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Identification of *Leptosphaeria biglobosa* 'canadensis' on *Brassica juncea* stubble from northern New South Wales, Australia

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Abstract. *Leptosphaeria biglobosa* 'canadensis' is reported for the first time in Australia. All 88 *Leptosphaeria* isolates cultured from *Brassica juncea* stubble from northern NSW were *L. biglobosa* 'canadensis' whilst all 55 isolates cultured from Victorian stubble of the same *B. juncea* lines were *L. maculans*. Both *L. biglobosa* 'canadensis' and *L. maculans* formed similar sized lesions on *B. juncea* cotyledons after 14 days. However, *L. biglobosa* 'canadensis' isolates colonised stems less effectively than *L. maculans* and consequently caused less crown cankering.

The two Dothideomycetes, *Leptosphaeria maculans* and *L. biglobosa*, comprise a species complex associated with disease of crucifers including *Brassica napus* (canola, oilseed rape). Through the use of molecular techniques, *L. biglobosa* has been divided into six subclades, namely 'canadensis', 'occiaustralensis', 'brassicae', 'australiensis', 'erysimii' and 'thlaspii'. *Leptosphaeria maculans* has been divided into two subclades, 'brassicae' and 'lepidii' (Mendes-Pereira *et al*. 2003; Voigt *et al*. 2005; Vincenot *et al*. 2008). In this paper we refer to *L. maculans* 'brassicae' as *L. maculans.*

During infection of *B. napus*, *L. maculans* produces grey/green large leaf lesions, followed by crown stem cankers. This disease, known as blackleg or Phoma stem canker, results in significant yield losses, particularly in Australia (Howlett 2004; Fitt *et al*. 2006*a*). In contrast, none of the subclades of *L. biglobosa* are reported to cause crown stem cankers. *Leptosphaeria biglobosa* 'brassicae' is the most common subclade. It is found in most canola growing areas with the exception of central Canada and Australia (Fitt *et al*. 2006*a*). These isolates cause small dark leaf lesions followed by pale brown stem lesions with a dark margin on the upper stem (known as Phoma or upper stem lesions) (West *et al*. 2002). In Australia, *L. biglobosa* 'australiensis' (cultured from *B. napus*) and more recently, *L. biglobosa* 'occiaustralensis' (cultured from *B. napus* and *Raphanus raphanistrum* (wild radish)) have been described in eastern and western Australia, respectively (Plummer *et al*. 1994; Vincenot *et al*. 2008).

Blackleg is primarily controlled by breeding varieties with resistance to *L. maculans* (Delourme *et al*. 2006). *Brassica juncea* (Indian mustard) is generally more resistant to *L. maculans* than *B. napus* (Purwantara *et al*. 1998; Li *et al*. 2008). Additionally, *B. juncea* is more drought-tolerant and can be grown in regions with short, warm to hot growing seasons in which soil water supply is unreliable (Oram *et al*. 2005). In Canada, *B. juncea* has been grown as a condiment mustard crop for 20 years, specifically in the hotter, drier areas of western Canada. In 2002, *B. juncea* varieties were released that produced canola quality oils (Burton *et al*. 2004). Over the past 20 years in Australia, small acreages in regions including northern New South Wales have produced condiment mustard. *Brassica juncea* varieties that produce canola-quality oils were commercially released in 2007 (Burton *et al*. 2008).

Since blackleg disease is a threat to the Australian oilseed industry, fungal populations are monitored for virulence changes in relation to both *B. juncea* and *B. napus* varieties released. During 2006 and 2007, 152 *Leptosphaeria* ascospore isolates were cultured from *B. juncea* stubble collected from trial sites in Victoria (Horsham and Beulah) and northern NSW (Tamworth, Burren Junction and Rowena). Fifty-five of these isolates were subjected to Polymerase Chain Reaction analysis and the resultant DNA fragments of four regions of the genome were sequenced. These genomic regions were the internal transcribed spacer (ITS) of rDNA (amplified with primers 5′-CCGTTGGTGAACCAGCGGAGGGATC-3′ and 5′-TCCGCTTATTGATATGCTTAAG-3′, Mendes-Pereira *et al*. 2003), actin (primers 5′-GAGCAGGAGATCCAGAC TGC-3′ and 5′-TTCGAGATCCACATCTGCTG-3′), β-tubulin (primers 5′-GTCGAGAACTCCGACGAGAC-3′ and 5′-ATC TGGTCCTCGACCTCCTT-3′) and the mating type allele, *MAT1–2* (primers 5′-GATGCCATGCACAAGAAGCTC-3′ and 5′-GCTTGGCCTTGCGCGACTGGC-3′). These latter three sets of primers were designed by aligning the sequences of *L. biglobosa* and *L. maculans* isolates (Voigt *et al*. 2005;

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Vincenot *et al*. 2008) and choosing a nucleotide region common to all isolates.

Sequences of the ITS, actin, β-tubulin and *MAT1–2* from isolates cultured from *B. juncea* stubble were compared with previously published sequences from *L. maculans* and *L. biglobosa* isolates (Table 1). On this basis, all 25 isolates from Victoria were identified as *L. maculans* (displaying 100% sequence similarity with previously published *L. maculans* sequences for each genomic region analysed) whilst all 30 northern NSW isolates were *L. biglobosa* 'canadensis' (displaying 100% sequence similarity with previously published *L. biglobosa* 'canadensis' sequences for each genomic region analysed). This is the first report of *L. biglobosa* 'canadensis' in Australia. Representative sequences from *L. maculans* and *L. biglobosa* 'canadensis' isolates have been deposited into GenBank (Accession numbers FJ172238, FJ172239, FJ172240, FJ172241, FJ172242, FJ172243 and FJ172244).

As well as by DNA sequencing, which is expensive and time-consuming, *L. maculans* and *L. biglobosa* 'canadensis' isolates can be distinguished by the size of the ITS fragments separated on a 2% agarose gel (580 and 555 bp, respectively) (Mendes-Pereira *et al*. 2003). On this basis, an additional 39 Victorian isolates cultured from *B. juncea* were classified as *L. maculans* and 58 isolates from northern NSW were classified as *L. biglobosa* 'canadensis'. Two *L. biglobosa* 'canadensis' isolates have been deposited into the New South Wales Plant Pathology Herbarium (DAR79245 and DAR79246).

Ascospore isolates (250) were also cultured from *B. napus* stubble collected from field trials in Victoria (Horsham, Geelong and Lake Bolac), southern NSW (Wagga Wagga and Illabo) and South Australia (Moyhall, Bordertown and Yeelena) between 2005 and 2007. All of these isolates were identified as *L. maculans*. Surprisingly, no *L. biglobosa* or *L. maculans* isolates were cultured from *B. napus* stubble collected from the same sites as the *B. juncea* stubble in northern NSW. This stubble contained isolates belonging to the genera *Alternaria*, *Pleospora* and *Embellisia*.

The infection pathway of the *L. maculans* and *L. biglobosa* 'canadensis' isolates cultured from *B. juncea* stubble was determined on three *B. juncea* advanced breeding lines, one *B. juncea* variety and three *B. napus* varieties. The *B. juncea* lines and variety were JC05002, JC05006, JC05007 and Dune, respectively. The resistance genes in these *B. juncea* lines and variety are uncharacterised. The *B. napus* varieties were Q2 (susceptible to most Australian isolates), Beacon (polygenic resistance) and Surpass 400 (*B. rapa* ssp. '*sylvestris*'-derived resistance) (Crouch *et al*. 1994). Cotyledons were wounded and inoculated with conidia (10^5) as described by Purwantara *et al*. (1998). Three *L. maculans* (06VTJ085, 06VTJ095 and 06VTJ112) and two *L. biglobosa* 'canadensis' (06VTJ140 and 06VTJ154) isolates were individually tested. Four cotyledons of each variety, inoculated with either *L. maculans* or *L. biglobosa* 'canadensis' were examined. After 8 days, small lesions (1–2 mm in diameter) formed on cotyledons of all plants inoculated with *L. maculans* isolates, whilst large necrotic lesions (5–7 mm in diameter) were observed on cotyledons inoculated with *L. biglobosa* 'canadensis' isolates (Fig. 1*a*, *b*). After 14 days, lesions of similar size were observed on all lines and varieties inoculated with either *L. maculans* or *L. biglobosa* 'canadensis' (Fig. 1*c*, *d*). Tissue was stained with lactophenol trypan-blue and mounted in glycerol (Keogh *et al*. 1980). The lesions caused by *L. maculans* isolates radiated symmetrically from the inoculation site whilst lesions caused by *L. biglobosa* 'canadensis' were spread asymmetrically across the cotyledon (Fig. 1*e*, *f*). Despite the differences in lesion appearance, hyphae of all isolates grew intercellularly within the cotyledons (Fig. 1 *g*, *h*) and petioles (Fig. 1*i*, *j*). A similar infection pattern was seen in the *B. napus* varieties inoculated with these isolates (data not shown).

Table 1. Published gene sequences (GenBank accession numbers) of *Leptosphaeria* spp. used in this study

Gene	*L. maculans*	*L. biglobosa* 'canadensis'
ITS	AJ550883	AJ550867
Actin	AY748971	AY748959
β-tubulin	AY749018	AY749007
MAT1–2	AY748928	AY748941

Ten weeks post-inoculation, two stems of each variety inoculated with either *L. maculans* or *L. biglobosa* 'canadensis' were stained with trypan blue. In both *B. juncea* and *B. napus* branched hyphae of *L. maculans* were growing intercellularly at the base of the stem (Fig. 1*k*, *l*). In contrast, no hyphae of *L. biglobosa* 'canadensis' were observed (data not shown). This is the first report of disease progression for *L. biglobosa* 'canadensis' on both *B. juncea* and *B. napus*.

To determine whether these isolates could form crown stem cankers after inoculation by a non-wounding method, plants were sprayed with conidia of individual *L. maculans* (64) and *L. biglobosa* 'canadensis' (88) isolates. Five plants of each variety or line were sprayed at the first leaf stage with conidia (10^7) suspended in 0.05% Tween 20. Internal infection was determined at maturity (6 months) by cross-sectioning stems at the crown and visually assessing the percentage of blackening (Marcroft *et al*. 2004). The internal stem infection data were averaged for all *L. maculans* isolates and for all *L. biglobosa* 'canadensis' isolates for each *Brassica* variety or line. Differences in averages were statistically analysed using one-way ANOVA. *Leptosphaeria biglobosa* 'canadensis' isolates caused significantly less internal stem infection at the crown of the stem than *L. maculans* isolates on all *B. juncea* lines and *B. napus* varieties tested ($P < 0.001$) (Table 2). As expected, less internal stem infection was observed in *B. juncea* lines compared with *B. napus* varieties when infected with *L. maculans* or *L. biglobosa* 'canadensis' isolates. Eighteen of 64 *L. maculans* isolates caused more than 30% internal stem infection in *B. juncea* lines, compared with only 7 of 88 *L. biglobosa* 'canadensis' isolates. For *B. napus* varieties, 49 of 64 *L. maculans* isolates caused more than 50% internal stem infection compared with 13 of 88 *L. biglobosa* 'canadensis' isolates.

Similar to our findings with *L. biglobosa* 'canadensis', *L. biglobosa* 'brassicae' has been shown to develop cotyledonary lesions more quickly on *B. napus* than *L. maculans* (Fitt *et al*. 2006*b*). In the present study, despite hyphae of *L. biglobosa* 'canadensis' and *L. maculans* growing intercellularly within the cotyledon and petiole, no *L. biglobosa*

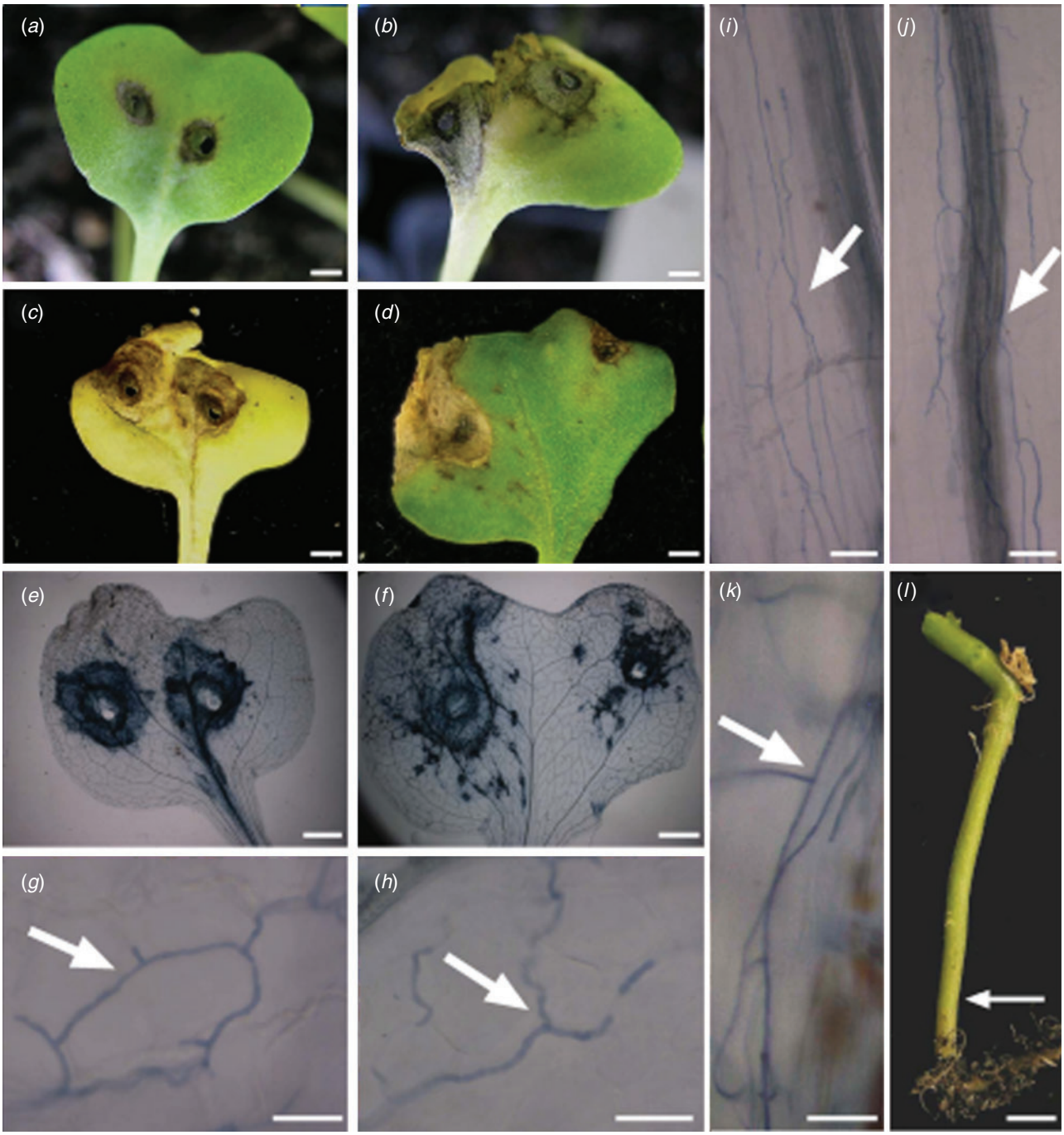

Fig. 1. Disease progression of *Leptosphaeria maculans* isolate 06VTJ095 and *L. biglobosa* 'canadensis' isolate 06VTJ154 after inoculation of wounded cotyledons of *Brassica juncea* var. Dune. (*a*, *b*) At 8 days post-inoculation lesions were smaller on cotyledons inoculated with *L. maculans* (*a*) than with *L. biglobosa* 'canadensis' (*b*). (*c*, *d*) After 14 days, lesions were of a similar size on cotyledons infected with either *L. maculans* (*c*) or *L. biglobosa* 'canadensis' (*d*). (*e*, *f*) Lesions caused by *L. maculans* (*e*) radiated symmetrically from the inoculation site whilst lesions caused by *L. biglobosa* 'canadensis' (*f*) were spread across the cotyledon. (*g*, *h*) Hyphae (white arrows) of *L. maculans* (*g*) and *L. biglobosa* 'canadensis' (*h*) grew intercellularly within the cotyledon. (*i*, *j*) Hyphae (white arrows) of *L. maculans* (*i*) and *L. biglobosa* 'canadensis' (*j*) grew down the petiole. (*k*, *l*) Ten weeks after inoculation, branched intercellular hyphae (white arrow) of *L. maculans* (*k*) were observed at the base of the stem (*l*) (white arrow) but hyphae of *L. biglobosa* 'canadensis' were not (data not shown). Three *L. maculans* and two *L. biglobosa* 'canadensis' isolates were individually inoculated onto four *B. juncea* and three *B. napus* varieties or lines. Four cotyledons and two stems from each variety or line inoculated with either *L. maculans* or *L. biglobosa* 'canadensis' were examined. Scale bars: (*a*–*f*) 5 mm; (*g*, *h*) 0.1 mm; (*i*–*k*) 0.5 mm; (*l*) 10 mm.

Table 2. Disease severity of *Leptosphaeria maculans* or *L. biglobosa* 'canadensis' isolates on *Brassica juncea* lines and *B. napus* varieties

Species	Variety/Line	Average internal stem infection (%)[A]	
		L. maculans	*L. biglobosa* 'canadensis'
Brassica juncea	JC05002	15 ± 2	9 ± 1
	JC05006	11 ± 1	4 ± 1
	JC05007	11 ± 1	6 ± 1
B. napus	Beacon	34 ± 3	18 ± 2
	Q2	65 ± 3	24 ± 2
	Surpass 400	16 ± 3	3 ± 1

[A]Five plants were sprayed with conidia of each isolate under controlled environmental conditions and disease severity (internal stem infection) determined at plant maturity (6 months). The average percentage internal stem infection caused by all *L. maculans* isolates (64) was compared with the average internal stem infection caused by all *L. biglobosa* 'canadensis' isolates (88) for all varieties and lines. These differences were statistically significant with $P < 0.001$ for all these comparisons. Values are presented as averages plus standard errors.

'canadensis' hyphae were observed in the eight stems of infected plants examined after 10 weeks. However, internal stem infection was evident at plant maturity, albeit significantly less than in plants inoculated with *L. maculans*. This reduced efficiency of *L. biglobosa* 'canadensis' to colonise the stem could be due to several factors including the inability to produce the toxin, sirodesmin PL, which significantly contributes to the virulence of *L. maculans* in *B. napus* stems (Elliott *et al*. 2007).

Previously in Australia two *L. biglobosa* 'australiensis' and 19 *L. biglobosa* 'occiaustralensis' isolates were identified amongst populations of *L. maculans* isolates (Plummer *et al*. 1994; Vincenot *et al*. 2008). In the present study, all 88 isolates identified on *B. juncea* stubble from northern NSW were *L. biglobosa* 'canadensis'. It is curious that these isolates were found in high frequency in this region and not in any other Australian region tested. One possible explanation is that northern NSW is a summer dominant rainfall region, which may influence pseudothecia development and affect timing of ascospore release resulting in more favourable conditions for *L. biglobosa* 'canadensis'. The epidemiology of this fungus is unknown.

Due to its ability to undergo sexual recombination, populations of *L. maculans* can rapidly adapt to selection pressures including exposure to major gene resistance (Sprague *et al*. 2006). All *L. biglobosa* 'canadensis' isolates cultured in this study were from ascospores, demonstrating that *L. biglobosa* 'canadensis' is undergoing sexual recombination in the field. Whether populations of *L. biglobosa* 'canadensis' will adapt to selection pressures and develop the ability to invade stems of *B. juncea* and *B. napus* is unknown.

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