Five *Stemphylium* spp. Pathogenic to Alfalfa: Occurrence in the United States and Time Requirements for Ascospore Production

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ABSTRACT

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Stemphylium isolates from alfalfa from 10 states in the U.S. were identified according to new taxonomic criteria. Seventeen of the 22 isolates were *S. alfalfae*/*Pleospora alfalfae* from California, Washington, Idaho, Utah, Kansas, Wisconsin, and New York. The other five isolates were *S. botryosum*/*P. tarda* from Minnesota, Wisconsin, Pennsylvania, and New York. All isolates produced similar symptoms on excised alfalfa leaves in tests to confirm pathogenicity. The time required for ascospore production, which has become more important in recent taxonomic revisions of *Stemphylium*/*Pleospora*, was determined under standardized conditions for isolates of five *Stemphylium* spp. obtained from E. G. Simmons. Culture plates of 0.1 strength potato-dextrose agar were seeded with conidia from 14-day-old cultures, sealed with Parafilm, and incubated under an 8-h photoperiod of 40–65 $\mu E \cdot m^{-2} \cdot s^{-1}$ of cool-white fluorescent lighting at 15±2 C. The numbers of days after dishes were seeded with conidia until mature ascospores were produced were: 12.7±3.3 for *S. alfalfae*/*P. alfalfae*, 26.4±3.3 for *S. globuliferum*/*Pleospora* sp., 27.3±2.8 for *S. vesicarium*/*Pleospora* sp., 28.9±2.5 for *S. herbarum*/*P. herbarum*, and 62.6±2.1 for *S. botryosum*/*P. tarda*. The time requirements for ascospore production determined under these standardized conditions complemented morphological taxonomic features in distinguishing some of the *Pleospora* pathogens of alfalfa, especially *P. alfalfae* and *P. tarda*, the two most common species in the U.S. These conditions also reduced ascospore production time for *P. tarda* to 2 mo.

Stemphylium leaf spot of alfalfa (*Medicago sativa* L.) occurs in humid climates wherever the crop is grown (5). In the U.S., the disease traditionally has been attributed primarily to *Stemphylium botryosum* Wallr. and its teleomorph, *Pleospora herbarum* (Pers.:Fr.) Rabenh. (18). In 1981, Cowling et al (3) described cool-temperature and warm-temperature biotypes of *S. botryosum* from California and eastern North America, respectively. The cool-temperature biotype produced bleached elongate lesions restricted by dark, narrow, sharply defined borders, whereas warm-temperature biotype isolates produced concentrically ringed, spreading, dark lesions as previously described by Smith (18). The biotypes also differed in growth characteristics on artificial media and in temperature and time requirements for ascospore production (3).

In 1984, Irwin (6) reported a new *Stemphylium* sp. that was widespread on alfalfa in Queensland, Australia, and that produced symptoms similar to those caused in California by the cool-temperature biotype of *S. botryosum*. However, the anamorph more closely resembled *S. vesicarium* than *S. botryosum* but had characteristics not common to either species. In 1985, Irwin et al (7) concluded that the disease in Australia was caused by the cool-temperature biotype reported by Cowling et al in California (3). In 1986, Irwin and coworkers (8) broadened Simmons's circumscription of *S. vesicarium* (16) to include the taxon from alfalfa in Australia (6) and, consequently, the cool-temperature biotype from California (3).

In 1985, Simmons (17) reported that *S. botryosum* and *P. herbarum* were not components of the same holomorph. He assigned *P. tarda* as the teleomorph of *S. botryosum* and *S. herbarum* as the anamorph of *P. herbarum*. Simmons also established a new holomorph, *S. alfalfae*/*P. alfalfae*, based on an isolate from Western Australia (17). These species, along with *S. globuliferum* and a member of the *S. vesicarium* complex pathogenic to a *Medicago* sp., were compared by Simmons in 1990 (5).

The reported time required for ascospore production varies greatly between and within *Stemphylium* spp. Simmons (17) included ascocarp maturation time as a factor in his recent description of *Pleospora* species. For example, Simmons's description of *P. alfalfae* (17) includes a time requirement of 17–25 days for ascospore production. Also, his description of *P. tarda* (17), as reflected by its specific epithet, includes an unusually long time requirement of about 8 mo for ascospore production. Cowling et al (3) reported mature ascospores by 8 wk at 18 C for the cool-temperature biotype of *S. botryosum*; the warm-temperature biotype required 8 wk at 18 C plus an additional 12 wk at 3 C, and then only 5% of the pseudothecia produced ascospores.

Leach (10–12) and Leach and Trione (13) demonstrated the effects of radiation (especially near UV light), temperature, and their interrelationships on conidial and sexual development of *S. botryosum*/*P. herbarum*. Most hosts of the species that they studied were grasses, although Leach (12) indicated that isolate host source did not appear to be a factor. Leach (11) reported that proto-pseudothecia were initiated by exposing growing colonies to near-UV radiation of less than 360 nm wavelength (290 nm was most effective). Subsequent maturation of pseudothecia was triggered by low temperature (5–10 C) and was hastened only slightly by light (12).

Because of the importance of time required for ascospore production in the identification of *Pleospora* spp. (17) and the sensitivity of *Pleospora* to radiation and temperature fluctuations and interactions (12), uniform environmental conditions are needed for comparing these species from alfalfa.

This study was initiated to determine which *Stemphylium*/*Pleospora* spp. occur on alfalfa in the U.S., based on Simmons's current taxonomic criteria, and to determine times required for ascospore production under standardized conditions by five *Stemphylium*/*Pleospora* spp. pathogenic to alfalfa.

MATERIALS AND METHODS

***Stemphylium* distribution.** Sources of *Stemphylium* isolates from alfalfa from the United States are given in Table 1. Isolates ID-H, WA-T, and UT (Jensen) were isolated from cool-temperature type lesions (3) from leaves collected 18–23 August 1990. Isolate MT SH2 SS#1 from D. G. Gilchrist also originated from a cool-temperature type lesion. However, the type of lesions from which the others

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were isolated is not known. Kansas isolates KS1 and KS8 (Table 1) were from cultures in stock and designated only as being from alfalfa. The other two isolates from Kansas were obtained in the spring of 1993 from nondistinct necrotic lesions that included other pathogens. A monoconidial isolate from each source was identified according to criteria of Simmons (5,17) and preserved in 15% glycerol at −80 C.

Pathogenicity of isolates was determined by their ability to produce lesions on excised alfalfa leaves using a modification of the methods described by Borges et al (1). Clones of cvs. CUF 101, Du Puits, Kanza, and Lahonton alfalfa plants selected for susceptibility to *S. alfalfae* were used. Clones were increased by rooting shoot cuttings in sand. The younger fully expanded leaves were inoculated by placing on each leaflet 1–4 2-μL drops of 0.05% water agar (to aid adhesion to leaflet), each containing 30–50 conidia. The point of inoculation on the leaflet was wounded slightly by pressing the edge of the micropipette tip against the leaflet at the time of inoculation. Inoculated leaves were floated on water in covered, plastic, petri dishes and placed at 20 C in a growth chamber. The dishes were kept in the dark for 48 h and then given a 12-h photoperiod of 81–88 $\mu E \cdot m^{-2} \cdot s^{-1}$ of cool-white fluorescent lighting. Seven to 10 days after inoculation, leaves were examined for disease development to confirm isolate pathogenicity.

Ascospore maturation. Isolates used to determine time requirements for ascospore production were provided by E. G. Simmons. These included type species isolates EGS 36-088 of *S. alfalfae*/*P. alfalfae*, EGS 04-118C of *S. botryosum*/*P. tarda*, and EGS 36-138.2 of *S. herbarum*/*P. herbarum* (17). Simmons also provided isolates 36-101 of *S. globuliferum*/*Pleospora* sp. and 37-065 of *S. vesicarium*/*Pleospora* sp.

The procedure chosen for determining ascospore maturation times for the five species is as follows: 1 ml of 0.5% Tween 20 containing $\approx 10^6$ conidia scraped from dishes of 14-day-old cultures was spread over the surface of a 9-cm-diameter, plastic, petri dish containing 25 ml of 0.1 strength potato-dextrose agar (Difco, 2% agar). Three sterile alfalfa stem pieces were placed on the agar surface of each dish. Stem pieces had no noticeable effect on the time required for ascospore production, but the population density of pseudothecia usually was greatest on and near the stem pieces. Also, pseudothecia on the stem pieces were more accessible for observation than those on or submerged in the agar. The stem pieces were from shoots collected in the early flowering stage in the greenhouse and were prepared by removing the leaves, washing in tap water, cutting stems to lengths of 2–3 cm, drying overnight at 50 C in an oven, and sterilizing with propylene oxide (4). Dishes were sealed with Parafilm and placed in a growth chamber at 15±2 C and an 8-h photoperiod of 40–65 $\mu E \cdot m^{-2} \cdot s^{-1}$ of cool-white fluorescent lighting.

In preliminary experiments using the same procedures but 25 C instead of 15 C, protopseudothecia production was greater at 25 C. However, none of the cultures produced ascospores at 25 C, even though the pseudothecia formed beaks. At 15 C, pseudothecia formed beaks only a few days before ascospore maturation. Thus, dishes were scanned under a stereomicroscope at 2-day intervals for pseudothecium beak formation. Cultures having pseudothecia with beaks then were examined daily for mature ascospores; four pseudothecia with beaks from each dish were crushed and examined with a compound light microscope. Ascospores were considered mature when they developed a yellowish-brown color, which occurred 2–4 days before they were discharged.

All experiments included at least four replications, and the mean numbers of days from seeding dishes with conidia until mature ascospores were observed were analyzed using the General Linear Models Procedure of SAS (14). Least square means were tested for significant differences.

RESULTS

***Stemphylium* distribution.** Seventeen of the 22 *Stemphylium* isolates from alfalfa were identified as *S. alfalfae* and the other five as *S. botryosum* (Table 1). *Stemphylium alfalfae* occurred in seven states across the U.S., whereas the *S. botryosum* isolates were from four states in the northeastern quarter. Isolates ID-H, WA-T, and UT (Jensen) from cool-temperature lesions (3,5) were *S. alfalfae* (Table 1). Cool-temperature lesions (3,5) developed on cv. Riley alfalfa plants in a plot at Manhattan, KS, after it was sprayed with a conidial suspension of isolate KS1 of *S. alfalfae* (Table 1). *Stemphylium alfalfae* was reisolated from these lesions.

All of the *Stemphylium* isolates recovered from alfalfa were pathogenic on excised leaves of alfalfa. Symptoms produced by isolates of *S. alfalfae* and *S. botryosum* were indistinguishable under the conditions used; both produced the warm-temperature type, concentric rings. Isolates of the five *Stemphylium* spp. provided by E. G. Simmons produced similar symptoms. However, the isolate of *S. vesicarium* was weakly pathogenic.

Ascospore maturation time. Mean days from seeding plates with conidia to mature ascospore formation and standard errors were: 12.7±3.3 for *S. alfalfae*, 26.4±3.3 for *S. globuliferum*, 27.3±2.8 for *S. vesicarium*, 28.9±2.5 for *S. herbarum*, and 62.6±2.1 for *S. botryosum*. These data are illustrated for selected isolates in Fig. 1. Least square means analysis indicated that the maturation time of ascospores of *S. alfalfae*/*P. alfalfae* was significantly ($P < 0.01$) shorter than that of all other species, and *S. botryosum*/*P. tarda* had a significantly ($P < 0.01$) longer maturation time than all other species. No significant differences were found among the maturation times of *S. herbarum*, *S. globuliferum*, and *S. vesicarium*.

The time required for ascospore maturation by additional isolates was determined. These included all *S. alfalfae* isolates in Table 1 and isolate 36-083 of *S. alfalfae* from E. G. Simmons (2); four isolates of *S. botryosum* (2E, ST-MN, ST-90-2 [Table 1] and isolate 08-069 from E. G. Simmons) (2); and isolate 30-181 of *S. herbarum* from E. G. Simmons

Table 1. Identification and origin of *Stemphylium* isolates from alfalfa in the United States

Contributor's code	Anamorph	Geographic origin	Contributors
AT (Altoona)	*S. alfalfae*	Kansas	D. L. Stuteville
BF	*S. alfalfae*	California	S. L. Nygaard
BF-83	*S. alfalfae*	California	S. L. Nygaard
CA-1	*S. alfalfae*	California	Pioneer Hi-Bred Int.
CA-4	*S. alfalfae*	California	Pioneer Hi-Bred Int.
2E	*S. alfalfae*	Pennsylvania	Pioneer Hi-Bred Int.
08-069S	*S. botryosum*	New Hampshire	E. G. Simmons
ID-H	*S. alfalfae*	Idaho	J. I. Edmunds
KS1	*S. alfalfae*	Kansas	D. L. Stuteville
KS8	*S. alfalfae*	Kansas	D. L. Stuteville
MT SH2 SS#1	*S. alfalfae*	California	D. G. Gilchrist
NY	*S. alfalfae*	New York	Pioneer Hi-Bred Int.
RP (Republic Co.)	*S. alfalfae*	Kansas	J. A. Appel
S-CA (Chino-1)	*S. alfalfae*	California	C. Chaisrisook
S-CA (Chino-2)	*S. alfalfae*	California	C. Chaisrisook
ST-90-2	*S. botryosum*	Wisconsin	S. L. Nygaard
ST-MN	*S. botryosum*	Minnesota	J. E. Tofte
WA (Pasco)	*S. alfalfae*	Washington	C. Chaisrisook
WA-T	*S. alfalfae*	Washington	J. I. Edmunds
WI (Arlington)	*S. alfalfae*	Wisconsin	C. Chaisrisook
UT (Jensen)	*S. alfalfae*	Utah	C. Chaisrisook
914	*S. botryosum*	Pennsylvania	K. T. Leath

(2). Time requirements of all of these isolates to produce ascospores were consistent with the times indicated in Fig. 1.

DISCUSSION

Based on Simmons's current taxonomic criteria (5,17), which were supported by random amplified polymorphic DNA analysis (2), *Stemphylium alfalfae* and *S. botryosum* accounted for all 22 *Stemphylium* isolates from alfalfa from 10 states in the United States (Table 1). Simmons (17) indicated that *S. herbarum,* although not abundant, was isolated from alfalfa from New Hampshire.

According to Simmons's current descriptions (5,17), *S. alfalfae*/*P. alfalfae* likely would include the cool-temperature (California) biotype of *S. botryosum*/*P. herbarum* described by Cowling et al (3), and *S. botryosum*/*P. tarda* would include the warm-temperature (eastern) biotype of *S. botryosum*/*P. herbarum* (3). The smaller ascocarp, shorter time required for ascospore production, and faster growth rate on agar with darker concentric rings (due to greater conidial density) all separate *S. alfalfae*/*P. alfalfae* from *S. botryosum*/*P. tarda. S. herbarum* occurs in the eastern U.S. (17) but is eliminated as the warm-temperature biotype (3) because of its shorter ascospore maturation time requirement, smaller ascocarp, and other morphological differences.

The times required for ascospore production by cultures of type species of *P. alfalfae* and *P. herbarum* (Fig. 1) were very similar to the times reported by Simmons (17) for those cultures. Simmons (17) reported that mature ascospores of *P. alfalfae* developed within 17 days, compared with 13 days in the present study (Fig. 1), and mature ascospores of *P. herbarum* developed within 25–30 days (17), compared with 29 days (Fig. 1). However, Simmons's description of *P. tarda* type species culture 04-118C includes 8 mo for ascospore production (in a refrigerator) (17) compared with only 2 mo under the conditions used here (Fig. 1).

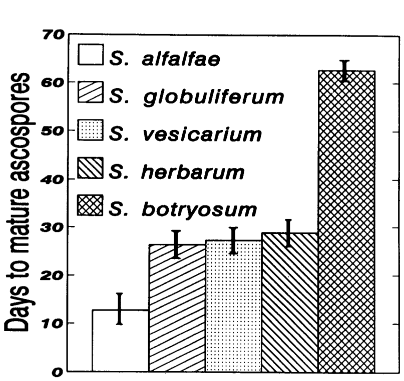

Fig. 1. Time required for ascospore development after seeding *Stemphylium* conidia onto plates of 0.1 strength potato-dextrose agar (Difco) and placing at 15±2 C under an 8-h photoperiod of 40–65 $\mu E \cdot m^{-2} \cdot s^{-1}$ of cool-white fluorescent lighting. Isolates provided by E. G. Simmons were: 36-088, *Stemphylium alfalfae*/*Pleospora alfalfae*; 36-101, *S. globuliferum*/*Pleospora* sp.; 37-065, *S. vesicarium*/*Pleospora* sp.; 36-138.2, *S. herbarum*/*P. herbarum*; and 04-118C, *S. botryosum*/*P. tarda*. Vertical bars represent standard errors of the mean.

Simmons noted that cultures of *S. globuliferum* produced ascospores within 3 mo at moderate temperatures and within 10 mo at 5 C (5), whereas we observed mature ascospores within 1 mo (Fig. 1).

Simmons reported ascospore production in *S. vesicarium* cultures in 3–6 mo at refrigerator temperatures, Lamprecht et al (9) reported them within 6 wk, and we observed them in 1 mo (Fig. 1).

The time requirement for ascospore production (Fig. 1) provides a complement to taxonomy that reduces reliance on the dimensions of morphological features, which are influenced by nutrition and environmental conditions and vary between host and artificial media (3). Also, the identification of *S. alfalfae* is complicated by the production of two general conidial types, cylindrical and ovoid (5,17), but under proper conditions is benefited by the production of ascospores within 2 wk.

The usefulness of the time requirement for ascospore production as a taxonomic aid would be enhanced greatly by a standard test that clearly defines light quality and intensity, photoperiod, and temperature. Our standardized procedure clearly separates *P. alfalfae, P. herbarum,* and *P. tarda* and provides ascospores of *P. tarda* ≈6 mo earlier than at refrigerator temperatures (17).

The association of specific *Stemphylium* leaf spot symptoms with specific *Stemphylium* spp. and geographic locations is not clear. Cowling et al (3) found that the cool-temperature biotype and the warm-temperature biotype of *S. botryosum,* which according to Simmons's changes (5,17) likely would be classified as *S. alfalfae* and *S. botryosum,* respectively, produced distinctly different symptoms. The cool-temperature biotype occurred in California in cool moist periods, especially early spring, whereas the warm-temperature types occurred in eastern North America during wet warm periods in the fall. *Stemphylium alfalfae* isolates ID-H, WA-T, and UT (Jensen) (Table 1) were isolated from cool-temperature bordered lesions with light centers from alfalfa collected in August 1990. In October 1992, alfalfa plants at Manhattan, KS, with the bordered, cool-temperature type lesions yielded *S. alfalfae.* D. L. Stuteville (*unpublished*) has observed warm-temperature symptoms in Kansas during rainy periods in late summer and early fall. However, we were unable to find these symptoms in Kansas fields during 1992 or 1993.

Lamprecht et al (9) and Thompson (19) associated environmental differences with differences in leaf spot symptoms on alfalfa caused by *S. vesicarium* in South Africa. Cool-temperature lesions, as reported by Cowling et al (3), were found in the cooler section of the south-western Cape, whereas warm-temperature type lesions (3) occurred on alfalfa under overhead irrigation in the warmer areas of the central Transvaal (9).

The recent changes in the taxonomy of *Stemphylium* pathogens of alfalfa make it difficult to compare the results of earlier reports (5,15). For example, Simmons (5) indicated that by current taxonomic criteria, Smith (18) was working with *S. botryosum*/*P. tarda* from sweet clover, *S. herbarum*/*P. herbarum* from red clover, and a third species from alfalfa, rather than *S. botryosum* on all three crops, as Smith reported. In describing the *S. vesicarium* species complex, Simmons (5) noted that mature ascospores he had examined from South Africa and Australia more closely resembled those of *P. herbarum* than those of the *S. vesicarium* teleomorph. Simmons (5) also predicted that additional *Stemphylium* spp. will be described as pathogens of alfalfa.

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