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FIRST STUDY ON FUSARIUM EQUISETI: CAUSES FUSARIUM WILT IN TOMATO CROP IN BALI, INDONESIA

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SUMMARY

Fusarium wilt causes losses to tomato crops, in Bali - Indonesia. This disease is caused by a pathogenic fungi. Research on the morphology and molecular pathogen of the fungi is very important to ensure the causes of plant diseases. Certainty about the causes of plant diseases is closely related to controlling the disease. Koch's postulate is done to confirm the agent of the disease. Based on observations of macroscopic, microscopic, and 18S rDNA characteristics, it can be identified that the cause of wilt in tomato plants in Bali is the *Fusarium equiseti*. This paper is the first report on one of the causes of wilt in tomato plants in Bali, *Fusarium equiseti*.

Key words: Fusarium wilt, tomato plants, microscopic, molecular study

Key findings: This study identifies pathogenic fungi that cause wilt in tomato plants based on morphological and molecular characters.

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INTRODUCTION

Tomato (*Lycopersicum esculentum* L.) is one of the most widely consumed vegetable species in Indonesia after kale and spinach vegetables (Central Bureau of Statistics, 2016). Tomato production in Indonesia is still low as compared to other countries in the world, which is only 0.8 million tons or 0.5% contributing to world tomato production of 161.79 million tons. However, China, India, and the United States, their production is 46.06 million tons or 30.02%, 8.89% and 8.54%, respectively (Secretary General of the Ministry of Agriculture, 2014). One of the causes of low

tomato production in Indonesia is the disease that attacks tomato plants.

Several types of diseases that commonly affect tomato plants caused by fungi and bacteria are Fusarium wilt (fungus: *Fusarium oxysporum* f. sp. *lycopersici*), Anthracnose (fungus: *Colletotrichum* spp), Buckeye rot (oomycetes: *Phytophthora nicotianae* var. *parasitica*, *P. capsici*, and *P. drechsleri*), Early blight (fungi: *Alternaria solani* and *A. tomatophila*, Gray leaf spot (fungi: *Stemphylium* spp.), Gray mold (fungus: *Botrytis cinerea*), Late blight (oomycete: *Phytophthora infestans*), Leaf mold (fungus: *Passalora fulva*, formerly *Cladosporium fulvum* and *Fulvia fulva*), Powdery mildew (fungi: *Leveillula taurica* and *Oidium neolycopersici*), Pythium damping-off and stem rot (oomycetes: *Pythium* spp.), Septoria leaf spot (fungus: *Septoria lycopersici*), bacterial canker caused by bacterium *Clavibacter michiganensis* subsp. *michiganensis*, Bacterial speck (bacterium: *Pseudomonas syringae* pv. tomato), bacterial spot (bacteria: *Xanthomonas* spp.), Bacterial wilt (bacterium: *Ralstonia solanacearum*) (Wani, 2011; Melanson, 2017).

Rozlianah and Sariah (2006) also reported that fungi can cause disease in tomato plants are *Fusarium verticillioides, F. oxysporum* and *F. equiseti*. This fungus attacks tomato plants during growth through the root system. The Fusarium fungus causes the most disease in tomato plants. It attacks tomato plants both in the greenhouse and in the field (Jones *et al.*, 1991). Pathogenic fungi cause symptoms of wilt on tomato plants, so the disease that attacks tomato plants is often called Fusarium wilt. Fusarium wilt in tomato plants is caused by *Fusarium oxysporun* f.sp. *lycopersici* (Stone *et al*., 2000). This wilt causes loss, decreases crop yields and even causes death in tomato plants (Amini and Sidovich, 2010).

Based on preliminary surveys Fusarium wilt often attacks tomato plants in Bali. This disease attacks tomato plants that live in lowland and highland areas, namely the villages of Sempidi, Petang- Badung Regency, and Songan Kintamani Village, Bangli. This disease attacks plants through the roots at the seedling stage and mature plants. Percentage of wilt disease ranges from 15-25%.

Molecular identification can be used to find out problems in the taxonomy of fungi (Raja *et al*., 2017). Comparison of sequences on ribosomal DNA coding genes can be used as characteristic traits for molecular identification of an organism such as fungi because these genes have conserved sequences (Kurtzman and Fell, 2006).

MATERIALS AND METHODS

Fungal pathogen isolation and virulence test

The stem above the root surface of a wilted tomato plant located in Cau Blayu village, Marga sub-district, Tabanan-Bali, was taken to the laboratory to be isolated. Wilted tomato stems are washed with running water, then washed with 70% alcohol, cut into small pieces measuring approximately 1.5 x 1.5 cm and planted in petri dishes with PDA media. This culture is incubated in a room temperature of 27 ± 2^{o} C for approximately 3 days so this culture will grow. Pure cultures that are inoculated on healthy tomato seedlings will show the same

symptomatic wilting in tomato plants on a wilted field due to pathogenic fungi. As the purpose of Koch's Postulate test. The pure culture was harvested to make fungal suspension 1.5 x 106 spores /mL inoculated on fresh tomato seeds that were 14 days old through the roots that had been injured by soaking for 1 and 12 hours. Three plants were treated with fungal isolates. For control, the roots of tomato seedlings were injured with a sterile needle then immersed in sterile water. Then the tomato plant seeds that have been inoculated with pathogenic fungal are planted in a polybag and then let stand a few days to see the development. Based on the preliminary test, those tomato seedlings that have been inoculated with pathogenic fungi will experience wilt after 6 days. Wilted tomato seeds are re-isolated in pure culture. The result of this pure culture must have characteristics and symptoms similar to pathogenic fungi that infect tomato plants in the field.

Morphological characterization

Fungal characterization based on morphological characteristics by observing macroscopic and microscopic characteristics. Macroscopic observation of pathogenic fungi, namely by observing the color of the colony and colonies reverse, growth, and flow of colonies. While the characteristic of microscopy is seeing hypa or fungal spores under a microscope.

Molecular identification

DNA extraction

Fusarium sp. fungus isolates were grown on PDA media for 3 days at room temperature (± 28^{o}C). *Fusarium* sp. genome DNA was extracted by taking hyphae on the edge of the fungal colony, then placing it in a centrifuge bottle and suspended with 100 µl PrepMan Ultra reagent (PrepMan Ultra Protocol, Applied Biosystem, USA). The sample was then vacuumed for 30 seconds and then placed in a heat block of 95-100°C for 10 minutes and placed at room temperature for 2 minutes. Samples were centrifuged at 10,000 rpm for 2 minutes and pellets were taken as DNA extracts and used for further analysis (Cano *et al*., 2004).

Amplification of DNA by PCR

The 18S rRNA gene was amplified by PCR using the Internal Transcriptional Spacer (ITS) 1 (5-TCCGTAGGTGAACCTGCGG-3) primer and ITS 4 (5-TCCTCCGCTTATTGATATGC-3). PCR tool used in this study are Takara PCR thermal cycler Personal (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) with pre-denaturation 94°C (4 minutes) followed by 35 cycles from denaturation 94°C (35 seconds), annealing 52°C (55 seconds), elongation 72°C (2 minutes) and post elongation 72°C for 10 minutes (Nishizawa *et al*., 2010).

Sequencing of ITS regions and computer analysis of DNA sequences

The nucleotide sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the guidelines of the tool and with the PE Applied Biosystems Automated DNA Sequencer (3130xl model, Applied Biosystems). Using *Genetyx* (version

11.0) and *Genetyx-ATSQ* (version 4.0) software (*Genetyx*, Tokyo, Japan), compared with the same DNA sequence from DDBJ / EMBL / GenBank through the NCBI BLAST program (Thompson *et al*., 1997). Phylogeny analysis is carried out using the MEGA 6.0 program (Kumar *et al*., 2001), the Maximum Parsimony (MP) method with bootstrap1000x, with the following steps: (1). Search for similarities between sequences. Sequence data stored in the notes in the FASTA format is analyzed using the Blast-WU facility available online at www.ebi.ac.uk/Clustalw. (2). Making a tree of phylogeny with the MEGA program. Data from processing using the ClustalW facility will then be used as basic data to make phylogeny trees using MEGA data facilities.

RESULTS AND DISCUSSION

Fungal isolate

Based on the Koch Postulate Test, isolates of *Fusarium* sp. from the withered tomato plants in the field caused the same symptoms of the disease, mushroom isolates are made pure culture on PDA media, and then pure culture is inoculated on fresh tomato seeds resulting in fresh tomato seeds wither (Figure 1).

Macroscopic and microscopic characteristic

Macroscopic observation by looking at the state of fungal colonies on PDA media. *Fusarium* sp. isolate in the field were then grown in Petri dishes with PDA media a lot of produced aerial mycelium, the color of the white colonies becomes yellowish with age, cream to light brown or dark brown colony reverse, yellowish white pigmentation, rapid colony growth was in 4 days the colony diameter 4.0 - 4.2 cm and in 11 days 9.2 cm. The same thing ever reported by Samson *et al*., 1981 that *Fusarium equiseti* (Corda) Sacc. colony on OA or PSA at 25^{o}C attaining a diameter of 5.8 cm in 4 days, culture whitish to buff, aerial mycelium floccose (Figure 2A).

The microscopic character seen is the size, septa, shape of fungal spores in PDA media. *Fusarium* sp. isolates have hyphae, microconidia, macroconidia, and chlamydospore. Microconidia consists of 1-2 cells, measuring 13-15 x 2-12 µm with 1-2 septates. Macroconidia have 3-7 septates, measuring 40-60 x 5-10 µm, shaped like a crescent or canoe. Clamidospores grows in the middle part of hypha (Figure 2 B-E). In accordance with the opinion of Barnett and Hunter (1998) that macroconidia fungi *Fusarium* is said to be in the form of canoe. Microconidia are smaller than macroconidia.

Molecular analysis

Samples of Fusarium sp.

This data is obtained based on molecular analysis. Based on the sequence alignment with the Genbank database, *Fusarium* sp. isolates have a similarity of 99% with *Fusarium equiseti* GGF2 isolates (Gen Bank Accession Number: HM008677.1), *Fusarium equiseti* strain F277882 (Gen Bank Accession Number: KC427030.1), *Fusarium equiseti* genomic DNA containing 18S (Gen Bank Accession Number: HF570011.1), Fusarium cf. equiseti MY-2011 AM-28 isolate (Gen Bank Accession Number: JN038469.1),

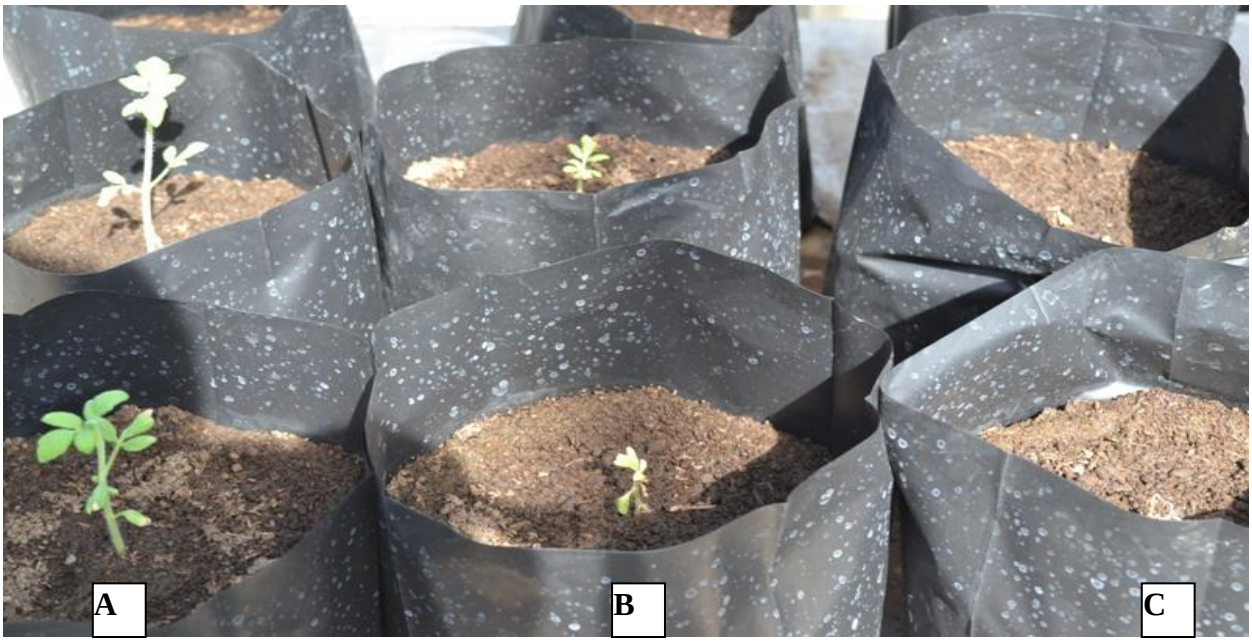

Figure 1. A. Control, B. The mushroom was inoculated on tomato seeds by soaking it for 1 hour resulting in wilting on tomato seeds (6 days after planting), C. mushrooms inoculated on tomato seeds by soaking 12 hours resulting in wilting until death in tomato seeds (6 days after planting).

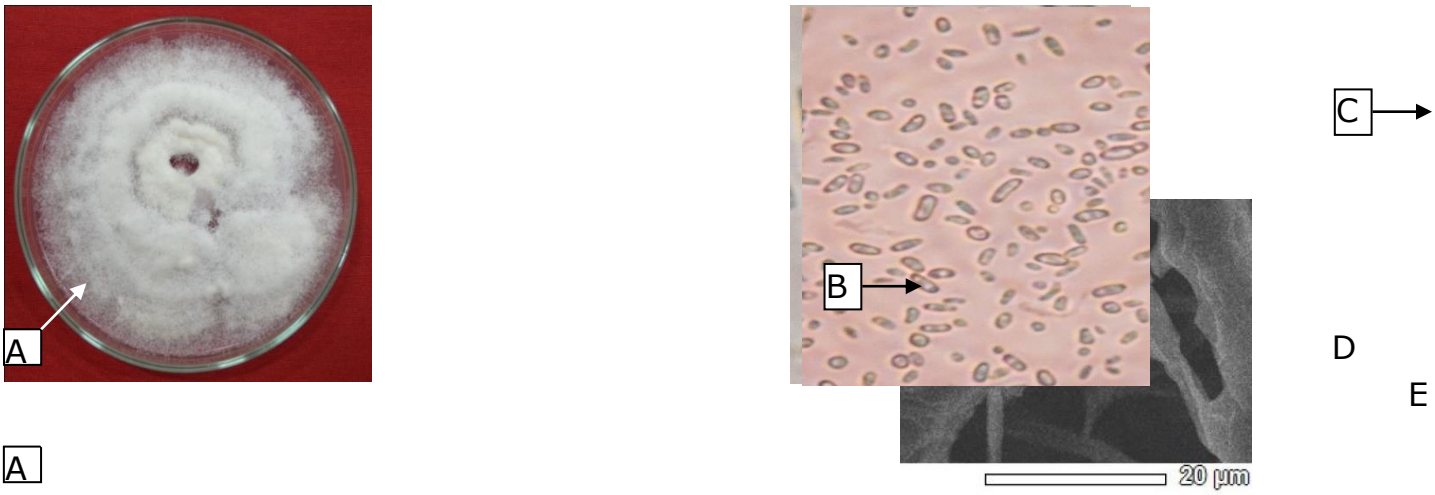

Figure 2. A. Macroscopic Characteristic: *Fusarium equiseti* fungal colonies, B-E. Microscopic Characteristic: B. Microconidia, C. Macroconidia, D. Hyphae with SEM (Scanning Electron Microscope), E. Clamydospore with SEM.

Table 1. Comparisons of 18S rDNA gene similarity levels of *Fusarium* sp. isolates with multiple sequences in Gen Bank using BLAST program.

Isolate	% Similarity	Accession Number
Fusarium equiseti isolate GGF2	89	HM008677.1
Fusarium equiseti strain F277882	90	KC427030.1
Fusarium equiseti genomic DNA containing 18S	91	HF570011.1
Fusarium cf. equiseti MY-2011 isolate AM-28	89	JN038469.1
Fusarium equiseti isolate dx-7	90	FJ441009.1
Fusarium equiseti strain 2013lg3	89	KF475770.1

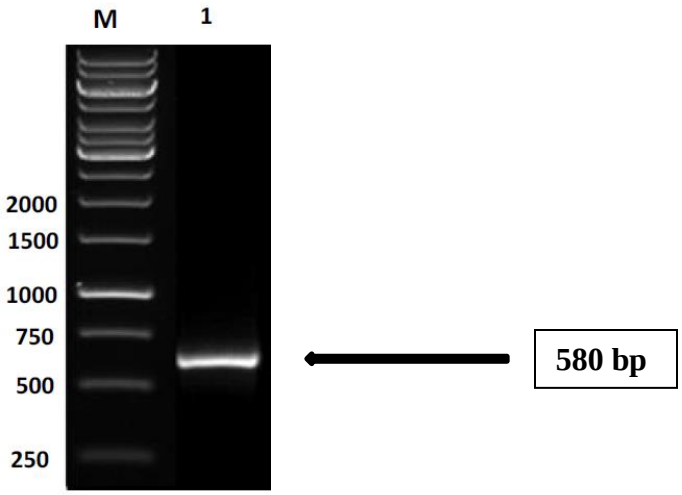

Figure 3. PCR amplification of ITS genes with ITS_5F and primary ITS_4R primers; M = 1 Kb ladder marker; 1 = PCR product sample *Fusarium* sp.

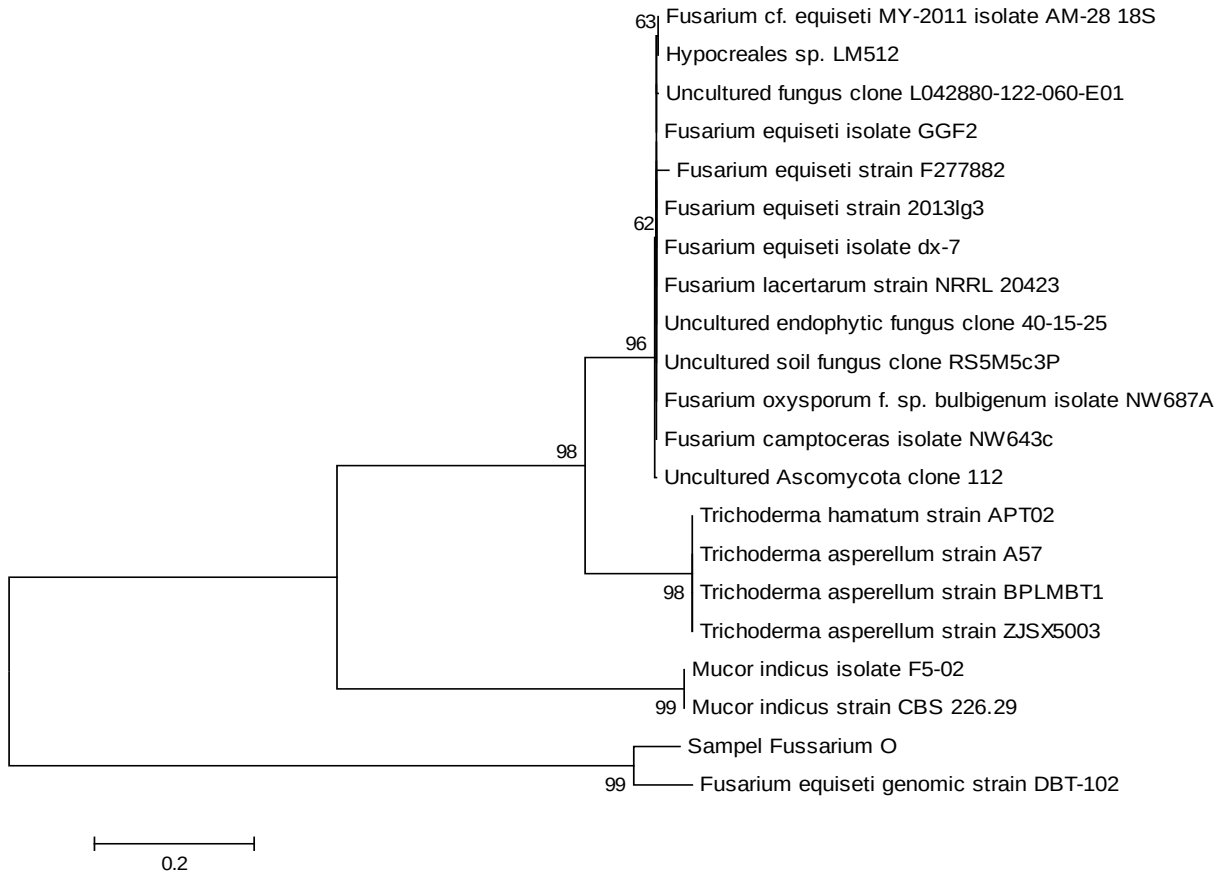

Figure 4. Phylogeny trees from Fusarium samples (*Fusarium* sp.) closely related to *Fusarium equiseti* by 99%.

Fusarium equiseti isolate dx-7 (Gen Bank Accession Number: FJ441009.1), *Fusarium equiseti* strain 2013lg3 (Gen Bank Accession Number: KF475770.1) (Table 1). This can also be seen in the resulting phylogenetic tree, with a 1000x bootstrap value that is shown to be 99% closely related to *Fusarium equiseti* (Figures 3 and 4).

Singha *et al*. (2016) reported a study of Fusarium causes of wilt in tomato plants. Fusarium isolates used were in this study from Assam, an area in north eastern India. The number of fusarium isolates used was 8 isolates. Analysis based on molecular characters, the eight Fusarium isolates were identified as three species of Fusarium causes of wilt in tomato plants, namely *Fusarium equiseti, Fusarium oxysporum*, *Fusarium proliferatum*. Chehri (2016) once reported that Fusarium fungi cause root rot in tomato plants in western Iran. Fusarium isolates collected in the field totaled 25 isolates. Isolates from several different regions in the western part of the Iran. Analysis based on morphological and molecular characteristics identified four species of fusarium namely *F. oxysporum, F. redolens, F. proliferatum* and *F. verticillioides.*

Fusarium fungus species in addition to the cause of wilt in tomato

plants can also infect other plants such as cucumber and melon plants. Research conducted by Chehri *et al*. (2011), the genus Fusarium is one of the disease-causing fungi in cucumber plants in Kermanshah province, an area of cucumber planting in Iran. Fusarium isolates infecting total of 100 isolates collected from cucumber plants, which were plant from several regions in Kermanshah Province, Iran. Morphological analysis result can be identified as five species of fungi that cause disease in cucumber plants namely *Fusarium oxysporum, F. proliferatum, F. equiseti, F. semitectum* and *F. solani.* The analysis of RFLP the five species of Fusariun were divided into two groups, namely the group consisting of *F. solani* and *F. proliferatum*, while the other groups consisted of *F. oxysporum, F. equiseti*, and *F. semitectum*. Babineau *et al.* (2018) have reported that *Fusarium oxysporum* fungi have several kinds of strains such as *Fusarium oxysporum* f.sp. *lycopersici* Tropical Race one which caused death in the Gross Michel banana plant in 1960.

CONCLUSION

Based on the results it can be concluded that one of the causes of wilt in tomato plants in Bali, Indonesia is the *Fusarium equiseti* fungus. Great efforts to control Fusarium Wilt of tomato plants to reduce losses in crop yields. Further research is needed to get other Fusarium species as wilt on tomato plants.

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