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Isolation and Identification of Endophytic Fungi from Leave and Stem of *Calopogonium mucunoides*

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A B S T R A C T

Thirty-seven isolates of endophytic fungi were isolated from leaves and stems of *Calopogonium mucunoides* collected from PTPN PTPN XII (Persero) Rubber Plantation, Klatakan, Kecamatan Tanggul, Kabupaten Jember, Jawa Timur. All isolates were identified based on morphological characteristics using the light microscope. The 37 isolates of endophytic fungi are members of Deuteromycota and Basidiomycota and classified to genera *Phoma, Phomopsis, Corynespora, Rhizoctonia, Helicosporium, Curvularia, Torulomyces, Gliocladium, Gloeosporium, Acremonium, Tripospermum, Aureobasidium, Colletotrichum, Humicola, Fusarium, Sclerotium*, and sterile hyphae.

1. Introduction

Endophytic fungi, also called plant hidden fungi, defined as fungi which part or all of their life cycle living naturally in the plant tissues without causing disease symptoms. Endophytic microbes are microbes that live inside plant tissues. Endophytic fungi are microorganisms most commonly found in plants (Petrini, 1991; Dreyfuss & Chapela, 1994). Endophytic fungi can be isolated from the leaves, stems, flowers, fruits, and seeds (Roza et al. 2011). The interaction between the fungal endophyte with plant involves a balance of antagonism (Schulz and Boyle, 2005). Endophytic fungi are one of the interesting microbial symbionts with a plant that give benefit for their host's defence from natural enemies (Faeth & Fagan, 2002). The presence of endophytic fungi increases the plant's defence system against environmental stress both abiotic and biotic. Endophytic fungi can become a saprophyte when host plants dying (Rodriguez et al. 2008). Some endophytic fungi produced toxic substances that can protect host plants from plant pathogens, insects, nematodes, and herbivores (Mousa et al, 2013).

In this study, we used *Calopogonium mucunoides*. This plant is a member of the family Fabaceae (Binggeli, 1996). *C. mucunoides* known to be a potential pharmacological plant because of their ability to produce secondary metabolites that can be used as a potential alternative medicine for treating skin diseases, diarrhoea, and antiulcer (Idu and Oniye, 2007; Enechi et al. 2013). The plant able to produce allelopathic which prevents weed growth on the plantation (Sihombing et al. 2012). Beside that, the plant also used as an agent of phytoremediation to clean metal contaminants in the gold mining waste (Hidayati *et al.* 2005). The purpose of this study is to identify the endophytic fungi from the leaves and stems of plants *C. mucunoides* based on morphological characters as a preliminary study to determine the diversity of fungi found in the leaves and stems of plants *C. mucunoides*.

2. Materials and methods

2.1. Isolation and culturing of endophytic Fungi

The isolation of fungi was performed according to Fernandez et al. (2011), Schulz et al. (1993), Katoch et al. (2014) and Buatong (2010) with modifications. *Calopogonium mucunoides* collected from PTPN XII (Persero) Rubber Plantation, Bagian Klatakan, Kecamatan Tanggul, Kabupaten Jember, Jawa Timur. The fresh leaves and stem parts were used for isolation of endophytic fungi. The plant samples were stored in the sealed plastic bags at 4°C until processed.

Healthy mature leaves and stems of *C. mucunoides* were washed thoroughly under running tap water, then the samples were sterilized by dipping them in 75% ethanol for 30 s, followed by immersing in 3 % sodium hypochlorite for several times, rinsed in sterile distilled water, and finally dried on sterile filter paper on petri dish. A piece of each leaves and stems were removed with a sterile scalpel then cut into small pieces about 1 to 1.5 cm, each piece was put on a Petri plate containing Potato Dextrose Agar (PDA) medium and incubated at room temperature (27-280C) to promote fungal growth and sporulation. After 7-8 days, individual hyphal tips of actively growing fungi were picked up for subculturing by

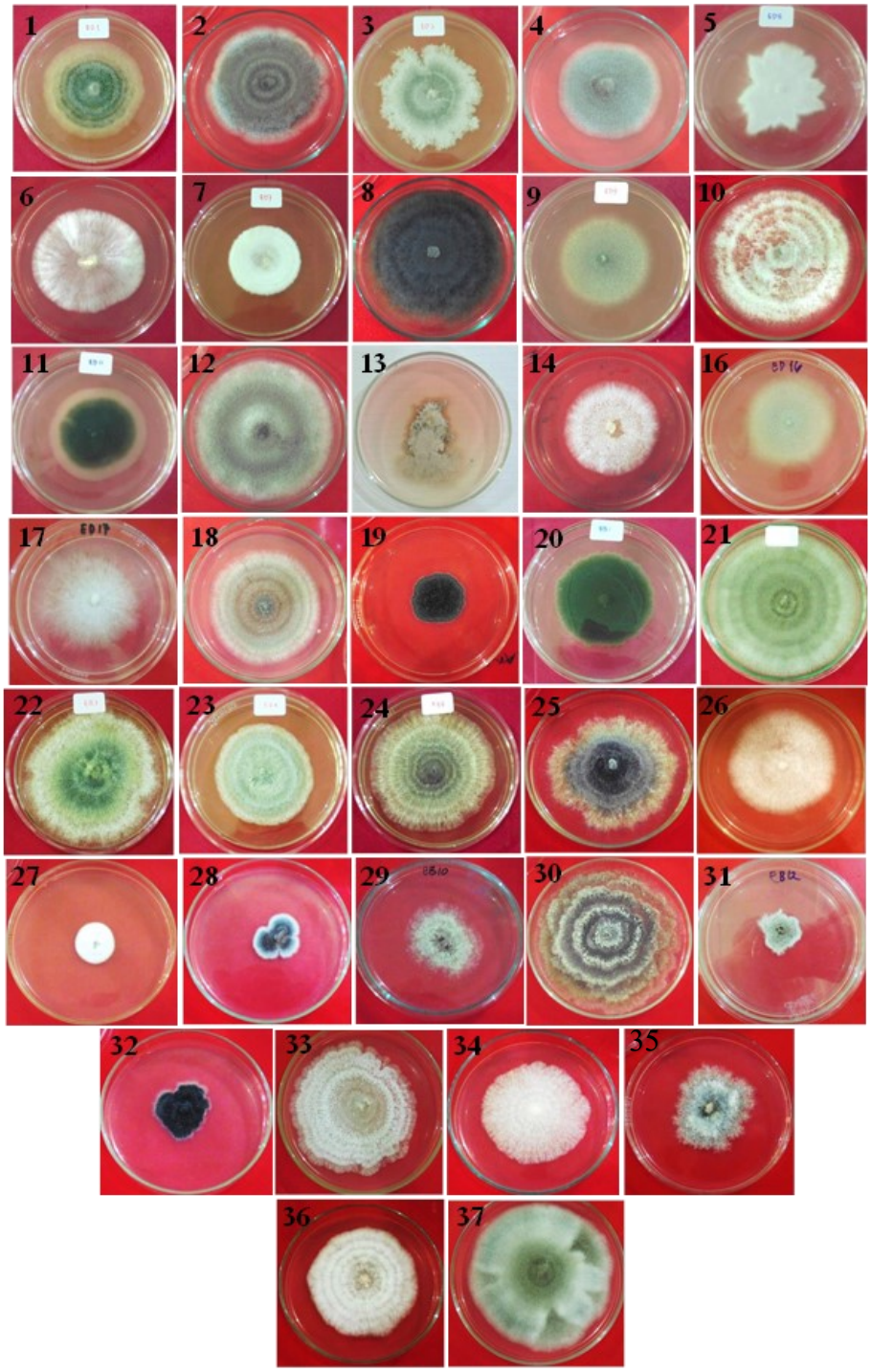

Figure 1. Colony morphology variations of endophytic fungi from *Calopogonium mucunoides* on PDA at day seven. Number 1 to 19 from leave (isolat 1:ED1, isolat 2:ED2, isolat 3:ED3, isolat 4:ED4, isolat 5:ED5, isolat 6:ED6, isolat 7:ED7, isolat 8:ED8, isolat 9:ED9, isolat 10:ED10, isolat 11:ED11, isolat 12:ED12, isolat 13:ED13, isolat 14:ED14, isolat 15:ED15, isolat 16:ED16, isolat 17:ED17, isolat 18:ED18, isolat 19:ED19). Number 20 to 37 from stem (isolat 20:EB1, isolat 21:EB2, isolat 22:EB3, isolat 23:EB4, isolat 24:EB5, isolat 25:EB6, isolat 26:EB7, isolat 27: EB8, isolat 28: EB9, isolat 29: EB10, isolat 30:EB11, isolat 31:EB12, isolat 32:EB13, isolat 33:EB14, isolat 34:EB15, isolat 35:EB16, isolat 36:EB17, isolat 37:EB18)

inoculating it onto new PDA medium plate individually and incubated at room temperature (27ºC) for one week. The purified fungal isolates were labelled for further use.

2.2. Morphological identification

Identification of endophytic fungal isolates was done by observing trypan blue stained slides prepared from stock cultures using a bright-field and phase contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, the colour of the colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and conidial characteristics. Obtained data were then compared with the descriptions of endophytic fungi and identified based on Selim et al. (2012) and Zuber (2011).

3. Results

A total of 25 fragments leaves and stem was used from *C. mucunoides*. In this study, a total of 37 endophytes were isolated (Figure 1). The endophytes were isolated using potato dextrose agar (PDA, Merck). The total numbers of colonies of endophytic fungi from the stem are lower than the ones from leaves.

Based on morphological characteristics identification (Figure 2 and 3), almost all endophytic fungi which were isolated are members of Deuteromycota group (Table 1). *Phoma* sp., *Acremonium* sp., *Phomopsis* sp., *Corynespora* sp., *Rhizoctonia* sp., *Aureobasidium* sp., *Colletotrichum* sp., *Gloeosporium* sp., *Humicola* sp., *Tripospermum* sp., and *Sclerotium* sp. were isolated from the leave samples. From stem samples, the fungi are *Curvularia* sp., *Colletotrichum* sp., *Humicola* sp., *Acremonium* sp., *Torula* sp., *Tripospermum* sp., *Fusarium* sp., *Helicosporium* sp., and *Gliocladium* sp. Other fungi identified by the presence of clamp connections are the member of the Basidiomycota. The mycelia sterillia group were found to be the highest occurrence.

4. Discussion

Identification of fungi was done based on Selim et al. (2012) and Zuber (2011) also by using standard protocol of Barnett and Hunter (1972). Identification is based on morphological characteristics of the fungi grown on the culture medium (PDA). The morphology including macroscopic and microscopic characteristics. The

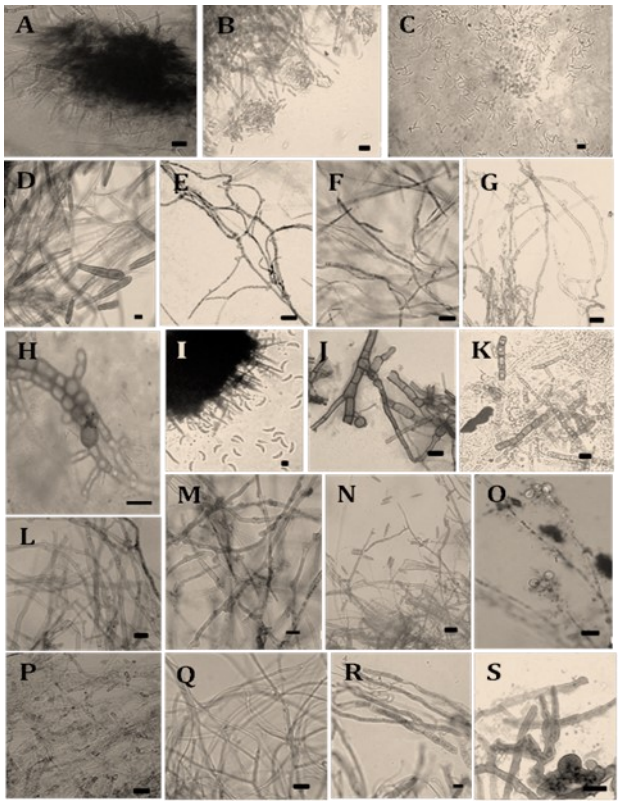

Figure 2. Light microscopic photographs of endophytic fungi from *Calopogonium mucunoides* leaves. A: *Phoma* sp., B: *Acremonium* sp., C: *Phomopsis* sp., D: *Corynespora* sp., E:*Sclerotium* sp., F:Sterile hypha, G: *Sclerotium* sp., H:*Aureobasidium* sp., I: *Colletotrichum* sp., J: *Rhizoctonia* sp., K: *Aureobasidium* sp., L: *Sclerotium sp*., M: *Sclerotium sp*., N:*Gloeosporium* sp., O: *Humicola* sp., P: Sterile hyphae, Q: Sterile hyphae, R: Sterile hyphae, S: *Tripospermum* sp. Bar: 10µm.

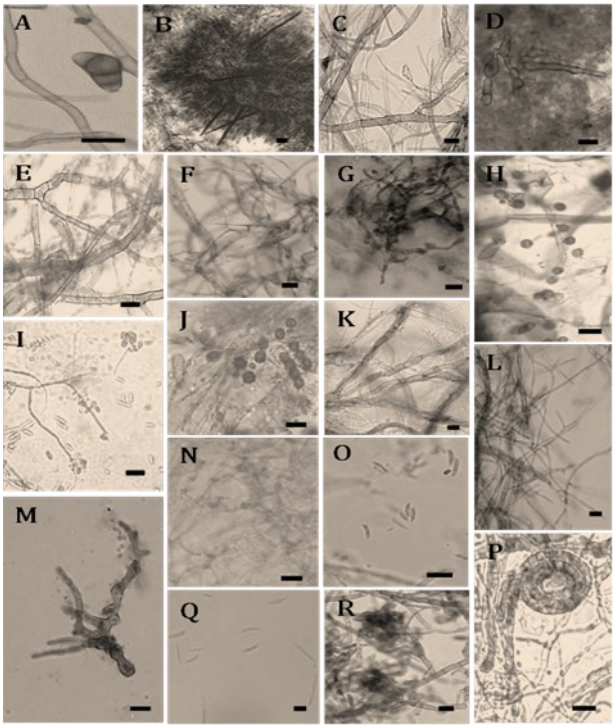

Figure 3. Light microscopic photograps of endophytic fungi from *Calopogonium mucunoides* stems. A: *Curvularia* sp., B: *Colletotrichum* sp., C: Sterile hyphae, D:unditified, E: Sterile hyphae, F: Sterile hyphae, G: Sterile hyphae, H: *Humicola* sp., I: *Acremonium* sp., J:*Torula* sp., K: Sterile hyphae, L: Sterile hyphae, M: *Tripospermum* sp., N: Sterile hyphae, O: *Fusarium* sp., P: *Helicosporium* sp., Q: *Fusarium* sp., R: *Gliocladium* sp. Bar 10µm.

Table 1. Endophytic isolates from *C. mucunoides*

Phylum	Leaves	Stems
Deuteromycota	*Phoma* sp. *Acremonium* sp. *Phomopsis* sp. *Corynespora* sp. *Rhizoctonia* sp. *Aureobasidium* sp. *Colletotrichum* sp. *Gloeosporium* sp. *Humicola* sp. *Tripospermum* sp. Mycelia sterile	*Curvularia* sp. *Colletotrichum* sp. *Humicola* sp. *Acremonium* sp. *Torula* sp. *Tripospermum* sp. *Fusarium* sp. *Helicosporium* sp. *Gliocladium* sp. Mycelia sterile
Basidiomycota	*Sclerotium* sp.	-

macroscopic identification of colony such as colour, diameter, colony growth, colony reverse, The observation was done for seven days during the fungal culturing.

Microscopic characterisation was done by observing shape and size of conidia, and hyphae. Observation of conidia including its arrangements (singular, chain or cluster), cell number (unicellular or multicellular), and conidial measurement. Observation of hyphae was also performed on the presence or absence of septa in hypha, its shape, morphology (racquet, nodular, pectinate, spiral, rhizoid, chandler), and modifications of hyphae (rhizoid, stolon, rhizomorph, haustorium, appressorium, chlamydospore, sclerotia). The results obtained were then compared with the literature and monographs.

Based on the results of isolation from leaves and stems of *C. mucunoides*, 37 isolates were obtained (Figure 6). The number of isolates from the leaves was 19 isolates, while from the stems was 18 isolates. The number of obtained isolates is similar to Anita and Sridhar (2009) which obtained 36 isolates of endophytic fungi from the *Canavalia carthatica*, but fewer than Phowtong et al. (2013) which obtained 69 isolates of endophytic fungi from *Sesbania grandiflora* (7 isolates from leaves and 62 isolates from stems). Santos et al. (2015) obtained 65 isolates of endophytic fungi as antibacterials from *Indigofera suffruticosa* Miller.

Identification of fungi can be done conventionally by observing the morphological characters and compare it to descriptions from the literature or monograph. The morphological characters which are observed including microscopic and macroscopic characteristics. Macroscopic observations based on the characteristics of fungal colony on agar medium like the color, texture, reverse side and margines (Gandjar et al. (1999)). Macroscopic and microscopic characteristics of fungal isolates can be observed in the presence or absence of conidia, conidia shape, conidia arrangement, and size, conidiophores, hyphae (septum or singular), and hypha's pigment (hyaline/nonpigmented). But If from the microscopic observation only the teleomorph is found then the fungi classified in the Ascomycota. When the teleomorph of such fungi is found, it is classified in the Ascomycota under a different name. If both types of conidia (teleomorph and anamorph) not found, then the fungi is placed to sterile hyphae group (Gandjar et al., 2006); Sedlář et al., 2009; Pitt and Hocking, 2009).

The results showed that most of fungal isolates were purified belonging to Ascomycota fungi and Basidiomycota fungi, but because it only shows the asexual form during the observation process so that all of fungi isolates belong to the Deuteromycota group. Deuteromycota group is a group of fungi that do not show feature of sexual phase. Ascomycota fungi classification based on asexual reproduction. Large number of this members did not show sexual structure so difficult to determine its class, therefore in practice this group of fungi was placed on Deuteromycota or fungi imperfecti (Alexopoulos et al., 1996). Members of the Ascomycota phyla can show both sexual and asexual phases, but if only found asexual without sexual form then belonging to Deuteromycota (White, 2009). Deuteromycota or fungi imperfecti is a group of fungi which sexual reproduction has not been identified. They are member of Ascomycota or some from Basidiomycota (Venkatesan and Suryanarayanan, 2013). The term of deuteromycota was not used anymore in present, due to the concept of DNA-based classification (Deoxyribose Nucleate Acid). However, some literature still uses this term to group all the fungi not belonging to other divisions (Taylor, 2011; Shenoy, 2007).

The result also discovered 12 fungal isolates that did not show the formation of conidia. These isolates were grouped as mycelia sterile. Isolates belonging to the mycelia sterile group because they did not show the form of anamorph or teleomorph during the observation process. Fungi isolates which were included in mycelia sterile group are *Sclerotium* sp., *Rhizoctonia* sp., and other fungi isolates

that can not identified because they present only a set of hyphae or hyphae with chlamydospores without showing conidia (Barnett and Hunter, 1972).

Previous studies about fungal endophyte from Legumes had been reported. Many endophytes were isolated from both leaves and stems of the plants, such as from Alfalfa (*Medicago sativa*), Soybean (*Glycine max*), *Rhynchosia beddomei*, *Clitoria ternatea*, *Sesbania grandiflora*, *Desmodium* sp., *Centrocema basilianum*, and *Canavalia cathartica* (Roza et al., 2011; Katoch et al., 2014; Ramesha & Srinivar, 2013; Powthong et al., 2012). Results showed that based on the morphological characteristics, the endophytic fungi isolated from legume species classified into Basidomycota, Ascomycota, and Deuteromycota (Dandu et al., 2014; Shankar & Shashikala, 2010; Phowtong et al., 2012; Senna & Sridhar, 2004). Powthong et al. (2013) reported that endophytic fungi isolates from legumes of *Sesbania grandiflora* classified as *Fusarium* sp., *Acremonium* sp., *Phaeoacremonium* sp., *Phomopsis* sp., and *Cladosporium* sp. Marcia González-Teuber et al. (2014) reported that endophytic fungi isolates from *Acacia hindsii* acquired as *Colletothricum* sp., *Fusarium* sp., *Cochliobolus* sp., *Phoma* sp., *Eupenicillium* sp., *Pichia* sp., and *Moesziomyces* sp. Pimentel et al. (2006) reported that endophytic fungi isolates from *Glycine max* classified as *Alternaria* sp., *Cladosporium* sp., *Chaetomium* sp., *Curvularia* sp., *Drecslera* sp., And non-dematiaceous fungi class *Acremonium* sp., *Aspergillus* sp., *Colletothicum* sp., *Fusarium* sp., *Paecilomyces* sp., and *Penicillium* sp., as well as the sterile hyphae.

The presence of an endophytic fungi of a plant depends on the species of plant, environment and isolation methods. Amount of endophytic fungi isolates that found depend on the diversity and distribution of endophytic fungi exist in the host plants. Each part of the plant will give different number of endophytic fungal isolates. The most dominant endophytic fungi present inside plants tissue indicated that the fungi are the most widely distributed species in the plant. This can be known by the percentage of endophytic fungi that have been found from isolation process.

A more realistic approach is needed to characterize the endophyte species from a single host or group of hosts. The number of samples required for the isolation of the endophytic fungus related on the distribution and abundance of fungi in the host plant and the tissue types (foliage, stem, bark, xylem, root). More intensive sampling method will increase the recovery of rare species, which are likely also to occur on many hosts, but the most common species on a specific host will be widely distributed on that host (Stone et al., 2004).

5. Conclusion

A total of 37 endophytes were isolated and assigned to 16 genera based on the morphological characteristics , in which 33 (56,7%) isolates are *Phoma* sp., *Phomopsis* sp., *Corynespora* sp., *Rhizoctonia* sp., *Helicosporium* sp., *Curvularia* sp., *Torulomyces* sp., *Gliocladium* sp., *Gloeosporium* sp., two isolates are *Acremonium* sp., two isolates are *Tripospermum* sp., two isolates are *Aureobasidium* sp., two isolates are *Colletotrichum* sp., two isolates are *Humicola* sp., two isolates are *Fusarium* sp., four (10,8%) isolates are *Sclerotium* sp., and 12 (32,43 %) isolates are mycelia sterile.

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