Histologic Observation, Identification and Secondary Metabolites Analysis of Endophytic Fungi Isolated from *Cananga odorata* (Lam.) Hook. F. & Thomson

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Abstract. The endophytic fungi could be isolated from the plant host to observed. The purpose of this research is to: 1) observe the endophytic fungi location in the *C. odorata* plant tissue; 2) identify each endophytic fungi isolated from *C. odorata* leaf, twig, and flower petal; 3) analyze the secondary metabolites contents of each endophytic fungi species. The location of endophytic fungi in the plant tissue observed by microscopic observation. The endophytic fungi were isolated from the leaf, twig, and flower petal of *C. odorata* plant by cutted these plant parts, then inoculated on Potato Dextrose Agar (PDA) medium and incubated in 27°C during 7 x 24 hours, each fungi were isolated, then the morphology and microscopic characteristic of each fungi colony isolates were described and identified. Each endophytic fungi isolates inoculated in Potato Dextrose Broth medium and shaked in 120 rpm during 7x24 hours, afterwards the secondary metabolites content were analyze using spectrophotometric method. The research result were: 1) the endophytic fungi location are on the epidermis cell wall and on the stomata guard cell wall of the leaf tissue, on the parenchymal cell wall of the twig tissue, and on the epidermis cell wall of the flower petal tissue; 2) Nine endophytic fungi species isolated from the *C. odorata* are: *Nigrospora sphaerica*, *Colletotrichum alienum*, *Mycellia sterilia* 1, *C. kahawae*, *Rhizoctonia* sp., *C. aotearoa*, *Micellia sterilia* 2, *C. alatae*, and *C. queenslandicum*. 3) the secondary metabolites contents produce by each endophytic fungi species: flavonoid (654 to 1793.13 mg kg⁻¹), tannin (29.36 to 52.21 mg kg⁻¹), saponin (2.31 to 3.35 mg kg⁻¹), alkaloid (23.05 to 40.28 mg kg⁻¹), and terpenoid (85.53 to 155.48 mg kg⁻¹).

Keywords : endophytic fungi, *Cananga odorata*, histologic observasion, identification, secondary metabolites

1. Introduction

*Cananga odorata* flower is oftenly harvested because of their sweet scent and in the traditional ritual ceremonies. The essential oil extracted from the flower have used mainly in perfume industry and aromatherapy. This plant extract contains some chemicals including: monoterpen, sesquiterpen, and phenylpropanoids [1]. The cananga plant extract could be used in some disease treatment, because it contains microbial compounds.

There is a symbiotic mutualism relationship between endophytic fungi and the host plant, i.e medicinal plants. The endophytic fungi produce some antimicrobial secondary metabolites that could protect the host plant from parasitic bacteria infection. On the other hand, the endophytic fungi get some
advantages from the host plant, i.e: nutrition, protection from environmental stress, and growth place [2][3]. The histologic observation have been done to observe the endophytic fungi location in their host plant tissue. The location of endophytic fungi in some plant parts based on the previous research result, i.e: in the leaf, bark, and twig tissue [4][5][6]. The observation result proved that the fungi mycelium found in epidermis cell wall of twig, root, and leaf of *Talinum paniculatum*[7]. In another previous research shows that the endophytic fungi mycelium was found on rhizome parenchyma cell wall and intercellular space, on the leaf sheath epidermis cell wall, and on the leaf epidermis cell wall of *Hedychium acuminatum* plant [8]. Some endophytic fungi isolated from *H. acuminatum*, i.e: *Rhizoctonia* sp., *Colletotrichum alienum*, *C. aotearoa*, *C. ti*, *C. coccodes*, *C. gloeosporioides*, *Fusarium solani*, *F. oxysporum*, *F. semitectum*, *Aspergillus parasiticus*, and *Hansfordia biophyla* could produces alkaloid, flavonoid, terpenoid, and tannin as well as *H. acuminatum* plant could produce. Based in this fact, the antimicrobial secondary metabolites could be obtained from the fungi liquid culture. People does not need to take the plant parts for take the antibacterial secondary metabolites as an antibiotic source. This research purpose are: 1) observe the endophytic fungi location in the *C. odorata* plant tissue; 2) identify each endophytic fungi isolated from *C. odorata* leaf, twig, and flower petal; 3) analyze the secondary metabolites contents of each endophytic fungi species.

2. Material and Methods

2.1. Material

*Canna odorata* plant parts: leaf, twig and flower petal, *Potato Dextrose Broth*, *Potato Dextrose Agar*, NaOCl 1%, alcohol 70%, chloramphenicol, lactophenol, and lactophenol cotton blue.

2.2. Method

2.2.1. Preparation of *C. odorata* Leaf, Twig and Flower Petal for Endophytic Fungi Location on Plant Tissue Microscopically Observation. The leaf, twig and flower petal of *C. odorata* were rinse in sterile destilled water, afterwards made microscopic slides by paradermal and longitudinal section. The endophytic in the *C. odorata* plant tissue location were observed microscopically.

2.2.2. Isolation and Identification of Endophytic Fungi. The *C. odorata* plant parts: leaf, twig, and flower petal were washed and soaked in NaOCl 1% for 1 minute, then washed with sterile destilled water. Then soaked in alcohol 70% for 1 minute and washed with sterile destilled water [9]. The flower petal and leaf were cutted in the thickness of 1x1 cm2 each, the twig were cutted in the thickness of 0.5 cm each. Each samples were inoculated on PDA medium contains chloramphenicol (100 mg/L), then incubated in 25°-27°C for 7 x 24 hours. Each endophytic fungi sorts isolated on PDA slant medium, then incubated in 25°-27°C for 3x24 hours for colony morphological observation. Afterwards each isolates were made slide cultures for microscopic observaton. Each isolates were identify.

2.2.3. Endophytic Fungi Liquid Culture Preparation. The secondary metabolites could be obtained from endophytic fungi liquid culture [10]. The endophytic fungi colony on PDA plate medium were cut 5x1cm in size, then put into 80 ml *Potato Dextrose Broth* (PDB) medium, incubated in 25°-27°C during 7 x 24 hours and shaken in 120 rpm rate at the same time. Then the liquid were centrifugated in 3000 rpm rate during 10 minutes. The supernatant were used to detect the content of secondary metabolites, i.e: alkaloid, flavonoid, terpenoid, and tannin produced by each endophytic fungi isolates.

2.2.4. Detection of Secondary Metabolites in The Liquid Culture of Each Endophytic Fungi. The alkaloid, flavonoid, terpenoid, and tannin contents in PDB medium were analyzed by spectrophotometry method at Chemistry Laboratory, Muhammadiyah University Malang.
3. Results and Discussion

3.1. The Histologic Location of Endophytic Fungi on C. odorata Leaf, Twig and Flower Petal

The endophytic fungi location in C. odorata tissue was observed microscopically. The endophytic fungi species were found in leaf, twig and flower petal tissue of C. odorata. The fungi mycelium was found in leaf epidermis cell wall and stomatal guard cell of leaf (fig 1a and 1b), in parenchyma cell wall of twig (fig 1c), and in epidermis cell wall of flower petal (fig 1d).

The mycelium does not make any damage to the plant tissue, there are no mycelium penetrate into cell wall nor cytoplasm. The fungi only take nutrition for their life in intercellular space of the plant tissue.

![Figure 1. Photomicroscopic of Endophytic Location in C. odorata Tissue.](image)

The endophytic fungi location in the host plant tissue observed microscopically by paradermal and transversal section. In some previous research, the mycelium found on epidermis, cortex, xylem, and floem tissue of Stevia rebaudiana (Bert) plant parts, i.e: root, stem, and leaf; [11]. The endophytic fungi mycelium also found on the epidermis cell, palisade cell, parenchymal sponda cell, xylem and phloem tissue of Azadirachta indica stem and leaf [12].

The histologic observation result show that the endophytic fungi on C. odorata found on the leaf, twig and flower petal. Although it is found the endophytic fungi, there is no damage effect on the C. odorata plant tissue. This fact proved the mutualism symbiotic interaction between the host plant and endophytic fungi.

3.2. The Endophytic Fungi Identification Result

The endophytic fungi isolation and identification result proved that it was found nine endophytic fungi species from C. odorata plant: Nigrospora sphaerica, Colletotrichum alienum, Mycelia sterilia 1, C. kahawae, Rhizoctonia sp., C. aotearoa, Mycelia sterilia 2, C. alatae, and C. queenslandicum (Fig 2).
**Figure 2.** The Light Micrograph of Each Endophytic Fungi Isolated from *C. odorata* leaf, twig and flower petal. (A) *Nigrospora sphaerica*, (B) *Colletotrichum alienum*, (C) *Mycelia sterilia* 1, (D) *Colletotrichum kahawae*, (E) *Rhizoctonia* sp., (F) *Colletotrichum aotearoa*, (G) *Mycelia sterilia* 2, (H) *Colletotrichum alatae*, (I) *Colletotrichum queenslandicum*. Notes: red arrow= conidia; yellow arrow = appresorium; green arrow= sclerotium.

Each endophytic fungi species are not specifically found in leaf, twig and flower petal parts. Some species found in leaf as well as in flower petal. The characteristics of these endophytic fungi species described in Table 1.

Table 1 shows the characteristics of each endophytic fungi species, five *Colletotrichum* spp. where found in *C. odorata*. *Colletotrichum* spp were also found as endophytic fungi in another plant, in *Piper nigrum* and *Michelia champaca* plant [13][14]. *Colletotrichum* spp. also found in *Hedychium acuminatum* [8]. *Colletotrichum* spp. have variation in conidia shape, i.e: cylindrical, cylindrical with round edge, ellipse. This fungi have appresorium with specific shape, i.e: cylindrical with round edge or irregular shape. *Mycelia sterilia* is oftenly found as endophytic fungi in Chinese medical plant in 27 from 29 sorts of medicinal plant were found *Mycelia sterilia* [15]. This fungi have a specific colony characters, the colony colour is brownish white with reverse colours is brownish white or greyish white, the texture is cotony or velvety. *Nigrospora sphaerica* is one of this endophytic fungi in *C. odorata*. This fungi is also found in *Catharanthus roseus*, a sort of medicinal plant [16].
Table 1. The Characteristics of Endophytic Fungi Species from *C. odorata* Plant Parts

| Species                  | Isolat Code | The Plant Part | Colony Morphology                                      | Vesicle (shape, size) | Conidia (shape, size) | Appresorium (shape, size) | Sclerotium (shape, size) |
|--------------------------|-------------|----------------|--------------------------------------------------------|------------------------|------------------------|---------------------------|--------------------------|
| *Nigrospora sphaerica*   | A           | Flower petal   | Greyish-white, reverse: grey-black, cottony           | Flat, 5 µm             | Globose, rough walled, 3 µm | -                         | -                        |
| *Colletotrichum alienum* | B           | Leaf, flower petal | Greish white, reverse: light yellow, cottony         | -                      | Cylindrical, smooth, 9-15 µm x 3-5 µm | Cylindrical with round edge, 12.5 µm x 7.5 µm | -                        |
| *Mycelia sterilia*       | C           | Twig, flower petal | Brownish-white, reverse: brownish-white, cottony     | -                      | -                      | -                         | Diam. 45-120 µm           |
| *Colletotrichum kahawae* | D           | Leaf           | Brownish-white, reverse: blackish-brown, cottony     | -                      | Cylindrical, smooth, 10-15 µm x 2-3 µm | Irregular, 9 µm x 10 µm    | -                        |
| *Rhizoctonia sp.*        | E           | Leaf           | Black, reverse: black, powdery                       | -                      | -                      | -                         | Diam. 37.5-50 µm          |
| *Colletotrichum aorearoa*| F           | Leaf, flower petal | Blackish white-pink, reverse: blackish pink, velvety | -                      | Cylindrical with round edge, smooth, 12.5-15 µm x 3.75-5 µm | Irregular, 10 µm x 7.5 µm | -                        |
| *Mycelia sterilia*       | G           | Leaf, twig, flower petal | Brownish-white, reverse: grey, velvety       | -                      | -                      | -                         | Diam. 20-100 µm           |
| *Colletotrichum alatae*  | H           | Leaf, flower petal | Blackish-orange, reverse: greyish orange, velvety   | -                      | Cylindrical, rough, 10-25 µm x 3-5 µm | -                         | -                        |
| *Colletotrichum queenslandicum* | I            | Leaf, twig, flower petal | Blackish-white, reverse: brownish-black, cottony | -                      | Cylindrical, 7-5 µm x 3 µm | Irregular, 10 µm x 5 µm | -                        |

3.3. Secondary Metabolites Content of Each Endophytic Fungi Species Isolates from *C. odorata* Plant Parts

The analysis result shows that each endophytic fungi species liquid culture contents: flavonoid, tannin, saponin, alkaloid, and terpenoid. It is proved that each of nine endophytic fungi species could produce these secondary metabolites compounds. There are differences of secondary metabolites compound content produced by each endophytic fungi species (Table 2).
Table 2. The Secondary Metabolites Content of The Endophytic Fungi in C. odorata Leaf, Twig, and Flower Petal Liquid Culture

| Isolate Code | Species                        | Secondary Metabolite (mg. kg⁻¹) |
|--------------|--------------------------------|---------------------------------|
|              |                                | Flavonoid        | Tannin        | Saponin   | Alkaloid   | Terpenoid  |
| A            | Nigrospora sphaerica           | 1666.56          | 49.59         | 3.17      | 36.75      | 148.25     |
| B            | Colletotrichum alienum         | 1002.50          | 36.29         | 2.60      | 27.99      | 106.80     |
| C            | Mycellia sterilia 1            | 1654.06          | 29.36         | 2.31      | 23.05      | 85.53      |
| D            | Colletotrichum kahawae         | 1261.88          | 41.47         | 2.82      | 32.34      | 124.34     |
| E            | Rhizoctonia sp.                | 785.31           | 31.99         | 2.36      | 24.28      | 87.06      |
| F            | Colletotrichum aotearoa        | 1411.88          | 44.53         | 2.97      | 35.44      | 133.11     |
| G            | Mycellia sterilia 2            | 838.44           | 33.17         | 2.44      | 25.22      | 94.96      |
| H            | Colletotrichum alatae          | 1793.13          | 52.21         | 3.35      | 40.28      | 155.48     |
| I            | Colletotrichum queenslandicum  | 1502.50          | 46.28         | 2.98      | 35.46      | 134.43     |

Each endophytic fungi isolated from C. odorata leaf, twig, and flower petal could produce flavonoid, tannin, saponin, alkaloid, and terpenoid. Colletotrichum alatae could produce all compound in the highest content compare with the other fungi species from C. odorata. C. alatae also found as one of the endophytic fungi isolate from leave and terminal branches of avocado (Persea americana Mil.) in the previous research [19]. It is need to make the next research about C. alatae liquid culture an antibiotic source specifically in antimicrobial effect.

Flavonoid, tannin, saponin, alkaloid, and terpenoid could be used as antimicrobe and antioxidant [10][17][18]. This research proved that nine endophytic fungi species isolated from C. odorata leaf, twig, and flower petal still produce some secondary metabolite although these fungi have been separated from the host plant tissue.

Based on this research, each endophytic fungi liquid culture could be used as the antimicrobial secondary metabolite source. This is another procedure to obtain the antimicrobial secondary metabolite from the endophytic fungi liquid culture, besides by extraction C. odorata plant parts as one of medicinal compound producer.

4. Conclusion
The conclusion are: 1) the endophytic fungi location are on the epidermis cell wall and on the stomata guard cell wall of the leaf tissue, on the parenchymal cell wall of the twig tissue, and on the epidermis cell wall of the flower petal tissue; 2) Nine endophytic fungi species isolated from the C. odorata are: Nigrospora sphaerica, Colletotrichum alienum, Mycellia sterilia 1, C. kahawae, Rhizoctonia sp., C. aotearoa, Micella sterilia 2, C. alatae, and C. queenslandicum. 3) the secondary metabolites contents produced by each endophytic fungi species: flavonoid (654 to 1793.13 mg · kg⁻¹), tannin (29.36 to 52.21 mg · kg⁻¹), saponin (2.31 to 3.35 mg · kg⁻¹), alkaloid (23.05 to 40.28 mg · kg⁻¹), and terpenoid (85.53 to 155.48 mg · kg⁻¹).
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