First report of *Fusarium concentricum* causing fruit blotch on roselle (*Hibiscus sabdariffa*)

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Abstract

Symptoms of fruit blotch were noticed on roselle (*Hibiscus sabdariffa*) in Relau, Penang and Karangan, Kedah. The aims of this study were to isolate and identify the suspected causal pathogen and to evaluate its pathogenicity towards the host. Two isolates of *Fusarium* sp. were isolated from the blotched fruits of *H. sabdariffa* and morphologically identified. Sequences analysis based on translation elongation factor (TEF1- α) and mitochondrial small subunit ribosomal DNA (mtSSU rDNA) showed that both isolates had 99.53% identity to AF333934 and 100% to AF333941, *Fusarium concentricum*. Phylogenetic analysis demonstrated that both isolates were in the same clade with the reference isolates of *F. concentricum* from GenBank, supported by 97% bootstrap value. The isolates were pathogenic toward *H. sabdariffa*, exhibiting typical blotch symptoms on the inoculated fruits. This is the first report of fruit blotch disease caused by *F. concentricum* on *H. sabdariffa* in Malaysia.

Keywords *Hibiscus sabdariffa* · Fruit blotch · *Fusarium concentricum*

Roselle (*Hibiscus sabdariffa*) belongs to the Malvaceae family. It is an annual herb and woody-based shrub (Nwaukwu and Ataga 2012). Over 300 species of *Hibiscus* are widely cultivated throughout the world. Roselle is one of the most commonly distributed *Hibiscus* species in tropical and sub-tropical regions including Malaysia (Mohamed et al. 2012). However, roselle is susceptible to a number of fungal infections which cause significant losses to farmers by reducing fruit production (Hassan et al. 2014). During preliminary observations in 2018, fruit blotch disease of roselle was noticed in Relau, Penang and Karangan, Kedah. Therefore, this study attempted to isolate and identify fungi associated with blotched fruit of *H. sabdariffa* and to confirm their pathogenicity through assay.

Symptomatic fruit samples were excised and surface sterilized with 70% ethanol and 1% sodium hypochlorite (1% NaOCl) for 3 min respectively, and rinsed in sterile distilled water three times consecutively for 1 min each. The samples were air-dried, transferred onto potato dextrose agar (PDA) and incubated at 25 ± 2 °C for 2 to 3 days. Pure cultures obtained from water agar (WA) plates were

used for further analysis. Colony appearance and pigmentation were observed from PDA plates. Carnation leaf agar (CLA) was used to observe the structure of macroconidia, microconidia, conidiogenous cells and sporodochia. The length and width of 30 randomly selected macroconidia and microconidia were measured and recorded.

Approximately 60 mg of fine mycelial powder was extracted using Invisorb Spin Plant Mini Kit (Stratec, Germany) according to the manufacturer's instruction. PCR amplifications of TEF1- α and mtSSU rDNA were carried out using primers pairs of EF1/EF2 (O'Donnell et al. 1998a) and MS1/MS2 (White et al. 1990). The amplification was carried out in a total volume of 50 μ l PCR mixture as described by Kee et al. (2017). The PCR reactions for TEF1- α and mtSSU were performed in a MyCycler™ Thermal Cycler (Bio-rad, Hercules, CA, USA) by following conditions as suggested by Kee et al. (2019) and Laurence et al. (2012), respectively. The PCR fragments were sent to a service provider for DNA purification and sequencing (First BASE Laboratories Sdn Bhd, Seri Kembangan, Malaysia).

The sequences obtained were analyzed and aligned using MEGA7 (Kumar et al. 2016) and BLAST was performed against sequences in the GenBank database (<https://blast.ncbi.nlm.nih.gov/>). The phylogenetic tree was constructed using maximum likelihood (ML) tree based on Tamura-3-parameter model (Tamura 1992) with 1000 bootstrap replications.

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Fig. 1 Morphological characteristics of *Fusarium concentricum* isolates from fruit blotch of roselle (*H. sabdariffa*) in Malaysia. **a** Colony appearance on PDA; **b** Reverse pigmentation; **c** Orange sporodochia on CLA; **d** Macroconidia; **e** Microconidia; **f** Microconidia on false head

The pathogenicity assay was employed on 12 attached healthy fruits on four plants of *H. sabdariffa*. Fungal mycelial plugs were prepared from 7-day-old PDA cultures using a sterile cork borer (6 mm diameter) while control plugs were prepared from an empty PDA plate. Two wound points were

made for each fruit, one for treatment and one for control. Both fungal mycelia and control plugs were aseptically inoculated on each fruit using a sterilized forcep and the plugs were covered with wet cotton to retain moisture. Each fungal isolate was tested in triplicate and the test was done twice. The

Table 1 GenBank accession numbers of *F. concentricum* and other *Fusarium* spp. used in the phylogenetic analysis

Species	Isolate	Host	Locality	GenBank accession No.		References
				TEF1- α	mtSSU	
<i>Fusarium concentricum</i>	NRRL29943	Soil of <i>Pinus densiflora</i> forest	Japan, Tsukuba, Ibaraki	AF333934	AF333942	Aoki et al. 2001
<i>F. concentricum</i>	NRRL26434	<i>Oryza sativa</i>	Japan, Tsukuba, Ibaraki	AF333933	AF333941	Aoki et al. 2001
<i>F. concentricum</i>	NRRL29944	<i>Oryza sativa</i>	Japan, Tsukuba, Ibaraki	AF333935	AF333943	Aoki et al. 2001
<i>F. concentricum</i>	NRRL 25303	<i>Oryza sativa</i>	Japan, Tsukuba, Ibaraki	AF160283	U61614	Aoki et al. 2001
<i>F. concentricum</i>	NRRL25181	<i>Musa sapientum</i>	Costa Rica	AF160282	U61600	O'Donnell et al. 1998b; Nirenberg and O'Donnell 1998
<i>F. concentricum</i>	FS3	<i>Hibiscus sabdariffa</i>	Penang, Malaysia	MN384965	MN384966	Present study
<i>F. concentricum</i>	FS32	<i>Hibiscus sabdariffa</i>	Kedah, Malaysia	MN384967	MN384968	Present study
<i>Fusarium proliferatum</i>	MUCL31970	Spanish forest soil	Canary Island	AF336913	AF336911	Kwon et al. 2001; Anderson et al. 2005
<i>F. proliferatum</i>	NRRL31071	<i>Triticum aestivum</i> cv. super dwarf	USA	AF291058	AF291056	Kwon et al. 2001; Anderson et al. 2005
<i>Fusarium fujikuroi</i>	NRRL13566	<i>Oryza sativa</i>	Taiwan	AF160279	U34499	O'Donnell et al. 1998b
<i>Fusarium fractiflexum</i>	NRRL28853	<i>Cymbidium</i> sp.	Japan, Hiroshima	AF333931	AF333939	Aoki et al. 2001
<i>F. fractiflexum</i>	NRRL28854	<i>Cymbidium</i> sp.	Japan, Komoro, Nagano	AF333932	AF333940	Aoki et al. 2001
<i>Fusarium sacchari</i>	NRRL13999	<i>Saccharum officinarum</i>	India	AF160278	U34498	Li-sha et al. 2017
<i>Fusarium subglutinans</i>	NRRL22016	<i>Zea mays</i>	United States	AF160289	U34501	O'Donnell et al. 1998b
<i>Fusarium verticillioides</i>	NRRL28893	<i>Musa cavendishii</i>	Mexico	AF273312	AF273305	Hirata et al. 2001
<i>Fusarium nygamai</i>	NRRL 13448	<i>Sorghum bicolor</i>	Australia	AF160273	U34510	Scauflaire et al. 2011
<i>Fusarium oxysporum</i>	NRRL 25356	Soil	France	AF008480	AF008446	O'Donnell et al. 1998b
<i>Fusarium circinatum</i>	NRRL 29945	<i>Pinus luchuensis</i>	Japan, Amami, Ohshima	AF333930	AF333938	Aoki et al. 2001
<i>Fusarium guttiforme</i>	NRRL22945	<i>Ananas comosus</i>	South America	AF160297	U34504	Nirenberg and O'Donnell 1998; O'Donnell et al. 1998b
<i>Fusarium hostae</i>	NRRL29647	<i>Hyacinthus</i> sp.	The Netherlands	AF324327	AF324288	Baayen et al. 2001
<i>Fusarium incarnatum</i>	CBS132907	<i>Triticum</i> sp.	Iran	MN170477	MH979695	Xia et al. 2019

Fig. 2 Maximum likelihood (ML) tree of *Fusarium* *concentricum* isolates (in bold) from fruit blotch of roselle (*H. sabdariffa*) in Malaysia and other *Fusarium* spp. generated based on combined dataset of TEF1- α and mtSSU DNA sequences. *Fusarium incarnatum* is used as an outgroup. Bootstrap values (> 85%) are displayed at the nodes and the number of substitutions per position is displayed in the scale bar

inoculated plants were placed in a plant house at the School of Biological Sciences, USM and incubated at 26 to 32 °C for 14 days. The disease signs and symptoms were looked for and noted every day. Fungal re-isolation was done from the symptomatic inoculated fruits and the resulting fungal isolates were re-identified morphologically.

Two isolates of *Fusarium* sp. (FS3 and FS32) were isolated from the blotched fruits of *H. sabdariffa*. Both isolates had pale orange to slightly velvety colonies on PDA plates (Fig. 1a) and pale orange with alternating velvet concentric rings on the reverse side (Fig. 1b). Formation of orange sporodochia was observed on CLA (Fig. 1c). Macroconidia were long, thin walled, 3–5 septate, with a sharp apical cell and foot-shaped basal cell, and were $46.3 \pm 6.8 \times 3.0 \pm 0.6 \mu\text{m}$ in size (Fig. 1d). Microconidia were 0–1 septate, oval shaped,

$9.4 \pm 1.5 \times 3.3 \pm 0.7 \mu\text{m}$ in size (Fig. 1e) and commonly formed in false heads (Fig. 1f). The conidiogenous cells were monophialides and polyphialides. Chlamydospores were absent. The isolates of the present study were presumed to be *Fusarium concentricum* in accordance with the description by Nirenberg and O'Donnell et al. (1998a) and Leslie and Summerell (2006). Both isolates of *F. concentricum* (MN384965– MN384968) were deposited in the Culture Collection Unit, Department of Plant Pathology, School of Biological Sciences, Universiti Sains Malaysia.

In the BLAST search, isolates FS3 and FS32 were 99.53% and 100% similar to the reference sequences of *Fusarium concentricum* AF333934 (TEF1- α) and AF333941 (mtSSU), respectively. Resultant sequences were deposited in Genbank as accession numbers, MN384965 and

Fig. 3 Pathogenicity assay of *Fusarium concentricum* isolates on healthy fruits of roselle (*H. sabdariffa*). **a** Irregular necrotic brown lesion developed on the inoculated fruit; **b** Presence of orange sporodochia and white mycelia on the lesion surface; **c** Control fruit remained healthy

MN384967 for TEF1- α and MN384966 and MN384968 for mtSSU. Several reference sequences of *F. concentricum* and other *Fusarium* spp. from GenBank used in the phylogenetic analysis are listed in Table 1. The maximum likelihood (ML) tree of the combined dataset of TEF1- α and mtSSU sequences demonstrated that the recovered isolates (FS3 and FS32) clustered in the one clade with the reference isolates of *F. concentricum* from GenBank, supported by 97% bootstrap value (Fig. 2). On the basis of morphological characteristics, DNA sequences and phylogenetic analysis, the identity of FS3 and FS32 isolates was confirmed as *F. concentricum*.

Both FS32 and FS3 isolates were pathogenic toward fruits of *H. sabdariffa*. After 7 days of the pathogenicity assay, the inoculated fruits displayed symptoms of irregular brown necrotic lesions at the wound points. As the disease progressed, the lesions became sunken, enlarged, and turned to darker brown, resulting in the appearance of blotch symptoms (Fig. 3a). The formations of white mycelia and orange sporodochia were observed on the lesion surface 12 days after inoculation (Fig. 3b) whilst the control produced no symptoms (Fig. 3c). Koch's postulates were fulfilled as the same fungal species were successfully re-isolated from the symptomatic inoculated fruits and morphologically re-identified.

Fusarium concentricum was first described by Nirenberg and O'Donnell et al. (1998a) from bananas in Central America and aphids in Korea. This pathogen has a wide host range, associating with multiple diseases on different hosts such as stem rot of *Paris polyphylla* var. *chinensis* and fruit rot of pepper and banana (Wang et al. 2013; Abd Murad et al. 2017; Xiao et al. 2019). The present study showed that *F. concentricum* was a causal pathogen of fruit blotch of roselle. Horst (2013) defined blotch as irregular or indefinite large or small necrotic areas on fruit, having intermediate symptoms between blight and spots. The described symptoms are in accordance with the symptoms displayed on the inoculated roselle fruits. Several different pathogens have been reported to threaten roselle cultivation worldwide and in Malaysia (Ooi and Salleh 1999; Swart and Langenhoven 2000; Amusa et al. 2001; McClintock and Tahir 2004; Ploetz et al. 2007; Eslaminejad and Zakaria 2011; Ansari et al. 2013; Pain et al. 2019).

Accurate identification of a fungal pathogen is crucial for implementing an effective disease management strategy. Morphological features are commonly used to identify fungi, a cost-effective and easy approach (Schmit and Lodge 2004). Nonetheless, the existence of overlapping morphological features between closely related species may lead to the misinterpretation of fungal identification. According to Leslie and Summerell (2006), *F. concentricum* has similar aerial microconidia which form in a false head as produced by *F. circinatum*, *F. guttiforme*, *F. sacchari*, *F. subglutinans* and *F. oxysporum*. However, based on a ML tree of combined dataset of TEF1- α and mtSSU genes, isolates of

F. concentricum in the present study were phylogenetically different from those *Fusarium* species. The TEF1- α gene was selected for phylogenetic analysis because it consists of informative sequences and non-orthologous copies which can distinguish isolates of *Fusarium* to species level (Geiser et al. 2004; Singh and Gupta 2017). In addition, the mtSSU gene has been widely used as an alternative to accurately delineate the identity of the *Fusarium* species (Bruns and Szaro 1992).

To our knowledge, this is the first report of *F. concentricum* causing fruit blotch on *H. sabdariffa* in Malaysia. The insights gained from this study can help to understand the disease etiology and further permit formulation of an integrated disease management to limit the disease.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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