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ASSESSMENT OF ECTOMYCORRHIZAL FUNGI MORPHOLOGY ON SEEDLINGS OF *SHOREA LEPIDOTA* AND *S. LEPROSULA* IN ELEVATED, CONTROLLED CARBON DIOXIDE ENVIRONMENT

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In response to rising atmospheric carbon dioxide (CO_2) levels, the dynamics of Dipterocarpaceae, a crucial tree family member, has undergone significant changes that influences the associated soil microorganisms including ectomycorrhizal (ECM) fungi. This study aimed to characterise ECM fungi morphologically, focusing on Dipterocarpaceae seedlings exposed to elevated CO_2 condition, by utilising morphotyping techniques. Root samples from *Shorea leprosula* and *S. lepidota* seedlings, each with three replicates, were collected from the Open Roof Ventilation System (ORVS) at the Universiti Kebangsaan Malaysia (UKM). The ECM-infected roots were isolated under a microscope and images of ECM morphotypes were captured and recorded for identification. A total of 13 ECM morphotypes were documented across both Dipterocarpaceae species. Eleven morphotypes were observed on *S. lepidota* seedlings, with six identifiable up to the genus level encompassing the genera *Russula* and *Lactarius*. Whilst, only two morphotypes on *S. leprosula* seedlings remained unidentifiable. Significant differences in root characteristics were observed between *S. lepidota* and *S. leprosula* seedlings, with the latter exhibiting larger, taller, and longer roots. The largest surface area was recorded in *S. leprosula* seedlings (19.2 cm^2), accompanied by a higher number of root tips (482 tips) compared to *S. lepidota* (233 tips). These observations highlighted the differences in root architecture between the two species that may influence mycorrhizal associations. This underscores the distinct root morphologies between the two *Shorea* species. In conclusion, subsequent research is imperative to ascertain the species of these ECM morphotypes to ensure the sustainability of Dipterocarpaceae species.

Keywords: Carbon dioxide, Dipterocarpaceae, ectomycorrhizas, open roof ventilation system, morphotype

INTRODUCTION

Dipterocarpaceae is one of the ecologically and economically significant plant families in Southeast Asia, serving as the backbone of tropical rainforests found in the majority of forests in this region. The Dipterocarpaceae family contributes significantly to the country's commodity yield by producing both wood and non-wood products. In Peninsular Malaysia alone, approximately 157 species have been recorded within this family (Saw & Sam 2000). Within the intricate tapestry of Malaysia's ecosystems, ectomycorrhizal (ECM) fungi stand out as microorganisms dwelling in the soil which play crucial roles in the symbiotic relationships they form with Dipterocarpaceae.

The interactions between ECM fungi and Dipterocarpaceae are multifaceted, encompassing both abiotic and biotic factors. Abiotic factors include nutrient exchange (Lee & Alexander 1994, Stuart & Plett 2020), water relations (Alexander et al. 1992, Sevanto et al. 2023), protection against stresses (Mohd Salleh et al. 2013, Essene et al. 2017), and broader contributions to forest ecosystem dynamics (Smith & Read 2008, Brearley et al. 2017). Whilst, biotic factors include root associations with mycorrhiza such as arbuscular mycorrhiza (AM) (Shi et al. 2014, Nur Aqilah 2018) and ECM (Becker 1983, Lee & Watling 1998). Further, underground microorganisms such as archaea and bacteria have also been reported to exist on Dipterocarpaceae roots (Nur Aqilah 2018).

Ectomycorhizal fungi, particularly in association with the Dipterocarpaceae family, play vital roles in nutrient exchange, water relations, and protection against stresses, contributing to the overall dynamics of tropical ecosystems. The identification of ECM has traditionally relied on the meticulous examination of fruiting bodies or sporocarps, as highlighted by recent studies (Nur Aqilah et al. 2020a, Nur Aqilah et al. 2020b). Additionally, advancements in molecular techniques, as evidenced in the works of Sirikantaramas et al. (2003) and Kaewgrajang et al. (2023), have significantly enriched our understanding of the diversity and ecological roles of ECM in these regions. Despite these commendable strides in the field, there exists a conspicuous gap in research, particularly concerning the morphology and anatomy of ECM fungi in tropical ecosystems. Moreover, the escalating levels of atmospheric carbon dioxide (CO_2) not only affect plant physiological activities but extend their influence to soil properties and the intricate underground relationships, especially the growth patterns of ECM fungi (Nur Aqilah et al. 2025).

Amidst these considerations, the escalating levels of CO_2 emerge as a primary factor with profound implications for climate dynamics. Beyond impacting plant physiological activities such as altered photosynthetic rates, stomatal conductance, and transpiration processes (Lloyd & Farquhar 2008, Wahidah et al. 2017, Alzate-Marin et al. 2021), this perturbation extends to influence nutrient availability, microbial activities, and overall soil health (Jin et al. 2019, Nottingham et al. 2020) as highlighted in previous studies. Notably, CO_2 concentration levels play a pivotal role in reshaping the growth patterns of ECM fungi, impacting critical parameters such as root extension, root diameter, biomass, and colonisation (Wang et al. 2015, Ostonen et al. 2007). The intricate dynamics of these responses underscore the interconnectedness of above-ground and below-ground components in the face of changing environmental conditions.

In light of these considerations, the present study directs its focus towards characterisation of the morphology of ECM fungi associated with selected dipterocarp seedlings (*S. leprosula* and *S. lepidota*) under conditions of heightened CO_2. Employing morphotyping methods, this investigation seeks to unravel the intricacies of structural adaptations and responses of ECM fungi to changing environmental conditions caused by elevated CO_2 levels. It is hoped that the knowledge gained from this study regarding ECM communities is indispensable for the effective conservation and restoration of the Dipterocarpaceae family. This is especially important for areas undergoing regeneration in the forest landscape in Malaysia.

MATERIALS AND METHODS

Study location

This research was carried out in a greenhouse known as the Open Roof Ventilation System (ORVS) located at the Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor (N 02° 55.145', E 101° 46.465') (Figure 1). The ORVS is a purpose-built artificial habitat designed to simulate an environment with elevated CO_2 concentrations, surpassing normal levels to mimic anticipated future conditions. The temperature, humidity, and CO_2 concentrations within the ORVS were meticulously regulated and monitored, with temperature inside the ORVS ranged between 25–45 °C, while humidity levels fluctuated between 37–87%. The CO_2 treatment was administered daily by continuously releasing pure CO_2 through spraying for two hours (9–11 am) at concentrations of 800–950 ppm. Following this two-hour period, the CO_2 level in the ORVS should approach those of the external environment (Wahidah et al. 2017, Nor-Atikah et al. 2020). The CO_2 gas was supplied through cylinders connected to the air handling system for the open-air space and diffused into the air.

Figure 1 (a) Study location at UKM Bangi, Selangor, (b) image showing *S. leprosula* tree, and (c) image showing *S. leprosula* leaves

Figure 2 (a) The tree seedling roots are placed in a petri dish filled with water against a white background and (b) morphological observations are conducted under a dissecting microscope using white light

Root ECM sampling from dipterocarp seedlings

The ECM root samples were obtained from *S. leprosula* and *S. lepidota* seedlings. Root samples were collected by excavating the soil using a soil auger until the root portion was exposed. Careful separation of the roots from the seedling stem was performed using a sharp, small knife before placing them into zip-lock plastic bags. Subsequently, the root samples were cleaned in the laboratory with a soft brush to minimize damage to the ECM roots (Seress et al. 2016, Defrenne et al. 2019). Finally, cleaned root samples were placed in square-shaped transparent containers and submerged in distilled water.

Sample analysis

Root samples were scanned using the Epson Perfection V800 Photo scanner along with WinRHIZO Pro software for data analysis. This includes surface area, width, height and morphological data (surface area, number of root tips, average diameter). The soil-free roots were then placed in large petri dishes and submerged in water until next procedure (Figure 2). Morphological characterisation was conducted by observing the roots under white

Table 1 Summary of the number of morphotypes found in seedlings of *S. lepidota* and *S. leprosula*

Host Plant	Morphotype	Family	Genera	Spesies (expected)	*Shorea lepidota*			*Shorea leprosula*		
					R1	R2	R3	R1	R2	R3
Shorea lepidota	M1	Russulaceae	*Russula*	*Russula* sp.	√	X	X			
	M2	Unidentified	Unidentified	Unidentified	√	X	X			
	M3	Russulaceae	*Lactarius*	*Lactarius* sp.	√	X	X			
	M4	Russulaceae	*Lactarius*	*Lactarius* sp.	√	X	X			
	M5	Russulaceae	*Lactarius*	*Lactarius* sp.	X	√	X			
	M6	Russulaceae	*Russula*	*Russula* sp.	X	√	X			
	M7	Russulaceae	*Russula*	*Russula* sp.	X	√	X			
	M8	Russulaceae	*Russula*	*Russula* sp.	X	X	√			
	M9	Unidentified	Unidentified	Unidentified	X	X	√			
	M10	Unidentified	Unidentified	Unidentified	X	X	√			
	M11	Unidentified	Unidentified	Unidentified	X	√	X			
Shorea leprosula	M12	Unidentified	Unidentified	Unidentified				√	X	X
	M13	Unidentified	Unidentified	Unidentified				√	X	X

light using a surgical microscope (Wild M3B Heerbrugg, Switzerland). Images of each ECM morphotype were captured using DinoCapture 2.0 software. Using forceps, ECMs were categorised based on morphological features such as branching, colour and size according to Agerer (1986), Defrenne et al. (2019) and Janowski & Leski (2023), and carefully placed into several petri dishes. Each ECM morphotype was labelled and recorded. ECM identification was carried out with reference to Agerer (1987–2006) and via DEEMY database (An Information System for Characterisation and Determination of EctoMYcorrhizae) (http://www.deemy.de/) (Agerer & Rambold 2013). The pictures of the morphotypes obtained in this study were deposited in the FRIM Fungarium.

RESULTS AND DISCUSSION

Morphotypes found in *Shorea* species

In this study, a comprehensive analysis of ectomycorrhizal associations in two *Shorea* species, *S. lepidota* and *S. leprosula*, was conducted. A total of 13 morphotypes were meticulously identified in the examined seedlings, and Table 1 presents a detailed tabulation of these morphotypes across three replicates for each *Shorea* species. Among the identified morphotypes, 11 were specifically associated with *S. lepidota*, while the remaining two morphotypes were found exclusively on *S. leprosula*. Through comparison with information from several textbooks (Agerer 1987–2006, Kumar & Atri 2019), reliable website (http://www.deemy.de/), discussion with experienced mycologist and further taxonomic scrutiny, it was determined that out of the 13 morphotypes, seven were classified at the genus level within the Russulaceae family. These were specifically identified as belonging to the genera *Lactarius* (comprising three morphotypes) and *Russula* (comprising four morphotypes). To enhance the understanding of these morphotypes, their morphological characteristics were meticulously detailed. This included aspects such as ramification, branching patterns, coloration, and size. This comprehensive description serves to illuminate the distinct attributes of each morphotype, contributing valuable information to the study. Figure 3 shows visual representation and a clearer comprehension of the observed differences between the morphotypes associated with *S. lepidota* and *S. leprosula*.

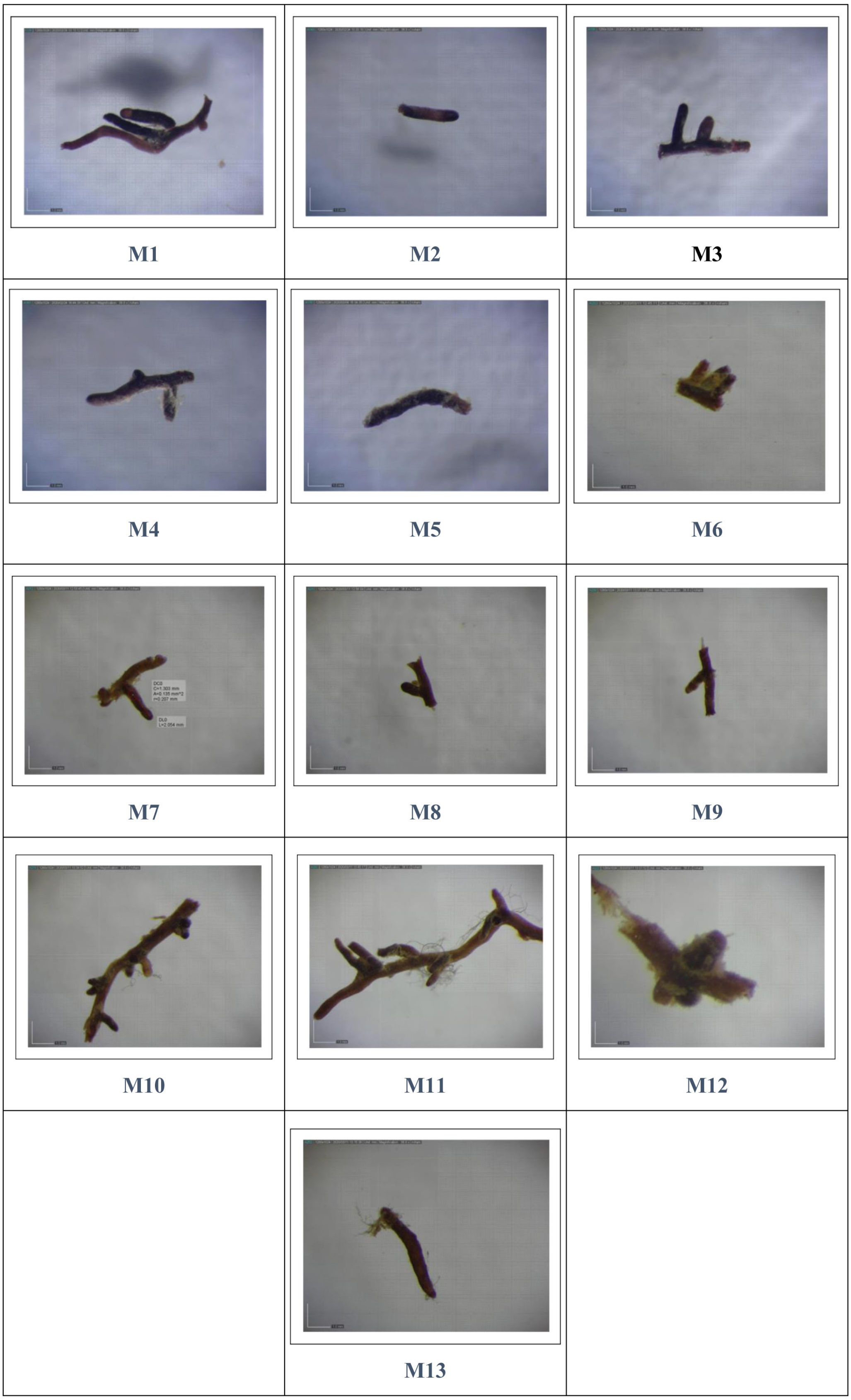
Characterisation of identified ectomycorrhizas morphotype associated with *Shorea lepidota* and *S. leprosula* seedlings

Of all 13 morphotypes identified to belong to either *S. lepidota* and *S. leprosula*, only seven were successfully identified at family and genus level (Figure 3). The morphological traits of the identified root morphotypes (M1 to M8) align with descriptions provided in the literature (Agerer 1986, Sakakibara et al. 2002, Janowski & Leski 2023). Among them, species like *Russula* and *Lactarius* stood out with unique features such as their branching patterns, rhizomorph structures, and mantle surface characteristics. These morphotypes also displayed a fascinating range of colors and shapes, reflecting their diversity and specialised roles in supporting the host plants. These findings shed light on the complex and vital relationships between dipterocarp species and their fungal partners in tropical forest ecosystems. The characterisation of each successful *Shorea* morphotypes were described in Table 2.

Physical root characteristics assessment between *Shorea lepidota* and *S. leprosula* seedlings

The root characteristics of both *S. lepidota* and *S. leprosula* seedlings were measured across three replicates (R1, R2, R3) for each species. The key results were tabulated in Table 3 below.

Our investigation uncovered significant variations in root characteristics between *S. lepidota* and *S. leprosula* seedlings. Specifically, *S. leprosula* exhibited larger root areas, taller roots, and longer roots compared to *S. lepidota*. Interestingly, the average diameter did not consistently differ between the two species. Notably, the largest surface area was recorded in *S. leprosula* seedlings, measuring 19.21 cm^2, with an overall average of 11.88 cm^2. Additionally, the number of tips on *S. leprosula* (482 tips) surpassed that of *S. lepidota* (233 tips). It is crucial to note that the number of tips found is influenced by the harvested root area. The mean number of tips extracted from *S. lepidota* roots was 77.7 tips,



M1 M2 M3

M4 M5 M6

M7 M8 M9

M10 M11 M12

M13

Figure 3 M1, M2, M3, M4, M5, M6, M7, M8, M9, M10 and M11 depict morphological images of morphotypes for ECM roots in seedlings of *S. lepidota*. M12 and M13 illustrate morphological images of morphotypes for ECM roots in seedlings of *S. leprosula*. M1, M7, M9, M10, M11, M12 and M13 could not be identified

Table 2 Description of morphotypes found in examined seedlings of *S. lepidota* and *S. leprosula*

Morpho-type	Predicted genus/ species	Branching type	Rhizo-morphs	Non-branched morphology	Distal tip morphology	Length (mm)	Diameter (mm)	Colour	Mantle surface	Hyphae	Other notes
1	*Russula* sp.	Monopodial pinnate	Present (-)	Straight/ curved	Cylindrical, Non-bulging	N/A	N/A	Beige to dark beige/ red	Visible, no cortical cells	Rare	No latisifiers, Carbonate mantle absent
3	*Lactarius* sp.	Monopodial pinnate	Rare	Straight/ slightly curved	Cylindrical, Non-bulging	0–5	0.35–0.6	White to gray/ yellowish-gray	Smooth/ silver	Absent	Non-translucent, no sclerotia
4	*Lactarius* sp.	Monopodial pinnate	Low	Straight	Non-bulging	0–3.3	0.43–0.53	Beige/ orange to grayish	Smooth	Not recorded	No mantle points, no sclerotia
5	*Lactarius* sp.	Monopodial pinnate	Present, no conical structures	Curved/ sinuous	Cylindrical, Non-bulging	0–10	0–0.2	Beige/ yellow/ orange/ white	Visible, shiny	Not recorded	Latisifiers present, no sclerotia
6	*Russula* sp.	Monopodial pinnate	Not visible	Straight/ sinuous	Non-bulging, cup-shaped	0–15	N/A	Beige/ orange to grayish	Smooth	Rare	Latisifiers visible, no sclerotia
8	*Russula* sp.	Monopodial pinnate/ monopodial pyramid	Rare	Straight/ curved	Cylindrical, non-bulging	0–3	0.25–0.35	Beige-yellow/ white	Wrinkled, same as mantle	Rare	No dimorphism, no sclerotia

Table 3 Physical characteristics of seedling roots of *S. lepidota* and *S. leprosula*

Root Characteristics	*Shorea lepidota*			*Shorea leprosula*		
	R1	R2	R3	R1	R2	R3
Area (cm^2)	64.18	56.76	59.13	96.68	94.08	139.36
Width (cm)	7.58	7.64	7.29	7.87	7.24	8.21
Height (cm)	8.47	7.43	8.10	12.24	12.99	16.97
Length (cm)	27.17	43.58	45.89	55.80	67.45	134.08
Surface area (cm^2)	4.58	5.55	4.95	5.87	10.58	19.21
Volume (cm^3)	0.06	0.06	0.04	0.05	0.13	0.22
Average diameter (mm)	0.54	0.41	0.34	0.47	0.49	0.46
Number of tip	61	84	88	147	14	321

whereas *S. leprosula* exhibited a higher mean with 160.7 tips.

The investigation into symbiotic relationships between ECM and Dipterocarpaceae has been a topic of research since its discovery in late 1960s (Singh 1996). The observed ECM associations in *Shorea* species may play a pivotal role in nutrient cycling within the studied ecosystem. One of key function of ECM fungi is to enhance nutrient uptake, particularly phosphorus (P) (Stuart et al. 2022, Yang et al. 2022), for host plants comprehending the ecological dynamics of these tropical forest ecosystems. This heightened nutrient acquisition can contribute to the overall health and vigor of *Shorea* species influencing their growth, survival, and ecological success. To ascertain the presence of ECM, roots undergo swelling and exhibit increased thickness compared to other lateral roots, owing to the mantle structures and hyphal systems on the root surface (Clowes 1950).

In this study, the morphotypes of ECM identified on *Shorea* seedling roots were characterised based on various morphological features, including the presence of rhizomorphs, hyphal systems, morphotype shape, tip characteristics, and colour. These morphological traits aligned consistently with the findings of previous researchers (Sakakibara et al. 2002, Defrenne et al. 2019, Janowski & Leski 2023). On average, the reported number of morphotypes is around 2–3 morphotypes per tree. This is supported by previous studies where the number of ECM morphotypes for individual dipterocarp trees is estimated to be up to five morphotypes, with 2–3 being the median number (Lee & Alexander 1996, Brearley et al. 2003, Brearley 2012). For temperate trees, Godbold et al. (2015) found that an average of 2.7 ECM species are found on each tree root, with an estimated range of 1-5 species per tree root.

Among the seven identifiable morphotypes, all belonging to the Russulaceae family, *Russula* (4 morphotypes) and *Lactarius* (3 morphotypes) genera were discerned on the roots of *S. lepidota* seedlings. The dominance of these two genera was consistent with previous studies reporting the prevalence of one or two ECM species in *Shorea* species (Lee & Alexander 1996, Ingleby et al. 1998). In line with Lee et al. (1997) and Ulfa et al. (2019), it is noteworthy to note that Dipterocarpaceae is frequently associated with the Russulaceae family.

The presence and diversity of ECM depend on various factors, including plant traits such as size (Glassman et al. 2017; Rudawska et al.

2018), genotype (Gehring et al. 2017, Lamit et al. 2016), along with soil conditions (Ding et al. 2020, Hicks Pries et al. 2023). In this investigation of *S. lepidota* seedlings, a greater abundance of ECM morphotypes was unveiled compared to *S. leprosula*. These findings aim to contribute to the limited research on ECMs in *S. lepidota* as the exploration for ECM morphotypes linked with *S. lepidota* in existing literature was limited, underscoring the necessity for a more comprehensive observation in our study. It is apparent that the results of this study display a lower number of morphotypes compared to previous reports in Malaysia. For instance, Becker (1983) described 10 ECM morphotypes from *S. leprosula* in the Pasoh Forest Reserve, Negeri Sembilan. Meanwhile, Lee et al. (1997) observed 24 ECM morphotypes on *S. leprosula* from various sites in Peninsular Malaysia and Lee et al. (1996) reported 36 ECM morphotypes in the Danum Valley, Sabah, East Malaysia.

There are other biotic factors that may influence these results. One such factor is the root structure of the plants. Previous studies suggested that root structure influences mycorrhizal associations (Alexander et al. 1992, Comas et al. 2014). This is consistent with our finding showing root architecture differences between *S. leprosula* and *S. lepidota* including larger root area and longer roots in *S. leprosula*. The average diameter did not consistently differ between the two species. The root system of angiosperms and gymnosperms comprises of short and long roots. The long roots near the stem base exhibit secondary thickening preventing the formation of mycorrhiza on the long roots. Short roots located at the branch ends lack in thickening and is considered as a better choice for mycorrhiza formation (Chilvers & Gust 1982, Waisel et al. 2002). This characteristic was also consistent with studies done by Jalonen et al. (2009) who described the advantage of abundant lateral roots in establishing mycorrhizal associations. Additionally, the morphological characters of *S. leprosula* roots characterised by a hard and slippery surface with root hairs on the distal part and having plenty of lateral roots (Harahap et al. 2018), contribute to the understanding of mycorrhizal associations in this species. However, it is crucial to note that currently there is a lack of information describing the morphology of *S. lepidota* roots.

Furthermore, substantial percentage (60–80%) of ECM morphotypes could not be identified at the family, genus or species levels in this study. This common challenge in identifying fungal species in tropical forests is attributed to insufficient references for tropical ECMs (Lee et al. 2010) and dependence on host species (Janowski & Leski 2023). Moreover, the growing interest and knowledge in tropical ECMs are evident from the increasing number of publications in the field (Corrales et al. 2018). Online tools facilitating the morphological identification of ECM roots such as the Ectomycorrhizae Descriptions Database (EDD) and the DEEMY platform, proved to be indispensable (Goodman et al. 2000, Agerer 1987–2006). In addition, molecular techniques such as those employed by Jumpponen et al. (2015) and Janowski & Leski (2023) have played a crucial role in confirming unidentified morphotypes, despite the many challenges which still persist (Landeweert et al. 2003, Lücking et al. 2020). Notably, the molecular database for tropical ECMs remains inadequate (Smith et al. 2013, Foster et al. 2022). Thus, our study emphasises the significance of morphological identification in the absence of molecular data. The challenges outlined here underscore the importance of continued research to enhance our understanding of tropical ECMs, contributing to valuable insights despite the existing constraints.

This study does not compare morphotypes under two different conditions—elevated CO_2 and ambient—directly but more accurately, serves as a preliminary guideline. An intriguing observation in our study was that a significant portion of the sampled dipterocarp seedling roots did not exhibit ECM presence. This was similarly reported by Godbold et al. (2015) in their observations on uncolonised roots in *Fagus sylvatica* trees in Bangor, UK, particularly in conditions with elevated atmospheric CO_2 levels. This phenomena can be attibuted to the effect of CO_2 on the difference in the identities of root-colonising fungal taxa between ambient and elevated CO_2 treatments as observed in the C4 grass species (Frew et al. 2021), or when large changes in atmospheric chemistry often affected ECM communities much less at the root tip level compared to the sporocarp level (Andrew & Lilleskov 2014).

CONCLUSION

This study characterised 13 distinct ectomycorrhizal (ECM) morphotypes from *S. lepidota* and *S. leprosula* seedlings, with seven identified at the genus level within the Russulaceae family. *Shorea leprosula* exhibited larger root surface areas and more root tips compared to *S. lepidota*, which may influence the diversity of ECM associations. These findings provide valuable insights into the morphological adaptations of ECM in response to elevated CO_2 conditions, contributing to the understanding of plant-fungal interactions in tropical ecosystems. Future research should incorporate molecular techniques to identify ECM morphotypes more precisely, as the challenges of unresolved fungal species remain significant. Furthermore, studies should investigate the functional roles of these associations in nutrient cycling and stress resistance under different environmental conditions. Examining the long-term impacts of elevated CO_2 on ECM community dynamics could yield insights critical for forest regeneration strategies. Expanding this research to include other *Shorea* species and members of the Dipterocarpaceae family will deepen our understanding of specificity and adaptability in ECM associations, ultimately contributing to the conservation and management of tropical forests in the face of climate change.

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