Aspects of Applied Biology ***127****, 2014*
Crop Production in Southern Britain: Precision Decisions for Profitable Cropping

Importance of *Leptosphaeria biglobosa* as a cause of phoma stem canker on winter oilseed rape in the UK

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Summary

Phoma stem canker is a major disease of oilseed rape in the UK, leading to annual yield losses worth more than £100M. The disease is caused by two closely related species, *Leptosphaeria maculans* and *L. biglobosa*. *L. maculans* is generally considered more damaging, causing stem base canker; *L. biglobosa* is generally less damaging, causing upper stem lesions. Therefore, previous work has mainly focused on *L. maculans* and there has been little work on *L. biglobosa*. This work investigated the contribution of *L. biglobosa* to stem canker epidemics by assessing the amounts of DNA of *L. maculans* and *L. biglobosa* in upper stem lesions or stem base cankers on winter oilseed rape cultivars with different types of resistance against *L. mac ulans*. Diseased upper stem and stem base samples were collected from nine oilseed rape cultivars in a 2011/2012 field experiment at Rothamsted. The presence of *L. maculans* or *L. biglobosa* in each stem sample was detected by species-specific PCR. The abundance of *L. maculans* or *L. biglobosa* in each stem sample was measured by quantification of *L. maculans* DNA and *L. biglobosa* DNA using quantitative PCR (qPCR). The amounts of *L. biglobosa* DNA were greater than those of *L. maculans* DNA in both upper stem and stem base samples. These results suggest that the severe upper stem lesions and stem base cankers in the 2011/2012 cropping season were mainly caused by *L. biglobosa*, suggesting that *L. biglobosa* can sometimes cause considerable yield loss in the UK. There were differences between cultivars in the amounts of *L. maculans* DNA and *L. biglobosa* DNA, with the susceptible cultivar Drakkar having more *L. maculans* DNA than *L. biglobosa* DNA while resistant cultivars had less *L. maculans* DNA than *L. biglobosa* DNA. These results suggest that *L. biglobosa* can be an important cause of phoma stem canker on oilseed rape in the UK.

Key words: *Leptosphaeria biglobosa, Leptosphaeria maculans*, oilseed rape, phoma stem canker, yield loss

Introduction

Phoma stem canker is a major disease problem on oilseed rape (*Brassica napus*) in the UK, causing losses worth more than £100M p.a. at price of £300 t^{-1}, despite use of fungicides costing £20M p.a. (Fitt *et al*., 2008; Stonard *et al*., 2010). The disease is caused by two closely related species, *L. maculans* and *L. biglobosa,* which co-exist on their oilseed rape host. *L. maculans* is generally considered more damaging than *L. biglobosa* because *L. maculans* is often associated with damaging stem base cankers and *L. biglobosa* is often associated with less damaging upper

stem lesions (Huang *et al*., 2005). Therefore, previous work has mainly focused on *L. maculans* and there has been little work on *L. biglobosa*. Although *L. biglobosa* can cause substantial yield losses in Poland (Huang *et al*., 2005) and China (Liu *et al*., 2014), there has been little work on yield losses caused by *L. biglobosa* in the UK.

Although there have been studies on the co-existence of *L. maculans* and *L. biglobosa* in the UK (Huang *et al*., 2005; Fitt *et al*., 2006*a*; Stonard *et al*., 2010), the potential yield losses that can be caused by *L. biglobosa* have not been evaluated. In the 2011/2012 oilseed rape growing season, the timing of the first major ascospore release was later than normal. When compared with the previous growing season (2010/2011), the first major release of ascospores was more than two months later. This was followed by severe upper stem lesions and raceme lesions before harvest in the 2011/2012 season. Severe upper stem lesions caused breakage of upper stems, which led to yield loss at harvest in 2012. Previous work has suggested that ascospores of *L. biglobosa* are released later than those of *L. maculans* (Huang *et al*., 2011). There is a need to investigate whether the severe upper stem lesions in 2012 were caused by *L. maculans* or *L. biglobosa* and to improve our understanding of the importance of *L. biglobosa* in relation to control of phoma stem canker on oilseed rape in the UK.

Materials and Methods

Preparation of stem samples

A field experiment with nine winter oilseed rape cultivars (with different combinations of *R* gene-mediated and quantitative resistance) was drilled on 30 August 2011 at Rothamsted, Harpenden. Before harvest, stems of the nine cultivars (Table 1) with upper stem lesions and/or stem base cankers were collected on 12 July 2012. A small piece of diseased stem tissue was excised from each stem lesion or canker and placed in a 2 mL tube. The stem samples were freeze-dried and stored at -20°C for further analysis. A total of 102 stem samples collected from nine cultivars in the 2011/2012 growing season were used for this study.

Table 1. *Cultivars and numbers of upper stem and stem base samples taken on 12 July 2012 from a winter oilseed rape field experiment at Rothamsted in the 2011/2012 growing season*

Cultivar	Qualitative resistance	Quantitative resistance	Number of samples	
			Upper stem	Stem base
Adriana	Yes (*Rlm*4)	Yes	7	6
Bilbao	Yes (*Rlm4*)	No	4	5
Excel	Yes (*Rlm7*)	Yes	8	8
Capitol	Yes (*Rlm*1)	No	6	4
Roxet	Yes (*Rlm*7)	?	5	6
DK Cabernet	Yes (*Rlm*1)	Yes	6	5
NK Grandia	No	Yes	6	5
ES-Astrid	No	Yes	6	5
Drakkar	No	No	5	5
Total			53	49

Extraction of DNA

The freeze-dried upper stem and stem base samples were ground separately into fine powder using a mortar and pestle. A 20 mg sub-sample from each ground sample was used for DNA extraction. The DNA was extracted using a DNA extraction kit (DNAMITE Plant Kit, Microzone Ltd, UK).

DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Labtech International, UK) and adjusted to a final concentration of 20 ng μL^{-1}.

Detection of L. maculans *and* L. biglobosa *by conventional PCR*

The presence of *L. maculans* and/or *L. biglobosa* in each of the diseased stem samples was determined by conventional PCR using species-specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*) (Liu *et al*., 2006). PCR reaction mixtures were prepared to a total volume of 20 µL, made up of 10 µL of Redtaq mix (Sigma Cat. No. R2532), 8.4 µL of sterile distilled water, 0.3 µL (10 µM) of forward primer and 0.3 µL (10 µM) of reverse primer and 1 µL (20 ng) of DNA sample. Positive and negative controls were also prepared by adding 1 µL (20 ng) of *L. maculans* (isolate ME24) DNA or *L. biglobosa* (isolate *L. biglobosa* 2003.2.8) DNA from pure cultures and 1 µL of sterile distilled water, respectively, for each set of PCR reaction mixtures prepared.

PCR reactions were done in a thermal cycler TC-5000 (Billy Scientific, TECHNE) with the following PCR conditions; initial denaturation period of 95°C for 2 min followed by 30 cycles of 30 s at 95°C, 30 s at 65°C (for *L. maculans*) or at 63°C (for *L. biglobosa*) and 1 min at 72°C, then 10 min at 72˚C and a hold at 4˚C. PCR products were visualised on 1.5% agarose gel stained with ethidium bromide.

Quantification of L. maculans *and* L. biglobosa *DNA by quantitative PCR*

The amounts of *L. maculans* or *L. biglobosa* DNA present in each of the stem samples were quantified by using SYBR Green qPCR with species-specific primers LmacF/LmacR (for *L. maculans*) and LbigF/LmacR (for *L. biglobosa*), as described by Huang *et al*. (2009). PCR reaction mixtures were prepared to a total volume of 20 µL made up of 10 µL of supermix (Sigma, SYBR green jumpstart TAG ready mix), 6.22 µL of sterile distilled water, 0.6 µL (10 µM) of forward primer and 0.6 µL (10 µM) of reverse primer, 0.08 µL of Rox dye and 2.5 µL (50 ng total) of sample 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. The thermocycling profile consisted of an initial cycle of 95°C for 2 min followed by 40 cycles of 30 s at 60°C, 45 s at 72°C and 15 s 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 s at 95°C. In each qPCR run, a standard dilution series consisting of 10000, 1000, 100, 10 and 1 pg of DNA of *L. maculans* (isolate ME24) or *L. biglobosa* (isolate 2003.2.8) was used to produce a standard curve. The amount of *L. maculans* DNA or *L. biglobosa* DNA for each unknown sample was estimated using the standard curve. Results were expressed as amount (pg) of *L. maculans* or *L. biglobosa* DNA in 50 ng total DNA from diseased plant tissue.

Results

Detection of L. maculans *and* L. biglobosa *DNA by* conventional *PCR*

The conventional PCR results showed a clear difference between *L. maculans* and *L. biglobosa*. *L. biglobosa* was more frequently observed than *L. maculans* in most of the upper stem and stem base samples of the cultivars Capitol, DK Cabernet (DKC), Adriana, Excel, NK Grandia (NKG), Roxet and Bilbao. In general, the percentage of stem samples in which *L. biglobosa* was detected was considerably greater than that in which *L. maculans* was detected (Fig. 1). For all the cultivars except Bilbao and Drakkar, the percentage of upper stem samples with *L. biglobosa* was greater than that with *L. maculans* (Fig.1a). For stem base samples, the percentage of samples with *L. biglobosa* was greater than percentage of samples with *L. maculans,* except for cultivars Drakkar and ES-Astrid (Fig. 1b).

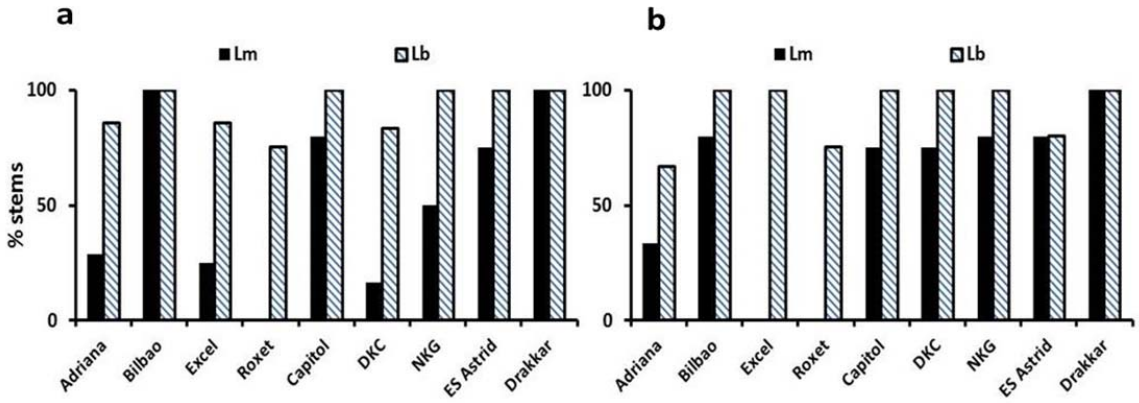

Fig. 1. Percentages of upper stem lesions (a) and stem base cankers (b) on different winter oilseed rape cultivars in which *Leptosphaeria maculans* (Lm) and/or *L. biglobosa* (Lb) were detected in samples collected on 12 July 2012 from a field experiment at Rothamsted in the 2011/2012 growing season.

Quantification of L. maculans *and* L. biglobosa *DNA by quantitative PCR*

In the 2011/12 growing season, the amount of *L. biglobosa* DNA was greater than that of *L. maculans* DNA for both upper stem lesion (Fig. 2a, c) and stem base canker (Fig. 2b, d) samples for all cultivars except for Drakkar, for which the amount of *L. maculans* DNA was greater than that of *L. biglobosa* DNA. For upper stem samples, differences in the amount of *L. biglobosa* DNA between cultivars were also observed, with the greatest amount of *L. biglobosa* DNA observed in Drakkar. The smallest amount of *L. biglobosa* DNA was detected in both upper and base stem samples of Bilbao. In addition, there were differences between cultivars in the amount of *L. maculans* DNA, with the greatest amount in Drakkar.

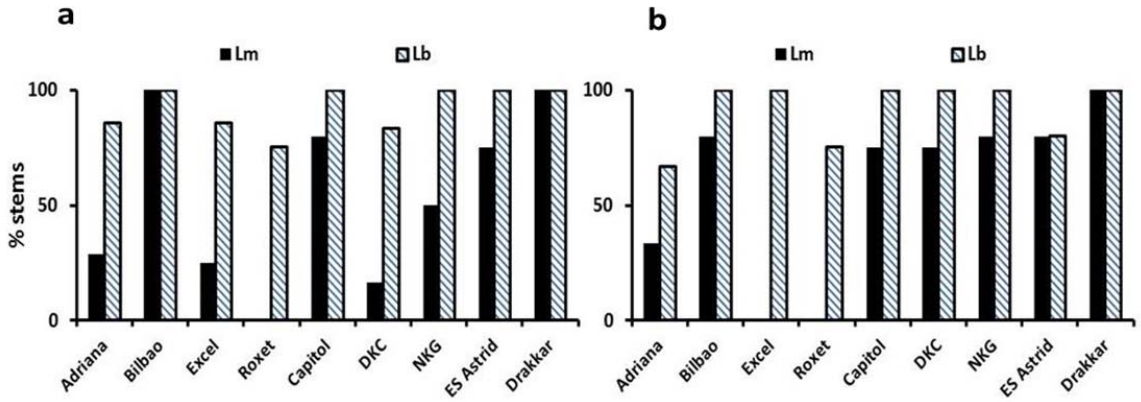

Fig. 2. The amounts of *Leptosphaeria biglobosa* (a, b) or *L. maculans* (c, d) DNA detected by qPCR in diseased upper stem (a, c) and stem base (b, d) samples of different winter oilseed rape cultivars collected on 12 July 2012 from a field experiment at Rothamsted in the 2011/2012 growing season.

Discussion

Results of this work suggest that in the UK *L. biglobosa* can cause severe upper stem lesions and stem base cankers that lead to serious yield losses. The results from both conventional PCR and quantitative PCR showed that *L. biglobosa* was dominant in both upper stem and stem base samples collected at Rothamsted in the 2011/12 growing season. The greater incidence of *L. biglobosa* than that of *L. maculans* in both upper stem and stem base samples suggests that both the upper stem lesions and stem base cankers were mainly caused by *L. biglobosa*. This conclusion is supported by the quantitative PCR results, indicating that the amount of *L. biglobosa* DNA was greater than

that of *L. maculans* DNA in both upper stem and stem base samples. Previously, *L. biglobosa* has been considered less damaging in the UK and its contribution to yield loss has been ignored. There is a need for further research on *L. biglobosa* to investigate the importance of *L. biglobosa* in relation to control of phoma stem canker on oilseed rape in the UK.

There were differences between cultivars in the incidence of *L. maculans* and *L. biglobosa* and in the amounts of *L. maculans* DNA and *L. biglobosa* DNA. This suggests that host resistance affects the co-existence between *L. maculans* and *L. biglobosa* (Fitt et al., 2006b). The smallest amount of *L. biglobosa* DNA was detected in cultivar Bilbao and the greatest amount of *L. biglobosa* DNA was detected in cultivars Roxet and Capitol. The greatest amount of *L. maculans* DNA was in the susceptible cultivar Drakkar and smallest in the resistant cultivars Roxet and Excel. These results suggest that there are effects of cultivar resistance on interactions between *L. maculans* and *L. biglobosa*.

Previous work has shown that ascospores of *L. biglobosa* are generally released later than ascospores of *L. maculans* (Huang *et al*., 2011). The first major release of ascospores was more than two months later in 2011/2012 than in previous season (2010/2011). The large amount of *L. biglobosa* DNA detected in diseased stems in the 2011/2012 season may have been due to the presence of large numbers of *L. biglobosa* ascospores in the inoculum in the 2011/2012 season. This can be confirmed by examining the spore samples using species-specific qPCR. The results of this study suggest that *L. biglobosa* can cause severe phoma stem canker and thus produce subsequent yield losses in the UK. Further work is needed to determine the relative importance of *L. maculans* and *L. biglobosa* in relation to severity of phoma stem canker epidemics over a number of sites and growing seasons.

Acknowledgements

The authors thank the UK Biotechnology and Biological Sciences Research Council (BBSRC, BB/I017585/1), the Home Grown Cereals Authority (HGCA), Felix Thornley Trust and the Chadacre Agricultural Trust for funding the work. We also thank breeding companies DSV, Elsoms, Limagrain, Grainseed, LS Plant Breeding, Monsanto, Pioneer, Saaten-Union and Syngenta for in-kind contributions to the work. We thank Georgia Mitrousia, Siti N Mohamed-Sidique and Mohammed Rafi for help with field sampling.

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