JOURNAL OF TROPICAL LIFE SCIENCE
2020, Vol. 10, No. 2, 89 – 95
http://dx.doi.org/10.11594/jtls.10.02.01

Research Article

Growth and Development of *Tristaniopsis merguensis* Seedling Inoculated by Natural Ectomycorrhiza

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Article history:
Submission October 2019
Revised December 2019
Accepted January 2020

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ABSTRACT

Tristaniopsis merguensis (*pelawan* tree, Indonesia) is a potential plant as a firewood, host for edible fungi, and nectar from the flower produce bitter honey. The existence of *T. merguensis* in the forest is important because the fungi grow under the tree, particularly in the above of the roots. However, it has not cultivated and conserved optimally due to slow growth and lack of information about the cultivation. Information about how to grow the *T. merguensis* seeds is very limited; therefore, research in growth and development of *T. merguensis* seedling was important. The aims of this study were to determine the appropriate planting medium for *T. merguensis* seed germination and to stimulate growth and development of *T. merguensis* seedling by using its fungi. Status of *T. merguensis* fungi was studied by analyzing root morphology. Fungi isolation was carried out from colonized root and fungi fruit body. The stimulated growth of *T. merguensis* seedling was carried out by using fungi isolated from colonization root in laboratory scale, as well as treated at a different phosphate concentration. The result showed that *T. merguensis* was associated with ectomycorrhizal fungi. Planting medium consists of sawdust and *T. merguensis* fine root resulted the highest percentage of germination. Seedlings were treated with ectomycorrhizal fungi grew better than without ectomycorrhizal fungi. The treatment of ectomycorrhizal fungi and phosphate 25% showed the highest growth rate.

Keywords: Ectomycorrhizal fungi, Sawdust, Heimiporus fungi, Tristaniopsis

Introduction

In Indonesia, *Tristaniopsis merguensis* (Myrtaceae), known as the Pelawan trees is a valuable tree have a strong and dy wood for firewood, furniture, build houses, and timber stands. Local people using the *T. merguensis* leaves for tea. The leaves have alkaloids, phenols, tannins, and flavonoids as antioxidants [1]. In addition, these trees produce bitter honey from flowers and edible fungi (*Heimiporus* sp) where growing under the tree canopy and above its roots. These fungi grow only at the beginning of the rainy season, once a year. The color of fungi is red when fresh and expensive in local market and for export.

T.merguensis grows in evergreen forests at low altitudes and on ridges up to 1,000 m altitude. It is a tree up to 30 m tall, with its smooth, light-brown, trunk bark flaking off in large, spiral, scroll-like pieces. The distribution of these plant is in Andaman Islands, Mergui Archipelago, Cambodia, Vietnam, Peninsular Malaysia, Singapore, Nepal, and Indonesia, especially found in Kalimantan [2, 3, 4]. *T. merguensis* tree in Indonesia also grows in the Province of Kepulauan Bangka Belitung. This species has been recognized as one of the key species for biodiversity sustainability in Kepulauan Bangka Belitung, since the *T. merguensis* tree can guarantee the growth of the fungi and as a feed trees by honey-bee [5]. Naturally, *T. merguensis* tree grows in the forest. Thus, *T. merguensis* is a tree with high economic value; however, it is not cultivated and conserved optimally due to slow growth and lack of information about cultivation. Currently, *T. merguensis* trees are rarely found in the Province of Kepulauan Bangka Belitung due to excessive exploitation. It is impor-

How to cite:
Triadiati T, Hidayanti AR, Sukarno N (2020) Growth and Development of *Tristaniopsis merguensis* Seedling Inoculated by Natural Ectomycorrhiza. Journal of Tropical Life Science 10 (2): 89 – 95. doi: 10.11594/jtls.10.02.01.

Table 1. Percentage of germination, plant height, and number of leaves of *T. merguensis* seedlings (8 WAP)

No. of Tray	Treatments	Germination (%)	Plant height (cm)	Number of leaves (individual)
1	Zeolite + *T. merguensis* fine roots	7[c]	3.5[a]	4[a]
2	Coco peat + *T. merguensis* fine roots	23[b]	3.2[a]	5[a]
3	Husk charcoal + *T. merguensis* fine roots	38[b]	3.8[a]	6[a]
4	Sawdust + *T. merguensis* fine roots	64[a]	4.0[a]	6[a]
5	Sand : soil (1:1)	0	0	0
6	Husk charcoal : soil (1:1)	0	0	0
7	husk charcoal : sand (1:1)	0	0	0

The data was followed by the same letter in the same column indicate not significantly different (Duncan-test; α=5%)

tant that this tree is retained to grow naturally for the purpose of fungi growth and bitter honey. Therefore, there is an important need to develop *ex situ* propagation to support replanting in natural habitats. On the other side, the study of *T. merguensis* tree propagation is very limited.

Myrtaceae is a family of plant that forming symbiotic relationships with ectomycorrhizal fungi [6]. *Tristaniopsis obovata* was reported to form a symbiotic association with *Cenococcum geophilum* ectomychorriza [7]. Because *T. merguensis* is included in Myrtaceae, we hypothesized that *T. merguensis* also has an opportunity to symbiosis with ectomycorrhizal. However, it is not known yet which fungi can form a symbiotic association with *T. merguensis* trees. Usually, the local people took *T. merguensis* seedlings from the forest for replanting. Until now, it is difficult to grow the *T. merguensis* seeds out-side the natural habitat *(ex-situ).* Therefore, it is important to find a method for germination and growth of *T. merguensis* seed *ex-situ* to support the sustainability diversity of *T. merguensis* biodiversity in the Province of Kepulauan Bangka Belitung. The purposes of this study are to discover the optimal media for germinating and cultivate of *T. merguensis* and optimizing the growth and development of seedlings by using natural ectomycorrhiza.

Material and Methods

Study Area

Materials used in this research were *T. merguensis* seeds, zeolite, coco peat, sand, husk charcoal, sawdust, soil, Johnson solution [8] and a soil-mycelium system (fine root and local inoculum from *T. merguensis* forest) taken from *T. merguensis* forest in Center Bangka Tengah District, Kepulauan Bangka Belitung Province where located at 2°19'18" S and 106° 09'51" E.

*Bioassay of symbiosis***.**

T. merguensis **s**eeds were surface sterilized by soaking in 70% alcohol (v/v) for one minute and then rinsed with sterile aquadest five times. *T. merguensis* **s**eed was dipped in 0.05% chlorox (v/v) for one minute and rinsed with sterile aquadest five times. Furthermore, sterilized seeds germinated in sterile zeolite media. Seedling of *T. merguensis* is transferred to pots with sterile zeolite and treated with top soil containing ectomycorrhizal mycelium collected from *T. merguensis* forest and Johnson solution. Johnson solution was used as a phosphate treatment. The plants without inoculation received phosphate treatments of 0% (P0), 25% (P25-k), 50% (P50-k), and 75% (P75-k), whereas the inoculated plants received phosphate treatments of 25% (P25-i), 50% (P50-i), and 75% (P75-i), respectively. Each treatment was carried out in four replicates. Parameters observed were the number of leaves and plant height weekly until 20 weeks after planting (WAP). Harvest of the root was carried out at 20 WAP (week after planted) for observation of ectomycorrhizal colonization.

Germination of T. merguensis seed

Seeds were collected from *T. merguensis* trees where grown in the forest. About 1,000 of *T. merguensis* seeds were spread in a tray with planting medium. The composition of planting medium consists of three layers. Seeds were spread on the surface of second layer of planting medium. The treatments of planting medium were shown in Ta-

ble 1. The design of this research was completely randomized design with five replicates. All treatments were placed under the plastic lid for 2 months until the seedlings have 4 leaves. The humidity of planting medium was maintained about 70-80 % by watering every day with sprayer. The parameters measured were percen-tage of germination, plant height, and number of leaves during 8 WAP.

Data analysis

All data were analyzed using general linear models (GLMs). All post hoc tests were carried out using DMRT. The standard level of significance was $p < 0.05$. All analyses were done using SPSS 20.0 software.

Results and Discussion

Germination of T. merguensis seeds

The *T. merguensis* seeds were spread in tray number 1 - 4 germinated a week after spreading, whereas no germination in tray number 5 – 7 (Table 1). Tray number 1 to 4 contained *T. merguensis* fine roots, on the other side, tray 5 to 7 did not contain fine roots. The tray no. 4 had the highest percentage of germination.

To analyze the *T. merguensis* seedlings growth rate we used treatments of natural ectomycorrhiza mycelium (isolated from *T. merguensis* roots in the forest) and different phosphate concentrations (Johnson solution) in the laboratory. Figure 1 shows that all treatments have a quite similar growth rate pattern during 19 WAP, but treatment of phosphate 25 % with inoculation (P25-i) has the best growth indicated by plant height. On the other side, leaves number of controls was significantly different ($p < 0.05$) with the inoculation and phosphate concentrations treatments during 19 WAP (Figure 2). The number of leaves and plant height of *T. merguensis* seedlings were influenced ($p < 0.05$) by interaction between ectomycorrhizal mycelium and different phosphate concentrations treatments. The leaves number and plant height of *T. merguensis* seedlings treated by phosphate 75 % with inoculation (P75-i) have the highest than others at 19 WAP (Table 2).

Symbiotic bioassay of T. merguensis seeds

Symbiotic bioassay of *T. merguensis* seeds was conducted to prove that symbiotic fungi affected *T. merguensis* seedlings growth. The plant

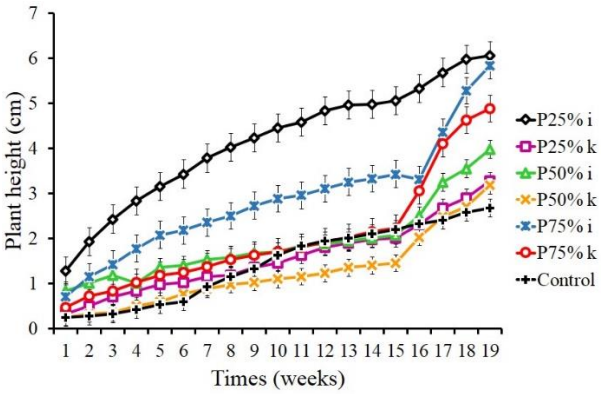

Figure 1. Growth rate of *T. merguensis* seedlings using plant height treated by ectomycorrhizal mycelium and different phosphate concentrations (Johnson solution) in laboratory

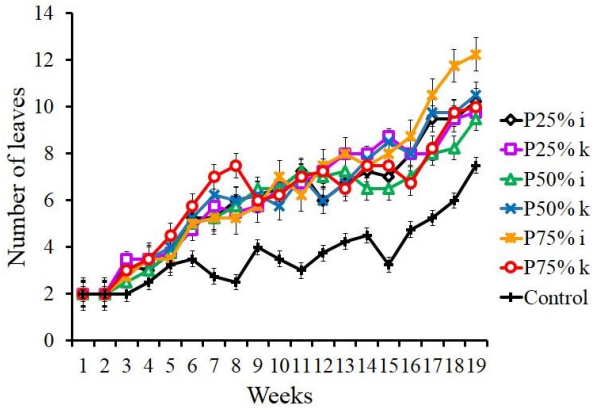

Figure 2. Leaves growth of *T. merguensis* seedlings treated by ectomycorrhizal mycelium and different phosphate concentrations (Johnson solution) in laboratory

height of *T. merguensis* seedlings treated by phosphate and without inoculation was not significantly different from control. The plant height of *T. merguensis* seedlings was not influenced by treatments of phosphate without inoculation (Figure 1 and 3). On the other side, the plant height of P25-i was higher than that of P50-i and P75-i (Figures 1 and 4). The number of leaves from *T. merguensis* seedling of all phosphate and with or without inoculation treatments were not significantly different, but significantly different from control (Figure 2).

The leave color of *T. merguensis* seedlings was treated by ectomycorrhizal fungi with phosphate greener than that of control. The treatment of P25% with ectomycorrhizal fungi i noculation P25-i) exhibited dark green (scale of 5-6) than all the phosphate treatments (Table 3).

Table 2. Number of leaves and plant height of *T. merguensis* seedlings treated by ectomycorrhizal mycelium and different phosphate concentrations (Johnson solution) in the laboratory (19 WAP)

Treatment of phosphate	Treatment of inoculation	Number of leaves	Plant height (cm)
Control		3^{c}	2.7^{c}
P25%	without inoculation (k)	10^{b}	3.3^{bc}
	with inoculation (i)	10^{b}	6.1a
P50%	without inoculation (k)	10^{b}	3.2^{bc}
	with inoculation (i)	11^{b}	4.0^{bc}
P75%	without inoculation (k)	10^{b}	4.9^{b}
	with inoculation (i)	13^{b}	5.8^{a}

The data was followed by the same letter in the same column indicate not significantly different (Duncan-test; $\alpha = 5\%$)

Table 3. Leaves color based on color chart* of *T. merguensis* (20 WAP)

Treatment of phosphate	Treatment of inoculation	Leaves color scale based on color chart *	Note
Control	-	2 to 3	Yellow to green yellowish
P25%	without inoculation (k)	4 to 5	Green to dark green
	with inoculation (i)	5 to 6	Dark green to very dark green
P50%	without inoculation (k)	4	Green
	with inoculation (i)	3 to 4	Green yellowish to light green
P75%	without inoculation (k)	3 to 4	Green yellowish to light green
	with inoculation (i)	3	Green yellowish

*Leaf Color Chart from IRRI (scale 1-6)

The root of seedlings was treated without inoculation (k) did not form a symbiosis with ectomycorrhizal mycelium. On the other side, the seedlings were treated with inoculation and 25% phosphate (P25-i) formed colonization. Treatment of inoculation with 50% phosphate (P50-i) and 75% phosphate (P75-i) did not have root colonization (Table 4).

The root of *T. merguensis* treated with ectomycorrhizal fungi has shown in initial colonization from mycelium structures surrounding the outer surface of the root (Figure 5).

The symbiotic between the *T. merguensis* and the fungus was analyzed using *T. merguensis* growth parameters. In this study, the plant height of the treatment 25% phosphate with inoculation (P25-i) was higher than that of the treatment 25 % phosphate without inoculation (P25-k) (Table 2). The increased plant height of the 25% phosphate treatment with inoculation (P25%-i) may have been influenced by successful of symbiosis between ectomycorrhiza and seedling roots of *T. merguensis.* This data supported by observations on the roots of *T. merguensis* inoculated by ectomycorrhizal fungi. The other study reported that the plant height of *Populus hopeiensis* (myco-rrhizal plants) was significantly stimulated with ectomycorrhiza (*Boletus edulis*) inoculation [9]. The root of *T. merguensis* which was colonized with ectomycorrhiza have hyphal growth on the outer surface of the root. This result is consistent with research conducted by Taylor and Alexander; Perrier *et al*. that colonization of plant roots by ectomycorrhiza was characterized by existing hypha in surface of root until penetrating the root epidermis [10, 11]. Therefore, the height of *T. merguensis* seedlings was inoculated with a soil-mycelium system was higher than that of seedlings without inoculated. The mutuality symbiosis between ectomycorrhizal fungi with plant's roots can increase the height of the host plant [12]. Mycorrhizal fungi regulate nutrient flow between the soil and plants [10], it helps in the absorption of the relatively immobile ions in soils such as phosphate, copper, and zinc [13]. This mutualistic symbiosis provides the fungi with relatively constant and direct access to carbohydrates, such as glucose and sucrose [14, 15]. The carbohydrates are translocated from leaves to root tissue and on to the plant's host. In return, the plant gains the bene-

Table 4. Colonization evaluation of *T. merguensis* seedling treated by phosphate (Johnson solution) and ectomycorrhiza inoculation for 5 months.

Treatment of phosphate	Treatment of inoculation	Colonization	Response
Control		no colonization	- (neg.)
P25%	without inoculation (k)	no colonization	- (neg.)
	with inoculation (i)	colonization	+ (pos.)
P50%	without inoculation (k)	no colonization	- (neg.)
	with inoculation (i)	no colonization	- (neg.)
P75%	without inoculation (k)	no colonization	- (neg.)
	with inoculation (i)	no colonization	- (neg.)

fits of the mycelium's higher absorptive capacity for water and mineral nutrients due to the comparatively large surface area of mycelium/root ratio, thus improving the plant's mineral absorption capabilities [16]. Most plants consist of two P-uptake pathways, namely the direct root P-uptake pathway and the mycorrhizal fungi P uptake pathway [17]. Most P fertilizers are immobilized in soils because P is strongly adsorbed to iron and aluminium cations at low soil pH and to calcium at high soil pH [18, 19]. Thus, root architectural features and the growth of mycorrhizal hyphae are important for maximizing the acquisition of P because the root and mycorrhizal systems with a relatively high surface area are able to effectively use a given volume of soil [20]. The mycelium of the ectomycorrhizal fungi can access these phosphorus sources and make them available for the host plants. Phosphate ions are required by the host plants to stimulate early growth and development and root formation [21].

In this study, the *T. merguensis* fine roots in planting media stimulated the germination of *T. merguensis* seeds. When compared with the treatments without *T. merguensis* fine roots in planting medium, it was assumed that fine roots of *T. merguensis* affected the germination of *T. merguensis* seeds. The *T. merguensis* fine roots is thought to be symbiotic with fungi forming ectomycorrhiza. Symbiotic association between plant roots and certain fungi is referred to as mycorrhiza [22].

Planting medium consisting of sawdust gave the best result because it can retain planting medium moisture which is suitable for *T. merguensis* seed germination. Moisture planting media are needed in the early stages of germination for seedling growth [23]. Moreover, sawdust is organic matter consists of carbon organic from cellulose, nitrogen, potassium, and phosphate. These nutrient mineral in sawdust are important for the seedling of *T. merguensis* growth.

Roots of seedling were treated without inoculation did not form a symbiosis with ectomycorrhizal fungi. On the other side, seedlings were treated with inoculation and P25% (P25-i) formed colonization. The treatment of inoculation and P50% (P50-i) and P75% (P75-i) did not produce root colonization. There was no colonization in the treatment of P50% (P50-i) and P75% (P75-i) was due to seedling were harvested too early (20 WAP). Therefore, there was no penetration yet of hyphae into the root epidermis, which subsequently could form the structure of colonization. Initial penetration of hyphae ectomycorrhizal *Tricholoma* sp. to the root epidermis of *Pinus densiflora* in greenhouse was found about 6 months after inoculated. At this stage, no formation of colonizing roots structures since hypha only penetrated upper layer of epidermis roots [24].

Seedling height treated with inoculation and P25% (P25-i) differed significantly from treatment without inoculation and P25% (P25-k). It is suspected that inoculation and P25% treatment (P25%-i) caused symbiotic formation between ectomycorrhiza with *T. merguensis*. Mycorrhizal fungi are able to form symbiotic relationship with the host plants when it’s grown in soil that contains low nutrients, thus, the host plant can grow well, even it planted in poor soil. The host plant will develop resistance to the fungal penetration when the concentration of phosphate in soil is too high, due to high soil phosphate levels have been found to be detrimental for the symbiotic association of mycorrhizal fungi with plants. The lower content of soil phosphate can increase the activity of fungi in the establishment of mycorrhizal symbiosis which can provide increased growth and development of the host plant [25].

Figure 3. Plant height of *T. merguensis* seedlings. (a) Seedling of control plant and without inoculation (b) with phosphate 25% (P25-i), (c) phos-phate 50% (P50-i), (d) phosphate P75% (P75-i). Scale bar = 5 cm

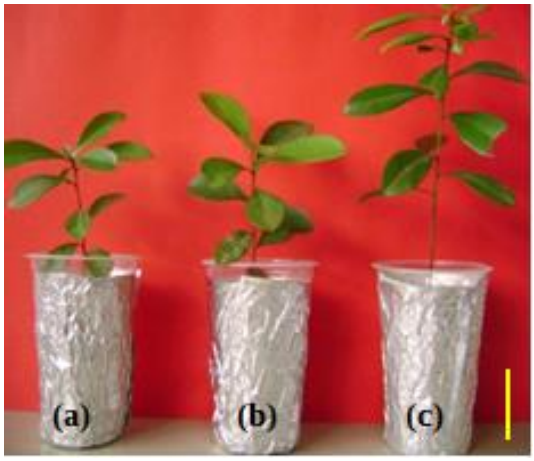

Figure 4. Plant height of *T. merguensis* seedlings. Seedling of *T. merguensis* treated with ectomycorrhiza fungi inoculation and Johnson solution contained (a) phosphate 50% (P50-i), (b) phosphate 75% (P75-i), and (c) phosphate 25% (P25-i) in 5 MAP. Scale bar = 5 cm.

The leaves number in all treatment was not significantly different until 20 WAP. However, although the leaves' number did not differ, they have different leaves color. Leaves of seedlings treated with inoculation and P25% (P25-i) has greener than the other treatments. On this treatment, the phosphate nutrient that play a role in the chlorophyll biosynthesis which affects photosynthesis. This result is consistent with Curtis and Clark which stated that the phoshate has an effect on plant growth and development through photosynthesis [26].

Figure 5 shows that roots of *T. merguensis* which inoculated by ectomycorrhizal mycelium

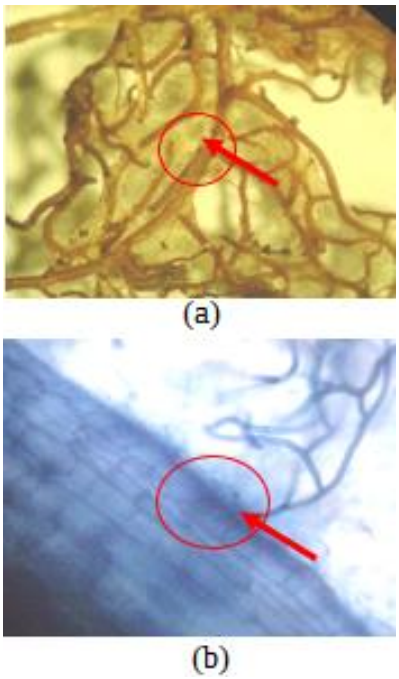

Figure 5. Root structure of *T. merguensis* seedling with fungi inoculation in the laboratory experiments. Initially structure (a) *T. merguensis* root after fungi inoculation, (b) ectomycorrhiza hyphae in outer surface of root

form colonization with mycorrhizas fungi. This data proves that the root of *T. merguensis* need ectomycorrhizal fungi since the beginning of growth. This study is supported by the previous studied that *T. guillainii*, *T. calobuxus*, and *T. whiteana* have mycorrhizal symbioses with arbuscular mycorrhizas fungi [11, 27]. It can be assumed that *T. merguensis* tree has a similar symbiotic association between ectomycorrhiza with others Tristaniopsis.

Conclusion

From pesent study it is concluded that *T. merguensis* is able to form a symbiosis with ectomycorrhizal fungi. Inoculation treatment with P25% (P25-i) improves plant growth and promote the ectomycorrhizal colonization. It was observed that formation of mycorrhizal colonization beginning at 20 WAP. Fine roots (soil-mycelium system) mixed with planting medium especially sawdust was important for the purpose of stimulating seed germination and seedling growth of *T. merguensis.*

Acknowledgment

We thank PEMDA Central Bangka for permission to sample of natural fungi in the Pelawan forest

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