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1 Evaluation of oilseed rape seed yield losses caused by *Leptosphaeria biglobosa*
2 in central China

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23 **Abstract**

24 Phoma stem canker of oilseed rape (*Brassica napus*), caused by *Leptosphaeria*
25 *maculans*/*L. biglobosa* is a globally important disease. Severe phoma stem canker symptoms
26 have been observed on winter oilseed rape in China but the seed yield loss caused by this
27 disease remains unknown. In May 2012 and May 2013, 17 and 13 crops were surveyed,
28 respectively, in seven counties of Hubei Province, central China. Stems with phoma stem
29 canker disease symptoms were sampled for pathogen isolation and identification. Only *L.*
30 *biglobosa* was identified by culture morphology and species-specific PCR; no *L. maculans*
31 was found. To evaluate the yield losses, yield components (number of branches per plant,
32 number of pods per plant, 1000-seed weight, number of seeds per pod) were assessed on
33 healthy and diseased plants sampled from crops in four counties and on plants from
34 inoculated pot experiments (plants of three cultivars were inoculated at the green bud stage by
35 injecting *L. biglobosa* conidia into the stem between the first and second leaf scars). Results
36 of the field surveys showed that diseased plants had 14-61% less branches and 32-83% less
37 pods than healthy plants, respectively. The estimated seed yield loss varied from 10% to 21%
38 and from 13% to 37% in 2012 and 2013, respectively. In the pot experiments, there were no
39 differences in numbers of branches or pods but there were differences in number of seeds per
40 pod between inoculated and control plants. For the three cultivars tested, the inoculated plants
41 had yield losses of 29 - 56% compared with the control. This study indicates that *L. biglobosa*
42 could cause substantial seed yield loss in China.

43 **Additional keywords:** *Brassica napus*, blackleg, disease control, crop disease survey

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47 **Introduction**

48 Phoma stem canker (also known as blackleg) is an important disease of oilseed rape (Fitt
49 et al. 2006a) and cruciferous vegetables (Rimmer and Berg 2007) worldwide. The disease
50 causes serious seed yield loss on oilseed rape in European countries, Australia and Canada.
51 The annual worldwide economic loss is estimated to be more than 1.6 billion USD despite use
52 of fungicides (Zhang et al. 2014). The pathogens that cause phoma stem canker are two
53 closely related *Leptosphaeria* species, *L. maculans* and *L. biglobosa* (Shoemaker and Brun
54 2001; Fitt et al., 2006a). *L. biglobosa* is often associated with upper stem lesions, whereas *L.*
55 *maculans* is often associated with stem base cankers (West et al. 2002). Generally, *L.*
56 *maculans* is considered to be more damaging than *L. biglobosa*. Air-borne ascospores
57 produced in pseudothecia on the diseased stem debris are the main sources of inoculum for
58 both pathogens (Huang et al. 2005). The ascospores germinate on leaves of oilseed rape and
59 germ tubes/hyphae penetrate through stomata, thereby causing phoma leaf spots (Huang et al.
60 2003). From the phoma leaf spots, the pathogens usually grow along the leaf petioles towards
61 the stems, where they cause upper stem lesions or stem base cankers (Toscano-Underwood et
62 al. 2003; Huang et al., 2014). Most studies on oilseed rape seed yield loss caused by phoma
63 stem canker did not distinguish whether the yield losses were caused by either *L. maculans* or
64 *L. biglobosa*. For example, in 1966, a severe phoma stem canker epidemic in central France
65 caused an average seed yield loss of 40%, compared with the yield of oilseed rape in 1964
66 (Lacoste et al. 1969). In 1979, the seed yield loss due to phoma stem canker was up to 50% in
67 UK (Gladders and Musa 1979). Since the 1980s, seed yield loss varying from 5.2% to 30%
68 have been reported in many other countries, including Canada (Gugel and Petrie 1992) and
69 the Netherlands (van der Spek 1981). In most regions where oilseed rape is grown (Australia,
70 Canada and western Europe), *L. maculans* and *L. biglobosa* coexist, with *L. maculans* being
71 reported to be isolated most frequently, although there is some spatial and temporal variation
72 in their prevalence (West et al. 2001). In Poland before 2000, phoma stem canker was

73 predominantly caused by *L. biglobosa*, with 97% of isolations from upper stem lesions and
74 67% from stem base cankers caused by *L. biglobosa* (Karolewski et al. 2002). There is good
75 evidence that *L. maculans* is spreading eastwards and *L. maculans* has now become
76 predominant in western Poland, whereas *L. biglobosa* is still predominant in eastern Poland
77 (Karolewski et al. 2002) and only *L. biglobosa* has been reported in Russia (Jedryczka et al.
78 2002). However, there is no information about yield losses caused by *L. maculans* or *L.*
79 *biglobosa* in these countries.

80 China is an important oilseed rape-producing country and the Chinese authorities are
81 very concerned about the potential damage to oilseed rape production from phoma stem
82 canker. This disease was first reported in China 50 years ago (Dai 1979) and the causal
83 pathogen was first identified as *L. biglobosa* in 2000 (West et al. 2000). Recent disease
84 surveys and pathogen identification have shown that phoma stem canker on both winter and
85 spring oilseed rape in China is caused by *L. biglobosa*; there was no *L. maculans* detected
86 from diseased stem samples (Liu et al. 2014; Zhang et al. 2014). Recently, *L. maculans* was
87 detected in imported oilseed rape seeds at ports in central China (Zhang et al. 2014). Although
88 methods for effective, rapid detection of *L. maculans* in infected seed lots of oilseed rape have
89 been developed (Song et al., 2016), there is a potential risk that *L. maculans* may spread into
90 China through importing of seeds of oilseed rape from countries where *L. maculans* is present.
91 Because there is evidence that *L. maculans* has been spreading into areas where only *L.*
92 *biglobosa* had been present (Fitt et al., 2008). Therefore, there is a need to continue phoma
93 stem canker disease surveys and pathogen identification in China.

94 Severe epidemics of phoma stem canker on Chinese oilseed rape have not generally been
95 observed, and only *L. biglobosa* has been found in China (Li et al. 2013; Zhang et al. 2014).
96 Since *L. biglobosa* is considered less damaging than *L. maculans*, there is no information
97 about oilseed rape seed yield loss caused by phoma stem canker in China. Assessment of seed

98 yield loss caused by phoma stem canker is very important for developing disease management
99 strategies to control this disease in China. Hubei province is the most important oilseed
100 rape-growing area in central China. The work reported in this paper aimed: (i) to identify the
101 pathogen(s) causing phoma stem canker on winter oilseed rape in central China, and (ii) to
102 assess the seed yield loss caused by phoma stem canker on winter oilseed rape in central
103 China.

104 **Materials and methods**

105 **Phoma stem canker crop survey and pathogen identification.** To investigate the
106 incidence of phoma stem canker, seventeen and thirteen crops of winter oilseed rape grown in
107 seven counties in Hubei Province of central China were surveyed in May 2012 and May 2013
108 before harvest, respectively (Fig. 1, Table 1). In each crop, 500 plants were sampled, from
109 five sites in a zig-zag pattern ('W' pattern) with 100 plants per site. The incidence (% of
110 plants affected) of phoma stem canker was calculated for each crop.

111 Stems with phoma stem canker symptoms were sampled for pathogen identification.
112 These stems were first classified as affected by phoma stem canker by observation of the
113 visible tissue discoloration and the presence of *Leptosphaeria* pycnidia. They were cut into
114 small pieces (about 0.5 × 0.5 cm). Each stem piece was divided into two. One half was used
115 for pathogen isolation and identification by morphology/pigment observation and PCR
116 confirmation. The other half was used for direct DNA extraction and identification by nested
117 PCR without isolation.

118 To isolate the causal pathogen(s), the stem pieces were surface-sterilised in 75% (v/v)
119 ethanol for 1 min, and then dipped in 5% (v/v) NaOCl for 1 min, followed by rinsing in water
120 three times. The surface-sterilized stem pieces were placed on Petri dishes containing potato
121 dextrose agar medium (PDA) amended with streptomycin at 100 µg mL⁻¹. The cultures were
122 incubated at 20°C for 7 days in darkness. Then, the hyphal tips from these colonies were

123 transferred to new PDA plates containing streptomycin and the cultures were incubated at
124 20°C for 7 days. This procedure was repeated three times until a pure culture of each isolate
125 was obtained.

126 Preliminary identification of the isolates was based on morphological characteristics of
127 the colonies, growth rate and pigment production on PDA (Fitt et al. 2006b). Then, the
128 isolates were cultured and the mycelia were collected for DNA extraction. Six PDA agar
129 plugs (2.5 mm in diameter) were inoculated into 10 ml PDB (potato dextrose broth) liquid
130 medium and maintained on an orbital shaker (25°C, 150 rpm) for 7 days. The mycelia were
131 harvested by filtration through filter papers in a Buchner-funnel and freeze-dried for 24 h.
132 Genomic DNA was extracted from the freeze-dried mycelia using a CTAB method (Möller et
133 al. 1992).

134 To confirm whether the isolates were *L. maculans* or *L. biglobosa*, *Leptosphaeria*
135 species-specific primers (Liu et al. 2006) were used in PCR with the DNA from each isolate.
136 The nested PCR amplification with the DNA from diseased stem pieces (DNA was extracted
137 from the freeze-dried stem samples using a CTAB method) consisted of two steps. In the first
138 step of PCR amplification, universal primers ITS1 and ITS4 were used. In the second step of
139 PCR amplification, *Leptosphaeria* species-specific primers were used. All the PCR products
140 were assessed by electrophoresis in 2% (w/v) agarose gel.

141 **Evaluation of the yield losses caused by phoma stem canker in winter oilseed**
142 **rapecrops.** To estimate the yield loss caused by phoma stem canker in winter oilseed rape
143 crops, healthy plants and diseased plants were sampled from four crops in May 2012 and May
144 2013. The four crops were located in four counties: Jingzhou (N30°11.3291', E111°58.2264',
145 cultivar: Deyou No.9); Xiangyang (N32°08.42', E112°09.51', cultivar: Zhongyou No.112);
146 Suizhou (N31°43.229', E112°9.432', cultivar: Zhongshuang No. 11) and Huanggang
147 (N31°11.382', E114°58.886', cultivar: Zhongshuang No. 10) (Fig.1).

148 In each crop, ten healthy plants without any phoma stem canker symptoms and ten
149 diseased plants with typical phoma stem canker symptoms were sampled randomly. Stems of
150 diseased plants were cut to examine the internal necrosis. The disease severity was scored
151 using a value scale of 0 to 4 (Zhou et al. 1999): 0, healthy, no disease; 1, less than 50% of the
152 stem cross-section area affected by the disease; 2, more than 50% and less than 90% of the
153 stem cross-section area affected by the disease; 3, the whole stem cross-section area affected by
154 the disease; 4, the whole plant dead. Disease index (DI) for the plants in each crop was
155 calculated by the formula: $DI = \{[\sum(Ni \times i)]/Nt \times 4\} \times 100$, where Ni is number of plants with
156 disease score i and Nt is the total number of plants assessed. For all the plants sampled, the
157 height (above ground), number of branches, and number of pods (P) of each plant were
158 assessed. Then, the pods from the ten healthy or ten diseased plants were each combined and
159 50 pods were randomly selected to assess the number of seeds in each pod (S). The seeds
160 from the ten healthy plants or ten diseased plants were each combined and the 1000-seed
161 weight (W) was estimated from 3000 seeds. Oil content of seeds from healthy or diseased
162 plants was measured by Foss NIR Systems 5000 (Foss NIR Systems Inc. Denmark) using the
163 standard procedure in the operation manual (<http://www.foss-nirsystems.com>). The yield/plant
164 (Y) and percentage crop yield loss (L) were estimated using the formulas: $Y = S \times P \times$
165 $W/1000$ and $L = (Y_H - Y_D) / Y_H \times D_{in}$ (Y_H : yield/plant for healthy plant; Y_D : yield/plant for
166 diseased plant; D_{in} : disease incidence, % plants affected).

167 **Yield losses caused by *L. biglobosa* in pot experiments.** To assess the yield loss caused
168 by *L. biglobosa*, plants of three winter oilseed rape cultivars were grown in pots and
169 inoculated with conidia of *L. biglobosa* isolate W10. The isolate W10 (isolated from oilseed
170 rape in Wuxue County, Hubei in 2010) was cultured on 20% V8 juice agar (Campbell's,
171 Camden, New Jersey, USA) and incubated at 20°C with a 12-h photoperiod for two weeks.
172 Sterilized water (10 ml) was added to each culture plate and scrubbed with a sterilized glass

173 spatula to dislodge the conidia. Conidial suspensions were filtered through four layers of
174 gauze to remove mycelial fragments. The concentration of the conidial suspensions was
175 measured using a haemocytometer and adjusted to 1.5×10^7 conidia ml⁻¹ using sterilized
176 water.

177 Three winter oilseed rape cultivars (Zhongyou 112, Zhongshuang 9 and Ningyou 7) were
178 initially grown in a growth cabinet at 20/16°C (day/night temperatures) with a 16-h
179 photoperiod for one month. In early October of each season, the seedlings were then
180 transplanted into pots (25 × 35 cm) containing yellow-brown clay soil (pH 6.0) amended with
181 a compound fertilizer (N: P: K = 15:15:15, Hubei Dong Sheng Chemicals Group Co., Ltd.,
182 Yuan An County, Hubei, China) at 0.5% (w/w). There were 40 pots with two seedlings per pot
183 for each cultivar. One week later, one seedling was removed from each pot. The pots were
184 kept outdoors under a shade and surrounded by nylon netting. They were arranged in a
185 randomized complete block design and were watered when required.

186 The oilseed rape plants at the green bud stage were inoculated with *L. biglobosa* on 12
187 March 2012 for the first experiment and on 10 March 2013 for the second experiment. To
188 inoculate the plants, 1-mm-diameter holes were made on the stems between the first and the
189 second lowest leaves with a sterilized needle, with one hole per plant. Conidial suspension of
190 *L. biglobosa* (10 µl) was injected into the hole using a microliter syringe (Gaoce Inc.,
191 Shanghai, China). The control treatment was injected with 10 µl of sterilized water. For each
192 cultivar, 25 plants were inoculated with *L. biglobosa* and 15 plants were inoculated with water.
193 To assess the success of the inoculation, five plants were sampled from each treatment at one
194 month after inoculation to assess the internal symptoms.

195 On 25 May 2012 or 20 May 2013, plants were harvested to assess the phoma stem canker
196 severity and yield. Phoma stem canker severity was assessed on each plant using a 0–4 scale
197 (Zhou et al. 1999) and then the disease index was calculated as described above. For each

198 plant, the yield components were assessed as in the field survey. The percentage of the plant
199 seed yield loss (P) was calculated using the formula: $P = (Y_C - Y_i)/Y_C \times 100$ (Y_C : seed
200 yield/plant for the control plant; Y_i : seed yield/plant for the inoculated plant).

201 **Data analysis.** Analysis of variance (ANOVA) by SAS software (version 9.0, SAS
202 Institute) was used for statistical analysis of data from field and pot experiments. Mean values
203 for different treatments in the pot experiments and crop disease surveys were compared at $P <$
204 0.05 according to the Student's t test. The data for oil content in each crop were
205 arcsin-transformed prior to analysis. To normalize the data, appropriate transformations were
206 determined empirically using normal probability plots, and the transformations were applied
207 before bivariate analysis was performed. To determine if significant correlations existed
208 between the severity of stem canker and yield components, a Pearson's correlation coefficient
209 was calculated by bivariate analysis using CORR Proc. Effects of season, cultivar (or location)
210 and treatment on yield components were determined using the general linear model (GLM)
211 procedure in ANOVA.

212 **Results**

213 **Phoma stem canker crop survey and pathogen identification.** In May 2012, 17
214 oilseed rape crops in seven counties and in May 2013, 13 oilseed rape crops in six
215 counties were surveyed (Table 1). These fields were representative of a large area of oilseed
216 rape grown in Hubei Province of China (Fig.1). In May 2012, 11 out of 17 crops had a low
217 incidence (< 1%) of phoma stem canker; disease incidence in the other 6 fields ranged from
218 12.8 to 75.2%. There were differences in disease incidence between the counties surveyed,
219 with the greatest disease incidence in Xiangyang (49%) and the smallest disease incidence in
220 Yichang (1%). Disease incidence was also significantly different between the crops surveyed
221 within the same county. The results of the disease survey in May 2013 showed a similar
222 pattern to those in May 2012. The disease incidence in seven out of 13 crops was less than 1%,

223 and ranged from 10.7% to 46.2% in the other six crops (Table 1). In May 2013, among the six
224 counties, the crops in Xiangyang had the greatest disease incidence (30.7%) (Fig. 2).

225 A total of 311 oilseed rape stems with phoma stem canker (208 stems in 2012, 103 stems
226 in 2013) were used for pathogen identification. For isolation and morphological identification,
227 a total of 141 *Leptosphaeria* isolates (115 isolates in 2012, 26 isolates in 2013) were obtained.
228 After 10 days of incubation on PDA, all the isolates produced black-brown globose pycnidia
229 with pink conidial ooze, suggesting that they were *L. biglobosa*. The isolates were further
230 identified to be *L. biglobosa* by species-specific PCR, in which a DNA fragment of 444 bp
231 was amplified. In nested PCR identification of the pathogens in these 311 diseased stems, 115
232 (in 2012) and 92 (in 2013) diseased stem samples were confirmed to be colonised by *L.*
233 *biglobosa* and no *L. maculans* was found. The frequency of *L. biglobosa* detected by nested
234 PCR in the diseased stem samples collected in May 2013 (89.3%) was greater than that in the
235 diseased stem samples collected in May 2012 (55.2%).

236 **Seed yield losses caused by phoma stem canker in winter oilseed rape crops.** There
237 were significant differences between the 2011-2012 season and the 2012-2013 season for
238 most of the seed yield components assessed. Therefore, the results are presented separately for
239 each season. In the 2011-2012 season, values for plant height, number of pods and
240 yield/plant of healthy plants were significantly greater ($P < 0.01$) than the corresponding
241 parameters for diseased plants of all the four crops in the four counties (Table 2). However,
242 the differences in the 1000-seed weight and oil content between the diseased and healthy
243 plants were not significant ($P > 0.05$). For the crops surveyed in Xiangyang, Suizhou and
244 Huanggang counties, number of branches per plant on healthy plants were significantly
245 greater ($P < 0.01$) than that on diseased plants. For example in Huanggang, diseased plants
246 had 61% less branches than healthy plants (Table 2). For the crops surveyed in Jingzhou and
247 Xiangyang counties, number of seeds per pod of healthy plants was also significantly greater

248 ($P < 0.01$) than that on diseased plants . When the four surveyed crops were analyzed together,
249 there were significant differences between healthy and diseased plants in plant height ($P <$
250 0.05), number of branches per plant ($P < 0.01$), 1000-seed weight ($P < 0.0001$) and seed yield
251 per plant ($P < 0.0001$). There were differences between the four crops surveyed in stem
252 canker severity, with the disease index being greater in Suizhou (25.1) than in Jingzhou (9.5),
253 Xiangyang (18.7) or Huanggang (6.9). The severe stem canker in Suizhou was associated
254 with greater crop seed yield loss (21.2%) than that for the diseased plants in Jingzhou (10.1%),
255 Xiangyang (16.2%) or Huanggang (12.5%) (Table 2).

256 For the crop disease survey in the 2012-2013 season, there were significant differences
257 ($P < 0.01$) between the diseased and healthy plants in number of branches, number of pods
258 and yield/plant for all four crops in the four counties (Table 2). However, the differences in
259 1000-seed weight and oil content between the diseased and healthy plants were not significant
260 ($P > 0.05$). For the crops surveyed in Xiangyang, Suizhou and Huanggang counties, plant
261 height differences between diseased and healthy plants were significant ($P < 0.01$). For the
262 crop in Jingzhou, there were significant differences in number of seeds per pod between
263 diseased and healthy plants (Table 2). When the four crops were analysed together, the
264 differences between diseased and healthy plants in number of branches, number of pods,
265 1000-seed weight and yield/plant reached the significant level ($P < 0.05$ or $P < 0.01$). For the
266 four crops sampled, the average disease index in 2012-2013 (16.9) was greater than that in
267 2011-2012 (15.1). The disease index differed between the four crops, with the disease index
268 being greater in Xiangyang (28.6) than those in Jingzhou (14.2), Suizhou (15.7) and
269 Huanggang (9.0). The severe stem canker in Xiangyang led to greater seed yield loss (37.5%)
270 than those losses for the diseased crops in Jingzhou (13.4%), Suizhou (27.3%) and
271 Huanggang (15.2%). The average seed yield loss in the 2012-2013 season (37.5%) was
272 greater than that in the 2011-2012 season (21.2%).

273 **Seed yield losses caused by *L. biglobosa* in pot experiments.** One month after
274 inoculation in each year (in April), the stem cross-section of the plants inoculated with *L.*
275 *biglobosa* showed blackened stem piths and vascular tissues (Fig. 3). By the end of the
276 experiment (at harvest in late May), all inoculated plants showed typical stem canker
277 symptoms (Fig. 4A), while the plants in the control treatment (inoculated with water) showed
278 no disease symptoms (Fig. 4B). When the plants were cut horizontally and vertically at the
279 inoculation sites, all the inoculated plants had blackened or rotted stem piths (Fig. 4C) and the
280 symptoms had spread along the stem (Fig. 4E), while the control plants had stem piths that
281 were healthy or had little blackening (Fig. 4D) that was only around the inoculation site and
282 did not spread along the stem (Fig. 4F).

283 There were differences between the experiments in the 2011-2012 season and the
284 2012-2013 season for all the seed yield components assessed. Therefore, the results are
285 presented separately for each experiment. For the experiment in the 2011-2012 season, the
286 number of seed/pods and yield/plant for all three cultivars were significantly different ($P <$
287 0.01) between the inoculated and control plants. However, there were no differences ($P > 0.05$)
288 between the inoculated plants and control plants in number of branches, number of pods,
289 1000-seed weight or oil content (Table 3). When the three cultivars were analyzed together,
290 the differences between inoculated plants and control plants in plant height, number of
291 seed/pods and yield/plant were significant ($P < 0.0001$). For inoculated plants, the disease
292 index was greater on cultivar Zhongshuang 9 (50.0) than that on cultivars Zhongyou 112 (38.1)
293 and Ningyou 7 (38.3). The seed yield loss caused by *L. biglobosa* was greater for cultivar
294 Zhongyou No.112 (42.1%) than that for cultivars Zhongshuang No. 9 (38.5%) and Ningyou
295 No. 7 (37.2%).

296 For the pot experiment in the 2012-2013 season, there were significant differences
297 between the inoculated and control plants in number of seeds/pod and yield/plant but no

298 differences in plant height, number of branches, number of pods, 1000-seed weight or oil
299 content for all the three cultivars (Table 3). When the three cultivars were analyzed together,
300 there were significant differences between inoculated plants and control plants in number of
301 seeds/pod ($P < 0.0001$) and yield/plant ($P < 0.0001$) but no differences in plant height,
302 number of branches, number of pods, 1000-seed weight or oil content. For inoculated plants,
303 the average disease index was greater in the 2012/2013 season (67.9%) than that in the
304 2011/2012 season (42.1%), with the disease index greater on Zhongyou 112 (70.0) than on
305 Zhongshuang 9 (66.3) and Ningyou 7 (66.5). The mean plant yield loss in the 2012/2013
306 growing season (44.1%) was greater than that in the 2011/2012 season (39.3%), with the plant
307 yield loss caused by *L. biglobosa* greater on Ningyou 7 (56.4%) than on Zhongyou 112
308 (47.1%) or Zhongshuang 9 (28.8%) (Table 3).

309 The combined data from the field surveys (four crops in two seasons) and the combined
310 data from the two pot experiments were used for analysis of the correlations between disease
311 levels and yield. Coefficient of correlation between crop yield and severity of stem canker
312 showed that crop yield was inversely correlated to the severity of stem canker both in the field
313 surveys ($r = -0.65$, $P < 0.0001$) and in the pot experiments ($r = -0.30$, $P < 0.01$). In the field
314 surveys, the severity of stem canker was negatively correlated with plant height ($r = -0.37$, P
315 < 0.0001), number of branches ($r = -0.49$, $P < 0.0001$), number of pods ($r = -0.65$, $P <$
316 0.0001), and number of seeds per pod ($r = -0.37$, $P < 0.005$). In contrast, the severity of stem
317 canker was not significantly ($P > 0.05$) correlated with any of these four yield components in
318 the pot experiments.

319 **Discussion**

320 Results of the disease surveys in May 2012 and May 2013 showed that phoma stem
321 canker occurred widely in the winter oilseed rape growing area in Hubei Province of China.
322 This is consistent with the previous surveys showing that phoma stem canker was commonly

323 observed in China, both on spring and on winter oilseed rape, with disease incidence varying
324 between crops, provinces and seasons (Li et al. 2013). In this study, a large variation in phoma
325 stem canker incidence (from < 1 to 75%) was observed between crops/counties and between
326 seasons. One reason for the variation may due to the differences in cultivar resistance because
327 different cultivars were used in different counties. This is supported by the results from pot
328 experiments showing that there were differences between cultivars in severity of stem canker
329 (Table 3). The large variation in phoma stem canker incidence between crops and seasons
330 suggests that there is a need to regularly monitor phoma stem canker in China in order to
331 assess the risk of phoma stem canker epidemics. Furthermore, phoma stem canker symptoms
332 have recently been found on many cruciferous vegetables in central China (Cai et al. 2014a,
333 b). Considering the pattern of phoma stem canker spread in Europe and Canada (Fitt et al.
334 2008; Zhang et al., 2014), there is a need to control this disease on oilseed rape and to prevent
335 it spreading to other cruciferous vegetables in China.

336 Based on colony morphology and/or species-specific PCR, the pathogen causing phoma
337 stem canker on winter oilseed rape crops in seven counties in Hubei province was identified
338 as *L. biglobosa*. Results from this study support the previous evidence that only *L. biglobosa*
339 (or B-type *L. maculans*) is currently associated with phoma stem canker on oilseed rape crops
340 in China (West et al. 2000; Fitt et al. 2008; Li et al. 2013; Liu et al. 2014). It is not clear why
341 *L. maculans* has not been found on oilseed rape and other cruciferous crops in China. One of
342 the reasons may be China has adopted effective quarantine measures. There is evidence that *L.*
343 *maculans* has been spreading into areas where only *L. biglobosa* was previously present (Fitt
344 2008). Previous studies showed that Chinese oilseed rape cultivars are very susceptible to *L.*
345 *maculans* (Li et al. 2008; Fitt et al. 2008; Zhang et al. 2014). Furthermore, the climatic and
346 agronomic conditions required for development of *L. maculans* stem canker epidemics appear
347 similar to those for *L. biglobosa* (West et al. 2002; Huang et al. 2003; Fitt et al. 2006b). *L.*

348 *biglobosa* is already present in China; if *L. maculans* spreads into China, phoma stem canker
349 may destroy the Chinese oilseed rape industry. Considering the potential risk of *L. maculans*
350 spreading into China through imported infected seeds together with infected crop debris (Fitt
351 et al. 2008; Zhang et al. 2014), quarantine restrictions on imported seeds of oilseed rape and
352 regular monitoring of the pathogen populations will help to reduce or avoid the risk of severe
353 phoma stem canker epidemics in China.

354 Results of the field disease surveys and the pot experiments suggest that *L. biglobosa* can
355 cause considerable seed yield loss on oilseed rape in China. Traditionally, *L. biglobosa* has
356 been considered to be ‘weakly virulent’ and its impact on seed yield loss in most oilseed rape
357 growing areas was ignored. Before 2000, phoma stem canker was rarely found in central
358 China and only *L. biglobosa* was isolated. The oilseed rape-rice rotation in central China may
359 suppress the production of the inoculum of *L. biglobosa*. However after 2000, the oilseed
360 rape-rice rotation has gradually been decreasing in central China. Meanwhile, Chinese
361 breeders have not made efforts to breed oilseed cultivars resistant to *Leptosphaeria* spp.
362 This may be one of the reasons why phoma stem canker was commonly observed recently on
363 oilseed rape in China (Li et al., 2013; Zhang et al., 2014). Our field survey data showed that *L.*
364 *biglobosa* could cause seed yield losses ranging from 10 to 37% in crops with the phoma stem
365 canker incidence ranging from 13 to 46%. Previous crop disease surveys showed that *L.*
366 *biglobosa* could cause seed yield losses up to 50% (Rong et al. 2015). In some crops, both
367 stem base cankers and upper stem lesions were observed. This result suggests that phoma
368 stem canker is currently a potential threat to oilseed rape production in China. Absence of *L.*
369 *maculans* might allow severe colonization of oilseed rape by *L. biglobosa*. However, it is
370 difficult to accurately estimate the seed yield loss caused by this pathogen under the field
371 conditions, because no fungicides are currently used to control phoma stem canker in China.
372 Furthermore, another important disease, namely sclerotinia stem rot caused by *Sclerotinia*

373 *sclerotiorum*, often co-exists with phoma stem canker caused by *L. biglobosa*, making it
374 difficult to estimate the yield loss caused by phoma stem canker alone and the severity of
375 phoma stem canker may be under estimated due to the co-infection by *S. sclerotiorum*. In this
376 study, the crop seed yield loss caused by phoma stem canker was estimated from yields of
377 diseased/healthy plants sampled from the same crop and the disease incidence (% plants
378 affected) in the crop. This may lead to overestimation of the seed yield loss. There is a need
379 for further investigation of the seed yield loss caused by *L. biglobosa* in field conditions in
380 China by doing field experiments with/without fungicides targeting *L. biglobosa* and *S.*
381 *sclerotiorum* and by inoculating the plants with naturally affected stem debris.

382 The severity of phoma stem canker differed between different cultivars both in the field
383 surveys and in the pot experiments. These results suggest that it is possible to use cultivar
384 resistance to control phoma stem canker in China. Use of host resistance to control phoma
385 stem canker caused by *L. maculans* has been well studied (Delourme et al. 2006; Larkan et al.
386 2015). However, there is no information on use of host resistance to control *L. biglobosa*.
387 Differences in disease index between the three cultivars were observed in the pot experiments.
388 It is necessary to examine a large number of cultivars using different inoculation methods to
389 screen for resistance against *L. biglobosa*. To guarantee the infection of plants, in this study,
390 conidial suspensions of *L. biglobosa* were injected into the stems of oilseed rape at the green
391 bud stage. Other inoculation methods used to screen for resistance against *L. maculans*, such
392 as cotyledon inoculation and petiole inoculation (Huang et al. 2014), should be used in future
393 work. It has been reported that inoculation at different times and on different tissues can
394 result in different seed yield losses on plants of oilseed rape. In south-eastern Australia, *B.*
395 *napus* plants inoculated with *L. maculans* conidia at the third- to fifth-true-leaf stage did not
396 develop phoma stem canker (Marcroft et al. 2005). Furthermore, the study by Li and
397 colleagues (2006) reported that the resistance response of *B. napus* plants to *L. maculans* was

398 affected both by the growth stage of the plant at inoculation and the temperature at which the
399 inoculated plants were grown. Late inoculation with *L. biglobosa* on the oilseed plants at the
400 green bud stage, rather than at the seedling stage, might be the reason for no significant
401 differences in plant height, number of branches, number of pods, 1000-seed weight or oil
402 content for all the three cultivars in these pot experiments. Ascospores of *L. biglobosa* and *L.*
403 *maculans* are the main inoculum in natural conditions (West et al. 2001; Huang et al. 2005).
404 Therefore, besides use of conidia as inoculum, ascospores should also be used as inoculum in
405 future work to screen cultivars of oilseed rape for resistant against *L. biglobosa* in China.

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520

521 **Figure Legends**

522 **Figure 1.** Sampling sites for the phoma stem canker survey on winter oilseed rape crops in
523 counties in Hubei province, central China. ‘○’: sites for disease survey only in May 2012;
524 ‘■’ sites for disease survey only in May 2013; ‘Δ’: sites for disease survey in both
525 seasons; ‘●’: sites for disease survey and yield loss assessment in both seasons.

526 **Figure 2.** Symptoms of phoma stem canker on stems of winter oilseed rape in Xiangyang
527 County in Hubei province in May of 2013. Symptoms (red arrows) of stem base cankers
528 and upper stem lesions were observed. The incidence of phoma stem canker was 46.2%
529 plants affected. Ten diseased and 10 healthy plants were sampled to estimate crop yield
530 loss.

531 **Figure 3.** Symptoms of phoma stem canker on stems of oilseed rape at one month after
532 inoculation in the pot experiments. Symptoms on cross-section of two oilseed rape stems
533 inoculated with *Leptosphaeria biglobosa* (right) or water (left) (A); Internal symptoms in
534 the stem pith of oilseed rape inoculated with *L. biglobosa* on cultivar Ningyou 7 (B),
535 cultivar Zhongshuang 9 (C) or with water on Ningyou 7 (D).

536 **Figure 4.** Symptoms of phoma stem canker on oilseed rape at 74 days after inoculation.
537 External and internal symptoms on stems of oilseed rape cultivar Ningyou 7 inoculated
538 with *Leptosphaeria biglobosa* (A, C, E) or with water (B, D, F).

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540

1 **Table 1** Incidence of phoma stem canker and its causal pathogen on winter oilseed
 2 rape crops in May 2012 and May 2013 in different counties in Hubei Province,
 3 central China.

County, year	Crop Nno.o. crops surveyed	Disease incidence (%) ^a	No. stems sampled stems	No. isolates obtained ^b	No. isolates of <i>L. biglobosa</i> ^c
Huanggang ^d , 2012	1	12.8	18	5	5
Huanggang, 2012	2	< 1	14	17	17
Jingmen, 2012	1	< 1	4	0	0
Jingmen, 2012	2	< 1	8	6	6
Jingzhou ^d , 2012	1	21.6	15	6	6
Jingzhou, 2012	2	< 1	3	1	1
Jingzhou, 2012	3	< 1	5	5	5
Suizhou, 2012	1	< 1	6	10	10
Suizhou, 2012	2	< 1	14	5	5
Suizhou ^d , 2012	3	41.6	12	10	10
Xiaogan, 2012	1	38.2	15	3	3
Xiangyang ^d , 2012	1	23.4	30	17	17
Xiangyang, 2012	2	75.2	24	17	17
Yichang, 2012	1	< 1	11	3	3
Yichang, 2012	2	< 1	5	2	2
Yichang, 2012	3	< 1	14	3	3
Yichang, 2012	4	< 1	10	5	5
Huanggang ^d , 2013	1	22.0	10	2	2
Huanggang, 2013	2	< 1	5	2	2
Jingmen, 2013	1	10.7	15	2	2
Jingzhou, 2013	1	< 1	4	2	2
Jingzhou, 2013	2	< 1	5	2	2
Jingzhou ^d , 2013	3	17.6	4	2	2
Jingzhou, 2013	4	< 1	9	2	2
Qianjiang, 2013	1	< 1	9	2	2
Qianjiang, 2013	2	< 1	7	2	2
Suizhou ^d , 2013	1	37.8	7	2	2
Xiangyang ^d , 2013	1	46.2	9	2	2
Xiangyang, 2013	2	15.2	15	2	2
Yichang, 2013	1	< 1	4	2	2
Total	30		311	141	141

4 ^a Incidence (% plants affected by phoma stem canker) was assessed by sampling 500
 5 plants from each crop.

6 ^b Identification of *L. biglobosa* was done based on morphological and cultural
 7 characteristics of isolates.

8 ^c Species-specific PCR was used to confirm identification of *L. biglobosa* (Liu et al.,
 9 2006).

1 ^d Disease and healthy plants were sample to estimate yield loss caused by phoma
2 stem canker.

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Table 2. Phoma stem canker disease index and effects of phoma stem canker on yield components of different oilseed rape cultivars sampled from four crops in each of four counties in Hubei Province, central China in May 2012 and May 2013.

Sample date	County (Cultivar ^a)		Disease index ^b	Height (cm)	No. branches	No. pods	No. seeds/pod	1000-seed weight (g)	Oil content (%)	Yield/plant (g)	Crop yield loss (%) ^c
May 2012	Jingzhou (DY9)	D ^d	9.5	177.0*** ^e	9.2	308.3**	19.0**	4.2	40.6	24.6**	10.1
		H	0	201.3	10.7	454.0	22.1	4.6	42.0	46.4	
	Xiangyang (ZY112)	D	18.7	114.4**	3.7**	77.1**	19.8**	4.0	40.1	6.0**	16.2
		H	0	161.7	8.0	360.7	29.2	3.6	39.1	38.3	
	Suizhou (ZS11)	D	25.1	140.7**	5.8**	149.0**	25.5	3.5	35.9	13.3**	21.2
		H	0	156.7	8.7	340.3	26.4	4.1	38.4	36.9	
	Huanggang (ZS10)	D	6.9	127.4**	3.2**	94.7**	22.3	3.3	39.4	6.9**	12.5
		H	0	166.3	8.3	509.3	26.4	3.9	39.9	52.1	
May 2013	Jingzhou (DY9)	D	14.2	147.2	4.8**	94.0**	18.7**	2.9	38.1	5.1**	13.4
		H	0	143.2	8.3	293.7	20.5	3.5	34.2	21.4	
	Xiangyang (ZY112)	D	28.6	63.8**	4.4**	87.0**	22.1	3.8	45.4	7.6**	37.5
		H	0	99.3	8.3	511.0	24.3	3.2	49.7	40.6	
	Suizhou (ZS11)	D	15.7	149.9**	4.4**	127.5**	24.4	3.0	40.6	9.6**	27.3
		H	0	174.0	7.3	550.0	25.2	2.4	37.7	34.9	
	Huanggang (ZS10)	D	9.0	103.4**	4.8**	102.8**	22.6	3.6	32.5	8.6**	15.2
		H	0	139.0	9.3	324.6	25.6	3.3	34	27.8	

^a The *Brassica napus* cultivars surveyed were Deyou 9 (DY9), Zhongyou 112 (ZY112), Zhongshuang 11 (ZS11) and Zhongshuang 10 (ZS10).

^b Disease index (DI) was calculated from assessments of disease severity score on a 0 – 4 scale (Zhou et al., 1999) using the formula: $DI = \{[\sum (Ni \times i)]/Nt \times 4\} \times 100$, where Ni is numbers of plants with disease score i and Nt is the total number of plants assessed.

^c Crop yield loss was estimated using the formula: $L = (Y_H - Y_D) / Y_H \times D_{in}$ (Y_H : yield/plant for healthy plant; Y_D : yield/plant for diseased plant; D_{in} : disease incidence in the crop, % plants affected).

^d Ten diseased (D) and ten healthy (H) plants were sampled from each crop.

^e Significant difference between diseased (D) and healthy (H) plants according to Student's T test ($P < 0.01$).

Table 3. Phoma stem canker disease index and effects of phoma stem canker on yield components of different oilseed rape cultivars inoculated with *L. biglobosa* in pot experiments

Harvest date	Cultivar		Disease index ^a	Height (cm)	No. branches	No. pods	No. seeds/pod	1000-seed weight (g)	Oil content (%)	Yield/plant (g)	Plant yield loss (%) ^b
May 2012 ^c	Zhongyou 112	Inoculated	38.1	195.6	9.4	490.1	10.6** ^d	4.1	27.2	21.1**	42.0
		Control	0	162.0	9.2	477.6	15.7	4.9	26.6	36.4	
	Zhongshuang 9	Inoculated	50	179.3	12.8	632.8	12.4**	3.8	25.4	29.0**	38.6
		Control	0	159.2	12.2	613.6	19.7	3.9	25.5	47.2	
	Ningyou 7	Inoculated	38.3	200.1	15.5	476.4	8.2**	4.7	30.6	18.3**	37.3
		Control	0	172.4	17.8	499.6	14.2	4.1	28.9	29.2	
May 2013 ^e	Zhongyou 112	Inoculated	70	154.3	7.8	327.4	11.0**	2.8	32.9	11.1**	47.1
		Control	0	159.4	7.2	338.0	20.0	3.1	32.4	21.0	
	Zhongshuang 9	Inoculated	66.3	135.8	8.4	279.0	17.8**	2.6	29.6	12.8**	28.9
		Control	0	138.8	8.2	296.0	21.8	2.8	33.3	18.0	
	Ningyou 7	Inoculated	67.5	151.3	9.7	279.0	9.7**	3.5	36.0	9.6**	56.4
		Control	0	132.7	10.8	330.0	17.1	3.7	36.6	22.0	

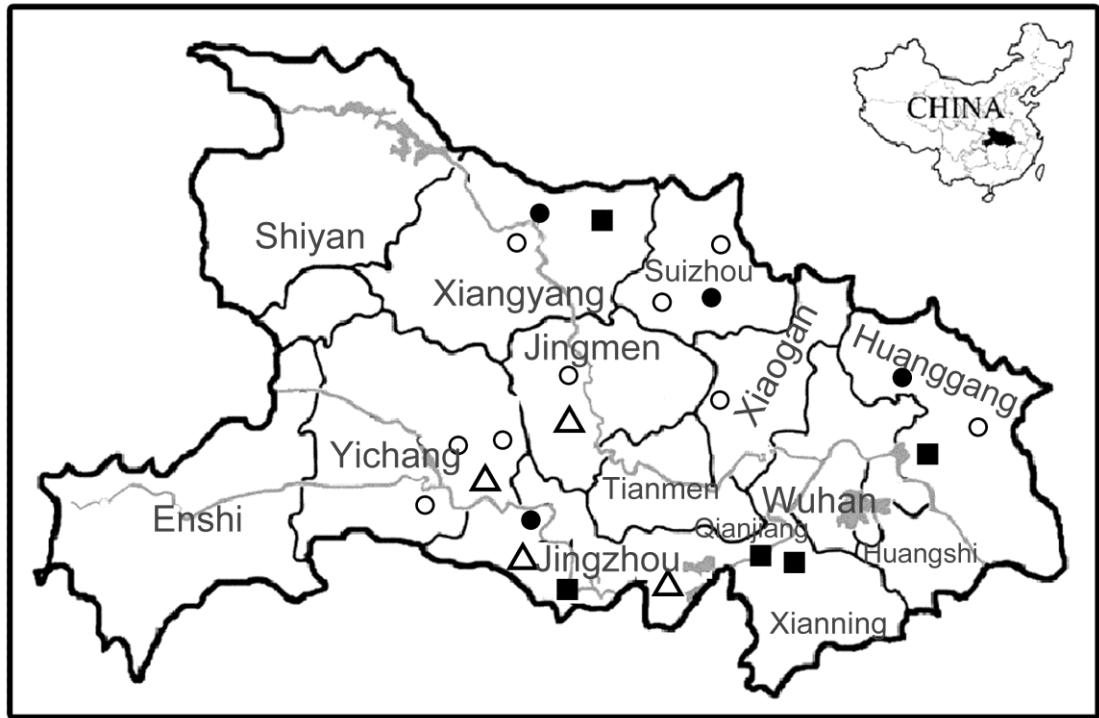
^a Disease index (DI) was calculated from assessments of disease severity score on a 0–4 scale (Zhou et al., 1999) using the formula: $DI = \{[\sum (Ni \times i)] / (Nt \times 4)\} \times 100$, where Ni is numbers of plants with disease score i and Nt is the total number of plants assessed.

^b Plant yield loss (%) was calculated using formula $(Y_c - Y_i) / Y_c \times 100\%$ (Y_c : yield/plant for control plant; Y_i : yield/plant for inoculated plant).

^c Plants were transplanted into pots on 12 October 2011, inoculated on 12 March 2012 and harvested on 25 May 2012.

^d Significant difference between the inoculated and non-inoculated (control) plants according to Student's *t* test ($P < 0.01$).

^e Plants were transplanted into pots on 10 October 2012, inoculated on 10 March 2013 and harvested on 20 May 2013.



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2 Fig. 1

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5 Fig. 2

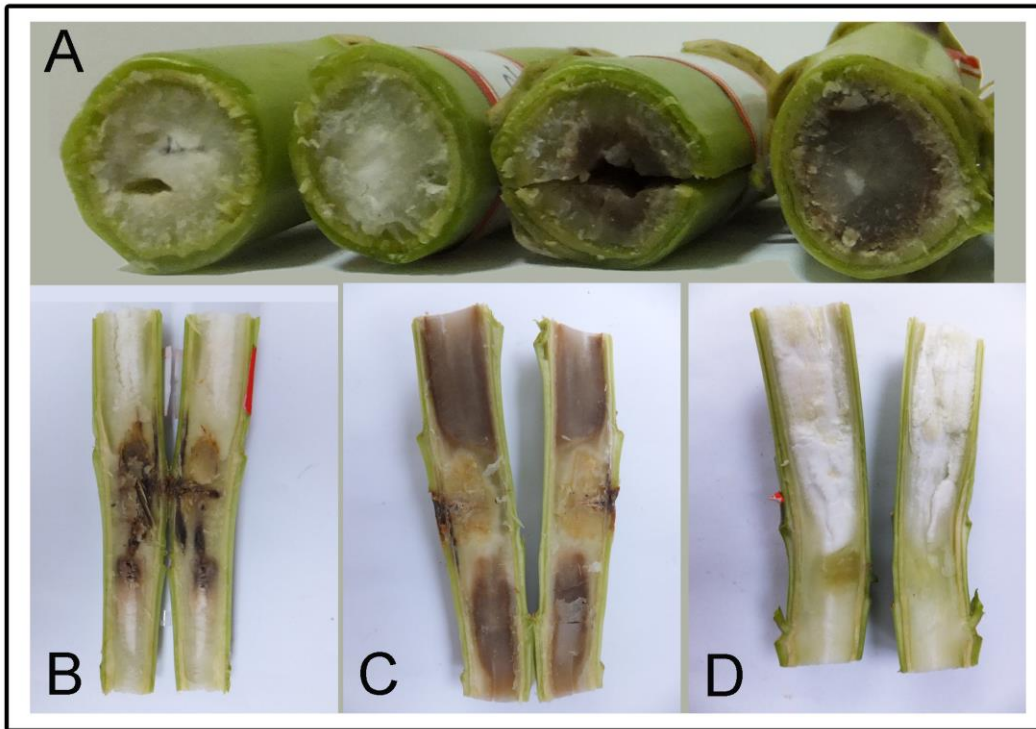
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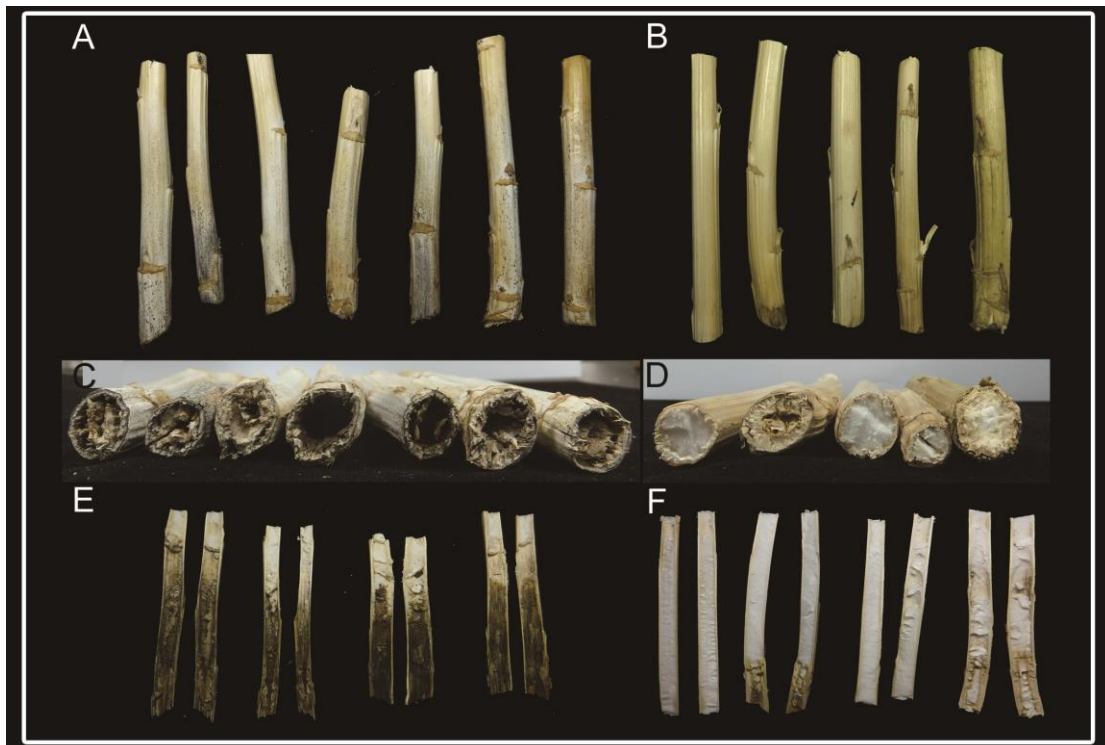
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Fig. 3



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Fig. 4