

Molecular Identification of Mushroom Causing Wilt Disease in Clove Plants (*Syzygium aromaticum* L.)

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ABSTRACT

Wilt disease in clove plants (*Syzygium aromaticum* L.) becomes one of the main factors inhibiting the production of cloves in Buleleng Regency Bali. This study aims to identify the pathogen causing wilt disease in clove plants in Buleleng Regency Bali. The identification of the pathogen causing wilt disease in clove plants was done macroscopically, microscopically, and molecularly. The macroscopic characteristic were that the color of the mushroom was shiny white when seen from the surface and the base of a Petri dish, colonies of fungi grew in a Petri dish walls, fungi formed aerial hyphae and fruit bodies. The microscopic characteristic was that the hyphae formed clamp connection. The molecular identification of the pathogen causing wilt disease was performed using PCR (Polymerase Chain Reaction) with primers internal transcribed spacer (ITS) to produce DNA fragment size of about 580 bp. The phylogenetic tree wilt disease-causing pathogen isolates was based on the method Neighbor Joining Tree with bootstrap values of 1000 was in a clade with *Schizophyllum commune*. Conclusion of the study results showed that the pathogen isolated at the clove root having a wilt disease symptom in Bali was *Schizophyllum commune* Fr. (*Schizophyllum* family, *Basidiomycota*).

Key words: molecular identification, wilt disease, internal transcribed spacer (ITS)

INTRODUCTION

Plant cloves (*Syzygium aromaticum* L.) from Family Myrtaceae is one of the plantation plants producing spice that has been used for centuries by the people of India as a traditional medicine¹, antibacterial², antifungals³ and largely as a raw material of cigarette industry⁴. Cloves have a fairly high economic value in plantation commodities in the province of Bali, because the price of dry cloves (moisture content 10-14%) in

the market is quite high at around Rp. 100,000 to Rp. 150,000/kg⁵. The high price of cloves causes clove farmers to be more enthusiastic in maintaining clove plantations with the expectation that production can be increased. Production of cloves in Bali experiences fluctuations and tends to decline, due to the attack of pests and plant diseases, such as wilt disease in clove plants.

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Clove farmers in Unggahan village, Seririt Sub-district and Busungbiu Village, Busungbiu Sub-district, Buleleng Regency Bali feel uneasy because thousands of clove plants that are still productive undergo sudden death due to pathogenic fungal attack causing wilt disease.

Clove plants that died were mostly plants that would bloom during the harvest season. The plants that were still small were also found to have been attacked by pathogenic fungi causing wilt disease, and even some had died with sudden wilting symptoms on the leaves then fell off, in the roots were white mycelium fungi and the stem dried, the plants eventually died. The *Kelias Subak* and Agricultural Extension Workers (PPL) of Seririt Sub-district stated that the pathogenic fungi causing wilt disease found in the clove plant roots began in April 2011. Based on the report of Bali Province Plantation Office¹⁹, the area of clove plants attacked by wilt disease in Buleleng regency Bali province until the month of July 2013 was 1413.03 ha of the total area of 7209 ha of clove plants with an attack percentage of 50%.

Research into white root fungal disease (JAP) on rubber, oil palm and cashew nuts that resemble the white root fungal disease has been widely reported^{6,7}, but the disease resembling white root fungal disease in clove plants in Bali, its pathogen has not yet been disclosed.

MATERIAL AND METHODS

Sampling of Sick Plants

Clove plants with fungal attacks causing wilt disease show early symptoms on the leaves that look pale, less shiny, edges or ends of the leaf fold towards the middle part and the leaves turn yellow, wilt and they finally die, white fungus is seen at the base of the stem and roots. The roots infected by white fungus were taken for the research sample. Samples were taken from the clove plant showing symptoms of wilt disease in the Unggahan Village and Busungbiu Village, Seririt Sub-district, Buleleng Regency Bali.

Pathogen isolation of sick plant roots

Isolation of the pathogen was done by cutting the clove plant root having wilt disease symptoms. Clove plant roots infected by pathogen were cut to the size of ± 0.5 cm and

disinfected by soaking them in 0.5% sodium hypochlorite solution for ± 30 seconds. The root piece was rinsed with flowing sterile water and dried over sterile tissue paper until completely dry. Root pieces were grown on PDA (Potato 200 g, Dextrose 20 g and the Agar of 21 g was added with anti-bacterial (Levofloxacin 250 mg) in 1000 mL of distilled water. All the stages were carried out in the Laminar Air Flow (LAF) to maintain aseptic conditions so as to avoid contamination, incubated for 3 days at room temperature (28°C). The growing colonies of fungi were purified back by growing them on PDA. Purification was done following the methods of Faen and Coffly⁸.

Pathogenicity test

Pathogenicity test was performed on Zanzibar clove seed varieties with the age of 12 months were grown in poly bags in the greenhouse. The fungi resulting from isolated purification were grown on stems of cassava with a diameter of 1 cm and a length of 3 cm as a food base, incubated for 7 days at a temperature of 28°C, then tested on plant seeds of clove some roots of which had been peeled with a knife (cutter), the peeled roots were affixed to cassava rod that has been grown with fungi.

Testing the pathogenicity of each isolate was conducted on 10 seedlings of clove and control treatment in the greenhouse until clove seedlings showed symptoms of wilt disease. Infected clove seedling showed symptoms of fungal wilt disease for 10 weeks after infestation. Clove seedlings showed symptoms of leaf wilting, leaf edges folded towards the middle part then they dried; the stem was grown with fungi and sometimes found the body of fruit, if clove seeds had been removed, mycelium fungi were seen spread on the roots.

Clove seeds showed wilt disease compared with those with control treatment, then matched with the clove plant having wilt disease symptoms in the field (Figure 1). Fungal pathogens that infected the roots of cloves having wilt disease symptoms were isolated back and were grown on PDA medium as isolates stock stored at temperatures of -10°C for future research.

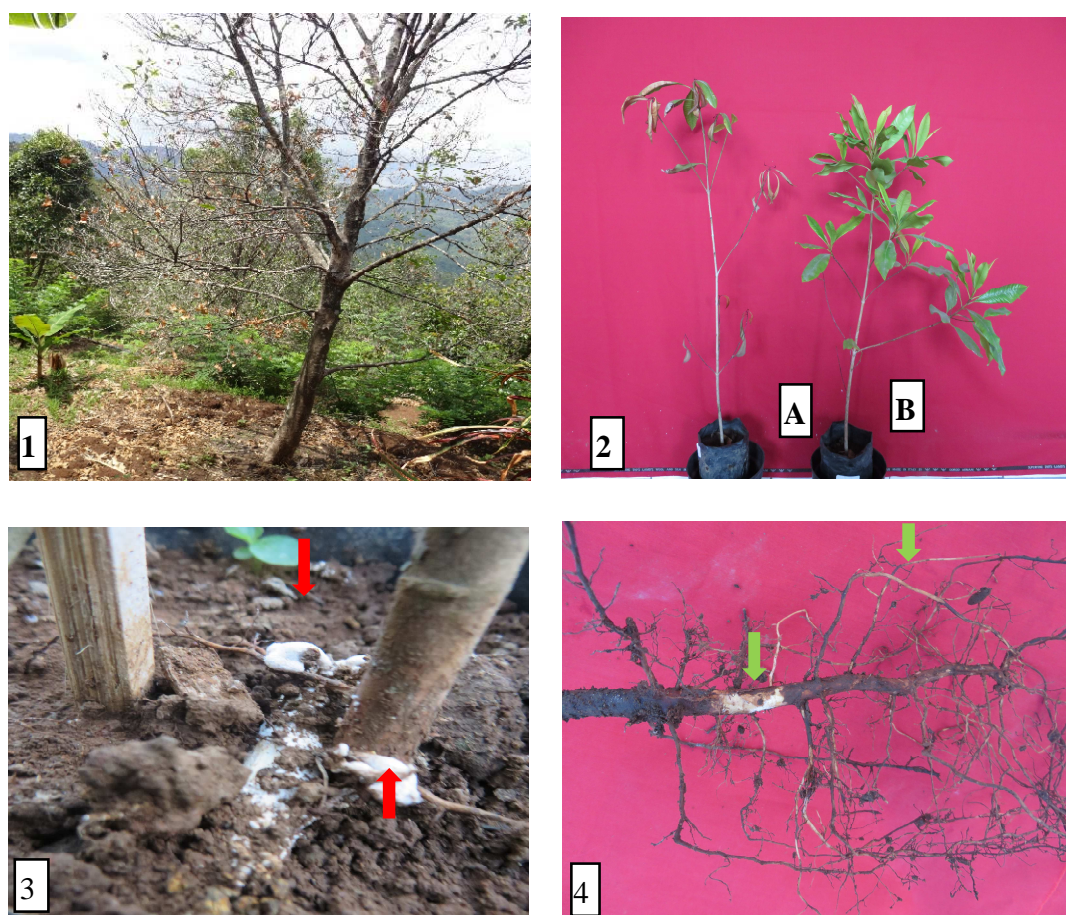


Fig. 1: Clove plant

1. Clove plants attacked by wilt disease in the field
2. *In vivo* testing A. Clove seedling having the symptom of wilt disease.
B. Control seedling
3. Clove fruit on plant roots infected by fungi (arrow).
4. Fungal mycelia spread in clove plant roots (without arrow).

Morphological Identification of Pathogens Causing Wilt Disease

Pure culture of pathogenic fungal isolates causing wilt disease in clove plants was identified macroscopically and microscopically, such as: color, form of colonies and mycelia under the microscope and then matched with the image on fungal identification book of Barnett and Hunter⁹; Fassati¹⁰. Morphological Identification was conducted at the Laboratory of Plant Pathology Faculty of Agriculture, University of Udayana.

DNA amplification and sequencing

PCR amplification used a primer ITS5 F: 5`--GGAAGTAAAAGTCGTAACAAGG--3` and primer ITS4 R: 5`--TCCTCCGCTTATTGATATGC--3`¹¹. DNA

amplification reaction was performed on a volume of 25 ml with a reaction composition, that is, Nuclease free water 10 μ L, Go taq green mastermixTM 12.5 μ L, Primer ITS5 and ITS4 each 0.5 μ L, 0.5 μ L of DMSO and 1 mL DNA template. DNA amplification to the area of ITS consists of: pre-denaturation of 95°C for 90 seconds, followed by 35 cycles of denaturation, annealing 55°C for 30 seconds, extension 72°C in 90 seconds, and a final extension for 5 minutes 72°C. DNA amplification result was analyzed by electrophoresis on agarose gel of 1%. DNA amplification product was subsequently used for sequencing nucleotides. Sequencing outcome data was used to analyze and compare the level of homology similarity to

existing data in GenBank using Bioedit software version 7.0.5.

Molecular Identification of Fungi Causing Wilt Disease

Identification of pure cultures of the fungus causing wilt disease was carried out molecularly based on genetic analysis using internal transcribed spacer (ITS) region, which consisted of ITS4 and ITS5 which aimed to identify the fungi to the species level. DNA extraction used Phythopure™ DNA Extraction Kit (GE Healthcare, UK). Some data sequence resulted from BLAST (Basic Local Alignment Search Tools) which is the closest species and a strains Type of each species were taken from GenBank data at NCBI (National Center for Biotechnology Information). The data were analyzed again by aligning the sequence using the program MEGA v.5.0 and bootstrap used was 1000 replicates¹².

RESULTS AND DISCUSSION

Identification Results of Pathogen Causing Wilt Disease on Clove Plants

1). Identification Morphology

Macroscopic observation of fungal isolates causing wilt disease show that fungal colonies were shiny white when seen from the surface and the base of a Petri dish. Fungal mycelia grow high along the wall of a Petri dish, mushroom formed aerial hyphae (hyphae root) and fruit bodies. Microscopic observations were conducted under the microscope in fungal hyphae forming clamp connection (Figure 2). The result of macroscopic observation was in the form of fruit bodies and the microscopic was in the form of clamp connection of fungi that cause wilt disease in clove plants that this fungus entered into the class Basidiomycetes and to determine up to the species level, it is necessary to make molecular identification.

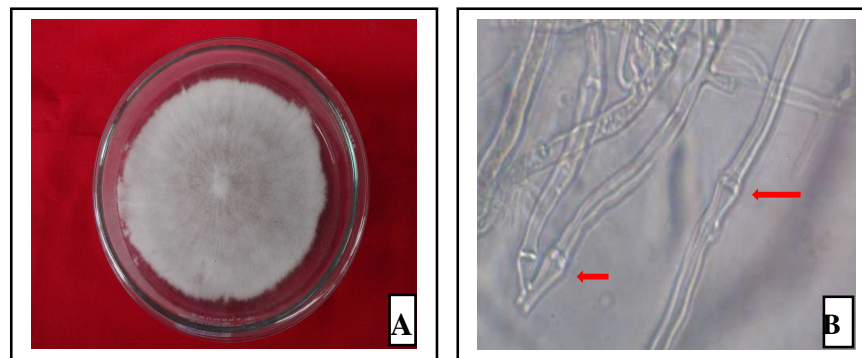


Fig. 2: Morphology of pathogenic fungi causing wilt disease
 A. Fungal colonies isolated from clove plants having the symptom of wilt disease grown in PDA medium for 10 days.
 B. Fungal mycelia and *clamp connection* (arrow) (100 x).

2). Molecular identification

DNA band Sized approximately 580 bp was successfully amplified from samples of fungi isolated from the roots of clove plants symptomatic of wilt disease (Figure 3). The amplification results proved the existence of fungal samples isolated from the roots of clove plants.

Nucleotide sequence reading analysis was carried out using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The raw data of sequencing results were then trimmed and assembled using ChromasPro program version 1.5. The data that had been assembled were then

blasted with genomic data that had been NCBI registered

(<http://www.ncbi.nlm.nih.gov/BLAST/>). Some data sequence was a result of the blast which is the closest species and is Strain Type of each species taken from the Genbank data in the NCBI. Next, phylogenetic tree construction was done using the program MEGA v. 5.0 and bootstrap used was 1000 replicates¹². Nucleotide sequence analysis showed that the isolates of the fungi causing wilt disease in clove plants had 99% homology in comparison with the *Schizophyllum commune* isolates available in GenBank (Table 1).

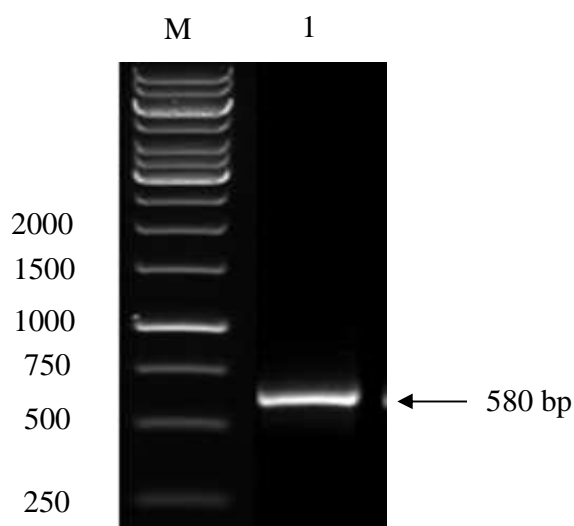


Fig. 3: PCR amplification of the ITS genes with Primer ITS5 F and Primer ITS R;

M = 1 kb DNA ladder marker; 1 = The PCR products of fungal samples.

Table 1 Homological level (%) of nucleotide sequence between fungi causing wilt disease in clove plants in Bali and some isolates of *Schizophyllum commune* available in Gen Bank

| Isolate | Level of Homology (%) |
|--|-----------------------|
| <i>Schizophyllum commune</i> strain xsd 08036 | 99 |
| <i>Schizophyllum commune</i> isolat Z3 | 99 |
| <i>Schizophyllum commune</i> isolat T28 | 99 |
| <i>Schizophyllum commune</i> genes for 18 S rRNA <i>Schizophyllum commune</i> genes for small subunit rRNA | 99 |
| <i>Schizophyllum commune</i> isolat HNO ₃ 4 | 99 |
| <i>Schizophyllum commune</i> isolat HNO ₃ 23 | 99 |
| Agaricaceae sp. 710 18 rRNA genes | 99 |

Nucleotide sequencing analysis results indicated that the isolated fungi causing wilt disease in clove plants in Bali had 99% homology to the *Schizophyllum commune* isolates available in GenBank. Phylogenetic tree of proximity of pathogenic isolates causing wilt disease pathogen was based on the method Neighbor Joining Tree with bootstrap value of 1000 replicates, isolates of pathogens were in a clade (group) with *Schizophyllum commune* Fr. (Schizophyllaceae, Basidiomycota) (Figure 3).

Pathogens of Wilt disease in clove plants in Bali which had been reported to farmers was caused by white root fungi (*Rigidoporus microporus*) synonymous with *Rigidoporus lignosus*. There were several possibilities why the white root fungi (JAP) were always identified with *R. lignosus* in

Indonesia: 1) identification was based solely on morphological data (possibility of misidentification was quite large), 2) contamination at the time of isolation, 3) DNA data that was less good.

Schizophyllum commune fungi were known as the cause of root rot or *Schizophyllum rot*, sap rot and heart rot in some plant species such as meranti (*Shorea smithiana*) in Kalimantan (Indonesia), *Fagus crenata* (Japan beech), *Ulmus* sp. (Elm), *Tilia* sp. (Lime), *Fagus* sp. (Beech), *Picea rubens* (red spruce), *Prunus salicina* (Japanese plum), and ornamental *Prunus* sp.^{13,14}. *S. commune* is also reported as a pathogen that has the ability of biodegradation of wood lignin degradation¹⁵, is sometimes capable of attacking the stems of plants that are still alive, especially in the pith of the wood

whose cells are dead or broken branches^{16,17}. Furthermore Snieskiene *et al.*¹⁸, report that *S. commune* is the most aggressive fungus that

infects the trees of type *Aesculus hippocastaneum* planted on roadsides in the city of Lithuania.

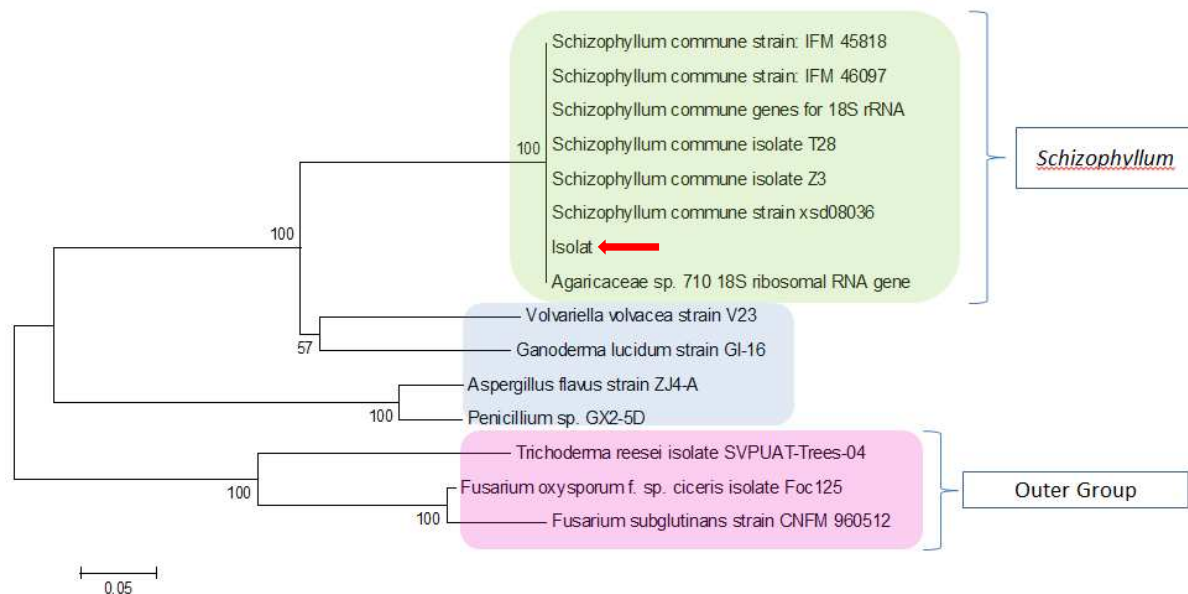


Fig. 3: Phylogenetic tree isolates of pathogens causing wilt disease in clove plants in Bali against other fungi in one clade or against another clade (outer group). Construction based on the method Neighbor Joining Tree with a value of 1000 bootstrap replicates.

CONCLUSION

Schizophyllum commune Fr. (Schizophyllaceae, Basidiomycota) is a pathogen that causes wilt disease in clove plants in Seririt Sub-district Buleleng Regency Bali.

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