Molecular Identification of Mushroom Causing Wilt Disease in Clove Plant: (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 dirty 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 500 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. (Schizophyllaceae, Basidiomycetes).

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

INTRODUCTION

Clove (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 medicine, antibiotic, antifungal and largely as a raw material of cigarette industry. 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/kg. 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.
Clove farmers in Unggahan Village, Sentani Sub-district and Bunungah Village, Bunungah 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. The roots were white mycelium fungi and the stem dried, the plants eventually died. The Klaten Sukoh and Agricultural Extension Workers (OPP) of Sentani 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 Office, the area of clove plants attacked by wilt disease in Buleleng regency Bali province until the month of July 2013 was 1,413.50 ha of the total area of 7,560 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 reported3, 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 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 samples. Samples were taken from the clove plant showing symptoms of wilt disease in the Unggahan Village and Bunungah Village, Sentani Sub-district, Buleleng Regency Bali.

Pathogen isolation of sick plant roots
Isolation of the pathogens was done by cutting the clove plant root having wilt disease symptoms. Clove plant roots infected by pathogens were cut to the size of 0.5 cm and disinfected by soaking them in 8.5% 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 (Penrose 100 g, Dextrose 20 g and the Agar of 21 g was added with anti-bacterial (Levesonacin 250 mg) in 1000 mL of distilled water. All the stages were carried out in the Luminar Air Flow (LAF) to maintain aseptic conditions so as to avoid contamination, incubated for 3 days at room temperature (25°C). The growing colonies of fungi were purified back by growing them on PDA. Purification was done following the methods of Fessia and Coffey.

Pathogenicity test
Pathogenicity test was performed on Zurubar 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 stem 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 25°C, then tested on plant seeds of clove some roots of which had been peeled with a knife (curter), the peeled roots were affixed to cassava rods 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 clove having wilt disease symptoms were isolated back and were grown on PDA medium as isolate stock stored at temperatures of -10°C for future research.
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 Hummer\textsuperscript{5,10}. 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'–GGAAGTAAAAGTGTAACAAAGG-3' and primer ITS4 R: 5'–TCCCTCCGCTATTGATATGC–3'. DNA amplification reaction was performed on a volume of 25 µl with a reaction composition, that is: Nuclease free water 10 µL, Go taq green mastermix\textsuperscript{11} 12.5 µL, Primer ITS5 and ITS4 each 0.5 µL, 0.5 µL of DMSO and 1 µL DNA template. DNA amplification to the area of ITS5 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
RESULTS AND DISCUSSION

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 space (ITS) region, which consisted of ITS1 and ITS5 which aimed to identify the fungus to the species level. DNA extraction used Phytopure™ DNA Extraction Kit (GE Healthcare, UK). Some data sequence resulted from BLAST (Basic Local Alignment Search Tools) which is the closest species and a strain 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 replicates.

2) Molecular identification

DNA band sequenced 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 Analyser) (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 a 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 replicates.

Fig 2: Morphology of pathogenic fungi causing wilt disease
A. Fungal colonies isolated from clove plants having the symptoms of wilt disease grown in PDA medium for 10 days.
B. Fungal mycelia and clamp connection (arrow) (100 x).
Nucleotide sequencing analysis results indicated that the isolated fungi causing wilt disease in clove plants in Bali had 99% homology to the *Schizopyllum commune* isolates available in GenBank. Phylogenetic tree of proximity of pathogenic isolates causing wilt disease pathogens was based on the method Neighbor Joining Tree with bootstrap value of 1000 replicates. Isolates of pathogens were in a clade (group) with *Schizopyllum commune* Fr. (Schizophyllaceae, Basidiomycota) (Figure 3).

Pathogens of WDI disease in clove plants in Bali which had been reported to farmers was caused by white root fungi (*Rigidoporus microporus*) synonymous with *Rigidosporidium*. 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.

*Schizopyllum commune* fungi were known as the cause of root rot or *Schizopyllum rot*, sap rot and heart rot in some plant species such as mesuri (*Dioscorea rotundata*) in Kalimantan (Indonesia), *Fungus crevans* (Japan beech), *Ulmus sp.* (Elm), *Tilia sp.* (Lime), *Fagus sp.* (Beech), *Picea abies* (red spruce), *Praunus lucidus* (Japanese plum), and ornamental *Prunus sp.*. 1 *Commune* is also reported as a pathogen that has the ability of biodegradation of wood lignin degradation, is sometimes capable of attacking the stems of plants that are still alive, especially in the path of the wood.
whose cells are dead or broken branches. Furthermore, Smeets et al. report that S. commune is the most aggressive fungus that infects the trees of type Aspergillus hippocastanum planted on road sidelines in the city of Lithuania.

![Phylogenetic tree of pathogens causing 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.](image)

**CONCLUSION**

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

**REFERENCES**


