Mycobiology

Research Note

Occurrence of Brown Blight Caused by *Waitea circinata* var. *zeae* on Cool Season Turfgrass in Korea

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Abstract In 2010, disease symptoms, including necrotic lesions on stems and leaves with circular yellow-brown or irregular brown color patches, were observed on cool-season turfgrass at golf courses (OHCC) and the Daegu University research farm in Gyeongbuk, Korea. We isolated the causal agent and identified it as *Waitea circinata* var. *zeae* by morphological characterization and molecular analysis. To the best of our knowledge, this is the first report of brown patch caused by *W. circinata* var. *zeae* on cool-season turfgrass in Korea.

Keywords Brown patch, Cool-season turfgrass, Molecular analysis, Pathogenicity

Cool-season turfgrass is very important to agronomists and horticulturists because it provides pasture and hay for livestock and adds beauty to our surroundings in lawns, gardens, parks, school grounds, and cemeteries. It also plays a vital role in nature by preventing soil erosion, filtering harmful substances, and providing habitat for wildlife. Turfgrass purifies the air by removing carbon dioxide and releasing oxygen. Turfgrasses have also been used as an attractive playing surface on football and cricket grounds and golf courses [1, 2].

The maintenance and management of cool-season turfgrasses is a multimillion dollar industry worldwide, including in South Korea. Fungal diseases, especially brown patch, leaf and sheath spot, and yellow patch, are the greatest threat to this industry [2]. Fungi of the genus *Rhizoctonia* and its teleomorph *Waitea* have been reported as the causal organisms for most turfgrass diseases. In South Korea, some diseases of cool-season turfgrass have been reported that are caused by new pathogenic agents. These new organisms may have been imported from another country along with turfgrass cultivars. Some of these fungal species may become endemic in turfgrass due to changing climate conditions, which has never happened before. The disease symptoms of infection with different pathogenic fungi are fairly similar; therefore, it is difficult to identify the causal strain based on disease symptoms alone. For the above mentioned reasons, the objective of this study was to identify the causal agent responsible for brown patch disease on cool-season turfgrass in Korea through morphological characterization and molecular analysis.

Brown patch disease was observed on cool-season turfgrass at a golf course (OHCC) and the Daegu University research farm in Gyeongbuk, Korea. Nineteen samples of diseased tissue (leaves and stems showing brown patch-like symptoms) from Kentucky bluegrass (*Poa pratensis* L.) and tall fescue (*Festuca arundinacea* Schreb.) were collected in May 2010. The disease symptoms observed in the field were necrotic lesions on the stems and leaves with circular yellow-brown or irregular brown patches 10~50 cm in diameter (Fig. 1A and 1B). Brown patch disease eventually developed, with a brownish color, in July. To isolate and identify the pathogen, collected diseased leaves and stems were surface sterilized with a 0.5% NaOCl solution for 2 min, washed with double

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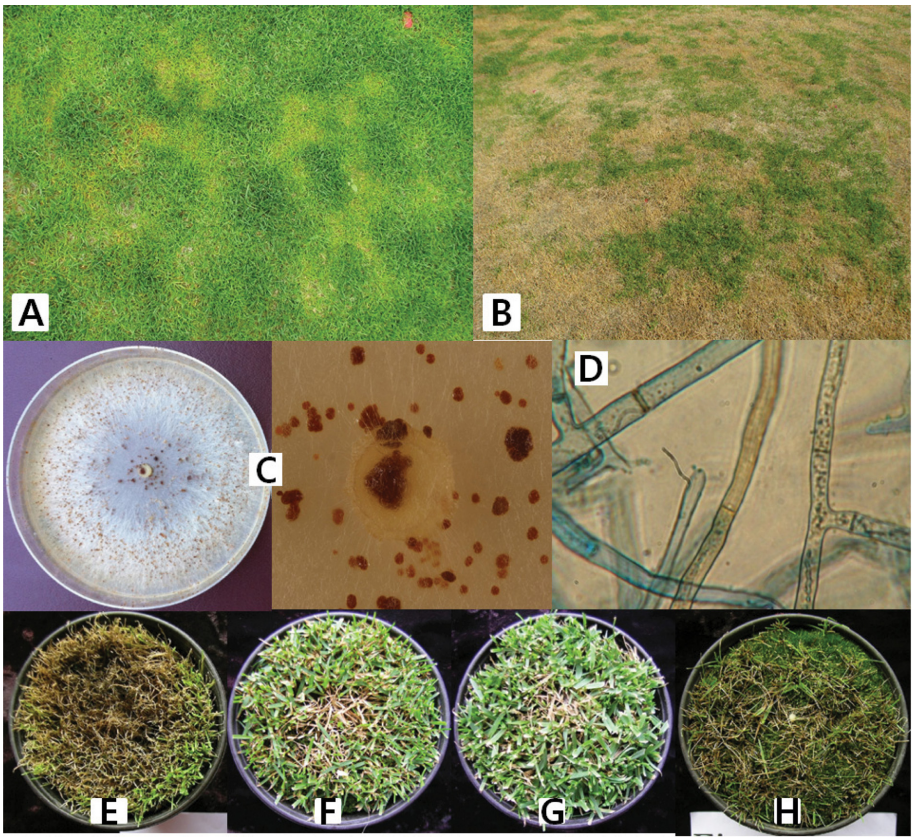

Fig. 1. Brown patch on cool-season turfgrass caused by *Waitea circinata* var. *zeae* (*Rhizoctonia zeae*). A, Disease symptoms developed in late May 2011; B, Severe disease symptoms on whole plants; C, 25-Day-old colony of *Waitea circinata* var. *zeae* growing on potato dextrose agar. Numerous sclerotia formed on the mycelia; D, Mycelia with setae; E~H, Disease symptoms on four species of turfgrass: E, Creeping bentgrass; F, Kentucky bluegrass; G, Tall fescue; H, Fine leaf fescue.

distilled water, dried by blotting, and then plated on potato dextrose agar (PDA) containing chloramphenicol (0.05 g/L), tetracycline (0.05 g/L), and streptomycin (0.05 g/L) to prevent bacterial contamination [3]. The petri plates were incubated for 2 days at 25°C. To obtain a pure culture, a hyphal tip of each isolate was aseptically transferred to a fresh PDA plate and incubated at 25°C. A total of 15 isolates were obtained from different locations, and we used isolate 17-1 for our morphological and molecular characterization. The colonies and sclerotia formation were examined during a 25-day incubation period (at 25°C). After 25 days of incubation, the observed colonies were colorless to buff-colored and fast growing with reddish-brown sclerotia that were ball shaped and 0.3~2.0 mm in diameter (Fig. 1C). The hyphae were multi septate (Fig. 1C). The color and size of the sclerotia, color of the mycelia, and pigment deposition of isolate 17-1 were distinctly different from those of other *Rhizoctonia* spp. [4].

The mycelial growth of 17-1 (KACC 45720), B-7 (AG-1 IB), and AG2-2(IV) (KACC 40132) on different fungal growth media (PDA, Oatmeal, V-8, and corn meal medium) was compared. We used the B-7 and AG 2-2(IV) isolates of *Rhizoctonia solani* as reference isolates for most of the experiments because *R. solani* is the most common causal agent for brown patch of turfgrass [5]. Isolates B-7 and AG2-2(IV) were collected in Gyeongbuk province, Korea [6]. A plug of mycelium (5-mm diameter) from the edge of a colony of each isolate grown on PDA was transferred to petri plates (90 × 15 mm) containing the above mentioned media and incubated in the dark at 25°C. The experiment was repeated three times. Colony diameter was measured after 48 hr of incubation. The mycelial growth of isolate 17-1 on PDA, V-8, and corn meal media was better and significantly different than that on oatmeal medium (Fig. 2).

There was no significant difference in the mycelial growth of isolate 17-1 on PDA, V-8, and corn meal media. Compared to that of isolates B-7 and AG2-2(IV), the mycelial growth of isolate 17-1 was significantly better on all tested media (Fig. 2). The mycelial growth of isolates B-7 and AG2-2(IV) was similar on PDA and corn meal media but was different on oat meal and V-8 media.

To determine the effect of pH on mycelial growth, a 5-mm diameter plug from the cultures of each isolate was placed onto 90-mm PDA plates adjusted to pH 4, 5, 6, 7, and 8. The plates were incubated at 25°C in the dark. The experiment was repeated three times. After 48 hr of incubation, colony diameter was measured. At all tested pH values, the mycelial growth of isolate 17-1 was significantly

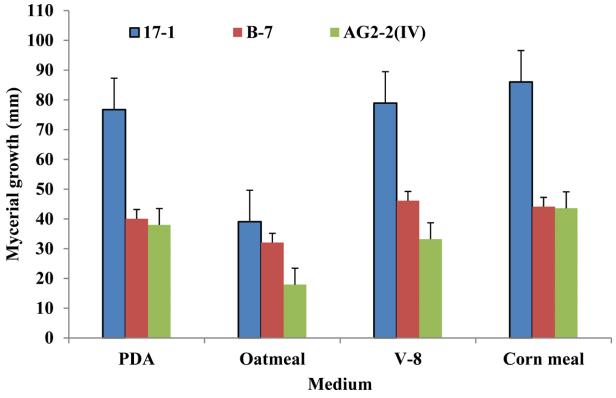

Fig. 2. Growth of isolate 17-1, B-7 (*Rhizoctonia solani* AG-1 IB), and AG2-2(IV) (*R. solani*) on different growth media. Mycelial growth was evaluated on four different media (potato dextrose agar [PDA], oatmeal media, V-8, and corn meal media). Bars indicate one standard deviation.

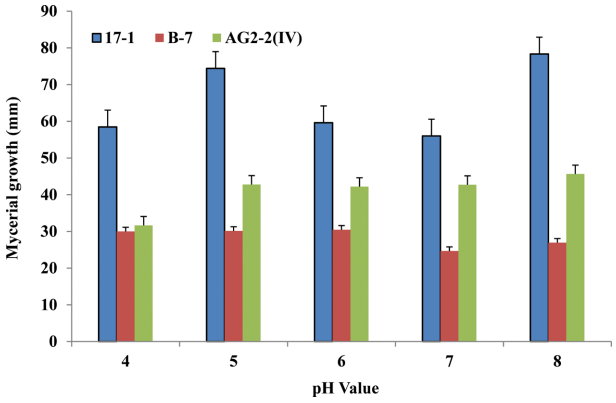

Fig. 3. Mycelial growth of isolate 17-1, B-7 (*Rhizoctonia solani* AG-1 IB), and AG2-2(IV) (*R. solani*) potato dextrose agar adjusted to pH 4, 5, 6, 7, and 8. Bars indicate one standard deviation.

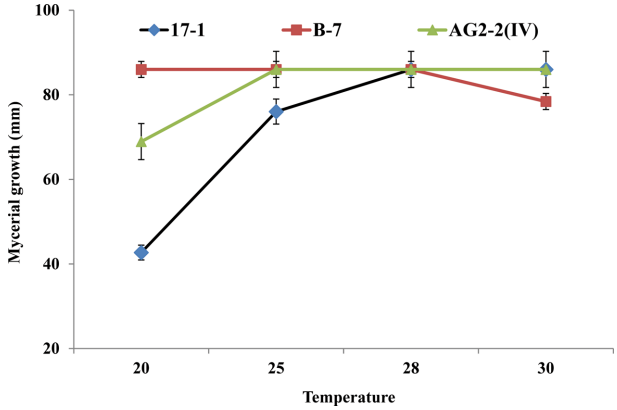

Fig. 4. Growth of isolate 17-1, B-7 (*Rhizoctonia solani* AG-1 IB), and AG2-2(IV) (*R. solani*) on potato dextrose agar plates incubated at different temperatures. Colony diameter was measured after 96 hr of incubation at 20°C, 25°C, 28°C, and 30°C. Error bars indicate one standard deviation.

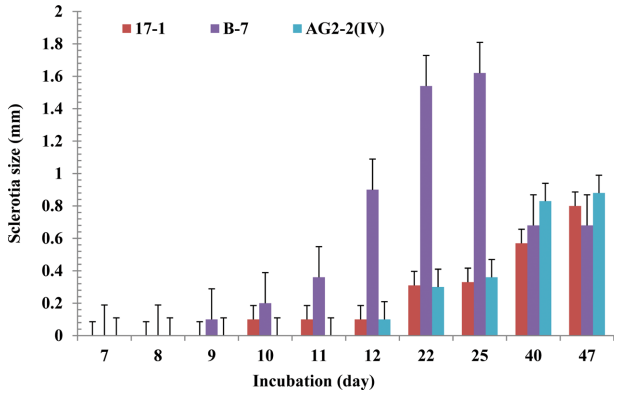

Fig. 5. Comparison of the sclerotia size of isolate 17-1, B-7 (*Rhizoctonia solani* AG-1 IB), and AG2-2(IV) (*R. solani*) on potato dextrose agar after different incubation periods. Bars indicate one standard deviation.

greater than that of isolates B-7 and AG2-2(IV) (Fig. 3). For isolate 17-1, maximum growth was observed at pH 8, and the second greatest growth was observed at pH 5, showing that isolate 17-1 can grow at a wide range of pH values.

The mycelial growth of these isolates at different temperatures was evaluated, and sclerotial size was measured after different incubation periods at 30°C. Three replicates of each isolate [17-1, B-7, and AG2-2(IV)] were randomly placed in the block and incubated at the four test temperatures. A 5-mm mycelial plug was placed at the center of the PDA plates, and the plates were incubated at 20°C, 25°C, 28°C, and 30°C in the dark. After 96 hr of incubation, colony diameter was measured. Simultaneously, the size of the sclerotia was measured on plates that were incubated at 30°C for different incubation periods (up to 47 days). Isolate 17-1 showed maximum mycelial growth at 28°C and 30°C. The mycelial growth of isolate 17-1 gradually increased from 15°C to 28°C, and was the same at 30°C (Fig. 4), indicating that isolate 17-1 grows optimally at 25~30°C.

The same optimal growth temperatures were reported for genus *Waitea* spp. by de la Cerda *et al.* [7]. There was no significant differences in the mycelial growth of isolates 17-1, B-7, and AG2-2(IV) at 28°C (Fig. 4). The size of the sclerotia of isolate 17-1 increased from 9 to 47 days of incubation (Fig. 5). Similar increasing trends were observed for isolates 17-1 and AG2-2(IV), which differed significantly from that of B-7 (Fig. 5).

For molecular identification, the total genomic DNA of morphologically similar isolates (16, 17-1, 22, and 24-2) was extracted with the HiGene Genomic DNA Prep Kit (Daejeon, Korea). The extracted genomic DNA was used as a template for amplification of the internal transcribed spacer (ITS) regions of rDNA with primers ITS1 and ITS4. To confirm the expected target size, PCR products were

separated by electrophoresis on a 1% agarose gel and purified with the HiGene PCR Purification Kit. Both strands of the amplified DNA were sequenced with by Macrogen Inc. (Seoul, Korea). The ITS region of rDNA was sequenced because this region has been extensively used for molecular identification of fungal species, and is probably the most widely sequenced region in fungi [8]. BLAST analysis (NCBI) of the ITS rDNA sequences showed that isolates 17-1 and 16 shared 97% (607/623 bp) similarity with the sequence from *Waitea c.* var. *zeae* HQ270161.1, which was isolated in China. The DNA sequences of the present isolate were deposited in GenBank (accession Nos. LC187268 and LC187269). To determine the phylogenetic relationships among the new isolate and other isolates, the nucleotide sequence of three *Waitea c.* var. *zeae*, three *Waitea c.* var. *circinata*, and three *Waitea c.* var. *oryzae* strains were retrieved from GenBank for use as reference sequences. The nucleotide sequences of four *Rhizoctonia* spp. and one *Rhizoctonia solani* isolate were used as an out group. The phylogenetic tree was constructed by MEGA 4.0.2 using the neighbor-joining method. In the phylogenetic tree, isolates 17-1 and 16 grouped in a clade containing *Waitea circinata* var. *zeae* (Fig. 6).

The pathogenicity of isolates 16, 17-1, 22, and 24-1 was tested on creeping bentgrass (*Agrostis palustris* Huds.), Kentucky bluegrass, tall fescue (*Festuca arundinacea* Schreb.), and Chewing fescue (*Festuca rubra* var. *commutata* Gaud.) plants using ground mycelia as inoculum. For the pathogenicity testing, a plastic container method was used. Plastic incubation containers were filled with 4 L of potting soil and 4 L of distilled water to maintain high humidity. Sterilized seeds of the bentgrass cultivar were sown in the plastic containers. Right after sowing the seeds, the plastic containers were placed under optimum climatic conditions so that the plants can grow, and all management activities were practiced. Then, 80-day-old bentgrass cultivars were directly inoculated with mycelia (powder) of isolates 16, 17-1, 22, and 24-1 (0.2 g per pot) using a micropipette. The plastic container was then covered with plastic to maintain high humidity, which allowed the isolate to become infectious and disease to develop, and it was incubated in a growth chamber at 25°C in the dark for 3 days and then brought back to the greenhouse. After 5 to 6 days of incubation, similar disease symptoms (necrotic lesions and irregular brown colored patches) were observed on all turf grass species (Fig. 1E~1H). There were no significant differences among the disease symptoms caused by isolates 16, 17-1, 22, and 24-1 (data not shown). Isolates 16, 17-1, 22, and 24 were successfully re-isolated from inoculated leaves and identified as the same fungus (confirming Koch's postulates). All the isolates were then preserved at −20°C in the Plant Pathology Laboratory of Kyungpook National University, and isolate 17-1 was deposited in the National Academy of Agricultural Science (KACC 45720), Suwon, Korea.

Isolate 17-1 (=16) was distinguished from other *Rhizoctonia* spp. based on ITS sequence data and morphological

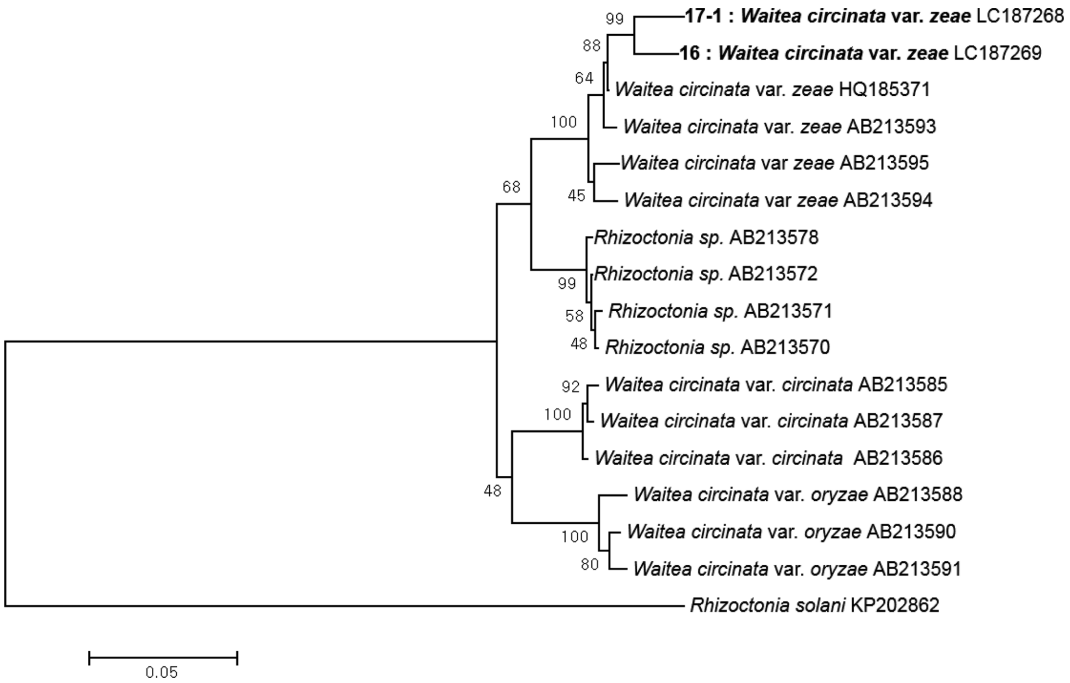

Fig. 6. Taxonomic position of isolate 17-1 based on the internal transcribed spacer (ITS) region of ribosomal DNA. Brown patch caused by a *Waitea circinata* var. *zeae* strain was compared to the disease caused by the strains isolated by Toda *et al.* [4]. ITS region sequences (amplified with primers ITS1 and ITS4) were analyzed using the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates, which were then used to construct a tree in MEGA ver. 4.0.2 with the neighbor-joining method. Numbers below the nodes are the bootstrap values.

characteristics and from *Waitea c*. var. *circinata* based on ITS sequence data. The percent sequence similarities among *W. circinata* strains were 88.4~94.6% [4]. Based on its morphological characteristics and molecular data, we identified isolate 17-1 (=16) as *Waitea c*. var. *zeae*.

Waitea circinata Warcup & Talbot has increasingly drawn the attention of plant pathologists as a causal agent of brown patch disease on cool-season turfgrasses [9, 10]. These fungi not only cause disease in turfgrasses but also in other graminaceous hosts as well as onions, sugar beets, and tall fescue grasses [11, 12]. *Waitea c*. var. *circinata* and *Waitea c*. var. *zeae* have been described as teleomorphs of *Rhizoctonia oryzae* Ryker & Gooch and *Rhizoctonia zeae* Voorhees, respectively [5, 10, 13, 14]. *Agrostis*, *circinata*, *oryzae*, *prodigus*, and *zeae* are the five different varieties of *W. circinata*, which can be distinguished by their biological and genetic differences [4, 15-18]. *Waitea c*. var. *zeae* has been detected as a causal agent of brown patch disease in various countries, including the USA, Japan, and Hungary [8, 13, 19]. *Waitea c*. var. *zeae* has not been detected in Korea. Therefore, ours is the first report of *Waitea circinata* var. *zeae* (*R. zeae*) on cool-season turfgrass in Korea.

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