Characterization of *Stemphylium* spp. from Spinach Based on Molecular Data, Host Response, and Azoxystrobin Sensitivity

K.A. Spawton[1], M.L. Derie[1], J.C. Correll[2], L.A. Stein[3], G. Olaya[4], R.N. Raid[5], G.V. Sandoya[5], T.L. Peever[6], and L.J. du Toit[1]

[1]Washington State University, Mount Vernon, WA; [2]University of Arkansas, Fayetteville, AR; [3]Texas A&M University, Uvalde, TX; [4]Syngenta Crop Protection, Vero Beach, FL; [5]University of Florida, Belle Glade, FL; [6]Washington State University, Pullman, WA

ABSTRACT (APS annual meeting, Cleveland, OH, USA, 3-7 Aug. 2019)

Stemphylium leaf spot of spinach causes losses under warm, humid conditions. The causal agent was described originally as *Stemphylium botryosum* based on anamorph and teleomorph morphology. As a result of increasing losses to this disease and evidence of diversity among *Stemphylium* isolates from spinach, molecular characterization, pathogenicity tests, and azoxystrobin resistance screening were performed on isolates sampled from symptomatic plants in Arizona, California, Florida, Oregon, Texas, and Washington as well as isolates from seeds grown in six countries, including the USA. The internal transcribed spacer 1 and 2 regions of ribosomal DNA, and regions of *glyceraldehyde-3-phosphate dehydrogenase* and *calmodulin* were sequenced. Two spinach cvs., Mandolin and Viroflay, were inoculated with each isolate. Isolates clustered into two phylogenetic lineages: 1) isolates most similar to *S. vesicarium*, and 2) isolates previously identified as *S. botryosum*. The former caused small (≤ 5 mm diameter) lesions on Mandolin 2 days after inoculation, but no symptoms on Viroflay, even after 21 days. The latter produced larger (5 to 15 mm diameter) lesions on both cultivars within 10 to 14 days. Isolates resistant to azoxystrobin (EC_{50} > 10 mg/liter) were detected, and mutations conferring resistance are being characterized. Differences in the biology, epidemiology, and management of these two causal agents of Stemphylium leaf spot of spinach are being investigated.

INTRODUCTION

- In 2001, the causal agent of Stemphylium leaf spot of spinach was first described in California and identified as *S. botryosum* based on morphology (5). Soon after, the disease was reported in Washington, Oregon, Delaware, Maryland, Florida, Arizona, and Texas (1,2,5,6,8,9). The fungus is seedborne and occurs in all regions of spinach seed production (4).
- In 2017 and 2018, other species of *Stemphylium* were reported to be pathogenic on spinach in Japan and Italy, including *S. beticola* (3,6).
- Recently, growers in Texas and Florida have reported reduced efficacy of QoI fungicides against Stemphylium leaf spot.
- The objectives of this study were to identify the causal agent causing Stemphylium leaf spot of spinach through molecular analyses, screen spinach cultivars for susceptibility to *Stemphylium* isolates, and assess the sensitivity of isolates to azoxystrobin.

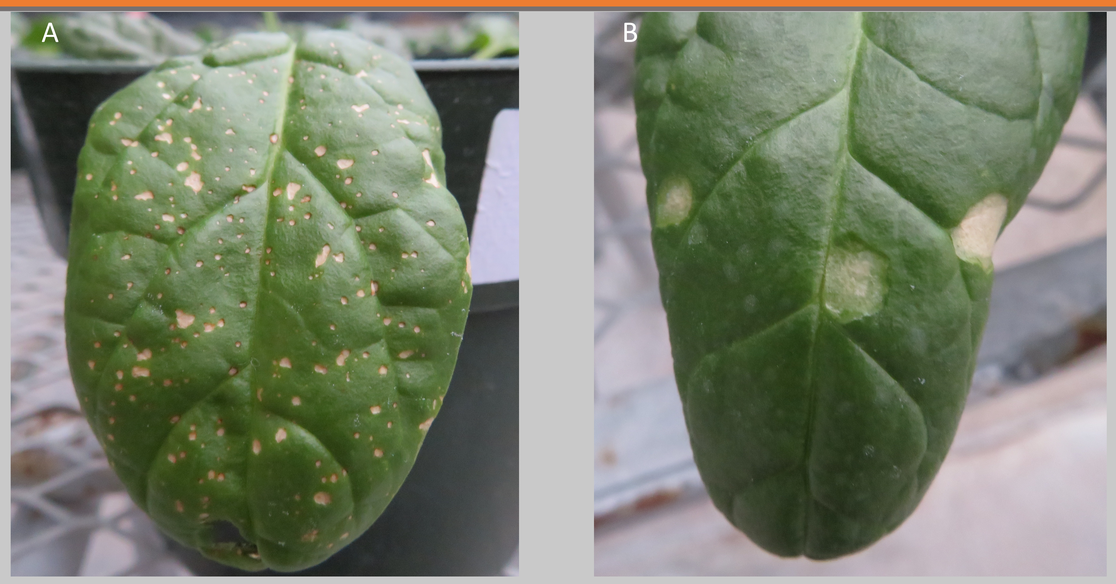

Fig. 1. Typical symptoms caused by lineage 1 (A) and lineage 2 (B) on the spinach cv. Mandolin.

MATERIALS AND METHODS

- Isolates of *Stemphylium* from symptomatic plants and seed lots were selected to represent a range of years, locations, and cultivars of spinach production.
- **Causal agent identification:** ITS rDNA, *gapdh*, and *cmdA* loci were sequenced. Phylogenetic lineages were determined based on BLAST results and phylogenetic analyses.
- **Pathogenicity:** Each isolate was inoculated onto two spinach cvs., Mandolin and Viroflay.
- **Azoxystrobin sensitivity:** Isolates were grown on medium amended with azoxystrobin at 0, 0.001, 0.01, 0.1, 0.1, 1, and 10 μg/ml (with 100 μg SHAM/ml) to assess sensitivity of mycelial growth and conidial germination to the fungicide. Some isolates were also tested at 100 μg azoxystrobin/ml.

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RESULTS

- **Causal agent identification:** Based on both *gapdh* and *cmdA* sequences, all isolates grouped in two phylogenetic lineages: isolates in lineage 1 (*n* = 147) most closely matched *S. vesicarium* and isolates in lineage 2 (*n* = 44) most closely matched *S. beticola* in the NCBI database with 99 to 100% identity scores. However, ITS rDNA sequences had 100% identity score to *S. vesicarium* or *S. globuliferum.* The latter is not a recognized species in recent studies (10). Therefore, spinach isolates previously identified morphologically as *S. botryosum* appear to be more closely related to *S. beticola*, as supported by phylogenetic analyses (*data not shown*).
- **Pathogenicity**:
  - Isolates of lineage 1 produced leaf spots on Mandolin only, while isolates of lineage 2 produced leaf spots on both cultivars (**Table 1**).
  - Isolates of lineage 1 produced numerous small lesions (≤ 5 mm diameter) that each developed a dark margin (**Fig. 1A**). Isolates of lineage 2 produced fewer, larger lesions (5 to 10 mm diameter), each with a diffuse, rapidly expanding margin (**Fig. 1B**).
- **Azoxystrobin sensitivity**: Mycelial growth of isolates of lineage 1 was sensitive to azoxystrobin at ≥ 10 μg/ml whereas isolates of lineage 2 were sensitive at ≥ 0.1 μg/ml (**Fig. 2**). Conidial germination of isolates of lineage 1 was reduced at 100 μg/ml compared to 1 μg/ml for isolates of lineage 2 (**Fig. 3**).

Table 1. *Stemphylium* isolates sequenced and tested for pathogenicity.

Isolate	Year	Location	DNA-based ID	Pathogenicity	
				Mandolin	Viroflay
St009	2001	WA, USA	lineage 2	+	+
St018	2002		lineage 2	+	+
St030	2002	WA, USA	lineage 2	+	+
St125	2004		lineage 2	+	+
St257	2004	AZ, USA	lineage 2	+	+
St354[a]	2005	Netherlands	lineage 2	+	+
St406	2012	OR, USA	lineage 2	+	+
St416	2014	AZ, USA	lineage 1	+	-
St428	2016	CA, USA	lineage 1	+	-
St430	2018	TX, USA	lineage 1	+	-
St468	2017	FL, USA	lineage 1	+	-
St469	2017	FL, USA	lineage 1	+	-
St480		FL, USA	lineage 1	+	-
St481	2019	TX, USA	lineage 1	+	-
St485	2019	TX, USA	lineage 1	+	-
St510	2018	FL, USA	lineage 1	+	-
St511	2018	FL, USA	lineage 1	+	-
St522	2019	TX, USA	lineage 1	+	-
St525	2019	TX, USA	lineage 1	+	-
St535	2019	TX, USA	lineage 1	+	-
St542	2019	TX, USA	lineage 1	+	-
St544	2019	TX, USA	lineage 1	+	-
St554	2019	TX, USA	lineage 1	+	-
St562	2019	TX, USA	lineage 1	+	-
St567	2019	TX, USA	lineage 1	+	-
St571	2019	TX, USA	lineage 1	+	-

[a] St354 was the only non-USA isolate tested to date for pathogenicity and sensitivity to azoxystrobin. Isolates of lineage 1 (*n* = 24) and lineage 2 (*n* = 6) were from seed lots grown in the European Union, Denmark, the Netherlands, Chile, Italy, China, and France ~ 15 years ago (4).

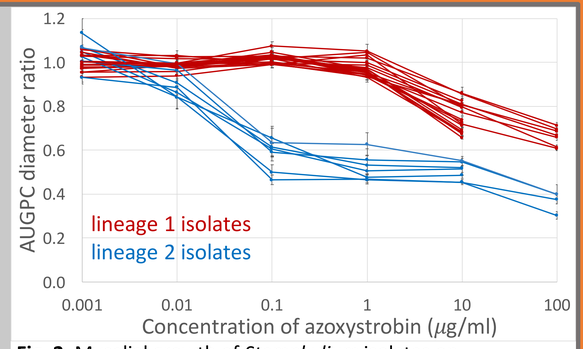

Fig. 2. Mycelial growth of *Stemphylium* isolates on azoxystrobin amended medium. AUGPC diameter ratio is the area under the growth progress curve of the mycelial diameter measured at 3, 5 and 7 days of growth on azoxystrobin amended medium divided by the AUGPC of the mycelial diameter on non-amended medium.

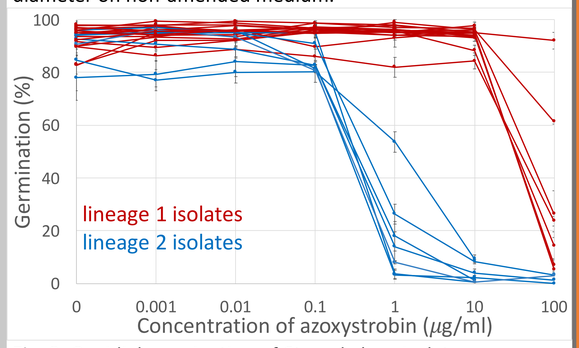

Fig. 3. Conidial germination of *Stemphylium* isolates on azoxystrobin amended medium.

CONCLUSIONS

- Symptoms caused by isolates of lineage 2 were typical of those previously described as being caused by *S. botryosum*. Molecular analyses suggest isolates previously described as *S. botryosum* from spinach are more closely related to *S. beticola* than *S. botryosum*.
- *cmdA* and *gapdh* sequences gave consistent lineages while ITS rDNA was inadequate.
- At least two phylogenetic lineages of *Stemphylium* cause Stemphylium leaf spots of spinach, although representatives of the two lineages caused different symptoms.
- Isolates of lineage 1 only caused leaf spots on Mandolin, but isolates of lineage 2 caused leaf spots on Mandolin and Viroflay. Additional cultivars need to be evaluated for susceptibility to both lineages.
- Isolates of lineage 1 were less sensitive to azoxystrobin than isolates of lineage 2, both in terms of conidial germination and mycelial growth. All isolates from spinach crops in southern states collected in 2016-19 were in lineage 1. This might account for the poor control of Stemphylium leaf spot with azoxystrobin reported by spinach growers in Texas and Florida.

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