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No Reported Species, *Botrytis aclada* Causing Gray Mold Neck Rot Disease on Onion Bulbs in Korea

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Gray mold neck rot was observed on onion bulbs (*Allium cepa* L.) in low–temperature warehouses in Changnyeong–gun, Korea. The causative pathogen was isolated from rotted onion bulb lesions and identified as *Botrytis aclada* based on morphological and culture characteristics, the sequences of three nuclear genes (*G3PDH, HSP60*, and *RPB2*), and polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) for *Botrytis* spp. identification. Although onion gray mold disease caused by *B. allii*, *B. cinerea*, and *B. squamosa* species has previously been reported in Korea, this, to the best of our knowledge, is the first report of *B. aclada* causing gray mold neck rot disease of onion bulbs in Korea.

Key words: Onion bulb, *Botrytis aclada*, gray mold neck rot

INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important commercial vegetable crops in Korea. Annually, more than 40% of the onions harvested are stored in low–temperature warehouses. However, a considerable portion of the crop is lost during storage because of diseases such as gray mold neck rot caused by *Botrytis allii*, *B. squamosal*, and *B. cinerea*; blue mold caused by *Penicillium* sp.; Fusarium basal rot caused by *Fusarium oxysporum* f. sp. *cepae*; and bacterial bulb rot caused by *Pseudomonas marginalis* pv. *marginalis* and *Burkholderia cepacia* (KSPP 2009; Kim *et al*., 2001; Lee *et al*., 2005). Gray mold rot is one of the major storage–related diseases. It has been reported low–temperature storage relate plant such as apple, grape, onion and seeds. However, it is difficult to efficiently control and manage grey mold disease in low–temperature storage facilities. In Korea, onion neck rot disease is reported to be mostly caused by three *Botrytis* species: *B. cinerea*, *B. allii*, and *B. squamosa* (Lee *et al*., 2005; Park *et al*., 1995; Kim *et al*., 1998) which have worldwide distribution. In 2010, *Botrytis sinoallii*, a new species on Allium crops, was reported in China. This pathogen occurred on green onion (*Allium fistulosum*) and garlic chives (*Allium tuberosum*) (Zhang *et al*., 2010). Between 2012 and 2013, rotted onion bulbs with gray mold neck rot symptoms were observed in a low–temperature storage facility in Changnyeong–gun, Korea. This study was conducted to identify the causal pathogen based on morphological and culture characteristics, phylogenetic studies, and PCR–RFLP analysis.

MATERIALS AND METHODS

Fungal collection and isolation

Onions harvested in early June were stored in warehouse at 0~1°C and 68~75% relative humidity. Generally, the primary symptom of gray mold neck rot on stored onions presented after 3 to 4 months of storage. From 2012 to 2013, infected onion bulbs were collected from a storage facility in Changnyeong–gun, after 5 to 8 months of storage. Sections containing lesions were cut from the rotted onion bulbs and transferred onto potato dextrose agar (PDA) plates and cultured at 20°C in darkness. Fourteen days after incubation, the isolates were transferred to fresh PDA plates and cultured for identification and determination of pathogenicity.

Pathogenicity test

To determine pathogenicity, the isolates were cultured on PDA plates for 10 days at 20°C. The cultured isolates were then suspended in sterile distilled water at a concentration of 1×10^5 conidia/mL, which was determined using a counting chamber (Marienfeld, Germany). Sterile filter paper disks (6 mm in diameter) soaked in the inoculum were placed on the outer surface of onion bulbs. Control bulbs were treated with sterile filter paper disks soaked in distilled water. The inoculated onion bulbs were placed in sterile plastic containers (30 × 10 × 20 cm in length, width, and height) containing a paper towel soaked with distilled water to maintain a high humidity (95% RH) and incubated for 10 days at 20°C.

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PCR amplification, phylogenetic study, and PCR–RFLP analysis

The isolates were grown in the dark for 7 days at 20°C on PDA plates. Genomic DNA was extracted from cultured colonies using an i–genomic BYF DNA extraction kit (INTRON Biotechnology, Korea) according to the manufacturer's instructions. PCR amplification of the internal transcribed spacer (ITS) region was performed using the primer set ITS1F (5'–CTT GGT CAT TTA GAG GAA GTA A–3') and ITS4 (5'–TCC TCC GCT TAT TGA TAT GC–3') using previously described conditions (White *et al*., 1990). The products were then sequenced. Although ITS region sequences have been widely used for species–level discrimination of fungi, variation in the ITS region within *Botrytis* is low, limiting its use to genus–level discrimination (Nielsen *et al*., 2001). Three nuclear protein–coding genes–glyceraldehyde–3–phosphate dehydrogenase (*G3PDH*), heat–shock protein 60 (*HSP60*), and DNA–dependent RNA polymerase subunit Ⅱ (*RPB2*)—were amplified using three sets of primers: G3PDHf (5'–ATT GAC ATC GTC GCT GTC AAC GA–3'), G3PDHr (5'–ACC CCA CTC GTT GTC GTA CCA–3'); HSP60f (5'–CAA CAA TTG AGA TTT GCC CAC AAG–3'), HSP60r (5'–GAT GGA TCC AGT GGT ACC GAG CAT–3'); and RPB2f (5'–GAT GAT CGT GAT CAT TTC GG–3'), RPB2r (5'–CCC ATA GCT TGC TTA CCC AT–3'). PCR amplification was performed using previously described conditions (Staats *et al*., 2005). These three genes, encoding enzymes involved in basic cellular processes, have been used for the identification of closely related *Botrytis* spp. (Staats *et al*., 2005). Isolates and reference strains representing seventeen recognized species of *Botrytis* were included in the phylogenetic analysis (Staats *et al*., 2005). *Sclerotinia sclerotiorum* was used as an outgroup. A combined gene sequence data set (*G3PDH, HSP60*, and *RPB2*), and three separate gene sequence data sets (*G3PDH, HSP60*, and *RPB2*) were established using the cloned partial gene sequences of *G3PDH, HSP60*, and *RPB2* reported in a previous study (Staats *et al*., 2005). Sequence similarities were evaluated using the program GENETYX–WIN version 3.2 (Soft Development, Japan). Phylogenetic analysis was performed using TreeView–Win 32 version 1.6.1, after multiple alignments of data by CLUSTAL W (http://clustalw.ddbj.nig.ac.jp, DNA Data Bank of Japan). Distances and clustering were determined with the neighbor–joining method using the program TreeView (Win32, ver. 1.6.1) (Saitou and Nei, 1987). Nielsen *et al*. developed sequence characterized amplified region (SCAR) primers (BA2f/BA1r) of the partial glycosyltransferase gene for the amplification of the DNA of *Botrytis* species associated with neck rot of onions (Nielsen *et al*., 2001). Digestion of the PCR amplicons with the restriction enzyme *Apo*I was used for distinguishing between the DNA of the *Botrytis* species *B. aclada*, *B. byssoidea*, *B. squamosa*, and *B. cinerea* (Nielsen *et al*., 2002). PCR amplification of six isolates and *B. cinerea* with the *Botrytis*–specific PCR primers (BA2f/BA1r) gave products. The amplicons were digested with the restriction enzyme *Apo*I in accordance with the manufacturer's instructions (Nielsen *et al*., 2002).

RESULTS AND DISCUSSION

Morphological characteristics and pathogenicity test

The infected tissues of the bulbs softened and discolored to brown as the decay advanced. The decayed tissues in the rotted bulbs lost firmness and became waterlogged. The upper portions of bulbs sank and gray mycelium and conidia formed on the outer surface of the bulbs (Fig. 1A and 1B). Colonies of isolates were gray or

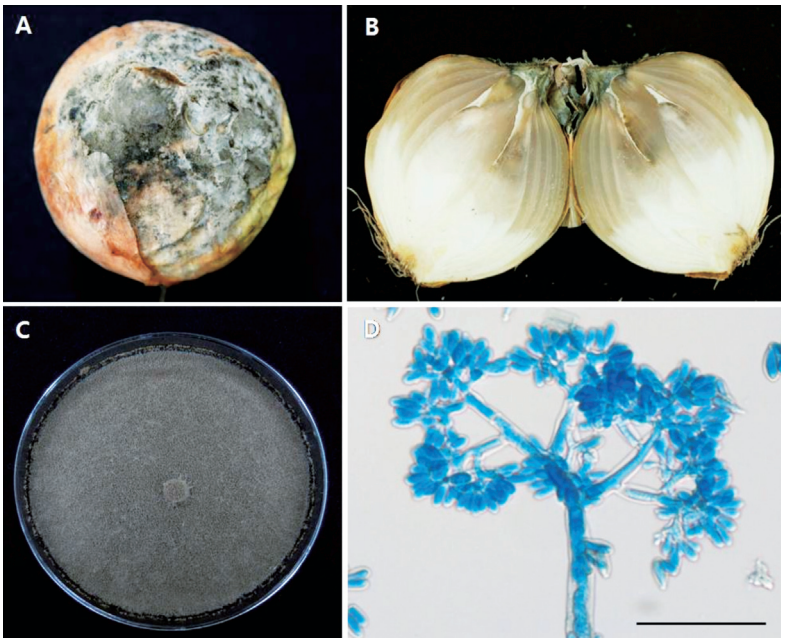

Fig. 1. Symptoms of gray mold neck rot on onion bulbs in storage. A: the outer surface of the bulb shows gray mycelium; B: vertical section of the lesion; C: colonies on PDA after 15 days; D: conidiophores and conidia (scale bar = 50 μm).

Table 1. Comparison of morphological and culture characteristics of the pathogen isolated from rotted onion bulbs with those of previously described *Botrytis* species

Characteristics		Present isolate	*Botrytis* species[a]			
			B. aclada	*B. allii*	*B. cinerea*	*B. squamosa*
Colony	Color	Gray or grayish brown	Gray or grayish brown	Gray or grayish brown	White mycelium, grayish brown	White mycelium
Conidia	Shape	ellipsoidal	ellipsoidal	ellipsoidal	ellipsoidal or obovoid	–
	Length (μm)	7.6–10.4	8.3–10.4	9.9–14.1	9.5–10.9	21.0–22.5
	Width (μm)	4.2–5.6	4.5–5.6	5.3–6.3	6.9–7.6	16.5–17.0
Sclerotia		No	No	No	Medium to large, dark brown	Small, black

[a] Described by Yohalem *et al*., Ellis and Waller, Chilvers and Toit, and Presly

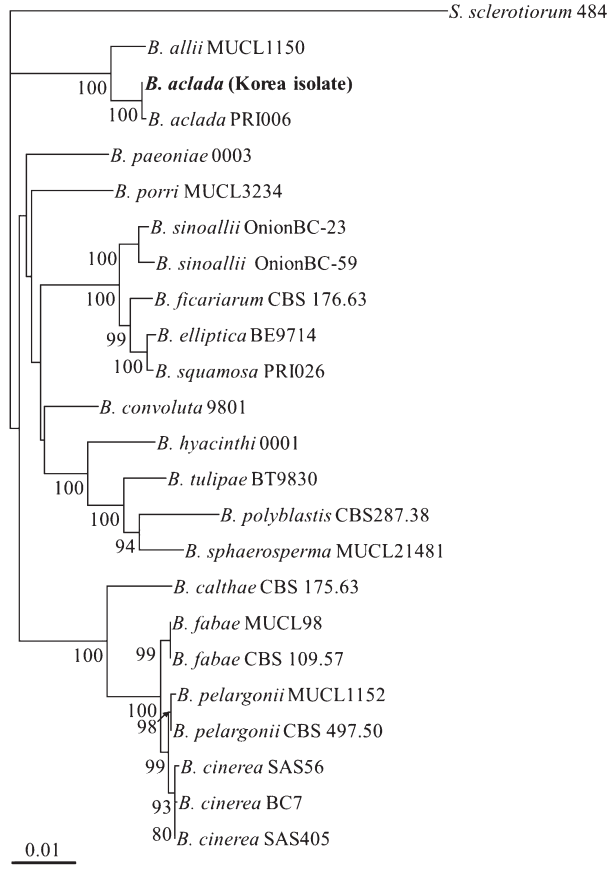

Fig. 2. Phylogenetic tree of members of the genus *Botrytis*, based on the combined data from *G3PDH, RPB2*, and *HSP60*. The tree was constructed using the neighbor–joining method and genetic distance was computed using Kimura's model. *Sclerotinia sclerotiorum* was used as an outgroup. Numbers on the branches are confidence value obtained for 100 replicates (only values above 80% are shown). The bar represents a phylogenetic distance of 1%.

grayish brown and covered with abundant ellipsoidal conidia with terminal branching conidiophores (Fig. 1C and 1D). The conidia of isolates were 7.6–10.4 μm in length and 4.2–5.6 μm in width, with an average dimension of 8.4 × 5.0 μm. No sclerotia were produced after 30 days of incubation at 20°C. The morphological and cultural characteristics of the isolates were similar to those of *B. aclada* and *B. allii* reported previously (Table 1) (Yohalem *et al*., 2003; Ellis and Waller, 1974; Chivers and Toit, 2006; Presly, 1985). However, the morphological characteristics of isolates were different from those of *B. cinerea* previously reported in Korea. To determine pathogenicity, the isolates were cultured on PDA plates for 10 days at 20°C. Symptoms similar to those of natural infection, including water–soaked lesions with gray mycelium and conidia, were observed on the surface of onion bulbs approximately 6 to 7 days after inoculation (data not shown). No lesions were induced in the control.

Phylogenetic analysis

PCR amplification of the ITS region was performed using the primer set ITS1F/ITS4, using previously described conditions. The resulting sequences of the isolates were 100% homologous. An NCBI BLAST search showed that the ITS region sequences of the isolates were > 99% homologous with the respective sequences in *B. cinerea* and *B. aclada*. In the neighbor–joining tree using the combined gene sequence data set (*G3PDH, HSP60*, and *RPB2*), strains of seventeen species of *Botrytis* and the isolates formed a clade with 100% bootstrap support, compared to the outgroup fungi (*S. sclerotiorum*) (Fig. 2). Within the *Botrytis* clade, the isolates were grouped with *B. aclada*, and were closely related to *B. allii*. All sequences of *B. aclada* were deposited in Genbank (Accession No. LC079036–G3PDH; LC0790377–RPB2; LC079038–HSP60).

PCR–RFLP analysis

The isolates were grouped as described by Nielsen *et al*. (Fig. 3A). The isolates corresponded to *B. aclada* (Fig. 3B). RFLP analysis helped distinguish this particular *Botrytis* species as being associated with neck rot of onions, since the morphological and culture characteristics were similar to those of *B. allii* isolated in Korea. Based on the sequence of three nuclear genes (*G3PDH, HSP60*, and *RPB2*) and RFLP analysis using *Apo*I, the isolates obtained from rotted onion bulbs collected from the low–temperature warehouse in Changnyeong–gun

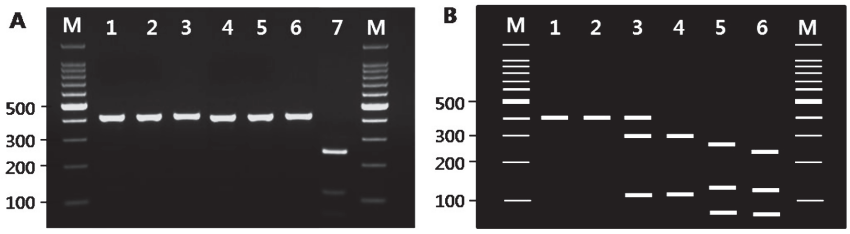

Fig. 3. *Apo*I restriction digest of PCR amplicons obtained using a *Botrytis*–specific primer set (BA2f/BA1r). A: *Apo*I restriction digest of the PCR amplification products: M: 100–bp ladder, lanes 1–6: isolates (this study), lane 7: *B. cinerea*; B: *Apo*I restriction digest of PCR amplification products of the isolate and other *Botrytis* species: M: 100–bp ladder, 1: isolate (this study), 2: *B. aclada*, 3: *B. allii*, 4: *B. byssoidea*, 5: *B. squamosal*, and 6: *B. cinerea*.

were identified as *B. aclada*. Although onion gray mold disease caused by *B. allii*, *B. cinerea*, and *B. squamosa* has previously been reported in Korea, *B. aclada* causing gray mold neck rot disease is a new pathogen in low–temperature warehouses. Moreover, *B. aclada* was dominant to *B. squamosa* in the low–temperature warehouse. This, to the best of our knowledge, is the first report of gray mold neck rot disease caused by *B. aclada* on onion bulbs in Korea.

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