Phytophthora sp a causal agent of leaf soft rot disease of nutmeg in Indonesia

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Abstract. Leaf soft rot symptom has been found in nutmeg germplasm collection of Indonesian Spices and Medicinal Crop Research Institute (ISMCRI), Sukabumi, West Java. The research was aimed to identify a causal agent of leaf soft rot disease and the effectiveness assay of fungicides on colonies growth. Based on morphological observations, Phytophthora was found consistently from the infected leaf samples and the fungus characteristic was identical to Phytophthora palmivora. Host range test showed the ability of the fungus to infect pepper and rubber plants. The effect of temperature on fungal growth was tested by planting the fungus on Carrot Agar Medium (CAM), and incubated at 25°C, 28°C, 31°C and 34°C. The efficacy of propineb and mancozeb on inhibiting the growth of fungal colonies was tested by food poisoning technique. Koch's postulate test showed that the first soft rot symptom on the inoculated leaves appeared on the second day and the infected leaves began to fall on the fourth day after inoculation. The optimum temperature for the fungus to grow is 28°C. The result of the efficacy test showed that mancozeb was more suppressing the growth of the Phytophthora than propineb.

Keywords: fungi, morphological identification, mancozeb, propineb

1. Introduction

Pests and diseases are important constraint in nutmeg (Myristica fragrans). Fruit soft rot (Colletotrichum), Dry fruit rot (Stigminna and Cercospora), Twilight die back (Botryodiplodia), and White rot root (Rigidoporus) are common diseases that have been reported in Indonesia [1,2,3]. Recently, soft rot symptom has been frequently seen from fallen nutmeg leaves in germplasm collection of ISMCRI in Sukabumi, West Java with a fungus morphologically similar to Phytophthora characteristics.

In Indonesia, leaf soft rot disease in nutmeg is still rarely studied. Previously, the occurrence of Phytophthora infection in cultivated nutmeg trees close to rubber plantation has been reported in 1968 in Central Java[2]. Since 1968 until now there have been no other reports about this disease [16,17], thus the information regarding the biological characteristic of the fungus is still limited. However, the incidence of Phytophthora infections in nutmeg trees has also been reported in India [4,14]. Of which, this disease will have an economic impact for the next three to four years if not managed properly. In India, the loss caused by this disease could reaches up to 35%[22]. Based on our observations in Sukabumi, the incidence of this disease in thousands of nutmeg seedlings could reach up to 10.81% which caused plants to languish, damaged on leaves, and reduced its quality as plant material.

The research aimed to identify a causal agent of leaf soft rot of nutmeg and the effectiveness assay of fungicides on fungal colonies growth.
2. Materials and method
Nutmeg leaves that showed leaf soft rot symptom were collected from nutmeg germplasm collection of ISMCRI, Cicurug, Sukabumi, West Java, from September 2018 to February 2019.

2.1. Observation of disease incidence and fungus confirmation
Observation of disease incidence (DI) was carried out on the nutmeg nursery (Banda variety: Ternate accession). Observation formula was DI = n.(N x 100%) / N, with n: number of infected plants and N: number of observed plants. Isolation of the fungus was performed by cutting the edges of freshly soft rot necrotic tissue of infected leaf, disinfected with alcohol 70% followed by rinse in sterilized distilled water. The infected leaf tissue were planted on water agar medium (WA) and incubated below white tube lamp (± 400 lux) for 24 - 48 hours at room temperature [5]. Fungal colonies bearing the sporangium were transferred onto the carrot agar medium (CAM) for purification, propagation and morphological characterization. The CAM was prepared according to Drenth and Sendall method [5].

2.2. Morphological characterization
The morphological characterization of asexual structure of isolates (Phy-Ta and Phy-Te) was initiated by planting the isolates on CAM for 6 – 8 days at room temperature with a photoperiod of 12 hours. The fungal structures were scrapped from the colony and put on a slide glass with a drop of lactic acid as mounting medium [6]. The presence of sporangium, shape, size, proliferation mode and sporangium branching pattern type were observed and documented under a Meiji compound microscope attached with Image Capture Software. Meanwhile, the presence and type of chlamydospore formation was detected by growing the isolates on WA medium [5]. The observation and measurement of the chlamydomospores were carried out under a compound microscope as described above.

Caducity characterization of the isolates was performed following a procedure described by [7]. The colony plugs (± 6 mm in diameter) of the isolates were put into a soil suspension made from reverse osmotic water for 3 days at room temperature. The colony plugs were taken out and shaken gently in the water on a slide glass for sporangial observation. Observations were conducted by counting the amount of sporangium released.

Mating type of the fungal isolates was tested by pairing each isolate with respective isolates of P. capsici from black pepper which is a culture collection of ISMCRI [8]. P. capsici (K10) isolate of black pepper was used as tester isolate for A1 mating type and S1 isolate for A2 mating type. The isolate that forming oospore as sexual spore during testing were further confirmed by growing it on polycarbonate membrane test [5]. The oospore size and antheridium formation attachment type were determined under the compound microscope.

The optimal growing temperature of fungi was tested by planting the fungi on CAM and incubated at 25°C, 28°C, 31°C, and 34°C. Observations were made on sporangial density, diameter, shape as well as the pattern of colony growth 7 days after incubation.

2.3. Pathogenicity test
Pathogenicity test were performed in the green house by artificial inoculation using zoospore suspension. Zoospore suspension was collected from the isolates grown on CMA for 7 days with a photoperiod of 12 hours, dislodged with deionized distilled water and adjusted at 10⁶ zoospores mL⁻¹. The suspension was then sprayed onto leaves of one year old nutmeg seedlings of Banda Variety. The inoculated seedlings were covered with transparent plastic for 48 hours to maintain air humidity and avoid direct contact with sunlight. The isolate (Phy-Te) was inoculated onto 5 seedlings as replication, on each plant there were wounded and unwounded leaves. The number of typical soft rot symptoms leaves was observed at 7 and 14 days after inoculation in the first to fifth fully expanded leaves.

2.4. Host range test
Host range tests were conducted using three or four detached leaves of cocoa, rubber, pepper, and nutmeg, which were then artificially inoculated using Phytophthora isolates (Phy-Te) obtained from...
nutmeg. Prior to inoculation, the detached leaves were washed with sterilized water and placed abaxial surface up in a moist chamber. Inoculation was conducted using attached culture disc of the respective isolates onto the wounded and unwounded leaves, a drop of aquades water placed upper the culture disc. Inoculated leaves were incubated at 25°C. Five days after inoculation, the necrotic area width was measured using a leaf area meter. The disease severity was estimated with modification scoring category by [9], i.e.: 0 = No lesions, 1 = 0.1–3%, 2 = 3.1–5%, 3 = 5.1–10%, 4 = 10.1–25%, 5 = 25.1–50%, 6 = 50.1–75%, 7 = 75.1–85%, 8 = 85.1–95% and 9 = 95.1–100% leaf area covered with soft rot symptom. The scoring category was used to determine the disease severity index (DSI), with the formula: DSI (%) = [sum (class frequency × score of rating class)] / [(total number of leaves). (maximal disease index)] ^ 1.100 [10].

2.5. In Vitro Fungicide Efficacy Test
Efficacy of propineb (systemic fungicide) and mancozeb (contact fungicide) on the growth of fungal colonies was tested by food poisoning technique [11]. The fungal colonies were inoculated onto PDA medium with fungicide concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, and 60 ppm, and then incubated for 7 days at room temperature. The diameter of colonies growth was measured after the incubation treatment and entered into the percentage inhibition formula: I = (D0-D1)/(D0 X 100%) ^ t, D0: diameter of control colony, D1: diameter of the colony treated with fungicide concentrations. Lethal concentrate50 (LC50) from each tested fungicide was obtained by performing probit-based analysis.

3. Results

3.1. Disease incidence and fungus confirmation
The symptom of severe leaf soft rot disease on seedlings were shown by the presence of dark brown soft rot on leaf midrib, soft rot enlarges to the lamina, and eventually the infected leaves would fall prematurely (Figure 1A). Disease incidence were varied between 8.30% and 13.41% in 2018. The disease incidence increased by 2.36% in November to December 2018 due to high humidity and rain intensity.

The results of planting infected leaves on WA and CAM media, respectively generated fungus with typical sporangium of *Phytophthora*. The characteristics of fungal colonies on CAM medium are white, thin, and forming star-like pattern (Figure 1B). Furthermore, two isolates (Phyt-Ta and Phyt-Te) have been collected and preserved as living culture in the culture collection of ISMCRI.

3.2. Morphological characterization
Morphological characteristics of the isolate showed that mycelia are hyaline and without hyphal swelling (Figure 1C). Chlamydospores are mostly raised from the tip of mycelia as swelling hypha look like hyaline and the size is vary from 11–(15.4)–27 x 6–(11.4)–20 µm (Figure 2D). Sporangia are dominated by obvious papillate, some with two papilate, lemon shape, pedicellate, and abundantly produced on solid medium. Sporangium branching patterns is simple. Sporangial size are 32.5–(41.9)–47.5 µm length, 18.8–(23.3)–30.0 µm width, papillate thickness, and papillate exit pore are 1–(3)–4 µm and 4–(4.9)–7 µm, respectively. Both isolates have similar morphology with external proliferation type and even some sporangium raised more than one new sporangium (Figure 1E and 1F). Caducity test showed that the sporangia was caducous. Sporangia which were released in water were 50% or more than the one that remain attached on the mycelial mats of colony in the medium.
Figure 1. Symptoms of leaf soft rot of nutmeg and morphological characteristics of *Phytophthora* (Phyt-Te). (A) Symptom, (B) Colony on CAM, (C) Mycelial tip, (D) Chlamydosporae, (E) Sporangia, (F) External proliferation, and (G) Oosporae with amphygenous antheridia.

The two isolates (Phyt-Ta and Phyt-Te) of nutmeg are heterothallic. The oosporae were produced after mating type assay of K10 isolate of *P. capsici* after a week in polycarbonate membrane mating type test but the oosporae weren’t produced after mating type assay of S1 isolate. The oosporae color are yellow to light brown, with amphygenous antheridium type and the size are 19–(25.0)–30 x 20–(25.9)–35 µm. On heterothallic species, the oosporae will only be produced if there were two isolates
with different matting types paired. Based on this assay, the two isolates of nutmeg are A2 mating type (Figure 1G).

Based on the presence of sporangium as a vegetative reproducing structures of the fungus and oospore as the sexual reproducing one [12,13,5], those two isolates were identified as *Phytophthora*. The characteristics of the sporangium were papillate type, hemisphere shape of apical thickening, and have external proliferation type as well as heterothallic mating type with amphygenous antheridium. Hence, both isolates are considered belong to group 2 of the *Phytophthora* grouping proposed by [13]; particularly having similar characteristics with *P. palmivora*.

Incubation temperature influenced colonies growth and sporulation of the fungus (Figure 2). Incubation at 25-28°C had the most optimal colonies growth. Meanwhile, sporulation was most abundant at 28°C. At 34°C, the colonies growth and sporulation were greatly inhibited. In addition, incubation temperature also affected the colonies shape and pattern. The colonies were thin and forming star-like patterns at 25°C, whereas at 28°C, the colonies were thin, smooth, and showed irregular pattern, and thicker like cotton and forming flower-like patterns at 31°C.

3.3. Pathogenicity test
Pathogenicity test showed the leaf soft rot symptom appeared on the second day as dot spots, often coalesce to form wider soft rot spot and the infected leaves fall four or five days after inoculation. Based on the observations, young leaves were more susceptible to *Phytophthora* infection. Wound leaves increases the severity of disease occurrence on both young and old leaves. Meanwhile, inoculation on unwounded mature leaves did not cause any symptoms (Figure 3).

![Figure 2](image-url)

**Figure 2.** *Phytophthora* (Phyt-Te) colonies growth and sporangial formation at different incubation temperatures. Same label on each histogram indicated non-significant difference based on Duncan test grade 5%
**Figure 3.** Disease severity in the first to fifth fully expanded leaves inoculated using *Phytophthora* (Phyt-Teisolate).

3.4. **Host Range Test**

Host range test can be used as additional information in identifying a pathogen. Some pathogenic species have specific hosts. Artificial inoculation showed that Phyt-Te isolate was pathogenic to nutmeg, pepper, and rubber, but the disease severity as indicated by size of necrotic spots was varied. The highest severity of the disease was seen in nutmeg and pepper leaves. In rubber the symptom appeared as spot but the severity was very low. In accordance with the results of the pathogenicity test, in various plant species, wounded leaves also increase the severity of the disease (Figure 4).

**Figure 4.** Host range test of *Phytophthora* (Phy-Te isolate)

3.5. **In Vitro Fungicide Test**

The treatment of mancozeb and propineb affected the growth of *Phytophthora* colonies. In comparison to propineb, mancozeb was more effective in suppressing the growth of *Phytophthora*. At 10 ppm concentration, the ability to inhibit the growth of colonies in mancozeb treatment ranged from 78.8% -
83.1%, while in propineb ranged from 60.47% - 61.3%. Probit analysis showed that LC$_{90}$ on Phytophthora (Phyt-Te isolate) was 16.98 ppm for mancozeb and 33.88 ppm for propineb (Figure 5 and 6).

**Figure 5.** Colonies growth of Phytophthora (Phyt-Te isolate) on mancozeb and propineb treatments. Same label on each histogram indicated non-significant difference based on Duncan test grade 5%.
- Control
- Mancozeb
- Propineb

**Figure 6.** LC$_{90}$ of mancozeb and propineb tested on Phytophthora (Phyt-Te isolate).

4. **Discussion**
In Indonesia, the first report on the occurrence of leaf soft rot of nutmeg was around 1968 in a nutmeg plantation close to rubber plantation which experienced an epidemic of fruit rot and leaf fall disease. $P$
*Phytophthora palmivora* fungus was believed as the causal agent of the disease [2]. In a location that is not close to rubber plantation in Sukabumi, leaf soft rot of nutmeg are found. Although further analysis of fungal identity is needed, morphologically the fungus has the same characteristics as *P. palmivora*. The host range test also indicated that the fungus was close to *P. palmivora*, since it able infecting other plant species. *P. palmivora*, is a species with a wide range of hosts [13]. In India, *Phytophthora* sp was also reported causing leaf fall disease of nutmeg [14,15]. *Phytophthora* found in nutmeg in India also pathogenic to rubber, vanilla, rose, coreopsis, eucalyptus, and citrus, but only showed hypersensitive reactions to cocoa and pepper [15].

Another species of *Phytophthora* obtained from leaf fall disease in nutmeg in India was identified as *Phytophthora ramorum* based on cultural, pathogenicity and morphological characters [4]. However, the morphology of *P. palmivora* is distinct from *P. ramorum*. *P. palmivora* has papillate sporangia, while *P. ramorum* has semipapillate sporangia[5,13]. This indicates that the cause of leaf soft rot disease in Indonesia is different from those in India.

How and where this fungus originated is still questionable. This fungus may persist in the garden by infecting young leaves that are available through the years on the collecting site of ISMCRI garden in Sukabumi. At that time, the impact and presence of the fungi were undetected because the young leaves were prevalently available at the upper part of plant canopy, and the fungus lacked the ability to infect mature leaves. However, when the garden mandate has been changed into nutmeg nurseries and planted thousands of nutmeg seedlings, young and unexpanded leaves become available on the canopy of seedling. Therefore, the presence of *Phytophthora* is detected from those infected leaves.

The results of fungicides efficacy test showed that mancozeb was known to be more effective than propineb in inhibiting the growth of *Phytophthora* in vitro. Mancozeb is a contact fungicide that has a mode of action to inhibit germination of spores attached to the plant surface [17]. In addition, Mancozeb is known to be effective in suppressing the development of various types of fungi that cause plant diseases [11]. Previous study reported that in vitro mycelia growth in *Phytophthora vignae* was significantly reduced in the treatment of mancozeb EC50 with a concentration of 12.47 ppm in P001 isolate and 19.32 ppm in P006 isolate[18]. Moreover, mancozeb was reported to be effective in controlling *Phytophthora infestans* the causal agent of potato late blight and *Phytophthora fragariae* that cause red core in strawberry [19,20,21].

*Phytophthora* infection in nutmeg, especially in the seedlings phases needs more attention. Even though, leaf soft rot is not a main disease of nutmeg, its appearance in seedlings phase affects its quality as a planting material. Therefore, further research on leaf soft rot of nutmeg is needed. In addition, a combination of several components, such as technical culture, exclusion, sanitation and integrated application of fungicides is recommended to prevent the emergence of this disease.

5. Conclusion

Leaf soft rot disease found in nutmeg germplasm collection at ISMCRI, Sukabumi is caused by *Phytophthora*. The result showed that the optimal temperature for the growth of *Phytophthora* colonies was 28°C. Young leaves or wounded leaves are more susceptible to this pathogen infection. The *Phytophthora* found in nutmeg is able to infect blackpepper and rubber leaves. The efficacy test demonstrated mancozeb suppresses the growth of pathogenic colonies better than propineb.

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