

Original Research Paper

The First Report of *Fusarium sacchari* Causing Yellow Leaf Spot Disease on *Rhynchostylis gigantea* Orchids in Thailand

Kanokwan Dekham and Krieng Kanchanawatee

Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

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Corresponding Author:

Krieng Kanchanawatee,
Department of Biology, Faculty
of Science, Chulalongkorn
University, Bangkok, Thailand;
Email: Krieng.K@chula.ac.th

Abstract: Yellow leaf spot disease was observed on *Rhynchostylis gigantea* orchids on an orchid farm in the Nonthaburi province of Thailand. Symptoms started with yellowish patches lacking definitive borders on the leaves of the orchids. The patches slowly expanded and merged, creating larger patches; a brown spot or black necrotic area then emerged at the center of some of the patches. *R. gigantea* leaves exhibiting these symptoms were used as the source for isolation studies. Based on the morphological characteristics of the fungal isolate (colony appearance, macroconidia, microconidia, conidiophore and chlamydo-spore structures), phylogenetic analysis of translation elongation factor-1 α sequence and fulfillment of Koch's postulates, the causative pathogen was identified as *Fusarium sacchari*. To our knowledge, this study is the first to report *F. sacchari* as a pathogen of *R. gigantea*.

Keywords: *Fusarium sacchari*, *Rhynchostylis gigantea*, Yellow Leaf Spot Disease

Introduction

Orchids are the most exported tropical ornamental crop of Thailand. Among the ornamental orchids, the genus *Rhynchostylis* is one of the most popular cultivations due to its unique fragrance and long tapering inflorescences (Thammasiri, 2016). However, fungal infestation is a major problem in cultivated *Rhynchostylis* species. This led us to survey fungal diseases on orchid farms in the central region of Thailand. On an orchid farm in Nonthaburi province, Thailand, we found many *Rhynchostylis gigantea* plants damaged by yellow leaf spot disease, leading to progressive death from the infected area.

Phytopathogenic *Fusarium* fungi can cause leaf spot diseases in various plants, from pteridophytes (Mali *et al.*, 2015) to angiosperms, including tobaccos (Li *et al.*, 2017), tomatoes (Gao *et al.*, 2016), mangos (Omar *et al.*, 2018), screw pines (Dehghan-Niri and Rahimian, 2016), lettuces (Garibaldi *et al.*, 2015), pineapples (Ibrahim *et al.*, 2017), onions (Parkunan and Ji, 2013), oleanders (Mirhosseini *et al.*, 2014) and palms (Ashfaq *et al.*, 2017). The yellow leaf spot disease caused by *Fusarium* species can only be found in orchids (Han *et al.*, 2015; Ichikawa and Aoki, 2000; Leslie and Summerell, 2006). Symptoms of the yellow spot disease described by Ichikawa and Aoki (2000) in *Cymbidium* orchids

infected with *Fusarium proliferatum* and *Fusarium subglutinans* began with small, water-soaked patches on the leaves. The patches then expanded and became reddish-brown, surrounded by yellowish swellings without definitive borders. The lesions then fused into a black necrotic area.

Fusarium species can be identified by their macroscopic and microscopic morphological characteristics, colony appearance, macroconidia and microconidia structures, conidiophore structures and chlamydo-spores (Leslie and Summerell, 2006). However, since many *Fusarium* belong to a species complex, accurate identification based solely on morphology is not reliable due to the difficulty in distinguishing between species (Omar *et al.*, 2018). Molecular identification is an additional tool for *Fusarium* identification that can be used with morphology. DNA sequence analysis of the translation elongation factor-1 α (TEF-1 α) gene has been successfully used to identify *Fusarium* species (Geiser *et al.*, 2004; O'Donnell *et al.*, 1998).

Although *Fusarium* species are known pathogens of orchids, knowledge of the species-specific interactions between host orchids and pathogenic fungi is still limited. The present study was conducted to identify *Fusarium* fungi isolated from *R. gigantea* exhibiting yellow leaf spot disease.



Fig. 1: Yellow leaf spot symptoms on *R. gigantea*. (A) Early symptoms appeared as yellowish patches without definitive borders, some containing a brown or black spot at the center. (B) The yellowish patches spread from the early-infected areas to other healthy leaf tissues. The wilted and brownish leaves clung to the stems of the late-infected leaves

Materials and Methods

Sampling and Fungal Isolation

Four infected *R. gigantea* plants were collected from an orchid farm in Nonthaburi province, Thailand. The plants were brought to the laboratory where the infected leaves were cut. The fungus isolation was performed from the infected young leaves. Leaves were washed three times with detergent and transferred to 20% (v/v) household bleach (Clorox) and 0.1% (v/v) Tween 80 for 20 min. The leaves were further washed with 70% (v/v) ethanol for 5 min and finally rinsed with sterile deionized water. To verify that the surface of the leaves was completely sterile, the deionized water used for rinsing the leaves in the final step was plated onto Luria-Bertani agar medium (HiMedia, India) and potato dextrose agar medium (PDA, HiMedia, India) and cultured at 27°C for 7-10 d to check for bacterial and fungal colonies.

The surface-sterilized orchid leaves were cut into 1×1 cm squares and then placed on the PDA plates and incubated at 27°C until mycelia appeared. Single-spore cultures were established using the dilution plating technique (Hansen and Smith, 1932) and then used in all experiments.

Morphological Identification

To assess macroscopic characteristics, fungi were cultured on PDA at 27°C for 7-10 d. The colors of the colonies on both sides of the petri dishes were determined according to Summerell *et al.* (2003). The color and appearance of the sporodochia from a culture grown on Carnation Leaf Agar (CLA) at 27°C for at least 2 weeks was also observed (Leslie and Summerell,

2006). CLA was prepared in accordance with Leslie and Summerell (2006). To assess microscopic characteristics, observations were performed on the 2- to 4-week-old cultures grown on CLA at 27°C (Hafizi *et al.*, 2013). The appearance, width and length of macroconidia and microconidia were recorded using a compound light microscope with a digital camera. Conidiophores (mono- or poly-phialides) were also observed (Summerell *et al.*, 2003) using the slide culture techniques in accordance with Johnson (1946). Briefly, 1.0×1.0 cm blocks of PDA were placed onto a sterile slide. The fungus was then inoculated at the margin of the PDA blocks with a sterile loop, followed by placement of a sterile coverslip over the block and incubation at 27°C for 3 d. After incubation, slide cultures were stained with lactophenol cotton blue and observed under a compound light microscope with a digital camera. All morphological characteristics were observed in the fungal cultures grown under a 12 h alternating period of light and dark.

Fungal DNA Extraction

DNA extraction was performed according to Cenis (1992) with minor modifications, where 400 µL extraction buffer (200 mM Tris pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate) was added to a loop-full of mycelia. The mycelia were ground using a plastic pestle in a 1.5-mL microcentrifuge tube and 8 µL of 10 mg/mL RNase A was added to the suspension and incubated at 65°C for 10 min. Then, 150 µL of 3 M sodium acetate (pH 5.2) was added and incubated at -20°C for 10 min, followed by centrifugation at 10,000 × *g* for 15 min. The supernatant was transferred to a new 1.5-mL microcentrifuge tube and 650 µL of isopropanol was

added, mixed and centrifuged at 10,000 x g for 10 min to precipitate the DNA. The DNA pellet was washed with 70% (v/v) ethanol, air-dried, dissolved in 100 µL of dH₂O and kept at -20°C until used.

PCR Amplification and DNA Sequencing

For fungal identification, the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) and TEF1-α gene of DNA were PCR-amplified and sequenced. The ITS1-5.8S-ITS2 region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White *et al.*, 1990) with thermal cycling at 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 50 s and then a final 72°C for 10 min. The TEF-1α region was PCR-amplified using EF-1 (5'-GTAAAGAGGCGCGGTGTCGGTGTG-3') and EF-2 (5'-GGAAGTACCAGTGATCATGTT-3') primers (O'Donnell *et al.*, 1998) with thermal cycling at 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 50 s and then a final 72°C for 10 min. The DNA fragments were amplified using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) thermocycler. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol and sent for commercial Sanger sequencing at AITbiotech, Singapore. Sequencing was performed on both strands using the same forward and reverse primers used for PCR amplification.

Phylogenetic Analysis of the Sequence Data

The ITS1-5.8S-ITS2 nucleotide sequences were compared to sequences in the GenBank database using the BLASTn algorithm (<http://blast.ncbi.nlm.nih.gov/>; Clark *et al.*, 2016). The TEF-1α nucleotide sequences of species related to the sample and outgroup were selected from the FUSARIUM-ID database to construct phylogenetic trees (Geiser *et al.*, 2004). Sequences were aligned by CLUSTAL W using multiple alignment in the Molecular Evolutionary Genetics Analysis 7 (MEGA 7) software (Kumar *et al.*, 2016). The distance of relationships among these same pathogens was constructed using the distance-based Neighbor-Joining (NJ) algorithm with Kimura's two-parameter calculation model in MEGA 7. For statistical support, a bootstrap of 1,000 times was implemented.

Detached Leaf Inoculations

Detached leaf inoculations were performed on fully-developed *R. gigantea* leaves. The leaves were washed five times with tap water followed by 70% (v/v) ethanol and sterile distilled water. Cotton wool soaked with sterile water was used to provide water and a moist environment for the leaves. Mycelium plugs cut from the

edge of growing colonies on PDA were placed on the adaxial surface. For wounded inoculations, the leaves were wounded by a sterile needle before placing the mycelium plugs at the injury locations. The inoculated leaves were kept in sealed clear plastic containers and held at 100% RH and 28°C with a 18 h photoperiod for 14 d. The cotton wool was changed every 3 d.

Results

Morphological Identification and Characterization of Pathogens

The diseased *R. gigantea* used in this study was selected from an orchid farm in Nonthaburi province, Thailand, where the *R. gigantea* were heavily infected with disease symptoms resembling yellow leaf spot disease on leaves. The symptoms started with 1-2 mm yellowish patches without definitive borders. The patches then slowly enlarged and merged with adjacent patches. A brown spot or black necrotic area then develop at the center of some of the yellowish patches while the disease slowly spread to the entire plant (Fig. 1A and 2A). Late infection turned whole leaves brown and wilted. The leaves still clung to the stem and were ready to drop off (Fig. 1B).

With regard to the macroscopic characteristics of fungal morphology, the colony color of the PDA culture was initially orange-yellow and became purple-white when aged (Fig. 2B and 2C). When the fungus was cultured on CLA, the sporodochia had a cream color and globular shape (Fig. 2D). With regard to microscopic characteristics, macroconidia production was largely induced on CLA. The morphology of the macroconidia was slender, slightly falcate and thin-walled. Although three septate macroconidia were the most common, two and four septate macroconidia were also observed. The mean length and width of macroconidia was 47.38±4.8 µm and 2.58±0.3 µm (N = 50), respectively (Fig. 2E). Macroconidia were also observed when the fungus was cultured on PDA, but at a very low frequency. The fungus grown on CLA or PDA produced abundant microconidia in the aerial mycelia. The shape of the microconidia was oval and slender with one or no septa. The mean length and width of microconidia were 8.85±2.3 µm and 1.74±0.3 µm (N = 50), respectively (Fig. 2F). The microconidia were produced on short mono- or polyphialides in false heads (Fig. 2G and 2H).

Chlamydospores were rarely found in cultures grown on PDA. Single verrucose chlamydospores were detected in slide cultures grown on PDA at 3 d after inoculation (Fig. 2I). Morphological characteristics of the fungus were consistent with the description of *Fusarium sacchari*, which is a member of the *Gibberella fujikuroi* species complex (Leslie and Summerell, 2006).

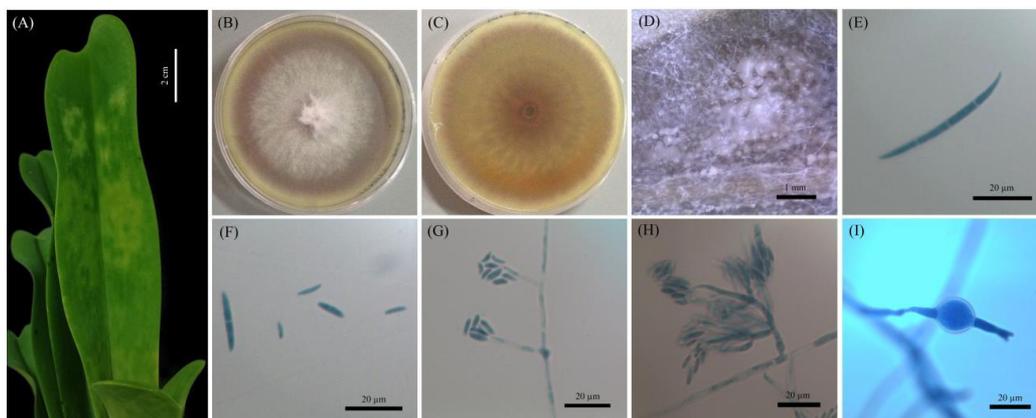


Fig. 2: Yellow leaf spot symptoms on *R. gigantea* leaves and the morphological characteristics of *F. sacchari*. (A) Symptoms on young leaves of *R. gigantea* showing yellowish patches without definitive borders. (B–I) Morphology of the cultured fungal isolate on PDA (B, C, G– I) or CLA (D– F) showing the (B) colony upper surface, (C) colony lower surface, (D) sporodochia, (E) macroconidia, (F) microconidia, (G) monophaealides, (H) polyphaealides and (I) chlamydospores

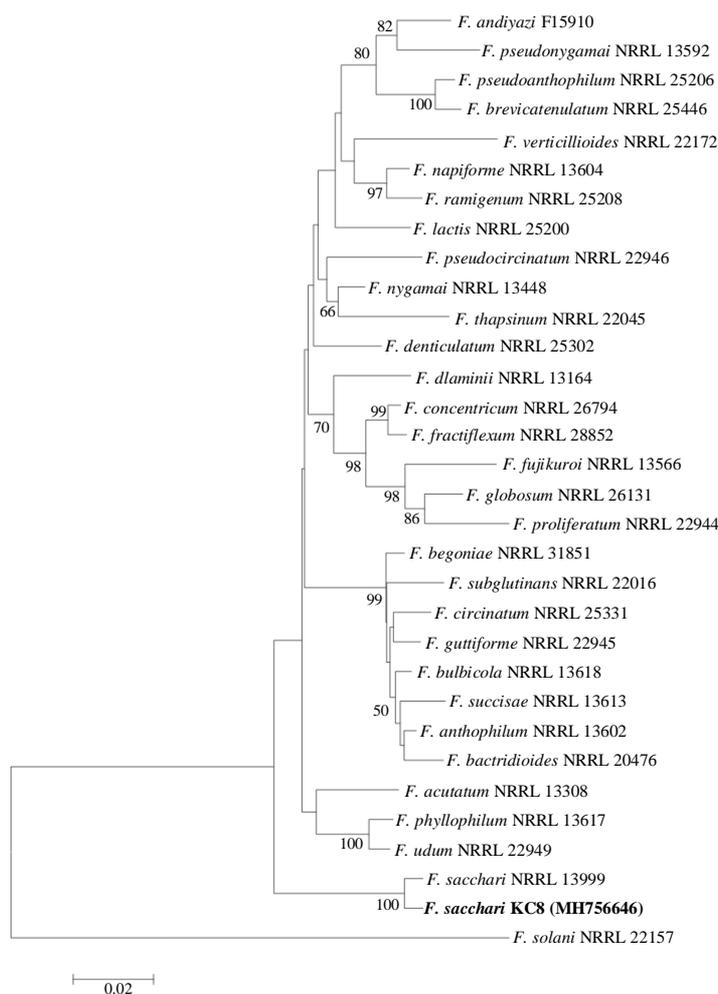


Fig. 3: Phylogenetic tree showing the inferred relationships of 30 *Fusarium* species from the *G. fujikuroi* species complex with the sample of this study (in bold) nested with *F. sacchari*. The tree was constructed using the NJ method (Kimura 2-parameter model). Accession numbers are given in parentheses. Bootstrap values of $\geq 50\%$ (1000 replicates) are shown. The *F. solani* strain NRRL 22157 was used as an outgroup. The scale bar represents 0.02 nucleotide substitutions per site

Molecular Identification and Phylogenetic Analysis

The BLASTn analysis of the ITS1-5.8S-ITS2 sequence confirmed that the fungal isolate was in the *G. fujikuroi* species complex (the closest species with 100% identity were *F. sacchari* and *F. verticillioides*), while the clean sequence suggested the presence of a single fungal isolate, or at least a single common isolate. The resulting ITS1-5.8S-ITS2 sequence was submitted to GenBank (accession No. MH752426). To construct phylogenetic relationships, the TEF-1 α sequences of 30 reference strains and the outgroup *F. solani* (NRRL 22157) were retrieved from the FUSARIUM-ID database. The TEF-1 α sequence of the present fungal isolate was submitted to GenBank (accession No. MH756646). The NJ phylogenetic tree showed that the fungal isolate was grouped in the same clade with the *F. sacchari* reference strain with a bootstrap value of 100% (Fig. 3). Thus, the combined phylogenetic and morphological data verified the identity of the isolate as *F. sacchari* strain KC8.

Detached Leaf Pathogenicity Test

The detached leaves were used to confirm the ability of *F. sacchari* strain KC8 to infect the leaves of *R. gigantea*. At 14 d post-inoculation, necrotic spots with yellow halos appeared on the adaxial surface of the wounded leaf. On the abaxial surface of the wounded leaf, yellow spots appeared which somewhat resembled the yellow spots on the adaxial surface of the unwounded leaf, but with stronger disease symptoms. No symptoms were observed on the wounded and unwounded leaves without mycelium plugs (Fig. 4). The re-isolated fungus from the inoculated leaves was morphologically identical to the *F. sacchari* in the present study, therefore fulfilling Koch's postulates.

Discussion

Fusarium sacchari is an economically important plant pathogen of sorghum (Leslie *et al.*, 2005), wheat (Wang *et al.*, 2014), maize (Hsuan *et al.*, 2011; Leslie *et al.*, 2005), banana (Tarnowski *et al.*, 2010), rice (Hsuan *et al.*, 2011; Petrovic *et al.*, 2013) and sugarcane (Egan *et al.*, 1997; Hsuan *et al.*, 2011; Poongothai *et al.*, 2014; Viswanathan *et al.*, 2017; 2011). In the present study, *F. sacchari* was isolated from *R. gigantea* leaves with yellow leaf spot disease, adding *R. gigantea* orchids to the host list of *F. sacchari*. The symptoms of the disease were somewhat similar to the yellow leaf spot disease observed in *Cymbidium* orchids infected with *F. proliferatum* and *F. subglutinans* (Ichikawa and Aoki, 2000), except the brown areas and black necrotic areas did not always develop. However, the black necrotic areas were observed on the detached leaves of *R. gigantea* at wounded inoculation sites (Fig. 4).



Fig. 4: Detached leaf pathogenicity test. Infection was performed by placing mycelium plugs of *F. sacchari* strain KC8 on fully-developed *R. gigantea* leaves. Untreated leaves were used as a control. Wounded leaves were pierced by a sterile needle. Photographs were taken 14 d after incubation in controlled environments at 100% RH and 28°C with a 18 h photoperiod

Morphological characteristics of the isolated fungus largely matched the descriptions in The *Fusarium* Laboratory Manual (Leslie and Summerell, 2006). However, the sporodochia color and the presence of chlamyospores disagreed with the manual's descriptions. The color of sporodochia in the present study was cream instead of orange. This might be due to the variation within the species, as observed in the *Fusarium oxysporum* sporodochia that may appear from cream to orange (Beale and Pitt, 1989; Smith *et al.*, 1988). In contrast to the absence of chlamyospores described in The *Fusarium* Laboratory Manual, we found single verrucose chlamyospores produced on the third day after inoculation onto PDA. This has also been reported previously (Poongothai *et al.*, 2014), where

three *F. sacchari* isolates out of 117 formed chlamydospores on PDA medium.

The fulfillment of Koch's postulates confirms that *F. sacchari* strain KC8 is the cause of yellow spot disease on *R. gigantea*. *F. sacchari* strain KC8 was able to infect both wounded and unwounded leaves, indicating pathogenic ability of the fungus to penetrate through the surface of the plant. Although the re-isolated fungi had all morphological characters resembling the inoculated *F. sacchari* strain KC8, the molecular characterization of TEF1- α gene should also be performed to confirm identification.

This present study not only provides information that can be applied to the disease management of *R. gigantea* orchids, but also serves as a precaution to protect persons in contact with the disease, as *F. sacchari* can cause mycotic keratitis and exogenous fungal endophthalmitis in non-immunocompromised farmers (Bansal *et al.*, 2016; Chander *et al.*, 2011).

Conclusion

This is the first report of *F. sacchari* causing yellow leaf spots on *R. gigantea* orchids, as supported by morphological characteristics, phylogenetic analysis of the TEF1- α gene and the fulfillment of Koch's postulates. Symptoms of the disease and the pathogen causing the disease are crucial to establish a disease management program, as this disease leads to the death of infected orchids.

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Author's Contributions

Kanokwan Dekham: Performed the experiments, analyzed the data and wrote the manuscript.

Krieng Kanchanawatee: Designed the study, analyzed the data and wrote the manuscript.

Ethics

The authors declare that there are no conflicts of interest.

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