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Identification and pathogenicity of *Fusarium* species causing dry rot disease associated with imported citrus fruits

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Abstract

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Dry rot disease is one of the main postharvest diseases caused by *Fusarium* species. Several pathogenic *Fusarium* species have the potential to be carried on imported citrus fruits. This study aims to detect, isolate, identify, and test the pathogenicity of *Fusarium* species from imported citrus fruits. *Fusarium* species were isolated using the Overnight Freezing Incubation technique (ONFIT) method by freezing citrus fruits at – 20°C for 15 hours. *Fusarium* species were identified morphologically and molecularly. Morphological identification was conducted macroscopically and microscopically by observing the characteristics of the colony on PDA media and conidia. Molecular identification was carried out by the Polymerase Chain reaction (PCR) method using universal primers of ITS1 and ITS4. Pathogenicity was tested by injecting 100 µl of fungal conidia suspension with a density of 10^5 conidia/ml into healthy citrus fruits. The results showed that 3 *Fusarium* species were successfully isolated and identified as *Fusarium equiseti*, *Fusarium lateritium,* and *Fusarium oxysporum*. The pathogenicity test proved that all *Fusarium* species were pathogenic to citrus fruits. The disease incidence of all *Fusarium* species was 100%. The disease severity caused by *F. equiseti*, *F. lateritium* and *F. oxysporum* were 73.33, 93.33 and 84.44%, respectively. These results are a new report of 3 *Fusarium* species causing dry rot disease in citrus fruits.

Keywords: detection; disease severity; fungal pathogen; molecular; morphological identification

Introduction

Citrus fruits are one of the most popular and widely consumed fruits in Indonesia (Andrini et al., 2021). Currently, Indonesia is still importing citrus fruits to supply market demand. This can be seen in a large number of imported citrus fruits in traditional and modern markets. Citrus fruits are imported from various countries such as Pakistan, China, America, Australia, and Argentina. The Ministry of Agriculture, Republic of Indonesia (2016) noted that Indonesia is the fourth largest citrus importing country among ASEAN countries with a contribution of 12.84%.

The importation of citrus fruits in international trade has the potential to bring non-native pathogenic fungal species that can invade local citrus fruits (Lichtenberg & Olson, 2018). This potential is based on the ability of pathogenic fungi to infect citrus fruits latently without showing disease symptoms (Johnston et al., 2005). Schaad et al. (2003) stated that latent infecting fungi are difficult to detect because the fungi cannot be isolated from the symptoms. This fact raises concerns that pathogenic fungi will be carried by imported citrus fruits, spreading and causing disease outbreaks in local citrus fruit plantations. Agrios (2005) emphasized that the spread of pathogens is an important stage in the process of pathogenesis.

Dry rot disease is one of the postharvest diseases that cause yield loss of citrus fruits in storage. Symptoms on the surface of infected fruit become rough or dry rot, light or dark brown, and sunken. White, gray, or pink mycelia appear on the fruit surface under moist conditions, depending on the *Fusarium* species (Whiteside et al., 1993). Hyun et al. (2000) found 5 *Fusarium* species associated with dry rot disease of citrus fruits in Jeju Island, namely *Fusarium moniliforme*, *Fusarium proliferatum*, *Fusarium solani*, *Fusarium sambucinum,* and *Fusarium equiseti*. Ezeibekwe & Unamba (2009) also reported that *Fusarium oxysporum* and *F. equiseti* were responsible for the rot disease of citrus fruits in Nigeria. *Fusarium* species that cause citrus fruit rot in Argentina are *Fusarium lateritium*, *F. moniliforme*, *F. oxysporum*, and *F. solani* (Fogliata et al., 2013). A recent report by Moosa et al. (2020) found *F. oxysporum* as the main cause of citrus fruit rot disease in Pakistan.

Plant health is important to be assessed to ensure that traded agricultural products are free of the pathogen (Turner et al., 2013). Therefore, fruit-borne pathogenic fungi are very important to identify. The identity of pathogenic fungi is used as the basis for formulating more appropriate crop protection measures, such as preventing the spread of pathogenic microorganisms, reducing disease incidence, increasing agricultural production, and ensuring sustainable agricultural practices (Doring et al., 2012; Droby & Wisniewski, 2018). Identification of pathogenic fungi can be done morphologically and molecularly. Morphological identification is carried out by isolating microorganisms from plants using culture media containing certain nutrients and growing them under conditions suitable for microorganisms (Turner et al., 2013). Molecular identification of eukaryotic microorganisms such as fungi is carried out by amplifying the Internal Transcribed Spacer (ITS) locus using the PCR technique (Sikdar et al., 2014).

This study aims to assess plant health by detecting, isolating, identifying, and testing the pathogenicity of *Fusarium* species associated with imported citrus fruits.

Materials and Methods

Research locations, time, and citrus fruits

The research was conducted at the Plant Mycology Laboratory, Department of Plant Protection, IPB University, Bogor Regency, and Biotechnology Laboratory, Applied Research Institute of Agricultural Quarantine, Bekasi Regency from September 2021 to July 2022. The citrus fruit species used in the study was *Citrus reticulata*. Citrus fruits come from imported samples taken at Tanjung Priok Port, Jakarta. Healthy citrus fruits without any disease symptoms with the same shape, size, and color were selected and used in this study.

Isolation of Fusarium species

Fusarium species were isolated by using the Overnight Freezing Incubation Technique (ONFIT). Citrus fruits are washed using tap water to remove dust or other materials that stick to the surface of the fruits. Citrus fruits were rinsed 2 times using sterile water and arranged on a rack in a 35 × 27 × 10 cm plastic container and tightly closed. The humidity in the container is maintained by adding sterile water. Citrus fruits were frozen in the freezer at – 20°C for 15 h and incubated at room temperature (modified Michailides et al., 2010). Isolation of *Fusarium* species was carried out on a total of 100 citrus fruits with the distribution of 10 citrus fruits in each plastic container. Fungal mycelia growing on the surface of citrus fruits were isolated and re-grown on PDA media. Isolation was based on differences in mycelia characteristics, such as color and texture, which lead to different fungal species. Fungal mycelia showing the same characteristics are grouped into one morphotype. Several colonies showing the same mycelia characteristics on the surface of citrus fruits were counted as 1 per 1 citrus fruit. The results of fungal isolation were quantified by calculating the percentage of the number of morphotypes and the emergence of the fungi on the surface of citrus fruits. The obtained fungi were made into single isolates to ensure the fungi really came from one species.

Morphological identification of Fusarium species

Fungi from a single isolate were identified based on the morphological characteristics of the colony on PDA media and conidia. Identification was done by observing the growth pattern of mycelia growing on PDA media. In addition, identification was also carried out morphometrically by observing the shape and size of the conidia. Carnation Leaf-Piece Agar (CLA) is used to emerge all morphological characteristics needed in the identification process. The identification process is based on the suitability of the observation results of morphological characteristics with the literature book on fungal identification to identify *Fusarium* species.

Molecular identification of Fusarium species

Molecular identification of *Fusarium* species was carried out through the isolation of DNA from fungal cultures, amplification of the fungal DNA ITS locus using the PCR technique, and identification of DNA sequences using the Basic Local Alignment Search Tool (BLAST) program on the NCBI website.

Isolation of DNA from Fusarium culture on PDA Media

DNA isolation of *Fusarium* species was done by following the protocol of Ausebel et al. (2003) with some modifications. 10 culture circles of *Fusarium* species were made by cork borer (4.5 mm in diameter). Then, the culture circles were placed on aluminum foil and stored in the freezer at – 80°C for 3 h. A total of 600 µl of 2% Mercapto Ethanol (ME) in CTAB extraction solution and 70 µl CTAB/NaCl solution were heated in a water bath at 65°C before being used. The culture circles in aluminum foil were taken from the freezer at – 80°C and crushed until smooth. 600 µl of warm 2% Mercapto Ethanol (ME) in CTAB extraction solution was added until dissolved. The mixed solution was put into a new 1.5 ml Eppendorf tube and heated in a water bath at 65°C for 60 min with occasional shaking or inverting. Chloroform: Isoamyl Alcohol / CI (24:1) with the same volume was added, then shaken and centrifuged at 10000 rpm, temperature 4°C for 5 min. The supernatant (liquid phase) is taken and measured. Then, it was transferred to a new 1.5 ml Eppendorf tube. As much as 1/10 of the volume of warm CTAB/NaCl solution was added and mixed well. CI (24:1) with the same volume was added again, then shaken and centrifuged at 10000 rpm, temperature 4°C for 5 min. The supernatant is taken and measured. Then, it was transferred to a new 1.5 ml Eppendorf tube. A total of 1 volume of CTAB precipitation solution was added and mixed well. The solution was centrifuged at 2700 rpm for 5 min at 4°C. The solution was added with 150 µl of TE buffer with high salt content. DNA pellets were precipitated by adding 0.6 volumes of isopropanol from the total solution. Then, it was centrifuged at 10000 rpm for 20 min at 4°C. Supernatant was discarded. The DNA pellet was washed with 500 µl of 80% ethanol. Then, it was centrifuged at 8000 rpm for 5 min at room temperature. The supernatant (remaining 80% ethanol) was discarded and the DNA pellet was dried. DNA pellet was dissolved or suspended by adding 100 µl of TE buffer. DNA was stored at – 20°C until being used for further testing (PCR).

DNA Quantification of Fusarium Species

DNA concentration of *Fusarium* species was measured by using Nanodrop One with serial number AZY2125665. This quantification aims to analyze the quality of DNA, because it will be used for the PCR testing process.

The Amplification of DNA ITS Locus from Fusarium Species

The amplification of the DNA ITS locus was carried out by a Thermal cycler using universal primer pairs of ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes & Bruns, 1993) and ITS4 (CCTCCGCTTATTGATATGC) (White et al., 1990) with an amplification product size of about 600 bp. DNA amplification was conducted in a volume of 50 µl containing 20 µl of PCR master mix, 2 µl of ITS1F primer, 2 µl of ITS4 primer, 4 µl of isolated DNA, and 22 µl of ddH_2O. DNA amplification was carried out in 35 cycles through denaturation of double-stranded DNA at 94°C for 35 s, annealing of the primers to the target DNA at 51°C for 1 min, and DNA extension at 72°C for 2 min. DNA amplification results were visualized on 1% agarose gel.

DNA Sequences of Fusarium Species

The PCR product was sent to First Base, Malaysia to be sequenced. The DNA sequences of ITS 1 and ITS4 DNA loci were compared with the DNA sequences from other countries by using the Basic Local Alignment Search Tool (BLAST) program on the NCBI website.

Phylogenetic Tree

The phylogenetic tree was created to analyze the relationship between *Fusarium* species found in imported citrus fruits, *Fusarium* species that already exist in Indonesia, and *Fusarium* species from other countries. Phylogenetic analysis was performed using Mega software version 10, based on the neighbor-joining approach with a bootstrap value of 1000 times.

Pathogenicity Test

Healthy citrus fruits without disease symptoms were used in the pathogenicity test. Citrus fruits were disinfected by immersion in 5.26% sodium hypochlorite for 4 min to remove surface contaminants and rinsed twice with sterile water. After drying, the citrus fruits were arranged on a rack in a 35 × 27 × 10 cm plastic container and tightly closed. The humidity in the container is maintained by adding sterile water. Ten citrus fruits in plastic containers were inoculated with *Fusarium* species. A total of 100 µl suspension of each *Fusarium* species (10^5 conidia/ml) was injected into the bottom of the calyx into the columella using a sterile syringe. Meanwhile, citrus fruits were inoculated with sterile water on control. Citrus fruits were incubated at 25°C in the dark for 21 days. The experiment was repeated 3 times (Camiletti et al., 2022).

Citrus fruits were observed by classifying dry rot symptoms caused by *Fusarium* species based on the rotten scale. Symptoms of dry rot were observed from the inside by splitting the citrus fruit. Scale 0 = no visible symptoms; 1 = 1–30% symptoms; 2 = 30–60% symptoms; and 3 = > 60% symptoms (Mojerlou & Safaie, 2012). The pathogenicity of *Fusarium* species was determined by assessing the incidence

and severity of disease based on the rotten scale in citrus fruits. The incidence and severity of the disease were calculated using the following formula:

$$DI = \frac{\text{Number of rotten fruit}}{\text{Total number of fruits observed}} \text{ x } 100\ \%$$

$$DS = \sum \frac{(n \text{ x } V)}{Z \text{ x } N} \text{ x } 100\ \%\ ,$$

where

DI = Disease Incidence;
DS = Disease Severity;
n = number of fruits showing the same scale;
V = symptom scale;
Z = the highest scale;
N = total number of fruits observed.

The study used a completely randomized design (CRD). Data were analyzed using Analysis of Variance (ANOVA). If the results of the analysis show a P-value < α = 0.05, then proceed with the Tukey test at level α = 0.05 to see the differences in incidence and severity of disease between *Fusarium* species pathogenicity and control (Kasiamdari & Sangadah, 2015). The analysis was performed using SAS software version 9.4.

Results

Isolation of Fusarium species

The ONFIT method succeeded in accelerating the emergence of fungi carried by imported citrus fruits. Figure 1 shows that the fungal mycelia were visible in 4 Days After Incubation (DAI). Mycelia growing from one colony was immediately isolated and transferred to PDA media. Isolation was carried out at 4 DAI when the fungal mycelia were visible. Fungal mycelia were continuously isolated on newly growing colonies, still separated, and have not merged with each other. Mycelia were isolated from colonies showing different characteristics. The emergence of new colonies increased and the old colonies expanded with increasing incubation days. Fungal mycelia almost covered the entire surface of citrus fruits at 6 DAI. The mycelia between the fungi have been merged with each other, so it is difficult to be isolated. Meanwhile, control without ONFIT treatment did not show fungal mycelia until 6 DAI.

Isolation of fungal mycelia produced 8 morphotypes from a total of 209 fungal colonies on the surface of citrus fruits. Morphotypes are formed as a result of grouping the same mycelial characteristics. The eight morphotypes had

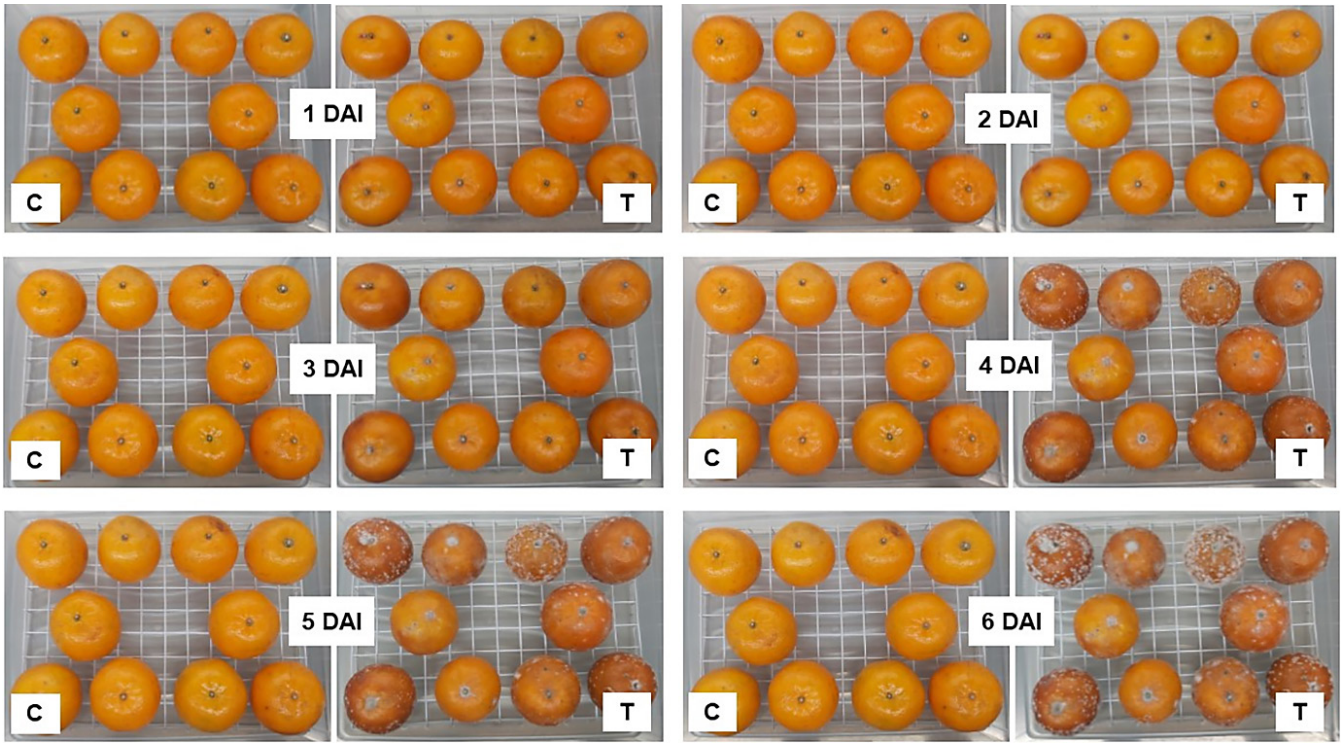

Figure 1

Table 1. Percentage of fungal morphotypes on citrus fruit surfaces

Morpho-type	Characterization of mycelia on the surface of citrus fruits	Amount	Percentage of morphotypes (%)[a]	Percentage of emergence (%)[b]
I	Mycelial colonies are green, like fine cotton, and grow flat	34	16.27	34
II	Mycelial colonies are yellowish-white, like coarse cotton, and grow convex	36	17.22	36
III	Mycelial colonies are white, like fine cotton, and grow convex	10	4.78	10
IV	Mycelial colonies are pink, like coarse cotton, and grow flat	14	6.70	14
V	Mycelial colonies are white, like coarse cotton, and grow flat	37	17.70	37
VI	Mycelial colonies are green, like fine powder, and grow convex	7	3.35	7
VII	Mycelial colonies are grayish green, like coarse cotton, and grow convex	21	10.05	21
VIII	Mycelial colonies are purplish white, like coarse cotton, and grow convex	50	23.92	50

Notes: [a] Percentage of morphotypes was obtained from the number of the same morphotypes per all morphotypes. [b] Percentage of emergences was obtained from the number of mycelial emergence in 100 citrus fruits. The emergence of the same mycelia in more than one of the same citrus fruit was counted as 1

different percentage values of number and emergence (Table 1). Morphotype VIII was the most emerging morphotype compared to other morphotypes with a percentage of 23.92%. A total of 50 fungal colonies of morphotype VIII were found from a total of 209 fungal colonies. In addition, the emergence of morphotype VIII was dominated by 50% in a total of 100 citrus fruits tested. A total of 50 fungal colonies of morphotype VIII were found in 50 of 100 citrus fruits. Meanwhile, morphotype VI was the least emerging morphotype with a percentage of 3.35%. A total of 7 fungal colonies of morphotype VI were found from a total of 209 fungal colonies. In addition, morphotype VI was the least at 7% in a total of 100 citrus fruits.

The ONFIT method causes the discoloration of the citrus fruits' skin to become darker brown with increasing incubation days (Figure 1). The discoloration was visually visible at 3 DAI. Meanwhile, control without ONFIT showed undiscoloration until 6 DAI. In addition, the ONFIT method resulted in the texture of citrus fruits becoming softer with increasing incubation days. Meanwhile, the texture on control was still hard even at 6 DAI.

Morphological identification of fungal morphotypes

Morphological identification determined that 3 of the 8 morphotypes were *Fusarium* species. Table 2 shows morphotype II identified as *Fusarium equiseti* (Leslie & Summerell, 2006; Hami et al., 2021), morphotype IV identified as *Fusarium lateritium* (Leslie & Summerell, 2006; Vitale et al., 2011), and morphotype VIII identified as *Fusarium oxysporum* (Leslie & Summerell, 2006; Ciampi et al., 2009). *F. equiseti* has yellowish white to brown colonies, irregular in shape, convex elevation, undulate margin, yellow pigmentation and mycelial growth rate of 5.83 ± 0.52 cm. Macroconidia is slightly curved and slender, apical cell morphology is tapered, basal cell morphology is pointed, mean length (35.04 ± 2.60) and mean width (4.03 ± 0.41), 5–7 septate (6-septate most dominant). *F. lateritium* has dark red colonies, circular in shape, flat elevation, entire margin, dark red pigmentation, and mycelial growth rate of 3.70 ± 0.03 cm. Macroconidia is relatively straight and slightly curved, apical cell morphology is tapered and hook, basal cell morphology is foot-shaped, mean length (46.78 ± 2.07) and mean width (4.29 ± 0.38), 5–7 septate (7-septate most dominant). *F. oxysporum* has purplish white colonies, circular in shape, flat elevation, entire margin, purple pigmentation, and mycelial growth rate of 5.80 ± 0.51 cm. Macroconidia are straight, slightly curved, and thick, apical cell morphology is tapered and curved, basal cell morphology is foot-shaped, mean length (44.56 ± 7.01) and mean width (4.46 ± 0.32), 3–4 septate (3 -septate most dominant) (Figure 2).

Molecular identification of fungal morphotypes

The use of the DNA isolation protocol from Ausebel et al. (2003) with several modifications succeeded in isolating DNA from all cultures of fungal morphotypes. Table 3 presents the results of measuring DNA quantity using nanodrop. Isolated DNA from morphotype II, IV, VIII was successfully measured with a concentration of 105.028, 64.580, 36.236 and the value of A260/A280 is 1.866, 1.829, 1.912, respectively.

The isolated DNA of all morphotypes had a good quality as shown in Figure 3. The DNA appeared intact without any smearing on the agarose gel. DNA was able to be amplified by ITS 1 and ITS 4 primers, which were characterized by the appearance of DNA bands according to the target size of ± 600 bp. The measuring results of the DNA quantity are in line with the DNA quality. DNA of morphotype VIII had the lowest concentration (36.236 ng/µl) and thinnest DNA band than other morphotypes.

Table 2. Morphological characterization of *Fusarium* species

Morphological characterization	Morphotype		
	II	IV	VIII
Macroscopic characteristics			
Colonies on PDA media[a]			
Color	Yellowish white	Dark red	Purplish white
Form	Irregular	Circular	Circular
Elevation	Convex (raised)	Flat	Flat
Margin	Undulate	Entire	Entire
Pigmentation[b]	Yellow	Dark red	Violet
Growth rate (cm)[c]	5.83 ± 0.52	3.70 ± 0.03	5.80 ± 0.51
Microscopic characteristics			
Macroconidia morphology	Slightly curved and slender	Relatively straight and slightly curved	Straight, Slightly curved, and thick
Apical cell morphology	Tapered	Tapered and hook	Tapered and curved
Basal cell morphology	Pointed	Foot-shaped	Foot-shaped
Mean length of macroconidia[d]	35.04 ± 2.60	46.78 ± 2.07	44.56 ± 7.01
Mean width of macroconidia[d]	4.03 ± 0.41	4.29 ± 0.38	4.46 ± 0.32
Septation	5–7 (6-septate most dominant)	5–7 (7-septate most dominant)	3–4 (3-septate most dominant)
Species identification			
Species	Fusarium equiseti	Fusarium lateritium	Fusarium oxysporum
References	Hami et al. (2021)	Vitale et al. (2011)	Ciampi et al. (2009)
	Leslie & Summerell (2006)	Leslie & Summerell (2006)	Leslie & Summerell (2006)

Notes: [a] Colonies were observed at the top of the culture of *Fusarium* species. [b] Pigmentation was observed on the bottom of the culture of *Fusarium* species. [c] The mycelial growth rate of *Fusarium* species was observed at 7 DAI. [d] The mean length and width of macroconidia were observed from 50 randomly selected macroconidia ± standard deviation

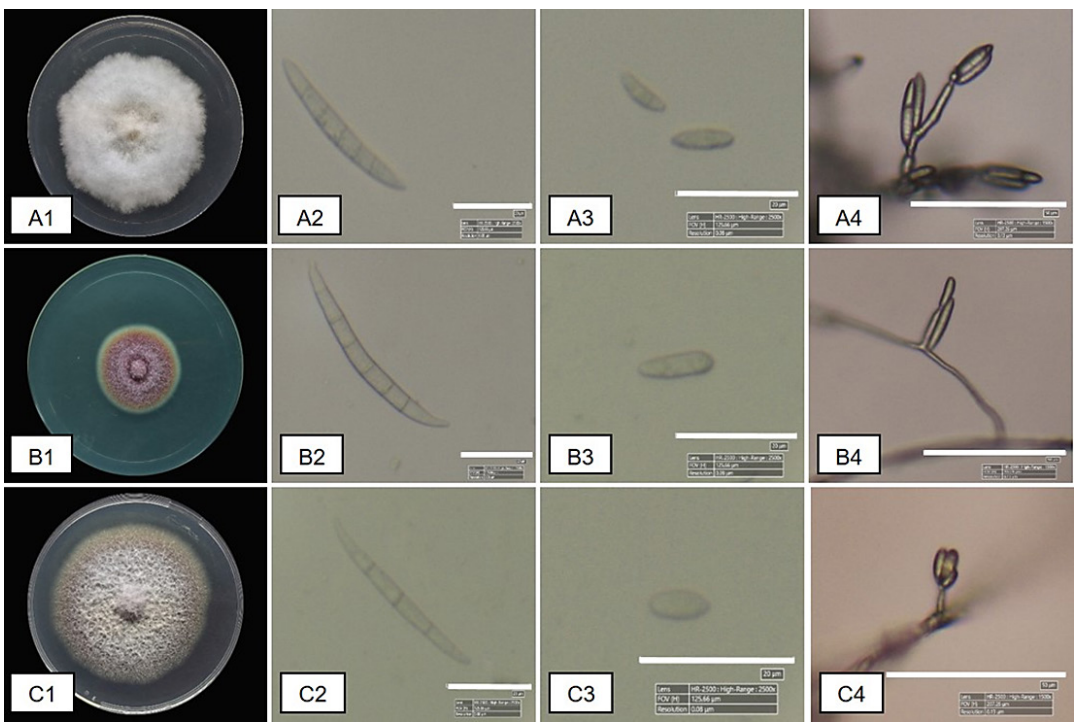

Fig. 2

Table 3. DNA quantification using nanodrop

Morphotype	Concentration (ng/µl)	A260/A280
II	105 028	1 866
IV	64 580	1 829
VIII	36 236	1 912

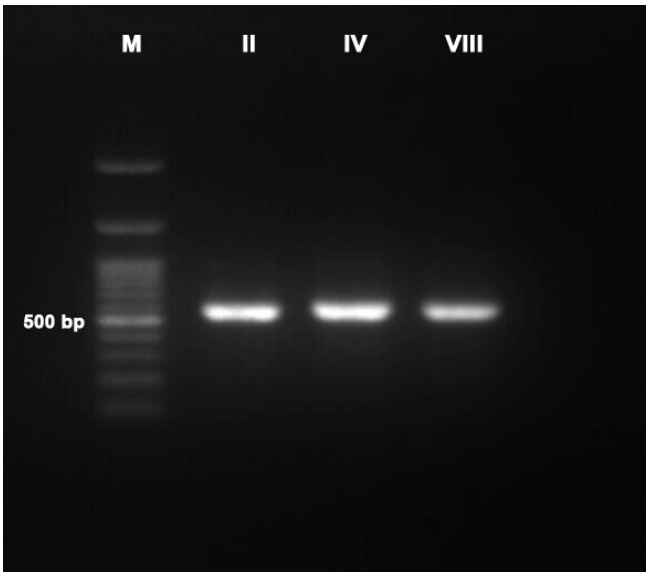

Fig. 3

BLAST results of all morphotypes sequences on the website https://www.ncbi.nlm.nih.gov/ showed that morphotype II had the highest similarity of 98.48% with *Fusarium equiseti* isolate from Pakistan at accession number MH879584.1, morphotype IV had the highest similarity of 98.02% with *Fusarium lateritium* isolate from Pakistan at accession number MH576418.1 and morphotype VIII had the highest similarity of 98.02% with *Fusarium oxysporum* isolate from Pakistan at accession number MH879861.1 (Table 4). These results also confirmed the suitability of the identification result of *Fusarium* species morphologically and molecularly.

The alignment of the ITS locus DNA sequences resulted in the differences in the DNA similarity percentage of *Fusarium* species (Table 5). The DNA sequences of *F. equiseti* had similar DNA sequences of 88.3% with

Table 5. Similarity matrix of ITS DNA nucleotide sequences between *Fusarium* species

No	*Fusarium* species	Similarity (%)[a]		
		F. equiseti	F. lateritium	F. oxysporum
1	*F. equiseti*	ID[b]		
2	*F. lateritium*	88.3	ID	
3	*F. oxysporum*	93.9	88.1	ID

Notes: [a] Similarity matrix of ITS DNA nucleotide sequences between *Fusarium* species was calculated using the Bioedit software version 7.2.5.
[b] ID (identity) indicates the same species

Table 4. Similarity of 3 morphotypes of *Fusarium* species with other isolates in GenBank

Morphotype	GenBank isolate identity	Accession number	Query cover, %	Similarity, %	Origin
II	*Fusarium equiseti* FUS-32	MH879584.1	100	98.48	Pakistan
	Fusarium equiseti PAK54	KY523100.1	100	98.31	Pakistan
	Fusarium equiseti N-32-1	MT560375.1	100	98.15	China
	Fusarium equiseti G388	KR094457.1	100	97.98	America
	Fusarium equiseti C1/32	KM246255.1	99	98.14	Brazil
IV	*Fusarium lateritium* G312	MH576418.1	100	98.02	Pakistan
	Fusarium lateritium BBA 63665	AF310982.1	99	95.72	Germany
	Fusarium lateritium FUS-29	MK091106.1	98	97.99	Pakistan
	Fusarium lateritium KCTC:46029	KC453998.1	98	97.3	South Korea
	Fusarium lateritium G312	AY266406.1	97	97.79	Thailand
VIII	*Fusarium oxysporum* FUS-33	MH879861.1	100	97.64	Pakistan
	Fusarium oxysporum	MH055398.1	100	97.64	United Arab Emirates
	Fusarium oxysporum PE-S1-15g	OL919443.1	100	97.64	Poland
	Fusarium oxysporum N-61-2	MT560381.1	100	97.64	China
	Fusarium oxysporum UFMGCB_529	FJ466709.1	99	98.15	Brazil

F. lateritium and 93.9% with *F. oxysporum*. Meanwhile, *F. lateritium* had a similar DNA sequence of 88.1% with *F. oxysporum*.

Phylogenetic Tree

The phylogenetic trees were created to analyze the genetic relationship of 3 *Fusarium* species found in imported citrus fruits with *Fusarium* species that already exist in

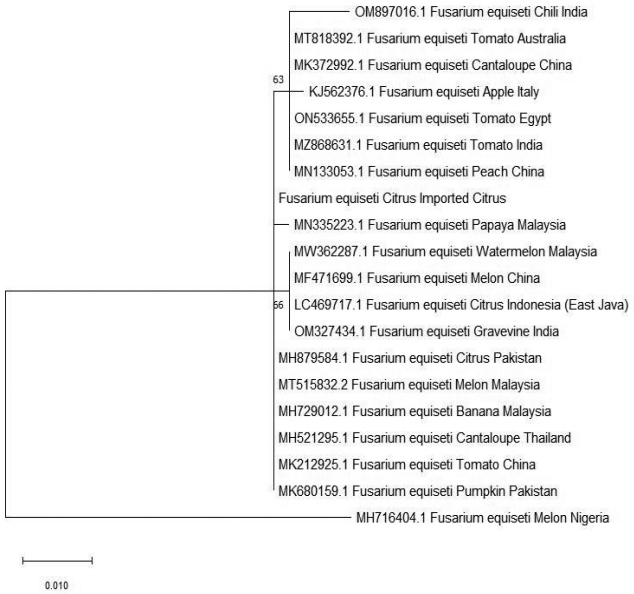

Fig. 4

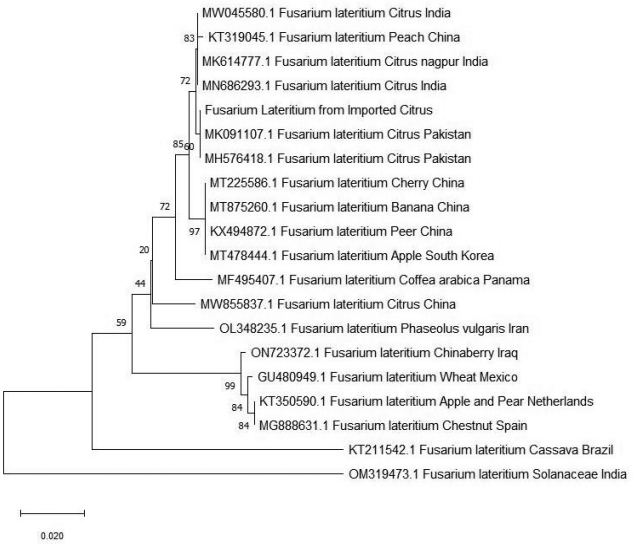

Fig. 5

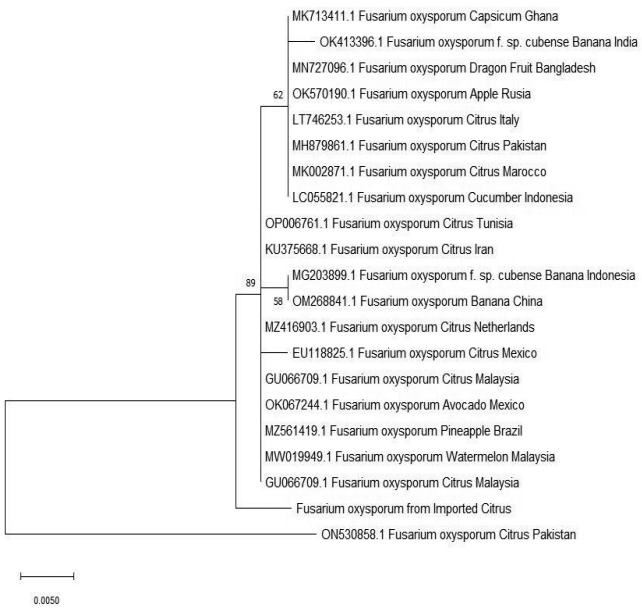

Fig. 6

Indonesia and several other countries. The phylogenetic tree in Figure 4 shows that *F. equiseti* from imported citrus fruits places a different position with *F. equiseti* which has been reported in Indonesia. *F. equiseti* from imported citrus fruits are genetically closer to *F. equiseti* from citrus fruits from Pakistan, melon fruits from Malaysia, banana fruits from Malaysia, cantaloupe fruits from Thailand, and pumpkin fruits from Pakistan. Figure 5 shows the phylogenetic tree of *F. lateritium* from imported citrus fruits and several other countries. Indonesia is not included in the phylogenetic tree because there is no data on the ITS sequence of *F. lateritium* from Indonesia at GenBank, NCBI. *F. lateritium* from imported citrus fruits is genetically closer to *F. lateritium* from citrus fruit from Pakistan. *F. oxysporum* from imported citrus fruits places a separate position with *F. oxysporum* from Indonesia and other countries (Figure 6).

Pathogenicity Test

The results of the pathogenicity test of 3 *Fusarium* species showed that all *Fusarium* species were pathogenic to the citrus fruits. Figure 7 shows that dry rot symptoms appeared in all fruits treated with *F. equiseti, F. lateritium* and *F. oxysporum*. There are differences in dry rot symptoms resulted from the pathogenesis process. Dry rot caused by *F. oxysporum* was dark red, while dry rot caused by *F. equiseti* and *F. lateritium* was dark brown. Meanwhile, controls injected with sterile water were still healthy without dry rot symptoms.

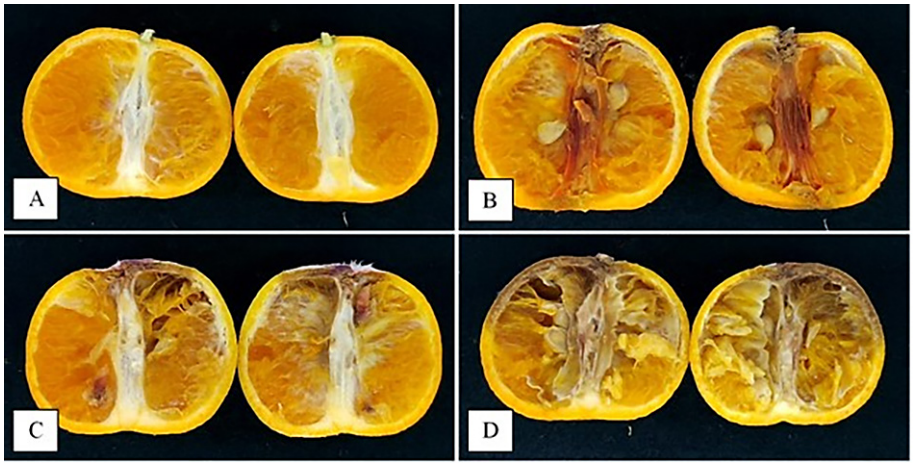

Fig. 7

Table 6. Incidence and severity of dry rot caused by *Fusarium* species

Treatment	Disease Incidence, %	Disease Severity, %
Control	0 ± 0.00 a	0 ± 0.00 a
Fusarium equiseti	100 ± 0.00 b	73.33 ± 6.67 b
Fusarium lateritium	100 ± 0.00 b	93.33 ± 3.33 c
Fusarium oxysporum	100 ± 0.00 b	84.44 ± 5.09 bc

Notes: Values are stated as mean ± SD. Mean values in the same column followed by different letters indicate a significant difference according to Tukey's test at $P < 0.05$

All *Fusarium* species caused a 100% incidence of dry rot (Table 6). This means that all citrus fruits tested were affected by dry rot disease. However, the disease severity of dry rot caused by each *Fusarium* species is different. The disease severity caused by *F. equiseti*, *F. lateritium, F. oxysporum* was 73.33%, 93.33% and 84.44%, respectively. The percentage values of disease severity indicate that all *Fusarium* species are capable of causing dry rot on a heavy scale, which is above 60%. *F. lateritium* resulted in the highest severity of dry rot disease compared to other *Fusarium* by 93.33%. Figure 7 shows that *F. lateritium* caused dry rot not only in the columella, but also in the carpel. *F. lateritium* caused dry rot in almost all parts of the fruit. *F. equiseti* resulted in the lowest severity of dry rot disease compared to other *Fusarium* species by 73.33%. However, the percentage value of severity was still not significantly different from dry rot caused by *F. oxysporum* of 84.44%.

Discussion

Fungi associated with imported citrus fruits were successfully isolated by using the ONFIT (Figure 1). The ONFIT method was chosen because it is able to activate and raise the presence of fungi in citrus fruits that look healthy without showing disease symptoms. The ONFIT method destroyed the structure of citrus fruits through the overnight freezing process. The freezing process causes damage to various defense factors in the fruits that were previously as inhibiting factors for fungal growth. In addition, the damage of citrus fruit structure is thought to cause changes in sugar macromolecules into simple sugar micromolecules as available nutrients that can be absorbed by the fungi. Therefore, this condition is favorable for the fungi to reactivate and grow until it is visible on the surface of citrus fruits. In this study, the ONFIT method was able to produce the fungal mycelia at 4 DAI after freezing at a temperature of -20°C. Meanwhile, the previous ONFIT method was able to produce the fungal mycelia of *Monilinia fructicola* on plums at 5 DAI after freezing at a temperature of -16°C (Michailides et al., 2010). Fungal mycelia did not appear in control without freezing up to 6 DAI. This is because the structure of the fruit is still strong to resist fungal infection.

The emergence of the fungal mycelia on the surface of the imported citrus fruit indicates that the fungi are already on the citrus fruits when being imported from the origin country to Indonesia. According to Michailides & Elmer (2000), pathogens have started the infection process since the flowering stage of the plant or when the fruit is

still young in the field. The absence of disease symptoms, even though the fungi were already present in citrus fruits was thought to be due to the status of these fungi as latent infecting pathogens or as non-pathogenic endophytes in citrus fruits. Sinclair (1991) defines latent infection as a parasitic relationship between the host and the pathogen, where the pathogen is in an inactive position in the plant tissue. Pathogens will become active when the host conditions are suitable for growth and development. In international trade, fruits are usually harvested before ripening and stored at low temperatures during transportation and marketing (Faisal et al., 2011). This condition causes the fungi in a latent position in the fruit.

Mehrota (2001) explained that immature fruit is difficult to be infected by pathogenic fungi because the potential important enzymes possessed by fungi are not sufficient to infect, immature fruit does not provide the nutrients needed by fungi, and some of the toxin metabolites present in fruit are able to resist fungal infection. Prusky & Lichter (2007) further explained that the host and pathogen are two important things that can determine the change from latent infection to active infection, or from biotrophic phase to necrotrophic phase. In the biotrophic phase, the pathogen infects latently because the pathogen does not have the enzymatic power to penetrate the host defenses. In this phase, the host has a strong defense structure. During the senescence process, the structure of host defense decreases. This is used by pathogens to actively infect the host. This condition is called the necrotrophic phase.

The isolated fungal mycelia were grouped into 8 morphotypes based on the different characteristics of the mycelia (Table 1). Differences in morphotypes indicate differences in fungal species. This means that imported citrus fruits are inhabited by several species of fungi. Morphotype VIII was the most commonly found in imported citrus fruits. This is due to the number of propagules or inoculum of morphotype VIII were greater than other morphotypes. Michailides et al. (2010) explained that the success of the pathogen in infecting the host latently depends on the amount of inoculum, host susceptibility, and favorable environmental conditions during the infection process.

The cells in citrus fruits that have been harvested are still actively doing various metabolisms (Brizzolara et al., 2020). The ONFIT method can cause cells or tissue death of citrus fruits, so that the skin color of the citrus fruit becomes dark brown. Died cells do not produce metabolites that inhibit fungal growth. Therefore, the fungi infect easily. Citrus fruits are protected by a fruit skin composed of epidermal and cuticle cells, which make the fruit structure hard (Ginzberg & Stern, 2016). Cell death causes damage to the epidermis and cuticle cells and the texture of citrus fruits become soft. Meanwhile, the texture of the citrus fruit still looks hard and the color of the skin is still orange in the control treatment (Figure 1).

Fusarium species were morphologically grouped into 3 morphotypes out of a total of 8 fungal morphotypes. Table 2 presents the results of the grouping of 3 fungal morphotypes based on morphological characterization. Morphotype II was identified as *Fusarium equiseti*, morphotype IV was identified as *Fusarium lateritium* and morphotype VIII was identified as *Fusarium oxysporum*. Figure 2 shows that each *Fusarium* species can clearly be distinguished morphologically based on macroscopic and microscopic characteristics. The most clearly distinguished morphological characterization macroscopically is the fungal colony. The colonies of *F. equiseti* were yellowish white with irregular margins, *F. lateritium* was dark red with circular margins and *F. oxyxporum* was purplish white with circular margins. The mycelial growth rate of *F. lateritium* was slower than *F. equiseti* and *F. oxysporum*. Hyun et al. (2000) explained that the growth characteristics of mycelia were different between *Fusarium* species.

Meanwhile, the most clearly distinguished morphological characterization microscopically is fungal macroconidia. Macronidia of *F. equiseti* had 5–7 septate (6-septate most dominant), *F. lateritium* had 5–7 septate (7-septa most dominant) and *F. oxysporum* had 3–4 septate (4-septate most dominant). *F. equiseti* and *F. lateritium* had the same number of septa. But, *F. equiseti* had shorter and slenderer macroconidia than *F. lateritium*. Leslie & Summerell (2006) noted that the morphological characteristics of macroconidia were more widely used to identify *Fusarium* species than microconidia. The number of septate on macroconidia is a specific morphological marker in identifying *Fusarium* species. Microconidia are not widely used in the identification process, because some *Fusarium* species do not produce microconidia.

DNA was successfully isolated from each culture of *Fusarium* species by using the protocol from Ausebel et al. (2003) with some modifications. The protocol was modified in order to be able to be used in this study. The original protocol was used to isolate plant DNA. In this study, the protocol was modified to isolate the fungal DNA of *Fusarium* species. Some of the modifications were proven to be able to isolate DNA of all *Fusarium* species with good quality, as shown in Table 3 and Figure 3. Motkova & Vytrasova (2011) explained that the amount of concentration and DNA purity were the main criteria in evaluating the quality of the produced DNA. Modification of Ausebel's protocol succeeded in isolating the DNA of all

Fusarium species with concentrations > 5 ng/µl (Table 3). Sikdar et al. (2014) explained that the minimum limit of fungal DNA concentration used in the PCR process was 5 ng per PCR reaction. Therefore, the total DNA concentration of all *Fusarium* species obtained is sufficient to be used in the Polymerase Chain Reaction (PCR) process. The value of the A260/A280 ratio of morphotypes II, IV, and VIII was 1.866, 1.829, and 1.912, respectively. These values indicate excellent DNA purity. Sambrook et al. (1989) stated that pure DNA has a range of A260/A280 ratio values between 1.8-2. Figure 3 visualizes the DNA of PCR product in a good quality, which is characterized by the intacity of the DNA band according to the target size of 600 bp without smearing on the agarose gel. Lorenz (2012) explained that smears occur due to differences in DNA size in agarose gel.

The results of the BLAST process showed that all morphotypes had similarities above 97% with several *Fusarium* species in GenBank (Table 4). The highest similarity value also confirms the species identity of each morphotype. Morphotype II was identified molecularly as *Fusarium equiseti*, morphotype IV was identified as *Fusarium lateritium* and morphotype VIII was identified as *Fusarium oxysporum*. Xu (2016) revealed that the similarity of fungal DNA above 97% in BLAST can be used to identify fungi to the species level because they lead to the same species.

The alignment of DNA sequences of all morphotypes determined the difference in the percentage of similarity between *F. equiseti*, *F. lateritium,* and *F. oxysporum* (Table 5). The similarity difference, which is quite far below 97% confirms that *F. equiseti*, *F. lateritium* and *F. oxysporum* are really different species. However, *F. equiseti* has a closer genetic relationship with *F. oxysporum* than *F. lateritium*. The significant differences in the percentage of DNA sequence similarity between *Fusarium* species confirmed the characterization of *F. equiseti*, *F. lateritium* and *F. oxysporum* were able to be clearly distinguished and identified morphologically.

The phylogenetic tree in Figures 4, 5, and 6 informs the genetic relationship of *F. equiseti*, *F. lateritium* and *F. oxysporum* from imported citrus fruits with the same species from Indonesia and other countries. *F. equiseti* and *F. oxysporum* from imported citrus fruits place clusters in a different clade with *F. equiseti* and *F. oxysporum* from Indonesia. According to Hossain et al. (2021), fungal species in a different clade from others in the phylogenetic tree show a far genetic relationship between fungal species. These results indicate that *F. equiseti* and *F. oxysporum* from imported citrus fruits are different strains with *F. equiseti* and *F. oxysporum* that already exist in Indonesia. Meanwhile, the DNA sequence of *F. lateritium* from Indonesia was not found in GenBank, so the genetic relationship with *F. lateritium* from imported citrus fruit could not be assessed.

Pathogenicity test results, as shown in Figure 7 revealed the fact that all *Fusarium* species from imported citrus fruits were pathogenic. *F. equiseti*, *F. lateritium* and *F. oxysporum* are fungal pathogens in citrus fruits. The pathogenic ability of all *Fusarium* species was proven by the emergence of dry rot symptoms in all citrus fruits at 21 DAI. However, citrus fruits were still healthy in the control at 21 DAI (Figure 7A). In this study, all *Fusarium* species caused a disease incidence of 100% (Table 6). Agrios (2005) explains that the emergence of disease symptoms is caused by a virulent pathogen, a susceptible host, and a conducive microenvironment. Disease incidence of 100% indicates that all *Fusarium* species are classified as pathogens with high virulence levels.

The disease severity caused by all *Fusarium* species has different percentage values. *F. lateritium* caused the highest disease severity of 93.33% (Table 6). According to Rauwane et al. (2020) virulence level of the pathogen is related to the pathogenicity. Thus, *F. lateritium* had the highest virulence level compared to *F. equiseti* and *F. oxysporum*. Meanwhile, *F. equiseti* has the lowest virulence level than other *Fusarium* species.

Conclusion

Fusarium species associated with imported citrus fruits have been detected, isolated, and identified. *Fusarium* species were successfully detected at 4 DAI by emerging mycelia on the surface of citrus fruits. *Fusarium* species were identified morphologically and molecularly as *Fusarium equiseti*, *Fusarium lateritium,* and *Fusarium oxysporum*. The pathogenicity test proved that all *Fusarium* species were pathogenic to citrus fruits. The disease incidence of all *Fusarium* species was 100%. The disease severity caused by *F. equiseti*, *F. lateritium* and *F. oxysporum* were 73.33, 93.33, and 84.44%, respectively. These results are a new report of 3 *Fusarium* species causing dry rot disease in citrus fruits.

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