Postharvest soft rot on *Citrullus vulgaris* caused by *Rhizopus oryzae* in South Korea

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Abstract During the summers of 2010 and 2011, a disease suspected to be *Rhizopus* soft rot was observed on watermelon at commercial markets in Jinju, South Korea. The infected parts of the mature fruits appeared water-soaked at first, and then softened and rotted rapidly. White mycelia grew from the primary infection site and gradually covered the fruit with tufted whisker-like gray sporangia and sporangiophores. To confirm the identity of the fungus, the complete internal transcribed spacer (ITS) rDNA sequence was amplified and sequenced. On the basis of the mycological characteristics, molecular identification and pathogenicity, the fungus was identified as *Rhizopus oryzae*. This is the first note of *R. oryzae* as a postharvest pathogen of watermelon in Korea.

Keywords *Rhizopus oryzae* · *Rhizopus* soft rot · Watermelon · rDNA spacer sequence

Watermelon (*Citrullus vulgaris*) is one of popular fruit consumed widely throughout the world for its fleshy, refreshing fruits. It is a natural and rich source of the antioxidant lycopene (Hall 2004) and non-essential amino acid citrulline, which is known to relax and dilate blood vessels (Rimando and Perkins-Veazie 2005). Watermelon is susceptible to a variety of fungal, bacterial and viral diseases at various stages of its growth (Sharma and Khan 1991; Roberts and Kucharck 2003).

During the summers of 2010 and 2011, a disease suspected to be *Rhizopus* soft rot was observed on watermelon at commercial markets in Jinju, South Korea. Wounded mature fruits were often affected, but undamaged or immature watermelons were not attacked. The infections started from cracks that occurred at harvest. The infected parts of the mature fruits appeared water-soaked at first, and then softened and rotted rapidly. White mycelia grew from the primary infection site and gradually covered the fruit with tufted whisker-like gray sporangia and sporangiophores (Fig. 1). Ten diseased fruit of watermelon were sampled, and isolation was performed on potato dextrose agar (PDA) as described previously (Kwon et al. 2010). Briefly, fungal mycelial tips produced on the diseased watermelon fruits were transferred to PDA. Ten *Rhizopus* isolates (one from each sample) were obtained from the diseased watermelon fruits. The colonies of fungus grown on PDA were white and cottony at first, then became heavily speckled with the appearance of sporangia and finally became brownish-grey to blackish-grey, and spread rapidly with stolons protruding from rhizoids to the substrate at various points. Sporangiospores were unequal, numerous, irregular, sub-globose or oval, angular with striations, and 4–11 µm in length. Sporangiophores were usually straight, smooth-walled, simple or branched, non-septate, long, and arose from stolons opposite rhizoids usually in groups of 3–5 or more, and 6–20 µm in diameter. Sporangia were globose, 50–200 µm in diameter, and white at first and later black with many spores. Columellae were globose to sub-globose, pale brown, and 80–110 µm in diameter. Rhizoids and stolons were dark brown. Two *Rhizopus* species, *R. stolonifer* and *R. oryzae*, are known to cause soft rots of cucurbit plants in South Korea (The Korean Society of Plant Pathology 2009; Kwon et al. 2010). Temperature growth studies are important to distinguish between the two species: *R. stolonifer* grows at 30 °C but not at 37 °C, whereas *R. oryzae* grows at 40 °C (Schipper and Stalpers 2003). The mycelium growth rate of all

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Fig. 1 Symptoms of soft rot on watermelon caused by *Rhizopus oryzae*. **a** Typical symptoms of *Rhizopus* soft rot on fruit and a longitudinal section of infected fruit; **b** symptom showing water-soaked appearance

and mycelium sporangia on the fruit surface; **c** symptoms induced by artificial inoculation after 4 days of incubation

fungal isolates was determined by propagation on PDA at different temperatures (30, 35, and 40 °C). The optimal temperature for mycelial growth was 30 °C and growth was still apparent at 37 °C. The measurements and taxonomic characteristics coincided with those of *Rhizopus oryzae* described previously (Lunn 1977; Schipper and Stalpers 2003) (Fig. 2). The representative fungal isolate has been deposited with the Korean Agricultural Culture Collection

(KACC 45160), National Academy of Agricultural Science, Rural Development Administration, Suwon, South Korea.

To test pathogenicity, inoculum of a representative fungal isolate (KACC 45160) was prepared as described previously (Kwon et al. 2010). The surfaces of six fruits were wounded by scratching lightly with a sterile scalpel and drop-inoculated with 100 µl of a conidium suspension (10^4 conidia/ml), and three control fruits were inoculated with 100 µl of sterile

Fig. 2 Morphological characteristics of *Rhizopus oryzae* isolated from watermelon. **a** Colony on PDA 7 days after inoculation; **b** sporangium and sporangiophore; **c** columella; **d** sporangiospores; **e** rhizoid

Fig. 3 Phylogenetic tree using ITS sequences showing closest known relatives of *Rhizopus oryzae*. DNA sequences from the NCBI nucleotide database were aligned using ClustalW and a phylogenetic tree was constructed using the neighbor-joining method and visualized with TreeView. Numbers above the branches indicate the bootstrap values. Bars indicate number of nucleotide substitutions per site. The culture isolated from watermelon is in **bold**

water. The inoculated fruits were placed in a vinyl bag at 25 °C with 100 % relative humidity for 48 h and then placed on a laboratory table at room temperature. Symptoms appeared 5 days after inoculation, whereas controls were asymptomatic. The symptoms were identical with those of the naturally-occurring disease. Morphological characteristics of the re-isolated fungus from the inoculated fruits were the same as those from the original isolate, fulfilling Koch's postulates.

Due to the high economic value of watermelon, identification of this progressing fungal disease is important. 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'-TCCGTAGG TGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTG ATATGC-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, 10 nM of each primer, and approximately 10 ng fungal genomic DNA with the total volume adjusted to 50 µl 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 products were extracted after agarose gel electrophoresis using a gel extraction kit (Geneall Biotechnology Co., Seoul, Korea). Purified PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) to generate the plasmid pJW84. Sequencing was performed using a Bigdye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with primers M13F and M13R, following the

manufacturer's instructions. The resulting 627-bp of the ITS rRNA gene sequence was deposited in GenBank (Accession No. JQ340027). 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 from *R. oryzae* strains were included for reference, and *Aspergillus flavus* ATCC 16883 (GenBank Accession No. AF13883) was used as an out-group (Fig. 3). The resulting sequence exactly matched sequences (GenBank Accession Nos. HQ285707 and AY803926) of *R. oryzae*.

On the basis of the mycological characteristics, molecular identification and pathogenicity, the fungus was identified as *R. oryzae*. This is the first report of the presence of *R. oryzae* on watermelon in South Korea.

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