Communication

Fusarium oxysporum Associated with Fusarium Wilt on Pennisetum sinese in China

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Abstract: Pennisetum sinese, a versatile and adaptable plant, plays an essential role in phytoremediation, soil reclamation, and fodder production. From 2018 to 2021, the occurrence of Fusarium wilt, with symptoms of foliar blight and internal discoloration of the stem, was observed in Chongqing, China. Pathogens were isolated from the symptomatic leaves. Based on morphological characteristics as well as DNA sequences of the 18S ribosomal RNA (SSU), translation elongation factor 1-α (EF1-α), RNA polymerase II subunit 1 (rpb1), and RNA polymerase II second largest subunit (rpb2) genes, the causal agents were identified as Fusarium oxysporum. Phylogenetic analysis of the combined dataset of EF1-α, rpb1 and rpb2 showed that pathogenic isolates clustered with F. oxysporum strains. The pathogen was reisolated from inoculated and diseased tissues; thus, Koch’s postulates were fulfilled. This is the first report of F. oxysporum causing Fusarium wilt on P. sinese in China and worldwide.

Keywords: Pennisetum sinese; Fusarium oxysporum; Fusarium wilt

1. Introduction

Pennisetum sinese (syn. Cenchrus flaccidus), a cross between Pennisetum purpureum and Pennisetum americanum [1], is extensively distributed in tropical and subtropical regions as a Gramineae perennial tall grass [2]. With high biomass production and rich protein and carbohydrates [3], P. sinese has been widely cultivated as silage forages, edible fungi culture medium, and shows a valuable potential for biomass energy [4]. Furthermore, P. sinese has strong adaptability, which enables its rapid growth in arid environments and contributes to its prominent role in phytoremediation and ecological restoration [5].

As one of the most critical soil-borne pathogens, Fusarium species limit plant growth and crop yield [6], and spawn massive agricultural financial losses worldwide [7]. For instance, Fusarium graminearum, F. culmorum, and F. avenaceum can infest Triticum aestivum and induce wheat scab. It is commonly found in wheat-producing regions throughout the northwest, southwest, middle, and southeast of China, severely reducing production [8]. Fusarium spp. produce carcinogenic mycotoxin that poses a threat to food security and human health [9]. In addition, Fusarium phytotoxicity is regarded as a relevant factor in plant disease severity and progression [10].

Pennisetum diseases are known to be induced by fungal species in genera, such as Curvularia, Tolyposporium, Puccinia, Colletotrichum, and Bipolaris, which lead to the main deterioration factors during cultivation and storage. In China, P. sinese production is particularly plagued by leaf blight issues due to being attacked by Gram-negative bacteria members Pantoea agglomerans in Chongqing [11] and fungi species Pyricularia pennisetigena in Zhanjiang, Guangdong Province [12]. The initial infection manifests as water-soaked brown spots and ultimately the whole leaf blast, consequently affecting the quantity and quality of the herbage.

In May 2018, foliar blight and stem wilt were first observed in the P. sinese field in Chongqing City, China, which may potentially harm forage yield. Our study aims to
describe this new *P. sinese* wilting disease and identify the causal agent. We confirmed the pathogen associated with *P. sinese* Fusarium wilt through morphological observation, molecular identification, and pathogenicity tests. To our knowledge, this is the first report of Fusarium wilt on *P. sinese* caused by *F. oxysporum* in China and worldwide. Our research will establish the groundwork for primary prevention, diagnosis, and treatment of Fusarium wilt diseases.

2. Materials and Methods

2.1. Disease Incidence and Sampling Collection

Every May, field surveys were carried out in the 400 m² *P. sinese* fields from 2018 to 2021. The planting base was located at Southwest University (29°22′ N, 105°33′ E), Rongchang District, Chongqing City, China. Typical symptoms of wilting *P. sinese* were recorded from all infected plants. The disease incidence was calculated according to the formula described by Zainudin et al. [13] Seven *P. sinese* fields were randomly selected, and five plots (20 m²) were investigated at every site. Approximately 70 plants per plot were included in the analysis. A total of 35 diseased plants were collected and brought to the laboratory.

2.2. Fungal Isolation and Morphological Observation

Infected leaf and stem samples were cut into 5–8 mm² pieces, then flashed with running tap water. The symptomatic fragments were immersed in 75% ethanol (v/v) for 30 s, 1% NaClO (w/v) for 10 s, and rinsed with sterile distilled water three times. Symptomatic tissues were then transferred onto 9-cm-diameter potato dextrose agar (PDA) and incubated in a biochemical incubator (Boxun BSP100, Shanghai, China) at 25 °C in the dark. Pure cultures of each isolate were obtained by subcultivating hyphal tips on PDA. Fungal colony characteristics were observed on PDA for 5–7 days. After the 10-day-inoculation, the shapes and sizes of the conidia were recorded from at least 100 conidia per isolate under a microscope (Nikon Eclipse E200, Shanghai, China).

2.3. Molecular Identification and Phylogenetic Analysis

Eight representative *F. oxysporum* strains isolated in different years were sequenced. Mycelia were scraped from 14-day-old colonies and genomic DNA was extracted according to the instructions of the PlantGen DNA Kit CW0553A (Cwbio, Taizhou, China).

Amplification reactions were carried out in a total volume of 50 µL, containing 2 µL of template DNA, 1 µL of each forward and reverse PCR primer, 21 µL of ultrapure water, and 25 µL of 2 × PCR Taq Master Mix PC1120 (Solarbio, Beijing, China), which included 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 250 µM dNTP, and 0.05 U Polymerease/µL. The 18S ribosomal RNA (SSU), translation elongation factor 1-α (EF1-α), RNA polymerase II subunit 1 (rpb1), and RNA polymerase II second largest subunit (rpb2) genes were amplified with primers NS1/fung [14], EF1-728F/EF-2 [15], RPB1-Fa/RPB1-G2R and RPB2-5f2/RPB2-7cr [16], respectively. The Applied Biosystems ProFlex PCR 4484075 was used for the amplification with the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 47 °C for 40 s, and extension at 72 °C for 150 s, with a final extension step at 72 °C for 10 min.

PCR products were detected by 1% agarose gel in 1 × TBE buffer stained with Ethidium Bromide (Thermo Fisher 15585011, Shanghai, China) using a Nucleic Acid Electrophoresis (Thermo Fisher PSC120M, Shanghai, China), and visualized under UVP Gelstudio plus (Analytik Jena, Shanghai, China), then sent to GENEWIZ, Inc. (Suzhou, China) for Sanger sequencing. Results of genome sequencing were manually edited using the software Chromas (v 2.6.6, Technelysium Pty, Ltd., Brisbane, Australia). Sequence similarity searches were analyzed by BLAST in the National Center for Biotechnology Information (NCBI) database. The obtained sequences and reference ones retrieved from NCBI GenBank were aligned by ClustalW. The maximum-likelihood tree was constructed on the three-gene (EF-1α + rpb1 + rpb2) combined datasets. Phylogenetic analysis was generated based
on the general time reversible model using MEGA 7 software, and branch strength was evaluated with 1000 replicates.

2.4. Pathogenicity Test

Pathogenicity assays were carried out in triplicate for eight *F. oxysporum* isolates. For in vivo experiment, stems of 6-week-old healthy *P. sinese* seedlings, which had been wounded by sterile needles, were dipped in a spore suspension of approximately $10^5$ conidia/mL for 30 min, while the negative control was treated with distilled water. All plants grew in sterilized soil and were placed in a greenhouse at $24 \pm 1 ^\circ C$ with a 12 h/12 h light and dark period. The in vitro test was conducted in the petri dish, which had been previously washed with 75% alcohol and padded with autoclaved filter paper moistened with sterilized distilled water to keep wet. Leaves were separated from *P. sinese*, wounded, and inoculated as previously described. Culture dishes were placed in the biochemical incubator. The temperature parameter was adjusted to $25 ^\circ C$ and the illumination condition was set to 24 h dark. Fungal pathogens were reisolated from symptomatic tissues of the infected plants and identified on the basis of cultural characteristics and molecular analysis to fulfill Koch’s postulates.

3. Results

3.1. Field Survey

All infected *P. sinese* showed typical symptoms as irregular deep reddish-brown lesions appearing on the blade tip; as the disease became worse, the whole leaf turned pale brown, then blighted, and the internal part of the stem gradually discolored. The infected plants dried out and defoliated at a severe stage. In 2018, the disease incidence was determined in the range of 54–71%, and the average among seven fields was calculated as 63%. The average disease incidence was recorded as 49%, 57%, and 52% in 2019, 2020, and 2021, respectively.

3.2. Morphological Identification

A total of 38 isolates of *Fusarium* were obtained from symptomatic samples. The colonies covered almost the whole petri dish in 6 days at 25 $^\circ C$. Aerial mycelia were abundant, white, and floccose initially. After 3 days of inoculation, yellow–orange pigmentation developed beneath the colonies.

Microconidia were single-celled, ellipsoidal, 0–1 septate, 5–14 $\times$ 2.5–4.5 $\mu$m. Macrocconidia were falcate, curved towards the ventral side, with apical cells papillate, basal cells foot-shaped, 1–4 septate (mostly 3 septate), hyaline, smooth, and thin-walled, 21–42 $\times$ 3–6 $\mu$m. Chlamydospores were globose to sub-globose with an average of 10 $\mu$m in diameter, and hyphae branched at acute angles (Figure 1). The observed morphological characteristics matched the descriptions of *F. oxysporum* [17].

3.3. Molecular Identification and Phylogenetic Analysis

BLAST results showed that the SSU, EF-1$\alpha$, rpb1, and rpb2 sequences of eight isolates attained 97–100% identity to the corresponding sequences of *F. oxysporum* strain T1-BH.1 (MN463097), FS11719a (MN417191), GR_FOA249 (MT305083), and VPR143194 (MN457545). Nucleotide sequences of eight strains were deposited in GenBank (Table 1).

A multilocus phylogenetic tree was constructed using the combined sequences of EF-1$\alpha$, rpb1 and rpb2 (Table 2). There were a total of 1932 positions in the final dataset. The tree with the highest log likelihood ($-7778.70$) is displayed in Figure 2. Bootstrap values were indicated at the nodes. In the maximum-likelihood tree, eight isolates were grouped into an independent clade, supported by the 100% bootstrap value. The combined three-locus dataset revealed that eight pathogenic isolates clustered strongly (100%) with *F. oxysporum* strains. Thus, based on morphology and sequence analysis, the associated fungus was determined as *F. oxysporum*. 
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Figure 1. Morphological characteristics of the pathogen on PDA medium. (a,b). The back and front of the colony; (c) microconidia; (d) hyphae with acute angled branch; (e) thick-walled chlamydospores; (f) monophialides producing macroconidia; (g) falcate-shaped macroconidia. Scale bars in (c-g) = 10 μm.

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Table 1. Isolates of Fusarium oxysporum obtained from Pennisetum sinese Fusarium wilt from 2018 to 2021 in China and GenBank accession numbers for DNA sequences used in this study.

| Isolate | Year | GenBank Accession Number |
|---------|------|--------------------------|
| PSFO1   | 2018 | MN954685 MN939683 MT314258 MT314259 |
| PSFO2   | 2018 | OP236575 OP243441 OP243434 OP243427 |
| PSFO3   | 2019 | OP236576 OP243442 OP243435 OP243428 |
| PSFO4   | 2019 | OP236577 OP243443 OP243436 OP243429 |
| PSFO5   | 2020 | OP236578 OP243444 OP243437 OP243430 |
| PSFO6   | 2020 | OP236579 OP243445 OP243438 OP243431 |
| PSFO7   | 2021 | OP236580 OP243446 OP243439 OP243432 |
| PSFO8   | 2021 | OP236581 OP243447 OP243440 OP243433 |

Table 2. Isolates of Fusarium spp. and GenBank accession numbers for DNA sequences used in this study.

| Fusarium Species       | Isolate | GenBank Accession Number |
|------------------------|---------|--------------------------|
| F. oxysporum GR FOAc   |         | MT305183 MT305069 MT305125 |
| F. oxysporum GR FOA247 |         | MT305196 MT305082 MT305138 |
| F. oxysporum NA FOA14  |         | MT568939 MT568955 MT568971 |
| F. oxysporum MA FOA25  |         | MT568947 MT568963 MT568979 |
| F. proliferatum MA FPA10 |       | MW091270 MW091290 MW091308 |
| F. redolens NA FRA02   |         | MW091278 MW091296 MW091316 |
| F. dimerum NRRL 36140  |         | HM347133 HM347203 HM347218 |
| F. mangiferae UMAF 0924 |       | KP753402 KP753435 KP753442 |
| F. aywerte RBG 5743    |         | KP083250 KP083273 KP083278 |
| F. commune NRRL 28387  |         | AF246832 JX171525 JX171638 |
| F. bulbicola NRRL 13618 |       | KF466415 KF466394 KF466404 |
| F. globosum NRRL 26131 |         | KF466417 KF466396 KF466406 |
| F. sacchari NRRL 13999 |         | AF160278 JX171466 JX171580 |
| F. konzum CBS: 119849  |         | LT996098 LT996200 LT996148 |

Figure 2. The maximum-likelihood tree was inferred from the concatenated sequences of the translation elongation factor 1-alpha (EF-1α), RNA polymerase II subunit 1 (rpb1), and RNA polymerase II second largest subunit (rpb2) genes. The scale bar represented 0.05 nucleotide substitutions per site.
3.4. Pathogenicity Test

The pathogenicity test showed *F. oxysporum* isolates were pathogenic to *P. sinese*, exhibiting typical symptoms as observed in the field. In the in vitro assay, the detached leaves became yellow–brown and eventually blighted after 7 days of inoculation with the spore suspension. The in vivo experiment demonstrated that reddish-brown regions formed at the apex of inoculated leaves. As the disease progressed, light brown areas began to emerge in the center of the expanding red–brown lesion and spread to the entire leaf. Plants treated with pathogenic inoculums all turned wilted, and their stems revealed vascular bundle browning 25 days later. The control groups did not develop any symptoms (Figure 3). The same fungal species (*F. oxysporum*) was successfully reisolated from symptomatic tissues, fulfilling Koch’s postulates.

![Symptoms of Fusarium wilt on *Pennisetum sinese*](image)

**Figure 3.** Symptoms of Fusarium wilt on *Pennisetum sinese*. (a) Typical symptoms on naturally infected *P. sinese* in the field; (b,c) stem discoloration on naturally infected *P. sinese*; (d) the detached leaf inoculated with *Fusarium oxysporum* isolate turned yellow–brown and withered; (e) the stem inoculated with *F. oxysporum* showed vascular bundle browning; (f,g) the control group stayed healthy; (h,i) the reddish-brown lesion appeared on leaves and infected leaves gradually wilted.

4. Discussion

*F. oxysporum* is a type of common soilborne fungi, which leads to vascular wilt and crown, stem, or root rot across a broad host range [18], including Canary Island date palm.
Fusarium wilt (Phoenix canariensis Hort. Ex Chabaud) [19], sweet basil wilt and crown rot [20], potato stem–end rot [21] and sugar beet lines root rot [22]. This fungus is also a considerable pathogen (clinically) that can infect both humans and animals [23].

Morphological observation provides clues to the pathogen’s identity as Fusarium spp. However, morphological features are not sufficient to distinguish many fusaria [24], and multi-locus sequence comparisons were proposed because they offer more credible information. The EF1-α sequence is extensively used to identify most Fusarium species [25]. In addition, combining portions of rpbl and rpbl2 is the most comprehensive phylogenetic assessment of Fusarium spp. as yet [6], receiving recognition for their high informativeness in analyzing Fusarium [26]. Thus, a phylogenetic tree was conducted consisting of concatenated partial sequences of genes for EF1-α, rpbl1 and rpbl2, which strongly verified that the associated pathogen was F. oxysporum.

When it comes to Fusarium wilt, F. oxysporum can colonize in the vascular bundles of the plant roots, resulting in vessel blockage, withering of the above-ground portion, along with brown necrotic spots appearing on the root epidermis [27–29]. Diseased crops exhibit root rot symptoms or even die. Our present work revealed that P. sinese was susceptible to Fusarium wilt; additionally, stem discoloration also provided evidence of vascular bundle infection.

As Pennisetum spp. is one of the most widely cultivated plants around the world [30], Fusarium wilt disease could cause a serious impact on forage. Located in Southwest China and the upper reaches of the Yangtze River, Chongqing enjoys a subtropical monsoon climate with four distinct seasons and abundant rainfall, leading to high pastures production. Since P. sinese is a primarily cultivated forage in China in the summer [31], growers of P. sinese should pay emphasis to P. sinese wilting. The identification of the causal pathogen provides a scientific basis for disease management, playing a major part in developing antifungal agents. For further research, management strategies will be investigated to minimize potential economic losses due to lower plant biomass production.

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Data Availability Statement: The sequence data generated in this study are deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank). All accession numbers are presented in Table 1.

Conflicts of Interest: The authors declare no conflict of interest.

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