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Isolation of *Geotrichum candidum* pathogenic to tomato (*Solanum lycopersicum*) in Washington State

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Abstract: *Geotrichum candidum* was isolated from fruit of tomato *(Solanum lycopersicum)* in Washington state. Cultural and morphological features, and rDNA sequence data, are reported. Koch's postulates were completed on tomato fruit. This is the first report of this fungus causing disease of tomato in Washington state.

Key words: Plant pathogenic fungus, yeast, molecular determination, fungus taxonomy, Pacific Northwest fungi.

Introduction: During ongoing surveys for naturally-occurring yeasts and yeast-like fungi in Washington State, a fungus was isolated from decaying fruit of tomato (*Solanum lycopersicum* L.). Characterization revealed it to be assignable to *Geotrichum candidum* Link: Fr. (anamorph of *Galactomyces candidus* de Hoog & M. Th. Smith). Although widely known as a pathogen of tomato and other crops, this fungus previously was reported in Washington only from rotten chicken meat (Njoku-Obi et al., 1957). This paper documents the occurrence of *G. candidum* on

tomato in Washington State, as well as ribosomal DNA (rDNA) sequence data, morphological features, and pathogenicity on tomato.

Materials and Methods: The strain was isolated from decaying fruit of *Solanum lycopersicum* collected in a home garden in Whitman Co., WA (N46 43.33 W117 19.11) in October, 2011. White exudate from soft-rotted plant tissue was streaked onto Difco Potato Dextrose agar (PDA) amended with 5 grams/L Difco yeast Extract and 100 ppm streptomycin sulfate (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Isolates for morphological characterization were incubated at ca. 20 °C under fluorescent room lighting (ca. 10 hrs light, 14 hrs dark).

DNA was extracted using "FastDNA® Spin Kit" (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) and the 5' end of the large subunit (LSU) rDNA containing regions D1, D2 and D3 were amplified using primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA, Gardes & Bruns 1993) and TW14 (GCTATCCTGAGGGAAACTTC, Hamby et al. 1998) in 25 µl reactions containing 5.0 µl of DNA template at 10.0 ng / µl, 1.0 µl of each primer at 4.0 pmol / µl, 4.0 µl of dNTP, 5.0 µl of 5x GoTaq ® Flexi Buffer (Promega, Madison, WI, USA), 1.5 µl of $MgCl^2$ at 25 mmol / µl (Promega, Madison, WI, USA) and 0.5 µl of GoTaq ® Taq Polymerase (Promega, Madison, WI, USA). Thermocycler settings were 4-min initial denature at 94 °C followed by 35 repeated cycles of 30 sec denaturation at 94 °C, 30 sec annealling at 54 °C, 1 min 72 °C extension and a final 10 min 72 °C extension. Agarose gel (1.4 %) electrophoresis was used to visualize the PCR product and ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA, USA) to clean PCR products. Four Sanger sequencing reactions were performed with ABI 3730 sequencers (Life Technologies, Carlsbad, CA, USA) by ELIM Biopharm (Hayward, CA, USA) using dye terminator methods and primers ITS1-F, TW14, ITS4 (TCCTCCGCTTATTGATATGC, White et al. 1990) and LR0R (ACCCGCTGAACTTAAGC, Moncalvo et al. 2000). The sequence was assembled using the programs phred 0.071220.c (Green & Ewing 2002) and phrap 1.090518 (Green 2009) within the Chromaseq 1.0 plugin to Mesquite 2.75 (Maddison DR & Maddison WP 2011, Maddison WP & Maddison DR 2011), and uploaded to GenBank (accession KF112070). Strain TOM_YEAST was preserved in a metabolically inactive state in a 15 % glycerol solution at -80 °C in the yeast collection in the Food Science Department at Washington State University.

The phylogenetic position was compared to those of strains of *Geotrichum* and *Galactomyces* represented in GenBank using maximum parsimony, neighbor joining and maximum likelihood methods. The D1/D2 LSU sequence of these strains were aligned using the rmcoffee mode of the program T-Coffee (Notredame et al. 2000) and edited manually. Maximum parsimony and neighbor joining trees were created using MEGA 5.05 (Tamura et al. 2011), with 10,000 bootstrap replications; the data set used for analysis was obtained using a partial deletion method with a 95% site coverage cutoff. The Close-Neighbor Interchange (CNI) tree search method with 10 initial trees and a search level of 3 was used for maximum parsimony, and the Kimura 2-parameter model of nucleotide evolution was used with uniform rates for neighbor joining. Maximum likelihood trees were inferred with GARLI 2.0 (Zwickl 2006) performing 1000 bootstrap replications with genthreshfortopoterm = 10,000. The best tree was obtained by performing 200 searchreps with genthreshfortopoterm = 20,000 and bootstrap results were summarized onto the best tree using the program sumtrees.py (Sukumaran & Holder 2010). The TrN+I+G nucleotide model was chosen for maximum likelihood analysis based on the lowest ranking AIC (Akaike 1974) score using the program jModeltest (Guindon & Gascuel 2003, Posada 2008). The ITS and D1-D3 LSU

sequences of TOM_YEAST were also compared with sequences in GenBank using the BLAST tool (Altschul et al. 1990).

Results and Discussion: For pathogenicity tests, six ripe, organically-grown tomatoes were purchased locally and submerged in 3% NaOCl for 30 seconds, rinsed in sterile water and placed individually in sterile plastic boxes. Each tomato was punctured 1-cm deep in two places on the shoulder with a pipette tip. Inoculum was prepared by scraping cells off of a two-day-old culture of *G. candidum* and suspending them in sterile water. Cells were washed once, and adjusted to 10^6 cells per mL. 25 uL of the cell suspension was inserted into one of the wounds on each tomato using a pipette, and 25 uL of sterile water applied to the other wounds to serve as a control. Inoculated tomatoes were incubated for four days at 20°C until lesions developed. This experiment was done twice using a total of twelve tomatoes.

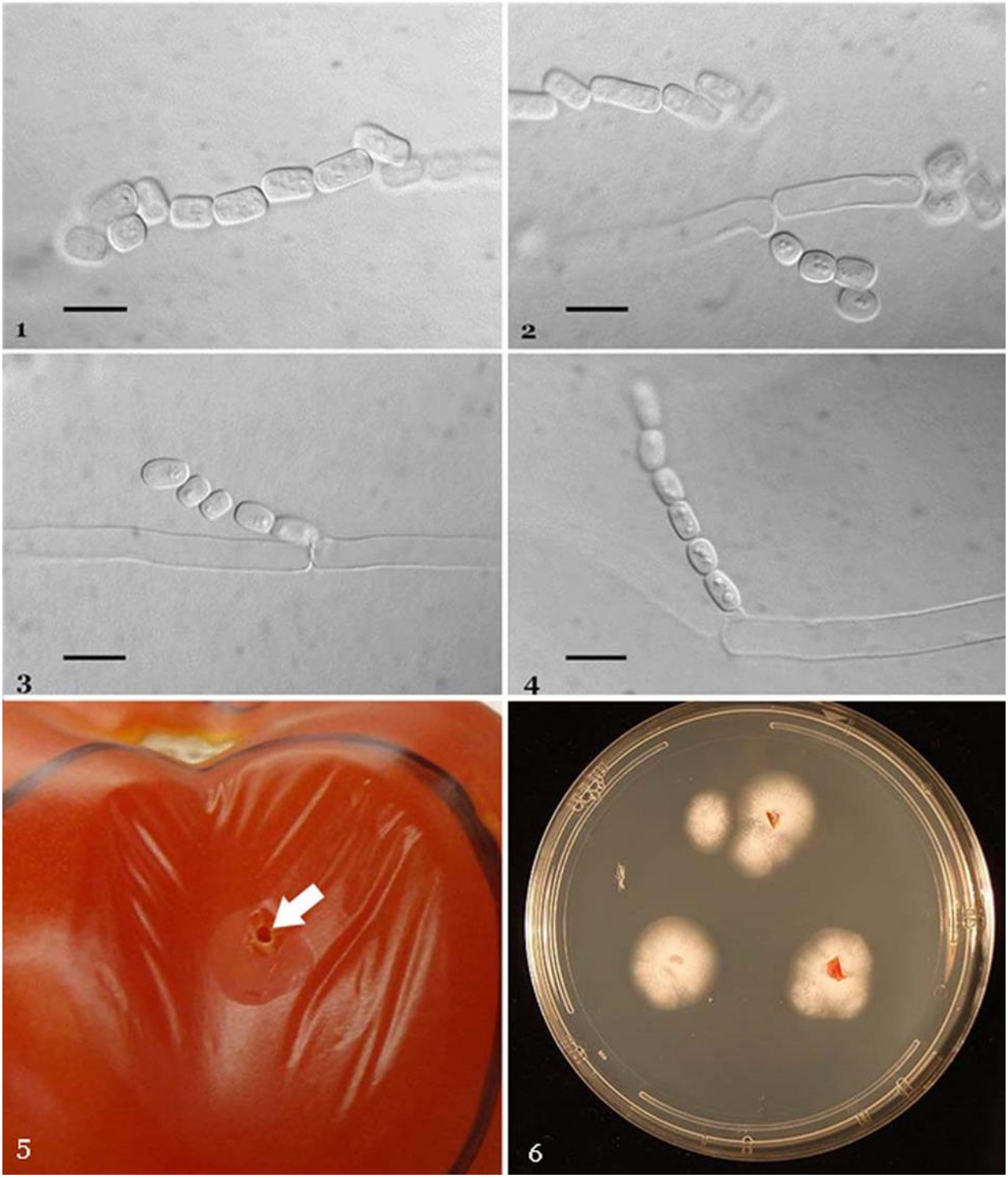
Morphological characterization was as follows: Colonies on PDA becoming ca. 3 cm diam in 72 hr, white, smooth (not shiny), with fruity odor. Reverse pale yellow. On Leonian's agar (Booth, 1971) growth rapid, very thin, hyphae-like to unaided eye, white, without reverse coloration. Hyphae rapidly disarticulating, arthrospores (Figs. 1-4) 2.5-11 X 3-3.5 µm. Large aseptate or sparingly septate hyphae abundant among disarticulating hyphae. No teleomorph was observed.

Characterization of the fungus determined it to be *Geotrichum candidum*. Morphological features of the fungus were consistent with those described for this species (de Hoog and Smith 2011). Analysis of sequence data suggested the fungus can be regarded conspecific with CBS 607.85, previously determined to be *Galactomyces candidus* based on phylogenetic analysis of D1/D2 and actin loci (Groenewald et al. 2012). ITS regions of TOM_YEAST and CBS 607.85 were indistinguishable (295/295 bp), and the D1-D3 LSU sequences differed at a single D3 position (869/870). Phylogenetically, TOM_YEAST was placed in a clade corresponding to *G. candidum* that received strong bootstrap support in all analyses (Fig. 7).

Characteristic sunken, water-soaked lesions developed at each of the sites inoculated with *G. candidum* (Fig. 5) but no symptoms developed at sites inoculated with water. For reisolation, three pieces of tissue were excised from each inoculation site or lesion edge and placed on PDA amended with 50ppm streptomycin sulfate. *Geotrichum candidum* was isolated (Fig. 6) from twelve out of twelve wounds receiving the spore suspension, and from zero out of twelve controls.

Geotrichum candidum was reported (Njoku-Obi et al., 1957) from spoiled chicken meat in Washington many years ago, but has not been associated with plant diseases in the state. This report is the first to document isolation of *G. candidum* from a decayed tomato fruit in Washington State. Completion of Koch's postulates with this fungus on tomato suggests that the fungus could have some economic significance as a tomato pathogen in the region.

Geotrichum candidum has been reported from a wide range of plant substrates and countries (Farr and Rossman, n.d.). In North America it was associated with various fruits in California, Florida, Georgia, Illinois, New Jersey, North Carolina, Pennsylvania, and in Mexico (Farr and Rossman, n.d.). It also has been associated with sour rot of *Daucus carota* in California, Canada, and Illinois, and has been reported from various tree species (Farr and Rossman, n. d.). Industrially, the fungus is important in cheese making, and also has been reported to cause geotrichosis in humans (de Hoog & Smith 2011).



Figs. 1-4. Arthrospores and hyphae produced by *Geotrichum candidum* viewed with differential interference contrast microscopy. Scale bar = 6 µm. Fig. 5. Lesion produced on tomato fruit inoculated with *G. candidum*. Arrow designates point of inoculation. Black line on fruit designates leading edge of lesion. Fig. 6. Colonies of *G. candidum* growing from tissue excised from leading edge of lesion on inoculated tomato fruit.

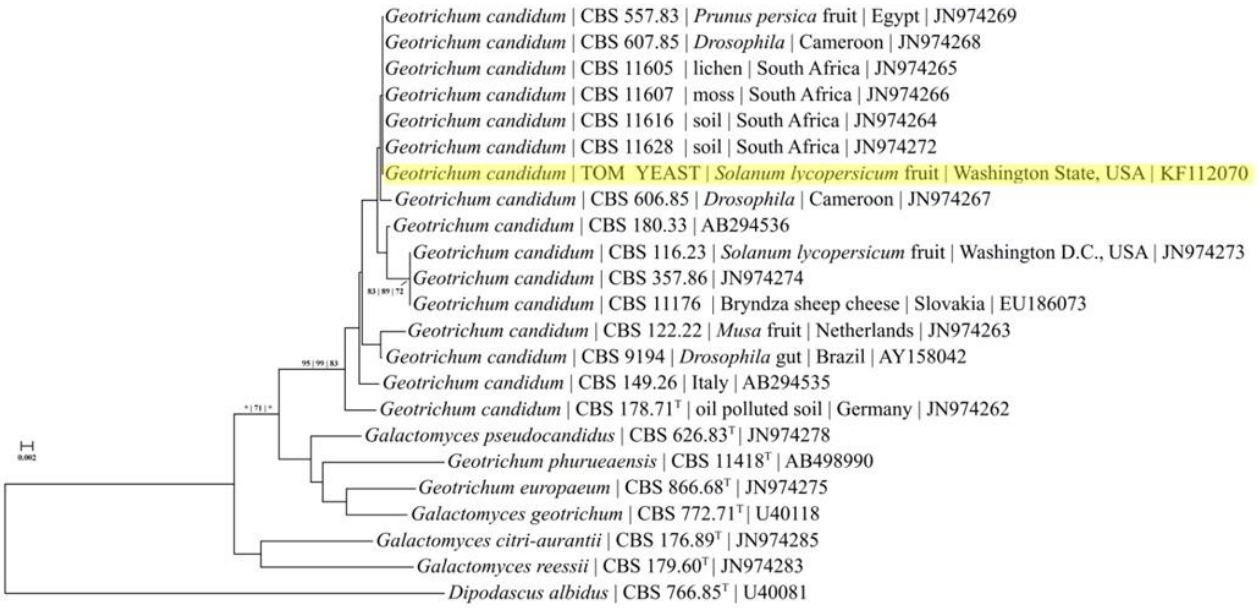


Fig. 7. Neighbor joining tree of *Geotrichum* and *Galactomyces* inferred from D1/D2 LSU rDNA sequences. Bootstrap support values greater than 70 from maximum parsimony, neighbor joining and maximum likelihood methods are indicated in order. Scale is substitutions per site. Names applied to CBS strains follow Groenewald et al. (2012).

Given the broad substrate and geographical range of *G. candidum* it is unsurprising that it now has been found in Washington State. Nonetheless, the ability of this fungus to rot tomato fruit (e.g., Pritchard & Porte, 1923; Moline, 1984; Oladiron and Iwu, 1993) suggests it may be worth further investigation in this region. Characterization of the fungus described in this report, using sequence as well as morphological data, should facilitate such further research.

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