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Vol. 7(13), pp. 1128-1135, 26 March, 2013
DOI: 10.5897/AJMR12.2069
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The occurrence of pink mold rot fungus *Trichothecium roseum* on tomatoes in Korea

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Accepted 15 March, 2013

***Trichothecium roseum* DUCC502 was isolated from leaves of tomato plants at a greenhouse located in Buyeo, Chungchungnamdo. The colony color of the isolate was white initially and became pale pink on potato dextrose agar and oatmeal agar. Conidiophores of the isolate were long and slender, unbranched, and 73 – 112 x 2.1 – 3.3 µm in size. Its conidia were two-celled, hyaline colored, and ovoid or ellipsoid shaped, and 11 – 18.3 x 6.1 – 8.5 µm in size. The 28S rDNA sequence analysis of the isolate showed it shared 99% similarity with that of *T. roseum* CBS113334. Mycelia of the isolate grew well on PDA plates under the conditions of pH 7–9 and temperature 20–25°C, respectively. A pathogenicity test showed the isolate caused necrotic regions and produced white to pale pink mycelia with spores on the surface of tomato fruits and leaves. This fungus was sensitive to benomyl and tebuconazole but less sensitive to dimethomorph, triflumizole and azoxystobin at 10 ug/ml concentration.**

Key words: Fungicide sensitivity, pink mold rot, postharvest disease, tomato, *Trichothecium roseum.*

INTRODUCTION

Tomato (*Solanum lycopersicum*) is one of the most popular vegetable crops in the world. In Korea, tomato is also one of economical vegetables for export to foreign countries. In 2011, the area for tomato cultivation in Korea was about 5,800 ha and tomato production reached 36 metric tons. Table and cherry tomatoes are cultivated year round in greenhouses across the nation. Diverse diseases and disorders can affect tomato production during its cultivation. Diseases, especially those caused by bacteria and fungi, are major factors in the depreciation of the quality of fresh fruits in tomato production. Regarding fungal diseases, Septoria leaf spot, early blight, late blight, gray mold, leaf mold and powdery mildew are well known in tomato (Jones et al., 1997).

Trichothecium fungi belong to the class Sordariomycetes of the phylum Ascomycota and are closely related to *Acremonium* spp. (Summerbell, 2011). The genus *Trichothecium* is comprised of six fungal species and distributed on decaying vegetables and in the soil. In general, fungi in this group have been considered as contaminants except for *T. roseum* which causes pink mold rot, one of the postharvest diseases of tomatoes reported in Argentina, Brazil and the United State of America (Bello, 2008; Inácio et al., 2011; Welch

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et al., 1975). *T. roseum* has also been isolated from apples (Zabka et al., 2006) and eggplants (Pandey, 2010). This species has been known to produce mycotoxins such as roseotoxin B and trichothecin (Engstrom et al., 1975; Ghosal et al., 1982). However, suitable fungicides and their efficient concentration for the control of pink mold rot have not yet been reported. In Korea, pink mold rot diseases caused by the species have been reported in melons and strawberries but not in tomatoes (Kwon et al., 1998, 2010). In this study we report its first occurrence in tomatoes in Korea together with its morphological and physiological properties.

MATERIALS AND METHODS

Fungal isolation and culture conditions

In May 2012, during consulting of tomato growers in a greenhouse located in Buyeo, Chungchungnamdo, Korea, we sampled leaves of tomato plants (cultivar Unicon). Conidia formed on the surface of the infected tomato leaves were detached under a stereomicroscope using a sterile inoculation needle and transferred into a 1.5 ml sterile micro-fuge tube containing 1 ml of sterile distilled water. After being mixed by voltexing, the conidial suspension was serially diluted with sterile distilled water. 200 ul of the diluted conidial suspension was spread on potato dextrose agar (PDA, BD company, Franklin Lakes, NJ, USA)). The conidia-inoculated PDA plate was incubated at 25°C for 3 days, and fungal hypha grown out from each single conidium was taken and transferred into a new PDA medium. In the same way, several single conidium isolates were obtained. The obtained single conidium isolates were maintained on PDA during the present study and stored either at -80°Cin 10% glycerol for long-term storage or in water at 4℃ for short-term storage.

Microscopic analysis

The DUCC502 isolate was subcultured on PDA at 25℃ for 5 days. A phase-contrast microscope (Axioskop 40, Carl Zeiss, Germany) and a scanning electron microscope (SEM, Hitachi S-430, Hitachi, Japan) were used for the observation of morphological characteristics. For the observation using a SEM, culture agar blocks were cut from the fungus grown PDA medium and fixed with 2% glutaraldehyde in a 0.1 M cacodylate buffer for 12 h and then 1% osmic acid for 1 h (Yun et al., 2009). The fixed sample was washed with a 0.05 M cacodylate buffer and followed by dehydration in a series of different concentrations of ethanol from 50 to 100% for 30 min each. The sample was dried with a critical point dryer (Hitachi, Japan) and coated with platinum palladium for 60 s using an ion sputter (Hitachi E-1030, Japan). The SEM was operated at 10 kV.

Growth test

To identify optimal growth conditions, pre-cultured DUCC502 isolate was transferred to the center of Petri plates. Difco™ media of potato dextrose agar(PDA; potato starch 4 g, dextrose 20 g, agar 15 g, and water 1 L), malt extract agar (MEA; maltose 12.75 g, dextrin 2.75 g, glycerol 2.35 g, peptone 0.78 g, agar 15 g, and water 1 L) and oatmeal agar (OA; oat meal 60 g, agar 12.5 g, and water 1 L) were used to evaluate the mycelia growth of the isolate DUCC502. These media were purchased from BD company (Franklin Lakes, NJ, USA) and prepared according to the manufacturer's instructions. For optimum temperature determination, incubations were carried out for 7 days on PDA (pH 7.0) at various temperatures (20, 25, 30 and 35°C). To determine optimum pH, a growth test was performed at 25°C on PDA at pH 5, 7 and 9 for 7 days. The colony diameter was measured for mycelia growth assessment. Three replicates were performed per each experiment. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 21.0. The significant differences between group means were compared using Duncan's multiple range test. Differences were considered significant at $p<0.01$.

Molecular analysis

For PCR amplification, genomic DNA was prepared from the fungal mycelia using the drilling method described by Kim et al. (1999). A primer set LROR (5'-ACCCGCTGAACTTAAGC-3') (White et al. 1990) and LR 4 (5'-ACCAGAGTTTCCTCTGG-3') (O'Donnell et al. 2000) were used to amplify the 28S rDNA D1/D2 region. Amplification of the 28S rDNA was performed as follows: 95°C for 3 min, followed by 30 cycles consisting of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s, and one final cycle of extension at 72°C for 5 min. The PCR products were purified with a High Pure PCR Purification Kit (Roche, Swiss) and ligated into pGEM T-easy vectors (Promega, USA). The ligated vectors were transformed into competent *Escherichia coli* DH5α cells according to the manufacturer's instructions. Sequencing of the amplified PCR product was carried out by Macrogen Inc. (Seoul, Korea). The obtained nucleotide sequences were manually edited and aligned using the Biological sequence alignment editor v7.0.5. Using BLASTN with the determined nucleotide sequences, the GenBank database was queried to search for similar nucleotide sequences (http://www.ncbi.nlm.nih.gov/BLAST). The determined 28S rDNA sequence was deposited to GenBank using BankIt. Reference sequences of related taxa were obtained from the GenBank database. A parsimony tree was constructed with related species using PAUP v.4.0b10 (Swofford, 2002). A heuristic search was performed for each dataset with 100 random taxon additions. Phylograms based on the 28S rDNA sequence were constructed by the neighbor-joining method (Kimura, 1980). Bootstrap values were generated with 1,000 replicates. *Bionectria epichloe* CBS118752 was used as an outgroup. A phylogenetic tree was drawn using FigTree v1.3.1(http://tree.bio. ed.ac.uk/ software/figtree/).

Pathogenicity test

For the pathogenicity test on tomatoes, *T. roseum* DUCC502 was grown on a PDA plate for 7 days at 25°C. To prepare the fungal inoculum, 1 ml of sterile water was added onto the fungus-grown PDA plate and conidia were suspended by swirling the plate. The concentration of conidia was adjusted using a haemocytometer to 10^6 conidia/ml and used for a pathogenicity test. A pathogenicity test was performed by the wound/drop inoculation method (Than et al., 2008) with five replications. Because *T. roseum* has been known as a postharvest disease pathogen, we carried out the pathogenicity test with detached tomato leaves and fruits (Cultivars Unicon and Summer King). Tomato seeds were sown in a commercial soil mixture in pots (80 mm in diameter), and grown in a plant growth room, in which temperatures ranged from 19-21°C at night and 26-28°C during the day. For the test on leaves, leaves from young tomato plants at ten leaf stages were surface sterilized with 0.05% sodium hypochlorite, pin pricked with a sterile needle and inoculated with 5 µl of conidia suspension. For the test on fruits, small tomato fruits were surface sterilized with 0.05% sodium hypochlorite. The fruit inoculations were performed in the same way

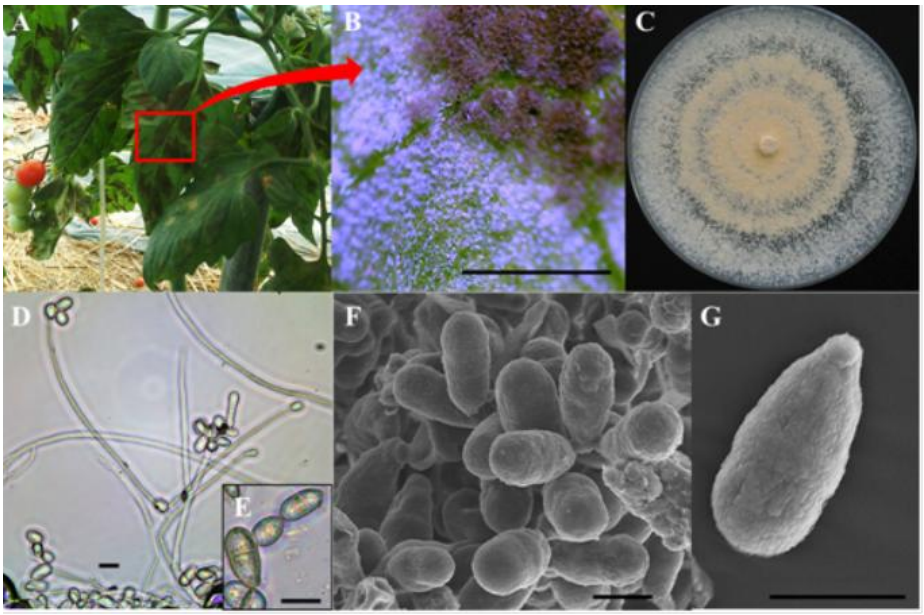

Figure 1. Symptom and morphology of Trichothecium roseum DUCC502. Tomato leaves with brown and yellow patches assumed to be infected with leaf mold (A). Back surface of a tomato leaf colonized by T. roseum with white conidiophores and by Cladosporium fulvum with dark brown conidiophores (B, bar = 1 mm). Colony morphology T. roseum DUCC502 grown on a PDA media plate (C). Conidiophores and conidia of T. roseum observed by a light microscope (D, E) and a scanning electron microscope (F, G). Bar = 10 µm.

as the leaf inoculations. Sterile water was used for a control inoculation. The inoculated fruits and leaves were placed on the surface of antiseptic gauzes that were moistened with sterile water and put in plastic containers (20 x 15 x 20 cm) which kept humidity above 85% during incubation at 25°C for 7 days. During the incubation period, the production of white to pale pink mycelia with conidia and necrotic regions on the surface of tomato fruits and leaves was examined. To verify the infection and colonization of the inoculated fungus, small pieces (0.3 x 0.2 mm) of plant tissues were dissected from the necrotic regions, surface sterilized with 0.02% sodium chlorite solution for 30 s, rinsed twice with sterile water, and placed on PDA plates. Mycelia and conidia grown out and formed from the small plant tissues were observed using a light microscope as mentioned above in the microscopic analysis section.

Extracellular enzyme activity test

Fungal isolate was precultured on PDA at 25°C for 5 days. To evaluate the ability of producing extracellular enzymes which could have a role in the degradation of plant tissues, *T. roseum* DUCC502 was grown on chromogenic media described by Yoon et al. (2007). The chromogenic media contained enzymatic carbon sources such as D-cellobiose (Sigma, USA) for β-glucosidase, polygalactronic acid (MP Biomedicals, USA) for pectinase, starch (Sigma, USA) for amylase, xylan (Sigma, USA) for xylanase , CM-cellulose (Sigma, USA) and avicel (Fluka, Ireland) for cellulase, and skim milk (Fluka, Ireland) for protease. After 10 days of culturing at 25°C, the formation of a clear zone which resulted from the enzymatic reaction of the carbon source substrate and extracellular enzymes produced by the fungus was assessed by measuring its size. The size of the clear zone was considered as relative enzyme activity. Each test was performed with three replicates.

Fungicide sensitivity test

To investigate fungicide sensitivity, the isolate DUCC502 was tested at 25°C on PDA plates supplemented with five different fungicides: azoxystobin, benomyl, dimethomorph, tebuconazole and triflumizole (Blixt et al., 2009). For the test concentration, 10, 20, 50, 100 and 200 µg/ml were used, respectively. After 7 days of culturing at 25°C, colony diameter was determined. Each test was performed with three replicates.

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) in SPSS version 21.0. The significant differences between treatment means were compared using Duncan's multiple range test. Differences were considered significant at $p<0.01$.

RESULTS AND DISCUSSION

Isolation of fungus

We sampled tomato leaves (cultivar Unicon) with severe symptoms of leaf mold disease (Figure 1A). For the diagnosis of the disease, we first observed the sampled leaves by a dissecting microscope (SZ2-ILST, Olympus, Japan). When we looked into the surface of the leaves, we found leaf mold fungus *Cladosporium fulvum* which occurred in the greenhouse under humid conditions (Yan et al., 2008). The leaf mold fungus formed dark brown conidiophores

Table 1. Comparison of morphological characters of the isolate DUCC502 with those of known *Trichothecium roseum.*

Characters		*Trichothecium roseum*[a]	Present study
Colony	color	pale rosy pink to	pale pink to
Conidiophore		hyaline or brightly colored	hyaline
	shape	long and slender	long and slender
	size	N.D	73 - 112 x 2.1-3.3 μm
Conidia	color	hyaline	hyaline
	shape	ovoid	ovoid or ellipsoid
	size	12 - 22 x 5 - 10 μm	11 - 18.3 x 6.1 - 8.5 μm
	no. of cell	2 cells	2 cells

[a]Data from Bello (2008). N.D : no description.

(Figure 1B). This fungal species was found to develop rapidly from lower leaves to the upper surface as seen in Figure 1A. But when we carefully observed the sampled leaves we also found conidiophores with white color on the back surface of the sampled leaves (Figure 1B). Thus, we sampled white conidiophores and performed spore isolation from the conidiophores. Several single spore isolates which showed very similar growth rates and colony patterns on PDA plates were obtained. One of the single-spore isolates was coded as DUCC502 and examined in detail for this study. The voucher specimen was deposited in the Dankook University Culture Collection (Cheonan, Korea).

Microscopic analysis

The fungal colonies were flat, granular, powdery, and formed concentric zonation on PDA at 25°C. The colony color was white initially and became pale pink to peach-colored; the reverse plate was pale (Figure 1C). Conidiophores of the isolate were long and slender, unbranched, 73 - 112 µm in length and 2.1 – 3.3 µm in width (Figure 1D). Conidia of the isolate were two-celled, hyaline colored, ovoid or ellipsoid shaped, 11 – 18.3 µm in length and 6.1 – 8.5 µm in width (Figure 1E-G). These morphological properties were similar to those of *T. roseum* reported by Bello (2008) (Table 1).

Growth test

A mycelial growth test showed that the isolate DUCC502 grew faster on oatmeal agar (OA) than on malt extract agar (MEA) and PDA (Figure 3A). The optimum pH and temperature for mycelial growth of the DUCC502 isolate on PDA were pH 7-9 and 20 or 25°C, respectively (Figure 3B-C). There was no significant difference in pH over the growth of the fungus. After 7 days of incubation on PDA, mycelia of the isolate grew to a diameter of 32.6 mm at 20°C, 33.8 mm at 25°C, 18.3 mm at 30°C and 8.5 mm at 35°C (Figure 3C). Kwon et al. (2010) reported that the optimum temperature of *T. roseum* in strawberries was 25°C. Their report agreed with our results. However, our results disagreed with the report of Hasija and Agarwal (1978) that optimal temperature and pH for both *T. roseum* isolates from apples (*Malus sylvestris*) and plums (*Prunus bokhariensis*) were 28°C and 6.0, respectively. It seems that different strains of *T. roseum* may have different growth properties.

Molecular analysis

To further confirm the identification of the DUCC502 isolate, molecular analysis of 28S rDNA was performed. We obtained PCR amplicon of a 791 bp-sized partial 28S rDNA sequence. A nucleotide sequence similarity search of the GenBank database using the Blast program revealed that the DUCC502 isolate's 28S rDNA shared 99% similarity with that of *T. roseum* CBS113334 (EU552162). A phylogenetic tree showed that the isolate DUCC502 positioned with *T. roseum* CBS113334 (Figure 4). These molecular results conformed to the morphological data (Table 1) that the DUCC502 isolate resembled *T. roseum.* Thus, we concluded that the DUCC502 isolate was identified as *T. roseum.* The 28S rDNA sequence of *T. roseum* DUCC502 was deposited in the GenBank DNA database under accession number JX458860.

Pathogenicity test

T. roseum has been mostly reported in tomato fruit. In this study it was isolated from leaves. Thus, it is interesting to know whether *T. roseum* DUCC502 is able to infect not only tomato fruits but also tomato leaves. Dark necrotic lesions were observed on tomato leaves inoculated with *T. roseum* DUCC502 conidia (Figure 3A). White mycelia with spores appeared on the surface of *T. roseum* DUCC502 conidia-inoculated tomato fruits (Figure 3B).

Table 2. Extracellular enzyme activities of the isolate DUCC502 shown on a chromogenic reaction medium containing each carbon substrate.

Extracellular enzyme						
AMY	**AVI**	**CB**	**CMC**	**XYL**	**PEC**	**PRO**
+	+	++	+	+	+	++

AMY, Amylase; AVI, avicelase; CB, β-glucosidase; CMC, CM-cellulase; XYL, xylanase; PEC, pectinase; PRO, protease; +, moderate activity; ++, strong activity.

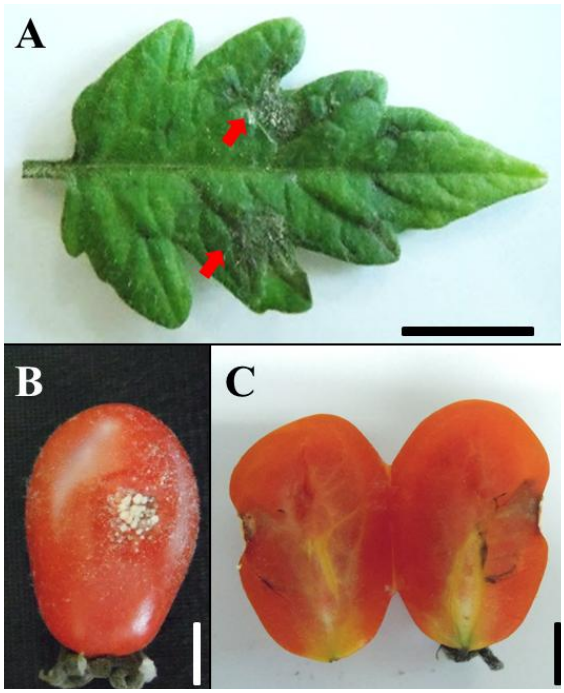

Figure 2. Pathogenicity test results of T. roseum DUCC502 on a young leaf and a tomato fruit. Arrows indicate necrotic lesions formed on a tomato leaf by the artificial inoculation of the fungal spore suspension (A). Symptoms of moldy rot on a tomato fruit by the artificial inoculation of the fungal spore suspension (B). Rotted lesion is shown near inoculation point in the vertically sectioned tomato fruit of Fig. 2 (C). Bar = 10 mm.

Symptoms on the tomato fruit were similar to those reported by Bello (2008). When we vertically sectioned the fruit with symptoms, rotted lesions were observed (Figure 3C). There were no changes on the leaves and fruits with the control inoculation (data not shown). *T. roseum* was re-isolated from the necrotic leaf lesion and rotted fruit lesion, fulfilling Koch's postulates. These *in vitro* results demonstrated that *T. roseum* DUCC502 was able to infect both leaves and fruits of tomatoes.

Extracellular enzyme activity

T. roseum DUCC502 showed amylase, avicelase, β-glucosidase, CM-cellulase, xylanase, pectinase and protease activities (Table 2). β-glucosidase and protease activities, especially, were stronger than other extracellular enzymes. These results showed that *T. roseum* DUCC502 has the ability to degrade some components of plant tissues such as cellulose, xylan and pectin. The reports on amylase, β-glucosidase, and pectinase by Janda-Ulfig et al. (2009), cellulase by Subash et al. (2005), and protease by Buckley and Jeffries (1981) support our results that *T. roseum* has the ability to produce plant cell degrading extracellular enzymes. This may explain how the *T. roseum* DUCC502 could cause necrotic and rotted lesions in tomatoes as shown in Figure 2.

Fungicide sensitivity

Currently, five kinds of fungicides are commercially available for ascomycete plant pathogens in Korea. The recommended concentration of these fungicides for field spray are 332 ug/ml in benomyl, 200 ug/ml in tuberconalzole, 217 ug/ml in azoxystobin, 375 ug/ml in triflumizole, and 250 ug/ml in dimethomorph, respectively. However, these fungicides has never been tested for *T. roseum* in Korea. Thus, we tested these five fungicides. In the dimethomorph-supplemented media, the DUCC502 isolate could grow at all the tested concentrations (Figure 3D). In the triflumizole-supplemented media, its growth was completely inhibited only at 200 µg/ml. In the media contained azoxystobin, the hindrance of the isolate's growth was apparent above 50 µg/ml. No growth was observed at any of the tested concentrations in the media which contained benomyl. The minimum concentration of benomyl was 10 µg/ml. This result confirmed that *T. roseum* is sensitive to benomyl (Luz et al., 2007). We also discovered that *T. roseum* is sensitive to tebuconazole at 10 µg/ml. Overall, our work provides fundamental data for fungicide selection. Except for dimethomorph, the four other fungicides can be used at 200 µg/ml which is below the recommended concentrations for field spray. It is suggested that among the four fungicides, either benomyl or tebuconazole is a better choice for the chemical control of *T. roseum* due to their effect at 40 times or 20 times lower concentration than the commercially recommended concentration.

In summary, we have isolated, identified, and characterized pink mold rot fungus *T. roseum* from tomatoes grown in Korea. This is the first report of a detailed description of *T. roseum* in Korea. At this point, we are not sure

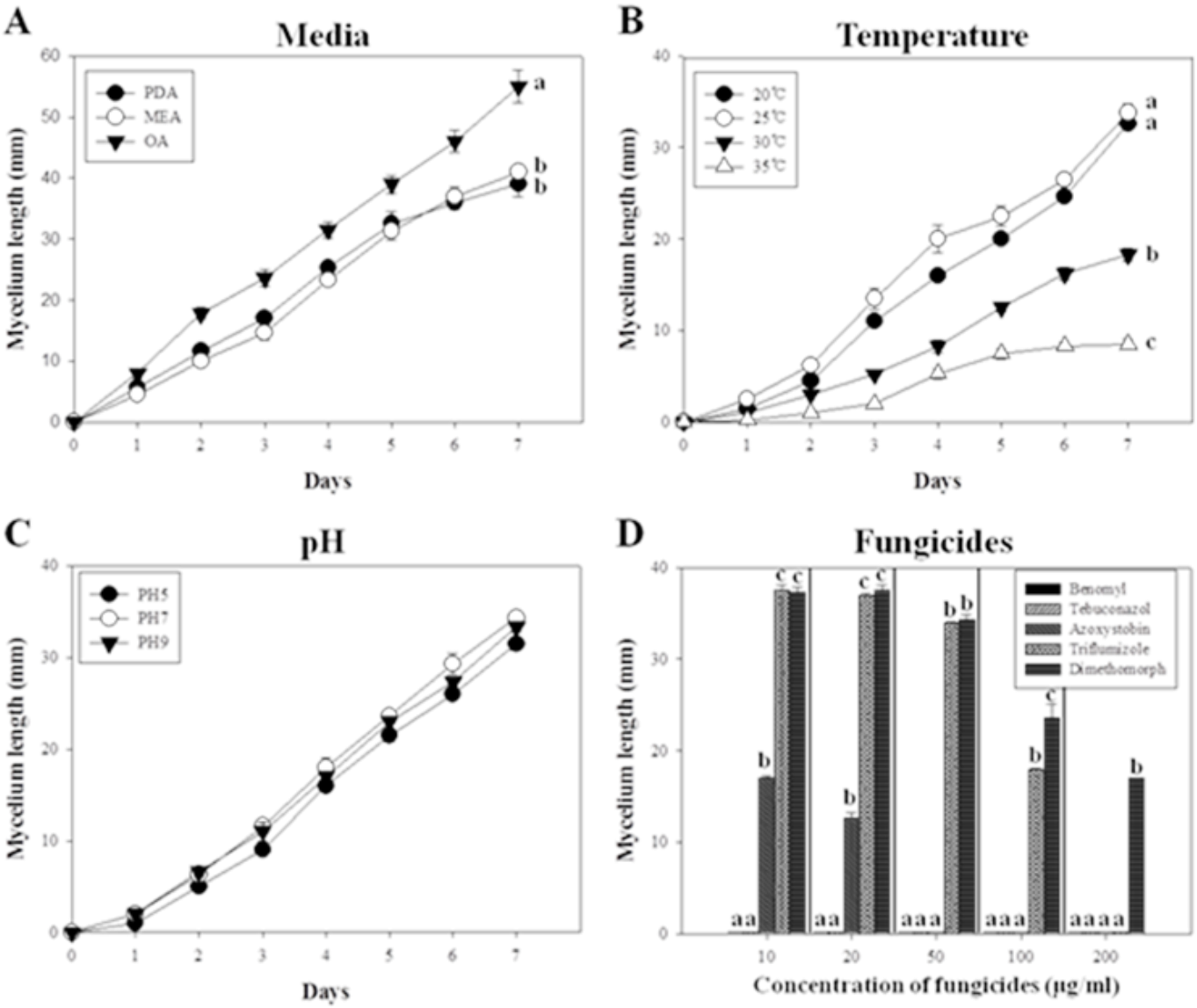

Figure 3. Mycelial growth of the fungal isolate DUCC502 under different growing conditions. Growth on three different media of PDA, MEA, and OA (oatmeal agar) (A), at five different temperatures on PDA (B), at three different pH on PDA (C) and on PDA containing five different fungicides (D). Growth was determined by measuring diameter of grown mycelia. Error bars indicate standard deviations. Mean separation by Duncan's multiple range test at $p < 0.01$. The same letter above or near bars represented no significant difference between treatments.

about the origin of this fungal species. Nowadays, tomato seeds have mostly been imported from Japan and European countries. Thus, one potent suspicion is that the pathogen could have been introduced with imported tomato seeds. The other suspicion is that it could have been introduced from other plant hosts such as melons or strawberries which are cultivated in domestic greenhouses. Because Korea has also imported melon seeds and strawberry seedlings from Japan and other countries, we also could not rule out the possibility that it could have been introduced with these imported plant seeds and seedlings. In Japan, the occurrence of pink mold rot in melons was reported in 1983 (Shinsu and Sakaguchi). To make clear its origin, inspection of imported seed and seedlings would be necessary. In addition, considering that this fungal species also has been known to be able to produce mycotoxins, further work needs to be performed regarding its distribution, yield loss, host range and food hygiene in tomato production.

ACKNOWLEDGEMENTS

This research was supported by the National Institute of Biological Resources, the Research Center of Tomato Export (RCTE), and the Institute of Planning and Evaluation

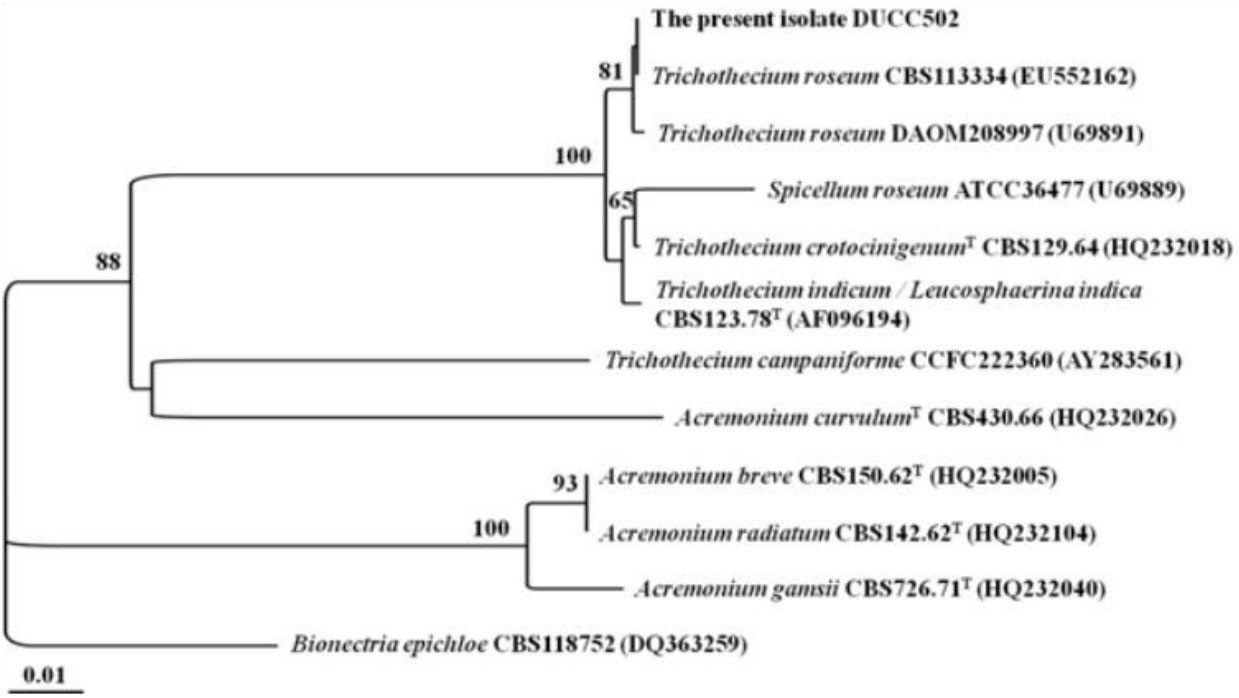

Figure 4. Phylogenetic tree based on partial 28S rDNA of the isolate DUCC502. Phylogram was constructed by the neighbor-joining method using PAUP v.4.0b10. Bootstrap values above 50% are shown at the nodes supported. Bionectria epichloe CBS118752 was used as an outgroup. The letter "T" indicates the type strain of the species.

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