The occurrence of Sclerotium rot on white clover (*Trifolium repens* L.) caused by *Sclerotium rolfsii* in Korea

Jin-Hyeuk Kwon · Hyeoun-Suk Cho · Shun-Shan Shen · Jinwoo Kim

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Abstract We observed an occurrence of sclerotium rot on white clover (*Trifolium repens* L.) grown as a green manure in sweet persimmon orchards of Jinju, Korea. The typical symptoms included gradual blighting, wilting, and rotting; and severely infected plants eventually died. Furthermore, mycelial mats spread over the infected white clover petioles and sclerotia formed near the soil surface. Sclerotia were globoid, 1–3 mm, and white to brown. The optimum temperature for growth on potato dextrose agar was 30 °C. White mycelium formed a typical clamp connection. Based on the morphological characteristics, internal transcribed spacer (ITS) sequence analysis, and pathogenicity tests, the causal agent was identified as *Sclerotium rolfsii* Saccardo. This is the first report of sclerotium rot from *S. rolfsii* on white clover in Korea.

Keywords *Sclerotium rolfsii* · Sclerotium rot · White clover · rDNA spacer sequence

Some legume manures, including white clover (*Trifolium repens* L.), can fertilize soils, supplying extra organic matter, and are more practical and economical than transporting

finished compost. White clover has been used as a green manure in the sweet persimmon orchards of Korea for the last 10 years.

In July 2011, an occurrence of sclerotium rot was observed on white clover in sweet persimmon orchards in Jinju, Korea. The rot was most developed on petioles near the soil line. Infected white clover plants eventually withered and died. White mycelial mats and numerous sclerotia were produced on petioles near the soil line (Fig. 1a and b).

Twenty diseased petioles of white clover plants were sampled, and isolation was performed on potato dextrose agar (PDA) as described previously (Kwon et al. 2012). Briefly, pieces of diseased petioles of white clover were dissected, subjected to surface-disinfection with 1 % sodium hypochlorite solution for 1 min, rinsed with sterilized distilled water and then air-dried. The dried samples were placed on water agar (WA) and incubated at 25 °C for 2 days. Mycelial tips of the fungal isolates grown on WA were transferred to PDA. A total of 20 *Sclerotium* isolates were collected from diseased plant samples. Mycelium growth rate of all fungal isolates was determined by propagation on PDA at different temperatures (20, 25, 30, 35, and 40 °C). The optimum temperature for growth was 30 °C. Aerial mycelia usually formed many narrow hyphal strands 4–8 µm wide. Sclerotia (1–3 mm) were white at first, and then turned dark brown (Fig. 1c). White mycelium formed typical clamp connections after 6 days of growth at 30 °C (Fig. 1d). The measurements and taxonomic characteristics coincided with those of *Sclerotium rolfsii* (Penz.) Saccardo described previously (Mordue 1974). Cultures of *S. rolfsii* were deposited with the Korean Agricultural Culture Collection (KACC 46991), National Academy of Agricultural Science, Rural Development Administration, Suwon, South Korea.

To test pathogenicity, inoculum of a representative fungal isolate was prepared as previously described (Kwon et al. 2012). Briefly, mycelial mats of the test fungus grown on PDA for 6 days were harvested and mixed thoroughly with sterilized soil.

J.-H. Kwon
Gyeongsangnam-do Agricultural Research and Extension Services, Jinju 660-360, Korea

H.-S. Cho
Crop Environment Research Division, National Institute of Crop Science, RDA, Suwon 441-857, Korea

S.-S. Shen
College of Plant Protection, Henan Agricultural University, Zhengzhou 450002, China

J. Kim (✉)
Institute of Agriculture and Life Sciences, Gyeongsang National University, Jinju 660-701, Korea
e-mail: jinwoo@gnu.ac.kr

Fig. 1 Symptoms of sclerotium rot on white clover (*Trifolium repens* L.) and mycological characteristics of the pathogenic fungus *Sclerotium rolfsii*: **a** infected plants in the field; **b** typical symptoms on petioles; **c** *S. rolfsii* on potato dextrose agar after 20 days of incubation; and **d** clamp connection (arrow). Bar=10 μ m

The soil mixture was used as an inoculum; 100 g soil inoculum was placed on top of the soil in Wagner's pots (Wagner's pot size 1/2,000a; 25 cm in diameter and 30 cm in height), after which five 1-month-old white clover plants were transplanted into each pot. Three pots containing non-infested soil inoculum were used as negative controls. All pots were kept separately in a greenhouse and observed for disease symptoms. Seven days following inoculation, the same disease symptoms as those witnessed in the field were observed, whereas no symptoms developed on the negative control plants. The fungus was re-isolated from artificially inoculated plants, fulfilling Koch's postulates.

To confirm the identity of the causal fungus, the complete internal transcribed spacer region (ITS) of the rRNA gene of the isolate was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). Total DNA was isolated using the Exgene Plant-Fungal SV Mini kit (Geneall Biotechnology Co., Seoul, Korea), following the manufacturer's instructions. The polymerase chain reaction (PCR) mixture contained 5 units Taq polymerase (Takara, Tokyo, Japan), 1×PCR buffer, 0.2 mM of each dNTP, 5 pmol of each primer, and approximately 10 ng fungal genomic DNA with the total volume adjusted to 50 μ l with

sterile water. PCR was performed using an Astec PC 802 thermal cycler (Astec, Fukuoka, Japan) with the following thermal profile: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s, 70 °C for 30 s and a final extension step of 72 °C for 4 min. Amplified products were separated by electrophoresis on a 0.8 % agarose gel in 1×TBE buffer at 100 V for 20 min. PCR amplicons were extracted after agarose gel electrophoresis using a gel extraction kit (Geneall Biotechnology Co., Seoul, Korea). Purified PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) to generate the plasmid pOR12. Sequencing was performed using a Bigdye Terminator Cycle Sequencing kit (Applied Biosystems) with primers M13F and M13R, following the manufacturer's instructions. The resulting 684-bp of ITS rRNA gene sequence was deposited in GenBank (Accession No. KC491874). Phylogenetic analysis was performed using MEGA4 software employing the neighbor-joining method and the Tajima-Nei distance model (Tamura et al. 2007). Previously published ITS sequences of *S. rolfsii* strains were included for reference. The phylogenetic tree was rooted to *Chloroscypha enterochrom* (Xu et al. 2010), and the representative isolate was placed within a clade comprising reference isolates of *S. rolfsii* (Fig. 2).

Fig. 2 Phylogenetic tree produced using internal transcribed spacer sequences, showing the closest known relatives of *Sclerotium rolfsii*. DNA sequences from the NCBI nucleotide database were aligned using ClustalW, and a phylogenetic tree was constructed using the neighbor-joining method, visualized with TreeView. The numbers above branches indicate bootstrap values. Bars indicate the number of nucleotide substitutions per site. The isolate studied in the present study is marked in bold

Due to the high economic value of green manure crops, identification of this progressing fungal disease is important. Based on the symptoms, mycological characteristics, ITS sequence analysis, and pathogenicity to the host plant, this fungus was identified as *Sclerotium rolfsii* Saccardo (Mordue 1974). To the best of our knowledge, this is the first report of sclerotium rot on white clover caused by *S. rolfsii* in Korea. This disease has been reported once previously; however, a species level identification was not made (Hong et al. 2004). The morphological characteristics observed by Hong et al. (2004) were similar to the description reported here. Therefore, we believe that the fungus reported by Hong et al. (2004) was also *S. rolfsii*.

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References

- Hong Y-K, Cho J-M, Lee B-C, Song S-B, Park S-T (2004) Pathogenicity and host range of a potential mycoherbicide, isolate BWC98-105, causing white root rot on *Trifolium repens*. Plant Pathol J 20:58–62
- Kwon JH, Kim J, Lee YH, Shim HS (2012) Sclerotium rot of sponge gourd caused by *Sclerotium rolfsii*. Res Plant Dis 18:54–56
- Mordue JE (1974) *Corticium rolfsii*. CMI descriptions of pathogenic fungi and bacteria. No. 410. Commonwealth Mycological Institute, Surrey
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
- White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322
- Xu Z, Harrington TC, Gleason ML, Batzer JC (2010) Phylogenetic placement of plant pathogenic *Sclerotium* species among teleomorph genera. Mycologia 102:337–346