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Effect of Mycorrhizal Inoculation and Frequency of Watering to The Seedling Growth of Agarwood (Aquilaria malacensis, Lamk)

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A B S T R A C T

Currently, many farmers have been trying to cultivate agarwood plants, after various studies have produced fungi that can be infected to agarwood plants to produce gaharu sap. However, the propagation of agarwood plants until now still has several inhibiting factors, especially problems with soil fertility and optimal water availability for seedling growth. Controlling soil fertility using synthetic fertilizers is no longer recommended due to pollution problems, so efforts towards using biological fertilizers are now the focus of utilization. Therefore, this study aimed to determine the effect of mycorrhizal fungus inoculation and frequency of watering on the growth of agarwood seedlings in nurseries with the growth parameters, namely: increase in plant height, stem diameter, number of leaves and percentage of mycorrhizal fungal infections in agarwood seedling roots. The research results showed that the treatment between mycorrhizal inoculation (I1) and the frequency of watering every 2 days (A2) resulted in good average growth of agarwood seedlings. This is because the highest mycorrhizal fungal infection, which is 93.3%, occurred in the treatment of the combination between mycorrhizal inoculation and the frequency of watering in every 2 days. The highest agarwood seedling was 25.70 cm, obtained from a variety of treatment between mycorrhiza inoculation and the frequency of watering in every 2 days. Meanwhile, the treatments without mycorrhizal inoculation at all levels of frequency of watering resulted low growth rates of agarwood seedlings.

Introduction

Agarwood (*Aquilaria malaccensis*, Lamk) is a forest tree known to produce gaharu sap. Gaharu sap is a sap produced by the Aquilaria plant to prevent the attack of Fusarium, Aspergillus and Penecillium fungi in the plant wounds. Gaharu sap is widely used as raw material for the perfume industry, cosmetics, incense, preservatives and various accessories. Gaharu sap has become one of the elite commodities of non-timber forest products with good prospects for development because the price is very high. The increasing demand in the world market today and the high selling price of gaharu sap have caused the hunting of agarwood plants in Indonesia to be more intensive and uncontrollable. This species in Indonesia is listed in appendix II of CITES as a rare plant caused by uncontrolled hunting in natural forests (Santoso and Sumarna, 2006).

Considering that gaharu sap production is still dependent on agarwood plants that grow naturally, efforts to increase public knowledge in agarwood cultivation need to be further developed, so it is hoped that gaharu sap products will become an alternative source of income for the community and can be a source of foreign exchange for the country (Salampessy et al., 2008). Propagation of agarwood plants can be done generatively or vegetatively. However, there are still many obstacles because it requires large quantities of vegetative material and care must be taken. Meanwhile, propagation by seed also encountered obstacles, namely: it took a long time to germinate, growth was not uniform and genetically had different characteristics from the parent, and seedlings that had been planted in the field at the age of 3-4 years were found dead due to lack of nutrients in the soil to support plant growth and development (Suhartati, 2013).

The supply of nutrients from the soil decreases due to low soil fertility. One of the causes of disturbed soil fertility is drought. Lack of water internally in plants results directly in decreased cell division and enlargement. According to Lubis (2000), if plants lack water, the growth process will be hampered and yields will decrease. The availability of water under optimum conditions for plant growth will result in plants being hampered or too late to enter the next vegetative phase. In addition to the water availability problem, dry land generally has marginal fertility, which has poor physical properties, nutrient deficiency, toxicity and high pest and disease attacks. Sujinah and Jamil (2016) suggest that drought stress can inhibit root growth, decreasing the shoot-root ratio.

In connection with that, one of the alternatives to produce agarwood seedlings in enough quantities that they can survive and grow, and develop well for the preservation of germplasm, so can be able to overcome the scarcity of plant by injection or inoculation with certain types of isolates in certain types of agarwood plants. The technology in agarwood nursery is needed to produce seeds that can grow fast and resist extreme environments such as on critical land, ex-mining land or acid soils (Fernando, et al., 2022).

The application of mycorrhizae in plants has proven to be very beneficial because it can accelerate and increase the uptake of nutrients, especially phosphorus, protect plants from root pathogens, prevent plants from drying out, and prevent plants from being poisoned by heavy metals as well as increasing the rate of growth and plant health both at the nursery and in the field (Suwandi et al, 2006, Prayoga and Budiprasetya, 2021). In general, mycorrhizal plants have better growth. Mycorrhiza as a type of biological fertilizer plays an important role in improving soil fertility, environmentally plant growth and soil nutrient status so crop yields are good. Therefore, the mycorrhizal inoculation can be said as 'biofertilizer' which is good for food crops, plantations, forestry and reforestation plants (Widada, 1994, Sri Wilarso Budi and Fatimah, 2020).

Environmental factors that influence soil water content, soil moisture and soil temperature. Ecological factors and plant factors influence water absorption by plants. According to Harjadi (1996) plants need water in optimal amounts to support their growth so the provision of water as needed throughout plant growth will always be ideal for these plants. In contrast, plant factors are root efficiency in absorbing water, pressure gradient for groundwater diffusion to roots, and the state of plant protoplasm. The ability of the roots to absorb water is influenced by the absorption capacity of the roots, the ability to translocate nutrients from the roots to the leaves, and the ability to expand the root system (Nirwanto et al., 2019).

This study aims to determine the effect of mycorrhizal inoculation and frequency of watering on the seedling growth of agarwood (*Aquilaria malaccensis*, Lamk) in the nursery

Research Methods

Time and Location

This research was carried out in February to April 2021 at the Greenhouse of the Faculty of Agriculture, Pattimura University, Ambon. The analysis of mycorrhizal infections in the seedlings was carried out at the Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Pattimura University, Ambon.

Materials and Equipments

The materials used in this study were agarwood seeds, mycorrhizal spores, water, plastic bags, label papers, 10% KOH, alkaline H_2O_2, Epson 664 (blue) printer ink, 25% commercial vinegar, staining solution (500ml acetoglycerol + 450 ml H2O2 + 50 ml commercial vinegar 25%. Meanwhile, the tools needed are a soil filter, iron drum/steam tub, stove, tub of sprouts, measuring cup, calliper, ruler, scissors, tweezers, glass slides, analytical balance, slide microscope, camera, and laptop.

Research Implementation

a. Preparation of planting media

The planting medium used a mixture of forest soil (topsoil) and river sand with a ratio of 2: 1 and sterilized in the iron drum for 2 hours.

b. Seed preparation

A. malaccensis seeds were sown for two months in a greenhouse for ± 500 seeds. Seedling was carried out in a seedling tank using a mixture of soil and sand media that had been sterilized, and then mixed again with husk charcoal. During the seeding growth process, the seedlings were watered with water according to the treatment being tested.

c. Preparation of inoculation materials

The inoculation material used in this study was mycorrhizal spores of the *Glomus etunicatum* and *Glomus fasciculatum* in the form of zeolite obtained from Biotrop Bogor.

d. Seed weaning and inoculation treatment

The number of seeds to be transferred to polybags is 54 seedlings. Weaning or transplanting of these seeds will be carried out after the seedlings are 2 months old, and the seedlings that are weaned or transferred are those with good growth (marked by healthy leaf growth and good seedling roots). Mycorrhizal inoculation treatment was carried out in 2 inoculation stages: the first stage of inoculation was carried out during the agarwood plant seed germination, and the second stage of inoculation was during weaning or transfer of seeds to polybags. The inoculation treatment used the hole technique, where the inoculum was inserted into the planting hole before the seeds were inserted into the planting hole. Each seedling was inoculated with 10 grams of inoculant per seedling.

e. Water anti-stress treatment

Drought stress treatment in the form of water stress was carried out after the seedlings were 2 months old from the second stage of mycorrhizal inoculation. The volume of water given is the same, namely 200 ml per polybag according to the water field capacity.

f. Harvest

Harvesting was carried out three months after the second inoculation and water treatment stage. At that time, secondary and tertiary roots were cut out to make root histology preparations for observing mycorrhizal infections in the roots of agarwood seedlings.

g. Observation of root infection

Observations of mycorrhizal infection variables were carried out by staining the roots using an ink-vinegar solution using the protocol of Vierheilig et al. (1998) modified by Nusantara (2011). The working stages of this method are as follows:

1. The roots are washed thoroughly with running water and repeated three times until it is quite clean from the remnants of soil dirt.
2. The roots are then soaked in 10% KOH for 24 hours
3. The roots are still dark in color, given a few drops of alkaline H_2O_2, then washed with running water three times.
4. The roots were immersed in a solution of Epson 664 ink and 25% commercial vinegar solution for 24 hours at room temperature.
5. To remove excess dye, the roots are immersed in acetoglycerol destaining solution.
6. After the staining, the roots were arranged on a glass slide: 5 pieces on the left and five on the right, then covered with a cover glass. Then observed under a microscope slide and recorded the number of mycorrhizal-infected roots from 10 samples.

Research Design

This study used a factorial experiment in a completely randomized design consisting of 2 factors: mycorrhizal inoculation (I) and watering (A) frequency. Each factor consists of the following treatments:

1. Inoculation of mycorrhizal fungi (I), which consists of 2 levels, namely:
   I_0 = without mycorrhizal fungi inoculation (control)
   I_1 = Inoculation of mycorrhizal fungi
2. Frequency of watering (A) which consists of 3 levels, namely:
   A_1 = Watering everyday morning
   A_2 = Watering every two days morning
   A_3 = Watering every three days morning

Therefore, six treatment combinations were obtained, namely I_0A_1, I_0A_2, I_0A_3, I_1A_1, I_1A_2, and I_1A_3, with three replications. Each replication consisted of 3 seedlings, so a total of 54 experimental units of seedlings were obtained.

Results And Discussion

The success of mycorrhiza inoculation and the effect of watering frequency on agarwood seedlings can be seen from plant growth results, including changes in height, stem diameter, and number of plant leaves. In comparison, the ability of mycorrhizal infection can be determined by the percentage of roots infected by arbuscular or hyphae of mycorrhiza.

Plant Height Increase

The results of the study in Table 1 show that the treatment of giving mycorrhizal inoculation and the frequency of watering as a single factor has a very significant effect on the increase in the height of agarwood seedlings. This means that agarwood seeds can produce good plant height growth without being combined with watering treatment or without mycorrhizal inoculation treatment. However, because the purpose of this study was to find the best combination of treatments, it was continued with Duncan's different test because the ANOVA table above had a significant effect on the increase in agarwood seedling height.

Table 1. ANOVA test to the effect of mycorrhizal inoculation (I) and frequency of watering (A) on average height of agarwood seedlings.

Source of Variance	db	SQ	MQ	F	F_{Table}	
					5%	1%
Mycorrhizal noculation (I)	1	371.7356	371.7356	28.413**	4.75	9.33
Frequency of watering (A)	2	235.5211	117.7606	9.00081**	3.89	6.93
I x A	2	115.9144	57.95722	4.430 *	3.89	6.93
Error	12	157	13.08333			
Total	17	901.86				

Notes: * Treatment has a significant effect at the 5% confidence level; ** Treatment has a very significant effect at the 1% confidence level

The results of the DMRT test at 5% level in Table 2 explain that the combination treatment of mycorrhizal fungi inoculation (I) and frequency of watering (A) on agarwood seedlings after 3 (three) months showed that treatment of I_1A_2 had the best average of seedling height, namely 25.70 cm. Still, it is not significantly different from the I_1A_1 and I_1A_3 treatments. This means that all the treatments that given by mycorrhiza inoculation produced the best average seedling height compared to treatments without mycorrhizal inoculation. Furthermore, Figure 1 showed that seedlings with inoculation of mycorrhizal fungi had better growth than those without inoculation of mycorrhiza.

Table 2. Different test results of DMRT (Duncan Multiple Range Test) to the average height of agarwood seedlings between the treatments

Treatment	Average heigh of seedling (cm)	Remark
I_0 A_3	**6.43**	a
I_0 A_2	**11.13**	ab
I_0 A_1	**17.07**	bc
I_1 A_3	**19.17**	cd
I_1 A_1	**21.50**	cd
I_1 A_2	**25.70**	d

Notes: The notation followed by the same letter indicates the effect is not significantly different at the 5% level DMRT test

Figure 1. The result growth of seedling with inoculation and without inoculation of mycorrhiza

Agarwood plant seeds inoculated with mycorrhizae have roots with a wider area of absorption of P and Ca nutrients, so that P plays a role in growth processes such as increasing the process of cell division and cell elongation. Plants without mycorrhizal treatment were not able to absorb P as well as plants treated with mycorrhizae because $H_2PO_4^-$ ions in the soil were susceptible to precipitation reactions so that roots had to exert greater energy in absorbing these ions (Salam et al., 1997). In addition, in the roots of plants with mycorrhizae, there was an increase in the activity of the phosphatase enzyme, which helps catalyze and hydrolyze phosphorus complexes that are insoluble in the soil, increasing available P. According to Musfal (2011), mycorrhizae can improve soil structure, increase nutrient availability, and assist the weathering process of parent material and soil organic matter. In addition, mycorrhizae can increase water and nutrient uptake and protect plants from attack by roots and toxic elements. They can also produce growth regulators such as auxins, cytokinins, and gibberellins to their host plants.

In general, mycorrhizal plants tend to be more resistant to drought stress, so the plant cortex is more resistant to damage than plants without mycorrhizae. In addition, mycorrhizal hyphae can also increase root resistance to decreased water movement so that water movement to the roots increases because mycorrhizal hyphae are consistently effective in aggregating soil grains so that the ability of the soil to store water will increase.

Furthermore, it can also be explained that the availability of water greatly affects the growth of plant height and the development of meristem tissues at the point of plant growth. Lubis (2000) states that if the plant lacks water, the growth process will be hampered, and the yield will decrease. The availability of water under optimum conditions for plant growth will result in stunted plant growth or stunted growth, or late entry into the next vegetative phase. The amount of water absorbed by the roots depends on the soil moisture content and the transpiration rate. When the soil water content is low or below field capacity, and the evapotranspiration rate exceeds the water absorption rate, the plant will be faced with water stress conditions, resulting in the plant experiencing a decrease in cell division and enlargement.

Stem Diameter and Number of Leaves

The study's stem diameter and the number of leaves of agarwood seedlings were not affected by the mycorrhizal inoculation treatment or the frequency of watering and the combination of the two treatments. This is presumably because the root infection by mycorrhizae takes a long process and time for the hyphae to infect and enter the cortical cells of the roots. This condition is so that the roots of agarwood seedlings can provide nutrients for the growth of stem cambium cells, affecting the increase in stem diameter. In addition, nutrients transported to meristem cells in plant shoots will be able to increase the number of leaves significantly. Nusantara (2002) explained that mycorrhizal fungi need a long time to form the necessary structures in their symbiosis with the host plant if the media conditions are not favorable.

The results showed that the average stem diameter achieved by the treatment of mycorrhizal inoculation and the frequency of watering once every two days was 4.3 mm on average. This is supported by Harahap (2018) who said that giving 10 grams of mycorrhizal inoculation on Jabon plant seedlings resulted in an average stem diameter of 4.12 mm for 3 months of plant seedling age.

Table 3. ANOVA test on the effect of mycorrhizal inoculation (I) and frequency of watering (A) on the average increase in diameter (mm) for 3 months in the nursery.

Source of Variance	db	SQ	MQ	F	F_{Tabel}	
					5%	1%
Mycorrhizal inoculation (I)	1	116.5356	116.5356	4.272 ^{tn}	4.75	9.33
Frequency of watering (A)	2	115.9433	57.97167	2.125 ^{tn}	3.89	6.93
I x A	2	106.7211	53.36056	1.956 ^{tn}	3.89	6.93
Error	12	327.32	27.27667			
Total	17	666.52				

Notes: ^{tn} Not significant different

Table 4. ANOVA test to the effect of mycorrhizal inoculation (I) and frequency of watering (A) on the average increase in leaves number for 3 months in the nursery.

Source of Variance	db	SQ	MQ	F	F_{Tabel}	
					5%	1%
Mycorrhizal inoculation (I)	1	4870.845	4870.85	4.5169 $_{tn}$	4.75	9.33
Frequency of watering (A)	2	199.3678	99.6839	0.0924 $_{tn}$	3.89	6.93
I x A	2	33.19	16.595	0.0154 $_{tn}$	3.89	6.93
Error	12	12940.26	1078.36			
Total	17	5472.24				

Notes : ^{tn} Not significant different

The increase in the stem diameter of the seedlings and the number of leaves of agarwood seedlings that were given a combination of mycorrhizal inoculation treatment and the frequency of watering every 2 days (I_1A_2) was the best to produce stem diameter and number of seedlings, which were 4.3 mm and 45 leaves, respectively, while the smallest increase in stem diameter and number of leaves was in seedlings without mycorrhizal fungi inoculation with a frequency of watering every 3 days (IoA_3) of 1.0 mm and 16.3 leaves. This condition indicated that the increase in diameter of agarwood seedlings inoculated with mycorrhizae had a better growth in stem diameter and number of leaves than plants without mycorrhizal inoculation.

Percentage of Root Infection

The results of the study in Table 4 show that the mycorrhizal fungi that were applied were able to symbiotically with the roots of the agarwood seedlings, where the seedlings that were inoculated with mycorrhizae and given a frequency of watering every 2 days had a better percentage of root infection, which reached 93.33%. This is because high soil moisture in wet soil will stimulate the development of spores and the formation of colonization with host plants (Delvian, 2004). The low percentage of mycorrhizal colonization on plant roots is due to the incomplete formation of associations and can also be influenced by the age of the plant. This statement is in line with Santoso et al. (2007), which showed that increased mycorrhizal colonization would occur with increasing plant age. The research results of Delvian (2003) stated that the response of hibiscus plants and jatropha to mycorrhizae could be seen within a period of 4 to 7 months.

Table 4. Effect of mycorrhizal inoculation (I) and frequency of watering (A) on root infection (%) in agarwood seedlings for 3 months in the nursery.

Treatments	Average infection of roots (%)
I_1A_1	91.11
I_1A_2	93.33
I_1A_3	77.78

Notes : I_1A_1 : Treatment of watering in every day; I_1A_2 : Treatment of watering in every two days; I_1A_3 : Treatment of watering in every three days

Muin (2009) stated that one factor that influences mycorrhizal fungi's association process is the amount of carbohydrates the host plant can send to the fungus. The association will occur perfectly, if the carbohydrates that the fungus can receive can be used optimally for the development of the fungus itself. Therefore, plants associated with mycorrhizal fungi must get sufficient sunlight to produce carbohydrates with high concentrations. Corryanti (2001) said that several factors influence mycorrhizal fungal infection, namely the host's sensitivity to infection and climatic and soil factors. Furthermore, it is said that plants with high phosphate dependence tend to be associated with mycorrhizae. Infection is thought to occur due to favorable conditions for spores and affects soil structure, air movement, water circulation, soil pH, nutrient content, and water storage capacity directly or indirectly. The detection method of infection is by looking at the presence or absence of ornaments that indicate mycorrhizal fungi infection, namely hyphae, vesicles, and arbuscules. If one of these ornaments is in the plant root's cortical tissue, it can be said that the root has been infected with mycorrhizae. The ornaments found in the observation of the roots of the agarwood seedlings in this study can be seen in Figure 2.

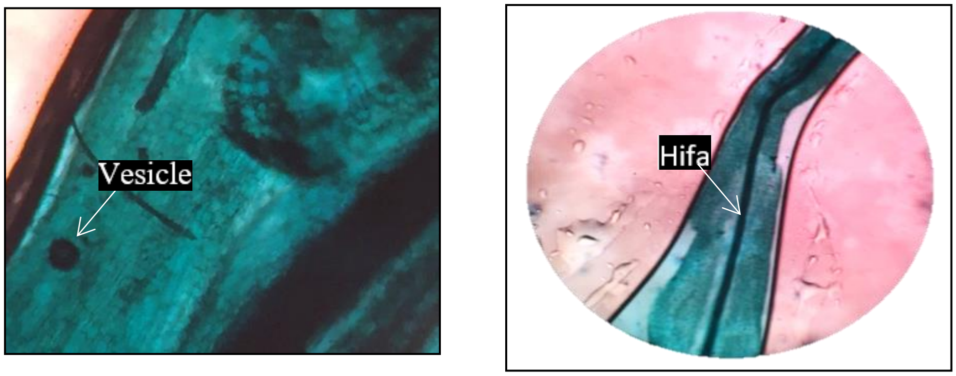

Figure 2. The structure of the mycorrhizal fungal infection in the root tissue of agarwood seedlings

Conclusion

1. The combination between the treatment of mycorrhizal inoculation and the watering frequency had a significant effect on the increase in the height of agarwood seedlings. Still, it did not significantly affect the increase in stem diameter and number of leaves in 3-month-old seedlings.

2. The highest agarwood seedling was 25.70 cm, obtained from a combination of treatment between mycorrhiza inoculation and the frequency of watering in every 2 days. Meanwhile, the treatments without mycorrhizal inoculation at all levels of frequency of watering resulted low growth rates of agarwood seedlings.

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