



Presence of Arbuscular Mycorrhizal Fungi on Fern from Tailing Deposition Area of Gold Mine in Timika, Indonesia

Suharno^{1*}, Rina Sri Kasiamdari², Endang Sutariningsih Soetarto³, Retno Peni Sancayaningsih⁴

¹Department of Biology, Faculty of Mathematics and Natural Science, University of Cenderawasih, Jayapura, Papua, Indonesia

²Laboratory of Plant Taxonomy, Faculty of Biology, Gadjah Mada University, Yogyakarta

³Laboratory of Microbiology, Faculty of Biology, Gadjah Mada University, Yogyakarta

⁴Laboratory of Plant Ecology, Faculty of Biology, Gadjah Mada University, Yogyakarta

*Corresponding author: harn774@yahoo.com

Abstract Detection of the presence of arbuscular mycorrhizal fungi (AMF) on marginal land within degrading reclamation effort land is very important to support the success of such reclamation. The purpose of this study is to determine the presence of AMF in the root rhizosphere of fern plants *Equisetum debile*, *Pteris radicans*, and *Nephrolepis hirsutula* that grow in the tailing area of gold mine Timika. The study used a survey method to isolate AMF from the root rhizosphere of fern plants in the gold mine tailing deposition area of the Modified Ajkwa Deposition Area (ModADA) of PT Freeport Indonesia (PTFI) in Mimika Regency, Papua, Indonesia. AMF infection was tested using a trypan blue staining method, while the calculation of the percentage of infection was carried out by a slide method. The presence of AMF spores was detected by a wet sieving method. The results of the study showed that there were AMFs in the root rhizosphere of the three types of fern. However, based on the observation of infection of the plant root system, there were only *P. radicans* indicating to interact with AMF, while *E. debile* and *N. hirsutula* were not associated with AMF. There were 54.44% of infection at the root rhizosphere found in *P. radicans* and number of spores ranged from 8 to 12 per 10 g of soil samples. Although there were no infection of AMF on the root rhizosphere of *E. debile* and *N. hirsutula*, however there were 4.33 and 11 spores per 10 g of soil samples of both plant rhizosphere respectively. Based on morphology, it can be identified that the types of AMF were member of genus *Scutelospora*, *Glomus*, *Claroideoglomus*, and *Acaulospora*.

Keywords: Arbuscular Mycorrhizal Fungi, Tailings, Ferns, Gold Mine, Timika

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1. Introduction

Arbuscular mycorrhizal fungi (AMF, Phylum Glomeromycota) as soil microorganism have an important role in the ecosystem, including on marginal land [1,2]. Mycorrhizal fungi is the key in facilitating the uptake of nutrients by plants, the increase of growth, and the yield of crop products [3,4,5]. Mycorrhiza increases plant growth at a low level of soil fertility [6,7], and degraded land [8]. AMF hyphae serve to help the expansion of the function of root systems to obtain nutrients [6]. In the last few decades, mycorrhiza also serves and is used as mycorrhizo-remediation in contaminated lands [9,10].

Fern plants are commonly found in a variety of habitats such as aquatic, wetland and terrestrial habitats [11,12]. Therefore, in some studies, environmental factors such as change in climate also affect activities of the growth of fern, photosynthesis and reproduction [13]. The discovery

of Glomeromycota fossils from the Ordovician period of Paleozoic era also confirms that AMF plays important role in the process [14,15]. The fossils were found in rhizome *Rhynia* and *Asteroxylon* [16]. The era was the transitional time of living creature development from aquatic to terrestrial habitats [14]. Fern plants are also known to make symbiosis with AMF, but it is reported that some plants were not detected as in water ferns of the familia *Marsileaceae*, *Salviniaceae* and *Isoetaceae* [17,18].

Horsetail (*Equisetum debile* Roxb; Equisetaceae) species is one of the fern groups found in water and wetland areas [19] that grow in sandy soil habitats containing sufficient, excessive, even stagnant water. *Nephrolepis hirsutula* G. Forst. (Nephrolepidaceae), has wet to dry habitats, even found as epiphytes, while *Pteris radicans* var *javanica* Alderw (Pteridaceae) was found in drier habitats [20]. Fern has a central role as a pioneer plants and a transitional ecosystem in the early succession of an area.

In addition to pioneer floras, ferns are also used as bio-indicators of metal pollution [21] with efficacy as

traditional medicine in China (as shown in horsetail species *E. debile*) [19]. The methanol extract of *E. debile* can inhibit the growth of canopy (hypocotyl) and roots of rice, acts as antioxidant and free radical catchers, and serve as antifungi, particularly in inhibiting the growth of *Aspergillus flavous* (42.26%) and *A. niger* (53.84 %) [22]. Some fern species of genus *Equisetum* can also be used as a diuretic herbal medicine [23], while the extract of *E. debile* stem is also believed to contain anti-tumor compounds and pesticide activity [24].

The types of fern widely spread, even many are prevalently found around watersheds. Some familial groups of the three types are found in Ajkwa watershed utilized as tailing deposition area of gold mine in Timika, Mimika Regency, Papua. There are at least 36 species *Pteridophyta* in the location of natural succession habitats in this area [25,26]. The tailing deposition areas resulted from the metal ore processing activities by PT. Freeport Indonesia (PTFI) reached 23,000 hectares or 230 km², while in the estuary area the areas are approximately 220 km². Every day PTFI are capable of processing approximately 220,000–240,000 tons of materials from the activities. Materials containing the concentrates of gold, copper and silver are only 3%, while the remaining 97% of tailing are discarded and not utilized [27,28]. Such wide areas need to be reclaimed to restore them into the original condition of ecosystem or at least near the condition as before any mining activities occurred in the areas [28]. To speed up the process, the land reclamation needs to be carried out using modern technologies, including the role of soil microorganisms. One of the factors in favor of defense and improvement of the growing quality of plant is the role played by mycorrhiza [8,10]. Therefore, according to Khan (2006), the types of AMF that are derived from the original areas can potentially be developed as the source of superior

microorganisms that can be developed as a source of inoculums. Hypothetically, the AMF associating with the ferns in this reclamation land would be potentially used as benefited inoculums. The objective of this study was to determine the presence of AMF in ferns *E. debile*, *P. radicans*, and *N. hirsutula* in the tailing deposition areas of gold mine in Timika, Papua.

2. Materials and Methods

2.1. Time and Sites of the Study

Sampling of ferns was carried out in November 2014, while the laboratory analysis was conducted for three months until February 2015. The selected samples of ferns were *E. debile*, *P. radicans*, and *N. hirsutula* (Figure 1). In other sources, some researchers included *N. hirsutula* into the family Nephrolepidaceae but some placed it into the family Davalliaceae or Dryopteridaceae. In addition to ferns, soil samples were also taken from rhizosphere of the site of the study.



Figure 1. Fern plant morphology. a. *Equisetum debile* Roxb., b. *Pteris radicans*, and c. *Nephrolepis hirsutula*

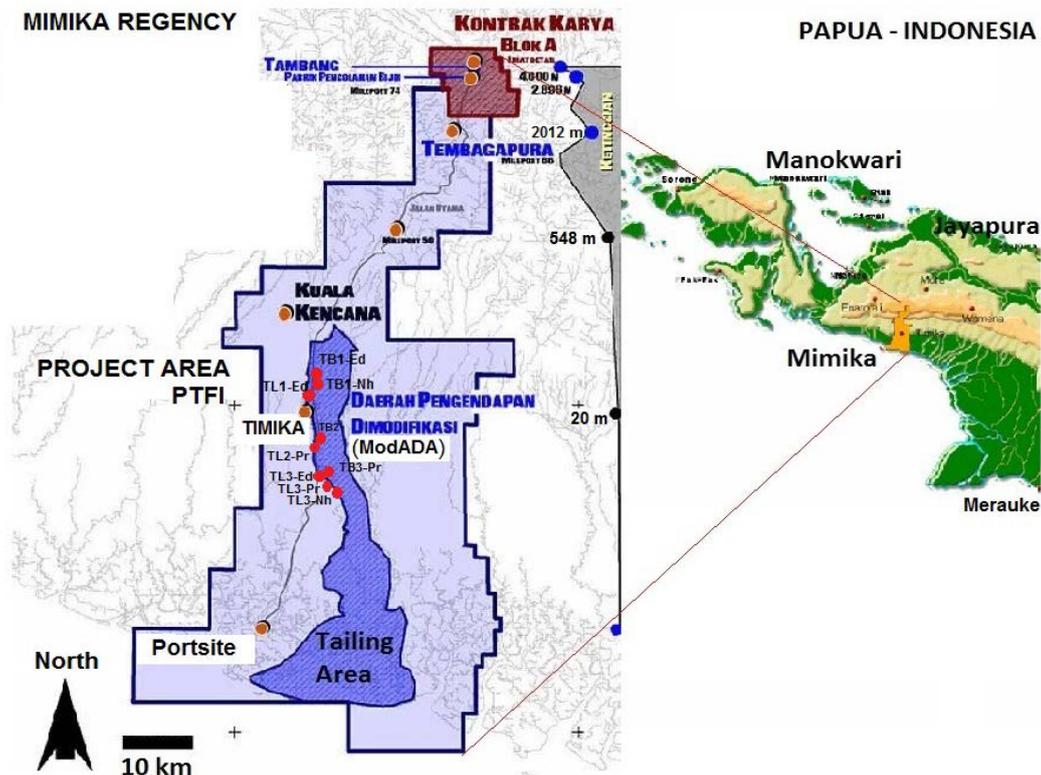


Figure 2. Sites of the study at tailing area in Timika, Mimika regency, Papua-Indonesia

The sites of the study were selected in three geographical position points, namely, the position S: 04°29'50.9" and E: 136°54'02.3" (at the top of the deposition areas), the position S: 04°35'24.1" and E: 136°54'32.9" (in the middle of the deposition areas), and the position S: 04°33'14.0" and E: 136°54'11.7" (at the bottom of the deposition areas) (Figure 2). The locations represented diverse sandy sediment particle size, where at the top it was >175 µm (rough), at the middle 150–175 µm (medium), at the bottom 38–75 µm (fine), and in estuary areas <38 µm (very fine) [27]. The three species of ferns were selected because from the observation of ferns, they were known to be dominant with diverse habitats. They were included into the tailing deposition areas, i.e. the Modified Ajkwa Deposition Area (ModADA) of PTFI in the old and new west embankment (double levee) of Timika, Mimika Regency, Papua with elevation of 30–52 m above sea level (Figure 1). Furthermore, the analyses of soil samples and fern roots were performed at the Laboratory of Plant Taxonomy, Faculty of Biology, Gadjah Mada University, Yogyakarta.

2.2. The Presence of AMF on Plant Rhizosphere

The presence of mycorrhiza was detected by taking both soil and plant root samples. The soil samples were taken for the isolation of spores, while the plant root samples were used to see AMF infection in plants. AMF spores were observed by a wet sieving method, i.e. filtering the spores stratified with the mesh sizes of 500, 250, 105, and 53 µm [29]. The soil samples of 10 g were filtered using the flowing water and the spores were filtered on the bottom mesh of 53 µm in size. The spore materials were then centrifuged at 3000 rpm for 5 minutes with the addition of 60% sucrose solution to separate the spores and other materials. The spores were transferred to the filter paper that was given the vertical and horizontal lines with the box size of 0.5 x 0.5 cm for ease of observation. The amount of the spores were observed and calculated under a microscope with a magnification of 40x. In the same way, a trapping method was also used to find out the diversity of AMF. In the trapping method, 300 grams of soil sample were mixed in a zeolite media in a pot with capacity of 3 kg. The plant used as host was *Sorghum bicolor*. The isolation of AMF was performed when the plant was 3 months old.

The data of AMF infection was important because AMF was obligate symbiosis with plants. The plant root samples were stained by a staining method [29]. The ways of plant root treatment were as follows: the plant roots were cleaned and fixed by using FAA (formalin– acetic acid– alcohol; v = 5: 5: 90) solution for 1 hour, soaked with 10% KOH solution for 24 hours, followed by 1% HCl for 24 hours. After washed with the solution of distilled water, the plant roots were stained by trypan blue 0.05% in lactoglycerol for 24 hours. The stained roots could be observed under a microscope or stored by the lactoglycerol solution of 50% up to several months [29,30].

2.3. The Percentage of AMF Infection in Plant Roots

The percentage of AMF infection was determined using a slide method. The stained roots were cut into pieces of 1

cm in length, and 30 pieces were observed microscopically (magnification of 100 or 400x) [28,31]. From the observations, the presence of AMF can be seen due to the structure of AMF in the roots, i.e. extraradical hyphae, intraradical hyphae, vesicles, arbuscles, or spores [29]. The arbuscular and vesicular structures are the main characteristics in AMF infection in the rhizosphere of plant roots. To calculate the percentage of mycorrhizal infection, the formula was used.

$$\begin{aligned} &\text{The percentage of root infection} \\ &= \frac{\text{The amount of infection roots}}{\text{The amount of all roots observed}} \times 100\% \end{aligned}$$

The percentage values indicating the presence of AMF were scored with scales of 0–5 [1]. Score of 0 indicates that plant roots were uninfected by AMF (0%); score of 1 the percentage of infection was very few (<1%); score of 2 it was few ranging from 1 to 10%; score of 3 it was moderate ranging from 10 to 50%, score of 4 it was large ranging from 50 to 90%, and score of 5 it was overwhelmed (>90%).

3. Results and Discussion

The observation of mycorrhizae status in the tailing deposition areas indicated the presence of AMF infection in *P. radicans* show, but there were no infection in *E. debile* and *N. hirsutul* (Table 1). The presence of AMF was identified based on the presence of symbiosis-forming structures such as intraradical hyphae, extraradical hyphae, vesicles, arbuscles, or intraradical spores in the plant root system. From the observation of root infection, fungal infection was found in *E. debile* and *N. hirsutula*, but the infection was not possibly from AMF. This is because the arbuscular and vesicular structures that characterize AMF were not found. These results were similar to Berch and Kendrick (1982) research, which did not find arbuscular structures in *Equisetum kyemale*, *E. hyemale* and *E. scirpoides*. On *E. arvense*, arbuscular structures were found in small amount and not found on all samples of plant roots. This condition also occurred in a fern group of the Order Lycopodiales. Arbuscular structure is one of the important criteria in identifying the presence of AMF that infects the plant root system.

Among 39 ferns observed in the areas of Ontario, Canada, arbuscular structures were not found in the group of the Order Lycopodiales and Equisetales, while they were overwhelmingly found up to 100% in Ophioglossales (*Botrychium oneidense* and *B. virginianum*) [32]. In fern *B. virginianum* (Ophioglossaceae), the AMF species identified as result based on molecular analysis was found. Some types of AMF found include those of the group of Glomus located in a cluster with *Glomus fasciculatum*. Other type found was from the genus *Scutellospora* and it is expected that it was a species of *Scutellospora gregaria* [33].

Results of the observation in Yunan (Southwest China), of 256 species Pteridophyta observed, the presence of AMF in saprophyte and leptosporophyte was very low, while that in eusporangiate was relatively high [16]. Based on habitat, various types of *Pteridophyta* were able to make a symbiosis with AMF, except water ferns such as Marsileaceae, Salviniaceae, and Isoetaceae [17]. This observation was reinforced by Cooper (1976)'s study in

New Zealand. Results of the observation on 31 fern species of the Order Filicales show that the presence of AMF based on arbuscular structures also varied. However, arbuscular structures were found in most species, except in

species *Asplenium trichomanes*, *Camptosaurus rhizophyllus*, *Cryptogamma stelleri* and *Osmunda regalis*. While arbuscular structures were not found in *O. regalis*, they were actually found in *O. cinnamomea* ranging from 35 to 90% [31].

Table 1. The status of AMF infection percentages in the ferns in the land of gold mine tailings in Timika, Papua.

No.	Species	Sample code	AMF infection at the root	Number of spores (10 g sample soil)	
				Rhizosphere soil	Trapping methods
1.	<i>E. debile</i>	TB1-Ed	-	2	19
		TL1-Ed	-	5	25
		TL3-Ed	-	6	24
		Average		4.33	19.67
2.	<i>P. radicans</i>	TB3-Pr	+	8	41
		TL2-Pr	+	12	89
		TL3-Pr	+	9	32
		Average		9.67	36.00
3.	<i>N. hirsutula</i>	TB1-Nh	-	8	102
		TB2-Nh	-	11	98
		TL3-Nh	-	14	141
		Average		11.00	113.67

Notes.: (+) arbuscular and vesicular structures were found; (-) not found.

The arbuscular structures were not found in the group of Equisetum and Nephrolepis possibly because the types of roots in the plants are rather different. The rhizome of *E. debile* was not only capable of growing well, but also the plant roots of *E. debile* and *N. hirsutula* were covered by the sufficiently abundant root hairs, so that the role played by the roots in performing their function is considered to sufficiently serve in meeting their needs. Brundrett (2004) argued that the AMF associated with terrestrial plants generally formed arbuscules and equipped with vesicles. Some longitudinally dominant intercellular hyphae structures were commonly found on AMF of *Arum*, while the dominance of intracellular hyphae often form hyphal coil in the roots and it was known as a type of *Paris*. On the association with the type of *Paris*, sometimes arbuscular structures were not formed, because the host plants with the type of microheterotrophic, arbuscular structures were often not formed [34]. This was also shown by Smith and Read (2008), indicating that when we make a classification based on morphology the categories used were relevant with a high degree of consistency both on host plants and fungi and not all resulted in the same results [2].

The ability of plant infection on the tailing ground also varies for each type of AMF [35,36]. In general, the equation of combined host plants and mycorrhizal fungi with different types of interaction appear during

development, active phase, and aging process for mycorrhizal associations. Duration of interaction between fungi and host is influenced by variation in the dependence of mycorrhizae on host plants and the effect of land condition on the ability of mycorrhiza to grow [34]. Diversity in arbuscular mycorrhiza is highly dependent on natural condition, but in general the presence of AMF in plants is able to make a symbiosis with 90% of high-level plants, including a group of ferns (Pteridophyta) [37].

The role of soil microorganism in the phytoremediation process is very important. Mycorrhizae also serve in remediation process by plants [9,38]. Mycorrhizae will support the growth of crops contaminated by various kinds of heavy metals. However, the interaction of AMF and plants should be mutually beneficial in terms of improving and maintaining optimal plant growth [9,39,40].

The percentage of AMF infection on *P. radicans* roots ranged from 40.0 to 63.3%, or averagely 54.4% (Table 2), while that of *E. debile* and *N. hirsutula* was similarly 0%. The ability of AMF infection on different types of ferns varied. In plants *Pityrogramma calomelanos* the percentage of AMF infection reached 75%, which was the highest among several other kinds of ferns [35]. The percentage of AMF infection in the plant roots was related to the ecological system of AMF and host plants in their habitats [36]. Table 2 also shows that arbuscular structures were not always found in the fern plant *P. radicans*.

Table 2. The percentage of AMF infection on plant roots *E. debile*, *P. radicans* and *N. hirsutula* in gold mine tailings area in Timika, Papua

No	Species of plants	Sample code	Structures of infection					Number of AMF infection	% infection	Score
			V	A	HI	HE	S			
1.	<i>E. debile</i>	TB1-Ed	0	0	5	0	0	0	0.00	0
		TL1-Ed	0	0	2	0	0	0	0.00	
		TL3-Ed	0	0	10	2	0	0	0.00	
		Average	0.00	0.00	5.67	0.67	0.00	0.00	0.00	
2.	<i>P. radicans</i>	TB3-Pr	16	5	18	2	5	18	60.00	4
		TL3-Pr	19	1	18	3	1	19	63.33	
		TL3-Pr2	12	0	12	0	1	12	40.00	
		Average	15.67	2.00	16.00	1.67	2.33	16.33	54.44	
3.	<i>N. hirsutula</i>	TB1-Nh	0	0	5	2	0	0	0.00	0
		TB2-Nh	0	0	2	0	0	0	0.00	
		TL3-Nh	0	0	10	0	0	0	0.00	
		Average	0.00	0.00	5.67	0.67	0.00	0.00	0.00	

Not all types of AMF are capable of forming a complete structure in the root cells simultaneously. Vesicular structures were often found, which are considered as food storage structures and the ramifications of auxiliary cells forming hyphae in ground, but sometimes were not found because it was not the right time [41]. Various dominant plant species were able to make a symbiosis in the gold mine tailing land in Timika. Plant *Bidens pilosa*, *Wedelia trilobata*, *Casuarina equisetifolia*, *Ficus adenosperma*, *Setaria* sp, *Brachiaria* sp, *Musaenda frondosa*, and *Amomum* sp, were found to be able to symbiosis by forming vesicular and arbuscular structures in root tissues, while several plant root samples uninfected by AMF were found in *Saccharum spontaneum* and *Phragmites karka* [28]. The structures formed by AMF on the plant root system were very important in the symbiosis process. Three important components in AMF association are the role of plant roots, intraradical hyphae, and extraradical hyphae in soil [2,41]. They played a great role in the adsorption of nutrient and plant resilience [2].

High or low level of the percentage of AMF infection, the growth of external hyphae and the production of spores depends on environmental conditions. When there is a drought, fungi tends to form spores and inversely the development of hyphae/ mycelium will be more intensive during the rainy season, although it is was not statistically significant. The spores were concentrated in the top layer of soil and significantly reduced in the deepest layer of soil profile. This condition is associated with the distribution of root system and the availability of carbon sources in their habitats [42].

The amount of AMF spores in the rhizosphere of plants *P. radicans* in the tailing area varied from 8-12 spores with an average of 9.7 spores per 10 g of soil samples. Meanwhile in *E. debile* 2-6 spores were found and in *N. hirsutula* 8-14 spores were found with average of 4.3 and 11.0 spores per 10 g of soil samples, respectively. The presence of spores in the rhizosphere of plants *E. debile* and *N. hirsutula* was because in natural habitats all plant roots were associated and interacted with various types of other plants in the ground. The number of spores increased rapidly with the treatment by a trapping method using *Sorghum bicolor* plant as host. The increase achieved 11-fold than the number of spores directly isolated from their natural habitat (Table 1) between 19.7 and 113.7 spores per 10 grams of soil samples. The number of spores in the root rhizosphere greatly varies, depending on local environmental conditions. According to Khade and Rodriguez (2002), the highest number of spores in the fern rhizosphere was found in *Adiantum tunulatum*, which reached an average of 15.5 spores per 10 g of soil samples.

All plants have the same opportunity to grow and develop in a natural habitat. The plant root system can grow indefinitely and can be associated with each other with or without the presence of a hyphae bridge formed by mycorrhiza [37,40]. Based on spore morphology, it is estimated that there were the types of AMF of the genus *Scutellospora*, *Glomus*, *Claroideoglomus*, and *Acaulospora* (Figure 3). Results of previous observations showed that some morphospecies of AMF were found in various plant species dominant in the tailing areas [28]. Even in a habitat, the types of AMF could be found and might not be found in other locations [43]. The difference was associated with AMF that was capable of growing on the

environmental conditions and the ability of fungi to form a symbiosis with various types of plants in the vicinity [35,37].

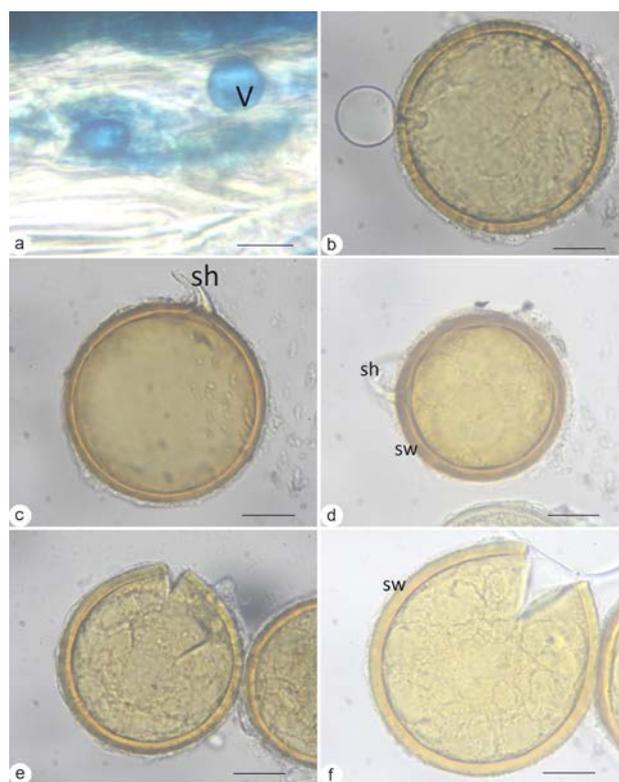


Figure 3. Infections and morphospecies AMF isolated from ferns. The structure of vesicles at the root *P. radicans* with trypan blue staining, b. *Scutellospora* spore morphology, c-d. *Glomus* spores, e. *Archaeospora* spore, and f. *Acaulospora* spore. v: vesicles, sh: subventing hyphae, sw: spore wall (scale bar: 25 μm)

Climate data in Timika in 2012 indicates that rainfall in this area was sufficiently high ranging from 280.5 to 726.3 mm/month, average temperature of 24.8 to 27°C, and humidity of 85-91% [28]. Decrease or increase in average temperature and humidity in the area is not too large possible because rainfall occur almost every day in every month. According to Cuenca and Lovera (2010), high rainfall affects the decreasing number of spores produced by AMF hyphae. Small change in weather throughout the year did not significantly affect the growth of AMF hyphae, although diverse fluctuations occurred. Fungi will form spores when the environmental conditions are not suitable for growth. If the availability of nutrients and water were met, the hyphae will grow well, while if the conditions did not allow growing, the AMF form spores [42].

In view of the role of AMF in land reclamation, the role of mycorrhiza and microbe in land reclamation and remediation processes are disturbed [8,40]. Microbe plays an important role in marginal land reclamation and remediation processes due to both natural processes and the influence of human activity. The symbiotic relationship between microbes and plants and their interactions in rhizosphere is important in determining the level of plant productivity and soil fertility [5,39]. The role of AMF is important in the process of mycorrhizal remediation [8,10], even increasing the role of fern plants in the hyperaccumulation of heavy metals, especially arsenic. Nevertheless, the response of AMF is highly

dependent on the type of plant and the type of heavy metals contained in the soil [4,10].

The presence of the type of AMF on mined land, particularly reclamation area, can be used as a source of inoculums for the rehabilitation of mined lands. This is because AMF is an essential component needed to help increase the vitality and growth of plants, especially in the post-mining location [38,40]. The types of AMF that were surely known to be able to adjust with the tailing habitat are able to serve in helping the land rehabilitation process [9,38,44]. The types of AMF are very important in land tailing revegetation process because they are tolerant to the appropriate conditions [10,44,45]. However, in order to identify the AMF to the level of species, much energy, time and cost are required. To date the method considered as appropriate to be used to identify the level of species is a molecular method. Therefore, the efforts to discover the types of AMF and prospects for the utilization of local AMF are more environment-friendly than other methods [8,9,10]. This process certainly run through long steps from isolation of the types of AMF, the selection of superior species, compatibility to plants, propagation and utilization in the field [3,46].

4. Conclusion

Fern *P. radicans* was able to make a symbiosis with AMF, while arbuscular structures and vesicles as main structures of AMF symbiosis process on plant root system were not found in *E. debile* and *N. hirsutula*. The percentage of infection on *P. radicans* roots was high enough, averagely 54.44%. The results of isolated spores in gold mine tailing deposition area of the Modified Ajkwa Deposition Area (ModADA) in Timika, Papua Province were found in *E. debile*, *P. radicans* and *N. hirsutula* of 4.33, 9.67 and 11, 00 spores per 10 g of soil samples, respectively. In view of morphological characteristics, some morphospecies of AMF was originated from the genus *Glomus*, *Claroideoglomus*, *Scutelospora*, *Archaeospora* and *Acaulospora*.

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