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

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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

87 available commercially, which further facilitated the use of LAMP in diagnostics. Thus,
88 in this study, we developed and evaluated a probe-based qPCR assay and a LAMP assay
89 for specific detection of *V. longisporum* from infected canola plant samples. The qPCR
90 assay was combined with a previously developed duplex qPCR for the two blackleg
91 pathogens (Fu et al. 2023) to constitute a triplex qPCR system, which was then tested for
92 capacity to specifically detect the three pathogens from canola samples collected from
93 various fields in Alberta.
94

95 **Materials and methods**

96 **Chemicals and standard techniques**

97 All chemicals and instruments were purchased from Fisher Scientific Canada (Ottawa,
98 ON) unless otherwise specified. All primers, probes, and gBlock were synthesized by
99 Integrated DNA Technologies (Coralville, IA). All DNA extraction was conducted using
100 the DNeasy Plant Pro kits (Qiagen Canada, Toronto, ON) with a Qiacube (Qiagen
101 Canada). The extracted DNA was dissolved in 50 µL water. When needed, DNA
102 concentration was measured with a Nanodrop 1000. The whole-genome sequenced *V.*
103 *longisporum* strains were identified by searching "Verticillium longisporum" in the
104 National Center for Biotechnology Information (NCBI) genome database
105 (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=100787). The average genome
106 size of all the whole-genome sequenced strains was calculated. The molecular weight of
107 the average genome was calculated using an online ssDNA Mass to Moles Convertor
108 (https://nebiocalculator.neb.com).
109

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

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

132

133 **Selection of genes for primer designing**

134 Candidate genes for qPCR and LAMP primer design were selected by the following
135 approach. All proteins of *V. longisporum* were accessed by searching the NCBI database
136 (https://www.ncbi.nlm.nih.gov) with the term 'Verticillium longisporum', which ended
137 up with 95,405 protein entries. From these proteins, those consisting of 50-60 amino
138 acids were retrieved, which ended up with 366 entries. After removing the 13 proteins
139 with predicted functions and 222 partial proteins, 131 protein entries remained. Blastp
140 was conducted with these 131 proteins as queries against NCBI protein database. After
141 removing the proteins that produced hit(s) from non-*V. longisporum* species, 66 proteins
142 remained. The genomic sequences (i.e. coding sequence and introns) were retrieved for
143 these 66 proteins (hereafter referred to as 66 genes). Two Blastn analyses were conducted
144 using the genomic sequences of these 66 genes as queries, one against the nucleotide
145 collection (nr/nt) database and the other against the whole-genome shotgun contigs (wgs)
146 database with the Organism set as Fungi (taxid:4751). After removing the genes that
147 produced hit(s) from non-*V. longisporum* species, four genes remained (GenBank
148 accession numbers CRK29573, CRK26624, CRK12486, and CRK45493). Among these
149 four genes, the first two are present in all the eight whole-genome sequenced *V.*
150 *longisporum* strains (strains PD589, VLB2, VL20, VL2, Vl32, VL1, Vl43, and Vl145c)
151 with 100% query cover and identical sequences. The other two were present in six whole-
152 genome sequenced strains with 100% query cover and identical sequences but were not
153 present in strains Vl32 and PD589. Both CRK29573 and CRK26624 had a coding
154 sequence of 180 nucleotides (nt), with the former having no intron and the latter having a
155 58-nt intron (thus the gene is 238 nt).

156

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

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

172

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

176

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

178 The specificities of P-VL and LAMP-VL were tested on one known *V. longisporum*
179 isolate (APHL 189), and 27 isolates of other species including 18 fungi and one
180 oomycete (*Pythium intermedium*). APHL 189 was isolated from a canola sample
181 provided by Dr. Manika Pradhan (Manitoba Agriculture, Food and Rural Development)
182 in 2016. Other species are common pathogens of canola or have been isolated by Alberta
183 Plant Health Lab (APHL) from either diseased canola samples or samples of crops
184 rotated with canola in Alberta. All fungal and oomycete species were grown on 4% (w/v)
185 potato dextrose agar (PDA) plates. For each species, approximately 50 mg of fresh
186 mycelia was harvested and subjected to DNA extraction. DNA was also extracted from a
187 canola clubroot gall sample infected by *Plasmodiophora brassicae* pathotype 5. In
188 addition, four DNA samples were prepared as negative controls, including one from 7-
189 day-old healthy canola leaves and one from 7-day-old healthy canola roots. The third and
190 fourth negative controls were prepared from a pellet from 50 mL of YPG media (1%
191 yeast extract, 1% peptone, 2% glucose, w/v) and LB broth, respectively, after incubating
192 10 g of canola root tissue collected from a *Verticillium*-free field plot at 150 rpm 30°C for
193 48 hours (the pellet was prepared by centrifuging the culture after the root tissue being
194 removed at 4,000 × *g* for 5 min). The concentration of all DNA samples was adjusted to
195 500 pg/µL. The qPCR primers/probe set P-VL and LAMP primer set LAMP-VL were
196 tested on all the above-mentioned DNA samples. In addition, to have a DNA quality and
197 quantity control, all DNA samples were tested with the universal primer pair ITS1/ITS2
198 (White et al. 1990; Table 1) by SYBR green-based qPCR. The qPCR analysis was
199 conducted twice.
200

201 **Sensitivity test of qPCR and LAMP**

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

207

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

217

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

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

224 subsample was subjected to DNA extraction. The obtained DNA samples were tested by
225 the triplex qPCR using P-VL, P-Lb and P-Lm and LAMP using LAMP-VL. The
226 experiment was conducted twice on DNA samples from alternative 100-mg subsamples
227 for each plant sample.
228

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

230 Selected field samples and the *V. longisporum* isolate APHL 189 were tested by the
231 multiplex PCR using the primer set P-Multi (Table 1), which was developed by
232 Inderbitzin et al. (2013) and included three primer pairs specific to the glyceraldehyde-3-
233 phosphate dehydrogenase gene (GAPDH), the elongation factor 1-alpha gene (EF1) and
234 the internal transcribed spacer region (ITS), respectively. According to Inderbitzin et al.
235 (2013), from the *V. longisporum* lineage A1/D1, the EF1 and ITS primers will produce a
236 310-base pair (bp) band and a 1020-bp band, respectively; from the lineage A1/D2, the
237 EF1 primers will produce the 310-bp band; from the lineage A1/D3, the GAPDH and
238 EF1 primers will produce a 490-bp band and the 310-bp band, respectively and from *V.*
239 *dahliae*, only the GAPDH primers will produce the 490-bp band. Thus, the primer set P-
240 Multi can be used to differentiate the three *V. longisporum* lineages and *V. dahliae* from
241 each other.
242

243 **Results**

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

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

247 which provided a reference for the quality and quantity of each DNA sample. P-VL
248 produced signals from the *V. longisporum* isolate APHL 189 (Table 2). In contrast, no
249 signal was produced from any of the triplicated qPCR reactions on DNA samples of all
250 the non-*V. longisporum* isolates and the four negative controls (Table 2). In the LAMP
251 analysis, LAMP-VL generated identical results as P-VL in the qPCR analysis: the signal
252 was produced from the *V. longisporum* isolate APHL 189 only (data not shown). Similar
253 results were obtained from the repeated experiment. These results confirmed that both P-
254 VL and LAMP-VL are specific to *V. longisporum*.
255
256 **Sensitivity of P-VL in singleplex qPCR**
257 The serial dilutions of the gBlock G-VL and *V. longisporum* genomic DNA were tested
258 in singleplex qPCR with P-VL. Based on the Cq values, standard curves were constructed
259 for P-VL against gBlock (Fig. 2a) and genomic DNA (Fig. 2b). The efficiencies of P-VL
260 against gBlock and genomic DNA were 1.01 (Fig. 2a) and 1.00 (Fig. 2b), respectively.
261 The low limit of P-VL for a positive detection in a 20-µL reaction was 24 ($10^{1.08} \times 2 = 24$)
262 single-stranded gBlock copies (Fig. 2a) or 1 pg genomic DNA (Fig. 2b). The average
263 genome size of the eight whole-genome sequenced *V. longisporum* stains is 69.7 Mb.
264 Based on the molecular weight calculation, one pg of the genomic DNA is composed of
265 28 genomes of *V. longisporum*. Thus the low detection limits of the qPCR assay using P-
266 VL were similar against gBlock and fungal genomic DNA.
267
268 **Sensitivity of LAMP-VL**

269 The serial dilutions of the *V. longisporum* genomic DNA were tested by LAMP using
270 LAMP-VL. The low limit of LAMP-VL for a positive detection in a 20-µL reaction was
271 1 pg genomic DNA (Fig. 3). This result indicated that the LAMP system has a similar
272 sensitivity as the qPCR system.
273

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

275 The serial dilutions of the *V. longisporum* genomic DNA were tested in triplex qPCR
276 using P-VL, P-Lb, and P-Lm. Based on the Cq values, standard curves were constructed
277 for each primer set (Fig. 4a-c). The efficiencies of P-VL, P-Lb, and P-Lm were 1.02, 1.00
278 and 0.97, respectively. The low limits of genomic DNA for a positive detection in a 20-
279 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
280 ($10^{-0.27}$ = 0.5) pg for P-Lm (Fig. 4c). This result indicated that the sensitivity of P-VL in
281 triplex qPCR was similar to that in singleplex qPCR.
282

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

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

292 LAMP results were identical to the qPCR results for the yes/no detection of *V.*
293 *longisporum* (Table 3). These results indicated that both the triplex qPCR and LAMP
294 developed in this study can be used on field samples.
295

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

297 With the Promega master mix, the multiplex PCR produced a single band close to 490 bp
298 from the two *V. dahliae* isolates, two bands close to 1020 bp and 310 bp, respectively,
299 from the *V. longisporum* isolate and three *V. longisporum*-infected canola samples (Fig.
300 5a). With the Thermo Fisher master mix, the multiplex PCR produced similar results as
301 with the Promega master mix except that two bands were produced from the two *V.*
302 *dahliae* isolates (Fig. 5b). Nevertheless, multiplex PCR confirmed the *V. longisporum*
303 identity of APHL 189 and the presence of *V. longisporum* in the three canola samples,
304 and also confirmed the specificity of our triplex qPCR and LAMP.
305

306 **Discussion**

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

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

315 available in the database (Verified Jan 22, 2024). Thus, based on the sequence analysis,
316 the two genes are specific to *V. longisporum* at the species level and ubiquitous at the
317 strain level. This is in contrast with the other two *Verticillium*-specific genes (CRK12486
318 and CRK45493) identified in our initial candidate selection, which are present only in the
319 six whole-genome sequenced A1/D1 strains (VLB2, VL20, VL2, VL1, Vl43 and Vl145c),
320 but not in the two A1/D3 strains (PD589 and Vl32). It is likely that CRK29573 and
321 CRK26624 are derived from the A1 parent and CRK12486 and CRK45493 are derived
322 from the D1 parent. The specificity of P-VL and LAMP-VL was further demonstrated by
323 the test on non-*V. longisporum* species. No signal was produced from any reaction.
324

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

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

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

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

384 expected to be useful for on-site diagnosis. However, successful LAMP reactions are
385 indeed highly dependent on the quality of template DNA, which has been overlooked by
386 many researchers. DNA extracted by many simplified protocols (i.e. meant to be
387 conducted in the field) employing high concentrations of detergents or alkalinity, should
388 be avoided when producing template DNA for LAMP assays. Therefore, at the current
389 time and for diagnostic labs equipped with qPCR cyclers, qPCR assays should be
390 considered preferable to LAMP. This recommendation is further highlighted in the case
391 of *V. longisporum* detection because successful extraction of good quality DNA from
392 canola stems by simplified methods is more difficult than from other plant tissues.
393
394 In summary, in this study, we developed a triplex qPCR system that can be used to detect
395 *V. longisporum*, *L. biglobosa,* and *L. maculans* in a single reaction. We confirmed the
396 usefulness of this system on canola samples collected from different locations in Alberta.
397 In addition, a LAMP assay was also developed, which can be used to verify the results
398 from the triplex qPCR. We recommend the use of this triplex qPCR/LAMP combo
399 system to others interested in detecting *V. longisporum* from plant samples.
400

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458
459

460 **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

461 [a]References of primers: P-VL and LAMP-VL, this study; P-Lb and P-Lm, Fu et al.
462 (2023); ITS, White et al. (1990); P-Multi, Inderbitzin et al. (2013).
463 [b]F = forward primer; R = reverse primer; P = probe.
464

465 **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

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

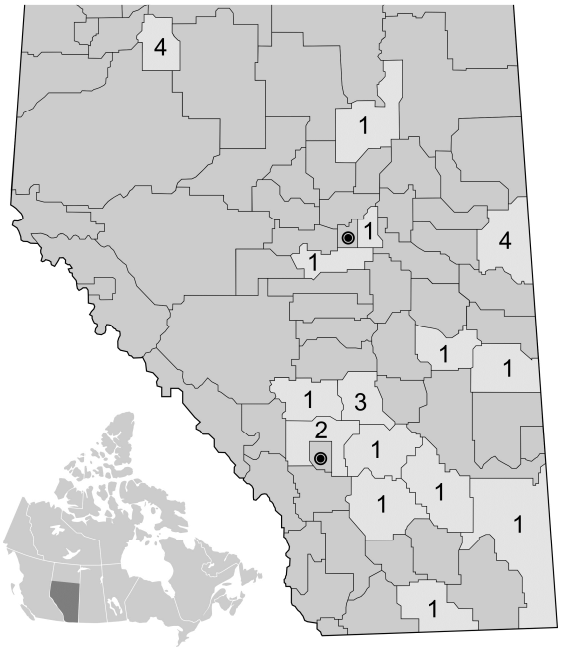
469 Agri-Food Canada, Saskatoon Research and Development Centre, and LB19-7 and
470 LM19-7 were purchased from University of Manitoba.
471 [b]SYBR Green qPCR using the primer set ITS (ITS1/ITS2) and probe-based qPCR using
472 the primer set P-VL. Quantification cycle (Cq) data were shown as mean ± standard
473 deviation (n = 3). NS = no signal from all the three replicates.
474 [c]DNA from microorganisms grown in LB broth or YPG media (1% yeast extract, 1%
475 peptone, 2% glucose, w/v) after incubating 200 mg healthy canola root (150 rpm, 30°C)
476 for 48 hours.
477

478 **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	-

479 [a]Triplex probe-based qPCR using the primer sets P-Lb, P-Lm and P-VL. Quantification
480 cycle (Cq) data were shown as mean ± standard deviation (n = 3). NS = no signal from all
481 the three replicates.
482 [b]LAMP analysis using the primer set LAMP-VL. +, positive reaction; -, negative reaction.
483 [c]Only one (without a standard deviation value) or two (with a standard deviation value)
484 of the three technical replicates generated Cq values.
485 [d]DNA from root of a 14-day old healthy canola seedling.
486

487



488

489 **Fig. 1.** Geographic locations in Alberta counties of the 24 canola stem samples that were
490 collected for *Verticillium longisporum* detection. In each county, the number of samples
491 was indicated.
492

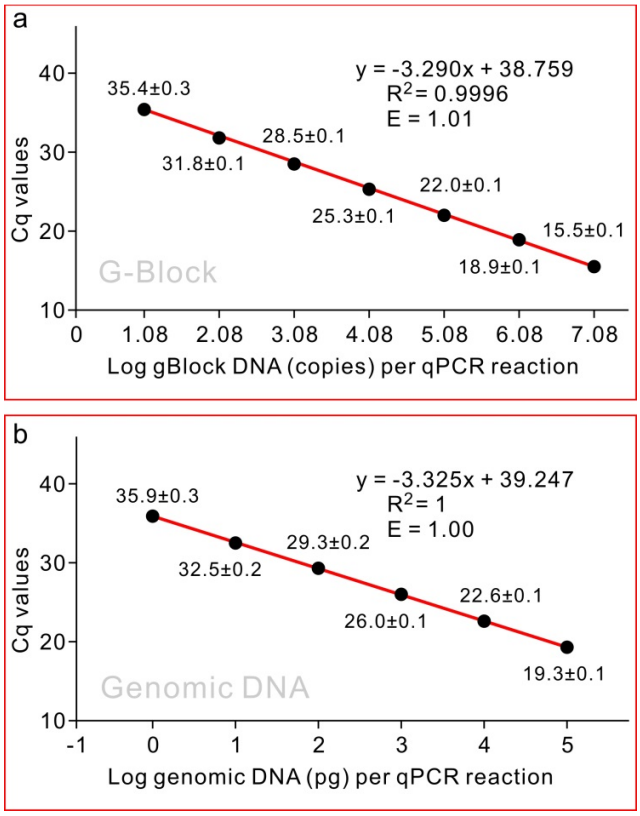


Fig. 2. Sensitivity of the qPCR primer set P-VL. a, on gBlock DNA. b, on *Verticillium longisporum* genomic DNA. The qPCR standard curves were generated from the mean of quantification cycle (Cq) values against log10 of gBlock double-stranded DNA copies (a) or picogram of genomic DNA (b) in one reaction. 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.

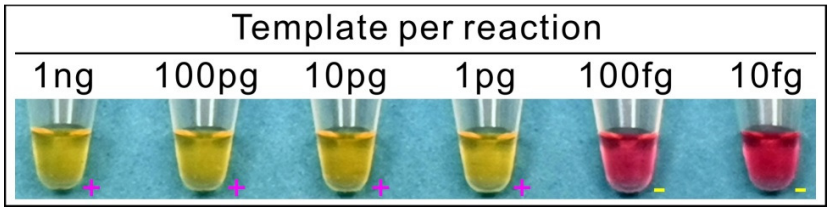


Fig. 3. Sensitivity of the LAMP primer set LAMP-VL. The LAMP reactions were conducted against a set of serial dilutions of *Verticillium longisporum* genomic DNA, from 1 ng per reaction to 10 fg per reaction. Changing of the color of the reaction mixture from red to yellow indicated a positive reaction.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

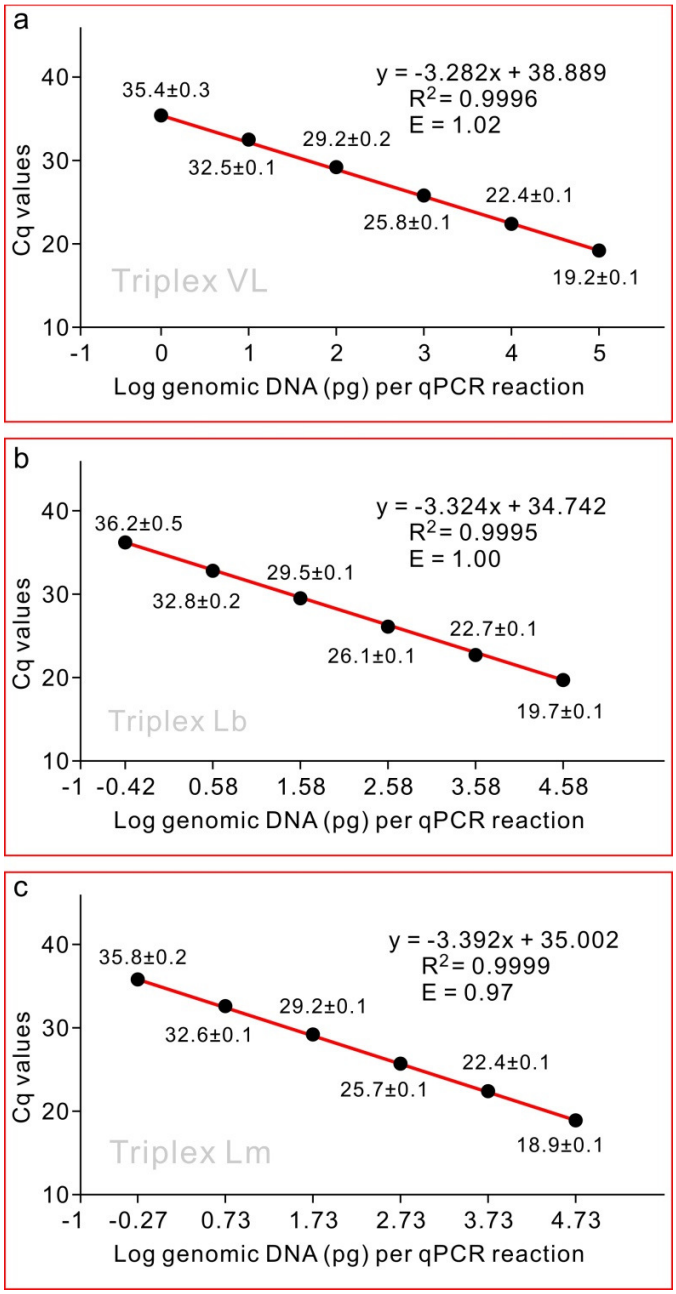


Fig. 4. Sensitivity of the triplex qPCR system for the detection of *Verticillium longisporum* by the primer set P-VL (a), *Leptosphaeria biglobosa* by the primer set P-Lb (b) and *L. maculans* by the primer set P-Lm (c) in one reaction. The qPCR standard curves were generated from the mean of quantification 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.

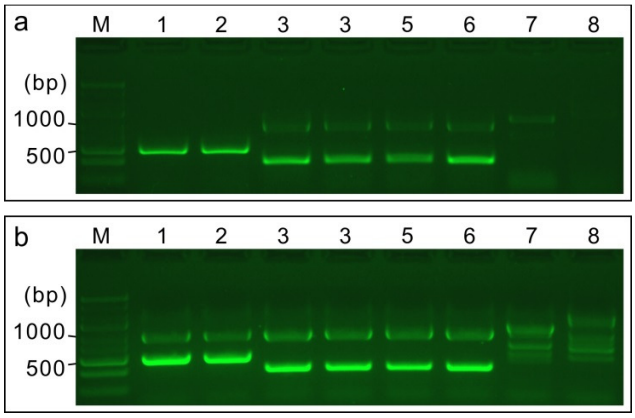


Fig. 5. Analysis of selected fungal strains and canola samples by a multiplex PCR system developed by Inderbitzin et al. (2013). The reactions were conducted in Promega Go Taq Master Mix (a) or Thermo Scientific Phire Plant Direct PCR Master Mix (b). M, GeneRuler Express DNA Ladder. 1 and 2, *Verticillium 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.