Etiology of white root disease of cashew (*Anacardium occidentale* L.)

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Abstract. White root disease is a significant disease of cashew in Indonesia. However, the identity of the causal agent of the disease remains unclear. The objective of the research was to determine the pathogen causing cashew white root disease. Procedures undertaken in this research were (1) field observation in cashew plantations in Karangasem, Bali; (2) isolation and characterization of the suspected fungus and (3) Koch’s postulate on cashew saplings in the greenhouse. Field observation showed that white root disease occurs in cashew plantations with trees prevalently at the age of 8-23 years, with the primary symptom wilting and the presence of rhizomorph on the basal stem. Morphologically, *Rigidoporus* sp. fungus was consistently isolated from the root of infected plants. An artificial inoculation on nine-month-old saplings showed that wilting symptoms and the presence of rhizomorph on the basal stem developed at 51-76 days after inoculation. The inoculated saplings finally died. Root colonization by *Rigidoporus* sp. ranged from 35-90%. *Rigidoporus* sp. as the causal agent of white root disease of cashew was confirmed.

Key words: colonization, pathogenicity, rhizomorph, *Rigidoporus* sp., symptom

1. Introduction

Cashew (*Anacardium occidentale* L.) is a major plantation crop in Indonesia, especially in Java, Bali, Nusa Tenggara, and Sulawesi. Because of its great flavor and numerous health benefits, the “real fruit” of this plant, also known as cashew nuts, is in high demand. Cashew nuts are used as a combination in some culinary sectors such as chocolate and milk to improve the taste of their products. People consume cashew nuts in a variety of meals, including cakes and ice cream [1]. Cashew nuts can be processed into a paste which can then be made into processed products such as pastries. Cashew nuts contain high unsaturated fatty acids, fiber, vitamins, and amino acids, beneficial to the body [2]. As a result, cashew nut demand will continue to rise in both home and foreign markets. As one of the cashew nuts-producing countries, Indonesia has many potentials to supply global demand. Until 2018, the total area of cashew in Indonesia was 494,268 hectares [3]. The area of the cashew plantation is still dominated (99.8%) by smallholder plantations [4].

Cashew commodities in Indonesia are steadily increasing in total area. However, this is not followed by a productivity increase [5]. This situation is caused by unoptimized cultivation techniques, including plant management and pests and diseases management. Moreover, smallholder plantations have several flaws, especially poorly managed plantations. As a consequence, pest and disease outbreaks spread rapidly.
White root disease is frequently observed in cashew trees currently. This disease was firstly recorded in Karangasem, Bali. The disease has also been reported in West Nusa Tenggara and Southeast Sulawesi [6]. Infected plants do not grow normally, and deteriorate roots led to dying. Fungi Rigidoporus microporus or Rigidoporus lignosus were assumed as the causal agents of this disease. However, the characteristics and identity of the causal agent are still unconfirmed.

The incidence of white root disease in cashew plantations in Indonesia, especially in Bali, has been increasing in the last few years, which caused many farmers or farmers groups to switch the commodities they cultivated with other crops. Farmer’s knowledge about this disease is limited so that the prevention and control for this disease are still not optimal. Farmers suffered a reduction in the number of productive plants resulting in low yields. The causal fungus persists for years in soils outside the host plant, so a source of inoculum is still available in the field. Moreover, this prominent factor becomes an obstacle to new planting activities, plant rehabilitation, or replanting. Other tropical plantation and forestry crops known to be affected by this disease include rubber (Hevea brasiliensis) [7–9], nutmeg (Myristica fragrans) [10], jackfruit (Artocarpus heterophyllus) [11], cloves (Syzygium aromaticum) [12], Artocarpus nobilis [13], and coffee (Coffea sp.) [14]. Furthermore, research was conducted to determine the pathogen causing white root disease of cashew.

2. Materials and methods
2.1 Field survey of white root disease
White root disease symptoms were observed on the cashew plantation in Sukadana Village, Kubu Subdistrict, Karangasem Regency, Bali, from August 2020-May 2021. Symptoms and signs of the disease appearing on cashew plants were observed and documented.

2.2. Sampling, isolation, and identification of the pathogenic fungus
Root samples were taken from infected cashew plants at smallholder plantation in Sukadana Village, Kubu District, Karangasem Regency (Latitude 8°15'08.8"S Longitude 115°31'41.3"E), Bali Province, on 27 August 2020. The samples were stored in cold conditions during transportation before then isolated in the laboratory. The root surface was cleaned with running water and sterilized by immersing it in 1% sodium hypochlorite solution for 30 seconds. Root samples were rinsed three times with sterile distilled water and dried. About 1 cm long root sections were placed on potato dextrose agar (PDA) medium with antibiotics to prevent bacterial growth. Five pieces of root sample were placed on PDA in a petri dish and incubated at 27°C for two days. Grown mycelia then recultured to the new PDA medium [8].

Characterization for identifying isolated pathogenic fungus was carried out by observing the morphological characters by binocular microscope (OLYMPUS BX51) and electron microscope (ZEISS EVO MA10 scanning electron microscope) and referred to the literature [15–17].

2.3. Molecular identification
Molecular identification of pathogenic fungi isolates was started with DNA isolation to referred methods [18]. PCR amplification of ITS (internal transcribed spacer) region used the pair of primer ITS1 (forward) (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (reverse) (5’-TCCTCCGCTTATTGATATGC-3’) [19] with 600 base pairs (bp) PCR product. PCR was carried out using the following program: initial denaturation for 2 minutes at 94 °C, followed by 35 cycles of 50 seconds denaturation at 94 °C, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, and final extension for 5 min at 72 °C. The amplification product was analyzed at 1% agarose gel electrophoresis and visualized with UV irradiation. DNA fragments were shipped to 1st BASE Malaysia for sequencing. The sequences of nucleotides were processed using the BioEdit application version 7.2.5. Analysis of DNA similarity compared with other nucleotides in the GenBank database was processed using the BLAST-N program on the national center for biotechnology information (NCBI) website. Phylogenetic analysis was carried out using the Neighbor-Joining method with 1000 bootstrap [20].
2.4. Koch's postulate test of Rigidoporus sp. on cashew saplings in greenhouse
Koch's postulate test was conducted to determine the causal agent of disease and pathogenicity of the Rigidoporus sp. on cashew saplings.

2.4.1 Cashew saplings. We used 12 saplings of B 02 cashew variety from the Cikampek Experimental Garden, Indonesian Spice and Medicinal Crops Research Institute, The Indonesian Agency for Agricultural Research and Development (IAARD) located in Cikampek, Karawang Regency, West Java. The 5-month-old saplings were taken to the greenhouse, transferred to larger polybags with a diameter of 25 cm with topsoil and manure (2:1, w/w) as planting media, and maintained until nine months old. Nine-month-old saplings were used for pathogenicity tests.

2.4.2 Inoculum of the pathogen. The pathogenic inoculum with Rigidoporus sp. characteristic was propagated by growing it in glass bottles together with 10 cm length wood sticks made from young cashew tree branches obtained from within IPB University Campus. The wood sticks were previously sterilized with 1% NaOCl solution for 3 minutes, rinsed with sterile water three times, then autoclaved at 121°C for 15 minutes. Ten pieces of sterilized wood sticks were put into a glass bottle containing a sterile PDA medium. After that, three to five mycelial discs of Rigidoporus sp. taken from the edge of seven-day-old cultures with 0.5 cm diameter cork borer were used to inoculate the sterilized wood sticks in each bottle.

2.4.3 Artificial inoculation of Rigidoporus sp. on cashew saplings. Artificial inoculation was carried out by attaching the inoculated wood sticks to the saplings in the greenhouse. Each sapling was inoculated with three to five inoculum sticks placed in a parallel position and in contact with the taproot, approximately to a depth of 10-15 cm. Uninoculated saplings were used as controls. The daily water need of saplings was reduced in intensity to provide an appropriate microclimate for the field conditions. Observation on saplings that have been inoculated with Rigidoporus sp. was conducted every day for three months. The observed variables were the incubation period for the first symptoms (days after inoculation / DAI), the first symptom on the canopy, sapling death (DAI), and the percentage of root surface colonization (%).

3. Results and discussion
3.1. White root disease on cashew plantation
Kubu is one of the eight sub-districts in Karangasem Regency, Bali Province, located in the eastern part of Bali Island (Figure 1). It is the largest sub-district with 27.6% of the Karangasem Regency total area [21]. Due to its agricultural ecosystems and climatic conditions, Kubu is very suitable for cashew plantations. This subdistrict is the center of cashew development in Karangasem Regency, where the majority of the cashew plantation area practicing organic farming and receiving organic certification from an international certification agency since 2008 [22]. The cashew plantation at Kubu Subdistrict is located at the foothills of Mount Agung, which is dominated by sandy soil, with an altitude of 200-250 meters above sea level. The foothills of Mount Agung are very rich with volcanic materials like andesite rock and pyroclastic sand, the typical topography of a volcano [23]. The daily temperature in Kubu sub-district is ranging from 30-33 °C due to its geographical location, which is right aside from the Bali Sea. This condition is ideal for the growth of the cashew plant, which requires up to 50% sunlight intensity.

Although planted in sandy soil at the foot of the mountain, cashews can grow well and productively. Instead of synthetic chemical fertilizers, farmers used manure as an organic material input. Manure is expected to increase the physical characteristics of the soil on the land and fertilize the plants. With a 10 m x 10 m planting distance applied for cashew (100 plants per ha), some farmers still planted various crops as relay cropping. They planted Borassus flabellifer, mango (Mangifera indica), guava (Psidium guajava), teak (Tectona grandis), srikaya (Annona squamosa), banana (Musa sp.), and coconut (Cocos nucifera). In some cases, this condition caused the vegetation on the
plantation to become very dense, thus affecting the microclimate of the plants and increasing the potential for infection by pathogens. Besides, the incidence of white root disease in cashew plantations is also influenced by soil conditions such as soil structure and moisture. Sandy soil has larger pores than soil with a dense texture, making it easier for mycelia or rhizomorphs of fungal pathogens to move and spread quickly.

Figure 1. The location map of field observation at Sukadana Village, Kubu, Karangasem, Bali (-8°15’16.0"S, 115°31’24.0"E). The sample of the infected root was taken from this location.

White root disease on cashew plantations is still present currently and has become a serious problem for farmers because infected productive plants might die in a short period. The plantation area infected with white root disease until May 2021 at the cashew-producing center of Bali is 14.1 ha. The infections were divided into a mild infection (not causing plant death) of 8.9 ha and severe (dead plant) of 5.2 ha. Observations in the field revealed that most of the cashew trees affected with white root disease were prevalently at the age of 8-23 years, found in less-maintained areas, such as the edge of the plantation away from the access road. Because land sanitation is poor, the pathogen inoculum spread quickly. Symptoms that appeared on infected plants were wilting, yellowing, and drying leaves on the plant canopy. When the soil around the roots is dug, signs of disease in the form of white rhizomorphs can be observed. Rhizomorphs can be observed up to the basal stem in a later phase. The roots and basal stems that have been infected with rhizomorphs will decay and turn black over time (Figure 2).
The fungal pathogen produced a fan-shaped fruiting body and was brown (Figure 3). There were radial stripes with a narrower border and a brown color on the upper surface. While on the lower surface, there was a layer of cream-colored pores. The fruiting bodies were attached to the dead stem of the tree and also tree trunk. Fruiting bodies overgrew in the rainy season. In terms of dry and hot land conditions, the fruiting bodies quickly dried out and turned thinner.

3.2 Pathogenic isolate of *Rigidoporus* sp. from infected cashew root

A fungal culture morphologically identical to the fungus *Rigidoporus* sp. was obtained based on the pathogenic fungus isolation from the infected cashew root. When grown on potato dextrose broth (PDB) medium, the mycelia formed a white solid like a jagged ball (Figure 4A). The purification of isolates on PDA medium showed bright white colony morphology with circular growth, slightly fibrous mycelium, and a smooth surface. The average growth of colony diameter in PDA medium is ± 12 mm per day so that the colonies have filled the entire surface of PDA in Petri dishes on the seventh day. On the tenth day, colonies had grown to a diameter of more than 90 mm, with mycelia covering the edges of the Petri dish (Figure 4B-C). The hyphae were hyaline and septate. Cystidioles were also present between hyphae (Figure 4D-E).
Electron microscopy observation confirmed microscopic structures characteristic of *Rigidoporus* sp., such as cystidioles and clamp connections (Figure 5). The samples analyzed under the scanning electron microscope were 18-day PDA medium cultures. The cystidioles structure was fusoid in shape (like a pole), while clamp connections were found between hyphae. A characteristic clamp connection of *Rigidoporus* sp. only formed when the fungus was cultured on synthetic media such as PDA. Clamp connection was known not to be found when observed from the fruiting body of *Rigidoporus* sp. Fungi of the genus *Rigidoporus* were characterized by monomitic and septate hyphae systems. The cystidioles were present between hyphae [15–17].

Figure 4. Morphological characteristics of *Rigidoporus* sp. isolated from infected cashew roots: Colony of *Rigidoporus* sp. on PDB (A), PDA (B-C) after ten days; hyphae (D) and cystidioles (E) with 100 x 10 optical zoom.

Figure 5. Scanning electron micrograph (SEM) of *Rigidoporus* sp. structure: cystidioles (A) and hyphae with clamp connection (B).

3.3. Molecular identification

Based on the results of amplification of the ITS region of rDNA visualized on agarose, the pathogenic fungus samples produced a single band at ± 600 bp. This size corresponded to the size of several *Rigidoporus* isolates registered in the GenBank database, ranging from 550-700 bp. Alignment of the amplified fungus's nucleotide sequences with the GenBank database showed that the pathogenic isolate (ARMB1) had 92.09% homology with *Rigidoporus* sp. E7081 from Java and *Rigidoporus* sp. E7089 from Sumatra (Figure 6). The fungus causing white root disease in cashew was identical to *Rigidoporus* sp. that stand in a different clade with *Rigidoporus microporus* from Malaysia and Indonesia that reported broadly infecting rubber plants.
3.4 Koch’s postulate test of Rigidoporus sp. on cashew saplings in greenhouse

The results of the test, including pathogenicity, the incubation period for first symptoms (DAI), first symptoms on the canopy, sapling death (DAI), and the percentage of root surface colonization (%) of cashew saplings, are presented in Table 1. White root disease incidence reached 100%, with the fastest incubation period for the emergence of disease symptoms was 51 days after inoculation (DAI). Meanwhile, the fastest period for sapling death was 56 DAI. The highest and the lowest root surface colonization were 90% and 35%, respectively. Early symptoms appearing on the plant canopy were yellowing and wilting of the leaves. The plant died at an advanced stage when the leaves got dry and brown. Some plants defoliated the canopy with the loss of all the leaves.

Table 1. Koch’s postulate test of Rigidoporus sp. on cashew saplings in the greenhouse.

| Cashew Sapling | Pathogenicity* | Incubation period (DAI) for first symptoms | First symptoms on canopy | Sapling death (DAI) | Colonization (%) on root surface |
|----------------|----------------|--------------------------------------------|-------------------------|---------------------|----------------------------------|
| 1              | +              | 53                                        | Yellowing               | 56                  | 85                              |
| 2              | +              | 56                                        | Wilting                 | 62                  | 90                              |
| 3              | +              | 60                                        | Yellowing+Wilting       | 71                  | 50                              |
| 4              | +              | 51                                        | Wilting                 | 56                  | 90                              |
| 5              | +              | 67                                        | Yellowing+Wilting       | 81                  | 90                              |
| 6              | +              | 49                                        | Yellowing+Wilting       | 56                  | 80                              |
| 7              | +              | 60                                        | Yellowing+Wilting       | 68                  | 85                              |
| 8              | +              | 69                                        | Wilting                 | 81                  | 35                              |
| 9              | +              | 72                                        | Wilting                 | 80                  | 90                              |
| 10             | +              | 61                                        | Yellowing               | 70                  | 50                              |
| 11             | +              | 76                                        | Yellowing+Wilting       | 84                  | 90                              |
| 12             | +              | 55                                        | Yellowing+Wilting       | 65                  | 50                              |

Average (± SD)  
60.75 ± 8.61  
69.17 ± 10.50  
73.75 ± 20.90

*) + = pathogenic to cashew saplings
All of the cashew saplings inoculated by *Rigidoporus* sp. showed similar symptoms to those found in the field. Symptoms on the plant canopy were wilting and yellowing of leaves (Figure 7). Signs on plant roots were the presence of rhizomorph and the root turned to black and decay.

![Figure 7. Nine-month-old cashew saplings inoculated with *Rigidoporus* sp.: symptoms on plants (A-D); control (E); and 50, 70, 90 root surface colonization (F-H), respectively; 90% root surface colonization causing root rot (I); and root of control (J).](image)

This study has successfully characterized and identified *Rigidoporus* sp. as the causal agent of white root disease on cashew, while there was no characterization of *Rigidoporus* infecting cashew before. Fungi from the *Rigidoporus* group have been widely reported as pathogens of plantation crops. Fungi of this group are wood decay fungi with essential ecological functions and economical [24]. Based on field observations, the fruiting bodies of *Rigidoporus* sp. were cream-colored pore surfaces. The fruiting bodies of the fungus grew on old infected plants and often appeared on fallen tree trunks. *Rigidoporus* sp. infected plant roots with rhizomorphs. Rhizomorphs attached and spread along the root surface. Furthermore, the infected productive plants will die. If the stump is left, it will be a source of inoculum in the soil. Based on the result of Koch’s postulate test, the colonization of *Rigidoporus* sp. on the cashew root surface ranged from 35%-90%. Although it needed varied incubation durations, the root surface colonization rate of 35%-50% percent was similarly able to kill cashew saplings, as with the colonization rate of 85%-90%. The results indicated that the isolate *Rigidoporus* sp. isolated from infected cashew roots is highly pathogenic. Therefore, early detection of this fungal infection in the field and at the nursery stage is necessary.

White root disease has become a severe problem of cashew plantations, especially in smallholder plantations. Yield reductions caused by the disease have not been documented or reported. So far, there has been no report on the incidence of white root disease on cashew plants outside Indonesia. Only a few investigations of white root disease on cashew plants have been conducted in Indonesia, such as in Bali and West Nusa Tenggara [25], where the disease was an endemic and main disease of cashew in these two areas. In Southeast Sulawesi, the disease also threatened cashew productivity and became one factor affecting the low yield of cashew nuts [26]. Research on white root disease is mainly concerned with rubber plants, especially Sumatra, because the yield loss is considerable. The white root disease of cashew displayed similar symptoms and signs to the white root disease of rubber
caused by *Rigidoporus microporus*, both in the field and in the greenhouse. Based on molecular identification using universal primers ITS1 and ITS4, the isolate obtained was not identical to *R. microporus* species from many countries registered in the GenBank database. The isolate obtained had a homology of 92.09% with *Rigidoporus* sp. E7081 from Java and *Rigidoporus* sp. E7089 from Sumatra. Both isolates were isolated from the acacia plant (*Acacia mangium*). These results are different from several previous studies [27, 28], which specifically stated that *R. microporus* was the causal agent of white root disease on cashew. To our knowledge, this is the first report of *Rigidoporus* determination on cashew.

4. Conclusion
White root disease on cashew in Bali is caused by the pathogenic fungus *Rigidoporus* sp. The disease has been threatening cashew since the sapling stages. Infected saplings showed the same symptoms as the infected productive plants in the field, characterized by fungal rhizomorphs colonizing the roots and basal stem. In advanced symptoms, the root of the infected plants decay and turn black.

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