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Performance of seedling and mixed-species planting test of three species of Rubiaceae treated by different seedling media and mycorrhizae application

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Abstract. Seedling growth in the nursery and early field performance of three fast-growing tree species from the Rubiaceae family, i.e., white jabon (*Neolamarckia cadamba*), red jabon (*Neolamarckia macrophylla*, and gempol (*Nauclea orientalis*), were tested by using different seedling media and arbuscular mycorrhizal fungi (AMF) treatments in the nursery and mixed-species field tests. The experiment design in the nursery and field test was laid out following a split-split plot design of a randomized complete block with four replications. The main factors were tree species, the sub-factors were seedling media, and the sub-sub factors consisted of the AMF inoculation, with a mixed species planting pattern. White jabon had a more dominant growth both at the seedling level in the nursery and at the field test level until the age of 2 years, followed by gempol and red jabon. AMF inoculation in those three species at the seedling level was strongly influenced by the characteristics of the seedling media like media with high fertility levels resulting in a very low percentage of AMF colonization. In the field trial, all tree species and treatments were infected with mycorrhizae with colonization percentages ranging from 27.6% to 57.7%, which was suspected to be an infection from native AMF in the planting site. The AMF application had a significant effect on the percentage of colonization and tree diameter growth of 2-year-olds with a percentage increase of 14.4% and 8.4%, respectively.

1 Introduction

Rubiaceae is one of the largest families of flowering plants comprising about 660 genera and 11,150 species, with most genera and species growing in humid tropical regions [1]. Several species of the Rubiaceae family in Indonesia are cultivated for wood production, such as white jabon (*Neolamarckia cadamba* (Roxb.) Bosser), red jabon (*Neolamarckia macrophylla*

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(Roxb.) Bosser), and gempol (*Nauclea orientalis* (L.) L.). Those species are categorized as fast-growing tree species that can be used for many purposes such as producing timber for pulp, plywood, and light construction, and using bioactive compounds from various parts of the plants in several traditional and folklore systems of medicine around the world [2].

White jabon and gempol are distributed on almost all islands in Indonesia, and it is often found in secondary forests along riverbanks and the transitional zone between swampy, permanently flooded areas, and periodically flooded areas [3, 4]. Red jabon naturally grows in eastern Indonesia, i.e., Sulawesi and Maluku Islands [5, 6].

The use of appropriate seedling media is an important factor to promote optimum seedling growth [7]. In this study, we combined the seedling media and arbuscular mycorrhizal fungi (AMF) inoculation to improve the seedling quality at the nursery and the field performance of three species of Rubiaceae, i.e., white jabon, red jabon, and gempol. Early inoculation of seedlings with AMF under nursery conditions can be beneficial in two ways i.e., the seedlings grow stronger and the better performance in fields [8].

However, the investigation of the effect of seedling treatments (growing media and AMF inoculation) in the nursery and its correlation with the field performance after out-planting, especially for tropical tree species, is still limited [9]. A study on the effectiveness of AMF to increase the success of planting white jabon, red jabon, and gempol is very necessary considering that these species are mostly planted in dry and marginal land [10-12], which is different from their natural habitat, i.e., moist soils. Some studies also revealed that these species are quite sensitive to drought stress [6, 13] and not suitable for critical land rehabilitation [10]. AMF helps plants to increase their nutrient uptake capacity and their resistance to drought and other abiotic stresses [14, 15]. The purpose of this study was to investigate the effect of seedling media and AMF inoculation on the early growth performance of white jabon, red jabon, and gempol.

2 Material and methods

2.1 Materials

Seeds of the three tree species were collected from different locations in Indonesia, i.e., Kampar, Sumatra for white jabon (00°18’ S, 100°05’ E, altitude of 50 m asl), Kolaka, Sulawesi Tenggara for red jabon (04°03’ N, 121°41’ E, altitude of 242 m asl), and Parungpanjang-Bogor, West Java for gempol (06˚20’ S, 106˚ 06’ E, altitude of 52 m asl). The seeds were extracted manually with the wet extraction method and dried at room condition (temperature 25-30°C, relative humidity 70-80%) until the seed moisture content reached 6%-8% [16].

2.2 Seedling preparation and experimental design in the nursery

The seedling preparation of all species was carried out at Nagrak Nursery Research Station in Bogor, West Java. Seeds were sown on mixed media consisting of sand, soil, and rice husk charcoal (5:3:1, by volume), which had been mashed. The media have previously been sterilized by steaming at a temperature of 120°C for 1 hour. Before sowing, the media were treated with 0.2% fungicide to avoid any chances of fungus infection attacking newly germinated seedlings. Media were sprayed with water using the fine sprayer to keep them wet and then the media were maintained in the greenhouse. Young four-leaf seedlings were transplanted in the two types of media, i.e., top-soil media and mixed media of compost, topsoil, rice husk charcoal, and lime (3:2:1:0.5, by volume). This mixed media has provided excellent growth for several forest tree species such as *Albizia chinensis* and *Calophyllum* *inophyllum* [17,18]. The whole media for seedling growing had been previously sterilized by drying directly in the sunlight for one day by flipping through the materials and put in a polybag with a size of 10 cm x 15 cm. Analysis of the chemical characteristics of the molded seedling media and polybag media was carried out at the Soil and Plant Nutrition Laboratory, SEAMEO BIOTROP, Bogor.

The experiment design in the nursery was laid out following a split-split plot design of a randomized complete block with four replications. The main factors were species (white jabon, red jabon, and gempol) and the sub-factors were seedling media (topsoil and mixed media). The sub-sub factors consisted of the AMF inoculation (with and without AMF). The AMF used in this research was a consortium of AMF (*Acaulospora* sp., *Gigaspora* sp., *Glomus* sp.1, and *Glomus* sp.2, a total spore of 626 spores per gram), because several studies have suggested that a consortium of AMF species give better results in terms of growth as compared to individual AMF species [19]. The AMF inoculation was conducted by adding 5 g of AMF at the same time as transplanting of seedling into polybags media. Each replication consisted of 100 seedlings so there were 400 seedlings for each treatment. The seedlings were further maintained for 3 months in the nursery, following the nursery conditions of two months in a shaded area (light intensity 50%) and one month in the open area. The seedling height, root collar diameter, and approximately 1 g subsample of roots from three seedling samples were taken randomly from each treatment extracted, cleared, and stained [20], and analyzed for AMF colonization percentage.

Table 1. Chemical characteristics of seedling media (topsoil and mixed media) and soil of planting test location at Parungpanjang Forest Research Station (PFRS), Bogor.

Parameter (method)	Topsoil media	Mixed organic media	The soil at PFRS
pH (SNI 03-6787-2002)			
H_2O (1:1)	6.0	6.1	4.58
$CaCl_2$(1:1)	5.7	6.0	4.18
C Org (SNI 13-4720-1998 (Walkey & Black))	2.12%	4.69%	1.92%
N Total (SNI 13-4721-1998 (Kjeldahl))	0.22%	0.56%	0.22%
Ratio C/N	9.63	8.37	8.77
P_2O_5 available (SL-MU-TT-05 (Bray I/II))	33.2 ppm	2080.5 ppm	13.75 ppm
Ca (SL-MU-TT-07C)	11.10 Cmol kg^{-1}	33.30 Cmol kg^{-1}	2.75 Cmol kg^{-1}
Mg (SL-MU-TT-07C)	7.08 Cmol kg^{-1}	9.41 Cmol kg^{-1}	1.80 Cmol kg^{-1}
K (SL-MU-TT-07C)	3.53 Cmol kg^{-1}	4.83 Cmol kg^{-1}	0.13 Cmol kg^{-1}
Na (SL-MU-TT-07C)	0.17 Cmol kg^{-1}	6.36 Cmol kg^{-1}	0.22 Cmol kg^{-1}
KTK (SL-MU-TT-07C)	26.23 Cmol kg^{-1}	10.68 Cmol kg^{-1}	24.42 Cmol kg^{-1}
KB (SL-MU-TT-07C)	83.42%	100%	20.04%

Notes: SNI = Indonesia National Standard

2.3 Experimental design in the field trial

The field trial was conducted at Parungpanjang Forest Research Station, Bogor (06°20'42” S, 106°06'15” E, 52 m asl altitude). The average annual rainfall and temperature are 2,440 mm and 27.8° C with a low soil nutrition content (low level of N, P, K, and C-organic with pH of 4.8) (Table 1). The site was an even area within state forest land covered with dense weeds, that grew rapidly even after cleaning [13]. Identification of the local inoculum potential in the soil at the field test was carried out before the out-planting using 12 sampling points from each treatment plot that will be established. Each sampling point consisted of 3 spot samples taken randomly from each treatment plot at depth of 0-20 cm. The soil samples from 3 spots were mixed homogeneously to represent a single sampling point. The total population of mycorrhizae was counted using the wet sieving method [21] using 10 g of soil sample. The filtered spores were observed under a stereo microscope and counted manually.

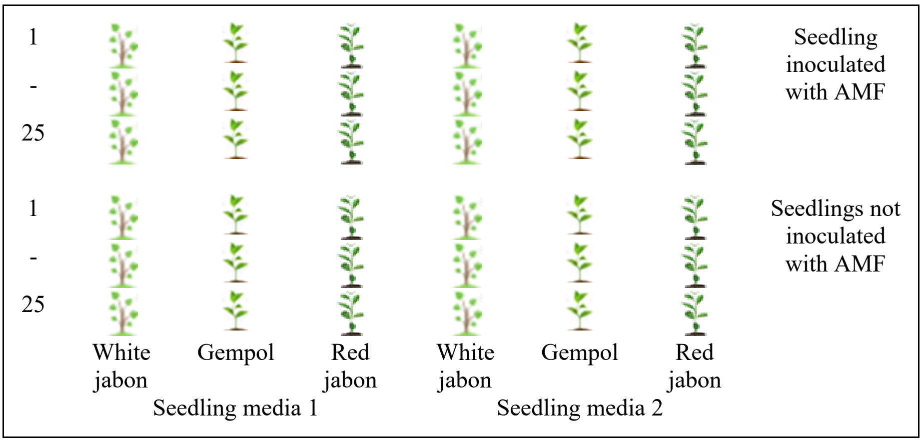

Fig. 1. Planting pattern of mixed tree species (white jabon, gempol, and red jabon) in a block in Parungpanjang, Bogor.

The design of seedling testing in the field trial is the same as the design at the seedling level in the nursery, i.e., a split-split plot design of a randomized complete block with four replications. The planting was carried out by mixed species tree plantation with the planting hole size 30 cm x 30 cm x 30 cm. Planting was carried out with a mixed pattern of species alternately, i.e., red jabon, gempol, and white jabon, with the spacing between species of 4 m and the planting line spacing within species of 2 m (Figure 1). Each replication consisted of 25 seedlings so there are 100 seedlings for each treatment. Weed competition was kept to a minimum by manual weeding. All seedlings in each plot constituted the measuring unit in all replications. The growth assessment was carried out at the age of 1 year and 2 years old after out-planting. The parameters assessed are seedling survival, total height (m), collar diameter (cm), and AMF colonization percentage [20].

2.4 Data analysis

Analysis of variance (ANOVA) was used to examine the effect of the seedling treatment using seedling growth media and AMF inoculation on the growth of three fast-growing tree species in the nursery and the field test at 2 years old. Treatment means were separated using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ whenever ANOVA showed significant treatment effects.

3 Result and discussion

3.1 Seedling performance and AMF colonization in the nursery

The analysis of variance showed that the interaction among species, seedling media and AMF inoculation had a significant effect on seedling height, sturdiness quotient, and AMF colonization percentage. Meanwhile, the root collar diameter was affected by the interaction between species and seedling media, as well as the interaction between seedling media and AMF inoculation. On the parameters of seedling biomass, species and seedling media had a significant effect on aboveground biomass, below-ground biomass, and total biomass, but their interaction has no significant effect, except for below-ground biomass (Table 2).

Table 2. Effect of species (S), seedling media (M), AMF inoculation (A), and their interactions on the seedling performances in nursery.

Source of variation	DF	SH	RCD	SQ	DF	AGB	BGB	TB	CP
Block (B)	3	29.04**	14.26**	8.73**	3	3.20*	3.99*	4.01*	0.88^{ns}
Species (S)	2	357.70**	50.18**	262.78**	2	19.80**	7.34**	18.18**	11.39**
B*S	6	12.91**	1.94^{ns}	6.31**	6	3.14*	2.98*	3.56*	0.39^{ns}
Error (S*B)	6				6				
Seedling media (M)	1	307.56**	62.40**	206.36**	1	33.93**	30.58**	39.01**	0.23^{ns}
S*M	2	16.73**	21.90**	4.26*	2	0.22^{ns}	9.68**	0.47^{ns}	0.50^{ns}
Error (S*M*B)	4544				24				
Mycorrhizae (A)	1	18.96**	5.78*	75.22**	1	0.84^{ns}	0.74^{ns}	0.89^{ns}	146.49**
S*A	2	18.86**	2.48^{ns}	33.35**	2	0.04^{ns}	0.06^{ns}	0.05^{ns}	3.03^{ns}
M*A	1	61.33**	9.93**	28.69**	1	1.16^{ns}	0.063^{ns}	1.07^{ns}	119.85**
S*M*A	2	4.40*	0.29^{ns}	7.04**	2	0.31^{ns}	0.08^{ns}	0.20^{ns}	3.16*
Error	4556				36				

Notes: DF = degree of freedom, SH = seedling height, RCD = root collar diameter, SQ = sturdiness quotient, AGB = above-ground biomass, BGB = below-ground biomass, TB = total biomass, CP = colonization percentage; The main plot factor was species (S) and the design was split with seedling media (M) as split-plot factor and AMF inoculation (A) as sub split plot factor; ** = significant at 99% confident level, * = significant at 95% confident level, ns= not significant at 95% confident level

Species gave a significant difference in the growth of the three forest tree species in the nursery until the age of 4 months. White jabon had the highest growth in height (31.2 cm), followed by gempol (28.1 cm), and the lowest was red jabon (22.3 cm). Likewise, with AMF inoculation treatment, seedlings with AMF inoculation gave the best height growth. In general, at the level of interaction between treatments, white jabon seedlings with mixed media, both inoculated (34.4 cm) and not inoculated with AMF (35.4 cm) gave the best height growth (Table 3). Molded organic seedling media also resulted in the best growth for root collar diameter and all biomass seedling parameters. In the percent colonization parameter, seedlings with topsoil media had the highest AMF colonization rates in the three tested species, namely 48.61% on red jabon, 74.44% on gempol, and 64.44% on white jabon (Table 3).

Table 3. Early growth performance of the three tree species on the different seedling media and AMF inoculation.

Species	Seedling media	Seedling height (cm)			Sturdiness quotient			Colonization percentage (%)		
		Without AMF	AMF	Mean	Without AMF	AMF	Mean	Without AMF	AMF	Mean
Red jabon *(N. macrophylla)*	M1	14.3 f	21.3 e	22.3 c	5.72 f	8.02 d	7.73 c	5.00 c	48.61 b	14.40
	M2	25.1 d	24.9 d		7.89 d	8.60 c		0.00 c	3.00 c	
Gempol *(N. orientalis)*	M1	24.8 d	27.9 c	28.1 b	7.46 e	7.88 d	8.30 b	0.75 c	74.44 a	17.29
	M2	29.2 c	29.1 c		8.62 c	8.82 c		2.50 c	5.00 c	
White jabon *(N. cadamba)*	M1	27.8 c	32.4 b	31.2 a	8.96 c	9.37 b	9.59 a	1.25 c	64.44 a	20.67
	M2	35.4 a	34.4 a		10.11 a	9.92 a		0.00 c	3.50 c	
Mean		27.1 b	27.8 a		8.35 b	8.87 a		1.75 b	33.17 a	

Notes: Means followed by the same letter in each column are not significantly different at ($P \leq 0.05$), M1 = topsoil media, M2 = mixed media, AMF = arbuscular mycorrhizal fungi

3.2 Seedling performance and AMF colonization in the field test

The interaction between species, seedling media, and AMF inoculation gave a significant difference in the height of the plants aged 1 year, and plant stem diameter at the age of 1 and 2 years. In seedling survival parameters, the species, seedling media, and the interaction between seedling media and AMF inoculation gave a significant effect on seedling survival. Meanwhile, for the percentage of colonization in the field test, the significant effect was only shown by differences in species and AMF inoculation treatments (Table 4).

Table 4. Effect of species, seedling media, AMF inoculation, and their interactions on the early seedling growth performance in the field.

Source of variation	DF	Seedling survival	Colonization percentage	DF	Height at 1 year	Diameter at 1 year	Height at 2 years	Diameter at 2 years
Block (B)	3	1.26ns	0.88ns	3	17.89**	4.57*	10.80**	10.35**
Species (S)	2	22.04**	11.39**	2	279.14**	167.41**	197.23**	145.01**
B*S	6	0.47ns	0.39ns	6	1.14ns	2.39*	13.30**	14.43**
Error (S*B)	6			6				
Seedling media (M)	1	14.13**	0.23ns	1	94.46**	151.39**	6.74*	8.46**
S*M	2	3.34ns	0.50ns	2	5.64*	9.06**	3.91*	1.05ns
Error (S*M*B)	24			1063				
Mycorrhizae (A)	1	3.85ns	5.71*	1	3.42*	9.04*	3.00ns	4.86*
S*A	2	0.84ns	1.38ns	2	9.44**	12.27**	0.78ns	1.40ns
M*A	1	6.67*	0.02ns	1	0.33ns	2.92ns	0.43ns	0.42ns
S*M*A	2	1.16ns	0.91ns	2	9.51**	9.76**	1.93ns	3.09*
Error	36			1075				

Notes: DF = degree of freedom; The main plot factor was species (S) and the design was split with seedling media (M) as a split-plot factor and AMF inoculation (A) as sub-split-plot factor; ** = significant at 99% confident level, * = significant at 95% confident level, ns= not significant at 95% confident level

Species with the best survival were shown by gempol (97.0%), followed by white jabon (91.0%) and red jabon (82.7%). The AMF inoculation treatment gave a better survival percentage for the three tested species, which was an average of 92.0% compared to seedlings without AMF inoculation with a survival of 88.8%. The interaction of species, seedling media and AMF inoculation provided a significant difference in the plant height at the age of 1 year, but at the age of 2 years, the interaction did not give a significant difference in the growth of plant height. The best height growth at 1-year-old of the plants was shown by interaction among white jabon with molded organic seedling media treated by both AMF inoculation (1.67 m) and without inoculation (1.65 m), and on the topsoil media with AMF inoculation (1.61 m) (Figure 1). At the age of 2 years, white jabon with molded organic seedling media gave the best height growth (6.02 m), while the interaction among species, seedling media, and AMF inoculation did not give a significant difference.

Figure 2 showed that species, seedling media, and AMF inoculation gave a significant difference in the tree diameter growth at 1 year and 2 years old. The largest diameter was shown by white jabon with molded organic seedling media both inoculated and uninoculated with diameters of 7.78 cm and 7.86 cm, respectively. AMF colonization was significantly different between inoculation treatments and at the species level with colonization percentages of 35.8% for red jabon, 53.6% for jabon, and 43.3% for white jabon. In the field test, AMF colonization occurred in almost every interaction of seedling treatment (Fig. 2). AMF infection in plants from uninoculated seedlings was caused by the presence of native AMF found at the study site. The results of the analysis of the AMF content from the soil taken before planting in the field test showed the presence of various mycorrhizal species, such as *Glomus* sp., *Acaulospora* sp., and *Gigaspora margarita* (Table 5).

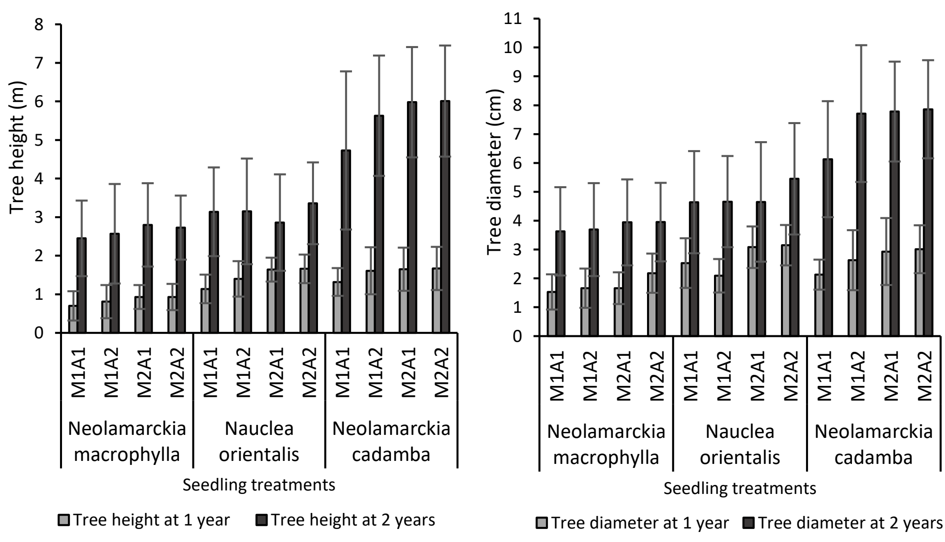

Fig. 2. Tree height and diameter of red jabon (*Neolamarckia macrophylla*), gempol (*Nauclea orientalis*), and white jabon (*Neolamarckia cadamba*) on the different media and AMF inoculation in the field test (Notes: M1 = topsoil, M2 = molded seedling media, A1 = without AMF inoculation, A2 = with AMF inoculation).

White jabon both in the nursery and in the field trial had better growth performance in height and diameter than gempol and red jabon. In the nursery, the three species were very responsive to the fertility of the seedling media, so the media with a high fertility level (mixed media) provided the best seedling growth. This study has similar results to study reported by [5,22]. In the field test at 2 years old, gempol had the highest survival 97.0%, followed by white jabon and red jabon with 91.0%, and 82.7%, respectively. The better growth performance and the survival of white jabon were thought to be related to the natural distribution of the two species which is relatively wider than that of red jabon [16]. In addition, the low growth of red jabon was thought to be due to differences in the characteristics of the location of seed collection which is at an altitude of 242 m asl, while the altitude of the location for the seed collection of white jabon and gempol is 50 m asl and 52 m asl, respectively, making it more adaptive to the field test location at Parungpanjang, Bogor (52 m asl). In addition, in mixed-species forest plantation establishment as in this study, it was also necessary to consider the pattern of height growth, root phenology and depth, foliar phenology, and crown structure for each species [23], so that there are no species whose growth is suppressed when planted together in a site. In this study, red jabon had a lower initial growth so it was suspected that it could not compete with white jabon and gempol in obtaining plant space, especially light needed for pioneer species.

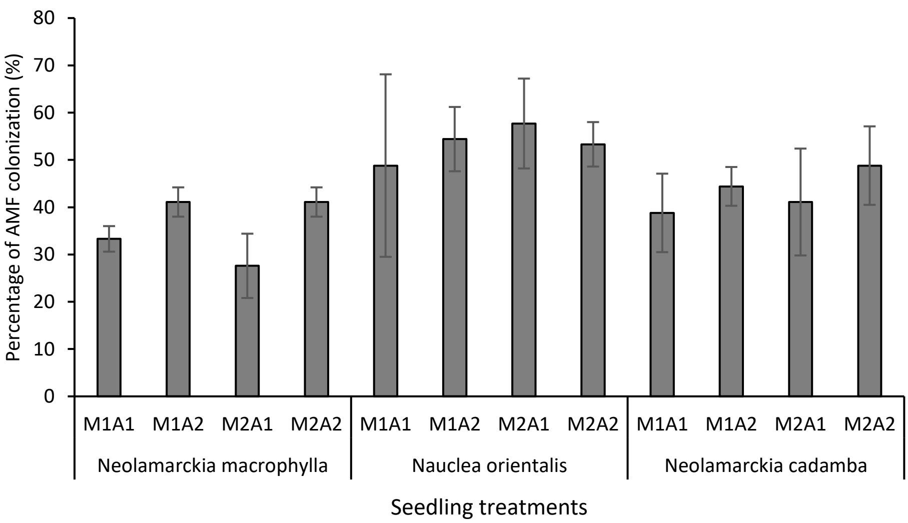

Fig. 3. Percentage of colonization of AMF inoculation on red jabon (*Neolamarckia macrophylla*), gempol (*Nauclea orientalis*), and white jabon (*Neolamarckia cadamba*) in the field test (Notes: M1 = topsoil, M2 = molded seedling media, A1 = without AMF inoculation, A2 = with AMF inoculation).

Table 5. Native mycorrhizae identified in the field test at Parungpanjang forest research station.

Block	Samples	Number of spora per 10 g	Mycorrhizae
I	1	4	*Acaulospora* sp.-2, *Acaulospora* sp.-3
	2	4	*Glomus* sp.-2
	3	1	*Glomus* sp.-2
II	1	0	-
	2	4	*Acaulospora* sp.-3
	3	0	-
III	1	6	*Acaulospora* sp.-2, *Acaulospora* sp.-3
	2	10	*Glomus* sp.-1, *Glomus* sp.-3
	3	5	*Gigaspora margarita*, *Glomus* sp.2, *Acaulospora* sp.-2, *Acaulospora* sp.-3
IV	1	6	*Glomus* sp.-2, *Acaulospora* sp.-2
	2	7	*Glomus* sp.2, *Glomus* sp.-3, *Acaulospora* sp.-2
	3	7	*Glomus* sp.-1, *Acaulospora* sp.-1, *Acaulospora* sp.-2

In this study, the average diameter growth of white jabon, gempol, and red jabon at 2 years old were 7.38 cm (3.69 cm year^{-1}), 4.84 cm (2.84 cm year^{-1}), and 3.81 cm (1.91 cm year^{-1}), while the results of other studies showed that the annual diameter increment was quite diverse, such as for white jabon in the range of 2.03 - 5.25 cm year^{-1} [10,24-26], gempol in the range of 1.83-2.84 cm year^{-1}[4], and red jabon in the range of 1.46-5.62 cm year^{-1} [27-29]. This difference may depend on the condition of soil fertility and sylvicultural practices and the level of soil fertility in Parungpanjang was relatively low. Several studies have also shown that jabon growth was very poor on soils with low fertility levels [12,26] so it was not suitable for rehabilitation of critical lands [10].

In this study, seedlings grown on mixed media had higher growth height and diameter for the three species tested. Mixed media was a mixture of several organic materials such as soil, compost rice husk charcoal, and lime so that the fertility rate was better [18,30]. Inoculation of AMF has been revealed to improve productivity in low-fertility soils [31]. In organic seedling media, the P content was very high (2080.5 ppm) compared to topsoil media which had a low P content (33.2 ppm). The colonization of AMF had a significantly negative correlation with soil P so the application of AMF in forest tree nurseries was more effective on seedling media with low P content. While the intensity of AMF colonization has a significantly negative correlation with total carbon, organic matter content, and soil moisture content [32].

At the field trial, all seed treatments, both inoculated and uninoculated with AMF after being planted in the field, had a percentage of AMF colonization that was not significantly different with a colonization range of 27.6-57.7%. *Acaulospora* and *Glomus* have been found as dominant genera in the field test. Some studies also reported a similar result of the domination of *Glomus* and *Acaulospora* in forest lands [33] indicating the AMF species are the most potential for application in reforestation. The species also have a small spore size and easily produced more spores in a short period [32]. In general, the AMF application had a significant effect on the percentage of colonization, growth in tree diameter, and height of 2-year-old with an increase of 14.4%, 8.4%, and 7.09%, respectively. The increase in plant height and diameter growth mainly occurred in seedlings with topsoil seedling media.

The implications of this study for the nursery practice and planting of forest tree species are the need to consider the fertility level of appropriate seedling media for the application of AMF so that AMF application can be more effective and planting a mixture of species needs to study the adaptation of each species, growth characteristics and appropriate planting patterns so that each species has optimal growth. In addition, the use of native AMF explored from the planting site is expected to be more effective in increasing plant adaptation and growth.

4 Conclusion

White jabon had a more dominant growth both at the seedling level in the nursery and at the field test level until the age of 2 years, followed by gempol and red jabon. AMF inoculation of these three species at the seedling level was strongly influenced by the characteristics of the seedling media, media with high fertility levels resulted in a very low percentage of colonization. In the field trial, all species and treatments were infected with mycorrhizae with colonization percentages ranging from 27.6-57.7% which was suspected to be an infection from native AMF in the planting site. The application of AMF was quite effective in increasing the percentage of seedling survival and growth, especially on the topsoil seedling media.

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