Wilt, Crown, and Root Rot of Common Rose Mallow (*Hibiscus moscheutos*) Caused by a Novel *Fusarium* sp.

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Abstract

A new crown and root rot disease of landscape plantings of the malvaceous ornamental common rose mallow (*Hibiscus moscheutos*) was first detected in Washington State in 2012. The main objectives of this study were to complete Koch's postulates, document the disease symptoms photographically, and identify the causal agent using multilocus molecular phylogenetics. Results of the pathogenicity experiments demonstrated that the *Fusarium* sp.

could induce vascular wilt and root and crown rot symptoms on *H. moscheutos* 'Luna Rose'. Maximum-likelihood and maximum-parsimony phylogenetic analyses of portions of translation elongation factor 1- α and DNA-directed RNA polymerase II largest and second-largest subunit indicated that the *Hibiscus* pathogen represents a novel, undescribed *Fusarium* sp. nested within the *Fusarium buharicum* species complex.

Hibiscus moscheutos L. (rose mallow or crimson-eyed rosemallow) is a malvaceous perennial that is used extensively as a landscape ornamental throughout the United States. This shrubby, cold-hardy perennial is native to wetlands in the central and southeastern United States, with a range that extends into southern Ontario (<http://plants.usda.gov/java/>). This species is the focus of recent management efforts (Environment Canada 2013) as well as research on propagation, genetics, and ecology (Hunter-Cario 2007; Klips et al. 2005; Kudoh and Whigham 2001). Many cultivars and hybrids have long been used in the ornamental landscape industry (Small 2004). Mature plants grow 0.6 to 2.4 meters tall, depending on the cultivar and environment, and produce several stems each year, with large flowers 10 to 25 cm in diameter (Winters 1970). Stems typically die back in the fall, with new growth emerging from the crown in the spring.

Hibiscus is the third highest-ranking deciduous shrub within the United States, based on sales, with an estimated wholesale value of \$23.2 million annually (Boyd and Cheatham 2004). Given its value to the ornamental industry and the long history of plant breeding efforts focused on developing resistant cultivars and hybrids, emergent diseases of *H. moscheutos* could have important implications for the industry. Therefore, the present research was initiated following the discovery of a putatively novel crown rot and vascular wilt disease of several *H. moscheutos* cultivars in a Washington State landscape during 2012 and two subsequent years. Thus, the main focus of this research was to attempt to complete Koch's postulates on *H. moscheutos* 'Luna Rose', compare the disease symptoms induced by the *Fusarium* sp. on rose mallow with those elicited by a closely related species, *Fusarium buharicum* Jacz., on kenaf (*H. cannabinus* L.) (Gerlach and Scharif 1970; Scharif and Akhaviadegan 1967), and identify the *Fusarium*

sp. isolated from symptomatic rose mallow using genealogical concordance phylogenetic species recognition (Taylor et al. 2000).

Materials and Methods

Acquisition and identification of plant material. Specimens of six different cultivars of *Hibiscus moscheutos* ('Plum Crazy', 'Disco Belle', 'Southern Bell White', 'Flare Rose', 'Rainer Red', and 'Mighty Big Pink') plus an unknown cultivar exhibiting symptoms of crown rot were submitted to the Washington State University Plant Pest Diagnostic Clinic from a home landscape in Benton County, WA from 2012 through 2014. Southern Bell White was purchased at a garden center in Pasco, WA; Disco Belle in Kennewick, WA; Flare Rose and Mighty Big Pink in Wagoner, OK; and Rainer Red in Glencoe, MO; however, the distributor of Plum Crazy was unknown. Species identity of submitted material was confirmed by the landscape owner's purchase records and by comparison of specimens with online photographs and descriptions (<http://plants.usda.gov/java/>; <http://www.missouribotanicalgarden.org>). The specimens submitted included living green stems with wilted leaves; some plants had dark-brown to black cankers extending from the crown into the stem. Cankers occasionally bore salmon-colored sporodochia with typical *Fusarium* fusiform, multiseptate macroconidia. Roots of infected crowns showed various levels of rot that usually was restricted to smaller roots.

Fungal isolation and preservation. Tissue from stem and root lesion margins was excised and surface disinfested for 4 min in 0.5% sodium hypochlorite, rinsed with sterile water, and then transferred to petri dishes containing half-strength V8 juice (1/2V8) agar (Stevens 1974) amended with antibiotics (streptomycin sulfate and tetracycline hydrochloride at 50 μ g/liter). Single-spore colonies were obtained by touching a sporodochium growing on 1/2V8 agar with a sterile loop, streaking the sporodochial conidia onto fresh 1/2V8, and then transferring single, well-isolated colonies onto fresh 1/2V8 agar. Cultures were then incubated at ambient room temperature (approximately 24°C) on 1/2V8 agar without antibiotics. Single-spored colonies on 1/2V8 agar grown under near ultraviolet and cool white lighting produced little aerial mycelium but frequently formed concentric rings of salmon-colored sporodochia and abundant dark-blue to black sclerotia. Two representative strains per year collected in 2012, 2013, and 2014 were archived in the Western Regional Plant Introduction Station (WRPIS) culture collection, Pullman, WA in 15% glycerol at -80°C and in the Agricultural Research Service Culture Collection (NRRL), Peoria, IL (Table 1).

Pathogenicity of isolates on *H. moscheutos*. Plants for the pathogenicity experiment were reared from *H. moscheutos* Luna Rose seed

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(Swallow Tail Gardens, Santa Rosa, CA), which had been disinfested with 0.5% sodium hypochlorite solution for 2 min and rinsed in sterile water prior to germination in Sunshine number 5 potting mix. Plants were grown for 7 weeks and were approximately 15 to 20 cm tall when they were inoculated. Inoculum for the pathogenicity experiments was grown on 1/2V8 agar for 3 to 4 weeks for 12 h/day under combined cool white and near ultraviolet light at 21 to 24°C. Conidia were gently washed from the surface of cultures with sterile water and quantified using a hemocytometer; then, sterile water was used to adjust the concentration to 1.0×10^5 and 5.0×10^5 conidia/ml for the first and second experiments, respectively. Each plant was punctured once on the stem just above the crown with a sterile 26-gauge needle to the depth of the needle bevel (2.5 mm). A sterile pipette tip was used to inoculate the wound site with 0.5×10^5 conidia in the first and 2.5×10^5 conidia in the second experiment. Negative controls were inoculated with sterile water. The negative controls (mock-inoculated plants) were established using potting mix from exactly the same fresh bags of Sunshine mix 5 as the inoculated plants. For the initial experiment, plants were placed in plastic trays and sealed in plastic canopies to maintain humidity. Each canopy had a 46-cm² window at each end made of Miracloth (22- to 25- μ m pore mesh; Calbiochem catalog number 475855) for ventilation. In the second experiment, each plant was placed in a tray and then covered with a Sun Bag (Sigma-Aldrich catalog number B7026) that had a single 4-cm², 0.02- μ m pore mesh window. After the inoculated plants had been enclosed in Sun Bags for 72 h, the bags were removed for the duration of the experiment.

Pathogenicity assays were conducted with the following three isolates: 2012-95 (= NRRL 66179), 2013-63 (= NRRL 66182), and 2014-53r (= NRRL 66184). The first experiment was initiated on 23 December 2014 in a WRPIS greenhouse using 24 plants randomly assigned to three inoculation treatments and a negative control. Each of the three treatments employed one of the three fungal isolates listed. The control was inoculated with sterile water. Each treatment and the control consisted of six plants (two replicates of three plants each). Plants were randomly assigned a treatment and position within the greenhouse canopies, using a random sequence function (<https://www.random.org/>). The experiment was repeated beginning on 6 August 2015 and conducted in a temperature-regulated growth room using the same experimental design and treatment

application as the first trial. HOBO data loggers (Onset Computer Corporation, Bourne, MA) were used to record temperature and humidity within the greenhouse canopies and within the growth room. The greenhouse was supplied with 16 h of supplemental daytime lighting and the growth room with 16 h of artificial lighting. During the first experiment, the plastic canopy interiors within the greenhouse ranged from 8 to 12°C (nights) and 16 to 27°C (days). The growth room temperature ranged from 17.5 to 19.5°C (nights) and 24 to 26°C (days) during the second experiment.

Lesion diameter, plant height, and the presence or absence of disease symptoms were recorded 5.5 weeks postinoculation in the initial experiment and 2.5 weeks postinoculation in the second experiment. The maximum diameter of stem lesions was measured near the crown of the plant, or the wound site was measured in the absence of lesion development in the negative controls. Lesion margins (or the wound site in the absence of rot) were excised aseptically, surface disinfested for 2 min in 0.5% sodium hypochlorite solution, rinsed with sterile water, and then plated on 1/2V8 agar amended with antibiotics for reisolation of the pathogen. The novel *Fusarium* sp. was reisolated from the inoculated wound sites but never from the mock-inoculated wound sites. To complete Koch's postulates, isolates recovered from plants used in the pathogenicity experiments were identified using a partial translation elongation factor 1- α (*TEF1*) sequence.

Statistical analysis. Each of the three isolates is regarded as a treatment. Analysis of variance (ANOVA; to detect overall differences between treatments and the control), *t* tests (to detect differences between individual treatments and the control), and general linear models (GLM; Fisher's least significant difference, model mean square error, to detect pairwise differences between treatments) were conducted in Systat 13 (Systat Software, Inc., Chicago).

Molecular phylogenetic identification of fungal isolates. Isolates were cultured in yeast-malt broth for 4 days and freeze dried overnight; then, a hexadecyltrimethyl-ammonium bromide (Sigma-Aldrich, St. Louis) protocol was used to extract total genomic DNA, as previously described (O'Donnell et al. 1998). Portions of the following three phylogenetically informative loci were polymerase chain reaction (PCR) amplified and sequenced following published protocols (O'Donnell et al. 2010, 2013): *TEF1* and DNA-directed RNA polymerase II largest (*RPB1*) and second-largest (*RPB2*) subunits. Platinum *Taq*

Table 1. Isolates used in the phylogenetic analysis

NRRL ^a	Equivalent number ^b	<i>Fusarium</i> spp.	Host, substrate	Geographic origin
13371	CBS 796.70	<i>Fusarium buharicum</i> Jacz.	<i>Hibiscus cannabinus</i> L.	Iran
13384	CBS 189.34	<i>F. subglutinatum</i> Reinking	Soil	Costa Rica
13622	FRC I-55	<i>F. lateritium</i> Wollenw.	<i>Ulmus</i> sp.	Louisiana
20429	ATCC 15662	<i>F. stilbioides</i> Wollenw.	Coffee	Nyasaland
20472	CBS 745.79	<i>F. sarcochromium</i> (Desm.) Sacc.	<i>Viscum album</i> L.	Switzerland
20897	CBS 190.34	<i>F. subglutinatum</i>	Soil	Costa Rica
25488	CBS 178.35	<i>F. buharicum</i>	<i>Gossypium</i> sp.	Russia
36148	CBS 109638	<i>F. buxicola</i> Sacc.	<i>Buxus</i> sp.	Belgium
53998	CBS 101734	<i>F. cyanostomum</i> (Sacc. & Flageolet) O'Donnell & Geiser	<i>Buxus sempervirens</i> L.	France
54149	JAS 481	<i>F. torreyae</i> T. Aoki, J. A. Smith, L. Mount, Geiser and O'Donnell	<i>Torreya taxifolia</i> Arn.	Florida
66179	WRPIS 2012-95	<i>Fusarium</i> sp.	<i>H. moscheutos</i> L. Southern Belle White	Washington
66180	WRPIS 2012-121	<i>Fusarium</i> sp.	<i>H. moscheutos</i> Flare Rose	Washington
66181	WRPIS 2013-62	<i>Fusarium</i> sp.	<i>H. moscheutos</i> Mighty Big Pink	Washington
66182	WRPIS 2013-63	<i>Fusarium</i> sp.	<i>H. moscheutos</i> Plum Crazy	Washington
66183	WRPIS 2014-53s	<i>Fusarium</i> sp.	<i>H. moscheutos</i> unknown cultivar	Washington
66184	WRPIS 2014-53r	<i>Fusarium</i> sp.	<i>H. moscheutos</i> unknown cultivar	Washington
–	F201114	<i>F. zanthoxyli</i> X. Zhou, T. Aoki, K. O'Donnell & Z. M. Cao	<i>Zanthoxylum bungeanum</i> Maxim.	China
–	F201126	<i>F. continuum</i> X. Zhou, T. Aoki, K. O'Donnell & Z. M. Cao	<i>Z. bungeanum</i>	China

^a Agricultural Research Service Culture Collection, Peoria, IL.

^b ATCC = American Type Culture Collection, Manassas, VA; CBS = CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; F = Northwest A&F University, Shaanxi, China; FRC = *Fusarium* Research Center, Department of Plant Pathology, The Pennsylvania State University, University Park; JAS = Jason A. Smith, University of Florida, Gainesville; and WRPIS = Western Regional Plant Introduction Station, Pullman, WA.

DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) was used for all PCR amplifications, amplicons were purified with Montage₉₆ filter plates (Millipore Corp., Billerica, MA), BigDye 3.1 Terminator reaction mix was used to sequence the amplicons, and sequencing reaction mixes were purified with BigDye Xterminator prior to running them on an ABI 3730 genetic analyzer. ABI sequence chromatograms were edited with Sequencher 5.2.4 (Gene Codes Corp., Ann Arbor, MI) and then exported as three separate partitions. The *RPB1* and *RPB2* partitions lacked indels (insertions and deletions), and, therefore, were well aligned; however, MUSCLE (Edgar 2004) was used to align the indel-containing *TEF1* partition. The NEXUS files were analyzed using maximum-likelihood (ML) (Zwickl 2006) and maximum-parsimony (MP) (Swofford 2003) methods as described by Crespo et al. (2016). BLASTn searches of GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), using the partial *RPB1* and *RPB2* sequences as the queries, suggested that the *H. moscheutos* crown rot pathogen likely represented a novel species closely related to *F. buharicum*, a malvaceous pathogen of kenaf (*H. cannabinus*) and cotton (*Gossypium* sp.). DNA sequences generated in the present study were deposited in GenBank as KX302912 to KX302935. The NEXUS and tree file were accessioned in TreeBASE as S19369 and Tr96424, respectively.

Results

Pathogenicity assays. Some wilt, root, and crown rot symptoms were observed on all of the experimental plants inoculated with the *H. moscheutos* pathogen, *Fusarium* sp. NRRL 66179, 66182, or 66184 in both experiments (Fig. 1 and 2A). However, disease symptoms were not observed on the mock-inoculated negative control (Fig. 2B). Colonies that grew from the advancing margins of stem lesions on 1/2V8 agar were morphologically identical to those used as inoculum; they were identified as the novel, undescribed *Fusarium* sp. by sequence analysis of the *TEF1* region (i.e., 100% match; data not shown).

Statistical analysis. ANOVA, *t* tests, and GLM pairwise comparisons grouped by treatment, using lesion diameter as the dependent variable, yielded $P = 0.000$ for inoculated treatments versus controls in all tests. Differences between treatments (isolates) under GLM were significant only in the first experiment. *P* values for *t* tests between treatments (isolates) and controls indicated that treatments and controls differed, thereby supporting pathogenicity of each isolate. However, the paired comparisons between treatments (isolates) conducted with GLM, when repeated, did not consistently result in $P \leq 0.05$ for any given pair; thus, there was no statistical support for differences in aggressiveness (virulence).

Identification of fungal isolates via phylogenetic analysis. In an attempt to identify the six strains isolated from symptomatic *H. moscheutos* stem and root tissue (Table 1), nucleotide sequence data were obtained from portions of *TEF1*, *RPB1*, and *RPB2* and used to conduct BLASTn queries of the National Center for Biotechnology Information (NCBI) GenBank, and *Fusarium* MLST (<http://www.cbs.knaw.nl>). Because the six strains shared identical *TEF1*, *RPB1*, and *RPB2* alleles, suggesting that they might represent clones, only sequences of NRRL 66179 were used to conduct BLASTn searches

Fig. 1. *Hibiscus moscheutos* Luna Rose 2.5 weeks after three plants to the left were inoculated with *Fusarium* sp. isolate 2013-63 (NRRL 66182) and three plants to the right were inoculated with sterile distilled water.

of the two web-accessible databases. Searches using the partial *TEF1* sequence as the query failed to identify any accessions producing significant alignments. However, searches of NCBI conducted with the partial *RPB1* and *RPB2* sequence data identified sequences of *F. buharicum* NRRL 13371 (JX171449 and JX171563) and *F. sublunatum* NRRL 13384 (JX171541 and JX171565) as the two best matches, with 93 and 90% identity, respectively. To obtain a definitive identification, the partial *TEF1*, *RPB1*, and *RPB2* sequence data obtained from the six *H. moscheutos* strains were aligned with those of closely related species within the *F. buharicum*, *F. torreyae*, and *F. lateritium* species complexes, based on our current understanding of phyletic relationships within the genus (O'Donnell et al. 2013), and analyzed phylogenetically using ML and MP methods. Analyses of the three individual partitions, in which the gene trees were midpoint rooted, indicated that the *Hibiscus* pathogen was nested within the *F. buharicum* species complex (FBSC) (ML/MP bootstrap support [BS] = 98 to 100%) and closely related to *F. buharicum* (ML-BS/MP-BS = 89 to 100%) (Fig. 3A to C). An identical topology was obtained when the partial *RPB1* + *RPB2* data were analyzed as a combined dataset and the phylogeny was rooted on sequences of *F. buxicola* and *F. cyanostomum* (Fig. 3D), based on more inclusive analyses (O'Donnell et al. 2013). The FBSC was strongly supported as sister to a clade comprising members of the *F. lateritium* and *F. torreyae* species complexes (Fig. 3A to D), which are primarily canker-inducing tree pathogens (Smith et al. 2011; Zhou et al. 2016).

Discussion

Pathogenicity experiments and multilocus molecular phylogenetics were used in the present study to characterize a novel *Fusarium* sp. responsible for a vascular wilt and crown and root rot of several *H. moscheutos* cultivars in a Washington State landscape planting. Results of the pathogenicity experiments show that the vascular wilt, root, and crown rot disease of the *H. moscheutos* cultivars in the Washington State landscape planting was caused by a novel *Fusarium* sp. closely related to two fusaria that are also pathogens of malvaceous hosts, *F. buharicum* and an undescribed species. The disease symptoms noted here are remarkably similar to those reported for *F. buharicum* on kenaf (Gerlach and Scharif 1970; Scharif and Akhavi-zadegan 1967). Both pathogens induce wilt together with root, crown, and stem rot on their respective hosts and blackening of the stem base, upon which abundant salmon-colored sporodochia were formed.

The *Hibiscus* pathogen was strongly supported by ML and MP bootstrapping as a genealogically exclusive species lineage (Taylor et al. 2000) closely related to *F. buharicum*, another malvaceous pathogen within the FBSC. Although partial *TEF1* sequence data are the most widely used region to identify fusaria via BLASTn queries of

Fig. 2. Stem base of *Hibiscus moscheutos*. A, Two and one-half weeks after it was inoculated with *Fusarium* sp. isolate 2013-63 (NRRL 66182), showing canker with salmon-colored sporodochia. B, Asymptomatic negative control.

GenBank, *Fusarium*-ID, or *Fusarium* MLST (Geiser et al. 2004; O'Donnell et al. 2010), we discovered that *TEF1* sequences from members of the FBSC were not represented in these web-accessible databases. This finding highlights the need for a concerted effort to populate these databases with reference *TEF1*, *RPB1*, and *RPB2*

sequences for all of the species complexes and monotypic lineages within *Fusarium* to facilitate identification of unknowns via the Internet.

Booth (1971) and Gerlach and Nirenberg (1982) accepted the placement of *F. buharicum* within the morphologically defined

Fig. 3. **A** to **C**, Maximum-parsimony (MP) phylograms inferred from portions of three phylogenetically informative protein-coding genes, which were rooted by the midpoint method. *EF1-α* = translation elongation factor 1-α; *RPB1* and *RPB2* = DNA-directed RNA polymerase II largest and second-largest subunits, respectively; MPT = most-parsimonious tree; CI = consistency index; and RI = retention index; **D**, Phylogram inferred from the combined *RPB1* + *RPB2* dataset rooted on outgroup sequences of *Fusarium buxicola* and *F. cyanostomum*. Note that strains of the novel *Fusarium* sp. pathogenic to *Hibiscus moscheutos* share identical *TEF1*, *RPB1*, and *RPB2* alleles and that it is strongly supported as a genealogically exclusive lineage (maximum-likelihood [ML] bootstrap [BS]MP-BS = 100%) within the *F. buharicum* species complex. This complex is sister to a clade comprising the *F. lateritium* and *F. torreyae* species complexes. The number above internodes represents ML-BS support based on 1,000 pseudoreplicates of the data. MP-BS is not indicated because it differed from the ML-BS value by ≤5%.

section Discolor. However, because molecular phylogenetic analyses have revealed that the sectional classification of *Fusarium* is largely artificial (Gräfenhan et al. 2011; O'Donnell et al. 2013), including that of section Discolor, we advocate referring to the genealogically exclusive lineages as species complexes. Currently, only two isolates of *F. buharicum* are housed in publically accessible culture collections and they include NRRL 13371 (= FRC R-4955 = CBS 796.70 = DSM 62165) from stem lesions of kenaf (*H. cannabinus* L.) in Iran (Gerlach and Scharif 1970) and NRRL 25488 (= CBS 178.35 = DSM 62166) from crown (collar) rot of cotton (*Gossypium* sp.) in Russia and Uzbekistan (Gerlach and Nirenberg 1982). In addition to *F. buharicum* and the *Fusarium* sp. reported here from *H. moscheutos*, a closely related, undescribed species within the FBSC appears to be pathogenic to several malvaceous weeds in North America, including velvetleaf (*Abutilon theophrasti* Medik.), spurred anoda (*Anoda crisata* (L.) Schldtl.), and prickly sida (*Sida spinosa* L.) (unpublished data). This finding suggests that these three fusaria may have evolved some host specialization on members of the Malvaceae.

Although we did not find any reports of *Fusarium* spp. causing disease of *H. moscheutos* (Farr et al. 1989; Jones and Benson 2001), a plant that is native to North America (Small 2004), it is possible that this pathogen might have been misidentified in the phytopathological literature. Therefore, future surveys of this pathogen are needed to assess its genetic diversity and to elucidate its area of endemism, host range, and epidemiology so that proactive measures can be taken to minimize its impact on the ornamental industry.

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