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MOLECULAR IDENTIFICATION OF FUNGI THE CAUSAL AGENT OF STRAWBERRY WILT DISEASE IN BALI

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ABSTRACT

The development of strawberry farming in Bali experiencing some obstacles that cause a decline in production, such as wilting disease. The disease was reported caused by the fungi base on morphological recognition. There are two fungi were recognized caused the strawberry wilt disease in Bali, they are from genus *Verticillium* and *Fusarium.* More specific information about causal agent of wilt disease in strawberry especially in Bali is needed. The one accurate identification is done through the molecular approach by analyzing DNA that encode the ribosomal DNA (rDNA). The 18S rDNA, including the internal areas of transcribed spacers (ITS), ITS1 and ITS4 have been widely used in phylogenetic studies. The amplification results of this area produce bands in different sizes that can be used to identify fungal species. Based on that the identification of strawberry wilt disease using molecular analysis was conducted. The 542 bp of Internal Transcribed Spacer (ITS) DNA was successfully amplified using PCR with pairing primers ITS 1 (5-TCCGTAGGTGAACCTGCGG-3'), and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). The sequences of three isolates were successfully obtained through sequencing. Homology levels were tested between sequences and showed that Candi Kuning sequence and Gobleg sequence had 95% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. While Pancasari sequence have 94% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. Candi Kuning, Gobleg, and Pancasari sequences had the same 86% with sequence of *Fusarium oxysporum* isolate C34-294 Brazil (KJ439088) and had 89% similarity with sequence *Fusarium oxysporum* f.sp. *fragariae* China (KT833080). Homology levels were tested between sequences and showed that Candi Kuning sequence and Gobleg sequence had 95% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. While Pancasari sequence have 94% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. Candi Kuning, Gobleg, and Pancasari sequences had the same 86% with sequence of *Fusarium oxysporum* isolate C34-294 Brazil (KJ439088) and had 89% similarity with sequence *Fusarium oxysporum* f.sp. *fragariae* China (KT833080). Based on phylogeny analysis of Pancasari, Gobleg and Candi Kuning isolates were obtained in one group with *Fusarium oxysporum* identified in America and Brazil, and also in one group with *Fusarium oxysporum* f. sp. *fragariae* that identified in China.

Keywords: strawberry, wilt disease, pathogenicity, molecular

INTRODUCTION

Strawberries entered Indonesia in the 1980s. Strawberries in Bali were first introduced in 1983 precisely in Candi Kuning Village, Tabanan Regency, Bali and were developed to Bukit Catu Village,

Pemuteran Village and Batu Sesa Village Tabanan Regency, Bali. Strawberry is one of potential fruit because it contains many phytochemicals mainly phenolic compounds that are beneficial to human health (Hannum, 2004). Strawberries in Indonesia within a year can produce up to five times and peak production occurs from July to August depending on environmental conditions (Sukumalanandana and Verheij, 1997). However recently the production of strawberry fruit in Bali has been decreased, and one of the contributing factors is strawberry wilt diseases. The strawberry wilt diseases was reported caused by the fungi from the genus *Verticillium* (Sari *et al*., 2018). In contras some researcher reported that the strawberry wilt disease caused by *Fusarium oxysporum* in many countries such as Carolina (Williamson *et al.*, 2012), Australia (Golzar *et al.*, 2007), Serbia (Stankovic *et al.*, 2014), and Spain (Arroyo *et al.*, 2009). Based on the data there are two fungi were reported cased the strawberry wilt disease in Bali, they are from genus *Verticillium* and *Fusarium.* More specific information about causal agent of wilt disease in strawberry especially in Bali is needed. The one accurate identification method is done through the molecular approach by analyzing DNA that encode the ribosomal DNA (rDNA). Therefore on this study the identification of strawberry wilt disease using molecular analysis was conducted.

MATERIALS AND METHODS

Sample collection and fungi isolation

This research was started from April 2018 to June 2018. The wilting strawberry plants samples were collected at the center of strawberry plantations in Bali (Pancasari Village, Sukasada District, Buleleng Regency; Gobleg Village, Banjar District, Buleleng Regency and Candikuning Village, Baturiti District, Tabanan Regency) and Plant Disease Laboratory, Faculty of Agriculture, Udayana University. The isolations have done by cutting the infected parts (leaf, trunk or root) with the size about (1x1) cm^2, then dipped it in alcohol for 2 minutes to remove contamination outside. Then, the pieces of plant were washed off by dipping it into the sterile water for 3 times, and then put it on Potato Dextrose Agar (PDA) medium which already contains chloramphenicol antibiotics (100mg/L) and grow on PDA medium which contained chloramphenicol antibiotics (100mg/L) (Ando *et al.*, 2003).

Molecular identification of fungi on strawberry that infected wilt disease

Pure cultures were rejuvenated to the PDA medium. A disc (4 mm diameters) of mycelium was put at the middle of petri dish

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that already contains PDA medium. After that, the cultures wereincubated for 24 hours and then used for DNA extraction.

DNA extraction and PCR

DNA extraction was done following a protocol based on ZR Fungal DNA Kit™ Catalog No. D6005 by ZYMO. The Genomic DNA extraction was conducted using ZR Fungal Bacteria DNA Kit (Zymo Research). The amplification odf DNA was conducted by PCR using MyTaq HS Red Mix that started by preparing the PCR master mix (9.5 µL ddH20, 12.5 µL MyTaq Red Mix [2x], 20 µmol primer ITS 1 [5-TCCGTAGGTGAACCTGCGG-3'], 20 µmol primer ITS 4 [5'-TCCTCCGCTTATTGATATGC-3'] (Singha *et al*., 2016), and I µL DNA template. The following cycling condition was used for PCR reaction: Initial denaturation at 94ºC for 60 s, then 35 cycles of 95ºC for 15 s, 52ºC for 15 s, 72ºC for 45 s, and 75ºC for 5 minutes. The PCR product was purified with ZymocleanÔ Gel DNA Recovery Kit (Zymo Research). Finally, bi-directional sequencing was conducted at Laboratory of Genetika Science in Kembangan Jakarta Barat.

Electrophoresis and sequencing

As much as 1 µL of PCR products were assessed by electrophoresis with 1% TBE agarose gel. Electrophoresis was done for 30 minutes at 100 Volt. The electrophoresed DNA then was visualized with a UV transiluminator.

Sequence Analysis

DNA sequences were analyzed to make the alignment which then used to determine the level of homology or alignment with the sequence of ITS gene that has been published in GenBank within Program Basic Local Alignment Tool (BLAST) (NCBI 2014).

Phylogenetic Analysis

The analysis was continued with the phylogenetic analysis by using software: ChromasPro, Molecular Evolutionary Genetics Analysis (MEGA 6.06), PAUP 4.0, and TreeGraph2.0.

RESULT AND DISCUSSION

Molecular Identification of Fungi on Strawberry that Infected Wilt Disease

Fungal cultures from Pancasari Village, Gobleg Village, and Candikuning Village, were successfully obtained, reproduced and identified morphologically based on macroscopic character (Fig. 1). Therefore the culture of the fungi can be used in the molecular identification phase.

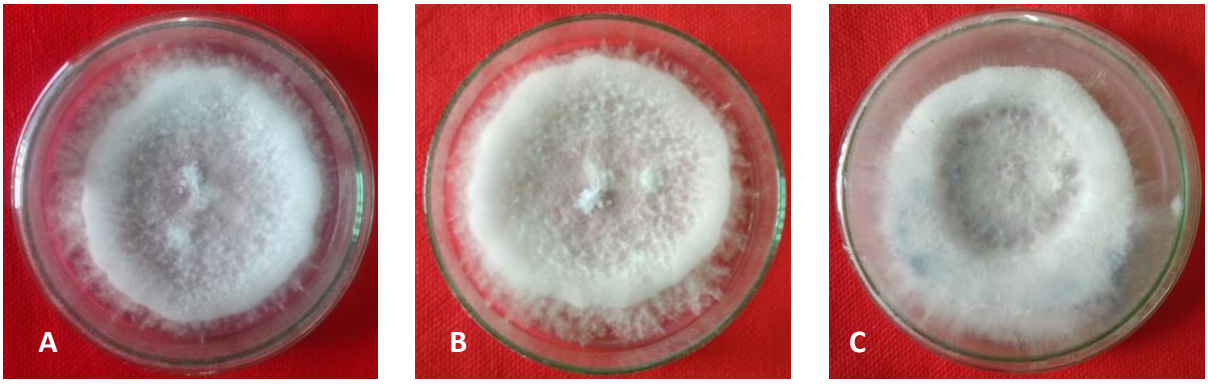

Fig. 1. Pure culture 7 days-old on PDA medium of A) Pancasari isolate, B) Gobleg isolate, and C) Candi Kuning isolate that isolated from strawberry plant on pathogenicity test

According to Sari *et al*., (2018), the decrease in strawberry production due to infection by fungi was very high. The decrease in strawberry production that occurred in Pancasari Village reached 95% and in Candi Kuning Village it reached 85%. The decrease in plant production due to the attack of *Fusarium* fungus is very dependent on the attack intensity, so the decline in production in each region will be different. This is because, *Fusarium* fungus is strongly influenced by environmental conditions. When the environmental conditions are suitable, the fungus will be able to reduce very high plant production. Environmental conditions that are not suitable for the development of Fusarium fungi, cause a decrease in plant production also decreases (Nagy *et al*., 2006). It was supported by Melysa *et al*. (2013), that plant infections carried out by *Fusarium* sp. affected by humid weather conditions and plant age. Younger plant ages tend to be more susceptible to disease than adults plant. Disease caused by *Fusarium* sp. easier to attack plants in the high rainy season.

In addition to environmental factors and plant age, the factor of varieties resistance may also affect the decrease in strawberry plant production. Considering, the development of strawberries in Bali is still depend on strawberry seedlings that taken from the former brood-stock. Brood-stock varieties of developed in strawberry production centers in Bali, especially in Pancasari Village, Gobleg Village and Candi Kuning Village are mixed varieties. Each variety for strawberry seedlings that taken from the previous brood-stock is certainly unknown. Therefore, resistant varieties and non-resistant varieties were mixed at a strawberry plantation that were mainly developed. The number of resistant and non-resistant varieties in each region certainly

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will not be the same, so the decreasing in strawberry production in each region will possibly different. It was compatible with the statement by Melysa *et al*. (2013), that strawberry plants of California varieties were more sensitive to disease. The results of the analysis of the disease intensity of the in vivo test on California varieties and Santung varieties can be seen that California varieties were more sensitive than Santung varieties. The intensity of pathogen attacks on California varieties were higher than Santung varieties.

Another research such as in Australia (Golzar *et al*., 2007; Fang *et al*., 2012), United States (Koike *et al*., 2009, 2013), Spain (Arroyo *et al*., 2009), Korea (Nagarajan *et al*., 2004) and China (Zhao *et al*., 2009) stated that *F. oxysporum* f. sp. *fragariae* has recently been considered as one of the most serious fungal threats to strawberry cultivation and it can also threaten strawberry fruit yield and transplant production in Bali.

Identification of pathogenic fungi that cause strawberry wilt disease was carried out molecularly by amplifying the Internal Transcribed Spacer (ITS) area by using two primers, namely ITS 1 and ITS 4 rDNA (ribosomal DNA). One of the molecular analysis that have been developed is using ribosomal DNA sequences in the Internal Transcribed Spacer area (Druzhirina and Kubicek, 2005). DNA amplification in this molecular analysis was carried out to multiply gene fragments located in the Internal Transcribed Spacer area. The DNS fragment size of the amplification target with the primer used was in accordance with the findings stated by White *et al*. (1990)The PCR product obtained and electrophoresed, showed the DNA band that appeared. The amplified DNA band showed the base length are 542 bp (Gobleg), 542 bp (Candi Kuning), and 544 bp (Pancasari) (Fig. 2).

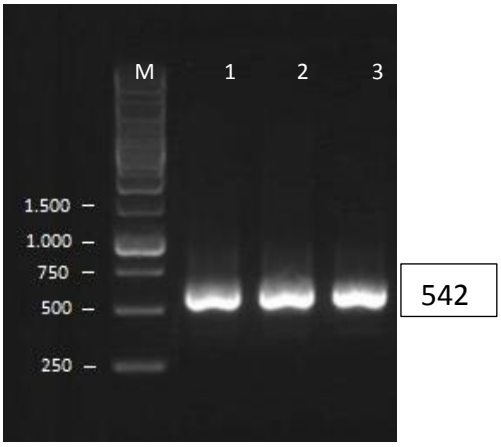

Fig. 2. 1 µL PCR Products were assessed by electrophoresis with 1% TBE agarose showed bands DNA 1st). Lane 1 is 542 bp band (Gobleg), 2nd). Lane 2 is 542 bp band (Candi Kuning), 3rd). Lane 3 is 544 bp band (Pancasari), and M= DNA marker 1kb

The appearance of DNA bands is an important information that shows that PCR has been successfully carried out on the DNA sample that was believed as *Fusarium oxysporum*. However, these results have not been able to ascertain the truth about whether the amplified is truly DNA from *Fusarium oxysporum* because the primer used is a universal primer. Although, in accordance with the statement from Singha *et al*. (2016), the *Fusarium oxysporum* species that amplified using ITS1 and ITS4 primers produced 380-620 bp sized bands. Therefore, sequencing was carried out to ensure the correctness of the information needed in this study.

Table 1. Homology of the fungus that causes strawberry wilt disease in Bali with its homologous sequence in GeneBank

No.	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Candi Kuning	ID												
2	Gobleg	100%	ID											
3	Pancasari	100%	100%	ID										
4	U34571	95%	95%	94%	ID									
5	KJ439088	86%	86%	86%	91%	ID								
6	KT833080	89%	89%	89%	94%	96%	ID							
7	LN809075	76%	76%	76%	79%	78%	80%	ID						
8	KR071709	36%	36%	36%	37%	40%	39%	37%	ID					
9	KR071713	36%	36%	36%	37%	40%	39%	38%	94%	ID				
10	KC874687	37%	37%	37%	38%	37%	37%	36%	85%	84%	ID			
11	KF641021	36%	36%	36%	37%	35%	35%	34%	80%	81%	86%	ID		
12	KM487196	34%	34%	34%	36%	39%	38%	36%	90%	91%	82%	77%	ID	
13	DQ266147	28%	28%	28%	29%	32%	31%	30%	38%	38%	35%	32%	37%	ID

Note: Candi Kuning: Candi Kuning Isolate; Gobleg: Gobleg Isolate; Pancasari: Pancasari Isolate; U34571: U34571 1 *Fusarium oxysporum* NRRL 13307 America; KJ439088: KJ439088 1 *Fusarium oxysporum* isolate C34-294 Brazil; KT833080: KT833080 1 *Fusarium oxysporum* f.sp. *fragariae* China; LN809075: LN809075 1 *Fusarium solani* Isolate 1111TES31E1; KR071709: KR071709 1 *Fusarium verticillioides* Strain CBS 108922; KR071713: KR071713 1 *Fusarium napiforme* Strain CBS 674-94; FFK: KC874687 1 *Fusarium fujikuroi* Isolate F148N4; KF641021: KF641021 1 *Fusarium nygamai* Isolate M42; KM487196: KM487196 1 *Fusarium subglutinans* strain MUCL 43485; DQ266147: DQ266147 1 *Verticillium alboatrum* Isolate V48I.

The similarity levels of samples sequences with sequences in GeneBank were showed in Table 1. Homology levels were tested between sequences and showed that Candi Kuning sequence and Gobleg sequence had 95% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. While Pancasari

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sequence have 94% similarity with sequence of *Fusarium oxysporum* NRRL 13307 (U34571) from America. Candi Kuning, Gobleg, and Pancasari sequences had the same 86% with sequence of *Fusarium oxysporum* isolate C34-294 Brazil (KJ439088) and had 89% similarity with sequence *Fusarium oxysporum* f.sp. *fragariae* China (KT833080).

Table 2. The genetic distance of the fungus that causes strawberry wilt disease in Bali with its homologous sequence in GeneBank

No.	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Candi Kuning	ID												
2	Gobleg	0.000	ID											
3	Pancasari	0.000	0.000	ID										
4	U34571	0.005	0.005	0.005	ID									
5	KJ439088	0.002	0.002	0.002	0.002	ID								
6	KT833080	0.002	0.002	0.002	0.002	0.000	ID							
7	LN809075	0.142	0.142	0.142	0.142	0.140	0.140	ID						
8	KR071709	0.578	0.578	0.578	0.573	0.576	0.576	0.607	ID					
9	KR071713	0.578	0.578	0.578	0.573	0.576	0.576	0.602	0.055	ID				
10	KC874687	0.573	0.573	0.573	0.573	0.571	0.571	0.604	0.057	0.071	ID			
11	KF641021	0.581	0.581	0.581	0.581	0.578	0.578	0.618	0.062	0.050	0.066	ID		
12	KM487196	0.576	0.576	0.576	0.571	0.573	0.573	0.607	0.059	0.052	0.076	0.066	ID	
13	DQ266147	0.637	0.637	0.637	0.642	0.640	0.640	0.645	0.590	0.583	0.581	0.592	0.597	ID

Note: Candi Kuning: Candi Kuning Isolate; Gobleg: Gobleg Isolate; Pancasari: Pancasari Isolate; U34571: U34571 1 *Fusarium oxysporum* NRRL 13307 America; KJ439088: KJ439088 1 *Fusarium oxysporum* isolate C34-294 Brazil; KT833080: KT833080 1 *Fusarium oxysporum* f.sp. *fragariae* China; LN809075: LN809075 1 *Fusarium solani* Isolate 1111TES31E1; KR071709: KR071709 1 *Fusarium verticillioides* Strain CBS 108922; KR071713: KR071713 1 *Fusarium napiforme* Strain CBS 674-94; FFK: KC874687 1 *Fusarium fujikuroi* Isolate F148N4; KF641021: KF641021 1 *Fusarium nygamai* Isolate M42; KM487196: KM487196 1 *Fusarium subglutinans* strain MUCL 43485; DQ266147: DQ266147 1 *Verticillium alboatrum* Isolate V48I.

Based on the data in Table 2, it showed that Candi Kuning sequence, Gobleg sequence and Pancasari sequence have the lowest genetic distance value with sequence of *Fusarium oxysporum* isolate C34-294 Brazil and sequence of *Fusarium oxysporum* f.sp. *fragariae* China are 0,002.

The genetic distance value of Candi Kuning sequence, Gobleg sequence and Pancasari sequence with sequence of *Fusarium oxysporum* NRRL 13307 America are 0,005. Genetic distance with a value of 0,002-0,005 are low. Low genetic distance was observed between the Candi Kuning

sequence, the Gobleg sequence and the Pancasari sequence with the sequence of *Fusarium oxysporum* isolate C34-294 Brazil, the *Fusarium oxysporum* f.sp. *fragariae* China, and sequence of *Fusarium oxysporum* NRRL 13307 America. This shows that the similarity level of the fifth sequences were very high. The genetic distance value of Candi Kuning sequence, Gobleg sequence and Pancasari sequence with sequence of *Verticillium alboatrum* isolate V48I as outgroup is 0,637. High genetic distance values indicate that the similarity between the two genes is very low. *Verticillium alboatrum* is used as an outgroup because it is a pathogenic fungus in strawberry plants and has characteristics similar to *Fusarium* when identified morphologically.

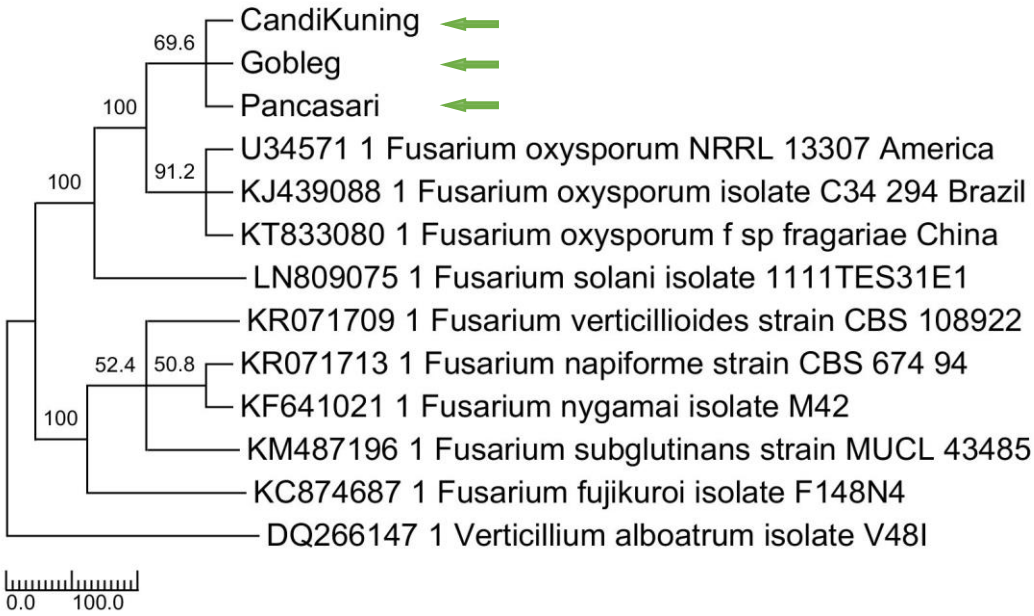

Fig. 3. Phylogenetic tree arranged based on DNA composition of Candi Kuning, Gobleg and Pancasari isolates with the Maximum Parsimony method. The number in the branch is the percentage of the level of trust in the group

Phylogeny tree which show the proximity of the Pancasari, Gobleg and Candi Kuning fungi isolates to several other species based on bootstrap 1,000 times replication are presented in Fig. 3. Based on the phylogeny analysis of Pancasari, Gobleg and Candi Kuning isolates were obtained in one group with *Fusarium oxysporum* that identified in America and Brazil, and also in one group with *Fusarium oxysporum* f.sp. *fragariae* that identified in China. This is consistent with the statement of Dharmayanti

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(2011) that in a phylogenetic topology branches becomes significant if the value of the data set is > 70%.

CONCLUSION

Based on molecular analysis of DNA sequences from Pancasari isolate, Gobleg isolate and Candi Kuning isolate are fungi from the genus of *Fusarium* and the species are *Fusarium oxysporum* that identified in America and Brazil, and also the species are *Fusarium oxysporum* f.sp. *fragariae* that identified in China.

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