Fungi identification and test on red calliandra (Calliandra calothyrsus) seed as potentially antagonist agent

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Abstract. Red calliandra (Calliandra calothyrsus) is an essential economic plant and used as a renewable energy source. Seed-borne fungi can be beneficial or detrimental. One of the beneficial roles of fungi is as an antagonistic agent. Red calliandra seed is commonly infected by pathogenic fungi such as Fusarium sp., Aspergillus sp., and Rhizopus sp. The purpose of this research was to identify the fungi in red calliandra seeds, which are potential as an antagonistic agent, and to study their ability to inhibit the growth of pathogenic Fusarium sp., Aspergillus sp. and Rhizopus sp. The seeds obtained are from Sukabumi, West Java, Indonesia. Fungi were isolated by soaking the seeds with 1% NaOCl for three minutes then washed with sterile aquadest. It was repeated three times. Furthermore, the seeds were sown on moist paper media and incubated until a fungus colony emerged, which is expected to be a potential antagonistic agent. The result showed that there was one isolate of fungi that was potential as an antagonistic agent; it was Talaromyces sp. It indicates that the antagonistic agent can be isolated from seeds. The inhibition percentages of Talaromyces sp. against Aspergillus sp., Fusarium sp., and Rhizopus sp. at seven days old were 67.92%, 59.4%, 8.89%, respectively. Talaromyces sp. can inhibit the growth of Fusarium sp. Rhizopus sp. and Aspergillus sp. in overgrowth at 14 days old. The antagonistic mechanism of Talaromyces sp. to Aspergillus sp., Fusarium sp. and Rhizopus sp. is microparasitism and competition. Talaromyces sp. isolated from red calliandra seeds were potential as antagonistic agents.

1. Introduction

Red calliandra (Calliandra calothyrsus) as one of the legume plants, has much utility, such as for fodder, apiculture, fuel, fiber, erosion control, shade, nitrogen-fixing, soil improver, ornamental, boundary, and also suitable for intercropping [1]. This species is an essential economic plant and used as a renewable energy source. For these reasons, this plant has been developed in the community forest. In planting programs, the limiting factor is disease in seeds, seedlings, plants and post-harvest products.

These diseases can harm the economy and need to be controlled. Control with chemical pesticides has adverse effects, such as pathogen resistance, costly, and hazardous to the environment. The use of antagonistic agents can be a solution as an environmentally friendly pest control. Seed-borne fungi can be beneficial or detrimental. One of the beneficial roles of fungi is as an antagonistic agent. Some types of fungi often found in forest plant seeds include Fusarium sp., which is a fungus often found in
the storage, and Aspergillus sp. as well as Rhizopus sp., which is the storage fungi [2]. Seed-borne fungi of red calliandra, among others, are Aspergillus sp. [3], Fusarium sp. [4] and Rhizopus sp.

Seed-borne pathogenic fungi can affect seed quality and the production of seedlings [5]. One of them, Fusarium sp. carried in red calliandra seeds, can cause damping off. Seed-borne fungi, both saprophytic and also pathogenic fungi, inhibited seed germination, and seedling emergence [6]. Antagonistic agents that are often used to control disease in plants can originate from fungi and bacteria. Some fungi genera serve as an antagonistic agent, namely Gliocladium sp., Penicillium sp. [7], Trichoderma sp., Talaromyces sp. [8].

Antagonistic agents are found in the soil, such as Trichoderma sp., Gliocladium sp. [9], Penicillium sp. Talaromyces sp. [10], but not much information on antagonistic agents from seed. The purpose of this research was to identify the fungi in red calliandra seeds, which are potential as antagonistic agents, and to study their ability to inhibit the growth of pathogenic fungi Fusarium sp., Aspergillus sp. and Rhizopus sp.

2. Materials and methods

2.1. Study site
The seeds obtained are from Sukabumi, West Java, Indonesia. The research was conducted at the pest and disease laboratory, BP2TPH, from January to May 2019.

2.2. Fungi identification at red calliandra seed as potentially antagonist agent
Fungi were isolated by soaking the seeds with 1% NaOCl for three minutes then washed with sterile aquadest. It was repeated up to three times. Furthermore, the seeds were sown on moist paper media and incubated until a fungus colony emerged, which is expected to be a potential antagonistic agent. Fungi identification was made using the identification key book [11] and other literature [12]. The potential fungi as an antagonist agent were isolated into pure cultured.

2.3. Antagonistic tests
The dual culture method was used to test between the antagonistic fungus and saprophytic/pathogenic fungi of seven-day-old culture. The saprophytic/pathogenic fungi of red calliandra seed included Fusarium sp., Aspergillus sp., and Rhizopus sp. The lower part of the Petri dish base was marked by two pieces, each with a distance of 3 cm from the edges of the petri dish. Furthermore, the mycelial plug (5 mm diameter) of the fungi colony was placed at one point, while the other was placed with the mycelial plugs of the saprophytic/pathogenic fungi colony. Then, the plates were incubated for 14 days in the ambient room.

2.4 Observations
Macroscopic observation consists of antagonist fungi colony observation in PDA medium, inhibition percentage measure, and observation of fungi colony interaction types in agar medium. While the microscopic observations are morphology observation of antagonist fungi (hyphae, spores) and observation hyphae interaction using the microscope. The inhibitory percentage observation was done by measuring the radius of Fusarium sp., Aspergillus sp., and Rhizopus sp. colony toward and against the fungi of antagonistic agents at the age of seven days.

\[
\text{Inhibitory percentage} = \frac{r_1 - r_2}{r_1} \times 100\% \tag{1}
\]

Remarks
\( r_1 \) = Colony radius of Fusarium sp., Aspergillus sp. and Rhizopus sp. were opposed to antagonistic agents
\( r_2 \) = Colony radius of Fusarium sp., Aspergillus sp. and Rhizopus sp. which are toward antagonistic agents
Interactions between fungal colonies on agar medium are based on Dickinson & Boardman (1971) in [13]:
A. Both fungi can grow mingle with each other without any microscopic signs of interaction.
B. Mixing up the growth where the fungus observed is growing into an opposing fungus either above or below the colony or the fungus observed has stopped growing and is overgrown by other colonies.
C. Inhibit each other at a distance of > 2 mm.

3. Results and discussions

3.1. Fungi identification at red calliandra seed as potentially antagonist agent

Observation results showed that fungus colonies appeared green in the incubation period of two weeks (Figure 1). Usually, saprophytic/pathogenic fungi appear before seven days. The macroscopic observation result showed that the fungus' characteristics on day three were light green with white edges (Figure 1a), colony diameter of 4.3-6.7 cm, (an average of 5.2 cm). However, some isolates were still colored white or greenish-white. On the 5th day, the colony diameter was 6-9 cm (7.75 cm on average). The edge of the colony was generally dark green, but some were light green or greenish-white (Figure 1b). On the 7th day, almost all isolates covered the cup (8-9 cm in diameter), with the edges were still white. The growths of fungus colonies on PDA media are listed in Table 1.

On the 3rd day, the colony was greenish-white; on the 4th day, several isolates covered the entire cup, and on the 7th day, all isolates covered the entire cup surface and were green in color. In line with research conducted by Achmad and Nurhayati [14], the fungus colonies are dark green, grow fast, spread and cover the entire surface of the cup. The observations showed that the fungus mycelium was white while the conidia was green.

![Figure 1](image)

(a) Fungal colony on PDA at the 3rd day (left), (b) the 5th day (center), (c) the 7th day (right).

| Days to- | Diameter (cm) | Color      |
|---------|---------------|------------|
|         | Range         | means      |            |
| 3       | 4.3-6.7       | 5.2        | white, green|
| 4       | 5-9           | 7.25       | green      |
| 5       | 6-9           | 7.75       | green      |
| 6       | 7-9           | 8          | green      |
| 7       | 8-9           | 8.5        | green      |

The identification result of this fungus based on [15] is *Talaromyces* sp., which is a sexual/teleomorphic stage of *Penicillium* sp. (Figure 2) [16] transferred all species of *Penicillium* subgenus *Biverticillium* to *Talaromyces*. As seen in Figure 1a, the characteristics of these fungi are green, penicilli biverticillate-symmetrical (phialides lanceolate, ascospores subglobose, or ellipsoidal,
ranging within the size 3.45 μm as in Figure 1b). *Talaromyces* sp. has white mycelium and green conidia [17,18]. Thus *Talaromyces* sp. can be isolated from the seeds of forest plants such as calliandra seeds.

![Figure 2. Morphology Talaromyces sp.](image)

*Talaromyces* sp is known as a type of fungi that is potential as an antagonistic agent [19]. Some types of fungi that their growth can be inhibited are *Phytophthora palmivora*, *P. parasitica*, *Peronophythora litchii*, *Colletotrichum capsici*, *C. gloeosporioides*, *Pestalotiopsis guepinii*, *Phyllosticta* sp.[20].

### 3.2. Antagonist test for *Talaromyces* sp. and *Fusarium* sp., *Aspergillus* sp., *Rhizopus* sp.

*Talaromyces* sp. can inhibit the growth of *Fusarium* sp. and *Aspergillus* sp. until seven days old with more than 50% of inhibition (Table 2). From Table 2, it can be seen that the percentage of inhibition of *Fusarium* sp. by *Talaromyces* sp. is relatively fixed while *Aspergillus* is increasing. It is because *Fusarium* growth tends to be slow. Growth of *Aspergillus* sp. is faster, but the size of the *Talaromyces* sp. approaching radius is shorter than those that are away from *Talaromyces* sp. The small inhibition percentage of *Rhizopus* sp by *Talaromyces* sp. is caused by the rapid growth of *Rhizopus* so that when the *Talaromyces* sp. approaches and is away from radius are almost the same until seven days old.

| Fungi species | Inhibition percentage (%) |
|---------------|---------------------------|
|               | The 3\textsuperscript{rd} day | The 4\textsuperscript{th} day | The 5\textsuperscript{th} day | The 6\textsuperscript{th} day | The 7\textsuperscript{th} day |
| *Fusarium* sp.| 57.81 | 63.37 | 60.43 | 61.71 | 59.4 |
| *Aspergillus* sp.| 18.31 | 44 | 57.05 | 67.78 | 67.92 |
| *Rhizopus* sp.| 23.63 | 16.67 | 12.5 | 10.83 | 8.89 |

Antagonism mechanism consisted of mycoparasitism, antibiosis, and competition. It can be seen in Figure 3 that after seven days old, *Talaromyces* can inhibit the growth of *Fusarium* sp. Even if *Talaromyces* is already dominant, *Aspergillus* sp. is still growing with almost the same growth rate. However, the two types of fungi still do not dominate each other.

Interactions among fungal isolates were categorized as antagonism and mutual antagonism [20]. Based on Dickinson & Boardman (1971) in [13], the interaction of *Talaromyces* s.p with *Fusarium* sp. and *Rhizopus* sp. is intermingling growth (overgrowth by the antagonist) as depicted in Figure 4. In contrast, the growing interaction of *Talaromyces* sp. with *Aspergillus* sp. is mutual intermingling, which means that there is no hyphal interaction with each other. According to [21], the dual culture of some *Penicillium* species with *Aspergillus* produced mutually intermingled growth without any zone of inhibition, showing that the production of the antibiotic by both fungi was failed.
From Figures 3 and 4, it appears that the highest inhibition is against *Fusarium* sp. This result is supported by [22] research, stating that the growth of *Talaromyces* sp. is fast and can inhibit the growth of pathogenic fungi within ten days.

![Figure 3](image1.jpg)

**Figure 3.** Antagonistic test between *Talaromyces* sp. and *Fusarium* sp., *Rhizopus* sp., *Aspergillus* sp. until seven days.

*Talaromyces* sp. was able to suppress the *Fusarium* sp. growth, *Aspergillus* sp., and *Rhizopus* sp. and it was even overgrowth at 14 days old (Figure 4). At 14 days, the dominant mechanism for the three types of pathogenic fungi was mycoparasitism, space and nutrition competition. According to [23], mycoparasitism is the main mechanism to control *Sclerotium rolfsii*, which is related to chitinase activity. In contrast, the activity of glucose-oxidase and antifungal compounds (antibiotics) plays a role in inhibiting the germination, growth, and melanization of *S. rolfsii* microsclerotia.

![Figure 4](image2.jpg)

**Figure 4.** Antagonistic test between *Talaromyces* sp. and *Fusarium* sp. (a), *Rhizopus* sp. (b), *Aspergillus* sp. (c)

Although *Rhizopus* sp. is still alive at the age of seven days, hyphae interference occurs, so this is categorized as antagonism (Figure 5a). According to [23], mycoparasitism involves cell wall-degrading enzymes, such as β1,3-, β-1,4-, and β-1,6-glucanases, cellulase, and chitinase. At the age of seven days, coagulation and granulation occur in the hypha of *Fusarium* sp., and the hyphal folding (Figure 5b). Coagulation and granulation in hypha of the pathogen showed the lysis [24]. In line with the research conducted by [21], during contact between *A. niger* hypha and *Penicillium* sp. hypha, there was no hyphae interaction. However, *Penicillium* sp. growing above *A. niger* would not completely inhibit the growth. It would be antagonistic if *Penicillium* sp. were overgrowth. According to [25], *T. convolutes* produces talaroconvolutins, which can inhibit the growth of *A. fumigatus* and *A. niger*. 
Based on the results, it is found out that *Talaromyces* sp. can inhibit overgrowth for *Fusarium* sp., at seven days old, while for *Rhizopus* sp. and *Aspergillus* sp., occur more than seven days. Thus, *Talaromyces* sp. has a high potential to suppress diseases caused by *Fusarium* sp., such as damping off.

![Figure 5.](image1.png)  
(a) Hyphal interference of *Rhizopus* sp. (b) Coagulation and granulation in the hyphae and hyphal folding of *Fusarium* sp.

### 4. Conclusion

One of the potential fungi as antagonist agent isolated from the seed of calliandra is *Talaromyces* sp. The main antagonistic mechanisms of *Talaromyces* sp. to *Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp. are mycoparasitism and competition.

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