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Note

Occurrence of Stem Canker on Rape Caused by *Leptosphaeria biglobosa* in Korea

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Stem canker symptoms were observed in a rape field in Muan, Korea during a disease survey in May 2006. A total of 15 isolates of *Phoma* sp. were obtained from the infected stems of the plant. All isolates were identified as *Leptosphaeria biglobosa* based on their morphological and cultural characteristics. The Korean isolates of *L. biglobosa* were assigned to 'brassicae' among six subclades of *L. biglobosa* complex based on the entire ITS sequences of rDNA. Pathogenicity of the fungal isolates was confirmed on leaves and stems of rape by artificial inoculation. This is the first report that *Leptosphaeria biglobosa* causes stem canker of rape in Korea.

Keywords : *Leptosphaeria biglobosa*, pathogenicity, rape, stem canker

Rape (*Brassica napus* L.) is cultivated as an oil seed crop or a vegetable in the world. In these days, its growing area was rapidly expanded owing to possibility as a bioenergy crop.

It has been reported that stem canker causes the most serious loss on rape plants in UK, Austuralia and North America (Fitt et al., 2006; West et al., 2001). The causal fungus of the disease was known as *Leptosphaeria maculans* (Desm.) Ces. & De Not., and assigned to two major groups by differences in pathogenicity, cultural and biochemical characteristics (Balesdent et al., 1992; Williams and Fitt, 1999). The one group is highly virulent, slowly grows and produces phytotoxin but not pigment in culture. Another group is weakly virulent, fast grows and produces pigment but not phytotoxin in culture. The former and the latter were distinguished as *L. maculans* [anamorph: *Phoma lingam* (Tode ex Fr.) Desm.] and *Leptosphaeria biglobosa* Shoemaker & Brun (anamorph: *Phoma* sp.), respectively (Shoemaker and Brun, 2001).

In Korea, *P. lingam* was recorded as a causal fungus of stem canker or blackleg of cabbage and Chinese cabbage (Cho and Shin, 2004). However, there has been no report on *Phoma* species causing stem canker in rape. This study was conducted to identify the casual fungus causing stem canker of rape in Korea based on their morphological characteristics and sequences of rDNA -ITS regions. Pathogenicity of the fungal isolates was tested to stems and leaves rape.

Disease incidence and symptoms. In May 2006, stem canker on rape was observed in a field in Muan, Korea. The symptoms were generally found on the upper parts of the plant stems. The symptoms appeared as whitish gray or dull brown spots with darker borders on which black dots were produced later (Figs. 1A-D). The dots were confirmed as pycnidia under a light microscope (Fig. 1D).

Identification of the pathogen. A total of 15 isolates of *Phoma* sp. were obtained from the lesions on diseased stems. To produce pycnidia of the fungus, the isolates were inoculated on oatmeal agar (OA) and incubated in darkness at 21°C for a week and then transferred in an incubator with alternating cycles of 12 h NUV light and 12h darkness for another week. Mycelial growth and cultural features of the isolates were examined after incubation on PDA, OA and malt extract agar (MEA) in darkness at 15-31°C for seven days. Pycinidia produced on OA had long cylindrical beaks and measured 240×290 μm in length (Figs. 2A-C). Conidia were hyaline but somewhat light tan in mass, smooth, straight, cylindrical and 5.7-3.0×2.2-1.5 μm (av. 4.4×1.7 μm) in size (Fig. 2D). The fungus grew well at the range of 20~27°C and showed optimum mycelial growth at 23°C. The colonies on OA were white to grayish white, produced diffusible pigment with pale straw to cinnamon. The colonies on PDA were grayish white and their growing patterns were regular at 15-23°C but irregular at higher temperatures than 27°C (Fig. 3A-D). The diffusible pigment was produced well at 31°C at 14 days after inoculation (Fig. 3D). The colonies on MEA were dark brown in color and showed slower growth than other two media. All the isolates were identified as *Leptosphaeria biglobosa* based on their morphological and cultural characteristics according to the description of previous worker (Table 1).

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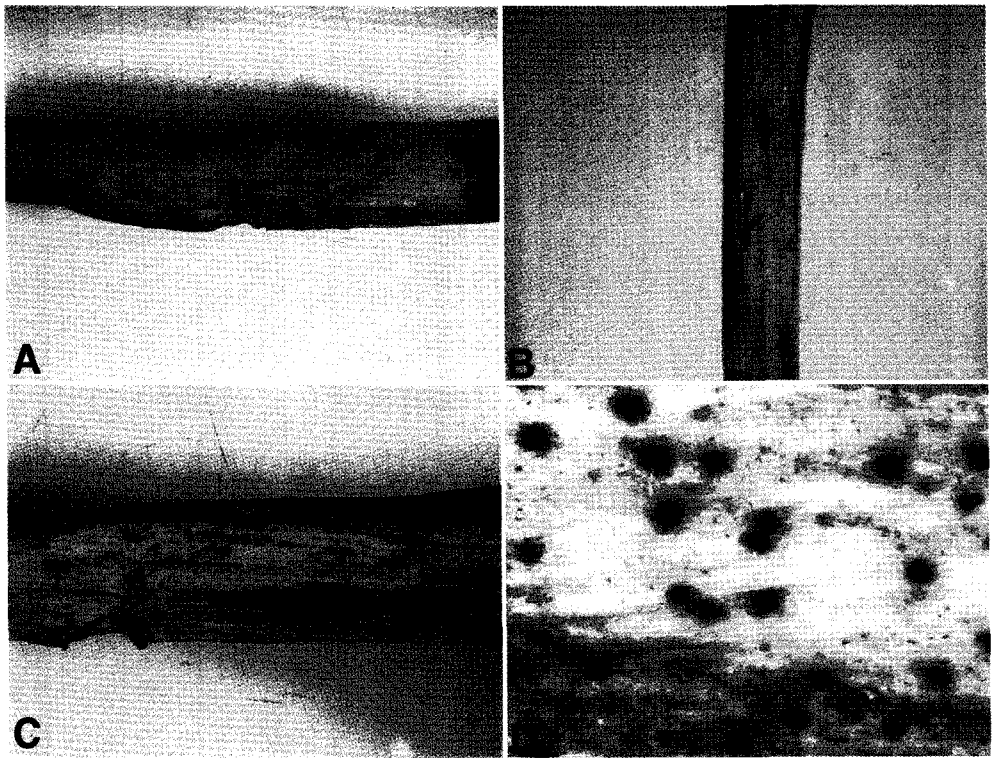

Fig. 1. Symptoms of rape stem canker. A-C, grayish lesions with small black dots on stems; D, Pycnidia shown as black dots.

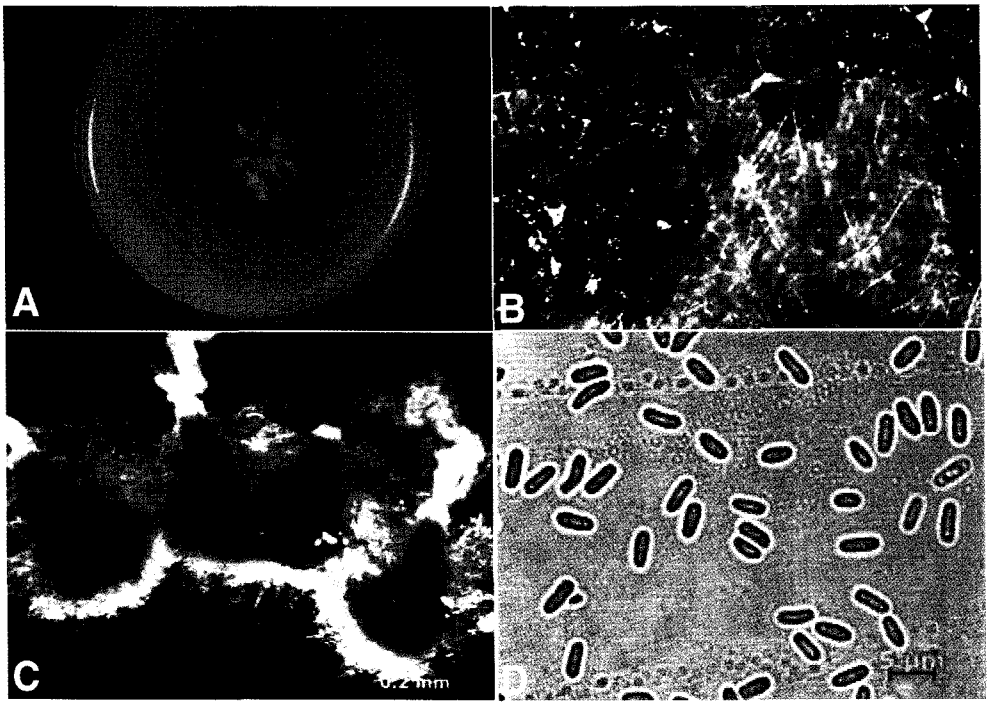

Fig. 2. Cultural and morphological features of *Leptosphaeria biglobosa* isolated from diseased rape stems. A, 14-day-old colony grown on OA at 24°C under alternating cycles of 12 hr NUV light and 12 hr darkness; B, Pycnidia produced on OA; C, pycnidia ejecting conidial mass (scale bar=0.2 mm); D, conidia (scale bar=5 μm).

L. maculans and *L. biglobosa* were reported to cause stem canker of rape (Fitt et al., 2006; Williams and Fitt, 1999). The former is associated with stem base canker leading to girdling and weakening of stem, lodging and

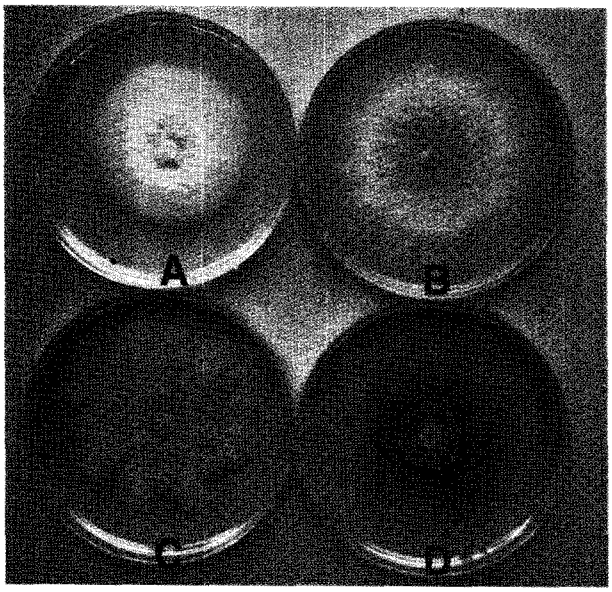

Fig. 3. Production of diffusible pigment of *L. biglobosa* on PDA at temperatures 20°C (A), 23°C (B), 27°C (C) and 31°C (D).

premature ripening of pod and plant death, while the latter is with less severe lesions on the upper stems that are light brown with a dark margin (Fitt et al., 2006; Williams and Fitt, 1999). *L. biglobosa* was considered as a weakly virulent or non-aggressive strain of *L. maculans* but recognized as a species differentiated from the latter in having a long neck that is usually swollen at the upper parts of scleroplectenchymatous pseudothecia (Shoemaker and Brun, 2001, Boerema et al., 2004). The present isolates of *L. biglobosa* could be easily differentiated from *L. maculans* in that the former fast grows on OA, producing yellowish brown diffusible pigment as other workers previously pointed out (Williams and Fitt, 1999).

Comparison of ITS sequences and phylogenetic tree. The region of ribosomal DNA internal transcribed spacer (ITS) 1, 5.8S and ITS2 was amplified by PCR with primers ITS1 and ITS4 designed by White et al. (1990). ITS sequences of three *Phoma* species isolates were generated and used for phylogenetic analysis.

The complete data set for analysis was obtained from sequences of the three isolates and GenBank databases. Sequences were edited using DNASTAR computer package (DNASTAR Inc.) and aligned by CLUSTAL W method (Thompson et al., 1994). A phylogenetic tree for ITS analysis was obtained from the data by neighbor-joining methods using MEGA v4.0 (Tamura et al., 2007) and sequence distance was calculated with Tamura-Nei parameter model. Bootstrap analysis was performed with 1,000 replications to determine the support for each clade. The ITS sequences of isolates P0601, P0602 and P0603 of *L. biglobosa* were aligned with those of *L. maculans* and *L. biglobosa* which are causal fungi of stem canker in rape. Sequences of the entire ITS regions of the Korean isolates were 502 bp and 504 bp (data not shown). This size was similar to that of *L. biglobosa* complex ranging from 496 bp to 499 bp but not to that of *L. maculans* complex having 468 bp as described by Mendes-Pereira (2003). The difference of sequences between the Korean isolates and *L. biglobosa* isolates was resulted from sequences of the ITS 2 region.

The phylogenetic tree generated from ITS sequences showed that the Korean isolates corresponded to *Leptosphaeria biglobosa* (Fig. 4). The Korean isolates were clearly differentiated from *L. maculans*. It has been reported that *L.*

Table 1. Morphological and cultural characteristics of Korean isolates, *Leptosphaeria biglobosa* and *Leptosphaeria maculans*

Structure		Characteristics		
		Korean isolates	Boerema et al. (2004)	
			Leptosphaeria biglobosa	Leptosphaeria maculans
Pycnidium	Shape	Subglobose	Globose-papillate	Subglobose
	Color	Blackish brown	Black or grayish brown	Black
	Diameter (μm) (neck length)	130~410 (240~290)	150~400 (150~200)	150~350(-400) (up to 600)
Conidium	Shape	Subcylindrical	Subcylindrical	Ellipsoidal to subcylindrical
	Color	Hyaline	Hyaline	-
	Size (μm)	3.0~5.7×1.5~2.2 (av. 4.4×1.7)	4~5×1.5~2	(2.5-)3.5~4.5(-5)×1~1.5(-2)
Colony	Diam. (cm)	4~5	5 (-7)	(1.5-) 2.5
	Growing pattern	Regular	Regular	Irregular or regular
	Color	White	White-grayish	Variable
	Pigmentation	Yellowish brown	Yellowish brown	No production

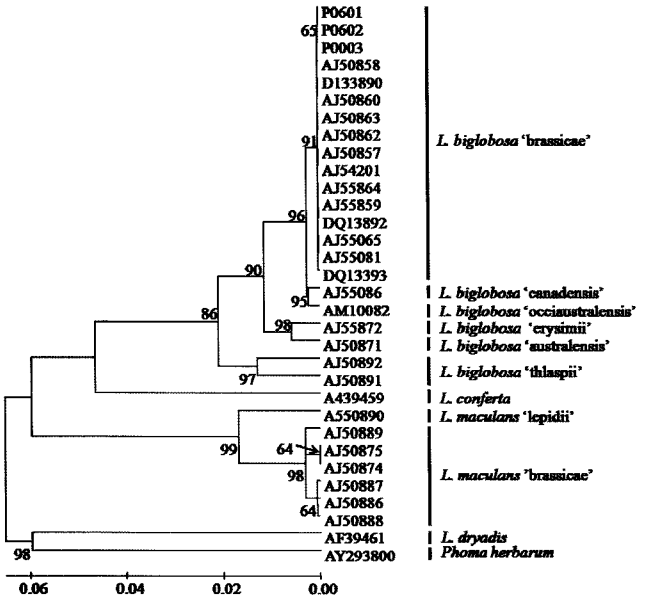

Fig. 4. Phylogenetic tree of *Leptosphaeria biglobosa* isolates from rape in Korea based on ITS1-5.8S and ITS2 rDNA sequences. The tree was generated using Neighbour-Joining analysis and Tamura-Nei option. The numbers at the branch node denote bootstrap values more than 60% using 1,000 replications. AJ, AF, AM, AY, DI and DQ numbers are DNA sequence accession number from NCBI GenBank.

biglobosa complex is genetically variable and can be further divided into subgroups. The subgroups were separated into NA1, NA2 and NA3 based on soluble protein, isozyme profiles and RFLP (Balesdent et al., 1992; Gall et al., 1995; Koch et al., 1991). Five subclades 'brassicae', 'erysimii', 'thlaspii', 'canadensis' and 'australensis' of *L. biglobosa* were reported based on several molecular tools including ITS-RFLP, Rep-PCR, sequences of ITS, *MAT*1-2, actin and β-tubulin genes which corresponded to specific host plants or geographic origins (Balesdent et al., 1998; Jedryczka et al., 1999; Mendes-Pereira et al., 2003; Voigt et al., 2001). Recently, a sixth novel subclade of *L. biglobosa*, 'occiaustralensis', was discovered in Australia (Vincenot et al., 2008). Among the 6 phylogenetic subclades of *L. biglobosa* complex, Korean isolates were assigned to *L. biglobosa* 'brassicae', (Fig. 4) which was known to be specialized on different *Brassica* species (Mendes-Pereira et al., 2003). Therefore, Korean isolates of *L. biglobosa* 'brassicae' might be obtained from different *Brassica* species including Chinese cabbage, mustard, and cabbage.

Pathogenicity. Three Korean isolates were tested for their pathogenicity to stems and leaves of rape. Seeds of rape (cv. Tammi) were sown in pots, and grown in the greenhouse at 20~30°C. For wound inoculation, the stems, and the third and fourth leaves of 30-day-old plants were wounded with sterilized needles. Each stem and leaf was inoculated with agar blocks containing fresh mycelia and pycnidia, and then placed in moisten plastic boxes. Water agar blocks were attached on wounded and unwounded stems and leaves as the control. The boxes placed in a incubator at 23°C for seven days and diameter of lesions was measured.

Table 2. Pathogenicity of *L. biglobosa* isolates to leaf and stem of rape by artificial inoculation

Isolate	Lesion diameter (mm)[a]			
	Leaf		Stem	
	Wounded	Unwounded	Wounded	Unwounded
P06001	6±3	0 or S[b]	4±2	0
P06002	5±2	0	3±1	0
P06003	5±2	0	3±1	0
Control	0	0	0	0

[a]Lesion diameter was measured seven days after inoculation. The data represents the average of three replicates±standard deviation.
[b]S = small spot.

All the isolates tested produced dark brown lesions with haloes on wounded stems and leaves of rape. However, only small spots or no symptoms were developed on unwound stems and leaves (Table 2). It has been reported that *L. biglobosa* is weakly pathogenic to leaves and stems of rape whereas *L. maculans* is strongly pathogenic (Fitt et al, 2006). The present study first reveals that *L. biglobosa* causes stem canker of rape in Korea.

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