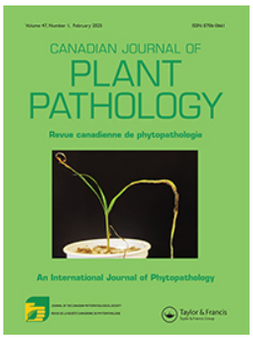

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Method / Méthode

A multiplex PCR assay for the detection of six foliar fungal pathogens of faba bean

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Abstract: Faba bean is susceptible to several foliar fungal diseases: ascochyta blight (*Ascochyta fabae*), alternaria leaf spot (*Alternaria* sp.), chocolate spot (*Botrytis cinerea* and *Botrytis fabae*), anthracnose (*Colletotrichum lentis*) and stemphylium blight (*Stemphylium* spp.). Under conducive conditions these diseases can cause significant economic losses. Visual diagnosis based on the appearance of lesions on faba beans is problematic, especially as they progress. The purpose of this study was to develop a multiplex polymerase chain reaction assay to detect the causal organisms of these diseases. Five pairs of primers, AFF7/AFR7 for *A. fabae*, BCF1/BCR1 for *B. cinerea*, BFF2/BFR2 for *B. fabae*, ClF2/ClR2 for *C. lentis* and Stem_F_S/Stem_R_S for *Stemphylium* spp. were developed. The previously developed primer pair aagpf1/aagpr1 was used to detect *Alternaria* sp. The primers were tested for specificity to their target pathogen and primer pairs AFF6/AFR6, aagpf1/aagpr1, BCF1/BCR1, BFF2/BFR2 and ClF2/ClR2 identified their target pathogens. The primer pair Stem_F_S/Stem_R_S identified *S. beticola*, *S. botryosum*, *S. eturmiunum* and *S. vesicarium*. Identification of pathogens with primers was evaluated using DNA from mycelia and from infected faba bean leaves generated through artificial inoculations under controlled conditions and collected from the field. A multiplex PCR assay with six primer pairs allowed for detection of the target pathogens to the species level for five pathogens, and to the genus level for species causing stemphylium blight. This assay enables quick diagnosis of leaf spots on faba bean, and minimizing time and effort needed to identify the primary cause of the infection through traditional isolation procedures.

Keywords: *Alternaria*, *Ascochyta fabae*, *Botrytis*, *Colletotrichum lentis*, multiplex PCR, *Stemphylium*, *Vicia faba*

Résumé: Le fèverole est susceptible à plusieurs maladies fongiques foliaires : la brûlure ascochytienne (*Ascochyta fabae*), la tache foliaire alternarienne (*Alternaria sp.*), la tache chocolat (*Botrytis cinerea* et *Botrytis fabae*), l'anthracnose (*Colletotrichum lentis*) et la brûlure Stemphylium (*Stemphylium spp.*). Dans des conditions favorables, ces maladies peuvent entraîner des pertes économiques importantes. Le diagnostic visuel basé sur l'apparence des lésions sur les fèves est problématique, en particulier à mesure qu'elles progressent. L'objectif de cette étude était de développer un test de réaction en chaîne par polymérase multiplex (PCR) pour détecter les agents pathogènes responsables de ces maladies. Cinq paires d'amorces ont été développées : AFF7/AFR7 pour *A. fabae*, BCF1/BCR1 pour *B. cinerea*, BFF2/BFR2 pour *B. fabae*, ClF2/ClR2 pour *C. lentis* et Stem_F_S/Stem_R_S pour les espèces de *Stemphylium*. La paire d'amorces précédemment développée, aagpf1/aagpr1, a été utilisée pour détecter *Alternaria* sp. Les amorces ont été testées pour leur spécificité envers leur pathogène cible, et les paires d'amorces AFF6/AFR6, aagpf1/aagpr1, BCF1/BCR1, BFF2/BFR2 et ClF2/ClR2 ont permis d'identifier leurs pathogènes cibles. La paire d'amorces Stem_F_S/Stem_R_S a permis d'identifier *S. beticola, S. botryosum, S. eturmiunum* et *S. vesicarium*. L'identification des pathogènes à l'aide des amorces a été évaluée en utilisant de l'ADN provenant de mycéliums et de feuilles de fève infectées, générées par inoculations artificielles dans des conditions contrôlées et collectées sur le terrain. Un test PCR multiplex avec six paires d'amorces a permis de détecter les pathogènes cibles au niveau de l'espèce pour cinq pathogènes, et au niveau du genre pour les espèces responsables de la brûlure de Stemphylium. Ce test permet un diagnostic rapide des taches foliaires sur la fève, tout en minimisant le temps et les efforts nécessaires pour identifier la cause principale de l'infection par les méthodes traditionnelles d'isolement.

Mots-clés: *Alternaria*, *Ascochyta fabae*, *Botrytis*, *Colletotrichum lentis*, PCR multiplex, *Stemphylium*, *Vicia faba*

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Introduction

Faba bean (*Vicia faba* L.) is a globally grown crop that has been produced commercially in Canada since 1972 (Bhatty 1974). It is a high-yielding crop and the seeds are rich in protein, making them ideal for human food and animal feed markets (Oomah et al. 2011). The plants also fix atmospheric nitrogen at a high rate, hence faba bean can be a valuable addition to a crop rotation. Furthermore, unlike other members of the Fabaceae, most genotypes of faba bean have high partial resistance to *Aphanomyces euteiches* Drechs., which causes severe root rot of lentil (*Lens culinaris* Med.) and pea (*Pisum sativum* L.) (Moussart et al. 2008). Nevertheless, they are susceptible to several pathogenic fungi and, under optimal conditions, foliar diseases can cause significant yield and quality losses (Tivoli et al. 2006). The common pathogens of faba bean are *Ascochyta fabae* Speg., *Alternaria* sp., *Botrytis fabae* Sard., *Botrytis cinerea* Pers., *Colletotrichum lentis* Damm, *Stemphylium botryosum* Walr., *Stemphylium eturmiunum* Simmons. and *Stemphylium vesicarium* Wallr.

Ascochyta blight (caused by *A. fabae*) is a common disease of faba bean, which can result in up to 90% yield loss in susceptible cultivars under ideal conditions (Avila et al. 2004). All above ground portions of faba bean can be affected. *Ascochyta fabae* is spread through infected seeds, plant debris and wind dispersal of ascospores (Kaiser 1997). Lesions caused by *A. fabae* are grey or light brown and irregular in shape. As the disease progresses, the lesions develop lighter rings around a darker centre and small black pyncidia may be present in the lesion. Failure to control ascochyta blight can result in vulnerability of the crop to other pathogens such as *Botrytis* spp. (Galloway 2017). Although breeding efforts are underway to develop resistant cultivars to ascochyta blight, the complex host–pathogen relationship has made it difficult (Avila et al. 2004).

Chocolate spot is the most widespread disease of faba bean and can cause yield losses of up to 80% (Bouhassan et al. 2004). This disease is caused by *B. cinerea* and *B. fabae*. Whereas *B. cinerea* can infect a wide range of plant species, pathogenicity of *B. fabae* appears to be restricted primarily to *V. sativa* and *V. faba*, on which it is more virulent than *B. cinerea* (Harrison 1988). Chocolate spot lesions begin as small rust-brown spots with a darker margin that increase in size and abundance as the disease progresses. As the lesions grow, they begin to merge causing large necrotic regions and defoliation.

Three diseases of faba bean that are not well studied in Canada are anthracnose (caused by *C. lentis*), alternaria leaf spot (caused by *Alternaria* sp.) and Stemphylium blight (caused by *S. botryosum*, *S. eturmiunum* and *S. vesicarium*). These pathogens are economically relevant and capable of infecting several species in the Fabaceae such as lentil, faba bean and, to a certain degree, pea (Sarantinos et al. 1996; Gossen et al. 2009). Lesions caused by *Stemphylium* spp. are described as large, dark lesions that begin at the edge of the leaf and move inwards (Bankina et al. 2021). Recent research revealed that *S. eturmiunum* may be the species of concern for faba bean in western Canada (Stratford and Banniza 2024). Lesions caused by *Alternaria* sp. are often brown and water soaked with concentric rings (Bankina et al. 2021). The lesions caused by *C. lentis* are not well characterized on faba bean. The economic impact of *Alternaria* sp., *C. lentis* and *Stemphylium* spp. on faba beans have been considered less than that of *A. fabae* and *B. fabae*; however, this may be due to misidentification in the field.

Diagnosis of foliar faba bean diseases can be challenging, as environmental factors, insect damage and the duration of infection impact lesion appearance. In addition, the coalescence of lesions as the diseases progress makes visual diagnosis less accurate. While isolating the pathogens from diseased faba bean tissue is possible, this method can be time consuming, less sensitive and may be confounded by saprophytic fungal growth. Polymerase chain reaction (PCR) identification of pathogens is often quicker and more accurate, while still cost-effective. The objective of this study was to develop a rapid multiplex PCR assay to detect six common pathogens in faba bean leaf samples.

Materials and methods

Isolates and plant materials

Single-spored isolates of *A. rabiei* (Pass.) Labr., *A. pisi* Lib., *C. coccodes* Wallr., *C. destructivum* O'Gara, *C. lindemuthianum* Sacc. et Magn., *C. linicola* Pethybr. et Laff., *C. truncatum* Schwein., *Fusarium avenaceum* Cook, *Phoma medicaginis* Malbr. & Roum. and *S. botryosum*, two isolates of *Alternaria* sp., *A. lentis* Vass., *A. fabae*, *B. fabae*, *Epicoccum nigrum* Link., Epicoccum sp. and Phoma sp., three isolates of *B. cinerea*, four isolates of *S. beticola* Woudenb. & Hanse, *S. vesicarium*, and *S. eturmiunum*, and *C. lentis* were included in this study (Table 1). *Botrytis fabae* isolate DAOM 137563A, *Colletotrichum destructivum* isolate DAOM 225 582 and *Stemphylium botryosum* isolate DAOM 195 299 were obtained from the Canadian Collection of Fungal Cultures at AAFC in Ottawa, ON. Field isolates were isolated between 1998

Table 1. Fungal species, isolate IDs, origin, plant host and specificity to primers of the multiplex PCR assay for the identification of foliar fungal pathogens of faba bean.

				Multiplex PCR primer pair specificity					
Species	Isolate ID	Origin	Plant Host	AFF7/ AFR7 (70 bp)	Aagpf1/ aagpr1 (663 bp)	BCF1/ BCR1 (343 bp)	BFF2/ BFR2 (470 bp)	ClF2/ ClR2 (124 bp)	Stem_F_S/ Stem_R_S (224 bp)
Ascochyta fabae	AF1[a]	Saskatoon, SK	*Vicia faba* L.	+	–	–	–	–	–
	AF4	Saskatoon, SK	*Vicia faba* L.	+	–	–	–	–	–
Ascochyta lentis	AL35	Lumsden, SK	*Lens culinaris* Med.	–	–	–	–	–	–
	AL61	Beechy, SK	*Lens culinaris* Med.	–	–	–	–	–	–
Ascochyta rabiei	AR3	Saskatoon, SK	*Cicer arietinum* L.	–	–	–	–	–	–
Ascochyta pisi	AP26	Saskatoon, SK	*Pisum sativum* L.	–	–	–	–	–	–
Alternaria sp.	Alt01	Saskatoon, SK	*Lens culinaris* Med.	–	+	–	–	–	–
	Alt04 [a]	Melfort, SK	*Vicia faba* L.	–	+	–	–	–	–
Botrytis cinerea	BC7	Moose Jaw, SK	*Lens culinaris* Med.	–	–	+	–	–	–
	BC11 [a]	Biggar, SK	*Lens culinaris* Med.	–	–	+	–	–	–
	BC13	Saskatoon, SK	*Lens culinaris* Med.	–	–	+	–	–	–
	BC20	Outlook, SK	*Lens culinaris* Med.	–	–	+	–	–	–
	BC35	Central Butte, SK	*Lens culinaris* Med.	–	–	+	–	–	–
Botrytis fabae	DAOM 137563A	Nova Scotia	*Vicia faba L.*	–	–	–	+	–	–
	BF5 [a]	Dundurn, SK	*Vicia faba* L.	–	–	–	+	–	–
	BF7	Humbolt, SK	*Vicia faba* L.	–	–	–	+	–	–
Colletotrichum lentis	CT30	Drinkwater, SK	*Lens culinaris* Med.	–	–	–	–	+	–
	CT39	Saskatchewan	*Lens culinaris* Med.	–	–	–	–	+	–
	CT450 [a]	Saskatoon, SK	*Vicia faba* L.	–	–	–	–	+	–
	CT458	Loverna, SK	*Lens culinaris* Med.	–	–	–	–	+	–
Colletotrichum coccodes	CC01	Saskatoon, SK	*Phaseolus vulgaris* L.	–	–	–	–	–	–
Colletotrichum destructivum	DAOM 225 582	Indian Head, SK	*Trifolium pretense* L.	–	–	–	–	–	–
Colletotrichum lindemuthianum	CL03	Saskatchewan	*Phaseolus sp.*	–	–	–	–	–	–
Colletotrichum linicola	CLa01	Saskatoon, SK	*Convolvulus arvensis* L.	–	–	–	–	–	–
Colletotrichum truncatum	CBS 151.35	USA	*Phaseolus lunatus* L.	–	–	–	–	–	–
Phoma medicaginis	Pm01	Minnesota, USA	*Medicago sativa* L.	–	–	–	–	–	–
Phoma sp.	Ax02	Saskatchewan	*Lathyrus ochroleucus* Hook.	–	-	–	–	–	–
	Ax03	RM of Douglas, SK	*Medicago sativa* L.	–	–	–	–	–	–
Stemphylium botryosum	DAOM 195 299, CBS 714.68, EGS 04-118C [a]	Ottawa, ON	*Medicago sativa* L.	–	–	–	–	–	+
Stemphylium beticola	SB11	Cabri, SK	*Lens culinaris* L.	–	–	–	–	–	+
	SB19	Bladworth, SK	*Lens culinaris* L.	–	–	–	–	–	+
	SB167	Saskatoon, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB168	Scott, SK	*Vicia faba* L.	–	–	–	–	–	+
Stemphylium eturmiunum	SB170	Melfort, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB173	Rosthern, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB187	Melfort, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB189	Rosthern, SK	*Vicia faba* L.	–	–	–	–	–	+
Stemphylium vesicarium	SB143	Saskatoon, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB174	Rosthern, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB178	Sutherland, SK	*Vicia faba* L.	–	–	–	–	–	+
	SB198	Melfort, SK	*Vicia faba* L.	–	–	–	–	–	+
Epiccocum nigrum	EN01	Saskatoon, SK	*Vicia faba* L.	–	–	–	–	–	–
	EN4	Outlook, SK	*Vicia faba* L.	–	–	–	–	–	–
Epiccocum sp.	SB26	Regina, SK	*Vicia faba* L.	–	–	–	–	–	–
	SB99	Bangledesh	*Lens sp.*	–	–	–	–	–	–
Fusarium avenaceum	Fav5	Assiniboia, SK	*Lens culinaris* L.	–	–	–	–	–	–

[a]Isolate DNA was tested individually and in a cocktail mix.

No amplification is indicated by –, amplification with + using primer pair AFF7/AFR7 with specificity for *Ascochyta fabae*, aagpf1/aagpr1 for *Alternaria* sp., BCF1/BCR1 for *Botrytis cinerea*, BFF2/BFR2 for *B. fabae*, ClF2/ClR2 for *Colletotrichum lentis* and Stem_F_S/Stem_R_S for four *stemphylium* species tested (*S. botryosum*, *S. beticola*, *S. eturmiunum* and *S. vesicarium*). Numbers in brackets are expected sizes of amplicons.

and 2023, single-spored and identified based on culture and conidia morphology on potato dextrose agar (PDA) (Difco, Becton Dickinson and Co., Franklin Lake, NJ, USA) before storage at −80°C.

Species identification of BC7, BC13 and BC20 as *B. cinerea* and DAOM 137563A as *B. fabae* were confirmed through sequence analysis by retrieving partial sequences of heat-shock protein 60 (*HSP60*), glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) and DNA-dependent RNA polymerase subunit II (*RPB2*) genes generated by Kuchuran et al. (2021) and comparing them to sequences for the same loci of *B. cinerea* isolates MUCL87 (type, CBS 131.28) and SAS56, of *B. fabae* isolates MUCL98 (type, CBS 120.29) and CBS 109.57 (Staats et al. 2005), of *B. euramericana* isolates B83 (type, CBS 14 169 919) (Brauna-Morževska et al. 2023) and B053–3 (Garfinkel et al. 2017), of *B. pseudocinerea* isolates VD110 (ex-type of type specimen PC0655988) (Walker et al. 2011) and 18B6 (Brauna-Morževska et al. 2023) and of *B. fabiopsis* isolates 3.13898 (ex-type of type specimen BroadbeanBC-2 deposited as HMAS 250 011) (Zhang et al. 2010) and B11 (Brauna-Morževska et al. 2023) (Supplementary Fig. S1). *Stemphylium eturmiunum* isolates SB170 and SB173 were previously confirmed through sequence comparisons with *S. eturmiunum* type isolate CBS 109 845 (Stratford and Banniza 2024).

All isolates were grown on PDA (Difco, Becton Dickinson and Co., Franklin Lake, NJ, USA) at room temperature for 5–7 days before transfer to glucose yeast broth (1.0 g ammonium phosphate monobasic, 0.2 g potassium chloride, 0.2 g magnesium sulphate heptahydrate, 1 mL of 0.5% cupric sulphate pentahydrate solution, 1 mL of 1% zinc sulphate heptahydrate solution, 10.0 g glucose, 5.0 g yeast extract [all Fisher Scientific, Ottawa, ON], 1.0 L distilled water) for incubation on a shaker (Labtech, Korea) at 24°C and 110 rpm for 48 h. The mycelia were filtered, lyophilized in a freeze-drier for 24 h and pulverized prior to DNA extraction.

Two types of infected plant material were used. Infected faba bean leaf samples were collected from Saskatchewan fields during August 2023 and artificially inoculated leaflets were produced indoors. Five field samples with lesions atypical for *A. fabae*, *B. fabae*, *C. lentis* and *Stemphylium* spp. were selected for identification (Fig. 1). The field samples were surface sterilized with a 10% bleach solution for 1 min, rinsed once with autoclaved-distilled water for 1 min, and freeze-dried within 24 h of collection.

For the generation of artificially inoculated leaflets, leaflets of 20-day-old faba bean plants cv. 'CDC 1310–5' were placed in 90 mm Petri dishes lined with moistened sterile filter paper. Cultures of *Alternaria* sp. isolate Alt04, *B. fabae* isolate BF5 and *C. lentis* isolate CT-450 were grown for 10 days on PDA. Plugs taken from the growing edge of the colonies were placed on the leaflets, the Petri dishes were sealed and leaflets were assessed every 24 h for lesion formation. Leaflets with PDA plugs containing no mycelia were included as a negative control. Alternatively, for the *A. fabae* isolate AF4, *B. cinerea* isolate BC11 and *S. botryosum* type-isolate DOAM 195 299 (CBS 714.68; IMI 135 456; EGS 04-118C), detached leaflets were inoculated with conidial suspensions of each isolate at a concentration of 1.0×10^5 conidia mL^{-1} and incubated in sealed Petri dishes with a moist filter paper for 72 h. Lesions were excised under sterile conditions, surface sterilized as previously described, frozen at −80°C and freeze dried for 24 h.

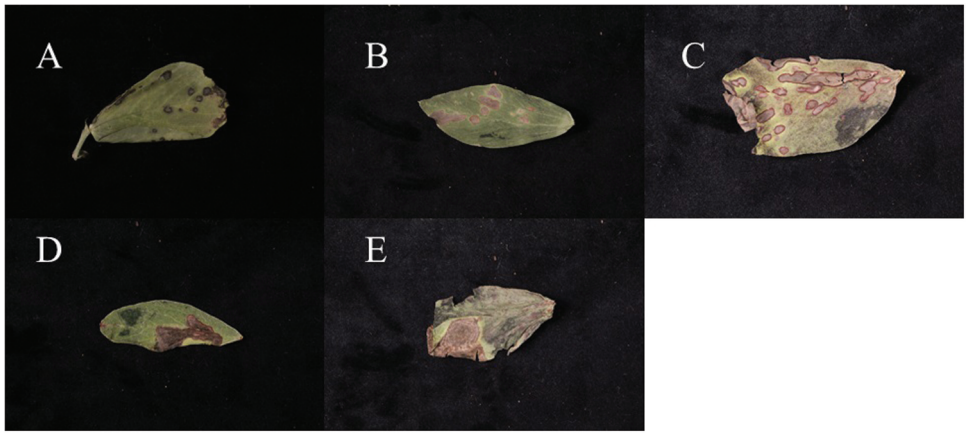

Fig. 1 Faba bean field samples collected in Saskatchewan in August 2023.

DNA extraction and primer development

A modified version of the Edwards' method was used to extract DNA from fungal mycelia (Edwards et al. 1991). DNA extraction from infected faba bean leaf tissue was performed using the E.Z.N.A. ® Plant DNA DS Mini Kit (Omega, Georgia, USA) following the manufacturer's protocol. The DNA was quantified using an IMPLEN NanoPhotometer® (Implen GmbH, Munich, Germany) and diluted to 50 ng μL^{-1} with sterile distilled water.

All primers were synthesized at Integrated DNA Technologies, Inc (Skokie, IL USA) (Table 2). The primer pair for *Alternata* sp. was designed from the mixed-linked glucanase (*MLG*) precursor gene previously described by Mmbaga et al. (Genbank accession number DQ026789, Mmbaga et al. 2011). Although these primers were described as being specific to *Alternaria alternata*, we referred to isolates identified with them as *Alternaria* sp. as no comparisons with ex-type isolates appeared to have been made (Mmbaga et al. 2011) and in view of the recent review of the genus (Woudenberg et al. 2015). The *A. fabae* (AFF7/AFR7), *B. cinerea* (BCF1/BCR1), *B. fabae* (BFF2/BFR2) and *C. lentis* (C1F2/ClR2) primer pairs were designed using the Primer-BLAST function from the National Centre for Biotechnology Information (NCBI) website (Ye et al. 2012). The *A. fabae* primers were designed based on the sequence of an unexpected amplicon obtained with primer pair BfabFS/BfabRS designed for the RNA polymerase II (*RPB2*) gene of *B. fabae* (GenBank accession number AJ745686.1, Staats et al. 2005) (BfabFS 5'-AGC CAT CGA CAC ATA CTG GT-3', BfabRS 5'-CTT TCT TTG TCT CGG CCC AC-3'). The sequence of the unexpected 100 bp amplicon was obtained by excising the band from a 2% agarose gel visualized with 1× GelRed™ and purifying the DNA using an EZ-10 Spin Column DNA Gel Extraction Minipreps Kit following the manufacturer's protocol (Bio Basic Inc., Burlington, ON). The purified DNA was sequenced at Eurofins Genomics (Louisville, KY, USA). The sequence obtained from the primer pair BfabFS/BfabRS was annotated using the GenBank database BLAST CDS feature (Ye et al. 2006) and deposited into GenBank under the accession number MT676770.

For *B. cinerea*, previously described primer pairs Bc-f/Bc-r amplifying a region of *Botryotinia fuckeliana* T4 SuperContig_10_1 genomic supercontig (Fan et al. 2015) did not amplify products for all isolates tested here, so a new pair of primers (BCF1/BCR1) was designed from the *Botryotinia fuckeliana* T4 SuperContig_10_1 genomic supercontig (GenBank accession number FQ790337.1, Amselem et al. 2011), sequences of which were adjacent to those of Bc-f/Bc-r. The *B. fabae* primer pair was designed from the necrosis and ethylene inducing peptide 1 gene (*NEP1*) (GenBank accession number AM087025.1, Staats et al. 2007). The *B. fabae* primer BFF2 was modified from the previously published primer Bfa-f (Fan et al. 2015), which produced an undesired amplicon when tested with other species in this study. The primer pair for *C. lentis* (ClF2/ClR2) was designed from the actin (*ACT*) coding region (GenBank accession number KX890331.1, Xu et al. 2017) which is an exact match to the type isolate of *C. lentis* (GenBank accession number KM105451, Damm et al. 2014). The *Stemphylium* spp. primers (Stem_F_S/Stem_R_S) where designed from the RPB2 coding region (GenBank accession number JQ905202.1, Geng et al. 2012) and were designed using Primer 3 software version 4.1.0 (Untergasser et al. 2012).

Table 2. Names, species/genus-specificity, sequences, target gene and amplicon size of primers optimized for the detection of foliar faba bean pathogens in a multiplex PCR assay.

Name	Species/genus	Sequence	Gene	Size (bp)
AFF7	*Ascochyta fabae*	CAGGCGGTCCTGGTGTCAA	Unknown	70
AFR7	*Ascochyta fabae*	CGAACGACGAGAGGAGTTGT	Unknown	
Aagpf1	*Alternaria* sp.	CGGCAACAACTACATCATCG	MLG	663
Aagpr1	*Alternaria* sp.	CTCCTGGTCAAAAGGAGCTG	MLG	
BCF1	*Botrytis cinerea*	AGCTCTAAATGCCGCAGAAGT	Unknown	343
BCR1	*Botrytis cinerea*	GAACCGACTCCTGACGTGAAA	Unknown	
BFF2	*Botrytis fabae*	CCTTTCTATCCTCGCTGCCG	NEP1	470
BFR2	*Botrytis fabae*	GCTGGTTGATCCTTGGGGAA	NEP1	
ClF2	*Colletotrichum lentis*	GGGCATCGAGATTCGGTCAG	ACT	149
ClR2	*Colletotrichum lentis*	TCAAGTGTTAGGTCGAGGCAG	ACT	
Stem_F_S	*Stemphylium spp.*	GTCACAGTCGTACAGGAGCT	RPB2	224
Stem_R_S	*Stemphylium spp.*	GTGCTGGTCTCTTGCTGTTC	RPB2	

End-point PCR

The primer sets were tested individually with DNA from all isolates (Table 1) and artificially infected faba bean tissue to assess specificity using conventional PCR. Each 20 µL PCR mixture contained 2 µL of 10× PCR buffer, 0.6 µL of $MgCl_2$, 0.25 µL of dNTPs (10 mm), 15.55 µL of sterile double-distilled water, 0.2 µL of each primer (10 µM), 1 U of Taq DNA polymerase (ThermoFisher Scientific, Burlington, ON) and 1 µL of DNA (50 ng μL^{-1}). DNA amplification was performed on a Bio-Rad S1000 thermal cycler (Mississauga, ON) as follows: 3 min at 95°C, 34 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min followed by a final extension step at 72°C for 5 min. DNA products were run on a 1.5% agarose gel and visualized using 1× GelRed™ (Biotium, Inc. Fremont, CA) cast into the gel.

Multiplex PCR assays

Once the specificity of each primer was confirmed individually, a multiplex assay was developed to detect the pathogens simultaneously. Each 25 µL PCR mixture contained 12.5 µL of Multiplex Master mix (Qiagen, Toronto, ON), 0.075 µM of BCF1 and BCR1, 0.05 µM of primers AFF7, AFR7, aapgf1, aagpr1, BFF2 and BFR2, 0.0313 µM of primers Stem_F_S and Stem_R_S and 0.025 µM of primers ClF2 and ClR2, 9 µL of sterile double-distilled water and 1 µL of DNA (50 ng μL^{-1}). The program consisted of 15 min at 95°C, 29 cycles of 94°C for 30 s, 62.4°C for 1 min and 72°C for 30 s followed by a final extension at 72°C for 10 s. DNA products were visualized on an agarose gel, as described above.

To validate the multiplex conditions, three separate assays were conducted using the multiplex PCR conditions described above. The first two of these were to ensure the multiplex assay would amplify the correct DNA regions when all isolates (Table 1) were tested individually. DNA of each isolate and faba bean leaf tissue infected with individual isolates were tested at a concentration of 50 ng μL^{-1} (Figs. 3 and 4). The third assay tested DNA from faba bean field samples collected in August 2023 using the conditions described above (Fig. 4). DNA from *A. fabae* (AF1), *Alternaria* sp. (Alt04), *B. cinerea* (BC11), *B. fabae* (BF5), *C. lentis* (CT-450) and *S. botryosum* (DAOMC 195 299) were combined to get a final concentration of 50 ng μL^{-1} and this cocktail of six isolates was used as a control.

Results and discussion

The specificity of each primer pair to its target species was determined using conventional and multiplex PCR. The primer pairs AFF7/AFR7, aagpf1/aagpr1, BCF1/BCR1, BFF2/BFR2 and ClF2/ClR2 demonstrated high specificity for their desired target species during both conventional and multiplex PCR experiments (Fig. 2). The primer pair Stem_F_S/Stem_R_S was highly specific to the target genus. None of the primer pairs amplified faba bean DNA (Fig. 3, lane 3).

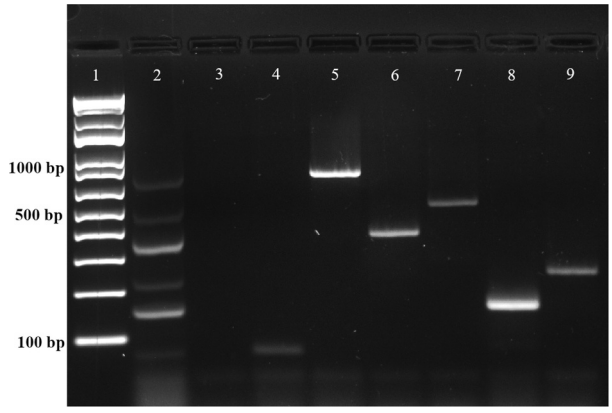

Fig. 2 Multiplex polymerase chain reactions using primer pairs AFF6/AFR6 (*Ascochyta fabae*), Aagpf1/Aagpr1 (*Alternaria* sp.), BCF1/BCR1 (*Botrytis cinerea*), BFF2/BFR2 (*Botrytis fabae*), ClF2/ClR2 (*Colletotrichum lentis*) and Stem_F_S/Stem_R_S (*Stemphylium* spp.) simultaneously to confirm specificity in identifying six foliar faba bean pathogens from fungal mycelia DNA. Lane 1: Invitrogen 1 kb plus DNA ladder, lane 2: DNA cocktail of *A. fabae* (AF1), *Alternaria* sp. (Alt04), *B. cinerea* (BC11), *B. fabae* (BF5), *C. lentis* (CT-450) and *S. botryosum* (DAOMC 195 299) at a total concentration of 50 ng μL^{-1}, lane 3: no template control, lane 4: *A. fabae* DNA, lane 5: *Alternaria* sp. DNA, lane 6: *B. cinerea* DNA, lane 7: *B. fabae* DNA, lane 8: *C. lentis* DNA, lane 9: *S. botryosum* DNA. DNA concentrations of individual target fungal species were at 50 ng μL^{-1} each.

The primer pair AFF7/AFR7 produced a 70 bp amplicon from DNA of the two *A. fabae* isolates (Table 1) and the *A. fabae* infected faba bean leaf tissue (Fig. 3, lane 4) but did not produce an amplicon for isolates of any other species. The primer pair aagpf1/aagpr1 produced a 663 bp amplicon for DNA from the two *Alternaria* sp. isolates (Table 1) and *Alternaria* sp. infected faba bean leaflets (Fig. 3, lane 5). The primer pair BCF1/BCR1 produced a 343 bp amplicon for DNA from the two isolates of *B. cinerea* (Table 1) and *B. cinerea* infected faba bean leaflets (Fig. 3, lane 6). The BFF2/BFR2 primer pair amplified a 470 bp amplicon and a faint secondary amplicon at 300 bp for DNA of the two *B. fabae* isolates (Table 1) as well as the *B. fabae* infected faba bean leaflets (Fig. 3, lane 7). The faint secondary amplicon was easily distinguishable from the five desired amplicons of the five fungal species in the assay based on size, so no further attempts were made to change amplification conditions. The primer pair ClF2/ClR2 generated a 149 bp amplicon for the four *C. lentis* isolates (Table 1) as well as

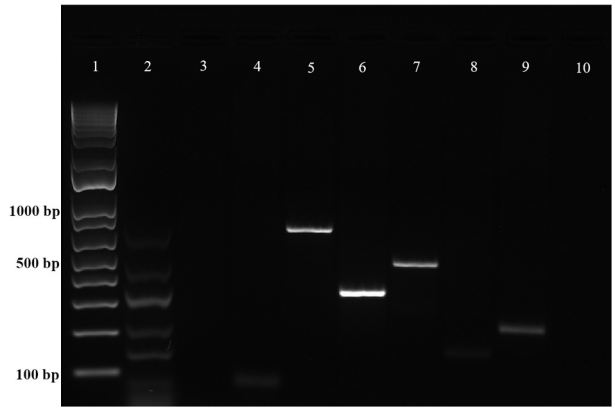

Fig. 3 Multiplex polymerase chain reaction using AFF6/AFR6 (*Ascochyta fabae*) , Aagpf1/Aagpr1 (*Alternaria* sp.), BCF1/BCR1 (*Botrytis cinerea*), BFF2/BFR2 (*Botrytis fabae*), ClF2/ClR2 (*Colletotrichum lentis*) and Stem_F_S/Stem_R_S (*Stemphylium* spp.) primer pairs simultaneously for the identification of five foliar faba bean pathogens from artificially inoculated faba bean leaflets of cv. 'CDC 1310–5'. Lane 1: invitrogen 1 kb plus DNA ladder, lane 2: DNA from each target fungal species simultaneously at a total concentrations of 50 ng μL^{-1}, lane 3: DNA of non-inoculated faba bean (50 ng μL^{-1}), lane 4: *A. fabae*-inoculated faba bean leaflets (50 ng μL^{-1}), lane 5: *Alternaria* sp.-inoculated faba bean leaflets (50 ng μL^{-1}), lane 6: *B. cinerea*-inoculated faba bean leaflets (50 ng μL^{-1}), lane 7: *B. fabae*-inoculated faba bean leaflets (50 ng μL^{-1}), lane 8: *C. lentis*-inoculated faba bean leaflets (50 ng μL^{-1}), lane 9: *S. botryosum*-inoculated faba bean (50 ng μL^{-1}) and lane 10: No template control.

the *C. lentis* infected faba bean leaflets (Fig. 3, lane 8) and did not produce an amplicon for any other species tested, including the other *Colletotrichum* spp. The Stem_F_S/Stem_R_S primer set amplified a 224 bp amplicon for the *S. botryosum* isolate, the *S. botryosum* infected faba bean leaflets, the four *S. beticola* isolates, the four *S. eturmiunum* isolates and the four *S. vesicarium* isolates but did not produce an amplicon for any other species tested (Table 1, Fig. 3, lane 9). To confirm absence of cross amplification among the primers, the multiplex assay was tested with a DNA cocktail comprised of one isolate of each of the six target species and all six amplicons visualized simultaneously on a 1.5% agarose gel (Fig. 2, lane 2).

Field samples with lesions distinct from those typically caused by *A. fabae*, *B. fabae* and *Stemphylium* spp. (Fig. 1) were collected to test the multiplex procedure revealing that sample A tested positive for *C. lentis*, sample B was positive for *Alternaria* sp. and *B. cinerea*, sample C was positive for *Alternaria* sp. and *Stemphylium* spp., sample D was positive for all six pathogens of interest= and sample E was positive for *Alternaria* sp., *B. cinerea* and *Stemphylium* spp. (Fig. 4). The multiplex thus successfully distinguished the fungal pathogens from naturally infected faba bean leaves. Five of these pathogens, *A. fabae, Alternaria* sp., *B cinerea*, *B. fabae* and *C. lentis* can be detected at the species level. *Stemphylium* can be detected at the genus level in the multiplex PCR test. Attempts to design primers for individual

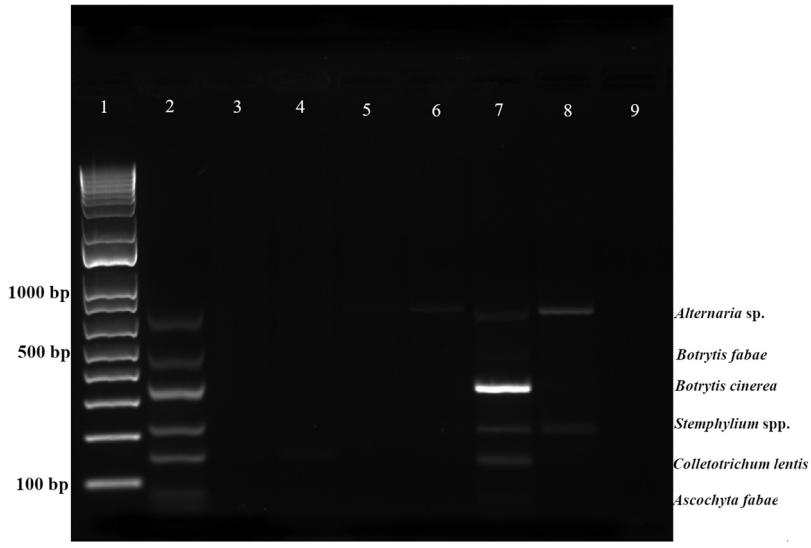

Fig. 4 Multiplex polymerase chain reaction using AFF6/AFR6 (*Ascochyta fabae*), Aagpf1/Aagpr1 (*Alternaria* sp.), BCF1/BCR1 (*Botrytis cinerea*), BFF2/BFR2 (*Botrytis fabae*), ClF2/ClR2 (*Colletotrichum lentis*) and Stem_F_S/Stem_R_S (*Stemphylium* spp.) primer pairs simultaneously for the diagnosis of faba bean field samples collected in Saskatchewan during July and August 2023. Lane 1: invitrogen 1 kb plus DNA ladder, lane 2: DNA cocktail of *A. fabae* (AF1), *Alternaria* sp. (Alt04), *B. cinerea* (BC11), *B. fabae* (BF5), *C. lentis* (CT-450) and *S. botryosum* (DAOMC 195 299) at a total concentration of 50 ng μL^{-1}; lanes 3: disease-free faba bean DNA (50 ng μL^{-1}), lanes 4–8: DNA (50 ng μL^{-1} each) from field sample leaves A–E (Fig. 1), lane 9: No template control.

Stemphylium spp. were unsuccessful and would require further evaluation of potentially diagnostic loci. At present, further sequencing would be required to conclusively determine the species of *Stemphylium* in leaf samples.

Testing of the few field samples from Saskatchewan evaluated here indicate that *Alternaria* sp. and *Stemphylium* spp. may be more prevalent pathogens than currently reported. Future work will encompass field samples and isolates from more diverse geographic locations to confirm this observation. To date, the assay has not been tested on faba bean plant parts other than leaves, nor has it been assessed for lesions from other species of the Fabaceae. In conclusion, the multiplex assay described in this study is a valuable tool for the quick diagnosis of foliar diseases on faba bean, eliminating the uncertainty of visual diagnosis based on lesion appearance and minimizing time and effort needed for isolation and diagnosis based on pathogen morphology.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary material

Supplemental data for this article can be accessed online here: https://doi.org/10.1080/07060661.2024.2448672

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