Development of a qPCR assay and a LAMP assay for *Verticillium longisporum* detection and a triplex qPCR assay for simultaneous detection of *V. longisporum*, *Leptosphaeria biglobosa* and *L. maculans* from canola samples

Heting Fu[1], Yalong Yang[1], Junye Jiang[1], Greg C. Daniels[2], Blake Hill[2], Shiming Xue[1], Kher Zahr[1], L. Stellar[2], Michael W. Harding[2], David Feindel[1], Carol Bvindi[3], Dilantha Fernando[3], and Jie Feng[1]

[1]Alberta Plant Health Lab, Alberta Agriculture and Irrigation (AGI), Edmonton, AB, T5Y 6H3, Canada.

[2]Crop Diversification Centre South, AGI, Brooks, AB, T1R 1E6, Canada.

[3]Department of Plant Science, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada.

Corresponding author:

Jie Feng, email: jie.feng@gov.ab.ca

Abstract

Verticillium wilt, Verticillium stem striping, and Verticillium stripe, are common disease names that all denote infection caused by *Verticillium longisporum*, on canola, or other Brassica crops. In this study, a quantitative PCR (qPCR) assay and a loop-mediated isothermal amplification (LAMP) assay were developed for the detection of *V. longisporum* from canola stem samples. Both assays are specific to *V. longisporum* at the species level and ubiquitous at the strain level. The low limit for positive detection of the two assays is 1 pg fungal DNA in a 20-µL reaction or 1,400 fungal cells in 100-mg plant tissue. The qPCR assay was combined with the duplex qPCR assay for the two blackleg pathogens, *Leptosphaeria biglobosa* and *L. maculans* to constitute a triplex qPCR system for simultaneous detection of all three pathogens. The usefulness of this triplex qPCR system was verified on canola samples collected from various locations in Alberta, Canada. Using this triplex qPCR system, *V. longisporum* was detected from one sample, while the two blackleg pathogens were detected at higher frequencies. Since it is sometimes difficult to differentiate Verticillium stripe and blackleg on Alberta canola samples based on visual symptoms, the triplex qPCR system is an important tool for the detection of *V. longisporum*, especially when its presence is masked or obscured by symptoms of blackleg.

Key words:

Verticillium stem striping, Stripe, Wilt, Canola, Blackleg, Multiplex qPCR

Introduction

Verticillium longisporum is a serious pathogen on brassicaceous plants (Depotter et al. 2016). It has been reported in Europe (Karapapa et al. 1997), Russia (Pantou et al. 2005), Japan (Ikeda et al. 2012), China (Yu et al. 2015), and Canada (CFIA 2016). On oilseed rape (canola), the disease caused by *V. longisporum* has been called Verticillium wilt but later renamed to Verticillium stem striping (Depotter et al. 2016). For consistency and brevity, we will use the name "Verticillium stripe" throughout the remainder of the paper understanding that any of the aforementioned common disease names may apply to infections by this fungus.

In Canada, *V. longisporum* was first found in Manitoba in 2014, and a nationwide survey conducted by the Canadian Food Inspection Agency and the Canola Council of Canada indicated the presence of the pathogen in canola stubble from five other provinces: British Columbia, Alberta, Saskatchewan, Ontario and Quebec (CFIA 2016). However, thereafter *V. longisporum* on canola has not been confirmed in Alberta in annual canola disease surveys. Inspectors observed symptoms reminiscent of Verticillium stripe but, blackleg pathogens, *Leptosphaeria maculans,* and *L. biglobosa*, rather than *V. longisporum*, were isolated from the suspect plant samples (Harding et al. 2022; 2023).

Different from many other fungal species, *V. longisporum* is amphidiploid that resulted from hybridization between two of the following four haploid ancestors: genotype A1 of an unknown species, genotype D1 of another unknown species, and genotypes D2 and D3 of *V. dahliae*. The currently identified *V. longisporum* isolates can be classified into

three lineages based on the parental combination: A1/D1, A1/D2, and A1/D3. Among the three lineages, A1/D1 is the most pathogenic lineage on canola, whereas lineage A1/D3 isolates are generally not pathogenic on this crop (Novakazi et al. 2015; Tran et al. 2013). Lineage A1/D2 is known only from horseradish in Illinois (USA) (Eastburn and Chang 1994). In Canada, a recent study indicated that *V. longisporum* isolates from canola and radish were in the A1/D1 group (Zou et al. 2020).

An effective disease management program is dependent on timely and proper identification of the causal agent of the disease. However, due to the diploid nature of *V. longisporum* and the fact that at least one of the parental species (i.e. *V. dahliae*) is a commonly occurring and widely distributed plant pathogen, accurate detection of *V. longisporum* has been difficult. Indeed, a DNA-based method for *V. longisporum* identification from plant samples has never been published. A multiplex PCR protocol developed by Inderbitzin et al. (2013) could be used to identify and differentiate *V. longisporum* into the lineage level by using DNA from pure fungal cultures. However, the use of this protocol for diagnosis of Verticillium stripe from plant samples has not been reported so far.

Quantitative PCR (qPCR) is an efficient method for plant disease diagnosis. qPCR especially probe-based qPCR is highly specific and sensitive. Another technique, loop-mediated isothermal amplification (LAMP), has gained popularity because of its capability for pathogen detection under a constant temperature and simple instrument requirements (Notomi et al. 2000). In recent years, LAMP reaction master mix became

84 available commercially, which further facilitated the use of LAMP in diagnostics. Thus,
85 in this study, we developed and evaluated a probe-based qPCR assay and a LAMP assay
86 for specific detection of *V. longisporum* from infected canola plant samples. The qPCR
87 assay was combined with a previously developed duplex qPCR for the two blackleg
88 pathogens (Fu et al. 2023) to constitute a triplex qPCR system, which was then tested for
89 capacity to specifically detect the three pathogens from canola samples collected from
90 various fields in Alberta.
91

92 **Materials and methods**

93 **Chemicals and standard techniques**

94 All chemicals and instruments were purchased from Fisher Scientific Canada (Ottawa,
95 ON) unless otherwise specified. All primers, probes, and gBlock were synthesized by
96 Integrated DNA Technologies (Coralville, IA). All DNA extraction was conducted using
97 the DNeasy Plant Pro kits (Qiagen Canada, Toronto, ON) with a Qiacube (Qiagen
98 Canada). The extracted DNA was dissolved in 50 µL water. When needed, DNA
99 concentration was measured with a Nanodrop 1000. The whole-genome sequenced *V.*
100 *longisporum* strains were identified by searching "Verticillium longisporum" in the
101 National Center for Biotechnology Information (NCBI) genome database
102 (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=100787). The average genome
103 size of all the whole-genome sequenced strains was calculated. The molecular weight of
104 the average genome was calculated using an online ssDNA Mass to Moles Convertor
105 (https://nebiocalculator.neb.com).
106

107 **Multiplex PCR, qPCR, and LAMP reactions**

108 Multiplex PCR was conducted in Promega Go Taq Master Mix or Thermo Scientific
109 Phire Plant Direct PCR Master Mix with a T100 thermocycler (Bio-Rad Canada,
110 Mississauga, ON). Each 20-µL reaction contained 0.25 µM of each primer and 1 ng DNA
111 from fungal cultures or 20 ng DNA from plants (infected or healthy). The PCR program
112 followed Zou et al. (2020). SYBR Green-based qPCR and probe-based qPCR were
113 conducted in SsoAdvanced universal SYBR green supermix (Bio-Rad Canada) and
114 PrimeTime gene expression master mix (Integrated DNA Technologies), respectively, in
115 a CFX96 touch real-time PCR detection system (Bio-Rad Canada). Each 20-µL qPCR
116 reaction contained 0.25 µM of each primer, 0.18 µM of each probe (probe-based qPCR
117 only), and 2 µL of each DNA template regardless of the concentrations. Each qPCR
118 reaction was conducted with three technical replicates. In each 96-well PCR plate, three
119 replicates of negative-control reactions were also included, in each of which 2 µL of
120 water was used as a template. The qPCR program consisted of an initial denaturation at
121 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and
122 annealing/extension at 60°C for 30 s. All LAMP reactions were conducted in the
123 WarmStart® Colorimetric LAMP Master Mix (NEB Canada, Whitby, ON). Each 20-µL
124 reaction contained 2 µL of template. Quantities of other components in each reaction
125 followed the NEB's instructions for the master mix. All reactions were conducted in
126 individual 200-µL PCR tubes. The reaction program consisted of only one step in which
127 the tubes were incubated at 65°C for 30 minutes. After the incubation, the reactions were
128 checked visually and the results were recorded by photography.

129

130 **Selection of genes for primer designing**

131 Candidate genes for qPCR and LAMP primer design were selected by the following
132 approach. All proteins of *V. longisporum* were accessed by searching the NCBI database
133 (https://www.ncbi.nlm.nih.gov) with the term 'Verticillium longisporum', which ended
134 up with 95,405 protein entries. From these proteins, those consisting of 50-60 amino
135 acids were retrieved, which ended up with 366 entries. After removing the 13 proteins
136 with predicted functions and 222 partial proteins, 131 protein entries remained. Blastp
137 was conducted with these 131 proteins as queries against NCBI protein database. After
138 removing the proteins that produced hit(s) from non-*V. longisporum* species, 66 proteins
139 remained. The genomic sequences (i.e. coding sequence and introns) were retrieved for
140 these 66 proteins (hereafter referred to as 66 genes). Two Blastn analyses were conducted
141 using the genomic sequences of these 66 genes as queries, one against the nucleotide
142 collection (nr/nt) database and the other against the whole-genome shotgun contigs (wgs)
143 database with the Organism set as Fungi (taxid:4751). After removing the genes that
144 produced hit(s) from non-*V. longisporum* species, four genes remained (GenBank
145 accession numbers CRK29573, CRK26624, CRK12486, and CRK45493). Among these
146 four genes, the first two are present in all the eight whole-genome sequenced *V.*
147 *longisporum* strains (strains PD589, VLB2, VL20, VL2, Vl32, VL1, Vl43, and Vl145c)
148 with 100% query cover and identical sequences. The other two were present in six whole-
149 genome sequenced strains with 100% query cover and identical sequences but were not
150 present in strains Vl32 and PD589. Both CRK29573 and CRK26624 had a coding
151 sequence of 180 nucleotides (nt), with the former having no intron and the latter having a
152 58-nt intron (thus the gene is 238 nt).

153

154 **Primer, probe, and gBlock design for qPCR and LAMP**

155 Based on the sequence of CRK29573, qPCR primer pairs were designed using Primer-
156 Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Each of the designed primer
157 pairs was tested in Primer 3 (https://primer3.ut.ee) for probe designing. Three
158 primers/probe sets were obtained. The sequences of the primers and the probe of each of
159 these three sets and the sequences of the primers/probe sets (P-Lm and P-Lb; Table 1)
160 used for the duplex qPCR for canola blackleg detection (Fu et al. 2023) were analyzed for
161 potential primer dimers using Multiple Primer Analyzer hosted by Thermo Fisher
162 (https://www.thermofisher.com). One set without any predicted primer dimer was
163 selected to be used in this study and named P-VL (Table 1). The probe in P-VL was
164 labeled with the fluorescent dye Cyanine5 (Cy5). A DNA sequence at 499 nt,
165 corresponding to nt 2669667 to 2670165 of the *V. longisporum* strain PD589 whole
166 genome sequence (GenBank accession number 200JAETXT010000011) and containing
167 the sequence of CRK29573, was retrieved from the NCBI database. A double-stranded
168 DNA fragment of this 499-nt sequence was synthesized as gBlock and named G-VL.

169

170 Based on the sequence of CRK26624, two sets of LAMP primers were designed using
171 Primer Explorer (https://primerexplorer.jp). After testing on *V. longisporum* genomic
172 DNA, only one set was used in further studies and named LAMP-VL (Table 1).

173

174 **Specificity test of P-VL and LAMP-VL**

175 The specificities of P-VL and LAMP-VL were tested on one known *V. longisporum*
176 isolate (APHL 189), and 27 isolates of other species including 18 fungi and one
177 oomycete (*Pythium intermedium*). APHL 189 was isolated from a canola sample
178 provided by Dr. Manika Pradhan (Manitoba Agriculture, Food and Rural Development)
179 in 2016. Other species are common pathogens of canola or have been isolated by Alberta
180 Plant Health Lab (APHL) from either diseased canola samples or samples of crops
181 rotated with canola in Alberta. All fungal and oomycete species were grown on 4% (w/v)
182 potato dextrose agar (PDA) plates. For each species, approximately 50 mg of fresh
183 mycelia was harvested and subjected to DNA extraction. DNA was also extracted from a
184 canola clubroot gall sample infected by *Plasmodiophora brassicae* pathotype 5. In
185 addition, four DNA samples were prepared as negative controls, including one from 7-
186 day-old healthy canola leaves and one from 7-day-old healthy canola roots. The third and
187 fourth negative controls were prepared from a pellet from 50 mL of YPG media (1%
188 yeast extract, 1% peptone, 2% glucose, w/v) and LB broth, respectively, after incubating
189 10 g of canola root tissue collected from a *Verticillium*-free field plot at 150 rpm 30°C for
190 48 hours (the pellet was prepared by centrifuging the culture after the root tissue being
191 removed at 4,000 × *g* for 5 min). The concentration of all DNA samples was adjusted to
192 500 pg/µL. The qPCR primers/probe set P-VL and LAMP primer set LAMP-VL were
193 tested on all the above-mentioned DNA samples. In addition, to have a DNA quality and
194 quantity control, all DNA samples were tested with the universal primer pair ITS1/ITS2
195 (White et al. 1990; Table 1) by SYBR green-based qPCR. The qPCR analysis was
196 conducted twice.
197

198 **Sensitivity test of qPCR and LAMP**

199 The gBlock G-VL was dissolved in water and a 10 pM stock solution was prepared,
200 which roughly equals 6×10^6 double-stranded DNA copies/µL. A set of 10-fold serial
201 dilutions was prepared from 6×10^6 copies/µL to 6 copies/µL. The serial dilutions were
202 tested by probe-based singleplex qPCR using P-VL. The experiment was conducted twice
203 with alternative preparations of gBlock serial dilutions.

204

205 The sensitivity of P-VL was further tested on genomic DNA of a known *V. longisporum*
206 isolate APHL 189. Genomic DNA of APHL 189 was adjusted to 50 ng/µL and then a set
207 of 10-fold serial dilutions was prepared from 50 ng/µL to 0.5 fg/µL. The serial dilutions
208 were tested by probe-based singleplex qPCR using P-VL and by triplex probe-based
209 qPCR using P-VL, P-Lb, and P-Lm. P-Lb and P-Lm were developed by Fu et al. (2023)
210 for the detection of the two canola blackleg pathogens *L. biglobosa* and *L. maculans*,
211 respectively. The serial dilutions of the APHL 189 genomic DNA were also tested by
212 LAMP using the primer set LAMP-VL. The qPCR and LAMP experiments were
213 conducted twice with alternative preparations of genomic DNA serial dilutions.

214

215 **Test of qPCR and LAMP on field samples**

216 Twenty-four canola stem samples showing black lesions were collected from 15 counties
217 in central and southern Alberta (Fig. 1). In addition, two canola stem samples (MB 1 and
218 MB 2) were collected from Manitoba, in which MB 2 was confirmed to be infected by *V.*
219 *longisporum* (Dilantha Fernando, unpublished data). The samples were cut into 2-mm
220 cubes and air-dried overnight at 23°C. From each of the air-dried samples, a 100-mg

221 subsample was subjected to DNA extraction. The obtained DNA samples were tested by
222 the triplex qPCR using P-VL, P-Lb and P-Lm and LAMP using LAMP-VL. The
223 experiment was conducted twice on DNA samples from alternative 100-mg subsamples
224 for each plant sample.
225

226 **Test of multiplex PCR on field samples and *V. longisporum* isolates**

227 Selected field samples and the *V. longisporum* isolate APHL 189 were tested by the
228 multiplex PCR using the primer set P-Multi (Table 1), which was developed by
229 Inderbitzin et al. (2013) and included three primer pairs specific to the glyceraldehyde-3-
230 phosphate dehydrogenase gene (GAPDH), the elongation factor 1-alpha gene (EF1) and
231 the internal transcribed spacer region (ITS), respectively. According to Inderbitzin et al.
232 (2013), from the *V. longisporum* lineage A1/D1, the EF1 and ITS primers will produce a
233 310-base pair (bp) band and a 1020-bp band, respectively; from the lineage A1/D2, the
234 EF1 primers will produce the 310-bp band; from the lineage A1/D3, the GAPDH and
235 EF1 primers will produce a 490-bp band and the 310-bp band, respectively and from *V.*
236 *dahliae*, only the GAPDH primers will produce the 490-bp band. Thus, the primer set P-
237 Multi can be used to differentiate the three *V. longisporum* lineages and *V. dahliae* from
238 each other.
239

240 **Results**

241 **Specificity of P-VL and LAMP-VL**

242 In the qPCR analysis, the primer set ITS1/ITS2 produced signals from all the DNA
243 samples, as indicated by the quantification cycle (Cq) value from each reaction (Table 2),

244 which provided a reference for the quality and quantity of each DNA sample. P-VL
245 produced signals from the *V. longisporum* isolate APHL 189 (Table 2). In contrast, no
246 signal was produced from any of the triplicated qPCR reactions on DNA samples of all
247 the non-*V. longisporum* isolates and the four negative controls (Table 2). In the LAMP
248 analysis, LAMP-VL generated identical results as P-VL in the qPCR analysis: the signal
249 was produced from the *V. longisporum* isolate APHL 189 only (data not shown). Similar
250 results were obtained from the repeated experiment. These results confirmed that both P-
251 VL and LAMP-VL are specific to *V. longisporum*.
252
253 **Sensitivity of P-VL in singleplex qPCR**
254 The serial dilutions of the gBlock G-VL and *V. longisporum* genomic DNA were tested
255 in singleplex qPCR with P-VL. Based on the Cq values, standard curves were constructed
256 for P-VL against gBlock (Fig. 2a) and genomic DNA (Fig. 2b). The efficiencies of P-VL
257 against gBlock and genomic DNA were 1.01 (Fig. 2a) and 1.00 (Fig. 2b), respectively.
258 The low limit of P-VL for a positive detection in a 20-µL reaction was 24 ($10^{1.08} \times 2 = 24$)
259 single-stranded gBlock copies (Fig. 2a) or 1 pg genomic DNA (Fig. 2b). The average
260 genome size of the eight whole-genome sequenced *V. longisporum* stains is 69.7 Mb.
261 Based on the molecular weight calculation, one pg of the genomic DNA is composed of
262 28 genomes of *V. longisporum*. Thus the low detection limits of the qPCR assay using P-
263 VL were similar against gBlock and fungal genomic DNA.
264
265 **Sensitivity of LAMP-VL**

266 The serial dilutions of the *V. longisporum* genomic DNA were tested by LAMP using
267 LAMP-VL. The low limit of LAMP-VL for a positive detection in a 20-µL reaction was
268 1 pg genomic DNA (Fig. 3). This result indicated that the LAMP system has a similar
269 sensitivity as the qPCR system.
270

271 **Sensitivity of P-VL in triplex qPCR**

272 The serial dilutions of the *V. longisporum* genomic DNA were tested in triplex qPCR
273 using P-VL, P-Lb, and P-Lm. Based on the Cq values, standard curves were constructed
274 for each primer set (Fig. 4a-c). The efficiencies of P-VL, P-Lb, and P-Lm were 1.02, 1.00
275 and 0.97, respectively. The low limits of genomic DNA for a positive detection in a 20-
276 µL reaction were 1 pg for P-VL (Fig. 4a), 0.4 ($10^{-0.42}$ = 0.4) pg for P-Lb (Fig. 4b) and 0.5
277 ($10^{-0.27}$ = 0.5) pg for P-Lm (Fig. 4c). This result indicated that the sensitivity of P-VL in
278 triplex qPCR was similar to that in singleplex qPCR.
279

280 **Triplex qPCR and LAMP analyses on field samples**

281 Twenty-six canola samples, 24 from Alberta and two from Manitoba were tested by the
282 triplex qPCR using the primer sets P-VL, P-Lb, and P-Lm (Table 3). *Verticillium*
283 *longisporum* was detected from one Alberta sample (23CAN-305B) and one Manitoba
284 sample (MB 2). Of the three pathogens, *V. longisporum*, *L. biglobosa,* and *L. maculans*,
285 at least one was detected from each of the 26 samples. From 13 of the 24 Alberta samples,
286 both *L. biglobosa* and *L. maculans* were detected. Among the 13 samples, the sample
287 positive for *V. longisporum* (23CAN-305B) was also positive for *L. biglobosa* and *L.*
288 *maculans*. In addition, all 26 canola samples were tested by LAMP using LAMP-VL. The

289 LAMP results were identical to the qPCR results for the yes/no detection of *V.*
290 *longisporum* (Table 3). These results indicated that both the triplex qPCR and LAMP
291 developed in this study can be used on field samples.
292

293 **Confirmation of the triplex qPCR and LAMP results by multiplex PCR**

294 With the Promega master mix, the multiplex PCR produced a single band close to 490 bp
295 from the two *V. dahliae* isolates, two bands close to 1020 bp and 310 bp, respectively,
296 from the *V. longisporum* isolate and three *V. longisporum*-infected canola samples (Fig.
297 5a). With the Thermo Fisher master mix, the multiplex PCR produced similar results as
298 with the Promega master mix except that two bands were produced from the two *V.*
299 *dahliae* isolates (Fig. 5b). Nevertheless, multiplex PCR confirmed the *V. longisporum*
300 identity of APHL 189 and the presence of *V. longisporum* in the three canola samples,
301 and also confirmed the specificity of our triplex qPCR and LAMP.
302

303 **Discussion**

304 **Specificity of P-VL and LAMP-VL**

305 The specificity of P-VL and LAMP-VL are reflected by the specificity of their target
306 genes, GenBank accession numbers CRK29573 and CRK26624, respectively. These two
307 genes are present in *V. longisporum* only. Blasting the NCBI nr/nt database with the two
308 genes did not produce any hit. When Blasting the two genes against the wgs database,
309 eight hits with 100% query cover and 100% similarity were produced for both genes.
310 These eight hits were from the eight whole-genome sequenced *V. longisporum* strains,
311 respectively, and these eight strains represent all the whole-genome sequenced strains

312 available in the database (Verified Jan 22, 2024). Thus, based on the sequence analysis,
313 the two genes are specific to *V. longisporum* at the species level and ubiquitous at the
314 strain level. This is in contrast with the other two *Verticillium*-specific genes (CRK12486
315 and CRK45493) identified in our initial candidate selection, which are present only in the
316 six whole-genome sequenced A1/D1 strains (VLB2, VL20, VL2, VL1, Vl43 and Vl145c),
317 but not in the two A1/D3 strains (PD589 and Vl32). It is likely that CRK29573 and
318 CRK26624 are derived from the A1 parent and CRK12486 and CRK45493 are derived
319 from the D1 parent. The specificity of P-VL and LAMP-VL was further demonstrated by
320 the test on non-*V. longisporum* species. No signal was produced from any reaction.
321

322 **Sensitivity of P-VL and LAMP-VL**

323 As a diploid, *V. longisporum* carries two copies of most genes, with each copy from one
324 parent (Tran et al. 2013). The exception is the nuclear ribosomal region (rDNA), for
325 which only one type is present in each lineage, as a result of DNA loss and/or concerted
326 evolution (Depotter et al. 2016). For some genes, the two copies may have similar
327 sequences that can be detected by the same set of primers. On the other hand, for many
328 other genes, the two copies may have significant differences. This would result in a
329 primer set for one copy unable to detect the other copy, but also Blast search of one copy
330 would fail to identify the other one. In addition, there may be other genes that are present
331 in only one parent and no homolog in the other parent. These genes are the real single-
332 copied genes. We do not know whether CRK29573 and CRK26624 are real single-copied
333 genes or genes with their homologs having different sequences. Nevertheless, based on
334 the Blast results, these two genes are the single targets of P-VL and LAMP-VL,

335 respectively, in the *V. longisporum* genome. Thus, the sensitivity of P-VL and LAMP-VL
336 was expected to be lower than primers that target multiple-copied genes or DNA regions.
337
338 In the sensitivity test, the low limits for P-VL and LAMP-VL in a positive reaction were
339 28 copies of genomic DNA. Given the fact that the efficiency of DNA extraction from
340 mycelia was not 100%, the low limit of the fungal cells for a positive reaction would be
341 larger than 28. According to Yang et al. (2021), the extraction efficiencies of pathogen
342 DNA from plant tissue was approximately 50%. Thus, we expected that the P-VL and
343 LAMP-VL could detect *V. longisporum* DNA from as less as 56 cells. With the
344 consideration that one DNA extraction generally results in 50 µL of DNA and 2 µL is
345 used in one qPCR or LAMP reaction, the low limit of *V. longisporum* cell numbers in a
346 sample from which the pathogen can be detected by our qPCR or LAMP assay is 1,400.
347
348 **The usefulness of the triplex qPCR and the LAMP**
349 Two typical Verticillium stripe symptoms are dark unilateral stripes on the stems and
350 microsclerotia in the stem cortex (Depotter et al. 2016). We have never observed these
351 two symptoms on a single stem sample collected in our surveys which sampled randomly
352 across Alberta and represented approximately 1% of canola acres in each County or
353 Municipal District (n = approximately 380 fields, annually). In 2021-2023, canola stem
354 samples showing symptoms suspicious for Verticillium stripe, such as discoloration on
355 the internal stem tissues in a 'starburst' pattern, rather than sectoring, or unilateral
356 striping on stems, were observed and tested for the presence of *V. longisporum*. However,
357 the results were always negative (Harding et al. 2021; 2022; 2023). The results presented

358 herein are the first confirmation of *V. longisporum* in our random Provincial survey.
359 However, even on the sample 23CAN-305B from which *V. longisporum* was detected,
360 the symptoms were not different from the symptoms of other samples from which only
361 one or both of the two blackleg pathogens were detected. This was largely because this
362 sample was infected by not only *V. longisporum* but also the two blackleg pathogens
363 (Table 3). On the other hand, this also reflected the difficulty of symptom-based
364 diagnosis of Verticillium stripe, at least on Alberta samples. Therefore, the triplex qPCR
365 will greatly aid in *V. longisporum* detection from canola samples in Alberta, even when
366 the two blackleg pathogens are not the detection targets. In our canola survey conducted
367 in 2023, we tested 306 canola stem samples (including the twenty 23CAN samples in
368 Table 2) with the triplex qPCR. All these samples had similar symptoms as the sample
369 23CAN-305B. *Verticillium longisporum* was detected from one sample only (23CAN-
370 305B). In contrast, *L. biglobosa* or *L. maculans* was detected from 235 samples. These
371 data again emphasized the difficulty in detecting *V. longisporum* visually when blackleg
372 symptoms are present and the importance of using the triplex qPCR to test canola
373 samples with ambiguous symptoms to discriminate between blackleg and Verticillium
374 stripe.
375
376 The LAMP assay developed in this study can be used to confirm the results from the
377 qPCR. Our data indicated that the LAMP had a similar sensitivity as the qPCR.
378 Compared to qPCR, LAMP was believed to be more specific because LAMP targets six
379 DNA regions by the six primers (Notomi et al. 2000). LAMP also has the advantage of
380 being simple to conduct and no requirement for special equipment. Thus, LAMP was

381 expected to be useful for on-site diagnosis. However, successful LAMP reactions are
382 indeed highly dependent on the quality of template DNA, which has been overlooked by
383 many researchers. DNA extracted by many simplified protocols (i.e. meant to be
384 conducted in the field) employing high concentrations of detergents or alkalinity, should
385 be avoided when producing template DNA for LAMP assays. Therefore, at the current
386 time and for diagnostic labs equipped with qPCR cyclers, qPCR assays should be
387 considered preferable to LAMP. This recommendation is further highlighted in the case
388 of *V. longisporum* detection because successful extraction of good quality DNA from
389 canola stems by simplified methods is more difficult than from other plant tissues.
390
391 In summary, in this study, we developed a triplex qPCR system that can be used to detect
392 *V. longisporum*, *L. biglobosa,* and *L. maculans* in a single reaction. We confirmed the
393 usefulness of this system on canola samples collected from different locations in Alberta.
394 In addition, a LAMP assay was also developed, which can be used to verify the results
395 from the triplex qPCR. We recommend the use of this triplex qPCR/LAMP combo
396 system to others interested in detecting *V. longisporum* from plant samples.
397

398 **References**

399 CFIA (Canadian Food Inspection Agency). 2016. Results of national survey for
400 *Verticillium longisporum*. Available at: https://inspection.canada.ca/plant-
401 health/invasive-species/plant-diseases/verticillium-stripe/notice-to-
402 industry/eng/1458134997626/1458135081349. Accessed: January 2024.

403 Depotter, J. R., Deketelaere, S., Inderbitzin, P., Von Tiedemann, A., Höfte, M., Subbarao,
404 K. V., Wood, T. A., and Thomma, B. P. 2016. *Verticillium longisporum*, the invisible
405 threat to oilseed rape and other brassicaceous plant hosts. Mol. Plant Pathol. 17:1004-
406 1016.
407 Fu, H., Yang, Y., Sarkes, A., Harding, M. W., Feindel, D., and Feng, J. 2023.
408 Development of a duplex qPCR system for detection and quantification of the two
409 canola blackleg pathogens *Leptosphaeria biglobosa* and *L. maculans*. Plant Dis.
410 107:2808-2815.
411 Harding, M. W., Daniels, G. C., Burke, D. A., Pugh, C. A., Hill, T. B., Zahr, K., Sarkes,
412 A., and Feng, J. 2021. Canola and mustard disease survey in Alberta, 2020. Can. Plant
413 Dis. Survey 101:109-113.
414 Harding, M. W., Daniels, G. C., Hill, T. B., Kennedy, M. A., Sarkes, A., Yang, Y., and
415 Feng, J. 2022. Canola disease survey in Alberta, 2021. Can. Plant Dis. Survey
416 102:110-113.
417 Harding, M. W., Daniels, G. C., Hill, T. B., Xue, S., Sarkes, A., Yang, Y., and Feng, J.
418 2023. Canola disease survey in Alberta, 2022. Can. Plant Dis. Survey 103:110-112.
419 Ikeda K., Banno S., Watanabe K., Fujinaga M., Ogiso H., Sakai H., Tanaka H., Miki S.,
420 Shibata S., Shiraishi T., and Fujimura M. 2012. Association of *Verticillium dahliae*
421 and *Verticillium longisporum* with Chinese cabbage yellows and their distribution in
422 the main production areas of Japan. J. Gen. Plant Pathol. 78:331-337.
423 Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. 2013. Identification and
424 differentiation of *Verticillium* species and *V. longisporum* lineages by simplex and
425 multiplex PCR assays. PLoS One 8:e65990.

426 Karapapa, V. K., Bainbridge, B. W., and Heale, J. B. 1997. Morphological and molecular
427 characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape.
428 Mycol. Res. 101:1281-1294.
429 Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and
430 Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nuc. Acids Res.
431 28:63-70.
432 Novakazi, F., Inderbitzin, P., Sandoya, G., Hayes, R.J., Tiedemann, A. V., and Subbarao,
433 K. V. 2015. The three lineages of the diploid hybrid *Verticillium longisporum* differ in
434 virulence and pathogenicity. Phytopathology 105:662-673.
435 Pantou, M. P., Strunnikova, O. K., Shakhnazarova, V. Y., Vishnevskaya, N. A.,
436 Papalouka, V. G., and Typas, M. A. 2005. Molecular and immunochemical
437 phylogenyof *Verticillium* species. Mycol. Res. 109:889-902.
438 Tran, V. T., Braus-Stromeyer, S. A., Timpner, C., and Braus, G. H. 2013. Molecular
439 diagnosis to discriminate pathogen and a pathogen species of the hybrid *Verticillium*
440 *longisporum* on the oilseed crop *Brassica napus*. Appl. Microbiol. Biotechnol.
441 97:4467-4483.
442 White, T. J., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of
443 fungal ribosomal RNA genes for phylogenetics. In: Innes, M. A., Gelfand, D. H.,
444 Sninsky, J. J., and White, T. J. (eds) PCR Protocols: A Guide to Methods and
445 Applications. San Diego, Academic Press, pp. 315-322.
446 Yang, Y., Zhou, Q., Zahr, K., Harding, M. W., Feindel, D., and Feng, J. 2021. Impact of
447 DNA extraction efficiency on the sensitivity of PCR-based plant disease diagnosis and
448 pathogen quantification. Eur. J. Plant Pathol. 159:583-591.

449 Yu, S., Su, T., Chen, J., Wang, W., Zhang, F., Yu, Y., Zhang, D., Zhao, X., and Lu, G.
450 2015. Real-time PCR as a diagnostic tool for evaluating the resistance of Chinese
451 cabbage cultivars to Verticillium wilt. Eur. J. Plant Pathol. 143:549-557.
452 Zou, Z., Bisht, V., and Fernando, W. D. 2020. Identification and characterization of
453 *Verticillium longisporum* lineage A1/D1 from Brassica crops in Manitoba, Canada.
454 Internat. J. Mol. Sci. 21:3499.
455
456

457 **Table 1.** Primers and probes used in this study

Name[a]	Sequences (5'-3')[b]	
P-VL	F	CGAGTCGTACTTGGATGGCT
	P	/5Cy5/CGTCCACTT/TAO/TACATAGCACGG/3IAbRQSp/
	R	CCAGGGAGAAGATGAGCTGT
P-Lb	F	GAAGAATGGCAAAATCACAGG
	P	/56-FAM/AGGAAGAAG/ZEN/CAGCCATAGGC/3IABkFQ/
	R	AGCTCTGCGCGACCTTTT
P-Lm	F	CCTCACACTCTCGACCCCTA
	P	/5HEX/CACAGCCAT/ZEN/ATCATCCTGCA/3IABkFQ/
	R	GCATGTTCTTGAACCGCTAC
LAMP-VL	F3	ATGAACTTACGCTGTCGA
	B3	AAACAAGCCCACAACGAC
	FIP	AGACGAACGTGACAGCATGCTCCACCTCAACAGAACAATG
	BIP	GTGTGACAGCCTCAACGAATACCTGATTACAGACTGTGTT
	LF	GCCTTATAAACCTTATCTCTGCGC
	LB	GTCCGCCTTGTGTAGAAAAGAA
ITS	ITS1	TCCGTAGGTGAACCTGCGG
	ITS2	GCTGCGTTCTTCATCGATGC
P-Multi	D1f	CCCCGGCCTTGGTCTGAT
	AlfD1r	TGCCGGCATCGACCTTGG
	A1f	AAGTGGAGCCCCGTATCTTGAAT
	A1r	CAACTGGCAACAGGGCTTGAAT
	Df	CCGGTCCATCAGTCTCTCTG
	Dr	CTGTTGCCGCTTCACTCG

458 [a]References of primers: P-VL and LAMP-VL, this study; P-Lb and P-Lm, Fu et al.
459 (2023); ITS, White et al. (1990); P-Multi, Inderbitzin et al. (2013).
460 [b]F = forward primer; R = reverse primer; P = probe.
461

462 **Table 2.** Specificity test of the qPCR primer set P-VL on canola pathogens

Species	Strain[a]	Cq values of qPCR[b] ITS1/ITS2	P-VL
Alternaria infectoria	APHL 002	24.2±0.1	NS
Alternaria solani	APHL 004	21.0±0.2	NS
Botrytis cinerea	APHL 199	19.0±0.1	NS
Colletotrichum coccodes	APHL 029	28.2±0.3	NS
Fusarium oxysporum	APHL 069	24.3±0.1	NS
Leptosphaeria biglobosa	APHL 216	21.1±0.1	NS
Leptosphaeria biglobosa	LB19-7	17.4±0.1	NS
Leptosphaeria biglobosa	APHL 1170	17.1±0.1	NS
Leptosphaeria biglobosa	LB19SK-11-2	18.0±0.1	NS
Leptosphaeria maculans	LM19-11	18.2±0.2	NS
Leptosphaeria maculans	LM19SK-11-21	18.3±0.1	NS
Leptosphaeria sclerotioides	APHL 101	18.2±0.1	NS
Phoma aliena	APHL 128	20.3±0.1	NS
Phoma betae	APHL 130	20.1±0.1	NS
Phoma medicaginis	APHL 134	19.4±0.1	NS
Plasmodiophora brassicae	Pathotype 5	25.8±0.1	NS
Pythium intermedium	APHL 159	18.0±0.2	NS
Rhizoctonia solani	APHL 234	22.9±0.2	NS
Sclerotinia sclerotiorum	APHL 244	19.7±0.1	NS
Stagonospora nodorum	APHL 120	21.6±0.1	NS
Trichoderma sp.	APHL 242	23.6±0.1	NS
Verticillium albo-atrum	APHL 190	18.3±0.2	NS
Verticillium albo-atrum	APHL 191	20.2±0.2	NS
Verticillium albo-atrum	APHL 192	18.3±0.1	NS
Verticillium dahliae	APHL 232	17.4±0.1	NS
Verticillium dahliae	APHL 303	23.0±0.2	NS
Verticillium lecanii	APHL 032	22.7±0.3	NS
Verticillium longisporum	APHL 189	21.5±0.2	25.8±0.1
Verticillium tricorpus	APHL 1117	22.8±0.1	NS
Negative control	Canola leaf	21.2±0.1	NS
Negative control	Canola root in LB[c]	24.2±0.1	NS
Negative control	Canola root in YPG[c]	26.6±0.1	NS
Negative control	Canola root	20.4±0.1	NS

463 [a]All strains belong to Alberta Plant Health Lab plant pathogen culture collection, a
464 project funded by the Canadian Agricultural Partnership (no. 601322) to JF except:
465 Lb19SK-11-2 and LM19SK-11-21 were provided by Dr. Gary Peng from Agriculture and

466 Agri-Food Canada, Saskatoon Research and Development Centre, and LB19-7 and
467 LM19-7 were purchased from University of Manitoba.
468 [b]SYBR Green qPCR using the primer set ITS (ITS1/ITS2) and probe-based qPCR using
469 the primer set P-VL. Quantification cycle (Cq) data were shown as mean ± standard
470 deviation (n = 3). NS = no signal from all the three replicates.
471 [c]DNA from microorganisms grown in LB broth or YPG media (1% yeast extract, 1%
472 peptone, 2% glucose, w/v) after incubating 200 mg healthy canola root (150 rpm, 30°C)
473 for 48 hours.
474

475 **Table 3.** Triplex qPCR and LAMP analyses on canola samples

		Cq values of triplex qPCR[a]			
Samples	Location	P-Lb	P-Lm	P-VL	LAMP[b]
2025A	Alberta	25.1±0.1	24.7±0.1	NS	-
2025B	Alberta	31.5±0.2	29.0±0.3	NS	-
2025C	Alberta	32.3±0.2	NS	NS	-
2025D	Alberta	26.5±0.1	NS	NS	-
23CAN-023	Alberta	NS	23.8±0.1	NS	-
23CAN-042	Alberta	NS	23.5±0.1	NS	-
23CAN-079	Alberta	28.0±0.1	33.2±0.4	NS	-
23CAN-083	Alberta	37.2±0.1	24.1±0.1	NS	-
23CAN-099	Alberta	28.3±0.1	25.1±0.1	NS	-
23CAN-135	Alberta	37.0±0.7	NS	NS	-
23CAN-152	Alberta	31.6±0.2	NS	NS	-
23CAN-197	Alberta	24.8±0.1	28.4±0.1	NS	-
23CAN-199	Alberta	27.0±0.1	NS	NS	-
23CAN-207	Alberta	30.3±0.1	38.5±0.9[c]	NS	-
23CAN-207	Alberta	28.3±0.1	27.6±0.1	NS	-
23CAN-305B	Alberta	26.9±0.1	26.7±0.1	30.0±0.1	+
23CAN-305C	Alberta	29.7±0.1	23.0±0.1	NS	-
23CAN-310	Alberta	NS	25.5±0.1	NS	-
23CAN-321	Alberta	37.2±0.8[c]	23.0±0.1	NS	-
23CAN-325	Alberta	24.2±0.1	37.2[c]	NS	-
23CAN-333A	Alberta	NS	25.3±0.1	NS	-
23CAN-342	Alberta	36.4±0.6	NS	NS	-
23CAN-348	Alberta	24.2±0.1	NS	NS	-
23CAN-363	Alberta	31.4±0.1	22.2±0	NS	-
MB 1	Manitoba	NS	22.7±0.1	NS	-
MB 2	Manitoba	24.1±0.1	NS	23.2±0.1	+
Negative control[d]		NS	NS	NS	-
Water		NS	NS	NS	-

476 [a]Triplex probe-based qPCR using the primer sets P-Lb, P-Lm and P-VL. Quantification
477 cycle (Cq) data were shown as mean ± standard deviation (n = 3). NS = no signal from all
478 the three replicates.
479 [b]LAMP analysis using the primer set LAMP-VL. +, positive reaction; -, negative reaction.
480 [c]Only one (without a standard deviation value) or two (with a standard deviation value)
481 of the three technical replicates generated Cq values.
482 [d]DNA from root of a 14-day old healthy canola seedling.
483

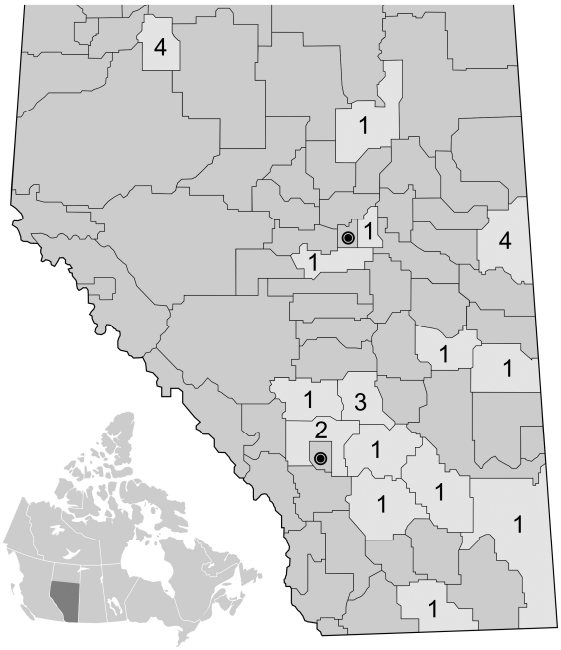

Fig. 1. Geographic locations in Alberta counties of the 24 canola stem samples that were collected for *Verticillium longisporum* detection. In each county, the number of samples was indicated.

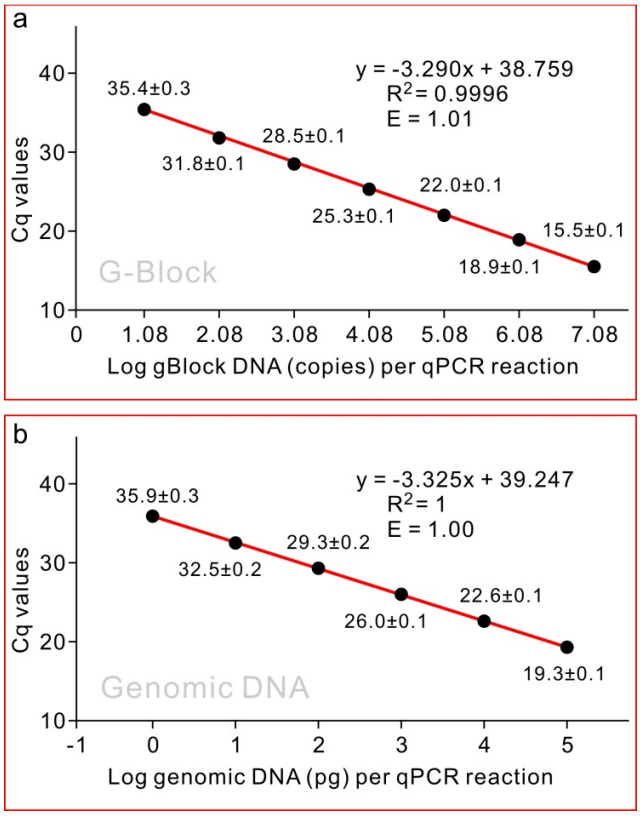

Fig. 2. Sensitivity of the qPCR primer set P-VL. a, on gBlock DNA. b, on *Verticillium longisporum* genomic DNA. The qPCR standard curves were generated from the mean of quantification cycle (Cq) values against log10 of gBlock double-stranded DNA copies (a) or picogram of genomic DNA (b) in one reaction. The R^2 score of the equation and the efficiency of the primers (E) are indicated over the curve. Efficiency was calculated as $E = -1+10^{(-1/slope)}$. Each data point is shown as mean of three technical replicates ± standard deviation.

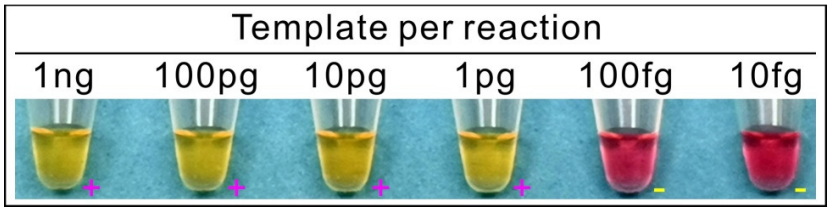

Fig. 3. Sensitivity of the LAMP primer set LAMP-VL. The LAMP reactions were conducted against a set of serial dilutions of *Verticillium longisporum* genomic DNA, from 1 ng per reaction to 10 fg per reaction. Changing of the color of the reaction mixture from red to yellow indicated a positive reaction. Each tube was also indicated by a + for positive reaction or a – for negative reaction.

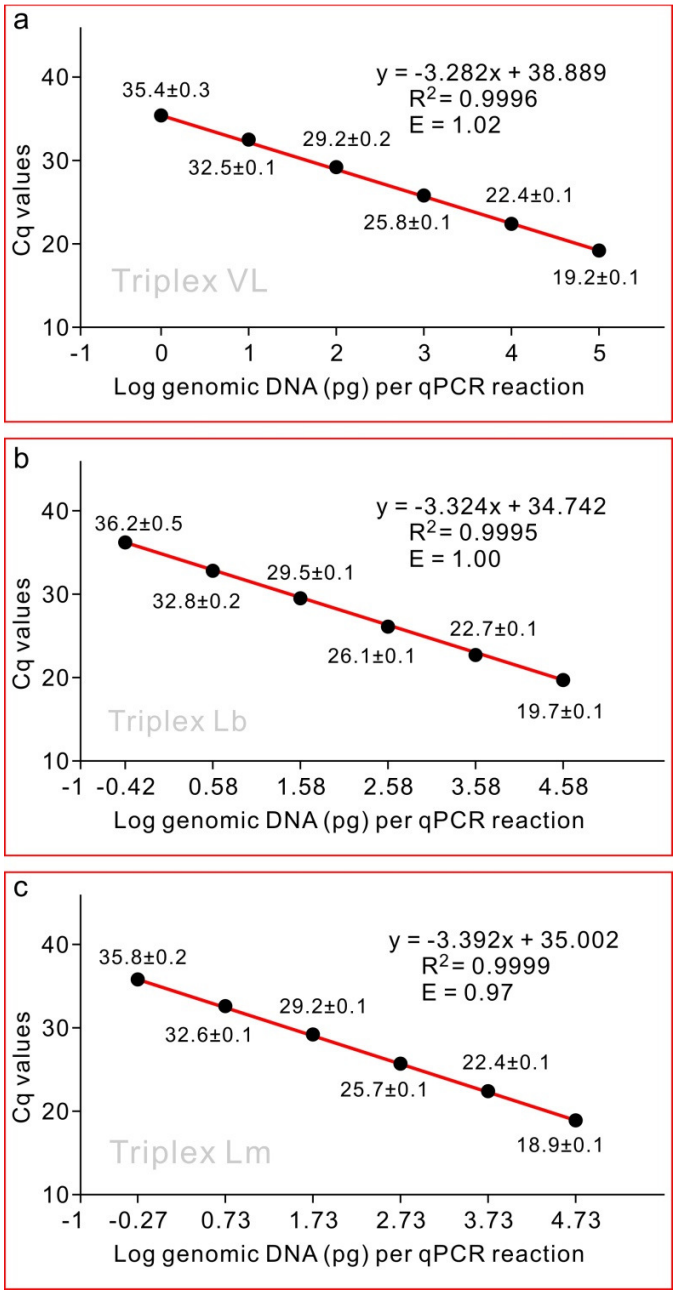

Fig. 4. Sensitivity of the triplex qPCR system for the detection of *Verticillium longisporum* by the primer set P-VL (a), *Leptosphaeria biglobosa* by the primer set P-Lb (b) and *L. maculans* by the primer set P-Lm (c) in one reaction. The qPCR standard curves were generated from the mean of quantification cycle (Cq) values against log10 of picograms of serial dilutions of genomic DNA from each of the three fungi. The R^2 score of the equation and the efficiency of the primers (E) are indicated over the curve. Efficiency was calculated as $E = -1+10^{(-1/slope)}$. Each data point is shown as mean of three technical replicates ± standard deviation.

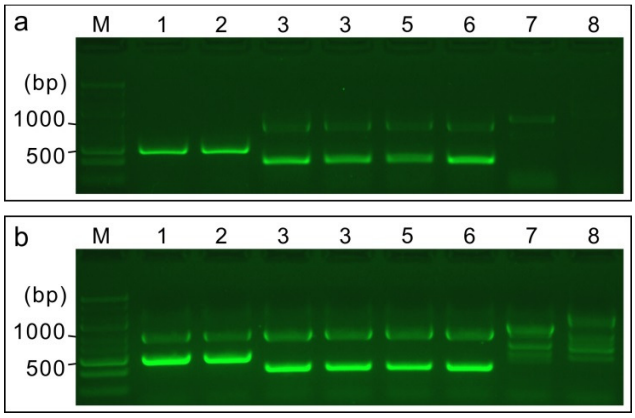

Fig. 5. Analysis of selected fungal strains and canola samples by a multiplex PCR system developed by Inderbitzin et al. (2013). The reactions were conducted in Promega Go Taq Master Mix (a) or Thermo Scientific Phire Plant Direct PCR Master Mix (b). M, GeneRuler Express DNA Ladder. 1 and 2, *Verticillium dahliae* isolates APHL 232 and APHL 303, respectively. 3, *V. longisporum* isolate APHL 189. 4, The canola sample from which APHL 189 was isolated. 5, Canola sample 23CAN-305B. 6, Canola sample MB 2. 7, Canola sample 23CAN023. 8, Healthy canola.