Research Article

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Effective control of *Leptosphaeria maculans* increases importance of *L. biglobosa* as a cause of phoma stem canker epidemics on oilseed rape

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Abstract

BACKGROUND: Phoma stem canker is a damaging disease of oilseed rape caused by two related fungal species, *Leptosphaeria maculans* and *L. biglobosa*. However, previous work has mainly focused on *L. maculans* and there has been little work on *L. biglobosa*. This work provides evidence of the importance of *L. biglobosa* to stem canker epidemics in the UK.

RESULTS: Quantification of *L. maculans* and *L. biglobosa* DNA using species-specific quantitative PCR showed that *L. biglobosa* caused both upper stem lesions and stem base cankers on nine oilseed rape cultivars in the UK. Upper stem lesions were mainly caused by *L. biglobosa*. For stem base cankers, there was more *L. maculans* DNA than *L. biglobosa* DNA in the susceptible cultivar Drakkar, while there was more *L. biglobosa* DNA than *L. maculans* DNA in cultivars with the resistance gene *Rlm7* against *L. maculans*. The frequency of *L. biglobosa* detected in stem base cankers increased from 14% in 2000 to 95% in 2013. Ascospores of *L. biglobosa* and *L. maculans* were mostly released on the same days and the number of *L. biglobosa* ascospores in air samples increased from the 2010/2011 to 2012/2013 growing seasons.

CONCLUSION: Effective control of *L. maculans* increased infection by *L. biglobosa*, causing severe upper stem lesions and stem base cankers, leading to yield losses. The importance of *L. biglobosa* to phoma stem canker epidemics can no longer be ignored. Effective control of phoma stem canker epidemics needs to target both *L. maculans* and *L. biglobosa*.

Supporting information may be found in the online version of this article.

Keywords: blackleg; canola; coexistent pathogens; disease control; oilseed rape; phoma stem canker; yield loss

1 INTRODUCTION

Crop losses due to diseases pose a very significant challenge to food security.[1] Phoma stem canker (blackleg) is a world-wide damaging disease on oilseed rape (*Brassica napus*, canola), globally causing >£1000 million yield losses per cropping season.[2] Considering the UK alone, oilseed rape growers had £60–80 million of annual yield losses due to phoma stem canker (www.cropmonitor.co.uk), in addition to the use of fungicides costing £20M.[3,4] Effective control of this disease is important for maintaining the yield stability, which is essential for food security since oilseed rape is the second most important source of vegetable oil worldwide and the meal after oil extraction is an important source of animal feed.[5,6] Furthermore, oilseed rape has an important role as a break crop within arable crop rotations.

In the UK, phoma stem canker is caused by two related fungal pathogen species, *Leptosphaeria maculans* and *L. biglobosa*,[7] which

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coexist on their host.[8] The proportions of the two species in local populations have been shown to affect the severity of stem canker epidemics.[4] *L. maculans* has been associated with stem base cankers and considered more damaging, while *L. biglobosa* has been associated with upper stem lesions and is generally considered less damaging.[9,10] Previous control of phoma stem canker, either by cultivar resistance and/or by fungicides, has mainly focused on *L. maculans*, without consideration of *L. biglobosa*. So far, all the major resistance (*R*) genes used in cultivars to control stem canker have been developed for control of *L. maculans*. Currently, the resistance gene *Rlm7* is the most effective gene used for control of phoma stem canker in the UK.[11,12] There have been at least 18 *R* genes for resistance against *L. maculans* identified and five of them have been cloned.[13–16] To date, no *R* genes have been identified or cloned for resistance against *L. biglobosa*. However, studies in China, where only *L. biglobosa* is present, showed that *L. biglobosa* can cause severe oilseed rape yield losses.[17–19] Studies in south-west Poland showed that *L. biglobosa* was more frequently isolated from stem cankers than *L. maculans*, and fungicide treatments increased seed yield and seed quality,[20,21] suggesting that *L. biglobosa* contributes to yield losses in Poland. In countries such as Canada, Australia and European countries, where both *L. maculans* and *L. biglobosa* are present,[22,23] the contribution of *L. biglobosa* to phoma stem canker epidemics and yield losses may be underestimated. Although infection by *L. maculans* and *L. biglobosa* on oilseed rape leaves can be distinguished by lesion phenotypes, with *L. maculans* causing larger grey leaf spots with many pycnidia and *L. biglobosa* causing smaller dark lesions with few pycnidia,[8,24] they cannot be distinguished once they have reached the stem. It is difficult to distinguish *L. maculans* and *L. biglobosa* by phoma stem canker symptoms; they can be distinguished only by species-specific PCR.[9,24,25] Recently, with the development of new species-specific quantitative PCR (qPCR), *L. biglobosa* was more frequently detected in stems with phoma stem canker than previously thought.[24] There is a need to investigate the contribution of *L. biglobosa* to phoma stem canker epidemics.

Methods used for control of diseases caused by related coexisting pathogens affect proportions of the related species. For example, use of fungicides to control cereal eyespot disease on stem bases caused by two related fungal pathogens, *Oculimacula yallundae* and *O. acuformis*, increased proportions of *O. acuformis* and decreased proportions of *O. yallundae* compared to proportions in untreated plots.[26] In the UK, in addition to host resistance, control of severe phoma stem canker epidemics is supplemented by foliar sprays with fungicides.[27,28] However, fungicide applications are mainly for control of *L. maculans*. In the autumn, the optimum timing for fungicide sprays is when a threshold of 10–20% of plants with *L. maculans* phoma leaf spots is reached[28] (https://ahdb.org.uk). Use of fungicides targeted to control *L. maculans* may increase infection by *L. biglobosa*. Furthermore, previous studies indicate that *L. maculans* is more sensitive to triazole fungicides (*e.g.* flusilazole and tebuconazole) than *L. biglobosa*.[12,29] Use of triazole fungicides to control phoma stem canker may affect the proportions of *L. maculans* and *L. biglobosa* in local pathogen populations. However, there has been little work on how the effects of fungicides on the coexistence of *L. maculans* and *L. biglobosa* relate to the severity of stem canker epidemics.

In Europe, phoma stem canker epidemics on winter oilseed rape are initiated in autumn by airborne ascospores of *L. maculans* and *L. biglobosa* released from pseudothecia that developed on crop debris from the previous cropping season.[10,30,31] Germ tubes of ascospores penetrate leaves mainly through stomata, causing phoma leaf spots from which the pathogen grows along petioles into stems to initiate stem base cankers or upper stem lesions.[32,33] The earlier that *L. maculans* and *L. biglobosa* reach the stem, the more severe is phoma stem canker that develops before harvest, therefore foliar fungicide sprays must be applied in autumn to prevent the pathogens from growing into the stems to cause stem cankers.[28,34,35] The optimal timing of fungicide applications depends on the timing of ascospore release. Previous studies showed that weather conditions affect the maturation of pseudothecia and the timing of ascospore release.[36–38] Pseudothecia of *L. biglobosa* matured more slowly at temperatures <10 °C than those of *L. maculans* but there was no difference between them in maturation rate at temperatures 15–20 °C.[36] Since *L. maculans* and *L. biglobosa* differ in aggressiveness and *in vitro* fungicide sensitivity,[12,29] the timing of *L. maculans* and *L. biglobosa* ascospore release may affect the efficacy of fungicide applications. However, it has been difficult to distinguish ascospores of *L. maculans* and *L. biglobosa* in air samples by visual methods; the timing and abundance of *L. maculans* and *L. biglobosa* ascospores released in the air can only be distinguished by species-specific qPCR.[12,20,31] Additionally, there is evidence that pre-infection by one of the *Leptosphaeria* species confers increased resistance to the other species,[39,40] which is likely to affect subsequent proportions of the two species in inoculum produced in a local population. This work aims to investigate the contribution of *L. biglobosa* to phoma stem canker epidemics by distinguishing (i) phoma stem canker caused by *L. maculans* and *L. biglobosa* on oilseed rape cultivars with different levels of resistance, and (ii) seasonal differences between *L. maculans* and *L. biglobosa* in the timing and abundance of release of ascospores.

2 MATERIALS AND METHODS

2.1 Phoma stem canker epidemics and seed yield

To investigate phoma stem canker epidemics in different growing seasons, nine oilseed rape cultivars were used for field experiments at Rothamsted, Harpenden, UK over three growing seasons (2010/2011, 2011/2012 and 2012/2013). Cultivars (cvs) Es-Astrid and NK Grandia had quantitative resistance (QR) without known major resistance (*R*) genes against *L. maculans* (*Rlm*). Six cultivars had an *Rlm* gene in a background with or without QR: Capitol (*Rlm1*), DK Cabernet (*Rlm1* + QR), Bilbao (*Rlm4*), Adriana (*Rlm4* + QR), Roxet (*Rlm7*) and Excel (*Rlm7* + QR). Cultivar Drakkar (with no known *Rlm* genes and no QR) was used as a susceptible control. The field experiments were sown on 13 September 2010, 30 August 2011 and 5 September 2012, with a plot size of 15 × 2 m. The field experiments were arranged in randomised block designs with three replicates. Severity of phoma leaf spot on each cultivar was assessed in autumn/winter and severity of phoma stem canker was assessed in summer before harvest. In each season, the weather data (*e.g.* total daily rainfall and mean temperature) were collected from the Rothamsted weather station, which was within 2 km of the field experiment sites.

2.2 Phoma leaf spots and phoma stem canker caused by *L. maculans* or *L. biglobosa*

To investigate the severity of phoma leaf spots caused by *L. maculans* or *L. biglobosa*, in autumn/winter in the 2011/2012 and 2012/2013 growing seasons, when 50% of Drakkar (susceptible control) plants had phoma leaf spots, 10 plants were randomly sampled from each plot. The numbers of *L. biglobosa* (small dark

lesions with no or few pycnidia) and *L. maculans* (large grey lesions with many pycnidia) lesions on each plant were counted. The severity of *L. maculans* and *L. biglobosa* phoma leaf spot was expressed as the number of phoma leaf spots per plant.

The severity of phoma stem canker was assessed in late June to early July, when plants were just starting to senesce, by pulling up 30 plants from each plot, cutting the stem base of each plant and scoring the necrotic tissue in the cross-section using a 0–6 scale: 0, no affected tissue; 1, 1–5% area affected, 2, 6–25% area affected; 3, 26–50% area affected; 4, 51–75% area affected; 5, 76–100% area affected, plant alive; 6, 100% area affected, stem broken or plant dead with a hollow or severely necrotic pith; modified from the 1–6 scale of Lô-Pelzer *et al.* (2009).[41] The data were then converted to a G2 disease index.[42] G2 index = $[(N0 \times 0) + ((N1 + N2) \times 1) + (N3 \times 3) + (N4 \times 5) + (N5 \times 7) + (N6 \times 9)]/Nt$, where $N0, 1, 2 \ldots 6$ are the numbers of stems with canker scores 0, 1, 2…6, respectively, and Nt is the total number of stems assessed. In addition to assessing stem base canker, in June/July 2012 and 2013 the upper part of the stem was assessed for severity of upper stem lesions. Symptoms were considered to be upper stem lesions if they were observed >10 cm above the root crown; they were also assessed on the 0–6 scale and a G2 index was calculated. At the end of the experiments, seeds were harvested from each plot to assess the effects of phoma stem canker severity on yield.

To investigate whether stem base canker or upper stem lesions were caused by *L. maculans* or *L. biglobosa*, stems of the nine cultivars with upper stem lesions or stem base cankers were collected. For each cultivar, five to eight plants with upper stem lesions or stem base cankers were sampled. Small pieces of diseased stem tissues were excised from each upper stem lesion or stem base canker and placed in a 2-mL tube. The stem samples were freeze-dried and stored at −20 °C for further DNA extraction and qPCR.

2.3 Air sampling to monitor the release of *L. maculans* and *L. biglobosa* ascospores

Release of ascospores of *L. maculans* and *L. biglobosa* in the air was monitored using a Burkard 7-day recording spore sampler (Burkard Manufacturing Company Ltd, Rickmansworth, UK). In each growing season, oilseed rape stems affected by phoma stem canker from the previous growing season were collected and placed radially around the Burkard spore sampler, as described by Huang *et al.*[10] The spore sampler was located 5 km from the field experiments and operated from September to February/March in each growing season. At 7-day intervals, the spore tape was removed from the sampler drum and cut into seven pieces, with each piece representing 1 day.[43] The daily spore tape was cut in half longitudinally, with one half stored in a 2-mL screw-topped tube for DNA extraction and the other half mounted on a microscope slide for counting *Leptosphaeria* ascospores.[12]

2.4 DNA extraction from plant and spore samples

The individual freeze-dried upper stem or stem base samples were ground into fine powder using a mortar and pestle. DNA was extracted from a 20-mg subsample of each ground sample using a DNA extraction kit (DNAMITE Plant Kit, Microzone Ltd, Stourbridge, UK). DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Labtech International, Stourbridge, UK) and adjusted to a final concentration of 20 ng μL^{-1} for qPCR. For the spore samples, DNA was extracted from each half spore tape using the cetyltrimethylammonium bromide (CTAB) protocol[12,31] with minor adaptations. All the spore DNA samples were diluted 1:10 using PCR water for qPCR.

2.5 Detection of *L. maculans* and *L. biglobosa* in stem and spore samples by qPCR

The amounts of *L. maculans* or *L. biglobosa* DNA in each stem or spore sample were quantified using a Sigma SYBR Green Jump Start Ready Mix (Sigma, Gillingham, UK) with species-specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*).[39] All reactions were done in 96-well PCR plates (ABgene) covered with cap strips, using a Stratagene Mx3000P quantitative PCR machine thermocycler as described by Huang *et al.*[12] In each qPCR run, a standard dilution series consisting of 10 000, 1000, 100, 10 and 1 pg of DNA of pure culture *L. maculans* (isolate ME24) or *L. biglobosa* (isolate 2003.2.8) was used to produce a standard curve. The amounts of *L. maculans* DNA or *L. biglobosa* DNA for each unknown sample were estimated using the standard curve. For stem samples, the results were expressed as the amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng total DNA from stem tissues. For spore samples, the results were expressed as absolute amounts of DNA on half-spore tape pieces.

2.6 Statistical analysis

Data from UK field experiments were analysed using ANOVA to determine whether there were significant differences between different cultivars or between growing seasons in severity of phoma leaf spots, severity of stem canker, the amount of *L. maculans* or *L. biglobosa* DNA in stem tissues or seed yield. For the DNA data, residual diagnostic plots for analysis on a natural scale indicated that transformation was needed to stabilize the variance, therefore the data for amounts of DNA of *L. maculans* or *L. biglobosa* were $\log_{10}$-transformed before ANOVA. To examine differences between *L. maculans* and *L. biglobosa* in the daily amounts of DNA quantified on tapes during autumn (September to November) or winter (December to February) within the three growing seasons or between the three seasons, paired *t*-tests were done assuming that the DNA measurements for *L. maculans* and *L. biglobosa* were paired on the date when samples were collected. All the analyses were done using GenStat 22nd edition.[44]

3 RESULTS

3.1 Phoma stem canker epidemics and seed yield

For stem base canker, there was a significant difference in severity of phoma stem canker between the three growing seasons ($P < 0.05$), with phoma stem canker more severe in 2010/2011 and 2012/2013 than in the 2011/2012 growing season (Table 1). There was also a significant difference in severity of phoma stem canker between cultivars ($P < 0.01$). Cultivar Drakkar had the greatest mean G2 score of 7.7 (Table 1). Cultivar DK Cabernet (1.72) had the smallest mean G2 index over the three seasons, followed by Excel (1.87) and Roxet (2.21). There was also a significant ($P < 0.01$) effect of interaction of season with cultivar. For example, there was no significant difference between Adriana (*Rlm4* + QR) and Bilbao (*Rlm4*) in G2 index in 2010/2011 and 2012/2013 but there was a significant difference between them in 2011/2012 (Table 1). However, for Capitol (*Rlm1*) and DK Cabernet (*Rlm1* + QR), there was a significant difference between them in all three seasons, with phoma stem canker less severe on DK

Table 1. Stem base canker severity (G2 index[a]) on different oilseed rape cultivars in the 2010/2011, 2011/2012 and 2012/2013 growing seasons[b]

Cultivar	2010/2011	2011/2012	2012/2013	Cultivar mean
Adriana	4.01b	4.01b	3.37bc	**2.65c**
Bilbao	4.57b	4.57b	3.92b	**4.01b**
Capitol	4.66b	4.66b	3.97b	**4.02b**
DK Cabernet	2.60c	2.60c	1.81 e	**1.72d**
Drakkar	8.12a	8.12a	7.44a	**7.73a**
Es-Astrid	4.17b	4.17b	3.64b	**2.81c**
Excel	2.28c	2.28c	2.75cd	**1.87d**
NK Grandia	3.87b	3.87b	1.97de	**2.56c**
Roxet	1.99c	1.99c	3.14bc	**2.21c**
Season mean	**4.03a**	**2.28b**	**3.56a**	

[a] G2 index was calculated as G2 index = $[(N0 \times 0) + ((N1 + N2) \times 1) + (N3 \times 3) + (N4 \times 5) + (N5 \times 7) + (N6 \times 9)]/Nt$, where $N0, 1, 2 \ldots 6$ are the numbers of stems with canker scores 0, 1, 2…6, respectively, and Nt is the total number of stems assessed.
[b] Average G2 indexes sharing the same letter were not statistically different at $P < 0.05$ in a multiple comparison with Fisher's least significant difference (LSD) test.

Table 2. Upper stem lesion severity (G2 index[a]) on different oilseed rape cultivars in the 2011/2012 and 2012/2013 growing seasons[b]

Cultivar	2011/2012	2012/2013	Cultivar mean
Adriana	0.38fg	0.23c	**0.31e**
Bilbao	4.07b	0.20c	**2.13b**
Capitol	1.88c	0.77ab	**1.33c**
DK Cabernet	0.34g	0.32bc	**0.33e**
Drakkar	6.74a	0.86a	**3.80a**
Es-Astrid	0.73ef	0.52abc	**0.63de**
Excel	0.78e	0.17c	**0.48e**
NK Grandia	0.63efg	0.33bc	**0.48e**
Roxet	1.28d	0.39abc	**0.84d**
Season mean	**1.87a**	**0.42b**	

[a] G2 index was calculated as G2 index = $[(N1 \times 0) + ((N1 + N2) \times 1) + (N3 \times 3) + (N4 \times 5) + (N5 \times 7) + (N6 \times 9)]/Nt$, where $N0, 1, 2 \ldots 6$ are the numbers of stems with canker scores 0, 1, 2…6, respectively, and Nt is the total number of stems assessed.
[b] Average G2 indexes sharing the same letter were not statistically different at $P < 0.05$ in a multiple comparison with Fisher's least significant difference (LSD) test.

Cabernet than on Capitol. For Roxet (*Rlm7*) and Excel (*Rlm7* + QR), there was a significant difference between them only in 2011/2012 with phoma stem canker more severe on Roxet than on Excel. For Es-Astrid (QR) and NK Grandia (QR), there was a significant difference between them in 2011/2012 and 2012/2013, with phoma stem canker more severe on NK Grandia than on Es-Astrid in 2011/2012 but less severe on NK Grandia than on Es-Astrid in 2012/2013 (Table 1).

For upper stem lesions, there was a significant difference in severity of upper stem lesions between growing seasons ($P < 0.05$) and between cultivars ($P < 0.01$) (Table 2). Upper stem lesions were more severe in 2011/2012 than in 2012/2013 (Table 1). Except for the susceptible cultivar Drakkar, cultivars Bilbao and Capitol had more severe upper stem lesions than other cultivars. There was also a significant ($P < 0.01$) interaction between season and cultivar. For example, there was a significant difference between Adriana (*Rlm4* + QR) and Bilbao (*Rlm4*) in the severity of upper stem lesions in 2011/2012 but there was no significant difference between them in 2012/2013 (Table 2).

For seed yield, there was a significant difference between growing seasons ($P < 0.01$), with the mean seed yield greater in 2010/2011 than in 2011/2012 and 2012/2013 (Table 3). There was a significant difference in seed yield between cultivars ($P < 0.01$); cultivar DK Cabernet (4.79 t/ha) had the greatest mean yield over the three seasons, followed by Bilbao (4.60 t/ha) and NK Grandia (4.56 t/ha). There was also a significant ($P < 0.01$) interaction of season with cultivar. For example, NK Grandia had more yield than Es-Astrid in 2010/2011 but had less yield in 2012/2013 and there was no significant difference between them in 2011/2012 (Table 3).

3.2 Phoma leaf spots caused by *L. maculans* or *L. biglobosa*

For phoma leaf spots caused by *L. maculans*, there was a significant difference between seasons in the number of *L. maculans* ($P < 0.01$) phoma leaf spots. There were more *L. maculans* phoma leaf spots in 2012/2013 than in 2011/2012 (Fig. 1 and Table S1). There was also a significant difference between cultivars in the number of *L. maculans* phoma leaf spots ($P < 0.01$). Cultivar Excel (3.24 leaf spots/plant) had the smallest and Drakkar (11.53 leaf spots/plant) had the greatest mean number of *L. maculans* phoma leaf spots over the two seasons. There were no significant differences between Capitol (*Rlm1*) and DK Cabernet (*Rlm1* + QR), Bilbao (*Rlm4*) and Adriana (*Rlm4* + QR) or Roxet (*Rlm7*) and Excel (*Rlm7* + QR) over the two seasons. However, there was a significant difference between Es-Astrid (QR) (8.8 leaf spots/plant) and NK Grandia (QR) (5.7 leaf spots/plant) in 2012/2013 but not in 2011/2012 (Fig. 1 and Table S1).

For phoma leaf spots caused by *L. biglobosa*, there was a significant difference between seasons ($P < 0.05$) in the number of *L. biglobosa* phoma leaf spots, with more *L. biglobosa* phoma leaf spots in 2012/2013 (12.72 leaf spots/plant) than in 2011/2012 (2.35 leaf spots/plant) (Fig. 1 and Table S2). However, there was no significant difference between cultivars in the number of *L. biglobosa* phoma leaf spots.

Table 3. Seed yield (t/ha) of different oilseed rape cultivars in the 2010/2011, 2011/2012 and 2012/2013 growing seasons[a]

Cultivar	2010/2011	2011/2012	2012/2013	Cultivar mean
Adriana	5.06abc	4.05bcd	3.79abc	**4.30b**
Bilbao	5.35ab	4.45ab	4.00abc	**4.60ab**
Capitol	3.94e	3.82cd	3.47cd	**3.75c**
DK Cabernet	5.36ab	4.78a	4.23ab	**4.79a**
Drakkar	3.06f	1.65e	2.84 e	**2.52d**
Es-Astrid	4.52cde	4.26abc	4.33a	**4.37b**
Excel	4.98bcd	4.30abc	3.61bcd	**4.30b**
NK Grandia	5.58a	4.44abc	3.65bcd	**4.56ab**
Roxet	4.43de	3.283d	3.14d	**3.62c**
Season mean	**4.70a**	**3.89b**	**3.68b**	

[a] Average seed yields sharing the same letter were not statistically different at $P < 0.05$ in a multiple comparison with Fisher's least significant difference (LSD) test.

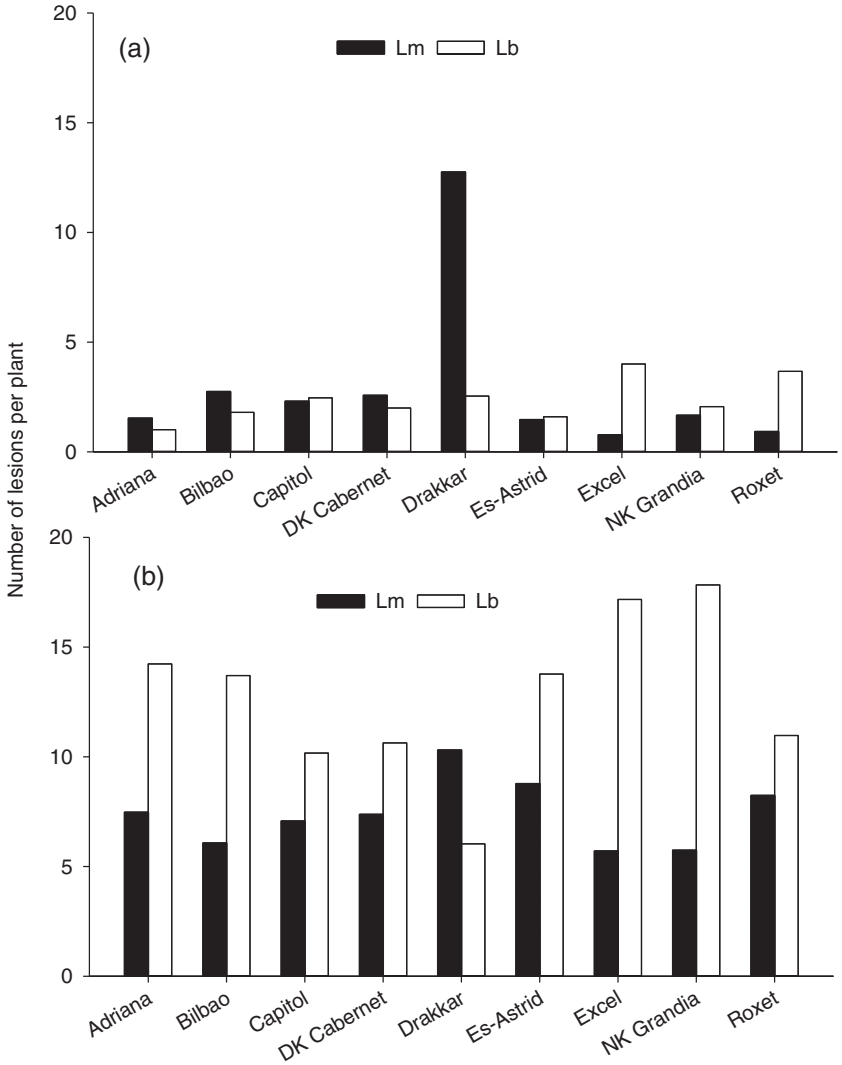

Figure 1. Numbers of leaf lesions caused by *Leptosphaeria maculans* (Lm) and *L. biglobosa* (Lb) on different oilseed rape cultivars assessed in the autumn of the 2011/2012 (a) and 2012/2013 (b) growing seasons in field experiments at Rothamsted, Harpenden, UK. For Lm lesion numbers, the effects of growing season ($P < 0.01$, standard error of difference (SED) = 0.292, df = 2), the effects of cultivar ($P < 0.01$, SED = 0.947, df = 8) and the interactions of cultivar with season ($P < 0.01$, SED = 1.296, df = 33) were significant. For Lb lesion numbers, the effects of growing season were significant ($P < 0.01$, SED = 0.929, df = 2), and the effects of cultivar ($P > 0.08$, SED = 1.90, df = 8) and the interactions of cultivar with season ($P > 0.07$, SED = 2.695, df = 33) were not significant.

3.3 Phoma stem catnker caused by *L. maculans* or *L. biglobosa*

The relative contributions of *L. maculans* or *L. biglobosa* to phoma stem canker were assessed by quantifying the amounts of *L. maculans* and *L. biglobosa* DNA in the stems using qPCR. In 2010/2011 and 2011/2012, the amount of *L. biglobosa* DNA was greater than that of *L. maculans* DNA for both upper stem lesion (Fig. 2(a),(c)) and stem base canker (Fig. 2(b),(d)) samples for all cultivars (Table S3). In 2012/2013, most cultivars had more *L. maculans* DNA than *L. biglobosa* DNA in upper stem lesions (Fig. 2(e)) and stem base cankers (Fig. 2(f)). For cultivars Excel and Roxet with the effective resistance gene *Rlm7* against *L. maculans*, there were large amounts of *L. biglobosa* DNA detected in both upper stem lesions and stem base cankers in 2010/2011 and 2011/2012, with a negligible amount of *L. maculans* (2010/2011) or no *L. maculans* DNA (2011/2012) detected in both upper stem lesions and stem base cankers.

For upper stem samples, there were significant differences between cultivars in the amounts of *L. maculans* DNA in 2010/2011 ($P < 0.01$) and 2011/2012 ($P < 0.01$) (Fig. 2(a),(c)). However, there were no significant differences between cultivars in the amount of *L. biglobosa* DNA in either 2010/2011 ($P > 0.42$) or 2011/2012 ($P > 0.08$) (Fig. 2(a),(c) and Table S3). By contrast, in 2012/2013, there was a significant difference between cultivars in the amount of *L. biglobosa* DNA ($P < 0.05$) but no difference in the amount of *L. maculans* DNA ($P > 0.09$) (Fig. 2(e) and Table S3).

For stem base samples, there were significant differences between cultivars in the amounts of *L. maculans* and *L. biglobosa* DNA in all three seasons. In 2010/2011, there were significant differences between cultivars in the amount of *L. maculans* ($P < 0.01$) or *L. biglobosa* ($P < 0.01$) DNA in stem base cankers (Fig. 2(b)). Similarly, there were significant differences between cultivars in the amount of *L. maculans* or *L. biglobosa* DNA in stem base cankers in 2011/2012 ($P < 0.01$ for *L. maculans*, $P < 0.01$ for *L. biglobosa*) and 2012/2013 ($P < 0.01$ for *L. maculans*, $P < 0.01$ for *L. biglobosa*) (Fig. 2(d),(f) and Table S3).

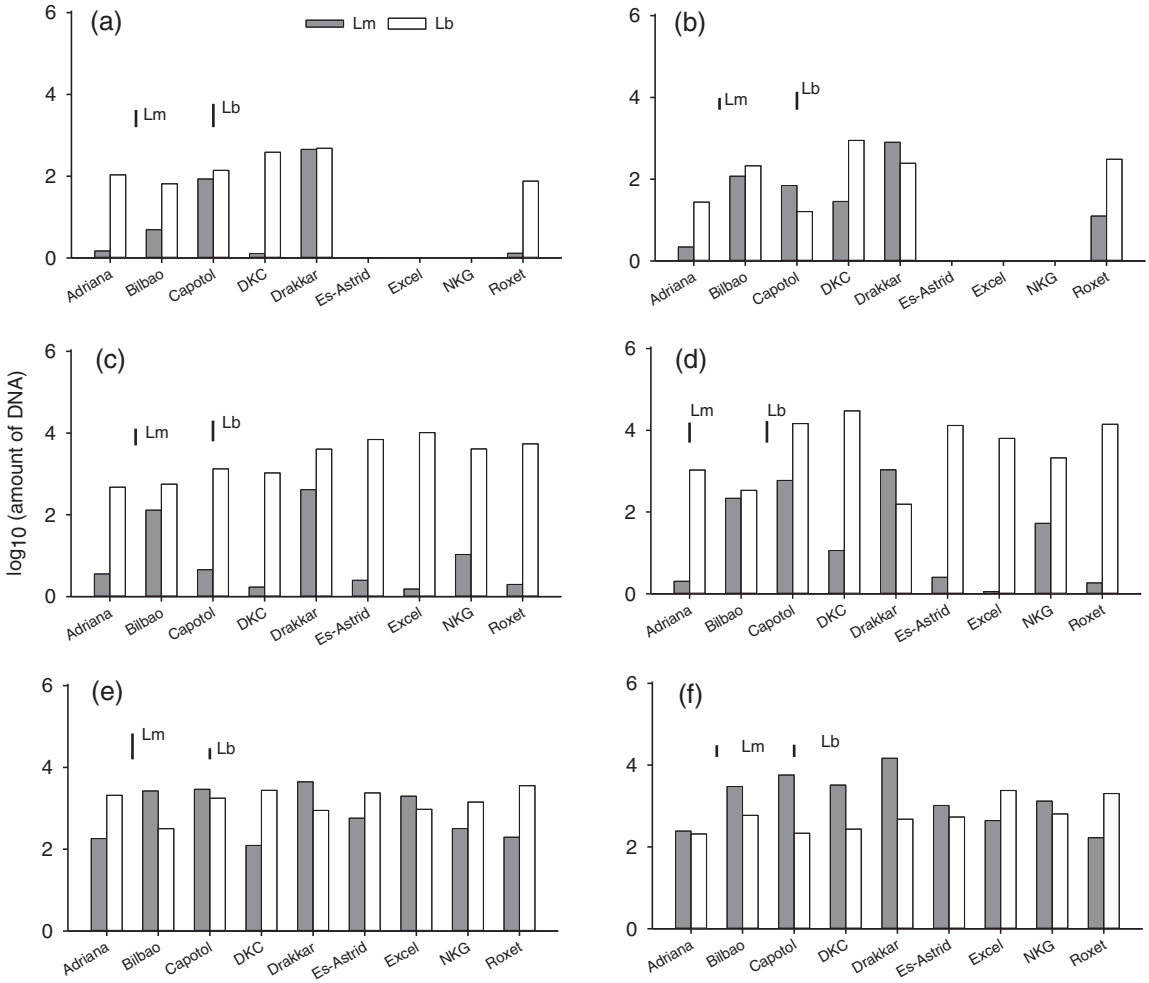

Figure 2. Amounts of *Leptosphaeria maculans* (Lm) and *L. biglobosa* (Lb) DNA (pg, $\log_{10}$-transformed) detected in upper stem lesions (a, c, e) or stem base cankers (b, d, f) of different oilseed rape cultivars in field experiments at Rothamsted in the 2010/2011 (a, b), 2011/2012 (c, d) and 2012/2013 (e, f) growing seasons. In each season before harvest, stems of each cultivar with upper stem lesions or stem base cankers were collected for DNA extraction and quantitative PCR. For statistical analysis, the DNA data were $\log_{10}$-transformed. The heights of vertical lines for each growing season are standard errors of differences (SED) for Lm and Lb between cultivars. Stems of Es-Astrid, Excel and NKG (NK Grandia) were not sampled in the 2010/2011 (a, b) growing season.

3.4 Changes in frequency of *L. maculans* and *L. biglobosa* detected in upper stem lesions and stem base cankers between different growing seasons

For upper stem lesions, the frequency of *L. biglobosa* detected by qPCR on the nine oilseed rape cultivars increased over the three growing seasons, with 85.7% in 2010/2011, 96.8% in 2011/2012 and 100% in 2012/2013. The frequency of *L. maculans* detected in upper stem lesions in these three seasons also increased, with 45.7% in 2010/2011, 51.6% in 2011/2012 and 91.7% in 2012/2013. The frequency of *L. biglobosa* detected in upper stem lesions increased more than that of *L. maculans*. However, the frequencies of *L. maculans* and *L. biglobosa* detected in stem base cankers were similar in 2010/2011, 2011/2012 and 2012/2013 (Fig. 3), suggesting that the stem base cankers were equally caused by *L. maculans* and *L. biglobosa* in those three seasons. Compared to 2000–2004, the frequency of *L. biglobosa* detected in stem base cankers had increased greatly by 2011–2013 (Fig. 3). While *L. biglobosa* was detected in stem base cankers since 2000 at the same location (Rothamsted), the frequencies of *L. biglobosa* detected in stem base cankers were 14% in 2000 and 34% in 2001 by hyphal tip isolation and morphology observation,[9] and 33% in 2003 and 13% in 2004 by species-specific PCR.[12] In this study using qPCR, the frequencies of *L. biglobosa* detected in stem base cankers were 91% in 2011 and 95% in 2013 (Fig. 3).

3.5 Release of *L. maculans* and *L. biglobosa* ascospores in different seasons

There were differences between growing seasons in numbers and patterns of ascospores released (Fig. 4). In all three growing seasons, few or no ascospores were observed before mid-September. After the first major release of ascospores was observed, ascospores continued to be released until spring in each season. In 2011/2012, when there was little rainfall in August/September (Fig. 4), ascospore release started about 1 month later than in the other two growing seasons. The late release of ascospores caused severe upper stem lesions (Fig. 5).

The periodic changes in amounts of *L. maculans* and *L. biglobosa* DNA (determined by qPCR) showed that there were differences between the three seasons in patterns of *L. maculans* and *L. biglobosa* ascospore release (Fig. 6). In 2010/2011, the daily amount of *L. maculans* DNA was greater than that of *L. biglobosa*

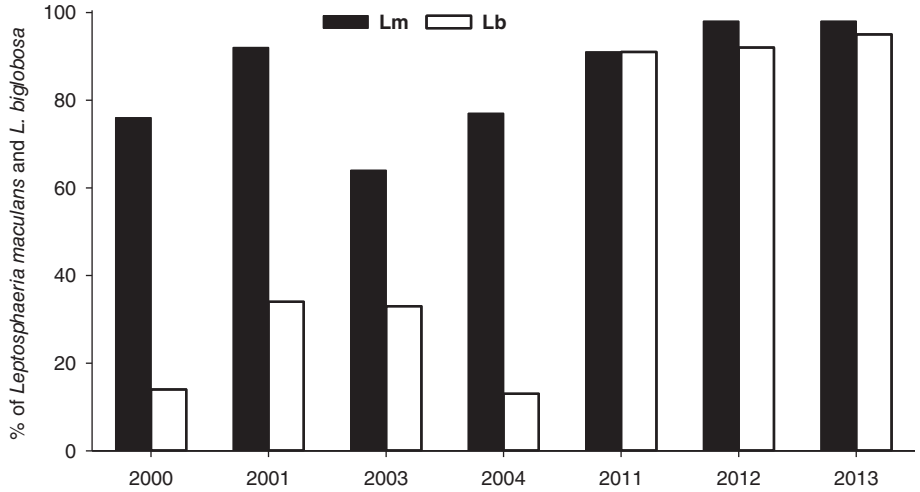

Figure 3. Frequency (%) of *Leptosphaeria maculans* (Lm) and *L. biglobosa* (Lb) detected in stem base canker of oilseed rape in field experiments at Rothamsted, Harpenden, UK. The detection of Lm and Lb was done by hyphal tip isolation in 2000 and 2001 (West *et al.*, 2002), by species-specific PCR in 2003 and 2004 (Huang *et al.*, 2011) and by quantitative PCR in 2011, 2012 and 2013 from this study.

DNA on most days. The mean amount of *L. maculans* DNA was significantly greater than that of *L. biglobosa* DNA both in autumn ($P < 0{\cdot}01$) and winter ($P < 0{\cdot}05$) (Table S4). This suggests that there were significantly more *L. maculans* ascospores than *L. biglobosa* ascospores. However, in 2011/2012 and 2012/2013, the daily amount of *L. maculans* DNA was less than that of *L. biglobosa* DNA on most days. In autumn, the mean amount of *L. maculans* DNA was not significantly different from that of *L. biglobosa* DNA in 2011/2012 ($P > 0{\cdot}64$) or 2012/2013 ($P > 0{\cdot}75$). However, in winter, the mean amount of *L. maculans* DNA was significantly greater than that of *L. biglobosa* DNA in 2011/2012 ($P < 0{\cdot}01$) but significantly less than that of *L. biglobosa* DNA in 2012/2013 ($P < 0{\cdot}01$). This suggests that there were significantly more *L. biglobosa* ascospores than *L. maculans* ascospores released in 2012/2013 (Fig. 6(c) and Table S4).

In 2011/2012 and 2012/2013, release of *L. biglobosa* ascospores was observed earlier and ended later than that of *L. maculans* ascospores (Fig. 6(b),(c)). However, after the first major release, ascospores of both species were released on most days in the three growing seasons.

4 DISCUSSION

The results of this work provide evidence that in the UK *L. biglobosa* can cause severe upper stem lesions and stem base cankers. Since *L. maculans* and *L. biglobosa* cannot be distinguished by symptoms on stems, the relative contributions of *L. maculans* and *L. biglobosa* to the severity of stem canker were determined by measuring the amounts of pathogen DNA using species-specific qPCR (Fig. 2 and Table S3). That more *L. biglobosa* DNA than *L. biglobosa* DNA was detected in both upper stem lesions and stem base cankers on the nine cultivars in 2010/2011 and 2011/2012 suggested that those upper stem lesions and stem base cankers were mainly caused by *L. biglobosa*. This was especially the case for cultivars Excel and Roxet with the effective resistance gene *Rlm7* against *L. maculans*; large amounts of *L. biglobosa* DNA were detected in both upper stem lesions and stem base cankers in 2010/2011 and 2011/2012, with a negligible amount or no *L. maculans* DNA, suggesting the upper stem lesions and stem base cankers on Excel and Roxet were mainly caused by *L. biglobosa*. Controlling *L. maculans* using major resistance genes provides an opportunity for infection by *L. biglobosa*. This is consistent with the study on the resistance gene *Rlm11*.[24] In field experiments using near-isogenic oilseed rape genotypes, with or without the effective resistance gene *Rlm11* in the Darmor genetic background (Darmor and Darmor-*Rlm11*), more *L. biglobosa* DNA than *L. maculans* DNA was found in both leaf and stem samples of Darmor-*Rlm11*, while more *L. maculans* DNA than *L. biglobosa* DNA was found in both leaf and stem samples of Darmor.[24] The contribution of *L. biglobosa* to phoma stem canker epidemics may be underestimated because at the leaf infection stage *L. maculans* causes larger grey leaf spots that can be easily seen, while *L. biglobosa* causes smaller dark lesions that are often ignored. At the stem canker stage, *L. maculans* and *L. biglobosa* cannot be distinguished by visible symptoms, therefore the contribution of *L. biglobosa* to phoma stem canker epidemics needs to be reconsidered and should not be ignored.

These results provide indirect evidence that *L. biglobosa* can cause yield losses in UK field conditions. Although previous studies showed that *L. biglobosa* can cause seed yield losses in China where only *L. biglobosa* is present,[17,18] it is difficult to examine the contribution of *L. biglobosa* to yield losses in field conditions where both *L. maculans* and *L. biglobosa* are present, such as in Europe. Studies in Poland showed that yield losses were observed in seasons with severe stem canker and pathogen isolation showed that these stem cankers were mainly caused by *L. biglobosa*[20,21]; these studies provide indirect evidence that *L. biglobosa* can cause yield losses in Polish field conditions. Using cultivars (Roxet and Excel) with the effective resistance gene *Rlm7* against *L. maculans*, this study indirectly assessed the yield losses caused by *L. biglobosa* in UK field conditions. Roxet and Excel both have *Rlm7*; there were no significant differences between them in severity of stem base canker or upper stem lesions in 2010/2011 and 2012/2013, and there were no significant differences between them in seed yield in 2010/2011 and 2012/2013 (Tables 1–3). However, in 2011/2012 Roxet had significantly more severe stem base cankers and upper stem lesions than Excel, and those upper stem lesions and stem base cankers were mainly caused by *L. biglobosa* (Fig. 2); consequently, Roxet had

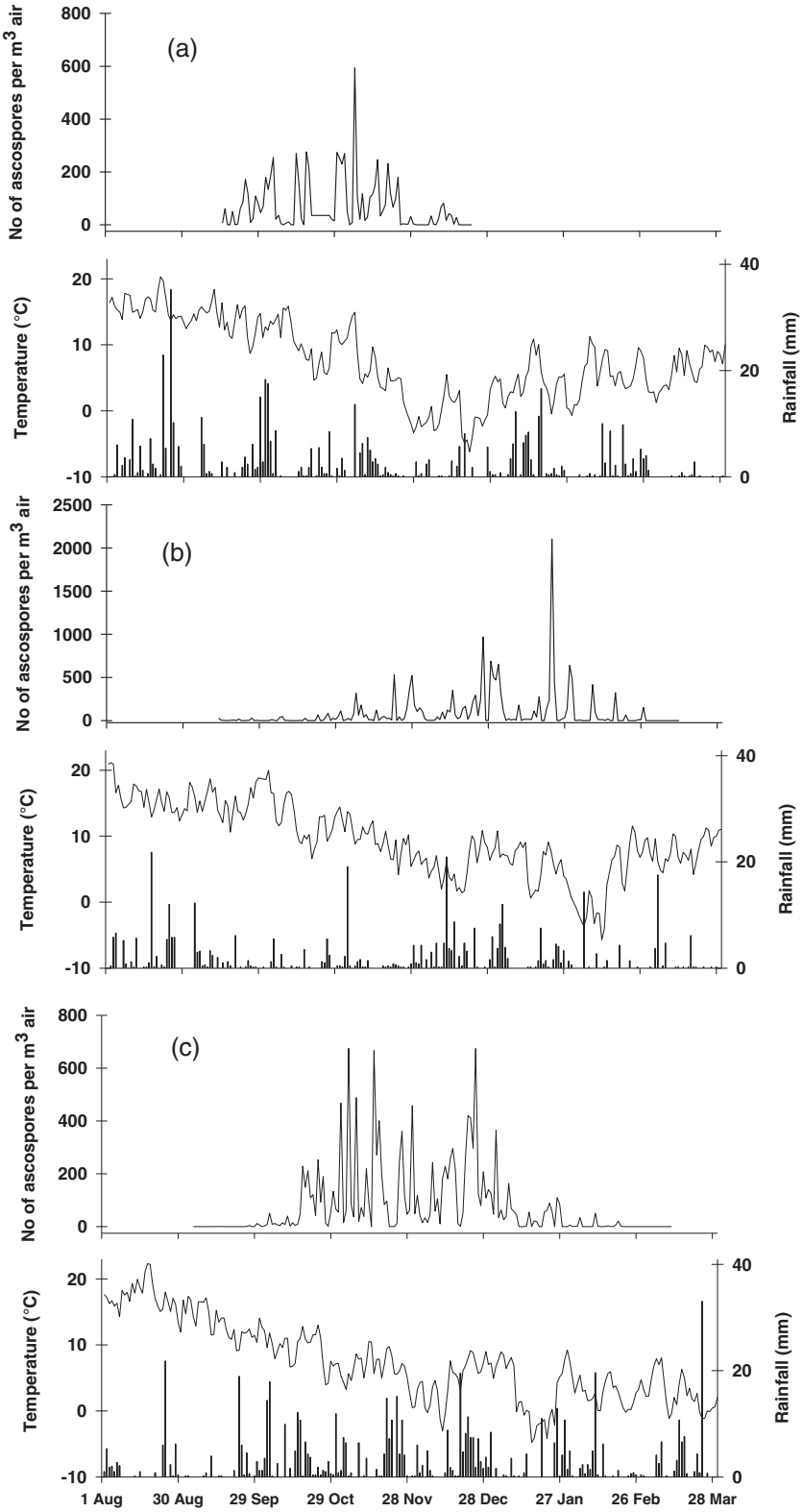

Figure 4. Changes in numbers of airborne ascospores of *Leptosphaeria* spp. in the 2010/2011 (a), 2011/2012 (b) and 2012/2013 (c) growing seasons detected by a Burkard spore sampler near oilseed rape field experiments, and the associated records of daily mean temperature and total rainfall at Rothamsted, Harpenden, UK.

significantly less seed yield than Excel. The results of this study suggest that severe upper stem infection can be an important contributor to yield losses. For example, the mean stem canker severity score of the nine cultivars was smaller in 2011/2012 than in 2010/2011, but the mean seed yield of the nine cultivars was also smaller in 2011/2012 than in 2010/2011, suggesting that

Figure 5. Upper stem lesions (white arrows) and stem base cankers (red arrows) on oilseed rape cultivar Bilbao in a field experiment in the 2011/2012 growing season at Rothamsted, Harpenden, UK. Photograph taken on 17 July 2012.

the decreased severity of stem base canker in 2011/2012 did not benefit seed yield because there was severe upper stem infection in 2011/2012. This suggested that severe upper stem infection made an important contribution to yield losses in 2011/2012. This is supported by a recent study reported in Australia where, in addition to stem base cankers, other symptoms such as infection of the lateral branches, the upper parts of the main stem, and individual flowers and pods were responsible for yield loss.[45] The study with near-isogenic genotypes Darmor and Darmor-*Rlm11* in field experiments showed that the stem canker severity scores were similar between them, with stem cankers on Darmor mainly caused by *L. maculans* and on Darmor-*Rlm11* mainly caused by *L. biglobosa*[24]; however, there was no information about the yield difference between Darmor and Darmor-*Rlm11*. To examine the contribution of *L. biglobosa* to yield losses, there is a need for controlled environment experiments using cultivars without *R* genes effective against *L. maculans* and inoculating them with *L. maculans* or *L. biglobosa*, which will provide direct evidence of yield losses caused by *L. biglobosa*.

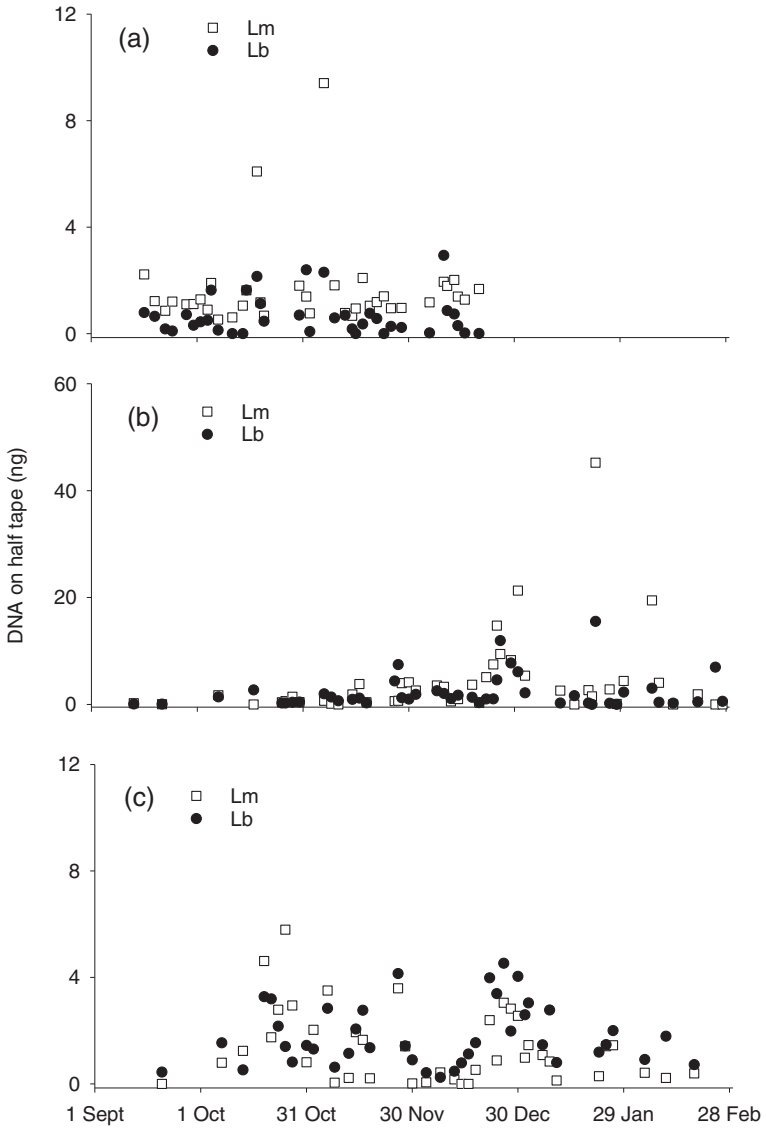

Figure 6. Daily amounts of DNA of *Leptosphaeria maculans* (Lm) or *L. biglobosa* (Lb) detected on half of a Burkard spore sampler tape by quantification of pathogen DNA using qPCR in the 2010/2011 (a), 2011/2012 (b) and 2012/2013 (c) growing seasons near oilseed rape field experiments at Rothamsted, Harpenden, UK.

Weather conditions, especially temperature and rainfall, have been shown to influence pseudothecial maturation of *L. maculans* and *L. biglobosa*.[36–38] Previous studies showed that under continuous wetness, there were no differences between *L. maculans* and *L. biglobosa* in pseudothecial maturation when the temperature was >15 °C, but *L. biglobosa* pseudothecia matured more slowly when the temperature was <10 °C.[36] With global warming, the UK winters may become wet and mild, which will favour *L. biglobosa* pseudothecial maturation. This may have been one of the reasons why *L. maculans* and *L. biglobosa* ascospores were first released on the same day in most of the seasons in this study, while previously *L. maculans* ascospores were released earlier, mainly in the autumn/winter, while *L. biglobosa* ascospores were released mainly in winter/spring.[12] Weather-based models have been developed to predict the effects of weather on pseudothecial maturation and the timing of ascospore release.[37,38] However, those models do not distinguish the release of ascospores between *L. maculans* and *L. biglobosa*. The results of this study showed that *L. biglobosa* can cause severe stem cankers and yield losses. There is a need to construct models specifically for the prediction of *L. maculans* or *L. biglobosa* ascospore release for guiding fungicide application and deployment of cultivar resistance for control of phoma stem canker.

Differences between cultivars in the amounts of *L. maculans* or *L. biglobosa* DNA suggest there are differences between cultivars in resistance against *L. maculans* or *L. biglobosa*. There are intensive studies on resistance against *L. maculans* using specific major resistance genes (*e.g. Rlm* and *LepR*).[14–16,46] However, there is little information on resistance against *L. biglobosa*. Roxet and Excel both have the resistance gene *Rlm7* against *L. maculans*; in the first two seasons (2010/2011, 2011/2012), little or no *L. maculans* DNA was detected in upper stem lesions or stem base cankers, suggesting *Rlm7* was very effective against *L. maculans*. However, in the third season (2012/2013), large amounts of *L. maculans* DNA were detected in upper stem lesions and stem base cankers (Fig. 2), suggesting the effectiveness of *Rlm7* in controlling *L. maculans* was reduced. This supports evidence that virulent *L. maculans* was detected in the UK in 2012/2013.[11] However, only 3% of the populations sampled in 2012/2013 were virulent against *Rlm7*,[11] suggesting that *Rlm7* was still effective against *L. maculans*. With *Rlm7* in Roxet and Excel effective against *L. maculans*, the differences between these two cultivars in upper stem lesions or stem base cankers were mainly caused by *L. biglobosa*, and the differences were mainly associated with genetic background quantitative resistance. To date, no *R* genes have been identified for control of *L. biglobosa*. Breeding for resistance against *L. biglobosa* may need to depend on quantitative resistance. Combining effective *R* genes against *L. maculans* with quantitative resistance against *L. biglobosa* will provide effective control of both phoma stem canker pathogens. The increased frequency of *L. biglobosa* detected in stem base cankers and the increased number of *L. biglobosa* ascospores detected in air samples from the results of this study, combined with the evidence that *L. biglobosa* is less sensitive to triazole fungicides,[12,29] suggest that there is a risk of severe phoma stem canker epidemics caused by *L. biglobosa*. Effective control of phoma stem canker epidemics needs to target both *L. maculans* and *L. biglobosa*.

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CONFLICT OF INTERESTS

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

YH, SS, CD, LG, JW and GM contributed to experimental work. AQ and YH statistically analysed the data. YH drafted the manuscript and BF, AQ, CD, JW and SS, assisted with editing the manuscript. The authors consent to the data policy of the journal.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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