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First Report on *Fusarium solani*, a Pathogenic Fungus Causing Stem Rot Disease on Dragon Fruits (*Hylocereus* sp.) in Bali

Wiwik Susanah Rita[1], Dewa Ngurah Suprapta[2*], I Made Sudana[2], I Made Dira Swantara[3]
1.Doctorate Program in Agricultural Science, School for Postgraduate Udayana University.
2.Laboratory of Biopesticide Faculty of Agriculture, Udayana University, Jl. PB. Sudirman Denpasar Bali Indonesia.
3.Laboratory of Chemistry, Faculty of Natural Science and Mathematic, Udayana University
*Email of corresponding author : biop@dps.centrin.net.id

Abstract
In recent years, dragon fruit crop (*Hylocereus* spp.) has become increasingly important in Bali Indonesia due to its high nutrient content and healing properties. However, the dragon fruit was reported to be seriously infected with several complex diseases caused by fungi and causing serious losses to the farmers. The study on morphological and molecular characterization the fungal pathogen was conducted to confirm the species of the fungi. Koch Postulate was applied to confirm the causal agent of the disease. There were two isolates of fungi isolated from the stems of diseased plants, namely isolate w1 (from stem of *H. undatus*) and isolate w2 (from stem of *H. polyrhizus*). Based on macroscopic and microscopic characteristics, and analysis of 18S rDNA, both of them were identified as *Fusarium solani*. This is the first report on the *F. solani* the cause of stem rot disease on dragon fruits in Bali.
Keywords : stem rot disease, dragon fruit, *Fusarium solani*

1. Introduction

Dragon Fruits (*Hylocereus* spp.), which are also known as pitaya, are the fruits of cactus species, especially of the genus *Hylocereus*. There are three species which have high commercial valuable fruits are the species of *Hylocereus undatus*, (red rind, white flesh), *Hylocereus polyrhizus* (red rind, red flesh), and *Hylocereus costaricensis* (red rind, super red flesh). *Hylocereus* spp. is grown commercially in Vietnam, Spain, Malaysia, Japan, Mexico and other tropical and subtropical areas because of its high nutrient content and healing properties. In recent years, this fruit has become increasingly important in Bali Indonesia. The dragon fruit is rich in vitamin, it helps the digestive process due to its fiber, prevent colon cancer and diabetes, neutralize toxic substances such as heavy metals, and helps to reduce cholesterol levels and high blood pressure (He *et al*., 2012; Zainoldin and Baba, 2009). Recently, dragon fruit has been reported to be seriously infected with several complex diseases caused by fungi and causing serious losses to farmers. Several dragon fruit plants grown in Sobangan Village, Bali Indonesia showed severe symptom of stem rot disease. The disease caused significant yield losses. Isolation of the fungi associated with the diseased-plants showed that *Fusarium* sp. was the most frequent found on the stems showing brown rot symptom.

Various diseases caused by fungi have been reported on dragon fruit in tropical and subtropical countries, such as fruit rot (*Bipolaris cactivora*) (Tarnowski *et al.*, 2010; He *et al*. 2012), stem rot (*Fusarium semitectum*, *Fusarium oxysporium*, *Fusarium moniliforme*) (Hawa *et al*., 2010), anthracnose (*Colletotrichum gloeosporioides*) (Masyahit *et al*., 2009), brown spot (*Botryodiplodia* sp.), basal rot (*Pythium* sp.) (Lin *et al*., 2006), wilt (*Fusarium oxysporium*), stem blight (*Diplodia* sp., *Ascochyta* sp., and *Phoma* sp.), black spot (*Alternaria* sp.), speck blight (*Nectriella* sp.), (Wang *et al*.,2007), and stem lesion (*Septogloeum* sp.) (Zheng *et al*., 2009).

In order to control the disease, it is necessary to identify the causal agent of the disease. The fungal pathogen can be identified based on cultural and morphological characters. However it could be highly variable depending on the media and cultural conditions that could be the problems in fungal identification. In recent years, the increasing use of molecular methods in fungal identification has emerged as a possible answer to the problems associated with the existing phenotypic identification systems (Mishra *et al*., 2003). One of the molecular approaches to fungal identification is based on Polymerase Chain Reaction (PCR).

The main area for the development of fungal identification is ribosomal genes, present in all organisms and at high copy numbers aiding detection and the sensitivity of the PCR reaction. The fungal nuclear ribosomal DNA (rDNA) consist of three genes, the large subunit gene (25S), the small subunit gene (18S), and the 5.8S gene, separated by internal transcribed spacer (ITS) regions, in a unit repeated many times. The ITS region is an area of particular importance to fungal identification. It has areas of high conservation and areas of high variability and is an ideal starter for the development of specific PCR primers for identification of fungal species (Atkins and Clark, 2004). Sequences of the ITS regions ITS1 and ITS2 have been used widely in molecular phylogenetic studies because of their relatively high variability and facility of amplification. For phylogenetic

applications, most researchers use sequence alignments that are based on nucleotide similarity (Tippery and Les, 2008).

Suga *et al*. (2000) has investigated phylogenetic relationships of *Fusarium solani* using sequences from the rDNA-ITS region. Mishra *et al*. (2003) has developed a fluorescent-based polymerase chain reaction in ITS region to identify five toxigenic and pathogenic *Fusarium* species. Abd-Elsalam *et al*. (2003) have developed two taxon-selective primers for quick identification of the *Fusarium* genus. These primers, ITS-Fu-f and ITS-Fu-r were designed by comparing the aligned sequences of internal transcribed spacer regions (ITS) of a range of *Fusarium* species. Zhang *et al*. (2006) and O'Donnell *et al*. (2008) have studied phylogeni of *Fusarium solani* Species Complex (FSSC) that cause infection in both humans and plants based on three genes of the ribosomal DNA. The ITS region including 5.8S rDNA sequence of 58 isolates *Candida parapsilosis* in Brazil and Japan was analyzed by Iida *et al*. (2005).

This paper reports on identification of fungal pathogen causing stem rot disease from dragon fruits planted in Bali based on morphological and molecular methods using sequences from rDNA-ITS region.

2. Materials and Methods

2.1. Fungal Pathogen Isolation and Virulence Test

Fungi were isolated from the diseased stem of *H. undatus* and *H. polyrhizus* from dragon fruit's orchard in Sobangan village, Mengwi Bali. Surface sterilization was carried out by cleaning the symptom margins with 70% ethanol and cut into small blocks (ca 1.5 x 1.5 x 1.5 cm), soaked in 1% sodium hypochlorite (NaOCI) for 3 min, and rinsed in several changes of sterile distilled water (each 1 min). All sterilized samples were placed onto Potato Dextrose Agar (PDA) and incubated at 25 ± 2°C for 7 days. Mycelium growing after 3 days of incubation was transferred to a new PDA to obtain pure fungal cultures. The pure culture was inoculated on healthy dragon fruit stems to confirm the similarity of symptoms in the field. For this purpose the Koch's postulates test was carried out. Dragon fruit stems (30 days in age) were injured with a sterile needle and inoculated by spraying with a suspension of spores of $1.5x10^6$ spores/mL. Three plants were inoculated with a fungal isolate. For control, dragon fruit stems were pierced with a sterile needle and sprayed with sterile water. Symptoms were observed for a week after inoculation. The symptoms were compared with the symptoms of the disease in the field. After that, the isolation of pathogenic fungi on infected stem was performed again. Pure cultures of pathogenic fungi were inoculated again on dragon fruit plants and the same inoculation procedure was done to obtain the similarity of symptoms. Fungal isolates obtained can be regarded as the cause of the disease and were used for further identification.

2.2. Morphological Characterization

Characterization of the main fungal pathogens was carried out macroscopically, by observing the fungal colony color, colony reverse color, no lines or concentric radier, issued exudates or not, media pigmentation, colony surface, and how the growth of fungi (fast or slow). Microscopic identification was also carried out by observing the shape of hyphae or spores under a microscope, and then the results were confirmed using fungal identification book (Pit and Hocking, 1997).

2.3. DNA Extraction

Fusarium sp. isolates w1 and w2 were grown on potato dextrose agar (PDA) medium for 3 days at room temperature. The mycelium grown was harvested and grown to a fine powder in a sterile mortar with liquid nitrogen. DNA was extracted by using PhythopureTM DNA Extraction Kit (GE Healthcare, UK) according to manufacturer's instructions.

2.4. Molecular identification and phylogenetic relationships of fungal pathogen

Identification of fungal isolates was performed based on molecular genetic analysis using the internal transcribed spacer region (ITS). PCR amplification used ITS 5 F: 5`- GGAAGTAAAAGTCGTAACAAGG - 3` and ITS 4 R: 5 `- TCCTCCGCTTATTGATATGC - 3 '(White *et al*. 1990). Amplification was performed on a volume of 25 L with the reaction mixture: 10 µL nuclease free water, 12.5 µL Go taq green master mix^{TM}, ITS5 and ITS4 each 0.5 µL, 0.5 µL DMSO, and 1 µL of DNA template. PCR amplification for regional ITS consists of: pre denaturation 95 ºC for 90 seconds, followed by 95 ºC for 30 seconds with 35 cycles, annealing 55 ºC in 30 seconds, extension 72 ºC in 90 seconds, and final extension 72 ºC for 5 minutes. The product was purified and then sequenced. The nitrogen base sequence was analyzed using automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

Sequencing raw data were trimmed and assembled using ChromasPro program version 1.5. The assembled data were BLASTED with genomic data that has been registered in NCBI / National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Some data sequence which is a result of blast nearest species and is a type strains of each species were taken from the data in the NCBI gene bank. Then the data were analyzed again with the sequence aligment using MEGA version5.0 program (Tamura *et al*. 2011) and boostrap used is 1000 replications (Felsenstein, 1985).

3. Results and Discussion

3.1. Fungal Isolates

There are 10 fungal isolates were obtained from isolation of fungi associated with the stem disease of dragon fruits grown in Sobangan Village, Bali Indonesia. Based on the Koch's postulates test, there were two isolates of *Fusarium* sp. namely isolate w1 (from *H. undatus*) and isolate w2 (from *H. polyrhizus*) caused the stem rot disease with the symptom similar to the symptom occurred in the field. The symptom appeared on stem as dark brown stem rot as shown in Figure 1.

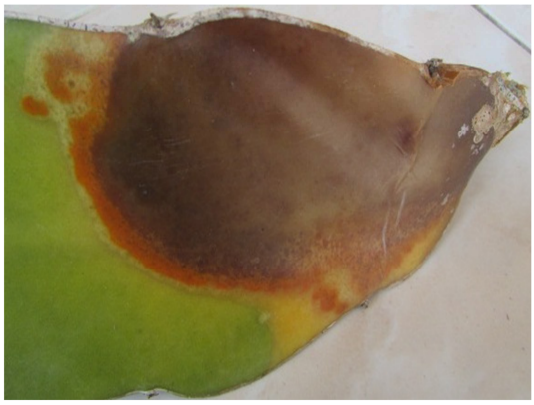

Figure 1. Symptoms of the stem rot diseases on *Hylocereus* sp. under field conditions.

3.2. Macroscopic Characteristic

Fusarium sp. isolate w1 grown on PDA produced abundant mycelium (sometimes in aerial, depends on cultural condition), white colony appearance, cream colony reverse, yellow to brown pigmentations, and fast growing (3.75-4.45 cm in 3 days) whereas *Fusarium* sp. isolate w2 produced abundant-powdery mycelium (sometimes in aerial), white colony appearance, yellow colony reverse, yellow pigmentations, and fast growing (4.35-5.25 cm in 3 days).

In a similar study, Chandran and Kumar (2012) studied for cultural, morphological variability in 13 isolates of *Fusarium solani* (Mart.) Sacc., incitant of dry root-rot of citrus, they reported that *F. solani* isolates were characterized as fast growing, moderately growing, and slow growing. The five isolates produced pale pink to dusky red color pigmentation in PDA medium and potato dextrose broth culture. The remaining all isolates produced pale yellow to dark yellow pigmentation. Most of the isolates produced profuse sporulation, whereas others produced moderate sporulation. Madhukeshwara (2000) has studied cultural variability among six isolates of *F. udum* causing wilt of pigeon pea. All the isolates varied with each other in terms of growth, mycelium, pigmentation, and sporulation. Most of the isolates produced cottony white raised mycelium, pale yellow to dusky red color pigmentation and moderate to profuse sporulation on PDA medium.

3.3. Microscopic Characteristics

Microscopic characters such as size, shape, septation, and color of conidia were studied using PDA medium. *Fusarium* sp. isolate w1 had longer macroconidia (1-4 septates; 18.4-31.7 µm in length), curved in shaped with hyaline in color, whereas *Fusarium* sp. isolate w2 had shorter macroconidia (1-3 septates; 15.3-24.8 µm in length), curved in shaped with hyaline in color. *Fusarium* sp. isolate w1 produced less microconidia while *Fusarium* sp. isolate w2 produces abundant microconidia (1 septate; 5.7- 8.5 µm in length), round to oval in shaped. Furthermore both the fungi had the septate hyphae and clamydospores in pairs with hyaline in color and located in the middle of hyphae as presented in Figure 2.

Pitt and Hocking (1997) reported that the main characters used to distinguish species of *Fusarium* are the size and shape of the macroconidia; the presence or absence of microconidia; and the presence of chlamydospora. Kawuri *et al*. (2012) reported that *Fusarium oxysporum* had macroconidia curved in shaped with four septates and foot cell, 31µm length and smooth surface, whereas microconidia has rough surface at 4.6 µm length. Chandran and Kumar (2012) reported that the number of septa in macro conidia and micro conidia of *Fusarium solani* (Mart.) Sacc. are 3-5 and 0-1 respectively and the color is hyaline. The shape of macro conidia is sickle shaped with blunt ends and micro conidia is round to oval shaped. The chlamydospores located in middle of hyphae (intercalary), on tip of the hyphae (terminal) and some chlamydospores were seen in middle of macro conidia.

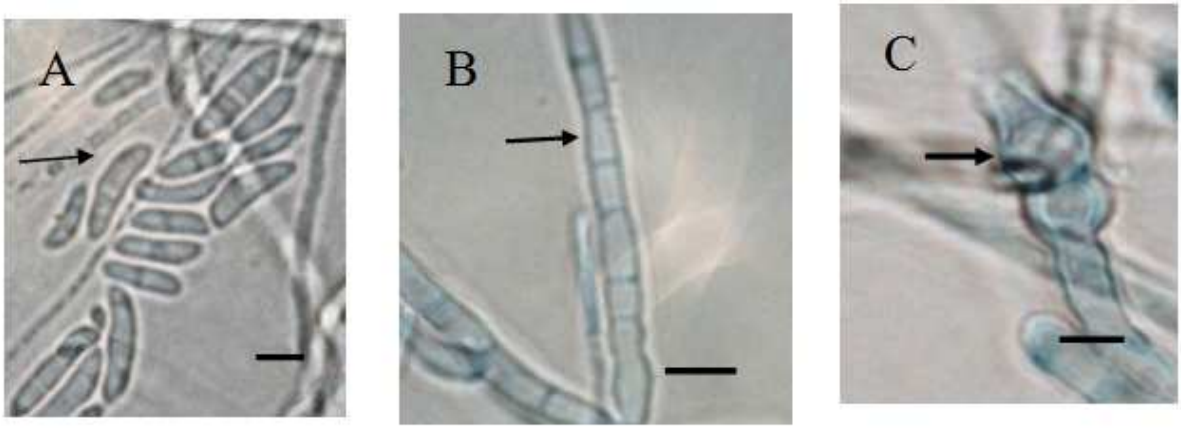

Figure 2. Microscopic characteristics of *Fusarium* sp. isolate w1 (A) macro conidia (B) septated hypae (C) Chlamydospore. Bars = 10 m.

3.4. Molecular Characteristics of the Pathogenic Fungus

PCR amplification of 18S rDNA of *Fusarium* sp. isolates w1 and w2 with primers ITS5 (F: 5`-GGAAGTAAAAGTCGTAACAAGG -3`) and ITS4 (R: 5`-TCCTCCGCTTATTGATATGC- 3') produced about 560 bp of DNA fragments, it is corresponding to 18S rDNA (Figure 3). The fragments then sequenced to determine the species of fungus based on the similarity with other references of identified species.

Based on the 18S rDNA analysis showed that *Fusarium* sp. isolates w1 and w2 have a close relationship with *Fusarium solani*. This can be seen in the phylogenetic tree shown in Figure 5. *Fusarium* sp. isolates w1 and w2 have 99% similarity with *Fusarium solani* strain 68 18S ribosomal RNA gene (Accession Number Gen Bank: JX897001.1), *Fusarium solani* strain H8 18S ribosomal RNA gene (Accesion Number Gen Bank: JF323002.1), and *Fusarium solani* genomic DNA containing partial 18S rRNA gene (Accession Number Gen Bank: FR691777.1). They have a larger similarity (100%) with *Fusarium* sp. r323 18S ribosomal RNA gene, partial sequence (Accession Number Gen Bank: HQ649839.1), and *Fusarium solani* strain M146 18S ribosomal RNA gene(Accession Number Gen Bank: JN127379.1) (Table 1).

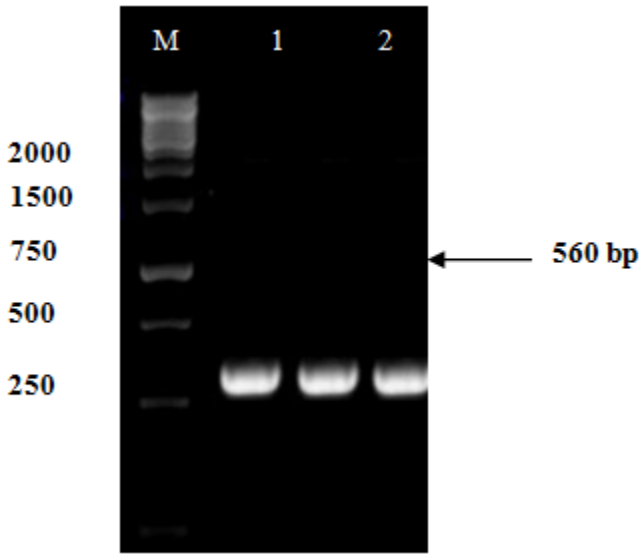

Figure 3. PCR amplification of the ITS gene with primer ITS_5F and primer ITS_4R. M = marker 1 Kb ladder (Fermentas), 1 = PCR product of *Fusarium* sp. isolate w2, and 2 = PCR product of *Fusarium* sp. isolate w2

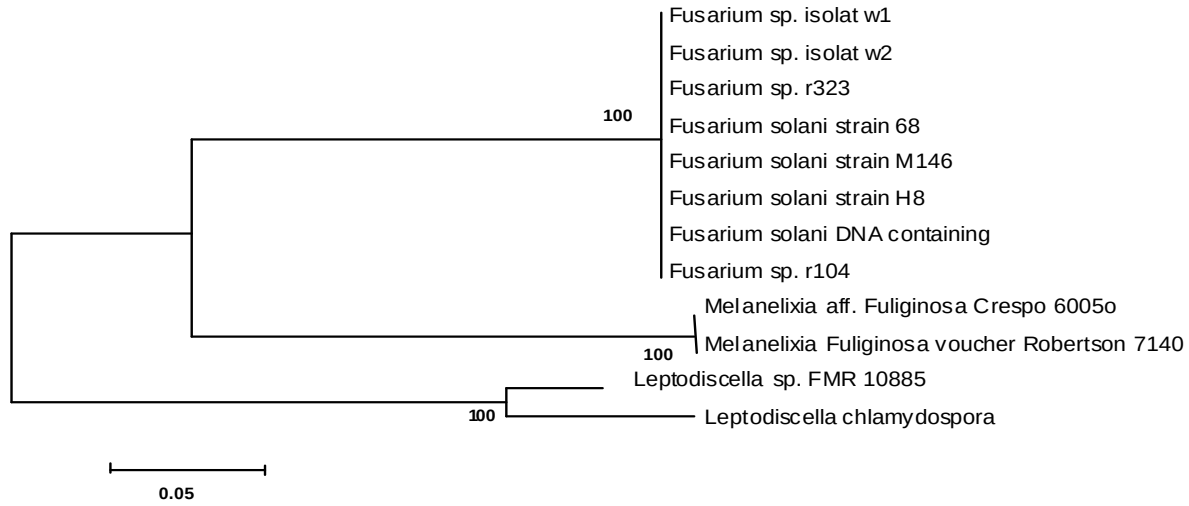

Figure 4. Phylogenetic relationship constructed from ITS sequences of the characterized clone library of *Fusarium*. Bootstrap value greater than 50% are shown at each node.

Table 1.comparisons of 18S rDNA gene similarity levels of *Fusarium* sp. isolates w1 and w2 with multiple sequences in GenBank using BLAST program

Isolates	% Similarity	Accession
Fusarium solani strain 68 18S ribosomal RNA gene,	99	JX897001.1
*Fusarium solan*i strain H8 18S ribosomal RNA gene,	99	JF323002.1
Fusarium solani genomic DNA containing partial 18S rRNA gene	99	FR691777.1
Fusarium sp. r323 18S ribosomal RNA gene, partial sequence	100	HQ649839.1
Fusarium solani strain M146 18S ribosomal RNA gene	100	JN127379.1

From the Table 1 and Figure 4, it can be seen that *Fusarium* sp. isolates w1 and w2 are closely related to the *Fusarium solani* strain 68 18S ribosomal RNA gene, *Fusarium solani* strain H8 18S, *Fusarium solani* genomic DNA containing partial 18S rRNA gene, *Fusarium* sp. r323 18S ribosomal RNA gene, and *Fusarium solani* strain M146 18S ribosomal RNA gene. Ronquillo (2012) reported that the *Fusarium solani* strain H8 18S caused bud rot in the oil palm in Ecuador. Shahnazi *et al*. (2012) investigated that *Fusarium solani* strain H8 18S caused yellowing disease of black pepper (*Piper nigrum* L.) in Malaysia. Sarmiento-Ramirez *et al*. (2010) reported that *Fusarium solani* genomic DNA containing partial 18S rRNA gene was responsible for mass mortalities in nests of logger head sea turtle. *Fusarium* sp. r323 18S ribosomal RNA gene was associated with Roots of Halophytic and Non-halophytic Plant Species was reported by Macia-Vicente *et al*. (2012). In addition, Rosado-Rodriguez *et al*. (2011) reported that *Fusarium solani* strain M146 18S ribosomal RNA gene was associated with Leatherback Sea Turtle (*Dermochelys coriacea*) Nests in the Mayaguez-Anasco Bay Coast, Western Puerto Rico.

Fusarium solani is one of the most frequently isolated fungi from soil and plant material, where they act as decomposers, but they are also host-specific pathogens of a number of agriculturally important plants, including sweet potato, cucurbits, and pea. Moreover, they are increasingly associated with opportunistic infections of humans and other animals, causing systemic infections with a high mortality rate, as well as localized infections in the skin and other body parts (Zhang*et al*., 2006). Mycotoxin trichothecenes produced by *Fusarium* is very toxic for human (Miller and Trenholm, 1994). This toxin can cause cancer, hemorrhage, edema and immune deficiency (Alexoupolos *et al*., 1996). WHO (1979) reported that mycotoxins are hazardous to human and animal health.

4. Conclusion

Based on the results of present study, it can be concluded that the causal agent of the stem rot disease on dragon fruits (*Hylocereus sp.*) in Bali is identified as *Fusarium solani*. Hard efforts must be done to control the disease in order to reduce the losses of dragon fruit production and the risk of mycotoxins contamination which are probably produced by *Fusarium solani*.

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