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Arbuscular mycorrhizal fungi status of *Nepenthes* spp. around Lore Lindu National Park, Central Sulawesi, Indonesia

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Abstract

Conservation of local and endemic biological resources of Lore Lindu National Park, Central Sulawesi Indonesia is of utmost importance as it is one of the most important ecosystem protected areas. Currently, the conservation area is threatened by forest encroachment, illegal logging and illegal mining which pose threat to the sustainability of the biological resources in it. Furthermore, there is still a lack of studies on endemic flora in this area, particularly research on the status of arbuscular mycorrhizal fungi (AMF) in Sulawesi's endemic flora in conservation areas. The present study aims to determine the species and colonisation levels of AMF on *Nepenthes* spp. plants that grow inside and outside the Lore Lindu National Park area. The results revealed that there were 3 species of *Nepenthes* in the study area, viz. *N. mirabilis*, *N. maxima* and *N. tentaculata* and as many as 4 species of AMF that have symbiosis with *Nepenthes* spp. namely, *Glomus* sp. 1, *Glomus* sp. 2, *Glomus* sp. 3 and *Gigaspora* sp. The degree of AMF colonisation on the roots of *Nepenthes* spp. varied from 2.4 % to 16.2 %. While the spore density varied between 4 and 60 per 15 g of soil. These results shows that *Nepenthes* spp. enter into symbiosis with AMF. Acquisition of more information is required in developing conservation plans and management strategies of *Nepenthes* species in the future.

Key words: colonisation, conservation, *Gigaspora*, *Glomus*, symbiosis.

Introduction

Nepenthes spp. ('pitcher' plants) are with the ability to prey on insects, where they use special traps called 'pouches' to attract, capture and digest insects as the main source of nutrition (Lee et al. 2016). Insect predation is a way for *Nepenthes* to overcome the lack of nutrients from the soil. This genus contains species that can grow as a liana or grow terrestrially (Mansur 2008). This plant has a unique way of trapping insects. Besides being able to trap insects, *Nepenthes* spp. are also able to trap frogs or birds that enter its pouch.

According to Sanusi et al. (2017), *Nepenthes* has long been used in tradi-

tional medicine in India and countries in Southeast Asia. Mansur (2006) reported that liquid in the pouch can be used as an eye, cough and burnt skin remedy. The stems are used to tie bird cages and fences (Dariana 2009) and root is used traditionally in the treatment of dysentery and colic (Schwallier et al. 2015). Some of the Miyanmin people at Yapsiei Station, West Sepik Province, Papua New Guinea, give the liquid from the plant pouches to infants and children as a treatment for cognitive disabilities and developmental delays (Hagwood 2019). In Malaysia, *Nepenthes* spp. which has been blended is used as an astringent drug (Aung et al. 2002).

It is estimated that there are 100 species of *Nepenthes* and 64 of them are found in Indonesia (Hariyadi 2013). It is known to be very well adapted to growing in nutrient-poor soils which have very low essential nutrients such as nitrogen, phosphorus and potassium as well as high soil acidity and the poor soil is a limiting factor for plant growth (Rembold et al. 2010, Bauer et al. 2012, Cheek and Jebb 2016). This plant grows in various ecological habitats, ranging from an altitude of 0–3500 m a.s.l., from fresh water swamps on the coast to high mountains. *Nepenthes* tends to grow in nutrient-poor, low pH and nitrogen-poor places (Wistuba and Fleischmann 2007) with an abundant supply of water and light (Givnish et al. 1984).

Research on the symbiotic association between carnivorous plants and arbuscular mycorrhizal fungi (AMF) in the world has been carried out, but similar studies have not been conducted in Indonesia. According to Macdougal (1899), Juniper et al. (1989) and Brundrett (2009) it is generally considered that carnivorous plants are non-mycorrhizal because there is no clear benefit for their association with fungal symbionts, for example through facilitating the acquisition of nutrients. However, Fuchs and Haselwandter (2004) and Chambers et al. (2008) reported sporadic colonisation by facultative arbuscular mycorrhizae on the roots of carnivorous plants. This is confirmed by the results of recent research reporting that *Nepenthes* plants can form symbiosis with AMF (Ducousso et al. 2008, Santiago and Darnowski 2012). Furthermore, Harikumar (2013) reported that two species of carnivorous plants, namely *Drosera burmanii* Vahl. and *Drosera indica* L., were also colonised by AMF.

Colonisation of *Nepenthes* spp. by AMF in Indonesia has never been reported, especially on the island of Sulawesi. Therefore, this study aims to determine the level of colonisation and species of AMF that are symbiotic with *Nepenthes* spp. located inside and outside the Lore Lindu National Park area, Central Sulawesi, Indonesia.

Material and Methods

Study sites

Observation and sampling of roots and rhizosphere soil of *Nepenthes* spp. was carried out in three locations, namely: Padang Padaeha (Inside the Lore Lindu National Park Area) with coordinates 01°19.986’ S and 120°19.581’ E, pine stands (Outside Lore Lindu National Park Area) with coordinates 01°35.156’ S and 120°25.822’ E and Padang Wanga (buffer zone of Lore Lindu National Park) with coordinates 01°29.784’ S and 120°19.273’ E. The maps and conditions of each research location are presented in figures 1 and 2.

a) b) c)

Fig. 1. The physical condition of each study location: a) Padang Padaeha, b) Padang Wanga, c) Pine stands.

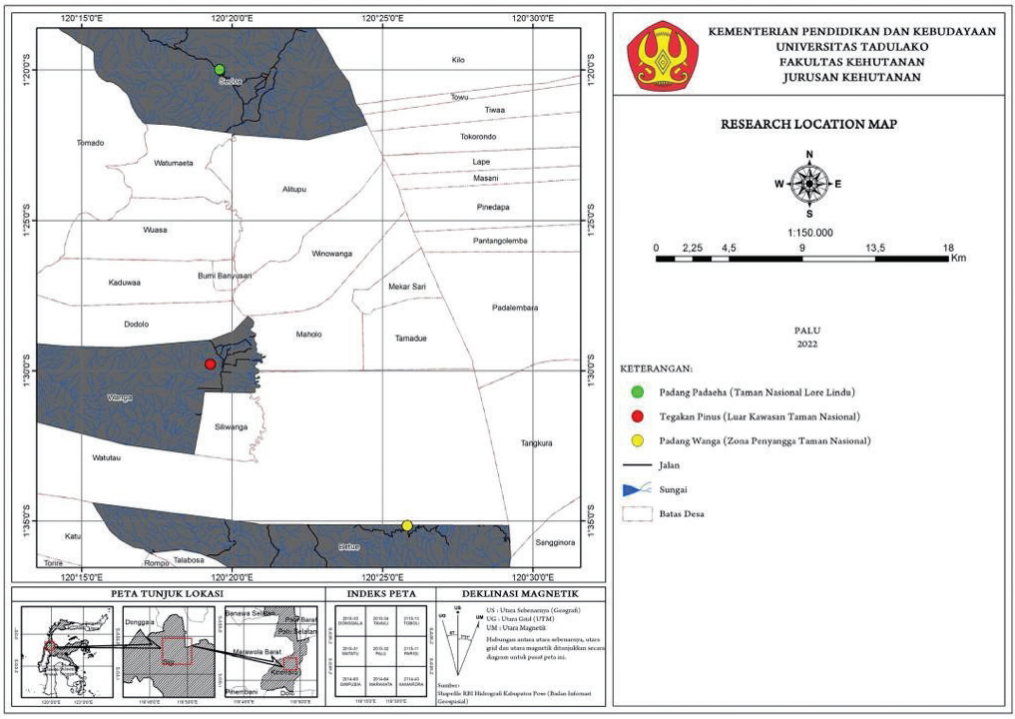


Fig. 2. Map of research locations.

Sample collection of *Nepenthes* spp. and other plants in the research location

In the field, initial identification was carried out to determine the species of *Nepenthes*. Its samples that differed morphologically from other plant species found in the study area were also collected and brought to the Celebence Herbarium (UPT, Sumberdaya Hayati Sulawesi), Tadulako University, Palu, Indonesia, for further identification.

Collection of *Nepenthes* spp. roots and rhizospheric soil samples

Collection of rhizosphere soil samples and *Nepenthes* roots was carried out in May–July 2022. Root and rhizosphere soil samples were collected from each *Nepenthes* species. From each study location, the roots were taken from 15 *Nepenthes* clumps that grew randomly. To represent the heterogeneous environmental conditions at each study location, the distance taken between them and others was approximately 25 m. Because *Nepenthes* is protected and is in a conservation area, the roots were taken carefully by digging up on one side to keep the plants from dying. The roots that appear were cut slowly and cleaned of soil or other impurities, then stored in a plastic box filled with water. After the roots were cleaned, the fine roots were selected and carefully cut into approximately 1 cm in size. These roots were then put into a plastic bottle containing 10 % ethanol. The root samples in these bottles were then taken to the laboratory to be analysed for AMF infection.

At each study location, 100 g of rhizosphere soil samples were collected from each *Nepenthes* clump at a depth of approximately 10 cm. In order to represent the heterogeneous environmental conditions of the study locations, soil samples were randomly collected from 25 *Nepenthes* clumps without differentiating species with a distance of approximately 25 m between the clumps. Soil samples from each *Nepenthes* clump were collected and composited to obtain one sample from each study location. Soil samples were collected separately in plastic bags, labeled, then brought to the laboratory and stored at room temperature.

Colonisation of *Nepenthes* root by arbuscular mycorrhizal fungi

The percentage of colonisation was calculated based on the 'presence and absence' of mycorrhizal structures (McGonigle et al. 1990) after the roots were stained with Trypan blue modified by Phillips and Hayman method (1970).

AMF colonisation enumeration used the infected root length method (Giovannetti and Mosse 1980). The roots that had been treated with Trypan blue were taken and arranged in a Petri dish, then observed carefully. Any field of view which showed colonisation was marked with (+) and (-) if there was no colonisation. There were four categories based on O'Connor et al. (2001), namely: not colonised (0 %), low (10 %), moderate (10–30 %), high (>30 %). Formula (1) is for calculation of AMF infection percentage (Brundrett et al. 1996).

$$\text{AMF infection} = \frac{\sum \text{infected roots}}{\text{Total of observed roots}} = 100, \%. \quad (1)$$

Spore isolation

The technique used in spore extraction was the pour-filter technique followed by centrifugation (Pacioni 1992). The working procedure for the pour-strain technique was to weigh 100 g of soil sample, then add enough water to make 1 L and stir for 1 min, then let it stand for ±15 min to precipitate. Then the soils were sieved in a set of sieves with a size of 325 µm, 50 µm and 40 µm sequentially from top to bottom. The top filter was removed and the second filter was sprayed with tap water. After the second filter was removed, the amount of residual soil left on the bottom filter was transferred into a measuring cup and then poured into a centrifuge tube.

Spore extraction was followed by centrifugation (Brundrett et al. 1996). The filter results were put into the centrifuge twice, the first at 2500 rpm for 5 min and the second at 1200 rpm for 2 min with the addition of 60% glucose solution to a third of the tube. Then the supernatant was washed in the third filter using running water for ±15 s to remove the glucose solution in the sample. The precipitate remaining on the filter was poured into a Petri dish and then observed under a microscope to identify any spores present. The spore density was calculated by the formula (2).

$$\text{Spore density} = \frac{\text{Number of spores}}{15\ \text{g soil}}. \quad (2)$$

Furthermore, spore preparations were made using Melzer dye. After counting the number of spores obtained from extraction and grouping them by type, they were placed on the preparation glass and then given PVLG and Melzer solutions. The spores were broken carefully by pressing the cover slip of the preparation using the tip of the needle. Spore color change in Melzer's solution was an indicator to determine the type of spore present. Identification of AMF spores was carried out based on The International Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM 2022).

Determination of AMF species was based on the characteristics shown by the spores (color, shape, size, hyphae, spore ornament) and grouping them into genera that have these characteristics. Observation of spores was carried out under an Axio Imager microscope with a magnification of 200X.

Data analysis

Data on the percentage of colonisation by AMF were analysed using analysis of variance with the SPSS software package (IBM SPSS Statistics 25, Armonk, NY, USA), and comparisons between study sites using the 5% honestly significant difference test.

Results and Discussion

Characteristics and species of *Nepenthes* spp. and the dominant plants at the research site

The results showed that only two species of *Nepenthes* were found in Padang Padaeha and pine stands, namely *N. tentaculata* Hook.f. and *N. maxima* Reinw. ex Nees, while in Padang Wanga three species were found, namely *N. tentaculata*, *N. maxima* and *N. mirabilis* (Lour.) Druce. In addition, several dominant plant species associated with *Nepenthes* were also found, such as *Imperata cilindrica* L., *Eucalyptus deglupta* Blume, *Selaginela plana* (Desv. ex Poir.) Hieron., *Mallotus* spp. and *Pinus merkussi* Jungh. et de Vriese. The characteristics of study locations are presented in Table 1.

Table 1. Characteristics and species of plants at each research location.

No	Study sites	Altitude, m a.s.l.	Temperature / Humidity	Dominant plants
1	Padang Padaeha (inside)	1607	25.8 °C/39 %	1. *Nepenthes tentaculate* 2. *Nepenthes maxima* 3. *Imperata cilindrica* *4. Eucalyptus deglupta* *5. Selaginela plana*
2	Padang Wanga (buffer zone)	1524	27.3 °C/53 %	1. *Nepenthes tentaculata* 2. *Nepenthes maxima* 3. *Nepenthes mirabilis* 4. *Imperata cilindrica* *5. Eucalyptus deglupta* *6. Selaginela plana*
3	Pine stand (outside)	1524	25.6 °C/40 %	1. *Nepenthes tentaculata* 2. *Nepenthes maxima* 3. *Pinus merkusii* *4. Imperata cilindrica* *5. Selaginela plana* *6. Mallotus* spp.

Based on the exploration results, it was found that three species were spread in the study locations, namely *N. tentaculata*, *N. mirabilis* and *N. maxima*. *N. tentaculata* and *N. maxima*, can be found in the three study locations. Both species were found living terrestrially on the ground, among reeds and without shade. This is in accordance to the results of Mansur (2006) and Khalid et al. (2015) that both have the same place to grow because they prefer open places with high light intensity and cold (humid) temperatures and can grow terrestrially on rocky soil and also between shrubs, ferns or other plants. Both can also interact with surrounding trees to support their growth because their length can reach 5–6 m (Khalid et al. 2015).

N. mirabilis was only found in Padang Wanga at an altitude of ±1524 m a.s.l. The location where this species was found is a field of reeds essentially a basin with water flow so that the soil tends to be moist and wet. Mansur (2006) explained that *N. mirabillis* has high adaptability to its environment, so that this species can live in various habitats in wet and dry places. Usually found in wet clearings and swamps, it also lives in clearings on roadside cliffs, riverbanks, secondary forest edges, and lakeshores and generally grows in red-yellow podzolic soils. The three species of *Nepenthes* found in the three study locations can be seen in Figure 3.

Spore density, colonisation and species of AMF that have symbiosis with *Nepenthes* spp.

The results of AMF colonisation level are presented in Table 2 and showed that the highest percentage was found in *Nepenthes* roots from Padang Padaeha, namely 16.2 % (medium category), followed by *Nepenthes* roots collected from Padang Wangga, namely 15.9 % (also in the category medium) while the lowest percentage was found in *Nepenthes* roots from

a) b) c)

Fig. 3. Species of *Nepenthes* found in three research sites: a) *N. mirabilis*, b) *N. tentaculata*, c) *N. maxima*

Table 2. AMF spore density, AMF species and their colonization on *Nepenthes* spp. roots.

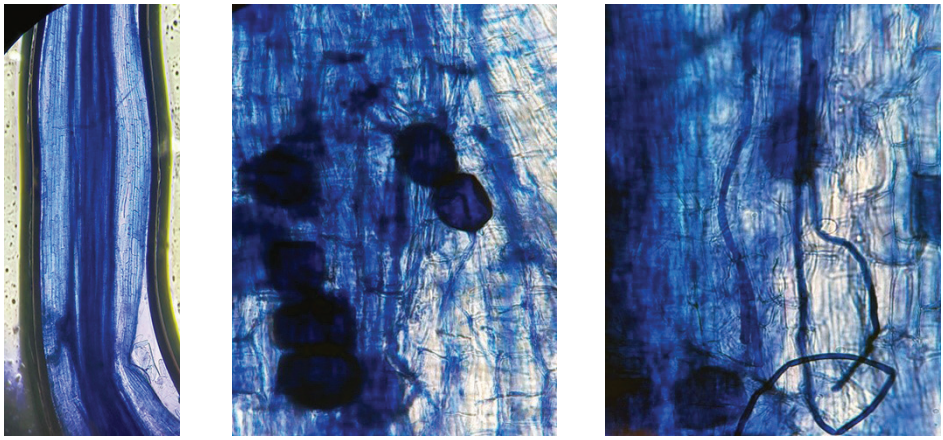
No	Study sites	Type of AMF	Spore density, per 15 g soils	Colonisation, %	Category
1	Padang Padaeha	*Gigaspora* spp.	4 ±1.73	16.2 ±0.85 a	Moderate
		Glomus sp. 1	4 ±2.0		
		Glomus sp. 2	60 ±3.60		
		Glomus sp. 3	5 ±1.0		
2	Padang Wanga	*Glomus* sp. 1	9 ±1.73	15.9 ±1.8 a	Moderate
3	Pine stand	-	-	2.4 ±0.3 b	Low

Note: Each value of AMF colonisation is the mean of three replications, ±Standard Deviation, means followed by same letter within a column are not significant at $p \leq 0.05$ level (Tukey's honestly significant test).

pine stands, namely 2.4 % (low category). The AMF structures found in colonised *Nepenthes* roots were in the form of hyphae and vesicles (Fig. 4). These results indicate that there are AMF that can be associated with *Nepenthes* roots.

Several species of *Nepenthes* are reported to have symbiosis with AMF, for example *N. madagascarensis* Poir. from Eastern Madagascar littoral forests (Ducousso et al. 2008) and *N. sanguinea* Lindl. from Corydon, Indiana, USA (Santiago and Darnowski 2012). Differences in the degree of AMF colonisation on host plant roots are caused by many factors. According to Permanasari et al. (2016), the number of spores is one of the factors that affect mycorrhizal colonies on plant roots. The higher the number of mycorrhizal spores, the higher the number of mycorrhizal colonies on plant roots in the soil. Furthermore, according to Yurisman et al. (2015), apart from being influenced by the number of spores, mycorrhizal colonisation is also influenced by the host plant roots. *Nepenthes* has slow and limited root development (Adamec 1997).

The results of this study showed four



a) b) c)

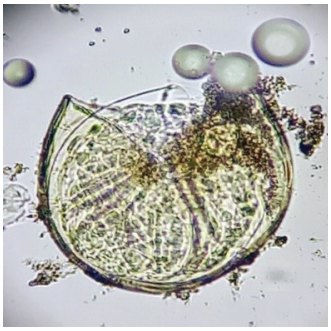
Fig. 4. Root cell structure without AMF infection a) and root cell structure with AMF infection such as hyphae, vesicles and spores b) and c).

AMF morpho-species found at the study site, namely *Glomus* sp. and *Gigaspora* sp. (Table 3, Fig. 5). The genus *Glomus* sp. has a spore development process that starts from the tip of the hyphae which enlarges to the maximum size and forms spores. Its spores are in the form of globos, subglobos, ovoid or obovoid with more than one layer of spore walls. Spores were found in loose aggregate form (Mukerji 1996, Patriyasari 2006). *Glomus* spores were round and oval or oval in shape with various colours, brown, yellowish brown to dark brown. AMF spores are formed as a result of swelling of one or more subtending hyphae in the soil or in roots (Brundrett et al. 1996). The spore shape is generally round to oval with vari-

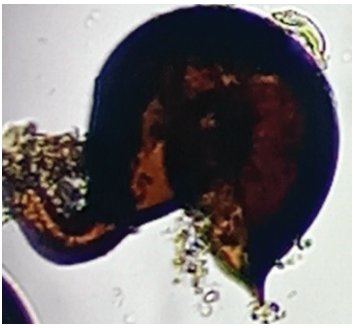
Table 3. Description of arbuscular mycorrhizal fungi spores found in study locations.

Species	Description	Location
Gigaspora sp.	Spores of the genus *Gigaspora* are translucent in colour, diameter is about 150 µm, there are boulbus suspensors, spores form spore-walls and germinal-walls.	A
Glomus sp. 1	*Glomus* genus with a distinctive character in the form of a spore-wall layer that connects what is found in the spore and the attached hyphae. The spore diameter is about 100 µm with a dark brown spore colour.	A, B
Glomus sp. 2	Spores of the genus *Glomus* are light yellow in colour with a diameter of more than 150 µm. Spores are characterised by the presence of several layers of spore-wall. The lipid-dropped spore can be seen very clearly.	A
Glomus sp. 3	Spores of the genus *Glomus* are bright yellow in colour with a diameter of 100–150 µm. Spores are characterised by the presence of a spore-wall layer that is continuous with attachment hyphae.	A

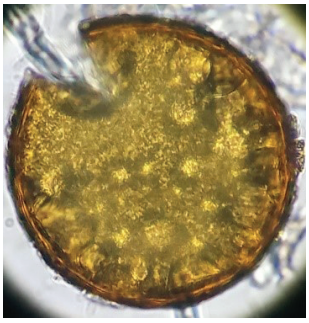
Note: A – Padang Padaeha; B – Padang Wanga (there were no spores in the pine stands).



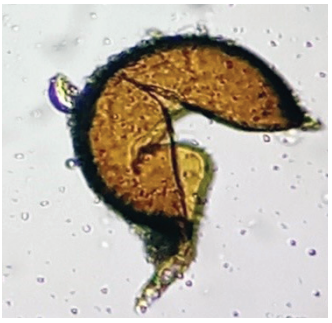
Gigaspora **sp.**



Glomus **sp. 1**



Glomus **sp. 2**



Glomus **sp. 3**

Fig. 5. Morpho-species of AMF found in *Nepenthes* spp. rhizosphere.

ous diameters. Each spore is bounded by one or more spore walls which have their own thickness. The colour varies from clear, yellow to black. The spore contains fat, cytoplasm and many nuclei. When environmental conditions allow, the spores will germinate by releasing hyphae which directly penetrate the spore wall or form a special germination structure (Nusantara et al. 2012). The development of AMF begins when it is in the soil in the form of spores so that it can infect plant roots (Solaiman and Hirata 1995).

Glomus was found the most because it has the widest distribution area and is the most tolerant of soil salinity conditions. The same results were obtained by Yusran et al. (2022), who found that it has more symbiosis with the plant of *Diospyros celebica* Bakh. compared to *Acaulospora*. The high presence of *Glomus* spores is because this genus has more species than other genera. According to Mukerji (1996), it is the largest genus with about 90 species.

Gigaspora sp. was only found in Padang Padaeha. The spores found were round in shape with a yellowish colour. This is in agreement with Alayya and Budi (2022) who stated that it spores are large, round in shape, creamy yellow, brownish yellow to blackish brown and have a rounded suspensor. *Gigaspora* sp. is a genus of AMF belonging to the family Gigasporaceae. This genus has distinctive features, including spores that are produced singly in the soil; they possess no inner spore wall layer and are globose or

sub-globose in shape, cream to yellow in colour, and 125–600 µm in size (Bentivenga and Morton 1994, INVAM 2022).

Soil condition is a factor that may influences the development and density of the number of AMF spores from the three soil samples. At the research sites in Padang Padaeha and Padang Wanga, *Glomus* sp. and *Gigaspora* sp. spores were found while in the pine stands there were no spores.

The results of physical and chemical properties analysis of the soil at the three study locations can be seen in Table 4.

Table 4. Physical and chemical properties of soil types in study sites.

No	Parameters	Study sites		
		A	B	C
1	Permeability, $cm \cdot h^{-1}$	5.36 ±0.04	1.12 ±0.10	0.49 ±0.03
2	Bulk density, $g \cdot cm^{-3}$	1.59 ±0.20	1.51 ±0.05	1.59 ±0.01
3	Porosity, %	39.92 ±0.40	43.09 ±0.20	40.09 ±0.04
4	Sand, %	76.4 ±0.15	22.3 ±0.40	45.7 ±0.26
5	Silt, %	18.7 ±0.20	50.0 ±0.19	26.0 ±0.10
6	Clay, %	4.9 ±0.20	27.7 ±0.20	28.3 ±0.20
7	pH	5.21 ±0.03	6.36 ±0.03	4.93 ±0.01
8	Organic C, %	0.48 ±0.02	4.49 ±0.02	2.79 ±0.01
9	Total N, %	0.05 ±0.001	0.38 ±0.03	0.17 ±0.01
10	CEC, cmol (+)·kg^{-1}	11.72 ±0.04	22.91 ±0.02	15.22 ±0.02
11	P_2O_5, ppm	4.51 ±0.02	5.12 ±0.10	3.32 ±0.01

Note: Value is the average from three replications, ±Standard Deviation; A = Padang Padaeha, B = Padang Wanga, C = pine stand.

Environmental conditions such as soil factors greatly determine the types of mycorrhiza that are able to develop (Hamel 2007, Helgason and Fitter 2009). Based on the results of soil analysis, the pH of soils ranged from 4.93–6.39 based on the criteria for assessing the chemical properties of the soil. pH 4.93 is classified as strongly acidic. According to Samsi et al. (2017), generally mycorrhizae are resistant to changes in soil pH so that even in alkaline or very acid soils they can be found but the amount depends on the adaptability of each AMF species. Maas and Nieman (1978) further explained that the optimum pH for the development of AMF varies. According to Tuheteru (2003) the optimum pH for AMF development ranges from 5.6–7 for *Glomus*, and pH 4–6 for *Gigaspora*.

The results of soil C-organic analysis showed that its content in Padang Padaeha was very low (0.48 %), in Padang Wanga was high (4.49 %), and in pine stands was moderate (2.79 %). C-organic is the content of organic matter in the soil which plays a role in the mineralisation process. The results of this mineralisation will produce inorganic compounds that can be directly absorbed by plants, so that nutrient needs are met. AMF spores were most commonly found in soil samples with very low organic C content, on the other hand very few in soils with high organic C content and were not found in soils with moderate organic C content. It is suspected that if there is a lot of organic matter in the soil, it will affect soil moisture so that the

AMF sporulation process is lower so that the number of spores will also be small (Burhanuddin 2012, Samsi et al. 2017). C-organic content of the soil in this study did not consistently affect the spore density in each study location. Besides C-organic, many factors affect the number of AMF spores in the soil, for example soil physico-chemical characteristics (Sivakumar 2013), host plant (Eom et al. 2000), season (Silva-Flores et al. 2019) and elevation (Melo et al. 2019).

According to Puspitasari et al. (2012), the number of spores is influenced by the interaction of several factors, including: the mycorrhiza itself, the host vegetation and soil chemical conditions such as pH, organic C and available P. Host vegetation has an influence on the formation of fine roots in the soil, where fine roots facilitate the occurrence of colonies by mycorrhizae.

Based on the results of the study, it was shown that *Glomus* sp. was the most common type of mycorrhizal spore. This shows that it has a higher level of adaptation to the environment than the genus *Gigaspora* sp. Further, explained by Alayya and Budi (2022) and Puspitasari et al. (2012), *Glomus* sp. is a mycorrhizal genus that has a wide distribution and high adaptation to environmental conditions. The physical and chemical properties of the soil that affect the AMF innoculant to infect plant roots are pH, macro- and micronutrients availability, texture, organic matter, moisture and temperature regime (Drouillon and Merckx 2005, Sjoberg 2005). When soil pH, the content of P and C-organic elements increases, the number and types of AMF fungi will increase (Muzakkir 2011).

The results of this study also support Harikumar's research (Harikumar 2013) which reported that carnivorous plants, namely *Drosera burmanii* and *D. indica*, are also in symbiosis with arbuscular mycorrhizal fungi. A fungal structure characteristic of AMF colonisation was found in both plants although the colonisation rate was low (less than 50 %). Root colonisation by AMF and spore density in the rhizosphere were higher in *D. burmanii*. Edappic factors such as soil pH and organic C content positively affect fungal development while soil moisture and P content have a negative effect.

Conclusions

Three species of *Nepenthes* were found in three study locations, namely *N. mirabilis*, *N. maxima* and *N. tentaculata,* spread of 2 species in Padang Padaeha, namely *N. mirabilis* and *N. tentaculata*, 2 species in the pine stand, namely *N. mirabilis* and *N. tentaculata*, and 3 species in Padang Wanga, namely *N. mirabilis, N. tentaculata* and *N. maxima*. There were 4 species of arbuscular mycorrhizal fungi which had symbiosis with *Nepenthes* spp., namely *Glomus* sp. 1, *Glomus* sp. 2, *Glomus* sp. 3 and *Gigaspora* sp. with the level of colonisation between 2.4 % to 16.2 %. The spore density of arbuscular mycorrhizal fungi also varied between 4–60 spores per 15 g of soil. These results imply that *Nepenthes* spp. is symbiotic with AMF and imparts useful information in the conservation management of these species in the future.

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