Cultural and DNA-based identification of *Sclerotinia trifoliorum* infecting chickpea in the U.S.

Njambere, E.N.[1], Chen, W.[2],
Frate, C.[3], Temple, S.R.[3],
Wu, B.M.[3] and Muehlbauer, F.J.[2]

[1]Washington State Univ., Pullman, WA, U.S.A.
[2]USDA-ARS, Pullman, WA, U.S.A.
[3]Univ. of California-Davis, CA, U.S.A.

Summary

Sclerotinia trifoliorum is identified for the first time to be one of the causal agents of white mold of chickpea in North America. Based on cultural characteristics, isolates exhibited 2 growth rates: fast growing and slow growing. Fast growing isolates were identified as *S. sclerotiorum* and slow growing isolates as *S. trifoliorum* based on ascospore morphlogy, and ability to induce pH change in growing media, presence and variation of group I introns in the nuclear small subunit rDNA, and ITS sequences. Intraspecific differences were also evident among isolates of *S. trifoliorum*. Both *S. sclerotiorum* and *S. trifoliorum* are pathogenic on chickpea.

Sclerotinia trifoliorum Erikss. is speculated to be one of the causal agents of white mold of chickpea in California (Njambere *et al.* 2006). Although three species of *Sclerotinia* (*S. sclerotiorum*, *S. minor* and *S. trifoliorum)* have been reported to cause the disease in various parts of the world, *S. trifoliorum* has never been reported infecting chickpea outside the Australia sub-continent. Identification of the three species has mainly been based on morphological characteristics such as sclerotial characteristics and growth in culture medium (Cother, 1977; Bretag and Mebald, 1987). More recently, scientists have used molecular tools to study members of this group of fungal species (Holst-Jensen *et al.* 1998, Powers *et al.* 2001). Nevertheless, ascospore dimorphism is still the most definitive character for identifying *S. trifoliorum*. Work was therefore undertaken to characterize the Californian isolates based on cultural characteristics, ascospore dimorphism and PCR-based techniques. The objective of this study was to develop reliable methods for identifying *S. trifoliorum* and to determine the efficiency of using the methods for population studies.

Isolates used in this study were obtained from sclerotia or segments of diseased stems sampled from infected chickpea plants from various locations in central California. Nine isolates were obtained. Four other previously identified isolates of *S. sclerotiorum* from lentil (*Lens culinaris* Medik) and pea (*Pisum sativum* L.) and *S. trifoliorum* from alfalfa (*Medicago sativa*) were also included in the study for comparison. To test for colony growth rates, PDA plates were centrally inoculated with a 7-mm diameter disc and maintained at room temperature (25°C) in the dark. Measurements were taken at 12 hours intervals until colonies covered the whole plate. Measurements were also taken on a pH indicator media with bromophenol blue (50mg per L) modified from Steadman *et al.* (1994). In order to observe ascospore morphology two methods were used to induce carpogenic germination: the method with preconditioning developed for *S. sclerotiorum* (Cobb and Dillard, 2004)), and the method without preconditioning developed for *S. trifoliorum* (Rehnstrom and Free, 1993). To determine the presence and sizes of group 1 introns in the nuclear small subunit rDNA, total DNA was extracted from sclerotia using the FastDNA® kit described by Chen *et al. (*1999). PCR amplifications were conducted using the primers ITS1/ITS4 and ITS5/ITS4 for the ITS regions containing the 5.8S rDNA and using various combinations of primers (NS5/NS6 and NS5/NS8), to amplify different regions of the small subunit rDNA. PCR products were separated and product sizes estimated using 1% agarose gel electrophoresis along with standard DNA size markers, to determine the presence of introns in the amplified DNA regions. 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, USA) at the Sequencing Core Facility of Washington State University. Sequence comparisons were carried out using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Among the nine isolates two were identified as *S. sclerotiorum* and the rest were *S. trifoliorum*. Isolates identified as *S. sclerotiorum* had fast growing rates and induced strong color change of the pH indicating medium, whereas slow growing isolates induced no color change or very faint color change and were identified as *S. trifoliorum*. Ascospore dimorphism was consistently observed among the slow growing isolates, but was not observed in fast growing isolates. Slow growing isolates exhibited 4 large and 4 small ascospores in the asci, whereas the fast growing isolates had all the 8 ascospores of similar size. All of the isolates of *S. trifoliorum* contain the introns located at the conserved DNA sequence region between primers ITS 1 and NS5. Based on the type of group I introns in the nuclear small subunit rDNA, intraspecific variation was evident among isolates of *S. trifoliorum*. We detected two of the five subgroups identified by Powers *et al.* (2001) among such a small sample size of nine isolates. The two base-pair differences in the ITS region between *S. trifoliorum* and *S. sclerotiorum* reported by Holst-Jensen *et al.* (1998) are also confirmed in this report. Furthermore, the ascospore dimorphism, ITS sequence variation, and group I introns in the small subunit rDNA correlated well with growth rates and possibly oxalic acid production in culture. This has important implications for using the above methods in species identifications for population studies.

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