Leptosphaeria maculans/L. *biglobosa* disease progression in oilseed rape and timing of ascospore release under New Zealand conditions

S. Lob, M.V. Jaspers, H.J. Ridgway and E.E. Jones

Lincoln University, PO Box 84, New Zealand

Corresponding author: Suhaizan.Lob@lincolnuni.ac.nz

Abstract *Leptosphaeria maculans*/L. *biglobosa* disease progression in oilseed rape and the timing of ascospore release from crop debris were studied in field experiments in 2011 and 2012. A similar pattern of disease progression was observed in both years, with the first leaf lesions characteristic of *L. maculans* observed 11.5 weeks after sowing and stem lesions after 25 weeks. Leaf lesions characteristic of *L. biglobosa* were observed after 17.5 weeks. *Leptosphaeria maculans* was isolated from non-symptomatic petioles and stems after 16.5 and 22 weeks, respectively, indicating endophytic growth of the pathogen from leaf lesions. Vaseline-coated slides did not trap ascospores in 2011, but in 2012 both Rotorod and Burkard spore samplers trapped ascospores from 29 May to 21 September. Nested PCR with ITS and species-specific primers detected both *L. maculans* and *L. biglobosa* ascospores, with *L. maculans* being more frequently detected. Peak ascospore numbers were detected in August and associated with periods of high rainfall.

Keywords Phoma black leg, Rotorod spore trapper, Burkard spore sampler, leaf lesion.

INTRODUCTION

Phoma blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces. & De Not. or *L. biglobosa* Shoemaker & Brun, is one of the most damaging diseases of oilseed rape (OSR)/canola worldwide (West et al. 2001). In New Zealand, forage brassica crops are also often severely infected. A survey carried out in 2009/2010 by Dr Ian Harvey (PLANTwise) and The Foundation for Arable Research (FAR) reported the disease from all brassica growing areas in New Zealand (Richard Chynoweth, FAR, personal communication). The disease was reported to be present in approximately 50% of OSR paddocks, some with over 40% of plants infected. Of the

two species, *L. maculans* is considered more important in most countries, since it is associated with the damaging stem base canker (West et al. 2001). However, both of these pathogens have a worldwide distribution (West et al. 2001) and often co-exist within the same fields. The first report of *L. maculans* in New Zealand was by Cunningham in 1927 (Cunningham 1927), with *L. biglobosa* only recently recognised as being present in New Zealand (Lob et al. 2013).

Phoma blackleg is generally considered to be a monocyclic disease (Salam et al. 2007). The primary inoculum is released from pseudothecia, which develop on infected stubble left after

wetting by rain or dew (West et al. 2001; Salam et al. 2003). The period and time of ascospore release varies between different regions but it usually coincides with seedling emergence. In Australia, eastern Canada and western Europe, ascospores are released in late autumn or early winter to infect winter OSR seedlings (McGee 1977; West et al. 2001). In contrast, the long cold winters in western Canada and eastern Europe cause an interruption in ascospore release, which starts in autumn, decreases in winter and reaches a peak in the following spring, when the ascospores infect new spring OSR crops (Jedryczka et al. 1999). In New Zealand, the timing of ascospore release has not been determined, although autumn-sown OSR and forage brassicas are more frequently infected than spring-sown OSR (Ian Harvey, PLANTwise, personal communication) indicating a similar situation to Australia, eastern Canada and western Europe.

After dispersal, ascospores adhere to the leaf, germinating in moist or humid conditions with the hyphae infecting the leaf via stomata or a wound. Leaf lesions are first observed 2 weeks later (Huang et al. 2005). The symptoms caused by *L. maculans* initially appear as pale grey spots, often turning pale brown with abundant black pycnidia. Leaf lesions caused by *L. biglobosa* are smaller, have dark margins with light brown centres and contain few, if any, pycnidia, which are formed only on old leaf lesions (Toscano-Underwood et al. 2001).

From the primary leaf lesions, the fungus grows endophytically in the vessels and cortex, down the petiole (Sexton & Howlett 2001) and finally colonises and kills the stem cells, where they produce blackened cankers that contain pseudothecia and pycnidia (West et al. 2001). Stem cankers are light brown in colour and often have distinct dark margins (West et al. 1999) with *L. maculans* characteristically producing lower stem cankers and *L. biglobosa* upper stem cankers. Conidia within the pycnidia are considered to be a source of secondary inoculum that leads to development of further leaf lesions but not stem lesions (Lô-Pelzer et al. 2009). Conidia are spread by rain splash to other leaves on the same

plant or adjacent plants in the same field mainly during the winter.

The aim of this study was to determine the timing of spore release of *L. maculans* and/or *L. biglobosa* from OSR stubble under natural field conditions. Disease progression from leaf lesions to stem canker was also determined.

MATERIALS AND METHODS

Investigation of the timing of ascospore release in the field

Spore-trapping was conducted in 2011 and 2012 in field plots at Lincoln University to determine timing of ascospores release for *L. maculans*/*L. biglobosa*. In 2011 a preliminary experiment was set up in a field site that had grown a previous OSR crop naturally infected with *L. maculans*/*L. biglobosa*. The existing crop was harvested in March leaving the stubble (10 cm height). The field site was replanted with OSR cv. 'Flash' by direct drilling on 11 April. Ascospore release was assessed at weekly intervals from 30 May to 27 September using Vaseline-coated slides secured onto bamboo sticks, one placed randomly in each of eight subplots but abandoned because no ascospores were detected.

Two other types of spore samplers were used to trap ascospores in 2012 under natural field conditions. In March 2012, the existing crop was harvested leaving the stubble (30 cm height) and the field site was replanted with OSR cv. 'Flash' by direct drilling on 30 April 2012. Three Rotorod spore samplers (Aerobiology Company, Nepean, Ontario, Canada) were placed 30 cm above the ground (Ghanbarnia et al. 2011) in the middle of three randomly selected subplots. The clear polystyrene rods used as a trapping surface in the Rotorod sampler were dipped in a Hexane Silicone suspension (manufacturer protocol) and allowed to dry before being placed in the retracting head of the spore sampler. The spore samplers were set up to operate at 10% sampling rate (6 min/h) and changed at weekly intervals, with the position of the three Rotorods randomly rotated between the nine subplots each week between 29 May and 1 October. The numbers of characteristic *L. maculans*/*L. biglobosa* ascospores

were counted on the whole trapping surface of the rods examined using a microscope (400× magnification). The mean number of ascospores trapped at weekly intervals was calculated from the three replicate samples.

A second type of spore sampler, a 7-day Burkard spore sampler (Burkard Scientific Ltd, Uxbridge, Middlesex, UK), was used to trap ascospores in a second field plot where a previous OSR crop was harvested to leave stubble (30 cm) and then direct drilled with a second OSR cv. 'Flash' crop. The Burkard spore sampler was placed in the middle of the plot, with the ascospores being trapped on the melinex tape wrapped around the sampler drum from 22 May to 7 September. Air was sampled at 10 litres/min with the standard orifice size 2 mm × 14 mm, with the drum rotating at 2 mm/h. The melinex tape was changed at weekly intervals, and the tape divided into seven equal sections (48 mm each), each representing a day. Each section was then cut in half along the direction of rotation. One half was mounted on a glass slide and the numbers of characteristic *L. maculans*/*L. biglobosa* ascospores were counted using a microscope (400× magnification) for two randomly selected longitudinal transects following the method described by Lacey & West (2006). These counts were used to calculate the number of ascospores trapped per week. The other half of melinex tape was placed in a 1.6 ml tube and stored at -20°C for DNA extraction and PCR analysis (Jedryczka et al. 2010).

Ascospore trapping data were compared with temperature and rainfall data obtained from the nearest weather station at Lincoln (Lincoln Broadfield EWS).

Disease progression in OSR field plants

Disease progression from the first characteristic leaf lesions to stem cankers was assessed weekly from autumn until spring of 2011 and 2012, in each of the field plots used for determine the timing of ascospore release. As soon as typical leaf lesions were first observed, isolations were made from lesion areas of ten plants from each subplot to verify the causal agent and to track progress of the pathogens. The diseased leaves/petiole/stems

tissues (~0.5 × 1.0 cm) were surface sterilised by immersion in 0.2% sodium hypochlorite solution for 1/1/2 min respectively and rinsed for 1/1/2 min respectively in sterile water. These tissues were placed on potato dextrose agar (PDA) (Difco™, New Jersey, USA) amended with streptomycin (5 mg/ml) and penicillin (5 mg/ml) and incubated at 15°C in darkness for 7 days after which they were examined for colonies characteristic of *L. maculans* and *L. biglobosa* growing from the tissue (West et al. 2002; Kuusk et al. 2002). At each assessment time, asymptomatic tissue from the cotyledon and adjoining stem region was also sampled to assess for systemic growth of the pathogen(s).

Molecular detection of *L. maculans* and/or *L. biglobosa* in spore traps

Since the ascospores of *L. maculans* and *L. biglobosa* cannot be distinguished by morphology, a nested PCR method was used to determine the presence of each species for selected sampling dates. DNA was extracted from the melinex tape of the Burkard spore sampler using the method of Kaczmarek et al. (2009). A nested PCR method was used, whereby genomic DNA was first amplified using the general primers ITS4 and ITS5 for the internally transcribed spacer (ITS) region of the rRNA (White et al. 1990). Each 25 µl PCR contained 2 µl of DNA template, 1× PCR buffer, 1.5 mM MgCl₂, 1 U Faststart Taq Polymerase (Roche), 200 µM of each dNTP, 5 µM ITS4 primer and 5 µM ITS5 primer. The DNA amplification was performed using a Bio-rad iCycler Thermal cycler (Hercules) with initial denaturing at 95°C for 2 min, followed by 40 cycles of 60°C for 30 s, 72°C for 2 min, and 95°C for 30 s followed by a final extension at 72°C for 10 min (White et al. 1990). After the first amplification, PCR products were diluted to 1:50. One µl of the diluted PCR products was used as template for second amplification using the same PCR conditions but using 10 µM of each species-specific primers (*LmacR*, *LmacF* and *LbigF*) (Liu et al. 2006). The thermal cycle comprised initial denaturing at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 70°C for 30 s and 72°C for 1 min, followed by a final-extension at 72°C

for 10 min (Liu et al. 2006). The PCR products were separated on 1.2% agarose gel stained with ethidium bromide (0.5 µg/ml) and photographed under UV light using the Versa Doc™ Imaging System Model 3000. A PCR product of 331 bp indicated the presence of *L. maculans* and 444 bp indicated the presence *L. biglobosa*. To confirm the identity, the PCR products were sequenced and the resulting sequences compared with those in GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the blast function.

RESULTS

Investigation of the timing of ascospore release in the field

No ascospores characteristic of *L. maculans*/*L. biglobosa* were trapped using Vaseline-coated slides in 2011. Ascospores characteristic of *L. maculans*/*L. biglobosa* were trapped in 2012 using both Rotorod and Burkard spore samplers. Ascospores were detected using the Rotorod sampler from 5 weeks after sowing (29 May–6 June 2012) until 21 weeks after sowing (17–21 Sept 2012). From 29 May to 29 June, the numbers of ascospores trapped were low, being

less than 1.0 ascospores/m³ at each assessment time. In general the numbers of ascospores trapped were higher throughout July and August, with the highest number of ascospores (9.2 ascospores/m³) trapped 16 weeks after sowing (13–17 August) (Figure 1). The release of ascospores appeared to be associated with rainfall, with peak numbers trapped during weeks with high rainfall or the week after the rain event.

Spore trapping with the Burkard spore sampler was done for 16 weeks, starting from 22 May (4 weeks after sowing) until 13 September (20 weeks after sowing). The numbers of ascospores trapped were low (<1.5 ascospores/m³) for all weeks except for 15 and 16 weeks after sowing (7–14 and 14–21 August) when ascospore numbers were 2.7 and 8.1 ascospores/m³, respectively (Figure 2). These two peaks in ascospore numbers coincided with high rainfall events (63.6 and 34.6 mm/week, respectively). The maximum daily temperature was 18.7–7.8°C and the minimum daily temperature -0.6–7.8°C during the experiment, and there seemed to be no clear relationship with ascospore numbers trapped (data not shown).

Figure 1 Seasonal pattern of the number of ascospores (bars) trapped using the Rotorod sampler in relation to the total rainfall (line) for the corresponding week for the period from 29 May to 5 October 2012 in an oilseed rape field experiment at Lincoln.

Figure 2 Seasonal pattern of the number of ascospores (bars) trapped using the Burkard spore sampler in relation to the total rainfall (line) for the corresponding week for the period from 22 May to 13 September 2012 in an oilseed rape field experiment at Lincoln.

Verification of presence of *L. maculans* and/or *L. biglobosa* ascospores

Low DNA concentrations were detected from the June and July samples corresponding to low ascospore counts on the same melinex tape assessed by microscopy, while higher DNA concentrations were detected from the August sample where microscopic assessments had indicated high ascospore numbers. Bands of 331 bp, indicating presence of *L. maculans* DNA, were observed for all samples tested (June-September), whilst bands of 444 bp, indicating presence of *L. biglobosa* DNA, were detected only in the June and July samples (Figure 3). Comparison of sequence obtained from the

331 bp and 444 bp products showed they were 100% identical to the reported sequence of *L. maculans* ‘brassicae’ (DQ133891.1) and *L. biglobosa* ‘brassicae’ (DQ133893.1) in GenBank.

Disease progression in OSR field plants

For 2011, the first leaf lesion typical of *L. maculans* infection was observed in the second week of July, 12 weeks after sowing (Table 1). During the first observation, 20% of the sampled plants had leaf lesions typical of *L. maculans* with the pathogen being isolated from these leaf lesions. Disease incidence increased to 80% and *L. maculans* was isolated from 100% of the lesions sampled. *Leptosphaeria maculans* was isolated from

Table 1 Disease progression (week after sowing) of *Leptosphaeria maculans*/*L. biglobosa* on oilseed rape field plants from the appearance of the first leaf lesions to stem canker.

Disease progression	Weeks after sowing (date)	
	2011	2012
First leaf lesion typical of <i>L. maculans</i> observed	12 (6 July)	12 (20 July)
<i>L. maculans</i> recovered from symptomless petiole	16 (3 August)	18 (31 August)
<i>L. maculans</i> recovered from symptomless stem	21 (6 September)	23 (5 October)
First lower stem lesion typical of <i>L. maculans</i> observed	24 (27 September)	27 (29 October)

the symptomless petioles from 44% of the plants with typical leaf lesions in the first week of August and from the symptomless stems from 33% of the plants with typical leaf lesions in the second week of September. The first lesion was observed on the lower stem at the end of September, 24 weeks after sowing. Very few leaf lesions typical of *L. biglobosa* were observed 16 weeks after sowing, and attempts to isolate *L. biglobosa* from these leaf lesions failed. Upper stem cankers characteristic of *L. biglobosa* were only observed 29 weeks after sowing, with *L. biglobosa* being isolated from these stem lesions. Disease incidence of *L. biglobosa* was low, being less than 10%.

A similar pattern of disease progression was observed in 2012 (Table 1). The first leaf lesion typical of *L. maculans* was visible in the third week of July, 12 weeks after sowing, with *L. maculans* being isolated from 100% of the plants with typical lesions. Seven weeks later (early September, 19 weeks after sowing) leaf lesions typical of *L. biglobosa* had been observed, but again, attempts to isolate the pathogen failed. At the end of August, 18 weeks after sowing, *L. maculans* was isolated from the symptomless petioles from 29% of plants with typical leaf lesions and from the symptomless stems from 100% of plants with typical leaf lesions in the first

week of October (23 weeks after sowing). The first lesions on the lower stems were observed 27 weeks after sowing, at the end of October. However, due to the time constraint to prepare the plot for the next trial, the crop was harvested once the lower stem cankers had been observed with no upper stem lesions being observed.

Leptosphaeria maculans grew asymptotically from the first leaf lesion to the petiole within 4–6 weeks and grew down to the stem without external stem symptoms within 5 weeks. Expression of stem base lesions happened within 12–15 weeks from the appearance of the first leaf lesion.

DISCUSSION

This is believed to be the first report of *Leptosphaeria maculans*/*L. biglobosa* disease progression in oilseed rape and timing of ascospore release under New Zealand conditions. This study is crucial to establishing an understanding of the pathogens' disease cycle and their relationship with environmental conditions. Spore trapping attempts in 2011 using Vaseline-coated slides were unsuccessful, possibly due to the passive nature of this sampling method. As shown by the other sampling methods (Rotorod and Burkard spore samplers), which actively draw in air, the ascospore numbers released are

Figure 3 Detection of *L. maculans* and/or *L. biglobosa* DNA trapped on melinex tape (Burkard spore sampler) by nested PCR with primary amplification of fungal DNA using ITS4 and ITS5 and secondary amplification using species-specific primers *LmacF*, *LmacR*, *LbigF*; L, 1 kb molecular marker; *Lm*, positive control for *L. maculans* isolate; *Lb*, positive control for *L. biglobosa* isolate; lanes 1–5, 26–30 June; lanes 6–7, 1–2 July; lanes 8–14, 10–16 July; lanes 15–18, 14–17 August; C negative control for PCR.

low and therefore there was a very low probability of the spores being trapped using the Vaseline-coated slides. However, disease symptoms characteristic of *L. maculans* and *L. biglobosa* were observed to develop on OSR plants in this plot, with *L. maculans* being isolated from symptomatic lesions indicating the presence of pathogen spores.

Leptosphaeria maculans was found to penetrate the host plant through the leaf lamina to reach the petiole 4 weeks after the first leaf symptoms were observed. At this stage, the pathogen did not express any visible symptoms on petioles, but was detected by isolation onto agar and subsequent identification based on colony morphology. From the petiole, the pathogen was found to move into the stem, resulting in the formation of a basal stem canker 12 weeks after the first leaf lesion symptom was observed. However, results from isolations showed that the pathogen had already infected the stem 3 weeks before stem lesions were observed. Similar results have been reported by Sexton & Howlett (2001) but these researchers did not provide any information on the timing of pathogen progression to infect the stem.

Leaf lesions typical of *L. biglobosa* (small light brown lesions with dark margins; Toscano-Underwood et al. 2001) were observed in the field in both 2011 and 2012. However, *L. biglobosa* was not isolated from these lesions, with fungi identified morphologically as *Alternaria* spp. and *Phoma exigua* being instead isolated. It is possible that these fungal species can cause leaf lesion symptoms similar to *L. biglobosa*, making early identification of *L. biglobosa* infections difficult. However, using the nested PCR method, *L. biglobosa* was detected in the June and July spore trapping samples indicating the presence of this pathogen, with *L. biglobosa* isolated from the upper stem cankers typical of *L. biglobosa*, which developed on a few plants, albeit in lower numbers than seen with *L. maculans*.

The chronological patterns of ascospores being trapped with the Rotorod and Burkard spore samplers were generally similar, although lower ascospore numbers were trapped using

the Burkard spore sampler. The results using the rotorod sampler showed that there was one peak in July, but most ascospores were detected in August. Ascospore release patterns were similar to those reported for Australia, eastern Canada and Western Europe. At four locations in Western Australia ascospore discharge started in July, reached a peak in August and declined in September, with no ascospores discharged in October (Khangura et al. 2007). Similarly, in another study in Western Australia the highest concentrations of ascospores were observed in June, July and August, with numbers declining in September and October (Bokor et al. 1975). In the present study, ascospore release appeared to coincide with rain events, with higher ascospore numbers trapped in the week during or immediately after high rainfall (above 4.5 mm/week). Guo & Fernando (2005) also reported peak ascospore release was associated with rain events, with ascospore dispersal continuing for up to 3 days after each rain event.

Although ascospores were first detected on 22 May, the first *L. maculans* leaf lesions were not observed until almost 2 months later on 20 July. In comparison, Huang et al. (2005) found the time taken from first ascospore release to expression of the initial leaf symptoms was only 1-3 weeks over the study period in 1998-2004. In the current study, although ascospores were detected in May, the environmental conditions may not have been conducive for infection. Also, in the present study visible stem lesions were observed 15 weeks after the appearance of leaf lesions, whereas Huang et al. (2005) reported that appearance of stem cankers took much longer to develop, being observed after 23-28 weeks. This may be related to differences in the relative susceptibility of the OSR cultivar used or related to differences in environmental conditions post infection.

The results from both the field experiments and the molecular method demonstrated that both *L. maculans* and *L. biglobosa* were present. Whilst microscopic analysis of the samples enabled the presence and numbers of ascospores

characteristic of *L. maculans*/*L. biglobosa* to be determined, the ascospores of the two species cannot be distinguished by morphology (Kaczmarek et al. 2009). However, using the nested PCR method and the species-specific primers for *L. maculans* and *L. biglobosa*, both species were detected. *Leptosphaeria maculans* and/or *L. biglobosa* were detected in each of the samples tested, i.e. one sample for each month. *Leptosphaeria maculans* was detected in the 27 June; 15, 16 and 31 August; and 1 and 3 September samples, with *L. biglobosa* detected in the 29 and 30 June and 15 July samples. In contrast Toscano-Underwood et al. (2003) reported that in the UK, ascospores of *L. biglobosa* were released in spring, later than those of *L. maculans*. Although the molecular method indicated the presence of *L. biglobosa* ascospores in June and July, no leaf lesion typical of *L. biglobosa* was observed. However, *L. biglobosa* stem cankers were observed later in the season. *Leptosphaeria biglobosa* has been reported to develop leaf lesions more slowly than *L. maculans*, with the time between ascospore release and appearance of leaf lesions reported to take at least 1 month and up to 18 weeks in comparison to 1–3 weeks for *L. maculans* (Huang et al. 2005).

Although the pattern of ascospore discharge was only recorded for one season (2012), the results of the study have shown an indication of the risk periods for infection under New Zealand conditions.

ACKNOWLEDGEMENTS

This study was conducted with financial support from the Foundation for Arable Research (FAR). The authors thank the Ministry of Higher Education Malaysia (MOHE) and University Malaysia Terengganu (UMT) for providing a scholarship to S. Lob, Ian Harvey (PlantWise) for providing advice on spore trapping and Lincoln University field staff for assistance in setting up the field experiments.

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