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Research Article
Identification and Pathogenicity of Fungal Dieback Disease on Sengon (*Paraserianthes falcataria* (L.) Nielsen) Seedling and Rice (*Oryza sativa*)

[1]Muhammad Alam Firmansyah, [2]Erfiani, [3]Anuraga Jayanegara, [1]Nurheni Wijayanto and [1]Achmad

[1]Department of Silviculture, Faculty of Forestry, Bogor Agricultural University, 16680 Bogor, Indonesia
[2]Department of Statistics, Faculty of Mathematics and Natural Science, Bogor Agricultural University, 16680 Bogor, Indonesia
[3]Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Bogor Agricultural University, 16680 Bogor, Indonesia

Abstract
Background and Objective: Sengon (*Paraserienthes falcataria* (L.) Nielsen) is a plant species in forestry plantation while rice (*Oryza sativa*) is an agricultural crop which potentially used in the agroforestry system. Both species are susceptible to dieback disease. This study aimed to isolate and identify the dieback disease on sengon seedlings to understand the pathogenicity of fungal dieback disease on the seedlings of both sengon and rice and to observe the symptom of dieback disease both macroscopically and microscopically. **Materials and Methods:** Pathogenicity test was conducted in a factorial completely randomized design (CRD). The treatments were control, wounded by carborundum, inoculated with pathogen as well as inoculated with pathogen and wounded by carborundum. **Results:** Results revealed that the greatest percentage of dieback disease on sengon and rice was occurred on the treatment of inoculation with wound both100%. While, the greatest percentage of dieback disease intensity of sengon and rice was obtained on the treatment of inoculation with wound by 98.2 and 40.6%, respectively. The PCR result identified that the pathogen was *Ceratobasidium ramicola* that form imperfect state as *Rhizoctonia* sp. **Conclusion:** This species of fungal pathogen is the major cause of dieback disease on sengon and rice seedlings due to seedlings death.

Key words: Sengon, rice, pathogenicity, *Rhizoctonia* sp., dieback, *Ceratobasidium ramicola*

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Corresponding Author: Achmad, Department of Silviculture, Faculty of Forestry, Bogor Agricultural University, 16680 Bogor, Indonesia
Tel: +62-251-8626806 Fax: +62-251-8626886

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Agroforestry is a cultivation system which combines the aspect of forestry and agriculture, it is able to integrate the forest enterprises and rural development in order to create a harmony between intensification of agricultural land and forest. Agroforestry is beneficial in terms of pest, disease and weed management. Although the relevance of pest and disease interactions with agroforestry measures has been recognized many years ago, few agroforestry studies have included detailed investigations on such interactions. Furthermore, quantitative analyses regarding the effects of agroforestry on disease control are still lacking[1,2].

Earlier studies have investigated the incidence of dieback disease to the plant species that used in the agroforestry system such as jabon (*Anthocephalus cadamba* (*Roxb.*) *Miq*)[3], *Fraxinus excelsior*[4] and olive trees (*Olea europaea*)[5]. However, all of these experiments only evaluated for single plant without investigating the relationship with other plant species. One of forestry trees that is usually cultivated together with agricultural crops is sengon (*Paraserienthes falcataria* (L.) Nielsen). Sengon is a species of forestry tree which generally grows in Indonesia and it is kind of fast growing species tree. One of agricultural crop that is able to be combined with forestry trees is rice (*Oryza sativa*). Rice is an agricultural crop that is consumed in a large amount in the world and easy to be cultivated.

Sengon and rice are expected to be susceptible to disease, so that the incidence and disease intensity of plants have to be elucidated. Disease inhibits the regeneration of plants at the classification of seedlings so that it will affect the quantity and quality of plant. Furthermore, Saeed *et al.*[6] explained that dieback disease potentially causes a high mortality rate of early stage of plant all over the world. This study therefore aimed to isolate and identify the dieback disease on sengon seedlings, to understand the pathogenicity of dieback fungus to the seedlings of sengon and rice and to observe the symptom of dieback disease both macroscopically and microscopically.

MATERIALS AND METHODS

Sample collection: This study was conducted in the laboratory of Forest Pathology, Department of Silviculture, Faculty of Forestry, Bogor Agricultural University and Permanent Nursery of Bogor Agricultural University, Dramaga, Indonesia from January-September, 2017. Sengon seedlings were obtained from Permanent Nursery of Bogor Agricultural University and rice seedlings were obtained from Experimental Field of Indonesian Rice Research Institute, Muara, Bogor. Seedlings were in healthy and good condition. Isolation of fungi was performed by picking up the part of diseased sengon due to surface sterilization with alcohol 90%. Sterilized leaf tissues then put into the petri dish containing medium of potato dextrose agar (PDA) and medium of potato dextrose broth (PDB). After that the sample was incubated for $\pm$7 days at a room temperature so that homogenous cultures can be obtained. During the experimental period, temperature and relative humidity were measured every day in the morning (7-8 am), in afternoon (12-1 pm) and in evening (4-5 pm) using thermometer and hygrothermometer, respectively.

Identification of fungi: Morphological identification of fungi was performed macroscopically and microscopically. The morphological identification was based on Barnett and Hunter[7]. Molecular identification of fungi was conducted by DNA sequencing according to Rahayu *et al.*[8]. The DNA was extracted from pure cultures of pathogen obtained from infected sengon plants sampled from Bogor Agricultural University Nursery. The internal transcribed spacer region (ITS), consisting of ITS1 and ITS4. A PCR-based protocol was developed to detect and identify the pathogen. The base sequence of DNA sequencing results processed with version 5.2.2 MEGA program[9]. The data obtained were analyzed by NCBI (National Center for Biotechnology Information). Koch's postulates test was performed to prove that the isolated fungus is a major cause of dieback disease symptom. This test consisted of inoculation of fungi to the sample, re-isolation of symptomatic plant tissue and identification of the result of re-isolation[10]. All the chemicals used were of pure analysis standard.

Pathogenicity test of fungi: Pathogenicity test was conducted on fungus which is able to generate the symptoms of dieback disease. Samples used for inoculation were sengon seedlings and rice which aged 2 months of weaning. Inoculated sample was incubated at the paranet house sized 3×2 m^2 with density of 65%. Every sample was covered with plastic to prevent external contamination as well as dissemination of disease to the environment. Seedling storage layout on paranet house used a completely randomized design (CRD) with two factors, i.e., type of seed and treatment.

Table 1: Disease severity and numeric value of disease scale

Score	Note
0	No symptom on leaves
1	Wilted leaves or 25% of leaves have necrosis
2	26-50% of leaves has necrosis
3	>50% of leaves has necrosis
4	Plant dead

The treatments were control sample, wounded by carborundum, inoculated with pathogen fungus as well as inoculated with pathogen fungus and wounded by carborundum at the same time.

Pathogenicity test parameters were disease incidence and dieback fungus attack intensity to the sengon and rice seedlings. Incidence of disease was determined as follow[11]:

$$\text{Disease incidence (KP)} = \frac{n}{N} \times 100\%$$

KP = Disease incidence (%)
n = Number of infected plant
N = Number of plant sample observed

Disease intensity was calculated by following a formula described in Stevic *et al.*[12]:

$$IS = \frac{\sum(nxv)}{N \times Z} \times 100\%$$

IS = Disease severity (%)
N = Number of leaf in every category
v = Numeric number of every attack category
N = Number of observed plant
Z = Numeric number of the highest attack category

Disease severity scale and the numeric value of disease used were based on Table 1[13].

Statistical analysis: Data obtained were analyzed using analysis of variance and followed by Duncan's multiple range test (DMRT). The statistical model was based on a factorial completely randomized design (CRD) as follow[14]:

$$Y_{ijk} = \mu + \alpha_i + \delta_{ik} + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

Y_{ijk} : Severity intensity at the repetition of k as well as level i from inoculation factor and level j from wound factor
μ : Mean of actual severity intensity
α_i : Main effect from level i of inoculation factor
δ_{ik} : Error effect that occurs on the level of i from inoculation factor in the repetition of k
β_j : Main effect of level j of wound factor
$(\alpha\beta)_{ij}$: Interaction effect between level i of inoculation factor and level j of wound factor
ε_{ijk} : Error effect on the repetition of k from level i of inoculation facor and level j of wound factor

All the statistical analyses were conducted by employing SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Level of significance was declared at $p<0.05$.

RESULTS

Based on the field observation results, management nursery of Bogor Agricultural University reported that there were >500 seedlings of sengon aged 1-2 months harmed by dieback disease with incidence percentage of 100% and intensity of 90%. These results showed that it can potentially be major problems for the production of seedlings in the next future.

The average values of temperature and relative humidity during 14 days of observation were 28°C and 82.9%, respectively. Based on the observations of dieback disease symptom on sengon seedlings at the nursery site, the seedlings were infected with dieback disease and the symptom was necrosis, mostly on the top of plants and leaves (Fig. 1a). Symptom of necrosis caused the leaves curled and dried, mycelium was found on the leaves as well (Fig. 1b) and caused wilt of the seedlings (Fig. 1c).

Fungus was successfully isolated from parts of the plant which harmed by dieback disease (Fig. 2). Both macroscopic and microscopic observations showed that the cause of the dieback fungus was similar to *Rhizoctonia* sp. Isolate had a rapid growth which could fulfill the petri dish (Ø 9 cm) after 3-4 days of incubation. Fungus isolate has microscopic characteristics included branches of hyphae which located perpendicular on the base of branch, septum and monilloid were found as well, there were no spores, either conidia or clamp connection (Fig. 3). The molecular identification based on DNA sequencing showed that the species name is *Ceratobasidium ramicola* that form imperfect state as *Rhizoctonia* sp.

In the pathogenicity test, similar symptoms like above were observed after re-inoculation of *Rhizoctonia* sp. to healthy seedlings of sengon and rice (Fig. 4). Figure 5 shows the difference between tissues of healthy rice leaves and rice

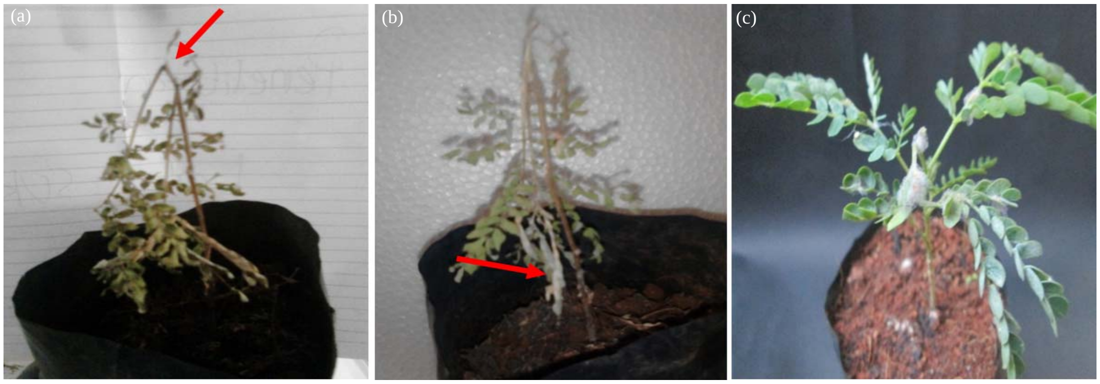

Fig. 1(a-c): Symptom of dieback disease on sengon seedlings at the permanent nursery (a) Yellow arrow shows necrosis on stem, (b) Yellow arrow shows necrosis on leaves and mycelium that founded on leaves and (c) Wilted leaves

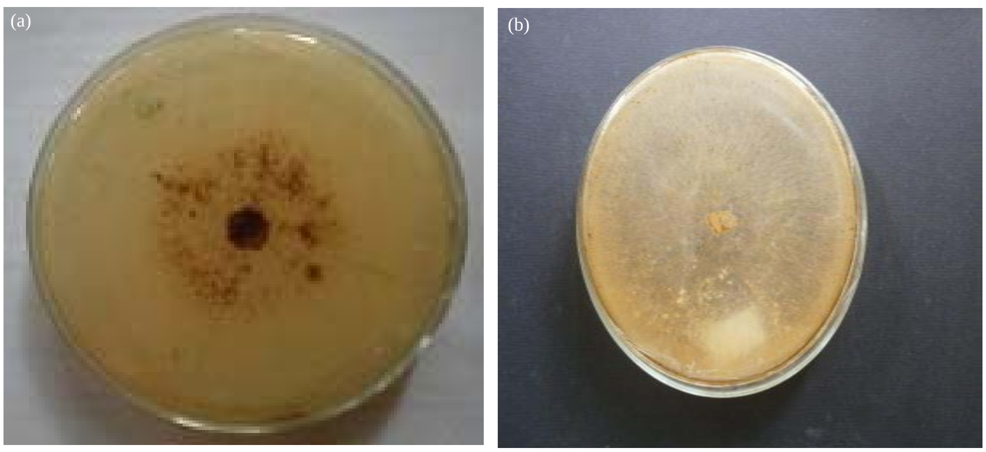

Fig. 2(a-b): Isolate of *Rhizoctonia* sp. fungus (a) Nosclerotia and (b) With unevenly dispersed sclerotia

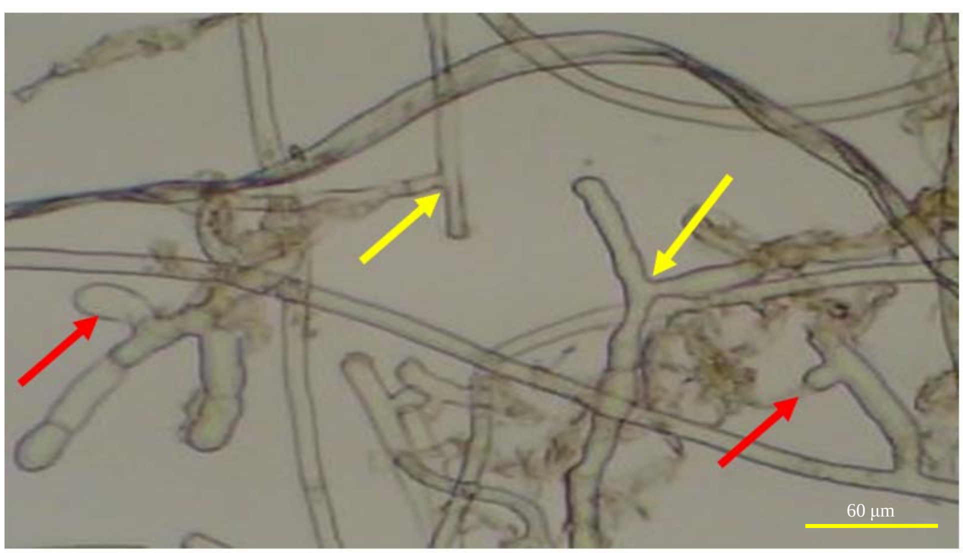

Fig. 3: *Rhizoctonia* sp. hyphae under microscope and 400x magnification. Red arrow shows monilloid cell, while yellow arrow shows the angle 90° on branching

Fig. 4(a-b): Yellow arrow shows wilted leaves and dead plants of (a) Rice and (b) Sengon seedlings

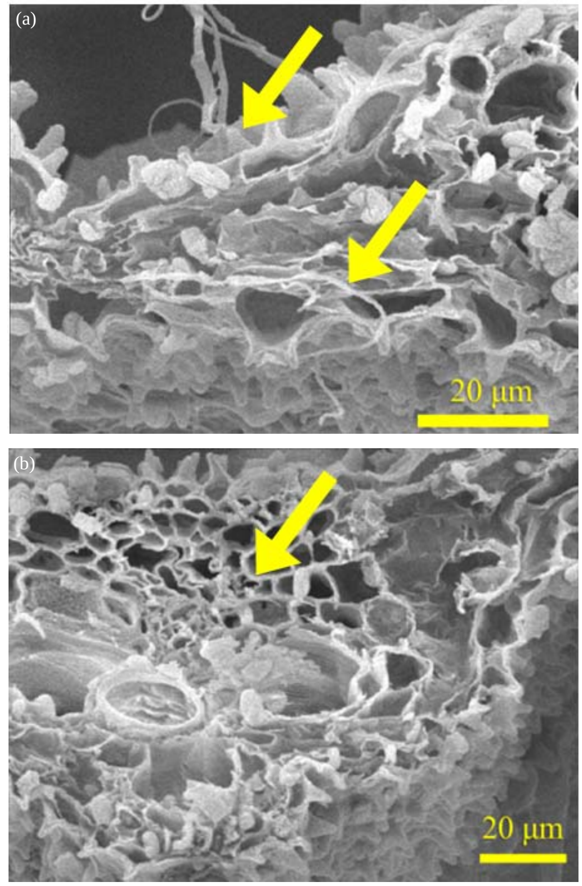

Fig. 5: Scanning electron microscope observation of rice seedlings leaves (a) Non inoculated leaves of *Rhizoctonia* sp. isolate, yellow arrow shows stomata was in a good condition and (b) Yellow arrow shows hyphae of *Rhizoctonia* sp. on rice leaves seedlings

leaves infected by *Rhizoctonia* sp. by using scanning electron microscope. Dieback disease incidence on plants inoculated with pathogen and wounded by carborundum treatments to both rice and sengon seedlings were 100% (Table 2). The treatment of wounded by carborundum to the rice and sengon were 0 and 7.6%, respectively and the last was control sample of 0% (Table 3).

Table 2: Dieback disease incidence to the seedlings of sengon and rice

		Treatment (%)			
Types	Repetition	Control	Wound	Inoculation	Inoculation, wound
Rice	5	0^b	0^b	100^a	100^a
Sengon	5	0^b	0^b	100^a	100^a

Number followed by different alphabet showed the sign of significantly different at a 5% of confidence interval based on Duncan's test

Table 3: Dieback disease severity intensity to the seedlings of rice and sengon

		Treatment (%)			
Types	Repetition	Control	Wound	Inoculation	Inoculation, wound
Rice	5	0^b	0.0^b	40.6^a	31.6^a
Sengon	5	0^b	7.6^b	98.2^a	96.3^a

Number followed by different alphabet showed the sign of significantly different at a 5% of confidence interval based on Duncan's test

DISCUSSION

Rice and sengon seedlings that harmed by dieback disease in the present study had a symptom of disease at the tip of the plants. Necrosis symptom spreaded along the leaves, the leaves dried, wilted and rolled, then deciduous. Besides that, necrosis would spread along the part of stem which was infected, at the first time it would shrink, dried and dead. It was consistent with the study that conducted by Aisah[13], who expressed the symptom of disease both at the nursery site and the location pathogenicity test, it is generally known that pathogen will initiate its activity to infect the succulent parts of plant first. It can be seen from the symptom of the disease which develops relatively fast towards the upper part of the plant compared to the base of stem. Stem tissue is relatively harder compared to internal tissue of the plant. Base of the stem experienced a process of lignifications so it was hardened, due to its phenomenon, pathogens would not infect the plants easily, because both of physical and chemical properties of lignin would act as barriers against diseases[13]. Symptom of the disease of plant is an abnormality of morphology or physiology as a response of pathogens disturbance[15]. For example, symptoms of dieback disease on mango crop and Jabon (*Anthocephalus cadamba* (Roxb.) Miq.) are the death of twigs, curled leaves and dried plant followed by deciduous leaves[16].

The identification results were consistent with the illustration of genera imperfect fungi by Barnett and Hunter[7]. They reported the characterization of the fungus which has no conidia but has hyphae and formed dark brown or blackish color of sclerotia in the microscopic observation is able to be classified as genus of *Rhizoctonia*. In addition, monilloid cell with ratio between diameter and length is 1:1 was founded, diameter of isolate hyphae obtained was 5-15 µm and it was consistent with Sneh *et al.*[17], who stated that commonly ratio of diameter: length is 1:1, the diameter of the hyphae isolate is commonly sized 3-17 µm with a length of 50-250 µm. These two statements support the statement that the classification of isolate fungus was identified as a type of *Rhizoctonia* sp. that form teleomorph state as *Ceratobasidium ramicola*.

Koch's postulates is an activity which performed to prove the major cause of a disease in plant. A microbe is able to be considered as the causative agent of the disease if it meets the law of Koch's postulates as follows: Pathogen forms a permanent association with the disease, pathogen can be isolated from diseased tissue and it is able to be grown on artificial media, the result of isolation is able to be inoculated in healthy plants and it will produce the same symptom as previous diseased plant, pathogen can be re-isolated from diseased plant and it will have similar characteristics to the previous pathogen[18]. Based on the results of inoculation in the series of Koch's postulates, it can be seen that the tested isolate was pathogenic to the seedlings of sengon. Generally, the fungus caused a symptom at the point of inoculation. Based on the observation of symptom, inoculation of the fungus generates an identical symptom with the natural symptom of dieback disease on sengon plant. The period of fungus to cause a symptom was relatively short. This condition showed that the fungus can develop faster in the tissue of host plant. This was supported by the radial growth of isolate of *Rhizoctonia* sp. on artificial media (PDA) that filled a petri dish with a short incubation period. Based on the Koch's postulates, the fungus which suspected as the major cause of dieback disease on sengon seedlings was an identified as *Ceratobasidium ramicola*.

The results of pathogenicity test showed a necrosis symptom which spreaded along the leaves and led to the death of entire organs of plant. Pathogenicity test is the capacity or the ability of a pathogen to cause a disease[18]. Pathogenicity test was performed on sengon and rice seedlings based on Achmad *et al.*[19]. Dieback disease symptom on both sengon and rice seedlings caused by biotic factor of *Ceratobasidium ramicola*. This fungus is able to enter the plant tissues of rice and sengon through wound or direct penetration with chemical wound such as carborundum. The wound treatments of rice and sengon seedlings were 0 and 7.5%, respectively. The results were significantly different because the wound treatment by carborundum on sengon leaves was abrasive. According to Achmad and Maisaroh[20], the addition of carborundum aims to add the abrasiveness of sample that produces microscopic wound on the inoculated plant, particularly on the surface of the cell wall.

Ceratobasidium ramicola required a relatively short period to cause a symptom on both rice and sengon seedlings, it was about one day after inoculation. After that, the disease was able to spread rapidly. According to the study which conducted by Achmad *et al.*[19], this kind of fungi has an ability to cause symptom rapidly. It was explained that the second day after inoculation, the symptom of leaf blight even death of marked leaves occurred and it led to the deciduous leaves. The developed disease leads to the activity of pathogen inoculums spreading, either through the air, water or vector. If the inoculum has reached or landed on the surface of the host, the pathogen will go through the next cycle of disease[18]. Based on observation results, dieback disease occurred on two treatments, inoculation and inoculation with wound. While the treatment without inoculation did not show any symptom of dieback disease. It proves that this fungus has the ability to cause disease or virulent as well as the statement that rice and sengon as hosts are susceptible to the disease due to visible symptoms of the disease after inoculation of this fungus.

Dieback disease may be caused by other fungi, according to Ahmad *et al*.[11] *Botryodiplodia theobromae* attack is able to cause dieback disease. The attack of *Ceratobasidium ramicola* that form imperfect state as *Rhizoctonia* sp. may produce different symptoms in different host, this fungus is a crucial fungus due to its comprehensive range of host plants. In addition, the fungus of *Rhizoctonia* sp. is able to cause leaf blight disease on suren seedlings[19,20]. Fungus of *Rhizoctonia* sp. may cause dumping off disease as well on sengon[6], pine and acacia seedlings[21]. This fungus may cause dumping off disease on agronomy and horticultural crops such as tomato[22] as well as becomes contaminant of seedlings surface from Solanaceae family which is able to reduce the germination of seedlings[23].

The development of disease is supported by three factors, such as the susceptible host, virulence of pathogen and supportive environment. Environment such as temperature and relative humidity are crucial to the development of disease. During 8 days of observation, sengon and rice seedlings were in the range of optimum temperature, so that the symptom occurred was the result of biotic factor, such as pathogen, not a biotic factors. The best average growth temperature for sengon and rice ranged from 22-29°C and 24-30°C, respectively[24,25]. Good quality seedlings will have a good vigor. Temperature and humidity are essential for growth of this fungus. According to Sneh *et al*.[17], it grows optimally at 25-30°C. A supportive environment according Henuk[26] is the condition of moisture, nutrients and high temperature which suitable for pathogen to germinate and then penetrate into the plant tissue. Different condition of temperature between day and night especially during dry season will facilitate the development of fungi, for example is the period of observation of this study. *Rhizoctonia* sp. is a soil borne pathogen which protected by warm condition and adequate soil moisture, it is able to lives in the soil as hyphae, sklerotia and basidiospores. *Rhizoctonia* sp. has ability to be a major cause of disease in a wide range of soil temperature, pH, type, fertility and moisture[27].

Since the fungus may attack both sengon and rice plants, people should consider if they want to plant these species simultaneously. Further investigations are required regarding the occurrence of *Rhizoctonia* sp. infection in various agroforestry systems.

CONCLUSION

This study has identified *Ceratobasidium ramicola* as the fungal pathogen causing dieback disease on sengon seedlings and rice. These plants are more susceptible to the fungal pathogen particularly after inoculation and wound treatment. Further studies are required to perform control measures in order to prevent spread of the disease in the plants.

SIGNIFICANCE STATEMENT

This study discovers level of pathogenicity and the species that causes disease on sengon seedling and rice that can be beneficial for studying the mechanism of attack so that the control can be effectively elucidated. This study will help researchers to uncover pathogenicity and identity of plant pathogent especially *Rhizoctonia* sp. that many researchers were not able to explore. Thus a new theory on the mechanism of *Rhizoctonia* sp. infection in sengon seedling and rice may be arrived at.

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