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Orchid Mycorrhizal Fungi: Identification of *Rhizoctonia* from West Kalimantan

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Orchid is an ornamental plants with high economic value. The excessive exploitation of orchids threatened or even endangered the species, especially those of the epiphytic orchids (*Appendiculla* sp., *Calanthe vestita,* and *Bulbophyllum beccarii*) in West Kalimantan. The discovery of the interaction between orchids and mycorrhizal fungi raises the possibility of ex situ conservation of orchids and it will ensure the success of orchid conservation. Orchid mycorrhizal fungi belongs to the group of *Rhizoctonia*-like, in which comprised of different genus such as *Ephulorhiza, Ceratoriza,* and *Tullasnela.* So far, there is no report on the identit of orchid mycorrhiza associated with the epiphytic orchids in West Kalimantan. The purpose of this study was to identify *Rhizoctonia*-like associated with *Appendiculata* sp., *Calanthe vestita,* and *Bulbophyllum beccarii* roots in the forest of Raya Pasi and Gunung Bawang, West Kalimantan. The methods were isolation and identification of *Rhizoctonia*-like from healthy orchid's root based on their morphological characteristics (such as the colony colour, hyphal cell size, sclerotial, concentric circles and monilioid cell, number of nuclei per cell), observation of peloton in root tissue and grouping of isolates. Based on identification of orchid mycorrhiza on the roots of the three species of orchids from West Kalimantan, it was observed that *Ceratorhiza* sp. was associated with *Appendiculla sp.,Ephuloriza* sp. with *C. vestita,* and *Tullasnela* sp. with *B. beccarii* roots, respectively.This result is preliminary information and it is still need to be further studied, especially on the role of *Rhizoctonia*-liker as orchid mycorrhizal fungi in association with the epiphytic orchid for conservation.

Key words: identification, morphology, orchid, orchid mycorrhizae, *Rhizoctonia*-like

Anggrek merupakan tanaman hias yang memiliki nilai ekonomi tinggi. Eksploitasi anggrek secaraberlebihan menyebabkan terancam jenis-jenis anggrek, khususnya anggrek epifit (*Appendiculla* sp., *Calanthe vestita* dan *Bulbophyllum beccarii*) di Kalimantan Barat. Penemuan adanya interaksi antara anggrek dengan jamur mikoriza, meningkatkan kemungkinan program konservasi anggrek di luat habitatnya dan menjamin keberhasilan konservasi. Jamur mikoriza-anggrek termasuk dalam kelompok *Rhizoctonia*-like, yang terdiri dari genus *Epulorhiza, Ceratoriza,* and *Tulalasnela*. Sejauh ini, tidak ada laporan tentang identifikasi mikoriza-anggrek yang berasosiasi dengan anggrek epifit di Kalimantan Barat. Tujuan penelitian ini adalah mengidentifikasi *Rhizoctonia*-like yang berasosiasi dengan perakaran *Appendiculata* sp., *Calanthe vestita,* and *Bullbophyllum beccarii* di hutan Raya Pasi dan Gunung Bawang di Kalimantan Barat. Metode penelitian yang digunakan adalah isolasi dan identifikasi *Rhizoctonia*-like dari akar anggrek yang sehat berdasarkan karakter morfologinya (seperti warna kolony, ukuran sel hifa, lingkaran kosentris, sel monilioid, dan jumlah inti per sel), observasi peloton pada jaringan akar, dan pengelompokan isolat. Berdasarkan identifikasi mikoriza-anggrek pada ketiga jenis anggrek spesifik Kalimantan Barat, masing-masing isolat menunjukkan bahwa *Ceratoriza* sp. berasosiasi dengan perakaran *Appendiculata* sp., *Ephuloriza* sp. berasosiasi dengan perakaran *C. vestita*, dan *Tullasnela* sp. berasosiasi dengan *B. beccarii.* Hasil ini merupakan informasi awal dan masih perlu dipelajari lebih lanjut tentang peranan *Rhizoctonia* sebagai jamur mikoriza pada anggrek dan agen pengendali hayati pada anggrek.

Kata kunci: anggrek, identifikasi, morfologi, mikoriza-anggrek, *Rhizoctonia*-like

Appendiculla sp, *Calanthe vestita*, and *Bullbophyllum beccarii* are orchid species, which are endemic in West Kalimantan tropical rain forests. They have high economic value, but difficult to cultivate, either vegetatively or generatively, including by tissue culture. In nature, cultivation of orchids require the presence of mycorrhizae to initiate germination and seedling development. Orchid mycorrhiza, which helps the absorption of essential ions, is a form of symbiosis between *Rhizoctonia* with Orchidaceae.

Rhizoctonia-like would penetrate and invade the plant embryo when the seed begins to germinate (Suryantini *et al. 20*11). Futhermore, fungal hyphae coiled around the plant root cells forming the peloton, which is one of the morphological characteristics of the orchid mycorrhizae.

Rhizoctonia-like was grouped based on the morphological characteristics, the number of cell nuclei per hyphal cell and anastomosis (fusion of hyphae) ability. Morphological characteristics of *Rhizoctonia*-like also comprises colony colour, pattern

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of concentric circles, sclerotia and hyphal cell size. Based on the number of the cell nuclei, *Rhizoctonia* is divided into three groups; uninucleate *(Ceratobasidium bicorne* strain), binucleate (*Ceratobasidium*), and multinucleate (*Rhizoctonia* sp.). Based on the ability to perform anastomosis, *Rhizoctonia* multinucleate includes 14 anastomosis group (AG) (AG 1-13 and AG BI), AG1-1D (subgroup of group AG 1) (Priyatmojo *et al. 20*01), *Waitea circinata, R. globulis* and *Tulasnella* sp. (orchid mycorrhizal fungi) (Athipunyakom *et al. 20*02). *Rhizoctonia* AG BI is multinucleate, non-pathogen, and obtained only from soil and plants in forest (the other name is AG 2-BI). *Rhizoctonia*-like is binucleate (BNR) consists of several groups; AG 1- AG 13, *W. circinata* var. *zeae* and var. *circinata* (Amaradasa 2011), as well as AG A – AG U, with theexception of AG BI (Hyakumachi *et al. 20*05), *C. cerealis, C. ramicola, E. repens* and *E. calendulina* (orchids mycorrhizal fungi) (Athipunyakom *et al*. 2002).

Rhizoctonia-like characterization is necessary to identify and differentiate between those that forms orchid mycorrhiza and those that are pathogenic. Specifity of the association between *Rhizoctonia*-like with orchid is affected by the vegetation diversity of the major forest component. Therefore, the orchid culture in ex situ conservation is influenced by the success of *Rhizoctonia*-like colonization (as mycorrhiza association) in root. The purpose of this study was to identify *Rhizoctonia*-like associated with *Appendiculla* sp., *C. vestita,* and *B. beccarii* roots in the forests of Hutan Raya Pasi and Gunung Bawang (West Kalimantan).

MATERIALS AND METHODS

Exploration and Isolation of Orchid Mycorrhizal Fungi (*Rhizoctonia*). Orchid mycorrhizal fungi was isolated from healthy roots of orchids obtained from forests of Hutan Raya Pasi and Gunung Bawang. Roots were cut (1 cm) thensurface sterilized with 70% ethanol (for 1 min), 2.5% sodium hypochlorite (for 30 s), and a final rinse with 70% ethanol (for 1 min). The six roots pieces were then cultured on a petri dish containing 10 ml potato dextrose agar (PDA) with 50 ppm streptomycin, and was incubated at 25 °C. There were five petri dishes for treatment. After three days incubation, the growth of fungi was observed under microscope to determine *Rhizoctonia* isolates. The hyphal tip of *Rhizoctonia* was cut and cultured in 10 mL soil extract (Johnson and Curl 1972). This step was repeated five to eight times to obtain pure isolates, and the culture was incubated at 25 - 28 °C to study the morphological characteristics.

Morphological Characteristics. *Rhizoctonia*-like isolates were observed based on the colony colour, sclerotia and hypha cell size (cell diameter), pattern of concentric circles, monilioid cell, and number of nuclei per cell **(**Windels *et al.* 1997; Carling *et al.* 1999**).** Mycelial plug was cultured on water agar (WA) on glass slide, incubated for 2 – 3 days. The number of nuclei were determined with fluorescence microscope after being stained with safranine O dye. This observation was conducted as many as 30 fields of view for each isolate.

Isolates Grouping of *Rhizoctonia*-like. Isolates grouping of *Rhizoctonia* spp. were performed following the procedure of Rauf *et al.* (2007) modified by Suryantini (2011), based on the criteria described by Mc.Nish *et al.* (1993). Isolate grouping was done by placing a mycelial plug of isolate within two – three cm from other mycelial plug in a petri dish (six cm diameter). The dish was incubated at 25 °C (2 – 3 days) until there was a contact between the hyphae of two isolates. Both ends the hyphae meet, observed under a microscope after being stained with safranin O dye. Criteria of ananstomosis ability are C0 (no reaction/contact between the two ends of hypha), C1 (contact fusion), C2 (imperfect fusion or hyphal fusion followed by cell lysis), C3 (perfect fusion).

RESULTS

Observation on the orchid roots showed that the root cells of *B. becarii*, *Appendicula* sp. and *C. vestita* were infected with peloton, which indicated that the roots were colonized by orchid mycorrhiza (Fig 1). We found three isolates of Rhizoctonia-like in orchid roots, each isolate was from each root of *B. becarii* (isolate RBBE), *Appendicula* sp. (isolate RAPP), and *C. vestita* (isolate RCVE).

Based on the morphological characters of the three isolates (RBEE, RAPP, and RCVE), it showed that the three isolates had different colony colors, which were white to light beige, light brown, and white (Fig 2 D, E, F). There were no significant differences on the length and width of hyphae, sclerotia colors were white or brown, but was not present in all isolates. The two isolates (RBEE and RAPP) had apparent concentric circles, whereas RCVE was less obvious (Table 2).

Rhizoctonia-like belongs to Basidiomycetes, in which monilioid cells (basidiospores) are one of the

important morphological characteristics for identification of the species. There were significant differences on the length of monilioid cell of RCVE isolate from the other two isolates (RBEE and RAPP). The length of monilioid cell in RCVE was 5.64 µm, whereas RBEE and RAPP were 15.83 and 19 µm, respectively. The diameter of monilioid cells of RCVE was 4.06 µm, and was significantly different from RBBE (8.94 µm) and RAPP (9.85 µm). The shapes of monilioid cell were either ellipsoidal (RBEE) or irregular (RAPP and RCVE) (Table 3, Fig 2 D, E, F).

The number of nuclei per cell determined the classification of *Rhizoctonia* into uninucleate, binucleate, or multinucleate. Table 3 showed that the average number of nuclei per cell in each isolate were 2.33 nuclei (RBBE), 2.10 nuclei (RAPP) and 2.30 nuclei (RCVE). Hyphal cells with one, three and four nuclei, were fewer than hypal cells with two nuclei. RBBE had sevens mononucleate (containing one nucleus), 14 binucleates (containing two nuclei), and nine multinucleate (containing several nuclei) cells. RAPP had eight mononucleate, 13 binucleates and nine multinucleate cells. RCVE had seven mononucleate, nine binucleates and 12 multinucleate cells. The three isolates found in the roots of *B. becarii*, *Appendicula* sp. and *C. vestita* were considered as the Binucleate Rhizoctonia (Table 3).

Rhizoctonia can be grouped based on the ability to anastomose with standard isolate (isolates with known AG group). In this report, the isolate groupings were only performed between isolates obtained from this study, without comparing with other isolates with known AG. It is showed that hyphal anastomis or hyphal contact was not observed between the isolates (C0). It was indicated that the three isolates (RBBE, RAPP and RCVE) were different species (Table 4).

DISCUSSION

The sustainability of epiphytic orchids (*C. vestita, Appendiculata*, and *Bulbophyllum*) are influenced by the presence of orchid mycorrhizae (*Rhizoctonia*). *Rhizoctonia*, as a cosmopolitan fungi, can be isolated from soil and plants (Suryantini 2011; Villajuan-Abgona *et al. 199*6; Matsumoto 2003; Paulitz and Schroeder 2005; Suryantini *et al. 20*11), but, as mycorrhizal fungi, they can only be isolated from healthy orchid roots. Batty *et al.* (2002) explained that

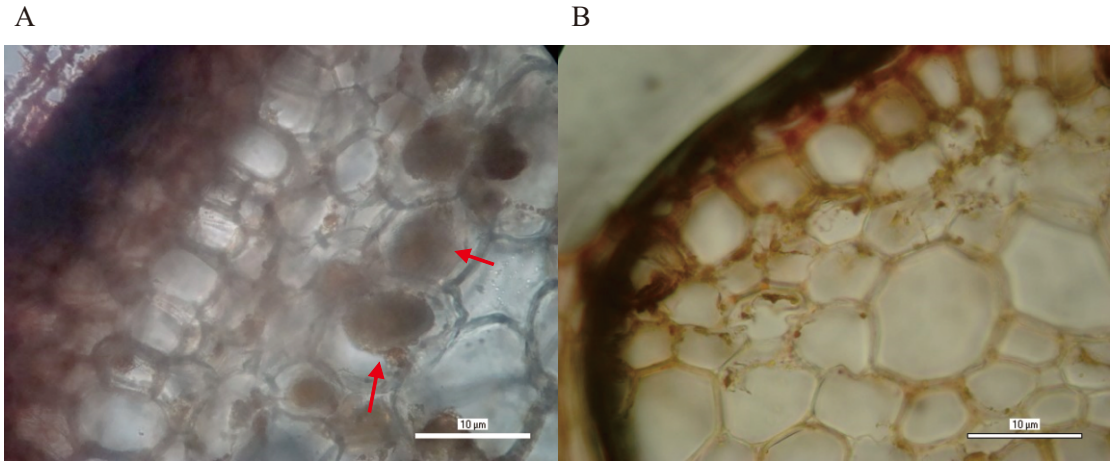

Fig 1 Orchid root cells (A and B); the peloton (red arrow).

Table 1 Morphology of orchid mycorrhizal fungi (*Rhizoctonia* spp.)

Isolates	Colony color	Hyphal size		Sclerotia colour	Concentric circles
		Length (µm)	Width (µm)		
RBBE	White to light beige	68.19 ± 36.78a*	4.79 ± 0.66 a*	Brown	Apparent
RAPP	Light brown	49.93 ± 38.20a	4.61 ± 1.20a	White	Apparent
RCVE	White	54.88 ± 30.70a	5.02 ± 1.09a	-	Less obvious

Note : RBBE (*Rhizoctonia* isolated from *B. beccarii* roots), RAPP (*Rhizoctonia* isolated from *Appendicula* sp. roots), RCVE (*Rhizoctonia* isolated from *C. vestita* roots). - = not present. * values followed by the same alphabet in the same column indicates no significant difference at 95% significance level.

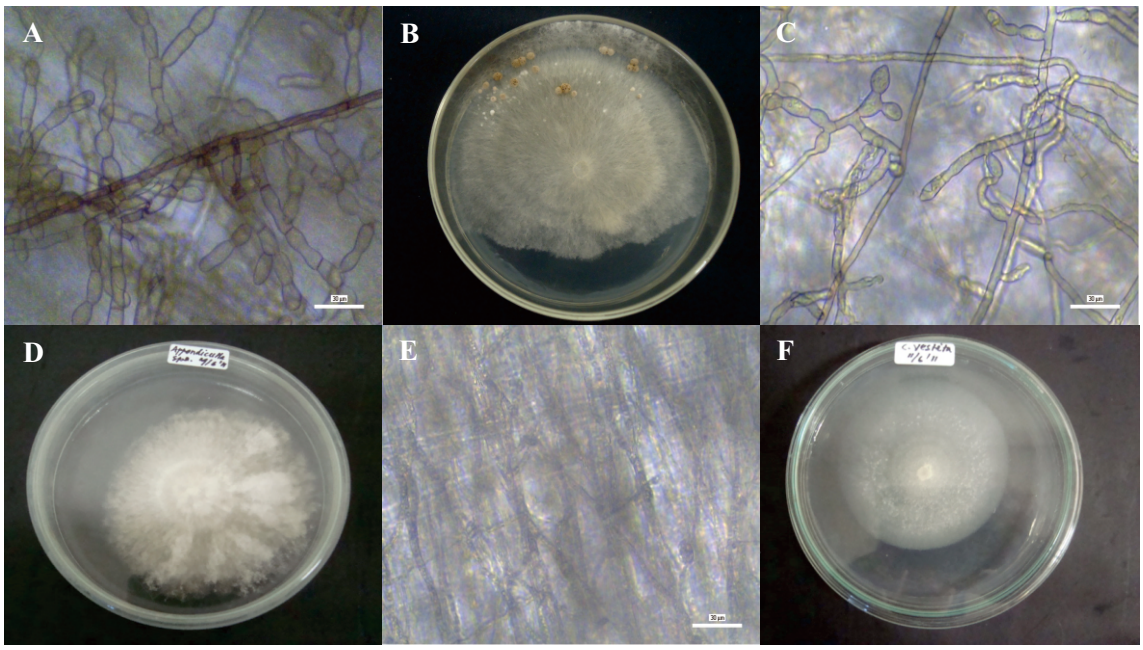

Fig 2 Monilioid cells (A, C, E) and isolate of *Rhizoctonia* (B, D, F). RBBE (A, B), RAPP (C, D), RCVE (E, F) (bar: 30 µm)

Table 2 Characteristics of *Rhizoctonia* monilioid cells

Isolates	Moniloid cells		Shape
	Length (µm)	Width (µm)	
RBBE	19.00 ± 5.88b*	8.94 ± 2.70b*	Ellipsoidal
RAPP	15.83 ± 3.82b	9.85 ± 1.20b	Irregular, formed at the tip cells of main hypha
RCVE	5.64 ± 1.82a	4.06 ± 0.44a	Irregular

Note : *values followed by the same alphabet in the same column indicates no significant difference at 95% significance level.

Table 3 The number of nuclei per cell *Rhizoctonia*

Isolates	Number of nuclei per cell				Average nuclei per hyphal cell	Note
	1 nuclei	2 nuclei	3 nuclei	4 nuclei		
RBBE	7	14	1	8	2.33	Binucleate
RAPP	8	13	7	2	2.10	Binucleate
RCVE	7	9	4	8	2.30	Binucleate

Note : RBBE (*Rhizoctonia* isolated from *B. beccarii* roots), RAPP (*Rhizoctonia* isolated from *Appendicula* sp. roots), RCVE (*Rhizoctonia* isolated from *C. vestita* roots).

Table 4 Isolate groupings based on the capability to perform anastomosis

Isolate	RBBE	RAPP	RCVE
RBBE	C3	C0	C0
RAPP		C3	C0
RCVE			C3

Note : : C0 is an indicator for the absence of hyphal contact (different AG); C3 is an indicator of hyphal contact and the perfect fusion (same AG and same clone). RBBE (*Rhizoctonia* sp. isolated from *B. beccarii* roots), RAPP (*Rhizoctoia* sp. isolated from *Appendicula* sp. roots), RCVE (*Rhizoctonia* sp. isolated from *C. vestita* roots).

they are fundamentally different from the highly specialized fungi forming other types of mycorrhizae.

One of the spesific characteristics of *Rhizoctonia*-like fungi is that the hypha branches at right angle, with a septum located near the right angles branches. *Rhizoctonia*-like fungi also form monilioid cells and sclerotia. The isolates from orchids were identified as *Ceratorhiza* (RBBE), *Epulorhiza* (RAPP) and *Tullasnela* (RCVE) (Identification based on Athipunyakom *et al. 20*04)**.** Orchid mycorrhizae could also be detected from the presence of pelotons formed in the orchid's roots. The peloton is hyphal masses of tightly-interwoven coils, of which the formation is influenced by the orchid's type (epiphytic or terrestrial) and orchid's habitat. Pereira *et al.* (2005) showed that 75% - 80% of the pelotons were found in the cortex cells of orchid's roots, whereas only 5% - 10% were found elsewhere in the orchid. The presence of hyphae penetrating the root cell wall and peloton in the cortical region indicated tolypophagy infection. Tolypophagy is an infection where the fungal coils of the mycelium enters the roots and are killed and digested by the plant. In these orchid, the fungal hyphae might have penetrated the cell wall, forming pelotons (young and old) and subsequently digested by the orchids, thus indicating the presence of mycorrhizal symbiosis between orchid plant roots with certain fungi.

Morphological property that can differentiate between genera is the isolate color. The difference in isolate color (Table 1) was influenced by age and species of *Rhizoctonia*. Some researchers showed that *Rhizoctonia* were white to dark brown (Suryantini *et al. 20*11; Kasiamdari 2000; Priyatmojo *et al. 20*01). Brown color shows the decomposition of melanin in the cell wall of hyphae.

There are wide variation of hyphal cell sizes presented by different researchers. The size variations reported include; diameter between 1.60 – 2.23 µm with lengths between 18.30 – 24.29 µm (Suryantini *et al.* (2011), 3 – 7 µm (diameter) (Sneh *et al. 19*91 *cit* Suryantini *et al. 20*11), 2.50 – 17.50 µm (diameter) and 15.00 – 382.50 µm (length) (Irawati 2004). Due to this wide variations, differences in the size of *Rhizoctonia* hyphae could not be used as a specific characteristic for grouping of *Rhizoctonia* (Suryantini *et al. 20*11). The difference was caused by genetic and environmental factors, such as growth media and temperature (Agrios 2005). On the other hand, the monilioid cell shape and size differ for each species of *Rhizoctonia*. The differences on monilioid cell shapes and sizes could be used to distinguish species of *Rhizoctonia.* However, the identification would be more complete with the addition of molecular identification.

Rhizoctonia can be classified by the number of nuclei in each hyphall cell into uninucleate (one nuclei), binucleate (two nuclei) and multinucleate (more than three nuclei). The number of nuclei in hyphal cells of *Rhizoctonia* ranged from two to three nuclei can be classified as binucleate. RBBE isolate had 2.33 nuclei per hyphal cell, which was the highest among the three isolates, while the smallest was RAPP with 2.10 nuclei/cell (Table 3). As reported before (Ogoshi *et al. 19*79), some of the cells had one nuclei at the tip of the hyphal cells or cells of young hyphae.

Generally, *Rhizoctonia* that form symbiosis with terrestrial orchid roots were binucleate *Rhizoctonia*, whereas the uninucleate and a few binucleate *Rhizoctonia* only formed symbiosis with epiphytic orchid (Bayman, *et al. 19*97; Pereira, *et al. 20*05). In this research three isolates binucleate *Rhizoctonia* were found associated with epiphytic orchid (in West Kalimantan). It would be very interesting to study further about the association specificity between uni-, bi- or multinucleate *Rhizoctonia* with its host of orchids (terrestrial or epiphytic).

The grouping of all binucleate *Rhizoctonia* isolates was based on the ability of anastomosis (cell fusion). The anastomis reaction between isolates which is shown by cell fusion, either followed or not followed cell lysis (the perfect fusion), indicated that they are the same species or clone (Ogoshi *et al*. 1979; Mc. Nish *et al*. 1995). This report showed that there was no contact between isolates when they were paired with each other (C0), indicating that they were not the same species. In conclusion, the identification of the three isolates based on the morphological characteristics were showed that they *Ceratorhiza* sp., *Epulorhiza* sp., and *Rhizoctonia* like. Those three isolates were associated with orchid roots isolated from West Kalimantan.

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