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New or Unusual Disease Reports

Plenodomus biglobosus on oilseed rape in Hungary

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Summary. The commonly occurring blackleg is an economically important disease in oilseed rape cultivation. This disease is caused by a complex of two closely related species, *Plenodomus lingam* and *P. biglobosus*. To date, only *P. lingam* (syn.: *Leptosphaeria maculans*) has been known in Hungary as the cause of blackleg in oilseed rape crops. The present study aimed to determine if *P. biglobosus* (syn.: *Leptosphaeria biglobosa*) was present in Hungary. The two fungus pathogens are difficult to distinguish using conventional morphological criteria. Reliable detection and differentiation of the two species can only be achieved using molecular methods. This is the first report describing the pathogen, *P. biglobosus*, in Hungary.

Keywords. *Brassica napus*, blackleg, multiplex PCR, ITS region.

INTRODUCTION

Blackleg or stem canker of oilseed rape is an internationally important disease of oilseed brassicas (oilseed rape, canola) (Rouxel and Balesdent, 2005). In Brassica-growing areas (especially in Australia, North America and Europe) this disease can cause severe yield losses (Henderson, 1918; Fitt *et al*., 2006). The disease is associated with two closely related fungi, *Plenodomus lingam* and *P. biglobosus* (Dilmaghani *et al*., 2009). These fungi have been referred to as *Leptosphaeria* species, but recent studies reveal that they belong in *Plenodomus* (de Gruyter *et al*., 2012; Wijayawardene *et al*., 2014). Co-existence of these two pathogens has been reported in different countries of Europe, including Poland (Kaczmarek and Jędryczka, 2011), Lithuania (Brazauskienė *et al*., 2011) and the Czech Republic (Mazáková *et al*., 2017).

Varga (2014) noted that the disease caused by *P. lingam* had become a significant factor in oilseed rape cultivation in the Carpathian Basin. Aggressive and non-aggressive isolates of *P. lingam* could be differentiated, based on restriction fragment length polymorphism (RFLP) analyses (Koch *et al*., 1991). Two groups of *P. lingam* isolates can be further separated, based on their abilities to produce phytotoxins *in vitro* (Koch *et al*., 1989). The isolates producing phytotoxin were defined as Tox^+, while non-toxin-producing isolates were defined as Tox^0 (Balesdent *et al*., 1992). The weakly virulent Tox^0 isolates (designated as the new species *P. biglobosus* since 2001) can cause necrotic lesions on the upper stems of plants, and are less likely to cause stem cankers than Tox^+ isolates (Shoemaker and Brun, 2001; West *et al*., 2002).

In 2010, samples from *Brassica napus* stems from near Rimski Šančevi, Serbia, with symptoms of canker were cultured, and some of the resulting isolates were identified as *P. biglobosus* (Mitrović *et al*., 2016). Due to the proximity of this region to Hungary, there was reason to assume that *P. biglobosus* could also be present in Hungary. Therefore, the aim of the present study was to determine if *P. biglobosus* was present in this country.

MATERIALS AND METHODS

Plant samples, fungus isolation and morphology

Leaves and stems of oilseed rape plants showing symptoms of blackleg were collected from oilseed rape hybrids in fields from four counties in Hungary in 2017 and 2018. Samples were transferred to the laboratory of the Department of Plant Pathology, Szent István University. Unsterilized samples were placed in humidity chambers made from 30 cm diam. Petri dishes each containing a layer of filter paper moistened with sterile distilled water to induce the pycnidia to exude cirri of conidia. Three days later, conidia were collected with sterile handmade glass needles, using the method of Goh (1999), and were suspended in sterile distilled water. Size and shape of 50 conidia per sample were characterized. Conidium size data were evaluated using multivariate analysis of variance (MANOVA). Normality of residuals was checked using the Shapiro-Wilk test, homogeneity of variances was assessed using Levene's test. Multivariate differences between samples were determined using the Wilk's-lambda (Tabachnick and Fidell, 2013).

Conidium suspensions were transferred onto potato dextrose agar (PDA, BioLab Zrt.), distributed using a spread plate technique, and then incubated for 3–4 d in the dark at 24 ± 1°C. Hyphal tips from germinating conidia (observed under an inverted microscope) were aseptically transferred onto fresh PDA plates using sterile dissection needles. Monoconidial isolates were characterized after 28 d. Macroscopic traits (growth rates, colour of mycelia, colony shapes, edges and patterns, amounts of aerial mycelium, presence of fruiting bodies, and pigment secretion) and microscopic traits (colour, shape and sizes of conidia) of fungal isolates were recorded.

Within two years, 188 plant samples were collected, and 54 *Plenodomus lingam* isolates were obtained. Three putative *P. biglobosus* isolates were chosen for detailed comparison with four *P. lingam* isolates.

Pathogenicity tests

Pathogenicity of the isolates was tested by inoculating the stems of live seedlings and detached leaves of oilseed rape seedlings. The seedlings were grown in potting mix soil from seeds that were untreated by fungicides, and were kept in the greenhouse of the Department of Plant Pathology (Buda Campus) for 2 months at 26 ± 3°C. Five seedlings were each injured above the cotyledons in V shape, using a sterile dissection needle, and were then inoculated with hyphal tip mycelium from 7–10 d-old PDA colonies. Five detached leaves were each inoculated at the main vein on the upper surface without puncturing, with each of seven different isolates. The detached leaves were then placed in sterile glass vessels containing sterilized glass beads and sterile distilled water in order, to maintain 95 ± 3% relative humidity. Control seedlings and control detached leaves were treated as for inoculated specimens with sterile pieces of PDA. The inoculated seedlings and the glass vessels containing the detached leaves were incubated under natural light conditions at room temperature for 10 d. Re-isolations from symptomatic tissues were made onto PDA to fulfil Koch's postulates. The pathogenicity test was repeated once.

DNA extraction from fungi, amplification in multiplex PCR and sequencing

Genomic DNA was extracted from the growing margins of single conidium colonies on PDA, using the cetyl-trimethyl-ammonium-bromide (CTAB) method (Maniatis *et al*., 1983), followed by phenol-chloroform extraction and isoamyl alcohol (24:1) precipitation. The concentration and purity of the DNA were evaluated using a NanoDropTM Spectrophotometer.

The ribosomal RNA region incorporating the internal transcribed spacer (ITS) regions and the 5.8S rRNA gene from *Plenodomus* isolates were amplified by multiplex PCR. The reverse primer LmacR (5' GCAAAATGTGCTGCGCTCCAGG 3') specific for *P. lingam* and *P. biglobosus*, and two species-specific forward primers; LmacF (5' CTTGCCCACCAATTGGATCCCCTA 3', for *P. lingam*) and LbigF (5' ATCAGGGGATTGGTGTCAGCAGTTGA 3', for *P. biglobosus*) were used (Liu *et al*., 2006). Two different PCR products were reliably detected: one of 331 bp from *P. lingam* isolates, and the other of 444 bp from *P. biglobosus* isolates.

PCR mixtures were prepared in a reaction volume of 50 μL, containing 15 ng genomic DNA, 0.2 μM forward and reverse primer, 200 μM dNTPs, 2.5 mM $MgCl_2$ and 0.4 U DreamTaq Polymerase (Thermo Scientific) in 10× Dream Taq Buffer (Fermentas). Amplifications were carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems), using amplification conditions consisting of denaturation at 95°C for 3 min, followed by 30 cycles of the following steps: denaturation at 95°C for 15 sec, annealing at 70°C for 30 sec, and extension at 72°C for 60 sec, with a final extension step at 72°C for 10 min. PCR products specific for *P. lingam* and *P. biglobosus* were electrophoretically separated in 1% agarose gel run in 1× TBE buffer. The gel was stained with EcoSafe stain and the products were visualized and photographed under UV light. The amplicons were purified using the High Pure PCR Product Purification Kit (Roche). Fragments were sequenced in both directions using the same primers as for PCR, in an ABI Prism automatic sequencer (BaseClear B.V.). Nucleotide sequence identities were determined by BLAST analyses.

RESULTS AND DISCUSSION

In 2017 and 2018, leaf and stem spots were observed on oilseed rape plants as specific symptoms of blackleg. Greyish lesions containing numerous small, black pycnidia, appeared on lower leaves of affected plants. The symptoms were identical to those for blackleg of oilseed rape described in Western Australia (Bokor *et al*., 1975). The pathogens causing the disease were identified in 54 samples from the isolates examined in this study, using morphology and molecular biology methods. *Plenodomus biglobosus* was identified from leaf samples from Kétpó (47°04'S/20°28'W), and stem samples from Sza-

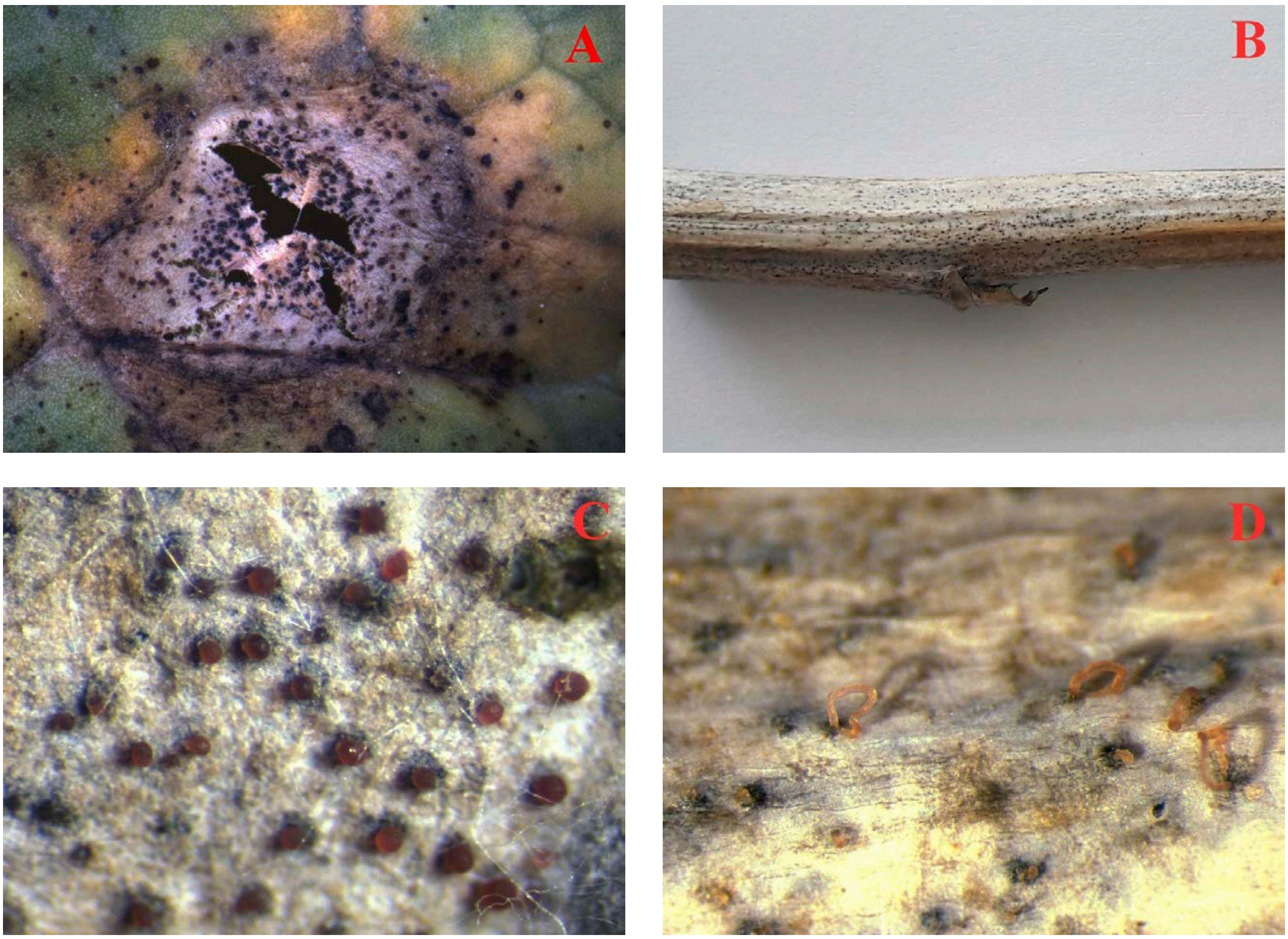

Figure 1. Leaf lesion on oilseed rape hybrid 'Marc KWS' (A), and stem necrosis with black pycnidia on hybrid 'Alvaro KWS' (B). Pycnidia on a leaf of hybrid 'Gordon KWS' (C) and a stem of hybrid 'Umberto KWS' (D).

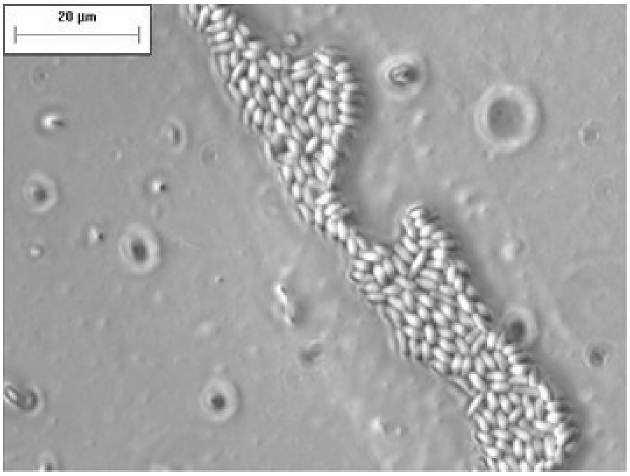

Figure 2. Conidia from *Plenodomus biglobosus*.

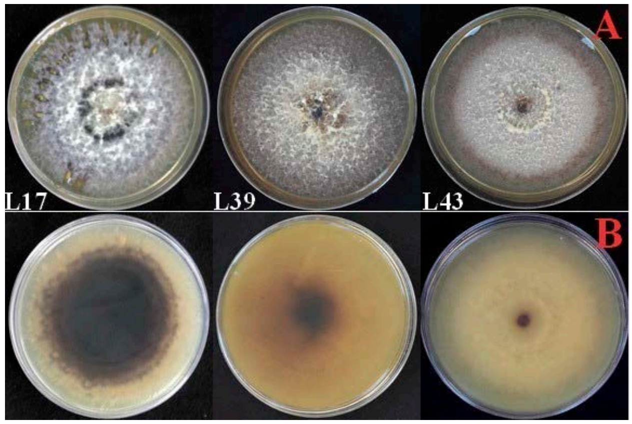

Figure 3. *Plenodomus biglobosus* colonies on PDA after 28 d. Upper surface (A) and lower surface (B).

lánta (45°93'S/18°23'W) and Tordas (47°33'S/18°76'W).

Plenodomus lingam and *P. biglobosus* caused small, circular patches on oilseed rape true leaves, that were 3–10 mm diam., with each lesion having a dark outer margin (Figure 1a). On diseased stems both pathogens produced broad, elongated necrotic lesions (Figure 1b). Dark pycnidia formed in leafspots. Conidial masses excreted from pycnidia, ranged in colour from claret to light brown (Figure 1c and d). It was not possible to distinguish the pathogens based on disease symptoms or morphology of pycnidia, as has been reported previously (Karolewski *et al*., 2007).

Conidia borne in pycnidia of *P. biglobosus* and *P. lingam* were hyaline, cylindrical, unicellular and were rounded at the ends (Figure 2). Dimensions of conidia are detailed in Table 1. Requirements were completed for the multivariate analysis of variance, Shapiro-Wilk test (P = 0.461 for conidium width; P = 0.745 for length) and Levene's test ($F_{(6;343)}$ = 0.568; P = 0.756 for width; $F_{(6;343)}$ = 1.198; P = 0.306 for length). Conidium dimensions for the different isolates did not differ significantly in length, or width (Wilk's-lambda = 0.96; P = 0.330), so conidium size was not reliable for identification of the isolates.

Eleven isolates out of the 54 samples were putatively identified on PDA medium as *P. biglobosus*, using the characteristics described by Mitrović *et al.* (2016) (Figure 3). The colour of colony upper surfaces was greyish, and the lower surfaces were dark greyish brown. Mitrović *et al.* (2016) detected yellow-brown pigments in substrate mycelia after 15 d. In the present study, yellow pigmentation was observed at the edges of the colonies and also in the PDA. In the middle of the colonies, there was abundant aerial mycelium. The colonies were round shape, with indeterminate edges. Calvert *et al.* (1949) described strains that were differed in production of pycnidia. In the present study, pycnidia were not observed in the colonies. Colony growth rates of the isolates are listed in Table 1. Colony form of *P. lingam* isolates was similar to that described by Mitrović and Marinković (2007).

Table 1. Host and plant organ sources, collection dates and regions of Hungary, average conidium dimensions and colony growth rates, for representative *Plenodomus* isolates assessed in this study.

Isolate	Host hybrid	Plant organ	Date of collection	Region of collection	Average conidium dimensions (µm)	Average colony growth rate (mm d^{-1})
L7	'Factor KWS'	stem	July 4th, 2017	Nagylózs	1.55×3.86	1.96
L17	'Marc KWS'	leaf	April 7th, 2018	Kétpó	1.57×3.73	2.71
L18	'Marc KWS'	leaf	April 7th, 2018	Kétpó	1.56×3.73	2.50
L26	'Hybrirock'	leaf	April 13th, 2018	Vadosfa	1.55×3.76	1.68
L34	'Hybrirock'	leaf	April 13th, 2018	Bősárkány	1.55×3.83	2.21
L39	'Umberto KWS'	stem	June 27th, 2018	Szalánta	1.57×3.75	2.64
L43	'Gordon KWS'	stem	June 28th, 2018	Tordas	1.55×3.78	2.67

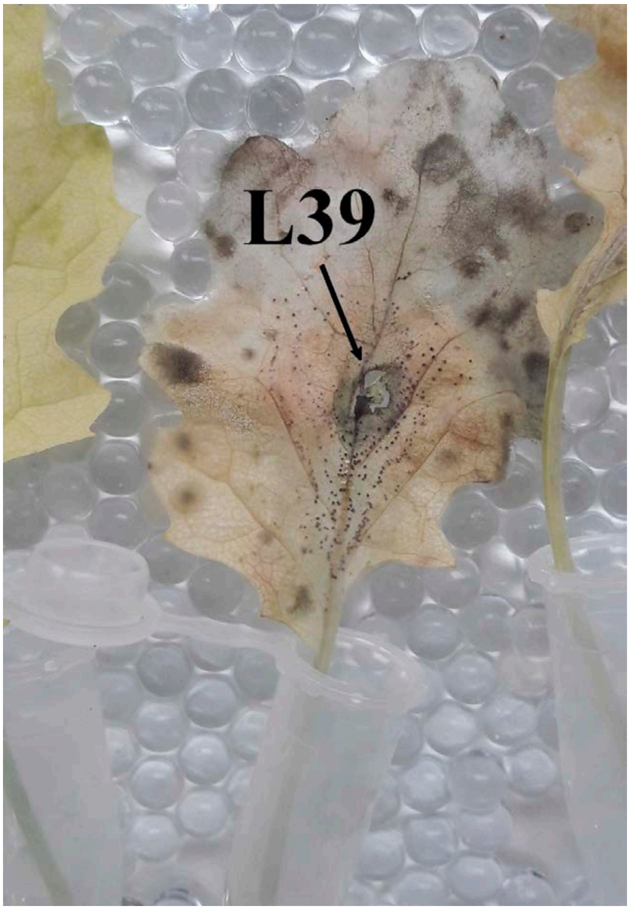

Figure 4. Necrotic lesion and pycnidia (arrow) caused by *Plenodomus biglobosus* (isolate L39) on an inoculated oilseed rape leaf after 10 d incubation.

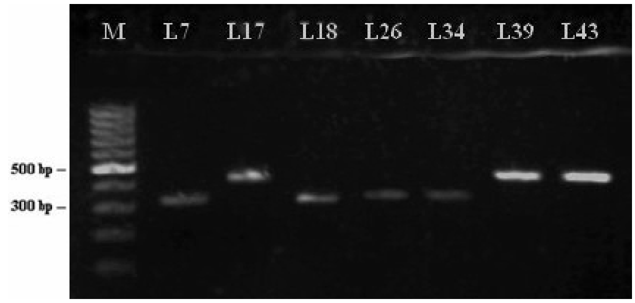

Figure 5. Gel electrophoresis pattern of some amplicons: a PCR product of 444 bp was amplified from *Plenodomus biglobosus* isolates L17, L39 and L43, and of 331 bp was amplified from *P. lingam* isolates L7, L18, L26 and L34. M indicates the GeneRuler 100 bp DNA ladder (Thermo Scientific).

In the pathogenicity tests *P. biglobosus* and *P. lingam* isolates caused browning and death of vascular bundles on oilseed rape seedlings at the inoculation points, after 2–3 d. After 10 d, necrosis was observed on inoculated seedlings, and on inoculated detached leaves. Pycnidia developed on some inoculated leaves (Figure 4). Control seedlings and control detached leaves did not show any symptoms. Koch's postulates were fulfilled by re-isolation of *P. biglobosus* from dark brown parts of the tissues of inoculated seedlings and detached leaves. The re-isolated isolates had the same cultural characteristics as the fungi used for the inoculations.

All of the samples were assessed by multiplex PCR with species specific primers: *P. biglobosus* was detected in 11 samples (20%), while *P. lingam* was detected in 43 samples (80%). Based on gel electrophoresis, the *P. biglobosus* isolates were clearly distinguishable from those of *P. lingam*, from their amplicon lengths (Figure 5). BLAST searches of the ITS sequences of the selected isolates matched the references with 99.7-100% similarity (Table 2).

The results presented here prove the presence of *P. biglobosus* associated with blackleg of oilseed rape

Table 2. Molecular identification and GenBank accession numbers of representative *Plenodomus* isolates assessed in this study.

Isolate	Molecular identification	DNA target[a]	GenBank accession No.	Blast match sequence		
				Reference accession No.	Coverage (%)	Identity (%)
L7	*Plenodomus lingam* (*Leptosphaeria maculans*)	ITS regions and the 5.8 rRNA gene	MK922353	*Leptosphaeria maculans* JF740234	100%	100%
L18			MK922979			
L26			MK922998			
L34			MK922973			
L17	*Plenodomus biglobosus* (*Leptosphaeria biglobosa*)		MK922972	*Leptosphaeria biglobosa* AJ542501	100%	99.7%
L39			MK922343			100%
L43			MK922971			

[a] ITS, internal transcribed spacer; rRNA, ribosomal RNA.

in Hungary. This pathogen has also been observed in neighbouring Serbia (Mitrovic *et al*., 2016). Further comprehensive monitoring and sampling is required to fully characterize distribution of this pathogen in Hungary. Both *Plenodomus* species initially infect the leaves of oilseed rape plants in autumn, leading to stem damage before harvest. However, little is known about role and the significance of *P. biglobosus* in the disease in Hungary, or the factors that influence host infection.

This is the first report of *P. biglobosus* infection of oilseed rape in Hungary.

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