Stem and Crown Rot of Chickpea in California Caused by *Sclerotinia trifoliorum*

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ABSTRACT

Njambere, E. N., Chen, W., Frate, C., Wu, B.-M., Temple, S. R., and Muehlbauer, F. J. 2008. Stem and crown rot of chickpea in California caused by *Sclerotinia trifoliorum*. Plant Dis. 92:917-922.

The identities of *Sclerotinia* isolates obtained from chickpea plants showing stem and crown rot were determined using morphological characteristics, variations in group I introns, and internal transcribed spacer (ITS) sequences. Isolates could be separated into two groups based on growth rates at 22°C, fast growing (about 40 mm per day) versus slow growing (about 20 mm per day). All fast-growing isolates induced stronger color change of a pH-indicating medium than did slow-growing isolates at 22°C. The slow-growing isolates contained at least one group I intron in the nuclear small subunit rDNA, whereas all fast-growing isolates lacked group I introns in the same DNA region. ITS sequences of the slow-growing isolates were identical to sequences of *Sclerotinia trifoliorum*. Those of the fast-growing isolates were identical to sequences of *S. sclerotiorum*. Finally, the slow-growing isolates showed ascospore dimorphism, a definitive character of *S. trifoliorum*, whereas the fast-growing isolates showed no ascospore dimorphism. Isolates of both species were pathogenic on chickpea and caused symptoms similar to those observed in the field. This study not only associated the differences between *S. sclerotiorum* and *S. trifoliorum* in growth rates, group I introns, ITS sequences, and ascospore morphology, but also represented the first report that *S. trifoliorum* causes stem and crown rot of chickpea in North America.

Additional keywords: apothecia, carpogenic germination, sclerotia

Sclerotinia spp. are destructive and cosmopolitan plant pathogens that cause stem and crown rot on various agronomic and horticultural crops and wild species. There are three economically important species of *Sclerotinia*. Of the three species, *Sclerotinia sclerotiorum* (Lib.) de Bary is the most ubiquitous, with a host range of over 400 plant species (2). *S. minor* Jagger causes diseases on a range of crops in at least 53 plant genera, and causes significant economic losses in peanut, sunflower, and lettuce, whereas *S. trifoliorum* Erikss. is reported to cause diseases in 21 genera, with major losses occurring mainly in legumes, particularly forage legumes like *Medicago* and *Trifolium* spp. (16). Although the three species all are reported to cause stem rot of chickpea in different parts of the world, the most commonly reported species is *S. sclerotiorum*. The

disease has been recorded in Australia (3,11), Canada (14), Chile, India, Iran, Morocco, Syria (13), the United States (7,18), and other countries (2,13). In the United States, *S. sclerotiorum* has been reported on chickpea in Arizona (18), California (4), North Dakota, and Washington (7). In addition to *S. sclerotiorum*, *S. minor* has been reported in chickpea fields in Arizona (18) and in Queensland, Australia (11). However, *S. trifoliorum* has not been reported infecting chickpea outside Australia, where it was reported to occur together with *S. sclerotiorum* (3).

Differentiation of the three *Sclerotinia* species is based on several criteria (16). *S. minor* can be relatively easily differentiated from the other two species based on its numerous, scattered small-sized sclerotia in culture and in the field. However, unequivocal differentiation between *S. sclerotiorum* and *S. trifoliorum* requires observation of ascospore morphology. *S. trifoliorum* shows dimorphism in ascospore size (two different-sized ascospores within a single ascus), whereas ascospores of *S. sclerotiorum* show no dimorphism (16). Induction of carpogenic germination of sclerotia of *Sclerotinia* spp. is a time-consuming process, and may take up to several months. To further complicate the matter, some isolates of *S. trifoliorum* are

heterothallic and require mating with a compatible strain for carpogenic germination and ascospore production (25,26). To facilitate identification of *S. trifoliorum* and differentiation from *S. sclerotiorum*, efforts have been made in searching for reliable and easily applied techniques. Restriction fragment length polymorphism was used to separate the three species of *Sclerotinia* (17). Tariq et al. (24) described differences in growth rates between *S. sclerotiorum* and *S. trifoliorum*. *S. sclerotiorum* grows faster than *S. trifoliorum* at 25°C. Powers et al. (21) reported that *S. trifoliorum* contains group I introns in the nuclear small subunit ribosomal (r)DNA, and separated isolates of *S. trifoliorum* into five subgroups based on number and position of introns, whereas *S. sclerotiorum* and *S. minor* do not contain any introns in the same DNA region. Sequences of the internal transcribed spacer (ITS) region of the nuclear rDNA show two nucleotide differences between *S. sclerotiorum* and *S. trifoliorum* (15). These studies documenting the different features between *S. sclerotiorum* and *S. trifoliorum* were performed on different sets of isolates. There is a need to relate these features on the same set of isolates.

In 2005 and 2006, widespread occurrence of stem and crown rot of chickpea was observed in the Central Valley and the Sacramento Valley of California. Disease symptoms in the field clearly exhibited two patterns of infection: infection of the stem near or below the soil line (crown infection) or infection of the stem that clearly started at least six inches above the soil line (stem infection). Most of the crown-infected plants initially were wilted, with occasional yellowing and subsequent death. Some plants showed white mycelial growth from around the infected stem on the soil surface (Fig. 1A). When the dying or dead plants were dug up, black, irregularly shaped sclerotia usually were visible on the stem or in soil adjacent to the plant (Fig. 1B). On plants showing stem infection, symptoms mainly included discoloration of stems followed by girdling of the entire stem as the lesion expanded, wilting above the lesion, and, finally, stem breakage. Mycelial growth was not always visible on the stem. Sclerotia usually were not found in plants with stem infections. The two symptom

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Accepted for publication 4 February 2008.

doi:10.1094/PDIS-92-6-0917

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types were characteristic of the previously reported pathogen *S. sclerotiorum*. It soon became apparent that the isolates from central California were very diverse, had different growth rates, and could be different species (20). The objective of this study was to determine the species identities of the causal agents, and to relate the three previously described features (growth rate, nuclear rDNA introns, and ITS sequence) differentiating be-

tween *S. sclerotiorum* and *S. trifoliorum* using the same set of isolates.

MATERIALS AND METHODS

Collection and isolation of the causal agent. Sclerotia were collected from stems of infected chickpea plants from various locations in central California (Table 1). Where sclerotia were absent, segments (5 to 10 cm) of diseased stems were collected and brought to the laboratory for isolation.

Ten isolates were obtained and used in this study. Four additional previously identified isolates of *S. sclerotiorum* from lentil (*Lens culinaris* Medik.) and pea (*Pisum sativum* L.) and *S. trifoliorum* from alfalfa also were included in the study for comparison (Table 1). For isolation, sclerotia or diseased stems were surface sterilized in 70% ethanol and then 3% sodium hypochlorite from commercial bleach for 1 min each. They then were rinsed in sterile

Fig. 1. Field symptoms of crown rot. **A**, White mycelium growth from diseased plants and **B**, black irregularly shaped sclerotia. **C**, Artificial inoculation in greenhouse produced similar symptoms.

Table 1. Isolates of *Sclerotinia* spp. used in the study along with their host origin, geographic location, and their cultural, molecular, and morphological characterizations

Species, isolate	Host	Location	Year of isolation	rDNA introns ^a	ITS haplotype		Apothecium production ^c	Ascospore dimorphism ^d	Growth rate (mm/day) ^e	pH medium color change
					SNP at position 120 ^b	SNP at position 376 ^b				
<i>Sclerotinia trifoliorum</i>										
05WM6 ^{f,g}	Chickpea	Robbins, CA	2005	Yes (2)	G	C	Yes	Yes	21.0	None
05WM8 ^g	Chickpea	Colusa, CA	2005	Yes (2)	G	C	Yes	Yes	18.7	Slight
05WM21 ^g	Chickpea	Five Points, CA	2005	Yes (2)	G	C	No	Nt	19.3	Slight
05WM42	Chickpea	Five Points, CA	2005	Yes (2)	Nt	Nt	Yes	Yes	21.3	Slight
06CWM-A8	Chickpea	Farmington, CA	2006	Yes (2)	Nt	Nt	Yes	Yes	22.0	Slight
06CWM-F2	Chickpea	Knights Landing, CA	2006	Yes (1)	Nt	Nt	No	Nt	12.7	None
06CWM-G3 ^f	Chickpea	Five Points, CA	2006	Yes (2)	Nt	Nt	Yes	Yes	21.0	Slight
06CWM-H6 ^f	Chickpea	Five Points, CA	2006	Yes (2)	Nt	Nt	Yes	Yes	20.5	Slight
05WMAL-1a ^g	Alfalfa	Visalia, CA	2005	Yes (2)	G	C	Nt	Nt	25.1	Slight
TP0506 ^g	Alfalfa	Prosser, WA	2006	Yes (2)	G	C	Nt	Nt	19.3	Slight
<i>S. sclerotiorum</i>										
05LWM-A5 ^g	Lentil	Colton, WA	2005	No (0)	T	T	Yes	No	46.2	Intense
05WM22 ^{f,g}	Chickpea	Five Points, CA	2005	No (0)	T	T	Yes	No	43.2	Intense
05WM33 ^{f,g}	Chickpea	Five Points, CA	2005	No (0)	T	T	Yes	No	38.7	Intense
WM-A1 ^g	Pea	Central WA	2002	No (0)	T	T	Yes	No	39.8	Intense
LSD _{0.05} ^h	...	...	...	...	...	...	...	...	1.6	...

^a Detection of introns in the nuclear small subunit ribosomal DNA (nss rDNA) with polymerase chain reaction (PCR). Yes: introns were detected; No: no introns were detected; number of introns detected in parentheses.

^b The single nucleotide polymorphism (SNP) position in the amplicon produced by PCR with the primer pair ITS1/ITS4. C: cytosine; G: guanine; T: thymine; and Nt: not tested.

^c Production of apothecia from sclerotia was induced either with conditioning in aerated water at 4°C or without conditioning; Nt: not tested.

^d Presence of ascospore dimorphism; Nt: not tested.

^e Colony growth rate in mm/day on potato dextrose agar at 22°C measured after inoculation with a 7-mm-diameter colonized agar disc.

^f Isolate selected for pathogenicity test in the greenhouse.

^g Isolate from which the ITS regions was sequenced.

^h LSD: least significant difference ($P < 0.05$).

distilled water and blotted dry in sterilized paper towels. Stems were aseptically cut into 1-cm pieces, then plated onto potato dextrose agar (PDA; Difco Laboratories, Detroit) containing 0.1% lactic acid (added after cooling). Sclerotia were cut in halves before plating. Plates then were incubated at room temperature (22°C) in the dark. Isolates were purified by hyphal tip isolation and placed onto new PDA plates. Only one isolate was obtained from each plant. Sclerotia were harvested from PDA plates, air dried, and stored in glass vials at -20°C for future use.

Morphological characterization. *Colony diameter and acid production.* To test for colony growth rates, a 7-mm-diameter disc was cut from the edge of a 3-day-old actively growing colony from each of the 13 isolates. The disc was transferred to the center of a 9-cm-diameter petri dish containing PDA. Plates were incubated at room temperature (22°C) in the dark. Colony diameters were measured every 12 h up to 84 h or until colonies covered the whole plates. Colony diameter was calculated from two measurements taken at right angles to each other. This study was arranged in a completely random design with three replicates (three plates per isolate) and was conducted two times. To test for possible production of oxalic acid, isolates were cultured on PDA plates containing the pH indicator bromophenol blue (50 mg/liter) modified by excluding pentachloronitrobenzene and antibiotics from the semiselective medium of Steadman et al. (23) in a manner similar to that described above. The intensity of color change of the pH indicator medium (none, slight, or intense) was recorded after 36 and 72 h of incubation at 22°C. Colony diameters also were measured every 12 h as mentioned above. Because there were no differences in growth rates between PDA and the pH-indicating medium, the data were combined for analyses. The growth rate of each isolate was determined from average of 12 plates per experiment.

Carpogenic germination. In order to observe ascospore morphology, carpogenic germination of sclerotia was induced in the laboratory. Two methods were used: the method with preconditioning developed for *S. sclerotiorum* (8) and the method without preconditioning developed for *S. trifoliorum* (22). Sclerotia were conditioned in aerated cold (4°C) water as described by Cobb and Dillard (8). The conditioning was done for 4, 6, 8, and 10 weeks to determine the optimum conditioning time. Conditioned sclerotia then were removed from water, air dried overnight, and subdivided for repeat experiments. Sclerotia not used immediately were stored in a refrigerator (4°C) in glass petri dishes. Subsequent production of apothecia and ascospores was done according to Cobb and Dillard (8). Conditioned apothecia were embedded on the

surface of autoclaved sand (approximately 45 cm³) in 9-cm-diameter glass petri plates (6 to 20 sclerotia per plate) and incubated at 20°C with 12 h of cool white fluorescent lights (100 to 132 microeinsteins m⁻² s⁻¹). Observations were made daily and additional sterile distilled water was added when necessary to maintain moisture. The time in days required for the first appearance of apothecia was noted before examining the apothecia and ascospores under a light microscope. In the method without preconditioning (22), sclerotia were placed directly in clean autoclaved sand as described above except that the plates were incubated at 15°C and light intensity was increased to about 200 to 300 microeinsteins m⁻² s⁻¹. Each treatment had two replicate plates, each containing 6 to 20 sclerotia. The preconditioning of sclerotia was done once and the experiment of carpogenic germination was performed twice.

Molecular characterization. *Group I introns.* To determine the presence of group I introns in the nuclear small subunit rDNA, total genomic DNA was extracted from sclerotia of the 14 isolates (Table 1) using the FastDNA kit as described by Chen et al. (6). Sclerotia were cut with razor blades into small pieces (2-mm slices) and placed into 2-ml centrifuge tubes containing a ceramic bead and garnet, following the manufacturer's instructions. DNA finally was eluted into 50 µl of water, and DNA quality initially was checked by agarose gel electrophoresis. DNA was diluted 100 times before being used in polymerase chain reaction (PCR). PCR amplifications were conducted using the primer pairs ITS1/ITS4 and ITS5/ITS4, described by White et al. (27), for the ITS regions containing the 5.8S rDNA. Various combinations of the rDNA primers

(NS3/NS6, NS5/NS6, and NS3/NS8) were used to amplify different regions of the small subunit rDNA. The reaction conditions were hot started for 10 min at 92°C prior to 35 amplification cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, and were followed with a final 10-min extension at 72°C. PCR products were separated and product sizes were estimated by 1% agarose gel electrophoresis along with standard DNA size markers to confirm the presence of introns in the amplified DNA regions. Gels were stained with ethidium bromide and then visualized and photographed under UV light.

Polymorphism in ITS sequences. The PCR products amplified with primer pair ITS1 and ITS4 were purified for direct DNA sequencing. Nucleotide sequences were determined from both strands using ABI PRISM 377 automatic sequencer (Applied Biosystems) at the Sequencing Core Facility of Washington State University. Sequence comparisons were carried out using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>).

Pathogenicity test. Five isolates, selected to represent the two different growth rates and acid production levels, were used in greenhouse pathogenicity assays (Table 1). This consisted of three isolates of *S. trifoliorum* and two of *S. sclerotiorum*. Inoculations were done on 3-week-old seedlings of chickpea cvs. Spanish White and Dwelley. A petiole inoculation method developed for soybean (10) was modified to inoculate a stem branch instead of a petiole. A branch of a stem just below the most newly expanded leaf node was cut at 3 cm from the main stem, and a 200-µl pipette tip bearing an agar plug on the wider end from the edge of actively growing colony was inserted over the cut through the agar plug. Control plants were

Fig. 2. Ascospore size dimorphisms and arrangement within the ascus. **A** and **B**, Isolates of *Sclerotinia trifoliorum* showing ascospore dimorphism of 4:4 and 2:2:2:2 tetrad arrangement respectively; and **C**, an isolate of *S. sclerotiorum* showing no ascospore size dimorphism.

inoculated with a plain agar block. Disease development was observed daily and disease severity recorded based on a 0-to-4 scoring system, where 0 = no disease, 1 = inoculated branch dried, 2 = up or downward movement of the disease from inoculation site on the main stem, 3 = both up and downward movement of the disease, and 4 = plant dead. The diseased plants were collected and used to isolate the

pathogen at the conclusion of the experiments. The experimental design was a randomized complete blocks with three replications (pots) consisting of three plants per pot. An observatory trial also was done whereby 4- to 6-week-old seedling were similarly inoculated and given the same treatment as the three old seedlings.

Statistical analysis. Analysis of variance with SAS (version 9.1) statistical

software was performed on data from the growth rate experiments and pathogenicity assays. Fisher's protected least significant difference ($P < 0.05$) was used to compare means of treatments.

RESULTS

Morphological characterization. All 14 isolates were confirmed to have cultural features similar to those of *Sclerotinia* spp.: white, dense mycelial growth, no conidial formation, and abundant black sclerotia of various sizes and shapes formed in a ring pattern. Isolates generally exhibited two growth rates, fast (approximately 40 mm diameter per day) and slow (approximately 20 mm diameter per day) (Table 1). Fast-growing isolates covered the entire agar surface within 48 h, whereas slow-growing isolates took more than 60 h to cover the plates. Of the 14 isolates tested, 10 were considered slow growing while the remaining 4 were considered to be fast growing (Table 1). The four fast-growing isolates induced strong color change from blue to bright yellow of the pH-indicating medium at 22°C, whereas slow-growing isolates induced no color change or very faint color change of the pH-indicating medium under the same temperature condition (Table 1).

Apothecium and ascospore production. Sclerotia from the four fast-growing isolates produced apothecia at 20°C after conditioning in water at 4°C. The time required for apothecium production decreased with increase of conditioning time period. The sclerotia took 7 days to start producing apothecia after 10 weeks of conditioning, 9 days after 8 weeks of conditioning, 14 days after 6 weeks of conditioning, and 18 days after 4 weeks of conditioning. No apothecia were formed within this time period by the slow-growing isolates. It was not until after almost 3 months that one slow-growing isolate (05WM6) conditioned for 10 weeks started to produce apothecia. However, most of the sclerotia of the other slow-growing isolates had rotted by that time. For those sclerotia incubated at 15°C without conditioning, two isolates from the fast-growing and two isolates from the slow-growing started forming apothecia within 4 weeks of incubation. It took isolates 05WM8 and 06CWM-A8 25 days to start forming apothecia, whereas the remaining isolates, except isolate 06CWM-F2, started germinating carpogenically within a period of 2 months. Similar trends were observed in the repeat experiments. The ascospore arrangement within the asci was observed under a light microscope for both the slow- and fast-growing isolates. Ascospore dimorphism was observed consistently among the slow-growing isolates (typical of *S. trifoliorum*) but was not observed in fast-growing isolates. Slow-growing isolates exhibited four large and four small ascospores within each ascus

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction products amplified with **A**, primers ITS1 and ITS4; **B**, primers ITS4 and ITS5; and **C**, primers NS5 and NS6. Larger product sizes in **B** and **C** indicate presence of introns. Lane L: DNA ladder; lanes 1, 4, 5, and 7: *Sclerotinia sclerotiorum* isolates 05LWM-A5, 05WM22, 05WM33, and WM-A1, respectively; lanes 2, 3, 6, 8, and 9: *S. trifoliorum* isolates 05WM6, 05WM21, 05WMAL-1a, TP-0506, and 05WM8, respectively; lane N: negative control.

(Fig. 2A and B), whereas the fast-growing isolates had all eight ascospores of the same size, as was expected for *S. sclerotiorum* (Fig. 2C). The arrangement of the large- and small-size ascospores varied, with the majority exhibiting a 4:4 tetrad arrangement. Ascospore size was in the range of 9.5 to 13.2 by 4.0 to 7.0 μm for *S. sclerotiorum* whereas, for *S. trifoliorum*, it varied from 13.3 to 19.7 by 7.7 to 8.8 μm for large ascospores and 11.1 to 14.8 by 6.1 to 7.7 μm for the small ascospores.

Molecular characterization. *Group 1 introns.* PCR with primer pair ITS1/ITS4 amplified a DNA fragment of about 540 bp and uniform size among all isolates tested. Seven of the isolates are shown in Figure 3A. However, when the primer ITS1 was replaced with the primer ITS5, which is 24 bp upstream of primer ITS1, PCR with primers ITS4/ITS5 amplified products of two different sizes, one of about 560 bp among the *S. sclerotiorum* isolates and another of about 1,000 bp from the *S. trifoliorum* isolates (Fig. 3B). The larger PCR amplicon from the *S. trifoliorum* isolates is due to the presence of an intron located between primers ITS1 and ITS5 (21). Similarly, PCR amplifications with primer pair NS5/NS6 (Fig. 3C; nine representative isolates are shown) indicated the presence of another intron in 8 of the 10 isolates of *S. trifoliorum*. PCR amplification with primer pairs NS3/NS6 and NS3/NS8 also showed the presence of the same intron in the eight isolates of *S. trifoliorum* (data not shown).

Single nucleotide polymorphism in the ITS region. PCR with primers ITS1 and ITS4 amplified a 540-bp DNA fragment of the ITS region. Sequencing both strands allowed unambiguous determination of sequences. The sequences of the four *S. trifoliorum* isolates were identical, and the sequences of the three *S. sclerotiorum* isolates also were identical among themselves. However, there were two single nucleotide polymorphisms (SNPs) between the sequences of the two groups. These two SNPs were located at position 120 (transversion T→G) and position 376 (transition T→C) of the amplicon (Table 1). BLASTn analysis of the ITS locus of slow-growing isolates (*S. trifoliorum*) showed 100% similarity to the sequence of *S. trifoliorum*, whereas the ITS locus of the fast-growing (*S. sclerotiorum*) isolates was identical to that of *S. sclerotiorum*. The ITS sequences of isolates 05WM6, 05WM21, and 05WM33 were deposited in GenBank and assigned accession numbers EU082464, EU082465, and EU082466, respectively.

Pathogenicity tests. All isolates tested were pathogenic to the two chickpea cvs. Spanish White and Dwelley, causing symptoms typical of stem and crown rot. Inoculation initially produced symptoms typical of stem rot, where lesion elongation, discoloration, and drying of the stem

from the point of inoculation were evident. The symptoms progressed indiscriminately in both directions on the main stem to other plant parts, causing stems to become discolored, tan, or bleached white. Expanding lesions often girdled the stem, causing foliage to wilt and stems to break. White, fluffy mycelium occasionally was formed on the soil surface next to an infected stem and at the point of inoculation. Inoculated plants died about a week after inoculation (Fig. 4). No sclerotia were formed on 3-week-old seedlings but sclerotia readily formed on plants inoculated when plants were 5 and 6 weeks old (Fig. 1C).

DISCUSSION

This is the first report that *S. trifoliorum* causes stem and crown rot of chickpea in North America. *S. trifoliorum* previously was reported to be associated with chickpea in Australia based on cultural characteristics in the absence of apothecia or ascospores (3,9). Our research provides a detailed characterization of the pathogen based on cultural characteristics and the definitive character of ascospore dimorphism. Furthermore, the morphological characters were associated with several distinctive molecular markers. One isolate (06CWM-F2) could be identified as *S. trifoliorum* based on colony morphology

and molecular markers but did not produce ascospores despite repeated attempts at inducing carpogenic germination. This isolate could be heterothallic and require mating with microconidia from a compatible strain for apothecium production (26) or it simply may be recalcitrant for carpogenic germination. Voucher specimens of isolates 05WM6, 06WM-A8, and 05WM33 used in this study were deposited in the Washington State University Herbarium and were assigned accession numbers 71364, 71365, and 71366, respectively.

Although *S. trifoliorum* usually is regarded as a pathogen of mainly the forage legumes, our study demonstrated that *S. trifoliorum* can infect and cause considerable damage on chickpea. The semiselective medium of Steadman et al. (23), from which the pH-indicating medium was derived, was designed for detecting airborne ascospores of *S. sclerotiorum* based on the fact that *S. sclerotiorum* produces oxalic acid in plants and in culture (12,19). Our study showed that this semiselective medium may not be as effective for detecting ascospores of *S. trifoliorum* when used at 22°C.

The modified petiole inoculation method with agar plugs gave uniform contact between the inoculum and the host plants and there were no escapes of inoculated plants. It gave more uniform inocula-

Fig. 4. Development of stem rot of chickpea following inoculation of 3-week-old plants of cv. Dwelley with isolates 05WM6, 06CWM-G3, and 06CWM-H6 of *Sclerotinia trifoliorum* and isolates 05WM22 and 06WM33 of *S. sclerotiorum*. The disease was rated on a scale of 0 to 4, where 0 = no disease, 1 = inoculated branch dried, 2 = up or downward movement of the disease from inoculation site on the main stem, 3 = both up and downward movement of the disease, and 4 = plant dead.

tion than did the colonized oat kernel method (1) in inoculating chickpea (*unpublished data*). When the inoculation was done near the crown of chickpea plants, the inoculation produced both stem rot and crown rot.

Powers et al. (21) reported that isolates of *S. trifoliorum* contain group I introns in the nuclear small subunit rDNA, whereas isolates of *S. minor* and *S. sclerotiorum* do not, and that isolates of *S. trifoliorum* could be separated into five subgroups based on the location and number of introns in the small subunit rDNA. A high level of intraspecific variation is evident among isolates of *S. trifoliorum*. We detected two of the five subgroups even in our small sample size of 10 isolates (Table 1). All *S. trifoliorum* isolates contained the intron located at the conserved DNA sequence region between primers ITS1 and ITS5. This is the same location of a group I intron that separated two formae speciales of *Phialophora gregata* (5). The two base-pair differences in the ITS region between *S. trifoliorum* and *S. sclerotiorum* reported by Holst-Jensen et al. (15) also are confirmed in the isolates obtained from chickpea in California. Furthermore, the ascospore dimorphism, ITS sequence variation, and group I introns in the small subunit rDNA were associated with growth rates and ability to induce pH changes in culture medium at 22°C. Willis (28) might have recognized this phenomenon of the differential growth rates much earlier when he reported that the difference in growth rates between *S. sclerotiorum* and *S. trifoliorum* was temperature dependent. *S. trifoliorum* grew more rapidly at low temperatures (5°C) and more slowly at higher temperatures (25°C) than did *S. sclerotiorum*.

We have shown that both *S. sclerotiorum* and *S. trifoliorum* infect chickpea in central California. However, the relative importance of each species at various locations is still unknown. It would be impractical to use ascospore dimorphism as the criterion to identify isolates into species for population studies because of the time required to induce carpogenic germination and the presence of heterothallism (hence, requiring mating) in some isolates of *S. trifoliorum* (25,26). Growth rate and group I intron and ITS sequence variations may be useful in separation of *S. sclerotiorum*

and *S. trifoliorum* in the *Sclerotinia* populations on chickpea from central California. This information can be used to investigate the relative importance of the two species at various geographic locations. Additionally, the two species can be investigated in relation to crown infection versus aboveground stem infection and other aspects of epidemiology.

ACKNOWLEDGMENTS

We thank T. Paulitz for providing an isolate of *S. trifoliorum*.

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