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Pathogen identification of sweet potato wilt in Bình Tân, Vĩnh Long

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ABSTRACT

This study aims at identifying the pathogen causing sweet potato wilt in Bình Tân district, Vĩnh Long province of Vietnam using morphological characterization, Koch's postulates and sequencing the ITS region amplified by primer set ITS1/ITS4. A total of seven fungal isolates were obtained from 14 infected stem samples collected from Bình Tân district. Based on morphological characterization, two isolates CD3 and CD5 were classified into the Fusarium genus, one of the major plant pathogen genera causing wilt diseases. CD5 exhibited typical wilt symptoms using Koch's postulates and was identified as Fusarium solani based on its ITS sequence. This result serves as a basis for further studies on disease management.

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1 INTRODUCTION

Sweet potatoes are mainly grown in developing countries. Annual production is around 106 million tons with cultivation area of 8.3 million hectares (FAOSTAT, 2014). In Vietnam, sweet potato is an essential component in agricultural production with an average yield of 10 tons/ha in 2014. It is the third most important food crop (after rice and maize) of the country in terms of production and cultivation area (Vu *et al.*, 2000; FAOSTAT, 2014).

Bình Tân district of Vĩnh Long province has the largest cultivation area (over 10 thousand hectares) of sweet potatoes in Vietnam with various types of sweet potatoes (Le Thi Thanh Hien *et al.*, 2014). Among them, purple sweet potatoes account for 70% of cultivation areas (Le Thi Thanh Hien *et al.*, 2014; Son, 2016) with high economic values due to wide consumption markets, mainly exported to China (accounting for 86%) (Son, 2016). However, sweet potatoes are susceptible to various pathogens, many of which, especially the wilt disease ("chạy dây" in Vietnamese), cause yield and quality losses. Application of chemicals is the most common means in disease management. In view of difficulties and problems associated with chemical control, a sustainable measure as biological control is at requirement. Although biological control has been effectively deployed for disease management (Khoa *et al.*, 2016; Quyen *et al.*, 2016), a significant concern is its host specificity (Brodeur, 2012). Therefore, pathogen identification is imperative to obtain the highest effects in disease management (Hoc, 2016). This study aims at identifying sweet potato wilt pathogen, which plays as a solid basis for further studies in disease management.

2 MATERIALS AND METHODS

2.1 Sample collection and isolation of the pathogens

Sample collection

Samples of sweet potato wilt (Fig. 1) were collected from seven fields in Bình Tân district of Vĩnh Long province. The typical symptoms were wilted plants with rot stems, vascular browning and necrosis. These stem samples were then wrapped with paper and stored in plastic bags with detailed information as sample location and collection date.

Fig. 1: Wilt symptoms on sweet potato field in Bình Tân of Vĩnh Long province

Isolation of the pathogens

The preliminary test was conducted to identify if the pathogen was bacteria or fungus using the method described by Burgess *et al*. (2008). The infected segments of stems then were cut into smaller pieces (2-3 cm in length), sterilized with ethanol 70% for 30 seconds (three times). Segments were put on the potato dextrose agar (PDA) plates [250 g potato, 20 g dextrose, 20 g agar and distilled water for 1,000 mL medium]. After 48-hour incubation at 28±2°C, fungi-like colonies were purified on new PDA plates.

2.2 Pathogen identification

Fungal pathogens were identified by morphology, pathogenicity using Koch's postulates and alignment of their internal transcribed spacer (ITS) sequencing.

Morphological identification

Fungal isolates were characterized for their sizes, shapes, structures of mycelia and spores with triple replication under a light microscope (Primo Star, Zeiss). Their species statuses were determined by comparison to the taxonomy classification of Campbell and Johnson (2013).

Pathogenicity test

Seven-day-old conidia of fungal isolates cultured on PDA medium were harvested and diluted with 200 mL of potato dextrose broth (PDB) in an Erlenmeyer flask. The flask was then incubated at 28±2°C on an orbital shaker with 80 rpm for 5-7 days. The number of conidia per mL in the suspension were counted with a hemocytometer, and then the suspension was diluted to obtain the 10^7 conidia/mL.

Healthy four-week-old sweet potato vines were used as plant materials for the experiment. The vines were cut just below a node and inoculated by soaking the severed ends in the conidial suspension for 30 minutes (Collins and Nielsen, 1976). Each vine was then planted in a 10×17-cm round pot. Each isolate was performed three replications. The plants were watered daily and fertilized with recommended dose instructed by Ngo and Loc (2005). Sterile distilled water was used as untreated control. The symptoms on leaves and stems were observed and recorded during five weeks. Reisolation of the pathogen from the inoculated plants was performed to test the presence of the pathogen.

Internal transcribed spacer (ITS) sequencing

Only fungal isolate CD5 exhibiting the typical symptoms was chosen for identification using a molecular technique where the ITS (internal transcribed spacer) region of pathogen was sequenced. DNA of isolate CD5 was extracted following the method described by Gardes and Bruns (1993) with some modifications. Mycelia of isolate CD5 were ground in 1.5 mL centrifuge tube with 600 µL lysis buffer containing 30 µL NaCl 0.5 M; 30 µL EDTA 0.5 M; 30 µL SDS 10%; 120 µL Tris-HCl 1 M, and 390 µL distilled water. After 10 minutes of incubation at 28±2°C, the sample was centrifuged at 13,000 rpm in 5 minutes and 400 µL of resulting supernatant was then transferred to a new tube. It was added with 400 µL ethanol 95% and centrifuged at 13,000 rpm for 5 minutes to precipitate DNA. The supernatant was discarded and the pellet was centrifuged again with 500 µL ethanol 75% at 13,000 rpm in 5 minutes. The pellet was then dried by vacuum centrifugation at 45°C in 10 minutes, suspended in 100 µL Tris-EDTA 0.1X and stored at −20°C. A volume of 2 µL extracted DNA was combined with a PCR mix containing 5 µL buffer 5X, 4 µL $MgCl_2$ 10X, 0.5 µL *Taq* polymerase 5X, 3 µL dNTPs 10 nM, 1 µL each of primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) 10 pmol and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) 10 pmol (White *et al*., 1990) and 33.5 µL sterile double-distilled water. Amplification was carried out using a programmable heat block (C1000™ Thermal cycler, Bio-Rad) with the following thermocycle, i.e., an initial denaturation step at 95ºC for 5 minutes, followed by 35 amplification cycles of denaturation at 95ºC for 1 minute and 30 seconds, annealing at 53ºC for 1 minute and extension at 72ºC for 1 minute and 30 seconds, and a final elongation at 72ºC for 5 minutes. The genomic DNA was then stored at 10ºC. The quality of amplified DNA was tested using electrophoresis. A volume of 10 µL products

was mixed with 1 µL loading dye and electrophoresed in a 1.5% agarose gel in TAE 1X buffer at 50 V for 30 minutes. The gel was then photographed under ultra-violet light using Biorad GelDoc. The gene was sequenced by PHUSA Biochem Co., Ltd. (Bình Minh, Vĩnh Long). The obtained sequence was aligned with other ITS sequences on the GenBank database (NCBI) using Standard Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST). The percent similarity were used as a basis for fungal identification.

3 RESULTS AND DISCUSSION

3.1 Morphological identification

Fourteen wilt stem samples were collected from seven fields in Bình Tân district, Vĩnh Long province. The preliminary test proved that wilt symptoms on sweet potato was caused by fungal pathogens (Fig. 2), and there were seven fungal isolates obtained from these infected samples.

Genera *Fusarium* and *Verticillium* are able to cause stem wilt on sweet potatoes (Burgess *et al.*, 2008). Observation of sizes, shapes, structures of mycelia and spores of seven isolates under light microscope indicated that the two isolates CD3 and CD5 had high similarities in morphological characteristics with the *Fusarium* genus. They had septate and branched mycelia (Fig. 3D and 4D) with three types of conidia (microconidia, macroconidia and chlamydospores). The microconidia were abundant with cylindrical shape, with non- or one septum. The microconidium size of isolate CD3 was 7.0-9.0 µm in length and 2.5-3.0 µm in width while those of isolate CD5 were 7.0-10.0 µm and 2.5-3.2 µm (Fig. 3A and 4A). The macroconidia are falcate with three to five septa. The macroconidium size of isolate CD3 was 25-40 µm in length and 2.5-3.2 µm in width while those of isolate CD5 were 27-41 µm and 2.5-3.2 µm (Fig. 3B and 4B). The chlamydospores were formed on hyphae with rough-walled, single or in chains of 2-3 spores (Fig. 3C and 4C). The other five isolates had different morphological characteristics compared to those of *Fusarium* and *Verticillium*. These isolates could be saprophytic fungi growing on infected tissues as secondary colonizers.

Fig. 2: Bacterial ooze did not come out from infected stems in distilled water in premilinary test

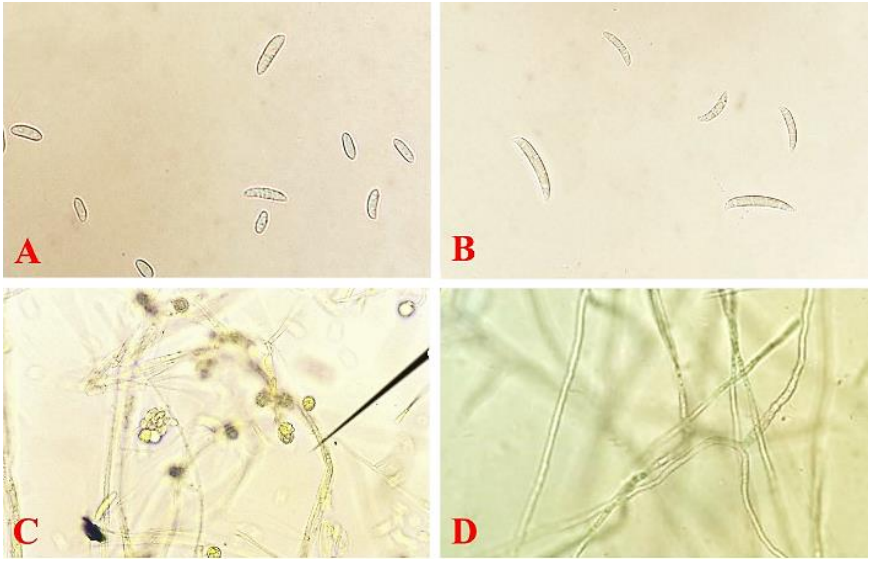

Fig. 3: Morphology of isolate CD3 under a light microscope (400×)

A: microconidia; B: macroconidia; C: chlamydospores and D: mycelia

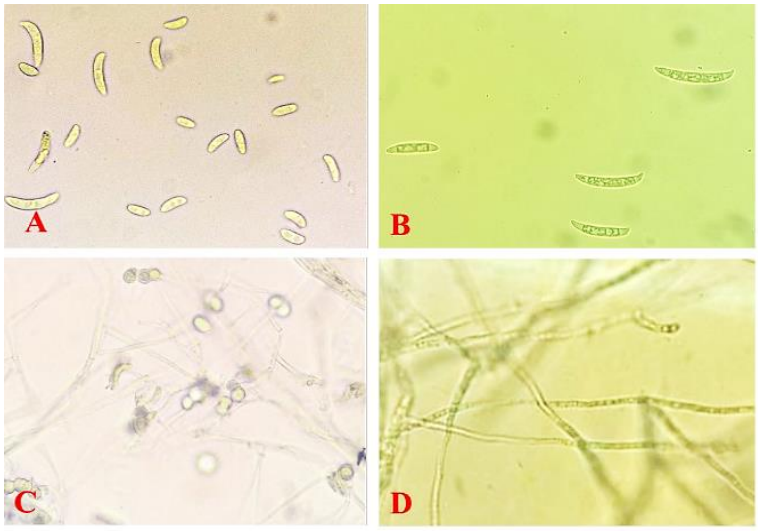

Fig. 4: Morphology of isolate CD5 under a light microscope (400×)

A: microconidia; B: macroconidia; C: chlamydospores and D: mycelia

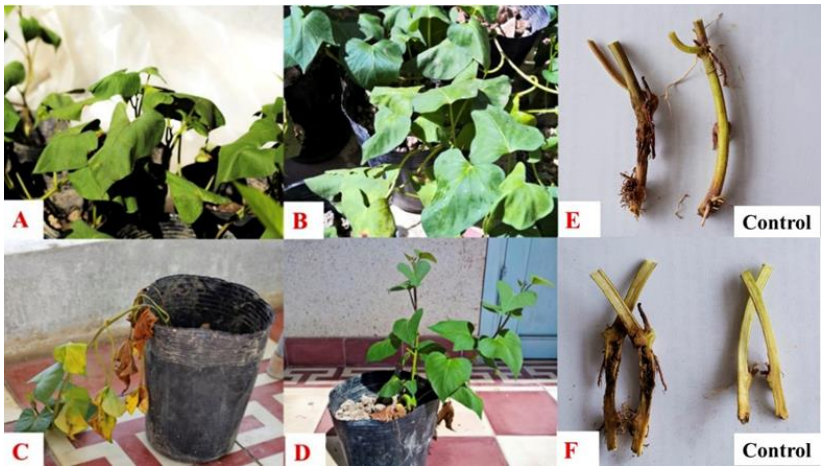

Fig. 5: The symptoms on sweet potato causing by isolate CD5

A and B: wilting leaves at 21 days after inoculation (DAI); C: yellowing of older leaves at 35 DAI; D: negative control at 35 DAI; E and F: stem canker with blackening lesion at 35 DAI

3.2 Identification using ITS sequencing

The ITS region of the isolate CD5 was extracted and electrophoresed on 1.5 % agarose gel for qualification (Fig. 6). It was then sequenced (537 bp) and aligned to other fungal ITS sequences on the GenBank database (NCBI). Isolate CD5 had ITS sequence with the highest similarity (99%) to fungus *F. solani* (Fig. 7). This result proved that isolate CD5 was fungus *F. solani*.

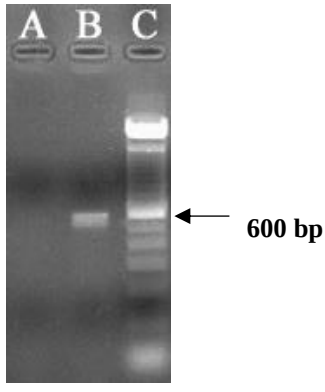

Fig. 6: The 600-bp PCR product of CD5 PCR amplified with universal primer set ITS1/ITS4 on 1.5% agarose gel

Lane A: negative control; lane B: CD5; lane C: Molecular marker (100 bp)

The result showed that the pathogen causing wilt on sweet potatoes in Bình Tân district of Vĩnh Long province was *F. solani*. In nature, *F. oxysporum* f.sp. *batatas* is the common pathogen causing wilt symptoms on sweet potato but this pathogen was not found in this study. However, it does not mean that *F. oxysporum* f.sp. *batatas* does not exist in the fields. *Fusarium* species can survive long time in soil as resistant chlamydospores (Clark *et al*., 2009). In this case, these two *Fusarium* species could persist in soil and infect sweet potato plants in the specific weather, cultivation and soil conditions. It could be speculated that the conditions of Bình Tân district are more favorable for *F. solani* than *F. oxysporum* f.sp. *batatas* to cause the disease.

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Fusarium solani isolate CBPPR0034 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; anc	950	950	97%	0.0	99%	KT211526.1
Fusarium solani strain EPM-012 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c	950	950	97%	0.0	99%	KC907714.1
Fusarium cf. solani CBS 2012 strain CBS 124892 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se	950	950	97%	0.0	99%	JX435189.1
Fusarium sp. KC-2010ba strain USMFSSC9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	950	950	97%	0.0	99%	JQ364976.1
Fusarium solani isolate FWC9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, con	944	944	97%	0.0	99%	KU097246.1
Fusarium solani isolate FSC90 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cor	944	944	97%	0.0	99%	JQ625574.1
Fusarium sp. KC-2010ba strain USMFSSC13 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	944	944	97%	0.0	99%	JQ364980.1
Fusarium solani isolate CBPPR0057 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; anc	941	941	95%	0.0	99%	KT211549.1
Uncultured fungus isolate MRRI24 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	939	939	96%	0.0	99%	KF060210.1
Fusarium sp. KC-2010ba strain USMFSSC12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	939	939	97%	0.0	99%	JQ364979.1
Fusarium sp. KC-2010ba strain USMFSSC4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	939	939	97%	0.0	99%	JQ364971.1
Fusarium solani isolate CBPPR0027 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; anc	937	937	95%	0.0	99%	KT211519.1
Uncultured Ascomycota isolate MRRI23 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	937	937	97%	0.0	99%	KF060209.1
Fusarium solani isolate RRI1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comp	937	937	97%	0.0	99%	KF060154.1
Fusarium sp. KC-2010ba strain USMFSSC10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribe	937	937	97%	0.0	99%	JQ364977.1
Fusarium sp. KC-2010ba strain USMFSSC8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	937	937	95%	0.0	99%	JQ364975.1

Fig. 7: Alignment results of ITS sequence of isolate CD5 with other ITS sequences on GenBank database (NCBI) using Blastn (accessed date 20 January 2017)

4 CONCLUSION

A total of seven fungal isolates were obtained from 14 infected stem samples collected from Bình Tân, Vĩnh Long of Vietnam. Two isolates CD3 and CD5 were classified into *Fusarium* genus based on morphological characterization. CD5 exhibited identical symptoms compared to field symptoms of sweet potato wilt ("chạy dây" in Vietnamsese) using Koch's postulates. CD5 was identified as *F. solani* based on its ITS sequence. This study serves as a basis for further studies to deploy biological control methods in the progress of developing sustainable and less chemical-depended cultivation.

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