Possibility to develop biological control agents for plant diseases on ramie plantation

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Abstract. Ramie is a kind of plant from the Urticaceae family that produces fiber from its stems. Currently, Wonosobo in Central Java is the largest location for ramie development in Indonesia. Generally, the development of ramie in this area is conducted in a monoculture which is potential for the emergence of plant diseases that may not have been considered previously, such as basal stem rot (BSR) and leaf spot (LS). This study aimed to obtain microorganisms that can be developed as biocontrol agents (BCA) for BSR and LS on ramie. Isolation of microbes from soil samples of ramie plantations was carried out using serial dilution methods on Martin Agar and Tryptic Soy Agar media. Furthermore, microbes with BCA potential were tested using the dual culture method with fungi associated with BSR and LS. The results revealed that were 11 bacteria and two fungi that have the potential as BCA. Of the total isolates, three bacteria and two fungi showed the ability to inhibit the growth of BSR and LS-associated fungi in the range of 10.78 - 73.33% in vitro. These results indicate that there is a potency to develop BCAs originating from ramie plantation soil itself.

Keywords: Boehmeria nivea L., microbial antagonists, basal stem rot disease, leaf spot disease

1. Introduction

Ramie (Boehmeria nivea L.) is a fiber-producing plant from the Urticaceae family. Ramie fiber is produced from the bark and widely used as home decoration. Currently, the center of the ramie development area in Indonesia is in Wonosobo Regency, Central Java Province. In this area, ramie is carried out in a monoculture for many years (even up to eight years). This cultivation system can trigger the emergence of some plant diseases that may not have been previously considered. A survey conducted in 2020 found that there were ramie lands that could no longer be planted due to rot on its basal stem, resulting in wilting. Apart from basal stem rot, another disease found was leaf spot. [1] reported that in India, there were several pathogens associated with ramie diseases including Cersospora boehmeriae, Colletotrichum gloeosporioides, and Curvularia. Moreover, in China, Pythium vexans is reported to be the causal agent of stunting disease of ramie [2]. Although up to now, ramie diseases have not been considered a major problem of ramie cultivation, this status can change when the environmental conditions are favorable for disease development. Therefore, it is important to prepare control methods for basal stem rot and leaf spot diseases, one of which is a biological control by harnessing microorganisms originating from ramie planting sites.

Biocontrol for plant pathogen infections is a control method that needs to be developed because physical or chemical methods are difficult to obtain satisfactory results. In addition, the application
of chemicals to the soil will result in the accumulation of toxic substances in the soil, resulting in pollution, which has adverse impacts on humans, animals, and the environment. Therefore, the use of biological agents is a common practice in biocontrol. In general, antagonist microorganisms can be isolated from soil or healthy plants surrounding infected plants. The mechanisms of antagonist microorganisms suppressing the development of pathogens that cause plant diseases include producing antibiotics for pathogens [3] and stimulating plants to produce toxic compounds [4].

The diversity of the agricultural soil microbiota is influenced by the type of plant, cropping system, and cultivation system. In ramie plantations with up to 8 years of productivity, the diversity of soil biota from the bacterial group is higher than that of fungi [4]. Meanwhile, planting peanuts with a continuous monoculture cropping system on the same land affects the diversity of antagonist fungi and has an effect on increasing pathogenic fungi, resulting in decreased plant health and production [5].

Agricultural land in tropical regions such as Indonesia is known to have mega biodiversity, including the microbial diversity of soil biota. This consensus for the development of antagonistic microbes in soil biota as biological agents. One of the agricultural land ecosystems that have the potential to find antagonist microbes from soil biota is ramie cultivation. Ramie is an annual plant that has been in the field for at least eight years and is reportedly susceptible to infection with soil-borne pathogens, such as white fungus (caused by Rosellinia necatrixPrill., seed rot caused by Thanatephorus cucumeris, a cane rot which is caused by Macrophomina phaseolina (Tassi) Gold., and eye rot caused by Myrothecium roridum Tode. Ex Fr. [6]. Therefore, this study aims to explore antagonist microbes in soil biota, identify them and complete the data as Indonesian genetics resources, and assess their antagonistic character against basal stem rot and leaf spot-associated pathogens.

2. Materials and methods
2.1. Exploration of soil microorganisms
Soil microbial exploration was conducted at ramie plantations in Wonosobo, Central Java (Lat 7°24'51” S, Long 109°58’28” E). A total of 14 soil samples were taken in a composite manner obtained from healthy ramie growing lands and on land where ramie plants showed symptoms of stem rot infection. The soil samples obtained were then air-dried, sieved, and ready for microbial isolation.

2.2. Isolation of soil microorganisms
Soil microbial isolation was carried out by serial dilution method. Ten g of soil sample plus 90 mL of sterile water was shaken for 15 minutes at 150 rpm. Further dilution is carried out by taking 1 mL of the initial soil solution and adding it to 9 mL of sterile water (10^1 to 10^3). At 10^3 dilution, 200 µL was taken to be grown on Tryptic Soy Agar (TSA) media as a medium for growing bacteria, actinomycetes, and fungi at seven days. Bacteria, actinomycetes, and fungi that have potential as antagonistic agents - characterized by the formation of a clear zone around them both on TSA and MA media - were transferred into both media for use in antagonism testing against pathogens which were associated with ramie stem rot and leaf spot. In addition, these isolates were also used for identification. Initial identification is based on morphological characters, while molecular identification will be carried out at Indonesian Culture Collection (InaCC), Indonesian Sciences Institute (LIPI).

**Antagonism test of soil microbial against fungi associated with basal stem rot and leaf spot**
The antagonism test was carried out by using the dual culture method. Both pathogenic fungi and antagonistic microbes were grown on two edges of the media with a distance of 3 cm each from the edge. The growth of pathogenic fungi was measured every day for seven days to calculate the percentage of inhibitory power possessed by antagonistic microbes as follows [7]:

\[
\text{Inhibition (\%)} = (1 - \text{(growth of tested pathogenic fungi/control growth)}) \times 100\%
\]
3. Results and discussion
The results of soil microbial isolation from 14 samples collected from ramie plantations showed a symptom of basal stem rot disease and plantation with healthy ramie plants obtained fungi, bacteria, and actinomycetes with a total population of 0.19-4.89x10^3, 3.93-9.57x10^3, and 1.07-8.67x10^3 cfu/g soil, respectively (Table 1). This microbial population is classified as a low population because, in general, the soil microbial population could reach 10^10 cfu/g soil [8]. This low population may be partly due to the continuous application of ramie cultivation for many years. [9] suggested that continuous planting of plants has the potential to have negative effects on soil health, both those related to biotic and abiotic aspects, soil fertility, and the product of cultivated plants. The biotic indicators that are affected can be in the form of composition, abundance, diversity, the role of soil micro and macro-organisms, enzyme activity, and the interaction of the food chain in the soil.

Table 1. Total populations of microbes in 14 soil samples collected from ramie plantation soil in Wonosobo, Central Java.

| Sample Number | Total population of soil microbes (cfu x 10^3 per g soil) |
|---------------|---------------------------------------------------------|
|               | Fungi | Bacteria | Actinomycetes |
| 1             | 0.62  | 9.53     | 0.00          |
| 2             | 0.28  | 7.43     | 0.00          |
| 3             | 3.27  | 4.97     | 0.00          |
| 4             | 0.36  | 7.00     | 8.67          |
| 5             | 0.29  | 5.33     | 2.33          |
| 6             | 1.33  | 5.20     | 2.33          |
| 7             | 2.83  | 6.83     | 2.33          |
| 8             | 4.89  | 7.63     | 3.00          |
| 9             | 0.44  | 4.33     | 4.00          |
| 10            | 0.99  | 8.43     | 5.67          |
| 11            | 0.69  | 6.37     | 2.33          |
| 12            | 1.68  | 9.57     | 1.07          |
| 13            | 0.32  | 3.93     | 0.00          |
| 14            | 0.19  | 4.67     | 0.00          |

From the screening for antagonistic characters of the isolated-soil microbes, we obtained 13 types of microbial antagonists, namely 11 types from the bacterial group and two types from the fungal group (Table 2). The indication of the antagonistic character of bacteria and fungi was shown by the presence of a clear zone around the antagonistic microbes on the agar medium (Figure 1). The two groups of microbes were further isolated and stored for antagonistic tests against the pathogens associated with basal stem rot and leaf spot diseases. Molecular identification for antagonistic microbes will be carried out at the Indonesian Culture Collection (InaCC). Identification of these antagonistic bacteria and fungi based on their morphological characters is presented in Table 2. Based on the morphological characters, we assumed that the antagonistic bacteria and fungi obtained were *Bacillus* spp. and *Trichoderma* spp., respectively.
Table 2. Potential microbes as biological agents isolated from 14 soil samples in ramie fields at Wonosobo, Central Java, and their morphological characters.

| Antagonistic microbes | Morphological characters                                      |
|-----------------------|---------------------------------------------------------------|
| FA2\(^1\)             | Fungal mycelia green color, fast growth                       |
| FA3                   | Fungal mycelia yellow color, fast growth                      |
| BA1\(^2\)             | Cream color, uneven edges, not slimy                          |
| BA2                   | Cream color, uneven edges, not slimy                          |
| BA3                   | Cream color, flat edge, slightly slimy                        |
| BA4                   | Cream color, uneven edges, not slimy                          |
| BA5                   | Cream color, uneven edges, slightly slimy                      |
| BA6                   | Cream color, uneven edges, slightly slimy                      |
| BA7                   | Cream color, flat edge, slightly slimy                        |
| BA8                   | Cream color, uneven edges, slightly slimy                      |
| BA9                   | Cream color, uneven edges, slightly slimy                      |
| BA10                  | Cream color, uneven edges, slightly slimy                      |
| BA11                  | Cream color, flat edge, slightly slimy                        |

1 FA: Putative fungal antagonist
2 BA: Putative bacterial antagonist

![Figure 1](image)

Figure 1. The formation of a clear zone (blue arrow) around the bacteria indicates bacterial antagonist character.

The bacterial isolates were cultured on TSA agar medium. The antagonistic bacterial isolates obtained were then tested for their antagonism ability against pathogens associated with basal stem rot disease. Bacterial and fungal isolates were tested for their antagonistic ability against ramie leaf spot pathogens. The test results showed that there were three bacterial antagonists, namely BA3, BA9, and BA11 which showed inhibition ability by 24.74%, 10.78%, and 10.78%, respectively, against fungi associated with basal stem rot disease (Table 3). For leaf spot disease, the inhibition ability of BA3, BA9, and BA11 was 49.44%, 31.67%, and 29.17%, respectively, and the inhibition of FA1 and FA2 was 66.67% and 73.33%, respectively (Table 3).

The inhibitory ability of microbial antagonists against pathogens classified into three groups, i.e., low (inhibition zone <10 mm), moderate (inhibition zone 10-15 mm), and high (inhibition zone > 20 mm) [10]. Based on these groupings, the potential inhibition of BA9 and BA11 was classified as
moderate against stem rot fungi but high against leaf spot fungi. The other antagonistic microbes, BA3, FA1, and FA2, have high inhibitory ability, both against stem rot fungi and leaf spot diseases. This indicates that the antagonistic microbes originating from ramie fields (indigenous microbes) could be developed as biocontrol agents, especially for pathogenic microbes associated with basal stem rot and leaf spot diseases and are likely to be used in disease-causing pathogens in other crops.

**Table 3.** Inhibition ability of antagonistic bacteria and fungi against fungi that cause basal stem rot and leaf spot disease on ramie.

| Isolate Code | Inhibition ability (%) on fungi associated with | Basal stem rot disease | Leaf-spot disease |
|--------------|---------------------------------------------|------------------------|------------------|
| BA3          |                                             | 24.74                  | 49.44            |
| BA9          |                                             | 10.78                  | 31.67            |
| BA11         |                                             | 10.78                  | 29.17            |
| FA1          | Not available                               |                        | 66.67            |
| FA2          | Not available                               |                        | 73.33            |

There are several antagonism mechanisms possessed by microbes in the process of inhibiting pathogens, namely competition, parasitation, and antibiosis. Based on the results of our antagonism testing, bacteria and fungi have the possible ability for antibiosis and to compete with pathogenic growth, respectively (Figures 2a and 2b). Microscopic observation of the fungal hyphae of stem rot disease showed malformations, i.e., swelling of the hyphae in the fungal mycelia that grew towards the bacteria, while in the control treatment, the fungus grew normally (Figures 3a and 3b). This indicated an antibiotic reaction in which bacteria produced certain antibiotic compounds that could inhibit fungal growth. The presence of swelling hyphae indicated that the hyphae did not develop normally [11]. Among the groups of antagonistic bacteria that can produce antibiotics is *Bacillus cereus* UW85 [12]. The antibiotics produced are zwittermicin A and antibiotic B, each of which plays a role in inhibiting elongation and causing inflammation of the buds of the *Phytophthora medicaginis*, so that these two antibiotics play a role in the process of suppressing root rot disease in legumes [12]. The mechanism of hyphae degradation by antagonistic bacteria was described by [13] in antagonistic bacteria *Fungivore collimonas* which has the ability to produces secondary metabolites and chitinase to damage the fungal cell walls of *Aspergillus niger*.

![Figure 2](image_url)

**Figure 2.** Inhibition of fungal growth in areas exposed to antagonistic bacteria (a); The occurrence of growing competition between antagonistic fungi and leaf spot fungi (b).
Figure 3. The occurrence of malformations in fungal hyphae associated with pathogen of stem rot disease (3a); Fungal hyphae in control showed normal hyphal formation (3b).

The mechanism of antagonism of *Trichoderma* spp. against leaf spot pathogenic fungi could be competition and also parasitism. This is indicated by the faster growth of *Trichoderma* spp. in the medium than the pathogenic fungi. In the competition mechanism, *Trichoderma* spp. have the ability to rapidly utilize nutrients and space available in the media so as to inhibit the growth of pathogens. [14] reported on the existence of competition, mycoparasitism, and antibiosis in the antagonism process of *Trichoderma* spp. against *Fusarium solani* as indicated by the faster growth of *Trichoderma* spp. than *F. solani* in agar medium. Microscopic observations also showed the presence of mycoparasitism, viz. *Trichoderma* hyphae penetrated *F. solani* hyphae, causing hyphal cells to die, as *Trichoderma* absorbs the nutrients in these cells. Hyphae of *Trichoderma* spp. can also wrap around the hyphae of *F. solani*, causing deformation of the hyphae and damaging the structure of the cell wall. In addition, *Trichoderma* spp. also produce various types of enzymes such as 1.3 glucanase, chitinase, protease, and cellulase, which might degrade the cell walls of pathogenic fungi that are mostly composed of chitin and glucan [15].

4. Conclusion
Exploration of soil-biota microbes in ramie field has obtained indigenous antagonist microbes that have a great opportunity to be used in biological plant-disease-management systems for agricultural, horticultural, and plantation crops. There are three bacteria (BA3, BA9, and BA11) that are potential to be developed as antagonist agents to basal stem rot. In addition, we did also obtain three bacteria (BA3, BA9, and BA11) and two fungi (FA1 and FA2) perform antagonistic activity against leaf spot disease.

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