 50% in Class D after 17 days of exposure (starting on 5 Sept 2012), after 15 days of exposure (starting on 9 Oct 2013) and after 14 days in controlled conditions (20^{o}C, continuous wetness, rain water pH 5.7, 75-80% RH and 12h day length) (Table 4.4, Fig. 4.6C). However, more days were taken to reach 50% of pseudothecia in Class D on stems that were exposed to lower temperatures; after 48 days of exposure (starting on 28 Nov 2012) and after 34 days of exposure (starting on 8 Jan 2014) (Table 4.4, Fig. 4.6C). On the upper stems, pseudothecia on cv. Drakkar reached 50% in Class D after 28 days of exposure (starting on 5 Sept 2012), after 17 days of exposure (starting on 9 Oct 2013) and after 16 days in controlled conditions (Table 4.4, Fig. 4.6C). However, more days were taken at lower temperatures; after 48 days of exposure (starting on 28 Nov 2012) and after 43 days of exposure (starting on 8 Jan 2014) (Table 4.4, Fig. 4.6C).

For other cultivars (Adriana, Bilbao and NK Grandia), stem bases and upper stems that were exposed to natural conditions at different times showed an earlier maturation of pseudothecia (50% pseudothecia in Class D), especially for stems exposed starting on 5 Sept 2012 or 9 Oct 2013 and those in controlled conditions (Fig. 4.6A, B, D, Table 4.4). The pseudothecial development was slower to reach 50% pseudothecia in Class D on stems that were exposed at lower temperatures (starting on 28 Nov 2012 and 8 Jan 2014 for all cultivars) (Fig. 4.6A, B, D, Table 4.4).

Results showed that more days were taken to reach 50% pseudothecia in Class D on stem bases and upper stems for both cultivars with QR (Adriana and NK Grandia) than for Bilbao (*Rlm4*) at different times of exposure (Table 4.4). Nevertheless, at low

Table 4.4: Number of days when 50% of pseudothecia of *Leptosphaeria* spp. on cvs Adriana (*Rlm4* + QR), Bilbao (*Rlm4*), Drakkar (no *R* gene) and NK Grandia (QR) of winter oilseed rape stem base and upper stem reach Class D (mature) differences in days taken between stem base and upper stem to reach 50% of *Leptosphaeria* spp. pseudothecia observed every two weeks (sample of 25 pseudothecia per cultivar) for 70 days in autumn/winter 2011/2012 (from 5 September 2012 to 14 November 2012 and from 28 November 2012 to 6 February 2013), in autumn/winter 2012/2013 (from 9 October 2013 to 18 December 2013 and from 8 January 2014 to 19 March 2014) and in controlled environment conditions[a].

Days taken to reach 50% pseudothecia in Class D												
[1]Growing season	Adriana (*Rlm4* + QR)			Bilbao (*Rlm4*)			Drakkar (No R gene)			NK Grandia (QR)		
	Stem bases	Upper stem	[2]Days	Stem bases	Upper stem	[2]Days	Stem bases	Upper stem	[2]Days	Stem bases	Upper stem	[2]Days
Autumn/winter 2011/2012												
[1]A	31	35	4	19	32	13	17	28	11	34	42	8
[1]B (Low temperature)	77	77	0	58	57	-1	48	48	0	77	77	0
Autumn/winter 2012/2013												
[1]C	20	30	10	17	19	2	15	17	2	19	21	2
[1]D (Low temperature)	58	56	-2	44	46	2	34	43	9	58	49	-9
Controlled environment conditions	29	32	3	17	20	3	14	16	2	20	34	14

[1]Stems that were exposed to natural conditions at different time points, **A**; from 5 September 2012 to 14 November 2012, **B**; from 28 November 2012 to 6 February 2013, **C**; from 9 October 2013 to 18 December 2013, **D**; from 8 January 2014 to 19 March 2014 and in controlled environment conditions (T5; 20^{o}C, continuous wetness, rain water pH 5.7, 70-80% RH, 205 µem^{-2}s^{-1}).

[2]Differences of days taken between basal stem and upper stem to reach Class D (mature pseudothecial) was calculated and negative value means that the pseudothecial mature (Class D) earlier and positive value means that the pseudothecial mature (Class D) was later.

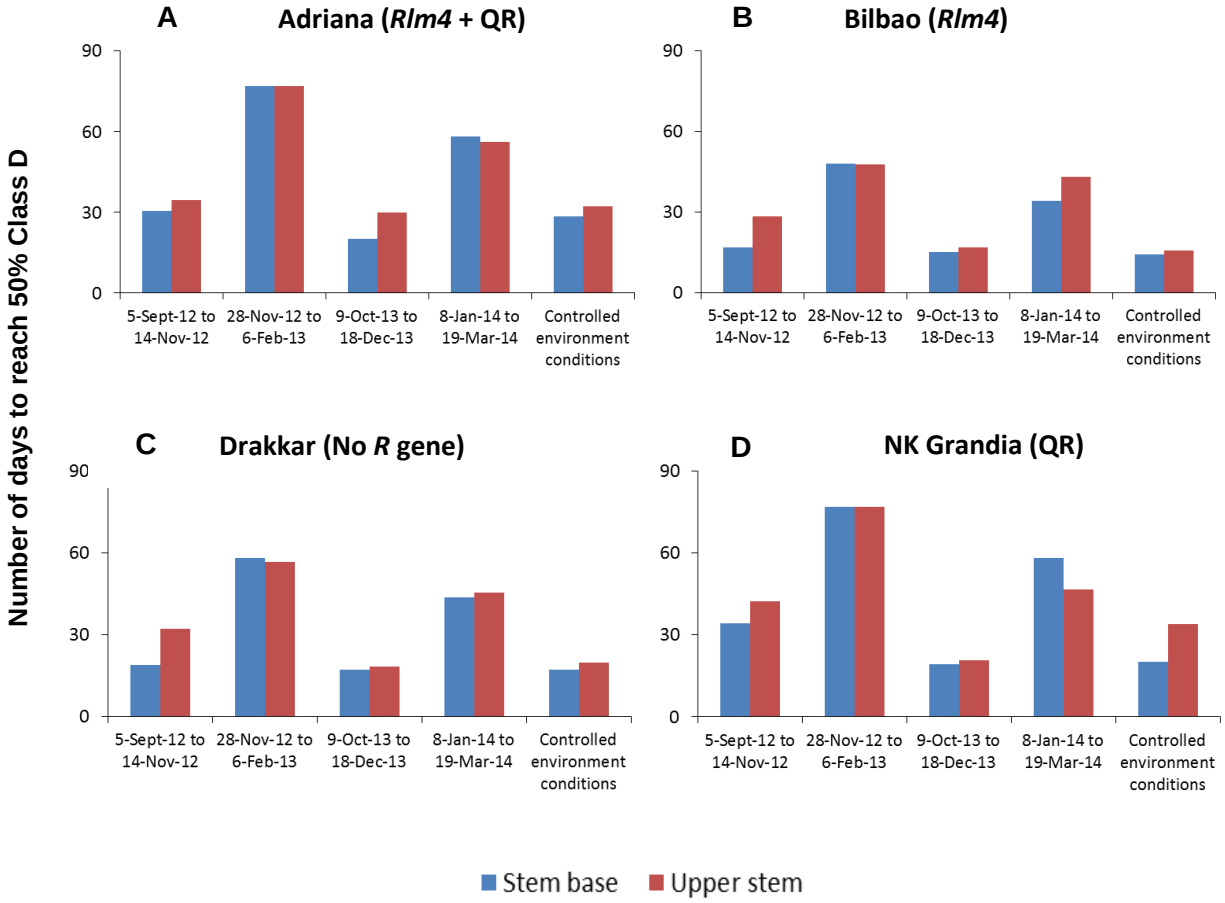


Fig. 4.6: Differences between stem bases and upper stems of winter oilseed rape in number of days to when 50% of pseudothecia of *Leptosphaeria* spp. reached Class D (mature) on cultivars (A) Adriana (*Rlm4* + QR), (B) Bilbao (*Rlm4*), (C) Drakkar (no *R* gene against *L. maculans*) and (D) NK Grandia (QR), for pseudothecia observed every two weeks (sample of 25 pseudothecia per cultivar) for a period of 70 days. In autumn/winter 2011/2012 from 5 September 2012 to 14 November 2012 and from 28 November 2012 to 6 February 2013; in autumn/winter 2012/2013 from 9 October 2013 to 18 December 2013 and from 8 January 2014 to 19 March 2014 and in controlled environment conditions (20^{o}C, continuous wetness, rain water pH 5.7 and 70-80% RH).

temperatures, cvs Adriana (*Rlm4* + QR) and NK Grandia (QR) were slower than cv. Bilbao (*Rlm4*) to reach 50% of pseudothecia in Class D on stems (stem bases and upper stems) (Table 4.4).

The maturation of pseudothecia on stems was much faster in controlled conditions than on stems that were exposed to natural conditions for all cultivars. However, pseudothecia on stems (stem bases and upper stems) of cvs Adriana (*Rlm4* + QR) and NK Grandia (QR) were slower than those on cvs Bilbao and Drakkar to reach 50% pseudothecia in Class D (Fig. 4.6, Table 4.4).

Generally, pseudothecial development on the upper stems was slower than pseudothecial development on the stem bases (Fig. 4.6, Table 4.4). Results showed that pseudothecia on the stem bases of all cultivars (Adriana, Bilbao, Drakkar and NK Grandia) generally reached 50% in Class D earlier than those on the upper stems (Table 4.4). However, pseudothecia on the upper stems of some cultivars matured much faster than those on stem bases, such as those on cvs Adriana and NK Grandia that were exposed to natural conditions starting on 8 Jan 2014 and those on cv. Bilbao that were exposed to natural conditions starting on 28 Nov 2012 (Table 4.4). There were no differences in the number of days taken for pseudothecia to reach 50% in Class D for both stem bases and upper stems for cvs Adriana, Drakkar and NK Grandia for pseudothecia that were exposed to natural conditions on 28 Nov 2012 (Table 4.4), when most of pseudothecia were in Class C (Appendix 4.10). In the controlled environment conditions, pseudothecia on upper stems reached 50% in Class D two to three days later than those on stem bases of cultivars Adriana, Bilbao and Drakkar but those on cv. NK Grandia reached 50% in Class D 14 days later than those on stem bases (Table 4.4).

Mean densities of pseudothecia differed significantly (P<0.05) between stem bases (Fig. 4.7A) and upper stems (Fig. 4.7B) for each cultivar at different times when stems were exposed to natural conditions (starting on 5 Sept 2012, 28 Nov 2012, 9 Oct 2013 and 8 Jan 2014) and in controlled conditions. The greatest mean density of pseudothecia was on the stem bases of all cultivars in natural conditions at different times and in controlled conditions (Fig. 4.7A). The greatest mean density of pseudothecia was on both stem bases and upper stems of cv. Drakkar (Fig. 4.7). Cultivars with QR (Adriana and NK Grandia) had the lowest density of pseudothecia (Fig. 4.7). Pseudothecial densities on stems were greater in controlled conditions than on stems that were exposed to natural conditions at different times for pseudothecia on both stem bases (Fig. 4.7A) and upper stems (Fig. 4.7B).

More *L. maculans* colonies than *L. biglobosa* colonies grew on PDA from 50 ascospores ejected from stem bases of most cultivars for stems exposed to natural conditions at different times except for cv. Adriana (stems exposed to natural conditions starting on 28 Nov 2012) (Fig. 4.8A, B, C & D). In controlled conditions, all stem bases of cvs Adriana, Bilbao, Drakkar and NK Grandia produced more *L. biglobosa* colonies than *L. maculans* colonies (Fig. 4.8E). On the upper stems (Fig. 4.9), there were more *L. biglobosa* colonies than *L. maculans* colonies that grew from 50 ascospores ejected onto PDA except for cv. NK Grandia (stems exposed to natural conditions starting on 28 Nov 2012) (Fig. 4.9B). All stems sampled from natural conditions at different times showed the greatest numbers of *L. maculans* or *L. biglobosa* colonies were from stem bases (Fig. 4.8A, B, C and D) or upper stems (Fig. 4.9A, B, C and D) of cv. Drakkar, respectively. In the controlled environment conditions, the greatest number of *L. maculans* colonies were from stem bases of cv. Adriana (Fig. 4.8E); more *L. biglobosa* than *L. maculans* colonies were isolated from

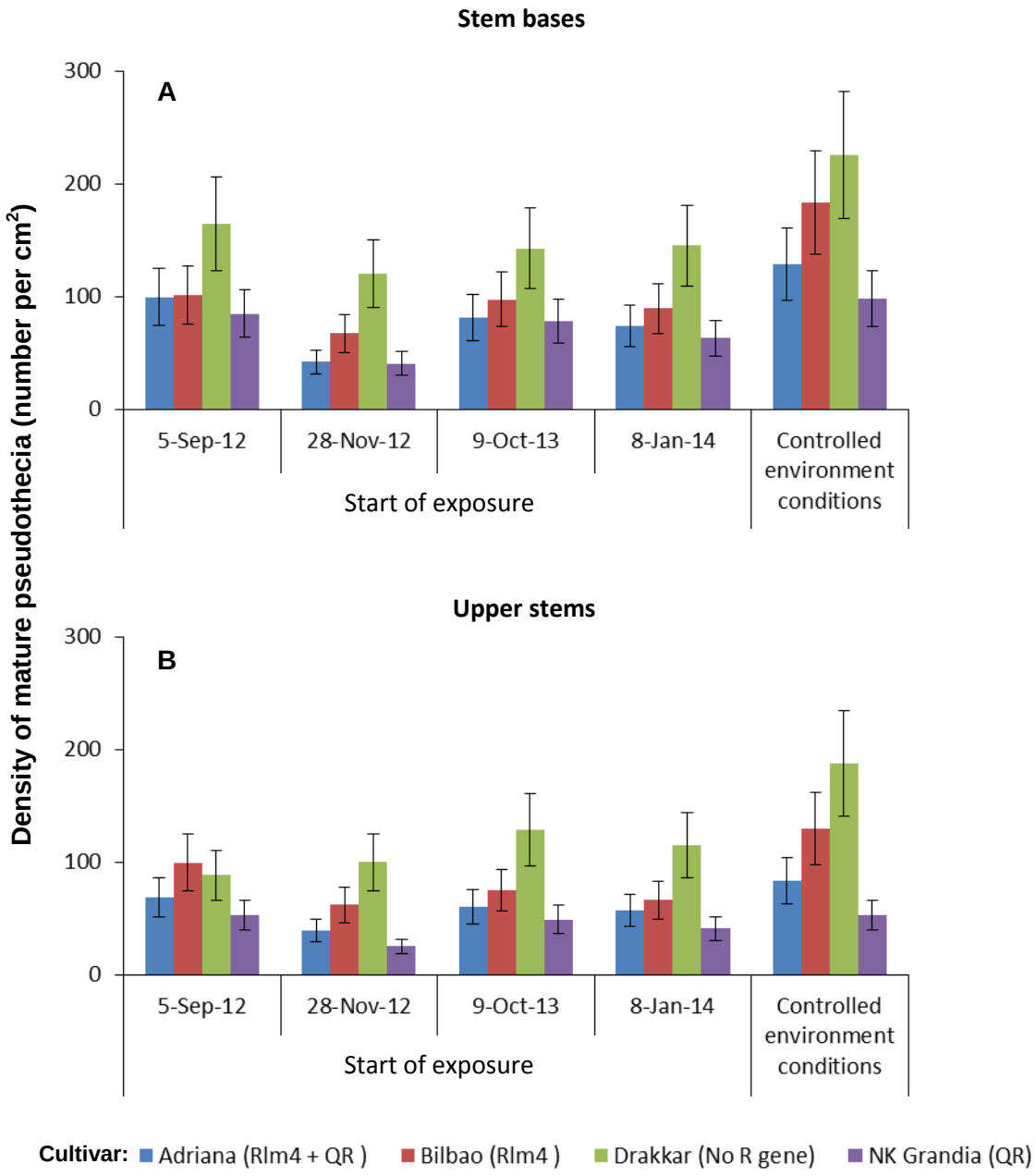


Fig. 4.7: The densities of mature pseudothecia (pseudothecia per cm^2) when 50% were in class D on winter oilseed rape (A) stem bases (<5 cm above ground level) and (B) upper stems (>10 cm above ground level) exposed at Bayfordbury at different times in autumn/winter 2012/2013 starting on 5 September 2012 and 28 November 2012, and in autumn 2013/2014 starting on 9 October 2013 and 8 January 2014 and in controlled environment conditions (20^{o}C, continuous wetness, rain water pH 5.7 and 70-80% RH).

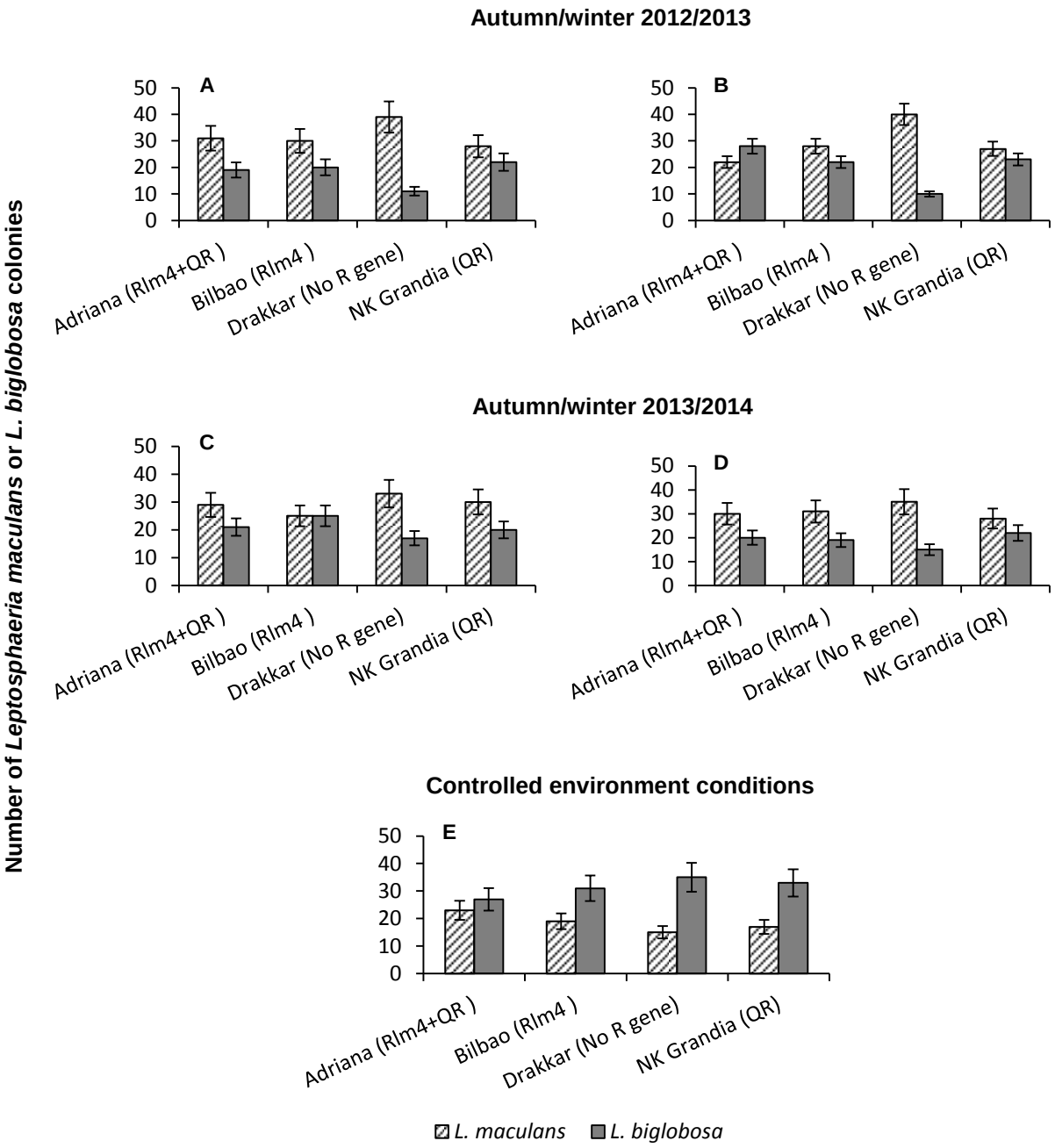


Fig. 4.8: Numbers of *Leptosphaeria maculans* or *L. biglobosa* colonies arising from ascospores discharged from stem bases exposed to natural conditions (A) from 5 Sept 2012 to 14 Nov 2012, (B) from 28 Nov 2012 to 6 Feb 2013, (C) from 9 Oct 2013 to 18 Dec 2013, (D) from 8 Jan 2014 to 19 March 2014 and (E) stems in a controlled environment cabinet (20^{o}C, continuous wetness, 12h day length). Only stem bases with 50% pseudothecia in Class D were selected and the ascospores were released from mature pseudothecia (Class D) onto PDA. A total of 50 ascospores per cultivar were released and colonies counted were identified based on morphological identification (refer chapter 2, Fig. 2.3).

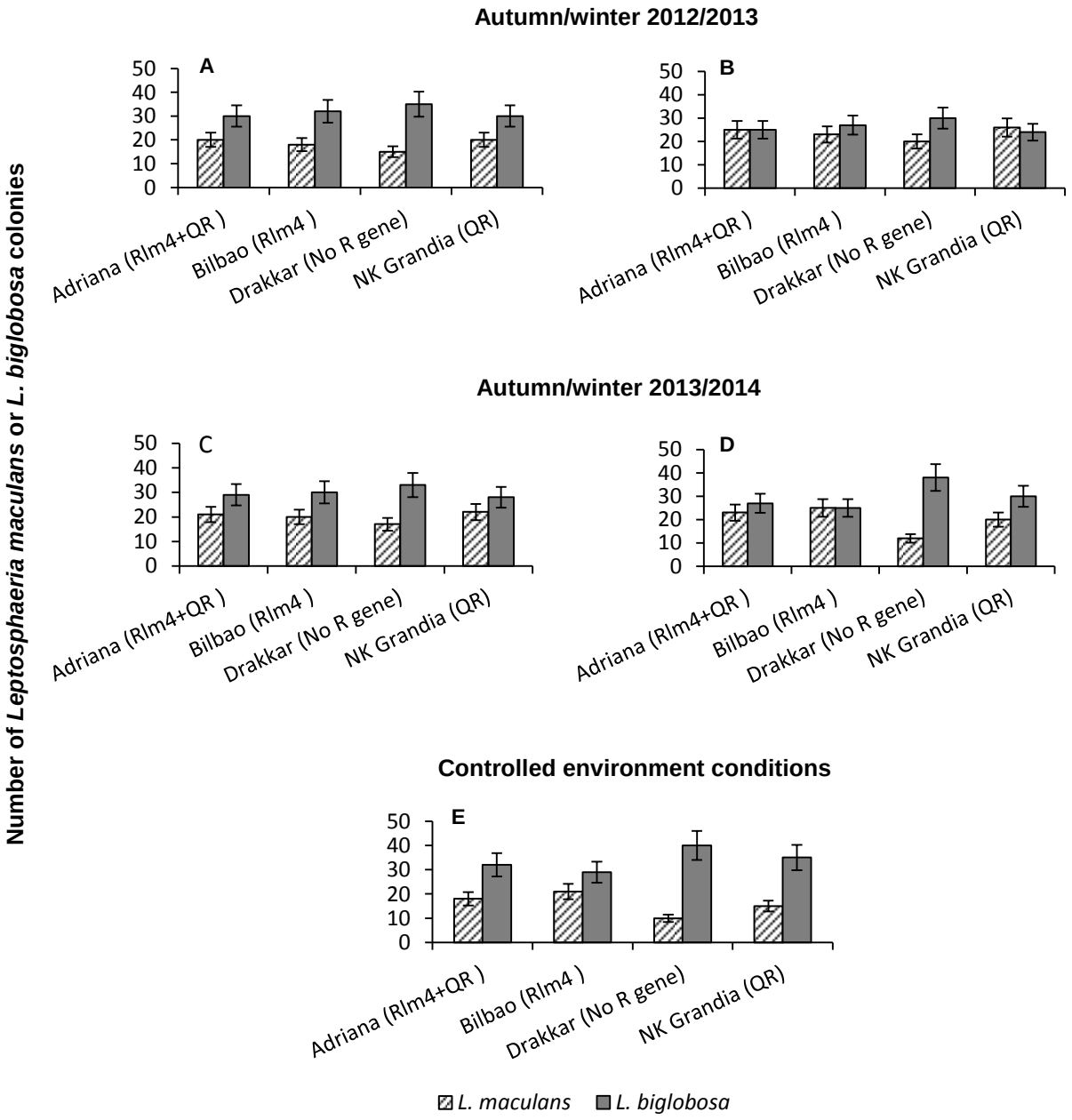


Fig. 4.9: Numbers of *Leptosphaeria maculans* or *L. biglobosa* colonies arising from ascospores discharged from upper stems exposed to natural conditions (A) from 5 Sept 2012 to 14 Nov 2012, (B) from 28 Nov 2012 to 6 Feb 2013, (C) from 9 Oct 2013 to 18 Dec 2013, (D) from 8 Jan 2014 to 19 March 2014 and (E) stems in controlled conditions. Only upper stems with 50% pseudothecia in Class D were selected and the ascospores were released from mature pseudothecia (Class D) onto PDA. A total of 50 ascospores per cultivar were released and colonies counted were identified based on morphological identification (refer chapter 2, Fig. 2.3).

the upper stems of all cultivars (Fig. 4.9E).

4.3.2 Effects of cultivar resistance and weather on maturation of pseudothecia of *Leptosphaeria* species under natural conditions in the 2011/2012, 2012/2013 and 2013/2014 growing seasons

In autumn/winter 2011/2012, 2012/2013 and 2013/2014, pseudothecial development was observed under natural conditions on stems of nine winter oilseed rape cultivars; with *R* genes [cvs Bilbao (*Rlm4*), Capitol (*Rlm1*), Roxet and Excel (both with *Rlm7*)], with QR [cvs Es-Astrid and NK Grandia], with both *R* genes and QR [cvs Adriana (*Rlm4* + QR) and DK Cabernet (*Rlm1* + QR)] and cv. Drakkar (susceptible to *L. maculans*). All stems were collected after harvest at Rothamsted and brought to the Bayfordbury experimental plot (Fig. 4.3).

The time until the first mature pseudothecia (Class D) were observed differed between cultivars on both stem bases (Fig. 4.10, 4.11) and upper stems (Fig. 4.12, 4.13). In autumn 2011, mature pseudothecia were first observed on stem bases of cvs Drakkar (susceptible to *L. maculans*), Bilbao (*Rlm4*) and Roxet (*Rlm7*) on 27 September 2011 (Fig. 4.10A), 2 weeks after stems were placed outside in the experimental plot at Bayfordbury, whereas pseudothecia on cvs Adriana (*Rlm4* + QR) (Fig. 4.11A), NK Grandia (QR) (Fig. 4.11A), Excel (*Rlm7*) and Capitol (*Rlm1*) (Fig. 4.10A) were first observed 3 weeks later on 4 October 2011. On cv. DK Cabernet (*Rlm1* + QR), mature pseudothecia were first observed on stem bases 4 weeks later (Fig. 4.11A). In autumn 2012, mature pseudothecia were first observed on cv. Drakkar on stem bases on 5 September 2012 (Fig. 4.10B), whereas on other cultivars mature pseudothecia were first observed 1 week later on 12 September 2012; cvs Bilbao, Excel and Roxet with *R*-gene (Fig. 4.10B), cvs Adriana and NK

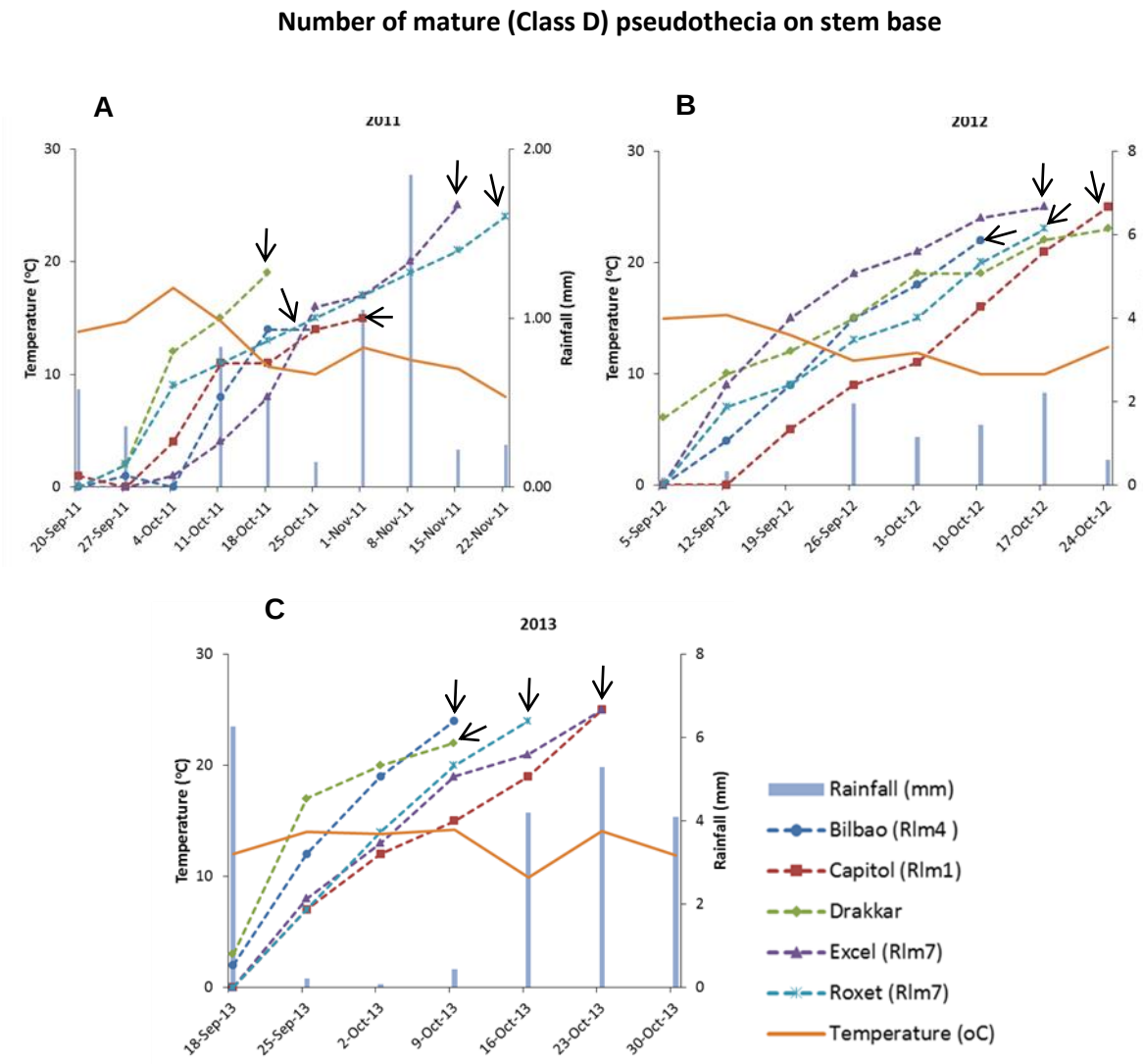


Fig. 4.10: Changes with time in the number of pseudothecia of *Leptosphaeria* spp. observed from a weekly sample of 25 pseudothecia per cultivar on winter oilseed rape stem bases of cultivars with *R* genes that were mature (Class D) in three growing seasons (autumn/winter) at Bayfordbury, (A) from September to November 2011, (B) from September to October 2012 and (C) from September to October 2013. Arrows showing the maximum number of pseudothecia that reached Class D (mature) for each five cultivars observed. The relationship between time to 50% maturation and temperature and rainfall is shown in Appendix 4.32. (Please refer to Appendix 4.14 – 4.22 for details on classes A to E of pseudothecial maturation).

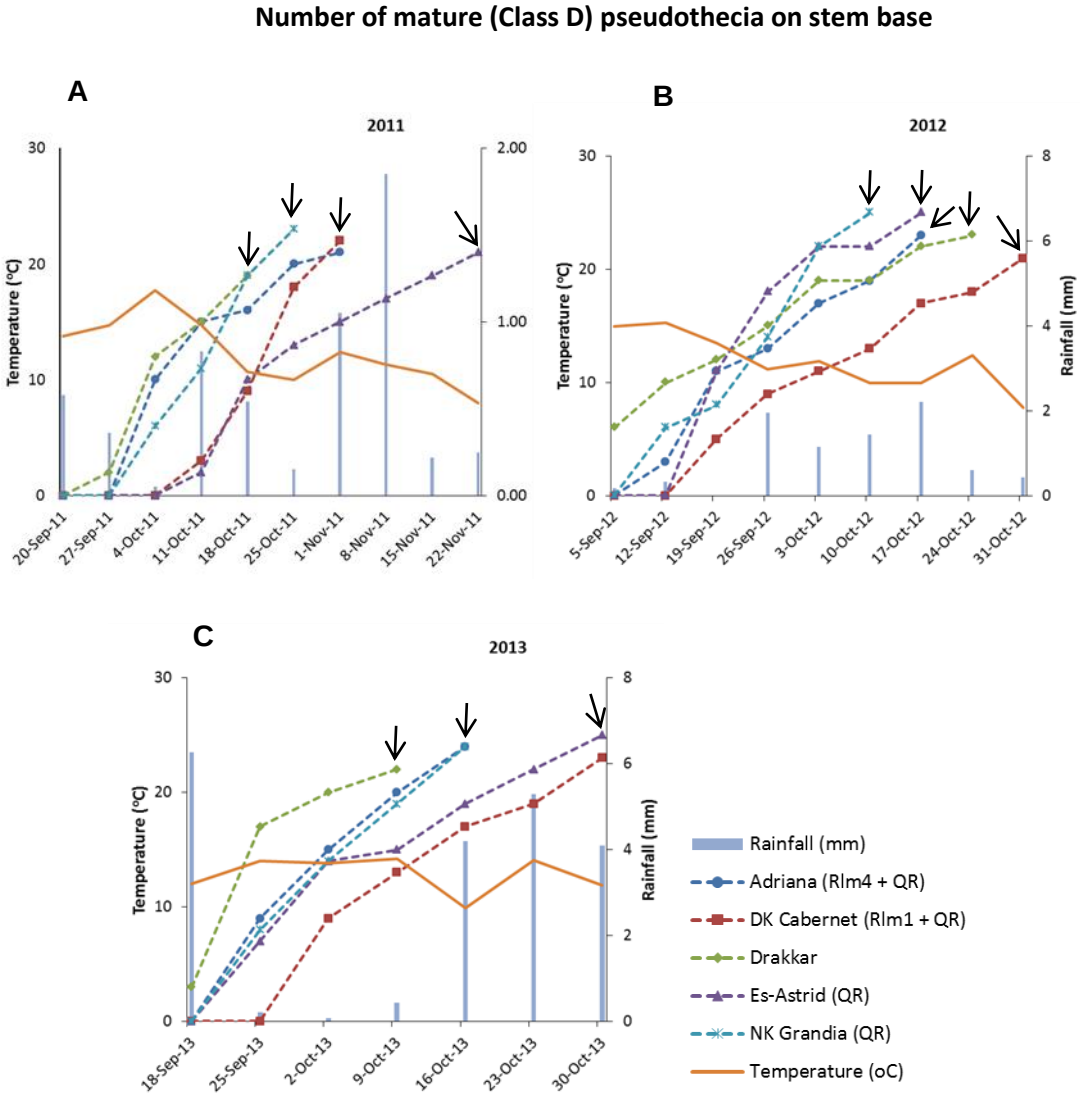


Fig. 4.11: Changes with time in the number of pseudothecia of *Leptosphaeria* spp. observed from a weekly sample of 25 pseudothecia per cultivar on winter oilseed rape stem bases of cultivars with *R* gene and/or without QR that were mature (Class D) in three growing seasons (autumn/winter) at Bayfordbury, (A) from September to November 2011, (B) from September to October 2012 and (C) from September to October 2013. Arrows showing the maximum number of pseudothecia that reached Class D (mature) for each five cultivars observed. The relationship between time to 50% maturation and temperature and rainfall is shown in Appendix 4.32. (Please refer to Appendix 4.14 – 4.22 for details on classes A to E of pseudothecial maturation).

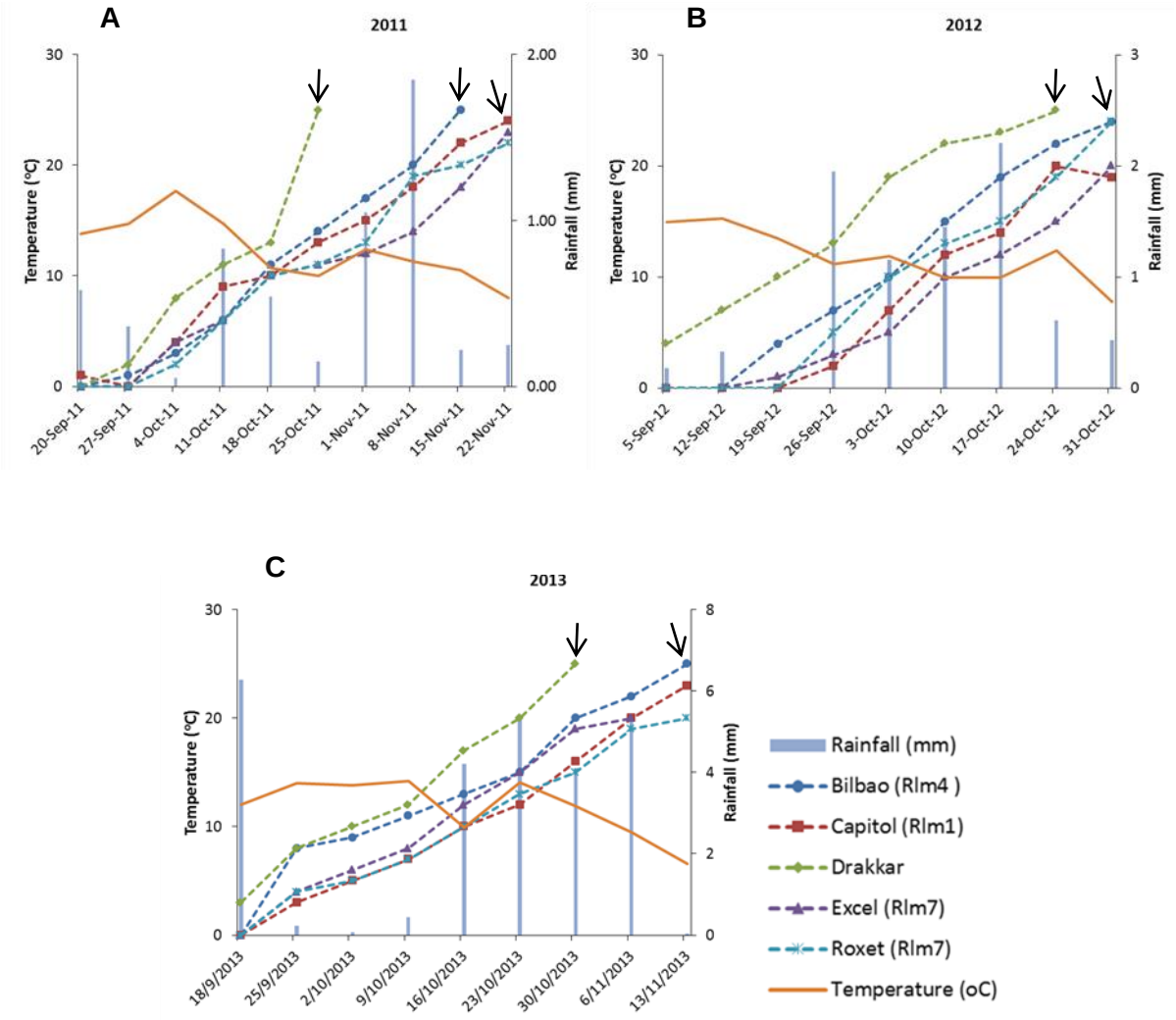


Fig. 4.12: Changes with time in the number of pseudothecia of *Leptosphaeria* spp. observed from a weekly sample of 25 pseudothecia per cultivar on winter oilseed rape upper stems of cultivars with *R* genes that were mature (Class D) in three growing seasons (autumn/winter) at Bayfordbury, (A) from September to November 2011, (B) from September to October 2012 and (C) from September to October 2013. Arrows showing the maximum number of pseudothecia that reached Class D (mature) for each five cultivars observed. The relationship between time to 50% maturation and temperature and rainfall is shown in Appendix 4.33. (Please refer to Appendix 4.23 – 4.31 for details on classes A to E of pseudothecial maturation).

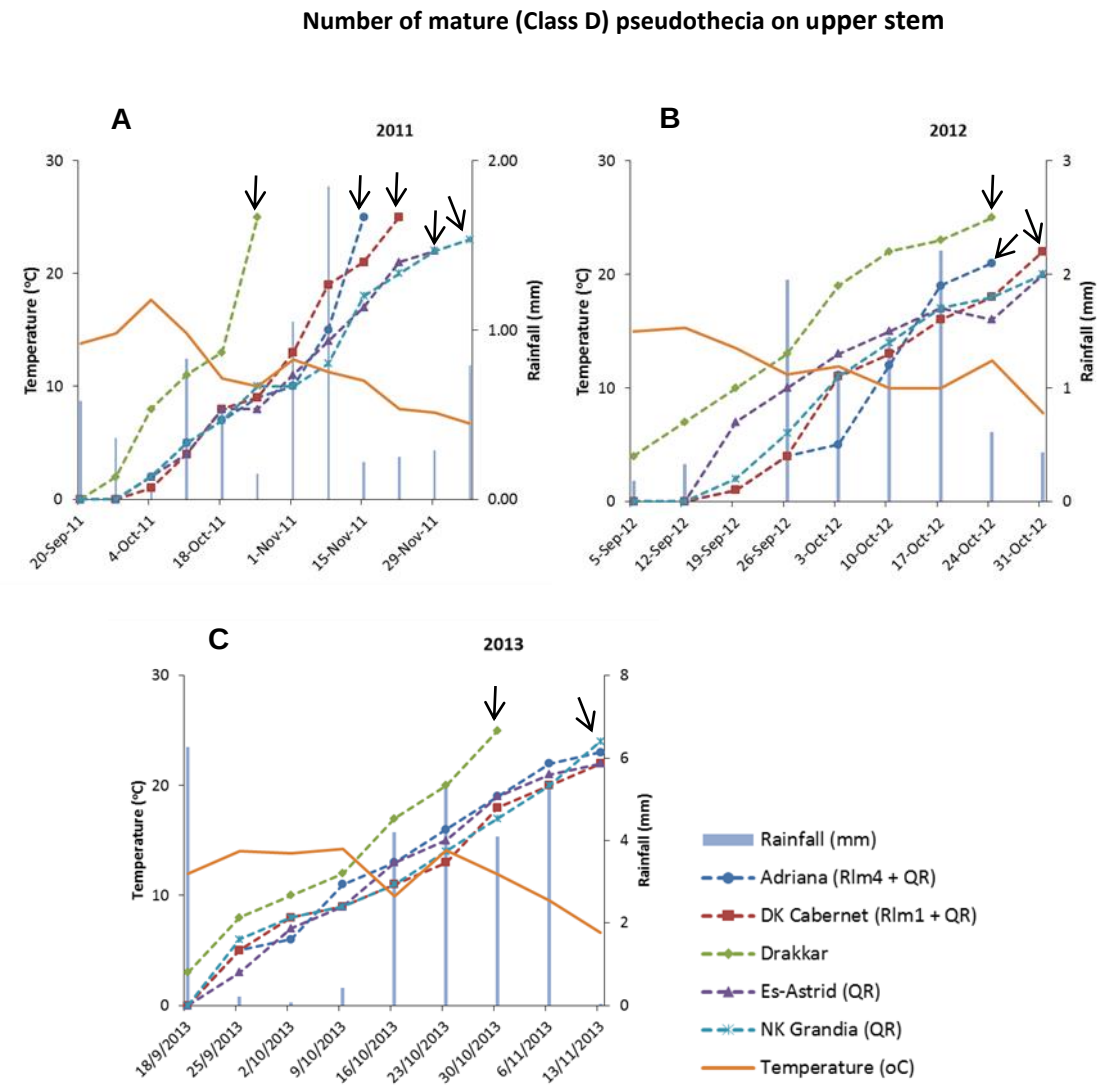


Fig. 4.13: Changes with time in the number of pseudothecia of *Leptosphaeria* spp. observed from a weekly sample of 25 pseudothecia per cultivar on winter oilseed rape upper stems of cultivars with *R* gene and/or without QR that were mature (Class D) in three growing seasons (autumn/winter) at Bayfordbury, (A) from September to November 2011, (B) from September to October 2012 and (C) from September to October 2013. Arrows showing the maximum number of pseudothecia that reached Class D (mature) for each five cultivars observed. The relationship between time to 50% maturation and temperature and rainfall is shown in Appendix 4.33. (Please refer to Appendix 4.23 – 4.31 for details on classes A to E of pseudothecial maturation).

Grandia with QR (Fig. 4.11B). Mature pseudothecia of cvs DK Cabernet, Es-Astrid (Fig. 4.11B) and Capitol (Fig. 4.10B) were first observed on stem bases on 19 September 2012, 2 weeks after mature pseudothecia were observed on cv. Drakkar. In autumn 2013, mature pseudothecia were first observed on stem bases of cvs Drakkar and Bilbao on 18 September 2013 (Fig. 4.10C). All other cultivars, such as Adriana, NK Grandia, Es-Astrid with QR (Fig. 4.10C), Excel, Roxet and Capitol with *R*-genes (Fig. 4.11C), were first observed with mature pseudothecia on stem bases 1 week later (25 September 2013), except that mature pseudothecia were first observed on cv. DK Cabernet (Fig. 4.11C) 2 weeks later (2 October 2013).

On upper stems in autumn 2011, mature pseudothecia were first observed on cvs Drakkar (susceptible to *L. maculans*) and Bilbao after 2 weeks of exposure to natural conditions on 27 September 2011 (Fig. 4.12A), whereas on all other cultivars (Adriana, DK Cabernet, NK Grandia, Es-Astrid) (Fig. 4.12A), (Excel, Roxet and Capitol) (Fig. 4.13A), mature pseudothecia were first observed 3 weeks later on 4 October 2011. In autumn 2012, mature pseudothecia were first observed on cv. Drakkar 1 week after stems were put outside in the experimental plot on 5 September 2012 (Fig. 4.13B), followed by pseudothecia on cvs Adriana, DK Cabernet, NK Grandia, Es-Astrid (Fig. 4.12B), whereas they were first observed on Excel and Bilbao after 3 weeks (on 19 September 2012) (Fig. 4.13B). It was after 4 weeks of exposure to natural conditions that mature pseudothecia were first observed on cvs Roxet (*Rlm7*) and Capitol (*Rlm1*) (Fig. 4.12B). In autumn 2013, mature pseudothecia were first observed on cvs Drakkar on 18 September 2013 (Fig. 4.12C) 1 week after stems were placed in the experimental plot and 2 weeks later mature pseudothecia were first observed on the other eight cultivars (on 25 September 2013) (Fig. 4.12C, Fig. 4.13C).

In mid-September 2011 and late October 2011, the majority of pseudothecia were at development stages Class A and Class B; after October, the majority of pseudothecia were at Class C and more Class D (mature) developed; in early November 2011 and afterwards, some pseudothecia were empty and the number of mature pseudothecia began to decrease, both on stem bases (Appendix 4.14 – 4.22) and on upper stems (Appendix 4.23 – 4.31). The development of pseudothecia in autumn 2012 and 2013 started earlier; the majority of pseudothecia were at Class C in September and by October the majority of pseudothecia were mature (Class D); in late October, most pseudothecia were empty (Class E) on stem bases (Appendix 4.14 – 4.22) and on upper stems (Appendix 4.23 – 4.31).

Pseudothecial development on stem bases of cv. Drakkar (susceptible to *L. maculans*) started earlier (Class D) and reached 50% of pseudothecia in Class D within 22 days, 15 days and 12 days in autumn 2011 (on 12 Oct), 2012 (on 20 Sept) and 2013 (on 29 Sept), respectively (Table 4.5). Pseudothecial development on other cultivars reached 50% of pseudothecia in Class D relatively earlier on the stem bases, for example on cv. Adriana in autumn 2011 (25 days on 14 Oct), cv. Adriana and Bilbao in autumn 2012 (25 days on 30 Sept) and in autumn 2013, cv. Bilbao (15 days on 3 Oct) (Table 4.5). From all nine cultivars, those on cv. Es-Astrid reached 50% of pseudothecia in Class D latest in autumn 2011 (41 days on 31 Oct), cv. Capitol in autumn 2012 (37 days on 12 Oct) and cv. DK Cabernet in autumn 2013 (27 days on 15 Oct) (Table 4.5).

In early October for each growing season, the greatest percentage of pseudothecia in Class D (mature) on stem bases was on cv. Drakkar (48% in autumn 2011) and no pseudothecia were observed in Class D for cvs Bilbao, DK Cabernet and Es-Astrid,

Table 4.5: Number of days when 50% of pseudothecia of *Leptosphaeria* spp. on stem bases (with or without *R* genes) were mature (Class D) for nine cultivars of winter oilseed rape observed from a weekly sample of 25 pseudothecia per cultivar in autumn 2011, 2012 and 2013 and the percentage (%) of pseudothecia that were mature (Class D) in early October 2011, 2012 and 2013.

	Stem base					
Growing season	2011/2012		2012/2013		2013/2014	
	20 Sept 2011[a]		5 Sept 2012[a]		18 Sept 2013[a]	
Cultivar	Days to reach 50% Class D (Date)	Early October (4 Oct 2011) (% Class D)[b]	Days to reach 50% Class D	Early October (3 Oct 2012) (% Class D)[b]	Days to reach 50% Class D	Early October (2 Oct 2013) (% Class D)[b]
Adriana (*Rlm4* + QR)	25 (14 Oct 2011)	40	25 (30 Sept 2012)	68	18 (6 Oct 2013)	60
Bilbao (*Rlm4*)	33 (23 Oct 2011)	0	25 (30 Sept 2012)	72	15 (3 Oct 2013)	60
Capitol (*Rlm1*)	39 (28 Oct 2011)	16	37 (12 Oct 2012)	44	22 (10 Oct 2013)	48
DK Cabernet (*Rlm1* + QR)	38 (28 Oct 2011)	0	33 (8 Oct 2012)	44	27 (15 Oct 2013)	36
Drakkar (No R gene)	22 (12 Oct 2011)	48	15 (20 Sept 2012)	76	12 (29 Sept 2013)	80
Es-Astrid (QR)	41 (31 Oct 2011)	0	31 (6 Oct 2012)	88	20 (8 Oct 2013)	56
Excel (*Rlm7*)	39 (29 Oct 2011)	4	31 (6 Oct 2012)	84	20 (8 Oct 2013)	52
NK Grandia (QR)	37 (27 Oct 2011)	24	26 (1 Oct 2012)	88	19 (7 Oct 2013)	56
Roxet (*Rlm7*)	33 (23 Oct 2011)	36	28 (3 Oct 2012)	60	20 (8 Oct 2013)	56

[a]Date when stems of all nine cultivars that were collected after harvest at Rothamsted Research, Harpenden were being exposed to natural conditions at the Bayfordbury experimental plot throughout autumn/winter 2011/2012, 2012/2013 and 2013/2014.

[b]Early autumn (October) is a crucial time to observe any phoma leaf spotting, therefore, ascospores released from mature pseudothecia (Class D) play an important role to decide control treatments (i.e. application of fungicides).

whereas in 2012, Es-Astrid and NK Grandia both had 88% mature pseudothecia (Class D) and all other cultivars had more than 50% mature pseudothecia, except for cvs Capitol (44%) and DK Cabernet (44%) (Table 4.5). In 2013, on cv. Drakkar 80% of pseudothecia were observed as mature (Class D) in early October 2013 and on other cultivars more than 50% of pseudothecia were observed as mature (Class D), except for cvs Capitol (48%) and DK Cabernet (36%) (Table 4.5).

The number of days required to reach 50% of pseudothecia in Class D showed that pseudothecia on stem bases of all cultivars matured earlier in autumn 2012 and autumn 2013 than in autumn 2011 (Table 4.6). In 2012/2013, pseudothecia on all nine cultivars reached 50% of pseudothecia in Class D from 2 to 11 days earlier whereas in 2013/2014, pseudothecia on all nine cultivars were from 10 to 21 days earlier to reach 50% of pseudothecia in Class D compared to the 2011/2012 growing season on stem bases (Table 4.6).

The result for pseudothecial development on stem bases suggests that the number of rain-days were related to the number of days to reach 50% of pseudothecia in Class D (mature). There was a greater correlation in the 2012/2013 growing season (r = 0.60) and 2013/2014 growing season (r = 0.81) than in 2011/2012 when there was a low correlation (r = 0.34). The number of days for 50% of pseudothecia to reach Class D (mature) showed a greater correlation to the total rain (mm) than to temperature in 2012/2013 (r = 0.59) and 2013/2014 (r = 0.76), whereas there was a poor correlation in 2011/2012 (r = 0.33) (Appendix 4.32). For all three growing seasons, there was a strong negative correlation between number of days for 50% of pseudothecia to reach Class D (mature) and temperature; in the 2011/2012 growing season (r = -0.67); compared with 2012/2013 (r = -0.58) and 2013/2014 (r = -0.65)

Table 4.6: Differences in number of days when 50% of pseudothecia of *Leptosphaeria* spp. were mature (Class D) on stem bases of nine cultivars of winter oilseed rape (with or without *R* genes) observed from a weekly sample of 25 pseudothecia per cultivar in autumn/winter 2011/2012, 2012/2013 and 2013/2014.

	Stem base				
Growing season	2011/2012	2012/2013		2013/2014	
	20 Sept 2011[a]	5 Sept 2012[a]		18 Sept 2013[a]	
Cultivar (*R* gene)	Days to reach 50% Class D	Days to reach 50% Class D	Days to reach 50% Class D compared to 2011/2012[b]	Days to reach 50% Class D	Days to reach 50% Class D compared to 2011/2012[b]
Adriana (*Rlm4* + QR)	25	25	0	18	-7
Bilbao (*Rlm4*)	33	25	-8	15	-18
Capitol (*Rlm1*)	39	37	-2	22	-17
DK Cabernet (*Rlm1* + QR)	38	33	-5	27	-11
Drakkar (No R gene)	22	15	-7	12	-10
ES Astrid (QR)	41	31	-10	20	-21
Excel (*Rlm7*)	39	31	-8	20	-19
NK Grandia (QR)	37	26	-11	19	-18
Roxet (*Rlm7*)	33	28	-5	20	-13

[a]Date when stems of all nine cultivars of that were collected after harvest at Rothamsted Research, Harpenden were put out to be exposed to natural conditions at the Bayfordbury experimental plot throughout autumn/winter 2011/2012, 2012/2013 and 2013/2014.

[b]The differences in number of days taken to reach Class D (mature pseudothecia) were calculated and negative value means that the pseudothecia matured (Class D) earlier and positive value means that the pseudothecia matured (Class D) later than in autumn/winter 2011/2012. The calculation: (number of days to reach 50% of pseudothecia mature (Class D) in 2012/2013 or 2013/2014) – (number of days to reach 50% of pseudothecia mature (Class D) in 2011/2012 (dry season).

(Appendix 4.32).

Pseudothecia on upper stems of cv. Drakkar (susceptible to *L. maculans*) matured earlier (Class D) than on those on other cultivars and reached 50% of pseudothecia in Class D within 33 days and 27 days in autumn 2011 (on 23 Oct) and 2012 (on 1 Oct), respectively (Table 4.7). On cv. Bilbao (*Rlm4*), the pathogen reached 50% of pseudothecia in Class D within 28 days and it was a day earlier than on cv. Drakkar on 16 October 2013 (Table 4.7). 50% of pseudothecia were in Class D on cvs Adriana and Bilbao in autumn 2011, both within 39 days (on 29 October 2011), whereas on cvs Adriana, Es-Astrid and Excel in autumn 2013, it was within 33 days, 34 days and 36 days, respectively (Table 4.7). In early October in each growing season, the greatest percentage of pseudothecia in Class D (mature) on upper stems were on cv. Drakkar (32% in autumn 2011, 76% in autumn 2012 and 40% in autumn 2013) (Table 4.7). It was only on cv. Es-Astrid in autumn 2012 that 52% of pseudothecia were in Class D in early October, whereas on other cultivars less than 50% were in Class D (mature pseudothecia) in all three growing seasons (Table 4.7).

The number of days required until 50% of pseudothecia were mature (in Class D) showed that in both autumn 2012 and autumn 2013 pseudothecia on upper stems of all cultivars matured earlier than in autumn 2011 (Table 4.8). In 2012/2013, on all nine cultivars the time to reach 50% of pseudothecia in Class D was from 2 to 18 days earlier whereas in 2013/2014, on all nine cultivars the time to reach 50% of pseudothecia in Class D was from 4 to 19 days earlier than on to the upper stems in the 2011/2012 growing season (Table 4.6).

Table 4.7: Number of days to when 50% of *Leptosphaeria* spp. pseudothecia on upper stems (with or without *R* genes) were mature (Class D) for nine cultivars of winter oilseed rape observed from a weekly sample of 25 pseudothecia per cultivar in autumn 2011, 2012 and 2013 and the percentage (%) of pseudothecia that were mature (Class D) in early October 2011, 2012 and 2013.

	Upper stem					
Growing season	2011/2012		2012/2013		2013/2014	
	20 Sept 2011[a]		5 Sept 2012[a]		18 Sept 2013[a]	
Cultivar (*R* gene)	Days to reach 50% Class D (Date)	Early October (4 Oct 2011) (% of Class D)[b]	Days to reach 50% Class D	Early October (3 Oct 2012) (% of Class D)[b]	Days to reach 50% Class D	Early October (2 Oct 2013) (% of Class D)[b]
Adriana (*Rlm4* + QR)	39 (29 Oct 2011)	8	43 (18 Oct 2012)	20	33 (21 Oct 2013)	24
Bilbao (*Rlm4*)	39 (29 Oct 2011)	12	39 (13 Oct 2012)	40	28 (16 Oct 2013)	36
Capitol (*Rlm1*)	41 (30 Oct 2011)	16	44 (19 Oct 2012)	28	43 (31 Oct 2013)	20
DK Cabernet (*Rlm1* + QR)	48 (7 Nov 2011)	4	40 (15 Oct 2012)	44	40 (28 Oct 2013)	32
Drakkar (No R gene)	33 (23 Oct 2011)	32	27 (1 Oct 2012)	76	29 (16 Oct 2013)	40
ES Astrid (QR)	53 (12 Nov 2011)	8	40 (14 Oct 2012)	52	34 (22 Oct 2013)	28
Excel (*Rlm7*)	51 (9 Nov 2011)	16	53 (27 Oct 2012)	20	36 (24 Oct 2013)	24
NK Grandia (QR)	57 (15 Nov 2011)	8	39 (13 Oct 2012)	44	39 (26 Oct 2013)	32
Roxet (*Rlm7*)	47 (6 Nov 2011)	8	47 (21 Oct 2012)	40	41 (28 Oct 2013)	20

[a]Date when stems of all nine cultivars that were collected after harvest at Rothamsted Research, Harpenden were being exposed to natural conditions at the Bayfordbury experimental plot throughout autumn/winter 2011/2012, 2012/2013 and 2013/2014.

[b]Early autumn (October) is a crucial time to observe any phoma leaf spotting, therefore, ascospores released from mature pseudothecial (Class D) will play an important role to decide control treatments (i.e. application of fungicides).

Table 4.8: Differences in number of days to when 50% of pseudothecia of *Leptosphaeria* spp. were mature (Class D) on upper stems of nine cultivars of winter oilseed rape (with or without *R* genes) observed from a weekly sample of 25 pseudothecia per cultivar in autumn/winter 2011/2012, 2012/2013 and 2013/2014.

	Upper stem				
Growing season	2011/2012	2012/2013		2013/2014	
	20 Sept 2011[a]	5 Sept 2012[a]		18 Sept 2013[a]	
Cultivar (*R* gene)	Days to reach 50% Class D (date)	Days to reach 50% Class D	Days to reach 50% Class D compared to 2011/2012[b]	Days to reach 50% Class D	Days to reach 50% Class D compared to 2011/2012[b]
Adriana (*Rlm4* + QR)	39	43	4	33	-6
Bilbao (*Rlm4*)	39	39	0	28	-11
Capitol (*Rlm1*)	41	44	3	43	2
DK Cabernet (*Rlm1* + QR)	48	40	-8	40	-8
Drakkar (No R gene)	33	27	-6	29	-4
ES Astrid (QR)	53	40	-13	34	-19
Excel (*Rlm7*)	51	53	2	36	-15
NK Grandia (QR)	57	39	-18	39	-18
Roxet (*Rlm7*)	47	47	0	41	-6

[a]Date when stems of all nine cultivars that were collected after harvest at Rothamsted Research, Harpenden were put out to be exposed to natural conditions at the Bayfordbury experimental plot throughout autumn/winter 2011/2012, 2012/2013 and 2013/2014.

[b]The differences in number of days taken to reach Class D (mature pseudothecia) were calculated and negative value means that the pseudothecia matured (Class D) earlier and positive value means that the pseudothecia matured (Class D) later than in autumn/winter 2011/2012. The calculation: (number of days to reach 50% of pseudothecia mature (Class D) in 2012/2013 or 2013/2014) – (number of days to reach 50% of pseudothecia mature (Class D) in 2011/2012 (dry season).

The results for pseudothecial development on upper stems suggest that the number of rain-days were related to the number of days for 50% of pseudothecia to reach Class D (mature) and showed a greater correlation for all three growing seasons (i.e. 2011/2012; r = 0.70, 2012/2013; r = 0.98 and 2013/2014; r = 0.86). The total rain (mm) also showed a greater correlation with number of days for pseudothecia to reach 50% Class D (mature) than temperature for all three growing seasons (i.e. 2011/2012; r = 0.72, 2012/2013; r = 0.95 and 2013/2014; r = 0.85). There was a low correlation between days for pseudothecia to reach 50% in Class D (mature) and temperature; (r = 0.13) in 2011/2012 growing season compared with 2012/2013 (r = -0.77) and 2013/2014 (r = 0.56). There is a weather pattern where the rain influences the maturation of pseudothecia (Appendix 4.33).

There were significant differences (P<0.05) in mean density of pseudothecia between stem bases and upper stems for each cultivar in all three growing seasons (autumn 2011/2012, 2012/2013 and 2013/2014) (Fig. 4.14). Generally, the mean density of pseudothecia was greater on the stem bases (< 5 cm above ground level) than the upper stems (> 10 cm above ground level) for all cultivars (Fig. 4.14). Cultivar Drakkar (susceptible to *L. maculans*) had the greatest density of pseudothecia on both stem bases and upper stems when sampled on 25 October 2011 (Fig. 4.14A), 3 October 2012 (Fig. 4.14B) or 9 October 2013 (Fig. 4.14C). The smallest densities of pseudothecia on stem bases for all three growing season were on cv. NK Grandia (QR) (Fig. 4.14). On the upper stems, the smallest densities of pseudothecia were on cultivar NK Grandia sampled on 3 October 2012 (Fig. 4.14B) and 9 October 2013 (Fig. 4.14C), whereas cv. Excel sampled on 25 October 2011 had the smallest density of pseudothecia (Fig. 4.14A).

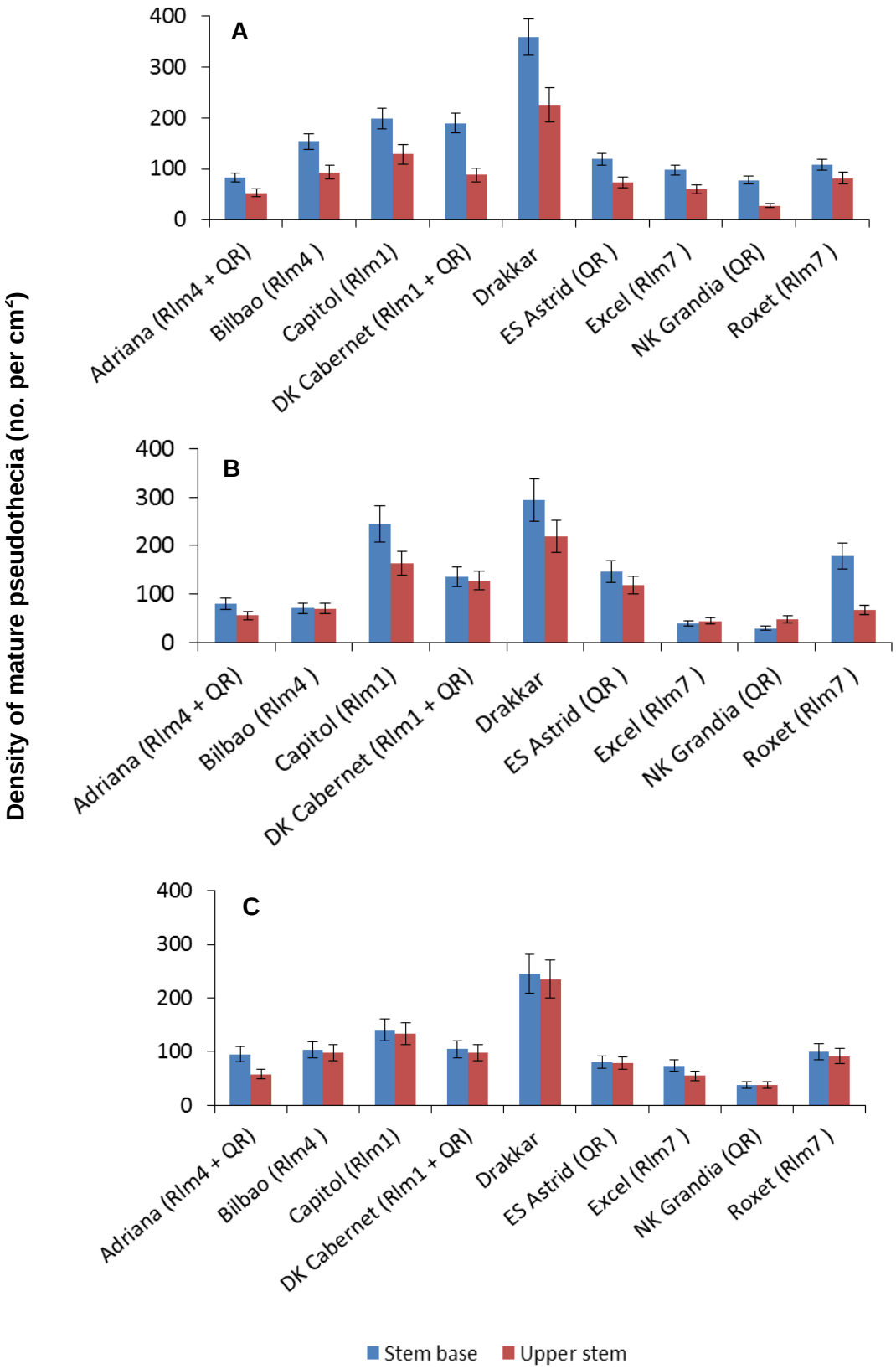


Fig. 4.14: The densities of mature pseudothecia (number pseudothecia per cm^2) produced on upper stems (>10 cm above ground level) and stem bases (<5 cm above ground level) of winter oilseed rape exposed at Bayfordbury sampled on (A) 25 October 2011, (B) 3 October 2012 and (C) 9 October 2013.

The stem bases (Fig. 4.15) and upper stems (Fig. 4.16) produced more *L. biglobosa* than *L. maculans* colonies from 50 ascospores in all three growing seasons from stems sampled on 25 October 2011, 30 October 2012 or 9 October 2013. In autumn 2011, for stem bases only cv. NK Grandia stems produced more *L. maculans* than *L. biglobosa* colonies from 50 ascospores (Fig. 4.15A). Cultivars that sometimes produced more *L. maculans* colonies from 50 ascospores were cv. Drakkar (in autumn 2012) (Fig. 4.15B) and cv. Excel (in autumn 2013) (Fig. 4.15C).

For the upper stems, some cultivars produced only *L. biglobosa* colonies from 50 ascospores [in autumn 2011, cvs Adriana and NK Grandia (Fig. 4.16A); in autumn 2012, cvs Adriana, Bilbao, Capitol, DK Cabernet, Es-Astrid and Excel (Fig. 4.16B); in autumn 2013, cvs Adriana, Capitol, Es-Astrid, Excel and NK Grandia (Fig. 4.16C)]. There were more *L. biglobosa* than *L. maculans* colonies produced from upper stems (Fig. 4.16). There were some cultivars that produced no *L. maculans* or *L. biglobosa* colonies from 50 ascospores but cultivar Drakkar produced both *L. maculans* and *L. biglobosa* from stem bases (Fig. 4.15) and upper stems (Fig. 4.16).

4.3.3 Assessment of ascospore release under natural conditions and proportions of *Leptosphaeria maculans* and *L. biglobosa* by using qPCR

In the Bayfordbury experimental plot in autumn 2011, the release of ascospores was later than in 2012/2013 and 2013/2014 growing seasons and many ascospores were released in December 2011 and January 2012 (Fig. 4.17A). The first ascospores were released on 22 October 2011 (126 spores/m^{-3}) (Fig. 4.17A). From 13 September until the end of October 2011, 27 spores/m^3 per day were released. More maxima were observed over the following few weeks separated by periods of far

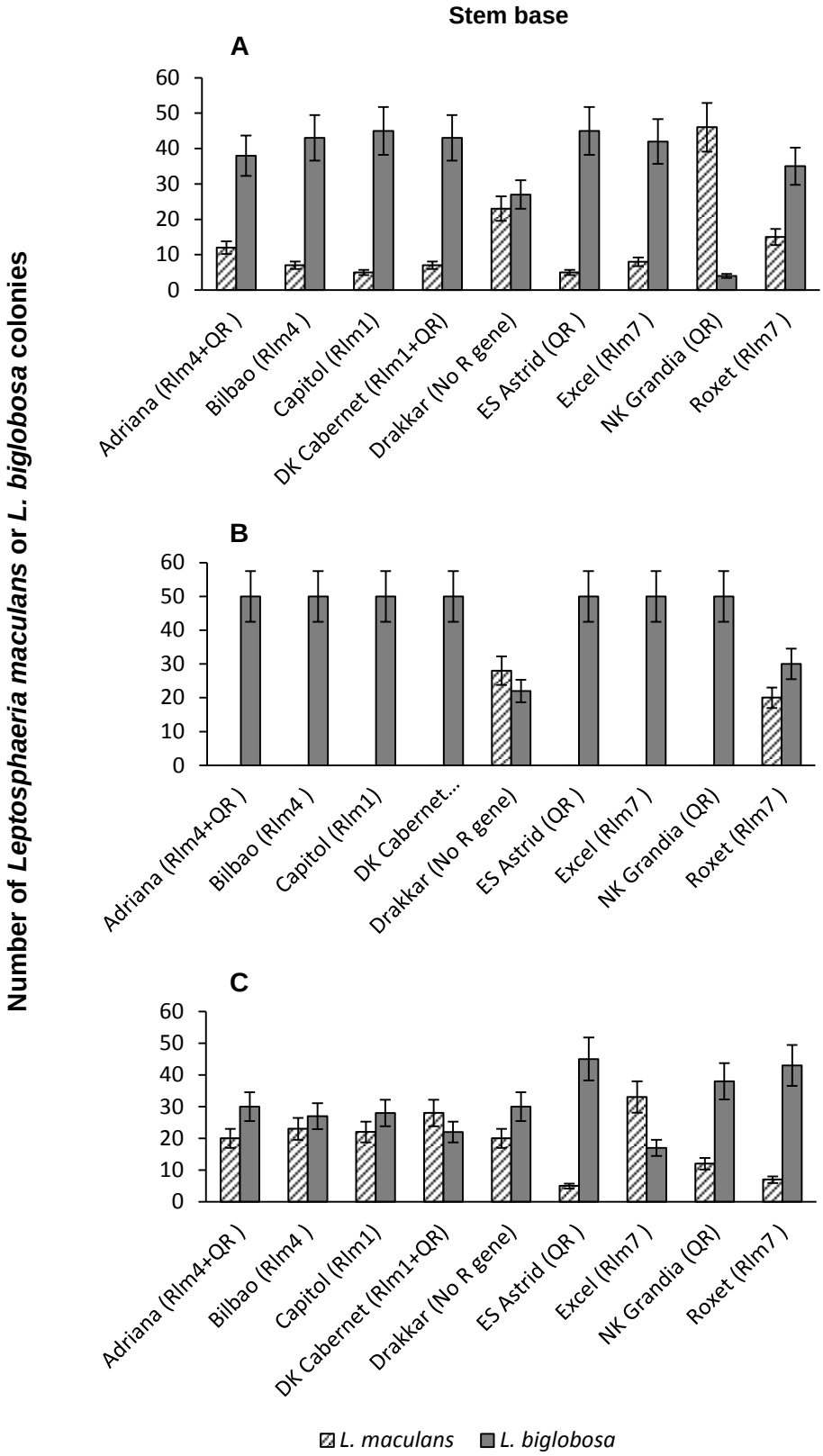


Fig. 4.15: Numbers of *Leptosphaeria maculans* or *L. biglobosa* colonies arising from ascospores released from mature pseudothecia (Class D) on stem bases onto PDA from stem bases sampled on (A) 25 October 2011, (B) 3 October 2012 and (C) 9 October 2013. A total of 50 ascospores per cultivar were released and colonies counted were identified based on morphological identification (refer chapter 2, Fig. 2.3).

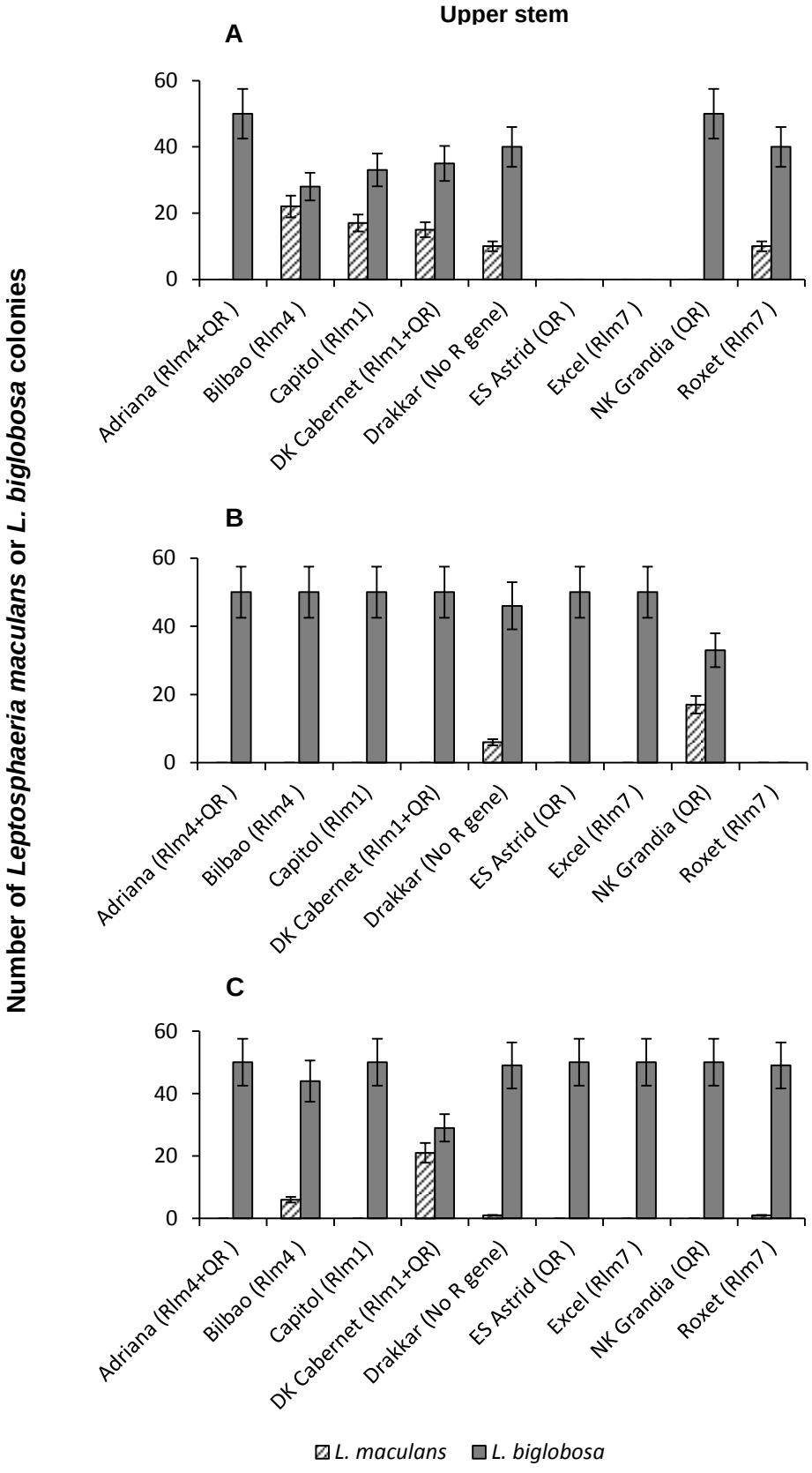


Fig. 4.16: Numbers of *Leptosphaeria maculans* or *L. biglobosa* colonies arising from ascospores released from mature pseudothecia (Class D) on upper stems onto PDA from upper stems sampled on (A) 25 October 2011, (B) 3 October 2012 and (C) 9 October 2013. A total of 50 ascospores per cultivar were released and colonies counted were identified based on morphological identification (refer chapter 2, Fig. 2.3).

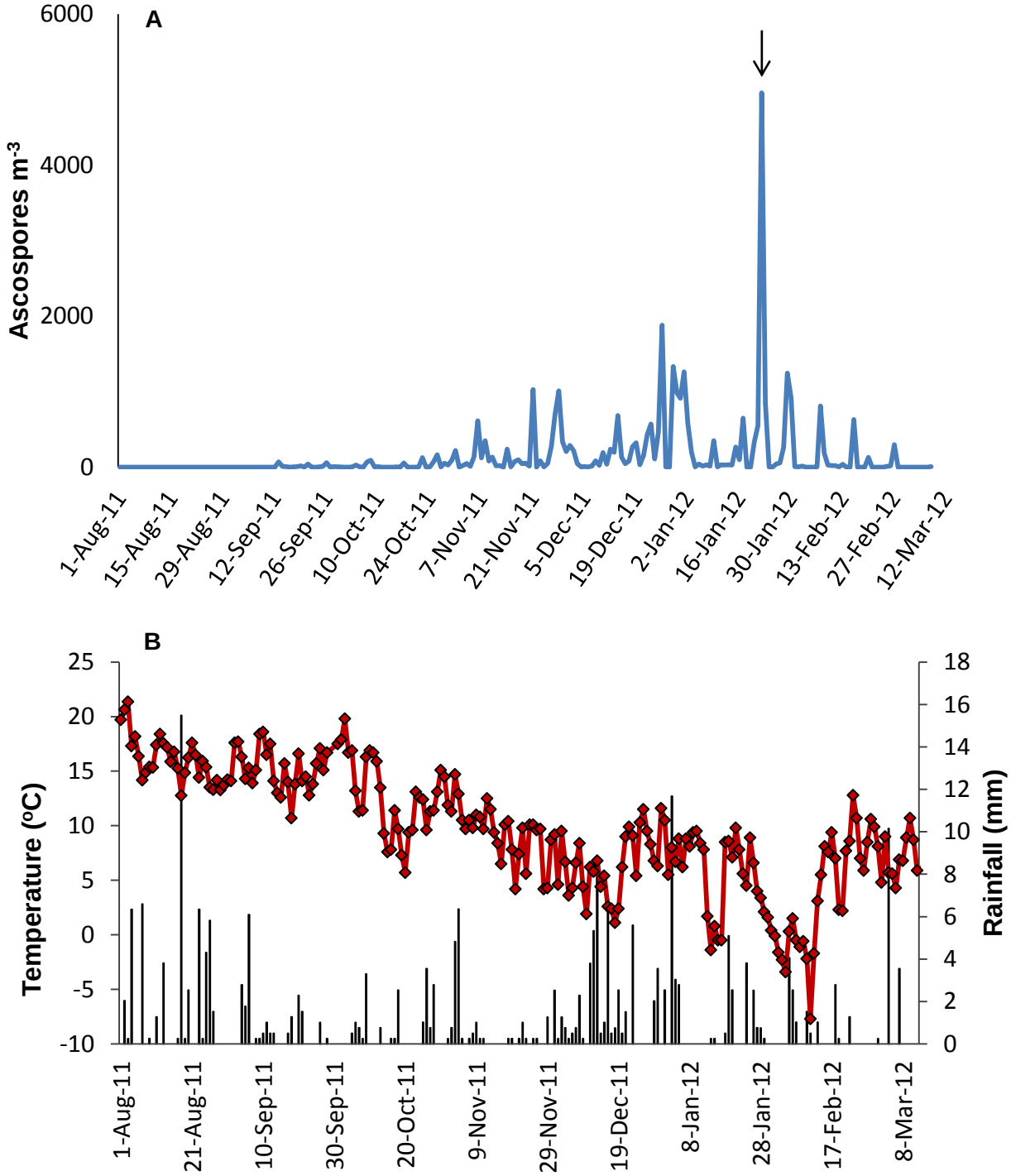


Fig. 4.17: Total daily concentrations of ascospores released in the period from (A) September 2011 to February 2012 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler and (B) weather parameters recorded by a Davis weather station; total daily rainfall (mm) and average temperature (^{o}C) from 1 August 2011 until end of March 2012. The maximum number of ascospores was on 22 January 2012 (4958 spores/m^{-3}) (arrow).

lower ascospore release (78-407 spores/m³). The greatest number of ascospores released was in the third week of January 2012; a maximum of 4958 spores/m^{-3} (22 January 2012) (Fig. 4.17A). After 28 November 2011 (total of 1010 spores/m^{-3}), there was a period with little (<682 spores/m³) or no ascospore release observed until 25 December 2011 (459 spores/m³) and ascospores release was 1881 spores/m³ on 26 December 2011. Then ascospore release was low again until a maximum ascospore release was observed on the 22 January 2012, after which numbers of ascospores released decreased steadily with some fluctuation from ca. 1618 to 0 spores/m³ (Fig. 4.17A). Ascospore release finished at the end of February 2012 (Fig. 4.17A).

The average daily temperature fluctuated but decreased slowly with time from a maximum of ca. 22°C in August to a minimum of *c*. -8°C in February and was <0°C three times (Fig. 4.17B). The temperature remained as high as in August during September and part of October during a prolonged, unusually warm period after which the temperature started to decrease. From September 2011 to November 2011, there was little rain (Fig. 4.17B). In the middle of December, above average temperature and above average rainfall occurred at the same time as a maximum ascospore release and a change to warmer, wetter days after many cold, dry days. In January 2012, there was substantial rainfall (Fig. 4.17B) and the weather conditions were suitable for the maturation and release of ascospores.

The ascospore release data for Bayfordbury showed little ascospore release at the start of measurement in September 2012 and the first ascospores were released on 30 September 2012 (23 spores/m-³) (Fig. 4.18A). From early September to October 2012, 66 spores/m³ per day were released. Three greatest maxima in ascospores

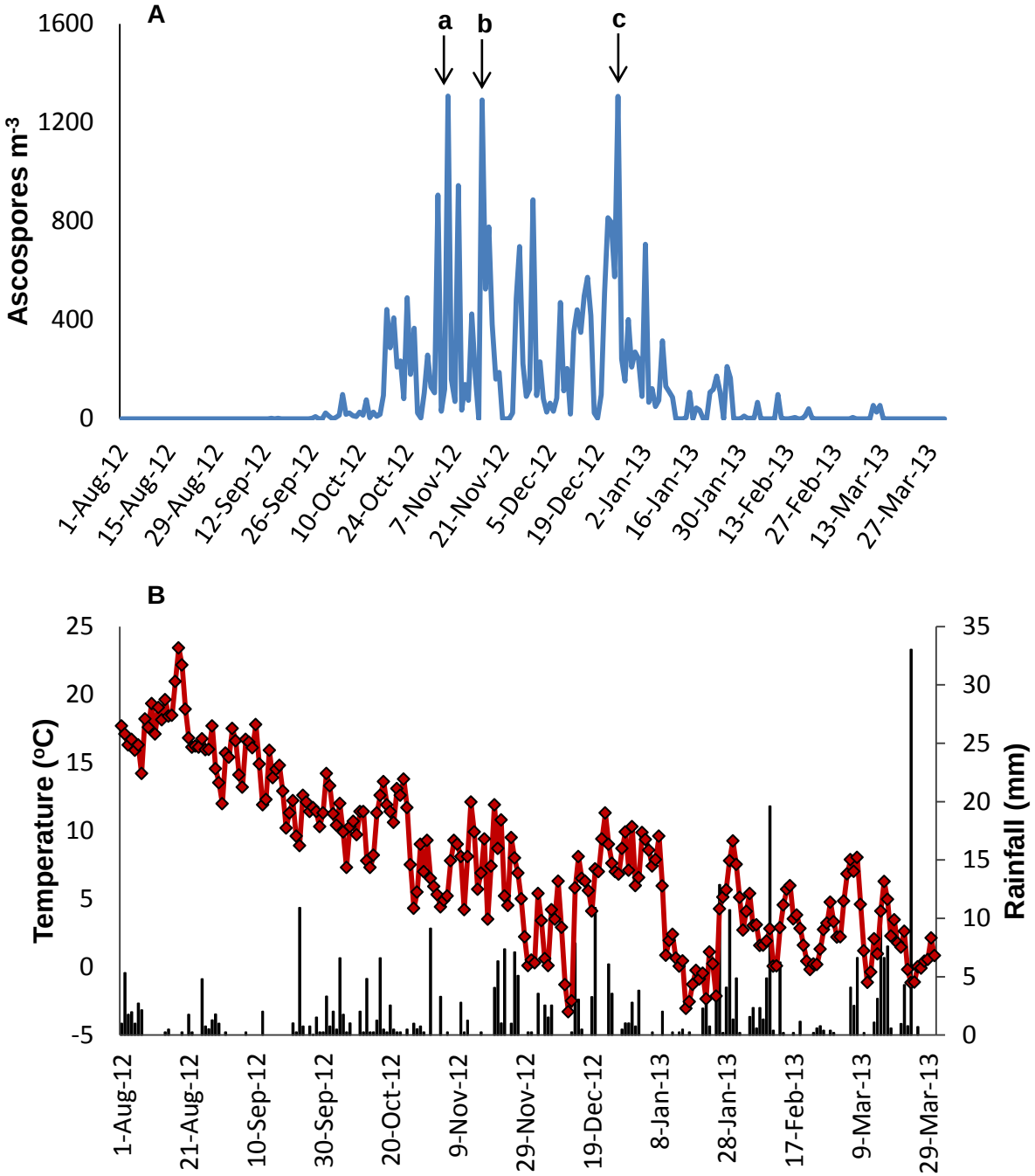


Fig. 4.18: Total daily concentrations of ascospores released in the period from (A) September 2012 to early March 2013 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler and (B) weather parameters recorded by a Davis weather station; total daily rainfall (mm) and average temperature (^{o}C) from 1 August 2012 until end of March 2013. The maximum number of ascospores was on (a) 5 November 2012 (1307 spores/m^{-3}), (b) on 15 November 2012 (1291 spores/m^{-3}) and (c) on 25 December 2012 (1306 spores/m^{-3}) (arrows).

release were recorded in November and in December (Fig. 4.18A). In November, the maximum was on 5 November 2012 (1307 spores/m^{-3}), then in the middle of November ascospore release decreased but was followed by a rapid increase to 1291 spores/m^{-3} on 15 November 2012 (Fig. 4.18A). Then ascospore release decreased to 814 and 0 spores/m^3 until the third maximum in ascospore release was on 25 December 2012 (1306 spores/m^{-3}) (Fig. 4.18A). At the end of December, ascospore release decreased to 407 spores/m^3. At the start of January, a final maximum of 706 spores/m^{-3} was observed on 2 January 2013, after which ascospore release decreased (Fig. 4.18A). Throughout January and February 2013, ascospore release remained low (<39 spores/m^{-3}) until early March 2013 when ascospore release measurement finished (Fig. 4.18A).

The average daily temperature fluctuated but decreased slowly with time from a maximum of 23°C in August 2012 to a minimum of -3°C in December 2012 and January 2013 with temperature <0°C three times (Fig. 4.18B). The greatest temperature of the period was recorded on two days in the middle of August when temperature was 23°C, significantly greater than the August average of 18°C. After being cold for several days, at the end of November it became warmer. This corresponded to maxima in ascospore release observed at the same time. The temperature increased towards the end of December and start of January, coinciding with a maximum in ascospore release (Fig. 4.18B). Rainfall in August was variable with high rainfall at the start and end of the month but many dry days in the middle, just before ascospore sampling began. Rainfall in September was low except at the end and October had much rain, followed by maximum ascospore release in early November 2012 (Fig. 4.18B). The start of November had many dry days while the

end of November was very wet with high rainfall continuing throughout December (Fig. 4.18B).

In autumn 2013, the first ascospores were released on 29 September 2013 (275 spores/m^{-3}) (Fig. 4.19A). From September 2013 until at the end of October, 219 spores/m^{-3} per day were released and more maxima were observed up to the third week of November 2013. There were three maximum in ascospore release recorded from late October until November, on 27 October 2013 (4575 spores/m^{-3}), 3 November 2013 (4619 spores/m^{-3}) and 9 November 2013 (3674 spores/m^{-3}) (Fig. 4.19A).

After the maximum release of ascospores, there was a period with little or no ascospore release observed, from 21 November 2013 until 11 December 2013 and by 12 December, maximum ascospores release was 3521 spores/m^{-3} (Fig. 4.19A). Then ascospore release was low again until a maximum ascospore release was observed on 18 January 2014 (2159 spores/m^{-3}), after which numbers of ascospores released decreased steadily to 0 spores/m^3 (Fig. 4.19A). The ascospore release finished in early March 2014 (Fig. 4.19A).

The average daily temperature fluctuated but decreased slowly with time from a maximum of *ca.* 24°C in August 2013 to a minimum of 1°C in November 2013 and was <0°C two times (Fig. 4.19B). From August 2013 to early of November 2013, there was high rainfall (a total of 254 mm) and high temperature occurred at the same time as maximum ascospore release (Fig. 4.19B). The second week of November 2013 had low rainfall continuing throughout March 2014. The average

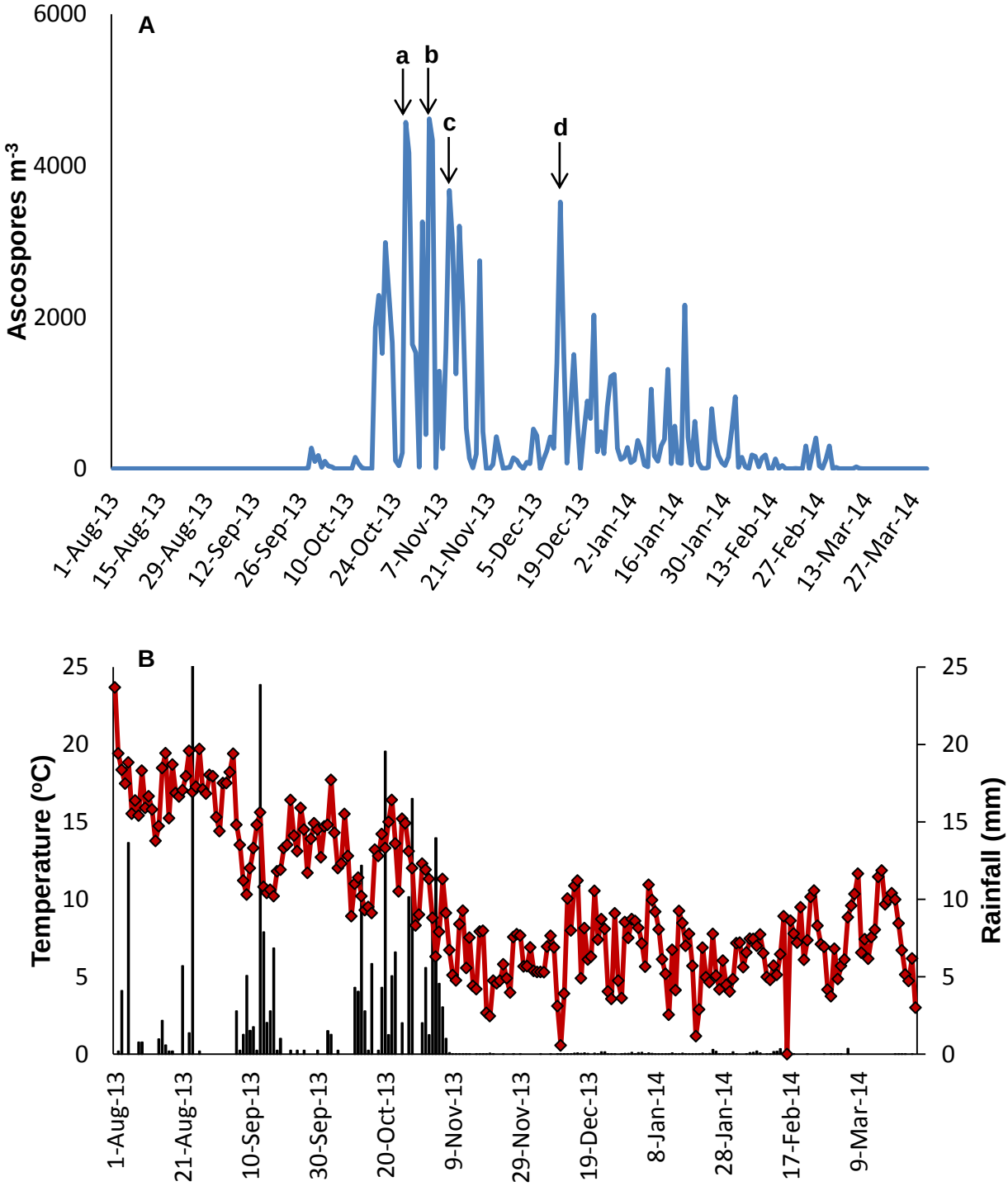


Fig. 4.19: Total daily concentrations of ascospores released in the period from (A) end of August 2013 to early March 2014 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler and (B) weather parameters recorded by a Davis weather station; total daily rainfall (mm) and average temperature (^{o}C) from 1 August 2013 until early March 2014. The maximum number of ascospores was on (a) 27 October 2013 (4575 spores/m^{-3}), (b) on 3 November 2013 (4619 spores/m^{-3}), (c) on 9 November 2013 (3674 spores/m^{-3}) and (d) on 12 December 2013 (3521 spores/m^{-3}) (arrows).

temperature was 6.6°C from November to December 2013 and 6.9°C from January to March 2014 (Fig. 4.19B). The phoma leaf spot forecast dates of 10% leaf spotting were predicted on 14 October 2011, 19 October 2012 and 5 October 2013, respectively. Over three growing seasons, the earliness of ascospore release at Bayfordbury experimental plot in autumn 2012 and autumn 2013 reflected an early prediction of the leaf spot model whereas in autumn 2011 the phoma leaf spot forecast date of 10% leaf spotting was a week earlier than the ascospore release.

DNA was extracted from a total of 36 spore tape samples from Bayfordbury for the 2013/2014 growing season. The quantity and quality of DNA of these samples were analysed by the NanoDrop ND-1000. In general, the purity of the DNA extracted from Burkard spore tapes was good with a mean of 1.76 (and range from 1.6 to 2.2) for the 260/280 value (refer to 4.2.3.5). There were large differences between samples in DNA concentration. DNA concentration ranged from 4.4 to 3006 ng/µl for the 2013/2014 DNA samples from Bayfordbury (Fig 4.20). The majority of this variation corresponded to the differences in the number of ascospores (assessed by light microscopy); the date on which the maximum amount of total DNA was detected (3 November 2013) corresponded to the date of maximum ascospore release. However, exceptions were also observed, when high ascospore counts appeared to be on dates with low total DNA concentration (e.g. 13 Dec) or *vice versa* (e.g. 5 Nov). In general, when there were large numbers of ascospores on half tapes, there were significant, positive correlations between ascospore numbers obtained by using visual assessment and amount of DNA (ng) from half tapes that were measured by qPCR (Fig. 4.20B) (Appendix 4.34).

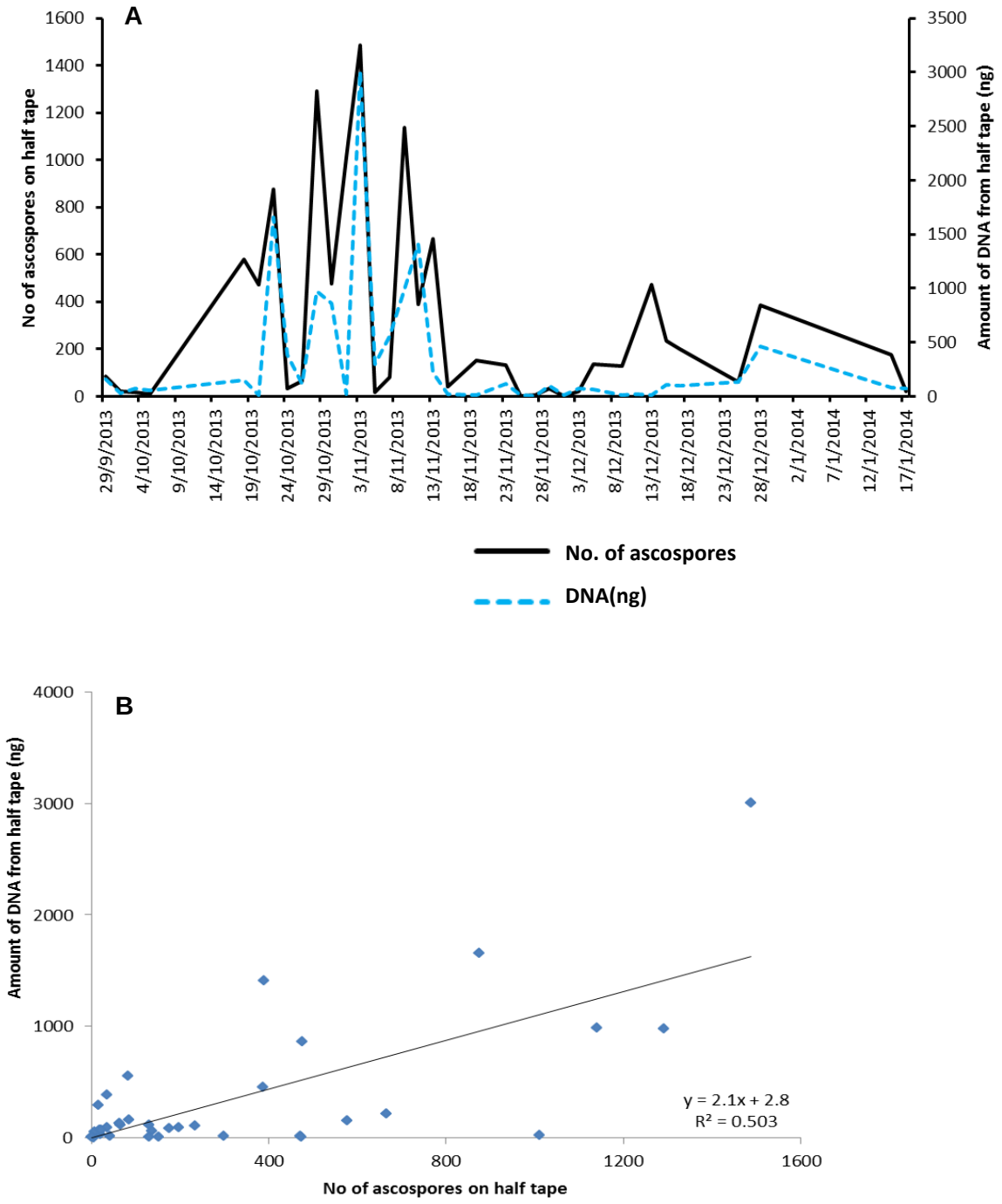


Fig. 4.20: (A) The total amount of DNA extracted from one half of the spore tape measured by the NanoDrop in relation to the number of ascospores on the other half of the spore tape assessed by light microscopy for spore samples collected from Bayfordbury in the 2013/2014 growing season. (B) Relationships between the total amount of DNA of fungi spores extracted from one half of the spore tape measured by the NanoDrop (y) and the number of ascospores on the other half of the spore tape (x) (y= 2.1x + 2.8) assessed by light microscopy for spore samples collected from Bayfordbury in the 2013/2014 growing season.

In the 2013/2014 growing season, at Bayfordbury changes with time in the amounts of *L. maculans* and *L. biglobosa* DNA revealed differences in the patterns of ascospore release between *L. maculans* and *L. biglobosa* (Fig. 4.21). On most days in October, the amount of *L. maculans* DNA detected was greater than that of *L. biglobosa* DNA, suggesting that the number of *L. maculans* ascospores was greater than that of *L. biglobosa* ascospores. From 1 November to 15 December, the amount of *L. biglobosa* DNA detected on most days (except for 19 and 23 of November) was greater than that of *L. maculans* DNA (Fig. 4.21), suggesting that the number of *L. biglobosa* ascospores released on those days was larger than that of *L. maculans* ascospores. The first maximum in *L. maculans* DNA (18 October) was detected earlier than that of *L. biglobosa* DNA (24 October) (Fig. 4.21). There was also a significant difference between months in the amounts of *L. maculans* and *L. biglobosa* DNA ($P<0.05$). Comparison of the data on number of ascospores assessed by microscopy and the data on amounts of *L. maculans/L. biglobosa* DNA assessed by qPCR showed a greater correlation between the numbers of ascospores and amounts of DNA for *L. maculans* than for *L. biglobosa,* where maxima in *L. maculans* DNA reflected maxima in ascospore count (e.g. 22 October). However, the opposite was also observed on 19 November, when a large amount of *L. maculans* DNA was detected but a small number of ascospores was counted by light microscopy (Fig. 4.21).

4.4 Discussion

These results suggest that quantitative resistance (QR) in cultivars may delay the pseudothecial maturation of *Leptosphaeria* species. It was observed that maturation was slowest on stems (stem bases and upper stems) of cultivars Adriana and NK Grandia (both with quantitative resistance) in both controlled environment conditions or when they were exposed at different time points in natural conditions during the

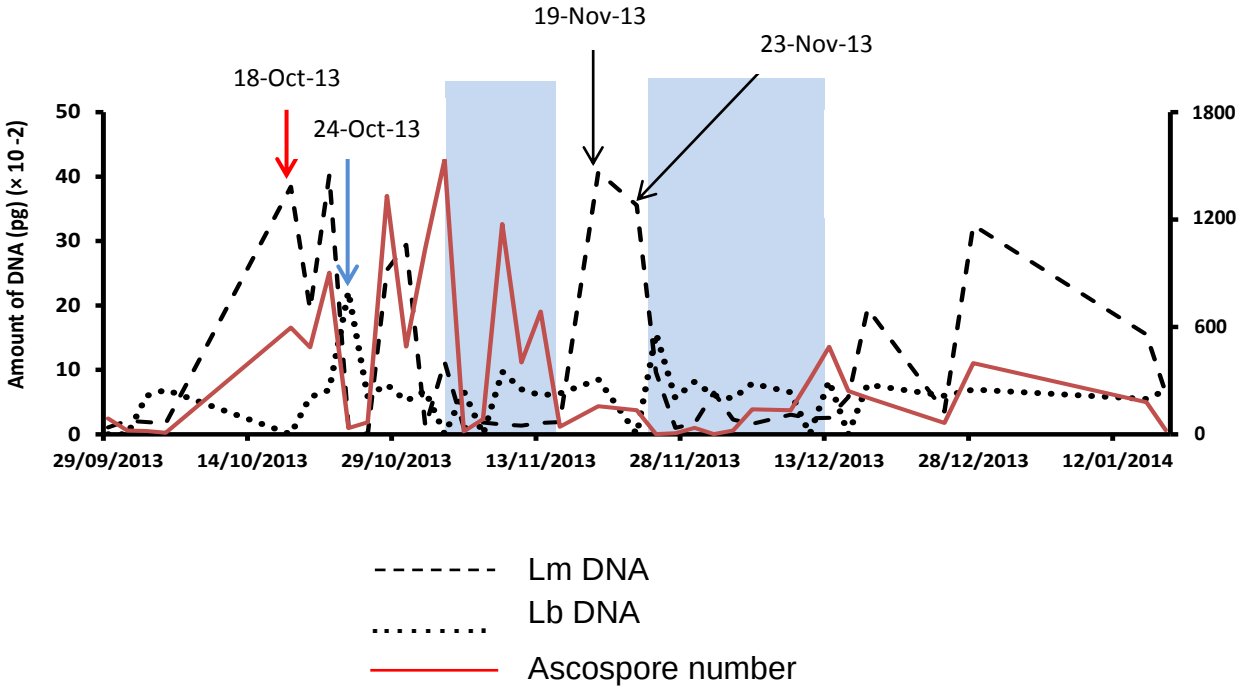


Fig. 4.21: Daily number of ascospores released counted on half of the spore tape compared with the amount of *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) DNA detected on the other half of the spore tape samples collected at Bayfordbury in the 2013/2014 growing season. Arrow showing the first maxima *of L. maculans* DNA (red arrow) and *L. biglobosa* DNA (blue arrow). The blue areas show the periods when a greater amount of *L. biglobosa* DNA was detected and the arrows (black) the period when a greater amount of *L. maculans* DNA was detected.

autumn/winter of 2012/2013 and 2013/2014 growing seasons (Table 4.2 & 4.3). The pseudothecia may have developed faster on cv. Bilbao than on cultivars Adriana (*Rlm4* + QR) and NK Grandia (QR) because Bilbao has no QR (Fig, 4.6, Table 4.4). This may be due to the fact that *R*-gene mediated resistance operates in leaf tissues while the QR operates later in the leaf petiole and stem tissues (Fitt *et al.,* 2006a; Huang *et al.,* 2009).

The consequence of the faster pseudothecial development on cv. Drakkar with no QR was that there is more *Leptosphaeria* inoculum for the next growing season whereas slower pseudothecial development on cultivars with QR decreased initial inoculum for the next growing season. Previous work had shown that the more severe the canker before harvest, the larger the number of pseudothecia and the faster the pseudothecial maturation (McGee, 1977; Petrie, 1995; Marcroft *et al.,* 2004a). In this work, pseudothecia on stems of cultivars with the combination of *R* gene resistance and QR consistently matured later than those on other cultivars (cultivars with only *R* genes or susceptible cv. Drakkar), regardless of the weather conditions. A combination of *R*-gene mediated resistance and effective deployment of quantitative resistance (QR) will limit the number of pseudothecia formed and thus reduce inoculum concentration in the next growing season. Similarly, the cultivar Darmor, with good QR, had less initial inoculum (ascospores) in autumn in France than cultivars without QR (Lô-Pelzer *et al*., 2009a; Brun *et al.,* 2010). Marcroft *et al.* (2004a) discovered that some *Brassica* species, such as *B. carinata*, *B. nigra*, *Sinapis alba* and *B. napus* cv. Surpass 400, had lower pseudothecial density and discharged fewer ascospores than other *B. napus* species (Marcroft *et al.,* 2004a). This could be linked to quantitative resistance (QR). It has been recommended that characters from these *Brassica* species that can influence the number of

pseudothecia produced on stems and subsequent ascospore discharge could be introgressed into commercial cultivars to reduce severity of phoma stem canker epidemics (Marcroft *et al.,* 2004a). Furthermore, cultivars with *R* genes and QR have stronger woody stems than the susceptible cv. Drakkar (i.e. cv. Drakkar had the smallest stem diameter) (refer Fig. 3.3.3 in Chapter 3) and may senesce later than susceptible cultivars. It would be more difficult for the fungus to colonise and recombine within these woody stems for sexual reproduction (Marcroft *et al.,* 2004).

Results from the pseudothecial density observations suggested that pseudothecia develop much faster at greater density on the basal part of the stems than on upper parts of stems for all cultivars exposed to natural conditions (Fig. 4.14) or controlled environment conditions (Fig. 4.7). After harvest, senescent stem tissues are rapidly colonised by *L. maculans* and *L. biglobosa* and a greater number of pseudothecia develop (West *et al.,* 2001), especially on severe stem base cankers. Moreover, there is a possibility that pseudothecia mature faster because the stem base tissues retain moisture longer than upper stem tissues (Thürwächter *et al.,* 1999). In this study, the observation that the susceptible cv. Drakkar had the greatest density of pseudothecia in all three growing seasons (Fig. 4.7 & 4.14) shows that pseudothecial density is strongly affected by cultivar factors. In the life cycle of hemibiotrophic pathogens such as *Leptosphaeria* spp., water is an important element to allow production of enzymes for degradation of the dead stem tissues to supply nutrients to the growing pathogen (Van Kan, 2006; Schumann & D'Arcy, 2010). Therefore, pseudothecial maturation was earlier in controlled conditions when stems were sprayed regularly to maintain continuous wetness than in natural conditions when there were sometimes dry periods.

The study of the proportions of *L. maculans* and *L. biglobosa* isolates from single ascospores ejected from stems showed that there were more than expected *L. biglobosa* isolates for all nine cultivars on both the upper stems and stem bases (Fig. 4.15 – 4.16). Previous work had reported that *L. maculans* isolates caused damaging basal stem cankers whereas *L. biglobosa* only penetrated the leaf lamella and petiole of the leaves and colonised the upper stem causing less damaging upper stem lesions (Hammond & Lewis, 1987; West *et al.,* 2002b; Salam *et al.,* 2007). However, in this study abundant pseudothecia of *L. biglobosa* were observed on stem bases. Therefore, there is a potential for the population structure of the pathogens in the UK to change during the season as pseudothecia on different parts of stem debris mature after harvest and for *L. biglobosa* to be predominant under some circumstances as it is in Poland (Jedryczka *et al.,* 2008).

These results support those of previous work on the effect of weather factors (e.g. temperature and rainfall) on pseudothecial maturation on stem stubble (Khangura *et al.,* 2001; Salam *et al.,* 2003; Toscano-Underwood *et al.,* 2003; Huang *et al.,* 2003b). In controlled environment conditions (at 20^{o}C under continuous wetness), pseudothecia mature faster than on stems exposed to natural conditions in autumn/winter at lower temperatures with periods of wetting and drying. Previous work showed that at 20^{o}C pseudothecial development for both pathogens is much faster than at lower temperatures (Toscano-Underwood *et al.,* 2003; Huang *et al.,* 2005). Stem debris wetness is essential for progress of each stage of the pseudothecial maturation to continue (Petrie, 1994; Pérès, *et al.,* 1999b; Kaczmarek & Jedryczka, 2011; Piliponytė-Dzikienė, *et al.,* 2014).

Pseudothecial development was slower on stems of all nine cultivars exposed to natural conditions in 2011/2012 compared to the 2012/2013 and 2013/2014 growing seasons because there was a period of dry weather in the 2011/2012 growing season that could explain the delay in maturation passing through its initial stages (classes A and B).

These results confirm that ascospore release of *Leptosphaeria* spp. is stimulated by wetness since release of ascospores was observed on days after rainfall. It has been observed that ascospore release can commence within 1h after the start of rainfall with >0.5mm rainfall (Hall, 1992; Huang *et al.,* 2005). West *et al.* (2002b) reported that on humid days without rain, high humidity, mist or dew may be sufficient to initiate or sustain spore release from pseudothecia of *Leptosphaeria* spp. The results presented in this study for three growing seasons (2011/2012, 2012/2013 and 2013/2014) support the findings of Toscano-Underwood *et al.* (2003) and Huang *et al.* (2007) in the UK, concerning the influence of temperature and rainfall on the release of ascospores of *Leptosphaeria* spp. More ascospores were collected earlier in the more consistently wet autumn/winters of 2012/2013 and 2013/2014 than in 2011/2012, when there was more dry weather in early autumn. However, in the winters of 2012/2013 and 2013/2014 there were several days with maximum numbers of ascospores in the air (Fig. 4.18 and Fig. 4.19), which means that weather factors allow many ascospores to be released when weather conditions were favourable. Furthermore, periods of cold winter weather largely prevent ascospore release leading to delayed ascospore release which led to development of upper stem lesions rather than stem base cankers (West *et al.,* 2002a). The influence of temperature, rainfall and humidity on ascospore release were also observed in Poland (Kaczmarek *et al.,* 2010), USA (Hershman and Perkins, 1995), Canada (Guo

and Fernando, 2005), Australia (Khangura *et al.,* 2007) and New Zealand (Lob *et al.,* 2013).

Results from the qPCR imply that ascospores of *L. maculans* were released earlier than ascospores of *L. biglobosa.* This is consistent with previous work which showed that ascospores of *L. maculans* are generally released earlier than ascospores of *L. biglobosa* (West *et al.,* 2002a; Huang *et al.,* 2011). The ascospores released early were mostly from stem base cankers (West *et al.,* 2001). Jedryczka (2007) and Kaczmarek *et al.* (2012) had observed a greater number of *L. biglobosa* ascospores than *L. maculans* ascospores in Poland. The qPCR allowed species identification between ascospores of *L. maculans* and *L. biglobosa* and quantification of the ascospores (Kaczmarek *et al.,* 2009). Results on 19 November 2013, when a large amount of *L. maculans* DNA was detected from the half tape but a small number of ascospores were counted on the other half tape, suggest that the presence on such tapes of fungal material other than ascospores, such as hyphal fragments and conidia (Kaczmarek *et al.,* 2012). This also suggests that continuous monitoring of ascospore release at one site will provide valuable data to validate and refine the phoma leaf spot forecasting system.

There is a need to analyse and understand the potential effect of quantitative resistance (QR) in limiting disease progression when QR could alter the sexual reproduction of the *L. maculans/L.biglobosa* species complex through different biochemical and/or biophysical properties of the infected tissues (Lô-Pelzer *et al.,* 2009a; Hayward *et al.,* 2012).

Chapter 5

Pathogenicity of *L. maculans* and *L. biglobosa* isolates to different cultivars in cotyledon assays

5.1 Introduction

Leptosphaeria maculans isolates were categorised into several pathogenicity groups (PG) (refer to 1.3.1) based on the interaction phenotype (IP) of the isolates on the cotyledons of a differential set of *Brassica* cultivars/lines (Mengistu *et al.,* 1991; Ansan-Melayah *et al.,* 1995). Interaction phenotype (resistance or susceptibility) depends on the presence of one major gene for resistance (*Rlm*) in the plant and one corresponding effector (*Avr*) gene in the pathogen. For example, the dominant single resistant allele *Rlm2* in cv. Glacier corresponds to *Avr* gene *AvrLm2* in PG2 isolates of *L. maculans*, resulting in an incompatible (resistant) interaction between cv. Glacier and PG2 isolates (Dilmaghani *et al.,* 2009). This is known as race-specific resistance and results from the interaction of a particular cultivar of the host plant with a particular pathogen race. *L. maculans* populations are composed of many races. Some of these races are capable overcoming some of the resistance genes in oilseed rape (Sprague *et al.,* 2006b; Canola Council, 2014).

Genetic resistance is often derived from intraspecific and interspecific breeding, especially by introgression into *B. napus* of resistance genes from other *Brassica* species, such as those with the B genome like mustard, *B. nigra*, *B. juncea* and *B. carinata* (refer Fig. 1.1). *Rlm1* in *Brassica napus* cv. Quinta gives resistance to PG3 isolates (Balesdent *et al.,* 2001) whereas *Rlm2* is in *B. napus* cv. Glacier gives resistance to PG2 isolates (Ansan-Melayah *et al.,* 1998; Larkan *et al.,* 2014). *Rlm3,* a single dominant allele derived from cv. Glacier, confers resistance to some European

races (Balesdent *et al.,* 2002) and *Rlm4*, controlling the resistance in *B. napus* cv. Jet Neuf was linked with *Rlm1* in Quinta (Balesdent *et al.,* 2001). Cultivar Jet Neuf has good quantitative resistance (QR) (Delourme *et al.,* 2008; Brun *et al.,* 2010). *Rlm5*, derived from the Indian mustard (*B. juncea*) lines 150-2-1, 151-2-1 and cv. Picra, confers resistance to some Australian isolates (Balesdent *et al.,* 2002). Moreover, *Rlm6*, like *Rlm5*, is also derived from mustard cv. Picra (Balesdent *et al.,* 2002). *Rlm7* in *B. napus* is linked to *Rlm3* and confers resistance to PG4 isolates (Balesdent *et al.,* 2002). *Rlm8* is present in *B. rapa* and confers resistance to PG4 isolates (Balesdent, *et al.,* 2002). *Rlm9* is a resistance gene in *B. napus* cv. Darmor against PG4 isolates (Delourme *et al.,* 2004). In this study, several cultivars with *Rlm1* (cv. Capitol) and *Rlm4* (cv. Bilbao) were susceptible (Balesdent *et al.,* 2006) but in the UK, the frequency of virulent *L. maculans* isolates differed between areas, with most isolates virulent against *Rlm1* and only a small number of isolates virulent against *Rlm4* (Stevens, 2014). The cultivar Es-Astrid with quantitative resistance (QR) was on the HGCA winter oilseed rape recommended list in 2005/2006, 2008/2009, 2009/2010, 2010/2011and 2011/2012 growing seasons, whereas the cultivar Excel with *Rlm7* was recommended from the 2007/2009 growing season until the 2011/2012 growing season. The cultivar DK Cabernet, with a combination of *Rlm1* and QR, has been recommended since the 2010/2011 growing season (http://www.hgca.com/varieties/hgca-recommended-lists).

The first effector (*Avr*) alleles and resistance (*R*) genes in the *Brassica napus* - *L. maculans* interaction were found through studying differential interactions between cultivar - isolate pairs in cotyledon infection assays (Mengistu *et al.,* 1991). These are rapid, widely used plant assays in which a differential set of *B. napus* cultivars/lines are inoculated with conidial suspensions applied to wounded cotyledons of *B. napus*

(Koch *et al.,* 1991; Mengistu *et al.,* 1991; Kutcher *et al.,* 1993). In natural conditions, infection is mostly from ascospores via stomatal pores but it is also via wounds (Hammond *et al.,* 1985; Chen & Howlett, 1996; West *et al.,* 2001). In controlled environment conditions, conidia are able to infect leaves only if they are wounded (Hammond, 1985). The race-specific resistance is easily assessed on cotyledons or leaves in controlled environment pathogenicity experiments (Ansan-Melayah *et al.,* 1997; 1998). Now, the race-specificity of isolates can sometimes be determined by using more rapid assays available, such as race-specific PCR (Kaczmarek *et al.,* 2012). However, cotyledon tests will still be useful and reliable to observe the phenotype results on gene-for-gene interactions (race-specific resistance gene). Moreover, researchers and plant breeders have adopted the cotyledon-inoculation test for screening for novel sources of resistance against *L. maculans* in *B. napus* due to the genetic diversity in response to *L. maculans* (Williams & Delwiche, 1979; Thurling & Venn, 1977; Rimmer & Van den Berg, 1992). Furthermore, complex PCR assays require skilled technicians and specialist equipment and extreme care to avoid false positives resulting from contamination or poor specificity of primers (Williams & Fitt, 1999).

Successful breeding done in France during the 1990's especially used the specific resistance gene *Rlm1* for which the pathogen population was mainly avirulent (Ansan-Melayah *et al.,* 1997). Race-specific resistance in *B. napus* against *L. maculans* isolates with the corresponding avirulent allele results in an incompatible interaction that inhibits colonisation and lesion development. However, severe epidemics have occurred on cultivars with race-specific resistance. Qualitative resistance is generally less durable than quantitative resistance because pathogen populations often rapidly evolve for virulence against the *R* genes so that plant hosts

with the corresponding resistance gene no longer detect the pathogen, which leads to development of disease. Breakdown of the major resistance gene *Rlm1* occurred in France in 1998 (Rouxel *et al.,* 2003). During the 1990's, winter oilseed rape cultivars with the resistance gene *Rlm1* (e.g. Capitol) were widely adopted and the frequency of virulent *avrLm1* isolates in the population increased to such an extent over five growing seasons that these cultivars were no longer resistant (Rouxel *et al.,* 2003). The most recent Europe-wide monitoring of the effector genes was done by Stachowiak *et al.* (2006). The frequency of *AvrLm1* allele was reported to be low (<20%) in Europe and the *AvrLm9* allele was not present in the pathogen population at all (Stachowiak *et al.,* 2006; Balesdent *et al.,* 2006).

It was exploitation of such resistance genes that produced extremely strong selection on *L. maculans* populations to develop mutations/deletions in the coding regions of these *Avr* genes, which are sufficient to evade detection by the corresponding major resistance gene so that it is no longer effective (Gout *et al.,* 2006a; Fudal *et al.,* 2007; Parlange *et al.,* 2009; Van de Wouw *et al.,* 2010). As a result, these cultivars are no longer considered to be highly resistant in France. A period of more than 10 years was needed for breeders to introduce this resistance source into commercial oilseed rape cultivars but the increase in virulent alleles leading to breakdown of resistance occurred after three cropping seasons (Rouxel *et al.,* 2003).

A similar breakdown of resistance occurred in Australia when spring oilseed rape cultivars with *B. rapa* ssp. *sylvestris* resistance was released to growers in 2000 (e.g. cv. Surpass 400) were grown extensively (Easton, 2001; Li and Cowling, 2003). However, the resistance was rendered ineffective within three years of its commercial release, resulting in 90% yield losses, and up to $10 million AUD in losses (Sprague

et al., 2006a; Van de Wouw *et al.,* 2010). Virulence evolves rapidly in the *L. maculans* populations because *Avr* genes are located in repetitive regions of the genome where RIP mutation and gene loss readily occur (Van de Wouw *et al.,* 2010). These plant pathogen interactions show a 'boom and bust' disease cycle defined as a cycle where a new resistant cultivar becomes so popular that it is grown as a homogeneous genotype, enabling the pathogen to overcome resistance and cause severe epidemics (McDonald & Linde, 2002a,b).

Recently, it has been suggested that *Rlm7* is more durable than other *R* genes commercially available against *L. maculans*. It has been widely deployed in new oilseed rape cultivars across Europe (e.g. DK Extrovert) (Clarke, 2014). However, virulence against *Rlm7* has been identified in French *L. maculans* populations (Daverdin *et al.,* 2012). A four-year field trial of *Rlm7* resistance in France showed a 36% increase in frequency of virulent isolates and results suggest that these changes occured due to the genomic environment (monoculture and lack of rotation) and reproductive system (infected stubble from the previous years) (Daverdin *et al.,* 2012). However, in the UK, only 3% of the *L. maculans* population has been reported to be virulent against the *Rlm7* gene (Stevens, 2014).

Many European commercial cultivars are known to carry major resistance genes which are no longer effective, as the corresponding avirulent alleles have been selected against within the *L. maculans* populations. This can happen when pathogen populations undergo rapid selection resulting in changes from avirulence to virulence, especially when cultivars with a particular *R* gene are grown extensively. *R* genes operating against *L. maculans* are often rendered ineffective after only a few seasons of commercial deployment, causing serious losses (Rouxel *et al.,* 2003;

Sprague *et al.,* 2006a). A total of thirteen *Avr* genes in *L. maculans* have now been identified (Fudal *et al.,* 2007; Parlange *et al.,* 2009; Balesdent *et al.,* 2013). The fifteen specific resistance genes that have been identified in the cultivated *Brassica* species map to three different linkage groups (Yu *et al.,* 2005, 2008; Delourme *et al.,* 2006; Rimmer 2006; Long *et al.,* 2011). In gene-for-gene interaction studies between *L. maculans* and *B. napus,* it has been demonstrated through the use of segregating plant and pathogen populations that the chromosome A07 carries five distinct *R* genes or alleles (*Rlm1*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*) and the A10 chromosome of *B. napus* harbours *Rlm2* and *LepR3,* whereas the resistance genes *Rlm5* and *Rlm6* were identified in *B. juncea*, *Rlm8* in *B. rapa* and *Rlm10* in *B. nigra* (Table 5.1). Very little is known about the number or types of QR genes conferring adult plant resistance in *B. napus* (Van de Wouw & Howlett, 2012).

Leptosphaeria species have a high evolutionary potential to adapt to cultivars with resistance genes and therefore it is crucial to monitor the frequency of avirulent and virulent isolates within fungal populations in order to predict changes. In addition, there is a lack of data about fungal *Avr* genes, which are generally defined by the resistance phenotype they induce on host plants and the modes of evolution under selection are generally unknown. Quantitative resistance does not produce strong selection on the pathogen (Zhu *et al.,* 1993; Delourme *et al.,* 2008). In this respect, quantitative resistance has been shown to increase the durability of qualitative resistance (Brun *et al.,* 2010), as the quantitative resistance genes do not provoke a rapid breakdown of their resistance (Kaur *et al.,* 2009).

Table 5.1: Relationship between *Brassica napus* resistance genes and *Leptosphareria maculans* effector (*Avr*) genes in the gene-for-gene interactions demonstrated through the use of segregating plant and pathogen populations.

Species	*Leptosphareria maculans* *Avr* gene	Resistance gene	[c]Location on *Brassica* chromosome	References
Brassica napus	[a]*AvrLm1*	[b]*Rlm1*	A07	Ansan-Melayah *et al.* (1998), Balesdent *et al.* (2002), Gout *et al.* (2006a), Delourme *et al.* (2004), Brun *et al.* (2010)
	alm1	*LEM1=Rlm4 or 1?*	A07	
	AvrLm2	*Rlm2*	A10	Delourme et *al.* (2004), Ansan-Melayah *et al.* (1998), Balesdent *et al.* (2002), Larkan *et al.* (2014)
	AvrLm3	[b]*Rlm3*	A07	Balesdent *et al.* (2002), Delourme *et al.* (2004)
	[a]*AvrLm4*	[b]*Rlm4*	A07	Balesdent *et al.* (2001), Delourme *et al.* (2004), Parlange *et al.* (2009), Brun *et al.* (2010)
	[a]*AvrLm7*	[b]*Rlm7*	A07	Balesdent *et al.* (2002), Delourme *et al.* (2004), Parlange *et al.* (2009)
	AvrLm9	[b]*Rlm9*	A07	Balesdent *et al.* (2002), Delourme *et al.* (2004)
Brassica rapa	*AvrLm8*	*Rlm8*	-	Balesdent *et al.* (2002)
Brassica rapa ssp. *sylvestris*	-	*LepR1* *LepR2* *LepR3* *LepR4*	A02 A10 A10 Unknown	Rimmer (2006), Larkan *et al.* (2013), Larkan *et al.* (2014)
Brassica juncea	*AvrLm5* [a]*AvrLm6*	*Rlm5* *Rlm6*	Unknown Unknown	Balesdent *et al.* (2002) Chèvre *et al.* (1997), Balesdent *et al.* (2002), Fudal *et al.* (2007), Brun *et al.* (2010)
Brassica nigra	-	*Rlm10*	Unknown	Chèvre *et al*. (1996)

[a]Three effector (*Avr*) genes, *AvrLm1*, *AvrLm6* and *AvrLm4-7*, have been cloned (Gout *et al.,* 2006a; Fudal *et al.,* 2007; Parlange *et al.,* 2009).

[b]Genetic mapping studies suggest that *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* are either a tight cluster of linked genes or different allelic versions of the same *R* gene. *Rlm4* and *Rlm3* are believed to be genetically linked to *Rlm1* (Balesdent *et al*. 2001; Delourme *et al*. 2004; 2006).

[c]These genes have been mapped to the *Brassica* A genome and *LepR3* and *Rlm2* have been cloned.

The proportions of *L. maculans* and *L. biglobosa* in a population is likely to be affected by cultivar choice, when cultivars resistant against *L. maculans* may be susceptible to *L. biglobosa* and *vice versa* (Fitt *et al.,* 2006a). Cultivar choice may also indirectly influence the severity of epidemics. However, there is little information about variation in cultivar resistance against *L. biglobosa*. *L. maculans* is classified as having a high probability of rendering *R* genes ineffective because it reproduces both sexually and asexually, is capable of mutation and has a large population size and extensive gene flow due to dispersal by air-borne ascospores (McDonald and Linde, 2002b). In France, researchers tested the durability of different sources of resistance by sowing both *B. juncea* and *B. nigra* back into their own stubble from the previous growing season for three seasons; the results showed that the *B. juncea* resistance was overcome by the pathogen in three seasons whereas resistance from *B. nigra* was not (Brun *et al.,* 2000).

Aim: To detect phenotypic responses of different *Brassica napus* cultivars to *L. maculans/L. biglobosa* in cotyledons inoculated with isolates obtained from phoma leaf spots or stem cankers.

Objectives:

1. To determine phenotypic responses of cotyledons against a range of *L. maculans* isolates inoculated onto a differential set of cultivars/lines of *Brassica napus*.

2. To determine phenotypic response of cotyledons against a range of *L. biglobosa* isolates inoculated onto a differential set of cultivars/lines of *Brassica napus.*

3. To inoculate the cotyledons of a differential set of cultivars with *L. maculans* for determination of alleles of *Avr* genes in isolates obtained from phoma leaf spots or stem cankers.

5.2 Materials and methods

5.2.1 Phoma leaf spot phenotype of *Leptosphaeria maculans* isolates derived from phoma leaf spots or stem cankers

L. maculans conidial inoculum was obtained from isolates from phoma leaf spots sampled in autumn/winter or from phoma stem cankers (basal cankers and upper stem lesions) sampled in the following summer. All of these isolates were obtained from samples from oilseed rape from Rothamsted Research, Harpenden experimental field plots of nine cultivars with different combinations of resistance (*R*) genes against *L. maculans* or without *R*-genes; some had quantitative resistance (QR). Refer to section 2.6 for the isolation procedures.

5.2.1.1 Preparation of inoculum of isolates from phoma leaf spots or stem cankers

Conidial suspensions were prepared for forty isolates of *L. maculans* isolated from phoma leaf spots and fifty-five isolates of *L. maculans* isolated from stems (31 isolated from upper stem lesions and 24 isolated from basal stem cankers) (Table 5.2). All of the conidial suspensions were prepared from 12-day-old cultures on V8 agar that were obtained from single pycnidial isolates (Balesdent *et al.,* 2001).

Conidial suspensions for inoculation were prepared. The cultures containing pycnidia were flooded with approximately 10 ml of sterile distilled water (SDW) and the surface of the agar was gently rubbed with a sterile Lazy-L spreader to release the

Table 5.2: Number of *L. maculans* isolates (confirmed by species-specific PCR) (Appendix 5.1) isolated from phoma leaf spots, upper stem lesions or basal stem cankers sampled at Rothamsted Research, Harpenden between June 2011 and June 2013 in autumn/winter or summer from different oilseed rape cultivars.

Cultivar	(*R*) – gene or quantitative resistance (QR)[a]	Isolates from leaf spots[b]	Isolates from upper stem lesions[b]	Isolates from basal stem cankers[b]
			Number	
Adriana	*Rlm 4* + QR	0	5	2
Bilbao	*Rlm 4*	5	3	4
Capitol	*Rlm 1*	3	4	3
Drakkar	no *R* genes	15	4	3
DK Cabernet	*Rlm 1* + QR	5	4	4
Es-Astrid	QR	2	5	5
Excel	*Rlm 7*	0	0	0
NK Grandia	QR	5	0	0
Roxet	*Rlm 7*	5	6	3
	Total	40	31	24

[a]Further information about these *R*-genes operating against *L. maculans* and quantitative resistance is given in Delourme *et al.* (2006) (refer to Chapter 1).

[b]Some isolates produced fewer pycnidia; then the inoculum with a small number of conidia was not usually sufficient for inoculation. Therefore, only isolates that produced more conidia were chosen.

conidia into the water. The conidial suspensions were then filtered through sterile 'Miracloth' into a 15ml tube. This was done inside a laminar air flow cabinet.

5.2.1.2 Preparation and storage of spore suspensions

The conidial concentrations were estimated with a Neubauer haemocytometer counting chamber. Firstly, the conidial suspension was shaken in a 15 ml tube and a 7 µl sample of the suspension was used to count the number of spores per small square at x400 magnification. The concentration of the conidial suspension was calculated from ten randomly chosen small squares (at 40x magnification) based on the formula;

$$\text{Number of spores/ml} = \text{number of spores /small square} \times 4 \times 10^6.$$

The adjusted conidial suspensions (10^6 conidia ml^{-1}) were transferred into sterile 1.5 ml vial tubes and frozen at -20^{o}C. Inoculum stored in this way remains viable for at least 2 years (Somda *et al.,* 1998) but in this study it was used about 12 months later.

5.2.1.3 Growth of a differential set of cultivars/lines

The cultivars or lines used in this study were cv. Drakkar (no *R* gene against *L. maculans*, used as control), cv. Columbus (*Rlm1*, *Rlm3*), cv. Bristol (*Rlm2*), line 02-22-2-1 (*Rlm3*), cv. Jet Neuf (*Rlm4*), line 99-150-2-1 (*Rlm5*), cv. Darmor-MX (*Rlm6*), line 01-23-2-1 (*Rlm7*) and line 01-190-1-1 (*Rlm9*). The plantlets were grown in seedling trays (5 x 8 cells) (Fig. 5.1) with a 50:50 mixture of John Innes potting compost No. 3 and Miracle-Gro all-purpose growing compost and seedling trays were placed in a glasshouse for 14 days before inoculation. All seedlings trays were placed on capillary matting (synthetic absorbent material) that soaked up water to

Fig. 5.1: Plantlets of *Brassica* with resistance (*R*) genes against *L. maculans* or without *R*-genes were grown in seedling trays (5 x 8 cells) for 14 days before inoculation.

provide water for the plantlets. Some plantlets were supported with a plastic stick to ensure that the cotyledons did not collapse on other cotyledons and that the inoculum drops stayed on the cotyledons. True leaves from all plantlets were removed to ensure that the cotyledons continued to expand and remained green until evaluation for interaction phenotypes between *Brassica* and *Leptosphaeria* species.

5.2.1.4 Inoculation of cotyledons

The experiment was done in a controlled environment cabinet (Conviron CMP6000® Series) at College Lane, Hatfield and a Bayfordbury controlled environment room (Fig. 5.2) at $20^{o}C/18^{o}C$ (day/night), 80-90% RH, photoperiod 12/12h with light intensity at $210\mu Em^{-2}s^{-2}$, using *Brassica* lines (section 5.2.1.3). Cotyledons were inoculated with conidial suspensions by using the wound and inoculate method (Huang *et al.,* 2006b). A sharp pin was used to gently wound the centre of each cotyledon lobe without passing through it. Using a Gilson pipette (p20) (Gilson Mediacal Electronics, SA, Villiers_le_Bel, France), a 10µl aliquot of spore suspension (1×10^{6}) was deposited onto each of four cotyledons of each plantlet of the cultivar/line that had already been wounded (Fig. 5.3) and the plantlets were put into a humidity box (Fig. 5.4a). The cotyledons were sprayed gently with sterile distilled water so that the inoculum remained on the cotyledon. Each cotyledon was assigned a number to identify the type of inoculum, which was useful later during the disease assessment (Fig. 5.4 b). Trays lids were kept covered with black polyethylene bags to provide complete darkness for 24 hours after inoculation in the controlled environment cabinet to encourage germination of *Leptosphaeria* conidia. The tray lid was also sprayed with sterile distilled water to maintain high humidity. After the 24

Fig. 5.2: Seedling trays in the controlled environment room at Bayfordbury.

Fig. 5.3: Plants in trays standing on a mat with moisture provided. Inoculum of *Leptosphaeria* spp. (arrows) was placed on cotyledons (wound inoculation) and the hole was for marking each plant.

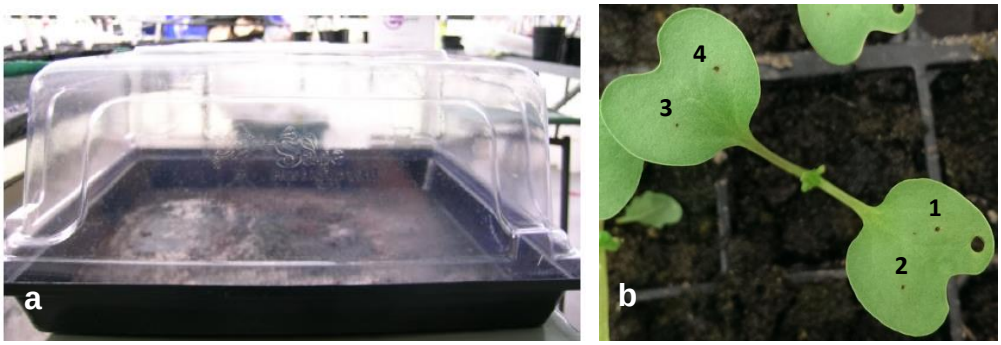


Fig. 5.4: The humidity box (a) and (b) inoculated sites were assigned numbers 1 to 4 prior to inoculation, so that they could be identified later during disease assessment of cotyledons 14 days post inoculation with *L. maculans* or *L. biglobosa*.

hours of darkness, covers were removed to provide light and cotyledons were sprayed with sterile distilled water twice daily. There were five replications for each isolate per cultivar or line and the experiment was done two times.

5.2.1.5 Disease assessment (lesion diameter)

Fourteen days post inoculation (dpi), the leaf spot lesions were observed and scored using a 0-9 scale modified from Li *et al.* (2004); 0- no visible symptoms, as in controls; 1- necrotic (lesion diameter 0.5–1.0 mm); 2- grey-green tissue (lesion diameter 1.0-1.5 mm) with a distinct margin; 3- dark necrotic lesion (lesion diameter 1.5-2.0 mm) with a distinct margin; 4- dark lesion (lesion diameter 2.0–3.0 mm) with diffuse margins; 5- dark lesion, brownish on lower surface (lesion diameter 3.0–4.0 mm) with a diffuse margin; 6- lesion with no or few pycnidia (lesion diameter 5.0–6.0 mm) with a diffuse margin; 7- lesion with a few pycnidia (lesion diameter > 6 mm) with a diffuse margin; 8- large lesion with a few pycnidia; 9- large lesion with profuse sporulation (Fig. 5.5). Cotyledons were evaluated for interaction phenotypes (IP) on a scale of 0-9 (1-3 was considered resistant (R), 4-5 was considered intermediate (I) and 6-9 was considered susceptible (S)) (Williams, 1985, modified by Li *et al.,* 2004) (Fig. 5.5). In addition, the diameters (mm) of leaf lesions were measured at 14 days post inoculation.

5.2.1.6 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. ANOVA was used to compare different treatments for the diameter of leaf lesions. Significant differences (95% confidence interval) were determined using analysis of variance (ANOVA) SPSS version 21 statistical packages.

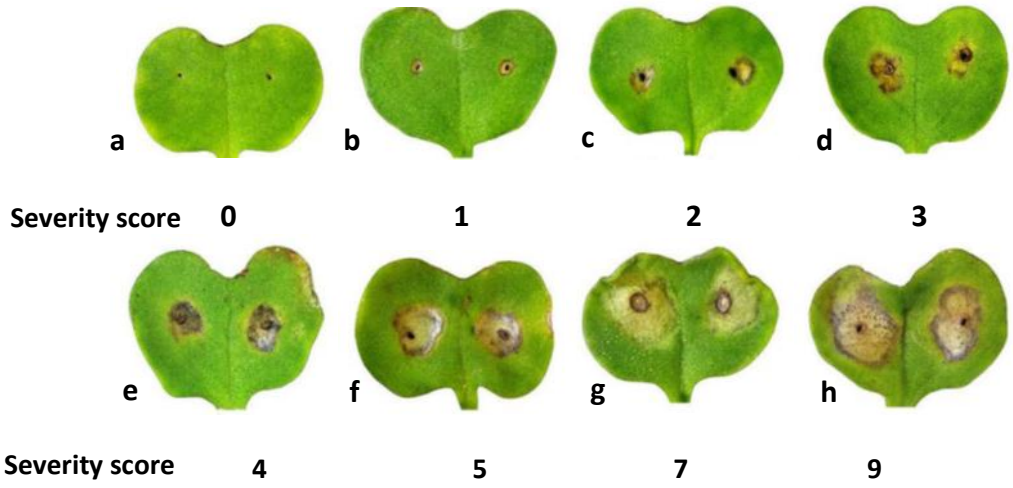


Fig. 5.5: Severity score that was used for *Leptosphaeria* species (*L. maculans* and *L. biglobosa)* (a) 0- no darkening around wounds; (b) 1- necrotic (lesion diameter 0.5–1.0 mm); (c) 2- grey-green tissue collapse (1.0-1.5 mm diameter) with a distinct margin; (d) 3- dark necrotic lesion (lesion diameter 1.5-2.0 mm) with a distinct margin; (e) 4- dark lesion (lesion diameter 2.0–3.0 mm) with diffuse margins; (f) 5- dark lesion, brownish on lower surface (lesion diameter 3.0–4.0 mm) with a diffuse margin; (g) 7- lesion with a few pycnidia (lesion diameter > 6 mm) with a diffuse margin and (h) 9- large lesion with profuse sporulation (Williams, 1985 modified by Li *et al.,* 2004).

5.2.2 Phoma leaf spot phenotype of *Leptosphaeria biglobosa* isolates derived from phoma leaf spots or stem cankers

5.2.2.1 Preparation of inoculum isolated from phoma leaf spots or phoma stem cankers

Forty-eight isolates of *L. biglobosa* (36 isolated from phoma leaf spots, 6 isolated from upper stem lesions and 6 isolated from basal stem cankers) were used to prepare the conidial suspensions (Table 5.3). The procedure for preparation of conidial suspensions was based on that described in sections 5.2.1.1 and 5.2.1.2.

5.2.2.2 Growth of cultivars with different resistance *(R)* genes against *Leptosphaeria maculans* or with QR

A set of 12 cultivars/lines was used for characterizing the *L. biglobosa* phoma leaf spot phenotype. The set comprised cv. Adriana (*Rlm 4* + QR), cv. Drakkar used as control (no *R* genes against *L. maculans*), cv. DK Cabernet (*Rlm 1* + QR), cv. Es-Astrid (QR) and a differential set of cultivars; Columbus (*Rlm1*, *Rlm3*), cv. Bristol (*Rlm2*), line 02-22-2-1 (*Rlm3*), cv. Jet Neuf (*Rlm4*), line 99-150-2-1 (*Rlm5*), cv. Darmor-MX (*Rlm6*), line 01-23-2-1(*Rlm7*) and line 01-190-1-1 (*Rlm9*). Please refer to 5.2.1.3 for cotyledon preparation.

5.2.2.3 Inoculation of cotyledons

Please refer to 5.2.1.4 for the procedure.

5.2.2.4 Disease assessment (lesion diameter)

Please refer to 5.2.1.5 for the procedure.

Table 5.3: Number of *L. biglobosa* isolates obtained from phoma leaf spots, upper stem lesions or basal stem cankers sampled at Rothamsted Research, Harpenden between June 2011 and June 2013 in autumn/winter or summer confirmed by species-specific PCR (Appendix 5.2).

Cultivar	(*R*) – gene or quantitative resistance (QR)[a]	Isolates from leaf spots[b]	Isolates from upper stem lesions[b]	Isolates from basal stem cankers[b]
			Number	
Adriana	*Rlm 4* + QR	4	0	0
Bilbao	*Rlm 4*	4	2	0
Capitol	*Rlm 1*	4	0	3
Drakkar	no R genes	4	2	0
DK Cabernet	*Rlm 1* + QR	4	0	0
Es-Astrid	QR	4	1	0
Excel	*Rlm 7*	4	0	0
NK Grandia	QR	4	1	2
Roxet	*Rlm 7*	4	0	1
	Total	36	6	6

[a]Further information about these *R*-genes operating against *L. maculans* and quantitative resistance is given in Delourme *et al.* (2006).

[b]Some isolates produced fewer pycnidia; then the inoculum with a small number of conidia was not usually sufficient for inoculation. Therefore, only isolates producing more conidia were chosen.

5.2.2.5 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. ANOVA was used to compare the diameter of leaf lesions for different treatments.

5.2.3 Determination of alleles of *Avr* genes in *Leptosphaeria maculans* isolates obtained from phoma leaf spots or stem cankers

5.2.3.1 Preparation of *Leptosphaeria maculans* inoculum

Please refer to 5.2.1.1 for the procedure.

5.2.3.2 Inoculation of cotyledons

Please refer to 5.2.1.4 for the procedure.

5.2.3.3 Disease assessment (lesion diameter)

The cotyledon assessment method (section 5.2.1.5) was used and scored using a 0-9 scale modified from Li *et al.* (2004) (Fig. 5.5). In addition, the diameters (mm) of leaf lesions were measured at 14 days post inoculation.

5.2.3.4 Statistical analysis

The data collected were saved as Microsoft Excel files and preliminarily analysed by Microsoft Excel. All differences in allele frequencies were statistically analysed using chi-squared analysis SPSS version 21 statistical packages. A 95% confidence interval was used to indicate significance. Margalef's index was used to provide a simple measure of race richness (Margalef, 1958). Where S = total number of races, N = total number of isolates in the sample and In = natural logarithm, Margalef's index = (S – 1) / In N.

5.3 Results

5.3.1 Phoma leaf spot phenotype produced by inoculation with *Leptosphaeria maculans* isolates derived from phoma leaf spots or stem cankers

The respective *Brassica* differential cultivars/lines used in this study were either resistant or susceptible when tested with different isolates of *L. maculans* (LM) isolated from phoma leaf spots, basal stem cankers or upper stem lesions. Only cv. Drakkar (susceptible to *L. maculans*) showed phoma leaf spot symptoms by 7 days post inoculation (Fig. 5.6) with a leaf lesion severity score ranging from 1 to 3 and lesion diameter ranging from 0.5 to 1.5mm, whereas other cultivars/lines (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7* and *Rlm9*) developed lesions by 9 days post inoculation, with a severity score <3 and lesion diameter ranging from 0.5 to 1.0mm (Fig. 5.7).

Based on the Interaction phenotype (IP), all *L. maculans* isolates from phoma leaf spots, basal stem cankers or upper stem lesions produced susceptible phenotypes at 14 days post inoculation on five *Brassica* differential genotypes (with *Rlm1*, *Rlm2*, *Rlm3*, *Rlm9* or cv. Drakkar) (Table 5.4, 5.5 and 5.6), whereas only *L. maculans* isolates from basal stem cankers inoculated on *Rlm5* line produced a susceptible phenotype (Table 5.5). There were different interaction phenotypes with resistant, intermediate or susceptible phenotypes at 14 days post inoculation for *L. maculans* isolates from phoma leaf spots inoculated onto *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7* cultivars/lines (Table 5.4). All *L. maculans* isolates from basal stem cankers produced a resistant (*R*) phenotype when inoculated on the *Rlm7* line and some isolates produced a resistant phenotype when inoculated on *Rlm4* or *Rlm6* cultivars; those isolates were from cv. Bilbao (Fig. 5.7). On *Rlm4*, *Rlm5* or *Rlm6* cultivars/lines,

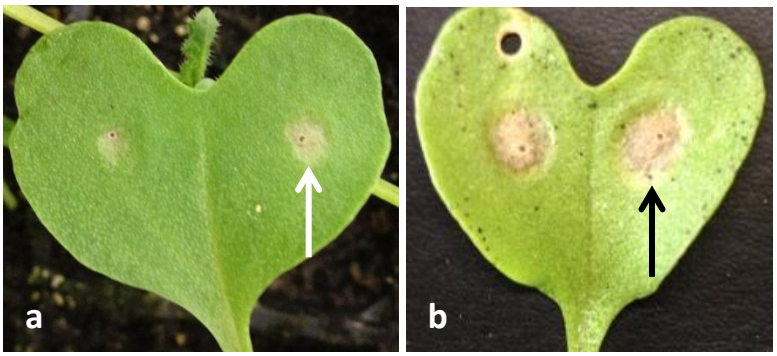


Fig. 5.6: *L. maculans* phenotypes on cv. Drakkar (susceptible to *L. maculans*) at 7 days post inoculation; (a) severity score 1 (white arrow) after inoculation with LM isolated from a phoma leaf spot on cv. Es-Astrid (IIKEA2) and (b) severity score 3 (black arrow) after inoculation with LM isolated from a phoma leaf spot on cv. DK Cabernet (IIK DKC 6) on seedlings grown in the controlled environment room at University of Hertfordshire, Bayfordbury.

Fig. 5.7: Early phoma leaf spotting symptoms at 9 days post inoculation with *L. maculans* on cv. Columbus (*Rlm1*) and severity score 1 to 2 (arrows) on seedlings grown in the controlled environment room at University of Hertfordshire, Bayfordbury.

Table 5.4: Differentiation of isolates of *L. maculans* obtained from phoma leaf spots from different source cultivars, based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

	Interaction phenotype (IP)								
	R gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar (No *R* gene)
From Bilbao (*Rlm 4*)									
IIKB2	S	S	S	I	I	I	R	S	S
II K B4	S	S	S	R	I	S	I	S	S
IIKB3	S	S	S	R	I	I	I	S	S
I3B1	S	S	S	R	I	S	R	S	S
I3B2	S	S	S	R	I	S	R	S	S
From Capitol (*Rlm1*)									
IIKC1	S	S	S	I	I	R	I	S	S
II K C6	S	S	S	S	S	I	S	S	S
IIKC2	S	S	S	I	I	R	R	S	S
From DKCabernet (*Rlm1* + QR)									
IIKDKC	S	S	S	R	I	R	R	S	S
II K DKC 2	S	S	S	R	R	R	R	S	S
II K DKC 4	S	S	S	R	S	S	R	S	S
II K DKC 1	S	S	S	R	S	I	R	S	S
IIKDKC 3	S	S	S	R	I	S	R	S	S
From Es-Astrid (QR)									
IIKEA1	S	S	S	I	R	I	R	S	S
IIKEA3	S	S	S	I	R	R	R	S	S
From Drakkar (No *R* gene)									
IIKD6	S	S	S	I	R	R	R	S	S
IIKD4	S	S	S	R	R	I	R	S	S

Table 5.4. Continued from preceding page

	Interaction phenotype (IP)								
	R gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar (No *R* gene)
IIKD3	S	S	S	I	R	R	R	S	S
IIKD1	S	S	S	R	R	R	R	S	S
IIKD2	S	S	S	R	R	R	R	S	S
II K D 5	S	S	S	S	R	I	R	S	S
II K Drakkar	S	S	S	S	R	R	R	S	S
IIK D7	S	S	S	R	R	R	R	S	S
IIKD8	S	S	S	R	R	R	R	S	S
IIKD10	S	S	S	R	R	I	R	S	S
IIKD11	S	S	S	R	R	I	R	S	S
IIKD12	S	S	S	R	R	R	R	S	S
IIKD13	S	S	S	I	R	R	R	S	S
IIKD15	S	S	S	R	R	R	R	S	S
D1	S	S	S	R	R	R	R	S	S
From NK Grandia (QR)									
IIKNKG1	S	S	S	R	R	R	R	S	S
IIKNKG4	S	S	S	R	R	R	R	S	S
IIKNKG6	S	S	S	R	R	R	R	S	S
IIKNKG5	S	S	S	R	R	R	R	S	S
IIKNKG3	S	S	S	R	R	R	R	S	S
From Roxet (*Rlm7*)									
IIKR1	S	S	S	R	I	R	R	S	S
IIKR2	S	S	S	R	R	R	R	S	S
IIKR3	S	S	S	R	R	R	R	S	S
IIKR5	S	S	S	R	R	R	R	S	S
IIKR7	S	S	S	R	I	R	R	S	S

Table 5.5: Differentiation of isolates of *L. maculans* obtained from basal stem cankers from different source cultivars, based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

	Interaction phenotype (IP)								
	R gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Adriana (*Rlm4* + QR)									
KA 70 B	S	S	S	S	S	S	R	S	S
ADR19B	S	S	S	S	S	S	R	S	S
From Bilbao (*Rlm4*)									
KB228B	S	S	S	R	S	R	R	S	S
KB229B	S	S	S	R	S	R	R	S	S
I31B	S	S	S	R	S	R	R	S	S
I32B	S	S	S	R	S	R	R	S	S
From Capitol (*Rlm1*)									
Cap1	S	S	S	S	S	S	R	S	S
Cap2	S	S	S	S	S	S	R	S	S
Cap3	S	S	S	S	S	S	R	S	S
From Drakkar (no *R* gene)									
KD136B	S	S	S	S	S	S	R	S	S
KD138B	S	S	S	S	S	S	R	S	S
KD139B	S	S	S	S	S	S	R	S	S
From DKCabernet (*Rlm1* + QR)									
KDK57B	S	S	S	S	S	S	R	S	S
KDK 55 B	S	S	S	S	S	S	R	S	S
KDK 61 B	S	S	S	S	S	S	R	S	S
DK59B	S	S	S	S	S	S	R	S	S

Table 5.5. Continued from preceding page

	Interaction phenotype (IP)								
	R gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Es-Astrid (QR)									
KEA 9 B	S	S	S	S	S	S	R	S	S
KEA 12 B	S	S	S	S	S	S	R	S	S
KEA2B	S	S	S	S	S	S	R	S	S
KEA11B	S	S	S	S	S	S	R	S	S
KEA14B	S	S	S	S	S	S	R	S	S
From Roxet (*Rlm7*)									
R5-1	S	S	S	S	S	S	R	S	S
R11	S	S	S	S	S	S	R	S	S
R7	S	S	S	S	S	S	R	S	S

Table 5.6: Differentiation of isolates of *L. maculans* obtained from upper stem lesions from different source cultivars based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*)following cotyledon inoculation.

	Interaction phenotype (IP)								
	R gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Adriana (*Rlm4* + QR)									
KA95U	S	S	S	I	S	S	R	S	S
KA72U	S	S	S	I	S	S	R	S	S
KA 98 U	S	S	S	I	S	S	R	S	S
KA85U	S	S	S	I	S	S	R	S	S
KA 99 U	S	S	S	R	S	S	I	S	S
From Bilbao (*Rlm4*)									
KB 217 U	S	S	S	S	R	R	R	S	S
B10P	S	S	S	S	R	R	R	S	S
B13P	S	S	S	S	R	R	R	S	S
From Capitol (*Rlm1*)									
KC250U	S	S	S	S	I	R	R	S	S
KC249U	S	S	S	S	R	R	R	S	S
KC255U	S	S	S	S	R	R	R	S	S
C10P	S	S	S	S	R	R	R	S	S
From Drakkar (no *R* gene)									
KD 122 U	S	S	S	S	R	R	R	S	S
KD123U	S	S	S	S	R	R	R	S	S
D1P	S	S	S	S	R	R	R	S	S
D1	S	S	S	S	R	R	R	S	S
From DKCabernet (*Rlm1* + QR)									
DKC4P	S	S	S	S	R	R	R	S	S

Table 5.6. Continued from preceding page

	Interaction phenotype (IP)								
	R genes on eight *Brassica* differential genotypes								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
DKCU1	S	S	S	S	R	R	R	S	S
DKC11P	S	S	S	S	R	R	R	S	S
DKC(J)11-26	S	S	S	S	R	R	R	S	S
From Es-Astrid (QR)									
KEA 24 U	S	S	S	S	S	S	R	S	S
KEA 18 U	S	S	S	S	S	S	R	S	S
KEA 25 U	S	S	S	S	S	S	R	S	S
KEA 17 U	S	S	S	S	S	S	R	S	S
KEA22U	S	S	S	S	S	S	R	S	S
From Roxet (*Rlm7*)									
KR158U	S	S	S	S	S	I	R	S	S
KR157U	S	S	S	S	S	I	R	S	S
KR159U	S	S	S	S	S	I	R	S	S
KR156U	S	S	S	S	S	I	R	S	S
KR164U	S	S	S	S	S	I	R	S	S
KR166U	S	S	S	S	S	I	R	S	S

isolates obtained from the upper stem lesions produced mixtures of interaction phenotypes (R, I or S) at 14 days post inoculation and on the *Rlm7* line all upper stem lesion *L. maculans* isolates produced a resistant phenotype. All *L. maculans* isolates from phoma leaf spots, basal stem cankers or upper stem lesions produced susceptible phenotypes when inoculated on cv. Drakkar (Tables 5.4, 5.5, 5.6) and showed differences in phenotype between 7 days post inoculation and 14 days post inoculation assessments (Fig. 5.8 and Appendix 5.1).

There were significant differences ($P<0.05$) in diameter of lesions (mm) between isolates of *L. maculans* from phoma leaf spots, basal stem cankers or upper stem lesions on *Brassica* differential genotypes (Fig. 5.9). For isolates of *L. maculans* from phoma leaf spots, the smallest lesions were observed on the *Rlm5* line inoculated with isolates from cvs Drakkar or NK Grandia (QR) (lesion diameter ranged from 0.5-1.2 mm) and the largest lesions were observed on the *Rlm9* line inoculated with isolates from cv. Capitol (*Rlm1*) (lesion diameter ranged from 6.5-9.0 mm) (Table 5.7; Fig. 5.9a). For isolates of *L. maculans* from basal stem cankers, the smallest lesions were observed on the *Rlm6* cultivar with isolates from cv. Bilbao (*Rlm4*) (lesion diameter ranged from 0.6-0.9 mm) and the largest lesions were observed on the *Rlm4* cultivar with isolates from cv. Es-Astrid (QR) (diameter ranged from 9.0-14.5 mm), on the *Rlm5* cultivar with isolates from cv. DK Cabernet (*Rlm1* + QR) (lesion diameter ranged from 11.5-13.5 mm) and on the *Rlm6* cultivar with isolates from cv. DK Cabernet (lesion diameter ranged from 12.5-13.5 mm) (Table 5.8; Fig. 5.9b). For isolates of *L. maculans* from upper stem lesions, the smallest lesions were observed on the *Rlm5* line with isolates from cv. Drakkar (lesion diameter ranged from 0.6-0.8 mm) and with isolates from cv. DK Cabernet (lesion 0.6-0.9 mm) and on the *Rlm7* cultivar with isolates from cv. Drakkar (lesion diameter ranged from 0.5-1.7 mm),

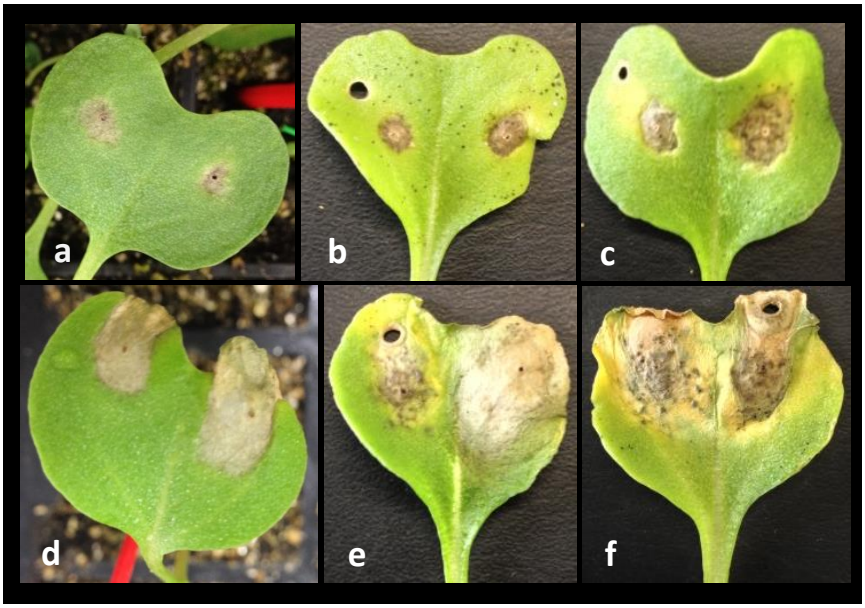


Fig. 5.8: *L. maculans* phenotypes on cv. Drakkar (susceptible to *L. maculans*) for isolates from phoma leaf spots on different cultivars; (a) severity score 0-1, isolate IIKB2 from cv. Bilbao at 7 days post inoculation; (b) severity score 3, isolate IIKR3 from cv. Roxet; (c) severity score 5, isolate IIKDKC from cv. DK Cabernet; at 14 days post inoculation on cv. Drakkar; (d) severity score 7, with no pycnidia produced isolate IIKD3 from cv. Drakkar; (e) severity score 8, with few pycnidia produced isolate IIKC1 from cv. Capitol and (f) severity score 9, with profuse pycnidial production isolate IIKD10 from cv. Drakkar. Refer to Appendix 5.1.

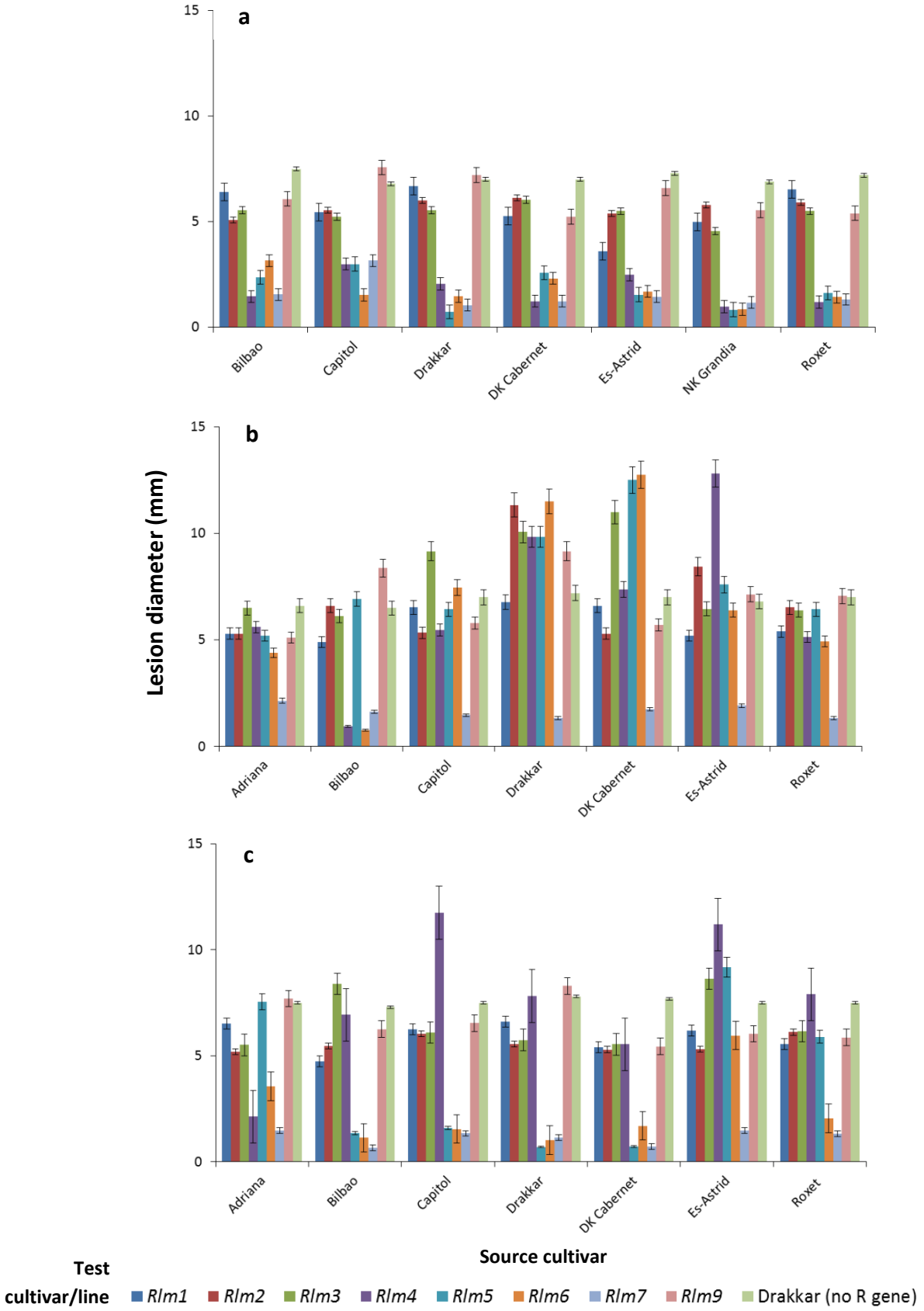


Fig. 5.9: Mean diameter of *L. maculans* phoma leaf spot lesions (mm) at 14 days post inoculation on a set of cultivars/lines for isolates from different cultivars isolated from (a) phoma leaf spots (b) basal stem cankers or (c) upper stem lesions.

Table 5.7: Differentiation of isolates of *L. maculans* obtained from phoma leaf spots from different source cultivars based on their leaf lesion diameter (mm) observed on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) 14 dpi.

	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Bilbao (*Rlm 4*)									
IIKB2	5.6	5.6	5.2	2.6	2.6	2.2	1.2	3.6	8
II K B4	9	6	6.5	1.1	2.2	3.8	2.1	8	7.5
IIKB3	5	3.6	5.4	1.4	2.2	2.8	1.9	6.5	7.5
I3B1	6.5	5	5.4	1.3	2.6	3.4	1.3	5.8	7
I3B2	6	5.2	5.2	0.9	2.2	3.6	1.3	6.5	7.3
From Capitol (*Rlm1*)									
IIKC1	5.2	5.6	9	2.6	2.8	1.1	2.4	7.2	6
II K C6	5.2	5.2	1.5	3.6	3.6	1.8	5.4	6.5	6.5
IIKC2	6	5.8	5.2	2.8	2.6	1.7	1.7	9	6.8
From DKCabernet (*Rlm1* + QR)									
IIKDKC	3.8	5	5.2	1.4	2.6	0.8	0.7	5.6	6.8
II K DKC 2	3.8	5.8	6	1.6	1.7	1.6	1.3	5	7
II K DKC 4	6.5	7.2	8	0.9	3.2	3.2	1.6	5	6.9
II K DKC 1	9	5.4	5.6	0.9	3	2.8	1.3	5	7.3
IIKDKC 3	3.2	7.2	5.4	1.4	2.4	3.2	1.3	5.6	7
From Es-Astrid (QR)									
IIKEA1	3.6	5.2	5.2	2.4	1.6	1.9	1.6	5.2	8.8
IIKEA3	3.6	5.6	5.8	2.6	1.5	1.5	1.3	8	9
From Drakkar (No *R* gene)									
IIKD6	5.4	5.8	3.8	1.9	0.5	1.4	1.1	7.2	6.5
IIKD4	5.4	5.4	5	1.7	0.5	1.9	1.2	7.2	7
IIKD3	5	5.4	5.6	2.2	0.5	1.7	0.5	5.8	5.8

Table 5.7. Continued from preceding page

Source cultivar/ (Isolate)	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
IIKD1	5	5.4	5	1.7	0.6	1.8	0.6	6	6
IIKD2	5	5.2	5.2	1.7	1.1	0.6	0.6	7.2	6.2
II K D 5	6	5.4	5.8	5.6	1.2	1.8	1.3	5.8	5.8
II K Drakkar	7.2	5.8	6	5.2	0.7	1.4	1.2	5.8	5.8
IIK D7	9	5.8	5	1.4	0.9	1.5	1.5	5.8	5.8
IIKD8	7.2	7.2	5.4	0.9	0.7	1.7	1.3	7.2	6
IIKD10	7.2	8	5.6	1.3	0.9	1.8	1.1	9	9
IIKD11	7.2	5.2	6	1.7	0.8	2.1	0.8	7.2	7.2
IIKD12	6.5	9	8	1.3	0.5	1.7	0.7	9	9
IIKD13	8	5.4	5.6	2.4	0.8	1.1	1.6	8	8
IIKD15	7.2	5.6	5.4	0.9	0.6	0.5	1.2	8	8
D1	9	5.4	5.6	0.9	0.6	1.1	1.1	9	9
From NK Grandia (QR)									
IIKNKG1	3.4	5.2	5.2	0.7	0.9	0.9	0.6	5.6	6.6
IIKNKG4	5.6	6.5	3.6	1.3	1.2	1.1	1.1	6	7
IIKNKG6	5.2	5.2	3.4	0.6	0.5	0.5	1.2	5.4	6.5
IIKNKG5	5.2	5.6	5.6	1.4	0.7	0.6	1.4	5.4	7.5
IIKNKG3	5.6	6.5	5	0.9	0.9	1.2	1.6	5.3	7
From Roxet (*Rlm7*)									
IIKR1	5.6	5.4	5	1.3	1.8	0.8	0.9	5.8	7
IIKR2	5.6	5.6	5.6	1.5	1.5	1.6	1.5	5.2	7.5
IIKR3	6.5	6.5	6.5	0.9	1.7	1.4	1.4	5	7
IIKR5	9	5.6	5.2	0.8	1.4	1.7	1.3	5.2	7.5
IIKR7	6	6.5	5.2	1.5	1.8	1.7	1.5	5.8	7

Table 5.8: Differentiation of isolates of *L. maculans* obtained from basal stem cankers from different source cultivars based on their leaf lesions diameter (mm) observed on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) 14 dpi.

	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Adriana (*Rlm4* + QR)									
KA 70 B	5	5.2	6.5	5.6	5.4	5.2	2.4	5	6.5
ADR19B	5.6	5.4	6.5	5.6	5	3.6	1.9	5.2	6.7
From Bilbao (*Rlm4*)									
KB228B	5.2	5.6	6.5	0.9	6	0.8	1.8	9	6.3
KB229B	5.4	5.6	5.6	1.4	7.2	0.9	1.8	9	6.4
I31B	5.2	7.2	5.2	0.7	8	0.8	1.6	6.5	6.6
I32B	3.8	8	7.2	0.8	6.5	0.6	1.3	9	6.7
From Capitol (*Rlm1*)									
Cap1	6	5	9	5.6	7.2	7.2	1.4	5.4	6.9
Cap2	8	5.4	8	5.6	6.5	8	1.6	6	7.2
Cap3	5.6	5.6	10.5	5.2	5.6	7.2	1.4	6	7
From Drakkar (no *R* gene)									
KD136B	5.8	9	7.2	8	6.5	10.5	1.3	8	7
KD138B	8	12.5	11.5	12.5	11.5	12.5	1.4	9	7.4
KD139B	6.5	12.5	11.5	9	11.5	11.5	1.3	10.5	7.2
From DKCabernet (*Rlm1* + QR)									
KDK57B	5.8	5	8	6.5	11.5	12.5	1.8	5.4	6.9
KDK 55 B	9	5.6	14.5	9	12.5	12.5	1.8	6	7.4
KDK 61 B	6	5.4	13.5	6	13.5	12.5	1.7	6	6.9
DK59B	5.6	5.2	8	8	12.5	13.5	1.7	5.4	6.8
From Es-Astrid (QR)									
KEA 9 B	5	9	5.6	9	6	5.2	2.2	5.6	6.9

Table 5.8. Continued from preceding page

	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
KEA 12 B	5	7.2	7.2	12.5	8	5.8	2.2	5.6	6
KEA2B	5.4	8	7.2	14.5	6	8	1.8	6.5	7
KEA11B	5.2	9	6.5	14.5	9	7.2	1.7	9	7
KEA14B	5.4	9	5.8	13.5	9	5.8	1.7	9	7
From Roxet (*Rlm7*)									
R5-1	5.2	5.8	5.6	5.2	5.6	5.4	1.3	8	7
R11	5.4	5.8	5.6	5	6.5	5.6	1.4	6	6.9
R7	5.6	8	8	5.2	7.2	3.8	1.3	7.2	7.2

isolates from cv. DK Cabernet (lesion 0.5-1.1 mm) and with isolates from cv. Bilbao (*Rlm4*) (lesion diameter ranged 0.6-0.7 mm). The largest lesions were observed on the *Rlm4* cultivar with isolates from cv. Capitol (*Rlm1*) (lesion diameter ranged from 10.5-12.5 mm) and with isolates from Es-Astrid (lesion diameter ranged from 9.0-12.5 mm) (Table 5.9; Fig. 5.9c). The *L. maculans* isolates from cvs Capitol (*Rlm1*) (isolated from phoma leaf spots or upper stem lesions) and Es-Astrid (QR) (isolated from basal stem cankers or upper stem lesions) produced larger lesions than other isolates of *L. maculans* from different cultivars (Fig. 5.9). All *L. maculans* isolates obtained from different cultivars produced larger lesions on the *Rlm9* line and on cv. Drakkar (no *R* gene) than on other lines/cultivars (Tables 5.7, 5.8 & 5.9).

Comparing the phenotype produced by all *L. maculans* isolates, most isolates from basal stem cankers produced larger lesions than isolates from upper stems or isolates from phoma leaf spots (Fig. 5.9 and Fig. 5.10). Some isolates from upper stem lesions (KR157U and KR158U) inoculated on the *Rlm4* cultivar produced larger lesions (diameter >7mm) than isolates from basal stem cankers (Fig. 5.11 and Table 5.9). Most of the *L. maculans* isolates from basal stems and upper stems produced larger lesions than *L. maculans* from phoma leaf spots.

5.3.2 Phoma leaf spot phenotype produced by inoculation with *Leptosphaeria biglobosa* isolates derived from phoma leaf spots or stem cankers

No symptoms were observed at 7 days post inoculation (dpi) with *L. biglobosa* (LB) isolates (Fig. 5.12) on 12 test cultivars after inoculation with 48 isolates derived from phoma leaf spots (36 isolates), basal stem cankers (6 isolates) or upper stem lesions

Table 5.9: Differentiation of isolates of *L. maculans* obtained from upper stem lesions from different source cultivars based on their leaf lesions diameter (mm) observed on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) 14 dpi.

	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
Source cultivar/ (Isolate)	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From Adriana (*Rlm4* + QR)									
KA95U	8	5.2	5	1.8	5.6	3.4	1.3	14.5	7
KA72U	5.6	5.2	5.2	1.8	8	3.4	1.2	6	7.5
KA 98 U	6	5.4	5.6	2.8	10.5	3.6	1.6	6	7.8
KA85U	5.8	5.2	6	2.6	5.6	3.8	1.5	6	7.7
KA 99 U	7.2	5	5.8	1.7	8	3.6	1.8	6	7.5
From Bilbao (*Rlm4*)									
KB 217 U	5.2	5.4	9	5.6	1.3	1.2	0.7	6.5	7
B10P	3.8	5.6	9	8	1.4	0.9	0.6	5.8	7.3
B13P	5.2	5.4	7.2	7.2	1.4	1.3	0.7	6.5	7.7
From Capitol (*Rlm1*)									
KC250U	5.6	5.4	5.2	10.5	1.9	1.5	1.5	6.5	7
KC249U	8	6.5	6	11.5	1.6	1.8	1.6	6	7.5
KC255U	5.6	6.5	7.2	12.5	1.6	1.7	1.5	6.5	7.5
C10P	5.8	5.8	6	12.5	1.3	1.2	0.8	7.2	7.8
From Drakkar (no *R* gene)									
KD 122 U	5.8	5.2	6	5.6	0.8	1.3	1.3	7.2	7.2
KD123U	5.5	5.4	5.4	10.5	0.6	1.3	1.7	8	8
D1P	8	5.6	5.8	8	0.7	0.8	0.5	9	8
D1	7.2	6	5.8	7.2	0.7	0.7	1.1	9	7.8
From DKCabernet (*Rlm1* + QR)									
DKC4P	5.6	5	5.8	5.2	0.9	2.4	0.7	5.6	7.5
DKCU1	5.6	5.4	5.6	5.4	0.8	2.4	0.6	5.6	7.8

Table 5.9. Continued from preceding page

Source cultivar/ (Isolate)	Lesion diameter (mm) *R* gene in test *Brassica* differential genotype								
	Columbus	Bristol	line 02-22-2-1	Jet Neuf	line 99-150-2-1	Darmor-MX	line 01-23-2-1	line 01-190-1-1	Drakkar (No *R* gene)
	Rlm 1	*Rlm 2*	*Rlm 3*	*Rlm 4*	*Rlm 5*	*Rlm 6*	*Rlm 7*	*Rlm 9*	
From DKCabernet (*Rlm1* + QR)									
DKC11P	5.4	5.4	5.6	5.8	0.6	0.8	0.5	5.4	7.5
DKC(J)11-26	5	5.4	5.2	5.8	0.6	1.2	1.1	5.2	7.9
From Es-Astrid (QR)									
KEA 24 U	5.4	5.2	5.6	11.5	5.6	5.4	1.3	6	7
KEA 18 U	6.5	5	9	9	10.5	6.5	1.7	6.5	7.5
KEA 25 U	5.8	5.4	11.5	12.5	12.5	5.8	1.7	5.6	7.6
KEA 17 U	7.2	5.6	11.5	10.5	11.5	6.5	1.4	5.6	7.8
KEA22U	6	5.4	5.6	12.5	5.8	5.6	1.3	6.5	7.8
From Roxet (*Rlm7*)									
KR158U	5.4	5.6	5.4	5.8	5.6	1.9	1.4	5.4	7.5
KR157U	5.6	5.4	5.4	8	5.6	2.1	1.5	5.2	7.7
KR159U	5.6	5.6	5.4	10.5	5.6	1.8	1.4	5.4	7.5
KR156U	5.4	6.5	5.6	5.8	5.4	2.3	1.3	5.2	7.4
KR164U	5.8	5.6	8	11.5	7.2	1.9	1.2	6	7.5
KR166U	5.6	8	7.2	5.8	6	2.3	1.2	8	7.4

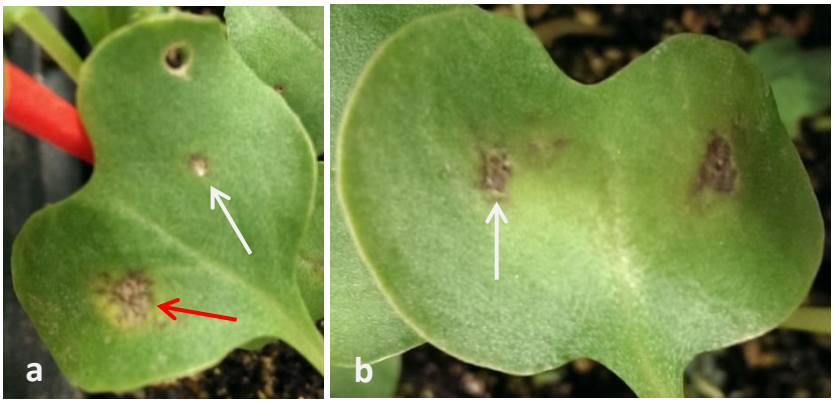


Fig. 5.10: Differences in lesion diameter at 14 days post inoculation on the test *Rlm7* line for *L. maculans* isolate from (a) upper stem lesion (cv. Drakkar; isolate D1P) (0.5 mm) (white arrow) and isolate from basal stem cankers (cv. Adriana; isolate ADR19B) (1.9 mm) (red arrow) or (b) isolates from phoma leaf spots (cv. Drakkar; isolate IIKD11) that produced lesions with diameter 0.8 mm (white arrow).

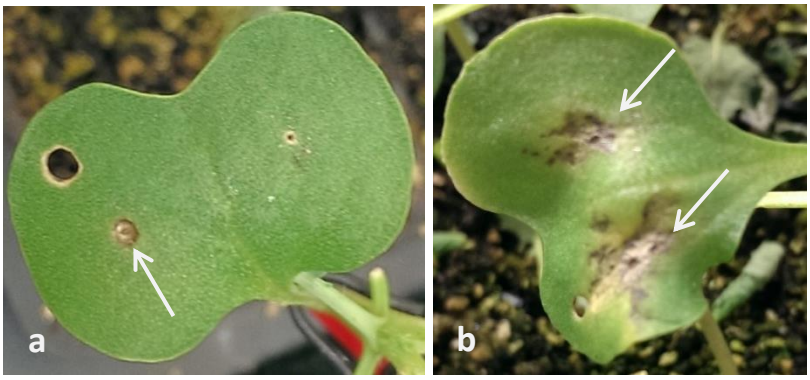


Fig. 5.11: Differences in lesion diameter between *L. maculans* isolates from basal stem cankers or upper stem lesions at 14 days post inoculation on the test *Rlm4* cultivar; (a) small lesions (0.9 mm) produced by isolates from basal stem cankers (cv. Bilbao; isolate KB228B) (arrow) and (b) larger lesions produced by isolates KR157U and KR158U from upper stem lesions (cv. Roxet), 8.0 mm and 5.8 mm, respectively (arrows).

Fig. 5.12: Early phoma leaf spot symptoms at 7 days post inoculation with *L. biglobosa* isolates from basal stem cankers (isolates from cvs Capitol (C6 and C117), Roxet (R11) and NK Grandia (NKG 63)) on cv. Adriana in a controlled environment room at University of Hertfordshire, Bayfordbury.

(6 isolates). However, by 14 days post inoculation, most of cultivars showed symptoms, with small lesions scoring ≥1 (Fig. 5.13). For *L. biglobosa* isolates from basal stem cankers, only isolates that were inoculated on cv. Drakkar (no *R* genes) produced leaf lesions with severity score >1 by 14 days post inoculation (Fig. 5.14a, Appendix 5.1), whereas some *L. biglobosa* isolates from upper stem lesions produced larger lesions when inoculated onto cultivars/lines with *Rlm4*, *Rlm7* or *Rlm4* + QR (Fig. 5.14b, Appendix 5.2). There was no significant difference (P>0.05) in lesion severity score on cv. Drakkar (no *R* genes) between *L. biglobosa* isolates from basal stem cankers and those from upper stem lesions, where *L. biglobosa* isolates from basal stem cankers produced lesions with score 1.0-2.2 and *L. biglobosa* isolates from upper stem lesions produced lesions with score 1.0-2.4 (Fig. 5.14). For some *L. biglobosa* isolates derived from phoma leaf spots, lesions with severity scores ≥1 were observed only on cultivars/lines with *Rlm1*, *Rlm4*, *Rlm7*, *Rlm4* + QR, *Rlm1* + QR, QR and cv. Drakkar (Table 5.10) (Fig. 5.15). There were also some *L. biglobosa* isolates that produced lesions with dark margins at 14 days post inoculation (Fig. 5.16).

The lesions sizes were different between isolates of *L. biglobosa* that were inoculated on cv. Drakkar than other cultivars/lines with or without *R* genes and QR either from leaf spots, basal stem cankers or upper stem lesions (Table 5.11, 5.12, 5.13). All isolates derived from phoma leaf spots that were inoculated on 12 test cultivars produced leaf spots with size ≤1mm (score 1) except for isolates that were inoculated on the *Rlm1* cultivar (isolates from cvs Bilbao, DK Cabernet, Excel and NK Grandia), the *Rlm4* cultivar (isolates from cvs Bilbao, DK Cabernet, Es-Astrid and Excel), the *Rlm7* line (isolates from cvs Drakkar, Es-Astrid, Excel and Roxet), the

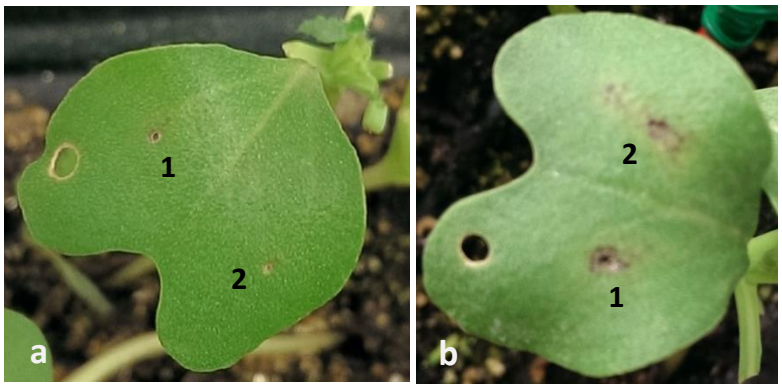


Fig. 5.13: Symptoms produced by *L. biglobosa* isolates from basal stem cankers on cv. Capitol (1- isolate C6 and 2- isolate C117) inoculated onto NK Grandia (QR), (a) no symptoms at 7 days post inoculation and (b) leaf lesions observed at 14 days post inoculation.

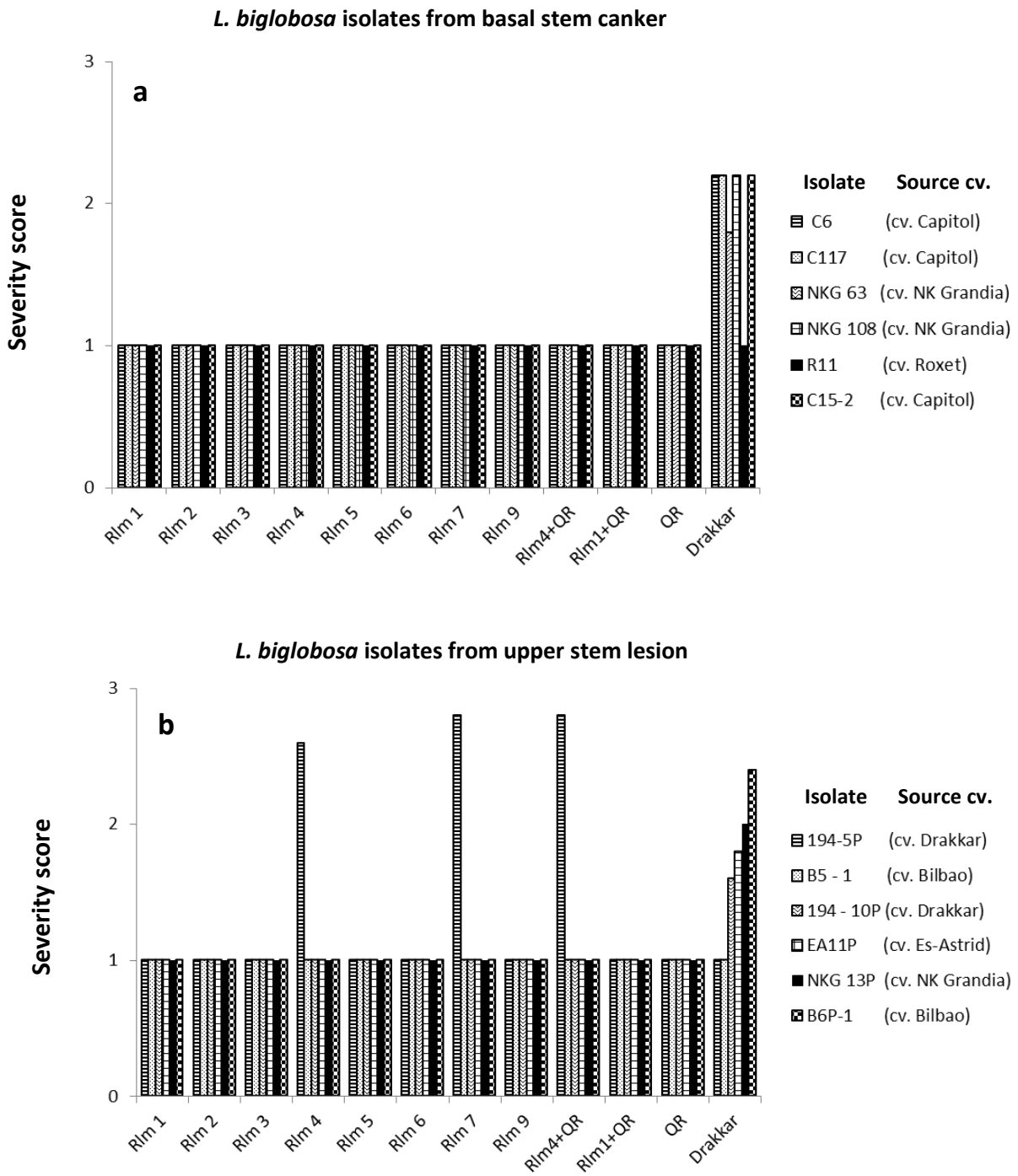


Fig. 5.14: Differentiation of isolates of *L. biglobosa* from (a) basal stem cankers (isolates C6, C117, NKG63, NKG108, R11 and C15-2) or (b) upper stem lesions (isolates 194-5P, B5-1, 194-10P, EA11P, NKG 13P and B6P1), based on their cotyledon lesion severity score (0-9 scale) observed at 14 days post inoculation on test cultivars with or without *R* genes against *L. maculans* and QR and cv. Drakkar used as control (susceptible to *L. maculans*).

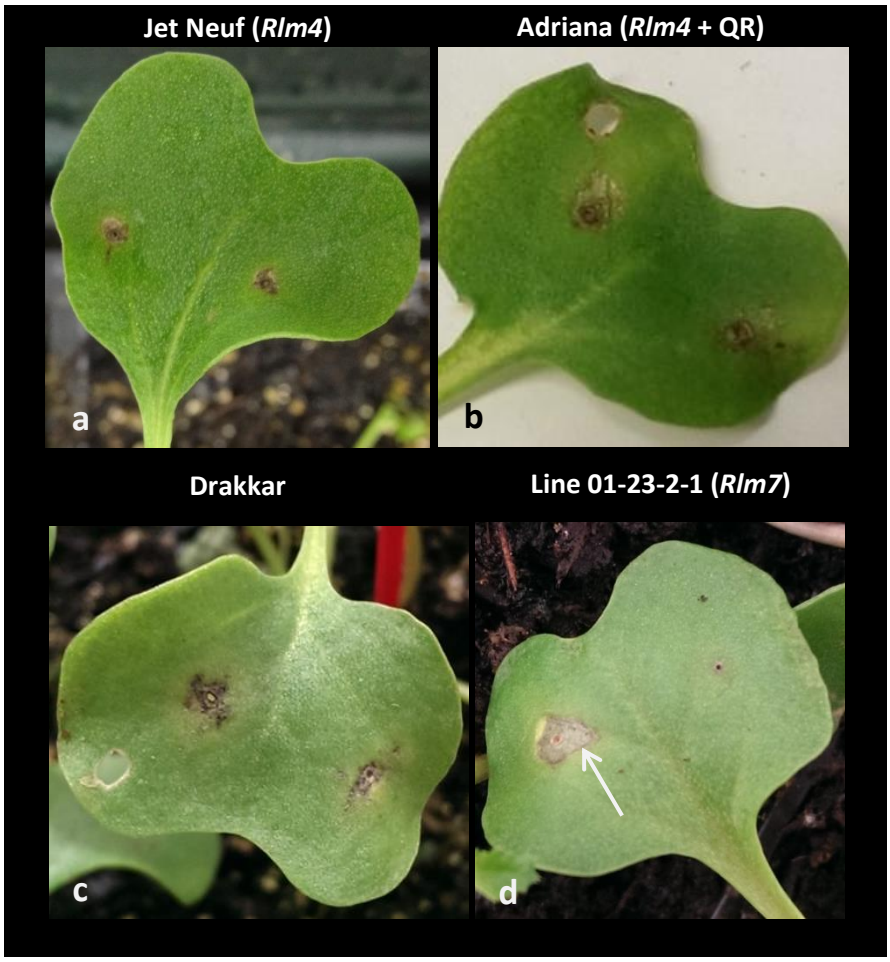


Fig. 5.15: Phoma leaf spot phenotype at 14 days post inoculation with *L. biglobosa* isolates derived from phoma leaf spots on different cultivars with (a) *Rlm4* (cv. Jet Neuf) (isolate IIKE1, severity score 1.2), (b) *Rlm4* + QR (cv. Adriana) (isolate Ex3, severity score 1.6), (c) cv. Drakkar (isolate IIKA4, severity score 2.2) or (d) *Rlm7* (line 01-23-2-1) (isolate IIKE2, severity score 2.4) (arrow). Refer to Appendix 5.2.

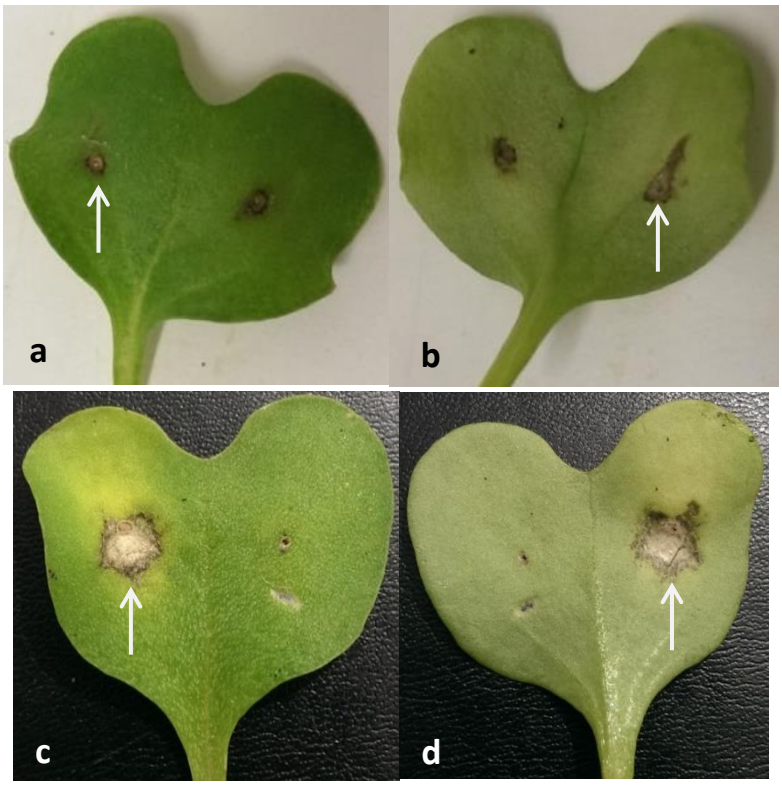


Fig. 5.16: Phoma leaf spots with dark margins (arrow) produced after inoculation of *B. napus* with *Rlm7* (line 01-23-2-1) with *L. biglobosa* isolate IIKEA2 on (a) upper surface or (b) lower surface and after inoculation with *L. biglobosa* isolate IIK E2 on (c) upper surface or (d) lower surface. Both isolates were isolated from phoma leaf spots.

Table 5.10: Differentiation of isolates of *L. biglobosa* derived from phoma leaf spots from different source cultivars based on their lesion severity score 14 dpi on cultivars/lines with or without *R* genes and QR and cv. Drakkar.

	Severity score (0-9)[a]											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
From Adriana												
IIK A4	1	1	1	1	1	1	1	1	1	1	1	2.2
IIK A2	1	1	1	1	1	1	1	1	1	1.6	1	2.6
IIK A3	1	1	1	1	1	1	1	1	1	1	1	2
Ax	1	1	1	1	1	1	1	1	1	1	1	2
From Bilbao												
IIK B1	1.6	1	1	1	1	1	1	1	1	1.2	1	1.6
IIK B2	1.4	1	1	2	1	1	1	1	1.4	1.4	2	2
IIK B3	1.6	1	1	1	1	1	1	1	1.2	1.4	1.2	1.8
Bx	1.6	1	1	1.4	1	1	1	1	1.4	1.4	1	1.8
From Capitol												
Cx1	1	1	1	1	1	1	1	1	1	1	1	1.4
Cx2	1	1	1	1	1	1	1	1	1	1	1	1.6
Cx3	1	1	1	1	1	1	1	1	1	1	1	1.4
Cx4	1	1	1	1	1	1	1	1	1	1	1	1.6
From Drakkar												
IIK D2	1	1	1	1	1	1	2	1	1	1	1	2
Dx2	1	1	1	1	1	1	1.6	1	1	1	1	2.8
Dx3	1	1	1	1	1	1	1.6	1	1	1	1	2.4
Dx4	1	1	1	1	1	1	2	1	1	1	1	2.2
From DK Cabernet												
IIK DKC7	1	1	1	1	1	1	1	1	1	1	1	2
IIK DKC 5	1.4	1	1	1	1	1	1	1	1	1	1.8	2.2

Table 5.10. Continued from preceeding page

	Severity score (0-9)[a]											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
From DK Cabernet												
IIK DKC 8	1	1	1	1	1	1	1	1	1	1	1	2.2
IIK DKC6	2.2	1	1	2.8	1	1	1	1	1.4	2.4	2.6	1
From Es-Astrid												
IIK EA3	1	1	1	1	1	1	1	1	1	1	1	1.4
IIK EA 4	1	1	1	1	1	1	1	1	1	1	1	1.8
IIK EA1	1	1	1	1	1	1	1	1	1	1	1.8	2.8
IIK EA2	1.6	1	1	2.6	1	1	1.6	1	1	2.2	2.4	1.8
From Excel												
IIK E1	1	1	1	1.2	1	1	1	1	1	1	1	2.2
IIK E2	1.6	1	1	2.4	1	1	2.4	1	2.2	1.8	1.8	2.6
Ex3	1	1	1	1.4	1	1	1.8	1	1.6	1.6	1	2
Ex4	1.4	1	1	1.6	1	1	2.2	1	1.8	1.6	1.4	2.2
From Roxet												
IIK R2	1	1	1	1	1	1	1.8	1	1	1	1	2.2
IIK R1	1	1	1	1	1	1	1	1	1	1	1	2.4
Rx3	1	1	1	1	1	1	1.4	1	1	1	1	1.8
Rx4	1	1	1	1	1	1	1.4	1	1	1	1	2.4
From NKGrandia (QR)												
IIK NKG 2	1.2	1	1	1	1	1	1	1	1	1	1	2.4
IIK NKG	1	1	1	1	1	1	1	1	1	1	1	2.2
NKGx3	1	1	1	1	1	1	1	1	1	1	1	2.4
NKGx4	1	1	1	1	1	1	1	1	1	1	1	2.2

[a]See Fig. 5.5

Table 5.11: Differentiation of isolates of *L. biglobosa* derived from phoma leaf spots from different source cultivars based on their lesion diameter (mm) 14 dpi observed on cultivars/lines with or without *R* genes and QR and cv. Drakkar.

	Lesion diameter (mm)											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
From Adriana												
IIK A4	0.8	0.7	0.6	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	1.5
IIK A2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.2	0.5	1.5
IIK A3	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.7	0.5	0.5	0.5	1.5
Ax	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	1.3
From Bilbao												
IIK B1	1	0.6	0.6	0.5	0.5	0.5	0.5	0.8	0.5	1	0.5	1.5
IIK B2	1.1	0.5	0.5	1.1	0.5	0.7	0.5	0.9	0.9	1	1.4	1.3
IIK B3	1.4	0.7	0.5	1	0.5	0.6	0.5	0.7	1.1	0.9	1	1.4
Bx	0.9	0.5	0.5	0.8	0.5	0.6	0.5	0.8	0.9	0.8	0.9	1.4
From Capitol												
Cx1	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.7	0.5	0.6	0.5	1
Cx2	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.5	1.1
Cx3	0.6	0.7	0.6	0.5	0.5	0.5	0.5	0.6	0.5	0.6	0.5	1.1
Cx4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	1.5
From DK Cabernet												
IIK D2	0.6	0.6	0.5	0.5	0.5	0.5	1.5	0.6	0.5	0.5	0.5	1.5
Dx2	0.7	0.6	0.5	0.5	0.5	0.5	1.4	0.8	0.5	0.5	0.5	1.7
Dx3	0.6	0.5	0.5	0.5	0.5	0.5	1.4	0.7	0.5	0.5	0.5	1.7
Dx4	0.6	0.5	0.5	0.5	0.5	0.5	1.5	0.6	0.5	0.5	0.5	1.5
IIK DKC7	0.6	0.6	0.5	0.5	0.5	0.6	0.5	0.6	1	1	1	1.5
IIK DKC 5	1.1	0.6	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1.5	1.6
IIK DKC 8	1	0.5	0.6	0.5	0.5	0.6	0.5	0.6	1	1	1	1.4

Table 5.11. Continued from preceeding page

	Lesion diameter (mm)											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
From DK Cabernet												
IIK DKC6	1.2	0.5	0.5	1.8	0.5	0.5	0.5	0.5	1	1.4	1.8	1.5
From Es-Astrid												
IIK EA3	0.6	0.6	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.5	0.5	1.4
IIK EA 4	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.7	0.5	0.6	0.5	1.5
IIK EA1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	1.5	1.8
IIK EA2	1.4	0.6	0.6	1.7	0.5	0.7	1.3	0.7	0.5	1.5	1.5	1.8
From Excel												
IIK E1	0.5	0.5	0.5	1.1	0.5	0.5	1	0.7	1	1	1	1.5
IIK E2	1.5	0.7	0.6	1.2	0.5	0.6	1.5	0.6	1.2	1.4	1.5	1.7
Ex3	1	0.5	0.5	1.1	0.5	0.6	1.4	0.6	1.5	1.3	1	1.5
Ex4	1.1	0.5	0.7	1.4	0.5	0.6	1.5	0.6	1.5	1.3	1.4	1.6
From Roxet												
IIK R2	0.6	0.6	0.6	0.5	0.5	0.5	1.5	0.6	0.5	0.5	0.5	1.5
IIK R1	0.6	0.5	0.6	0.5	0.5	0.5	1	0.7	0.5	0.6	0.5	1.6
Rx3	0.5	0.6	0.5	0.6	0.5	0.5	1.1	0.6	0.6	0.5	0.5	1.5
Rx4	0.6	0.6	0.6	0.5	0.5	0.5	1.1	0.6	0.5	0.5	0.5	1.5
From NK Grandia												
IIK NKG 2	1	0.7	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.8	1.6
IIK NKG	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.7	0.6	0.5	0.6	1.6
NKGx3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.5	0.5	0.6	1.5
NKGx4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5

Table 5.12: Differentiation of isolates of *L. biglobosa* from basal stem canker from different source cultivar based on their leaf lesion diameter (mm) 14 dpi observed on cultivars/lines with or without *R* genes and QR and cv. Drakkar.

	Lesion diameter (mm)											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
C6	0.5	0.5	0.5	0.6	0.5	0.5	0.7	0.5	0.6	0.5	0.5	1.4
C117	0.5	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5
NKG 63	0.6	0.5	0.5	0.7	0.5	0.5	0.6	0.5	0.6	0.6	0.5	1.5
NKG 108	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.5	1.5
R11	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.5	0.5	0.5	0.8
C15-2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.3

Table 5.13: Differentiation of isolates of *L. biglobosa* from upper stem lesions from different source cultivar based on their leaf lesion diameter (mm) 14 dpi observed on cultivars/lines with or without *R* genes and QR and cv. Drakkar.

	Lesion diameter (mm)											
	Cultivar/line											
Source cultivar/ Isolate	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no *R* gene)
194-5P	0.5	0.5	0.5	1.5	0.5	0.5	1.5	0.5	1.5	0.6	0.5	1
B5 - 1	0.5	0.6	0.5	0.6	0.5	0.5	0.5	0.5	0.7	0.7	0.5	1
194 - 10P	0.5	0.5	0.5	1	0.5	0.6	0.5	0.5	0.8	0.5	0.5	1.2
EA11P	0.5	0.6	0.5	0.6	0.6	0.6	0.5	0.5	0.6	0.6	0.5	1.3
NKG 13P	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	1	0.5	0.5	1.5
B6P-1	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	1	0.5	0.5	1.5

Rlm4 + QR cultivar (isolates from cvs Bilbao, DK Cabernet and Excel), the *Rlm1* + QR cultivar (isolates from cvs Adriana, Bilbao, DK Cabernet, Es-Astrid and Excel) or the QR cultivar (isolates from cvs Bilbao, DK Cabernet, Es-Astrid and Excel) (Table 5.10).

5.3.3 Determination of alleles of *Avr* genes in *Leptosphaeria maculans* isolates obtained from phoma leaf spots or stem cankers

In total, 95 *L. maculans* (LM) isolates were collected from different cultivars with or without *R* genes against *L. maculans*; isolates were derived from phoma leaf spots (40 isolates), basal stem cankers (24 isolates) or upper stem lesions (31 isolates).

For *L. maculans* isolates derived from phoma leaf spots, *L. maculans* isolates from cultivars Bilbao, Capitol, DK Cabernet, Es-Astrid, Drakkar, NK Grandia or Roxet were virulent when inoculated onto cvs Columbus (*Rlm1*), Bristol (*Rlm2*), line 02-22-2-1 (*Rlm3*) or line 01-190-1-1 (*Rlm9*) (Fig. 5.17). Most isolates did not produce symptoms on cv. Jet Neuf (*Rlm4*), with 97.5% of isolates possessing the avirulent allele *AvrLm4*, or line 99-150-2-1 (*Rlm5*), with 97.5% of isolates possessing the avirulent allele *AvrLm5*, or cv. Darmor-MX (*Rlm6*), with 90% of isolates possessing the avirulent allele *AvrLm6* or line 01-23-2-1 (*Rlm7*), with 100% of isolates possessing the avirulent allele *AvrLm7* (Fig. 5.17). There were no isolates of *L. maculans* that possessed avirulent alleles of *AvrLm1, AvrLm2, AvrLm3* or *AvrLm9* (Fig. 5.17). The avirulent alleles *AvrLm4, AvrLm5, AvrLm6* and *AvrLm7* were observed in the isolates from phoma leaf spots from cultivars Bilbao, Capitol, DK Cabernet, Es-Astrid, Drakkar, NK Grandia and Roxet (Fig. 5.17). A total of thirty-nine isolates that carried *AvrLm4* and *AvrLm5* that were from cvs Bilbao (5 isolates), Capitol (2 isolates), DK

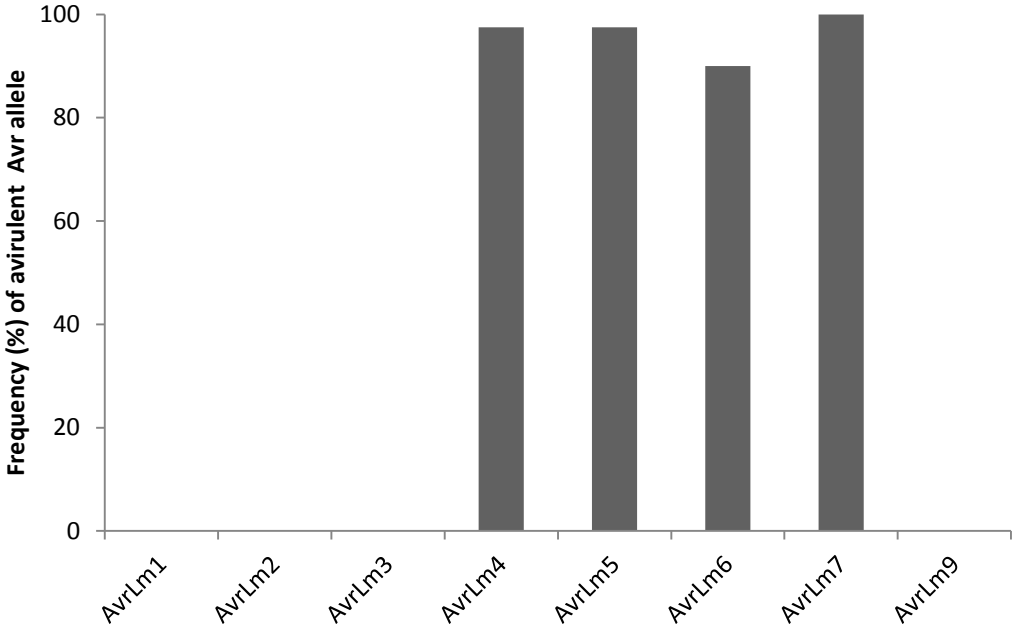


Fig. 5.17: Frequencies (%) of avirulent *Avr* alleles in 40 *Leptosphaeria maculans* isolates sampled by isolating from phoma leaf spots on nine winter oilseed rape grown at Rothamsted Research. Thirty-nine isolates carried *AvrLm4* (97.5%) and *AvrLm5* (97.5%), 36 isolates carried *AvrLm6* (90%) and 40 isolates carried *AvrLm7* (100%).

Cabernet (5 isolates), Es-Astrid (2 isolates), Drakkar (15 isolates), NK Grandia (5 isolates) and Roxet (5 isolates). Thirty-six isolates that carried *AvrLm6* were from cvs Bilbao (3 isolates), Capitol (3 isolates), DK Cabernet (5 isolates), Es-Astrid (2 isolates), Drakkar (13 isolates), NK Grandia (5 isolates) and Roxet (5 isolates). Forty isolates that carried *AvrLm7* were from cvs Bilbao (5 isolates), Capitol (3 isolates), DK Cabernet (5 isolates), Es-Astrid (2 isolates), Drakkar (15 isolates), NK Grandia (5 isolates) and Roxet (5 isolates).

L. maculans isolates derived from basal stem cankers were from cvs Adriana, Bilbao, Capitol, Drakkar, DK Cabernet, Es-Astrid and Roxet. They were all virulent when inoculated on cvs Columbus (*Rlm1*), Bristol (*Rlm2*), line 02-22-2-1 (*Rlm3*), line 99-150-2-1 (*Rlm5*) or line 01-190-1-1 (*Rlm9*) (Fig. 5.18). Some isolates were avirulent on cv. Jet Neuf (*Rlm4*), with 16.7% of isolates possessing the avirulent allele *AvrLm4*, or cv. Darmor-MX (*Rlm6*), with 16.7% of isolates possessing the avirulent allele *AvrLm6*, or line 01-23-2-1 (*Rlm7*), with 100% of isolates possessing the avirulent allele *AvrLm7* (Fig. 5.18). There were no isolates of *L. maculans* that possessed avirulent alleles of *AvrLm1, AvrLm2, AvrLm3, AvrLm5* or *AvrLm9* (Fig. 5.18). The avirulent alleles *AvrLm4, AvrLm6* and *AvrLm7* were observed in the isolates from basal stem cankers from the cultivars Adriana, Bilbao, Capitol, DK Cabernet, Es-Astrid, Drakkar and Roxet (Fig. 5.18). Four isolates that carried *AvrLm4* and *AvrLm6* were from cv. Bilbao and 24 isolates that carried *AvrLm7* were from cvs Adriana (2 isolates), Bilbao (4 isolates), Capitol (3 isolates), DK Cabernet (4 isolates), Es-Astrid (5 isolates), Drakkar (3 isolates) or Roxet (3 isolates).

For *L. maculans* derived from upper stem lesions, all *L. maculans* isolates from cultivars Adriana, Bilbao, Capitol, Drakkar, DK Cabernet, Es-Astrid and Roxet were

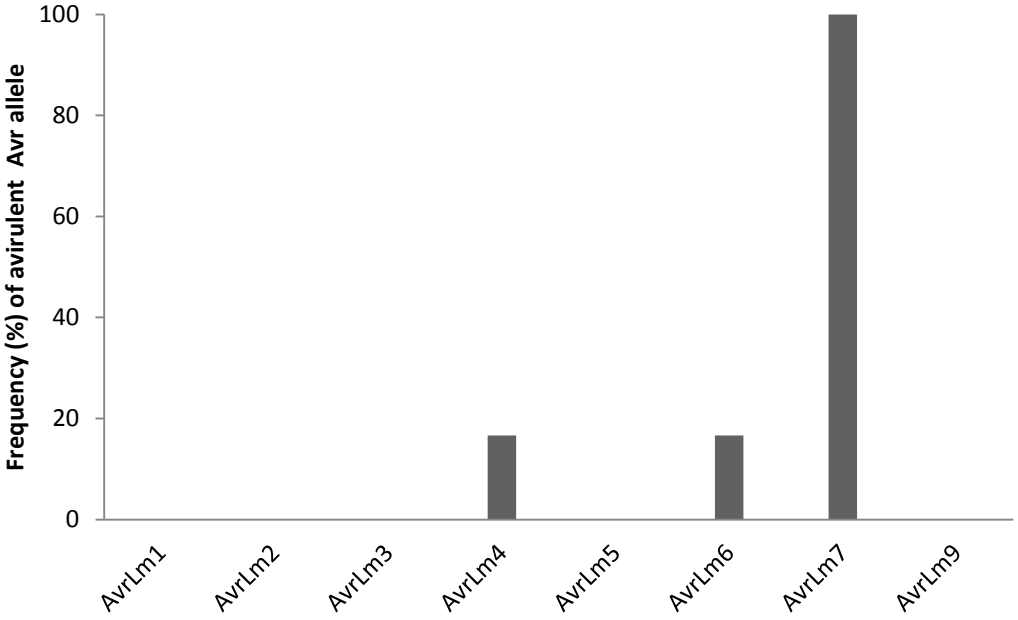


Fig. 5.18: Frequencies (%) of avirulent *Avr* alleles in 24 *Leptosphaeria maculans* isolates sampled by isolating from basal stem cankers on nine winter oilseed rape grown at Rothamsted Research. Four isolates carried *AvrLm4* (16.7%) or *AvrLm6* (16.7%) and 24 isolates carried *AvrLm7* (100%).

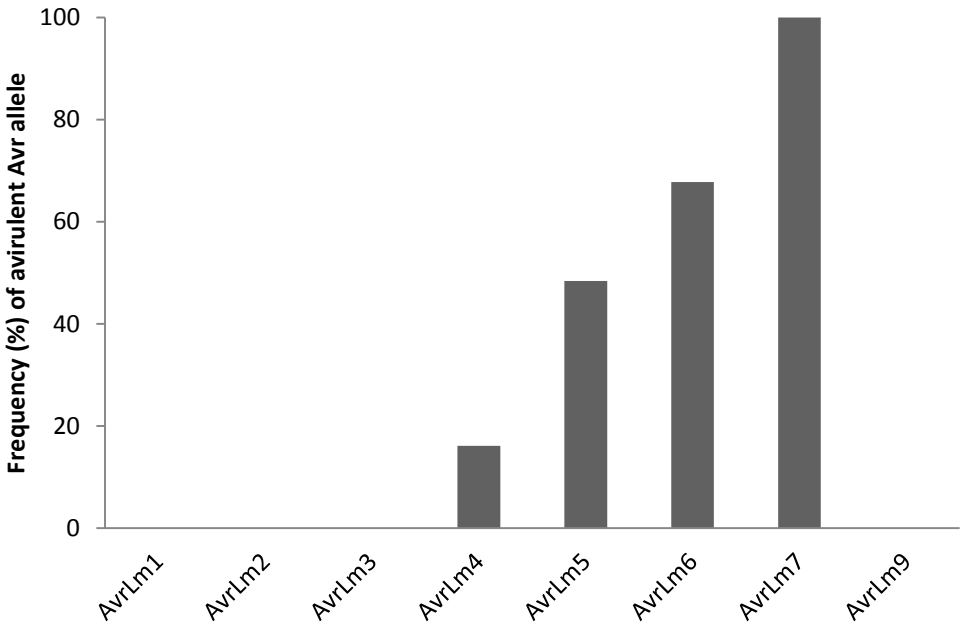


Fig. 5.19: Frequencies (%) of avirulent *Avr* alleles in 31 *Leptosphaeria maculans* isolates sampled by isolating from upper stem cankers on nine winter oilseed rape grown at Rothamsted Research. Five isolates carried *AvrLm4* (16.1%), 15 isolates carried *AvrLm5* (48.4%), 21 isolates carried *AvrLm6* (67.7%) and 31 isolates carried *AvrLm7* (100%).

virulent when inoculated on cvs Columbus (*Rlm1*), Bristol (*Rlm2*), line 02-22-2-1 (*Rlm3*) or line 01-190-1-1 (*Rlm9*) (Fig. 5.19). Some isolates were avirulent on cv. Jet Neuf (*Rlm4*) with 16.1% of isolates possessing the avirulent allele *AvrLm4*, or line 99-150-2-1 (*Rlm5*) with 48.4% of isolates possessing the avirulent allele *AvrLm5*, or cv. Darmor-MX (*Rlm6*) with 67.7% of isolates possessing the avirulent allele *AvrLm6* or line 01-23-2-1 (*Rlm7*) with 100% of isolates possessing the avirulent allele *AvrLm7* (Fig. 5.19). There were no isolates of *L. maculans* that possessed avirulent alleles of *AvrLm1, AvrLm2, AvrLm3* and *AvrLm9* (Fig. 5.19). The alleles *AvrLm4, AvrLm5, AvrLm6* and *AvrLm7* were observed in the isolates from upper stem lesions from the cultivars Adriana, Bilbao, Capitol, DK Cabernet, Es-Astrid, Drakkar and Roxet (Fig. 5.19). Five isolates that carried *AvrLm4* were from cvs. Adriana and 15 isolates that carried *AvrLm5* were from cvs Bilbao (3 isolates), Capitol (4 isolates), Drakkar (4 isolates) or DK Cabernet (4 isolates). Twenty-one isolates that carried *AvrLm6* were from cvs Bilbao (3 isolates), Capitol (4 isolates), Drakkar (4 isolates), DK Cabernet (4 isolates) or Roxet (6 isolates). Thirty-one isolates that carried *AvrLm7* were from cvs Adriana (5 isolates), Bilbao (3 isolates), Capitol (4 isolates), Drakkar (4 isolates), DK Cabernet (4 isolates), Es- Astrid (5 isolates) or Roxet (6 isolates).

Most *L. maculans* isolates from phoma leaf spots or upper stem lesions possessed avirulent Avr alleles of *AvrLm4 , AvrLm5, AvrLm6* and *AvrLm7* (Fig. 5.17 & Fig. 5.19) whereas *L. maculans* isolates from basal stem cankers possessed only avirulent alleles *AvrLm4, AvrLm6* and *AvrLm7* (Fig. 5.18).

Only seven allele combinations (Table 5.14) were found in this study and the most frequent *L. maculans* race amongst isolates derived from phoma leaf spots, basal stem cankers or upper stem lesions, with the combination of alleles *avrLm1-avrLm2-*

avrLm3-avrLm4-AvrLm5-AvrLm6-AvrLm7-avrLm9 was race Av(6)-7-(8) using the terminology proposed by Balesdent *et al.* (2005). The next most frequent race was Av4-5-6-7-(8) (Table 5.14). The most diverse population of isolates of *L. maculans* was derived from upper stem lesions, with four races, followed by isolates derived from phoma leaf spots, with three races and the most frequent race (87.5%) was Av4-5-6-7-(8) (Table 5.14).

5.4 Discussion

These results suggest (refer section 5.3.1) that there were differences in phenotypic response on cotyledons between *L. maculans* isolates from different cultivars and from different sources (phoma leaf spots, upper stem lesions or basal stem cankers), whereas fewer differences were observed in phenotypic response between *L. biglobosa* isolates from different cultivars and different sources. This may have been due to specific interactions with *L. maculans* isolates; the resistance or susceptibility of winter oilseed rape cultivars to *L. maculans* depends on gene-for-gene interactions between the resistance (*R*) genes in the *B. napus* host and the corresponding effector (*Avr*) genes in the isolate of *L. maculans*. The results indicate that most *L. maculans* isolates from basal cankers were more virulent (produced larger lesions) than *L. maculans* isolates from upper stems (Table 5.5, 5.6, 5.8 and 5.9). However, there were some *L. biglobosa* isolates that produced larger lesions (>1.0mm) when inoculated on different cultivars, such as isolates from phoma leaf spots (isolates from cvs Bilbao, DK Cabernet, Es-Astrid, Excel and Roxet) and isolates from basal stem cankers (isolated from cv. Drakkar). This suggests that a difference between cultivars in susceptibility to specific *L. biglobosa* isolates can be detected by the cotyledon test. This study also indicated that isolates from stems infected more cultivars than isolates from phoma leaf spots (Table 5.14). Therefore, this study

Table 5.14: The frequency of *Leptosphaeria maculans* races on winter oilseed rape sampled by isolating from phoma leaf spots, basal stem cankers or upper stem lesions on nine cultivars grown in Rothamsted Research experimental plots.

Race[a]	Frequency (%)			
	Phoma leaf spots	Basal stem canker	Upper stem lesion	Mean
Av4-5-6-7-(8)	87.5	0.0	0.0	29.2
Av4-5-(6)-7	10.0	0.0	0.0	3.3
Av4-6-7-(8)	0.0	16.7	0.0	5.6
Av5-6-7-(8)	0.0	0.0	48.4	16.1
Av6-7-(8)	2.5	0.0	19.4	7.3
Av4-(5)-(6)-7	0.0	0.0	16.1	5.4
Av(6)-7-(8)	0.0	83.3	16.1	33.1
Number of isolates	40	24	31	31.7
Number of races	3	2	4	3
Margalef index	0.54	0.31	0.87	0.57

[a]Race nomenclature according to Balesdent *et al.* (2005). The numbers indicate the AvrLm loci for which the isolate is avirulent and has been characterised, and numbers in parentheses indicate Avr loci for which the isolate allele has not been characterised.

suggests considering stem as the best tissues to sample for re-survey of the frequency of avirulence alleles in field populations of *L. maculans* to update the previous survey by Stachowiak *et al.* (2006) in Europe.

These results showed that the avirulent allele of *AvrLm7* was predominant for all *L. maculans* isolates sampled from phoma leaf spots, upper stem lesions or basal stem cankers. The other avirulent alleles that were predominant were *AvrLm4*, *AvrLm5* (isolates from phoma leaf spots or upper stem lesions) and *AvrLm6* (isolates from phoma leaf spots, basal stem cankers or upper stem lesions). Therefore, cultivars with resistance genes *Rlm7* (cvs Roxet and Excel) and *Rlm4* (cv. Bilbao) still have effective resistance against *L. maculans*. Four avirulent alleles were observed to be at a high frequency in this study: *AvrLm4*, *AvrLm5*, *AvrLm6* and *AvrLm7*. Therefore the corresponding *R* genes could be good candidates for use in new cultivars to provide resistance against *L. maculans*. To be more effective, by adding QR into resistant cultivars with *R* genes, the *R* genes will be more durable (Brun *et al.,* 2010).

These results are consistent with the French and European surveys that showed that virulent alleles of *AvrLm2*, *AvrLm3* and *AvrLm9* (i.e. *avrLm2, avrLm3* and *avrLm9*) are fixed at 100% in the *L. maculans* populations in Europe (Balesdent *et al.,* 2006; Stachowiak *et al.,* 2006). Therefore, from these results it is evident that the avirulent alleles *AvrLm1*, *AvrLm2*, *AvrLm3* and *AvrLm9* were absent, indicating that cultivars relying only on *Rlm1*, *Rlm2*, *Rlm3* or *Rlm9* no longer have effective resistance against *L. maculans* in the UK. Previous studies stated that even though the cultivars with *R* genes *Rlm1* and *Rlm4* have been used commercially for more than 10 years, the avirulent alleles *AvrLm1* and *AvrLm4* are still present in the *L. maculans* populations (Huang *et al*., 2010). Avirulent *AvrLm4* alleles were observed in isolates

from phoma leaf spots (isolates from cvs Bilbao, Capitol, DK Cabernet, Es-Astrid, Drakkar, NK Grandia and Roxet), in isolates from basal stem cankers (isolates from cv. Bilbao) and in isolates from upper stem lesions (isolates from cv. Adriana) but no avirulent allele *AvrLm1* was observed in this study.

Single resistance genes do not always provide durable resistance, as has been shown in field experiments using the *Rlm6* gene introgressed into *B. napus* from *B. juncea* (Brun *et al.,* 2010). Several other cultivars have been reported to have ineffective race-specific resistance genes, for example Surpass 400 (*LepR3*), Capitol (*Rlm1*) and the *Rlm6* gene in *Brassica* cultivars when they were grown extensively (Sprague *et al.,* 2006a; Balesdent *et al.,* 2006; Rouxel *et al.,* 2003). The breakdown of resistance in *B. napus* cultivars containing single dominant *R* gene-based resistance confirms that there is a broad spectrum of variation in virulence within *L. maculans* populations, giving *L. maculans* the potential to rapidly adapt to challenges from a new resistance gene (Kuswinanti *et al.,* 1999; Li *et al.,* 2003; 2004; Rouxel *et al.,* 2003).

Therefore, there is a need to combine *R* gene resistance against *L. maculans* with QR in oilseed rape as a primary objective to reduce stem canker severity and substantially control pseudothecial development (less initial inoculum). In European countries, the *Rlm7* gene (in this study in cvs Excel and Roxet) has been widely deployed in new oilseed rape cultivars. It has been suggested that *Rlm7* is more durable than other *R* genes commercially available (Clarke, 2014). The European survey suggested that the *Rlm6* and *Rlm7* resistance genes were still effective sources of resistance against *L. maculans* (Baledent *et al.,* 2005; Stachowiak *et al.,* 2006). There was no virulence against *Rlm6* or *Rlm7* identified in this study but it has been reported that in French populations there are *L. maculans* isolates that are

virulent against *Rlm7* (Daverdin *et al.,* 2012). Thus, breeders need to be aware that continuous use of *Rlm7* is likely to select for isolates virulent against *Rlm7*. The presence of the avirulent *AvrLm5* allele was identified in isolates from phoma leaf spots and upper stems lesions. Until now, the *Rlm5* resistance gene has not been used in commercial cultivars (Stachowiak *et al*., 2006). However the presence of virulent alleles of *AvrLm5* may have occurred because of the widespread use of *B. juncea* (source of *Rlm5*). Thus, isolates with this virulent allele may have occured before the use of this resistance gene in commercial oilseed rape cultivars (Stachowiak *et al*., 2006). Balesdent *et al.* (2005) had reported that all European isolates did not carry the avirulent *AvrLm3* allele, *AvrLm9* allele or *AvrLm2* allele but did carry the avirulent *AvrLm6* allele and *AvrLm7* allele. In support of that statement, in this study all the isolates from different cultivars possessed the avirulent *AvrLm6* and *AvrLm7* alleles (Table 5.14).

Brun *et al.* (2010) demonstrated that a major *R*-gene (*Rlm6*) is more durable when expressed in a genetic background that also has quantitative resistance. The cultivar resistance is not so easily rendered ineffective by rapid changes in pathogen populations. *Leptosphaeria maculans* and *L. biglobosa* have a high evolutionary potential to adapt to resistance genes and population shifts associated with the sexual recombination of *L. maculans* are well known across the world (Chen and Fernando, 2005; Bradley *et al.,* 2005). Therefore, it is important to continuously monitor the frequency of avirulent and virulent *L. maculans* isolates in populations to predict and manage the risk of disease epidemics. In order to fully understand the nature of the *Brassica–Leptosphaeria* interaction, it is important to consider the genes that are currently involved in both qualitative and quantitative resistance against phoma stem canker pathogens (Hayward *et al.,* 2012).

Chapter 6
General discussion

Most research on phoma stem canker of winter oilseed rape has focused on *L. maculans*, which was considered to be the main cause of yield loss (West *et al.*, 2001, Fitt et *al.,* 2006a). Therefore, this study aimed to give more information on the response of different cultivars to *L. biglobosa* and *L. maculans* in three growing seasons (2010/2011, 2011/2012 and 2012/2013) (Chapter 3). In the UK and other countries that grow winter oilseed rape, growers need resistant cultivars to fully control phoma stem canker (Fitt *et al.,* 2006a). Durable crop resistance against pathogens is a priority for breeders with breeding and dissemination of resistant *B. napus* cultivars is considered to be the most efficient and economical methods of disease control (Marcroft, *et al.,* 2004a; McDonald, 2010; Canola Council, 2014). As a consequence, when one *R* gene has failed, breeders solution is to introduce a new *R* gene and this leads to a boom-and-bust cycle of disease (refer to 5.1). However, Brun *et al.,* (2010) demonstrated that a major *R*-gene (Rlm6) is more durable when expressed in a genetic background that also has quantitative resistance. Nevertheless, there is a lack of understanding of *R* gene and QR effects on the development of pseudothecia on stems of different cultivars with or without *R* genes and QR. Thus, the effects of cultivar resistance on maturation of pseudothecia of these *Leptosphaeria* species were observed (Chapter 4). *L. maculans* can evolve to overcome disease resistance conveyed by *R* genes bred into oilseed rape within three years of commercial release of a cultivar (Howlett *et al.,* 2015). In addition, the ability of *L. biglobosa* to respond to selection pressure is not well understood. Therefore, isolates of both pathogens were sampled from different sources (phoma leaf spots, upper stem lesions or stem cankers) to identify their phenotypic interaction with different cultivars in cotyledon tests (Chapter 5).

Objective 1: To investigate influence of cultivar resistance (*R*-gene/quantitative resistance) on the proportions of *L. maculans* and *L. biglobosa* at the phoma leaf spot and stem canker stages.

These results show that there was a difference in the ratio of *Leptosphaeria maculans* to *L. biglobosa* leaf spots on leaves between the nine winter oilseed rape cultivars in each of two growing seasons (2011/2012 and 2012/2013). Most cultivars with *R* genes against *L. maculans* (Bilbao, Capitol, Excel and Roxet) or with QR (NK Grandia and Es-Astrid) or with *R* genes combined with QR (Adriana and DK Cabernet) had more *L. biglobosa* leaf spots than cultivar Drakkar (refer section 3.3.1, Fig. 3.10 and Fig. 3.11). Cultivar Drakkar (susceptible to *L. maculans*) was significantly different from all other cultivars in every growing season (2010/2011, 2011/2012, 2012/2013) with more *L. maculans* leaf spots than other cultivars (refer to section 3.3.1). These results show that there was a greater proportion of *L. biglobosa* than *L. maculans* in the basal stem cankers and upper stem lesions in all growing seasons (refer section 3.3.2). The results differ from those of previous work, where West *et al.* (2002b) reported that there was more *L. maculans* than *L. biglobosa* in severe basal stem cankers. In particular in this work (refer section 3.3.1 & 3.3.3), there was more *L. biglobosa* in the basal stem cankers than previously reported. Severe damage in the upper stems, where more *L. biglobosa* was isolated in 2012 and 2013 suggests that *L. biglobosa* can cause severe epidemics (Fig. 3.14, Fig. 3.37a) (Huang *et al.,* 2014b).

Since *L. maculans* and *L. biglobosa* have previously been shown to coexist in the same diseased leaf or the same basal stem canker (Mahuku *et al.,* 1996; Fitt *et al.,* 2006b; Liu *et al.,* 2007; Stonard *et al.,* 2010), there may be competition between *L.*

maculans and *L. biglobosa*. Liu (2007) and Stonard *et al.* (2010) reported that there may be niche differences and competition for nutrients between the two species (Toscano-Underwood *et al.,* 2003) that gives *L. biglobosa* an advantage when *L. maculans* is controlled. For example, if *R* genes impede *L. maculans* by recognizing the effector product of the corresponding *AvrLm* gene, they may give an opportunity for *L. biglobosa* to colonise the tissues. While quantitative resistance (QR) normally provides partial resistance (Huang *et al.,* 2014a), it does not select strongly for particular pathotypes in the pathogen population and may also be resistant to *L. biglobosa* that contributes towards slow disease development characteristics (i.e. retards pseudothecial development of *L. biglobosa*). However, it is unlike qualitative resistance where diversity of resistance genes among cultivars enhances its effect.

A previous study showed that *L. biglobosa* grows much faster than *L. maculans* on nutrient agar (Huang *et al.,* 2001). There is little effect of triazole fungicides against *L. biglobosa* (Eckert, 2005), which may give *L. biglobosa* an additional competitive advantage when such fungicides are used against *L. maculans* (Stonard *et al*., 2010). There is evidence of population changes when the cultivars that are most resistant against *L. maculans* [Excel (*Rlm7*) and Roxet (*Rlm7*)] are grown since they then have the greatest proportion of *L. biglobosa* (Fig. 3.13 and Fig. 3.14). Cultivars Excel (*Rlm7*) and Roxet (*Rlm7*) have been bred against *L. maculans* and did not respond to *L. biglobosa*. This explains why there is a need for further investigation on *L. biglobosa* that may sometimes cause considerable yield losses in the UK (Huang *et al.,* 2014); *L. biglobosa* has the potential to increase in importance every season when these cultivars with *R* genes and fungicides are used to control *L. maculans*. In addition, it has been hypothesised that *L. maculans* and *L. biglobosa* are descended from a common ancestor and that *L. biglobosa* is the older of the two species (Gudelj

et al., 2004; Fitt *et al.,* 2006a, b; Fitt *et al.,* 2008). Evolutionary separation dates between *L. maculans* and *L. biglobosa* were estimated as 22 MYA (Grandaubert *et al.,* 2014).

There is evidence that infection by *L. biglobosa* may induce resistance against *L. maculans*. Both controlled environment and field experiments have shown that pre- or co-inoculation with *L. biglobosa* may cause induced resistance to subsequent infection by *L. maculans* (Liu *et al.,* 2006; Liu *et al.,* 2007). A decrease in size of *L. maculans* leaf lesions has also been observed in controlled environment experiments following inoculation or pre-treatment (24-48 hr before) with *L. biglobosa* before inoculation with *L. maculans* (Liu *et al.,* 2006). Inoculation with *L. biglobosa* onto the second true leaf was also shown to reduce of size *L. maculans* lesions on the same leaf (Liu *et al.,* 2006; Liu *et al.,* 2007; Mahuku *et al.,* 1996). Liu *et al.* (2006) reported that, under field conditions, pre-treatment with *L. biglobosa* in the October resulted in a decrease in incidence of phoma leaf spotting (December to March) and subsequent stem canker severity (June/July).

In addition, agricultural practices such as ploughing affect *Leptosphaeria* populations (Fitt *et al.,* 2006b; Schneider *et al*., 2006). Both *L. maculans* and *L. biglobosa* are able to survive on upper stem and basal stem debris on the soil surface. Previous work on buried stem debris showed that *L. maculans* can survive on the basal stem debris whereas *L. biglobosa* cannot survive so long on upper stem or basal stem debris (Huang *et al.,* 2003a), suggesting that ploughing will favour *L. maculans*. However, there are changes in agricultural practises including less ploughing and more direct drilling or non-inversion tillage that may directly influence evolution of populations (sexual recombination of virulence characters on stem debris and as the

source of primary inoculum). Therefore, when the proportion of *L. biglobosa* was greater in the three growing seasons (2010/2011, 2011/2012 and 2012/2013), there is a need to investigate survival of *L. biglobosa* on oilseed rape stems.

The results in chapter 3 (refer section 3.3.6) showed that there were effects of background quantitative resistance (QR) on the effectiveness of an *R* gene against *L. maculans*. Cultivars with *R* genes combined with QR, such as DK Cabernet (*Rlm1* + QR) and Adriana (*Rlm4* + QR), appeared to have less severe cankers than cultivars with the same *R* gene without the QR. Thus, the severity of stem canker on DK Cabernet (*Rlm1* + QR) was less than that on Capitol (*Rlm1*), whereas Adriana (*Rlm4* + QR) had less severe stem canker than Bilbao (*Rlm4*). These results suggest that the durability of cultivar *R* gene resistance against *L. maculans* may be enhanced when the gene is introduced into a host background with QR. In addition, cv. DK Cabernet (*Rlm1* + QR) has been the best yielding cultivar for two seasons (2009/2010 and 2010/2011) at a Nottinghamshire farm (Farmers weekly, 24 February 2012). This study showed the cultivars with *Rlm7* (Excel and Roxet) were effective for three growing seasons against *L. maculans* and suggests that by combining QR in resistant cultivars containing *Rlm7* may be effective to control *L. biglobosa*. Therefore, combining QR and *R* gene-mediated resistance can increase durability of that *R*-gene resistance (Sprague *et al.,* 2006b; Brun *et al.,* 2010; McDonald, 2010). *R* gene-mediated qualitative resistance is known to operate in cotyledons and leaves in autumn (Balesdent *et al.*, 2001), while quantitative resistance (QR) operates in the leaf stalk and stem tissues, in the period until harvest in summer (Pilet *et al.,* 1998, Delourme *et al.,* 2006, Huang *et al.,* 2009).

The availability of cultivars with *R* genes against *L. maculans* has given farmers a false sense of security and encouraged them to plant such cultivars more intensively. In the future, the management of phoma stem canker should rely on quantitative resistance (QR) that has proved to be more durable (Marcroft *et al.,* 2004b; Palloix *et al.,* 2009; Brun *et al.,* 2010). Brun *et al.* (2010) have shown that resistance conferred by *Rlm6* in an oilseed rape background that included quantitative resistance was still effective after three years, but isogenic lines with *Rlm6* that lacked quantitative resistance were severely diseased after only three years. However, those studies were focused on *L. maculans* and in this study *L. biglobosa* was observed on different cultivars with *R* genes or QR that showed cultivar QR can be effective against *L. biglobosa*. QR is more difficult to work with but the advantage is that QR is more durable to provide long-lasting disease resistance and normally only one gene is overcome at a time resulting in the variety having its resistance eroded rather that going from immune to very susceptible in one season. Thus ensuring that cultivars retain quantitative resistance should be an important aim of breeding programs and it should be combined with cultural practices (e.g. crop rotation and separation of new crops from previous crops) (Marcroft *et al.,* 2004b) for sustainable management of phoma stem canker.

This work identifies some important issues in relation to further understanding of phoma stem canker epidemics on winter oilseed rape. The biology of *L. biglobosa* and the response of cultivars to *L. biglobosa* and the role of QR all need to be better understood. It is also important to investigate the role of QR in giving resistance to *L. biglobosa*.

Objective 2: To determine effects of cultivar resistance on maturation of pseudothecia of these *Leptosphaeria* species (*L. maculans* and *L. biglobosa*) in natural and controlled environment conditions.

Pseudothecial development on stems of the susceptible cultivar Drakkar may have been much faster than that on other cultivars (refer section 4.3.1) because it developed more severe cankers. Previous studies showed that the more severe the canker, the faster the maturation of pseudothecia (West *et al.,* 2001). The production of more pseudothecia in cultivars increased the quantity of primary inoculum available (McGee, 1977; Petrie, 1995; Lô-Pelzer *et al.,* 2009a). The work reported in chapter 4 (refer section 4.3.1 & 4.3.2, Fig. 4.6) also suggests that resistance (*R*) genes and QR in a cultivar may delay pseudothecial development by slowing down the maturation of pseudothecia. This may have been related to differences between cultivars in severity of disease at harvest, since there is evidence that after harvest the number of pseudothecia increase and that they mature faster with increasing severity of disease at harvest (Lô-Pelzer *et al.,* 2009a). Marcroft *et al.* (2004a) also showed that cultivation of cultivars with *R* genes produced fewer pseudothecia than cultivation of susceptible cultivars. Therefore, if cultivars are grown that impede the ability of the pathogens (*L. maculans* and *L. biglobosa*) to complete sexual reproduction on stems so that they produce fewer pseudothecia, this may improve the control of stem canker by reducing the initial inoculum, especially in autumn.

Results of pseudothecial maturation and ascospore release work in natural conditions (refer section 4.3.3) suggest that weather factors (temperature and rainfall) affected the maturation of pseudothecia and the timing of the first major ascospore release. Autumn/winter 2011/2012 was unusual with several months of

dry weather before wet weather in December 2011, when there was rainfall with a temperature <10^{o}C until January 2012. Subsequently, ascospores were not released in large numbers until the third week of January 2012. However, the first ascospores were observed on 22 October 2011 and this may have been due to dew, as in the absence of rainfall, dew may stimulate release of ascospores (Huang *et al.,* 2005). In the 2012/2013 and 2013/2014 growing seasons, with more rainfall recorded than in 2011/2012, the first ascospores were released much earlier, from 30 September 2012 and from 29 September 2013, respectively. These results support previous work reporting that ascospore discharge (pseudothecial maturation) is influenced by weather conditions (temperature and rainfall) (Toscano-Underwood *et al.,* 2003; Khangura *et al.,* 2007; Salam *et al.,* 2007; Huang *et al.,* 2007).

The exact timing of ascospore release is important in predicting the optimum timing for fungicide application to control phoma leaf spot development which influences subsequent stem canker development (Eckert *et al.,* 2010; Stonard *et al.,* 2010; Huang *et al.*, 2011). The weather-based forecast model takes account only of conditions from August to mid-October (Evans *et al.,* 2008) to predict when 10% of plants in a crop will be affected by phoma leaf spotting. Therefore, the phoma leaf spot forecast did not work well in 2011/2012 when ascospores were released later (in January 2012), because there was a very dry autumn in 2011 and the phoma leaf spot forecast only uses data from August to October. Therefore, fungicide application to control phoma stem canker was not necessary. Only 2010/2011 and 2012/2013 data provided a reasonable fit with the existing model. When plants are small, the pathogen can quickly spread from the leaf petiole to reach the stem (West *et al.,* 2001; Aubertot *et al.,* 2006).

In the UK, fungicide usage against phoma stem cankers is decided in the autumn and application of fungicides is recommended when the leaves show phoma leaf spot symptoms (10% plants affected) (Gladders *et al.,* 1998; West *et al*., 2001). The spray regime depends on when the phoma leaf spotting begins and it is recommended to use a two-spray autumn fungicide regime if phoma leaf spotting occurs early in the autumn (before mid-October), while a single autumn spray is effective if phoma leaf spotting is late (appearing after mid-October) and no spraying needed if the epidemic starts after late November (http://croprotect.rothamsted.ac.uk/information/phoma-stem-canker-oilseed-rape).

However, fungicides have targeted only *L. maculans*. Studies have confirmed that *L. maculans* and *L. biglobosa* differ in sensitivity to specific fungicides (Kaczmarek *et al.,* 2009; Eckert *et al.,* 2010), where control of *L. biglobosa* requires higher doses of triazole fungicides than control of *L. maculans* (Kaczmarek & Jedryczka, 2011). This use of triazole fungicides against *L. maculans* will give a competitive advantage to *L. biglobosa* and an opportunity for *L. biglobosa* to colonise the crop. There is a possibility that effects of fungicide on relative amounts of *L. maculans* and *L. biglobosa* on stems from the previous season may contribute to pseudothecial development and to differences in production of ascospores on stem debris between cropping seasons (Huang *et al.,* 2010). Stonard *et al.* (2010) and Huang *et al.* (2011) have shown that the effects of fungicides on interactions between *L. maculans* and *L. biglobosa* in autumn play an important role in determining the effectiveness of autumn fungicide usage for decreasing severity of stem cankers in the following summer.

This work contrasts with previous work suggesting that *L. maculans* colonises within cortex, wood and pith tissues causing severe cankers in basal stems and that *L.*

biglobosa is usually in the cortex upper stem tissues causing less harm (West *et al.,* 2002b). The results in this study suggest that *L. biglobosa* can cause severe cankers in both basal stems and upper stems.

These results suggest that combining *R* gene-mediated resistance and quantitative resistance (QR) commercial oilseed rape cultivars can decrease inoculum (ascospores) and thus reduce the subsequent severity of phoma stem cankers. An example is the introduction of the cultivar DK Cabernet (*R* + QR), which has proven on farm performance and has been on the HGCA recommended list since 2011. Therefore, there is a need to further understand how the QR that can help to reduce initial inoculum by slowing down pseudothecial development on stems of winter oilseed rape.

Objective 3: To detect phenotypic response of different cultivars to *L. maculans* and *L. biglobosa* in cotyledons inoculated with isolates obtained from phoma leaf spots or stem cankers.

L. maculans isolates characterised for phenotype response on different cultivars indicated that the there were differences in phenotypic response on cotyledons between *L. maculans* isolates from different cultivars and from different sources (phoma leaf spots, upper stem lesions and basal stem cankers) (refer to 5.3.1). This result suggests that *L. maculans* isolates from basal stems were more virulent than those from upper stems. The aggressiveness of *L. biglobosa* isolates obtained from phoma leaf spots, upper stem lesions and basal stem cankers was tested on a differential set of cultivars. The results showed that some *L. biglobosa* isolates obtained from upper stem lesions (isolates 194-5P, 194-10P, EA11P, NKG 13P and

B6P-1) and basal stem cankers (isolates C6, C117, NKG 63, NKG 108 and C15-2) were more aggressive than *L. biglobosa* isolates obtained from phoma leaf spots (Table 5.11, 5.12, 5.13 and Fig. 5.14). These results indicate that breeding for resistance against *L. maculans* may affect the susceptibility of the cultivars to *L. biglobosa* when *R* genes only recognizing the effector product of the corresponding *AvrLm* gene and giving an opportunity for *L. biglobosa* to colonise the tissues.

The results showed that avirulent alleles of the effector gene *AvrLm7* were present in all *L. maculans* isolates obtained from phoma leaf spots, upper stem lesions and basal stem cankers (Fig. 5.17, Fig. 5.18 and Fig. 5.19). By contrast, some other avirulent alleles were at a low frequency, especially those related to effector genes *AvrLm4* and *AvrLm6* in *L. maculans* isolates obtained from basal stem cankers. However, avirulent alleles of effector genes *AvrLm4*, *AvrLm5* and *AvrLm6* were at high frequency (≥90%) in *L. maculans* isolates from phoma leaf spots (Fig. 5.17). This indicates that the corresponding *R* genes can be deployed effectively in cultivars. The winter oilseed rape cvs Excel and Roxet carrying *Rlm7* are still effective against *L. maculans*. Similarly, cultivars carrying *Rlm4*, *Rlm5* and *Rlm6* would have effective resistance due to the high frequency of the corresponding avirulent alleles of effector genes in the *L. maculans* populations. Other *R*-genes such as *Rlm1*, *Rlm2, Rlm3* and Rlm9, do not appear to be useful for control of *L. maculans.* Therefore, these *R*-genes would not be good candidates for breeding programmes.

However, it is possible that there is a fitness cost to the pathogen if it carries virulent alleles and that the frequency of the avirulent alleles could increase again over time in the absence of selection pressure imposed by *R* genes. This would result in the corresponding *R* genes again becoming effective against the pathogen. For example,

the virulent allele *avrLm4* has been shown to have a fitness cost in Europe and in the absence of cultivars with *Rlm4* the *L. maculans* population may change over time to carry a high frequency of the avirulent allele *AvrLm4* (Huang *et al.,* 2006a).

The resistance gene *Rlm7* is commonly used in European cultivars, especially in France; based on the survey results, the *Rlm7* resistance gene remains an effective source of resistance against *L. maculans* and is still effective after more than 7 years since its release (Balesdent *et al.,* 2005; Stachowiak *et al.,* 2006; Clarke, 2014). The frequency of the avirulent allele *AvrLm7* was high in this study, suggesting that the cultivars carrying *Rlm7* (Excel and Roxet) are still effective against *L. maculans*. However, intensive use of this gene in many cultivars grown under short rotations will probably result in it being rendered ineffective. Breakdown of oilseed rape resistance to *L. maculans*, resulting from changes in *L. maculans* populations, has been reported in Europe (Rouxel *et al.,* 2003). The effector gene *AvrLm7* is reported to be the same as *AvrLm4*, as this gene produces products recognized by both *Rlm4* and *Rlm7* (Parlange *et al.,* 2009).

Although the avirulent alleles of effector genes *AvrLm4*, *AvrLm5* and *AvrLm6* were observed at high frequencies, they were less than 100%, which suggests that reliance on cultivars with only *Rlm*4, *Rlm5* or *Rlm6* would probably be short-lived. This suggests that these *R* genes should be used strategically to maintain their usefulness (e.g. to deploy cultivars regionally according to their complement of resistance genes with differences between different regions) or in combination with quantitative sources of resistance. Under experimental conditions in France, a cultivar carrying *Rlm6* was grown repeatedly for 3 cropping season at a site inoculated with its own stubble; this resulted in the detection of isolates of *L.*

maculans that could overcome *Rlm6* (Brun *et al.,* 2000). Similarly, in Australia cultivars dependent on *LepR3* broke down very quickly (Li *et al.,* 2003). There is little knowledge about cultivar resistance to *L. biglobosa* and therefore there is a great need to understand the risk of severe phoma stem canker caused by *L. biglobosa* on winter oilseed rape in the UK.

Conclusions:

The recent focus on using *R* genes in breeding for resistance against *L. maculans* may have overlooked the impact of *L. biglobosa,* which may cause considerable damage, especially in cultivars with *R* genes that are effective against *L. maculans*. It is predicted that global warming will continue to increase the range and severity of phoma stem canker epidemics in the UK (Evans *et al.,* 2008). Furthermore, there are new threats as many of the most effective fungicides are no longer permitted by new EU legislation. This means there is a greater need to produce cultivars with effective resistance to control phoma stem cankers caused by *L. maculans* and *L. biglobosa*. Thus, it is crucial that the agricultural industry (farmers, advisors, breeders) understand how *R* gene/QR and fungicides affect *L. biglobosa* as well as *L. maculans* so that appropriate strategies are developed for management of phoma stem canker. Breeders need to consider *L. biglobosa* because no cultivars have been tested for resistance to *L. biglobosa* until now. Therefore, the breeding programmes need to change their screening programmes to include *L. biglobosa*. Further studies need to target control of *L. biglobosa* as well as *L. maculans.*

This study also suggests that both weather factors (temperature and rainfall) and host resistance affect the maturation of pseudothecia and the timing of the first major ascospore release. The differences between cultivars in pseudothecial maturation

have implications for the subsequent severity of stem canker. Therefore, it may be necessary to modify the current forecasting web-site system (http://www.rothamsted.ac.uk/Content-Section=Leafspot.html) to include a factor for cv. resistance.

Breeders may need to use other tests for resistance rather than just cotyledon tests. Growers are advised to use cultivars with combinations of *R* gene resistance against *L. maculans* and QR. In addition, there is relatively good quantitative resistance in many UK cultivars and by combining QR with *R* gene it will reduce the inoculum concentration and subsequently in summer less severe phoma stem cankers epidemics will develop.

Future work:

This work identifies some important issues in relation to further understanding of phoma stem canker epidemics on winter oilseed rape:

1. Optima conditions for infection and colonisation by *L. biglobosa* (temperature and different types of cultivar resistance against *L. maculans*).
2. The roles of QR in giving durable resistance against *L. maculans* for resistance such as *Rlm7*.
3. Need to have genes of QR gives resistance to *L. biglobosa* as well as *L. maculans*.
4. Need to investigate any *R* genes against *L. maculans* also give resistance against *L. biglobosa*.
5. Study if any *R* genes or QR give resistance against both *L. maculans* and *L. biglobosa*
6. Further understanding of the nature of the QR.

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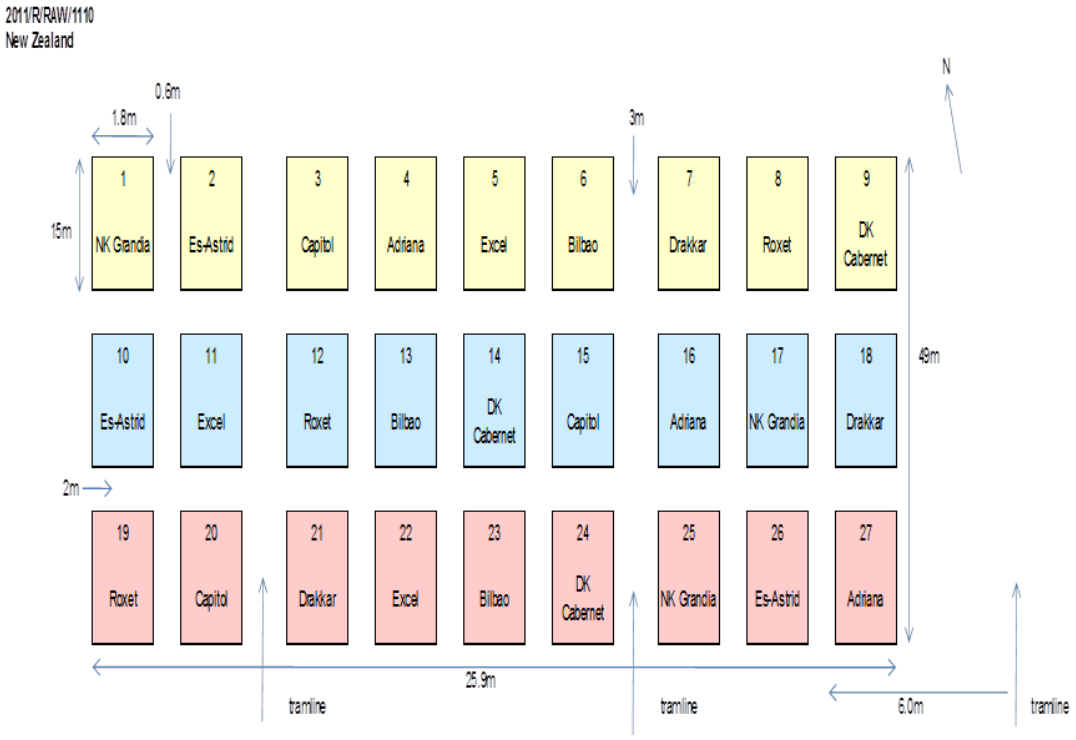
Appendices

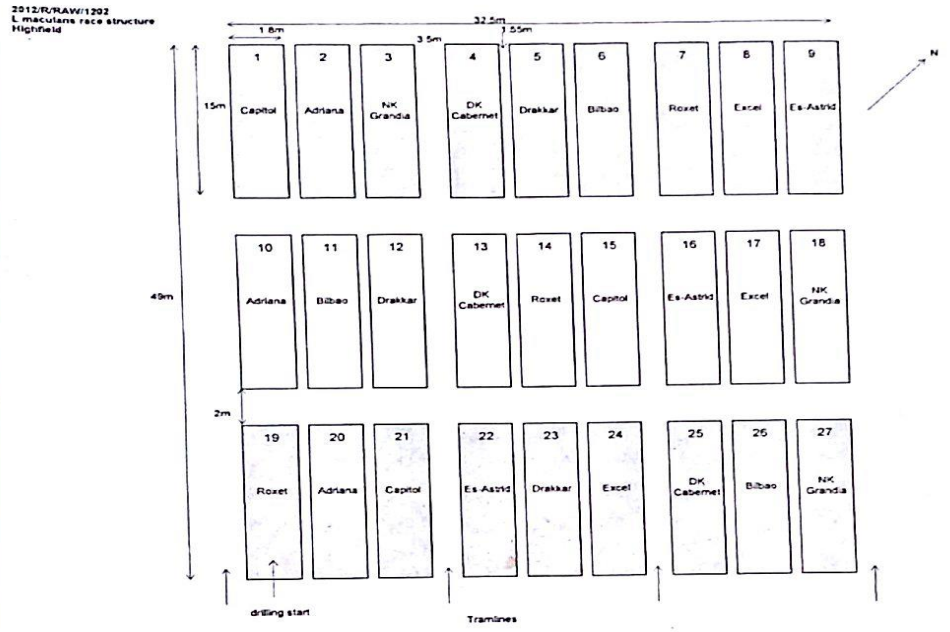
Appendix 2.1

Figure: The randomised block design of the winter oilseed rape experiments at Rothamsted Research, Harpenden for the (a) 2010/2011 (b) 2011/2012 growing seasons.

a



b



Appendix 3.1

ANOVA of numbers of leaves per plant, number of leaves per plant with *L. maculans* (Lm) spots and number of Lm phoma leaf spots on leaves from 10 plants per cultivar with 3 replicates of winter oilseed rape (with *R*-genes against *L. maculans* or no *R*-genes or with quantitative resistance (QR)) sampled from an experiment at Rothamsted on 9 December 2010 (2010/2011 growing season).

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Number of LM spots on leaves Mean±SD
Adriana	5.20±0.05^{a}	2.09±0.23^{a}	5.79±0.01^{bc}
Bilbao	4.27±0.04^{c}	2.09±0.00^{a}	8.21±0.79^{a}
Capitol	4.50±0.00^{bc}	2.23±0.11^{a}	4.50±0.20^{d}
DK Cabernet	4.49±0.00^{bc}	2.01±0.38^{a}	5.80±0.10^{bc}
Drakkar	4.69±0.24^{bc}	2.20±0.10^{a}	6.71±0.21^{b}
ES-Astrid	4.33±0.34^{c}	2.14±0.57^{a}	6.31±0.19^{b}
Excel	4.83±0.06^{ab}	1.88±0.32^{a}	4.91±0.42^{cd}
NK Grandia	4.88±0.06^{ab}	2.09±0.27^{a}	4.75±0.26^{d}
Roxet	4.67±0.15^{bc}	2.05±0.40^{a}	7.93±0.07^{a}

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

Appendix 3.2

ANOVA of number of leaves per plant, number of leaves per plant with *L. maculans* (Lm) spots and with *L. biglobosa* (Lb) spots, number of Lm and Lb phoma leaf spots on leaves from 30 plants per cultivar (10 plants per plot x three replicates) of winter oilseed rape sampled in the 2011/2012 growing season (25 January 2012) at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)).

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Leaves number per plant with LB spots Mean±SD	Number of LM spots on leaves Mean±SD	Number of LB spots on leaves Mean±SD
Adriana	9.93±0.89^{a}	1.50±0.70^{ab}	0.56±0.41^{b}	4.80±5.20^{b}	0.83±0.66^{b}
Bilbao	7.40±0.52^{bc}	1.30±0.26^{b}	1.03±0.20^{ab}	3.41±2.21^{b}	1.80±0.10^{ab}
Capitol	7.66±0.30^{bc}	1.30±0.26^{b}	1.36±0.25^{a}	3.30±2.60^{b}	2.80±1.05^{ab}
DK Cabernet	6.76±0.25^{c}	0.96±0.28^{b}	0.90±0.10^{ab}	4.24±3.96^{b}	2.00±1.13^{ab}
Drakkar	9.31±1.12^{ab}	3.18±0.48^{a}	1.20±0.20^{ab}	14.41±1.59^{a}	2.54±0.08^{ab}
ES-Astrid	8.23±0.55^{abc}	0.83±0.66^{b}	0.90±0.10^{ab}	2.80±3.90^{b}	1.60±0.40^{ab}
Excel	6.56±0.45^{c}	0.53±0.25^{b}	0.81±0.01^{ab}	0.79±0.64^{b}	1.48±0.80^{ab}
NK Grandia	8.86±1.22^{ab}	1.20±0.69^{b}	1.23±0.40^{ab}	2.37±1.95^{b}	2.53±1.72^{ab}
Roxet	7.93±0.37^{abc}	1.13±1.27^{b}	1.50±0.20^{a}	1.98±2.53^{b}	4.03±1.72^{a}

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

Appendix 3.3

ANOVA of number of leaves per plant, number of leaves per plant with *L. maculans* (Lm) spots and with *L. biglobosa* (Lb) spots, number of Lm and Lb phoma leaf spots on leaves from 30 plants per cultivar (10 plants per plot x three replicates) of winter oilseed rape sampled in the 2012/2013 growing season (24 Oct 2012, 21 Nov 2012, 11 Dec 2012 and 30 Jan 2013) at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without *R*-genes against *L. maculans* and with or without quantitative resistance (QR)).

24 Oct 2012

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Leaves number per plant with LB spots Mean±SD	Number of LM spots on leaves Mean±SD	Number of LB spots on leaves Mean±SD
Adriana	5.40±0.20^{ab}	0.60±0.20^{a}	0.86±0.61^{ab}	0.53±0.30^{a}	1.87±1.75^{ab}
Bilbao	5.33±0.30^{ab}	0.80±0.00^{a}	0.60±0.20^{ab}	1.26±0.30^{a}	1.00±0.20^{b}
Capitol	5.46±0.11^{ab}	0.46±0.30^{a}	0.33±0.30^{ab}	0.40±0.34^{a}	0.40±0.40^{b}
DK Cabernet	6.26± 0.11^{a}	0.60±0.40^{a}	0.53±0.30^{ab}	0.93±0.94^{a}	0.80±0.72^{b}
Drakkar	4.80±0.20^{b}	0.86±0.11^{a}	0.06±0.11^{b}	0.93±0.11^{a}	0.06±0.11^{b}
ES-Astrid	5.60±0.34^{ab}	0.80±0.34^{a}	0.26±0.23^{b}	0.73±0.23^{a}	0.33±0.30^{b}
Excel	6.26±0.80^{a}	1.00±0.20^{a}	0.40±0.00^{ab}	1.33±0.23^{a}	0.40±0.00^{b}
NK Grandia	6.06±0.80^{a}	0.40±0.00^{a}	0.40±0.02^{ab}	0.46±0.11^{a}	0.40±0.20^{b}
Roxet	5.26±0.11^{ab}	0.86±0.30^{a}	1.13±0.23^{a}	1.06±0.61^{a}	3.20±1.03^{a}

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

21 Nov 2012

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Leaves number per plant with LB spots Mean±SD	Number of LM spots on leaves Mean±SD	Number of LB spots on leaves Mean±SD
Adriana	6.63±0.32[a]	1.70±0.52[a]	1.60±0.85[a]	6.70±4.57[a]	10.50±13.99[a]
Bilbao	5.33±0.25[b]	1.63±0.35[a]	1.26±1.05[a]	7.87±4.68[a]	10.90±9.27[a]
Capitol	5.06±0.11[b]	1.46±0.32[a]	0.70±0.17[a]	4.06±1.25[a]	1.33±0.51[a]
DK Cabernet	5.56±0.15[ab]	1.16±0.15[a]	1.00±0.26[a]	4.50±0.70[a]	2.33±0.98[a]
Drakkar	5.06±0.28[b]	1.73±0.15[a]	1.13±0.77[a]	6.56±0.85[a]	2.73±2.59[a]
ES-Astrid	5.70±0.52[ab]	1.30±0.36[a]	1.36±0.35[a]	4.60±2.15[a]	4.23±1.97[a]
Excel	5.76±0.41[ab]	1.80±0.50[a]	1.56±0.37[a]	6.90±4.86[a]	13.93±10.08[a]
NK Grandia	6.56±0.56[a]	1.50±0.62[a]	1.06±0.15[a]	4.73±0.98[a]	3.10±0.45[a]
Roxet	5.66±0.58[ab]	1.66±0.58[a]	1.93±0.98[a]	7.17±3.97[a]	12.23±9.31[a]

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

11 Dec 2012

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Leaves number per plant with LB spots Mean±SD	Number of LM spots on leaves Mean±SD	Number of LB spots on leaves Mean±SD
Adriana	6.70±0.55[a]	1.83±0.28[a]	2.00±0.52[a]	7.33±2.14[a]	13.93±5.00[ab]
Bilbao	5.56±0.73[a]	1.56±0.20[a]	2.00±0.26[a]	5.90±2.54[a]	14.93±2.72[ab]
Capitol	5.93±0.60[a]	1.53±0.05[a]	1.86±0.40[a]	7.60±1.17[a]	10.47±2.68[ab]
DK Cabernet	6.20±0.60[a]	1.73±0.11[a]	2.03±0.45[a]	7.67±1.96[a]	11.00±1.30[ab]
Drakkar	5.26±0.50[a]	1.80±0.20[a]	1.16±0.47[a]	9.97±3.12[a]	6.03±4.28[b]
ES-Astrid	6.23±0.30[a]	1.70±0.26[a]	1.66±0.47[a]	8.93±0.35[a]	14.00±5.62[ab]
Excel	6.06±0.83[a]	1.50±0.34[a]	1.83±0.20[a]	6.30±3.62[a]	17.80±5.73[ab]
NK Grandia	6.50±0.55[a]	1.66±0.11[a]	1.96±0.37[a]	5.73±0.77[a]	11.20±0.62[ab]
Roxet	5.86±0.40[a]	1.70±0.36[a]	2.06±0.47[a]	6.80±0.75[a]	19.20±6.59[a]

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

30 Jan 2013

Cultivar	Leaves number per plant Mean±SD	Leaves number with LM spots per plant Mean±SD	Leaves number per plant with LB spots Mean±SD	Number of LM spots on leaves Mean±SD	Number of LB spots on leaves Mean±SD
Adriana	8.96±0.70[a]	1.33±0.20[a]	0.56±0.05[a]	5.86±0.77[a]	0.86±0.20[a]
Bilbao	7.33±0.66[ab]	1.66±0.77[a]	0.86±0.23[a]	8.23±3.14[a]	2.23±0.90[a]
Capitol	7.43±0.15[ab]	1.53±0.64[a]	0.56±0.25[a]	7.40±4.25[a]	1.50±1.21[a]
DK Cabernet	7.13±0.20[b]	1.53±0.05[a]	0.73±0.20[a]	6.90±0.40[a]	1.33±0.28[a]
Drakkar	7.20±0.43[b]	1.73±0.45[a]	0.33±0.32[a]	8.73±1.51[a]	0.76±1.06[a]
ES-Astrid	7.30±0.75[b]	1.46±0.25[a]	0.50±0.26[a]	7.86±0.80[a]	1.13±0.75[a]
Excel	7.90±0.87[ab]	1.43±0.15[a]	0.56±0.30[a]	6.20±1.60[a]	1.06±0.83[a]
NK Grandia	8.36±0.55[ab]	1.70±0.72[a]	0.53±0.20[a]	6.73±2.20[a]	1.36±0.55[a]
Roxet	7.53±0.32[ab]	2.10±0.36[a]	0.83±0.41[a]	7.56±0.25[a]	1.83±1.45[a]

Means within column that do not share a letter are significantly different according to Tukey's HSD test at P=0.05.
The mean difference is significant at the 0.05 level.

Appendix 3.4

ANOVA of Stem diameter (cm) of nine winter oilseed rape cultivars assessed in summer before harvest in each of the three growing seasons.

Cultivar	30 June 2011 Mean±SD	13 July 2012 Mean±SD	26 July 2013 Mean±SD
Adriana	0.95±0.28^{abc}	1.41±0.21^{a}	1.32±0.18^{a}
Bilbao	1.10±0.26^{ab}	1.38±0.21^{a}	1.35±0.31^{a}
Capitol	0.62±0.17^{c}	1.46±0.49^{a}	1.39±0.23^{a}
DK Cabernet	1.13±0.11^{a}	1.39±0.44^{a}	1.26±0.23^{a}
Drakkar	0.75±0.23^{bc}	0.78±0.27^{b}	1.12±0.17^{a}
ES-Astrid	0.85±0.20^{abc}	1.34±0.19^{a}	1.29±0.29^{a}
Excel	1.17±0.24^{a}	1.39±0.31^{a}	1.21±0.24^{a}
NK Grandia	1.03±0.37^{ab}	1.36±0.30^{a}	1.28±0.39^{a}
Roxet	1.08±0.37^{ab}	1.43±0.30^{a}	1.19±0.16^{a}

Means within column that do not share a letter are significantly different.
The mean difference is significant at the 0.05 level.

Appendix 3.5

ANOVA of stem canker severity score (0-6 scale) on basal part of the stem for three growing seasons observed in summer (30 June 2011, 13 July 2012 and 26 July 2013) at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without R-genes against *L. maculans* and with or without quantitative resistance (QR)).

Cultivar	30 June 2011 Mean±SD	13 July 2012 Mean±SD	26 July 2013 Mean±SD
Adriana	3.37±0.17^{bc}	0.76±0.20^{b}	3.05±0.28^{ab}
Bilbao	3.69±0.29^{b}	2.37±1.17^{b}	3.29±0.55^{ab}
Capitol	3.81±0.11^{b}	2.59±0.80^{b}	3.52±0.90^{ab}
DK Cabernet	2.41±0.11^{d}	0.74±0.38^{b}	2.37±0.87^{ab}
Drakkar	5.07±0.17^{a}	5.76±0.25^{a}	5.37±0.77^{a}
ES-Astrid	2.10±0.20^{de}	0.92±0.32^{b}	2.29±1.67^{ab}
Excel	3.17±0.17^{c}	1.23±1.20^{b}	2.02±1.50^{b}
NK Grandia	3.07±0.07^{c}	1.51±0.30^{b}	2.63±1.16^{ab}
Roxet	1.91±0.11^{e}	1.43±0.64^{b}	2.43±1.27^{ab}

Means within column that do not share a letter are significantly different.
The mean difference is significant at the 0.05 level.

Appendix 3.6

ANOVA of phoma stem canker severity score for upper part of the stem for two growing seasons observed in summer on samples collected on 13 July 2012 or 26 July 2013 at Rothamsted Research, Harpenden for nine winter oilseed rape cultivars (with or without R-genes against *L. maculans* and with or without quantitative resistance (QR)).

Cultivar	13 July 2012 Mean±SD	26 July 2013 Mean±SD
Adriana	0.48±0.37[b]	0.16±0.26[a]
Bilbao	2.59±1.64[ab]	0.14±0.22[a]
Capitol	1.56±0.65[b]	0.58±0.45[a]
DK Cabernet	0.42±0.42[b]	0.17±0.22[a]
Drakkar	5.18±0.48[a]	0.63±0.60[a]
ES-Astrid	0.91±0.06[b]	0.40±0.48[a]
Excel	1.98±1.57[b]	0.17±0.23[a]
NK Grandia	1.65±0.93[b]	0.29±0.25[a]
Roxet	1.78±0.67[b]	0.28±0.30[a]

Means within column that do not share a letter are significantly different.
The mean difference is significant at the 0.05 level

Appendix 3.7

The amounts of *L. maculans* and *L. biglobosa* DNA in basal stems and upper stems of winter oilseed rape from the 2011/2012, 2012/2013 and 2013/2014 growing seasons.

Growing season	Cultivar	Basal stems				Cultivar	Upper stems			
		qPCR results		Data were skewed and Log_{10}-transformed			qPCR results		Data were skewed and Log_{10}-transformed	
		L. maculans DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA		*L. maculans* DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA
2011/2012	Adriana	774.6	4733.24	2.89	8.46	Adriana	6469.2	133.4	8.77	4.89
		188.312	762.848	2.28	6.64		504.72	1392.36	6.22	7.24
		3.028	153.512	0.61	5.03		0.67408	3.464	-0.39	1.24
		0.892	12128	0.28	9.4		0.988	5666.4	-0.01	8.64
		0.736	887.96	0.24	6.79		0.152	5642.4	-1.88	8.64
		2.168	1310.4	0.5	7.18		0.28	233.748	-1.27	5.45
	Bilbao	251.04	4070	2.4	8.31	Bilbao	0.036	118.92		4.78
		230.16	7540	2.36	8.93		0.88	3.68	-0.13	1.3
		2008	32272	3.3	10.38		93.4	15.416	4.54	2.74
		11488	7142.8	4.06	8.87		13.252	42.324	2.58	3.75
		1232	569.84	3.09	6.35		9.46	628.04	2.25	6.44
		146.56	1042.4	2.17	6.95		0.208	602	-1.57	6.4
		0.024	62.8	0.01	4.14	Capitol	9000	169.484	9.1	5.13
		441.6	1938	2.65	7.57		18.56	3.928	2.92	1.37
		57.24	926.8	1.77	6.83		814.32	3726.8	6.7	8.22
		39.212	2583.6	1.6	7.86		257.68	2111.6	5.55	7.66
		152.52	7615.6	2.19	8.94		324.44	1178.48	5.78	7.07
	Capitol	265.2	20.372	2.43	3.01		0.124	5.304	-2.09	1.67
		8114.8	1751.8	3.91	7.47		2.516	183.48	0.92	5.21
		971.6	36.412	2.99	3.59	DK Cabernet	0.42752	263.2	-0.85	5.57
		56.74	334.76	1.76	5.81		0.16128	563.864	-1.82	6.33
		3877.6	1606.32	3.59	7.38	Drakkar	1972.8	325.48	7.59	5.79
		115.84	400.92	2.07	5.99		60.772	213.12	4.11	5.36
		3.28	42.624	0.63	3.75		527.8	433.12	6.27	6.07

		18.36	16.508	1.29	2.8		3062.4	4472	8.03	8.41
2011/2012	Capitol	655.88	41.176	2.82	3.72	Drakkar	21.336	17.928	3.06	2.89
		27.72	14.568	1.46	2.68		3214	6048.8	8.08	8.71
		9.728	30.4	1.03	3.41		1893.6	1792	7.55	7.49
		1.092	13.468	0.32	2.6		67.212	107.2	4.21	4.67
	DK Cabernet	390.24	13212	2.59	9.49	Es-Astrid	40.064	299.24	3.69	5.7
		204.8	2525.28	2.31	7.83	Excel	46.548	12556	3.84	9.44
		9066.4	1456.68	3.96	7.28	NK Grandia	370.4	2395.6	5.91	7.78
		903.44	495.28	2.96	6.21	Roxet	1.676	83.916	0.52	4.43
		425.08	379.28	2.63	5.94		0.188	1884.8	-1.67	7.54
		8862	10136.8	3.95	9.22		7.836	701.2	2.06	6.55
		921.52	5538.76	2.96	8.62		0.064	2.228		0.8
		10976	814.12	4.04	6.7		0.204	19.792	-1.59	2.99
	Drakkar	40.064	210.92	1.61	5.35		0.152	79200	-1.88	11.28
		4941.6	10646.4	3.69	9.27		0.0472	38.316		3.65
		10.872	5292.84	1.07	8.57					
		275.8	2010.08	2.44	7.61					
		49.74	118.08	1.71	4.77					
		299.24	3451.2	2.48	8.15					
		1869.6	20720	3.27	9.94					
		675.72	3091.2	2.83	8.04					
		319.32	6520	2.51	8.78					
		239.52	25.256	2.38	3.23					
		2314.8	4586.4	3.36	8.43					
		643.6	31.296	2.81	3.44					
		661.84	8122.4	2.82	9					
		13.292	33.236	1.16	3.5					
	Es-Astrid	1458.4	7358	3.16	8.9					
		2297.6	6922.4	3.36	8.84					
	Excel	216.508	42572	2.34	10.87					
	NK Grandia	7844.8	389.52	3.89	5.96					
	Roxet	5636.4	9303.24	3.75	9.14					
		93.92	25.784	1.98	3.25					
		68.128	1227.2	1.84	7.11					

		557.12	440.52	2.75	6.09					
2011/2012	Roxet	3.888	1967.2	0.69	7.58					
		6.04	5548.4	0.85	8.62					
		0.08	61.588	0.03	4.12					
		0.008	967.924	0.95	6.88					

Growing season	Cultivar	Basal stems				Cultivar	Upper stems			
		qPCR results		Data were skewed and Log_{10}-transformed			qPCR results		Data were skewed and Log_{10}-transformed	
		L. maculans DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA		*L. maculans* DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA
2012/2013	Adriana	5.02	38311	1.61	17.46	Adriana	31.696	23535.2	3.46	10.07
		563.864	189.12	6.33	5.24		2098.8	1157.6	7.65	7.05
		8.552	40560	2.15	12.91		839.852	45.216	6.73	3.81
		4.924	251.84	1.59	5.53		2.232	97.5	0.8	4.58
		1.036	207.92	0.04	5.34		0.5344	5867.6	-0.63	8.68
		0.0252	6.244	-3.68	1.83		0.2016	8772	-1.6	9.08
		0.908	15980	-0.1	9.68		329	519.72	5.8	6.25
		2980.4	5.976	8	1.79		0.0552	57.452		4.05
		1.712	35128	0.54	10.47		544.88	75.428	6.3	4.32
		0.992	17244	-0.01	9.76		0.00876	0.13352		-2.01
		0.06268	15.448	-2.77	2.74	Bilbao	245.64	12264	5.5	11.72
	Bilbao	1987.6	46660	7.59	15.36		1.66	31679	0.51	12.67
		523.36	6.488	6.26	1.87		10.9	15575.24	2.39	9.65
		10.412	4245.16	2.34	8.35		5.768	10601	1.75	13.87
		23.336	2394	3.15	7.78		252.56	32300	5.53	14.99
		2121.2	25.232	7.66	3.23		316.88	4762	5.76	8.47
		202	151.64	5.31	5.02		58.908	55592	4.08	13.23
		0.0568	117.68		4.77		17.188	23.736	2.84	3.17
		1.068	9368	0.07	9.15		85.96	49.644	4.45	3.9
		0.02716	115.32	-3.61	4.75		0.0488	460.04		6.13
		1603.6	73.124	7.38	4.29		291.04	3716.8	5.67	8.22

	Capitol	1338.4	12716	7.2	9.45	Capitol	1.896	124.36	0.64	4.82
2012/2013	Capitol	2.156	58748	0.77	10.98	Capitol	2.568	3946.4	0.94	8.28
		1652	9264	7.41	9.13		39.516	39952	3.68	10.6
		94.6	26672	4.55	10.19		0.02476	15.392		2.73
	DK Cabernet	3.612	12669	1.28	11.75		0.00064	163.28		5.1
		1906	24674	7.55	12.42	DK Cabernet	907.88	6295	6.81	11.05
		5118.4	5.91	8.54	20.2		0.16992	20708	-1.77	9.94
		14.808	13080	2.7	11.78		0.00464	0.09056	-5.37	2.2
		8.984	39608	2.2	10.59		0.078	29.132	-2.55	3.37
		0.00612	0.01744		0.56		9.916	6952	2.29	11.15
		0.652	19576	-0.43	9.88		0.7132	16744	-0.34	9.73
		1757.2	79200	7.47	11.28		0.0284	20.356		3.01
		757.44	79200	6.63	11.28	Drakkar	13.504	17.116	2.6	2.84
	Drakkar	5508.8	18.624	8.61	2.92		599.84	2849.16	6.4	7.95
		7291.6	71.008	8.89	4.26		630.6	14957	6.45	11.92
		2416.8	10.556	7.79	2.36		20.032	46868	3	10.76
		24.076	11004	3.18	9.31		7371.6	15808	8.91	9.67
		28.7	31368	3.36	12.66		5.492	18064	1.7	9.8
		1812.4	417.92	7.5	6.04		3844.8	469.04	8.25	6.15
		1744	15.224	7.46	2.72		0.5732	13.108	-0.56	2.57
		35.824	47.616	3.58	3.86		1083.04	19977.2	6.99	9.9
		5712.4	2018	8.65	7.61		0.658	7866	-0.42	11.27
	Es-Astrid	0.2948	29160	-1.22	10.28		3471.2	6987.6	8.15	8.85
		0.1452	21012	-1.93	9.95		2838.4	40972	7.95	10.62
		24.42	8.372	3.2	2.12	Es-Astrid	7.024	39106	1.95	10.35
		0.6004	64000	-0.51	11.07		0.2376	7920	-1.44	11.28
		0.7184	75824	-0.33	11.24		0.0916	2654		7.88
	Excel	5.396	19059	1.69	16.76		0.17	7920	-1.77	11.28
		3.132	5.39	1.14	20.1		6.548	3202.4	1.88	8.07
		2.368	9161	0.86	16.03	Excel	4.448	31968	1.49	10.37
		2.6	5524	0.96	13.22		7.416	33788	2	12.73
		4.8	24270.76	1.57	10.1		0.7664	8824	-0.27	9.09
		4.032	49800	1.39	15.42		0.2168	2663.6	-1.53	7.89
		11.956	15688	2.48	11.96		0.0836	17944		9.8

2012/2013		44.552	43982	3.8	15.3		0.404	27428	-0.91	10.22
		0.1212	5065.6	-2.11	8.53	Excel	0.06044	10088		9.22
		0.242	26684	-1.42	10.19		0.03032	3295.14		8.1
		0.0192	334.32		5.81		0.1	28.552	-2.3	3.35
		0.14372	29480	-1.94	10.29		0.1244	9.628	-2.08	2.26
		0.09028	5726		10.96	NK Grandia	5778.8	33900	8.66	12.73
		0.1432	7136.8	-1.94	8.87		7.32	4177.2	1.99	8.34
	Excel	0.094	6294	-2.36	8.75		1073.2	1.39512	6.98	0.33
	NK Grandia	4479.6	8009	8.41	13.59		198.76	16977	5.29	16.65
		9.96	61340	2.3	17.93		23.164	29033	3.14	12.58
		1124.8	5.70572	7.03	1.74		39.62	3186.4	3.68	8.07
		29.652	27584	3.39	17.13		28.84	12428.4	3.36	9.43
		743.86	12878	6.61	11.77		17.204	9568	2.85	9.17
		33.388	57348	3.51	13.26		0.0188	12416		9.43
		1100.2	389.52	7	5.96		11.124	16528	2.41	9.71
		0.0164	15548		9.65		5.18	4361.6	1.64	8.38
		1870.4	173.16	7.53	5.15		4.1	1591.2	1.41	7.37
		14.696	32148	2.69	10.38		25.076	144.84	3.22	4.98
		1.288	41776	0.25	10.64	Roxet	11.652	6105.56	2.46	8.72
		12.608	64.716	2.53	4.17		1770.4	34088	7.48	10.44
	Roxet	1.132	6265	0.12	11.05		0.1112	11076	-2.2	9.31
		14.316	5.224	2.66	1.65		0.242	4627.6	-1.42	8.44
		2.196	11967	0.79	11.69		0.27	14904	-1.31	9.61
		34.776	16788	3.55	12.03		0.1064	1042.636	-2.24	6.95
		8.584	834.36	2.15	6.73					
		0.2332	29208	-1.46	10.28					
		0.1636	37072	-1.81	10.52					
		0.3416	43264	-1.07	10.68					
		0.1724	7920	-1.76	11.28					

Growing season	Cultivar	Basal stems				Cultivar	Upper stems			
		qPCR results		Data were skewed and Log_{10}-transformed			qPCR results		Data were skewed and Log_{10}-transformed	
		L. maculans DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA		*L. maculans* DNA	*L. biglobosa* DNA	*L. maculans* DNA	*L. biglobosa* DNA
2013/2014	Adriana	1258.8	299680	7.14	12.61	Adriana	13580	121192	9.52	11.71
		6082	21.064	8.71	3.05		80.08	7.144	4.38	1.97
		10.248	1701.28	2.33	7.44		26.656	31156	3.28	10.35
		56.56	3471.6	4.04	8.15		2714.4	33096	7.91	10.41
	Bilbao	388.96	9958.4	5.96	9.21	Bilbao	1975.2	361880	7.59	12.8
		1241.92	52688	7.12	10.87		54.084	124458.4	3.99	11.73
		273.28	26655.6	5.61	10.19		10784	7.344	9.29	1.99
	Capitol	4327.6	11488	8.37	9.35	Capitol	1130.276	2449.92	7.03	7.8
		4947.6	8114.8	8.51	9		0.21272	207.92	-1.55	5.34
		19.92	971.6	2.99	6.88		1554.4	415.776	7.35	6.03
		2066.4	4245.16	7.63	8.35		10108	106440	9.22	11.58
		4811.2	8517.12	8.48	9.05	DK Cabernet	8656.8	53111	9.07	13.89
		1330.8	2098.8	7.19	7.65		1274.8	398520	7.15	12.9
	DK Cabernet	2275.2	723280	7.73	13.49		1498	119624.4	7.31	11.69
		1456.44	18.972	7.28	2.94	Drakkar	1648	72248.4	7.41	11.19
		5450	17728	8.6	9.78		304.44	1287200	5.72	14.07
		6233.6	54596	8.74	10.91		5405.6	2962.4	8.6	7.99
		9576	1554.88	9.17	7.35		8158.4	28744	9.01	10.27
	Drakkar	97.92	24724000	4.58	17.02		1627.607	8814.4	7.39	9.08
		5082.8	126656	8.53	11.75	Es-Astrid	235.56	728760	5.46	13.5
		7337.6	62492	8.9	11.04		1982	6817600	7.59	15.74
		11228	135880	9.33	11.82		2.4892	1032.8	0.91	6.94
	Es-Astrid	175.56	2.7	5.17	0.99	Excel	3440.4	22788.32	8.14	10.03
		3.716	96560	1.31	11.48		0.8557	868440	-0.16	13.67
2013/2014	Excel	1225.8	85.648	7.11	4.45		10044	68212	9.21	11.13
		313.36	5800	5.75	15.57		2157.2	17.032	7.68	2.84
		91.92	44936	4.52	10.71	NK Grandia	2.412	5465.144	0.88	8.61
		2650	44776	7.88	15.31		5996.8	598920	8.7	13.3

	Excel	10068	48204	9.22	15.39	NK Grandia	1269.2	83401.78	7.15	11.33
	NK Grandia	1409.6	8252	7.25	13.62		202.8	1571200	5.31	14.27
		3746.8	11408	8.23	11.64	Roxet	6.388	7253.12	1.85	8.89
	Roxet	73.328	19312	4.29	12.17		5767.2	737.216	8.66	6.6
		376.32	563.864	5.93	6.33		2.164	340.064	0.77	5.83
		142.96	204.8	4.96	5.32		6.404	7089.6	1.86	8.87
		149.2	4839.04	5.01	8.48					
		63.068	1433.6	4.14	7.27					
		54.852	2121.2	4	7.66					

Appendix 3.8

Relationships between severity of basal stem canker or upper stem lesion and amount of *L. maculans* (LM) DNA, *L. biglobosa* (LB) DNA and combined (LM and LB) in the 2010/2011, 2011/2012 and 2012/2013 growing seasons.

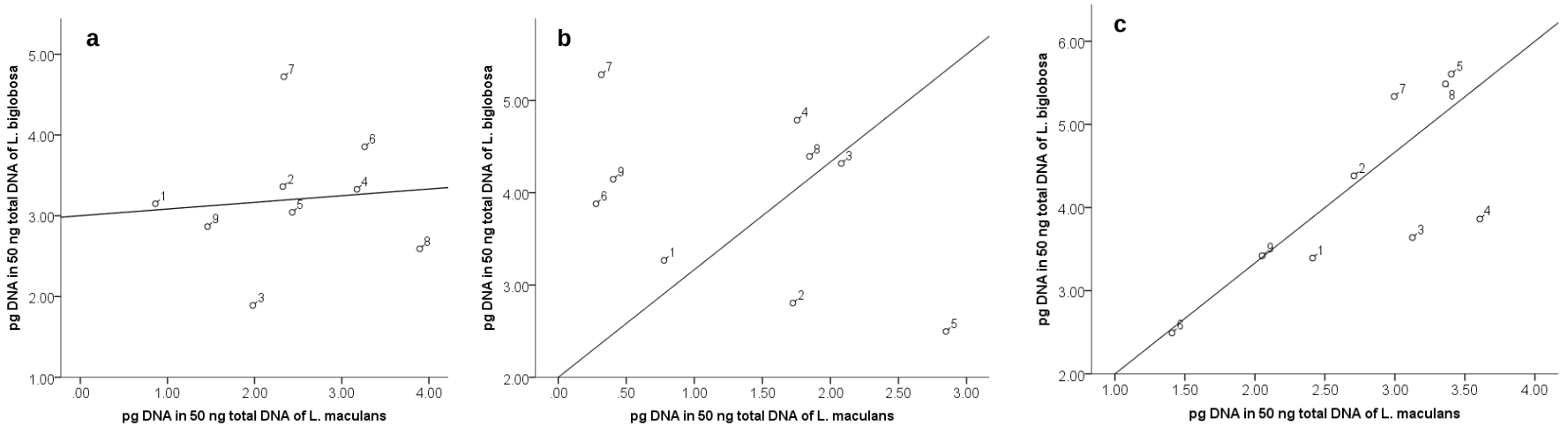
	Regression parameters			
Stem canker assessment	Stem canker with amount LM DNA	Stem canker with amount LB DNA	Stem canker with amount LM and LB DNA	Amount LM and LB DNA
2010/2011	r= -0.14 R^2= 0.02 *P*= 0.72	r= -0.22 R^2= 0.05 *P*= 0.53	r= -0.24 R^2= 0.06 *P*= 0.53	r= 0.98 R^2= 0.96 *P*= 0.80
2011/2012	r= 0.74 R^2= 0.55 *P*= 0.02	r= -0.63 R^2= 0.39 *P*= 0.71	r= 0.11 R^2= 0.01 *P*= 0.78	r= 0.43 R^2= 0.18 *P*= 0.25
2012/2013	r= 0.35 R^2= 0.12 *P*= 0.35	r= 0.37 R^2= 0.14 *P*= 0.32	r= 0.39 R^2= 0.15 *P*= 0.29	r= 0.73 R^2= 0.53 *P*= 0.03
Upper stem lesions	Upper stem lesions with amount LM DNA	Upper stem lesions with amount LB DNA	Upper stem lesions with amount LM and LB DNA	Amount LM and LB DNA
2010/2011	#	#	#	r= 0.43 R^2= 0.18 *P*= 0.24
2011/2012	r= 0.65 R^2= 0.42 *P*= 0.06	r= 0.37 R^2= 0.14 *P*= 0.32	r= 0.68 R^2= 0.46 *P*= 0.04	r= 0.16 R^2= 0.02 *P*= 0.69
2012/2013	r= 0.16 R^2= 0.03 *P*= 0.68	r= 0.01 R^2= 0.0001 *P*= 0.98	r= 0.09 R^2= 0.01 *P*= 0.81	r= 0.29 R^2= 0.08 *P*= 0.45

not assessed in the experiment

P= significance level and *r*= Pearson's r correlation test for relationship between basal stem canker or upper stem lesions and the amounts of *L. maculans* (LM) DNA, *L. biglobosa* (LB) DNA and combined (LM and LB)

Appendix 3.9

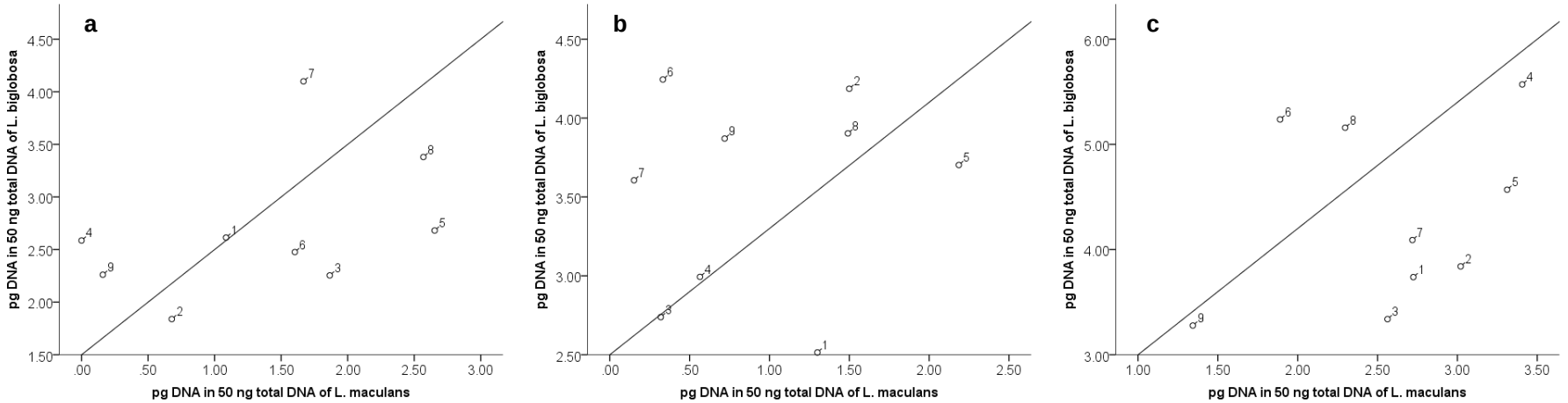
Relationships between the amount of *L. maculans* DNA and amount of *L. biglobosa* DNA in the (a) 2010/2011; y= 3 + 0.08x, (b) 2011/2012; y= 1.17x + 2 and (c) 2012/2013; y= 1.33x + 0.67 growing seasons from basal stem canker.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.10

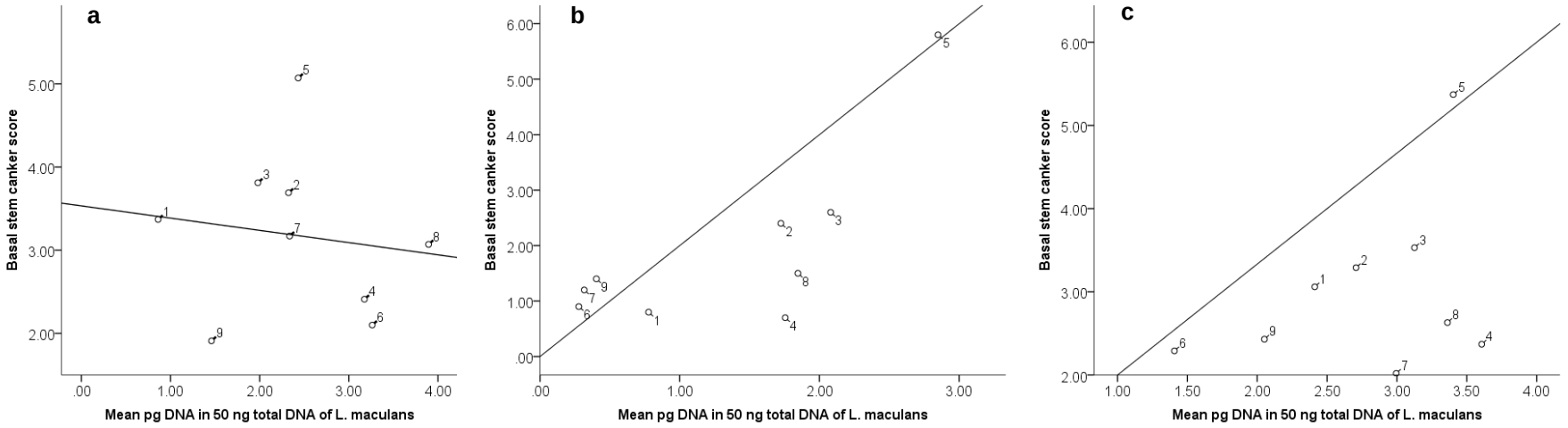
Relationships between the amount of *L. maculans* DNA and amount of *L. biglobosa* DNA in the (a) 2010/2011; y= 1x + 1.5, (b) 2011/2012; y= 0.8x + 2.5 and (c) 2012/2013; y= 1.2x + 1.8 growing seasons from upper stem lesions.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.11

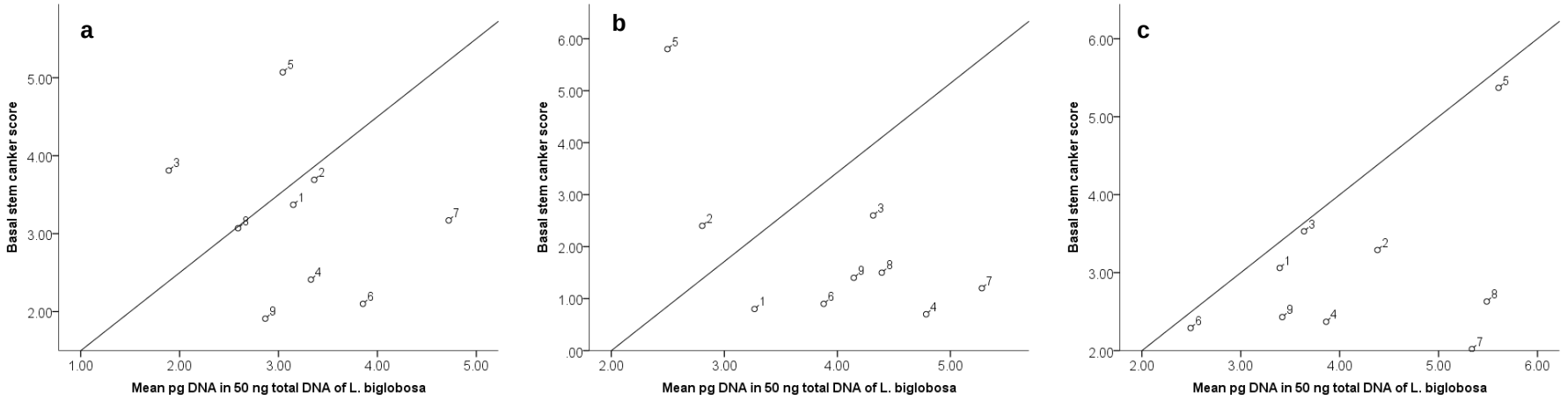
Relationships between severity of basal stem canker and amount of *L. maculans* DNA (pg DNA out of 50 ng of DNA) in the (a) 2010/2011; y= 3.53 – 0.15x, (b) 2011/2012; y= 0.5x + 0and (c) 2012/2013; y= 1.33x + 0.67 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.12

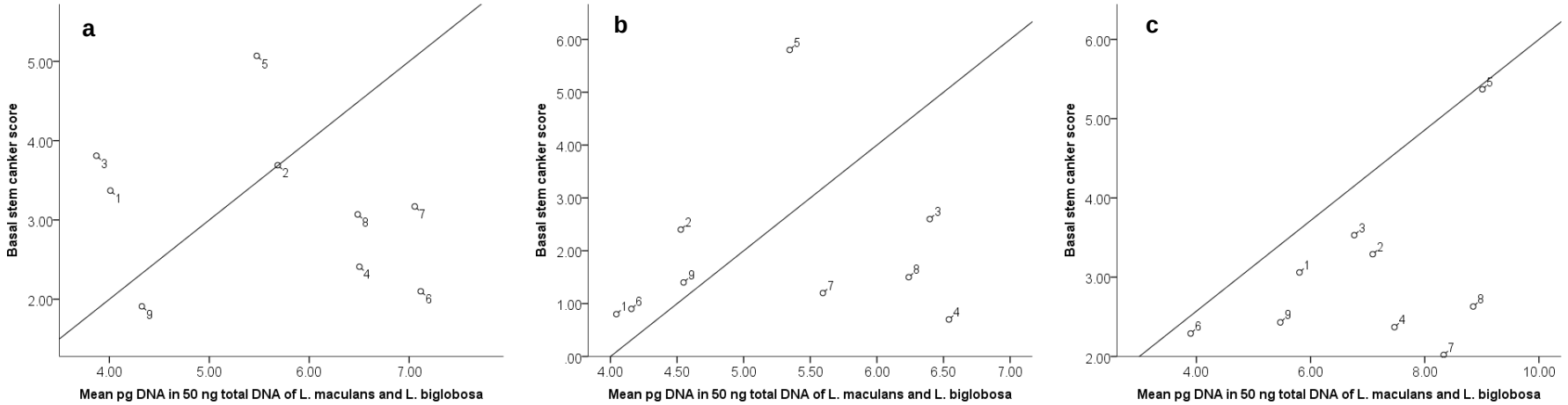
Relationships between severity of basal stem canker and amount of *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) in the (a) 2010/2011; y= y= 1x + 0.5, (b) 2011/2012; 1.71x + 3.43 and (c) 2012/2013; y= y= 1x + 0 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.13

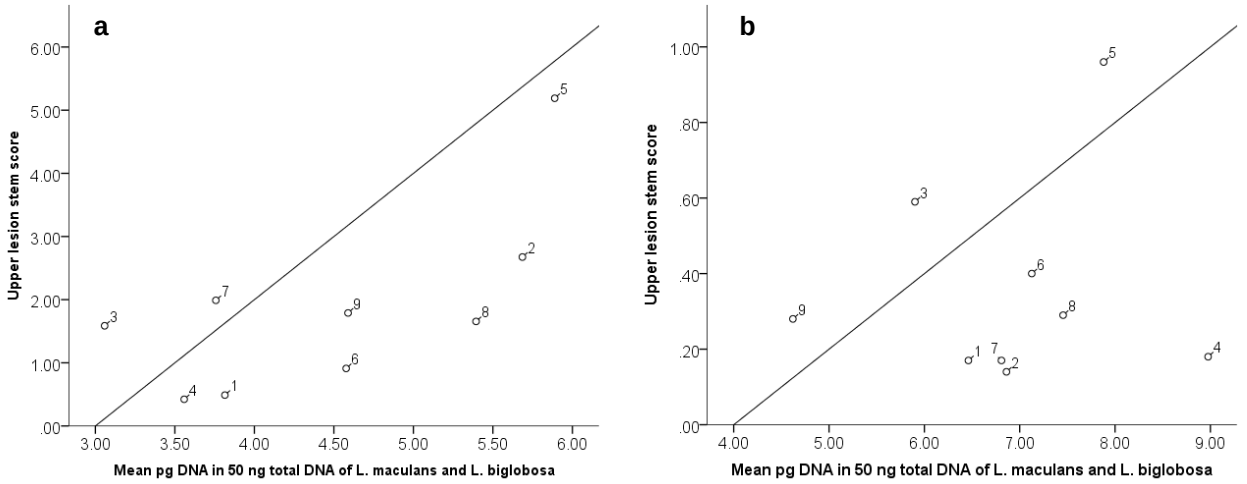
Relationships between severity of basal stem canker and the combined amount of *L. maculans* and *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) in the (a) 2010/2011; y= y= y= 1x + 2, (b) 2011/2012; y= 2x + 8 and (c) 2012/2013; y= 0.57x + 0.29 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.14

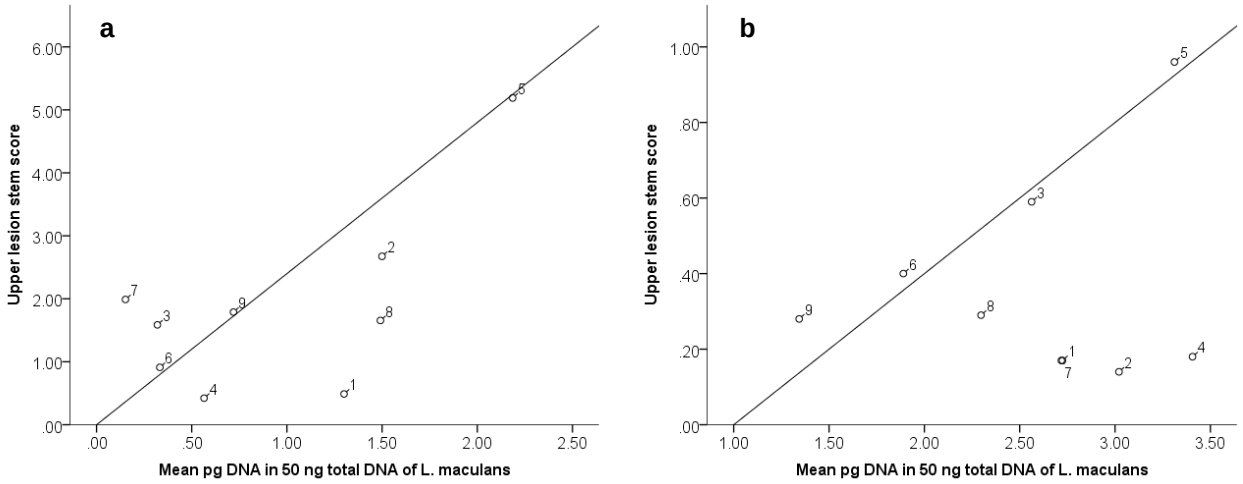
Relationships between severity of upper stem lesions and the amount of combined (*L. maculans* and *L. biglobosa*) DNA (pg DNA out of 50 ng of DNA) in the (a) 2011/2012; y= 2x + 6 and (b) 2012/2013; y= 0.2x + 0.8 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.15

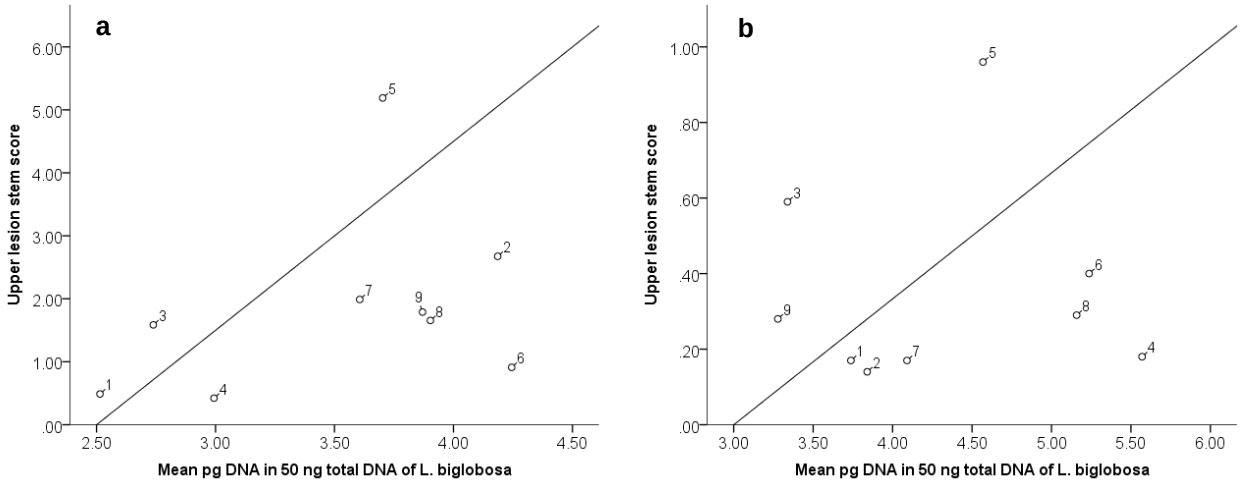
Relationships between severity of upper stem lesions and the amount of *L. maculans* DNA (pg DNA out of 50 ng of DNA) in the (a) 2011/2012; y= 2.4x + 0 and (b) 2012/2013; y= 0.4x + 0.4 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.16

Relationships between severity of upper stem lesions and the amount of *L. biglobosa* DNA (pg DNA out of 50 ng of DNA) in the (a) 2011/2012; y= 3x + 7.5 and (b) 2012/2013; y= 0.33x + 1 growing seasons.



1= Adriana (*Rlm 4* + QR), **2**= Bilbao (*Rlm 4*), **3**= Capitol (*Rlm 1*), **4**= DK Cabernet (*Rlm 1* + QR), **5**= Drakkar (no R gene), **6**= Es-Astrid (QR), **7**= Excel (*Rlm 7*), **8**= NK Grandia (QR), **9**= Roxet (*Rlm 7*)

Appendix 3.17

Principal components analysis 2010/2011

Latent roots

1	2	3
14.068	7.520	0.071

Percentage variation

1	2	3
64.95	34.72	0.33

Trace

21.66

Latent vectors (loadings)

	1	2	3
LM_spots	0.98864	0.15007	0.00808
Leaf_NoLM	0.00061	0.04976	-0.99876
Stemcanker	-0.15029	0.98742	0.04910

Principal component scores

	1	2	3
1	-0.3255	0.1448	0.0059
2	1.9992	0.8210	0.0410
3	-1.6768	0.3912	-0.1228
4	-0.1813	-0.8071	0.0387
5	0.3089	1.9645	-0.0232
6	0.3597	-1.0312	-0.1123
7	-1.1853	-0.1982	0.1986
8	-1.2690	-0.3015	-0.0169
9	1.9701	-0.9836	-0.0089

Principal components analysis 2011/2012

Latent roots

1	2	3	4
158.07	13.16	4.78	0.73

Percentage variation

1	2	3	4
89.33	7.44	2.70	0.41

Trace

177.0

Latent vectors (loadings)

	1	2	3	4
LBspot	0.01769	0.53223	0.78948	0.17548
LMspot	0.89118	-0.35955	0.19584	0.13071
LeafNoLB	0.01038	0.18091	0.20787	-0.17380
LeafNoLM	0.16744	0.01658	0.07271	-0.15456
Stemcanker	0.32773	0.46648	-0.22373	-0.74894
Upperlession	0.26445	0.58038	-0.48971	0.58061

Principal component scores

	1	2	3	4
1	-0.223	-2.327	-0.129	-0.067
2	-0.376	0.801	-0.981	-0.034
3	-0.669	0.896	0.366	-0.730
4	-0.877	-1.532	0.798	0.107
5	11.535	0.323	-0.079	0.103
6	-1.967	-0.861	-0.096	0.025
7	-3.413	0.560	-1.215	0.224
8	-1.829	0.567	0.101	0.032
9	-2.179	1.573	1.236	0.339

Principal components analysis 2012/2013

Latent roots

1	2	3	4
140.68	10.41	3.34	0.21

Percentage variation

1	2	3	4
90.89	6.73	2.16	0.14

Trace

154.8

Latent vectors (loadings)

	1	2	3	4
LBspot	-0.95527	0.25900	0.14262	-0.00098
LMspot	0.21590	0.93375	-0.24739	0.12117
LeafNoLB	-0.04317	-0.12559	-0.07746	0.74186
LeafNoLM	0.00957	0.02798	0.00068	0.34707
Stemcanker	0.19075	0.18269	0.94970	0.15035
Upperlession	0.05010	0.10531	0.10263	-0.54027

Principal component scores

	1	2	3	4
1	-0.707	0.124	0.185	0.251
2	-1.933	-0.903	0.854	0.095
3	2.704	-0.357	0.054	-0.044
4	2.015	-0.384	-0.992	0.162
5	7.936	1.204	0.719	-0.094
6	-0.587	1.554	-0.913	-0.041
7	-4.852	0.016	-0.041	-0.292
8	1.436	-2.153	-0.269	-0.104
9	-6.011	0.897	0.402	0.065

Appendix 4.1

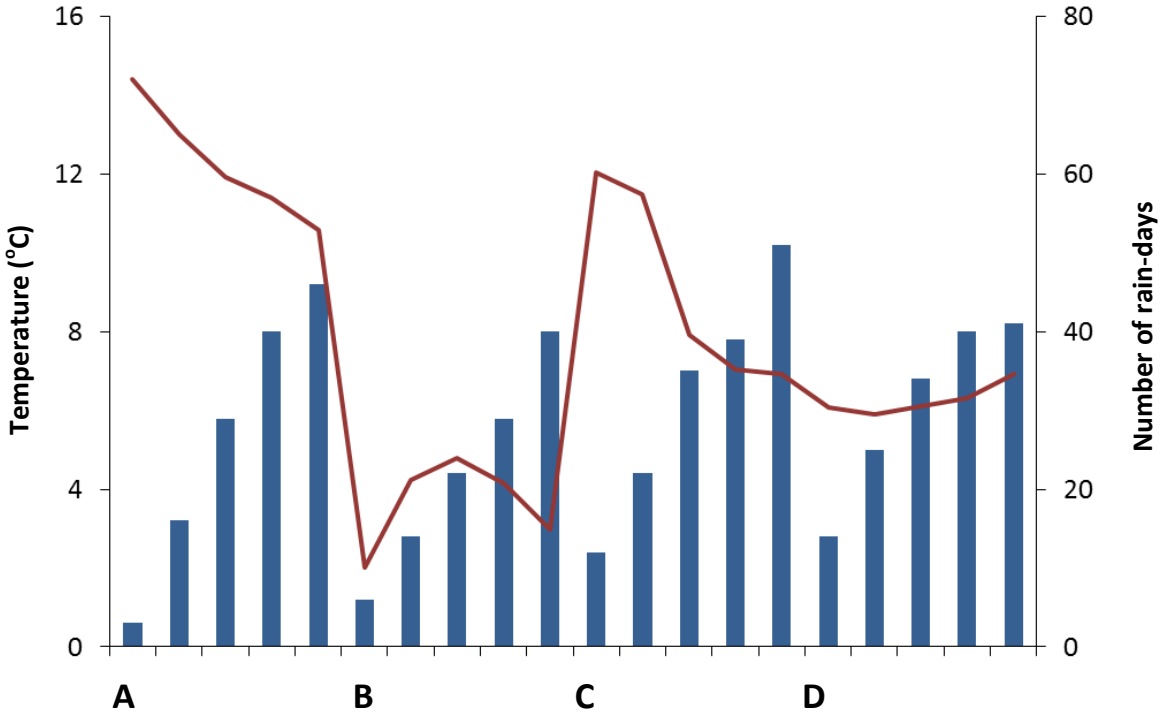


Fig.: Average temperature (°C) and number of rain-days for every two weeks from the day when the stem base debris was put outside until 70 days later in autumn/winter 2012/2013 (from 5 September 2012 to 14 November 2012 and from 28 November 2012 to 6 February 2013), in autumn/winter 2013/2014 (from 9 October 2013 to 18 December 2013 and from 8 January 2014 to 19 March 2014). The total rainfall for 70 days was 74.94 mm, 146.96 mm, 216.67 mm and 251.60 mm for A (from 5 September 2012 to 14 November 2012), B (from 28 November 2012 to 6 February 2013), C (from 9 October 2013 to 18 December 2013) and D (8 January 2014 to 19 March 2014), respectively. Weather conditions for both stem base cankers and upper stem lesions that were exposed in natural conditions in autumn/winter 2012/2013 (A and B) and 2013/2014 (C and D).

Appendix 4.2

Weather conditions (average temperature, rain-days and total rainfall) from the first day that stem bases of nine cultivars of winter oilseed rape (with or without *R* genes) were exposed to natural conditions at Bayfordbury until 50% of pseudothecia were mature (Class D) observed from a weekly sample of 25 pseudothecia per cultivar in autumn 2011, 2012 and 2013.

Cultivar (*R* gene)	Pseudothecial development (Class D) on stem base				
	Days to reach 50% Class D	Date 50% pseudothecia mature (Class D)	[a]Temperature (^{o}C)	[b]Rain days	[c]Total rain (mm)
2011/2012 (Day 1, stem base exposed to natural conditions: 20 Sept 2011)					
Adriana (Rlm4 + QR)	25	14 Oct 2011	15.2	25	11.64
Bilbao (Rlm4)	33	23 Oct 2011	13.5	31	14.68
Capitol (Rlm1)	39	28 Oct 2011	13.2	45	22.79
DK Cabernet (Rlm1 + QR)	38	28 Oct 2011	13.2	45	22.79
Drakkar (No R gene)	22	12 Oct 2011	15.6	23	10.88
ES Astrid (QR)	41	31 Oct 2011	13.3	45	22.79
Excel (Rlm7)	39	29 Oct 2011	13.2	45	22.79
NK Grandia (QR)	37	27 Oct 2011	13.3	45	22.79
Roxet (Rlm7)	33	23 Oct 2011	13.5	31	14.68
2011/2012 (Day 1, stem base exposed to natural conditions: 5 Sept 2012)					
Adriana (Rlm4 + QR)	25	30 Sept 2012	13.0	26	18.99
Bilbao (Rlm4)	25	30 Sept 2012	13.0	26	18.99
Capitol (Rlm1)	37	12 Oct 2012	12.3	57	37.49
DK Cabernet (Rlm1 + QR)	33	8 Oct 2012	12.5	53	35.21
Drakkar (No R gene)	15	20 Sept 2012	14.2	3	2.28
ES Astrid (QR)	31	6 Oct 2012	12.8	49	33.95
Excel (Rlm7)	31	6 Oct 2012	12.8	49	33.95
NK Grandia (QR)	26	1 Oct 2012	13.1	32	22.30
Roxet (Rlm7)	28	3 Oct 2012	13.0	36	25.09
2012/2013 (Day 1, stem base exposed to natural conditions: 18/9/2013)					
Adriana (Rlm4 + QR)	18	6 Oct 2013	14.0	15	5.29
Bilbao (Rlm4)	15	3 Oct 2013	13.9	12	3.77
Capitol (Rlm1)	22	10 Oct 2013	13.7	15	5.29
DK Cabernet (Rlm1 + QR)	27	15 Oct 2013	13.1	45	28.85
Drakkar (No R gene)	12	29 Oct 2013	13.7	6	2.01
ES Astrid (QR)	20	8 Oct 2013	14.0	15	5.29
Excel (Rlm7)	20	8 Oct 2013	14.0	15	5.29
NK Grandia (QR)	19	7 Oct 2013	13.9	15	5.29
Roxet (Rlm7)	20	8 Oct 2013	14.0	15	5.29

[a]Average temperature (^{o}C) from the day when the stem bases were put outside to the day when the 50% of pseudothecia were mature (Class D).

[b]Number of days with rainfall >0.2mm from the day when the stem bases were put outside to the day when the 50% of pseudothecia were mature (Class D).

[c]Total rainfall (mm) from the day when the stem bases were put outside to the day when 50% of pseudothecia were mature (Class D).

Appendix 4.3

Weather conditions (average temperature, rain-days and total rainfall) from the first day that upper stems of nine cultivars of winter oilseed rape (with or without *R* genes) were exposed to natural conditions at Bayfordbury until 50% of pseudothecia were mature (Class D) observed from a weekly sample of 25 pseudothecia per cultivar in autumn 2011, 2012 and 2013.

Cultivar	Pseudothecial development (Class D) on upper stem				
	Days to reach 50% Class D	Date 50% pseudothecia mature (Class D)	[a]Temperature (^{o}C)	[b]Rain days	[c]Total of rain (mm)
2011/2012 (Day 1 stem base exposed to natural conditions: 20 Sept 2011)					
Adriana (Rlm4 + QR)	39	29 Oct 2011	13.2	45	22.79
Bilbao (Rlm4)	39	29 Oct 2011	13.2	45	22.79
Capitol (Rlm1)	41	30 Oct 2011	13.3	45	22.79
DK Cabernet (Rlm1 + QR)	48	7 Nov 2011	13.1	63	35.47
Drakkar (No R gene)	33	23 Oct 2011	13.5	31	14.68
ES Astrid (QR)	53	12 Nov 2011	12.8	70	37.48
Excel (Rlm7)	51	9 Nov 2011	12.9	68	36.98
NK Grandia (QR)	57	15 Nov 2011	12.7	70	37.48
Roxet (Rlm7)	47	6 Nov 2011	13.1	62	35.22
2011/2012 (Day 1 stem base exposed to natural conditions: 5 Sept 2012)					
Adriana (Rlm4 + QR)	43	18 Oct 2012	12.0	75	51.18
Bilbao (Rlm4)	39	13 Oct 2012	12.2	61	42.32
Capitol (Rlm1)	44	19 Oct 2012	11.9	76	51.43
DK Cabernet (Rlm1 + QR)	40	15 Oct 2012	11.9	63	42.82
Drakkar (No R gene)	27	1 Oct 2012	13.1	32	22.30
ES Astrid (QR)	40	14 Oct 2012	12.0	62	42.57
Excel (Rlm7)	53	27 Oct 2012	11.7	90	56.47
NK Grandia (QR)	39	13 Oct 2012	12.2	61	42.32
Roxet (Rlm7)	47	21 Oct 2012	11.9	82	54.42
2012/2013 (Day 1 stem base exposed to natural conditions: 18 Sept 2013)					
Adriana (Rlm4 + QR)	33	21 Oct 2013	13.1	67	60.06
Bilbao (Rlm4)	28	16 Oct 2013	13.0	51	34.69
Capitol (Rlm1)	43	31 Oct 2013	12.9	105	102.42
DK Cabernet (Rlm1 + QR)	40	28 Oct 2013	13.2	101	100.4
Drakkar (No R gene)	29	16 Oct 2013	13.0	51	34.69
ES Astrid (QR)	34	22 Oct 2013	13.2	72	65.12
Excel (Rlm7)	36	24 Oct 2013	13.1	77	71.72
NK Grandia (QR)	39	26 Oct 2013	13.2	81	73.74
Roxet (Rlm7)	41	28 Oct 2013	13.2	101	100.4

[a]Average temperature (^{o}C) from the day when the upper stems were put outside to the day when the 50% of pseudothecia were mature (Class D).

[b]Number of days with rainfall >0.2mm from the day when the upper stems were put outside to the day when the 50% of pseudothecia were mature (Class D).

[c]Total rainfall (mm) from the day when the upper stems were put outside to the day when 50% of pseudothecia were mature (Class D).

Appendix 4.4

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem bases (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 5 September 2012 until 14 November 2012 (70 days).

Stem base						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	5	10	8	2	0
	28	2	7	6	10	0
	42	0	2	6	17	0
	56	0	0	0	20	5
	70	0	0	0	15	10
Bilbao (*Rlm4*)	14	3	5	11	6	0
	28	0	2	5	15	0
	42	0	0	0	23	2
	56	0	0	0	15	10
	70	0	0	0	9	16
Drakkar (No R gene)	14	0	6	11	8	0
	28	0	0	6	19	0
	42	0	0	0	22	3
	56	0	0	0	12	13
	70	0	0	0	4	21
NK Grandia (QR)	14	12	6	4	3	0
	28	4	8	6	7	0
	42	0	4	8	13	0
	56	0	1	3	19	2
	70	0	0	1	12	12

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.5

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem bases (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 28 November 2012 until 6 February 2013 (70 days).

Stem base						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	15	7	3	0	0
	28	6	10	9	0	0
	42	3	8	11	3	0
	56	0	4	13	8	0
	70	0	0	14	11	0
Bilbao (*Rlm4*)	14	13	9	3	0	0
	28	7	11	7	0	0
	42	0	6	15	4	0
	56	0	2	12	11	0
	70	0	0	9	16	0
Drakkar (No R gene)	14	8	12	5	0	0
	28	2	6	13	4	0
	42	0	3	13	9	0
	56	0	0	12	13	0
	70	0	0	0	19	6
NK Grandia (QR)	14	16	7	2	0	0
	28	8	10	7	0	0
	42	0	13	10	2	0
	56	0	5	14	6	0
	70	0	0	13	12	0

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.6

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem bases (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 9 October 2013 until 18 December 2013 (70 days).

Stem base						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (Rlm4 + QR)	14	7	8	7	3	0
	28	2	4	5	14	0
	42	0	0	8	17	0
	56	0	0	0	20	5
	70	0	0	0	13	12
Bilbao (Rlm4)	14	4	6	7	8	0
	28	0	2	3	18	2
	42	0	0	0	21	4
	56	0	0	0	15	10
	70	0	0	0	9	16
Drakkar (No R gene)	14	0	2	13	10	0
	28	0	0	0	24	1
	42	0	0	0	16	9
	56	0	0	0	10	15
	70	0	0	0	3	22
NK Grandia (QR)	14	5	9	6	5	0
	28	0	4	6	15	0
	42	0	0	7	18	0
	56	0	0	0	22	3
	70	0	0	0	15	10

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.7

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem bases (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 8 January 2014 until 19 March 2014 (70 days).

Stem base						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	16	4	5	0	0
	28	4	8	10	3	0
	42	0	6	11	8	0
	56	0	1	13	11	0
	70	0	0	8	16	1
Bilbao (*Rlm4*)	14	12	9	4	0	0
	28	3	7	10	5	0
	42	0	3	11	11	0
	56	0	0	8	17	0
	70	0	0	2	21	2
Drakkar (No R gene)	14	10	7	8	0	0
	28	2	9	6	8	0
	42	0	2	10	13	0
	56	0	0	6	18	1
	70	0	0	0	22	3
NK Grandia (QR)	14	17	4	4	0	0
	28	6	11	6	2	0
	42	0	8	10	7	0
	56	0	0	15	10	0
	70	0	0	6	18	1

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.8

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem bases (cvs Adriana, Bilbao, Drakkar and NK Grandia) in controlled environment conditions (20^{o}C, 75-80% RH, with continuous wetness, rain water pH 5.7) observed every two weeks until 70 days.

Stem base						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	4	7	8	6	0
	28	0	3	10	12	0
	42	0	0	6	18	1
	56	0	0	0	23	2
	70	0	0	0	6	19
Bilbao (*Rlm4*)	14	5	3	9	8	0
	28	0	0	5	18	2
	42	0	0	0	22	3
	56	0	0	0	11	14
	70	0	0	0	2	23
Drakkar (No R gene)	14	1	3	9	12	0
	28	0	0	0	21	4
	42	0	0	0	14	11
	56	0	0	0	7	18
	70	0	0	0	0	25
NK Grandia (QR)	14	4	7	6	8	0
	28	0	3	9	13	0
	42	0	0	4	19	2
	56	0	0	0	20	5
	70	0	0	0	8	17

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). Data are the average from two experiments.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.9

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 5 September 2012 until 14 November 2012 (70 days).

Upper stem						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	11	6	8	0	0
	28	2	9	12	2	0
	42	0	4	8	13	0
	56	0	0	0	20	5
	70	0	0	0	15	10
Bilbao (*Rlm4*)	14	14	4	6	1	0
	28	1	8	9	7	0
	42	0	2	7	16	0
	56	0	0	0	22	3
	70	0	0	0	9	16
Drakkar (No R gene)	14	5	9	7	4	0
	28	0	4	9	12	0
	42	0	0	0	21	4
	56	0	0	0	13	12
	70	0	0	0	4	21
NK Grandia (QR)	14	14	4	7	0	0
	28	4	6	11	4	0
	42	0	1	12	12	0
	56	0	0	0	20	5
	70	0	0	0	13	12

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled upper stem (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.10

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 28 November 2012 until 6 February 2013 (70 days).

Upper stem						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	18	6	1	0	0
	28	8	11	6	0	0
	42	3	7	14	1	0
	56	0	3	14	8	0
	70	0	0	15	10	0
Bilbao (*Rlm4*)	14	14	6	5	0	0
	28	7	4	14	0	0
	42	0	6	15	4	0
	56	0	0	13	12	0
	70	0	0	9	16	0
Drakkar (No R gene)	14	8	12	5	0	0
	28	1	9	12	3	0
	42	0	1	18	6	0
	56	0	0	11	14	0
	70	0	0	0	18	7
NK Grandia (QR)	14	19	4	2	0	0
	28	11	8	8	0	0
	42	6	10	9	0	0
	56	0	6	10	9	0
	70	0	4	9	12	0

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled upper stem (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.11

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 9 October 2013 until 18 December 2013 (70 days).

Upper stem						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	10	4	9	2	0
	28	2	6	6	11	0
	42	0	1	8	16	0
	56	0	0	0	19	6
	70	0	0	0	14	11
Bilbao (*Rlm4*)	14	4	7	8	6	0
	28	0	4	5	16	0
	42	0	0	2	20	3
	56	0	0	0	15	10
	70	0	0	0	10	15
Drakkar (No R gene)	14	4	6	7	8	0
	28	0	2	4	19	0
	42	0	0	0	22	3
	56	0	0	0	11	14
	70	0	0	0	6	19
NK Grandia (QR)	14	13	7	3	2	0
	28	3	4	5	13	0
	42	0	0	10	15	0
	56	0	0	0	21	4
	70	0	0	0	16	9

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled upper stem (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.12

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem (cvs Adriana, Bilbao, Drakkar and NK Grandia) exposed to natural conditions observed every two weeks from 8 January 2014 until 19 March 2014 (70 days).

Upper stem						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	16	7	2	0	0
	28	7	10	8	0	0
	42	0	5	16	4	0
	56	0	0	8	12	0
	70	0	0	0	19	6
Bilbao (*Rlm4*)	14	8	10	7	0	0
	28	2	7	16	0	0
	42	0	2	15	8	0
	56	0	0	8	17	0
	70	0	0	0	21	4
Drakkar (No R gene)	14	7	8	10	0	0
	28	0	5	16	4	0
	42	0	0	14	11	0
	56	0	0	0	20	5
	70	0	0	0	13	12
NK Grandia (QR)	14	16	6	3	0	0
	28	9	10	6	0	0
	42	2	6	12	5	0
	56	0	2	10	13	0
	70	0	0	0	20	5

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled upper stem (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.13

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem (cvs Adriana, Bilbao, Drakkar and NK Grandia) in controlled environment conditions (20^{o}C, 75-80% RH, with continuous wetness, rain water pH 5.7) observed every two weeks until 70 days.

Upper stem						
Cultivar	Days of incubation	Pseudothecial development				
		Class A	Class B	Class C	Class D	Class E
Adriana (*Rlm4* + QR)	14	5	9	6	5	0
	28	0	3	12	10	0
	42	0	0	11	14	0
	56	0	0	0	21	4
	70	0	0	0	13	12
Bilbao (*Rlm4*)	14	4	6	7	8	0
	28	0	3	8	14	0
	42	0	0	4	19	2
	56	0	0	0	21	4
	70	0	0	0	8	17
Drakkar (No R gene)	14	2	4	8	11	0
	28	0	0	4	19	2
	42	0	0	0	21	4
	56	0	0	0	16	9
	70	0	0	0	4	21
NK Grandia (QR)	14	9	4	6	6	0
	28	0	8	7	10	0
	42	0	2	10	13	0
	56	0	0	0	20	5
	70	0	0	0	10	15

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled upper stem (i.e. 25 pseudothecia were observed at each assessment). Data are the average from two experiments.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

Appendix 4.14

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Adriana (*Rlm4* + QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	22	3	0	0	0
27-Sep	9	15	1	0	0
4-Oct	6	9	0	10	0
11-Oct	2	6	2	15	0
18-Oct	3	0	6	16	0
25-Oct	0	0	4	20	1
1-Nov	0	0	0	21	4
8-Nov	0	0	0	17	8
15-Nov	0	0	0	13	12
22-Nov	0	0	0	11	14
29-Nov	0	0	0	8	17
6-Dec	0	0	0	6	19
13-Dec	0	0	0	5	20
20-Dec	0	0	0	4	21

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5-Sep	18	3	4	0	0
12-Sep	8	6	8	3	0
19-Sep	3	8	3	11	0
26-Sep	0	6	6	13	0
3-Oct	0	0	6	17	2
10-Oct	0	0	3	19	3
17-Oct	0	0	0	23	2
24-Oct	0	0	0	11	14
31-Oct	0	0	0	5	20
7-Nov	0	0	0	2	23
14-Nov	0	0	0	0	0
21-Nov	0	0	0	0	0
28-Nov	0	0	0	0	0

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18-Sep	15	5	5	0	0
25-Sep	6	2	8	9	0
2-Oct	1	3	6	15	0
9-Oct	0	0	5	20	0
16-Oct	0	0	0	24	1
23-Oct	0	0	0	17	8
30-Oct	0	0	0	14	11
6-Nov	0	0	0	6	19
13-Nov	0	0	0	1	24
20-Nov	0	0	0	0	0
27-Nov	0	0	0	0	0
4-Dec	0	0	0	0	0
11-Dec	0	0	0	0	0

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.15

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Bilbao (*Rlm4*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	21	4	0	0	0
27-Sep	13	7	4	1	0
4-Oct	17	8	0	0	0
11-Oct	1	6	10	8	0
18-Oct	1	2	1	14	7
25-Oct	0	0	0	14	11
1-Nov	0	0	0	13	12
8-Nov	0	0	0	10	15
15-Nov	0	0	0	7	18
22-Nov	0	0	0	4	21
29-Nov	0	0	0	2	23
6-Dec	0	0	0	2	23
13-Dec	0	0	0	0	25
20-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	11	10	4	0	0
12-Sep	4	12	5	4	0
19-Sep	0	3	13	9	0
26-Sep	0	0	9	15	1
3-Oct	0	0	3	18	4
10-Oct	0	0	0	22	3
17-Oct	0	0	0	19	6
24-Oct	0	0	0	15	10
31-Oct	0	0	0	9	16
7-Nov	0	0	0	2	23
14-Nov	0	0	0	1	24
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	5	8	10	2	0
25-Sep	0	3	10	12	0
2-Oct	0	0	5	19	1
9-Oct	0	0	0	24	1
16-Oct	0	0	0	14	11
23-Oct	0	0	0	11	14
30-Oct	0	0	0	6	19
6-Nov	0	0	0	3	22
13-Nov	0	0	0	2	23
20-Nov	0	0	0	0	25
27-Nov	0	0	0	0	25
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.16

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Capitol (*Rlm1*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	16	1	0	1	7
27-Sep	23	2	0	0	0
4-Oct	11	5	5	4	0
11-Oct	0	3	8	11	3
18-Oct	9	1	1	11	3
25-Oct	0	0	4	14	7
1-Nov	0	0	0	15	10
8-Nov	0	0	0	13	12
15-Nov	0	0	0	11	14
22-Nov	0	0	0	9	16
29-Nov	0	0	0	5	20
6-Dec	0	0	0	2	23
13-Dec	0	0	0	2	23
20-Dec	0	0	0	0	25

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	9	6	10	0	0
12-Sep	3	4	18	0	0
19-Sep	0	1	19	5	0
26-Sep	0	0	16	9	0
3-Oct	0	0	14	11	0
10-Oct	0	0	9	16	0
17-Oct	0	0	4	21	0
24-Oct	0	0	0	25	0
31-Oct	0	0	0	18	7
7-Nov	0	0	0	8	17
14-Nov	0	0	0	2	23
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	6	8	11	0	0
25-Sep	1	3	14	7	0
2-Oct	0	0	13	12	0
9-Oct	0	0	10	15	0
16-Oct	0	0	6	19	0
23-Oct	0	0	0	25	0
30-Oct	0	0	0	16	9
6-Nov	0	0	0	11	14
13-Nov	0	0	0	8	17
20-Nov	0	0	0	4	21
27-Nov	0	0	0	2	23
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.17

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar DK Cabernet (*Rlm1* + QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	23	2	0	0	0
27-Sep	16	9	0	0	0
4-Oct	19	4	2	0	0
11-Oct	12	6	4	3	0
18-Oct	0	7	9	9	0
25-Oct	0	0	5	18	2
1-Nov	0	0	0	22	3
8-Nov	0	0	0	17	8
15-Nov	0	0	0	17	8
22-Nov	0	0	0	16	9
29-Nov	0	0	0	14	11
6-Dec	0	0	0	12	13
13-Dec	0	0	0	11	14
20-Dec	0	0	0	8	17

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	19	6	0	0	0
12-Sep	16	8	1	0	0
19-Sep	12	4	4	5	0
26-Sep	4	2	10	9	0
3-Oct	0	0	14	11	0
10-Oct	0	0	9	13	3
17-Oct	0	0	6	17	2
24-Oct	0	0	4	18	3
31-Oct	0	0	0	21	4
7-Nov	0	0	0	13	12
14-Nov	0	0	0	8	17
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	20	5	0	0	0
25-Sep	13	8	4	0	0
2-Oct	9	4	3	9	0
9-Oct	1	4	7	13	0
16-Oct	0	0	8	17	0
23-Oct	0	0	5	19	1
30-Oct	0	0	0	23	2
6-Nov	0	0	4	12	9
13-Nov	0	0	0	7	18
20-Nov	0	0	0	3	22
27-Nov	0	0	0	0	25
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).
[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.18

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Drakkar (susceptible and no *R* gene) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	10	14	1	0	0
27-Sep	8	8	5	2	2
4-Oct	3	3	7	12	0
11-Oct	1	3	6	15	0
18-Oct	1	0	1	19	4
25-Oct	0	0	0	15	10
1-Nov	0	0	0	9	16
8-Nov	0	0	0	6	19
15-Nov	0	0	0	5	20
22-Nov	0	0	0	3	22
29-Nov	0	0	0	1	24
6-Dec	0	0	0	3	22
13-Dec	0	0	0	1	24
20-Dec	0	0	0	0	25

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	4	6	9	6	0
12-Sep	1	2	12	10	0
19-Sep	0	0	13	12	0
26-Sep	0	0	10	15	0
3-Oct	0	0	4	19	2
10-Oct	0	0	0	19	6
17-Oct	0	0	0	22	3
24-Oct	0	0	0	23	2
31-Oct	0	0	0	11	14
7-Nov	0	0	0	10	15
14-Nov	0	0	0	3	22
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	2	3	17	3	0
25-Sep	0	0	7	17	1
2-Oct	0	0	3	20	2
9-Oct	0	0	0	22	3
16-Oct	0	0	0	20	5
23-Oct	0	0	0	15	10
30-Oct	0	0	0	8	17
6-Nov	0	0	0	4	21
13-Nov	0	0	0	2	23
20-Nov	0	0	0	0	25
27-Nov	0	0	0	0	25
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.19

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Es-Astrid (QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	22	3	0	0	0
27-Sep	16	9	0	0	0
4-Oct	6	11	8	0	0
11-Oct	0	13	10	2	0
18-Oct	0	6	9	10	0
25-Oct	0	3	9	13	0
1-Nov	0	2	8	15	0
8-Nov	0	0	7	17	1
15-Nov	0	0	4	19	2
22-Nov	0	0	2	21	2
29-Nov	0	0	0	24	1
6-Dec	0	0	0	17	8
13-Dec	0	0	0	11	14
20-Dec	0	0	0	3	22

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	17	5	3	0	0
12-Sep	13	7	5	0	0
19-Sep	2	5	7	11	0
26-Sep	0	3	4	18	0
3-Oct	0	1	2	22	0
10-Oct	0	0	3	22	0
17-Oct	0	0	0	25	0
24-Oct	0	0	0	19	6
31-Oct	0	0	0	17	8
7-Nov	0	0	0	13	12
14-Nov	0	0	0	9	16
21-Nov	0	0	0	5	20
28-Nov	0	0	0	3	22

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).
[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	19	3	3	0	0
25-Sep	10	5	3	7	0
2-Oct	4	3	4	14	0
9-Oct	0	1	9	15	0
16-Oct	0	0	6	19	0
23-Oct	0	0	3	22	0
30-Oct	0	0	0	25	0
6-Nov	0	0	0	17	8
13-Nov	0	0	0	12	13
20-Nov	0	0	0	8	17
27-Nov	0	0	0	6	19
4-Dec	0	0	0	2	23
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).
[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.20

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Excel (*Rlm7*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	17	8	0	0	0
27-Sep	14	11	0	0	0
4-Oct	15	5	4	1	0
11-Oct	0	7	14	4	0
18-Oct	0	0	14	8	0
25-Oct	0	0	9	16	0
1-Nov	0	0	8	17	0
8-Nov	0	0	5	20	0
15-Nov	0	0	0	25	0
22-Nov	0	0	0	17	8
29-Nov	0	0	0	13	12
6-Dec	0	0	0	11	14
13-Dec	0	0	0	6	19
20-Dec	0	0	0	0	25

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	18	7	0	0	0
12-Sep	11	5	0	9	0
19-Sep	7	1	2	15	0
26-Sep	0	0	6	19	0
3-Oct	0	0	4	21	0
10-Oct	0	0	1	24	0
17-Oct	0	0	0	25	0
24-Oct	0	0	0	18	7
31-Oct	0	0	0	7	18
7-Nov	0	0	0	3	22
14-Nov	0	0	0	0	25
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	17	7	1	0	0
25-Sep	11	6	0	8	0
2-Oct	4	2	6	13	0
9-Oct	0	0	6	19	0
16-Oct	0	0	4	21	0
23-Oct	0	0	0	25	0
30-Oct	0	0	0	15	10
6-Nov	0	0	0	9	16
13-Nov	0	0	0	5	20
20-Nov	0	0	0	4	21
27-Nov	0	0	0	0	25
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.21

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar NK Grandia (QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	19	6	0	0	0
27-Sep	11	9	5	0	0
4-Oct	6	9	4	6	0
11-Oct	0	4	10	11	0
18-Oct	0	0	5	19	1
25-Oct	0	0	0	23	2
1-Nov	0	0	0	19	6
8-Nov	0	0	0	18	7
15-Nov	0	0	0	15	10
22-Nov	0	0	0	11	14
29-Nov	0	0	0	7	18
6-Dec	0	0	0	5	20
13-Dec	0	0	0	0	25
20-Dec	0	0	0	0	25

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	20	5	0	0	0
12-Sep	16	3	0	6	0
19-Sep	11	4	2	8	0
26-Sep	4	3	4	14	0
3-Oct	0	1	2	22	0
10-Oct	0	0	0	25	0
17-Oct	0	0	0	16	9
24-Oct	0	0	0	13	12
31-Oct	0	0	0	7	18
7-Nov	0	0	0	3	22
14-Nov	0	0	0	1	24
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	19	6	0	0	0
25-Sep	14	5	0	6	0
2-Oct	7	4	6	8	0
9-Oct	2	2	7	14	0
16-Oct	0	1	3	22	0
23-Oct	0	0	0	25	0
30-Oct	0	0	0	16	9
6-Nov	0	0	0	13	12
13-Nov	0	0	0	7	18
20-Nov	0	0	0	3	22
27-Nov	0	0	0	1	24
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment).

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.22

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape stem base of cultivar Roxet (*Rlm7*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Stem base (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
20-Sep	17	8	0	0	0
27-Sep	12	11	0	2	0
4-Oct	9	3	4	9	0
11-Oct	0	1	13	11	0
18-Oct	0	4	8	13	0
25-Oct	0	2	8	15	0
1-Nov	0	0	8	17	0
8-Nov	0	0	6	19	0
15-Nov	0	0	3	21	1
22-Nov	0	0	0	24	1
29-Nov	0	0	0	17	8
6-Dec	0	0	0	11	14
13-Dec	0	0	0	5	20
20-Dec	0	0	0	0	25

[a]Stem base (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
5/9/2012	19	6	0	0	0
12-Sep	11	6	1	7	0
19-Sep	5	3	8	9	0
26-Sep	0	2	10	13	0
3-Oct	0	1	9	15	0
10-Oct	0	0	5	20	0
17-Oct	0	0	2	23	0
24-Oct	0	0	0	13	12
31-Oct	0	0	0	6	19
7-Nov	0	0	0	2	23
14-Nov	0	0	0	0	25
21-Nov	0	0	0	0	25
28-Nov	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Stem base (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
18/9/2013	21	4	0	0	0
25-Sep	15	3	0	7	0
2-Oct	7	2	2	14	0
9-Oct	0	2	3	20	0
16-Oct	0	0	0	24	1
23-Oct	0	0	0	12	13
30-Oct	0	0	0	10	15
6-Nov	0	0	0	7	18
13-Nov	0	0	0	5	20
20-Nov	0	0	0	2	23
27-Nov	0	0	0	0	25
4-Dec	0	0	0	0	25
11-Dec	0	0	0	0	25

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.
[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.23

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Adriana (*Rlm4* + QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	15	5	3	2	0
11-Oct	5	6	9	5	0
18-Oct	0	8	10	7	0
25-Oct	0	5	11	9	0
1-Nov	0	0	15	10	0
8-Nov	0	0	14	10	1
15-Nov	0	0	8	15	2
22-Nov	0	0	3	19	3
29-Nov	0	0	0	21	4
6-Dec	0	0	0	23	2
13-Dec	0	0	0	18	7
20-Dec	0	0	0	12	13

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	20	4	0	1	0
26-Sep	11	6	4	4	0
3-Oct	6	8	6	5	0
10-Oct	0	10	8	7	0
17-Oct	0	0	14	11	0
24-Oct	0	0	13	12	0
31-Oct	0	0	10	15	0
7-Nov	0	0	4	19	2
14-Nov	0	0	0	21	4
21-Nov	0	0	0	18	7
28-Nov	0	0	0	11	14

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	16	3	1	5	0
2-Oct	6	8	5	6	0
9-Oct	0	9	8	8	0
16-Oct	0	5	9	11	0
23-Oct	0	0	12	13	0
30-Oct	0	0	9	16	0
6-Nov	0	0	5	19	1
13-Nov	0	0	0	22	3
20-Nov	0	0	0	23	2
27-Nov	0	0	0	19	6
4-Dec	0	0	0	10	15
11-Dec	0	0	0	9	16

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.24

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Bilbao (*Rlm4*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	18	4	0	3	0
11-Oct	9	6	4	6	0
18-Oct	2	8	4	11	0
25-Oct	0	5	6	14	0
1-Nov	0	0	8	17	0
8-Nov	0	0	5	20	0
15-Nov	0	0	0	25	0
22-Nov	0	0	0	20	5
29-Nov	0	0	0	17	8
6-Dec	0	0	0	13	12
13-Dec	0	0	0	11	14
20-Dec	0	0	0	8	17

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	16	4	1	4	0
26-Sep	6	7	5	7	0
3-Oct	0	5	11	9	0
10-Oct	0	2	13	10	0
17-Oct	0	0	15	10	0
24-Oct	0	0	10	15	0
31-Oct	0	0	6	19	0
7-Nov	0	0	2	22	1
14-Nov	0	0	0	24	1
21-Nov	0	0	0	20	5
28-Nov	0	0	0	16	9

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	14	3	0	8	0
2-Oct	6	8	2	9	0
9-Oct	0	10	4	11	0
16-Oct	0	4	8	13	0
23-Oct	0		10	15	0
30-Oct	0	0	5	20	0
6-Nov	0	0	3	22	0
13-Nov	0	0	0	25	0
20-Nov	0	0	0	18	7
27-Nov	0	0	0	12	13
4-Dec	0	0	0	8	17
11-Dec	0	0	0	3	22

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.25

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Capitol (*Rlm1*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	10	3	8	4	0
11-Oct	3	4	9	9	0
18-Oct	0	7	8	10	0
25-Oct	0	3	9	13	0
1-Nov	0	0	10	15	0
8-Nov	0	0	7	18	0
15-Nov	0	0	2	22	1
22-Nov	0	0	0	24	1
29-Nov	0	0	0	19	6
6-Dec	0	0	0	16	9
13-Dec	0	0	0	12	13
20-Dec	0	0	0	10	15

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	14	7	4	0	0
26-Sep	4	5	14	2	0
3-Oct	0	2	19	4	0
10-Oct	0	0	18	7	0
17-Oct	0	0	15	10	0
24-Oct	0	0	13	12	0
31-Oct	0	0	9	14	2
7-Nov	0	0	0	20	5
14-Nov	0	0	0	19	6
21-Nov	0	0	0	14	11
28-Nov	0	0	0	10	15

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	16	4	2	3	0
2-Oct	7	8	5	5	0
9-Oct	0	4	14	7	0
16-Oct	0	1	14	10	0
23-Oct	0	0	13	12	0
30-Oct	0	0	9	16	0
6-Nov	0	0	4	20	1
13-Nov	0	0	0	23	2
20-Nov	0	0	0	19	6
27-Nov	0	0	0	14	11
4-Dec	0	0	0	10	15
11-Dec	0	0	0	5	20

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.26

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar DK Cabernet (*Rlm1* + QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	19	2	3	1	0
11-Oct	10	4	7	4	0
18-Oct	0	3	14	8	0
25-Oct	0	0	16	9	0
1-Nov	0	0	15	10	0
8-Nov	0	0	14	11	0
15-Nov	0	0	13	12	0
22-Nov	0	0	6	19	0
29-Nov	0	0	4	21	0
6-Dec	0	0	0	25	0
13-Dec	0	0	0	20	5
20-Dec	0	0	0	16	9

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	18	2	4	1	0
26-Sep	10	6	5	4	0
3-Oct	3	8	6	8	0
10-Oct	0	5	11	9	0
17-Oct	0	2	12	11	0
24-Oct	0	0	12	13	0
31-Oct	0	0	7	16	2
7-Nov	0	0	4	18	3
14-Nov	0	0	0	22	3
21-Nov	0	0	0	17	8
28-Nov	0	0	0	11	14

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	12	3	5	5	0
2-Oct	5	4	8	8	0
9-Oct	0	7	9	9	0
16-Oct	0	2	12	11	0
23-Oct	0	0	12	13	0
30-Oct	0	0	7	18	0
6-Nov	0	0	3	20	2
13-Nov	0	0	0	22	3
20-Nov	0	0	0	18	7
27-Nov	0	0	0	14	11
4-Dec	0	0	0	10	15
11-Dec	0	0	0	8	17

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.27

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Drakkar (susceptible and no *R* gene) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	7	3	7	8	0
11-Oct	3	5	6	11	0
18-Oct	0	4	8	13	0
25-Oct	0	2	5	18	0
1-Nov	0	0	4	21	0
8-Nov	0	0	0	25	0
15-Nov	0	0	0	25	0
22-Nov	0	0	0	19	6
29-Nov	0	0	0	15	10
6-Dec	0	0	0	11	14
13-Dec	0	0	0	8	17
20-Dec	0	0	0	5	20

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	6	5	9	5	0
26-Sep	3	2	13	7	0
3-Oct	0	1	14	10	0
10-Oct	0	0	13	12	0
17-Oct	0	0	6	19	0
24-Oct	0	0	3	22	0
31-Oct	0	0	2	23	0
7-Nov	0	0	0	25	0
14-Nov	0	0	0	18	7
21-Nov	0	0	0	14	11
28-Nov	0	0	0	9	16

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	4	6	7	8	0
2-Oct	0	8	7	10	0
9-Oct	0	0	10	12	0
16-Oct	0	0	8	17	0
23-Oct	0	0	5	20	0
30-Oct	0	0	0	25	0
6-Nov	0	0	0	25	0
13-Nov	0	0	0	20	5
20-Nov	0	0	0	11	14
27-Nov	0	0	0	9	16
4-Dec	0	0	0	3	22
11-Dec	0	0	0	1	24

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.28

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Es-Astrid (QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	14	5	4	2	0
11-Oct	9	7	5	4	0
18-Oct	3	5	9	8	0
25-Oct	0	6	11	8	0
1-Nov	0	2	14	9	0
8-Nov	0	0	14	11	0
15-Nov	0	0	11	14	0
22-Nov	0	0	7	17	1
29-Nov	0	0	2	21	2
6-Dec	0	0	1	22	2
13-Dec	0	0	0	19	6
20-Dec	0	0	0	11	14

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	18	4	3	0	0
26-Sep	11	7	7	0	0
3-Oct	4	5	9	7	0
10-Oct	0	7	8	10	0
17-Oct	0	2	10	13	0
24-Oct	0	1	9	15	0
31-Oct	0	0	8	17	0
7-Nov	0	0	7	16	2
14-Nov	0	0	0	20	5
21-Nov	0	0	0	16	9
28-Nov	0	0	0	12	13

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	16	3	3	3	0
2-Oct	9	5	4	7	0
9-Oct	0	7	9	9	0
16-Oct	0	3	9	13	0
23-Oct	0	0	10	15	0
30-Oct	0	0	6	19	0
6-Nov	0	0	3	21	1
13-Nov	0	0	0	22	3
20-Nov	0	0	0	16	9
27-Nov	0	0	0	12	13
4-Dec	0	0	0	10	15
11-Dec	0	0	0	8	17

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.29

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Excel (*Rlm7*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	16	2	3	4	0
11-Oct	9	3	7	6	0
18-Oct	3	5	7	10	0
25-Oct	0	3	11	11	0
1-Nov	0	0	13	12	0
8-Nov	0	0	11	14	0
15-Nov	0	0	6	18	1
22-Nov	0	0	1	23	1
29-Nov	0	0	0	19	6
6-Dec	0	0	0	12	13
13-Dec	0	0	0	10	15
20-Dec	0	0	0	7	18

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	11	7	6	1	0
26-Sep	7	6	9	3	0
3-Oct	0	9	11	5	0
10-Oct	0	3	15	7	0
17-Oct	0	1	14	10	0
24-Oct	0	0	15	10	0
31-Oct	0	0	13	12	0
7-Nov	0	0	9	15	1
14-Nov	0	0	3	20	2
21-Nov	0	0	0	22	3
28-Nov	0	0	0	17	8

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	10	8	3	4	0
2-Oct	4	7	8	6	0
9-Oct	0	8	9	8	0
16-Oct	0	3	12	10	0
23-Oct	0	1	12	12	0
30-Oct	0	0	9	15	1
6-Nov	0	0	4	19	2
13-Nov	0	0	0	20	5
20-Nov	0	0	0	18	7
27-Nov	0	0	0	14	11
4-Dec	0	0	0	11	14
11-Dec	0	0	0	7	18

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.30

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar NK Grandia (QR) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	13	4	6	2	0
11-Oct	6	5	9	5	0
18-Oct	0	6	12	7	0
25-Oct	0	2	13	10	0
1-Nov	0	0	15	10	0
8-Nov	0	0	13	12	0
15-Nov	0	0	6	18	1
22-Nov	0	0	3	20	2
29-Nov	0	0	0	22	3
6-Dec	0	0	0	23	2
13-Dec	0	0	0	16	9
20-Dec	0	0	0	11	14

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	14	5	4	2	0
26-Sep	10	3	6	6	0
3-Oct	3	6	10	6	0
10-Oct	0	6	11	8	0
17-Oct	0	3	11	11	0
24-Oct	0	0	11	14	0
31-Oct	0	0	8	17	0
7-Nov	0	0	4	18	3
14-Nov	0	0	0	20	5
21-Nov	0	0	0	18	7
28-Nov	0	0	0	12	13

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	12	2	5	6	0
2-Oct	7	4	6	8	0
9-Oct	2	3	11	9	0
16-Oct	0	3	11	11	0
23-Oct	0	1	10	14	0
30-Oct	0	0	8	17	0
6-Nov	0	0	5	20	0
13-Nov	0	0	0	24	1
20-Nov	0	0	0	19	6
27-Nov	0	0	0	16	9
4-Dec	0	0	0	11	14
11-Dec	0	0	0	8	17

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

Appendix 4.31

Table: Development of pseudothecia (as numbers of pseudothecia at different developmental stages) on infected winter oilseed rape upper stem of cultivar Roxet (*Rlm7*) exposed to natural conditions in three growing seasons (2011/2012, 2012/2013 and 2013/2014) observed every week.

[a]Upper stem (2011/2012 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
4-Oct	12	3	8	2	0
11-Oct	7	1	11	6	0
18-Oct	3	4	8	10	0
25-Oct	0	0	15	10	0
1-Nov	0	0	14	11	0
8-Nov	0	0	12	13	0
15-Nov	0	0	5	19	1
22-Nov	0	0	3	20	2
29-Nov	0	0	0	22	3
6-Dec	0	0	0	18	7
13-Dec	0	0	0	12	13
20-Dec	0	0	0	9	16

[a]Upper stem (2012/2013 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
19-Sep	16	2	7	0	0
26-Sep	11	4	7	3	0
3-Oct	3	6	11	5	0
10-Oct	0	4	14	7	0
17-Oct	0	0	15	10	0
24-Oct	0	0	12	13	0
31-Oct	0	0	10	15	0
7-Nov	0	0	5	19	1
14-Nov	0	0	0	24	1
21-Nov	0	0	0	20	5
28-Nov	0	0	0	16	9

[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

[a]Upper stem (2013/2014 growing season)					
	[b]Classes				
Sample date	A	B	C	D	E
25-Sep	16	1	4	4	0
2-Oct	9	6	5	5	0
9-Oct	2	7	9	7	0
16-Oct	0	2	13	10	0
23-Oct	0	0	12	13	0
30-Oct	0	0	10	15	0
6-Nov	0	0	4	19	2
13-Nov	0	0	0	20	5
20-Nov	0	0	0	16	9
27-Nov	0	0	0	13	12
4-Dec	0	0	0	8	17
11-Dec	0	0	0	5	15

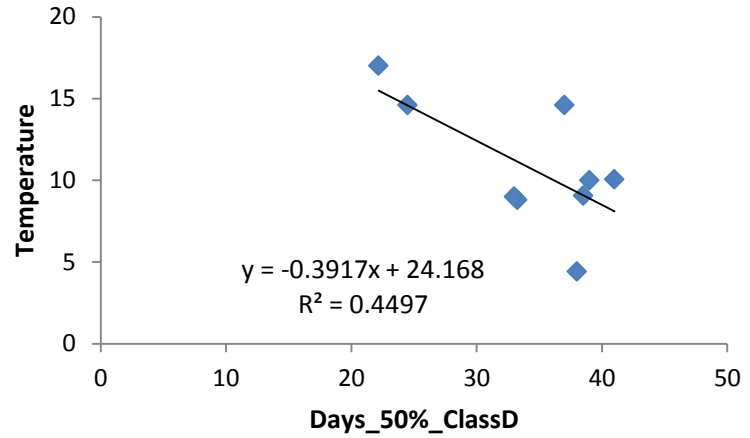
[a]Five stems were sampled at each assessment and five pseudothecia were excised from each of the 5 sampled stem bases (i.e. 25 pseudothecia were observed at each assessment). [b]Classification of pseudothecial maturation is described in section 4.2.1.3.

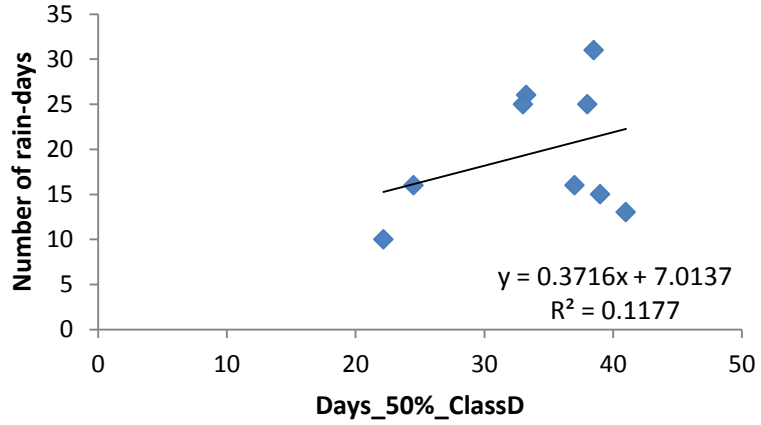
[b]Classification of pseudothecial maturation is described in section 4.2.1.3.

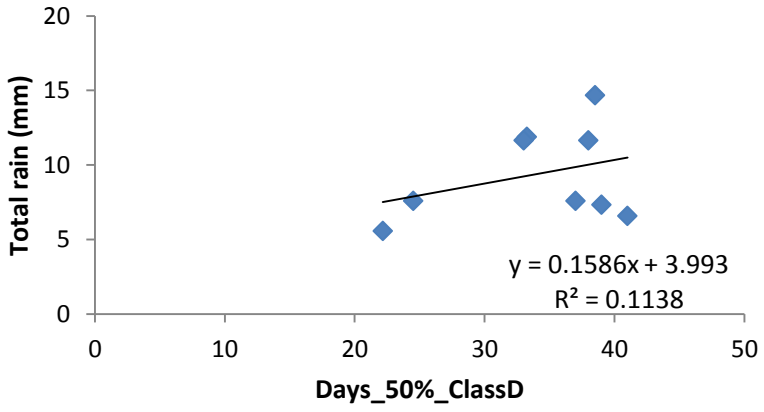
Appendix 4.32: Relationships between number of days for 50% of pseudothecia to reach Class D (mature) on basal stem canker for each of nine different cultivars and the total rain (mm) and temperature in the 2010/2011, 2011/2012 and 2012/2013 growing seasons (data from stems exposed to natural conditions).

Stem base

2011/2012 growing season

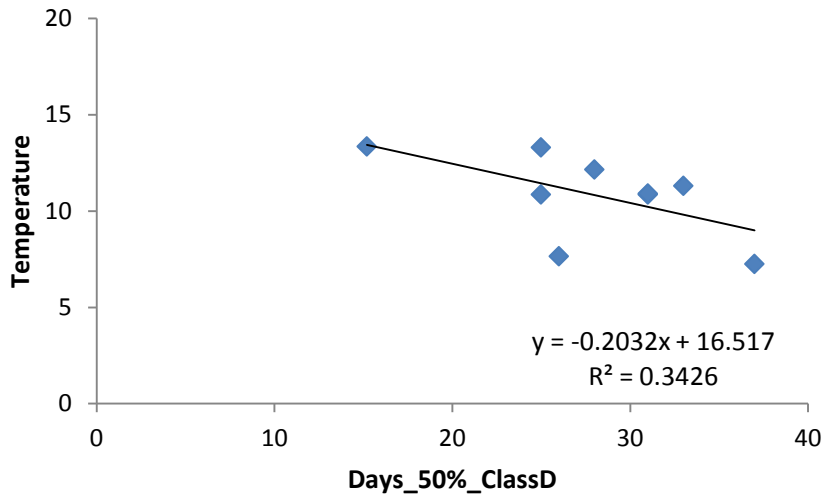


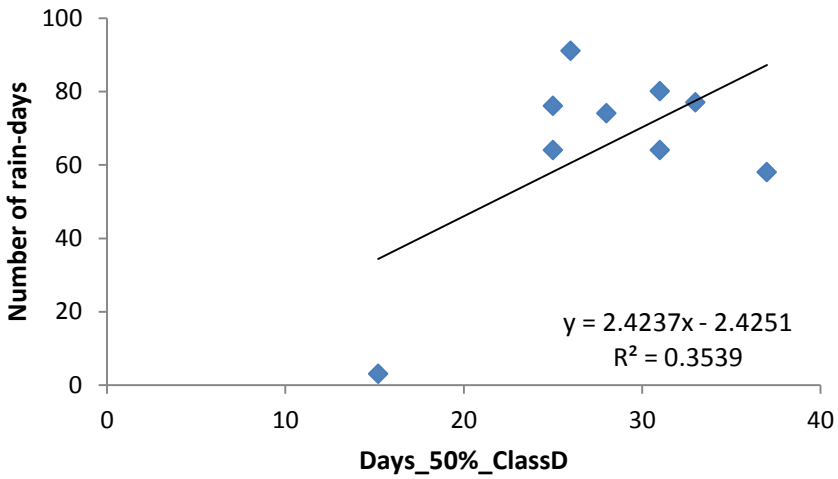


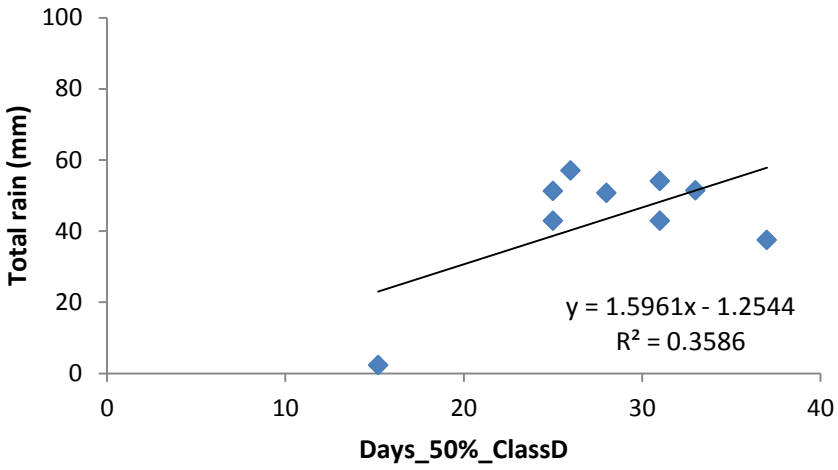


Stem base

2012/2013 growing season

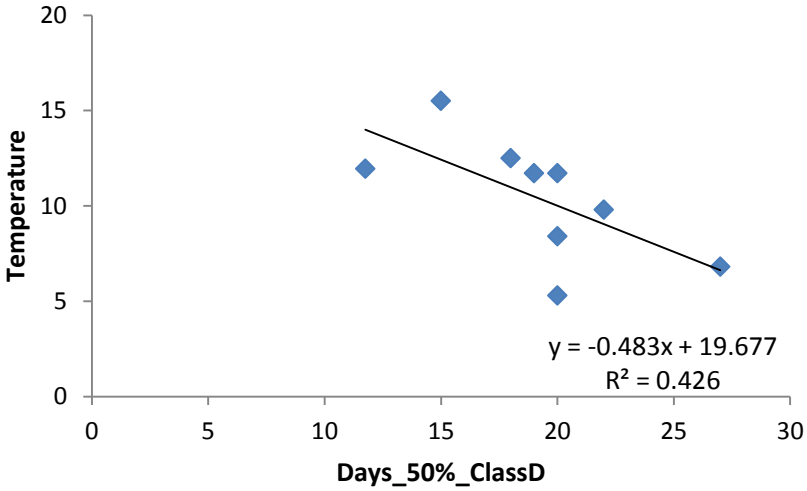


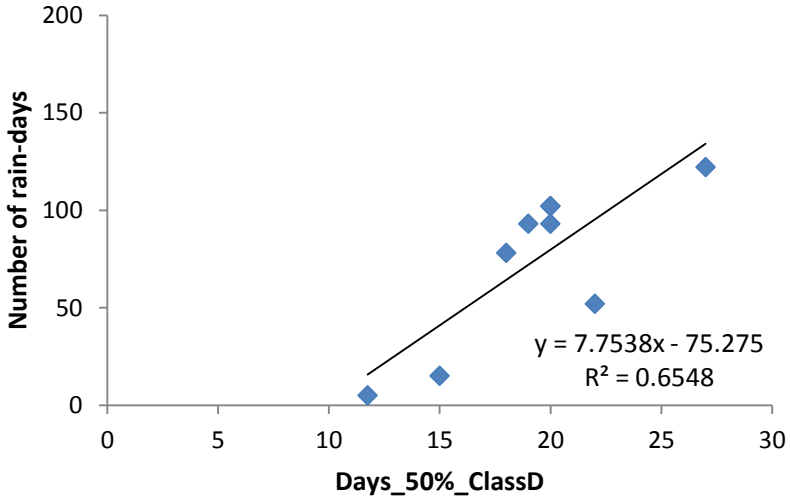


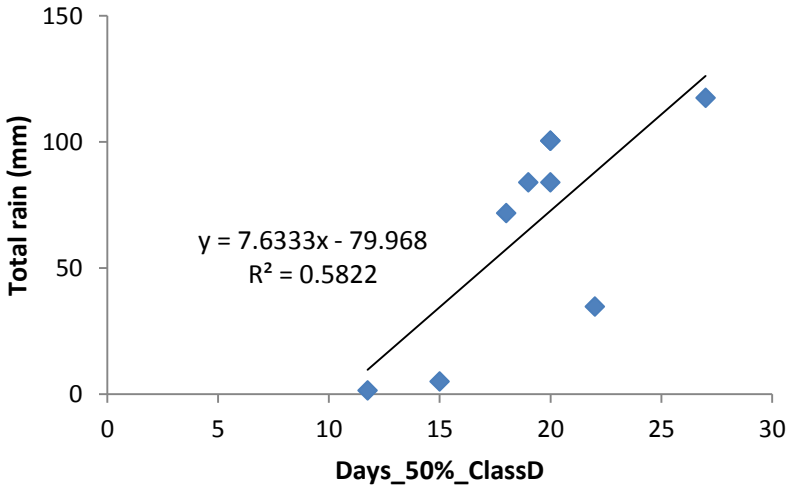


Stem base

2013/2014 growing season



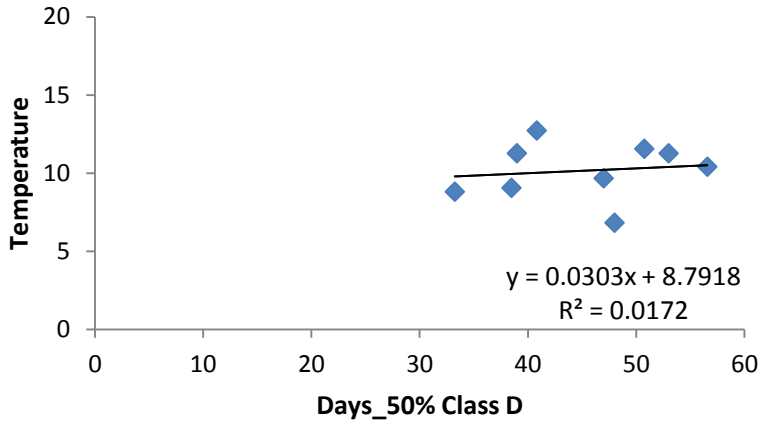


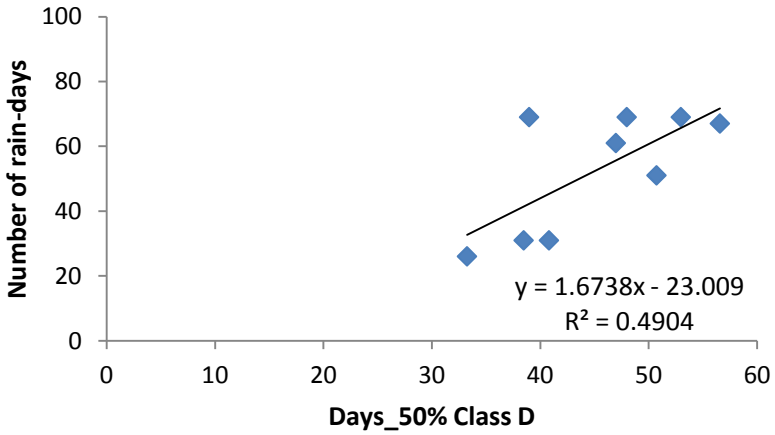


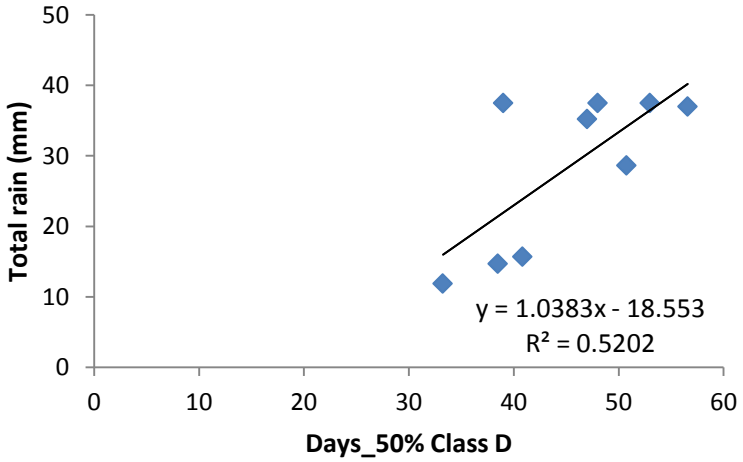
Appendix 4.33: Relationships between number of days for 50% of pseudothecia to reach Class D (mature) on upper stem lesions for each of nine different cultivars and the total rain (mm) and temperature in the 2010/2011, 2011/2012 and 2012/2013 growing seasons (data from stems exposed to natural conditions).

Upper stem

2011/2012 growing season

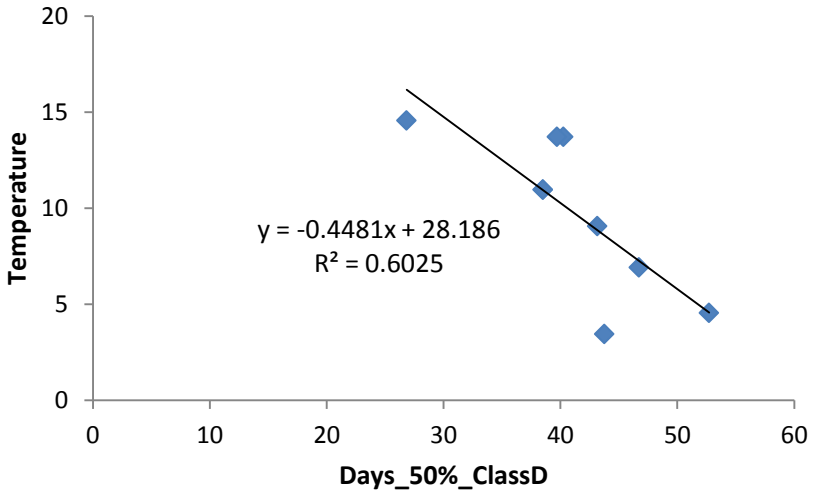


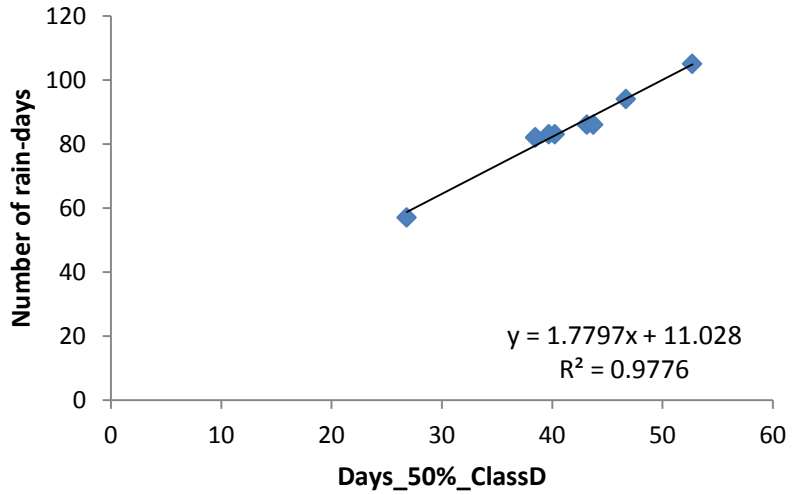


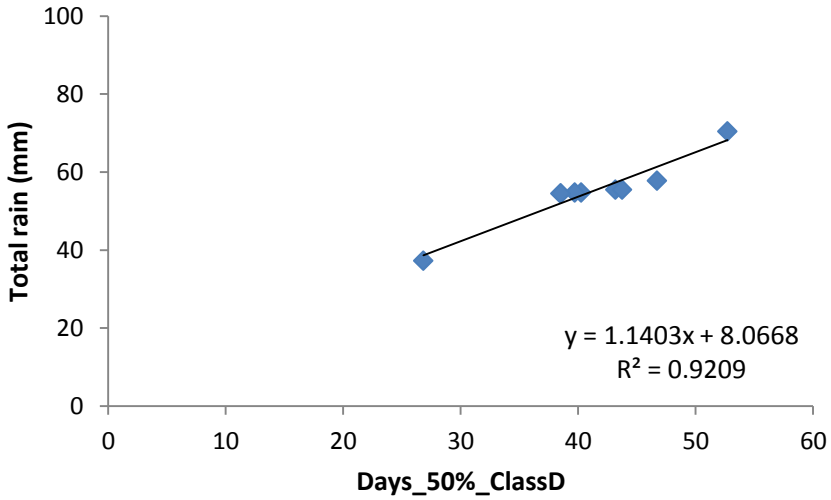


Upper stem

2012/2013 growing season

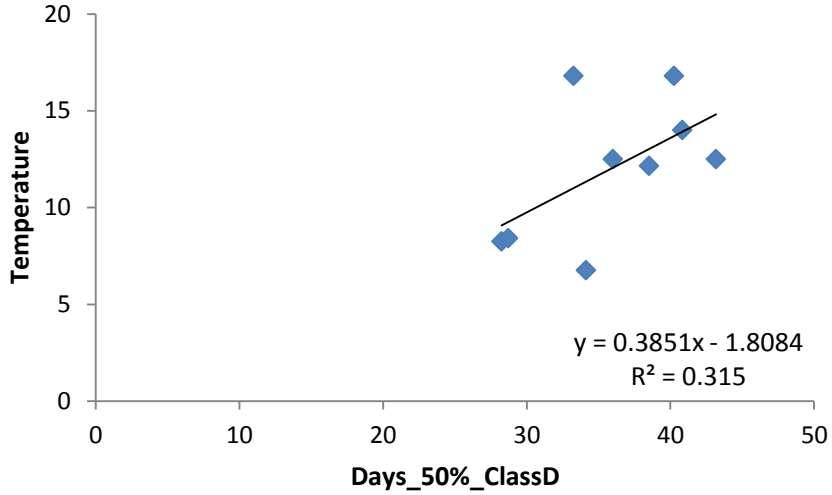


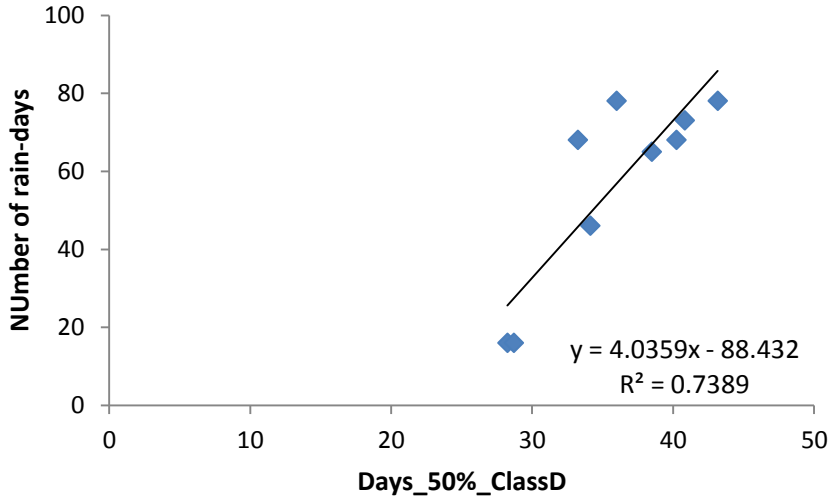


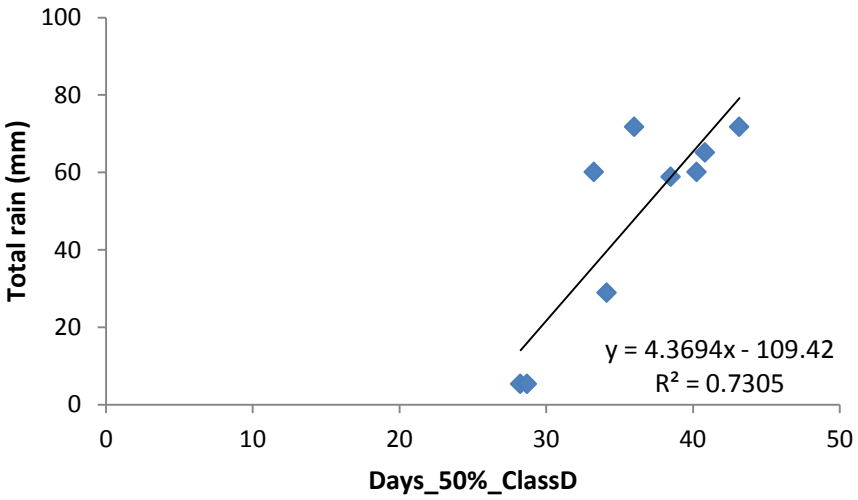


Upper stem

2013/2014 growing season







Appendix 4.34

	No. of ascospores	*DNA (ng)*
No. of ascospores	1	
DNA (ng)	0.709405	1

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.708708
R Square	0.502267
Adjusted R Square	0.487184
Standard Error	148.3518
Observations	35

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	732889.1	732889.1	33.30063	1.90E-06
Residual	33	726272.7	22008.26		
Total	34	1459162			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	81.57663	28.85439	2.827183	0.007919	22.87193	140.2813	22.87193	140.2813
159.82	0.236449	0.040974	5.77067	1.9E-06	0.153087	0.319812	0.153087	0.319812

Appendix 5.1

Table: Differentiation of isolates of *L. maculans* from phoma leaf spots, basal stem cankers and upper stem lesions, based on their leaf spot scoring (0-9) with different *R* genes on eight *Brassica* differential genotypes and cv. Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

			R gene in test *Brassica* differential genotype									
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar	*Avr*
Source: phoma leaf spots												
IIKB2	2012/2013	Bilbao	6.6	6.6	6.2	4.6	4.6	4.2	2.2	5.6	6.6	A4,5,6,7
II K B4	2012/2013	Bilbao	7.8	7.0	7.2	2.0	4.2	5.8	4.0	7.6	6.2	A4,5,7
IIKB3	2012/2013	Bilbao	6.0	5.6	6.4	2.6	4.2	4.8	3.8	7.2	6.6	A4,5,6,7
I3B1	2011/2012	Bilbao	7.2	6.0	6.4	2.4	4.6	5.4	2.5	6.8	6.4	A4,5,6,7
I3B2	2011/2012	Bilbao	7.0	6.2	6.2	1.8	4.2	5.6	2.4	7.2	6.6	A4,5,7
IIKC1	2012/2013	Capitol	6.2	6.6	7.8	4.6	4.8	2.0	4.4	7.4	9.0	A4,5,6,7
II K C6	2012/2013	Capitol	6.2	6.2	7.2	5.6	5.6	3.6	6.4	7.2	9.0	A6,7
IIKC2	2012/2013	Capitol	7.0	6.8	6.2	4.8	4.6	3.2	3.4	7.8	8.8	A4,5,6,7
IIKDKC	2012/2013	DK Cabernet	5.8	6.0	6.2	2.6	4.6	1.6	1.4	6.6	7.6	A4,5,6,7
II K DKC 2	2012/2013	DK Cabernet	5.8	6.8	7.0	3.0	3.2	3.0	2.4	6.0	7.6	A4,5,6,7
II K DKC 4	2012/2013	DK Cabernet	7.2	7.4	7.6	1.8	5.2	5.2	3.0	6.0	7.2	A4,5,6,7
II K DKC 1	2012/2013	DK Cabernet	7.8	6.4	6.6	1.8	5.0	4.8	2.3	6.0	7.4	A4,5,6,7
IIKDKC 3	2012/2013	DK Cabernet	5.2	7.4	6.4	2.6	4.4	5.2	2.5	6.6	7.6	A4,5,6,7
IIKEA1	2012/2013	Es-Astrid	5.6	6.2	6.2	4.4	3.0	3.8	3.0	6.2	7.2	A4,5,6,7
IIKEA3	2012/2013	Es-Astrid	5.6	6.6	6.8	4.6	2.8	2.8	2.4	7.6	6.6	A4,5,6,7
IIKD6	2012/2013	Drakkar	6.4	6.8	5.8	3.8	1.0	2.6	2.0	7.4	8.5	A4,5,6,7
IIKD4	2012/2013	Drakkar	6.4	6.4	6.0	3.2	1.0	3.8	2.2	7.4	9.0	A4,5,6,7
IIKD3	2012/2013	Drakkar	6.0	6.4	6.6	4.2	1.0	3.2	1.0	6.8	9.0	A4,5,6,7
IIKD1	2012/2013	Drakkar	6.0	6.4	6.0	3.2	1.2	2.8	1.2	7.0	8.7	A4,5,6,7
IIKD2	2012/2013	Drakkar	6.0	6.2	6.2	3.2	2.0	1.2	1.2	7.4	8.0	A4,5,6,7
II K D 5	2012/2013	Drakkar	7.0	6.4	6.8	6.6	2.2	3.6	2.4	6.8	8.5	A4,5,7

R gene in test *Brassica* differential genotype												
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar	*Avr*
II K Drakkar	2012/2013	Drakkar	7.4	6.8	7.0	6.2	1.4	2.6	2.2	6.8	9.0	A4,5,7
IIK D7	2012/2013	Drakkar	7.8	6.8	6.0	2.6	1.8	2.8	2.8	6.8	8.8	A4,5,6,7
IIKD8	2012/2013	Drakkar	7.4	7.4	6.4	1.8	1.4	3.2	2.4	7.4	9.0	A4,5,6,7
IIKD10	2012/2013	Drakkar	7.4	7.6	6.6	2.4	1.8	3.6	2.0	7.8	9.0	A4,5,6,7
IIKD11	2012/2013	Drakkar	7.4	6.2	7.0	3.2	1.6	4.0	1.6	7.4	8.8	A4,5,6,7
IIKD12	2012/2013	Drakkar	7.2	7.8	7.6	2.4	1.0	3.4	1.4	7.8	8.5	A4,5,6,7
IIKD13	2012/2013	Drakkar	7.6	6.4	6.6	4.4	1.6	2.0	3.0	7.6	8.5	A4,5,6,7
IIKD15	2012/2013	Drakkar	7.4	6.6	6.4	1.8	1.2	1.0	2.2	7.6	9.0	A4,5,6,7
D1	2011/2012	Drakkar	7.8	6.4	6.6	1.8	1.2	2.0	2.0	7.8	8.5	A4,5,6,7
IIKNKG1	2012/2013	NK Grandia	5.8	6.2	6.2	1.4	1.8	1.8	1.2	6.6	7.4	A4,5,6,7
IIKNKG4	2012/2013	NK Grandia	6.6	7.2	5.6	2.4	2.2	2.0	2.0	7.0	7.8	A4,5,6,7
IIKNKG6	2012/2013	NK Grandia	6.2	6.2	5.4	1.2	1.0	1.0	2.2	6.4	7.4	A4,5,6,7
IIKNKG5	2012/2013	NK Grandia	6.2	6.6	6.6	2.6	1.4	1.2	2.6	6.4	7.8	A4,5,6,7
IIKNKG3	2012/2013	NK Grandia	6.6	7.2	6.0	1.8	1.8	2.2	3.0	6.3	7.4	A4,5,6,7
IIKR1	2012/2013	Roxet	6.6	6.4	6.0	2.4	3.6	1.6	1.8	6.8	7.0	A4,5,6,7
IIKR2	2012/2013	Roxet	6.6	6.6	6.6	2.8	2.8	3.0	2.8	6.2	6.8	A4,5,6,7
IIKR3	2012/2013	Roxet	7.2	7.2	7.2	1.8	3.4	2.6	2.6	6.0	6.8	A4,5,6,7
IIKR5	2012/2013	Roxet	7.8	6.6	6.2	1.6	2.6	3.4	2.5	6.2	7.4	A4,5,6,7
IIKR7	2012/2013	Roxet	7.0	7.2	6.2	2.8	3.6	3.2	2.8	6.8	7.2	A4,5,6,7
Source: basal stem cankers												
KA 70 B	2012/2013	Adriana	6.0	6.2	7.2	6.6	6.4	6.2	4.4	6.0	7.8	A7
ADR19B	2012/2013	Adriana	6.6	6.4	7.2	6.6	6.0	5.6	3.8	6.2	7.8	A7
KB228B	2012/2013	Bilbao	6.2	6.6	7.2	1.8	7.0	1.6	3.6	7.8	8.2	A4,6,7
KB229B	2012/2013	Bilbao	6.4	6.6	6.6	2.6	7.4	1.8	3.6	7.8	8.4	A4,6,7
I31B	2011/2012	Bilbao	6.2	7.4	6.2	1.4	7.6	1.6	3.0	7.2	8.0	A4,6,7
I32B	2011/2012	Bilbao	5.8	7.6	7.4	1.6	7.2	1.2	2.4	7.8	8.2	A4,6,7
Cap1	2011/2012	Capitol	7.0	6.0	7.8	6.6	7.4	7.4	2.6	6.4	8.5	A7

R gene in test *Brassica* differential genotype												
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar	*Avr*
Cap2	2011/2012	Capitol	7.6	6.4	7.6	6.6	7.2	7.6	3.0	7.0	8.5	A7
Cap3	2011/2012	Capitol	6.6	6.6	8.0	6.2	6.6	7.4	2.6	7.0	8.8	A7
KD136B	2012/2013	Drakkar	6.8	7.8	7.4	7.6	7.2	8.0	2.4	7.6	9.0	A7
KD138B	2012/2013	Drakkar	7.6	8.4	8.2	8.4	8.2	8.4	2.6	7.8	9.0	A7
KD139B	2012/2013	Drakkar	7.2	8.4	8.2	7.8	8.2	8.2	2.4	8.0	9.0	A7
KDK57B	2012/2013	Drakkar	6.8	6.0	7.6	7.2	8.2	8.4	3.6	6.4	8.5	A7
KDK 55 B	2012/2013	Drakkar	7.8	6.6	8.8	7.8	8.4	8.4	3.6	7.0	8.2	A7
KDK 61 B	2012/2013	Drakkar	7.0	6.4	8.6	7.0	8.6	8.4	3.4	7.0	8.5	A7
DK59B	2012/2013	Drakkar	6.6	6.2	7.6	7.6	8.4	8.6	3.2	6.4	8.5	A7
KEA 9 B	2012/2013	Es-Astrid	6.0	7.8	6.6	7.8	7.0	6.2	4.2	6.6	8.8	A7
KEA 12 B	2012/2013	Es-Astrid	6.0	7.4	7.4	8.4	7.6	6.8	4.2	6.6	8.5	A7
KEA2B	2012/2013	Es-Astrid	6.4	7.6	7.4	8.8	7.0	7.6	3.6	7.2	8.5	A7
KEA11B	2012/2013	Es-Astrid	6.2	7.8	7.2	8.8	7.8	7.4	3.4	7.8	8.4	A7
KEA14B	2012/2013	Es-Astrid	6.4	7.8	6.8	8.6	7.8	6.8	3.2	7.8	8.5	A7
R5-1	2011/2012	Roxet	6.2	6.8	6.6	6.2	6.6	6.4	2.4	7.6	7.8	A7
R11	2011/2012	Roxet	6.4	6.8	6.6	6.0	7.2	6.6	2.6	7.0	7.4	A7
R7	2011/2012	Roxet	6.6	7.6	7.6	6.2	7.4	5.8	2.4	7.4	7.8	A7
Source: upper stem lesions												
KA95U	2012/2013	Adriana	7.6	6.2	6.0	3.6	6.6	5.4	2.4	8.8	9.0	A4,7
KA72U	2012/2013	Adriana	6.6	6.2	6.2	3.6	7.6	5.4	2.2	7.0	8.8	A4,7
KA 98 U	2012/2013	Adriana	7.0	6.4	6.6	4.8	8.0	5.6	3.0	7.0	9.0	A4,7
KA85U	2012/2013	Adriana	6.8	6.2	7.0	4.6	6.6	5.8	2.8	7.0	9.0	A4,7
KA 99 U	2012/2013	Adriana	7.4	6.0	6.8	3.2	7.6	5.6	3.6	7.0	8.8	A4,7
KB 217 U	2012/2013	Bilbao	6.2	6.4	7.8	6.6	2.4	2.2	1.4	7.2	8.4	A5,6,7
B10P	2011/2012	Bilbao	5.8	6.6	7.8	7.6	2.6	1.8	1.2	6.8	8.6	A5,6,7
B13P	2011/2012	Bilbao	6.2	6.4	7.4	7.4	2.6	2.4	1.4	7.2	8.4	A5,6,7

R gene in test *Brassica* differential genotype												
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Drakkar	*Avr*
KC250U	2012/2013	Capitol	6.6	6.4	6.2	8.0	3.8	2.8	2.8	7.2	8.2	A5,6,7
KC249U	2012/2013	Capitol	7.6	7.2	7.0	8.2	3.0	3.6	3.0	7.0	8.2	A5,6,7
KC255U	2012/2013	Capitol	6.6	7.2	7.4	8.4	3.0	3.4	2.8	7.2	8.5	A5,6,7
C10P	2011/2012	Capitol	6.8	6.8	7.0	8.4	2.4	2.2	1.6	7.4	8.2	A5,6,7
KD 122 U	2012/2013	Drakkar	6.8	6.2	7.0	6.6	1.6	2.4	2.4	7.4	9.0	A5,6,7
KD123U	2012/2013	Drakkar	7.2	6.4	6.4	8.0	1.2	2.4	1.4	7.6	8.5	A5,6,7
D1P	2011/2012	Drakkar	7.6	6.6	6.8	7.6	1.4	1.6	1.0	7.8	8.8	A5,6,7
D1	2011/2012	Drakkar	7.4	7.0	6.8	7.4	1.4	1.4	2.0	7.8	9.0	A5,6,7
DKC4P	2011/2012	DK Cabernet	6.6	6.0	6.8	6.2	1.8	4.4	1.4	6.6	7.8	A5,6,7
DKCU1	2011/2012	DK Cabernet	6.6	6.4	6.6	6.4	1.6	4.4	1.2	6.6	8.0	A5,6,7
DKC11P	2011/2012	DK Cabernet	6.4	6.4	6.6	6.8	1.2	1.6	1.0	6.4	7.6	A5,6,7
DKC(J)11-26	2011/2012	DK Cabernet	6.0	6.4	6.2	6.8	1.2	2.2	2.0	6.2	7.8	A5,6,7
KEA 24 U	2012/2013	Es-Astrid	6.4	6.2	6.6	8.2	6.6	6.4	2.4	7.0	7.8	A7
KEA 18 U	2012/2013	Es-Astrid	7.2	6.0	7.8	7.8	8.0	7.2	3.2	7.2	7.6	A7
KEA 25 U	2012/2013	Es-Astrid	6.8	6.4	8.2	8.4	8.4	6.8	3.4	6.6	7.6	A7
KEA 17 U	2012/2013	Es-Astrid	7.4	6.6	8.2	8.0	8.2	7.2	2.6	6.6	8.0	A7
KEA22U	2012/2013	Es-Astrid	7.0	6.4	6.6	8.4	6.8	6.6	2.4	7.2	7.8	A7
KR158U	2012/2013	Roxet	6.4	6.6	6.4	6.8	6.6	3.8	2.6	6.4	7.2	A6,7
KR157U	2012/2013	Roxet	6.6	6.4	6.4	7.6	6.6	4.0	2.8	6.2	7.2	A6,7
KR159U	2012/2013	Roxet	6.6	6.6	6.4	8.0	6.6	3.5	2.6	6.4	7.6	A6,7
KR156U	2012/2013	Roxet	6.4	7.2	6.6	6.8	6.4	4.3	2.4	6.2	7.4	A6,7
KR164U	2012/2013	Roxet	6.8	6.6	7.6	8.2	7.4	3.8	2.2	7.0	7.2	A6,7
KR166U	2012/2013	Roxet	6.6	7.6	7.4	6.8	7.0	4.3	2.2	7.6	7.2	A6,7

Appendix 5.2

Table: Differentiation of isolates of *L. biglobosa* derived from phoma leaf spots from different source cultivars based on their leaf spot scoring (0-9) 14 dpi on cultivars/lines with or without *R* genes and/or QR and cv. Drakkar (susceptible to *L. maculans*).

Isolates	Growing season	Severity score (0-9)											
		Cultivar/line											
		Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar
IIK A4	2012/2013	Adriana	1	1	1	1	1	1	1	1	1	1	1	2.2
IIK A2	2012/2013	Adriana	1	1	1	1	1	1	1	1	1	1.6	1	2.6
IIK A3	2012/2013	Adriana	1	1	1	1	1	1	1	1	1	1	1	2
Ax	2013/2014	Adriana	1	1	1	1	1	1	1	1	1	1	1	2
IIK B1	2012/2013	Bilbao	1.6	1	1	1	1	1	1	1	1	1.2	1	1.6
IIK B2	2012/2013	Bilbao	1.4	1	1	2	1	1	1	1	1.4	1.4	2	2
IIK B3	2012/2013	Bilbao	1.6	1	1	1	1	1	1	1	1.2	1.4	1.2	1.8
Bx	2013/2014	Bilbao	1.6	1	1	1.4	1	1	1	1	1.4	1.4	1	1.8
Cx1	2013/2014	Capitol	1	1	1	1	1	1	1	1	1	1	1	1.4
Cx2	2013/2014	Capitol	1	1	1	1	1	1	1	1	1	1	1	1.6
Cx3	2013/2014	Capitol	1	1	1	1	1	1	1	1	1	1	1	1.4
Cx4	2013/2014	Capitol	1	1	1	1	1	1	1	1	1	1	1	1.6
IIK D2	2012/2013	Drakkar	1	1	1	1	1	1	2	1	1	1	1	2
Dx2	2013/2014	Drakkar	1	1	1	1	1	1	1.6	1	1	1	1	2.8
Dx3	2013/2014	Drakkar	1	1	1	1	1	1	1.6	1	1	1	1	2.4
Dx4	2013/2014	Drakkar	1	1	1	1	1	1	2	1	1	1	1	2.2
IIK DKC7	2012/2013	DK Cabernet	1	1	1	1	1	1	1	1	1	1	1	2
IIK DKC 5	2012/2013	DK Cabernet	1.4	1	1	1	1	1	1	1	1	1	1.8	2.2

Isolates	Growing season	Severity score (0-9)												
		Cultivar/line												
		Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar
IIK DKC8	2012/2013	DK Cabernet	1	1	1	1	1	1	1	1	1	1	1	2.2
IIK DKC6	2012/2013	DK Cabernet	2.2	1	1	2.8	1	1	1	1	1.4	2.4	2.6	1
IIK EA3	2012/2013	Es-Astrid	1	1	1	1	1	1	1	1	1	1	1	1.4
IIK EA 4	2012/2013	Es-Astrid	1	1	1	1	1	1	1	1	1	1	1	1.8
IIK EA1	2012/2013	Es-Astrid	1	1	1	1	1	1	1	1	1	1	1.8	2.8
IIK EA2	2012/2013	Es-Astrid	1.6	1	1	2.6	1	1	1.6	1	1	2.2	2.4	1.8
IIK E1	2012/2013	Excel	1	1	1	1.2	1	1	1	1	1	1	1	2.2
IIK E2	2012/2013	Excel	1.6	1	1	2.4	1	1	2.4	1	2.2	1.8	1.8	2.6
Ex3	2013/2014	Excel	1	1	1	1.4	1	1	1.8	1	1.6	1.6	1	2
Ex4	2013/2014	Excel	1.4	1	1	1.6	1	1	2.2	1	1.8	1.6	1.4	2.2
IIK R2	2012/2013	Roxet	1	1	1	1	1	1	1.8	1	1	1	1	2.2
IIK R1	2012/2013	Roxet	1	1	1	1	1	1	1	1	1	1	1	2.4
Rx3	2013/2014	Roxet	1	1	1	1	1	1	1.4	1	1	1	1	1.8
Rx4	2013/2014	Roxet	1	1	1	1	1	1	1.4	1	1	1	1	2.4
IIK NKG 2	2012/2013	NK Grandia	1.2	1	1	1	1	1	1	1	1	1	1	2.4
IIK NKG	2012/2013	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	2.2
NKGx3	2013/2014	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	2.4
NKGx4	2013/2014	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	2.2

Table: Differentiation of isolates of *L. biglobosa* derived from basal stem cankers from different source based on their leaf spot scoring (0-9) 14 dpi on cultivars/lines with or without *R* genes and/or QR and cv. Drakkar (susceptible to *L. maculans*).

		Severity score (0-9)												
		Cultivar/line												
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar
C6	2011/2012	Capitol	1	1	1	1	1	1	1	1	1	1	1	2.2
C117	2011/2012	Capitol	1	1	1	1	1	1	1	1	1	1	1	2.2
NKG 63	2011/2012	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	1.8
NKG 108	2011/2012	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	2.2
R11	2011/2012	Roxet	1	1	1	1	1	1	1	1	1	1	1	1
C15-2	2010/2011	Capitol	1	1	1	1	1	1	1	1	1	1	1	2.2

Table: Differentiation of isolates of *L. biglobosa* derived from upper stem lesions from different source cultivars based on their leaf spot scoring (0-9) 14 dpi on cultivars/lines with or without *R* genes and/or QR and cv. Drakkar (susceptible to *L. maculans*).

		Severity score (0-9)												
		Cultivar/line												
Isolates	Growing season	Cultivar	Columbus *Rlm 1*	Bristol *Rlm 2*	line 02-22-2-1 *Rlm 3*	Jet Neuf *Rlm 4*	line 99-150-2-1 *Rlm 5*	Darmor-MX *Rlm 6*	line 01-23-2-1 *Rlm 7*	line 01-190-1-1 *Rlm 9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar
194-5P	2011/2012	Drakkar	1	1	1	2.6	1	1	2.8	1	2.8	1	1	1
B5 - 1	2010/2011	Bilbao	1	1	1	1	1	1	1	1	1	1	1	1
194 - 10P	2011/2012	Drakkar	1	1	1	1	1	1	1	1	1	1	1	1.6
EA11P	2010/2011	Es-Astrid	1	1	1	1	1	1	1	1	1	1	1	1.8
NKG 13P	2010/2011	NK Grandia	1	1	1	1	1	1	1	1	1	1	1	2
B6P-1	2011/2012	Bilbao	1	1	1	1	1	1	1	1	1	1	1	2.4

Appendix 5.3

Table: Differentiation of isolates of *L. biglobosa* derived from phoma leaf spots from different source cultivars based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cvs with QR (Adriana, DK Cabernet and Es-Astrid) and Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

	Interaction phenotype (IP)											
	Cultivar/lines											
Source cultivar/ Isolates	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no R genes)
From Adriana												
IIK A4	R	R	R	R	R	R	R	R	R	R	R	R
IIK A2	R	R	R	R	R	R	R	R	R	R	R	R
IIK A3	R	R	R	R	R	R	R	R	R	R	R	R
Ax	R	R	R	R	R	R	R	R	R	R	R	R
From Bilbao												
IIK B1	R	R	R	R	R	R	R	R	R	R	R	R
IIK B2	R	R	R	R	R	R	R	R	R	R	R	R
IIK B3	R	R	R	R	R	R	R	R	R	R	R	R
Bx	R	R	R	R	R	R	R	R	R	R	R	R
From Capitol												
Cx1	R	R	R	R	R	R	R	R	R	R	R	R
Cx2	R	R	R	R	R	R	R	R	R	R	R	R
Cx3	R	R	R	R	R	R	R	R	R	R	R	R
Cx4	R	R	R	R	R	R	R	R	R	R	R	R
From Drakkar												
IIK D2	R	R	R	R	R	R	R	R	R	R	R	R
Dx2	R	R	R	R	R	R	R	R	R	R	R	R
Dx3	R	R	R	R	R	R	R	R	R	R	R	R
Dx4	R	R	R	R	R	R	R	R	R	R	R	R

Table Continued from preceding page

	Severity score (0-9)											
	Cultivar											
Source cultivar/ Isolates	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no R genes)
From DKCabernet												
IIK DKC7	R	R	R	R	R	R	R	R	R	R	R	R
IIK DKC 5	R	R	R	R	R	R	R	R	R	R	R	R
IIK DKC 8	R	R	R	R	R	R	R	R	R	R	R	R
IIK DKC6	R	R	R	R	R	R	R	R	R	R	R	R
From Es-Astrid												
IIK EA3	R	R	R	R	R	R	R	R	R	R	R	R
IIK EA 4	R	R	R	R	R	R	R	R	R	R	R	R
IIK EA1	R	R	R	R	R	R	R	R	R	R	R	R
IIK EA2	R	R	R	R	R	R	R	R	R	R	R	R
From Excel												
IIK E1	R	R	R	R	R	R	R	R	R	R	R	R
IIK E2	R	R	R	R	R	R	R	R	R	R	R	R
Ex3	R	R	R	R	R	R	R	R	R	R	R	R
Ex4	R	R	R	R	R	R	R	R	R	R	R	R
From Roxet												
IIK R2	R	R	R	R	R	R	R	R	R	R	R	R
IIK R1	R	R	R	R	R	R	R	R	R	R	R	R
Rx3	R	R	R	R	R	R	R	R	R	R	R	R
Rx4	R	R	R	R	R	R	R	R	R	R	R	R
From NKGrandia (QR)												
IIK NKG 2	R	R	R	R	R	R	R	R	R	R	R	R
IIK NKG	R	R	R	R	R	R	R	R	R	R	R	R
NKGx3	R	R	R	R	R	R	R	R	R	R	R	R
NKGx4	R	R	R	R	R	R	R	R	R	R	R	R

Appendix 5.4

Table: Differentiation of isolates of *L. biglobosa* derived from basal stem cankers from different source cultivars based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cvs with QR (Adriana, DK Cabernet and Es-Astrid) and Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

	Lesions diameter (mm)											
	Cultivar											
Source cultivar/ Isolates	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no R genes)
C6	R	R	R	R	R	R	R	R	R	R	R	R
C117	R	R	R	R	R	R	R	R	R	R	R	R
NKG 63	R	R	R	R	R	R	R	R	R	R	R	R
NKG 108	R	R	R	R	R	R	R	R	R	R	R	R
R11	R	R	R	R	R	R	R	R	R	R	R	R
C15-2	R	R	R	R	R	R	R	R	R	R	R	R

Appendix 5.5

Table: Differentiation of isolates of *L. biglobosa* derived from upper stem lesions from different source cultivars based on their interaction phenotype (R=resistant, I=intermediate and S=susceptible) (refer 5.2.1.5) with different *R* genes on eight *Brassica* differential genotypes and cvs with QR (Adriana, DK Cabernet and Es-Astrid) and Drakkar (susceptible to *L. maculans*) following cotyledon inoculation.

	Lesions diameter (mm)											
	Cultivar											
Source cultivar/ Isolates	Columbus *Rlm1*	Bristol *Rlm2*	Line02-22-2-1 *Rlm3*	Jet Neuf *Rlm4*	Line99-150-2-1 *Rlm5*	Darmor-MX *Rlm6*	Line01-23-2-1 *Rlm7*	Line01-190-1-1 *Rlm9*	Adriana *Rlm4*+QR	DK Cabernet *Rlm1*+QR	Es-Astrid QR	Drakkar (no R genes)
194-5P	R	R	R	R	R	R	R	R	R	R	R	R
B5 - 1	R	R	R	R	R	R	R	R	R	R	R	R
194 - 10P	R	R	R	R	R	R	R	R	R	R	R	R
EA11P	R	R	R	R	R	R	R	R	R	R	R	R
NKG 13P	R	R	R	R	R	R	R	R	R	R	R	R
B6P-1	R	R	R	R	R	R	R	R	R	R	R	R

List of publications

1. Siti Nordahliawate M Sidique, Yong-Ju Huang, Avice M Hall and Bruce D L Fitt, (2012), Maturation of *Leptosphaeria maculans and L. biglobosa* pseudothecia and first appearance of phoma leaf spots on winter oilseed rape, *Aspects of Applied Biology, 117,* 209-215.

2. Aiming Qi, Yong-Ju Huang, Martin Malcolm-Brown, Siti Nordahliawate M. Sidique and Bruce D.L. Fitt, (2014), Predicting timing of release of ascospores of *Leptosphaeria* spp. to improve control of phoma stem canker on oilseed rape in the UK. *Aspects of applied biology, 125,* 109 -116.

Aspects of Applied Biology 117, 2012
Crop Protection in Southern Britain

Maturation of *Leptosphaeria maculans* and *L. biglobosa* pseudothecia and first appearance of phoma leaf spots on winter oilseed rape

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Summary

After harvest in August 2011, stem debris of nine winter oilseed rape cultivars were collected and incubated under natural conditions at Bayfordbury, Hertfordshire. From September 2011 to February 2012, the average temperature was 8.8°C and total rainfall was 15.9 mm, with 84 rain days recorded. There were differences between cultivars in early stages of pseudothecial maturation, with pseudothecia on susceptible cultivars maturing faster than those on cultivars with quantitative resistance with or without *R* genes in September and October 2011. The greatest mean densities of pseudothecia were on Capitol (*Rlm 1*) on the stem bases (449 per cm^2) and on Drakkar (no *R*-genes) on upper stems (431 per cm^2). From September to November 2011, there was little rainfall and few ascospores were released. In December 2011, there was more rainfall (23 days recorded) with temperature <10°C. Subsequently, more ascospores were released, with a maximum in late January 2012 (1224 spores per m^3) and phoma leaf spots were observed in early February 2012.

Key words: Winter oilseed rape, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, pseudothecia, phoma leaf spots

Introduction

In European countries, *Leptosphaeria maculans* and *L. biglobosa* co-infect winter oilseed rape plants to cause phoma stem canker disease. Surveys by Crop Monitor show that incidence of the disease in summer before harvest was greater in 2011 (96% plants affected) than in 2010 (74%) (http://www.cropmonitor.co.uk). Ascospores released from mature pseudothecia on crop debris are the main inoculum for the initial colonisation of UK crops in autumn. The number of ascospores released is influenced by rain and temperature (Huang *et al*., 2007). A large number of plants with leaf lesions have often been observed before the maximum ascospore discharge in the UK and France (West *et al*., 1999), confirming the ability of the first ascospores released to produce a high incidence of leaf spotting due to the susceptibility of the crop to infection in

early growth stages. The objectives of this work are to determine effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia of *L. maculans* and L. *biglobosa* on stem debris and to determine the first appearance of phoma leaf spotting after ascospore release.

Materials and Methods

Winter oilseed rape field experiments, 2010/2011 and 2011/2012

The winter oilseed rape field experiments were situated at Rothamsted Research, Harpenden, Hertfordshire, for the 2010/2011 and 2011/2012 growing seasons. After harvest (July) for the 2010/2011 growing season, the winter oilseed rape crop was sown in August 2011 for the 2011/2012 growing season. There were nine cultivars of winter oilseed rape planted in the field experiments. The cultivars had different combinations of resistance (*R*) genes or were without *R* genes; some had quantitative resistance (QR) (Delourme *et al*., 2006). The cultivars were Adriana (*Rlm 4* + QR), Bilbao (*Rlm 4*), Capitol (*Rlm 1*), Drakkar (no *R* gene), DK Cabernet (*Rlm 1* + QR), Es-Astrid (QR), Excel (*Rlm 7*), NK Grandia (QR) and Roxet (*Rlm 7*). In autumn/winter, an assessment of phoma leaf spots was done for the 2011/2012 growing season. In both growing seasons, the field experiments were in randomised block designs with three replicate blocks, each with nine plots.

Assessment of maturation and density of pseudothecia on crop debris

Stems of the nine winter oilseed rape cultivars that were collected after harvest in August 2011 from the Rothamsted field experiment were incubated under natural conditions at Bayfordbury, the University of Hertfordshire field station, starting in September 2011. From each of the nine cultivars, five stem bases and upper stems were sampled weekly at random to monitor pseudothecial maturation and development of ascospores by microscopic examination. Five pseudothecia were randomly excised from each stem by using forceps and placed on a glass slide in a drop of distilled water. Then a cover slip was placed on the slide and pressure was applied to force the asci out. The pseudothecial maturation Classes A–D were based on the stage of development of asci and ascospores; Class A: asci and ascospores undifferentiated, Class B: asci differentiated and ascospores undifferentiated, Class C: asci and ascospores differentiated and Class D: asci mature with eight spores per ascus (Fig. 1) (Toscano-Underwood *et al*., 2003). The final stage of maturation was Class E when the pseudothecia are empty. The numbers of pseudothecia in each maturity class were recorded for all nine cultivars. At the end of the experiments (when all the pseudothecia were in Class D or Class E), three stems with abundant pseudothecia were chosen per cultivar. All stems were cut into small pieces (0.8 cm × 5 cm) at the stem bases (< 5 cm above ground level) and upper stems (> 10 cm above ground level). The number of pseudothecia on each piece was counted under a binocular stereo-microscope (at ×60 magnification). The density of pseudothecia on each stem was calculated by taking the mean from the stem pieces from each of the three stems per cultivar.

Air sampling and ascospore counting

Approximately 150 stems per cultivar were placed around a Burkard spore sampler at Bayfordbury in September 2011. A Burkard spore sampler was used to collect ascospores released from pseudothecia that developed on the stems, with daily monitoring of the weather parameters. The exposed tape was removed from the sampler drum at 7-day intervals. Each tape was cut into 48 mm long pieces that each represented a 24-h period. Then the tape pieces were mounted on a microscope slide and stained with 0.1% (w/v) tryphan blue in lactophenol. The daily release of ascospores was calculated from the average numbers of ascospores counted in two longitudinal traverses of each piece of tape under a microscope (×100 magnification). The daily

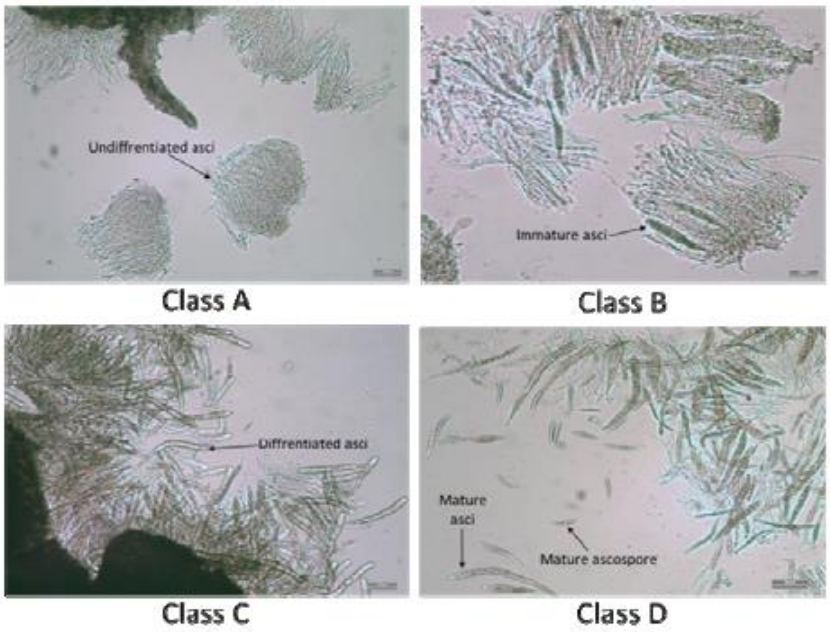

Fig. 1. Pseudothecial maturation; Classes A – D were based on the stage of development of asci and ascospores. Class A: asci and ascospores undifferentiated, Class B: asci differentiated and ascospores undifferentiated, Class C: asci and ascospores differentiated and Class D: asci mature with eight spores/ ascus (Toscano-Underwood *et al*., 2003).

mean concentration of ascopores per cubic metre of air was calculated (Lacey & West, 2006) as (N × 0.28/ width of one traverse (mm)), where N was the daily total count of ascospores.

Phoma leaf spot assessments (autumn/winter), 2011/2012

Ten plants selected at random were collected from each plot at Rothamsted on 6 February 2012 for the 2011/2012 growing season. The the numbers of leaf spots caused by *L. maculans* or *L. biglobosa*, respectively, on each of the true leaves were recorded. The first symptoms, which are the phoma leaf spots that lead to early stem cankers, were differentiated visually between *L. maculans* and *L. biglobosa*. In general, *L. biglobosa* causes small darker leaf lesions than *L. maculans*, which produces large pale grey lesions with many pycnidia.

Results

There were differences in pseudothecial maturation between the susceptible cultivar Drakkar and the other eight more resistant cultivars with or without *R* genes. Mature pseudothecia (Class D) were first observed on harvested stems of the susceptible cultivar Drakkar, with eight ascospores per ascus in the first week of September 2011 (Table 1). In early October, many pseudothecia were mature (Class D) on stems of cultivars Drakkar (48%), ES Astrid (44%) and Adriana (40%), whereas on stems of other cultivars they were still at an early stage of maturation. By 13 December 2011, most cultivars had some empty pseudothecia (Class E). The greatest mean density of pseudothecia on the stem base (< 5 cm above ground level) was on Capitol (*Rlm 1*, 449 per cm^2), whereas on upper stems (> 10 cm above ground level) the greatest density was on Drakkar (no *R* gene, 431 per cm^2) (Table 2).

Table 1. *Number of mature (Class D) pseudothecia observed from a weekly sample of 25 pseudothecia obtained from stem base of each of nine winter oilseed rape cultivars exposed at Bayfordbury, Hertfordshire, September to December 2011*

Sample date	Number of mature pseudothecia								
	Adriana (*Rlm4*+ QR)	Bilbao (*Rlm4*)	Capitol (*Rlm1*)	DK Cabernet (*Rlm1*+ QR)	Drakkar (No *R* gene)	ES Astrid (QR)	Excel (*Rlm7*)	NK Grandia (QR)	Roxet (*Rlm7*)
13 Sep	0	0	0	0	8	0	0	0	0
20 Sep	0	0	1	0	0	0	0	0	0
27 Sep	0	1	0	0	2	0	0	0	0
4 Oct	10	0	4	0	12	11	8	2	1
11 Oct	15	8	11	7	15	18	20	21	11
18 Oct	16	14	11	22	19	22	16	19	17
25 Oct	20	14	14	14	15	22	12	9	19
1 Nov	21	13	15	17	9	25	25	19	18
8 Nov	17	10	13	17	6	19	22	18	16
15 Nov	13	7	11	19	5	17	19	15	13
22 Nov	11	4	9	16	3	13	17	11	10
29 Nov	8	2	5	14	1	9	13	9	6
6 Dec	6	2	2	11	3	5	11	7	4
13 Dec	5	0	2	8	1	3	9	2	3

Table 2. *The densities of pseudothecia in Class D or Class E (pseudothecia per cm2) produced on winter oilseed rape stem base and upper stem debris exposed at Bayfordbury, Hertfordshire, UK on 13 December 2011*

Cultivar	Pseudothecial density (number per cm^2)	
	Upper stem (> 10 cm above ground level)	Stem base (< 5 cm above ground level)
Adriana (*Rlm4*+QR)	175.0	97.3
Bilbao (*Rlm4*)	124.3	90.7
Capitol (*Rlm1*)	363.0	449.0
DK Cabernet (*Rlm1*+QR)	179.7	164.3
Drakkar (No *R* gene)	431.0	230.3
ES Astrid (QR)	217.3	264.7
Excel (*Rlm7*)	83.0	40.3
NK Grandia (QR)	85.7	45.0
Roxet (*Rlm7*)	79.7	244.7

In the Bayfordbury experimental plot in autumn 2011, the release of ascospores was later than in many growing seasons and many ascospores were released in December 2011 and January 2012 (Fig. 2). The greatest number of ascospores released was in late January 2012, with an average 1224 spores per m^3. From September 2011 to November 2011, there was little rain. More rainfall occurred in December 2011. In January 2012, there was substantial rainfall and the weather conditions were suitable for the maturation and release of ascospores. The average daily temperature was 8.8°C and total rainfall was 15.9 mm, with 84 rain days recorded in the period from the start of September 2011 to end of February 2012.

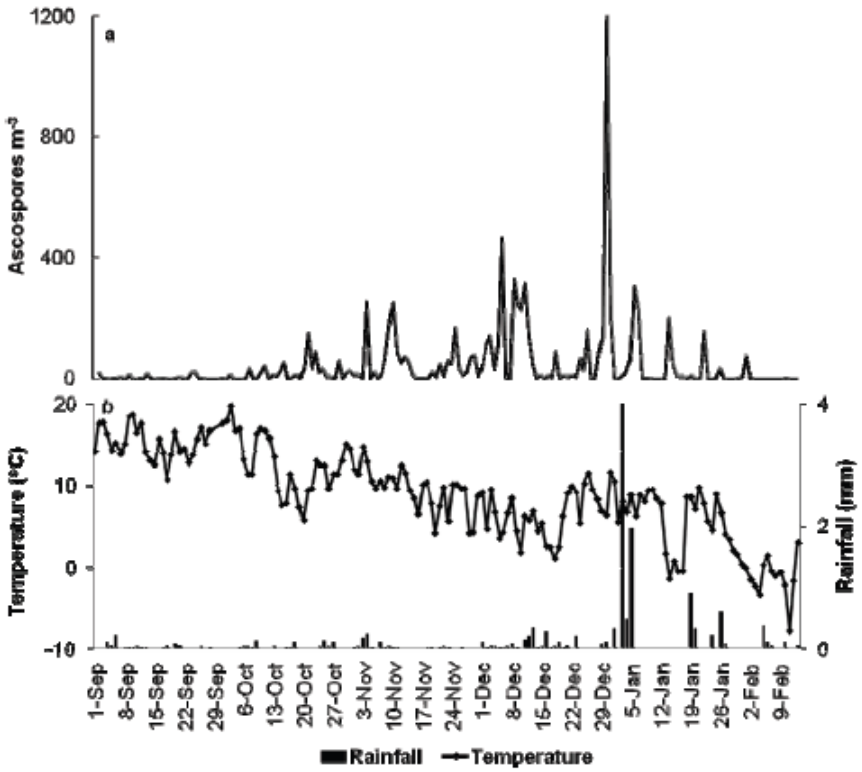

Fig. 2. Daily concentrations of ascospores released in the period from September 2011 to February 2012 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury, Hertfordshire, UK and collected by a Burkard spore sampler (a) in relation to daily total rainfall (mm) and average temperature (°C) (b).

Table 3. *Numbers of phoma leaf spots caused by L. maculans or L. biglobosa on leaves of 30 plants per cultivar of winter oilseed rape sampled for the 2011/2012 growing season on 6 February 2012*

Cultivar	Average number of leaves plant^{-1}	Number phoma leaf spots found on leaves of 30 plants	
		L. maculans (large pale lesions)	*L. biglobosa* (small dark lesions)
Adriana (*Rlm 4* + QR)	10	15	4
Bilbao (*Rlm 4*)	7	10	5
Capitol (*Rlm 1*)	8	10	8
DK Cabernet (*Rlm 1*+ QR)	7	13	6
Drakkar (no *R* gene)	9	43	8
Es-Astrid (QR)	8	8	5
Excel (*Rlm 7*)	7	2	4
NK Grandia (QR)	9	8	7
Roxet (*Rlm 7*)	8	7	12
Total	-	116 (66.3%)	59 (33.7%)

In the 2011/2012 growing season, phoma leaf spotting appeared in early February 2012 in the Rothamsted field experiment on leaves of all nine cultivars with or without different combinations of resistance (*R*) genes and quantitative resistance (QR). Results from the assessment of plants sampled on 6 February 2012 showed that there were differences between cultivars in number of phoma leaf spots (Table 3). There was more leaf spotting caused by *L. maculans* than by *L. biglobosa*, with 66% of the total number of leaf lesions being large pale grey lesions and 34% being small darker lesions from a total of 175 lesions found on leaves of 270 plants (10 per plot with three plots per cultivar) observed. From all the nine cultivars, only Excel (*Rlm 7*) and Roxet (*Rlm 7*) had more leaf spotting caused by *L. biglobosa* than by *L. maculans*.

Discussion

The results suggest that there were differences in pseudothecial maturation and density between the cultivars, with pseudothecia on the susceptible cultivar (Drakkar) maturing faster than those on cultivars with quantitative resistance with or without *R* gene-mediated resistance (Adriana, Bilbao, Capitol, DK Cabernet, Es-Astrid, Excel, NK Grandia and Roxet) in September and October 2011 (Table 1). This may have been related to differences between cultivars in severity of disease at harvest, since there is evidence that after harvest the numbers of pseudothecia increase and that they mature faster with increasing severity of disease at harvest (Lo-Pelzer *et al*., 2009).

The observation that the release of ascospores in this season was later than in many growing seasons suggests that weather factors (temperature and rainfall) affected the maturation of pseudothecia and the timing of the first major ascospore release. The wetness provided by rainfall is essential for pseudothecial maturation of both *L. maculans* and *L. biglobosa* and more ascospores are released on days with rain (Huang *et al*., 2005; Toscano-Underwood *et al*., 2003). *L. biglobosa* naturally matures more slowly than *L. maculans* and therefore most *L. maculans* ascospores are usually released early in autumn/winter (Fitt *et al*., 2006). These differences may explain why there was less *L. biglobosa* leaf spotting than *L. maculans* leaf spotting observed on 6 February 2012.

The late appearance of phoma leaf spotting in February 2012 (usually leaf spotting appears in October/November) was associated with a low incidence of phoma stem base canker in summer 2012 (data not presented). Previous work has showed that a high incidence of phoma leaf spots (100%) in winter 2000/2001 is associated with a high incidence of stem canker (100%) in the following spring/summer (Huang *et al*., 2002). Timing of the first phoma leaf spots of this monocyclic disease is an important factor determining the severity of phoma stem canker in the following spring/summer (West *et al*., 2002; Huang *et al*., 2005).

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Predicting timing of release of ascospores of *Leptosphaeria spp.* to improve control of phoma stem canker on oilseed rape in the UK

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Summary

Phoma stem canker is an economically damaging disease of oilseed rape crops, causing an annual loss estimated at more than £87M in the UK at a price of £390/t. It is caused by two related pathogens: *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, with *L. maculans* being more damaging to the stem bases of infected crops. The rate of maturation of pseudothecia on previous infected crop residues is dependent on wetness-adjusted daily temperature and therefore dates of release of significant number of ascospores from pseudothecia are affected by weather conditions each year. Accurate estimates of timing of ascospore release are essential for guiding timing of sprays on autumn-sown crops to manage severe phoma stem canker epidemics the next spring. Effective control of this disease is critical to protect the potential yield and therefore to achieve high, stable oilseed rape yields. Daily observations of numbers of ascospores of *Leptosphaeria* species, and records of daily mean temperature and rainfall at four separate sites from 2010/2011, 2011/2012 and 2012/2013 cropping seasons were used to validate existing weather-based models developed to predict the date of the first major release of ascospores after harvest of the previous crops. The usefulness and merits of these weather-based models were then evaluated for control of phoma stem canker epidemics in relation to annual uncertainties in the controlling weather variables both in the past and under future climate change. These assessments were considered in relation to methods that were proposed and are currently used to guide fungicide control of phoma stem canker disease in the UK.

Key Words/phrases: oilseed rape, climate change, phoma stem canker, *Leptosphaeria maculans, Leptosphaeria biglobosa,* disease forecast, optimal timing of fungicide spray

Introduction

Phoma stem canker is an important disease of oilseed rape and can cause appreciable economic losses, depending on the disease incidence and severity in the given cropping season. The disease is caused by the related *Leptosphaeria* species: *L. maculans* and *L. biglobosa*. In the UK, where oilseed rape is autumn-sown and harvest starts in July/August the following year, both pathogens produce airborne ascospores that develop in pseudothecia on infected crop stems. After landing on oilseed rape leaves, ascospores of both species infect the leaf through stomata. They then grow in leaf tissues and produce phoma leaf spot lesions. Subsequently, both pathogens grow symptomlessly along leaf petiole tissues to reach the stem base where stem cankers are formed.

Effective management of phoma stem canker relies on growing resistant cultivars and spraying fungicides when oilseed rape crops are at a vulnerable stage. When fungicides are used, it is critical that growth of pathogens from leaves into the stems can be stopped. For example, optimal timings of fungicide applications were recommended to be on dates when 10% of plants

sampled had phoma leaf spots in autumn in the UK (West *et al*. 1999). The rate of progress towards pseudothecial maturity and the subsequent timing of ascospore release on affected crop debris are governed by temperature and rainfall (Toscano-Underwood *et al*. 2003). Weather-based models using temperature and rainfall were developed to predict the date of release of ascospores of *Leptosphaeria* spp. in order to improve control of the disease (Salam *et al*., 2007; Huang *et al*., 2007).

The objectives of this paper were to assess the accuracy in the estimation of first dates when ≥10% of the maximum daily spore count occurred at four sites over three cropping seasons using the weather-based model by Huang *et al* (2007) and the weather-based model – SporacleEzy by Salam *et al* (2007). Meanwhile, 200 years of daily future weather data generated with a stochastic weather generator - LARS-WG version 5 (http://www.rothamsted.ac.uk/mas-models/larswg.php) at Rothamsted Research, near Harpenden, were used to assess the likely changes in distribution of the date of first major ascospore release during the cropping season under a medium CO_2 emission scenario in the time periods of 2020s, 2050s and 2080s with baseline weather records from 1975 to 2005 as a reference.

Materials and methods

Numbers of airborne ascospores of *Leptosphaeria* species were monitored and counted with a Burkard volumetric spore sampler (Burkard Manufacturing Company Ltd) surrounded by affected oilseed rape debris for three consecutive cropping seasons, 2010/2011, 2011/2012 and 2012/2013 at Cowlinge, Suffolk, Rothwell, Lincolnshire, Whittlesford, Cambridgeshire, and Rothamsted Research or Bayfordbury, Hertfordshire (Fig. 1). Weather data were recorded using on-site weather stations at Rothamsted, Bayfordbury and Cowlinge, while those weather data were obtained from the nearest synoptic weather station - Waddington for Rothwell and Andrewsfield for Whittlesford.

Fig. 1. Locations where airborne ascospores of *Leptosphaeria* species were collected using Burkard volumetric spore samplers.

Huang *et al.* (2007) reported that the first date with ascospore counts ≥10% of the maximum daily ascospore release was closely related to date when either 30% or 50% of pseudothecia reached maturity. Therefore, they developed models for predicting dates when 30% or 50% of pseudothecia reached maturity to estimate the first date with at least 10% of the

maximum daily ascospore release. For predicting the date when 30% of pseudothecia were mature (P_{30}), the following equation was established and is used:

$$P_{30} = \sum_{i=1}^{n} [\frac{1}{11.6 + 69.3(0.89)^{T_i}}] R_{bi} \qquad (1)$$

where P_{30} was the accumulated daily rainfall-adjusted rate of progress towards 30% pseudothecial maturity, T_i was the mean daily temperature of the day(i) and R_{bi} was the threshold daily rainfall adjustment factor of the day(i). The threshold daily rainfall was set at 0.5 mm. Thus, if the rainfall for the day is > 0.5 mm, R_{bi} =1 otherwise R_{bi} =0. The starting date for accumulating P_{30} was from 1 August, and the date with 30% of pseudothecia matured was when the accumulated P_{30} reaches one or above (i.e. $P_{30} \geq 1$).
Similarly, the predicted date when 50% pseudothecia are mature (P_{50}) was calculated with the following equation:

$$P_{50} = \sum_{i=1}^{n} [\frac{1}{10.5 + 72.09(0.92)^{T_i}}] R_{bi} \qquad (2)$$

Salam *et al.* (2007) developed a suitable model (SporacleEzy) for predicting the first date with ascospore count ≥10% of the maximum daily ascospore release under the UK conditions. This model was based on the accumulated number of days favourable for pseudothecial maturation. A day was considered as a day favourable for pseudothecial maturation when the rainfall was ≥ 1 mm and the mean temperature was >6 and <22°C. To predict the first date with at least 10% of the maximum daily ascospore release, a total of 18 days favourable for pseudothecial maturation should be accumulated from 1 August.

To assess the impact of future climate change on the distribution of first date with a first major ascospore release, 200 years of daily maximum and minimum temperature and rainfall were generated for 2020s, 2050s and 2080s under a medium CO_2 emission scenario at Rothamsted using the stochastic weather generator LARS-WG and site parameters derived from weather records during the period 1975-2005 treated as baseline weather. Future climate change scenarios were projected by altering the baseline site parameters using change factors derived from climate projections with the global climate model HadCM3 of UK Met Office (Semenov and Stratonovitch, 2010).

Results

Daily temperature and rainfall and daily release of ascospores of Leptosphaeria. species

Records of weather were data logged on sites at Rothamsted, Bayfordbury and Cowlinge during the three consecutive cropping seasons, but were obtained from synoptic weather stations at Waddington and Andrewsfield for Rothwell and Whittlesford, respectively. Counts of numbers of ascospores released daily by *Leptosphaeria* species were complete from the starting date to the finishing date, except for some days in late December and January in 2011/2012 and 2012/2013 at Whittlesford. The ascospore counts showed distinctive peaks of maximum daily ascospore release for most cropping season and site combinations (e.g. at Rothamsted in 2010/2011, shown in Fig. 2), but maxima were less clear for few of them (e.g. at Bayfordbury in 2011/2012, shown in Fig. 3). The first date with ≥10% of the maximum daily ascospore release was identified and used to validate the weather-based models developed by Huang *et al.* (2007) and Salam *et al.* (2007).

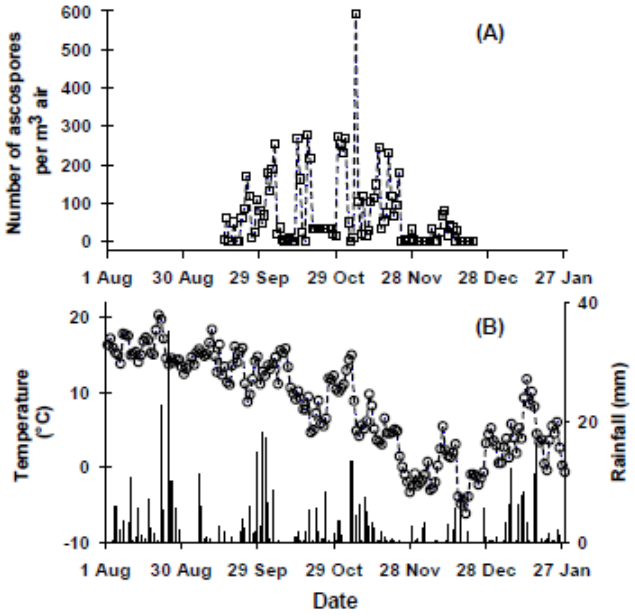

Fig. 2 The daily number of ascospores released by *Leptosphaeria* species (A) (open squares) in relation to daily mean air temperature (open circles) and daily rainfall (vertical bars) (B) for the 2010/2011 cropping season at Rothamsted, Hertfordshire.

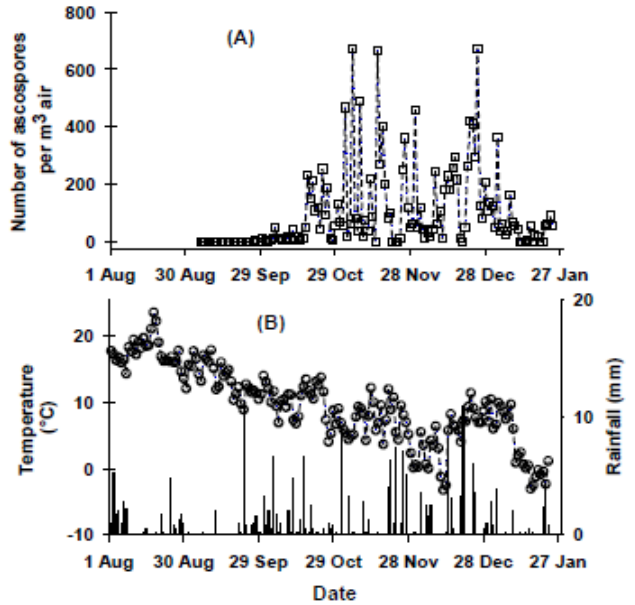

Fig. 3 The daily number of ascospores released by *Leptosphaeria* species (A) (open squares) in relation to daily mean air temperature (open circles) and daily rainfall (vertical bars) (B) for the 2011/2012 cropping season at Bayfordbury, Hertfordshire

The observed and estimated first date with ≥10% of the maximum daily ascospore release

Table 1 The observed first date with ≥10% of the maximum daily ascospore release, the estimated dates when either 30% or 50% pseudothecia were mature using equations developed by Huang *et al.* (2007), and the estimated date corresponding to first date with ≥10% of the maximum daily ascospore release using the SporacleEzy model developed by Salam *et al.* (2007).

Site	Crop season	First 10% of maximum ascospore release date	Estimated 30% pseudothecia mature date	Estimated 50% pseudothecia mature date	SporacleEzy-estimated first ≥10% of maximum ascospore release date
Rothamsted	2010/2011	16-Sep-2010	13-Sep-2010	27-Sep-2010	14-Sep-2010
Bayfordbury	2011/2012	4-Nov-2011	23-Oct-2011	22-Nov-2011	26-Sep-2011
	2012/2013	18-Oct-2012	5-Oct-2012	1-Nov-2012	8-Oct-2012
Cowlinge	2010/2011	13-Oct-2010	24-Sep-2010	4-Oct-2010	19-Sep-2010
	2011/2012	19-Nov-2011	6-Oct-2011	3-Nov-2011	27-Oct-2011
	2012/2013	21-Oct-2012	16-Oct-2012	10-Nov-2012	14-Oct-2012
Rothwell	2010/2011	15-Oct-2010	12-Sep-2010	30-Sep-2010	6-Sep-2010
	2011/2012	24-Nov-2011	11-Oct-2011	7-Nov-2011	8-Oct-2011
	2012/2013	10-Oct-2012	25-Sep-2012	15-Oct-2012	23-Sep-2012
Whittlesford	2010/2011	15-Oct-2010	23-Sep-2010	13-Oct-2010	26-Sep-2010
	2011/2012	10-Oct-2011	19-Sep-2011	25-Oct-2011	17-Oct-2011
	2012/2013	10-Nov-2012	12-Oct-2012	25-Oct-2012	1-Oct-2012

Deviation of estimated from observed date with ≥10% of the maximum daily ascospore release

Table 2 shows the deviation in days between the predicted and the observed first date with ≥10% of the maximum daily ascospore release for each cropping season at different sites using weather-based models by Huang *et al.* (2007) and Salam *et al.* (2007). It was clear that the model for predicting 30% pseudothecial maturity date and the SporacleEzy model both estimated the first date with ≥10% of the maximum daily ascospore release earlier than the observed date in all cases. However, the model for predicting 50% pseudothecial maturity date estimated dates that were both earlier and later than the observed dates (Table 2). Calculated root mean square deviation (RMSD) indicates that the first dates with ≥10% of the maximum daily ascospore release estimated by the model for predicting 50% pseudothecial maturation date deviated least from the observed dates (Table 2).

Table 2 Deviation of estimated from observed first date with ≥10% of the maximum daily ascospore release using models of predicting dates when 30% and 50% pseudothecia were mature by Huang *et al.* (2007) and using the SporacleEzy model by Salam *et al.* (2007)

Site	Crop season	Deviation of estimated date from observed date with ≥10% of maximum daily ascospore release*		
		Using 30% pseudothecia mature date	Using 50% pseudothecia mature date	Using SporacleEzy
Rothamsted	2010/2011	-3	11	-2
Bayfordbury	2011/2012	-12	18	-39
	2012/2013	-13	14	-10
Cowlinge	2010/2011	-19	-9	-24
	2011/2012	-44	-16	-23
	2012/2013	-5	20	-7
Rothwell	2010/2011	-33	-15	-39
	2011/2012	-44	-17	-47
	2012/2013	-15	5	-17
Whittlesford	2010/2011	-22	-2	-19
	2011/2012	-21	15	7
	2012/2013	-29	-16	-40
RMSD**		25.2	14.2	27.1

*Deviation is calculated as (estimated date – observed date), so negative and positive values mean that the predicted dates are earlier and later than observed dates, respectively.
**RMSD is root mean square deviation and smaller values of RMSD mean better accuracy than larger ones.

The impact of climate change on distributions of first date with ≥10% of a maximum daily ascospore release

The 200 years of daily maximum and minimum temperature, and rainfall generated by LARS-WG weather generator were used to examine the changes in these weather variables *per se* and their impact on distributions of the first date with ≥10% of a maximum daily ascospore release with reference to the baseline weather from 1975 to 2005 under a medium CO_2 emission scenario at Rothamsted, Hertfordshire. Since the weather conditions in autumn (September to November inclusive) are most relevant to the epidemics of phoma stem canker, the changes in temperature and rainfall conditions were calculated and are shown in Table 3. It is in concordance with projections by the 2009 UK Climate Projection programme (© UK Climate Projections 2009) (http://ukclimateprojections.metoffice.gov.uk) that autumnal maximum, minimum and average temperatures will be warmer in the future. However, unlike the changes projected for reduced rainfall in summer and increased rainfall in winter, both the number of rain days and total rainfall in autumn showed little change in the future (Table 3).

The first date with ≥10% of a maximum daily ascospore release was calculated using the model for predicting 50% pseudothecial maturation date by Huang *et al.* (2007) and the SporacleEzy model of Salam *et al.* (2007) and its distribution is shown in Fig. 4 for baseline weather and in 2020s, 2050s and 2080s. Because of the importance in accumulating rainfall-adjusted rate of progress towards pseudothecial maturity in the model by Huang *et al.* (2007), the median date of a major ascospore release changed from the current date 11 November to 1 November in 2080s (i.e. it is likely to occur earlier). However, the variability in first date with ≥10% of a maximum daily ascospore release started to decrease a little in 2080s. Since the number of rain days showed little change in the future and this value had a controlling role in accumulating the necessary number of days favourable for pseudothecia maturation in the

SporacleEzy model by Salam *et al.* (2007), there was not much change in the distribution and associated variability of the first date of a major ascospore release in any future time period compared with the baseline weather conditions (Fig. 4). The median first major ascospore release date estimated by the SporacleEzy model changed from the current date 11 October in the baseline weather to 18 October in 2080s (i.e. it is likely to be later).

Table 3 The median maximum (Tmax), minimum (Tmin) and average (Tave) air temperature, number of rain days when daily rainfall above zero (Rain>0.0) and above 0.5 mm (Rain>0.5) and total rainfall in Autumn (September to November inclusive) in the baseline weather (1975-2005), 2020s, 2050s and 2080s under a medium CO_2 emission scenario at Rothamsted, Hertfordshire.

Variable	Time period			
	Baseline	2020s	2050s	2080s
Tmax (°C)	13.8	14.5	16.1	17.1
Tmin (°C)	6.7	7.3	8.9	9.9
Tave (°C)	10.2	10.9	12.5	13.5
Rain>0.0 (day)	49.0	49.0	50.0	50.0
Rain>0.5 (day)	37.0	37.0	37.0	37.0
Total rainfall (mm)	199.6	202.3	204.4	200.0

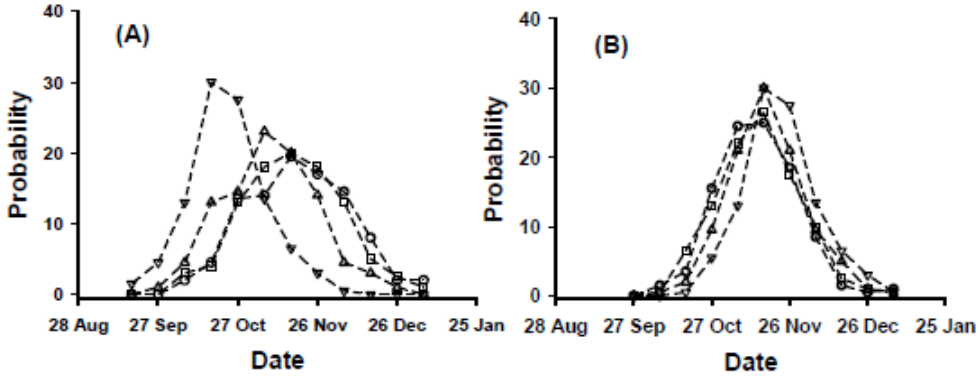

Fig. 4 The distribution of first date with ≥10% of a maximum daily ascospore release based on the date when 50% pseudothecia are mature using the model by Huang *et al.* (2007) (A) and predicted with the SporacleEzy model by Salam *et al.* (2007) for the baseline weather (1975-2005) (open circle) and in 2020s (open squares), 2050s (upper triangle) and 2080s (down triangles) under a medium CO_2 emission scenario at Rothamsted, Hertfordshire.

Discussion

Although severe and apparent symptoms of stem cankers are developed and noticeable on infected oilseed rape crops in spring and summer in the UK, effective control of phoma stem canker epidemics mostly depends on the optimal timing of fungicide applications during the

phoma leaf spotting stage in the previous autumn when crop plants are still in early vegetative phase. Although the model to predict 50% pseudothecia maturity date was better able to estimate the onset of a first ≥10% of the maximum daily ascospore release than the model to predict 30% pseudothecia maturity date and the SporacleEzy model, the accuracy of these models may have been compromised by the quality of both weather and ascospore data and also by the use of weather data of synoptic weather stations distant from experimental fields at Rothwell and Whittlesford. It was assumed that sub-zero temperatures (i.e. <0°C) stopped pseudothecial maturation process (Huang *et al*., 2007). However, ascospore release from mature pseudothecia continued when daily mean temperatures were <-3°C (Fig. 2).

Because the temperature and rainfall conditions vary with locations and from season to season, these variations then lead to variability in start of a major release of *Leptosphaeria* airborne inoculums to infect oilseed rape leaves. Thus, use of accurate and reliable models with these weather variables to estimate the timing of onset of a major ascospore release can guide decisions on spraying fungicides to manage phoma stem canker epidemics. The UK current system to determine the time of spraying fungicides was based on estimating the date when a threshold of 10% of plants were shown or likely to show phoma leaf spots in autumn by weekly crop inspections or using a model developed by Evans *et al*.(2006). Furthermore, varieties with different resistant genes can lead to various dates with 10% of plants showing phoma leaf spots even airborne ascospores land on the leaf at the same time. This can have bad consequences if the current model is used to predict the date with a threshold of 10% of plants infected with phoma leaf lesions irrespective of effects of varieties. Therefore, if the validity of a given model to predict the onset of a first major ascospore release can be proved as an effective substitute for the threshold of 10% of plants infected with phoma leaf spots, this new model can then provide a much earlier warning and a greater control of this disease.

The impact of future climate change on the distribution of a first major onset of ascospore release depended on the use of weather-based models (Fig. 4). The median date of a first major ascospore release was not likely to be much affected by climate change based on the use of the SporacleEzy model. However, when the model to predict 50% pseudothecial maturity date was used, the median date of the first major ascospore release tended to be earlier in the future relative to the baseline weather as a reference.

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List of Conferences

Year 2011

1. British Society of Plant Pathology Meetings 2011: The Impact of Bioactive Small Molecules in Plant Pathology., 15/12/2011 - 16/12/2011, Clare College, University of Cambridge. (Poster).

Year 2012

2. Research Day School of Life and Medical Sciences, 15/05/2012 -15/05/2012, University of Hertfordshire. (Poster).

3. Crop Protection and Conservation and Communication Conference, Participant, 20/09/2012 - 20/09/2012, University of Hertfordshire.

4. Crop Protection in Southern Britain Conference. Poster presenter, 27/11/2012 - 28/11/2012, Peterborough Arena, UK. (Poster).

5. British Society of Plant Pathology Meetings 2012: Fitness Costs and Trade-offs in Plant-Parasite Interactions., 16/12/2012 - 18/12/2012, Norwich, UK. (Poster).

Year 2013

6. Postgraduate Seminar School of Life and Medical Sciences., 16/04/2013 - 16/04/2013, University of Hertfordshire. (Oral presentation).

7. UK Brassica research community Annual meeting, Participant, 09/05/2013 - 09/05/2013, Rothamsted research, Harpenden.

8. 11th Annual Life Sciences Research Day, 15/05/2013 - 15/05/2013, University of Hertfordshire. (Oral presentation).

9. Crop Protection, principles and practice, Participant, 27/02/2013 - 01/03/2013, University of Hertfordshire.

10. Crop Pathogens, Pests & Weeds, Participant, 23/05/2013 - 25/05/2013, University of Hertfordshire and Rothamsted Research.

11. 10th International Congress of Plant Pathology (ICPP 2013, 25/08/2013 - 30/08/2013, Beijing, China. (Oral Presentation).

12. Agriculture and Horticulture Development Board (AHDB) Crop Research Conference, Poster presenter, 25/09/2013 - 25/09/2013, Queen Elizabeth II Centre, London. (Poster).

13. British Society of Plant Pathology Meetings 2013: Visions of plant disease management in 2050: metropolis, arcadia or dystopia?, 17/12/2013 - 18/12/2013, Conference Aston, Conference Centre and Hotel, University of Aston, Birmingham. (Oral presentation).

Year 2014

14. School of Life and Medical Sciences Research Conference, 09/04/2014 - 10/04/2014, University of Hertfordshire. (Oral Presentation).

Abstract (Oral presentation)

1. Postgraduate Seminar School of Life and Medical Sciences, 16 April 2013, University of Hertfordshire.
2. 11th Annual Life Sciences Research Day, 15 May 2013, University of Hertfordshire.

Abstract:

Effects of host resistance on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* (cause of phoma stem canker) in *brassica napus* (oilseed rape)

Siti Nordahliawate M.Sidique, Yong-Ju Huang, Avice M. Hall and Bruce D.L. Fitt
School of Life and Medical Sciences, University of Hertfordshire, UK

Introduction: *Leptosphaeria maculans* and *L. biglobosa* co-infect winter oilseed rape plants to cause phoma stem canker disease. The sexual spores of both species are produced in pseudothecia on infected winter oilseed rape stem debris after harvest and this is the most important source of inoculum for infection of newly-emerged plants in autumn. Current surveys by Crop Monitor showed that the proportion of UK crops affected by the disease was high in both 2012 (83%) and 2011(96%). This study investigates effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia on stem debris. **Methods:** Stems of nine oilseed rape cultivars with *R*-genes or without *R*-genes that were collected after harvest in August 2011 and 2012 from the Rothamsted field trial were incubated under natural conditions at Bayfordbury starting in September 2011 and 2012. Then five stems were sampled weekly at random to monitor the pseudothecial maturation and develop of the ascospores by microscopic examination. Each pseudothecium was characterized into Class A and B for early stage, Class C and D for mature stage with ascospores produce and Class E, empty pseudothecium. Stems for pseudothecial maturation were placed around the Burkard spore sampler that was used to collect ascospores released from pseudothecia that developed on the stems with daily monitoring of the weather parameters. The exposed tape was removed from the sampler drum every 7-day intervals. Each tape was cut into 48 mm long pieces that represented a 24-h period and were mounted on a microscope slide. The number of ascospores was counted in two longitudinal traverses of each piece of tape. **Results:** Results of pseudothecial maturation and ascospore release in natural conditions suggest that weather factors (temperature and rainfall) affected the maturation of pseudothecia and the timing of the first major ascospore release and subsequent phoma leaf spot development for both growing seasons. In December 2011, there was more rainfall (total rainfall 51.9 mm) with temperature $<10^{o}$C. Subsequently ascospores released more in the third week of January 2012 after having enough wet and the phoma leaf spots were observed in early February 2012. For 2012/2013 growing season the first ascospores released was much earlier in early October 2012 and phoma leaf spots were observed in 24 October 2012 with more rainfall recorded than previous season starting from early September until end of December 2012 (total rainfall 158.9 mm). **Conclusions:** The results suggest that there were differences in pseudothecial maturation between the cultivars. The wetter September in 2012 demonstrates a difference between pseudothecial maturation on different cultivars, with the fastest maturation occurring on the most susceptible cultivar. This has implications for the subsequent severity of stem canker. There is a need to investigate impact of cultivar resistance on the maturation of pseudothecia and also the possibility that *L. maculans* retards the development of pseudothecia from *L. biglobosa*.

3. 10th International Congress of Plant Pathology (ICPP 2013, 25 August 2013 – 30 August 2013, Beijing, China.

Abstract:

Effects of host resistance on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* (cause of phoma stem canker) in *Brassica napus* (oilseed rape).

S.N.M. Sidique [1,2], Y.J. Huang [1], A.M. Hall[1] and B.D.L. Fitt[1]
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In the UK, phoma stem canker epidemics on winter oilseed rape are initiated in autumn by air-borne ascospores produced in pseudothecia on infected crop debris from previous crops. This study aimed to investigate the effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia on stem debris. In autumn 2011 (2011/2012 growing season), there were no differences between cultivars in pseudothecial maturation after a period of dry weather in September (total rainfall 20.5 mm). After more rainfall in December 2011 (total rainfall 51.9 mm), pseudothecia matured and more ascospores were released. After a major release of ascospores in the third week of January 2012, phoma leaf spots were observed in 6 February 2012. However in autumn 2012 (2012/2013 growing season), when there was more rainfall from September to December (total rainfall 158.9 mm), there were differences between different cultivars in pseudothecial maturation, with the maturation on the most susceptible cv. Drakkar faster than on the other cultivars. The first ascospores were released in early October and phoma leaf spots were observed in 24 October 2012. The early appearance and severity of phoma leaf spotting in autumn 2012 suggests that there will be severe phoma stem canker in the summer of 2013. Results of this study suggest that both weather factors (temperature and rainfall) and host resistance affect the maturation of pseudothecia and the timing of the first major ascospore release. The role of host resistance in pseudothecial maturation requires further investigation.

Topic area: Airborne plant diseases

4. British Society of Plant Pathology Meetings 2013: Visions of plant disease management in 2050: metropolis, arcadia or dystopia?, 17 December 2013, Conference Aston, Conference Centre and Hotel, University of Aston, Birmingham.

5. School of Life and Medical Sciences Research Conference, 9 April 2014 – 10 April 2014, University of Hertfordshire.

Abstract:

Effects of host resistance on maturation of pseudothecia of *Leptosphaeria* spp. (cause of phoma stem canker) in *Brassica napus* (oilseed rape)

The severity of phoma stem canker epidemics on winter oilseed rape in summer is greatly influenced by timing of ascospore release in the previous autumn. It is known that weather (temperature and rainfall) influences timing of ascospore release. This work investigated effect of cultivar resistance on pseudothecial maturation (ascospore release) in natural conditions for two seasons and in controlled environment conditions. In autumn 2011, there were no differences between cultivars in pseudothecial maturation after a period of dry weather in September. The first ascospores were released in the third week of January 2012 after 51.9 mm rainfall in December 2011. In autumn 2012, however, there was more rainfall from September to December (total rainfall 158.9 mm) than in 2011 and the pseudothecia of the susceptible cv. Drakkar matured much faster than those of the more resistant cultivars. The first ascospores were released in early October 2012. In autumn 2013, the major release of ascospores started on 18 October and the observation of pseudothecial maturation is in progress. In controlled environment conditions, pseudothecia matured much faster on cv. Drakkar than on other cultivars. The differences between cultivars in pseudothecial maturation have implications for the subsequent severity of stem canker. Therefore, it may be necessary to modify the current forecasting web-site system (http://www.rothamsted.ac.uk/Content-Section=Leafspot.html) to include a factor for cv. resistance.

Poster
Year 2011; British Society of Plant Pathology Meetings 2011

Effects of host resistance on interactions between the hemibiotrophic pathogens *Leptosphaeria maculans* and *L. biglobosa* (cause of phoma stem canker) in *Brassica napus* (oilseed rape)

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(*Corresponding author: s.mohamed-sidique@herts.ac.uk*)

University of Hertfordshire
The School of Life Sciences

INTRODUCTION

- Phoma stem canker is one of the most economically important diseases of winter oilseed rape in UK and other European countries and has spread across Canada and Mexico (Fitt et al., 2006).
- Current surveys by crop monitor showed that the proportion of UK crops affected by the disease was greater in 2011 (96%) than in 2010 (74%).
- Yield loss in diseased crop may be up to 30-50%, due to lodging of the plants
- It is caused by two closely related hemibiotrophic fungal pathogens; *Leptosphaeria maculans* and *L. biglobosa*.
- In the initial colonisation of leaves, the fungus has a biotrophic life style. Later, the necrotrophic phase when the stem cortex is killed results in a dark visible canker.

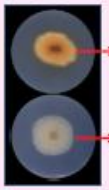
This study aims to investigate effects of different sources of host resistance on interactions between *L. maculans* and *L. biglobosa*.

MATERIALS & METHODS

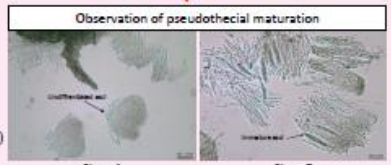
Disease assessment; stems from field sampling

Single pycnidial isolation (on V8 agar with antibiotics for 5 days at 20°C)

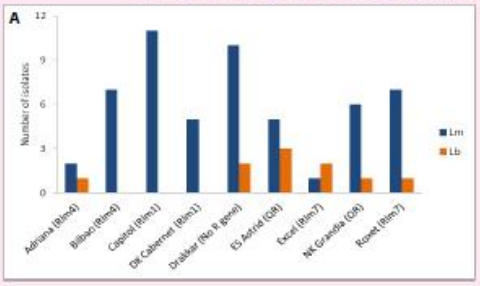
Culture with pycnidia and deep pink spore masses (arrow)



Observation of pseudothecial maturation



RESULTS & DISCUSSION



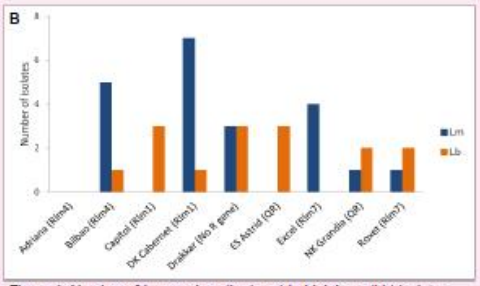


Figure 1: Number of *L. maculans* (Lm) and *L. biglobosa* (Lb) isolates obtained from (A) upper stem and (B) stem base of nine oilseed rape cultivar with *R*-genes or no *R*-genes or quantitative resistance (QR)

- 64 isolates from stem base; 37 isolates from upper stem.
- Proportions of *L. maculans* and *L. biglobosa* differed between stem base (more *L. maculans*) and upper stem (more *L. biglobosa*).
- Proportions of *L. maculans* and of *L. biglobosa* differed between cultivars.

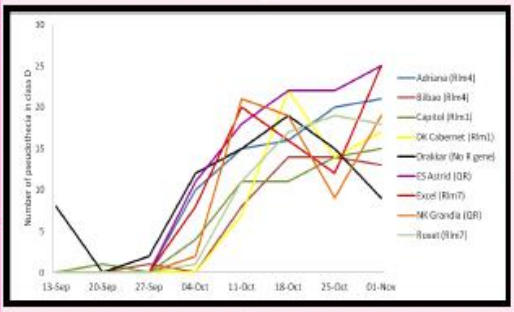


Figure 2: Changes in number of mature pseudothecia observed on nine cultivars, September to November 2011.

- 13th September 2011 -first mature pseudothecia (Class D) observed on Drakkar.
- Differences in pseudothecial maturation between cultivars.

FUTURE WORK

- Continue with disease assessment in winter oilseed rape crops in autumn/winter of 2011/2012 and 2012/2013 and confirm identification using species-specific PCR.
- Characterisation of pseudothecial/ascospore characteristics of *L. maculans* and *L. biglobosa* isolated from different cultivars.
- Further investigation of interactions between *L. maculans*/*L. biglobosa* and host resistance.

ACKNOWLEDGEMENTS

We thank the staff in School of Life Sciences, University of Hertfordshire for their technical assistance and the Ministry of Higher Education (MOSTI), Malaysia for the scholarship.

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E. Lô-Pelzer, et al., (2009). *Plant Pathology* 58, 61-70.
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BSPP Presidential Meeting 2011: The Impact of Bioactive Small Molecules in Plant Pathology, Clare College, Cambridge, UK, 15 to 16 Dec 2011

Maturation of *Leptosphaeria maculans* and *L. biglobosa* pseudothecia and first appearance of phoma leaf spots on winter oilseed rape

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INTRODUCTION

Phoma stem canker symptoms before harvest on winter oilseed rape cv. Drakkar

- Phoma stem canker is one of the most economically important diseases of winter oilseed rape in UK and other European countries (Fitt et al., 2006).
- It is caused by two closely related hemibiotrophic fungal pathogens; *Leptosphaeria maculans* and *L. biglobosa*.
- Current surveys by Crop Monitor showed that the proportion of UK crops affected by the disease was less in 2012 (83%) than in 2011(96%).
- Ascospores produced in mature pseudothecia on crop debris are the main source of inoculum for the initial colonisation of UK crops in autumn.
- In the initial colonisation of leaves, the pathogens have biotrophic life styles. Later, the necrotrophic phase when the stem cortex is killed results in dark visible cankers.

This study aims to determine whether there are differences in pseudothecial maturation on stem debris between cultivars with different types of resistance in relation to effects of environment conditions on the maturation.

MATERIALS & METHODS

Phoma leaf spot assessments (autumn/winter), 2011/2012

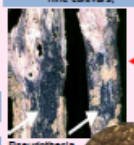
Sampled on 6 February 2012, field experiments: Rothamsted Research, Harpenden, Hertfordshire

Ten plants selected at random were collected from each plot

Numbers of leaf spots caused by *L. maculans* and *L. biglobosa* were recorded

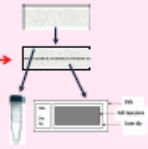
Maturation of pseudothecia on crop debris

Five stems were sampled weekly at random to monitor the pseudothecial maturation and development of the ascospores by microscopic examination on each of the nine cultivars.

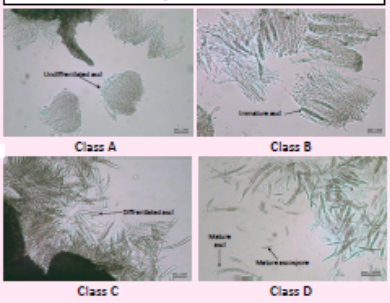


Pseudothecia (arrow) produced on stem bases

Stem debris of Adriana (*Rlm 4* + QR), Bilbao (*Rlm 4*), Capitol (*Rlm 1*), Drakkar (no *R* genes), DK Cabernet (*Rlm 1* + QR), ES-Astrid (QR), Excel (Rlm 7), NK Grandia (QR) and Roxet (Rlm 7) at Bayfordbury, Hertfordshire for air sampling and ascospore counting with daily monitoring of the weather parameters



Observation of pseudothecial maturation



RESULTS

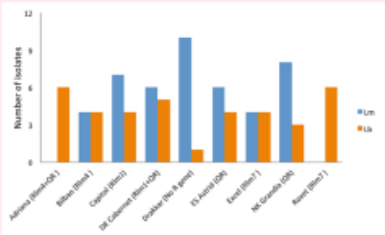


Figure 1: Numbers of *L. maculans* (Lm) and *L. biglobosa* (Lb) isolates obtained from phoma leaf spots on nine oilseed rape cultivars with *R*-genes or no *R*-genes or quantitative resistance (QR).

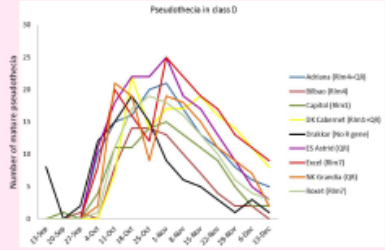


Figure 2: Changes in number of mature (Class D) pseudothecia observed from weekly sample of 25 pseudothecia obtained from stems of each of nine winter oilseed rape cultivars.

- Phoma leaf spotting appeared in early February 2012 in the Rothamsted field experiment
- 66% of leaf lesions were large pale grey lesions (*L. maculans*) and 34% were small darker lesions (*L. biglobosa*) from a total of 175 lesions found on leaves of 270 plants (10 per plot with three plots per cultivar)
- Differences in pseudothecial maturation between cultivars
- In early October, on most cultivars pseudothecia were still in early stage of maturation except on Cv. Drakkar (48%), ES-Astrid (44%) and Adriana (40%) in Class D
- First release of ascospores in late October 2011
- Maximum number of ascospores released was in late January 2012 with an average 1224 spores per m³
- From September 2011 to November 2011, there was little rain. More rainfall occurred in December 2011

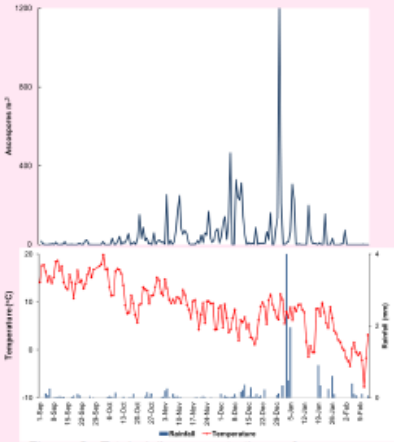


Figure 2: Total daily concentrations of ascospores released from September 2011 to February 2012 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler (A) in relation to total daily rainfall (mm) and average temperature (°C) (B).

DISCUSSION

The results suggest that there were differences in pseudothecial maturation between the cultivars, with pseudothecia from susceptible cultivars maturing faster than those from cultivar with or without *R*-genes.

The late appearance of phoma leaf spotting in February 2012 (usually leaf spotting appears in October/November) was associated with a low incidence of phoma stem base canker in summer 2012 (data not presented).

Observation of pseudothecial development until ascospore release and continuous monitoring the weather is essential to forecast phoma leaf spot occurrence and decrease use of fungicides when the risk of phoma stem canker is low for winter oilseed rape.

ACKNOWLEDGEMENTS

We thank the staff in School of Life and Medical Sciences, University of Hertfordshire for their technical assistance and the Ministry of Higher Education (MOSTI), Malaysia for the scholarship.

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Huang et al., 2005. *Eur. J. Plant Pathol* 111: 253-277
Huang et al., 2007. *Annals of Applied Biology* 151, 99-111
West et al., 2002. *Plant Pathology* 51: 311-321

Effects of host resistance on maturation of *Leptosphaeria maculans* and *L. biglobosa* (cause of phoma stem canker) in *Brassica napus* (oilseed rape)

Siti Nordahliawate M. Sidique, Yong-Ju Huang, Avice M. Hall and Bruce D.L. Fitt
School of Life and Medical Sciences, University of Hertfordshire, Hatfield, Herts AL10 9AB, UK
(*Corresponding author: s.n.mohamed-sidique@herts.ac.uk*)

University of Hertfordshire

INTRODUCTION

- *Leptosphaeria maculans* and *L. biglobosa* co-infect winter oilseed rape plants to cause phoma stem canker disease.
- The sexual spores of both species are produced in pseudothecia on infected winter oilseed rape stubble in autumn after harvest and this is the most important source of infection for newly-emerged plants.
- Current surveys by Crop Monitor showed that the proportion of UK crops affected by the disease was less in 2012 (83%) than in 2011(96%).

This study investigates effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia on stem debris.

MATERIALS & METHODS

Phoma stem canker were assessed on 30 June 2011 and 19 July 2012 field experiments: Rothamsted Research, Harpenden, Hertfordshire

↓

Stem debris were collected after harvest from each plot in August 2011 and 2012

↓

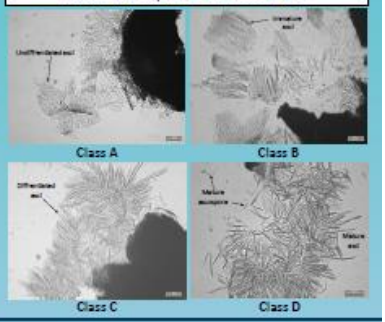
Stem debris were incubated under natural conditions at Bayfordbury starting in September 2011 until March 2012 (stem debris from 2011/2012 growing season) and August 2012 (stem debris from 2012/2013 growing season; on going)

Stem debris of Adriana (*Rlm* 4 + QR), Bilbao (*Rlm* 4), Capitol (*Rlm* 1), Drakkar (no *R* genes), DK Cabernet (*Rlm* 1 + QR), Es-Astrid (QR), Excel (*Rlm* 7), NK Grandia (QR) and Roxet (*Rlm* 7) at Bayfordbury, Hertfordshire for air sampling and ascospore counting with daily monitoring of the weather parameters

Five stems were sampled weekly at random to monitor the pseudothecial maturation and development of the ascospores by microscopic examination on each of the nine cultivars from: September 2011 to March 2012 for the 2011 harvest and August still on going for the 2012 harvest.

Pseudothecia produced on stem bases

Observation of pseudothecial maturation



RESULTS

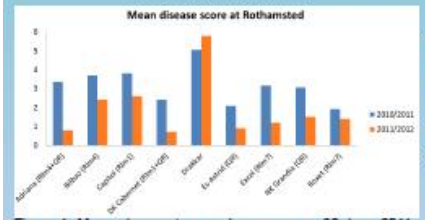


Figure 1: Mean phoma stem canker scores on 30 June 2011 and 19 July 2012 at Rothamsted Research, Harpenden for the 2011/2012 and 2012/2013 growing seasons.

- There were differences between summer 2011 and summer 2012 in severity of phoma stem canker in the field experiment at Rothamsted, which is probably a reflection of the different weather conditions in the two seasons.
- Differences in pseudothecial maturation between cultivars.
- In 2011 there are few differences between cultivars and maturity was delayed because of very dry weather in September 2011 with very little maturation of pseudothecia
- However in 2012, the pseudothecia on cv. Drakkar mature much earlier and faster than on the other cultivars ($P<0.05$) and also the slowest maturation occurs in the cv. NK Grandia which has QR

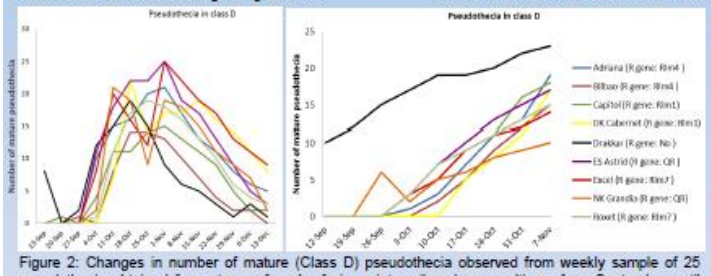


Figure 2: Changes in number of mature (Class D) pseudothecia observed from weekly sample of 25 pseudothecia obtained from stems of each of nine winter oilseed rape cultivars from September until December 2011 (A) and September 2012 until November 2012 (on going) (B).

- First release of ascospores in late October 2011
- Maximum number of ascospores released was in late January 2012 with an average 1224 spores per m^3
- Pseudothecia maturation and ascospores counting still on going for the 2012 harvest

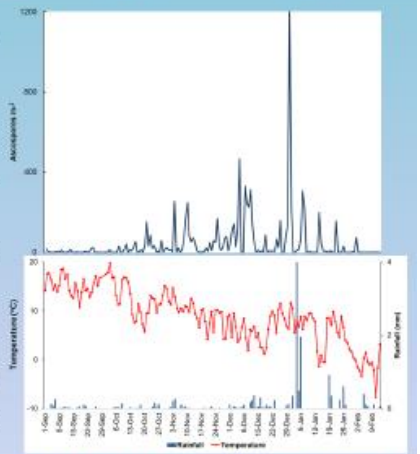


Figure 3: Total daily concentrations of ascospores released from September 2011 to February 2012 from stems of nine winter oilseed rape cultivars spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler (A) in relation to total daily rainfall (mm) and average temperature (°C) (B).

DISCUSSION

The results suggest that there were differences in pseudothecial maturation between the cultivars. The wetter September in 2012 (data not presented) demonstrates a difference between pseudothecial maturation on different cultivars, with the fastest maturation occurring on the most susceptible cultivar. This has implications for the subsequent severity of stem canker.

There is a need to investigate impact of cultivar resistance on the maturation of pseudothecia and also the possibility that *L. maculans* retards the development of pseudothecia from *L. biglobosa*.

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Toscano-Underwood *et al.*, *Journal of Plant Pathology* 82, 726-736

Effects of host resistance on maturation of *Leptosphaeria maculans* and *L. biglobosa* (cause of phoma stem canker) in *Brassica napus* (oilseed rape)

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INTRODUCTION

- Worldwide losses from phoma stem canker are estimated at £1000M per growing season (Fitt *et al.*, 2008).
- The sexual spores of *L. maculans* and *L. biglobosa* species are produced in pseudothecia on infected winter oilseed rape stem debris after harvest and this is the most important source of inoculum for infection of newly-emerged crops in autumn.
- Surveys by Crop Monitor showed that the proportion of UK crops affected by the disease was large in both 2011(96%) and 2012 (83%).
- This study aims to determine effects of cultivar resistance and environmental conditions (precipitation and temperature) on maturation of pseudothecia of *L. maculans* and *L. biglobosa* on stem debris. There were 1) Controlled environment experiments 2) Field experiments, 2011/2012 & 2012/2013

MATERIALS & METHODS

Phoma stem canker was assessed on 30 June 2011, 19 July 2012 and 29 July 2013 in field experiments at Rothamsted Research, Harpenden, Hertfordshire

Stem debris were collected after harvest from each plot in August 2011 and 2012

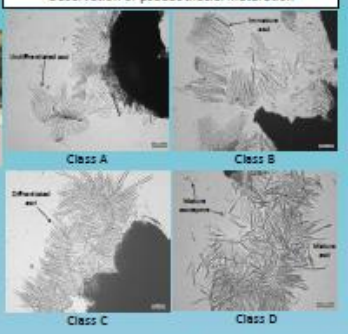
Stem debris were incubated under natural conditions at Bayfordbury from September 2011 until March 2012 (stem debris from 2010/2011 growing season) and from August 2012 until March 2013 (stem debris from 2011/2012 growing season); Controlled environment experiment November 2012 until March 2013.

Controlled environment conditions (20°C, 50 – 80% humidity, rainfall water pH: 5.7 and stems were sampled two weekly).
Five stems were sampled weekly at random to monitor pseudothecial maturation and development of ascospores by microscopic examination from: September 2011 to March 2012 for the 2011 harvest and from August 2012 to February 2013 for the 2012 harvest

Stem debris at Bayfordbury field station were placed around a Burkard spore sampler used to collect ascospores released from pseudothecia on the stems.

Pseudothecia produced on stem bases

Observation of pseudothecial maturation



RESULTS

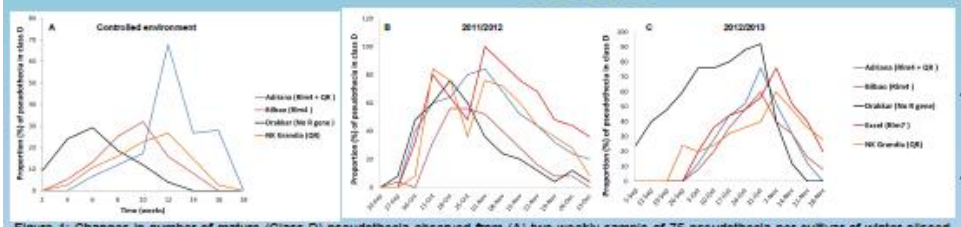


Figure 1: Changes in number of mature (Class D) pseudothecia observed from (A) two weekly sample of 75 pseudothecia per cultivar of winter oilseed rape stem debris from November 2012 until March 2013 and weekly sample of 25 pseudothecia per cultivar obtained from winter oilseed rape stem debris from (B) September until December 2011 (C) and September 2012 until November 2012.

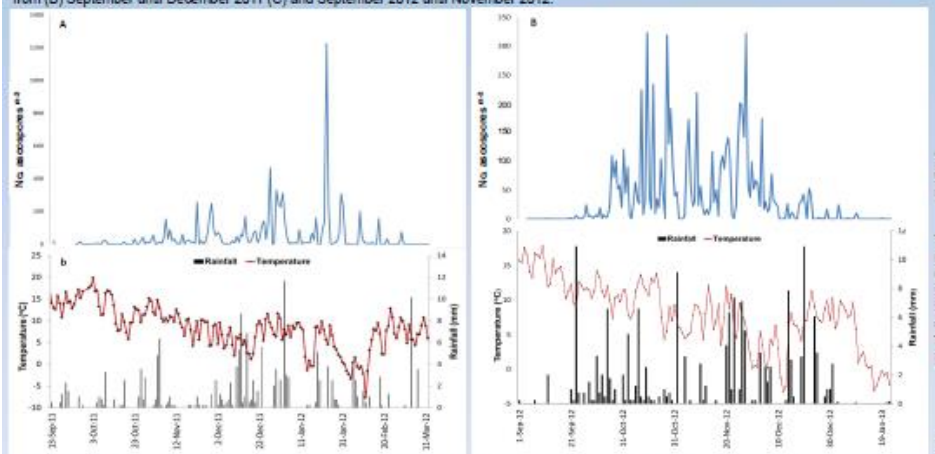


Figure 2: Total daily concentrations of ascospores released from (A) September 2011 to March 2012 and (B) September 2012 to January 2013 from stems debris of winter oilseed rape spread in an experimental plot at Bayfordbury and collected by a Burkard spore sampler in relation to total daily rainfall (mm) and average temperature (°C).

- Maturity was late because of very dry weather in September 2011 with very little maturation of pseudothecia.
- First release of ascospores in late October 2011 and phoma leaf spot lesion observed in February 2012.
- However in 2012, pseudothecia on cv. Drakkar matured much earlier and faster than on the other cultivars ($p<0.05$).
- First ascospores release was much earlier, phoma leaf spot lesions observed in October 2012.

DISCUSSION

The results suggest that there were differences in pseudothecial maturation between the cultivars.

There is an important role of resistance in a cultivar may be to delay pseudothecial maturation that requires further investigation to improve forecasting and disease control strategies; optimise forecasting (include factor for cultivar resistance) and fungicide treatment.

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Generic Training for Researchers (GTR) and courses undertaken in the past year:

1. Information gathering
2. Information management – EndNote
3. Initial Registration Assessment
4. Literature review
5. Research ethics
6. Statistics - Getting started with statistics
7. Statistics - Comparing groups
8. Statistics - Relationships in data
9. Teaching for research students
10. British PhD, how to bag one
11. Doctoral Review Assessment
12. Experimental design
13. Health and safety in research
14. Getting to the end on time
15. Getting published and promoting your research
16. How to be an effective researcher
17. If you fail to plan, you are planning to Fail
18. Plagiarism and how to avoid it
19. What thesis?
20. The Viva and process of Research Degree Examination
21. Presenting your research (oral presentations)
22. Rapid reading
23. Technical writing

Courses:

1. Crop Protection, principles and practice, 27/02/2013 - 01/03/2013, University of Hertfordshire.

2. Crop Pathogens, Pests & Weeds, 23/05/2013 - 25/05/2013, University of Hertfordshire and Rothamsted Research.