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Progress report of *Stemphylium*

Executive Summary

Leaf spot diseases have been increasing in frequency in California and Arizona production areas as well as in other production areas of the U.S. in recent years (Table 1). In an effort to examine the frequency of the various spinach leaf spot pathogens, approximately 20-50 lesions from various areas have been examined. The predominant leaf spot diseases identified have been Stemphylium leaf spot (caused by *S. versicarium* and *S. beticola*) and anthracnose of spinach (caused by *Colletotrichum spinaciae*). As Stemphylium leaf was the more predominate leaf spot pathogen observed, an effort was made to determine if the two species causing leaf spot could be distinguished based on a molecular test and if the species could be identified directly from symptomatic infected spinach tissue. The molecular tests developed can identify if the lesion is caused by Stemphyilium and can discriminate the two species. This information can facilitate improving management decisions in real time as the test takes eight hours to complete versus 1-2 weeks for culture isolation and identification.

Progress report of *Stemphylium*

Leaf spot diseases of spinach, caused by *Stemphylium vesicarium* and *S. beticola*, are important leaf spot pathogens of spinach (Correll et al., 1994; Hernandez-Perez and du Toit, 2006; and Koike et al., 2001). Although isolates were initially characterized as *S. botryosum*, more recent studies indicate that the isolates can be identified as *S. vesicarium* or *S. beticola* based on a multi-locus gene sequencing (Liu et al., 2020). Field observations of the typical symptoms caused by *S. beticola* showed that a brown ring(s) was often observed inside the lesions, but not for leaf spots caused by isolates of *S. vesicarium*; however, this distinction was much less evident on young spinach leaves. Accurate detection and identification is necessary for epidemiological studies and effective disease management. The objective of this research was to (1) develop the species-specific primers using PCR assay for detection of *S. vesicarium* and *S. beticola*, and (2) to apply this technique for field samples diagnosis and pathogen identification.

(1) We developed a quick DNA extraction method by extracting DNA directly from mycelia and lesion on the leaves, and designed the species-specific primers based on the sequences of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and calmodulin (*cmdA*) genes (Liu et al., 2021), we used these species-specific primers for PCR amplification for different pathogens, the PCR condition was described earlier (Liu et al., 2012). Results using pure isolate DNA showed that species-specific primers of *S. vesicarium* based on *gapdh* (Fig. 1A) or *cdmA* (Fig. 1B) can only amply *S. vesicarium*, and the primers of *S. beticola* based on *gapdh* (Fig. 1A) or *cdmA* (Fig. 1B) can only amply *S. beticola*. Both primers cannot amply the species of *Colletotrichum spinaciae*, *C. truncatum*, *C. coccodes*, *Cercospora* spp., *Myrothecium verrucaria*, *Cladosporium variabile*, and spinach (Fig. 1A and 1B). The size of the amplified fragments is around 190 bp for *Stemphylium* spp. based on *gapdh* and 220 bp based on *cdmA* (Fig. 1A and 1B).

(2) We also tested this molecular technique with field spinach samples. We sampled a diversity of biotic and abiotic lesions from spinach leaves in a baby leaf field trial in Texas infected with *Stemphylium* on February, 2022. Forty lesions were chose for pathogen identification (Table 1). Symptoms were first observed to determine disease type. Further, the lesions were cut in half, with one half used for isolations on Potato Dextrose Agar (PDA) media (Liu et al., 2020) and the other half used for direct DNA extraction and testing with the species-specific PCR assays (Liu et al., 2021). The half lesions were surface- sterilized in 10% household bleach (0.525% sodium hypochlorite) for 2 min, rinsed in sterilized water, air-dried, and placed into petri dishes containing PDA. The pure culture was obtained after 7 days and was transferred to a different PDA plate; the conidia were checked under compound microscope (Olympus BX41) after 7 to 14 days on PDA to determine the species based on conidia morphology (Table 1 and Fig. 1). We also extracted the DNA from the diseased lesions using Quick DNA extraction method, half lesion was used for DNA extraction followed the early protocol (Liu et al., 2021). Meanwhile, we identify the disease based on symptoms and pathogen morphology. Based on symptoms for 40 lesions, 28 are Stemphylium leaf spots, 9 are white rust, 1 downy mildew, and 2 abiotic. Based on conidia morphology on PDA, 8 lesions were *S. vesicarium*. Based on species-specific primers, 23 lesions are *S. vesicarium* based on the standard PCR amplification pattern (Table 2 and Fig. 2A and 2B). Based on lesions phenotyped as *S. vesicarium*, isolations and PCR based assays confirmed the presence of the pathogen from 33% and 100% of the samples, respectively.

In general, species-specific primers based on *gapdh* and *cdmA* genes can directly detect *Stemphylium* from the lesions, which is more accurate and quicker than media culturing. This technique can be used for species identification before *Stemphylium* spp. developed asexual or sexual structures, the whole process can be completed in a single day, which could be very useful for routine pathogen inspection.

References

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Table 1. Summary of predominant pathogens isolated from spinach leaf spot samples collected from four states in the U.S. from 2016 to 2018.

Pathogen	Samples positive for that pathogen/total number of samples examined[a]	Incidence (%)
Colletotrichum spinaciae	28/63[a]	44
Stemphylium vesicarium and *S. beticola*	39/63	62
Cercospora beticola	5/63	8
Colletotrichum coccodes and *C. truncatum*	4/63	6
Myrothecium verrucaria	2/63	3

[a] Samples identified with pathogens/total samples identified with pathogens.

Table 2. Molecular test using species-specific PCR primers for *S. vesicarium* and *S. beticola* directly from lesions of the field samples.

Isolate	Phenotype		Colony Morphology	Conidia[h]	PCR Based					
					S. v.				*S. b.*	
	WA Diag. (02/15/22)	AR Diag. (02/18/22)			cdmA[i]	cdmA[j]	Gapdh[k]	Gapdh[l]	cdmA	gapdh
Water		Control	-	-	-	-	-	-	-	-
Asymptomatic tissue		Control	-	-	-	-	-	-	-	-
Viroflay		Control	-	-	-	-	-	-	-	-
TX-8 (S. b.)		Control	-	-	-	-	-	-	+	+
St432 (+)		Control	-	-	+	+	+	+	-	-
Viroflay+TX8		Control	-	-	-	-	-	-	+	+
Viroflay+St432		Control	-	-	+	+	+	+	-	-
Viroflay+TX8+St432		Control	-	-	+	+	+	+	-	-
1	White rust[a]	White rust	-	-	-	-	-	-	-	-
2	Insect damage	S. v.	*Cladosporium*[b]	*Cladosporium*	-	-	+	-	-	-
3	S. v.	S. v.	-	-	+	+	+	+	-	-
4	Abiotic	White rust	*Alternaria* +[c]	*Alternaria*	-	-	-	-	-	-
5	S. v.	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-
6	Unknown	Abiotic	-	-	-	-	-	-	-	-
7	S. v.	S. v.	*Alternaria*	*Alternaria*	+	+	+	+	-	-
8	S. v.	S. v.	-	-	-	+	+	+	-	-
9	S. v.	S. v.	-	-	+	+	+	+	-	-
10	White rust	White rust +[d]	*Alternaria*	*Alternaria*	-	-	-	-	-	-
11	S. v.	S. v.	-	-	+	+	+	+	-	-
12	White rust	White rust	-	-	-	-			-	-
13	S. v.	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-
14	S. v.	S. v.	-	-	+	+	+	+	-	-
15	S. v.?	S. v.	-	-	-	+	+	+	-	-
16	White rust +[e]	White rust	-	-	-	-	-	-	-	-
17	S. v.	S. v.	-	-	+	+	+	+	-	-
18	S. v.	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-
19	Hole/abiotic	S. v.	*Alternaria*	*Alternaria*	-	-	-	-	-	-
20	White rust	White rust	-	-	-	-	-	-	-	-
21	S. v.	S. v.	-	-	+	+	+	+	-	-
22	S. v.	S. v.	-	-	+	+	+	+	-	-
23	S. v.	S. v.	-	-	+	+	+	+	-	-
24	Downy mildew[g]	White rust	-	-	-	-	-	-	-	-
25	Downy mildew	White rust +[d]	*Alternaria*	*Alternaria*	-	-	-	-	-	-

26	Abiotic	S. v	-	-	-	-	-	-	-	-
27	Abiotic/ mechanical	S. v.	*Alternaria*	*Alternari*a	-	-	-	-	-	-
28	White rust	White rust +[d]	*Alternaria*	-	-	-	-	-	-	-
29	S. v.	S. v.	*Stemphylium*	-	+	+	+	+	-	-
30	Downy mildew	Downy mildew	-	-	-	-	-	-	-	-
31	Abiotic	Abiotic	-	-	-	-	-	-	-	-
32	S. v.	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-
33	S. v.	S. v.	-	-	+	+	+	+	-	-
34	S. v.	S. v.	Unknown	-	-	-	-	-	-	-
35	S. v.	S. v.	-	-	+	+	+	+	-	-
36	Abiotic	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-
37	S. v.	S. v.	*Stemphylium* +[f]	S. v. +[f]	+	+	-	-	-	-
38	S. v./abiotic	S. v.	Alternaria	*Alternaria*	-	-	-	-	-	-
39	S. v	S. v.	-	-	+	+	+	+	-	-
40	S. v.	S. v.	*Stemphylium*	S. v.	+	+	+	+	-	-

[a]: White rust was observed under dissecting and compound microscopes.
[b]: A putative isolate of a non-pathogenic *Cladosporium* was observed.
[c]: *Alternaria* colony morphology was observed in addition to bacterium.
[d]: A combination of White rust + *S. v.*
[e]: A combination of White rust + cold damage
[f]: Colony morphology of *Stemphylium* sp. in addition to unknown fungal morphology
[g]: Downy mildew was observed under dissecting and compound microscopes.
[h]: Conidia produced after 1-month culture on PDA.
[i]: Confirmed species based on PCR with *cdmA* gene species-specific primers with regular PCR condition.
[j]: Confirmed species based on PCR with *cdmA* gene species-specific primers with optimized PCR condition.
[k]: Confirmed species based on PCR with *gapdh* gene species-specific primers with regular PCR condition.
[l]: Confirmed species based on PCR with *gapdh* gene species-specific primers with optimized PCR condition.
+: positive and -: negative band amplification.
S.v. is *Stemphylium vesicarium* and S.b. is *S. beticola*.
AR diag.: diagnosis by Arkansas scientist; WA diag: diagnosis by Washington scientist.

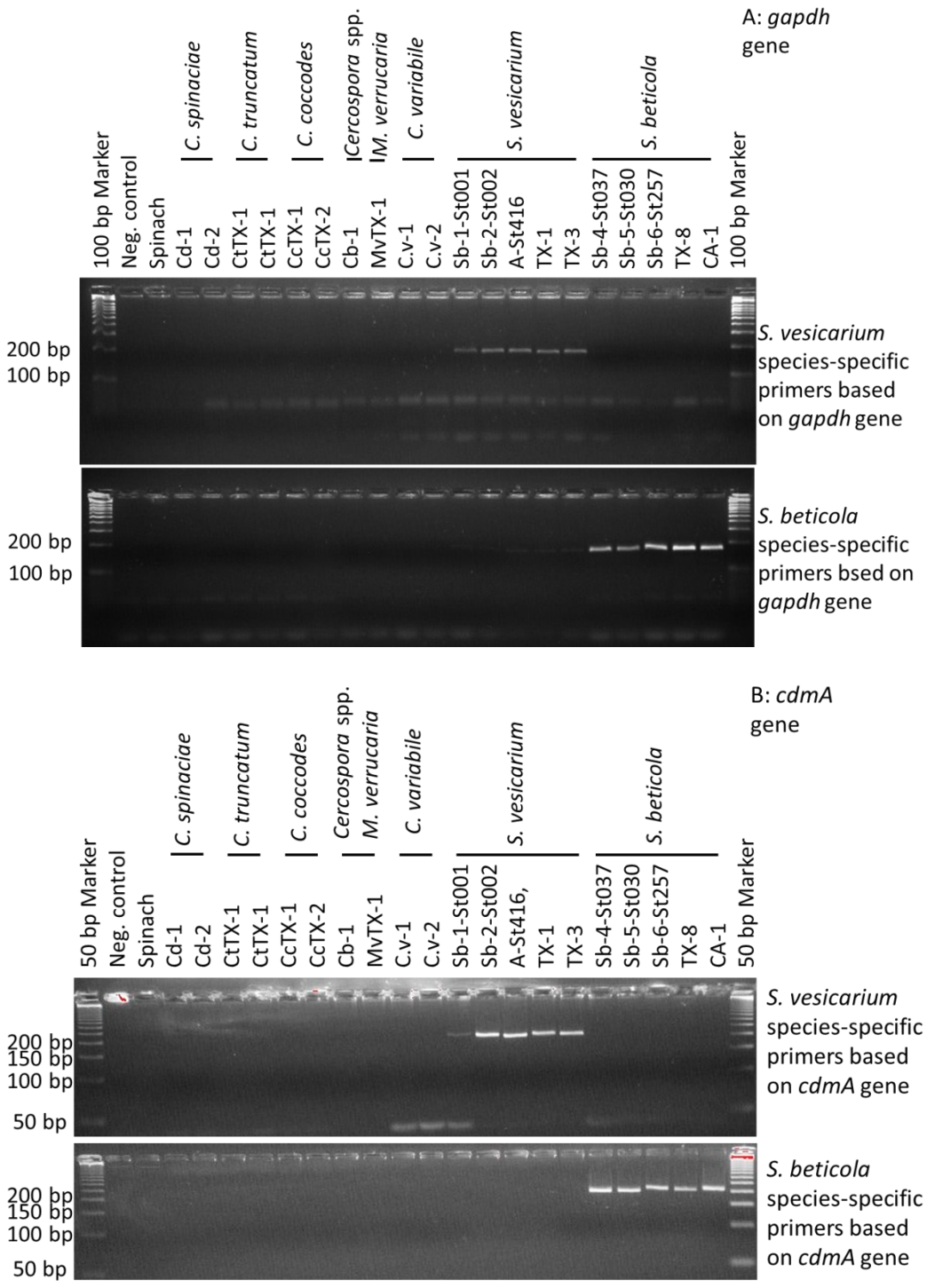
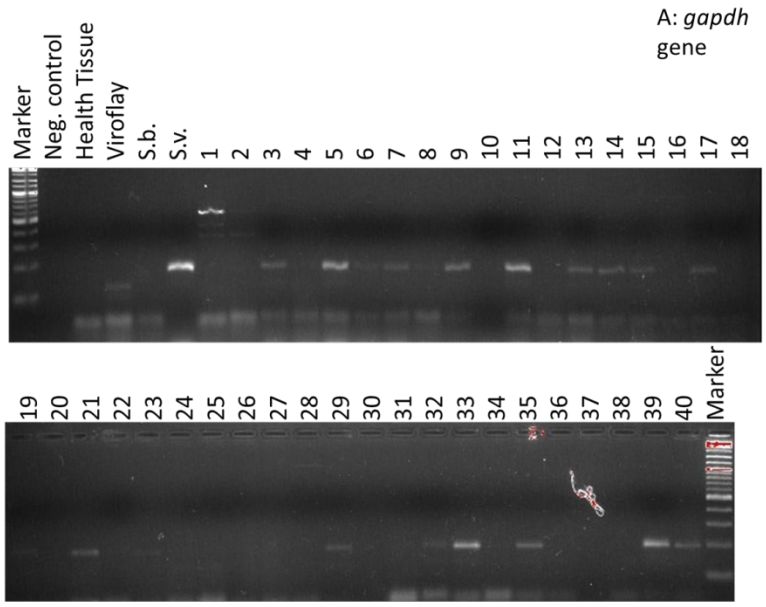


Fig. 1. Species-specific primers of *S. vesicarium* based on *gapdh* (A) and *cdmA* (B) can only amply *S. vesicarium*, and the primers of *S. beticola* can only amply *S. beticola*.



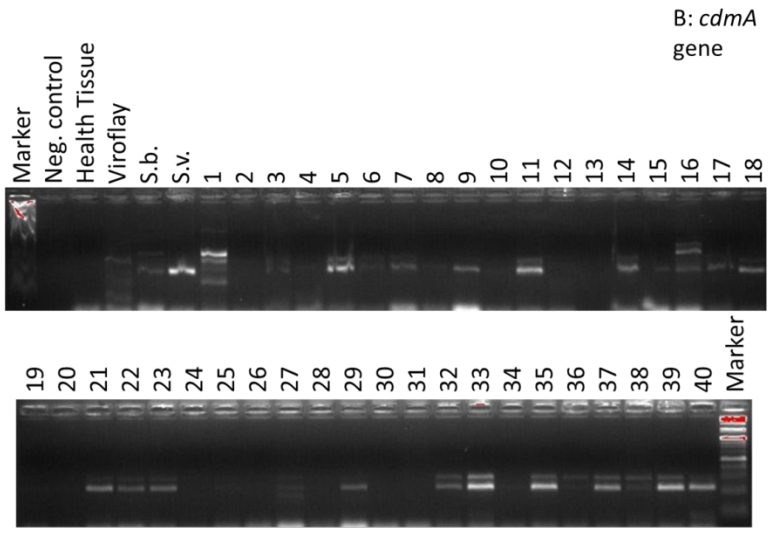


Fig. 2. Species-specific primers of *S. vesicarium* based on *gapdh* (A) and *cdmA* (B) can only amply *S. vesicarium* from the DNA directly extracted from diseased lesions. On the gel, S.b. is S. *beticola* (TX-8) and S.v. is *S. vesicarium* (St432).